# The speckling domain of the Wilms tumor suppressor WT1 overlaps with the transcriptional repression domain

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**Abstract The Wilms tumor suppressor gene *WT1* encodes a zinc finger protein, expressed as different splicing variants, that has all the hallmarks of a transcription factor. The −KTS form of WT1 displays a homogeneous localization within the nucleus and has been shown to activate or repress the activity of various target genes. In contrast, the WT1(+KTS) variant demonstrates a speckled pattern of expression within the nucleus. This and its association with factors of the splicing machinery has led to the hypothesis that WT1(+KTS) might play a role in post-transcriptional processes. By the generation of a series of deletion constructs and subsequent immunofluorescence analysis, we have identified and characterized the domain which is responsible for the localization of WT1 variants in nuclear speckles. The speckling domain comprises amino acids 76–120 within the N-terminus of WT1 and is sufficient to target other proteins into distinct nuclear domains. Interestingly the WT1 speckling domain does not overlap with the domain required for interaction with the splicing factor U2AF65 but overlaps with the transcriptional repression domain. Thus our data challenge the view that association of WT1 with spliceosomes is responsible for the speckling phenotype.** 

*Key words:* Wilms tumor protein; Zinc finger protein; Transcriptional repression; Nuclear focus; Splicing factor

## 1. Introduction

The Wilms tumor suppressor gene *WT1* encodes a four zinc finger containing protein which is essential for the development of various organs including the kidneys, gonads, heart and spleen [1–3]. Mutations in *WT1* predispose humans to the generation of Wilms tumor, a pediatric kidney cancer [4,5], as well as other, more complex diseases like the Denys–Drash [6] and Frasier syndromes [7–9]. The latter are both characterized by progressive nephropathy and genital malformations. Based on these phenotypes and the expression pattern, it is generally assumed that WT1 plays a pivotal role in mesenchymal–epithelial transitions [10].

WT1, which is encoded by 10 exons, is expressed as a 36–62 kDa protein family that arises because of three alternative sites of translation initiation [11,12]. There are four main WT1 protein isoforms with molecular masses of 52–54 kDa which are generated by two alternative splicing events of the *WT1* mRNA. Alternative splice I comprises exon 5, which encodes 17 amino acids in the central part of the protein. The functional consequences of the inclusion of exon 5 have not yet been defined clearly. Alternative splice II results from use of an alternative splice donor sequence between exons 9 and 10. Its insertion leads to three additional amino acids (the KTS sequence) that disrupt the spacing between zinc fingers 3 and 4. These splice variants are expressed at a constant ratio (+KTS:−KTS ≈ 2:1) [13].

There is biochemical and genetic evidence that points to different functions for the −KTS and +KTS variants of WT1 [14]. Most notably, the alternatively spliced forms of WT1 localize to different subnuclear compartments. While WT1(−KTS) is diffusely localized throughout the nucleus, WT1(+KTS) is expressed in a speckled pattern within the nucleus [15,16]. These observations suggest that different splice forms of WT1 exert their role at different sites in the nucleus.

Although WT1 has many properties that are typical of a transcription factor including a glutamine/proline-rich N-terminus, activation and repression domains [17–19], nuclear localization signals [20] and four $Cys_2$–$His_2$ zinc fingers at the C-terminus, it is primarily the −KTS form of WT1 which has been shown to be transcriptionally active [14]. The +KTS form on the other hand has been demonstrated to be associated with splice factors and could be colocalized and coimmunoprecipitated with antisera against proteins found in spliceosomes or coiled bodies [15]. Localization of speckling WT1 variants, however, was clearly distinct from that of the essential splicing factor SC35 [16]. Despite this controversy it remains a possibility that +KTS variants of WT1 are involved in post-transcriptional processes, a hypothesis which is underscored by the recent finding that WT1, particularly the +KTS variant, specifically and directly associates with the splicing factor U2AF65 [21]. By gel filtration and sedimentation analysis, WT1 has also been shown to be present in ribonucleoprotein complexes [22].

There are several reports about the ability of WT1 to bind RNA. Computer modeling has demonstrated that WT1 possesses in its N-terminus a potential RNA recognition motif similar to that in constitutive splicing factors [23]. Also, the zinc finger domain of WT1 has been shown to bind to RNA derived from exon 2 of the insulin-like growth factor II gene (*Igf-2*). In this case the WT1(+KTS) form bound the RNA probe with greater affinity than WT1(−KTS) [24]. In a more systematic search, the SELEX (systematic evolution of ligands by exponential enrichment) method has been employed to

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identify WT1 binding sequences from a random RNA pool. Three different groups of RNA ligands which were specifically bound by WT1 were selected. In contrast to earlier work, however, in this case the WT1(+KTS) isoform was unable to bind the identified RNAs [25]. Given the partly contradicting results, the significance of these findings has to remain elusive until a functional role for WT1 in RNA processing has been defined.

