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Wilms Tumor Chromatin Profiles Highlight Stem Cell Properties and a Renal Developmental Network

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Abstract

Wilms tumor is the most common pediatric kidney cancer. To identify transcriptional and epigenetic mechanisms that drive this disease, we compared genomewide chromatin profiles of Wilms tumors, embryonic stem (ES) cells and normal kidney. Wilms tumors prominently exhibit large active chromatin domains previously observed in ES cells. In the cancer, these domains frequently correspond to genes that are critical for kidney development and expressed in the renal stem cell compartment. Wilms cells also express ‘embryonic’ chromatin regulators and maintain stem cell-like p16 silencing. Finally, Wilms and ES cells both exhibit ‘bivalent’ chromatin modifications at silent promoters that may be poised for activation. In Wilms tumor, bivalent promoters correlate to genes expressed in specific kidney compartments and point to a kidney-specific differentiation program arrested at an early-progenitor stage. We suggest that Wilms cells share a transcriptional and epigenetic landscape with a normal renal stem cell, which is inherently susceptible to transformation and may represent a cell-of-origin for this disease.

Introduction

The fundamental role of genetic changes in cancer progression is now unquestioned. These aberrations are being cataloged at an unprecedented pace through the application of high-throughput genomic tools (Stratton et al., 2009). In contrast, the extent to which epigenetic events and chromatin environments contribute to cellular transformation remains controversial.

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The genomic instability present in many cancers complicates the study of DNA methylation and chromatin. Moreover, the most relevant self-renewing cell populations are frequently obscured by tumor heterogeneity. Nonetheless, there is increasing evidence that aberrant DNA methylation and chromatin regulation profoundly contribute to specific types of cancer (Feinberg et al., 2006; Jones and Baylin, 2007). The advent of new epigenomic tools provides an opportunity to investigate their contributions broadly (Barski et al., 2007; Lister et al., 2009; Mikkelsen et al., 2007). Pediatric cancers represent attractive models to study as their relatively normal genomic background facilitates epigenomic characterization and suggests that epigenetic factors may make play particularly critical roles in pathogenesis.

Wilms tumor is characterized by a multipotent "triphasic" histology that includes an undifferentiated 'blastemal' component and varying amounts of epithelial and stromal elements (Rivera and Haber, 2005). These tumors can also be associated with developmental abnormalities, including persistent embryonic tissue known as nephrogenic rests, and are thus believed to be intimately connected to kidney organogenesis.

Genetic abnormalities described in Wilms tumor involve genes that regulate the metanephric mesenchyme, a kidney-specific stem cell population that resembles blastemal tumor cells and gives rise to most epithelia in adult kidneys. The tumor suppressor *WT1* has been linked to survival and differentiation of these cells (Call et al., 1990; Gessler et al., 1990; Kreidberg et al., 1993; Moore et al. 1999), and the activation of β-catenin is a crucial step in epithelialization (Koesters et al., 1999). Similarly, the recently identified tumor suppressor *WTX* is expressed in kidney stem cells and has been linked to Wnt signaling and WT1 transcriptional control (Major et al., 2007; Rivera et al., 2007; Rivera et al., 2009). Yet known mutations account for less than 50% of Wilms tumors, leaving a majority without any known causal genetic alteration.

Two aspects of Wilms tumor suggest that epigenetic alterations also play critical roles in pathogenesis. First, the classical imprinted gene *IGF2*, normally expressed only from the paternal allele, frequently exhibits bi-allelic expression in sporadic Wilms tumors (Ogawa et al., 1993). *IGF2* imprinting is also lost in Beckwith-Wiedeman, an overgrowth syndrome associated with an elevated risk of Wilms tumors (Weksberg et al., 1993). Second, similarities in gene expression between Wilms tumor and fetal kidney raise the possibility that pathways active in organ-specific stem cells may be shared by the tumor (Li et al., 2002). This relationship is further supported by the occasional spontaneous regression of nephrogenic rests, which may reflect the re-activation of kidney differentiation pathways (Beckwith et al., 1990).

Whole genome analysis of chromatin state is now feasible by combining chromatin immunoprecipitation (ChIP) with sequencing – 'ChIP-Seq' (Barski et al., 2007; Mikkelsen et al., 2007). Of particular interest are specific histone modifications that relate closely to transcriptional programs, cellular state and epigenetic processes (Kouzarides, 2007). Maps of histone H3 trimethylated at lysine 4 (K4me3), lysine 36 (K36me3) or lysine 27 (K27me3) identify promoters, transcripts or sites of Polycomb repression, respectively (Barski et al., 2007; Li et al., 2007; Mikkelsen et al., 2007). In embryonic stem (ES) cells, 'bivalent domains' with overlapping K27me3 and K4me3 are associated with developmental genes that are presently silent, but poised for activation upon differentiation (Azuara et al., 2006; Bernstein et al., 2006). Bivalent domains have been proposed to predispose gene promoters to DNA methylation in cancer (Ohm et al., 2007). However, the global role of such marks in cancer has not been explored. Such an analysis could provide insight into the developmental state of tumor cells and how they relate to non-malignant counterparts.

Here, we present a whole genome analysis of chromatin in primary Wilms tumors. We focused on Wilms as an initial model because (i) resected tumors provide a ready source of homogeneous, undifferentiated 'blastemal' cells which resemble embryonic renal tissue and

may represent a model of tumor stem cells (Rivera and Haber, 2005) and (ii) Wilms cells exhibit relatively normal genetic backgrounds with few copy number alterations or known mutations, thus facilitating and highlighting the importance of epigenomic analysis (Rivera et al., 2007). We mapped K4me3, K27me3 and K36me3 in Wilms tumors, normal kidneys, and fetal kidneys, and compared the maps to analogous data for human ES cells. These chromatin data were integrated with published transcript profiles, and complemented by mutation and copy number analyses.

The data reveal an interconnected network of genes that appear to drive Wilms tumor phenotype and proliferation. Many of these genes correspond to known regulators of kidney development, but some may be novel master regulators of this process. The maps also point to critical roles for Polycomb repression in both fully silenced and bivalent patterns, an important feature of ES cell biology that is recapitulated in Wilms tumor. For example, the p16 tumor suppressor is repressed by Polycomb in a manner reminiscent of normal stem cells, but distinct from many adult tumors. Similarly, markers of epithelial differentiation are maintained in a bivalent, poised state that may signal a latent differentiation potential akin to a normal renal stem cell. In summary, in depth analysis of Wilms tumor chromatin points to a transformed phenotype that is sustained through the precise control of developmental and proliferative mechanisms shared with early kidney precursors and ES cells.

Results

Genome-wide maps of chromatin state in Wilms tumor

Genomewide chromatin maps were generated for three *WTX* mutant Wilms tumors, normal kidney and fetal kidney using ChIP-Seq. We selected tumors with pronounced blastemal compartments and few large-scale copy number changes in order to enrich for homogeneous tumor cells with defined genetic abnormalities. These tumors did not contain alterations in *WT1* or β-*catenin*. After manually dissecting blastemal compartments, we performed ChIPs for K4me3, K27me3 or K36me3 (see Methods). ChIP DNA was sequenced on an Illumina Genome Analyzer and reads were aligned to the human genome (hg18) to produce density maps and identify genomic intervals enriched for a given modification (Mikkelsen et al., 2007). These data were integrated with maps of ES cell chromatin (Ku et al., 2008), with sequence-based annotations for promoters, transcripts and other genomic features, and with transcript profiles for Wilms tumors and other renal tissues (Brunskill et al., 2008; Yusenko et al., 2009). Although we focused our analysis on these *WTX* mutant tumors, we also mapped K4me3 in a *WT1* mutant tumor for comparison. Chromatin datasets are summarized in Tables S1–5 and are publicly available at http://www.broadinstitute.org/cgibin/seq_platform/chipseq/shared_portal/clone/Wilms.py, and at Gene Expression Omnibus (GEO) GSE20512.

