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XXV CICLO

"New implications for the LKB1/AMPK pathway: regulation of apoptosis and circadian rhythm"

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SUMMARY

Acute Lymphoblastic Leukemia (ALL), the most common pediatric cancer, is an aggressive malignancy of lymphopoietic cells characterized by a clonal proliferation of blast cells originated from lymphoid precursors arrested at early stages of differentiation. Nowadays the introduction of risk-directed treatment and intensified cure protocols has improved the outcome of ALL pediatric patients. Although current therapies achieve five-year event-free survival rate of about 80% in children, the rest of patients experience treatment resistance and risk of early relapse. In order to develop new additional therapeutic supports and to assemble specific and personalized therapies, improving drug response and reducing drug toxicity, it is extremely important to identify novel therapeutic targets and new prognostic biomarkers on leukemic cells.

To pursue this aim, we first explored through Reverse Phase Protein Arrays (RPPA) the phosphoproteomic profile of B-cell precursor (BCP)-ALL pediatric patients. This technique represents a powerful tool to profile protein pathways activation in order to identify novel diagnostic and prognostic biomarkers and new therapeutics targets. RPPA approach leads to measure the activation levels/phosphorylation of large numbers of signalling proteins and to identify critical interactions within the protein network. This is a potential starting point for drug development and/or design of individual therapy regimens.

The principal aim of my PhD research was the discovery of new candidate biomarkers and therapeutic targets involved in the leukemic process starting from the study of the phosphoproteomic profiles of 118 BCP-ALL pediatric patients through RPPA. We screened the expression/activation of 92 different proteins/phophoproteins part of key signalling “hubs”. Correlation of signalling activation with clinical response and known genetic information enabled us to identify new protein pathway biomarkers that, when validated in larger clinical sets, could be used for patients stratification and targeted therapy trials. In detail, we observed an increased activation/expression of several pathways involved in cell proliferation in patients with a clinical poor phenotype. First, MLL-rearranged leukemia patients revealed BCL-2 hyperphosphorylation caused by AMPK activation, indicating that AMPK could be responsible of apoptosis inhibition in these patients and thus it could be considered as a new potential therapeutic target. Second, in patients with poor clinical response to Prednisone we observed the up-modulation of LCK activity with respect to patients with good response. This tyrosine-kinase can be down-modulated with clinically used inhibitors that could be considered

for further studies as a new additional therapy for Prednisone-resistant patients. Further we also found an association between high levels of CYCLIN E and relapse incidence. Moreover, CYCLIN E is more expressed in patients with early relapse, who usually show an unfavourable prognosis. We decided to first explore in deep the functional significance of AMPK activation in MLL-rearranged patients. We showed that the AMPK pathway contributes directly to the survival of MLL-rearranged BCP-ALL cells and AMPK can be considered a new druggable target in MLL-rearranged leukemias. Indeed its inhibition, using a commercial inhibitor (Compound C), induces cell death through the intrinsic apoptosis pathway in both cell lines and primary samples. Moreover, Compound C is able to synergize with drugs commonly used in pediatric ALL therapy.

The second aim was thus to study in deep the AMPK principal activator that was also observed to be hyperphosphorylated in the previous study, LKB1. LKB1 was found to be overexpressed in MLL-rearranged leukemia at protein level, but not at mRNA level. Its specific silencing brought cell cycle block at S phase and apoptosis. Very interestingly we also found that LKB1 is hyperphosphorylated in cells during mitosis and is localized at centrosome. This result opens the possibility that LKB1 could play a key role in the control of cell cycle through regulation of elongation and stability of microtubules probably following the AMPK-MRLC pathway. Moreover LKB1 probably controls also DNA damage in MLL-rearranged leukemia, indeed its inhibition induces mitotic catastrophe.

LKB1 is a master kinase and has broad known functions, even in cellular metabolism and circadian rhythm. The third aim of my PhD was thus focused on the role played by LKB1 in circadian rhythm. We report that LKB1 is a key factor in circadian control, interacting with clock-proteins. LKB1 is able to regulate clock-proteins phosphorylation, function and localization in the cell in a circadian manner. Therefore, a comprehensive understanding of the molecular links that connect LKB1 and the circadian clock could provide therapeutic benefit against cancer. For example, specific LKB1 inhibitors or activators can be more effective in the treatment of cancer if administered at a particular time of the day (chronotherapy).

In my PhD thesis I showed that identification and functional studies of new aberrant molecular players involved in cancer will be useful in order to highlight the connection between these novel markers and the goal to discover more effective patient-tailored treatments.

RIASSUNTO

La Leucemia linfoblastica acuta (LAL), il tumore pediatrico più diffuso, è una patologia delle cellule linfopoietiche caratterizzata da una proliferazione clonale di blasti originati dai precursori linfoidi bloccati a stati differenziativi precoci. Ad oggi un miglioramento della prognosi dei pazienti affetti da LAL è stao portato dall'introduzione di trattamenti differenziati sulla base del rischio di ricaduta e da protocolli di cura più intensi. Le terapie attualmente usate permettono di ottenere una sopravvivenza dell'80% a 5 anni dalla diagnosi, tuttavia esiste ancora circa un 20% di pazienti che presenta rischio di ricaduta precoce e resistenza alla terapia. Risulta dunque fondamentale identificare nuovi target terapeutici e biomarcatori delle cellule leucemiche al fine di sviluppare trattamenti di supporto innovativi e di individuare terapie più specifiche, migliorando la risposta alle terapie e riducendone la loro tossicità. Per perseguire questo obiettivo, per prima cosa tramite la tecnica di Reverse Phase Protein Arrays (RPPA) abbiamo mappato il profilo fosfoproteomico di pazienti pediatrici BCP-LAL. Questa tecnica rappresenta uno strumento potente per studiare il profilo proteico delle vie di trasduzione del segnale con lo scopo di identificare nuovi marker prognostici e target terapeutici. Con la metodica degli RPPA è possibile misurare il livello di attivazione/espressione di un ampio numero di proteine di segnale e di identificare alterazioni nei punti chiave dei network cellulari. Questo risulta essere un punto di partenza potenziale per lo sviluppo di nuovi farmaci e/o l'ideazione di nuovi regimi terapeutici.

Lo scopo principale del mio dottorato di ricerca è stato la scoperta di nuovi biomarcatori e target terapeutici coinvolti nel processo leucemico partendo dallo studio del profilo fosfoproteomico di 118 pazienti pediatrici BCP-LAL attraverso la tecnica di RPPA.
Abbiamo analizzato i livelli di espressione/attivazione di 92 differenti proteine/fosfoproteine di segnalazione chiave. La correlazione sulle vie di trasduzione attivate con la risposta alla terapia e con le caratteristiche genetiche dei pazienti ci ha permesso l'identificazione nuovi biomarcatori che, una volta validati in un gruppo allargato di campioni di LAL-B, potranno permettere una migliore stratificazione dei pazienti e potranno essere usati per lo sviluppo di cure paziente-specifiche.
In particolare, in pazienti a prognosi infausta, abbiamo osservato un aumento dell'attivazione/espressione di diverse vie di trasduzione del segnale coinvolte nella proliferaziione cellulare. In primo luogo, pazienti leucemici con traslocazione del gene MLL presentano l'iperfosforilazione di BCL-2 conseguente all'attivazione di AMPK, indicando che AMPK possa essere responsabile dell'inibizione dell'apoptosi in questi

pazienti e quindi potrebbe essere un nuovo target terapeutico. Il secondo risultato osservato è una up-modulazione dell'attività di LCK nei pazienti con scarsa risposta al trattamento con Prednisone. Questa tirosina-chinasi potrebbe essere down-modulata tramite l'utilizzo di inibitori utilizzati in clinica e, quindi, potrebbe essere considerata per ulteriori studi al fine di identificare terapie addizionali per i pazienti resistenti al Prednisone. Da questa analisi fosfoproteomica è emersa, inoltre, un'associazione tra gli elevati livelli di CICLINA E e l'incidenza di ricaduta. In aggiunta la CICLINA E è risultata essere maggiormente espressa in pazieni con ricaduta precoce, generalmente associata ad una prognosi sfavorevole.
Successivamente abbiamo approfondito, come prima cosa, il significato funzionale dell'attivazione di AMPK nei pazienti con riarrangiamento del gene MLL. Abbiamo dimostrato che il pathway di AMPK contribuisce direttamente alla sopravvivenza delle cellule BCP-LAL MLL-riarrangiate, suggerendo AMPK come nuovo target terapeutico per questo tipo di leucemia. Infatti l'inibizione di AMPK, tramite l'utilizzo del suo inibitore commerciale (Compound C), porta a morte cellulare attraverso la via intrinseca di apoptosi sia nelle linee cellulari sia in colture cellulari primarie. Inoltre il Compound C ha azione sinergica con farmaci utilizzati in clinica per la terapia di pazienti pediatrici LAL.

Il secondo scopo del mio dottorato di ricerca è stato quello di focalizzarmi su LKB1, principale attivatore di AMPK, risultato essere anch'esso iperfosforilato nello studio di proteomica. LKB1 è stato trovato overespresso a livello proteico, ma non a livello di mRNA nelle leucemie con riarrangiamento del gene *MLL*. Il suo silenziamento specifico porta ad un blocco del ciclo cellulare in fase S e conseguente apoptosi.
Molto interessante è stata la scoperta che LKB1 risulta essere iperfosforilato nelle cellule in mitosi, con localizzazione centrosomica. Questo data apre la possibilità che LKB1 possa avere un ruolo chiave nella regolazione del ciclo cellulare tramite la regolazione dell'allungamento e della stabilità dei microtubuli, probabilmente attraverso il pathway AMPK-MRLC. In aggiunta LKB1 potrebbe avere un ruolo anche nel controllo del danno al DNA nelle leucemie con riarrangiamento del gene *MLL*, in quanto la sua inibizione porta a catastrofe mitotica.

LKB1 è una chinasi molto importante con funzioni note diffuse anche nel metabolismo cellulare e nel ritmo circadiano. Il terzo scopo del mio dottorato di ricerca è stato focalizzare lo studio del ruolo di LKB1 nel ritmo circadiano. Abbiamo scoperto che LKB1 gioca un ruolo chiave nel controllo circadiano, interagendo con le proteine clock.

LKB1, infatti, è in grado di fosforilare le proteine circadiane, regolandone funzione e localizzazione cellulare in modo circadiano.
Una compresione globale del collegamento molecolare che collega LKB1 e il sistema circadiano può portare a benefici nella terapia tumorale. Per esempio, specifici inibitori o attivatori di LKB1 potrebbero essere più efficaci nella terapia tumorale se somministrati in un particolare momento del giorno (cronoterapia).

Nella corso del mio dottorato ho potuto dimostrare che l'identificazione e lo studio funzionale di nuovi biomarcatori aberranti coinvolti nei tumori, possono essere utilizzati per sottolineare il collegamento tra questi con lo scopo ultimo di scoprire nuove efficaci terapie pazienti-specifiche.

AIM OF THE STUDY

Acute Lymphoblastic Leukemia (ALL) is an aggressive malignancy of lymphopoietic cells characterized by a clonal proliferation of blast cells originated from lymphoid precursors arrested at early stages of differentiation. ALL is the most common pediatric cancer, accounting for a quarter of all malignancies among children aged less than 15 years. This potentially catastrophic disease was once fatal in majority of patients but nowadays the introduction of risk-directed treatment and intensified cure protocols have improved the outcome of ALL pediatric patients. Indeed, current therapies achieve five-year event-free survival rate of about 80% in children receiving protocol-based therapy in the developed countries. Advances in genomics and molecular analysis of tumours have improved the ability to select patients that are likely to respond to a particular therapeutic agent, but substantial numbers of patients still experience relapse and have poor outcomes, failing to ever achieve a complete remission.

The goal of my PhD was to discover new candidate biomarkers and therapeutic targets involved in the leukemic process and, to this aim, to investigate the aberrant activation or expression of proteins involved in cellular signal transductions pathways of leukemic cells. The tyrosine kinases (TK) are attractive targets for anti-neoplastic therapy, as very often the alteration of their signaling can be connected with tumor growth and development, and they could be easily targeted with specific agents. The constitutive activation of TK stimulates multiple signaling pathways responsible for defective DNA repair, apoptosis block and increased cell proliferation.

We thus employed the innovative technique of the Reverse Phase Protein Arrays to investigate signal transduction pathways activation in 120 pediatric patients affected by Acute Lymphoblastic Leukemia (ALL). Results obtained in this study prompted us to further evaluate the role of the AMPK pathway hyperactivation in MLL-rearranged ALL. The LKB1-AMPK axis has several functions ranging from metabolism regulation to cell growth, proliferation, apoptosis, autophagy, cell polarity, oxidative stress and cancer. However, the role of LKB1/AMPK pathway in cancer is not yet fully understood considering that, so far, data reported in literature are contradictory. The effects of LKB1/AMPK activation are determined by the cell type investigated, depending on signalling alterations in related pathways. Our second aim thus was to assess whether AMPK hyperactivation supports the survival of MLL-rearranged BCP-ALL cells, and whether its inhibition could affect leukemic cell growth and drug resistance thus pointing out AMPK as a possible new therapeutic target in this high-risk ALL patients.

Due to the relevance of AMPK activation coming from our studies, we decided to explore also expression and function of LKB1, the principal AMPK kinase, in pediatric ALL. LKB1 is a multitasking kinase with great potential in orchestrating cell activity, found to play a role in cell cycle, polarity, apoptosis, cell cycle arrest and cell proliferation. To date, LKB1 has been considered as a tumor suppressor because the hereditary and somatic loss of function mutations of this gene are associated with an increased risk of cancer development. But recent evidences show that LKB1 can also play a potentially oncogenic role in some cells and this opens for LKB1, as for AMPK, the possibility of a dual role in a cell-dependent way.

During the last year of my PhD I had the possibility to collaborate with Prof. Paolo Sassone Corsi and to spend nine months in his laboratory at the University of California, Irvine (CA, USA). Here I started to evaluate the role played by LKB1 in metabolism and circadian rhythm regulation. As described in literature, mutations in circadian clock proteins have been linked to cancer and metabolic syndromes. Although LKB1 has been reported to play a role in the circadian clock, the mechanism is not yet clear. A comprehensive understanding of the molecular links that connect LKB1 and the circadian clock could provide therapeutic benefit against cancer.

CHAPTER I

"AMPK in pediatric Acute Lymphoblastic Leukemia"

I. INTRODUCTION

I.I LKB1-AMPK axis

The connection between cellular metabolism and tumorigenesis was first proposed 100 years ago by Warburg O., but the molecular mechanisms interconnecting metabolism signaling pathways and cell growth begun to be decoded only in the past decade, making this an active area of investigation in cancer research.

One of the newly uncovered links directly connecting cell metabolism and cancer is represented by the discovery that that the serine/threonine kinase LKB1 (Liver Kinase B1; also known as Serine/Threonine Kinase 11 - *STK11*), a known tumor suppressor, is the key upstream activator of the AMP-activated protein kinase (AMPK) (Hong S.P. *et al.*, 2003; Hawley S.A., *et al.*, 2003; Woods A., *et al.* 2003; Shaw R.J., *et al.*, 2004).

LKB1 is a kinase originally identified as the tumor suppressor gene responsible for the inherited cancer disorder called Peutz-Jeghers Syndrome (PJS), an autosomal dominantly inherited disorder that predisposes to a wide spectrum of benign and malignant tumors (Hemminki A., 1999; Westerman A., *et al.*, 1999; Jenne D.E., *et al.*, 1998). To date, over 60 different mutations have been mapped to LKB1, many of which would be expected to impair LKB1 activity. These discoveries aroused great interest because they indicate that LKB1 is likely to function in cells as a tumor suppressor, and consistent with this, overexpression of LKB1 in a number of tumor cell lines has been shown to suppress cell growth by inducing a G_1 cell cycle block (Tiainen M., *et al.*, 1999). However, little is known regarding the mechanism by which LKB1 activity is regulated in cells.

The search for LKB1 substrates led to the identification of AMPK (Hong S.P., *et al.*, 2003; Hawley S.A., *et al.*, 2003; Woods A., *et al.* 2003; Shaw R.J., *et al.*, 2004), a serine-threonine kinase that acts as a cellular fuel sensor activated under conditions of ATP depletion and elevated AMP levels. The LKB1/AMPK axis has several function, that are described below, ranging from metabolism to cell growth, proliferation, apoptosis, autophagy, cell polarity and oxidative stress (Wang W. and Guan K.L. 2009; Fogarty S. and Hardie D.G. 2010; Kottakis F. and Bardeesy N. 2012).

I.II AMP-activated protein kinase

The *AMPK* gene maps in the chromosomal region 5p12 and spans 39kb. The protein encoded by this gene belongs to a ser/thr protein kinase family highly conserved from yeast to human. The physiological role ascribed to AMPK is the inactivation of ATP-consuming metabolic processes, including fatty acid, cholesterol and protein synthesis, and the activation of ATP-generating pathways such as glycolysis and fatty acid oxidation. This is initially accomplished by direct phosphorylation of key metabolic enzymes, followed by effects on gene expression.

AMPK is an heterotrimeric complex composed of an α-catalytic subunit, a β-subunit important both for complex formation and glycogen binding, and a γ-regulatory subunit, which binds AMP (Davies S.P., *et al.*, 1995). Under conditions of metabolic stress, the ratio of AMP to ATP is increased and AMP binds to the γ-subunit of AMPK, serving as an allosteric activator and protecting AMPK against phosphatases (Davies S.P., *et al.*, 1995; Carling D., *et al.*, 2008). The phosphorylation of AMPK at Thr172 in the α subunit is indeed critical for its activation. Thus far, several AMPK kinases have been identified, but the most important are LKB1 (Hawley S.A., *et al.*, 2003; Hong S.P., *et al.*, 2003; Shaw R.J., *et al.*, 2004) and the calmodulin-dependent protein kinase-β (CaMKK-β) (Hawley S.A., *et al.*, 2005; Woods A., *et al.*, 2005;). Biochemical and genetic analyses in worms, flies and mice have revealed that LKB1 is the major kinase phosphorylating the AMPKα activation loop under conditions of energy stress (Carling D., *et al.*, 2008). From tissue-specific knockouts of LKB1 in mice, it appears that LKB1 dictates most of the AMPK activation in all tissues examined thus far, with the exception of some hypothalamic neurons (Anderson K.A., *et al.*, 2008), T-cells (Tamas P., *et al.* 2006), and endothelial cells (Stahmann N., *et al.*, 2006) in which CAMKK2 appears to play a key role, although only in response to changes in the concentration of calcium (Hawley S.A., *et al.*, 2005) .

Other kinases, including TGF-β-activating kinase 1 (TAK1) and ataxia-telangiectasia mutated (ATM) (Xie M., *et al.*, 2006; Momcilovic M., *et al.*, 2006; Suzuki A., *et al.*, 2004), are bona fide kinases that remains to be further confirmed. Interestingly, a recent study reported that TAK1 mediates TNF-related apoptosis by inducing its ligand to activate AMPK, inducing autophagy independent of LKB1 and CaMMK (Herrero-Martin G., *et al.*, 2009). AMPK can also be activated by hormones and cytokines, including leptin (e.g., in skeletal muscle), adiponectin secreted from adipocytes, IL-6 and ciliary neurotrophic factor (CNTF) (Steinberg G.R., *et al.*, 2009; Zhang B.B., *et al.*, 2009). Interestingly, leptin could exert opposite effects on AMPK depending on cell types (Minokoshi Y., *et al.*, 2002; Minokoshi Y., *et al.*, 2004). Moreover, AMPK can be activated by a variety of pharmacological agents. The prototypical activator is 5-aminoimidazole-4-carboxamide 1-D-ribonucleoside (AICAR), a cell permeable agent that is phosphorylated and converted

to ZMP, an AMP analog, after entering the cell. Importantly, two clinically used antidiabetic drugs, metformin and thia-zolidinediones (TZDs), have been described to activate AMPK (Zhang B.B., *et al.*, 2009). Upon activation, AMPK phosphorylates a plethora of substrates and regulates their functions with final effects depending on cell types and on signaling alterations in related pathways.

I.III Downstream targets of AMPK pathway

The number of identified AMPK targets has grown rapidly in recent years (Steinberg G.R., *et al.*, 2009; Shackelford D.B., *et al.*, 2009a; Hardie D.G. 2007; Carling D., *et al.*, 2008), here we decided to highlight only some the best characterized ones.

I.III.a Enzymes for fatty acid & cholesterol synthesis

AMP-activated protein kinase was first identified as a kinase that phosphorylates and inhibits acetyl CoA carboxylase (ACC) and HMG-CoA reductase, rate-limiting enzymes for *de novo* synthesis of fatty acid and cholesterol, respectively. Interestingly, both of these enzymes have important roles in tumorigenesis. Statins, HMG-CoA reductase inhibitors that lower cholesterol levels, have been suggested to prevent cancer in experimental models and reduce cancer risk in humans (Katz M.S. 2005; Farwell W.R., *et al.*, 2008). FASN, ACC and other enzymes required for *de novo* synthesis of free fatty acid and cholesterol are highly expressed in several types of cancers, including those arising from breast, prostate, colon and ovary (Kuhajda F.P. 2000; Baron A., *et al.*, 2004). The increase of these enzymes is attributed to augmented expression and maturation of the transcription factors SREBP-1 and SREBP-2. AMPK has been shown to inhibit SREBP1 (Zhou G., *et al.*, 2001), however the mechanism is not fully understood. Interestingly, pharmacological inhibition of FASN in human ovarian cancer cells leads to a rapid activation of AMPK, followed by the induction of cytotoxicity (Zhou W., *et al.*, 2007). The cytotoxic effect is suppressed by compound C, a chemical inhibitor of AMPK, thus these findings suggest that AMPK mediates the effect of pharmacological inhibition of FASN.

I.III.b p53 & other tumor suppressors

Another important partner of AMPK is the tumor suppressor p53, with which AMPK is mutually regulated. On one hand, AMPK activation by AICAR or glucose deprivation leads to up-regulation of p53 as well as its phosphorylation at Ser15 (Jones R.G., *et al.*, 2005; Imamura K., *et al.*, 2001). Mouse embryonic fibroblasts (MEFs) bearing wild-type p53 are arrested at G_1/S phase by glucose deprivation, AICAR and expression of a constitutively activated AMPK mutant, whereas the metabolic checkpoint is ineffective in

the p53-deficient MEF cells (Jones R.G., *et al.*, 2005). Expression of a dominant negative mutant of the AMPK α1-subunit in prostate cancer cells accelerates their growth, with a concomitant decreases in mRNA and protein levels of p53 (Zhou J., *et al.*, 2009). On the other hand, AMPK is regulated by p53 (Feng Z., *et al.*, 2007; Budanov A.V., *et al.*, 2008) resulting in inhibition of mTOR. These findings suggest that their mutual regulation enhances their tumor suppressive functions.

I.III.c LKB1-AMPK-mTORC1 pathway

Work from several laboratories in the past 5 years has revealed that one of the major growth regulatory pathways controlled by LKB1-AMPK is the mammalian target-of-rapamycin (mTOR) pathway. mTOR is a central integrator of nutrient and growth factor inputs that controls cell growth in all eukaryotes and is deregulated in most human cancers (Guertin D.A., *et al.*, 2007).

mTOR is found in two biochemically and functionally discrete signaling complexes: mTORC1 and mTORC2. mTOR complex 1 (mTORC1) includes raptor, which acts as a scaffold to recruit downstream substrates such as 4EBP1 and ribosomal S6 kinase (p70S6K1) that contribute to mTORC1-dependent regulation of protein translation (Holz M.K., *et al.*, 2005). mTORC1 controls the translation of a number of cell growth regulators, including cyclin D1, hypoxia inducible factor 1a (HIF-1α), and c-myc, which in turn promote processes including cell cycle progression, cell growth and angiogenesis, all of which can become deregulated during tumorigenesis (Guertin D.A., *et al.*, 2007). In contrast, mTORC2 contains the rictor subunit and is neither sensitive to nutrients nor acutely inhibited by rapamycin (Guertin D.A., *et al.*, 2007).

Cancer genetics and Drosophila genetics led to the discovery of upstream components of mTORC1 including the tuberous sclerosis complex 2 (TSC2) tumor suppressor and its obligate partner TSC1 (Shaw R.J. and Cantley L.C., 2006). TSC2 inhibits mTORC1 indirectly via regulation of the small GTPase Rheb, such that loss of TSC1 or TSC2 leads to hyperactivation of mTORC1 (Huang J., and Manning B.D. 2008). When levels of ATP, glucose or oxygen are low, AMPK directly phosphorylates TSC2 on conserved serine sites (Inoki K., *et al.*, 2003; Shaw R.J., *et al.*, 2004; Liu L., *et al.*, 2006) and primes serine residues close by for subsequent phosphorylation by GSK-3 (Inoki K., *et al.*, 2006). While TSC2 is clearly a central receiver of inputs that regulate mTORC1, cells lacking TSC2 still partially suppress mTORC1 following AMPK activation (Hahn-Windgassen A., *et al.*, 2005; Gwinn D.M., *et al.*, 2008). In agreement with these data, raptor has been identified as a direct substrate of AMPK *in vivo*. Phosphorylation of two conserved serines in raptor by AMPK induced binding to 14-3-3 and resulted in suppression of mTORC1 kinase activity (Gwinn D.M., *et al.*, 2008). Importantly, mTORC1 is currently the only signaling

pathway downstream of LKB1 that has been shown to be deregulated in tumors arising in humans and mouse models of both PJS (Shaw R.J., *et al.*, 2004; Shackelford D.B., *et al.*, 2009b) and NSCLC (Ji H., *et al.*, 2007; Carretero J., *et al.*, 2007).

I.III.d eNOS and BCL2

Activation of AMPK is also known to stimulate Nitric Oxide (NO) production at least in part due to phosphorylation and activation of endothelial nitric oxide synthase (eNOS) (Morrow V.A., *et al.*, 2003; Drew B.G., *et al.*, 2004). Indeed it was demonstrated that AMPK co-precipitates with and phosphorylates eNOS at Ser-1179 and Thr-497 (Chen Z.P., *et al.*, 1999), and Ser-1179 and Ser-116 (Drew B.G., *et al.*, 2004).

NO is a multifunctional transcellular messenger that can play a dual role in cancer with both pro- and anti-apoptotic effects (Choi B.M., *et al.*, 2002; Xu W., *et al.*, 2002). Interestingly, a prominent NO production has been observed in undifferentiated tumors (Thomsen L.L., *et al.*, 1995). In addition, it has been reported that NO can prevent apoptosis by elevating BCL-2 expression both at mRNA and protein levels in B-lymphocytes (Mannick J.B., *et al.*, 1994).

This is not one of the best characterized AMPK activated signaling pathways, but is the one we found to be key for MLL-rearranged leukemia cell survival as further described in the two published papers.

I.IV AMPK and cancer

The role of AMPK in cancer is not yet fully understood considering that, so far, data reported in literature are contradictory. The effects of AMPK activation are determined by the cell type investigated, depending on signaling alterations in related pathways. Indeed, results presented in 2012 by Jeon *et al.* suggest that targeting the LKB1-AMPK axis in cancer should be done with caution and with attention to specific contexts (Jeon S.M., *et al.*, 2012).

AMPK has been frequently proposed as a potential therapeutic target in cancer either because is the major downstream target of the tumor suppressor LKB1 or because of its known role in the regulation of cell proliferation, cell cycle progression and autophagy via mTORC1, p53 and p27 (Fogarty S. and Hardie D.G. 2010). As previously described, AMPK can be activated through different pharmacological agents, and data in the literature demonstrated that treatment with Metformin or AICAR can suppress cell proliferation and induce apoptosis in many cell types such as melanoma (Zhang W.B., *et al.*, 2010), glioblastoma (Liu B., *et al.*, 2009), astrocytoma (Rattan R., *et al.*, 2005), breast cancer (Woodard J., *et al.*, 2010), renal cell carcinoma (Sengupta T.K., *et al.*, 2007), acute

myeloid leukemia (Green A.S., *et al.*, 2010), ovarian cancer, (Zhou W., *et al.*, 2007) and osteosarcoma (Okoshi R., *et al.*, 2008). Two studies (Kuznetsov J.N., *et al.*, 2011; Shaw M.M., *et al.*, 2007) also showed that AMPK activation by AICAR can induce apoptosis in some BCP-ALL cell lines. In addition, reduced levels of adiponectin have been found in the plasma of patients with some cancers (e.g., breast and prostate cancers), and treatment of cancer cells with adiponectin attenuated their growth, an event that is blocked by the dominant negative mutant of AMPK (Barb D., *et al.*, 2007; Grossmann M.E., *et al.*, 2008; Kim K.Y., *et al.*, 2009). Finally, a large number of studies have shown that maneuvers that activate AMPK, such as treatment with pharmacological agents (e.g., AICAR, metformin and TZDs), exercise and dietary restriction can attenuate cancer cell growth *in vitro* and inhibit tumor development *in vivo* in mammary carcinomas, mammary gland, and liver (Jiang W., *et al.*, 2008; Rattan R., *et al.*, 2005; Dowling R.J., *et al.*, 2007; Buzzai M., *et al.*, 2007).

Rising data however highlight the dual role of AMPK. Its activation can also result in anti-apoptotic effects in multiple myeloma (Baumann P., *et al.*, 2007), prostate cancer (Park H.U., *et al.*, 2009) and glioma (Vucicevic L., *et al.*, 2009). AMPK depletion by small interfering RNA was reported to induce growth arrest and apoptosis in human and rat glioma (Vucicevic L., *et al.*, 2009) and pheochromocytoma cell lines (Mizrachy-Schwartz S., *et al.*, 2011). AMPK activity is higher also in OVCAR3 and A431 cells than in primary keratinocytes (Byekova Y.A., *et al.*, 2011). Moreover, increased expression of LKB1 and AMPK was observed in UVB-induced murine basal cell carcinoma (Lee J.H., *et al.*, 2007).

Interestingly, retrospective investigations reported that patients with Type 2 diabetes taking metformin display a reduced risk of cancer as compared with the normal population or with the patients who have never taken metformin (Evans J.M., *et al.*, 2005; Libby G., *et al.*, 2009), and a reduced trend of mortality compared with patients who take sulfonylureas or insulin (Bowker S.L., *et al.*, 2006). This underlines the intriguing emerging evidence that AMPK activation has a dual role in cancer depending on alterations of interconnected signaling pathways, thus its effects are likely to be cell type and tissue specific.

To reconcile these two opposing functions of AMPK, Jeon *et al.* suggested a reflection about the temporal manner of AMPK activation that, at physiological levels, is essential for the survival of tumor cells during energy stress (starvation or matrix detachment) but it is quickly followed by inactivation (Jeon S.M., *et al.*, 2012). According to this model, use of AMPK inhibitors in acute regimens could prove beneficial for cancer therapy by sensitizing cells to energy stress. Moreover, the acute nature of the treatment could potentially cause metabolic stress, sensitizing cells to other chemotherapy regiments. Sustained inactivation of the LKB1-AMPK pathway on the other hand, could result in

long-term stress, promote rewiring of intracellular metabolic processes, and tip the balance towards increased proliferation due to activation of mTOR and other pathways (Jeon S.M., *et al.*, 2012).

A more complex inter-relationship between the mitogenic and metabolic pathways is highlighted by the recent discovery of the interaction between kinase suppressor of ras 2 (KSR2) and AMPK (Costanzo-Garvey D.L., *et al.*, 2009). KSR1 and KSR2 are best known for their scaffold function for the Raf/MEK/ERK signaling cascade, facilitating the activation of Raf and MEK (Kortum R.L. and Lewis R.E. 2004; Dougherty M.K., *et al.*, 2009). Costanzo-Garvey *et al.* have found that AMPK associates with KSR1 and KSR2 with more preference toward the latter (Costanzo-Garvey D.L., *et al.*, 2009). Hence, it is tempting to speculate that KSR2 functions as a switching point of cell fate for mitogenesis and metabolic checkpoint if all these proteins coexist in the same complex. In proliferating cells, KSR2 brings an active MEK/ERK complex to AMPK, preventing its activation by LKB1. Conversely, under metabolic stress, binding of AMPK to KSR2 prevents Raf/MEK to be targeted to the plasma membrane for their activation. Alternatively, KSR2 could form independent complexes with AMPK and Raf/MEK.

I.V AMPK and Glucose metabolism in cancer cells

One of the prominent traits of cancer cells is aerobic glycolysis, which was first described by Otto Warburg in the 1920s (Warburg O., 1956). Regardless of adequate oxygen supply, cancer cells rely on the glycolysis that takes place in the cytosol over oxidative phosphorylation in the mitochondria, although the former is much less efficient in generating ATP. Complex mechanisms are involved in the adaptation of cancer cells to glycolysis. Two important modulators relevant to AMPK are HIF-1 and p53 (**Fig. I.1**).

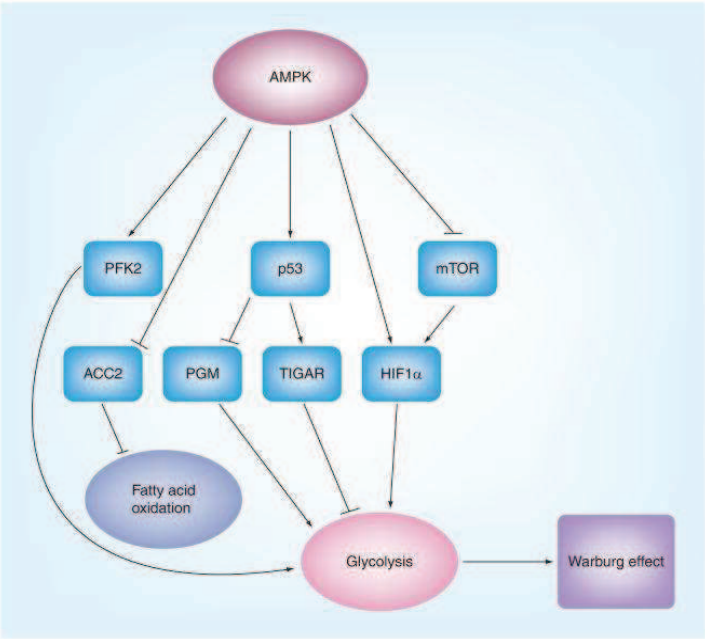

Fig I.1: AMPK plays a role in reprogramming energy metabolism

Therefore, the characteristic increases of glucose uptake and glycolysis in tumors distinguish them from normal tissues, thereby offering a tracing advantage by ^{18}F 2-fluoro-2-deoxy-D-glucose PET scanning, and these increases are also targeting points for cancer therapy (Pelicano H., *et al.*, 2006).

AMP-activated protein kinase has been implicated in the regulation of HIF1α. This may be mediated by its action on mTORC1 (Shaw R.J. 2006). Thus, in LKB1- or AMPK-deficient fibroblasts, the levels of HIF1α and its downstream targets are elevated and could be diminished by rapamycin (Shackelford D.B., *et al.*, 2009b). A similar increase of HIF1α is also found in the epithelia of gastrointestinal hamartomas from *LKB1*$^{+/-}$ mice (Shackelford D.B., *et al.*, 2009b). These studies suggest that AMPK suppresses glycolysis in tumor cells by inhibiting mTOR. In addition, AMPK activation upregulates HIF-1α and VEGF expression in hypoxia (Hwang J.T., *et al.*, 2004; Lee M., *et al.*, 2006). Thus, it remains to be clarified whether these findings are reflective of acute stress response and examined whether the net effect of these opposite regulations by AMPK depends on cellular context and metabolic state of the cells.

Cell metabolism is also emerging as a key regulator of T-cell homeostasis and adaptive immunity. Recent work investigating the role of LKB1/AMPK signaling in T-cells has uncovered a novel function for this energy-sensing pathway in both T-cell development and peripheral T-cell function. Evidence to date suggests that LKB1 and AMPK regulate a metabolic switch in T cells that functions to antagonize anabolic pathways (glycolysis and mTORC1) and promote mitochondrial oxidative metabolism (lipid oxidation) (Blagih

J., *et al.*, 2012). Through the coordinated control of cellular metabolism, LKB1 and AMPK regulate a "metabolic checkpoint" in T cells that restrict T-cell growth in a fashion similar to that observed in cancer cells (Shackelford D.B., *et al.*, 2009a). However, it is clear that LKB1 and AMPK can also mediate independent functions in T cells and may operate in distinct signaling pathway (Blagih J., *et al.*, 2012). Future work focusing on exploring the role of AMPK and LKB1 in lymphoid B-cell and T-cell function is needed.

I.VI AMPK as a new therapeutic target

The first aim of my study, as previously described, is to identify new therapeutic targets in MLL rearranged pediatric B acute lymphoblastic leukemia, in order to improve therapy efficacy and reduce toxicity. The tyrosine kinase (TK) are attractive targets for anti-neoplastic therapy, as very often the alteration of their signaling can be connected with tumor growth and development, and they could be easily targeted with specific agents. The constitutive activation of TK stimulates multiple signaling pathways responsible for defective DNA repair, apoptosis block and increased cell proliferation. Recent publications put specific protein signalling pathways activation at a central point in the effective treatment of cancer (Jones S., *et al.*, 2008; Cancer Genome Atlas Research Network. 2008). In this light, post-translational modifications (PTMs) that control activation and function of specific signaling pathways play a central role. One of the most important PTMs, as we will discuss in the IIIrd Chapter, is phosphorylation underpinned by ongoing activity of protein kinases and phosphatases. Mapping the activation state of hundreds of proteins in signalling transduction pathways (STPs) will lead to the identification of altered STPs in tumor cells and consequently to the discovery of new therapeutic targets. Newly discovered target can be used for rational, molecular-targeted drug design and could be useful to identify cancer patients that may benefit from the use of specific kinase inhibitors. These molecules are remarkably effective in the treatment of various cancers including brain tumors, cervical, gastric, prostate, breast cancers, and leukemia. One of the most effective examples of these inhibitors is Imatinib, the inhibitor of the Bcr/Abl kinase (Pytel D., *et al.*, 2009).

The goal is thus to find new therapeutic targets for recently developed kinase inhibitors, thus increasing the response to treatment and reducing toxicity.

I.VII Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia (ALL) is a clonal malignant disease of the bone marrow that originates from lymphoid progenitor cells often carrying specific genetic and epigenetic alterations. It is characterized by an accumulation of malignant blast cells resulting in the suppression of normal haematopoiesis and infiltration of various extramedullary sites. ALL is the most common pediatric cancer, accounting for a quarter of all malignancies among children aged <15 years. This potentially catastrophic disease was once fatal in four-fifths of patients, but thanks to the introduction of risk-directed treatment and improved supportive care clinical outcome has improved remarkably over the past 50 years. Today the 5-year event-free survival rate for ALL is approximately 80% in children receiving protocol-based therapy in the developed countries (Pui C.H., 2010). The majority of ALLs are of B-cell lineage in both children (>80%) and adults (>75%) (Pui C.H., 2010; Pui C.H., *et al.*, 2004). In B cell precursor ALL (BCP-ALL) blast cells are regarded as malignant counterparts of normal B-cell precursors.

Current approaches of risk assessment rely on a number of key clinical and laboratory findings such as the initial leukocyte count, age at diagnosis and early treatment response. Children aged 1–9 years have a better outcome than infants and adolescents. Leukocyte count is a continuous variable, with decreasing counts conferring a better outcome (Pui C.H., 2010).

The most informative prognostic factor is the response to early treatment, as determined by measurements of the rate of clearance of leukemic cells from the blood or bone marrow. This estimate of minimal residual disease accounts for the drug sensitivity or resistance of leukemic cells and the pharmacodynamics of the drugs, which is affected by the pharmacogenetics of the host. Flow cytometric profiling of aberrant immunophenotype markers and polymerase chain reaction amplification of fusion transcripts or antigen-receptor genes, which are at least 100-fold more sensitive than conventional morphological determinants, have allowed minimal residual disease to be detected at very low levels (< 0.01%). This provides a useful means to identify patients at very low or high risk of relapse (Pui C.H., *et al.*, 2004). Minimal residual disease can be measured by the current techniques in nearly all patients, and has become a key factor for risk stratification in childhood ALL.

In spite of treatment and risk stratification improvement, 20% of children with ALL ultimately relapse, and cure rate after relapse reaches only 25% to 40%. Very high remains the interest to understand the mechanisms of relapse. Genome-wide studies using matched diagnosis and relapse samples from the same patients have shown that both sets of samples are clonally related, and that the relapse clones are often present as minor populations at diagnosis, which suggests that they are selected during treatment.

Indeed, many of the genetic alterations that emerge in the dominant clone at relapse involve genes that have been implicated in treatment resistance (e.g. *CDKN2A/B* or *IKZF1*) (Pui, C.H., *et al.*, 2004; Pieters, R., *et al.*, 2007) and gene expression studies have identified a proliferative gene signature that emerges at relapse with consistent up-regulation of genes, such as survivin, that could provide useful targets for novel therapeutic intervention (Pui, C.H., *et al.*, 2004).

Childhood acute leukemia have long been the best characterized malignancies from a genetic viewpoint. In BCP-ALL, individual chromosomal abnormalities are strong independent indicators of outcome, especially to indicate risk of relapse (Hjalgrim L.L., *et al.*, 2003). The most significant impact for risk stratification for treatment are t(9;22)(q34;q11)/*BCR-ABL1* and rearrangements of the *MLL* gene. The most frequently found MLL fusion genes are MLL-AF9 t(9;11), MLL-AF4 t(4;11) and MLL-ENL t(11,19) (Secker-Walker L.M., 1997). In ALL, the partner genes are limited and *AF4* is the most common one, whereas in AML the partner genes are much more diverse.
Translocations of the *MLL* gene located at chromosome band 11q23 define a subset of aggressive acute leukemia with distinctive clinical and biological features (Gilliland D.G., *et al.*, 2004). *MLL* rearrangements can be found in 5-10% of childhood and adult acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) (Pui C.H., *et al.*, 2003; Bacher U., *et al.*, 2005; Mancini M., *et al.*, 2005), and are frequently seen in infants (Rowley J.D. and Olney H.J. 2002) and in secondary leukemia following treatment with topoisomerase II poisons (Pui C.H., *et al.*, 2003). As a consequence of the translocation, *MLL* undergoes fusion with a wide variety of partner genes (up to 50) and the chimeric proteins that are originated lead to either myeloid or lymphoid leukemia (hence the name MLL). Biologically, *MLL* translocations determine differentiation, lineage and immunophenotype of leukemia blast cell populations. For example, the blast populations in the ALL cases exhibit an early CD10- CD24- pro-B cell immunophenotype and uniquely co-express the myeloid associated antigen CD15 (Borkhardt A., *et al.*, 2002). Observed morphologic and phenotypic heterogeneity is influenced by the partner genes involved (Hunger S.P., *et al.*, 1992). In all of these patient populations, *MLL* translocations are poor prognostic factors with significant adverse effects on response to treatment. For an ultra high-risk population within infant ALL, the constellation of poor prognostic features including age <3 months at diagnosis, WBC count >100,000/µL, early pro-B CD10- immunophenotype and t(4;11) translocation, is associated with event free survival of ~5% (20,21). Infant leukemia with *MLL* translocations often are resistant to common chemotherapeutic agents (Pieters R., *et al.*, 1998; Pui C.H., *et al.*, 2002) and infants also are more vulnerable to toxicities, thus more intensive treatment for infant

ALL has increased treatment complications without improving outcome (Hilden J.M., *et al.*, 2006). Furthermore, MLL-rearranged leukemia display a highly distinct gene expression profile that is consistent with an early hematopoietic progenitor expressing selected multi-lineage markers. Clustering algorithms revealed that lymphoblastic leukemia with *MLL* translocations can clearly be separated from conventional acute lymphoblastic and acute myelogenous leukemia (Armstrong S.A., *et al.*, 2003).

Other recurrent structural abnormalities in BCP ALL include t(12;21)(p13;q22)/*ETV6-RUNX1* fusion, as well as t(1;19)(q23;p13.3)/*TCF3-PBX1* fusion. However, these are not used in risk stratification on all protocols. The *ETV6-RUNX1* fusion occurs in approximately 25% of younger children and these patients have an extremely good prognosis. Among patients with *TCF3* rearrangements, those with *TCF3-PBX1* were originally regarded as poor risk on some treatment protocols, but on modern therapy they are classified as standard risk. In contrast, the rare variant t(17;19)(q22;p13)/*HLF-TCF3* fusion has a dismal outcome (Pui C.H., *et al.*, 2006). Translocations involving *IGH@* at 14q32 are emerging as a significant subgroup in childhood ALL (Pui C.H., *et al.*, 2006; Campana D. 2009; Stow P., 2010; Yang J.J., *et al.*, 2008). It is of interest that they occur more frequently in adolescents and, although numbers are small, they appear to have an inferior outcome.

Intra-chromosomal amplification of chromosome 21 (iAMP21) consists of an abnormal chromosome 21 with highly variable morphology between patients and with a common amplified region that in all cases includes the *RUNX1* gene (Pui C.H., *et al.*, 2006; Campana D. 2009; Stow P., *et al.*, 2010). This abnormality was originally described as poor risk factor (Pui C.H., *et al.*, 2006; Campana D., 2009; Stow P., *et al.*, 2010; Yang J.J., *et al.*, 2008), although the outcome has been shown to be protocol dependent (Pui C.H., *et al.*, 2006). Other numerical cytogenetic abnormalities include: high hyperdiploidy (51–65 chromosomes) (Pui C.H., *et al.*, 2006), near-haploidy (24–29 chromosomes) and low hypodiploidy (31–39 chromosomes) (Pui C.H., *et al.*, 2005; Campana D., 2009). High hyperdiploidy accounts for approximately 30% of childhood BCP-ALL and is characterized by the gain of specific chromosomes. It is associated with a good prognosis in children. Near-haploidy and low hypodiploidy are rare, comprising <1% of childhood ALL. Their characteristic features are the gain of specific chromosomes onto the haploid chromosome set and, in the majority of patients, the presence of a population of cells with an exact doubling of this chromosome number (Pui C.H., *et al.*, 2006). Both are linked to a poor outcome and are used to stratify patients as high risk.

I. REFERENCES

Anderson K.A., Ribar T.J., Lin F., Noeldner P.K., Green M.F., Muehlbauer M.J., Witters L.A., Kemp B.E., Means A.R. "Hypothalamic CaMKK2 contributes to the regulation of energy balance". Cell. Metab. 2008, 7:377–88.

Armstrong S.A., Golub T.R., Korsmeyer S.J. "MLL-rearranged leukemias: insights from gene expression profiling". Semin. Hematol. 2003, 40:268-73.

Bacher U., Kern W., Schnittger S., Hiddemann W., Haferlach T., Schoch C. "Population-based age-specific incidences of cytogenetic subgroups of acute myeloid leukemia". Haematologica. 2005, 90:1502-10.

Barb D., Williams C.J., Neuwirth A.K., Mantzoros C.S. "Adiponectin in relation to malignancies: a review of existing basic research and clinical evidence". Am. J. Clin. Nutr. 2007, 86:S858–S866.

Baron A., Migita T., Tang D., Loda M. "Fatty acid synthase: a metabolic oncogene in prostate cancer?" J. Cell. Biochem. 2004, 91:47–53.

Baumann P., Mandl-Weber S., Emmerich B., Straka C., Schmidmaier R. "Inhibition of adenosine monophosphate-activated protein kinase induces apoptosis in multiple myeloma cells". Anticancer Drugs. 2007, 18: 405-410.

Blagih J., Krawczyk C.M., Jones R.G. "LKB1 and AMPK: central regulators of lymphocyte metabolism and function". Immunol. Rev. 2012, 249:59-71.

Borkhardt A., Wuchter C., Viehmann S., Pils S., Teigler-Schlegel A., Stanulla M., Zimmermann M., Ludwig W.D., Janka-Schaub G., Schrappe M., Harbott J. "Infant acute lymphoblastic leukemia - combined cytogenetic, immunophenotypical and molecular analysis of 77 cases". Leukemia. 2002, 16:1685-90.

Bowker S.L., Majumdar S.R., Veugelers P., Johnson J.A. "Increased cancer-related mortality for patients with Type 2 diabetes who use sulfonylureas or insulin: response to Farooki and Schneider". Diabetes Care. 2006, 29:1990–1991.

Budanov A.V. and Karin M. "p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell. 2008;134(3):451–460.

Buzzai M., Jones R.G., Amaravadi R.K., Lum J.J., DeBerardinis R.J., Zhao F., Viollet B., Thompson C.B. "Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth". Cancer Res. 2007, 67:6745–6752.

Byekova Y.A., Herrmann J.L., Xu J., Elmets C.A., Athar M. "Liver kinase B1 (LKB1) in the pathogenesis of UVB-induced murine basal cell carcinoma". Arch. Biochem. Biophys. 2011, 508: 204-211.

Campana D. "Minimal residual disease in acute lymphoblastic leukemia". Semin. Hematol. 2009, 46:100–6.

Cancer Genome Atlas Research Network. "Comprehensive genomic characterization defines human glioblastoma genes and core pathways". Nature. 2008, 455:1061–1068.

Carling D., Sanders M.J., Woods A. "The regulation of AMP-activated protein kinase by upstream kinases". Int. J. Obes. (Lond) 2008, 32:S55–9.

Carretero J., Medina P.P., Blanco R., Smit L., Tang M., Roncador G., Maestre L., Conde E., Lopez-Rios F., Clevers H.C., Sanchez-Cespedes M. "Dysfunctional AMPK activity, signalling through

mTOR and survival in response to energetic stress in LKB1-deficient lung cancer". Oncogene. 2007, 26:1616–25.

Chen Z.P., Mitchelhill K.I., Michell B.J., Stapleton D., Rodriguez-Crespo I., Witters L.A., Power D.A., Ortiz de Montellano P.R., Kemp B.E. "AMP-activated protein kinase phosphorylation of endothelial NO synthase". FEBS Lett. 1999, 443: 285–289

Choi B.M., Pae H.O., Jang S.I., Kim Y.M., Chung H.T. "Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator". J. Biochem. Mol. Biol. 2002, 35: 116–126.

Costanzo-Garvey D.L., Pfluger P.T., Dougherty M.K., Stock J.L., Boehm M., Chaika O., Fernandez M.R., Fisher K., Kortum R.L., Hong E.G., Jun J.Y., Ko H.J., Schreiner A., Volle D.J., Treece T., Swift A.L., Winer M., Chen D., Wu M., Leon L.R., Shaw A.S., McNeish J., Kim J.K., Morrison D.K., Tschöp M.H., Lewis R.E. "KSR2 is an essential regulator of AMP kinase, energy expenditure, and insulin sensitivity". Cell. Metab. 2009, 10:366–378.

Davies S.P., Helps N.R., Cohen P.T., Hardie D.G. "5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine proteinphosphatase-2AC". FEBS Lett. 1995, 377:421-5.

Dowling R.J., Zakikhani M., Fantus I.G., Pollak M., Sonenberg N. "Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells". Cancer Res. 2007, 67:10804–10812.

Dougherty M.K., Ritt D.A., Zhou M., Specht S.I., Monson D.M., Veenstra T.D., Morrison D.K. "KSR2 is a calcineurin substrate that promotes ERK cascade activation in response to calcium signals". Mol. Cell. 2009, 34:652–662.

Drew B.G., Fidge N.H., Gallon-Beaumier G., Kemp B.E., Kingwell B.A. "Highdensity lipoprotein and apolipoprotein AI increase endothelial NO synthase activity by protein association and multisite phosphorylation". Proc. Natl. Acad. Sci. USA. 2004, 101: 6999–7004.

Evans J.M., Donnelly L.A., Emslie-Smith A.M., Alessi D.R., Morris A.D. "Metformin and reduced risk of cancer in diabetic patients". BMJ. 2005, 330:1304–1305.

Farwell W.R., Scranton R.E., Lawler E.V., Lew R.A., Brophy M.T., Fiore L.D., Gaziano J.M. "The association between statins and cancer incidence in a veterans population". J. Natl. Cancer. Inst. 2008, 100:134–139.

Feng Z., Hu W., de Stanchina E., Teresky A.K., Jin S., Lowe S., Levine A.J. "The regulation of AMPK β1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways". Cancer Res. 2007, 67:3043–3053.

Fogarty S. and Hardie D.G. "Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer". Biochim. Biophys. Acta. 2010, 1804: 581-591.

Gilliland D.G., Jordan C.T., Felix C.A. "The molecular basis of leukemia". Hematology (Am. Soc. Hematol. Educ. Program) 2004, 80-97.

Green A.S., Chapuis N., Maciel T.T., Willems L., Lambert M., Arnoult C., Boyer O., Bardet V., Park S., Foretz M., Viollet B., Ifrah N., Dreyfus F., Hermine O., Moura I.C., Lacombe C., Mayeux P., Bouscary D., Tamburini J. "The LKB1/AMPK signaling pathway has tumor suppressor activity in acute myeloid leukemia through the repression of mTOR-dependent oncogenic mRNA translation". Blood. 2010, 116: 4262-4273

Grossmann M.E., Nkhata K.J., Mizuno N.K., Ray A., Cleary M.P. "Effects of adiponectin on breast cancer cell growth and signaling". Br. J. Cancer. 2008, 98:370–379.

Guertin D.A. and Sabatini D.M. “Defining the role of mTOR in cancer”. Cancer Cell. 2007, 12:9–22.

Gwinn D.M., Shackelford D.B., Egan D.F., Mihaylova M.M., Mery A., Vasquez D.S., Turk B.E., Shaw R.J. “AMPK phosphorylation of raptor mediates a metabolic checkpoint”. Mol. Cell. 2008, 30:214–26.

Hahn-Windgassen A., Nogueira V., Chen C.C., Skeen J.E., Sonenberg N., Hay N. “Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity”. J. Biol. Chem. 2005, 280:32081–9.

Hardie D.G. “AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy”. Nat. Rev. Mol. Cell. Biol. 2007, 8:774–785.

Hawley S.A., Boudeau J., Reid J.L., Mustard K.J., Udd L., Mäkelä T.P., Alessi D.R., Hardie D.G. “Complexes between the LKB1 tumor suppressor, STRADalpha/beta and MO25alpha/beta are upstream kinases in the AMP-activated protein kinase cascade”. J. Biol. 2003, 2:28.

Hawley S.A., Pan D.A., Mustard K.J, Ross L., Bain J., Edelman A.M., Frenguelli B.G., Hardie D.G. “Calmodulin-dependent protein kinase kinase-β is an alternative upstream kinase for AMP-activated protein kinase”. Cell. Metab. 2005, 2:9–19.

Hemminki A., Avizienyte E., Roth S., Loukola A., Aaltonen L.A., Järvinen H., de la Chapelle A. “A serine/threonine kinase gene defective in Peutz-Jeghers syndrome”. Nature. 1998, 391:184–7.

Herrero-Martin G., Hoyer-Hansen M., Garcia-Garcia C., Fumarola C., Farkas T., Lopez-Rivas A., Jäättelä M. “TAK1 activates AMPK-dependent cytoprotective autophagy in TRAIL-treated epithelial cells”. EMBO J. 2009, 28:677–685.

Hemminki A. “The molecular basis and clinical aspects of Peutz-Jeghers syndrome”. Cell. Mol. Life Sci. 1999, 55: 735–50.

Hilden J.M., Dinndorf P.A., Meerbaum S.O., Sather H., Villaluna D., Heerema N.A., McGlennen R., Smith F.O., Woods W.G., Salzer W.L., Johnstone H.S., Dreyer Z., Reaman G.H., Children's Oncology Group. “Analysis of prognostic factors of acute lymphoblastic leukemia in infants: report on CCG 1953 from the Children's Oncology Group”. Blood. 2006, 108:441-51.

Hjalgrim L.L., Rostgaard K., Schmiegelow K., Söderhäll S., Kolmannskog S., Vettenranta K., Kristinsson J., Clausen N., Melbye M, Hjalgrim H., Gustafsson G. “Age- and sex-specific incidence of childhood leukemia by immunophenotype in the Nordic countries”. J. Natl. Cancer Inst. 2003, 95:1539–1544.

Holz M.K., Ballif B.A., Gygi S.P., Blenis J. “mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events”. Cell. 2005, 123:569–80.

Hong S.P., Leiper F.C., Woods A., Carling D., Carlson M. “Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases”. Proc. Natl. Acad. Sci. USA. 2003, 100:8839–43.

Huang J. and Manning B.D. “The TSC1-TSC2 complex: a molecular switchboard controlling cell growth”. Biochem. J. 2008, 412:179–90.

Hunger S.P., Sklar J., Link M.P. “Acute lymphoblastic leukemia occurring as a second malignant neoplasm in childhood: report of three cases and review of the literature”. J. Clin. Oncol. 1992, 10:156-63.

Hwang J.T., Lee M., Jung S.N., Lee H.J., Kang I., Kim S.S., Ha J. “AMP-activated protein kinase activity is required for vanadate-induced hypoxia-inducible factor 1α expression in DU145 cells”. Carcinogenesis. 2004, 25:2497–2507.

Imamura K., Ogura T., Kishimoto A., Kaminishi M., Esumi H. “Cell cycle regulation via p53 phosphorylation by a 5′-AMP activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, in a human hepatocellular carcinoma cell line”. Biochem. Biophys. Res. Commun. 2001, 287:562–567.

Inoki K., Zhu T., Guan K.L. “TSC2 mediates cellular energy response to control cell growth and survival”. Cell. 2003, 115:577–90.

Inoki K., Ouyang H., Zhu T., Lindvall C., Wang Y., Zhang X., Yang Q., Bennett C., Harada Y., Stankunas K., Wang C.Y., He X., MacDougald O.A., You M., Williams B.O., Guan K.L. “TSC2 Integrates Wnt and Energy Signals via a Coordinated Phosphorylation by AMPK and GSK3 to Regulate Cell Growth”. Cell. 2006, 126:955–68.

Jenne D.E., Reimann H., Nezu J., Friedel W., Loff S., Jeschke R., Muller O.,Back W., Zimmer M. “Peutz-Jeghers syndrome is caused by mutations in a novel serine threonine kinase”. Nat. Genet. 1998, 18: 38–43.

Jeon S.M., Chandel N.S., Hay N. “AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress”. Nature. 2012; 485:661–665.

Ji H., Ramsey M.R., Hayes D.N., Fan C., McNamara K., Kozlowski P., Torrice C., Wu M.C., Shimamura T., Perera S.A., Liang M.C., Cai D., Naumov G.N., Bao L., Contreras C.M., Li D., Chen L., Krishnamurthy J., Koivunen J., Chirieac L.R., Padera R.F., Bronson R.T., Lindeman N.I., Christiani D.C., Lin X., Shapiro G.I., Jänne P.A., Johnson B.E., Meyerson M., Kwiatkowski D.J., Castrillon D.H., Bardeesy N., Sharpless N.E., Wong K.K. “LKB1 modulates lung cancer differentiation and metastasis”. Nature. 2007, 448:807–10.

Jiang W., Zhu Z., Thompson H.J. “Dietary energy restriction modulates the activity of AMP-activated protein kinase, Akt, and mammalian target of rapamycin in mammary carcinomas, mammary gland, and liver”. Cancer Res. 2008, 68:5492–5499.

Jones R.G., Plas D.R., Kubek S, Buzzai M., Mu J., Xu Y., Birnbaum M.J., Thompson C.B. “AMP-activated protein kinase induces a p53-dependent metabolic checkpoint”. Mol. Cell. 2005, 18:283–293.

Jones S., Zhang X., Parsons D.W., Lin J.C., Leary R.J., Angenendt P., Mankoo P., Carter H., Kamiyama H., Jimeno A., Hong S.M., Fu B., Lin M.T., Calhoun E.S., Kamiyama M., Walter K., Nikolskaya T., Nikolsky Y., Hartigan J., Smith D.R., Hidalgo M., Leach S.D., Klein A.P., Jaffee E.M., Goggins M., Maitra A., Iacobuzio-Donahue C., Eshleman J.R., Kern S.E., Hruban R.H., Karchin R., Papadopoulos N., Parmigiani G., Vogelstein B., Velculescu V.E., Kinzler K.W. “Core signaling pathways in human pancreatic cancers revealed by global genomic analyses”. Science. 2008, 321:1801–1806.

Katz M.S. “Therapy insight: potential of statins for cancer chemoprevention and therapy”. Nat. Clin. Pract. Oncol. 2005, 2:82–89.

Kim K.Y., Baek A., Hwang J.E., Choi Y.A., Jeong J., Lee M.S., Cho D.H., Lim J.S., Kim K.I., Yang Y. “Adiponectin-activated AMPK stimulates dephosphorylation of AKT through protein phosphatase 2A activation”. Cancer Res. 2009, 69:4018–4026.

Kortum R.L. and Lewis R.E. “The molecular scaffold KSR1 regulates the proliferative and oncogenic potential of cells”. Mol. Cell. Biol. 2004, 24:4407–4416.

Kottakis F. and Bardeesy N. “LKB1-AMPK axis revisited”. Cell Research. 2012, 12: 1617-20.

Kuhajda F.P. “Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology”. Nutrition. 2000, 16:202–208.

Kuznetsov J.N., Leclerc G.J., Leclerc G.M., Barredo J.C. “AMPK and Akt Determine Apoptotic Cell Death following Perturbations of One-Carbon Metabolism by Regulating ER Stress in Acute Lymphoblastic Leukemia”. Mol. Cancer Ther. 2011, 10: 437-447.

Lee J.H., Koh H., Kim M., Kim Y., Lee S.Y., Karess R.E., Lee S.H., Shong M., Kim J.M., Kim J., Chung J. “Energy-dependent regulation of cell structure by AMP-activated protein kinase”. Nature. 2007, 447: 1017-1020.

Lee M., Hwang J.T., Yun H., Kim E.J., Kim M.J., Kim S.S., Ha J. “Critical roles of AMP-activated protein kinase in the carcinogenic metal-induced expression of VEGF and HIF-1 proteins in DU145 prostate carcinoma”. Biochem. Pharmacol. 2006, 72:91–103.

Libby G., Donnelly L.A., Donnan P.T., Alessi D.R., Morris A.D., Evans J.M. “New users of metformin are at low risk of incident cancer: a cohort study among people with Type 2 diabetes”. Diabetes Care. 2009, 32:1620–1625.

Liu B., Fan Z., Edgerton S.M., Deng X.S., Alimova I.N., Lind S.E., Thor A.D. “Metformin induces unique biological and molecular responses in triple negative breast cancer cells”. Cell Cycle. 2009, 8: 2031-2040.

Liu L., Cash T.P., Jones R.G., Keith B., Thompson C.B., Simon M.C. “Hypoxia-induced energy stress regulates mRNA translation and cell growth”. Mol. Cell. 2006, 21:521–31.

Mancini M., Scappaticci D., Cimino G., Nanni M., Derme V., Elia L., Tafuri A., Vignetti M., Vitale A., Cuneo A., Castoldi G., Saglio G., Pane F., Mecucci C., Camera A., Specchia G., Tedeschi A., Di Raimondo F., Fioritoni G., Fabbiano F., Marmont F., Ferrara F., Cascavilla N., Todeschini G., Nobile F., Kropp M.G., Leoni P., Tabilio A., Luppi M., Annino L., Mandelli F., Foà R. “A comprehensive genetic classification of adult acute lymphoblastic leukemia (ALL): analysis of the GIMEMA 0496 protocol”. Blood. 2005, 105:3434-41.

Mannick J.B., Asano K., Izumi K., Kieff E., Stamler J.S. “Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation”. Cell. 1994, 79: 1137–1146

Minokoshi Y., Kim Y.B., Peroni O.D., Fryer L.G., Müller C., Carling D., Kahn B.B. “Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase”. Nature. 2002, 415:339–343.

Minokoshi Y., Alquier T., Furukawa N., Kim Y.B., Lee A., Xue B., Mu J., Foufelle F., Ferré P., Birnbaum M.J., Stuck B.J., Kahn B.B. “AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus”. Nature. 2004, 428:569–574.

Mizrachy-Schwartz S., Cohen N., Klein S., Kravchenko-Balasha N., Levitzki A. “Up-regulation of AMPK in cancer cell lines is mediated through c-Src activation”. J. Biol. Chem. 2011, 286: 15268-15277.

Momcilovic M., Hong S.P., Carlson M. “Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase *in vitro*”. J. Biol. Chem. 2006, 281:25336–2534.

Morrow V.A., Foufelle F., Connell J.M., Petrie J.R., Gould G.W., Salt I.P. “Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells”. J. Biol. Chem. 2003, 278: 31629–31639.

Okoshi R., Ozaki T., Yamamoto H., Ando K., Koida N., Ono S., Koda T., Kamijo T., Nakagawara A., Kizaki H. “Activation of AMPactivated protein kinase induces p53-dependent apoptotic cell death in response to energetic stress”. J. Biol. Chem. 2008, 283: 3979-3987.

Park H.U., Suy S., Danner M., Dailey V., Zhang Y., Li H., Hyduke D.R., Collins B.T., Gagnon G., Kallakury B., Kumar D., Brown M.L., Fornace A., Dritschilo A., Collins S.P.
“AMP-activated protein kinase promotes human prostate cancer cell growth and survival”. Mol. Cancer Ther. 2009, 8: 733- 41.

Pelicano H., Martin D.S., Xu R.H., Huang P. “Glycolysis inhibition for anticancer treatment”. Oncogene. 2006, 25:4633–4646.

Pieters R., den Boer M.L., Durian M., Janka G., Schmiegelow K., Kaspers G.J., van Wering E.R., Veerman A.J. "Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia-implications for treatment of infants". Leukemia. 1998, 12:1344-8.

Pui C.H., Gaynon P.S., Boyett J.M., Chessells J.M., Baruchel A., Kamps W., Silverman L.B., Biondi A., Harms D.O., Vilmer E., Schrappe M., Camitta B. "Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region". Lancet. 2002, 359:1909-15.

Pui C.H., Chessells J.M., Camitta B., Baruchel A., Biondi A., Boyett J.M., Carroll A., Eden O.B., Evans W.E., Gadner H., Harbott J., Harms D.O., Harrison C.J., Harrison P.L., Heerema N., Janka-Schaub G., Kamps W., Masera G., Pullen J., Raimondi S.C., Richards S., Riehm H., Sallan S., Sather H., Shuster J., Silverman L.B., Valsecchi M.G., Vilmer E., Zhou Y., Gaynon P.S., Schrappe M. "Clinical heterogeneity in childhood acute lymphoblastic leukemia with 11q23 rearrangements". Leukemia. 2003, 17:700-6.

Pui C.H., Relling M.V., Downing J.R. "Mechanisms of Disease: acute lymphoblastic leukemia". N. Engl. J. Med. 2004, 350:1535–1548.

Pui CH, Evans WE: Treatment of acute lymphoblastic leukemia. N Engl J Med 2006;354:166–78

Pui C.H. „Recent research advances in childhood acute lymphoblastic leukemia". J. Formos. Med. Assoc. 2010, 109:777-87.

Rattan R., Giri S., Singh A.K., Singh I. "5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside inhibits cancer cell proliferation *in vitro* and *in vivo* via AMP-activated protein kinase". J. Biol. Chem. 2005, 280:39582–39593.

Rowley J.D. and Olney H.J. "International workshop on the relationship of prior therapy to balanced chromosome aberrations in therapy-related myelodysplastic syndromes and acute leukemia: overview report". Genes Chromosomes Cancer. 2002, 33(4):331-45.

Secker-Walker L.M. "General Report on the European Union Concerted Action Workshop on 11q23, London, UK, May 1997". Leukemia. 1998, 12:776-8.

Sengupta T.K., Leclerc G.M., Hsieh-Kinser T.T., Leclerc G.J., Singh I., Barredo J.C. –"Cytotoxic effect of 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) on childhood acute lymphoblastic leukemia (ALL) cells: implication for targeted therapy". Mol. Cancer. 2007, 6: 46.

Shackelford D.B. and Shaw R.J. "The LKB1-AMPK pathway: metabolism and growth control in tumour suppression". Nat. Rev. Cancer. 2009a, 9:563-75.

Shackelford D.B., Vasquez D.S., Corbeil J., Wu S., Leblanc M., Wu C.L., Vera D.R., Shaw R.J. "mTOR- and HIF-1α mediated tumor metabolism in an LKB1 mouse model of Peutz-Jeghers syndrome". Proc. Natl. Acad. Sci. USA. 2009b, 106:11137-42.

Shaw M.M., Gurr W.K., McCrimmon R.J., Schorderet D.F., Sherwin R.S. "5'AMP-activated protein kinase alpha deficiency enhances stress-induced apoptosis in BHK and PC12 cells". J. Cell. Mol. Med. 2007, 11: 286-29.

Shaw R.J., Kosmatka M., Bardeesy N., Hurley R.L., Witters L.A., DePinho R.A., Cantley L.C. "The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress". Proc. Natl. Acad. Sci. USA. 2004, 101:3329–35.

Shaw R.J., Lamia K.A., Vasquez D., Koo S.H., Bardeesy N., Depinho R.A., Montminy M., Cantley L.C. "The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin". Science. 2005, 310:1642–6.

Shaw R.J. and Cantley L.C. “Ras, PI(3)K and mTOR signalling controls tumour cell growth”. Nature. 2006, 441:424–30.

Shaw R.J. “Glucose metabolism and cancer”. Curr. Opin. Cell. Biol. 2006, 18:598–608.

Stahmann N., Woods A., Carling D., Heller R. “Thrombin activates AMP-activated protein kinase in endothelial cells via a pathway involving Ca2+/calmodulin-dependent protein kinase kinase beta”. Mol. Cell. Biol. 2006, 26:5933–45.

Steinberg G.R. and Kemp B.E. “AMPK in health and Disease”. Physiol. Rev. 2009, 89(3):1025–1078.

Stow P., Key L., Chen X., Pan Q., Neale G.A., Coustan-Smith E., Mullighan C.G., Zhou Y., Pui C.H., Campana D. “Clinical significance of low levels of minimal residual disease at the end of remission induction therapy in childhood acute lymphoblastic leukemia”. Blood. 2010, 115:4657–63.

Suzuki A., Kusakai G., Kishimoto A., Shimojo Y., Ogura T., Lavin M.F., Esumi H. “IGF-1 phosphorylates AMPK-α subunit in ATM-dependent and LKB1-independent manner”. Biochem. Biophys. Res. Commun. 2004, 324:986–992.

Tamas P., Hawley S.A., Clarke R.G., Mustard K.J., Green K., Hardie D.G., Cantrell D.A. “Regulation of the energy sensor AMP-activated protein kinase by antigen receptor and Ca2+ in T lymphocytes”. J. Exp. Med. 2006, 203:1665–70.

Thomsen L.L., Miles D.W., Happerfield L., Bobrow L.G., Knowles R.G., Moncada S. “Nitric oxide synthase activity in human breast cancer”. Br. J. Cancer. 1995, 72: 41–44.

Tiainen M., Ylikorkala A., Makela T.P. “Growth suppression by Lkb1 is mediated by a G(1) cell cycle arrest”. Proc. Natl. Acad. Sci. U.S.A. 1999, 96: 9248–9251.

Vucicevic L., Misirkic M., Janjetovic K., Harhaji-Trajkovic L., Prica M., Stevanovic D., Isenovic E., Sudar E., Sumarac-Dumanovic M., Micic D., Trajkovic V. “AMPactivated protein kinase-dependent and -independent mechanisms underlying in vitro antiglioma action of compound C”. Biochem. Pharmacol. 2009, 77: 1684-1693.

Wang W. and Guan K.L. “AMP-activated protein kinase and cancer”. Acta Physiol. (Oxf). 2009, 196: 55-63.

Warburg O. “On respiratory impairment in cancer cells”. Science. 1956, 124:269–270.

Westerman A.M., Entius M.M., de Baar E., Boor P.P., Koole R., van Velthuysen M.L., Offerhaus G.J., Lindhout D., de Rooij F.W., Wilson J.H. “Peutz-Jeghers syndrome: 78-year follow-up of the original family”. Lancet. 1999, 353: 1211–1215.

Woodard J., Joshi S., Viollet B., Hay N., Platanias L.C. “AMPK as a therapeutic target in renal cell carcinoma”. Cancer Biol. Ther. 2010, 10: 1168-1177.

Woods A., Johnstone S.R., Dickerson K., Leiper F.C., Fryer L.G., Neumann D., Schlattner U., Wallimann T., Carlson M., Carling D. “LKB1 is the upstream kinase in the AMP-activated protein kinase cascade”. Curr. Biol. 2003, 13:2004–8.

Woods A., Dickerson K., Heath R., Hong S.P., Momcilovic M., Johnstone S.R., Carlson M., Carling D. “Ca^{2+}/calmodulin-dependent protein kinase kinase-β acts upstream of AMP-activated protein kinase in mammalian cells”. Cell. Metab. 2005, 2:21–33.

Xie M., Zhang D., Dyck J.R., Li Y., Zhang H., Morishima M., Mann D.L., Taffet G.E., Baldini A., Khoury D.S., Schneider M.D. “A pivotal role for endogenous TGF-β-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway”. Proc. Natl. Acad. Sci. USA. 2006, 103:17378–17383.

Xu W., Liu L.Z., Loizidou M., Ahmed M., Charles I.G. “The role of nitric oxide in cancer”. Cell Res. 2002, 12: 311–320.

Yang J.J., Bhojwani D., Yang W., Cai X,, Stocco G., Crews K., Wang J., Morrison D., Devidas M., Hunger S.P., Willman C.L., Raetz E.A., Pui C.H., Evans W.E., Relling M.V., Carroll W.L. “Genome-wide copy number profiling reveals molecular evolution from diagnosis to relapse in childhood acute lymphoblastic leukemia”. Blood. 2008, 112:4178–83.

