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Genetics

Truncated WT1 mutants alter the subnuclear localization of the wild-type protein

(Wilms tumor/dominant-negative mutants/alternative splicing/spliceosomes/nuclear speckling)

CHRISTOPH ENGLERT*, MARC VIDAL*, SHYAMALA MAHESWARAN*, YIMIN GE†, ROBERT M. EZZELL†, KURT J. ISSELBACHER*, AND DANIEL A. HABER*‡

*Massachusetts General Hospital Cancer Center and †Surgical Research Unit, Harvard Medical School, Charlestown, MA 02129

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ABSTRACT ***WT1* encodes a zinc-finger protein, expressed as distinct isoforms, that is inactivated in a subset of Wilms tumors. Both constitutional and somatic mutations disrupting the DNA-binding domain of WT1 result in a potentially dominant-negative phenotype. In generating inducible cell lines expressing wild-type isoforms of WT1 and WT1 mutants, we observed dramatic differences in the subnuclear localization of the induced proteins. The WT1 isoform that binds with high affinity to a defined DNA target, WT1(−KTS), was diffusely localized throughout the nucleus. In contrast, expression of an alternative splicing variant with reduced DNA binding affinity, WT1(+KTS), or WT1 mutants with a disrupted zinc-finger domain resulted in a speckled pattern of expression within the nucleus. Although similar in appearance, the localization of WT1 variants to subnuclear clusters was clearly distinct from that of the essential splicing factor SC35, suggesting that WT1 is not directly involved in pre-mRNA splicing. Localization to subnuclear clusters required the N terminus of WT1, and coexpression of a truncated WT1 mutant and wild-type WT1(−KTS) resulted in their physical association, the redistribution of WT1(−KTS) from a diffuse to a speckled pattern, and the inhibition of its transactivational activity. These observations suggest that different WT1 isoforms and WT1 mutants have distinct subnuclear compartments. Dominant-negative WT1 proteins physically associate with wild-type WT1 *in vivo* and may result in its sequestration within subnuclear structures.**

Wilms tumor is a pediatric kidney cancer that can present either sporadically or in the presence of genetic susceptibility. The *WT1* tumor suppressor gene was identified by its localization to the chromosome 11p13 Wilms tumor locus, its inactivation in a subset of Wilms tumors (reviewed in ref. 1), and its ability to suppress the growth of cultured Wilms tumor cells (2). *WT1* encodes a transcription factor, with four DNA-binding zinc fingers at the C terminus, and a proline- and glutamine-rich transactivation domain at the N terminus. In transient transfection assays, WT1 represses transcription from numerous promoter constructs containing the G+C-rich early growth response 1 (EGR1) consensus sequence (3). A potentially physiological target gene, the epidermal growth factor receptor (EGFR), has recently been identified. Expression of *WT1* by using an inducible promoter results in suppression of endogenous EGFR synthesis and induction of apoptosis, an effect that is prevented by constitutive expression of EGFR (4). The transactivational properties of *WT1* are differentially mediated by alternatively spliced variants that are present in constant relative proportion in normal tissues expressing *WT1* (5). The function of alternative splice I, inserted between the transactivation and DNA-binding domains, has not been clearly defined. However, alternative splice II, which is present in ≈80% of the *WT1* transcripts, leads to the insertion of three amino acids (KTS) between zinc fingers 3 and 4, abolishing binding to the EGR1 consensus sequence (3).