Shared patterns of active chromatin in Wilms tumor and ES cells

We began our analysis by comparing promoter states across the different samples, focusing on K4me3 as a marker of transcriptional initiation (Li et al., 2007). We annotated promoters based on the presence of K4me3 and performed unsupervised clustering of the various samples. As expected, all three *WTX* mutant Wilms tumors cluster closely (Fig 1a, S1). Remarkably, K4me3 patterns in the tumors resemble those in ES cells to a significantly greater extent than those in normal kidney. This relationship does not extend to other precursor populations, as Wilms tumors more closely resembled ES cells than hematopoietic progenitors (Cui et al., 2009) (Fig S1).

Wilms tumors also resembled ES cells with respect to the chromatin patterns seen at developmental loci. Both cell types exhibit an overabundance of broad K4me3 intervals or

'domains' that contrast markedly with the punctate K4me3 peaks seen at typical promoters (Fig 2a, 2b).

Active chromatin domains mark kidney development genes in Wilms tumor

In ES cells, the largest K4me3 domains coincide with master regulators of pluripotency, including *OCT4* and *SOX2* (Mikkelsen et al., 2007). Initial examination of our data suggested a similar effect in Wilms tumor. For instance, one of the largest K4me3 domains in Wilms tumor overlaps *SIX2* which encodes a transcription factor (TF) with essential functions in maintaining kidney stem cells in an undifferentiated state (Kobayashi et al., 2008; Self et al., 2006) (Fig 2b, 2c). *SIX2* is expressed at high levels in Wilms tumor relative to other renal tumors and adult kidneys (Yusenko et al., 2009) (Fig 2d). We also detected a strong signal for SIX2 protein in the blastemal compartments of tumor sections by immunofluorescence (Fig 2e). A similar signal is detected in the metanephric mesenchyme, the renal stem cell compartment in developing kidneys. Another K4me3 domain coincides with *GDNF*, which encodes a growth factor that is expressed in kidney stem cells and which is an essential regulator of kidney development (Moore et al., 1996; Sanchez et al., 1996) (Fig 1b).

This observation prompted us to test whether the presence of K4me3 domains could be used to identify genes that underlie Wilms tumor biology. We therefore examined the set of genes which, like *SIX2* and *GDNF*, are marked by the broadest K4me3 domains in Wilms tumor (K4me3 interval >3.5 kb) but are unmarked in normal kidney (K4me3 interval < 0.7 kb). This produced a list of 114 genes (Set 1; Table S2). Set 1 genes show higher expression in Wilms tumor relative to normal kidney tissue (Fig 3a), as expected from their active chromatin states. In addition, genes in this set exhibit relatively strong enrichment for K36me3, a chromatin mark which typically coats active transcripts (Fig S2).

Using the Gene Ontology Database, we confirmed that Set 1 is highly enriched for genes with functions in kidney and mesoderm development (Fig 1b). In addition to *GDNF* and *SIX2*, Set 1 contains 23 other well-characterized nephrogenesis regulators such as *EYA1* and *OSR1* (Brodbeck and Englert, 2004;Mugford et al., 2008) (Table S6). Set 1 genes are highly expressed in fetal kidney, consistent with the enrichment for developmental functions. Importantly, Set 1 genes do not appear to be general features of cancer, as they are expressed at much lower levels in adult renal cell carcinoma (Yusenko et al., 2009) (Fig 3a). Taken together, these findings suggest that Wilms tumor cells may be driven by a transcriptional regulatory network analogous to renal progenitors.

Identification of potentially novel regulators of Wilms tumor and kidney development

Since many Set 1 genes have not been previously linked to kidney development, we explored whether these candidates might also play roles in this process. In fact, many Set 1 genes without previously described kidney functions are expressed in a pattern similar to *SIX2*, with high levels in Wilms tumor and fetal kidney and considerably reduced signals in other renal tumors and adult kidney. One interesting candidate is *SOX11*, which encodes a TF with defined roles in neurons and mesenchymal stem cells (Kubo et al., 2009) (Fig 2f, 2g). Using immunofluorescence, we confirmed that SOX11, like SIX2, is expressed in the blastemal compartment of Wilms tumor (Fig 2h). In fetal kidney, SOX11 protein is detected adjacent to the surface of the organ, where mesenchymal and epithelial precursors reside. This suggests a role for this TF in renal precursors during embryogenesis. Thus, an unbiased screen for K4me3 domains in Wilms tumor reveals genes with previously unrecognized functions in tumor biology and early kidney development.

Roughly 10% of the large K4me3 domains in Wilms tumor do not correspond to annotated protein coding genes. Recently, a variety of non-coding RNAs have been identified by

genomewide screens, in part through their associations with K4me3 and other histone modifications (Guttman et al., 2009; Marson et al., 2008; Mikkelsen et al., 2007). We therefore asked whether chromatin domains that do not correspond to classical gene annotations could reflect the regulation of non-coding RNAs in Wilms tumor. We identified several striking examples (Table S7). One prominent K4me3 domain corresponds to a cluster of three microRNAs on chromosome 19: mir-99b, let-7e and mir-125a (Fig 2i). Though they remain poorly characterized, these miRNAs have been implicated in several malignancies (Li et al., 2009; Wu et al., 2008; Wu et al., 2009). *HOTAIR*, a large non-coding RNA in the HOXC cluster with known associations to cancer (Rinn et al., 2007), is also marked by a large K4me3 domain in Wilms tumor (Fig 2j). Interestingly, *HOTAIR* also shows variable degrees of K27me3 in the tumors. These signature chromatin patterns suggest possible roles for these and other non-canonical elements in Wilms tumor biology and/or kidney development.

An ‘embryonic’ repertoire of chromatin regulators in Wilms tumor

To further explore commonalities between Wilms and ES cells, we examined genes whose promoters have compact K4me3 peaks in both cell types, but which lack this mark in normal kidney (Fig 1b). This produced a list of 793 genes (Set 2; Table S3). Set 2 is statistically enriched for genes with annotated functions in chromatin, epigenetic and transcriptional regulation (Fig 1b). It includes numerous chromatin regulators that are highly expressed in embryonic tissues and stem cells, and which could contribute to a stem cell-like chromatin state in the tumor. We compiled a list of 60 chromatin regulators in Set 2 (Fig 4a; Table S8). As expected, these genes tend to be expressed at high levels in Wilms and ES cells. They also show high expression in fetal kidney, but are relatively silent in adult kidney. The regulators do not appear to be simply markers of proliferation as they are expressed at much lower levels in renal cell carcinoma (Fig S3) (Yusenko et al., 2009).

