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Wilms Tumor and the WT1 Gene

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Wilms tumor or nephroblastoma is a pediatric kidney cancer arising from pluripotent embryonic renal precursors. Multiple genetic loci have been linked to Wilms tumorigenesis; positional cloning strategies have led to the identification of the *WT1* tumor suppressor gene at chromosome 11p13. *WT1* encodes a zinc finger transcription factor that is inactivated in the germline of children with genetic predisposition to Wilms tumor and in a subset of sporadic cancers. When present in the germline, specific heterozygous dominant-negative mutations are associated with severe abnormalities of renal and sexual differentiation, pointing to the essential role of *WT1* for normal genitourinary development. The role of this tumor suppressor in normal organ-specific differentiation is also supported by the highly restricted temporal and spatial expression of *WT1* in glomerular precursors of the developing kidney and by the failure of kidney development in *wt1*-null mice. Of two major alternative splicing products encoded by *WT1*, the (−KTS) isoform appears to mediate transcriptional activation of genes implicated in cellular differentiation, possibly also repressing proliferation-associated genes. The (+KTS) isoform, whose DNA-binding domain is disrupted by the insertion of three amino acids, may be involved in some aspect of mRNA processing. In addition to its function in genitourinary development, a role for *WT1* in hematopoiesis is suggested by its aberrant expression and/or mutation in a subset of acute human leukemias. *WT1* is also expressed in mesothelial cells; a specific oncogenic chromosomal translocation fusing the N-terminal domain of the Ewing sarcoma gene *EWS* to the three C-terminal zinc fingers of *WT1* underlies desmoplastic small round cell tumor, an abdominal tumor thought to arise from the peritoneal lining. Understanding the distinct functional properties of WT1 isoforms and tumor-associated variants will provide unique insight into the link between normal organ-specific differentiation and malignancy.

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GENETICS OF WILMS TUMOR

Sporadic Wilms Tumor

Wilms tumor, or nephroblastoma, is a pediatric kidney cancer thought to originate from pluripotent embryonic renal precusors [1]. It arises in 1/10,000 children, usually around age 5, although children with genetic predisposition may develop bilateral Wilms tumors by age 2. In very rare cases, Wilms tumor may also occur in adults, presumably arising from persistent embryonic rests. Wilms tumor itself is composed of distinct histological elements, and is often described as "triphasic," reflecting the presence of epithelial and stromal components, as well as "blastemal" or undifferentiated mesenchymal cells [1]. In some tumors, further differentiation along myogenic or neural cell lineages is evident, further supporting the derivation of Wilms tumor from transformed pluripotent cells [2]. The clinical treatment of Wilms tumor involves surgical resection of the affected kidney, which may be followed by chemotherapy and, in cases with a poor prognosis, radiation therapy. With modern therapeutic approaches, cure is achieved in ~80% of cases [3]. However, so-called anaplastic Wilms tumor remains refractory to treatment, a characteristic that has been linked to the frequency of p53 mutations in this rare variant [4]. The genetic abnormalities underlying most cases of Wilms tumor remain unknown, although three genes have been implicated in a subset of tumors. *WT1*, the subject of this review, is inactivated in ~15% of sporadic Wilms tumors (see below). *Insulin-like growth factor 2 (IGF2)*, a gene that is normally imprinted and expressed only from the paternal allele, may show "relaxation of imprinting" and hence increased dosage resulting from biallelic expression (see below). Finally, constitutive activation of β-catenin, a component of the Wnt pathway implicated in renal differentiation, is observed in ~15% of tumors [5]. The relationship of these mutations to each other and potentially to distinct histological subtypes of Wilms tumor is unknown. In addition to the *WT1* locus at chromosome 11p13 [6–9] and the imprinted locus which is telomeric to it at 11p15 [10, 11], loss of heterozygosity (LOH) in sporadic Wilms tumors has also been demonstrated at chromosomes 1p, 16q, and 7p, pointing to the presence of

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TABLE 1

Human Syndromes Associated with Predisposition to Wilms Tumor

Syndrome	Clinical manifestations	Germline abnormality
WAGR	Wilms tumor, aniridia, genitourinary defects, mental retardation	Interstitial deletion at 11p13 (including *WT1, PAX6*)
Beckwith–Wiedemann	Organomegaly, hypoglycemia, umbilical hernia, predisposition to adrenal cortex carcinoma, hepatoblastoma, Wilms tumor	Duplication of paternal 11p15: uniparental isodisomy (Increased IGF2 expression) Inactivation of $p57^{KIP2}$
Denys–Drash	Wilms tumor, genitourinary defects, renal mesangial sclerosis, intersex disorder	Missense mutations of WT1 (dominant negative) (e.g., Arg to Trp at codon 394)
Frasier	Genitourinary defects, focal glomerular sclerosis, intersex disorder (no Wilms tumor)	KTS splice donor site [reduction in WT1(+KTS)] (e.g., position +2 in intron 9) TG → T**C**

additional tumor suppressors that have not been identified [12–14]. Cases of Wilms tumor showing LOH at chromosome 16q may be associated with a worse prognosis than those without evidence of allelic losses at that locus [15, 16].

Genetic Predisposition

To date, insight into the genetic causes of Wilms tumor has been derived primarily from the study of germline abnormalities conferring predisposition to this tumor, often along with developmental abnormalities (see Table 1). Together with retinoblastoma and neuroblastoma, Wilms tumor was one of the pediatric cancers first used by Knudson and Strong in modeling the number of rate-limiting genetic events required to initiate tumorigenesis [17]. Five to 10% of Wilms tumors present as bilateral disease, indicating a likelihood of genetic predisposition. Consistent with the two-hit model, these cases arise at an earlier age. Virtually all such cases also show persistent nephrogenic rests, precursor lesions composed of undifferentiated blastemal cells [18, 19]. Nephrogenic rests may be present during kidney development, but they normally disappear shortly after birth; their persistence in the kidneys of children with Wilms tumor points to a constitutional abnormality in renal differentiation. However, unlike classical inherited cancer syndromes like retinoblastoma, familial Wilms tumor is rare [20]. This may result in part from the lethality of this cancer prior to the advent of modern chemotherapy, and in fact, cases with familial transmission of *WT1* mutations may become more common with the curative treatment of mutation carriers. In addition, reduced fertility may be associated with genitourinary malformations that may be present in carriers of *WT1* mutations. However, many children with bilateral Wilms tumor do not have a family history of cancer and presumably carry a *de novo* germline mutation in a tumor suppressor gene [21]. Only a subset of these have demonstrated germline mutations in *WT1,* pointing to additional genes that are likely to contribute to genetic susceptibility to Wilms tumor. One such gene may be on chromosome 17, a locus implicated in at least one large family associated with late-onset of Wilms tumor in the absence of genitourinary malformations [22].

WAGR and Beckwith–Wiedemann Syndromes

While familial Wilms tumor is rare, the study of constitutional syndromes associated with developmental abnormalities has yielded two genetic loci on chromosome 11. The initial discovery that two rare conditions, Wilms tumor and aniridia, a malformation or absence of the iris, occur together far more frequently than predicted by chance alone suggested that the responsible genes might be closely linked [23]. Indeed, both *WT1* and *Pax6* were subsequently shown to reside within a locus on chromosome 11p13 [24, 25], where gross cytogenetic deletions are present in children with WAGR syndrome (for Wilms tumor, aniridia, genitourinary malformations, and mental retardation) [26–28]. Constitutional hemizygosity for WT1 is associated with malformations of genitourinary development, and Wilms tumor results from the somatic acquisition of a point mutation within the second WT1 allele in renal precursors [29–32]. Reduced gene dosage for *Pax6,* a master gene for eye development, is responsible for aniridia [33].

A second Wilms tumor predisposition syndrome has been linked to chromosome 11p15 [34–36]. Beckwith–Wiedemann syndrome (BWS) is characterized by asymmetric organomegaly, umbilical hernia, and hypoglycemia, along with a modestly increased risk for various cancers, including adrenal carcinoma, hepatoblastoma, and Wilms tumor [37, 38]. Some cases of BWS show constitutional duplication of the paternally derived chromosome 11p15, and others show inheritance of two paternally derived chromosomes 11 without maternal contribution (uniparental isodisomy) [39, 40]. These unusual genetic features are suggestive of genomic imprinting. Much interest has focused on *IGF2,* which resides at that chromosomal locus, since its restricted expression from the paternally derived

allele would lead to increased gene expression in cases with BWS [41–44]. *IGF2* encodes a potent growth factor, consistent with its contribution to a fetal overgrowth syndrome such as BWS, and the relaxation or loss of *IGF2* imprinting in some sporadic Wilms tumors supports a role in tumorigenesis [45–48]. Nonetheless, other genes located at chromosome 11p15 may also contribute to BWS and Wilms tumorigenesis. The cyclin-dependent kinase inhibitor p57 is also imprinted, but expressed from the maternally derived allele [49, 50]. Inactivating mutations in p57 have been reported in the germline of some cases with BWS [50, 51]. In these cases, loss of cellular growth control associated with inactivation of p57 may contribute to organomegaly. Mouse models with either overexpression of IGF2 or disruption of p57 recapitulate both shared and distinct features of BWS [52–54], suggesting that the human syndrome, which is known to be pleiomorphic in its manifestations and its severity, may in fact result from the accumulation of distinct genetic events. Other imprinted genes at chromosome 11p15 have also been implicated in Wilms tumorigenesis, including the nontranslated RNA H19, which regulates *IGF2* expression and may suppress cellular proliferation [55, 56]. The link between germline abnormalities at chromosome 11p15 and sporadic Wilms tumorigenesis also appears to be complex. Tumors in which LOH is confined to 11p15, excluding the *WT1* gene at 11p13, suggest the existence of a second classical tumor suppressor gene at that locus [10, 57].

GERMLINE AND SOMATIC INACTIVATION OF *WT1*

WT1 was initially isolated by virtue of its chromosomal location, within the minimal deleted region at chromosome 11p13 [24, 25]. Its amino acid sequence revealed a presumptive transcription factor, with four C-terminal Cys_2-His_2 zinc fingers mediating sequence-specific DNA binding, and an N-terminal proline/glutamine-rich domain implicated in transcriptional activation. Alternative pre-mRNA splicing leads to the generation of distinct WT1 isoforms (see Fig. 1) [58] (see below). A subset of cases with genetic predisposition to Wilms tumor harbor germline mutations inactivating *WT1*. In WAGR syndrome, where one germline *WT1* allele is lost by a gross chromosomal deletion, somatic inactivation of the second allele in the Wilms tumor occurs via point mutation [59, 60]. In cases with a germline point mutation within one *WT1* allele, loss of the remaining allele typically occurs by a chromosomal mechanism, including nondisjunction or interstitial deletion. Of note, hemizygosity for *WT1* is associated with genitourinary malformations, suggesting that gene dosage may be critical for normal development [31].