Zhang B.B., Zhou G., Li C. “AMPK: an emerging drug target for diabetes and the metabolic syndrome”. Cell. Metab. 2009, 9:407–416.

Zhang W.B., Wang Z., Shu F., Jin Y.H., Liu H.Y., Wang Q.J., Yang Y. “Activation of AMP-activated protein kinase by temozolomide contributes to apoptosis in glioblastoma cells via p53 activation and mTORC1 inhibition”. J. Biol. Chem. 2010, 285: 40461-40471.

Zhou G., Myers R., Li Y., Chen Y., Shen X., Fenyk-Melody J., Wu M., Ventre J., Doebber T., Fujii N., Musi N. “Role of AMP-activated protein kinase in mechanism of metformin action”. J. Clin. Invest. 2001, 108:1167–1174.

Zhou J., Huang W., Tao R., Ibaragi S., Lan F., Ido Y., Wu X., Alekseyev Y.O., Lenburg M.E., Hu G.F., Luo Z. “Inactivation of AMPK alters gene expression and promotes growth of prostate cancer cells”. Oncogene. 2009, 28:1993–2002.

Zhou W., Han W.F., Landree L.E., Thupari J.N., Pinn M.L., Bililign T., Kim E.K., Vadlamudi A., Medghalchi S.M., El Meskini R., Ronnett G.V., Townsend C.A., Kuhajda F.P. “Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells”. Cancer Res. 2007, 67:2964–2971.

AIM OF STUDY -CHAPTER I-

Acute Lymphoblastic Leukemia (ALL) is the most common form of pediatric cancer with a worldwide incidence of about 1–4.75 per 100 000 persons. Remarkable progress has been made in treatment of childhood ALL but therapy is not yet effective in all cases. Current research interest focuses on identifying new specific molecular drug targets for new patient-tailored approaches that can improve therapy efficacy and reduce toxicity.

The tyrosine kinases (TK) are attractive targets for anti-neoplastic therapy, as very often the alteration of their signaling can be connected with tumor growth and development, and they could be easily targeted with specific agents. The constitutive activation of TK stimulates multiple signaling pathways responsible for defective DNA repair, apoptosis block and increased cell proliferation.

The aim of Chapter I is to discover new candidate biomarkers and therapeutic targets involved in the leukemic process and, to this aim, to investigate the aberrant activation or expression of proteins involved in cellular signal transductions pathways of leukemic cells.

We will apply the RPPA technology (Reverse Phase Protein Array) to directly quantitatively measure protein pathway function and activation and thus to profile 120 pediatric patients affected by BCP-ALL in order to identify groups with specific molecular aberrations and to identify new targets for therapy.

Correlation of signalling activation with clinical response/follow-up and known genetic information (e.g. gene rearrangements) will enable us to identify new protein pathway biomarkers that could be used for patient stratification and targeted therapy trials.

Results that will be obtained in this part of the study will be subjected to extensive functional validation in order to point out new therapeutic targets and new diagnostic and prognostic biomarkers for pediatric leukemia patients.

"Functional Protein Network Activation Mapping Reveals New Potential Molecular Drug Targets for Poor Prognosis Pediatric BCP-ALL"

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Complete Pdf is attached in Appendix 2.

Abstract

Background. In spite of leukemia therapy improvements obtained over the last decades, therapy is not yet effective in all cases. Current approaches in Acute Lymphoblastic Leukemia (ALL) research focus on identifying new molecular targets to improve outcome for patients with a dismal prognosis. In this light phosphoproteomics seems to hold great promise for the identification of proteins suitable for targeted therapy.

Methodology/Principal Findings. We employed Reverse Phase Protein Microarrays to identify aberrantly activated proteins in 118 pediatric B-cell precursor (BCP)-ALL patients. Signal transduction pathways were assayed for activation/expression status of 92 key signalling proteins. We observed an increased activation/expression of several pathways involved in cell proliferation in poor clinical prognosis patients. MLL-rearranged tumours revealed BCL-2 hyperphosphorylation through AMPK activation, which indicates that AMPK could provide a functional role in inhibiting apoptosis in MLL-rearranged patients, and could be considered as a new potential therapeutic target. Second, in patients with poor clinical response to prednisone we observed the up-modulation of LCK activity with respect to patients with good response. This tyrosine-kinase can be down-modulated with clinically used inhibitors, thus modulating LCK activity could be considered for further studies as a new additional therapy for prednisone-resistant patients. Further we also found an association between high levels of CYCLIN E and relapse incidence. Moreover, CYCLIN E is more expressed in early relapsed patients, who usually show an unfavourable prognosis.

Conclusions/Significance. We conclude that functional protein pathway activation mapping revealed specific deranged signalling networks in BCP-ALL that could be potentially modulated to produce a better clinical outcome for patients resistant to standard-of-care therapies.

Introduction

Acute Lymphoblastic Leukemia (ALL) is the most common form of pediatric cancer with a worldwide incidence of about 1–4.75 per 100000 persons[1]. Remarkable progress has been made in treatment of childhood ALL but therapy is not yet effective in all cases. Current research interest focuses on identifying new specific molecular drug targets for new patient-tailored approaches that can improve therapy efficacy and reduce toxicity. Knowledge of deregulation of cell signalling pathways in cancer that regulate and control cell proliferation, differentiation, survival and death forms the basis for understanding tumour progression. Recent publications have placed elucidation of protein signalling pathways at the central point in the effective treatment of cancer[2,3]. Pathway activation and function is controlled by post-translational modifications, mainly by phosphorylation, underpinned by ongoing activity of protein kinases and phosphatases. Consequently, functional pathway mapping technology that can directly measure the activation state of hundreds of proteins in signalling transduction pathways (STPs), can hold great promise for the identification of altered STPs in tumour cells. Such efforts promise to potentially provide new targets for rational, molecular-targeted drug design and could identify cancer patients that may benefit from the use of specific targeted inhibitors[4,5]. Protein activation status can not be directly analyzed through gene expression profiling, since post-translational modifications, such as phosphorylation, are not predictable from gene expression levels. Here, Reverse Phase Protein Microarray (RPMA) technology had been used to profile the activation state of 92 key molecules in a cohort of 118 newly diagnosed precursor pediatric BCP-ALL patients, in order to identify and map pathway activation changes associated with clinical characteristics. This innovative technique can measure the activation levels/phosphorylation of large numbers of signalling proteins at once from small clinical samples in a very reproducible, precise, sensitive and high-throughput manner. The RPMA format immobilizes in spots dozens of different patient samples on one array and each array is then incubated with a specific antibody, thus a single endpoint is measured and directly compared across multiple samples without introduction of experimental variability. This cutting-edge technology has already been applied with success to profile the cellular STPs activity in several cancers[4-9]. We observed an increase or decrease in activation/phosphorylation state of signalling proteins within specific protein networks in clinical poor prognosis patients cohorts. In particular, here we show the inhibition of the LCK kinase in Prednisone Good Responder (PGR) patients, and a hyperactivated pathway in the MLL-rearranged cohort of patients that leads to BCL-2 activation through LKB1 and AMPK phosphorylation. Moreover, we found a correlation between CYCLIN E expression and

Relapse Free Survival (RFS) rates: patients who show high levels of CYCLIN E expression have a more elevated probability to relapse. These new informations on pediatric BCP-ALL activated protein patterns provided by phosphoproteomic analyses with RPMA will be the start for future functional studies with specific protein inhibitors, in order to point out new drugs for patient tailored therapies.

Results

Correlation between Protein Expression and Clinical Characteristics

We first searched for correlation between protein expression/activation and patients clinical characteristics. In particular we considered the followings: age (1-9 years vs > 9 years), sex, white blood cell count (WBC > vs < of 50 x 109/L), DNA index (1-1.15 vs ≥ 1.16), chromosomal translocations (non-translocated, t(9;22), t(12;21), t(1;19) and MLL rearrangements), Minimal Residual Disease (Low Risk, Medium Risk, High Risk), immunophenotype (Prepre-B, Pre-B, CALL, Prepre-B/CALL) and prednisone response through Wilcoxon tests or two-sample Welch t-tests implemented in multtest package. No correlation was found between protein expression/activation and age, sex, WBC, DNA index, Minimal Residual Disease (MRD) and immunophenotype (data not shown), but we observed differentially activated/expressed proteins in MLL-rearranged vs non-translocated and in Prednisone Good Responder (PGR) vs Prednisone Poor Responder (PPR) patients comparisons.

AMPK Pathway is Hyperactivated in MLL-rearranged Patients

We compared primary leukemia samples isolated from 8 MLL-rearranged patients (5 with t(4;11), 2 with t(9;11), and one with t(11;19)) with 36 patients without known genomic aberrancies. Statistical analysis (Wilcoxon test with Benjamini-Hochberg multiplicity corrections) revealed different expression or activation of 9 proteins between the MLL-rearranged patients and the non-translocated ones. Our results show that 4 proteins were statistically significantly elevated in the MLL-rearranged patients group: CYCLIN E (p= 0.02425), ANNEXIN 2 (p= 0.02910), AMPKβ (S108) (p= 0.02910) and AMPKα (S485) (p= 0.03686). Furthermore a set of 3 more proteins, eNOS/NOS III (S116 – corresponding to S114 in human), LKB1 (S428) and BCL-2 (S70), was found to be differentially activated in the MLL-rearranged cohort using a Global Test analysis (p=0.003) (**Fig. 1A**). These 3 proteins are all known members of the AMPK pathway (see Discussion) and, along with the findings that AMPK itself was activated in the MLL-rearranged cohort,

form the basis of a comprehensive pathway derangement in MLL-rearranged patients (presented schematically in **Fig. 1B**). We thus identified a singular MLL-specific hyperactivated pathway that through AMPK phosphorylation leads to the activation of BCL-2. A heatmap was generated to highlight the relationships between clustering and protein expression levels (**Fig. 1C**).

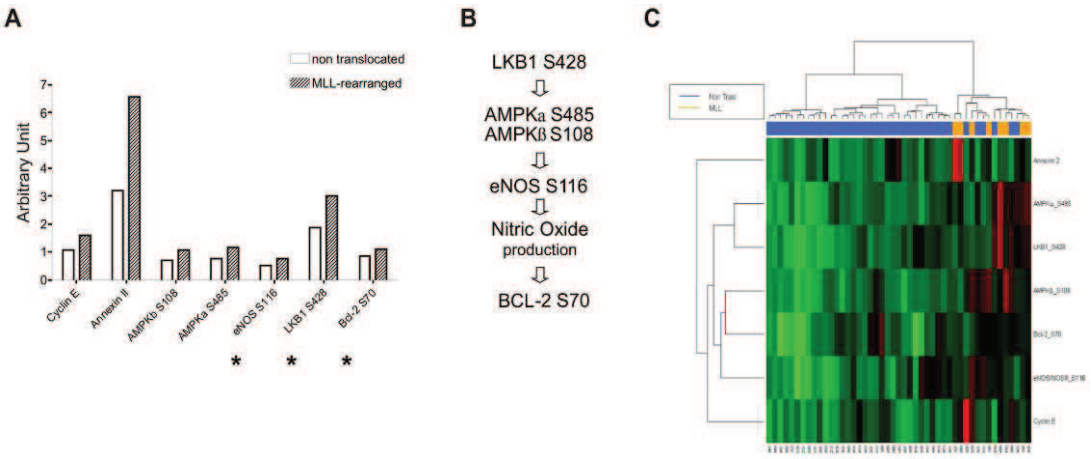

Fig. 1:Hyperactivation of the AMPK pathway in MLL-rearranged patients.
(A) Histogram of proteins, that are part of the AMPK pathway, found to be differentially activated (Wilcoxon testwith Benjamini-Hochberg multiplicity correction, $p<0.05$) between the MLL- and non-translocated cohorts. * indicates proteins that are part of the AMPK pathway, but that did reach statistical significance using Global Test analysis ($p= 0.003$). (B) Scheme of the AMPK pathway. (C) Heatmap with hierarchical clustering. The heatmap was generated with R Project using the proteins differentially expressed/phosphorylated in the "MLL-rearranged patients" group vs "non-translocated patients" group comparison. MLL-rearranged patients are highlighted in orange.

RPMA results were validated by Western Blot in an independent set of patients (**Fig. 2A**). Of note, total forms of the AMPK pathway proteins do not show substantial differences between MLL-rearranged and non-translocated patients (**Fig. 2B**), corroborating the observation that the higher phosphorylation levels of the proteins in the AMPK pathway are the peculiar molecular derangement characteristic of MLL-rearranged BCP-ALL.

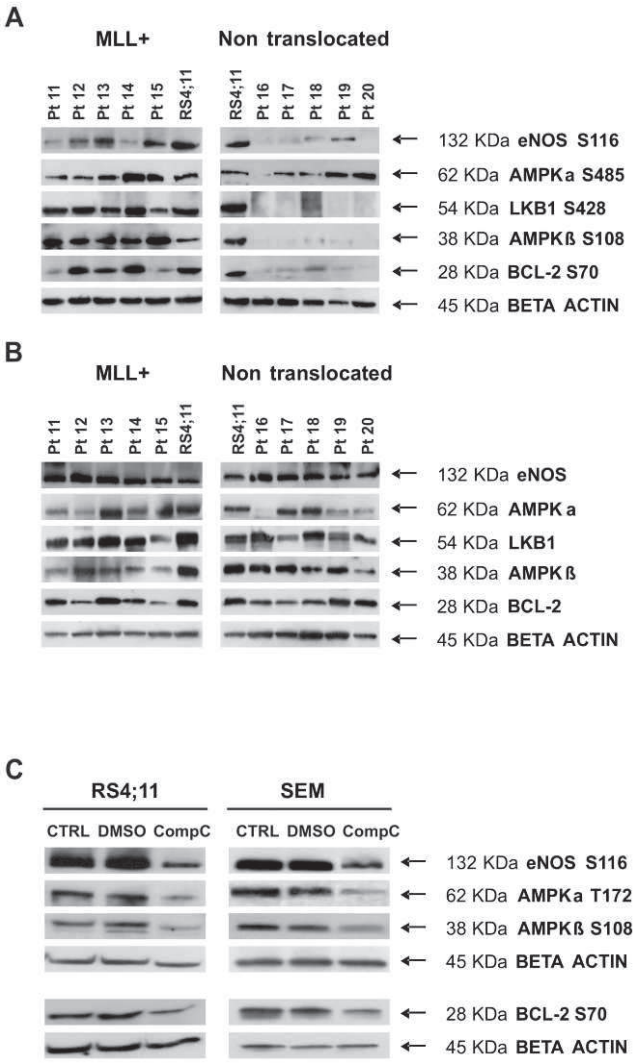

Fig. 2:Validation of RPMA results through Western Blot.
(A) Hyperactivation of the AMPK pathway in MLL-rearranged patients vs non-translocated ones (independent sets of pediatric BCP-ALL at diagnosis: patients 11-15 are MLL-rearranged -all MLL-AF4-, patients 16-20 are non-translocated). RS4;11 cell lysate was used as positive control for antibody staining. (B) Total forms of the AMPK pathway proteins in previously described patients: 11-15 are MLL-rearranged and 16-20 are non-rearranged. RS4;11 cell lysate was used as positive control for antibody staining. There are no substantial differences on total protein form levels between MLL-rearranged and non-translocated patients. (C) AMPK pathway inhibition after Compound C treatment. RS4;11 and SEM cells (both MLL-rearranged) were treated with the AMPK inhibitor Compound C 8μM for 48 hours. Phosphorylation of AMPK pathway proteins was evaluated through WB in control, DMSO treated and Compound C treated cells.

Additionally, we asked whether it was possible to identify a difference in the gene expression levels of the same AMPK-related genes that we identified through RPMA analysis among MLL-rearranged and non-translocated patients. We analyzed the gene expression profiles of 29 MLL-rearranged and 41 non-translocated pediatric BCP-ALL patients. All the patients included in this analysis were part of a larger cohort of samples analyzed by gene expression profiling during the international "Microarray Innovation in Leukemia" (MILE) study[10].

The unsupervised analysis with the 15 probe sets corresponding to Lkb1, Ampkα and β, eNos and Bcl-2 genes was not able to accurately separate MLL-rearranged and non-translocated patients (**Fig. S1A**). Moreover, when performing a comparative analysis between MLL-rearranged and non-translocated patients using the 15 probe sets of the AMPK-related genes, only one probe set (PRKAA 214917_at, corresponding to Ampkα) resulted to be differentially expressed between the two groups with a fold change more than 2.0 (**Fig. S2B**). It is of note that this probe set resulted to be upregulated in the non-translocated group of patients.

In order to verify the relationships kinase-substrate within the AMPK pathway, we treated the two MLL-rearranged cell lines SEM and RS4;11 with the commercial AMPK inhibitor Compound C. As shown in **Fig 2C**, after AMPK inhibition the activation levels of AMPKα and β and all the downstream targets are markedly decreased, confirming the functional link between these proteins. In addition, apoptosis is induced in MLL-rearranged cell lines after Compound C treatment (LC50 8μM, 48h), while other two human non-translocated BCP-ALL cell lines are insensitive to AMPK inhibition (data not shown).

Our data provide evidence that in MLL-rearranged patients a number of directly connected kinase-substrates are activated, and this can contribute to the chemotherapy resistance observed in these patients.

LCK Activity is Down-Modulated in Prednisone Good Responder Patients

We compared the phosphoproteomic profile of 9 Prednisone Poor Responder (PPR) patients vs 109 Prednisone Good Responder (PGR) patients. Statistical analyses (two-sample Welch t-statistics -unequal variances- with Bonferroni multiplicity corrections) revealed that in PGR patients the inhibited form of the kinase LCK (LCK phosphorylated at Y505) was higher than in PPR patients (p=0.0388) (**Fig. 3A**). We confirmed the overexpression of the inhibited form of LCK in PGR patients by Western Blot in an independent set of patients (**Fig.3B**). In order to provide biochemical evidence of a hyperactivation state of LCK in PPR cells compared to PGR cells, we performed radioactive autophosphorylation assay of LCK. To this purpose we selected REH cells as a model for PPR patients and NALM6 cells as a model for PGR patients[11]. We first confirmed that LCK is more phosphorylated in Y505 in NALM6 cells than in REH cells (**Fig. 3C**): when similar amounts of total LCK were analyzed, the LCK Y505 phosphorylation was evident almost exclusively in NALM6 cells. As expected, on the contrary, the autophosphorylation activity of immunoprecipitated LCK was significantly higher in REH cells (**Fig. 3D**).

This tyrosine-kinase, important in the regulation of growth and differentiation of eukaryotic cells, resulted to be more activated in cells that do not respond to glucocorticoids treatment, and thus activation of LCK could be considered as a putative marker for prednisone resistance.

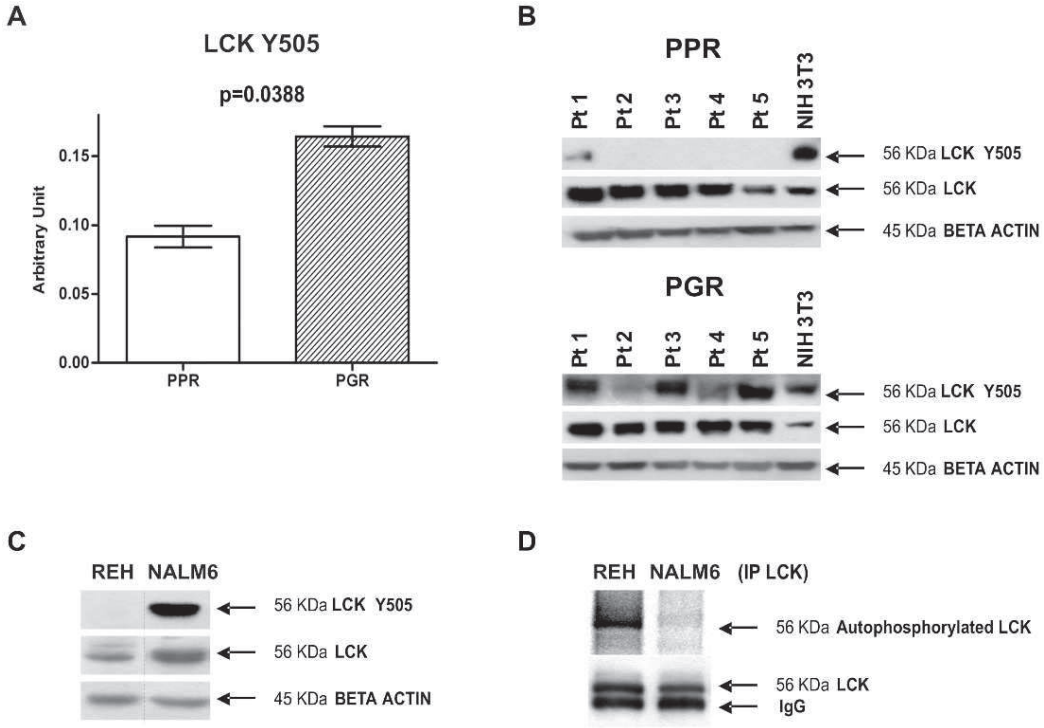

Fig. 3:Hyperactivation of LCK in PPR patients.
(A) LCK Y505 expression measured with RPMA is higher in Prednisone Good Responder (PGR) (0.164 ± 0.007) than in Prednisone Poor Responder (PPR) (0.092 ± 0.008) patients (two-sample Welch t-statistics -unequal variances- with Bonferroni multiplicity corrections p=0.0388). (B) LCK hyperphosphorylation at Y505 in PGR patients was confirmed by Western Blot in an independent set of specimens (pediatric BCP-ALL at diagnosis: patients 1-5 are Prednisone Good Responders, patients 6-10 are Prednisone Poor Responders). NIH3T3 commercial cell lysate (BD Biosciences) was used as positive control for antibody staining. Expression of the total form of LCK does not differ between the patients. (C) LCK activation state in the cell lines chosen as in vitro model. REH cell lines are a model for PPR patients, while NALM6 are a model for PPR patients. The LCK activation state was evaluated through WB. (D) In vitro LCK autophosphorylation assay. LCK was immunoprecipitated from REH and NALM6 cells, and then incubated in a phosphorylation mixture at 30°C for 40min. Autophosphorylation of LCK was analyzed by digital autoradiography on Cyclone Plus (upper gel), and by WB with anti-LCK-antibody to assess the LCK amount (lower gel).

CYCLIN E is Upregulated in Relapsed Patients

We performed a Relapse Free Survival (RFS) analysis considering patients included in AIEOP LLA 88, 91, 95 and 2000 therapy protocols in order to identify proteins related to a more aggressive disease. Relapse Cumulative Incidence were obtained through Kaplan-Meier estimates and the difference between two curves was assessed by log rank test. CYCLIN E, that regulates cell cycle steps critical for growth control, had been demonstrated to be overexpressed in many malignant tumors (for a review see Möröy *et al.*[12]), and in particular it could be useful to assess malignancy of blasts in adult B- and T-ALL[13]. Similarly to van Rhenen *et al.*[14] we thus searched for a threshold value for CYCLIN E that resulted in the largest difference in survival between the two groups defined by that threshold. We considered 10 equispaced cutoff values between the first quartile and the third quartile. A threshold was deemed valid only if the difference in the Relapse Cumulative Incidence curves was statistically significant (CYCLIN E threshold 1.228, log rank test after Bonferroni corrections p=0.000075) (**Fig. 4A**). We found that patients with CYCLIN E levels higher than 1.228 have a higher probability to relapse: 15 of 28 (53%) patients with CYCLIN E higher than 1.228 relapsed, while among the 84 patients with CYCLIN E lower than 1.228 only 14 (17%) relapsed. We confirmed RPMA CYCLIN E levels by Western Blot (**Fig. 4B**). Moreover, we did observe that 6 of 7 MLL-rearranged patients and 3 of 3 patients with t(9;22) are in the high CYCLIN E expression group. Furthermore, 16 of 19 patients MRD-Standard Risk have low levels of CYCLIN E, and 4 of 5 patients MRD-High Risk have high levels of CYCLIN E. In the groups determined by the above threshold we searched for differences in age, sex, immunophenotype, chromosomal translocations, DNA index, WBC count, prednisone response and MRD. From this multivariate analysis, CYCLIN E expression resulted independent from all other considered variables. We also compared CYCLIN E expression in early vs late relapsed patients. By definition, early relapsed patients are those patients who suffered of a relapse within 6 months after stopping front-line treatment, and they show a worst prognosis with respect to late relapsed patients[15]. CYCLIN E resulted significantly higher in early relapsed patients (Wilcoxon test p=0.002) **(Fig. 4C**). Because CYCLIN E in conjunction with its kinase subunit CDK2 regulates essential processes for entering into the S-phase, we also looked if differences between relapsed and non-relapsed patients could be associated to CYCLIN E together with CDK2. After Global Test analysis, the two proteins resulted to be correlated (p=0.009395) in our patients cohort. These observations taken together strongly suggest that CYCLIN E could be considered as a marker for the aggressiveness of the disease.

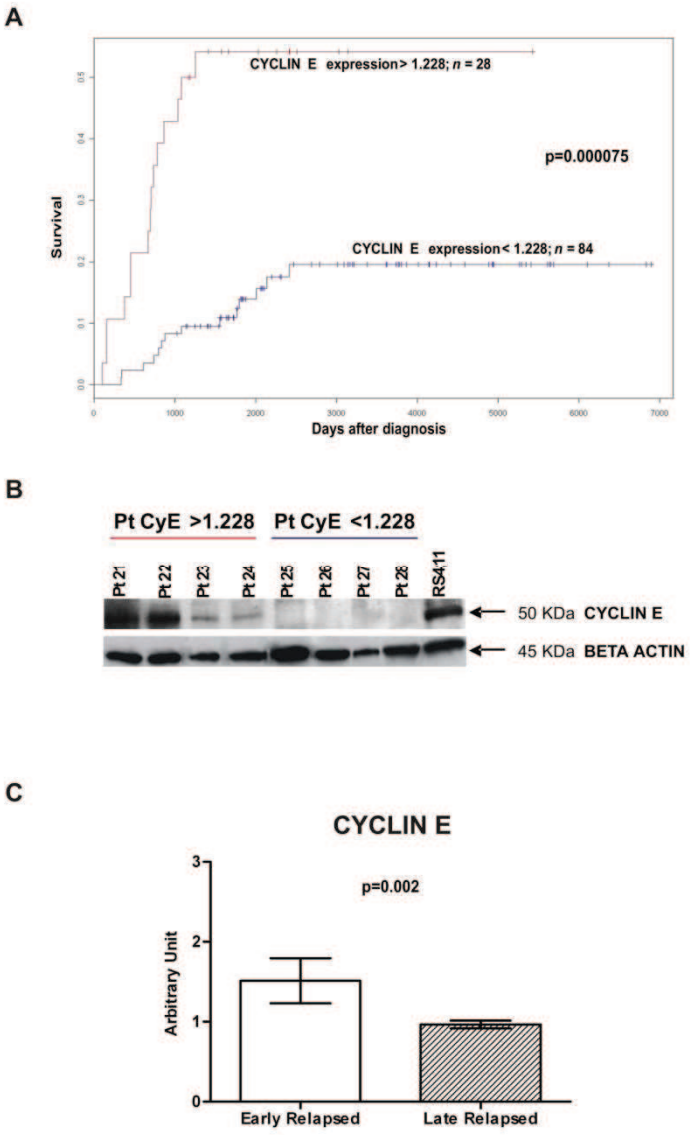

Fig. 4:CYCLIN E expression and Relapse Free Survival.
(A) Relapse Cumulative Incidence comparison between patients with CYCLIN E expression levels < 1.228 (n=84, blue line) and patients with CYCLIN E levels > 1.228 (n=28, red line). Patients with elevated CYCLIN E levels have a higher probability to relapse (CYCLIN E threshold 1.228, log rank test after Bonferroni corrections p=0.000075). (B) RPMA results validation with Western Blot. Patients 21-24 showed high levels of CYCLIN E after RPMA analysis: 1.974, 1.896, 1.537, 1.506 respectively. Patients 25-28 showed low levels of CYCLIN E after RPMA analysis: 0.463, 0.470, 0.298, 0.292 respectively. RS4;11 cell lysate was used as positive control for antibody staining. (C) Early relapsed patients (relapsed within 910 days from diagnosis) show higher levels of CYCLIN E expression with respect to late relapsed patients (Wilcoxon test p=0.002).

Discussion

Despite dramatic improvements in leukemia therapy over the last decades, about 20% of patients does not achieve a stable complete disease remission. In the past few months, new findings using genome-wide mutational analysis have placed central importance on protein pathway deregulation as the principal driving force in many human malignancies[2,3]. We have originated the RPMA technology to directly quantitatively measure protein pathway function and activation to broadly map signal transduction networks and profile human cancers in order to identify groups of patients with specific molecular aberrations and to identify new targets for therapy. In this study we used the RPMA global pathway mapping technique in pediatric BCP-ALL patients to map the pathway activation status of 92 different phosphorylated and total proteins of key signalling "hubs" known to be involved in human tumorigenesis and metastasis. Correlations of signalling activation with clinical response/follow-up and known genetic information (e.g. gene rearrangements), enabled us to identify new protein pathway biomarkers that, when validated in larger clinical sets, could be used for patient stratification and targeted therapy trials.

Our first main finding concerns infants with MLL (Mixed Lineage Leukemia) rearrangements. MLL translocations are present in about 6% of pediatric leukaemia patients, especially in infants with ALL where about 75% of patients are MLL-rearranged, and their presence predicts early relapse and poor prognosis (event-free survival of 28–45%)[16,17]. We identified a singular MLL-specific hyperactivated pathway that through AMPK phosphorylation leads to the activation of BCL-2, a well known anti-apoptotic regulator crucial for chemotherapy resistance already found to be over-expressed both at mRNA and protein levels in MLL-rearranged leukemias[18]. This pathway derangements appear to emanate from LKB1, a serine/threonine kinase that has been shown to phosphorylate AMPK[19]. AMP-activated protein kinase (AMPK) is a serine/threonine kinase that acts as a cellular fuel sensor, activated under conditions that deplete ATP and elevate AMP levels such as metabolic or environmental stresses. AMPK is known to activate endothelial nitric oxide synthase (eNOS)[20,21] and thus to stimulate Nitric Oxide (NO) production. NO is a multifunctional transcellular messenger that can play a dual role in cancer with both pro- and anti-apoptotic effects[22,23]. Interestingly, a prominent NO production has been observed in undifferentiated tumours[24] such as the MLL-rearranged leukemias. In addition, it has been reported that NO can prevent apoptosis by elevating BCL-2 expression both at mRNA and protein levels in B-lymphocytes[25,26]. We showed that treatment of two MLL-rearranged cell lines with the AMPK inhibitor Compound C not only brings to AMPK deactivation, but also of its described downstream targets. This

confirms the kinase-substrate relationship between these proteins, and highlights the essential contribute of AMPK in sustaining the activation of this pathway. Activation of AMPK had been studied in several tumour types because it usually leads to antitumor effects[27], but we found AMPK phosphorylation to be at the highest relative levels in the MLL-rearranged subgroup. We speculate that the role of AMPK pathway in leukemias and other hematological tumours may be different from solid epithelial malignancies, as already reported by Baumann *et al.*[28] in multiple myeloma cells. Indeed, it will be of primary interest to inhibit AMPK, and thus BCL-2, activity using commercially available AMPK inhibitors in order to more fully elucidate the functional role of AMPK activation in MLL-rearranged patients, and to evaluate AMPK as a potential new therapeutic target for this specific subgroup of patients. The mRNA results, indicating that the AMPK pathway genes are not upregulated in MLL-rearranged patients, sustain the importance to deepen with protein activation analyses in order to better define disease-related disorders in cellular metabolism, and thus to identify new molecular drug targets.

One of the strongest independent predictive factors for therapy outcome in childhood ALL is the response to initial prednisone treatment. Prednisone response is considered a backbone in Berlin-Frankfurt-Münster (BFM)-oriented protocols, and is defined by the number of peripheral leukemic blasts on day 8 of therapy[29,30]. The threshold value for distinction between good and poor response is 1000 blasts/µl. In trial ALL-BFM 90, PGR patients showed a 6-years Event Free Survival (EFS) of 82%, while for PPR patients this was only 34%[31]. Here, we observed that in PGR patients LCK is less activated than in PPR patients (LCK Y505, p=0.0388), and we exploited the autophosphorylation assay of LCK in order to biochemically confirm its higher activity in PPR cells. LCK is a non receptor protein-tyrosine kinase of the Src oncogene family mostly expressed in T cells, where plays an essential role in activation and development, and in some B cells and other cancer tissues[32,33]. Its activity is primarily regulated by phosphorylation, catalyzed by the kinase CSK, at the tyrosine residue Y505, located near the C-terminus, leading to protein deactivation[34]. In the future, if ongoing validation continues to implicate LCK activation as a predictive marker for prednisone resistance, we will investigate the causal significance of this and possibly implicate this molecule as a therapeutic target that could modulate prednisone response mechanisms. Very interestingly, inhibition of LCK through treatment with kinase inhibitors currently used in clinical practice for other indications such as BMS-354825 (Dasatinib) and STI-571 (Imatinib)[35] had already been demonstrated to be able to induce apoptosis in human T cells. Thus, it will be of interest to establish if LCK inhibitors could be useful as a possible additional support in BCP-ALL PPR patients treatment.

Our third main result is that high levels of CYCLIN E expression are indicator of a more aggressive disease. Patients who show elevated CYCLIN E expression have a higher probability to relapse. CYCLIN E had been demonstrated to be overexpressed in many malignant tumors (for a review see Möröy *et al.*[12]), and, in particular, Scuderi *et al.*[13] reported that BCP-ALL blasts of adult patients had high CYCLIN E levels and relapsed samples displayed additional accumulation of the protein. CYCLIN E regulates cell cycle progression through the restriction point R, at the end of the G_1-phase, to allow cells to enter S-phase inducing S-phase specific genes. The restriction point R has been recognized to be critical for growth control and thus also for the prevention of unrestricted cell proliferation, malignant transformation and tumorigenesis. We found a cutoff value of CYCLIN E expression able to distinguish patients who have a higher probability to relapse (53% vs 17%), and this is independent from all other clinical and molecular variables. Interestingly, the elevated CYCLIN E expression group is anyhow enriched in poor prognosis patients (MLL-rearranged, t(9;22) translocated and MRD-High Risk), and CYCLIN E is more expressed in early relapsed (within 910 days from the diagnosis) patients who have a worst prognosis[15] with respect to late relapsed patients. This indicates that CYCLIN E expression could correlate with the malignant potential of the cells, and thus could be considered as a marker of the aggressiveness of the disease and a new therapeutic target in pediatric BCP-ALL.

This study emphasizes the importance of protein pathway activation mapping analysis of clinical specimens as a route for discovery of functional derangement that may be functional, causative agents of the cancer. Proteins related to proliferation and survival such as LCK, AMPK and CYCLIN E were found to be hyperactivated or overexpressed in poor prognosis patients with BCP-ALL, and could represent new molecular drug targets in pediatric B-ALL. When further validated in functional studies, specific kinase inhibitors that target AMPK pathway, LCK-mediated signalling and CYCLIN E activation could be evaluated in prospective clinical trials whereby patients who are in need of better therapeutic options could be selected and stratified for targeted therapeutics tailored to the molecular defect.

Materials and Methods

Ethics Statement

The study was approved by the Ethical Committee board of the University of Padova, the Padova Academic Hospital and the Italian Association of Pediatric Onco-Hematology (AIEOP). Patient's parents or their legal guardians provided written informed consent following the tenets of the Declaration of Helsinki.

Patients

Bone marrow samples from 118 children with newly diagnosed BCP-ALL were analyzed. Diagnosis was made according to standard cytomorphology, cytochemistry and immunophenotypic criteria[36]. The study was approved by the local ethics committees and informed consent was obtained for all patients. Samples were collected at the Pediatric Oncohematology Laboratory (Padua, Italy), between 1990 and 2006 and stored in the BioBank in liquid nitrogen in FCS+DMSO. Bone marrow mononuclear cells from patients were separated by Ficoll-Hypaque technique (Pharmacia, Uppsala, Sweden) and frozen within 3hours after collection. The whole blood blast percentage for all samples was between 70% and 98%. Patients molecular and clinical characteristics are resumed in Table S1.

Cell lines

Human leukemia cell lines SEM, RS4;11, REH and NALM6 were purchased from DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). SEM and RS4;11 cell lines derive from BCP-ALLs carrying the t(4;11) MLL-AF4 translocation. REH cell line derives from a BCP-ALL carrying the t(12;21) TEL-AML1 translocation. NALM6 cells derive from a BCP-ALL without recurrent chromosomal translocations. Cells were cultured in RPMI 1640 (Biochrom AG, Berlin, Germany) with 10% FCS, penicillin (100U/ml) and streptomycin (100µg/ml), and maintained at 37°C in a humidified atmosphere with 5% CO_2.

Reverse Phase Protein Microarrays

Cell Lysis. Cells were washed with ice–cold PBS 1X and lysed on ice for 20minutes in an appropriate lysis buffer: TPER Reagent (Pierce, Rockford, IL), 300mM NaCl, 1mM Na orthovanadate, 200mM PEFABLOC (AEBSF) (Roche, Basel, Switzerland), 1ug/mL Aprotinin (Sigma, St. Louis, MO), 5mg/mL Pepstatin A (Sigma), 1mg/mL Leupeptin (Sigma). Cell lysates were then cleared by centrifugation and supernatants were collected and assayed for protein concentration with the Coomassie Protein Assay Reagent Kit (Pierce). Cell lysates were diluted to 1mg/ml in a mixture of 2x Tris-Glycine SDS Sample

Buffer (Invitrogen Life Technologies, Carlsbad, CA) plus 5% of β-Mercaptoethanol. Lysates were stored at -80°C and boiled 8 minutes immediately prior to arraying.

RPMA Printing. Lysates were loaded into a 384-well plate and serially diluted with lysis buffer into four-point dilution curves ranging from undiluted-1:8. As positive controls for antibody staining we added also 3 commercial cell line lysates: A431+EGF, Hela+Pervanadate and Jurkat Apoptotic cell lysates (BD Biosciences, Franklin Lakes, NJ). We divided the 118 samples in 2 set of arrays, thus 59 and 59 samples were printed in duplicate in each array set onto nitrocellulose-coated slides (FAST slides, Whatman Schleicher & Schuell, Florham Park, NJ) with the 2470 Arrayer (Aushon BioSystems, Burlington, MA). On each set of arrays also the above mentioned cell lines and 2 bridge samples were printed for antibody signal normalization between the 2 sets. Printed slides were stored dessicated (Drierite, Sigma) at -20°C until use.

RPMA Staining. Selected slides were stained with Sypro Ruby (Invitrogen) according to the manufacturer's instruction, in order to estimate the total protein amount of each printed sample. Before antibody staining the arrays were treated with ReBlot Plus Mild Antibody Stripping Solution (Chemicon, Temecula, CA) 1X for 15minutes at room temperature, rinsed 2 times for 5minutes in PBS 1X, and then blocked for 1hour at room temperature in blocking solution (2gr I-Block - Tropix, Bedford, MA - and 0.1% Tween-20 in 1l of PBS 1X). Blocked arrays were stained with antibodies on an automated slide stainer (Dako Autostainer Plus, Dako Cytomation, Carpinteria, CA) using the CSA kit (Dako Cytomation) as described previously[6]. Slides were air dried and scanned on a PowerLook 1000 flatbed scanner (UMAX, Dallas, TX) at 600dpi. For an example of antibody-stained slides please see (**Fig. S2**).
For the complete list of the 92 stained antibodies with RPMA, please see Table S2. Each antibody was previously subjected to extensive validation for single band specificity by Western Blot (WB). For phospho-specific antibodies, each antibody was checked for specificity using cell extracts with and without appropriate ligand induction. The 92 antibodies used in this study were carefully selected based on both their extensive validation for specificity as well as detecting key signalling molecules known for their involvement in motility, invasion, pro-survival, and growth factor signalling.

Image Analysis. The TIF images of antibody- and Sypro Ruby-stained slides were analyzed using Microvigene Software (VigeneTech Inc, Boston, MA) to extract numeric intensity values from the arrays images. Briefly this software, specifically developed for

RPMA analysis, localizes the spots and subtracts the local background, calculating pixel intensity for each spot. The software calculates these values in the antibody-stained slides, the corresponding negative control slides (secondary antibody alone) and the total protein slide. Then, for each sample, the signal of the negative control array is subtracted from the antibody slide signal and then the resulting value is normalized to the total protein value, to ensure that intensity values were not dependent on changes in concentration of printed lysate. The data values were normalized to either one of the bridging cases to facilitate comparison of sample values between paired arrays stained with the same antibody. The data processing generates normalized single value for each protein measured for each sample.

Western Blot

The following antibodies were used for WB at the concentrations reported in parentheses. Primary antibodies: anti-AMPKα (23A3) (1:1000), anti-phospho-AMPKα S485 (1:1000), anti-AMPKß1 (1:1000), anti-phospho-AMPKα T172 (1:1000), anti-AMPKß1 (1:1000), anti-phospho-AMPKß S108 (1:1000), anti-BCL-2 (1:1000), anti-phospho-BCL-2 S70 (1:1000), anti-LCK (L22B1) (1:1000), anti-LCK Y505 (1:500), anti-β-ACTIN (1:1000) (all from Cell Signaling Technology, Inc, Danvers, MA), CYCLIN E (BD Biosciences) (1:500), anti-eNOS/NOS III CT (1:1000), anti-phospho-eNOS/NOS III S116 (both from Upstate – Millipore, Billerica, MA) (1:1000). Secondary antibodies: HRP-Goat anti-rabbit and anti-mouse IgG-conjugate (Zymed Laboratories, Inc., South San Francisco, CA) (1:50000). Total cell lysates were analyzed by SDS-PAGE under reducing conditions, and transferred to a nitrocellulose sheet (Hybond-P, GE Healthcare, Chalfont St. Giles, UK) following standard methods. Membranes were saturated for 3hours with 2% Amersham ECL Advance Blocking Reagent (GE Healthcare), primary antibodies were incubated overnight at 4°C and secondary antibodies for 1hour at room temperature. The immunoreactivity was determined by an enhanced chemiluminescent reaction (Amersham ECL ADVANCE Western Blotting Detection Kit, GE Healthcare). For the stripping, membranes were incubated for 30minutes in constant rocking in a solution 25mM Glycin, 1% SDS and pH2, then washed in T-PBS 1X and resaturated.

AMPK inhibition in MLL-rearranged cell lines

AMPK was specifically inhibited in SEM and RS4;11 cell lines using Compound C (Calbiochem, Darmstadt, Germany) 8μM for 48 hours. Proteins were extracted from treated and control cells as described for RPMA. Of note, in order to carefully determine the activation level of AMPKα, in these experiments we measured the phosphorylation of the main activation site that is the T172.

Immunoprecipitation and in vitro kinase assay

REH and NALM6 cell lines were chosen as in vitro model for PPR and PGR patients respectively. Cells were lysed in a buffer containing 20mM Tris-HCl, pH 7.5, 150mM NaCl, 2mM EDTA, 2mM EGTA, 0.5% (v/v) Triton X-100, 2mM dithiothreitol, protease inhibitor cocktail Complete (Roche), 10mM NaF, 1μM okadaic acid, 1mM Na orthovanadate. LCK was immunoprecipitated with limiting amount (20ng) of anti-LCK 3A5 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), for 4h at 4°C, followed by addition of protein A-Sepharose (Sigma). 100μM Na vanadate was present throughout the incubations. Immunoprecipitates were washed once with NET buffer (50mM Tris–HCl pH 8.0, 150mM NaCl, 5mM EDTA, 0.05% (v/v) Nonidet P-40, 2mg/ml bovine serum albumin) and twice with 50mM Tris–HCl pH 7.5, then incubated at 30°C for 40min with a phosphorylation mixture containing 50mM Tris-HCl, pH 7.5, 10mM MgCl2, 10mM MnCl2 10μM [γ-33P]ATP (specific radioactivity ~5000cpm/pmol) and 50μM Na orthovanadate, in a total volume of 20ml. Samples were then boiled for 5min, loaded onto 11% SDS–PAGE followed by blotting to Immobilon-P membranes (Millipore); autophosphorylation of LCK was analyzed by digital autoradiography on Cyclone Plus (PerkinElmer, Waltham, MA) to detect the radioactivity and by WB with anti-LCK to assess the LCK amount.

Statistical Analysis

Statistical analyses were performed with R. Identification of activated proteins was obtained through Wilcoxon tests or two-sample Welch t-tests implemented in multtest package[37]. Pearson's chi-squared test was used for clinical variables. Pathways were identified using global test[38]. P-values were corrected for multiplicity using Benjamini-Hochberg method to control false discovery rate or with Bonferroni method to control family wise error rate; therefore the reported p-values are adjusted p-values. Survival curves were obtained through Kaplan-Meier estimates and the difference between two curves was assessed by log rank test. Finally, a heatmap was generated to highlight the relationships between clustering and protein expression levels.

Supporting information

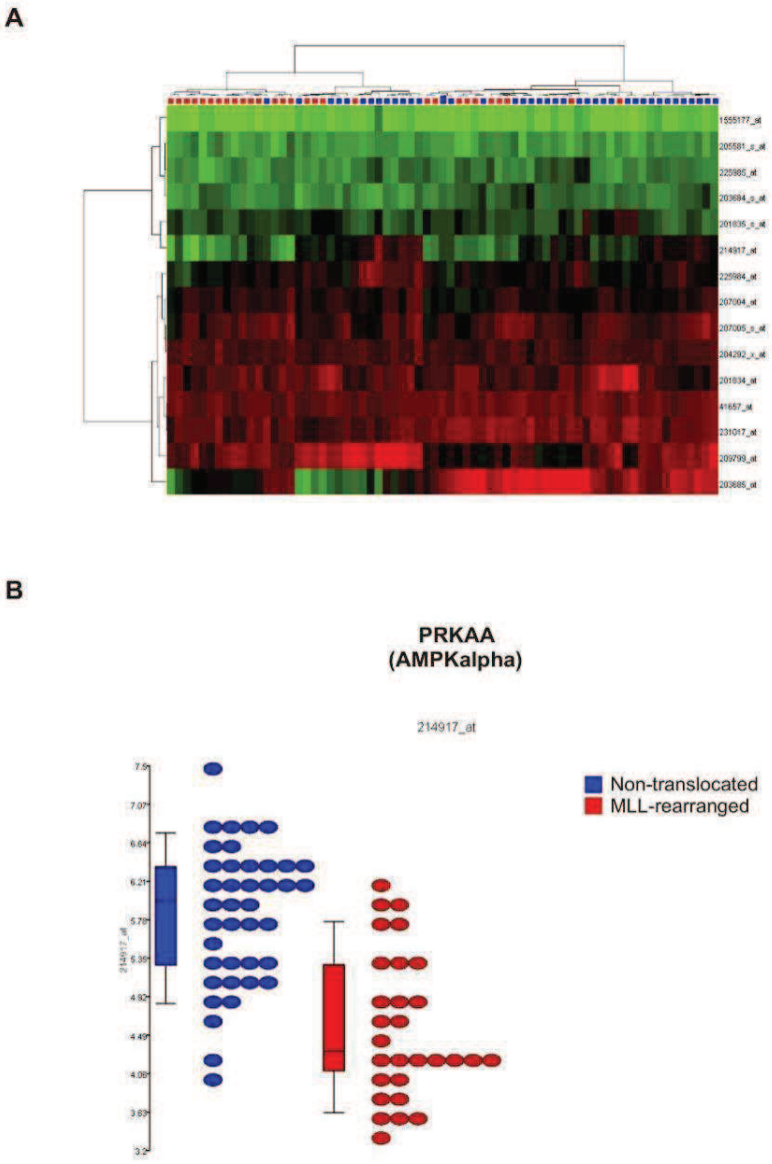

Fig. S1:AMPK-related genes are not upregulated in MLL-rearranged patients.
(A) Heatmap generated with Partek Genomics Suite software using the probe sets corresponding to Lkb1, Ampkα and β, eNos and Bcl-2 genes. The unsupervised analysis is not able to accurately separate MLL-rearranged and non-translocated patients. MLL-patients are highlighted in red. (B) Dot Plot representing the raw expression data of PRKAA probe set (214917_at). The comparative analysis, performed with Partek Genomics Suite software, between MLL-rearranged (red) and non-translocated (blue) patients, using the 15 probe sets of the AMPK-related genes, shows only one probe set (PRKAA, corresponding to Ampkα) differentially expressed between the two groups with a fold change more than 2.0. AMPKα results upregulated in the non-translocated patients. Each dot represents a patient, and the boxes represent the median expression values for each group of specimens. The expression values on y axis are reported on log2 scale.

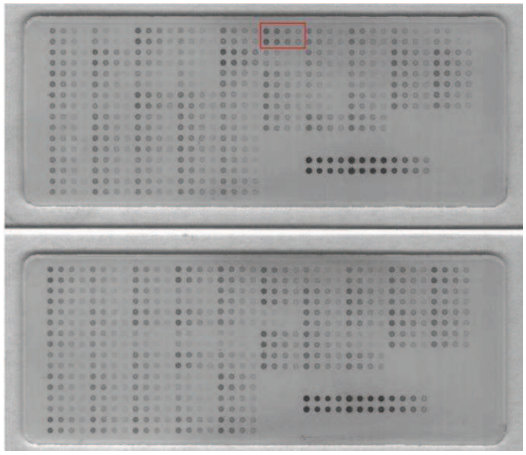

Fig. S2: Example of RPMA stained slides.
Slides in the picture are stained with LCK Y505 antibody. Each patient lysate was printed in a four-point dilution curve ranging from undiluted to 1:8 in duplicate (an example is framed in red). Samples were divided in 2 set of arrays, thus 59 and 59 samples were printed in duplicate in each array set onto nitrocellulose-coated slides. As positive controls for antibody staining, in the right lower part of the slides we added 3 commercial cell line lysates: A431+EGF, HeLa+Pervanadate and Jurkat Apoptotic cell lysates. On each set of arrays the above mentioned cell lines and 2 bridge samples were used for antibody signal normalization between the 2 sets.

Table S1: Patients clinical and molecular characteristics.

Characteristic	*Number (%)*
Sex	
Male	62 (52.5)
Female	56 (47.5)
Age (years)	
<1	6 (5.1)
1-9	92 (78.0)
>9	20 (16.9)
AIEOP[a] therapy protocol	
LLA[b] 88	1 (0.8)
LLA 91	16 (13.6)
LLA 95	35 (29.6)
LLA 2000	62 (52.5)
Interfant	4 (3.4)
Immunophenotype	
Prepre-B	10 (8.5)
Pre-B	30 (25.4)
CALL	76 (64.4)
Prepre-B/CALL	2 (1.7)
WBC Count	
>50 x 10^9/L	34 (28.8)
<50 x 10^9/L	84 (71.2)
DNA Index	
1-1.15	88 (74.6)
≥1.16	19 (16.1)
ND[c]	11 (9.3)
Prednisone Response	
Good	109 (92.4)
Poor	9 (7.6)
Chromosome Translocation	
Neg[d]	36 (30.5)
t(12;21)	21 (17.8)
t(1;19)	7 (5.9)
t(9;22)	3 (2.5)
MLL rearrangements	8 (6.8)
ND	43 (36.5)
MRD (only AIEOP LLA 2000 patients)	
Standard Risk	19 (30.6)
Medium Risk	38 (61.3)
High Risk	5 (8.1)
Outcome	
Relapsed	32 (27.1)
Dead	15 (12.7)
Complete Remission	71 (60.2)

[a]AIEOP= Italian Association of Pediatric Oncohematology.
[b]LLA= Acute Lymphoblastic Leukemia.
[c]ND= No Data.
[d]Neg= negative for MLL-rearrangements, 12;21, 1;19 and 9;22.

Table S2: Primary antibodies for RPMA staining.

Antibody	***Company***	***RPMA Dilution***
14_3_3	Upstate Biotechnology Inc.	1:20000
4EBP1 S65	Cell Signaling Technology, Inc.	1:100
AKT S473	Cell Signaling Technology, Inc	1:100
AKT T308	Cell Signaling Technology, Inc	1:100
AMPKα S485	Cell Signaling Technology, Inc	1:50
AMPKβ S108	Cell Signaling Technology, Inc	1:50
Annexin 1	BD Transduction Laboratories	1:5000
Annexin 2	BD Transduction Laboratories	1:500
Ask1 S83	Cell Signaling Technology, Inc	1:50
ATF2 T71	Cell Signaling Technology, Inc	1:100
Bad S112	Cell Signaling Technology, Inc	1:100
Bad S136	Cell Signaling Technology, Inc	1:50
Bad S155	Cell Signaling Technology, Inc	1:100
Bak	Cell Signaling Technology, Inc	1:50
Bax	Cell Signaling Technology, Inc	1:250
Bcl-2 S70	Cell Signaling Technology, Inc	1:250
Bcl-xL	Cell Signaling Technology, Inc	1:500
Cdk2	Cell Signaling Technology, Inc	1:100
Chk1 S345	Cell Signaling Technology, Inc	1:50
Chk2 S33/35	Cell Signaling Technology, Inc	1:50
c-Kit Y703	Zymed Laboratories, Inc.	1:500
Cleaved Casp 3 D175	Cell Signaling Technology, Inc	1:50
Cleaved Casp 6 D162	Cell Signaling Technology, Inc	1:50
Cleaved Casp 7 D198	Cell Signaling Technology, Inc	1:1000
Cleaved Casp 9 D315	Cell Signaling Technology, Inc	1:250
Cleaved Casp 9 D330	Cell Signaling Technology, Inc	1:50
Cleaved PARP D214	Cell Signaling Technology, Inc	1:100
CREB S133	Cell Signaling Technology, Inc	1:100
Cyclin A	Cell Signaling Technology, Inc	1:20

Cyclin D1	BD Transduction Laboratories	1:20
Cyclin E	BD Transduction Laboratories	1:100
EGFR Y1068	Cell Signaling Technology, Inc	1:50
EGFR Y1148	Cell Signaling Technology, Inc	1:200
EGFR Y992	Cell Signaling Technology, Inc	1:100
eIF4G S1108	Cell Signaling Technology, Inc	1:1000
Elk1 S383	Cell Signaling Technology, Inc	1:100
eNOS-eNOSIII S116	Upstate Biotechnology Inc.	1:500
ERK1/2 T202/Y204	Cell Signaling Technology, Inc	1:2000
Estrogen Rec α S118	Cell Signaling Technology, Inc	1:1000
FADD S194	Cell Signaling Technology, Inc	1:250
FAK Y397	BD Transduction Laboratories	1:50
FHKR S256	Cell Signaling Technology, Inc	1:50
FHKR/L1 T24/T32	Cell Signaling Technology, Inc	1:200
Grb2	Cell Signaling Technology, Inc	1:1000
GSK3αβ S21-9	Cell Signaling Technology, Inc	1:100
GSK3αβ S279/216	BioSource	1:500
HIF1α	BD Transduction Laboratories	1:20
HSP70	Stressgen	1:200
IKBα S32/36	BD Transduction Laboratories	1:50
IRS-1 S612	Cell Signaling Technology, Inc	1:100
Jak1 Y1022/1023	Cell Signaling Technology, Inc	1:100
Lck Y505	Cell Signaling Technology, Inc	1:500
LKB1 S428	Cell Signaling Technology, Inc	1:100
MARCKS S152/156	Cell Signaling Technology, Inc	1:50
MEK1/2 S217/221	Cell Signaling Technology, Inc	1:400
MSK1 S360	Cell Signaling Technology, Inc	1:50
mTOR S2448	Cell Signaling Technology, Inc	1:100
p27 Kip1	BD Transduction Laboratories	1:100
p38 MAPK T180/Y182	Cell Signaling Technology, Inc	1:100
p70 S6 T412	Upstate Biotechnology Inc.	1:500
p70 S6 T389	Cell Signaling Technology,	1:20

	Inc	
p90 RSK S380	Cell Signaling Technology, Inc	1:400
PAK1 S199/204-PAK2 S192/197	Cell Signaling Technology, Inc	1:50
PDGFRβ Y716	Upstate Biotechnology Inc.	1:250
PDK1 S241	Cell Signaling Technology, Inc	1:200
PKA C T197	Cell Signaling Technology, Inc	1:200
PKCα S657	Upstate Biotechnology Inc.	1:2000
PKCαβII T638/641	Cell Signaling Technology, Inc	1:100
PKCδ T505	Cell Signaling Technology, Inc	1:100
PKCθ T538	Cell Signaling Technology, Inc	1:100
PKCζ/λ T410/403	Cell Signaling Technology, Inc	1:50
PRAS40 T246	BioSource	1:3000
PTEN S389	Cell Signaling Technology, Inc	1:500
PYK2 Y408	Cell Signaling Technology, Inc	1:200
A-Raf S299	Cell Signaling Technology, Inc	1:50
B-Raf S445	Cell Signaling Technology, Inc	1:50
C-Raf S338	Cell Signaling Technology, Inc	1:200
Ras-GRF1 S916	Cell Signaling Technology, Inc	1:50
S6 Ribosomal Protein S235/236	Cell Signaling Technology, Inc	1:200
SAPK/JNK T183/Y182	Cell Signaling Technology, Inc	1:200
SGK1	Upstate Biotechnology Inc.	1:200
Shc Y317	Cell Signaling Technology, Inc	1:250
SHIP Y1020	Cell Signaling Technology, Inc	1:50
Smad2 S465/467	Cell Signaling Technology, Inc	1:250
Src Y416	Cell Signaling Technology, Inc	1:100
Src Y527	Cell Signaling Technology, Inc	1:500
STAT1 Y701	Cell Signaling Technology, Inc	1:50
STAT3 S727	Cell Signaling Technology, Inc	1:200
STAT3 Y705	Upstate Biotechnology Inc.	1:100
STAT5 Y694	Cell Signaling Technology, Inc	1:50
STAT6 Y641	Cell Signaling Technology, Inc	1:100
Tuberin-TSC2 Y157	Cell Signaling Technology, Inc	1:50

References

1. Redaelli A, Laskin BL, Stephens JM, Botteman MF, Pashos CL (2005) A systematic literature review of the clinical and epidemiological burden of acute lymphoblastic leukaemia (ALL). Eur J Cancer Care (Engl) 14: 53–62.

2. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, et al. (2008) Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 321: 1801–1806.

3. Cancer Genome Atlas Research Network (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 455: 1061–1068.

4. Liotta LA, Espina V, Mehta AI, Calvert VS, Rosenblatt K, et al. (2003) Protein microarrays: meeting analytical challenges for clinical applications. Cancer Cell 3: 317–325.

5. Petricoin EF, 3rd, Bichsel VE, Calvert VS, Espina V, Winters M, et al. (2005) Mapping molecular networks using proteomics: a vision for patient-tailored combination therapy. J Clin Oncol 23: 3614–3621.

6. Wulfkuhle JD, Aquino JA, Calvert VS, Fishman DA, Coukos G, et al. (2003) Signal pathway profiling of ovarian cancer from human tissue specimens using reverse-phase protein microarrays. Proteomics 3: 2085–2090.

7. Petricoin EF, 3rd, Espina V, Araujo RP, Midura B, Yeung C, et al. (2007) Phosphoprotein pathway mapping: Akt/mammalian target of rapamycin activation is negatively associated with childhood rhabdomyosarcoma survival. Cancer Res 67: 3431–3440.

8. Kornblau SM, Tibes R, Qiu YH, Chen W, Kantarjian HM, et al. (2009) Functional proteomic profiling of AML predicts response and survival. Blood 113: 154–164.

9. Pierobon M, Calvert V, Belluco C, Garaci E, Deng J, et al. (2009) Multiplexed cell signaling analysis of metastatic and nonmetastatic colorectal cancer reveals COX2-EGFR signaling activation as a potential prognostic pathway biomarker. Clin Colorectal Cancer 8: 110–117.

10. Haferlach T, Kohlmann A, Wieczorek L, Basso G, Kronnie GT, et al. (2010) Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group. J Clin Oncol 28: 2529–2537.

11. Bachmann PS, Gorman R, Papa RA, Bardell JE, Ford J, et al. (2007) Divergent mechanisms of glucocorticoid resistance in experimental models of pediatric acute lymphoblastic leukemia. Cancer Res 67: 4482–4490.

12. Moroy T, Geisen C (2004) Cyclin E. Int J Biochem Cell Biol 36: 1424–1439.

13. Scuderi R, Palucka KA, Pokrovskaja K, Bjo¨rkholm M, Wiman KG, et al. (1996) Cyclin E overexpression in relapsed adult acute lymphoblastic leukemias of Bcell lineage. Blood 87: 3360–3367.

14. van Rhenen A, Feller N, Kelder A, Westra AH, Rombouts E, et al. (2005) High stem cell frequency in acute myeloid leukemia at diagnosis predicts high minimal residual disease and poor survival. Clin Cancer Res 11: 6520–6527.

15. Henze G, Fengler R, Hartmann R, Kornhuber B, Janka-Schaub G, et al. (1991) Six-year experience with a comprehensive approach to the treatment of recurrent childhood acute lymphoblastic leukemia (ALL-REZ BFM 85). A relapse study of the BFM group. Blood 78: 1166–1172.

16. Pui CH, Evans WE (2006) Treatment of acute lymphoblastic leukemia. N Engl J Med 354: 166–178.

17. Pui CH, Chessells JM, Camitta B, Baruchel A, Biondi A, et al. (2003) Clinical heterogeneity in childhood acute lymphoblastic leukemia with 11q23 rearrangements. Leukemia 17: 700–706.

18. Robinson BW, Behling KC, Gupta M, Zhang AY, Moore JS, et al. (2008) Abundant anti-apoptotic BCL-2 is a molecular target in leukaemias with t(4;11) translocation. Br J Haematol 141: 827–839.

19. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, et al. (2004) The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc Natl Acad Sci U S A 101:3329–3335.

20. Morrow VA, Foufelle F, Connell JM, Petrie JR, Gould GW, et al. (2003) Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells. J Biol Chem 278: 31629–31639.

21. Drew BG, Fidge NH, Gallon-Beaumier G, Kemp BE, Kingwell BA (2004) Highdensity lipoprotein and apolipoprotein AI increase endothelial NO synthase activity by protein association and multisite phosphorylation. Proc Natl Acad Sci U S A 101: 6999–7004.

22. Choi BM, Pae HO, Jang SI, Kim YM, Chung HT (2002) Nitric oxide as a proapoptotic as well as anti-apoptotic modulator. J Biochem Mol Biol 35: 116–126.

23. Xu W, Liu LZ, Loizidou M, Ahmed M, Charles IG (2002) The role of nitric oxide in cancer. Cell Res 12: 311–320.

24. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, et al. (1995) Nitric oxide synthase activity in human breast cancer. Br J Cancer 72: 41–44.

25. Mannick JB, Asano K, Izumi K, Kieff E, Stamler JS (1994) Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. Cell 79: 1137–1146.

26. Genaro AM, Hortelano S, Alvarez A, Martı´nez C, Bosca´ L (1995) Splenic B lymphocyte programmed cell death is prevented by nitric oxide release through mechanisms involving sustained Bcl-2 levels. J Clin Invest 95: 1884–1890.

27. Schneider A, Younis RH, Gutkind JS (2008) Hypoxia-induced energy stress inhibits the mTOR pathway by activating an AMPK/REDD1 signaling axis in head and neck squamous cell carcinoma. Neoplasia 10: 1295–1302.

28. Baumann P, Mandl-Weber S, Emmerich B, Straka C, Schmidmaier R (2007) Inhibition of adenosine monophosphate-activated protein kinase induces apoptosis in multiple myeloma cells. Anticancer Drugs 18: 405–410.

29. Reiter A, Schrappe M, Ludwig WD, Hiddemann W, Sauter S, et al. (1994) Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. Blood 84: 3122–3133.

30. Lauten M, Stanulla M, Zimmermann M, Welte K, Riehm H, et al. (2001) Clinical outcome of patients with childhood acute lymphoblastic leukaemia and an initial leukaemic blood blast count of less than 1000 per microliter. Klin Padiatr 213: 169–174.

31. Schrappe M, Reiter A, Ludwig W-D, Harbott J, Zimmermann M, et al. (2000) Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German- Austrian-Swiss ALL-BFM Study Group. Blood 95: 3310–3322.

32. Rudd CE, Trevillyan JM, Dasgupta JD, Wong LL, Schlossman SF (1988) The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc Natl Acad Sci U S A 85: 5190–5194.

33. Amundadottir LT, Leder P (1998) Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. Oncogene 16: 737–746.

34. Bergmen M, Mustelin T, Oetken C, Partanen J, Flint NA, et al. (1992) The human p50csk tyrosine kinase phosphorylates p56lck at Tyr-505 and down regulates its catalytic activity. EMBO J 11: 2919–2924.

35. Blake S, Hughes TP, Mayrhofer G, Lyons AB (2008) The Src/ABL kinase inhibitor dasatinib (BMS-354825) inhibits function of normal human Tlymphocytes in vitro. Clin Immunol 127: 330–339.

36. Basso G, Buldini B, De Zen L, Orfao A (2001) New methodologic approaches for immunophenotyping acute leukemias. Heamatologica 86: 675–692.

37. Pollard KS, Ge Y, Taylor S, Dudoit S (2008) Multtest: Resampling-based multiple hypothesis testing. R package version 1.18.0.

38. Goeman JJ, van de Geer SA, de Kort F, van Houwelingen HC (2004) A global test for groups of genes: testing association with a clinical outcome. Bioinformatics 20: 93–99.

"AMPK Inhibition enhances Apoptosis in MLL-Rearranged Pediatric B-Acute Lymphoblastic Leukemia Cells "

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Abstract
The serine/threonine kinase AMPK and its downstream effectors, including eNOS and BCL-2, are hyperactivated in B-cell precursor-acute lymphoblastic leukemia (BCP-ALL) cells with *MLL* gene rearrangements. We investigated the role of activated AMPK in supporting leukemic cell survival and evaluated AMPK as a potential drug target. Exposure of leukemic cells to the AMPK inhibitor Compound C resulted in cell cycle block at G_2/M phase followed by massive apoptosis, an effect that was seen only in cells with *MLL* rearrangements. These results were confirmed by targeting AMPK with specific shRNA. Compound C-induced apoptosis was associated with mitochondrial membrane depolarization, reactive oxygen species production, cytochrome C release and caspases cleavage, indicating intrinsic apoptosis pathway activation. Compound C treatment resulted in a strong antileukemic activity also in MLL-rearranged primary BCP-ALL samples. By Western blotting we demonstrated the dephosphorylation of AMPK, eNOS and BCL-2 after treatment both in cell lines and primary samples. Moreover, AMPK inhibition synergistically enhanced the antiproliferative effects of vincristine, daunorubicin and cytarabine. Taken together these results indicate that the activation of the AMPK pathway directly contributes to the survival of MLLrearranged BCP-ALL cells. AMPK inhibitors could represent a new therapeutic strategy for this high-risk leukemia.

Keywords Acute Lymphoblastic Leukemia, MLL rearrangements, AMPK, apoptosis

Introduction

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that acts as a cellular fuel sensor activated under conditions of ATP depletion and elevated AMP levels such as heat-shock, nutrient deprivation, hypoxia and other metabolic or environmental stresses.[1,2] AMPK is an heterotrimeric complex composed of an α catalytic subunit, a β subunit important both for complex formation and glycogen binding, and a γ regulatory subunit which binds AMP.[3] AMPK activity is enhanced by phosphorylation of the α-subunit at Threonine 172 by LKB1, a serine/threonine kinase encoded by the tumor-suppressor gene *STK11* which is mutated in patients with Peutz-Jeghers syndrome.[4,5] In addition, AMPK can also be activated by several hormones and cytokines such as adiponectin[6] and leptin.[7] The physiological role ascribed to AMPK is the inactivation of ATP-consuming metabolic processes, including fatty acid, cholesterol and protein synthesis, and the activation of ATP-generating pathways such as glycolysis and fatty acid oxidation.[8,9] This is initially accomplished by direct phosphorylation of key metabolic enzymes, followed by effects on gene expression.

While AMPK is traditionally regarded as a sensor of cellular energy status and a regulator of metabolism, recently it has been reported to be involved in the regulation of several biological processes including cell growth, proliferation, apoptosis, autophagy and cell polarity.[10,11] In cancer, the role of AMPK is not yet fully understood and data reported in literature so far are contradictory. The effects of AMPK activation are determined by the cell type investigated, depending on signalling alterations in related pathways. AMPK activation results in pro-apoptotic effects reported in acute myeloid leukemia,[12] ovarian cancer,[13] astrocytoma,[14] and osteosarcoma,[15] and in antiapoptotic effects, observed in multiple myeloma,[16] prostate cancer[17] and glioma.[18] We found that B-cell acute lymphoblastic leukemia (BCP-ALL) with rearrangements of the *MLL* gene have an hyperactivated signal transduction pathway that leads from phosphorylation of LKB1 and AMPK to phosphorylation of BCL-2, through downstream endothelial nitric oxide synthase (eNOS) activation.[19] In the present study, we assessed whether this hyperactivation supports the survival of MLL-rearranged BCP-ALL cells, and whether its inhibition affects leukemic cell growth and drug resistance.

Materials and Methods

Cell lines and culture

Human leukemia cell lines SEM, RS4;11, MHH-CALL-2 and MHH-CALL-4 were purchased from DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). SEM and RS4;11 cell lines were derived from BCP-ALLs carrying the t(4;11) MLL-AF4 translocation. MHH-CALL-2 and MHH-CALL-4 cell lines were derived from BCP-ALLs without recurrent chromosomal translocations. Cells were cultured in RPMI 1640 (Biochrom AG, Berlin, Germany) with 10% FCS, glutamine (2mM/L) (GIBCO, Invitrogen Life Technologies, Carlsbad, CA, USA), penicillin (100U/ml) (GIBCO) and streptomycin (100µg/ml) (GIBCO), and maintained at 37°C in a humidified atmosphere with 5% CO2. Cells were treated with Compound C (Calbiochem, Darmstadt, Germany) dissolved in DMSO at different times and concentrations.

MTT assay

Cell proliferation was assessed by MTT assay before and after treatment. Equal concentrations of cells were plated in triplicate in a 96-well plate and incubated with 10µl MTT (Sigma-Aldrich, St. Louis, MO, USA) for 4 hours. Absorbance was measured at 560nm using Victor3 TM 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA). The GI50 (Growth Inhibition50 = compound concentration required to inhibit cell proliferation by 50%) was calculated by plotting the data as a logarithmic function of (x) when viability was 50%. DMSO treated cells viability was set to 100%.

Cytofluorimetric assays

Apoptosis was determined using the Annexin-V-FLUOS staining kit (Roche, Basel, Switzerland), following manufacturer's instructions. Samples were analyzed by flow cytometric analysis (Cytomics FC500, Beckman Coulter, Fullerton, CA, USA). DMSO treated cells viability was set to 100%. The LC50 (Lethal Concentration50 = compound concentration required to induce cell mortality by 50%) was calculated by plotting the data as a logarithmic function of (x) when viability was 50%.

The mitochondrial membrane potential (ψmt) was measured with the lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine (JC-1, Molecular Probes, Invitrogen Life Technologies), as already described.20 Briefly, before and after treatment (Compound C 8µM, 48 hours - LC50 -), cells were collected by centrifugation

and resuspended in Hank's balanced salt solution (HBSS) containing 1μM JC-1. Cells were then incubated for 10 minutes at 37°C, centrifuged, resuspended in HBSS and analyzed by flow cytometric analysis. Mitochondrial membrane depolarization is associated with mitochondrial production of Reactive Oxygen Species (ROS). The production of ROS was measured, before and after treatment (LC50 Compound C 8μM, 48 hours), by flow cytometry using hydroethidine (HE, Molecular Probes, Invitrogen Life Technologies) 2.5μM. Cells were incubated for 10 minutes at 37 °C, centrifuged, resuspended in HBSS and analyzed by flow cytometric analysis.[20] Caspase-3 activation was evaluated by flow cytometry using a human active caspase-3 fragment antibody conjugated with FITC (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, before and after treatment (Compound C 8μM, 48 hours), cells were collected by centrifugation and resuspended in Cytofix (BD Pharmingen) buffer for 20 minutes, washed with Perm/Wash (BD Pharmingen), and then incubated for 30 minutes with the antibody. Cells were then washed and analyzed by flow cytometry (Cytomics FC500, Beckman Coulter).

Lentiviral vector-mediated transduction of shRNA in leukemia cells

The lentiviral plasmids containing AMPKα1 shRNA expression cassette or an un-relevant shRNA sequence were purchased from Sigma-Aldrich. The lentiviral vector stocks were generated by a transient three-plasmid vector-packaging system.[21] One μg of p24 equivalent of lentiviral vectorcontaining supernatant was used to transduce 1 x 106 target cells in 35 mm-diameter Petri dish. After 6-9 hours at 37°C, the supernatant was replaced with complete medium. Evaluation of AMPKα1 silencing, through Sybr Green Real-Time Quantitative PCR (RQ-PCR) and Western Blot experiments, and quantification of apoptosis, through AnnexinV-PI staining, were carried out 72-96 hours after transduction.

