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Tumor-associated WT1 Missense Mutants Indicate That Transcriptional Activation by WT1 Is Critical for Growth Control*

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The *WT1* gene encodes a zinc finger DNA binding transcription factor and is mutated in up to 15% of Wilms tumor cases. The WT1 protein binds to the promoters of many genes through GC- or TC-rich sequences and can function both as a transcriptional repressor and an activator in co-transfection assays depending on the cell type, the structure of the test promoter, and even the expression vectors used. Engineered expression of WT1 can lead to growth suppression by both cell cycle arrest and induction of apoptosis. However, the transcriptional activity of WT1 that is required for growth control was not defined. We found that three N-terminal tumor-associated missense mutations of WT1 were defective for activation of both a synthetic reporter containing WT1-binding sites as well as the promoter of a WT1 responsive gene, p21. These mutants failed to inhibit cell growth but still retain their ability to repress several putative WT1 target promoters. These results indicate that activation and not repression by WT1 is the critical transcriptional activity of the protein responsible for its growth suppressing properties.

Wilms tumor, an embryonic malignancy of the kidney, affects approximately 1 in 10,000 live births making it the most common solid tumor of children (1). Histologically, this tumor develops from malignant transformation of renal stem cells, metanephric blastema, that persist in the developing fetal kidney where they may appear as undifferentiated metanephric mesenchyme cell, stromal cells, or epithelial cells (2). The association of Wilms tumor with the WAGR (Wilms Aniridia Genitourinary malformation, and mental Retardation) syndrome with concomitant constitutional deletions of chromosome 11p13 led to positional cloning of the *WT1* gene (3, 4). WT1 is critical for normal renal development. It is expressed in the condensing mesenchyme, renal vesicle, and glomerular epithelium of the developing kidney. Furthermore, mice homozygous for disruption of *wt1* die before birth with complete agenesis of the kidney (reviewed in Refs. 5–8).

WT1 encodes a zinc finger transcription factor and undergoes alternative splicing at two positions yielding four isoforms (9). WT1-A refers to an isoform that excludes all alternative splices while WT1-B is spliced to include exon 5, encoding a 17-amino acid sequence immediately N-terminal to the zinc finger domain. WT1-C contains a 3-amino acid insertion (KTS) between zinc fingers three and four and WT1-D contains both the 17- and the 3-amino acid insertions (10). Zinc fingers 2–4 of WT1 are 67% identical to the EGR-1[1] protein and both proteins can bind to a GC-rich sequence and affect transcription (9, 11, 12). WT1 can also bind to alternate sequences including TC-rich sequences (13–16) and was shown to activate or repress transcription of many genes whose promoter contain these sequences. These include early growth response 1 (EGR-1) (9, 12) insulin like-growth factor 2 (IGF-II) (17), platelet-derived growth factor A chain (18, 19), insulin-like growth factor 1 receptor (IGF-IR) (20, 21), epidermal growth factor receptor (EGFR) (22), WT1 (23, 24), and others (25–34).

Mutations in *WT1* occur in about 15% of Wilms tumor cases (reviewed in Refs. 5 and 35–37). Most WT1 point mutations are localized to the zinc finger region and are predicted to produce proteins incapable of binding to DNA (38–44). These are null mutants when present in the homozygous or hemizygous state. When present in the heterozygous state, these mutations can function as dominant negative alleles, inhibiting the function of the wild-type protein (5, 45–47). Point mutations of WT1 can also occur in the N-terminal effector regions of the protein. Two such mutations, F112Y and P129L, were isolated from kidney tumors of newborn rats treated with *N*-nitroso-*N*′-methylurea (NMU) (48). These animals developed tumors that were histopathologically very similar to human Wilms tumors suggesting the involvement of the *wt1* gene. The codon 112 mutation was found in four rat nephroblastomas where in each case, the wild-type *wt1* allele was lost. The codon 129 mutation was found in a mesenchymal rat kidney tumor although in this case, the wild-type allele was still present (48). Park *et al.* (49) also described a WT1 mutation outside of the zinc finger domain. This mutation, F154S, was identified in nephrogenic rest from two patients with Wilms tumor and was not further characterized until now.

