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LKB1 DEFICIENCY SENSITIZES CANCER CELLS TO OXIDATIVE STRESS

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Index

Abstract……1

Riassunto……3

1. Introduction……5

1.1 Lung cancer: pathology and treatment options……5

1.2 Liver Kinase B1 (LKB1)……12

1.2.1 LKB1 gene: an overview……12

1.2.2 LKB1 in cancer……13

1.2.3 LKB1 and energy homeostasis……15

1.2.4. LKB1-driven susceptibilities in lung cancer……19

1.3 Redox state regulation and oxidative stress……22

2. Aim of the study……31

3. Materials and methods……33

3.1 Cell lines and culture conditions……33

3.2 Retroviral vectors production……34

3.3 Isogenic cell lines generation……34

3.4 Gene silencing through RNAi……35

3.5 ROS-inducing treatments……36

3.6 Inhibitors……37

3.7 Measurement of intracellular ROS levels……37

3.8 Apoptosis analysis……38

3.9 Cytotoxicity evaluation……38

3.10 Clonogenic potential evaluation……39

3.11 Cell cycle analysis.....40
3.12 RNA extraction, reverse transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR).....40
3.13 Mutational analysis.....42
3.13.1 Sanger sequencing.....42
3.13.2 Next-Generation Sequencing.....43
3.14 Western blot analysis.....43
3.15 Immunohistochemistry analysis (IHC).....44
3.16 Immunofluorescence analysis (IFA).....45
3.17 Characterization of aqueous metabolites using Nuclear Magnetic Resonance (NMR).....46
3.18 Measurement of total and reduced glutathione using High-Performance Liquid Chromatography (HPLC).....46
3.19 Statistical analysis.....46

4. Results.....49
4.1 Characterization of isogenic cell lines.....49
4.2 LKB1+ cells are characterized by reduced expression of *NOX1* and *PPARGC1A*.....51
4.3 LKB1 deficiency and basal oxidative stress.....53
4.4 LKB1 reconstitution in LKB1-deficient cell lines confers resistance to H_2O_2-induced oxidative stress.....54
4.5 LKB1 regulates glutathione homeostasis following oxidative stress.....56
4.6 Insights into the mechanism linking LKB1 to regulation of oxidative stress.....58
4.7 LKB1 deficiency sensitizes cancer cells to cisplatin- and to irradiation-mediated cytotoxic effects.....62
4.8 Cisplatin treatment sensitizes cancer cells to irradiation.....67
4.9 *LKB1* silencing increased sensitivity to oxidative stress.....68

4.10 LKB1+ cells are relatively resistant to chemotherapeutics inducing oxidative stress.....70

4.11 Evaluation of LKB1 status as predictive biomarker of response to ROS-inducing cancer therapies.....72

5. Discussion.....77

6. References.....87

Abbreviations

ACC1/2	Acetyl-coA carboxylase 1/2
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated Protein Kinase
ATP	Adenosine triphosphate
CC	Compound C
CDDP	*cis*-diamminedichloroplatinum(II) (cisplatin)
EGFR	Epidermal growth factor receptor
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
IFA	Immunofluorescence analysis
IHC	Immunohistochemistry
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LKB1	Liver Kinase B1
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOX1	NADPH oxidase 1
NSCLC	Non-small cell lung cancer
OxPhos	Oxidative phosphorylation
pACC	Phosphorylated ACC
pAMPK	Phosphorylated AMPK
PKC-δ	Protein kinase C-delta
pLKB1	Phosphorylated LKB1
PPARGC1A (PGC-1α)	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
ROS	Reactive oxygen species
SRB	Sulforhodamine B
TP53	Tumor protein 53
VEGF	Vascular endothelial growth factor

Abstract

Increased oxidative stress is a common feature of cancer cells. Uncontrolled proliferation, in fact, requires a profound metabolic remodelling, accompanied by altered redox status. Moreover, reactive oxygen species (ROS) are involved in cell signalling, tumor growth and metastasis. Recent studies suggest that increased oxidative stress in cancer cells could be exploited for therapeutic purposes. Understanding biochemical mechanisms involved in ROS generation and maintenance of the cellular antioxidant potential raises the possibility of therapeutic targeting these pathways. In our study, we investigated the role of LKB1/AMPK pathway in response to oxidative stress. LKB1 is a serine threonine kinase whose germ-line mutations are associated with the Peutz-Jeghers syndrome and somatically mutated in certain tumor types, including non-small cell lung cancer (NSCLC) and cervical carcinoma. Through the activation of AMP-activated protein kinase (AMPK), LKB1 modulates both anabolic and catabolic metabolic processes. We observed that LKB1 reconstitution in LKB1-deficient cancer cells significantly reduced expression of NADPH oxidase 1 (NOX1), a ROS-producing enzyme. LKB1+ cells showed reduced endogenous oxidative stress and increased resistance to exogenous oxidative stress, induced by hydrogen peroxide, cisplatin or irradiation, compared to LKB1- cells. Moreover, we observed that AMPK inhibition sensitized LKB1+ cells to oxidative stress, whereas NOX1 inhibition reduced ROS generation and increased resistance to exogenous oxidative stress. In lung cancer samples, *LKB1* mutations were strongly associated with loss of LKB1 protein but the LKB1 status was not associated with response to bevacizumab and platinum-based chemotherapy in advanced NSCLC patients. Overall, these results indicate that LKB1, via the activation of AMPK and the down-regulation of NOX1, confers resistance to oxidative stress and impairs response of cancer cells to some chemotherapeutics and irradiation *in vitro*.

Riassunto

Una caratteristica comunemente riscontrata nel cancro è un aumento dello stress ossidativo, rispetto al corrispondente tessuto sano. Infatti, la proliferazione incontrollata delle cellule tumorali richiede un importante rimodellamento metabolico, che si accompagna ad alterazioni dell'equilibrio redox. Le specie reattive dell'ossigeno (ROS) risultano, inoltre, coinvolte nelle vie di segnalazione cellulare, nella crescita tumorale e nella metastatizzazione del cancro.

Recenti studi suggeriscono che l'aumento dello stress ossidativo possa essere sfruttato a scopi terapeutici, per eliminare selettivamente le cellule tumorali. La comprensione dei meccanismi molecolari che governano la produzione di ROS o il mantenimento del potenziale antiossidante delle cellule potrebbe, infatti, rendere possibile un'azione diretta su questi stessi meccanismi a scopo terapeutico.

In questo lavoro di tesi abbiamo valutato il ruolo della via di segnalazione LKB1/AMPK nella risposta allo stress ossidativo. LKB1 è una serina/treonina chinasi, le cui mutazioni sono state associate alla sindrome ereditaria di Peutz-Jeghers; risulta, inoltre, mutata sporadicamente in alcuni tipi di cancro, tra cui il carcinoma polmonare non a piccole cellule (NSCLC) e il carcinoma della cervice uterina. Attraverso l'attivazione della chinasi AMPK, LKB1 modula sia processi anabolici, sia processi catabolici. I nostri dati hanno dimostrato come la re-introduzione di *LKB1* in cellule tumorali *LKB1*-mutate riduce significativamente l'espressione della NADPH ossidasi 1 (NOX1), un enzima coinvolto nella produzione di ROS. Le cellule LKB1+ manifestano, inoltre, uno stress ossidativo endogeno ridotto, rispetto alle cellule LKB1-, che si accompagna ad un'aumentata resistenza allo stress ossidativo esogeno, indotto dal perossido di idrogeno, dal cisplatino o dall'irradiazione. Abbiamo osservato che l'inibizione di AMPK porta ad una sensibilizzazione delle cellule LKB1+ allo stress ossidativo, suggerendo che la loro ridotta sensibilità sia mediata da AMPK; inoltre,

l’inibizione di NOX1 nelle cellule LKB1- si accompagna ad una ridotta produzione di ROS e ad una maggiore resistenza allo stress ossidativo.

Infine, in campioni di carcinoma polmonare, le mutazioni di *LKB1* sono risultate fortemente associate alla perdita della proteina LKB1; tuttavia, lo *status* di LKB1 non è risultato associato alla risposta al bevacizumab o alla chemioterapia con derivati del platino in pazienti affetti da NSCLC avanzato.

Nel loro insieme, questi risultati indicano che LKB1, attraverso l’attivazione di AMPK e la ridotta espressione di NOX1, conferisce resistenza allo stress ossidativo nelle cellule tumorali, compromettendo la risposta ad agenti chemioterapici o all’irradiazione *in vitro*, i quali modulano l'equilibrio ossidativo della cellula.

1. Introduction

1.1 Lung cancer: pathology and treatment options

Lung cancer is a malignant tumor accounting for many deaths, as it is often diagnosed in advanced stage and is relatively resistant to cytotoxic therapy. Three main types of lung cancer are recognized: non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC) and lung carcinoid. NSCLC, the most common histological type of lung cancer (accounting for 85% of cases), is any type of epithelial lung cancer other than SCLC. NSCLC is further divided in three main histological subtypes: adenocarcinoma, squamous cell carcinoma and large cell carcinoma. SCLC originates from neuroendocrine cells in the bronchus, which may lead to ectopic production of hormones like ADH and ACTH, resulting in paraneoplastic syndromes. This type of lung cancer is rare (accounting for 10-15% of lung cancer cases) and more rapidly metastatic than NSCLC; however, compared to the main lung cancer histological type, it is initially more sensitive to chemotherapy and radiotherapy. Lung carcinoid, finally, is a very rare neuroendocrine tumor (less than 5% of lung cancer cases), that grows slowly and rarely metastasizes.

Lung cancer symptoms are principally coughing, poor appetite, weight loss and dyspnoea. In many patients, when symptoms appear, lung cancer has already spread from the original site. About 10% of patients do not present symptoms at diagnosis. The principal cause of lung cancer is smoking. Cigarette smoke contains at least 73 carcinogens (Hecht, 2012), including benzo[a]pyrene and radioisotope polonium-210. Nevertheless, about 10% of lung cancer cases occur in nonsmokers. These cases are usually caused by passive smoking, radon inhalation, asbestos (O'Reilly *et al.*, 2007), air pollution (Chen *et al.*, 2008) and inherited factors.

From an epidemiological point of view, lung cancer is the most common cancer type in men (in term of incidence and mortality), while it is the third in incidence and the second in mortality (after breast cancer) among women. In 2012, 1.82

million new cases of lung cancer were reported globally, along with 1.56 million deaths, representing 19.4% of all deaths from cancer (World Cancer Report 2014). The incidence of lung cancer is higher in Europe, North America and East Asia, while is much lower in Africa and South Asia. People most likely to develop lung cancer are aged over 50, with a history of smoke. From the 1960s, lung adenocarcinoma started to become more frequent than other types of lung cancer. The cause of this event is partly the introduction of filter cigarettes. Filters remove larger particle from tobacco smoke – thus reducing deposition in larger airways – but increase particle deposition in small airways, where adenocarcinoma arises (Charloux *et al.*, 1997). Considered the high incidence of lung cancer and the prevalence of the histological type NSCLC, the latter constitutes a considerable social problem, with hundred thousand new cases every year and a very low 5-year survival rate, ranging from 9% to 15%, depending on the stage of disease (Wang et al., 2010b).

As other cancers, lung cancer is initiated by mutations (caused by carcinogens) that activate oncogenes or inactivate tumor suppressor genes. In Caucasian population, the four most mutated genes in lung adenocarcinoma (representing 40% of all lung cancer cases) are *TP53*, *KRAS*, *LKB1* and *EGFR* (relative frequencies of mutations vary based on molecular subtype). From a molecular point of view, lung adenocarcinoma can be divided in three subtypes: magnoid, bronchioid and squamoid. Magnoid molecular subtype is mutated principally in *TP53*, *KRAS*, *LKB1* and *EGFR*. Bronchioid subtype is enriched in *EGFR* mutations and less frequently mutated in *TP53*, *KRAS* and *LKB1*. Squamoid subtype, finally, is mutated principally in *TP53*, while mutation frequencies of other genes are low (Wilkerson *et al.*, 2012). Based on these mutation frequencies, lung cancer is principally driven by inactivation of two tumor suppressor genes (*TP53* and *LKB1*) and by activation of two oncogenes (*KRAS* and *EGFR*). Several other genes are less frequently mutated in NSCLC: *LRP1B*, *NF1*, *ATM*, *APC*, *EPHA3*, *PTPRD*, *CDKN2A*, *ERBB4*, *KDR*, *FGFR4*, *NTRK1*, *RB1*, *NTRK3*, *EPHA5*, *PDGFRA*, *GNAS*, *LTK*, *INHBA*, *PAK3*, *ZMYND10*, *NRAS* and *SLC38A3* (Ding *et al.*, 2008). Along with the four most mutated genes, these are the hypothetical 26 driver genes of NSCLC. Moreover, 3%-7% NSCLC cases harbour the fusion oncogene EML4-ALK (Pillai and Ramalingam, 2012). Further mutated genes, albeit with a very low

frequency, are *BRAF*, *HER2*, *PIK3CA*, *MET*, *MEK1* and *AKT1* (Lung Cancer Mutation Consortium, 2011). Specific mutations are associated with specific clinical characteristics of NSCLC and define different molecular subtypes of the pathology. For instance, *EGFR* mutations are prevalent in lung adenocarcinoma of nonsmoker patients (Ren *et al.*, 2012), whereas *KRAS* mutations occur most commonly in adenocarcinoma of life-long smokers (D'Arcangelo and Cappuzzo, 2012).

Treatment of lung cancer depends on the type and stage of tumor. Therapeutic options are surgery, chemotherapy, radiotherapy and targeted therapies. In early-stage NSCLC, surgery – in particular lobectomy – is the treatment of choice. In advanced NSCLC (the vast majority of cases at diagnosis), chemotherapy is used as first-line treatment, improving survival of patients. Usually, a combination of two drugs is used, of which one is a platinum-based drug (cisplatin or carboplatin). The second drug is gemcitabine, paclitaxel, docetaxel, vinorelbine (Clegg *et al.*, 2002), etoposide or pemetrexed (Fuld *et al.*, 2010). Adjuvant chemotherapy – i.e. the use of chemotherapy after surgery – is beneficial in NSCLC, as neoadjuvant chemotherapy – i.e. chemotherapy before surgery. In 1980s *cis*-diamminedichloroplatinum(II) (CDDP or cisplatin) was recognized as the most effective chemotherapeutic in the treatment of lung cancer. The introduction of new anticancer drugs in 1990s prompted platinum doublet therapy, namely the combination of a platinum-based drug with a different chemotherapeutic. Cisplatin kills cancer cells by forming DNA crosslinks as monoadduct, interstrand crosslink, intrastrand crosslink or DNA-protein crosslink. Intrastrand crosslink is the major type of DNA damage induced by cisplatin. The crosslinks generated by cisplatin adducts block DNA replication and induce distortions in the double helix. Once inside the cells, cisplatin is activated through aquation reactions, which substitute the cis-chloro ligands of the drug with water molecules (Kelland, 2000). Aquated cisplatin is a highly reactive species that interacts with many endogenous nucleophiles, such as methionine, metallothionein and proteins, thus being susceptible to cytoplasmic inactivation. Cisplatin that avoids neutralization in the cytoplasm reaches the nucleus and reacts preferentially with guanines in DNA. The consequent DNA damage induces cell cycle arrest in S-phase and elicits DNA repair systems (Wagner and

Karnitz, 2009), which activate apoptosis if repair proves impossible. Cisplatin resistance is mediated by different mechanisms: reduced cellular uptake, increased cellular efflux, increased detoxification of the drug, inhibition of apoptosis or increased DNA repair (Stordal and Davey, 2007).

Radiotherapy is used in combination with chemotherapy in high-grade NSCLC with curative intent. Continuous hyperfractionated accelerated radiotherapy (CHART), consisting of a high dose of radiations given in a short time period (Hatton and Martin, 2010), is the most used in the clinic. In case of bronchus obstruction by cancer, brachytherapy (localized internal radiotherapy) is given to open the passage, allowing reduction in treatment time. As cisplatin, radiotherapy causes both direct and indirect DNA damage. Energy is transferred to tumor tissue through photon (X-radiation and γ-radiation) or charged particles (β-radiation). Both photons and charged particles are ionizing radiations, which directly ionize the atoms of the DNA molecule and indirectly damage cellular genetic material as a result of the ionization of water, producing highly reactive free radicals, principally hydroxyl radicals. Photon radiotherapy causes DNA damage principally through free radicals. Exposure of cells to ionizing radiations induces single-stranded and double-stranded DNA breaks, the latter being much more difficult to repair and leading to dramatic chromosomal aberrations and genetic deletions, thus inducing cell death. Interestingly, radiotherapy is combined with platinum doublet chemotherapy because cisplatin and other platinum-based drugs act as radiosensitizers. The ability of cisplatin to enhance cancer cells killing by ionizing radiation has been attributed to the inhibition of non-homologous end joining (NHEJ), a pathway involved in the repair of double-stranded DNA breaks induced by radiotherapy (Boeckman *et al.*, 2005). Nevertheless, the mechanism of specificity towards cancer cells of this synergistic effect has not been fully understood.

The advent of the genomic era, following completion of the Human Genome Project, led to the introduction of targeted therapies among the treatment options for NSCLC. The outcome in specific subsets of advanced NSCLC patients has been significantly improved by drugs targeted to mutated *EGFR* and *EML4-ALK* fusion oncogene. *EGFR*-mutated cancer cells develop oncogene addiction towards epidermal growth factor receptor (EGFR) signaling, which becomes

constitutively activated in absence of the ligand (epidermal growth factor, EGF). EGFR is a tyrosine kinase receptor controlling a complex signalling pathway, leading to cell proliferation and survival, through the activation of RAS/RAF/MAPK pathway and PI3K pathway (Scaltriti and Baselga, 2006). Activating mutations in exons 18-21 (corresponding to kinase domain of the protein) cause the ligand-independent activation of the receptor through autophosphorylation and the sensitivity to the drug gefitinib (Lynch *et al.*, 2004). Gefitinib (trade name Iressa) is an EGFR-specific inhibitor that binds to the adenosine triphosphate (ATP)-binding site in the kinase domain, inhibiting the activation of anti-apoptotic RAS pathway. Erlotinib (trade name Tarceva) is another tyrosine kinase inhibitor specific to EGFR. Gefitinib and erlotinib, by inhibiting the constitutive activation of EGFR, induce apoptosis in EGFR-addicted (mutated) cancer cells and significantly improve the outcome in NSCLC patients harbouring activating *EGFR* mutations (Costanzo *et al.*, 2011). Importantly, as RAS pathway mediates the anti-apoptotic effect of constitutively activated EGFR, activating mutations of the oncogene *KRAS* predict resistance to EGFR inhibition. In any case, *EGFR* and *KRAS* mutations are mutually exclusive. Unfortunately, *KRAS* mutations are not druggable. Moreover, a missense mutation in exon 20 (T790M) induces secondary resistance to EGFR-inhibiting drugs.

EML4-ALK fusion is the result of an inversion in chromosome 2 that joins the *echinoderm microtubule-associated protein-like 4* (*EML4*) gene with the *anaplastic lymphoma kinase* (*ALK*) gene. *EML4-ALK* fusions occur principally in adenocarcinomas arising in nonsmokers (Martelli *et al.*, 2009) and rarely co-occur with *EGFR* or *KRAS* mutations. ALK is a tyrosine kinase receptor controlling cell proliferation and survival, thus sustaining malignant phenotype when fused to EML4 (Soda *et al.*, 2007). Crizotinib (trade name Xalkori) is a small kinase inhibitor specific to ALK and ROS1. As gefitinib and erlotinib, crizotinib competitively binds to the ATP-binding pockets of targeted kinases. ALK inhibition by crizotinib results in reduced growth, migration and invasion of malignant cells.

A further treatment option for NSCLC is antiangiogenic therapy, a targeted therapy not related to specific mutations. Tumor neongiogenesis is the formation of new blood vessels from pre-existing vasculature towards the

tumor. As cancer grows, in the initial phase of tumorigenesis, the existing vasculature becomes insufficient to provide oxygen and glucose to cells. The switch between a slow-growing tumor and a highly aggressive tumor is mediated by an angiogenic switch. During the angiogenic switch, balance between proangiogenic and antiangiogenic factors is perturbed in favour of the former. Two general mechanisms could induce neovascularization of tumors: (I) angiogenic activity is stimulated by the release of angiogenic molecules such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) by the cancer cells; (II) angiogenic activity arises from host cells recruited by the tumor (e.g. macrophages) or by the mobilization of proangiogenic molecules (produced by host cells) bound to the extracellular matrix (Folkman, 1992). Several pro- and antiangiogenic factors have been described. Proangiogenic factors comprise VEGF, acidic fibroblast growth factor (aFGF), bFGF, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), placenta growth factor (PIGF), insulin-like growth factor, IL-1, hepatocyte growth factor (HGF), transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), angiogenin, angiopoietins, angiotensin II, ceruloplasm, fibrin, IL-8, plasminogen activator, polyamines, urokinase. Antiangiogenic factors comprise angiostatin, endostatin, eosinophilic major basic protein, high-molecular-weight hyaluronan, interferon-γ, interferon-α, IL-4, IL-12, laminin and fibronectin peptides, nitric oxide, platelet factor 4, somatostatin, thrombospondin I, tissue inhibitor of metalloproteinases and retinoids (Sakurai and Kudo, 2011). All these pro- and antiangiogenic factors, along with many others not reported here, constitute a balance finely regulating angiogenesis. Imbalance towards proangiogenic or antiangiogenic factors determines whether endothelial cells proliferate to originate new blood vessels or rest in a non-proliferative state.

Angiogenesis is required for invasive tumor growth and metastasis and its inhibition is a strategy of cancer therapy. The development of antiangiogenic agents followed two main strategies: inhibition of proangiogenic factors as well as therapy with endogenous antiangiogenic factors, such as endostatin and angiostatin (Folkman, 2002). The first approach applied in the clinic was the use of molecules with antiangiogenic activity, such as interferon-α (IFN-α). Inhibition

of tumor angiogenesis is achieved, currently, using neutralizing antibodies directed against specific proangiogenic factors or their receptors on tumor cells, or by inhibitors that interfere with receptor signalling. Thus, antiangiogenic therapy has become a targeted therapy. The most used and the first clinically available antiangiogenic molecule was the VEGF neutralizing monoclonal antibody bevacizumab (trade name Avastin). As VEGF is required for angiogenesis induction and is up-regulated in cancer (Plate *et al.*, 1992), bevacizumab effectively inhibits tumor angiogenesis without affecting physiologic angiogenesis, as endothelium of normal tissues is usually quiescent. Bevacizumab is currently approved by the Food and Drug Administration and by European Medicines Agency for the treatment of certain metastatic tumors. In 2004, bevacizumab was approved for the treatment of metastatic colorectal cancer in combination with standard chemotherapy. Moreover, it was approved for the treatment of inoperable or metastatic non-small cell lung cancer in combination with platinum-based chemotherapy, for the treatment of advanced metastatic renal carcinoma, and for the treatment of epithelial ovarian carcinoma in combination with paclitaxel and carboplatin. Several phase II and phase III clinical trials were conducted to evaluate efficacy and safety of bevacizumab plus chemotherapy in different types of metastatic cancer. In several clinical trials, bevacizumab improved progression-free survival in many solid malignancies when combined to standard chemotherapy, but had little effect on overall survival (Jubb and Harris, 2010).

According to one hypothesis, the combination of bevacizumab with cytotoxic chemotherapy is more effective than chemotherapy alone because VEGF blockade, in addition to causing metabolic stress, induces vascular normalization ((Jain, 2005); Figure 1.1). Blood vessels in tumors are dilated, irregular and excessively tortuous. The dysregulated sprouting of new blood vessels paradoxically causes inhomogeneous delivery of nutrient and oxygen to cancer cells, as angiogenic vessels are irregularly perfused. VEGF blockade by bevacizumab does not destroy tumor vasculature, but transiently normalizes the abnormal function and structure of tumor blood vessels, increasing the efficiency for oxygen and drug delivery.

