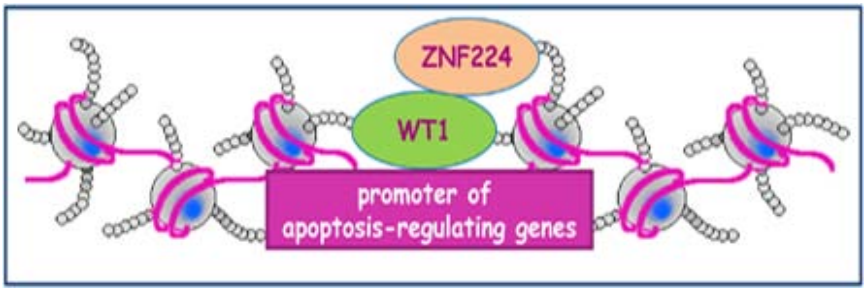
UNIVERSITA' DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA IN BIOCHIMICA
E BIOLOGIA CELLULARE E MOLECOLARE
XXV CICLO

Giorgia Montano

Role of WT1-ZNF224 interaction in the regulation of leukemia cells apoptosis



Academic Year 2011/2012

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Role of WT1-ZNF224 interaction in the regulation of leukemia cells apoptosis

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"DEDICATO A QUELLO CHE SI CELA NEL PROFONDO...PER SEMPRE"
L.F.

Riassunto

WT1 é un fattore trascrizionale a dita di zinco che regola l'espressione di un elevato numero di geni coinvolti nel differenziamento, nel ciclo cellulare e nell'apoptosi. Originariamente è stato identificato come oncosoppressore coinvolto nella patogenesi del tumore di Wilms, un tumore renale pediatrico. Successivamente è stato descritto anche un ruolo di oncogene per WT1 in diversi tumori solidi e leucemie. La capacità di WT1 di attivare e/o reprimere la trascrizione dei suoi geni bersaglio è fortemente influenzata dal contesto cellulare e dall'interazione con alcuni cofattori. Recentemente abbiamo identificato la *Kruppel-like zinc finger protein* ZNF224, come nuovo interattore molecolare di WT1 e abbiamo dimostrato che ZNF224 può promuovere l'attivazione trascrizionale da parte di WT1 sul gene per il recettore della vitamina D (VDR), noto gene bersaglio di WT1. Questi dati ci hanno spinto ad indagare l'effetto dell' interazione ZNF224/WT1 su altri geni coinvolti nel processo apoptotico in cellule di leucemia mieloide cronica K562 che esprimono elevati livelli di WT1 endogeno. I nostri esperimenti dimostrano che ZNF224 esercita un duplice effetto sulla espressione dei geni bersaglio di WT1, agendo da co-attivatore di WT1 nella regolazione dei geni pro-apoptotici Bax, Bak e VDR e impedendo a WT1 di attivare i geni anti-apoptotici bag3 e A1-Bfl-1. Successivamente, avendo osservato che ZNF224 è poco espressa in linee cellulari di leucemia mieloide rispetto a WT1, abbiamo deciso di approfondire il ruolo di ZNF224 nel processo apoptotico. A tale scopo abbiamo valutato se ZNF224 venisse

modulata in cellule K562 in seguito a trattamento con citarabina (ara-C), un farmaco chemioterapico comunemente impiegato nel trattamento delle leucemie mieloidi. Abbiamo osservato che l'espressione di ZNF224 è indotta da citarabina e che tale induzione aumenta la sensibilità delle cellule K562 al farmaco.

I nostri risultati dimostrano l'importante ruolo che il complesso trascrizionale ZNF224/WT1 svolge nella modulazione di geni apoptotici e che l'aumento di ZNF224 indotto da ara-C può rappresentare uno dei meccanismi attraverso cui il farmaco induce apoptosi in cellule leucemiche.

Summary

The transcription factor Wilms' tumour gene 1, WT1, is implicated both in normal developmental processes and in the generation of a variety of solid tumors and hematological malignancies. Physical interactions of other cellular proteins with WT1 are known to modulate its function. We previously identified the Krüppel-like zinc finger protein, ZNF224, as a novel human WT1-associating protein that enhances the transcriptional activation of the human vitamin D receptor promoter by WT1. Here, we have analyzed the effects of WT1/ZNF224 interaction on the expression of apoptosis-regulating genes in the chronic myelogenous leukemia (CML) K562 cell line. The results demonstrated that ZNF224 acts in fine tuning of WT1-dependent control of gene expression, acting as a co-activator of WT1 in the regulation of proapoptotic genes and suppressing WT1 mediated trans-activation of antiapoptotitc genes. Moreover, the DNA damaging drug cytosine arabinoside (ara-C) induces expression of ZNF224 in K562 cells and this induction enhances cell apoptotic response to ara-C. These findings suggest that ZNF224 can be a mediator of DNA damage-induced apoptosis in leukemia cells.

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as control (-). G3PDH was used to monitor equal loading conditions. Molecular weight to the left. (B) Western blot analysis of WT1 protein level in pool of K562 cells stably transfected with the expression vector p3XFLAG-ZNF224 and transiently transfected with WT1 shRNA (+) or with non silencing scrambled shRNA used as control (-). G3PDH was used to monitor equal loading conditions. Molecular weight to the left. (C) RT-qPCR analysis of mRNA levels in pool of K562 cells over-expressing ZNF224 (pool ZNF224-FLAG), or in pool of K562 cells over-expressing ZNF224 and transiently transfected with WT1 shRNA (pool ZNF224-FLAG + WT1 shRNA). The empty vector used as control was: pool of K562 stably transfected with p3XFLAG for the pool ZNF224-FLAG; pool of K562 cells over-expressing ZNF224 and transiently transfected with scrambled shRNA for the pool ZNF224-FLAG + WT1 shRNA. Error bars represent standard deviations of three independent experiments. *****P<0.05 versus control; ******P=0.004 versus control; ns: not significant.

Chromatin immunoprecipitation assay was performed with anti-FLAG antibody (panel A and C) or anti-WT1 antibody (panel B). Immunoprecipitation with non-specific IgG were used as negative control. Non-immunoprecipitated chromatin was used as total input control. PCR analysis was used to evaluate the WT1 and ZNF224 binding to AldA, A1/Bfl-1, bag3, Bax and Bak promoter regions, using specific primers. As control of specificity of immunoprecipitated chromatin, PCR was performed using G3PDH primers. Results are representative of two independent experiments. Size, in base pairs, of the PCR products to the left. (D) Total protein extracts from HEK293 transfected with p3XFLAG-ZNF224 and from HEK293 co-transfected with p3XFLAG-ZNF224/pcDNA3WT1(-KTS) were analyzed by western blot with the indicated antibodies. G3PDH was used to monitor equal loading conditions. Molecular weight to the left.

Expression of ZNF224 and WT1 mRNAs in different human myeloid leukemic cell lines and in normal human CD34+/CD38- hematopoietic progenitors cells used as control. Endogenous levels of ZNF224 and WT1 were evaluated by quantitative real time PCR. The internal control was GAPDH. Error bars represent standard deviations of two independent experiments.

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Figure 13: K562 apoptosis induction by different doses of ara-C. K562 cells were treated with 0.25 μM, 0.50 μM and 1μM ara-C for 72h. NT: untreated K562 cells collected at 72h used as control. (A) Cell death as determined by 4',6-diamidino-2-phenylindole (DAPI) positivity. (B) Apoptosis as determined by annexin V-APC positivity. Error bars represent standard deviations of three independent experiments. *****P<0.05 versus untreated (NT).

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NT: untreated cells collected at 72h. The cell viability was determined by trypan blue exclusion. The cell viability was calculated as a percentage from the viability of the untreated cells (NT) set as 100%. Apoptosis was determined by annexin V-APC positivity followed by flow cytometry. Error bars represent standard deviations of three independent experiments. *P<0.05, **P<0.005 versus untreated (NT) cells.

1.Introduction

1.1 Wilms' tumor gene 1 protein (WT1)

WT1 was originally defined as a tumor suppressor gene, whose mutations and deletions were associated with urogenital disease and the development of kidney tumors [1]. During embryogenesis, WT1 is necessary for the development of kidneys, gonads, spleen and mesothelial tissues as judged by severe malformations in transgenic mice with deleted WT1, which die in utero [2].

WT1 consists of ten exons, encoding an mRNA transcript of about 3.2 kb. The mRNA translates into a 429 amino acid zinc finger transcription factor containing an amino terminal proline glutamine-rich domain, and four carboxyl terminal zinc finger domains. The proline glutamine-rich domain (exons 1-6) is critical for the transcriptional regulatory function of WT1, being characterized by repression, activation, self-association, RNA-recognition domains and nuclear localization signal. The carboxyl terminal region contains four C2H2 zinc finger, encoded by exons 7-10, conferring specific DNA binding [3,4] (fig.1).

Alternative splicing of the WT1 pre-mRNA results in four major isoforms: WT1(-17AA/-KTS), WT1(+17AA/-KTS), WT1(-17AA/+KTS) and WT1(+17AA-+KTS), ranging in size between 52-54 kDa (fig.2). The first alternative splicing event affects the entire exon 5, leading to presence or absence of 17 amino acids (+/-17AA).

The second alternative splicing event generates an insertion or a deletion of three amino acids, lysine, threonine and serine (+/-KTS),

encoded by exons 9, between the third and fourth zinc finger domains (fig.2). The +17AA isoform of WT1 has been shown to produce variable effects on the transcriptional function of WT1. In some cell types, it acts as an transcriptional activation domain, and in others it has been shown to constitute an independent transcriptional repression domain. Mice homozygous for a wt1 allele lacking exon 5 have no salient phenotype: in transgenic mice, an exon 5 knockout model showed no functional requirement for this insert, since the mice developed normally [5].

By contrast, a variety of data point to a profound functional importance of the alternative KTS splicing. In fact, mice carrying either a KTS-positive or a KTS-negative WT1 transgene, display distinct phenotypes, and X-ray crystallographic and structural studies along with DNA binding studies indicate that the KTS insertion destabilizes the interaction of WT1 with DNA [6,7].

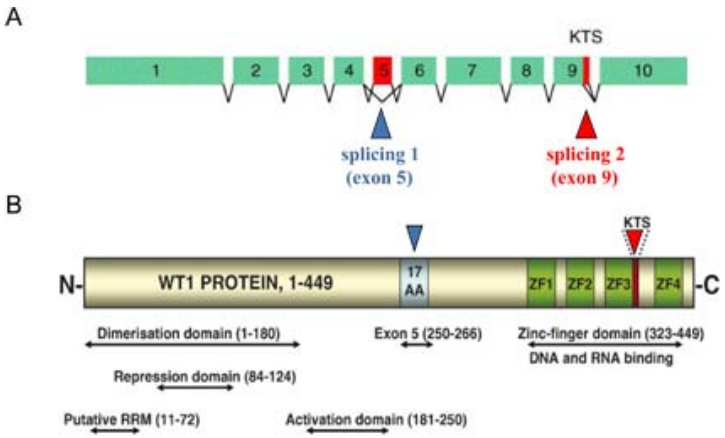

Figure 1. Schematic representation of WT1 mRNA and protein.
(A) Structure of the WT1 mRNA. WT1 is encoded by ten exons. Alternative splicing of exon 5 (splicing 1) and alternative splicing of exon 9 (splicing 2) are shown.
(B) Structure of WT1 protein. The N-terminal region (exons 1-6) comprises a Pro/Gln-rich dimerisation domain (aa 1-180), a transcriptional activation (aa 181-250) and repression domain (aa 84-124), and a putative RNA Recognition Motif (RRM) (aa 11-72), encoded by exons 1-6.
C-terminal region contains four zinc finger domains, encoded by exons 7-10.
The inclusion of exon 5 leads to an insertion of 17 amino acid residues into the N-terminal region of WT1. The splicing 2 in the exon 9 leads to the presence or absence of the tripeptide KTS, between zinc finger 3 and 4.
(A: Modified from Biochim.Biophys.Acta (BBA) 2008; 1785:55-62)
(B: Modified from Trends Biochem Sci. 1998; 23:389-393)

Furthermore, some experimental evidence indicate that the WT1(+KTS) and WT1(–KTS) variants have different functions: WT1(–KTS) isoforms act mainly as transcriptional factors, binding GC-rich DNA sequences with high affinity. These variants can work as activators or repressors of a great variety of cellular genes, depending on cellular context and cofactor interactions [8]. In contrast, WT1(+KTS) isoforms are mainly involved in pre-mRNA splicing [9]. Indeed, there is evidence that these variants bind DNA with little affinity, instead showing affinity for RNA [7]. Moreover, WT1(+KTS) isoforms are present in nuclear domains rich in splicing factors and have been found associated with proteins involved in mRNA-splicing [9]. On the contrary, the WT1(–KTS) isoforms are present in diffuse domains of transcriptional activity in the nucleus [10,11].

Additionally, since each protein isoform may play specific and different biological roles, generation of specific isoform ratio and maintenance of this ratio is critical to the overall biological effects of WT1 [12].

A new isoform, termed sWT1 due to its small size (35-37 kDa), was identified a few years ago. A second promoter, located in intron one, generates the mRNA coding for the sWT1 protein, which lacks the repression domain found in the N-terminus of the full-length protein. The sWT1 was reported to be over-represented in leukemic samples and to confer oncogenic properties due to strong activation of certain WT1 target genes, as compared to full-length WT1 [13].

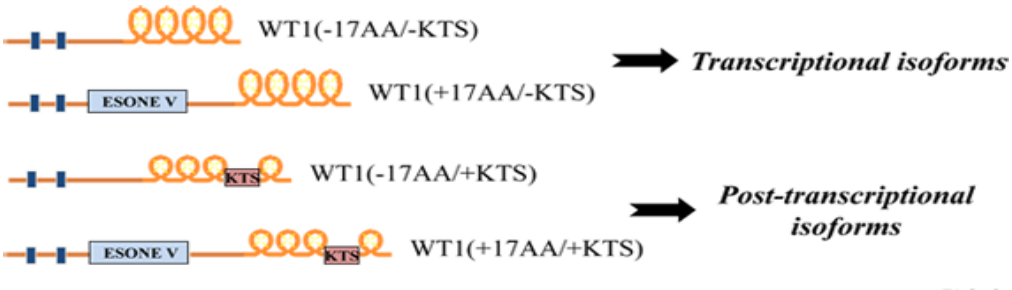

Figure 2. Schematic representation of the WT1 major isoforms. The blue box represents the exon V; the red box indicates the residue KTS.

1.2 The role of WT1 in leukemia

The Wilms' tumor gene 1 (WT1) was identified as a tumor suppressor gene whose mutations are found associated with urogenital disease and the development of Wilms' tumor, a pediatric kidney cancer [1]. In recent years, the study of the involvement of WT1 in tumorigenesis has unexpectedly revealed a potential role for WT1 as an oncogene [3]. The first evidence supporting this role was the over expression of wild-type WT1 in a variety of human cancers of both hematological and non-hematological origin [14] (fig.3). Leukemia is the most studied of these malignancies and there is a growing body of evidence demonstrating WT1's biological and clinical importance in cell survival, differentiation and proliferation of leukemic cells[14]. WT1 gene is highly expressed in the majority of acute myeloid leukemias (AML) and acute lymphoid leukemias (ALL) (fig.3). Thus, WT1 mRNA and protein serve as promising tumor markers for the detection of leukemia and monitoring of disease progression [14-17]. In chronic myelogenous leukemia (CML), WT1 levels are usually low in the chronic phase but frequently increase in blast crisis phase of the disease [15]. In myelodisplastic syndrome (MDS), WT1 mRNA expression levels increase along with disease progression [18, 19].
Eighty percent of the lymphoid leukemias and ninety percent of the myeloid leukemias, both adult and childhood, have elevated WT1 expression [20, 21].

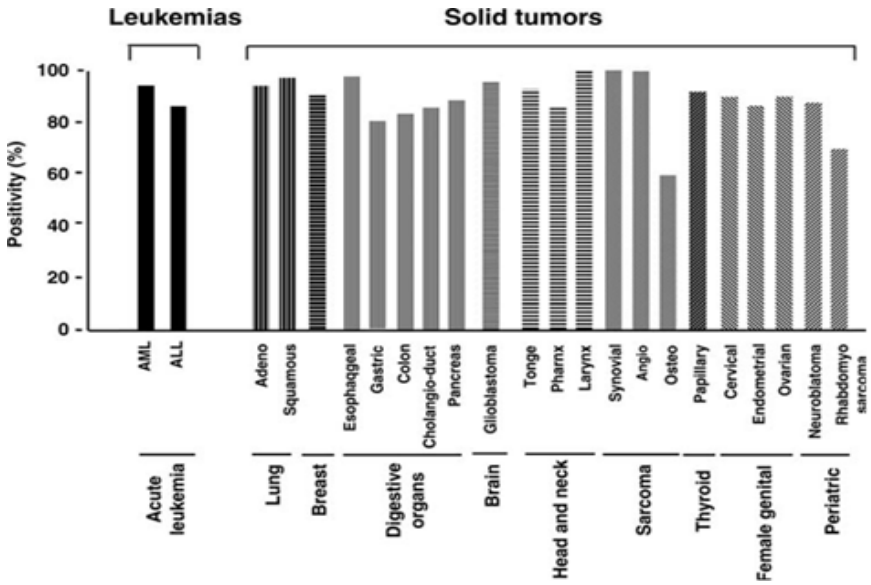

Figure 3. WT1 expression in various types of leukemia and solid cancers. Positivity of WT1 overexpression in leukemias and solid tumors. AML, acute myeloid leukemias; ALL, acute lymphoid leukemias. (From Jpn J.Clin.Oncol. 2010; 40:377-387).

Elevated levels of WT1 in leukemia are associated with poor response to therapy. The majority of AML patients express WT1 at diagnosis with frequencies varying from 73-93%, a significant dominance of the WT1 (+17AA) isoform and increased amount of WT1 at relapse [22]. The expression of WT1 in the majority of human acute leukemias but not in normal mononuclear blood cells and normal $CD34^+$ hematopoietic progenitors, qualifies the wt1 gene transcript as a marker useful in monitoring minimal residual disease (MRD) after chemotherapy [23-26].

Some reports show no significant correlation between WT1 expression and different myeloid leukemia FAB subtypes, while there are other reports indicating higher WT1 expression in M3 AML, harbouring the PML/RARα fusion protein, and less WT1 transcripts in M5 AML [27-29]. Thus, a majority of leukemias express high levels of WT1 with no strong correlation to phenotype, suggesting that WT1 may positively affect proliferation and/or viability of the leukemic cells, rather than interfere with specific differentiation mechanisms.

WT1 mRNA levels in bone marrow at diagnosis may be used as a predictor of clinical outcome, since several studies show a positive correlation between high WT1 expression and a worse long-term outcome. Altogether, the positive correlation between WT1 expression and poor clinical outcome further support the notion of WT1 as an oncogene in leukemia [3].

Furthermore, down-regulation of WT1 gene expression in leukemic cell lines induces apoptosis indicating that WT1 is important for viability of leukemic cells [30]. Moreover, constitutive expression of WT1 inhibits apoptosis in K562 leukemic cell line (CML) treated with apoptosis inducing agents etoposide, doxorubicin and imatinib, further supporting that WT1 may antagonize cell death [30-32].

WT1 is expressed in a small subset of hematopoietic stem cells (1.2%) [14]. These results could indicated that WT1-expressing $CD34^+$ cells are the normal counterparts of leukemic cells and that leukemic cells are mainly generated as a result of leukemic transformation of the

WT1 expressing $CD34^+$ cells. Since it is known that progenitor cells of various types of tissues express WT1, we may assume that WT1-expressing progenitor cells can differentiate into tissue-specific cells by down-regulation of WT1 expression, but that if this down-regulation is impaired, the WT1-expressing progenitor cells continue to proliferate and transform into leukemic and solid tumor cells as a result of accumulation of secondary, tertiary, or further genetic events [14] (Fig.4). One of the mechanisms at the basis of the involvement of WT1 in cancer diseases, is the indirect regulation of programmed cell death, through the transcriptional regulation of its target genes [33].

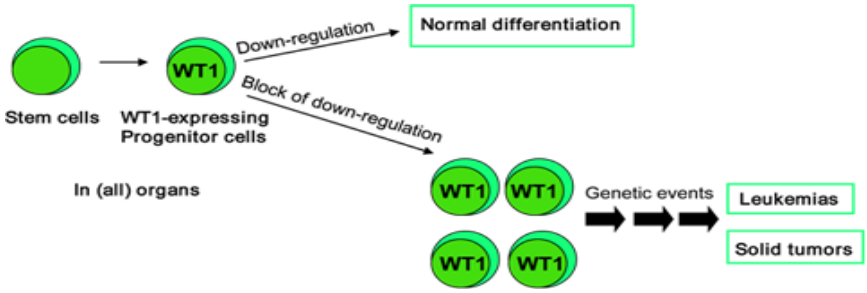

Figure 4. WT1 implication in leukemogenesis and tumorigenesis. Progenitor cells that express WT1 exist in almost all organs and differentiate into organ-specific cells along with down-regulation of WT1 expression. When this down-regulation is impaired, the WT1-expressing progenitor cells continue to proliferate and transform into leukemic and solid tumor cells.

1.3 Transcriptional regulation of the WT1 target genes

As transcription factor, WT1 mediates transcriptional regulation of various genes involved in divergent processes as growth, cell cycle control, development and apoptosis **(Table 1)**. WT1 can either activate or repress transcription of its target genes. Usually, WT1 target genes are most efficiently trans-activated by the WT1(–KTS) isoform of WT1.

Several genes playing a central role in the control of apoptosis have been proposed as targets of WT1, including the pro-apoptotic bcl-2 family members, Bax and Bak [33-35], bcl-2 itself [36], and the anti-apoptotic genes A1/Bfl-1 [37].

Recently, we identified the anti-apoptotic bag 3 gene, involved in apoptosis regulation in leukemia cells, as a novel WT1 target gene [38]. We reported that WT1 positively regulates bag3 gene expression through a transcriptional mechanism. Moreover, we demonstrated that WT1 knockdown affects bag3 expression and enhances apoptosis level in the human erythroleukemia cell line K562 [38].

The expression of specific bcl-2 family members regulated by WT1 vary by cell type and by WT1 isoforms. For example, in some cell types WT1 promotes apoptosis through up-regulation of pro-apoptotic proteins such as Bak and Bax [33-35]. WT1(-17AA/-KTS) up-regulated transcription of pro-apoptotic gene Bak, inducing apoptosis in osteosarcoma cells Saos-2; on the contrary, the down-regulation of the pro-apoptotic Bak gene in K562 cells by over-expression of the WT1(+17AA/-KTS) isoform was demonstrated [33, 34].

Table 1. Target genes of WT1.

Target gene	Regulation of transcript
Growth factor receptors	
Epidermal growth factor receptor	Repression
Insulin growth factor I receptor	Repression
Growth factors	
Amphiregulin	Activation
Erythropoietin	Activation
Insuli-like growth factor II	Activation
Connective tissue growth factor	Repression
Transcription factor genes	
Dax-1	Activation
SRY	Activation
Pou4f2	Activation
Apoptosis regulators	
A1-Bfl-1	Activation
Bak	Activation/Repression
Bax	Activation
Bcl-2	Activation/Repression
Bag3	Activation

Cell cycle regulators	
Cyclin E	Repression
c-myc	Activation/Repression
p21	Activation
Others	
E- cadherin	Activation
Syndecan-1	Activation
Nephrin	Activation
Podocalyxin	Activation
Vitamine D Receptor (VDR)	Activation

In other cell types WT1 inhibits apoptosis and up-regulates anti apoptotic target genes such as bcl-2 and A1/Bfl-1. For example, WT1 (–17AA/–KTS) over-expression results in increased expression of the endogenous A1/Bfl-1 gene in murine, hematopoietic, immature myeloid 32D cl3 cells [37]. Moreover, WT1 (–17AA/–KTS) activates bcl-2 promoter in Saos-2 cells, and suppresses bcl-2 promoter in HeLa cells [33]. These different reports with positive and negative effects of WT1 on target genes may seem conflicting. However, WT1-mediated suppression or activation of target genes is most likely explained by different cellular environment, by isoform ratio of WT1variants and by physical interactions of WT1 with other cellular proteins. Thus, it is possible that the expression of each target gene may be either positively or negatively regulated by WT1 [39]. An increasing number of interacting partners has been proposed to regulate WT1 transcriptional function, acting as either co-activators or as co-repressors **(Table 2)**. These factors include DNA-binding proteins, such as members of the p53 family, and proteins that do not directly contact DNA, such as Par-4 (Prostate Apoptosis Response factor 4) and BASP1 (Brain Acid Solubile Protein) [40-43]. Par-4 can act as either a transcriptional co-activator or as a co-repressor for WT1, depending on the splice isoform of WT1 [40-41].

Indeed, Par4 was originally identified as a WT1-interacting protein, able to enhance the transcriptional repression by WT1 [40] This study showed that the zinc fingers of WT1 mediated the interaction with Par4. Another study demonstrated the presence of an independent

binding site within WT1 for Par4, the 17AA motif present in only specific splice isoforms of WT1. Par4 was able to act as a co-activator for this splice isoform–specific transcriptional domain [41]. Thus, Par4 can mediate both positive and negative interactions with WT1 via these domains that lead to either transcriptional activation or repression. BASP1 was identified as a WT1 transcriptional co-suppressor. It interacts with the repression domain of WT1, inhibiting the WT1 transcriptional regulation on target genes in podocyte cells. [42] Recently, we identified a novel human WT1 cofactor, the Krüppel-like zinc finger protein, ZNF224, and demonstrated that ZNF224 may act as a transcriptional co-regulator of WT1 in order to modulate WT1 transcriptional activity on apoptotic target genes [44].

Table 2. WT1 binding proteins.

PROTEIN	WT1-INTERACTING DOMAIN	REMARKS
WT1	N-terminus (1-180aa)	Dominant negative function of WT1 mutants
Hsp70	N-terminus (1-180aa)	Decreased proliferation
hUBC9	N-terminus (85-179aa)	Involved in sumoylation of WT1
BASP1	N-terminus (71-101aa)	WT1 transcriptional co-suppressor
Pax-2	N-terminus (1-466 aa)	Joint co-expression in renal development
Par-4	N-terminus (+17AA)	Involved in apoptosis/coactivator with WT1(+17AA)
	ZN-fingers	Induces WT1 TR
STAT3	N-terminus (1-281aa)	Promotes cell proliferation
U2AF65	ZN-fingers	Role in pre-mRNA splicing
Ciao 1	ZN-fingers	Reduces WT1 TA on EGF receptor
ZNF255	ZN-fingers	Reduces WT1 TA
p53	ZN-fingers	Repress the WT1 TA
P73	ZN-fingers	Ihibits DNA-binding and TA of WT1
CBP	ZN-fingers	Induces WT1 TA
SRY	ZN-fingers	WT1 nad SRY acts synergistically to activate transcription
E1B55K	ZN-fingers	Inhibits WT1-mediated cell death
ZNF224	ZN-fingers	Enhances activation mediated by WT1 on pro-apoptotic genes and inhibiting WT1-mediated transcriptional activation of anti-apoptotic genes.

The abbreviations used; TA, transcriptional activation; TR, transcriptional repression; ZN-fingers, Zinc fingers; Hsp70, Heat shock protein 70; Par-4, Prostate apoptosis respone 4; UBC9, Ubiquitin-conjugating enzyme 9; BASP1, Brain acid soluble protein 1; CBP, CREB binding factor.

1.4 The zinc-finger protein ZNF224

ZNF224 is a member of the KRAB Zinc-Finger family of transcription factor [45]. The Zinc Finger Protein family (ZFP), is one of the main groups of proteins with DNA-binding activity. One third of ZFPs, the KRAB-ZFPs, contain a potent transcriptional repression domain defined as the Kruppel-associated box (KRAB). The KRAB-ZFPs are mostly localized in the nucleus and work as repressors of transcription mediated by RNA polymerases I, II and III. Although the function of KRAB-ZFPs is largely unknown, they appear to play important roles in controlling cell differentiation, apoptosis, and cancer.

The ZNF224 protein consists of 707 aminoacids, contains 19 tandemly repeated C2H2 zinc finger domains at the C-terminal region, and at the NH2 terminus ZNF224 contains a KRAB repression domain of 45 aminoacids [45] (fig.5).

Figure 5. Schematic representation of ZNF224. ZNF224 protein contains 19 tandemly repeated C2H2 zinc finger domains at the C-terminal region and a KRAB repression domain of 45 aminoacids at the NH2 terminus.

ZNF224 is ubiquitously expressed in several human adult and fetal tissues, albeit with quantitative differences [46].

The human aldolase A was the first gene identified as target of ZNF224 transcriptional repression [47,48]. To repress gene transcription, ZNF224 recruits, through its KRAB domain, the KAP-1 corepressor and enzymes activities, in accordance with repression mechanism of KRAB-ZFP family [46]. Recently, the arginine methyltransferase PRMT5, was demonstrated to play a crucial role in the transcriptional ZNF224 repressor complex [49].

Subsequently, the mitochondrial carrier citrate gene was indicated as a target of ZNF224-mediated transcriptional repression [50]. The finding that the two so-far-known target genes of ZNF224 repressor activity (aldolase A and the mitochondrial carrier citrate) both encode proteins involved in carbohydrate oxidation in humans leads to suppose a role for ZNF224 in controlling the energy metabolism.

An alternatively spliced isoform arising from the ZNF224 gene, namely ZNF255, was identified. ZNF255, shows a different expression pattern in human tissues, a distinctive subcellular localization and a reduced repression activity compared with ZNF224 [46]. These distinctive feartures might indicate a divergent function of ZNF224 and ZNF255. This suggestion was strengthened by the identification of specific interaction between ZNF224 or ZNF255 and WT1 splice variants (-KTS) and (+KTS). We demonstrated a specific nuclear interaction of ZNF224 with the (–KTS) transcriptional isoform. On the contrary, ZNF255 interacts preferentially with the

+KTS isoform, colocalizes with WT1 in translating ribosomes and is present in ribonuclear protein complexes (RNP), strongly indicating a role for this interaction in post-transcriptional control [44].

The interaction ZNF224/WT1(-KTS) was involved in the transcriptional regulation as demonstrated by co-transfection and ChIP experiments using a known WT1 target gene, the vitamin D receptor (VDR). These findings indicate that ZNF224 acts as a transcriptional co-activator of WT1 on VDR promoter, providing the first example of a KRAB-ZPF protein that does not act simply as a transcriptional repressor. In addition, siRNA-mediated knockdown of ZNF224 in the human erythroleukemia cell line K562 resulted in a decreased expression of VDR [44]. Since it is known that vitamin D can induce apoptosis in leukemia cells [51], our finding suggests a role for ZNF224/WT1 interaction in this pathway through the transcriptional activation of VDR.

1.5 Aim of the work

Recently, we demonstrated that the zinc-finger protein ZNF224 is a novel transcriptional cofactor of WT1(-KTS) isoform [44].

According to the evidence that both WT1(-KTS) and ZNF224 are transcriptional factors, we reported their specific nuclear interaction and demonstrated that this interaction results in an increase of transcriptional activation mediated by WT1 on vitamin D receptor promoter (VDR). VDR, a known target of the WT1 transcriptional activation, elicits anti-tumor effects mainly through the induction of cancer cell apoptosis and cell cycle arrest. We demonstrated that ZNF224 acts as a co-activator of WT1 in order to modulate VDR transcription in chronic myeloid leukemia K562 cell line [44].

The aim of this thesis was to identify other genes potentially regulated by the WT1/ZNF224 interaction and to clarify the role of this interaction in the control of apoptosis in leukemic cells. In particular, we decided to analyze the consequences of this interaction on WT1-dependent transcriptional regulation of pro-apoptotic genes Bax and Bak, and anti-apoptotic genes A1/Bfl-1 and bag3 in K562 cell line. In fact, it is known that WT1 exerts its antiapoptotic effects, at least partially, through the regulation of expression of the bcl-2 family members [34]. Our results demonstrated that ZNF224 acts in fine tuning of WT1-dependent control of apoptotic gene expression.

These findings prompted us to investigate if ZNF224 itself could be modulated by chemioterapeutic agents, in order to clarify its role in apoptosis regulation.