In this study, we used these three WT1 tumor-associated mutations, WT1-A(F112Y), WT1-A(P129L), and WT1-A(F154S), as genetic tools to further gain insight into the molecular action of WT1 and its involvement in Wilms tumor. We found that all three mutants were defective for WT1-mediated transcriptional activation and were also unable to suppress growth in colony formation assays. Surprisingly, all these mutants were still competent for transcriptional repression of reporter genes in co-transfection assays, suggesting that the transcriptional activation function of WT1, and not transcriptional repression, is most critical for its ability to suppress growth.

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[1] The abbreviations used are: EGR-1, early growth response-1; IGF, insulin growth factor; EGFR, epidermal growth factor receptor; PCR, polymerase chain reaction; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; CDK, cyclin-dependent kinase; NMU, *N*-nitroso-*N*′-methylurea.

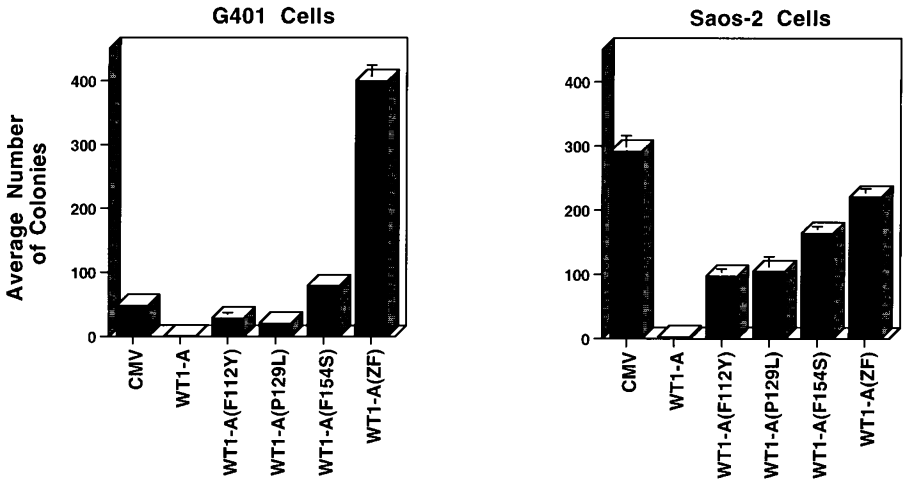

FIG. 1. **Mutant WT1-A proteins are defective for growth suppression.** G401 and Saos-2 cells were transfected with 20 μg of the CMV-murine (*m*) WT1-A expression vectors and selected in media containing 0.5 mg/ml G418 for 2 to 3 weeks. Shown are the average number of G418-resistant colonies for each quadruplicate set of plates (± S.D.) for each vector tested.

MATERIALS AND METHODS

Transfection Assay—NIH-3T3 cells, grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1 × penicillin/streptomycin, plated at a density of 2×10^5 (6-well dishes) or 0.9×10^5 (12-well dishes), were transfected utilizing SuperFect Transfection Reagent (Qiagen) according to the manufacturer's directions. The amount of reporter and WT1 expression vectors used in each transfection are indicated in the figure legends. The cells were harvested 48 h after transfection and assayed for CAT activity using an enzyme-linked immunosorbent assay (Roche Molecular Biochemicals) according to the manufacturer's directions. Luciferase activity was measured using the Dual-Reporter Assay System (Promega).

Immunoblotting—293T cells, plated at a density of 4×10^6 in 10-cm dishes, were transfected with 20 μg of WT1-A expression vectors by calcium phosphate method as described previously (50). Western blots were performed using 0.1 μg/ml rabbit polyclonal anti-WT1 C19 antibody (Santa Cruz Biotechnology) as described (51).

Immunofluorescence—To detect WT1-A proteins by immunofluorescence, 2×10^5 NIH-3T3 cells plated in 35-mm dishes were transfected by SuperFect reagent as above. WT1 expressing cells were detected using 0.1 μg/ml anti-WT1 C19 antibody diluted in a 3% bovine serum albumin/phosphate-buffered saline solution as described previously (50).