In order to eventually clarify the biochemical properties of the WT1 variants we have identified and characterized the domain which is responsible for the speckling phenotype of WT1. This domain which comprises amino acids 76–120 overlaps with the transcriptional repression domain of WT1 and is sufficient to confer 'speckling' to other proteins.

## 2. Materials and methods

### 2.1. Generation of expression constructs

All constructs used in this study are based on the murine WT1 sequence which is more than 95% identical at the amino acid level to its human counterpart [26]. *WT1* deletion constructs were generated by polymerase chain reaction (PCR)-mediated amplification of the indicated fragments and subsequent cloning into the *Eco*RI and *Apa*I sites of the eukaryotic expression vector Rc/CMV (Invitrogen). The vector had previously been modified so that the second *Eco*RI site (position 1744) was deleted. In addition, two complementary oligonucleotides had been inserted between the *Hin*dIII and *Eco*RI sites constituting an HA-epitope tag. A sequence encoding a nuclear localization signal (KKKRKVD) was inserted into the 3′-end of each deletion construct. Construct CHD was made by amplifying the full-length *WT1* cDNA (harboring both alternative splice sequences) by PCR using primers with *Eco*RI and *Apa*I sites at the 5′- and 3′-termini, respectively. The resulting fragment was then cloned into the *Eco*RI and *Apa*I sites of Rc/CMV which also carried an HA-tag. CHDΔ was generated by digestion of CHD with *Bss*SI and subsequent religation. By this procedure amino acids 84–177 of WT1 are deleted.

For the generation of the glutathione *S*-transferase (GST) fusion construct, a fragment of WT1 encompassing codons 76–160 was amplified by PCR and ligated into the *Bam*HI and *Kpn*I sites of the vector pEBG (described in [27]). A sequence encoding a nuclear localization signal (KKKRKVD) was inserted into the 3′-end of the expression construct. As a control a construct was used in which a pair of oligonucleotides encoding a nuclear localization signal were ligated into the *Bam*HI and *Kpn*I sites of pEBG.

### 2.2. Cell culture and transfections

The human osteosarcoma cell line U2OS was obtained from the American Type Culture Collection (ATCC), COS-7 cells from the European Collection of Cell Cultures (ECACC). Cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transient transfections were done using the Super-Fect reagent (Qiagen) according to the manufacturer's recommendations. For the colocalization analysis the SD3 cell line has been used [28] which harbors an inducible wild-type *WT1* allele containing both alternative splice sequences.

### 2.3. Antibodies and immunofluorescence analysis

For immunofluorescence analysis, cells were grown on coverslips, fixed with 4% (w/v) paraformaldehyde, permeabilized with 1% Nonidet P-40 in 10 mM glycine, preadsorbed with 3% (w/v) bovine serum albumin and exposed to antibodies C-19 (rabbit polyclonal anti-WT1, Santa Cruz), 12CA5 (mouse monoclonal anti-HA, Roche) or to a monoclonal anti-GST antibody (Sigma Immunochemicals). Coverslips were then exposed to rhodamine-conjugated goat anti-rabbit or fluorescein-conjugated goat anti-mouse antibody. Samples were examined by using a Zeiss Axiovert 135 microscope. For colocalization analysis the Openlab digital imaging system (Improvision, UK) was used.

### 2.4. Western blotting analysis

For Western blotting, cellular lysates were extracted with RIPA buffer (10 mM Tris–HCl, pH 7.4/150 mM NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% sodium dodecyl sulfate (SDS)), electrophoretically separated on a 12% SDS–PAGE and transferred onto a PVDF membrane (Immobilon-P; Millipore). Blots were probed with the anti-HA antibody 12CA5, followed by horseradish peroxidase-coupled goat anti-mouse antibody and enhanced chemiluminescence analysis (ECL; Amersham).