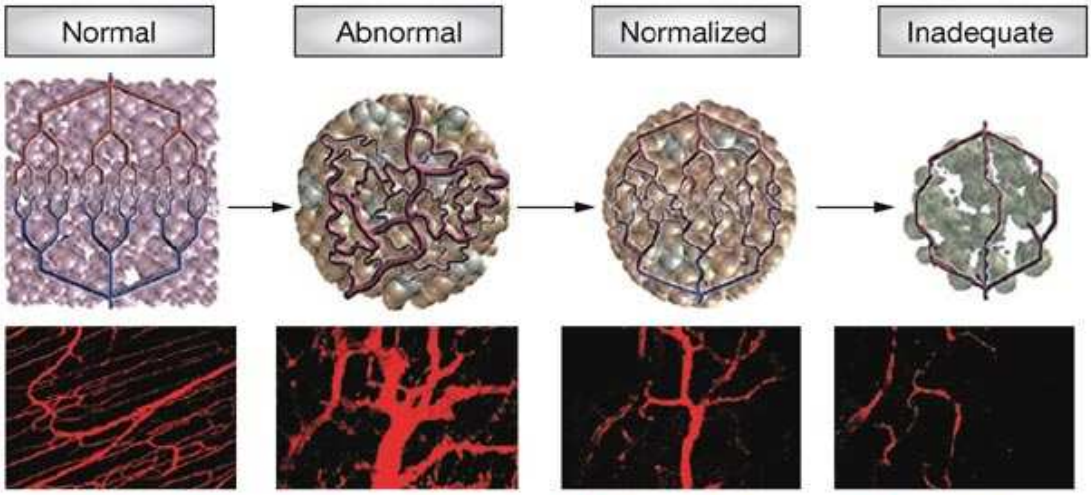

Figure 1.1. While in normal tissues blood vessels are harmonically distributed, tumor vasculature is abnormally dilated and irregular. The addition of an antiangiogenic drug as bevacizumab transiently normalizes tumor blood flow resulting in improved drug delivery of cytotoxic chemotherapy. Continuation of antiangiogenic therapy eventually makes tumor vasculature inadequate to sustain tumor viability. **Bottom panels:** visualization of normal, abnormal, normalized and inadequate vasculature. Adapted from Jain, 2005.

1.2 Liver Kinase B1 (LKB1)

1.2.1 LKB1 gene: an overview

Liver Kinase B1 (*LKB1*, also known as Serine Threonine Kinase 11, *STK11*) is a tumor suppressor gene localized on chromosome 19p13.3. Germline inactivating mutations in *LKB1* are recognized as causative of the inherited disorder Peutz-Jeghers syndrome (Hemminki *et al.*, 1998), a rare autosomic dominant disease characterized by abnormal hyperpigmented macules on the lips and oral mucosa, hamartomatous polyps in the gastrointestinal tract and increased risk of cancer. The involvement of *LKB1* mutations in this cancer-susceptibility syndrome highlights its role as a tumor suppressor gene.

The protein encoded by *LKB1* gene is a serine threonine kinase primarily involved in energy homeostasis control, as discussed below, as well as control of cell proliferation and polarity (Boudeau *et al.*, 2003). Effects of LKB1 activation

are mediated by phosphorylation of AMP-Activated Protein Kinase (AMPK) and at least 12 additional AMPK-related kinases (Liu et al.): BRSK1, BRSK2, NUAK1, NUAK2, QIK, QSK, SIK, MARK1, MARK2, MARK3, MARK4 and MELK (Hardie, 2004). The role of most of these ARKs and their targets in LKB1 signalling is currently largely unknown.

LKB1 protein presents a nuclear localization sequence and it is localized both in the nucleus and in the cytoplasm. Unlike the majority of kinases, activation of LKB1 is phosphorylation-independent and is mediated by direct interaction with the pseudokinase STRADα and an armadillo-repeat containing protein, known as MO25α (Baas *et al.*, 2004). In particular, STRADα assumes a conformation typical of active kinases in presence of ATP and MO25α; this conformation is essential for activation of LKB1 through an allosteric mechanism (Zeqiraj *et al.*, 2009). Although LKB1 activation does not require protein phosphorylation, in human at least eight residues of LKB1 can be phosphorylated. LKB1 phosphorylation is required for the nucleocytoplasmic transport of the protein. The best characterized upstream kinases of LKB1 are Ataxia Telangiectasia Mutated (ATM), the ribosomal protein kinase p90RSK (S6K) and Protein Kinase A (PKA). Recently, however, novel kinases were described as upstream regulators of LKB1, as PKCζ (phosphorylating LKB1 at Ser307; (Xie *et al.*, 2009) and at Ser428; (Song *et al.*, 2008)) and Fyn (Yamada and Bastie, 2014). Moreover, other proteins lacking kinase activity are also able to regulate LKB1 activation, such as the orphan nuclear receptor Nur77 (Zhan *et al.*, 2012) and Poly-ADP Ribose Polymerase (PARP; (Shin et al., 2009)). Considering LKB1 upstream regulators, it is clear that stimuli that lead to LKB1 activation are primarily stressors, like DNA damage and metabolic impairment. Thus, LKB1 phosphorylation represents a rapid response of cells to various type of stress, in order to maintain homeostasis and avoid cell death.

1.2.2 LKB1 in cancer

Somatic mutations of *LKB1* are rarely described in human cancer. The only two exceptions are cervical carcinoma, in which *LKB1* is the most frequently mutated

gene (20% of cases; (Wingo et al., 2009)) and lung cancer (Sanchez-Cespedes, 2007), (Matsumoto *et al.*, 2007), (Strazisar *et al.*, 2009), (Okuda *et al.*, 2011) (Osoegawa *et al.*, 2011), (Zhong *et al.*, 2006), (Launonen, 2005). *LKB1* mutations are very rare in SCLC, but are more frequent in NSCLC, at least in Caucasian population (*LKB1* mutations are rare in Asian lung cancer patients; (Koivunen *et al.*, 2008)). In particular, about 30% of lung adenocarcinoma cases bear *LKB1* inactivating mutations, whereas *LKB1* genetic alterations are less frequent in the other NSCLC subtypes (Carretero *et al.*, 2004).

LKB1 mutations in NSCLC are significantly associated with smoking habit and are more prevalent in males. Genetic alterations in *LKB1* gene comprise missense, splicing sites and nonsense mutations, as well as promoter alterations and homozygous deletion of the entire gene; considering all these genetic and epigenetic events, loss of LKB1 expression is estimated to occur in 90% of NSCLC (Gill *et al.*, 2011).

LKB1 is the third most mutated gene in NSCLC, after *TP53* and *KRAS*. *LKB1* mutations are significantly correlated with *KRAS* mutations (Matsumoto *et al.*, 2007) and mutually exclusive with *EGFR* mutations. The co-occurrence of *LKB1* and *KRAS* mutations drives aggressiveness of lung cancer, with shorter latency and more frequent metastasis (Ji *et al.*, 2007). Given the high prevalence of *LKB1* mutations in NSCLC, a great effort has been put to understand its role in lung tumorigenesis.

Inhibition of mammalian target-of-rapamycin (mTOR) pathway through AMPK activation is one of the most recognized mechanisms of tumor suppression by LKB1 (Shaw *et al.*, 2004), (Shaw, 2009). mTOR pathway sustains and regulates protein synthesis, thus is an anabolic process required to promote cell proliferation and tumorigenesis. AMPK directly phosphorylates two proteins involved in mTORC1 complex formation and activity, TSC2 tumor suppressor and Raptor (Shaw, 2009). The classical AMPK activator metformin, however, failed to phosphorylate AMPK and suppress cell growth in a *LKB1*-deficient background, indicating the requirement of LKB1 to activate AMPK following AMP/ATP ratio increase caused by metformin treatment (Huang *et al.*, 2008). Energy stress is a condition largely present in human tumors, determining AMP increase and ATP decrease. Consequent AMPK activation leads to mTOR inhibition and growth

arrest only in a *LKB1*-proficient milieu. As demonstrated *in vitro* by Dong and colleagues, NSCLC cell lines treated with the glycolysis inhibitor 2-deoxyglucose undergo energy stress, with a consequent phosphorylation of AMPK in *LKB1* wild-type cells and inhibition of mTOR activity. *LKB1* mutant cells, however, are highly resistant to mTOR activity inhibition caused by 2-deoxyglucose and continue to proliferate (Dong et al., 2013b). In light of these data, LKB1/AMPK pathway links metabolism homeostasis and cancer growth control.

Although the role of AMPK in cancer metabolism is widely investigated, however, it is not currently clear whether AMPK activation has anti- or pro-tumorigenic effects. Various findings indicate it has both activities, depending on the cellular context (Faubert *et al.*, 2015), (Liang and Mills, 2013), raising the possibility that also LKB1 could be both a tumor suppressor and a contextual oncogene. Notwithstanding the great majority of published studies indicate a tumor suppressor role for LKB1, in fact, it is also known that LKB1 promotes cancer cells resistance to chemotherapy and radiotherapy (Shin *et al.*, 2014), (Saigusa *et al.*, 2013). Moreover, two AMPK-related kinases, NUAK1 and NUAK2, have been described as oncogenes (Suzuki *et al.*, 2003), (Kusakai *et al.*, 2004), (Namiki *et al.*, 2011a), (Namiki *et al.*, 2011b).

The role of LKB1 in tumor resistance to metabolic stress induced by chemotherapy and irradiation, as well as by antiangiogenic therapy, will be addressed in this thesis.

1.2.3 LKB1 and energy homeostasis

ATP is the key molecule for energy storage in all living cells. It is essential to all processes constituting the anabolic metabolism, like protein and lipid synthesis, and to other processes as intracellular transport of small molecules and organelles and function of antiport pumps that maintain ion gradients and membrane potential. ATP is obtained through catabolic reactions that degrade and finally oxidize nutrients assumed from the diet. The major biochemical processes that constitute the catabolic metabolism are glycolysis, Krebs cycle and oxidative phosphorylation (OxPhos) in mitochondria. Energy is released by

ATP when the last phosphate group is hydrolysed, obtaining one molecule of adenosine diphosphate (ADP). Two molecules of ADP can be condensed to obtain one molecule of ATP and one molecule of adenosine monophosphate (AMP).

Given the importance of ATP, cells developed different mechanisms to control the AMP/ATP ratio and modulate metabolism to maintain energy homeostasis. The principal "energy status guardian" in mammalian cells is AMPK (Hardie and Carling, 1997). AMPK is a heterotrimeric protein composed by three subunits, α, β and γ. The latter subunit contains four Cystathionine Beta Synthase (CBS) repeat sequences, constituting two AMP binding sites called Bateman domains (Adams *et al.*, 2004). When ATP/AMP ratio decreases due to energy stress, AMP binds both Bateman domains; the γ subunit undergoes a conformational change that exposes the catalytic domain in the α subunit. AMPK is activated when threonine 172 in the α subunit is phosphorylated by LKB1 or by Ca^{2+}/Calmodulin-dependent protein kinase kinase β (CAMKKβ; (Witters *et al.*, 2006)). In mammals, the α subunit can exist as either the α1 or α2 isoform, the β subunit can exist as either the β1 or β2 isoform, and the γ subunit can exist as either the γ1, γ2 or γ3 isoform (Kemp *et al.*, 2003). AMPK pathway is depicted in Figure 1.2.

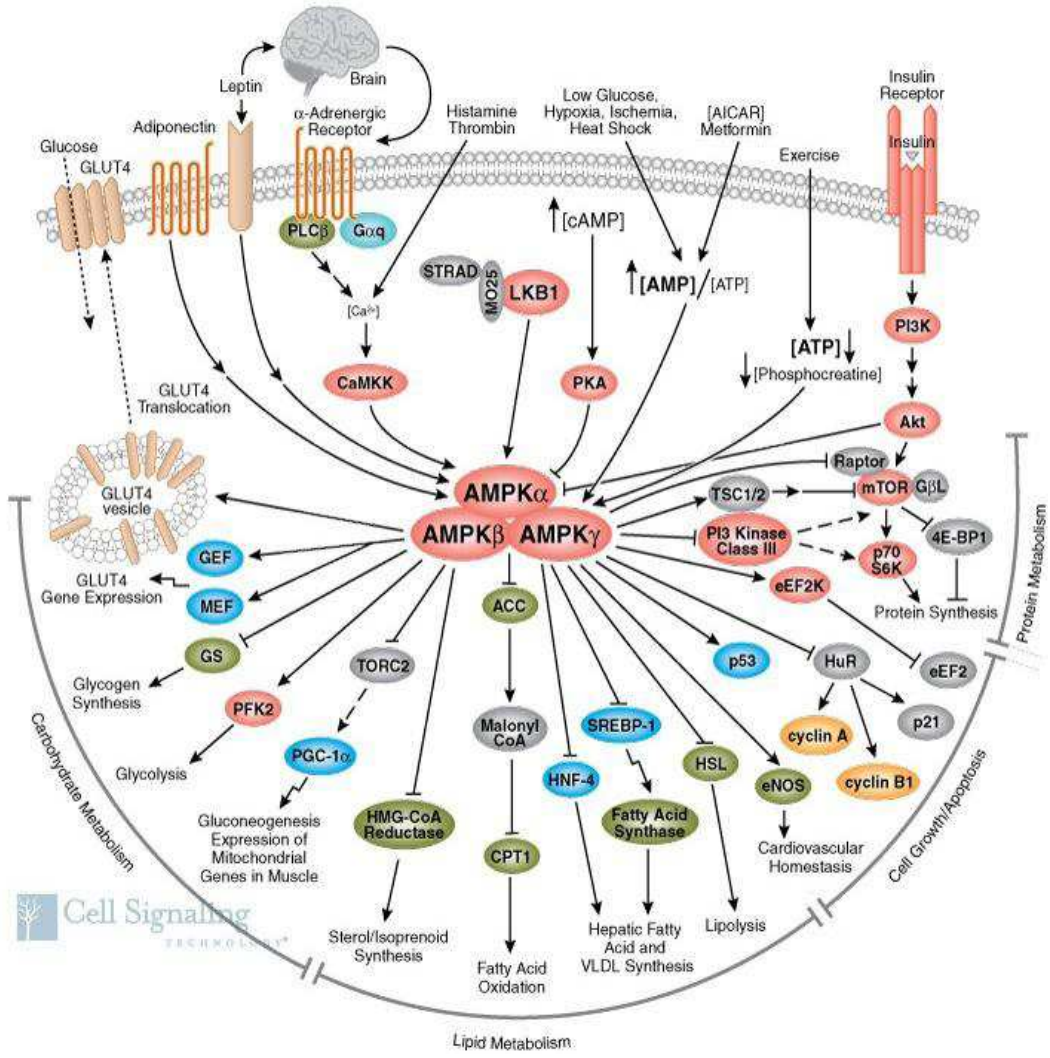

Figure 1.2. AMPK pathway. Source: Cell Signaling Technology.

AMPK switches off biosynthetic pathways consuming ATP and activates catabolic processes generating ATP. Initially described as an inhibitor of fatty acid and cholesterol synthesis, AMPK is an evolutionarily conserved energy stress sensor, since a very similar protein cascade is present in higher plants (Hardie and MacKintosh, 1992). AMPK promotes glucose transport, 6-phosphofructo-2-kinase activity and glycolysis, cell survival, β-oxidation of fatty acids and eNOS activity, while inhibiting glucose-regulated gene transcription, fatty acid synthesis, ion channels, cholesterol synthesis and protein synthesis (Kemp *et al.*, 2003). Moreover, phosphorylated AMPK enhances mitochondrial activity through the activation of several transcription factors, including nuclear respiratory factors (NRF) 1 and 2, PPAR-α, mitochondrial transcription factor A

and myocyte-enhancing factor 2A in skeletal muscle cells. The consequent expression of proteins of the respiratory chain, enzymes of β-oxidation, proteins of the mitochondrial genome and the glucose transporter GLUT4 is coordinated by the activation of PPAR-γ co-activator (PGC) 1, a protein that maximizes transcriptional activity of several transcription factors (Ojuka, 2004), (Reznick and Shulman, 2006).

AMPK mediates LKB1 effects on the ability of a cell to respond to low energy conditions, acting, finally, as a tumor suppressor (Alessi *et al.*, 2006). Tumor cells, in fact, have a great demand of lipids, nucleotides and proteins to sustain rapidly and uncontrolled proliferation, that finally causes energy stress. The consequent activation of LKB1/AMPK pathway inhibits anabolic processes required to biosynthesis and activates catabolic pathways in order to restore the physiologic ATP/AMP ratio. Noteworthy, AMPK is a negative regulator of the Warburg effect and its ablation promotes a metabolic switch to aerobic glycolysis, lipid synthesis and biomass accumulation (Faubert *et al.*, 2013). AMPK inhibition of the Warburg effect is a further mechanism through which LKB1 exerts its tumor suppressor properties. NSCLC cell lines are dependent on glutaminolysis to sustain aerobic glycolysis, in order to maintain Krebs cycle and biomass accumulation (van den Heuvel *et al.*, 2012). Inhibition of aerobic glycolysis by AMPK restores glucose utilization in energy obtaining through Krebs cycle and OxPhos. Hence, glutamine use in reverse Krebs cycle is inhibited and biomass accumulation, is, therefore, repressed. Thus, inhibition of aerobic glycolysis by AMPK accounts, partly, for the anti-proliferative activity of LKB1.

Notwithstanding the role of LKB1/AMPK pathway in metabolic reprogramming, connections between LKB1 and the Warburg effect are poorly described in the literature. Recently, Faubert and colleagues observed that cells lacking LKB1 display increased glucose and glutamine uptake and utilization, in order to provide biosynthetic intermediates for growth. This metabolic reprogramming, resembling the Warburg effect, is dependent on the stabilization of Hypoxia-Inducible Factor-1α (HIF-1α) under normoxia (Faubert *et al.*, 2014).

Notably, LKB1 governs metabolic remodelling through several AMPK-related kinases. NUAK1, for instance, has been described as an inhibitor of glucose uptake through insulin signalling in oxidative muscle (Inazuka *et al.*, 2012).

NUAK2, also known as SNARK, is involved in cellular stress responses linked to obesity and type 2 diabetes (Rune *et al.*, 2009). QIK (SIK2) regulates whole body glucose metabolism decreasing lipolysis from white adipocytes (Park *et al.*, 2014). MARK2 and MARK4 negatively regulate insulin sensitivity in peripheral tissues (Klutho *et al.*, 2011), (Sun *et al.*, 2012). Although AMPK-related kinases are poorly characterized as metabolic modulators, the above-cited data indicate that LKB1 coordinates a complex cellular network to regulate cell metabolism.

1.2.4. LKB1-driven susceptibilities in lung cancer

Recent studies identified specific vulnerabilities driven by LKB1 loss in lung cancer cells.

In the effort to identify novel actionable mutations in NSCLC, Kim and colleagues tested 230,000 synthetic small molecules and 2 whole-genome small interfering RNA (siRNA) libraries in a panel of 91 lung tumor-derived cell lines. Toxicity patterns identified in at least 30% of the NSCLC cell lines were correlated with genomic profiles in order to identify somatic mutations that predict sensitivity or resistance to the tested compounds. They found that co-occurring *KRAS* and *LKB1* mutations drive coatomer complex I (COPI)-dependent lysosome acidification. COPI is a protein complex associated with non-chlatrin coated vesicles and is required for lysosomal maturation. LKB1 depletion was found sufficient to sensitize *KRAS* mutant/*LKB1* wild-type cell lines to COPI depletion. Lysosomal maturation is necessary to support mitochondrial function in *KRAS*/*LKB1* double mutant cell lines. This genetic background promotes energetic stress, as oncogenic RAS induces reactive oxygen species (ROS) accumulation, prevented by LKB1/AMPK-dependent repression of acetyl-coA carboxylases (ACC1/2; (Jeon *et al.*, 2012)). LKB1 loss in a *KRAS*-mutated background probably drives dependency upon hydrolysis of lysosomal macromolecules for supply of mitochondrial Krebs cycle substrates. In fact, the ablation of COPI in *KRAS*/*LKB1* mutated cell lines caused mitochondrial malfunction and cell death. According to Kim and colleagues, lysosome addiction in *KRAS*/*LKB1* double mutant cell lines is driven by ACC1 activation. Co-

occurrence of *KRAS* and *LKB1* mutations is estimated to affect 6-18% of all NSCLC cases, thus the possibility to exploit a treatment based on chemical perturbation of lysosome maturation is of great clinical interest (Kim *et al.*, 2013).

KRAS/*LKB1* double mutant lung cancer cell lines were used also by Liu and colleagues in order to define novel therapeutic targets. Through high-throughput RNA interference screens in lung cancer cell lines from genetically engineered mouse models, the authors identified the deoxythymidilate kinase (DTYMK) as synthetically lethal with *Lkb1* deficiency. DTYMK catalyses the conversion of deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP), the first step in deoxythymidine triphosphate (dTTP) biosynthesis. Metabolomics analysis in *Lkb1*-null cells revealed that *LKB1*-mutated lung cancers have deficits in nucleotide metabolism that confer hypersensitivity to DTYMK inhibition. Short hairpin RNA (shRNA) screens identified 13 genes that confer a growth disadvantage in *Lkb1*-null cells when silenced. The top four candidate genes were *Dtymk*, *Chek1*, *Pdhb* and *Cmpk1*, all involved in anabolic pathways leading to nucleotide synthesis and DNA replication. *Dtymk* knockdown inhibited the growth of *Lkb1*-null cells, while producing a weaker effect in *Lkb1* wild-type cells. Moreover, *Lkb1*-null cells were more prone to DNA damage, as decreased expression of DTYMK respect to *Lkb1* wild-type cells caused dUTP misincorporation in DNA. These results suggest that DTYMK is a potential therapeutic target in *LKB1*-mutated NSCLC. The expression of DTYMK is increased in lung adenocarcinomas in comparison with normal lung, but is decreased in *LKB1*-mutated cancers, in which DTYMK inhibition would cause a decrease in dTTP pool, DNA damage and, finally, cell death. Moreover, through the induction of DNA damage, DTYMK inhibition would synergize with conventional chemotherapeutic drugs, such as cisplatin, that block DNA replication and induce DNA damage (Liu *et al.*, 2013).

Shackelford and colleagues identified the metabolism drug phenformin as selectively inducing apoptosis in *KRAS*/*LKB1* mutated NSCLC cells lines. As metformin, phenformin inhibits complex I of the mitochondria, resulting in increase of AMP and ADP levels. Phenformin, but not metformin, induced apoptosis in *LKB1*-deficient cell lines, as a result of ATP levels drop. In fact, AMPK pathway is not activated by phenformin in a *LKB1*-mutated background, thus

cells are not able to react to ATP decrease caused by mitochondrial complex I inhibition. *LKB1*-deficient NSCLC cell lines do not induce mitophagy, thus accumulating damaged mitochondria. This causes the complete loss of mitochondrial potential following phenformin treatment, while in *LKB1* wild-type cells mitochondrial potential is preserved (Shackelford *et al.*, 2013).

Inge and colleagues discovered that *LKB1* inactivation sensitizes NSCLC cell lines to endoplasmic reticulum (ER) stress induced by pharmacological treatment. In eukaryotic cells, some proteins are synthesized in the endoplasmic reticulum. Nutrient deprivation and hypoxia cause the accumulation of unfolded and misfolded proteins in the ER, inducing ER stress. 2-deoxyglucose (2-DG) showed anticancer activity in *KRAS*/*LKB1* double mutant tumors. By inhibiting glucose dependent protein glycosylation, 2-DG aggravates ER stress in cancer cells. Moreover, *LKB1*-mutated cell lines were more sensitive to ER stress-inducing drugs, such as tunicamycin and brefeldin A. While unfolded protein response is protective in case of low ER stress, it is pro-apoptotic in case of acute ER stress. Thus, in *LKB1*-deficient cell lines ER stress induced by tunicamycin or brefeldin A activates pro-apoptotic unfolded protein response, which causes cell death through ROS production. As tunicamycin and brefeldin A are not currently used in the clinic, the authors tested bortezomib and celecoxib, known to induce ER stress. Both these drugs preferentially reduced the survival of LKB1-deficient cell lines compared to LKB1-proficient cell lines. These results imply that *LKB1*-mutated NSCLC could be hypersensitive to ER stress aggravators (Inge *et al.*, 2014).

In conclusion, the above-presented studies indicate that *LKB1* mutations in NSCLC confer sensitivity to lysosomal maturation inhibiting treatments, to DNA damage inducing agents, to inhibitors of mitochondrial respiration and to ER stress inducing drugs, thus envisaging novel therapeutic interventions in a large number of cancer cases.

1.3 Redox state regulation and oxidative stress

Energy obtaining from nutrients in eukaryote cells requires the activity of the mitochondrial electron transport chain. Even in cancer cells displaying the Warburg effect, which use prevalently glycolysis in order to obtain energy and biochemical intermediates, inhibition of the electron transport chain with mitochondrial poisons, as cyanide, is lethal. Oxidative phosphorylation (OxPhos) is a catabolic reaction that couples the transfer of electrons derived from Krebs cycle through the electron transport chain subunits to the reconstitution of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate. The final acceptor of electrons is the oxidant molecule dioxygen.

