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Ubiquitin specific protease 18 (*Usp18*) is a WT1 transcriptional target

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Abstract

Wilms tumor gene *WT1* encodes a zinc finger containing transcription factor which is required for renal development. Mutations in *WT1* are observed in 20% of Wilms tumor (a pediatric kidney cancer), but the *in vivo* WT1 targets and associated molecular pathways involved in the etiology of Wilms tumor are still elusive. To identify WT1 targets we performed genome wide comprehensive expression profiling using Affymetrix Gene Chip Mouse Genome 430 2.0 Array, comparing E13.5 mouse kidneys in which *Wt1* had been somatically ablated with littermate controls. We identified *Usp18* as the most differentially expressed gene in mutant kidney. Using tetracycline inducible cells we demonstrated a repressive effect of WT1 on *USP18* expression. Conversely, knockdown of WT1 led to the upregulation of *Usp18*. Furthermore, direct binding of WT1 to the *Usp18* promoter was demonstrated by ChIP assay. Overexpression of *USP18* in murine and human cell lines resulted in cell proliferation. Additionally, *Usp18* upregulation was observed in a mouse model of Wilms tumor. Taken together our data demonstrate that *Usp18* is a transcriptional target of WT1 and suggest that increased expression of *USP18* following WT1 loss contributes to Wilms tumorigenesis.

Keywords

USP18; WT1; Wilms tumor; Transcription; Target

Introduction

The *Wilms tumor gene 1* (*WT1*) encodes a four zinc finger-containing transcription factor and was originally identified by virtue of its inactivation in Wilms tumor (WT), a pediatric kidney neoplasm. WT is thought to arise during early kidney development from a mesenchymal cell that fails to differentiate but instead continues to proliferate abnormally (reviewed in reference [1]). Germline *WT1* mutations can result in WT, developmental

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Supplementary data

Supplementary Data is available at ECR Online: Excel file

anomalies or renal failure, and somatic *WT1* mutations are present in approximately 20% of all WT [2]. Somatic genetic ablation of *Wt1* in cells of the developing kidney, in conjunction with biallelic expression of *Igf2*, produces WT in mice, demonstrating that WT1 ablation is a critical alteration for tumorigenesis [3]. This role is presumably primarily due to the dysregulation of genes normally transcriptionally regulated by WT1.

Four major isoforms of the WT1 protein are produced by alternative splicing of exon 5 which encodes 17 amino acids and the terminal 9 nucleotides (three amino acids, lysine, threonine, and serine "KTS") of exon 9. In mammals the presence or absence of exon 5 has no known physiological significance. In contrast, the presence of the KTS amino acids significantly diminishes the DNA binding ability of WT1 and thereby alters WT's function as a transcription factor. The +KTS isoforms has been shown to localize with the spliceosome complex in the nucleus suggesting that +KTS is involved in post-transcriptional processes (reviewed in reference [4]) [5,6].

The binding of WT1 zinc finger domains to DNA is essential for its role as a transcription factor, and several WT1 consensus binding sites have been identified, notably the EGR-1 like consensus site, the WTE site, and a TCC rich motif [7,8,9]. Dozens of putative WT1 target genes have been identified based on the presence of these consensus sites and subsequently tested using *WT1*-inducible cell lines (reviewed in reference [10]). These putative target genes include those encoding transcription factors, growth factors and receptors, cell cycle regulators, cell adhesion molecules, extracellular proteins and cell polarity proteins. However, the identity of target genes that play a role in kidney development and whose dysregulation is an important step leading to tumor development is still not clear. The goal of the present study was to carry out an *in vivo* gene expression analysis to begin to identify WT1 target genes in fetal kidney and subsequently to confirm their transcriptional regulation by WT1 and assess their possible role in tumorigenesis.

Using a *Wt1* conditional knockout allele (*Wt1^{fl}*) and a tamoxifen TM -inducible Cre transgene (*Cre-ERTM*), we ablated WT1 in developing mouse kidney and compared the gene expression profile of mutant kidneys soon after ablation with kidneys from littermate controls. From this we identified *Usp18* as being dramatically upregulated following WT1 ablation. Subsequent experiments using two *in vitro* systems demonstrated that *Usp18* is a *bona fide* WT1 target gene, being upregulated following WT1 ablation and downregulated following WT1 over-expression. By luciferase reporter assay we identified the shortest promoter fragment responsible for WT1-mediated repression and also demonstrated direct binding of WT1 to the promoter of *Usp18*. Overexpression of *Usp18* in HEK293 or M15 cells promoted cell proliferation, consistent with the model that the increased expression of *Usp18* following *Wt1* mutation is a critical step in tumorigenesis. Additionally, *Usp18* upregulation was observed in a mouse model of Wilms tumor. Our study therefore establishes *Usp18* as a biologically relevant target of WT1 transcriptional function in the developing kidney, provides new insight into the potential mechanism by which WT1 ablation results in tumorigenesis and defines USP18 as a potential pharmacological target for antineoplastic treatment.