Colony Suppression Assay—G401 and Saos-2 cells, grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were plated in 10-cm dishes at a density of 1×10^6/ml and transfected in duplicate with 20 μg of WT1-A expression vectors or empty expression vector by calcium phosphate precipitation. Cells were incubated with the DNA-calcium phosphate complex for 16–18 h, washed twice with phosphate-buffered saline, and refed with fresh media for an additional 12 h. 72 h after transfection, duplicate plates of cells were washed with phosphate-buffered saline, trypsinized, pooled and diluted 1:11 into media containing 0.5 mg/ml G418. Five plates for each duplicate pair of transfectant were cultured for 2–3 weeks under selection. The cells were stained with Giemsa dye and the number of G418-resistant colonies were counted.

Plasmid Construction—The RSV-WT1-A expression vector (45), murine p21-CAT (52) (gift of B. Vogelstein), human WT1-Luc (53) (gift of M. Eccles), hIGF-II-(17) (gift of I. Drummond), mEGR1-CAT (12), and the Zif_3tk-CAT (45) constructs were previously described. The G_5tk-Luc reporter construct was generously provided by Peter Traber, University of Pennsylvania. WT1-A mutants were constructed by mutagenic PCR using wild-type and mutant WT1 oligonucleotides and RSV-WT1-A as a DNA template (54). The top strand of the 112 mutant was made by PCR using the 5′ wild-type primer (O195), 5′-CGCGGATCCGAGACAGTGCCTGAGCGCCTT-3′ and the 3′ mutant primer (O191), 5′-GGGAGGACCGTAGGGTCCGTA-3′. This generated a 90-base pair fragment. The bottom strand was made with the 5′ mutant primer (O190) 5′-TACGGACCCTACGGTCCTCCC-3′ and the 3′ wild-type primer (O127) 5′-GTCGACGTCGACTTATCC*ACTCTCGTACCCTATAC*-3′. This generated a 474-base pair fragment that was combined with the 90-base pair product of the first PCR reaction and re-amplified using wild-type primers O195 and O127 to produce a DNA fragment encoding amino acids 86–256. The 129 mutant was made in a similar way. The top strand was made using the 5′ wild-type primer O195 and the 3′ mutant primer (O282) 5′-TAGGGCGCATTGAGGAACATCCT-3′ while the bottom strand was made with the 5′ mutant primer (O296) 5′-AGGATGTTCCTCAATGCGCCCTA-3′ and the 3′ wild-type primer O127. The products of the PCR reactions containing both the 112 and 129 mutations were digested with *Age*I/*Nco*I and inserted into pSP64-WT1-A digested with *Age*I/*Nco*I. The top strand of the 154 mutation was made in a reaction with the wild-type 5′ primer 5′-GGCCATTCACCGGACA-3′ and a mutant 3′ primer 5′-GCCCCGTCG*G*AAGTGACCGT-3′. The second PCR reaction was performed with a mutant 5′ primer 5′-ACGGTCACTTCCGACGGGGC-3′ and a wild-type 3′ primer 5′-GAATTCGAATTCTCAAAGCGCCACGTGGGAGTTT-3′. The products from these reactions were then re-amplified with wild-type primers to produce a DNA fragment encoding amino acids 100–429 of WT1-A containing the codon 154 mutation. This fragment was digested with *Age*I/*Eco*RI and inserted into *Age*I/*Eco*RI digested pSP64-WT1-A to produce the full-length WT1 coding region containing the 154 mutation. To construct RSV-WT1-A mutants, the appropriate pSP64-WT1-A mutants were digested to release the entire WT1-A coding region and inserted into an expression vector containing the Rous sarcoma virus (RSV) long terminal repeat. All WT1-A mutations were confirmed by sequencing with an internal primer (O138) 5′-GGCCAGTTCACCGGTACA-3′ using dideoxy sequencing method and Sequenase version 2.0 (U. S. Biochemical Corp.).

