The role of Wilms' Tumor gene product (WT1)

in CD95-mediated apoptosis

in T-cell leukaemias

Dissertation

submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

of the

Ruperto-Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

BSc-Genetics/MSc-Human Genetics

Konstantina Bourkoula

Born in: Kalamata, Greece

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Referees: Prof. Dr. Krammer P.H.

              Prof. Dr. Angel P.

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Abstract

Apoptosis, also known as programmed cell death, is a very critical process for the maintenance of tissue homeostasis in multicellular organisms. In our immune system, which is comprised of different cell types with different but interconnected functions, apoptosis plays a crucial role in preserving homeostasis during development, throughout adult life and upon termination of immune responses. Any defects in apoptosis can potentially lead to autoimmunity or cancer.

CD95 (Fas/APO-1) belongs to the family of the so-called death receptors, and upon binding with its ligand, the CD95L, can instruct CD95-bearing cells to undergo apoptosis. The CD95/CD95L system is crucial for homeostasis of the immune system, and deregulation of CD95-mediated apoptosis results in several diseases including autoimmune syndromes, leukaemias or AIDS.

CD95-mediated apoptosis is used by the T-lymphocytes of the immune system at several stages during their development and after the termination of an immune response, so that the quality and quantity of activated T cells are controlled. In these cases, the T cells being activated through their TCR die via CD95-mediated apoptosis upon restimulation, a process collectively known as activation-induced cell death (AICD).

One characteristic of cancer is its resistance to apoptosis. Leukaemias are no exception. One of the ways a cancerous T-lymphocyte evades death is by deregulating its CD95/CD95L system by e.g., downregulating the CD95L. NF-AT and Egr proteins (1-3) are amongst the different transcription factors that take part in the regulation of the CD95L. Upon TCR stimulation different signalling pathways lead to the activation of NF-AT and the transcriptional activation of Egr factors. These then bind on the CD95L promoter and activate it.

Wilms' tumor gene 1 (*WT1*) encodes a four-zinc-finger protein. Alternative splicing at the pre-mRNA level yields several transcript variants, of which the best characterized are the so called WT1-KTS and WT1+KTS isoforms. The WT1-KTS isoform, which lacks three amino acids between zinc-fingers three and four, is suggested to be a transcription factor that belongs to the Egr family and has target sequences similar to Egr1.

WT1 is expressed during the early stages of haematopoiesis and has been implicated in leukaemogenesis. Its role in leukaemias is controversial as it is either found to be mutated or overexpressed, and overexpression is linked with more immature leukaemias. It is therefore uncertain whether WT1 is a tumor suppressor or tumor promoter.

In this study the expression of WT1 is analyzed in eight leukaemic T-cell lines (Jurkat 16, Jurkat 282, CEM, Myla, Hut78, SeAx, Molt4 and HH) and in primary T cells. It is shown that WT1 is not expressed in mature T cells. Also, contrary to previous reports that support overexpression of WT1 in Jurkat and Molt4, no expression of WT1 is seen in these cell lines in this study. Expression of WT1 is detected in only two of the leukaemic cell lines (CEM and Hut78). In the latter, no mutations are detected in WT1. The expression of WT1 coincides with CD95L expression and high AICD in these cell lines.

Moreover, ectopic expression of WT1-KTS in a WT1-non-expressing cell line upregulates the CD95L and enhances AICD. Silencing of WT1-KTS in a WT1-expressing cell-line reduces CD95L transcription and dramatically decreases the levels of AICD. Examination of three putative WT1 binding sites on the CD95L promoter (Egr sites) indicates that WT1-KTS acts through the CD95L promoter Egr sites. Mutations of these sites completely abolish the effect of WT1-KTS. Furthermore, a detailed analysis of individual binding sites and electrophoretic mobility shift assays reveal that WT-KTS can directly bind on the CD95L promoter at position -120, relative to the transcription start site (+1).

Taken together, the results of this piece of work disagree with previous reports on the overexpression status of WT1 in the leukaemic T cell lines Jurkat and Molt4. Expression of full-length WT1 is only supported for the cell lines CEM and Hut78. In agreement with previous work, WT1 is not expressed in healthy mature T cells. This work also supports the transcription factor function of WT1-KTS, as it can regulate CD95L transcription. Finally, it shows for the first time the ability of WT1-KTS to bind on the CD95L promoter and upregulate the CD95L upon T cell activation in leukaemic T-cell lines.

The correlation for the need of WT1 during haematopoiesis, its loss in leukaemias and its ability to cause CD95L-mediated apoptosis in the leukaemic cell lines that express it, support a possible function for WT1 as a tumor suppressor in the course of leukaemogenesis.

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I Introduction

1. Apoptosis - Programmed cell death

During development and in adult life a vast number of cells are known to die in many different tissues in both vertebrates and invertebrates. Naturally occurring cell death has been investigated since the nineteenth century, highlighting its importance and until nowadays a significant research effort has been directed towards unravelling the role of cell death, the signals that regulate it, and the mechanisms by which cell destruction is actually achieved. Although cell death was probably already considered by Aristotle for the regression of the ductus arteriosus after birth (Barclay *et al*, 1944), it was not until 1842, when the 'cell theory' was established (Schwann, 1839) and cells could be visualized under a microscope, that Vogt could actually detect the disappearance of cells while he was studying the development of the midwife toad (*Alytes obstetricians*) (Vogt, 1842). Gradually, evidence for the existence of naturally occurring cell death accumulated (Clarke & Clarke, 1996), and it was subsequently realized that there is not only one way a cell can die (Lockshin & Williams, 1965). The roots of the morphological distinctions between different cell death types go back to 1972 when the term 'apoptosis' was also introduced (Kerr *et al*, 1972). It is now known that the cell death that Vogt saw is what is called 'programmed cell death' or apoptosis and along with necrosis, they comprise the best characterized ways of cell death. Interestingly enough, there is no precise definition for either process and distinction between the two types is based on morphological features and the cause of death.

Necrosis is generally considered as a passive process and takes place upon physical or chemical injury such as oxygen deprivation in the heart muscle during a heart attack (Roeske *et al*, 1977). In necrosis, the damaged cell enlarges with consequent plasma membrane

disruption, followed by the release of cytosolic components in the extracellular space, an event that causes inflammation (Proskuryakov *et al*, 2003). Contrary to necrosis, apoptosis is thought to be an active genetically encoded process. The apoptotic process is initiated by several stimuli including growth factor deprivation, UV- or γ- irradiation (Kasibhatla *et al*, 1998), chemotherapeutic agents (Friesen *et al*, 1999; Eichhorst *et al*, 2000) and a family of transmembrane proteins the so called 'death receptors' upon engagement with their appropriate ligand (Li-Weber & Krammer, 2003). After initiation, an executionary phase follows during which the apoptotic cell shrinks, the plasma membrane inverts exposing phosphatidylserine, blebbing (zeiosis) occurs, the nucleus is fragmented, chromatin condenses and eventually endonucleases produced by the dying cell cleave the DNA between nucleosomes (Garcia-Martinez *et al*, 1993; Nagata *et al*, 2003). In this way the DNA is fragmented into 200 bp pieces, which upon electrophoresis produce the characteristic 'DNA ladder' found in apoptotic cells (Wyllie *et al*, 1980; Montague & Cidlowski, 1996). Finally, the apoptotic process is terminated by the appearance of 'apoptotic bodies', which are membrane-enclosed vesicles containing the remainders of the cell and are engulfed by phagocytic cells, hence preventing inflammation (Krammer, 1999). Apoptosis is crucial for tissue homeostasis and remodelling during development, in the nervous system and in the immune system (Krammer, 1999; Vaux & Korsmeyer, 1999; Arnold *et al*, 2006)

2. T-lymphocyte apoptosis and activation-induced cell death (AICD)

2.1 T-lymphocytes

The human immune system is a community that consists of different cell types, namely T- and B- lymphocytes, natural killer (NK) cells, macrophages, antigen-presenting cells (APCs) and their various subclasses, which have to work together, interact and communicate with one another in order for the system to function properly and the organism to remain healthy. The

T-lymphocytes (T cells) develop in the bone marrow from haematopoietic stem cells into immature T cells. They then emigrate from the bone marrow into the thymus, where they mature and are positively or negatively selected, depending on the affinity of their T-cell antigen receptors (TCR) for self major histocompatibility complex (MHC) antigens class I and II (Sebzda *et al*, 1999). The TCR is a multiprotein complex made up of clonally variable antigen-binding chains (heavy TCRα and light TCRβ immunoglobulin chains) that are associated with invariant accessory proteins, which are collectively called CD3 (Call & Wucherpfennig, 2005). Signaling from the TCR requires not only the presence of CD3 but also the help of a co-receptor namely CD4 or CD8. T cells that interact with MHC class II molecules develop into T cells that express CD4 on their surface (CD4$^+$) and T cells that have high affinity for MHC class I express CD8 on their surface (CD8$^+$) (Basson & Zamoyska, 2000; Basson & Zamoyska, 2001). Only the mature T cells that have a functional TCR are allowed to leave the thymus and home secondary lymphoid organs such as the spleen and lymph nodes. Mature CD4$^+$ T cells function as helper T cells, and by secreting cytokines they can either regulate cellular immune responses [T helper1 (Th1) cells] or antibody responses [T helper 2 (Th2) cells] (Jankovic *et al*, 2001). Mature CD8$^+$ T cells function as cytotoxic effector cells (T effector/T killer) (Sebzda *et al*, 1999).

When the organism is threatened by an infectious agent, APCs present antigen-specific peptides from the infectious agent under inspection to the T cells (Drakesmith *et al*, 2000). Communication between APCs and T cells through antigen-MHC complexes, co-stimulatory cell-surface molecules and cytokine production, activates T cells. Activated T cells then proliferate by clonal expansion, and in their own turn contact other T cells (T killer cells) or B cells to instruct them so that the infectious agent is quickly and effectively eliminated with as few casualties as possible.

2.2 T-lymphocyte death

Death of T cells occurs via apoptosis and is destined to control the quality of the T cells so as to prevent autoreactive events that could lead to autoimmunity. In addition, apoptosis functions to control the quantity of the T cells especially after an immune response so that homeostasis is maintained and both autoimmunity and leukaemias are prevented. After having entered the thymus, immature T cells undergo rearrangement of their TCR genes in favour of their differentiation. Successful TCR rearrangement leads to further maturation and TCR-affinity based positive and negative selection of the T cells (Surh & Sprent, 1994). Failure of proper TCR rearrangement leads to the inability of stimulation by self-MHC-peptide complexes and these T cells die by neglect. Only T cells that express a TCR that can interact with self-MHC are positively selected and rescued (Marrack & Kappler, 1997). T cells that express self-reactive antigen receptors that bind to self-MHC peptide complexes with high affinity are negatively selected and are eliminated by apoptosis (Nossal, 1994). These check points during T-lymphocyte development ensure that self-MHC restriction and self-tolerance are successfully accomplished before the T cells finally get to the periphery (Kishimoto & Sprent, 2000a; Kishimoto & Sprent, 2000b). Therefore, only 2-4% of the T cells that develop in the thymus will finally leave the thymus as mature T cells (Chen *et al*, 1983). In the periphery, if a T cell can not be efficiently stimulated by growth signals it also dies by neglect (Nelson & Willerford, 1998).

2.3 Activation-induced cell death

In the periphery, upon infection, resting mature T cells are activated by antigens via their TCR and the adaptive immune response is initiated. In order for the immune effect to be augmented activated T cells undergo clonal expansion and their numbers increase dramatically. However, after the clearance of infection, such high numbers are not needed any more and activated T cells die by apoptosis. Only a small fraction of antigen-specific T

cells survive as long lived memory T cells (Sprent & Surh, 2002). This results in a drop in the numbers of activated T cells so that homeostasis is maintained (Fig.I.1).

Figure I.1. Graph showing the course of a T cell immune response and the apoptosis phenotype for each phase (Krammer, 1999).
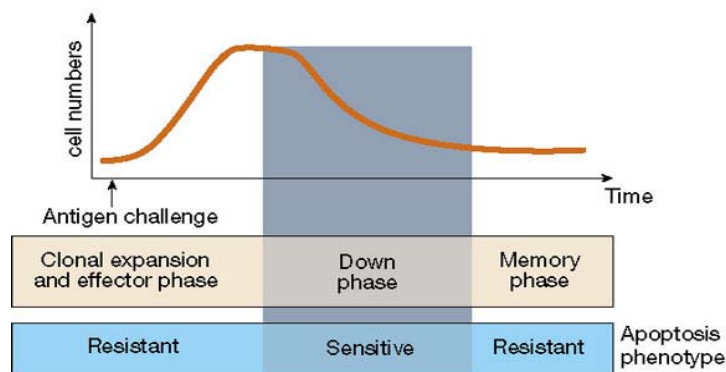
There are currently two mechanisms by which apoptosis of pre-activated T cells takes place in the periphery. One is 'activated T cell autonomous death' (ACAD) caused by cytokine withdrawal (Lenardo *et al*, 1999; Hildeman *et al*, 2002). The other one is called activation induced cell death (AICD) (Shi *et al*, 1989) and has the special characteristic that it requires the involvement of TCR-stimulation induced pathways of death receptors and death ligands. The death receptors belong to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily. They are type I transmembrane receptors, characterized by the presence of two to five cysteine-rich extracellular repeats that are important for ligand binding (Orlinick *et al*, 1997a & 1997b) and an intracellular 80 amino-acid long death domain (DD), which is necessary for the transduction of the apoptotic signal (Itoh & Nagata, 1993).

The receptors recognized so far include TNF-R1 (CD120a), CD95 (APO-1/Fas), DR3 (APO-3, LARD, TRAMP, WSL1), TNF-related apoptosis-inducing ligand (TRAIL)-R1 (APO-2, DR4), TRAIL-R2 (DR5, KILLER, TRICK2), and DR6 (Schulze-Osthoff *et al*, 1998). The

Ectodysplasin A receptor (EDAR) and the nerve growth factor receptor (NGFR) are also sometimes referred to as death receptors (French & Tschopp, 2003; Wajant, 2003). Death receptors are activated by their ligands that have co-evolved as a death ligand family. Among the death receptors, the best characterized system for AICD induction is the CD95 and its ligand the CD95Ligand (CD95L) (Dhein *et al*, 1995; Ju *et al*, 1995; Alderson *et al*, 1995).

3. The CD95/CD95L system

3.1 CD95/CD95L in apoptosis signalling

The apoptosis pathway via the CD95 system is initiated when CD95 binds to its ligand. This can occur in an autocrine manner, where the cell produces its own CD95L which can then bind to CD95 located on the same cell's surface leading to the cell committing suicide. Another possibility is that it can take place in a fratricide/paracrine fashion, where one cell produces CD95L that binds to the CD95 on a neighbouring cell's surface and hence instructs that cell to die (fratricide) (Krammer, 2000) (Fig.I.2).

Figure I.2. Schematic diagram showing the different modes of AICD
Upon TCR stimulation with antigen-specific MHC complex, the CD95-bearing T cell gets activated and produces CD95L. CD95L can either bind on CD95 of a neighbouring T cell passing on the death signal (1), or it can be recognized by self-receptor leading to the suicide death of the T cell (2), or it can be passed on a CD95-bearing cell further away and cause activation of the death pathway (3).
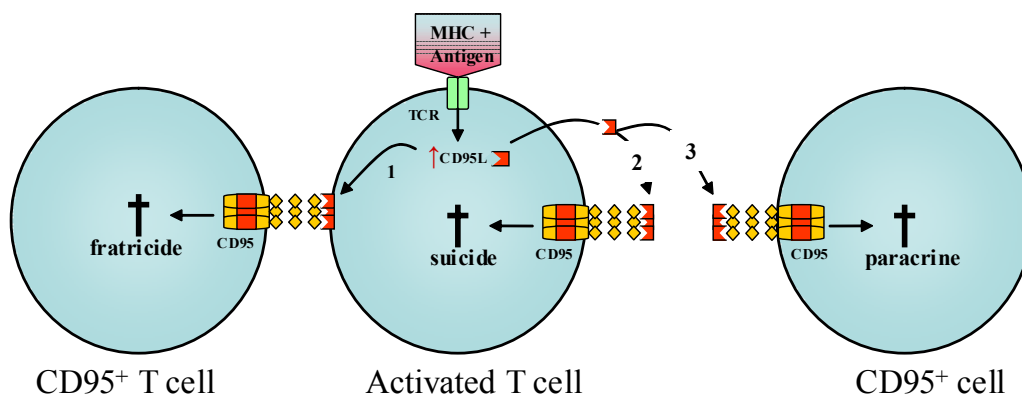
In any case, binding of CD95L to CD95 triggers the formation of the death-inducing signalling complex (DISC) (Kischkel *et al*, 1995), from which the apoptotic signals emanate (Fig.I.3). The exact stoichiometry of the DISC is not fully understood but the protein interactions that take place through homotypic protein domains are well characterized. The CD95 DISC is composed of oligomerized, most probably trimerized CD95, which through its DD recruits the serine phosphorylated adapter Fas-associated DD (FADD)/Mort1 (Chinnaiyan *et al*, 1996). FADD, which also has a death-effector domain (DED), in turn reacts with the DED of two isoforms of caspase-8 [caspase-8/a (FADD-like interkeukin-1β (IL-1β)-converting enzyme (FLICE, Mach-α1) and caspase-8/b (Mach-α2)] (Muzio *et al*, 1996; Boldin *et al*, 1996; Golks *et al*, 2006), caspase 10 (Sprick *et al*, 2002) and c-FLIP$_{L/S/R}$ (Thome & Tschopp, 2001; Golks *et al*, 2005).

Caspases are aspartate-specific cysteine proteases that are produced in a pro-enzyme (zymogene) form, which release active enzyme after proteolytic cleavage. As such, binding of caspase-8 and -10 to FADD leads to their activation by autoproteolysis (Martin *et al*, 1998). The active caspase works as a heterotetramer containing two large subunits with the active site (catalytic domain) and two small subunits (prodomain) (Earnshaw *et al*, 1999). After activation of caspase-8, the prodomain remains at the DISC and the active caspase-8 heterotetramer $p10_2$-$p18_2$ dissociates from the DISC to initiate a cascade of activation of other caspases, an event that marks the executionary phase of apoptosis (Lavrik *et al*, 2003; Golks *et al*, 2006).

Two different pathways for CD95-mediated apoptosis have been described, the mitochondrial-dependent and mitochondrial–independent pathways, corresponding to the quantity of DISC formation and caspase-8 activation. Accordingly, the cells can be categorized by the pathway they follow in type I (mitochondrial-independent) and type II

(mitochondrial-dependent) cells (Scaffidi *et al*, 1998). In type I cells, high amounts of active caspase-8 are produced at the DISC, which can process the effector caspase-3. Active caspase-3 in turn activates caspases -6 and -7, whose activation is responsible for the cleavage of several cellular substrates. This finally leads to morphological changes of the cell and to DNA fragmentation. In type II cells, DISC formation is low and as a result little active caspase-8 is produced. In these cells apoptosis induction depends on the activation of the pro-apoptotic molecule BID, which is a Bcl-2 homology domain-3 (BH3) containing Bcl-2 family member (Luo *et al*, 1998). When BID is activated by cleavage, the active form, tBID ('t' for truncated) is produced, which migrates to the mitochondria, where it sequesters and co-operates with Bax and Bak proteins. This protein aggregation is thought to result in the loss of the mitochondrial membrane potential ($\Delta\Psi$m) and the release of pro-apoptotic molecules like cytochrome *c* (Korsmeyer *et al*, 2000). Cytochrome *c* associates with the apoptotic protease-activating factor-1 (Apaf-1), deoxyadenosine triphosphate and caspase-9 forming a large protein complex, the apoptosome (Li *et al*, 1997). The apoptosome can then activate caspase-9, which in turn activates caspase-3 and -7 and finally apoptosis via the mitochondrial pathway can be brought to completion.

T cells that are not activated or memory T cells are resistant to apoptosis and behave like type II cells, forming CD95 DISC in smaller amounts. In type II cells apoptosis can be blocked at the DISC level via cellular FLICE inhibitory protein (c-FLIP$_{L/S/R}$) (Schmitz *et al*, 2004) and also at the mitochondrial level by upregulation of the anti-apoptotic Bcl-2 family members; Bcl-2 and Bcl-x$_L$ (Peter *et al*, 1997). T cells which are sensitive towards apoptosis, like the T cells at the termination of an immune response, behave like type I cells, since they form a lot of CD95 DISC complexes which can activate a strong caspase cascade independent of the mitochondria. Therefore, it has been suggested that T cells after activation switch from

apoptosis resistant type II phenotype to apoptosis sensitive type I phenotype (Krueger *et al*,

2003).

Figure I.3. Signalling pathways induced by CD95. CD95 signalling pathway (including DISC formation) used in type I and type II cells. DISC formation leads to caspase-8 activation. In type I cells the extrinsic apoptosis pathway is followed (shown in yellow). Active caspase-8 directly cleaves caspase-3, which in turn activates other effector caspases. The effector caspases-6 and -7 along with caspase-3 cleave other protein targets an event that finally leads to death. In type II cells (shown in light green) the intrinsic apoptosis pathway is followed where the cascade is amplified through the mitochondria. Truncated Bid (tBid) mediates cytochrome c release from the mitochondria leading to the formation of the apoptosome, in which caspase-9 is activated. Active caspase-9 in turn activates caspase-3 and other effector caspases, finally causing death. The caspase activation is inhibited at the DISC level by c-FLIP proteins, at the cytoplasmic level by IAPs and at the mitochondrial level by Bcl-2 proteins.
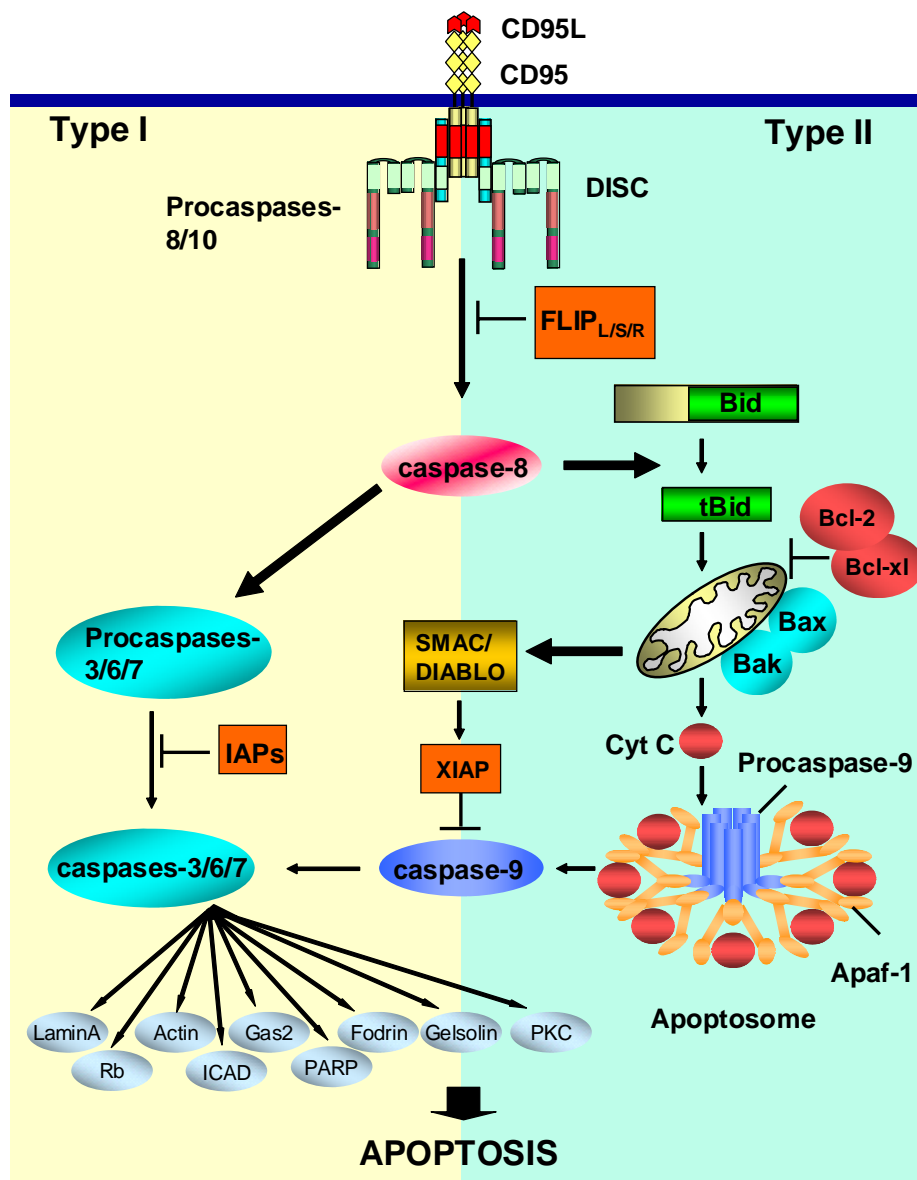
3.2 CD95/CD95L in disease

The CD95/CD95L system plays a major role in apoptosis induction and more particularly in AICD of T cells. Any malfunctions in this system can prove to be detrimental for the fate of T cells and subsequently for the organism. This is best understood when describing the phenotype of *lpr* (for lymphoproliferation) mice that lack a functional CD95, as well as *gld* (for generalized lymphoproliferative disease) mice that bear a mutant CD95L, which exhibit various autoimmune phenomena resembling systemic lupus erythematosus in humans. Both mouse strains produce autoantibodies and accumulate abnormal CD4$^-$ CD8$^-$ T cells leading to lymphadenopathy, splenomegaly, and other autoimmune symptoms (Cohen & Eisenberg, 1991; Nagata & Suda, 1995).