Carbohydrate and lipid catabolism produce acetyl-CoA, a small molecule that enters the mitochondria and is condensed with oxaloacetate to produce citrate, the first intermediate of Krebs cycle. Krebs cycle or citric acid cycle is an amphibolic metabolic pathway (participating in both catabolic and anabolic processes), which finally oxidizes the two carbon atoms from acetyl-CoA in two carbon dioxide molecules and derives a GTP molecule (directly used to reconstitute ATP), three NADH molecules and a $FADH_2$ molecule. These reduced cofactors are able to transport high-energy electrons to the mitochondrial respiratory chain. The transfer of electrons through the four complexes constituting the mitochondrial electron transport chain is coupled with the transfer of protons across the mitochondrial inner membrane. The electrochemical proton gradient constituted through the activity of mitochondrial respiratory chain drives ATP synthesis by the complex V, ATP synthase. NADH transfers two electrons to complex I of the mitochondrial respiratory chain and is oxidized to NAD^+; $FADH_2$ transfers two electrons to complex II of the mitochondrial respiratory chain and is oxidized to FAD. Moreover, succinate produced by Krebs cycle is oxidized to fumarate at the level of complex II, also known as succinate dehydrogenase. Electrons derived from NADH, $FADH_2$ and succinate are transported through complex III to complex IV, where molecular oxygen accepts electrons and is converted into water. The reactions of oxidation and reduction that sustain the activity of the

mitochondrial electron transport chain contribute to regulate the cellular redox state.

A small percentage of transported electrons reacts prematurely with oxygen, generating superoxide ($O_2^{\cdot-}$), a reactive oxygen species (ROS), principally at complex I and complex III ((Li *et al.*, 2013); Figure 1.3). Thus, OxPhos is the principal site of ATP production in eukaryote cells, but also a potential source of oxidative stress.

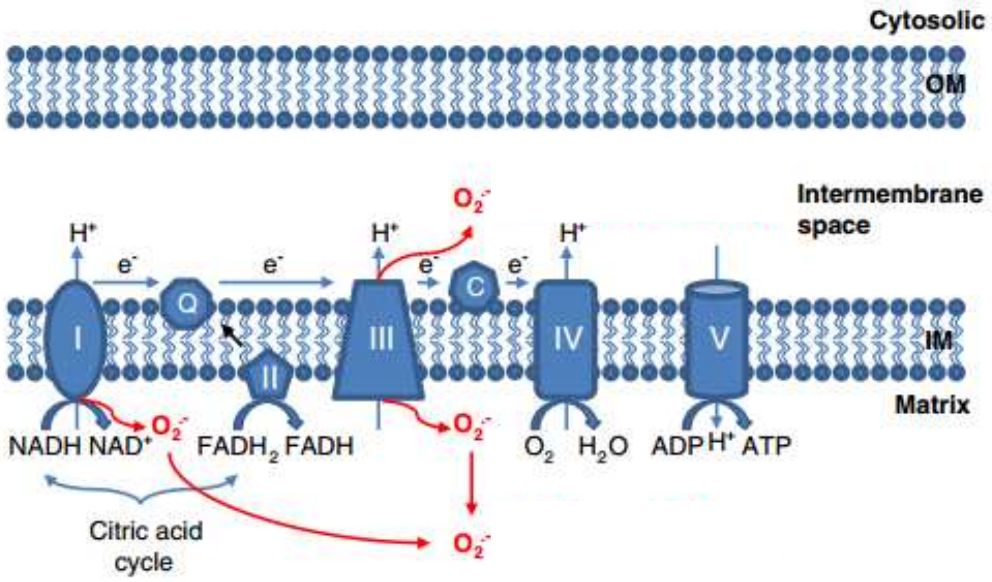

Figure 1.3. Electron leakage to oxygen from complex I in the mitochondrial matrix and from complex III both in the intermembrane space and in the matrix. Adapted from Li *et al.*, 2013.

Superoxide rapidly reacts with water generating oxygen and two other ROS, hydrogen peroxide (H_2O_2) and hydroxyl ion (OH-). The latter ROS is very reactive and oxidizes every molecule in its path, causing damage to protein and principally to lipids (lipid peroxidation), but not to DNA, as its high reactivity prevents it from reaching the nucleus. H_2O_2, instead, is less reactive and has a longer half-life. Thus, this ROS is able to cause oxidative damage to DNA molecule. Both H_2O_2 and hydroxyl ion are able to initiate lipid peroxidation, a chain reaction mechanism causing important damage to cell membrane. In this process, a reactive oxygen species removes a hydrogen atom from a polyunsaturated fatty acid, producing water and a fatty acid radical. Fatty acid

radicals are not stable and rapidly react with molecular oxygen to produce peroxyl-fatty acid radicals. These radicals, subsequently, react with other polyunsaturated fatty acids, producing fatty acid radicals and lipid peroxides, thus establishing a chain reaction (Figure 1.4). The final products of lipid peroxidation are reactive aldehydes, as malondialdehyde and 4-hydroxynonenal, which may be mutagenic and carcinogenic. For instance, malondialdehyde reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts (Marnett, 1999). Thus, use of oxygen by mitochondria is advantageous as it allows a great efficiency of energy derivation from nutrients, but at the same time is harmful, as metabolic by-products (ROS) generated by mitochondrial respiration cause self-propagating oxidative stress.

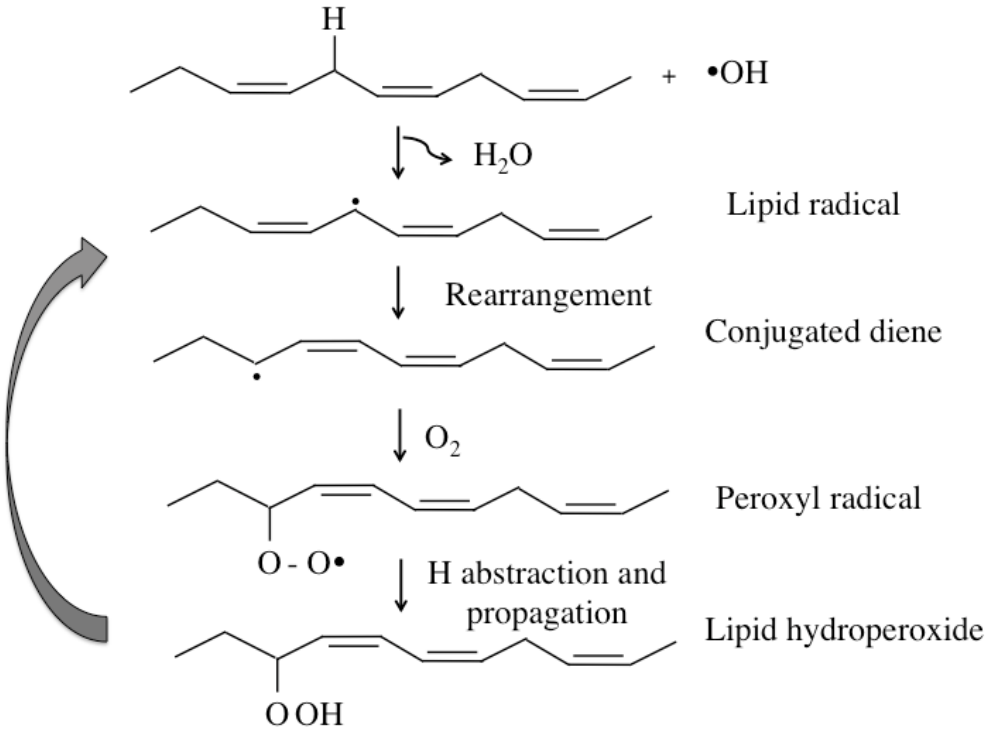

Figure 1.4. Scheme of lipid peroxidation sequential reactions. Adapted from (Jairam *et al.*, 2012).

Noteworthy, ROS are not only metabolic by-products. Immune system cells, principally neutrophils, produce ROS (through the non-mitochondrial respiratory burst catalyzed by NADPH oxidase) in order to kill pathogens (Segal, 2005). Moreover, low levels of ROS are physiologically important in intracellular

signalling (Remacle *et al.*, 1995). In fact, many human proteins, such as the transcription factor NF-κB, through their thiol groups, are redox-sensitive. Protein phosphorylation, one of the most important mediators of cell signalling, is a redox-sensitive process (Sen, 2000). ROS production during hypoxia is necessary to HIF-1α stabilization and to many of the cellular responses to hypoxia (Hamanaka and Chandel, 2010). Moreover, ROS are able to inhibit different phosphatases, such as PTP1B, PTEN and MAPK phosphatases, through oxidation of the reactive cysteine in the catalytic domain (Hamanaka and Chandel, 2010). Given the importance of ROS as signalling second messengers, excessive oxidative stress is detrimental as it disrupts intracellular signalling.

In order to cope with oxidative stress, cells have developed different antioxidant systems. An antioxidant is a molecule that inhibits oxidation of other molecules, by scavenging ROS. Antioxidants terminate chain reactions involved in ROS generation, as lipid peroxidation, by removing free radical intermediates. Cells maintain different types of antioxidants, such as glutathione, vitamin C, vitamin A, vitamin E, as well as enzymes such as catalase, superoxide dismutase and various peroxidases.

One of the most important antioxidants, with a central role in maintaining cellular redox state, is glutathione (GSH). GSH is a tripeptide constituted by glutamate, glycine and cysteine, with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and the amine group of cysteine. By acting as an electron donor, GSH reduces disulfide bonds within proteins to cysteine. In this process, glutathione is converted to its oxidized form (GSSG). Once oxidized, glutathione can be reduced back by glutathione reductase, using NADPH as an electron donor. Under physiological conditions, glutathione is prevalently present in cells in its reduced form.

GSH is not an essential nutrient and is synthesized by cells from the amino acids L-cysteine, L-glutamic acid and glycine. Cells produce glutathione in two ATP-dependent steps: (I) γ-glutamylcysteine is synthesized via the enzyme glutamate cysteine ligase (GCL). This reaction is the rate-limiting step in glutathione synthesis (Lu, 2009); (II) glycine is added to the C-terminal of γ-glutamylcysteine via the enzyme glutathione synthase (GSS). GSH participates directly in neutralization of reactive oxygen species and in maintenance of exogenous

antioxidants, such as vitamin C and E, in their reduced form. Moreover, glutathione is involved in DNA synthesis (Suthanthiran *et al.*, 1990) and in iron metabolism (Kumar *et al.*, 2011). Given the central role of glutathione in different functions essential to cellular viability, cells need to maintain an elevated GSH/GSSG ratio. This can be achieved through *de novo* glutathione synthesis and by reduction of GSSG to GSH. Therefore, two cellular processes are central in reduced glutathione replenishment: (I) methionine conversion to cysteine through methionine cycle (dependent on folate cycle) and transsulfuration pathway are essential for *de novo* glutathione synthesis, whereas (II) the pentose phosphate pathway, producing NADPH, is critical for GSSG reduction to GSH.

Oxidative stress is induced both by increased ROS production and by antioxidants depletion ((Scandalios, 2002); Figure 1.5). Cell proliferation is the principal cause of excess endogenous ROS production, as a consequence of anabolic reactions necessary to DNA, protein and lipid synthesis, which require mitochondrial metabolism. Thus, cancer cells are characterized by an increased steady-state ROS level compared to normal cells. Moreover, Warburg effect is associated with increased ROS levels through decreasing cancer cells antioxidant capacities (El Sayed *et al*.). In fact, glucose has an essential antioxidant function, acting as substrate of the pentose phosphate pathway (Cardone *et al.*, 2012), and its excessive use in glycolysis causes a decrease of reducing equivalents (i.e. NADPH). Furthermore, pyruvate (but not lactate) has a potent antioxidant function (El Sayed *et al.*, 2012).

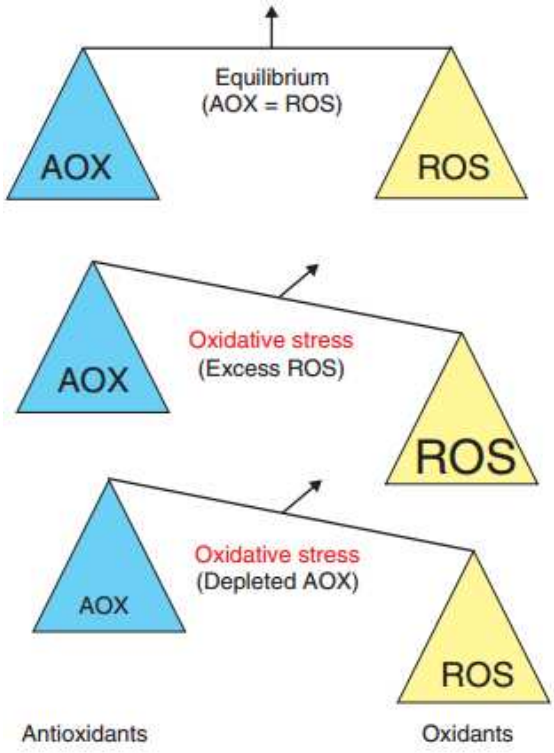

Figure 1.5. Under physiological conditions, cells are able to balance levels of ROS and antioxidants, resulting in redox equilibrium. Cells are subjected to oxidative stress when ROS production is increased or antioxidant species are depleted. Adapted from Scandalios, 2002.

Consequently, cancer cells are subjected both to increased ROS production and to antioxidant species depletion. Nevertheless, cancer cells redox state is paradoxical: increased ROS production is associated with increased antioxidant species production, as adapting response becoming essential to cancer progression and chemoresistance (Traverso *et al.*, 2013). Thus, redox equilibrium in cancer cells is shifted to a higher level. Cancer cells develop an antioxidant species addiction and are more susceptible to a further increase in oxidative stress. This biochemical property of tumor cells can be exploited for therapeutic purposes (Trachootham *et al.*, 2009). In fact, the vast majority of cancer therapies works in part causing oxidative stress (either by inducing ROS production or by depleting glutathione and other antioxidant species). As steady-state oxidative stress is higher in cancer cells than in normal cells, exogenous ROS stress or antioxidants depletion increase oxidative stress overcoming the threshold separating cell survival from cell death only in cancer cells (Figure 1.6).

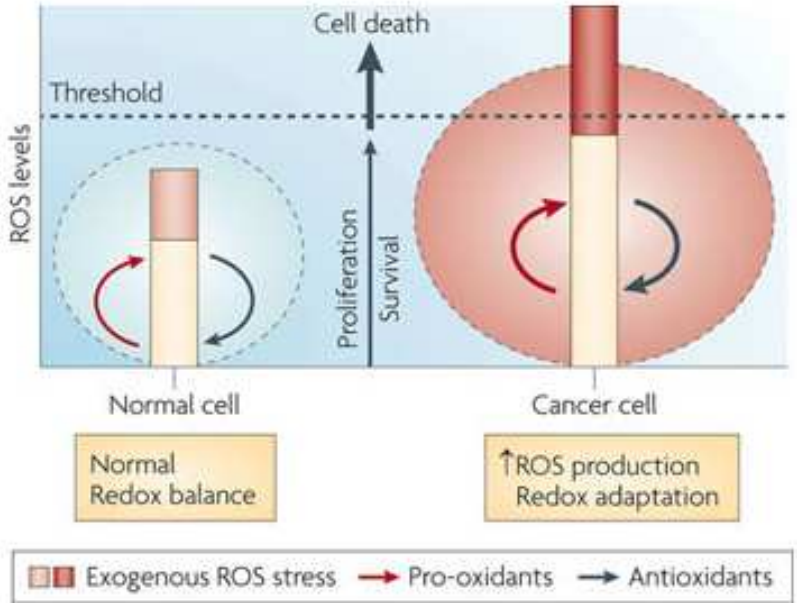

Figure 1.6. Exogenous ROS stress increases ROS levels both in normal and in cancer cells. In the latter, however, ROS levels exceed the cell death threshold, as basal oxidative stress is higher. Adapted from Trachootham *et al.*, 2009.

Antioxidants supplementation in diet has always been considered beneficial, as ROS may have a pathogenetic role in many diseases, like cancer. In fact, oxidative damage of DNA causes mutations and plays an important role in the initiation and progression of multistage carcinogenesis (Waris and Ahsan, 2006). Nevertheless, given the role of antioxidant species in chemoresistance and in cancer cells survival to oxidative stress, antioxidants supplementation could be detrimental and, as sustained by the Nobel Prize James Watson, "free-radical-destroying antioxidative nutritional supplements may have caused more cancers than they have prevented" (Watson, 2013). Conversely, as mentioned, the vast majority of cancer therapies kills cancer cells by increasing oxidative stress beyond the cell death threshold. This mechanism of action is shared by different chemotherapeutic agents and by radiotherapy (Borek, 2004). Some of the traditional anticancer drugs that induce lethal oxidative stress in cancer cells are cisplatin (Casares *et al.*, 2012), paclitaxel and other taxanes (Alexandre *et al.*, 2007), doxorubicin (Tsang *et al.*, 2003) and cytarabine (Iacobini *et al.*, 2001). Moreover, new anticancer drugs inducing oxidative stress have been developed, as elesclomol (Kirshner *et al.*, 2008), a small molecule that exerts its anticancer

function selectively through the induction of ROS, unlike the above-mentioned drugs.

Cancer cells react to oxidative stress inducing drugs by up-regulating antioxidant systems, principally glutathione and thioredoxin. GSH is essential to neutralize ROS induced by cancer treatments and to detoxify anticancer compounds by conjugating to electrophilic drugs through the enzyme glutathione-S-transferase (Townsend and Tew, 2003). For instance, the role of glutathione in the regulation of cisplatin resistance has been well documented (Chen and Kuo, 2010). During chemotherapy and radiotherapy, glutathione is continuously depleted. Oxidative stress induces activation of Nrf2 (Nuclear Factor Erythroid 2 – Related Factor 2; NFE2L2) – Keap1 (Kelch-like Erythroid cell-derived protein with CNC homology Associated Protein 1) signalling pathway and NF-κB pathway (Schreck *et al.*, 1992). Nrf2-Keap1 pathway is one of the most important cell defense and survival pathways (Jaramillo and Zhang, 2013). Nrf2 transcription factor (TF) protects cancer cells from oxidative stress by increasing the expression of different antioxidant genes. Among them, GCLC (glutamate cysteine ligase, catalytic subunit), GSR (glutathione reductase) and xCT (cystine transporter), involved in glutathione metabolism, TXN1 (thioredoxin) and TXNRD1 (thioredoxin reductase 1), involved in thioredoxin production and utilization, GPX2 (glutathione peroxidase 2), involved in glutathione utilization, G6PD (glucose-6-phosphate dehydrogenase) and PGD (phosphogluconate dehydrogenase), involved in NADPH production (Gorrini *et al.*, 2013). Nrf2 binds to the Antioxidant Response Elements (ARE) in the upstream regulatory region and enhances the expression of the above-cited genes in cancer cells treated with pro-oxidants or certain chemotherapeutic agents. Interestingly, as other TFs, Nrf2 has a dual role: its expression in normal cells maintains redox equilibrium by eliminating carcinogens and, thus, contributing to cancer prevention; its common overexpression in cancer cells, instead, is associated with acquired chemoresistance and an aggressive phenotype (Lau *et al.*, 2008).

Considering the anticancer properties of ROS-inducing therapies and the pro-tumorigenic role of a sustained antioxidant response in treated cancer cells, the combination of oxidative stress-inducing therapy with an antioxidant-depleting therapy would be promising. An instance is the combination of the thioredoxin

and glutathione inhibiting and ROS generating agent arsenic trioxide (As_2O_3) with ascorbic acid, which is converted in its oxidizing form dehydroascorbic acid (Watson, 2013). Unfortunately, clinically usable drugs able to lower glutathione levels in cancer cells are not yet available. Use of the glutathione synthesis inhibitor L-buthionine-(S,R)-sulfoximine (BSO) causes up-regulation of Nrf2 transcription factor that, consequently, enhances glutathione synthesis (Lee *et al.*, 2008). Motexafin gadolinium is a porphyrin molecule that, through a futile redox recycling, transfers hydrogen atoms from antioxidants to produce ROS. Unfortunately, its combination with radio- and chemotherapies has shown only modest life extensions in clinical trials (Watson, 2013). Recently, the natural product piperlongumine from the *Piper longum* plant was revealed as a potential anticancer drug. Most interesting, this molecule exerts its activity binding to the active sites of several key cellular antioxidant enzymes, as glutathione-S-transferase, thus blunting antioxidant response of cancer cells to oxidative stress inducing therapies (Raj *et al.*, 2011). The discovery, in the future, of antioxidants blocking agents, as piperlongumine, with a clinical efficacy and safety and the combination of these molecules with traditional – such as cisplatin or doxorubicin – or novel – such as elesclomol – ROS-inducing drugs may improve the outcome of cancer treatments.

In this thesis work, we investigated the role of *LKB1* tumor suppressor gene, commonly mutated in certain types of human cancer, in the response to oxidative stress induced by different agents, such as some chemotherapeutics – comprising cisplatin, paclitaxel, doxorubicin and arsenic trioxide – and γ-irradiation. Moreover, we explored a novel mechanism of action accounting for differential sensitivity to exogenous oxidative stress in LKB1-deficient and LKB1-proficient cancer cell lines.

2. Aim of the study

Lung cancer is the primary cause of cancer death worldwide in men and the second in women, after breast cancer. Non-small cell lung cancer (NSCLC), the most frequent histologic type, accounts for 85-90% of diagnosed lung cancer cases. Incidence of lung cancer in 2012 was 1.82 million new cases globally, with 1.56 million deaths (World Cancer Report 2014). NSCLC represents, hence, a remarkable socio-economic problem. Treatment of inoperable NSCLC comprises a combination of chemotherapy with radiotherapy. With the advent of the genomic era, several oncogene mutations were identified in NSCLC and exploited for the development of novel targeted therapies. For instance, gefitinib and erlotinib are used to treat *EGFR*-mutated non-small cell lung cancer, while the inhibitor crizotinib is beneficial in a subset of NSCLC patients characterized by the *EML4-ALK* fusion oncogene. Moreover, the anti-VEGF monoclonal antibody bevacizumab, in combination with carboplatin and paclitaxel, is one of the targeted therapy options available for advanced NSCLC, improving the progression-free survival of treated patients.

Regarding targeted therapies, stratification of patients, that is the identification of patients that can benefit from the administration of a targeted drug, is necessary. Nevertheless, stratification criteria for antiangiogenic therapy and for radio-chemotherapy are currently unavailable. These therapies share the induction of metabolic perturbations and oxidative stress as mechanisms of action. Energy stress and the related induction of reactive oxygen species (ROS) production activate the tumor suppressor LKB1, the third most mutated gene in NSCLC (30% of adenocarcinoma cases, corresponding to hundred thousand new events every year). LKB1 controls a complex metabolic reprogramming coordinated by the downstream kinase AMPK. Our previous findings indicated that AMPK modifies the pathologic response of tumor xenografts to anti-VEGF therapy (Nardo *et al.*, 2011). In particular, experimental tumors bearing a dysfunctional LKB1/AMPK pathway developed large necrotic areas following VEGF blockade. These results raise the possibility that LKB1/AMPK pathway

could protect cancer cells from energy stress induced by antiangiogenic treatment and that *LKB1*-mutated tumors could be more sensitive to antiangiogenic therapy. Moreover, previous findings suggest that activation of LKB1/AMPK pathway may lead to acquired chemoresistance and radioresistance in treated cancer cells, through decreased oxidative stress.

In light of these considerations, the aim of this study is to determine, in an *in vitro* model, the role of the tumor suppressor gene LKB1 in the response of tumor cells to oxidative stress induced by some chemotherapeutics and by radiation therapy. Moreover, a second aim of this project is to establish whether *LKB1* mutational status can be used as a stratification criterion for NSCLC patients treated with a combination of chemotherapy and radiotherapy and whether *LKB1*-mutated patients could benefit from an oxidative stress-inducing therapeutic regimen. In parallel, we propose to investigate the possible correlation between *LKB1* mutational status and AMPK activation with clinical outcome in bevacizumab-treated advanced NSCLC patients.

3. Materials and methods

3.1 Cell lines and culture conditions

In this thesis work, the following cell lines were used:

- ✓ A549: NSCLC cell line, purchased from ATCC (Manassas, VA, USA) and cultured in DMEM-F12 medium (Sigma-Aldrich; Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (EuroClone; Milan, Italy), 2 mM Ultraglutamine 1 (Lonza; Basel, Switzerland) and 1% Anti-Anti (antibiotic-antimycotic mix, Life Technologies; Carlsbad, CA, USA).

- ✓ H460: NSCLC cell line, purchased from ATCC and cultured in RPMI-1640 medium (EuroClone) supplemented with 10% fetal bovine serum (EuroClone), 10 mM Hepes buffer (Lonza), 2 mM Ultraglutamine 1 (Lonza), 1 mM Na Pyruvate (Lonza) and 1% Anti-Anti (Life Technologies).

- ✓ HeLa: cervical carcinoma cell line, purchased from ATCC and cultured in DMEM High Glucose medium (EuroClone) supplemented with 10% fetal bovine serum (EuroClone), 2 mM Ultraglutamine 1 (Lonza) and 1% Anti-Anti (Life Technologies).