RESULTS

Biological Activity of WT1-A Missense Mutations—Previous studies have shown that WT1 can suppress growth when expressed in RM1 cells (a Wilms tumor cell line) (55), Saos-2 cells (22), and *ras* transformed NIH-3T3 cells (56). Therefore, to determine the biological activity of the WT1-A mutants, we performed colony suppression assays in both G401 (an embryonic kidney tumor cell line) and Saos-2 cells (osteosarcoma cells), both of which do not express any detectable WT1 protein. As expected, wild-type WT1-A inhibited growth in both cell lines, suppressing colony formation up to 100-fold when compared with the empty WT1 expression vector. In contrast, all three WT1-A mutants only suppress growth 2–3-fold (Fig. 1). To confirm that the growth suppressive properties of WT1 was specifically due to the effector domain of the protein and not the zinc finger domain, we also performed colony suppression assay with a construct expressing only the zinc finger region of WT1. Fig. 1 also shows that the zinc finger domain of WT1-A was not sufficient for growth suppression. In fact, cells transfected with an expression vector for the zinc finger domain promoted growth especially in G401 cells where there were at least 10 times more colonies when compared with the empty expression vector (Fig. 1). From this assay, we can conclude that these WT1-A tumor-associated mutations are functionally deficient for at least one WT1 biological activity. These results also confirm that the role of WT1 in growth control is a critical point in tumorigenesis.

WT1-A Mutants Are Competent for Transcriptional Repres-

sion—Like other transcription factors, WT1 has been shown to be a bifunctional transcription factor capable of both activating and repressing target genes. The initial characterization of WT1 showed that it was a transcriptional repressor whose potential target genes including many growth-related genes. To investigate the repressive function of both wild-type and mutant WT1-A proteins, we determined their effect on natural WT1 responsive promoters. We utilized a WT1 expression vector containing the CMV promoter as we and others showed that it facilitates the ability of WT1 to act as a repressor (51). We first examined the effect of WT1-A proteins on the human WT1 promoter. Fig. 2*A* shows that CMV-WT1-A repressed transcription of this promoter by nearly 90% of control levels. The codon 112 and 129 CMV-WT1-A mutants repressed this promoter to comparable levels while CMV-WT1-A(F154S) repressed it by as much as 98% being an even stronger repressor. We next examined the effect of WT1 proteins on the EGR-1 promoter, the first gene whose expression was shown to be down-regulated by WT1. As shown in Fig. 2*B*, all three WT1-A mutant proteins repressed this promoter to similar levels when compared with wild-type protein. Similarly, both wild-type and mutant WT1-A proteins repressed the human IGF-II P3 promoter to equal levels (Fig. 2*C*). Last, we determined the effect of WT1-A proteins on the EGFR promoter, another gene whose transcription is down-regulated by WT1 expression (22, 57). As with the other promoters tested, the EGFR promoter was similarly repressed by all WT1-A proteins (data not shown). As a further test of transcriptional repression, all three mutations were engineered into the N-terminal portion of WT1-A (amino acids 1–182), fused to the DNA-binding domain of Gal4, and examined for their ability to affect transcription of a reporter gene containing Gal4-binding sites. In this assay, all the fusion proteins were able to repress transcription as well as the wild-type fusion protein (data not shown). Taken together, these data indicate that all three WT1-A mutants are still competent for transcriptional repression. They also suggest that the repression function of WT1 is not the critical activity of the protein responsible for its growth suppressive properties.

WT1-A Mutations Fail to Activate Transcription of an Artificial Reporter Gene—As noted, WT1 is a bifunctional transcription factor capable of both repressing and activating gene transcription. We previously showed that WT1-A can activate a reporter gene containing upstream WT1/EGR-1-binding sites (45). We therefore tested the ability of the WT1 mutants to activate this reporter in 3T3 cells. As demonstrated in Fig. 3, wild-type WT1-A activated this reporter 7-fold while all three mutants, (WT1-A(F112Y), WT1-A(P129L), and WT1-A(F-154S)) activated this reporter at most 2-fold. These results were seen in multiple experiments as well as in 293T cells (data not shown).