Materials and methods

Isolation of mouse embryonic kidney and microarray analysis

Wt1^{flox/flox} female mice were crossed with *Wt1^{-flox};Cre-ERTM* males. At day E11.5 pregnant females were intraperitoneally injected with 3mg/40gm body weight (BW) tamoxifen (Sigma) and sacrificed two days later. E13.5 kidneys were isolated and stored at -80°C. Genotyping was performed as described [11]. For microarray analysis RNA were prepared for each genotype from five pools of fetal kidneys, each pool consisting of kidney

pairs from four embryos. The microarray analysis was performed using Affymetrix GeneChip Mouse Genome 430 2.0 Arrays and carried out at the UT MD Anderson Microarray core facility.

Quantitative RT-PCR and Western Blot

cDNA was synthesized from 1.0 µg total RNA using TaqMan Reverse Transcriptase Reagents (Applied Biosystems). Real-time PCR reactions were carried out employing the SYBR PCR Master Mix (Applied Biosystems, UK) on a ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) in a 96-well format. PCR conditions were: 95°C for 15 min, followed by 40 cycles of three-step PCR including melting for 30 s at 95°C, annealing for 30 s at 60°C and elongation for 30 s at 72°C. Primer sequences are given in a supplementary Excel file. Expression levels were determined in one plate for all samples simultaneously and normalized to the corresponding amounts of *-Actin* cDNA measured within the same plate. Relative expression levels were calculated using the 2^{-CT} method [12]. Western blots were performed as described previously [15] using anti HA antibody (Cell Signalling), anti EGFR (Cell Signaling) and anti α -actin antibody (Sigma).

Cell culture, transfections, reporter assays and proliferation assay

Mouse mesonephric M15, MM and human embryonic kidney HEK293 cells were maintained in DMEM supplemented with 10% FCS (HyClone) at 37°C with 5% CO₂. T-REx™-293 (-KTS) and T-REx™-293 (+KTS) cells harboring the *WT1* episome were further supplemented with Zeocin and Blasticidin in the DMEM medium. To induce *WT1* expression, cells were grown in 1 µg/ml tetracycline for the indicated time.

Transfections in all cell lines were performed using SuperFect transfection reagent (Qiagen) in a 24-well format for reporter assay and 10 cm dishes for proliferation assays. For reporter assays, M15 and HEK293 cells were seeded at a density of 2×10^4 and 4×10^4 /well, respectively, a day in advance and transfected with 200 ng of reporter construct and an increasing amount of the *WT1* expression construct in a dose dependent experiment as indicated in the legend. In the luciferase reporter gene assays where *Usp18* promoter deletion constructs were used, 200 ng of the *WT1* expression construct was used. Each well was supplemented with empty vector to make the concentration of input DNA equal when necessary. Each well was also transfected with 5 ng of a renilla construct for normalization. Firefly and Renilla luciferase activities were measured 36 h after transfection using the Dual-Luciferase Assay kit (Promega) as recommended by the manufacturer.

For proliferation assays, HEK293 and M15 cells were transfected with *EGFP* or *EGFP-USP18* expression constructs. 36 hrs post transfection cells were cultured in the presence of 600 mg/ml or 2000 mg/ml G418 respectively for 2 weeks. Stably integrated cells were pooled and 40,000 cells (HEK293) or 20,000 cells (M15) were seeded into 6 well plates in the selection media. Each day cells were washed and trypsinized and counted in a hemacytometer to measure the cell growth.

RNA interference

For WT1 knockdown experiments M15 cells were seeded at 1×10^5 cells per well in a 6-well or 2×10^4 cells in a 24 well plate 1 day prior to transfection. siRNA (ON-TARGET plus SMARTpool duplex, Dharmacon, Lafayette, Colorado, USA) directed against WT1 was diluted to 100 µl or 60 µl in serum-free media to achieve a final concentration of 50 nM and 100 nM. 10 µl or 4 µl HyperFect Transfection Reagent (Qiagen) was added, respectively. Samples were vortexed, incubated at room temperature for 20 min and then added drop-wise to the cells. Three hours posttransfection media was replaced and cells were grown additionally for 24 or 36 hrs. siRNA against luciferase gene was used as a negative control.