The study of the role of the proteins WT1 and ZNF224 in regulation of apoptotic genes expression and in apoptosis in leukemic cells, will provide novel insight into the understanding the molecular mechanism by which these proteins are involved in the apoptosis regulation, paving the way to new potential strategies for the treatment of leukemia

2. Materials and methods

2.1 Cell culture and stable transfection

HEK293 human cell line were cultured in Dulbecco's modified Eagle's medium (Bio-Whittaker, Verviers, Belgium) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin-penicillin mix (Bio-Whittaker) at 37°C in 5% CO_2. The stable clone from HEK293 expressing p3XFLAG-ZNF224 (CL13) and clone from HEK293 cells transfected with empty p3XFLAG (CN2), were obtained as described [49].

The chronic myeloid leukemia cell lines K562, MEG-01, and acute myeloid leukemia cell lines HEL, JK1, PLB989, KG1, PL21, HL60 and NB4, were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 100 μg/ml penicillin-streptomycin mix (Bio-Whittaker) at 37°C in 5% CO_2. Acute myeloid leukemia cell lines F36-P and UT7 were cultured in RPMI 1640 containing 40ng/ml GM-CSF, supplemented with 10% fetal calf serum and 100 μg/ml penicillin-streptomycin mix (Bio-Whittaker) at 37°C in 5% CO_2. To obtain pools of clones stably expressing 3XFLAG-ZNF224, K562 cells were transfected with p3XFLAG-ZNF224 G418-selectable plasmid using HiPerFect Reagent (Qiagen, Hilden, Germany). Transfected cells were selected in medium containing 800 μg/ml G418 (Promega, Madison, WI) for 4 weeks. Dead cells were removed by Ficoll density gradient centrifugation (GE Healthcare Bio Science AB, Sweden, Uppsala). The positive pool of clones were analyzed by Western Blotting using M2 anti-FLAG antibody (Sigma-Aldrich, MO,

USA). As negative control we selected pools of K562 cells stably transfected with the empty p3XFLAG G418-selectable plasmid.
We used pool of K562 cells transduced with a retroviral vector encoding for WT1(-KTS) or with the control empty vector (pMIG), as described [52].

2.2 Isolation of CD34$^+$ cells from umbilical cord blood

Umbilical cord blood was, after ethical approval and informed consent, collected from mothers giving birth to normal full-term infants, from which mononuclear cells were isolated by separation on Lymphoprep (Nycomed Pharma, Oslo, Norway); CD34$^+$ cells were enriched by labeling with magnetic beads (CD34 Progenitor Cell Isolation Kit, Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. CD34$^+$ cell purity was always >90% as determined by flow cytometry. CD34$^+$ cells were cultured in StemSpan medium (StemCell Tech) supplemented with 20% fetal calf serum containing 100ng/ml SCF, 100ng/ml flt3-ligand, 100ng/mnl TPO.

2.3 ShRNA mediated knockdown

To obtain stable ZNF224 gene silencing (clone C3.3), we transfected 2x10^6 HEK293 cells with 10 μg of short interfering RNA plasmid SH2351C3 (ZNF224 shRNA) (Open Biosystems, Huntsville,

Alabama, USA), using Lipofectamine Reagent (Invitrogen, Carlsbad, CA, USA), according to the manifacturer's protocol. The cells were grown in selective medium until colony formation. Positive clones were analyzed by western blotting using anti-ZNF224 antibody (T3). Pools of K562 clones silenced for ZNF224 (ZNF224 shRNA) were obtained by transfection of 1.5 μg of short interfering RNA plasmid SH2351C3 (Open Biosystem) using the HiPerFect Reagent (Qiagen), according to the manifacturer's protocol; transfection of a non-silencing shRNA (scrambled shRNA) was used as negative control (Open Biosystem). Transfected cells were selected in medium containing 500 μg/ml Puromycin (Promega) for 4 weeks. Dead cells were removed by Ficoll density gradient centrifugation (GE Healthcare). Positive clones were analyzed by western blotting using anti-ZNF224 antibody (T3).

K562 cells were transiently transfected using the HiPerFect Reagent (Qiagen) in 12-well plates with 1.5 μg of the short interfering RNA plasmid SH2351C3 to silence ZNF224, or 1.5 μg of the short interfering RNA plasmid SH25dE-10 to silence WT1, or 1.5 μg of non-silencing negative control (scrambled shRNA). All plasmids were purchased from Open Biosystems. Cells were collected 48h after transfection.

2.4 RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen,

Carlsbad, CA, USA), according to the manifacturer's protocol. 1μg of each RNA was reverse transcripted using QuantiTect Reverse Transcription Kit (Qiagen), according to the manifacturer's protocol. Real-time PCR was carried out in a iCycler Thermal Cycler (Bio-Rad, Berkeley, CA, USA) using the SYBR Green I Master Mix (Bio-Rad) and specific primers for: ZNF224 (Fw 5'-gggctgtcttggcacaattc-3', Rev 5'-ttgcctccttgaacgtggtc-3'); WT1 (Fw 5'-gtgaaaccattccagtgtaaaac-3', Rev 5'-gccaccgacagctgaagggc-3'); bag3 (Fw 5'-cctgttagctgtggttg-3', Rev 5'-aacatacagatattcctatggc-3'); VDR (Fw 5'-tccagttcgtgtgaatgatg-3', Rev 5'-gaagattcagatgacccttc-3'); A1/Bfl-1 (Fw 5'-cggcatcattaactggggaag-3', Rev 5'-ctagaagttacaggaaagatc-3'); Bak (Fw 5'-tgaaaaatggcttcggggcaa-3', Rev 5'-ctctcaaacggctggtggcaatc-3'); Bax (Fw 5'-tcaggatgcgtccaccaagaaag-3', Rev 5'-gcaaagtagaaaagggcgacaacc-3'); matrin3 (Fw 5'-cattctaataaggagtggagtc-3', Rev 5'-tgctagtttccactctgcctt-3'); aldolase A (Fw 5'-tcaaccacactccgtccacg-3', Rev 5'-gtagcaagttccggtgcttc-3'); -actin (Fw 5'-cgacaggatgcagaaggaga-3', Rev 5'-cgtcatactcctgcttgcttgctg-3'). β-actin house-keeping gene was used as a reference gene for relative quantification. All real-time PCR reactions were performed in triplicates.

To analyze the expression of ZNF224 mRNA levels in K562 cells treated with ara-C and in the untreated controls, total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. 0.5μg of total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied

Biosystems Inc., Foster City, CA, USA) with random hexamer primers according to the manufacturer's instructions. Quantitative PCR was carried out using TaqMan probe-based chemistry (Applied Biosystems); the probe for ZNF224 (Hs00273760) and the endogenous control β-actin (Hs99999903_m1) were purchased as Assay-on-Demand (Applied Biosystems). The amplification reactions were all performed in duplicates in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Data were collected and analyzed using the Sequence Detector v.1.1 software (Applied Biosystems).

The relative quantification in gene expression was determined using the $\Delta\Delta C_T$ method [53]. Normalization: $\Delta C_T = C_T$ (sample)-C_T (endogenous control); $\Delta\Delta C_T = \Delta C_T$ (sample1)-ΔC_T (sample2). Relative quantification= $2^{-\Delta\Delta CT}$. Efficacy of the PCR amplification of controls and test was identical; parallelism of standard curves of the control and test was confirmed.

P-value was determined by Student's *t* test. All *P* values were considered significant when $P<0.05$.

2.5 Transient transfection and luciferase-reporter assays

The HEK293 stable clones CL13, CN2 and C3.3 were transiently transfected using Lipofectamine Reagent (Invitrogen) in 12-well plates with 0.2 μg of a luciferase reporter plasmid containing the proximal bag3 promoter (PromBag3-Luc) and 0.4, 0.8 and 1.2 μg of

WT1(-KTS) expression plasmid. The pGL4-null empty vector (0.2 μg) was used to evaluate the background luciferase activity, whereas the pCMV-LUC (50ng) was the positive control for the assay. To normalize the luciferase assay a pRL-CMV plasmid (20ng) coding for the *renilla* luciferase was used. The Dual-Luciferase Reporter Assay System (Promega Corporation, WI, USA) was performed 48h after the transfection according to manufacturer's instructions. Results are representative of two independent experiments.

2.6 Western blot analysis

Total protein extract was obtained as follow: cells were washed twice with cold PBS, then were lysed in lysis buffer (10 mM Hepes pH 7.8, 250 mM NaCl, 5 mM EDTA pH 8.0, 1% Nonidet P-40, 1 mM sodium orthovanadate and 50 mM sodium fluoride) and protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1μg/ml aprotinin and 1μg/ml pepstatin A) for 30 min on ice. The extract was centrifuged in a microfuge at maximum speed for 10 min and the supernatant was quantified using the Bio-Rad protein assay reagent, resolved on SDS–PAGE and then transferred to Hybond membranes (Amersham Biosciences, NJ, USA). Non-specific binding sites were blocked for 2h with 5% milk in Tris-Tween buffered saline (tTBS) (5 mM Tris pH 7.5, 15 mM NaCl, 0.1% Tween-20), washed three times with tTBS and incubated with the following antibodies:
anti-FLAG (M2) from Sigma-Aldrich, 1:1000

anti-VDR (D-6) from Santa Cruz Biotechnology, 1:500

anti-GAPDH (7-B) from Santa Cruz Biotechnology, 1:1000

anti-BAX (B-9) from Santa Cruz Biotechnology, 1:500

For these primary antibodies the secondary antibody was goat-anti-mouse IgG (H+L)-HRP conjugated (BioRad170-6516) antibody, 1:3000, detected with an ECL western blot detection system (Amersham Biosciences).

anti-WT1 (C19) from Santa Cruz Biotecnology, 1:500

anti-BAG3 (TOS-2) from BioUniverSa s.r.l., Fisciano, Italy, 1:10000

anti-A1/BFL-1 (PRS3873) from Sigma-Aldrich, 1:1000

anti-Cleaved caspase-3 (Asp175) from Cell Signaling, Denvers, USA, 1:1000

anti-BAK (B5897) from Sigma-Aldrich, 1:2000

For these primary antibodies the secondary antibody was goat-anti-rabbit IgG (H+L)-HRP conjugated (BioRad170-6515) antibody, 1:3000, detected with an ECL western blot detection system (Amersham Biosciences).

The Western blot for ZNF224 was performed using the anti-ZNF224 (T3) antibody (Rabbit polyclonal antibody) [47]. Non-specific binding sites were blocked for 2h in SuperBlock blocking solution buffer in PBS (Thermo Scientific) with 0.1% Tween-20. SuperBlock blocking solution buffer was used in incubation steps with primary and secondary antibody. The primary antibody was diluted in a ratio 1:500.

The secondary antibody was Goat-anti-rabbit IgG (H+L)-HRP

conjugated (BioRad170-6515) antibody (1:3000), detected with an ECL western blot detection system (Amersham Biosciences).

2.7 Chromatin Immunoprecipitation (ChIP) assay

The ChIP assay was performed as previously described [49]. Briefly, cross-linked chromatin was prepared from HEK293 cells transfected with 3XFLAG-CMV-7.1-ZNF224 or pcDNA3WT1(-KTS), or co-transfected with 3XFLAG-CMV-7.1-ZNF224 and pcDNA3WT1(-KTS). Briefly, $5x10^7$ cells were cross-linked with 1% of fixing solution (11% HCHO, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Hepes pH 8.0) and harvested in 1.5 ml of cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Nonidet P-40) with protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1μg/ml aprotinin and 1μg/ml pepstatin A). Nuclei were collected and resuspended in 300 μl of nuclear lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.8% SDS, and protease inhibitors). The DNA was sheared by sonication to an average length between 200 and 1000 base pairs. Sonicated DNA was diluted 3-fold in dilution buffer (10 mM Tris-HCl, pH 8.0, 0.5 mM EGTA, 1% Triton X-100, 140 mM NaCl, protease inhibitors) and precleared with protein A/G plus-agarose (Santa Cruz Biotechnology) at 4°C for 1h. The precleared extract obtained from cells transfected with 3XFLAG-CMV-7.1-ZNF224 or co-transfected with 3XFLAG-CMV-7.1-ZNF224 and pcDNA3WT1(-KTS) was divided in two aliquots. One aliquot was

incubated with 3μg of FLAG M2 mouse antibody (Sigma-Aldrich), and the other was incubated with 3μg of anti-mouse IgG (Sigma-Aldrich), used as control. The precleared extract obtained from cells transfected with pcDNA3WT1(-KTS) was divided in two aliquots and incubated with 5μg of rabbit anti-WT1 antibody (C19 Santa Cruz Biotechnology), or with 5 μg of anti-rabbit IgG (Sigma-Aldrich), used as control. Beads were collected by centrifugation and then washed three times with washing buffer P1 (30mM NaCl, 50mM Tris-HCl pH 8.0, 1mM EDTA, 10% glycerol, 0.1% Nonidet P-40, protease inhibitors), twice with washing buffer P2 (50Mm Tris-HCl pH 8.0, 150 mM NaCl, 1mM EDTA, 10% glycerol, 0.1% Nonidet P-40, protease inhibitors) and twice with TE buffer (1mM Na-EDTA, 10mM Tris-HCl pH8.0). The first supernatant of the IgG control served as the total input control for the PCR analysis effectiveness. The beads were taken up in 100 μl of TE. 1 μg of RNase was added, and cross-links were reversed at 65°C overnight. The next day, samples were adjusted to 0.5% SDS and 0.5 mg/ml proteinase K and incubated for 3h at 50°C. DNA was purified by phenol-chloroform extraction. ChIP samples were then analyzed by PCR using 2μl of immunoprecipitated material, the input control diluted in a ratio 1:200, and specific primers for: Bax (Fw 5'-agatgctcattggacagtcacg-3', Rev 5'-gagtttctgcccctcagtgct-3'); Bak (Fw 5'-gatggagtcttgcactgtcacc-3', Rev 5'-ttctggctaacatggtgaaacc-3'); bag3 (Fw 5'-agtccagctcgtccggcttc-3', Rev 5'-ggccagttgctacctccct-3'); A1/Bfl-1 primer sequences are described in [37]. Aldolase A primer sequences are described in [48];

as control of immunoprecipitated chromatin specificity, PCR was performed using G3PDH primers (Fw 5'-ggtcgtattgggcgcctggtcacca-3', Rev 5'-cacacccatgacgaacatgggggc-3'). Results are representative of two independent experiments.

2.8 Analysis of viability and apoptosis by flow cytometry

Apoptosis of K562 cells, pools of K562 cells stably overexpressing ZNF224 (ZNF224-FLAG) or stably transfected with ZNF224 shRNA, pool of K562 cells retrovirally transduced for WT1(-KTS), was induced by cytosine arabinoside (ara-C) (Sigma-Aldrich). To this aim, cell number was counted in a Bürker chamber and viability was determined by trypan blue exclusion. For assessment of apoptosis, cells were plated at a density of $2.5x10^5$/well in 12-well plates and were stained with propidium iodide (Sigma-Aldrich) and analyzed by flow cytometry as described [54].

For annexin V staining, $3x10^5$ cells were rinsed twice in cold PBS containing Ca^{2+} and Mg^{2+}, than were resuspended in cold annexin V-binding buffer (5M NaCl, 1M $CaCl_2$, 1M Hepes buffer/NaOH pH 7.4) and stained with Annexin V-APC (550474, BD Pharmingen) and DAPI (4',6-diamidino-2-phenylindole) (D9564, Sigma-Aldrich). This allows for the discrimination of live cells (DAPI impermeable) from apoptotic cells (annexin V and DAPI positive cells). Following incubation on shaker in the dark for 15 min at 4°C, cells were analyzed on a FACS Aria flow cytometer (BD Biosciences Immunocytometry System).

3.RESULTS

3.1 ZNF224 represses WT1-mediated bag3 expression

We recently reported a specific nuclear interaction between ZNF224 and WT1 and demonstrated that this interaction enhances WT1-mediated trans-activation of VDR promoter, thus implying that ZNF224 acts as a transcriptional co-regulator of WT1 [44].

In this work we investigated if ZNF224 also affects WT1-dependent transcriptional regulation of the anti-apoptotic bag3 gene expression, recently identified as a novel target of WT1 [38]. To this aim we used a HEK293 cell clone, which does not express endogenous WT1, while stably expressing ZNF224 (CL13 clone) [49] and a C3.3 clone stably expressing a small hairpin RNA (shRNA) specific for ZNF224. As control, we used a clone generated by stable transfection of HEK293 cells with the empty vector (CN2 clone) [49]. The expression level of ZNF224 in these clones is shown in figure 6A. CN2, CL13 and C3.3 clones were transfected with increasing amounts of the WT1(-KTS) expression vector and bag3 expression was evaluated at mRNA and protein levels by quantitative real-time-PCR and western blot analyses, respectively. A progressive increase in bag3 mRNA and protein levels was observed in CN2 clone (Fig.6B lanes 1, 4, 7 and Fig.6C left panel), thus confirming that WT1 activates bag3 gene expression [38]. Conversely, in CL13 clone, overexpression of ZNF224 considerably abolished WT1-mediated bag3 induction, as indicated by down-modulation of bag3 transcript and protein

expression (Fig.6B lanes 2, 5, 8 and Fig.6C middle panel). In C3.3 clone, silenced for ZNF224, the increase in bag3 mRNA and protein was higher than in CN2 clone (Fig.6B lanes 3, 6, 9 and Fig.6C right panel), thus indicating an increased transactivation of WT1 on bag3 gene in the absence of ZNF224.

To further investigate the effects of ZNF224 on bag3 gene transcription, we introduced a luciferase reporter plasmid containing bag3 gene promoter into CN2, CL13 and C3.3 clones, in presence of increasing amounts of WT1(-KTS) expression plasmid. Analysis of luciferase activity shows that WT1 was able to activate bag3 promoter in a dose-dependent manner in CN2 (Fig.7B lanes 10, 13, 16) as opposed to CL13 (Fig.7B lanes 11, 14, 17), in which we observed a dose-dependent decrease of WT1-mediated transcriptional activity. As expected, in C3.3 clone an increased luciferase activity with respect to CN2 clone (Fig.7B lanes 12, 15, 18 versus lanes 10, 13, 16) was observed. These findings indicate that ZNF224 suppresses bag3 gene expression by a transcriptional mechanism.

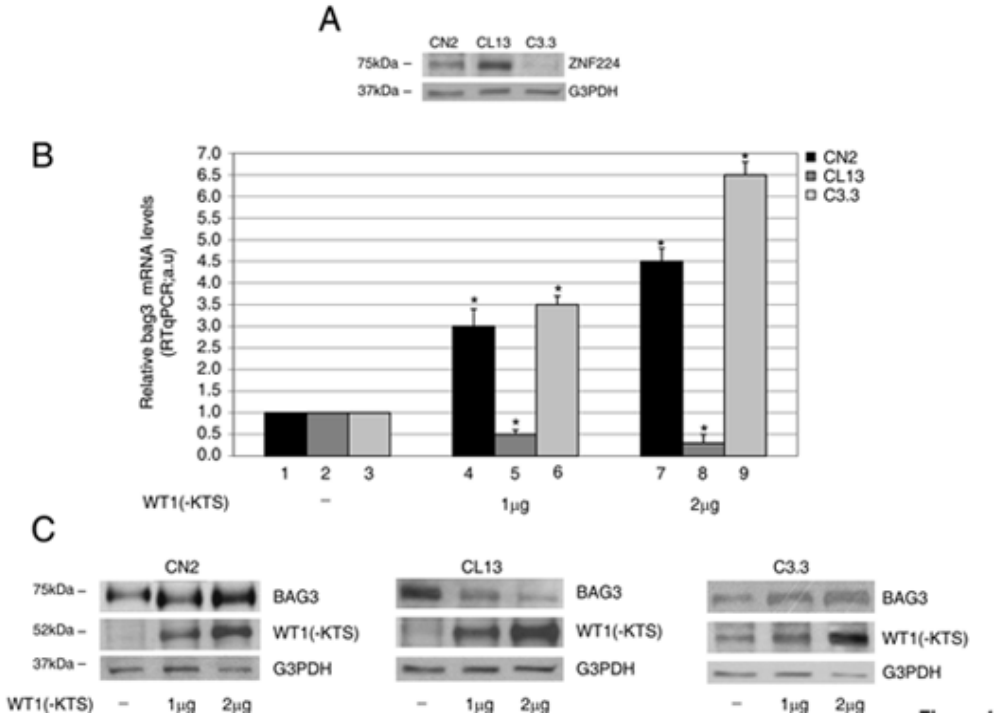

Figure 6. **ZNF224 overexpression negatively regulates WT1-mediated bag3 expression.** (A) Western blot analysis of the ZNF224 protein level in CL13, CN2 and C3.3 HEK293 clones, using anti-ZNF224 antibody (T3). G3PDH is shown as equal loading control. Molecular weight to the left. (B) RT-qPCR analysis of the bag3 mRNA expression in CN2, CL13 and C3.3 clones transiently transfected for 48h with increasing amount of WT1(-KTS) expression vector. mRNA levels of untransfected clones were referred to as 1 (lanes 1-3). Error bars represent standard deviations of three independent experiments. *P<0.05 versus control. (C) Western blot analysis of BAG3 and WT1 protein levels in total protein lysates obtained from CN2, CL13 and C3.3 clones transiently transfected with increasing amount of WT1(-KTS) expression vector for 48h. G3PDH was used to monitor equal loading conditions. One representative result out of three performed is presented. Molecular weight to the left.

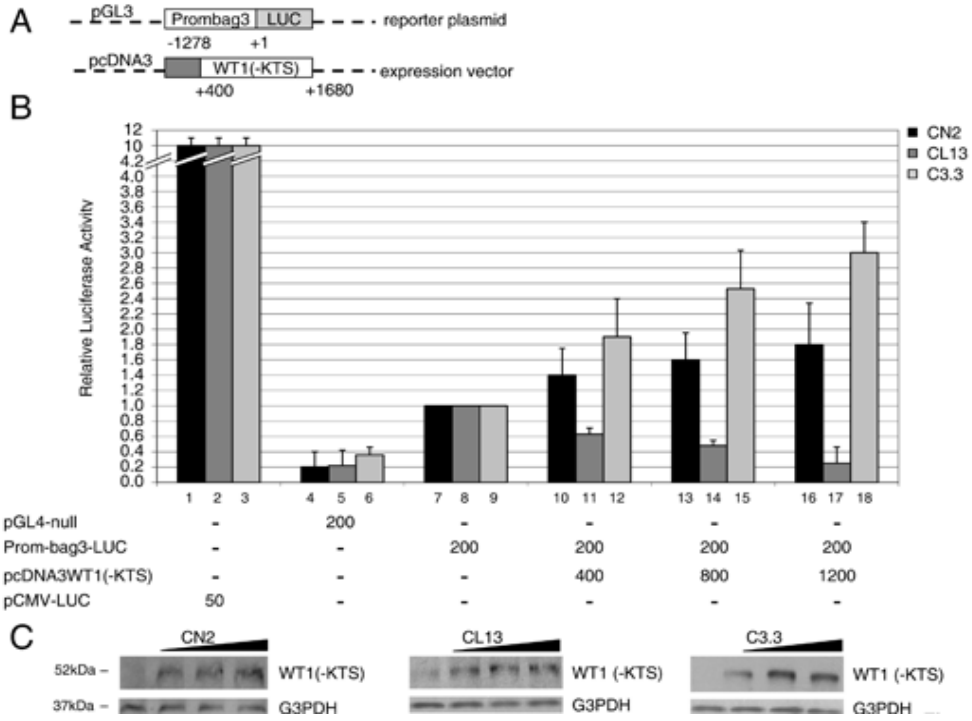

Figure 7: **ZNF224 suppresses WT1-mediated transcriptional activation of bag3 promoter.** (A) Schematic representation of the reporter plasmid containing the bag3 promoter region (-1278 to +1) and of the WT1(-KTS) expression vector containing the illustrated region (+400 to +1680) used in transient transfection experiments. (B) CN2, CL13 and C3.3 HEK293 clones were co-transfected with prom-bag3 reporter and increasing amount of WT1(-KTS) expression vector (lanes 10-18) or trasfected with prom-bag3 alone as control (lanes 7-9). After 48h cells were collected and relative luciferase activities were calculated, with the activity of control cells set as 1 (lanes 7-9) (triplicate determinations). The effect of WT1 on bag3 promoter in CN2, CL13 and C3.3 clones is shown. pGL4-null empty vector activity indicates the background (lanes 4-6) and pCMV-LUC was the positive control (lanes 1-3). Error bars represent standard deviations of three independent experiments. (C) WT1(-KTS) protein overexpression was evaluated by western blot analysis. G3PDH was used as loading control. Molecular weight to the left.

3.2 ZNF224/WT1 interaction modulates the expression of apoptosis-regulating genes in K562 cells

To further confirm that WT1/ZNF224 complex modulates bag3 expression and to better characterize its role in leukemic cells, we evaluated the effects of the knockdown of ZNF224 on the expression of WT1 target genes. To this aim, K562 cells were transfected with two different shRNA plasmids targeting ZNF224 and a negative control shRNA. The knockdown of ZNF224 leads to a dramatic increase in bag3 and A1/Bfl1 anti-apoptotic mRNAs expression, whereas it causes a decrease in Bak and Bax mRNAs expression, as already previously observed for VDR [44], another pro-apoptotic WT1 target gene [51] (Fig.8A). The aldolase A mRNA was used as control, being known the role of transcriptional repression exerted by ZNF224 on aldolase A gene transcription [47,48]; as expected, ZNF224 silencing produced a considerable increase in aldolase A mRNA expression (Fig.8A). The changes observed in mRNAs expression after ZNF224 RNAi were confirmed at the protein levels by western blotting (Fig.8B). To further verify that ZNF224, besides its role as transcriptional repressor, may act as a cofactor of WT1, we generated a pool of K562 cells stably transfected with ZNF224 (Fig.9A) and evaluated the mRNA levels of WT1 target genes. As shown in Fig.9C, ZNF224 overexpression is accompanied by a decrease in bag3 and A1/Bfl1 mRNAs expression and an increase in Bak, Bax and VDR mRNAs expression. The decrease in aldolase A mRNA expression was used as a control of ZNF224 overexpression

(Fig.9C). We next confirmed that the ZNF224-mediated modulation of apoptotic genes was dependent on WT1 expression in K562 cells. To this aim we transiently transfected a pool of K562 cells over-expressing ZNF224 with WT1 shRNA plasmid (Fig.9B). The depletion of WT1 in this pool of cells affects the apoptotic gene expression, demonstrating that ZNF224 requires WT1 to modulate the expression of bag3, A1/Bfl1, Bax, Bak and VDR (Fig.9C). On the other hand, the expression of aldolase A, a direct target of ZNF224 transcriptional repression, was not influenced by WT1 knock-down (Fig.9C). The above reported experiments indicate that ZNF224 cooperates with WT1 in the transcriptional modulation of apoptosis-regulating genes, enhancing gene activation mediated by WT1 on pro-apoptotic genes and inhibiting WT1-mediated transcriptional activation of anti-apoptotic genes.

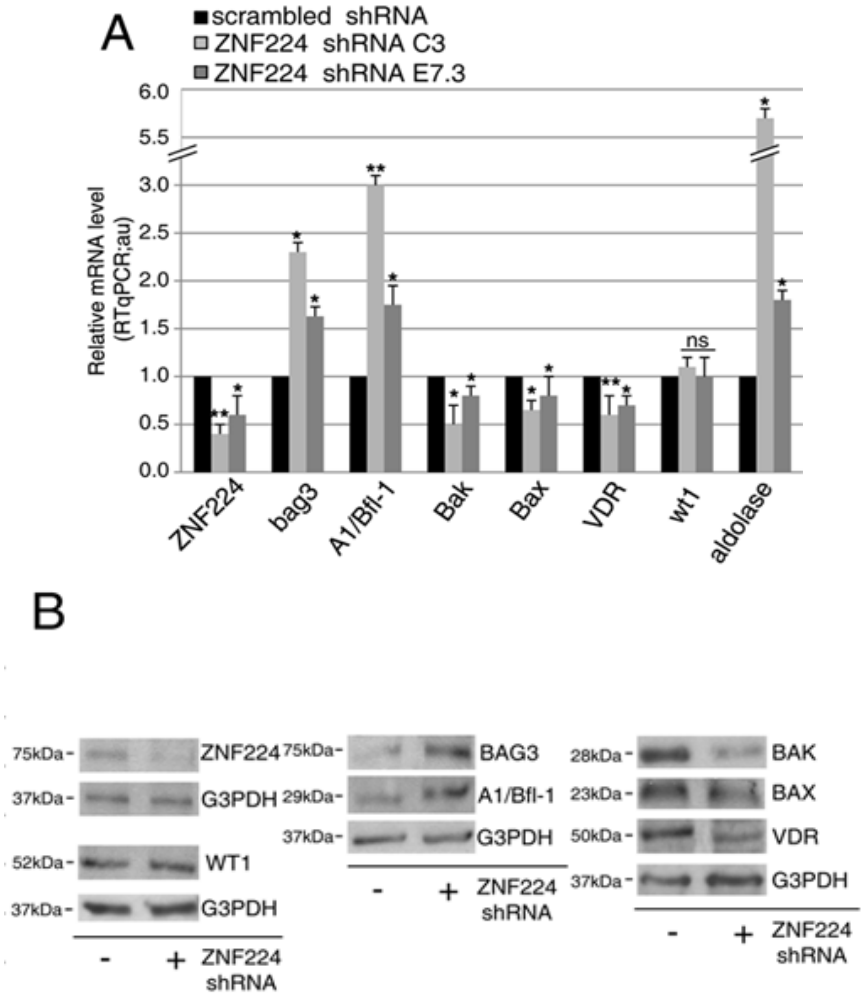

Figure 8: **ZNF224 depletion in K562 cells affects the expression of WT1 target genes.** (A) RT-qPCR analysis of mRNA levels in K562 cells transfected for 48h with two different ZNF224 shRNAs (shRNA C3 and shRNA E7.3) or non silencing scrambled shRNA, as negative control. Error bars represent standard deviations of three independent experiments. *****P=0.05 versus scrambled shRNA; ******P<0.002 versus scrambled shRNA; ns, not significant. (B) Total protein extracts were analyzed by western blot with the indicated antibodies. G3PDH was

used to monitor equal loading conditions. One representative result out of three performed is presented. Molecular weight to the left.

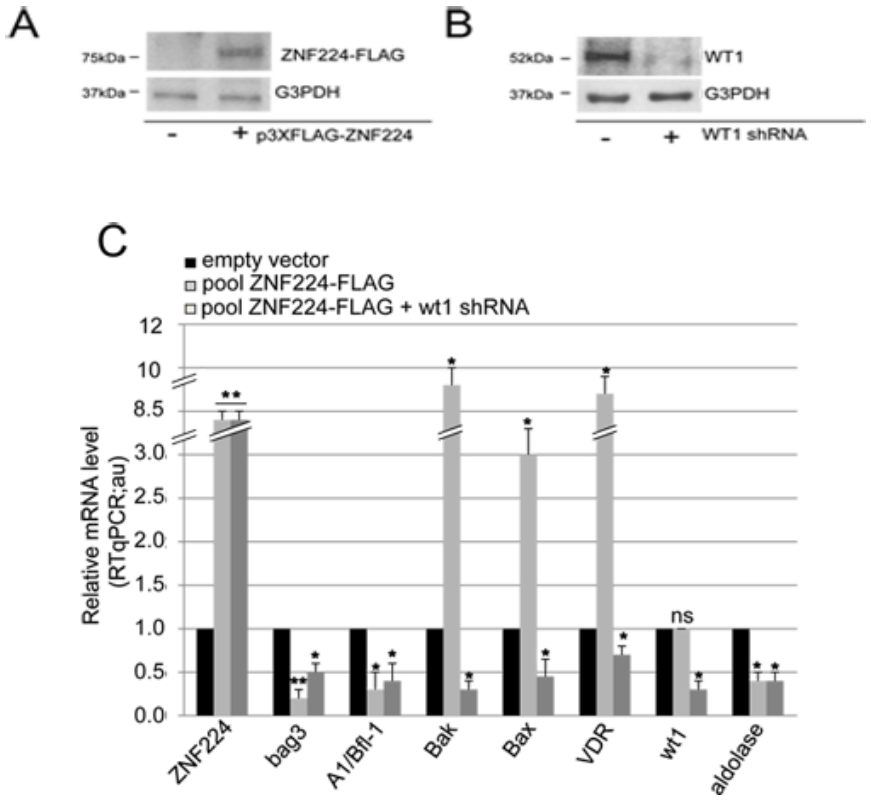

Figure 9: **ZNF224 and WT1 cooperate in the modulation of apoptotis-regulating genes.** (A) Western blot analysis showing the expression of ZNF224-FLAG protein in pool of K562 cells stably transfected with the expression vector p3XFLAG-ZNF224 (+) or with p3XFLAG empty vector used as control (-). G3PDH was used to monitor equal loading conditions. Molecular weight to the left. (B) Western blot analysis of WT1 protein level in pool of K562 cells stably transfected with the expression vector p3XFLAG-ZNF224 and transiently transfected with WT1 shRNA (+) or with non silencing scrambled shRNA used as control (-). G3PDH was used to monitor equal loading conditions. Molecular weight to the left. (C) RT-qPCR analysis of mRNA levels in pool of K562 cells over-expressing ZNF224 (pool ZNF224-FLAG), or in pool of K562 cells over-expressing ZNF224 and transiently transfected with WT1 shRNA

(pool ZNF224-FLAG + WT1 shRNA). The empty vector used as control was: pool of K562 stably transfected with p3XFLAG for the pool ZNF224-FLAG; pool of K562 cells over-expressing ZNF224 and transiently transfected with scrambled shRNA for the pool ZNF224-FLAG + WT1 shRNA. Error bars represent standard deviations of three independent experiments. *****P<0.05 versus control; ******P=0.004 versus control; ns: not significant.