Many of these 60 chromatin regulators have documented roles in embryogenesis, kidney development and self-renewal. *JARID2*, *FBXL10* and *CBX2* are all linked to Polycomb silencing. *JARID2* and *FBXL10* encode members of the jumonji protein family, several of which have histone demethylase activity (Kouzarides, 2007). *JARID2* is a component of PRC2, the Polycomb repressive complex that catalyzes K27me3 (Li et al., 2010; Peng et al., 2009; Shen et al., 2009). *FBXL10* and *CBX2* encode subunits of Polycomb repressive complex 1 (Sanchez et al., 2007). Polycomb repressors play particularly important and dynamic roles in the maintenance of lineage-specific gene expression programs during early development (Sparmann and van Lohuizen, 2006). In addition, *FBXL10* and *PR-Set7*, another Set 2 member, have been implicated in proliferation and anti-senescence through the regulation of p15 and p53-dependent pathways (He et al., 2008; Shi et al., 2007).

NSD1 and *WHSC1*, which encode K36 methyltransferases, have been previously linked to Wilms tumor and developmental kidney disorders (Bergemann et al., 2005; Kurotaki et al., 2002; Nimura et al., 2009; Wang et al., 2007). *NSD1* is inactivated in Sotos syndrome, a congenital condition associated with increased susceptibility to Wilms tumors, while *WHSC1* is named for Wolf-Hirshhorn syndrome, a genetic condition associated with kidney hypoplasia.

Taken together, these finding suggest that ‘embryonic’ regulators common to Wilms tumor and ES cells maintain a stem-like chromatin environment conducive to proliferation and essential for developmental plasticity and specification.

Patterns of bivalent chromatin suggest a developmental arrest in Wilms tumor

Bivalent domains, which are simultaneously enriched for both K4me3 and K27me3, correlate to genes that are silent but poised within a given cell population (Azuara et al., 2006; Bernstein

et al., 2006). In contrast, K27me3-specific domains correspond to genes that are more stably silenced. As a result, genomewide analysis of K27me3 and K4me3 has been useful in defining the developmental potential of stem cells, including ES cells, neural stem cells and hematopoietic progenitors (Cui et al., 2009; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007).

We defined a set of 470 'bivalent' genes whose promoters are enriched for both K27me3 and K4me3 in Wilms tumor (Set 3; Fig 1c; Table S4). Set 3 genes exhibit low levels of expression in Wilms tumor; unlike genes in Set 1 and Set 2, their transcripts lack K36me3 (Fig S2).

Nearly all Set 3 genes are also bivalent in ES cells, but few are bivalent in normal kidney. Because bivalent domains tend to resolve progressively during differentiation, this suggests that Wilms tumor cells correspond to a developmental stage along the normal kidney differentiation pathway. This conclusion is also consistent with the observation that Set 1 and Set 2 genes are highly expressed in both Wilms and fetal kidney, while Set 3 genes are more frequently expressed in adult kidney (Fig 3b) (Yusenko et al., 2009).

Remarkably, when compared to a recently published gene expression atlas for developing mouse kidney (Brunskill et al., 2008), a subset of Set 3 genes shows robust expression in specific epithelial structures (Fig S4). Of the 207 genes with the most structure-specific expression in the kidney, 18 map to promoters that are bivalent in Wilms tumor. In contrast, just 3 map to K27me3-specific promoters (see below). We selected specific examples of bivalent genes for protein level analysis using immunofluorescence. While the blastemal Wilms tumor cells exhibited little or no protein signal for these genes, expression could be readily identified in specific epithelial structures of normal kidneys (Fig 5).

To gain insight into developmental pathways that are more stably inactivated, we collated 188 genes whose promoters lack K4me3 but are enriched for K27me3 in Wilms tumor (Set 4; Fig 1c; Table S5). As expected, these genes are largely silent in Wilms tumor and their transcripts show little K36me3 (Fig S2).

Set 4 is enriched for genes involved in distinct non-kidney developmental processes, including brain, limb, ear and blood vessel formation (Fig 1c). Nearly all Set 4 genes are bivalent in ES cells, but they appear to have become stably inactivated in Wilms. Indeed, the genes show little expression in both fetal and adult kidneys. Thus, Wilms tumor cells exhibit a degree of lineage commitment that is distinct from ES cells, but consistent with a stalled kidney-specific differentiation program.

Wilms tumors maintain native epigenetic silencing of the p16 locus

One of the best studied targets of Polycomb silencing and K27me3 is the tumor suppressor p16 (Fig 6a). Polycomb repressors silence p16 during normal development, playing a particularly key role in proliferating stem cells (Bracken et al., 2007;Jacobs et al., 1999). The p16 locus frequently undergoes homozygous deletion or inactivation by DNA methylation in malignant cells (Herman and Baylin, 2003). P53 inactivation has been documented in anaplastic Wilms tumors (Bardeesy et al., 1994), a small subset with poor prognosis, but the mechanism by which most Wilms tumors evade p16-mediated apoptosis remains undefined.

We used comparative genomic hybridization to evaluate the p16 locus in the tumors studied here – in all cases we found both alleles intact. Additionally, methylation-specific PCR confirmed that the locus remains unmethylated at the DNA level in the tumors (Fig 6b). However, the Wilms tumor chromatin maps reveal a K27me3 domain spanning the p16 locus in a pattern similar to ES and other primary cells (Cui et al., 2009;Ku et al., 2008) (Fig 6a). We speculate that ‘embryonic’ Polycomb proteins and other chromatin regulators expressed

in Wilms tumors underlie a native chromatin environment that maintains p16 silencing, thereby facilitating tumor proliferation and anti-senescence pathways. It will be important to ascertain whether Wilms progression is associated with loss-of-function of these chromatin regulators, and whether this could account for increased reliance of anaplastic tumors on deletion or DNA methylation of p16, and p53 inactivation (Bardeesy et al., 1994;Natrajan et al., 2008).

Chromatin patterns of imprinted genes in Wilms tumor

One of the largest K4me3 domains in the Wilms genome coincides with the *IGF2* promoter, with no analogous domain present in ES cells, fetal kidney or normal kidney. Since *IGF2* loss-of-imprinting has been implicated as a causal event in Wilms tumorigenesis (Ogawa et al., 1993; Weksberg et al., 1993), it is likely that the active domain reflects this epigenetic event. We thus tested whether alterations in other imprinted genes could be detected in our data. We found several other imprinted genes, including *NNAT*, *PEG3*, and *ZIM2*, that showed enrichment of K4me3 relative to normal kidney (Fig 6c). In the case of *PEG3* and *ZIM2*, similar signals are seen in fetal kidney, and thus the marks do not appear tumor specific. Interestingly, *NNAT* has been previously shown to be expressed at high levels in Wilms tumors (Dekel et al., 2006; Li et al., 2002). These data suggest that imprinting defects in this disease can be detected at the level of chromatin, but appear confined to a limited set of loci.

Aberrant chromatin patterns in early kidney differentiation genes

Our data suggest that Wilms tumor reflects a stalled kidney-specific differentiation program. To narrow down the developmental stage of the tumor cells, we examined the chromatin states of the earliest known markers of kidney differentiation.

The differentiation program of the metanephric mesenchyme proceeds along separate stromal and epithelial lineages (Mugford et al., 2008). The stromal lineage is characterized and regulated by *FOXD1*. Key genes in the earliest epithelial differentiation stages include *PAX8*, *FGF8*, *WNT4* and *LHX1*, which are induced following β-catenin activation (Park et al., 2007). Genetically, *PAX8* and *FGF8* appear to act upstream of *WNT4*, while *LHX1* is downstream of this gene (Kobayashi et al., 2005).