RQ-PCR

Total RNA was isolated and retrotranscribed as previously described.[22] Reactions were performed in a total volume of 20μl, containing 1μl of cDNA, 10μl of SYBR Green (Invitrogen Life Technologies), and 150nM forward and reverse primers, on an ABI Prism 7900HT Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA) with the annealing temperature set at 60 °C for all tested genes. Relative quantification was done using the ΔΔCt method, normalizing to GUS mRNA. Primers used for Real-Time Quantitative PCR analysis: AMPKα1-fwd: 5′- GGAGCCTTGATGTGGTAGGA-3′; AMPKα1-rev: 5′-GTTTCATCCAGCCTTCCATTC-3′; AMPKα2- fwd 5′-ACCAGCTTGCAGTGGCTTAT-3′;

AMPKα2-rev: 5′-CAGTGCATCCAATGGACATC-3′; GUSfwd: 5′-GAAAATATGTGGTTGGAGAGCTCATT-3′; GUS-rev: 5′-CGGAGTGAAGATCCCCTTTTTA-3′.

Western Blotting

The following antibodies and final concentrations were used for Western blotting. Primary antibodies: anti-phospho-AMPKα T172 (1:500), anti-AMPKα (23A3) (1:1000), anti-AMPKα1 (1:1000), anti-phospho-AMPKß S108 (1:1000), anti-phospho-BCL-2 S70 (1:500), anti-β-ACTIN (1:10000) (all from Cell Signaling Technology, Inc, Danvers, MA, USA), anti-phospho-eNOS/NOS III S116 (1:1000) (Millipore, Billerica, MA, USA). Secondary antibodies: HRP-Goat anti-rabbit and anti-mouse IgG-conjugate (Zymed Laboratories, Inc., South San Francisco, CA, USA) (1:50000). Total cell lysates were analyzed by SDS-PAGE under reducing conditions, and transferred to a nitrocellulose sheet (Hybond-P, GE Healthcare, Chalfont St. Giles, UK) following standard methods. Membranes were saturated for 3 hours with 2% Amersham ECL Advance Blocking Reagent (GE Healthcare), primary antibodies were incubated overnight at 4°C and secondary antibodies for 1 hour at room temperature. The immunoreactivity was determined by an enhanced chemiluminescent reaction (Amersham ECL ADVANCE Western Blotting Detection Kit, GE Healthcare). For the stripping, membranes were incubated with Restore PLUS Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) following manufacturer's instructions and then washed in T-PBS 1X and resaturated.

Cell Cycle analysis

Cells were synchronized 24 hours prior to treatment by starvation without FBS. Compound C was then added 6 hours after the end of starvation. Before and after treatment (8μM, 24 hours) cells were centrifuged, fixed with ice-cold ethanol (70%), treated with lysis buffer containing RNAseA (Qiagen, Hilden, Germany) and propidium iodide (PI). Samples were analyzed on a Cytomic FC500 flow cytometer (Beckman Coulter), and cell cycle analyses were performed using Multicycle Wincycle software (Phoenix Flow Systems, San Diego, CA, USA).

Immunofluorescence

Compound C treated (8μM) and control human MLL-rearranged leukemia cell lines were placed on the slide by cytospin and then fixed with formaldehyde (4%) for 15 minutes. Cells were permeabilized with 0.1% Triton X-100/PBS 1X and incubated first with blocking buffer (5% BSA in PBS 1X) and than with primary antibody (1:100) diluted in blocking buffer overnight. Cells were then incubated for 1 hour with species-specific secondary antibodies conjugated to Alexa dyes (1:2000) (Invitrogen Life Technologies), and for 10 minutes with DAPI (1:10000) (Sigma-Aldrich). Primary antibodies: Cytochrome c, Cleaved PARP (Asp214) and BCL-XL (from Cell Signaling Technology, Inc), Caspase 7 active and Caspase 9 active (from GeneTex Inc, Irvine, CA, USA).

Primary leukemia cell cultures

BCP-ALL patient samples were obtained after informed consent following the tenets of the Declaration of Helsinki. The study was approved by the Ethical Committee board of the University of Padova, the Padova Academic Hospital and the Italian Association of Pediatric Onco-Hematology (AIEOP). Diagnosis was made according to standard cytomorphology, cytochemistry and immunophenotypic criteria.[23] All analyzed BCP-ALL samples were obtained at the time of diagnosis before treatment, after Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) separation of mononuclear cells. Mononuclear cells were frozen as viable cells in FCS and 10% DMSO and stored in liquid nitrogen. The percentage of CD19+ cells ranged from 84% to 96%. We included in the study 4 patients positive for the 11q23 MLL-rearrangement MLL-AF4 and 4 patients negative for the 11q23 MLL-rearrangement and the chromosomal translocations t(12;21) TEL-AML1, t(9;22) BCR-ABL and t(1;19) E2A-PBX1. Chromosomal translocation analyses were performed for standard diagnostic procedures. Human bone marrow–derived mesenchimal stromal cells (MSCs) immortalized by telomerase reverse transcriptase transduction were kindly donated by Dr. Dario Campana (Yong Loo Lin School of Medicine, National University of Singapore, Singapore).[24] MSCs were maintained in RPMI 1640 medium supplemented with 10% FCS, glutamine (2mM/L), penicillin (100U/ml), streptomycin (100μg/ml) and 10-6M hydrocortisone (Sigma-Aldrich). MSCs were seeded in 24-well flat-bottomed plates (Costar Corning, Cambridge, MA, USA) and grown until confluence. To prepare cultures of primary samples, culture media was removed and adherent cells were washed 7 times with AIM-V tissue culture medium (Invitrogen Life Technologies) with 10% FCS. Leukemic cells were then resuspended in AIM-V medium with 10% FCS, and 1 x 105 leukemic cells were placed on the MSCs layer in each well.

Compound C was added 24 hours after seeding at different concentrations (1, 5 and 8µM). Quantification of apoptosis was carried out 24 and 48 hours after treatment, downregulation of the AMPK pathway was tested after 48 hours.

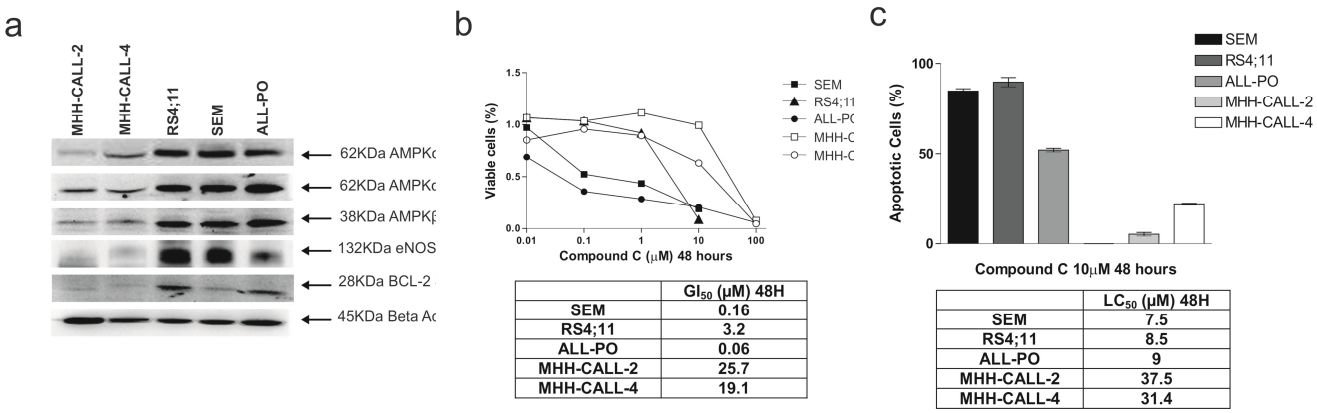
Combined drugs analysis

To test potential synergistic, additive, or antagonistic effects of the combination of AMPK inhibition and drugs commonly used in ALL treatment, we performed MTT experiments as follows. SEM cells were treated for 48 hours with the different chemotherapeutic cytarabine (Aractyn, Pfizer) (0.001-100µM), daunorubicin (Pfizer) (0.0001-10µM) and vincristine (Pfizer) (0.0001-1µM). Compound C was also added to drug solutions at fixed combination ratios. Cell viability was determined after 48 h of treatment by MTT test as described above. To determine the synergistic, additive, or antagonistic effects of the drug combinations, we used CalcuSyn software (version 2.0, Biosoft, Cambridge, United Kingdom), which is based on the method of the combination index (CI) of Chou and Talalay.[25] Synergy, additivity, and antagonism were defined by a CI<1, CI=1, or CI>1, respectively.

Results

AMPK inhibition decreases proliferation and survival in MLL-rearranged cells

To study the functional role of AMPK in the survival of MLL-rearranged cells, we used five BCP-ALL cell lines, three with MLL gene rearrangements (RS4;11, SEM and ALL-PO) and two without (MHH-CALL-2 and MHH-CALL-4). As shown in **Figure 1a**, AMPK was activated in MLL-rearranged cells, similarly to what was observed in primary cells from patients.[19] By contrast, AMPK activation was not detected in MHH-CALL-2 and MHH-CALL-4 (**Fig. 1a**). We treated the five BCP-ALL cell lines with the AMPK inhibitor Compound C. The capability of compound C to inhibit the AMPK pathway in SEM and RS4;11 cells was previously demonstrated.[19] Downregulation of the AMPK pathway in ALL-PO cells is shown in **Supplementary Figure S1**. Cells were exposed to the inhibitor for different time periods (6–96 h) at concentrations ranging from 0.001 to 100 μM. The three MLL-rearranged cell lines were much more sensitive to AMPK inhibition than the two non-rearranged ones. The GI50 measured by MTT assay was 0.16 μM for SEM, 3.2 μM for RS4;11 and 0.06 μM for ALL-PO cells, whereas it resulted to be 25.7 μM and 19.1 μM for the non-translocated cell lines (**Fig. 1b**). **Supplementary Figure S2a** shows the results obtained at all tested times and concentrations. MLL-rearranged cell lines exposed to the inhibitor also underwent significantly more apoptosis, as measured with Annexin V and PI staining. LC50 was 7.5 μM for SEM, 8.5 μM for RS4;11 and 9 μM for ALL-PO, whereas it was 37.5 μM for MHH-CALL-2 and 31.4 μM for MHH-CALL-4 (**Fig. 1c**, for more detailed results please see **Supplementary Figure S2b**).



	GI_{50} (μM) 48H
SEM	0.16
RS4;11	3.2
ALL-PO	0.06
MHH-CALL-2	25.7
MHH-CALL-4	19.1

	LC_{50} (μM) 48H
SEM	7.5
RS4;11	8.5
ALL-PO	9
MHH-CALL-2	37.5
MHH-CALL-4	31.4

Fig. 1. AMPK inhibition induces a decrease in cell proliferation and survival. (a) AMPK pathway activation status in BCP-ALL cell lines. MHH-CALL-2 and MHH-CALL-4 are non-translocated cell lines, RS4;11, SEM and ALL-PO carry the (4;11) MLL-AF4 translocation. (b) Cell proliferation rates were determined through MTT assay after treatment with compound C at different times and concentrations. Here the 48 h are shown. DMSO-treated cells viability was set to 100%. GI50=compound concentration required to inhibit cell proliferation by 50%. (c) Cell viability was determined by flow cytometry with Annexin V–PI staining after treatment with compound C at different times and concentrations. Here the 10 mM 48 h results are shown. DMSO-treated cells viability was set to 100%. Results represent the mean of three independent experiments ±s.e.m. LC50=compound concentration required to induce cell mortality by 50%. eNOS, endothelial nitric oxide synthase.

Therefore, activation of the AMPK pathway appears to be essential for sustaining the survival of MLL-rearranged cells. To validate the association between AMPK inhibition and apoptosis, we suppressed AMPKα1 expression using two different shRNAs (shAMPKalpha1 1 and 2) in SEM, ALL-PO and MHH-CALL-2 cells. AMPKα1 mRNA expression was efficiently reduced after lentiviral vector transduction in the three cell lines (**Fig. 2a**, control AMPKa mRNA expression was set to 100%). In line with the previous results, SEM and ALL-PO cells underwent more apoptosis than MHH-CALL-2 cells **(Fig. 2b**, control cell viability was set to 100%; t-test, SEM vs MHH-CALL-2 P=0.004 and P=0.0003 for shAMPKalpha1 1 and 2, respectively, ALL-PO vs MHH-CALL-2 P=0.006 and P=0.0001). AMPKα1 protein expression was also reduced in MLL-rearranged and non-translocated silenced cells, but the downregulation of the downstream pathway was observed only in SEM and ALL-PO cells (**Fig. 2c**). shRNA silencing has effects on MLL-rearranged cells survival similar to compound C, therefore what we observed after compound C treatment in these cells can be mainly attributed to AMPK deactivation. These experiments also demonstrated that the specific silencing of AMPK has no consequences on nontranslocated cells survival, meaning that the apoptosis we previously observed with 30–40 μM of compound C probably derived from off-target effects because of high concentration of the inhibitor. For this reason, to investigate cell cycle modifications and apoptosis driven by AMPK inhibition, we performed the following experiments only on MLL-rearranged cells.

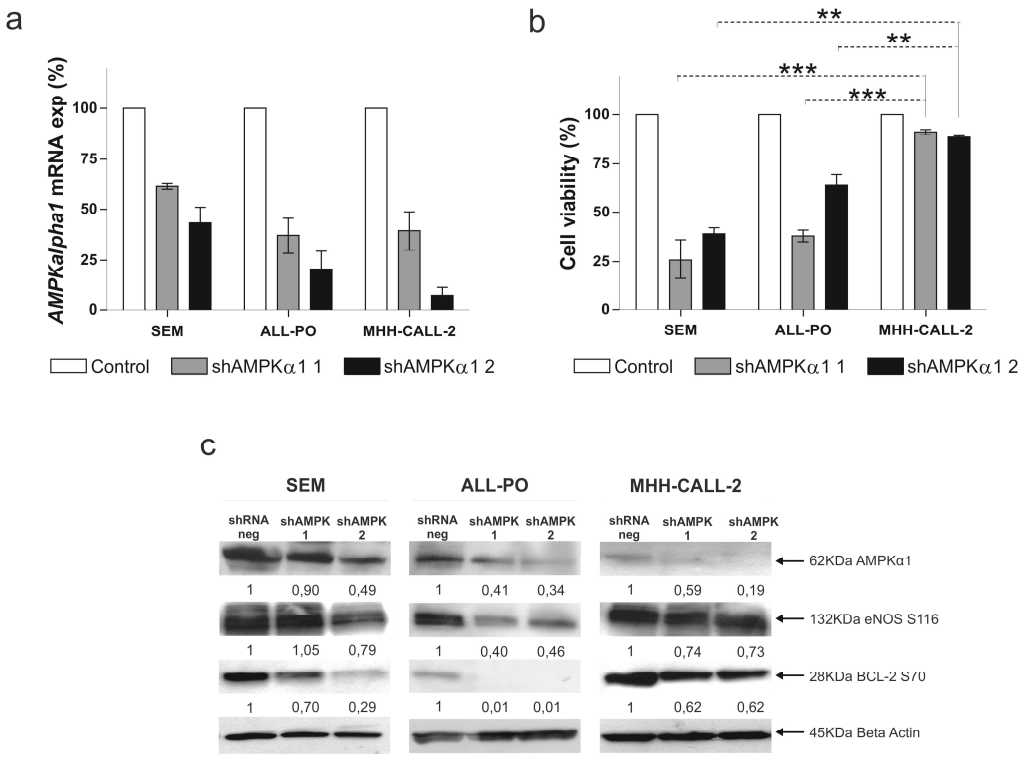


Fig. 2. AMPK-specific silencing brings to the same effects of compound C treatment. (a) Inhibition of AMPKα1 mRNA expression in SEM,ALL-PO and MHH-CALL-2 cells with both shAMPKalpha1 1 and 2. AMPKα1 mRNA expression in control cells (transfected with un-relevant shRNA sequence) was set to 100%. Results represent mean±s.e.m. of three experiments. mRNA expression levels were measured through Sybr Green real-time quantitative PCR. (b) Cell viability after AMPKα1 silencing in SEM, ALL-PO and MHH-CALL-2 cells was evaluated through Annexin V–PI staining. Control cells viability was set to 100%. Results represent mean±s.e.m. of three experiments. Silenced MLL-rearranged SEM and ALL-PO cells undergo more apoptosis than non-translocated MHH-CALL-2 cells (**P<0.01, ***P<0.001). (c) Inhibition of AMPKα1 protein levels and decrease in the downstream AMPK pathway activation was examined using western blot in control and silenced SEM, ALL-PO and MHH-CALL-2 cells. Representative results are shown with densitometric analysis results indicated under gel images.

Compound C treatment induces cell cycle alterations

Representative cell cycle histograms based on flow cytometric analyses of MLL-rearranged cells exposed to compound C are shown in **Figure 3**. After 24 h of compound C treatment (8 μM), an accumulation in G_2/M phase was observed in SEM and RS4;11 cell lines. SEM cells in G2/M phase increased from about 13 up to 34%, whereas cells in G_1 decreased from about 72 to 56% and cells in S phase from about 15 down to 10% (**Fig. 3a**). Similarly, after treatment RS4;11 cells in G_2/M phase increased from about 17 up to 38%, whereas cells in G_1 decreased from about 53 to 43% and cells in S phase from about 30 down to 19% (**Fig. 3b**). Differently, in ALL-PO cells an accumulation in G_1 was observed after AMPK inhibition. The percentage of cells in G_1 phase increased from about 48 up to 91%, whereas G_2/M and S phase cells decreased from about 19 to 5% and from 33 to 4%, respectively (**Fig. 3c**).

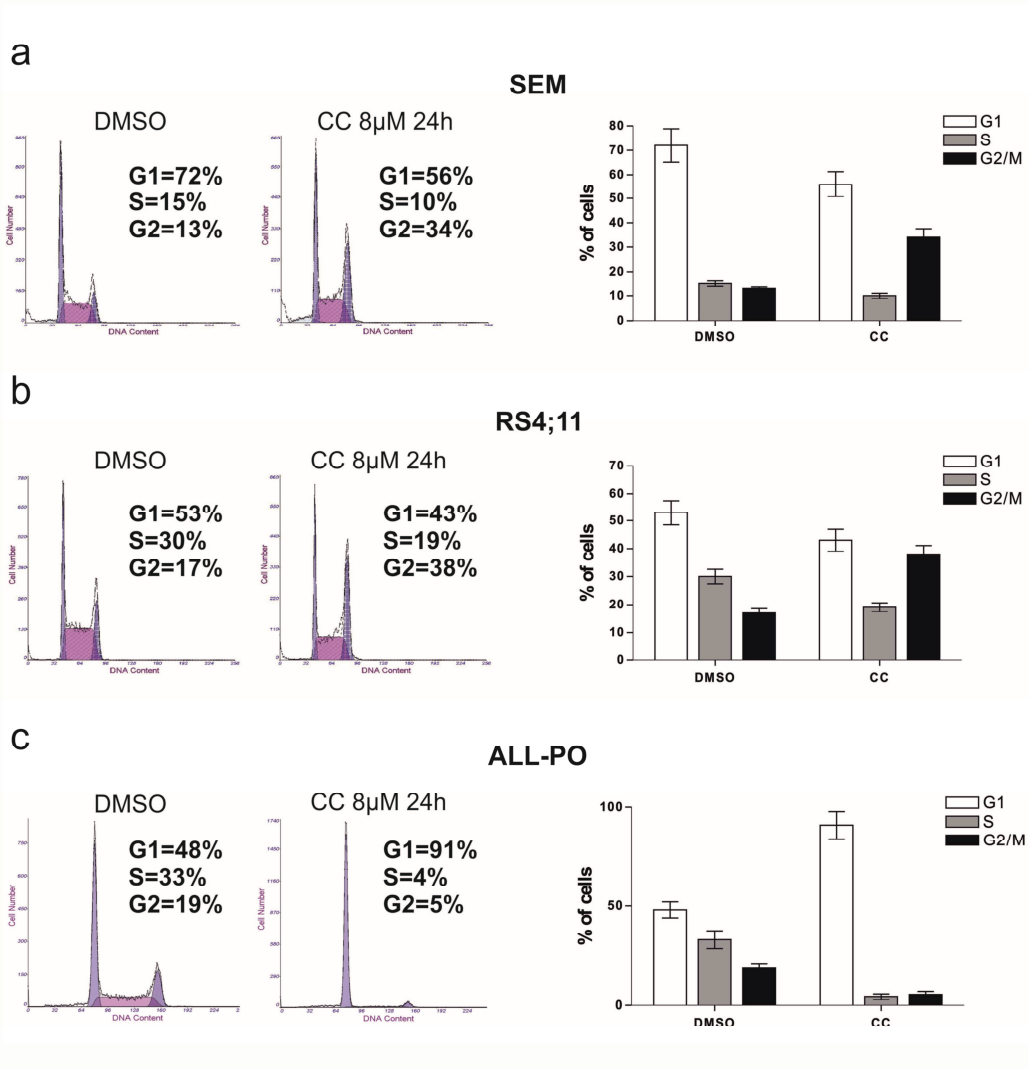


Fig. 3. Cell cycle alterations after AMPK inhibition. Results are shown in (a) for SEM cells, (b) for RS4;11 cells and (c) for ALL-PO cells. DMSO and CC (8 μM, 24 h) samples were analyzed by flow cytometry and cell cycle analyses were performed using Multicycle Wincycle software. Representative histograms are shown. The percentage of each phase of the cell cycle (G_1, S, G_2/M) was calculated. Results come from three independent experiments.

Compound C induces apoptosis through the mitochondrial pathway

Next, we examined whether apoptosis occurs through the extrinsic or the intrinsic pathway. For this purpose, we treated MLL-rearranged cell lines SEM, RS4;11 and ALL-PO with 8 μM compound C for 24, 48 and 72 h. Results are resumed in **Figure 4a** (SEM cells), **Figure 4b** (RS4;11 cells) and **Figure 4c** (ALL-PO cells). Both FAS and FAS-L were present at low levels in control and DMSO-treated cells and did not increase after AMPK inhibition (data not shown). We also examined if exposure to compound C for 24 or 48 h could induce BCP-ALL cell differentiation using a panel of B-lymphocyte differentiation markers including CD58/10/45/19/34/20; and no change in marker expression was

detected (data not shown). Thus, apoptosis induced by compound C does not involve FAS and AMPK inhibition does not promote B-cell differentiation.

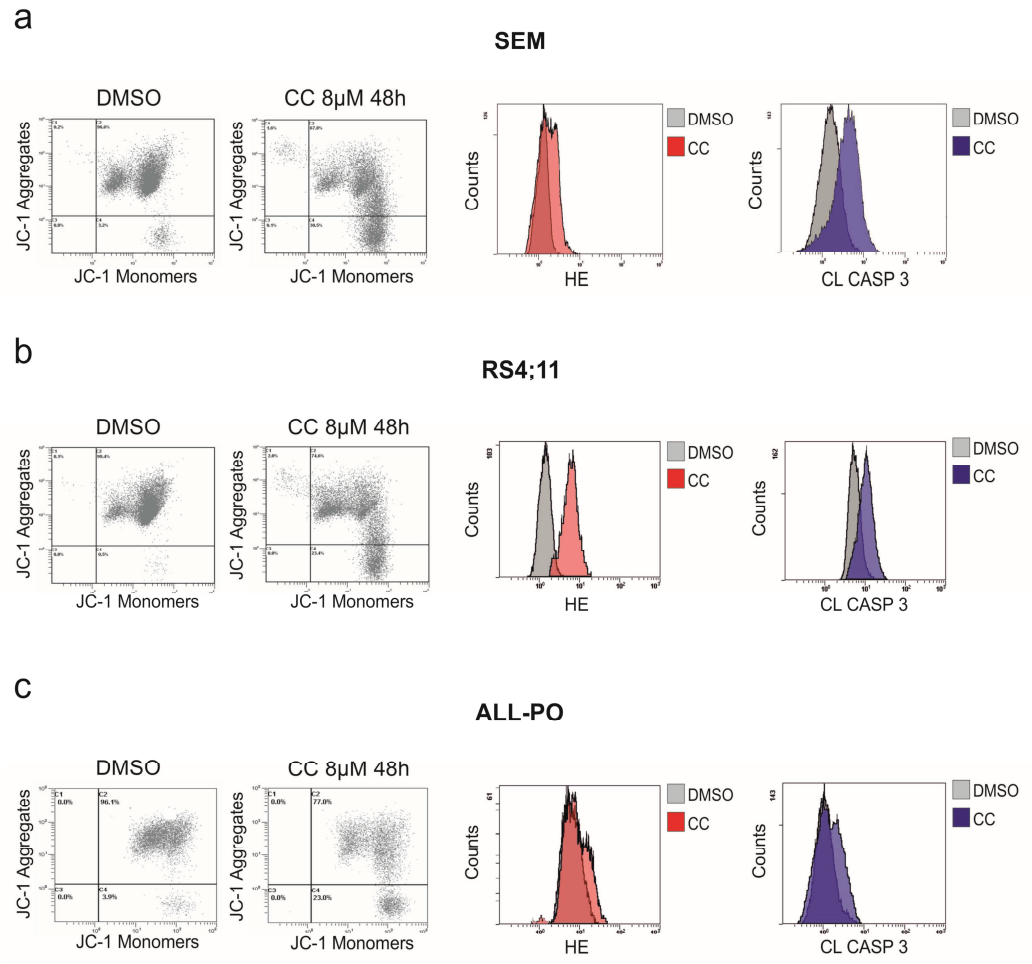


Fig. 4. Compound C (CC)-mediated cell death follows the mitochondrial pathway (I). Results are shown in (a) for SEM cells, (b) for RS4;11 cells and (c) for ALL-PO cells. In the left panels, depolarization of the mitochondrial transmembrane potential, monitored by the fluorescent dye JC-1, in MLL-rearranged cells treated with CC C (8 μM, 48 h) is reported. The method is based on the ability of this fluorescent probe to enter selectively into the mitochondria, and its color changes reversibly from green to orange as membrane potential increases. This property is due to the reversible formation of JC-1 aggregates on membrane polarization. Aggregation causes a shift in the emitted light from 530nm (emission by JC-1 monomers) to 590nm (emission by JC-1 aggregates) following excitation at 490 nm. In middle panels, shown are the mitochondrial production of reactive oxygen species in MLL-rearranged cells treated with CC (8 μM, 48 h). The fluorescence indicator hydroethidine (HE), that is oxidized by superoxide anion into ethidium ion, which emits red fluorescence, was measured. Gray: DMSO-treated cells, red: CC-treated cells. In the right panels, shown are the cleavage of caspase-3 measured by flow cytometry in MLL-rearranged cells after CC (8 μM, 48 h) treatment. Gray: DMSO-treated cells, blue: CC-treated cells.

Apoptotic stimuli may alter the mitochondrial transmembrane potential Ψmt monitored by the fluorescent dye JC-1. JC-1 fluorescence shifted in MLL-rearranged cells exposed to compound C for 48 h, indicating depolarization of mitochondrial membrane potential (**Fig. 4**, left panels). As mitochondrial membrane depolarization is associated with

mitochondrial production of ROS, we investigated whether ROS production was increased after compound C treatment. We used the fluorescent indicator hydroethidine that is oxidized by superoxide anion into ethidium ion, which emits red fluorescence; superoxide is produced by mitochondria when cytochrome c is released. As shown in **Figure 4** middle panels, exposure to compound C for 48 h induced ROS production in treated MLL-rearranged cells, and we also observed the activation of caspase-3 (**Fig. 4**, right panels) and decreased levels of the anti-apoptotic protein BCL-XL (**Fig. 5**). This leads to mitochondrial outer membrane permeabilization through BAX/BAK action, with the consequent release of cytochrome c into the cytoplasm, a necessary event for downstream caspases activation. As shown in **Figure 5**, the release of cytochrome c in the cytoplasm of SEM, RS4;11 and ALL-PO cells after 48 h of AMPK inhibition is particularly evident. Release of cytochrome c results in activation of caspase-9 through self-cleavage at Asp315. Cleaved caspase-9 further processes other caspase members, including caspase-3 and caspase-7 that are effector caspases responsible for the proteolytic cleavage of many key proteins such as PARP. When cleaved, PARP is no longer able to initiate DNA repair and cells undergo apoptosis. In treated MLL-rearranged cells we observed at 48 h the activation of caspase-7 and caspase-9, and at 72 h the cleavage of PARP (**Fig. 5**). Finally, after 72 h of AMPK inhibition we also observed an increase in chromosomal DNA fragmentation into multiples of the 180-bp nucleosomal unit (**Supplementary Fig. S3**).

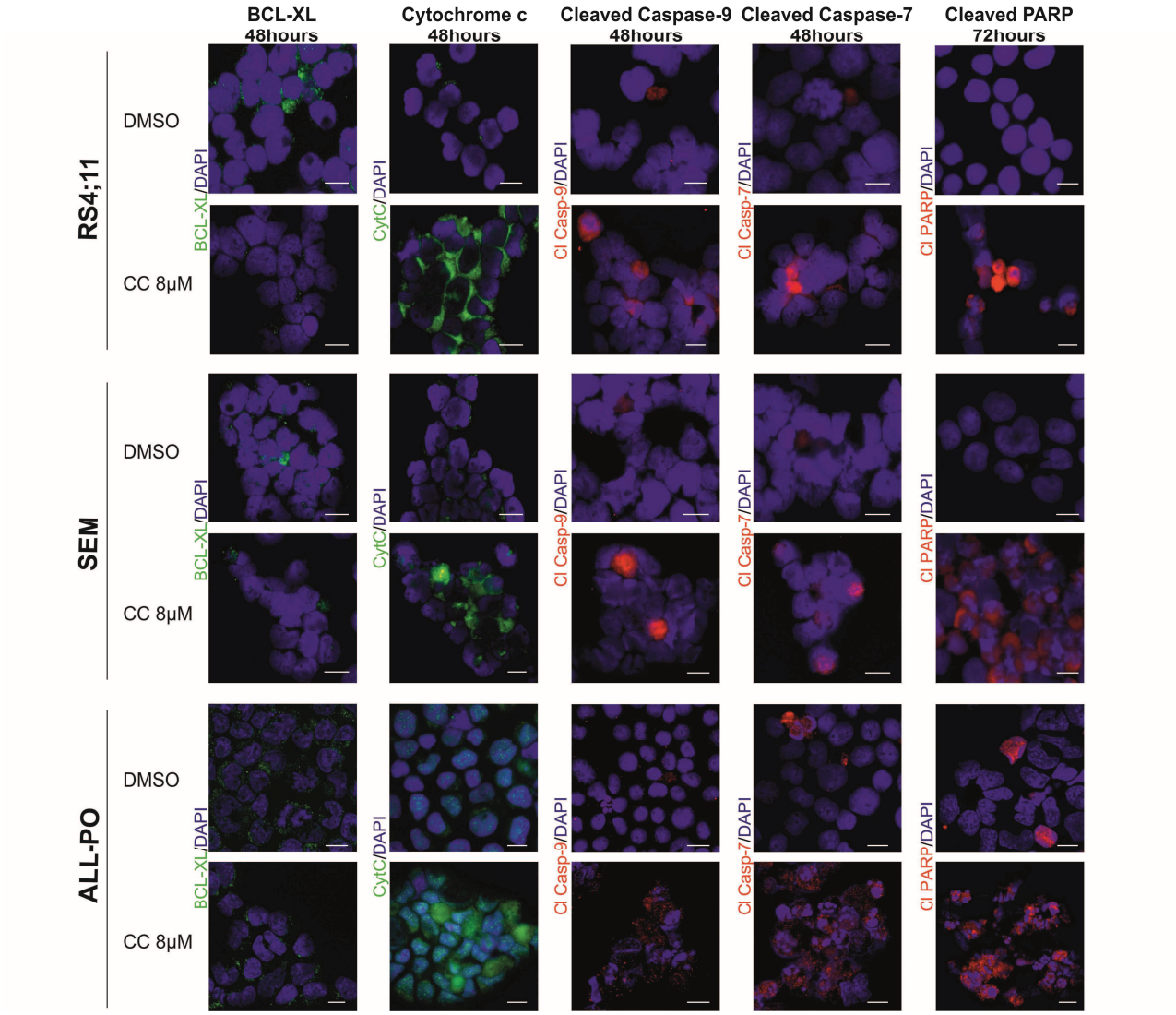


Fig. 5. Compound C (CC)-mediated cell death follows the mitochondrial pathway (II). Apoptotic proteins expression was determined by immunofluorescence in MLL-rearranged cells after DMSO or CC (8 μM, 48 and 72 h) treatment. Panel shows pseudo-color merged images of cell lines decorated with 4,6-diamidino-2-phenylindole (DAPI), Alexa-fluor 488 and 594. Scale bar 5 μm.

AMPK inhibition induces cell death in primary leukemia cultures

We treated nine primary BCP-ALL cultures (five with MLL gene rearrangements and four non-translocated) with DMSO alone or compound C. Cells with MLL rearrangements underwent apoptosis when exposed to low concentrations (1 and 5 μM) of compound C more extensively than cells lacking MLL gene rearrangements (t-test, 1 μM 24 h P=0.005, 5 μM 24 h P=0.007, 1 μM 48 h P=0.03, 5 μM 48 h P=0.01; **Fig. 6a**). We also show that AMPK and its downstream targets were dephosphorylated in two MLL-rearranged samples after 48 h of treatment with 5 μM of compound C (**Fig. 6b**). Moreover we observed, as for MLL-rearranged cell lines, the decrease in BCL-XL, the release of cytochrome c and the cleavage of caspase-7, caspase-9 and PARP (**Fig. 6c**).

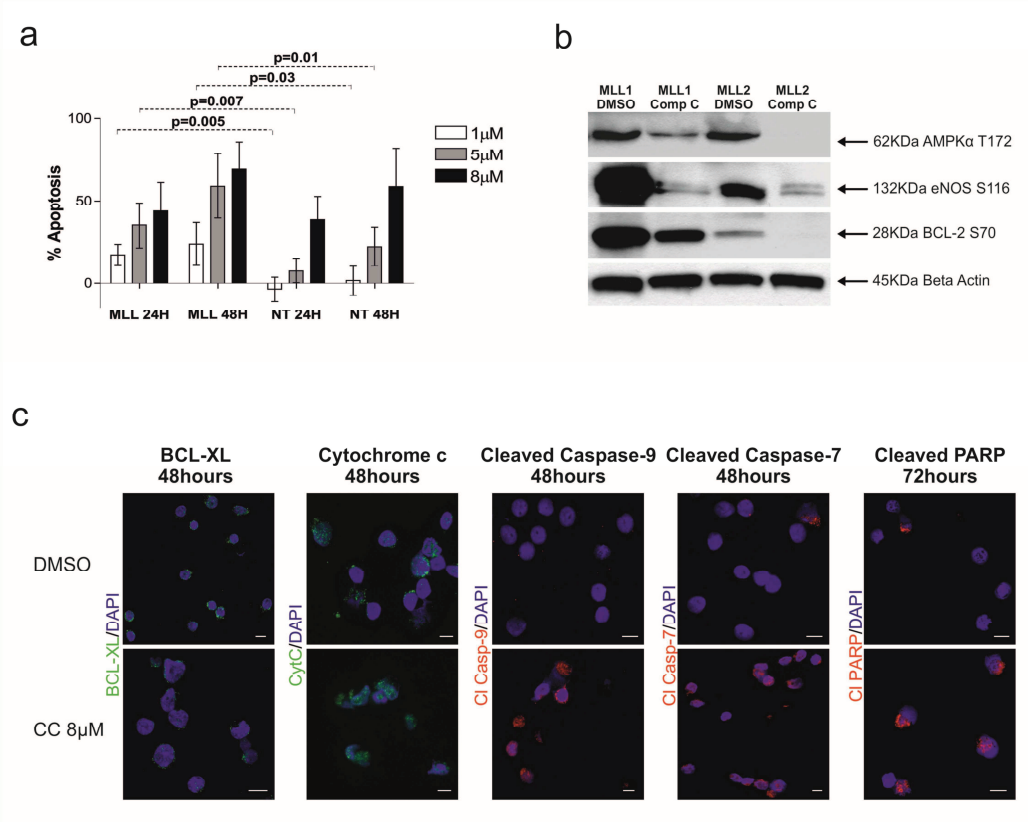


Fig. 6. AMPK inhibition induces apoptosis in primary leukemia cultures. (a) Primary cells from nine patients (five MLL-rearranged and four non-translocated) were cultured on MSCs layer and treated with compound C (CC, 1–5–8 μM) for 24 and 48 h. Apoptosis was determined by flow cytometry with Annexin V–PI staining. DMSO-treated cells viability was set to 100%. Results represent mean±s.e.m. MLL-rearranged primary cells undergo more apoptosis than the non-translocated ones at 24 and 48 h after treatment with 1 and 5 μM CC. (b) Inhibition of the AMPK pathway activation was analyzed by western blot in control and CC (5 μM, 48 h) treated patients'cells. Representative results of two MLL-rearranged patients are shown. (c) Apoptotic proteins expression was determined by immunofluorescence after CC (8 μM, 48 and 72 h) treatment in one MLL-rearranged primary culture. Panel shows pseudo-color merged confocal images of cells decorated with 4,6-diamidino-2-phenylindole (DAPI), Alexa-fluor 488 and 594. Scale bar 5 μm.

AMPK inhibition synergizes with drugs used in ALL chemotherapy MLL-rearranged SEM, RS4;11 and ALL-PO cells were exposed to five drugs, namely daunorubicin, vincristine, cytarabine, dexamethasone and L-asparaginase in the presence or absence of the AMPK inhibitor compound C. The complete dose-response curves are shown in **Supplementary Figures S4a** (SEM), b (RS4;11) and c (ALL-PO). We estimated the concentration of each drug that caused 50% of growth inhibition with or without the compound C added at fixed combination ratio as indicated in **Table 1** and searched for synergistic interactions. As can be observed, the addition of compound C significantly lowered the GI50 of all drugs in ALL-PO cells, whereas in RS4;11 we did not observe any variation of the efficacy of vincristine and dexamethasone. The addition of compound C failed to increase the efficacy of dexamethasone also in SEM cells. For the other conditions, the calculated combination indexes showed values <1, indicating that compound C strongly synergizes with these chemotherapeutic drugs, and at the same time suggesting the potential synergistic effect of AMPK inhibition in combination with chemotherapeutic drugs with different mechanisms of action in the treatment of MLL-rearranged BCP-ALL.

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Table 1 Treatment of MLL-rearranged BCP-ALL cell lines with Compound C and drugs used in ALL chemotherapy

	Vcr	*Vcr+CC (1:100)*	*Ara-C*	*Ara-C+CC (1:0.1)*	*Dex*	*Dex+CC (1:1)*	*Dauno*	*Dauno+CC (1:1)*	*L-Asp**	*L-Asp+CC* (1:0.1)*
SEM										
GI_{50} (nM)	18.9±4.9	6.5±5.7	133.0±47.9	4.1±0.86	2.2±0.2	5.5±0.6	171.0±44.1	5.8±2.1	0.61±0.09	0.12±0.02
CI at GI_{50}		0.012		0.007		9.3		0.12		0.196
RS4;11										
GI_{50} (nM)	7.0±1.1	7.8±1.2	69.7±6.5	54.5±5.3	0.51±0.2	0.41±0.14	171.0±44.1	4.5±1.5	0.13±0.02	0.00023±0.00002
CI at GI_{50}		1.18		0.48		1.18		0.04		0.03
ALL-PO										0.19±0.08
GI_{50} (nM)	11.0±3.1	0.91±0.42	59.7±5.6	12.8±4.3	2.3±0.9	0.48±0.1	221.3±35.2	36.4±4.2	0.70±0.05	0.105
CI at GI_{50}		0.021		0.202		0.196		0.143		

* Data expressed in UI/ml
Abbreviations: Vcr, vincristine; Ara-C, cytarabine; Dauno, daunorubicin; Dex, dexamethasone; L-Asp, L-asparaginase; CC, Compound C; CI, Combination Index.
Synergy, additivity and antagonism are defined by a CI<1, CI=1, or CI>1 respectively.

Table 1: Treatment of MLL-rearranged BCP-ALL cell lines with compound C and drugs used in ALL chemotherapy

Discussion

In this study we report that the activation of the serine/threonine kinase AMPK is a key factor for the survival of BCP-ALL MLL-rearranged cells. Inhibition of AMPK activity caused a decrease in cell proliferation and survival both in cell lines and primary cultures, and it synergistically enhance the antiproliferative activity of ALL chemotherapeutic drugs, thus pointing out this cellular fuel sensor as a promising new therapeutic target.

AMPK has been frequently proposed as a potential therapeutic target in cancer because of its position downstream of tumour suppressor LKB1 and its known role in the regulation of cell proliferation, cell cycle progression and autophagy via mTORC1, p53 and p27.11 Its activation through Metformin or AICAR treatment can suppress cell proliferation and induce apoptosis in many cell types, i.e. melanoma,[26] glioblastoma,[27] breast cancer[28] and renal cell carcinoma.[29] Two studies[30,31] showed that AMPK activation by AICAR can induce apoptosis in some BCP-ALL cell lines, but MLL-rearranged cells were not included in these studies. Interestingly, there is emerging evidence that AMPK activation has a dual role in cancer depending on alterations of interconnected signalling pathways, thus its effects are likely to be cell-type and tissue specific. Recent papers report that AMPK is hyperactivated in some cancers and increased cell death is observed after inhibition of its activity. Compound C treatment or AMPK depletion by small interfering RNA induce growth arrest and apoptosis in human prostate cancer,[17] multiple myeloma,[16] human and rat glioma[18] and pheochromocytoma cell lines.[32] AMPK activity was also reported to be higher in some cancer cell lines such as OVCAR3 and A431 than in primary keratinocytes.[33] Moreover, increased expression of LKB1 and AMPK was observed in UVB induced murine basal cell carcinoma.[34] These findings, together with our previous observations that the AMPK pathway is hyperactivated in MLL-rearranged BCP-ALL cells and that the AMPK inhibitor Compound C induces dephosphorylation of AMPK and its downstream targets,19 prompted us to study the effects of AMPK inhibition on the survival of MLL-rearranged blast cells. We first used the commercially available AMPK inhibitor Compound C to treat MLL-rearranged and non-translocated cell lines. MLL-rearranged cells show decrease of proliferation and survival at really low concentrations of Compound C with respect to non translocated cells, with very different levels of GI50 and LC50. The specific silencing of AMPK by two different shRNA induced a decrease of cell survival similarly to Compound C treatment, thus what we observed after Compound C treatment can be mainly attributed to AMPK inhibition. Of note, in non-translocated cells the activation of the pathway downstream AMPK is not modified by specific silencing, confirming our idea that this pathway is specific for MLL-rearranged cells. These results prove that AMPK activation has a very important role in

supporting the survival of MLL-rearranged cells, thus AMPK could be specifically targeted in order to induce cell death. We then described that Compound C-induced apoptosis follows the mitochondrial pathway. We observed mitochondrial membrane depolarization, ROS production, Cytochrome C release and activation of Caspases followed by PARP cleavage. We previously reported that Compound C treatment also brings to a reduction of BCL-2 S70 levels.[19] Compound C-induced apoptosis was already shown to be related to ROS production and BCL-2 downregulation also in multiple myeloma[16] and in glioma cells,[18] thus supporting our findings. Of note, not only cell lines but also MLL-rearranged primary leukemia cells responded to AMPK inhibition with Compound C. MLL-rearranged BCP-ALL primary cultures underwent much more apoptosis than those lacking MLL rearrangements after exposure to low concentrations of Compound C, indicating that AMPK is critical for cell survival also in patients' primary cells. Interestingly, apoptosis occurred after an initial cell cycle block at the G_2/M phase. In agreement with these findings, inhibition of the AMPK pathway in glioma cells18 also brings to G_2/M arrest. Of note, in *Drosophila melanogaster* the activation of the LKB1-AMPK pathway is strictly required for an accurate mitosis and chromosome segregation.[35,36] We also investigated the effects of the simultaneous treatment of SEM cells with Compound C vincristine, daunorubicin and cytarabine. Our results show that AMPK inhibition enhanced the chemotherapeutic effects of these drugs significantly lowering their GI50 values. These results suggest that AMPK inhibition could augment the effects of conventional chemotherapy for MLLrearranged BCP-ALL. Further studies are needed to better understand the molecular mechanism(s) involved in the synergistic effect.

In conclusion, this study provides new insights into the role of AMPK in cancer and, in particular, in BCP-ALL. AMPK activation is required for MLL-rearranged cell survival and its inhibition is able to induce cell death. For these reasons, AMPK could be considered as a new drug target in MLLrearranged leukemias and kinase inhibitors targeting AMPK should be further studied to make new therapeutic options available for this high-risk form of leukemia.

Supplementary information

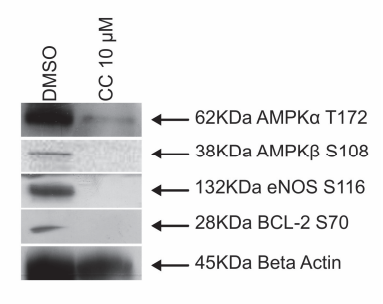
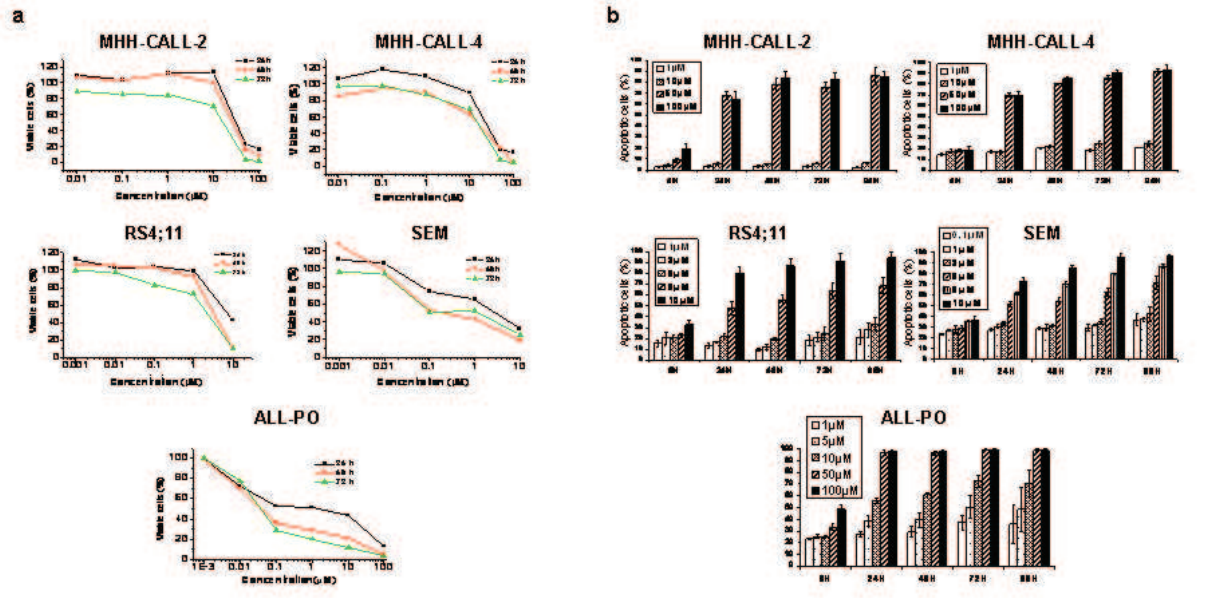


Fig. S1: Inhibition of the AMPK pathway in ALL-PO cell treated with Compound C (CC 10µM, 48 hours).

Supplementary Figure S2



GI_{50} (µM)	MHH-CALL-2	MHH-CALL-4	RS4;11	SEM	ALL-PO
24 hours	31.4	24.8	7.1	2.8	2.27
48 hours	**25.7**	**19.1**	**3.2**	**0.16**	**0.062**
72 hours	16.6	16.3	2.3	0.09	0.061

LC_{50} (µM)	MHH-CALL-2	MHH-CALL-4	RS4;11	SEM	ALL-PO
24 hours	41.6	38.7	9	9.8	16.7
48 hours	**37.5**	**31.4**	**8.5**	**7.5**	**9.7**
72 hours	37.3	29.2	8	6.4	7.1

Fig. S2: (a) Cells were treated with Compound C at different concentrations ranging from 0.001µM up to 100µM for different times (24-72 hours). Cell proliferation rates were determined through MTT assay. DMSO treated cells viability was set to 100%. The GI50 (Growth Inhibition50= compound concentration required to inhibit cell proliferation by 50%) was calculated by plotting the data as a logarithmic function of (x) when viability was 50%. (b) Cells were treated with Compound C at different concentrations ranging from 0.1µM up to 100µM for different times (6-96 hours). Cell viability was determined by flow cytometry with Annexin V-PI staining. DMSO treated cells viability was set to 100%. Results represent Mean of 3 independent experiments ± SEM. The LC50 (Lethal Concentration50= compound concentration required to induce cell mortality by 50%) was calculated by plotting the data as a logarithmic function of (x) when viability was 50%.

Supplementary Figure S3

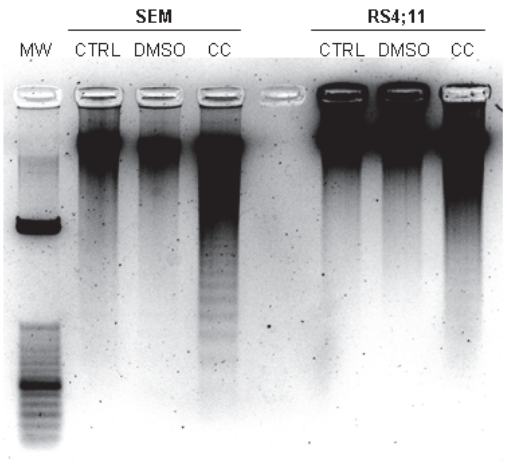


Fig. S3: DNA Laddering. DNA fragmentation in MLL-rearranged cell lines after Compound C (CC 8µM, 72 hours) treatment. DNA was extracted from Compound C (CC) treated (8µM, 72 hours) and control human MLL-rearranged leukemia cell lines with the Puregene Cell and Tissue Kit (Qiagen) following manufacturer's instructions. DNA was extracted from 1 X 106 cells and finally resuspended in 50µL of DNA hydratation solution. 1µg of DNA was loaded on 1% agarose gel and the gel run for 2 hours at 40V. DNA fragments were visualized using the ImageQuant 300 (GE Healthcare).

Supplementary Figure S4

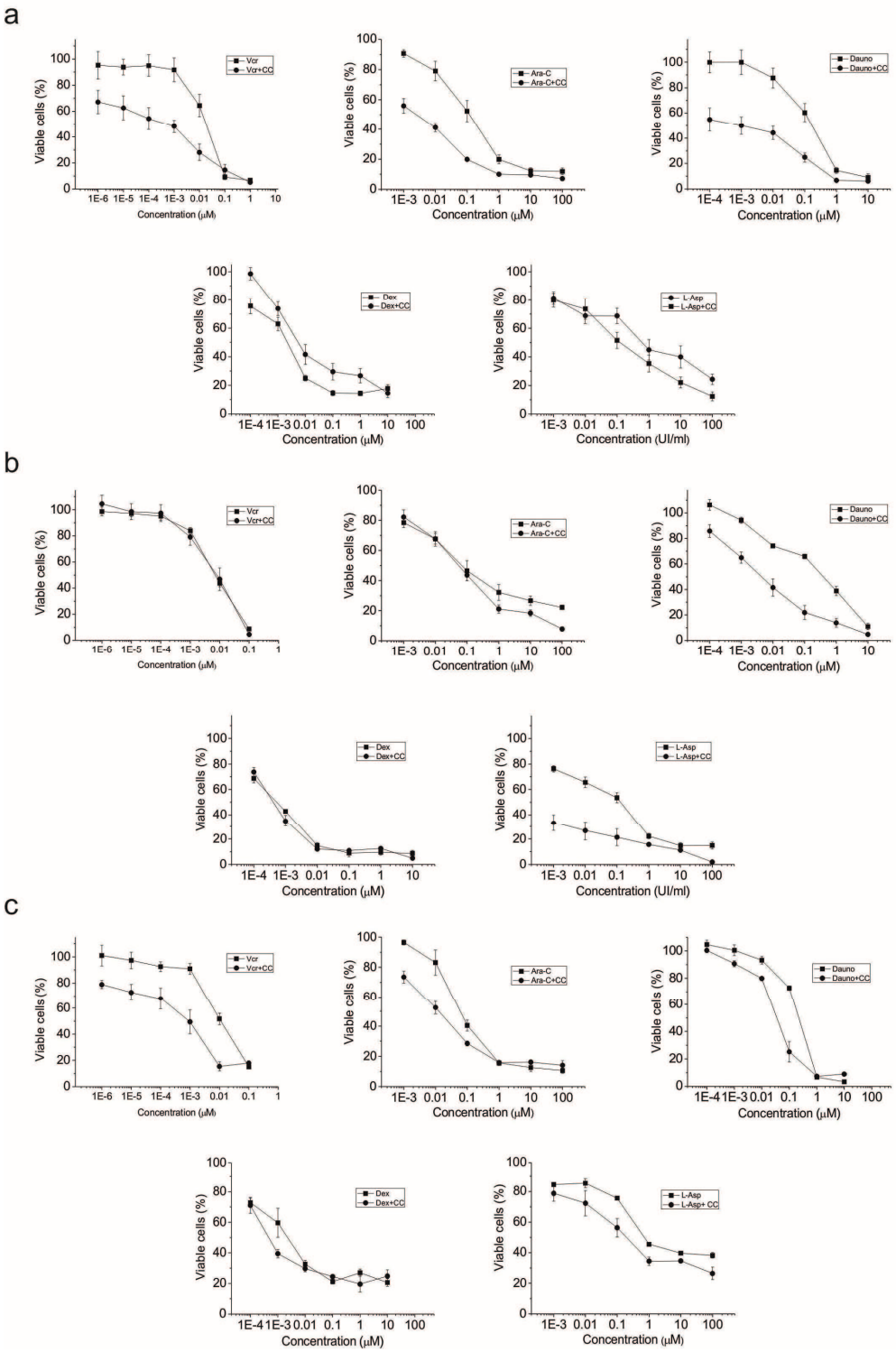


Fig. S4: SEM (a), RS4;11 (b) and ALL-PO (c) cells were treated with the indicated concentrations of drugs in the presence of CC at the molar ratio (drug:CC) of 1:100 for vincristine (Vcr), 1:0.1 for cytarabine (Ara-C), 1:1 for daunorubicin (Dauno), 1:1 for dexamethasone (Dex) and 1:0.1 for L-asparaginase (L-Asp). Cell viability was determined by MTT test after 48 hours of treatment. Data are expressed as mean±SEM of 4-5 independent experiments performed in triplicate.

References

1. Hardie DG, Carling D, Carlson M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* 1998; 67: 821-855.
2. Hardie DG, Hawley SA, Scott JW. AMP-activated protein kinase--development of the energy sensor concept. *J Physiol* 2006; 574: 7-15.
3. Xiao B, Heath R, Saiu P, Leiper FC, Leone P, Jing C *et al.* Structural basis for AMP binding to mammalian AMP-activated protein kinase. *Nature* 2007; 449: 496-500.
4. Alessi DR, Sakamoto K, Bayascas JR. LKB1-dependent signaling pathways. *Annu Rev Biochem* 2006; 75: 137–163.
5. Motoshima H, Goldstein BJ, Igata M, Araki E. AMPK and cell proliferation--AMPK as a therapeutic target for atherosclerosis and cancer. *J Physiol* 2006; 574: 63-71.
6. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, *et al.* Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 2002; 8: 1288-1295.
7. Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Müller C, Carling D, *et al.* Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 2002; 415: 339-343.
8. Hardie DG, Carling D. The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* 1997; 246: 259-273.
9. Hardie DG. The AMP-activated protein kinase pathway--new players upstream and downstream . *J Cell Sci* 2004; 117: 5479-5487.
10. Wang W, Guan KL. AMP-activated protein kinase and cancer. *Acta Physiol (Oxf)* 2009; 196: 55-63.
11. Fogarty S, Hardie DG. Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. *Biochim Biophys Acta* 2010; 1804: 581-591.
12. Green AS, Chapuis N, Maciel TT, Willems L, Lambert M, Arnoult C *et al.* The LKB1/AMPK signaling pathway has tumor suppressor activity in acute myeloid leukemia through the repression of mTOR-dependent oncogenic mRNA translation. *Blood* 2010; 116: 4262-4273.
13. Zhou W, Han WF, Landree LE, Thupari JN, Pinn ML, Bililign T *et al.* Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells. *Cancer Res* 2007; 67: 2964-2971.
14. Rattan R, Giri S, Singh AK, Singh I. 5-Aminoimidazole-4-carboxamide-1-beta-Dribofuranoside inhibits cancer cell proliferation in vitro and in vivo via AMP-activated protein kinase. *J Biol Chem* 2005; 280: 39582-39593.

15. Okoshi R, Ozaki T, Yamamoto H, Ando K, Koida N, Ono S *et al.* Activation of AMPactivated protein kinase induces p53-dependent apoptotic cell death in response to energetic stress. *J Biol Chem* 2008; 283: 3979-3987.

16. Baumann P, Mandl-Weber S, Emmerich B, Straka C, Schmidmaier R. Inhibition of adenosine monophosphate-activated protein kinase induces apoptosis in multiple myeloma cells. *Anticancer Drugs* 2007; 18: 405-410.

17. Park HU, Suy S, Danner M, Dailey V, Zhang Y, Li H *et al.* AMP-activated protein kinase promotes human prostate cancer cell growth and survival. *Mol Cancer Ther* 2009; 8: 733-741.

18. Vucicevic L, Misirkic M, Janjetovic K, Harhaji-Trajkovic L, Prica M, Stevanovic D *et al.* AMPactivated protein kinase-dependent and -independent mechanisms underlying in vitro antiglioma action of compound C. *Biochem Pharmacol* 2009; 77: 1684-1693.

19. Accordi B, Espina V, Giordan M, VanMeter A, Milani G, Galla L *et al.* Functional protein network activation mapping reveals new potential molecular drug targets for poor prognosis pediatric BCP-ALL. *PLoS One* 2010; 5: e13552.

20. Viola G, Fortunato E, Cecconet L, Del Giudice L, Dall'Acqua F, Basso G. Central role of mitochondria and p53 in PUVA-induced apoptosis in human keratinocytes cell line NCTC-2544. *Toxicol Appl Pharmacol* 2008; 227: 84-96.

21. Indraccolo S, Tisato V, Tosello V, Habeler W, Esposito G, Moserle L *et al.* Interferon-alpha gene therapy by lentiviral vectors contrasts ovarian cancer growth through angiogenesis inhibition. *Hum Gene Ther* 2005; 16: 957-970.

22. Accordi B, Pillozzi S, Dell'Orto MC, Cazzaniga G, Arcangeli A, Kronnie GT *et al.* Hepatocyte growth factor receptor c-MET is associated with FAS and when activated enhances drug-induced apoptosis in pediatric B acute lymphoblastic leukemia with TELAML1 translocation. *J Biol Chem* 2007; 282: 29384-29393.

23. Basso G, Buldini B, De Zen L, Orfao A. New methodologic approaches for immunophenotyping acute leukemias. *Heamatologica* 2001; 86: 675–692.

24. Mihara K, Imai C, Coustan-Smith E, Dome JS, Dominici M, Vanin E *et al.* Development and functional characterization of human bone marrow mesenchymal cells immortalized by enforced expression of telomerase. *Br J Haematol* 2003;120: 846-849.

25. Chou TC, Talalay P . Analysis of combined drug effects: a new look at a very old problem.*Trends Pharmacol Sci* 1983;4*:* 450–454.

26. Chen MB, Shen WX, Yang Y, Wu XY, Gu JH, Lu PH. Activation of AMP-activated protein kinase is involved in vincristine-induced cell apoptosis in B16 melanoma cell. *J Cell Physiol* 2011; 226: 1915-1925.

27. Zhang WB, Wang Z, Shu F, Jin YH, Liu HY, Wang QJ *et al.* Activation of AMP-activated protein kinase by temozolomide contributes to apoptosis in glioblastoma cells via p53 activation and mTORC1 inhibition. *J Biol Chem* 2010; 285: 40461-40471.
28. Liu B, Fan Z, Edgerton SM, Deng XS, Alimova IN, Lind SE *et al.* Metformin induces unique biological and molecular responses in triple negative breast cancer cells. *Cell Cycle* 2009; 8: 2031-2040.
29. Woodard J, Joshi S, Viollet B, Hay N, Platanias LC. AMPK as a therapeutic target in renal cell carcinoma. *Cancer Biol Ther* 2010; 10: 1168-1177.
30. Sengupta TK, Leclerc GM, Hsieh-Kinser TT, Leclerc GJ, Singh I, Barredo JC. Cytotoxic effect of 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) on childhood acute lymphoblastic leukemia (ALL) cells: implication for targeted therapy. *Mol Cancer* 2007; 6: 46.
31. Kuznetsov JN, Leclerc GJ, Leclerc GM, Barredo JC. AMPK and Akt Determine Apoptotic Cell Death following Perturbations of One-Carbon Metabolism by Regulating ER Stress in Acute Lymphoblastic Leukemia. *Mol Cancer Ther* 2011; 10: 437-447.
32. Shaw MM, Gurr WK, McCrimmon RJ, Schorderet DF, Sherwin RS. 5'AMP-activated protein kinase alpha deficiency enhances stress-induced apoptosis in BHK and PC12 cells. *J Cell Mol Med* 2007; 11: 286-29
33. Mizrachy-Schwartz S, Cohen N, Klein S, Kravchenko-Balasha N, Levitzki A. Up-regulation of AMPK in cancer cell lines is mediated through c-Src activation. *J Biol Chem* 2011; 286: 15268-15277.
34. Byekova YA, Herrmann JL, Xu J, Elmets CA, Athar M. Liver kinase B1 (LKB1) in the pathogenesis of UVB-induced murine basal cell carcinoma. *Arch Biochem Biophys* 2011; 508: 204-211.
35. Lee JH, Koh H, Kim M, Kim Y, Lee SY, Karess RE *et al.* Energy-dependent regulation of cell structure by AMP-activated protein kinase. *Nature* 2007; 447: 1017-1020.
36. Bonaccorsi S, Mottier V, Giansanti MG, Bolkan BJ, Williams B, Goldberg ML *et al.* The Drosophila Lkb1 kinase is required for spindle formation and asymmetric neuroblast division. *Development* 2007; 134: 2183-2193.

CHAPTER II

“New implication for the LKB1 pathway: regulation of apoptosis”

II. INTRODUCTION

II.I LKB1

The *LKB1* gene (also known as serine/threonine kinase 11, *STK11*) was discovered only in 1998 as a new tumor suppressor gene by linkage analysis of Peutz-Jeghers syndrome (PJS) (Hemminki A. *et al.,* 1998; Jenne D.E. *et al.,* 1998). The *LKB1* gene maps in the chromosomal region 19p13.3, spans 23kb and is made up of nine coding exons and a final non-coding exon (Hemminki A. *et al.,* A 1998). *LKB1* encodes for a mRNA of ~2.4kb transcribed in telomere-to-centromere direction and for a protein of 433 amino acids and ~48kDa (Hemminki A. *et al.,* A 1998). LKB1 has now been classified as a member of the calcium/calmodulin regulated kinase-like family, which is part of the Ca^{2+}/calmodulin kinase group (http://www.kinase.com), and is a master kinase that can potentially activate several downstream kinases by phosphorylating a conserved threonine in their activation loops (Lizcano J.M., *et al.*, 2004). Among LKB1 targets there are the two extensively characterized kinases partitioning defective gene 1 (Par1)/ microtubule affinity-regulation kinases (MARKs) and AMP-activated protein kinase (AMPK) (Benton R. and St Johnston D. 2003; Cohen D., *et al.,* 2004; Kahan B.B., *et al,.* 2005; Shaw R., *et al,.* 2005; Liang J., *et al.*, 2007; Hemminki A., 1999). LKB1 can also phosphorylate and activate 13 more protein kinases part of the AMPK family (Lizcano J.M., *et al.*, 2004). Therefore, LKB1 is a multitasking kinase, with great potential in orchestrating cell activity. Indeed LKB1 has been found to play a role in cell cycle polarity (Baas A.F., *et al,.* 2004), energy metabolism (Hurov J.B., *et al.*, 2007), apoptosis, cell cycle arrest and cell proliferation (Marignani P.A., *et al.,* 2001; Alessi D.R., *et al.*, 2006).

LKB1 gene encodes for two splice variants, $LKB1_l$ and $LKB1_s$, the latter being recently discovered. Both isoforms contain the same eight first coding exons but they differ in the ninth and last one: $LKB1_s$ has a shorter C-terminal with 39 instead of 63 residues, and lacks the serine 428 (Ser428) phosphorylation site and the cysteine 430 (Cys430) farnesylation site (Denison F.C., *et al.*, 2009). However the significance of these differences is not well understood as both proteins have similar cellular localization and as these residues are not essential for the kinase activity (Alessi D.R., *et al.,* 2006). Both isoforms are ubiquitously expressed, but $LKB1_s$ is the dominant isoform in spleen and testis where it seems to be involved in spermatogenesis and male knockout mice for this isoform are sterile (Denison F.C., *et al.*, 2009; Towler M.C., *et al.*, 2008).

LKB1 mRNA transcript is translated in a protein of 433 amino acids that weights 48.6kDa (reference Q15831 in Uniprot). The protein, which has a serine-threonine kinase

activity, possesses a nuclear localization signal in the N-terminal non catalytic region (residues 38-43) and a kinase domain (residues 49-309) (Alessi D.R., *et al.,* 2006). The protein is localized predominantly in the cytoplasm (Alessi D.R., *et al.,* 2006), but a fraction localizes also in the nucleus, membrane and mitochondria (Baas A.F., *et al.,* 2003; Karuman P., *et al.,* 2001; Boudeau J., *et al.,* 2003; Zeng P.Y., *et al.,* 2006).

Human LKB1 shows strong homology with the cytoplasmic serine/threonine kinases of several organisms, including *Xenopus laevis* egg and embryonic kinase I (XEEK1), mouse LKB1, *Caenorhabditis elegans* portioning defective gene 4 (Par-4) and *Drosophila* LKB1 (Su J.Y., *et al.,* 1996; Smith D.P., *et al.,* 1999; Watts J.L., *et al.,* 2000; Martin S.G., *et al.,* 2003).

The C- and N-terminals non-catalytic regions of LKB1 are not related to any other proteins and posses no identifiable functional domains (Hemminki A., *et al.,* 1998; Jenne D.E., *et al.,* 1998).

LKB1 contains at least four auto-phosphorylation sites on Thr185, Thr189, Thr336 and Thr402, and five phosphorylation sites, Ser31, Ser307, Ser325, Ser428 and Thr363 (which also may be autophosphorylated), one farnesylation site, Cys430, and one acetylation site on Lys48 (**Fig. II.1**).

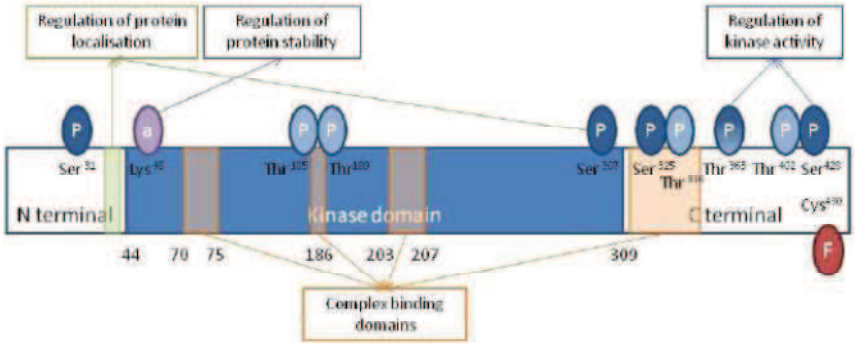


Fig. II.1: LKB1 protein domains. Farnesylation site indicated by F, acetylation by a, phosphorylation by P (dark blue for phosphorylation by other kinases and pale blue for autophosphorylation sites).

II.II The LKB1 complex

In vivo, LKB1 forms an heterotrimeric complex with two proteins termed STE20-related adaptor (STRAD) and mouse-protein 25 [MO25 or calcium binding protein 39 (CAB39)], that play a crucial role in regulating LKB1 protein stability, kinase activity and cellular localization (Boudeau J., *et al.,* 2003a; Boudeau J., *et al.,* 2004; Baas A.F., *et al.,* 2003). STRAD is a pseudokinase so lacks any activity; however, once in the complex, its closed conformation with an ordered activation loop is typical of active kinases. MO25 stabilizes the complex by binding both STRAD and LKB1 (Boudeau J., *et al.,* 2003a), as a scaffold

protein; more precisely, MO25 interacts with STRAD regulatory helix and with LKB1 activation loop in the C-terminus (Zeqiraj E., *et al.,* 2009a). The interaction between STRAD and MO25 facilitates the allosteric conformational change of STRAD into a closed conformation and ATP binding. Both these proteins are required to fully activate LKB1 (Zeqiraj E., *et al.,* 2009b), indeed LKB1 is barely active as a monomer as demonstrated by mutations in the STRAD-binding domain or by the use of siRNA against STRAD (Baas A. F., *et al.,* 2003). The binding of STRAD to LKB1 substantially activates through conformational change the autophosphorylation ability of LKB1 and hence increasing of 100-fold its capacity to phosphorylate downstream substrates (Hawley S.A., *et al.,* 2003).
The structure of the complex was discovered recently by crystallography (PDB reference 2WTK). Once in the complex, LKB1 stably acquires a canonical closed conformation with a reorder of its activation loop, which is typical of activated kinases (Zeqiraj E., *et al.,* 2009a). To achieve this, STRAD acquires a closed conformation and binds to LKB1 kinase domain. Once bound, the two proteins are phosphorylated by LKB1, on Thr329 and Thr419 STRAD, and on Thr336 and Thr363 LKB1 itself. Mutations on those 4 residues do not affect the complex formation and the activity of LKB1 kinase.
Once synthesized, LKB1 protein is imported into the nucleus by importins α and β. Instead, STRAD and MO25 diffuse passively to the nucleus. By binding to its complex, LKB1 is re-localized to the cytoplasm, where it mainly plays its role (Baas A.F., *et al.,* 2003). STRAD was identified to play an important role in the LKB1 localization, promoting the complex export from the nucleus through its binding to CRM1 and exportin-7 (Dorfman J. and Macara I.G. 2008). It also prevents the re-localization to the nucleus competing with the importins binding site of LKB1. In conclusion, the formation of the LKB1 complex is the essential step in the kinase regulation: it promotes its activity both directly, by an allosteric mechanism, and indirectly, by the control of its localization.
However, yet little is known on how the formation of the complex is regulated. One hypothesis involves the chaperones Hsp90 and Cdc37 (cell division cycle 37 homolog, on chr 19p13.2). As the LKB1 complex does not spontaneously arise when mixing the three proteins (Boudeau J., *et al.,* 2003a), the two chaperones may be required to facilitate its assembly. Moreover, Hsp90 can stabilize LKB1: use of Hsp90 inhibitors like geldanamycin induced degradation of LKB1 by the proteosome (Boudeau J., *et al.,* 2003b). No other protein has been described to interact with the complex, and complex formation is not dependent on phosphorylation of any of the three kinases. The regulation of LKB1 complex activity could also be achieved by regulation of STRAD and MO25 protein expression.

II.III Function of LKB1

The main function of LKB1 is to activate by phosphorylation the AMPK subfamily of kinases (or AMPK-related kinases). This subfamily contains 14 identified members: AMPK 1 and 2, SIK 1 and 2, NUAK 1 and 2, MARK 1, 2, 3 and 4, BRSK 1 and 2, QSK and SNRK (Jaleel M., *et al.,* 2005; Lizcano J.M., *et al.,* 2004). Upon phosphorylation by LKB1, their activity is enhanced of 50-fold (Alessi D.R., *et al.,* 2006). They have been associated with pathways implicated in cell metabolism, cell polarity and apoptosis as detailed below. They have been identified by sequence homology in their kinase domain. However, they have different length and different functional domains. It is important to note that many of the known substrates are expressed in a tissue-specific manner and may not explain ubiquitous effects of LKB1 and its downstream kinases in all cell types. Importantly, to date there is no substantive mutational data from human tumors to specifically support any of the downstream kinases, including the two AMPK catalytic genes, as being a particularly critical target of LKB1 in tumor suppression. One confounding issue with the lack of mutations found in these downstream kinases is that there is a great deal of redundancy among them, suggesting that loss of any one of them may be compensated for by other family members, unlike the case for LKB1 for which no other specific kinase has been shown to compensate in vivo (Alessi D.R., *et al.,* 2006).

LKB1 has also been reported to interact with other pathways regulating cell differentiation, via an unknown mechanism. This multi-functional role is illustrated in **Figure II.2**.