- ✓ NCI-H1975: NSCLC cell line, purchased from Banca Biologica e Cell Factory (IRCCS San Martino, Genova) and cultured in RPMI-1640 medium (EuroClone) supplemented with 10% fetal bovine serum (EuroClone), 10 mM Hepes buffer (Lonza), 2 mM Ultraglutamine 1 (Lonza), 1 mM Na Pyruvate (Lonza) and 1% Anti-Anti (Life Technologies).

- ✓ NCI-H1650: NSCLC cell line, purchased from Banca Biologica e Cell Factory and cultured in RPMI-1640 medium (EuroClone) supplemented with 10% fetal bovine serum (EuroClone), 10 mM Hepes buffer (Lonza), 2 mM Ultraglutamine 1 (Lonza), 1 mM Na Pyruvate (Lonza) and 1% Anti-Anti (Life Technologies).

Cells were cultured in humidified atmosphere at 37°C, with 5% CO_2. In all experiments, cells were trypsinized and counted using Countess™ Automated Cell Counter (Life Technologies).

3.2 Retroviral vectors production

Viral vectors were generated in our laboratory using HEK 293T human embryonic kidney cell line, due to its high transfection capacity. In particular, cells were transfected with 3 plasmids using calcium phosphate transfection method. The viral vectors produced in this way belong to the HIV-based vectors called SIN (self-inactivating). The following plasmids were used in retroviral vectors production:

- ✓ A plasmid coding the transgene of interest
- ✓ A packaging plasmid (gag-pol gpt), coding HIV *gag* and *pol* genes
- ✓ pHCMV-G plasmid driving the expression of Vesicular Stomatitis Virus protein G (VSV-G), which allows to extend the tropism of the virus.

3.3 Isogenic cell lines generation

A549, H460 and HeLa cells present inactivating mutations in *LKB1* gene, causing the loss of the protein. In order to generate isogenic pairs for LKB1 expression,

LKB1-deficient cells were transduced with a retroviral vector coding for LKB1 wild-type (RV-LKB1 wt) or with the empty vector (RV-pBABE).
Cells transduced with RV-pBABE retroviral vector were renamed "LKB1-", whereas cells transduced with RV-LKB1 wt retroviral vector were renamed "LKB1+".
Retroviral vectors contained, in addition to insert sequence, a puromycin resistance gene; this allows to select transduced cells with the aminonucleoside antibiotic puromycin, using the following concentrations:

- ✓ A549 and H460 cells: 4 µg/ml
- ✓ HeLa cells: 2 µg/ml

3.4 Gene silencing through RNAi

$3 \cdot 10^5$ cells per well were plated in 6-wells tissue culture plates and incubated, in order to allow adhesion. The following day, a mix of Opti-MEM® I Medium with GlutaMAX™ (Life Technologies) and Stealth RNAi™ siRNA 20 nM (Life Technologies) was blended, in equal volumes, with a mix of Opti-MEM® I Medium with GlutaMAX™ and Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies), after 5 minutes of incubation at room temperature. Following additional 20 minutes of incubation, in order to permit the formation of cationic liposomes covered with siRNA molecules, 500 µl of final mix were added, drop by drop, to adherent cells, along with 1.5 ml of culture medium.
Lipofection was blocked after 6 hours, through removal of medium with liposomes, two washings with medium without serum and addition of culture medium.
Sequences of Stealth RNAi™ siRNAs are reported in Table I.

Stealth siRNA name	Stealth siRNA code	Sense sequence
siRNA	Negative Control Low GC Duplex	AGCUACACUAUCGAGCAAUUAACUU
siLKB1$^{(1)}$	STK11HSS110329	UCUACAACAUCACCACGGGUCUGUA
siLKB1$^{(2)}$	STK11HSS110330	GGAAAUUCAACUACUGAGGAGGUUA
siLKB1$^{(3)}$	STK11HSS110331	CCACGGGUACUUCUGUCAGCUGAUU
siAMPKα1$^{(1)}$	PRKAA1HSS108454	CCCAUCCUGAAAGAGUACCUUCUU
siAMPKα1$^{(2)}$	PRKAA1HSS108455	CCCUCAAUAUUUAAAUCCUUCUGUG
siAMPKα1$^{(3)}$	PRKAA1HSS108456	ACCAUGAUUGAUGAUGAAGCCUUAA
siAMPKα2$^{(1)}$	PRKAA2HSS108457	ACCGAGCUAUGAAGCAGCUGGAUUU
siAMPKα2$^{(2)}$	PRKAA2HSS108458	GCAGGUCCUGAAGUUGAUAUCUGGA
siAMPKα2$^{(3)}$	PRKAA2HSS108459	CCCACUGAAACGAGCAACUAUCAAA
siNOX1$^{(1)}$	NOX1HSS178285	CCGGUCAUUCUUUAUAUCUGUGAAA
siNOX1$^{(2)}$	NOX1HSS178286	GGUCUCACUGGAGUGAUCAUGACAA
siNOX1$^{(3)}$	NOX1HSS178287	UGGGAUGAUCGUGACUCCCACUGUA

Table I. Stealth siRNAs used in this thesis work.

3.5 ROS-inducing treatments

Cells used in this thesis work were treated with hydrogen peroxide (H_2O_2, Sigma-Aldrich), or with different chemotherapeutic agents known to induce ROS production, or with ionizing radiations, in order to study cell response to oxidative stress.

Chemotherapeutics comprised:

- ✓ Cisplatin (Santa Cruz Biotechnology; Santa Cruz, CA, USA), purchased in powder, was resuspended in NaCl 0.9% to 3 mM concentration, stored in the dark and used within 24 hours from preparation
- ✓ Paclitaxel (trade name Anzatax®, Hospira Italia; Naples, Italy), purchased as a solution with concentration 7 mM
- ✓ Doxorubicin (Accord Healthcare Italia; Monza, Italy), purchased as a solution with concentration 2 mg/ml
- ✓ Arsenic trioxide (As_2O_3), kindly gifted by Dr. Luigi Quintieri (Department of Pharmacology and Anaesthesiology, University of Padua) as a solution with concentration 25 mM.

γ-irradiation was carried out using the biological irradiator IBL-437C (CIS Bio International; Gif-sur-Yvette, France), subjecting cells to different doses of γ-radiations: 12 Gy for H460 and HeLa cells, 14 Gy for A549 cells.

In order to confirm ROS involvement in the cellular responses observed, analysis were performed using the glutathione precursor N-acetyl-L-cysteine (NAC, Sigma-Aldrich). Adherent cells were pre-treated for 16 hours with 2 mM NAC and, subsequently, treated with different ROS-inducing agents.

3.6 Inhibitors

In this thesis work, the following inhibitors were used in functional studies:

- ✓ Compound C (Merck Millipore; Billerica, MA, USA), an AMPK inhibitor, was purchased in powder and resuspended in DMSO
- ✓ Apocynin (Abcam; Cambridge, UK), a NADPH oxidase inhibitor, was purchased in powder and resuspended in DMSO.

3.7 Measurement of intracellular ROS levels

Cells treated with different ROS-inducing treatments were stained with 10 µM CM-H_2DCFDA (Life Technologies), according to manufacturer's instructions. DCFDA is a ROS detection cell-permeable probe, which is oxidized to a fluorescent non-permeable probe by endogenous H_2O_2. Following a staining of 30 minutes, cells were washed, resuspended in PBS and analysed using a FACSCalibur™ flow cytometer (BD Biosciences; San Jose, CA, USA).
ROS production over time was measured using CellROX® Reagent (Life Technologies), according to manufacturer's instructions. Fluorescence was assayed every 15 minutes for 1 hour using a BD LSRII Flow Cytometer (BD Biosciences).

3.8 Apoptosis analysis

$3 \cdot 10^5$ cells per well were plated in 6-wells tissue culture plates and incubated, in order to allow adhesion. The following day, cells were treated with 20 µM cisplatin or 2 mM H_2O_2. Irradiated cells were plated immediately after γ-irradiation.

Apoptosis was evaluated following 24, 48 and 72 hours of treatment with cisplatin or γ-irradiation and following 30, 90, 180, 270 and 360 minutes of treatment with H_2O_2, using Annexin-V-FLUOS Staining Kit (Roche Applied Sciences; Penzberg, Germany). Cells were stained with 2 µl Annexin V, 2 µl propidium iodide and 100 µl Hepes buffer, according to the manufacturer's instruction. Following an incubation of 15 minutes in the dark, staining was blocked with 200 µl Hepes buffer and apoptosis was evaluated by flow cytometry using an EPICS Coulter XL with Expo32 software (Beckman Coulter; Brea, CA, USA).

3.9 Cytotoxicity evaluation

Cytotoxicity following treatment with cisplatin, paclitaxel, doxorubicin and arsenic trioxide was evaluated using Sulforhodamine B (SRB) assay. SRB is a dye that specifically binds and, thus, stains, cellular proteins, whose content is directly proportional to cell number and viability.

$7.5 \cdot 10^3$ cells per well were plated in 96-wells tissue culture plates and incubated, in order to allow adhesion. Subsequently, cells were treated with increasing doses of different chemotherapeutics for 24, 48 and 72 hours. Cell proteins were fixed by culture medium removal and addition, in each well, of 125 µl cold trichloroacetic acid (TCA, Sigma-Aldrich), 10% w/v, diluted in culture medium without serum. Following 1 hour of incubation at 4°C, plates were washed 5 times with water, in order to remove TCA traces. Proteins in each well were, then, stained with 50 µl SRB (Sigma-Aldrich) 0.4% diluted in acetic acid 1% for 15 minutes. After 4 washings with acetic acid 1% and 1 washing with water, in order to completely remove excess SRB, plates were left to dry over-night.

Stained proteins were dissolved in 150 µl Tris 10 mM, leaving the plates in agitation for 15 minutes at room temperature. Finally, absorbance at 540 nm was measured using VICTOR™ X4 Multilabel Plate Reader with WorkOut 2.5 software (PerkinElmer; Waltham, MA, USA).

3.10 Clonogenic potential evaluation

Clonogenic potential of isogenic cell lines differing in LKB1 expression was measured by counting colonies formed after plating cells at limiting dilutions, following treatments with cisplatin, arsenic trioxide, γ-irradiation and the combination of cisplatin and γ-irradiation.

Cells were plated at very low density in 6-wells tissue culture plates (125 cells per well in standard culture conditions or 500 cells per well in culture condition with chemotherapeutics) and incubated, in order to allow adhesion. The following day, cells were treated with different concentrations of cisplatin or arsenic trioxide, with or without 2 mM NAC. After 2 hours, drugs were removed and cells were maintained in standard culture medium.

Irradiated cells were plated at very low density in 6-wells tissue culture plates (125 cells per well) immediately after γ-irradiation.

Regarding the combination of cisplatin treatment and γ-irradiation, $3 \cdot 10^6$ cells were plated in T75 tissue culture flasks and incubated, in order to allow adhesion. The following day, cells were treated with low concentration of cisplatin (5 mM for A549 cells, 1 mM for H460 cells). After 24 hours of treatment, cells were trypsinized, counted and γ-irradiated (14 Gy for A549 cells, 12 Gy for H460 cells). Immediately after γ-irradiation, cells were plated at very low density in 6-wells culture plates (500 and 2500 cells per well).

Colonies (obtained after about 15-20 days) were fixed in cold methanol for 10 minutes at 4°C. Following 1 washing with phosphate buffered saline (PBS), colonies were stained with crystal violet 33%, diluted in PBS, for 2 minutes at room temperature. Excess crystal violet was removed with 1 washing with water and stained colonies were left to completely dry.

Colonies were counted by two independent operators and number of colonies obtained from different treatments was normalized on number of colonies obtained from standard culture conditions.

3.11 Cell cycle analysis

The distribution in the different phases of cell cycle was determined in cisplatin-treated or in γ-irradiated cells using flow cytometry analysis with propidium iodide. A549 cells were synchronized through culture in serum deprivation for 24 hours and, subsequently, treated with 20 µM cisplatin or γ-irradiated with 14 Gy. After 24 and 48 hours, $1 \cdot 10^6$ cells were trypsinized, washed and resuspended in 1 ml of GM solution (glucose 1.1 mM, NaCl 0.14 M, KCl 5 mM, Na_2HPO_4 1.5 mM, KH_2PO_4 1.1 mM, EDTA 0.5 mM). Cells were fixed and permeabilized by adding 3 ml of cold 100% ethanol, drop by drop, and preserved at -20°C until the staining. Fixed cells were washed and resuspended in 500 µl of propidium iodide (100 µg/ml), containing DNase-free RNase (12 µg/ml; Sigma-Aldrich). Following 1 hour of incubation, cells were analysed using a FACSCalibur™ flow cytometer with ModFit software (BD Biosciences).

3.12 RNA extraction, reverse transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using RNeasy® Mini Kit (Qiagen; Venlo, Netherlands), according to manufacturer's instruction. cDNA was synthesized from 0.3 to 1 µg of total RNA using High Capacity RNA-to-cDNA Kit (Life Technologies). Reverse transcription was followed by quantitative PCR using Platinum® SYBR® Green (Life Technologies). Real-time PCRs were performed in an ABI Prism 7900 HT Sequence Detection System (Life Technologies). Results were analyzed using the ΔΔCt method with normalization against HMBS gene expression. Primers used for qRT-PCR are reported in Table II.

Gene	Forward primer	Reverse primer
LKB1	5'-CTGACCTGCTGAAAGGGATG-3'	5'-CAGCCGGAGGATGTTTCTT-3'
AMPKα1	5'-GGAGCCTTGATGTGGTAGGA-3'	5'-GTTTCATCCAGCCTTCCATTC-3'
AMPKα2	5'-ACCAGCTTGCAGTGGCTTAT-3'	5'-CAGTGCATCCAATGGACATC-3'
NOX1	5'-GGGGTCAAACAGAGGAGAGC-3'	5'-CCACTTCCAAGACTCAGGGG-3'
HMBS	5'-GGCAATGCGGCTGCAA-3'	5'-GGGTACCCACGCGAATCAC-3'

Table II. List of primers used in this thesis work.

Expression levels of 21 genes involved in oxidative metabolism were analysed by RealTime Ready Custom Panels (Roche Applied Sciences). Genes included in Custom Panels were selected based on their role in oxidative metabolism:

- ✓ Glutathione synthesis: *GCLC* (*glutamate-cysteine ligase, catalytic subunit*), *GCLM* (*glutamate-cysteine ligase, modifier subunit*), *GSS* (*glutathione synthetase*)
- ✓ Glutathione degradation: *GGT6* (*gamma-glutamyltransferase 6*), *GGT7* (*gamma-glutamyltransferase 7*)
- ✓ Transcription factors involved in glutathione metabolism: *NFE2L2* (*nuclear factor erythroid 2-like 2*; NRF2), *PPARGC1A* (*peroxisome proliferator-activated receptor gamma coactivator 1 alpha*; PGC-1α), *JUN* (*jun proto-oncogene*)
- ✓ Glutathione adducts transporters: *ABCC1* (*ATP-binding cassette sub-family C member 1*), *ABCC2* (*ATP-binding cassette sub-family C member 2*)
- ✓ Enzymes involved in antioxidant defenses: *CAT* (*catalase*), *TXN* (*thioredoxin*), *SOD2* (*superoxide dismutase 2*), *GPX1* (*glutathione peroxidase 1*), *GSR* (*glutathione reductase*), *MSRA* (*methionine sulfoxide reductase A*)
- ✓ Enzymes involved in ROS production: *NOX1* (*NADPH oxidase 1*)
- ✓ Enzymes involved in transsulfuration pathway: *CBS* (*cystathionine β-synthase*), *CTH* (*cystathionine γ-lyase*)

- ✓ Cystine and cysteine transporters: *SLC7A11* (*solute carrier family 7 member 11*; xCT), *SLC7A10* (*solute carrier family 7 member 10*; ASC1).

3.13 Mutational analysis

3.13.1 Sanger sequencing

LKB1 gene was sequenced in a small cohort of advanced NSCLC patients treated with a combination of bevacizumab and chemotherapy, using Sanger sequencing method. Genomic DNA (gDNA) was obtained from sections of formalin fixed – paraffin embedded (FFPE) biopsies using QIAamp® DNA Micro Kit (Qiagen), according to manufacturer's instructions. *LKB1* exons were amplified through PCR, starting from 50 ng of gDNA, using primers listed in Table III.

Exon	Forward primer	Reverse primer
1	5'-GAAGGGAAGTCGGAACACAA-3	5'-GGAGAGAAGGAAGGAAGACAGA-3'
2	5'-TTCTCTCTAGGGAAGGGAGGAG-3'	5'-ATTGCCACAATGGCTGACTT-3'
3	5'-CTCCAGAGCCCCTTTTCTG-3'	5'-CAGTGTGGCCTCACGGAAAGGA-3'
4	5'-AGCTGGGCCTGTGGTGTT-3	5'-AACGGGTGCAGTGCCTGT-3'
5	5'-ACCCTCAAAATCTCCGACCT-3'	5'-TCCATAAAGTAAGCACCCCCTA-3'
6	5'-CTCCTAGGGCGTCAACCAC-3'	5'-ACACCCCCAACCCTACATTT-3'
7	5'-CTTAGGAGCGTCCAGGTATCAC-3'	5'-CTCAACCAGCTGCCCACAT-3'
8	5'-GAGCTGGGTCGGAAAACTG-3'	5'-AGAAGCTGTCCTTGTTGCAGA-3'
9	5'-TAAGTGCGTCCCCGTGGT-3' 5'-CAGGACAGGTCCCAGAAGAG-3'	5'-AGCCTCACTGCTGCTTGC-3' 5'-CGGTCACCATGACTGACTAGC-3'

Table III. Primers used in PCR and Sanger sequencing reactions.

Exons were amplified through Taq enzyme activation for 10 minutes at 95°C and 45 cycles, composed of 1 minute at 95°C, 1.5 minutes at 60°C for exon 3 or at 62°C for exons 1, 2, 4-9, and 2 minutes at 72°C, followed by 10 minutes at 72°C. PCR products were purified with illustra™ ExoProStar™ Kit (GE Healthcare Life Sciences; Pittsburgh, PA, USA). Purified amplicons were sequenced using BigDye® Terminator v1.1 Cycle Sequencing Kit (Life Technologies) and PCR

primers ten-fold diluted. Finally, sequences were purified using BigDye XTerminator® Purification Kit (Life Technologies), according to manufacturer's instructions, and separated on a 3730xl DNA Analyzer (Life Technologies) capillary sequencer. Obtained electropherograms were analysed using Chromas software (Technelysium Pty Ltd.; South Brisbane, Australia) and identified mutations were mapped on LKB1 protein, using ExPASy Translate Tool (http://web.expasy.org/translate).

3.13.2 Next-Generation Sequencing

High-coverage sequencing of *TP53*, *KRAS*, *LKB1* and *EGFR* genes was performed using Ion Torrent™ technology (Life Technologies). gDNA was quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies), in order to provide 50 ng of high-quality double-stranded gDNA to GeneDX (Gaithersburg, MD, USA), which performed sequencing analysis using a custom Ion AmpliSeq™ panel. Identified variants were mapped using a free online mapping tool (SNPnexus; http://www.snp-nexus.org).

3.14 Western blot analysis

Proteins from cells treated under different conditions were extracted using a lysis buffer (NP-40 0.2%, NaCl 30 mM, Tris HCl pH 7.5 10 mM, EDTA 0.4 mM, NaF 50 mM, Na_3VO_4 1 mM and protease inhibitor cocktail) and lysates obtained were quantified using QuantumMicroProtein Bicinchoninic Acid Protein Assay Kit (EuroClone). 35 µg of proteins were denaturated and loaded in a NuPAGE® Novex® 4-12% Bis-Tris Midi Protein Gel (Life Technologies). Separated proteins were blotted on a nitrocellulose membrane (Amersham; Buckinghamshire, UK) for 2 hours at 400 mA. Membranes were saturated for 1 hour with PBS - 0.1% Tween - 5% milk and incubated over night with primary antibodies at 4°C, according to manufacturer's instructions. The following primary antibodies were used in this thesis work:

- ✓ Rabbit anti pAMPK^{Thr172} (Cell Signaling Technology; Danvers, MA, USA)
- ✓ Rabbit anti AMPK (Cell Signaling Technology)
- ✓ Rabbit anti pACC^{Ser79} (Cell Signaling Technology)
- ✓ Rabbit anti ACC (Cell Signaling Technology)
- ✓ Rabbit anti pLKB1^{Ser428} (Cell Signaling Technology)
- ✓ Mouse anti LKB1 (Santa Cruz Biotechnology)
- ✓ Mouse anti α-tubulin (Sigma-Aldrich).

Membranes were washed 2 times for 15 minutes (un-phosphorylated antigens) or 3 times for 5 minutes (phosphorylated antigens) and incubated for 1 hour with horseradish-conjugated secondary antibodies (Amersham-Pharmacia; Little Chalfont, UK). Detection was obtained using Western Lightning plus ECL reagents (PerkinElmer), containing Luminol, which is oxidized by horseradish peroxidase, resulting in light emission at 425 nm. Signals emitted were acquired using ChemiDoc™ XRS system (Bio-Rad; Hercules, CA, USA).

3.15 Immunohistochemistry analysis (IHC)

Sections of 5 µm were cut from FFPE biopsies, deparaffinazed in xylene for 30 minutes and, subsequently, rehydrated with decreasing concentrations of ethanol (100%, 95% and 80%) and finally washed with water. Antigen retrieval was achieved using a 10 mM citrate buffer pH 6 for 20 minutes at 95°C. Endogenous peroxidases were inactivated with a solution of 3% H_2O_2 for 5 minutes. Subsequently, nonspecific binding sites were saturated by incubating sections for 1 hour in PBS - 5% goat serum and primary antibodies were added for 1 hour. The following primary antibodies were used:

- ✓ Mouse anti LKB1 (Ley 37D/G6, Santa Cruz Biotechnology)

Incubation with biotinylated secondary antibody (Vector Laboratories; Peterborough, UK) for 30 minutes was followed by incubation for 30 minutes with VECTASTAIN® ABC Kit (Vector Laboratories), composed of avidin and biotinylated horseradish peroxidase. Antigens were stained by incubating sections with 3,3'-diaminobenzidin (DAB, DAKO; Glostrup, Denmark), diluted according to manufacturer's instructions. Finally, sections were counterstained with Mayer's haematoxylin (Sigma-Aldrich) for 1 minute and staining was fixed with increasing concentrations of ethanol (80%, 95% and 100%) and with xylene. Slides were mounted with Entellan™ New (Merck Millipore) and analysed by two anatomopathologists with a semiquantitative method, attributing a score ranging from 0 to 18, based on percentage of stained cells and on intensity of staining. In the statistical analysis, LKB1 staining was considered negative if score=0, positive if score ≥1.

3.16 Immunofluorescence analysis (IFA)

Cells were plated ($5 \cdot 10^4$ per well) in 4-wells BD Falcon™ CultureSlides (BD Biosciences) and incubated, in order to allow adhesion. The following day, cells were fixed in 1% formaldehyde for 20 minutes, washed with PBS and permeabilized in PBS - 0.2% Triton X-100 (Sigma-Aldrich) for 10 minutes. Cells were, then, washed with PBS and saturated with PBS - 0.1% Triton X-100 - 1% BSA - 5% goat serum for 1 hour. Primary antibody, diluted in PBS - 0.1% Triton X-100 according to manufacturer's instructions, was added and incubated over night at 4°C. The following primary antibodies were used in this thesis work:

- ✓ Rabbit anti NOX1 (Abcam, ab55831)
- ✓ Rabbit anti NOX1 (Sigma-Aldrich, HPA035299)

Following three washings in PBS, cells were incubated with Alexa Fluor® conjugated secondary antibody (Life Technologies) diluted in PBS - 0.1% Triton X-

100 for 1 hour in the dark. Nuclei were stained with TO-PRO®-3 Iodide (Life Technologies), diluted 1:1000 in PBS, for 15 minutes in the dark.
Immunofluorescence signals were visualized on a Zeiss Axiovert 100M confocal microscope (Carl Zeiss AG; Oberkochen, Germany).

3.17 Characterization of aqueous metabolites using Nuclear Magnetic Resonance (NMR)

Metabolic changes following LKB1 reconstitution and treatment with 2 mM H_2O_2 were investigated by measuring aqueous metabolites through NMR. $1 \cdot 10^7$ cells were lysed with 1 ml of water, fixed with 10 ml of cold 77% ethanol and frozen at -80°C. Samples were analysed through NMR in collaboration with Dr. Egidio Iorio (Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome).

3.18 Measurement of total and reduced glutathione using High-Performance Liquid Chromatography (HPLC)

Total and reduced glutathione levels were measured in H_2O_2 – treated cells using HPLC. $4 \cdot 10^5$ cells were washed thrice with cold PBS and lysed with 100 µl of cold HCl 0.01M. Lysates were preserved at -80°C until analysis. HPLC were performed in collaboration with Dr. Luigi Quintieri (Department of Pharmacology and Anaesthesiology, University of Padua).