Both *FOXD1* and *LHX1* are bivalent (Set3) in Wilms tumor, suggesting that the bulk of blastemal cells are arrested at a stage preceding the specification of stromal or epithelial lineages. The bivalent state suggests that these genes remain poised, and this may relate to the partial differentiation capacity of blastemal cells, which is evident histologically. *FGF8* did not meet the stringent criteria for inclusion in Set 3, but exhibits a moderate degree of bivalency. In contrast, chromatin at the *PAX8* locus is active while *WNT4* exhibits neither active nor inactive chromatin marks.

In summary, although a limited amount of epithelial commitment may be detected at the chromatin level, Wilms cells appear to maintain the full capacity of the undifferentiated metanephric mesenchyme, and thus may represent an earlier developmental stage than previously appreciated through transcriptional studies.

Discussion

Although the genetic basis of Wilms tumor has yet to be fully characterized, the fact that these tumors are genomically stable and occur at a young age suggests that relatively few mutations are involved. The narrow developmental window in which the tumors arise is thought to reflect the presence of immature kidney tissue and, in particular, of a renal stem cell population subject to transformation. These factors suggest that aberrant retention of stem cell-like transcriptional programs and chromatin regulation may play a critical role in this pediatric cancer.

In fact, our analyses highlight a set of loci with active chromatin profiles in Wilms tumor that form an easily recognizable network reflecting transcriptional programs in early kidney development. Examples include *SIX2*, *GDNF* and *EYA1*, all of which are essential for the survival of the organ-specific stem cell population (Brodbeck and Englert, 2004). Other genes, such as *SOX11*, exhibit similar chromatin and expression patterns, suggesting that they may function during kidney development, and perhaps play oncogenic roles in Wilms tumor.

Our results also provide evidence of a specialized chromatin network in Wilms tumor that is reminiscent of ES cells. The factors and pathways are largely distinct from those defined in adult cancers (Ben-Porath et al., 2008; Wong et al., 2008) (Table S9). Some of the implicated chromatin regulators, including *NSD1* and *WHSC1*, have already been linked to Wilms tumorigenesis and kidney development (Bergemann et al., 2005; Kurotaki et al., 2002; Nimura et al., 2009; Wang et al., 2007). While these genes may play roles akin to oncogenes in adult cancer, we speculate that their role in Wilms tumor, and possibly other pediatric cancers, is to maintain an embryonic chromatin environment conducive to self-renewal and developmental plasticity.

Further evidence of an arrested developmental program emerges from an analysis of K27me3 patterns. Our data suggest that, as in ES cells, bivalent domains in Wilms tumor associate with genes that may be induced upon differentiation. In fact, the repertoire of bivalent genes in the tumor includes both early and late kidney differentiation genes. This suggests that the blastemal tumor cells maintain a latent differentiation potential that is akin to normal renal progenitors, but aberrantly kept in check. In contrast, developmental genes associated with other lineages are more frequently enriched for K27me3 alone, and thus appear more stably inactivated.

Although our study focused on *WTX* mutants, which represent the largest subset of genetically defined Wilms tumors, there is some evidence that these findings also apply to other genotypes (Fukuzawa et al., 2009; Li et al., 2004). First, the gene sets defined here based on chromatin patterns in *WTX* tumors have relatively consistent expression patterns across multiple Wilms tumors that were not selected by genotype (Fig 2d, 2g, 3). Second, when we subsequently analyzed K4me3 patterns in a *WT1* mutant tumor we found them to be highly concordant with the *WTX* mutant tumors (Fig S1).

In summary, our data suggest that Wilms tumor cells diverge from a normal developmental course, maintaining self-renewal capacity and an arrested differentiation program through the activities of select chromatin regulators and a transcriptional regulatory network akin to renal progenitors. Although Wilms tumors could conceivably arise from reprogramming of mature renal tissue, the narrow developmental window in which these tumors arise and the maintenance of an array of pathways active in renal progenitors suggest a close association with a normal stem cell population. We speculate that kidney stem cells present early in development represent the cell-of-origin for Wilms tumors, and that the transcriptional regulatory network and chromatin environment of these cells makes them uniquely susceptible to transformation through the acquisition of minimal further genetic and epigenetic alterations. Similar mechanisms may play roles in other pediatric tumors, where transformation occurs in a defined developmental window and is associated with a small number of genetic changes. By contrast, cellular reprogramming may play a more substantial role in adult tumors, which are associated with numerous mutations including alterations in chromatin modifying enzymes (Dalgliesh et al., 2010).

Methods

Tumor and tissue samples

Surgical samples of primary Wilms tumors, normal kidney, and fetal kidney were obtained from Boston Children's Hospital with institutional review board approval. DNA was prepared using the Puregene DNA Purification Kit (Gentra, Minneapolis, MN) and RNA was prepared using Trizol (Invitrogen, Carlsbad, CA) or the RNeasy Mini Kit (Qiagen, Valencia, CA). Tumor tissues were genotyped for WTX, WT1, and beta-catenin mutations. DNA methylation at the p16 locus was measured by methylation specific PCR after bisulfite conversion with the EZ DNA Methylation Kit (Zymo Research) using PCR primers described previously (Herman et. al., 1996).

Chromatin Immunoprecipitation and High-Throughput Sequencing

ChIP assays for tumor and kidney tissues were performed using a variation of previously described procedures (Kirmizis et al., 2003; Mikkelsen et al., 2007). Frozen tissues corresponding to blastemal compartments were thawed, chopped into small fragments, crosslinked in 1% formaldehyde, washed and disaggregated into single cells using 50μm medicones on the Medimachine (Becton Dickinson). Cells were lysed and sonicated with a Branson 250 to a size range of 200 to 700bp. Chromatin was immunoprecipitated with antibody against K4me3 (Abcam 8580), K27me3 (Upstate 07-449) or K36me3 (Abcam 9050).

Libraries were prepared from ~5 nanograms of ChIP DNA, loaded onto flow cells and sequenced on the Illumina Genome Analyzer by standard procedures (Mikkelsen et al., 2007). Reads were aligned to the reference (hg18) human genome using Maq (http://maq.sourceforge.net/maqman.shtml). Statistics on read numbers and alignment are detailed in Table S1.

Chromatin Maps and Initial Data Analysis

Enrichment profiles were generated for each histone modification – cell type combination as described (Mikkelsen et al., 2007). Briefly, aligned reads were extended to 300 bases to approximate the average ChIP fragment. Signal was then estimated at any given position (25-bp resolution) as the number of sequenced ChIP fragments that overlap that position.

A sliding window approach was used to identify significantly enriched intervals or 'peaks' from each dataset. Thresholds were defined based on a background model of randomly positioned reads. Peaks of histone modification were defined by sliding a 700 bp window across the genome, and identifying windows with a minimum average height of 4 reads. We confirmed that these thresholds yielded few significant peaks when applied to data for control input samples. Broad K4me3 domains were identified by repeating this procedure with a 1400 bp sliding window and the same average threshold. In both cases, enriched intervals separated by less than 100 bp were merged into a single interval.

Genes from the hg18 RefSeq database were classified based on the presence of K4me3 and/or K27me3 peaks in their promoter regions (defined as +/− 2.5 kb of the transcriptional start sites). Genic K36me3 signals were calculated based on the average signal across the length of the annotated transcript. In addition, we collated a set of domains that are further than 10 kb from the start site of any annotated gene which could correspond to non-coding RNA transcripts or other elements.