In humans, CD95 and CD95L mutations are found in autoimmune lymphoproliferative syndrome (ALPS) patients. ALPS type Ia patients carry mostly heterozygous dominant negative mutations in CD95 and ALPs type Ib patients have mutations in CD95L. These patients display lymphadenopathy and splenomegaly due to impaired apoptosis (Rieux-Laucat *et al*, 1995; Fisher *et al*, 1995). Deregulated expression of CD95 and CD95L is also linked with systemic lupus erythematosus (SLE) (Kovacs *et al*, 1997) and CD95L has been shown to be mutated in a patient with SLE (Wu *et al*, 1996). In addition, the involvement of CD95 and its ligand in AIDS has been hypothesized, as the levels of CD95 and CD95L have been shown to correlate with disease stage (Bahr *et al*, 1997; Bohler *et al*, 1997). Also, the HIV protein TAT was recently shown to induce CD95L expression and might play a role in T cell loss during AIDS disease progression (Li-Weber *et al*, 2000; Gulow *et al*, 2005). Except for CD95 itself, another equally important player in the system is inevitably its ligand, as when there is CD95L there is no initiation for apoptotic signalling in AICD. Therefore, the ability of a T cell to express CD95L determines its ability to commit suicide upon AICD and/or to kill neighbouring CD95-bearing cells.

4. The CD95L and its transcriptional regulation

CD95L is a 40kDa glycoprotein with a cytosolic N-terminus and an extracellularly oriented C-terminus. Membrane-bound CD95L can be cleaved by metalloproteases producing a soluble form of CD95L (Mariani *et al*, 1995) but the functional differences between the two forms are not very well characterized (Fig.I.2). Although CD95 is widely expressed, CD95L expression is very restricted. It is expressed on activated T cells (Suda *et al*, 1995), NK cells (Oshimi *et al*, 1996), at immune-privileged sites of the corneal epithelium and the retina of the eye (Griffith *et al*, 1995), on Sertoli cells of the testis (Bellgrau *et al*, 1995) and on certain tumors (Hahne *et al*, 1996; O'Connell *et al*, 1999). It is important for AICD of mature T cells and it is also suggested that it is involved in the apoptosis of T cells during their development in the thymus (Ogasawara *et al*, 1995; Castro *et al*, 1996; Fisher *et al*, 1996). Deregulation in CD95L expression can be detrimental and constitutive expression has been linked with lymphoproliferative disorders in animal studies (Watanabe *et al*, 1995)

The pathway from the TCR that leads to CD95L upregulation is summarized in Fig.I.4 and it can be pointed out, that chemotherapeutic drugs, oxidative stress and UV or γ- irradiation can also cause CD95L upregulation. Depending on the stimulus, different or sometimes overlapping pathways are followed that lead to the activation/transcriptional activation/assembly of specific transcription factors, which can eventually bind on their target sequences on the promoter of CD95L and hence activate its expression. Transcriptional activation of the *CD95L* gene in T cells occurs rapidly. CD95L mRNA can be detected as early as 1h following anti-CD3/anti-CD28 or phorbol myristate acetate (PMA)/ionomycin stimulation and reaches a maximum after 4h (Li-Weber *et al*, 2002). The CD95L promoter spans 960 bp and regulatory factors have been shown to bind from -860 to +100 relative to the transcription start site (+1) (Table I.1) (Li-Weber & Krammer, 2003).

Figure I.4. Main signalling pathways leading to CD95L upregulation. Schematic representation of the different stimuli and the pathways they stimulate for CD95L mRNA production. Each signalling cascade results in the production of active transcription factors targeting the CD95L promoter. Signals from the TCR, activate NF-AT and NF-κB transcription factors and in turn NF-AT upregulates Egr, which can bind on the CD95L promoter. TCR signalling also goes through the Ras pathway, which produces Jun/Fos dimers (AP-1). Cancer drug treatment, PMA, UV and Hydrogen peroxide affect NF-κB production and UV/stress can also act through MEK for AP-1 production.
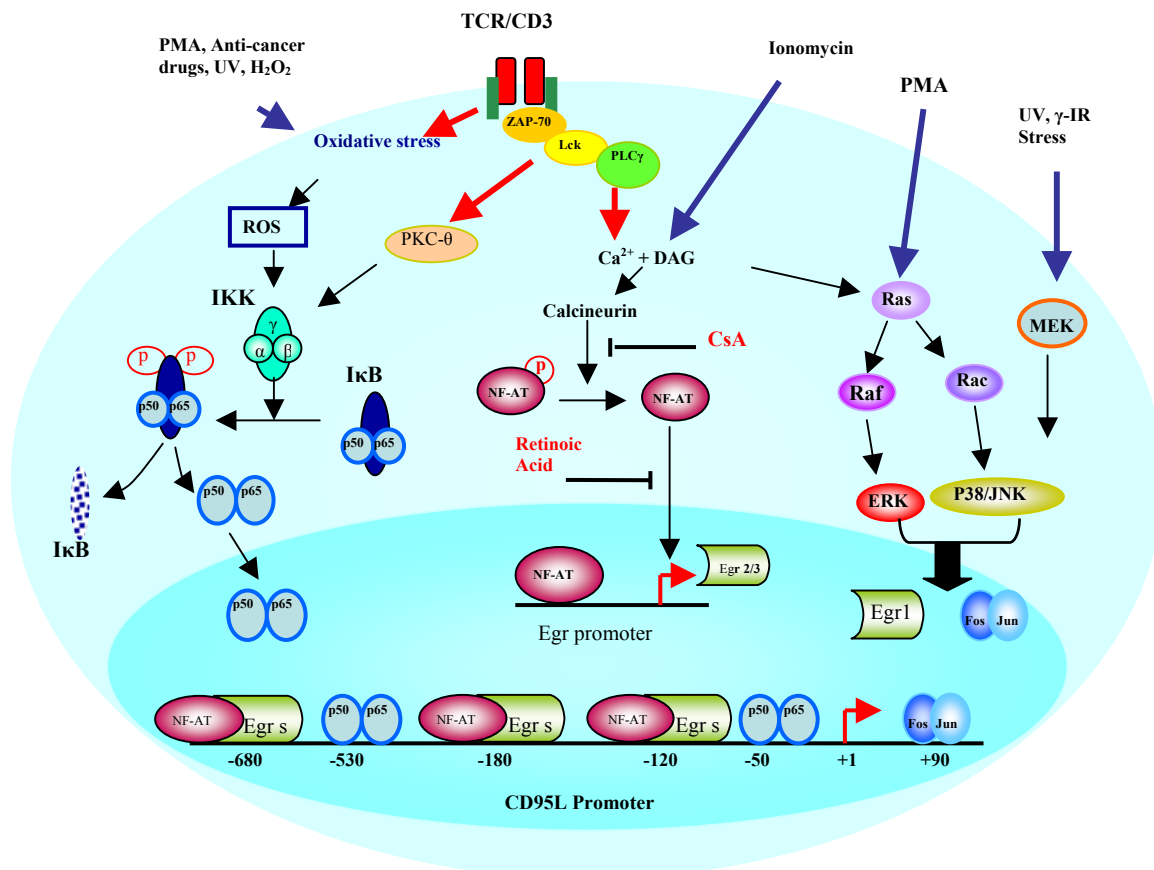
Table I.1. Transcription factors (TF) and their target sequences on the human CD95L promoter in T cells

CD95L promoter	TF	Binding sites	Reference
+90	AP-1 (Jun/Fos)	CCTGACTCACC	Eichhorst *et al*, 2000
+65	IRF-1/vIRFs	GAGAAGAAGTAAACCGTTTG	Kirchhoff *et al*, 2002
+1			
-30	c-Myc	TGGAAACTCTATAAGAGAGATCC	Kasibhatla *et al*, 2000
	NF-AT	TGGAAACTCTATAAGAGAGATCC	Latinis *et al*, 1997
-50	NF-κB/SP-1	GAGGTGTTTCCCTT	Li-Weber *et al*, 2000
-65	IRF-1/vIRFs	AGAGAAAGAGAAAGACAGAGG	Kirchhoff *et al*, 2002
-120	Egr/SP-1	GCTGCAAAGTGAGTGGGTGTTTCTTTG	Mittelstadt & Ashwell, 1998;1999
	NF-AT	GCTGCAAAGTGAGTGGGTGTTTCTTTG	Li-Weber *et al*, 1998; 1999
	IRF-1/vIRFs	GCTGCAAAGTGAGTGGGTGTTTCTTTG	Chow *et al*, 2000
-180	NF-AT	ATTGTGGGCGGAAACTTCC	Holtz-Heppelmann *et al*, 1998
	Egr/SP-1	ATTGTGGGCGGAAACTTCC	Li-Weber *et al*, 1999
-230	Jun/ATF2	TTGGGTAGCACAGCGA	Faris *et al*, 1998a and 1998b
-530	NF-κB/SP-1	ATGGTCTCTCCCCTC	Li-Weber *et al*, 2000
-680	Egr/SP-1	TTTCTAAAGTGGGTGTAGCAGGTTTTTAAC	Li-Weber *et al*, 1999
	NF-AT	TTTCTAAAGTGGGTGTAGCAGGTTTTTAAC	
-980	NF-κB	ATAG A/C AAGTCCCCA	Kasibhatla *et al*, 1999

(TATA box)

One of the major interactions that take place on the CD95L promoter is mediated by the nuclear factor of activated T cells (NF-AT) and early growth response (Egr1-4) proteins. Three binding sites for NF-AT have been identified on the CD95L promoter at positions -120, -180 and -680 relative to transcription start site (+1) (Latinis *et al* 1997; Holtz-Heppelmann *et al*, 1998; Li-Weber *et al*, 1998). Also, it has been shown that both -120 and -180 are composite Egr/NF-AT binding sites where both NF-AT and Egr1/2/3 proteins can bind (Li-

Weber *et al*, 1999). Interestingly, Egr2 and Egr3 are NF-AT targets themselves (Mittelstadt & Ashwell, 1998; 1999). Moreover, another composite Egr/NF-AT site at -680 is now known to be recognized by both NF-AT and Egr1/Egr3 proteins (Li-Weber *et al*, 1999). One transcription factor that belongs to the Egr family and shares a great degree of homology with Egr1 is the Wilms' tumor 1 protein (WT1). WT1 has been suggested to be required during haematopoiesis, and aberrant expression or mutations have been linked to impaired apoptosis and leukaemogenesis.

5. The Wilms' Tumor gene (*WT1*)

The Wilms' tumor gene was first identified by positional cloning and sequencing in patients with Wilms' tumor (Call *et al*, 1990; Gessler *et al*, 1990). Wilms' tumor is a paediatric kidney malignancy, first described by Max Wilms in 1899, affecting 1 in 10,000 children below the age of 5 (Matsunaga, 1981). Wilms' tumor is thought to arise through the uncontrolled proliferation of undifferentiated mesenchyma cells initially destined to become glomerular podocytes, which form the filtration barrier within the glomerolus (Beckwith, 1998). Wilms' tumor is often observed in association with several rare malformation syndromes, of which most common are the WAGR syndrome (Wilms' tumor, Aniridia, Genitourinary abnormalities and mental Retardation) (Pendergrass, 1976), the Denys-Drash syndrome (diffuse mesangial sclerosis, male pseudohermaphroditism and Wilms' tumor) (Pelletier *et al*, 1991) and the Beckwith-Wiedemann syndrome (gigantism, macroglossia, omphalocele, hyperinsulinism and predisposition to several tumors like Wilms' tumor) (Henry *et al*, 1991).

The *WT1* gene is located at 11p13, spans 50kb and is comprised of ten exons (Call *et al*, 1990; Gessler *et al*, 1990). Its expression is highest during embryogenesis, where it is found in

multipotent progenitor cells of a restricted range of cells and tissues, mainly the genitourinary system (Pritchard-Jones *et al*, 1990; van Heyningen *et al*, 1990), the epicardium and subepicardial mesenchyme (Moore *et al*, 1999), the neurons (Wagner *et al*, 2002) and at early stages of haematopoiesis (Ellisen *et al*, 2001). In adults, expression continues in specific cell types of the kidney and gonad, and in CD34$^+$ progenitor cells of the bone marrow (Inoue *et al*, 1997). Mature human resting or activated T cells do not produce WT1. The important role of WT1 during development is realized once WT1 is homozygously knocked out. WT1 null mice die *in utero* at embryonic day 13 and they display complete agenesis of the kidneys, gonad, spleen and adrenal glands (Kreidberg *et al*, 1993; Herzer *et al*, 1999).

A very interesting aspect of *WT1* is that its 10 exons can undergo pre-mRNA splicing in several ways. Also, other alternative isoforms are derived from the use of an upstream and in frame CTG start codon, an internal ATG start codon at the end of exon 1 and a residue in exon 6 which is subject to RNA editing (Bruening & Pelletier, 1996; Scharnhorst *et al*, 1999; Discenza & Pelletier, 2004). Moreover, an alternative promoter within intron 1 was recently identified, which gives rise to the *AWT1* transcript (Dallosso *et al*, 2004). This transcript is paternally imprinted and has overlapping pre-mRNA splicing variants with *WT1*. All these splicing and translational events overall yield a great amount of splicing isoforms, with 36 identified at the time of printing (Hohenstein & Hastie, 2006).

6. Wilms' Tumor-The Protein

The function of the majority of these transcript variants is so far unknown, and different variants are produced in different cell types and tissues. However, four major splice variants are always encountered in almost all cells where WT1 is expressed. These arise from differential splicing of two splicing sites. The splicing site I is mammal-specific and involves

the whole of exon 5, which encodes a stretch of 17 amino-acids. The splicing site II involves

the last three codons of exon 9, which encode for the three amino-acids lysine, threonine and

serine (KTS) (Haber *et al*, 1991). Overall these splicing events give rise to four splicing

variants (Fig.I.5), which are attributed different but questionable cellular functions.

Figure I.5. Schematic representation of the WT1 mRNA. The mRNA of WT1 before
splicing is depicted on the upper part. The bottom part contains the resulting transcripts after
differential splicing of two major splicing sites, one including or excluding exon5 and one
including or excluding the last three codons of exon 9 (KTS) (bottom). Exons are shown in
blue blocks and splicing sites in green.
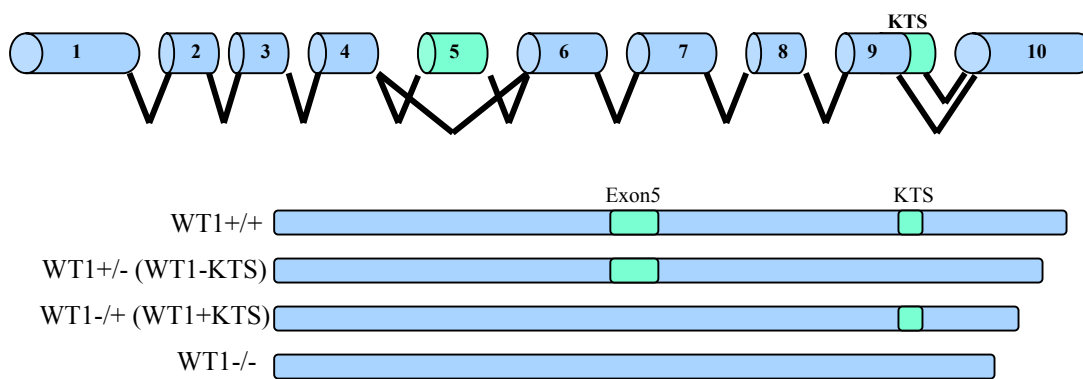
Depending on the absence or presence of the two splice inserts, WT1 encodes a protein of 52-

54 kDa that shares a high degree of structural homology with the early growth response (Egr)

family of transcription factors (Rauscher *et al*, 1990). At the C-terminus, WT1 has four C_2H_2

zinc fingers at the, a pattern typical for DNA binding factors and a nuclear localization

domain (Call *et al*, 1990; Gessler *et al*, 1990; Bruening *et al*, 1996). The N-terminus of WT1

is proline/glutamine rich and serves as a transactivation domain. In addition, the N-terminus

includes a self-association domain, a repressor domain and an RNA recognition motif (Wang

et al, 1995; Herzer *et al*, 2001) (Fig.I.6).

Considering the different domains that the WT1 protein possesses, it becomes apparent that

different splice variants could have different functions. Therefore, the WT1-KTS variant,

containing all the prerequisites for a DNA binding protein with transcriptional regulatory abilities, is thought to be a transcription factor (Davies *et al*, 1998). Several studies have shown that WT1-KTS can act both as a transcriptional activator and repressor depending on the promoter, cell type and cell cycle stage (see below). The addition of the three amino-acids between zinc fingers three and four in the WT1+KTS variant, changes the spacing between the last two zinc fingers, reduces its affinity for DNA binding and as a result WT1+KTS is not attributed transcription factor activities (Laity *et al*, 2000). However, it is thought that WT1+KTS is involved in RNA processing as it has a higher affinity for RNA (Kennedy *et al*, 1996). Also, it has been shown to co-localize with RNA speckles and splicing factors in the nucleus (Larsson *et al*, 1995). Not much is known about the 17 amino-acid inclusion or exclusion in the protein but it has recently been suggested that the WT1+/- variant has an anti-apoptotic function on the intrinsic apoptosis pathway in some leukaemias (Ito *et al*, 2006). However, mice homozygous for exon 5 deletion develop normally and are fertile (Eggenschwiler *et al*, 1997).

Figure I.6. Schematic representation of the WT1 protein. (A) WT1 protein annotated for the different domains of interest. The splicing sites are also depicted in turquoise. (B) Pdb Representation of the putative way WT1 can coil around the DNA. The zinc ions are depicted as blue spheres and an iron atom in green.
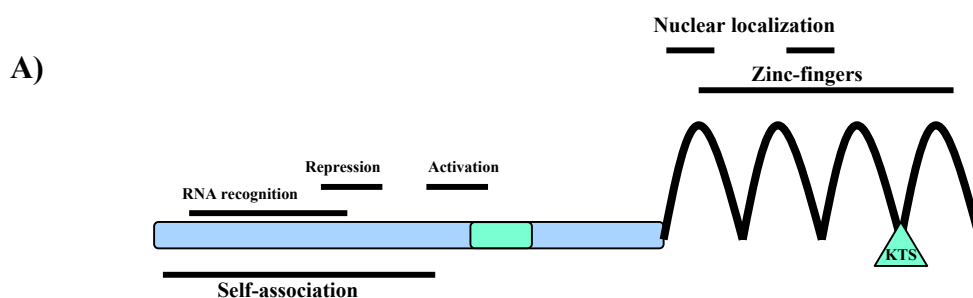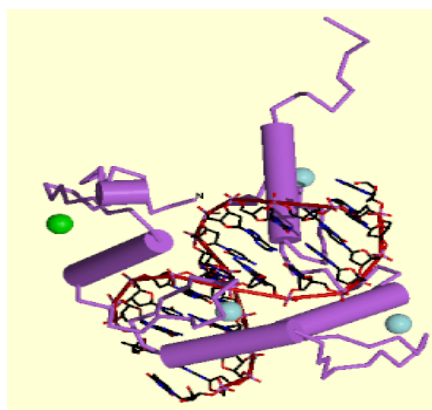
Moreover, the ratio between the WT1-/+ KTS is of importance. It is conserved among tissues (Haber *et al*, 1991) and any imbalanced expression will lead to developmental abnormalities (Hammes *et al*, 2001). This is reflected in transgenic mice experiments. When the WT1+KTS isoform is selectively omitted the animals display a phenotype reminiscent of Frasier syndrome in humans (glomerulopathy and male-to-female sex reversal). When the WT1-KTS isoform is selectively deleted, the malformations in the mice are more severe, characterized by hypoplastic kidneys and streak gonads. It is worth pointing out though, that unlike the completely WT1 null mice, both the WT1+KTS and WT1-KTS transgenic mice survive to birth, indicating a functional overlap between the two isoforms (Hammes *et al*, 2001).

7. WT1-KTS transcription targets and protein collaborators

Belonging to the Egr family of transcription factors, WT1-KTS usually binds to the GC-rich canonical Egr1 DNA binding motif -GCG(5)CG- (Rauscher *et al*, 1990) but also to TC-rich regions -(TCC)4TCTCC- (Wang *et al*, 1993). The identification of *bona fide* WT1-KTS target genes, however, is a difficult process as a result of isoform and context-specific regulatory activities of WT1. The transcription factor WT-KTS variant has a growing list of candidate target genes. The best characterized transcriptional relationships of WT1-KTS and its target promoters are summarized in table I.2.

Table I.2. Summary of the best studied putative genes that WT1-KTS regulates at the transcriptional level

Gene promoter target	Reference
Gonad	
Sf1$\updownarrow$	Wilhelm & Englert, 2002
Wnt4	Sim *et al*, 2002
*Dax1↑	Kim *et al*, 2002
MIS↑	Nachtigal *et al*, 1998
Kidney	
AREG↑	Lee *et al*, 1999
*CDH1↑	Hosono *et al*, 2000
Podocytes	
PODX↑	Palmer *et al*, 2001
NPHS1↑	Wagner *et al*, 2004
Cell cycle	
p21↑	Englert *et al*, 1997
cyclinD1↑	Tuna *et al*, 2005
cyclinE↓	Loeb *et al*, 2002
Apoptosis	
Bcl-2$\updownarrow$	Mayo *et al*, 1999 ;
	Cheema *et al*, 2003
*Bak↑	Morrison *et al*, 2005
EGFR↓	Englert et al, 1995a
Transcription	
*c-Myc↓	Han *et al*, 2004
*WT1↓	Rupprecht *et al*, 1994
*Egr1$\updownarrow$	Madden *et al*, 1991
Pax2↓	Ryan *et al*, 1995

Genes marked with an asterisk are target at an Egr1 binding site. Arrows depict the transcriptional effect, ↑ activation, ↓suppression and $\updownarrow$ for both.