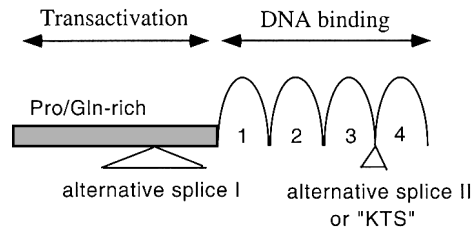

FIG. 1. Schematic representation of WT1 gene product. The N-terminal transactivation domain and the C-terminal zinc finger domain are noted, along with the two alternative splicing events. Alternative splice I inserts 17 amino acids between the transactivation and DNA-binding domains; alternative splice II results from the use of an alternative splice donor site and introduces 3 amino acids (KTS) between zinc finger 3 and 4, altering the DNA-binding properties of the encoded protein.

Denys–Drash Syndrome

The severity of these genitourinary defects, which primarily affect males, is variable; they include ureteral abnormalities, horseshoe kidneys, and urethral malformations. Far more striking developmental defects are present in Denys–Drash syndrome (DDS), a constellation of symptoms including pseudohermaphroditism, renal mesangial sclerosis, and predisposition to Wilms tumor, that is associated with specific mutations in *WT1* [30]. The most common mutation is Arg to Trp at codon 394, within the third zinc finger, but other point mutations have a similar phenotype, including mutations in the second zinc finger domain [61–63]. It is possible that these specific point mutations lead to a gain-of-function phenotype, associated with altered DNA-binding affinity and target gene specificity. However, the presence of rare DDS cases due to an N-terminal truncation of *WT1* suggests a loss-of-function mechanism [64, 65]. The severity of the genitourinary malformations associated with DDS, compared with the more subtle developmental defects caused by *WT1* hemizygosity in WAGR syndrome, has been taken as evidence of a dominant-negative effect mediated by these mutant proteins. Dimerization of WT1 through the N-terminal domain has been documented, raising the possibility that mutant DDS proteins may disrupt the function of the wild-type gene product [66–69]. However, association of the dysfunctional WT1 proteins with other gene products is also possible. Remarkably, when Wilms tumors arise in DDS patients, LOH analysis indicates somatic loss of the remaining wild-type allele [30]. This suggests that complete inactivation of WT1 function provides a further selective advantage in tumor formation, beyond the dominant-negative properties of the protein encoded by the mutant allele.

The abnormalities in sexual differentiation that are evident in patients with DDS highlight the critical role of WT1 in normal gonadal as well as renal

development. In addition to Wilms tumor, children with DDS are at risk for developing undifferentiated germ cell tumors called gonadoblastomas, pointing to the consequences of disrupted gonadal differentiation in carriers of these specific mutations [30]. In contrast to DDS, Frasier syndrome is associated with sex reversal and renal abnormalities, but apparently without predisposition to Wilms tumor [70–72]. This syndrome has been linked to an intronic mutation that alters the ratio between two alternatively spliced *WT1* transcripts (see below). The apparent dissociation between the developmental and tumorigenic consequences of germline *WT1* alterations may reflect the fact that WT1 isoforms have overlapping but not identical functions. An alteration in their relative expression levels may lead to aberrant genitourinary development, but full gene inactivation may be required for tumorigenesis.

WT1 Mutations in Sporadic Wilms Tumor

In sporadic Wilms tumors, *WT1* is inactivated in a subset, estimated at 10–15% [73–77]. Mutations include nonsense as well as missense changes, and they are distributed throughout the coding region, without particular hotspots. Another ~10% of sporadic Wilms tumors express elevated levels of an in-frame deletion of exon2, that may alter the transactivational function of the encoded protein [78]. In the remaining cases, *WT1* appears to be wild-type and is expressed at high levels. Presumably, these tumors have alterations in downstream targets of WT1 or result from genetic alterations in other cellular pathways. To date, no clear histological features distinguish Wilms tumors with *WT1* mutations from those with wild-type transcripts, although some evidence suggests that myogenic differentiation may be more common in tumors with *WT1* mutations [2]. The topological location of nephrogenic rests associated with Wilms tumor has also been suggested to have some predictive value, with rests present within the renal lobule more likely to be associated with a *WT1* mutation than those at the periphery [79]. In two cases of sporadic Wilms tumor associated with adjacent nephrogenic rests, these precursor lesions were found to harbor the same *WT1* mutation as the neighboring tumor, suggesting that inactivation of this tumor suppressor is an early somatic event, leading to the persistence of these undifferentiated cells [29]. In addition to Wilms tumor, somatic mutations inactivating *WT1* have been demonstrated in human acute leukemias and rare cases of mesothelioma (see below).

DEVELOPMENTAL REGULATION AND DISRUPTION OF *WT1* IN ANIMAL MODELS

Expression in the Developing Kidney

During embryogenesis, *WT1* is expressed at high levels in the developing kidneys, gonads, spleen, and the mesothelial lining of abdominal organs [80–84]. A common feature of these tissues is their mesenchyme to epithelial transition during embryonic development, raising the possibility that WT1 may contribute either to the induction or to the response to such developmental signals. During normal kidney differentiation, signals from the metanephric mesenchyme induce growth and invasion by the ureteric bud, which in turn, triggers condensation of the metanephric blastema around the tip of the ureteric bud [85, 86]. WT1 is present at low levels in undifferentiated metanephric mesenchyme, but not in the ureteric bud [80]. Higher levels of *WT1* are expressed in the condensing mesenchyme as it differentiates to form the epithelial structures of the mature kidney, specifically in the comma-shaped and S-shaped bodies that constitute the precursors of glomeruli (see Fig. 2). In the adult kidney, *WT1* expression is maintained within podocytes, the specialized glomerular structures responsible for filtration of blood.

Phenotype of wt1-Null Mice

Hemizygous inactivation of *wt1* in the mouse does not appear to have a significant phenotype [87], while *wt1*-null mice die at an early stage of embryonic development, day E13.5 [88]. The cause of death may be related to malformations of the heart and diaphragm, structures that themselves do not express *wt1* but are lined by *wt1*-expressing mesothelial surfaces (see below). Kidney development is absent. The ureteric bud fails to invade the metanephric mesenchyme, which undergoes programmed cell death. *In vitro*, *wt1*$-/-$ metanephric mesenchyme fails to differentiate when cocultured with wild-type ureteric bud or spinal cord, an efficient inducer of metanephric differentiation. Given the reciprocal inductive signals between mesenchyme and ureteric bud, it is therefore possible that *wt1* expression in undifferentiated mesenchymal cells is required to initiate signals from metanephric mesenchyme that promote ureteric bud invasion, as well as for its intrinsic capacity to respond to signals from the ureteric bud [89]. The apoptosis of blastemal cells in *wt1*-null mice may result either from a direct survival effect of *wt1* itself or, indirectly, as a consequence of disrupted survival signals from the ureteric bud. Of note, the early developmental arrest mediated by *wt1* inactivation in the mouse precludes studying its effect at later stages of renal differentiation, including its peak expression during glomerular differentiation. The

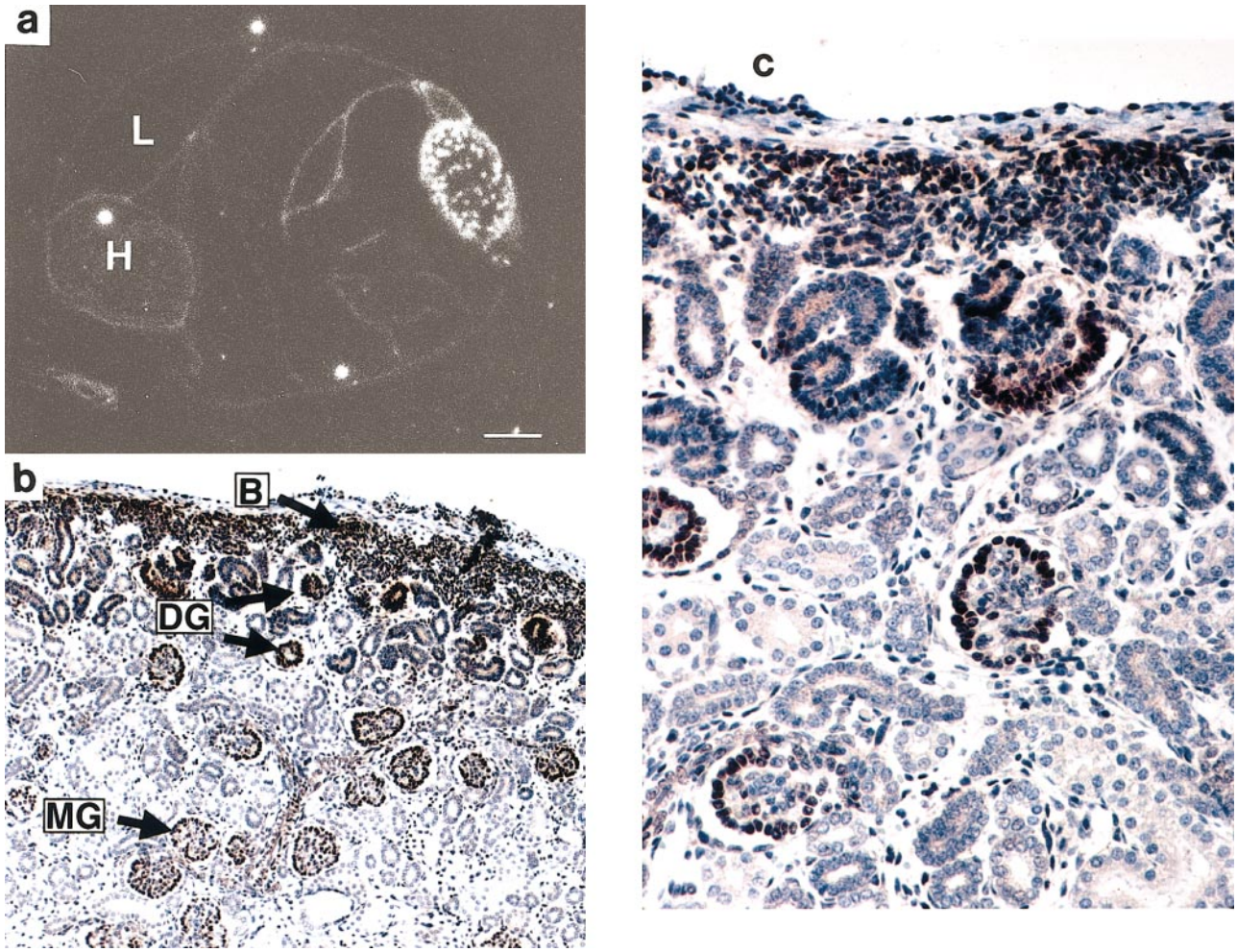

FIG. 2. Developmentally regulated expression of WT1 (a) RNA *in situ* hybridization of a mouse embryo (18 day; cross-section), demonstrating expression of the *wt1* transcript in the developing kidney (bright staining). Expression of *wt1* mRNA is also apparent in the thin layer of mesothelium lining the heart (H), lungs (L), and peritoneal cavity. (Reproduced from Park *et al., Nature Genet.* **4,** 415, 1993, with permission.) (b) Immunohistochemical analysis of WT1 expression in the developing human kidney (18 week). Expression is noted in the blastemal cells (B) that comprise the condensed mesenchyme in the subcortical zone; highest expression is observed as these cells give rise to developing glomeruli (DG). Lower levels of WT1 expression are present in the podocytes of mature glomeruli (MG), located more centrally in the differentiating kidney. (Courtesy of Dr. W. Gerald, Memorial Sloan Kettering Cancer Center, New York, NY.) (c) Higher magnification of developing kidney, demonstrating expression of WT1 in the "S-shaped body," a precursor of the glomerulus, and in the podocyte layer of developing glomeruli. (Courtesy of Dr. W. Gerald.)

timing of *wt1* inactivation during renal differentiation may in fact be critical to its consequences for tumorigenesis, possibly explaining why this is not well modeled in the mouse knockout.