We clustered cell types by K4me3 promoter patterns using the hierarchical clustering package in GenePattern package (Reich et al., 2006). Clustering was performed for all three WTX

tumors, one WT1 tumor, ES cells and normal kidney across all genes using Pearson correlations.

Gene Set Definition

To define Set 1, we identified in each *WTX* tumor the 3500 most enriched, broad K4me3 intervals, as defined above. We then retained domains that appear in all three tumors, but do not correspond to a K4me3 peak in normal kidney. The nearest promoter to each domain, up to a distance of 2.5 kb, was included in Set 1. Set 2 includes genes with K4me3 promoter peaks in the three tumors and in ES cells, but without K27me3 in Wilms or ES cells, or corresponding K4me3 peaks in normal kidney. Set 3 includes genes with overlapping K4me3 and K27me3 peaks in at least two out of the three *WTX* tumors. Set 4 contains genes with K27me3 promoter peaks but without K4me3 in all three tumors. Genes that appeared in more than one set were included in the earlier set (e.g., if a gene met criteria for Set 1 and Set 2, it was only counted in Set 1). A list of genes with annotated functions in chromatin or epigenetic regulation was compiled from genes in Set 1 and Set 2 and also includes a small number of additional genes with active chromatin in Wilms and ES cells, as in Set 2, but allowing for minimal K4me3 signal in normal kidney.

Expression Analysis

Gene expression datasets for renal tumors, normal kidney and developing kidney structures were downloaded from the Gene Expression Omnibus (Brunskill et al., 2008; Yusenko et al., 2009). Data were normalized with GCRMA, quantile normalization and background correction using the GenePattern package (Reich et al., 2006). For heat maps, only probe sets with at least one present ("P") call across samples and a minimum 1.5-fold difference between averages of the sample groups were selected. Hierarchical clustering by Pearson correlation and heat map visualizations used GenePattern. Human genes were mapped to mouse orthologs as described (Ku et al., 2008).

Immunofluorescence

Immunofluorescence was performed for Wilms tumors and fetal kidneys using standard protocols and the following antibodies: SIX2 (Proteintech), SOX11 (Sigma Prestige HPA000536), KCNJ3/GIRK1 (Abcam ab61191), and NR4A2 (Sigma Prestige HPA000543). Frozen sections were fixed with 4% formaldehyde for 15 min and permeabilized with 0.1% Triton-X for 10 min. Incubation with primary antibodies was performed overnight at 4°C. This was followed by incubation with Alexa Fluor conjugated secondary antibodies for 1 hour at room temperature (1:2000 concentration).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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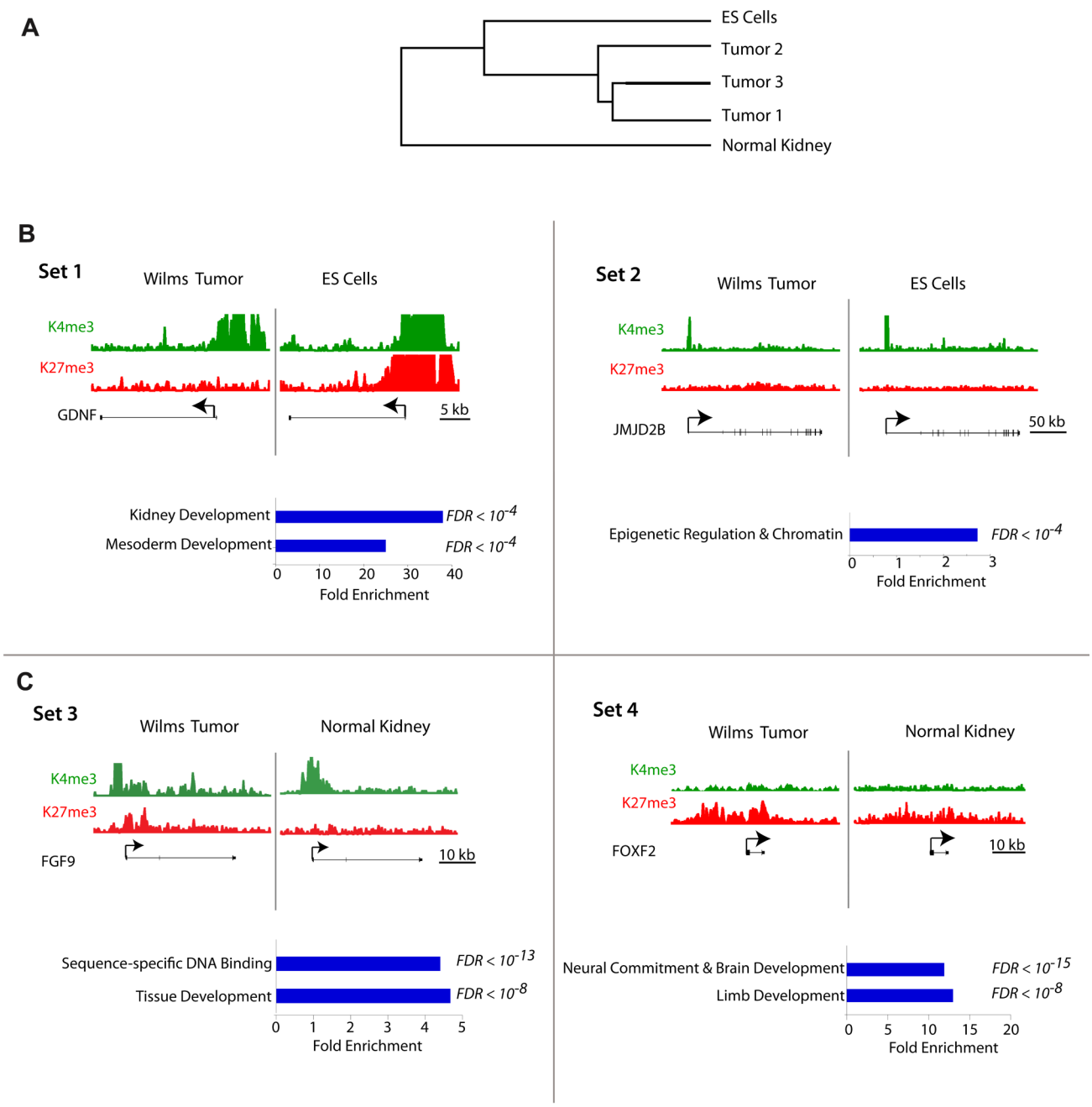

Figure 1. Gene classification based on chromatin provides insights into the developmental state of Wilms tumor

(a) Wilms tumors, normal kidney and ES cells were clustered by K4me3 promoter states. The cluster tree depicts relationships among these different tissues. (b, c) Four gene sets were defined based on promoter chromatin states. For each set, chromatin profiles are shown for a typical gene. These profiles depict ChIP-Seq signals for the indicated modification. The x-axis corresponds to genome position. The y-axis corresponds to the number of sequenced ChIP fragments that overlap a given position (range = 0–12).

Set 1 contains genes with broad K4me3 domains in Wilms tumor; it is enriched for known regulators of kidney and mesoderm development (top two categories shown). **Set 2** contains

genes with promoter K4me3 peaks in Wilms tumor and ES cells, but not in normal kidney; it is enriched for regulators of chromatin, transcription and epigenetic processes. **Set 3** contains genes with overlapping K4me3 and K27me3, a subset of which is differentially-expressed in kidney (see text and Fig 3). **Set 4** contains genes with promoter K27me3 peaks; it is enriched for genes involved in distinct developmental processes. See also Fig S1, Tables S1–S5.

