The Predominant WT1 Isoform (+KTS) Encodes a DNA-Binding Protein Targeting the Planar Cell Polarity Gene Scribble in Renal Podocytes

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Abstract

WT1 encodes a tumor suppressor first identified by its inactivation in Wilms’ Tumor. Although one WT1 splicing variant encodes a well-characterized zinc finger transcription factor, little is known about the function of the most prevalent WT1 isoform, whose DNA binding domain is disrupted by a three-amino acid (KTS) insertion. Using cells that conditionally express WT1(+KTS), we undertook a genome-wide chromatin immunoprecipitation and cloning analysis to identify candidate WT1(+KTS)-regulated promoters. We identified the planar cell polarity gene Scribble (SCRB) as the first WT1(+KTS) target gene in podocytes of the kidney. WT1 and SCRB expression patterns overlap precisely in developing renal glomeruli of mice, and WT1(+KTS) binds to a 33-nucleotide region within the Scribble promoter in mouse and human cell lines and kidneys. Together, our results support a role for the predominant WT1(+KTS) isoform in transcriptional regulation and suggest a link between the WT1-dependent tumor suppressor pathway and a key component of the planar cell polarity pathway. Mol Cancer Res; 8(7): 975–85. ©2010 AACR.

Introduction

WT1 is a tumor suppressor gene initially identified due to its inactivation in Wilms’ tumor (nephroblastoma), the most common pediatric kidney tumor (1–6). Since its initial discovery, WT1 is mutated in ~10% of sporadic Wilms tumors, to be translocated in tumors of mesothelial-derived cell types (the EWS-WT1 fusion in desmoplastic small round cell tumor), and to be both mutated and aberrantly expressed in a subset of acute leukemias (7, 8). WT1 is required for the normal development of the kidneys and gonads, and contributes to the development of the spleen, retina, and the mesothelial lining of the heart, diaphragm, and peritoneum (9–12). Consistent with its role during development, WT1 null mice die at day E13.5 due to malformations of the heart and diaphragm, and they lack development of both the kidneys and gonads. A detailed understanding of the function of WT1 would provide important insight into the molecular relationship between normal organ development and carcinogenesis.

WT1 is a 50-kDa protein that contains an amino terminal transactivation domain and a carboxy terminal DNA binding domain consisting of four zinc fingers. Alternative splicing of WT1 leads to distinct isoforms, of which the most significant results from the variable insertion of three amino acids, lysine, threonine, and serine (KTS), in the critical linker between zinc fingers three and four (13). WT1(−KTS), with an uninterrupted zinc finger domain, is an extensively characterized transcription factor. Insertion of the KTS splice, however, dramatically alters the DNA binding properties of the WT1 zinc finger, such that no DNA binding sequence and no transcriptional target genes have been identified. Instead, WT1(−KTS) has been postulated to play a role in RNA metabolism, based on its partial subnuclear colocalization with interchromatin granules and association with splicing components (14–16), yet no clear function of WT1(−KTS) in RNA processing has been successfully shown.

The physiologic importance of WT1(+KTS) function is illustrated by Frasier syndrome, a sex reversal syndrome associated with severe developmental defects of the kidneys and gonads, caused by a splice donor mutation resulting in a decreased WT1(+KTS) to WT1(−KTS) ratio (17–19). WT1 isoform–specific mouse knockouts have been generated, revealing similar but not identical genitourinary defects [e.g., streak gonads in WT1(−KTS) null mice versus male to female sex reversal in WT1(+KTS) null mice; ref. 20]. Taken together, these data indicate that the two isoforms have overlapping but distinct functions and that...
maintaining the proper ratio of the two isoforms is critical for normal kidney and urogenital development.

Microarray-based searches for target genes of WT1 (+KTS) have failed to identify viable candidate target genes, and in vitro binding site selection screens have been unsuccessful (7). Therefore, we undertook a genome-wide in vivo DNA binding site screen to search for candidate target gene promoters physically bound by this WT1 isoform. We identified in vivo WT1(+KTS) binding sites within the promoters of several genes, of which the most striking was the planar cell polarity (PCP) gene SCRIBBLE (SCRIB). The remarkable temporal and spatial coexpression of WT1 and SCRIB in the developing kidney supports a previously unappreciated link between this developmentally regulated tumor suppressor and the PCP pathway. The identification of SCRIBBLE as a potential WT1(+KTS) target gene also define this critical WT1 isoform as a bona fide transcriptional regulator and identify key DNA sequences implicated in its specific DNA binding activity.

