UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Università degli Studi di Padova

Dipartimento di *Scienze Chirurgiche, Oncologiche e Gastroenterologiche*

SCUOLA DI DOTTORATO DI RICERCA IN
ONCOLOGIA E ONCOLOGIA CHIRURGICA
XXVIII CICLO

WILMS' TUMOR SUPPRESSOR GENE (*WT1*) LOSS IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA PROMOTES CELL SURVIVAL AND RESISTANCE TO DNA DAMAGE

Direttore della Scuola : Ch.mo Prof. PAOLA ZANOVELLO

Supervisore : Dott. ERICH PIOVAN

Co-Supervisore : Dott. VALERIA TOSELLO

Dottorando : FULVIO BORDIN

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Riassunto

La leucemia linfoblastica acuta a cellule T (LLA-T) è un tumore ematologico derivante dalla trasformazione neoplastica dei progenitori dei linfociti T. Grazie ai numerosi progressi effettuati nelle tecniche di biologia molecolare, numerose alterazioni genetiche sono state identificate nei pazienti affetti da LLA-T, generando nuove opportunità per la messa a punto di una terapia più mirata. Delezioni e mutazioni a carico del gene *WT1* sono state identificate nel 10-12% dei pazienti affetti da LLA-T, ma tuttora gli effetti derivanti da tali alterazioni non sono stati ancora ben chiariti. Il gene *WT1* codifica per un fattore di trascrizione caratterizzato da diverse isoforme derivante da splicing alternativo. Le isoforme sono conservate in tutti i vertebrati ma differiscono tra loro per la presenza o assenza dell'esone 5 e di un tripeptide composto dagli amminoacidi lisina-trenonina-serina (KTS), a cavallo tra il terzo e quarto dominio zinc finger. Le isoforme prive del KTS (KTS–) possiedono una forte affinità nel legare il DNA e quindi sono in grado di regolare la trascrizione dei geni bersaglio. La nostra ipotesi è che *WT1* agisca come oncosopressore in condizioni di aplo-insufficienza nella LLA-T e che la sua assenza comporti un'alterata regolazione del suo assetto trascrizionale. In questo studio sono stati analizzati gli effetti della sovra-espressione di *WT1*, sia delle isoforme normali che di un caratteristico mutante trovato nella LLA-T, sulla sopravvivenza e proliferazione delle cellule leucemiche. Abbiamo osservato che esclusivamente le isoforme (KTS–) erano in grado di influenzare negativamente la crescita delle cellule leucemiche e di diminuire la loro capacità di formare colonie in soft-agar. La sovra-epressione delle isoforme derivate da una caratteristica mutazione frameshift (E384Stop) nell'esone 7 del gene *WT1*, invece, non produceva alcun effetto sulla crescita delle cellule di LLA-T. Per mimare la deplezione di *WT1* abbiamo indotto il silenziamento del gene nella linea cellulare di leucemia MOLT4 e abbiamo valutato l'effetto sulla crescita delle cellule mediante saggio clonogenico. Abbiamo riscontrato che le cellule leucemiche con bassi livelli di espressione di *WT1* presentavano un significativo incremento di colonie rispetto alle cellule di controllo. Questi risultati suggeriscono che le mutazioni di *WT1* possano determinare l'aplo-insufficenza del gene nelle cellule di LLA-T, favorendo la

crescita e lo sviluppo tumorale. Allo scopo di valutare se le mutazioni a carico di *WT1* potessero indurre un'alterazione del suo assetto trascrizionale in cellule di LLA-T, ci siamo focalizzati nell'analisi dei suoi bersagli dopo deplezione di *WT1* in una linea cellulare di LLA-T. Mediante l'analisi di immunoprecipitazione della cromatina su tecnologia microarray (Chromatin Immunoprecipitation on chip; ChIP-chip), effettuata nella linea cellulare MOLT4, abbiamo ottenuto circa 800 geni regolati direttamente da WT1 nella LLA-T. Analisi bioinformatiche hanno dimostrato un arricchimento di questi geni in alcune vie di segnale implicate nella risposta allo stress cellulare: la via di attivazione di p53, le vie coinvolte nella riparazione del DNA dopo danno cellulare e la via di segnalazione della Mitogen-Activated Protein (MAP) chinasi. Integrando l'analisi ChIP-chip con l'analisi del profilo di espressione genica, ottenuta dopo silenziamento di *WT1* nelle cellule MOLT4, abbiamo ulteriormente definito i bersagli diretti di WT1 che risultano anche de-regolati nella LLA-T. Questi risultano arricchiti nella via di segnalazione delle MAP chinasi. Prendendo spunto da questi risultati, abbiamo infine verificato se la ridotta espressione di *WT1* nelle cellule di LLA-T, sia in linee cellulari che in campioni primari derivanti da pazienti affetti da LLA-T, ne favorisca la sopravvivenza in seguito a danno al DNA, per esempio dopo trattamento con radiazioni ionizzanti o farmaci chemioterapici. Le analisi della vitalità cellulare e dell'apoptosi di queste cellule hanno chiaramente mostrato come le alterazioni di *WT1* inducano una maggior resistenza a condizioni di stress, in quanto vanno ad interferire con la trascrizione di importanti geni apoptotici, soprattutto quelli a valle di p53. In particolare l'induzione del gene *BBC3/PUMA*, un fattore chiave nella risposta apoptotica, è significativamente più elevata nelle cellule in cui WT1 e p53 risultano funzionali. In conclusione, analizzando l'effetto della perdita dell'espressione di *WT1* nella LLA-T, abbiamo riscontrato un'alterata regolazione di numerosi geni coinvolti nella patogenesi della LLA-T, in particolare quelli responsabili della risposta cellulare in seguito al danno al DNA, suggerendo ulteriormente un ruolo di *WT1* come gene oncosopressore in questa neoplasia.

Summary

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic tumor, resulting from the transformation of T-cell progenitors. Thanks to advances in molecular techniques, many alterations have been identified in T-ALL cells opening new opportunities for targeted therapy. *WT1* gene deletions and mutations have been reported in 10-12% of T-ALL patients, but the mechanisms downstream of *WT1* alterations in T-ALL have not been elucidated. The *WT1* gene encodes a zinc-finger transcription factor which is characterized by multiple alternative isoforms. The isoforms that lack the three amino acids lysine-threonine-serine (KTS–) between zinc finger 3 and 4, are conserved throughout vertebrate evolution and have high DNA-binding affinity and transcriptional activity. Most of *WT1* mutations found in T-ALL are heterozygous frameshifts in exon 7 predicted to produce a truncated protein which lacks the DNA binding domain. Our main hypothesis is that *WT1* acts as a haplo-insufficient tumor suppressor gene in T-ALL and that *WT1* loss in T-ALL leads to de-regulation of pathway in T-ALL. In this study, we first analyzed the effects of full-length and mutant WT1 isoform over-expression on the survival and proliferation of T-ALL cells. We observed that only the (KTS–) isoforms negatively affected growth of T-ALL and impaired colony formation in soft-agar. Importantly, the truncated WT1 proteins, derived from a characteristic frameshift mutation in exon 7 (E384Stop), had no effects. In parallel, we also analyzed the effects of *WT1* loss in T-ALL cells. We found that *WT1* knockdown in MOLT4 cells significantly increased the number of colonies in clonogenic assays in comparison with control cells. Overall these results indicated that *WT1* most probably works as an haplo-insufficient tumor suppressor gene in T-ALL. In order to evaluate if mutations in *WT1* locus are most likely responsible for an impaired transcriptional program we mainly focused on the analysis of WT1 deregulated targets following *WT1* loss in T-ALL cells. To define the structure of the transcriptional network activated by loss of function of *WT1*, we performed ChIP-chip and gene expression analysis in MOLT4 T-ALL cells. ChIP-chip analysis showed that WT1 direct targets were enriched in pathways responsible for cellular response to stress, such as p53, nucleotide excision repair and Mitogen-Activated Protein Kinases (MAPK)

signalling pathways. Integration of ChIP-chip data with gene expression analysis performed under *WT1* loss of function conditions in MOLT4 cells provided an enrichment in the MAPK pathway. Stemming from these results, we finally evaluated if the loss of *WT1* conferred increased survival after DNA damage, such as ionizing radiation or chemotherapeutic drugs, in MOLT4 T-ALL cells and primary T-ALL xenografts. Analysis of cell viability and apoptosis showed that *WT1* alterations induced increased survival following DNA-damaging conditions, mainly affecting directly the transcription of important mediators of p53 apoptotic response. A master regulator of these effects was *BBC3/PUMA*, whose induction was augmented in the presence of both WT1 and p53 proteins. In conclusion, analyzing *WT1* loss in T-ALL cells we determined a deregulation of several genes involved in the pathogenesis of T-ALL, in particular genes responsible for cellular response to stress, strongly suggesting *WT1* acts as tumor suppressor gene in T-ALL cells.

1. Introduction

1.1 T-cell Acute Lymphoblastic Leukemia

1.1.1 Molecular pathogenesis of T-cell Acute Lymphoblastic Leukemia

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic tumors resulting from the uncontrolled clonal proliferation of an immature lymphoid cell committed to the T-cell lineage. Acquisition by the precursor of a series of genetic abnormalities leads to differentiation arrest and high proliferation of the transformed cell resulting in infiltration of a neoplastic clone in the bone marrow and in the suppression of normal hematopoiesis in various extramedullary sites[1,2]. The malignant transformation is a multistep process that includes several oncogenes and tumor suppressor genes which collaborate to disorganize the normal gene network that controls T-cell development leading to differentiation arrest, uncontrolled cell cycle progression, abnormal cellular metabolism and excessive cell proliferation. The most prominent genetic alteration is the deletion of the *CDKN2A* locus in chromosome band 9p21 affecting the *p16/INK4A* and *p14/ARF* suppressor genes which regulate cell cycle progression and p53 mediated apoptosis, respectively[3]. In addition, activation of NOTCH1 signaling is the most prominent oncogenic pathway in T-cell transformation and is aberrantly activated in over 60% of cases[4]. The members of NOTCH protein family are essential regulators of the commitment of haematopoietic progenitors to the T-cell lineage. NOTCH1 activating mutations result in a ligand-independent release of the intracellular domain of NOTCH1 (ICN), which subsequently translocates to the nucleus where it acts as a transcription factor. Alternatively, NOTCH1 mutations in the proline, glutamic acid, serine, threonine-rich (PEST) domain or inactivating mutations in the E3-ubiquitin ligase gene *FBXW7* mutations preserve ICN from ubiquitin-mediated degradation by the proteasome[5]. The activation of many other oncogenes and oncogenic fusions also occur at defined stages in T-cell development, again providing a close link between T-cell ontogeny and leukemogenesis. In fact, approximately 50% of T-ALL cases are characterized by chromosomal translocations which frequently involve the juxtaposition of

promoter and enhancer elements from T-cell receptor (TCR) genes with several transcription factor genes[1]. Hence, translocations between *TCR* and *HOX* genes (*TLX1/HOX11*, *TLX3/HOX11L2*, *NKX2.1*, *NKX2.2*, *NKX2.5*, and *HOXA*)[6-13], basic helix-loop-helix (bHLH) family members *TAL1*, *TAL2*, *LYL1*[14-19], *BHLHB1*[20], LIM-only domain (LMO) genes (*LMO1*, *LMO2*)[21-23], *MYC*[24-28], *MYB*[29] or *TAN1*[30] are recurrently found in T-ALL. In some cases, these factors can also be activated in the context of other non-TCR-associated chromosomal abnormalities. This is the case for small deletions activating *TAL1*[31] and *LMO2*[32]; duplications of the *MYB* oncogene[33,34] and the *t(5;14)(q32; q11)* translocation which activates the *TLX3* oncogene in chromosome 5 by relocating it to the vicinity of the BCL11B locus in chromosome 14[35]. Additional molecular alterations present in T-ALL include transcription factor fusion oncogenes such as *PICALM/MLLT10/CALM-AF10*[36-38], *MLL-MLLT1/MLL-ENL*[39,40], *SET/NUP214*[41], *NUP98-RAP1GDS1*[42,43]; activation of signaling factors driving proliferation such as *LCK*[44], *CCND2*[45,46]; *JAK1*[47], *NUP214-ABL1*[48], *EML1-ABL1*[49] and *NRAS*[50]. Another important step in T-cell transformation is the loss of tumor suppressor genes in some signaling pathways, such as the RAS[51] and PI3K signaling pathways[52]: in particular, cryptic deletions and/or mutations are present in the neurofibromatosis type 1 (*NF1*) gene, which encodes a negative regulator of the RAS pathway, occur in 3% of T-ALL[53], and *PTEN* loss, a critical negative regulator of the PI3K-AKT signaling pathway, through nonsense, missense mutations or deletions occurs in 10-20% of human T-ALL cases[54,55]. Finally loss-of-function mutations have also been identified in other tumor suppressor genes in T-ALL. Deletions and mutations in the *WT1* gene are present in about 10% of T-ALLs and are frequently associated with oncogenic expression of the *TLX1*, *TLX3*, or *HOXA* oncogenes[55,56]. Monoallelic or biallelic deletions involving the *LEF1* locus and mutations in the *LEF1* gene are present in about 15% of T-ALL cases[57]. *ETV6* mutations, a transcriptional repressor strictly required for the development of hematopoietic stem cells, produce truncated proteins with dominant-negative activity[58,59]. Loss-of-function mutations and heterozygous deletions of the BCL11B are recurrently found in T-ALL, suggesting that *BCL11B* haplo-insufficiency may be an important pathogenetic event in T cell leukemogenesis[60-62]. Moreover, loss-of-function mutations in

RUNX1 can be found in immature T-ALL samples, suggesting a tumor suppressor role for *RUNX1* in T-cell transformation[62,63]. Recurrent somatic *GATA3* missense mutations, an important regulator of T cell differentiation, cluster in the zinc finger DNA-binding protein domain, and may be responsible for the early block in T-cell development of leukemia[64].

Classification of other recurrent genetic alterations in T-ALL

Category	Gene target	Genetic rearrangement	Outcome (ref.)	Frequency (ref.)
NOTCH1 pathway	*NOTCH1*	t(7;9)(q34;p13)	NA	<1% (27)
		Activating mutation	Good (51, 129)	>60% (6)
			GPR (45, 49, 50)	
			No impact (48)	
	FBXW7	Inactivating mutation	NA	8%–30% (37, 38)
Cell cycle defects	*CDKN2A/2B*	9p21 deletion methylation	Good (60)	70% (7)
	CCND2	t(7;12)(q34;p13)	NA	1% (57)
		t(12;14)(p13;q11)		
	RB1	13q14 deletion	No impact (33)	4% (58)
	CDKN1B	12p13 deletion	NA	2% (59)
Cell growth transcription factor	*MYC*	t(8;14)(q24;q11)	NA	1% (25)
tumor suppressors	*WT1*	Inactivating mutation/deletion	No impact (80)	10% (80)
	LEF1	Inactivating mutation/deletion	NA	10%–15% (93)
	ETV6	Inactivating mutation/deletion	No impact (33)	13% (32, 33)
	BCL11B	Inactivating mutation/deletion	No impact (33)	10% (98)
	RUNX1	Inactivating mutation/deletion	No impact (33)	10%–20% (100, 101)
			Poor (101)	
	GATA3	Inactivating mutation/deletion	Poor (33)	5% (33)
Signal transduction	PTEN	Inactivating mutation	No impact (106, 130)	10% (33, 106, 130)
		10q23 deletion	Poor (33)	10% (33, 106, 130)
	NUP214-ABL1	Episomal 9q34 amplification	Poor (108)	4% (108)
			No impact (131)	
	EML1-ABL1	t(9;14)(q34;q32)	NA	<1% (109)
	ETV6-ABL1	t(9;12)(q34;p13)	NA	<1% (110)
	BCR-ABL1	t(9;22)(q34;q11)	Poor (132)	<1% (132)
	NRAS	Activating mutation	No impact (33)	5%–10% (32, 113)
	NF1	Inactivating mutation/deletion	No impact (33)	3% (114)
	JAK1	Activating mutation	No impact (33)	4%–18% (118, 119)
	ETV6-JAK2	t(9;12)(p24;p13)	NA	<1% (117)
	JAK3	activating mutation	No impact (33)	5% (33)
	FLT3	activating mutation	No impact (33)	2%–4% (133, 134)
	IL7R	activating mutation	No impact (33)	10% (120, 121)
Chromatin remodeling	*EZH2*	Inactivating mutation/deletion	Poor (33)	10%–15% (33, 135)
	SUZ12	Inactivating mutation/deletion	No impact (33)	10% (33)
	EED	Inactivating mutation/deletion	No impact (33)	10% (33)
	PHF6	Inactivating mutation/deletion	No impact (81)	20%–40% (81)

GPR, good prednisone response.

Table 1. Classification of recurrent genetic alterations in T-ALL [65].

More recently, alterations in some epigenetic modifiers, such as the polycomb repressive complex 2 (*PRC2*) have been found. Loss-of-function mutations and deletions have been reported up to 25% of T-ALLs in two critical components of the PRC2 complex, *EZH2* and *SUZ12* genes[66]. In addition, NOTCH1 activation was shown to specifically induce loss of the repressive *H3K27me3* mark by antagonizing PRC2 complex activity during T cell transformation, suggesting a

dynamic interplay between oncogenic NOTCH1 activation and loss of PRC2 function in the pathogensis of T-ALL[66]. In addition, mutations and deletions in *PHF6* gene, a factor with a proposed role in epigenetic regulation, are present in about 16% of pediatric and 38% of adult T-ALL cases[67].

Currently, much emphasis has been put on the role of non-coding RNAs, such as miRNAs, lncRNAs, and circular RNAs, in normal development and disease, including cancer. With respect to T-ALL, Mavrakis and colleagues[68] identified a set of five microRNAs (*miR-19b, miR-20a, miR-26a, miR-92, and miR-223*), small noncoding RNAs with regulatory functions, that cooperatively suppress a network of tumor suppressor genes, including *PHF6*, *PTEN*, *BIM*, and *FBXW7* in a NOTCH1-induced murine bone marrow transplant model of T-ALL. Another work identified the *miR-17-92* cluster as one of the most prominent oncogenic miRNA clusters able to induce T-cell leukemia in concert with activated NOTCH1[69,70]. Finally, other studies identified the roles of *miR-223*[71] and *miR-128-3p*[72] as novel oncogenic miRNAs in T-ALL that cooperate with activated NOTCH1 signaling to accelerate T-ALL formation *in vivo*.

Besides the emerging importance of microRNAs in many malignant diseases, long non-coding RNA (lncRNA) have been reported to play important roles in the pathogenesis of some tumors, although their role in the pathogenesis of ALL or other hematological system cancers remains poorly characterized[73]. Long noncoding RNAs are transcripts with a length of at least 200 nucleotides that lack protein-coding potential [74]. They act mainly in concert with chromatin modifier enzymes and serve as scaffolds bridging between multiple proteins, guides to target chromatin remodelers to their target sites, or control devices that can induce protein conformational changes and thereby activate/inactivate the interacting protein complex[75]. In the context of T-ALL, Trimarchi and colleagues published the first landmark study on the identification of a set of lncRNAs under control of aberrant NOTCH1 signaling in T-ALL[76]. They identified *LUNAR1* as an oncogenic lncRNA, localized in the nucleus, that is over-expressed in primary T-ALLs, with higher expression in T-ALL cases that harbor activating *NOTCH1* mutations. *LUNAR1* is located in cis to the insulin-like growth factor 1 receptor (*IGF1R*) locus and promotes its expression through a direct interaction between

an intronic *IGF1R* enhancer. Moreover, in vitro knockdown of *LUNAR1* significantly affected leukemic cell growth owing to decreased IGF1R signaling. These studies collectively show that lncRNAs act as an additional layer of complexity in T-ALL disease biology, suggesting that NOTCH signaling is able to shape also the lncRNA landscape in this disease[77]. These findings open the possibility that such previously uncharacterized transcripts are key modulators of cellular transformation, through their interaction with oncogenic and tumor suppressor programs in leukemia.