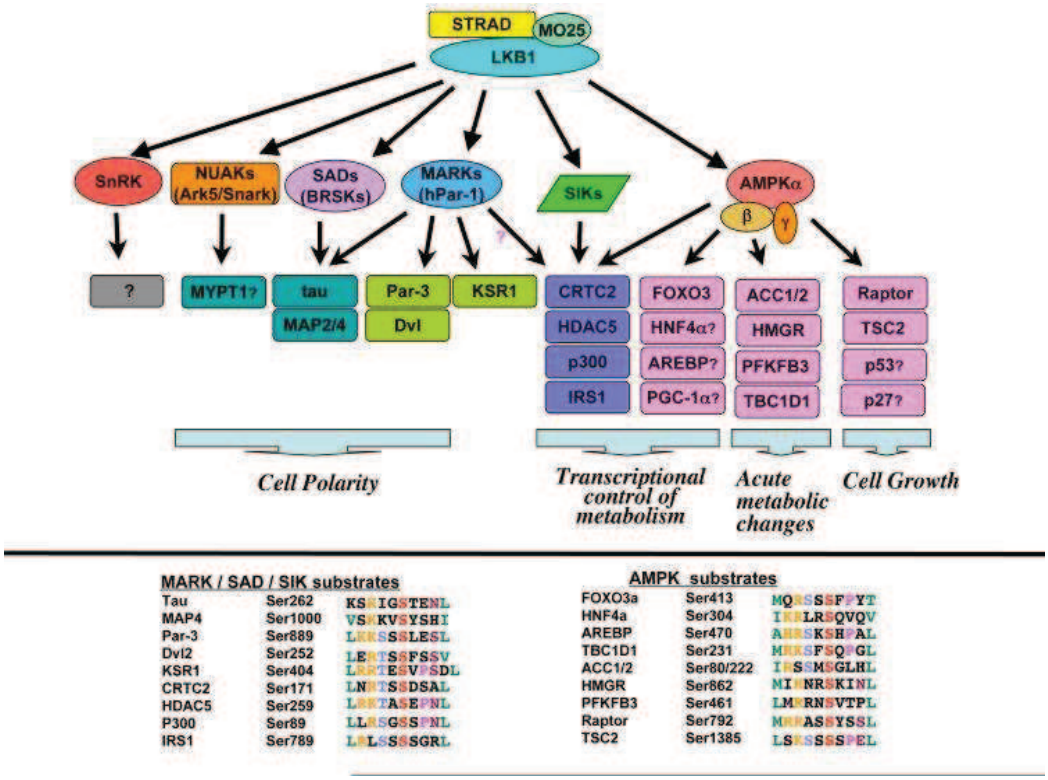


Fig. II.2: LKB1 in complex with its two regulatory subunits STRAD and Mo25 directly phosphorylates and activates a family of 14 AMPK-related kinases. All well-established substrates of AMPK and its related family members are shown, and those for which further in vivo data is needed are shown with a question mark. Bottom: The sequences flanking the best-characterized phosphorylation site in each substrate with those residues selected for from in vitro peptide library and alanine scanning peptide mutagenesis studies highlighted.

The first identified and best known effector of LKB1 is AMPK. AMPK is first activated by the binding to AMP in its γ subunit (Carling D., *et al.*, 2008). This enforces an allosteric mechanism and eventually inhibits the dephosphorylation of AMPK Thr172 site on the catalytic subunit, required for complete activation. This phosphorylation can be performed either by LKB1 as shown by biochemical assays (Hawley S.A., *et al.*, 2003; Shaw R.J., *et al.*, 2004) and by the Ca2+/calmodulin-dependent protein kinase β (CaMKKβ). LKB1-mediated activation takes place in response to AMP/ATP increased ratio, whereas CaMKKβ-mediated activation happens in the presence of increased Ca2+ levels in the cytoplasm (Carling D., *et al.*, 2008; Jansen M., *et al.*, 2009).

SIK1 and 2 (salt-inducible kinase 1, on chr 21q22.3 and salt-inducible kinase 2 or QIK, on chr 11q23.1) have also been reported to play a role in cell metabolism by phosphorylation of CRTC, like AMPK. SIK1 was shown to be active in adrenocortical cells (Katoh Y., *et al.*, 2004), and SIK2 in adipose tissue (Horike N., *et al.*, 2003) and pancreatic islet cells (Screaton R.A., *et al.*, 2004), and both were reported to act in the liver (Shaw R.J., *et al.*, 2005). The SIK kinases can be inactivated by hormones (Katoh Y., *et al.*, 2004). This could explain how the action of AMPK and SIK kinases on the same

targets is coordinated: one is responding to cellular energy levels, and one to external hormonal stimuli. In addition to its part in cell metabolism, a novel role of SIK1 has been discovered recently, linking it to p53 and anoikis (Cheng H., *et al.*, 2009). SIK1 was identified as a regulator of p53 by phosphorylation in anchorage independent conditions by siRNA screening in a soft agarose assay. Loss of SIK1 enabled cells to survive in those conditions and promoted metastasis *in vivo*. Similarly, AMPK has been reported to control a p53-mediated apoptosis response but in conditions of energy stress (Jones R. G., *et al.*, 2005; Shaw R.J., *et al.*, 2004).

NUAK 1 (NUAK family SNF1-like kinase 1 or ARK5, on chr 12q23.3) and NUAK 2 (NUAK family SNF1-like kinase 2 or SNARK, on chr 1q32.1) have also been associated with resistance to apoptosis, one in nutrient starvation and the other in glucose starvation (Suzuki A., *et al.*, 2003) or in tumor cell lines (Legembre P., *et al.*, 2004).

The role of LKB1 in cell polarity has been first discovered by genetic screening of its orthologs, PAR in *Caenorhabditis elegans* and dLKB1 in *Drosophila melanogaster* (Alessi D.R., *et al.*, 2006). The PAR family can define different membrane domains, hereby polarizing the cell; PAR1, (MARK and BRSK in humans) for the lateral domain, and PAR3 and 6 for the apical domain, (Jansen M., *et al.*, 2009). The protein PAR5 (14-3-3 protein) and PAR4 (LKB1) can respectively inhibit or activate by phosphorylation the effectors, PAR1, 3 or 6. They regulate this polarization mechanism and control asymmetric division, which is an important process in several epithelial tissues. Even though these mechanisms are quite different in invertebrates and vertebrates, these studies suggest that LKB1 plays a similar role in humans. This was demonstrated in intestinal epithelial cells as they became polarized following LKB1 expression (Baas A.F., *et al.*, 2004). Mammalian epithelial cells mutants in the C-terminal of LKB1 also failed to become polarized, further confirming this link (Forcet C., *et al.*, 2005). AMPK was shown to be one of the LKB1 effectors implicated in cell polarization (Zhang L., *et al.*, 2006; Zheng B. and Cantley L.C., 2007), by potentially targeting myosin regulatory light chains. It is of note that NUAK1 may also play a role in cell adhesion by interacting with myosin phosphatases (Zagorska A., *et al.*, 2010).

MARK 1 to 4 (MAP/microtubule affinity-regulating kinase 1 to 4, respectively on chr 1q41, 11q13.1, 14q32.32 and 19q13.32) and BRSK 1 and 2 (BR serine/threonine kinase 1 and 2 or SAD-A and B, respectively on chr 19q13.42 and 11p15.5) share homology with the PAR1 proteins. There is little evidence of the role of MARK proteins other than by homology. MARK proteins were reported to phosphorylate Tau proteins, which are

best known for their major role in the onset of Alzheimer's disease. This may lead to the destabilization of microtubules and, overall, cell polarity (Katajisto P., *et al.*, 2007). They are also thought to phosphorylate the microtubule-associated proteins, and can then re-shape microtubules skeleton, centrosome positioning and cell polarization (Drewes G., *et al.*, 1998; Kojima Y., *et al.*, 2007). BRSK proteins have been detected mainly in the brain. Mice lacking those two isoforms cannot form axons and dendrites due to a deficiency in their neuron polarization (Kishi M., *et al.*, 2005).

The roles of other kinases activated by LKB1 are not well defined. QSK (or SIK family kinase 3 on chr 11q23.3) has been identified to play a role in cell cycle progression by a knock-down experiment in *Drosophila* (Bettencourt-Dias M., *et al.*, 2004). SNRK has been reported to be only present in the testis (Jaleel M., *et al.*, 2005), where the alternative splice variant of LKB1 is present. This may indicate that LKB1 and SNRK cooperate to regulate spermatogenesis. Further studies will possibly uncover novel functions of LKB1.

Beyond its role in the activation of its downstream kinases, LKB1 has been shown to regulate other key signalling pathways by mechanisms that are not fully understood and which may bring to indirect effects. In addition to its role via the interaction with its downstream kinase AMPK, LKB1 is thought to regulate the mTOR pathway by directly phosphorylating PTEN (Mehenni H., *et al.*, 2005b).
Similarly, LKB1 role on migration has not been restricted to its action on cell polarity via MARK and BRSK. It has also been demonstrated to inhibit cell motility (Deguchi A., *et al.*, 2010) via the phosphorylation of PAK1 (p21 protein (Cdc42/Rac)-activated kinase 1 on chr 11q14.1). The interaction of LKB1 with PAK1 and Cdc42, which activates PAK1, has been mentioned before (Zhang S., *et al.*, 2008) and the protein has been shown to co-localize at the leading edge of migrating cells. Furthermore, an integrated genetic and proteomic screen have shown the association of *LKB1* loss with invasion and metastasis in lung cancer, via the activation of EMT and focal adhesion markers (Carretero J., *et al.*, 2010). The authors established that LKB1 and Src were cooperating in an antagonistic fashion for the regulation of both cell growth via the mTOR pathway, and migration via the PAK1 pathway. More evidence of LKB1 role in the p53-pathway has been uncovered with its interaction with p21, downstream target of p53, and with its binding to p53 in the nucleus, stabilizing it by phosphorylation (Zeng P.Y. and Berger S.L., 2006). LKB1 is also recruited by p53 to activate the transcription of p21 by binding to the gene promoter region. This study suggests that LKB1 complex could have a function in the nucleus and not only in the cytoplasm. LKB1 and p21 low expression were correlated in another study to pancreatic cancer. This confirmed the potential interaction between LKB1 and

the p53 pathways to mediate cell cycle arrest (Morton J.P., *et al.*, 2010). In *Xenopus*, inhibition of the ortholog of LKB1, XEEK1, led to developmental abnormalities characteristic of defective Wnt signalling, and XEEK1 was shown to phosphorylate GSK3β, thereby activating the Wnt-β-catenin pathway (Ossipova O., *et al.*, 2003). It was previously shown that Wnt and the mTOR pathway were co-regulated in human due to the involvement of GSK3β and AMPK (Inoki K., *et al.*, 2006); the discovery of Ossipova *et al.* implies that LKB1 could partecipate to this mechanism. On the contrary, LKB1 was shown to inhibit the Wnt pathway as its expression *de novo* induced a dephosphorylation of GSK3β (Lin-Marq N., *et al.*, 2005). It is also possible that LKB1 action on Wnt pathway may be species-dependent, context-dependent (dependent on energy level in the cell), or development-dependent.
Finally, LKB1 has been associated in the cross-talk between cell types via the TGF-β pathway, which regulates cell growth and death. LKB1 expression in endothelial cells promotes vascular smooth muscle recruitment via TGF-β, linking LKB1 and angiogenesis (Londesborough A., *et al.*, 2008). Similarly, *LKB1* loss was shown to disrupt the cross-talk between mesenchymal and epithelial compartment mediated in the intestines by TGF-β, but the exact mechanism leading to the deregulation of TGF-β signalling by LKB1 has to be elucidated (Katajisto P., *et al.*, 2008).

Ultimately, LKB1 can be seen as a master pathway regulator due to its high number of downstream targets and its involvement in many major cell regulatory functions. How LKB1 is directed among its many function is not yet fully understood, but it seems to be cell type dependent and context dependent. The study of LKB1 function suggests that it can integrate environmental signals to activate proper downstream effectors.

II.IV Regulation of LKB1

The regulation of LKB1 activity is a complex process. Although essential, it is not restricted to its binding to STRAD and MO25 as described in paragraph II.II. The study of LKB1 function revealed that its effect is cell type dependant and controlled by environmental stimuli. As little is known on the downstream kinases of LKB1, the regulation of LKB1 has been mainly investigated by the study of its interaction with other molecules in its environment.

There was any paper reporting evidence of direct regulation of LKB1 expression by miRNA since when Godlewski *et al.*, 2010 showed that miR-451 can repress MO25α and

hence regulate the LKB1-AMPK pathway in glioma cells in response to metabolic stress by shifting the equilibrium between cell proliferation and cell migration (Godlewski J., *et al.*, 2010a; Godlewski J., *et al.*, 2010b). miR-451 overexpression reduces the availability of the active LKB1 complex but does not influence the level of expression of LKB1 itself.

Another mechanism for LKB1 regulation can be through the regulation of its localization. LKB1 plays its role mainly in the cytoplasm and is only present there when bound to its complex. But the regulation of this transport has not been fully deciphered.
One hypothesis is that LKB1 re-localization to the cytoplasm is controlled by its phosphorylation by PKC-ζ (or protein kinase C zeta, *PRKCZ*, on chr 1q36.33) on Ser307, a novel phosphorylation site recently discovered (Xie Z., *et al.*, 2009). As AMPK stimulates PKC-ζ activity, this mechanism uncovers a feedback between LKB1 and its activating kinases.
STK11IP (serine/threonine kinase 11 interacting protein or LIP1 on chr 2q35) is reported to play a role in LKB1 localization, but neither its function nor its mechanism of interaction with the LKB1- STRAD-MO25 complex are understood (Smith D.P., *et al.*, 2001). Co-expression of the two proteins (STRADα and MO25) increased the amount of LKB1 in the cytoplasm, and the two proteins may play a role in TGF-β signalling via binding of both STK11IP and LKB1 to SMAD4. The control of LKB1 activity by regulation of its localization is not restricted to the mechanism of transport of the complex to the cytoplasm. LKB1 is also reported to co-localize with E-cadherins at adherens junctions, where it can mediate its action on cell polarization (Sebbagh M., *et al.*, 2009).

Recently, a new mechanism of regulation of LKB1 expression by SIRT1 (sirtuin 1 or silent mating type information regulation 2 homolog 1, on chr 10q21.3) has been reported. SIRT1 is a class III NAD-dependent deacetylase enzyme. It regulates histone H3 or other protein acetylation status in correlation with the cells NAD+/NADH ratio, e.g. with the cells metabolic status. SIRT1 is therefore a metabolic sensor, as is AMPK whose activity depends on the AMP/ATP ratio. Interaction between SIRT1 and LKB1, which is upstream of AMPK, is therefore not surprising. One study showed that the activation of SIRT1 using polyphenols lead to the increase of LKB1 phosphorylation at Ser428 and AMPK activity (Hou X., *et al.*, 2008). The actual mechanism of this regulation was uncovered in another study which showed that LKB1 was acetylated on various lysine residues, Lys48 seeming to be the most important one as assessed by point mutation (Lan F., *et al.*, 2008). Overexpression of SIRT1 in the 293T cell line induced LKB1 deacetylation, and conversely SIRT1 downregulation by shRNA promoted LKB1 acetylation. The deacetylation of LKB1 increased its binding to STRAD, its transport to the nucleus and

its activation of downstream kinases such as AMPK. SIRT1 overexpression led to the deacetylation of LKB1 in primary porcine aortic endothelial cells (Lan F., *et al.*, 2008). However LKB1 regulation mechanism via SIRT1 may be cell type and context dependent.

II.V LKB1 role in cancer

As mentioned above, the *LKB1* gene was discovered only in 1998 as a new tumor suppressor gene in Peutz-Jeghers syndrome (PJS), an autosomal dominant disease. LKB1 has been associated with PJS by CGH analysis then by linkage analysis (Hemminki A., *et al.*, 1997). Mutations leading to inactivation of *LKB1* were then discovered in 80% of patients with PJS (Jenne D.E., *et al.*, 1998; Aretz S., *et al.*, 2005), and the syndrome is not only a familial disease as 10 to 20% of patients acquire PJS by spontaneous germline mutation of *LKB1*.

The disease is characterized by the presence of mucocutaneous melanin pigmentation appearing in early childhood and the development of hamartomatous polyposis in the gastrointestinal tract. Although LKB1 expression is ubiquitous, the tissues expressing LKB1 at higher level in normal adult seem to be the ones where patients with PJS develop tumors most frequently (Sanchez-Cespedes M., 2007).

Patients with PJS have usually lost only one copy of *LKB1*, mostly by germline mutation, although some cases also lose the remaining allele. They can display mutations throughout the gene, and over 100 different ones have been reported; but interestingly, they target the kinase activity domain of LKB1, the C-terminal one but not the N terminal. In the C-terminal, a frequently observed mutation is a stop codon to suppress the last 20 amino acids that are regulate LKB1 localization. A set of mutations in the C-terminal of LKB1 that disrupt the kinase ability to control intestinal epithelial polarity have also been uncovered (Forcet C., *et al.*, 2005). Single AA substitutions that prevent the binding of LKB1 with STRAD have also been found in patients with PJS (Zeqiraj E., *et al.*, 2009a).

Mouse models have been developed to understand the role of LKB1 in PJS. Lkb1 null mice do not survive beyond embryonic day 10. This shows that LKB1 is essential for embryonic development, probably due to the interaction between LKB1 and Wnt signalling (Ossipova O., *et al.*, 2003), and its role in axon development (Kishi M., *et al.*, 2005). However, mice presenting only a monoallelic loss of LKB1 develop polyps that mirror the human PJS condition (Jansen M., *et al.*, 2009). This confirms the observation made in patients with PJS: loss of only one allele of LKB1 is pathogenic. The Lkb1 +/- mouse model, developed to study PJS, was also reported to spontaneously develop

tumors, mainly hepatocellular carcinoma late in age (Nakau M., *et al.*, 2002), confirming this status.

Initially LKB1 was described as a tumor suppressor, indeed functionally regulating processes in cancer cells such as: cell polarity, cell proliferation in response to environmental stimuli and apoptosis. Cell migration is a polarized phenomenon that demands cytoskeletal reorganization; loss of LKB1 was demonstrated to promote cell migration and metastasis (Carretero J., *et al.*, 2010; Taliaferro-Smith L., 2009). The disruption of cell polarity also leads to abnormal and chaotic tissue structure and can also impair asymmetric division, which is important in the regulation of stem cell differentiation or renewal during its cell division. LKB1 has also been linked to angiogenesis (Londesborough A., *et al.*, 2008), essential for tumor maintenance. It interacts with pathways that have been associated with cancer development, for example the p53, Wnt-β- catenin or Akt-mTOR pathways. Taken together, this underlines that LKB1 is relevant to cancer biology.

LKB1 function in lung cancer has been well studied. In this tumor the loss of the chromosomal arm 19p, and in particular the 19p13 region where LKB1 locus is, is frequently reported; about 80% of non small cell lung carcinoma (NSCLC) and 30% of small cell lung carcinoma have this abnormality (Rodriguez-Nieto S. and Sanchez-Cespedes M., 2009). LKB1 was then associated with this locus (Sanchez-Cespedes M., 2007) and mutation screening confirmed that the gene was biallelically lost.
Most of the mutations observed in PJS were also detected in lung carcinoma (Sanchez-Cespedes M., 2007). Promoter methylation was rarely observed (Sanchez-Cespedes M., 2007). Interestingly, *LKB1* loss was associated to the NSCLC type, and especially to adenocarcinoma (Fernandez P., *et al.*, 2004), smoking, absence of EGFR mutations (Rodriguez-Nieto S. and Sanchez-Cespedes M., 2009).

Surprisingly, *LKB1* was reported to be lost in a low number of sporadic tumors in the past. Even though patients with PJS develop pancreatic, intestinal and breast cancer, there are few reports of somatic mutations in these cancers (Sanchez-Cespedes M., 2007). In other studies of sporadic cancer, some cases with mutations of *LKB1* in head and neck cancer (Kenanli E., *et al.*, 2010; Qiu W., *et al.*, 2006), cervical cancer (Wingo S., *et al.*, 2009) hepatocellular carcinoma (Kim C.J., *et al.*, 2004) and gastric tumors (Park W.S., *et al.*, 1998), some cases with promoter methylation in colorectal cancer (Trojan J., *et al.*, 2000) and some cases with loss of *LKB1* chromosomal region or LOH in colorectal (Forster L.F., *et al.*, 2000; Trojan J., *et al.*, 2000), ovarian (Wang Z.J., *et al.*, 1999a) sex

cord-stromal (Kato N., *et al.*, 2004), breast (Forster L.F., *et al.*, 2000; Yang T.L., *et al.*, 2004) and brain cancers (Sobottka S.B., *et al.*, 2000) were reported. In these cases, LKB1 loss of function was not necessarily a homozygous event, and the effect of this loss was not always investigated. LOH in the 19p13.3 locus is the most commonly reported event and usually affects a high proportion of the cases. Loss at the protein level was found in a subset of pancreatic and biliary tumors (Sahin F., *et al.*, 2003).The development of conditional mouse models helped to understand the role of LKB1 in tumorigenesis by mimicking the onset of disease observed in patients with PJS in a specific tissue. There are very few reports on natural cancer occurrence in $lkb1^{+/-}$ mice, which is unexpected when compared to the data obtained with conditional models. Patients with PJS develop cancer late in life, whereas the polyps appear in adolescence. It is possible that the $lkb1^{+/-}$ mice studied for polyp formation were not kept long enough or died of complications from the polyps before the onset of these cancers. As such, a mouse strain with *LKB1* loss restricted to the mammary gland developed grade 2 ductal or solid papillary breast carcinoma that recapitulated the features of PJS breast cancers (McCarthy A., *et al.*, 2009). Only a few mice with monoallelic loss of lkb1 developed tumors after 120 weeks, but nearly 30% with homozygous loss of lkb1 died of the disease within 80 weeks. Also, the cancer developed late in the lifetime of the mice, after 10 months of age. Overall, this demonstrated a role for LKB1 in breast cancer. Similar findings were reported in gynaecological cancer, with female $lkb1^{+/-}$ mice that survived past 40 weeks of age spontaneously developing endometrial carcinomas (Contreras C.M., *et al.*, 2008). This confirmed the hypothesis that the $lkb1^{+/-}$ mice develop cancer late in life, making it difficult to study. To conclude, in light of the possible haploinsufficient effect of LKB1 in tumorigenesis, there is evidence that this gene plays a role in gynaecological, pancreatic, intestinal, breast and liver cancers, in addition to lung cancer.

To date, LKB1 has been considered a tumor suppressor because the hereditary and somatic loss of function mutations of this gene are associated with an increased risk of cancer development. Current analysis of LKB1 function has focused on its regulation of AMPK and mTOR signaling. Because LKB1/AMPK signaling inhibits mTOR, but activated Akt stimulates mTOR activity, LKB1 and Akt are thought to play opposing roles with regard to mTOR regulation (Shaw R.J., *et al.*, 2004; Corradetti M.N., *et al.*, 2004). Recently was shown that LKB1 is necessary for cancer cell survival in the context of aberrant Akt activation (Zhong D., *et al.*, 2008). In this paper, the authors carried out a detailed analysis on Thr^{32} phosphorylation of FoxO3a and demonstrated that upregulation of FoxO3a phosphorylation at Thr^{32} by Akt required the function of wild-

type LKB1. This is as a novel finding because it provides the first evidence that LKB1 and Akt play co-operative roles with regard to the phosphorylation of FoxO3a on Thr^{32}. In addition their data indicated that LKB1 is required for the suppression of multiple pro-apoptotic signaling molecules by an aberrantly activated Akt.
It is unknown whether LKB1 directly or indirectly participates in the phosphorylation of Akt target proteins. Direct interaction between LKB1 and Akt has not been demonstrated previously, and it is possible that LKB1 mediates this effect through its downstream substrates. Therefore, it will be important to determine in the future whether LKB1 or its downstream target(s) directly participate in the phosphorylation of Akt substrates.
The first evidence that LKB1 plays a potentially oncogenic role in cells having activated Akt, opens the direction to a double role for LKB1 in a cell-dependent way.

In addition a recent paper highlights this new perspective as they demonstrate that LKB1-AMPK pathways can have a positive role in tumorigenesis, acting to maintain metabolic homeostasis and attenuate oxidative stress thereby supporting the survival of cancer cells (Jeon S.M., *et al.*, 2012).

II.VI LKB1 role in haematological diseases

LKB1 is essential for the maintenance of haematopoietic stem cell (HSC) homeostasis (Nakada D., *et al.*, 2010; Gurumurthy S., *et al.*, 2010; Gan B., *et al.*, 2010). As known, LKB1 regulates metabolism of many adult cell types, so the authors of these three studies decided to determine whether it can also affect metabolic control of HSCs. A requirement for LKB1 in haematopoiesis was revealed *in vitro* and *in vivo*. Using *in vivo* murine model, in which LKB1 was conditionally deleted from haematopoietic tissues, they observed that loss of LKB1 eventually led to a decline in bone marrow cellularity, progressive pancytopenia and animal death. Moreover, HSCs transiently increased in number, an effect associated with enhanced proliferation, and then markedly decreased. This suggests that LKB1 is necessary to maintain quiescence specifically in HSCs. *In vitro*, *LKB1*-deficient HSCs formed fewer colonies than controls in culture; and *LKB1*-deficient bone marrow showed a markedly decreased ability to repopulate the haematopoietic system of irradiated mice. The decrease in HSC numbers could be caused by cell death; indeed, all three groups observed increased levels of apoptosis in *LKB1*-deficient HSCs. Furthermore, Gurumurthy *et al.* observed increased autophagy in haematopoietic tissues of lkb1-deficient mice, as well as an enhanced expression of phosphorylated histone H2AX, a marker of DNA damage. These findings, together with the observation by Nakada *et al.* that many lkb1-deficient HSCs are aneuploid, suggest that increased apoptosis could be caused by metabolic or genotoxic stress.

In addition, Gan *et al.* observed that lkb1-deficient HSCs have reduced levels of PPARγ co-activator 1 (PGC1), a transcriptional co-activator with known roles in metabolism and mitochondrial biogenesis (Gan B. *et al.*, 2010). Consistent with this, all three studies show that loss of LKB1 leads to reduced ATP levels and decreased mitochondrial potential.

The three studies also found that most of the effects of LKB1 on HSC homeostasis are independent from AMPK and mTORC1, as treatment with the mTORC1 inhibitor rapamycin did not rescue the effects of *LKB1* deficiency, including HSC depletion. Furthermore, Gurumurthy *et al.* and Gan *et al.* observed that treatment with an AMPK activator did not restore the depleted cells in the bone marrow and thymus, and Nakada *et al.* found that AMPK-deficient HSCs were neither rapidly depleted nor unable to reconstitute irradiated mice, in contrast to *LKB1*-deficient HSCs (Gan B. *et al.*, 2010; Gurumurthy S. *et al.*, 2010; Nakada D. *et al.*, 2010).

So, it seems that LKB1 balances proliferation and quiescence in HSCs by regulating cell survival, cell cycle progression and mitochondrial function. Candidate downstream effectors of LKB1 signalling include AMPK-related kinases, and further studies are needed to determine whether they are involved in this LKB1-mediated function.

In Acute Myeloid Leukemias (AML), the LKB1/AMPK/TSC2 tumor-suppressor pathway is consistently functional in primary cells and, when specifically activated by metformin, blocks the catalytic activity of mTOR (Green A.S., *et al.*, 2010). As previously described, the loss of LKB1 function is common in cancer, due to either genetic defects or other mechanisms, including epigenetic silencing (Shackelford D.B., *et al.*, 2009).

However, Green A.S., *et al.,* consistently detected the expression of the LKB1 protein in human AML cell lines and in primary AML samples, and metformin treatment for LKB1/AMPK activation results in pro-apoptotic effects (Green A.S., *et al.*, 2010). Metformin activates AMPK by at least two LKB1-dependent mechanisms. First, metformin inhibits complex I of the mitochondrial respiratory chain, which results in the generation of reactive nitrogen species that activate PKCξ, which, in turn, phosphorylates LKB1 at S428. Phosphorylation of LKB1 at this residue is required for its translocation from the nucleus to the cytoplasm and subsequent AMPK activation (Memmott R.M. and Dennis P.A., 2009). Metformin also increases intracellular AMP levels, which activates AMPK by inducing changes in the AMPK γ-subunit conformation that exposes the T172 residue of AMPK α-subunit, thus generating a good substrate for LKB1 (Dowling R.J., *et al.*, 2007; Zhang L., *et al.*, 2007) Hence, the major increase in AMPKα T172 phosphorylation consistently detected in metformin-treated primary AML cells indicates the adequate activity of the serine/threonine kinase, LKB1 (Shaw R.J. *et*

al., 2004). Overall, current results in literature demonstrate a potent tumor-suppressor role for the LKB1/AMPK pathway in AML, which is markedly activated by metformin.

In acute lymphoblastic leukemia there are only few studies regarding the LKB1/AMPK pathway. Two of these showed that LKB1/AMPK activation by AICAR can induce apoptosis in some BCP-ALL cell lines (NALM6 and SupB15), T-ALL (CCRF-CEM) and BPC-ALL with TEL/AML1 (REH). They found that treatment with AICAR inhibited cell proliferation, induced cell cycle arrest in G_1-phase, and apoptosis through the mitochondrial pathway as revealed by the release of cytochrome C and cleavage of caspase 9 (Sengupta T.K., *et al.*, 2007; Kuznetsov J.N., *et al.*, 2011). They also shown that AICAr induces dose- and time-dependent growth inhibition in acute lymphoblastic leukemia (ALL) cell lines (Kuznetsov J.N., *et al.*, 2011).

Moreover they underline an emerging evidence that LKB1/AMPK activation has a dual role in cancer depending on alterations of interconnected signalling pathways, thus its effects are likely to be cell-type and tissue specific (Kuznetsov J.N., *et al.*, 2011).

AIM OF STUDY -CHAPTER II-

Starting from our previous findings that AMPK is hyperactivated in pediatric MLL-rearranged B-Cell Precursor-Acute Lymphoblastic Leukemia (BCP-ALL) patients supporting the survival of leukemia cells, we decided to focus our studies on the AMPK activator, the serine/threonine kinase LKB1.

LKB1 is a multitasking kinase with great potential in orchestrating cell activity, found to play a role in cell cycle, polarity, apoptosis, cell cycle arrest and cell proliferation. To date, LKB1 has been considered as a tumor suppressor because the hereditary and somatic loss of function mutations of this gene are associated with an increased risk of cancer development. But recent evidences show that LKB1 can also play a potentially oncogenic role in some cells and this opens for LKB1, as for AMPK, the possibility of a dual role in a cell-dependent way.

We already showed that LKB1 is hyperphosphorylated in MLL-rearranged BCP-ALL and in this study we aim to explore also its expression and function in pediatric ALL. Through proteomic and gene expression analyses we will evaluate its expression, opening also to further experiments in order to understand the mechanisms of its transcriptional or post-transcriptional regulation. Moreover, we will explore LKB1 cellular localization and, through specific silencing experiments, we will assess its possible role in supporting leukemia cells survival.

Thanks to these studies we aim to contribute to the elucidation of LKB1 role in cancer and, in particular, in pediatric BCP-ALL. We will evaluate if LKB1 could be considered as a new candidate biomarker or a new therapeutic target in MLL-rearranged BCP-ALL cells.

II. MATERIALS AND METHODS

II.VII *Cell lines and culture*

Human BCP-ALL cell lines SEM, RS4;11 and MHH-CALL2 (DSMZ) were cultured in RPMI 1640 (Invitrogen-Life Technologies) supplemented with 10% fetal bovine serum (FBS; Invitrogen-Life Technologies), glutamine (2mM/l; GIBCO, Invitrogen Life Technologies), penicillin (100U/ml; GIBCO) and streptomycin (100mg/ml; GIBCO), and maintained at 37°C in a humidified atmosphere with 5% CO2. Human ALL cell line MHH-CALL4 was cultured in the same conditions but supplemented with 20% FBS. SEM and RS4;11 cell lines were derived from BCP-ALLs carrying the t(4;11) MLL-AF4 translocation. MHH-CALL-2 and MHH-CALL-4 cell lines were derived from BCP-ALLs without recurrent chromosomal translocations.

II.VIII *Lentiviral vector-mediated transduction of shRNA in leukemia cells*

The lentiviral plasmids containing LKB1 shRNA expression cassette or an un-relevant shRNA sequence were purchased from Sigma-Aldrich. The lentiviral vector stocks were generated by a transient three-plasmid vector-packaging system (Indraccolo S. *et al.*, 2005). One µg of p24 equivalent of lentiviral vector-containing supernatant was used to transduce 1 x 10^6 target cells in 35 mm-diameter Petri dish. After 6-9h at 37°C, the supernatant was replaced with complete medium. Evaluation of LKB1 silencing and quantification of apoptosis were carried out 72-96h after transduction.

II.IX *RNA isolation and SYBR Green quantitative real-time PCR assays (RQ-PCR)*

Total RNA was isolated from cell lines (2 to 5 × 10^6 per sample) using Trizol (Invitrogen Life Technologies). RNA (1µg) was retrotranscribed using the SuperScript II system (Invitrogen Life Technologies) in 20µL final volume following the manufacturer's instructions. RQ-PCR was performed with 3µL of cDNA in 20µL using the SYBR Green method (Invitrogen Life Technologies) and analyzed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Annealing temperature was set at 60°C for all tested genes. Experiments were carried out in triplicate. Relative quantification was done using the ΔΔCt method and expression values for target genes were normalized to the concentration of the housekeeping gene GUS. Primers: LKB1-fwd: 5′-GCCGGGACTGACGTGTAGA-3′; LKB1-rev: 5′-CCCAAAAGGAAGGGAAAAACC-3′; GUS-fwd: 5′-GAAAATATGTGGTTGGAGAGCTCATT-3′; GUS-rev: 5′-CGGAGTGAAGATCCCCTTTTTA-3′.

II.X *Annexin V/PI assays and MTT Assay*

Apoptosis was determined using the Annexin-V-FLUOS staining kit (Roche), following manufacturer's instructions. Samples were analyzed by flow cytometric analysis (Cytomics FC500, Beckman Coulter).

The cell viability was assayed by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). This assay was performed by the Mosmann method [Mosmann T., 1983]. Briefly, cells were placed in 96wells plates at different time points after shRNA transfection (24h, 48h and 72h). At all time points, 10µL of MTT reagent (5 mg/ml) was added to each well. The plate was then incubated at 37°C for 3h, and after this incubation, acidic isopropanol (100µl of 0.08N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the formazan crystals. The plate was read in the Victor3™ 1420 Multilabel Counter (PerkinElmer) using a test wavelength of 570nm and a reference wavelength of 630nm. Absorbance was corrected by subtracting the mean value obtained from non-seeded wells.

II.XI *Western blot*

Western blot analyses were carried out as previously described (Accordi B., *et al.,* 2012). Briefly, cells were lysated in an appropriate lysis buffer (Biosource International) following manufacturer's instructions. Protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce). Antibodies used were anti-LKB1 (1:1000, Sigma Aldrich), anti-phospho-LKB1 S428 (1:1000, Cell Signaling), anti-AMPK (1:1000, Cell Signaling), anti-phospho-AMPK T172 (1:500, Cell Signaling), anti-MRLC (1:1000, GeneTex), anti-phospho- MRLC S19 (1:1000, GeneTex), anti-MAPK activation loop (1:1000, Cell Signaling), anti-MO25 (1:20000, Abcam), anti-p53 (1:1000, Cell Signaling), anti-PARP (1:1000, Cell Signaling), and anti-β-actin (1:10000, Sigma-Aldrich). The secondary antibody was horseradish peroxidase–conjugated goat anti-rabbit or mouse IgG (Millipore). Total cell lysates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, and transferred to a PVDF sheet (Hybond-P, GE Healthcare) following standard methods. Membranes were saturated for 3h with 2% Amersham ECL Advance Blocking Reagent (GE Healthcare), primary antibodies were incubated overnight at 4°C and secondary antibodies for 1h at room temperature. The immunoreactivity was determined by an enhanced chemiluminescent reaction (Amersham ECL ADVANCE Western Blotting Detection Kit, GE Healthcare). For the stripping, membranes were incubated with Restore PLUS Western Blot Stripping Buffer (Pierce) following the manufacturer's instructions and then washed in T-phosphate-buffered saline 1X and resaturated.

II.XII *Immunofluorescence*

Cells were placed on the slides by citospin and then fixed with formaldehyde (4%) for 15 min at room temperature (RT). After 3 washes of 5min with PBS 1X, cells were permeabilized with 0.1% Triton X-100/PBS 1X for 10min at RT. After that cells were incubated first with blocking buffer (5% BSA in PBS 1X) for 30min and then with primary antibody diluted in blocking solution overnight at +4°C. The next day cells were incubated with secondary antibodies conjugated to fluorophore (1:2000) for 1h and with DAPI (1:10000) (Sigma-Aldrich) for 10min at RT. After 3 washes of 5min, slides were covered with the coverslip and analyzed with a fluorescence microscope (VICO, Nikon).
Antibodies: anti-LKB1 (1:200, Sigma-Aldrich), anti-phospho-LKB1 S428 (1:200, Cell Signaling), anti-AMPK (1:200, Cell Signaling), anti-phospho-AMPK T172 (1:200, Cell Signaling), anti-MRLC (1:300, GeneTex), anti-phospho-MRLC S19 (1:300, GeneTex), anti-PCM1 (1:300, AbNova), anti-MO25 (1:20000, Abcam), anti-AURORA A (1:300, Cell Signaling) and anti-α-Tubulin (1:400, Sigma-Aldrich).

II.XIII *Cell cycle analysis*

After 24h from shRNA transfection, treated cells were centrifuged, fixed with ice-cold ethanol (70%) overnight at 4°C. Cells were washed twice with PBS 1X, lysed with lysis buffer containing RNAseA (Qiagen, Hilden, Germany) and treated with 50µg/mL propidium iodide (PI) in 1mL PBS1X. Samples were analyzed using Cytomics FC500 (Beckman Coulter). Cycle analyses were performed using Multicycle Wincycle software (Phoenix Flow Systems, San Diego, California, USA).

II.XIV *Data analysis*

Data were analysed with Mann-Whitney or unpaired two-tailed *t* test. A *P* value of <0.05 was considered significant.

II. RESULTS

II.XV *LKB1 characterization in pediatric BCP-ALL MLL-rearranged cells: proteomic and gene expression profiles*

Considering our previous findings on LKB1 hyperphosphorylation in pediatric MLL-rearranged leukemia, we analysed the LKB1 total protein expression in the four cell lines taken as a model for our *in vitro* experiments. We used SEM and RS4;11 cell lines carrying t(4;11) as a model for MLL-rearranged patients, and MHH-CALL-2 and MHH-CALL-4 as a model for non-translocated BCP-ALL patients. In the SEM and RS4;11 cells total LKB1 is more expressed than in MHH-CALL-2 and MHH-CALL-4 cells. The same trend was detected for total MO25, a LKB1 partner protein in the activation complex (**Fig. II.Ia**). These data demonstrate that there is a difference in total protein levels that could explain the LKB1 hyperphosphorylation previously observed in MLL-rearranged patients. To understand if total protein expression is driven directly by gene expression, we evaluated LKB1 gene expression levels in 43 MLL-rearranged patients vs 139 B-Others through Affymetrix HGU133 Plus 2.0 assays already performed in our laboratory (Haferlach T., *et al.*, 2010). *STK11* specific probes resulted to be not significantly different between the two subgroups of patients (**Fig. II.Ib,** in red MLL-rearranged patients, in light blue B-Others). Thus LKB1 hyperexpression in MLL-rearranged patients seems not to be derived from a different gene expression, but maybe to a different post transcriptional regulation.

The potential role of miR-451 in post-transcriptional regulation of LKB1 expression will be investigated, as described in paragraph "Ongoing studies and future directions".

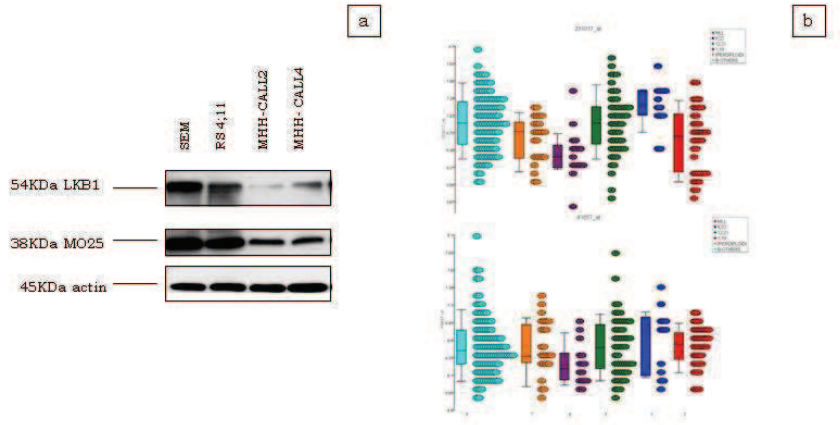


Fig. II.I: a) Western Blot analysis revealed higher protein expression of LKB1 and MO25 in SEM and RS4;11 (MLL-rearranged cell lines) compared to MHH-CALL-2 and MHH-CALL-4 (not translocated cell lines). b) Affymetrix array showed that *STK11* specific probes are not significantly different between MLL-rearranged patients (in red) and B-Others (in light blue).

II.XVI *Cytoplasmatic localization of LKB1 and the eye-catching spots*

To characterize LKB1 localization and function in leukemia cells with MLL-translocation, we performed immunofluorescence staining. We observed that LKB1 total protein is predominantly detectable at a cytoplasmatic level in both cell lines (**Fig. II.IIa upper panels**). The phosphorylated form instead seems to be strongly upregulated only in some cells (**Fig. II.IIb lower panels**). Very interestingly, a deeper analysis of the images revealed that all these eye-catching cells were in mitosis (**Fig. II.IIb**), suggesting that probably LKB1 has an important role in this phase of the cell cycle.

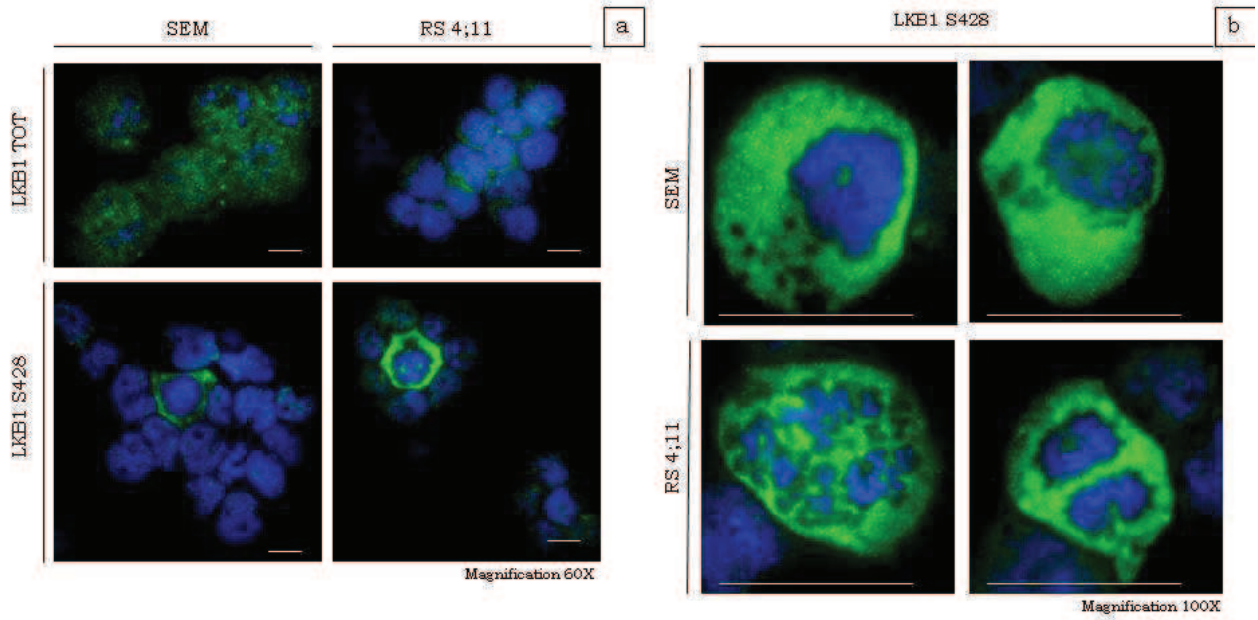


Fig. II.II: a.) Immunofluorescence staining revealed that total-LKB1 and ph-LKB1 (S428) were predominantly expressed at a cytoplasmatic level but also detectable in the nucleus. b) Further analysis in the immunofluorescence staining revealed multiple eye-catching spots. ph-LKB1 is present in all mitosis phases. Panel shows pseudo-color merged images of cell lines decorated with 4,6-diamidino-2-phenylindole (DAPI), Alexa-fluor 594.Scale bar 5µm.

In order to examine the role of LKB1 in leukemia cell cycle, we performed co-immunofluorescence staining with other proteins involved in mitosis. We found a co-localization between ph-LKB1 (S428) and α-tubulin at centrosome (**Fig. II. IIIa**). We also found that ph-LKB1 (S428) localizes near Aurora A kinase, an important regulator of cytokinesis, mitosis and G_2/M transition (Marumoto T., *et al.*, 2002). Moreover, we found also that ph-LKB1 (S428) and the pericentriolar material 1 (PCM1) protein localizes in the cytoplasm (**Fig. II. IIIb**). The protein PCM-1 localizes to cytoplasmic granules known as "centriolar satellites" and is required for centrosome assembly, function and

microtubules anchoring (Dammermann A. and Merdes A., 2002). PCM-1 is tightly associated with the centrosome complex through G_1, S, and a portion of G_2. However, late in G_2, as cells prepare for mitosis, PCM-1 dissociates from the centrosome and then remains dispersed throughout the cell during mitosis before re-associating with the centrosomes in the G_1 phase progeny cells. These results demonstrate that the pericentriolar material is a dynamic substance whose composition can fluctuate during the cell cycle (Balczon R., *et al.,* 1994). Further studies through co-immunoprecipitation experiments will be needed to assess if they physically interacts. We also performed co-immunofluorescence staining between α-tubulin and AMPK, the major downstream target of LKB1. Even in this staining we found a co-localization between α-tubulin and AMPK, lighter in SEM cells, but probably due to a different cell cycle stage. Also we found co-localization between α-tubulin and myosin regulatory light chain S19 (ph-MRLC S19), direct downstream target of AMPK, implicated in cytokinesis and cell locomotion (Thaiparambil J.T., *et al.*, 2012) (**Fig. II. IIIc**).

In summary, the localization at centrosome of ph-LKB1, ph-AMPK and ph-MRLC may suggest a role for this pathway in the regulation of elongation and stability of microtubules during mitosis.

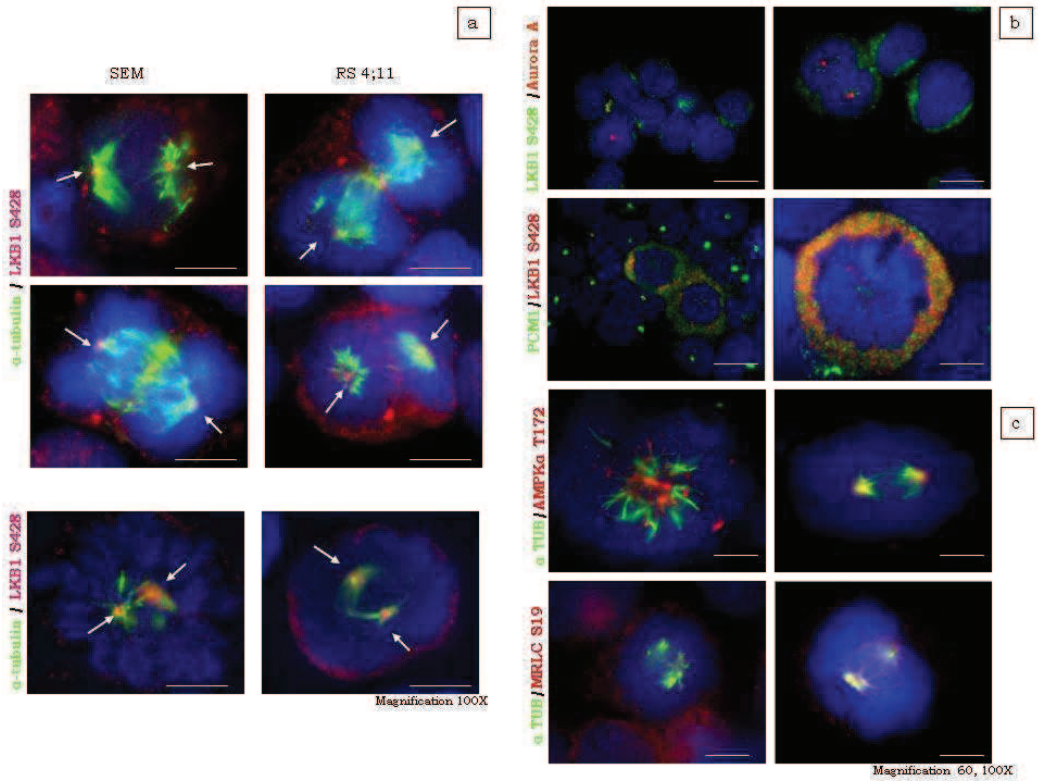


Fig. II.III: a) Immunofluorescenze staining revealed co-localization of ph-LKB1 (S428) and α-tubulin at the centrosome. b) ph-LKB1 (S428) is located near Aurora A kinase (upper panels) and in the cytoplasm together with PCM1 (lower panels). c) Co-immunofluorescence staining shows co-localization of α-tubulin and AMPK (upper panels) and co-localization of α-tubulin and p-MRLC (S19) (lower panels). Panel shows pseudo-color merged images of cell lines decorated with 4,6-diamidino-2-phenylindole (DAPI), Alexa-fluor 488 and 594.Scale bar 5μm.

II.XVII *LKB1 silencing decreases survival in MLL-rearranged cells*

In our previous paper (Accordi B., *et al.*, 2012) we investigated the role of the AMPK pathway in MLL-rearranged cells and we assessed that the AMPK pathway is switched on in the MLL-rearranged cells and switched off in cells without recurrent translocations. The same trend is found for LKB1. In order to investigate the functional role of LKB1 in leukemia cells we silenced *LKB1* in two different cell lines chosen as *in vitro* model: SEM carrying the t(4;11) MLL-AF4 translocation and MHH-CALL-2 derived from a BCP-ALL without recurrent chromosomal translocations. First we tested five lentiviral vectors encoding different LKB1-specific short-hairpin RNA (shRNA) [all the sequence of shLKB1 (pLKO.1-puro) are reported in **Table II.I**].

shLKB1 (pLKO.1-puro)	Sequence
shLKB1 407	CCGGGAGTGTGCGGTCAATATTTATCTCGAGATAAATATTGACCGCACACTCTTTTT
shLKB1 408	CCGGGCCAACGTGAAGAAGGAAATTCTCGAGAATTTCCTTCTTCACGTTGGCTTTTT
shLKB1 409	CCGGGATCCTCAAGAAGAAGAAGTTCTCGAGAACTTCTTCTTCTTGAGGATCTTTTT
shLKB1 410	CCGGGAAGAAGAAGTTGCGAAGGATCTCGAGATCCTTCGCAACTTCTTCTTCTTTTT
shLKB1 411	CCGGCATCTACACTCAGGACTTCACCTCGAGGTGAAGTCCTGAGTGTAGATGTTTTT

Table II.I: shLKB1 (pLKO.1-puro) sequence (Sigma Aldrich).

After mRNA and protein analysis we choose two different shRNA (sh408 and sh410) that brought to the best silencing. Following a double transduction of the lentiviral vector, LKB1 was efficiently silenced. MLL-rearranged cells showed a LKB1 mRNA expression reduced of 40% respect to the control (**Fig. II.IVa**), and underwent significantly more apoptosis (shRNA/sh408 p=1.43138E-05; shRNA/sh410 p=0.000708005)**,** (**Fig. II. IVb** control cell viability was set to 100%).

Otherwise, despite of a reduction of 50% of LKB1 mRNA expression (**Fig. II.IVc**), MHH-CALL-2 cells didn't undergo apoptosis (**Fig. II.IVd**).

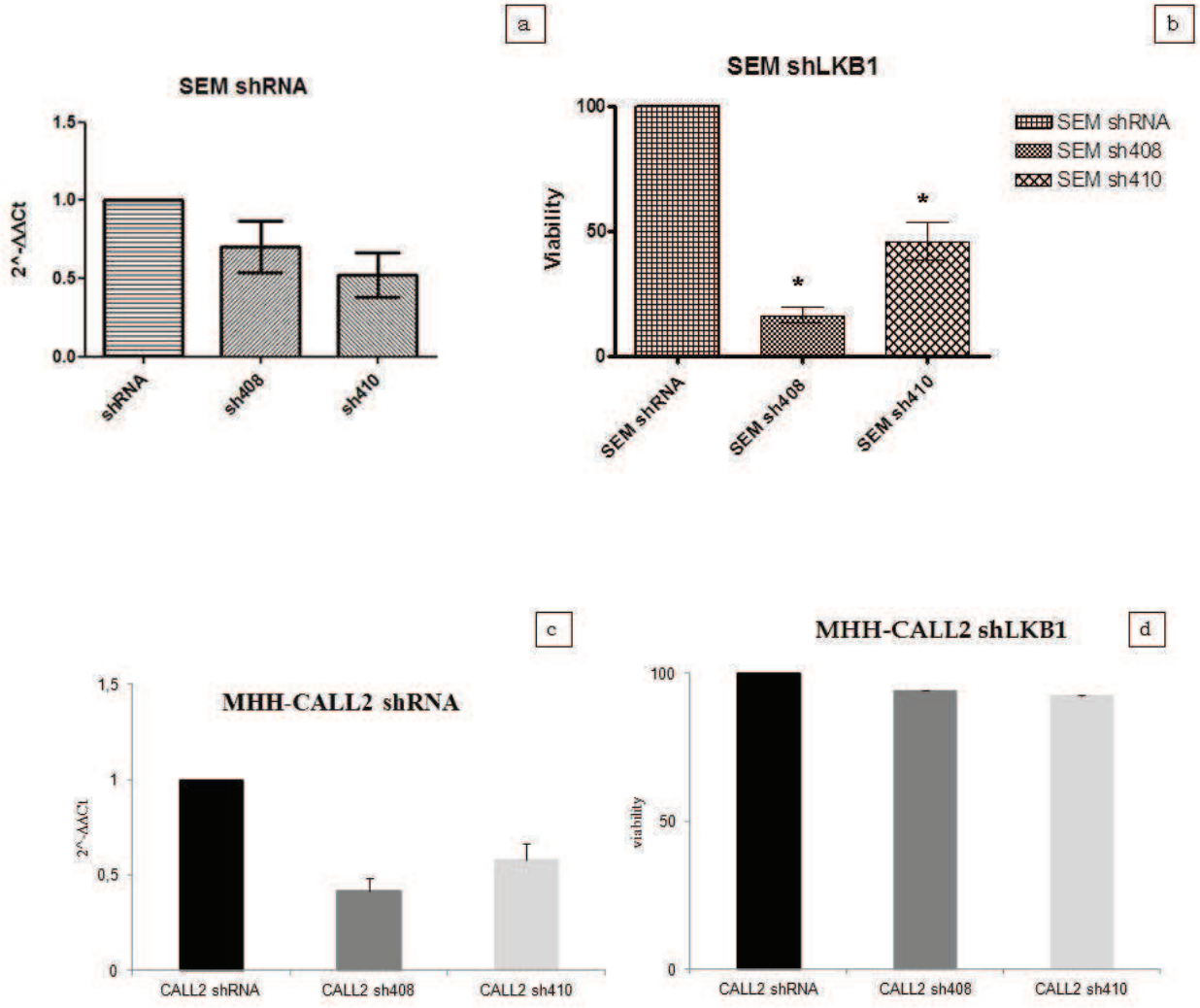


Fig. II.IV: a) RQ-PCR analysis, following a double transduction with shRNA408 and shRNA410 in SEM cells, LKB1 was efficiently silenced obtaining LKB1 mRNA expression reduction of 50% compared to control (shRNA).
b) Annexin V/PI assay revealed that silenced MLL-rearranged cells undergo significatively more apoptosis than the control (shRNA) (Test t, SEM shRNA/SEM sh408 p=1.43138E-05; SEM shRNA/SEM sh410 p=0.000708005) (control cell viability was set to 100%).
c) RQ-PCR analysis following a double transduction with shRNA408 and shRNA410 in MHH-CALL2 cells, LKB1 was efficiently silenced obtaining LKB1 mRNA expression reduction of 50% compared to control (shRNA). d) MHH-CALL2 didn't undergo apoptosis (Test t, MHH-CALL-2 shRNA/ MHH-CALL-2 sh408 p=0,434; MHH-CALL-2 shRNA/MHH-CALL-2 sh410 p=0,429) (control cell viability was set to 100%). (Test t, SEM sh408/MHH-CALL-2 sh408 p=0,022, SEM sh410/MHH-CALL-2 sh410 p=0,029).

II.VIII *LKB1 silencing decreases the activation of the downstream proteins*

Considering that LKB1 induced apoptosis only in MLL-rearranged cells, we then focused our attention for the following experiments only to SEM cells. After transduction of shRNA, as expected, LKB1 total protein and ph-LKB1 (S428) expression are reduced in silenced cells (**Fig. II.Va**). Intriguingly, western blot analysis showed also a clear reduction of MO25, indicating the loss of the active LKB1 complex (**Fig. II.Va**). In terms of the other partner of LKB1, STRADα, the reduction after silencing was not so evident (**Fig. II.Va**). Moreover the protein levels of downstream targets were markedly decreased, compared to controls (**Fig. II.Vb,** WB ph-AMPK T172, ph-MARK family activation loop, total MRLC, ph-MRLC S19).

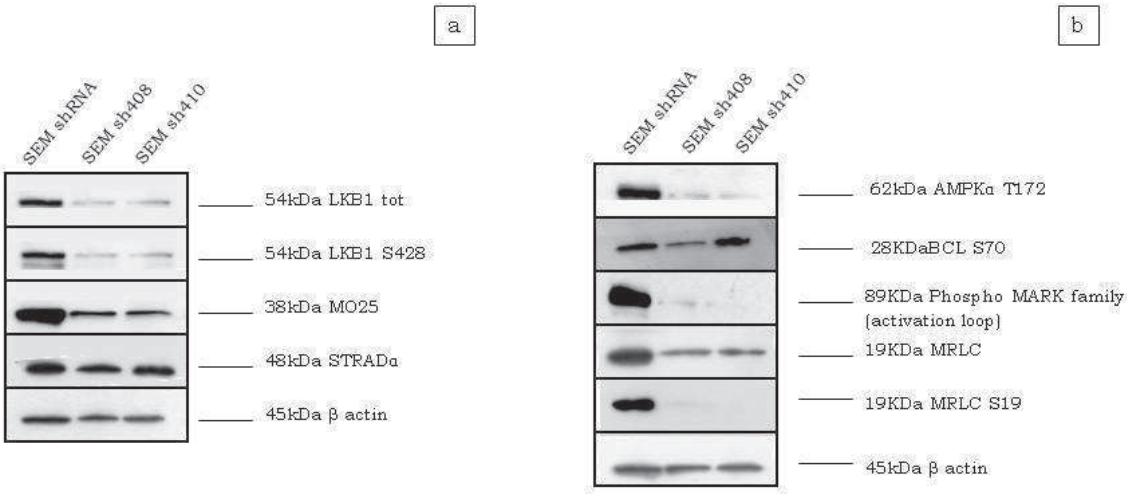


Fig. II.V: a) Western Blot analysis after LKB1 silencing revealed a significant drop in the protein level of total LKB1 and ph-LKB1 (S428) in silenced cells versus control. A clearl reduction of MO25 was also evident. b) Western Blot analysis showed that protein levels of downstream targets were markedly decreased versus vector control.

II.XIX *LKB1 silencing arrests the cell cycle at the S phase and destroys the G_2/M phase*

We next performed cell cycle analysis in LKB1 silenced SEM cells to deeper understand the role of LKB1 in cell cycle regulation. 48 hours after transduction we can appreciate an increase of subG_1 population, an increase of the S phase and a marked decreased of G_2/M phase (**Fig. II.VI).** After LKB1 silencing the S phase increased from 49% in the control to 67% in sh408 silenced cells and to 57% in sh410 silenced cells. The G_2/M population decreased from 14% in the control to only 2% in sh408 silenced cells and to 4.8% in sh410 silenced cells (**Fig. II.VI)**. Our data indicate that cells with low LKB1 expression can't cross the S phase and this causes the accumulation of cells in this phase of the cycle with a simultaneous loss of cells in the G_2/M phase.

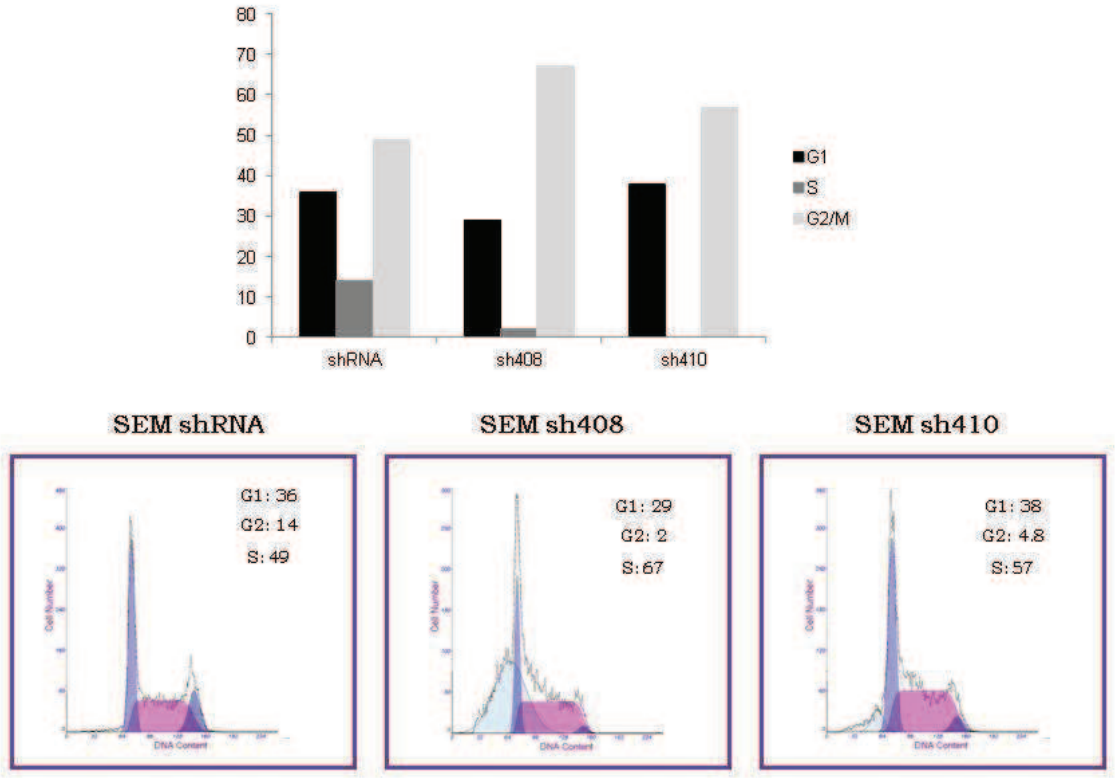


Fig. II.VI: Cell cycle histograms based on flow cytometric analyses of treated cells: our data suggest that without LKB1 cells don't cross S phase causing stacking of cells in this phase of the cycle with reduction of cells in G_2/M phase.

II.XX *LKB1 silencing leads to mitotic catastrophe*

The mitotic catastrophe is a type of cell death that occurs during mitosis or results from mitotic failure. Mitotic catastrophe results from a combination of deficient cell-cycle checkpoints (in particular the DNA structure checkpoints and the spindle assembly checkpoint) and cellular damage. Failure to arrest the cell cycle before or at mitosis triggers an attempt of aberrant chromosome segregation, which culminates in the activation of the apoptotic default pathway and cellular demise.

At least two subtypes of mitotic catastrophe can be distinguished. First, mitotic catastrophe can kill the cell during or close to the metaphase, in a p53-independent manner. Second, mitotic catastrophe can occur after failed mitosis, during the activation of the polyploidy checkpoint, in a partially p53-dependent manner. Mitotic catastrophe is accompanied by chromatin condensation, mitochondrial release of proapoptotic proteins (in particular Cyt. *c* and AIF), caspase activation and DNA degradation. This implies that mitotic catastrophe is accompanied by the key molecular events defining apoptosis, namely, capase activation and MMP (Matrix metalloproteinases) (Green D. and Kroemer G., 1998; Zamzami N. and Kroemer G., 2003).

LKB1 has been shown to regulate cell growth and death, and these functions have been linked to the tumor suppressor p53 (Karuman P., *et al.*, 2001; Zeng P.Y. and Berger S.L., 2006). LKB1-dependent phosphorylation of p53 in S15 and in S392 has been reported to be essential for cell cycle arrest in G_1 phase (Zeng P.Y. and Berger S.L., 2006). Here, we investigated the involvement of p53 in LKB1 pathway (**Fig. II.VIIa**). After LKB1 silencing total p53 was markedly decreased, compared to the control.
Moreover, we observed that in sh408 and sh410 silenced cells there is an increase of cleaved PARP levels (**Fig. II.VIIa**)**,** indicating DNA damage (Ahel I., *et al.*, 2008). Furthermore, we analysed other targets that accompany the mitotic catastrophe such as caspases 2 activation and DNA degradation (Ahel I., *et al.*, 2008). We discovered, as expected, in SEM sh408, an increase of Caspase 2 and increase of γH2Ax protein levels (**Fig.II.VIIa**). In SEM sh410 these results are not very clear, but probably because this shRNA was less efficient than sh408.

Interestingly, after failure to activate the G_2/M checkpoint(s), cells with DNA lesions (or incomplete DNA replication) activate an apoptotic program that leads to the phenotypic manifestation of mitotic catastrophe, during the metaphase of the cell cycle. Suppression of the apoptotic program (and of the spindle checkpoint) then may lead to asymmetric cell division or mitotic slippage, resulting into the generation of tetraploid cells which,

when the polyploidy checkpoint is inactivated, can generate an aneuploid offspring (Castedo M., *et al.*, 2004). Analysis of mitotic index revealed that, after LKB1 silencing, leukemia cells with MLL-rearrangement underwent mitotic catastrophe with the presence of chromatin condensation and asymmetric cell division that generated polyploidy cells (**Fig. II.VIIb**).

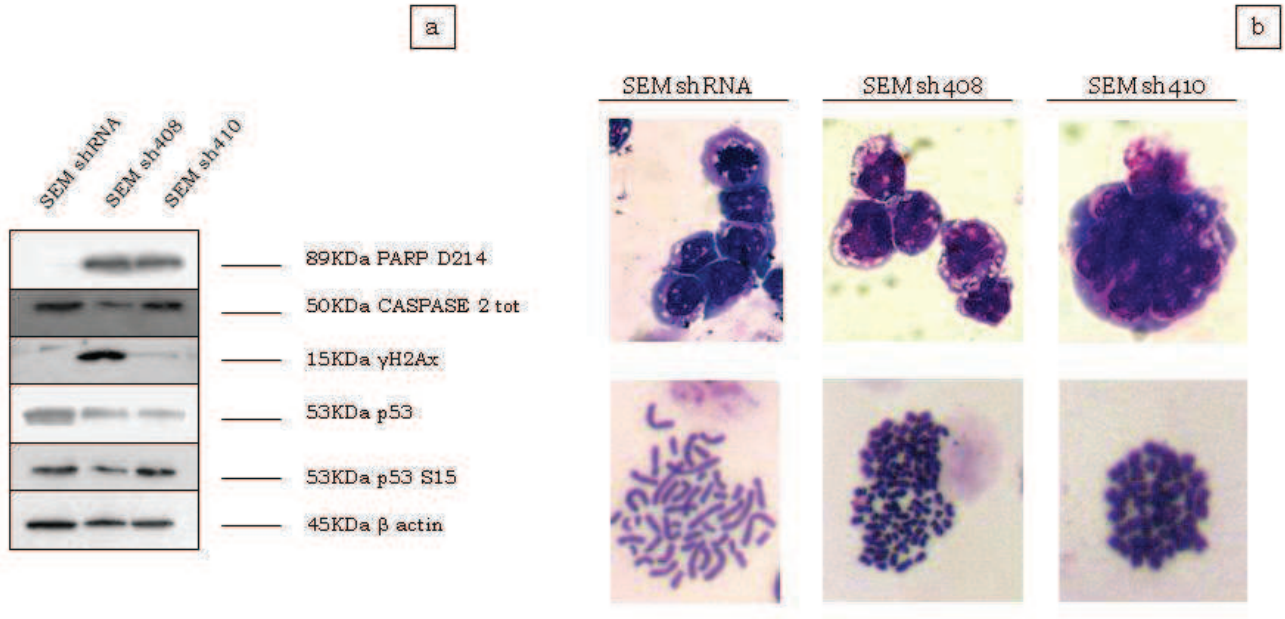


Fig. II.VII: a) Western blot analysis revealed caspase activation and DNA degradation (increase of γH2Ax protein levels and decrease of p53). b) Mitotic index analysis reveal that, after LKB1 silencing, leukemia cells with MLL-rearrangement undergo mitotic catastrophe with chromatin condensation and asymmetric cell division that generates polyploidy cells. Scale bar 5 µm.

II. DISCUSSION

In this study we report the implication of LKB1 as a survival regulator in B-Cell Precursor-Acute Lymphoblastic Leukemia (BCP-ALL) MLL-rearranged cells. We recently discovered an hyperactivated signal transduction pathway in pediatric MLL-rearranged BCP-ALL patients, that starts from phosphorylated LKB1/AMPK and, through downstream eNOS activation, leads to the phosphorylation of BCL-2 (Accordi B., *et al.*, 2010). AMPK activation was demonstrated to be necessary for the survival of MLL-rearranged BCP-ALL both in cell lines and patients primary cultures, and its inhibition induces cell death following the mitochondrial pathway (Accordi B., *et al.*, 2012).
In this study we focused on the AMPK activator, the serine/threonine kinase LKB1. LKB1 can also phosphorylate and activate 13 more protein kinases part of the AMPK family (Lizcano J.M., *et al.*, 2004). Therefore, LKB1 is a multitasking kinase, with great potential in orchestrating cell activity. Indeed LKB1 has been found to play a role in cell cycle polarity (Baas A.F., *et al,*. 2004), energy metabolism (Hurov J.B., *et al.*, 2007), apoptosis, cell cycle arrest and cell proliferation (Marignani P.A., *et al.*, 2001; Alessi D.R., *et al.*, 2006). In literature little is known regarding LKB1 in haematological disease and only in the context of haematopoietic stem cell where plays an essential for the maintenance homeostasis, according to three studies published in Nature (Nakada D., *et al.*, 2010; Gurumurthy S., *et al.* 2010; Gan B., *et al.*, 2010).

As previously described, LKB1 is hyperphosphorylated in MLL-rearranged BCP-ALL cells (Accordi B., *et al.*, 2010) and here we found that also the total form of the protein is overexpressed in these leukemia subgroup, but this isn't due to a different gene expression. RNA expression levels do not change between MLL-rearranged and not translocated patients. This means that higher expression/activation of the LKB1 protein in MLL-rearranged patients is probably due to a different post transcriptional regulation. In this light we hypothesized that miR-451, already demonstrated in literature to control MO25 and LKB1 expression in glioma cells (Godlewski, J., et al., 2010a ; Godlewski, J., et al., 2010b), could be implicated in this altered regulation, and we are performing experiments to verify its involvement. Through miRNA analysis with Affymetrix arrays we determined that in MLL-rearranged patients miR-451 is less expressed than in B-Others patients and we validated this data with RQ-PCR. Further studies will follow to confirm that MO25 and LKB1 are direct targets of miR-451 in BCP-ALL MLL-rearranged cells.

We then observed that in MLL-rearranged leukemia cell lines LKB1 total protein is predominantly detectable at a cytoplasmatic level, while the phosphorylated form seems

to be strongly upregulated only in some cells. Very interestingly, cells expressing high levels of activated LKB1 are all in mitosis. Thus, we decided to further explore LKB1 involvement in the mitotic phase and we found that it co-localizes with α-tubulin at centrosome. Moreover, also activated AMPK and MRLC are at centrosome, suggesting that probably the LKB1 pathway plays a key role in the control of the cell cycle through regulation of elongation and stability of microtubules. In agreement with these findings Kojima Y., *et al.*, 2007, shown that LKB1, in HepG2 cells (liver hepatocellular carcinoma) is involved in the regulation of microtubule dynamics through the activation of MARKs (Kojima Y., et al., 2007). In addition the activation of the LKB1-AMPK pathway was demonstrated to be strictly required for an accurate mitosis and chromosome segregation in *Drosophila melanogaster* (Bonaccorsi S., et al., 2007) and also to directly binds the mitotic apparatus and travels from centrosomes to the spindle midzone during mitosis and cytokinesis in human cancer cell lines [A431 (human epidermoid cancer cells), HeLa (human cervical cancer cells), A459 (human lung carcinoma cells), MCF10A (human breast epithelial cells), MDA-MB-231 (human breast carcinoma cells), NIH-3T3 (mouse embryonic fibroblasts), and 3T3-L1 (mouse pre-adipocytes fibroblasts)] (Vazquez-Martin A., *et al.*, 2009).

To further investigate the role of LKB1 in MLL-rearranged cells survival, we knocked down *LKB1* expression through lentiviral vectors encoding LKB1-specific short-hairpin RNA (shRNA) (shLKB1 pLKO.1-puro). After LKB1 silencing, MLL-rearranged cells presented an increase of subG_1 and S populations with a consistent decrease of cells in G_2/M phase. These data prompted us to speculate that in the absence of LKB1 these cells can't cross the S phase and this caused the accumulation of cells in this phase of the cycle with loss of cells at the G_2/M. Interestingly, in support of our previous conclusions about the role of LKB1 in the mitotic phase, we also found that after LKB1 silencing activated and total MRLC are strongly reduced.
Moreover, LKB1 silencing induced significantly more apoptosis in MLL-rearranged cells than in not rearranged ones. Loss of LKB1 led to an increase of cleaved PARP levels, of Caspase 2 and of γH2Ax, decrease of p53 protein levels, chromatin condensation and asymmetric cell division with generation of polyploidy cells.