The developmental role of *WT1* has been inferred from its normal expression pattern and from defects in *WT1*-null mice. *WT1* is expressed in the developing kidney and in the gonads and mesothelium, organs that are defective or that fail to form in mice lacking *WT1* (6, 7). Children harboring one deleted *WT1* allele, so-called WAGR syndrome (Wilms Tumor/Aniridia/Genitourinary abnormalities/Retardation), also develop mild genitourinary defects. However, severe abnormalities of renal and sexual development are observed in children with Denys–Drash syndrome (DDS), who have a dysfunctional, potentially dominant-negative *WT1* mutation (8). Most *WT1* mutations in DDS children are missense mutations affecting hot spots within zinc fingers 3 or 4. However, some DDS mutations encode truncated proteins, lacking part of or even the entire zinc-finger domain (9), suggesting that expression of the N terminus of WT1 is sufficient to induce the DDS phenotype. The possibility that disruption of the WT1 zinc-finger domain leads to a dominant-negative effect is supported by the observation that these mutations may be heterozygous in Wilms tumor specimens (10, 11) and may display oncogenic activity in baby rat kidney transformation assays (12). Functional studies of *WT1* have been limited by the absence of cultured cell lines expressing detectable levels of WT1 protein. We therefore established osteosarcoma cell lines with inducible expression of wild-type WT1 isoforms and WT1 mutants by using the tetracycline-regulated promoter. In these embryonal cell types expressing low levels of endogenous wild-type *WT1* transcript, induction of WT1(−KTS) triggers apoptosis (4). This effect is attenuated following expression of WT1(+KTS) and is not observed following induction of a truncated WT1 mutant. Thus, different WT1 isoforms have distinct effects in these cells, suggesting that they provide a valuable model to study *WT1* function.

MATERIALS AND METHODS

Generation of Inducible Cell Lines and Chloramphenicol Acetyltransferase (CAT) Assays. U2OS and Saos-2 cell lines were generated with inducible constructs encoding wild-type *WT1* splice variants, WT1(−KTS) and WT1(+KTS), and two mutant constructs, WTAR (in-frame deletion of zinc finger 3)

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Abbreviations: EGR1, early growth response 1; EGFR, epidermal growth factor receptor; snRNP, small nuclear ribonucleoprotein; IG, interchromatin granules; CAT, chloramphenicol acetyltransferase; DDS, Denys–Drash syndrome; CMV, cytomegalovirus; HA, hemagglutinin; 3AT, 3-aminotriazole; AD, transactivation domain; DB, DNA-binding domain.

‡To whom reprint requests should be addressed at: Laboratory of Molecular Genetics, Massachusetts General Hospital Cancer Center, CNY 7, Building 149, 13th Street, Charlestown, MA 02129.

and WT1-del Z (encoding aa 1–326 and lacking the entire zinc-finger domain) (4). For analysis of WT1 domains required for speckling, Cos-7 cells were transiently transfected by calcium chloride/DNA precipitation with cytomegalovirus (CMV)-driven plasmids encoding deleted constructs tagged with a hemagglutinin (HA) epitope. For CAT assays, cells with inducible WT1-del Z were transfected with the EGR1-CAT reporter construct (3), together with WT1(−KTS). The total amount of CMV promoter sequence transfected into each dish was equalized, and transfection efficiencies were standardized by cotransfection of a human growth hormone reporter construct.

Antibodies and Immunological Analyses. For immunofluorescence analysis, cells were grown on coverslips, fixed with 4% (wt/vol) paraformaldehyde, permeabilized with 1% Nonidet P-40 in 10 mM glycine, preadsorbed with 3% (wt/vol) bovine serum albumin (BSA), and exposed to rabbit anti-WT1 antibody WTc8 (4) [1/100 dilution]. Coverslips were then exposed to rhodamine-conjugated goat anti-rabbit antibody (1/100 dilution; Jackson ImmunoResearch). For identification of IGs, cells were stained with antibody against SC35 (a gift from T. Maniatis, Harvard University), followed by fluorescein-conjugated goat anti-mouse antibody. Samples were examined by using a laser confocal microscope (Bio-Rad MRC600 imaging system attached to a Zeiss axiovert microscope) using ×63 and ×100 planeofluar objectives. 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% SDS) and blots were probed with antibody WTc8 (1/1000 dilution), followed by goat anti-rabbit antibody and enhanced chemiluminescence analysis (ECL; Amersham). For coimmunoprecipitation studies, U20S cells with inducible WT1-del Z were transiently transfected with HA-tagged WT1(−KTS) and radiolabeled, and cellular lysates were prepared in RIPA buffer, immunoprecipitated by using either antibody WTc8 or the monoclonal antibody 12CA5 directed against the HA epitope, and fractionated by SDS/PAGE.