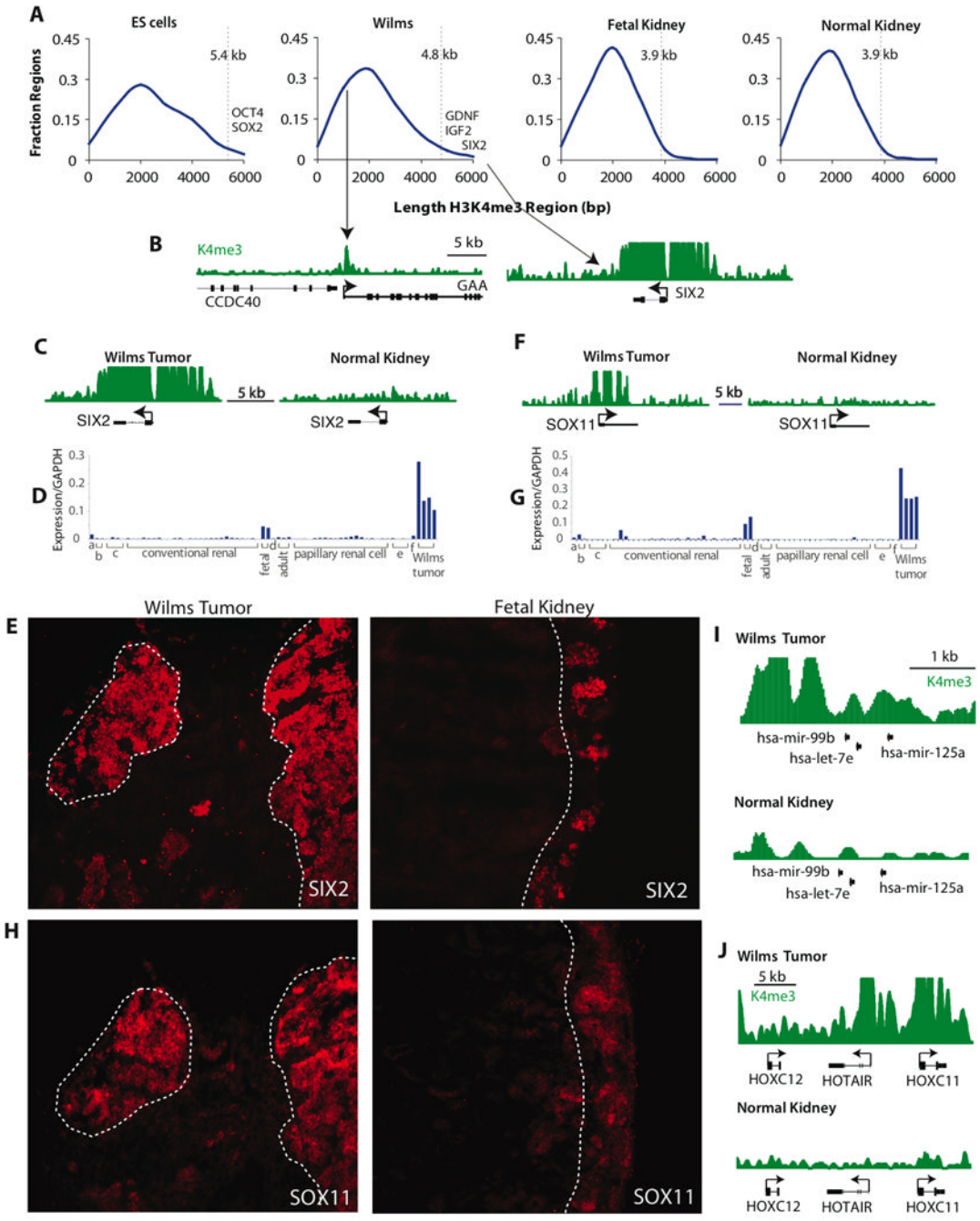

Figure 2. Domains of modified histones identify developmental regulators in Wilms tumor

(a) Histograms depict the size distribution of K4me3 enriched regions in ES cells, Wilms tumor, fetal kidney and normal kidney. In both ES cells and Wilms tumor, the most expansive regions correspond to developmental genes with critical roles in the respective cell types. 95th percentile values (5.4 kb for ES cells, 4.8 for Wilms tumor, and 3.9 kb for both fetal and normal kidney) are indicated on each plot. (b) Genomic views show K4me3 signal in Wilms tumor for a typical promoter (*GAA*) and for the kidney stem cell regulator *SIX2*. (c–d) K4me3 signal at the *SIX2* and *SOX11* loci in Wilms tumor compared to normal kidney. (e–f) Expression of *SIX2* and *SOX11* mRNA shown for a panel of renal tumors and tissues [a: clear cell sarcoma of the kidney; b: collecting duct carcinoma; c: chromophobe renal cell cancer; conventional

renal cell carcinoma; fetal kidney; d: renal lipoma; adult kidney; papillary renal cell carcinoma; e: renal oncocytoma; f: rhabdoid tumor of kidney; Wilms tumor]. (g–h) Immunofluorescence images depict *SIX2* and *SOX11* protein expression in a WTX mutant Wilms tumor and fetal kidney. In Wilms tumor, *SIX2* and *SOX11* have similar expression in the blastemal compartment (dotted outlines). In fetal kidney both markers are adjacent to the surface of the organ where mesenchymal and epithelial precursors are located (dotted lines). Original magnification 200×. (i,j) Genomic views show non-coding RNA genes with broad K4me3 domains in Wilms tumor. See also Tables S6,S7.

