

RESEARCH ARTICLE

EED, a member of the polycomb group, is required for nephron differentiation and the maintenance of nephron progenitor cells

Le Zhang^{1,2,*}, Sandrine Ettou^{1,3,**}, Myda Khalid^{2,4,**,‡}, Mary Taglienti¹, Dhawal Jain⁵, Youngsook L. Jung⁵, Catherine Seager^{1,3}, Yongqing Liu^{4,§}, Kar-Hui Ng^{4,¶}, Peter J. Park⁵ and Jordan A. Kreidberg^{1,2,3,4,6,‡‡}

ABSTRACT

Epigenetic regulation of gene expression has a crucial role allowing for the self-renewal and differentiation of stem and progenitor populations during organogenesis. The mammalian kidney maintains a population of self-renewing stem cells that differentiate to give rise to thousands of nephrons, which are the functional units that carry out filtration to maintain physiological homeostasis. The polycomb repressive complex 2 (PRC2) epigenetically represses gene expression during development by placing the H3K27me3 mark on histone H3 at promoter and enhancer sites, resulting in gene silencing. To understand the role of PRC2 in nephron differentiation, we conditionally inactivated the *Eed* gene, which encodes a nonredundant component of the PRC2 complex, in nephron progenitor cells. Resultant kidneys were smaller and showed premature loss of progenitor cells. The progenitors in *Eed* mutant mice that were induced to differentiate did not develop into properly formed nephrons. *Lhx1*, normally expressed in the renal vesicle, was overexpressed in kidneys of *Eed* mutant mice. Thus, PRC2 has a crucial role in suppressing the expression of genes that maintain the progenitor state, allowing nephron differentiation to proceed.

KEY WORDS: Epigenetics, Histone methylation, Kidney development, Nephron, Stem cell

INTRODUCTION

The nephrons of the metanephric kidney contain many distinct types of highly differentiated epithelial cells. Nephrons are derived from groups of mesenchymal progenitor stem-like cells (nephron progenitor cells or NPCs) that both self-renew and are induced to differentiate into aggregates (pretubular aggregates, PTAs). Subsequent mesenchymal to epithelial transformation (MET) of PTAs results in formation of a simple tubule (the renal vesicle, RV), which then undergoes segmentation to ultimately form the nephron (reviewed in McMahon, 2016). It is increasingly recognized that the number of nephrons in a kidney is a major determinant of kidney function and overall cardiovascular health in humans (Hoy et al.,

2008; Puelles et al., 2014). Nephron number is determined by the successful induction of sufficient numbers of NPCs during the development of the kidney. Development of the metanephric kidney depends on the interaction of the ureteric bud and the metanephric mesenchyme at the inception of kidney development, and thereafter by the maintenance of appropriate numbers of NPCs, also referred to as the ‘cap mesenchyme’ (Short et al., 2014). If appropriate numbers of NPCs are maintained, human kidneys each harbor several hundred thousand to nearly a million nephrons, whereas mouse kidneys contain ~15,000 nephrons (Zimanyi et al., 2009).

Factors such as BMP7, WNT9B, FGF9 and FGF20 are expressed by the ureteric bud derivatives and the cap mesenchyme itself and regulate the balance between self-renewal and differentiation of kidney progenitors (McMahon, 2016). Additionally, a set of transcription factors expressed by NPCs in the cap mesenchyme, including WT1, PAX2, EYA1, and SIX1, are required to maintain viability of the metanephric mesenchyme (Kreidberg et al., 1993; Torres et al., 1995; Xu et al., 1999, 2003; Li et al., 2003). By contrast, the transcription factor SIX2 is involved in maintaining the balance between self-renewal and differentiation (Self et al., 2006). The absence of SIX2 results in the failure of self-renewal after the initial interaction of the ureteric bud and metanephric mesenchyme, resulting in the premature differentiation of a small number of nephrons, concomitant loss of the progenitor population, and a failure to develop normal kidneys (Self et al., 2006). Importantly, the successful induction of a nephron requires not only expression of these factors in NPCs, but also their subsequent repression as cells differentiate.