As research on the field of WT1 progresses, it becomes clearer that the distinct effects of WT1 are mediated by the association of WT1 with other factors. WT1-KTS is usually found to be contained in complexes composed of either DNA-binding proteins or non-DNA-binding proteins.   One example of the interaction of WT1 with a DNA-binding protein is its collaboration with p53. Both proteins co-immunoprecipitate and WT1 enhances p53 DNA

binding by stabilizing p53 (Maheswaran *et al*, 1995). Transgenic mice experiments have also shown that p53 enhances WT1-mediated transcriptional repression (Menke *et al*, 2002). Others include SF1 (Nachtigal *et al*, 1998), CBP and p300 (Wang *et al*, 2001). A non-DNA binding protein that indirectly, by interacting with WT1, affects gene transcription, is Par-4. Par-4 has been shown to interact with WT1 by the yeast-two-hybrid system (Johnstone *et al*, 1996) and that a direct interaction with the zinc fingers of WT1 augments transcriptional repression by WT1 (Richard *et al*, 2001). Other non-DNA-binding proteins that interact with WT1 include Hsp70 (Maheswaran *et al*, 1998), WTIP (Srichai *et al*, 2004) and BASP1 (Carpenter *et al*, 2004).

8. WT1 and leukaemia: a tumor suppressor or an oncogene?

The complexity in understanding the biology of WT1 is not only due to the existence of different splice isoforms, which have different functions and are differentially expressed, but is also reflected in WT1's controversial role in leukaemogenesis. Although WT1 was first identified in Wilms' tumors because of mutations (Haber *et al*, 1990), it is now known that only 10% of the Wilms' tumors actually have mutations in the gene (Coppes *et al*, 1993; Gessler *et al*, 1994). For this reason it is thought that other loci may be involved, e.g., *FWT1* (Rahman *et al*, 1996). An interesting observation was that Wilms' tumor patients develop leukaemia as a second primary tumor (Moss *et al*, 1989; Pritchard-Jones *et al*, 1994). In addition, leukaemias are also common in relatives of children with Wilms' tumor. The involvement of WT1 during early haematopoiesis and its very high expression in more undifferentiated progenitor $CD34^+/CD33^-$ cells (100-fold more than in differentiated precursors $CD33^+$) (Maurer *et al*, 1997) raises the question on whether WT1 is also implicated in leukaemogenesis.

Research trying to provide an answer has been contradictory. On one hand, WT1 has been found to be mutated in sporadic leukaemia at a rate of 10%, which is comparable to Wilms' tumors and at a rate of 15% in AML patients (King-Underwood *et al*, 1996). Most of the mutations identified resulted in zinc-finger loss. Such a C-terminally truncated WT1 protein would lose its DNA binding ability but would retain its protein-protein interaction capacity, having, however, its usual function altered. Interestingly, it has been suggested that even heterozygous mutations are sufficient to contribute to leukaemogenesis (King-Underwood & Pritchard-Jones, 1998). This can be explained, if the result of the mutation is a truncated protein that can still self-associate. Such an event has been shown to alter the subnuclear localization of the wild-type protein and produce WT1 proteins that act in a dominant-negative manner (Englert *et al*, 1995b). These characteristics give WT1 a tumor suppressor function, which results in leukaemogenesis when lost due to mutation during early haematopoiesis.

On the other hand, WT1 has been awarded an oncogenic role, as it has been shown to be overexpressed in acute leukaemic cell lines and acute leukaemias of myeloid and lymphoid origin (Inoue *et al*, 1997, Menssen *et al*, 1995). High expression of WT1 has been found to correlate with less-differentiated phenotypes (Pritchard-Jones & King-Underwood, 1997). This overexpression has been suggested to be isoform-dependent as it has been connected with an excess of the exon-5 containing WT1 variant (Renshaw *et al*, 1997).

9. WT1 and its controversial role in apoptosis

The oncogenic potential of WT1 is further supported by the indications that WT1 promotes cell proliferation and inhibits apoptosis (Yamajami *et al*, 1996, Loeb & Sukumar, 2002),

hypothetically through upregulation of anti-apoptotic Bcl-2 (Mayo *et al*, 1999) and cyclin D1 (Tuna *et al*, 2005). In other cases, when WT1 expression is lost in leukaemic cell lines or in the knock-out mouse, apoptosis is enhanced (Algar *et al*, 1996; Herzer *et al*, 1999). In contrast, expression of the WT-KTS variant has induced G1 arrest and apoptosis in myeloblastic leukaemia cells (Murata *et al*, 1997) and has been shown to induce apoptosis by upregulating pro-apoptotic Bak (Morrison *et al*, 2005). Therefore, WT1 may act either as a 'survival factor' by preventing apoptosis of transformed cells or it may induce apoptosis and hence prevent the proliferation of leukaemic cells. However, there are no clearly described pathways that explain how WT1 is able to affect such opposing mechanisms.

10. Aim of study

The role of WT1 in leukaemogenesis is unclear. However, it is apparent that WT1 plays a very important role in apoptosis. Therefore, depending on its expression profile during haematopoiesis, it can direct the cell either towards apoptosis or towards proliferation. In the unlucky event of a cell turning cancerous, the eventual expression of WT1 could act as a prevention mechanism by instructing apoptosis. On the contrary, the presence of loss of function mutations in WT1 would abolish its latter ability and then the cell would be free to survive and continue its carcinogenesis, eventually leading to leukaemia. Furthermore, if WT1 expression continues or restarts after the haematopoietic stages where it is actually required, then this would give the cell a proliferative advantage, promoting in this way leukaemogenesis. As already mentioned, all these events have been described, making it difficult for researchers to characterize WT1's exact function. Currently, opinions tend to support WT1 overexpression in leukaemias, so that WT1 is being considered as a target marker for immunotherapy (Inoue et al, 1994; Inoue et al, 1996; Oka *et al*, 2000; Gao *et al*, 2000). One should be cautious, as most research is based on detecting WT1 expression by

reverse-transcriptase polymerase chain reaction against the C-terminus of the WT1 transcript, and not by protein analysis.

The role of WT1 in apoptosis induction of human T-cell leukaemias or leukaemic cell lines has not been extensively studied. It provides a very interesting and challenging field of research, taking into consideration that WT1 is a transcription factor that binds to the Egr1 consensus and is involved in haematopoiesis and apoptosis. The CD95L is important for the apoptosis of mature T cells and there is also evidence that it participates in intrathymic apoptosis during T cell development. In addition the CD95L contains three sites where Egr1 has been shown to bind on its promoter. Therefore, the aim of this work was to study the expression of WT1 in leukaemic T-cell lines and its mutation status. In addition, analysis of the connection between WT1 presence/absence with T cell apoptosis was implemented and finally the role of WT1 in the function of the CD95/CD95L system was studied.

II Materials and Methods

II.1 Materials

1.1 Buffers.
All chemical substances used, were obtained from Sigma-Aldrich unless otherwise stated.

Buffer	Composition
Buffer A (nuclei extract)	10mM HEPES pH8.0, 0.5M sucrose, 50mM NaCl, 0.25mM EGTA, 1mM EDTA, 0.5mM spermidine, 0.5% Triton x-100, 1μg/ml trypsin and a 100mM solution in isopropanol of 0.5mM PMSF, 0.5μg/ml leupeptin, 0.7μg/ml pepstatin, 1μg/ml aprotinin and 40μg/ml bestatin
Buffer B (nuclei extract)	10mM HEPES pH8.0, 25% glycerol, 500mM NaCl, 0.1mM EGTA, 0.1mM EDTA, 05mM spermidine, 0.25mM DTT and a 100mM solution in isopropanol of 0.5mM PMSF, 0.5μg/ml leupeptin, 0.7μg/ml pepstatin, 1μg/ml aprotinin and 40μg/ml bestatin
TE	10mM Tris pH 8.0, 1mM EDTA
Solution 1 (plasmid isolation)	50mM glucose, 25mM Tris pH 8.0 and 10mM EDTA
Lower Gel buffer (western blot)	1.5M Tris pH 8.8, 0.4% SDS
Upper Gel buffer (western blot)	0.5M Tris pH 6.8, 0.4% SDS
10xSDS Running buffer	0.025M Tris, 0.19M glycin, 1% SDS
Semi Dry buffer	0.045M Tris, 0.035M glycin, 5% MeOH
SDS sample buffer (loading)	0.0625M Tris, 50mM DTT, 2.3% SDS, pH 6.8, 0.1% Bromophenol blue
(SDS) wash-buffer	0.02M Tris pH 7.3, 0.15M NaCl, 0.01% Tween-20
DNA-binding buffer (EMSA)	50mM HEPES pH 7.5, 100mM NaCl, 2mM EDTA, 20% glycerol
Luciferase Buffer	1.07mM $(MgCO_3)_4$ $Mg(OH)_2 \cdot 5H_2O$, 668mM tricine, 100mM $MgSO_4$, 10mM EDTA, 1M DTT, 4mM CoAOAc, 100mM ATP, 5mg luciferin
PBS	8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4
TBE (10x)	108g Tris, 55g Boric acid, 40ml EDTA pH 8.0 (0.5M)
ACK	41.45g NH_4Cl, 5g $KHCO_3$, 0.186g EDTA, in 500ml volume with H_2O, pH 7.27
RIPA	120mM NaCl, 50mM Tris/HCl (pH 8.0), 1% NP-40, 0.5% Desoxycholate, 1mM PMSF, 25mM NaF, 0.1% SDS, 200μM Na_3VO_4, 1mM DTT, protease inhibitors (tablet of complete) and H_2O to the appropriate volume
Nicoletti buffer	PBS buffer, 0.1% SodiumCitrate, 0.1% TritonX-100
Propidium Iodide	Propidium Iodide in PBS 2mg/ml

1.2 Media

Medium	Composition
Bacterial Culture	
LB (10x)	
	90g Bacterial Tryptone (Roth), 45g Yeast extract (Gerbu),
	90g NaCl, 900ml H_2O
LB-Agar	
	450μl (1x) LB, 9g Agar
Human Cell Culture	
RPMI	
	900ml RPMI (Invitrogen), 10% FCS (Gibco), 10mg/ml Gentamicin (Gibco)
DMED	
	500ml DMED (Invitrogen), 10% FCS (Gibco), 1μg/ml Puromycin, 1μg/ml Tetracyclin, 0.5mg/ml G418

1.3 Antibodies

Protein Target	Antibody	Secondary Antibody
Western Blot		
WT1 N-terminus	(F-6) sc-7385 (Santa Cruz Biotechnology)	Mouse monoclonal (Santa Cruz Biothechnology)
WT1 C-terminus	(C-19) sc-192 (Santa Cruz Biotechnology)	Rabbit Polyclonal (Santa Cruz Biotechnology)
YY1	(H-10) sc-7341 (Santa Cruz Biotechnology)	Mouse monoclonal (Santa Cruz Biotechnology)
Tubulin	Monoclonal anti-α-tubulin (Sigma)	Mouse monoclonal (Santa Cruz Biotechnology)
EMSA		
Egr-1	(C-19) sc-189 (Santa Cruz Biotechnology)	-
WT1	(F-6)X sc-7385 (Santa Cruz Biotechnology)	-
Sp1	(PEP2) X sc-59 (Santa Cruz Biotechnology)	-
Fas-ligand	(Nok-1) Purified mouse anti-human fas ligand (BD Pharmingen)	Mouse monoclonal IgG1 (BD Pharmingen)
IgG1	(anti-TNP) Purified mouse IgG1,κ, isotype control (BD Pharmingen)	-

1.4 Cell Lines

Jurkat16, Jurkat 282, Molt4, CEM, Myla, SeAx and HH are human T-cell leukaemic cell lines. Hut78 is a human T-cell lymphoma. All cell lines were cultured in RPMI medium. The cell line U2OS is an osteosarcoma cell line in which WT1 expression is inhibited by the presence of tetracycline (Haber *et al*, 1996). U2OS cells were cultured in DMEM medium. Tetracycline was removed from the medium for at least 48h prior of experiments to allow WT1 expression. All cells were grown at 37ºC in a humidified 5% CO_2 incubator.

1.5 Oligonucleotide primers used for cDNA amplification (Qiagen)

Target Gene	Sequence	PCR Parameters	
		Annealing Temp	No of Cycles
WT1-A	5'-GCCCAATACAGAATACACA-3' forward 5'-TCACACACTGTGCTGCCT-3' reverse	56.5°C	30
WT1-B	5'-CCCAACCACTCATTCAAG-3' forward 5'-TATTCTGTATTGGGCTCCGC-3' reverse	67°C	25
WT1-C	5'-CTAACGCGCCCTACCTGCCC-3' forward 5'-GGTGCAGCTGTCGGTGGGGG-3' reverse	57°C	40
CD95	5'-CAAGTGCAGATGTAAAC-3' forward 5'-TGAAGTTGATGCCAATTACG-3' reverse	58°C	30
CD95L	5'-ATAGGATCCATGTTTCTGCTCTTCCACCTACAGAAGGA-3' forward 5'-ATAGAATTCTGACCAAGAGAGGCTCAGATACGTTGAC-3' reverse	58°C	30
β-actin	5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' forward 5'-CTAGAATTTGCGGTGGACGATGGAGGG-3' reverse	60°C	20

1.6 Oligonucleotide primers used for quantitative real-time PCR (Eurogentec)

Target Gene	Sequence
CD95	5'-ACTGTGACCCTTGCACCAAAT-3' forward
	5'-GCCACCCCAAGTTAGATCTGG-3' reverse
	5'-AATCATCAAGGAATGCACACTCACCAGCA-3' probe
CD95L	5'-AAAGTGGCCCATTTAACAGGC-3' forward
	5'-AAAGCAGGACAATTCCATAGGTG-3' reverse
	5'-TCCAACTCAAGGTCCATGCCTCTGG-3'probe
β-actin	5'-ACCCACACTGTGCCCATCTACGA-3' forward
	5'-CAGCGGAACCGCTCATTGCCAATGG-3' reverse
	5'-ATGCCCTCCCCCATGCCATCCTGCGT-3'probe

1.7 Oligonucleotide Probes used for EMSA (Qiagen)

Gene promoter site	Sequence
CD95L -120	5'-TCAGCTGCAAAGTGAGTGGGTGTTTCTTTGAG
CD95L -180	5'-ATTGTGGGCGGAAACTTCCAGGGG
CD95L -680	5'-GATCTAATTTCTAAAGTGGGTGTAGCAGGTTTTTAAC
NFY	5'-CACCTTTTAACCAATCAGAAAAAT

1.8 Vectors

Vector	Origin
pRc/CMV	Invitrogen
pCR-Blunt II-TOPO	Invitrogen
CMV_WT1-CA	Englert C. (Herzer *et al*, 2001)
CMV_WT1-CC	Englert C. (Herzer *et al*, 2001)
pTATA_Luc	Li-Weber *et al*, 1998

II. 2 Methods

2.1 Plasmid isolation

The plasmids that were screened for positive clone content were purified according to the following protocol: 2ml of an overnight LB culture were centrifuged at 6000rpm for 5 mins and the cell pellet was resuspended in 350μl of solution 1 with the addition of 25μl of lysozyme (10mg/ml in 10mM Tris pH 8.0). Cell lysis was allowed to take place for 2 mins at room temperature and then the lysozyme was heat-inactivated at 95°C for 60 sec. The samples were then spun down at 13.000rpm for 10mins and the supernatant was mixed with 40μl Na-acetate (2.5M) (Roth) and 420μl isopropanol and stored at -80°C for 15mins. The samples were then allowed to warm up to room temperature and a centrifugation step at 13.000rpm for 15mins produced a DNA containing pellet. The pellet was washed once with 70% ethanol and the samples were vacuum dried. The final pellet was resuspended in 30μl of TE buffer.

The plasmid constructs destined for sequencing of their inserts were purified and isolated with a Qiagen Plasmid Midi Kit according to manufacturer's instructions.   All other plasmid constructs were isolated with a Nucleobond Plasmid Maxi purification kit following the manufacturer's protocol.

2.2 Total mRNA extraction

The mRNA isolation and purification was carried out using the NucleoSpin RNA II kit (Macherey-Nagel). Briefly, $1x10^6$-$1x10^7$ cell were used. The cell pellets were washed once in PBS and then homogenized in 350μl of RA1 buffer and 3.5μl of β-mercaptoethanol. The mixture

was applied on a filter provided and after 1 min centrifugation the flowthrough was mixed with 350µl 70% Ethanol and transferred into a fresh tube. After a short spin, 350µl of membrane dissolving buffer (MDB) were added on the filter and the samples were again spun down for 1 min. The samples were then incubated at room temperature for 15mins in 95µl of DNase solution (90µl DNase buffer with 10µl DNase). 200µl of RA2 buffer were added (inactivating the DNase) followed by another round of brief centrifugation. The samples were washed using 600µl of RA3 buffer. After a short spin another 250µl of RA3 buffer were added and 2 min centrifugation followed. Finally, mRNA was eluted from the filter with 40µl of RNase-free water. This last step was repeated to optimize the concentration of the eluate. The mRNA samples were stored at −80°C.

2.3 Reverse-Transcription reaction for cDNA generation (RT)

1µg of DNase treated RNA was reversed transcribed into cDNA with 50 U MuLV reverse transcriptase (Roche) in the presence of 50µM oligo-d(T) and 200nM dNTPs at 42°C for 45mis. Aliquots (¼) of the resulting cDNA were then used as template for PCR amplifications.

2.4 Polymerase Chain Reaction (PCR)

2.4.1 PCR for plasmid DNA amplification

All PCRs on plasmid DNA were carried out using Taq DNA polymerase (BioLabs) in a typical PCR (total 40µl) as follows:

31.5µl	Sterile H_2O
4µl	10xPCR Buffer
1µl	Forward Primer
1µl	Reverse Primer
1µl	plasmid DNA (2µg)
1µl	dNTPs
0.5µl	Taq DNA polymerase

2.4.2 PCR for cDNA amplification

The amplification of the C-terminus of WT1 and all other cDNA amplifications were carried out

using Taq DNA polymerase in a 40µl reaction according to the following protocol:

28.5µl	Sterile H_2O
4µl	10xPCR Buffer
1µl	Forward Primer
1µl	Reverse Primer
5µl	cDNA
0.5µl	Taq DNA polymerase (2.5U)

For the amplification of the GC-rich N-terminus of WT1, the GC-Rich PCR system (Roche) was

used according to manufacturer's protocol. This system uses a combination of two polymerase

enzymes, the *Taq* DNA and *Tgo* DNA polymerases, providing higher fidelity, yield and

specificity than using *Taq* alone. Two different reactions (I and II see below) were carried out

separately and mixed just before amplification.

GC-Rich PCR reaction I (Total 35µl):

1µl	dATP (200µM)
1µl	dCTP (200µM)
1µl	dTTP (200µM)
1µl	dGTP (200µM)
1µl	Forward primer (200nM)
1µl	Reverse primer (200nM)
10µl	GC-Rich resolution solution (1M)
xµl	cDNA (25-50ng)
xµl	H_2O

GC-Rich PCR reaction II (Total 15µl):

1µl	GC-Rich PCR System Enzyme mix (2U/50µl)
4µl	H_2O
10µl	5xGC-Rich buffer with DMSO (1.5mM $MgCl_2$)

2.5 Cloning

2.5.1 Cloning reaction

Cloning of the amplified transcripts for subsequent sequencing was done using the Zero Blunt Topo PCR Cloning Kit (Invitrogen life technologies), which provides the direct insertion of blunt-end PCR products in the pCR-Blunt II-TOPO vector using the topoisomerase I enzyme from *Vaccinia* virus. All reactions were carried out according to the manufacturer's instructions and incubated at room temperature for 5mins.

Cloning Reaction (Total 6μl):

2μl	Blunt-end PCR product
1μl	Salt solution (1.2M NaCl, 0.06M MgCl$_2$)
2μl	Sterile water
1μl	pCRII-Blunt-TOPO vector

2.5.2 Transformation

Transformation of the plasmids was done in chemically competent DH5a-*E.coli*. 2μl of the TOPO cloning reaction were introduced into 50μl of competent bacteria and the mixture was incubated on ice for 30mins. The cells were then heat-shocked at 42°C for 30sec prior to transfer to ice. 250μl of S.O.C. medium were added and the reactions were incubated at 37°C for 1h with horizontal shaking. Finally, 50μl from each reaction were spread on agar plates containing 50μg/ml kanamycin. The plates were incubated overnight at 37°C to allow colony formation.

2.5.3 Positive clone selection

After the overnight incubation and the appearance of clones, approximately 24 clones were selected from each reaction plate for analysis. Each single colony was allowed to grow overnight in a liquid agar culture containing kanamycin (50 μg/ml), followed by isolation of the plasmid.

After plasmid isolation, successful insertion was tested by restriction analysis using the *Eco*R1
enzyme (Fermentas GMBH), which has two restriction sites on the vector flanking the PCR
insertion position. This directly allows the screening of the clones, as agarose gel electrophoresis
of the enzyme digested reactions would yield a product of the exact size as the original PCR
amplified product in a positive clone.

2.5.4 Fill-in reaction to produce "blunt ends"

The amplified PCR reactions of the cDNA destined for cloning contained mostly product with
sticky ends. Since the cloning vector is designed for blunt-end ligation, the sticky ends needed to
be converted into blunt ends. This was done by filling in the sticky ends with the complementary
nucleotides. A typical fill-in reaction is described below (total 20µl) and samples were incubated
at room temperature for 30mins:

> 5µl PCR product
>
> 2µl Buffer (200mM Tris, 100mM $MgCl_2$, pH 7.6)
>
> 2µl dNTPmix (10mM)
>
> 1µl Klenow fragment (Fermentas)
>
> 10µl H_2O

2.5.5 Restriction digestion

Restriction digestion of plasmid DNA was carried out at 37°C for 1h and 20 mins using the
following protocol:

> 3µl plasmid DNA (mini-lysate) (20ng)
> 2µl Buffer (10x)
> 0.02µl RnaseA
> 11µl H_2O
> 0.5µl enzyme

2.5.6 Competent cell preparation

The strain to be made competent was set at 5ml in an overnight liquid Agar culture. After the overnight growth, 2ml of the culture were inoculated with 100ml of L-Broth and allowed to grow further at 37°C with shaking. Samples of the culture were taken at regular intervals and OD600 of the cells was measured. Growth was stopped when the OD600 reached a value of 0.42-0.45 (indicating sufficient growth). The cells were then placed on ice for 5 mins and 100ml of the culture were spun at 6000rpm for 5 mins. The pellet was resuspended in 40ml of ice-cold $CaCl_2$ (0.1M). The cells were re-pelleted, gently resuspended in 25ml of ice-cold $CaCl_2$ (0.1M) and incubated on ice for 30 mins. The cells were pelleted again prior to resuspension in 1ml of ice-cold $CaCl_2$ (0.1M). Finally, 15% (v/v) sterile glycerol was added and 50μl aliquots were frozen in liquid N_2 and stored at -80°C.

2.6 Western blot analysis

2.6.1 Protein extraction

Whole cell lysates were prepared for protein extraction for western blot analysis. Usually $1x10^6$-$1x10^7$ cells were used, the pellet of which was washed once in ice cold PBS and then resuspended in 100-250μl of radioimmunoprecipitation assay (RIPA) buffer. An incubation of 20 mins at 4°C was followed with vortexing, and the cells were centrifuged for 20mins at 4°C at 13.000rpm. The supernatant was then transferred to a fresh tube and th protein concentration was determined using the BCA Protein Assay kit according to manufacturer's instructions (PerBio, Pierce). 20-50μl of 5x sample loading buffer were added to the sample, corresponding to 20-25μg protein in each sample. Finally, the samples were incubated at 95°C for 10mins and were stored at -20°C.

2.6.2 Nuclear protein extraction

1×10^8 cells were used for each preparation. The cell pellet was recovered by centrifugation at 1200rpm for 10mins and used immediately. All reactions were performed at 4°C. The cell pellet was resuspended in Buffer A (10x volume) and after vigorous vortexing the nuclei were collected by centrifugation at 3500 rpm for 5mins. Nuclei were resuspended again in Buffer A (10x volume) and another step of centrifugation at 4000rpm for 5mins was carried out. Then the supernatant was carefully removed and the pellet was resuspended in 500μl of Buffer B. Each sample was incubated at 4°C with stirring for 30mins and the supernatant was collected by centrifugation at 13000rpm for 10mins, and it was stored at -80°C for future usage. Determination of protein concentration was carried out with the BCA Protein Assay kit according to manufacturer's instructions (PerBio, Pierce).