Plasmids

3.5 kb murine *Usp18* promoter was originally cloned into *pXp2* vector which was a kind gift from Dr Zhang [13]. This plasmid was used as a template to amplify 3.4, 0.76 and 0.226 kb *Usp18* promoter fragments that contained the transcription start site by using the primers listed in Table 1. The 1.2 kb human *USP18* promoter was amplified by PCR and ligated into *pGL3-Basic* vector. Mutant constructs *0.226kbUsp18 A, B, C* and human *USP18* mutant promoter were created by PCR-based mutagenesis with oligonucleotide pairs listed in Table 1, using QuikChange II Site Directed Mutagenesis Kit (Stratagene) according to the manufacturer's recommendations. All plasmid constructs were verified by sequencing.

The *WT1* expression vectors, *FLAG WT1(-KTS)* and *(+KTS)* with exon 5 were constructed by inserting the coding region of *WT1* obtained from RT-PCR products from normal human RNA, engineered with a Kozak consensus translation initiation signal and an amino terminal FLAG tag sequence, into the polylinker of pBK-CMV (Agilent Technologies, Santa Clara, CA, USA) under the control of the CMV promoter. The *pEYFP-USP18* expression construct was a kind gift from Dr Alexander Sorkin [14]. *WT1(-KTS)* deletion constructs were generated by PCR using the primer pairs listed in Table 1, using the *FLAG WT1(-KTS)* plasmid construct as a template. All forward primers contained HA tag encoding sequence. PCR products were ligated into *pcDNA3.3 TOPO* expression vector (Invitrogen). The primer HA WT1F was used in combination of WT1fullR, WT1aR, WT1bR and WT1cR to generate full (full length), a (Zn 2–4), b (Zn 3–4) and c (Zn 1–4) expression constructs. Similarly, forward primers WT1g1F and HAN-delfor1 were used in combination with WT1fullR to generate the d (256–447) and e (180–447) expression constructs.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously with modifications [15]. A total of 6×10^6 M15 cells or 1×10^7 induced T-REx™-293 (-KTS) cells were incubated with 1% formaldehyde for 10 min at 37°C. The reaction was stopped by the addition of glycine, and cells were washed in 1X PBS and harvested. Cells were resuspended in 500 µl SDS lysis buffer (0.1% SDS, 30 mM Tris, pH 8, 10 mM EDTA, supplemented with protease inhibitors) followed by sonication to an average DNA length of 200–1000 bp. The sample was cleared by centrifugation and diluted to 3 ml in the same SDS lysis buffer containing 250 mM NaCl and 1% Triton X100. Aliquots of the diluted samples were kept as input controls. The lysate was precleared and then WT1 protein-DNA complexes were immunoprecipitated at 4°C overnight using antibodies to WT1 (agarose conjugated) (sc-192 AC; Santa Cruz), Histone H3 (Millipore), FLAG®M2 or anti-rabbit IgG antibody (Santa Cruz). To recover immune complexes, protein A agarose beads were added and incubated for 2 hrs at 4°C. Beads were washed thoroughly and the complexes were eluted and cross-links were reversed by adding 300 mM NaCl and overnight incubation at 65°C. Samples were treated with proteinase K and DNA was recovered in 40 µl elution buffer.

In situ hybridization

E13.5 kidneys from wild-type and *Wt1* mutant mice were fixed with 4% paraformaldehyde/phosphate buffered saline at 4°C, embedded into paraffin and cut into 5-µm-thick sections. For *in situ* hybridizations a 697-bp cDNA fragment of *Usp18* was amplified using the primers *mUsp18in1F* and *mUsp18in1R* and cloned into *pCRII-TOPO* (Invitrogen). *In situ* hybridizations on 6-µm paraffin sections were performed as described previously using digoxigenin-labeled (Roche) *Usp18* riboprobes [16].

Results

***Usp18* expression is increased in the *Wt1* mutant mouse kidney**

In order to identify WT1 target genes that might have a role in Wilms tumorigenesis, we performed genome-wide gene expression analysis comparing E13.5 kidneys in which *Wt1* was genetically ablated in ~85% of cells (*Wt1*^{-fl}; *ER-Cre*TM embryos) following TM treatment at E11.5 and kidneys from littermate controls (*Wt1*^{-fl}). With this dose of TM treatment we did not observe any histological differences between mutant and control kidneys [3]. Therefore, it is very likely that the changes in the gene expression profile detected in *Wt1*-mutant kidney at this timepoint is a direct consequence of WT1 ablation, not a consequence of histologic changes. Of the eight genes identified by microarray analysis at a false discovery rate of 0.001, *Usp18* (a.k.a. *Ubp43*) was the most significantly dysregulated. This result was confirmed by subsequent qPCR data which demonstrated *Usp18* to be up-regulated 120X in WT1-ablated kidney (Fig. 1A).