1.1.2 Genetic subgroups in T-ALL: molecular characteristics and prognostic relevance

In the last years gene expression profiling studies identified molecular sub-types of T-ALL, which share unique immuno-phenotypic markers and gene expression signatures reflecting distinct stages of arrest during T cell development caused by deregulation in specific cellular processes, including cell cycle signaling, cell growth and proliferation, chromatin remodeling, and self-renewal[78]. There are at least four distinct genetic T-ALL subgroups: the TAL/LMO, the TLX3/HOX11L2, the TLX1/HOX11 and the HOXA subgroups. The TLX3 and TLX1 subgroups are exclusively characterized by rearrangements of the *TLX3* and *TLX1* oncogenes, respectively[10,79-83]. In contrast, the TAL/LMO and HOXA subgroups seem to be characterized by the presence of various rearrangements affecting several, but functionally equivalent oncogenes. For instance, the TAL/LMO subgroup is predominantly characterized by ectopic expression of TAL1 or LMO2 as a consequence of chromosomal rearrangements driven by T-cell receptor (TCR) recombinatory events[14,21,22,32,84-89]. TAL1 and LMO2 normally participate in the same transcriptional complex that regulates the activity of the important E2A/HEB transcription factors[90-92]. This may explain why *TAL1*- or *LMO2*-rearranged T-ALL cases have highly similar if not identical gene expression profiles[41]. The HOXA T-ALL subgroup is characterized by various chromosomal aberrations that all drive ectopic HOXA expression[41,93-95]. These include *CALM-AF10* translocations, *MLL*-rearrangements or an inversion on chromosome 7 between the *HOXA* gene cluster and the *TCRβ* locus, and the *SET-NUP214* gene fusion due to the *del(9) (q34.11q34.13)* [41]. SET-NUP214 recruits histone modifying enzymes boosting the activation of the entire *HOXA* locus.

In parallel to the genetic classification, T-ALL cases can be further characterized on the basis of immuno-phenotypic features. These immuno-phenotypic categories include two classification systems: the European Group for the Immunological Characterization of Leukemias (EGIL) classification system and the more recent T-cell receptor (TCR) based classification system. The EGIL classification system distinguishes pro-/pre-T-cell subgroup ($CD7^+$, $CD2^+$ and/or $CD5^+$ and/or $CD8^+$, but $CD1^-$ and $sCD3^-$), the cortical T ($CD1^+$) or the mature T

(sCD3$^+$/CD1$^-$) subgroup based on the cluster of differentiation (CD) markers indicated[96]. The TCR system distinguishes the immature (IM) stage (Cytoplasmic-beta (Cytß)$^-$, sCD3$^-$, TCRαß$^-$ or TCRγδ$^-$), the pre-aß stage (Cytß$^+$, sCD3$^-$, TCRαß$^-$ or TCRγδ$^-$) and the TCRαß (sCD3$^+$, TCRαß$^+$) or TCRγδ (sCD3$^+$, TCRαß$^+$) stages[79].

Recognition of specific immuno-phenotypic and genetic characteristics may have prognostic relevance in T-ALL. The *TAL*- or *LMO2*-rearranged cases are exclusively arrested at the αß-lineage and share a similar gene expression profile; the *TAL*-rearranged cases are predominantly associated with mature TCRαß expressing blasts, while the *LMO2*-rearranged cases seemed to have a less mature phenotype[89]. Importantly, *TAL1*-rearranged T-ALLs demonstrate a trend to better outcome in various studies compared to the *LMO2*-rearranged leukamias, even if these findings need to be confirmed in larger T-ALL cohorts [82,84,97].

TLX1 and *TLX3* are related homeobox genes but they constitute different genetic subgroups. Notably, *TLX1*- and *TLX3*-rearranged cases are arrested at different developmental stages: about 40% of cases have an immature immuno-phenotype, 40% of cases are committed to the αß-lineage[89] and finally, 20% of cases have a mature immuno-phenotype committed to the TCRγδ-lineage. In general, the *TLX3*-rearranged cases are arrested at early stages of differentiation and have an adverse outcome respect to the *TLX1*-rearranged cases at least in some studies [98,99]. The HOXA subgroup consists of T-ALL cases that have different types of rearrangements resulting in *CALM-AF10*, *SET-NUP214* or *MLL*-fusion products or rearrangements that directly activate *HOXA* genes[100,101]. However, the number of T-ALL subtypes defined by gene expression profiling data is quite limited, and the possible explanation is that various molecular aberrations can lead to over-expression of the same oncogenes. Recently a novel immature subtype of pediatric T-ALL has been identified. This subtype is named early T-cell precursor (ETP-ALL) and it is characterized by unique immuno-phenotypic properties, including lack of CD1a and CD8 expression, weak CD5 positivity, and expression of one of the stem-cell/myeloid markers CD13, CD117, CD33[102]. The ETP-ALL cells arise from early T-cell progenitors arrested at the double-negative stage of thymocyte development, show higher levels of *LYL1* and *LMO2* expression and

mutations targeting genes implicated in epigenetic regulation and cell signaling such as *FLT3*, *NRAS*, *DNMT3A*, *IDH1* and *IDH2*. This subtype of T-ALL typically presents a myeloid expression pattern together with inactivation of important transcription factors such as RUNX1, GATA3, and ETV6[103]. The initial study that defined ETP- ALL reported an extremely poor clinical course for these patients, with 10-year overall survival rates of 19% for ETP-ALL patients, as compared with 84% for other T-ALL subtypes[104].

Genetic lesions that define molecular-genetic subtypes in T-ALL

Gene category	Gene target	Genetic rearrangement	Outcome (ref.)	Frequency[A] (ref.)
bHLH family members	*TAL1*	t(1;14)(p32;q11)	Good (66–68)	3% (28)
		t(1;7)(p32;q34)	Good (66–68)	3% (28)
		1p32 deletion	Good (66–68)	16%–30% (61)
	TAL2	t(7;9)(q34;q32)	NA	1% (11, 30)
	LYL1	t(7;19)(q34;p13)	NA	1% (12)
	BHLHB1	t(14;21)(q11.2;q22)	NA	1% (13)
LMO family members	*LMO1*	t(11;14)(p15;q11)	NA	1% (15, 30)
		t(7;11)(q34;p15)	NA	1% (15, 30)
	LMO2	t(11;14)(p13;q11)	NA	6% (126)
		t(7;11)(q34;p13)	NA	6% (126)
		11p13 deletion	NA	3% (29)
	LMO3	t(7;12)(q34;p12)	NA	<1% (21)
Homeobox family members	*TLX1*	t(11;14)(p15;q11)	Good (76)	5%–10% (126)
				30% (76, 88)
	TLX3	t(11;14)(p15;q11)	Poor (83, 88, 126)	20% (20)
			No impact (67)	5% (84)
			Good (127)	5% (84)
	HOXA	Inv(7)(p15q34)	NA	3% (23, 128)
		t(7;7)(p15;q34)	NA	3% (23, 128)
	HOXA (*CALM-AF10*)	t(10;11)(p13;q14)	Poor (87, 126)	5%–10% (87)
	HOXA (*MLL-ENL*)	t(11;19)(q23;p13)	NA	1% (88)
	HOXA (*SET-NUP214*)	9q34 deletion	No impact (33)	3% (30)
		inv(14)(q11.2q13)	NA	
	NKX2.1	inv(14)(q13q32.33)	NA	5% (21)
		t(7;14)(q34;q13)	NA	
	NKX2.2	t(14;20)(q11;p11)	NA	1% (21)
Proto-oncogene	*c-MYB*	t(6;7)(q23;q34)	NA	3% (26)

[A]Frequency refers to the frequency in which each mutation occurs in the patient population.

Table 2. Genetic lesions that define molecular-genetic subtypes in T-ALL [65].

1.1.3 **Opportunities for targeted therapy in T-ALL**

High dose multi-agent chemotherapy is the current standard therapy for T-ALL and is highly effective in the majority of childhood leukemias, reaching almost 85% of survival rates at ten years. Unfortunately, the aggressive regiments are very often associated with acute toxicities and long-term side-effects. Moreover, at least 40% of adult patients still fail the current induction therapy.

In the last couple of years numerous studies have elucidated numerous genetic defects that drive T-ALL, opening numerous opportunities for multiple target therapies. The identification of activating NOTCH1 mutations in a large percentage of T-ALL patients created enormous interest in developing new therapies for T-ALL and prompted the initiation of clinical trials to test the effectiveness of agents blocking NOTCH1 signaling. The combination of γ-secretase inhibitors (GSIs) and glucocorticoids was shown in preclinical models to increase efficacy and decrease toxicity in the treatment of T-ALL[105]. Alternative strategies to target the NOTCH1 pathway are still being developed and include specific NOTCH1- inhibitory antibodies, stapled peptides that target the NOTCH1 transcriptional complex[106,107] or pharmacologic inhibition or genetic ablation of IGF1R, a direct NOTCH1 target gene, inhibiting growth and viability of T-ALL cells[108]. Finally, Schnell and colleagues[109] confirmed the critical role of Hes family BHLH transcription factor 1 (HES1) as downstream component of NOTCH1 signaling in T-ALL and revealed that perhexiline could evoke a strong *in vitro* and *in vivo* antileukemic response by reverting the *HES1*-driven gene expression signature, providing a new lead for targeted T-ALL treatment linked to hyperactive NOTCH1. Another possibility for a target therapy relies on the *BCR-ABL* translocations that characterizes approximately 6% of adults and children with T-ALL[48]. BCR-ABL fusion exhibits aberrant sub-cellular localization and constitutive kinase activity. Therapy with tyrosine kinase inhibitors designed to target BCR-ABL, including imatinib, dasatinib and nilotinib, inhibit proliferation and induce apoptosis in T-ALL cell lines[110] leading to a new therapeutic option in a subset of cases of T-ALL. Moreover, 10% of all T-ALL show gain-of- function mutations in IL7R that causes cytokine-independent receptor activation[111], while JAK1 or JAK3 gain-of-function mutations induce a constitutive activation of

JAK/STAT signaling[47]. A number of inhibitors of JAK/STAT signaling are currently under clinical development, and the determination of their clinical activity against JAK1 mutated T-ALL will be of great interest. Pharmacologic inhibition of BCL-2 has been suggested as a promising new therapeutic strategy in immature subtypes of human T-ALL, in particular in ETP-ALL[112]: recent studies[113,114] showed an increased sensitivity toward the highly specific BCL-2 inhibitor ABT-199, and synergistic effects were reported between ABT-199 and conventional chemotherapeutics that are currently used in T-ALL. Finally, constitutive activation of the PI3K/AKT/ mTOR signal transduction pathway is achieved by deletions or mutations targeting PTEN (15% of T-ALL cases)[115,116] or PTEN post-transcriptional inactivation[117]. Given the aberrant activation of the PI3K/AKT/mTOR pathway, this signaling cascade has been evaluated as a novel therapeutic target in T-ALL. The mTOR inhibitor rapamycin showed promising results in preclinical models[118] and might modulate glucocorticoid resistance in T-ALL[119]. However, inhibition of mTOR can hyperactivate AKT by a feedback loop between mTOR, PI3K, and AKT[120]. Therefore, dual PI3K/mTOR small-molecule inhibitors have been evaluated and demonstrated to have cytotoxic activity against T-ALL cell lines and lymphoblasts obtained from primary human leukemia patients[121]. In addition, direct AKT inhibition leads to rapid cell death in some T-ALL cell lines and primary patient samples[122].

In conclusion, the identification and molecular characterization of new oncogenes and tumor suppressors has uncovered much of the mechanisms involved in the pathogenesis of T-ALL. The development of representative and well-characterized xenografts and genetic animal models of T-ALL for preclinical testing, the identification of solid biomarkers of treatment response to standard therapies, and the development of a dynamic framework of clinical trials that facilitates testing of new and emerging drugs and drug combinations in the clinic have been essential in ensuring an effective translation of molecular to the clinic for the treatment of T-ALL.

1.1.4 **Mechanisms of resistance in T-ALL**

T-ALL is an aggressive hematologic cancer for which limited therapeutic options are available for patients with primary resistant or relapsed disease. Unfortunately, the specific mechanisms mediating escape from therapy, disease progression and leukemia relapse are still largely unknown.

Glucocorticoids play a fundamental role in the treatment of all lymphoid tumors because of their capacity to induce apoptosis in lymphoid progenitor cells. Glucocortcoids are included in the 4-6 weeks of remission-induction therapy that is crucial to eradicate the initial leukemic cell burden and restores normal hematopoiesis. Unfortunately, primary glucocorticoid resistance is particularly frequent in T-ALL and is significantly associated with a poor clinical outcome [123, 124]. Recent studies explored the possibility that the activation of one or more oncogenic signaling pathways implicated in T cell transformation could be driving primary glucocorticoid resistance in T-ALL directly by interfering with glucocorticoid receptor function or indirectly via inhibition of glucocorticoid-induced apoptosis. In this context, AKT1 emerged as a plausible candidate as PI3K-AKT activation plays a major role in the pathogenesis of T-ALL, particularly in leukemias harboring mutations and deletions in the PTEN tumor suppressor gene[115]. In fact, AKT1 can induce glucocorticoid resistance by phosphorylation of the glucocorticoid receptor impairing its nuclear relocalization and blocks transcriptional regulation of glucocorticoid target genes. In addition to direct inactivation of the glucocorticoid receptor, AKT1 favors resistance to glucocorticoid therapy by promoting cell growth, metabolism, and survival in T-ALL: mTOR phosphorylation by AKT impairs glucocorticoid-induced apoptosis by increasing the expression of MCL1; AKT-mediated phosphorylation of XIAP prevents degradation of this anti-apoptotic factor, and increased metabolism induced by AKT activation can antagonize metabolic inhibition induced by glucocorticoids. The convergent effects of direct and indirect mechanisms downstream of AKT1 antagonizing the anti-leukemic effects of glucocorticoids further support the role of the PI3K-AKT pathway as therapeutic target for the reversal of primary glucocorticoid resistance in T-ALL[125].

Mutations of the tumor suppressor gene *p53* have been associated with resistance to treatment and poor prognosis of patients in several tumor entities. p53 is

considered the "*guardian of the genome*": it regulates a range of physiologic functions including ageing, development, cell metabolism, differentiation, and tissue homeostasis. Depending on the cellular context, p53 activation and stabilization leads to the transcription of numerous genes controlling cell cycle arrest or apoptotic cell death[126]. In the hematopoietic system, p53 is primarily expressed in hematopoietic stem cells (HSCs) and regulates their quiescence and self-renewal. Genetically engineered mice that lack *p53* have a 2- to 3-fold increase in their HSCs pool. This is probably the result of the higher rate of cellular proliferation of *p53*-deficient HSCs. Un-regulated proliferation of HSCs in the absence of p53 can make them prone to accumulate mutations leading to leukemogenesis[127]. Given its pleiotropic activity, it comes as no surprise that disruption of the p53 pathway is a common denominator in many malignancies. More than 50% of solid tumors have loss of wild type *p53* expression due to deletions or point mutations[128,129]. Surprisingly, in contrast to solid tumors, hematologic malignancies present a rather low incidence of genetic alterations in *p53* (10%-20%)[130,131]. *p53* mutations/deletions have been reported in chronic lymphocytic leukemia (CLL), marginal zone lymphoma, follicular lymphoma, and diffuse large B-cell lymphoma[132]. Nonetheless, aberrations in *p53* correlate with an inferior clinical outcome in hematologic cancers in particular in patients with CLL and AML[133-136]. In contrast, in ALL patients, mutations or deletions of the *p53* gene are rare at presentation of the disease (2% to 3%) and are restricted to a small subset of cases[137]. Nevertheless, presence of *p53* mutation correlated with poor clinical outcome[138,139] and an increased incidence of *p53* mutations occurs more often in relapsed childhood ALL cases (20-30%) and are strongly predictive for non-response to treatment and poor outcome, suggesting the importance of this alteration in progressive disease[51,140]. Moreover, mutations of the *p53* gene may induce drug resistance by interfering with normal apoptotic pathway in leukemic cells[141]. Finally, although the *p53* tumor suppressor gene can be inactivated by *p53* gene mutations, also defects in pathways that regulate p53 levels or inhibit p53 function can induce cell transformation. Thus, at least in a subset of T-cell lymphomas, increased levels of MDM2, the physiological p53 regulator, can inhibit p53 transcriptional activity and targets p53 for degradation

through the ubiquitin–proteasome system, resulting in cell cycle deregulation[142]. More recently, using whole-exome sequencing Tzoneva and colleagues identified mutations in the 5'-nucleotidase cytosolic II gene (*NT5C2*) which encodes for a 5'-nucleotidase enzyme that is responsible for the inactivation of nucleoside-analog chemotherapy drugs in about 19% of relapsed T-ALLs[143]. NT5C2 mutant proteins showed increased nucleotidase activity *in vitro* and conferred resistance to chemotherapy with 6-mercaptopurine and 6-thioguanine, two nucleoside analogs commonly used to maintain durable remissions in the treatment of ALL. This data is an example of a relapse-associated gain-of-function mutation that plays an important role in the progression and chemoresistance in T-ALL.

1.2 The Wilms' Tumor suppressor (*WT1*) gene

1.2.1 *WT1* gene structure

The *WT1* gene is located on the short arm of chromosome 11 (position 11p13) and spans about 50kb of genomic DNA. It consists of 10 exons encoding an mRNA of approximately 3.2kb. The first six exons encode for a proline-glutamine(Pro-Gln)-rich region, activation and repression domains, nuclear localization signals, and at least two self-association domains, while exons seven to ten code for four Cys_2-His_2 zinc finger domains at the C-terminus. These motifs are employed by several transcription factors that play an important role in cellular signal transduction and in particular, they have a high degree of structural homology to the early growth response (EGR) transcription factors family, where the (Pro-Gln)-rich region form the transactivation domain and zinc fingers bind to DNA in a sequence-specific manner[144,145].

WT1 is expressed as a 52 kDa protein in a multitude of isoforms that are produced by a combination of alternative splicing, RNA editing and alternative translation start sites, leading to at least 24 different variants[146]. Of particular interest are two alternative splice sites in *WT1* gene:

- exon 5, which is a cassette exon mammal specific that encodes 17 amino acids that are included or omitted in the middle of the Pro/Glu-rich domain. These residues are required for the interaction of WT1 with Par-4 (Prostate apoptosis response factor 4), a coactivator of transcription. The degree of exon 5 inclusion was shown to be altered in Wilms' tumours and acute myeloid leukemia. However, mouse knock-out of exon 5 does not appear to affect development or fertility[147].
- exon 9, conserved from zebrafish to man, presents an alternative splice donor site that results in omission or inclusion of a lysine-threonine-serine tripeptide (KTS) in the canonical TGEKP linker sequence between zinc fingers 3 and 4[145]. The presence of these three amino acids alter the conformation of the zinc finger domain, changing the DNA-binding affinity of the isoforms[148].

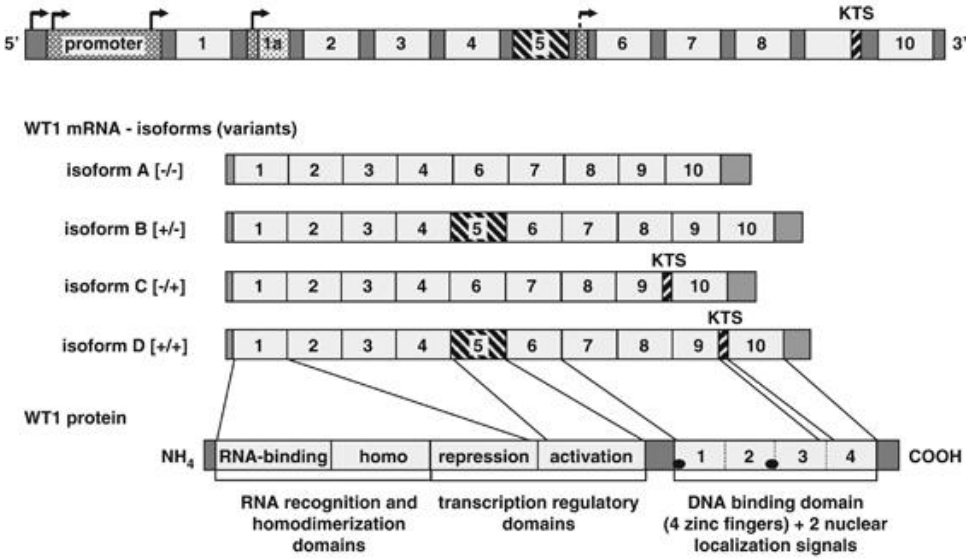


Figure 1. Scheme of *WT1* gene and its protein products.[149]

A) *WT1* gene structure: alternative splice sites are crosshatched, balck arrows indicate alternative transcription initiation sites. B) The four major WT1 isoforms are generated by alternative splicing and vary in the presence or absence of 17 aminoacids encoded by exon 5 (Ex5) and KTS insert: WT1 variants A[Ex5−/KTS−], B [Ex5+/KTS−], C [Ex5−/KTS+] and D [Ex5+/KTS+].