Wt1-null embryos also lack gonads, demonstrating an early requirement for *wt1* expression for development of the undifferentiated gonad, although migration of primodial germ cells is unaffected. Wt1 is expressed in stromal cells of the spleen [80, 82] and its role in development of the spleen has been demonstrated by breeding into mouse strains in which the null phenotype is not lethal until birth [90]. The role of WT1 in the differentiation of gonadal and mesothelial structures is discussed in subsequent sections.

Partial Rescue of Null Phenotype Using Transgenic YACs

Rescue of the *wt1*-null phenotype has been attempted, using a yeast artificial chromosome (YAC) containing 280 kb of genomic sequence flanking human *WT1* [91]. This approach was designed to identify regulatory sequences required for the striking expression pattern of endogenous *wt1* during development [92], as well as to ensure physiological expression of the various WT1 isoforms that result from alternative pre-mRNA splicing. Of particular interest, crossing transgenic mice harboring one or several copies of the YAC transgene with wt1+/− mice produced wt1−/−;YAC+

mice that were viable for up to 48 h following birth [91]. Complete rescue of epicardial and diaphragmatic abnormalities was achieved, but not of the defects in kidney development. While most of these mice lacked kidneys, some showed evidence of early renal differentiation, including branching of the ureteric bud, condensation of mesenchyme, and in very rare cases, small numbers of comma- and S-shaped bodies. Given the high degree of sequence conservation between human and mouse WT1 proteins, failure of the transgenic YAC to rescue the *wt1*-null renal phenotype may result from absence of essential regulatory sequences that direct its temporal and spatial expression pattern in the kidney and/or lower expression levels of the transgenic construct compared with endogenous *wt1*.

Developmental Regulation of WT1 Expression

The requirement for large YACs to encompass the regulatory sequences implicated in the temporal and spatial expression pattern of *WT1* within the developing kidney indicates that these sequences are likely to be located at significant distance from the *WT1* transcriptional start site [92]. Indeed, these regulatory sequences remain to be identified, despite analysis of the presumptive *WT1* promoter. Reporter constructs containing up to 25 kb of genomic sequence upstream of the *WT1* transcriptional start site appear to be ubiquitously expressed, including cell lines in which endogenous *WT1* is not expressed [93]. As noted above, a YAC containing 280 kb of genomic sequences encompassing the *WT1* gene appears to be required to replicate the tissue-specific expression pattern of the endogenous gene [91, 92].

A number of transcription factors have been shown to regulate the proximal *WT1* promoter in reporter assays, including activation by PAX2 [94–97], PAX8 [98, 99], and SP1 [93], and possibly repression by WT1 itself [100–102]. However, regulation of the endogenous *WT1* transcript has been inconsistent, with the possible exception of PAX8. PAX2, a gene implicated in renal tubular development, is coexpressed with WT1 in undifferentiated precursors, but expressed in distinct cell types following glomerular and tubular differentiation [103]. As noted below, loss of Pax2 expression in zebrafish is associated with a broadening of the WT1 expression pattern, suggesting that it may normally repress, rather than activate WT1 expression [104]. The relative contribution of PAX genes to the developmental regulation of WT1 therefore remains to be determined. In addition to its tightly regulated expression during renal development, WT1 is also expressed in hematopoietic cells and in human acute leukemias (see below) [82, 105, 106]. Potential enhancer sequences that contain a GATA-1 binding site and may contribute to its expression in leukemic cell line have been identified within the third intron of WT1 [107], as well as 50 kb downstream of the promoter [108]. However, endogenous *WT1* is expressed in early hematopoetic progenitors and in myeloid and lymphoid, rather than erythroid, precursors. The factors regulating the expression of endogenous *WT1* in hematopoietic cells therefore remain uncertain. Similarly, the regulation of WT1 expression during gonadal differentiation is not well understood (see below).

While direct transcriptional regulators of *WT1* has been difficult to identify, some clues have arisen from factors that trigger renal differentiation, including *WT1* expression, in culture. These factors are presumably indirect, but may point to signaling pathways that culminate in *WT1* activation. Treatment of the murine myelobast cell line M1 with leukemia inhibitory factor (LIF) results in upregulation of the endogenous WT1 transcript [109]. LIF and the related interleukin 6 (IL6) are also expressed in the ureteric bud but not in metanephric mesenchyme [110]. Addition of LIF/IL6 to metanephric mesenchyme *in vitro* triggers nephrogenic differentiation and *WT1* expression. In this assay, WNT signaling also initiates nephrogenic differentiation and *WT1* expression [111, 112]. However, we have not observed direct induction of the *WT1* promoter following activation of WNT signaling (R. Palmer and D. Haber, unpublished).

Other Model Organisms

Nephroblastomas have not been reported in mice bearing one inactivated *wt1* allele, either wt1+/− strains generated by homologous recombination [88] or the naturally occurring Sey^{Dey} mutation that recapitulates the human WAGR chromosomal deletion [87]. This may reflect interspecies differences in pathways leading to renal tumorigenesis, or alternatively, it may result from differences in the size of the target cell population and the likelihood of somatic inactivation of the remaining wild-type *wt1* allele. The number of nephrons in the mouse is estimated to be 100-fold smaller than in humans [87], and kidney differentiation occurs over a shorter interval, potentially reducing the probability of additional genetic events. In the rat, an embryonal kidney cancer model has been developed, based upon treatment of newborns with the chemical carcinogen *N*-nitroso-*N′*methylurea (NMU) [113–116]. Mutational analysis of the endogenous rat *wt1* transcript revealed missense mutations in 7/18 tumors, most of which were homozygous for the mutant allele [116].

The high degree of evolutionary conservation in WT1 sequence has also allowed studies in *Xenopus* and zebrafish. Zebrafish *wt1* mutants have not been described, but some insight can be derived from fish lacking the orthologue of *PAX2,* another transcription

factor implicated in early kidney development [104]. The zebrafish mutant termed *no isthmus* (*noi*) has a mutation in *pax2.1,* one of two orthologues and the only one expressed in the pronephros. *Noi* mutants demonstrate normal glomerular structures, but lack differentiated pronephric tubules and have an abnormal pronephric duct [104]. Loss of pax2.1 is associated with the caudal expansion of *wt1* expression beyond its physiological pattern, consistent with absence of an inhibitory signal from pax2.1. A possibly complementary observation is derived from microinjection experiments in *Xenopus,* where ectopic expression of *wt1* inhibits pronephric tubule differentiation [117, 118].

STRUCTURE AND ALTERNATIVE SPLICING OF *WT1*

Distinct WT1 Isoforms

WT1 encodes a protein migrating around 50 kDa, which contains two domains with apparent functional properties: a C-terminal C_2H_2 zinc finger domain involved in DNA binding and an N-terminal proline/glutamine-rich transactivational domain (see Fig. 1). The coding sequence is comprised of 10 exons, with each zinc finger encoded by an individual exon [58]. Two alternative pre-mRNA splicing events give rise to distinct gene products. Exon 5 encodes 17 amino acids that are inserted between the transactivation and DNA-binding domains (alternative splice I). The 17 amino acids encoded by alternative splice I can mediate some transcriptional repression activity in a reporter assay, when fused to a heterologous DNA-binding domain [119], although similar transactivational properties are observed using native WT1 proteins containing or lacking this insertion [120]. The precise function of this 17-amino-acid insertion therefore remains uncertain.

Alternative splice II results from the use of a variable splice donor site between exons 9 and 10, leading to the insertion of three amino acids, lysine–threonine–serine (commonly referred to as KTS), between the third and the fourth zinc fingers [58]. This insertion disrupts the critical spacing between these zinc fingers resulting in loss of DNA binding to the consensus WT1 DNA-binding sequence [121]. This observation, and the fact that WT1(+KTS) proteins appear to be colocalized with elements of the pre-mRNA splicing machinery [122], have led to the suggestion that this isoform plays a role in RNA processing rather than transcriptional activation (see below). Whatever its function, the importance of the ratio between the (−KTS) and (+KTS) isoforms is highlighted by Frasier syndrome, a severe developmental defect affecting kidneys and gonads that has been linked to presence of a germline mutation in the exon 9 splice donor consensus, associated with reduced expression of WT1(+KTS) [70–72]. Measurements of the relative expression of *WT1* splice variants have differed depending on the assays used; overall, transcripts encoding the (+KTS) insertion appear to comprise ~80% of the cellular *WT1* mRNA, while those encoding exon 5 account for ~60% [58]. While some cell lines appear to have different ratios of these isoforms, Frasier syndrome remains the only clear link between altered expression of *WT1* splicing variants and functional abnormalities. Of the two alternative splice variants, only the (KTS) insertion is conserved in lower vertebrates, including fish, amphibian, and birds [123–125].

Additional isoforms of WT1 have been reported, resulting from RNA editing (leucine to proline at codon 280) [126] and use of an upstream CUG initiation codon [127], but the functional consequences resulting from these differences remain undefined. Recently, a truncated form of WT1 has been detected in several cell lines and in Wilms' tumor specimens due to an internal initiation at an in-frame AUG codon at position 127, giving rise to a 36- to 38-kDa protein [128]. This internal initiation site, which is evolutionarily conserved, is also predicted to generate all four alternative splicing variants of WT1. Absence of the N-terminal domain may lead to enhancement of transcriptional activation over transcriptional repression, although the truncated products are expressed at considerably lower levels than the full-length protein, and their functional significance remains to be determined. Most studies of WT1 function have focused on the conventional AUG-initiated transcripts encoding or lacking alternative splices I and II (KTS). Ectopic expression of constructs encoding the various isoforms of WT1 in a Wilms tumor cell line inhibits cellular proliferation, consistent with a tumor suppressor effect [78] (see Fig. 3). However, it is possible that the development of physiologically relevant assays for WT1 function will bring insight into the relevance of these additional low abundance products.