Expression and Cellular Localization of Wild-type and Mutant WT1-A Proteins—To be assured that all of the mutant proteins were produced to similar levels in the cells, immunoblot analysis on extracts from transfected cells was performed. Fig. 4 shows that all three mutant proteins were expressed to comparable levels. Interestingly, the wild-type protein was expressed to a lesser level compared with all three mutant proteins (Fig. 4). To examine the cellular localization of the WT1-A proteins, we performed indirect immunofluorescence on transiently transfected cells. Our data shows that the mutant WT1-A proteins were correctly localized to the cell nucleus similar to the wild-type protein (Fig. 5). These results thus confirmed that the transcriptional difference seen between the wild-type and mutant WT1-A proteins were in fact due to a difference in their innate transcriptional activity and not to a lack of expression or to incorrect cellular localization.

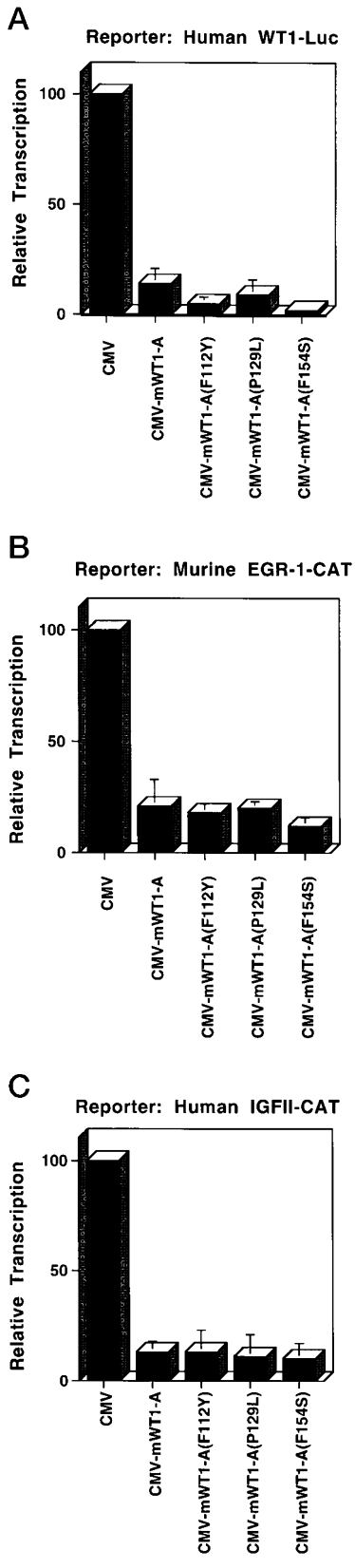

FIG. 2. **All WT1-A mutant proteins can still repress transcription.** *A,* 3T3 cells were co-transfected with a 0.2 μg of human WT1 promoter luciferase reporter, 5 ng of tk-renilla, and 2.0 μg of empty CMV, WT1-A, and mutant WT1-A expression vectors. *B,* 293T cells were co-transfected with 0.2 μg of murine EGR-I promoter-CAT construct. *C,* 0.4 μg of hIGF-II P3 promoter-CAT construct, 5 ng of tk-renilla, and 2.0 μg of empty CMV, WT1-A, and mutant WT1-A expression vectors. Following luciferase assay, the averages (± S.D. of three independent experiments are shown.

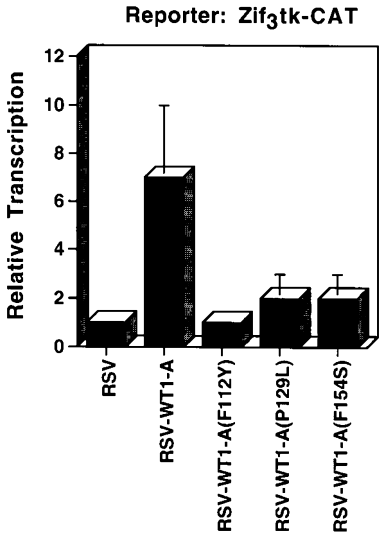

FIG. 3. **WT1-A mutants are deficient in activating the Zif$_3$tk-CAT reporter.** NIH-3T3 cells were transfected with 0.4 μg of Zif$_3$tk-CAT reporter vector, 0.1 μg of tk-GH as an internal control, and 2.0 μg of wild-type of mutant RSV-WT1-A expression vectors. At 48 h after transfection, the cells were harvested and assayed for CAT activity. The data represent the average (±S.D.) of eight independent experiments.