Yeast Two-Hybrid Assay. The yeast strain, MaV103 (*GAL1*::*HIS3*), and the construction of plasmids are described elsewhere (13). Full-length murine *WT1* was inserted into a vector encoding the GAL4-transactivation domain (AD; 768–881) and full-length wild-type, mutant, or truncated *WT1* was introduced into another construct containing the GAL4 DNA-binding domain (DB; 1–147). Plasmids used contain the *CEN6* centromeric sequence to ensure low copy number, and selection was based on presence of the *HIS3* gene cloned downstream of GAL4 DNA-binding sites. Increasing concentrations of 3-aminotriazole (3AT) were used to titrate the strength of the protein interaction bringing the GAL4 AD in proximity to the GAL4 DB. The growth of DB-WT1 plus AD-WT1 transformants was compared with that of yeast containing DB plus AD, DB-WT1 plus AD, intact GAL4, and fusion constructs encoding known protein partners, including retinoblastoma protein (Rb) plus E2F1, Fos plus Jun, and *Drosophila* DP plus E2F.

RESULTS

Distinct Subnuclear Localization of WT1 Isoforms and Mutants. Endogenous WT1 protein in the developing kidney and in the testis demonstrates a nuclear speckling pattern (14), although transient overexpression of WT1(−KTS) in Cos-7 cells results in a diffuse nuclear pattern. This difference in apparent subnuclear localization could result from different expression levels, from the presence of interacting proteins restricted to specific cell types, or from differences in the properties of WT1 isoforms, all of which are expressed in normal WT1-expressing tissues (5). To test the functional properties of each WT1 isoform when expressed at comparable levels in the same cell type, we analyzed osteosarcoma cells containing a tightly regulated, tetracycline-repressable promoter (4). Expression of WT1 following withdrawal of tetracycline is shown for four representative cell lines containing inducible WT1(−KTS); WT1(+KTS); the naturally occurring