Functional Domains of WT1

Two different domains of WT1 appear sufficient to confer nuclear localization. Truncated WT1 proteins containing only the zinc finger domain [129] or only the N-terminal domain [69] both demonstrate expression in the nucleus, although the precise identity of the nuclear localization signals (NLS) has not been defined. In addition, the first 182 residues of WT1 encode a dimerization or oligomerization domain, as demonstrated by yeast two-hybrid analysis and coimmunoprecipitation studies [67, 69, 130]. The physiological significance of WT1 self-association has not been elucidated, although it has been postulated to contribute to the effect of dominant-negative mutations [67, 69, 130]. In addition, the possibility of either dimerization

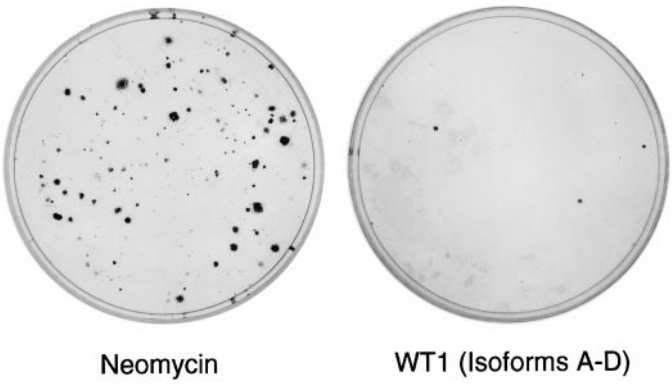

FIG. 3. WT1-mediated inhibition of colony formation in a Wilms tumor cell line. Representative plates showing the formation of colonies by the Wilms tumor cell line, RM1, harboring an altered endogenous *WT1* transcript. Transfection of cells with a construct conferring neomycin resistance gives rise to drug resistant colonies, while cotransfection of wild-type WT1 isoforms suppresses colony formation. Isoforms A through D refer to the four combinations containing and/or lacking the two alternatively spliced insertions in the *WT1* transcript. The inhibition of cellular proliferation by WT1 has been linked to its mediation of a G_1 phase cell cycle arrest in some cells and apoptosis in other cell types.

or cooperative DNA binding has been raised by the observation that transcriptional repression of the *platelet-derived growth factor-A* (*PDGF-A*) promoter requires two WT1 binding sites flanking the transcriptional start site [131].

The effect of WT1 domains on transcriptional activation have been studied primarily through the use of transient transfection and promoter–reporter assays [132]. Both transcriptional activation and repression of reporter constructs have been reported, depending upon the number of binding sites within the promoter, cellular context, and even the choice of expression vector [133]. Wang and co-workers [131] observed transcriptional repression of the *PDGF-A* promoter by WT1 when the two DNA-binding sites are present, but activation of a reporter containing either site alone. In these assays, use of WT1 deletion constructs indicated that residues 84–179 were required for the transcriptional repression, and residues 180–294 contained a transcriptional activation domain, while deletion of the first 84 residues had no effect on transcriptional regulation. However, other mapping studies identified amino acids 1–180 as critical for transcriptional activation of the endogenous $p21^{Cip1}$ and *Syndecan-1* promoters [134, 135]. As noted below, structure–function analyses of WT1 transactivational domains have been hampered by the discrepancy between promoter–reporters that are either activated or repressed by WT1 in transient transfection assays, yet whose endogenous genes do not appear to be physiologically regulated by WT1 *in vivo* (see Table 2). The apparent loss of DNA-binding specificity that accompanies overexpression of WT1 in promoter–reporter assays and the variable activation or repression of such constructs have made it all the more important to identify functionally relevant transcriptional targets of WT1.

TRANSACTIVATIONAL PROPERTIES OF WT1(−KTS)

DNA-Binding Sequence

The four Kruppel-like C_2H_2 zinc fingers of WT1 mediate DNA binding by the (−KTS) isoform. Zinc fingers 2–4 have a high degree of amino acid homology to those of the immediate early gene *early growth response 1* (*EGR1*) [136], although the three zinc fingers of *EGR1* are encoded by a single exon, a genomic structure that is distinct from that of *WT1* and suggests that these genes do not share a common evolutionary origin. The transactivational domain of WT1 and EGR1 are not related. The WT1 zinc finger domain binds to the characteristic GC-rich EGR1 DNA-binding element, although with ~40-fold less affinity than EGR1 itself [121, 137]. Most WT1-responsive promoters identified to date contain one or more EGR1 sites within their promoters (see below). A second potential DNA-binding site for WT1 consists of TCC repeats, which have been mapped by analysis of the WT1-responsive promoters of *PDGF-A* [138] and *epidermal growth factor receptor* (*EGFR*) [139]. More recently, PCR selection of genomic DNA sequences with high affinity for the WT1(−KTS) zinc fingers, coupled with extensive mutational studies, led to the characterization of an optimized binding site, 5′-GCGTGGGAGT-3′ [140]. This binding site, called WTE, displays 20- to 30-fold higher affinity for WT1 than does the EGR1 sequence. This high-affinity WTE site has recently been reported to mediate binding by WT1(−KTS) to the promoters of *Amphiregulin* [141] and *Bcl2* [142], two genes that appears to be regulated by WT1 *in vivo*. Analysis of additional WT1(−KTS)-target genes will be required to determine whether presence of the WTE sequence within a promoter is more useful in predicting regulation of the native gene by WT1 than does presence of the EGR1 or TCC-repeat motifs. Some evidence suggests a potential DNA recognition sequences for WT1(+KTS), but the precise binding site remains uncertain [143, 144].

Transcriptional Repression

Transcriptional repression by WT1 was first demonstrated through fusion of the N-terminal domain to a heterologous DNA-binding domain [120]. The observation that the WT1(−KTS) isoform binds to the EGR1 DNA-binding consensus sequence subsequently made it possible to test the transactivational properties of the full-length protein using promoter–reporter assays. The characterization of WT1 as a transcriptional

TABLE 2

Proposed *WT1* Transcriptional Target Genes

Genes	Promoter–reporter assays	Target sequence[a]	Regulation of endogenous transcript[b]	References
EGR-1	Repression/activation	EGR1 consensus	—	[120, 161]
PDGF-A	Repression/activation	EGR1 consensus TCC repeats	—	[131, 146, 147]
EGFR	Repression	TCC repeats	—	[139]
IGF-II	Repression	EGR1 consensus	—	[148]
IGF-1R	Repression	EGR1 consensus	—	[149, 150]
Midkine	Repression	EGR1 consensus	—	[248]
C-myc	Repression	EGR1 consensus	—	[154]
N-myc	Repression	EGR1 consensus	—	[155]
WT1	Repression	EGR1 consensus	—	[100–102]
PAX2	Repression	EGR1 consensus	—	[94]
TGF-β	Repression	EGR1 consensus	—	[152]
RAR-α	Repression	EGR1 consensus	—	[153]
MDR-1	Repression	EGR1 consensus	—	[249]
NOVH	Repression	ND	—	[250]
SRY	Activation	EGR1 consensus	—	[203]
Androgen receptor	Repression	EGR1 consensus	—	[203]
$G\alpha_i$*-2*	Repression	EGR1 consensus	—	[158]
CTGF	Repression	ND	—	[177]
$p21^{Cip1}$	Activation	ND	Activation (I)	[163, 173]
Amphiregulin	Activation	WTE	Activation (I)	[141]
Syndecan-1	Activation	EGR1 consensus	Activation (I)	[135]
E-cadherin	Activation	EGR1 consensus	Activation (S)	[167]
BCL-2	Repression/activation	WTE	Activation (S)	[142, 154, 156]
DAX-1	Activation	EGR1 consensus	Activation (I)	[165]
MIS	Activation	SF-1 site (indirect)	—	[166]
CSF-1	Repression/activation	EGR1 consensus	Activation (I)	[151, 160]
RbAp46	Activation	ND	Activation (S)	[172]
VDR	Activation	WTE	Activation (S)	[178]
ODC	Repression	EGR1 consensus	Repression (I)	[251]
TERT	Repression	EGR1 consensus	Repression (S)	[159]

[a] DNA sequences responsible for WT1-mediated transcriptional effects: EGR-1 consensus, GC-rich binding site; TCC repeats, TC-rich binding site; WTE, *in vitro* optimized site; and ND, not determined.

[b] (I), inducible expression of WT1 associated with target gene regulation; (S), constitutive expression of WT1 in stably transfected clones associated with changes in target gene expression.

repressor of genes containing the GC-rich EGR1 sequence within their promoter led to the identification of a large number of potential target genes, whose promoters are repressed by ectopic expression of WT1 in transient transfection assays [145]. These genes, listed in Table 2, include *EGR1* [120], *WT1* itself [100–102], *PDGF-A* [146, 147], *IGF2* [148], *insulin-like growth factor receptor* (*IGFR*) [149, 150], *Pax2* [94], *colony stimulating factor 1* (*CSF1*) [151], *transforming growth factor-β* (*TGF-β*) [152], *retinoic acid receptor-α* [153], *C-myc* [154], *N-myc* [155], *Bcl2* [154, 156], *Inhibin α* [157], *G protein* α_i*-2* [158], and *telomerase RT (TERT)* [159] among others. Some of these genes encode embryonic growth factors, providing an attractive hypothesis to explain the tumor suppressor properties of WT1, but most were identified by virtue of their GC-rich promoters, a relatively common characteristic. Moreover, few of these WT1-responsive promoters are associated with evidence that the endogenous gene itself is regulated by WT1. In fact, the generation of cells with inducible expression of WT1 isoforms demonstrated that induction of WT1(−KTS) was not accompanied by altered expression of the native genes, despite potent repression of corresponding promoter–reporter constructs in transfection assays [139, 160]. A possible exception is the *EGFR*, in which downregulation of its WT1-responsive promoter following induction of WT1(−KTS) is associated with reduced synthesis of the endogenous protein [139]. However, repression of endogenous *EGFR* mRNA is not observed in all cell lines [160]. The discrepancy between promoter–reporter studies and physiological regulation of endogenous genes by WT1 may have a number of explanations, including the loss of specificity in DNA binding that accompanies transient overexpression of both WT1 and the target promoters, as well as the context of WT1-binding sites within a chromatinized promoter template. To date, WT1 has not been shown

to associate with bona fide transcriptional corepressors. Confirmation of WT1′s role as a transcriptional repressor therefore awaits the identification of endogenous genes whose downregulation by WT1 is linked to its functional properties.