Materials and Methods

Cell culture

UB27 and UD28 cell lines, which express WT1(−KTS) and WT1(+KTS), respectively, were grown as previously described (21). Murine podocytes were also as previously described and induced to differentiate by removal of mouse IFN γ followed by thermoshifting from 32°C to 37°C (22). UB27 and UD28 cell lines were created in the laboratory of Dr. Daniel Haber (Massachusetts General Hospital, Charlestown, MA) in 1994, whereas podocytes were obtained from Dr. Jochen Reiser (Massachusetts General Hospital, Charlestown, MA) in 2005. None of these cell lines have been authenticated or tested.

Tissue collection

Kidneys were collected from C57BL/6J mice within 18 hours of birth. Animals were euthanized by decapitation, and kidneys were harvested from both male and female pups. Human kidneys (18-wk gestation) were collected following an Institutional Review Board–approved protocol.

Chromatin immunoprecipitation, and chromatin immunoprecipitation and cloning

WT1-bound chromatin fragments were isolated from UB27 and UD28 cells 16 hours after the withdrawal of tetracycline as previously described (23). Modifications included substituting cell lysis buffer (5 mmol/L piperazine-N,N’-bis[2-ethanesulfonic acid];1,4-Piperazinediethanesulfonic acid, 85 mmol/L potassium chloride, 0.5% NP40, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 100 ng/mL of leupeptin and aprotinin) for swelling buffer and using 1 μg of anti-WT1 antibody C19 conjugated to protein A agarose (Santa Cruz Biotechnology) for the first immunoprecipitation step. Anti-WT1 antibody WTc8 (21) was used for the second immunoprecipitation step. The resulting DNA fragments were digested with Sau3A1, passed over a ChromaSpin-200 size fractionation column (BD Biosciences), and ligated into the BamH1 site of puc19 vector (New England Biolabs).

Standard chromatin immunoprecipitation (ChIP) assays were done as previously described for tissues (24). Antibodies used for ChIP analysis included WT1 (C19; Santa Cruz), WT1 WTc8 (21), RNA polymerase (N20; Santa Cruz), acetylated histone H3 (06-599, Upstate), and rabbit IgG (Santa Cruz).

Reverse transcription-PCR

RNA was isolated from various mouse tissues using Trizol (Invitrogen) followed by purification using RNeasy columns (Qiagen) according to the manufacturer’s protocols. For each reaction, 1 μg of RNA was reverse transcribed and amplified by PCR using gene-specific primers and EZ rTth (Applied Biosystems) according to the manufacturer’s protocol.

In situ hybridization

Neonatal mouse kidneys were cross-linked overnight in 4% paraformaldehyde at 4°C then successively washed in 10%, 15%, and 20% sucrose and embedded in OCT (Sakura). Embedded tissues were sectioned, mounted on glass slides, and hybridized to digoxigenin (DIG)-labeled riboprobes according to standard methods (25). The probe used for SCRIB has been previously described (26), whereas the probe for murine WT1 spanned from nt1321 to nt1756 of the WT1 mRNA sequence.

Quantitative PCR

RNA was extracted from the kidneys of C57BL/6 mice as described for reverse transcription-PCR. Expression levels of Scribble and WT1 were measured with gene-specific primers and were normalized to glyceraldehyde-3-phosphate dehydrogenase as previously described (27).

Western blots

Whole-cell extracts were prepared from UB27 and UD28 grown in the presence of tetracycline or 16 hours after tetracycline removal, from murine podocytes grown at 32°C or at specified times after thermoshift to 37°C, and from murine podocytes after infection with small hairpin RNA (shRNA) expressing lentiviruses followed by 48 hours of puromycin selection. Equal amounts of protein from each extract were separated by electrophoresis on 10% or gradient Tris-HCl gels (Bio-Rad), transferred to membrane, and incubated with appropriate antibodies. Antibodies against WT1 (C19), β-tubulin (H235), Scribble (H300), and Actin (H300) were purchased from Santa Cruz Biotechnology.