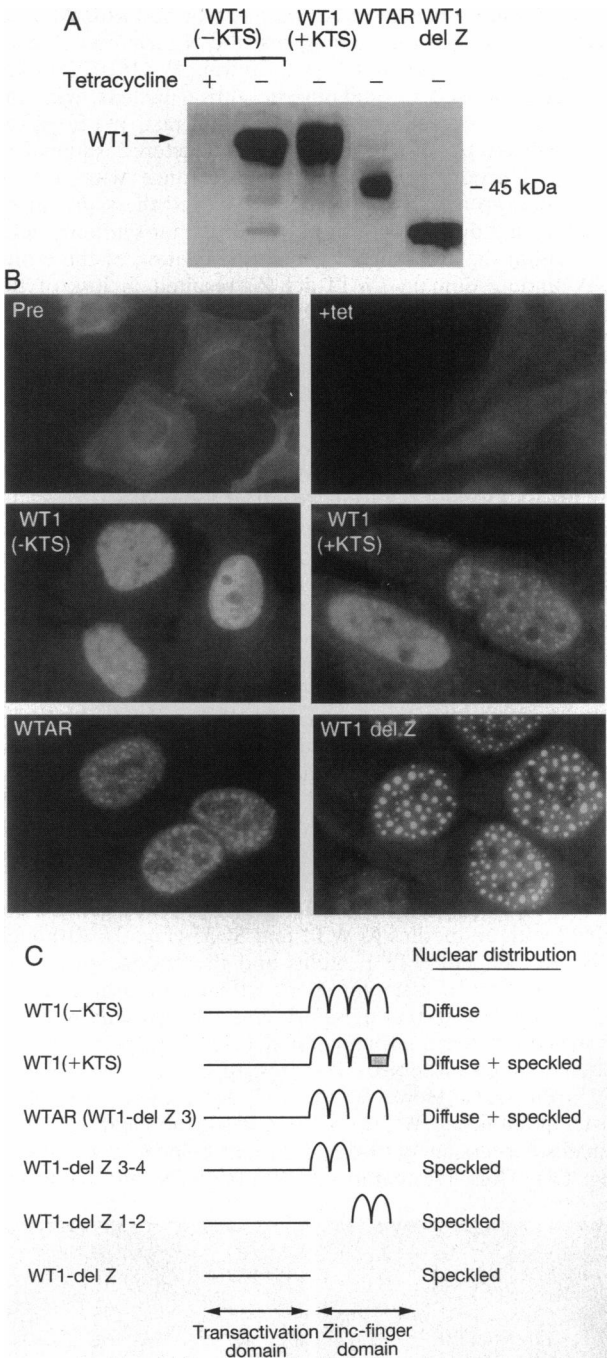

FIG. 1. Distinct subnuclear localization of WT1 variants. (*A*) Inducible expression of WT1. Immunoblot analysis of cell extracts from osteosarcoma cells stably transfected with wild-types WT1(−KTS) or WT1(+KTS) or mutants WTAR (in-frame deletion of zinc finger 3) or WT1-del Z (lacking the entire zinc-finger domain) under control of a tetracycline-repressable promoter. Baseline endogenous WT1 expression was undetectable in the presence of tetracycline, and induction of the transgene was observed following withdrawal of tetracycline. (*B*) Subnuclear localization of WT1 proteins determined by staining with anti-WT1 antibody WTc8 followed by indirect immunofluorescence analysis. Osteosarcoma cells were grown in the presence of tetracycline (+tet) to demonstrate tight regulation of the inducible promoter. Tetracycline was withdrawn, and cells expressing WT1(−KTS), WT1(+KTS), WTAR, or WT1-del Z were stained with WTc8. Induced cells were also stained with preimmune serum (Pre) to demonstrate the specificity of antibody WTc8. (*C*) Schematic representation of WT1 isoforms and truncation mutants and the observed subnuclear localization.

mutant WTAR, with an in-frame deletion of zinc finger 3 (10); or the truncated mutant WT1-del Z, lacking the entire zinc-finger domain (Fig. 1*A*).

Immunofluorescence studies were performed with WTc8, a polyclonal antibody directed against the N terminus of WT1 (4), 36 h after tetracycline withdrawal. WT1(−KTS) was diffusely expressed throughout the entire nucleus, with the exception of the nucleoli (Fig. 1*B*). In contrast, WT1(+KTS) was localized primarily within 30–50 clustered subnuclear structures, producing a speckled appearance with a faint diffuse background. Mutant WTAR showed the same mixed speckling and diffuse nuclear pattern. Further synthetic deletions within the zinc-finger domain or deletion of the entire DNA-binding domain (WT1-del Z) resulted in loss of the diffuse nuclear component, leading to enhanced definition of the subnuclear clusters (Fig. 1 *B* and *C*). Thus, localization of WT1 protein to these subnuclear structures requires the N terminus and appears to be independent of the DNA-binding domain. However, presence of the uninterrupted WT1 zinc fingers 1–4 appears to override this effect, leading to the diffuse nuclear expression pattern of WT1(−KTS).

WT1(+KTS) and WT1-del Z Do Not Colocalize with Splicing Factor SC35. The number and size of WT1-associated speckled structures are similar to the clusters of interchromatin granules (IGs) that contain components of spliceosomes (15–18). These structures are currently thought to play a role in the storage and possibly the preassembly of spliceosomal components rather than in the splicing process itself (19). IGs are identified by the presence of SC35 (16, 17), a spliceosome assembly factor that is required for the initial step of pre-mRNA splicing and is localized to the central core of IG domains (20). The structures that are recognized by antibody against SC35 are also identified by antibody against Sm, which recognizes the small nuclear ribonucleoproteins (snRNPs), which are localized both within IGs and more diffusely in the nucleus (15, 17).

Cells expressing either WT1(+KTS) or WT1-del Z were stained with antibodies to WT1 and SC35 (Fig. 2). Although both the induced WT1 proteins and the endogenous SC35 were expressed in a similar speckled pattern, laser confocal microscopy showed that these proteins were present in different structures. A small fraction of WT1(+KTS), primarily that present in finely dispersed structures, appeared to overlap with SC35 (Fig. 2*A*). However, the well-demarcated subnuclear clusters containing WT1(+KTS) and all the clearly defined domains expressing WT1-del Z did not colocalize with SC35 (Fig. 2*B*). Thus, the majority of WT1(+KTS) and truncated mutant WT1 proteins are localized in discrete subnuclear clusters that are distinct from those containing pre-mRNA splicing factors and defined by the presence of SC35. However, we cannot exclude their colocalization with snRNPs outside these well-demarcated structures.