Transcriptional Activation

Like transcriptional repression, activation of promoter–reporters by WT1 has been described, an effect that appears to be modulated by both promoter and cellular contexts [131, 133, 161, 162]. Recently, the physiological relevance of WT1-mediated transcriptional activation has been supported by a number of observations: (1) induction of cell cycle arrest by WT1(−KTS) is linked to its induction of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ [163, 173]; (2) a Wilms-tumor-associated point mutation in *WT1* abrogates transcriptional activation but not repression [163]; (3) *in vivo* physical association of WT1 with the transcriptional coactivator CBP/P300 [W. Wang and D. Haber, unpublished]; and (4) use of expression profile analysis to identify endogenous genes, such as *Amphiregulin* that are transcriptionally induced by WT1(−KTS) [141].

Ectopic expression of WT1(−KTS) in a number of cell types leads to a G_1 cell cycle arrest, an observation that is correlated with induction of the endogenous $p21^{Cip1}$ gene, and activation of its promoter in reporter assays [163, 164, 173]. Of note, deletion of the N-terminal domain of WT1 results in loss of its ability to induce $p21^{Cip1}$ as well as growth arrest in Saos-2 cells, but unimpaired transcriptional repression of an EGR1-containing promoter–reporter [134]. A naturally occurring point mutation abrogating transcriptional activation of the $p21^{Cip1}$ promoter, but not transcriptional repression of a GC-rich promoter, has also been identified in a Wilms tumor specimen [163].

Additional target genes that may be induced by WT1(−KTS) include *Amphiregulin* [141], *Dax1* [165], *Müllerian inhibiting substance* (*MIS*) [166], *Bcl-2* [142], *Syndecan-1* [135], *E-cadherin* [167], and *CSF1* [160]. The induction of *Amphiregulin, Dax1,* and *MIS* are discussed in detail in subsequent sections. The promoter of *Bcl-2* is either repressed or activated by WT1(−KTS), depending on cell type [142, 154, 156]. The *Bcl-2* promoter contains the optimal WT1-binding site, WTE, and increased levels of endogenous Bcl-2 expression are observed in the rhabdoid tumor cell line G401, following stable transfection of *WT1(−KTS)* [142]. Induction of *Bcl-2* by WT1 may contribute to its survival function in renal precursors, although the kidney defects in *Bcl*-2-null mice primarily involve abnormalities in the proximal and distal tubules and collecting ducts, which are not sites of wt1 expression [168–170]. Syndecan-1 is a mesenchymal proteoglycan thought to play a role in epithelialization of the kidney [171]. The *Syndecan-1* promoter appears to be activated by both the (−KTS) and (+KTS) isoforms of WT1 through a region that is GC rich, although the precise DNA sequences required for this effect remain undefined [135]. Induction of endogenous Syndecan-1 is observed following zinc-inducible expression of WT1 in NIH 3T3 cells, but not in the human embryonic kidney 293 cells or the osteosarcoma Saos2 cells [160]. *E-cadherin* is also a cell surface protein, which is expressed in the condensing metanephric mesenchyme. WT1(−KTS) mediates activation of the *E-cadherin* promoter and induction of the endogenous transcript is detectable following ectopic expression in NIH 3T3 cells [167]. Loss of E-cadherin expression is common to many human cancers, although this has not been correlated with the presence of *WT1* mutations in Wilms tumors. Finally, expression of the hematopoietic growth factor *CSF1* is increased following inducible expression of *WT1(−KTS)* in osteosarcoma cells [160], but this has not been confirmed in blood cells.

Identification of Transcriptional Targets Using Differential Expression Screening

A number of efforts have been made to search for WT1 target genes using unbiased screens for transcripts whose expression is altered following ectopic expression of WT1(−KTS). These attempts have been particularly successful when combined with inducible expression of WT1(−KTS), since the selection of individual stably transfected clones may be accompanied by nonspecific clonal variation or even by secondary compensatory changes in cell types where WT1(−KTS) inhibits proliferation. Techniques used have included differential display [160], subtractive hybridization [172], representative differential analysis [167], and more recently, hybridization to high-density oligonucleotide microarrays [141].

Inducible expression of WT1(−KTS) in osteosarcoma cells, using a tightly regulated, tetracycline-repressible promoter, triggers a G_1 cell cycle arrest followed by apoptosis [139, 173]. Analysis of early time points following WT1 induction therefore made it possible to search for transcriptional targets that might be linked to a biological effect. In an initial study [141], Affymetrix chips carrying markers for 6800 genes were used, with analytic criteria that included a >5-fold change in expression level in duplicate experiments, followed by independent confirmation using Northern blotting. A small number of potential WT1(−KTS) target genes were identified. Of note, no target genes were confirmed to be reduced in expression levels following WT1(−KTS) expression. The transcript found to be most dramatically increased in expression level was *Amphiregulin* [174, 175], a member of the *epidermal*

growth factor (*EGF*) gene family, which was induced 70-fold by microarray hybridization and 10-fold by Northern analysis [141]. In addition $p21^{Cip1}$ and HSP70, two transcripts previously known to be induced following WT1 expression were identified. Another potentially novel WT1 target gene, *acidic fibroblast growth factor* (*aFGF*), was identified by the array analysis and confirmed to be moderately induced (2- to 3-fold) as determined by Northern blotting analysis. Of note, no target genes were identified for WT1(+KTS), even after screening Affymetrix chips carrying markers for 40,000 genes (R. Palmer and D. Haber, unpublished).

The induction of endogenous *Amphiregulin* by WT1(−KTS) was confirmed in rat embryonic kidney cells with tetracycline-regulated expression of WT1 [141]. Further studies of the *Amphiregulin* promoter mapped the WT1-responsive site to a sequence that closely resembles the optimal DNA-binding site, WTE, predicted by PCR selection experiments [140]. The physiological significance of this target gene was further supported by expression studies, demonstrating that Amphiregulin is specifically expressed in condensed mesenchyme and in glomerular precursors, correlating spatially and temporally with WT1 expression in the developing kidney. Furthermore, in renal organ cultures, purified Amphiregulin enhances ureteric branching by 2-fold compared with untreated control [141]. Amphiregulin produced by the renal mesenchyme would presumably signal through the epidermal growth factor receptor, which is present in the ureteric bud. However, significant redundancy in this pathway is likely, since recently generated *Amphiregulin*-null mice, like *EGF*-null mice, are viable without any gross defects [176].

Another microarray study was recently performed using the Wilms tumor-derived cell line, WiT49, which was stably transfected with a potential dominant-negative *WT1* mutant [177]. A gene encoding connective tissue growth factor (CTGF) was expressed at higher levels in a clone expressing the WT1 mutant, but repression of this target gene by wild-type WT1 was not demonstrated. cDNA arrays have also been used to demonstrate a correlation between expression of endogenous *WT1* and *vitamin D receptor* (*VDR*) genes in different tissue types [178]. Induction of VDR by WT1 was subsequently demonstrated in HT29 colon carcinoma and HEK293 cells, along with increased sensitivity toward 1,25-dihydroxyvitamin D3, but a physiological interaction between VDR and WT1 remains to be demonstrated.

The identification of native genes that are regulated by WT1(−KTS) may eventually provide some insight into common features of their promoters that are correlated with their transcriptional regulation. Native WT1-binding sites can be classified as either a GC-rich (EGR1-like) sites, which are present in the promoters of *Syndecan-1, E-cadherin,* and *Dax1* [135, 165, 167], or the higher affinity WTE sequences, such as those present in the promoters of *Bcl-2, Amphiregulin,* and *VDR* [141, 142, 178]. The *Bcl-2* and *VDR* promoters contain a single WT1(−KTS)-binding site [142, 178], while those of *E-cadherin, Dax-1,* and *Amphiregulin* have two elements both of which appear to contribute to WT1-mediated activation of reporter constructs [141, 167]. In addition, some target genes may be induced indirectly by WT1. The MIS promoter does not contain a WT1-binding site, but it may be induced following expression of WT1(−KTS) by virtue of a protein–protein interaction with the orphan nuclear receptor steroidogenic factor 1 (SF1), which does bind to the MIS promoter [166]. Induction of Hsp70 following WT1 expression results from the activation of heat shock factor (HSF) sites [134], presumably reflecting the displacement of HSF from Hsp70 by WT1 (see below). Finally, the context of WT1-responsive sites in native promoters may provide clues to other transcriptional regulators that may interact with WT1. Thus it is of interest that the WT1-binding site in the *Amphiregulin* promoter is adjacent to a cAMP-responsive element (CRE) box [141], while that in the *E-cadherin* promoter is close to a CAAT box [167]. While these sites appear to contribute to WT1-mediated transactivation in reporter assays, cooperative binding or protein–protein interactions between WT1 and other transcriptional regulators have not been demonstrated.

POTENTIAL ROLE OF WT1(+KTS) IN RNA PROCESSING

The first clue suggesting a nontranscriptional role for WT1(+KTS) emerged from its subnuclear expression pattern [122]. Confocal microscopy revealed that this isoform is present within 30–50 nuclear dots or speckles [122], in contrast to WT1(−KTS), which appears to be expressed diffusely throughout the nucleoplasm (see Fig. 4). Analysis of cell lines and tissues expressing endogenous WT1 supports a nuclear expression pattern with both diffuse and speckled components [179]. Of note, WT1 mutants with a disrupted zinc finger domain show a more pronounced speckled nuclear expression pattern [69], leading to the suggestion of competition between an N-terminal "speckling domain" and the zinc finger C-terminal domain for localization of WT1 protein.