Taking together all these findings can say that LKB1 silencing in MLL-rearranged leukemia cells leads to mitotic catastrophe, a type of cell death that occurs during mitosis or resulting from mitotic failure.
We thus conclude that LKB1 has a key role for survival of MLL-rearranged BCP-ALL cells, because its silencing brings to cell cycle block and apoptosis in these cells. Very

likely LKB1 exerts its role contributing to the regulation of elongation and stability of microtubules during mitosis. This seems to be accomplished through the AMPK-MRLC pathway. These data provide new insights into the role of LKB1 in cancer rendering this kinase an attractive target for future targeted therapies. Moreover our study also contributes to the elucidation of the biology of MLL-rearranged BCP-ALL, a very high risk subtype of leukemia with few available therapeutic options.

Work presented at AACR Conference "Metabolism and Cancer" 2011, Baltimore (MD) October 16-19, 2011. Abstract n. B67 and Poster, Appendix 1.

II. ONGOING STUDIES AND FUTURE DIRECTIONS

As previously described LKB1 is hyperphosphorylated in pediatric MLL-rearranged B-Cell Leukemia (**Fig. 1,** Accordi B., *et al.*, 2010). Interestingly, we didn't found a correlation between gene expression and proteomics profiles, in fact, STK11 and MO25 specific probes in Affymetrix experiments weren't significantly upregulated in MLL-rearranged patients (**Fig. II.Ib** for *STK11*, **Fig. II.VIIIa** for *MO25*). Thus LKB1 hyperphosphorylation isn't due to a different gene expression, but maybe to a different post transcriptional regulation.

Another explanation for LKB1 hyperactivation in MLL-rearranged B-Cell could be found in a different formation of its heterotrimeric complex. Boudeau J. et al., showed that in cells LKB1 is found in a 1:1:1 heterotrimeric complex with the pseudokinase STRAD (STe20-Related ADaptor) and the scaffolding MO25 (MOuse protein 25). Unlike the majority of protein kinases, which are regulated by phosphorylation, LKB1 is activated by binding to STRAD and MO25 through an unknown, phosphorylation-independent, molecular mechanism. The binding of STRAD to LKB1 substantially activates the autophosphorylation of LKB1 and hence its ability to phosphorylate downstream substrates is increased by 100-fold through conformational change (Hawley S.A. et al., 2003). MO25 stabilizes the complex by binding both STRAD and LKB1 (Boudeau J., et al., 2003a), as a scaffold protein. This prompted us to wondering if LKB1 hyperactivation in MLL-rearranged leukemic B-Cells could be explained by an altered formation of its complex (altered stechiometry).

As shown in the results of this chapter, in the cell lines carrying the t(4;11) chosen as a model for MLL rearranged patients (SEM and RS4;11), total LKB1 and total MO25 are higher than in cell lines model for non-translocated BCP-ALL patients (MHH-CALL-2 and MHH-CALL-4) (**Fig. II.Ia**). These data suggest that there is a difference in protein levels that, considering the equal gene expression, can be the result of a regulation miRNA-dependent.

miRNAs are currently a well established class of ~22 nt endogenous, noncoding small RNAs that influence mRNA stability and translation (Kloosterman W.P. and Plasterk R.H., 2006). Recent evidences showed that miRNA mutations or mis-expression correlate with various human cancers and indicate that miRNAs can function both as tumour suppressors or oncogenes. miRNAs have been shown to repress the expression of important cancer-related genes and might be useful in the diagnosis and

treatment of cancer (Esquela-Kerscher A. and Slack F.J., 2006; Lawler S. and Chiocca E.A., 2009).

In literature it was recently described a link between miR-451 and MO25/LKB1 by Godlewski J. et al., 2010. They show that miR-451 can repress MO25α and hence regulate the LKB1-AMPK pathway and glioma cells response to metabolic stress, by shifting cellular equilibrium and increasing cell proliferation and cell migration (Godlewski J., et al., 2010a; Godlewski J., et al., 2010b). miR-451 overexpression reduces the availability of the active LKB1 complex but does not influence the level of expression of LKB1 itself. This finding uncovers another possible mechanism of regulation of LKB1 activity through the regulation of complex expression by miRNA.

This data all together led us to wonder if this miRNA-dependent regulation of LKB1 is present also in BCP-ALL leukemia, thus elucidating the difference between MLL-rearranged and not translocated patients (B-Others). We hypothesized that patients MLL, with an higher protein expression of LKB1 and MO25, had a lower expression of miR-451 with respect to patients without rearrangements. Through miRNA analysis with Affymetrix arrays, we determined that miR-451 is expressed at different levels in MLL-rearranged and B-Others patients. Indeed, we found that in MLL-rearranged patients miR-451 is less expressed than in B-Others patients (**Fig. II.VIIIb**).

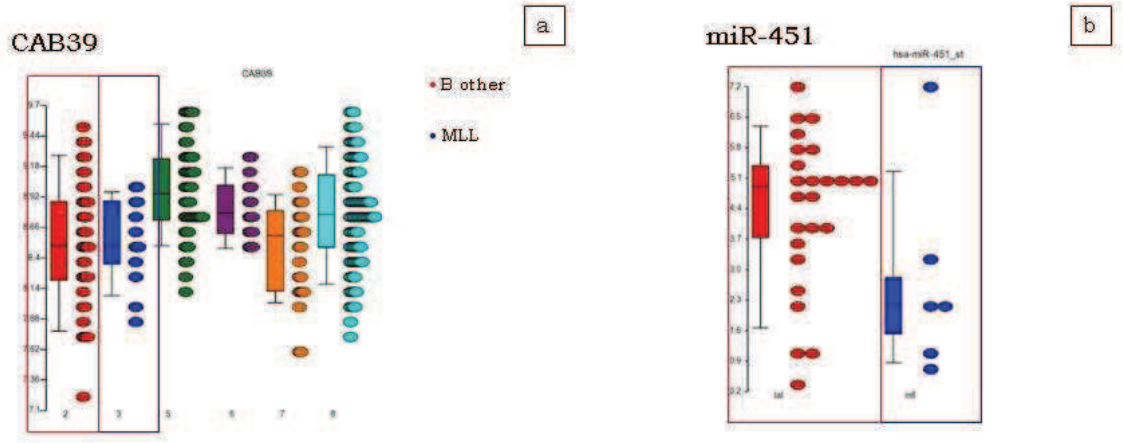


Fig. II.VIII: a) *MO25 (CAB39)* specific probes in Affymetrix experiments weren't significantly upregulated in MLL-rearranged patients (in blu) with respect to B-others (in red). b) miRNA analysis with Affymetrix arrays shown that miR-451 is expressed at different levels in MLL-rearranged (blue) and B-Others patients (red). In MLL-rearranged patients miR-451 is less expressed than in B-Others patients.

This is a very intriguing result that prompted us to proceed in this study.
We then validated this data with RQ-PCR (High Capacity cDNA Reverse Transcription Kit 43768814-Applied Biosystems) comparing miR-451 expression in 24 MLL-rearranged patients vs 19 B-Others patients (p=0.0008426, Wilcoxon test) (**Fig. II. IX**).

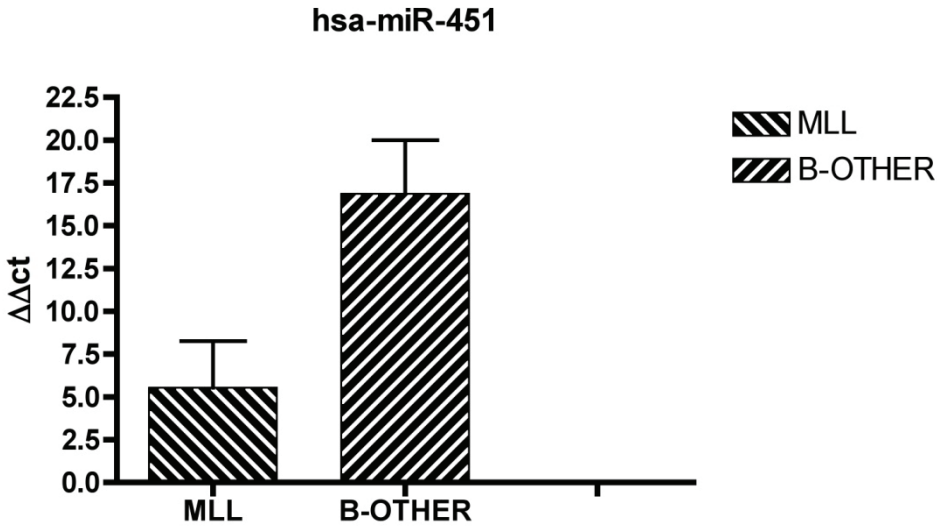


Fig. II.IX: Validation of miRNA Affymetrix arrays with RQ-PCR (High Capacity cDNA Reverse Transcription - Applied Biosystems) comparing miR-451 expression in 24 MLL-rearranged patients vs 19 B-Others patients (p=0.0008426, Wilcoxon test).

Preliminary conclusions based on these results encouraged our hypothesis that there is a different regulation of the LKB1 complex among MLL-rearranged and B-Others patients probably through miR-451.
To verify that LKB1 and MO25 are target of miR-451 in our cells, we will need first to find an appropriate in vitro model through RNA and protein screening in different commercial cell lines representative for the two subgroup of BCP-ALL patients. Successively we want to connect miR451 expression level with our target (LKB1 and MO25) through miR-451 mimic transfection experiments and luciferase assay. We will then perfom functional experiments as apoptosis and proliferation tests in treated cells in order to verify our hypothesis that leukemic cell regulate their survival through MO25-mediated LKB1 activation.

II. REFERENCES

Accordi B., Espina V., Giordan M., VanMeter A., Milani G., Galla L., Ruzzene M., Sciro M., Trentin L., De Maria R., te Kronnie G., Petricoin E., Liotta L., Basso G. “Functional Protein Network Activation Mapping Reveals New Potential Molecular Drug Targets for Poor Prognosis Pediatric BCP-ALL”. PLoS One. 2010, 5:e13552.

Ahel I., Ahel D., Matsusaka T., Clark A.J., Pines J., Boulton S.J., West S.C. “Poly(ADP-ribose)-binding zinc finger motifs in DNA repair/checkpoint proteins”. Nature. 2008, 451: 81-85.

Alessi D.R., Sakamoto K., Bayascas J.R., "LKB1-dependent signaling pathways". Annu. Rev. Biochem. 2006, 75:137-163.

Aretz S., Stienen D., Uhlhaas S., Loff S., Back W., Pagenstecher C., McLeod D.R., Graham G.E., Mangold E., Santer R., Propping P., Friedl W., "High proportion of large genomic STK11 deletions in Peutz-Jeghers syndrome". Hum. Mutat. 2005, 26:513-519.

Baas A.F., Kuipers J., Van der Wel N.N., Batlle E., Koerten H.K., Peters P.J., Clevers H.C. “Complete polarization of single intestinal epithelial cells upon activation of LKB1 by STRAD”. Cell. 2004, 116:457-466.

Baas A.F., Boudeau J., Sapkota G.P., Smit L., Medema R., Morrice N.A., Alessi D.R., Clevers H.C. “Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD”. EMBO J. 2003, 22:3062-3072.

Balczon R., Bao L., Zimmer W.E. “PCM-1, A 228-kD centrosome autoantigen with a distinct cell cycle distribution”.J. Cell Biol. 1994, 124: 783-793.

Bettencourt-Dias M., Giet R., Sinka R., Mazumdar A., Lock W.G., Balloux F., Zafiropoulos P.J., Yamaguchi S., Winter S., Carthew R.W., Cooper M., Jones D., Frenz L., Glover D.M. "Genome-wide survey of protein kinases required for cell cycle progression". Nature. 2004, 432:980-987.

Bonaccorsi S., Mottier V., Giansanti M.G., Bolkan B.J., Williams B., Goldberg M.L., Gatti M. “The Drosophila Lkb1 kinase is required for spindle formation and asymmetric neuroblast division”. Development. 2007, 134: 2183–2193.

Boudeau J., Scott J.W., Resta N., Deak M., Kieloch A., Komander D., Hardie D.G., Prescott A.R., van Aalten D.M., Alessi D.R. "Analysis of the LKB1-STRAD-MO25 complex". J. Cell. Sci. 2004, 117:6365-6375.

Boudeau J., Baas A.F., Deak M., Morrice N.A., Kieloch A., Schutkowski M., Prescott A.R., Clevers H.C., Alessi D.R. "MO25alpha/beta interact with STRADalpha/beta enhancing their ability to bind, activate and localize LKB1 in the cytoplasm". EMBO J. 2003a, 22:5102-5114.

Boudeau J., Deak M., Lawlor M.A., Morrice N.A., Alessi D.R., "Heat-shock protein 90 and Cdc37 interact with LKB1 and regulate its stability". Biochem. J. 2003b, 370:849-857.

Benton R., St Johnston D. “Drosophila PAR1 and 14-3-3 inhibit Bazooka/Par-3 to establish complementary cortical domains in polarized cells”. Cell. 2003, 115:691-704.

Carretero J., Shimamura T., Rikova K., Jackson A.L., Wilkerson M.D., Borgman C.L., Buttarazzi M.S., Sanofsky B.A., McNamara K.L., Brandstetter K.A., Walton Z.E., Gu T.L., Silva J.C., Crosby K., Shapiro G.I., Maira S.M., Ji H., Castrillon D.H., Kim C.F., Garcia-Echeverria C., Bardeesy N., Sharpless N.E., Hayes N.D., Kim W.Y., Engelman J.A., Wong K.K. "Integrative Genomic and Proteomic Analyses Identify Targets for Lkb1-Deficient Metastatic Lung Tumors". Cancer Cell. 2010, 17:547-559.

Carling D., Sanders M.J., Woods A. "The regulation of AMP-activated protein kinase by upstream kinases". Int.J Obes. 2008, 32:S55-S59.

Castedo M., Perfettini J.L., Roumier T., Andreau K., Medema R., Kroemer G. “Cell death by mitotic catastrophe: a molecular definition”. Oncogene. 2004, 23:2825-37.

Cheng H., Liu P., Wang Z.C., Zou L., Santiago S., Garbitt V., Gjoerup O.V., Iglehart J.D., Miron A., Richardson A.L., Hahn W.C., Zhao J.J. "SIK1 couples LKB1 to p53-dependent anoikis and suppresses metastasis". Sci.Signal. 2009, 2(80):ra35.

Cohen D., Brennwald P.J., Rodriguez-Boulan E., Müsch A., “Mammalian PAR-1 determines epithelial lumen polarity by organizing the microtubule cytoskeleton”. J Cell Biol. 2004, 164:717-727.

Contreras C.M., Gurumurthy S., Haynie J.M., Shirley L.J., Akbay E.A., Wingo S.N., Schorge J.O., Broaddus R.R., Wong K.K., Bardeesy N., Castrillon D.H. "Loss of Lkb1 provokes highly invasive endometrial adenocarcinomas". Cancer Research. 2008, 68:759-766.

Corradetti M.N., Inoki K., Bardeesy N., DePinho R.A., Guan K.L. “Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome”. Genes Dev. 2004, 18:1533–1538.

Dammermann A. and Merdes A. “Assembly of centrosomal proteins and microtubule organization depends on PCM-1”. J. Cell Biol. 2002, 159: 255-266.

Denison F.C., Hiscock N.J., Carling D., Woods A. "Characterization of an alternative splice variant of LKB1". J Biol.Chem. 2009, 284:67-76.

Deguchi A., Miyoshi H., Kojima Y., Okawa K., Aoki M., Taketo M.M., "LKB1 suppresses p21-activated kinase-1 (PAK1) by phosphorylation of Thr109 in the p21-binding domain". J Biol.Chem. 2010, 285:18283-18290.

Dorfman J., Macara I.G. "STRADalpha regulates LKB1 localization by blocking access to importin-alpha, and by association with Crm1 and exportin-7". Mol. Biol. Cell. 2008, 19:1614-1626.

Dowling R.J., Zakikhani M., Fantus I.G., Pollak M., Sonenberg N. “Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells”. Cancer Res. 2007, 67:10804-10812

Drewes G., Ebneth A., Mandelkow E.M. "MAPs, MARKs and microtubule dynamics". Trends Biochem. Sci. 1998, 23:307-311.

Esquela-Kerscher A. and Slack F.J. “Oncomirs-microRNAs with a role in cancer” Nature Reviews Cancer. 2006, 6: 259-269.

Fernandez P., Carretero J., Medina P.P., Jimenez A.I., Rodriguez-Perales S., Paz, M.F., Cigudosa J.C., Esteller M., Lombardia L., Morente M., Sanchez-Verde L., Sotelo T., Sanchez-Cespedes M. "Distinctive gene expression of human lung adenocarcinomas carrying LKB1 mutations". Oncogene. 2004, 23:5084-5091.

Forcet C., Etienne-Manneville S., Gaude H., Fournier L., Debilly S., Salmi M., Baas A., Olschwang S., Clevers H., Billaud M. "Functional analysis of Peutz-Jeghers mutations reveals that the LKB1 C-terminal region exerts a crucial role in regulating both the AMPK pathway and the cell polarity". Hum. Mol. Genet. 2005, 14:1283-1292.

Forster L.F., Defres S., Goudie D.R., Baty D.U., Carey F.A. "An investigation of the Peutz-Jeghers gene (LKB1) in sporadic breast and colon cancers". J. Clin. Pathol. 2000, 53:791-793.

Gan B., Hu J., Jiang S., Liu Y., Sahin E., Zhuang L., Fletcher-Sananikone E., Colla S., Wang Y.A., Chin L., Depinho R.A. “Lkb1 regulates quiescence and metabolic homeostasis of haematopoietic stem cells”. Nature. 2010, 468:701–704.

Godlewski J., Bronisz A., Nowicki M.O., Chiocca E.A., Lawler S. "microRNA-451: A conditional switch controlling glioma cell proliferation and migration". Cell Cycle. 2010a, 9:2742-2748.

Godlewski J., Nowicki M. O., Bronisz A., Nuovo G., Palatini J., De L.M., Van B.J., Ostrowski M. C., Chiocca E A., Lawler S.E. "MicroRNA-451 regulates LKB1/AMPK signaling and allows adaptation to metabolic stress in glioma cells". Mol. Cell. 2010b, 37:620-632.

Green A.S., Chapuis N., Maciel T.T., Willems L., Lambert M., Arnoult C., Boyer O., Bardet V., Park S., Foretz M., Viollet B., Ifrah N., Dreyfus F., Hermine O., Moura I.C., Lacombe C., Mayeux P., Bouscary D., Tamburini J. "The LKB1/AMPK signaling pathway has tumor suppressor activity in acute myeloid leukemia through the repression of mTOR-dependent oncogenic mRNA translation. Blood 2010; 116: 4262–4273"

Green D. and Kroemer G. "The central executioners of apoptosis: caspases or mitochondria?" Trends Cell Biol. 1998, 8:267-71.

Gurumurthy S., Xie S.Z., Alagesan B., Kim J., Yusuf R.Z., Saez B., Tzatsos A., Ozsolak F., Milos P., Ferrari F., Park P.J., Shirihai O.S., Scadden D.T., Bardeesy N. "The Lkb1 metabolic sensor maintains haematopoietic stem cell survival". Nature. 2010, 468:659–663.

Haferlach T., Kohlmann A., Wieczorek L., Basso G., Kronnie G.T., Béné M.C., De Vos J., Hernández J.M., Hofmann W.K., Mills K.I., Gilkes A., Chiaretti S., Shurtleff S.A., Kipps T.J., Rassenti L.Z., Yeoh A.E., Papenhausen P.R., Liu W.M., Williams P.M., Foà R. "Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group". J. Clin. Oncol. 2010, 28: 2529-37.

Hawley S.A., Boudeau J., Reid J.L., Mustard K.J., Udd L., Makela T.P., Alessi D.R., Hardie D.G. "Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP activated protein kinase cascade". J Biol. 2003, 2:28.

Hemminki A., Tomlinson I., Markie D., Jarvinen H., Sistonen P., Bjorkqvist A.M., Knuutila S., Salovaara R., Bodmer W., Shibata D., De la C.A., Aaltonen L.A. "Localization of a susceptibility locus for Peutz-Jeghers syndrome to 19p using comparative genomic hybridization and targeted linkage analysis". Nat Genet. 1997, 15:87-90.

Hemminki A., Avizienyte E., Roth S., Loukola A., Aaltonen L.A., Järvinen H., De la Chapelle A. "A serine/threonine kinase gene defective in Peutz-Jeghers syndrome". Nature. 1998, 391:184-187.

Hemminki A. "The molecular basis and clinical aspects of Peutz-Jeghers syndrome". Cell. Mol. Life Sci. 1999, 55:735-750.

Horike N., Takemori H., Katoh Y., Doi J., Min L., Asano T., Sun X.J., Yamamoto H., Kasayama S., Muraoka M., Nonaka Y., Okamoto M. "Adipose-specific expression, phosphorylation of Ser794 in insulin receptor substrate1,and activation in diabetic animals of salt-inducible kinase-2". J Biol. Chem. 2003, 278:18440-18447.

Hou X., Xu S., Maitland-Toolan K.A., Sato K., Jiang B., Ido Y., Lan F., Walsh K., Wierzbicki M., Verbeuren T.J., Cohen R.A., Zang M. "SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase". J Biol. Chem. 2008, 283:20015-20026.

Hurov J.B., Huang M., White L.S., Lennerz J., Choi C.S., Cho Y.R., Kim H.J., Prior J.L., Piwnica-Worms D., Cantley L.C., Kim J.K., Shulman G.I., Piwnica-Worms H. "Loss of the Par-1b/MARK2 polarity kinase leads to increased metabolic rate, decrease adiposity, and insulin hypersensitivity *in vivo*". Proc Natl Acad Sci USA. 2007, 104:5680-5685.

Indraccolo S., Tisato V., Tosello V., Habeler W., Esposito G., Moserle L. Stievano L., Persano L., Chieco-Bianchi L., Amadori A. "Interferon-alpha gene therapy by lentiviral vectors contrasts ovarian cancer growth through angiogenesis Inhibition". Hum. Gene Ther. 2005, 16: 957-970.

Inoki K., Zhu T., Guan K.L. "TSC2 mediates cellular energy response to control cell growth and survival". Cell. 2003, 115:577-590.

Inoki K., Ouyang H., Zhu T., Lindvall C., Wang Y., Zhang X., Yang Q., Bennett C., Harada Y., Stankunas K., Wang C.Y., He X., MacDougald O.A., You M., Williams B.O., Guan K.L. "TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth". Cell. 2006, 126:955-968.

Jaleel M., McBride A., Lizcano J.M., Deak M., Toth R., Morrice N.A., Alessi D.R. "Identification of the sucrose non-fermenting related kinase SNRK, as a novel LKB1 substrate". FEBS Lett. 2005, 579:1417-1423.

Jansen M., ten Klooster J.P., Offerhaus G.J., Clevers H. "LKB1 and AMPK family signaling: the intimate link between cell polarity and energy metabolism". Physiol Rev. 2009, 89:777-798.

Jenne D.E., Reimann H., Nezu J., Friedel W., Loff S., Jeschke R., Muller O., Back W., Zimmer M. "PeutzJeghers syndrome is caused by mutations in a novel serine threonine kinase". Nat Genet. 1998, 18:38-43.

Jeon S.M., Chandel N.S., Hay N. “AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress”. Nature. 2012, 485:661-5.

Jones R.G., Plas D.R., Kubek S., Buzzai M., Mu J., Xu Y., Birnbaum M.J., Thompson C.B. "AMP-activated protein kinase induces a p53-dependent metabolic checkpoint". Mol. Cell. 2005, 18:283-293.

Kahan B.B., Alquier T., Carling D., Hardie D.G. “AMP-activated protein kinase:ancient energy gauge provides clues to modern understanding of metabolism”. Cell Metab. 2005, 1:15-25.

Karuman P., Gozani O., Odze R.D., Zhou X.C., Zhu H., Shaw R., Brien T.P., Bozzuto C.D., Ooi D., Cantley L.C., Yuan J. “The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death”. Mol. Cell. 2001, 7:1307-1319.

Katajisto P., Vallenius T., Vaahtomeri K., Ekman N., Udd L., Tiainen M., Makela T.P. "The LKB1 tumor suppressor kinase in human disease". Biochim. Biophys. Acta. 2007, 1775:63-75.

Katajisto P., Vaahtomeri K., Ekman N., Ventela E., Ristimaki A., Bardeesy N., Feil R., DePinho R. A., Makela T.P. "LKB1 signaling in mesenchymal cells required for suppression of gastrointestinal polyposis". Nat. Genet. 2008, 40:455-459.

Kato N., Romero M., Catasus L., Prat J. "The STK11/LKB1 Peutz-Jegher gene is not involved in the pathogenesis of sporadic sex cord-stromal tumors, although loss of heterozygosity at 19p13.3 indicates other gene alteration in these tumors". Hum. Pathol. 2004, 35:1101-1104.

Katoh Y., Takemori H., Horike N., Doi J., Muraoka M., Min L., Okamoto M. "Salt-inducible kinase (SIK) isoforms: their involvement in steroidogenesis and adipogenesis". Mol. Cell Endocrinol. 2004, 217:109-112.

Kenanli E., Karaman E., Enver O., Ulutin T., Buyru N. "Genetic Alterations of the LKB1 Gene in Head and Neck Cancer". DNA Cell Biol. 2010, 29:735-8.

Kim C.J., Cho Y.G., Park J.Y., Kim T.Y., Lee J.H., Kim H.S., Lee J.W., Song Y.H., Nam S.W., Lee S. H., Yoo N.J., Lee J.Y., Park W.S. "Genetic analysis of the LKB1/STK11 gene in hepatocellular carcinomas". Eur. J Cancer. 2004, 40:136-141.

Kishi M., Pan Y.A., Crump J.G., Sanes J.R. "Mammalian SAD kinases are required for neuronal polarization". Science. 2005, 307:929-932.

Kloosterman W.P. and Plasterk R.H. “The diverse functions of microRNAs in animal development and disease”. Dev. Cell. 2006, 11:441-50

Kojima Y., Miyoshi H., Clevers H.C., Oshima M., Aoki M., Taketo M.M. "Suppression of tubulin polymerization by the LKB1-microtubule-associated protein/microtubule affinity-regulating kinase signaling". J Biol.Chem. 2007, 282:23532-23540.

Koo S.H., Flechner L., Qi L., Zhang X., Screaton R.A., Jeffries S., Hedrick S., Xu W., Boussouar F., Brindle P., Takemori H., Montminy M. "The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism". Nature. 2005, 437:1109-1111.

Kuznetsov J.N., Leclerc G.J., Leclerc G.M., Barredo J.C. "AMPK and Akt determine apoptotic cell death following perturbations of one-carbon metabolism by regulating ER stress in acute lymphoblastic leukemia". Mol. Cancer Ther. 2011; 10: 437–447

Lan F., Cacicedo J.M., Ruderman N., Ido Y. "SIRT1 modulation of the acetylation status, cytosolic localization,and activity of LKB1. Possible role in AMP-activated protein kinase activation", J Biol. Chem. 2008, 283:27628-27635.

Lawler S. and Chiocca E.A. "Emerging functions of microRNAs in glioblastoma". J. Neurooncol. 2009, 92:297-306.

Liang J., Shao S.H., Xu Z.X., Hennessy B., Ding Z., Larrea M., Kondo S., Dumont D.J., Gutterman J.U., Walker C.L., Slingerland J.M., Mills G.B. "The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis". Nat Cell Biol. 2007, 9:218-224.

Lin-Marq N., Borel C., Antonarakis S.E. "Peutz-Jeghers LKB1 mutants fail to activate GSK-3beta, preventing it from inhibiting Wnt signaling". Mol.Genet Genomics. 2005, 273:184-196.

Lizcano J.M., Goransson O., Toth R., Deak M., Morrice N.A., Boudeau J., Hawley S.A., Udd L., Makela T.P., Hardie D.G., Alessi D.R. "LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1". EMBO J. 2004, 23:833-843.

Legembre P., Schickel R., Barnhart B.C., Peter M.E. "Identification of SNF1/AMP kinase-related kinase as an NF-kappaB-regulated anti-apoptotic kinase involved in CD95-induced motility and invasiveness". J Biol.Chem. 2004, 279:46742-46747.

Londesborough A., Vaahtomeri K., Tiainen M., Katajisto P., Ekman N., Vallenius T., Makela T.P. "LKB1 in endothelial cells is required for angiogenesis and TGFbeta-mediated vascular smooth muscle cell recruitment". Development. 2008, 135:2331-2338.

Marignani P.A., Kanai F., Carpenter C.L. "LKB1 associates with Brg1 and is necessary for Brg1-induced growth arrest". J Biol Chem 2001, 276:3241532418.

Martin S.G., St Johnston D. "A role for *Drosophila* LKB1 in anterior–posterior axis formation and epithelial polarity". Nature. 2003,421:379-384.

Marumoto T., Hirota T., Morisaki T., Kunitoku N., Zhang D., Ichikawa Y., Sasayama T., Kuninaka S., Mimori T., Tamaki N., Kimura M.,Okano Y., Saya H. "Roles of aurora-A kinase in mitotic entry and G_2 checkpoint in mammalian cells". Genes Cells. 2002, 7: 1173-1182.

McCarthy A., Lord C.J., Savage K., Grigoriadis A., Smith D.P., Weigelt B., Reis-Filho J.S., Ashworth A. "Conditional deletion of the Lkb1 gene in the mouse mammary gland induces tumour formation". J Pathol. 2009, 219:306-316.

Mehenni H., Lin-Marq N., Buchet-Poyau K., Reymond A., Collart M.A., Picard D., Antonarakis S.E. "LKB1 interacts with and phosphorylates PTEN: a functional link between two proteins involved in cancer predisposing syndromes". Hum. Mol. Genet. 2005, 14:2209-2219.

Memmott R.M. and Dennis P.A. "LKB1 and mammalian target of rapamycin as predictive factors for the anticancer efficacy of metformin". J. Clin. Oncol 2009, 27:e226

Mosmann T. “Rapid colorimetric assay for cellular growth and survival: application to proliferation and citotoxic assay”. J. Immunol. Meth. 1983, 65: 55–63.

Morton J.P., Jamieson N.B., Karim S.A., Athineos D., Ridgway R.A., Nixon C., McKay C.J., Carter R., Brunton V.G., Frame M.C., Ashworth A., Oien K.A., Evans T.R., Sansom O.J. "Lkb1 Haploinsufficiency Cooperates with Kras to Promote Pancreatic Cancer via Suppression of p21 Dependent Growth Arrest". Gastroenterology. 2010, 139:586-97.

Nakada D., Saunders T.L., Morrison S.J. “Lkb1 regulates cell cycle and energy metabolism in haematopoietic stem cells”. Nature. 2010, 468:653–658.

Nakau M., Miyoshi H., Seldin M.F., Imamura M., Oshima M., Taketo M.M. "Hepatocellular carcinoma caused by loss of heterozygosity in Lkb1 gene knockout mice". Cancer Research. 2002, 62:4549-4553.

Ossipova O., Bardeesy N., DePinho R.A., Green J.B. "LKB1 (XEEK1) regulates Wnt signalling in vertebrate development". Nat Cell Biol. 2003, 5:889-894.

Park W.S., Moon Y.W., Yang Y.M., Kim Y.S., Kim Y.D., Fuller B.G., Vortmeyer A.O., Fogt F., Lubensky I.A., Zhuang Z. "Mutations of the STK11 gene in sporadic gastric carcinoma". Int. J Oncol. 1998, 13:601-604.

Qiu W., Schonleben F., Thaker H.M., Goggins M., Su, G.H. "A novel mutation of STK11/LKB1 gene leads to the loss of cell growth inhibition in head and neck squamous cell carcinoma". Oncogene. 2006, 25:2937-2942.

Rodriguez-Nieto S., Sanchez-Cespedes M. "BRG1 and LKB1: tales of two tumor suppressor genes on chromosome 19p and lung cancer". Carcinogenesis. 2009, 30:547-554.

Sahin F., Maitra A., Argani P., Sato N., Maehara N., Montgomery E., Goggins M., Hruban R. H., Su G.H. "Loss of Stk11/Lkb1 expression in pancreatic and biliary neoplasms". Mod. Pathol. 2003, 16:686-691.

Sanchez-Cespedes M. "A role for LKB1 gene in human cancer beyond the Peutz-Jeghers syndrome". Oncogene. 2007, 26:7825-7832.

Screaton R.A., Conkright M.D., Katoh Y., Best J.L., Canettieri G., Jeffries S., Guzman E., Niessen S., Yates J.R., Takemori H., Okamoto M., Montminy M. "The CREB coactivator TORC2 functions as a calcium- and cAMPsensitive coincidence detector". Cell. 2004, 119:61-74.

Sebbagh M., Santoni M.J., Hall B., Borg J.P., Schwartz M.A. "Regulation of LKB1/STRAD localization and function by E-cadherin". Curr. Biol. 2009, 19:37-42.

Sengupta T.K., Leclerc G.M., Hsieh-Kinser T.T., Leclerc G.J., Singh I., Barredo J.C. “Cytotoxic effect of 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) on childhood acute lymphoblastic leukemia (ALL) cells: implication for targeted therapy”. Mol. Cancer. 2007, 6: 46;

Shackelford D.B., Shaw R.J. “The LKB1-AMPK pathway: metabolism and growth control in tumor suppression”. Nat. Rev. Cancer. 2009, 9:563-575

Shaw R.J., Kosmatka M., Bardeesy N., Hurley R.L., Witters L.A., DePinho R.A., Cantley L.C. "The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress". Proc. Natl. Acad. Sci. U.S.A. 2004a, 101:3329-3335.

Shaw R.J., Bardeesy N., Manning B.D., Lopez L., Kosmatka M., DePinho R.A., Cantley LC. “The LKB1 tumor suppressor negatively regulates mTOR signaling”. Cancer Cell. 2004b, 6:91–99.

Shaw R.J., Lamia K.A., Vasquez D., Koo S.H., Bardeesy N., DePinho R.A., Montminy M., Cantley L.C. "The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin". Science. 2005, 310:1642-1646.

Sobottka S.B., Haase M., Fitze G., Hahn M., Schackert H.K., Schackert G. "Frequent loss of heterozygosity at the 19p13.3 locus without LKB1/STK11 mutations in human carcinoma metastases to the brain". J. Neurooncol. 2000, 49:187-195.

Smith D.P., Spicer J., Smith A., Swift S., Ashworth A. “The mouse Peutz–Jeghers syndrome gene Lkb1 encodes a nuclear protein kinase”. Hum Mol Genet. 1999, 8:1479-1485.

Smith D.P., Rayter S.I., Niederlander C., Spicer J., Jones C.M., Ashworth A. "LIP1, a cytoplasmic protein functionally linked to the Peutz-Jeghers syndrome kinase LKB1". Hum. Mol. Genet. 2001, 10:2869-2877.

Su J.Y., Erikson E., Maller J.L. “Cloning and characterization of a novel serine/threonine protein kinase expressed in early *Xenopus* embryos”. J Biol Chem. 1996, 271:14430-14437.

Suzuki A., Kusakai G., Kishimoto A., Lu J., Ogura T., Esumi H. "ARK5 suppresses the cell death induced by nutrient starvation and death receptors via inhibition of caspase 8 activation, but not by chemotherapeutic agents or UV irradiation". Oncogene. 2003, 22:6177-6182.

Taliaferro-Smith L., Nagalingam A., Zhong D., Zhou W., Saxena N.K., Sharma D., "LKB1 is required for adiponectin-mediated modulation of AMPK-S6K axis and inhibition of migration and invasion of breast cancer cells". Oncogene. 2009, 28:2621-2633.

Thaiparambil J.T., Eggers C.M., Marcus A.I. “AMPK regulates mitotic spindle orientation through phosphorylation of myosin regulatory light chain”. Mol. Cell. Biol. 2012, 32: 3203-17.

Towler M.C., Fogarty S., Hawley S.A., Pan D.A., Martin D.M., Morrice N.A., McCarthy A., Galardo M.N., Meroni S.B., Cigorraga S.B., Ashworth A., Sakamoto K., Hardie D.G. "A novel short splice variant of the tumour suppressor LKB1 is required for spermiogenesis". Biochem. J. 2008, 416:1-14.

Trojan J., Brieger A., Raedle J., Esteller M., Zeuzem S. "5'-CpG island methylation of the LKB1/STK11 promoter and allelic loss at chromosome 19p13.3 in sporadic colorectal cancer". Gut. 2000, 47:272-276.

Vazquez-Martin A., Oliveras-Ferraros C., Menendez J.A. “The metabolic rheostat AMP-activated protein kinase (AMPK) is unexpectedly required for proper cell division and faithful chromosomal segregation during mitosis”. Cell Cycle. 2009, 8: 2385-98.

Wang Z.J., Churchman M., Campbell I.G., Xu W.H., Yan Z.Y., McCluggage W.G., Foulkes W.D., Tomlinson I.P. "Allele loss and mutation screen at the Peutz-Jeghers (LKB1) locus (19p13.3) in sporadic ovarian tumours". Br. J. Cancer. 1999, 80:70-72.

Watts J.L., Morton D.G., Bestman J., Kemphues K.J. “The *C. elegans* par-4 gene encodes a putative serine–threonine kinase required for establishing embryonic symmetry”. Development. 2000, 127:1467-1475.

Wingo S.N., Gallardo T.D., Akbay E.A., Liang M.C., Contreras C.M., Boren T., Shimamura T., Miller D.S., Sharpless N.E., Bardeesy N., Kwiatkowski D.J., Schorge J.O., Wong K.K., Castrillon D.H. "Somatic LKB1 mutations promote cervical cancer progression". PLoS ONE. 2009, 4:e5137.

Xie Z., Dong Y., Zhang J., Scholz R., Neumann D., Zou M.H. "Identification of the serine 307 of LKB1 as a novel phosphorylation site essential for its nucleocytoplasmic transport and endothelial cell angiogenesis". Mol. Cell Biol. 2009, 29:3582-3596.

Yang T.L., Su Y.R., Huang C.S., Yu J.C., Lo Y.L., Wu P.E., Shen C.Y. "High-resolution 19p13.2-13.3 allelotyping of breast carcinomas demonstrates frequent loss of heterozygosity". Genes Chromosomes. Cancer. 2004, 41:250-256.

Zagorska A., Deak M., CampbelL D.G., Banerjee S., Hirano M., Aizawa S., Prescott A.R., Alessi D.R. "New roles for the LKB1-NUAK pathway in controlling myosin phosphatase complexes and cell adhesion". Sci. Signal. 2010, 3:ra25.

Zamzami N. and Kroemer G. “Apoptosis: mitochondrial membrane permeabilization--the (w)hole story?” Curr Biol. 2003, 13:R71-3.

Zeng P.Y., Berger S.L. “LKB1 is recruited to the p21/WAF1 promoter by p53 to mediate transcriptional activation”. Cancer Res.2006, 66:10701-10708.

Zeqiraj E., Filippi B.M., Deak M., Alessi D.R., Van Aalten D.M. "Structure of the LKB1-STRAD-MO25 complex reveals an allosteric mechanism of kinase activation". Science. 2009a, 326:1707-1711.

Zeqiraj E., Filippi B.M., Goldie S., Navratilova I., Boudeau J., Deak M., Alessi D.R., Van Aalten D.M. "ATP and MO25alpha regulate the conformational state of the STRADalpha pseudokinase and activation of the LKB1 tumour suppressor". PLoS Biol. 2009b, 7:e1000126.

Zhang L., Li J., Young L.H., Caplan M.J. "AMP-activated protein kinase regulates the assembly of epithelial tight junctions". Proc Natl. Acad. Sci. U.S.A. 2006, 103:17272-17277.

Zhang L., He H., Balschi J.A. “Metformin and phenformin activate AMP-activated protein kinase in the heart by increasing cytosolic AMP concentration”. Am. J. Physiol. Heart Circ. Physiol. 2007, 293:H457-466

Zhang S., Schafer-Hales K., Khuri F.R., Zhou W., Vertino P.M., Marcus A.I. "The tumor suppressor LKB1 regulates lung cancer cell polarity by mediating cdc42 recruitment and activity". Cancer Research. 2008, 68:740-748.

Zheng B., Cantley L.C. "Regulation of epithelial tight junction assembly and disassembly by AMP-activated protein kinase". Proc Natl. Acad. Sci. U.S.A. 2007, 104:819-822.

Zhong D., Liu X., Khuri F.R., Sun S.Y., Vertino P.M., Zhou W. “LKB1 is Necessary for Akt-mediated Phosphorylation of Pro-apoptotic Proteins”. Cancer Res. 2008, 68:7270–7277.

CHAPTER III:

“LKB1 regulates the circadian rhythm”

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III. INTRODUCTION

III.I Circadian rhythms in mammals

Circadian rhythms occur with a periodicity of about 24 hours and regulate a wide array of metabolic and physiologic functions. The Earth's rotation around its axis leads to day-night cycles, which affects the physiology of most living organisms. Circadian (from the Latin *circa diem* meaning "about a day") clocks are intrinsic, time tracking systems that enable organisms to anticipate environmental changes (such as food availability and predatory pressure), and allow them to adapt their behavior and physiology to the appropriate time of day (Schibler U., and Sassone-Corsi P., 2002). This system controls a wide variety of physiological functions, including sleep-wake cycles, body temperature, hormone secretion, feeding behaviour and locomotory activity (Schibler U. and Sassone-Corsi P., 2002). Accumulating epidemiological and genetic evidence indicates that disruption of circadian rhythms can be directly linked to many pathological conditions, including sleep disorders, depression, metabolic syndrome and cancer. Intriguingly, a number of molecular gears constituting the clock machinery have been found to establish functional interplays with regulators of cellular metabolism and cell cycle.

The three integral parts of circadian clocks are: an input pathway that includes detectors to receive environmental cues (or Zeitgebers) and transmits them to the central oscillator; a central oscillator that keeps circadian time and generates rhythm; and output pathways through which the rhythms are manifested via control of various metabolic, physiological and behavioral processes. Distinguishing characteristics of circadian clocks include that they are entrainable (synchronizable by external cues); self-sustained (oscillations can persist even in the absence of zeitgebers); and temperature compensated (moderate variations in ambient temperature does not affect the period of circadian oscillation) (Roenneberg T., Merrow M., 2005).

Circadian clocks are present in almost all of the tissues in mammals. The master or 'central' clock is located in the hypothalamic suprachiasmatic nucleus (SCN), which contains 10-15,000 neurons. Peripheral clocks are present in almost all other mammalian tissues such as liver, heart, lung and kidney, where they maintain circadian rhythms and regulate tissue-specific gene expression.

Peripheral tissues also contain functional circadian oscillators that are self-sustained at the single cell level, but they do not respond to light-dark cycles and appear to require other physiological stimuli in order to sustain their circadian rhythms. Importantly, lesion of the SCN in rodents disrupts the circadian periodicity in peripheral tissues and SCN transplantation into SCN ablated and thus arrhythmic animals restores this

dysfunction (Lehman M.N. *et al.*, 1987; Ralph M.R. *et al.*, 1991). Additional experiments, in which the transplantation approach of fibroblasts was applied to peripheral tissues demonstrates a hierarchical dominance of the SCN over peripheral clocks (Pando M.P. *et al.*, 2002). To date, however, the means by which the SCN communicates with the other tissues to sustain and synchronize their cycles is still not clear. Several observations support the idea that communication might be exerted by a combination of neuronal signals through the autonomic nervous system and humoral factors, of which glucocorticoids and retinoic acid are the most likely candidates (Antle M.C. and Silver R., 2005). Furthermore, peripheral rhythms in mammals are affected by other SCN-independent stimuli (Yoo S.H. *et al.*, 2004). Although light is the main stimulus that entrains the central pacemaker, peripheral clocks themselves can be entrained by food (Stokkan K.A. *et al.*, 2001), probably through modifications of hormonal secretion or metabolite availability. Conversely, restricted access to food can reset the phase of peripheral oscillators with little, if any, effects on the central pacemaker in the SCN (Damiola F. *et al.*, 2000). Another important environmental cue is temperature (Roenneberg T. and Merrow M., 2005). Temperature compensation is one of the most prominent features of the circadian system as it allows the integration of moderate variations in ambient temperature that do not affect the period length of circadian oscillation. Nevertheless, low-amplitude temperature cycles can synchronize the circadian clocks in peripheral tissues in mammals independently of the central clock (Brown S.A. *et al.*, 2002).

Additional evidence demonstrated the presence of circadian oscillators even in established cell lines; in cultured fibroblasts the endogenous clock system needs a simple serum shock to be resynchronized, whereas the pacemaker of zebrafish embryonic cell starts ticking upon exposure to a short pulse of light (Hunt T. and Sassone-Corsi P., 2007).

III.II The molecular machinery

The clock machinery is based on a group of complex transcriptional–translational feedback loops (Glossop N.R. and Hardin P.E., 2002; Morse D. and Sassone-Corsi, 2002; Reppert S.M. and Weaver D.R., 2002; Sahar S. and Sassone-Corsi P., 2009) (**Fig. III.1**). In mammals, the proteins circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1) form the positive branch of the feedback loop (Gekakis N. *et al.*, 1998; Vitaterna M.H. *et al.*, 1994). These proteins are members of the family of basic-helix-loop-helix (bHLH)-PAS transcription factors that heterodimerize through their PER–ARNT–SIM (PAS) domains and bind to DNA elements, the so-called E-boxes with the nucleotide sequence CACGTG. E-boxes are among the most frequent

promoter elements in mammalian genomes and are present in either one or multiple copies in the regulatory regions of CCGs (clock-controlled genes).

Among the CLOCK–BMAL1 target genes are the period genes (*Per1*, *Per2* and *Per3*) and cryptochrome genes (*Cry1* and *Cry2*). PER and CRY proteins form a heterodimeric repressor complex that translocates into the nucleus to inhibit CLOCK–BMAL1- mediated activation of CCGs – that include *Per* and *Cry* genes (Darlington T.K. *et al.*, 1998; Griffin E.A. *et al.*, 1999; Kume K. *et al.*, 1999; Sato T.K. *et al.*, 2006). A cyclic proteolytic degradation of the repressor complex has an essential role in the maintenance of the amplitude of the circadian oscillation. Degradation occurs upon phosphorylation of the PER proteins by casein kinase Iε (CKIε) and recruitment of the specialized F-box protein FBXL3 (Busino L. *et al.*, 2007; Godinho S.I., *et al.*, 2007; Siepka S.M., *et al.*, 2007) that ubiquitylates CRY1. These events are thought to allow the CLOCK–BMAL1 activator complex to start a new transcriptional cycle within 24 hours (Eide E.J., *et al.*, 2005).

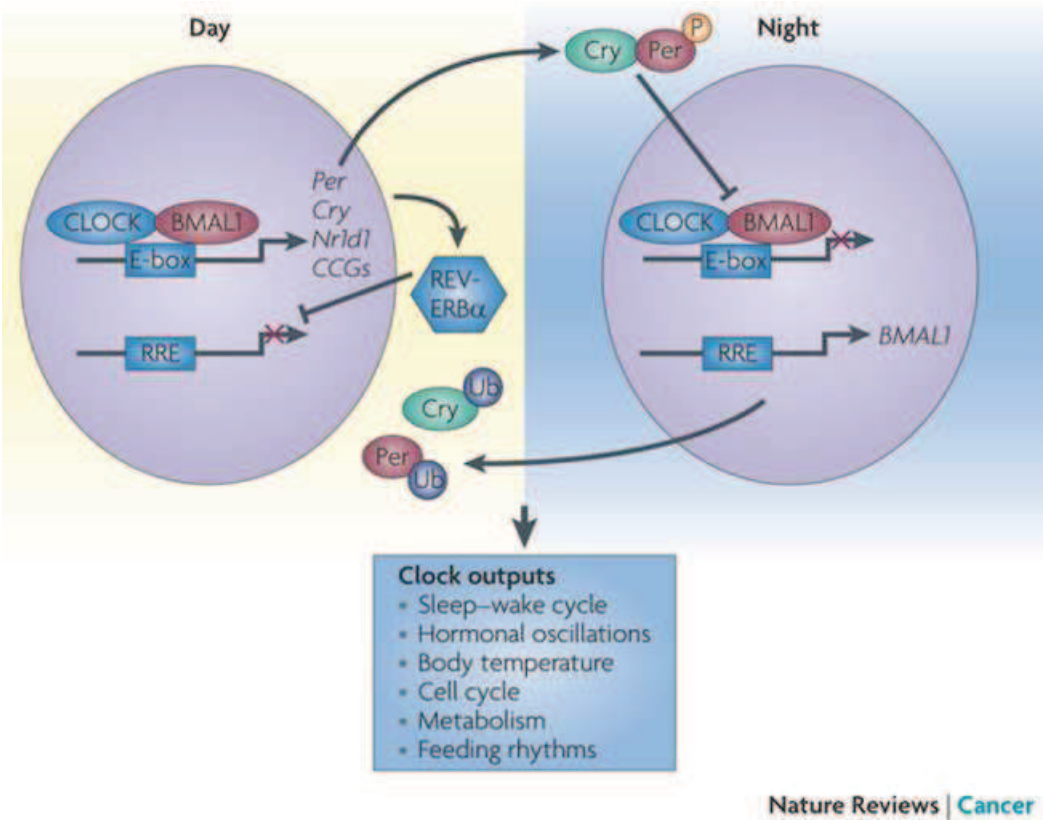

Fig. III.1: CLOCK and BMAL1 increase the transcription of Period (Per), Cryptochrome (Cry) and other clockcontrolled genes (CCGs) during the day. In the classic view, the levels of Per and Cry proteins increase during the night, after qhich they dimerize and translocate to the nucleus to repress CLOCK-BMAL1-mediated transcription. Per and Cry proteins are the ubiquitylated and degraded to initiate a new circadian cycle. Conversely, REVERBα protein levels are high during the day and inhibit *BMAL1* transcription at this time. At night, REVERBα protein levels are low, allowing *BMAL1* transcription to take place. P, phosphorylation; RRE, REVERBα/ROR response elements; Ub, ubiquitylation (Sahar S. and Sassone-Corsi P., 2009).

Gene expression profiles obtained from microarray studies have revealed that up to 10% of all transcripts in different tissues display circadian oscillation (Akhtar R.A., *et al.*, 2002; Duffield G.E., *et al.*, 2002; Panda S., *et al.*, 2002; Storch K.F., *et al.*, 2002). Considering that the overlap of circadian genes in various tissues is limited, the proportion of genes that are expressed in a rhythmic manner is actually much higher. Among these, many are genes directly regulated by CLOCK–BMAL1 through E-boxes contained in their promoters, such as vasopressin (*Avp*) or D site albumin promoter-binding protein (*Dbp*) (Jin X., *et al.*, 1999; Ripperger J.A., *et al.*, 2000; Ripperger J.A. and Schibler U., 2006). Other genes encode transcription factors that, in turn, regulate the cyclic expression of additional CCGs. This mode of regulation occurs through binding of these transcription factors to regulatory consensus sequences that differ from E-boxes, such as D-boxes or receptor-related orphan receptor (ROR) response elements (ROREs) (Ueda H.R., *et al.*, 2005). Additional transcriptional–translational regulatory loops contribute to the function of the circadian clock. Specifically, the transcription factors reverse erythroblastosis virus-α (REV-ERBα) and retinoic acid receptor-related orphan receptor-α (RORα) are nuclear receptors that constitute an additional loop and therefore have an essential role in driving rhythmic *Bmal1* expression. These two transcription factors bind to ROREs in the *Bmal1* promoter and either activate (in the case of RORα binding) or repress (by the binding of REV-ERBα) *Bmal1* transcription (Preitner N., *et al.*, 2002; Sato T.K., *et al.*, 2004). Completion of this feedback loop occurs through transcription of the gene encoding REV-ERBα that is directly activated by CLOCK–BMAL1 and through its expression with robust rhythmicity.

III.III Post-translational modifications (PTMs) on circadian control

Various core clock proteins undergo post-translational modifications (PTMs), a feature that is likely to contribute significantly to the plasticity of the circadian system.

It has been shown that control of circadian clock components operates not only at the post-transcriptional level (Cermakian N., and Sassone-Corsi P., 2000; So W.V., and Rosbash M., 1997), but also at the level of protein stability (Sahar S., *et al.*, 2010) and through intracellular localization (Takano A., *et al.*, 2004; Vielhaber E.L., *et al.*, 2001). The crucial importance of post-transcriptional regulation becomes more apparent when one considers the delay in the negative-feedback loop that is required to give the clock a 24-hour period. Transcription–translation feedback cycles generally operate on a timescale of up to a few hours. If, following synthesis, the repressor proteins PER and CRY translocated to the nucleus to repress CLOCK and BMAL1, the whole cycle would take just a few hours rather than one day. To maintain the daily oscillations of clock

proteins, a significant delay between the activation and repression of transcription is required; the delay between the activation and repression of transcription is ensured by regulation through post-translational modifications. Reversible phosphorylation, for example, regulates important processes such as nuclear entry, formation of protein complexes and protein degradation (Gallego M. and Virshup D.M., 2007). Each of these can individually contribute to introduce the delay that keeps the period at ~24 hours.
Various PTMs have been described for most clock proteins to participate in controlling the timing between the activation and the repression of circadian transcription (Gallego M.and Virshup D.M., 2007), and many of these occur in a time-specific manner.

III.III.I Phosphorylation

Phosphorylation is a PTM that is widely used by eukaryotic (but also prokaryotic) cells to regulate, activate or modify various cellular processes. Therefore it is not surprising that phosphorylation also regulates many critical steps within the circadian mechanism. The rhythmic phosphorylation of a protein is achieved by the regulated action of kinases and phosphatases that counterbalance their activity with different activities at different windows (Reischl S. and Kramer A., 2011).
Many clock proteins contain several putative phosphoacceptor sites for a number of possible candidate kinases (Hirayama J. and Sassone-Corsi P., 2005). A paradigmatic example of the importance of this modification for circadian function is the phosphorylation of PER proteins by CKIε. This kinase has been closely linked to circadian rhythms, as the phenotype of the hamster *tau* mutant, which is characterized by impaired circadian rhythmicity, is caused by a mutation in the gene encoding CKIε (Lowrey P.L., *et al.*, 2000). CKIε-mediated phosphorylation of PER proteins targets them for ubiquitin mediated degradation, thereby controlling the duration of their function (Eide E.J., *et al.*, 2005; Gallego M. and Virshup D.M., 2007). Further evidence for the importance of CKIε comes from *Drosophila*, in which drastic disruptions in circadian rhythms are caused by mutations in the fly ortholog of CKIε, *Doubletime* (*dbt*) (Kloss B., *et al.*, 1998; Price J.L., *et al.*, 1998). CKIε also phosphorylates and activates BMAL1 (Eide E.J., *et al.*, 2002). BMAL1 is a target for various kinases and displays a remarkable profile of circadian phosphorylation in a range of tissues and in serum-shocked synchronized fibroblasts. Glycogen synthase kinase 3β (GSK3β) has recently been shown to phosphorylate mammalian BMAL1 and to control its stability and activity (Sahar S., *et al.*, 2010). Interestingly, the *Drosophila* ortholog of GSK3, *Shaggy* (*sgg*), also phosphorylates the fly clock proteins PERIOD and TIMELESS (Martinek S., *et al.*, 2001). Moreover, a high-throughput approach recently demonstrated that inhibition of GSK3β

expression and activity leads to a shortening of the circadian period (Hirota T., *et al.*, 2008). The involvement of GSK3β in circadian physiology is of interest as this kinase has been implicated in the molecular mechanism of lithium-based therapy of depression (Doble B.W. and Woodgett J.R., 2003), a syndrome in humans linked to disruption of circadian rhythms (Bunney W.E. and Bunney B.G., 2000; Perreau-Lenz S., *et al.*, 2007). Finally, in addition to BMAL1, GSK3β phosphorylates other clock proteins, including CRY2 (Harada Y., *et al.*, 2005), PER2 (Iitaka C., *et al.*, 2005), REV-ERBα (Yin L., *et al.*, 2006) and CLOCK (Spengler M.L., *et al.*, 2009), resulting in different effects on the protein stability of each substrate. BMAL1 is also targeted by other kinases, for example by mitogen-activated protein kinases (MAPKs) (Sanada K., *et al.*, 2002) and CK2α (Tamaru T., *et al.*, 2009). An important site of CK2α activity is the serine residue at position 90 (S90) in BMAL1, the mutation of which leads to disruptions in circadian rhythmicity (Tamaru T., *et al.*, 2009). The ortholog of CK2 kinase in *Drosophila* also exerts a crucial circadian function by phosphorylating PER and regulating its nuclear entry (Akten B., *et al.*, 2003; Lin J.M., *et al.*, 2002; Lin J.M., *et al.*, 2005).

As described above, a variety of kinases involved in circadian regulation have been identified. Considering the complexity of the circadian system and the amount of multiply phosphorylated clock components, it is reasonable to speculate that more kinases are involved in fine-tuning the circadian oscillator (Reischl S. and Kramer A., 2011).

III.III.II Sumoylation, acetylation and ubiquitylation

Among clock proteins, BMAL1 has been shown to undergo extensive PTMs in addition to phosphorylation. BMAL1 is a substrate for sumoylation (Cardone L., *et al.*, 2005), ubiquitylation (Kwon I., *et al.*, 2006; Lee J., *et al.*, 2008; Sahar S., *et al.*, 2010) and acetylation (Hirayama J., *et al.*, 2007). The role of acetylation is discussed further below.

Sumoylation and ubiquitylation are PTMs that directly influence the intracellular pathways that control protein stability. Sumoylation in particular is involved in various cellular processes, such as transcriptional regulation, nuclear transport and protein stability. BMAL1 is sumoylated by the small ubiquitin-like modifier 1 (SUMO-1) at a specific, conserved lysine residue at position 259 (K259) located in the PAS-domains linker. BMAL1 shows a circadian pattern of sumoylation in mouse liver that closely follows its activation. Sumoylation of BMAL1 is controlled by the circadian clock and requires CLOCK, the heterodimerization partner of BMAL1. Ectopic expression of a SUMO-1-deficient form of BMAL1 by using a viral vector demonstrated that sumoylation has an important role in the rhythmic expression of BMAL1. Sumoylation of BMAL1 is

enhanced by ubiquitin conjugating enzyme 9 (UBC9), an E2 ligase that activates the SUMO pathway. These findings, and the reported BMAL1 ubiquitylation (Lee J., *et al.*, 2008; Sahar S., *et al.*, 2010), stress the important role the proteasome has in regulating clock proteins.

III.IV A chromatin remodeling clock

Because the fraction of mammalian transcripts that oscillates in a circadian manner is remarkably high (Akhtar R.A., *et al.*, 2002; Duffield G.E., *et al.*, 2002; Panda S., *et al.*, 2002; Storch K.F., *et al.*, 2002), it follows that there must be an equally widespread program of dynamic changes in chromatin remodeling that accompany and allow for circadian gene expression. What could be defined as the 'circadian epigenome' probably involves cycles of chromatin transitions that allow a highly dynamic chromatin structure with the DNA to be locally and temporally permissive to transcription. Epigenetic control can be exerted through a variety of mechanisms, including DNA methylation, microRNA-mediated metabolic pathways, histone variants and histone PTMs (Bernstein E., and Allis C.D., 2005; Berger S.L., 2007; Borrelli E., *et al.*, 2008; Cheung P., *et al.*, 2000a; Miranda T.B. and Jones P.A., 2007; Strahl B.D. and Allis C.D., 2000). The presence of an "indexing epigenetic code" suggests that the coordinated and progressive combination of these processes allows the epigenome to move from an "unlocked" transcriptionally active to a "locked" silenced state, thereby determining the fate and physiology of a given cell (Borrelli E., *et al.*, 2008). Histone PTMs are largely responsible for the plasticity of chromatin remodeling (Cheung P., *et al.*, 2000a). Histones can be modified at multiple amino acid residues and at more than 30 sites within their N-terminal tails. Many different PTMs (acetylation, methylation, phosphorylation, sumoylation, ubiquitylation, ADP ribosylation and biotinylation) can occur, some involving modification of the same residue but at different times. Therefore, the number of possible PTM combinations is enormous, an observation that inspired the concept of the 'epigenetic code', suggesting that modifications can be coupled functionally and occur interdependently in specific combinations (Strahl B.D. and Allis C.D., 2000). For example, acetylation of lysine residue at positions 9 (K9) or 14 (K14) of histone H3 is known to be associated with an open chromatin conformation that leads to active gene transcription (Lee D.Y., *et al.*, 1993). Phosphorylation of H3 at the nearby serine residue at position 10 (S10) appears to function as a priming event that allows acetylation of K14 with a much higher efficacy (Cheung P., *et al.*, 2000b; Lo W.S., *et al.*, 2000), probably owing to the physical interaction of enzymes that are thought to elicit these combined modifications (Merienne K., *et al.*, 2001; Lo W.S., *et al.*, 2000).

Several studies have indicated that histone modifications at CCG promoters occur in a circadian manner (Crosio C., *et al.*, 2000; Curtis A.M., *et al.*, 2004; Doi M., *et al.*, 2006; Etchegaray J.P., *et al.*, 2003; Naruse Y., *et al.*, 2004). For example, the rhythmic (rather than constitutive) recruitment of CLOCK–BMAL1 to the *Dbp* promoter is associated with the acetylated status of the H3 tail at that locus (Ripperger J.A. and Schibler U., 2006). In addition to phosphorylation and acetylation, methylation is thought to contribute to regulating circadian transcription. Whereas trimethylation of lysine residue at position 4 of H3 (H3K4 trimethylation), a marker of open chromatin, has been associated with H3K9 acetylation and therefore activation of the *Dbp* promoter, H3K4 dimethylation at the same promoter correlates with the repressive phase of its cycle (Ripperger J.A. and Schibler U., 2006). The finding that CLOCK has intrinsic histone acetyl transferase (HAT) activity (Doi M., *et al.*, 2006) revealed the long-sought molecular link between epigenetic control and the circadian clock. CLOCK is more than merely a transcription factor and, indeed, reveals it to be a new type of DNA-binding HAT (Doi M., *et al.*, 2006). CLOCK is a bona fide HAT with a preference for acetylating lysine residues in H3 and H4. Importantly, CLOCK-mediated H3K14 acetylation at CCG promoters follows a circadian rhythmicity (Doi M., *et al.*, 2006; Nakahata Y., *et al.*, 2008). Interestingly, studies of mouse embryonic fibroblasts (MEFs) derived from homozygous *Clock* mutant (*c/c*) mice demonstrate that the HAT activity of CLOCK is essential for circadian gene expression (Doi M., *et al.*, 2006).

Similar to other HATs (Glozak M.A., *et al.*, 2005), CLOCK also elicits the acetylation of non-histone substrates, such as of its heterodimerization partner BMAL1, which is acetylated at a single, highly conserved lysine residue (K537) (Hirayama J., *et al.*, 2007).

This event is crucial for the circadian transcriptional program. Indeed, BMAL1 acetylation increases the affinity of CLOCK–BMAL1 for the repressor CRY, an association that is crucial for the negative feedback loop of the clock (Hirayama J., *et al.*, 2007). Finally, recent reports show that CLOCK can acetylate non-histone proteins other than BMAL1; for example, the glucocorticoid receptor is a target of CLOCK and its acetylation appears to regulate its function (Nader N., *et al.*, 2009).

III.V Role of CCGs in cancer

Core circadian clock genes (CCGs) seem to be important in tissue homeostasis and tumorigenesis. Several studies using human subjects lend additional support to the epidemiological data that link defects in circadian rhythms to increased susceptibility for developing cancer and poor prognosis. For example, the expression of all three *Per* genes is deregulated in breast cancer cells (Lamia K.A., *et al.*, 2008). *Per1* expression is downregulated in most patients, possibly owing to methylation of its promoter. In

addition, mutations in *NPAS2* have been associated with an increased risk of breast cancer and non-Hodgkin's lymphoma (Sadacca L.A., *et al.*, 2011). Importantly, several studies using mouse models have established convincing links between some clock genes and tumorigenesis. Specifically, *Per1* and *Per2* seem to function as tumour suppressors in mice (Shimba S., *et al.*, 2005; Fontaine C., *et al.*, 2003). Targeted ablation of *Per2* leads to the development of malignant lymphomas (Fontaine C., *et al.*, 2003), whereas its ectopic expression in cancer cell lines results in growth inhibition, cell cycle arrest, apoptosis and loss of clonogenic ability (Preitner N., *et al.*, 2002). Interestingly, *Per2* mRNA levels are downregulated in several human lymphoma cell lines and in tumour cells from patients with acute myeloid leukaemia (Preitner N., *et al.*, 2002). *Per1* mRNA levels are downregulated, as well, in non-small cell lung cancer tissues compared with matched normal tissues, and overexpression of PER1 can suppress the growth of human cancer cell lines (Shimba S., *et al.*, 2005). In addition, knockdown of CKIε induces the growth inhibition of cancer cells, and CKIε expression is increased in various human cancers, such as leukemia and prostate cancer (Alenghat T., *et al.*, 2008).

Bmal1 epigenetic inactivation, via cytosine-guanine (CpG) island promoter hypermethylation, contributes to the development of hematologic malignancies, non-Hodgkin lymphoma and acute lymphocytic leukemia, by distrupting cellular circadian clock, leading to loss of circadian rhythmicity of target genes such as *c-myc*, catalase and p300 (Taniguchi H., *et al.*, 2009).

Further, the expression levels of *Cry1* and *Bmal1* are correlated with clinicopathological parameters in epithelial ovarian cancer, and combination of low expression levels of both genes is an independent prognostic factor, as are stage and histological subtype (Tokunaga H., *et al.*, 2008). These results consistently point to a direct link between the dysfunction of key circadian regulators and cancer (Karlsson B., *et al.*, 2001)

A matter of debate is whether circadian rhythmicity *per se* or only certain core clock genes are involved in cell cycle regulation. So far, only two core clock genes, *Per1* and *Per2*, have been shown to function as tumour suppressors in mice. It should be stressed that individual mutation of either *Per1* or *Per2* in the mouse has a mild effect on circadian behavior (Cermakian N., *et al.*, 2001; Zheng B., *et al.*, 2001; Bae K., *et al.*, 2001; Zheng B., *et al.*, 1999). It has been showed that *Per2*-mutant mice carrying a deletion in the PAS-b domain have a higher incidence of spontaneous salivary gland hyperplasia than wild-type mice (Fu L., *et al.*, 2002). Moreover, *Per2*-mutant mice are more sensitive to γ-irradiation, with significantly higher rates of malignant lymphoma formation after irradiation. Following irradiation, thymocytes from *Per2*-mutant mice have reduced accumulation of p53, and thus have reduced apoptosis. Furthermore, the

reported γ-irradiation induced increase in circadian gene transcription is attenuated in *Per2*-mutant mice (Fu L., *et al.*, 2002).

An interesting link between the circadian clock and cancer was established in breast through a study demonstrating that PER2 can bind to and destabilize estrogen receptor-α (ERα) (Fu L., *et al.*, 2002), a key transcription factor that promotes the growth of mammary epithelial cells, deregulation of which has been shown to cause breast cancer (Green K.A. and Carroll J.S, 2007). Consequently, *Per2* overexpression leads to reduced ERα levels and transcriptional activity. Interestingly, all three mammalian *Per* proteins are structurally similar, with large regions of homology, but with striking differences in specific domains (Hirayama, J. & Sassone-Corsi, P., 2005). It is possible that specialized protein interactions with these domains could lead to the different regulatory pathways through which PER1 and PER2 exert their tumour suppressor function. This might constitute a system of redundancy so that the two proteins can compensate for each other not only in circadian function but also in tumour suppression activity. There is no apparent correlation between the disruption of circadian behaviour and increased tumorigenesis in mouse models. Indeed, *Cry1–/–Cry2–/–* mice (Gauger M.A. and Sancar A., 2005) or *Clock/Clock*-mutant mice (which lack exon 19 of *Clock*) (Antoch M.P., *et al.,* 2008), the circadian rhythms of which are highly compromised, do not show a predisposition to cancer upon irradiation. Moreover, MEFs derived from *Clock/Clock* mutant mice have lower levels of DNA synthesis and cell proliferation than wild-type MEFs (Miller B.H., *et al.*, 2007). Somewhat unexpectedly, ablation in the mouse of both *Cry* genes in a *Trp53–/–* background delays the onset of cancer (Ozturk N., *et al.*, 2009). These observations suggest that other regulatory features intrinsic to clock regulators and independent of their circadian function could participate in carcinogenesis. Is the disruption of circadian rhythms linked to carcinogenesis? It seems that individual core circadian clock proteins (such as PER1 and PER2) might have acquired multiple roles and so control both circadian rhythms and the cell cycle. Also, the consequence of circadian disruption for cancer predisposition might be dependent on how the rhythm is disrupted. Indeed, the molecular mechanism whereby the circadian clock influences cancer development and progression could be explained by its regulation of the cell cycle, DNA damage responses and cellular metabolism.

III.VI Metabolism

Cancer cells have a wide array of metabolic abnormalities. Accumulating evidence has revealed an intimate relationship between circadian rhythms and metabolism (Eckel-Mahan K. and Sassone-Corsi P., 2009). Although the circadian clock regulates multiple metabolic pathways, metabolite availability and feeding behavior can in turn regulate the circadian clock (Damiola F., *et al.*, 2000). Several genes (the protein products of which have crucial roles in metabolic processes) exhibit a circadian expression pattern; these include glucose-6-phosphatase and PCK2 (gluconeogenesis), pyruvate kinase (glycolysis), glucokinase (glycogen synthesis), glucose transporter 2 (glucose transport) and HMG-CoA reductase (cholesterol metabolism).

Forced misalignment of behavioural and circadian cycles in human subjects was recently shown to cause a decrease in leptin and an increase in glucose and insulin levels (Shea S.A., *et al.*, 2009). Moreover, metabolic defects are observed in animals lacking core clock genes such as *Clock* and *Bmal1* or clock-controlled genes such as *Nocturnin* (a deadenylase that is highly expressed at night and which specifically removes poly(A) tails from mRNAs) (Green C.B., *et al.*, 2007) *Clock/Clock*-mutant mice become hyperphagic and obese and develop classic signs of metabolic syndrome, such as hyperglycaemia, dyslipidemia and hepatic steatosis (Shirogane T., *et al.*, 2005). Further demonstrating a close association between the circadian clock and metabolism, the expression of several nuclear receptors, including peroxisome proliferator- activated receptor (PPAR) family members (PPARα, PPARγ and PPARδ) and estrogen-related receptor (ESRR) family members (ESRRα, ESRRβ and ESRRγ), is rhythmic in a tissue-specific manner (Yang X., *et al.,* 2006). PPARγ co-activator 1α (PPARGC1α) has a circadian expression pattern in metabolic tissues, and mice lacking *Ppargc1a* have defects in locomotor activity, body temperature regulation and metabolic rate (Liu C., *et al.*, 2007). Although we are still lacking a satisfactory molecular understanding of how clock proteins modulate metabolism, emerging evidence implicates chromatin remodeling as a key control mechanism, suggesting that some clock regulators may be at the crossroad between epigenetics and metabolism (Bunger M.K., *et al.*, 2000; Alenghat T., *et al.*, 2008). Perturbations in the expression or activities of these regulators (such as CLOCK and SIRT1) could contribute to cancer by causing higher proliferation and defects in metabolic pathways, for example in the modulation of glycolytic and gluconeogenic enzymes.

III.VII LKB1/AMPK in the circadian rhythm

LKB1/AMPK pathway is a central mediator of metabolism. AMPK has the capability to monitor and respond to nutrient and energy fluctuations. Upon activation, AMPK phosphorylates its targets, either promoting catabolic energy-producing pathways or inhibiting anabolic energy-consuming pathways (Hardie D.G., *et al.*, 2007). To maintain metabolic homeostasis, the body relies on metabolic sensors such as nuclear receptors (NRs) and AMPK to coordinate cellular energy usage with the fluctuations in metabolites that occur owing to daily fed/fasting cycles. NRs sense hormones and metabolites such as lipids, steroids, retinoids, and xenobiotics, whereas conversely, AMPK senses the energy state of the body through its ability to respond to changes in the AMP:ATP ratio that take place during rest and exercise. These characteristics make them ideal candidates to transmit metabolic input signals to other aspects of physiology, including circadian rhythm. On the other hand, the ability of NRs and AMPK to directly regulate metabolism also makes it possible for their activities to feed back circadian signals to metabolism (Fan W., *et al.*, 2011). Recently, it was demonstrated that AMPK has a role in the regulation of circadian rhythms (Lamia K., *et al.*, 2009).

NRs and AMPK are regulators that couples metabolism with circadian rhythm. AMPK is activated when energy supplies are low, such as during fasting or exercising. When activated, AMPK promotes catabolic pathways to burn glucose or fatty acids to produce ATP or inhibits anabolic pathways to limit ATP consumption. It functions by directly phosphorylating metabolic enzymes or regulating gene transcription. The targets of AMPK cover many aspects of metabolism including glucose uptake, glycolysis, gluconeogenesis, glycogen synthesis, fatty acid oxidation and synthesis, steroid synthesis, protein synthesis, and mitochondrial biogenesis (Hardie D.G., 2007).