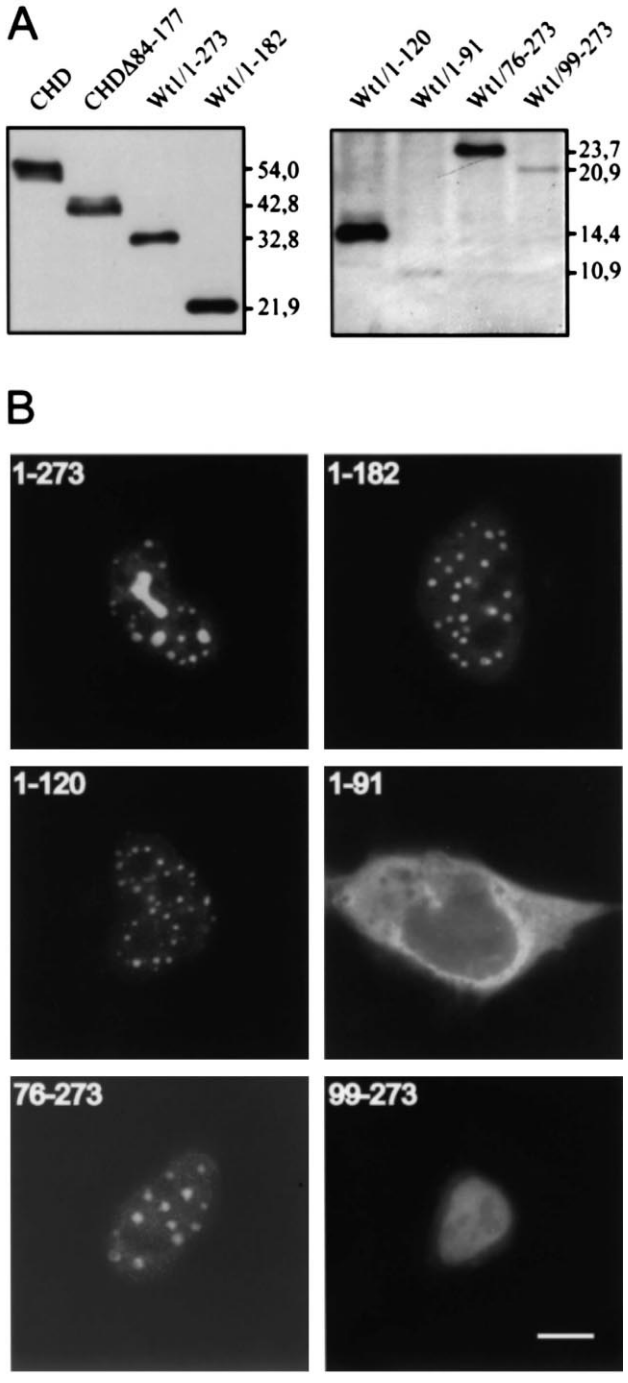


Fig. 1. Identification of the WT1 speckling domain. A: Western blot analysis using protein extracts from U2OS cells transfected with the constructs indicated. An anti-HA antibody was used for protein detection. Positions of marker proteins (in kDa) are indicated. B: Subnuclear localization of WT1 proteins determined by staining with anti-HA antibody followed by indirect immunofluorescence analysis. U2OS cells were transfected with the constructs indicated. Except where construct *WT1/1–91* has been used, only the nuclei are stained. Bar, 5 μm.

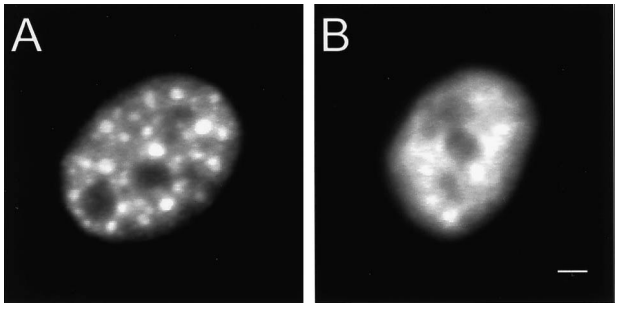


Fig. 2. Localization of wild-type and speckling domain-deficient mutant WT1 protein. Immunofluorescence analysis of the WT1(+KTS) isoform (A) and a mutant variant lacking amino acids 84–177 (B). Human U2OS cells were used for this experiment. Protein detection was carried out using an anti-WT1 antibody. Bar, 2 μm.