Costaining of WT1(+KTS)-associated speckles with antibodies against Sm antigens has suggested at least partial colocalization of this isoform with interchromatin granules (IGs), potential nuclear assembly sites that contain splicing factors and small nuclear ribonucleoproteins (snRNPs) [122]. RNase treatment alters the expression pattern of WT1(+KTS) supporting

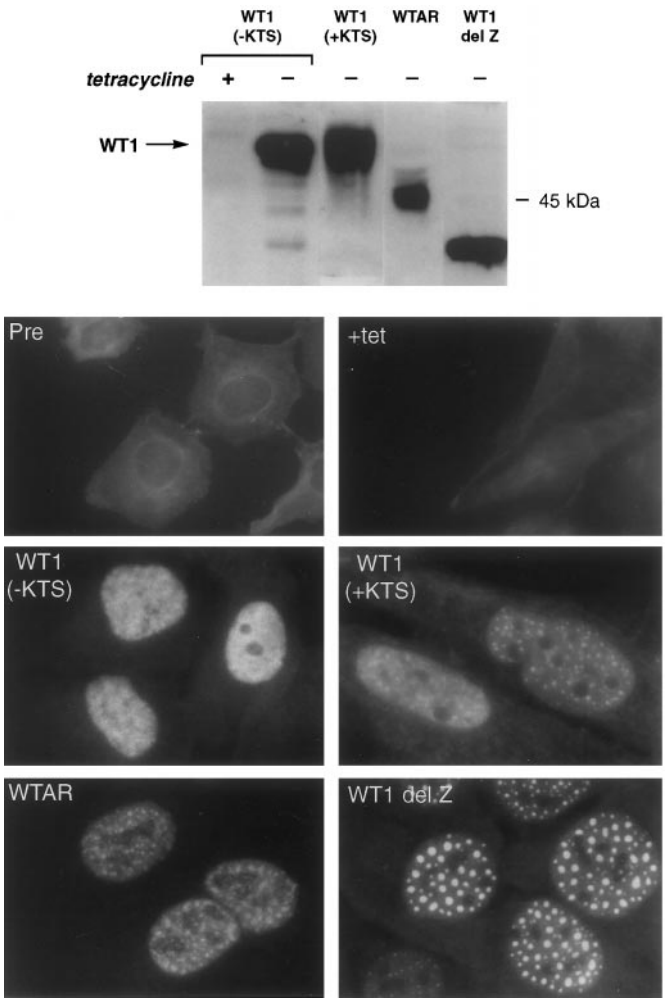

FIG. 4. Distinct subnuclear expression of WT1 variants. Tetracycline-regulated inducible expression of WT1 variants in the U2OS osteosarcoma cell line gives rise to distinct expression patterns. Staining using antibody WTc8 demonstrates a diffuse nuclear expression pattern for the WT1(−KTS) isoform, and a mixed diffuse and microspeckled pattern for the WT1(+KTS) isoform. Disruption of the zinc finger DNA-binding domain leads to enhanced speckling, as demonstrated by WTAR, a naturally occurring, Wilms-tumor-associated mutation with an in-frame deletion of zinc finger 3, and WT1 delZ, a synthetic construct lacking the entire zinc finger domain, which is analogous to a mutation associated with Denys–Drash syndrome. Antibody specificity is demonstrated by absent staining of cells expressing WT1 using preimmune serum (Pre); tightly regulated inducible expression is shown by WTc8-staining of cells grown in the presence of tetracycline. The top panel demonstrates comparable inducible expression of the WT1 variants, using immunoblotting analysis of cells grown in the presence or absence of tetracycline. (Reproduced from Englert *et al., Proc. Natl. Acad. Sci. USA* **92,** 11960, 1995, with permission.)

its colocalization with elements of the splicing machinery. However, the larger nuclear speckles apparent in WT1 proteins with a disrupted DNA-binding domain do not colocalize with the essential splicing factor SC35, suggesting that some WT1-associated nuclear structures may be distinct from IGs [69]. WT1(+KTS)-associated speckles are also distinct from those associated with PML bodies, as well as other transcriptional regulators. Recent studies demonstrating a protein–protein interaction between WT1 and the essential splicing factor U2AF65 have provided evidence that WT1 may in fact be involved in some aspect posttranscriptional regulation [180]. WT1 protein is also detectable, along with snRNPs, in the poly(A)$^+$ fraction of cellular nuclear extracts isolated using oligo(dT) chromatography [181]. This coelution with the poly(A)$^+$ fraction requires the zinc finger domain of WT1. WT1 has also been shown to bind, via the zinc finger domain, to RNA sequences present in exon 2 of the *IGF2* transcript [182]. However, a biological role for WT1(+KTS) in the posttranscriptional regulation of *IGF2* mRNA has not been demonstrated. *IGF2* mRNA-binding required zinc finger 1 but not zinc finger 4 and showed some enhanced affinity for WT1(+KTS) over the (−KTS) isoform. In contrast, a requirement for zinc finger 4 has been suggested by studies involving PCR selection of a pool of random 20-mer RNA species, leading to the identification of RNA sequences capable of competing with WT1–DNA complexes [183]. However, these studies were performed using the (−KTS) isoform of WT1, which unlike WT1(+KTS), has a more clearly defined role in transcriptional regulation. RNA binding was also demonstrated in these studies for the *bona fide* transcription factor EGR1, whose DNA-binding domain is closely related to that of WT1(−KTS). While the zinc finger domain of WT1 may have some affinity for RNA as well as DNA, the possibility of an RNA recognition motif (RRM) in the N-terminal domain of WT1 has also been suggested on the basis of computer modeling [184]. Taken all together, the distinct nuclear localization of WT1(+KTS) has raised the possibility that this isoform may mediate some aspect of mRNA processing, although further studies are required to demonstrate a clear role for WT1 in posttranscriptional regulation and RNA metabolism and to identify potentially relevant mRNA targets.

WT1-BINDING PROTEINS AND MODIFICATIONS

Potential protein partners of WT1 have been identified using a number of different approaches, including yeast two-hybrid assays and coimmunoprecipitation experiments (see Table 3). In baby rat kidney cells, most of the endogenous 50-kDa WT1 protein migrates between 100 and 150 kDa by gel filtration analysis, suggesting that it may be associated with one or more proteins [161]. These protein–protein interactions may point to potentially relevant cellular pathways, although no obligate partner protein has been identified to date, and the functional importance of many candidate interactors remains to be demonstrated.

TABLE 3

Candidate WT1-Interacting Proteins

Gene products	WT1 interaction domain	Yeast two-hybrid or GST pull-down	Coimmunoprecipitation		Developmental coregulation	Subnuclear (speckles) colocalization (with WT1 + KTS)
			Ectopic expression	Endogenous proteins		
WT1	1–180 aa	+	+	ND[a]	NA[b]	NA
HSP70	1–180 aa	ND	+	+	+ (kidney)	+
hUBC9	85–179 aa	+	−	−	−	−
SF1	N-terminus	+	−	−	+ (gonad)	−
p53	Zinc fingers 1–2	+	+	+	−	−
p73	Zinc fingers	+	+	−	−	−
p63	Zinc fingers	ND	+	−	−	−
Par-4	Zinc fingers	+	+	−	−	−
Ciao-1	Zinc fingers	+	+	−	−	−
U2AF65	Zinc fingers 3–4	+	+	+	−	+
WTAP	Zinc fingers	+	+	+	−	Partial
CBP	Zinc fingers	+	+	−	−	−

[a] ND, not determined.
[b] NA, not applicable.

CBP/P300 and p53

Probably the most readily understood protein partner for WT1 is the transcriptional coactivator CREB-binding protein (CBP). Recent studies indicate that WT1 and CBP interact directly, through the first two zinc finger domains of WT1 and the E1A-binding domain of CBP (residues 1625–1991), and the two proteins are stoichiometrically coimmunoprecipitated from cellular lysates (W. Wang and D. Haber, unpublished). The association of WT1 with CBP contributes to the transcriptional activation of WT1 target genes, particularly the *Amphiregulin* promoter, in which the WT1-binding site is within 10 nucleotides of a consensus sequence for CRE binding proteins (CREB) [141]. Both WT1(−KTS) and (+KTS) are capable of interacting with CBP, raising the possibility that both isoforms may potentially modulate transcription, even in the absence of direct DNA binding. The WT1–CBP interaction is the first demonstration of an association with a modifier of chromatin structure and paves the way for future investigations of WT1-mediated transcriptional regulation in a native chromatin context.

While the WT1–CBP association is consistent with the transactivational properties of WT1, it is also of interest in that it may explain the coimmunoprecipitation of WT1 and the tumor suppressor protein p53 [161, 185]. WT1 and p53 are coimmunoprecipitated from cellular lysates, an interaction that is also mediated by WT1 zinc fingers 1–2, but these two proteins do not appear to bind directly *in vitro* [161]. Ectopic expression of these two transcription factors results in the reciprocal modulation of their transactivational properties and protein stability [161, 185], which may be explained by their binding to two distinct domains of CBP. WT1 has also been reported to interact functionally with the p53 homologs p73 and p63, through its zinc finger domain, leading to modulation of transactivational properties [186]. In cells expressing adenovirus E1B 55K protein, a multimeric protein complex containing p53 and WT1 appears to be sequestered in the cytoplasm [187].

HSP70

A potentially functional protein interaction between WT1 and the inducible heat shock protein Hsp70, was also identified by protein sequencing of bands coimmunoprecipitated with WT1 from cellular lysates [134]. Hsp70 binds to the proline-rich N-terminal domain of WT1. The biological relevance of this protein association is suggested by the stoichiometric coimmunoprecipitation of the two endogenous proteins and the observation that Hsp70 and WT1 are colocalized in nuclear speckles, both in cultured cell lines and in developing glomerular podocytes. Remarkably, the loss of transcriptional activation of endogenous $p21^{Cip1}$ that accompanies deletion of the 180 N-terminal amino acids of WT1 is restored if this domain is replaced with a heterologous Hsp70-binding J-domain, but not a mutant J-domain [134]. This suggests that recruitment of Hsp70 to the proline-rich N-terminus of WT1 may contribute to appropriate protein folding and to its transactivational properties. Ectopic expression of WT1 leads to induction of endogenous Hsp70 transcription through the HSF sites in the Hsp70 promoter, consistent with the standard model for Hsp70 induction [188–190], whereby the binding of WT1 to Hsp70 leads to displacement of HSF from its complex with Hsp70.

PAR-4, CIAO-1, UBC9, and WTAP

Additional potential WT1-interacting proteins have been identified by yeast two-hybrid analyses. Par-4 (for prostate apoptosis response) and Ciao-1, a novel protein containing seven WD40 motifs, bind to the zinc finger domain of WT1 [191, 192]. Coimmunoprecipitation of ectopically expressed proteins has been demonstrated, although both Par-4 and Ciao-1 appear to be diffusely expressed in the nucleus and cytoplasm. Cotransfection of either protein along with WT1(−KTS) leads to inhibition of WT1-mediated transactivation of a synthetic promoter containing tandem WT1-binding sites, and Par-4 represses transcription when fused to a heterologous DNA-binding domain [191]. Another yeast two-hybrid screen, using the N-terminal domain of WT1 as bait, identified a novel WT1-interacting protein, hUBC9, encoding the human homolog of the yeast ubiquitin-conjugating enzyme 9 [193]. In yeast, UBC9 is a nuclear protein involved in degradation of S- and M-phase cyclins [194], but no function has yet been ascribed to the WT1–hUBC9 interaction. Recently, a third yeast two-hybrid analysis identified another novel protein, WTAP (WT1-associating protein), that interacts with the C-terminal zinc finger domain of WT1 [195]. *WTAP* encodes an ubiquitously expressed protein of unknown function. The coimmunoprecipitation of endogenous proteins supports a potentially biological interaction between these two proteins, but the functional consequences of this protein association remain to be defined.