3.3 WT1 recruits ZNF224 to the promoter of apoptosis-regulating genes

To determine whether WT1 recruits ZNF224 on endogenous WT1 target genes we performed chromatin immunoprecipitation (ChIP) experiments. HEK293 cells were transfected with an expression vector for ZNF224 (p3xFLAG-ZNF224) (Fig.10A), or an expression vector for WT1 (pcDNA3WT1(-KTS) (Fig.10B), or co-transfected with pcDNA3WT1(-KTS) and p3xFLAG-ZNF224 (Fig.10C) and chromatin was immunoprecipitated with FLAG (fig.10A and C) or with WT1 antibodies (Fig.10B). As expected, we observed by PCR analysis that ZNF224 was bound to the aldolase A gene promoter (AldA) and not to the promoter regions of WT1 target genes (Fig.10A), thus indicating that ZNF224 alone was unable to directly bind to these WT1 target promoters; furthermore, WT1 was bound to Bax, Bak, A1/Bfl1 and bag3 promoter regions and not to AldA (Fig.10B). When ZNF224 was over-expressed along with WT1, it bound WT1 target gene promoters, thus demonstrating that WT1 recruits ZNF224 on these promoters (Fig.10C). No bands were observed when a G3PDH fragment was amplified, using the same chromatin samples immunoprecipitated with FLAG (fig.10A, C) or with WT1 antibodies (Fig.10B), thus demonstrating the specificity of chromatin immunoprecipitation.

This result provides experimental evidence that WT1 and ZNF224 interact at chromatin level and this interaction does not involve direct DNA binding of ZNF224.

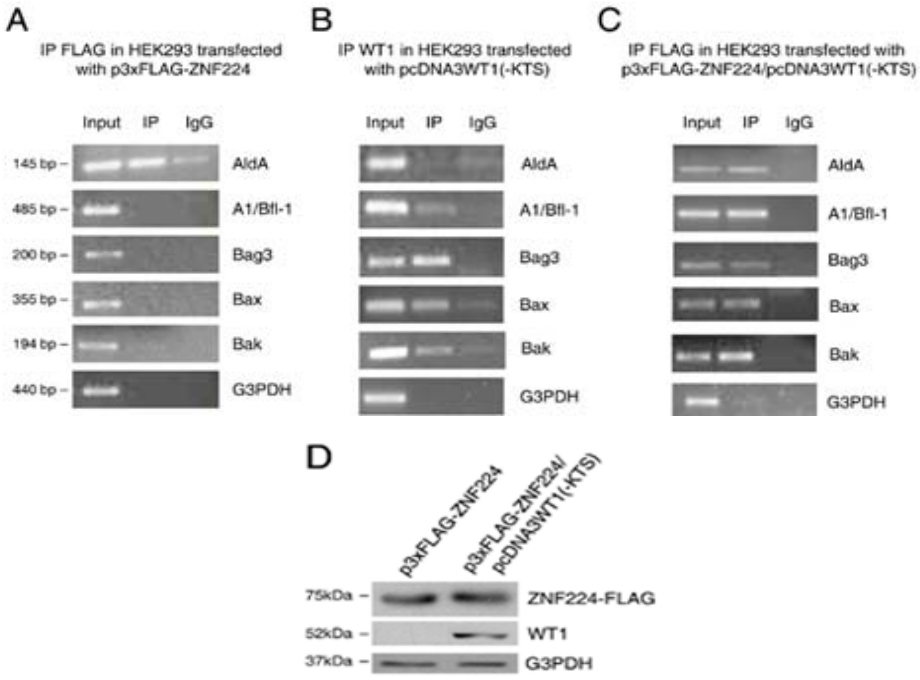

Figure 10: ZNF224 is recruited by WT1 to apoptotic target gene promoters. Chromatin immunoprecipitation assay was performed with anti-FLAG antibody (panel A and C) or anti-WT1 antibody (panel B). Immunoprecipitation with non-specific IgG were used as negative control. Non-immunoprecipitated chromatin was used as total input control. PCR analysis was used to evaluate the WT1 and ZNF224 binding to AldA, A1/Bfl-1, bag3, Bax and Bak promoter regions, using specific primers. As control of specificity of immunoprecipitated chromatin, PCR was performed using G3PDH primers. Results are representative of two independent experiments. Size, in base pairs, of the PCR products to the left. (D) Total protein extracts from HEK293 transfected with p3XFLAG-ZNF224 and from HEK293 co-transfected with p3XFLAG-ZNF224/pcDNA3WT1(-KTS) were analyzed by western blot with the indicated antibodies. G3PDH was used to monitor equal loading conditions. Molecular weight to the left.

3.4 ZNF224 and WT1 expression in myeloid leukemia cell lines

Our previous findings demonstrated that ZNF224 cooperates with WT1 in the transcriptional modulation of apoptosis-regulating genes inducing pro-apoptotic gene expression in CML K562 cells. These results suggested a pro-apoptotic role of ZNF224 in myeloid leukemia. So, we decided to evaluate the expression of ZNF224 and WT1 mRNAs in different myeloid leukemic cell lines and in normal human $CD34^+/CD38^-$ hematopoietic progenitors cells. The endogenous levels of ZNF224 and WT1 were evaluated in exponentially growing cells by quantitative real time PCR (Fig 11). In accord with a large amount of clinical and preclinical data, indicating an oncogenic role for WT1 in leukemogenesis [3], we observed that WT1 is highly expressed in all myeloid cell lines analyzed with respect to $CD34^+$ cells (Fig.11A). At the contrary, low expression levels of ZNF224 mRNA were detected in most of the cells analyzed: acute myeloid leukemia cells (AML) JK1, PL21, NB4 (AML M3), PLB985 (AML M4), MV4-11 (AML M5), HEL, F36-P (AML M6) and chronic myeloid leukemia (CML) MEG-01 (CML), K562 (CML) (fig.11B).

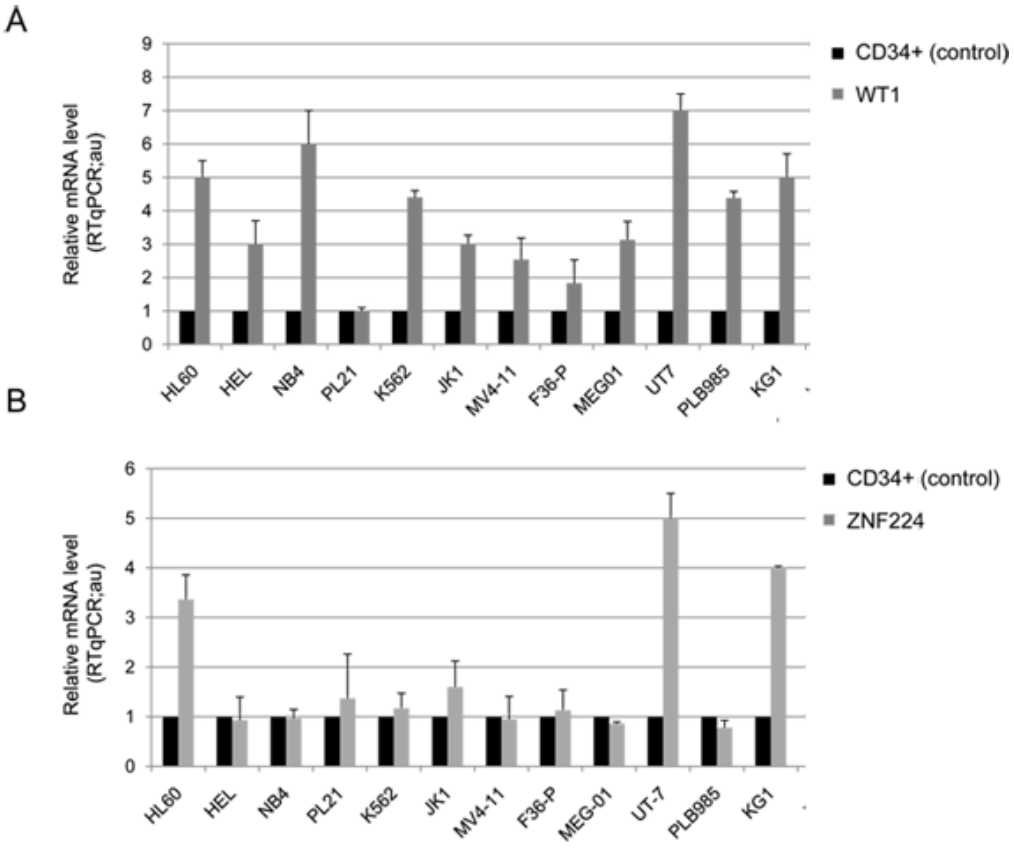

Figure 11: ZNF224 and WT1 expression in mieloyd leukemia cell lines. Expression of ZNF224 and WT1 mRNAs in different human myeloid leukemic cell, lines and in normal human $CD34^{+}/CD38^{-}$ hematopoietic progenitors cells used as control. Endogenous levels of ZNF224 and WT1 were evaluated by quantitative real time PCR. The internal control was GAPDH. Error bars represent standard deviations of two independent experiments.

3.5 Ara-C induces ZNF224 expression in K562 cells and human hematopoietic progenitors cells

To clarify the role of ZNF224 in apoptosis, we decided to investigate if ZNF224 itself could be modulated by chemotherapeutic agents. We chose cytosine arabinoside (ara-C) because it is one of the key drugs for treatment of leukemia.

The above-reported experiment indicates that K562 cells express endogenous ZNF224 at low level, so we decided to expose these cells to increasing concentrations of ara-C (0.25μM, 0.50μM, 1μM) for 24, 48 and 72h after which cell viability and ZNF224 mRNA levels were measured (Fig.12). Exposure of K562 to ara-C induced a time-dependent cell death (Fig.12A, B and C). Then, we evaluated ZNF224 mRNA levels by quantitative real-time-PCR (Fig.12D, E, F). In accordance with our previous findings that ZNF224 induced pro-apoptotic genes expression, we observed that levels of this factor also increased in a time and dose dependent fashion and, apparently, an inverse relationship subsisted between cell viability and ZNF224 levels.

Surprisingly, a dose dependent effect of ara-C on cell viability was not observed, even if a dose dependent increase of ZNF224 was measured. Measurement of cell death by DAPI incorporation and annexin V binding confirmed lack of a dose dependent effect of ara-C on K562 death (Fig.13).

To confirm that leukemic cells died by apoptosis, K562 cells were cultured with or without 1μM ara-C for 72h after which we evaluated

sub-G1 DNA content and annexin V binding by FACS analysis and the caspase 3 by western blot. We found that time-dependent increase in ZNF224 mRNA expression was accompanied by time-dependent induction of apoptosis and caspase-3 activation (Fig.14A, B, C), thus suggesting that caspase-3 activation was a direct consequence of ZNF224 induction.

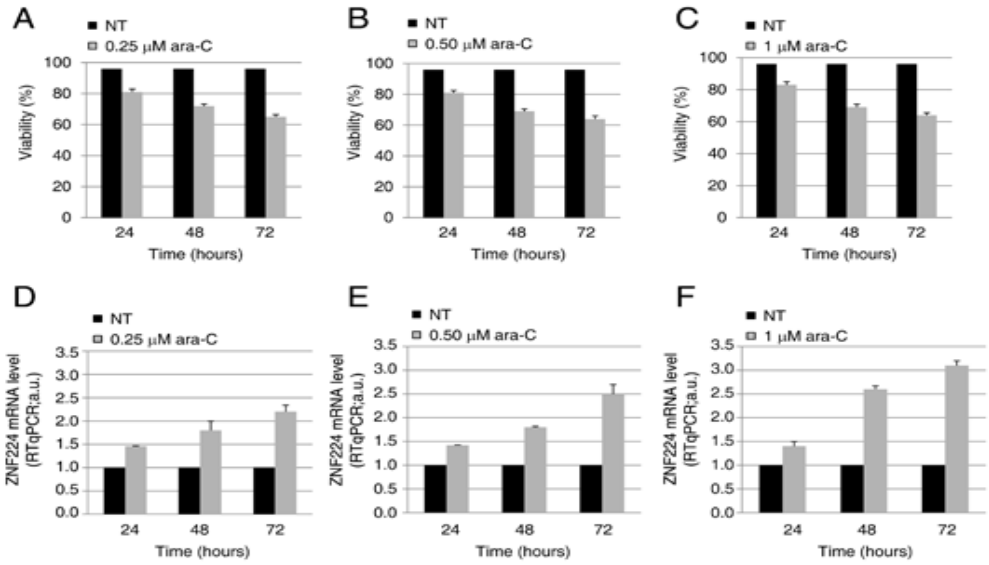

Figure 12: Dose-response and time-course of ara-C induced ZNF224 expression. K562 cells were treated with 0.25 μM, 0.50 μM, 1μM ara-C for 24, 48 and 72h. NT: untreated K562 cells collected at the indicated times. (A, B, C) Viability as determined by trypan blue exclusion. The cell viability was calculated as a percentage from the viability of the untreated cells (NT). The results are means +/- SD from two impendent experiments. (D, E, F) RT-qPCR analysis of ZNF224 mRNA expression in K562 cells treated with 0.25μM, 0.50 μM, 1μM ara-C for the indicated times. Error bars represent standard deviations of two independent experiments.

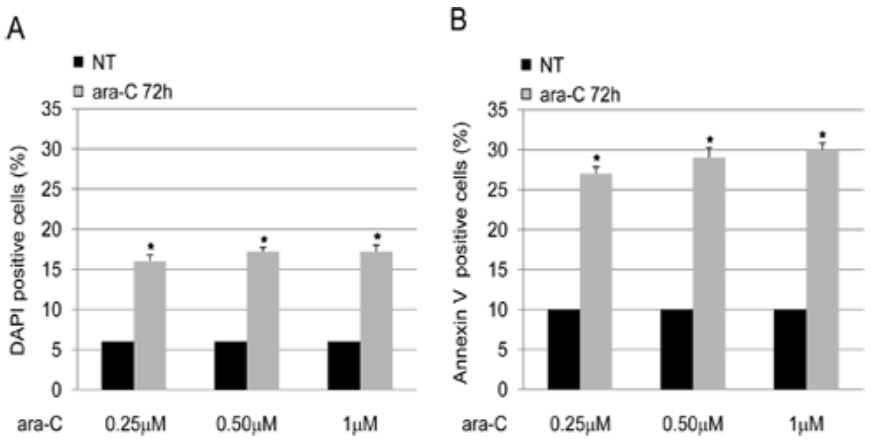

Figure 13: K562 apoptosis induction by different doses of ara-C. K562 cells were treated with 0.25 μM, 0.50 μM and 1μM ara-C for 72h. NT: untreated K562 cells collected at 72h used as control. (A) Cell death as determined by 4',6-diamidino-2-phenylindole (DAPI) positivity. (B) Apoptosis as determined by annexin V-APC positivity. Error bars represent standard deviations of three independent experiments. *****P<0.05 versus untreated (NT).

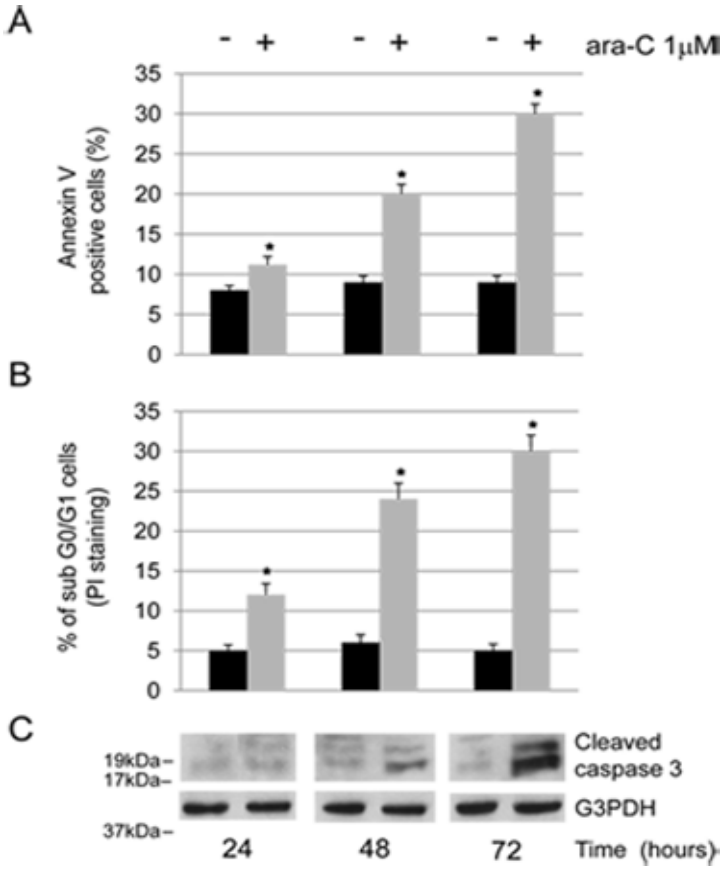

Figure 14: Time-course of ara-C induced apoptosis. K562 cells were treated with 1μM ara-C and analysed after 24, 48 and 72 h. (A) Apoptosis as determined by annexin V-APC staining followed by flow cytometry. Error bars represent standard deviations of three independent experiments. *P<0.05 versus untreated (NT) cells. (B) Cell apoptosis was measured by staining with propidium iodide and flow cytometric analysis. Error bars represent standard deviations of three independent experiments. *P<0.05 versus untreated (NT) cells. (C) Western blot analysis of cleaved caspase-3. One representative result out of three performed is presented. Molecular weight to the left.

ZNF224 induction by ara-C was confermed in human $CD34^+$ hematopoietic progenitors cells. We exposed these cells to increasing concentrations of ara-C (0.05μM, 0.1μM, 0.25μM, 0.50μM, 1μM) for 24 and 48h and then cell viability and ZNF224 mRNA levels were measured (Fig.15). We observed that the exposure to ara-C induced a time and dose-dependent cell death (fig.15A). Then, we evaluated ZNF224 mRNA levels by quantitative real-time PCR (fig.15B). Interestingly, we observed ZNF224 induction also at low concentrations of drug (0.05μM); this is in accordance with the higher sensitivity to the drug of $CD34^+$ cells compared to K562.

Next, by western blot analysis we confirmed the induction of ZNF224 in K562 and $CD34^+$ cells treated with 1μM ara-C at protein level (fig.16A, B).

Finally, we confirmed that the increase of ZNF224 in ara-C-treated K562 cells is accompanied by an increase of proapoptotic VDR, Bax and Bak, and decrease of anti-apoptotic bag3 and A1/Bfl-1, as measured by quantitative real-time-PCR and western blot analysis (fig.17A, B).

In addition, ara-C was ineffective in inducing pro-apoptotic gene expression in pools of K562 cells silenced for ZNF224 (Fig.17C), notwithstanding the decrease in WT1 mRNA expression (Fig.17A, B), thus confirming the role of ZNF224 as important mediator of the ara-C-induced activation of pro-apoptotic genes.

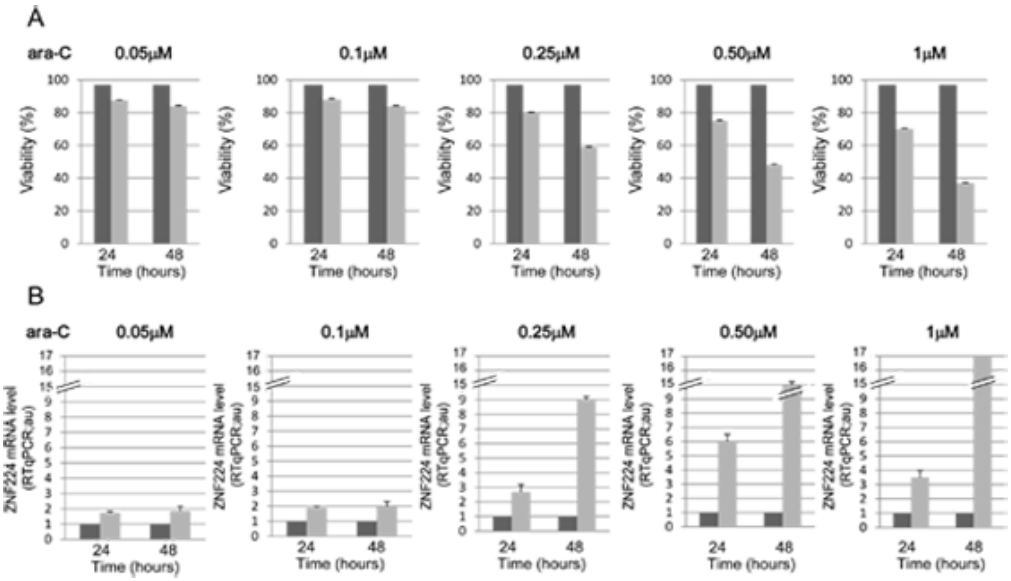

Figure 15. Dose-response and time-course of ara-C induced ZNF224 expression in human CD34$^+$ hematopoietic progenitors cells. CD34$^+$ cells were treated with 0.05µM, 0.1µM, 0.25µM, 0.50µM, 1µM ara-C for 24 and 48 h. NT: untreated CD34$^+$ cells collected at the indicated times. (A) Viability as determined by trypan blue exclusion. The cell viability was calculated as a percentage from the viability of the untreated cells (NT). The results are means +/- SD from two impendent experiments. (B) RT-qPCR analysis of ZNF224 mRNA expression in CD34$^+$ cells treated with 0.05µM, 0.1µM, 0.25µM, 0.50µM, 1µM ara-C for the indicated times. Error bars represent standard deviations of two independent experiments.

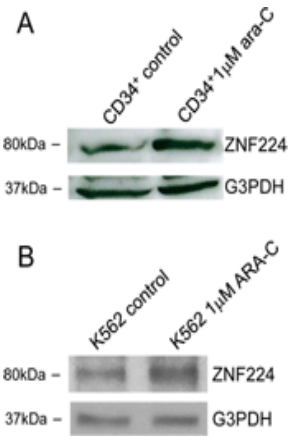

Figure 16. Ara-C induces ZNF224 expression in CD34$^+$ cells and K562 cells. (A) Western blot analysis of ZNF224 in CD34$^+$ cells and (B) K562 cells treated with 1μM ara-C for 48h. One representative result out of two performed is presented. Molecular weight to the left.

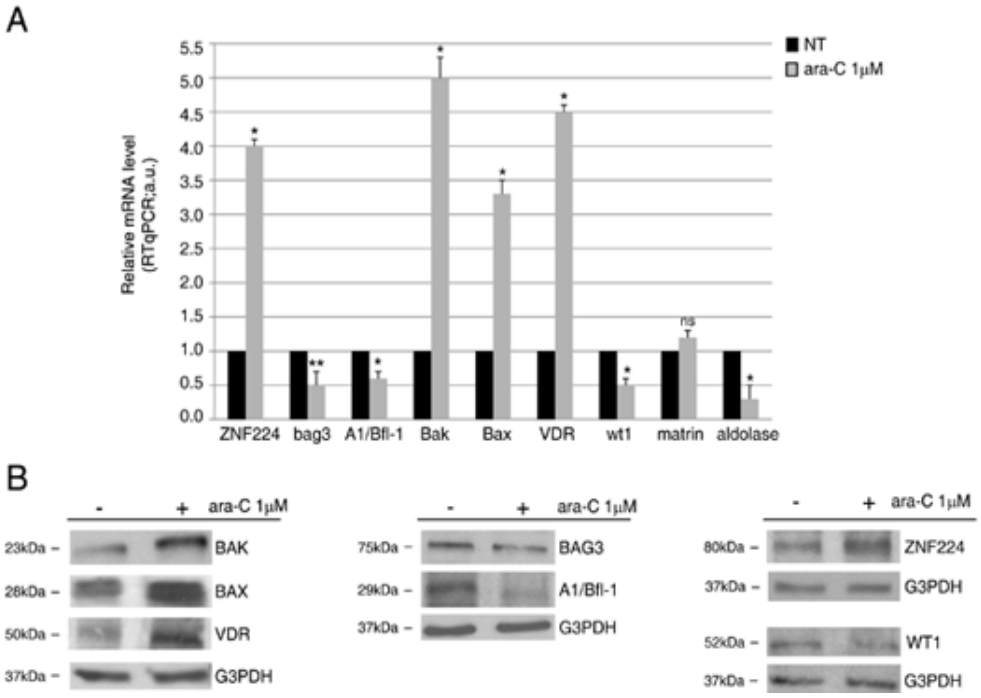

Figure 17: Modulation of apoptosis regulating genes by ara-C. (A) RT-qPCR analysis of mRNA levels in K562 cells treated with 1μM ara-C for 72hr. NT: untreated K562 cells collected at 72h used as control. mRNA level of matrin3, used as negative control, was measured to demonstrate the specificity of ara-C effect on apoptosis regulating genes. Error bars represent standard deviations of three independent experiments. *P<0.05 versus control; **P=0.006 versus control; ns, not significant. (B) Total protein extracts were analyzed by western blot with the indicated antibodies. G3PDH was used to monitor equal loading conditions. One representative result out of three performed is presented. Molecular weight to the left.

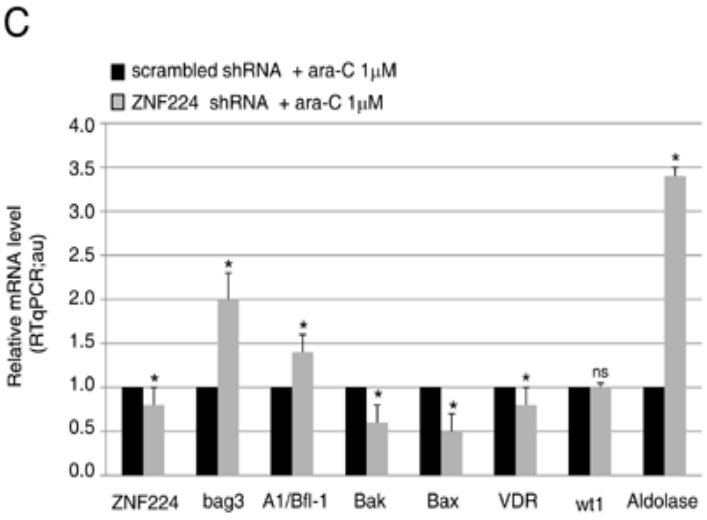

Figure 17: Modulation of apoptosis regulating genes by ara-C.
(C) RT-qPCR analysis of mRNA levels of pools of K562 cells silenced for ZNF224 and treated with 1μM ara-C for 72h (ZNF224 shRNA+ara-C 1μM) or of pools of K562 cells stably transfected with scrambled shRNA treated with 1μM ara-C for 72h (scrambled shRNA+ara-C 1μM) used as negative control. Error bars represent standard deviations of three independent experiments. *P<0.05 versus scrambled shRNA treated with ara-C.

3.6 ZNF224 overexpression enhances the apoptotic effect mediated by ara-C in K562 cells

To confirm our previous findings that there is a strong correlation between ZNF224 induction by ara-C and pro-apoptotic gene expression, we evaluated the apoptotic response to ara-C in pools of K562 cells stably overexpressing ZNF224 (ZNF224-FLAG) or ZNF224-silenced (ZNF224 silencing) or overexpressing WT1(-KTS).

These pools of clones and their corresponding controls were cultured with or without 1μM ara-C for 72h and then viability and apoptosis were analyzed.

As shown in figures 18A and 18D, K562 cells expressing ZNF224-FLAG were significantly more sensitive to the effects of ara-C on cell viability and apoptosis with respect to K562 cells not expressing ZNF224-FLAG. According to these findings, silencing of ZNF224 increased cell viability and reduced the number of annexin V-positive cells after exposure to ara-C (Fig.18B and E). These results support a role for ZNF224 in the apoptotic response to ara-C.

Moreover, we observed that WT1(-KTS) overexpressing K562 cells are protected, at least partly, from cell death further confirming the antiapoptotic role of WT1 (Fig.18C and F).

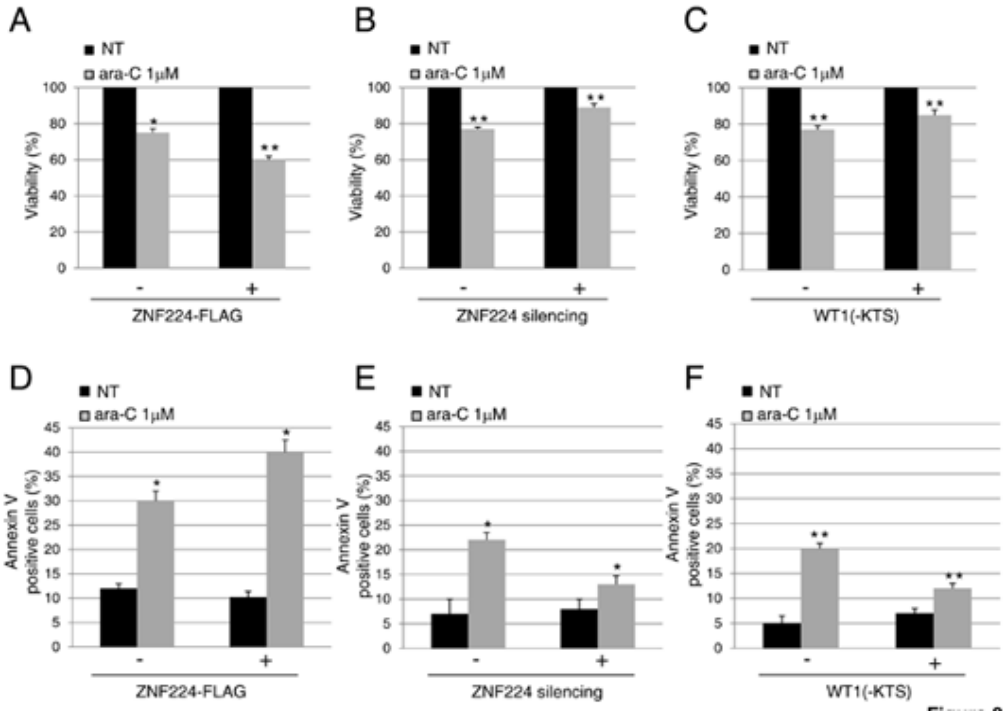

Figure 18: ZNF224 enhances ara-C induced apoptosis.
A, D: The cell viability and the apoptosis of K562 cells expressing or not expressing ZNF224-FLAG, measured after treatment with 1μM ara-C for 72h. B, E: The cell viability and the apoptosis of pools of K562 cells stably transfected with ZNF224 shRNA or scrambled shRNA, measured after treatment with 1μM ara-C for 72h. C, F: The cell viability and the apoptosis of pools of K562 cells retrovirally transduced with WT1(-KTS) or control empty vector (pMIG), measured after treatment with 1μM ara-C for 72h. NT: untreated cells collected at 72h. The cell viability was determined by trypan blue exclusion. The cell viability was calculated as a percentage from the viability of the untreated cells (NT) set as 100%. Apoptosis was determined by annexin V-APC positivity followed by flow cytometry. Error bars represent standard deviations of three independent experiments. *P<0.05, **P<0.005 versus untreated (NT) cells.