3.19 Statistical analysis

Results were expressed as mean value ± SD. Statistical analysis of data was performed using Student's t-test or Mann-Whitney test. Survival analysis was performed using Kaplan-Meier estimator and Log-rank test. Fisher's exact test

was used to study correlation between mutational status and IHC score. Differences were considered statistically significant when $p \leq 0.05$.

4. Results

4.1 Characterization of isogenic cell lines

To study the role of LKB1 in response to oxidative stress - inducing agents, isogenic tumor cell lines were generated. Genetic heterogeneity in cancer cells complicates any attempt to correlate genetic traits with cellular behaviour, since several mutations in oncogenes or in tumor suppressor genes could account for observed phenomena. Isogenic cell lines can overcome this issue, as only the gene of interest is differently expressed, whereas the genetic background is identical.

In this thesis work, three different LKB1-deficient cancer cell lines were used: A549 and H460 (derived from NSCLC) and HeLa (derived from cervical carcinoma). These cell lines bear inactivating mutations in *LKB1* gene (p.Q37* in A549 and H460 cell lines, a 25 kb deletion in HeLa), resulting in protein loss. LKB1 expression was restored in these cancer cell lines by transduction with a retroviral vector coding for wild-type *LKB1* cDNA whereas transduction with an empty retroviral vector provided a control comparable with parental (LKB1-deficient) cells.

LKB1 is known to be the master kinase that phosphorylates and activates AMPK under stress conditions, such as glucose deprivation, as demonstrated in (Hardie, 2004). To evaluate whether LKB1/AMPK pathway was functional in *LKB1*-reconstituted cells, LKB1+ and LKB1- cell lines were cultivated in tissue culture medium with or without glucose. Western blot analysis revealed pathway activation in LKB1+ cells, as evidenced by phosphorylation of AMPK and of its downstream target ACC, following glucose deprivation for 24 hours. Moreover, LKB1- cells showed higher cell death following glucose withdrawal compared to LKB1+ cells, as demonstrated by apoptosis evaluation (Figure 4.1).

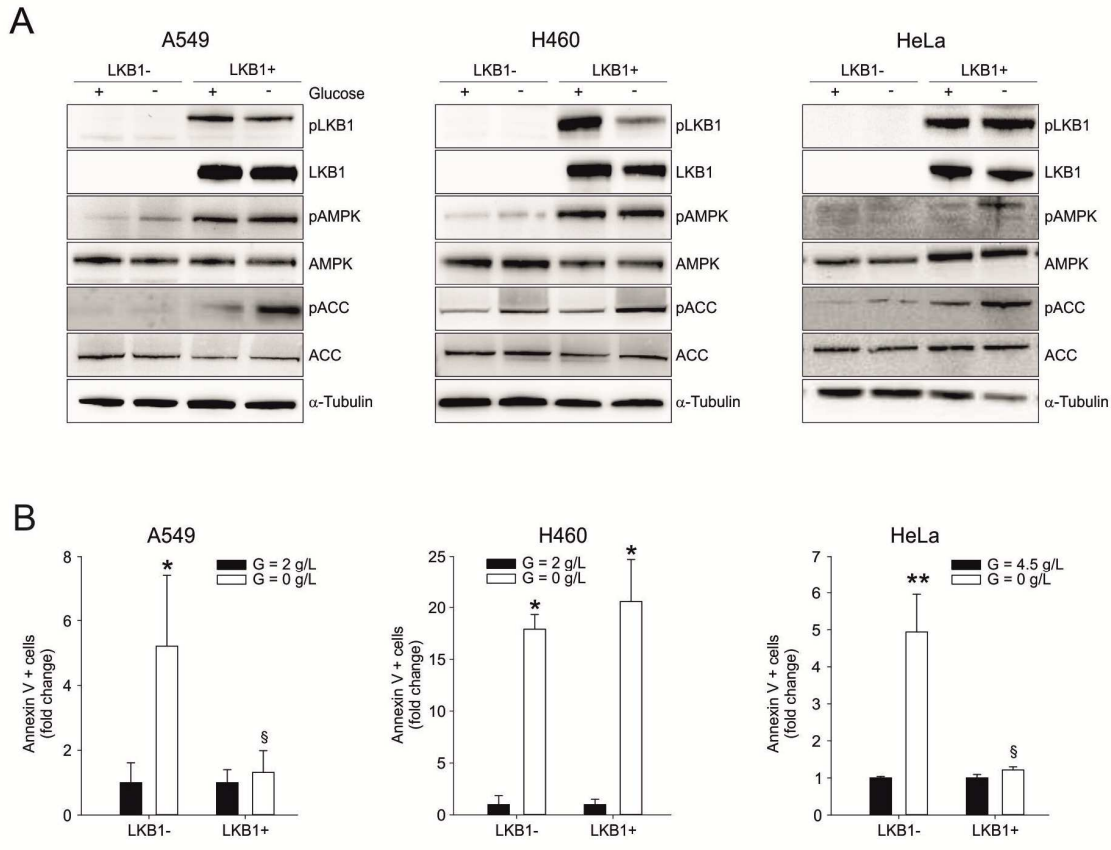

Figure 4.1. *LKB1* reconstitution in LKB1-deficient cell lines A549, H460 and HeLa. **A.** Western blot analysis following glucose deprivation revealed AMPK pathway activation in LKB1+ cells, as demonstrated by AMPK and ACC phosphorylation. **B.** Apoptosis evaluation by Annexin V staining showed increased sensitivity to glucose deprivation in LKB1-, compared to LKB1+ cells, in A549 and HeLa - but not H460 - cell lines. Annexin V positivity is calculated relatively to staining of cells cultivated with glucose, equal to 1. The average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. *p<0.05 and **p<0.01 compared with cells cultivated with glucose. §p<0.05 compared to LKB1- cells cultivated under glucose starvation.

Therefore, *LKB1* wild-type gene transfer restored LKB1/AMPK responsiveness to energy stress, leading to increased cell survival under nutrient starvation conditions.

4.2 LKB1+ cells are characterized by reduced expression of *NOX1* and *PPARGC1A*

Recent studies suggest that the LKB1/AMPK pathway regulates cellular redox state. AMPK is necessary to maintain NADPH homeostasis, thus controlling redox balance (Jeon *et al.*, 2012). Moreover, glucose deprivation promotes ROS generation by mitochondria, as glucose has antioxidant functions (Cardone *et al.*, 2012). Hence, high levels of cell death under glucose starvation could be due to increased oxidative stress in LKB1- tumor cells.

To evaluate the implications of LKB1 loss on redox homeostasis and resistance to oxidative stress, we assessed by quantitative PCR the expression of 21 genes involved in redox homeostasis in LKB1- and LKB1+ cell lines. Remarkably, in all cell lines tested several antioxidant genes were relatively down-regulated in LKB1+ compared to LKB1- cells (Figure 4.2).

Moreover, each cell line showed increased expression of some antioxidant genes in LKB1+ variant, but such genes were not shared among all cell lines analysed. The most consistently down-regulated genes in LKB1+, compared to LKB1- cells, were *NOX1* and *PPARGC1A*. *NOX1* encodes for NADPH oxidase 1, a homologue of the macrophage NOX2, responsible for the respiratory burst in response to pathogens. NADPH oxidases catalyse the transfer of one electron from NADPH to oxygen, generating superoxide or H_2O_2, thus increasing oxidative stress. *PPARGC1A* encodes for the mitochondrial master regulator PPAR-γ Coactivator 1 Alpha (PGC-1α), a transcriptional coactivator that promotes mitochondria biogenesis and improved oxidative metabolism. By stimulating mitochondria biogenesis and improving electron transport chain activity, PGC-1α accelerates ROS production as by-products of cellular respiration.

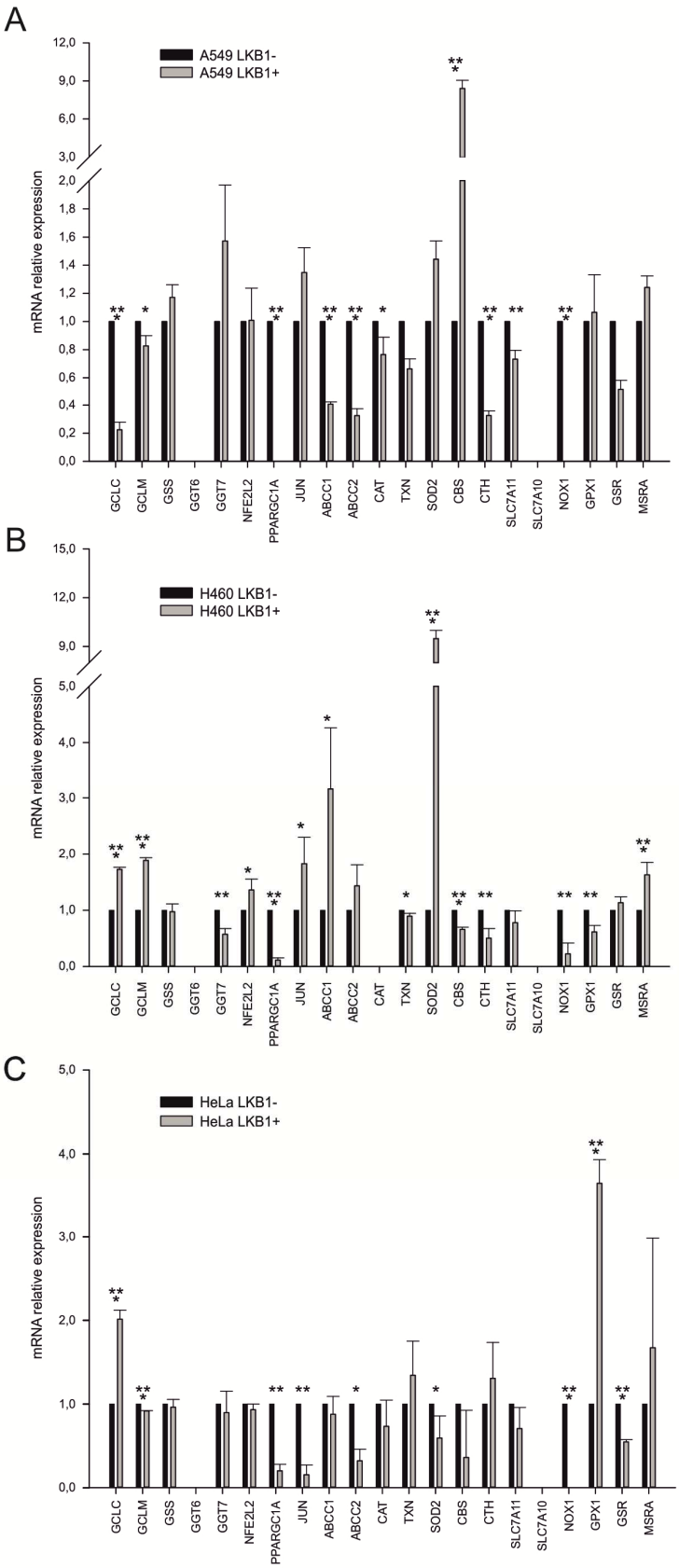

Figure 4.2. Gene expression analysis in A549 **(A)**, H460 **(B)** and HeLa cells **(C)**. Globally, oxidative metabolism related genes were down-regulated in LKB1+, compared to LKB1- cells. *NOX1* and

PPARGC1A were the most down-regulated genes in all cell lines tested. The mRNA relative expression is calculated relatively to LKB1- cells (equal to 1) and is indicated as mean ± standard deviation of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared with mRNA relative expression in LKB1- cells.

Higher expression of NOX1 and PGC-1α in LKB1- cells, compared to LKB1+ cells, suggested that these cells might have increased endogenous oxidative stress.

4.3 LKB1 deficiency and basal oxidative stress

Increased expression of NOX1 and PGC-1α in LKB1- cells, compared to LKB1+ cells, could result in an over-production of ROS. To test this hypothesis, we measured basal ROS levels by flow cytometry using CellROX® Reagent. Oxidative stress was measured every 15 minutes for 1 hour under standard culture conditions. In A549 and HeLa cell lines, fluorescence increased more rapidly in LKB1- cells, indicating heightened ROS production (Figure 4.3A). In line with these findings, we detected increased amounts of oxidized glutathione (GSSG) [calculated as total - reduced glutathione (GSH)] in LKB1- cells (Figure 4.3B).

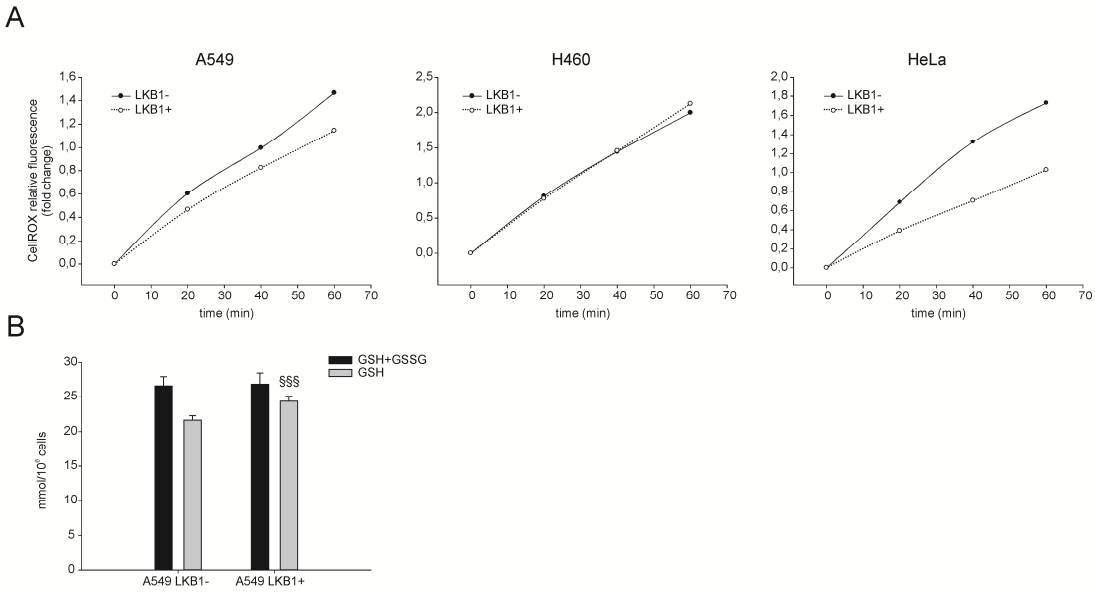

Figure 4.3. ROS generation is increased in some LKB1- cells, compared to their LKB1+ counterpart. **A.** Measurement of steady-state ROS generation over time in isogenic cell lines by CellROX® Reagent. **B.** Determination of total and reduced glutathione by HPLC in A549 isogenic cell line. §§§p<0.001 compared with reduced GSH amount in LKB1- cells.

4.4 LKB1 reconstitution in LKB1-deficient cell lines confers resistance to H_2O_2-induced oxidative stress

Sensitivity of cancer cells to ROS-inducing agents is determined by their higher basal oxidative stress, compared to normal cells. In order to test whether the higher endogenous oxidative stress in LKB1-deficient cells conferred sensitivity to exogenous oxidative stress, we treated LKB1- and LKB1+ cell lines with 2 mM H_2O_2. ROS production was higher in LKB1- cells, compared to LKB1+ cells, as measured by DCFDA staining (Figure 4.4A). Moreover, results indicated that H_2O_2 caused apoptosis in a time-dependent manner in both LKB1- and in LKB1+ cells. LKB1 reconstitution, however, protected cells from H_2O_2-induced oxidative stress, leading to a significant decrease in cell death (Figure 4.4B).

Pre-treatment of cells with N-acetyl-L-cysteine (NAC), a precursor of glutathione, significantly decreased cell death in LKB1- cells, whereas it did not affect survival

of LKB1+ cells (Figure 4.4C). This finding showed that apoptosis following H_2O_2 treatment was due to oxidative stress.

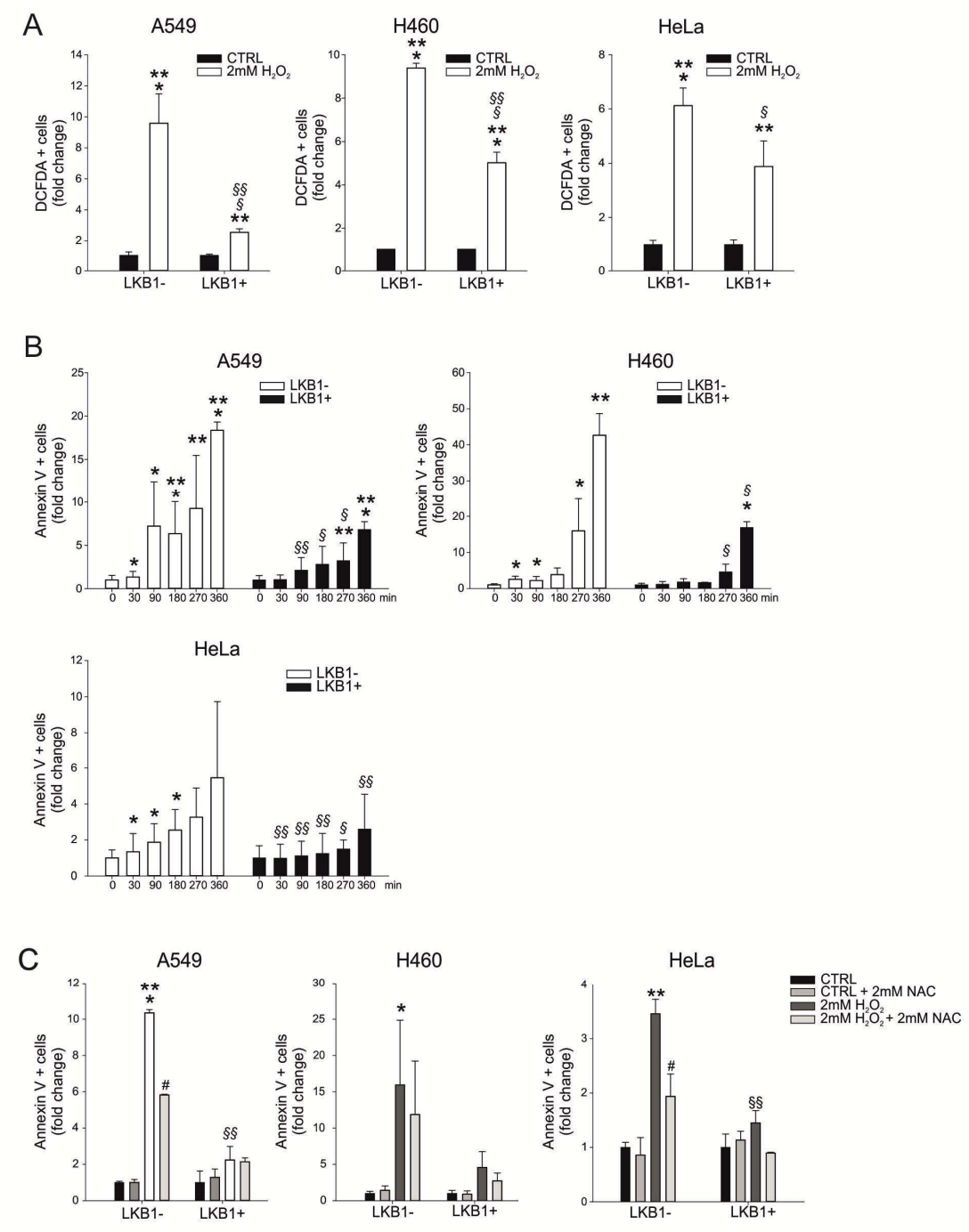

Figure 4.4. LKB1 protected cancer cells from oxidative stress induced by H_2O_2. **A.** ROS production, measured by DCFDA staining, was invariably higher in LKB1- compared to LKB1+ cells. DCFDA positivity is calculated relatively to staining of untreated cells, equal to 1. The average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. **p<0.01 and ***p<0.001 compared with untreated (CTRL) cells. §p<0.05 and

§§§p<0.001 compared to LKB1- cells. **B.** Apoptosis levels in LKB1- and in LKB1+ cells following 2 mM H_2O_2 treatment for 30, 90, 180, 270 and 360 minutes. Annexin V positivity is calculated respect to untreated cells staining, set equal to 1. The average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to untreated (0 minutes) cells. §p<0.05 and §§p<0.01 compared to LKB1- cells treated with H_2O_2 for the same time. **C.** Pre-treatment with NAC reduced apoptosis in LKB1- cells. The average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared with untreated (CTRL) cells. §§p<0.01 compared to LKB1- cells treated with 2 mM H_2O_2. $^{\#}$ p<0.05 compared to cells treated with 2 mM H_2O_2 in absence of pre-treatment with NAC.

4.5 LKB1 regulates glutathione homeostasis following oxidative stress

To get a broader view of the metabolites altered following oxidative stress, we measured cytoplasmic aqueous metabolites levels in LKB1-deficient and in LKB1-proficient cells. A549 LKB1- and LKB1+ cells were treated with 2 mM H_2O_2 for 90 minutes and lysed in water. Cellular extracts were delivered to Dr. Egidio Iorio (Istituto Superiore di Sanità, Rome) and analysed by Nuclear Magnetic Resonance (NMR) spectroscopy. NMR analysis revealed marked reduction of glutathione level following H_2O_2 treatment in LKB1- cells (54.2%), compared to LKB1+ cells (28.6%; Figure 4.5A). Besides glutathione, LKB1- and LKB1+ cells also differed in phosphocholine levels. With regard to glutathione, these findings were confirmed by HPLC in A549 LKB1- and LKB1+ cells treated with 2 mM H_2O_2 for 90 and 270 minutes (Figure 4.5B).

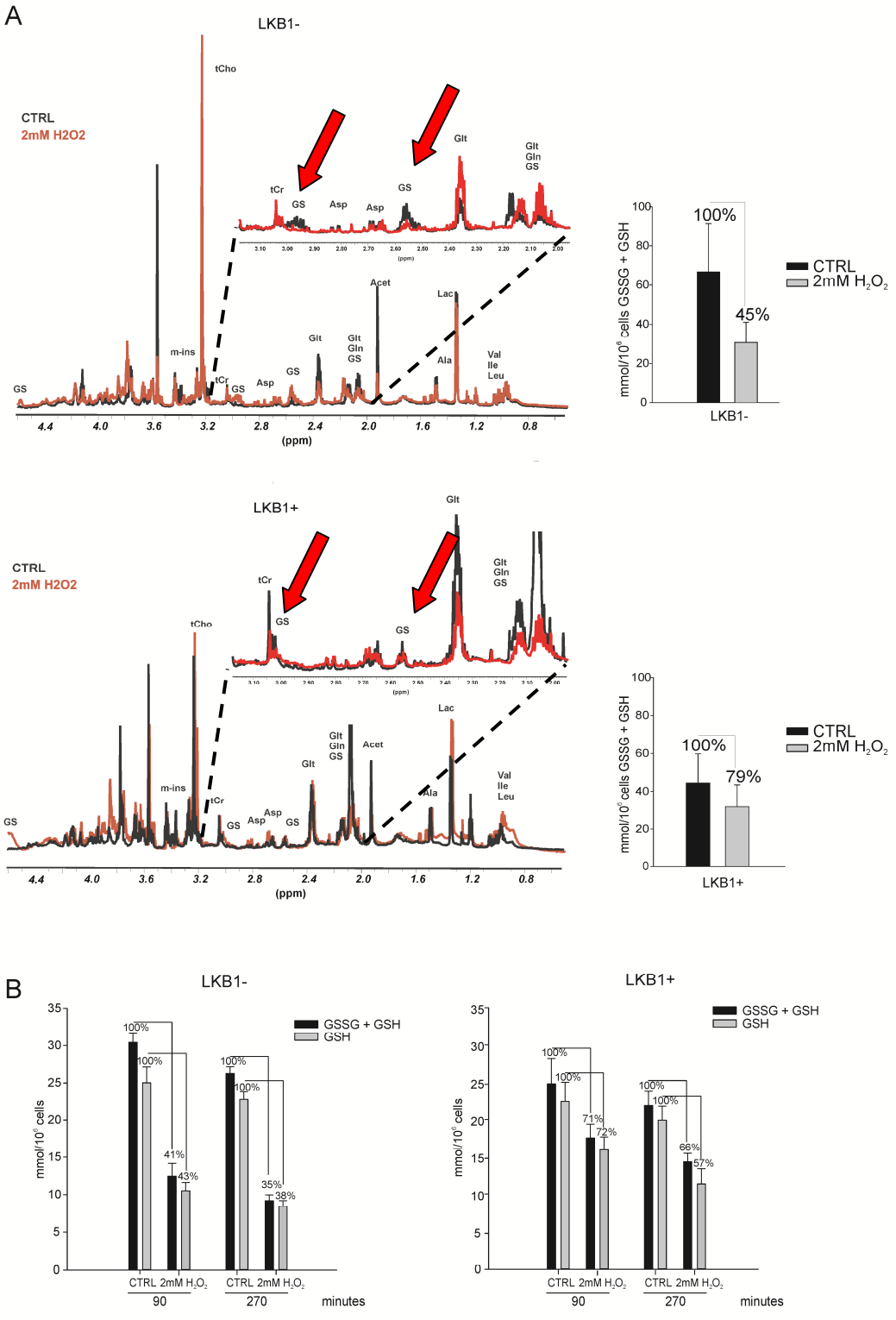

Figure 4.5. **A.** NMR spectroscopy analysis of aqueous metabolites in untreated cells (black) and in H_2O_2–treated cells (red). Glutathione (indicated by GS) is evidenced by red arrows. **Inserts:** quantification of glutathione levels in untreated cells (black bars) and in H_2O_2–treated cells (light grey bars). Indicated metabolites are: glutathione (GS), myo-inositol (m-ins), total choline (tCho), total creatine (tCr), aspartate (Asp), glutamate (Glt), glutamine (Gln), acetate (Acet), alanine (Ala),

lactate (Lac), valine (Val), isoleucine (Ile) and leucine (Leu). **B.** Determination of total and reduced GSH levels by HPLC in LKB1- and in LKB1+ cells following 90 and 270 minutes of H_2O_2 treatment.