2.6.3 SDS-PAGE

For a 10% SDS-page gel, 2ml of lower gel buffer were mixed with 3.3ml of H_2O, 2.6ml of 30% Acrylamide, 50μl of 10% APS and 5μl of TEMED. Approximately 7ml were poured in the glass plates where the gel would be mounted to set. On top of the set lower gel, a stacking gel was poured which was made of 1.25ml of upper gel buffer, 3ml H_2O, 0.8ml of 30% Acrylamide, 50μl of 10% APS and 5μl of TEMED. Combs of the required well size and number were added and the gel was allowed to set.

2.6.4 Western blot

For nuclear extract protein analysis 20-25μg of protein were mixed with 5xSDS-sample loading buffer, and the samples were boiled for 5mins at 95°C. For whole cell lysates, 20-25μg of already pre-mixed with loading buffer were boiled for 10mins at 95°C. The boiled samples were

loaded in the well of a mini-gel (20-25µg of protein) along with 15µl of pre-stained SDS-protein marker (NewEngland BioLabs) and one mini-gel was run at 35mA. The gel was then blotted with semi-dry buffer on a transfer membrane in a blotting chamber (BioRad) for 1h and 20 mins at 60mA. After transfer the membrane containing the proteins was removed from the chamber and blocked with 5% milk (milk poweder in wash-buffer) for 1h at room temperature. Then it was washed three times (10mins each) with wash-buffer and the antibody was applied in 5% BSA for 1h at room temperature(WT1-C19, YY1, Tubulin) or overnight at 4°C (WT1-F6 antibody) on a horizontal rocker. After another round of washes as before, the appropriate secondary antibody was applied in 5% milk in a dilution 1:10.000 for 1h at room temperature. The membrane was washed with wash-buffer once more and placed in a film cassette for developing. The chemiluminescence reagents 'enhanced luminol' and 'oxidizing reagent' (1:1) (PerkinElmer Life Sciences) and an X-ray film (Kodak) was applied.

2.7 Electrophoretic Mobility Shift Assay (EMSA)

2.7.1 Primer annealing

First, oligonucleotide probes were annealed for labeling. This was carried out by mixing 20µg of sense and anti-sense oligonucleotides with 5µl of Tris/MgCl$_2$ (200mM pH 7.5), 10µl of KCl (800mM) (AppliChem) and H$_2$O to a final volume of 100µl. The mixture was incubated at 90°C for 2 mins and then placed in a water bath of 70°C initial temperature that was allowed to cool down to room temperature.

2.7.2 Labeling

300ng of annealed oligonucleotides were labeled radioactively in a reaction using 2μl of Tris (200mM pH 7.5)/ MgCl$_2$ (100mM), 17μl of ddH$_2$O, 0.25μl dGTP, dCTP, dTTP (4mM), 1μl of Klenow fragment and 2.5μl of ^{32}P-dATP. The reaction was allowed to take place at 37°C for 40 mins. The unlabeled probe was removed by precipitation with 10μg/μl tRNA, 4M NH$_4$Ac (1:1 v/v) and 70% ethanol (2v), the resulting pellet was washed in 70% ethanol and after drying the labeled oligonucleotides were resuspended in 50μl of TE buffer.

2.7.3 Binding reaction

The labeled and purified oligonucleotides were set for protein binding in a reaction that contained 1-1.5μl of labeled oligonucleotides (30-50 counts), 1.5μl of dI-dC (200ng/μl), 8μl of DNA-binding buffer, 12μl of ddH$_2$O and 8-20μg of protein (nuclear extract) or recombinant protein. The WT1+/- KTS recombinant proteins have been described before (Elser *et al*, 1997). The binding reaction was carried out at room temperature for 30 mins. For supershift assays, before the addition of the radioactively labeled oligonucleotides, the sample was incubated with the antibody of interest (1-2μg) on ice for 30mins.

2.7.4 EMSA loading

The samples were run onto a 30% polyacrylamide gel [7.5ml of 30% polyacrylamide (0.8% Bis) with 5ml TBE (5x), 37,5ml ddH$_2$O, 0.4ml 10% APS and 50μl TEMED]. The gel had a thickness of 1.5mm and was run at 150V of constant voltage, for 1.4-1.6h. The gel was then transferred onto a Whatman membrane and vaccum-dried at 80°C for 1-2h. Afterwards, the membrane with the gel were placed in cassette for radioactive exposure along with an X-ray film (Kodak) and exposure was carried out overnight at -80°C. The film was developed the following day.

2.8 T cell preparation

2.8.1 T cell isolation

For primary T cell isolation, 30ml of blood were poured gently on 15ml Biocoll Separating solution and were centrifuged for 20mins at 2420rpm at 20°C. The supernatant (containing T cells) was carefully removed with a pipette and placed in a fresh tube where the volume was added up to 50ml with RPMI medium (without additives). The cells were centrifuged for 10mins at 1500rpm and the T cells now present in the pellet were cleared off the supernatant.   Next was the monodepletion step, which would permit all monocytic cells to adhere and hence B and T cell would remain in solution. This was done by resuspending the previous cell pellet in RPMI and allowing to rest overnight.   After monodepletion, the supernatant was isolated and the total concentration was brought down to 8×10^6 cells/ml by spinning down at 1500rpm for 5mins and dissolving the pellet in fresh medium (50ml). This was then divided in two tubes of 25ml each and then an equal volume of Erythrocytes was added. The cells were mixed well and centrifuged at 1000rpm, low brake, for 10mins at 20°C. Most of the supernatant was removed and only 20ml were left, in which the pellet was resuspended. Then 15ml of Ficoll were added on top carefully and the sample was spun down at 2420rpm for 20mins at 20°C. The supernatant (containing B cells) was discarded and the erythrocytes in the pellet (containing T cells and erythrocytes) were lyzed with ACK lysis buffer. Lysis was stopped when the characteristic clear cherry red colour appeared, with RPMI medium. The mixture was centrifuged for a last time at 1500rpm for 5mins and only T cells are left.

2.8.2 Erythrocyte preparation (2% sheep Erythrocytes)

25ml of Erythrocytes were washed 3-4 times with RPMI after the final spin at 1200rpm for 10 mins at 20°C, the pellet was resuspended in AET solution and incubated for 15 mins at 37°C, rolling. Then the pellets were washed 4-5 times in PBS and the final pellet was resuspended in RPMI (FCS added) yielding a 2% erythrocyte solution.

2.9 Transfection of Jurkat T cells

Jurkat T cells were transfected with two different protocols depending on the aim of the transfectants. For luciferase analysis Jurkat cells were transfected with electroporation and for transient transfection with WT1 expressing constructs, Jurkat cells were transfected with Amaxa nucleofection.

2.9.1 Electroporation

For electroporation $1x10^7$ cells were used for each transfection, and each transfection was carried out in duplicates. The cells were spun down at 1500rpm for 5 mins at 20°C and the cell pellet was resuspended in 400μl of RPMI. The appropriate plasmid DNA was added (two plasmids for co-transfection) experiments and after mixing gently with the pipette the sample was poured in a cuvette for this volume. Electroporation was done in a Gene Pulser II (BioRad) at 0.240kV and 0.950μFx1000. After the procedure was finished, another 400μl of RPMI were added in the cuvette and all 800μl were added in a 6-well plate. Fresh RPMI was added to a final volume of 2ml. Before any other treatment, transfectants were allowed to recover overnight in a humidified 37°C/5%CO_2 incubator.

2.9.2 Nucleofection

Nucleofection was done for Jurkat cells to study apoptosis induction or CD95/CD95L expression after transfection because it is a more gentle procedure and results in less dead cells, an obvious advantage when studying cell death. Generally the protocol provided by the manufacturer was followed, but every time before transfection the cells were checked for optimal conditions with as little death as possible and as higher transfection efficiency as possible.   For each nucleofection, $5x10^6$ cells were used.   The cell pellet was resuspended in 100μl of room temperature Nucleofector Solution and then either 5μg of plasmid DNA or 2μg of pmaxGFP (for transfection efficiency analysis) were added.   After mixing gently, the samples were poured in a cuvette provided by the manufacturer and after setting the correct program on the Nucleofector I device (Amaxa biosystems), nucleofection could take place.   The sample was then removed from the cuvette and was transferred in a culture plate containing 2ml of RPMI.   For establishing the successful production of transient transfectants which express the proteins of interest, Western blot analysis had to be carried out.   Because the protein of interest in a nuclear protein and nuclear extracts had to be made, a large number of cells was required.   For this reason, for each sample, 2-3 nucleofection reactions were pooled in 10ml of RPMI. All transfectants were allowed to recover overnight in a humidified 37°C/5%CO_2 incubator.

2.10 HiPerfect transfection (siRNA)

For the introduction of siRNA in CEM cells, the HiPerfect system from Qiagen was used. $2x10^5$ cells were resuspended in 90μl of fresh RPMI with 9μl of HiPerfect transfection solution and the appropriate amount of siRNA. Each sample was transferred in a well of a 96 well-plate and incubated for 6h in a humidified 37°C/5%CO_2 incubator. After incubation, the samples were

transferred in a 24 well-plate and 400µl of RPMI were added. The cells were then further

cultured in a humidified 37°C/5%CO$_2$ incubator until efficient knock-down was achieved. The

efficiency of the procedure was checked with a negative control (siRNA Alexa Fluor 488) with

target sequence AATTCTCCGAACGTGTCACGT (Qiagen), which provides green fluorescence

when transfection is successful and the % of positively transfected cells was measured by flow

cytometry after the pellets were resuspended in 200µl PBS. Then the needed concentrations of

the siRNA in question could be calculated. Western blot analysis was used to determine the time

point after siRNA introduction and the exact siRNA concentration that yielded the most efficient

knock-down. Because the number of the cells used for each transfection is low and Western blot

with nuclear extracts requires more protein, 2-3 reactions were pooled after harvesting prior to

analysis (Davies *et al*, 2004).

2.11 Apoptosis assay

2.11.1 Cell Stimulation

Cells were stimulated with PMA and ionomycin. End concentration of PMA was 10ng/µl and

ionomycin 0.5µM. Both substances were diluted from a stock of PMA 100ng/µl and ionomycin

10mM. For T cell stimulation, day 6 T cells were transferred into wells coated with 30 µg/ml

anti-CD3 Ab (pb-anti-CD3) and were cultured for 24 h.

2.11.2 Apoptosis detection

For apoptosis analysis, cells were centrifuged at 2000rpm for 10mins, the cell pellet was washed

once in 1xBPS and after another centrifugation the pellet was resuspended in 200µl of propidium

iodide in Nicoletti buffer (50µg/ml) and the samples were incubated overnight at 4°C. The

following day staining was detected by flow cytometry (FCS canto) after gating on the cells at a forward- to side- scatter profile and look for staining positivity at PE-A. Statistical analysis provided the percentage of fragmented nuclei out of the total of gated cells. Specific apoptosis was calculated by the following formula: %specific apoptosis = [% dead cells-% dead cells (untreated control)] / [100-% dead cells (untreated control)] x100.

2.12 Cell surface staining

For the CD95 cell surface staining, 10^5 cells were used for each reaction. All reactions were carried out at 4°C. The cells were spun down and the pellet was washed with 500µl of freshly made PBS supplemented with 2% FSC. The cells were then incubated for 15mins on ice with either the antibody of interest (human anti-APO-1) or isotype control antibody (IgG3, Pharmingen), diluted 1:100 in PBS/FSC. After antibody incubation was finished, the cells were recovered by centrifugation at 2000rpm for 3mins and they were washed twice with PBS/FCS. Then 3µl of the secondary antibody were added in 100µl of PBS and the reactions were incubated for 20mins on ice in the dark. Maintaining the reactions in the dark for as much as possible, the cells were washed three times in PBS/FCS and the final pellet was resuspended in 100µl of PBS/FCS to be analyzed by flow cytometry (Facs canto).

2.13 Agarose gel electrophoresis

All DNA products were separated using 0.7-2.5% (w/v) agarose in ½ x TBE buffer, according to the expected fragment size. Separations were performed at a voltage ranging from 50-135V according to the concentration of the gel and the time duration was ranging from 20-40mins

according to the gel concentration and the expected fragment size. Gels were visualized by UV

illumination in a BioRad GelDocumentation system (BioRad life sciences).

2.14 Quantitative real-time PCR

For quantitative real-time PCR a PCR-Taqman Kit from Eurogentec was used. A PCR mixture

was prepared for each sample consisting of:

<div style="border:1px solid">

PCR-Mix

1.25µl PCR buffer (2x)
2.50µl MgCl$_2$ (10mM)
1.00µl dNTPs (200µM)
0.0625µl Hot Gold Star enzyme (0.05U)
0.125µl Uracil-N-glycosylase (0.02U)
1.30µl H$_2$O

</div>

The different reactions carried out for quantitative real-time PCR were as follows and each

sample yielded enough amount for triplicates of 25µl:

CD95 Ligand	CD95 Receptor	β-actin
0.88µl forward primer	5.30µl forward primer	5.30µl forward primer
15.80µl reverse primer	5.30µl reverse primer	15.80µl reverse primer
3.50µl probe Taq	3.50µl probe Taq	3.50µl probe Taq
4.40µl cDNA template	4.40µl cDNA template	4.40µl cDNA template
43.80µl PCR mix	43.80µl PCR mix	43.80µl PCR mix
20.30µl H$_2$O	25.40µl H$_2$O	14.90µl H$_2$O

The aliquots of 25µl were placed in a 96-optical well plate along with a triplicate of a control

sample containing only water. The reactions took place in a GeneAmp 5700 real-time PCR

machine for 40-45 cycles and fold-increase in the mRNA levels of the gene in question was

normalized with respect to β-actin levels for each sample.

2.15 Bioinformatics systems

The WT1 mRNA sequence used for sequence comparisons was obtained from the NCBI nucleotide database for transcript variant D (http://www.ncbi.nlm.nih.gov). Alignment of the sequenced clones against the known WT1 mRNA sequence was done with the ClustalW tool available at the European bioinformatics institute at http://www.ebi.ac.uk/. Translation of the sequenced clones was done by the Translate tool at the proteomics server ExPASy at http://www.expasy.org/. Alignment of the translated sequence with the known WT1 amino acid sequence retrieved from the NCBI-protein database was done with the BLAST protein-protein alignment tool available at http://www.ncbi.nlm.nih.gov/BLAST/.

III Results

## 1. WT1 expression in leukaemic T-cell lines

1.1 Analyzing WT1 expression at the mRNA level

As already mentioned, WT1 has been reported to be either overexpressed or mutated in both AML and ALL. We therefore looked into whether WT1 is expressed in T-cell leukaemias. To characterize the expression patterns of WT1 in T-cell leukaemias seven leukaemic T-cell lines and primary T cells were investigated by RT-PCR with three different WT1-specific primers. For each set of primers, PCR conditions were initially optimized by carrying out a gradient PCR using mRNA from U2OS cells. The optimal conditions were then used to amplify the mRNA from the cell lines under investigation. To avoid mutations caused by the amplification procedure, a combination of proofreading polymerases was used to amplify the transcript from the leukaemic T-cell lines and primary T cells. The region between nucleotides 784-1800 (end of transcript) was the region amplified using three different sets of primers (Fig.III.1A).

The experiment showed that the mRNA corresponding to the C-terminus of WT1 was detected in most of the leukaemic cell lines studied, which is in accordance with a previous report (Inoue *et al*, 1994). However, analysis of the N-terminus showed that not all of the leukaemias expressed full length WT1 transcript. Only Hut78 and CEM expressed full length WT1. Molt4 did not express exons 1-3 and Jurkat did not express exons 1-6. Myla, SeAx and HH did not express WT1 at all (Fig.III.1B). Healthy, mature resting T cells or T cells stimulated with either anti-CD3 or PMA and ionomycin did not produce WT1 (Fig.III.1C).

Figure III.1. WT1 mRNA expression analysis in leukaemic T-cell lines.
(A) Diagrammatic representation of the WT1 transcript indicating the major splicing sites and the exons encoding the four zinc-fingers. Labelled arrows A-C show the position of the three different sets of primers used to amplify the mRNA in RT-PCR. (B) RT-PCR on total mRNA from the indicated leukaemic cell lines either induced with PMA and ionomycin or non-induced, using WT1 specific primers. The two bands appearing in lines B and C contain the equivalent splicing isoforms. (C) RT-PCR on total mRNA from primary T cells either non-induced (lane 1) or induced with anti-CD3 (lane 2) or PMA and ionomycin (lane 3) with WT1-specific primer set C. U2OS mRNA is shown for positive control. For loading control β-actin is shown.

A)
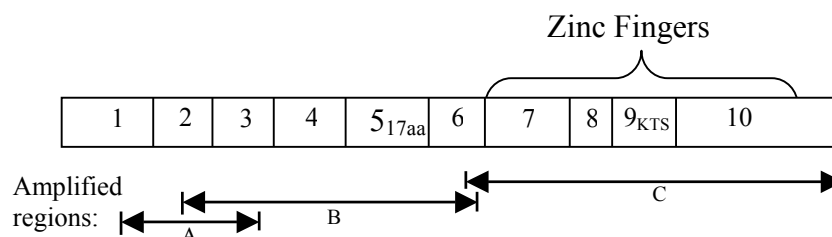
B)
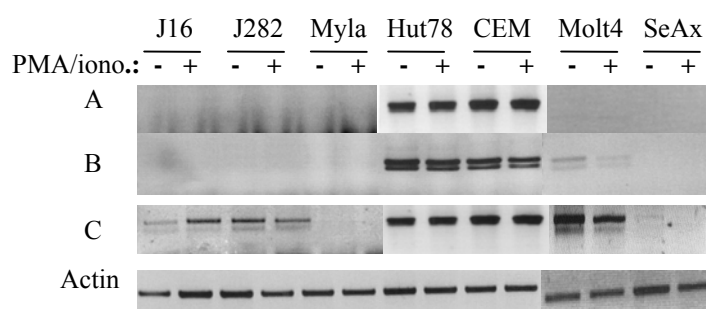
C)
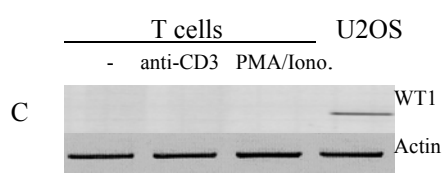
Within this work it was not possible to amplify nucleotides 1-783 of exon 1 although several sets of published primers (also at the genomic level) were used (Makrigiannakis *et al*, 2001; Schumacher *et al*, 2003; Oji *et al*, 2004). Interestingly none of these researchers has been able to provide an optimized PCR product for exon 1. This was due to the high GC content of the transcript, which impeded optimized amplification.

1.2 WT1 mutation screening

The WT1 cDNAs produced by RT-PCR, with the three sets of primers, were cloned and sequenced. From each cloning experiment, approximately 24 samples were randomly selected and screened for presence of insert. After positive clone selection, between five and ten different positive clones were randomly selected from each experiment and sent for sequencing (SeqLab). To screen for mutations in the WT1 transcript, the sequences obtained were then aligned against the known WT1 transcript sequence (NCBI, WT1 variant D, which contains both exon 5 and KTS splicing sequences) in a ClustalW alignment (EBI) (Fig.III.2). The alignment results indicated that the mRNA sequences amplified had no mutations. Only in the C-terminal part of the gene, a single nucleotide difference was seen (Fig.III.2C), G in cDNA at position 1525 compared to A in the published genomic DNA sequence (NCBI). However, this difference had no effect on the protein, since translation of the amplified cDNA sequence (ExPasy) and alignment with the known WT1 amino acid sequence gave 100% match (Fig.III.2D).

Sequencing of exons 2-6 at the N-terminal part of the gene showed that the sequences for both exon 5 splice variants were obtained in a ratio of 1:2.3 for WT1-Exon5:WT1+Exon5. Moreover, for the C-terminal part of the gene, both WT1-KTS and WT1+KTS sequences were obtained at an equal proportion. All positive clones (5-10) that were sequenced from each experiment produced the same sequencing result. The above results showed that the leukaemic cell lines CEM and Hut78 expressed full-length wild-type WT1 transcript, whereas mature healthy resting or activated T cells and the leukaemic cell lines, Myla, SeAx and HH did not produce WT1 transcript at all. Contrary to previous reports, the leukaemic cell lines Jurkat16, Jurkat282 and Molt4 (Inoue *et al*, 1994) did not produce full-length WT1 transcript (Fig.III.3).

Figure III.2. Mutation screening in the WT1 transcript. Representative ClustalW alignment (EBI) of the cloned and sequenced WT1 transcripts against the known WT1 mRNA sequence for the WT1 (+/+) variant (NCBI). (A) Alignment of exons 1-3, (B) alignment of exons 2-6 and (C) alignment of exons 6-10. The blue rectangle points a position where a G always appears in the cDNA but an A has been given to the genomic sequence (NCBI). (D) BLAST alignment (NCBI) of the translated C-terminal cloned sequence against the WT1 protein amino acid sequence until the translation stop (STP) codon.

A)

B)

C)

```
CAGCACAGGGTACGAGAGCGATAACCACACAACGCCCATCCTCTGCGGAGCCCAATACAG 1260
--------------------------------------------------------CCCAATACAG 10
                                                        **********

AATACACACGCACGGTGTCTTCAGAGGCATTCAGGATGTGCGACGTGTGCCTGGAGTAGC 1320
AATACACACGCACGGTGTCTTCAGAGGCATTCAGGATGTGCGACGTGTGCCTGGAGTAGC 70
************************************************************

CCCGACTCTTGTACGGTCGGCATCTGAGACCAGTGAGAAACGCCCCTTCATGTGTGCTTA 1380
CCCGACTCTTGTACGGTCGGCATCTGAGACCAGTGAGAAACGCCCCTTCATGTGTGCTTA 130
************************************************************

CCCAGGCTGCAATAAGAGATATTTTAAGCTGTCCCACTTACAGATGCACAGCAGGAAGCA 1440
CCCAGGCTGCAATAAGAGATATTTTAAGCTGTCCCACTTACAGATGCACAGCAGGAAGCA 190
************************************************************

CACTGGTGAGAAACCATACCAGTGTGACTTCAAGGACTGTGAACGAAGGTTTTCTCGTTC 1500
CACTGGTGAGAAACCATACCAGTGTGACTTCAAGGACTGTGAACGAAGGTTTTCTCGTTC 250
************************************************************

AGACCAGCTCAAAAGACACCAAAGGAGACATACAGGTGTGAAACCATTCCAGTGTAAAAC 1560
AGACCAGCTCAAAAGACACCAAAGGGGACATACAGGTGTGAAACCATTCCAGTGTAAAAC 310
************************************  ************************

TTGTCAGCGAAAGTTCTCCCGGTCCGACCACCTGAAGACCCACACCAGGACTCATACAGG 1620
TTGTCAGCGAAAGTTCTCCCGGTCCGACCACCTGAAGACCCACACCAGGACTCATACAGG 370
************************************************************

TAAAACAAGTGAAAAGCCCCTTCAGCTGTCGGTGGCCAAGTTGTCAGAAAAAGTTTGCCCG 1680
TAAAACAAGTGAAAAGCCCCTTCAGCTGTCGGTGGCCAAGTTGTCAGAAAAAGTTTGCCCG 430
************************************************************

GTCAGATGAATTAGTCCGCCATCACAACATGCATCAGAGAAACATGACCAAACTCCAGCT 1740
GTCAGATGAATTAGTCCGCCATCACAACATGCATCAGAGAAACATGACCAAACTCCAGCT 490
************************************************************

GGCGCTTTGAGGGGTCTCCCTCGGGGACCGTTCAGTGTCCCAGGCAGCACAGTGTGTGAA 1800
GGCGCTTTGAGGGGTCTCCCTCGGGGACCGTTCAGTGTCCCAGGCAGCACAGTGTGTGAA 550
************************************************************
```

D)

```
MGSDVRDLNALLPAVPSLGGGGGCALPVSGAAQWAPVLDFAPPGASAYGSLGGPAPPPAP 60
------------------------------------------------------------


PPPPPPPPHSFIKQEPSWGGAEPHEEQCLSAFTVHFSGQFTGTAGACRYGPFGPPPPSQA 120
------------------------------------------------------------


SSGQARMFPNAPYLPSCLESQPAIRNQGYSTVTFDGTPSYGHTPSHHAAQFPNHSFKHED 180
------------------------------------------------------------


PMGQQGSLGEQQYSVPPPVYGCHTPTDSCTGSQALLLRTPYSSDNLYQMTSQLECMTWNQ 240
------------------------------------------------------------


MNLGATLKGVAAGSSSSVKWTEGQSNHSTGYESDNHTTPILCGAQYRIHTHGVFRGIQDV 300
------------------------------------------------------------


RRVPGVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGEKPYQCDFKDC 360
------------SASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGEKPYQCDFKDC 48
            ************************************************


ERRFSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKTHTRTHTGKTSEKPFSCRWPS 420
ERRFSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKTHTRTHTGKTSEKPFSCRWPS 108
************************************************************


CQKKFARSDELVRHHNMHQRNMTKLQLAL--- 449
CQKKFARSDELVRHHNMHQRNMTKLQLALSTP 140
****************************
```
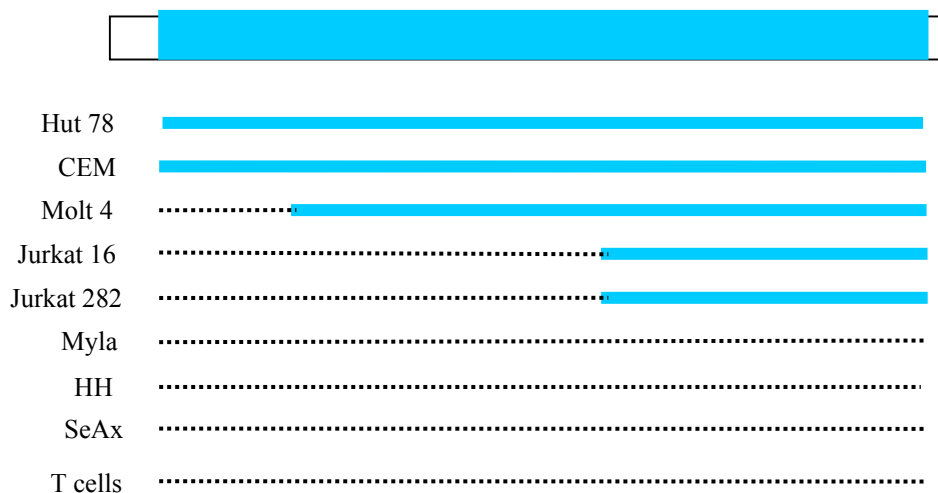
48

Figure III.3. Summary of WT1 expression pattern. The top diagram shows the amplified WT1 transcript (highlighted in blue). At the bottom, the overall length of the transcript that was amplified by RT-PCR in the indicated leukaemic cell lines and in primary T cells is shown in blue lines. Dotted lines represent absence of transcript.