4.DISCUSSION

Regulation of apoptosis is a critical function of WT1 and is due at least in part to the modulation of expression of bcl-2 family members [33]. WT1 is highly expressed in leukemia cells and its overexpression is associated with a poor response to therapy [55]. We recently showed that WT1 positively regulates the expression of the antiapoptotic gene bag3, by a transcriptional mechanism. WT1-mediated increase in BAG3 protein levels contributes to the pro-survival role of WT1 in leukemic cells [38]. The effects of WT1 on target gene expression appear to be isoform-specific, strongly influenced by different cellular contexts and by physical interactions with other proteins that are known to modulate WT1 transcriptional function [39-43]. We previously identified the Krüppel-like zinc finger protein, ZNF224, as a novel WT1-interacting factor involved in WT1 transcriptional regulation of VDR promoter [44]. In this study, we provide further evidence of ZNF224 acting as a transcriptional co-regulator of WT1 and playing a relevant role in the control of WT1-mediated expression of apoptosis-regulating gene in the CML cell line K562, thus pointing to a role for the ZNF224/WT1 interaction in leukaemia. Indeed, chromatin immunoprecipitation assays revealed that ZNF224 binds to the promoter of WT1 target genes only if recruited by WT1 itself, thus indicating that ZNF224 acts as a transcriptional co-factor of WT1, without directly binding to promoter DNA sequences. A combination of over-expression and knockdown

analyses of both ZNF224 and WT1 in K562 cell line revealed that ZNF224 exerts a dual effect on the expression of WT1 target genes, acting as a co-activator of WT1 in the regulation of pro-apoptotic genes and suppressing WT1-mediated trans-activation of anti-apoptotic genes.

These data demonstrate that ZNF224, beyond its known role of DNA binding transcriptional repressor [49], may act in a DNA binding-independent mode of transcriptional regulation, through its ability to interact with another trascription factor, i.e. WT1. Our finding thus reveals a still undiscovered function for ZNF224 as transcriptional co-regulator.

However, we cannot rule out that ZNF224 could participate in the regulation of apoptosis also through the direct binding to promoters of not yet identified target genes, where it acts as a repressor.

Other Krüppel-like zinc finger proteins are involved in cell proliferation, apoptosis and neoplastic transformation [56]. For instance, ZBP-89 has been show to be an important regulator in apoptosis and cell growth and can induce apoptosis through mechanisms both dependent and independent of p53 [57]; ZNF23 inhibits cell cycle progression via up-regulation of p27kip-1 [58]; APAK (ATM ad p53 associated KZNF protein) specifically inhibits p53-mediated apoptosis [59]; ZNF382 exerts a pro-apoptotic role by repressing NF-KB and AP1 signaling and by inhibiting the expression of multiple oncogenes, including the NF-kB upstream factors STAT3, STAT5B, ID1 and IKBKE [60].

Although KRAB-containing zinc-finger proteins are thought to function mainly as transcriptional repressors, it has been recently reported that ZBRK1 and ZNF263 display activating as well as repressing effects on target gene transcription, by yet unknown mechanisms and co-regulators [61,62]. Indeed, several lines of evidence indicate that specific transcription factors can use distinct combinations of co-regulator complexes and enzymatic activities required for modifying chromatin machinery, in a cell-, gene-, and promoter-specific manner.

Deciphering the role of ZNF224 as a transcriptional regulator of WT1, identifying additional components of the WT1/ZNF224 complex and elucidating how alterations of the WT1/ZNF224 complex affect the expression of apoptosis-regulating genes will shed further light into the molecular mechanisms of apoptosis regulation.

Furthemore, in this study we observed that (CML) K562 cells express low level of ZNF224, and that cytosine arabinoside (ara-C) induced ZNF224 expression in K562 cells; ara-C is the key agent for the treatment of acute myeloid leukemia [63]. After being transported into leukemic cells, ara-C is phosphorylated by several enzymes including deoxycytidine kinase (dCK) to ara-C triphosphate (ara-CTP), an active metabolite, and then incorporated into DNA, thereby inhibiting DNA synthesis. Ara-C induced DNA damage triggers a wide spectrum of intracellular signaling elements that may contribute to cytotoxicity [64,65]. However, the molecular mechanism coupling ara-C-induced DNA damage to the initiation of cell death have not been yet well

disclosed.

We demonstrated that exposure of K562 cells and human hematopoietic progenitors cells $CD34^{+}/CD38^{-}$ to ara-C increased ZNF224 transcript in a time- and dose-dependent manner, suggesting an important role for the anti-leukemic drug in regulating expression of this protein.

ZNF224 induction is associated with an increase in apoptotic cell death, that correlates with downregulation of antiapoptotic WT1 target genes such as bag3 and A1/Bfl1 and upregulation of proapoptotic molecules such as VDR, Bax and Bak.

On the other hand, lack of correlation between cell death values and ara-C doses may be due to a concomitant activation mechanisms, responsible for apoptosis resistance, that hamper the expected cell death increase. Such an hypothesis is in accordance with the notion that K562 are resistant to ara-C induced cell death [66,67].

Finally, notwithstanding the heterogeneity of pool of clones, the increased sensitivity to ara-C induced apoptosis in pools of clones overexpressing ZNF224, is a further element in support of the pro-apoptotic role of this zinc finger protein. WT1(-KTS) overexpression was able to counteract, at least in part, the proapoptotic effect of ZNF224 following ara-C stimulation, thus confirming the relevant role of ZNF224/WT1 interaction in fine tuning of apoptotic-gene expression.

Taken together, our findings establish a relevant role for ZNF224 in apoptotic processes in CML thus indicating that ZNF224 could

provide a critical link between ara-C induced DNA-damage and the expression of pro-apoptotic genes in leukemia cells.

A better definition of the molecular mechanism coupling ara-C-induced DNA damage to the initiation of cell death could lead to the development of new cancer chemotherapy strategies used for the treatment of myeloid leukemia.

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LIST OF PAPERS

I "WT1 protein is a transcriptional activator of the antiapoptotic bag3 gene"
Cesaro E*, **Montano G***, Rosati A, Crescitelli R, Izzo P, Turco MC, Costanzo P.
***These authors contributed equally to this work.
Leukemia. 2010. 24, 1204-1206.

II "Biochemical and functional interaction between ZNF224 and ZNF255, two members of the Kruppel-like zinc-finger protein family, and WT1 protein isoforms"
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Hum.Mol.Genet, 2010. 19, 3544-3556.

III "ZNF224: Structure and role of a multifunctional KRAB-ZFP protein"
Lupo A, Cesaro E, **Montano G**, Izzo P, Costanzo P.
Int.J.Biochem.Cell Biol. 2011. 43, 470-473.

IV "Role of WT1-ZNF224 interaction in the expression of apoptosis-regulating genes"
Montano G, Cesaro E, Fattore L, Vidovic K, Palladino K, Crescitelli R, Izzo P, Turco M.C, Costanzo P.
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Paper I

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LETTER TO THE EDITOR

WT1 protein is a transcriptional activator of the antiapoptotic *bag3* gene

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WT1 gene, originally identified as a tumor suppressor involved in the formation of Wilms' tumor of the kidney, was subsequently described to have an oncogenic role in a variety of tumors from different origins, including leukemias.[1] In comparison to normal progenitor cells, it is overexpressed in acute lymphoblastic and myeloblastic leukemia, and in the blast crisis phase of chronic myelogenous leukemia;[1,2] high

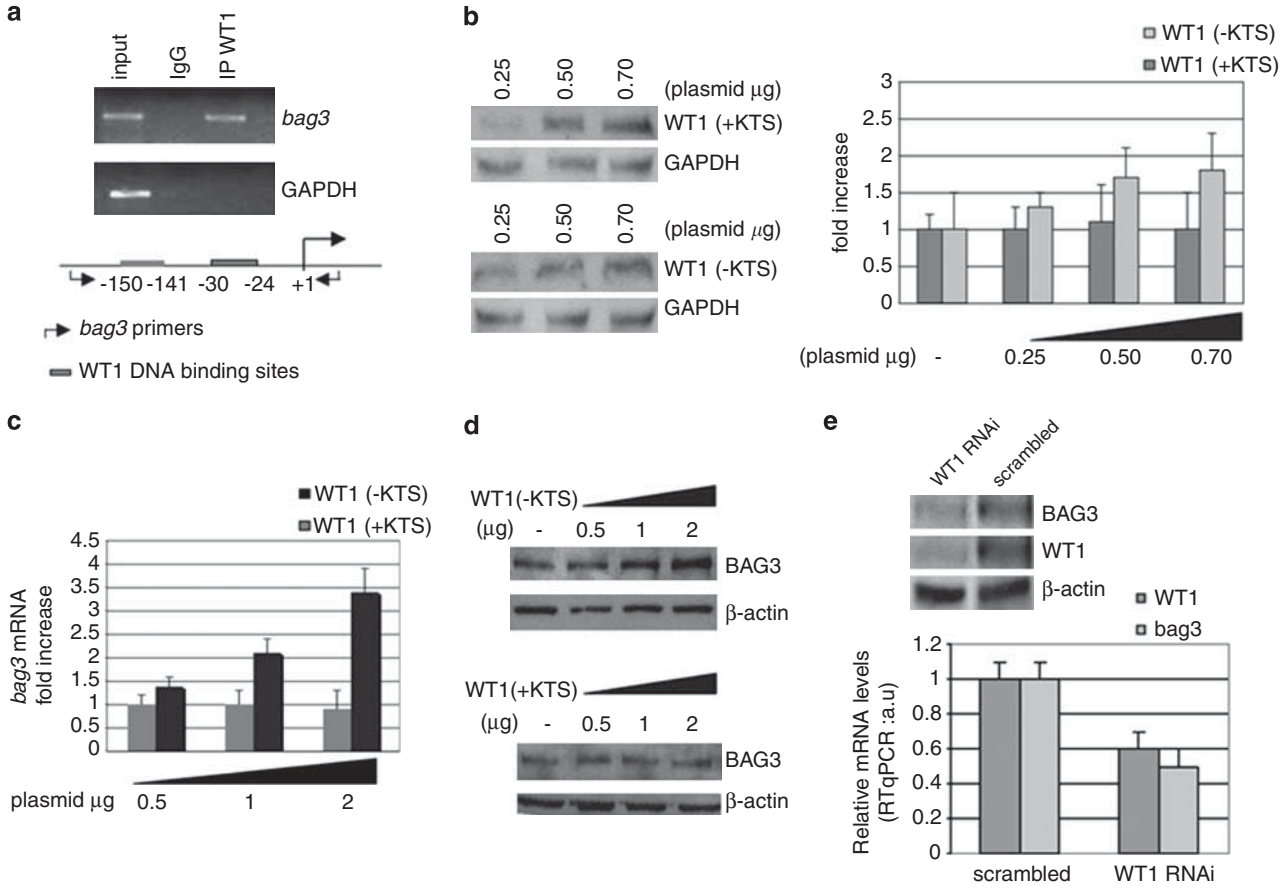

Figure 1 WT1 positively regulates *bag3* expression through a transcriptional mechanism. (**a**) Chromatin immunoprecipitation (ChIP) was performed in K562 cells with anti-WT1 antibody (C19; Santa Cruz Biotechnology, Santa Cruz, CA, USA), as described,[8] or with nonspecific IgGs as control. The resultant samples were analyzed by standard PCR using specific *bag3* primers (forward: 5′-tcggcgcaaaggagaggga-3′; reverse: 5′-ggccagttgctacctccct-3′). As a negative control, PCR was performed using GAPDH-specific primers (forward: 5′-aggtcatccatgacaactttgg-3′; reverse: 5′-ttgtcataccaggaaatgagct-3′). Schematic representation of the *bag3* promoter region that contains the WT1 DNA-binding sites is reported in the lower part of the panel. (**b**) HEK293 cells were transiently transfected using Lipofectamine Reagent (Invitrogen, Carlsbad, CA, USA) in six-well plates with 0.2 μg of a luciferase reporter plasmid containing *bag3* promoter and 0.25, 0.5 or 0.7 μg of WT1(−KTS) or WT1(+KTS) expression plasmid. The Dual-Luciferase Reporter Assay (Promega Corporation, Madison, WI, USA) was performed 24h after the transfection. Bar graphs depict the means of fold increase of transcription activities with respect to *bag3* promoter activity (triplicate determinations). WT1(−KTS) and WT1(+KTS) protein overexpression was evaluated by western blot analysis and GAPDH was used as loading control. (**c**) HEK293 cells were transiently transfected using Lipofectamine Reagent (Invitrogen) in six-well plates with 0.5, 1 or 2 μg of a WT1(−KTS) or WT1(+KTS) expression plasmid. At 48 h after transfection, total RNA was isolated using TRIzoL reagent (Invitrogen) and cDNA was synthesized from 1 μg of each RNA using MMLV reverse transcriptase (Invitrogen). RNA levels were measured by real-time PCR in a Bio-Rad iCycler using Master Mix (Bio-Rad, Berkeley, CA, USA) and specific primers for *bag3* or β-actin (forward: 5′-cgacaggatgcagaaggaga-3′; reverse: 5′-cgtcatactcctgcttgcttgctg-3′), respectively. Standard deviations were calculated from triplicate measurements. (**d**) HEK293 cells were treated as described in (**c**); total protein lysates were obtained and resolved by 10% SDS–PAGE gels and were immunoblotted. Extracts were analyzed for their content of BAG3 protein in western blotting using a polyclonal anti-BAG3 antibody (TOS-2; Enzo Biochem, New York, NY, USA). An antibody recognizing β-actin was used to monitor equal loading conditions. (**e**) K562 cells were transiently transfected using Lipofectamine 2000 Reagent (Invitrogen) with 2 μg of a short interfering RNA plasmid (RNAi) to silence WT1, or 2 μg of a scrambled RNA; after 48 h cell RNA and total protein extracts were prepared. RNA levels were measured by real-time PCR in a Bio-Rad iCycler using Master Mix (Bio-Rad) and primers specific for WT1, *bag3* or β-actin. Proteins were analyzed by western blot analysis using anti-WT1 and anti-BAG3 (TOS-2) antibodies. Anti β-actin antibody was used as control.

levels of the protein are associated with a poor response to therapy.[1,3] *WT1* knockdown by antisense oligonucleotides or RNA interference was shown to induce apoptosis; conversely, its overexpression in myeloid leukemia cells protected against cell death. Modulation of some members of the *bcl-2* family has been associated with apoptosis inhibition by *WT1*.[1,3]

Among proteins that regulate apoptosis in leukemia cells, a role is assigned to BAG3, a member of the family of proteins that, through their BAG domain, interact with HSC70/HSP70 heat shock proteins. *bag3* gene expression is constitutive in some tumor types, including leukemias; in these cells, BAG3 protein has been shown to sustain cell survival and downmodulate cell apoptotic response to drugs, by either HSP70-dependent or -independent mechanisms.[4–7]

Here we report that WT1 induces *bag3* gene expression. This finding identifies a novel target of WT1 protein involved in apoptosis regulation in leukemia cells.

As using *in silico* analysis we had found two putative WT1 binding sites on the *bag3* promoter, we decided to investigate whether WT1 is directly recruited onto the *bag3* promoter using a chromatin immunoprecipitation assay (ChIP) in K562 cells, which express significant amounts of endogenous WT1 and BAG3. Chromatin was immunoprecipitated with anti-WT1 antibodies. Subsequent PCR analysis, performed using oligonucleotides covering the putative WT1 binding sites, revealed that WT1 bound to the *bag* promoter sequences (350-bp band), whereas rabbit IgG antibody controls did not (Figure 1a). WT1 protein has two major isoforms, designated WT1(-KTS) and WT1(+KTS), containing an extra three aminoacids (KTS) between the third and fourth zinc fingers; WT1(−KTS) appears to exert its effect mainly as a transcriptional factor, whereas WT1(+KTS) is involved in RNA processing.[1] To investigate the transcriptional modulation of *bag3* promoter by WT1, we introduced a luciferase reporter plasmid containing the *bag3* promoter into HEK293 cells and analyzed luciferase levels in the presence of increasing amounts of WT1(−KTS) or WT1(+KTS) expression plasmids. As shown in Figure 1b, the 'transcriptional isoform', WT1(−KTS), enhanced *bag3* promoter activity in a dose-dependent manner, whereas transfection of the 'post-transcriptional isoform', WT1(+KTS), did not influence the transcriptional activity.

To assess whether forced expression of WT1 isoforms could modulate endogenous *bag3*, we transfected HEK293 cells with increasing concentrations of the expression vectors encoding WT1(−KTS) or WT1(+KTS) and evaluated *bag3* mRNA and BAG3 protein levels by quantitative real-time-PCR and western blot analyses, respectively. We observed a progressive increase in the levels of *bag3* RNA and BAG3 protein in cells transfected with increasing amount of WT1(−KTS), and not in those transfected with WT1(+KTS) (Figures 1c–d), confirming the transcriptional activation of endogenous *bag3* gene by WT1.

To provide a further argument for *bag3* regulation by WT1, we evaluated *bag3* expression in K562 cells following small interfering RNA (RNAi)-mediated knockdown of WT1. As shown in Figure 1e, the levels of *bag3* mRNA in WT1 knockdown cells were decreased by about 40%; western blotting confirmed downmodulation of BAG3 protein.

Altogether, these observations ascribe a role to WT1 in *bag3* gene regulation.

We next evaluated the effect of WT1 silencing on apoptosis. In K562 cells treated with the proapoptotic agent phenethyl isothiocyanate (PEITC), WT1 silencing significantly enhanced apoptosis, as assessed by flow cytometry, with respect to control scrambled RNA (Figure 2a). Conversely, BAG3 protein appeared to exert an antiapoptotic effect against PEITC treatment, since its overexpression obtained by cell transduction with a *bag3* cDNA-carrying adenovirus (AdhBAG3) downmodulated PEITC-induced apoptosis (Figure 2b). We therefore investigated whether the enhancement of apoptosis induced by WT1 knockdown was reversed by *bag3* overexpression. Indeed, in cells infected by AdhBAG3, cell survival was rescued by more than 25% compared to cells treated with WT1 RNAi alone (Figure 2a). Therefore, WT1 inhibition of apoptosis appeared to be in part mediated by BAG3 protein.

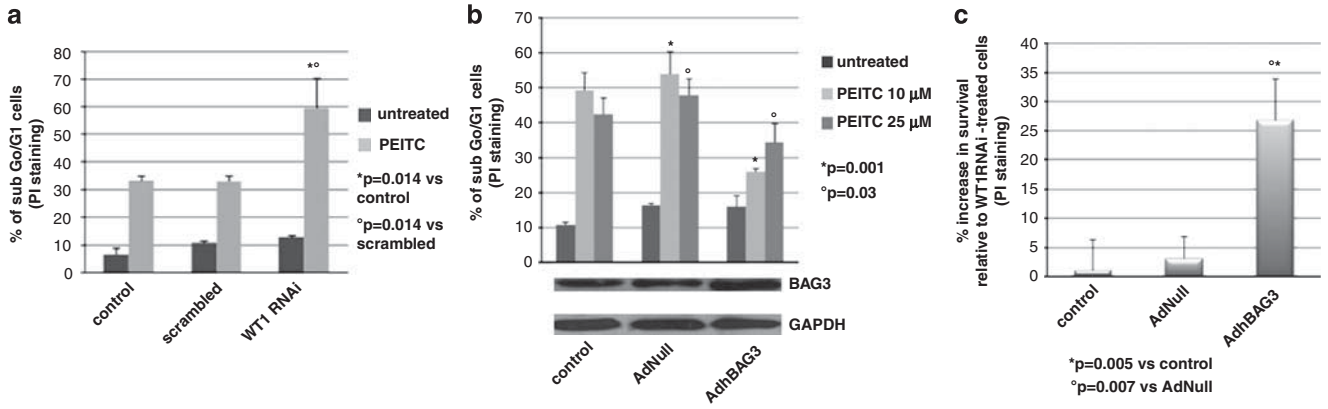

Figure 2 WT1 knockdown affects *bag3* (gene) expression and enhances apoptosis. (**a**) K562 cells were transiently transfected using Lipofectamine 2000 Reagent (Invitrogen) with a plasmid expressing WT1 RNAi or a scrambled sequence. After 48 h, cells were exposed to PEITC (10μM) for 18 h and percentage of apoptotic nuclei was measured by cell permeabilization and staining with propidium iodide, in flow cytometry, as described.[6] (**b**) Adenoviral constructs were generated using the BD Adeno-X Expression Systems 2 (BD Biosciences-Clontech, Palo Alto, CA, USA). For AdhBAG3 construction, *bag3* full-length cDNA from human brain was cloned in pDNR-CMV vector and the gene expression cassette was transferred to the Adenoviral Acceptor Vector pLP-Adeno-X-PRLS viral DNA, containing ΔE1/ΔE3 Ad5 genome, by Cre-loxP-mediated recombination. AdNull, devoid of *bag3* cDNA, was used as a control. K562 were transduced with AdhBAG3 or with control AdNull using a multiplicity of infection of 100 (100 MOI); after 48 h cells were exposed to PEITC at the indicated concentrations for 18 h, then apoptosis was measured by flow cytometry. Graph depicts the percentages of apoptotic nuclei. Significance between two groups was calculated by Student's *t*-test. For western blot analysis, whole-cell lysates were obtained and analyzed for BAG3 content. GAPDH was assessed to verify equal loading conditions. (**c**) K562 were transfected with WT1 RNAi or a scrambled sequence; after 24 h cells were transduced with AdhBAG3 or AdNull and, after additional 24 h, exposed to PEITC (10 μM) for 18 h. The graph depicts the percentage increase in cell survival with respect to WT1 RNAi-treated cells.

The above-reported findings show that WT1 protein regulates *bag3* expression and that WT1-mediated increase in BAG3 protein levels contributes to the prosurvival role of WT1 in leukemic cells. The identification of *bag3* as a target gene of WT1 improves our understanding of apoptosis regulation by this factor. We believe that the associated impacts of BAG3 and WT1 in leukemia cell survival and response to therapy deserve further investigation.

Conflict of interest

The authors declare no conflict of interest.

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Paper II

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Biochemical and functional interaction between ZNF224 and ZNF255, two members of the Krüppel-like zinc-finger protein family and WT1 protein isoforms

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Wilms' tumour suppressor gene, WT1, is mutated/deleted in ~15% of Wilms' tumours, highly expressed in the majority of other cancers and is essential for normal embryonic development. The gene encodes multiple isoforms of a zinc-finger (ZF) protein with diverse cellular functions, in particular participating in both transcriptional and post-transcriptional gene regulation. Physical interactions of other cellular proteins with WT1 are known to modulate its function. However, despite the isolation of several WT1-binding proteins, the mechanisms involved in regulating WT1 activities are not clearly understood. In this study, we report the identification of the Krüppel-like ZF protein, ZNF224, as a novel human WT1-associating protein and demonstrate that ZNF224 and its isoform ZNF255 show a specific pattern of interaction with the WT1 splicing variants WT1(−KTS) and WT1(+KTS). These interactions occur in different subcellular compartments and are devoted to control different cellular pathways. The nuclear interaction between ZNF224 and WT1(−KTS) results in an increase in trascriptional activation mediated by WT1, implying that ZNF224 acts as a co-regulator of WT1, whereas, on the contrary, the results obtained for ZNF255 suggest a role for this protein in RNA processing together with WT1. Moreover, our data give the first functional information about the involvement of ZNF255 in a specific molecular pathway, RNA maturation and processing.

INTRODUCTION

The KRAB (Krüppel-associated box) domain-containing zinc-finger (ZF) genes are part of the poly-ZF family that constitutes the largest mammalian transcriptional regulatory gene family. Almost 50% of transcription factor genes in the human genome encode ZFs, of which ~40% contains KRAB domains (1). Typically, KRAB-ZF proteins mediate transcriptional repression yet, to date, little else is known of their function as many *KRAB-ZF* genes are human-specific, so loss-of-function approaches using gene targeting in mouse embryonic stem cells are not possible. Recently, we have shown that the ZNF224 protein functions as a transcriptional repressor of the human *aldolase A* gene, consistent with the presumed function of the KRAB-ZF family (2,3). Furthermore, we have demonstrated the existence of an alternatively spliced isoform arising from the *ZNF224* gene, known as ZNF255 or bone marrow zinc finger 2. These two isoforms have distinct pattern of distribution within the cell, implying that they perform different cellular functions. Unlike ZNF224 that shows a homogenous nuclear distribution, ZNF255 is distributed throughout the cell and is present also in nucleoli and cytosol. This splicing isoform displays little affinity for the DNA consensus sequence bound by ZNF224

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(4). A transcriptional repression role for ZNF255 has previously been suggested, due to the presence of a Krüppel-related novel transcriptional repressor module, nominated as KRNB: ZNF255 has been shown to interact with Wilms' tumour 1 (WT1) protein and inhibit WT1-mediated transcriptional activation (5). Although the study of *KRAB-ZF* genes is in its infancy, ZNF224 and ZNF255 display typical features of this group of ZF proteins that originate from a single gene by alternative splicing or different promoter usage, giving rise to multiple isoforms with distinct subcellular localizations and strong functional differences (6,7).

In contrast to the KRAB-ZF family, the WT1 ZF protein, WT1, has been conserved throughout vertebrate radiation and has been extensively studied. Initially identified as a tumour suppressor gene, whose mutations and deletions are associated with urogenital disease and the development of Wilms' tumour (8), *WT1* has been shown to be essential not only for the development of the urogenital system, but also for numerous other tissues/organs, including the heart (9,10). The *WT1* gene encodes at least 24 different isoforms through a combination of alternative splicing or translation initiation sites (11,12). Many of the isoforms are found only in mammals and have yet to be associated with a specific function (13,14). However, the variable splicing of exon 9, leading to the insertion or exclusion of the amino acids lysine, threonine and serine (KTS) between the third and fourth ZFs gives rise to the WT1 isoforms more extensively studied, WT1(+KTS) and WT1(−KTS). This splicing site is conserved in all vertebrates and is essential for normal development (15). Moreover, the correct ratio of (+KTS)/(−KTS) isoforms must be strictly maintained as heterozygous mutations affecting this alternative splicing event have been shown to be the cause of the Frasier syndrome, a urogenital disorder characterized by glomerulosclerosis and gonad dysgenesis (16). Although showing redundancy *in vivo*, these two major WT1 isoforms have distinct properties at the molecular level. The (−KTS) isoform acts mainly as a transcriptional factor, binding GC-rich DNA sequences with a high affinity (17,18). It can work as an activator, co-activator or repressor depending on cellular context and cofactor interactions (19). As a transcriptional factor, WT1 is able to modulate the expression of a wide number of genes involved in tissue differentiation, as *podocalyxin* and *VDR* (20,21), in apoptosis, e.g. *bcl-2* (22), bax and bak (23), or in cell cycle control, as *Cyclin E* (24). Moreover, the transcriptional regulatory properties of WT1 are thought to be influenced by an interaction with an increasing number of interacting partners, such as par-4 or BASP1 (19,25,26). WT1 can also act as a transcription cofactor for other DNA-binding proteins (27,28). In contrast, there is evidence that the WT1(+KTS) isoform displays little affinity for DNA (29–31), instead showing affinity for RNA and has been implicated in transcript processing. The WT1(+KTS) isoform is present in nuclear domains rich in splicing factors and has been found associated with snRNPs (17,32,33) and two proteins involved in splicing, U2AF65 and WTAP (34,35). Recently, it has been found that WT1 binds the mRNA of alpha-actinin 1 (36), is also a component of mRNP complexes (37) and is associated with translating polysomes (38). This observation establishes a link between WT1 and the regulation of translation and extends its potential range of functions in the cell. The recent finding that WT1 shuttles between the nucleus and cytoplasm (38) places WT1 at every stage of gene expression from initiating transcription through mRNA splicing and translocation to translation. The concept of transcription factors shuttling between nucleus and cytoplasm and acquiring new functions has been proposed and is likely to occur via additional interacting protein partners (39).

In the present study, we characterized physical and functional interactions of ZNF224 and its isoform ZNF255 with WT1 splice variants (+KTS) and (−KTS). Our results show a specific interaction of ZNF224 with the (−KTS) isoform and suggest that ZNF224 may act as a transcriptional co-regulator of WT1(−KTS) (as shown by co-transfection experiments using a known WT1 target gene, *VDR*). On the contrary, ZNF255 interacts and co-localizes with both WT1 isoforms, with a preference for the (+KTS) isoform, suggesting a role for this interaction in RNA maturation and post-transcriptional control. As well as extending our understanding of the molecular interactions of WT1 isoforms in different subcellular compartments, our results allow us to suggest, for the first time, a cellular role for ZNF255 and provide a striking example of how recently evolved ZF proteins, such as ZNF255 and ZNF224, can function to modulate the activity of an ancient, evolutionary conserved ZF protein, WT1.

RESULTS

WT1 interacts *in vivo* with ZNF224 and ZNF255

The protein ZNF255 was previously identified as an interacting partner of WT1 by affinity chromatography and shown to inhibit WT1 transactivation (5). We recently demonstrated that ZNF255 is in fact an alternatively spliced variant of the transcriptional repressor ZNF224 that lacks the KRAB domain while retaining 19 ZFs. Furthermore, ZNF255 displays a markedly reduced transcriptional repressor activity and decreased affinity for the AldA-NRE motif, when compared with the effects of its KRAB-containing isoform ZNF224 (4).

In order to investigate whether WT1 also binds to the ZNF224 protein, we performed co-immunoprecipitation assays of the endogenous proteins in the leukaemia cell line K562, which express high levels of WT1. Total protein extracts were immunoprecipitated with an anti-WT1antibody (F6) directed at the N-terminus and the subsequent western blot probed with an antibody that recognizes ZNF224 and ZNF255 isoforms (T3), revealing that both proteins are able to interact with WT1 (Fig. 1A). As a negative control, neither ZNF224 nor ZNF255 is immunoprecipitated with rabbit IgG.