## 3. Results

### *3.1. The minimal speckling domain of WT1 comprises amino acids 76–120*

Previous work has demonstrated that the domain responsible for the localization of WT1 in speckles must be contained in the N-terminus of the molecule. This is based on the observation that a WT1 mutant form with a deletion of the C-terminal zinc finger domain can also be found in these subnuclear compartments [16]. We have therefore generated a series of N- and C-terminal deletions of the WT1 amino-terminal domain. All constructs harbored an HA-tag as well as a nuclear localization signal and were driven from a CMV promoter. Following Western blot analysis to ensure correct expression of all constructs (Fig. 1A), the localization of the respective gene products was analyzed by immunofluorescence. As can be seen in Fig. 1B, proteins comprising the first 273, 182 or 120 amino acids of WT1 are localized in nuclear speckles. Although clearly most of the cells expressing the latter construct showed a speckled expression, the localization to those nuclear domains was more stringent when construct WT1/1–182 was used. Further deletion (WT1/1–91) results in an ubiquitous distribution in both the nucleus and the cytoplasm. Most likely the polypeptide is too small to be efficiently kept in the nucleus. These results demonstrate a requirement for amino acid residues between positions 91 and 120 for WT1 to be targeted to nuclear speckles. In the case of the N-terminally deleted WT1 mutants, WT1/76–273 demonstrated a prominent speckling pattern whereas the WT1/99–273 mutant showed a homogeneous nuclear expression pattern. From these analyses we conclude that a minimal domain containing amino acids 76–120 of WT1 is required for the localization in nuclear speckles. Interestingly this domain almost perfectly overlaps with the WT1 domain responsible for transcriptional repression which has been mapped between amino acids 85 and 124 [17,19].

We next wanted to address the function of the speckling domain in the context of the full-length WT1 molecule. Start-

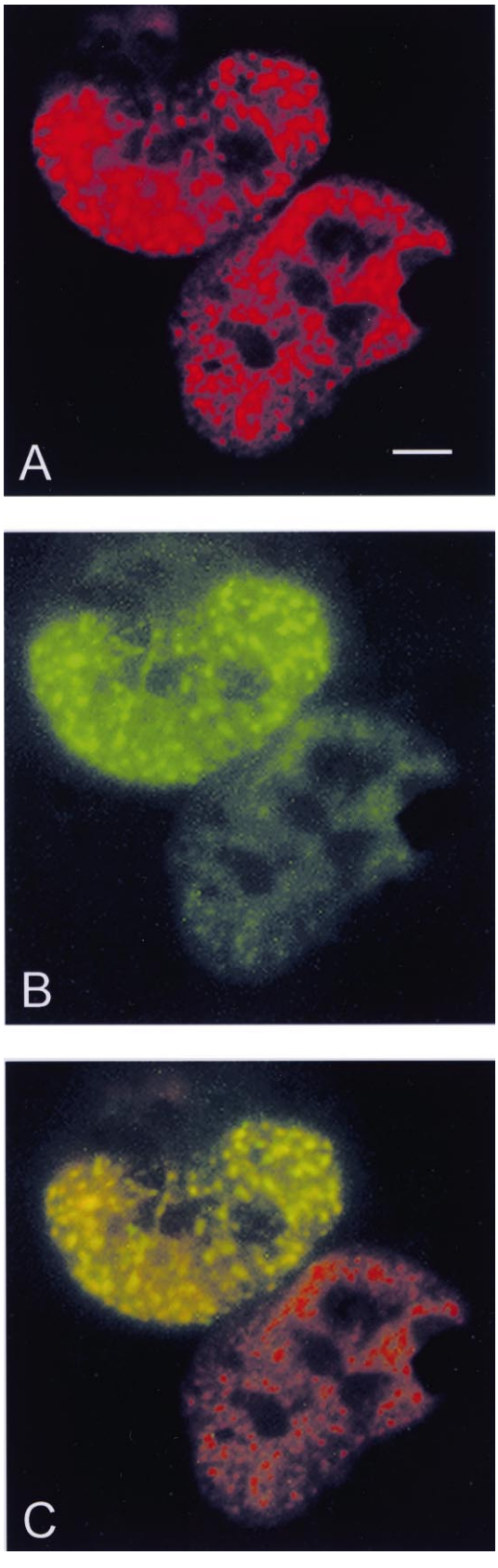


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Fig. 3. Colocalization of wild-type WT1(+KTS) and a WT1 mutant form using digital deconvolution microscopy. A Saos-2 cell line which stably expresses a WT1(+KTS) isoform (SD3, see [28]) was transfected with the WT1/1–273 construct. Detection of the wild-type WT1 protein was carried out with the anti-WT1 antibody C-19 (A), the mutant WT1 protein was visualized using the anti-HA antibody 12CA5 (B). In C the upper two frames have been merged. The yellow color indicates colocalization of the wild-type and mutant proteins. Note that only the upper cell has been successfully transfected with the mutant construct, the bottom cell expresses only wild-type protein. Bar, 2 μm.