1.3 Analyzing WT1 expression at the protein level

The finding that some leukaemic cell lines produce WT1 transcript does not imply that they necessarily produce WT1 protein. To further investigate whether WT1 mRNA expression corresponds to the WT1 protein expression in these cell lines, Western blot analysis for WT1 protein was carried out. Two different antibodies were used, one directed against the C-terminus of the protein (C19) and one against the N-terminus of the protein (F6). In accordance to the mRNA analysis, the full length WT1 was detected only in Hut78 and CEM cells (Fig.III.4). As mentioned above, one of the special characteristics of WT1 is its ability to produce many splice variants. The WT1 transcripts previously amplified by RT-PCR in the leukaemic cell lines Jurkat and Molt4 could be translated into truncated protein products whose functions are not known yet. However, in the Jurkat cell lines no full-length protein products were detected. This does not exclude the existence of very low molecular weight proteins which were non-detectable with the antibodies used. Moreover, in the leukaemic cell line Molt4, not all the transcript was detectable at the mRNA level. The C-terminus but not

the complete N-terminus could be amplified. Consistent with this result, at the protein level, WT1 was recognized with the antibody directed against the C-terminus. However, the antibody that recognizes the first 180 amino acids of the protein (F6) did not detect any protein product. This data demonstrates that not all T-cell lines, which have been reported to overexpress *WT1*, such as Jurkat and Molt4 (Inoue *et al*, 1994; Inoue *et al*, 1997), actually produce WT1 protein.

Figure III.4. WT1 protein expression in T-cell leukaemias. Western blot analysis of nuclear extracts from the indicated cell lines either without treatment (A) or after 24h of PMA and ionomycin stimulation (B) using two different WT1 specific antibodies, F6 directed against the N-terminus and C19 against the C-terminus as shown on the diagram on the top. The two bands indicate the exon5 splicing isoforms. U2OS was used as a positive control.
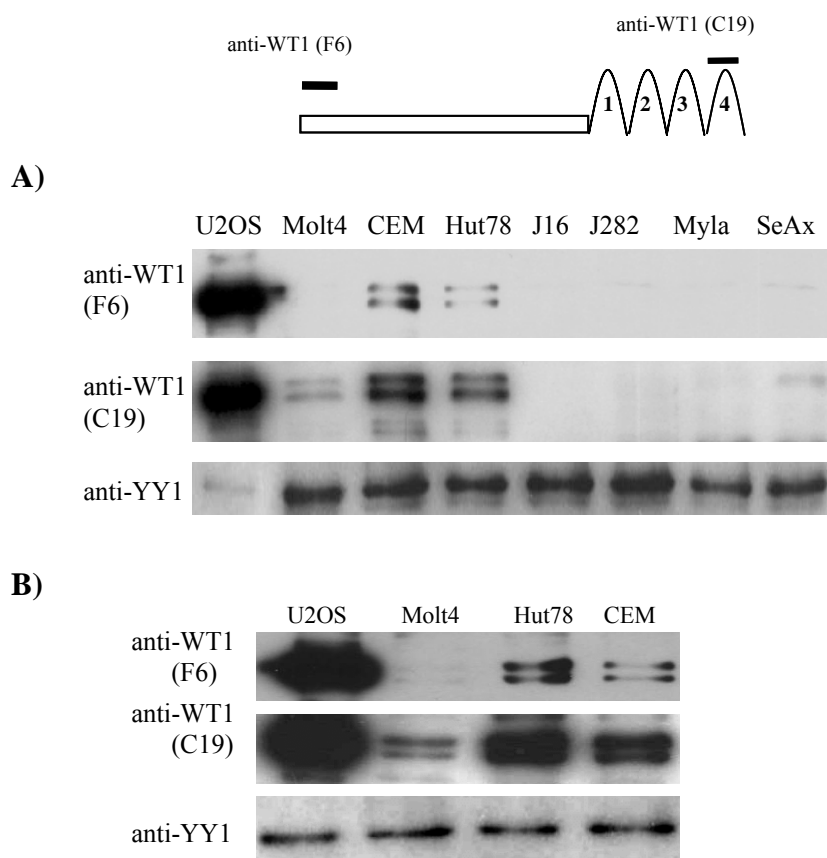
2. WT1 is involved in AICD

2.1 WT1 expression coincides with higher AICD

One of the goals of this study was to understand the role WT1 plays in the apoptosis of T cells. For this reason, all leukaemic T-cell lines were analyzed for apoptosis upon PMA and ionomycin stimulation, which mimics TCR stimulation. All the cell lines were either left untreated or were treated with PMA and ionomycin for 24h. Then they were stained with propidium iodide and DNA fragmentation was analyzed by flow cytometry the following day. After 24h of stimulation with PMA and ionomycin high levels of apoptosis were seen for the WT1-expressing cell lines Hut78 and CEM (Fig.III.5). To determine whether the elevated apoptosis detected in Hut78 and CEM (24h after stimulation) was mediated by the CD95 system, an antibody against the CD95L (anti-FasL) was used to inhibit the effect of CD95/CD95L-mediated apoptosis. The apoptosis in Hut78 and CEM induced by PMA and ionomycin was completely blocked in the presence of the antibody against CD95L (Fig.III.6A and B). These experiments indicate that WT1 might play an important role in CD95-mediated AICD in T cells.

Figure III.5. Apoptosis in leukaemic T-cell lines correlates with WT1 expression. Leukaemic T-cell lines were induced with PMA and ionomycin for 24h before flow cytometry analysis of propidium iodide staining for DNA fragmentation. Non-induced T-cell lines were used as controls. One representative of five experiment is shown.
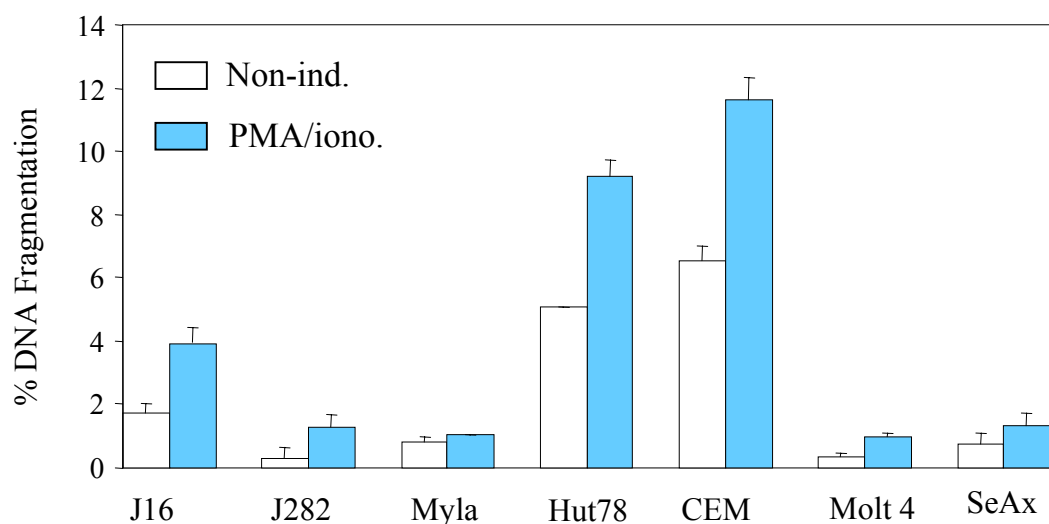
Figure III.6.   Apoptosis of WT1-expressing leukaemias upon TCR stimulation is CD95L-dependent.   (A) CEM apoptosis and (B) Hut78 apoptosis could efficiently be blocked with anti-CD95L antibody.   Cells were either left untreated (open bars) or were stimulated with PMA and ionomycin for 24h (coloured bars).   Antibody against the CD95L (anti-CD95L) (pink bars) and an isotype IgG1 antibody (grey bars) were added 30mins before PMA and ionomycin treatment.

| A) CEM					B) Hut78				
PMA/Iono	-	+	+	+	PMA/Iono	-	+	+	+
Anti-CD95L	-	-	+	-	Anti-CD95L	-	-	+	-
IgG1	-	-	-	+	IgG1	-	-	-	+
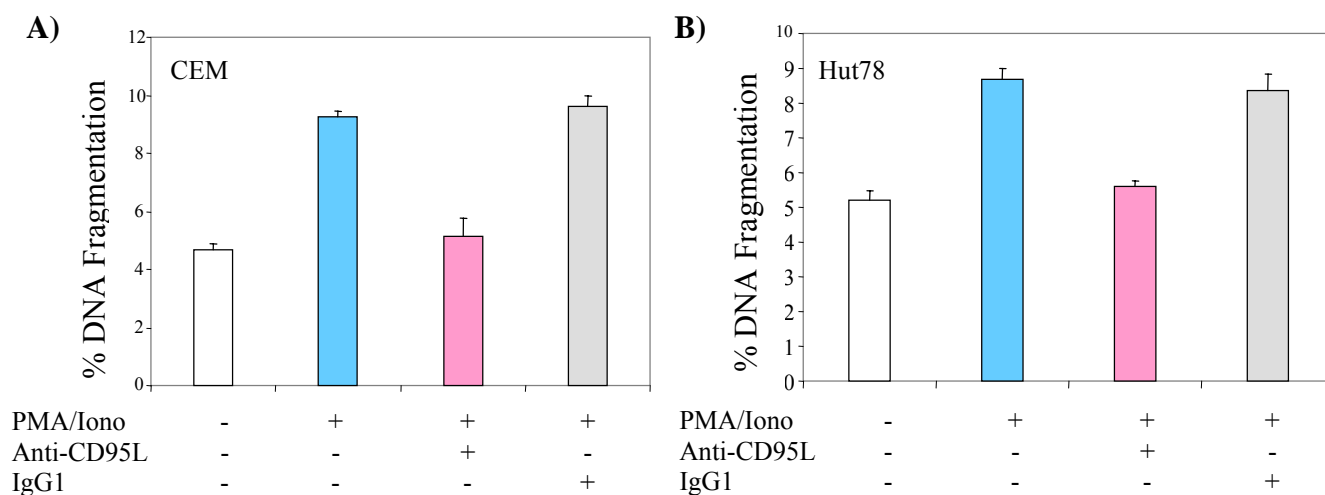
2.2 Ectopic expression of WT1 in WT1-non-expressing T-cell leukaemia enhances AICD

To further analyze the actual role of WT1 in AICD of T-cell leukaemias, two different approaches were followed. In the first approach, WT1 was introduced into J16 cells, which do not express WT1, to examine if WT1 would enhance apoptosis. In the second approach, the *WT1* gene was silenced by *WT1* siRNA in CEM cells that express WT1, to see whether knocking down *WT1* would lead to a reduction in AICD.

For the first approach, J16 cells had to be transfected and then apoptosis would be analyzed. J16 cells, however, are very difficult to transfect and very sensitive to such treatments. Therefore, careful analysis for transfection methods was necessary and nucleofection by Amaxa proved to be one of the best.   Still, optimization had to be carried out before each experiment to ensure high transfection efficiency (50-80%) with as low background cell death as possible (Fig.III.7).

Figure III.7. Optimized transfection conditions for J16 cells. Jurkat 16 cells were transfected with a GFP-expressing construct and analyzed for cell death after transfection by flow cytometry. The living cells were gated (left panel, shown in red) and percent fluorescence was calculated on the gated living cells (right panel). A >50-80% fluorescence was used for further experiments.
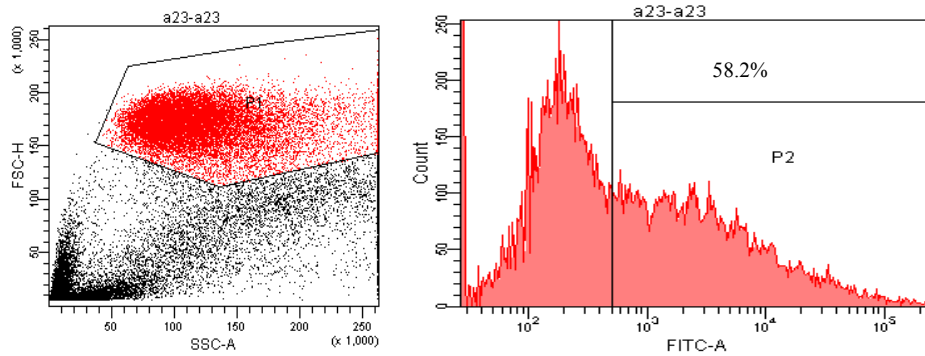
Once nucleofection conditions were optimal, J16 cells were transiently transfected with empty

vector pCMV or WT1+KTS or WT1-KTS. After overnight recovering, the cells were either

left untreated or were stimulated with PMA and ionomycin and apoptotic cell death was

measured 24h after stimulation by flow cytometry. Stimulated cells transfected with WT1-

KTS were also treated with an antibody against the CD95L or isotype control antibody. The

expression of WT1-KTS in J16 cells resulted in more than a 3-fold increase in the AICD

levels, whereas WT1+KTS had no effect. Blocking with the antibody against the CD95L

confirmed that the apoptosis induced was CD95L-mediated (Fig.III.8A). Calculation of the

percentage of specific apoptosis showed that WT1-KTS brought about a 5-fold increase when

compared to empty vector or to WT1+KTS (Fig.III.8B). In addition, Western blot analysis of

nuclear extracts from the transfectants confirmed successful expression of both variants

indicating that the effect seen was not due to unequal expression but was the result of the

WT1-KTS action (Fig.III.8C).

Figure III.8. Ectopic expression of WT1-KTS increases AICD in Jurkat leukaemia.
(A) J16 cells were transfected with empty vector CMV, WT1+KTS or WT1-KTS and then either left untreated (open bars) or stimulated with PMA/ionomycin for 24h (coloured bars). The stimulated cells were also treated with anti-CD95L or isotype control. % DNA fragmentation was analyzed by flow cytometry for propidium iodide positive cell staining. (B) % Specific apoptosis of J16 cells transfected with either empty vector CMV, WT1-KTS or WT1+KTS. (C) Western blot analysis with WT1-C19 antibody on nuclear extracts from the transfectants showing that both WT1 constructs are expressed in J16 cells after transfection. CEM nuclear extract is shown for positive control and YY1 for loading control. One of three experiments is shown.

A)
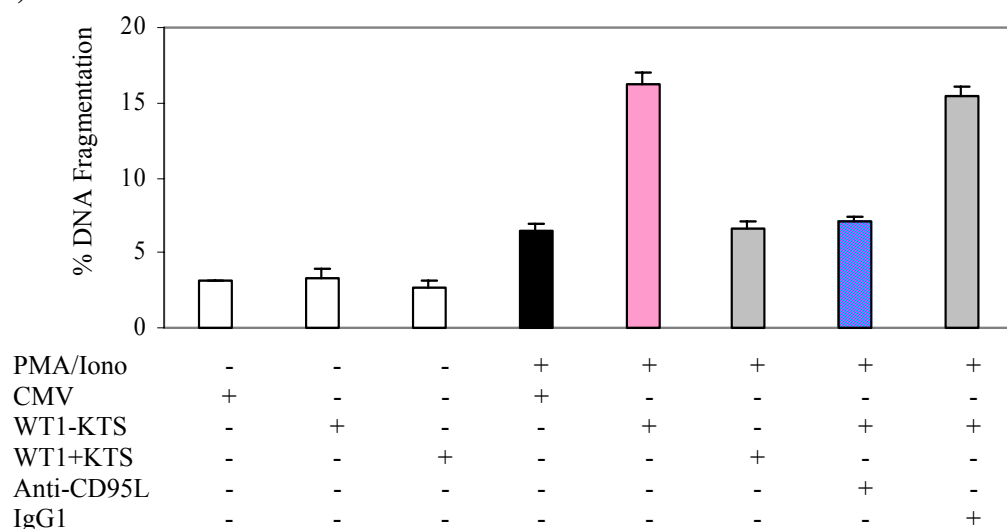
B)
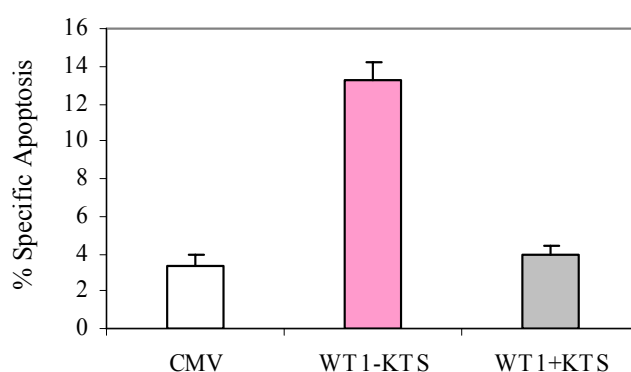
C)
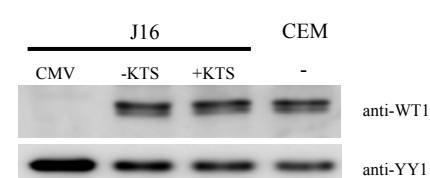
2.3 Silencing of *WT1* in WT1-expressing T-cell leukaemia reduces AICD

In the second approach, to verify that WT1 is an important player in AICD of CEM cells, *WT1* had to be silenced. CEM cells constitute another sensitive cell line that requires gentle handling and successful transfection without massive death is almost inevitable. Several

transfection methods for the introduction of the siRNA were tested but the HiPerfect system (Qiagen) proved to be the only system that allowed efficient knock-down avoiding cell death due to the procedure itself. The efficiency of the transfection was determined by introducing different concentrations of fluorescent oligonucleotides and analyzing the transfectants 24h later by flow cytometry. Non-transfected cells or cells transfected with buffer alone did not produce any fluorescence (Fig.III.9A). Addition of increasing amounts of fluorescent oligonucleotides resulted in a dose-dependent increase in fluorescence. Furthermore, the aim of this experiment was to look for the apoptotic pattern of CEM cells after stimulation when *WT1* is knocked-down. Therefore, it was necessary to certify that any effects seen would not be due to the siRNA introduction procedure. For this reason, CEM cells were either not-transfected, control-transfected with scrambled oligonucleotides or transfected with increasing concentrations of *WT1*-siRNA. Apoptosis was assayed 24h post-transfection, when no decrease in the WT1 levels could be detected thus far (Fig.III.9B). The transfection procedure resulted in an increase in apoptosis which was unaffected by the addition of increasing concentrations of siRNA. These results suggest that the procedure indeed sensitized CEM cells, an event that could not be avoided, but it also shows that the oligonucleotide against *WT1* did not lead to an increase nor did it lead to a decrease in apoptosis. Therefore any effects seen after the knock-down of *WT1* are expected to be entirely due to the absence of WT1.

Once the silencing transfection system was optimized, the siRNA against *WT1* was introduced into CEM cells. After 48 hrs of recovering, the cells were stimulated with PMA and ionomycin. Activation induced apoptotic cell death was measured 24 hrs after stimulation and the WT1 expression levels were controlled by Western blot. The introduction of scrambled oligonucleotides in CEM cells had no effect in WT1 expression whereas the *WT1*-siRNA resulted in the down-regulation of WT1 expression in a dose dependent manner (Fig.III.10A).

Since best down-regulation of WT1 expression was achieved with siRNA concentrations of

120 and 180nM, these were used in the subsequent experiments, which showed that AICD

was suppressed by the *WT1*-siRNA in a dose-dependent manner (Fig.III.10B). Moreover, the

negative values obtained for the specific apoptosis assay of CEM cell transfected with 180nM

of *WT1*-siRNA indicated that not only apoptosis induction was refrained, but that there was

also proliferation. These results further confirm an important role of WT1 in AICD of T cells.