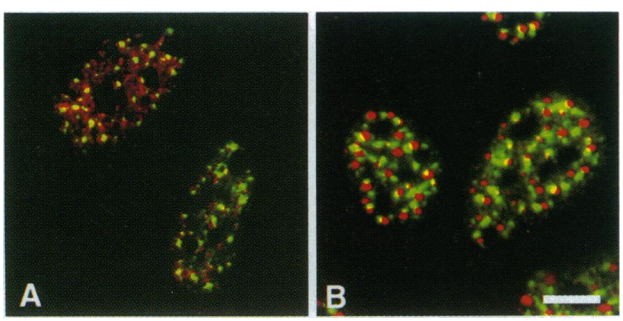

FIG. 2. Distinct localization of SC35 and WT1(+KTS) or WT1-del Z. Confocal imaging of osteosarcoma cells expressing inducible WT1(+KTS) (*A*) or WT1-del Z (*B*). Samples were stained with an antibody to WT1 (red fluorescence) and an antibody to SC35 (green fluorescence), a splicing factor present within IG clusters. The distinct localization of these proteins is confirmed by the absence of overlapping red and green fluorescence, which would produce a yellow signal. WT1(+KTS)-expressing cells contain a small number of yellow clusters associated with areas in which the distribution of this isoform is more diffuse. (Bar = 10 μm.)

***In Vivo* Physical Association, Sequestration, and Functional Inactivation of WT1(−KTS) by WT1-del Z.** The prominent speckling pattern observed with WT1-del Z made it possible to test whether the diffuse expression of wild-type WT1(−KTS) was affected by the presence of this mutant protein. Osteosarcoma cells containing inducible WT1-del Z were transfected with CMV-driven WT1(−KTS) tagged with the HA epitope. Transfected cells were then grown in the presence or absence of tetracycline and examined by indirect immunofluorescence by using the 12CA5 antibody directed against the HA epitope. In the presence of tetracycline, WT1(−KTS) was present in the expected diffuse pattern within the nucleus of transfected cells. However, withdrawal of tetracycline and induction of WT1-del Z expression resulted in the redistribution of WT1(−KTS), producing a prominent speckling pattern (Fig. 3*A*). Thus, coexpression of WT1(−KTS) and mutant WT1-del Z alters the physical localization of the wild-type protein, leading to its recruitment to a subnuclear compartment.

To determine whether this relocation of WT1(−KTS) alters its functional properties, cells containing inducible WT1-del Z were cotransfected with WT1(−KTS) and EGR1-CAT, a reporter construct containing the EGR1 promoter driving the CAT gene (3). This reporter is either activated or repressed by WT1(−KTS), depending upon the cellular context. Osteosarcoma cells lacking WT1-del Z expression showed 3-fold activation of EGR1-CAT by WT1(−KTS), consistent with our previous observations (4). However, induction of WT1-del Z expression 3 h before transfection of WT1(−KTS) completely abolished its ability to transactivate this target promoter (Fig. 3*B*). Our results are consistent with the observations of Reddy *et al.* (21), who reported that potential dominant-negative WT1 mutants abrogate transcriptional activation by WT1(−KTS), and they suggest that this effect may result from the physical sequestration of wild-type WT1.

To test whether WT1-del Z associates directly with HA-tagged WT1(−KTS), cells expressing both proteins were radiolabeled, and extracts were immunoprecipitated by using the 12CA5 antibody directed against the HA epitope. WT1-del Z coprecipitated with HA-tagged WT1(−KTS) in cells expressing both proteins (Fig. 3*A*). This protein–protein association was observed when using the stringent RIPA extraction buffer, and no WT1-del Z was precipitated in the absence of HA-WT1(−KTS), despite expression of high levels of WT1-del Z. The interaction between these two proteins appeared to be stoichiometric, with the amount of coprecipitated WT1-del Z equal to that of the directly immunoprecipitated HA-WT1(−KTS), although the high levels of WT1-del Z expression achieved when using the inducible promoter may favor dimerization (Fig. 3).