These results indicated that LKB1 controlled glutathione homeostasis and that LKB1-deficient cells had reduced capacity to restore glutathione levels under oxidative stress.

4.6 Insights into the mechanism linking LKB1 to regulation of oxidative stress

As AMPK is a key mediator of LKB1 activities on cellular physiology, we first investigated the possible involvement of AMPK in response to oxidative stress. To this end, we used both pharmacological inhibition and gene silencing approaches. Inhibition of AMPK activity with Compound C (CC) sensitized LKB1+ cells to H_2O_2-induced cell death. Unexpectedly, CC increased apoptosis also in LKB1- cells (Figure 4.6A), which should have reduced AMPK activation. However, Western blot analysis evidenced that H_2O_2 treatment caused AMPK activation also in LKB1- cells, likely via a LKB1-independent pathway, thus explaining the effects of CC in these cells. siRNA-mediated *AMPK* silencing sensitized both LKB1+ and LKB1- cells to H_2O_2 treatment (Figure 4.6B). Collectively, these data indicated that AMPK mediates the effects of LKB1 on oxidative stress.

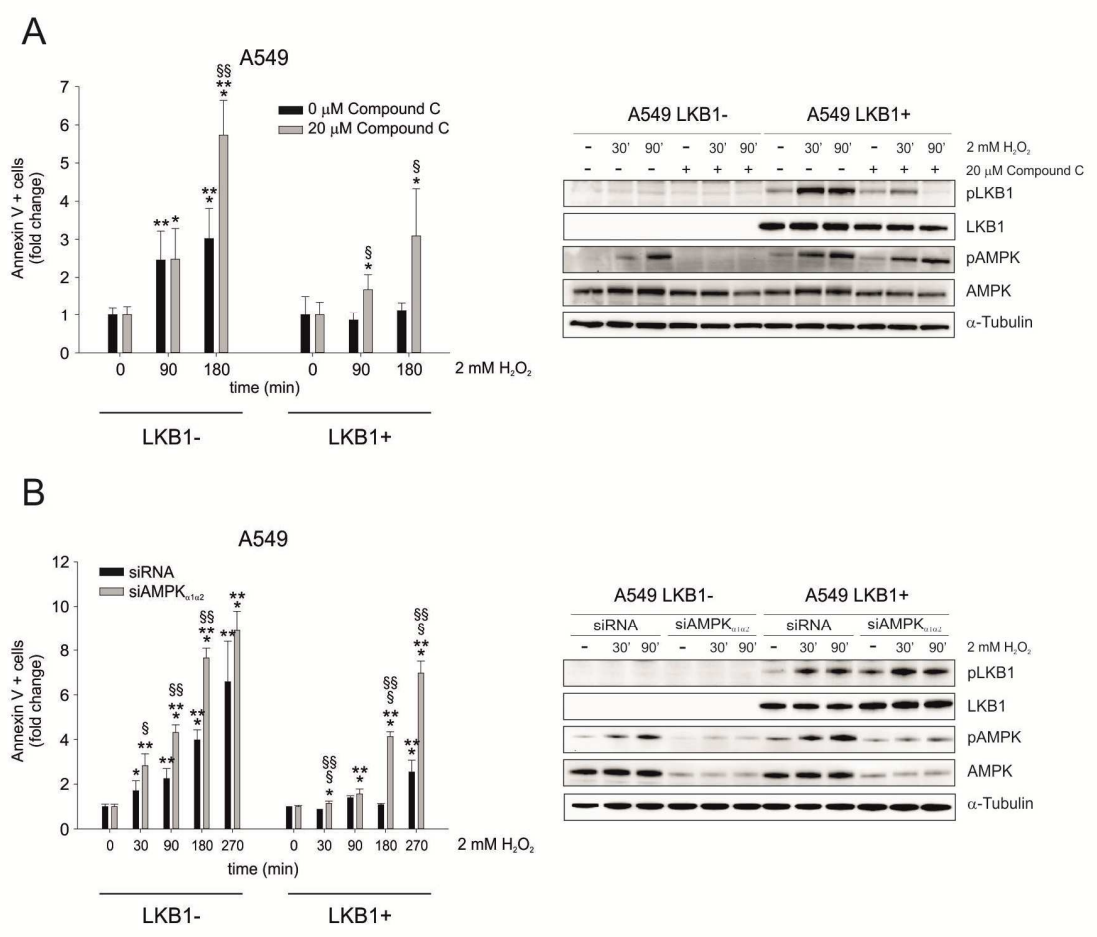

Figure 4.6. AMPK is involved in LKB1-dependent response to oxidative stress. **A.** AMPK inhibition with Compound C (CC) sensitized both LKB1+ and LKB1- cells to H_2O_2-induced cell death. Annexin V positivity is calculated relatively to untreated cells staining, set equal to 1. Average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to untreated (0 minutes) cells. §p<0.05 and §§p<0.01 compared to untreated (0 μM CC) cells. Western blot analysis confirmed inhibition of AMPK both in LKB1- and in LKB1+ cells following H_2O_2 treatment. **B.** siRNA-mediated *AMPK* silencing increased cell death in both LKB1- and LKB1+ cells following H_2O_2 treatment. The average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to untreated (0 minutes) cells. §p<0.05, §§p<0.01 and §§§p<0.001 compared to siRNA cells. Western blot analysis disclosed reduced AMPK activation in siAMPK-treated cells.

Next, we examined whether NOX1 accounted for increased sensitivity to exogenous oxidative stress in LKB1- cells. Immunofluorescence analysis revealed that NOX1 protein expression was strongly reduced in LKB1+, compared to LKB1- cells (Figure 4.7).

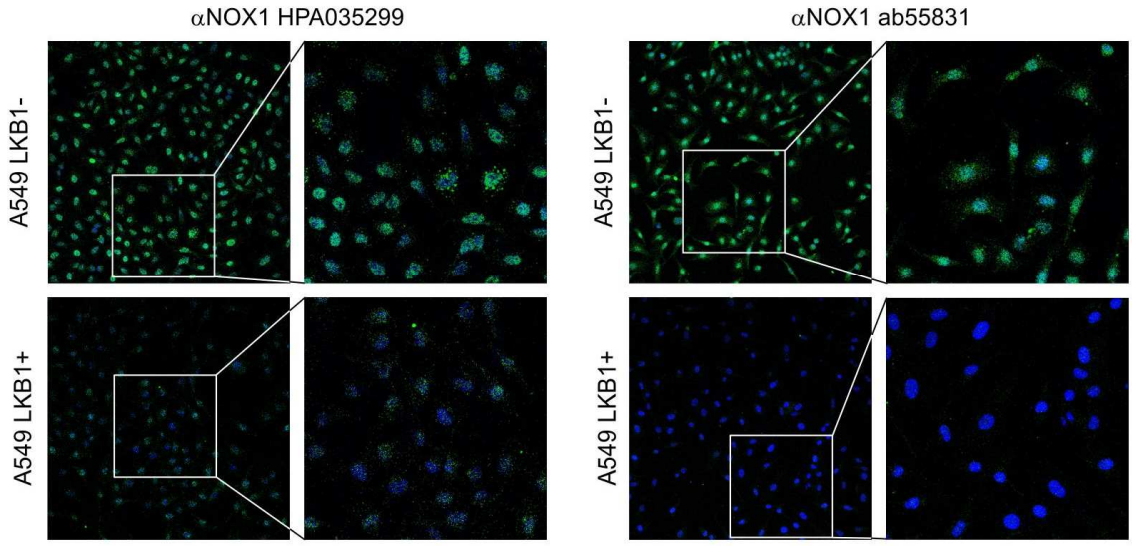

Figure 4.7. NOX1 protein assessment in A549 LKB1- and LKB1+ cells through immunofluorescence analysis using two different αNOX1 antibodies.

NOX1 silencing using two different siRNAs reduced steady-state ROS generation in A549 LKB1- cells, whereas it had negligible effects in LKB1+ cells (Figure 4.8).

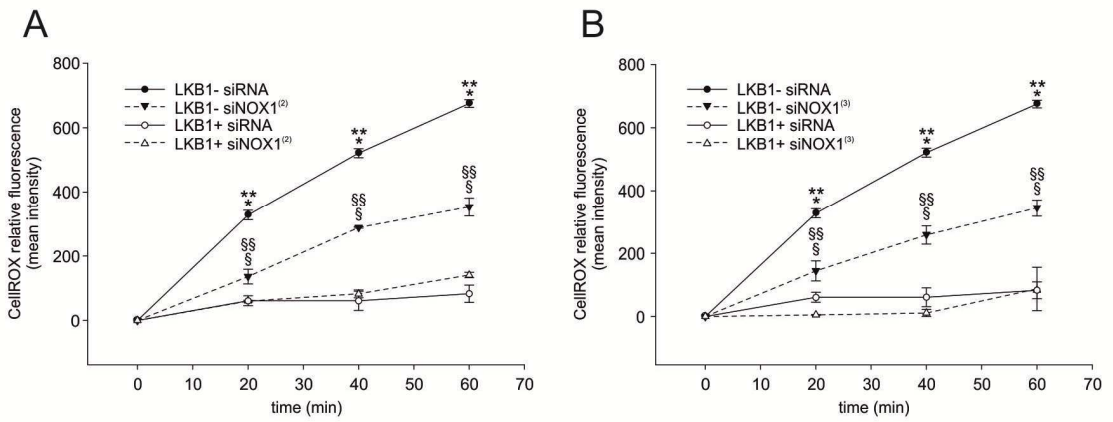

Figure 4.8. NOX1 silencing with siNOX1$^{(2)}$ (**A**) and siNOX1$^{(3)}$ (**B**) reduced endogenous ROS production in A549 LKB1- cells, whereas it had minimal effects in LKB1+ cells. ***p<0.001 compared to 0 minutes. §§§p<0.001 compared to LKB1- siRNA cells.

Intriguingly, *NOX1* silencing conferred resistance to exogenous oxidative stress in LKB1- cells (Figure 4.9A). In line with these findings, inhibition of NOX1 with the

specific inhibitor apocynin significantly increased resistance of LKB1- cells to H_2O_2 - induced cell death, whereas leaving unaffected response to oxidative stress in LKB1+ cells (Figure 4.9B).

Altogether, these data indicated that NOX1 - through the generation of endogenous ROS - is implicated in increased sensitivity to exogenous oxidative stress observed in LKB1- cells. It remains to be established whether the suppressive effects of LKB1 on NOX1 expression is AMPK-mediated.

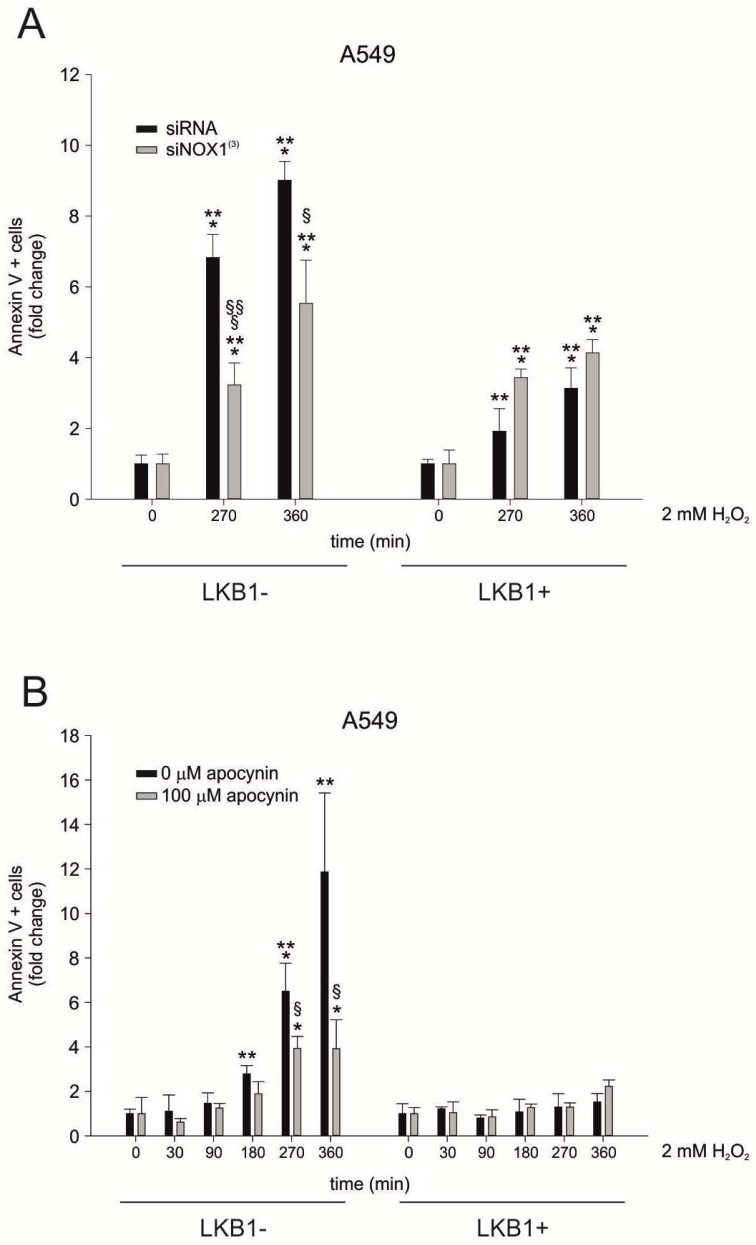

Figure 4.9. NOX1 contributes to apoptosis following oxidative stress. **A.** *NOX1* silencing reduced apoptosis of LKB1- cells following treatment with H_2O_2. The average fluorescence intensity is

indicated as mean ± standard deviation of three independent experiments. **p<0.01 and ***p<0.001 compared to untreated (0 minutes) cells. §p<0.05 and §§§p<0.001 compared to siRNA cells. **B.** NOX1 inhibition with apocynin significantly attenuated cytotoxic effects of H_2O_2 in LKB1- cells, with no effects in LKB1+ cells. Annexin V positivity is calculated relatively to untreated cells, set equal to 1. Average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to untreated (0 minutes) cells. §p<0.05 compared to untreated (0 μM apocynin) cells.

Down-regulation of *PPARGC1A* in LKB1+ cells could be a parallel mechanism accounting for resistance of LKB1+ cells to exogenous oxidative stress. However, future studies are needed to investigate this hypothesis.

4.7 LKB1 deficiency sensitizes cancer cells to cisplatin- and to irradiation-mediated cytotoxic effects

Cisplatin-based chemotherapy is the first-line therapeutic regimen in many cancers, including NSCLC. Besides damaging DNA by forming adducts, cisplatin induces oxidative damage by inducing ROS production (Casares *et al.*, 2012). Moreover radiotherapy, another therapeutic option for cancer patients, is known to kill cancer cells by ionization of water that produces highly reactive hydroxyl ions. In order to test whether LKB1 influenced cellular response to cisplatin, LKB1- and LKB1+ isogenic cell lines were treated with different concentrations of cisplatin. Cytotoxicity assessment using sulphorhodamine B (SRB) assay revealed that A549, H460 and HeLa LKB1+ cells were more resistant to cisplatin than their LKB1- counterpart (Figure 4.10A). These results were confirmed by apoptosis evaluation following a single dose of cisplatin. NAC supplementation rescued cell viability following cisplatin, supporting the involvement of ROS in cisplatin-induced cell death (Figure 4.10B). Moreover, acute cisplatin treatment reduced clonogenic potential more markedly in LKB1- cells, compared to LKB1+ cells, as evidenced by the much reduced colony number in LKB1- cells 15 days after cisplatin administration.

The cytotoxic effects of cisplatin in LKB1- cells were partially due to oxidative stress, as suggested by restoration of clonogenic potential in the presence of 2

mM NAC, whereas antioxidant supplementation did not improve the clonogenic potential of LKB1+ cells (Figure 4.10C). Cisplatin, both through ROS generation and by forming adducts, induces DNA damage and cell cycle arrest. We assessed distribution in the different phases of cell cycle following 24 and 48 hours of treatment with 20 μM cisplatin in A549 LKB1- and LKB1+ cells. In both cell lines, cisplatin treatment blocked cell cycle in S phase. However, LKB1+ cells accumulated rapidly and almost completely in S phase, increasing the percentage of cells in G1/G0 phase at 48 hours, whereas LKB1- cells accumulated slowly in S phase, maintaining an elevated proportion of cells in G1/G0 phase (Figure 4.10D). These results suggested that LKB1+ cells might engage promptly DNA damage response systems, repairing DNA damage induced by the drug, whereas LKB1- cells might be less prompt to respond to DNA damage.

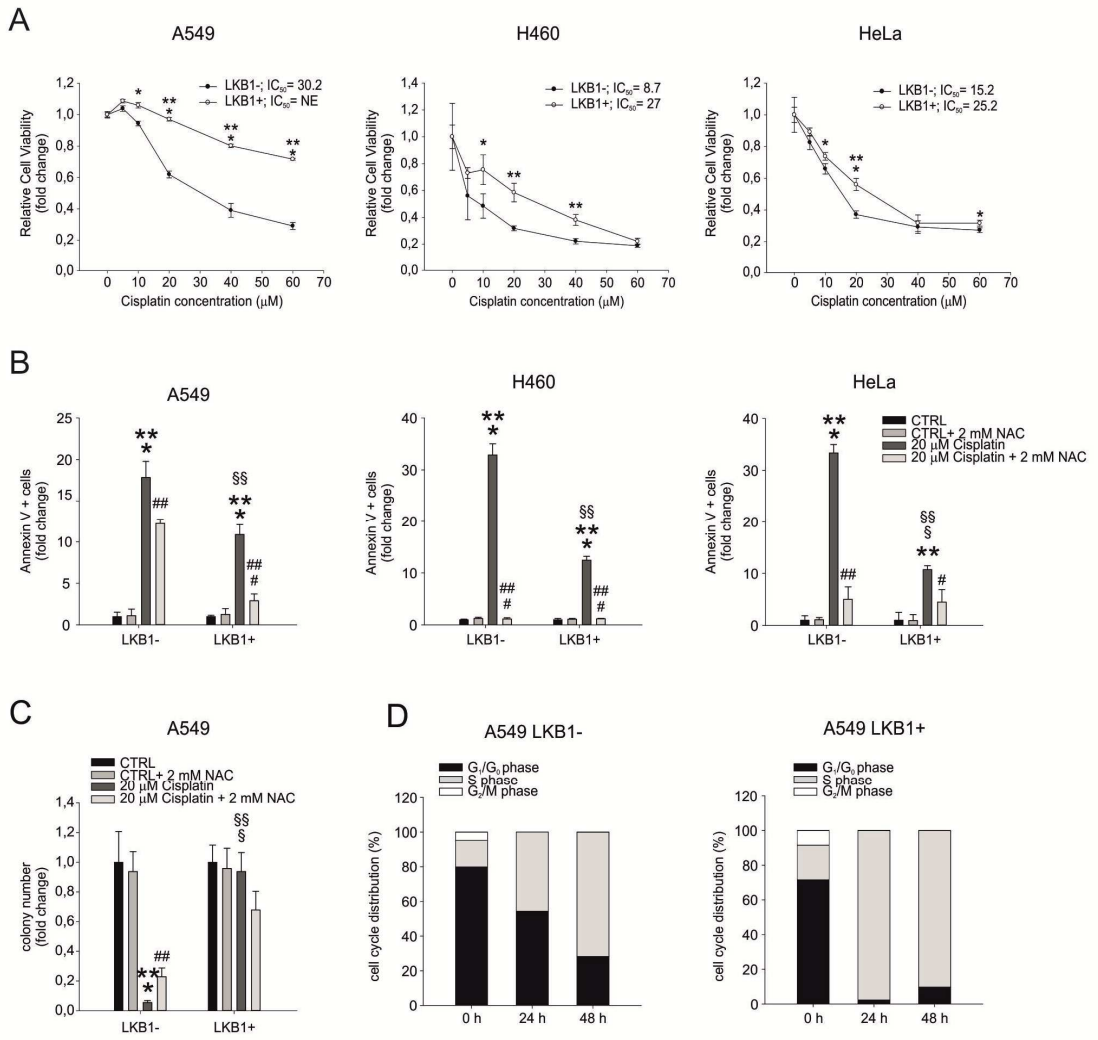

Figure 4.10. LKB1- cells are highly responsive to cisplatin. **A.** Measurement of cytotoxicity following different doses of cisplatin by the SRB assay. Representative images of three independent experiments are shown. *p<0.05, **p<0.01 and ***p<0.001 compared to LKB1- cells. IC_{50} values indicate the concentration of cisplatin that reduced cell viability to 50%. NE: not evaluable. **B.** Apoptosis evaluation following a single dose of cisplatin. Annexin V positivity is expressed relatively to untreated cells (CTRL), set equal to 1. Average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. **p<0.01 and ***p<0.001 compared with untreated (CTRL) cells. §§p<0.01 and §§§p<0.001 compared to LKB1- cells treated with 20 μM cisplatin. $^{##}$p<0.01 and $^{###}$p<0.001 compared to cells treated with 20 μM cisplatin in absence of pre-treatment with NAC. **C.** Clonogenic assay in A549 cells following 20 μM cisplatin treatment. Colony number is calculated relatively to untreated (CTRL) cells. Average colony number is indicated as mean ± standard deviation of three independent experiments. ***p<0.001 compared with untreated (CTRL) cells. §§§p<0.001 compared to LKB1- cells treated with 20 μM cisplatin. $^{##}$p<0.01 compared to cells treated with 20 μM cisplatin in absence of pre-treatment with NAC. **D.** Cell cycle profile of A549 cells 24 and 48 hours after cisplatin treatment.

Ionizing radiation is known to induce oxidative stress by ionization of water. We tested the response of LKB1- and LKB1+ cells to γ-irradiation. DCFDA staining of irradiated cells revealed that ROS generation was significantly increased in LKB1- cells, compared to LKB1+ cells (Figure 4.11A). Clonogenic potential was significantly greater in irradiated LKB1+ versus LKB1- cells and the detrimental effects of irradiation were attenuated by pre-treatment with 2 mM NAC, but this effect was limited to LKB1- cells (Figure 4.11B). Moreover, γ-irradiation was more cytotoxic to LKB1- cells, compared to LKB1+ cells, as evidenced by apoptosis evaluation. NAC pre-treatment partly rescued viability of irradiated LKB1- cells (Figure 4.11C). Irradiation induces severe DNA damage, eventually leading to cell cycle arrest. We assessed the cell cycle profile both 24 and 48 hours after γ-irradiation (14 Gy) of A549 LKB1- and LKB1+ cells. Irradiation blocked the cell cycle in G_2/M phase in both cell lines. However, irradiated LKB1+ cells accumulated more rapidly and extensively in G_2/M phase, reducing the percentage of cells in S phase, compared to LKB1- cells (Figure 4.11D). Altogether, these results suggested that LKB1+ cells might activate DNA damage response systems more efficiently than LKB1- cells.