Figure III.9. Establishing the *WT1*-specific siRNA system. (A) CEM cells were either not transfected (-), mock transfected (buffer) or transfected with increasing concentrations of green fluorescent oligonucleotide for 24h and fluorescence was detected with flow cytometry. (B) CEM cells were either not transfected (-), mock transfected (buffer) or transfected with the indicated increasing concentrations of *WT1*-siRNA. 24h post-transfection specific apoptosis was assayed by flow cytometry for nuclei fragmentation.
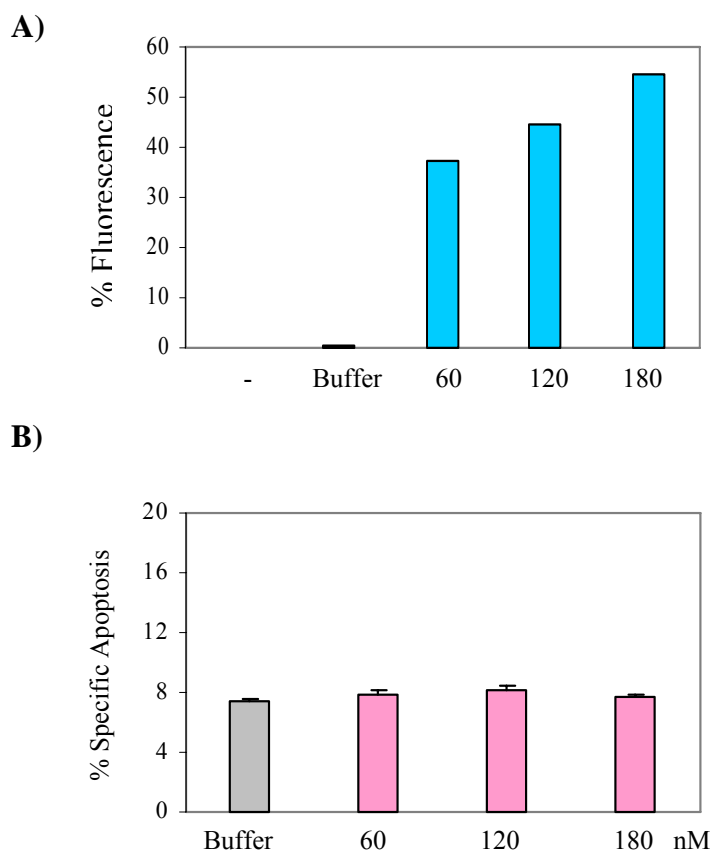
A)

B)

Figure III.10. ***WT1*** **specific siRNA decreases AICD in CEM leukaemia.** (A) Western blot analysis with WT1-C19 antibody of nuclear extracts from CEM cells either control transfected with scrambled oligonucleotide (-) or transfected with the indicated increasing amounts of *WT1* specific siRNA. Transcription factor YY1 was used as a loading control. (B) Specific apoptosis was assayed 24h after PMA and ionomycin stimulation of CEM cells transfected for 48h with different amounts of WT1 specific siRNA or scrambled oligonucleotides for control. The experiment shown is representative of five.
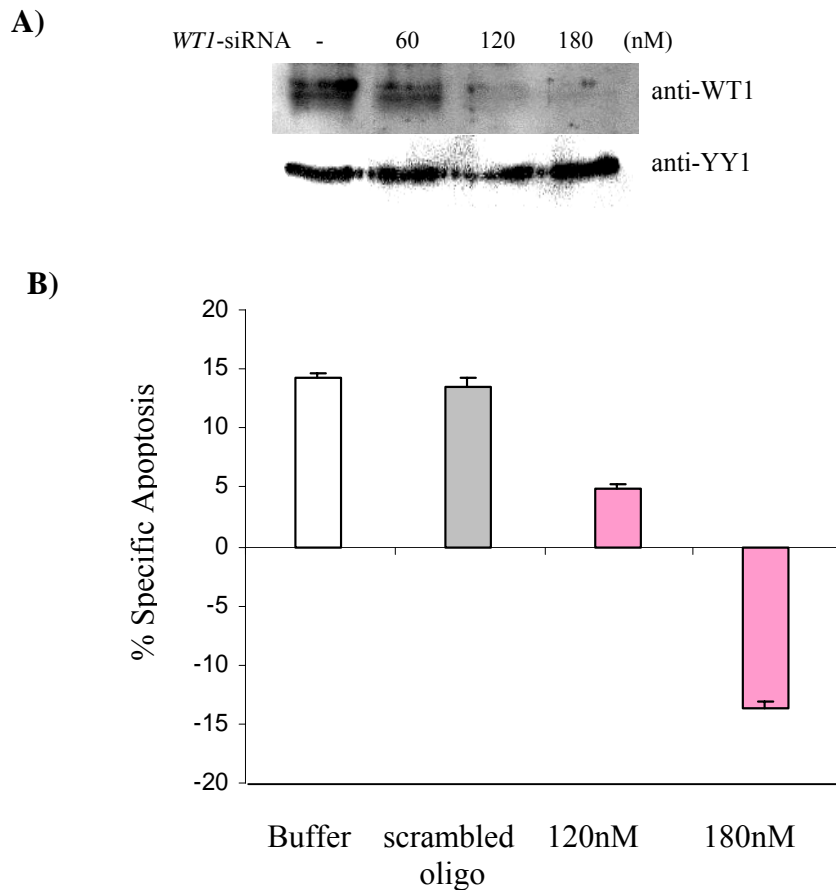
3. WT1 is involved in CD95L expression

3.1 WT1 expression coincides with higher CD95L expression

WT1 is a zinc-finger transcription factor with target sequences similar to Egr1 (Lee & Haber, 2001). As it has been previously shown that Egr1 binds and up-regulates the CD95L promoter after T cell stimulation (Li-Weber *et al*, 1999) and that the apoptosis seen in WT1-expressing T-cell leukaemias is CD95L-mediated (Fig.III.11), it was of interest to see whether WT1 plays a role in the regulation of the CD95L gene expression. To investigate this, the CD95L

mRNA expression levels were analyzed by reverse-transcriptase PCR and quantitative real-time PCR in all the leukaemic cell lines. The results showed higher basal expression levels of CD95L mRNA in the WT1-expressing cell lines Hut78 and CEM when compared with the WT1-non-expressing cell lines. The CD95L mRNA expression levels were further up-regulated upon PMA and ionomycin stimulation in all cell lines, but the increase in the WT1-expressing cell lines Hut78 (4-fold) and CEM (5-fold) was notably stronger than in the WT1-non-expressing cell lines (Fig.III.11). This data indicates that WT1 may promote the expression of CD95L at the transcriptional level.

Figure III.11. WT1 expression coincides with higher CD95L expression.
(A) Amplification by RT-PCR of the CD95L cDNA for the different leukaemias either without stimulation (-) or induced for 1.5h with PMA and ionomycin (+). (B) Respective quantitative real-time PCR analysis of the CD95L for the different leukaemias either without stimulation or induced for 1.5h with PMA and ionomycin.
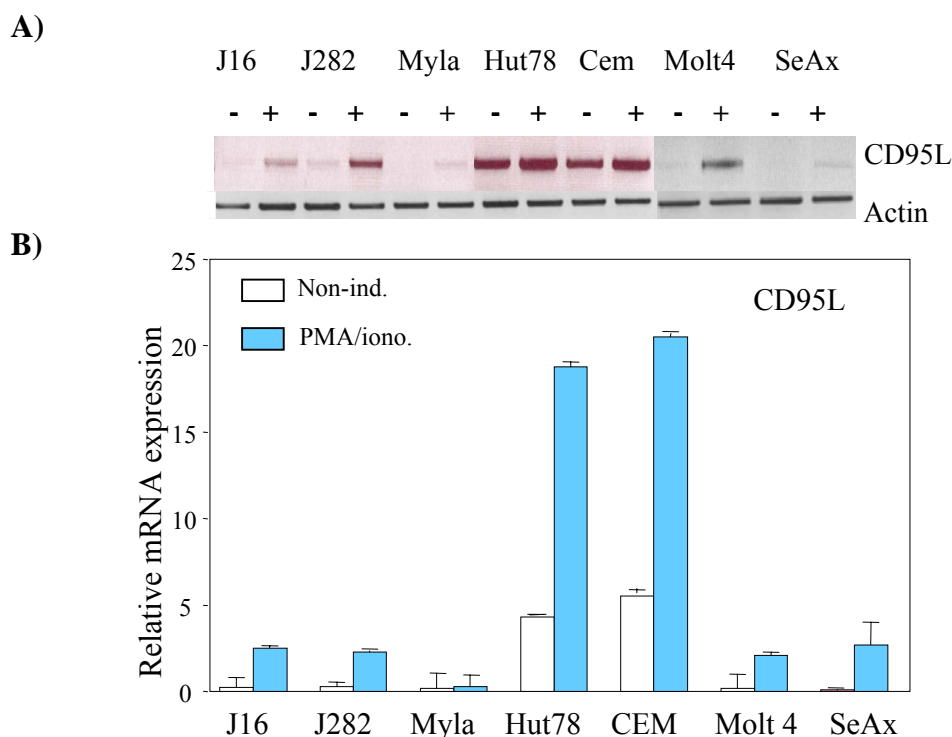
3.2 WT1 expression does not correlate with CD95 expression

To verify that the apoptosis induction was due to CD95L up-regulation and was not dependent on increased levels of CD95, CD95 mRNA levels were examined with reverse transcriptase PCR. This experiment showed that CD95 levels remained almost unchanged among the different cell lines (Fig.III.12A). Quantitative real-time PCR was then carried out in order to obtain more detailed expression levels, since small changes would not have been clearly detected by a non-quantitative method. The quantitative real-time PCR showed that the receptor's mRNA levels varied between non-stimulated and stimulated conditions, but the overall differences in expression did not correlate with WT1 expression (Fig.III.12B). For example, in both J16 and CEM, CD95 levels were increased after stimulation, but only CEM was shown to express WT1. Since mRNA levels 1h30mins after stimulation did not necessarily reflect the amount of receptor expressed on the surface when apoptosis was examined, CD95 protein levels present on the surface of the cells were analyzed. Therefore, cell surface staining for CD95 was carried out in all cell lines 24h after stimulation. The CD95 levels on the surface of the leukaemic cell lines studied were very high and remained high after induction with PMA and ionomycin irrespective of WT1 expression (Fig.III.12C). These results suggest that WT1 did not have an effect on CD95 and the increase in the apoptosis levels upon PMA and ionomycin stimulation in the WT1-expressing cell lines, hence depends on CD95L expression.

Figure III.12. WT1 expression does not correlate with CD95 expression.
(A) Amplification by RT-PCR of the CD95 cDNA for the different leukaemic cell lines either without stimulation (-) or induced for 1.5h with PMA and ionomycin (+). (B) Quantitative real-time PCR analysis of CD95 for the different leukaemias either without stimulation or induced for 1.5h with PMA and ionomycin and (C) All the cell lines either non-treated or induced overnight with PMA and ionomycin were stained for cell surface CD95 with a CD95-specific antibody (anti-APO) and staining was detected by flow cytometry. Non-stained cells and isotype controls are shown.

A)

B)

C)
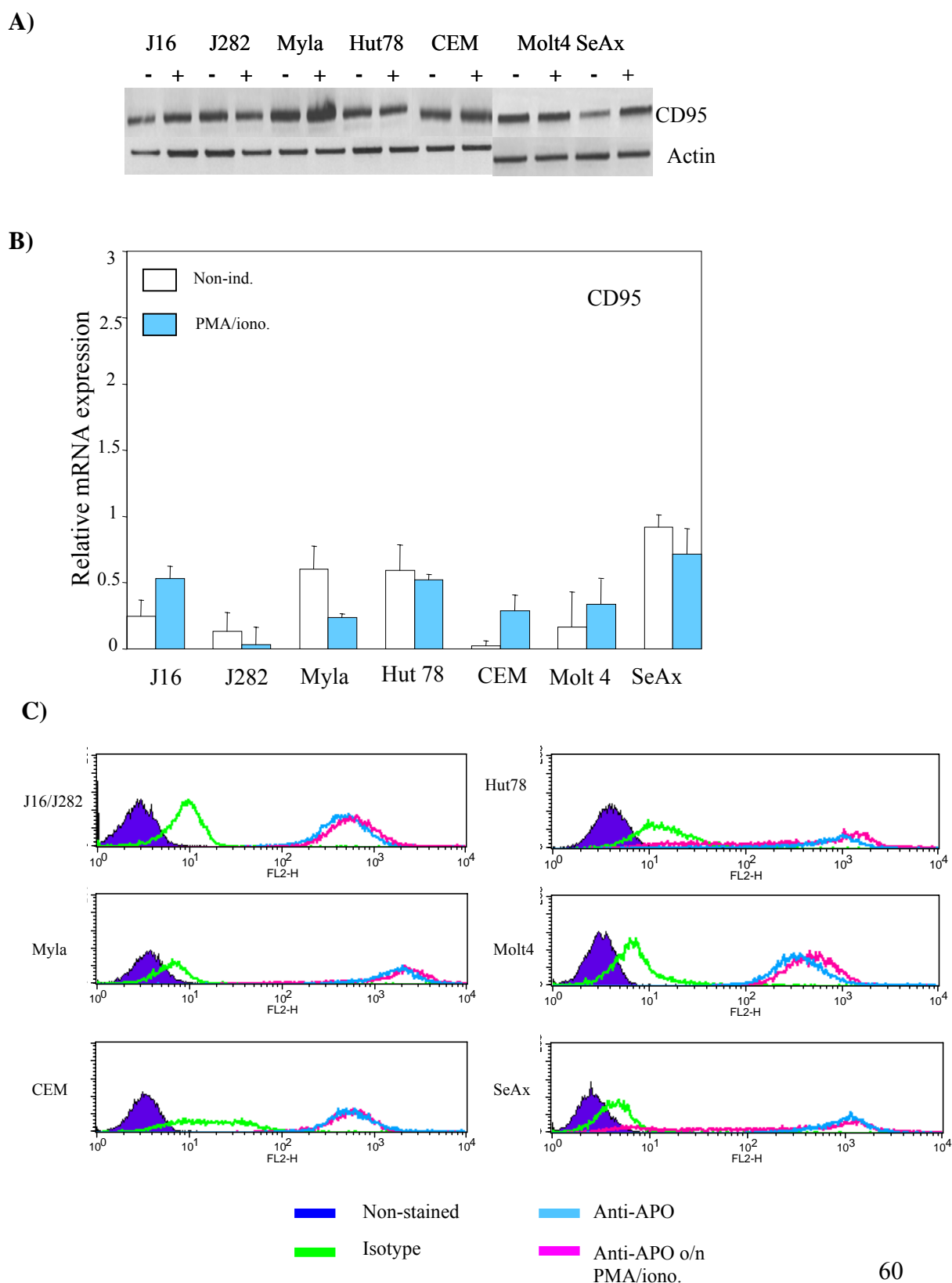
3.3 Ectopic expression of WT1 in a WT1-non-expressing T-cell leukaemia enhances CD95L mRNA expression

In order to gain a deeper insight in the relation between WT1 and CD95L expression, WT1 was introduced into the WT1-non-expressing Jurkat T cells by transient transfections. Two WT1 expressing plasmids, one encoding the variant form of WT1 with high DNA-binding-affinity (WT1-KTS) and one encoding the variant form of WT1 with low DNA-binding-affinity (WT1+KTS), were used to transfect J16 cells and the CD95L expression levels were then analyzed by quantitative real-time PCR. Ectopic expression of the WT1-KTS variant resulted in a 9-fold increase in CD95L mRNA expression, whereas only a 2-fold increase in the CD95L mRNA was seen in the WT1+KTS transfected cells (Fig.III.13A). The different effects of the two plasmids in the CD95L mRNA expression were not due to unequal expression in the transfected cells, since both WT1 variants were equally expressed 24h post-transfection (Fig.III.13B). These results indicate that WT1 may enhance the CD95L mRNA expression.

Figure III.13. Ectopic expression of WT1-KTS upregulates the CD95L. (A) Quantitative real-time PCR of CD95L and CD95 after transient transfection of J16 cells with WT1+KTS, WT1-KTS or empty vector CMV. The experiment shown is one of three reproducible ones. (B) Western blot analysis of whole cell lysates from the transfected cells using the anti-WT1 C19 antibody to detect the expression of WT1 protein 24h post-transfection. The two bands appearing in the control empty vector pCMV transfection are due to unspecific binding of the polyclonal antibody used on whole cell lysates.
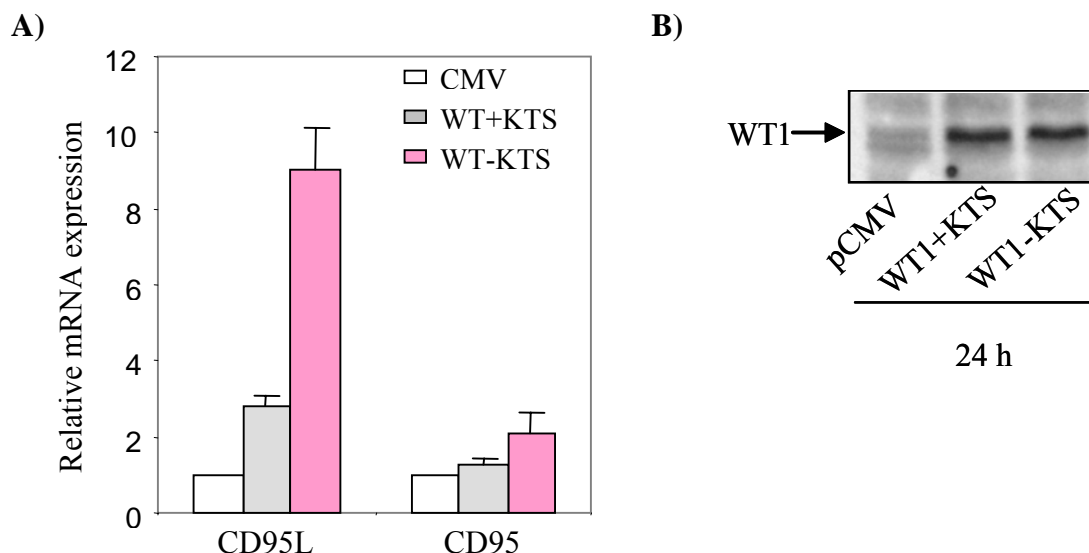
3.4 Silencing of *WT1* in WT1-expressing T-cell leukaemia reduces CD95L mRNA expression

In order to study the effect of *WT1* silencing on CD95L expression, CEM cells were left untreated or were transfected with either scrambled oligonucleotides or with *WT1*-siRNA. After an incubation of 70h to allow silencing to take place, the cells were stimulated with PMA and ionomycin for 1h30mins and total mRNA was extracted. The mRNA was then reverse-transcribed and the cDNA was used for quantitative real-time PCR of the CD95L. The introduction of *WT1*-specific siRNA, resulted in a drop of CD95L expression levels in a dose-dependent manner (Fig.III.14). Addition of scrambled oligonucleotides did not alter CD95L expression compared to untreated CEM control cells.

Figure III.14. Silencing of *WT1* decreases expression of CD95L mRNA. Real-time PCR for CD95L mRNA expression 70h after transfection of CEM cells with either scrambled oligonucleotides or the indicated concentrations of *WT1*-specific siRNA and 1.5h of PMA and ionomycin stimulation.
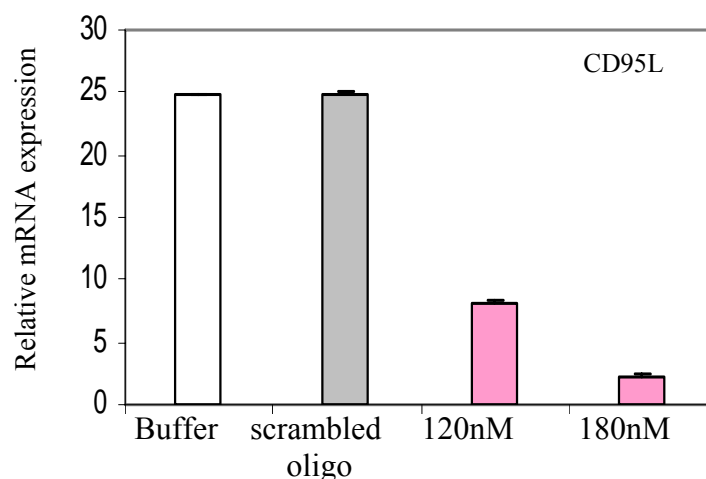
4. WT1 activates the CD95L promoter

4.1 Ectopic expression of WT1 increases CD95L promoter activity

The most putative way that WT1 could affect CD95L transcription would be by directly acting on its promoter through the Egr1 binding sites and hence altering its transcriptional levels. To examine whether WT1 enhances CD95L expression at the transcriptional level, J16 cells were co-transfected with a CD95L promoter-luciferase construct (wt) (Fig.III.15) together with either the WT1+KTS or the WT1-KTS plasmid construct. Analysis of the luciferase production showed that both WT1 constructs enhanced the CD95L promoter activity. The effect was higher when the WT1-KTS expression plasmid was co-transfected (Fig.III.16). To confirm that WT1 enhances promoter activity through the Egr-binding sites, a CD95L promoter-luciferase construct where all three Egr binding sites were mutated (Egr-mut) was included in the same setting. The data obtained showed that mutations at the Egr sites significantly diminished the effects of WT1 on the CD95L promoter (Fig.III.16).

Figure III.15. Egr1 binding sites on the CD95L promoter. Schematic representation of the composite Egr1/NF-AT binding sites on the CD95L promoter relative to the transcription start site (top) and respective DNA binding sequences of Egr1 (highlighted in pink) and NF-AT (highlighted in blue) used in luciferase reporter constructs and for which oligonucleotides were designed (bottom).
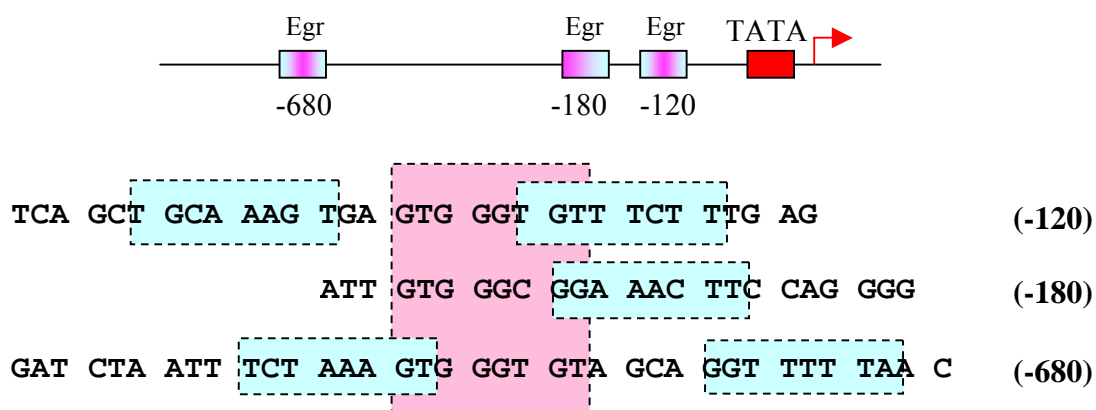
Figure III.16. WT1 expression increases CD95L promoter activity. Luciferase activity of the CD95L promoter after co-transfection of J16 cells with either a wild-type CD95L promoter-luciferase construct or an Egr1-mutated CD95L promoter-luciferase construct in combination with WT1+KTS, WT1-KTS or empty vector as control (-). Cells were either non-induced or induced for 8h with PMA and ionomycin.
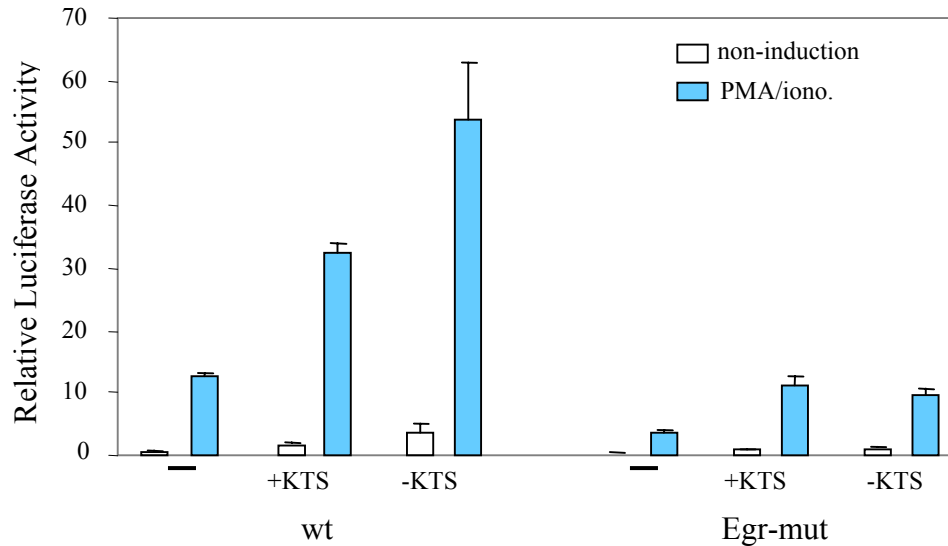
4.2 WT1-KTS driven CD95L promoter activity occurs through Egr1 sites

To further verify that the CD95L Egr sites could confer the WT1-mediated transcriptional activation, co-transfection experiments in J16 cells were performed using luciferase reporter plasmids containing 3 copies of each Egr binding site. Each luciferase construct was transfected along with the WT1+KTS, the WT1-KTS plasmid or an empty vector as a control. In all experiments, the WT1+KTS had no effect on CD95L induction, which was similar to the empty vector. The WT1-KTS however, induced high luciferase production from all binding sites (Fig.III.17A). This effect was specific to the Egr binding sites, since WT1-KTS did not have any effect on a luciferase reporter construct containing 5 copies of the Sp1 binding site (Fig.III.17B).