1.2.2 *WT1* gene expression and function

The *WT1* gene encodes a zinc finger tissue specific transcription factor that was originally identified through its involvement in the development of Wilms' tumor, a pediatric kidney tumor. The ratio of exon 5 splice variants differs between cell types, species and developmental stage whereas the ratio between (KTS+) and (KTS–) proteins is 2:1 and is nearly constant in all cell types[147]. The balance between (KTS+/−) isoforms is regulated by an unknown mechanism. Disruption of this mechanism results in severe urogenital abnormalities characteristic of Frasier Syndrome, as was confirmed in transgenic mice that carry mutations that affect KTS splicing[150]: in fact, mice that express either only the (KTS+) or only the (KTS–) isoforms were found to die soon after birth because of kidney defects[146].

The WT1 proteins are found only in the nucleus in all of the cell types which express the gene. However, the sub-nuclear localization is different for the four alternatively spliced isoforms: the (KTS–) isoforms, which have high DNA

affinity, co-localize preferentially with ubiquitous trascription factors whereas the majority of the (KTS+) isoforms are found in a speckled pattern and co-localize with nucleoriboproteins in splicing complexes, suggesting a role in RNA processing[144,151]. The biology of WT1 is complex, and, in addition to its function as a tumor suppressor, this gene has multiple roles during development and tissue homeostasis. WT1 is expressed in a tissue-specific pattern and also depends on the growth stage of the organism: during mammalian embryonic development, WT1 is needed in the urogenital system to induce the mesenchymal-epithelial transition during nephon formation[152,153]; in the developing heart it is required for proliferation of vascular progenitors, mantaining then in an undifferentiated state[146]; it was also found expressed in spleen, certain areas of the brain, spinal cord, mesothelial organs, diaphragm, limb, and other developing tissues and organs[154-156].

Mice deleted for *WT1* lack kidneys, gonads and spleen and die *in utero* at mid-gestation mainly due to defective coronary vasculature [157]. Due to the premature death of *WT1*-deleted embryos, this model is not informative regarding the role of WT1 in hematopoiesis. The role of WT1 in hematopoiesis has been investigated by Hosen N. and colleagues. They generated a knock-in green fluorescent protein (GFP)-reporter mouse (WT1GFP/+), in which GFP was expressed under the endogenous transcriptional regulatory elements of the *WT1* gene. Using these WT1GFP/+ mice, they examined *WT1* expression in normal hematopoietic precursors and leukemic cells from mouse leukemia models. In normal hematopoietic cells, *WT1* was expressed in none of the long-term (LT) hematopoietic stem cells (HSC) and very few (<1%) of the multipotent progenitor cells. In contrast, in murine leukemias induced by acute myeloid leukemia 1 (*AML1)/ETO+TEL/PDGFβR* or *BCR/ABL*, *WT1* was expressed in c-kit^{+}lin^{-}Sca-1^{+} (KLS) cells, which contained a subset, but not all, of transplantable leukemic stem cells (LSCs). More recently, using a tamoxifen-inducible *WT1* knockout mouse model Chou Y.(2011) and colleagues deleted *WT1* gene in young and adult mice. Within 7-9 days they observed rapid kidney failure, spleen and pancreas atrophy, loss of bone and body fat and importantly dramatic reduction of red blood cells due to lack of megakaryocyte and megakaryocyte-eritrocyte progenitors. Other hematopoietic compartments were not affected indicating either that WT1 is not

important for hematopoietic compartments other that the eritrocytes or that the observation window of this mouse model was not sufficient to detect other defects.

1.2.3 WT1 as a transcription factor: its role in regulating gene expression

WT1 has been shown to act as a transcriptional repressor or activator of several target genes depending on cellular and promoter context. Among the targets are genes important for cellular growth and metabolism, including extracellular matrix component, growth factors and other transcription factors[158]: for example the PDGF-A, CSF-1, PAX-3, IGF-IR and WT1 itself[159]. By its zinc finger domains, (KTS–) isoforms were shown to bind the consensus sequence, identical for EGR-1, GCGG-GGGCG and several in vitro experiments using target promoters linked to a reporter gene have been used to identify putative WT1 targets, many of them involved in cell growth (Wnt signaling pathway[160]or c-myc[161]), cell cycle control and apoptosis (CDKN1A/p21[162], GADD45A[163], Bcl-2[164] and some components of the MAPK pathway[165]).

WT1 transcriptional activity is also influenced by several interacting protein partner, such as Par-4, Hsp70 and p53[151]. The functional importance of these interaction is in many cases unclear, but it is interesting that WT1 lacking the entire DNA-binding domain at the C-terminus still retains some cellular effects, indicating functions mediated through the N-teminal domain[166]. Particular intriguing is the interaction between WT1 and p53. *p53* is a tumor suppressor gene expressed ubiquitously and is considered the guardian of genomic integrity. Ionizing irradiation and other insults causing genetic damage determine an increase of p53 levels, which induce cell cycle arrest or apoptosis after transcription of its target genes. The physical interaction between WT1 and p53 proteins in rat kidney cells and primary Wilms' tumor induces p53 stabilization after association with the first-second zinc finger domains of WT1[167,168]. On the other hand, p53 can also influence WT1's ability to regulate IGF1R gene expression: (KTS–) isoforms significantly repressed IGF1R transcription only in presence of a wild type p53[169]. However, a functional interaction between WT1

and p53 was observed only with the wild-type proteins. In some experiments, co-transfection of p53 with wild type WT1 enhanced p53 target induction but this mechanism was abolished in the presence of mutant-WT1. These experiments suggest that WT1 mutants, with a disrupted DNA-binding domain cannot mediate their effect because of a non-productive interaction with p53. On the other hand, WT1 repressor activity on EGR1 promoter is 10-fold higher in cells with a functional p53 compare to cells with mutated p53. In conclusion, WT1 exerts a cooperative effect on p53, enhancing the ability to transactivate their respective targets.

1.2.4 **Molecular genetics of *WT1***

WT1 was initially discovered mutated in pediatric patients with Wilms' tumor. Mutations of *WT1* associated with Wilms' tumor are found in a subset of patients, estimated at 10–15%. Mutations include nonsense as well as missense changes, and they are distributed throughout the coding region, without particular hotspots. Another 10% of sporadic Wilms' tumors express elevated levels of an in-frame deletion of exon2, that may alter the transactivational function of the encoded protein. In the remaining cases, *WT1* appears to be wild-type and is expressed at high levels. Presumably, these tumors have alterations in downstream targets of WT1 or result from genetic alterations in other cellular pathways[158,170]. In contrast, other Wilms' tumor congenital syndromes, the WAGR (Wilms' tumor, aniridia, genitourinary abnormalities, mental retardation), the Denys-Drash syndrome (DDS), and Frasier Syndrome (reviewed by [171]) present an increased risk of Wilms' tumors caused by inherited germline deletions in the *WT1* locus. In these cases *WT1* mutations are point mutations in the zinc finger domains, which results in inactive or truncated protein, or at the second splice donor site in intron 9, resulting in the loss of the (KTS–) isoforms in the mutated allele and offsetting of the isoform ratio[172].

1.2.5 **WT1 in leukemia**

In recent years, the study of WT1's role in malignancy evidenced a biological and clinical importance of WT1 in cell survival, differentiation and proliferation and opened a discussion on its behavior as tumor suppressor or oncogene.

In addition to its prominent role as tumor suppressor in the pathogenesis of Wilms' tumors, aberrantly high expression of *WT1* is detected in several forms of cancer, including breast cancer and lymphoid and myeloid leukemias, paradoxically suggesting an oncogenic role for WT1, although the mechanism underlying this effect remains unexplained[173]. In particular it was showed that 73-93% of AML patients had elevated *WT1* expression at diagnosis[174], and *WT1* over-expression was used as a marker of prognosis or minimal residual disease to predict relapse. Despite these reports, controversy still exists with other ones showing no predictive value[158,174,175]. Importantly, also mutations in the *WT1* gene are found in leukemias. The first report of *WT1* mutations associated with development of AML was published in 1994 by King-Underwood and colleagues[176] , who identified *WT1* mutations in 15% of AML patients and 1 case of bi-phenotypic leukemia, noting that the patients were refractory to chemotherapy and showed worse overall survival rate. Since then, *WT1* alterations have been reported in 10% of cases of AML and ALL and 20% of bi-phenotypic leukemias. Notably, previous reports have shown the absence of *WT1* mutations in pre-B-ALL, suggesting that mutational loss of *WT1* may contribute to transformation of T-cell and myeloid but not B-cell progenitor cells. In T-ALL as in AML, *WT1* alterations mainly consist on mono-allelic deletions or heterozygous frame-shift insertions that cluster in exon 7, predicted to produce a truncated protein which lacks the DNA binding domain. In addition, some patients showed mono-allelic deletions associated with heterozygous mutations or compound heterozygous mutations. Additional frameshift mutations predicted to encode N-terminal truncations in WT1 were detected in exons 1 and 2 and few cases presented missense mutations in exon 9, which encodes the third zinc finger of WT1 protein. The functional relevance of these mutations is highlighted by the fact that they have been described as the most frequent genetic abnormality in patients with DDS and in a sporadic Wilms' tumor sample[55,56]. Consistent with

these results, allelic expression analysis in *WT1*-mutated T-ALL samples demonstrated the presence of both wild-type and mutant *WT1* transcripts in leukemic lymphoblasts. The presence of *WT1* mutations predicted to encode truncated WT1 proteins devoid of DNA-binding activity led us to hypothesize that *WT1* loss could lead to haplo-insufficiency or have a dominant negative effects towards the wild type WT1 proteins, implying that wild-type WT1 acts as a tumor suppressor in leukemia. Interestingly, *WT1* mutations were particularly prevalent in T-ALL cases harboring chromosomal rearrangements associated with the expression of TLX1/HOX11, TLX2/HOX11L2, and HOXA9 transcription factor oncogenes, suggesting that *WT1* loss and aberrant expression of oncogenic homeobox factors may be mechanistically linked in the pathogenesis of T-ALL[56, 177].

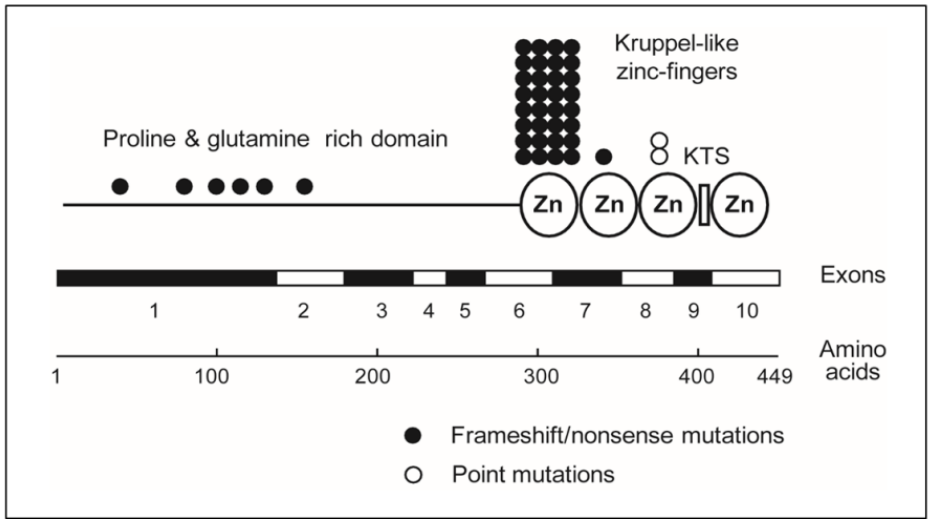


Figure 2. Somatic mutations in *WT1* in T-ALL.
Schematic representation of *WT1* mutations identified in primary T-ALL samples[56].

The importance of *WT1* mutations in the context of leukemia patients is highlighted by the fact that these alterations may have an impact on the prognosis of both T-ALL and AML cases, even if many groups reported conflicting results. In fact *WT1* mutations have been shown to be a poor prognostic factor in specific genetic subgroups or either they have been shown to have no impact on clinical outcome. More specifically, in T-ALL, even if no significant difference were

found in the relapse-free survival (RFS) and overall survival (OS) between the mutated *WT1* and wild type *WT1* patients in the overall cohort, within the standard risk group of thymic T-ALL, the mutated *WT1* subgroup showed an inferior RFS as compared to wild type *WT1* thymic patients[175]. In the case of AML, the outcome of patients is highly heterogeneous and depends, among others, on the cytogenetic and molecular profiles. In this disease context, mutated *WT1* samples had an inferior OS and RFS in the total cohort of AML patients or at least in a genetic subgroup of CN-AML (cytogenetically normal acute myeloid leukemia) patients[178,179]. More recently, an important role of WT1 as epigenetic modulator was demonstrated in AML [180]. It was reported that *WT1* mutations are inversely correlated with *TET2* gene mutations in AML, and that *WT1* mutant AML samples are characterized by significantly marked reductions in global and site-specific DNA hydroxymethylation and disordered DNA hydroxymethylation potentially representing a convergent mechanism of leukemic transformation. These mutations induce *TET2* loss of function and reduce genomic 5-hydroxymethylcytosine (5hmC) with a reciprocal increase in 5-methylcytosine (5mC), featuring extensive promoter hypermethylation. *WT1* loss also led to marked reductions in 5hmC levels and a defect in hematopoietic differentiation, a phenotype similar to that observed with loss of *TET2*. Taken together, these results suggest that the hydroxymethylation pathway may be affected by these mutations and, in addition to its role as a sequence-specific transcription factor, WT1 may act as a cofactor for TET enzymes recruiting or stimulating their activity at specific sites in the genome.

2. Aim of the thesis

WT1 has been described to act as both a tumor suppressor and an oncogene in leukemia. The presence of mutations in about 10% of patients with AML and ALL resulting in the truncation of the C-terminus WT1 domain lacking the major DNA binding portion supported a role as tumor suppressor in leukemia. Apart from *WT1* mutations, over-expression of *WT1* has also been observed in leukemia supporting a role as an oncogene even if the mechanisms of this over-expression have not been elucidated yet. Moreover, *WT1* mutated samples generally over-express *WT1* transcript suggesting that the truncated protein could have a dominant negative effect or that mutations lead to gain-of function.

Very recently, the role of *WT1* mutations were defined in AML. WT1 was described as a tumor suppressor in AML cooperating with TET2 in epigenetic regulation. In T-ALL the role of WT1 is still unclear and many questions are still open.

This study focused on the role of *WT1* alterations in T-ALL. Our main hypothesis was that WT1 works a tumor suppressor in T-ALL and that *WT1* alterations impair its transcriptional program leading to tumor progression.

The following principal aims were pursued:

- *What are the effects of WT1 over-expression and loss in T-ALL cells?*

  In order to answer this question we over-expressed the four most abundant WT1 isoforms in T-ALL cells and we asked whether this over-expression affects cell growth and survival. We then analyzed the effects of *WT1* loss in T-ALL cells. We used the short hairpin RNA technology to significantly lower *WT1* expression in T-ALL cells and we studied the effects on cell growth and survival. Finally, the effects of over-expression of a common WT1 mutant was analyzed in wild-type T-ALL cells.

- *What are the de-regulated genes and pathways following WT1 loss in T-ALL cells?*

  To this end, we performed Chromatin-immuno-precipitation combined with microarrays technology (ChIP-chip) to identified the direct WT1 targets in T-ALL cells. This analysis was also combined with gene expression analysis performed in a loss of function system to identify WT1-regulated targets and to allowed the identification of specific pathways controlled by WT1 in T-ALL cells.

- *What are the effects of WT1 alterations in T-ALL primary samples?*

  An important part of this study included the characterization of primary human T-ALL xenografts for *WT1* mutations. We characterized wild-type and mutant *WT1* T-ALL xenografts for *WT1* expression both at the transcript and protein level. We explored if *WT1* mutated T-ALL xenografts showed common features with T-ALL cell lines engineered to lose *WT1* expression. Considering the huge heterogeneity of primary T-ALL samples, this is a an ambitious aim to pursuit but also a first important attempt to understand WT1's role in T-ALL.

3 . Materials & Methods

3.1 Cell lines and primary T-ALL xenograft samples

CCRF-CEM, JUKAT, MOLT4 T-ALL cells were obtained from the ATCC. The PF382 and P12-Ichikawa T-ALL cells were from the DSMZ repository. T-ALL cell lines were cultured in RPMI 1640 (Euroclone) medium supplemented with 10% fetal bovine serum, 1% Ultraglutamine, 1% Na-Piruvate, 1% Hepes, 100U/ml penicillin G and 100 μg/ml streptomycin (Lonza) at 37°C in a humidified atmosphere under 5% CO_2. HeLa, U2OS and Human Embryonic Kidney 293T (HEK293T) cells were grown in Dulbecco's modified Eagle's (DMEM, Euroclone) medium supplemented with 10% fetal bovine serum, 1% Ultraglutamine, 1% Na-Piruvate, 1% Hepes, 100U/ml penicillin G, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere under 5% CO_2. Primary xenografts T-ALL (PDTALL) cells were obtained from the bone marrow of newly diagnosed ALL pediatric patients, with informed consent according to the guidelines of the local ethics committee. For xenograft establishment, 6- to 8-weeks-old mice NOD Rag1^{null} IL2Rγ^{null} immune-deficient mice (Charles River, Wilmington, MA, USA) were retro-orbitally injected with 10x10^6 T-ALL cells in 200 μl. For short-term *in vitro* experiments, primary T-ALL xenograft derived cells were maintained in RPMI-1640 media supplemented with 20% FBS.

3.2 Ionizing irradiation and chemotherapeutic drugs

Cells were irradiated using γ-radiation derived from an ^{137}Cs source. For drug treatments, cells were subjected to different doses of etoposide, cytarabine, vincristine and methotrexate (Sigma).

3.3 Plamids and constructs

pGipz and pTripz shRNA constructs targeting different regions of human *WT1* transcript (V2LHS_270428 and V2THS_202981) and pGipz shRNA construct targeting different regions of human *p53* transcript (V3LHS_333917, V3LHS_333919 and V3LHS_333920) were used for knockdown experiments (Open Biosystem). pGipz and pTripz non-silencing shRNA (sh-Scramble) were

used as negative controls (RHS4348 and RHS4743, Open Biosystem). Migr1 vectors (gently provided by Prof. Warren Pear, University of Pennsylvania, Philadelphia PA) were used to clone full length and mutant *E384Stop WT1* constructs using BglII and BglII/EcoRI restriction sites, respectively. Full length and mutant *E384Stop WT1* sequences were cloned in pcDNA3.1 vector (Life Technologies) for transient WT1 over-expression in Hela and U2OS cells. *LEF1*, *LST1*, *RB1*, *MBNL1* and *BBC3/PUMA* reporter constructs were generated cloning a short nucleotide sequence, that resulted significantly bound by WT1 from the Agilent probes in our ChIP-on Chip analysis, into the pGL4.23[luc2/minP] vector (Promega) using the NheI/HindIII restriction sites. For luciferase experiments, plasmid which expresses *Renilla* luciferase was also used (pGL4.74 [*hRluc*/TK]; Promega).