Glutathione S-transferase fusion proteins, whole-cell extracts, and electrophoretic mobility shift assays

Glutathione S-transferase (GST) fusion protein constructs expressing the zinc finger domains of WT1(−KTS) and WT1(+KTS) were expressed in Escherichia coli BL21 as previously described (28). Whole-cell extracts of UB27 and UD28 cells were made as previously described (29). Electrophoretic mobility shift assay (EMSA) probes
>50 bp in length were generated by PCR, cloned into the pUC19 vector, and sequenced. EMSA probes <50 bp were synthesized and annealed. All probes were end labeled with $^{32}$P and 1 ng of probe was used per reaction. Binding reactions were done as previously described (28) with the exception that labeled probes were not added until the last 20 minutes of incubation. Competitor probes, if used, were added during the first 10 minutes of incubation.

**Luciferase constructs and assays**

Scribble reporter constructs were generated by cloning the identified 23-bp WT1(+KTS) binding site plus 5 nt each of 5′ and 3′ native flanking sequence into pGL3 Basic (Promega). For luciferase reporter assays, 25 ng reporter were cotransfected into NIH3T3 cells in 12-well format, along with transfected WT1B (21), WT1D (21), SP1 (OriGene), AP2 α (OriGene), AP2 (OriGene) β, or empty vector. Luciferase activity was measured using the Dual-Glo Luciferase Assay system (Promega) and was normalized by Renilla luciferase that is cotransfected for transfection efficiency control. All experiments were done in quadruplicate. $P$ values were calculated by performing pairwise Student’s $t$ test analysis.

**Lentiviral vectors and infection**

pLKO.1-shRNA lentiviruses targeting WT1 were obtained from the Broad collection (30). pLKO.1-shRNA lentiviruses targeting Scribble were created by annealing primers and cloning into the Age I-EcoRI site of pLKO.1 vector. The sequences of all constructs were verified by sequencing. Murine podocytes were infected with shRNA lentiviruses as previously described (30), and a detailed protocol is available at http://www.broadinstitute.org/rnai/trc/lib. Lentivirus constructs expressing WT1(+KTS) or WT1(+KTS) were created by cloning full-length murine WT1 into pLKO.1 vector. These constructs were used in an identical manner as the shRNA constructs to produce WT1-expressing lentivirus.

**Small interfering RNA**

Knockdown of WT1 and Scribble expression in murine podocytes was accomplished using Stealth RNAi small interfering RNA (siRNA) constructs (Invitrogen). Several constructs were designed for each gene using the manufacturer’s software. Constructs that effectively knocked down the expression of the same target gene were pooled. Stealth RNAi siRNA–negative control hi GC (Invitrogen) was used as a negative control. Murine podocytes were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol.

The sequences of the primers used for various techniques and constructs are listed in Supplementary Table S2.

**Results**

To identify target genes for WT1(+KTS), we performed a genome-wide binding site screen using “ChIP-cloning,” involving sequencing of anti-WT1–immunoprecipitated formaldehyde–cross-linked chromatin fragments. We chose not to use hybridization to known promoter fragments or tiling arrays (ChIP-chip) due to uncertainty as to whether WT1(+KTS) might bind outside of traditional promoter fragments. ChIP followed by high-throughput sequencing (ChIP-seq) was not available when this project was initiated. For our screen, we used two well-characterized U2OS-derived cell lines previously used to study WT1 function, UD28 and UB27, which conditionally express tetracycline-regulated WT1(+KTS) or WT1(−KTS), respectively (21). WT1(−KTS)–inducible cells were used as a positive control for this binding site screen aimed at identifying candidate WT1(+KTS) target genes. Sixteen hours after the removal of tetracycline, cells were fixed with formaldehyde, lysed, sonicated; and the resulting chromatin fragments were sequenatively immunoprecipitated with two different antibodies against WT1 (C19 and WtC8), cloned into pUC19, and sequenced. Of 364 inserts isolated from WT1(+KTS)-bound chromatin, 109 corresponded to 92 different known genes; 194 represented uncharacterized genomic regions; 57 were repetitive DNA elements; and 4 did not have a database match. Similarly, we sequenced a total of 140 inserts from WT1(−KTS)–expressing cells. Of these, 32 inserts corresponded to 27 unique known genes; 80 were uncharacterized genomic regions; 27 were repetitive DNA elements; and 1 had no database match. Although none of the 27 candidate WT1(+KTS) binding sites were associated with previously characterized WT1 target genes, we were able to amplify the known WT1 target gene AMPHIREGULIN (AREG) from the pool of immunoprecipitated chromatin fragments, indicating that our screen was effective albeit not saturating.