Unlike some organs that maintain a population of stem cells throughout life, and use these cells to regularly replenish populations of differentiated epithelial cells, the mammalian kidney does not maintain adult stem cells, according to our present understanding (McMahon, 2016). Rather, once a sufficient number of nephrons are induced, the cap mesenchyme disappears, and no additional nephrons can be formed once these NPCs are gone (Short et al., 2014). The molecular events that regulate the disappearance of this progenitor population are not well understood, although it might result, in part, from a steady decrease in their number during kidney development (Short et al., 2014). Based on our knowledge of how stem cell populations are specified in other organs, as well as recent studies on histone modification in the developing kidney (Adli et al., 2015; Hilliard and El-Dahr, 2016), it is likely that epigenetic regulation of gene expression has an important role in regulating the expression of transcription factors that determine progenitor self-renewal and/or nephron differentiation. Here, we investigate the role of the polycomb repressive complex (PRC) in the maintenance of NPCs in the kidney and in nephron differentiation. PRC refers to two complexes, PRC1 and PRC2 (Entrevan et al., 2016). PRC2 places the H3K27me3 mark at promoter and enhancer regions, resulting in transcriptional

¹Department of Urology, Boston Children’s Hospital, Boston, MA 02115, USA.

²Division of Nephrology, Boston Children’s Hospital, Harvard Medical School, Boston, MA 02115, USA.

³Department of Surgery, Harvard Medical School, Boston, MA 02115, USA.

⁴Department of Pediatrics, Harvard Medical School, Boston, MA 02115, USA.

⁵Biomedical Informatics, Harvard Medical School, Boston, MA 02115, USA.

⁶Harvard Stem Cell Institute, Cambridge, MA 02139, USA.

*Present address: Department of Neurology, Yale University, New Haven, CT 06520, USA.

‡Present address: James Whitcomb Riley Hospital for Children, Indianapolis, IN 46202, USA.

§Present address: The Second Hospital of Shandong Inv. Jinan, PR China.

¶Present address: National University of Singapore, Singapore.

**These authors contributed equally to this work

‡‡Author for correspondence (jordan.kreidberg@childrens.harvard.edu)

© L.Z., 0000-0002-4860-831X; Y.L., 0000-0003-3535-7416; J.A.K., 0000-0002-9085-2420

repression (Conway et al., 2015). Repression of gene expression by PRC1 and PRC2 is believed to be a major mechanism whereby gene expression is negatively regulated during development, the other major mechanism being histone deacetylation (Margueron et al., 2005). We found that nephron progenitors were prematurely lost from the developing kidney in the absence of functional PRC2, even though low-level SIX2 expression was detected beyond the time point at which it should cease. Additionally, we found that nephrons were unable to form properly in the absence of PRC2 function during nephron differentiation. *Lhx1* (previously *Lim1*), a transcription factor involved in nephron differentiation (Kobayashi et al., 2005), is overexpressed in the absence of PRC2 function, suggesting that PRC2 is required to repress a progenitor program of gene expression and allow nephron differentiation to proceed.

RESULTS

Analysis of kidneys in *Eed* mutant mice

To determine the role of PRC2 in the developing kidney, we inactivated expression of *Eed* in NPCs using a previously reported bacterial artificial chromosome (BAC) *Six2-GFP/Cre* transgene (hereafter referred to as *Six2-TGC*) (Kobayashi et al., 2008), mated to mice carrying a floxed allele of *Eed* (Xie et al., 2014). EED is a nonredundant component of PRC2 (Cao et al., 2014), such that its inactivation should prevent deposition of H3K27me3. *Eed fl/fl*, *Six2-TGC* mice (referred to herein as '*Eed* mutants') were runted and rarely survived more than 10 days. Kidneys of postnatal *Eed* mutant mice were smaller than those of control mice (Fig. 1A and Fig. S1). We used fluorescence-activated cell sorting (FACS) to assess the overall numbers of cells derived from SIX2-expressing progenitors, by combining an enhanced yellow fluorescent protein