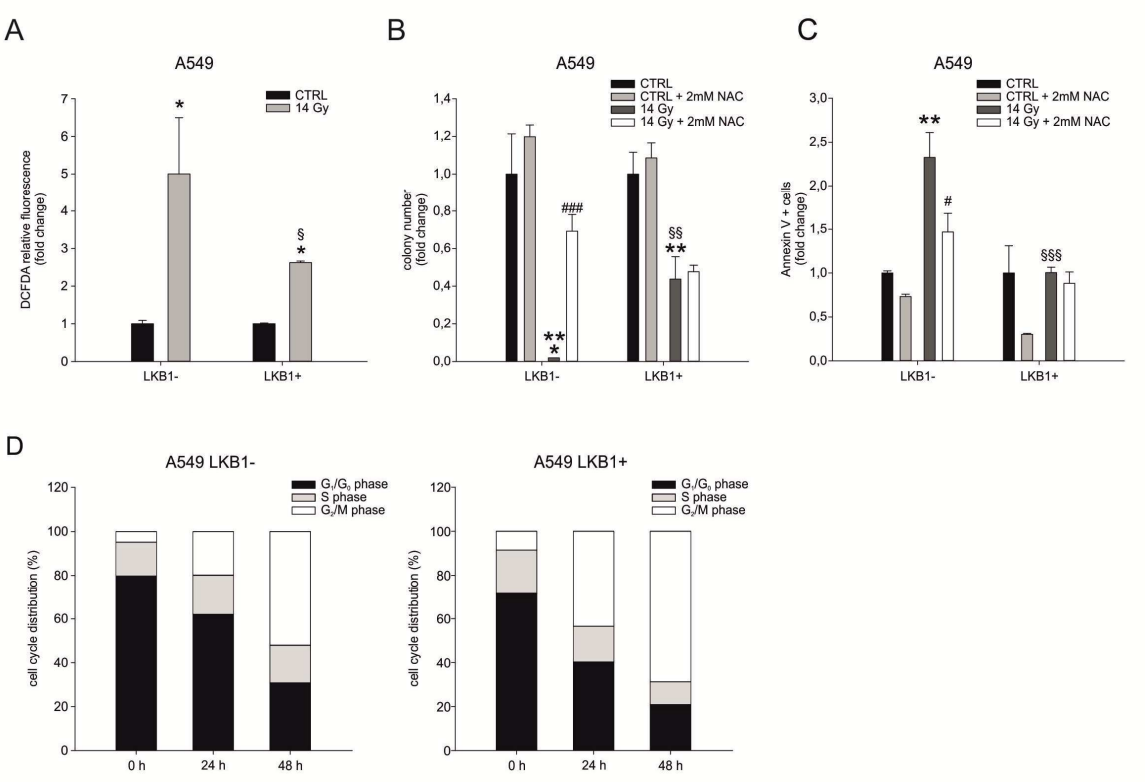

Figure 4.11. LKB1+ cells are relatively resistant to irradiation. **A.** ROS generation in irradiated A549 LKB1- and LKB1+ cells. DCFDA positivity is calculated relatively to staining of untreated cells (CTRL), set equal to 1. The average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. *p<0.05 compared with untreated (CTRL) cells. §p<0.05 compared to irradiated LKB1- cells. **B.** Clonogenic potential evaluation in A549 cells following 14 Gy γ-irradiation. Colony number is calculated respect to untreated (CTRL) cells. The average colony number is indicated as mean ± standard deviation of three independent experiments. **p<0.01 and ***p<0.001 compared with untreated (CTRL) cells. §§p<0.01 compared to irradiated LKB1- cells. $^{\#\#\#}$p<0.001 compared to irradiated cells in absence of pre-treatment with NAC. **C.** Apoptosis evaluation following γ-irradiation in A549 LKB1- and LKB1+ cells. Annexin V positivity is calculated relatively to untreated cells (CTRL). The average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. **p<0.01 compared to untreated (CTRL) cells. §§§p<0.001 compared to irradiated LKB1- cells. $^{\#}$p<0.05 compared to irradiated cells without pre-treatment with NAC. **D.** Cell distribution in G_1/G_0, S and G_2/M phases of the cell cycle in A549 cells 24 and 48 hours post-irradiation.

Overall, these results indicated that LKB1 protects cancer cells from cytotoxic effects caused by cisplatin or ionizing radiation, likely by reduction of oxidative stress and by repairing DNA damage induced by these treatments.

4.8 Cisplatin treatment sensitizes cancer cells to irradiation

Cisplatin is endowed with radiosensitizing ability and is often used in clinical practice in order to improve anticancer activity of radiotherapy (Liu *et al.*, 2014), (Toulany *et al.*, 2014). In order to test the role of LKB1 in response to the combination of cisplatin and irradiation, we determined the clonogenic potential of cisplatin-treated and γ-irradiated A549 and H460 LKB1- and LKB1+ cells. Cisplatin pre-treatment was carried out for 24 hours with a low non-cytotoxic dose of drug (5 μM for A549, 1 μM for H460), based on results of the SRB assay (see Figure 4.10A). Cisplatin and γ-irradiation reduced clonogenic potential of LKB1- cells, without or weakly affecting the clonogenic potential of LKB1+ cells. Combination of cisplatin and irradiation almost completely abolished clonogenic potential of LKB1- cells and markedly reduced the number of colonies generated from LKB1+ cells (Figure 4.12). Apoptosis evaluation did not reveal increased cell death following combination of cisplatin and irradiation, compared to single treatments. This finding indicated that cells were subjected to a sub-lethal damage, not detectable with apoptosis evaluation.

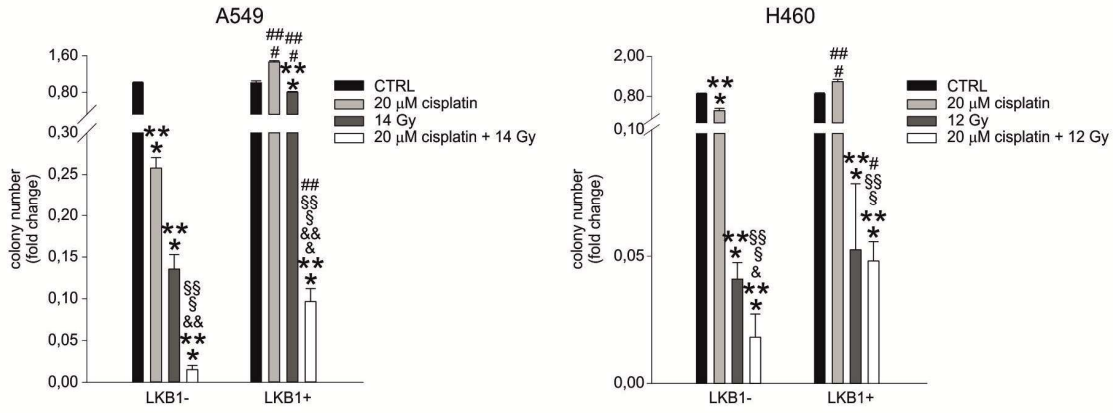

Figure 4.12. Combination of cisplatin and irradiation dramatically reduced the clonogenic potential of both LKB1- and LKB1+ cells. Colony number is calculated relatively to untreated (CTRL) cells. The average colony number is indicated as mean ± standard deviation of three independent experiments. ***p<0.001 compared with untreated (CTRL) cells. $^{\&}$p<0.05, $^{\&\&}$p<0.01 and $^{\&\&\&}$p<0.001 compared to irradiated cells. §§§p<0.001 compared to cells treated with cisplatin. $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared with LKB1- cells with the same treatment.

These results indicated that the combination of cisplatin and irradiation was much more effective than the single treatments and significantly reduced clonogenic potential of cisplatin- and radiation-resistant LKB1+ cells. Cisplatin, by reducing cellular antioxidant potential, could sensitize cancer cells to the damaging effects of oxidative stress induced by ionizing radiations. Future studies are needed to determine whether the combination of cisplatin and irradiation increases oxidative stress more than the single treatments. Another possible explanation is that radiosensitizing ability of cisplatin could be due to excessive DNA damage induced by the combination of this chemotherapeutic with ionizing radiations.

4.9 *LKB1* silencing increased sensitivity to oxidative stress

To rule out the possibility that resistance to oxidative stress observed in LKB1+ cells might be due to over-expression of LKB1 protein, we used a complementary approach: *LKB1* silencing in two NSCLC cell lines, H1975 and H1650, which had physiological LKB1 expression levels. RT-qPCR analysis indicated an average silencing of *LKB1* transcripts of 80%. Inhibition of LKB1-AMPK pathway activity by *LKB1* silencing was assessed by Western blot analysis (Figure 4.13A). We evaluated whether *LKB1* silencing increased sensitivity of LKB1-proficient cells to oxidative stress. To this aim, we treated H1975 and H1650 siRNA and siLKB1 cells with 2 mM H_2O_2. *LKB1* silencing increased sensitivity of LKB1-proficient H1650 cells to H_2O_2, as measured by apoptosis evaluation (Figure 4.13B).

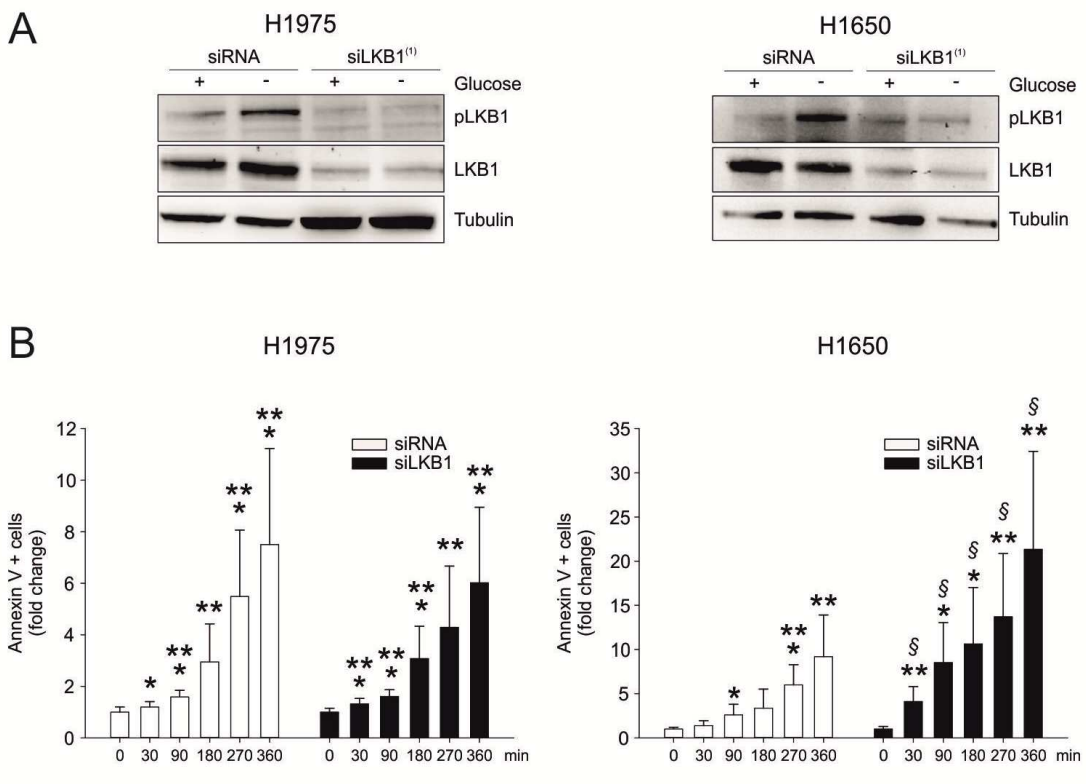

Figure 4.13. *LKB1* silencing in H1975 and in H1650 cancer cell lines impaired LKB1-AMPK pathway activity and sensitized H1650 cells to H_2O_2 - induced oxidative stress. **A.** Western blot analysis following glucose deprivation. **B.** Annexin V positivity is calculated respect to staining of untreated (0 minutes) cells, set equal to 1. The average fluorescence intensity is indicated as mean ± standard deviation of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared with untreated (0 minutes) cells. §p<0.05 compared to siRNA cells treated with H_2O_2 for the same time.

Sensitivity of *LKB1*-silenced cells to cisplatin was measured by the SRB cytotoxicity assay. Results showed that siLKB1 cells - particularly H1650 cells - were more sensitive to cisplatin treatment, compared to control siRNA cells (Figure 4.14).

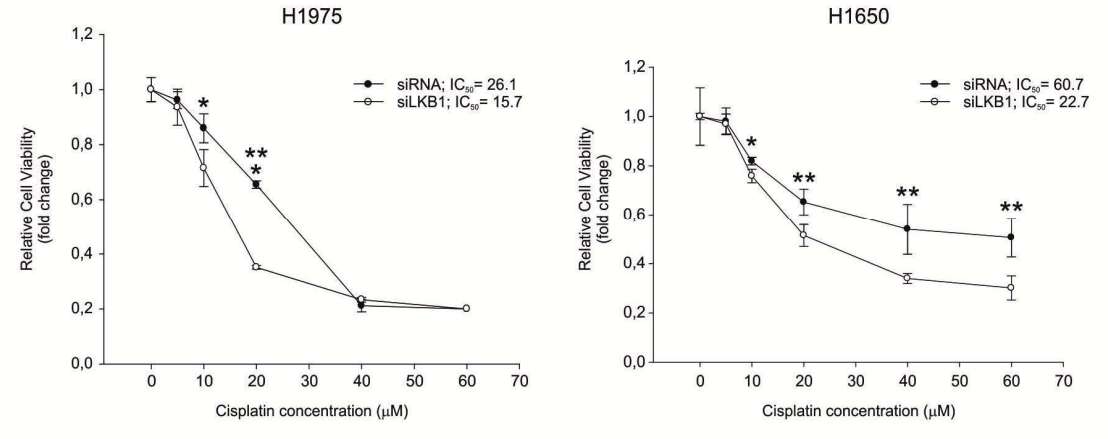

Figure 4.14. *LKB1* silencing sensitized H1975 and H1650 cells to cisplatin-mediated cytotoxic effects. Representative images of three independent experiments are shown. *p<0.05, **p<0.01 and ***p<0.001 compared with siRNA cells.

Collectively, these data indicated that *LKB1* silencing increased cytotoxic effects of oxidative stress induced by H_2O_2 and by cisplatin treatment.

4.10 LKB1+ cells are relatively resistant to chemotherapeutics inducing oxidative stress

Increased sensitivity to cisplatin and to γ-irradiation prompted us to investigate whether LKB1 deficiency defines a subset of cancer cells more responsive to ROS-inducing chemotherapeutics. ROS induction has been previously reported following treatment of tumor cells with several chemotherapeutic drugs, including taxanes (Alexandre *et al.*, 2007), anthracyclines (Sterba *et al.*, 2013) and arsenic trioxide (You and Park, 2012). We evaluated cytotoxicity following treatment with paclitaxel (taxane), doxorubicin (anthracycline), or arsenic trioxide. Results of SRB assay revealed that H460 and HeLa LKB1+ variants were more resistant than their LKB1- counterparts to all chemotherapeutics tested. In contrast, no significant difference was observed in A549 LKB1- and LKB1+ cells (Figure 4.15).

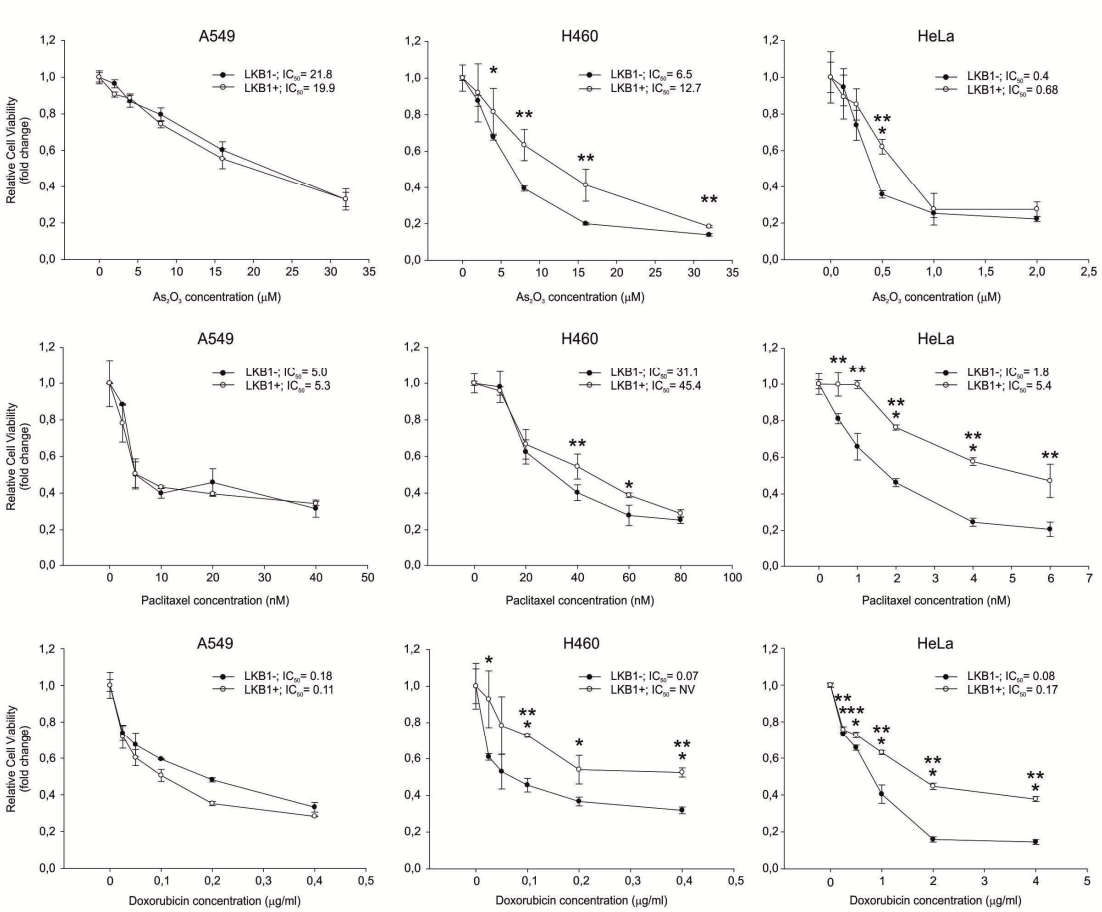

Figure 4.15. Cytotoxic effects following treatment with arsenic trioxide, paclitaxel or doxorubicin in tumor cell lines. Representative images of three independent experiments are shown. *p<0.05, **p<0.01 and ***p<0.001 compared with LKB1- cells.

These results indicated that the LKB1 status was associated with different response to several ROS-inducing drugs commonly used in the clinic, thus suggesting that LKB1 status could predict tumor response to several chemotherapeutic regimens.

4.11 Evaluation of LKB1 status as predictive biomarker of response to ROS-inducing cancer therapies

In vitro data demonstrating that LKB1 expression confers resistance to oxidative stress in cancer cells prompted us to investigate the role of LKB1 in a retrospective study in advanced NSCLC patients treated with platinum-based chemotherapy plus bevacizumab.

Antiangiogenic therapy, by inducing a condition of starvation and hypoxia in tumors, leads to overproduction of ROS in cancer cells (Fack *et al.*, 2015). To study the possible predictive role of LKB1 status in response to antiangiogenic therapy, we assessed *LKB1* mutational status and evaluated expression of LKB1 protein by IHC in a cohort of 40 advanced NSCLC patients treated with the antiangiogenic drug bevacizumab plus platinum-based chemotherapy.

LKB1 gene was sequenced by either Sanger sequencing and next-generation sequencing, through Ion Torrent technology. As no hotspot mutations were described in *LKB1*, we sequenced all 9 coding exons. Mutations in *LKB1* gene were identified in 7 out of 21 analysable samples (33.3%; about 50% of samples were not analysable due to DNA low quality). IHC analysis identified LKB1 loss in 10 samples out of 32 analysed samples (31.25%). Among mutations in *LKB1* gene, we identified 6 truncating mutations, involving aminoacids 63, 152, 158, 279, 290 and 293 of LKB1 protein, and a missense mutation, substituting valine at position 372 with glutamic acid (Table I). *LKB1* mutations were strongly associated with IHC negative staining (Fisher's exact test $p=7{,}145 \cdot 10^{-5}$). Pt 10 and Pt 11 showed IHC negative staining even in absence of *LKB1* mutations.

Sample ID	*LKB1* mutational status	IHC score
Pt 1	WT	5
Pt 2	non-amplifiable	10
Pt 3	WT	6
Pt 4	**c.474_480delTCAGCTG (p.C158*)**	**0**
Pt 5	**c.190_218del29 (p.Q152*)**	**0**
Pt 6	WT	12
Pt 7	WT	5
Pt 8	WT	5
Pt 9	non-amplifiable	3
Pt 10	WT	0
Pt 11	WT	0
Pt 12	non-amplifiable	0
Pt 14	non-amplifiable	12
Pt 15	non-amplifiable	5
Pt 16	non-amplifiable	4
Pt 17	WT	5
Pt 18	WT	NE
Pt 19	WT	6
Pt 20	non-amplifiable	NE
Pt 21	non-amplifiable	12
Pt 22	**c.849_850delTG (p.L290*)**	**0**
Pt 23	WT	12
Pt 24	WT	2
Pt 25	**c.1115T>A (p.V372D)**	**0**
Pt 26	WT	3
Pt 27	non-amplifiable	NE
Pt 28	non-amplifiable	4
Pt 29	non-amplifiable	0
Pt 30	**c.641_662del22 (p.G279*)**	**0**
Pt 31	non-amplifiable	18
Pt 32	non-amplifiable	12
Pt 33	**c.157delG (p.V63*)**	**0**
Pt 34	WT	18
Pt 35	non-amplifiable	2
Pt 36	non-amplifiable	NP
Pt 37	non-amplifiable	NP
Pt 38	non-amplifiable	NP
Pt 39	non-amplifiable	NP
Pt 40	non-amplifiable	NP
Pt 41	**c.877G>T (p.E293*)**	NP

Table I. *LKB1* mutational status and IHC staining of advanced NSCLC patients treated with bevacizumab + chemotherapy. WT: wild-type. NE: not evaluable. NP: not performed.

LKB1 status, defined as either *LKB1* mutational status or IHC staining, was not associated with response to therapy (Fisher's exact test p=0.3784).

We subsequently assessed progression free survival (PFS) and overall survival (OS) according to LKB1 status. Median PFS was 7.8 months (min. 1.7 months, max. 24.1 months). 1-year PFS was 34.1% in LKB1 - wild-type patients and 36.4% in LKB1-mutated patients. Kaplan-Meier curves related to PFS are reported in Figure 4.16. Results indicated that LKB1 status was not correlated to PFS following bevacizumab + chemotherapy treatment (Log-rank test p=0.9753).

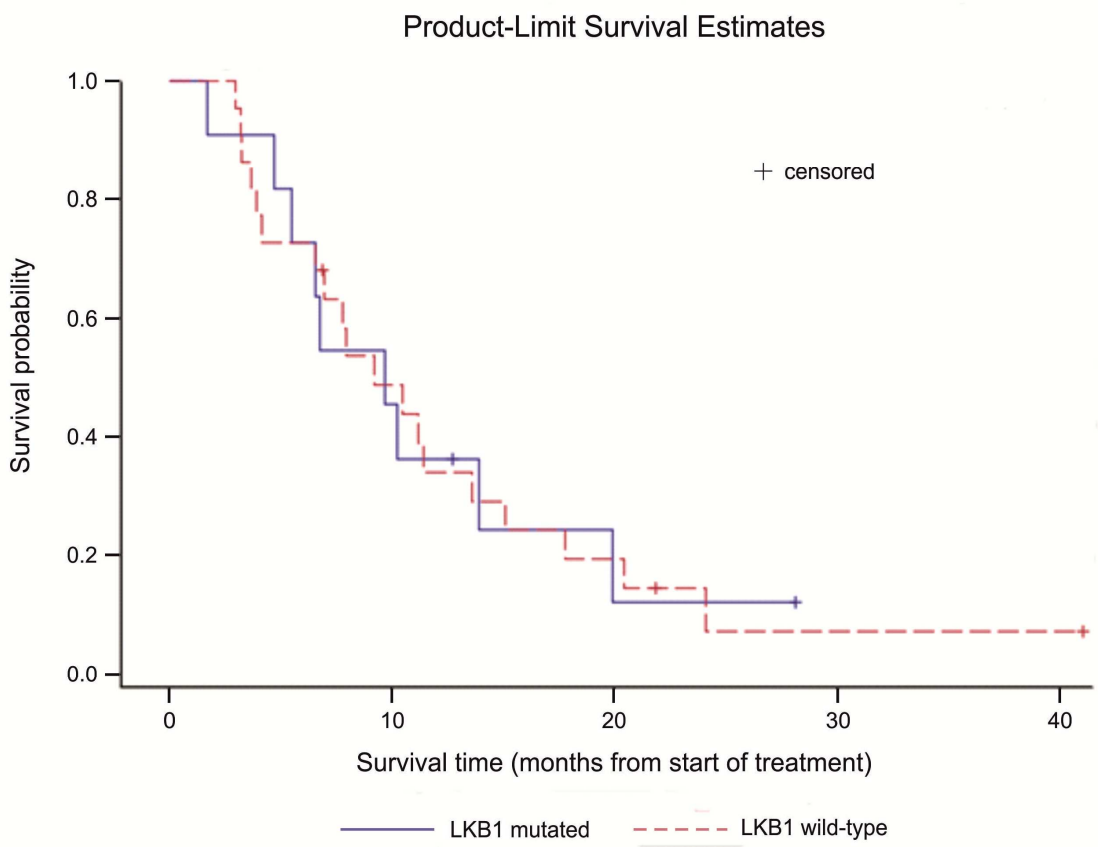

Figure 4.16. Kaplan-Meier curves related to PFS of 33 advanced NSCLC patients treated with bevacizumab + chemotherapy.