By *in-situ* hybridization *Wt1* expression was detectable in the condensed mesenchyme and developing nephrons in E13.5 mouse kidney. *Usp18* expression was detectable in developing structures of the kidney at this time. Interestingly, in kidneys from tamoxifentreated embryos, *Usp18* expression increased significantly following recombination and loss of WT1 function (Fig. 1C). To rule out the possibility of non-specific staining of *Usp18* we concomitantly used sagittal section of E13.5 mouse embryo and performed *in situ* hybridization using sense and antisense probes (Fig. 1B). No staining was observed following hybridization with a sense probe, while hybridization with the antisense *Usp18* probe revealed specific staining in the brain, olfactory epithelium, tongue, corpus straitum, gut anal canal and dorsal root ganglion.

***Usp18* expression is altered upon overexpression or knockdown of WT1**

To examine whether *USP18* expression could be regulated by WT1 in renal cells, we used the T-REXTM-293 cell line, a human embryonal kidney cell line modified to express WT1 upon tetracycline treatment. Upon induction of the WT1(-KTS) isoform for 24 hrs, *USP18* transcript was reduced 60% compared to the uninduced control (Fig. 2A left). However, a similar amount of induced expression of the WT1(+KTS) isoform had no effect on *USP18* expression. This suggests that the WT1(-KTS) isoform modulates *USP18* expression but not the WT1(+KTS) isoform. This conclusion further supports previous studies which established the role of WT1(-KTS) as a transcription factor (reviewed in reference [17]).

To validate the induction study, reciprocal knock-down studies were performed in MM cells, a murine embryonic cell line which robustly expresses WT1. siRNA knock-down of *Wt1* resulted in a ~90% reduction in WT1 protein (Fig. 2A right, inset). *Usp18* expression was upregulated in a dose dependent manner following treatment with 50 and 100nM *Wt1* siRNA, with 3.5 fold *Usp18* upregulation being observed at the higher concentration of *Wt1* siRNA (Fig. 2A right). These two independent experiments suggest that WT1 represses *Usp18* expression in mammalian cells.

To demonstrate that this regulation is mediated through the *Usp18* promoter, we examined the activity of the 3.4 kb *Usp18* promoter using a luciferase reporter construct. Induction of WT1(-KTS) followed by transfection of the promoter luciferase construct significantly reduced the luciferase activity in WT1(-KTS) cells but not in the WT1(+KTS) overexpressing cells (Fig. 2B left). In a reciprocal study in which WT1 was knocked down by siRNA, increased luciferase activity was observed (Fig. 2B right). To evaluate the dose dependent repression of the *Usp18* promoter, we ectopically overexpressed WT1(-KTS) in HEK293 (Fig. 2C left) and M15 (Fig. 2C right) cells carrying the *Usp18* luciferase reporter construct. Luciferase activity was down-modulated in a dose dependent manner in both cell

lines. Moreover, this effect was not seen with WT1(+KTS) isoform. Cumulatively, these data indicate that WT1(-KTS) down-regulates *Usp18* expression via direct or indirect interaction with the *Usp18* promoter. This was observed using two different cell lines.

WT1 Zn finger domain is necessary for *Usp18* repression

The zinc finger domain of WT1 is known to be critical for its function as a transcriptional regulator. To verify the importance of this domain in the regulation of the *Usp18* promoter, we generated and tested the repressive function of several WT1 mutant constructs. Consistent with the studies above, co-expression of full length WT1 with the 3.4 kb *Usp18* promoter-luciferase reporter significantly reduced luciferase activity. However deletion mutants a (Zn 3–4), b (Zn 2–4) and c (Zn 1–4) failed to repress the *Usp18* promoter suggesting that zinc finger 3–4 are essential for the repression function (Fig. 3A). Interestingly, but very unexpectedly, mutant d, in which the N-terminal 256 amino acids were deleted, repressed *Usp18* promoter activity 2 fold more than the full length protein, suggesting that this short fragment can function as an autonomous domain. Construct e, in which the NH-terminal 180 amino acids were deleted, failed to repress the promoter activity significantly. This suggests that the mid-region of WT1 inhibits the repression function of the zinc finger domain, at least in the context of the *Usp18* promoter. Expression levels of full length WT1 and various WT1 deletion constructs were generally comparable with the exception of mutant b which was more highly expressed (Fig. 3B). Despite this increased expression, this truncated WT1 still was unable to repress the expression of the *Usp18* reporter construct.