The proteins ZNF224 and ZNF255 show different subcellular localization patterns: ZNF224 is predominantly a nuclear protein, whereas ZNF255 is present in the nucleus and in the cytoplasm. WT1 localization both in the nucleus and in the cytoplasm has been interpreted as reflecting distinct functional roles in the different subcellular compartments (37). These findings led us to investigate whether the interaction between the ZNF224/ZNF255 isoforms and WT1 occurred in specific compartments. To look at this, we conducted an immunoprecipitation assay for endogenous proteins on nuclear and cytoplasmic extracts of K562 cells. Western blot

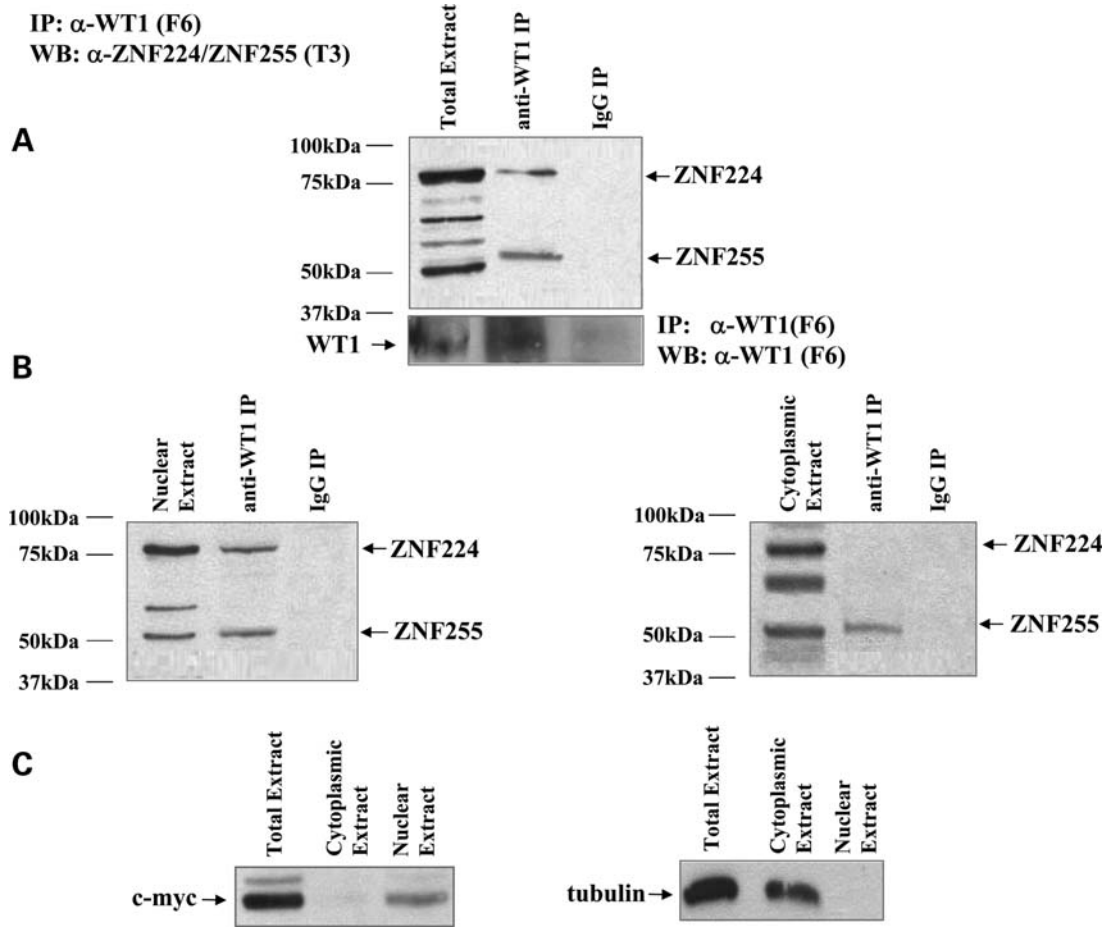

Figure 1. Immunoprecipitation of ZNF224 and ZNF255 with the Wilms' tumour protein WT1. (**A**) The anti-WT1 F6 antibody or control immunoglobulin (IgG) was used in immunoprecipitation assay on K562 total lysates. Western blot with the antibody against ZNF224 and ZNF255 (T3) shows that both proteins interact with WT1. Lower panel shows western blot with anti-WT1 F6 antibody. (**B**) The anti-WT1 F6 antibody was used in immunoprecipitation assay on nuclear and cytoplasmic extract of K562 cells. Western blot with the antibody T3 shows that ZNF224 interacts with WT1 only in the nucleus, whereas ZNF255 is present in nuclear and cytoplasmic immunoprecipitates. The additional bands detected by the T3 antibody in extracts may represent additional isoforms of ZNF224/255 or closely related, cross-reacting KRAB-ZNF proteins. (**C**) The purity of fractioned extract was checked by western blot with anti-c-myc and anti-tubulin.

analysis of immunoprecipitated proteins performed with T3 antibody shows that ZNF224 interacts with WT1 only in the nucleus, whereas the interaction between WT1 and ZNF255 occurs both in the nucleus and in the cytoplasm (Fig. 1B). The extract purity was controlled with anti-myc and anti-tubulin antibodies (Fig. 1C), confirming that the cytoplasmic extracts were free from nuclear contamination and vice versa. These specific interactions were further validated in HEK293 cells, where ectopic WT1 and ZNF224 or ZNF255 proteins fused to a FLAG epitope were co-expressed and subsequently immunoprecipitated with the anti-WT1 antibody in a similar manner (data not shown).

ZNF224 and ZNF255 interact with specific WT1 isoforms

Given the differential interactions of ZNF224/ZNF255 with WT1 in particular subcellular compartments and increasing evidence that WT1 isoforms are involved in different stages of gene expression regulation, we decided to consider whether the proteins ZNF224 and ZNF255 interact specifically with the WT1(+KTS) and (−KTS) isoforms, in order to investigate potential biological roles of these interactions. Specific antibodies to the WT1 isoforms do not exist, so we performed immunoprecipitation assays with the C-terminal anti-WT1 antibody (C19) on cell lysates from HEK293 cells co-transfected with plasmids coding for WT1(−KTS) or WT1(+KTS) together with plasmids coding for ZNF224-FLAG or ZNF255-FLAG, respectively. ZNF224-FLAG was specifically co-immunoprecipitated by anti-WT1 antibodies in the presence of the WT1(−KTS) isoform (Fig. 2A), but not with the WT1(+KTS) isoform (Fig. 2C). On the contrary, ZNF255 was co-immunoprecipitated with both isoforms, WT1(−KTS) and WT1(+KTS) (Fig. 2B and D). Moreover, this experiment is consistent with the subcellular localization of the isoforms: the interaction between ZNF224 and WT1(−KTS) occurs in the nucleus, whereas ZNF255 and WT1(+/−KTS) interact in both the nucleus and the cytoplasm.

In order to visualize these interactions and confirm the result of the immunoprecipitation experiments, immunofluorescence under confocal microscopy was performed on COS7 cells transfected with the expression plasmids described above.

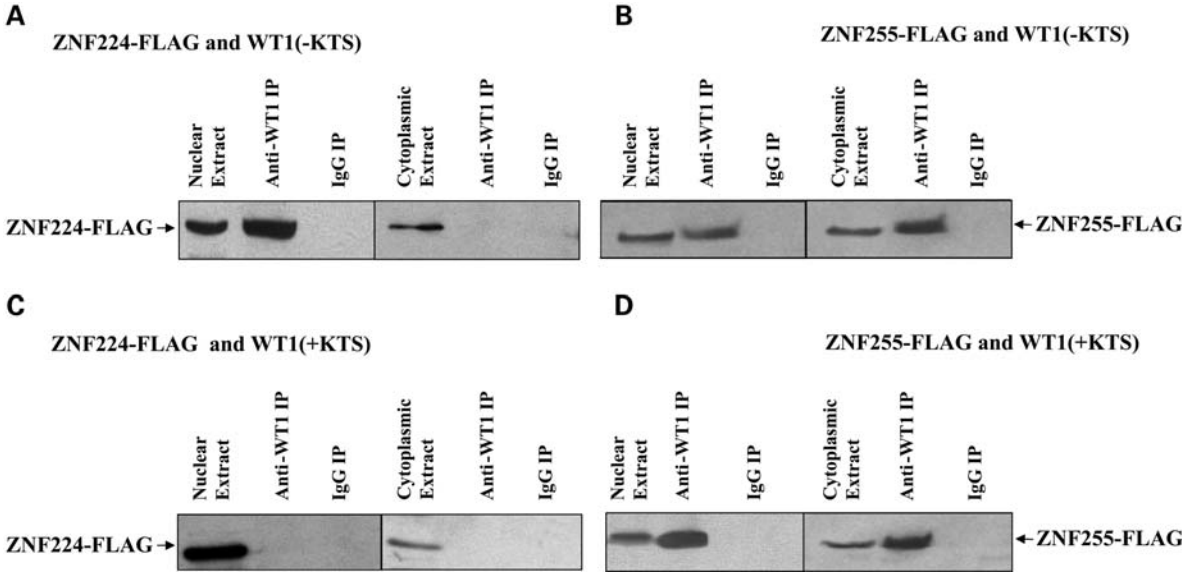

Figure 2. ZNF224 and ZNF255 show different affinity for the (+/−KTS) WT1 isoforms. HEK293 cells were co-transfected with p3XFLAG-ZNF224 and pcDNA3-WT1(−KTS) (**A**) or with p3XFLAG-ZNF224 and pcDNA3-WT1(+KTS) (**C**) C19 antibody was used to immunoprecipitate the different isoforms of WT1 from nuclear and cytoplasmic lysate. The immunoprecipitated complexes were probed with the anti-FLAG antibody. In (A), a specific interaction of ZNF224 with WT1(−KTS) in the nucleus is shown. No interaction was observed between ZNF224 and WT1(+KTS) (C). The same experiment was performed on HEK293 transfected with p3XFLAG-ZNF255 and pcDNA3-WT1(−KTS) (**B**) or pcDNA3-WT1(+KTS) (**D**). Western blot shows that ZNF255 interacts with both WT1 isoforms in nuclear and cytoplasmic compartments.

Transfected cells, observed under confocal microscopy, show the expected subnuclear localization patterns of WT1(+KTS) and (−KTS) isoforms, consistent with their proposed roles: the 'transcriptional isoform', WT1(−KTS), gave rise to the expected diffuse nuclear localization apart from nucleoli (Fig. 3A and C), typical of a transcription factor, whereas the 'RNA processing isoform', WT1(+KTS), is present in the nuclear speckles, a pattern commonly associated with nuclear splicing factors (Fig. 3B and D). Merging of the double stained planes revealed the signal of ZNF224-FLAG protein (red) overlapping strikingly with the WT1(−KTS) signal (green) (Fig. 3A), but showing no overlap with the WT1(+KTS) signal (Fig. 3B), in accordance with the immunprecipitation results. Furthermore, consistent with the immunoprecipitation, ZNF255-FLAG partially overlaps with the WT1(−KTS) signal (Fig. 3C) and shows a nuclear speckled pattern that strikingly overlaps with the signal of WT1(+KTS) isoform (Fig. 3D), such that almost all WT1(+KTS) speckles (green) are associated with ZNF255. Note that the diffuse versus speckled localization of WT1 isoforms is not 100% discrete, as reported by Larsson *et al*. (17).

As a whole, the results from the immunoprecipitation and immunofluorescence assays demonstrate that both ZNF224 and ZNF255 interact with WT1 in an isoform-specific manner. Since WT1(−KTS) and WT1(+KTS) have been suggested to control separate aspects of gene expression regulation, it is tempting to speculate that there are distinct functional roles for these protein complexes in the cell. The specific nuclear interaction between ZNF224 and WT1(−KTS) suggests that these proteins cooperate in transcriptional regulation. The results obtained for ZNF255 and WT1, on the contrary, suggest an involvement in a different cellular process, such as RNA processing.

ZNF224 enhances WT1-mediated transcriptional activation

It is well documented that physical interactions of WT1 with other cellular proteins can modulate its transcriptional activity. Therefore, as both WT1(−KTS) and ZNF224 are transcriptional factors, we first hypothesized that their specific nuclear interaction would be involved in transcriptional regulation.

To determine the effect of the WT1(−KTS)/ZNF224 interaction on WT1-dependent transcriptional regulation, we carried out transfection experiments in HeLa cells. We chose a reporter plasmid (phVDR-LUC) containing the human *vitamin D receptor* promoter, a known target of the WT1 transcriptional activation, cloned upstream of the Firefly Luciferase gene (21). As shown in Figure 4A, the co-transfection of a fixed amount of pcDNA3WT1(−KTS) and increasing concentrations of p3XFLAG-ZNF224 causes a dose-dependent up-regulation of the phVDR-LUC transcriptional activity (lanes 5–7). The specificity of the effect of ZNF224 on the activation function of WT1 was demonstrated by co-transfection of a construct expressing Nrf2 as a negative control (p3XFLAG-Nrf2), which does not influence the luciferase activity (lanes 11–13). Furthermore, increasing concentrations of p3XFLAG-ZNF224 had no effect on phVDR-LUC in the absence of WT1 (lanes 8–10). On the contrary, as shown in Figure 4B, co-transfection of increasing amount of p3XFLAG-ZNF255 (lanes 5–7) with WT1(−KTS) does not significantly affect phVDR-LUC transcriptional activity.

Given that ZNF224 only activates transcription in the presence of WT1, we performed chromatin immunoprecipitation (ChIP) experiments to investigate the nature of this interaction, at the molecular level, on endogenous target genes

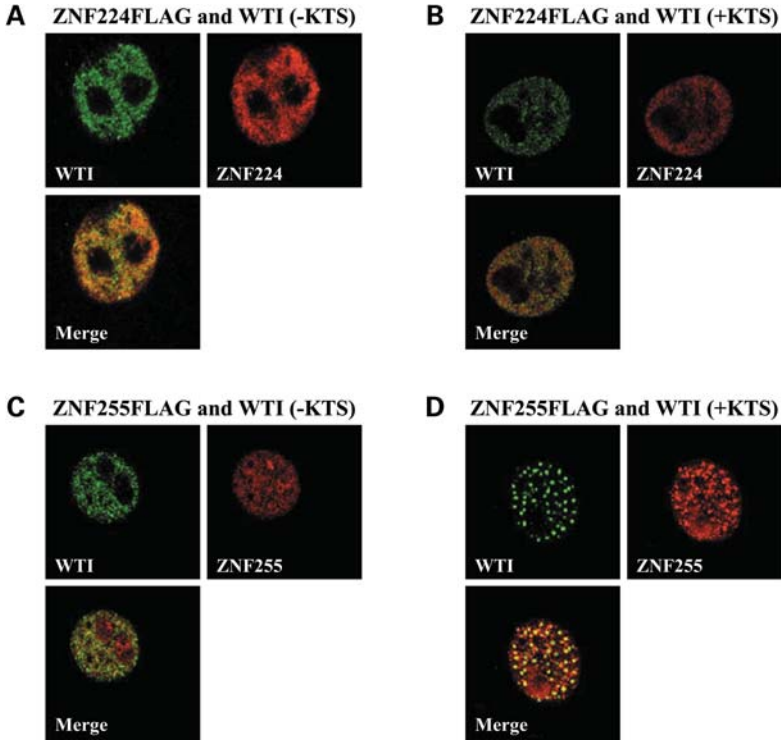

Figure 3. ZNF224 and ZNF255 co-localize with different WT1 isoforms. COS7 cells were transfected with p3XFLAG-ZNF224 plus pcDNA3-WT1(−KTS) (**A**) or pcDNA3-WT1(+KTS) (**B**) and with p3XFLAG-ZNF255 plus pcDNA3-WT1(−KTS) (**C**) or pcDNA3-WT1(+KTS) (**D**). Twenty-four hours after transfection, the cells were fixed, permeabilized and the immunofluorescence was performed with anti-FLAG and anti-WT1 (C-19) as primary antibodies. Panel A shows the overlap of ZNF224-FLAG (red) and WT1(−KTS) signals (green) whereas ZNF224-FLAG does not overlap with the signal of WT1(+KTS) (B). ZNF255-FLAG has a nuclear localization (red) that partially overlaps with WT1(−KTS) signal (green) (C) but shows a nuclear speckled pattern that precisely overlaps with the signal of WT1(+KTS) isoform (D).

of both WT1 (*VDR*) and ZNF224 (*AldA*). Chromatin was prepared from HEK293 cells transfected with pcDNA3WT1 (−KTS), or p3XFLAG-ZNF224, or co-transfected with pcDNA3WT1(−KTS) and p3XFLAG-ZNF224, respectively. Chromatin was immunoprecipitated with anti-WT1 (Fig. 4C, left panel) or anti-FLAG (Fig. 4C middle and right panels) antibodies. Real-time PCR analyses were performed using oligonucleotides flanking the WT1-binding sites on the *VDR* promoter or the *AldA*-NRE element, which we had previously characterized as a ZNF224-binding site (2). As expected, WT1 was bound to *VDR* promoter region and not to *AldA*-NRE element (Fig. 4C, left panel), whereas ZNF224 was bound to *AldA*-NRE element and not to *VDR* promoter region (Fig. 4C, middle panel), thus confirming that ZNF224 alone has no effect on *VDR*. When ZNF224 was over-expressed along with WT1, it was found to bind the *VDR* promoter, thus demonstrating that recruitment of ZNF224 to *VDR* promoter requires WT1 (Fig. 4C, right panel). These results provide experimental evidence that WT1 and ZNF224 interact at the chromatin level in a kidney cell line.

In order to determine the consequences of this interaction in a more physiologically relevant setting, we carried out siRNA-mediated knockdown of ZNF224 in K562 cells and measured the expression of endogenous VDR and of aldolase A mRNA, taken as a control. Figure 5 shows that knockdown of ZNF224 leads to a considerable increase in aldolase A mRNA expression, according to the ZNF224 repression role on *aldolase A* gene transcription (40); at the same time, ZNF224 silencing leads to an appreciable reduction in VDR expression both at mRNA (Fig. 5A) and protein levels (Fig. 5B), demonstrating that not only can exogenous ZNF224 augment WT1-mediated VDR expression but also ZNF224 contributes to the normal physiological expression of the *VDR* gene.

These experiments demonstrate that the protein ZNF224 can cooperate with WT1 in the transcriptional regulation of the *VDR* promoter, enhancing the gene activation mediated by WT1, and that this cooperation does not involve direct DNA binding of ZNF224. On the other hand, the poor transcriptional effect of p3XFLAG-ZNF255 supports the notion that the isoform ZNF255 is mainly involved in a different process, such as RNA processing.

Conversely, employing similar transient transfection experiments, we did not observe functional consequences of the WT1(−KTS)/ZNF224 interaction on the transcriptional regulation of the only known ZNF224 target sequence (*AldA*-NRE), suggesting that WT1 does not play a role in ZNF224-dependent transcriptional repression (data not shown). This raises the intriguing possibility that KRAB-ZF proteins are not simply transcriptional repressors, as is widely thought, given that ZNF224 appears to have both repression and activation functions with the latter modulated via interaction with WT1.

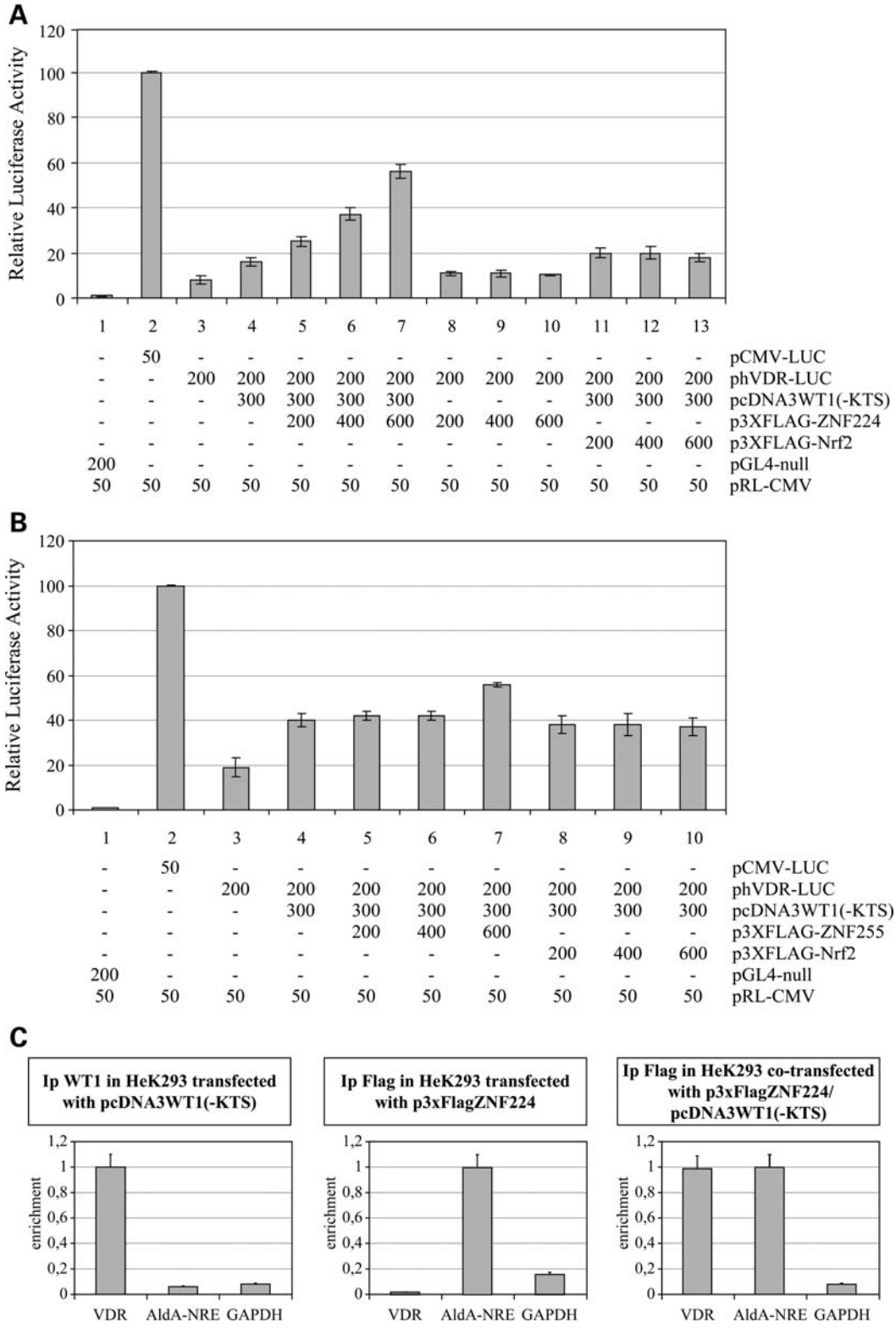

Figure 4. ZNF224 specifically enhances WT1-mediated transcriptional regulation and is recruited by WT1 to *VDR* promoter. **A-B**: The -960phVDR-LUC was used as reporter plasmid in transfection assays performed in HeLa cells [(**A**) lanes 3–13 and (**B**) lanes 3–10]. The reporter gene is activated by the pcDNA3 WT1(−KTS) (A and B, lane 4). The co-transfection of p3XFLAG-ZNF224 plasmid activates the reporter gene in a dose-dependent manner (A, lanes 5–7), whereas the p3XFLAG-ZNF255 co-transfection has no effect (B, lanes 5–7). Moreover, the reporter gene is not activated by transfection of p3XFLAG-ZNF224 plasmid alone (A, lanes 8–10). pRL-CMV plasmid was used to normalize the results, pGL4-null empty vector activity indicate the background (A and B, lane 1) and the pCMV-LUC was the positive control (A and B, lane 2). The p3XFLAG-Nrf2 plasmid was used as a negative control. [(A) lanes 11–13 and (B) lanes 8–10]. (**C**) ChIP assay was performed with anti-WT1 antibody (left panel) or anti-FLAG antibody (middle and right panels). Analysis of WT1 and ZNF224 binding to *VDR* and *AldA*-NRE regions was conducted by quantitative real-time PCR. *Error bars* indicate the mean value ± SD of two independent experiments.

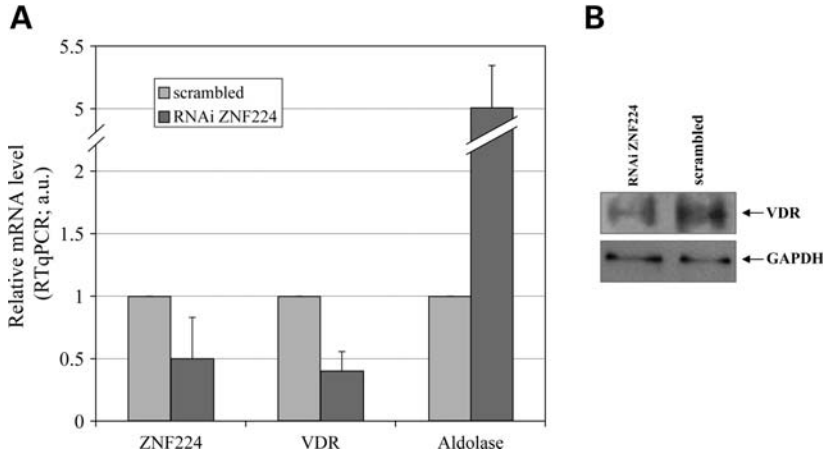

Figure 5. ZNF224 knockdown affects the mRNA expression of VDR, an endogenous Wt1 target gene in K562 cells. (**A**) K562 cells were transfected with ZNF224 shRNA or scrambled RNA. Forty-eight hours after transfection, RNA and total protein extracts were prepared. ZNF224, aldolase A and VDR mRNA levels were measured by real-time PCR. Error bars indicate the mean value ± SD of two independent experiments. (**B**) Protein extracts were analysed by western blot with anti-VDR and anti-GAPDH antibodies.

ZNF255 is associated with actively translating polyribosomes

Evidence is emerging that WT1 is a shuttling protein with roles in RNA metabolism and possibly in translation (37,38). The interaction and co-localization of ZNF255 with the WT1(+KTS) isoform prompted us to evaluate the possibility that ZNF255 co-localizes with WT1 in the translating ribosomes. To this aim, we performed a sucrose gradient analysis, allowing the separation of various components of the RNA processing machinery. HEK293 cells were transfected with plasmids coding for ZNF255-FLAG and WT1(+KTS) proteins and the cellular extract was loaded on a 10–50% sucrose gradient. Following ultracentrifugation, the collected fractions were analysed by western blot with specific antibodies. The antibody against L7A, an integral major ribosome subunit protein, was used as a marker for the ribosome profile on the gradient. The anti-Hsp90β antibody, a protein that does not associate with the ribosomes, was used as a negative control. As shown in Figure 6A, the distribution pattern of ZNF255 overlapped with WT1 and L7A localization in polysomal fractions. On the contrary, endogenous ZNF224 protein was not associated with the ribosomes, being present in the same fractions as Hsp90β.

The sucrose gradient was also performed on extracts treated with ethylenediamine tetra-acetic acid (EDTA), leading to the dissociation of polysomes and 80S ribosomes and delocalizing the proteins associated with the translational complex. Staining with the anti-L7a antibody and monitoring the absorbance at 260 nm of the gradient fractions (Figure 6B, upper panel) show that EDTA treatment was effective in inducing polyribosome dissociation. As shown in Figure 6B, the ZNF255-FLAG and WT1 sedimentation profiles were disrupted in EDTA-treated extracts. Under these conditions, the two proteins were redistributed to lighter fractions of the gradient, sedimenting primarily with the 60S and 40S subunits. By comparison, Hsp90β and ZNF224 localization were not affected by EDTA treatment.

Taken together, these data indicate that cytoplasmic ZNF255 is associated with the translation machinery, in a manner similar to WT1, suggesting an involvement in protein synthesis or in translational regulation.

ZNF255 co-purifies with poly(A) RNP

Given that cytoplasmic WT1 is associated with actively translating ribosomes and with poly(A) RNP complexes (37,38), we decided to test whether cytoplasmic ZNF255 could also be detected in RNP complexes and was capable of binding to poly(A) RNPs. For this purpose, HEK293 cells were transfected with ZNF255-FLAG and WT1(+KTS) coding plasmids, and 48 h after transfection, cellular extracts were incubated with oligo(dT) cellulose beads, to precipitate mRNA and its binding proteins. After extensive washing, the RNA was eluted from the beads with hot diethyl pyrocarbonate (DEPC)-treated water. As shown in Figure 7, both ZNF255-FLAG and WT1 proteins were detected in the eluate by the western blot analysis (lane 2, middle panels), confirming an association of these two proteins with the mRNPs. As expected, the Hsp90 protein is not eluted with the oligo(dT)-binding complexes (lane 2, lower panel). Interestingly, the ZNF224 protein again shows a different behaviour from its isoform, remaining totally unbound by the beads, as is Hsp90 (lane 2, upper panel). In the control experiments, to exclude the possibility of a non-specific binding of ZNF255-FLAG and WT1 protein to the oligo(dT) cellulose, the beads were saturated with oligonucleotides containing stretches of 10–18 adenines prior to incubation with the extract (Fig. 7, lanes 3 and 4). Under these conditions, WT1 and ZNF255-FLAG are not detected in these eluates (lane 4, middle panels). These results indicate that the binding of WT1 and ZNF255 to the beads is mediated by poly(A) RNA and once again that ZNF224 has a different behaviour with respect to the isoform.

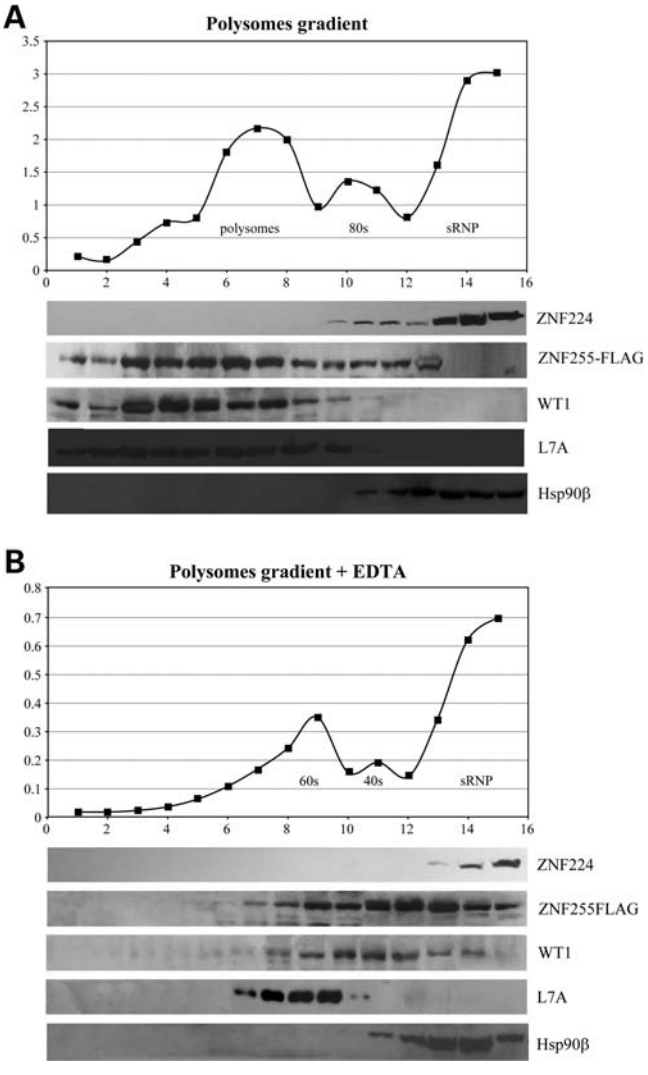

Figure 6. ZNF255 and WT1 are present in the polysome fractions. The polysome gradient was performed in HEK293 cells transfected with the plasmids coding for ZNF255-FLAG and WT1(+KTS). The protein extracts were loaded on a 10–50% sucrose gradient. Fifteen fractions were collected from the top and analysed by western blot using anti-WT1, anti-FLAG and T3 antibodies. As a marker for the ribosome profile, the antibody anti-L7A was used. The anti-Hsp90β antibody was used as a negative control. The upper panel in (**A**) and (**B**) shows the optical density profiles of the gradient fractions at 260 nm in the absence and in the presence of EDTA, respectively. The analysis shows that a large proportion of ZNF255 and WT1 proteins are found in polysome fractions, whereas ZNF224 is not (A, upper panels). Polyribosome dissociation by EDTA modifies L7A, ZNF255-FLAG and WT1 localization in the gradient fractions, whereas the localization of Hsp90β (B, lower panel) and ZNF224 (B, upper panel) is not modified. Polysomes, ribosomal subunits 80s, 60s and 40s and RNP are indicated.

Altogether our data point towards a striking specificity of differential interaction: ZNF255 may participate with WT1(+KTS) in RNA maturation and post-transcriptional control, whereas the ZNF224 interaction with WT1(−KTS) is involved in WT1-mediated transcriptional regulation.

DISCUSSION

Alternative splicing/initiation of the *ZNF224* gene can give rise to at least two different proteins: ZNF224, which is

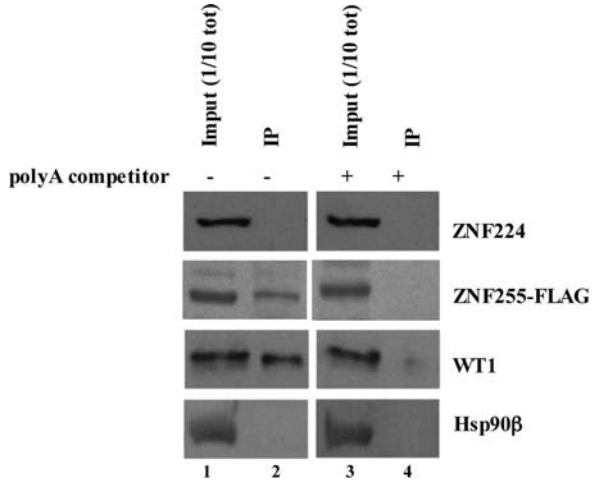

Figure 7. ZNF255 binds the RNA poly A. The HEK293 cells were transfected with p3XFLAG-ZNF255 and pcDNA3-WT1(+KTS) and the protein extract was incubated with oligo(dT) cellulose for 2 h. The poly(A)$^+$-binding proteins were eluted, loaded on an SDS–PAGE and analysed by western blot using anti-WT1, anti-FLAG and T3 antibodies. ZNF255-FLAG and WT1 proteins are detected in the poly(A)$^+$-eluted samples (lane 2, middle panels), whereas ZNF224 and Hsp90β, used as negative control, are not present (lane 2, upper and lower panels, respectively). As further control, the oligo(dT) beads were incubated with poly(A) oligonucleotides. Under this condition, WT1 and ZNF255-FLAG signals were not observed (lane 4, middle panels). Lanes 1 and 3 represent 1/10 input samples hybridized with the same antibodies as control.

shown to function as a transcriptional repressor of the *aldolase A* gene (2,3,40), and ZNF255, which shows a different subcellular localization and reduced affinity for the *aldolase A* promoter (4). ZNF255 has been shown to interact with the ZFs of WT1 to modulate WT1 transcriptional activity (5). Since ZNF224 shares its ZF domains with the ZNF255 isoform, we sought to investigate whether ZNF224 also interacted with WT1.