From our list of candidates, we selected five genes for further analysis based on their proposed functions and/or knockout mouse phenotypes: (a) Scribble (SCRIBE), a PCP gene initially shown to function as a tumor suppressor protein in Droso phila; (b) the Bardet-Biedl syndrome 1 (BBS1) gene, another PCP gene associated with a pleiotropic genetic disease characterized by obesity, retinal degeneration, kidney malformations, olfactory deficits, and polydactyly; (c) insulin-like growth factor 2 mRNA binding protein 2 (IMP2), a regulator of insulin-like growth factor II biosynthesis, a key growth factor implicated in Wilms tumorogenesis; (d) heparan sulfate 2-sulfotransferase (HS2ST1); and (e) fibroblast growth factor 18 (FGF18), two genes thought to function in the same signaling pathway. Hs2st1 null mice are remarkable for kidney agenesis. The identity of all known genes isolated in this screen is listed in Supplementary Table ST1.

We first measured the expression levels of the five candidate target genes using reverse transcriptase-PCR of RNA isolated from various mouse tissues and a cultured mouse renal podocyte cell line. The expression patterns for all five genes correlated well with the expression pattern for WT1, with the highest RNA levels in testes, kidney, and podocytes (Fig. 1A). Next, we confirmed and mapped the binding of WT1 to the promoters of the five candidate...
genes using standard ChIP assays in UB27 and UD28 cells. In all five cases, anti-WT1 antibodies immunoprecipitated DNA sequences within 1 kb of known initiator codons, either within the putative promoter or first intron of the target genes (Fig. 1B). The known WT1 binding site within the promoter of AREG served as a positive control for binding of WT1(−KTS), whereas the Albumin promoter was used as a negative control. To best characterize the DNA binding properties of WT1(+KTS), we selected SCRIB for further detailed analysis.

ChIP assays of both neonatal mouse kidneys (Fig. 1C) and embryonic (18 wk) human kidneys (Fig. 1D) showed that WT1 is physiologically bound to the SCRIB promoter.

To define the precise binding site for WT1(±KTS) within the SCRIB promoter, we performed ChIP analysis of UD28 and UB27 cells, 16 hours after the induction of WT1 expression. Binding of WT1(±KTS), but not WT1(−KTS), was observed to a SCRIB promoter region that was amplified by primers located −250 bp upstream of the initiator ATG (primer set 2; Fig. 2A, lane 6), whereas no WT1 binding was detected to sequences amplified by primers located either −400 bp upstream (primer set 1) or −460 bp downstream (primer set 3) of primer set 2 (Fig. 2A, lanes 1 and 11). The WT1 binding site is therefore located within the 1.2-kb region located between primer set 1 and primer set 3. Radiolabeled probes spanning this 1.2-kb region were