Identification of minimal *Usp18* promoter responsible for WT1-mediated repression

Having demonstrated that WT1 regulation of *Usp18* expression is mediated by a 3.4kb region upstream of the transcription start site (TSS), we sought to identify the minimal WT1-responsive promoter region. 5' truncation deletions were generated from the 3.4 kb *Usp18* luciferase reporter construct such that each deletion construct contained the TSS and 1.5, 0.7 or 0.226 kb 5' upstream of the TSS. These constructs were transfected alone or together with WT1(-KTS) into HEK293 or M15 cells. Although the promoter activity of different deletion constructs varied (white bars), each were significantly repressed 2–3 fold upon expression of WT1(-KTS) (asterisks). This was observed in both HEK293 and M15 cells (Fig 4A). These results indicate that a WT1 response element(s) resides in the 226 bp fragment.

Although WT1 has a loose consensus binding site, it is known to bind to GC rich and (TCC)n sequences. In order to study further the interaction between WT1 and the *Usp18* promoter, we attempted to identify regions in the promoter conserved between mouse and human. Pairwise alignment of the 300 bases upstream of the TSS revealed only a very modest degree of conservation (Fig 4B). However, use of the Biobase MATCH™ transcription factor binding prediction tool identified three potential WT1 binding sites (A,B and C) in the murine 226 bp minimal promoter region (dashed box) (Fig 4B). Similar analysis of the region 5' of the human gene identified at least one potential WT1 binding site (Hu) 186 bp upstream of the TSS (dashed box) (Fig 4B).

In order to determine whether WT1 binds to the *Usp18* promoter, cross-linked protein-DNA fragments were immunoprecipitated by anti-WT1 antibody from M15 cell lysates. As shown in Fig 4 (C left), we were able to specifically amplify (PCR product indicated by the star) sequences corresponding to the *Usp18* promoter region from non-precipitated input cell lysate ("input") (positive control) and also the DNA fragments that were coimmunoprecipitated with anti-WT1 antibody or with an anti-H3 antibody (positive control). In contrast, immunoprecipitation with an anti-IgG antibody (IgG) did not pull down any DNA

fragments from which *Usp18* promoter region sequences could be amplified (negative control). Furthermore, to demonstrate WT1 binding to the *USP18* promoter in human cells, we used T-REx™-293 WT1(-KTS) cells in which FLAG-tagged WT1 (-KTS) expression can be induced. PCR products corresponding to the *USP18* promoter were detected in the WT1 induced cells (+ lane) when WT1 was immunoprecipitated with anti-FLAG antibody but not in uninduced cells (Fig 4C right). As expected, a PCR product was detected in both induced and uninduced cells following precipitation with anti-H3 antibody. Similarly, PCR products were detected in non-immunoprecipitated cell lysates (a second positive control). Collectively these results indicate that WT1 specifically binds to the endogenous *Usp18* promoter. Furthermore, they are consistent with the regulation by WT1 of *Usp18* expression we observed in both mouse and human cell lines.

Mutational analysis of murine *Usp18* promoter identifies two functional WT1 binding sites

The murine 226 bp *Usp18* promoter sequence possesses three potential WT1 binding sites. To determine whether all these binding sites are important or whether they function independently of each other, we generated promoter luciferase constructs in which the three binding sites (A, B and C) were mutated alone or in combination. In binding sites A sequence GGGCG, in B sequence GCCCG and in C sequence GGGGC were replaced with TTTT (Fig 4B). These constructs were co-transfected with vector control or WT1(-KTS) and luciferase assays were performed. As shown in Fig. 5A, in the absence of WT1 no significant repression was observed in mutated promoter constructs. The expression of the construct containing the wild type 226 bp *Usp18* fragment (Fig. 5A, three open circles) was repressed upon co-transfection of WT1, however mutation of either of the putative WT1 binding sites A or B (indicated by circles with an X), singly or in combination, resulted in no *Usp18* repression. In contrast, mutation of binding site C had no effect on the ability of WT1 to repress the expression of the construct. These results suggest that A and B are WT1 binding sites and both are required for effective gene modulation.

Mutational analysis of the human *USP18* promoter identifies one functional WT1 binding site

There is only 42% homology between human and mice in the 300 bp region 5' of the *USP18* genes, and the two functional WT1 binding sites we identified in the mouse promoter are not well conserved in the human promoter region (Fig. 4B). There is, however, in the human *USP18* promoter a 10 bp GC-rich motif similar to a canonical WT1 binding site (Fig. 4B). When a reporter construct containing the 1.2 kb human *USP18* promoter was transfected with WT1(-KTS), strong transcriptional repression was observed (Fig. 5B, open circles). However, substitution of CGGGC by TTTT in the putative WT1 binding motif resulted in the inability of WT1 to transcriptionally repress the expression of the construct (Fig. 5B, circles with X).