Our data, derived from endogenous immunoprecipitation, isoform-specific transfection, confocal immunofluorescence localization and siRNA knockdown of endogenous expression, reveal a striking isoform- and subcellular compartment-specific interaction between the major WT1 isoforms and ZNF224/255. ZNF224 interacts with WT1 only in the nucleus and only with the (−KTS) isoform, whereas ZNF255 can interact with both WT1 isoforms in the nucleus and cytoplasm, but predominantly with WT1(+KTS), implying diverging functional roles for the ZNF224/WT1 and ZNF255/WT1 interactions. Based upon the proposed roles of the WT1(−KTS) and WT1(+KTS) isoforms, it is tempting to speculate that the ZNF224/WT1 interaction is involved in transcriptional regulation, whereas the ZNF255/WT1 interaction mediates post-transcriptional regulation, extending into the cytoplasm. This notion is supported by transfection assays on a known WT1-regulated promoter, *VDR*, which revealed a ZNF224 enhancement of WT1(−KTS)-mediated transactivation and ChIP experiments, demonstrating that ZNF224 is recruited into *VDR* promoter by WT1. Moreover, siRNA knockdown of ZNF224 resulted in a reduced expression of endogenous VDR. However, under similar transfection conditions, ZNF255 had no effect on WT1(−KTS)-mediated transactivation of *VDR*, consistent

with the weaker association observed under confocal microscopy. Typically, KRAB-ZF proteins are considered as transcriptional repressors, recently implicated in imprinting and disease, acting via KRAB-mediated recruitment of KAP-1 co-repressor complexes containing histone deacetylases and methyltransferases (40–42). However, our data indicate that, even in the presence of the KRAB domain, ZNF224 can also function as a transcriptional co-activator, without directly binding promoter DNA.

With the identification of factors that can modulate the ability of WT1 to regulate transcription, the complexity of the role of WT1 in transcriptional regulation is beginning to be revealed. These factors include DNA-binding proteins, such as members of the p53 family, and proteins that do not directly contact DNA, such as BASP1 and Par-4 (19,25,26). BASP1 was identified as a WT1 transcriptional co-suppressor (25), whereas Par-4 can act as either a transcriptional co-activator or as a co-repressor for WT1, depending on the splice isoform of WT1 (26).

In our co-transfection experiments, ZNF255 displayed a poor activity in WT1-mediated transcriptional regulation, supporting the hypothesis that ZNF255 interaction with WT1 could be involved in a different molecular process. However, although our findings differ from the previously reported inhibition of WT1-mediated transactivation of *VDR* by ZNF255 (5), there are several differences between the two experiments, in particular that they make use of different cell lines. Although there are numerous examples in the literature of cell line-dependent differences in WT1 activity (10,12), the differences between these two studies may have a biological explanation: our data show that ZNF255 can interact with WT1(−KTS) in the nucleus, albeit less strongly than the ZNF224 interaction; thus, if, as we propose, WT1/ZNF255 complexes function at a post-transcriptional level, the overexpression of ZNF255 might sequester WT1(−KTS) from a transcriptional compartment, thereby inhibiting WT1 transactivation. Furthermore, as ZNF255 does not contain the KRAB domain, any repressive function is unlikely to occur via the classical KRAB-mediated KAP1 co-repressor pathway. Notwithstanding experimental differences, our direct comparison demonstrates a clear difference between the abilities of ZNF224 and ZNF255 to modulate the transcriptional activity of WT1.

Whereas the ZNF224/WT1(−KTS)-specific interaction suggests a role in transcriptional regulation, the lack of transcriptional activity associated with the ZNF255/WT1(+/−KTS) interaction and the continued interaction in the cytoplasm raises the intriguing possibility that ZNF255 is involved in conferring post-transcriptional function on WT1, leading us to investigate a role for ZNF255 in post-transcriptional control. Previously, it has been demonstrated that WT1 could be present in actively translating polysomes and bound to polyadenylated RNA (37,38). Our experiments demonstrate that ZNF255, but not ZNF224, co-localizes with WT1 in the translating ribosomes and is associated with WT1 in poly(A)$^+$ transcript-containing complexes.

To consider WT1(−KTS) as the 'transcriptional isoform' and WT1(+KTS) as the 'post-transcriptional isoform' is likely to be an oversimplification as there is clearly redundancy at the molecular and the genetic levels (10,43). It is likely that the insertion of KTS affects the binding affinity of the ZF region for nucleic acids and/or proteins rather than switching between two distinct states. Such a view is consistent with our findings and with several other studies, including: Larsson *et al*. (17) first demonstrated the specific localization of WT1(+KTS) and WT1(−KTS) and also showed that this was not 100% discrete; Caricasole *et al*. (29) showed that both (+KTS) and (−KTS) isoforms could bind RNA; Niksic *et al*. (38) provided evidence that both isoforms could associate with polysomes and shuttle between the nucleus and cytoplasm. However, the demonstration that ZNF255 interacts with WT1 in the nucleus but does not affect transcription, yet interacts with both isoforms in the cytoplasm provides an example of how the activity of WT1 isoforms may be modulated by a switch in interacting partner and that ZNF255 may be part of a WT1-containing shuttling complex. Furthermore, it is intriguing to note that the expression of ZNF255 may augment the nuclear speckled localization of WT1 (+KTS) over that seen in the presence of ZNF224, although this would require further investigation. It has been postulated that WT1 may be involved in all stages of gene expression, being physically associated with, first, the promoter, then the splicing pre-mRNA and ultimately present during translation. However, our findings imply that, if this is the case, different isoforms of ZNF224/ZNF255 participate at specific steps.

Despite their prevalence in the human genome, little is known of the role of human *KRAB-ZF* genes in development and disease. Many human *KRAB-ZF* genes, among which *ZNF224*, have no murine homologue, precluding the use of mouse genetics (gene knockout) to elucidate their function. One well-characterized *KRAB-ZF* gene, *ZNF74*, was identified as a developmental gene hemizygously deleted in the majority of patients with DiGeorge syndrome (44,45) and, recently, polymorphisms within the *ZNF74* gene have been strongly correlated with age-at-onset of schizophrenia (46). It is tempting to speculate that the recently evolved primate-specific KRAB-ZF factors are involved in higher, neurological functions. Intriguingly, specific functions of the individual WT1(+KTS) and WT1(−KTS) isoforms in neuronal development are beginning to emerge: WT1(+KTS) is specifically required for the development of olfactory neuronal progenitor cells, whereas WT1(−KTS) is specifically required for the development of neuronal cells of the embryonic retina (47,48). In addition, our finding that ZNF224 modulates endogenous VDR expression in the chronic myelogenous leukaemia (CML) cell line, K562, may point to a role for this interaction in leukaemia: The majority of CMLs express high levels of WT1 (49), and it has recently been demonstrated that vitamin D signalling is important for stimulating the Hox-A10/MafB pathway controlling haematopoietic differentiation (50). Although we have found no strong correlations within publicly available databases, more powerful bioinformatic interrogations focusing on ZNF224/255, WT1 and vitamin D signalling may prove fruitful, as may an extended analysis searching for other genes in leukaemic cells potentially regulated by the WT1/ZNF224 interaction.

A major unresolved question surrounding KRAB-ZF proteins is why they contain more ZFs than necessary to provide

sequence-specific DNA-binding diversity. Most genomic studies tend only to consider ZF binding to DNA as a driving force for the evolution of poly-ZF proteins; however, the example presented herein demonstrates that protein:protein interaction is likely to be a significant factor. The complex, isoform-specific interactions of ZNF224/255 with an ancient ZF transcription factor described herein suggest that they have evolved in a specific way to interact with specific isoforms of a protein conserved throughout vertebrate radiation.

MATERIALS AND METHODS

Cell lines and transfection

HeLa, HEK293 human cell lines and the COS7 monkey cell line were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% fetal calf serum and 1% penicillin and streptomycin (Bio Whittaker). K562 cells were cultured in RPMI 1640 with 10% fetal calf serum and 1% penicillin and streptomycin (Bio-Whittaker). HeLa and HEK293 cells were transfected with FuGENE Reagent (Roche, Basilea, Switzerland), as recommended by the manufacturer. COS7 cells used in immunofluorescence assay and K562 cells used in RNA interference experiments were transfected using Lipofectamine 2000 Reagent (Invitrogen, CA, USA).

Immunoprecipitation assay

For the exogenous proteins, HEK293 cells were co-transfected with 3 μg of pcDNA3-WT1 (+/−KTS) together with 3 μg of p3XFLAG-ZNF224 or p3XFLAG-ZNF255 in a 100 mm plate. Total protein extract was obtained as follows: cells were lyzed in lysis buffer (25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic hepes acid (HEPES) pH 7.4, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2.5 mM EDTA, 2.5 mM ethylene glycol tetraacetic acid (EGTA), 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 mM NaF and 5 mM sodium pyrophosphate) for 30 min on ice. The extract was centrifuged in a microfuge at the maximum speed for 10 min and the supernatant was used in the immunoprecipitation. K562 cell lysates for immunoprecipitation assays of endogenous proteins were prepared as above. Nuclear and cytoplasmic extracts from K562 and HEK293 cells for the fractioned lysates were prepared by resuspending pelletted cells in the Cytoplasm Lysis Buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenyl methane sulfonyl fluoride (PMSF), 10 μg/ml aprotinin, leupeptin and pepstatin) and incubated for 15 min on ice. After the addition of 0.6% NP-40, the lysate was vigorously vortexed and centrifugated for 30 s at the maximum speed. The supernatant (cytoplasm) was collected and the nuclear pellet were transferred in a fresh tube and incubated on ice for 15 min in the Nuclei Lysis Buffer (20 mM HEPES pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, leupeptin and pepstatin). The nuclear extract was then cleared with 5 min of centrifugation at the maximum speed.

An anti-WT1 antibody (F6 Santa Cruz Biotechnology, CA, USA) or the IgG rabbit (Santa Cruz Biotechnology) were incubated overnight with 600 μg of protein lysate. The immune complexes were collected with Protein A/G PLUS Agarose (Santa Cruz Biotechnology), washed four times with the lysis buffer and loaded on 8% SDS–PAGE.

To evaluate the interaction of ZNF224 and ZNF255 with different WT1 isoforms, HEK293 cells were transfected with 3 μg of the plasmids p3XFLAG-ZNF224 or p3XFLAG-ZNF255 and pcDNA3-WT1(-KTS) or pcDNA3-WT1(+KTS). The cells were lyzed as described previously and incubated overnight with C19 antibody or rabbit IgG. ProSep-VA High CAPACITY beads (Millipore Corporation, MA, USA) were then added to the samples and incubated for 1 h. The immunoprecipitated samples were washed and loaded on 8% SDS–PAGE for western blot analysis with the anti-FLAG antibody.

Antibodies and western blot

Protein samples from the assays were quantified using the Bio-Rad protein assay reagent, resolved on SDS–PAGE and then transferred to Hybond membranes (Amersham Biosciences, NJ, USA). Non-specific binding sites were blocked for 2 h with 5% milk in tris-tween buffered saline (tTBS) (5 mM Tris pH 7.5, 15 mM NaCl, 0.1% Tween-20), washed three times with tTBS and incubated with the following antibodies:

anti-ZNF224/ZNF255 1:1000 (Rabbit polyclonal antibody T3)
anti-WT1 (C19 Santa Cruz Biotecnology) 1:1000
anti c-Myc (Amersham Pharmacia) 1:500
anti-FLAG, M2 (Sigma Aldrich, MO, USA) 1:5000
anti-L7a serum 1:2500
anti-VDR antibody (Santa Cruz Biotechnology) 1:100
anti-GAPDH (OriGene Technologies, Rockville, USA) 1:1000
anti-tubulin (Upstate, Lake Placid, NY, USA) 1:1000

The secondary antibodies were protein A, IgG anti-mouse and IgG anti-rabbit (Amersham) conjugated to horseradish peroxidase 1:5000 and detected with ECL western blot detection system (Amersham).

Luciferase assay

To normalize the luciferase assay pRL-CMV (50 ng) coding for the *renilla* luciferase was co-transfected in HeLa cells plated at a density of 3×10^5 per well in 24-well dishes. The pGL4-null empty vector (200 ng) was used to evaluate the background lucyferase activity, whereas the pCMV-LUC (50 ng) was the positive control for the assay. The −960phVDR-LUC (200 ng) coding for the *firefly* luciferase gene controlled by the *VDR* promoter was used as a reporter plasmid. The transcriptional activity of the reporter plasmid is activated by the co-transfection of the pcDNA3 WT1(−KTS) (300 ng). To evaluate the effect of ZNF224 and ZNF255, 200, 400 or 600 ng of the p3XFLAG-ZNF224 and the p3XFLAG-ZNF255 plasmids were transfected. The p3XFLAG-Nrf2 plasmid coding for the transcriptional factor Nrf2 was used as a negative control. The Dual-Luciferase Reporter Assay System (Promega Corporation, WI, USA) was performed 48 h after the transfection according to manufacturer's instructions.

ChIP assay and real-time PCR

HEK293 cells were transfected with pcDNA3WT1(-KTS), or p3XFLAG-ZNF224, or co-transfected with pcDNA3WT1 (-KTS) and p3XFLAG-ZNF224, respectively. Chromatin was purified and immunoprecipitated with anti-Flag (Sigma), anti-WT1 (C19 Santa Cruz Biotechnology) and immunoglobulin G (IgG Sigma) antibodies, as described previously (40). ChIP samples were then analysed by quantitative real-time PCR using a Master Mix SYBR Green (Bio-Rad, CA, USA) and specific primers: for VDR TB3 Fw, 5-CACCTGGCTCA GCGTCC-3 and TB3 Rev, 5-GCCAGGAGCTCCGTTGGC-3. For AldA-NRE: AldA-NRE Fw, 5-CCCTCTGTTCCACTG GGCAAGTGAG-3 and AldA-NRE Rev, 5-CCATTCCAGTT CCCAGGCCTGGGTG-3.

As a negative control, real-time PCR was done using *GAPDH*-specific primers (GAPDH For: 5′-GGTCGTATTGG GCGCCTGGTCACCA-3′ and GAPDH Rev: 5′-CACACCC ATGACGAACATGGGGGC-3′). The amount of the immunoprecipitated DNA fragments derived from *VDR* promoter or *AldA*-NRE region were compared with the negative IgG control. Real-time PCR data analysis followed the methodology previously described (51).

siRNA knockdown and real-time PCR

K562 leukaemia cells were transiently transfected using Lipofectamine 2000 Reagent (Invitrogen) in six-well plates with 2 μg of the short interfering RNA plasmid SH2351C3 (Open Biosystems, Huntsville, AL, USA) to silence ZNF224/255 or 2 μg of a scrambled RNA. Forty-eight hours after transfection, the total RNA was isolated by TRIzol Reagent (Invitrogen). One microgram of each RNA was used for cDNA synthesis with MMLV reverse transcriptase (Invitrogen). RNA levels were measured by real-time PCR in a Bio-Rad iCycler using Master Mix (Bio-Rad) and specific primers for β-actin, ZNF224 and VDR mRNAs, respectively. For β-actin Fw: 5′-CGA CAG GAT GCA GAA GGA GA-3′ and Rev, 5′-CGT CAT ACT CCT GCT TGC TTG CTG-3′; for ZNF224 Fw: 5′-GGGCTGTCTTGGCACAATTC-3′ and Rev, 5′-TTGCCTCCTTGAACGTGGTC-3′; for VDR Fw: 5′-TCCAGTTCGTGTGAATGATG-3′ and Rev, 5′-GAAGGG TCATCTGAATCTTC-3′.

β-Actin was used as a reference gene for relative quantifications.

Immunofluorescence assay

COS7 cells were plated on cover slips at a concentration of 2×10^5 per cover slip and co-transfected with 2 μg of p3XFLAG-ZNF224 or p3XFLAG-ZNF255, with 2 μg of pcDNA3-WT1(−KTS) or pcDNA3-WT1(+KTS). Twenty-four hours after transfection, the cells were washed three times with cold phosphate buffered saline (PBS) and then fixed with 2% paraformaldehyde (Sigma) for 10 min. The slides were washed again and incubated for 10 min in 0.2% of Triton X-100 to permeabilize the cellular membrane. The cells were incubated for 20 min in Donkey Serum (Sigma) diluted 1:20 in PBS to block the aspecific interaction sites of the antibody. The antibodies used were: anti-FLAG (Sigma) diluted 1:200 and anti-WT1 (C19 Santa Cruz Biotechnology) diluted 1:100 in PBS with 1% bovine serum albumin (BSA) and 0.5% Tween-20 and incubated for 1 h. The secondary antibodies were the rhodopsin-conjugated donkey anti-mouse and the fluorescein-conjugated donkey anti-rabbit both diluted 1:500 in PBS with 1% BSA and 0.5% Tween-20 and incubated for 30 min. For the visualization of nuclei and to mount the slides on the cover slips, a Mounting Medium (Vector Laboratories, Vectashield, CA, USA) with 4′,6-diamidino-2-phenylindole (DAPI) was used. The samples were analysed using the Carl Zeiss LSM510 confocal microscope using oil immersion lenses.

Polysome extraction and sucrose gradient fractionation

HEK293 cells (2×10^6 cells per 10 cm dish) were transfected with 3 μg of pcDNA3-WT1(+KTS) and 3 μg of p3XFLAG-ZNF255. Twenty-four hours after transfection, the cells were treated for 3 min with 100 μg/ml of cycloheximide, then washed twice with PBS containing cycloheximide (100 μg/ml) and harvested and pelletted in a microfuge. The cellular pellet was resuspended in polysome extraction buffer (PEB), (20 mM Tris pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.3% NP-40; 1 mg/ml heparin, 1 mM PMSF, 10 μg/ml aprotinin, leupeptin and pepstatin) and incubated for 20 min on ice. Lyzed cells were pelleted by centrifugation for 10 min at 12 000 rpm (Eppendorf microfuge) at 4°C and the supernatant was layered onto 5 ml of 10–50% sucrose gradient prepared in PEB. The samples were centrifuged in a Beckman MSL 50 rotor at 37 000 rpm in a Beckman Coulter Ultracentrifuge (SW50.1 rotor) for 2 h and 30 min at 4°C. The gradients were fractionated from the top and the absorbance of cytosolic RNA (A_{260}) was monitored with a recording spectrophotometer. Proteins were precipitated by the addition of trichloacetic acid at 20% final concentration, washed with ice-cold acetone and resuspended in the loading buffer. The resulting fractions were then loaded on a 12–8% gradient SDS–PAGE and analysed by western blot. As a positive control for the Polysome fractions, the anti-L7a antibody that recognizes the major ribosome subunit protein L7a was used. The EDTA treatment was performed by adding 20 mM EDTA to the extract and incubating for 20 min on ice prior to the sample being loaded onto the gradient that had been supplemented with the same EDTA concentration.

Oligo(dT) chromatography

HEK293 cells (2×10^6 cells per 10 cm dish) were transfected with 3 μg of p3XFLAG-ZNF255 and 3 μg of pcDNA3-WT1(+KTS). Forty-eight hours after transfection, the cells were harvested, washed twice with cold PBS and resuspended in 2 ml of lysis buffer (10 mM Tris–HCl pH 7.5, 5 mM NaCl, 2 mM $MgCl_2$, 8% glycerole, 1 mM DTT, 0.1% NP-40, 0.1 mg/ml heparin, 1 mM PMSF, 10 μg/ml aprotinin, leupeptin and pepstatin). The lysate was incubated for 10 min on ice, passed through a 22G needle 10 times on ice and centrifugated in a microfuge for 20 min at 12 000*g*. The supernatant was collected and the NaCl concentration was adjusted to 250 mM. Fifty milligrams of oligo(dT) cellulose (Amersham) was pre-hydrated for 30 min in Lysis Buffer before adding

to the lysate and incubating with rotation for 2 h at 4°C. The sample was then washed four times with Wash Buffer (10 mM Tris–HCl pH 7.5, 250 mM NaCl, 2 mM $MgCl_2$), gently vortexed and finally the poly(A)$^+$ binding proteins were eluted with 1 ml of 60°C DEPC H_2O and precipitated with isopropanol. One-tenth of the supernatant representing the input was precipitated with isopropanol and loaded on SDS–PAGE together with the eluted poly(A)$^+$ sample. For the negative control, the pre-swollen oligo(dT) beads were saturated with poly(A) oligonucleotides [25 μg poly(A)/10 mg oligo(dT) cellulose] by 2 h of incubation at 4°C and then incubated with the protein extract.

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Paper III

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Molecules in focus

ZNF224: Structure and role of a multifunctional KRAB-ZFP protein

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ABSTRACT

The Kruppel-like zinc finger protein ZNF224 was originally identified as the transcriptional repressor of the human aldolase A gene. ZNF224 transcriptional repression depends on interaction with the corepressor KAP-1 and the recruitment of enzyme activities modifying chromatin, in accordance with repression mechanism of KRAB-ZFP family. Recently, the arginine methyltransferase PRMT5 was demonstrated to play a crucial role in the transcriptional ZNF224 repressor complex. An alternatively spliced isoform, ZNF255, arises from the ZNF224 gene. ZNF224 and ZNF255 have a distinct pattern of distribution within the cell and display a specific pattern of interaction with different molecular partners. These isoform-specific interactions seem to control different cellular pathways. These findings suggest that ZNF224 is a multifunctional protein and that alternative splicing, sub-cellular compartmentalization and isoform-specific interactions may modulate its activity.

1. Introduction

The zinc finger protein (ZNF) family, including about 800 members, is one of the main groups of proteins with DNA-binding activity. Several ZFPs show additional domains that specifically regulate the chromatin structure through the recruitment of other regulatory proteins on the gene promoters (Urrutia, 2003). One third of ZNFs, the KRAB-ZFPs, contain a potent transcriptional repression domain defined as the Kruppel-associated box (KRAB). This domain usually consists of an A box and a B box and is located in the amino-terminal sequence (Friedman et al., 1996; Huntley et al., 2006). Three closely related subfamilies of KRAB-ZFPs have been found so far: one carrying the A box only, another having both A and B boxes, and the third carrying the A box and a divergent "b" box (Urrutia, 2003). The KRAB-ZFPs are mostly localized in the nucleus and work as repressors of transcription mediated by RNA polymerases I, II and III (Grondin et al., 1996; Lorenz et al., 2001). Although the function of KRAB-ZFPs is largely unknown, they appear to play important roles in controlling cell differentiation, apoptosis, and cancer.

ZNF224, identified as the transcriptional repressor of the human aldolase A gene, is a member of the KRAB-ZFPs (Medugno et al., 2003). This protein is one of the few examples of KRAB-ZFPs whose gene target has been identified and, therefore, is a useful molecular tool for studying the biological functions of this protein family. To repress gene transcription, ZNF224 recruits, through its KRAB domain, the KAP-1 corepressor and the enzymes modifying chromatin on the promoter targets (Medugno and Florio, 2005; Sripathy et al., 2006). Recently, we identified PRMT5, an arginine methyltransferase type II, as part of the transcriptional repression complex located on the promoter of the human aldolase A gene (Cesaro et al., 2009). This finding provided novel insight into the chromatin modifications required for the regulation of transcription mediated by KRAB-ZFPs.

We also demonstrated the existence of an alternatively spliced isoform arising from the ZNF224 gene, namely ZNF255 and already known as BMZF2 (Bone Marrow Zinc Finger 2), which shows a different expression pattern in human tissues, a distinctive subcellular localization and a reduced repression activity compared with ZNF224 (Lee et al., 2002; Medugno et al., 2007). ZNF224 and its isoform ZNF255 show a specific pattern of interaction with the Wilms' tumor protein (WT1) isoforms, suggesting a role in controlling different cellular pathways (Florio et al., 2010).

The study of some of the known features of ZNF224, such as structure, expression and interactions with other proteins, might elucidate its function in physiological and pathological processes.

2. Structure

The ZNF224 protein sequence, which consists of a 707 amino acid open reading frame, originates a protein product of 82 kDa and contains 19 tandemly repeated C2H2 zinc finger domains. The domain motifs are separated by a seven-amino acid-long consensus

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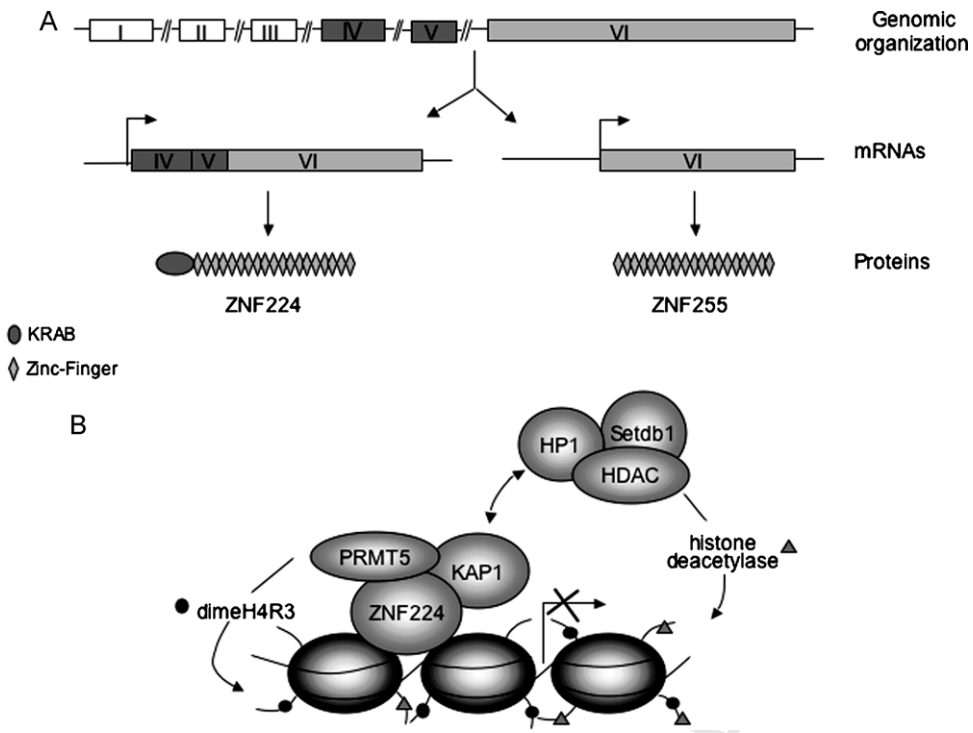

Fig. 1. Structure of ZNF224 gene and molecular model of its transcriptional repression complex. (A) Schematic representation of ZNF224 genomic organization, related mRNAs and proteins. The 6 exons are depicted as boxes. The 5′ UTR of ZNF224 mRNA is formed by fusion of exons I, II and III, whereas the complete coding region is generated by fusion of exons IV, V and VI. The 5′ UTR of ZNF255 mRNA corresponds to intron 5 of the ZNF224 gene and the complete coding region is contained within exon VI. The arrows in mRNA structures indicate the translation start sites. Translation of ZNF255 mRNA generates a protein of 622 amino acids, while a protein of 707 amino acids arises from translation of ZNF224 mRNA. (B) Formation of the ZNF224 transcriptional repression complex through the recruitment of the corepressor KAP1and the histone modifying activities. Gray triangles and black circles represent the histone modification sites.

sequence (TGEKPYX), called H/C link, which, in turn, connects consecutive finger domains (Urrutia, 2003). Electrophoretic mobility shift assays (EMSA) suggest that the domain of ZNF224 that binds to the aldolase A promoter is located within the zinc finger motifs 5–8 (Medugno et al., 2003). At the NH_2 terminus ZNF224 contains a canonical KRAB A motif of 45 amino acids and a divergent b box (Medugno and Florio, 2005).

The genomic organization of the ZNF224 gene is shown in Fig. 1A. Comparison between ZNF224 genomic sequences and its mRNA showed that the mRNA is divided into six exons. The 5′ UTR is formed by fusion of exons I, II and III, whereas the complete coding region is generated by fusion of exons IV, V and VI. The KRAB-A and KRAB-b domains are encoded by different exons (IV and V). Inspection of the mRNA sequence of a second transcript, namely ZNF255, revealed that the 5′ UTR corresponded to intron 5 of the ZNF224 gene and the complete coding region was contained within exon VI. Translation of ZNF255 mRNA generates a protein of 622 amino acids. Interestingly, ZNF255 lacks the KRAB domain, but contains the 19 zinc finger domains (Medugno et al., 2007). The aforementioned data indicate that the two protein species ZNF224 and ZNF255, differing in the presence or absence of the KRAB domain, arise from a single gene, in accordance with the results obtained for other KRAB-containing ZFPs (Mark et al., 1999).

3. Expression

The KRAB repression module, the main feature of the KRAB-ZFPs, is specific of the genomes of vertebrate tetrapods. The expression profile suggests that the KRAB domain is a rather recent product of evolution and that the expansion of KRAB-ZFPs in vertebrate tetrapods is due to new functions related to differentiation and speciation. In the human genome the genes encoding KRAB-ZFPs are organized in clusters and one of the most extensive clusters is located on human chromosome 19 (Shannon et al., 2003).

The ZNF224 gene is mapped at the human gene locus 19q13.2 and has no orthologs in mice. This is a common feature of many human KRAB-ZFPs, suggesting that events of duplication and loss of common ancestral genes occurred independently after the divergence of the lineages in humans and mice and leading to the hypothesis that sequence and functional variations in these duplicated proteins play a role in establishing species-specific traits (Shannon et al., 2003; Hamilton et al., 2006).

By Northern blotting analysis and semi-quantitative PCR assay, we found that ZNF224 is ubiquitously expressed in several human adult and fetal tissues, albeit with quantitative differences. On the contrary, ZNF255 mRNA appears to be expressed at higher levels in fetal tissues than in the adult counterparts (Medugno et al., 2007).