3.4 **Retrovirus and lentivirus production.**

MigR1 retroviral vectors and pGipz and pTripz (non-silencing shRNA control or *WT1*-shRNA) lentiviral vectors (3 µg) were transfected in HEK293T using JetPEI® (Polyplus Transfection) transfection agent and the correspondent packaging plasmids, pCMV-Δ8.9 (Addgene) plasmid (2.7 µg), containing *gag*, *pol* and *rev* genes, and VSV-G (Addgene) plasmid (300 ng), expressing envelope genes. The viral surnatants were collected 48 hours after transfection, filtered and used to infect target cells. Infection of T-ALL cell lines were performed by spinoculation of viral supernatants produced in HEK293T cells: target cells were resuspended in medium containing virus and distributed 1-2x10^6 cells per well in 24 well plates. Hexadimethrine bromide (Polibrene®, Sigma) was added at 0.8 µg/ml final concentration. Plates were centrifuged at 2200 rpm for 90 minutes at room temperature. After infection cells were placed at 37°C over night and then positive selected by adding puromycin 1 µg/ml (Sigma) in the medium for a period of about 3-5 days.

3.5 ChIP-chip analysis

ChIP-chip analysis of the WT1 target genes was performed in MOLT4 T-ALL cells. Briefly, immune-precipitation was performed using a combination of a rabbit polyclonal antibody (ab15429, Abcam), and a mouse monoclonal antibody (6F-H2, Millipore) specific respectively for the C-terminus and the N-terminus of WT1 protein. ChIP-chip was performed following the standard protocols provided by Agilent Technologies using Agilent Human Proximal Promoter Microarrays (244.000 features per array), as previously described[181]. This platform analyzes ~17,000 of the best-defined human genes sourced from UCSC hg18 (NCBI Build 36.1, March 2006) and covers regions ranging from −5.5 kb upstream to +2.5 kb downstream of their transcriptional start sites. We scanned the arrays with an Agilent scanner and extracted the data using Feature Extraction 8 software. Genes that were direct targets of WT1 were identified using a ChIP-chip significance analysis, as previously described[181].

3.6 Gene expression analysis

RNA was isolated from triplicate cultures of MOLT4 infected with either sh-scramble or sh-*WT1* pGipz vectors using Trizol reagent (Life Technologies). Following purification with RNAasy kit (Life Technologies), 1µg of RNA was amplified and labeled using 3' IVT Express Kit and hybridized on GeneChip Human U133 Plus 2.0 (Affymetrix). Inter-array normalization was performed with the GC-RMA algorithm using open-source Bioconductor software. Group differences were evaluated using t-test and fold change. For the Pathway Enrichment analyses, gene sets of interest, were tested for functional annotations enrichment using the web-based DAVID bioinformatics tools available at http://david.abcc.ncifcrf.gov. GSEA analysis was performed in MATLAB to test for enrichment of gene sets from the WT1 direct targets and the ranked list of genes sorted by t-score comparing sh-*WT1* versus sh-Scramble.

3.7 Statistical analysis

Student's t test (two tailed, unpaired) was used for statistical comparison in functional experiments *p<0.05, **p<0.01, ***p<0.001.

3.8 Cell viability assays and flow cytometric analysis

T-ALL cell lines ($3x10^5$ cells per well) and primary T-ALL xenografts ($1x10^6$ cells per well) were seeded in 24 well plates and treated either with γ-radiation or chemotherapeutic drugs for 24 hours. Cell viability analysis was performed using the bioluminescent method Vialight plus (Lonza). Apoptosis analysis was performed by flow cytometry (FACS) after cell staining with Annexin-V-FLUOS Staining Kit (Roche) and Propidium Iodide (Sigma) according to manufacturer's instructions. Analysis of proliferation after 12 hours of γ-radiation (combined with cell cycle profile and DNA synthesis was performed using the Click-iT™ EdU Flow Cytometry Assay Kit (Life Technologies). All the analysis were performed in triplicate. The samples were collected on a FACSCalibur (BD Biosciences) using Cell Quest software (BD Biosciences), and analyzed with FlowJo software (Tree Star).

3.9 Clonogenic assay

Colony assays were performed by resuspending CCRF-CEM T-ALL cells over-expressing WT1 full length and E384Stop mutant isoforms, and MOLT4 T-ALL cells expressing sh-Scramble and sh-*WT1*, in RPMI 1640 medium supplemented with 10% fetal bovine serum and Methocult H4100 (Stemcell Technologies) composed of 2.6% methylcellulose in Iscove's MDM. The cell suspensions were seeded in 35x10mm dishes, in triplicate, at a concentration of 1000 cells per dish and incubated at 37°C in a humidified atmosphere under 5% CO_2, for 7 to 14 days. At the end of the incubation period colonies were counted. using an inverted microscope (Leica) and a scoring grid. Two biological independent experiments were performed.

3.10 **Human Apoptosis and MAPK pathway Arrays.**

We analyzed the modulation and the expression profiles of 35 apoptosis-related proteins and phosphorylation of 24 MAP kinases simultaneously hybridizing approximately 200 µg of MOLT4 cell lysates into the Human Apoptosis Array and the Human MAPK Array kits (R&D Systems). Whole cell extracts were incubated overnight with the arrays, according with the kit instructions. Following the incubation, the arrays were washed, hybridized with a cocktail of biotinilated detection antibodies before applying the Streptavidin-HRP and chemiluminescent detection reagents. The BioRad ChemiDoc XRS Imager was used to capture the signals from the arrays. The density of each spot was quantified by Quantity One software (BioRad) and processed data was normalized to negative control probe (PBS) to be comparable between arrays.

3.11 **Western blotting.**

Total cell lysates were prepared using lysis buffer (NaCl 150mM, TrisHCl 50 mM, EDTA 2mM, 1% NP40) supplemented with phosphatase inhibitors (NaF 50mM, Na_3OV_4 1mM) and protease inhibitors cocktail tablets (Roche) and normalized for protein concentration using the Micro BCA™ Protein Assay Kit (Thermo Scientific). For Western blotting, protein samples were separated on 4-12% gradient NuPAGE® Bis-Tris poly-acrylamide or 3-8% gradient NuPAGE® Tris-Acetate SDS-PAGE gels (Life Technologies) and transferred to nitrocellulose membranes (Protran). Membranes were then blocked in PBS-Tween-20 0.1% containing 5% nonfat milk, incubated over night with primary antibodies according to the antibody manufacturer's instructions, and finally incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse IgG (Perkin Elmer). The BioRad ChemiDoc XRS Imager was used to capture membrane images following incubation with Western Lightning® Plus-ECL substrate (Perkin Elmer). Antibodies against total p53 (DO-1) were from Santa Cruz Biotechnology; antibodies recognizing β-actin, PARP, Cleaved Caspase3, phospho-p53 (S15), acetylated-p53 (K382), phospho-Histone H2AX (S139), phospho-ATM (S1981), total ATM, phospho-Chk2 (Thr68), total Chk2, XIAP,

PUMA, p21, BAX and Survivin were from Cell Signaling Technologies; antibody against WT1 (6F-H2) was from Millipore; antibody against HO-2/HMOX2 was from Novus.

3.12 Low Density Arrays and Real Time quantitative PCR (RT-qPCR)

Total RNA was isolated using Trizol reagent, c-DNA was obtained from 1µg of RNA using the SuperScript® First-Strand Synthesis System (Life Technologies). Analysis of 94 genes related to DNA repair, apoptosis and cell cycle following DNA damaging conditions, was performed using Custom TaqMan® Array Cards (Life Technologies) with the TaqMan® Universal PCR Master Mix (Life Technologies) and ABI Prism 7900 Sequence Detection System. Arrays were performed in duplicate. Relative quantification was done using the ΔΔCt method[182], normalizing to β2M and GAPDH genes. The list of genes contained in the array is presented in the following table:

MAPK	APOPTOSIS	DNA REPAIR		CELL CYCLE	OTHERS
CCNE1	ATM	APEX1	POLE4	14-3-3 σ	B2M
MAPK10	ATR	BARD1	POLG	CCND2	GAPDH
MAPK11	BAX	BRCA1	POLH	CCND3	WT1
MAPK12	BBC3	BRCA2	POLK	CCNE1	
MAPK14	CASP9	DCLRE1A	POLQ	CDC25A	
MAPK8	CD82	DDB1	PRKDC	CDC25C	
MAPK9	CHEK1	ERCC5	SIRT1	CDK6	
PPM1D	CHEK2	GTF2H1	XPA	CDKN1A	
	EI24	H2AFX	XPC	FANCA	
	FAS	LIG1	XPC	FANCC	
	HSPB1	LIG3	XRCC1	GADD45A	
	MDM2	LIG4	XRCC1	GADD45B	
	MDM2	MBD4	XRCC4	GADD45G	
	MDM4	MGMT	XRCC4	MDC1	
	NOXA	MLH1	XRCC5	PCNA	
	PARP1	MSH2	XRCC5	RAD1	
	PARP1	MSH3	XRCC6	RAD17	
	PERP	MSH6		RAD23B	
	PRKCD	NBN		RAD50	
	PSMB10	NTHL1		RAD51	
	PSMB5	OGG1		RAD52	
	TP53	PNKP		RAD9A	
	TP53I3	POLA1		RPA3	
	TP73	POLD4		RRM2	
		POLE3		TP63	

Regular RT-qPCR experiments were performed using SensiMix™ SYBR® No-ROX Kit (Bioline) and then the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Relative expression levels were obtained using the ΔΔCt method[182] and normalizing to β2M gene. Primer sequences used in the RT-qPCR are reported below:

Gene		Sequence
BAX	Forward	5'-CATGTTTTCTGACGGCAACTTC-3'
BAX	Reverse	5'-AGGGCCTTGAGCACCAGTTT-3'
BBC3/PUMA	Forward	5'-TTGTGCTGGTGCCCGTTCCA-3'
BBC3/PUMA	Reverse	5'-AGGCTAGTGGTCACGTTTGGCT-3'
DUSP10	Forward	5'-CCCTCTACCACTATGAGAAAG-3'
DUSP10	Reverse	5'-TCAATGAACTCAAAAGCCTC-3'
DUSP6	Forward	5'-TTCTACCTGGAAGATGAAGC-3'
DUSP6	Reverse	5'-CAATGTCATAGGCATCGTTC-3'
FAS	Forward	5'-TGCAGAAGATGTAGATTGTGTGATGA-3'
FAS	Reverse	5'-GGGTCCGGGTGCAGTTTATT-3'
GADD45A	Forward	5'-GCCTGTGAGTGAGTGCAGAA-3'
GADD45A	Reverse	5'-CCCCACCTTATCCATCCTTT-3'
LIG1	Forward	5'-CCTGCGAATACAAATATGACGGGC-3'
LIG1	Reverse	5'-CTTGGAATGGCTGGATCTGCTTCT-3'
CDKN2A/p21	Forward	5'-AGACTCTCAGGGTCGAAAAC-3'
CDKN2A/p21	Reverse	5'-TTCCAGGACTGCAGGCTTC-3'
POLH	Forward	5'-GGGAGCAGTGATTGTGGAGGAAAT-3'
POLH	Reverse	5'-CCTCCAAGACTACGGATTTTGCGA-3'
PPM1D	Forward	5'-AGAGAATGTCCAAGGTGTAGTC-3'
PPM1D	Reverse	5'-TCGTCTATGCTTCTTCATCAGG-3'
PTPN7	Forward	5'-AGGAGAAATGTGTCCACTAC-3'
PTPN7	Reverse	5'-AAAGAGGATGTGCTTTACTG-3'
WT1	Forward	5'-GTTCCCCAACCACTCATTCAAG-3'
WT1	Reverse	5'-GGCTCCTAAGTTCATCTGATTCCA-3'
XPC	Forward	5'-CATGAGGACACACACAAGGT-3'
XPC	Reverse	5'-CAGGTTTGAGAGGTAGTAGG-3'

3.13 **Luciferase assay**

HeLa and U2OS cells were seeded 5x104 cells per well in 24 well plates and transfected with increasing concentrations of pcDNA3.1 plasmids (100, 250, 500 ng) over-expressing the four wild type full length WT1 isoforms, that differ for the presence or absence of exon 5 (defined as 5+ or 5– respectively) or for the presence or absence of the KTS insert (KTS+ and KTS– respectively), and two WT1 mutants isoforms (WT1 E384Stop with exon 5+ or 5–) together with pGL4.23 [luc2/minP] vector (150 ng) carrying the insertion of a specific response element upstream of a minimal promoter and a luc2 gene (Promega). For internal normalization of transfection efficiency, cells were also co-transfected with pGL4.74 [hRluc/TK] plasmid (100 ng), which allowed *Renilla* luciferase expression. Experiments were performed in triplicates. We measured luciferase activity 48 hours after transfection with the Dual-Luciferase Reporter® assay kit (Promega). Transfected cells were gently rinsed with PBS from culture medium, homogeneously lysated for 20 minutes in 150 µl of passive lysis buffer and finally harvested. Cell lysates (5 µl per well) were added in 96 well plate and luciferase assay reagent (50 µl) was added to each well, measuring firefly luciferase activity with VICTOR™ X5 Multilabel Plate Reader (Perkin Elmer). *Renilla* luciferase activity was then measured adding Stop & Glo® reagent (50 µl) to each well.

4 . Results

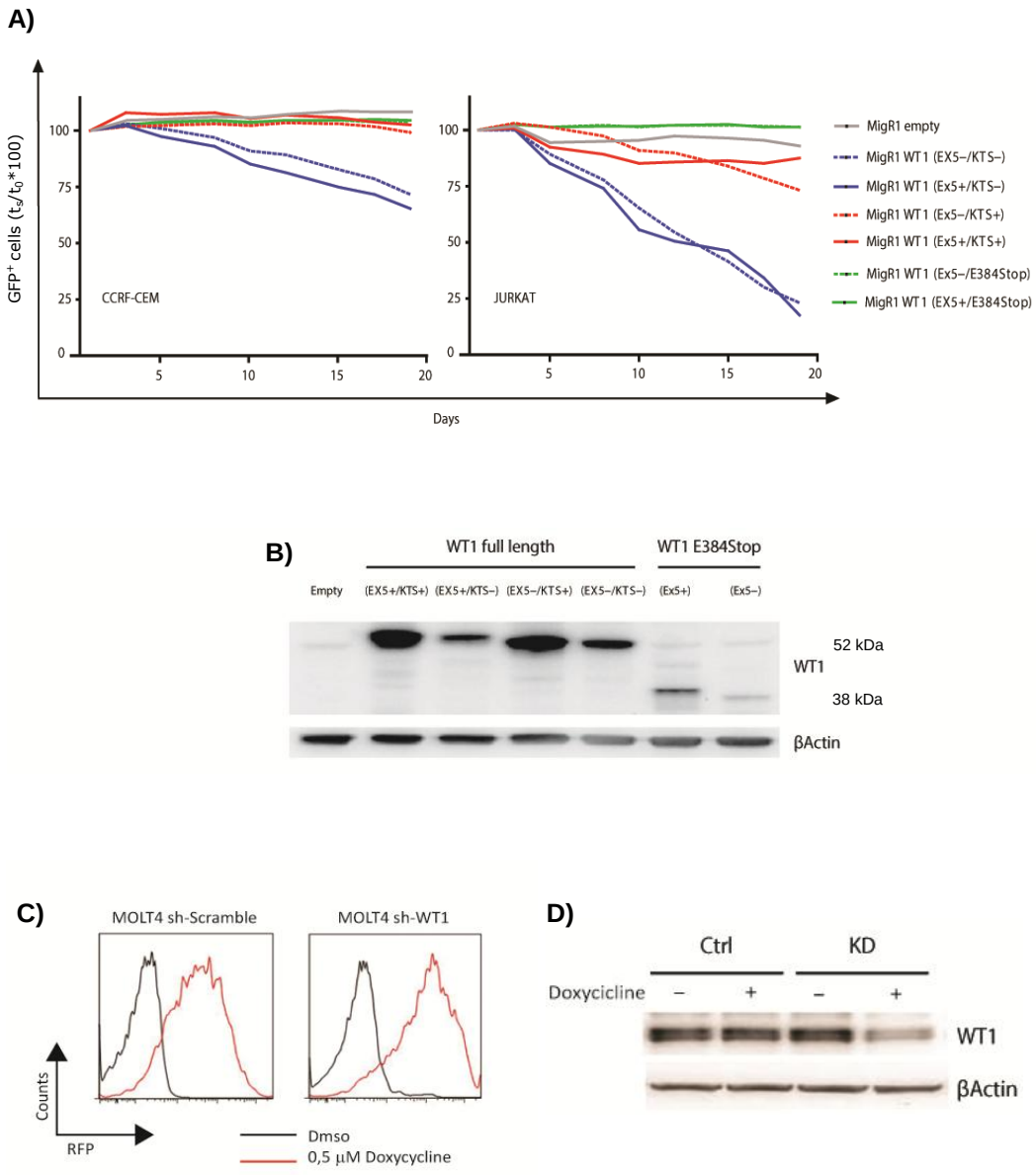
4.1 *WT1* acts as a tumor suppressor gene in T-ALL cells.

WT1 protein has been reported to have opposing roles depending upon the cell type in which it is expressed. It was seen to act both as a tumor suppressor or as an oncogene. To elucidate the role of WT1 in T-ALL we first tested the effects of over-expression of different WT1 isoforms in CCRF-CEM and Jurkat T-ALL cell lines. CCRF-CEM, which lack WT1 protein expression due to heterozygous nonsense point mutations in exon 1 of *WT1* gene[56], and Jurkat, which show very low WT1 expression[183], were infected with Migr1-IRES GFP retroviral vectors expressing the four full length WT1 isoforms that differ for the presence (Ex5+) or absence (Ex5−) of exon 5 or for the presence (KTS+) or absence (KTS−) of the KTS tripeptide. Moreover, in order to study a possible role of truncated WT1 protein, cells were also infected with Migr1 encoding two mutated isoforms containing or lacking exon 5. GFP was expressed through an internal ribosomal entry site (IRES) and allowed infected cells to be tracked by flow cytometry. As shown in one of the two replicates represented in Figure 3.A), we observed that only over-expression of the (KTS−) isoforms negatively affected growth of T-ALL cells, as indicated by progressive loss of GFP positive cells, while other WT1 isoforms and the mutants had no effect. Over-expression of full length and mutant WT1 isoforms in CCRF-CEM was confirmed by western blot analysis (Figure 3.B).

Clonogenic assays have been widely used as an *in vitro* marker for transformation and as a method to monitor tumor cells growth and proliferation. We thus analyzed the effects of WT1 over-expression in T-ALL cells, performing clonogenic assays in soft-agar medium. We found that CCRF-CEM cells over-expressing the (KTS−) full length isoforms showed significant reduction in colony formation in methylcellulose compared with cells expressing the empty vector and other WT1 isoforms (mutants included).

Subsequently, we set out to determine the functional consequence of *WT1* loss in T-ALL. To this end we thus used pTRIPZ lentiviral vectors to silence *WT1* in MOLT4 T-ALL cells, that express moderate levels of WT1 protein, upon treatment with doxycycline. After 6 days of doxycycline treatment, expression of the shRNAs, both in sh-Scramble (Ctrl) and sh-*WT1* cells (KD), was confirmed by

detection of red-fluorescent protein (RFP) co-expressed with the short hairpin by flow cytometry analysis. *WT1* knockdown was verified by RT-qPCR and western blot analysis (Figure 3.C-D). Cell growth and cell cycle regulation was then analyzed and compared between Ctrl and KD cells, but no differences were found. Cells were then tested in colony formation assays, and, as expected, *WT1* loss in MOLT4 cells favored the growth of a significant higher number of colonies compared to MOLT4 cells expressing an irrelevant hairpin (Figure 3.E).