WT1 Domains Required for Dimerization in the Yeast Two-Hybrid System. To confirm the ability of WT1 protein to self-associate, WT1 homodimerization was tested in the yeast two-hybrid assay. A modified version of this assay was used, allowing estimation of the strength of interaction between hybrid proteins encoded by low copy number, centromeric plasmids (ref. 13 and M.V. and E. Harlow, unpublished data). The *HIS3* gene, inserted downstream of GAL4-binding sites, was used as reporter, allowing growth in the absence of histidine, which is inhibited by titratable concentrations of 3AT. Transfection of full-length WT1 fused to the GAL4 DB suppressed the background growth of colonies in the absence of histidine and the presence of 10mM 3AT, indicating that WT1 alone functions as a transcriptional repressor in yeast (data not shown). Cotransfection of full-length WT1 fused to

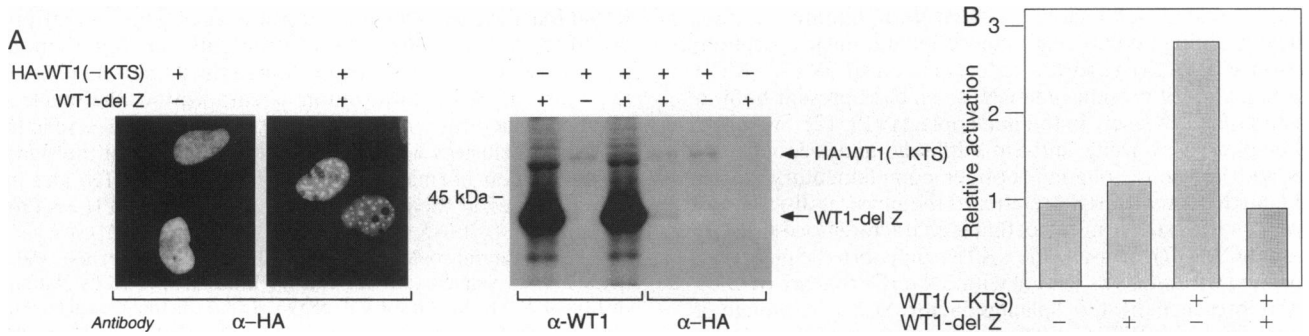

FIG. 3. Physical association of WT1(−KTS) and WT1-del Z. (*A Left*) WT1(−KTS) is recruited to subnuclear structures following coexpression of WT1-del Z. Osteosarcoma cells with inducible WT1-del Z expression were transiently transfected with HA-tagged WT1(−KTS) and then analyzed by immunofluorescence by using 12CA5 antibody directed against the HA epitope. The expression pattern of WT1(−KTS) is shown in the absence of WT1-del Z expression and following induction of WT1-del Z expression. Antibody 12CA5 does not recognize WT1-del Z (see below). (*Right*) Coimmunoprecipitation of WT1-del Z and WT1(−KTS). Radiolabeled extracts from the cells described above, with or without expression of WT1-del Z and HA-tagged WT1(−KTS) were immunoprecipitated with either antibody WTc8, directed against the N terminus of WT1, or antibody 12CA5, directed against the HA epitope. (*B*) Expression of WT1-del Z inhibits transactivation by WT1(−KTS). Relative CAT activity from the EGR1-CAT reporter construct containing the WT1-responsive sites within the native EGR1 promoter in the presence or absence of WT1(−KTS) or WT1-del Z.

the GAL4 AD led to enhanced growth, demonstrating homodimerization of WT1, which brings GAL4 AD in proximity to GAL4 DB and overrides the transcriptional repression by WT1 (Fig. 4). Growth of these transformants in the absence of histidine was inhibited above 30 mM 3AT, suggesting a relatively weak protein association within the yeast background. However, the yeast assay may underestimate the strength of WT1 homodimerization since it requires compensation for transcriptional repression by WT1 itself.

The yeast two-hybrid assay was used to define the domain of WT1 involved in homodimerization. Naturally occurring WT1 mutations and overlapping synthetic deletions were constructed in the WT1 component of the chimera containing GAL4 DB. Wild-type WT1 isoforms (WT1-A–WT1-D), containing combinations of alternative splices I and II (KTS) demonstrated dimerization in yeast, as did naturally occurring point mutations in exon 3 (WT1/201; ref. 22), exon 6 (WT1/273; ref. 23), and zinc finger 3 (WTAR; ref. 10) (Fig. 4). However, WT1 dimerization was abolished by disruption of either exon 1 or 2. Minimal deletions of aa 1–67 within exon 1 and aa 147-188 constituting exon 2 were sufficient to disrupt dimerization. The requirement for exon 2 is particularly interesting since an aberrantly spliced WT1 transcript with an in-frame deletion of this exon is found in ≈10% of Wilms tumor specimens, and it encodes a protein with altered transactivation properties (2). Thus, the WT1 domain required for homodimerization is contained within the extreme N terminus of WT1, a domain that has been implicated in transcriptional repression by WT1.