USP18 positively regulates cell proliferation

To determine the functional consequence of WT1-mediated repression of *Usp18*, we performed cell proliferation assays using pools of HEK293 and M15 cells stably expressing YFP or YFP-*USP18*. By qPCR, 15X and 22X YFP-*USP18* overexpression was detected in M15 and HEK293 cells, respectively compared to the vector control cells. (Fig. 6C). As previously reported, YFP-*USP18* expression was shown predominantly in the cytoplasm (Fig 6 A right and 6B right) [14]. In the proliferation assay no significant difference was observed until day three. However, post day three *USP18* over-expressing cells proliferated faster than the control cells (Fig 6 A left and 6B left). This suggests that *USP18* positively modulates cell proliferation. Previously it has been reported that *Usp18* regulates EGFR. Western blot analysis of the *USP18* overexpressing HEK293 cell lysates revealed a modest increase in EGFR expression compared to the vector control cells (Fig 6D).

***Usp18* upregulated in mouse Wilms tumors with *Wt1* ablation**

The positive role of USP18 in cell proliferation prompted us to investigate *Usp18* expression in tumors from a mouse model in which Wilms tumors arise following somatic *Wt1* ablation [3]. We compared *Usp18* expression in mouse tumor vs. E13.5, E18.5 and adult kidney. A three to seven fold *Usp18* upregulation was observed in the tumors (Fig. 6E).

Discussion

WT1 ablation is critical for the development of at least a subset of Wilms tumors. This event presumably results in the dysregulation of key genes normally regulated by WT1 and whose aberrant up- or down-regulation, cumulatively, leads to a neoplastic phenotype. While a multitude of genes have been reported to be transcriptionally regulated by WT1, with few exceptions, none of these studies have been carried out in the developing kidney from which tumors arise. Therefore we used our *Wt1^{flox}* strain to ablate WT1 *in vivo* during kidney development and identified genes whose expression changed within ~24 – 36 hours. Because there was no detectable histologic difference between control and WT1-ablated embryonic kidneys at this early time point, gene expression changes are most likely due to loss of WT1 function itself rather than changes in histology/kidney development that are, ultimately, observed following WT1 ablation [3].

We identified *Usp18* as the most up-regulated mRNA observed following *in vivo* ablation of WT1 in developing mouse kidney. This up-regulation was also observed by *in situ* analyses of WT1-ablated embryonic kidneys when compared with control. A variety of subsequent, *in vitro* experiments consistently supported the notion that WT1 suppresses *Usp18* expression, and we therefore conclude that *Usp18* is a bona fide target for WT1 transcriptional regulatory function in the embryonic kidney.

While we were able to demonstrate robust binding of WT1 to the *Usp18* promoter by ChIP-Seq, this promoter was not identified by a ChIP-chip analysis of chromatin from a later stage of mouse kidney development (E18.5) [18]. The differing times of analyses along with the different approaches used to identify WT1 targets (i.e. DNA binding versus differential expression analysis) likely impact the differing results. Interestingly, other members of the ubiquitin specific protease family were identified at these latter stages but not in our microarray analysis [18]. In addition to the differing times of experimental analysis, a possible functional redundancy of these proteases may also be a factor in the results.

WT1 functions as a transcriptional activator as well as repressor, and the zinc finger domain is critical for these roles. The zinc finger domain alone has been shown to bind DNA, but in experiments using two different promoter constructs (the EGR consensus and PDGF-CAT reporter) this domain alone did not repress transcription, although a domain consisting of aa 84–179 did [8,19,20]. Unexpectedly, our data indicate that the zinc finger domain itself can also act to repress transcription. Mutant d whose N-term 250 aa are deleted, repressed *USP18* promoter more strongly than the full length WT1 protein even though the expression level of mutant protein was slightly less than the full-length protein (Fig. 3B). Whether this unexpected result is unique to the *Usp18* promoter or is generalizable to a subset of WT1-responsive promoters will require extensive additional studies.

In both human and murine cell lines we demonstrated that WT1 overexpression results in reduced *USP18* expression and that WT1 ablation results in increased *Usp18* expression. Interestingly, while there is over 96% homology between the human and mouse WT1 proteins [21], there is only 42% homology between human and mice in the 300 bp region 5' of the *USP18* genes. There is, however, a GC-rich motif in the human USP18 promoter that, when mutated, results in a dramatic decrease in the ability of WT1 to repress transcription.