ing from a wild-type *WT1*(+*KTS*) cDNA (named CHD) we have deleted amino acids 84–177 harboring those amino acids required for speckling. This construct was named CHDΔ and was expressed as efficiently as the wild-type version (Fig. 1A). The human osteosarcoma cell line U2OS was transfected with both constructs and localization of the gene products was analyzed by immunofluorescence (Fig. 2). The CHD protein could be found throughout the nucleus but also in defined nuclear speckles whereas CHDΔ harboring the internal deletion was uniformly distributed within the nucleus. This observation was independent of the cell line used and has been confirmed in COS-7 cells as well as in the murine mesonephric M15 cell line (data not shown). Thus deletion of an internal WT1 fragment encompassing amino acids 84–177 renders the protein unable to localize to nuclear speckles confirming the previous deletion analysis.

### 3.2. Wild-type and mutant WT1 localize to identical subnuclear structures

As can be seen in Fig. 1, the shape, size and number of WT1-associated speckles are not always identical. We therefore wanted to investigate whether the appearance of the speckles is dependent on the structure and length of the WT1 protein and whether wild-type and mutant WT1 forms localize to the same nuclear foci. For this analysis we have transfected a cell line harboring an inducible full-length *WT1*(+*KTS*) transgene with a construct encoding WT1/1–273. Nuclear localization of the different WT1 forms was examined by immunofluorescence analysis using digital deconvolution microscopy (Fig. 3). For the wild-type form an antibody was used which is directed against the C-terminal amino acids of WT1, a region missing in the mutant form (Fig. 3A). The mutant protein was detected with an HA antibody which does not recognize the wild-type protein (Fig. 3B). Cells which expressed both the mutant as well as the wild-type WT1 form demonstrated colocalization of both proteins in subnuclear structures (Fig. 3C). This observation confirms the conclusions drawn from the experiments described above in that the localization of WT1 in nuclear speckles does not depend on the presence of the zinc finger region which is responsible for nucleic acid binding. Also, the structure of the WT1 protein does not seem to influence the shape, size and number of speckles it localizes to. We conclude from this experiment that the WT1-associated speckles do not reflect properties of the respective WT1 protein but are defined nuclear compartments.

### 3.3. The speckling domain of WT1 is sufficient for the localization in subnuclear structures

After having defined the amino acids which are necessary for the localization of WT1 in speckles we wanted to investigate whether this particular domain of WT1 was also sufficient for the localization. To address this question we have fused WT1 amino acids 76–160 together with a nuclear localization signal to GST. Here we have used a slightly extended form of the minimal speckling domain defined above because WT1/1–160 was localized in speckles in nearly 100% of all nuclei examined, whereas the minimal domain (amino acids 76–120) was less stringent in that regard. As GST is normally localized in both the cytoplasm as well as the nucleus (data not shown) we have used a GST carrying the same nuclear localization signal as the GST–WT1 fusion protein as a control for this experiment. Immunofluorescence analysis of cells transfected with a GST–NLS encoding construct revealed a uniform nuclear staining pattern (Fig. 4A,B). In contrast, the fusion protein of WT1 and GST could be found in clearly demarcated nuclear speckles (Fig. 4B,D). Number and appearance of these structures were very similar to the ones associated with the WT1 forms that can be seen in Fig. 1 and independent of the cell type used. The localization of a fusion protein consisting of GST and the N-terminal fragment of WT1 indicates that the WT1 speckling domain is not only necessary but also sufficient for the localization in nuclear speckles. In addition, this domain can be transferred onto other proteins which are then also targeted to this nuclear compartment.