U2AF65

The subnuclear speckling pattern of WT1(+KTS) and its proposed interaction with pre-mRNA splicing factors [122] led to the demonstration of a protein interaction between WT1 and the ubiquitous splicing factor U2AF65 [180]. Both isoforms of WT1 are coimmunoprecipitated with U2AF65, although this interaction appears to be stronger for the (+KTS) variant. Two WT1 domains appear to be required: the region immediately N-terminal to the zinc fingers and the zinc finger domain itself, but excluding the zinc finger 1. Coimmunoprecipitation of endogenous WT1–U2AF65 proteins has been demonstrated, although the functional implications of this protein interaction for either protein remain to be determined. Recently, p116, another protein involved in pre-mRNA splicing which appears to function as a U5 snRNP-associated GTPase, has been proposed as a possible WT1-interacting protein, based on their coelution on sucrose and Nycodenz density gradients and their overlapping expression in subnuclear speckles [181].

WT1 Self-Association

Finally, WT1 has been shown to dimerize through its N-terminal domain, based on yeast two-hybrid and *in vitro* GST pull-down assays, as well as coimmunoprecipitation analyses [67–69, 130]. The stoichiometry of WT1 self-association is uncertain, and high levels of ectopic expression are required to demonstrate *in vivo* protein self association. Nonetheless, the potential dimerization or oligomerization of WT1 would have significant implications for the effect of naturally occurring *WT1* mutations. For instance, DDS mutations that impair DNA binding but leave the self-association domain intact may function in a dominant negative manner [67–69, 130]. The altered nuclear localization of these *WT1* mutant products might also contribute to sequestration of the wild-type protein [69].

Protein Phosphorylation

In addition to the effect of protein–protein interactions, WT1 function may be altered by posttranslational modification. Protein kinase A (PKA) phosphorylates WT1 *in vitro* at two serine residues within zinc fingers 3 and 4 (Ser-365 and Ser-393), leading to loss of DNA-binding activity, but persistent RNA binding [196, 197]. Substitution of alanine for the two serine residues alleviates PKA-mediated inhibition of WT1-dependent transactivation of a reporter construct [197], and treatment of cells with the PKA activator forskolin may cause cytoplasmic accumulation of WT1 [196]. The two serines are evolutionarily conserved in all WT1 orthologues, and the potential significance Ser-365 phosphorylation is suggested by the mutation of this residue to phenylalanine in a human pre-B cell line [24] and NMU-induced rat nephroblastoma [116]. However, *in vivo* phosphorylation of WT1 by PKA, or potentially other kinases, remains to be demonstrated.

ROLE OF WT1 IN GONADAL DEVELOPMENT

The importance of WT1 for gonadal differentiation is underscored by both human Denys–Drash and Frasier syndromes that are associated with intersex disorders [30, 70] and by the failure of gonadal development in *wt1*-null mice [88]. WT1 is expressed diffusely in the undifferentiated gonads [80, 81, 198, 199]; its expression becomes more restricted following sexual differentiation, namely, to the pre-Sertoli and Sertoli cells of the testis, and the granulosa and epithelial cells of the ovary [81]. Taken together, these observations have suggested that WT1 is required at the earliest stage of the undifferentiated gonad, before sexual differentiation occurs, with a later role within supportive cells of the mature gonad. WT1 mutations have been reported in undifferentiated gonadoblastomas arising in pa-

tients with DDS [30], but not in more mature ovarian or testicular tumors.

The coexpression of WT1 and the orphan nuclear receptor, SF1 in Sertoli cells of the developing gonad, along with their similar knockout phenotype, prompted analysis of a potential interaction between these two transcription factors, leading to the regulation of MIS expression [166]. WT1(−KTS) and SF1 synergistically activate the MIS promoter through an SF1-binding site. Interestingly, WT1(+KTS) does not show a similar effect suggesting some requirement for the WT1 zinc finger domain, although the enhancement of SF1-dependent transactivation appears to be independent of DNA binding by WT1. SF1 and WT1 proteins interact in both yeast and mammalian two-hybrid analyses and GST pull-down assays, although coimmunoprecipitation of WT1 and SF1 has yet to be demonstrated *in vivo*. Reduced expression of MIS resulting from inactivation of WT1(−KTS) is consistent with the male to female sex reversal phenotype associated with DDS [30]. However, the similar abnormalities of sexual differentiation present in Frasier syndrome are linked to a selective reduction in WT1(+KTS) transcripts [70] and presumably reflect additional functions linked to this isoform.

Another orphan nuclear receptor potentially linked to the role of WT1 in ovarian development is Dax1. The *Dax-1* gene resides on the X chromosome; individuals with an XY karyotype but a deletion in *Dax-1* develop normally as males, but duplication of the *Dax-1* locus (Xp21.2-p21.3) is associated with male to female sex reversal [200]. Dax-1 has been identified as a transcriptional target of WT1(−KTS) [165], but loss of WT1-dependent activation of Dax-1 would not be expected to cause sex reversal, based on the Dax-1 null phenotype. However, it is conceivable that the imbalance in the two WT1 isoforms present in Frasier syndrome might be associated with increased Dax-1 expression. Of note, Dax-1 can also interact with SF1 and interfere with SF1-mediated transactivation by recruiting the corepressor N-CoR [201]. Increased expression of Dax-1 may therefore modulate the induction of MIS by SF1 and WT1 proteins.

Finally, a potential interaction between WT1 and the male-determining factor SRY has been suggested [202], although conflicting data have been generated in different models [203]. Stable transfection of ES cell lines with a XX karyotype using a construct encoding SRY is associated with the induction of the *WT1* transcript, but not that of other genes implicated in male sexual differentiation, including SOX9, MIS, SF1, and P450 aromatase [202]. However, since WT1 is expressed in both testis and ovary, SRY is unlikely to be the only determinant of WT1 expression in the gonads. In transient transfection assays, WT1 itself activates the promoters of SRY, while it represses the MIS and androgen receptor promoters, although regulation of endogenous genes has not been demonstrated [203]. Taken all together, the role(s) of WT1 isoforms in sexual differentiation is likely to involve interactions with these well-characterized regulators of gonadal development, but the critical targets of WT1 itself remain to be identified.

DESMOPLASTIC SMALL ROUND CELL TUMOR (DSRCT)

WT1 Expression in Mesothelial Cells

In addition to its striking expression pattern in the kidney and gonads, *WT1* is expressed at high levels in the mesothelial cells that line visceral organs and the pleural, peritoneal, and pericardial surfaces [80, 82]. The mesothelial lining undergoes a mesenchymal to epithelial conversion, consistent with that observed in other *WT1*-expressing tissues. Of note, *wt1*-null mice succumb during early embryonic development to abnormalities of the heart and diaphragm [88]; while these tissues themselves do not express *wt1*, failure of inductive signals from the surrounding mesothelial lining might lead to malformations in these organs. Although *WT1* mutations have not been detected in asbestos-induced mesotheliomas [204–206], homozygous inactivation has been observed in a multicystic peritoneal mesothelioma that was not associated with exposure to this carcinogen [82].

EWS–WT1 Translocation in DSRCT

DSRCT is a recently recognized embryonic neoplasm with a predilection for young males, associated with an aggressive clinical course and poor prognosis [207]. The tumor typically arises within the abdominal cavity, and while its precise tissue of origin is uncertain, it frequently arises from the peritoneal lining, suggesting that it may originate from a primitive mesothelial cell type [208]. Histologically, the tumor belongs to the "small blue cell" class that includes Ewing sarcoma and other undifferentiated cancers of childhood, thought to be derived from immature progenitors. Tumor cells express a complex pattern of markers suggestive of epithelial, muscle, and neural lineages, consistent with their derivation from pluripotent progenitors. Of particular interest, however, is the marked desmoplasia that is characteristic of DSRCT, but not of other similar childhood cancers. Desmoplasia denotes the abundance of reactive stroma (including fibroblasts and endothelial cells) surrounding small nests of tumor cells. This striking histological characteristic of DSRCT is presumably the result of an interaction between the tumor and surrounding normal tissues, with secretion by cancer cells of factors that may stimulate fibroblast recruitment and angiogenesis, which in turn support tumor growth. In addition to its unique histological

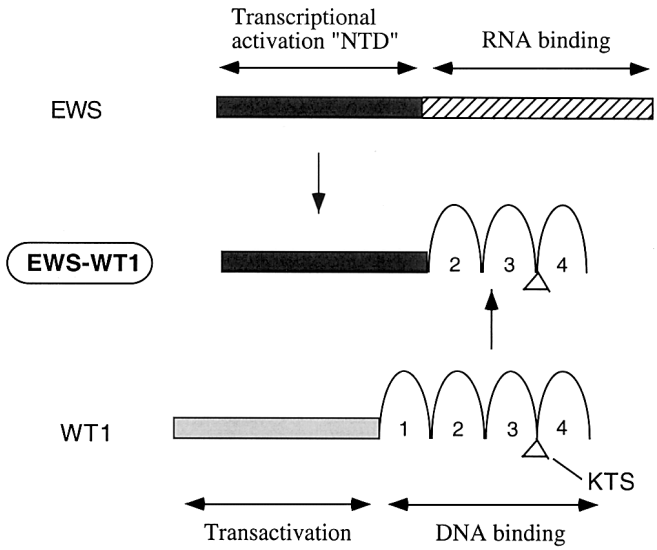

FIG. 5. EWS–WT1 translocation in desmoplastic small round cell tumor (DSRCT). Schematic representation of the chimeric translocation product that underlies DSRCT. The EWS gene product contains an N-terminal domain (NTD) that confers transcriptional activation properties, following its translocation to a heterologous DNA binding domain. The WT1 zinc fingers 2–4, including the KTS alternative splice, are fused to the NTD of EWS to generate a chimeric transcription factor.

appearance, DSRCT is remarkable for a defining genetic abnormality, the t(11;22) (p13;q12) chromosomal translocation, which generates a chimeric fusion of the Ewing sarcoma gene *EWS* and *WT1* [209]. To date, virtually all cases of pathologically confirmed DSRCT demonstrate the *EWS–WT1* translocation, marking this as an essential genetic event for development of this tumor [209, 210] (see Fig. 5).