FIGURE 1. Confirmation of candidates from WT1 binding site screen. A, reverse transcription-PCR analysis of expression levels of Wt1, Scrib, Bbs1, Imp2, Fgf18, Hs2st1, and Gapdh in RNA isolated from various mouse tissues and a podocyte cell line. B, ChIP analysis of protein binding to candidate target gene promoters in U2OS-derived cell lines 16 hours after induction of either WT1(−KTS) or WT1(+KTS). Protein binding to the Amhiregulin promoter is shown as a positive control, whereas analysis of protein binding to the Albumin control is shown as a negative control. Binding of RNA polymerase II (RNAP) and acetylated histone H3 (AcH3) were included as positive controls and IgG as a negative control. C, ChIP analysis of protein binding to the promoters for Scribble, Amhiregulin, and Dihydrofolate reductase (Dhfr) in neonatal mouse kidneys and (D) to the promoters for Scribble, Amhinegulin, and Albumin in embryonic (18 wk) human kidneys.
FIGURE 2. Localization of the WT1(+KTS) binding site within the Scribble promoter. A, ChIP analysis of WT1 binding to the Scribble promoter in WT1 (+KTS)- and WT1(-KTS)-expressing cells 16 h after the induction of WT1 expression. We also monitored binding of RNA polymerase II (RNAP) and acetylated histone H3 (AcH3) as positive controls and IgG as a negative control. Three sets of primers were used to localize the binding of WT1 to within a ∼1.2-kb region of the Scribble promoter. B, alignment of the mouse and human sequences of the region encompassed by gel shift probe #2. Shaded nucleotides are conserved between the two species, and the region containing the WT1 binding site is underlined. C, binding of GSTWT1 fusion proteins (left) and whole-cell extracts of WT1(+KTS)- and WT1(-KTS)-expressing cells (middle and right) to a 33-bp radiolabeled probe of the WT1 binding site within the Scribble promoter as measured by EMSA. Addition of one of two different antibodies against WT1, C19, and the mixed monoclonals WT12+56 diminishes the amount of protein binding to the WT1 site probe. The far right measures expression of WT1 in the cells grown in the presence or absence of tetracycline as measured by Western blot analysis. The level of β-tubulin expression is measured as a loading control.
used in EMSAs to further refine the potential WT1(+KTS) binding site to an 843-bp region (data not shown; Supplementary Fig. S1). Incubation of whole-cell extracts derived from induced and uninduced UD28 and UB27 cells, and GST-WT1 fusion proteins to overlapping probes within this 843-bp region combined with anti-WT1 antibody supershifts narrowed the WT1 binding site to a 128-bp fragment, containing a 23-bp region that was perfectly conserved between human and mouse (data not shown; Fig. 2B). EMSA of radiolabeled probes containing this site with various amounts of native 3′ and 5′ flanking sequences defined the minimal WT1 binding site as consisting of this conserved 23-bp sequence plus 5 bp of both the 3′ and 5′ native flanking sequence (data not shown; Fig. 2C). The binding activity observed with extracts from UD28 cells to a probe containing this 33-bp sequence was dependent on the induction of WT1(+KTS) and was reduced following incubation with a polyclonal antibody against WT1 (lane 10) or a mixture of two monoclonal anti-WT1 antibodies (lane 11).

The binding specificity of WT1 for this site was confirmed by EMSA, using GST fusion proteins and competition with excess unlabeled probe (Fig. 3). To identify nucleotides critical for binding of GST-WT1(+KTS) fusion protein, we used a panel of probes with each nucleotide individually mutated to a T residue, with the exception of the one wild-type T residue (nucleotide #12) that was mutated to an A residue (Fig. 3B). This analysis revealed a central stretch of 10 nucleotides, from nucleotides #8 to #17, which are critical for GST-WT1(+KTS) binding. Mutation of any one of these nucleotides abrogated binding (Fig. 3B, lanes 10-19). In addition to these 10 nucleotides, mutation of nucleotides #1, #31, and #32 also abolished binding (Fig. 3B, lanes 3, 33, and 34), whereas mutation of either nucleotide #24 or #25 diminished binding activity. The same nucleotides, #1, #8 to #17, #31, and #32, are required for in vitro binding by GST-WT1(+KTS) (Fig. 3C, lanes 3, 10-19, 34, and 35). In contrast to GST-WT1(+KTS), however, binding of GST-WT1(−KTS) was greatly diminished by mutation of nucleotides #18 to #23 and #25 to #28 (Fig. 3C, lanes 20-25 and 27-30). Overall, binding of WT1(−KTS) to this sequence was weaker than binding of WT1(+KTS), but specific binding of WT1(−KTS) was enhanced by mutation of nucleotide 7 (Fig. 3C, lane 9), presumably because this mutation renders the sequence more comparable with the consensus WT1(−KTS) binding site WTE. Through ChIP and EMSA analysis, combined with mutational analysis, we propose 5′-ACCAAGCGGATGGCGAGCGGCCGCCGCCGCCGCCGCCG-3′ as a candidate WT1(+KTS) binding site (WKE) within the SCRIB promoter. Of note, the striking selectivity of DNA binding by WT1(+KTS) observed in vivo was clearly relaxed in vitro, thus limiting the detailed analysis of binding differences between (+KTS) and (−KTS) variants of WT1.