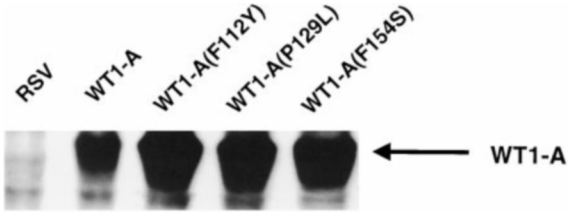

FIG. 4. **Expression of mutant WT1-A proteins.** 293T cells were transfected with 20 μg of RSV-WT1-A expression vectors by the calcium phosphate method. Forty-eight hours after transfection, cells were harvested and protein concentration was determined using Bio-Rad protein reagent. Whole cell extracts (100 μg) were then separated on a 10% SDS-polyacrylamide electrophoresis gel, transferred to nylon membrane, and probed with anti-WT1 C19 antibody.

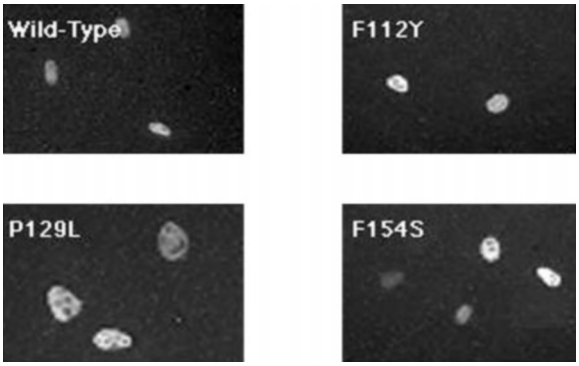

FIG. 5. **Cellular localization of WT1-A proteins.** 3T3 cells were transfected with 2.0 μg of empty RSV expression vector, wild-type, or mutant RSV-WT1-A expression vectors and immunostained for WT1 using the C19 antibody.

WT1-A Mutant Proteins Are Unable to Activate the Murine p21$^{WAF1/CIP1}$ Promoter—Cell growth is regulated at many different points within the cell cycle, one being regulation of the cyclin-dependent kinase (CDK) inhibitor p21$^{WAF1/CIP1}$. Recently, Englert *et al.* (58) showed that induction of WT1-A led to up-regulation of endogenous p21$^{WAF1/CIP1}$ in an inducible Saos-2 cell line (58) and stimulated the p21 promoter in co-transfection assays. We therefore speculated that these mutant WT1-A proteins may be defective for WT1 mediated activation

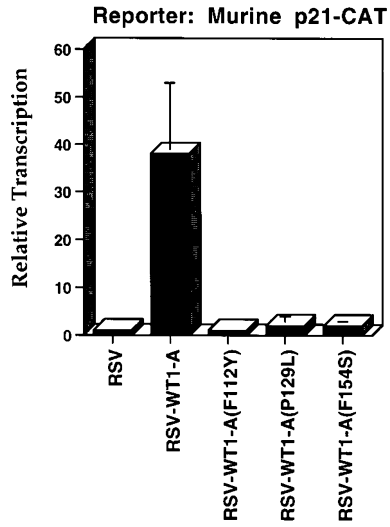

FIG. 6. **Mutant WT1-A proteins cannot activate the murine p21 promoter.** 3T3 cells were co-transfected with 50 ng of murine p21 promoter-CAT reporter vector, 0.1 μg of tk-GH, and 2.0 μg of wild-type or mutant RSV-WT1-A expression vectors. CAT activity was measured and the results of three independent experiments are shown.

of the p21 promoter. To investigate this possibility, we co-transfected NIH-3T3 cells with a full-length murine p21 promoter-CAT reporter construct along with either wild-type or mutant RSV-WT1-A expression vectors. As shown in Fig. 6, wild-type WT1-A was able to activate this promoter by a factor of nearly 40 while all three mutants could only activate it up to 2-fold. This result is consistent with a model in which these tumor-derived WT1-A mutations may have led to tumor formation due to their inability to up-regulate the CDK inhibitor, p21$^{WAF1/CIP1}$, and control cell cycle progression.