Median OS was 26.9 months (min. 26.2 months, max. 28.0 months). 2-year OS was 36.4% in LKB1 - wild-type patients and 36.4% in LKB1-mutated patients. Kaplan-Meier curves related to OS are reported in Figure 4.17. Results indicated that LKB1 status was not correlated to OS (Log-rank test p=0.7105). In conclusion, this pilot translational study showed that it is equivalent to

determine the LKB1 status by IHC or at the genetic level. Likely due to the small number of samples analysed or the possible confounding effect of mutations in additional genes that were not scored (i.e. KRAS and TP53), LKB1 status did not correlate with response to chemotherapy + antiangiogenic therapy, nor was a prognostic factor.

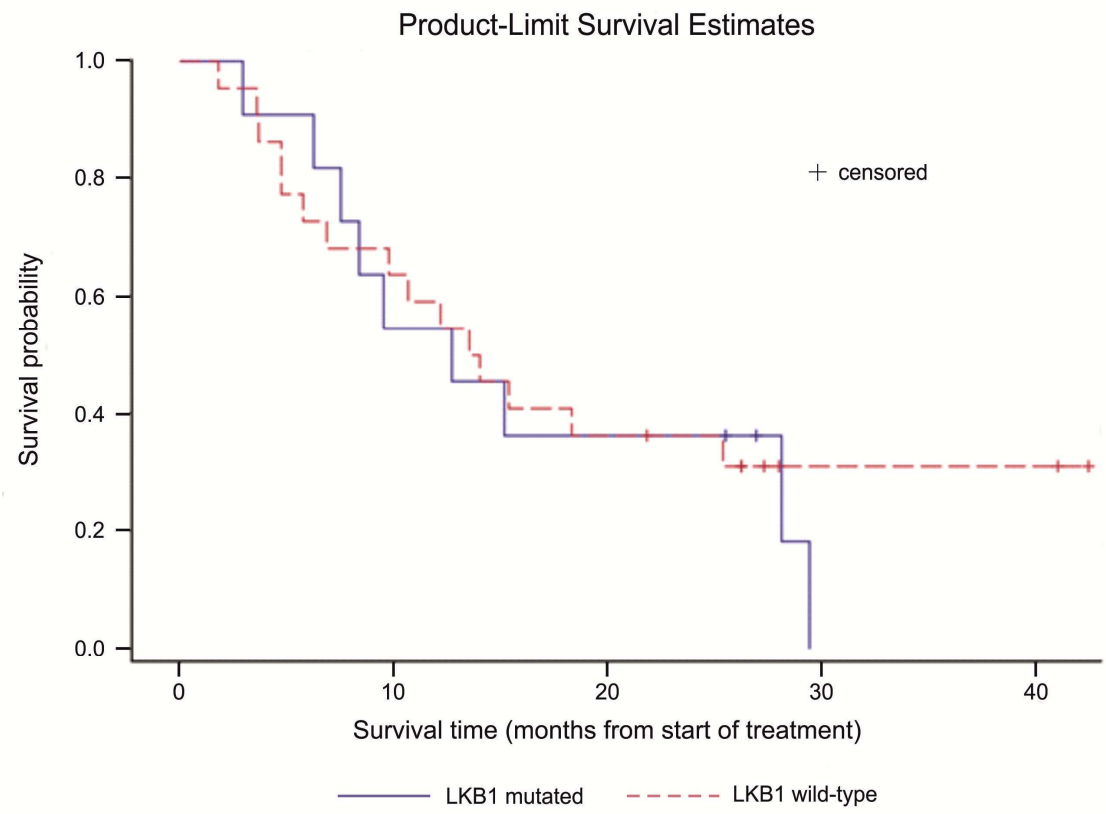

Figure 4.17. Kaplan-Meier curves related to OS of 33 advanced NSCLC patients treated with bevacizumab + chemotherapy.

5. Discussion

Lung cancer is one of the most lethal malignancies, owing to its very high prevalence and aggressiveness. Non-small cell lung cancer (NSCLC), the major histological type, accounts for 85-90% of lung cancer cases, representing a continuously growing socio-economic problem. By 2030, in fact, cancer is estimated to become the first cause of death and NSCLC, characterized by an elevated morbidity and mortality, by a currently increasing incidence among women and by outstanding resistance to therapies, might become the principal public health issue, with a huge economic impact on National Health Systems in Western Countries.

Given the elevated prevalence and mortality of lung cancer, a great effort has been put to unravel the molecular mechanisms underlying lung carcinogenesis. About 30% of NSCLC cases bear an inactivating mutation in the gene encoding for the threonine/kinase Liver Kinase B1 (LKB1). *LKB1* gene was initially identified as the tumor suppressor gene responsible for the Peutz-Jeghers syndrome, an autosomal dominant disorder characterized by hamartomatous polyps of the gastrointestinal tract and increased risk of cancer. *LKB1* mutations are rare in sporadic cancers, except in NSCLC and in cervical carcinoma. LKB1 coordinates a signalling pathway controlling energy homeostasis through AMP-activated Protein Kinase (AMPK) and 12 AMPK-related kinases. The tumor suppressor activity of LKB1/AMPK pathway and the frequent inactivation of LKB1 in NSCLC suggest an important role of LKB1 in lung carcinogenesis. Recently, several studies identified different molecular pathways dis-regulated in lung cancer following *LKB1* loss: the MZF1/c-MYC pathway (Tsai *et al.*, 2014), VEGF signaling (Liang *et al.*, 2014), Wnt/β-catenin (Jian *et al.*, 2014), increased phosphorylation of certain tyrosine kinase receptors (i.e. EGFR, ErbB2, c-Met, EphA2, RET and IGF1R; (Okon *et al.*, 2014)), the CRTC1-NEDD9 signalling axis (Feng *et al.*, 2012), and p53 and p16 (Liang *et al.*, 2010). Noteworthy, several studies in the literature focused on the role of LKB1 following stress conditions that alter cancer cell physiology. The consequent AMPK activation, caused by energy imbalance, contributes to tumor suppression. Nevertheless, LKB1/AMPK

pathway could reasonably control lung carcinogenesis even in absence of exogenous perturbing agents, when energy homeostasis is maintained. Moreover, LKB1/AMPK activity could influence tumor growth following endogenous metabolic stress, caused by uncontrolled proliferation of cancer cells. Oxidative stress is the principal endogenous stress that cancer cells must cope with, both generated as collateral effect of mitochondrial respiration (Li *et al.*, 2013) and required to sustain proliferation and invasive phenotype of cancer (Kumar *et al.*, 2008). Reactive oxygen species (ROS) are a "double-edged sword" on cancer, as low levels are pro-tumorigenic, whereas high levels induce an extensive damage to cellular structures, leading to cell death. We found that LKB1-expressing cancer cells are more resistant to exogenous oxidative stress and this is at least in part attributable to reduced steady-state ROS production following NOX1 inhibition.

Recently, Jeon and colleagues identified a role for AMPK in NADPH homeostasis maintenance (Jeon *et al.*, 2012). NADPH is necessary to preserve the reducing potential of cells, which is fundamental to counteract the damaging effect of excessive ROS on nucleic acids, lipids and proteins. In particular, NADPH acts as electron donor during reduction of oxidized antioxidant systems, such as glutathione and thioredoxin. According to this study, AMPK regulates NADPH homeostasis through the phosphorylation (inhibition) of ACC1 and ACC2, with the consequent decrease of NADPH-consuming fatty acid synthesis and the increase of NADPH-generating β-oxidation of fatty acids. Conceivably, compromised NADPH homeostasis - as would occur in cells lacking LKB1 activity - could cause vulnerability to oxidative stress. The observations by Jeon *et al.* also imply that LKB1+ cancer cells would contain higher levels of reduced glutathione compared with LKB1- cells, especially under oxidative stress. In our experiments, however, we did not find such a difference nor did we observe significant differences in ACC phosphorylation in LKB1- versus LKB1+ cells (data not shown). Probably, the fact that oxidative stress can also trigger LKB1-independent AMPK activation - mediated by Ca^{2+}/Calmodulin-dependent protein kinase kinase β (CaMKKβ) (Witters *et al.*, 2006) - could in part explain these findings. Finally, in light of our findings linking LKB1 to NOX1, it should be considered that the reducing potential of NADPH is also used by NOX1 to generate ROS in a futile

redox cycling reaction. Therefore, increased amount of NADPH in LKB1+ cells described by Jeon *et al.* could be a consequence of reduced ROS generation, rather than be the cause of increased resistance to oxidative stress.

In any case, several lines of evidence support a role of LKB1/AMPK in the cellular response to oxidative stress. First, LKB1/AMPK activation inhibits cell proliferation, which is followed by reduced ROS production by mitochondria, as anabolic processes generally boost oxidative stress. Second, LKB1 can decrease KRAS-induced ROS production (Weinberg *et al.*, 2010) and this may explain the frequent co-occurrence of *LKB1* inactivating mutations and *KRAS* activating mutations in NSCLC. Third, glutathione synthesis requires two ATP-dependent steps (γ-glutamylcysteine synthesis and glycine addition). During energy stress, induced by glucose deprivation (such as antiangiogenic therapy) or by radio-chemotherapy, cancer cells with a functional LKB1/AMPK pathway are more likely to maintain ATP levels, thus preserving glutathione synthesis. In contrast, *LKB1*-mutated cells would undergo shortage of ATP levels and a subsequent drop of glutathione synthesis. Fourth, antioxidant and chemopreventive effects of red ginseng have recently been attributed to mitochondria protection mediated by LKB1/AMPK (Dong *et al.*, 2013a). Finally, during metabolic stress LKB1/AMPK activation induces autophagy, a double-edged sword process in cancer. In fact, autophagy can have an anti-tumorigenic role by inhibiting accumulation of damaged proteins and organelles, as well as a pro-tumorigenic role by acting as a cell survival mechanism (Yang *et al.*, 2011). By recycling of cellular proteins and organelles, autophagic cancer cells are able to maintain energy production during stress conditions, such as chemotherapy. Therefore, autophagy in cancer cells is associated with chemoresistance and radioresistance. In particular, mitophagy (autophagy of mitochondria) is a clearance mechanism that eliminates damaged or hyperactive mitochondria, more prone to ROS production (Soengas, 2012). Hence, LKB1/AMPK activation provides a survival advantage to treated cancer cells, both by reducing ROS production and protecting from oxidative damage through autophagy.

Recently, Xu and colleagues reported that LKB1 protects cells from ROS-mediated cell damage through p38 activation (Xu *et al.*, 2014). The authors observed that cells lacking LKB1 exhibit increased ROS generation, but attributed

the protective role of LKB1 to an elegant and complex mechanism involving enhanced antioxidant systems activity through the mitogen-activated protein kinase p38. The consequent increased activity of superoxide dismutase 2 (SOD2) and catalase protects cells from DNA damage and loss of viability. We did not assess the activity of SOD2 and catalase, but we observed that genes encoding for these two antioxidant enzymes were not up-regulated in LKB1+ cells. Moreover, we evaluated phosphorylation of p38 in A549 LKB1- and LKB1+ cells and we did not observe any increase of phospho-p38 levels in LKB1+, compared to LKB1- cells (data not shown). Importantly, according Xu and colleagues, LKB1 protective role from endogenous oxidative stress is independent of AMPK, whereas we observed that AMPK inhibition through Compound C and siRNA-mediated *AMPK* silencing abolish resistance of LKB1+ cells to oxidative stress and exacerbate sensitivity of LKB1- cells. It is noteworthy that Xu and colleagues generated most of their data working with mouse embryonic fibroblasts. Conceivably, cell-type differences could explain the discrepancies between these two studies. In our model, sensitivity to oxidative stress associated with LKB1 loss likely depends from increased NOX1-mediated ROS generation under standard cell culture conditions, as demonstrated by inhibiting NOX1 with apocynin and by *NOX1* gene silencing.

A somewhat unexpected finding of our study was the apparent up-regulation of several antioxidant genes in LKB1- cells. This may be explained considering some known paradoxical features of redox balance in cancer cells: cancer cells that produce large amount of ROS need adaptive mechanisms to survive, thus increasing synthesis of antioxidant systems (Traverso *et al.*, 2013).

Interestingly, we observed a marked down-regulation of *NOX1* and *PPARGC1A* in LKB1+ variant of all isogenic cell lines analysed. NADPH oxidase 1 (also known as GP91-2), the protein encoded by *NOX1* gene, is an homolog of the catalytic subunit of the superoxide-generating NADPH oxidase of phagocytes, gp91phox (NOX2). NADPH oxidases are multi-subunit enzyme complexes that use NADPH as an electron donor in the reduction of molecular oxygen to produce superoxide anion. Until recent years, it was believed that cytochrome b-245, known as gp91phox, was the only catalytic subunit of NADPH oxidase and that was peculiar of professional phagocytes. Recently, however, six homologs of

gp91phox were found, including NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2 (Bedard and Krause, 2007). NADPH oxidases are composed of 6 subunits: a Rho GTPase (usually Rac1 or Rac2), and 5 phox subunits (the catalytic subunit, p22phox, p40phox, p47phox and p67phox). In neutrophils, the complex is latent and is activated to assemble in the membranes during respiratory burst. NADPH oxidase transfers electrons derived by NADPH across the membrane and couples them to molecular oxygen to produce superoxide anions and kill bacteria and fungi contained in phagosomes. All NADPH oxidases share the ability to transfer electrons across the plasma membrane and to produce superoxide outside the cells, rapidly dis-mutated in H_2O_2, which easily permeates cell membranes. As NADPH oxidases are present not only in immune cells, the physiological functions of NOX family enzymes include, besides host defence, also posttranslational modification of proteins, cellular signalling, regulation of gene expression and cell differentiation. Increased NADPH oxidase activity has been linked particularly to cardiovascular diseases and neurodegeneration. In fact, a role of NADPH oxidase has been described in atherosclerosis (Violi *et al.*, 2009) and in Alzheimer's disease (Ansari and Scheff, 2011). Considered the involvement of ROS in both early and late stages of tumorigenesis, NADPH oxidase dysregulation has been linked also to cancer initiation (Parzefall *et al.*, 2014) and progression (Gupta *et al.*, 2014). Thus, we hypothesized that *NOX1* down-regulation in LKB1+ cells could account for reduced ROS generation and, ultimately, for increased resistance to exogenous oxidative stress. We demonstrated that LKB1- cells undergo increased basal ROS generation, heightened oxidation of glutathione and, finally, increased sensitivity to H_2O_2–induced cell damage. In this regard, it should be pointed out that, as cited above, NOX1 is not the only catalytic subunit of NADPH oxidases. Thus, down-regulation of other NOX subunits expressed in the lung and in the uterine cervix (such as NOX2, NOX4 and NOX5) could also contribute to resistance to oxidative stress observed in LKB1+ cells. The vanillin-related compound apocynin is a NOX1 and NOX2 complex formation inhibitor, thus it should not be excluded that apocynin rescued LKB1- cells viability partly through NOX2 inhibition. On the other hand, results obtained with siRNA-mediated specific *NOX1* silencing seem to rule out the possible involvement of NOX2. Notably, decreased NADPH

oxidase activity could be more important than reduced expression of catalytic subunits. Hence, down-regulation of regulatory subunits, such as Rac1 or non-catalytic phox subunits, could also account for reduced ROS production in LKB1+ cells. For instance, up-regulation of p22phox mediates NADPH oxidase activation in pancreatic cancer cells (Edderkaoui *et al.*, 2013) and H_2O_2 production downstream of FLT3 (Woolley *et al.*, 2012).

With regard to the mechanism linking LKB1 to NOX1 in cancer cells, no connection was described in the literature. However, some evidences from the fields of inflammation and cardiovascular disorders suggested the involvement of LKB1/AMPK in modulation of ROS production by NADPH oxidases. In macrophages, for instance, the protective role of adiponectin against ethanol-induced ROS production has been ascribed to AMPK-mediated NOX2 down-regulation, via inhibition of NF-κB (Kim *et al.*, 2014). Moreover, a role of AMPK in protection against ROS-induced vascular dysfunction was recently identified (McCarty *et al.*, 2009), (Wang *et al.*, 2010a), again involving inhibition of NF-κB. In line with these findings, we demonstrated that AMPK has a fundamental role in LKB1-mediated resistance to oxidative stress and that AMPK inhibition through Compound C or siRNA-mediated *AMPK* silencing sensitize LKB1+ cells to H_2O_2 treatment and further increase LKB1- cells sensitivity to exogenous oxidative stress. It remains to be investigated whether AMPK silencing caused perturbations in NF-κB activity and NOX1 expression in our models.

Alternatively, AMPK activity could be linked to NOX1 down-regulation through Protein Kinase C (PKC)-δ (Kong *et al.*, 2012), as observed in ischemic heart diseases. In particular, reduced AMPK activity in LKB1- cells could increase PKC-δ activation, leading to EGFR transactivation, ERK1/2 phosphorylation and subsequent NOX1 up-regulation, as described in (Fan *et al.*, 2005). Of note, increased EGFR autophosphorylation and, in general, receptor tyrosine kinase phosphorylation, has been observed in NSCLC following LKB1 loss ((Okon *et al.*, 2014); see above). Thus, further investigation is needed to study the possible role of EGFR transactivation in increased generation of ROS. Moreover, as PKC-δ is activated by diacylglycerol, it should be interesting to investigate whether inhibition of PKC-δ activation is mediated by AMPK ability to mediate lipid processing in the plasma membrane. The complex mechanism that putatively

links AMPK to NOX1 delineates a possible role of the LKB1/AMPK pathway in the control of inflammation, which also supports cancer initiation and growth.

With regard to PGC-1α, the co-activator encoded by *PPARGC1A* gene also found up-regulated in LKB1- cells, its role in response to oxidative stress is far more difficult to dissect. In fact, PGC-1α, by fostering mitochondrial biogenesis and enhancement of electron transport chain activity, increases mitochondrial ROS generation, but, at the same time, contributes to ROS detoxification, thus providing a protective role against ageing and neurodegenerative disorders (Austin and St-Pierre, 2012). Moreover, PGC-1α, together with AMPK and SIRT1, takes part in an energy-sensing network that mediates cell survival following energy stress (Canto and Auwerx, 2009) and is necessary to increase mitochondrial activity following AMPK activation. Thus, up-regulation of *PPARGC1A* in LKB1- cells is counterintuitive. Probably, in cancer cells with a functional LKB1/AMPK pathway, PGC-1α down-regulation would be advantageous, as enhanced activity of damaged cancer mitochondria could lead to generation of lethal amounts of ROS. However, PGC-1α could be directly involved in cancer phenotype, supporting glutamine metabolism and reverse citric acid cycle to sustain anabolic processes, as described in breast cancer (McGuirk *et al.*, 2013). By providing a surviving mechanism or by modulating the Warburg phenotype, PGC-1α down-regulation in LKB1+ cells could be linked to reduced ROS generation. Although currently unknown, the possible mechanism that could account for reduced PGC-1α expression in LKB1+ cells appears to be mediated by Signal Transducer and Activator of Transcription (STAT)3 activation (work in progress).

Increased resistance of LKB1+ cells to H_2O_2–induced oxidative stress suggests that LKB1/AMPK pathway could influence response to several cancer treatments known to induce ROS generation. We initially tested cisplatin and irradiation, as platinum-based chemotherapy is a first-line treatment in NSCLC and radiotherapy is one of the therapeutic options for this type of cancer. We demonstrated that LKB1+ variants of all isogenic cell lines tested were more resistant than their LKB1- counterparts were, and that oxidative stress was involved in response to treatment, as confirmed by effects of NAC supplementation. Moreover, we found that LKB1+ cells blocked cell cycle more

rapidly and extensively than LKB1- cells. The rapid block of LKB1+ cells in S phase after cisplatin treatment and in G2/M phase after irradiation suggests recruitment of DNA repair systems, whereas LKB1- cells continue to proliferate, even though their DNA is damaged, and block cell cycle belatedly. Recently, it has been proposed that AMPK acts as genomic stress sensor and promotes DNA damage response (Sanli *et al.*, 2014), (Ui *et al.*, 2014). Thus, it could be postulated that relative resistance to cisplatin, irradiation, paclitaxel or doxorubicin treatment observed in LKB1+ cells is mediated by enhanced DNA repair, compared to LKB1- cells. However, the pro-survival effect of NAC supplementation in cisplatin-treated or irradiated LKB1- cells indicates that resistance to cytotoxic cancer treatments does not depend only on DNA repair. In conclusion, we hypothesize that resistance to oxidative stress in LKB1+ cells is multifaceted, involving both anti-proliferative, ROS-inhibiting, pro-autophagic and DNA repair mechanisms.

Radiotherapy is often given in combination with cisplatin or carboplatin, as platinum compounds are radio-sensitizers. One explanation of this property is that platinum compounds, by forming DNA adducts, inhibit non-homologous end joining (NHEJ) DNA repair system, thus sensitizing cancer cells to double-strand breaks induced by radiations (Boeckman *et al.*, 2005). However, these findings do not explain the specificity for cancer cells, as cisplatin-based chemotherapy and radiotherapy induce DNA damage even in normal cells. An alternative explanation takes into account the redox balance of cancer cells. In fact, aquated cisplatin is a very reactive compound that easily interacts with many nucleophiles in the cytoplasm. Glutathione is a nucleophilic molecule involved in phase II detoxification reactions through the enzyme glutathione-S-transferase (GST). Thus, glutathione conjugates to cisplatin in order to eliminate this cytotoxic compound outside the cytoplasm. Therefore, cisplatin treatment induces glutathione depletion (Siddik, 2003). The consequent decrease of antioxidant defence could sensitize cancer cells to ROS-inducing radiations, while normal cells, less subjected to endogenous ROS generation, could better tolerate glutathione depletion. We tested the radio-sensitizing ability of cisplatin on A549 and H460 LKB1- and LKB1+ cells. Apoptosis evaluation at 24 and 48 hours post-irradiation revealed no increase in cytotoxicity compared to single

treatments. However, clonogenic potential evaluation indicated that a low dose of cisplatin was able to effectively kill cancer cells in combination with irradiation, at doses where cisplatin alone and irradiation as single agents were much less effective. These effects were stronger in LKB1- than in LKB1+ tumor cells. Speculatively, the increased amount of reduced glutathione available in LKB1+ cells counteracts the damaging effects of ROS induced by irradiation. Quantification of glutathione levels following cisplatin treatment will be required to clarify whether cisplatin leads to severe glutathione depletion in LKB1- cells. Interestingly, the combination of cisplatin with irradiation did not increase apoptosis, compared with single treatments. This suggests that combined treatment caused a sub-lethal damage, which probably involves permanent cell cycle arrest and cellular senescence. In line with this hypothesis, optical microscope analysis during the clonogenic assay evidenced, besides colonies, the presence of numerous single cells characterized by altered morphology.

In the final part of this work, encouraged by these *in vitro* data, we tested the possible predictive role of LKB1 in response to antiangiogenic therapy and platinum-based chemotherapy in a cohort of advanced NSCLC patients. We assessed mutational status of *LKB1* gene by sequencing and expression levels of LKB1 protein by IHC, thus identifying a robust correlation between inactivating mutations and lack of protein. Unfortunately, LKB1 status was not predictive of response to treatment, considering both progression free survival (PFS) and overall survival (OS) parameters. However, it should be considered the small number of samples analysed as well as the intrinsic limitations of a retrospective study. Moreover, other genetic factors could influence the therapeutic response. In particular, *LKB1* inactivating mutations often co-occur with *KRAS* activating mutations in NSCLC. Oncogenic RAS signalling, by promoting uncontrolled cell proliferation, anabolic metabolism and ROS generation, may potentiate the effects of LKB1 loss on ROS homeostasis. In this regard, it is important to note that both A549 and H460 lung cancer cell lines utilized in our study carried both *KRAS* and *LKB1* mutations. Thus, KRAS status - which was unknown in our retrospective study - could be important to modulate therapeutic responses to this drug combination. Future studies are planned to investigate this possibility

by genetic screening of mutations in lung cancer-associated genes using a next generation sequencing panel.

In conclusion, this study reinforce the evidence connecting LKB1/AMPK to regulation of oxidative stress and identified a novel NOX1-mediated mechanism accounting for increased ROS production in LKB1-deficient tumor cells. As LKB1 loss confers increased sensitivity of cancer cells to γ-irradiation and treatment with several cytotoxic drugs *in vitro*, it will be important to validate these findings in clinical studies.

6. References

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