4. Biological function

4.1. Transcriptional repressor

Biochemical and functional studies identified ZNF224 as the transcriptional repressor of the human aldolase A gene (Medugno et al., 2003; Medugno and Florio, 2005). It specifically interacts through the zinc finger motifs to its cognate DNA-binding sequence, AldA-NRE, and represses transcription of the human aldolase A gene. By immunoprecipitation and co-transfection experiments, we demonstrated that ZNF224-mediated repression requires the KRAB-A domain and depends on specific interaction with the KAP1 corepressor molecule. Moreover, treatment with the histone deacetylase inhibitor trichostatin A demonstrated that ZNF224-mediated repression depends on histone deacetylase activity (Medugno and Florio, 2005) (Fig. 1B). We recently identified the arginine methyltransferase PRMT5 as an additional component

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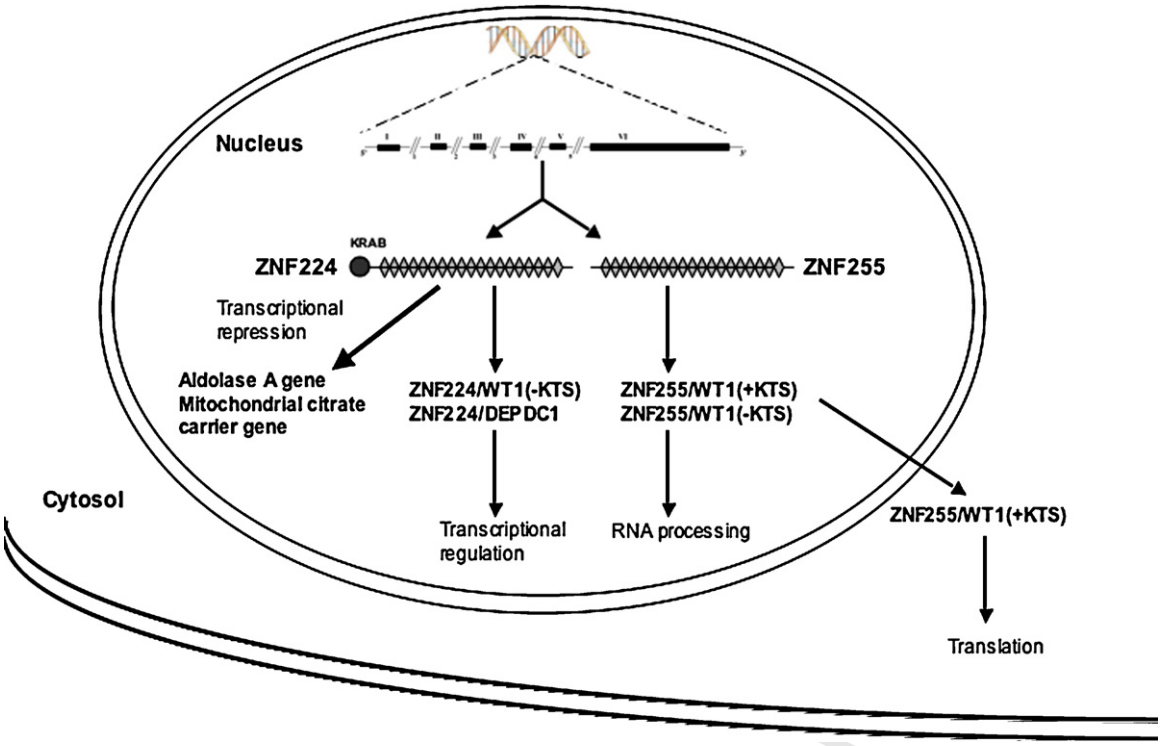

Fig. 2. Known biological functions of the multifunctional ZNF224 protein. While ZNF224 function is restricted to regulating transcription in the nucleus, ZNF255 isoform may play distinct roles in RNA processing in the nucleus and cytoplasm.

of the ZNF224 repressor complex (Cesaro et al., 2009). PRMT5 is involved in the regulation of the gene expression by methylation of histones H3 and H4, transcription factors and co-regulators (Pollack et al., 1999; Richard et al., 2005). In addition, PRMT5 negatively regulates the expression of genes involved in the cell cycle (Fabbrizio et al., 2002). In the ZNF224 repressor complex, we demonstrated by ChIP assays that PRMT5 methylates arginine 3 of histone H4 on the nucleosomes surrounding the promoter region (Fig. 1B). This chromatin modification is associated with the repression of aldolase A gene expression. Moreover, the association of ZNF224/PRMT5 repression complex with the aldolase A promoter and the methylation status of H4R3 both vary during the cell cycle, confirming that ZNF224 plays a significant role in the cell-cycle modulation of aldolase A gene expression and suggesting that PRMT5-mediated methylation of histones is required to elicit repression of genes whose function correlates to the cell cycle. The ZNF224/PRMT5 interaction region was mapped between the second and third zinc finger motifs of ZNF224. These domains are conserved in several KRAB-ZFPs and are evolutionarily related, indicating that PRMT5 may be a key mediator for the regulation of KRAB-ZFP-mediated repression (Cesaro et al., 2009).

Recently, the mitochondrial carrier citrate gene was indicated as a target of ZNF224-mediated transcriptional repression (Iacobazzi et al., 2009). The finding that the two so-far-known target genes of ZNF224 repressor activity (aldolase A and the mitochondrial carrier citrate) both encode proteins involved in carbohydrate oxidation in humans leads to suppose a role for ZNF224 in controlling the energy metabolism.

4.2. Interacting proteins

While it has been demonstrated that ZNF224 acts as a transcriptional repressor, the biological function of ZNF255 isoform is still largely unknown. Transient transfection and ChIP experiments demonstrated that ZNF255 shows reduced transcriptional repression on aldolase A gene compared with ZNF224. Unlike ZNF224, which shows a homogeneous nuclear distribution, ZNF255 is distributed throughout the cell and is also present in the nucleoli (Medugno et al., 2007). The distinctive cellular localization and differential repression might indicate a divergent function of ZNF224 and ZNF255 (Fig. 2). This suggestion was strengthened by the identification of specific interaction between ZNF224 or ZNF255 and WT1. Two major isoforms of this zinc finger protein exist, arising from the insertion or exclusion of the amino acids lysine, threonine and serine (KTS) between the third and fourth zinc finger. The WT1(−KTS) isoform is a transcriptional factor that modulates the expression of a number of genes involved in tissue differentiation, cell cycle control and apoptosis (Morrison et al., 2005; Loeb et al., 2002). In contrast, the WT1(+KTS) isoform displays high affinity for RNA and functions in the RNA metabolism and possibly splicing (Caricasole et al., 1996; Davies et al., 1998).

By co-immunoprecipitation experiments, we observed the specific nuclear interaction between ZNF224 and the WT1 transcriptional isoform (−KTS). The involvement of this interaction in the transcriptional regulation was demonstrated by cotransfection and ChIP experiments using a known WT1 target gene, the vitamin D receptor (VDR). These findings indicate that ZNF224 acts as a transcriptional co-activator of WT1 on VDR promoter, providing the first example of a KRAB-ZPF protein that does not act simply as a transcriptional repressor. In addition, siRNA-mediated knockdown of ZNF224 in the human erythroleukemia cell line K562 resulted in a decreased expression of VDR (Florio et al., 2010). Since it is known that vitamin D can induce apoptosis in leukemia cells (Kizildag et al., 2009), our finding suggests a role for ZNF224/WT1 interaction in this pathway through the transcriptional activation of VDR. Conversely, ZNF255 interacts preferentially with the +KTS isoform, colocalizes with WT1 in translating ribosomes and is present in ribonuclear protein complexes (RNP), strongly indicating a role for this interaction in post-transcriptional control (Florio et al., 2010). Recently, another molecular partner of ZNF224, the cancer testis antigen DEPDC1, was identified (Harada et al., 2010). ZNF224/DEPDC1 complex plays a critical role in bladder carcino-

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genesis, repressing the transcription of the A20 gene, a negative regulator of the NF-kB-mediated antiapoptotic pathway.

5. Possible medical applications

ZNF224 has been indicated as a candidate gene in influencing intermediate phenotypes associated with Alzheimer's disease, suggesting a further role of the multifunctional protein ZNF224 in the progression of degenerative diseases (Shulman et al., 2010). Moreover, ZNF224, through the interaction with different molecular partners, takes part in the control of cell cycle-regulated transcription (Cesaro et al., 2009), carcinogenesis (Harada et al., 2010) and apoptosis (Florio et al., 2010).

Particularly, our recent finding that ZNF224/WT1 interaction modulates VDR expression in K562 cell line may point to a role for this interaction in leukemia (Florio et al., 2010). WT1 is overexpressed in leukemia and plays an oncogenic role through the transcriptional upregulation of anti-apoptotic genes such as bag3 (Cesaro et al., 2010). The study of ZNF224/WT1 interaction in the vitamin D signalling in leukemic cells, as well as the identification of other genes potentially modulated by the WT1/ZNF224 complex, could therefore provide novel insight into the understanding of apoptotic process and pave the way to novel molecular treatments in cancer.

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Paper IV

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Role of WT1–ZNF224 interaction in the expression of apoptosis-regulating genes

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The transcription factor Wilms' tumor gene 1, WT1, is implicated both in normal developmental processes and in the generation of a variety of solid tumors and hematological malignancies. Physical interactions of other cellular proteins with WT1 are known to modulate its function. We previously identified the Krüppel-like zinc-finger protein, ZNF224, as a novel human WT1-associating protein that enhances the transcriptional activation of the human vitamin D receptor promoter by WT1. Here, we have analyzed the effects of WT1–ZNF224 interaction on the expression of apoptosis-regulating genes in the chronic myelogenous leukemia (CML) K562 cell line. The results demonstrated that ZNF224 acts in fine tuning of WT1-dependent control of gene expression, acting as a co-activator of WT1 in the regulation of proapoptotic genes and suppressing WT1 mediated transactivation of antiapoptotitc genes. Moreover, the DNA damaging drug cytosine arabinoside (ara-C) induces expression of ZNF224 in K562 cells and this induction enhances cell apoptotic response to ara-C. These findings suggest that ZNF224 can be a mediator of DNA damage-induced apoptosis in leukemia cells.

INTRODUCTION

Wilms' tumor gene 1, WT1, which is essential for the embryonic development of several organs and structures, encodes multiple isoforms of DNA-binding protein with zinc-finger domain (1,2). Two major isoforms of WT1 protein are designed WT1(+KTS) and WT1(−KTS), because of the insertion or exclusion of three amino acids (KTS) between the third and fourth zinc fingers; WT1(−KTS) appears to act mainly as a transcriptional factor, whereas WT1(+KTS) is involved in RNA processing (3,4).

WT1 is expressed in many tissues, including a small subset of hematopoietic stem cells (5). Overexpression of WT1 has been observed in a wide range of solid tumors and hematopoietic malignancies, including acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) in blastic phase (6–8), as well as in myelodysplastic syndrome (MDS) (9,10).

The expression of the transcription factor WT1 in leukemic cells was proposed to exert anti-apoptotic effects (11,12). Several genes playing a central role in the control of apoptosis have been proposed as targets of WT1, including the proapoptotic bcl-2 family members, Bax and Bak (13,14), bcl-2 itself (15), and the anti-apoptotic genes A1/Bfl-1 (16) and bag3 (17).

However, despite the identification of these target genes, little is still known about the molecular mechanism through which WT1 exerts its regulatory effect on leukemia cell apoptosis.

The regulatory properties of WT1 are complex since this transcription factor elicits both activator and repressor functions, depending on the cellular context and interactions with co-factors. An increasing number of interacting partners has been proposed to regulate its function, acting as either co-activators or co-repressors (18). We recently identified the Krüppel-like zinc-finger protein, ZNF224, as a novel WT1-associating protein that enhances the transcriptional activation of the vitamin D receptor (VDR) promoter by WT1 in the chronic myelogenous leukemia (CML) K562 cell line (19). This finding prompted us to search for other genes potentially regulated by the WT1/ZNF224 complex in leukemic cells.

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In this study, we demonstrated that ZNF224 inhibits WT1-mediated transcriptional activation of the anti-apoptotic bag3 gene, recently identified as a novel WT1 target gene (17). Moreover, by short hairpin RNA (sh-RNA)-mediated knock-down and overexpression of ZNF224 in K562 cells, we demonstrated that ZNF224 regulates WT1 activity at several WT1 target genes, acting as a co-activator of WT1 in the regulation of proapoptotic genes and suppressing WT1-mediated transactivation of anti-apoptotic genes.

Furthermore, we observed that ZNF224 itself could be modulated by cytosine arabinoside (ara-C), a drug widely used in the treatment of myeloid leukemia (20) and that ZNF224 overexpression increases susceptibility to apoptosis of K562 cells. Our findings demonstrated the role of ZNF224–WT1 interaction in the modulation of apoptotic genes expression and suggest that the increased expression of ZNF224 may represent an important event in the ara-C mediated-induction of apoptosis. Collectively, data from this paper disclose a previously unrecognized, novel function of ZNF224 in the regulation of WT1-mediated transcription of genes involved in apoptosis, paving the way to potential strategies for treatment of leukemia.

RESULTS

ZNF224 represses WT1-mediated bag3 expression

We recently reported a specific nuclear interaction between ZNF224 and WT1 and demonstrated that this interaction enhances WT1-mediated transactivation of VDR promoter, thus implying that ZNF224 acts as a transcriptional co-regulator of WT1 (19).

In this work, we investigated whether ZNF224 also affects WT1-dependent transcriptional regulation of the anti-apoptotic bag3 gene expression, recently identified as a novel target of WT1 (17). To this aim we used an HEK293 cell clone, which does not express endogenous WT1, while stably expressing ZNF224 (CL13 clone) (21) and a C3.3 clone stably expressing a small hairpin RNA (shRNA) specific for ZNF224. As control, we used a clone generated by stable transfection of HEK293 cells with the empty vector (CN2 clone) (21). The expression level of ZNF224 in these clones is shown in Figure 1A. CN2, CL13 and C3.3 clones were transfected with increasing amounts of the WT1(-KTS) expression vector and bag3 expression was evaluated at mRNA and protein levels by quantitative real-time-PCR and western blot analyzes, respectively. A progressive increase in bag3 mRNA and protein levels was observed in CN2 clone (Fig. 1B lanes 1, 4, 7 and Fig. 1C left panel), thus confirming that WT1 activates bag3 gene expression (17). Conversely, in CL13 clone, overexpression of ZNF224 considerably abolished WT1-mediated bag3 induction, as indicated by down-modulation of bag3 transcript and protein expression (Fig. 1B lanes 2, 5, 8 and Fig. 1C middle panel). In C3.3 clone, silenced for ZNF224, the increase in bag3 mRNA and protein was higher than in CN2 clone (Fig. 1B lanes 3, 6, 9 and Fig. 1C right panel), thus indicating an increased transactivation of WT1 on bag3 gene in the absence of ZNF224.

To further investigate the effects of ZNF224 on bag3 gene transcription, we introduced a luciferase reporter plasmid containing bag3 gene promoter into CN2, CL13 and C3.3 clones, in the presence of increasing amounts of WT1(−KTS) expression plasmid. Analysis of luciferase activity shows that WT1 was able to activate bag3 promoter in a dose-dependent manner in CN2 (Fig. 2B lanes 10, 13, 16) as opposed to CL13 (Fig. 2B lanes 11, 14, 17), in which we observed a dose-dependent decrease of WT1-mediated transcriptional activity. As expected, in C3.3 clone an increased luciferase activity with respect to CN2 clone (Fig. 2B lanes 12, 15, 18 versus lanes 10, 13, 16) was observed. These findings indicate that ZNF224 suppresses bag3 gene expression by a transcriptional mechanism.

ZNF224/WT1 interaction modulates the expression of apoptosis-regulating genes in K562 cells

To further confirm that WT1/ZNF224 complex modulates bag3 expression and to better characterize its role in leukemic cells, we evaluated the effects of the knock-down of ZNF224 on the expression of WT1 target genes. To this aim, K562 cells were transfected with two different shRNA plasmids targeting ZNF224 and a negative control shRNA. The knock-down of ZNF224 leads to a dramatic increase in bag3 and A1/Bfl1 anti-apoptotic mRNAs expression, whereas it causes a decrease in Bak and Bax mRNAs expression, as already previously observed for VDR, another pro-apoptotic WT1 target gene (19) (Fig. 3A). The aldolase A mRNA was used as a control, being known the role of transcriptional repression exerted by ZNF224 on aldolase A gene transcription (22); as expected, ZNF224 silencing produced a considerable increase in aldolase A mRNA expression (Fig. 3A). The changes observed in mRNAs expression after ZNF224 RNAi were confirmed at the protein levels by western blotting (Fig. 3B).

To further verify that ZNF224, besides its role as transcriptional repressor, may act as a cofactor of WT1, we generated a pool of K562 cells stably transfected with ZNF224 (Fig. 3C) and evaluated the mRNA levels of WT1 target genes. As shown in Figure 3E, ZNF224 overexpression is accompanied by a decrease in bag3 and A1/Bfl1 mRNAs expression and an increase in Bak, Bax and VDR mRNAs expression. The decrease in aldolase A mRNA expression was used as a control of ZNF224 overexpression (Fig. 3E).

We next confirmed that the ZNF224-mediated modulation of apoptotic genes was dependent on WT1 expression in K562 cells. To this aim we transiently transfected a pool of K562 cells overexpressing ZNF224 with WT1 shRNA plasmid (Fig. 3D). The depletion of WT1 in this pool of cells affects the apoptotic gene expression, demonstrating that ZNF224 requires WT1 to modulate the expression of bag3, A1/Bfl1, Bax, Bak and VDR (Fig. 3E). On the other hand, the expression of aldolase A, a direct target of ZNF224 transcriptional repression, was not influenced by WT1 knock-down (Fig. 3E).

The above-reported experiments indicate that ZNF224 cooperates with WT1 in the transcriptional modulation of apoptosis-regulating genes, enhancing gene activation mediated by WT1 on pro-apoptotic genes and inhibiting

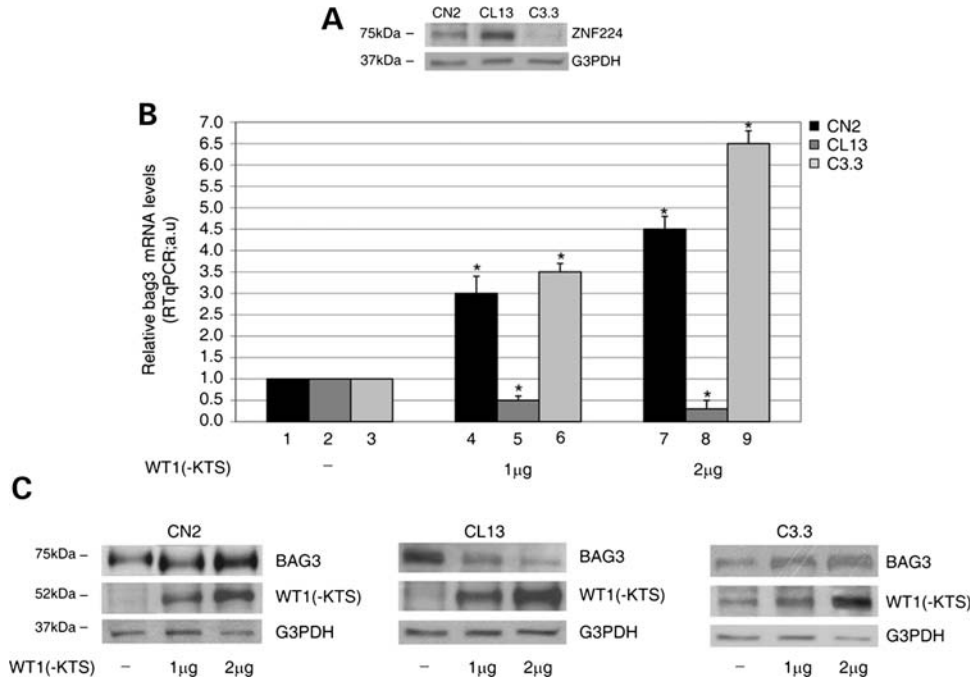

Figure 1. ZNF224 overexpression negatively regulates WT1-mediated bag3 expression. (**A**) Western blot analysis of the ZNF224 protein level in CL13, CN2 and C3.3 HEK293 clones, using anti-ZNF224 antibody (T3). G3PDH is shown as equal loading. Molecular weight to the left. (**B**) RT-qPCR analysis of the bag3 mRNA expression in CN2, CL13 and C3.3 clones transiently transfected for 48 h with increasing amount of WT1(−KTS) expression vector. mRNA levels of untransfected clones were referred to as 1 (lanes 1–3). Error bars represent standard deviations of three independent experiments. $^{*}P < 0.05$ versus control. (**C**) Western blot analysis of BAG3 and WT1 protein levels in total protein lysates obtained from CN2, CL13 and C3.3 clones transiently transfected with increasing amount of WT1(−KTS) expression vector for 48 h. G3PDH was used to monitor equal loading conditions. One representative result out of three performed is presented. Molecular weight to the left.

WT1-mediated transcriptional activation of anti-apoptotic genes.

WT1 recruits ZNF224 to the promoter of apoptosis-regulating genes

To determine whether WT1 recruits ZNF224 on endogenous WT1 target genes we performed chromatin immunoprecipitation (ChIP) experiments. HEK293 cells were transfected with an expression vector for ZNF224 (p3xFLAG-ZNF224) (Fig. 4A), or an expression vector for WT1 (pcDNA3WT1 (-KTS) (Fig. 4B), or co-transfected with pcDNA3WT1(-KTS) and p3xFLAG-ZNF224 (Fig. 4C) and chromatin was immunoprecipitated with FLAG (Fig. 4A and C) or with WT1 antibodies (Fig. 4B). As expected, we observed by PCR analysis that ZNF224 was bound to the aldolase A gene promoter (AldA) and not to the promoter regions of WT1 target genes (Fig. 4A), thus indicating that ZNF224 alone was unable to directly bind to these WT1 target promoters; furthermore, WT1 was bound to Bax, Bak, A1/Bfl1 and bag3 promoter regions and not to AldA (Fig. 4B). When ZNF224 was overexpressed along with WT1, it binds WT1 target gene promoters, thus demonstrating that WT1 recruits ZNF224 on these promoters (Fig. 4C). No bands were observed when a G3PDH fragment was amplified, using the same chromatin samples immunoprecipitated with FLAG (Fig. 4A and C) or with WT1 antibodies (Fig. 4B), thus demonstrating the specificity of chromatin immunoprecipitation.

This result provides experimental evidence that WT1 and ZNF224 interact at chromatin level, and this interaction does not involve direct DNA binding of ZNF224.

Ara-C induces ZNF224 expression in K562 cells

Because the above-reported findings suggest that WT1–ZNF224 interaction plays a relevant role in apoptotic genes expression, we decided to investigate whether ZNF224 itself could be modulated by chemotherapeutic agents. We chose cytosine arabinoside (ara-C) because it is one of the key drugs for treatment of leukemia.

K562 cells were exposed to increasing concentrations of ara-C (0.25, 0.50, 1 μM) for 24, 48 and 72 h after which cell viability and ZNF224 mRNA levels were measured (Fig. 5). We observed that exposure to ara-C induced a time-dependent cell death (Fig. 5A–C).

Then, we evaluated ZNF224 mRNA levels by quantitative real-time-PCR (Fig. 5D–F). In accordance with our previous findings that ZNF224 induced pro-apoptotic genes expression, we observed that levels of this factor also increased in a time dependent fashion and, apparently, an inverse relationship subsisted between cell viability and ZNF224 levels. Surprisingly, a dose-dependent effect of ara-C on cell viability was not observed, even if a dose-dependent increase of ZNF224 was measured. Measurement of cell death by DAPI incorporation and annexin V binding confirmed the lack of a

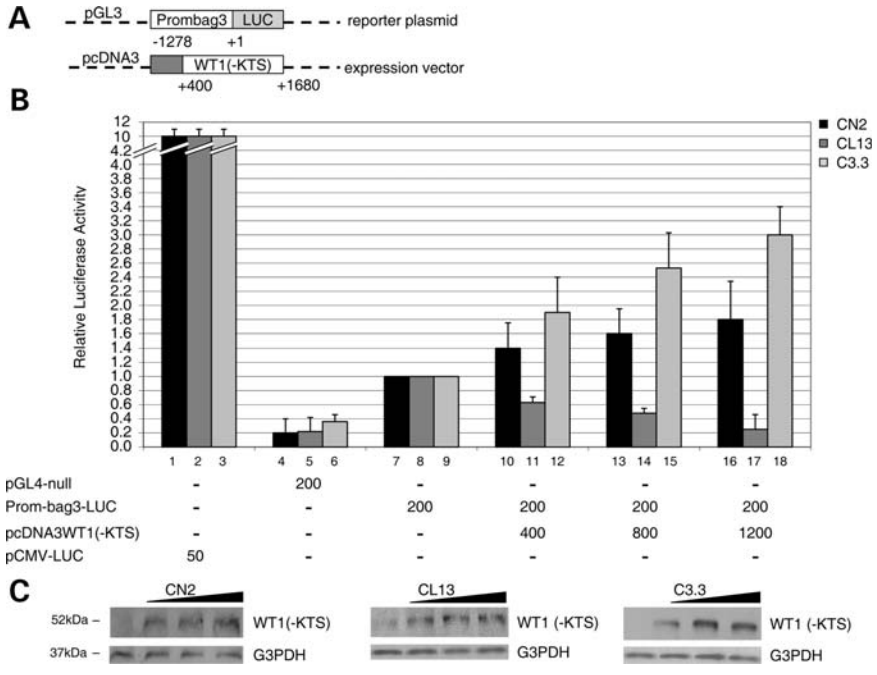

Figure 2. ZNF224 suppresses WT1-mediated transcriptional activation of bag3 promoter. (**A**) Schematic representation of the reporter plasmid containing the bag3 promoter region (−1278 to +1) and of the WT1(-KTS) expression vector containing the illustrated region (+400 to +1680) used in transient transfection experiments. (**B**) CN2, CL13 and C3.3 HEK293 clones were co-transfected with prom-bag3 reporter and increasing amount of WT1(-KTS) expression vector (lanes 10–18) or trasfected with prom-bag3 alone as control (lanes 7–9). After 48 h cells were collected and relative luciferase activities were calculated, with the activity of control cells set as 1 (lanes 7–9) (triplicate determinations). The effect of WT1 on bag3 promoter in CN2, CL13 and C3.3 clones is shown. pGL4-null empty vector activity indicates the background (lanes 4–6) and pCMV-LUC was the positive control (lanes 1–3). Error bars represent standard deviations of three independent experiments. (**C**) WT1(−KTS) protein overexpression was evaluated by western blot analysis. G3PDH was used as loading control. Molecular weight to the left.

dose-dependent effect of ara-C on K562 death (Supplementary Material, Fig. S1).

To confirm that leukemic cells died by apoptosis, K562 cells were cultured with or without 1 μM ara-C for 72 h after which we evaluated sub-G1 DNA content and annexin V binding by FACS analysis and the caspase 3 activition by western blot. We found that time-dependent increase in ZNF224 mRNA expression was accompanied by time-dependent induction of apoptosis and caspase-3 activation (Fig. 6A–C), thus suggesting that caspase-3 activation was a direct consequence of ZNF224 induction.

Next, we confirmed that the increase of ZNF224 in ara-C-treated K562 cells is accompanied by an increase of proapoptotic VDR, Bax and Bak, and the decrease of anti-apoptotic bag3 and A1/Bfl-1, as measured by quantitative real-time-PCR and western blot analysis (Fig. 7A and B).

In addition, ara-C was ineffective in inducing pro-apoptotic gene expression in pools of K562 cells silenced for ZNF224 (Fig. 7C), notwithstanding the decrease in WT1 mRNA expression (Fig. 7A and B), thus confirming the role of ZNF224 as an important mediator of the ara-C-induced activation of pro-apoptotic genes.

ZNF224 overexpression enhances the apoptotic effect mediated by ara-C in K562 cells

Next, we evaluated the apoptotic response to ara-C in pools of K562 cells stably overexpressing ZNF224 (ZNF224-FLAG) or ZNF224-silenced (ZNF224 silencing) or overexpressing WT1(−KTS).

These pools of clones and their corresponding controls were cultured with or without 1 μM ara-C for 72 h and then viability and apoptosis were analyzed.

As shown in Figure 8A and D, K562 cells expressing ZNF224–FLAG were significantly more sensitive to the effects of ara-C on cell viability and apoptosis with respect to K562 cells not expressing ZNF224–FLAG. According to these findings, silencing of ZNF224 increased cell viability and reduced the number of annexin V-positive cells after exposure to ara-C (Fig. 8B and E). These results support a role for ZNF224 in the apoptotic response to ara-C.

Moreover, we observed that WT1(−KTS) overexpressing K562 cells are protected, at least partly, from cell death further confirming the anti-apoptotic role of WT1 (Fig. 8C and F).

DISCUSSION

Regulation of apoptosis is a critical function of WT1 and is due at least in part to the modulation of expression of bcl-2 family members (23). WT1 is highly expressed in leukemia cells and its overexpression is associated with a poor response to therapy (24,25). We recently showed that WT1 positively regulates the expression of the anti-apoptotic gene bag3, by a transcriptional mechanism. WT1-mediated increase in

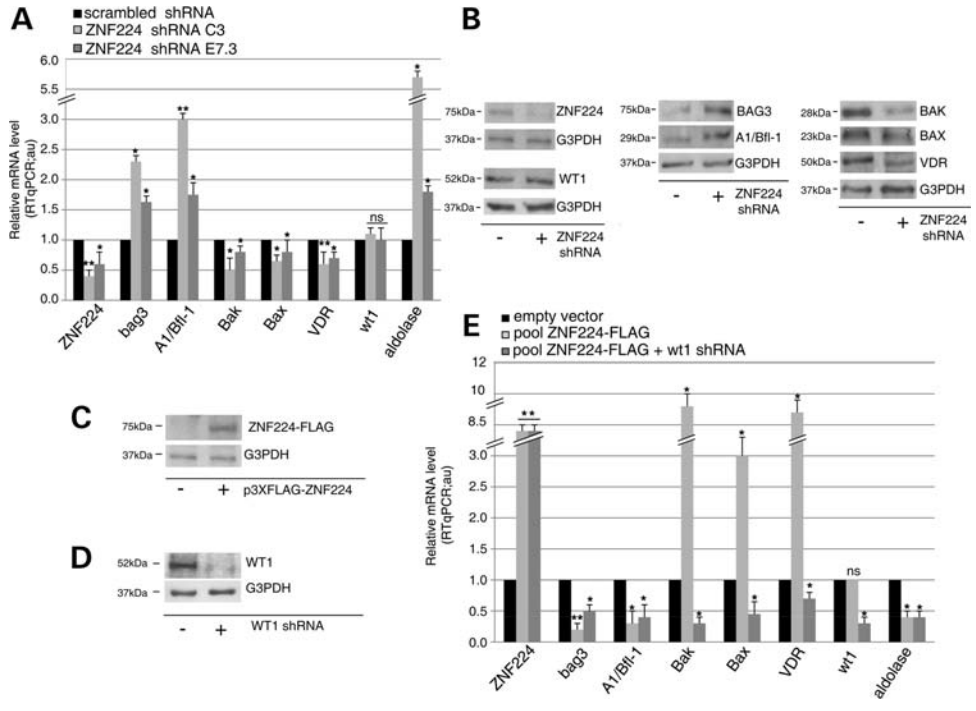

Figure 3. ZNF224 and WT1 cooperate in the modulation of apoptotis-regulating genes. (**A**) RT-qPCR analysis of mRNA levels in K562 cells transfected for 48 h with two different ZNF224 shRNAs (shRNA C3 and shRNA E7.3) or non-silencing scrambled shRNA, as negative control. Error bars represent standard deviations of three independent experiments. $^{*}P = 0.05$ versus scrambled shRNA; $^{**}P < 0.002$ versus scrambled shRNA; ns, not significant (**B**) Total protein extracts were analyzed by western blot with the indicated antibodies. G3PDH was used to monitor equal loading conditions. One representative result out of three performed is presented. Molecular weight to the left. (**C**) Western blot analysis showing the expression of ZNF224–FLAG protein in pool of K562 cells stably transfected with the expression vector p3XFLAG-ZNF224 (+) or with p3XFLAG empty vector used as control (−). G3PDH was used to monitor equal loading conditions. Molecular weight to the left. (**D**) Western blot analysis of WT1 protein level in pool of K562 cells stably transfected with the expression vector p3XFLAG-ZNF224 and transiently transfected with WT1 shRNA (+) or with non-silencing scrambled shRNA used as control (−). G3PDH was used to monitor equal loading conditions. Molecular weight to the left. (**E**) RT-qPCR analysis of mRNA levels in pool of K562 cells overexpressing ZNF224 (pool ZNF224-FLAG), or in pool of K562 cells overexpressing ZNF224 and transiently transfected with WT1 shRNA (pool ZNF224-FLAG + WT1 shRNA). The empty vector used as control was: pool of K562 stably transfected with p3XFLAG for the pool ZNF224-FLAG; pool of K562 cells overexpressing ZNF224 and transiently transfected with scrambled shRNA for the pool ZNF224-FLAG + WT1 shRNA. Error bars represent standard deviations of three independent experiments. $^{*}P < 0.05$ versus control; $^{**}P = 0.004$ versus control; ns: not significant.

BAG3 protein levels contributes to the pro-survival role of WT1 in leukemic cells (17).

The effects of WT1 on target gene expression appear to be isoform-specific, strongly influenced by different cellular contexts and by physical interactions with other proteins that are known to modulate WT1 transcriptional function (18,26–28). We previously identified the Krüppel-like zinc-finger protein, ZNF224, as a novel WT1-interacting factor involved in WT1 transcriptional regulation of VDR promoter (19). In this study, we provide further evidence of ZNF224 acting as a transcriptional co-regulator of WT1 and playing a relevant role in the control of WT1-mediated expression of apoptosis-regulating gene in the CML cell line K562, thus pointing to a role for the ZNF224/WT1 interaction in leukemia. Indeed, chromatin immunoprecipitation assays revealed that ZNF224 binds to the promoter of WT1 target genes only if recruited by WT1 itself, thus indicating that ZNF224 acts as a transcriptional co-factor of WT1, without directly binding to promoter DNA sequences. A combination of over-expression and knock-down analyses of both ZNF224 and WT1 in K562 cell line revealed that ZNF224 exerts a dual effect on the expression of WT1 target genes, acting as a co-activator of WT1 in the regulation of pro-apoptotic genes and suppressing WT1-mediated transactivation of anti-apoptotic genes.

These data demonstrate that ZNF224, beyond its known role of DNA-binding transcriptional repressor (22), may act in a DNA binding-independent mode of transcriptional regulation, through its ability to interact with another transcription factor, i.e. WT1. Our finding thus reveals a still undiscovered function for ZNF224 as transcriptional co-regulator.