Recently it was discovered that the expression of the AMPKβ2 subunit and phosphorylation of the AMPK substrates Raptor and ACC are rhythmic in mouse liver (Davies S.P., *et al.*, 1992; Fan W. *et al.*, 2011). Nuclear localization of the AMPKα1 subunit was also found to be oscillating in a circadian manner (Lamia K., *et al.*, 2009). On the other hand, AMPK also directly regulates circadian rhythm through the degradation of PER2 or CRY1 (Um J.H., et al., 2007; Lamia K., *et al.*, 2009). When activated by metformin, AMPK can phosphorylates either Casein Kinase 1ε (CK1ε) (Um J.H., *et al.*, 2007) or CRY1 (Fan W., *et al.*, 2011), inducing PER2 or CRY1 degradation, respectively, which results in an increase in circadian period and reduced amplitude of BMAL1 expression (Lamia K., *et al.*, 2009). Therefore, the circadian clock regulates AMPK activity to fulfill the body's different energy requirements during the diurnal physiological cycles. In addition, various metabolic energy states also regulate AMPK activity and influence the circadian rhythm by controlling the degradation of PER2 and CRY1.

Also LKB1 has been reported to play a role in the circadian clock, but the mechanism is not yet clear. Indeed it has been shown that liver-specific deletion of LKB1 in mouse abolished AMPK activation and significantly increase the amount of CRY1 present in liver nuclei across the circadian cycle (Lamia K., *et al.*, 2009).

It was reported that the kinase LKB1 is biochemically sufficient to activate AMPK *in vitro* and is genetically required for AMPK activation by energy stress in a number of mammalian cell lines (Hawley S.A., *et al.*, 2003; Shaw R.J., *et al.*, 2004; Woods A., *et al.*, 2003). Because of this potent connection to AMPK, we began to consider the possibility that LKB1 might play a role in the circadian rhythms and explored whether this is AMPK-dependent. Since LKB1 is a central regulator of organism metabolism (Shaw R., *et* al., 2005) we wondered if LKB1 could couple cellular metabolism with circadian rhythm.

AIM OF THE STUDY –CHAPTER III-

The aim of this study is to investigate the role played by LKB1 in metabolism and circadian rhythm. A comprehensive understanding of the molecular links that can connect LKB1 and the circadian clock could provide therapeutic benefit against cancer.

Circadian rhythms occur with a periodicity of about 24 hours and regulate a wide array of metabolic and physiologic functions. Circadian control of physiology and behavior is required for a healthy life. Disruption of circadian rhythms has been considered as a causative factor for development of several diseases. As described in literature, mutation in circadian clock proteins that either have histone modifying ability (such as CLOCK) or associate with histone modifiers (such as BMAL1, PER2 and REV-ERBα) has been linked to cancer and metabolic syndromes.

Recently, it was demonstrated that AMPK has a role in the regulation of circadian rhythms. When activated by metformin, AMPK can phosphorylates either Casein Kinase 1ε (CK1ε) or CRY1, inducing PER2 or CRY1 degradation, respectively, which results in an increase in circadian period and reduced amplitude of BMAL1 expression. Although LKB1 has been reported to play a role in the circadian clock, the mechanism is not yet clear.

We thus began to consider the possibility that LKB1, the principal AMPK activator, might play a role in the circadian rhythms and to explore whether this is AMPK-dependent. Since LKB1 is a central regulator of organism metabolism, we wondered if LKB1 could couple cellular metabolism with circadian rhythm.

II. MATERIALS AND METHODS

III.VIII *Plasmids*

FLAG-tagged, Myc-tagged and GDB-tagged plasmids (Doi M., *et al.*, 2006) and (Travnickova-Bendova Z., *et al.*, 2002). Flag–Myc–BMAL1/pCS2 (Hirayama J., *et al.*, 2007), full and truncated mouse *Clock* ORFs (Nakahata Y., *et al.*, 2008) were already described. pSG/FLAG-LKB1 vector encodes the human LKB1 fused N-terminally to the Flag epitope; the pSG/FLAG-D243 plasmid encodes a truncated mutant LKB1 (1-243 amino acids); the pSG/FLAG-D88 plasmid encodes a truncated form of LKB1 (88-433 amino acids); the pSG/FLAG-D146-186 and the pSG/FLAG-D317 plasmid were kindly gifts of M. Billaud (Nony P., *et al.*, 2003).

III.IX *Antibodies*

Antibodies against LKB1 was from CellSignaling (1:1000), antibodies against CLOCK (1:1000) and rabbit IgG (1:5000) from Santa Cruz Biotechnology, antibodies against Flag (M2) (1:5000) and α-tubulin (1:20000) from Sigma-Aldrich. Antibodies against BMAL1 (1:4000) and Myc (1:5000) were already described (Cardone L., *et al.*, 2005), antibodies against GAL4 (Santa Cruz Biotechnology, 1:10000), antibodies against H3K4 monomethylase (Abcam, 1:500), p84 was from Santa Cruz Biotechnology (1:5000).

III.X *Cell Culture*

HEK 293 were grown in DMEM (4.5 g/l glucose) supplemented with 10% FBS, glutamine (2mM/L) (GIBCO, Invitrogen Life Technologies, Carlsbad, CA, USA), penicillin (100U/ml) (GIBCO) and streptomycin (100µg/ml) (GIBCO). MEFs WT or homozygous *Lkb1*$^{-/-}$ sibling mice cells were kind gifts of Alessi D. and were cultured in DMEM (4.5 g/l glucose) supplemented with 10% FBS, and antibiotics. JEG3 cells were grown in Basal Medium Eagle (GIBCO, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS and antibiotics.

III.XI *Preparation of Cell Extracts and Nuclear Extracts from Cultured Cell Lines*

Cells were washed twice with phosphate-buffered saline (PBS) and lysed in RIPA buffer (50 mM Tris/HCL [pH 8.0], 150 mM NaCl, 5 mM EDTA, 15 mM MgCl2, 1% NP40, 1× protease inhibitor cocktail [Roche], 1 mM DTT (Fisher Scientific), 1 µM trichostatin A [TSA, Sigma Aldrich], 10 mM NAM (Sigma Aldrich), 10 mM NaF (Sigma Aldrich), 1 mM PMSF (Sigma Aldrich). For nuclear extracts (Andrews and Faller, 1991), after washing

cells with cold PBS, cells were lysed with hypotonic buffer (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1× protease inhibitor cocktail, 1 mM DTT, 1 µM TSA, 10 mM NAM, 10 mM NaF, 1 mM PMSF). Following a brief centrifugation, supernatant was the cytoplasmic fraction and pellet was resuspended in hypertonic buffer (20 mM HEPES-KOH [pH 7.9], 25% glycerol, 420 mM NaCl 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1× protease inhibitor cocktail, 1 mM DTT, 1 µM TSA, 10 mM NAM, 10 mM NaF, 1 mM PMSF). Supernatants were recovered as nuclear extracts.

III.XII *ChIP Assays*

Conventional ChIP assay was used for histones from MEFs (Yamamoto T., *et al.*, 2004). After serum shock, cells were washed two times with room temperature PBS, then PBS/1 mM $MgCl_2$ was added. Disuccinimidyl Glutalate (DSG) was added to a final concentration of 2 mM for crosslinking. After 45 min at room temperature, formaldehyde was added to a final concentration of 1%(v/v) and cells were incubated for 15 min. After dual crosslinking, glycine was added to a final concentration of 0.1 M and incubated for 10 min to quench formaldehyde out. After harvesting, cells were lysed in 500 µl ice-cold cell lysis buffer (50 mM Tris/HCl [pH 8.0], 85 mM KCl, 0.5% NP40, 1 mM PMSF, 1× protease inhibitor cocktail) for 10 min on ice. Nuclei were precipitated by centrifugation (3000 g for 5 min), resuspended in 600 µl ice-cold RIPA buffer (50 mM Tris/HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM PMSF, 1× protease inhibitor cocktail), and incubated on ice for 30 min. Sonication was done to obtain DNA fragments 100–600 bp in length.

III.XIII *Preparation of GST–BMAL1 proteins*

The GST fusion vector, pGEX-BMAL1(WT)) was transformed in Escherichia coli BL21(DE3). Cells were grown at 25 °C to D_{600} = 0.5–0.8 by induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 12 h, and collected by centrifugation. The cell pellets were resuspended in phosphate-buffered saline (PBS) containing 0.5 mM EDTA, and then sonicated. Insoluble material was removed by centrifugation. GST-fusion proteins were purified from the soluble extracts in a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech), according to the manufacturer's instructions.

III.XIV *In Vitro Kinase Assay*

Glutathione-S-transferase (GST) or GST-BMAL1 proteins were purified from BL21 strain of *Escherichia coli* using glutathione- sepharose beads (GE Healthcare). Purified proteins were washed in kinase buffer (20mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 5mM DTT) and

then incubated with or without 25 ng recombinant LKB1 (New England Biolabs) in presence of 200 μM ATP and 5 μCi γ^{32}p ATP at 30°C for 30 min. Reaction was terminated by boiling in presence of Laemmli buffer. Samples were resolved by SDS-PAGE, stained by Coomassie blue, destained, dried and then phosphorylated BMAL1 was detected by autoradiography.

III.XV *Quantitative Real-Time PCR*

Total RNA was isolated from cell lines (2 to 5 × 10^6 per sample) using Trizol (Invitrogen Life Technologies). RNA (1μg) was retrotranscribed using the SuperScript II system (Invitrogen Life Technologies) in 20μL final volume following the manufacturer's instructions. Each quantitative real-time PCR was performed using the Chromo4 real time detection system (BIO-RAD). The PCR primers are mbmal1 mRNA, mDBP mRNA, mCLOCK mRNA, mREVERBα mRNA, RORα mRNA, actin mRNA (Hirayama J., *et al.*, 2007; Sahar S., *et al.*, 2010). For a 20 μl PCR, 50 ng of cDNA template was mixed with the primers to final concentrations of 200 nM and 10 μl of SYBR Green Supermix (BIO-RAD), respectively. The reaction was first incubated at 95°C for 3 min, followed by 40 cycles at 95°C for 30 s and 60°C for 1 min.

III.XVI *Cell lysis, immunoprecipitation and western blot analysis*

Cells were cultured 48hr after trasfection with plasmids and then lysed with RIPA-1 (150 mM NaCl, 5 mM EDTA, 1% NP-40, 50 mM Tris-HCl, pH 8.0, MgCl 1.5mM, 1× protease inhibitor, deacetylase inhibitors, and 1 mM PMSF). Liver samples (1 g) were manually homogenated by pestle in RIPA-1. Immunoprecipitation experiments were carried out in the RIPA-1 buffer with Flag, GDB, myc, BMAL1, CLOCK or LKB1 antibodies. Western analysis were performed as previously described (Accordi B., *et al.*, 2010). Protein samples were resolved on 8% or 6% SDS–PAGE and immunoblotted with the antibodies previously mentionated.

III.XVII *Mice, cell line transfections and Dexamethasone treatment*

Wild-type mice were entrained in 12 h light:12 h dark for 14 days before placement in constant darkness. For luciferase assays, cells growing in 24-well plates were transfected with various combinations of expression plasmids. The total amount of DNA applied per well was adjusted by adding pSG5 vector. Cell extracts were subjected to luminometry-based-luciferase assay (Promega), and luciferase activity was normalized by β-galactosidase activity. Real-time luciferase activities were monitored using Kronos

(ATTO). All experiments were repeated at least three times. For synchronization, LKB1-deficient MEFs were treated with 0.1 mM dexamethazone (Dex) for 2 hr and then the culture medium was changed to DMEM containing 20% FBS.

III.XVIII *Chromatin fractionation*

Cells were washed twice with PBS and lysed in Complete Buffer A (Buffer H: 100mM Hepes, pH 7.8, 250mM KCl, 10mM EGTA, 10mM EDTA; 2.2M sucrose, H_20; 3% triton, 1× protease inhibitor cocktail, 1 mM DTT, 10 mM NaF, 1 mM PMSF). After a centrifugation at 2000rpm for 5min the cytoplasm was saved. The pellet was washed once with Complete Buffer A, than twice with Complete Low Salt Buffer (Buffer H: 100mM Hepes, pH 7.8, 250mM KCl, 10mM EGTA, 10mM EDTA; 80% glycerol, H_20; 1× protease inhibitor cocktail, 1 mM DTT, 10 mM NaF, 1 mM PMSF). Nuclear pellet were resuspended with 1X Complete Low Salt Buffer and 2X Complete High Salt Buffer (Buffer H: 100mM Hepes, pH 7.8, 250mM KCl, 10mM EGTA, 10mM EDTA; 80% glycerol; 4M KCl; H_20; 1× protease inhibitor cocktail, 1 mM DTT, 10 mM NaF, 1 mM PMSF). Nutate for 1h and than, after 20min of centrifugation at 12000rpm supernatant was recovered as nuclei fraction. The remaining pellet was resuspended with RIPA buffer, sonicated and after 5min of centrifugation supernatants was recovered as chromatin fraction.

III. RESULTS:

III.XIX *LKB1 elevates CLOCK–BMAL1-mediated transcriptional activation*

To test whether LKB1 has a role in circadian gene transcription, we analyzed CLOCK-BMAL1-mediated activation of a *Per1* promoter-based luciferase reporter vector in JEG3 cells. Together with CLOCK-BMAL1, we co-expressed either wild type (WT) LKB1 or different kinase-deficient LKB1 mutants (K78A, Δ243, Δ317, Δ88, Δ146-188) (Nony P., *et al.*, 2003, **Fg. III.Ia**). Co-expression of WT LKB1 elicited a robust and significant increase in the transcriptional activity of CLOCK-BMAL1 from *Per1* promoter (**Fig. III.Ib**, p:0,002), which was abolished when the LKB1 kinase domain was deleted (kinase-deficient LKB1 mutants) (**Fig. III.Ic**, C-B/C-B+ LKB1 K78A p=0,8962; C-B/C-B+ LKB1 Δ88 p=2,16726E-05; C-B/C-B+ LKB1 Δ137 p=5,52525E-07; C-B/C-B+ LKB1 Δ243 p=0,01225; C-B/C-B+ LKB1 Δ146–186 p=1,27266E-08), demonstrating the critical role of kinase activity. Similar results were obtained using *Dbp* reporters (data not shown). Again, use of the kinase-deficient LKB1 mutants demonstrated the absolute requirement of the LKB domain for full activity and cooperation with CLOCK-BMAL1. These results show that LKB1potentiates CLOCK-BMAL1-dependent gene activation in a synergistic, rather than additive, manner.

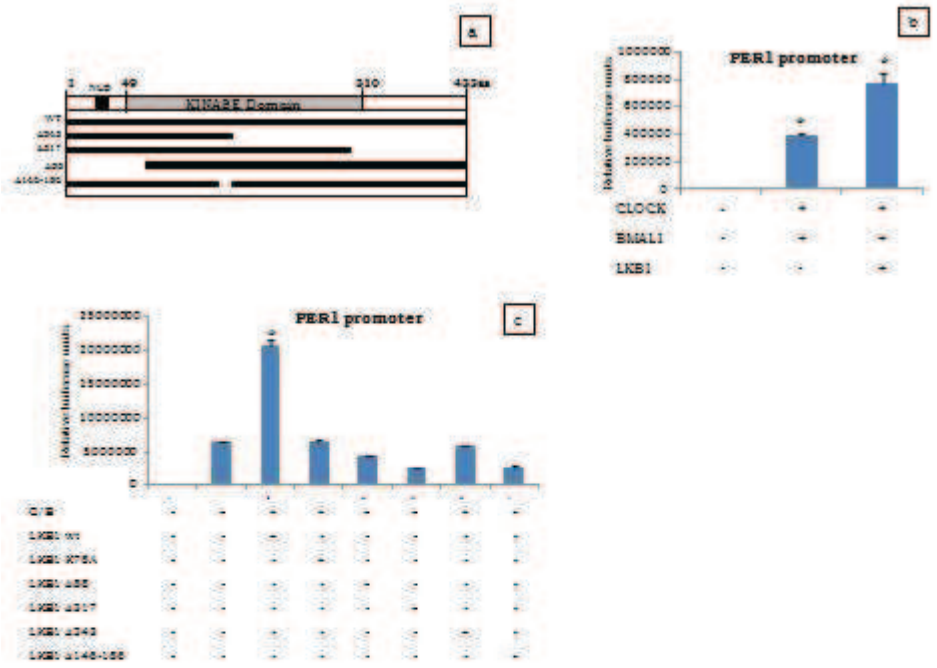

Fig. III.I: a) Different kinase-deficient LKB1 mutants (K78A, Δ243, Δ317, Δ88, Δ146-188). b) Luciferase assays shows that co-expression of WT LKB1 elicited a robust and significant increase in the transcriptional activity of CLOCK-BMAL1 on *Per1* promoter (Test t, p=0,002). c) kinase-deficient LKB1 mutants didn't increase the transcritptional activity of CLOCK-BMAL1.

III.XX *LKB1 interacts with CLOCK and BMAL1*

The transcriptional enhancing property of LKB1 on CLOCK-BMAL1 (**Fig. III.Ia**) suggested that these proteins may interact. To test this possibility, we used HEK 293 cells to coexpress tagged proteins, Flag-LKB1 with Myc-tagged CLOCK and/or BMAL1. Immunoprecipitation assays using Flag antibody–coupled agarose beads revealed that CLOCK and BMAL1 physically interact with LKB1, either when they are in a complex together or when they are expressed alone (**Fig. III.IIa**). Interaction of CLOCK-BMAL1 and kinase-deficient LKB1 mutant was also observed, indicating that more than one site of LKB1 are implicated in the interaction (**Fig. III.IIb**).

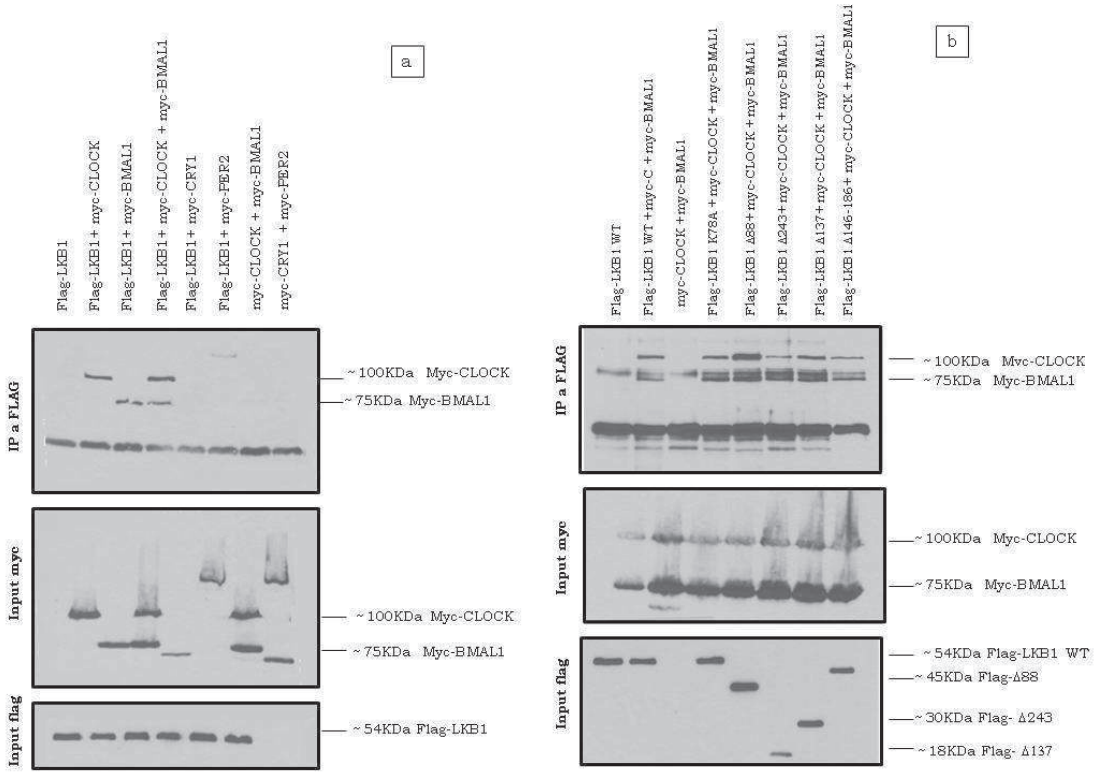

Fig. III.II: a) Immunoprecipitation assays using Flag antibody–coupled agarose beads revealed that CLOCK and BMAL1 physically interact with LKB1 either when they are in a dimeric complex or expressed alone . The association of such proteins is specific as shown by the lack of interaction with CRY1 and PER1. b) Interaction of CLOCK-BMAL1 and kinase-deficient LKB1 mutant was also observed, indicating that more than one site of LKB1 is implicated in this interaction.

But the interaction should be in the N-terminal of CLOCK, as demonstrated by immunoprecipitation after co-expression of plasmids expressing various deletions of CLOCK (**Fig. III.IIIa**). Association of these proteins is specific, as shown by the lack of interaction with CRY1 and PER1 (**Fig. III.IIa**).

To investigate whether native, endogenous LKB1 protein interacts with CLOCK and BMAL1, we performed the immunoprecipitation assay *in vivo* in liver of WT mice at various circadian times (ZT3, ZT9, ZT15, ZT21). We confirmed the physical interaction between LKB1, CLOCK and BMAL1 also *in vivo* not at all circadian times, but only at ZT 15 (**Fig III.IIIb,c**). Thus cellular CLOCK and BMAL1 interacts with endogenous LKB1 in a specific, circadian manner.

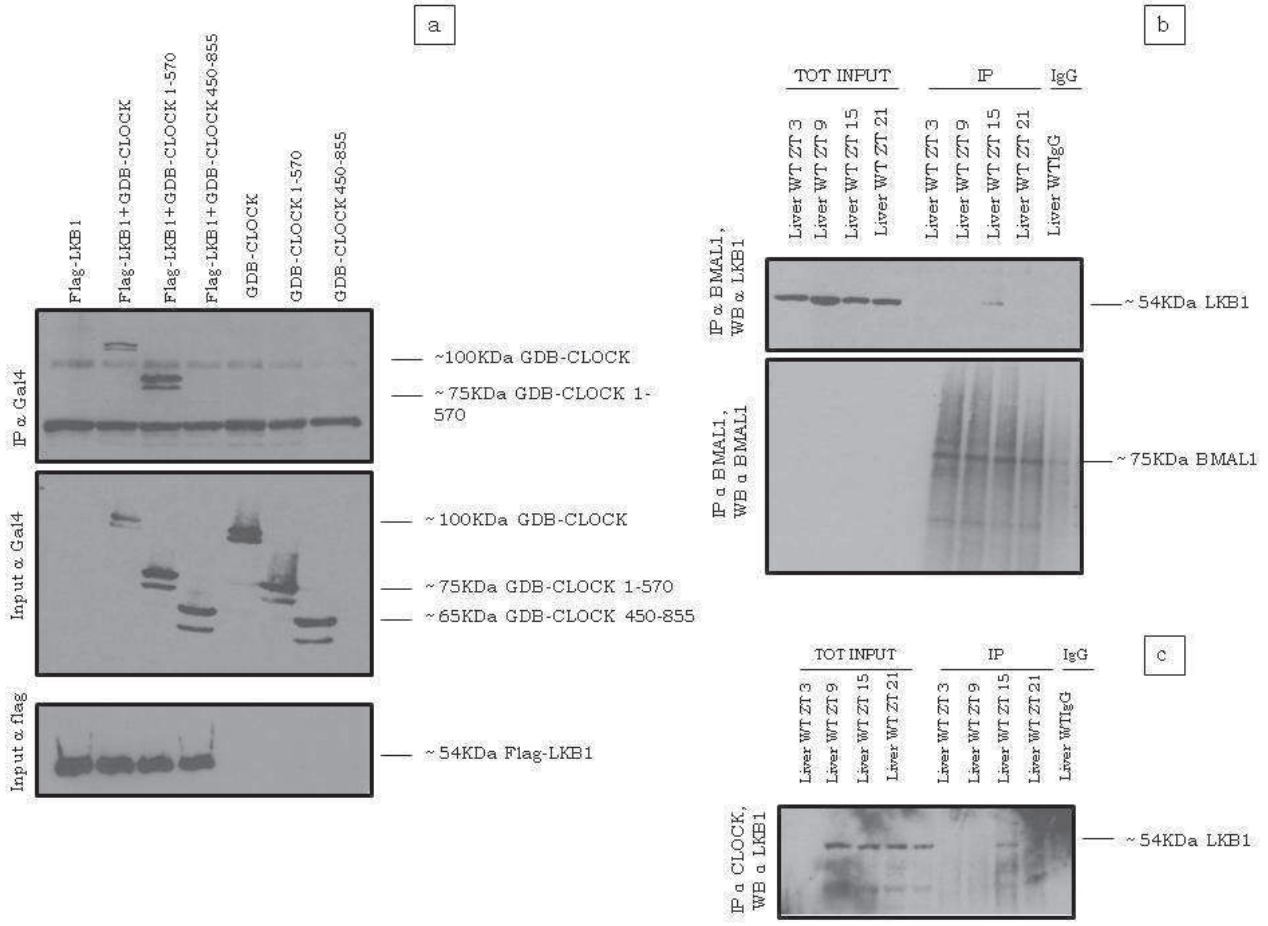

Fig. III.III: a) Immunoprecipitation experiments after co-expression of plasmids expressing various deletions of CLOCK demonstrate that interaction between CLOCK and LKB1 should take place in the N-terminal of CLOCK.
b) Immunoprecipitation assay *in vivo* in liver of WT mice at various circadian times (ZT3, ZT9, ZT15, ZT21). We confirmed the physical interaction between LKB1 and BMAL1 also *in vivo* only at ZT 15. c) We also found physical interaction between LKB1 and CLOCK *in vivo* only at ZT 15.

III.XXI *LKB1 regulates BMAL1 phosphorylation and localization*

BMAL1 is a phosphoprotein targeted by various kinases (Sanada K., *et* al., 2002; Tamaru T., *et* al., 2009; Eide E.J., *et al.*, 2002). Our results demonstrating that LKB1 increases the transcriptional activity of CLOCK-BMAL1 and also physically interacts with them prompted us to determine whether LKB1 can phosphorylate BMAL1. We performed *in vitro* kinase assays on bacterially purified GST-BMAL1 or GST alone by incubating them with recombinant LKB1 in presence of γ^{32}P-ATP. Our results demonstrate that GST-BMAL1 was readily phosphorylated by LKB1, whereas GST alone, although expressed at much greater levels, was not phosphorylated under equivalent conditions (**Fig. III.IVa**). Thus, BMAL1 appears to be an efficient substrate of LKB1.

To investigate whether BMAL1 phosphorylation would be influenced in the absence of LKB1, we used total lysate of WT and LKB1 MEFs (kindly gift of Alessi D.), and analyzed BMAL1 phosphorylation levels by Western analysis. Interestingly, we found that in WT MEFs BMAL1 is present mostly in the hyper-phosphorylated form, whereas in LKB1$^{-/-}$ MEFs BMAL1 is not present in the hyper-phosphorylated form (**Fig. III.IVb**).

In addition, through cytoplasm and nuclear fractionation we noticed that in WT MEFs, BMAL1 is mostly localized in the nucleus, while in LKB1$^{-/-}$ MEFs it is mostly present in the cytoplasmic fraction (**Fig. III.IVb**). When BMAL1 is in the cytoplasm and is not hyper-phosphorylated, it is not active as a transcription factor.

Probably LKB1 plays a role in the control of BMAL1 activity. These results suggest that LKB1 can phosphorylate BMAL1 and through this it can regulate the localization of BMAL1 in the cell.

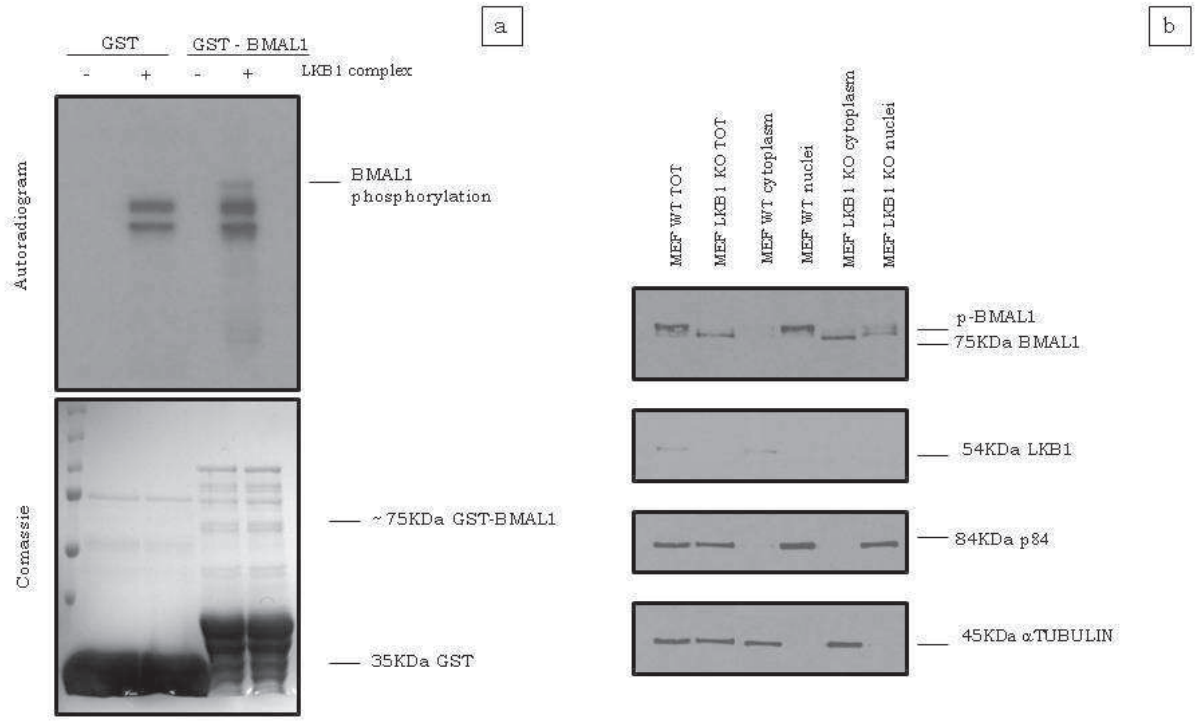

Fig. III.IV: a) *In vitro* kinase assays with recombinant LKB1 in presence of γ^{32}P-ATP. GST-BMAL1 was readily phosphorylated by LKB1. b) Western Blot analysis of WT and LKB1$^{-/-}$ MEFs. In WT MEFs, BMAL1 is mostly present in the hyper-phosphorylated form while in LKB1$^{-/-}$ MEFs BMAL1 is not present in the hyper-phosphorylated form. Cytoplasm and nuclear fractionation show that in WT MEFs BMAL1 is mostly localized in the nucleus, but in LKB1$^{-/-}$ MEFs it is mostly present in the cytoplasmic fraction.

III.XXII *Oscillation in BMAL1 Activity correlates with BMAL1 phosphorylation status and is dependent on LKB1*

Our results show that LKB1 regulates BMAL1 phsphorylation levels. Thus, we questioned whether BMAL1 phosphorylation oscillation would be influenced in the absence of LKB1. To address this question, we entrained WT and LKB1$^{-/-}$ MEFs with dexamethasone and prepared cellular extracts at various circadian times and analyzed BMAL1 levels by Western analysis.

BMAL1 phosphorylation oscillates as expected in WT MEFs, peaking at 12 hours post-synchronization with lowest levels at 30 hours post-synchronization (**Fig. III.Va**). Furthermore, in LKB1$^{-/-}$ MEFs, BMAL1 phosphorylation displays only a mild oscillation, with total levels of the protein being higher at all time points (**Fig. III.Va**). In addition we found again that in WT MEFs, BMAL1 is mostly localized in the nucleus and in LKB1$^{-/-}$ MEFs is mostly present in the cytoplasmic fraction (**Fig. III.Vb**). These results indicate that LKB1 is critical for the circadian oscillation of BMAL1 phosphorylation.

Furthermore in WT MEFs BMAL1 phosphorylation oscillates from the hyper-phosphorylated form to the hypo-phoshorylated form. In LKB1$^{-/-}$ MEFs, as previously noticed, BMAL1 is present always not in the hyper- phosphorylated form, but only in the hypo-phosphorylated form.

Taken together these results with the data from the kinase assay prompted us to investigate which is the LKB1 phosphorylation site on BMAL1. To do this we are planning to perform Mass Spectrometric analysis.

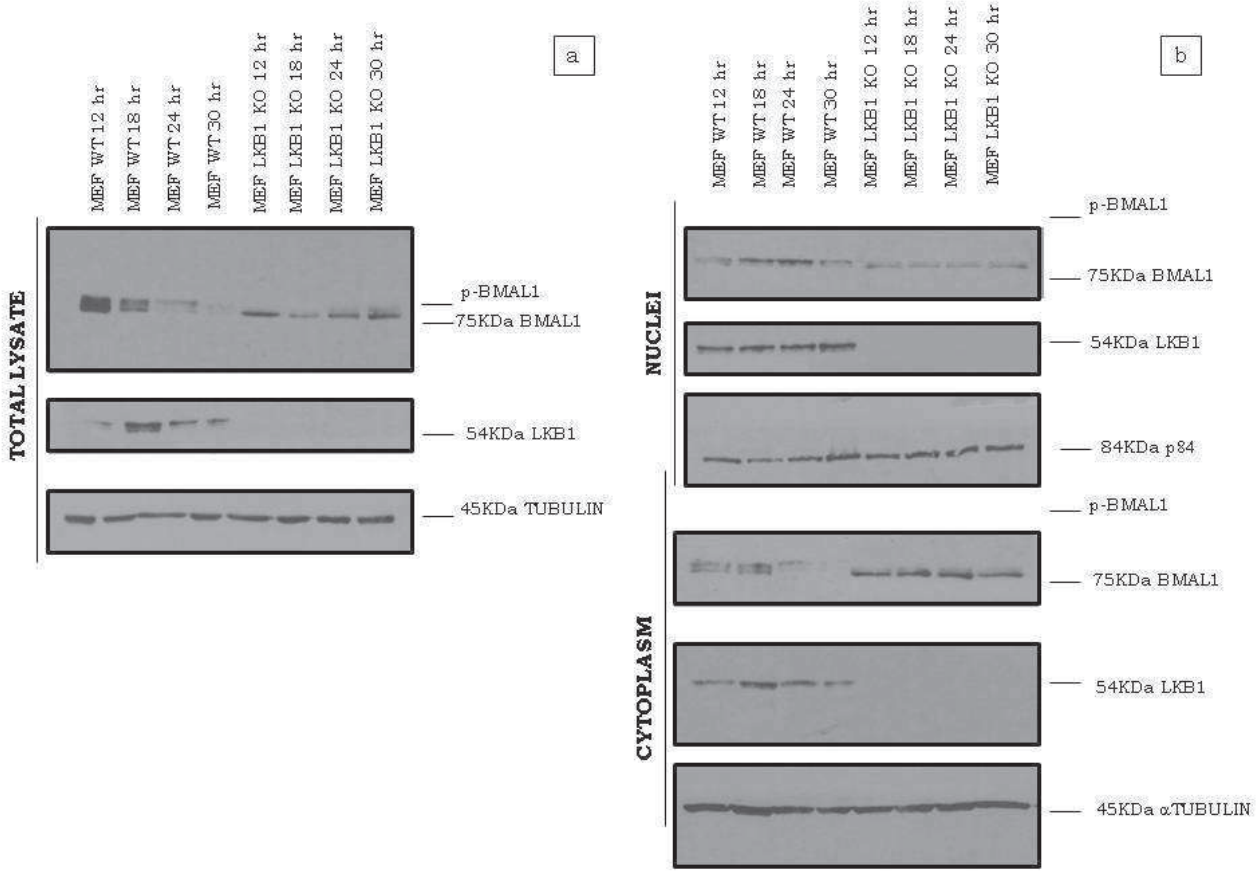

Fig. III.V: a) BMAL1 phosphorylation oscillates as expected in WT MEFs, peaking at 12 hours with lowest levels at 30 hours post-synchronization. In LKB1$^{-/-}$ MEFs, BMAL1 phosphorylation displays only a mild oscillation, with total levels of protein being higher at all time points. b) We found that in WT MEFs, BMAL1 is mostly localized in the nucleus (upper panels) and in LKB1$^{-/-}$ MEFs is mostly present in the cytoplasmic fraction (lower panels).

III.XXIII *LKB1$^{-/-}$ MEFs Display Dampened Circadian Oscillation of CCGs.*

The regulation of BMAL1 phosphorylation by LKB1 may impact on the cyclic expression of clock-controlled genes. We thereby compared the expression profile of the *clock-controlled* genes in WT and LKB1$^{-/-}$ MEFs synchronized by dexamethasone. Lack of LKB1 resulted in a significant dampening of the clock-controlled genes (*Dbp, Cry1, Bmal1, Per2*) oscillation as compared to WT MEFs (**Fig. III.VI**), featuring a decreased amplitude.

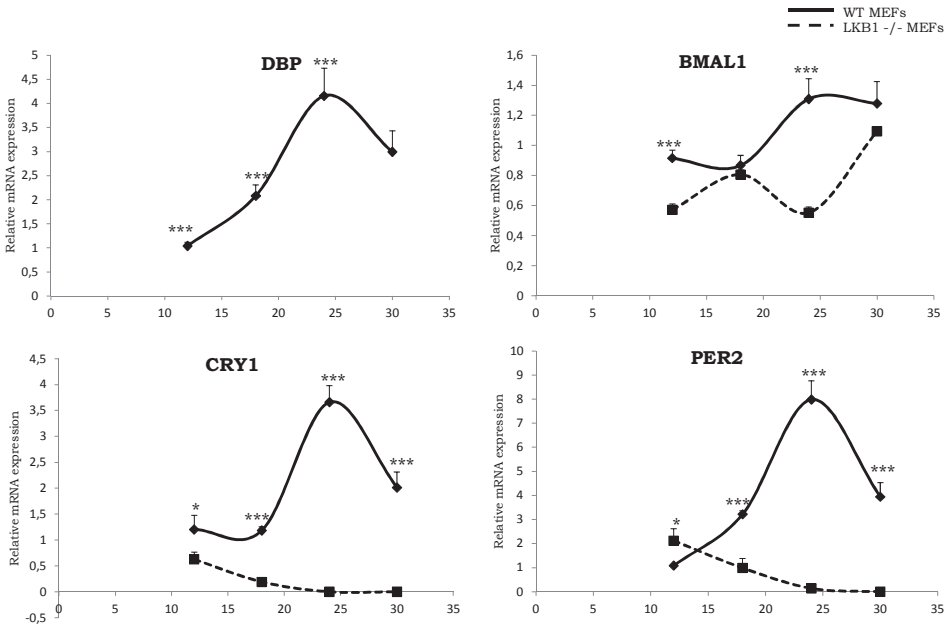

Fig. III.VI: We compared expression profiles of *clock-controlled* genes (CCGs) in WT and LKB1$^{-/-}$ MEFs synchronized by dexamethasone. Lack of LKB1 resulted in a significant dampening of clock-controlled genes (*Dbp, Cry1, Bmal1, Per2*) oscillation as compared to WT MEFs, featuring a decreased amplitude.

III.XXIV *LKB1 is recruited to CCG promoter*

Our previous results show that BMAL1 phosphorylation is higher in WT MEFs nucleus fraction and disappear in LKB1$^{-/-}$ MEFs. This drove us to another question: does LKB1 get recruited to CCG promoters in *vitro* (MEFs) and also *in vivo* (Liver)? Moreover does BMAL1 get recruited to DBP or PER promoters in WT and LKB1 KO MEFs? To answer to these questions, we synchronized WT and LKB1$^{-/-}$ MEFs with dexamethasone and we perfomed ChIP assay at two different time points (12h and 24h based on *Dbp* mRNA relative expression). We found that LKB1 is recruited on the *per*1 promoter E-box region (E1) being low at circadian time (CT) 12 and high at CT 24 (**Fig. III.VIIa**).

Furthermore with the aim of reinforce this data, we performed chromatin fractionation at two different CT (12h and 24h) in WT and LKB1$^{-/-}$ MEFs after synchronization with dexamethasone (**Fig. III.VIIb**). We also performed chromatin fractionation at 4 different ZT (3 ,9 ,12 ,15) in mouse liver (**Fig. III.VIIc**).

We found either *in vivo* (**Fig. III.VIIc**) than *in vitro* (**Fig. III.VIIb**) that, as expected, LKB1 is mostly localized in the cytoplasm fraction, but we can also appreciate LKB1 in the nuclei and in the chromatin fraction (**Fig. III.VIIb,c**).

These finding suggest that LKB1 does get recruited to CCG promoters in *vitro* (MEFs) and also *in vivo* (Liver), but additional experiment must be performed to confirm the data.

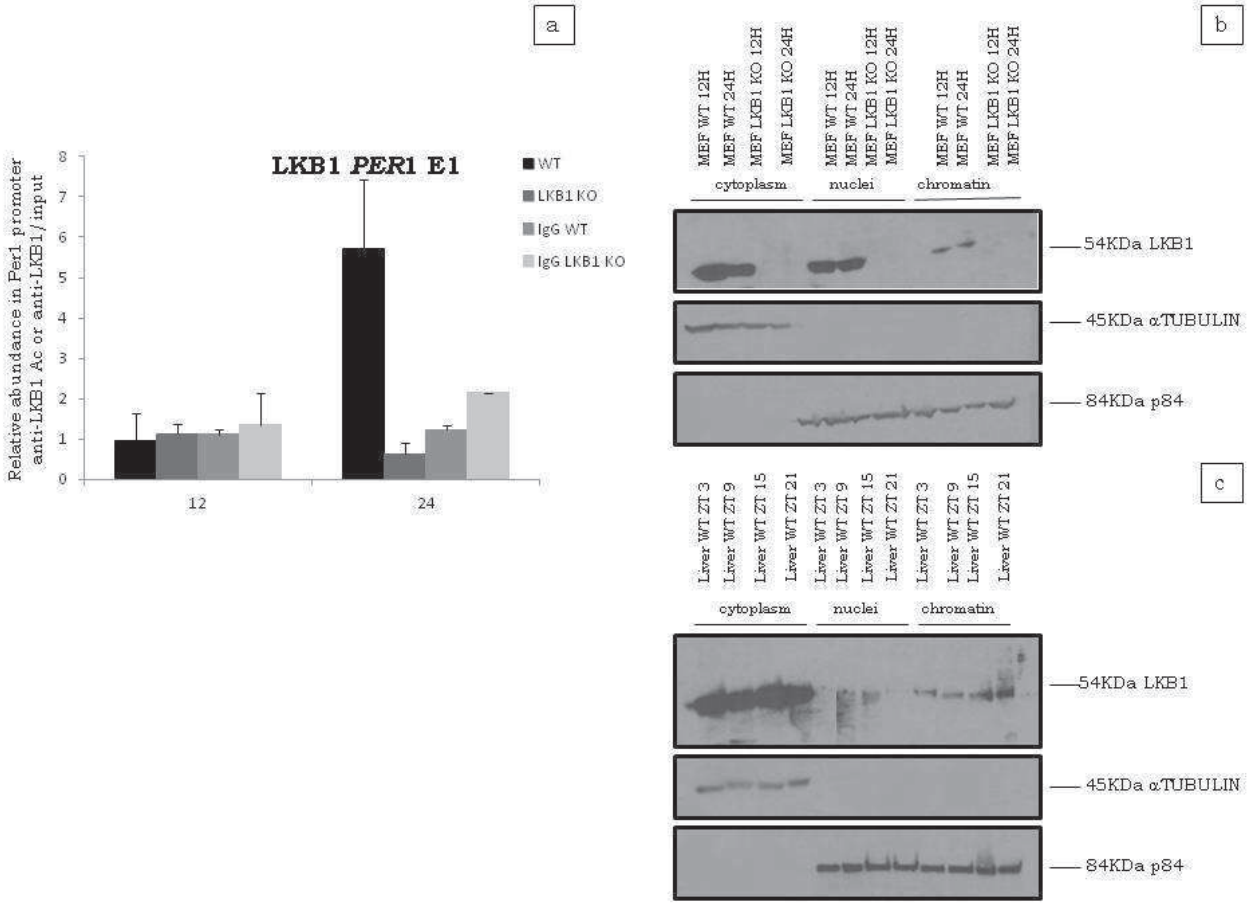

Fig. III.VII: a) ChIP assay after synchronization with dexamethasone at different time points (12h and 24h based on *Dbp* mRNA relative expression). LKB1 is recruited on the *per*1 promoter E-box region being low at circadian time (CT) 12 and high at CT 24. b) Chromatin fractionation at CT 12h and 24h in WT and LKB1$^{-/-}$ MEFs after synchronization with dexamethasone. LKB1 is mostly localizated in the cytoplasm fraction. We can also appreciate LKB1 in nuclei and chromatin fraction. c) Chromatin fractionation at 4 different Zeitgeber (ZT 3, 9, 12, 15) in mouse liver. LKB1 is mostly localizated in the cytoplasm fraction but we can also appreciate LKB1 in nuclei and chromatin fraction.

III. DISCUSSION

In this study we report that the the serine/threonine kinase LKB1 is a key factor in circadian control. First of all we found that LKB1 elevates CLOCK–BMAL1-mediated transcriptional activation in the *PER1* promoter (**Fig. III.Ia**) and in *DBP* promoter (data not shown). Our results show that LKB1 potentiates CLOCK-BMAL1-dependent gene activation in a synergistic, rather than additive, manner. Interestingly, the LKB1 activity and kinase function is necessary for the enhancement of CLOCK-BMAL1 activity. The transcriptional enhancing property of LKB1 on CLOCK-BMAL1 (**Fig. III.Ia**) suggests a specific association of these proteins, as we revealed with immunoprecipitation assays *in vitro* (**Fig. III.IIa**) and *in vivo* (**Fig III.IId,e**).

BMAL1 is a critical regulator of the circadian clock. Its ablation in the mouse, in addition to causing arrhythmicity (Bunger M.K., *et al.*, 2000), leads to premature aging and reduced life-span (Kondratov R.V., *et al.*, 2006). BMAL1 undergoes various PTMs, including phosphorylation, acetylation (Hirayama J., *et al.*, 2007), sumoylation (Cardone L., *et al.*, 2005; Lee J., *et al.*, 2008) and ubiquitylation (Kwon I., *et al.*, 2006). The functional interactions between these PTMs have not been deciphered, but phosphorylation by distinct kinases appears to regulate unique activities (Kwon I., *et al.*, 2006; Kondratov R.V., *et al.*, 2003). BMAL1 phosphorylation has a dual role: activation of BMAL1-mediated trascription for example by Casein Kinase Iε (CKIε) (Eide E.J., *et al.*, 2002), or inhibition by MAPK (Sanada K., *et al.*, 2002). Furthermore BMAL1 CK2α-mediated phosphorylation carry effect on its intracellular localization (Tamaru T., *et al.*, 2009). Our present results indicate that LKB1-mediated phosphorylation of BMAL1 controls either the activity and/or localization of BMAL1. Our data demonstrating that LKB1 increases the transcriptional activity of CLOCK-BMAL1 and also physically interacts with them, prompted us to determine whether LKB1 can phosphorylate BMAL1. Our results demonstrate that BMAL1 was readily phosphorylated by LKB1 (**Fig. III.IIIa**), thus, BMAL1 appears to be an efficient substrate of LKB1. To confirm our kinase assay data we found that BMAL1 phosphorylation changes in presence or absence of LKB1. LKB1 seems to play an important role in the phosphorylation status and probably also in the cellular localization of BMAL1. Indeed, WT MEFs are characterized, as expected, by hyper-phosphorylated form of BMAL1, which in not present in LKB1$^{-/-}$ MEFs (**Fig. III.IIIb**). In addition BMAL1 is mostly localized in the nucleus in WT MEFs, and on contrary it is mostly present in the cytoplasmic fraction in LKB1$^{-/-}$ MEFs (**Fig. III.IIIb**) meaning that it is not active as a transcription factor. This particular trend was found also post-synchronization with dexamethasone. These suggest that LKB1, through phosphorylation of BMAL1, can regulate its localization in the cell in a circadian manner.

Next we are planning to identify the site of phosphorylation on BMAL1 by performing a Mass Spectrometric analysis. This regulation of BMAL1 phosphorylation by LKB1 has a strong impact on the cyclic expression of clock-controlled genes (CCGs) and the absence of LKB1 resulted in a significant dampening of the CCGs (*Dbp, Cry1, Bmal1, Per2*) oscillation (**Fig. III.IV**). In order to determinate the extent of LKB1 function on circadian gene regulation we are carrying out a comparative microarray analysis of global gene expression levels in WT and LKB1-deficient MEFs at four different circadian time points (CT 12, CT 18, CT 24 and CT 30) after synchronization with dexamethasone.

This regulation of BMAL1 phosphorylation by LKB1 could be at chromatin level, indeed, LKB1 seems to be recruited on the *per1* promoter E-box region with a lower expression at CT 12 and higher at CT 24 (**Fig. III.Va**). To further support our hypothesis, chromatin fractionation *in vitro* (CT 12 and CT 24) and *in vivo* (ZT 3 , ZT 9 , ZT 12 , ZT 15) prove that although LKB1 is mostly localizated in the cytoplasm fraction, LKB1 was also detected in the nuclear and the chromatin fractions (**Fig. III.Vc**).

These finding suggest that LKB1 does get recruited to CCG promoters in *vitro* (MEFs) and also *in vivo* (Liver), but additional experiment must be performed to confirm the data.

Our results suggest that the implication of LKB1 in circadian control is significant especially considering that this kinase has been linked to various diseases such as PJS, metabolic disorders and cancer.

In conclusion this study provides new insights into the role of LKB1, finding additional function in the circadian system. Considering the role of LKB1 in cancer, our results also suggest that abnormality in cancer could be a consequence of a disrupted circadian clock. Therefore, a comprehensive understanding of the molecular links that connect LKB1 and the circadian clock could provide therapeutic benefit against cancer. For example, specific LKB1 inhibitors or activators can be more effective in the treatment of cancer if administered at a particular time of the day (chronotherapy).

III. ONGOING STUDIES AND FUTURE DIRECTIONS

We have demonstrated that CLOCK/BMAL1 transcriptional activity is enhanced by LKB1-mediated signaling and also that LKB1 regulates BMAL1 phosphorylation in a circadian manner with consequent strong impact on the cyclic expression of clock-controlled genes (CCGs). Lack of LKB1, indeed, resulted in a significant dampening of the clock-controlled genes (*Dbp, Cry1, Bmal1, Per2*) oscillation as compared to WT MEFs (**Fig. III.IV**).

In order to determinate the extent of LKB1 function on circadian gene regulation we are carrying out a comparative microarray analysis of global gene expression levels in WT and LKB1$^{-/-}$ MEFs at four different circadian time points (CT 12, CT 18, CT 24 and CT 30) after synchronization with dexamethasone.

To determine the *in vivo* role played by LKB1 in connecting circadian system and metabolism and to analyze the effect of inactivating the LKB1 pathway *in vivo* we are planning *in vivo* experiment with liver-specific *stk11*−/− mice. As an essential modulator of metabolism *in vivo*, the liver provides a cardinal domain in which to study interactions between the clock and metabolism. Much of the liver transcriptome and proteome oscillates in expression or activity and this rhythmicity is largely dependent on food as the zeitgeber (ZT) (Reddy A.B., *et al.* 2006; Yang X., *et al.* 2006). To analyze the effect of specific inactivation of the LKB1 pathway *in vivo*, we want to use the tamoxifen-inducible Albumin-CRE recombinase system that will allow us to generate time- and tissue-specific mouse mutants. We are presently crossing *stk11*$^{-/-}$mice (FVB; 129S6-*Stk11tm1Rdp*) with tamoxifen-inducible albumin-CRE mice.

Fig. : *stk11* strain

F1 heterozygotes will be mated to obtain an F2 generation, which will be genotyped to select male stk11/ stk11 × $cre^{-/-}$ and WT/WT × $cre^{-/-}$ littermates that will be used for experiments. Fourteen mice from each group will be used for circadian experiment and Cre recombinase activity will be observed in liver tissues following tamoxifen administration. We will harvest the liver and snap-freeze in liquid nitrogen and then we are planning to perform proteomics, transcriptomics and metabolomics analysis.

All research involving vertebrate animals has been performed under protocol approved by the Institutional Animal Care and Use Committee (IACUC). Animals are monitored on a daily basis by both the lab and University Lab Animal Resources (ULAR) veterinary staff for signs of distress, pain, and/or infection, and are given ad libitum access to food and water. Cages were cleaned on a weekly basis and when visibly soiled to maintain a clean environment. All husbandry procedures and welfare policies are conducted according to the Guide for the Care and Use of Laboratory Animals, set forth by the Institute of Laboratory Animal Resources, Commission on Life Sciences, and National Research Council.

III. REFERENCES

Akhtar R.A., Reddy A.B., Maywood E.S., Clayton J.D., King V.M., Smith A.G., Gant T.W., Hastings M.H., Kyriacou C.P. "Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus". Curr. Biol. 2002, 12:540-50.

Akten B., Jauch E., Genova G.K., Kim E.Y., Edery I., Raabe T., Jackson F.R. "A role for CK2 in the Drosophila circadian oscillator". Nat. Neurosci. 2003, 6:251-7.

Alenghat T., Meyers K., Mullican S.E., Leitner K., Adeniji-Adele A., Avila J., Bućan M., Ahima R.S., Kaestner K.H., Lazar M.A. "Nuclear receptor corepressor and histone deacetylase 3 govern circadian metabolic physiology". Nature. 2008, 456:997-1000.

Antle M.C., Silver R. "Orchestrating time: arrangements of the brain circadian clock". Trends Neurosci. 2005, 28:145-51.

Antoch M.P., Gorbacheva V.Y., Vykhovanets O., Toshkov I.A., Kondratov R.V., Kondratova A.A., Lee C., Nikitin A.Y. "Disruption of the circadian clock due to the Clock mutation has discrete effects on aging and carcinogenesis". Cell Cycle. 2008, 7:1197–1204.

Bae K., Jin X., Maywood E.S., Hastings M.H., Reppert S.M., Weaver D.R. "Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock". Neuron. 2001, 30:525–536.

Berger S.L. "The complex language of chromatin regulation during transcription". Nature. 2007, 447:407-12.

Bernstein E., Allis C.D. "RNA meets chromatin". Genes Dev. 2005, 19:1635-55.

Borrelli E., Nestler E.J., Allis C.D., Sassone-Corsi P. "Decoding the epigenetic language of neuronal plasticity". Neuron. 2008, 60:961-74.

Brown S.A., Zumbrunn G., Fleury-Olela F., Preitner N., Schibler U. "Rhythms of mammalian body temperature can sustain peripheral circadian clocks". Curr Biol. 2002, 12:1574-83.

Bunney W.E. and Bunney B.G. "Molecular clock genes in man and lower animals: possible implications for circadian abnormalities in depression". Neuropsychopharmacology. 2000, 22:335-45.

Bunger M.K., Wilsbacher L.D., Moran S.M., Clendenin C., Radcliffe L.A., Hogenesch J.B., Simon M.C., Takahashi J.S., Bradfield C.A. "Mop3 is an essential component of the master circadian pacemaker in mammals". Cell. 2000, 103:1009-17.

Busino L., Bassermann F., Maiolica A., Lee C., Nolan P.M., Godinho S.I., Draetta G.F., Pagano M. "SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins". Science. 2007, 316:900-4.

Cardone L., Hirayama J., Giordano F., Tamaru T., Palvimo J.J., Sassone-Corsi P. "Circadian clock control by SUMOylation of BMAL1". Science. 2005, 309:1390-4.

Cermakian N. and Sassone-Corsi P. "Multilevel regulation of the circadian clock". Nat. Rev. Mol. Cell. Biol. 2000, 11:59-67.

Cermakian N., Monaco L., Pando M.P., Dierich A. Sassone-Corsi P. "Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene". EMBO J. 2001, 20:3967–3974.

Cheung P., Allis C.D., Sassone-Corsi P. "Signaling to chromatin through histone modifications". Cell. 2000a, 103:263-71.

Cheung P., Tanner K.G., Cheung W.L., Sassone-Corsi P., Denu J.M., Allis C.D. “Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation”. Mol. Cell. 2000b, 5:905-15.

Crosio C., Cermakian N., Allis C.D., Sassone-Corsi P. “Light induces chromatin modification in cells of the mammalian circadian clock”. Nat. Neurosci. 2000, 3:1241-7.

Curtis A.M., Seo S.B., Westgate E.J., Rudic R.D., Smyth E.M., Chakravarti D., FitzGerald G.A., McNamara P. “Histone acetyltransferase-dependent chromatin remodeling and the vascular clock”. J. Biol. Chem. 2004, 279:7091-7.

Damiola F., Le Minh N., Preitner N., Kornmann B., Fleury-Olela F., Schibler U. “Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus”. Genes Dev. 2000, 14:2950-61.

Darlington T.K., Wager-Smith K., Ceriani M.F., Staknis D., Gekakis N., Steeves T.D., Weitz C.J., Takahashi J.S., Kay S.A. “Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim”. Science. 1998, 280:1599-603.

Davies S.P., Carling D., Munday M.R., Hardie D.G. “Diurnal rhythm of phosphorylation of rat liver acetyl-CoA carboxylase by the AMP-activated protein kinase, demonstrated using freeze-clamping. Effects of high fat diets”. Eur. J. Biochem. 1992, 203:615-23.

Doble B.W. and Woodgett J.R. “GSK-3: tricks of the trade for a multi-tasking kinase”. J. Cell. Sci. 2003, 116:1175-86.

Doi M., Hirayama J., Sassone-Corsi P. “Circadian regulator CLOCK is a histone acetyltransferase”. Cell. 2006, 125:497-508.

Duffield G.E., Best J.D., Meurers B.H., Bittner A., Loros J.J., Dunlap J.C. “Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells”. Curr. Biol. 2002, 12:551-7.

Eckel-Mahan K. and Sassone-Corsi P. “Metabolism control by the circadian clock and vice versa”. Nature Struct. Mol. Biol. 2009, 16:462–467.

Eide E.J., Vielhaber E.L., Hinz W.A., Virshup D.M. “The circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase Iepsilon”. J. Biol. Chem. 2002, 277:17248-54.

Eide E.J., Woolf M.F., Kang H., Woolf P., Hurst W., Camacho F., Vielhaber E.L., Giovanni A., Virshup D.M. “Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation”. Mol Cell Biol. 2005, 25:2795-807.

Etchegaray J.P., Lee C., Wade P.A., Reppert S.M. “Rhythmic histone acetylation underlies transcription in the mammalian circadian clock”. Nature. 2003, 421:177-82.

Fan W., Downes M., Atkins A., Yu R., Evans R.M. “Nuclear receptors and AMPK: resetting metabolism”. Cold Spring Harb. Symp. Quant. Biol. 2011, 76:17-22.

Fontaine C., Dubois G., Duguay Y., Helledie T., Vu-Dac N., Gervois P., Soncin F., Mandrup S., Fruchart J.C., Fruchart-Najib J., Staels B. “The orphan nuclear receptor Rev-Erbalpha is a peroxisome proliferator-activated receptor (PPAR) gamma target gene and promotes PPARgamma-induced adipocyte differentiation”. J. Biol. Chem. 2003, 278:37672-80.

Fu L., Pelicano H., Liu J., Huang P., Lee C., “The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo”. Cell. 2002, 111:41–50.

Gallego M. and Virshup D.M. “Post-translational modifications regulate the ticking of the circadian clock”. Nat. Rev. Mol. Cell. Biol. 2007, 8:139-48.

Gauger M.A. and Sancar A. “Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer”. Cancer Res. 2005, 65:6828–6834.

Gekakis N., Staknis D., Nguyen H.B., Davis F.C., Wilsbacher L.D., King D.P., Takahashi J.S., Weitz C.J. “Role of the CLOCK protein in the mammalian circadian mechanism”. Science. 1998, 280:1564-9.

Godinho S.I., Maywood E.S., Shaw L., Tucci V., Barnard A.R., Busino L., Pagano M., Kendall R., Quwailid M.M., Romero M.R., O'neill J., Chesham J.E., Brooker D., Lalanne Z., Hastings M.H., Nolan P.M. “The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period”. Science. 2007, 316:897-900.

Glossop N.R. and Hardin P.E. “Central and peripheral circadian oscillator mechanisms in flies and mammals”. J. Cell. Sci. 2002, 115:3369-77.

Glozak M.A., Sengupta N., Zhang X., Seto E. “Acetylation and deacetylation of non-histone proteins”. Gene. 2005, 363:15-23.

Green C.B., Douris N., Kojima S., Strayer C.A., Fogerty J., Lourim D., Keller S.R., Besharse J.C. “Loss of Nocturnin, a circadian deadenylase, confers resistance to hepatic steatosis and diet-induced obesity”. Proc. Natl. Acad. Sci. U S A. 2007, 104:9888-93.

Green K.A. and Carroll J.S. Oestrogen-receptor mediated transcription and the influence of co-factors and chromatin state. Nature Rev. Cancer. 2007, 7:713–722.

Griffin E.A. Jr, Staknis D., Weitz C.J. “Light-independent role of CRY1 and CRY2 in the mammalian circadian clock”. Science. 1999, 286:768-71.

Harada Y., Sakai M., Kurabayashi N., Hirota T., Fukada Y. “Ser-557-phosphorylated mCRY2 is degraded upon synergistic phosphorylation by glycogen synthase kinase-3 beta”. J. Biol. Chem. 2005, 280:31714-21.

Hardie D.G. “AMP-activated/SNF1 protein kinases: Conserved guardians of cellular energy”. Nat. Rev. Mol. Cell. Biol. 2007, 8: 774–785.

Hawley S.A., Boudeau J., Reid J.L., Mustard K.J., Udd L., Mäkelä T.P., Alessi D.R., Hardie D.G. “Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade”. J. Biol. 2003, 2:28.

Hirayama J. and Sassone-Corsi P. “Structural and functional features of transcription factors controlling the circadian clock”. Curr. Opin. Genet. Dev. 2005, 15:548-56.

Hirayama J., Sahar S., Grimaldi B., Tamaru T., Takamatsu K., Nakahata Y., Sassone-Corsi P. “CLOCK-mediated acetylation of BMAL1 controls circadian function”. Nature. 2007, 450:1086-90.

Hirota T., Lewis W.G., Liu A.C., Lee J.W., Schultz P.G., Kay S.A. “A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3beta”. Proc. Natl. Acad. Sci. U S A. 2008, 105:20746-51.

Hunt T. and Sassone-Corsi P. ”Riding tandem: circadian clocks and the cell cycle”. Cell. 2007, 129:461-4.

Iitaka C., Miyazaki K., Akaike T., Ishida N. “A role for glycogen synthase kinase-3beta in the mammalian circadian clock”. J. Biol. Chem. 2005, 280:29397-402.

Jin X., Shearman L.P., Weaver D.R., Zylka M.J., de Vries G.J., Reppert S.M. “A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock”. Cell. 1999, 96:57-68.

Karlsson B., Knutsson A., Lindahl B. “Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27,485 people”. Occup. Environ. Med. 2001, 58:747-52.

Kloss B., Price J.L., Saez L., Blau J., Rothenfluh A., Wesley C.S., Young M.W. “The Drosophila clock gene double-time encodes a protein closely related to human casein kinase Iepsilon”. Cell. 1998, 94:97-107.

Kondratov R.V., Chernov M.V., Kondratova A.A., Gorbacheva V.Y., Gudkov A.V., Antoch M.P. “BMAL1-dependent circadian oscillation of nuclear CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system”. Genes Dev. 2003 17: 1921–1932.

Kondratov R.V., Kondratova A.A., Gorbacheva V.Y., Vykhovanets O.V., Antoch M.P. “Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock”. Genes Dev. 2006, 20: 1868–1873.

Kwon I., Lee J., Chang S.H., Jung N.C., Lee B.J., Son G.H., Kim K., Lee K.H. “BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer”. Mol. Cell. Biol. 2006, 26:7318-30.

Kume K., Zylka M.J., Sriram S., Shearman L.P., Weaver D.R., Jin X., Maywood E.S., Hastings M.H., Reppert S.M. “mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop”. Cell. 1999, 98:193-205.

Lamia K.A., Storch K.F., Weitz C.J. “Physiological significance of a peripheral tissue circadian clock”. Proc. Natl. Acad. Sci. U S A. 2008, 105:15172-7.

Lamia K.A., Sachdeva U.M., DiTacchio L., Williams E.C., Alvarez J.G., Egan D.F., Vasquez D.S., Juguilon H., Panda S., Shaw R.J., Thompson C.B., Evans R.M. “AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation”. Science. 2009, 326:437-40.

Lee J., Lee Y., Lee M.J., Park E., Kang S.H., Chung C.H., Lee K.H., Kim K. “Dual modification of BMAL1 by SUMO2/3 and ubiquitin promotes circadian activation of the CLOCK/BMAL1 complex”. Mol. Cell. Biol. 2008, 28:6056-65.

Lee D.Y., Hayes J.J., Pruss D., Wolffe A.P. “A positive role for histone acetylation in transcription factor access to nucleosomal DNA”. Cell. 1993, 72:73-84.

Lehman M.N., Silver R., Gladstone W.R., Kahn R.M., Gibson M., Bittman E.L. “Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain”. J. Neurosci. 1987, 7:1626-38.

Lin J.M., Kilman V.L., Keegan K., Paddock B., Emery-Le M., Rosbash M., Allada R. “A role for casein kinase 2alpha in the Drosophila circadian clock”. Nature. 2002, 420:816-20.

Lin J.M., Schroeder A., Allada R. “In vivo circadian function of casein kinase 2 phosphorylation sites in Drosophila PERIOD”. J. Neurosci. 2005, 25:11175-83.

Liu C., Li S., Liu T., Borjigin J., Lin J.D. “Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism”. Nature. 2007, 447:477-81.

Lo W.S., Trievel R.C., Rojas J.R., Duggan L., Hsu J.Y., Allis C.D., Marmorstein R., Berger S.L. “Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14”. Mol. Cell. 2000, 5:917-26.

Lowrey P.L., Shimomura K., Antoch M.P., Yamazaki S., Zemenides P.D., Ralph M.R., Menaker M., Takahashi J.S. “Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau”. Science. 2000, 288:483-92.

Martinek S., Inonog S., Manoukian A.S., Young M.W. “A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock”. Cell. 2001, 105:769-79.

Merienne K., Pannetier S., Harel-Bellan A., Sassone-Corsi P. “Mitogen-regulated RSK2-CBP interaction controls their kinase and acetylase activities”. Mol. Cell. Biol. 2001, 21:7089-96.

Miller B.H., McDearmon E.L., Panda S., Hayes K.R., Zhang J., Andrews J.L., Antoch M.P., Walker J.R., Esser K.A., Hogenesch J.B., Takahashi J.S. “Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation”. Proc. Natl. Acad. Sci. USA. 2007: 104:3342–3347.

Miranda T.B., Jones P.A. “DNA methylation: the nuts and bolts of repression”. J. Cell. Physiol. 2007, 213:384-90

Morse D. and Sassone-Corsi P. “Review Time after time: inputs to and outputs from the mammalian circadian oscillators”. Trends Neurosci. 2002, 25:632-7.

Nader N., Chrousos G.P., Kino T. “Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications”. FASEB J. 2009, 23:1572-83.

Nakahata Y., Kaluzova M., Grimaldi B., Sahar S., Hirayama J., Chen D., Guarente L.P., Sassone-Corsi P. “The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control”. Cell. 2008, 134:329-40.

Naruse Y., Oh-hashi K., Iijima N., Naruse M., Yoshioka H., Tanaka M. “Circadian and light-induced transcription of clock gene Per1 depends on histone acetylation and deacetylation”. Mol. Cell. Biol. 2004, 24:6278-87.

Nony P., Gaude H., Rossel M., Fournier L., Rouault J.P., Billaud M. “Stability of the Peutz–Jeghers syndrome kinase LKB1 requires its binding to the molecular chaperones Hsp90/Cdc37”. Oncogene. 2003, 22: 9165–9175.

Ozturk N., Lee J.H., Gaddameedhi S., Sancar A. “Loss of cryptochrome reduces cancer risk in p53 mutant mice”. Proc. Natl. Acad. Sci. USA. 2009, 106:2841-6.

Panda S., Hogenesch J.B., Kay S.A. “Circadian rhythms from flies to human”. Nature. 2002, 417:329-35.

Pando M.P., Morse D., Cermakian N., Sassone-Corsi P. "Phenotypic rescue of a peripheral clock genetic defect via SCN hierarchical dominance”. Cell. 2002, 110:107-17.

Perreau-Lenz S., Zghoul T., Spanagel R. “Clock genes running amok. Clock genes and their role in drug addiction and depression”. EMBO Rep. 2007, 8:S20-3.

Preitner N., Damiola F., Lopez-Molina L., Zakany J., Duboule D., Albrecht U., Schibler U. “The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator”. Cell. 2002, 110:251-60.

Price J.L., Blau J., Rothenfluh A., Abodeely M., Kloss B., Young M.W. “double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation”. Cell. 1998, 94:83-95.

Ralph M.R. and Lehman M.N. “Transplantation: a new tool in the analysis of the mammalian hypothalamic circadian pacemaker”. Trends Neurosci. 1991, 14:362-6.

Reddy A.B., Karp N.A., Maywood E.S., Sage E.A., Deery M., O'Neill J.S., Wong G.K., Chesham J., Odell M., Lilley K.S., Kyriacou C.P., Hastings M.H. “Circadian orchestration of the hepatic proteome”. Curr. Biol. 2006, 16: 1107–1115.

Reppert S.M. and Weaver D.R. “Review Coordination of circadian timing in mammals”. Nature. 2002, 418:935-41.

Roenneberg T. and Merrow M. “Circadian clocks - the fall and rise of physiology”. Nat. Rev. Mol. Cell. Biol. 2005, 6:965-71.

Reischl S. and Kramer A. “Kinases and phosphatases in the mammalian circadian clock”. FEBS Lett. 2011, 585:1393-9.

Ripperger J.A., Shearman L.P., Reppert S.M., Schibler U. “CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP”. Genes Dev. 2000, 14:679-89.

Ripperger J.A. and Schibler U. “Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions”. Nat Genet. 2006, 38:369-74.

Sadacca L.A., Lamia K.A., deLemos A.S., Blum B., Weitz C.J. “An intrinsic circadian clock of the pancreas is required for normal insulin release and glucose homeostasis in mice”. Diabetologia. 2011, 54:120-4.

Sahar S. and Sassone-Corsi P. "Metabolism and cancer: the circadian clock connection” Nature Reviews Cancer. 2009, 9:886-96.

Sahar S., Zocchi L., Kinoshita C., Borrelli E., Sassone-Corsi P. “Regulation of BMAL1 protein stability and circadian function by GSK3beta-mediated phosphorylation”. PLoS One. 2010, 5:e8561.

Sanada K., Okano T., Fukada Y. “Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1”. J. Biol. Chem. 2002, 277:267-71.

Sato T.K., Panda S., Miraglia L.J., Reyes T.M., Rudic R.D., McNamara P., Naik K.A., FitzGerald G.A., Kay S.A., Hogenesch J.B. “A functional genomics strategy reveals Rora as a component of the mammalian circadian clock”. Neuron. 2004, 43:527-37.

Sato T.K., Yamada R.G., Ukai H., Baggs J.E., Miraglia L.J., Kobayashi T.J., Welsh D.K., Kay S.A., Ueda H.R., Hogenesch J.B. “Feedback repression is required for mammalian circadian clock function”. Nat Genet. 2006, 38:312-9.

Schibler U., and Sassone-Corsi P. “A web of circadian pacemakers”. Cell. 2002, 111:919-22.

Scheer F.A., Hilton M.F., Mantzoros C.S., Shea S.A. “Adverse metabolic and cardiovascular consequences of circadian misalignment”. Proc. Natl. Acad. Sci. USA. 2009, 106:4453–4458.

Shaw R.J., Kosmatka M., Bardeesy N., Hurley R.L., Witters L.A., DePinho R.A., Cantley L.C. “The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress”. Proc. Natl. Acad. Sci. U S A. 2004, 101:3329-35.

Shaw R.J., Lamia K.A., Vasquez D., Koo S.H., Bardeesy N., Depinho R.A., Montminy M., Cantley L.C. “The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin”. Science. 2005, 310:1642-6.

Shimba S., Ishii N., Ohta Y., Ohno T., Watabe Y., Hayashi M., Wada T., Aoyagi T., Tezuka M. “Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis”. Proc. Natl. Acad. Sci. U S A. 2005, 102:12071-6.

Shirogane T., Jin J., Ang X.L., Harper J.W. “SCFbeta-TRCP controls clock-dependent transcription via casein kinase 1-dependent degradation of the mammalian period-1 (Per1) protein”. J. Biol. Chem. 2005, 280:26863-72.

Siepka S.M., Yoo S.H., Park J., Song W., Kumar V., Hu Y., Lee C., Takahashi J.S. “Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression”. Cell. 2007, 129:1011-23.

So W.V. and Rosbash M. “Post-transcriptional regulation contributes to Drosophila clock gene mRNA cycling”. EMBO J. 1997, 1623:7146-55.

Spengler M.L., Kuropatwinski K.K., Schumer M., Antoch M.P. “A serine cluster mediates BMAL1-dependent CLOCK phosphorylation and degradation”. Cell Cycle. 2009, 8:4138-46.

Stokkan K.A., Yamazaki S., Tei H., Sakaki Y., Menaker M. “Entrainment of the circadian clock in the liver by feeding”. Science. 2001, 291:490-3.