The unique pattern of WT1 expression in the developing kidney provides a compelling measure of developmental regulation. A single section of fetal kidney contains subcortical aggregates of condensing mesenchyme (blastemal precursors), with progressive stages of differentiated structures, from comma-shaped bodies to S-shaped and immature glomeruli, as differentiation progresses internal to the cortical border. RNA in situ hybridization revealed a nearly complete overlap in the expression patterns of WT1 and SCRIB, with both genes expressed in the blastemal cells of the condensed mesenchyme, comma- and S-shaped bodies of the kidney cortex, and in the more developed mature glomeruli (Fig. 4A). Expression of WT1 is tightly regulated during renal development, peaking ~4 days after birth in mice and declining thereafter. As measured by quantitative PCR, SCRIB levels are also high in the kidneys of neonatal mice and decline thereafter, leveling off ~3 weeks after birth (Fig. 4B). Thus, SCRIB gene expression mirrors that of WT1 with an ~1-week delay.

In mature kidneys, expression of both WT1 and SCRIB is confined to podocytes. We analyzed temperaturesensitive SV40 T antigen–immortalized mouse podocytes that differentiate upon temperature shift from 32°C to 37°C. Western blot analysis showed that the levels of both WT1 and SCRIB proteins declined in parallel as cells were induced to differentiate following thermostimulation to the restrictive temperature (Fig. 4C). To test for functional regulation of the SCRIB promoter by WT1, Northern blot analysis of UD28 and UB27 cells grown in the absence or presence of tetracycline (Supplementary Fig. S2) were done. Induction of neither WT1(−KTS) nor WT1(−KTS) expression resulted in altered expression levels of SCRIB mRNA. Next, we cloned the 33-bp binding site identified by EMSA into a promoter containing a luciferase reporter construct. Cotransfection of NIH-3T3 cells with this construct plus a WT1(+KTS) expression construct resulted in an average 3-fold induction, whereas cotransfection with a WT1(−KTS) expression construct resulted in a 2-fold induction (Fig. 5A). The identified WT1 binding site within the SCRIB promoter also contains consensus binding sites for the transcription factors Sp1 and AP-2. Cotransfection of neither Sp1, AP-2 α, nor AP-2 β expression constructs affected WT1-mediated transactivation (data not shown), indicating that these proteins are unlikely to be functional cofactors in SCRIB gene regulation.

We studied the effects of WT1 overexpression on SCRIB gene expression by infecting murine podocytes with lentivirus constructs expressing empty vector, WT1(−KTS), or WT1(+KTS). Overexpression of WT1(+KTS) in podocytes resulted in a modest but consistent increase in SCRIB protein levels, whereas WT1(−KTS) had no effect (Fig. 5B). Knockdown of WT1 expression in murine podocytes, either by pools of three WT1 siRNA constructs or by infection with lentiviral shRNA constructs resulted in decreased baseline mRNA levels of SCRIB (Fig. 5C and D). Taken together with the DNA binding site definition, ChIP, and precise coexpression of these transcripts in specific structures of the developing kidney, these results suggest that WT1(+KTS) directly regulates SCRIB gene expression.

Published OnlineFirst June 22, 2010; DOI: 10.1158/1541-7786.MCR-10-0033
Discussion

Using a ChIP cloning strategy applied to cells with inducible expression of WT1 isoforms, we identified candidate target genes, including some whose promoters are bound by the WT1(-KTS), WT1(+KTS), or both variants. Among these, SCRIB is of particular interest in that it is bound exclusively by WT1(+KTS), displays a precise

FIGURE 3. Mutational analysis of the WT1 binding site within the Scribble promoter. A, GSTWT1 fusion proteins, but not GST alone, bind to the Scribble promoter WT1 binding site and can be competed for by the addition of a 500-fold excess of unlabeled WT1 site probe (WT). A 500-fold excess of unlabeled nonspecific (NS) probe does not compete for binding. Binding of GSTWT1(+KTS) (B) and GSTWT1(-KTS) (C) to Scribble promoter WT1 binding site probe (lane 2) and to probes in which one nucleotide has been mutated to T (lanes 3-35), with the exception of the only wild-type T residue that has been mutated to an A residue (lane 13). GST alone does not bind to the probe (lane 1).
temporal and spatial colocalization of expression with WT1 in differentiating structures of the developing kidney, and functionally links the PCP pathway to the Wilms tumor suppressor gene WT1. Detailed analysis of the SCRIB promoter identifies a DNA binding site for WT1(+KTS), the predominant splicing variant of WT1, which had not been previously implicated in transcriptional regulation.