E)

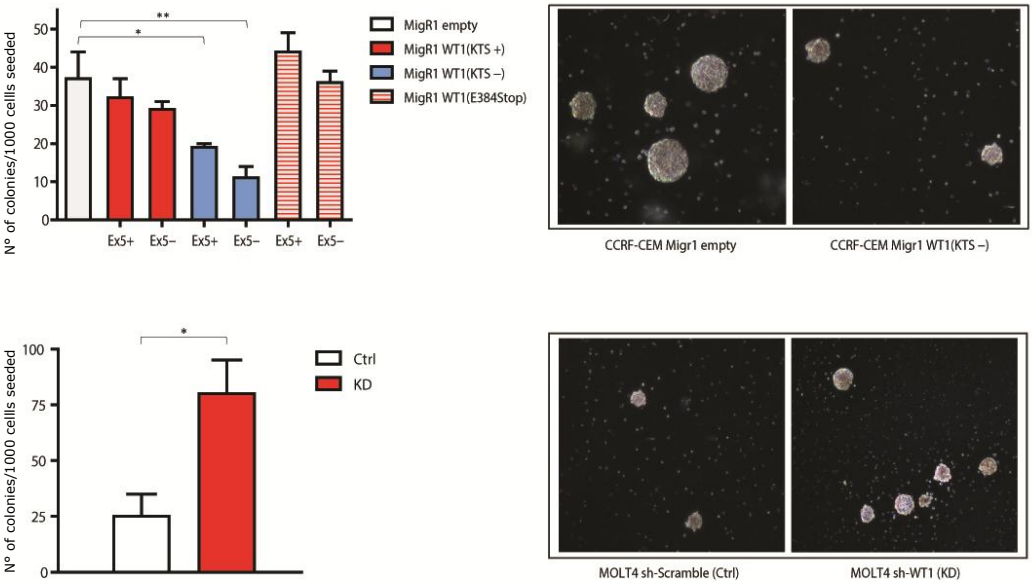


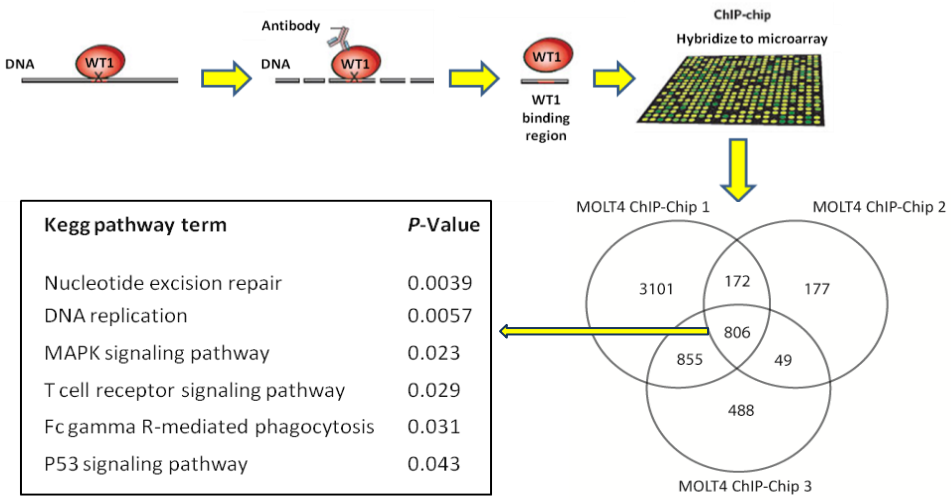
Figure 3. *WT1* over-expression induces cell growth arrest, while its knockdown increases colony formation in T-ALL cells.

A) Proliferation of JURKAT and CCRF-CEM cells carrying the MigRI GFP retrovirus with different WT1 isoforms; percentage of GFP positive cells was monitored every three days by flow cytometry for 20 days. Percentages of GFP positive cells were normalized to the level on day 3 (t=0) post-transduction. B) Immunoblot of the over-expression of full length and mutant WT1 isoforms in CCRF-CEM. Wild type CCRF-CEM were used as negative control for *WT1* expression. βActin is shown as loading control. C) Flow cytometry analysis of red fluorescent protein (RFP) in MOLT4 cells infected with pTripz lentivirus vector co-expressing both RFP and a specific sh-*WT1* hairpin (KD). An irrelevant hairpin was used as control (Ctrl). Analysis was performed after 6 days treatment with doxycycline at 0.5 μM. D) Immunoblot analysis of WT1 protein expression in Ctrl and KD MOLT4 cells treated for 6 days with doxycycline or with the vehicle control (DMSO). E) Optical microscope images of methylcellulose colonies from CCRF-CEM over-expressing WT1 isoforms and MOLT4 cells infected with sh-Scramble and sh-*WT1*. The histogram plots on the left represent mean and SD of colony number per 1000 cells seeded, for MOLT4 cells in triplicate plates.

4.2 Analysis of ChIP-chip and gene expression data identify WT1 direct targets to be enriched in genes involved in cellular response to stress conditions.

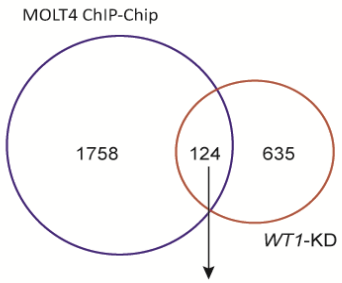
WT1 is a transcription factor that acts as activator or repressor depending on cellular context. However, little is known about the targets and the transcriptional program regulated by WT1 in T-ALL. To define the structure of its transcriptional network, ChIP-Chip analysis was performed in MOLT4 T-ALL cells (which express moderate level of WT1 protein). ChIP-Chip analysis in MOLT4 cells identified 806 regulatory elements bound in three independent experiments (three arrays with FDR 5%). Among the 806 WT1 targets, we found enrichment in pathways involved in cellular response to stress such as nucleotide excision repair, MAPK, and p53 signaling pathways (Figure 4.A). To identify genes differentially regulated by WT1, we performed gene expression profile in MOLT4 cells under loss of function conditions using shRNA technology to knockdown *WT1* gene (KD). Microarray analysis showed 758 genes significantly regulated in the three replicates ($p<0.05$; Fold change ≥ 1.4). Comparing genes differentially regulated by WT1 in the KD system with genes identified in at least two of three ChIP-chip experiments we identified a total of 124 genes both bound and regulated by WT1 in the context of T-ALL. Functional annotation of these genes showed that they were enriched in the MAPK signaling pathway (Figure 4.B). This pathway has been previously found to be regulated by WT1 in different cellular systems by other groups. Finally, Gene Set Enrichment Analysis (GSEA) was performed with the ChIP-chip and gene expression profile data sets and this analysis showed a significant enrichment of genes whose promoters were bound by WT1 in the expression signature associated with KD in MOLT4 cells. Most notably, we found several genes involved in the pathogenesis of T-ALL (such as *LEF1*, *LMO2* and *RB1*) to be bound and regulated by WT1. These were found to be characteristically down-regulated.

A)



Kegg pathway term	*P*-Value
Nucleotide excision repair	0.0039
DNA replication	0.0057
MAPK signaling pathway	0.023
T cell receptor signaling pathway	0.029
Fc gamma R-mediated phagocytosis	0.031
P53 signaling pathway	0.043

B)



Gene Ontology	
Kegg Pathway	Genes
MAPK Pathway	*DUSP10, DUSP6, FGFR1, JUND, PPM1A, PTPN7*

Figure 4. Identification of WT1-bound promoter regions and functional classification of genes bound and differentially regulated by WT1.

A) ChIP-chip experiments scheme performed in MOLT4 cells: cells were lysed and using anti-WT1 antibodies, protein-DNA complexes were immune-precipitated, DNA isolated, labeled and hybridized to genomic microarrays. Genes identified by the analysis are shown in Venn diagrams (FDR 5%). Gene ontology was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov). B) Venn diagram representation showing the overlap between the WT1 deregulated targets, obtained from the gene expression profile after *WT1* knockdown in MOLT4 cells, and the ChIP-chip data set. Gene ontology of genes both bound and regulated by WT1 showed enrichment in MAPK signaling pathway.

4.3 **Functional validation of WT1 targets.**

Bioinformatic analysis provided us a ranking of genes both bound and regulated after *WT1* loss in our cellular model. Next we set out to functionally validate some of these targets by luciferase assays in Hela cells. The targets we chose belonged both to the top genes of our lists, with the best P-value score (for example *LST1*), and genes that are known to be involved in the pathogenesis of T-ALL (*LEF1* and *RB1*), even if they presented a lower P-value. We cloned their regulatory elements upstream of the luciferase reporter gene and over-expressed all the four full length isoforms of WT1, with or without exon 5 and with or without KTS, in Hela cells (which do not express WT1). The luciferase activity is directly correlated with the binding of WT1 isoforms to the target sequences. We observed that the (KTS−) isoforms induced significant luciferase activity in all the gene targets analyzed, because of higher affinity for the promotorial regions. On the other hand, the (KTS+) isoforms were less effective in luciferase induction, given their low affinity for DNA. Furthermore, luciferase induction was dose-responsive: increasing the amount of DNA, determined a higher induction of luciferase activity. We verified WT1 isoform protein expression by western blot analysis (Figure 5.A). Interestingly, the more effective (KTS–) isoforms were less expressed compared with the (KTS+) isoforms.

We hypothesized that WT1 mutant isoforms may have an impaired transcriptional activity due to the lack of a DNA binding domain. Mutants (E384Stop) were expressed both alone and in combination with the (KTS–) isoforms, using *LEF1* promoter region, which showed the highest reporter activity. (KTS–) isoforms had a high transcriptional activity, while mutant alone induced very low levels of luciferase, in accordance with our hypothesis, and in combination did not modify the transcriptional activity of the (KTS−) isoforms. However at the protein level, mutant isoforms, notwithstanding a comparable quantity of transfected DNA with full length isoforms, had a very low expression, probably due to a high protein instability (Figure 5.B). Thus, at least in our system, it seems that WT1 mutants do not exert neither a transcriptional activity or a dominant negative effect on wild-type isoforms.

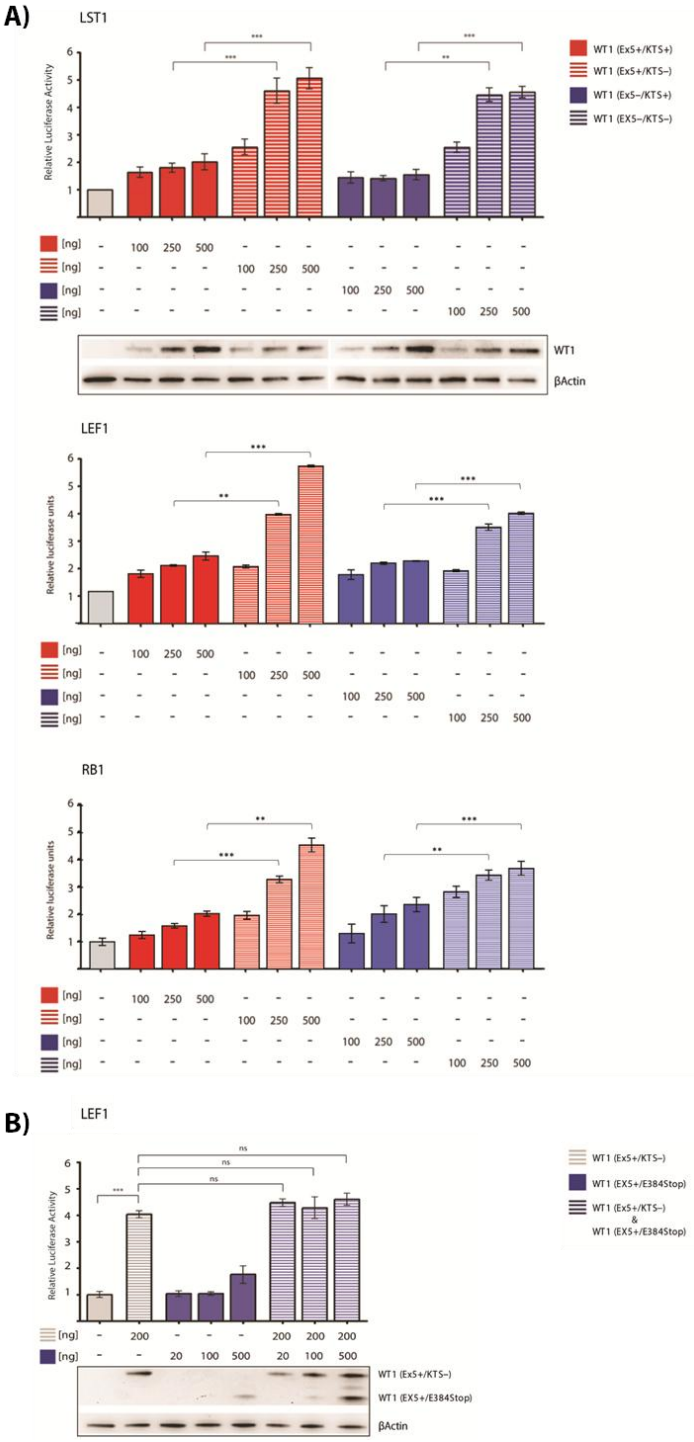


Figure 5. Validation of putative WT1 binding regions by luciferase reporter assays in Hela cells.

A) Luciferase induction by WT1 isoforms using the WT1 binding regions of *LST1*, *RB1* and *LEF1* promoters. The red bars represent the WT1 isoforms with exon 5, the blue bars the isoforms without exon 5. The stripped bars indicate the (KTS–) isoforms. Error bars represent standard deviation for triplicate experiments (**P<0.01; *** P<0.001). The panel under the graphs shows a representative immunoblot. B) Luciferase induction by WT1(E384Stop/Ex5+) mutant alone (blue) and in combination with WT1(Ex5+/KTS–) isoform (stripped blue) using the WT1 binding region in *LEF1* promoter. Error bars represent SD for triplicate experiments (*** P<0.001). The panel under the bars shows a representative immunoblot for WT1 and βActin.

4.4 *WT1* loss confers resistance to DNA damage-induced apoptosis in T-ALL cells with a functional p53 pathway.

Stemming from bioinformatical analysis of ChIP-on-chip data and gene expression profile data, we hypothesized that WT1 may be involved in DNA damage response. We used p53 proficient MOLT4 cells as a model for study the effects of *WT1* loss in T-ALL cells. After doxycycline induction, MOLT4 cells were subjected to increasing doses of γ-radiation for 24 hours. Analysis of cell viability and apoptosis (Figure 6.A) showed that KD cells had significantly higher cell viability compared to control cells after γ-irradiation. Cell cycle analysis, revealed that after 12 hours of γ-radiation treatment (2 Gy), both control and *WT1* knockdown cells showed a comparable G2/M cell cycle arrest while the S phase resulted strongly decreased in control cells but not in KD cells. This observation was further strengthened by combining DNA content analysis with 5-ethynyl-2'-deoxyuridine (EDU) labeling (Figure 6.B).

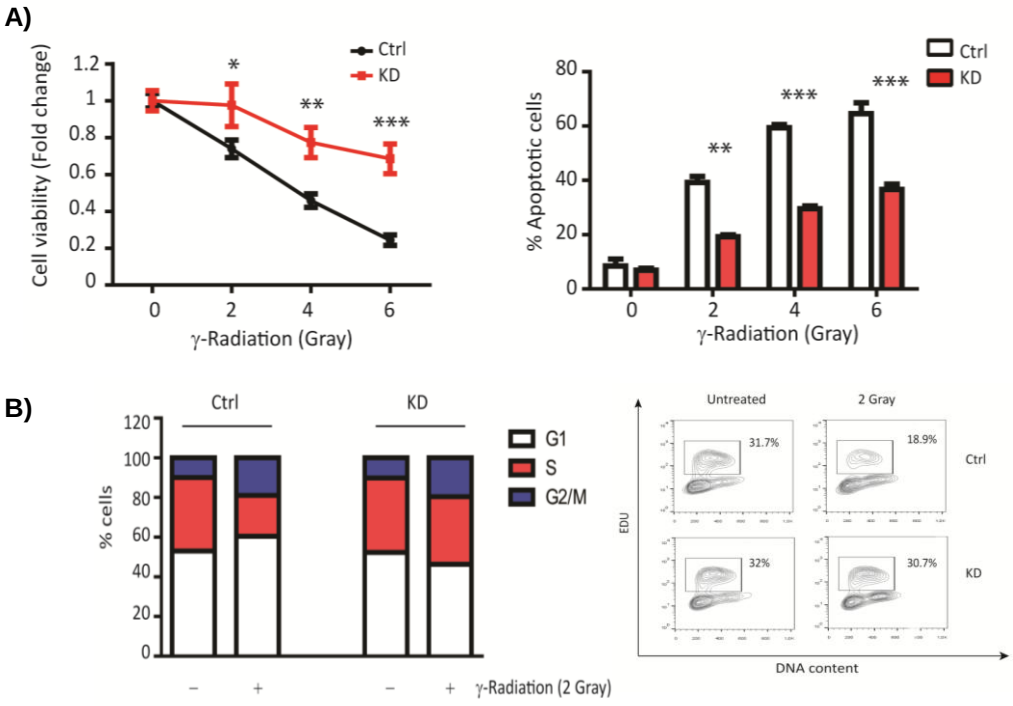


Figure 6. *WT1* knockdown protects from γ-radiation induced apoptosis in MOLT4 T-ALL cells.
A) Cell viability and apoptosis analysis in MOLT4 cells infected either with sh-scramble (Ctrl) or sh-*WT1* (KD) after 24h treatment with increasing doses of γ-radiation. Error bars represent SD for triplicate experiments (* P<0.05; **P<0.005; *** P<0.001). B) Analysis of cell cycle and proliferation in sh-Scramble (Ctrl) and sh-*WT1* (KD) MOLT4 cells through direct measurement of DNA synthesis. The Click-iT® EdU Flow Cytometry Assay kit was used in combination with cell cycle analysis. Analysis was performed after 12h of γ-radiation (2 Gy). On the right, plots of a representative experiment are shown. Square regions identify cells in active proliferation (S-phase).

Since our Chip-Chip data also indicated that WT1 target genes were enriched in pathways involved in DNA repair and cellular stress response, we focused on the analysis of the apoptotic response at the molecular level performing protein arrays in MOLT4 cells (Ctrl and KD) treated for 6 and 24 hours with 6 Gray (Gy) of irradiation. At 6 hours we observed minimal differences between *WT1* knockdown and control cells except for cleaved Caspase3 expression that was considerably higher in the sh-Scramble cells compared to sh-*WT1*. After 24 hours, differences in apoptotic protein expression became more evident: in fact KD cells, both under basal conditions and after irradiation, showed significantly lower levels of cleaved Caspase3 compared to control cells, but higher levels of anti-apoptotic proteins such as Survivin, XIAP and HO-2, that resulted strongly down-regulated in sh-Scramble cells. Surprisingly, KD cells had higher levels of phosphorylated p53 at serine 15, serine 46 and serine 392, compared to sh-scramble control cells, suggesting future further analysis to evaluate p53 activation and post translational modification early after DNA damage. Changes in protein expression (Cleaved Caspase3, XIAP, Survivin, HO-2) identified by protein arrays were validated by western blot (Figure 7).

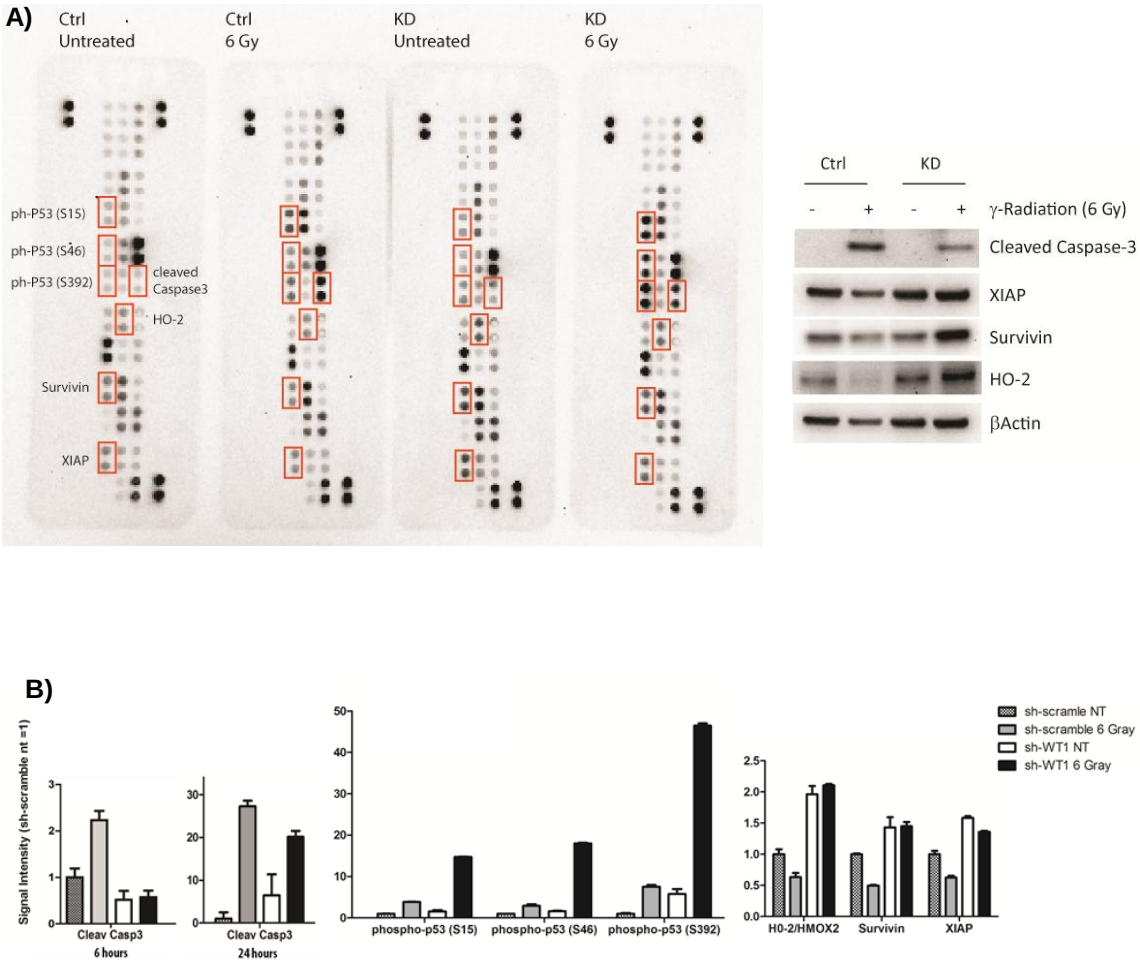


Figure 7. Apoptosis protein arrays in MOLT4 cells after 24 hours from 6 Gy of γ-radiation.

