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 transcriptional 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
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/organisms, 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 Fraser syndrome, a urogenital disorder characterized by glomerulosclerosis and gonadal 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 BASPI (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-WT1 antibody (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
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.
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 immunoprecipitation 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.

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.
of both WT1 (VDR) and ZNF224 (AldA). Chromatin was prepared from HEK293 cells transfected with pcDNA3-WT1 (-KTS), or p3XFLAG-ZNF224, or co-transfected with pcDNA3-WT1(-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 transcripational 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.
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-Hsp90 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 polyosomal fractions. On the contrary, endogenous ZNF224 protein was not associated with the ribosomes, being present in the same fractions as Hsp90B.

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, Hsp90B 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 with respect to the isoform.

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.
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(2KTS) 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 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 (2KTS) 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 its role in post-transcriptional regulation.

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. 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.

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.
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 polyosomes 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 polyosomes 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% CO2 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 lysed 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 Na3VO4, 1 mM DTT, 1 mM phenyl methane sulfonyl fluoride (PMSF), 10 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 fractionated lysates were prepared by resuspending pelleted cells in the Cytoplasm Lysis Buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenyl methane sulfon 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 lysed 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 Biotechnology) 1:1000
- anti-e-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 x 10^5 per well in 24-well dishes. The pGL4-null empty vector (200 ng) was used to evaluate the background luciferase 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) 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′-CACTGGCTCA GCGTCC-3′ and TB3 Rev, 5′-GCCAGAGCTCCGTTGGC-3′. For AldA-NRE: AldA-NRE Fw, 5′-CCCTCTGTTCCACTG GGAAGTGAG-3′ and AldA-NRE Rev, 5′-CCATTCAGTT CCCAGCCCTGGTG-3′. As a negative control, real-time PCR was done using GAPDH-specific primers (GAPDH Fw: 5′-GGTCGTATTGG GCGTCC-3′ and AldA-NRE Rev, 5′-GCCAGAGCTCCGTTGGC-3′). The amount of the immuno-precipitated 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 GAPDH For: 5′-GGTCGTATTGG GCGTCC-3′ and AldA-NRE Rev, 5′-GCCAGAGCTCCGTTGGC-3′. The amount of the immuno-precipitated 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).

Immunofluorescence assay

COS7 cells were plated on cover slips at a concentration of 2 × 105 per cover slip and co-transfected with 2 μg of p3XFLAG-ZNF224 or pcDNA3WT1(-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 1:200 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-diamino-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 × 106 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 pelleted in a microfuge. The cellular pellet was resuspended in polysome extraction buffer (PEB), (20 mM Tris pH 7.5, 100 mM KCl, 5 mM MgCl2, 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. Lysed cells were pelleted by centrifugation for 10 min at 12 000 rpm (Eppendorf microfuge) at 4°C and the supernatant was layered onto a sucrose gradient prepared in 2 ml of lysis buffer (10 mM Tris–HCl pH 7.5, 5 mM MgCl2, 0.3% NP-40; 1 mg/ml heparin, 1 mM PMSF, 10 μg/ml aprotinin, leupeptin and pepstatin). 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 1 ml of the 20% sucrose fraction, and the mixture incubated for 15 min on ice. The samples were centrifuged 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 (A260) was monitored with a recording spectrophotometer. Proteins were precipitated by the addition of trichloroacetic 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 × 106 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 MgCl2, 8% glycerol, 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 000g. 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 MgCl₂), gently vortexed and finally the poly(A)⁺ binding proteins were eluted with 1 ml of 60°C DEPC H₂O and precipitated with isopropanol. One-tenth of the supernatant representing 8 × 10 mg oligo(dT) cellulose] by 2 h of incubation at 4°C and then incubated with the protein extract.

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