In DSRCT, the translocation breakpoint within the EWS gene may occur in introns 7–10, resulting in fusion of the so-called N-terminal domain or "NTD" of EWS to the chimeric product [209]. This domain exhibits strong transactivational properties *in vitro,* and its recurrent fusion to heterologous DNA-binding domains underlies a number of malignancies, including Ewing sarcoma [211]. The C terminus of EWS (which is lost in the EWS–WT1 chimera) contains an 85-amino-acid RNA recognition motif, leading to the suggestion that EWS itself may be an RNA-binding protein [212]. The N terminus of EWS, which is translocated to the WT1 DNA-binding domain in DSRCT, has significant homology to the TLS protein (another chromosomal translocation target) [213, 215] and more modest homology to the C-terminus of RNA polymerase II and the TATA-binding protein-associated factor-68 ($TAF_{II}68$) [214]. These and other studies have led to the suggestion that EWS may be associated with some aspect of basic transcriptional regulation and processing. Distinct translocations involving the NTD of EWS have been implicated as the initiating genetic lesion in a number of different cancers. Thus, the NTD of EWS is translocated to the DNA-binding domain of ETS-family transcription factors (FLI1, ERG, or ETV1) in the Ewings sarcoma/PNET family of tumors [216–218]. In melanoma of soft parts, the same domain of EWS is fused to the DNA-binding domain of the transcription factor ATF1 [219], and in myxoid chondrosarcoma, to the DNA binding domain of CHN [220]. The EWS-related gene TLS is also translocated to the DNA-binding domain of CHOP in myxoid liposarcoma [213]. In all these cases, a consistent somatic chromosomal rearrangement leads to a novel chimeric transcription factor, whose regulation of specific target genes presumably drives malignant transformation.

The breakpoint within the *WT1* gene is invariant, between exons 7 and 8 [210]. This effectively removes the first DNA-binding zinc finger of WT1 and fuses zinc fingers 2–4 to the NTD of EWS. The EWS–WT1 chimera thus encodes a novel transcription factor, with a potent transcriptional activation domain derived from EWS and a DNA-binding domain derived from a subset of the WT1 zinc fingers. The reciprocal product of the t(11;22) (p13;q12) chromosomal fusion is not expressed, confirming that EWS–WT1 is the underlying molecular lesion [209]. Of note, the KTS alternative splice, between zinc fingers 3 and 4 of WT1, is preserved in EWS–WT1. Synthetic fusion of the NTD of EWS to the DNA-binding domain of GAL4 results in a potent transcriptional activator of GAL4-dependent promoters [221]. The translocation of this domain onto a heterologous DNA-binding domain thus provides the resulting chimera with a potent transactivator domain. The frequency with which the NTD of EWS is translocated to the DNA-binding domains of distinct transcription factors in different tumor types suggests that oncogenic tissue specificity is directed by the DNA-binding domain provided by the translocation partner. However, the chimeric protein is placed under control of the native EWS promoter, which is likely to alter its expression profile. Distinct protein interactions mediated by the NTD of EWS may also alter the identity of responsive promoters.

As for WT1 itself, the identification of transcriptional targets for EWS–WT1 has been complicated by the difficulty in predicting the regulation of endogenous genes based on transient transfection studies using EWS–WT1-responsive promoter–reporters. Most GC-rich promoter constructs that are repressed by WT1(−KTS) show potent activation by EWS–WT1(−KTS) [222, 223]. While this might suggest an appealing model, whereby growth-inducing genes may be repressed by the WT1 tumor suppressor and induced by the EWS–WT1(−KTS) oncogenic product, this has largely not been supported by analysis of native genes following inducible expression of EWS–WT1

[224]. Nonetheless, analysis of osteosarcoma cells with tetracycline-regulated expression of EWS–WT1(−KTS) has identified one potential target gene, *PDGF-A* [224]. The *PDGF-A* promoter is repressed by WT1(−KTS), although the endogenous transcript is not affected by inducible expression of wild-type WT1 in these cells. In contrast, induction of EWS–WT1(−KTS) triggers expression of the endogenous *PDGF-A* mRNA, specifically the 2.3- and 2.8-kb species whose transcription is driven from the WT1-responsive promoter. The presence of the EGR1 and TC-rich sites flanking the start site appears to be essential for activation of the promoter by EWS–WT1(−KTS) in transfection assays. Significantly, endogenous PDGF-A is expressed at high levels in primary DSRCT tumors. Its expression is restricted to the nests of tumor cells, where secretion of this potent chemoattractant may contribute to the recruitment of stromal fibroblasts and endothelial cells. Further insight into the biological properties of EWS–WT1 awaits the identification of additional transcriptional targets, potentially using differential expression screening. Functional studies have also been limited by the absence of cultured tumor cell lines and mouse models and even by uncertainty about the specific cell of origin for DSRCT. In primary rodent cells, expression of EWS–WT1 does not cooperate with classical combinations of oncogenes in achieving cell transformation (K. Nichols and D. Haber, unpublished). However, retroviral expression of EWS–WT1(−KTS) in NIH 3T3 cells does appears to confer transformed properties [225].

ROLE OF *WT1* IN HUMAN ACUTE LEUKEMIA

The high levels of *WT1* expression in most human acute leukemias raised the possibility that this tumor suppressor may also contribute to hematopoietic malignancies and, by implication, with normal hematopoiesis itself. Data in this field have been controversial with some observations suggesting an oncogenic role for wild-type *WT1*. A subset of acute leukemias, approximately 10%, have inactivating mutations in WT1 [226–228] while the majority express high levels of the wild-type transcript [82, 105, 106]. This discrepancy is similar to that observed with sporadic Wilms tumor. As proposed in Wilms tumor, it is possible that leukemias expressing wild-type *WT1* have mutations in downstream pathways or that expression of *WT1* in these cells is simply a marker of their original cell lineage. While mature hematopoietic cells do not express *WT1*, RT-PCR analysis of very early bone marrow precursors does suggest presence of the transcript [229]. Other investigators have suggested that *WT1* is aberrantly expressed in human leukemia and that it may in fact contribute to the malignant phenotype. This hypothesis is supported by some [230–232], but not all clinical studies [233], suggesting a possible correlation between *WT1* expression and more primitive and refractory leukemias. In addition some *in vitro* studies using antisense technology [234], as well as stable transfection of *WT1* constructs have raised the possibility that *WT1* expression abrogates differentiation in leukemia-derived cell lines [109, 235–239]. These studies have been limited by their reliance on individual stably selected *WT1*-expressing clones, which may have lost their responsiveness to differentiation agents during the selection process or as a direct adaptation to *WT1* expression. In fact, we have recently achieved high titer retroviral infection of bone marrow progenitors, as well as leukemia-derived cells using constructs encoding the different WT1 isoforms (L. Ellisen and D. Haber, unpublished). The potent and immediate induction of myelomonocytic differentiation following ectopic expression of *WT1* strongly supports its role as a tumor suppressor in human leukemia and argues against an oncogenic effect. Studies in mouse leukemic models also support a tumor suppressor effect of ectopic WT1 (239, 240). The possible contribution of *WT1* to physiological hematopoiesis is less clearly defined. While the *in vitro* induction of differentiation in leukemic cell lines is associated with downregulation of endogenous *WT1* (229, 241–244), this may simply reflect the differentiation of precursor cells to more mature hematopoietic cell types in which *WT1* is no longer expressed. *Wt1*-null mice die before mature hematopoiesis is achieved, but no gross abnormalities have been reported in blood islands in these mice [88] or in mice whose developmental arrest is delayed by expression of transgenic WT1-containing YACs [91].

FUNCTIONAL PROPERTIES OF *WT1*

As described in this review, many lines of evidence support the concept of *WT1* as a critical regulator of organ-specific differentiation, whose inactivation or disruption triggers malignant transformation in embryonic cell types. These observations include the striking developmentally regulated expression pattern of *WT1* in the kidney, the consequences of its inactivation in human syndromes and mouse models, and the identification of downstream target genes implicated in cellular differentiation pathways. Additional insight has emerged from ectopic expression of WT1 isoforms, although these have been limited by the nature of the cell types studied. As for other tumor suppressor genes that affect cellular proliferation, caution must be exercised in interpreting experiments using stably selected clonal cell lines, since they are likely to have undergone additional genetic changes that allow them to tolerate *WT1* expression. For these reasons, inducible gene expression systems or acute retroviral infection of uncloned cell populations provide more reliable ap-

proaches to dissect the effects of ectopic WT1 expression.

Transfection of WT1 isoforms into the Wilms tumor-derived cell line RM1, harboring an altered *WT1* transcript, leads to suppression of colony formation and reduced tumorigenesis in nude mice [78]. Similar observations have been made using the G401 cell line, which is derived from a rhabdoid tumor of the kidney and does not express endogenous *WT1*, and NIH 3T3 and F9 embryonal carcinoma cell lines [158, 245–247]. In osteosarcoma cell lines expressing low levels of the wild-type transcript, inducible expression of *WT1*, particularly the (−KTS) isoform, triggers an initial G_1 cell cycle arrest, followed by apoptosis [139]. The cell cycle effect is correlated with the direct transcriptional activation of $p21^{Cip1}$ [164, 173]; apoptosis in these cells is associated with downregulation of EGFR expression and is rescued by constitutive expression of EGFR, suggesting that it results from the withdrawal of growth-factor-mediated survival signals. However, these and other models based on cancer-derived cell lines are limited in their ability to respond to potential differentiation signals. More recently, we have succeeded in expressing WT1 isoforms in primary blood cell precursors and differentiation-competent leukemic cell lines (L. Ellisen and D. Haber, unpublished). In this setting, ectopic expression of *WT1* is a potent and immediate inducer of lineage-specific cellular differentiation.

The ability of *WT1* to directly induce cellular differentiation provides an attractive model that may explain the consequences of its inactivation, namely aberrant genitourinary development and persistence of the pluripotent renal precursors that give rise to Wilms tumor. However, other functional properties of *WT1* remain mysterious, including its importance to the survival of undifferentiated renal blastemal cells, as demonstrated by their apoptosis in *wt1*-null mice. It is possible that *WT1* mediates distinct effects in different cell types and that it directly enhances survival of these renal stem cells, while promoting differentiation of more mature renal precursors. Alternatively, *WT1* may promote cell survival indirectly, by promoting reciprocal differentiation signals between mesenchyme and ureter, which themselves serve to prevent apoptosis of blastemal stem cells. Also unknown are the precise transcriptional targets of *WT1* that are essential for its tumor suppressor function and for its roles in renal, gonadal, and potentially hematopoietic differentiation. While most studies have focused on the identification of potential transcriptional targets, the possible role of the most abundant isoform, WT1(+KTS), in some aspect of pre-mRNA splicing has raised important questions as to its precise function and its physiological targets. The multiple isoforms of WT1 that are generated through alternative splicing of this zinc finger gene are likely to provide new insight into the coordination of transcriptional and nontranscriptional mechanisms that may contribute to genitourinary differentiation. Finally, the identification of additional Wilms tumor genes, and of their potential interactions with *WT1*, will provide a better understanding of the cellular pathways that lead to this pediatric kidney cancer.

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