DISCUSSION

We have shown that wild-type WT1 is expressed both diffusely throughout the nucleus and within discrete subnuclear structures. The localization of WT1 is modulated by its alternative splicing, with insertion of three amino acids (KTS) between zinc fingers 3 and 4 sufficient to cause a shift from a diffuse to a predominantly speckled expression pattern. Truncated WT1 proteins with a disrupted zinc-finger domain show exclusive localization to subnuclear clusters. Their dimerization with wild-type WT1 is associated with the redistribution of WT1(−KTS) from a diffuse to a speckled pattern and its functional inactivation. This potential sequestration of wild-type WT1 protein in cells coexpressing a truncated WT1 mutant may underlie the dominant-negative effect of WT1 proteins with a disrupted DNA-binding domain.

The localization of transcription factors within defined subnuclear structures has been of considerable interest, although its functional implications are unknown. Our confocal microscopic analysis indicates that WT1 proteins are not colocalized with SC35, a splicing factor that identifies IGs (16, 17). The distinct localization of WT1 and spliceosomal components, despite a similarly speckled expression pattern, was most clearly demonstrated when using WT1-del Z and SC35, since the expression of both these proteins is restricted to

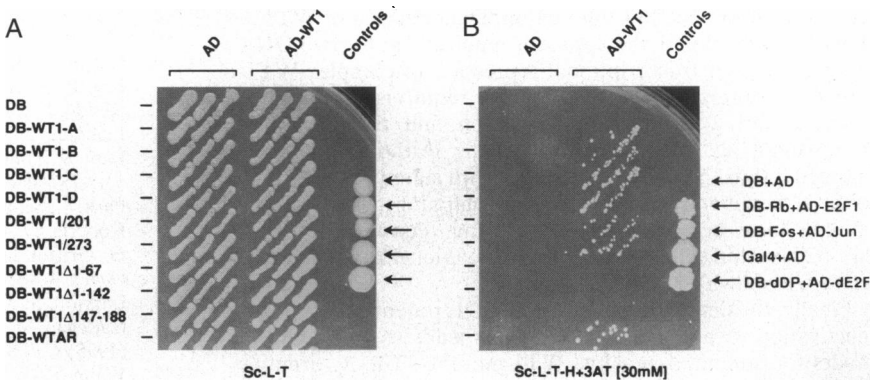

FIG. 4. Dimerization of WT1 in the yeast two-hybrid system. Transformants (strain MaV103; *GAL1::HIS3*) in synthetic complete medium lacking leucine and tryptophan (Sc-L-T) (*A*) and replica-plated cells on plates containing the *His3* competitive inhibitor 3AT (Sc-L-T-H+3AT [30mM]) (*B*). Three individual transformants were tested with combinations of plasmids encoding GAL4 AD, alone or fused with full-length WT1 (AD-WT1), and GAL4 DB, alone or fused with WT1 constructs (DB-WT1). WT1 constructs are wild-type isoforms [WT1-A, lacking both alternative splices; WT1-B, encoding splice I, lacking splice II (KTS); WT1-C, lacking splice I, encoding splice II (KTS); and WT1-D, encoding both alternative splices], naturally occurring point mutations (WT1/201 and WT1/273), overlapping deletions within exon 1 (WT1Δ1–67 and WT1Δ1–143), a naturally occurring in-frame deletion of exon 2 WT1Δ147–188, and a naturally occurring in-frame deletion of zinc finger 3 (WTAR). As controls, five patches represent transformation with separate DB plus AD, intact GAL4 plus AD plasmid, and DB plus AD together with three known protein partners: Rb plus E2F1, Fos plus Jun, and *Drosophila* homologue of DP (dDP) plus E2F. The plates were incubated at 30°C for 6 days.