However, we cannot rule out that ZNF224 could participate in the regulation of apoptosis also through the direct binding to promoters of not yet identified target genes, where it acts as a repressor.

Other Krüppel-like zinc-finger proteins are involved in cell proliferation, apoptosis and neoplastic transformation (29). For instance, ZBP-89 has been show to be an important regulator in apoptosis and cell growth and can induce apoptosis through mechanisms both dependent and independent of p53 (30); ZNF23 inhibits cell cycle progression via up-regulation of p27kip-1 (31); APAK (ATM ad p53 associated KZNF protein) specifically inhibits p53-mediated apoptosis (32); ZNF382 exerts a pro-apoptotic role by repressing NF-KB and AP1 signaling and by inhibiting the expression of multiple oncogenes, including the NF-kB upstream factors STAT3, STAT5B, ID1 and IKBKE (33).

Although KRAB-containing zinc-finger proteins are thought to function mainly as transcriptional repressors, it has been

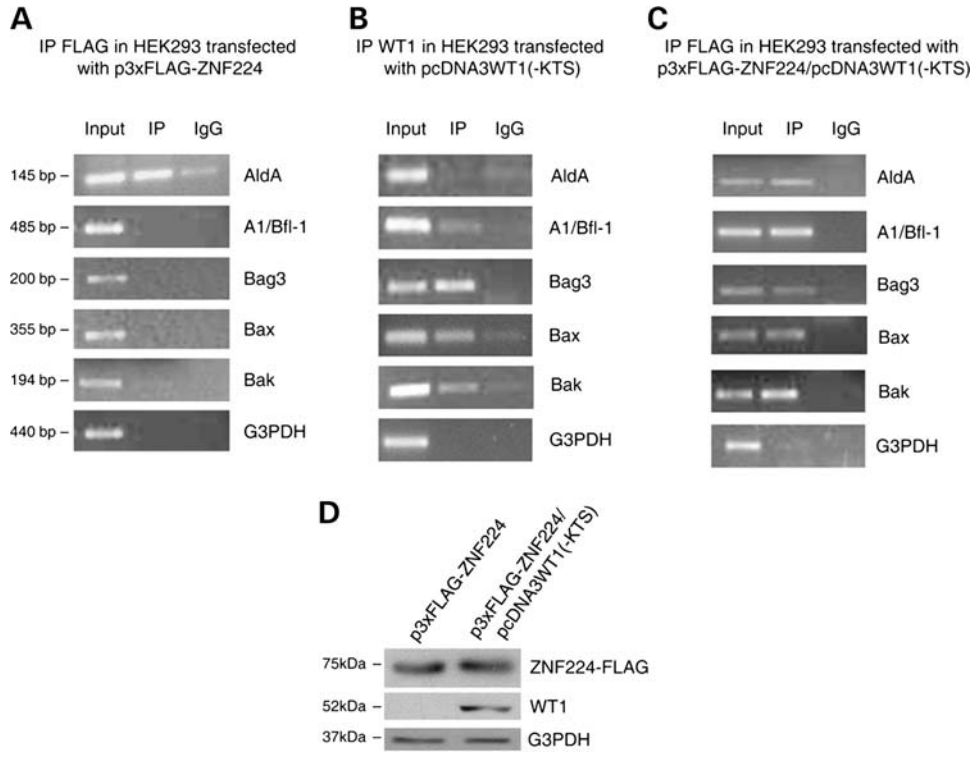

Figure 4. ZNF224 is recruited by WT1 to apoptotic target gene promoters. Chromatin immunoprecipitation assay was performed with anti-FLAG antibody (**A** and **C**) or anti-WT1 antibody (**B**). Immunoprecipitation with non-specific IgG were used as negative control. Non-immunoprecipitated chromatin was used as total input control. PCR analysis was used to evaluate the WT1 and ZNF224 binding to AldA, A1/Bfl-1, bag3, Bax and Bak promoter regions, using specific primers. As control of specificity of immunoprecipitated chromatin, PCR was performed using G3PDH primers. Results are representative of two independent experiments. Size, in bp, of the PCR products to the left. (**D**) Total protein extracts from HEK293 transfected with p3XFLAG-ZNF224 and from HEK293 co-transfected with p3XFLAG-ZNF224/pcDNA3WT1(-KTS) were analyzed by western blot with the indicated antibodies. G3PDH was used to monitor equal loading conditions. Molecular weight to the left.

recently reported that ZBRK1 and ZNF263 display activating as well as repressing effects on target gene transcription, by yet unknown mechanisms and co-regulators (34,35). Indeed, several lines of evidence indicate that specific transcription factors can use distinct combinations of co-regulator complexes and enzymatic activities required for modifying chromatin machinery, in a cell-, gene-, and promoter-specific manner.

Deciphering the role of ZNF224 as a transcriptional regulator of WT1, identifying additional components of the WT1/ZNF224 complex and elucidating how alterations of the WT1/ZNF224 complex affect the expression of apoptosis-regulating genes will shed further light into the molecular mechanisms of apoptosis regulation.

Furthermore, our study revealed that exposure of K562 cells to ara-C increased ZNF224 transcript in a time- and dose-dependent manner, suggesting an important role for the anti-leukemic drug in regulating expression of this protein. ZNF224 induction is associated with an increase in apoptotic cell death, which correlates with down-regulation of anti-apoptotic WT1 target genes such as bag3 and A1/Bfl1 and upregulation of proapoptotic molecules such as VDR, Bax and Bak.

On the other hand, the lack of correlation between cell death values and ara-C doses may be due to a concomitant activation mechanisms, responsible for apoptosis resistance, that hamper the expected cell death increase. Such an hypothesis is in accordance with the notion that K562 are resistant to ara-C induced cell death (36).

Finally, notwithstanding the heterogeneity of pool of clones, the increased sensitivity to ara-C induced apoptosis in pools of clones overexpressing ZNF224 is a further element in support of the pro-apoptotic role of this zinc-finger protein. WT1(−KTS) overexpression was able to counteract, at least in part, the proapoptotic effect of ZNF224 following ara-C stimulation, thus confirming the relevant role of ZNF224/WT1 interaction in fine tuning of apoptotic-gene expression.

Taken together, our data support a relevant role for ZNF224 in ara-C induced apoptosis of leukemia cells and suggest that ZNF224 could provide a critical link between ara-C induced DNA-damage and the expression of pro-apoptotic genes in leukemia cells.

A better definition of the molecular mechanism coupling ara-C-induced DNA damage to the initiation of cell death could lead to the development of new therapeutic drugs used for the treatment of leukemia.

MATERIALS AND METHODS

Cell culture and stable transfection

HEK293 human cell lines were cultured in Dulbecco's modified Eagle's medium (Bio-Whittaker, Verviers, Belgium) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin-penicillin

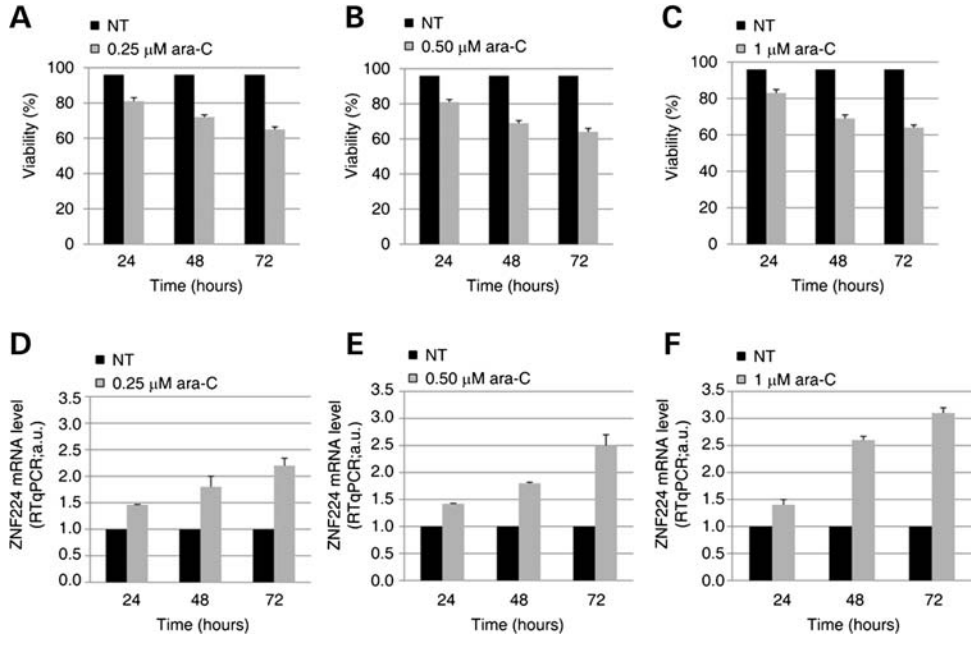

Figure 5. Dose and time-course of ara-C induced ZNF224 expression. K562 cells were treated with 0.25 μM, 0.50 μM, 1 μM ara-C for 24, 48 and 72 h. NT: untreated K562 cells collected at the indicated times. (**A**–**C**) Viability as determined by trypan blue exclusion. The results are means + - SD from two impendent experiments. (**D**–**F**) RT-qPCR analysis of ZNF224 mRNA expression in K562 cells treated with 0.25 μM, 0.50 μM, 1 μM ara-C for the indicated times. Error bars represent standard deviations of two independent experiments.

mix (Bio-Whittaker) at 37°C in 5% CO_2. The stable clone from HEK293 expressing p3XFLAG-ZNF224 (CL13) and clone from HEK293 cells transfected with empty p3XFLAG (CN2) were obtained as described (21).

The chronic myeloid leukemia cell lines K562 were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 100 μg/ml penicillin-streptomycin mix (Bio-Whittaker) at 37°C in 5% CO_2. To obtain pools of clones permanently expressing 3XFLAG-ZNF224, K562 cells were transfected with p3XFLAG-ZNF224 G418-selectable plasmid using HiPerFect Reagent (Qiagen, Hilden, Germany). Transfected cells were selected in medium containing 800 μg/ml G418 (Promega, Madison, WI, USA) for 4 weeks. Dead cells were removed by Ficoll density gradient centrifugation (GE Healthcare Bio Science AB, Sweden, Uppsala). The positive pool of clones was analyzed by western blotting using M2 anti-FLAG antibody (Sigma-Aldrich, MO, USA). As negative control, we selected pools of K562 cells stably transfected with the empty p3XFLAG G418-selectable plasmid.

We used pool of K562 cells transduced with a retroviral vector encoding for WT1(−KTS) or with the control empty vector (pMIG), as described (37).

ShRNA-mediated knock-down

To obtain stable ZNF224 gene silencing (clone C3.3), we transfected 2×10^6 HEK293 cells with 10 μg of short interfering RNA plasmid SH2351C3 (ZNF224 shRNA) (Open Biosystems, Huntsville, AL, USA), using Lipofectamine Reagent (Invitrogen, Carlsbad, CA, USA), according to the manifacturer's protocol. The cells were grown in selective medium until colony formation. Positive clones were analyzed by western blotting using anti-ZNF224 antibody (T3). Pools of K562 clones silenced for ZNF224 (ZNF224 shRNA) were obtained by transfection of 1.5 μg of short-interfering RNA plasmid SH2351C3 (Open Biosystem) using the HiPerFect Reagent (Qiagen), according to the manufacturer's protocol; transfection of a non-silencing shRNA (scrambled shRNA) was used as negative control (Open Biosystem). Transfected cells were selected in medium containing 500 μg/ml Puromycin (Promega) for 4 weeks. Dead cells were removed by Ficoll density gradient centrifugation (GE Healthcare). Positive clones were analyzed by western blotting using anti-ZNF224 antibody (T3).

K562 cells were transiently transfected using the HiPerFect Reagent (Qiagen) in 12-well plates with 1.5 μg of the short-interfering RNA plasmid SH2351C3 to silence ZNF224, or 1.5 μg of the short-interfering RNA plasmid SH25dE-10 to silence WT1, or 1.5 μg of non-silencing negative control (scrambled shRNA) as control. All plasmids were purchased from Open Biosystems. Cells were collected 48 h after transfection.

RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. One microgram of each RNA was reverse transcripted using QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's protocol. Real-time PCR was carried out in a iCycler Thermal Cycler (BioRad, Berkeley, CA, USA) using the SYBR Green I Master Mix (BioRad) and specific primers for: ZNF224 (Fw 5′-gggctgtcttggcacaattc-3′, Rev 5′-ttgcctccttgaacgtggtc-3′); WT1 (Fw 5′-gtgaaaccattccagtgtaa aac-3′, Rev 5′-gccaccgacagctgaagggc-3′); bag3 (Fw 5′-cctgttag ctgtggttg-3′, Rev 5′-aacatacagatattcctatggc-3′); VDR (Fw

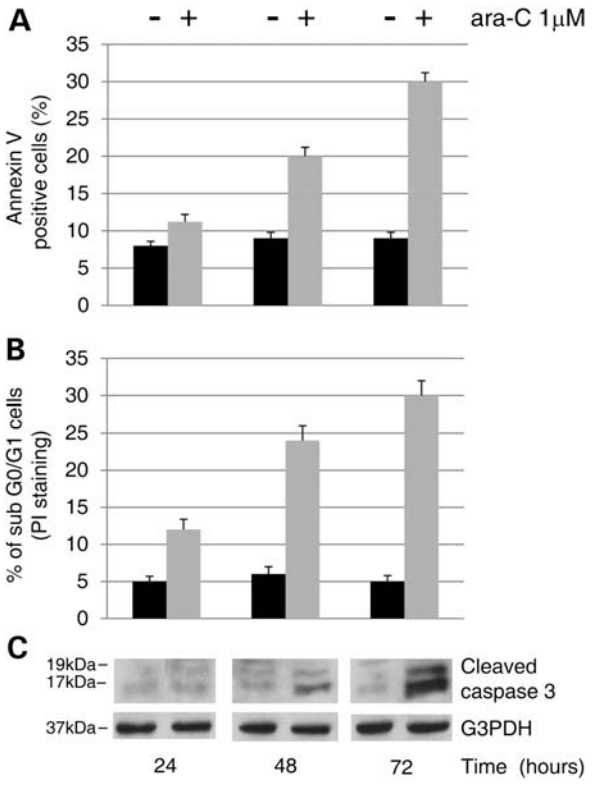

Figure 6. Time-course of ara-C induced apoptosis. K562 cells were treated with 1 μM ara-C and analyzed after 24, 48 and 72 h. (**A**) Apoptosis as determined by annexin V-APC staining followed by flow cytometry. Error bars represent standard deviations of three independent experiments. (**B**) Cell apoptosis was measured by staining with propidium iodide and flow cytometric analysis. Error bars represent standard deviations of three independent experiments. (**C**) Western blot analysis of cleaved caspase-3. One representative result out of three performed is presented. Molecular weight to the left.

5′-tccagttcgtgtgaatgatg-3′, Rev 5′-gaagattcagatgacccttc-3′); A1/Bfl-1 (Fw 5′-cggcatcattaactggggaag-3′, Rev 5′-ctagaagt tacaggaaagatc-3′); Bak (Fw 5′-tgaaaaatggcttcggggcaa-3′, Rev 5′-ctctcaaacggctggtggcaatc-3′); Bax (Fw 5′-tcaggatgcgtc caccaagaaag-3′, Rev 5′-gcaaagtagaaaagggcgacaacc-3′); matrin3 (Fw 5′-cattctaataaggagtggagtc-3′, Rev 5′-tgctagtttccac tctgcctt-3′); aldolase A (Fw 5′-tcaaccacactccgtccacg-3′, Rev 5′-gtagcaagttccggtgcttc-3′); β-actin (Fw 5′-cgacaggatgcaga aggaga-3′, Rev 5′-cgtcatactcctgcttgcttgctg-3′). β-actin housekeeping gene was used as a reference gene for relative quantification. All real-time PCR reactions were performed in duplicate.

To analyze the expression of ZNF224 mRNA levels in K562 cells treated with ara-C and in the untreated controls, total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. 0.5 μg of total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, USA) with random hexamer primers according to the manufacturer's instructions. Quantitative PCR was carried out using TaqMan probe-based chemistry (Applied Biosystems); the probe for ZNF224 (Hs00273760) and the endogenous control β-actin (Hs99999903_m1) were purchased as Assay-on-Demand (Applied Biosystems). The amplification reactions were all performed in triplicates in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Data were collected and analyzed using the Sequence Detector v.1.1 software (Applied Biosystems).

The relative quantification in gene expression was determined using the $\Delta\Delta C_T$ method (38). Normalization: $\Delta C_T = C_T(\text{sample}) - C_T(\text{endogenous control})$; $\Delta\Delta C_T = \Delta C_T(\text{sample1}) - \Delta C_T(\text{sample2})$. Relative quantification $= 2^{-\Delta\Delta CT}$. The efficacy of the PCR amplification of controls and test was identical; parallelism of standard curves of the control and test was confirmed.

Statistical analysis

P-value was determined by Student's *t* test. All *P*-values were considered significant when $P < 0.05$.

Transient transfection and luciferase-reporter assays

The HEK293 stable clones CL13, CN2 and C3.3 were transiently transfected using Lipofectamine Reagent (Invitrogen) in 12-well plates with 0.2 μg of a luciferase reporter plasmid containing the proximal bag3 promoter (PromBag3-Luc) and 0.4, 0.8 and 1.2 μg of WT1(−KTS) expression plasmid. The pGL4-null empty vector (0.2 μg) was used to evaluate the background luciferase activity, whereas the pCMV-LUC (50 ng) was the positive control for the assay. To normalize the luciferase assay a pRL-CMV plasmid (20 ng) coding for the *renilla* luciferase was used. The Dual-Luciferase Reporter Assay System (Promega Corporation, WI, USA) was performed 48 h after the transfection according to manufacturer's instructions. Results are representative of two independent experiments.

Western blot analysis

Total protein extract was obtained as follow: cells were washed twice with cold PBS, then were lysed in lysis buffer (10 mM HEPES pH7.8, 250 mM NaCl, 5 mM EDTA pH 8.0, 1% Nonidet P-40, 1 mM sodium orthovanadate and 50 mM sodium fluoride) and protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin and 1 μg/ml pepstatin A] for 30 min on ice. The extract was centrifuged in a microfuge at maximum speed for 10 min and the supernatant was quantified using the BioRad protein assay reagent, resolved on SDS–PAGE and then transferred to Hybond membranes (Amersham Biosciences, NJ, USA). Non-specific binding sites were blocked for 2 h with 5% milk in Tris–Tween buffered saline (tTBS) (5 mM Tris pH 7.5, 15 mM NaCl, 0.1% Tween-20), washed three times with tTBS and incubated with the following antibodies:

anti-FLAG (M2) from Sigma-Aldrich, 1:1000
anti-VDR (D-6) from Santa Cruz Biotechnology, 1:500
anti-GAPDH (7-B) from Santa Cruz Biotechnology, 1:1000
anti-BAX (B-9) from Santa Cruz Biotechnology, 1:500
For these primary antibodies the secondary antibody was goat-anti-mouse IgG (H+L)-HRP conjugated (BioRad170-6516) antibody, 1:3000, detected with an ECL western blot detection system (Amersham Biosciences).

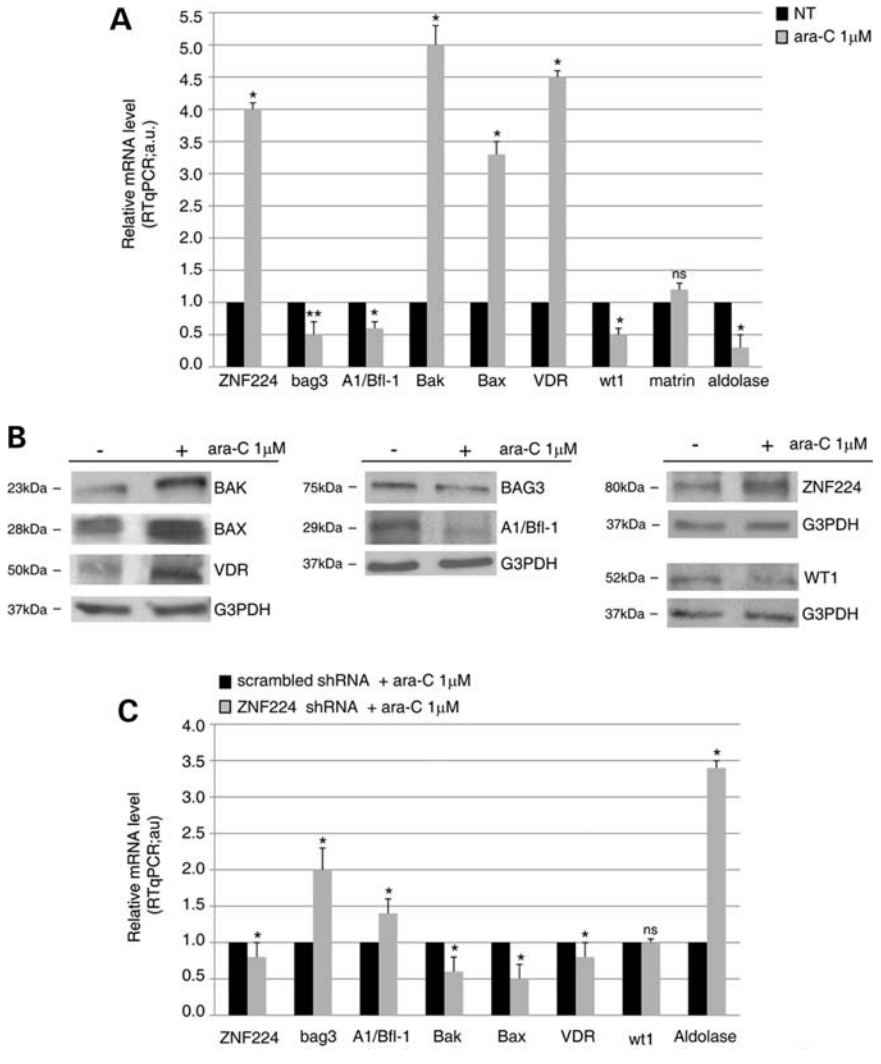

Figure 7. Modulation of apoptosis-regulating genes by ara-C. (**A**) RT-qPCR analysis of mRNA levels in K562 cells treated with 1 μM ara-C for 72 h. NT: untreated K562 cells collected at 72 h used as control. mRNA level of matrin3 was measured to demonstrate the specificity of ara-C effect on apoptosis-regulating genes. Error bars represent standard deviations of three independent experiments. $^{*}P < 0.05$ versus control; $^{**}P = 0.006$ versus control; ns, not significant. (**B**) Total protein extracts were analyzed by western blot with the indicated antibodies. G3PDH was used to monitor equal loading conditions. One representative result out of three performed is presented. Molecular weight to the left. (**C**) RT-qPCR analysis of mRNA levels of pools of K562 cells silenced for ZNF224 and treated with 1 μM ara-C for 72 h (ZNF224 shRNA+ara-C 1 μM) or of pools of K562 cells stably transfected with scrambled shRNA treated with 1 μM ara-C for 72 h (scrambled shRNA+ara-C 1 μM) used as negative control. Error bars represent standard deviations of two independent experiments. $^{*}P < 0.05$ versus scrambled shRNA treated with ara-C.

anti-WT1 (C19) from Santa Cruz Biotecnology, Santa Cruz, CA, USA, 1:500
anti-BAG3 (TOS-2) from BioUniverSa s.r.l., Fisciano, Italy, 1:10000
anti-A1/BFL-1 (PRS3873) from Sigma-Aldrich, 1:1000
anti-Cleaved caspase-3 (Asp175) from Cell Signaling, Denvers, USA, 1:1000
anti-BAK (B5897) from Sigma-Aldrich, 1:2000

For these primary antibodies, the secondary antibody was goat-anti-rabbit IgG (H+L)-HRP conjugated (BioRad170-6515) antibody, 1:3000, detected with an ECL western blot detection system (Amersham Biosciences).

The western blot for ZNF224 was performed using the anti-ZNF224 (T3) antibody (Rabbit polyclonal antibody) (39). Non-specific binding sites were blocked for 2 h in SuperBlock blocking solution buffer in PBS (Thermo Scientific) with 0.1% Tween-20. SuperBlock blocking solution buffer was used in incubation steps with primary and secondary antibodies. The primary antibody was diluted in a ratio 1:500.

The secondary antibody was goat-anti-rabbit IgG (H+L)-HRP conjugated (BioRad170-6515) antibody (1:3000), detected with an ECL western blot detection system (Amersham Biosciences).

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as previously described (21). Briefly, cross-linked chromatin was prepared from HEK293 cells transfected with 3XFLAG-CMV-7.1-ZNF224 or

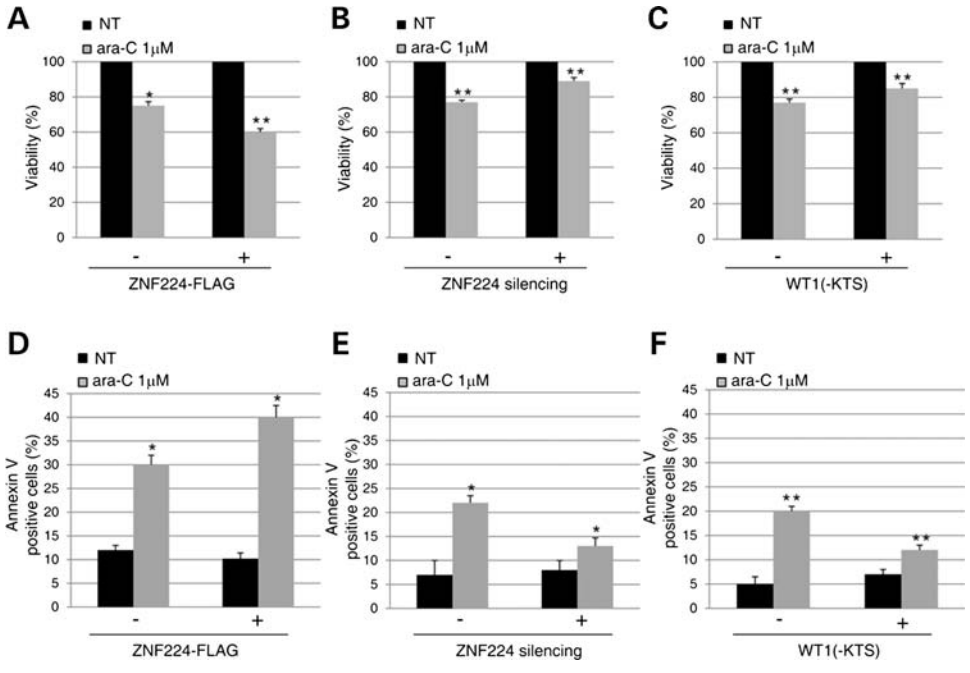

Figure 8. ZNF224 enhances ara-C induced apoptosis. (**A** and **D**) The cell viability and the apoptosis of K562 cells expressing or not expressing ZNF224-FLAG, measured after treatment with 1 μM ara-C for 72 h. (**B** and **E**) The cell viability and the apoptosis of pools of K562 cells stably transfected with ZNF224 shRNA or scrambled shRNA, measured after treatment with 1 μM ara-C for 72 h. (**C** and **F**) The cell viability and the apoptosis of pools of K562 cells retrovirally transduced with WT1(−KTS) or control empty vector (pMIG), measured after treatment with 1 μM ara-C for 72 h. NT, untreated cells collected at 72 h. The cell viability was determined by trypan blue exclusion. The cell viability was calculated as a percentage from the viability of the untreated cells (NT) set as 100%. Apoptosis was determined by annexin V-APC positivity followed by flow cytometry. Error bars represent standard deviations of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.005$ versus untreated (NT) cells.

pcDNA3WT1(-KTS), or co-transfected with 3XFLAG-CMV-7.1-ZNF224 and pcDNA3WT1(-KTS). Briefly, 5×10^7 cells were cross-linked with 1% of fixing solution (11% HCHO, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES pH 8.0) and harvested in 1.5 ml of cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Nonidet P-40) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin and 1 μg/ml pepstatin A). Nuclei were collected and resuspended in 300 μl of nuclear lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 0.8% SDS and protease inhibitors). The DNA was sheared by sonication to an average length between 200 and 1000 bp. Sonicated DNA was diluted 3-fold in dilution buffer (10 mM Tris–HCl, pH 8.0, 0.5 mM EGTA, 1% Triton X-100, 140 mM NaCl, protease inhibitors) and precleared with protein A/G plus-agarose (Santa Cruz Biotechnology) at 4°C for 1 h. The precleared extract obtained from cells transfected with 3XFLAG-CMV-7.1-ZNF224 or co-transfected with 3XFLAG-CMV-7.1-ZNF224 and pcDNA3WT1(-KTS) was divided in two aliquots. One aliquot was incubated with 3 μg of FLAG M2 mouse antibody (Sigma-Aldrich), and the other was incubated with 3 μg of anti-mouse IgG (Sigma-Aldrich), used as control. The precleared extract obtained from cells transfected with pcDNA3WT1(-KTS) was divided in two aliquots and incubated with 5 μg of rabbit anti-WT1 antibody (C19 Santa Cruz Biotechnology), or with 5 μg of anti-rabbit IgG (Sigma-Aldrich), used as control.

Beads were collected by centrifugation and then washed three times with washing buffer P1 (30 mM NaCl, 50 mM Tris–HCl pH 8.0, 1 mM EDTA, 10%glycerol, 0.1% Nonidet P-40, protease inhibitors), twice with washing buffer P2 (50 Mm Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, protease inhibitors) and twice with TE buffer (1 mM Na-EDTA, 10 mM Tris–HCl pH8.0). The first supernatant of the IgG control served as the total input control for the PCR analysis effectiveness. The beads were taken up in 100 μl of TE. 1 μg of RNase was added, and cross-links were reversed at 65°C overnight. The next day, samples were adjusted to 0.5% SDS and 0.5 mg/ml proteinase K and incubated for 3 h at 50°C. DNA was purified by phenol–chloroform extraction. ChIP samples were then analyzed by PCR using 2 μl of immunoprecipitated material, the input control diluted in a ratio 1:200, and specific primers for: Bax (Fw 5′agatgctcattggacagtcacg-3′, Rev 5′-gagtttctgcccctcagtgct-3′); Bak (Fw 5′-gatggagtcttgcactgt cacc-3′, Rev 5′-ttctggctaacatggtgaaacc-3′); bag3 (Fw 5′-agtcc agctcgtccggcttc-3′, Rev 5′-ggccagttgctacctccct-3′); A1/Bfl-1 primer sequences are described in (16). Aldolase A primer sequences are described in (22); as control of immunoprecipitated chromatin specificity, PCR was performed using G3PDH primers (Fw 5′-ggtcgtattgggcgcctggtcacca-3′, Rev 5′-cacacc catgacgaacatgggggc-3′). Results are representative of two independent experiments.

Analysis of viability and apoptosis by flow cytometry

Apoptosis of K562 cells, pools of K562 cells stably overexpressing ZNF224 (ZNF224-FLAG) or stably transfected with

ZNF224 shRNA, pool of K562 cells retrovirally transduced for WT1(−KTS), was induced by cytosine arabinoside (ara-C) (Sigma-Aldrich). To this aim, cells were plated at a density of 2.5×10^5/well in 12-well plates. Cell number was counted in a Bürker chamber and viability was determined by trypan blue exclusion.

For assessment of apoptosis, 2×10^5 cells were stained with propidium iodide (Sigma-Aldrich) and analyzed by flow cytometry as described (40).

For annexin V staining, 3×10^5 cells were rinsed twice in cold PBS with Ca^{2+} and Mg^{2+}, than was resuspending in cold annexin V-binding buffer (5 M NaCl, 1 M $CaCl_2$, 1 M HEPES buffer/NaOH pH 7.4) and stained with Annexin V-APC (550474, BD Pharmingen), DAPI (4′,6-diamidino-2-phenylindole) (D9564, Sigma-Aldrich), and 7AAD (7-amino-actinomycin) (559925, BD Pharmingen). This allows for the discrimination of live cells (DAPI impermeable) from apoptotic cells (annexin V and DAPI positive cells) and necrotic cells (7AAD and DAPI positive cells). Following incubation on shaker in the dark for 15 min at 4°C, cells were analyzed on an FACS Aria flow cytometer (BD Biosciences Immunocytometry System).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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