Storch K.F., Lipan O., Leykin I., Viswanathan N., Davis F.C., Wong W.H., Weitz C.J. “Extensive and divergent circadian gene expression in liver and heart”. Nature. 2002, 417:78-83.

Strahl B.D., Allis C.D. “The language of covalent histone modifications”. Nature. 2000, 403:41-5.

Takano A., Isojima Y., Nagai K. “Identification of mPer1 phosphorylation sites responsible for the nuclear entry”. J. Biol. Chem. 2004, 27931:32578-85.

Tamaru T., Hirayama J., Isojima Y., Nagai K., Norioka S., Takamatsu K., Sassone-Corsi P. “CK2alpha phosphorylates BMAL1 to regulate the mammalian clock”. Nat. Struct. Mol. Biol. 2009, 16:446-8.

Taniguchi H., Fernández A.F., Setién F., Ropero S., Ballestar E., Villanueva A., Yamamoto H., Imai K., Shinomura Y., Esteller M. “Epigenetic inactivation of the circadian clock gene BMAL1 in hematologic malignancies”. Cancer Res. 2009, 69:8447-54

Tokunaga H., Takebayashi Y., Utsunomiya H., Akahira J., Higashimoto M., Mashiko M., Ito K., Niikura H., Takenoshita S., Yaegashi N. “Clinicopathological significance of circadian rhythm-related gene expression levels in patients with epithelial ovarian cancer”. Acta Obstet. Gynecol. Scand. 2008, 87:1060-70.

Travnickova-Bendova Z., Cermakian N., Reppert S.M., Sassone-Corsi
P. “Bimodal regulation of mPeriod promoters by CREB-dependent signaling
and CLOCK/BMAL1 activity”. Proc. Natl. Acad. Sci. USA. 2002, 99: 7728–7733.

Tremblay A.M., Wilson B.J., Yang X.J., Giguère V. “Phosphorylation-dependent sumoylation regulates estrogen-related receptor-alpha and -gamma transcriptional activity through a synergy control motif”. Mol. Endocrinol. 2008, 22:570-84.

Ueda H.R., Hayashi S., Chen W., Sano M., Machida M., Shigeyoshi Y., Iino M., Hashimoto S. “System-level identification of transcriptional circuits underlying mammalian circadian clocks”.
Nat Genet. 2005, 37:187-92.

Um J.H., Yang S., Yamazaki S., Kang H., Viollet B., Foretz M., Chung J.H. “Activation of 5'-AMP-activated kinase with diabetes drug metformin induces casein kinase Iepsilon (CKIepsilon)-dependent degradation of clock protein mPer2”. J. Biol. Chem. 2007, 282:20794-8.

Vielhaber E.L., Duricka D., Ullman K.S., Virshup D.M. “Nuclear export of mammalian PERIOD proteins”. J. Biol. Chem. 2001, 276:45921-7.

Vitaterna M.H., King D.P., Chang A.M., Kornhauser J.M., Lowrey P.L., McDonald J.D., Dove W.F., Pinto L.H., Turek F.W., Takahashi J.S. “Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior”. Science. 1994, 264:719-25.

Woods A., Johnstone S.R., Dickerson K., Leiper F.C., Fryer L.G., Neumann D., Schlattner U., Wallimann T., Carlson M., Carling D. “LKB1 is the upstream kinase in the AMP-activated protein kinase cascade”. Curr. Biol. 2003, 13:2004-8.
Yamamoto T., Nakahata Y., Soma H., Akashi M., Mamine T.,Takumi T. “Transcriptional oscillation of canonical clock genes in mouse peripheral Tissues”. BMC Mol. Biol. 2004, 5: 18.

Yang X., Lamia K.A., Evans R.M. “Nuclear receptor expression links the circadian clock to metabolism”. Cell. 2006, 126: 801–810.
Yoo S.H., Yamazaki S., Lowrey P.L., Shimomura K., Ko C.H., Buhr E.D., Siepka S.M., Hong H.K., Oh W.J., Yoo O.J., Menaker M., Takahashi J.S. “PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues”. Proc Natl Acad Sci U S A. 2004, 101:5339-46.

Yin L., Wang J., Klein P.S., Lazar M.A. “Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock”. Science. 2006, 311:1002-5.

Zheng B., Larkin D.W., Albrecht U., Sun Z.S., Sage M., Eichele G., Lee C.C., Bradley A. “The mPer2 gene encodes a functional component of the mammalian circadian clock”. Nature. 1999, 400:169–173.

Zheng B., Albrecht U., Kaasik K., Sage M., Lu W., Vaishnav S., Li Q., Sun Z.S., Eichele G., Bradley A., Lee C.C. “Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock”. Cell. 2001, 105:683–694.

APPENDIX 1

Pubblicazioni

Accordi B, **Galla L**, Milani G, Curtarello M, Serafin V, Lissandron V, Viola, G, te Kronnie G, De Maria R, Petricoin 3rd EF, Liotta LA, Indraccolo S, Basso G.
AMPK inhibition enhances apoptosis in MLL-rearranged pediatric B-acute lymphoblastic leukemia cells.
Leukemia doi:10.1038/leu.2012.338; accepted article preview online November 21, 2012. Accepted for publication.

Accordi B, Espina V, Giordan M, VanMeter A, Milani G, **Galla L**, Ruzzene M, Sciro M, Trentin L, De Maria R, te Kronnie G, Petricoin E, Liotta L, Basso G.
Functional protein network activation mapping reveals new potential molecular drug targets for poor prognosis pediatric BCP-ALL.
PLoS One 2010 Oct 21; 5(10):e13552.

Abstracts

B. Accordi, V. Espina, M. Giordan, A.J. VanMeter, **L. Galla**, G. Milani, M. Sciro, R. De Maria, G. te Kronnie, E.F. Petricoin, L.A. Liotta, G. Basso. AMPK inhibition induces apoptosis in pediatric B-ALL cells with MLL gene rearrangements.
Convegno Annuale della Associazione Italiana di Colture Cellulari, Firenze, 2-4 Dicembre 2009. (Oral Presentation).

B. Accordi, V. Espina, M. Giordan, A.J. VanMeter, **L. Galla**, G. Milani, M. Sciro, R. De Maria, G. te Kronnie, E.F. Petricoin, L.A. Liotta, G. Basso.
AMPK inhibition induces apoptosis in pediatric B-ALL cells with MLL gene rearrangements.
Convegno Annuale dell'Associazione di Biologia Cellulare e del Differenziamento, Fiesole (Fi), 26-27 Marzo 2010. (Poster)

G. Milani, B. Accordi, M. Giordan, S. Bresolin, M. Sciro, **L. Galla**, G. Te Kronnie, G. Basso. Phosphoproteomic profiling of pediatric T-lineage acute lymphoblastic leukemia.
ESH-EHA Scientific Workshop T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL) MEETS NORMAL T-CELL DEVELOPMENT; Mandelieu La Napoule (France) May 7-9 2010.

B. Accordi, V. Espina, M. Giordan, A.J. VanMeter, **L. Galla**, G. Milani, M. Sciro, R. De Maria, G. te Kronnie, E.F. Petricoin, L.A. Liotta, G. Basso.
AMPK inhibition induces apoptosis in pediatric B-ALL cells with MLL gene rearrangements.
15° Congress of the European Hematology Association; Barcelona (Spain) June 10-13, 2010.
Heamatologica Vol.95, Supplement no.2, 2010. (Abstract n.0244, Poster).

B. Accordi, G. Milani, L. Marchioretto, **L. Galla**, M. Sciro, G. te Kronnie, G. Basso. Promoter region Hypermethylation contributes to c-MET downregulation in pediatric MLL-rearranged BCP-ALL.
15° Congress of the European Hematology Association; Barcelona (Spain) June 10-13, 2010.
Heamatologica Vol.95, Supplement no.2, 2010. (Abstract n.0013, Poster).

G. Milani, B. Accordi, M. Giordan, S. Bresolin, M. Sciro, **L. Galla**, G. Te Kronnie, G. Basso. Phosphoproteomic profiling of pediatric T-lineage acute lymphoblastic.
15° Congress of the European Hematology Association; Barcelona (Spain) June 10-13, 2010.
Heamatologica Vol.95, Supplement no.2, 2010. (Abstract n.0003, Poster).

B. Accordi, V. Espina, M. Giordan, AJ. VanMeter, G. Milani, M. Sciro, L. **Galla, L**. Trentin, R. De Maria, G. te Kronnie, LA. Liotta, EF. Petricoin III, G. Basso.
Functional Protein Network Activation Mapping Reveals New Potential Molecular Drug Targets for Poor Prognosis Pediatric B-ALL.
Italy-USA Oncoproteomics Meeting, Roma, 12-13 Luglio 2010. (Oral Presentation).

L. Galla, B. Accordi, G. Milani, M. Sciro, G. te Kronnie, G. Basso.
AMPK inhibition induces mitochondrial apoptosis in pediatric B-ALL cells with MLL gene rearrangements.
Cancer Metabolism, Barcelona BioMed Conferences, Barcelona (Spain) November 8-10, 2010. (Poster).

B. Accordi, **L. Galla**, G. Milani, M. Curtarello, G. Viola, G. te Kronnie, S. Indraccolo, G. Basso.
L'inibizione di AMPK induce apoptosi mitocondriale nelle BCP-ALL con riarrangiamento del gene MLL.
Secondo Workshop AIEOP IN LAB, Catania 19-20 Maggio 2011. (Poster P26).

L. Galla, B. Accordi, M. Curtarello, G. Milani, S. Bresolin, G. te Kronnie, S. Indraccolo, G. Basso G.
A new perspective for LKB1 as pediatric MLL-rearranged B-ALL cells survival promoter. Metabolism and Cancer 2011, Baltimore (MD) October 16-19, 2011. (Abstract n. B67, Poster).

G. Milani, B. Accordi, M. Giordan, S. Bresolin, **L. Galla**, M. Sciro, E.Vendramini, G.Cazzaniga, G. te Kronnie, G. Basso.
Lack of Protein Kinase C Alpha Is Associated with Poor Prognosis in Pediatric T-Lineage Acute Lymphoblastic Leukemia.
53° American Society of Hematology (ASH) Annual Meeting; San Diego (CA) December 10-13, 2011. (Abstract n.744, Oral Presentation).

F. Seyfried, B. Accordi, M. Queudeville, SM. Eckhoff, G. Milani, **L. Galla**, M. Giordan, JM. Kraus, G. Basso, H. Armin Kestler, G. te Kronnie, KM. Debatin, LH. Meyer.
High Risk Acute Lymphoblastic Leukemia with Rapid NOD/SCID Engraftment Is Characterized by High Protein Expression of CYCLIN B, Beta- CATENIN, ANNEXIN I and Decreased PKC Alpha Activation.
53° American Society of Hematology (ASH) Annual Meeting; San Diego (CA) December 10-13, 2011. (Poster).

B. Accordi, **L. Galla**, G. Milani, M. Giordan, M. Curtarello, G. Viola, G. Te Kronnie, R. De Maria, V. Espina, EF. Petricoin, LA. Liotta, S. Indraccolo, G. Basso.
Reverse Phase Protein Microarrays for biomarker and therapeutic targets discovery in pediatric BCP-ALL: focus on the AMPK pathway.
XIII Congress of the Italian Society of Experimental Hematology, Roma 17-19 Ottobre 2012.
(Poster PO-179).

“AMPK inhibition induces mitochondrial apoptosis in pediatric B-ALL cells with MLL gene rearrangements.”

Galla L, Accordi B, Milani G, Sciro M, te Kronnie G, Basso G

Presented as poster at “Cancer Metabolism, Barcelona BioMed Conferences, Barcelona (Spain) November 8-10, 2010”

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer. In spite of therapy improvements obtained over the last decades, therapy is not yet effective in all cases. Infants with MLL (Mixed Lineage Leukemia) rearrangements (11q23) form the most striking example of ALL patients who have not benefited from the improved treatment regimen. The presence of MLL-rearrangements predicts early relapse and poor prognosis with few therapeutic possibilities. Through RPPA (Reverse Phase Protein Arrays) studies performed in MLL-rearranged B-ALL patients, it was found an hyperactivated pathway that leads to BCL2 activation through LKB1, AMPK and eNOS phosphorylation. In this study we evaluated the effective role of the AMPK pathway in supporting MLL-rearranged B-ALL cells survival, and we explored the mechanism by which leukemia cells undergo apoptosis.
AMPK was inhibited using the AMPK inhibitor Compound C in MLL-rearranged cell lines (RS4; 11, SEM) and not-rearranged cell lines (MHH-CALL2, MHH-CALL4). We found that only MLL-rearranged cells undergo apoptosis after inhibition of proliferation and cell cycle block at the $G0/G_1$ phase. Moreover, we observed mitochondrial depolarization, reactive oxygen species (ROS) production, Caspase-3 cleavage and DNA fragmentation.
Our results demonstrate that the AMPK pathway activation appears to directly contribute to the survival of MLL-rearranged leukemia cells, and Compound C treatment induces apoptosis following the mitochondrial pathway. Therefore AMPK can be suggested as a potential new molecular drug target, and AMPK inhibitors could actually represent a new option for improving treatment outcome in pediatric MLL-rearranged leukemia patients.

AMPK INHIBITION INDUCES MITOCHONDRIAL APOPTOSIS IN PEDIATRIC B-ALL CELLS WITH MLL GENE REARRANGEMENTS

Galla L, Accordi B, Milani G, Sciro M, te Kronnie G, Basso G

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer. The development of effective therapies for children with ALL has greatly improved long-term event-free survival rates, but about 20% of patients is still not able to reach a stable complete remission. Infants with ***MLL*** (Mixed Lineage Leukaemia) gene rearrangements form the most striking example of ALL patients who did not benefit from the improved treatments (4-yr EFS 40%).

We used **Reverse Phase Protein Microarrays** (RPMA) to better map the biology of the MLL disease and to identify aberrantly activated phosphoproteins specific for *MLL* rearranged patients.

Through RPMA studies performed in MLL-rearranged B-ALL patients it was found an **hyperactivated pathway** that leads to BCL2 activation through LKB1, AMPK and eNOS phosphorylation (Accordi et al., Plos One 2010).

AIM

The discovery of the molecular signal pathway portrait in subgroups of patients could play an important role in improving treatment outcome through tailored therapy.

The goal of our studies was to evaluate the **effective role of the AMPK pathway in supporting MLL-rearranged B-ALL cells survival** and to explore the mechanism by which leukemia cells undergo apoptosis.

METHODS AND RESULTS

We thus performed a **pharmacological assay** treating 4 selected human cell lines with a commercial **AMPK inhibitor** (Compound C, Calbiochem) at different times, 6-96 hours, and at different concentrations ranging from 0.001μM up to 100μM.

We measured the proliferation rate and the induced apoptosis through MTT assay and Annexin V-PI staining.

We then investigated the effect of AMPK inhibition after Compound C treatment using western blot, cell cycle analysis and cytofluorimetric assays.

Cell lines	Cell phenotype	Cytogenetics
SEM	Human B cell precursor leukemia (ALL)	Carries t(4;11)
RS 4;11	Human B cell precursor leukemia (ALL L2)	Carries MLL-AF4 fusion gene
MHH CALL 2	Human B cell precursor leukemia (cALL)	-
MHH CALL 4	Human B cell precursor leukemia (pre B-ALL)	-

MTT assay

GI50 (μM)

	SEM	RS4;11	CALL2	CALL4
24h	2.8	7.1	31.4	24.8
48h	**0.16**	**3.2**	**25.7**	**17.1**
72h	0.09	2.3	16.6	16.3

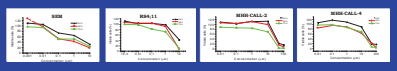

Fig. 1a: MTT assay.

Annexin V-PI staining

LC50 (μM)

	SEM	RS4;11	CALL2	CALL4
24h	9.8	9	41.6	38.7
48h	**7.5**	**8.4**	**37.5**	**31.4**
72h	6.4	8	37.3	29.2

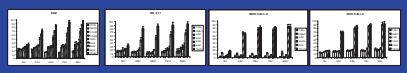

Fig. 1b: Annexin V-PI staining.

Inactivation of the AMPK pathway

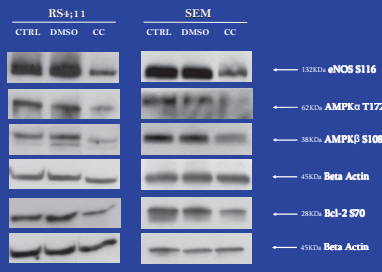

Fig. 2: AMPK pathway inactivation. As previously described (Accordi et al. Plos One, 2010), in MLL-rearranged cells, the AMPK pathway is constitutively activated and lead to BCL-2 activation through AMPK and eNOS phosphorylation.
eNOS activation lead to the production of nitric oxide (NO). NO is a multifunctional messanger with a dual role in cancer: pro- and anti-apoptotic.
In undifferentiated tumors, such as ALL with MLL-rearrangments, NO in able to activate BCL2 thus preventing apoptosis.
As shown in this figure, treatment with Compound C is able to induce dephosphorylation and thus deactivation of AMPK and of its downstream targets eNOS and BCL-2.
This result proves the kinase-substrate relationship between these proteins in MLL-rearranged BCP-ALL cells, and highlights the crucial role of AMPK in the activation of this pathway.

Cell cycle analyses reveal an arrest at the G2/M phase

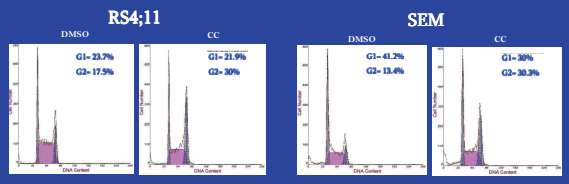

Fig. 3: Cell cycle analysis. The effect of AMPK inhibition after Compound C treatment on cell cycle progression of MLL-rearranged SEM and RS4;11 cells was studied. After treatment a G2/M arrest pattern was observed with a concomitant decrease of all the other phases of the cell cycle. The G2/M cell population increased from 13% in the control to 34% in SEM cells, and from 17% to 38% in RS4;11 cells. Under the same conditions the G1 cells population decreased from 72% to 56% in SEM cells, and from 53% to 43% in RS4;11 cells, whereas the S phase decreased from 15% to 10% (SEM) and from 30% to 19% (RS4;11).

Compound C-induced apoptosis follows the mithochondrial pathway

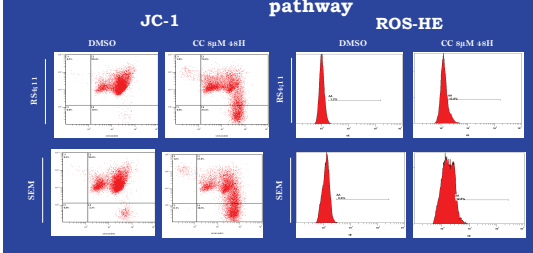

Fig. 4: Treatment with Compound C induces mithocondrial membrane depolarization (JC1) and reactive oxygen species (ROS-HE) production in MLL-rearranged cell lines.

Release of Cytochrome C

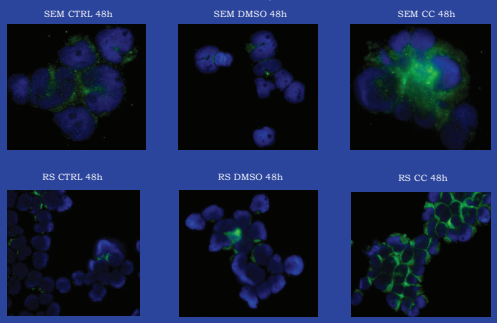

Fig. 5: Cytochrome C immunostaining.
BCL 2 proteins are found in the cytosol where often act as sensor of cellular damage or stress. Following cellular stress they relocate to the surface of the mithocondria where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic bcl-2 proteins and can lead to the formation of pores in the mitochondria and the release of cytochrome C and other pro-apoptotic molecules from the intermembrane space. This in turn leads to the formation of the apoptosome and the activation of the caspase cascade.
Damage to mitochondria leads to release of cytochrome C.

Cleaved Casp 3

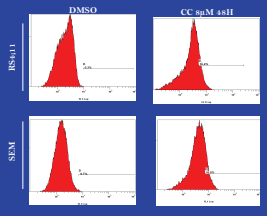

Fig. 6: Apoptosis induced by Compound C leads to Caspase 3 cleavage.
In this figure it is shown an increase of 16% of caspase 3 cleavage after 48h treatment with Compound C in MLL-rearranged cell lines.

DNA Fragmentation

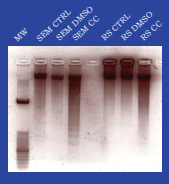

Fig. 7: Compound C causes DNA damage visualized through laddering assay.

CONCLUSIONS

The AMPK pathway activation appears to directly contribute to the survival of MLL-rearranged leukemia cells, and Compound C treatment induces apoptosis following the mitochondrial pathway.

Therefore our data strongly encourage further studies of AMPK as a potential new molecular drug target, and AMPK inhibitors could actually represent a new option for improving treatment outcome in pediatric MLL-rearranged leukemia patients.

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“A new perspective for LKB1 as pediatric MLL-rearranged B-ALL cells survival promoter.”

Galla L, Accordi B, Curtarello M, Milani G, Bresolin S, te Kronnie G, Indraccolo S, Basso G.

Metabolism and Cancer 2011, Baltimore (MD) October 16-19, 2011. (Abstract n. B67, Poster).

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer. In spite of therapy improvements obtained over the last decades, diagnosis of ALL with MLL (Mixed-Lineage Leukemia) rearrangements (11q23) predicts early relapse and poor prognosis with few therapeutic options. Proteomics technologies hold the promise to provide better insights into protein activation state in cancer tissues and are expected to bring to more effective therapies for these patients.
Through RPPA (Reverse Phase Protein Arrays) studies performed on MLL-rearranged B-ALL patients, we previously identified a hyperactivated pathway that leads to BCL-2 activation through LKB1, AMPK and eNOS phosphorylation in these patients.
BCL-2 is a well known anti-apoptotic regulator associated with chemotherapy resistance, thus the activation of the LKB1 pathway seems to support the survival of MLL-rearranged leukemia cells. The same pathway was not activated in non-translocated patients.
The LKB1 pathway is an energy sensing pathway activated in cells in response to low energy levels. More recently, LKB1 and AMPK have been linked also to many other fundamental cellular processes including regulation of cell proliferation, cell polarity, transcription and damage responses.
Gene Expression analysis of this pathway did not show differences between these two groups of patients. We investigated the presence of mutations in the LKB1 gene in different leukemia cell lines by sequence analysis and no mutation was found.
In SEM and RS4;11 cells (MLL-rearranged B-ALL derived cells) immunofluorescence staining for activated LKB1 revealed hyperactivation of the kinase with a predominant cytoplasmatic localization in dividing cells. Further analysis revealed that LKB1 has a specific localization during the M phase of the cell cycle at spindle microtubules and at centrosomes. Furthermore, we found that LKB1 co-localizes with PCM1 (pericentriolar material 1) suggesting that LKB1 plays a key role in the control of the cell cycle through regulation of elongation and stability of microtubules.
To more thoroughly investigate the role of LKB1 in MLL-rearranged cells we silenced LKB1 in two different cell lines chosen as *in vitro* model: SEM carrying the t(4;11) MLL-AF4 translocation, as a model for MLL-rearranged leukemias and MHH-CALL2 derived from a BCP-ALL without recurrent chromosomal translocations. Following transduction of 2 different lentiviral vectors encoding LKB1-specific short-hairpin RNA (shRNA), mRNA expression of LKB1 was reduced of 50% compared to control (cells transduced with a scramble shRNA vector). Western blot analysis confirmed in silenced cells the reduction of LKB1 also at protein level. We observed that after LKB1 silencing MLL-rearranged cells underwent apoptosis, while the viability of non-rearranged cells was similar to the control. Intriguingly, western blot analysis showed also the reduction of MO25, indicating the loss of the active LKB1 complex. Indeed MO25 is a LKB1 partner in the heterotrimeric active complex LKB1/STRADα/MO25 and functions as a scaffold protein that promotes LKB1 activity through stabilization of the complex. Also the expression of the other pathway components, MAPK and AMPK, was decreased by shRNA transduction.
Our conclusions are that LKB1 pathway activation directly contributes to the survival of MLL-rearranged leukemia cells since shRNA silencing induces apoptosis. Activated LKB1 very likely exerts its action through controlling mitotic spindle microtubules formation and stability.

A new perspective for LKB1 as pediatric MLL-rearranged B-ALL cells survival promoter

Galla L[1], Accordi B[1], Curtarello M[2], Milani G[1], Bresolin S[1], te Kronnie G[1], Indraccolo S[2], Basso G[1]

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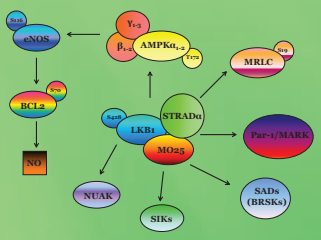
[2]Immunology and Diagnostic Molecular Oncology, IOV, Padua, Italy

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer. The development of effective therapies for children with ALL has greatly improved long-term event-free survival rates, but about 20% of patients is still not able to reach a stable, complete remission. Infants with ***MLL*** (Mixed Lineage Leukaemia) gene rearrangements are the most striking example of ALL patients who did not benefit from improved treatments (4-yr EFS 40%).

We used **Reverse Phase Protein Microarrays** (RPMA) to better map the biology of MLL disease and to identify aberrantly activated phosphoproteins specific for *MLL* rearranged patients.
Through RPMA studies, performed in MLL-rearranged B-ALL patients, we found an **hyperactivated pathway** that leads to BCL2 activation through LKB1, AMPK and eNOS phosphorylation (Accordi et al., Plos One 2010).



The LKB1 pathway is an energy sensing pathway activated in cells in response to low energy levels, linked to many fundamental cellular processes including regulation of cell proliferation, cell polarity, transcription and damage responses.

AIM

The discovery of the molecular signal pathway portrait in different subgroups of patients could play an important role in improving treatment outcome through tailored therapy. The goal of our studies was to evaluate the **effective role of the LKB1 pathway in supporting MLL-rearranged B-ALL cells survival.**

METHODS AND RESULTS

Gene Expression analysis of this pathway did not show differences between these two groups of patients. We investigated the presence of mutations in the LKB1 gene in different leukemia cell lines by sequence analysis and no mutation was found.

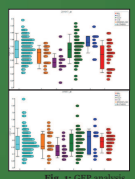


Fig. 1: GEP analysis.

We used different cell lines chosen as *in vitro* model: **SEM** and **RS4;11** as a model for MLL-rearranged leukemias; **MHH-CALL2** and **MHH-CALL4** as a model for leukemias without recurrent chromosomal translocations.
First of all we used Immunofluorescence and Western Blot staining for quantification and localization of LKB1 protein and pathway components.

Cell lines	Cell phenotype	Cytogenetics
SEM	Human B cell precursor leukemia (ALL)	Carries t(4;11)
RS 4;11	Human B cell precursor leukemia (ALL L2)	Carries MLL-AF4 fusion gene
MHH CALL 2/4	Human B cell precursor leukemia (cALL)	

Tab. 1: Cell lines.

Eyes Catching spot

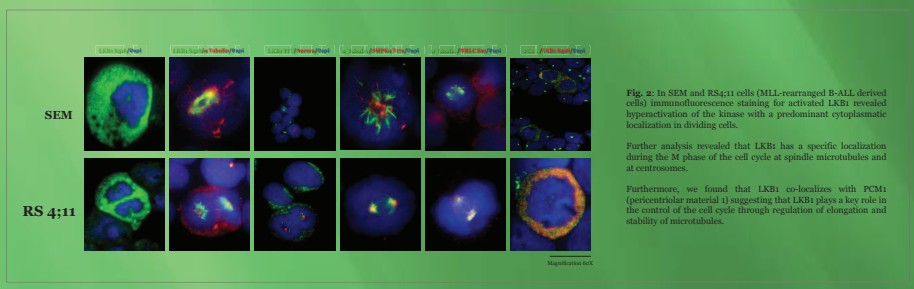


Fig. 2: In SEM and RS4;11 cells (MLL-rearranged B-ALL derived cells) immunofluorescence staining for activated LKB1 revealed hyperactivation of the kinase with a predominant cytoplasmatic localization in dividing cells.

Further analysis revealed that LKB1 has a specific localization during the M phase of the cell cycle at spindle microtubules and at centrosomes.

Furthermore, we found that LKB1 co-localizes with PCM1 (pericentriolar material 1) suggesting that LKB1 plays a key role in the control of the cell cycle through regulation of elongation and stability of microtubules.

Fig. 2: IF staining.

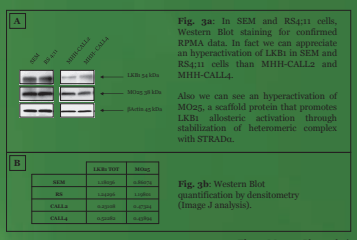


Fig. 3a: In SEM and RS4;11 cells, Western Blot staining for confirmed RPMA data. In fact we can appreciate an hyperactivation of LKB1 in SEM and RS4;11 cells than MHH-CALL2 and MHH-CALL4.

Also we can see an hyperactivation of MO25, a scaffold protein that promotes LKB1 allosteric activation through stabilization of heteromeric complex with STRADα.

Fig. 3b: Western Blot quantification by densitometry (Image J analysis).

Fig. 3: Western Blot analysis.

We silenced LKB1 with shLKB1 and we analysed the effective silencing with Western Blot analysis and RQ-PCR, we measured the induced apoptosis through Annexin V-PI staining, Western blot, cell cycle analysis and cytofluorimetric assays.

LKB1 silencing with shLKB1

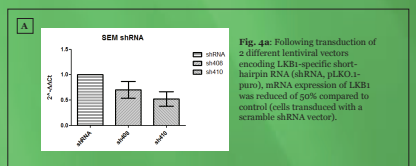


Fig. 4a: Following transduction of 2 different lentiviral vectors encoding LKB1-specific short-hairpin RNA (shRNA, pLKO.1-puro), mRNA expression of LKB1 was reduced of 50% compared to control (cells transduced with a scramble shRNA vector).

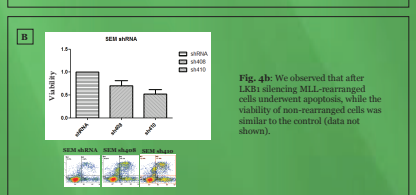


Fig. 4b: We observed that after LKB1 silencing MLL-rearranged cells underwent apoptosis, while the viability of non-rearranged cells was similar to the control (data not shown).

Fig. 4: LKB1 silencing.

Inactivation of the LKB1 pathway

Fig.5a: LKB1 pathway inactivation.
Western blot analysis confirmed in silenced cells the reduction of LKB1 also at protein level. Also the expression of the other pathway components, MAPK and AMPK, was decreased by shRNA transduction.
Under the figure of Western Blot the quantification by densitometry is report (Image J analysis).

Fig.5b : Intriguingly, western blot analysis showed also the reduction of MO25, indicating probably the loss of the active LKB1 complex. Indeed MO25 is a LKB1 partner in the heterotrimeric active complex LKB1/STRADα/MO25 and functions as a scaffold protein that promotes LKB1 activity through stabilization of the complex.
Under the figure of Western Blot the quantification by densitometry is report (Image J analysis).

Fig. 5: LKB1 pathway inactivation.

Cell Cycle

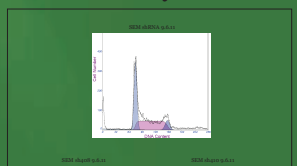


Fig.6: Cell cycle analysis revealed increases in G1 phase in the silencing cells (SEM sh408 and sh410), despite the S phase. This data harmonized with our hypothesis for which LKB1 have a key role for LKB1 in the control of the cell cycle.

Fig. 6: Cell cycle analysis.

CONCLUSIONS

Our conclusions are that LKB1 pathway activation directly contributes to the survival of MLL-rearranged leukemia cells since shRNA silencing induces apoptosis. Activated LKB1 very likely exerts its action through controlling mitotic spindle microtubules formation and stability.

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APPENDIX 2

OPEN ACCESS Freely available online

PLoS one

Functional Protein Network Activation Mapping Reveals New Potential Molecular Drug Targets for Poor Prognosis Pediatric BCP-ALL

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1 Oncohematology Laboratory, Department of Pediatrics, University of Padova, Padova, Italy, **2** Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, Virginia, United States of America, **3** Department of Biological Chemistry and Venetian Institute of Molecular Medicine (VIMM), University of Padova, Padova, Italy, **4** Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Roma, Italy

Abstract

Background: In spite of leukemia therapy improvements obtained over the last decades, therapy is not yet effective in all cases. Current approaches in Acute Lymphoblastic Leukemia (ALL) research focus on identifying new molecular targets to improve outcome for patients with a dismal prognosis. In this light phosphoproteomics seems to hold great promise for the identification of proteins suitable for targeted therapy.

Methodology/Principal Findings: We employed Reverse Phase Protein Microarrays to identify aberrantly activated proteins in 118 pediatric B-cell precursor (BCP)-ALL patients. Signal transduction pathways were assayed for activation/expression status of 92 key signalling proteins. We observed an increased activation/expression of several pathways involved in cell proliferation in poor clinical prognosis patients. MLL-rearranged tumours revealed BCL-2 hyperphosphorylation through AMPK activation, which indicates that AMPK could provide a functional role in inhibiting apoptosis in MLL-rearranged patients, and could be considered as a new potential therapeutic target. Second, in patients with poor clinical response to prednisone we observed the up-modulation of LCK activity with respect to patients with good response. This tyrosine-kinase can be down-modulated with clinically used inhibitors, thus modulating LCK activity could be considered for further studies as a new additional therapy for prednisone-resistant patients. Further we also found an association between high levels of CYCLIN E and relapse incidence. Moreover, CYCLIN E is more expressed in early relapsed patients, who usually show an unfavourable prognosis.

Conclusions/Significance: We conclude that functional protein pathway activation mapping revealed specific deranged signalling networks in BCP-ALL that could be potentially modulated to produce a better clinical outcome for patients resistant to standard-of-care therapies.

Citation: Accordi B, Espina V, Giordan M, VanMeter A, Milani G, et al. (2010) Functional Protein Network Activation Mapping Reveals New Potential Molecular Drug Targets for Poor Prognosis Pediatric BCP-ALL. PLoS ONE 5(10): e13552. doi:10.1371/journal.pone.0013552

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Competing Interests: Dr. Accordi, Dr. Basso, Dr. te Kronnie, Dr. Petricoin and Dr. Liotta have filed for a patent related to the AMPK pathway activation finding (Provisional Patent Application Number 61/064,692). The remaining authors declare no conflict of interest.

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Introduction

Acute Lymphoblastic Leukemia (ALL) is the most common form of pediatric cancer with a worldwide incidence of about 1–4.75 per 100 000 persons [1]. Remarkable progress has been made in treatment of childhood ALL but therapy is not yet effective in all cases. Current research interest focuses on identifying new specific molecular drug targets for new patient-tailored approaches that can improve therapy efficacy and reduce toxicity. Knowledge of deregulation of cell signalling pathways in cancer that regulate and control cell proliferation, differentiation, survival and death forms the basis for understanding tumour progression. Recent publications have placed elucidation of protein signalling pathways at the central point in the effective treatment of cancer [2,3]. Pathway activation and function is controlled by post-translational modifications, mainly by phosphorylation, underpinned by ongoing activity of protein kinases and phosphatases. Consequently, functional pathway mapping technology that can directly measure the activation state of hundreds of proteins in signalling transduction pathways (STPs), can hold great promise for the identification of altered STPs in tumour cells. Such efforts promise to potentially provide new targets for rational, molecular-targeted drug design and could identify cancer patients that may benefit from the use of specific targeted inhibitors [4,5]. Protein activation status can not be directly analyzed through gene expression profiling, since post-translational modifications, such as phosphorylation, are not predictable from gene expression levels.

Here, Reverse Phase Protein Microarray (RPMA) technology had been used to profile the activation state of 92 key molecules in a cohort of 118 newly diagnosed pediatric BCP-ALL patients, in order to identify and map pathway activation changes associated with clinical characteristics. This innovative technique can measure the activation levels/phosphorylation of large numbers of signalling proteins at once from small clinical samples in a very reproducible, precise, sensitive and high-throughput manner. The RPMA format immobilizes in spots dozens of different patient samples on one array and each array is then incubated with a specific antibody, thus a single endpoint is measured and directly compared across multiple samples without introduction of experimental variability. This cutting-edge technology has already been applied with success to profile the cellular STPs activity in several cancers [4–9]. We observed an increase or decrease in activation/phosphorylation state of signalling proteins within specific protein networks in clinical poor prognosis patients cohorts. In particular, here we show the inhibition of the LCK kinase in Prednisone Good Responder (PGR) patients, and a hyperactivated pathway in the MLL-rearranged cohort of patients that leads to BCL-2 activation through LKB1 and AMPK phosphorylation. Moreover, we found a correlation between CYCLIN E expression and Relapse Free Survival (RFS) rates: patients who show high levels of CYCLIN E expression have a more elevated probability to relapse. These new information on pediatric BCP-ALL activated protein patterns provided by phosphoproteomic analyses with RPMA will be the start for future functional studies with specific protein inhibitors, in order to point out new drugs for patient tailored therapies.

Results

Correlation between Protein Expression and Clinical Characteristics

We first searched for correlation between protein expression/activation and patients clinical characteristics. In particular we considered the followings: age (1–9 years vs >9 years), sex, white blood cell count (WBC > vs < of 50×10^9/L), DNA index (1–1.15 vs ≥1.16), chromosomal translocations (non-translocated, t(9;22), t(12;21), t(1;19) and MLL rearrangements), Minimal Residual Disease (Low Risk, Medium Risk, High Risk), immunophenotype (Prepre-B, Pre-B, CALL, Prepre-B/CALL) and prednisone response through Wilcoxon tests or two-sample Welch t-tests implemented in multtest package. No correlation was found between protein expression/activation and age, sex, WBC, DNA index, Minimal Residual Disease (MRD) and immunophenotype (data not shown), but we observed differentially activated/expressed proteins in MLL-rearranged vs non-translocated and in Prednisone Good Responder (PGR) vs Prednisone Poor Responder (PPR) patients comparisons.

AMPK Pathway is Hyperactivated in MLL-rearranged Patients

We compared primary leukemia samples isolated from 8 MLL-rearranged patients (5 with t(4;11), 2 with t(9;11), and one with t(11;19)) with 36 patients without known genomic aberrancies. Statistical analysis (Wilcoxon test with Benjamini-Hochberg multiplicity corrections) revealed different expression or activation of 9 proteins between the MLL-rearranged patients and the non-translocated ones. Our results show that 4 proteins were statistically significantly elevated in the MLL-rearranged patients group: CYCLIN E ($p = 0.02425$), ANNEXIN 2 ($p = 0.02910$), AMPKβ (S108) ($p = 0.02910$) and AMPKα (S485) ($p = 0.03686$). Furthermore a set of 3 more proteins, eNOS/NOS III (S116 – corresponding to S114 in human), LKB1 (S428) and BCL-2 (S70), was found to be differentially activated in the MLL-rearranged cohort using a Global Test analysis ($p = 0.003$) (**Figure 1A**). These 3 proteins are all known members of the AMPK pathway (see Discussion) and, along with the findings that AMPK itself was activated in the MLL-rearranged cohort, form the basis of a comprehensive pathway derangement in MLL-rearranged patients (presented schematically in **Figure 1B**). We thus identified a singular MLL-specific hyperactivated pathway that through

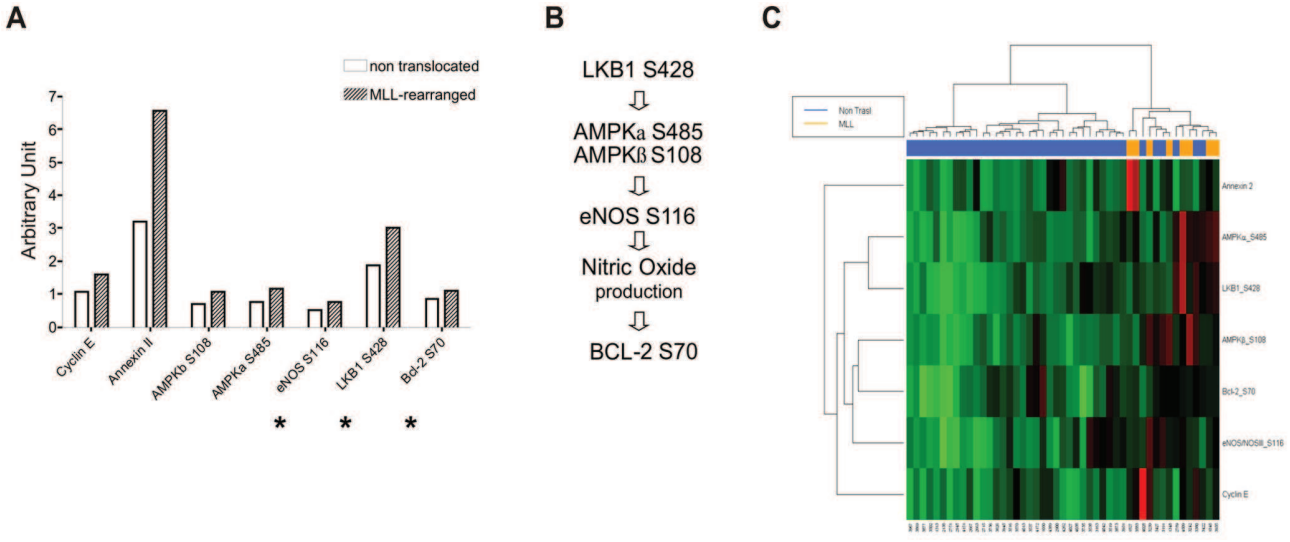

Figure 1. Hyperactivation of the AMPK pathway in MLL-rearranged patients. (A) Histogram of proteins, that are part of the AMPK pathway, found to be differentially activated (Wilcoxon test with Benjamini-Hochberg multiplicity correction, $p<0.05$) between the MLL- and non-translocated cohorts. * indicates proteins that are part of the AMPK pathway, but that did reach statistical significance using Global Test analysis ($p = 0.003$). (B) Scheme of the AMPK pathway. (C) Heatmap with hierarchical clustering. The heatmap was generated with R Project using the proteins differentially expressed/phosphorylated in the "MLL-rearranged patients" group vs "non-translocated patients" group comparison. MLL-rearranged patients are highlighted in orange.
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AMPK phosphorylation leads to the activation of BCL-2. A heatmap was generated to highlight the relationships between clustering and protein expression levels (**Figure 1C**). RPMA results were validated by Western Blot in an independent set of patients (**Figure 2A**). Of note, total forms of the AMPK pathway proteins do not show substantial differences between MLL-rearranged and non-translocated patients (**Figure 2B**), corroborating the observation that the higher phosphorylation levels of the proteins in the AMPK pathway are the peculiar molecular derangement characteristic of MLL-rearranged BCP-ALL.

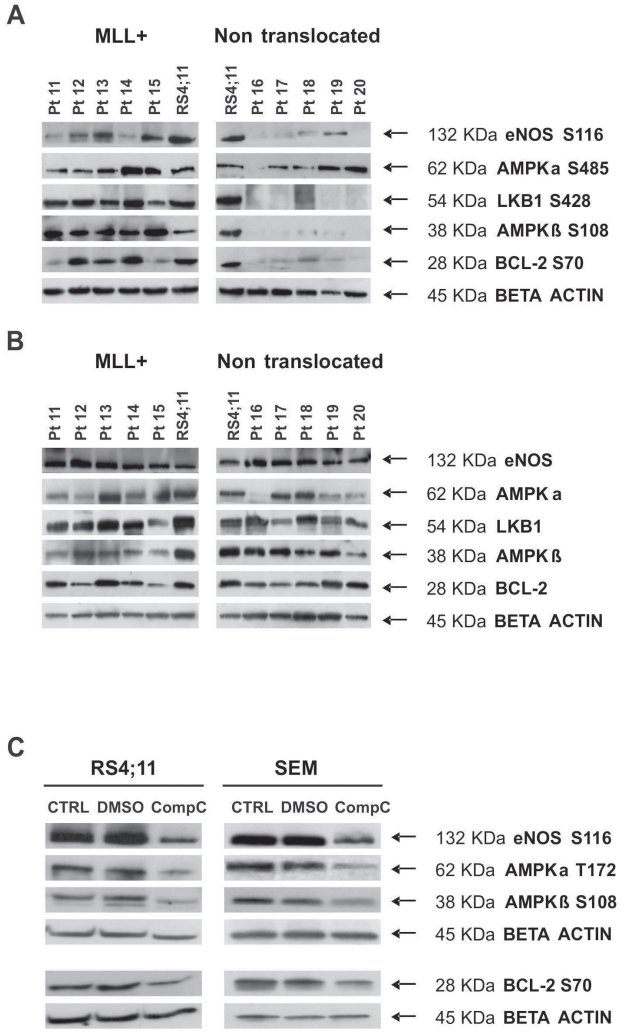

Figure 2. Validation of RPMA results through Western Blot. (A) Hyperactivation of the AMPK pathway in MLL-rearranged patients vs non-translocated ones (independent sets of pediatric BCP-ALL at diagnosis: patients 11–15 are MLL-rearranged -all MLL-AF4-, patients 16–20 are non-translocated). RS4;11 cell lysate was used as positive control for antibody staining. (B) Total forms of the AMPK pathway proteins in previously described patients: 11–15 are MLL-rearranged and 16–20 are non-rearranged. RS4;11 cell lysate was used as positive control for antibody staining. There are no substantial differences on total protein form levels between MLL-rearranged and non-translocated patients. (C) AMPK pathway inhibition after Compound C treatment. RS4;11 and SEM cells (both MLL-rearranged) were treated with the AMPK inhibitor Compound C 8 μM for 48 hours. Phosphorylation of AMPK pathway proteins was evaluated through WB in control, DMSO treated and Compound C treated cells.
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Additionally, we asked whether it was possible to identify a difference in the gene expression levels of the same AMPK-related genes that we identified through RPMA analysis among MLL-rearranged and non-translocated patients. We analyzed the gene expression profiles of 29 MLL-rearranged and 41 non-translocated pediatric BCP-ALL patients. All the patients included in this analysis were part of a larger cohort of samples analyzed by gene expression profiling during the international "Microarray Innovation in Leukemia" (MILE) study [10]. The unsupervised analysis with the 15 probe sets corresponding to Lkb1, Ampkα and β, eNos and Bcl-2 genes was not able to accurately separate MLL-rearranged and non-translocated patients (**Figure S1A**). Moreover, when performing a comparative analysis between MLL-rearranged and non-translocated patients using the 15 probe sets of the AMPK-related genes, only one probe set (PRKAA 214917_at, corresponding to Ampkα) resulted to be differentially expressed between the two groups with a fold change more than 2.0 (**Figure S1B**). It is of note that this probe set resulted to be upregulated in the non-translocated group of patients.

In order to verify the kinase-substrate relationships within the AMPK pathway, we treated the two MLL-rearranged cell lines SEM and RS4;11 with the commercial AMPK inhibitor Compound C. As shown in **Figure 2C**, after AMPK inhibition the activation levels of AMPKα and β and all the downstream targets are markedly decreased, confirming the functional link between these proteins. In addition, apoptosis is induced in MLL-rearranged cell lines after Compound C treatment (LC50 8 μM, 48 h), while other two human non-translocated BCP-ALL cell lines are insensitive to AMPK inhibition (data not shown).

Our data provide evidence that in MLL-rearranged patients a number of directly connected kinase-substrates are activated, and this can contribute to the chemotherapy resistance observed in these patients.

LCK Activity is Down-Modulated in Prednisone Good Responder Patients

We compared the phosphoproteomic profile of 9 Prednisone Poor Responder (PPR) patients vs 109 Prednisone Good Responder (PGR) patients. Statistical analyses (two-sample Welch t-statistics -unequal variances- with Bonferroni multiplicity corrections) revealed that in PGR patients the inhibited form of the kinase LCK (LCK phosphorylated at Y505) was higher than in PPR patients ($p = 0.0388$) (**Figure 3A**). We confirmed the overexpression of the inhibited form of LCK in PGR patients by Western Blot in an independent set of patients (**Figure 3B**).

In order to provide the biochemical evidence of an hyperactivation state of LCK in PPR cells compared to PGR cells, we performed a radioactive autophosphorylation assay of LCK. To this purpose we selected REH cells as a model for PPR patients and NALM6 cells as a model for PGR patients [11]. We first confirmed that LCK is more phosphorylated in Y505 in NALM6 cells than in REH cells (**Figure 3C**): when similar amounts of total LCK were analyzed, the LCK Y505 phosphorylation was evident almost exclusively in NALM6 cells. As expected, on the contrary, the autophosphorylation activity of immunoprecipitated LCK was significantly higher in REH cells (**Figure 3D**).

This tyrosine-kinase, important in the regulation of growth and differentiation of eukaryotic cells, resulted to be more activated in cells that do not respond to glucocorticoids treatment, and thus activation of LCK could be considered as a putative marker for prednisone resistance.

CYCLIN E is Upregulated in Relapsed Patients

We performed a Relapse Free Survival (RFS) analysis considering patients included in AIEOP LLA 88, 91, 95 and

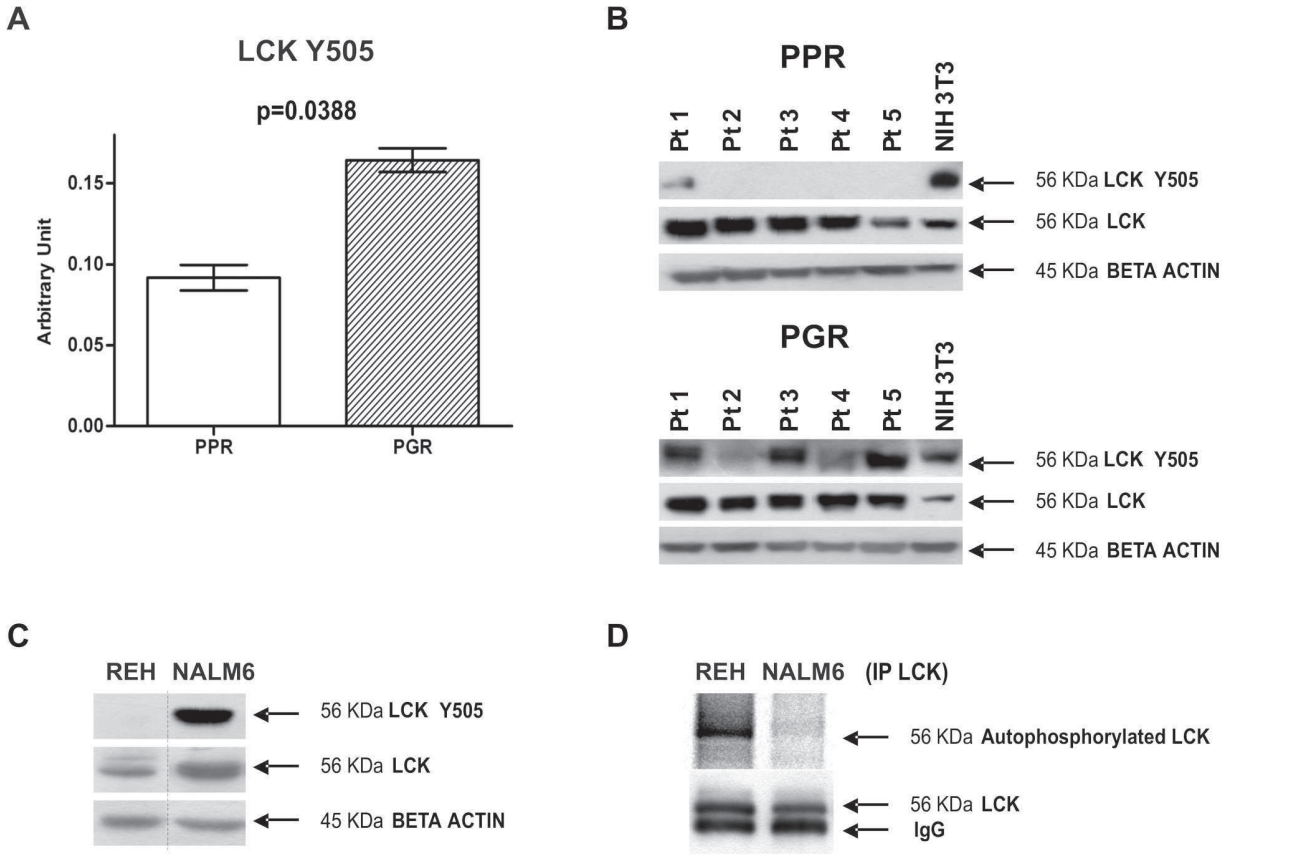

Figure 3. Hyperactivation of LCK in PPR patients. (A) LCK Y505 measured with RPMA is higher in Prednisone Good Responder (PGR) (0.164±0.007) than in Prednisone Poor Responder (PPR) (0.092±0.008) patients (two-sample Welch t-statistics -unequal variances- with Bonferroni multiplicity corrections p = 0.0388). (B) LCK hyperphosphorylation at Y505 in PGR patients was confirmed by Western Blot in an independent set of specimens (pediatric BCP-ALL at diagnosis: patients 1–5 are Prednisone Good Responders, patients 6–10 are Prednisone Poor Responders). NIH3T3 commercial cell lysate (BD Biosciences) was used as positive control for antibody staining. Expression of the total form of LCK does not differ between the patients. (C) LCK activation state in the cell lines chosen as *in vitro* model. REH cell lines are a model for PPR patients, while NALM6 are a model for PPR patients. The LCK activation state was evaluated through WB. (D) In vitro LCK autophosphorylation assay. LCK was immunoprecipitated from REH and NALM6 cells, and then incubated in a phosphorylation mixture at 30°C for 40 min. Autophosphorylation of LCK was analyzed by digital autoradiography on Cyclone Plus (upper gel), and by WB with anti-LCK antibody to assess the LCK amount (lower gel).
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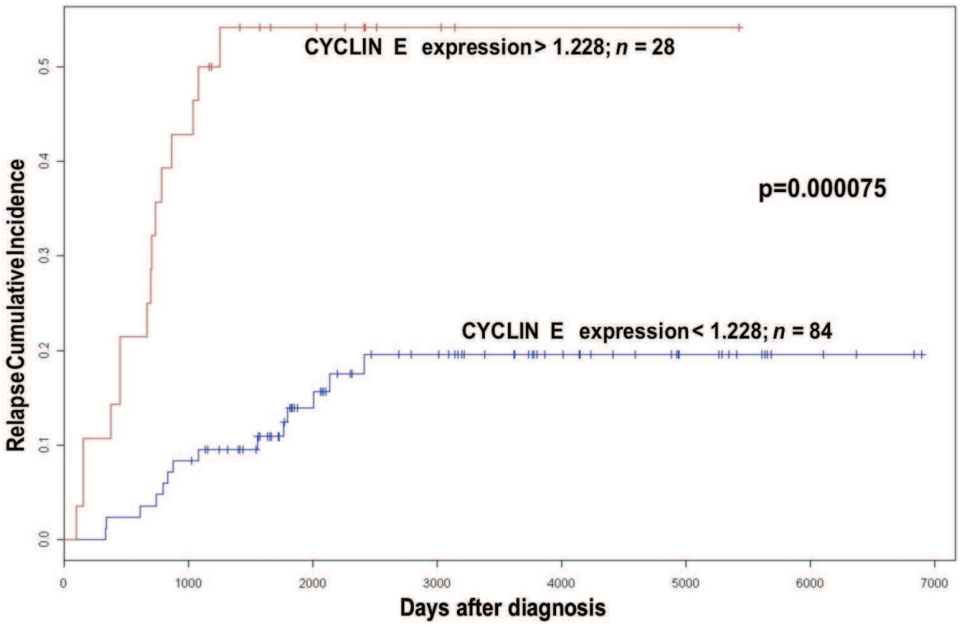
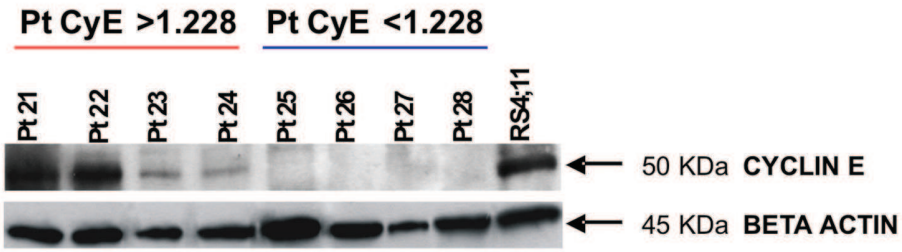
2000 therapy protocols in order to identify proteins related to a more aggressive disease. Relapse Cumulative Incidence were obtained through Kaplan-Meier estimates and the difference between two curves was assessed by log rank test. CYCLIN E, that regulates cell cycle steps critical for growth control, had been demonstrated to be overexpressed in many malignant tumours (for a review see Möröy et al. [12]), and in particular it could be useful to assess malignancy of blasts in adult B- and T-ALL [13]. Similarly to van Rhenen et al. [14] we thus searched for a threshold value for CYCLIN E that resulted in the largest difference in survival between the two groups defined by that threshold. We considered 10 equispaced cutoff values between the first quartile and the third quartile. A threshold was deemed valid only if the difference in the Relapse Cumulative Incidence curves was statistically significant (CYCLIN E threshold 1.228, log rank test after Bonferroni corrections p = 0.000075) (**Figure 4A**). We found that patients with CYCLIN E levels higher than 1.228 have a higher probability to relapse: 15 of 28 (53%) patients with CYCLIN E higher than 1.228 relapsed, while among the 84 patients with CYCLIN E lower than 1.228 only 14 (17%) relapsed. We confirmed RPMA CYCLIN E levels by Western Blot (**Figure 4B**). Moreover, we observed that 6 of 7 MLL-rearranged patients and 3 of 3 patients with t(9;22) are in the high CYCLIN E expression group. Furthermore, 16 of 19 patients MRD-Standard Risk have low levels of CYCLIN E, and 4 of 5 patients MRD-High Risk have high levels of CYCLIN E. In the groups determined by the above threshold we searched for differences in age, sex, immunophenotype, chromosomal translocations, DNA index, WBC count, prednisone response and MRD. From this multivariate analysis, CYCLIN E expression resulted independent from all other considered variables. We also compared CYCLIN E expression in early vs late relapsed patients. By definition, early relapsed patients are those patients who suffered of a relapse within 6 months after stopping front-line treatment, and they show a worst prognosis with respect to late relapsed patients [15]. CYCLIN E resulted significantly higher in early relapsed patients (Wilcoxon test p = 0.002) (**Figure 4C**). Because CYCLIN E in conjunction with its kinase subunit CDK2 regulates essential processes for entering into the S-phase, we also looked if differences between relapsed and non-relapsed patients could be associated to CYCLIN E together with CDK2. After Global Test analysis, the two proteins resulted to be correlated (p = 0.009395) in our patients cohort. These

A


CYCLIN E expression> 1.228; n = 28
p=0.000075
CYCLIN E expression< 1.228; n = 84
Relapse Cumulative Incidence
Days after diagnosis
0.0
0.1
0.2
0.3
0.4
0.5
0
1000
2000
3000
4000
5000
6000
7000

B


Pt CyE >1.228
Pt CyE <1.228
Pt 21
Pt 22
Pt 23
Pt 24
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Pt 27
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RS4;11
50 KDa CYCLIN E
45 KDa BETA ACTIN

C

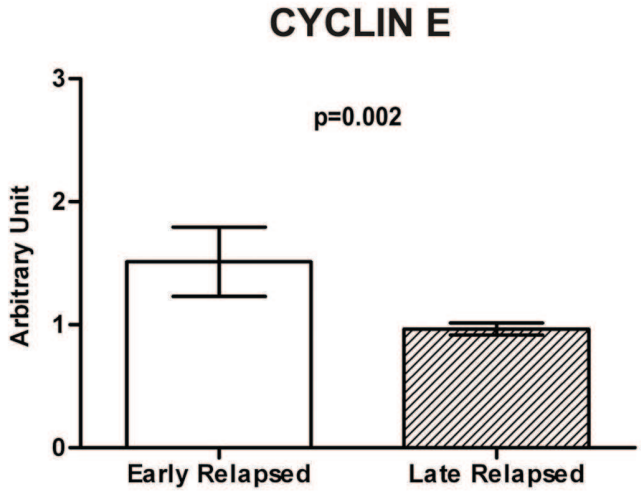
CYCLIN E
p=0.002
Arbitrary Unit
0
1
2
3
Early Relapsed
Late Relapsed

Figure 4. CYCLIN E expression and Relapse Cumulative Incidence. (A) Relapse Cumulative Incidence comparison between patients with CYCLIN E expression levels <1.228 (n = 84, blue line) and patients with CYCLIN E levels >1.228 (n = 28, red line). Patients with elevated CYCLIN E levels have a higher probability to relapse (CYCLIN E threshold 1.228, log rank test after Bonferroni corrections p = 0.000075). (B) RPMA results validation with Western Blot. Patients 21–24 showed high levels of CYCLIN E after RPMA analysis: 1.974, 1.896, 1.537, 1.506 respectively. Patients 25–28 showed low levels of CYCLIN E after RPMA analysis: 0.463, 0.470, 0.298, 0.292 respectively. RS4;11 cell lysate was used as positive control for antibody staining. (C) Early relapsed patients (relapsed within 910 days from diagnosis) show higher levels of CYCLIN E expression with respect to late relapsed patients (Wilcoxon test p = 0.002).
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observations taken together strongly suggest that CYCLIN E could be considered as a marker for the aggressiveness of the disease.

Discussion

Despite dramatic improvements in leukemia therapy over the last decades, about 20% of patients does not achieve a stable complete disease remission. In the past few months, new findings using genome-wide mutational analysis have placed central importance on protein pathway deregulation as the principal driving force in many human malignancies [2,3]. We have originated the RPMA technology to directly quantitatively measure protein pathway function and activation to broadly map signal transduction networks and profile human cancers in order to identify groups of patients with specific molecular aberrations and to identify new targets for therapy. In this study we used the RPMA global pathway mapping technique in pediatric BCP-ALL patients to map the pathway activation status of 92 different phosphorylated and total proteins of key signalling "hubs" known to be involved in human tumourigenesis and metastasis. Correlations of signalling activation with clinical response/follow-up and known genetic information (e.g. gene rearrangements) enabled us to identify new protein pathway biomarkers that, when validated in larger clinical sets, could be used for patient stratification and targeted therapy trials.

Our first main finding concerns infants with MLL (Mixed Lineage Leukemia) rearrangements. MLL translocations are present in about 6% of pediatric leukaemia patients, especially in infants with ALL where about 75% of patients are MLL-rearranged, and their presence predicts early relapse and poor prognosis (event-free survival of 28–45%) [16,17]. We identified a singular MLL-specific hyperactivated pathway that through AMPK phosphorylation leads to the activation of BCL-2, a well known anti-apoptotic regulator crucial for chemotherapy resistance already found to be over-expressed both at mRNA and protein levels in MLL-rearranged leukemias [18]. This pathway derangements appear to emanate from LKB1, a serine/threonine kinase that has been shown to phosphorylate AMPK [19]. AMP-activated protein kinase (AMPK) is a serine/threonine kinase that acts as a cellular fuel sensor, activated under conditions that deplete ATP and elevate AMP levels such as metabolic or environmental stresses. AMPK is known to activate endothelial nitric oxide synthase (eNOS) [20,21] and thus to stimulate Nitric Oxide (NO) production. NO is a multifunctional transcellular messenger that can play a dual role in cancer with both pro- and anti-apoptotic effects [22,23]. Interestingly, a prominent NO production has been observed in undifferentiated tumours [24] such as the MLL-rearranged leukemias. In addition, it has been reported that NO can prevent apoptosis by elevating BCL-2 expression both at mRNA and protein levels in B-lymphocytes [25,26]. We showed that treatment of two MLL-rearranged cell lines with the AMPK inhibitor Compound C not only brings to AMPK deactivation, but also of its described downstream targets. This confirms the kinase-substrate relationship between these proteins, and highlights the essential contribute of AMPK in sustaining the activation of this pathway. Activation of AMPK had been studied in several tumour types because it usually leads to antitumour effects [27], but we found AMPK phosphorylation to be at the highest relative levels in the MLL-rearranged subgroup. We speculate that the role of AMPK pathway in leukemias and other hematological tumours may be different from solid epithelial malignancies, as already reported by Baumann *et al.* [28] in multiple myeloma cells. Indeed, it will be of primary interest to inhibit AMPK, and thus BCL-2, activity using commercially available AMPK inhibitors in order to more fully elucidate the functional role of AMPK activation in MLL-rearranged patients, and to evaluate AMPK as a potential new therapeutic target for this specific subgroup of patients. The mRNA results, indicating that the AMPK pathway genes are not upregulated in MLL-rearranged patients, sustain the importance to deepen with protein activation analyses in order to better define disease-related disorders in cellular metabolism, and thus to identify new molecular drug targets.

One of the strongest independent predictive factors for therapy outcome in childhood ALL is the response to initial prednisone treatment. Prednisone response is considered a backbone in Berlin-Frankfurt-Münster (BFM)-oriented protocols, and is defined by the number of peripheral leukemic blasts on day 8 of therapy [29,30]. The threshold value for distinction between good and poor response is 1000 blasts/μl. In trial ALL-BFM 90, PGR patients showed a 6-years Event Free Survival (EFS) of 82%, while for PPR patients this was only 34% [31]. Here, we observed that in PGR patients LCK is less activated than in PPR patients (LCK Y505, p = 0.0388), and we exploited the autophosphorylation assay of LCK in order to biochemically confirm its higher activity in PPR cells. LCK is a non receptor protein-tyrosine kinase of the Src oncogene family mostly expressed in T cells, where it plays an essential role in activation and development, and in some B cells and other cancer tissues [32,33]. Its activity is primarily regulated by phosphorylation, catalyzed by the kinase CSK, at the tyrosine residue Y505, located near the C-terminus, leading to protein deactivation [34]. In the future, if ongoing validation continues to implicate LCK activation as a predictive marker for prednisone resistance, we will investigate the causal significance of this and possibly implicate this molecule as a therapeutic target that could modulate prednisone response mechanisms. Very interestingly, inhibition of LCK through treatment with kinase inhibitors currently used in clinical practice for other indications such as BMS-354825 (Dasatinib) and STI-571 (Imatinib) [35] had already been demonstrated to be able to induce apoptosis in human T cells. Thus, it will be of interest to establish if LCK inhibitors could be useful as a possible additional support in BCP-ALL PPR patients treatment.

Our third main result is that high levels of CYCLIN E expression are indicator of a more aggressive disease. Patients who show elevated CYCLIN E expression have a higher probability to relapse. CYCLIN E had been demonstrated to be overexpressed in many malignant tumours (for a review see Möröy *et al.* [12]), and, in particular, Scuderi *et al.* [13] reported that BCP-ALL blasts of adult patients had high CYCLIN E levels and relapsed samples displayed additional accumulation of the protein. CYCLIN E

regulates cell cycle progression through the restriction point R, at the end of the G1-phase, to allow cells to enter S-phase inducing S-phase specific genes. The restriction point R has been recognized to be critical for growth control and thus also for the prevention of unrestricted cell proliferation, malignant transformation and tumourigenesis. We found a cutoff value of CYCLIN E expression able to distinguish patients who have a higher probability to relapse (53% vs 17%), and this is independent from all other clinical and molecular variables. Interestingly, the elevated CYCLIN E expression group is anyhow enriched in poor prognosis patients (MLL-rearranged, t(9;22) translocated and MRD-High Risk), and CYCLIN E is more expressed in early relapsed (within 910 days from the diagnosis) patients who have a worst prognosis [15] with respect to late relapsed patients. This indicates that CYCLIN E expression could correlate with the malignant potential of the cells, and thus could be considered as a marker of the aggressiveness of the disease and a new therapeutic target in pediatric BCP-ALL.

This study emphasizes the importance of protein pathway activation mapping analysis of clinical specimens as a route for the discovery of functional derangement that may be functional, causative agents of the cancer. Proteins related to proliferation and survival such as LCK, AMPK and CYCLIN E were found to be hyperactivated or overexpressed in poor prognosis patients with BCP-ALL, and could represent new molecular drug targets in pediatric B-ALL. When further validated in functional studies, specific kinase inhibitors that target AMPK pathway, LCK-mediated signalling and CYCLIN E activation could be evaluated in prospective clinical trials whereby patients who are in need of better therapeutic options could be selected and stratified for targeted therapeutics tailored to the molecular defect.

Materials and Methods

Ethics Statement

The study was approved by the Ethical Committee board of the University of Padova, the Padova Academic Hospital and the Italian Association of Pediatric Onco-Hematology (AIEOP). Patient's parents or their legal guardians provided written informed consent following the tenets of the Declaration of Helsinki.

Patients

Bone marrow samples from 118 children with newly diagnosed BCP-ALL were analyzed. Diagnosis was made according to standard cytomorphology, cytochemistry and immunophenotypic criteria [36]. The study was approved by the local ethics committees and informed consent was obtained for all patients. Samples were collected at the Pediatric Oncohematology Laboratory (Padova, Italy), between 1990 and 2006 and stored in the BioBank in liquid nitrogen in FCS+DMSO. Bone marrow mononuclear cells from patients were separated by Ficoll-Hypaque technique (Pharmacia, Uppsala, Sweden) and frozen within 3 hours after collection. The whole blood blast percentage for all samples was between 70% and 98%. Patients molecular and clinical characteristics are resumed in **Table S1**.

Cell lines

Human leukemia cell lines SEM, RS4;11, REH and NALM6 were purchased from DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). SEM and RS4;11 cell lines derive from BCP-ALLs carrying the t(4;11) MLL-AF4 translocation. REH cell line derives from a BCP-ALL carrying the t(12;21) TEL-AML1 translocation. NALM6 cells derive from a BCP-ALL without recurrent chromosomal translocations. Cells were cultured in RPMI 1640 (Biochrom AG, Berlin, Germany) with 10% FCS, penicillin (100U/ml) (GIBCO, Invitrogen Life Technologies, Carlsbad, CA) and streptomycin (100 μg/ml) (GIBCO), and maintained at 37°C in a humidified atmosphere with 5% CO_2.

Reverse Phase Protein Microarrays

Cell Lysis. Cells were washed with ice–cold PBS 1X and lysed on ice for 20minutes in an appropriate lysis buffer: TPER Reagent (Pierce, Rockford, IL), 300 mM NaCl, 1 mM Na orthovanadate, 200 mM PEFABLOC (AEBSF) (Roche, Basel, Switzerland), 1ug/mL Aprotinin (Sigma, St. Louis, MO), 5 mg/mL Pepstatin A (Sigma), 1 mg/mL Leupeptin (Sigma). Cell lysates were then cleared by centrifugation and supernatants were collected and assayed for protein concentration with the Coomassie Protein Assay Reagent Kit (Pierce). Cell lysates were diluted to 1 mg/ml in a mixture of 2X Tris-Glycine SDS Sample Buffer (Invitrogen Life Technologies) plus 5% of β-Mercaptoethanol. Lysates were stored at −80°C and boiled for 8minutes immediately prior to arraying.

RPMA Printing. Lysates were loaded into a 384-well plate and serially diluted with lysis buffer into four-point dilution curves ranging from undiluted to 1:8. As positive controls for antibody staining we added also 3 commercial cell line lysates: A431+EGF, Hela+Pervanadate and Jurkat Apoptotic cell lysates (BD Biosciences, Franklin Lakes, NJ). We divided the 118 samples in 2 set of arrays, thus 59 and 59 samples were printed in duplicate in each array set onto nitrocellulose-coated slides (FAST slides, Whatman Schleicher & Schuell, Florham Park, NJ) with the 2470 Arrayer (Aushon BioSystems, Burlington, MA). On each set of arrays also the above mentioned cell lines and 2 bridge samples were printed for antibody signal normalization between the 2 sets. Printed slides were stored desiccated (Drierite, Sigma) at −20°C until use.

RPMA Staining. Selected slides were stained with Sypro Ruby (Invitrogen Life Technologies) according to the manufacturer's instruction, in order to estimate the total protein amount of each printed sample. Before antibody staining the arrays were treated with ReBlot Plus Mild Antibody Stripping Solution (Chemicon, Temecula, CA) 1X for 15minutes at room temperature, rinsed 2 times for 5minutes in PBS 1X, and then blocked for 1 hour at room temperature in blocking solution (2gr I-Block - Tropix, Bedford, MA - and 0.1% Tween-20 in 1l of PBS 1X). Blocked arrays were stained with antibodies on an automated slide stainer (Dako Autostainer Plus, Dako Cytomation, Carpinteria, CA) using the CSA kit (Dako Cytomation) as described previously [6]. Slides were air dried and scanned on a PowerLook 1000 flatbed scanner (UMAX, Dallas, TX) at 600dpi. For an example of antibody-stained slides please see **Figure S2.**

For the complete list of the 92 stained antibodies with RPMA, please see **Table S2**. Each antibody was previously subjected to extensive validation for single band specificity by Western Blot (WB). For phospho-specific antibodies, each antibody was checked for specificity using cell extracts with and without appropriate ligand induction. The 92 antibodies used in this study were carefully selected based on both their extensive validation for specificity as well as detecting key signalling molecules known for their involvement in motility, invasion, pro-survival, and growth factor signalling.