well-demarcated subnuclear clusters, with minimal diffuse nuclear staining. While this manuscript was in preparation, Larsson *et al.* (24) reported colocalization of WT1(+KTS) with Sm, a motif present in snRNPs, which is present both in IGs and more diffusely in the nucleoplasm (15, 17). WT1 was coprecipitated by using anti-Sm antibody, and treatment of cells with antisense oligonucleotides complementary to U1 and U6 RNA or with RNAse altered the physical distribution of WT1(+KTS) (24), an effect that is characteristic of snRNPs but not SC35 (17). Thus, WT1(+KTS) appears to be physically associated with snRNPs but not within the IGs that are defined by the presence of the splicing factor SC35. Although a potential role for WT1(+KTS) in some aspect of pre-mRNA splicing is possible, the different localization of WT1(+KTS) and an essential splicing factor leads us to suggest an alternative explanation—i.e., that subnuclear clusters may represent a storage site for WT1 isoforms and mutants with reduced DNA-binding affinity.

Our observations using deletion mutants of WT1 suggest that the speckled distribution of WT1(+KTS) is unlikely to result from novel RNA binding affinity conferred by the KTS alternative splice, but rather from a loss of function, such as DNA-binding activity. Localization to subnuclear structures requires the N terminus of WT1 and is enhanced in mutants containing disruptions of the C-terminal DNA-binding domain. Thus, the diffuse localization of WT1(−KTS) suggests that presence of the uninterrupted zinc fingers 1–4 overrides association with subnuclear clusters, while subtle modifications in the zinc-finger domain [WT1(+KTS) and WTAR] demonstrate a mixed diffuse and speckled distribution, and major zinc-finger deletions (WT1-del 1-2, WT1-del 3-4, and WT1-del Z) show an exclusively speckled pattern. The possibility that subnuclear clusters constitute a transcriptionally inactive, potentially sequestered pool of WT1 is supported by the loss of WT1(−KTS) transactivational activity following its recruitment to these structures by coexpression of a WT1 truncation mutant (Fig. 3). This relocation of WT1(−KTS) is explained by our observation that WT1 can dimerize *in vivo*, and it suggests an intriguing mechanism whereby the amount of WT1(−KTS) transactivational activity may be titrated by its physical association with either WT1(+KTS) or WT1 mutants.

Self-association of WT1 has recently been observed *in vitro* and in the yeast two-hybrid assay (21). Our use of a modified assay using low copy number centromeric plasmids and a titratable growth inhibitor demonstrates that this is a relatively weak protein–protein interaction in yeast. The strength of the interaction in yeast was not increased by deletion of the C terminus of WT1, as in WT1-del Z. In contrast, the interaction of WT1 with WT1-del Z in mammalian cells appears to be stoichiometric and resistant to stringent extraction buffer, suggesting that protein modification or additional interacting factors may stabilize this interaction. Dimerization of WT1 *in vivo* is consistent with the apparent requirement for two DNA binding sites for transcriptional repression of complex WT1-target promoters (4, 25) and with the requirement for WT1 exons 1 and 2 for both dimerization and transcriptional repression (Fig. 4 and refs. 2 and 25). Although we have mapped a third function, association with subnuclear clusters, to the N terminus of WT1, attempts at mapping a more specific domain have been complicated by the requirement of aa 267–326 (within exons 6 and 7) for nuclear localization of WT1 (data not shown).

Finally, the dimerization of WT1 and its potential functional inactivation within subnuclear clusters leads us to propose a model for dominant-negative WT1 mutants. The N terminus of WT1 is the minimal domain required in dominant-negative mutants associated with DDS (9). Like WT1-del Z, these mutants may therefore bind wild-type WT1(−KTS), resulting in its redistribution to subnuclear structures and in its functional inactivation. This mechanism is analogous to that proposed for dominant-negative p53 mutants; but while mutant p53 may sequester wild-type protein in the cytoplasm (26, 27), the shift in WT1 localization occurs within the nucleus. Further structural characterization of these WT1-associated subnuclear clusters will be required to understand the significance of their normal association with WT1(+KTS) and the consequences of their forced interaction with WT1(−KTS).

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