DISCUSSION

The ability of WT1 to both activate and repress transcription has made it difficult to decipher the molecular mechanism by which it functions as a tumor suppressor gene and to understand what are the WT1 target genes relevant to this process. Here, we described three tumor-derived WT1 mutations and their use as a genetic tool to understand the critical transcriptional function of WT1. We showed that, unlike the wild-type protein: 1) mutant WT1-A proteins were unable to activate transcription of WT1 responsive promoters. 2) Mutant WT1-A proteins were unable to suppress growth in colony forming assay. 3) Mutant WT1-A proteins, like the wild-type protein, were still competent for transcriptional repression of putative WT1 target promoters.

When initially identified, WT1 was shown to be a transcriptional repressor. Since then, our group as well as others showed that WT1 can also function as a transcriptional activator (19, 51). Interestingly, almost all of the studies that showed WT1 to act as a repressor of transcription used a CMV expression vector. We demonstrated that the empty CMV expression vector alone was capable of strongly repressing basal transcription of target promoters suggesting that this potent promoter may be sequestering *trans*-activating factors from the transcription machinery (51). This indicated that the choice of expression vector used would determine whether WT1 would act as a repressor or an activator in *in vitro* assays and called into question the notion that WT1 acted as a pure repressor.

The use of an inducible or stable systems for WT1 expression have, in some cases, suggested that down-regulation of gene expression by WT1 is related to its function as a growth suppressor. Werner *et al.* (59) showed that in G401 cells, expression of WT1-A led to decreased levels of IGF-1R protein and

reduced cellular proliferation. WT1 (expressed from the CMV promoter) was also capable of repressing a reporter gene containing IGF-1R promoter sequences, suggesting that the growth suppressive nature of WT1 may be due, at least in part, to the repression of IGF-1R (59). Englert *et al.* (22) and Menke *et al.* (57) showed that induction of WT1-A in Saos-2, Hep3B, and HepG2 cells led to p53 independent apoptosis. Apoptosis was associated with reduced expression of the EGFR protein and was partially rescued by constitutive expression of EGFR but not IGF-1R. Of note, EGFR mRNA levels were not examined in this study. However, a recent study showed that, when expressed in an inducible manner in HEK293 cells, WT1 did not affect the mRNA levels of a number of putative WT1 target genes including the EGFR (60). Given the recent observations that WT1 can bind RNA (61) and associate with splicing factors (62, 63) it is possible that WT1 down-regulates the expression of EGFR and some other genes at the post-transcriptional level.

Recent studies have highlighted the ability of WT1 to activate transcription of target genes. One group found that induction of WT1 expression was associated with up-regulation of the cell cycle inhibitor p21 (58). Furthermore, in transient transfection experiments, WT1 activated the p21 promoter. Another group created a 3T3 cell line which inducibly expressed WT1 and found that WT1 expression was correlated with activation of the endogenous syndecan gene, a marker of renal differentiation. Co-transfection experiments showed that WT1 activated the syndecan promoter (32). We found that stable expression of WT1 in 3T3 cells leads to the acquisition of a partial epithelial phenotype and up-regulation of a number of markers of renal differentiation (64), some of which contain WT1-binding sites in their promoters and are activated by co-transfected WT1.[2]

Previous analyses of tumor-derived WT1 mutants have indicated that single amino acid changes can alter the transcriptional function of the protein. Two such mutations (S273G and G201D) were described by Park *et al*. (49, 65). Utilizing the CMV promoter in co-transfection assays, this group found that these mutant WT1-A proteins activated rather than repressed expression of the EGR-1 reporter gene. However, there was no correlation of transcription with a functional growth suppression assay. It is possible that these proteins, when expressed from the RSV promoter may also have altered transcriptional activation function. Nevertheless, this information suggests that an imbalance in WT1 transcriptional function may be sufficient to contribute to tumor development. Our study confirms that missense WT1 mutations in the effector region of the protein can alter its transcriptional activity. Furthermore, we showed a close correlation between the inability of three WT1 mutants to activate transcription and their inability to suppress cell growth. This information also suggests that WT1 functions in large part as an activator of genes that in turn suppress cell growth.