Figure III.17. WT1-KTS driven CD95L promoter activity occurs through Egr1 sites.
(A) J16 cells were co-transfected with a CD95L promoter-luciferase construct (containing three copies of either the -120, the -180 or the -680 Egr1 sites) and WT1+KTS, WT1-KTS or empty vector CMV. (B) Co-transfection of J16 cells with a CD95L promoter-luciferase construct containing five copies of the Sp1 binding site along with either WT1+KTS, WT1-KTS or empty vector CMV.
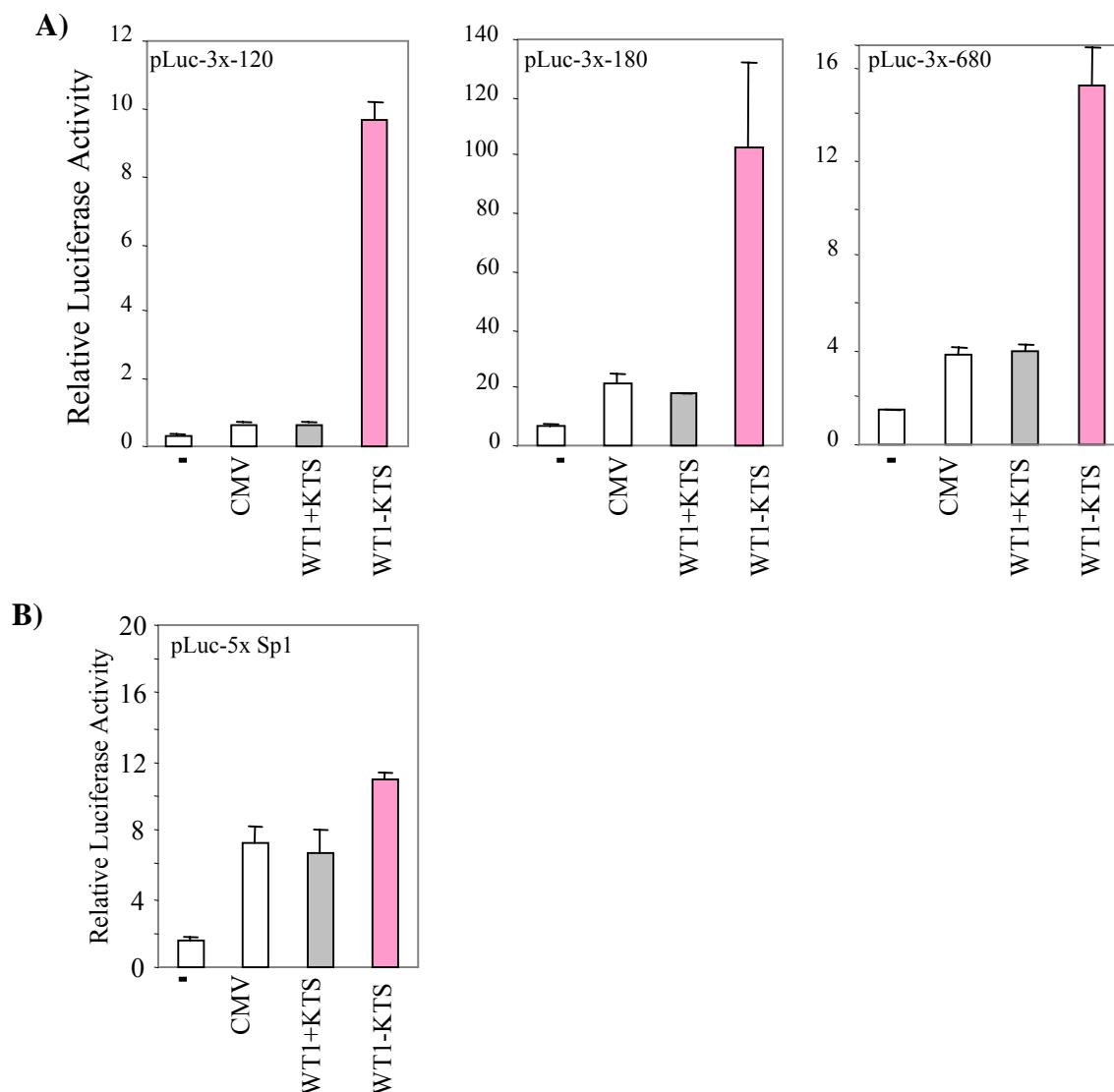
To further confirm that the CD95L Egr sites could confer the WT1-mediated transcriptional activation during AICD, the same co-transfection experiments were performed using luciferase reporter plasmids containing 3 copies of each Egr binding site, but the cells were also stimulated with PMA and ionomycin for 24h (Fig.III.18). As it was seen, WT1+KTS had no effect on the CD95L promoter after stimulation since the levels of luciferase

production were similar to the background activation levels of the promoter, which occured

with empty vector CMV on all three Egr1 binding sites. On the contrary, all three Egr1

binding sites were responsive to WT1-KTS with a higher effect detected at the -120 and -180

sites (Fig.III.18). This data further supports WT1-KTS action on the CD95L promoter

through the Egr1 binding sites.

**Figure III.18. WT1-KTS driven CD95L promoter activity occurs through Egr1 sites after T cell activation.** J16 cells were co-transfected with a CD95L promoter-luciferase construct containing three copies of either the -120, the -180 or the -680 Egr1 sites along with a plasmid expression construct of either WT1+KTS (grey bars) or WT1-KTS (blue bars) or with empty vector CMV (empty bars) for control. The transfectants were either left untreated (-) or were stimulated with PMA and ionomycin for 24h (+).
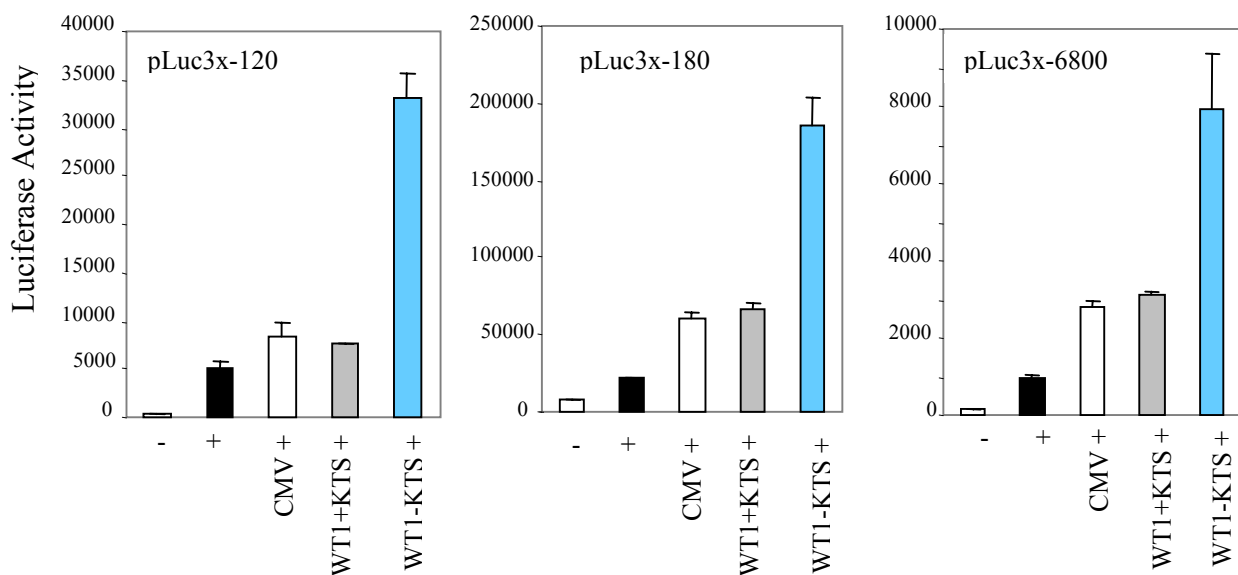
5. WT1 binds directly on the CD95L promoter

5.1 Recombinant WT1-KTS binds on the CD95L promoter

To further investigate whether WT1 directly binds to the CD95L Egr sites, recombinant

WT1+KTS and WT1-KTS proteins were analyzed for their ability to associate with

oligonucleotides encoding each of the three Egr binding sites (Fig.III.15) by an

electrophoretic mobility shift assay (EMSA) (Fig.III.19). All three sites showed binding of

the WT1-KTS but not of the WT1+KTS. In agreement with the luciferase reporter results, the

strongest binding was shown at the -120 site. This data demonstrates that WT1 enhances

CD95L transcription by directly interacting with at least one of the Egr binding sites of the

CD95L promoter.

Figure III.19. WT1-KTS can bind on the CD95L promoter.
Recombinant WT1+KTS or WT1-KTS proteins were used to probe oligonucleotides representing the three Egr1 binding sites on the CD95L promoter at an electrophoretic mobility shift assay.
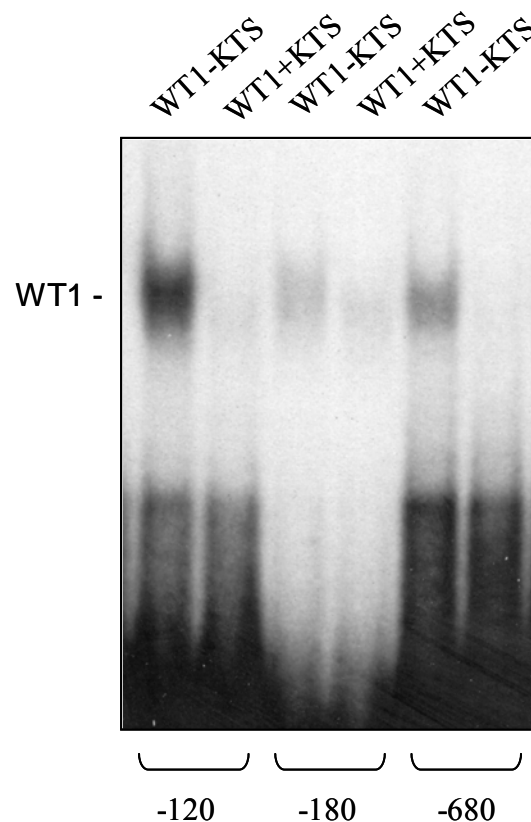
5.2 WT1 binds on the CD95L promoter at -120 in WT1-expressing T-cell

leukaemia

As WT1 has already been shown to be expressed in CEM leukaemia (Fig.III.1& 4) and to

bind directly to the CD95L promoter (Fig.III.19), it was of importance to check whether WT1

was also able to bind on the CD95L promoter in CEM. For this reason, nuclear proteins from

CEM cells either stimulated with PMA and ionomycin for 3h or left untreated, were analyzed

for their ability to interact with oligonucleotides representing the Egr binding sites on the

CD95L promoter by EMSA. The EMSA carried out with oligonucleotide probes representing

the −120 site, clearly showed, that after stimulation differential banding patterns appear, with

two new protein complexes being prominently formed on that site of the promoter

(Fig.III.20). To define whether the nuclear proteins of interest were part of these complexes,

a supershift assay was carried out, in the presence of antibodies against Egr1 and WT1. With

the antibody against Egr1 the bottom complex was shifted such that it disappeared completely

and the top complex was highly reduced. Therefore, after stimulation Egr1 was involved in

the formation of at least two complexes at -120 on the CD95L promoter. In addition, in the

presence of the antibody against WT1, it was seen that both bands were again reduced in their

intensity, indicating that WT1 was also involved in the formation of these complexes at -120

(Fig.III.20). However, the addition of an antibody against Sp1 had no effect in band shifting

or intensity, highlighting binding specificity for Egr1 and WT1 at -120 on the CD95L

promoter.

Figure III.20. WT1-KTS binds on the CD95L promoter at -120 in WT1-expressing-CEM leukaemia. Oligonucleotide probes representing the -120 Egr1 binding site on the CD95L promoter were used to detect binding of nuclear proteins from CEM cells either without stimulation (-) or after 3h of PMA and ionomycin stimulation (+). Egr1 and WT1-F6 antibodies were used for supershift assay. Sp1 antibody controls the supershift reaction and NF-Y is shown for loading control. New prominent complex formation after stimulation is indicated by blue clammer and the position of the WT1-binding complex by red clammer. Stars depict non-specific complexes. Shown, is one experiment representative of three reproducible ones.
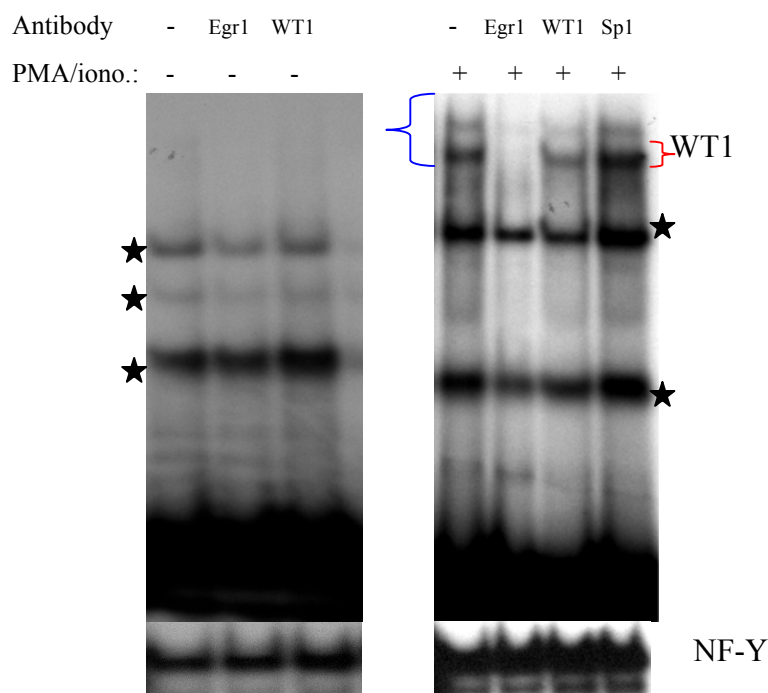
IV Discussion

The role of WT1 in leukaemogenesis has been very controversial. Several studies have shown that it is mutated in leukaemias (King-Underwood *et al*, 1996; King-Underwood & Pritchard-Jones, 1998), whereas others have indicated that it is overexpressed (Inoue *et al*, 1997; Menssen *et al*, 1995). These discrepancies have resulted in two different opinions on WT1's role in leukaemogenesis. On one hand it is thought that it is a tumor suppressor, and on the other hand it is thought that it acts as a tumor survival factor. It is likely that WT1 can be attributed both characteristics depending on the cell context, its expression pattern and mutation status during development. In combination, these factors can eventually lead to contradictory but understandable effects (Fig.IV.1).

Figure IV.1. Schematic representation of the differential effects of WT1 on leukaemogenesis. WT1 is expressed during the early stages of haematopoiesis (red line). If its expression is lost prematurely or if loss of function mutations occur, then this can lead to leukaemia and gives WT1 a tumor suppressor function (blue pathway). If however, its expression continues after the normal stages (red dotted line) or a dominant-negative mutant protein is produced then this can also lead to leukaemia (red pathway) giving however WT1 a tumor promoter function.
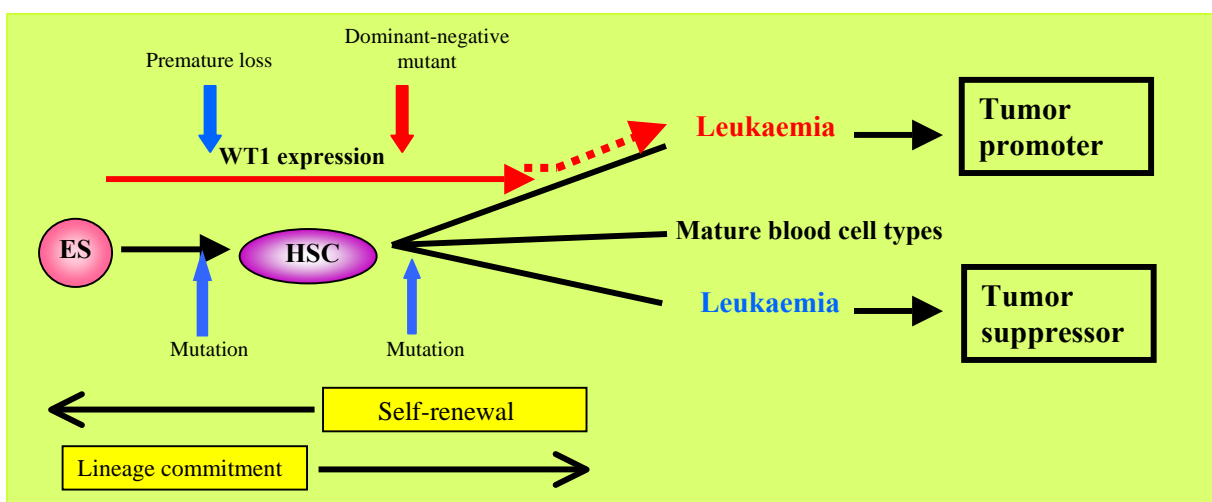
It is known that WT1 expression is restricted during the early stages of haematopoiesis (Maurer *et al*, 1997). Also, it is suggested that WT1 is involved in apoptosis induction by upregulating the pro-apoptotic protein Bak (Morrison *et al*, 2005) and in apoptosis inhibition by upregulating the anti-apoptotic Bcl-2 (Mayo *et al*, 1998). Bearing this in mind, two different scenarios can be explained. In the first case, if WT1 is mutated (loss of function mutations) at an early stage during haematopoiesis 'when its expression might be essential for apoptosis induction (e.g., negative selection)' this would lead to leukaemia, and hence WT1 could be characterized as a tumor suppressor. In the second case, if expression of WT1 continues after the appropriate time, this would probably affect the proliferation status of the WT1-expressing cells, giving them a growth advantage (through Bcl-2 upregulation) and hence promoting tumor growth. In addition, if a mutation which gives rise to a dominant-negative effect occurs at early stages of development, then this would also have a tumor growth promoter function.

However, WT1's role in T cell leukaemogenesis and T cell apoptosis is not well studied. Therefore, it was of interest to study its expression patterns and mutation status in T cell leukaemias and characterize its involvement in CD95/CD95L-mediated apoptosis. Because WT1 is not expressed in mature T cells the study was initiated in leukaemic T-cell lines, of which some were already reported to overexpress WT1.

1. WT1 expression analysis in leukaemic T-cell lines

In this analysis, eight leukaemic T-cell lines were used, namely CEM, Hut78, Molt4, Myla, SeAx, HH and two Jurkat sub-clones, Jurkat 16 and Jurkat 282. The expression patterns of WT1 were analyzed both at the mRNA and at the protein level. At the mRNA level, RT-PCR

reactions which amplified the WT1 transcript from nucleotide 784 (exon1) to nucleotide 1800 (end of transcript) were carried out. The RT-PCR showed that only CEM and Hut78 express full-length WT1 transcript. Jurkat 16, Jurkat 282 and Molt4 express part of the transcript (C-terminal part) and Myla, SeAx and HH do not express WT1 at all (Fig.III.1). Cloning and sequencing of the amplified regions showed that all parts of the transcript expressed were wild-type and no mutations could be identified (Fig.III.2).

Because mRNA expression does not necessarily imply protein expression, to confirm WT1 expression, Western blot analysis for the WT1 protein was carried out (Fig.III.4). WT1 protein could not be detected at all in the cell lines Jurkat 16 and Jurkat 282, in Myla, SeAx and HH. The finding that Jurkat cells do not produce WT1 is contradictory to a previous report, whereby RT-PCR alone on the C-terminus of the transcript suggested that Jurkat cells overexpress WT1 (Inoue *et al*, 1997). It is possible, however, that in Jurkat where C-terminal transcript is detected, that a truncated WT1 protein is produced, but was not detected in this piece of work. Further investigation of this was beyond the scope of this study as the aim was to screen for the presence or absence of wild-type WT1 expression and these results indicate that Jurkat cells do not produce wild-type WT1 protein.

Moreover, in the cell line Molt4, the N-terminus of the transcript (exons 1-3) was not detected. The protein analysis showed a WT1 protein that could be detected with the antibody against the C-terminus but not with the antibody against the N-terminus (which detects the first 180 amino acids of the protein). It is questionable whether this is indeed a WT1 protein product, as the C-terminal antibody is polyclonal and sometimes detects some protein at the same molecular weight of WT1, even in the cell lines that do not express WT1 at all, e.g., SeAx and HH. This is one of the reasons the antibody against the N-terminus was used. It can be seen that with this antibody, no actual WT1 protein is detected in Molt4. The

finding that Molt4 does not produce WT1 disagrees with the previous report in which only an RT-PCR against the C-terminus of the transcript was performed. In that study, it was therefore mistakenly suggested that WT1 is overexpressed in Molt4 (Inoue *et al*, 1997). Collectively, the RT-PCR and the Western blot analysis results in my work supported the conclusion that wild-type WT1 is expressed only in the leukaemic cell lines CEM and Hut78. This analysis of WT1 expression is by now one of the most representative, as previous research was based mainly on reverse-transcriptase PCR done only on the C-terminal part of the transcript (exons 6-10), and in most cases no protein analysis was ever done.

The above findings highlight the importance of a more careful WT1 expression analysis before making any conclusions on its overexpression status in leukaemias. WT1 is lately considered as a good target for immunotherapy based on its expression or overexpression (Inoue *et al*, 1994; Gao *et al*, 2000). As shown above, leukaemic T-cell lines previously thought to overexpress WT1 actually do not produce WT1 at all. Therefore, expression of WT1 should not be carried out solely through reverse-transcriptase PCR on the C-terminus of the transcript, even if it is difficult to amplify the N-terminus. Additionally, protein analysis or staining for WT1 should be implemented. Otherwise, the results of WT1-targeted immunotherapy may prove to be detrimental.

2. WT1 is involved in AICD

After having identified the leukaemic cell lines that actually produce WT1, a comparison of the apoptotic behaviour of these cell lines upon T cell receptor stimulation was feasible. Without any stimulation, the apoptosis levels of the leukaemic cell lines were very low. All the cell lines responded to the apoptosis induction, however, the Hut78 and CEM reaction to

stimulation was distinguishably more prominent (Fig.III.5). The apoptosis levels after PMA and ionomycin treatment in Hut78 and in CEM showed a more than 2-fold increase, when compared to a less than 1-fold increase in the other cell lines. This result is intriguing, as full-length WT1 was only detected in Hut78 and CEM.

In order to verify that the apoptosis detected in Hut78 and CEM was mediated by the CD95/CD95L system, the CD95L was blocked with an antibody (Fig.III.6). Indeed, blockage of the CD95L action, recovered survival in these cell lines, as the apoptosis levels under PMA and ionomycin treatment dropped down to background apoptosis levels. The above data for the first time indicates that WT1 expression in the leukaemic cell lines coincides with higher apoptotic cell death.

To confirm that the enhanced AICD is influenced by the presence of WT1, two different systems were designed. In the first system, WT1 was transfected into a WT1-non-expressing cell line with low apoptotic death after 24h of stimulation. As such, Jurkat cells provided a good system and WT1-KTS or WT1+KTS was ectopically expressed in either non-treated cells or in cells treated with PMA and ionomycin for 24h. Both WT1 splice variants could be expressed and any effects seen were therefore not the result of uneven expression (Fig.III.8C). In addition, the WT1-KTS is the variant which has transcription factor abilities, and the presence of WT1+KTS would therefore act as a control in the effects seen. Specific apoptosis analysis of Jurkat cells treated as described above showed that WT1 greatly increased AICD (Fig.III.8B). This increase was attributed only to the WT1-KTS variant as the WT1+KTS had absolutely no effect. Moreover, blockage of the WT1-KTS –mediated AICD with an antibody against the CD95L, reconfirming that the CD95/CD95L system was involved (Fig.III.8A). This experiment indicates that WT1-KTS brings about apoptosis and is in

agreement with previous reports that support an apoptotic role for the WT1-KTS variant (Morrison *et al*, 2005; Murata *et al*, 1997).