A) Whole cell extracts from sh-Scramble (Ctrl) and sh-*WT1* (KD) MOLT4 cells after 24h from γ-radiation (6 Gy) were hybridized to human apoptosis protein arrays. Expression of apoptotic proteins was evaluated in separate immunoblots (panel on the right). B) Apoptotic proteins that were consistently modulated (Cleaved Caspase3, Survivin, HO-2, XIAP, phospho-p53) were labeled and their relative quantification, calculated respect to untreated Ctrl cells, is shown in the histogram plots.

Similarly, we evaluated if *WT1* loss also protects from DNA damage induced by chemotherapeutic drugs used in the treatment of leukemia. Analysis of cell viability and apoptosis of MOLT4 cells treated with increasing doses of etoposide (from 0.1 to 5 µM) for 24 hours showed that KD cells were significantly less sensitive to lower doses of etoposide than control cells. In fact, western blot analysis of pro- and anti-apoptotic factors (PARP, cleaved Caspase3, XIAP, Survivin, HO-2) following etoposide treatment showed a similar trend to that observed after γ-radiation. A similar trend was also observed in cell viability assays after treatment with increasing doses of other anti-neoplastic drugs, such as cytarabine (ARA-C), vincristine and methotrexate, currently used in the therapy of T-ALL.

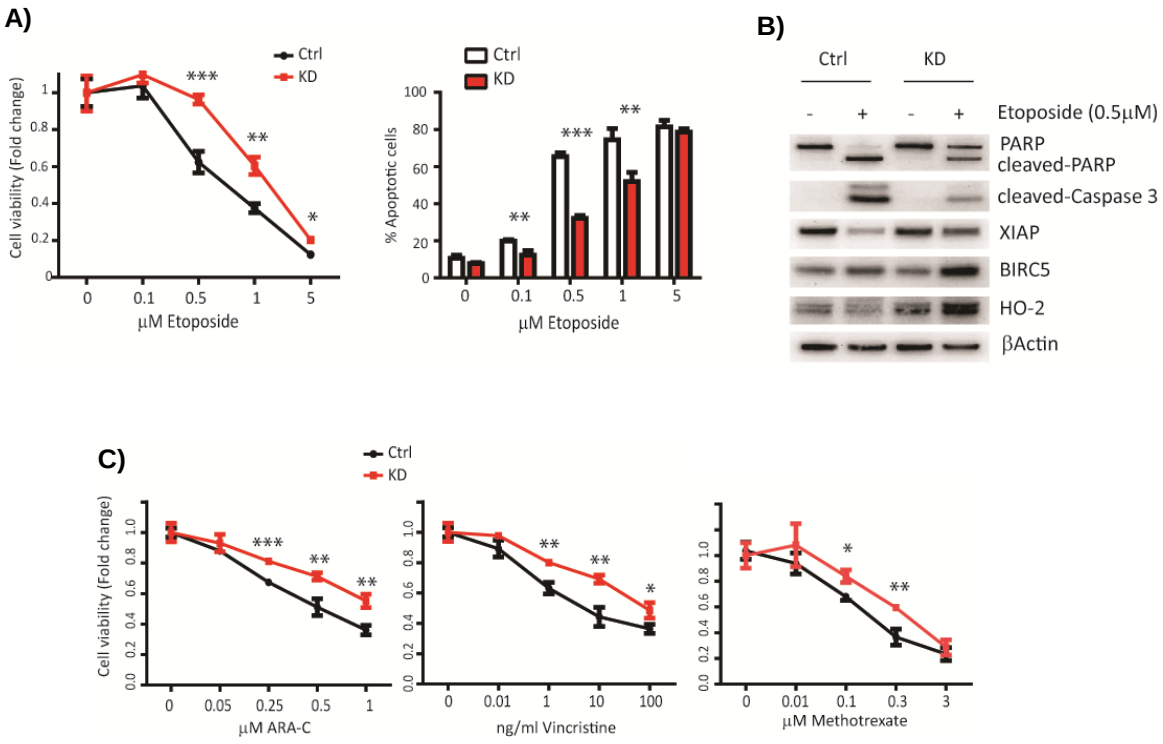


Figure 8. *WT1* knockdown protects from chemotherapic drug-induced apoptosis in MOLT4 T-ALL cells.
(A) Cell viability assays and apoptosis analysis using AnnexinV staining in MOLT4 cells infected either with sh-scramble (Ctrl) or sh-*WT1* (KD) after 24 hours treatment with increasing doses of etoposide. Error bars represent SD for triplicate experiments (* P<0.05; **P<0.01; *** P<0.001). (B) Western blot analysis of PARP, Cleaved Caspase-3, Survivin, Xiap and HO-2 in MOLT4 cells infected either with sh-scramble (Ctrl) or sh-*WT1* (KD); analysis was performed after 24 hours treatment with 0.5 µM etoposide. βactin is shown as loading control. (C) Cell viability assays in MOLT4 cells infected either with sh-scramble (Ctrl) or sh-*WT1* (KD) after 24 hours treatment with increasing doses of cytarabine (ARA-C), vincristrine and methotrexate. Error bars represent SD for triplicate experiments (*P<0.05; **P<0.01; *** P<0.001).

4.5 *WT1* loss does not confer resistance to DNA damage in T-ALL cells with a defective p53 pathway.

To evaluate if *WT1* loss may confer resistance to DNA damaging agents interfering with p53 pathway, we further extended our analysis to T-ALL cell lines that have an impaired p53 function due to mutations in the DNA binding motif, such as PF382 (p53 mutation: R273C; Cosmic database) and P12 Ichikawa (p53 mutation: R248Q/P; Cosmic database). As for MOLT4 cells, *WT1* knockdown was successfully induced following treatment with doxycycline (Figure 9.A-B). Treatment of PF382 and P12-Ichikawa infected cells with increasing doses of γ-radiation and etoposide showed that after 24 hours both Ctrl and KD cells had a high viability, indicating that these cells were more resistant than MOLT4 cells to these treatments. Further extending cell viability analysis to 48 hours disclosed that *WT1* knockdown does not confer protection from apoptosis compared to control cells. In addition, cleaved PARP, cleaved Caspase-3, XIAP, and Survivin were not differentially regulated between Ctrl and KD cells upon DNA damage (Figure 9.C-F). Western blot analysis of p53 stabilization and activation showed high level of basal p53 protein as expected for cells carrying mutations in the *p53* locus. Notably, the ratio between PARP and cleaved PARP was identical between sh-Scramble and sh-*WT1* cells further supporting the fact that *WT1* loss does not affect the apoptotic response in p53 mutated T-ALL cells.

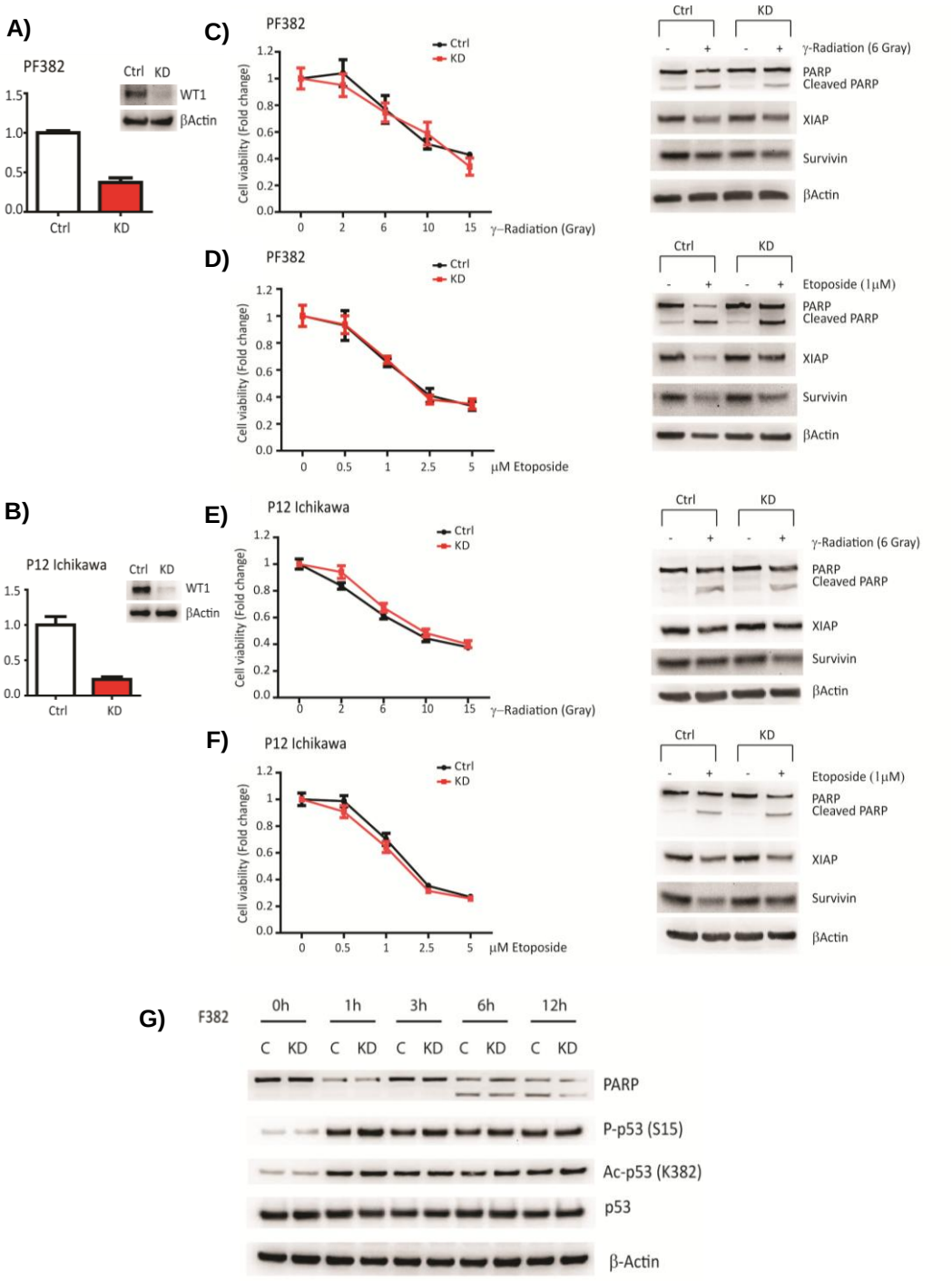


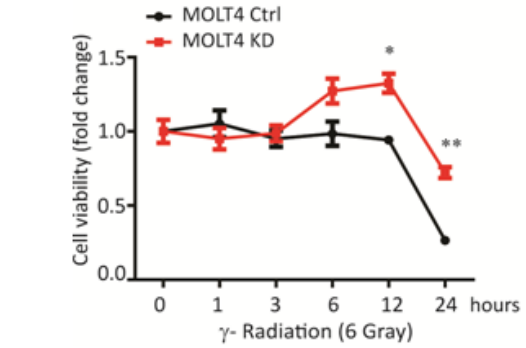
Figure 9. *WT1* knockdown does not protect from γ-radiation and etoposide induced-apoptosis in PF382 and P12 Ichikawa cells.

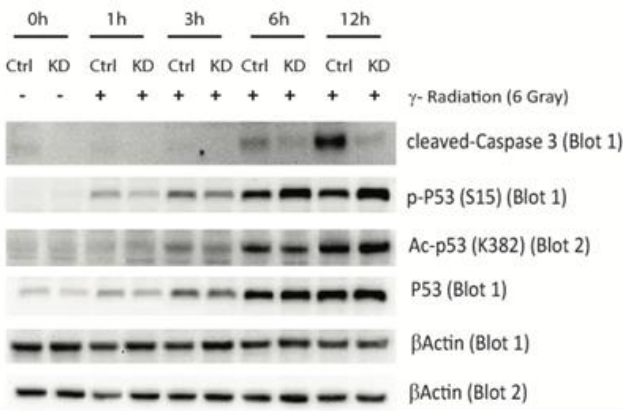
A-B) Relative expression of *WT1* gene transcript in Ctrl and KD PF382 and P12 Ichikawa cells treated 6 days with doxycycline as assessed by RT-qPCR. Upper right, immunoblot analysis of WT1 protein. C-F) Cell viability assay analysis in PF382 (C and D) and P12 Ichikawa (E and F) T-ALL cells infected either with sh-scramble (Ctrl) or sh-*WT1* (KD) after 24 hours from γ-radiation and etoposide treatment. Right panels: immunoblot analysis of PARP, XIAP, Survivin. βActin is shown as loading control. G) Immune-blot of PARP, phosphorylated-p53 (S15), acetylated-p53 (K382), and total p53 at different time points after 6 Gy of γ-irradiation in PF382 cells. βActin is shown as loading control.

4.6 *WT1* loss promotes survival dampening the p53 apoptotic response

Given the dependence of WT1 function with a functional p53 response we evaluated if *WT1* loss affected p53 activation and stabilization following DNA damage. p53 activation is a complex process and requires recognition of DNA damage. After ionizing radiation, the serine/threonine kinase ATM acts at an early stage in damage sensing phosphorylating p53 protein, in a serine residue (S15), and the serine/threonine kinase Chk2. Chk2 triggers stabilization of p53 by phosphorylating another serine residue (S20). MOLT4 cells, infected either with sh-Scramble or sh-*WT1*, were treated with 6 Gy of γ-radiation. Western blot analysis of phosphorylated p53 (p-P53, S15), acetylated p53 (Ac-P53, K382) and of total p53 at different time points (0,3,6,12 hours) demonstrated that sh-Scramble MOLT4 cells had similar levels of p53 induction and activation compared to sh-*WT1* MOLT4 cells. Moreover, phosphorylation of ATM, Chek2 and γH2AX, involved in the initial phases of DNA damage recognition, was not severely affected by *WT1* loss (Figure 10.C).

A)





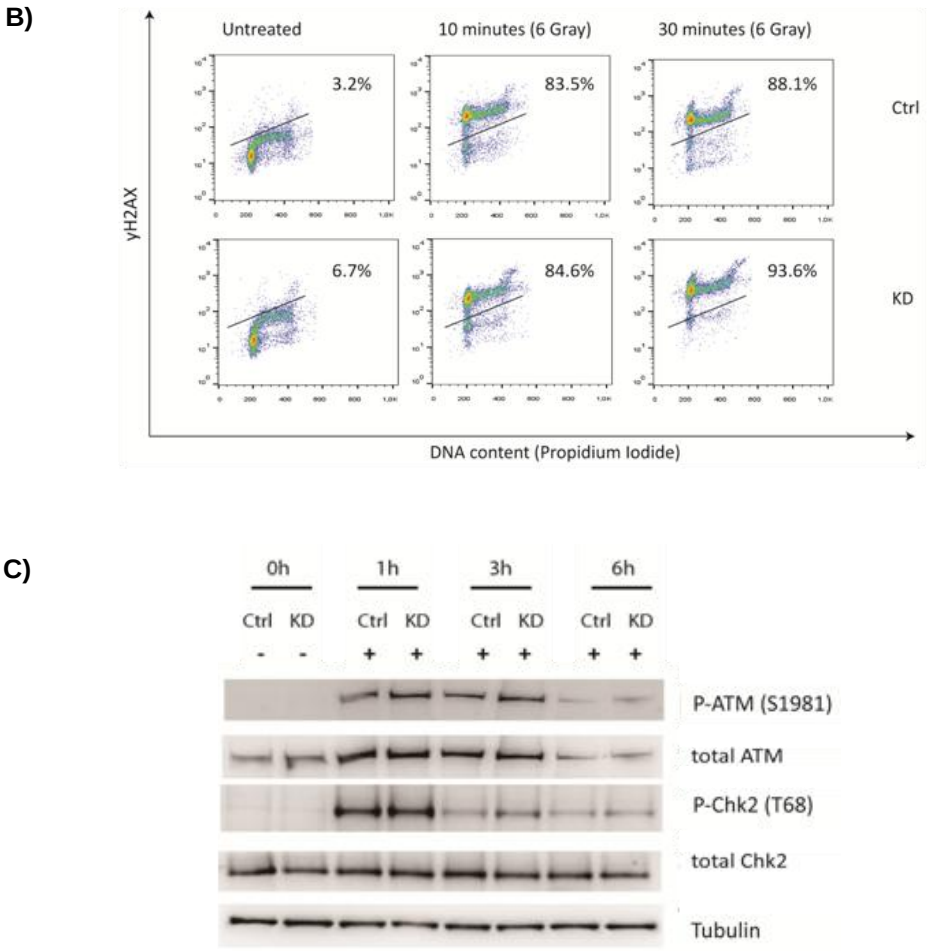


Figure 10. *WT1* loss does not impair DNA damage recognition in MOLT4 T-ALL cells.

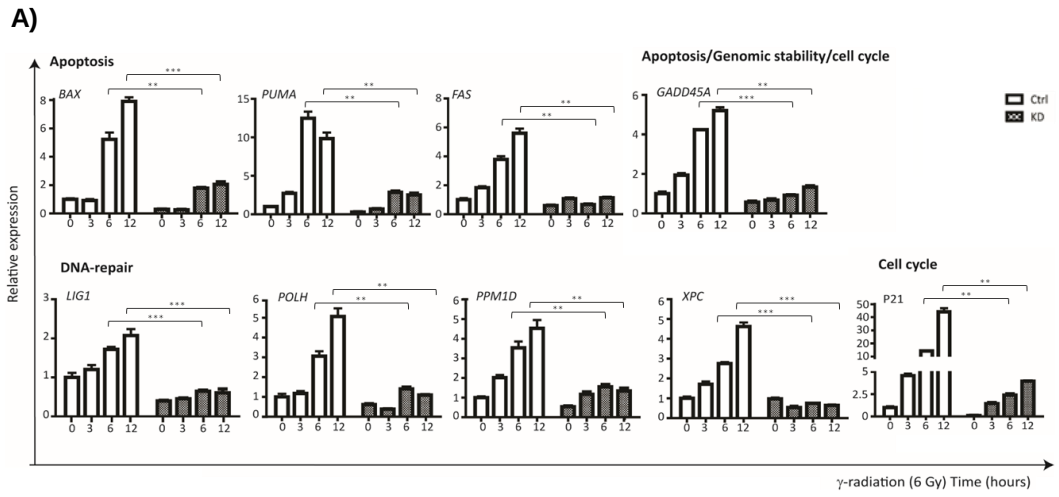
A) Left panel: **c**ell viability analysis in MOLT4 cells infected either with sh-scramble (Ctrl) or sh-*WT1* (KD) after 1, 3, 6 and 12 hours from γ-radiation (6 Gy). Right panel: analysis of Cleaved Caspase3, phosphorylated p53 (S15), acetylated p53 (K382), and total p53 expression levels by western blot. βactin is shown as loading control. B) Flow cytometry analysis of histone H2AX phosphorylation (S139), in combination with Propidium Iodide (PI) incorporation. sh-Scramble (Ctrl) and sh-*WT1* cells (KD) cells were treated with 6 Gy of γ-radiation for 10 and 30 minutes. One representative experiment is shown. C) Immunoblot analysis of phosphorylated and total ATM (S1981) and Chk2 (T68) in sh-Scramble (Ctrl) and sh-*WT1* (KD) MOLT4 cells following 1,3 and 6 hours from 6 Gy of γ-radiation. βactin is shown as loading control.