Image Analysis. The TIF images of antibody- and Sypro Ruby-stained slides were analyzed using Microvigene Software (VigeneTech Inc, Boston, MA) to extract numeric intensity values from the arrays images. Briefly this software, specifically developed

for RPMA analysis, localizes the spots and subtracts the local background, calculating pixel intensity for each spot. The software calculates these values in the antibody-stained slides, the corresponding negative control slides (secondary antibody alone) and the total protein slide. Then, for each sample, the signal of the negative control array is subtracted from the antibody slide signal and then the resulting value is normalized to the total protein value, to ensure that intensity values were not dependent on changes in concentration of printed lysate. The data values were normalized to either one of the bridge cases to facilitate comparison of sample values between paired arrays stained with the same antibody. The data processing generates a normalized single value for each protein measured for each sample.

Western Blot

The following antibodies were used for WB at the concentrations reported in parentheses. Primary antibodies: anti-AMPKα (23A3) (1:1000), anti-phospho-AMPKα S485 (1:1000), anti-AMPKß1 (1:1000), anti-phospho-AMPKα T172 (1:1000), anti-AMPKß1 (1:1000), anti-phospho-AMPKß S108 (1:1000), anti-BCL-2 (1:1000), anti-phospho-BCL-2 S70 (1:1000), anti-LCK (L22B1) (1:1000), anti-LCK Y505 (1:500), anti-β-ACTIN (1:1000) (all from Cell Signaling Technology, Inc, Danvers, MA), CYCLIN E (BD Biosciences) (1:500), anti-eNOS/NOS III CT (1:1000), anti-phospho-eNOS/NOS III S116 (both from Upstate – Millipore, Billerica, MA) (1:1000). Secondary antibodies: HRP-Goat anti-rabbit and anti-mouse IgG-conjugate (Zymed Laboratories, Inc., South San Francisco, CA) (1:50000). Total cell lysates were analyzed by SDS-PAGE under reducing conditions, and transferred to a nitrocellulose sheet (Hybond-P, GE Healthcare, Chalfont St. Giles, UK) following standard methods. Membranes were saturated for 3 hours with 2% Amersham ECL Advance Blocking Reagent (GE Healthcare), primary antibodies were incubated overnight at 4°C and secondary antibodies for 1 hour at room temperature. The immunoreactivity was determined by an enhanced chemiluminescent reaction (Amersham ECL ADVANCE Western Blotting Detection Kit, GE Healthcare). For the stripping, membranes were incubated for 30minutes in constant rocking in a solution 25 mM Glycin, 1% SDS and pH2, then washed in T-PBS 1X and resaturated.

AMPK inhibition in MLL-rearranged cell lines

AMPK was specifically inhibited in SEM and RS4;11 cell lines using Compound C (Calbiochem, Darmstadt, Germany) 8 μM for 48 hours. Proteins were extracted from treated and control cells as described for RPMA. Of note, in order to carefully determine the activation level of AMPKα, in these experiments we measured the phosphorylation of the main activation site that is the T172.

Immunoprecipitation and *in vitro* kinase assay

REH and NALM6 cell lines were chosen as *in vitro* model for PPR and PGR patients respectively. Cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% (v/v) Triton X-100, 2 mM dithiothreitol, protease inhibitor cocktail Complete (Roche), 10 mM NaF, 1 μM okadaic acid, 1 mM Na orthovanadate. LCK was immunoprecipitated with limiting amount (20 ng) of anti-LCK 3A5 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), for 4 h at 4°C, followed by addition of protein A-Sepharose (Sigma). 100 μM Na orthovanadate was present throughout the incubations. Immunoprecipitates were washed once with NET buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.05% (v/v) Nonidet P-40, 2 mg/ml bovine serum albumin) and twice with 50 mM Tris–HCl pH 7.5, then incubated at 30°C for 40minutes with a phosphorylation mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$ 10 μM [γ-^{33}P]ATP (specific radioactivity ~5000cpm/pmol) and 50 μM Na orthovanadate, in a total volume of 20 ml. Samples were then boiled for 5minutes, loaded onto 11% SDS–PAGE followed by blotting to Immobilon-P membranes (Millipore); autophosphorylation of LCK was analyzed by digital autoradiography on Cyclone Plus (PerkinElmer, Waltham, MA) to detect the radioactivity and by WB with anti-LCK to assess the LCK amount.

Statistical Analysis

Statistical analyses were performed with R. Identification of activated proteins was obtained through Wilcoxon tests or two-sample Welch t-tests implemented in multtest package [37]. Pearson's chi-squared test was used for clinical variables. Pathways were identified using global test [38]. P-values were corrected for multiplicity using Benjamini-Hochberg method to control false discovery rate or with Bonferroni method to control family wise error rate; therefore the reported p-values are adjusted p-values. Survival curves were obtained through Kaplan-Meier estimates and the difference between two curves was assessed by log rank test. Finally, a heatmap was generated to highlight the relationships between clustering and protein expression levels.

Supporting Information

Figure S1 AMPK-related genes are not upregulated in MLL-rearranged patients. (A) Heatmap generated with Partek Genomics Suite software using the probe sets corresponding to Lkb1, Ampkα and β, eNos and Bcl-2 genes. The unsupervised analysis is not able to accurately separate MLL-rearranged and non-translocated patients. MLL-patients are highlighted in red. (B) Dot Plot representing the raw expression data of PRKAA probe set (214917_at). The comparative analysis, performed with Partek Genomics Suite software, between MLL-rearranged (red) and non-translocated (blue) patients, using the 15 probe sets of the AMPK-related genes, shows only one probe set (PRKAA, corresponding to Ampkα) differentially expressed between the two groups with a fold change more than 2.0. AMPKα results upregulated in the non-translocated patients. Each dot represents a patient, and the boxes represent the median expression values for each group of specimens. The expression values on y axis are reported on log2 scale.
Found at: doi:10.1371/journal.pone.0013552.s001 (9.54 MB TIF)

Figure S2 Example of RPMA stained slides. Slides in the picture are stained with Lck Y505 antibody. Each patient lysate was printed in a four-point dilution curve ranging from undiluted to 1:8 in duplicate (an example is framed in red). Samples were divided in 2 set of arrays, thus 59 and 59 samples were printed in duplicate in each array set onto nitrocellulose-coated slides. As positive controls for antibody staining, in the right lower part of the slides we added 3 commercial cell line lysates: A431+EGF, Hela+Pervanadate and Jurkat Apoptotic cell lysates. On each set of arrays the above mentioned cell lines and 2 bridge samples were used for antibody signal normalization between the 2 sets.
Found at: doi:10.1371/journal.pone.0013552.s002 (7.47 MB TIF)

Table S1 Patients clinical and molecular characteristics.
Found at: doi:10.1371/journal.pone.0013552.s003 (0.05 MB DOC)

Table S2 Primary antibodies for RPMA staining.
Found at: doi:10.1371/journal.pone.0013552.s004 (0.12 MB DOC)

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Author Contributions

Conceived and designed the experiments: BA VE MR GtK EP LL GB. Performed the experiments: BA AV GM LG MS LT. Analyzed the data: BA MG. Wrote the paper: BA MG MR GtK EP. Interpreted the data: BA VE MR GtK EP LL GB. Final approval: VE MR RDM GtK EP LL GB.

References

1. Redaelli A, Laskin BL, Stephens JM, Botteman MF, Pashos CL (2005) A systematic literature review of the clinical and epidemiological burden of acute lymphoblastic leukaemia (ALL). Eur J Cancer Care (Engl) 14: 53–62.
2. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, et al. (2008) Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 321: 1801–1806.
3. Cancer Genome Atlas Research Network (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 455: 1061–1068.
4. Liotta LA, Espina V, Mehta AI, Calvert VS, Rosenblatt K, et al. (2003) Protein microarrays: meeting analytical challenges for clinical applications. Cancer Cell 3: 317–325.
5. Petricoin EF, 3rd, Bichsel VE, Calvert VS, Espina V, Winters M, et al. (2005) Mapping molecular networks using proteomics: a vision for patient-tailored combination therapy. J Clin Oncol 23: 3614–3621.
6. Wulfkuhle JD, Aquino JA, Calvert VS, Fishman DA, Coukos G, et al. (2003) Signal pathway profiling of ovarian cancer from human tissue specimens using reverse-phase protein microarrays. Proteomics 3: 2085–2090.
7. Petricoin EF, 3rd, Espina V, Araujo RP, Midura B, Yeung C, et al. (2007) Phosphoprotein pathway mapping: Akt/mammalian target of rapamycin activation is negatively associated with childhood rhabdomyosarcoma survival. Cancer Res 67: 3431–3440.
8. Kornblau SM, Tibes R, Qiu YH, Chen W, Kantarjian HM, et al. (2009) Functional proteomic profiling of AML predicts response and survival. Blood 113: 154–164.
9. Pierobon M, Calvert V, Belluco C, Garaci E, Deng J, et al. (2009) Multiplexed cell signaling analysis of metastatic and nonmetastatic colorectal cancer reveals COX2-EGFR signaling activation as a potential prognostic pathway biomarker. Clin Colorectal Cancer 8: 110–117.
10. Haferlach T, Kohlmann A, Wieczorek L, Basso G, Kronnie GT, et al. (2010) Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group. J Clin Oncol 28: 2529–2537.
11. Bachmann PS, Gorman R, Papa RA, Bardell JE, Ford J, et al. (2007) Divergent mechanisms of glucocorticoid resistance in experimental models of pediatric acute lymphoblastic leukemia. Cancer Res 67: 4482–4490.
12. Möröy T, Geisen C (2004) Cyclin E. Int J Biochem Cell Biol 36: 1424–1439.
13. Scuderi R, Palucka KA, Pokrovskaja K, Björkholm M, Wiman KG, et al. (1996) Cyclin E overexpression in relapsed adult acute lymphoblastic leukemias of B-cell lineage. Blood 87: 3360–3367.
14. van Rhenen A, Feller N, Kelder A, Westra AH, Rombouts E, et al. (2005) High stem cell frequency in acute myeloid leukemia at diagnosis predicts high minimal residual disease and poor survival. Clin Cancer Res 11: 6520–6527.
15. Henze G, Fengler R, Hartmann R, Kornhuber B, Janka-Schaub G, et al. (1991) Six-year experience with a comprehensive approach to the treatment of recurrent childhood acute lymphoblastic leukemia (ALL-REZ BFM 85). A relapse study of the BFM group. Blood 78: 1166–1172.
16. Pui CH, Evans WE (2006) Treatment of acute lymphoblastic leukemia. N Engl J Med 354: 166–178.
17. Pui CH, Chessells JM, Camitta B, Baruchel A, Biondi A, et al. (2003) Clinical heterogeneity in childhood acute lymphoblastic leukemia with 11q23 rearrangements. Leukemia 17: 700–706.
18. Robinson BW, Behling KC, Gupta M, Zhang AY, Moore JS, et al. (2008) Abundant anti-apoptotic BCL-2 is a molecular target in leukaemias with t(4;11) translocation. Br J Haematol 141: 827–839.
19. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, et al. (2004) The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc Natl Acad Sci U S A 101: 3329–3335.
20. Morrow VA, Foufelle F, Connell JM, Petrie JR, Gould GW, et al. (2003) Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells. J Biol Chem 278: 31629–31639.
21. Drew BG, Fidge NH, Gallon-Beaumier G, Kemp BE, Kingwell BA (2004) Highdensity lipoprotein and apolipoprotein AI increase endothelial NO synthase activity by protein association and multisite phosphorylation. Proc Natl Acad Sci U S A 101: 6999–7004.
22. Choi BM, Pae HO, Jang SI, Kim YM, Chung HT (2002) Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. J Biochem Mol Biol 35: 116–126.
23. Xu W, Liu LZ, Loizidou M, Ahmed M, Charles IG (2002) The role of nitric oxide in cancer. Cell Res 12: 311–320.
24. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, et al. (1995) Nitric oxide synthase activity in human breast cancer. Br J Cancer 72: 41–44.
25. Mannick JB, Asano K, Izumi K, Kieff E, Stamler JS (1994) Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. Cell 79: 1137–1146.
26. Genaro AM, Hortelano S, Alvarez A, Martínez C, Boscá L (1995) Splenic B lymphocyte programmed cell death is prevented by nitric oxide release through mechanisms involving sustained Bcl-2 levels. J Clin Invest 95: 1884–1890.
27. Schneider A, Younis RH, Gutkind JS (2008) Hypoxia-induced energy stress inhibits the mTOR pathway by activating an AMPK/REDD1 signaling axis in head and neck squamous cell carcinoma. Neoplasia 10: 1295–1302.
28. Baumann P, Mandl-Weber S, Emmerich B, Straka C, Schmidmaier R (2007) Inhibition of adenosine monophosphate-activated protein kinase induces apoptosis in multiple myeloma cells. Anticancer Drugs 18: 405–410.
29. Reiter A, Schrappe M, Ludwig WD, Hiddemann W, Sauter S, et al. (1994) Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. Blood 84: 3122–3133.
30. Lauten M, Stanulla M, Zimmermann M, Welte K, Riehm H, et al. (2001) Clinical outcome of patients with childhood acute lymphoblastic leukaemia and an initial leukaemic blood blast count of less than 1000 per microliter. Klin Padiatr 213: 169–174.
31. Schrappe M, Reiter A, Ludwig W-D, Harbott J, Zimmermann M, et al. (2000) Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German- Austrian-Swiss ALL-BFM Study Group. Blood 95: 3310–3322.
32. Rudd CE, Trevillyan JM, Dasgupta JD, Wong LL, Schlossman SF (1988) The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc Natl Acad Sci U S A 85: 5190–5194.
33. Amundadottir LT, Leder P (1998) Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. Oncogene 16: 737–746.
34. Bergmen M, Mustelin T, Oetken C, Partanen J, Flint NA, et al. (1992) The human p50csk tyrosine kinase phosphorylates p56lck at Tyr-505 and down regulates its catalytic activity. EMBO J 11: 2919–2924.
35. Blake S, Hughes TP, Mayrhofer G, Lyons AB (2008) The Src/ABL kinase inhibitor dasatinib (BMS-354825) inhibits function of normal human T-lymphocytes in vitro. Clin Immunol 127: 330–339.
36. Basso G, Buldini B, De Zen L, Orfao A (2001) New methodologic approaches for immunophenotyping acute leukemias. Heamatologica 86: 675–692.
37. Pollard KS, Ge Y, Taylor S, Dudoit S (2008) Multtest: Resampling-based multiple hypothesis testing. R package version 1.18.0.
38. Goeman JJ, van de Geer SA, de Kort F, van Houwelingen HC (2004) A global test for groups of genes: testing association with a clinical outcome. Bioinformatics 20: 93–99.

APPENDIX 3

Leukemia (2012), 1–9

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ORIGINAL ARTICLE

AMPK inhibition enhances apoptosis in MLL-rearranged pediatric B-acute lymphoblastic leukemia cells

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The serine/threonine kinase AMP-activated protein kinase (AMPK) and its downstream effectors, including endothelial nitric oxide synthase and BCL-2, are hyperactivated in B-cell precursor-acute lymphoblastic leukemia (BCP-ALL) cells with *MLL* gene rearrangements. We investigated the role of activated AMPK in supporting leukemic cell survival and evaluated AMPK as a potential drug target. Exposure of leukemic cells to the commercial AMPK inhibitor compound C resulted in massive apoptosis only in cells with *MLL* gene rearrangements. These results were confirmed by targeting AMPK with specific short hairpin RNAs. Compound C-induced apoptosis was associated with mitochondrial membrane depolarization, reactive oxygen species production, cytochrome c release and caspases cleavage, indicating intrinsic apoptosis pathway activation. Treatment with low concentrations of compound C resulted in a strong antileukemic activity, together with cytochrome c release and cleavage of caspases and poly(ADP-ribose) polymerase, also in MLL-rearranged primary BCP-ALL samples. Moreover, AMPK inhibition in MLL-rearranged cell lines synergistically enhanced the antiproliferative effects of vincristine, daunorubicin, cytarabine, dexamethasone and L-asparaginase in most of the evaluated conditions. Taken together, these results indicate that the activation of the AMPK pathway directly contributes to the survival of MLL-rearranged BCP-ALL cells and AMPK inhibitors could represent a new therapeutic strategy for this high-risk leukemia.

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Keywords: acute lymphoblastic leukemia; MLL rearrangements; AMPK; apoptosis

INTRODUCTION

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that acts as a cellular fuel sensor activated under conditions of ATP depletion and elevated AMP levels such as heat-shock, nutrient deprivation, hypoxia and other metabolic or environmental stresses.[1,2] AMPK is an heterotrimeric complex composed of an α-catalytic subunit, a β-subunit important both for complex formation and glycogen binding, and a γ-regulatory subunit, which binds AMP.[3] AMPK activity is enhanced by phosphorylation of the α-subunit at threonine 172 by LKB1, a serine/threonine kinase encoded by the tumor-suppressor gene *STK11*, which is mutated in patients with Peutz–Jeghers syndrome.[4,5] In addition, AMPK can also be activated by several hormones and cytokines such as adiponectin[6] and leptin.[7] The physiological role ascribed to AMPK is the inactivation of ATP-consuming metabolic processes, including fatty acid, cholesterol and protein synthesis, and the activation of ATP-generating pathways such as glycolysis and fatty acid oxidation.[8,9] This is initially accomplished by direct phosphorylation of key metabolic enzymes, followed by effects on gene expression.

Although AMPK is traditionally regarded as a sensor of cellular energy status and a regulator of metabolism, recently it has been reported to be involved in the regulation of several biological processes including cell growth, proliferation, apoptosis, autophagy and cell polarity.[10,11] In cancer, the role of AMPK is not yet fully understood and data reported in literature so far are contradictory. The effects of AMPK activation are determined by the cell type investigated, depending on signaling alterations in related pathways. AMPK activation results in pro-apoptotic effects reported in acute myeloid leukemia,[12] ovarian cancer,[13] astrocytoma,[14] and osteosarcoma[15] and in anti-apoptotic effects, observed in multiple myeloma,[16] prostate cancer[17] and glioma.[18]

We previously found that pediatric B-cell precursor-acute lymphoblastic leukemia (BCP-ALL) patients with rearrangements of the *MLL* gene display the hyperactivation of a signal transduction pathway that leads from phosphorylation of LKB1 and AMPK to phosphorylation of BCL-2, through downstream endothelial nitric oxide synthase activation.[19] In this study, we assessed whether this hyperactivation supports the survival of MLL-rearranged BCP-ALL cells, and whether its inhibition affects leukemic cell growth and drug resistance.

MATERIALS AND METHODS

Cell lines and culture

Human leukemia cell lines SEM, RS4;11, MHH-CALL-2 and MHH-CALL-4 were purchased from DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Human leukemia cell line ALL-PO was kindly donated by Professor Andrea Biondi (University of Milano-Bicocca, Monza, Italy).[20] SEM, RS4;11 and ALL-PO cell lines were derived from BCP-ALLs carrying the t(4;11) MLL-AF4 translocation. MHH-CALL-2 and MHH-CALL-4 cell lines were derived from BCP-ALLs without recurrent chromosomal translocations. Cells were cultured in RPMI 1640 (Biochrom

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AG, Berlin, Germany) with 10% fetal calf serum (FCS), glutamine (2 mM/l; GIBCO, Invitrogen Life Technologies, Carlsbad, CA, USA), penicillin (100 U/ml; GIBCO) and streptomycin (100 μg/ml; GIBCO), and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Cells were treated with compound C (Calbiochem, Darmstadt, Germany) dissolved in dimethylsulphoxide (DMSO) at different times and concentrations, or with DMSO alone.

MTT assay

Cell proliferation was assessed by MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay after treatment. Equal concentrations of cells were plated in triplicate in a 96-well plate and incubated with 10 μl MTT (Sigma-Aldrich, St Louis, MO, USA) for 4 h. Absorbance was measured at 560 nm using Victor$^3_{TM}$ 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA). The growth inhibition$_{50}$ (GI_{50} = compound concentration required to inhibit cell proliferation by 50%) was calculated by plotting the data as a logarithmic function of (*x*) when viability was 50%. DMSO-treated cells viability was set to 100%.

Cytofluorimetric assays

Apoptosis was determined using the Annexin-V–FLUOS staining kit (Roche, Basel, Switzerland), following the manufacturer's instructions. Samples were analyzed by flow cytometric analysis (Cytomics FC500, Beckman Coulter, Fullerton, CA, USA). DMSO-treated cells viability was set to 100%. The lethal concentration$_{50}$ (LC_{50} = compound concentration required to induce cell mortality by 50%) was calculated by plotting the data as a logarithmic function of (*x*) when viability was 50%.

The mitochondrial membrane potential (ψ_{mt}) was measured with the lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine (JC-1, Molecular Probes, Invitrogen Life Technologies), as already described[21] in DMSO alone and compound C (8 μM, 48 h–LC_{50})-treated cells.

Mitochondrial membrane depolarization is associated with mitochondrial production of reactive oxygen species (ROS). The production of ROS was measured in DMSO alone and compound C (LC_{50} compound C 8 μM, 48 h) treated cells by flow cytometry using hydroethidine (Molecular Probes, Invitrogen Life Technologies) 2.5 μM.[21]

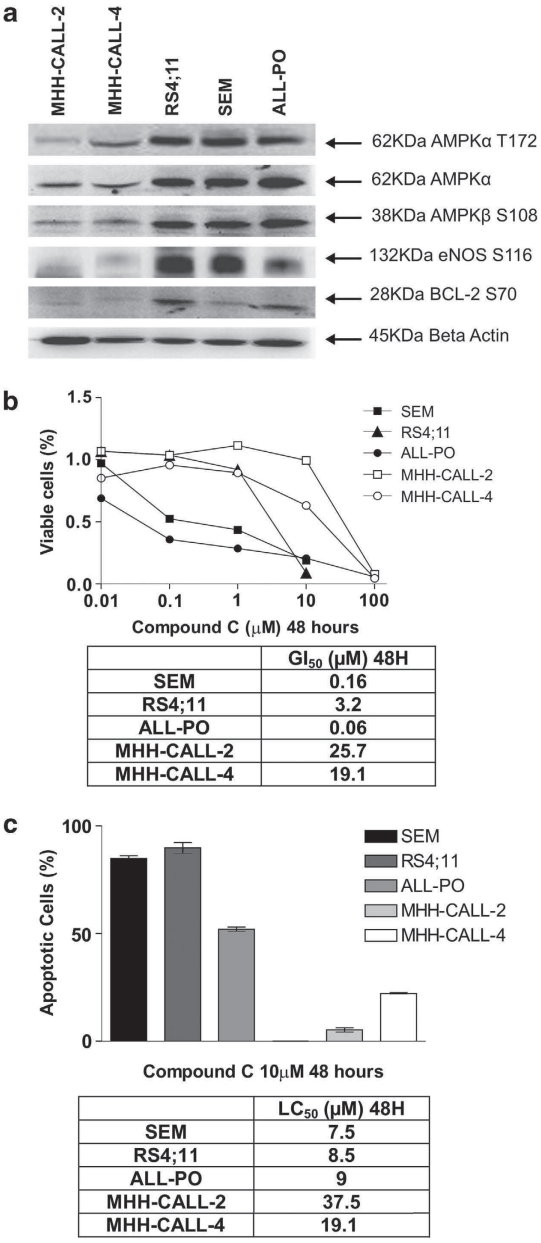
Caspase-3 activation was evaluated by flow cytometry using a human active caspase-3 fragment antibody conjugated with fluorescein isothiocyanate (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, DMSO alone and compound C (8 μM, 48 h) treated cells were collected by centrifugation and resuspended in Cytofix (BD Pharmingen, BD Biosciences) buffer for 20 min, washed with Perm/Wash (BD Pharmingen, BD Biosciences), and then incubated for 30 min with the antibody. Cells were then washed and analyzed by flow cytometry.

Lentiviral vector-mediated transduction of shRNA in leukemia cells

The lentiviral plasmids containing AMPKα1 short hairpin RNA (shRNA) expression cassette or an un-relevant shRNA sequence were purchased from Sigma-Aldrich. The lentiviral vector stocks were generated by a transient three-plasmid vector-packaging system.[22] One microgram of p24 equivalent of lentiviral vector-containing supernatant was used to transduce 1×10^6 target cells in 35-mm diameter Petri dish. After 6–9 h at 37 °C, the supernatant was replaced with complete medium. Evaluation of AMPKα1 silencing, through Sybr Green real-time quantitative PCR and western blot experiments, and quantification of apoptosis, through Annexin V–propidium iodide (PI) staining, were carried out 72–96 h after transduction.

Real-time quantitative PCR

Total RNA was isolated and retrotranscribed as previously described.[23] Reactions were performed in a total volume of 20 μl, containing 1 μl of complementary DNA, 10 μl of SYBR Green (Invitrogen Life Technologies), and 150 nM forward and reverse primers, on an ABI Prism 7900HT Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA) with the annealing temperature set at 60 °C for all tested genes. Relative quantification was done using the ΔΔCt method, normalizing to *GUS* mRNA. Primers used for real-time quantitative PCR analysis: *AMPKα1*-fwd: 5′-GGAGCCTTGATGTGGTAGGA-3′; *AMPKα1*-rev: 5′-GTTTCATCCAGCCTTC CATTC-3′; *AMPKα2*-fwd 5′-ACCAGCTTGCAGTGGCTTAT-3′; *AMPKα2*-rev: 5′-C AGTGCATCCAATGGACATC-3′; *GUS*-fwd: 5′-GAAAATATGTGGTTGGAGAGCT CATT-3′; GUS-rev: 5′-CGGAGTGAAGATCCCCTTTTTA-3′.



	GI_{50} (μM) 48H
SEM	0.16
RS4;11	3.2
ALL-PO	0.06
MHH-CALL-2	25.7
MHH-CALL-4	19.1

	LC_{50} (μM) 48H
SEM	7.5
RS4;11	8.5
ALL-PO	9
MHH-CALL-2	37.5
MHH-CALL-4	19.1

Figure 1. AMPK inhibition induces a decrease in cell proliferation and survival. (**a**) AMPK pathway activation status in BCP-ALL cell lines. MHH-CALL-2 and MHH-CALL-4 are non-translocated cell lines, RS4;11, SEM and ALL-PO carry the (4;11) *MLL-AF4* translocation. (**b**) Cell proliferation rates were determined through MTT assay after treatment with compound C at different times and concentrations. Here the 48 h are shown. DMSO-treated cells viability was set to 100%. GI_{50} = compound concentration required to inhibit cell proliferation by 50%. (**c**) Cell viability was determined by flow cytometry with Annexin V–PI staining after treatment with compound C at different times and concentrations. Here the 10 μM 48 h results are shown. DMSO-treated cells viability was set to 100%. Results represent the mean of three independent experiments ± s.e.m. LC_{50} = compound concentration required to induce cell mortality by 50%. eNOS, endothelial nitric oxide synthase.

Western blotting

The following antibodies and final concentrations were used for western blotting. Primary antibodies: anti-phospho-AMPKα T172 (1:500), anti-AMPKα (23A3) (1:1000), anti-AMPKα1 (1:1000), anti-phospho-AMPKß S108

(1:1000), anti-phospho-BCL-2 S70 (1:500), anti-β-ACTIN (1:10 000; all from Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phospho-endothelial nitric oxide synthase/NOS III S116 (1:1000; Millipore, Billerica, MA, USA). Secondary antibodies: horseradish peroxidase-goat anti-rabbit and anti-mouse immunoglobulin G-conjugate (Zymed Laboratories, Inc., South San Francisco, CA, USA; 1:50 000). Total cell lysates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, and transferred to a nitrocellulose sheet (Hybond-P, GE Healthcare, Chalfont, St Giles, UK) following standard methods. Membranes were saturated for 3 h with 2% Amersham ECL Advance Blocking Reagent (GE Healthcare), primary antibodies were incubated overnight at 4 °C and secondary antibodies for 1 h at room temperature. The immunoreactivity was determined by an enhanced chemiluminescent reaction (Amersham ECL ADVANCE Western Blotting Detection Kit, GE Healthcare). For the stripping, membranes were incubated with Restore PLUS Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) following the manufacturer's instructions and then washed in T-phosphate-buffered saline 1 × and resaturated.

To perform densitometric analysis shown in Figure 2c, western blot images were analyzed by ImageJ software (NIH, Bethesda, MA, USA). The intensity of the bands was normalized to the intensity of the bands corresponding to the β-ACTIN protein. Control cells (shRNA neg) ratio was set as 1.

Cell cycle analysis

Cells were synchronized 24 h before treatment by starvation without FBS. Compound C was then added 6 h after the end of starvation. DMSO alone and compound C (8 μM, 24 h) treated cells were centrifuged, fixed with ice-cold ethanol (70%), treated with lysis buffer containing RNAseA (Qiagen, Hilden, Germany) and PI. Samples were analyzed on a Cytomic FC500 flow cytometer (Beckman Coulter), and cell cycle analyses were performed using Multicycle Wincycle software (Phoenix Flow Systems, San Diego, CA, USA).

Immunofluorescence

Compound C (8 μM) and DMSO-treated human MLL-rearranged BCP-ALL cell lines and one primary BCP-ALL cell culture were placed on the slide by cytospin and then fixed with formaldehyde (4%) for 15 min. Slides were treated with NH_4Cl (Sigma-Aldrich) 50 mM for 15 min to reduce background. Cells were permeabilized with 0.1% Triton X-100/phosphate-buffered saline 1 × for 3 min and incubated first with blocking buffer (5% bovine serum albumin in phosphate-buffered saline 1 ×) and then with primary antibody (1:100) diluted in blocking buffer overnight at 4 °C. Cells were then incubated for 1 h with species-specific secondary antibodies conjugated to Alexa-fluor 488 or 594 (1:1000–1:2000; Invitrogen Life Technologies), and for 10 min with 4,6-diamidino-2-phenylindole (1:10 000; Sigma-Aldrich). Primary antibodies: cytochrome c, cleaved poly(ADP-ribose) polymerase (PARP) (Asp214) and BCL-XL (from Cell Signaling Technology, Inc.), caspase-7 active and caspase-9 active (from GeneTex Inc., Irvine, CA, USA). Images of decorated cells were acquired by the videoconfocal system ViCo microscope (Nikon Eclipse 80i, Nikon, Japan). Images were acquired with ImageProPlus (Media Cybernetics, Rockville, MD, USA) and collected at magnification × 60.

Primary leukemia cell cultures

BCP-ALL patient samples were obtained after informed consent following the tenets of the Declaration of Helsinki. The study was approved by the ethical committee board of the University of Padova, the Padova Academic Hospital and the Italian Association of Pediatric Onco-Hematology (AIEOP). Diagnosis was made according to standard cytomorphology, cytochemistry and immunophenotypic criteria.[24] All analyzed BCP-ALL samples were obtained at the time of diagnosis before treatment, after Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) separation of mononuclear cells. Mononuclear cells were frozen as viable cells in FCS and 10% DMSO and stored in liquid nitrogen. The percentage of CD19 + cells ranged from 84 to 96%. We included in the study five patients positive for the 11q23 MLL-rearrangement MLL-AF4 and four patients negative for the 11q23 MLL-rearrangement and the chromosomal translocations t(12;21) TEL-AML1, t(9;22) BCR-ABL and t(1;19) E2A-PBX1. Chromosomal translocation analyses were performed for standard diagnostic procedures.

Human bone marrow-derived mesenchimal stromal cells (MSCs) immortalized by telomerase reverse transcriptase transduction were kindly donated by Dr Dario Campana (Yong Loo Lin School of Medicine, National

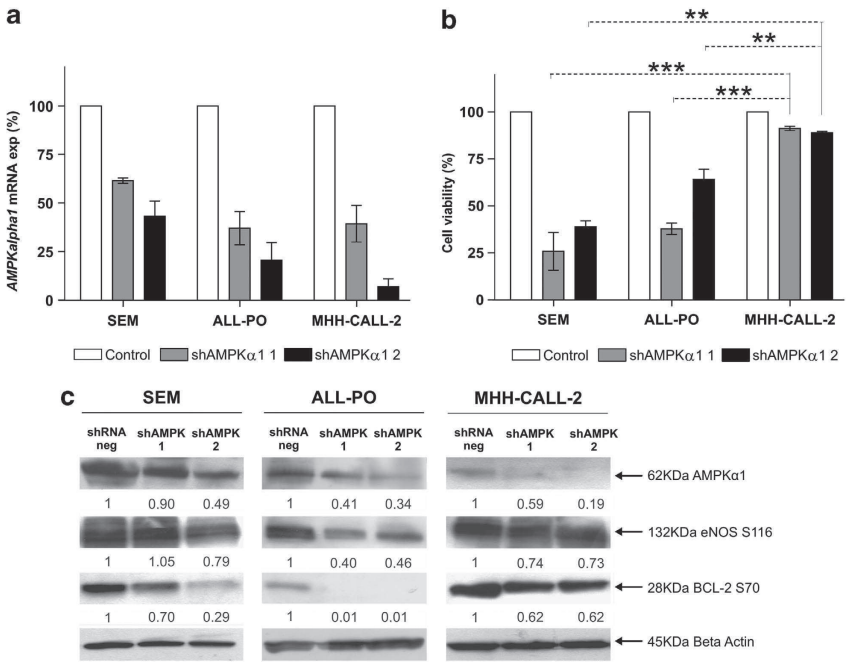


Figure 2. AMPK-specific silencing brings to the same effects of compound C treatment. (**a**) Inhibition of *AMPKα1* mRNA expression in SEM, ALL-PO and MHH-CALL-2 cells with both shAMPKalpha1 1 and 2. *AMPKα1* mRNA expression in control cells (transfected with un-relevant shRNA sequence) was set to 100%. Results represent mean ± s.e.m. of three experiments. mRNA expression levels were measured through Sybr Green real-time quantitative PCR. (**b**) Cell viability after *AMPKα1* silencing in SEM, ALL-PO and MHH-CALL-2 cells was evaluated through Annexin V–PI staining. Control cells viability was set to 100%. Results represent mean ± s.e.m. of three experiments. Silenced MLL-rearranged SEM and ALL-PO cells undergo more apoptosis than non-translocated MHH-CALL-2 cells (**$P<0.01$, ***$P<0.001$). (**c**) Inhibition of AMPKα1 protein levels and decrease in the downstream AMPK pathway activation was examined using western blot in control and silenced SEM, ALL-PO and MHH-CALL-2 cells. Representative results are shown with densitometric analysis results indicated under gel images.

University of Singapore, Singapore).[25] MSCs were maintained in RPMI 1640 medium supplemented with 10% FCS, glutamine (2 mM/l), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10^{-6}M hydrocortisone (Sigma-Aldrich). MSCs were seeded in 24-well flat-bottomed plates (Costar Corning, Cambridge, MA, USA) and grown until confluence. To prepare cultures of primary samples, culture media was removed and adherent cells were washed seven times with AIM-V tissue culture medium (Invitrogen Life Technologies) with 10% FCS. Leukemic cells were then resuspended in AIM-V medium with 10% FCS, and 1×10^5 leukemic cells were placed on the MSCs layer in each well. Compound C was added 24 h after seeding at different concentrations (1, 5 and 8 μM). Quantification of apoptosis was carried out 24 and 48 h after treatment, downregulation of the AMPK pathway was tested after 48 h.

Combined drugs analysis

To test potential synergistic, additive or antagonistic effects of the combination of AMPK inhibition and drugs commonly used in ALL treatment, we performed MTT experiments as follows.

SEM, RS4;11 and ALL-PO cells were treated for 48 h with the different chemotherapeutics cytarabine (Aractyn, Pfizer, New York, NY, USA; 0.001–100 μM), daunorubicin (Pfizer; 0.0001–10 μM), vincristine (Pfizer; 0.0001–1 μM), dexamethasone (Sigma-Aldrich; 0.0001–10 μM) and L-asparaginase (Kidrolase, Eusa Pharma, Jazz Pharmaceuticals plc, Dublin, Ireland; 0.001–100 IU/ml). Compound C was added to drug solutions at fixed combination ratios. Cell viability was determined after 48 h of treatment. To determine the synergistic, additive or antagonistic effects of the drug combinations, we used CalcuSyn software (version 2.0, Biosoft, Cambridge, UK), which is based on the method of the combination index (CI) of Chou and Talalay.[26] Synergy, additivity and antagonism were defined by a $CI < 1$, $CI = 1$ or $CI > 1$, respectively.

RESULTS

AMPK inhibition decreases proliferation and survival in MLL-rearranged cells

To study the functional role of AMPK in the survival of MLL-rearranged cells, we used five BCP-ALL cell lines, three with *MLL* gene rearrangements (RS4;11, SEM and ALL-PO) and two without (MHH-CALL-2 and MHH-CALL-4). As shown in Figure 1a, AMPK was activated in MLL-rearranged cells, similarly to what was observed in primary cells from patients.[19] By contrast, AMPK activation was not detected in MHH-CALL-2 and MHH-CALL-4 (Figure 1a).

We treated the five BCP-ALL cell lines with the AMPK inhibitor compound C. The capability of compound C to inhibit the AMPK pathway in SEM and RS4;11 cells was previously demonstrated.[19] Downregulation of the AMPK pathway in ALL-PO cells is shown in Supplementary Figure S1. Cells were exposed to the inhibitor for different time periods (6–96 h) at concentrations ranging from 0.001 to 100 μM. The three MLL-rearranged cell lines were much more sensitive to AMPK inhibition than the two non-rearranged ones. The GI_{50} measured by MTT assay was 0.16 μM for SEM, 3.2 μM for RS4;11 and 0.06 μM for ALL-PO cells, whereas it resulted to be 25.7 μM and 19.1 μM for the non-translocated cell lines (Figure 1b). Supplementary Figure S2a shows the results obtained at all tested

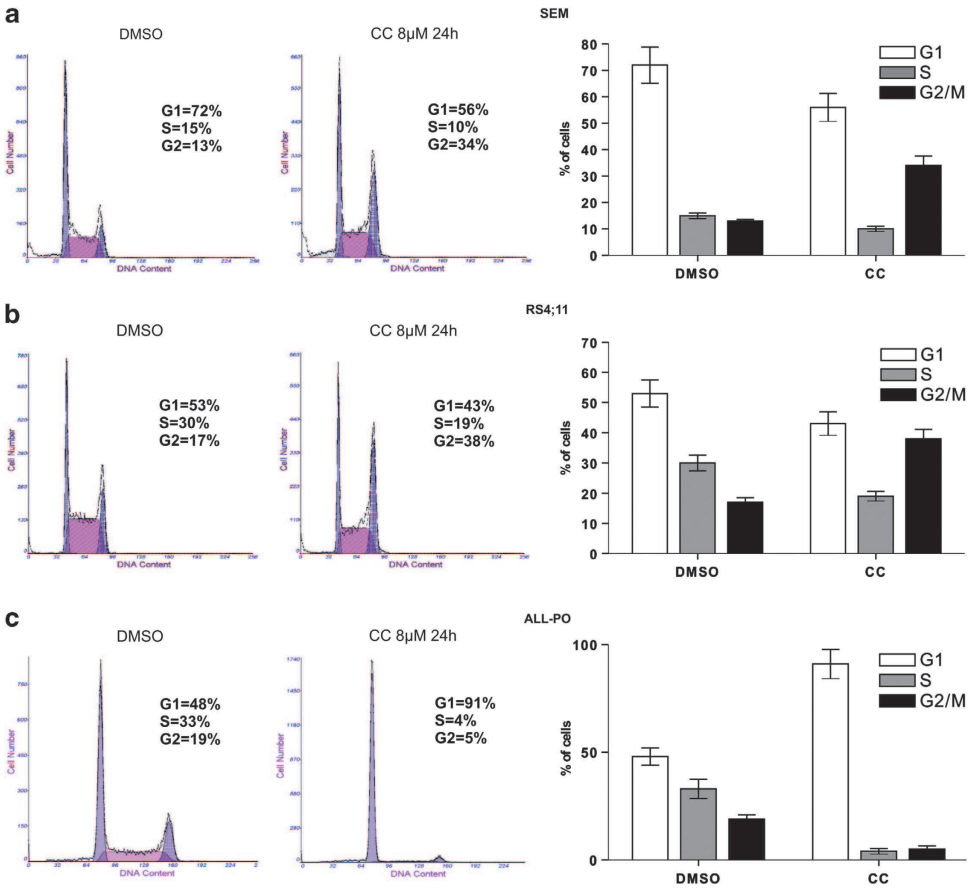


Figure 3. Cell cycle alterations after AMPK inhibition. Results are shown in **(a)** for SEM cells, **(b)** for RS4;11 cells and **(c)** for ALL-PO cells. DMSO and CC (8 μM, 24 h) samples were analyzed by flow cytometry and cell cycle analyses were performed using Multicycle Wincycle software. Representative histograms are shown. The percentage of each phase of the cell cycle (G1, S, G2/M) was calculated. Results come from three independent experiments.

times and concentrations. MLL-rearranged cell lines exposed to the inhibitor also underwent significantly more apoptosis, as measured with Annexin V and PI staining. LC_{50} was 7.5 μM for SEM, 8.5 μM for RS4;11 and 9 μM for ALL-PO, whereas it was 37.5 μM for MHH-CALL-2 and 31.4 μM for MHH-CALL-4 (Figure 1c, for more detailed results please see Supplementary Figure S2b). Therefore, activation of the AMPK pathway appears to be essential for sustaining the survival of MLL-rearranged cells.

To validate the association between AMPK inhibition and apoptosis, we suppressed AMPKα1 expression using two different shRNAs (shAMPKalpha1 1 and 2) in SEM, ALL-PO and MHH-CALL-2 cells. AMPKα1 mRNA expression was efficiently reduced after lentiviral vector transduction in the three cell lines (Figure 2a, control AMPKα mRNA expression was set to 100%). In line with the previous results, SEM and ALL-PO cells underwent more apoptosis than MHH-CALL-2 cells (Figure 2b, control cell viability was set to 100%; *t*-test, SEM vs MHH-CALL-2 $P=0.004$ and $P=0.0003$ for shAMPKalpha1 1 and 2, respectively, ALL-PO vs MHH-CALL-2 $P=0.006$ and $P=0.0001$). AMPKα1 protein expression was also reduced in MLL-rearranged and non-translocated silenced cells, but the downregulation of the downstream pathway was observed only in SEM and ALL-PO cells (Figure 2c).

shRNA silencing has effects on MLL-rearranged cells survival similar to compound C, therefore what we observed after compound C treatment in these cells can be mainly attributed to AMPK deactivation. These experiments also demonstrated that the specific silencing of AMPK has no consequences on non-translocated cells survival, meaning that the apoptosis we previously observed with 30–40 μM of compound C probably derived from off-target effects because of high concentration of the inhibitor. For this reason, to investigate cell cycle modifications and apoptosis driven by AMPK inhibition, we performed the following experiments only on MLL-rearranged cells.

Compound C treatment induces cell cycle alterations

Representative cell cycle histograms based on flow cytometric analyses of MLL-rearranged cells exposed to compound C are shown in Figure 3. After 24 h of compound C treatment (8 μM), an accumulation in G2/M phase was observed in SEM and RS4;11 cell lines. SEM cells in G2/M phase increased from about 13 up to 34%, whereas cells in G1 decreased from about 72 to 56% and cells in S phase from about 15 down to 10% (Figure 3a). Similarly, after treatment RS4;11 cells in G2/M phase increased from about 17 up to 38%, whereas cells in G1 decreased from about 53 to 43% and cells in S phase from about 30 down to 19% (Figure 3b). Differently, in ALL-PO cells an accumulation in G1 was observed after AMPK inhibition. The percentage of cells in G1 phase increased from about 48 up to 91%, whereas G2/M and S phase cells decreased from about 19 to 5% and from 33 to 4%, respectively (Figure 3c).

Compound C induces apoptosis through the mitochondrial pathway

Next, we examined whether apoptosis occurs through the extrinsic or the intrinsic pathway. For this purpose, we treated MLL-rearranged cell lines SEM, RS4;11 and ALL-PO with 8 μM

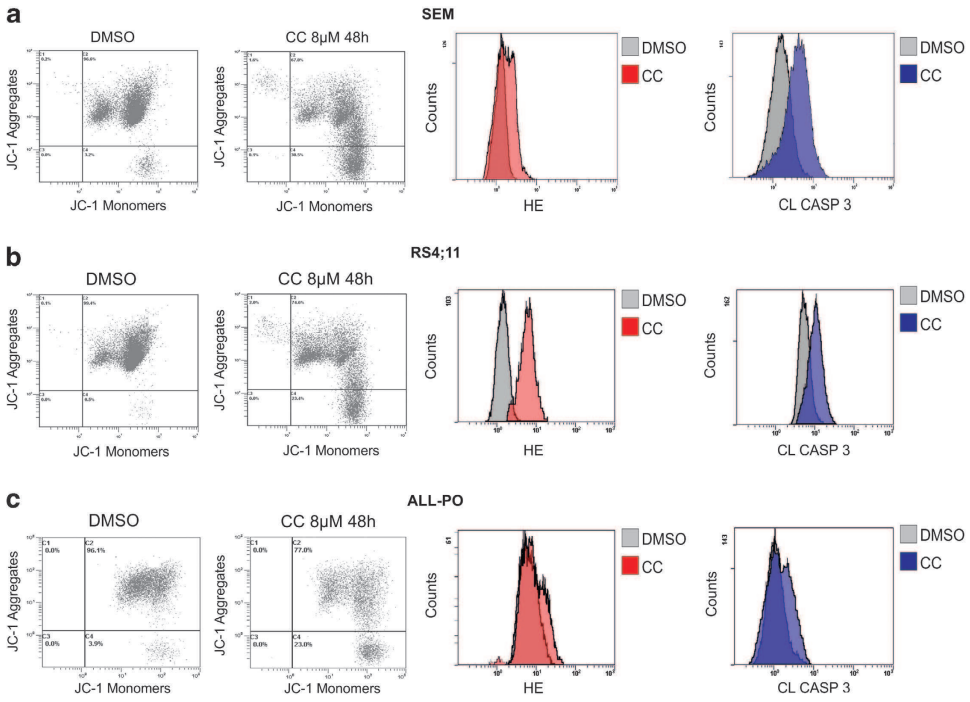


Figure 4. Compound C (CC)-mediated cell death follows the mitochondrial pathway (I). Results are shown in (**a**) for SEM cells, (**b**) for RS4;11 cells and (**c**) for ALL-PO cells. In the left panels, depolarization of the mitochondrial transmembrane potential, monitored by the fluorescent dye JC-1, in MLL-rearranged cells treated with CC C (8 μM, 48 h) is reported. The method is based on the ability of this fluorescent probe to enter selectively into the mitochondria, and its color changes reversibly from green to orange as membrane potential increases. This property is due to the reversible formation of JC-1 aggregates on membrane polarization. Aggregation causes a shift in the emitted light from 530 nm (emission by JC-1 monomers) to 590 nm (emission by JC-1 aggregates) following excitation at 490 nm. In middle panels, shown are the mitochondrial production of reactive oxygen species in MLL-rearranged cells treated with CC (8 μM, 48 h). The fluorescence indicator hydroethidine (HE), that is oxidized by superoxide anion into ethidium ion, which emits red fluorescence, was measured. Gray: DMSO-treated cells, red: CC-treated cells. In the right panels, shown are the cleavage of caspase-3 measured by flow cytometry in MLL-rearranged cells after CC (8 μM, 48 h) treatment. Gray: DMSO-treated cells, blue: CC-treated cells.

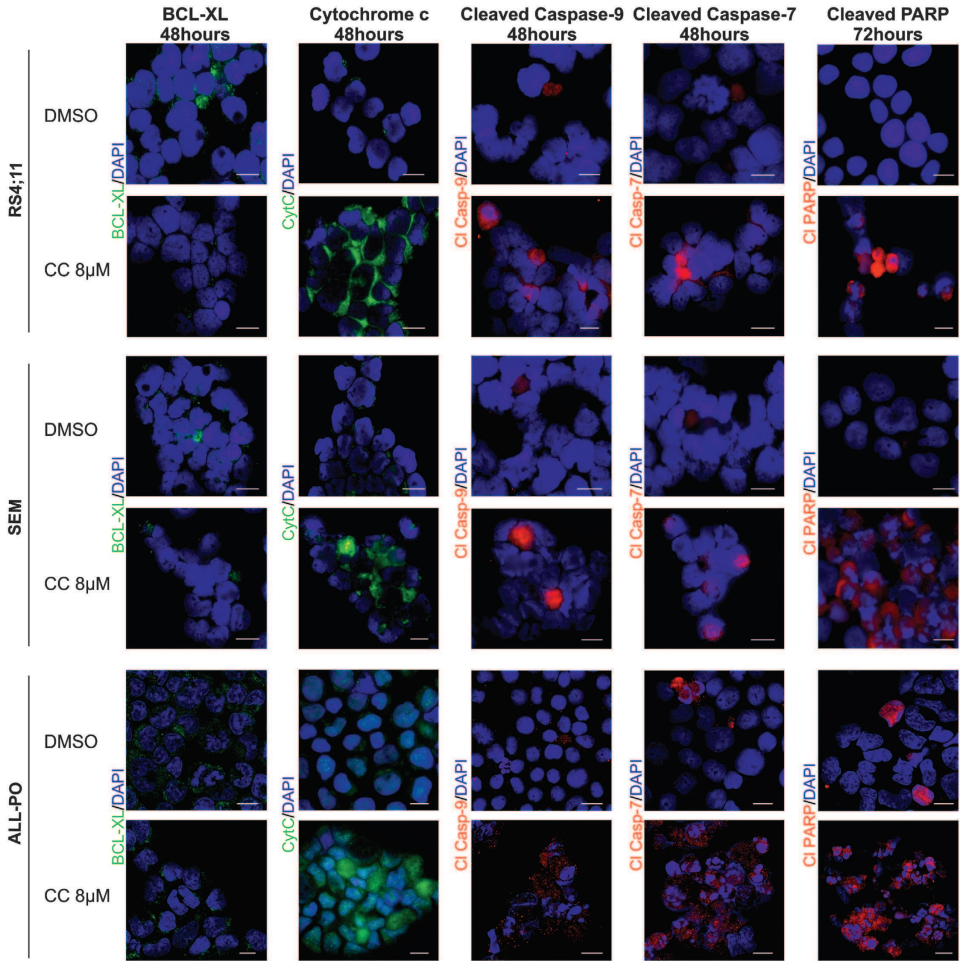


Figure 5. Compound C (CC)-mediated cell death follows the mitochondrial pathway (II). Apoptotic proteins expression was determined by immunofluorescence in MLL-rearranged cells after DMSO or CC (8 μM, 48 and 72 h) treatment. Panel shows pseudo-color merged images of cell lines decorated with 4,6-diamidino-2-phenylindole (DAPI), Alexa-fluor 488 and 594. Scale bar 5 μm.

compound C for 24, 48 and 72 h. Results are resumed in Figure 4a (SEM cells), Figure 4b (RS4;11 cells) and Figure 4c (ALL-PO cells).

Both FAS and FAS-L were present at low levels in control and DMSO-treated cells and did not increase after AMPK inhibition (data not shown). We also examined if exposure to compound C for 24 or 48 h could induce BCP-ALL cell differentiation using a panel of B-lymphocyte differentiation markers including CD58/10/45/19/34/20; and no change in marker expression was detected (data not shown). Thus, apoptosis induced by compound C does not involve FAS and AMPK inhibition does not promote B-cell differentiation.

Apoptotic stimuli may alter the mitochondrial transmembrane potential Ψ_{mt} monitored by the fluorescent dye JC-1. JC-1 fluorescence shifted in MLL-rearranged cells exposed to compound C for 48 h, indicating depolarization of mitochondrial membrane potential (Figure 4, left panels). As mitochondrial membrane depolarization is associated with mitochondrial production of ROS, we investigated whether ROS production was increased after compound C treatment. We used the fluorescent indicator hydroethidine that is oxidized by superoxide anion into ethidium ion, which emits red fluorescence; superoxide is produced by mitochondria when cytochrome c is released. As shown in Figure 4 middle panels, exposure to compound C for 48 h induced ROS production in treated MLL-rearranged cells, and we also observed the activation of caspase-3 (Figure 4, right panels) and decreased levels of the anti-apoptotic protein BCL-XL (Figure 5). This leads to mitochondrial outer membrane permeabilization through BAX/BAK action, with the consequent release of cytochrome c into the cytoplasm, a necessary event for downstream caspases activation. As shown in Figure 5, the release of cytochrome c in the cytoplasm of SEM, RS4;11 and ALL-PO cells after 48 h of AMPK inhibition is particularly evident. Release of cytochrome c results in activation of caspase-9 through self-cleavage at Asp315. Cleaved caspase-9 further processes other caspase members, including caspase-3 and caspase-7 that are effector caspases responsible for the proteolytic cleavage of many key proteins such as PARP. When cleaved, PARP is no longer able to initiate DNA repair and cells undergo apoptosis. In treated MLL-rearranged cells we observed at 48 h the activation of caspase-7 and caspase-9, and at 72 h the cleavage of PARP (Figure 5). Finally, after 72 h of AMPK inhibition we also observed an increase in chromosomal DNA fragmentation into multiples of the 180-bp nucleosomal unit (Supplementary Figure S3).

AMPK inhibition induces cell death in primary leukemia cultures

We treated nine primary BCP-ALL cultures (five with *MLL* gene rearrangements and four non-translocated) with DMSO alone or compound C. Cells with MLL rearrangements underwent apoptosis when exposed to low concentrations (1 and 5 μM) of compound C more extensively than cells lacking MLL gene rearrangements

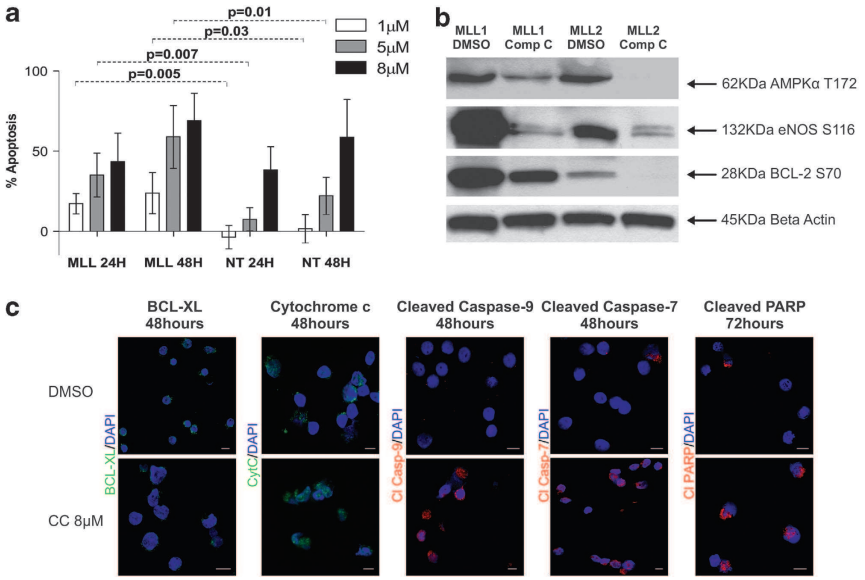


Figure 6. AMPK inhibition induces apoptosis in primary leukemia cultures. (**a**) Primary cells from nine patients (five MLL-rearranged and four non-translocated) were cultured on MSCs layer and treated with compound C (CC, 1–5–8 μM) for 24 and 48 h. Apoptosis was determined by flow cytometry with Annexin V–PI staining. DMSO-treated cells viability was set to 100%. Results represent mean ± s.e.m. MLL-rearranged primary cells undergo more apoptosis than the non-translocated ones at 24 and 48 h after treatment with 1 and 5 μM CC. (**b**) Inhibition of the AMPK pathway activation was analyzed by western blot in control and CC (5 μM, 48 h) treated patients'cells. Representative results of two MLL-rearranged patients are shown. (**c**) Apoptotic proteins expression was determined by immunofluorescence after CC (8 μM, 48 and 72 h) treatment in one MLL-rearranged primary culture. Panel shows pseudo-color merged confocal images of cells decorated with 4,6-diamidino-2-phenylindole (DAPI), Alexa-fluor 488 and 594. Scale bar 5 μm.

(*t*-test, 1 μM 24 h $P = 0.005$, 5 μM 24 h $P = 0.007$, 1 μM 48 h $P = 0.03$, 5 μM 48 h $P = 0.01$; Figure 6a). We also show that AMPK and its downstream targets were dephosphorylated in two MLL-rearranged samples after 48 h of treatment with 5 μM of compound C (Figure 6b). Moreover we observed, as for MLL-rearranged cell lines, the decrease in BCL-XL, the release of cytochrome c and the cleavage of caspase-7, caspase-9 and PARP (Figure 6c).

AMPK inhibition synergizes with drugs used in ALL chemotherapy

MLL-rearranged SEM, RS4;11 and ALL-PO cells were exposed to five drugs, namely daunorubicin, vincristine, cytarabine, dexamethasone and L-asparaginase in the presence or absence of the AMPK inhibitor compound C. The complete dose-response curves are shown in Supplementary Figures S4a (SEM), b (RS4;11) and c (ALL-PO). We estimated the concentration of each drug that caused 50% of growth inhibition with or without the compound C added at fixed combination ratio as indicated in Table 1 and searched for synergistic interactions. As can be observed, the addition of compound C significantly lowered the GI_{50} of all drugs in ALL-PO cells, whereas in RS4;11 we did not observe any variation of the efficacy of vincristine and dexamethasone. The addition of compound C failed to increase the efficacy of dexamethasone also in SEM cells. For the other conditions, the calculated combination indexes showed values <1, indicating that compound C strongly synergizes with these chemotherapeutic drugs, and at the same time suggesting the potential synergistic effect of AMPK inhibition in combination with chemotherapeutic drugs with different mechanisms of action in the treatment of MLL-rearranged BCP-ALL.

DISCUSSION

In this study, we report that the activation of the serine/threonine kinase AMPK is a key factor for the survival of BCP-ALL MLL-rearranged cells. Inhibition of AMPK activity caused a decrease in cell proliferation and survival both in cell lines and primary cultures, and it synergistically enhanced the antiproliferative activity of ALL chemotherapeutic drugs, thus pointing out this cellular fuel sensor as a promising new therapeutic target.

AMPK has been frequently proposed as a potential therapeutic target in cancer because of its position downstream of tumor-suppressor LKB1 and its known role in the regulation of cell proliferation, cell cycle progression and autophagy via mTORC1, p53 and p27.[11] Its activation through Metformin or AICAR (5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide) treatment can suppress cell proliferation and induce apoptosis in many cell types, that is, melanoma,[27] glioblastoma,[28] breast cancer[29] and renal cell carcinoma.[30] Two studies[31,32] showed that AMPK activation by AICAR (5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide) can induce apoptosis in some BCP-ALL cell lines, but MLL-rearranged cells were not included in these studies.

Interestingly, there is emerging evidence that AMPK activation has a dual role in cancer depending on alterations of interconnected signaling pathways, thus its effects are likely to be cell-type and tissue specific. Recent papers report that AMPK is hyperactivated in some cancers and increased cell death is observed after inhibition of its activity. Compound C treatment or AMPK depletion by small interfering RNA induce growth arrest and apoptosis in human prostate cancer,[17] multiple myeloma,[16] human and rat glioma[18] and pheochromocytoma cell lines.[33] AMPK activity was also reported to be higher in some cancer cell lines such as OVCAR3 and A431 than in primary keratinocytes.[34] Moreover, increased expression of LKB1 and AMPK was observed in UVB (Ultraviolet B)-induced murine basal cell carcinoma.[35]

These findings, together with our previous observations that the AMPK pathway is hyperactivated in MLL-rearranged BCP-ALL cells and that the AMPK inhibitor compound C induces dephosphorylation of AMPK and its downstream targets,[19] prompted us to study the effects of AMPK inhibition on the survival of MLL-rearranged blast cells. We first used the commercially available AMPK inhibitor compound C to treat MLL-rearranged and non-translocated cell lines. MLL-rearranged cells show decrease in proliferation and survival at really low concentrations of

Table 1. Treatment of MLL-rearranged BCP-ALL cell lines with compound C and drugs used in ALL chemotherapy

	Vcr	*Vcr + CC (1:100)*	*Ara-C*	*Ara-C + CC (1:0.1)*	*Dex*	*Dex + CC (1:1)*	*Dauno*	*Dauno + CC (1:1)*	*L-Asp*[a]	*L-Asp + CC*[a] *(1:0.1)*
SEM										
GI_{50} (nM)	18.9 ± 4.9	6.5 ± 5.7	133.0 ± 47.9	4.1 ± 0.86	2.2 ± 0.2	5.5 ± 0.6	171.0 ± 44.1	5.8 ± 2.1	0.61 ± 0.09	0.12 ± 0.02
CI at GI_{50}		0.012		0.007		9.3		0.12		0.196
RS4;11										
GI_{50} (nM)	7.0 ± 1.1	7.8 ± 1.2	69.7 ± 6.5	54.5 ± 5.3	0.51 ± 0.2	0.41 ± 0.14	171.0 ± 44.1	4.5 ± 1.5	0.13 ± 0.02	0.00023 ± 0.00002
CI at GI_{50}		1.18		0.48		1.18		0.04		0.03
ALL-PO										
GI_{50} (nM)	11.0 ± 3.1	0.91 ± 0.42	59.7 ± 5.6	12.8 ± 4.3	2.3 ± 0.9	0.48 ± 0.1	221.3 ± 35.2	36.4 ± 4.2	0.70 ± 0.05	0.19 ± 0.08
CI at GI_{50}		0.021		0.202		0.196		0.143		0.105

Abbreviations: Ara-C, cytarabine; BCP-ALL, B-cell precursor-acute lymphoblastic leukemia; CC, compound C; CI, combination index; Dex, dexamethasone; Dauno, daunorubicin; GI_{50}, growth inhibition$_{50}$; L-Asp, L-asparaginase; Vcr, vincristine. Synergy, additivity and antagonism are defined by a $CI < 1$, $CI = 1$ or $CI > 1$, respectively. [a]Data are expressed in IU/ml.

compound C with respect to non-translocated cells, with very different levels of GI_{50} and LC_{50}. The specific silencing of AMPK by two different shRNA induced a decrease in cell survival similarly to compound C treatment, thus what we observed after compound C treatment can be mainly attributed to AMPK inhibition. Of note, in non-translocated cells the activation of the pathway downstream AMPK is not modified by specific silencing, confirming our idea that this pathway is specific for MLL-rearranged cells. These results prove that AMPK activation has a very important role in supporting the survival of MLL-rearranged cells and can be specifically targeted in order to induce cell death. Of note, we treated with compound C also the acute monocytic leukemia cell line carrying the t(4;11) MV4;11. These cells showed, as MLL-rearranged BCP-ALL cells, very low levels of GI_{50} (0.62 μM) and LC_{50} (0,77 μM) after 48 h of treatment (data not shown). We then described that compound C-induced apoptosis follows the mitochondrial pathway. We observed mitochondrial membrane depolarization, ROS production, cytochrome C release and activation of caspases followed by PARP cleavage. We previously reported that compound C treatment also brings to a reduction of BCL-2 S70 levels.[19] Compound C-induced apoptosis was already shown to be related to ROS production and BCL-2 downregulation also in multiple myeloma[16] and in glioma cells,[18] thus supporting our findings. Of note, not only cell lines but also MLL-rearranged primary leukemia cells responded to AMPK inhibition with compound C following the mitochondrial apoptotic pathway. MLL-rearranged BCP-ALL primary cultures underwent much more apoptosis than those lacking MLL rearrangements after exposure to low concentrations of compound C, indicating that AMPK is critical for cell survival also in patients' primary cells.

Interestingly, apoptosis occurred after an initial cell cycle block at the G2/M phase in SEM and RS4;11 cells. In agreement with these findings, inhibition of the AMPK pathway in glioma cells[18] also brings to G2/M arrest. In addition, in *Drosophila melanogaster* the activation of the LKB1-AMPK pathway was demonstrated to be strictly required for an accurate mitosis and chromosome segregation.[36,37] Further investigation will be performed in order to understand the mechanisms underlying the G2/M cell cycle block in these cells. On the other hand, ALL-PO cells showed an accumulation in G1 phase after treatment. Very little is known about this cell line, thus further studies, that is, cell cycle proteins mutation status, will be required in order to explain their different cell cycle variation in response to AMPK inhibition. Nevertheless, a decrease in S phase, which reflects a proliferation decrease is a common signature after compound C treatment.

We also investigated the effects of the simultaneous treatment of SEM, RS4;11 and ALL-PO cells with compound C and vincristine, daunorubicin, cytarabine, dexamethasone and L-asparaginase. Our results show that AMPK inhibition enhanced the chemotherapeutic effects of these drugs significantly lowering their GI_{50} values in most of the evaluated conditions. These results suggest that AMPK inhibition could augment the effects of conventional chemotherapy for MLL-rearranged BCP-ALL and pointed towards the potential clinical utility of AMPK inhibitors, although further studies are needed to better understand the molecular mechanism(s) involved in the synergistic effect.

In conclusion, this study provides new insights into the role of AMPK in cancer and, in particular, in BCP-ALL. AMPK activation is required for MLL-rearranged cell survival and its inhibition is able to induce cell death. For these reasons, AMPK could be considered as a new drug target in MLL-rearranged leukemias and kinase inhibitors targeting AMPK should be further studied to make new therapeutic options available for this high-risk form of leukemia.

CONFLICT OF INTEREST

BA, LL, EP, and GB are co-inventors on pending patent applications that cover findings within this paper and the authors could receive royalties as a consequence. These applications have been licensed to Theranostics Health, Inc., and LL and EP are equity shareholders. The other authors declare no competing financial interests.

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REFERENCES

1 Hardie DG, Carling D, Carlson M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* 1998; **67**: 821–855.
2 Hardie DG, Hawley SA, Scott JW. AMP-activated protein kinase--development of the energy sensor concept. *J Physiol* 2006; **574**: 7–15.
3 Xiao B, Heath R, Saiu P, Leiper FC, Leone P, Jing C *et al.* Structural basis for AMP binding to mammalian AMP-activated protein kinase. *Nature* 2007; **449**: 496–500.
4 Alessi DR, Sakamoto K, Bayascas JR. LKB1-dependent signaling pathways. *Annu Rev Biochem* 2006; **75**: 137–163.
5 Motoshima H, Goldstein BJ, Igata M, Araki E. AMPK and cell proliferation--AMPK as a therapeutic target for atherosclerosis and cancer. *J Physiol* 2006; **574**: 63–71.
6 Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S *et al.* Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 2002; **8**: 1288–1295.

7 Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Müller C, Carling D *et al.* Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 2002; **415**: 339–343.
8 Hardie DG, Carling D. The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* 1997; **246**: 259–273.
9 Hardie DG. The AMP-activated protein kinase pathway--new players upstream and downstream. *J Cell Sci* 2004; **117**: 5479–5487.
10 Wang W, Guan KL. AMP-activated protein kinase and cancer. *Acta Physiol (Oxf)* 2009; **196**: 55–63.
11 Fogarty S, Hardie DG. Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. *Biochim Biophys Acta* 2010; **1804**: 581–591.
12 Green AS, Chapuis N, Maciel TT, Willems L, Lambert M, Arnoult C *et al.* The LKB1/AMPK signaling pathway has tumor suppressor activity in acute myeloid leukemia through the repression of mTOR-dependent oncogenic mRNA translation. *Blood* 2010; **116**: 4262–4273.
13 Zhou W, Han WF, Landree LE, Thupari JN, Pinn ML, Bililign T *et al.* Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells. *Cancer Res* 2007; **67**: 2964–2971.
14 Rattan R, Giri S, Singh AK, Singh I. 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside inhibits cancer cell proliferation in vitro and in vivo via AMP-activated protein kinase. *J Biol Chem* 2005; **280**: 39582–39593.
15 Okoshi R, Ozaki T, Yamamoto H, Ando K, Koida N, Ono S *et al.* Activation of AMP-activated protein kinase induces p53-dependent apoptotic cell death in response to energetic stress. *J Biol Chem* 2008; **283**: 3979–3987.
16 Baumann P, Mandl-Weber S, Emmerich B, Straka C, Schmidmaier R. Inhibition of adenosine monophosphate-activated protein kinase induces apoptosis in multiple myeloma cells. *Anticancer Drugs* 2007; **18**: 405–410.
17 Park HU, Suy S, Danner M, Dailey V, Zhang Y, Li H *et al.* AMP-activated protein kinase promotes human prostate cancer cell growth and survival. *Mol Cancer Ther* 2009; **8**: 733–741.
18 Vucicevic L, Misirkic M, Janjetovic K, Harhaji-Trajkovic L, Prica M, Stevanovic D *et al.* AMP-activated protein kinase-dependent and -independent mechanisms underlying in vitro antiglioma action of compound C. *Biochem Pharmacol* 2009; **77**: 1684–1693.
19 Accordi B, Espina V, Giordan M, VanMeter A, Milani G, Galla L *et al.* Functional protein network activation mapping reveals new potential molecular drug targets for poor prognosis pediatric BCP-ALL. *PLoS One* 2010; **5**: e13552.
20 Gobbi A, Di Berardino C, Scanziani E, Garofalo A, Rivolta A, Fontana G *et al.* A human acute lymphoblastic leukemia line with the T(4;11) translocation as a model of minimal residual disease in SCID mice. *Leuk Res* 1997; **21**: 1107–1114.
21 Viola G, Fortunato E, Cecconet L, Del Giudice L, Dall'Acqua F, Basso G. Central role of mitochondria and p53 in PUVA-induced apoptosis in human keratinocytes cell line NCTC-2544. *Toxicol Appl Pharmacol* 2008; **227**: 84–96.
22 Indraccolo S, Tisato V, Tosello V, Habeler W, Esposito G, Moserle L *et al.* Interferon-alpha gene therapy by lentiviral vectors contrasts ovarian cancer growth through angiogenesis inhibition. *Hum Gene Ther* 2005; **16**: 957–970.
23 Accordi B, Pillozzi S, Dell'Orto MC, Cazzaniga G, Arcangeli A, Kronnie GT *et al.* Hepatocyte growth factor receptor c-MET is associated with FAS and when activated enhances drug-induced apoptosis in pediatric B acute lymphoblastic leukemia with TEL-AML1 translocation. *J Biol Chem* 2007; **282**: 29384–29393.
24 Basso G, Buldini B, De Zen L, Orfao A. New methodologic approaches for immunophenotyping acute leukemias. *Heamatologica* 2001; **86**: 675–692.
25 Mihara K, Imai C, Coustan-Smith E, Dome JS, Dominici M, Vanin E *et al.* Development and functional characterization of human bone marrow mesenchymal cells immortalized by enforced expression of telomerase. *Br J Haematol* 2003; **120**: 846–849.
26 Chou TC, Talalay P. Analysis of combined drug effects: a new look at a very old problem. *Trends Pharmacol Sci* 1983; **4**: 450–454.
27 Chen MB, Shen WX, Yang Y, Wu XY, Gu JH, Lu PH. Activation of AMP-activated protein kinase is involved in vincristine-induced cell apoptosis in B16 melanoma cell. *J Cell Physiol* 2011; **226**: 1915–1925.
28 Zhang WB, Wang Z, Shu F, Jin YH, Liu HY, Wang QJ *et al.* Activation of AMP-activated protein kinase by temozolomide contributes to apoptosis in glioblastoma cells via p53 activation and mTORC1 inhibition. *J Biol Chem* 2010; **285**: 40461–40471.
29 Liu B, Fan Z, Edgerton SM, Deng XS, Alimova IN, Lind SE *et al.* Metformin induces unique biological and molecular responses in triple negative breast cancer cells. *Cell Cycle* 2009; **8**: 2031–2040.
30 Woodard J, Joshi S, Viollet B, Hay N, Platanias LC. AMPK as a therapeutic target in renal cell carcinoma. *Cancer Biol Ther* 2010; **10**: 1168–1177.
31 Sengupta TK, Leclerc GM, Hsieh-Kinser TT, Leclerc GJ, Singh I, Barredo JC. Cytotoxic effect of 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) on childhood acute lymphoblastic leukemia (ALL) cells: implication for targeted therapy. *Mol Cancer* 2007; **6**: 46.
32 Kuznetsov JN, Leclerc GJ, Leclerc GM, Barredo JC. AMPK and Akt determine apoptotic cell death following perturbations of one-carbon metabolism by regulating ER stress in acute lymphoblastic leukemia. *Mol Cancer Ther* 2011; **10**: 437–447.
33 Shaw MM, Gurr WK, McCrimmon RJ, Schorderet DF, Sherwin RS. 5'AMP-activated protein kinase alpha deficiency enhances stress-induced apoptosis in BHK and PC12 cells. *J Cell Mol Med* 2007; **11**: 286–298.
34 Mizrachy-Schwartz S, Cohen N, Klein S, Kravchenko-Balasha N, Levitzki A. Up-regulation of AMPK in cancer cell lines is mediated through c-Src activation. *J Biol Chem* 2011; **286**: 15268–15277.
35 Byekova YA, Herrmann JL, Xu J, Elmets CA, Athar M. Liver kinase B1 (LKB1) in the pathogenesis of UVB-induced murine basal cell carcinoma. *Arch Biochem Biophys* 2011; **508**: 204–211.
36 Lee JH, Koh H, Kim M, Kim Y, Lee SY, Karess RE *et al.* Energy-dependent regulation of cell structure by AMP-activated protein kinase. *Nature* 2007; **447**: 1017–1020.
37 Bonaccorsi S, Mottier V, Giansanti MG, Bolkan BJ, Williams B, Goldberg ML *et al.* The Drosophila Lkb1 kinase is required for spindle formation and asymmetric neuroblast division. *Development* 2007; **134**: 2183–2193.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)