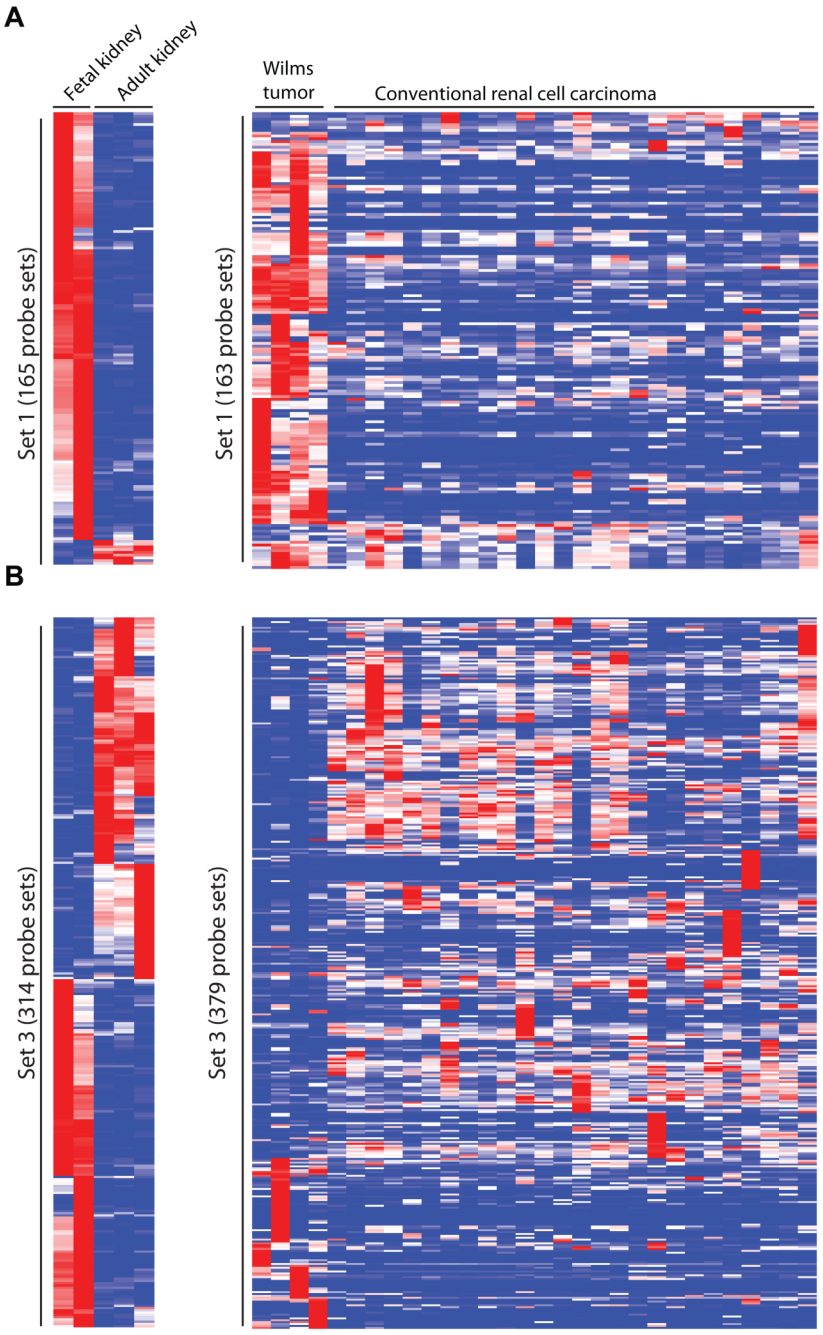

Figure 3. Gene sets defined by chromatin state in Wilms tumor show differential expression patterns in normal and malignant kidney tissues

Row-normalized heat maps depict relative expression of genes in Set 1 and Set 3. Red indicates high levels of expression; blue indicates low or no expression. (a) Genes in Set 1 (broad K4me3) are preferentially expressed in Wilms tumor and fetal kidney (Yusenko et al., 2009). (b) Genes in Set 3 show relatively greater expression in adult kidney, and heterogeneous expression in conventional renal cell carcinoma (cRCC). See also Fig S2.

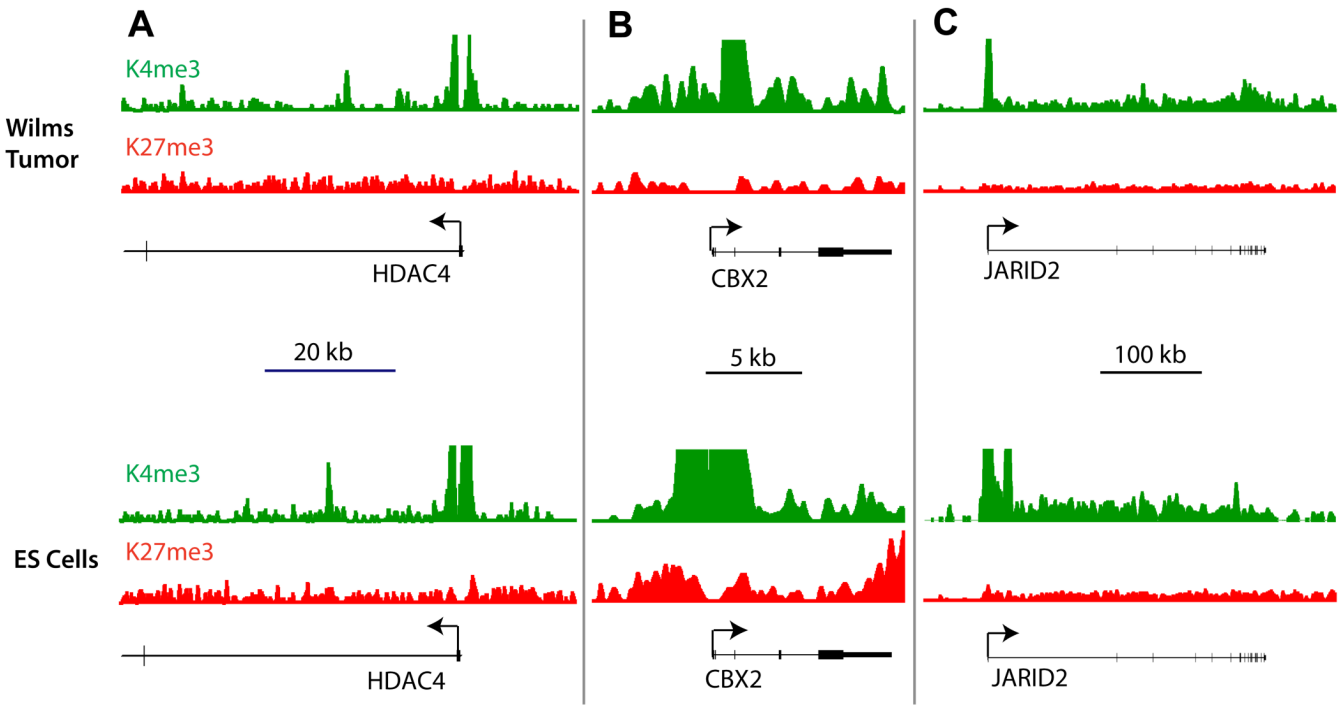

Figure 4. Chromatin regulators in Wilms tumor and ES cells

(a) Genomic views depict K4me3 and K27me3 signals for genes encoding key chromatin regulators that are common to Wilms tumor and ES cells, but inactive in normal kidney and adult renal cancers. The highlighted genes are a subset of 'embryonic' chromatin regulators active in Wilms tumor. See also Fig S3, Table S8, S9.