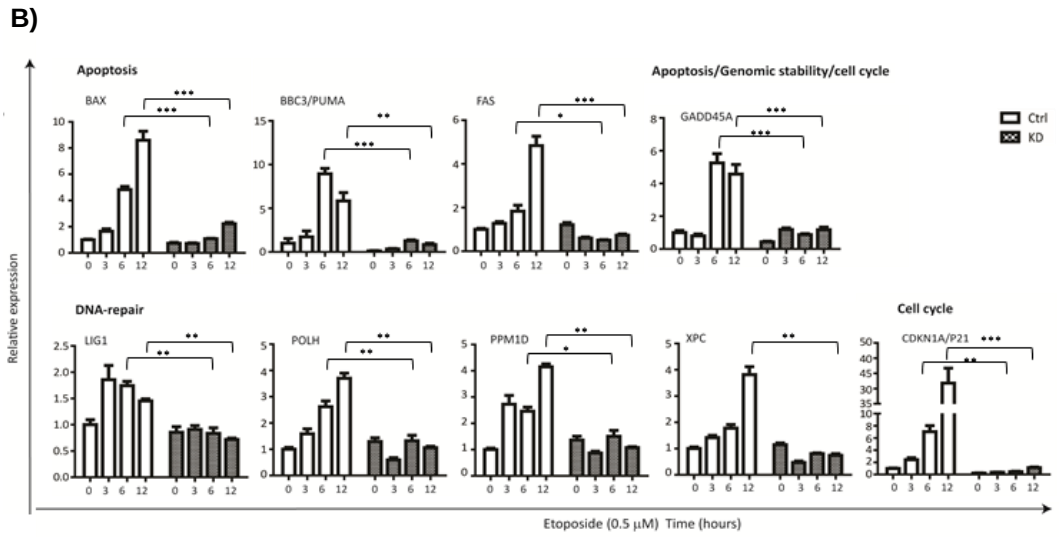
Given these results, we hypothesized that WT1 may affect the apoptotic response downstream of p53 activation. Using low-density arrays and RT-qPCR we analyzed the expression of genes involved in DNA repair, apoptosis and cell cycle progression under basal conditions and after 3, 6 and 12 hours following γ-radiation treatment, both for KD and Ctrl MOLT4 cells. We found that *WT1* knockdown markedly lowered the transcript levels of genes involved in DNA damage response both basally and after irradiation (Figure 11.A): interestingly

most of them were involved in p53 response such as *CDKN1A/p21, BBC3/PUMA, GADD45A, BAX* and *FAS*. Surprisingly, these genes resulted significantly bound by WT1 in at least one of our ChIP-chip replicates (*CDKN1A/p21*, $P=1.53x10^{-7}$; *BBC3/PUMA* $P=1.06x10^{-7}$; *GADD45A*, $P=7.38x10^{-5}$; *BAX*, $P=1.4x10^{-4}$; *FAS*, $P=2.13x10^{-2}$). For the pro-apoptotic factors *BAX* and *BBC3/PUMA* and the cell cycle regulator *CDKN1A/p21*, a potent cyclin-dependent kinase inhibitor, differences in their induction were particularly clear at 12 hours from γ-radiation, resulting more expressed in sh-Scramble compared to sh-*WT1* MOLT4 cells. Their regulation at protein level was also confirmed by western blot analysis. In addition, *WT1* knockdown cells failed to up-regulate genes involved in DNA repair, such as *LIG1, POLH, PPM1D* and *XPC*, that were instead significantly induced in sh-Scramble cells at 6 and 12 hours after 6 Gy of γ-radiation. Interestingly, *LIG1* gene was significantly bound in our ChIP-chip data set in 3 out of 3 replicates with a P value of $2.37x10^{-9}$. Similar results were also found after treatment of MOLT4 cells with 0.5 μM of etoposide (Figure 11.B).

Among the WT1 regulated targets identified by the integration of ChIP-chip data and gene expression analysis, the MAPK pathway resulted particularly enriched. We thus verified by RT-qPCR gene expression of some important regulators of the MAPK pathway, obtained from bioinformatic analysis, such as *DUSP6*, *DUSP10*, *PPM1A* and *PTPN7*. However, no significant differences in gene expression were found upon γ-radiation treatment between sh-Scramble and sh-*WT1* cells (data not shown). Considering that MAPK pathway regulation mainly occurs at the post-transcriptional level we analyzed MAPK components in response to DNA damage using MAPK pathway peptide arrays. Analysis was performed at 3 and 6 hours from 6 Gy of γ-radiation and it showed that critical regulators of cellular stress such as p38 and JNK proteins were not induced upon DNA damage, indicating that, at least in our context, the MAPK pathway is not crucial for DNA damage response (data not shown).

Finally we verified gene expression changes of genes involved in DNA damage response following γ-radiation treatment in cells with an impaired p53 response. Thus, sh-Scramble and sh-*WT1* PF382 cells were subjected to 6 Gy of γ-radiation and we evaluated gene expression changes by RT-qPCR. We found no significant differences in the gene expression profile of most genes involved in DNA damage response, both in control and KD cells (Figure 11.C), except for *GADD45A* and *PPM1D*, that had a higher expression in control cells, especially at early time points after damage.





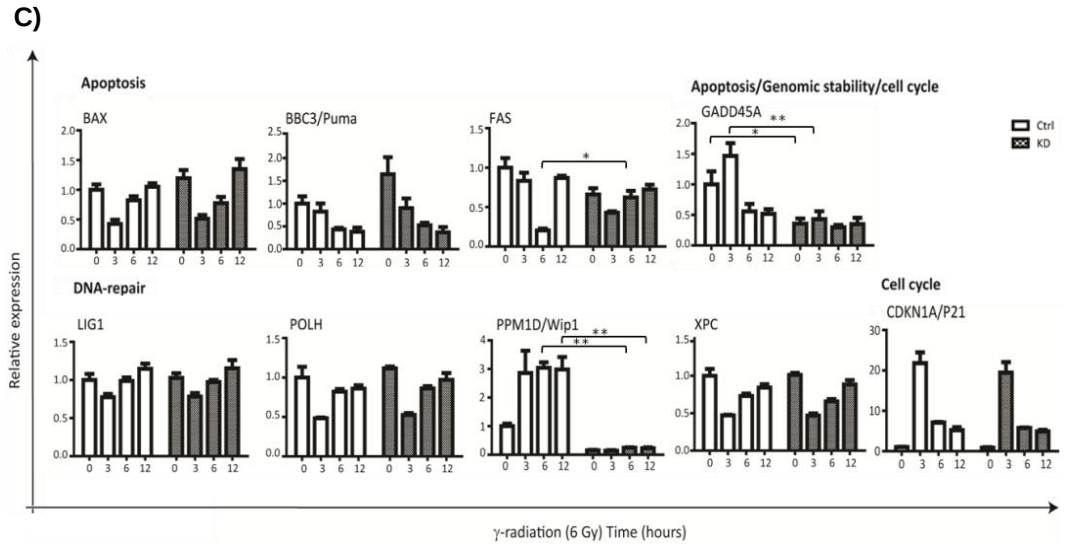


Figure 11. *WT1* knockdown impairs p53 DNA damage response in MOLT4 cells but not in PF382 cells.
Relative expression of specific genes involved in the DNA damage response in MOLT4 cells after different times (3, 6 and 12 hours) from 6 Gy of γ-radiation (A) and 0.5 μM etoposide (B) treatments. C) Relative expression of specific genes involved in the DNA damage response after different times (3, 6 and 12 hours) from 6 Gy of γ -radiation in PF382 cells. Analysis was performed in both sh-Scramble (Ctrl) and sh-*WT1* cells (KD). Expression is calculated relative to untreated sh-Scramble cells (Ctrl 0h) fixed as 1.

4.7 *WT1* alterations are associated with increased resistance to DNA damage in human primary T-ALL xenografts

Since deficiency in WT1 protein resulted in increased resistance to DNA damage in MOLT4 T-ALL cell line, we wondered if this phenotype could also be present in human primary T-ALL samples carrying alterations in *WT1* locus. Amongst forty T-ALL xenograft samples generated in our laboratory, three xenografts resulted mutated in exon 7 (PDTALL13, PDTALL40R, PDTALL51R,) with frameshift or nonsense mutations: PDTALL13 was obtained from a newly diagnosed T-ALL patient, whereas PDTALL40R and PDTALL51R were obtained from relapsed (R) patients.

PDTALL	Phenotype	*WT1* status	*p53* status
8	Thymic	wild type	wild type
9	Early-T	wild type	wild type
10	Early-T	wild type	wild type
11	Thymic	wild type	wild type
12	Early-T	wild type	wild type
13D	Thymic	ins GGGCCGG at position 1110; predicted to produce truncated protein	wild type
15	T-mature	wild type	wild type
16	Thymic	wild type	wild type
19R	Early-T	wild type	R248Q
40R	Early-T	C1142A; predicted to produce truncated protein	R213Q; 236_237insC
51R	Early-T	insCGGCCACTCCCCGGGGGTCC/delGTG at position 1102; predicted to produce truncated protein	wild type

Table 3. Human primary T-ALL xenograft characteristics.

WT1 expression was analyzed by RT-qPCR and resulted quite similar among PDTALL xenograft samples even if one of the *WT1* mutated samples, PDTALL13, showed higher levels of *WT1* expression respect to all the other samples (Figure 12.A). On the other hand, mutant *WT1* samples showed a trend towards lower levels of WT1 protein expression respect to the wild-type *WT1* xenograft samples, as shown by western blot analysis in Figure 12.B. Surprisingly, the mutant WT1 protein was only clearly detectable in CCRF-CEM infected with a plasmid carrying the mutant WT1 (E384Stop) but not as native protein in mutant *WT1* PDTALL xenografts.

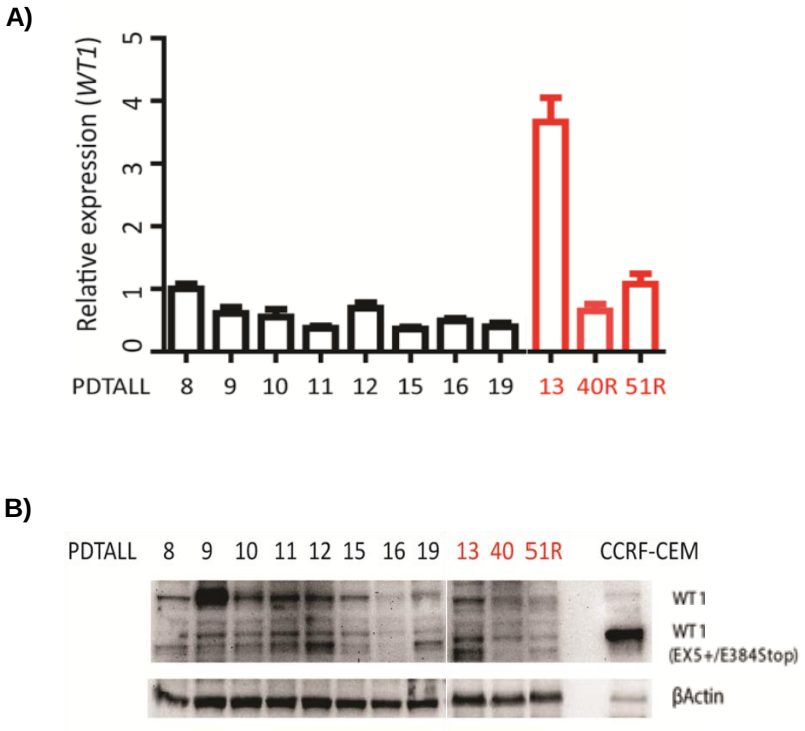


Figure 12. *WT1* mRNA and protein expression in primary T-ALL xenograft samples

A) Relative expression of *WT1* mRNA in primary T-ALL xenografts (*WT1* expression of PDTALL8 is fixed at 1).

B) Immunoblot analysis of WT1 protein levels in primary T-ALL xenograft samples. βActin is shown as loading control. WT1 mutated samples are indicated in red.

We compared the effects of increasing doses of γ-radiation (0.5 to 6 Gy) between mutant *WT1* xenografts and wild type *WT1* xenografts (PDTALL8, 9, 10, 11, 12, 15, 16 and 19R) that were in use in our laboratory. Cell viability and apoptosis assays showed that PDTALL xenografts could be divided into two subgroup: sensitive (IC50 < 1.5 Gy) and resistant (IC50 > 1.5 Gy) and, among these last ones, all the WT1 mutated xenografts were included. Interestingly, two xenografts, PDTALL19R and 40R were particularly resistant due to mutations in *p53* locus.

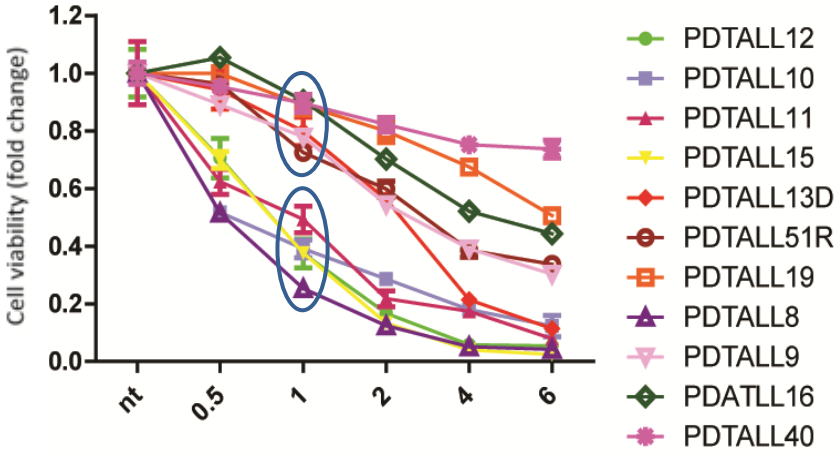


Figure 13. Primary T-ALL xenograft samples with *WT1* alterations are resistant to γ-radiation-induced apoptosis
Cell viability analysis of primary T-ALL xenograft samples after 24h of treatment with increasing doses of γ-radiation (0.5 to 6 Gy).

In order to determine if xenograft response to γ-radiation was possibly related to an altered expression of genes involved in DNA damage response, we performed RT-qPCR analysis for numerous genes involved in the DNA damage response. For this analysis wild-type *WT1* and the mutant *WT1* samples were exposed to 2 Gy γ-radiation for 3 hours. Among the genes analyzed, the most differentially regulated ones after damage were the pro-apoptotic factor *BBC3/PUMA* and cell cycle regulator *CDKN1A/p21*. Both RT-qPCR and western blot analysis confirmed that these genes were nicely induced in the wild type *WT1* xenografts PDTALL8, 9, 10 ,11 and 12 after γ-radiation, most probably due to efficient activation and stabilization of p53. Differently from the other *WT1* wild type samples PDTALL16 showed high basal level of BBC3/PUMA that resulted further up-regulated following DNA damage. Surprisingly, this sample showed minimal induction in BBC3/PUMA expression at the transcript level. PDTALL13D xenograft, which resulted mutated for *WT1* gene, showed p53 stabilization similar to wild type WT1 xenografts but both RT-qPCR and western blot analysis showed an impaired expression of p53 targets BBC3/PUMA and CDKN1A/p21. On the other hand, in PDTALL51R, both pro-apoptotic genes were highly induced after DNA damage, although it showed resistance to γ-radiation in cell viability assays (Figure 13). Finally, no BBC3/PUMA or CDKN1A/p21 induction was found in PDTALL19R and 40R samples, where

mutations in *p53* locus impaired the canonical p53 activation after DNA damage (Figure 14). Considering the molecular and genetic heterogeneity of PDTALL xenograft samples, this study is a first attempt to associate *WT1* mutations with resistance to therapy. Interestingly, PDTALL13D showed an impaired *BBC3/PUMA* induction similar to that observed in MOLT4 KD cells following p53 activation.

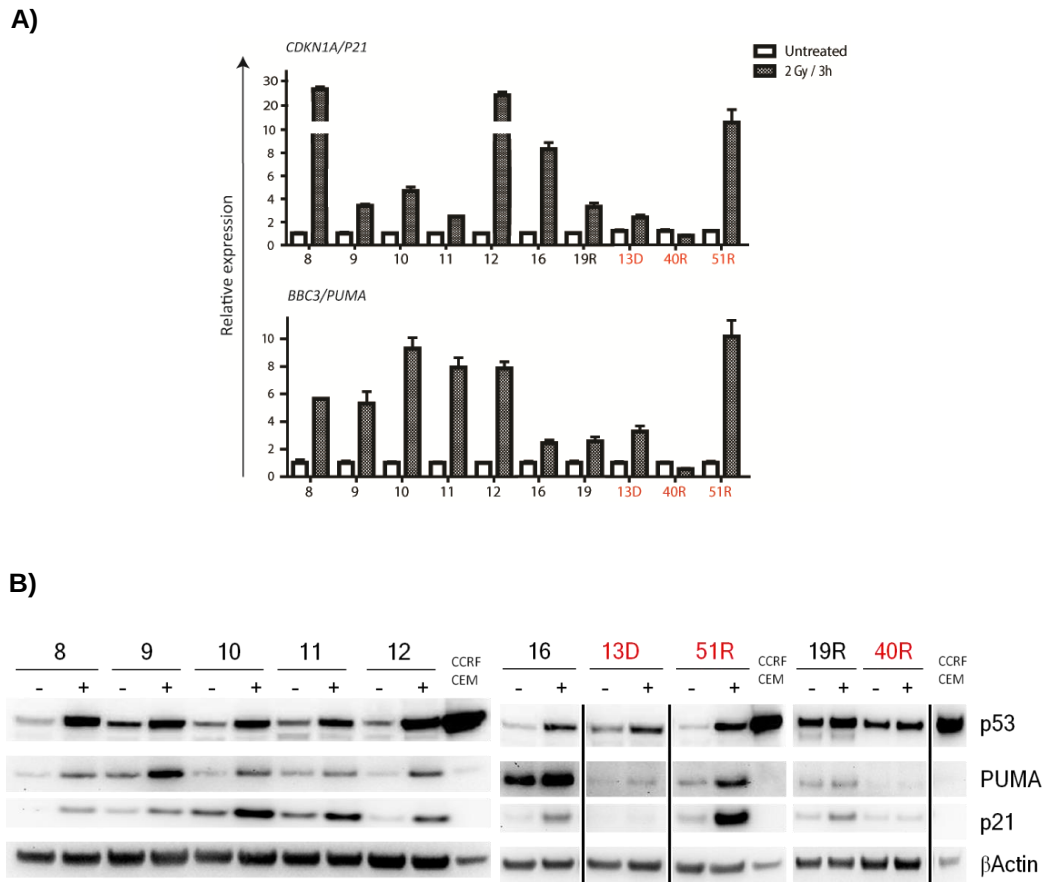


Figure 14. *BBC3/PUMA* and *CDKN1A/p21* genes are the most differentially regulated genes between *WT1* wild type and mutated xenograft samples following DNA damage.

A) Relative expression of *BBC3/PUMA* and *CDKN1A/p21* genes in primary xenografts samples. The *WT1* mutated xenografts are highlighted in red. Analysis was performed after 3h from 2 Gy of γ-radiation. Expression of specific genes for each sample is relative to the corresponding untreated samples. B) Western blot analysis of p53, BBC3/PUMA and CDKN1A/p21 protein expression after 3h hours from 2 Gy γ-radiation. βActin is shown as loading control. The mutated *WT1* xenografts are highlighted in red.