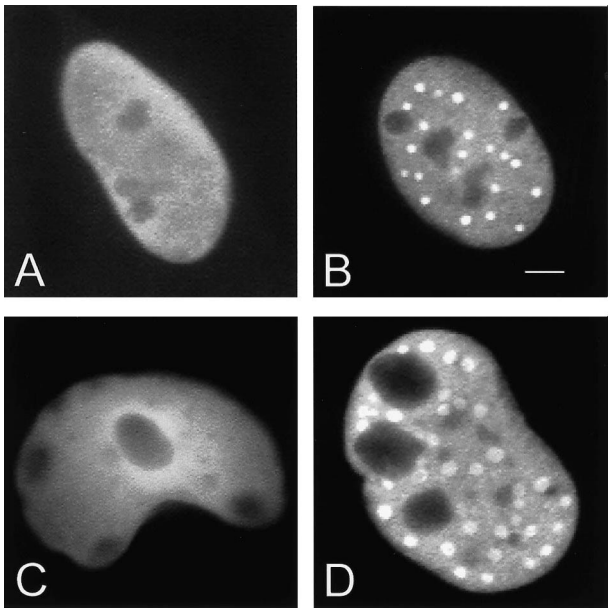


Fig. 4. The WT1 speckling domain is sufficient to target GST into nuclear speckles. U2OS (A,B) and COS cells (C,D) were transfected with a construct encoding either GST alone (A,C) or a fusion between GST and amino acids 76–160 of WT1 (B,D). Both gene products carry a C-terminal nuclear localization signal sequence. The proteins were visualized using an anti-GST antibody. Only the nuclei of transfected cells are shown. Bar, 2 μm.

## 4. Discussion

Using a series of WT1 deletions we have identified the domain which is responsible for the speckling phenotype of WT1. This domain is located within the N-terminus of the protein and comprises amino acids 76–120. It has been shown earlier that a small fraction of WT1 in a cell, preferentially the +KTS variants, are physically associated with the splicing factor U2AF65 [21]. Since factors involved in splicing often localize in nuclear speckles, this would suggest that the speckling phenotype of WT1 is caused by its association with U2AF65. In the same report, however, it is stated that the binding to U2AF65 is not a prerequisite for the speckled distribution of WT1. This is confirmed by the observation that binding of U2AF65 required the C-terminal domain of WT1 (amino acids 181–449, [21]), whereas our data clearly demonstrate localization of the speckling domain within the N-terminal part of the molecule. Thus, the localization in speckles and the association with factors of the splicing machinery, at

least with regard to U2AF65, are separable features of WT1. This observation challenges the view that WT1's association with the mRNA splicing machinery is responsible for the speckling phenotype.

WT1 has been shown to possess distinct transcriptional activation and repression domains. A detailed analysis revealed that residues 85–124 are responsible for the transcriptional repression function of WT1 whereas amino acids 181–250 are required to activate transcription [17–19]. It is interesting to note that the domain which we have identified as the speckling domain of WT1 shows significant overlap with the repression domain of this molecule. Given that only a fraction of WT1 seems to be associated with splicing factors [21] or in other instances colocalization with these factors cannot be observed [16] one could speculate that there are other factors which WT1 might be associated with in nuclear speckles. These could be factors mediating transcriptional repression. Although there is accumulating evidence that WT1 can function as a transcriptional activator [29–31], WT1 had initially been characterized as a transcriptional repressor and there are a number of reports in which the −KTS and the +KTS variants repress transcription equally well [32]. How transcriptional repression and the localization in speckles relate to each other (whether for example speckles serve as storage sites for repressor molecules) is at present unclear and must await further functional characterization of these subnuclear structures. Interestingly, some members of the family of human histone deacetylases, which serve as components of transcriptional repressor complexes, have also been found to be localized in discrete nuclear foci [33].

Our results demonstrate that the speckling domain of the WT1 protein is located within the N-terminus of the molecule. The original observation with regard to the differential localization, however, was made with the +KTS and −KTS variants of WT1. The difference between those splice forms is limited to the C-terminus of the molecule. How can one envisage that a structural motif within the zinc finger region of WT1 can affect the differential localization of the molecule for which an N-terminal domain is required? One possibility is that the localization of WT1 is guided by differential affinities. The −KTS form of WT1 might for example have a much higher affinity for DNA than the +KTS form and therefore override an association mediated by the speckling domain. Conversely, the +KTS form might preferentially be associated with speckles since its DNA binding affinity is not sufficiently high. It has recently been reported that the introduction of a point mutation into the zinc finger region of a −KTS variant of WT1 leading to the loss of DNA binding results in enhanced colocalization with splice factors, i.e. speckling [21]. This observation supports the 'competition' hypothesis. Alternatively there might be an intramolecular interaction between the N- and the C-terminus of WT1 which might influence its biochemical properties.

The identification of the speckling domain of WT1 should prove useful since it can serve as a tool to identify components with which WT1 is associated. This in turn will help to clarify whether the different splice forms of WT1 exert their function at the transcriptional and/or post-transcriptional level.

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