WT1(+KTS) has been implicated in mRNA metabolism, based on several experimental observations including association with active splice complexes (15, 31). However, the nuclear localization of WT1(+KTS) is complex, and the precise subnuclear structures harboring this splice variant are not fully defined. WT1(+KTS) has also been reported to bind to RNA encoding exon 2 of insulin-like growth factor 2 and a 34-bp fragment of α-actinin 1, suggesting a role for this isoform in regulating gene expression at the posttranscriptional level (32, 33).

Our first indication that (+KTS)-containing zinc finger domains might mediate DNA binding came from studies of the EWS-WT1 translocation that defines another
pediatric cancer, desmoplastic small round cell tumor. This chromosomal rearrangement fuses the transactivational domain of the EWS gene to the three COOH-terminal zinc fingers of WT1 (omitting zinc finger 1), but retaining the (+/−KTS) splice. In expression-based screens, we identified distinct transcriptional targets for the two predominant chimeric oncogenes, EWS-WT1(−KTS) and EWS-WT1(+KTS), associated with distinct DNA binding sites (34, 35). Thus, although the EWS transactivational domain may have unmasked the DNA binding activity of the modified WT1(+KTS) zinc fingers, it suggests that insertion of (+KTS) may alter, rather than abrogate, DNA binding. Although expression-based screens for targets of the full WT1(+KTS) zinc finger domain were not successful, we were able to identify a DNA binding sequence through a genome-wide ChIP screen. Our results suggest that this isoform may indeed encode a DNA binding transcriptional regulator, albeit one with distinct binding specificity and transcriptional activation requirements from the well characterized WT1(−KTS).

The 5′-ACCAAGCGGATGCGAGCCGCGCCGC-3′ sequence identified here is the first physiologically validated WT1(+KTS) binding site, which we have named WKE for WT1(+KTS) binding element. This sequence bears little resemblance to WTE (28), the consensus WT1(−KTS) binding site, to alternative binding sites proposed for WT1(−KTS) (7), or even for E(+KTS) RE (34) or E-WRE (28, 36-39), defined as a binding sites for the EWS-WT1(+KTS) and EWS-WT1(−KTS) chimeras (lacking zinc finger 1), respectively (Table 1). It is noteworthy that the binding specificity of WT1 isoforms is context dependent: both WT1(−KTS) and WT1(+KTS) bind to WKE in vitro, whereas ChIP assays only identify WT1(+KTS) at the SCRIB promoter. Such in vitro context dependence may reflect the presence of other interacting proteins that may stabilize or destabilize the zinc finger/DNA interactions or the contribution of chromatin context. Furthermore, binding alone is insufficient for transactivation as robust binding of WT1(+KTS) to the SCRIB promoter in U2OS-based cell lines did not result in a

**FIGURE 5.** WT1 regulates Scribble expression. A, luciferase assay (B) Western blot analysis of murine podocytes infected with lentivirus expressing WT1(−KTS), WT1(+KTS), or empty vector. Blots were probed for WT1, Scribble, and, as a loading control, β-tubulin. C, RNA was isolated from untransfected murine podocytes and podocytes transfected with either a pool of three siRNA constructs targeting WT1 or a high GC negative control. Isolated RNA was amplified by reverse transcription-PCR with primers against either the WT1 or SCRIB promoter. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were measured as a control. D, Western blot analysis of WT1 and Scribble protein levels in murine podocytes infected with lentivirus hairpin constructs targeting Wt1 or Scrib. Levels of β-tubulin were measured as a loading control.
tumor risk papillomavirus oncoviral protein E6, the mammary targeted for proteasome-mediated degradation by the high-required for wound healing (44-49). Additionally, tumorigenesis, in which it has been proposed as a tumor