4.8 WT1 directly induces the pro-apoptotic factor BBC3/PUMA

We focused on BBC3/PUMA, a crucial pro-apoptotic factor downstream of p53 in DNA regulatory damage signaling, given that it has not been previously described to be a WT1 target, and some of its regulatory elements were found enriched in our ChIP-chip analysis. We thus determined the effects of *WT1* expression on the activity of a regulatory element obtained from our ChIP-on-chip analysis, located in the *BBC3/PUMA* intron 1-2, using luciferase reporter assays in U2OS cells. To this end, we over-expressed the four full length WT1 isoforms (both KTS+/– and exon 5+/–) and the mutant WT1(E384Stop, exon 5+/–) in U2OS cells (which do not express WT1 protein). These experiments showed that the (KTS−) isoforms were more efficient in inducing *BBC3/PUMA* reporter activity compared to (KTS+) and the mutant isoforms (Figure 15.B). Western blot analysis of WT1 protein expression showed that the full length WT1 isoforms were efficiently expressed following transfection while mutant WT1 proteins were expressed to much lower levels and were clearly detectable only after high exposure (Figure 15.B).

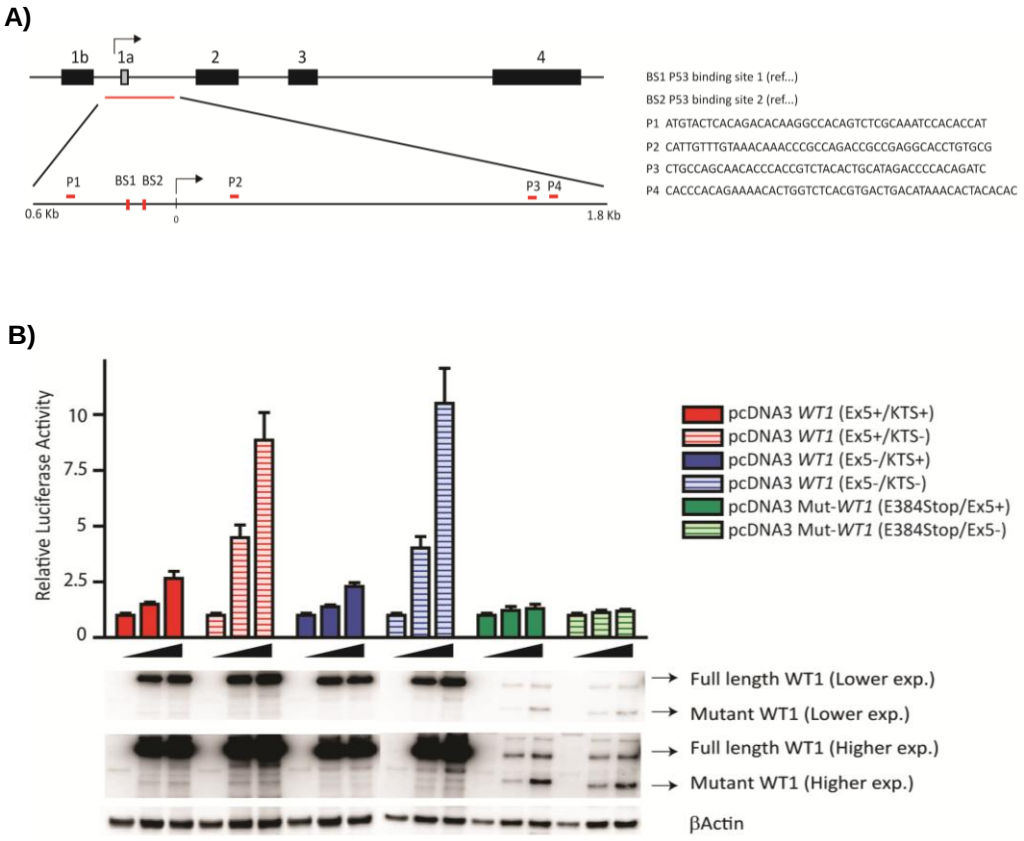


Figure 15. *BBC3/PUMA* is a WT1 target.

A) Schematic representation of WT1 binding sites in the promoter region and intron 1-2 of BBC3 gene. BS1 and BS2 are previously identified p53 binding sites. P1, P2, P3 and P4 are Agilent probes that resulted significantly bound by WT1 in ChIP-chip analysis. The P4 binding site had the best score and it was cloned into the pGL4.23[*luc2*/minP] vector expressing the Luciferase gene under a minimal promoter region. B) Luciferase reporter activity of the *BBC3/PUMA* reporter construct in U2OS cells in response to increasing doses (250 and 500 ng) of full length or mutant WT1 isoforms. Western blot analysis of full length WT1 or mutant WT1 isoform expression is shown. βActin is shown as loading control.

Since *BBC3/PUMA* is a known p53 target, and we found that WT1 is able to directly influence *BBC3/PUMA* reporter activity, we evaluated if WT1 transcriptional activity could be influenced by a functional p53 pathway. We identified that U2OS cells to be p53 proficient following γ-radiation and to lack *WT1* expression (Figure 16.A). We initially screened three *p53* shRNAs to determine which one induced the most efficient sh-*p53*. Silencing was evaluated under basal conditions and after γ-radiation. As shown in Figure 16.A, we found sh-*P53*(19) and sh-*P53*(20) to be the most efficient in knocking down *p53* expression. Subsequently, we performed luciferase assays in U2OS cells stably expressing an irrelevant hairpin (sh-Scramble) or a specific *p53* shRNA (sh-*P53*

(20)) to silence *p53* expression and transfected then with *BBC3/PUMA* reporter construct and WT1(Ex5+/KTS–) construct. We found that the full length WT1 (Ex5+/KTS–) isoform nicely induced *BBC3/PUMA* regulatory element and this induction was significantly augmented after 24h of γ-radiation at 10 Gy (Figure 16.B). On the contrary, the same regulatory element activity was not significantly affected by γ-radiation in U2OS cells transfected with the empty vector. Notably, the activation of the *BBC3/PUMA* regulatory element induced by the full length WT1 isoform (Ex5+/KTS–) in the presence of γ-radiation was significantly reduced in U2OS cells carrying the sh-*P53*(20) hairpin (Figure 16.B). Western blot analysis of U2OS cells transfected either with the empty vector or the vector expressing full length WT1 (Ex5+/KTS–) isoform in the presence or absence of γ-radiation demonstrated stabilization of p53 protein following γ-radiation which was remarkably decreased in cells expressing sh-*P53*(20) (Figure 16.B). Similar results were obtained using U2OS cells expressing sh-*P53*(19). Overall these results suggest that p53 directly or indirectly augments transcriptional activity of WT1 with respect to the pro-apoptotic factor BBC3/PUMA.

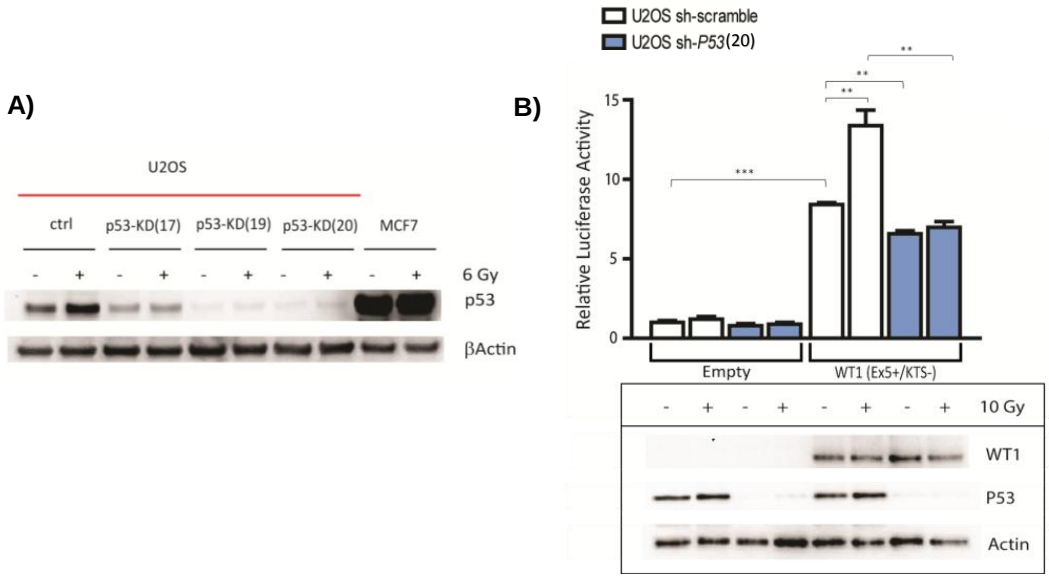


Figure 16. p53 cooperates with WT1 to induce high levels of *BBC3/PUMA* reporter activity.

A) Immuno-blot analysis of p53 induction in U2OS cells wild-type, infected with sh-scramble or three different sh-*P53* ((17),(19),(20)) in the presence or absence of 10 Gy of γ-radiation. As positive control for p53 expression, MCF7 cells were used, since they present a mutant p53 protein. B) Luciferase reporter activity of the *BBC3/PUMA* reporter construct in response to over-expression of the full length WT1(Ex5+/KTS-) isoform in the presence or absence of 10 Gy of γ-radiation in U2OS cells infected with the sh-Scramble and the sh-*P53*(20) vector. Immuno-blot analysis of p53 and WT1 protein expression in infected cells is showed under the graph.

5 . Discussion

T-lineage acute lymphoblastic leukemia (T-ALL) accounts for 10% to 15% of pediatric and 25% of adult ALL cases. The introduction of intensive combination chemotherapy protocols has led to remarkable improvements in survival of this disease; however, chemotherapy is non-specific and highly toxic and patients suffer strong side effects from these treatments. WT1 transcription factor is described to behave both as a tumor suppressor or an oncogene in tumor formation depending on the tumor type. This is supported by the fact that WT1 may act both as an activator and a repressor of the same targets under different cellular conditions. In particular, in leukemia cells WT1 is described both an oncogene and as tumor suppressor. Higher *WT1* mRNA levels in leukemia cells respect to normal bone marrow and progenitor cells support its role as an oncogene and aberrant *WT1* levels in acute leukemia (both under and over-expressed) are reported to be associated with a poor prognosis[175,184-186]. Moreover, *WT1* expression levels are used as a marker to detect minimal residual disease[187] in AML. WT1 is also a suitable candidate for targeted therapy, and has been developed as a target for immunotherapy because of presentation of WT1 peptides on the cell surface[188]. On the contrary, the role of WT1 as tumor suppressor is supported by mutations and deletions in *WT1* locus. In both AML and T-ALL mutations account for 8-12% of patients and are mainly frameshifts located in exon 7 predicted to result in truncated proteins lacking the four zinc fingers that not only carry the major DNA binding portion of the WT1 protein, but also contain the nuclear localization signal and binding domains for interacting proteins predicted to regulate WT1 function, such as p53, and homologous p63 and p73[158].

Studies evaluating the impact of *WT1* mutations in all T-ALL cohorts in both pediatric and adult cohorts failed to detect any significant differences in response to therapy and survival between *WT1* mutated and *WT1* wild type patients even if patients with mutations in *WT1* were doing worse in term of survival than wild type patients[56]. Moreover in AML, *WT1* mutated samples were found to have a poor outcome at least in cytogenetically normal AML patients[178,179]. These clinical findings have suggested that *WT1* mutations may confer a selective advantage in tumor progression or relapse.

Our data support that *WT1* acts as a tumor suppressor gene in T-ALL. Over-expression of full length and mutated WT1 isoforms (exon 5+/– and KTS+/–; E384Stop exon 5+/–) in CCRF-CEM and Jurkat T-ALL cells showed that only the (KTS−) isoforms, which are presumed to be transcriptionally more active, negatively affected growth of T-ALL cells most probably inducing cell cycle arrest. Very low levels of *WT1* mutant expression, as demonstrated by western blot analysis, suggested that truncated WT1 mutant may be unstable and easily targeted for degradation further supporting that WT1 mainly acts as an haplo-insufficient tumor suppressor. Interestingly, using clonogenic assays in soft-agar we demonstrated that CCRF-CEM cells over-expressing the (KTS−) isoforms had an impaired capacity of colony formation compared to the other full length and mutant isoforms. Consistently with our previous results, WT1 loss determined a significant increase in colony formation compared to MOLT4 cells expressing an irrelevant hairpin. The fact that (KTS−) isoforms, which are considered the most efficient in transcriptional regulation, negatively impacted on T-cell growth, supported the hypothesis that loss of *WT1* can significantly affect its transcriptional program leading to tumor progression.

We thus performed gene expression profiling in a loss of function system in MOLT4 cells which express moderate levels of WT1 protein. Data set of genes regulated following WT1 loss were combined with ChIP-chip experiment results performed in MOLT4 cells. This analysis allowed the identification of WT1-regulated targets in T-ALL cells. We obtained more than a hundred WT1 targets that were both directly bound and deregulated by WT1 in our leukemia model. From this analysis we found that there was an enrichment in genes involved in response to cellular stress and in particular in the Mitogen-Activated Protein Kinase (MAPK) pathway, as previously found also by other groups[165,189]. Moreover, genes bound by WT1 were found characteristically down-regulated, strongly suggesting that WT1 primarily functions as a transcriptional activator in T-ALL cells. Interestingly, among these WT1 direct targets, we found *LEF1* ($P=4{,}96\times10^{-18}$), *LMO2* ($P=2{,}30\times10^{-06}$) and *RB1* ($4{,}53\times10^{-5}$) that have been previously involved in the pathogenesis of T-ALL. Importantly, the (KTS−) isoforms were the most efficient in transcriptional regulation as demonstrated for the regulatory regions of specific selected targets (*LST1, LEF1* and *RB1*). As

expected, over-expression of a typical WT1 mutant (E384Stop) failed to induce luciferase activity. Notably, WT1 mutant was not effective in altering the transcriptional activity of the (KTS−) isoform when both were co-expressed in the same cellular context.

ChIP-chip analysis identified about 800 WT1 direct targets that were enriched in in pathways responsible for cellular response to stress, such as p53, nucleotide excision repair and MAPK signalling pathways. This analysis suggested that WT1 may be involved in resistance to DNA damage.

We used MOLT4 cells as a model of p53 proficient T-ALL cell line. Sequencing analysis of *p53* locus showed that MOLT4 cells carry an heterozygous *p53* nonsense mutation (p.R306X). This mutation was not detectable at the cDNA level probably due to nonsense-mediated decay[190]. We demonstrated that specific ablation of *WT1* in MOLT4 cells, induced resistance to DNA damage following γ-radiation and chemotherapy. *WT1* deficiency in MOLT-4 cells significantly increased survival, affecting the expression, as confirmed both at mRNA and protein levels, of crucial members of the Bcl2 family, such as *BBC3*/PUMA and *BAX*. Moreover, the induction of other important genes involved in cell cycle regulation, such as *CDKN1A* and genes involved in DNA repair were affected in MOLT4 that had lower levels of WT1 protein. Importantly, *BBC3/PUMA, CDKN1A, GADD45, CD82* and *LIG1* presented significant WT1 binding sites in their regulatory regions at least in 2 out of three ChIP-chip replicates ($p<0.05$). This suggests that loss of *WT1* may directly affect expression of p53 targets and/or genes involved in DNA damage response and consequently induce resistance to DNA damage. Importantly, increased resistance to DNA damage following *WT1* loss depended on *p53* status. Indeed, *p53* mutated T-ALL cell lines such as PF382 and P12 Ichikawa, were not affected by *WT1* loss. This supports the idea that WT1 may directly affect transcription of genes involved in DNA damage response interacting with a functional p53 protein. The possibility that WT1 and p53 can interact and that this interaction may modulate transcription of their reciprocal targets has been already described elsewhere [167,169,191]. In particular, it was previously shown that the *GADD45A* promoter contains binding sites for WT1 transcription factor and that *GADD45A* promoter activity is augmented by co-expression of p53 and WT1, but not by p53 alone[191]. Here we

found that WT1 may regulate crucial pro-apoptotic Bcl2-like members through a mechanism previously described for *GADD45A*. Notably, our experiments demonstrated for the first time that DNA damage response and p53 stabilization could augment the transcriptional activity of WT1 on *BBC3/PUMA* regulatory element influencing *BBC3/PUMA* expression.

Considering that T-ALL cell lines are generally mutated for *p53* most probably due to *in vitro* culture selection, we extended our analysis to primary human T-ALL xenograft samples that are characterized by a significantly lower incidence of *p53* mutations. We characterized our primary T-ALL xenograft samples for WT1 and *p53* status. Among the 10 xenografts used in our study, 7 were wild-type (PDTALL8, 9, 10, 11, 12, 15, 16, 19R) and 3 were mutated for *WT1* (PDTALL13D, 40R, 51R); samples PDTALL 19R and 16R also presented mutations in *p53* locus (Table 3).

In coherence with previous data in literature[175], we showed that primary T-ALL xenografts carrying mutations in *WT1* locus, express comparable or higher levels of WT1 transcript respect to wild type *WT1* samples. However, mutant *WT1* samples showed a trend towards lower levels of WT1 protein expression respect to the wild type *WT1* xenograft samples, as shown by western blot analysis in Figure 12.B, and the presumed truncated WT1 proteins were not detectable in freshly isolated primary T-ALL xenografts. These findings enforce the hypothesis that *WT1* mutations in T-ALL most probably lead to a haplo-insufficiency rather than to gain of function effects. Cell viability and apoptosis assays showed that PDTALL xenografts could be divided into two subgroup: sensitive (IC50 < 1.5 Gy) and resistant (IC50 > 1.5 Gy) and, among these last ones, all the WT1 mutated xenografts were included. Interestingly, two xenografts, PDTALL19R and 40R were particularly resistant due to mutations in *p53* locus. PDTALL9 and 16 were instead wild type for *p53* gene and showed a regular p53 protein induction after DNA damage, suggesting that their resistance was probably due to other mechanisms. Importantly, the *WT1* mutated sample PDTALL13D was the only T-ALL xenograft that showed, both at the transcript and protein levels, impaired BBC3/PUMA and CDKN2A/P21 induction even in the presence of canonical p53 activation, recapitulating the phenotype described for MOLT4 cells. Interestingly, PDTALL13D belongs to the thymic subgroup according to the

phenotype classification[96]. Notably it was reported that in the standard risk group of thymic T-ALL cases, *WT1* mutated patients had an inferior RFS respect to the *WT1* wild-type patients[175], suggesting that in thymic samples *WT1* mutations may significantly impact on tumor progression. Differently, PDTALL51R is a relapse sample with an early-T/ETP-like phenotype, characteristics that may explain its complexity at both molecular and functional level.

Primary xenograft samples are very interesting models to study the impact of molecular alterations in tumor pathogenesis. However, the functional and molecular interpretation of data generated in primary tumor xenograft models is generally particularly challenging. In our experimental setting, the major problem was represented by the low number of *WT1* mutated T-ALL xenografts mainly due to the moderate incidence of *WT1* mutations in T-ALL. This issue negatively impacted on data management. Another obstacle was represented by the high heterogeneity of primary T-ALL samples, as showed by the immune-phenotypic characterization, leading to difficulties in data interpretation. Finally, T-ALL xenografts derived from primary samples are generally difficult to manipulate *in vitro* with the classical tools used to knockdown or to over-express specific genes, thus impairing data confirmation through rescue experiments. Our study is a first attempt to approach the analysis of WT1 function in T-ALL primary xenografts. Increasing the number of xenografts collected will help to move from hypothesis or speculation to the generation of a cleared picture of WT1 function in T-ALL.

In conclusion, we found that *WT1* loss in T-ALL cells conferred increased resistance to DNA damage, affecting the expression of crucial pro-apoptotic proteins and other important genes involved in cell cycle regulation and DNA repair. These data were in agreement with our ChIP-chip analysis performed on MOLT4 cells, which demonstrated that *WT1* loss altered its transcriptional network, through the modulation of targets enriched in pathways involved in cellular stress response. In particular, the p53 pathway was significantly affected, suggesting a functional WT1-p53 interaction in the transcription of important cell cycle and apoptosis regulators after DNA damage, such as *BBC3/PUMA* gene. Thus, understanding the cellular mechanisms of resistance will be critical to the successful treatment for T-ALL, opening numerous possibilities of future

therapeutic intervention, especially in patients who relapse or become refractory to traditional chemotherapeutic treatment regimens.

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