Thus, despite poor cross-species homology, both mouse and human promoters contain functional WT1 binding sites.

Our data strongly suggest that WT1 regulates *Usp18*, implying a role for Usp18 in tumorigenesis. Besides the kidney, *Usp18* is expressed in several fetal tissues. USP18 protein is a member of a large family of proteases and functions to remove ubiquitin-like moieties from proteins [22,23]. Consonant with our observation of enhanced cell proliferation of HEK293 and M15 cells upon exogenous Usp18 expression, a similar promotion of cellular growth by Usp18 has recently been reported in an acute promyelocytic leukemic cell lines [24]. A role for USP18 in regulating protein, but not RNA, levels of EGFR, which is overexpressed in various human cancers has been reported (reviewed in [25] [14]). Interestingly, we observed a modest increase in EGFR protein upon Usp18 overexpression. These data in sum suggest that enhancement of cell proliferation may be a common role for USP18 in a variety of cell types and that this may be effected by its positive regulation of EGFR protein levels. A role for Usp18 in negatively regulating apoptosis induced by BCR-ABL has also been reported [28]. We however did not assess the anti-apoptotic effect of Usp18 expression in either HEK293 nor M15 cells upon expression of Usp18, although the data on cell viability following USP18 overexpression in HEK293 and M15 cell lines indirectly suggest that such overexpression does have a major impact on cell apoptosis. In contrast to these data regarding the effect of *Usp18* expression on proliferation of mesenchymal cell lines, analysis of cultured kidney rudiments following *Wt1* ablation demonstrated no increase in cell proliferation in rudiments after three days in culture. In this study, however, proliferation was quantified for all cell types in the kidney, and a modest increase in the proliferation of a mesenchymal component may have not been robust enough to detect. Additionally, proliferation in the explant cultures was assessed at only one time-point (three days in culture) which may have been too early to note an affect on cell proliferation.

To date, the best characterized role of USP18 has been in the host immune response in which it negatively regulates interferon signaling [26]. *Usp18*^{-/-} cells show prolonged STAT phosphorylation and induction of numerous interferon inducible genes [22]. *Usp18* knock out mice are viable, but have a decreased life span due to neuron-degenerative disease [27]. No salient kidney phenotype has been reported in mice with loss of function of Usp18. However the phenotypic effect of increased Usp18 expression *in vivo* has not been studied. Addressing this question will require generation of a transgenic mouse model.

In conclusion, this study identified *USP18* as a direct target of WT1 and demonstrated that it is negatively regulated by WT1 at the level of transcription. We further demonstrated that overexpression of USP18 modestly enhances proliferation of kidney cells and *Usp18* is overexpressed in a mouse model of Wilms tumor. Taken together our data suggest that increased expression of USP18 following WT1 loss contributes to Wilms tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TSG	Tumor suppressor gene
USP18	Ubiquitin specific protease 18
WT1	Wilms tumor gene 1
TSS	Transcription start site
ChIP	Chromatin Immunoprecipitation

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Wt1 is a transcriptional repressor of *Usp18* gene.

Usp18 expression is increased in the *Wt1* mutant mouse kidney.

ChIP analyses demonstrate that WT1 binds to and represses *USP18* expression.

Usp18 expression is upregulated in mouse Wilms tumors with Wt1 ablation.

Figure 1.

Usp18 expression is up-regulated *in vivo* in fetal kidney following somatic WT1 ablation. *Wt1^{-fl}; Cre-ERTM* embryos and littermate controls were treated in utero with tamoxifen at E11.5, and kidneys were assessed at E13.5. A, qPCR analysis of *Usp18* RNA expression in mutant kidney relative to littermate controls. B *Usp18* expression analysis by *in situ* hybridization in the sagittal section of mouse E13.5 embryo using anti-sense and sense probe. C, *in situ* hybridization of *Wt1* and *Usp18* expression at E13.5 control and mutant kidney. Means and SD of three independent experiments are shown (* $P < 0.005$; paired Student's *t*-test). MB= Mid Brain, D= Diencephalon, CC= Cerebral Cortex, CS= Corpus Striatum, OE= Olfactory Epithelium, T= Tongue, G= Gut, DU= Duodenum, AC= Anal Canal, L= Lung, DRS= Dorsal Root Ganglion.

Figure 2.