| Table 1. Comparison of identified binding sites for WT1 isoforms and fusion proteins |
|----------------|-----------------|---------------------------------|
| Element        | Specificity      | Sequence                        |
| WKE            | WT1(+KTS)        | 5′-AN6GGATGCGGAN13-3′            |
| WTE            | WTE             | 5′-GGCTGGAGGT-3′                 |
| WRE            | WRE             | 5′-CCGTGGTTGG-3′                 |
| E(+KTS)RE      | EWS-WT (+KTS)   | 5′-GGAGG(A/G)-3′                 |
| E-WRE          | EWS-WT (-KTS)   | 5′-(G/C)(G/C)(G/C)              |
|                |                  | TGGGGG-3′                       |

detectable change in SCRIB gene expression. According to this model, WT1(+KTS) is contributory or permissive for transactivation, but requires additional factors, accounting for the failure of expression-based screens, and pointing to the relevance of genomic binding approaches.

The interaction between the predominant isoform of WT1 and SCRIB links this key PCP gene to the regulation of renal development and tumorigenesis. Scribble contains four PDZ domains (26) that presumably function as scaffolds for protein-protein interactions localized to the adherens junctions and tight junctions of cellular membranes (40). In Drosophila, Scribble interacts both genetically and physically with the tumor suppressors lethal giant larvae (Lg) and discs large (DL) to regulate apical-basal polarity (41, 42), with Scribble loss of function mutations disrupting polarity and leading to impaired cell cycle exit and tissue overgrowth (43). In mammalian cells, SCRIB regulates epithelial cell migration in vitro and in vivo, and is required for wound healing (44-49). Additionally, SCRIB is targeted for proteasome-mediated degradation by the high-risk papillomavirus oncoviral protein E6, the mammary tumor–associated Adenovirus 9 E4-region, and the human T-cell leukemia virus type 1, suggesting that loss of cell polarity and disruption of cell junctions contributes to epithelial-derived cancers (40). Misexpression and mislocalization of SCRIB has recently been implicated in breast tumorigenesis, in which it has been proposed as a tumor suppressor (50). Interestingly, another key regulator of the PCP pathway, PAR3, has recently been implicated by inactivating mutations in diverse human cancers (51, 52). As such, induction of SCRB by WT1(+KTS) is consistent with both of their roles as suppressors of tumorigenesis.

Although a full knockout of Scribble has not been reported, in circletail (scribble<sup>Coc/Coc</sup>) mice, Scribble is truncated after the second PDZ domain (amino acid 971; ref. 26), and rumpelstilzchen (scribble<sup>rumz/rumz</sup>) mice contain an iso-lucene to lysine substitution at residue 285 (53). Both scribble<sup>Coc/Coc</sup> and scribble<sup>rumz/rumz</sup> mice have severe neural tube defects (craniorachischisis) and exencephaly, but no evidence of renal abnormalities, implicating either partial persistent gene function or functional redundancy in the role of Scribble during renal development. In zebrafish, however, Scribble is required for normal pronephros development as knockdown of zScrib resulted in the formation of pronephric cysts (54). As a target of WT1(+KTS), SCRIB may therefore be one of several downstream targets that together define the functional properties of the most abundant WT1 isoform.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Dr. Daphne Bell and members of her laboratory for assistance with sequencing, Dr. Keith Joung for the helpful discussions, and Dr. Jochen Reiser for the generous gift of cultured podocytes.

**Grant Support**

NIH grants R37CA58596 (D.A. Haber), K08DK080175 (M.N. Rivera), T32CA009216 (W.J. Kim); the Burroughs Wellcome Fund (M.N. Rivera), the Howard Hughes Medical Institute, Massachusetts General Hospital (M.N. Rivera), and a Tosteson postdoctoral fellowship from the Massachusetts Biomedical Research Corp. (J. Wells).

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Received 01/25/2010; revised 05/25/2010; accepted 05/26/2010; published OnlineFirst 06/22/2010.

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The Predominant $WT1$ Isoform (+KTS) Encodes a DNA-Binding Protein Targeting the Planar Cell Polarity Gene $Scribble$ in Renal Podocytes

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*Mol Cancer Res* 2010;8:975-985. Published OnlineFirst June 22, 2010.