The exact identity of the genes responsible for the ability of WT1 to suppress cell growth is not completely known but the $p21^{WAF1/CIP1}$ gene is an excellent candidate. Kudoh *et al.* (66) showed that micro-injection of WT1 inhibited cell cycle progression in serum-starved and released NIH 3T3 cells, an effect that was mapped mid to late G_1 phase. Cell cycle arrest was associated with down-regulation of both CDK2 and CDK4 kinase activities (66) and was completely abrogated by co-injection of CDK4 and cyclin D1 or CDK2 and cyclin E cDNAs. This is consistent with induction of the p21 CDK inhibitor. Englert *et al.* (58) showed that induction of WT1 led to up-regulation of p21 protein, and found that WT1 could activate the p21 promoter. This group later found that an N-terminal deletion of WT1 [WT1 Δ6–180] was unable to suppress growth and could not activate the p21 promoter but was still competent for transcriptional repression of both the EGFR and EGR promoter (67). These results are further supported by our the observation that the three tumor-associated missense WT1 mutants localized in the same N-terminal region of WT1 we analyzed were unable to up-regulate the expression of p21 promoter in co-transfection studies. Taken together, these results indicate that activation of p21 by WT1 is important for the growth suppressive properties of WT1. Our data are consistent with the notion that it is the ability of WT1 to activate and not repress transcription that is most critical for the role of the protein in growth control. However, it remains likely that other WT1 target genes required for growth suppression have not yet been identified. The mutant WT1 proteins may still be able to regulate the expression of some of these genes to the same degree as the wild-type protein. Alternatively, the mutant WT1 proteins might also be defective for transcriptional repression of WT1 genes important for growth suppression that were not examined in our study. Nevertheless, these mutants represent important tools that can be used to further understand the transcriptional function of WT1 and its involvement in Wilms tumor.

The three WT1 mutations characterized here are located in what was previously identified by Gal4 fusion assay as the repression domain of WT1 (85–179) (68). However, we found that these mutants did not affect the ability of WT1 to repress transcription either in its native conformation or as a Gal4-fusion protein. This suggests that there may be overlapping but functionally distinct domains in the N-terminal domain of WT1 (amino acids 1–180) that interact with transcriptional co-activators and co-repressors. All three mutations may significantly alter the secondary or tertiary structure of WT1 and thus their interaction with partner proteins. Indeed, the mutations F112Y and F154S introduce a potential phosphorylation site into the protein while P129L removes a potential helix breaking residue.

The identity of putative WT1 co-activators are not yet know. p53 (69), Par-4 (70), and Ciao-1 (71) were all identified as WT1-binding proteins that affect its transcriptional function through interaction with the zinc finger domains of WT1. Hence these proteins are unlikely to be co-factors for WT1-mediated activation whose interaction is disrupted by the N-terminal mutations. The only identified proteins interacting with the N terminus of WT1 to date are human ubiquitin conjugating enzyme (hUBC9) (68, 72) and Hsp-70 (67). Hsp70 binds to the N-terminal 180 amino acids of WT1. WT1/Hsp-70 interaction is required for WT1 mediated growth inhibition and activation of the p21 promoter. We are currently determining if the three WT1 missense mutations we characterized are defective for this interaction.

In summary, the results presented here are consistent with a model in which the mutant WT1 proteins are defective for transcriptional activation because of failure to interact with some critical component(s) of the transcriptional machinery. Moreover, we found that transcriptional activation by WT1 is most closely correlated with its ability to function as a growth suppressor. This suggests that loss of WT1 function in Wilms tumors leads to tumor development due to insufficient activation of genes involved in cell cycle control, differentiation, and apoptosis.

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[2] S. Hosono and J. D. Licht, unpublished observations.

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