In the second system used in this direction, a WT1-expressing cell line with high AICD was chosen. In this setting, *WT1* was silenced in CEM cells by siRNA. The *WT1* silencing oligonucleotide could reduce WT1 protein levels in a dose-dependent manner and itself had no effect on the apoptosis status of the cells (Fig.III.10A and III.9B). After introducing the appropriate siRNA amounts to achieve maximum *WT1* knock-down efficiency and stimulating the cells for 24h, but within the time-range of the knock-down effect, specific apoptosis was calculated. Silencing of WT1 in CEM T cells not only brought down AICD levels in a dose-dependent manner, but even resulted in cell proliferation (Fig.III.10B). This finding is in disagreement with reports showing growth inhibition of fresh human leukaemic cell and cell lines by a *WT1*-siRNA (Yamagami *et al*, 1996; Hubinger *et al*, 2001). It must be taken into account that the effects seen in our work were only detectable after cell stimulation. These two systems described above provide evidence for the first time that WT1 mediates AICD in leukaemic T cells that express it.

3. WT1 interferes with CD95L expression

As mentioned above, WT1 expression in the leukaemic cell lines investigated coincided with higher apoptotic cell death, which could be blocked with an antibody against the CD95L. This suggests that the CD95/CD95L system is involved. To elucidate the connection between WT1 expression and CD95/CD95L system-mediated AICD, the CD95 system had to be analyzed. In this context, expression of CD95 and CD95L were analyzed by RT-PCR in all the cell lines, either without any treatment or 3h after stimulation with PMA and ionomycin, a

time point when the stimulation effects are clearly seen on the CD95L (Li-Weber *et al*, 1998). RT-PCR for the CD95L mRNA revealed that it was inducible in all the cell lines (Fig.III.11A). To be more accurate, quantitative real-time PCR was also carried out, which confirmed that all cell lines were responsive to PMA and ionomycin treatment by upregulating the CD95L, but it was clear that the induction levels in WT1-non-expressing cell lines (e.g., 2-fold increase in Jurkat) were very low when compared to WT1-expressing cell lines (e.g., 5-fold increase in Hut78 and 4-fold increase in CEM) (Fig.III.11B). This is the first time that CD95L expression is reported to coincide with WT1 expression.

The CD95 was also analyzed. Changes in CD95 levels would indicate that the WT1 effect on AICD was not due to CD95L changes alone. In this direction, CD95 was analyzed at the transcriptional level by RT-PCR and quantitative real-time PCR (Fig.III.12A and B). The RT-PCR analysis showed that the receptor was expressed in all the cell lines and at almost constant levels. The quantitative analysis supported further the expression of the receptor in all the cell lines. There were small changes seen in either upregulation or downregulation after stimulation with PMA and ionomycin, but these changes did not coincide with WT1 expression. Moreover, cell surface staining for the receptor provided further evidence that CD95 is unaffected by WT1 expression (Fig.III.12C). CD95 expression on the surface was detected in all the cell lines. Surprisingly, in agreement with the mRNA analysis, the receptor amounts present on the surface remained equally high and unaffected after stimulation. Collectively, the above data indicates for the first time that WT1 affects the CD95/CD95L system-mediated AICD by interfering with CD95L expression.

4. WT1 upregulates the CD95L at the transcriptional level

To better understand the relationship between CD95L expression and WT1, both WT1 splice variants (WT1-KTS and WT1+KTS) were ectopically expressed in WT1-non-expressing Jurkat cells and the mRNA levels of CD95 and CD95L were analyzed by quantitative real-time PCR. The CD95 mRNA levels were almost unaffected by the presence of either splicing isoform. The CD95L levels however, were notably affected by the presence of WT1-KTS. The WT1-KTS brought about a 9-fold increase in the CD95L mRNA levels, whereas the WT1+KTS had only a very small effect (Fig.III.13A). Western blot analysis of the transfected cells confirmed that both variants were expressed at equal levels, so the effects seen were attributed to the actual function of WT1-KTS (Fig.III.13B). In accordance, silencing of *WT1* in the WT1-expressing CEM cells (as described before) resulted in a decrease of the CD95L mRNA levels as seen by quantitative real-time PCR. This drop in the CD95L mRNA levels was siRNA dose-dependent (Fig.III.14). Therefore, WT1 expression in the panel of the leukaemic cell lines studied coincided with CD95L expression, the presence of WT1 upregulated the CD95L and the absence of WT1 resulted in a reduction of CD95L mRNA levels. This is the first report that shows the CD95L being upregulated upon stimulation in the presence of WT1 expression. This suggests that WT1 interferes with CD95L expression.

WT1-KTS is known to be a transcription factor which belongs to the Egr family of transcription factors and shares DNA binding specificity with the Egr family member, Egr1. It has been shown that the CD95L is a target for Egr factors, having three putative Egr1 binding sites on its promoter (Li-Weber *et al*, 1999; Rengarajan *et al*, 2000) (Fig.III.15). Therefore, the most putative way that WT1 could interfere with CD95L expression would be

by directly acting on the CD95L promoter. The first step to investigate this was by examining the whole promoter of the CD95L. A luciferase reporter construct driven by the CD95L promoter (-680 to +100) was introduced in Jurkat cells along with WT1-KTS or WT1+KTS. Both WT1 splice variants could activate the promoter at very low levels in non-stimulation conditions, with the WT1-KTS causing a slightly higher activity. After stimulation with PMA and ionomycin the promoter activity was greatly enhanced by both variants. The effect of the WT1-KTS variant which has a higher affinity for DNA binding (Haber *et al*, 1991) was more prominent as it resulted in an 11-fold increase in the luciferase activity when compared to the 7-fold increase caused by the WT1+KTS (Fig.III.16). In addition, a mutant CD95L promoter luciferase reporter construct was introduced in Jurkat cells followed by the introduction of WT1-KTS or WT1+KTS. The mutant CD95L promoter has been shown to inhibit binding of Egr factors (Li-Weber *et al*, 1999) but it would also inhibit binding of WT1 if WT1 would act on the CD95L promoter through the Egr1 sites. Under non-stimulation conditions, none of the splice variants of WT1 had an effect. After stimulation, both variants had very small and similar effects, and the luciferase activity of the mutant promoter was of levels similar to those produced from the wild-type promoter without the presence of WT1 (Fig.III.16). Although it has been shown that Egr factors are necessary for TCR-mediated CD95L expression and work co-operatively with NF-AT (Latinis *et al*, 1997; Li-Weber & Krammer, 2002), the above results suggest for the first time that WT1 is also important and that it functions through the Egr1 DNA binding sites found on the CD95L promoter.

Furthermore, analysis of the individual putative Egr1 binding site of the CD95L promoter provided more evidence for the need of WT1-KTS. In this system a luciferase reporter construct under the influence of a minimal promoter containing a triplicate of each Egr1 binding site from the CD95L promoter (i.e., 3x-120, 3x-180 and 3x-680) was transfected in Jurkat leukaemic cells along with WT1-KTS or WT1+KTS expression plasmids (Fig.III.17).

The WT1-KTS splice variant could activate luciferase production from all Egr1 sites with highest activity detected at the -180 site (Fig.III.17A). To validate the specificity of WT1 for the Egr1 target sequences, a luciferase reporter construct under the influence of a minimal promoter containing five copies of the Sp1 binding sequence was introduced in Jurkat cells with WT1-KTS or WT1+KTS plasmid constructs. Not surprisingly, the effect of WT1-KTS was negligible on the Sp1 target sequences (Fig.III.17B). Based on the same transfection system, the luciferase reporter construct under the influence of a minimal promoter containing a triplicate of each Egr1 binding site from the CD95L promoter was co-transfected in Jurkat cells with WT1-KTS or WT1+KTS. In addition to the previous setting, the cells were stimulated with PMA and ionomycin. Stimulation conditions would give a better idea of how WT1 behaves on the CD95L promoter upon T cell stimulation. Like at the non-stimulation conditions, the presence of WT1-KTS resulted in higher luciferase activity than when absent from all constructs. The activity of the promoter was much higher after simulation when compared to no-stimulation for all three sites, but the Egr1 binding sites at -120 and -180 showed a higher induction (Fig.III.18). These results strongly support the ability of WT1-KTS to influence CD95L expression at the promoter level for the first time and once more points out the regulatory abilities of the WT1-KTS variant (Davies *et al*, 1998).

5. WT1 binds on the CD95L promoter at -120

The capability of WT1 to upregulate the CD95L at the promoter level was therefore shown. The proof of which Egr1 binding site(s) is important for WT1 binding was given by an electrophoretic mobility shift assay with recombinant WT1-KTS and WT1+KTS proteins. Only the WT1-KTS proteins were able to form a complex with the oligonucleotides

representing Egr1 binding sites, confirming once more the transcription factor function of this splice variant (Fig.III.19) and its higher affinity for DNA binding (Haber *et al*, 1991). Although the previous experiments indicated that both the -120 and -180 could strongly respond to WT1-KTS with the -180 site reacting better, the actual protein interaction experiment showed very strong binding only on the -120 site. The high inducible activity of the -180 site could be the result of NF-AT and Egr factors, as it the -180 site is a strong composite NF-AT/Egr binding site (Li-Weber *et al*, 1999; Holtz-Heppelmann *et al*, 1998). The fact that the results from the experiment of the luciferase reporter containing the -120 Egr1 binding site agreed with the ability of recombinant WT1-KTS to form a complex with oligonucleotides representing this site, suggests for the first time that WT1-KTS binds on the CD95L promoter with high affinity at -120 and with lower affinities at the -180 and -860 sites.

The use of the recombinant WT1 protein might not exactly represent what is taking place within the nucleus, as other factors e.g., Egr2 and 3 might compete with WT1-KTS for binding on the CD95L promoter. For this reason, an electrophoretic mobility shift assay with nuclear extracts from CEM, which express WT1, was carried out (Fig.III.20). At non-induction conditions three complexes were formed which were unspecific DNA-protein interactions as it has been shown before (Li-Weber *et al*, 1999). After stimulation the band patterns clearly changed, as the formation of two new complexes, with slower mobility, was visible. The new bands disappeared completely after addition of the antibody against Egr1, showing that Egr1 was involved in these complexes and an antibody against it pulled out all the proteins participating in the complex. This finding agrees with a previous report that suggested a co-operative binding of Egr1 and NF-AT at that site (Li-Weber *et al*, 1999).
The antibody against WT1 reduced the intensity of the bands substantially, indicating that WT1 was also involved in these complexes. The persistence of the complexes could either be

due to the weak effect of the antibody or more likely due to a co-operative binding of Egr1 and WT1 on that site of the promoter, which would strengthen WT1 binding on the promoter. The latter case is supported by the finding that there is no WT1 binding-complex at non-stimulation conditions, when Egr1 is also not bound. The specificity of the antibodies and the efficiency of the system were controlled with the addition of an antibody against Sp1, which as expected did not have any effects on the formed complexes. Moreover, the reductions in band intensities were not due to unequal loading as this was checked with the constitutively expressed factor NF-Y and it showed that all samples contained the same amount of nuclear proteins. Therefore, according to these results, it is shown that WT1 can bind directly on the CD95L promoter.

Collectively, this data indicates that WT1 binds on the CD95L promoter with higher affinity for the -120 and acting in concert with Egr1, they upregulate the CD95L. In the system under investigation, when WT1 is expressed in a leukaemic cell line, it can bind to the CD95L promoter after TCR stimulation and activate CD95L expression. CD95L in turn can react with CD95 and the apoptosis pathway is initiated (Fig.IV.2).

Figure IV.2. WT1's mode of action in leukaemic T cells. Upon TCR stimulation (1), the signals emanating lead to dephosphorylation of NF-AT (2). Active NF-AT translocates into the nucleus where it upregulates Egr factors (3). Binding of NF-AT and Egr proteins on the CD95L promoter in the presence of WT1 (4) lead to increased expression of CD95L (5) which upon binding with CD95 (6) signals for apoptosis (7). In WT1 expressing cells (A) CD95L overexpression by WT1 overcomes resistance to apoptosis and the cell can then commit suicide. In WT1-non-expressing cells, CD95L expression is lower, anti-apoptotic signals are stronger and cells are more resistant to apoptosis (B).
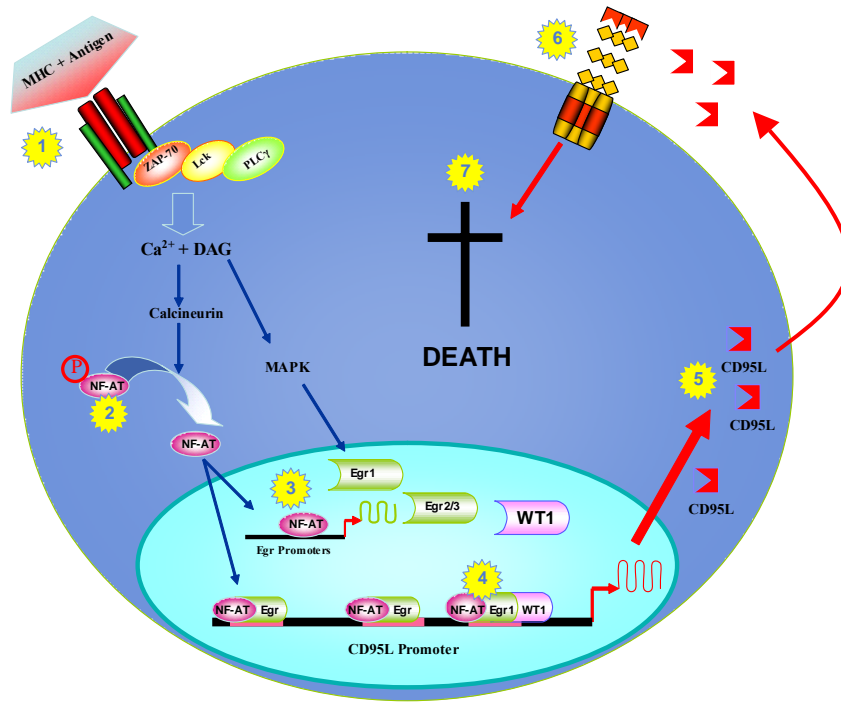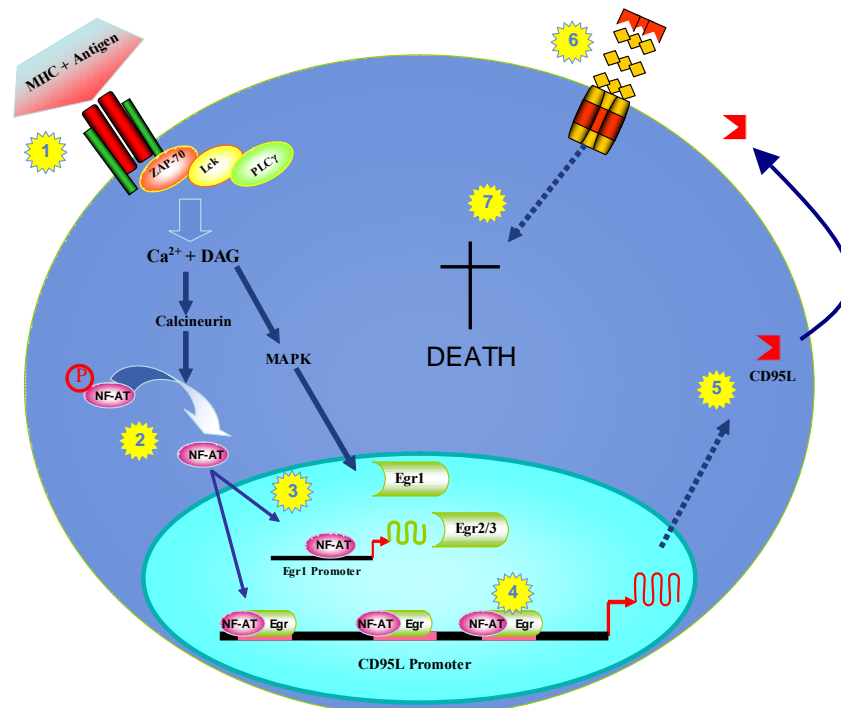
6. Future perspectives

After establishing a trustworthy RT-PCR and protein analysis system for detecting full-length WT1, it is necessary to re-screen all leukaemic cell lines that were previously reported to overexpress WT1 by using only RT-PCR against its C-terminus (Inoue *et al*, 1996; 1997). This will be the only way to clearly identify the percentage of the leukaemias that actually express WT1. In relation to this, the exact stages at which WT1 is expressed during haematopoiesis should be re-assessed to make sure when WT1 is needed.

Once the stages of haematopoiesis that express WT1 are determined, then the role of WT1 in the CD95 system has to be analyzed. It is possible that WT1 expression is not only needed for differentiation but that it is also required to upregulate the CD95L in mediating apoptosis when there is a system malfunctioning in the cell (carcinogenesis) or even in the case of a T cell being autoreactive (negative selection). Interestingly, WT1 belongs to the Egr family, and Egr factors have been shown to be important during thymic selection (Shao *et al*, 1997; Basson *et al*, 2000).

Moreover, WT1 and Egr1-3 are zinc-finger transcription factors which in many cases have common binding sites on the DNA and this is also the situation on the CD95L promoter. As shown in this piece of work, it would be of further interest to see what is happening in the absence of Egr proteins. Therefore, the different Egr proteins can be silenced in for example CEM cells or in T cells at different stages of development and then the ability of WT1 to bind on the CD95L promoter can be checked. In addition, as mentioned in section I.7, WT1 has been shown to interact with several other proteins. Therefore, the possibility for Egr and WT1 interaction can be analyzed by immunoprecipitation experiments and the yeast-two-hybrid system in combination with the usage of wild-type and mutant proteins.

Finally, not to neglect the complicated splicing patterns of WT1 (section I.5), it is worth checking for the presence of different splice variants in the leukaemic cell lines in which part of the WT1 transcript was identified by RT-PCR (e.g., Jurkat). For this, a northern blot analysis would be more suitable. In the case of a truncated protein being expressed in these cells then its role should be analyzed. This system may help identify the function of WT1 transcript variants more easily due to the absence of full-length WT1, which when present could interfere and mask the effect of the other splice variants. It is also possible that an N-terminally truncated/zinc-finger containing WT1 protein (e.g., in Molt4) could have a dominant negative effect, acting on the promoter of the CD95L and hence suppressing its expression. This event would in turn repress CD95L-mediated apoptosis. Such a situation has been described before for an N-terminally truncated WT1 protein which lacked the repressor domain and was reported to have oncogenic properties (Hossain *et al*, 2006).

7. Summary

The aim of this work was to analyze the expression patterns of WT1 in human T-cell leukaemias and its role in apoptosis, in an attempt to understand its connection with leukaemogenesis. This work was based on the study of leukaemic T-cell lines, some of which were previously reported to overexpress WT1, as the knowledge of WT1's complex behaviour in primary T-cell leukaemias was very limited and this would not have provided a good starting point. The expression analysis showed that only two out of the eight cell lines tested expressed WT1. Two cell lines (Jurkat and Molt4) previously suggested to overexpress WT1 by RT-PCR on the C-terminus of the transcript (Inoue *et al*, 1997) actually did not, as RT-PCR on the whole transcript and Western blot analysis did not detect full-length WT1 transcript and protein respectively. This argues against several reports which support WT1

overexpression in leukaemias by carrying out RT-PCR on parts of the transcript (Inoue *et al*, 1994; Inoue *et al*, 1996; Inoue *et al*, 1997). In addition, it points out the need for a more careful examination for the presence of both the whole transcript and protein by using an antibody against the N-terminus of WT1 protein, before jumping into such conclusions. One of the problems that most researchers have to face when analyzing WT1 mRNA expression is its very high GC content (NCBI), which impedes amplification of the N-terminus. This problem was also encountered in this piece of work but with a lot of screening, optimization and persistence reliable results were obtained. This also raises the question of how trustworthy WT1 expression can be for targeted immunotherapy (Oka *et al*, 2000; Gao *et al*, 2000), highlighting the need for a more careful examination of WT1 expression.

WT1 is not expressed in the majority of the leukaemic cell lines studied and in the two cell lines that express it (Hut78 and CEM) it is wild-type. So how can it be involved in leukaemogenesis? WT1 (like Egr proteins) is expressed during the early stages of haematopoiesis (Maurer *et al*, 1997), and its expression is usually connected with more immature leukaemias (Sekiya et al, 1994; Patmasiriwat *et al*, 1996). As in kidney development, it is most likely necessary for differentiation and important for apoptosis induction in the unlucky event of system malfunctioning. Thus, premature cessation of expression would leave the cells in a non-differentiated phenotype. Accumulation of such undifferentiated cells would end up in leukaemogenesis, a situation similar to Wilms' tumors which gives WT1 a tumor suppressor function. On the contrary, if WT1 expression continues after the appropriate time point during haematopoiesis, then this would result in a proliferative advantage of these cells through upregulation of anti-apoptotic Bcl-2 (Mayo *et al*, 1998), again leading to leukaemogenesis. The latter case, gives WT1 an oncogenic character.

The second interesting finding in this work was that WT1 is involved in AICD by upregulating the CD95L in leukaemic cells. Under normal circumstances mature resting T cells do not express WT1. Also, T cells do not express WT1 when they are activated. That shows that WT1 is not involved in CD95L transcriptional regulation of mature T cells. Why does it then upregulate the CD95L in leukaemic cells? This is presumably an attempt of the cell to sense that there is something wrong and it has to commit suicide. This idea is also supported by the finding that WT1 can mediate apoptosis by upregulating Bak (Morrison *et al*, 2005). WT1 expression being either a remnant of the oncogenic process or deliberate would aim in T cell death upon activation (Fig.IV.2).

The findings that WT1 is not overexpressed in leukaemic T-cell lines and that it mediates AICD by upregulating the CD95L, support a tumor suppressor function of WT1 in T cell leukaemias.

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Abbreviations

Ab	Antibody
AICD	Activation induced cell death
AIDS	Acquired immunodeficiency syndrome
APS	Ammonium peroxidisulfate
AREG	Amphiregulin
ATP	Adenosine tri-phosphate
FCS	Foetal calf serum
CDH1	Cadherin-1 (E-Cadherin)
c-FLIP	Cellular FLICE-inhibitory protein
cDNA	Complementary desoxyribozonucleic acid
CsA	Cyclosporine A
DAG	Diacylglycerol
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signalling complex
DTT	Dithiotreitol
Egr	Early growth response proteins
EGFR	Epidermal growth factor receptor
ES	Embryonic stem cell
FADD	Fas-Associated death domain protein
FLICE	FADD-like ICE
Gas2	Growth arrest-specific 2
GFP	Green fluorescent protein
h	Hour
HSC	Haematopoietic stem cell
IAP	Inhibitor of apoptosis
ICAD	Inhibitor of CAD (Caspase-activated DNase)
ICE	Interleukin-1β converting enzyme
IgG	Immunoglobulin of G class
IκB	Inhibitor of κB proteins
IR	Irradiation

IKK	IκB-Kinase
JNK	c-Jun N-terminal kinase
IRF	Interferon regulatory factor
kDa	Kilodalton
LcK	Lymphocyte-specific protein tyrosine kinase
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK Kinase
mRNA	Messenger ribonucleic acid
min	Minute
NF-AT	Nuclear factor of activated T cells
NFκB	Nuclear factor κB
NPHS1	Nephrin
PAGE	Polyacrylamide gel electrophoresis
PARP	poly(adenosine diphosphate-ribose) polymerase
PKC	Protein kinase
PKC-θ	Protein kinase C-θ
PCR	Polymerase chain reaction
PODX	Podocalyxin
Rb	Retinoblastoma
ROS	Reactive oxygen species
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
sec	Seconds
TCR	T cell receptor
TEMED	Tetramethyl-ethyl diamin
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UV	Ultraviolet
V	Volt
XIAP	X-linked inhibitor of apoptosis protein
ZAP-70	Zeta-chain (TCR) associated protein kinase 70kDa