Usp18 expression is altered upon induction or knockdown of WT1. A, Induced expression of WT1(-KTS) but not WT1(+KTS) results in downregulation of endogenous *USP18* expression in T-REx™-293 cells as assessed by qPCR. Expression is shown relative to that in uninduced controls. Efficient silencing of WT1 protein expression upon transfection of MM cells with a siRNA-Wt1 pool (Right, inset). Silencing of WT1 protein results in upregulation of endogenous *Usp18* expression in same MM cells. Control was siRNA-luciferase transfected cells (A Right). B left, Induced expression of WT1(-KTS) but not WT1(+KTS) results in reduced expression of *Usp18*-luciferase reporter construct in HEK293 cells. Silencing of WT1 protein results in upregulation of *Usp18*-Luc expression

in MM cells (B, Right). C , Dose-dependent downregulation of *Usp18-luc* reporter with transfection of increasing (50, 100, 200 and 400 ng) amounts of *WT1(-KTS)* expressing plasmid. This was observed in both HEK293 (left) and M15 (right) cells. No down-regulation of the reporter construct was observed using 200 ng of *WT1(+KTS)* plasmid. * $P < 0.05$; ** $P < 0.005$; paired Student's *t*-test.

Figure 3. Zn finger is responsible for WT1 mediated repression. Schematic drawing of WT1 mutants. (A) WT1 full length or mutants were co-transfected in HEK293 cells with 3.4 kb *Usp18* promoter luciferase construct. (B) Expression of WT1 full length and various deletion constructs are shown by Western blot.

Figure 4.

Identification of minimal *Usp18* promoter responsible for WT1 mediated repression.

(A) Comparative analysis of transcriptional activity using deletion mutants of *Usp18* promoter. A series of deletion mutants of *Usp18* promoter is shown in the schematic. The smallest (226 bp) *Usp18* luc promoter was significantly repressed upon co-expression of WT1. Renilla luciferase was used for the normalization of transfection efficiencies. Values represent the means $\pm$ SD from three independent experiments. (* P < 0.05; ** P < 0.005; paired Student's t -test).

(B) The mouse *Usp18* promoter sequence (Ensemble ID ENSMUST00000032198) was aligned with human sequence (Ensemble ID ENST00000215794) using the T-coffee

program (<http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee.cgi/index.cgi>). Predicted WT1 binding sites A, B and C in mouse and Hu in human are indicated by the dashed boxes with exchange of nucleotides by site directed mutagenesis. Rectangle box represents the transcription start site.

(C) ChIP-PCR analysis following immunoprecipitation of endogeneous WT1 from M15 murine mesenchymal cells (left panel) or human T-REx™ -293 WT1(-KTS) cells (right panel) in which WT1 expression was induced (+) or not induced (-) Antibodies used were WT1 (c-19) antibody (Wt1), antibody against the FLAG domain of the FLAG-tagged WT1 from T-REx™ -293 WT1(-KTS) (-FLAG), -histone H3 antibody (H3) (positive control), or antibody against IgG (IgG) (negative control). The presence of the *Usp18* promoter region (-5bp to -153bp upstream of the transcription start site in the murine gene and -137 to -308bp upstream of the human gene transcription start site) in the coprecipitated DNA fragments was assessed by PCR. As a negative control, PCR primers corresponding to a region 3.5 kb upstream of both genes were used. represents PCR specific band, below which are primer dimer bands.

A

B

Figure 5.

Mutational analysis of *Usp18* promoter demonstrates importance of the proximal-most WT1 binding site in the negative regulation of *Usp18* by WT1. Expression of mouse and human mutated and unmutated *USP18* promoter (A and B respectively) of luciferase reporter construct with (+) or without (-) co-transfection of *WT1(-KTS)* expression construct into HEK293 cells is displayed relative to expression of the renilla luciferase internal control using . (* $P < 0.05$; paired Student's t -test).

Figure 6.

Overexpression of USP18 results in an increase in cell number. HEK293 (A, left) or M15 (B, left) cells were transfected with *YFP* or *YFP-Usp18* expression construct. Pools of stable *YFP* or *YFP-USP18* expressing cells were seeded into 6-well plates and cell proliferation was measured each day for 7 days. *YFP* or *YFP-USP18* overexpressing cells were fixed in PFA and cytosolic expression of *YFP* was visualized under the microscope (A and B right). (C) *Usp18* expression was measured by qPCR in HEK293 and M15 cells, overexpressing *Usp18* (D) EGFR expression was detected in HEK293 cells at day 5 in presence or absence of *Usp18* overexpression. (E) *Usp18* expression in a mouse model of WT. *-Actin* was used as an internal control. (* $P < 0.05$; paired Student's *t*-test).