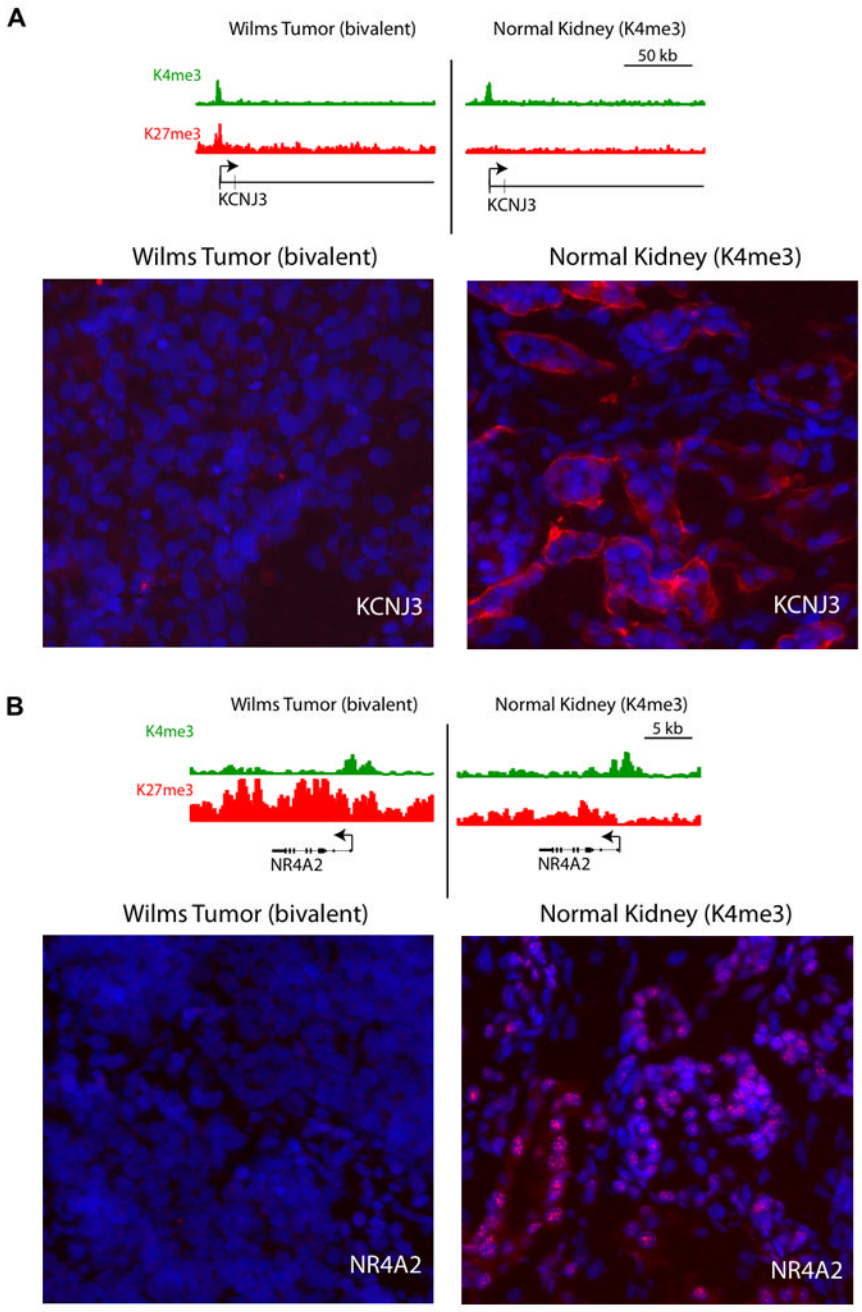

Figure 5. Protein expression patterns of bivalent genes in Wilms tumor and kidney tissue

Genomic views depict K4me3 and K27me3 signals for (a) *KCNJ3* and (b) *NR4A2* which are bivalent in the tumor cells (top panels). Variable transition to a more active chromatin state is evident in normal kidney which is heterogeneous. Immunofluorescence images show expression of the corresponding proteins in Wilms tumor and normal kidney (bottom panels). The images reveal weak expression in tumor, consistent with the chromatin patterns, but strong staining of specific epithelial compartments of normal kidney (red signals). Nuclei are stained with DAPI (blue signal). Original magnification 400×. See also Fig S4.

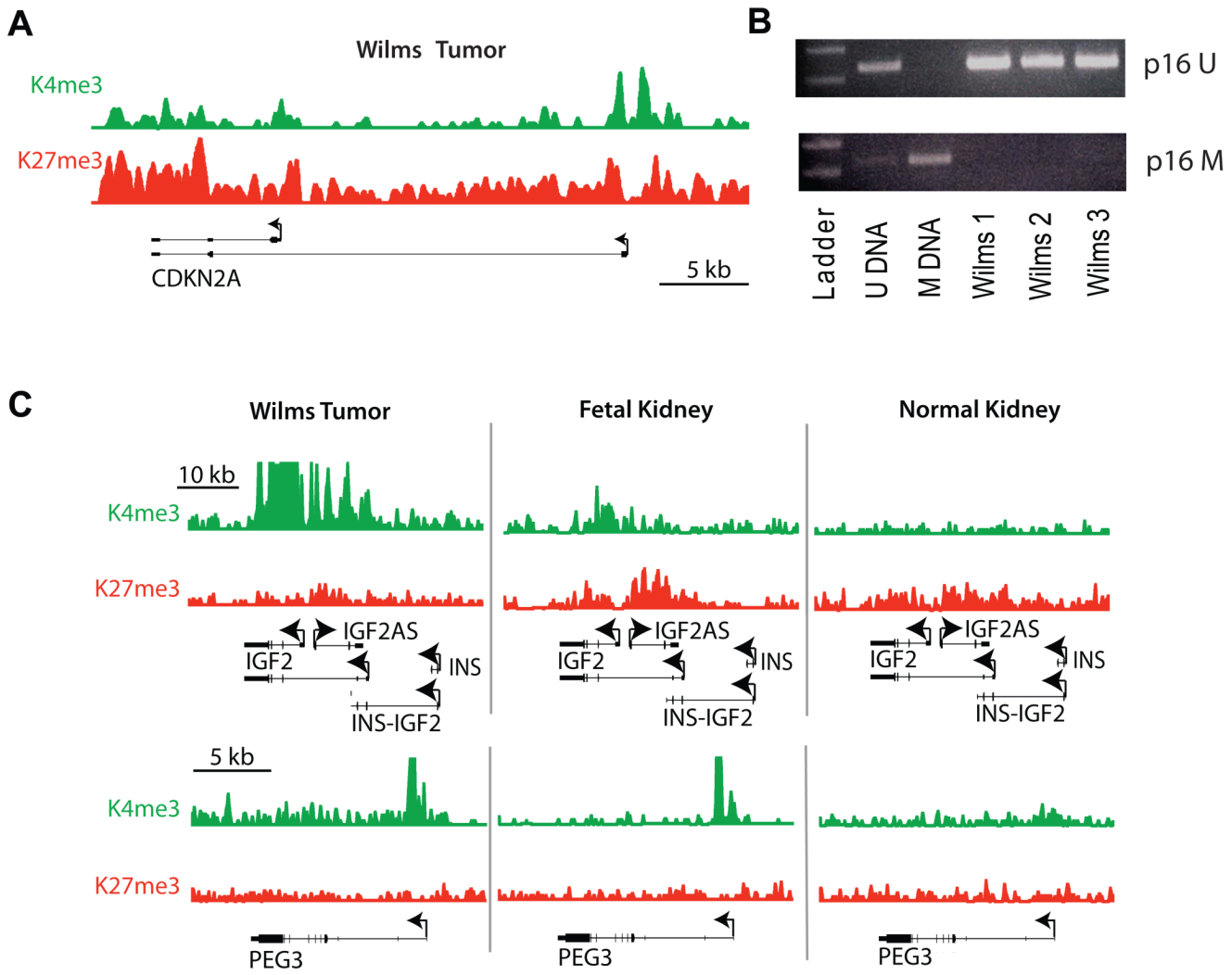

Figure 6. Epigenetic regulation in Wilms tumor

(a) Genomic view depicts K4me3 and K27me3 signals for the p16 tumor suppressor locus (*CDKN2A*). This locus remains intact in the tumor, but is silenced by expansive K27me3 in a pattern reminiscent of normal stem cells. (b) Methylation Specific PCR (MSP) shows that the p16 locus remains unmethylated at the DNA level, a pattern typical of normal stem cells but distinct from many adult cancers. Bisulfite converted DNA was amplified using primers specific for unmethylated (p16 U) or methylated (p16 M) versions of the locus. Gels show PCR products for unmethylated (U) and methylated (M) genomic DNA controls and three Wilms tumor samples. (c) Genomic views depict K4me3 and K27me3 signals for imprinted gene loci. *IGF2*, which has been causally implicated in Wilms tumor, is associated with a uniquely broad K4me3 domain in Wilms tumor. *PEG3* also shows substantially more K4me3 in the tumor.