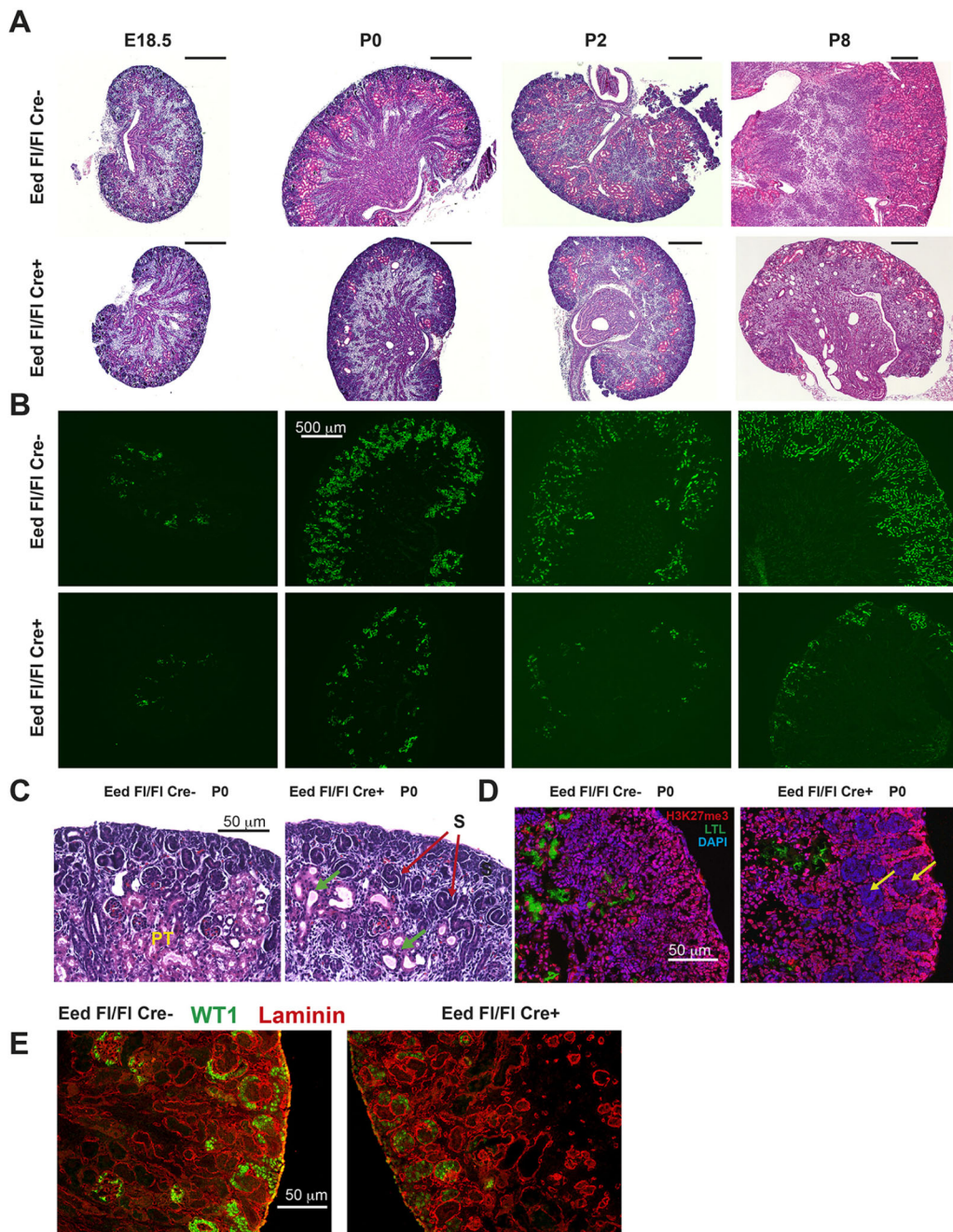


Fig. 1. Histology and characterization of *Eed* mutant kidneys. (A) Hematoxylin and Eosin stains of E18.5, P0, P2 and P8 kidneys as indicated at the top of each column. Genotypes: top row, *Eed fl/fl*; bottom row, *Six2-TGC*, *Eed fl/fl*. (B) LTL lectin staining at equivalent time points as in (A). Top and bottom row genotypes as in (A). (C) Higher-power histology of P0 kidneys showing S-shaped tubules in *Eed* mutants (red arrows) and proximal tubules (PT) abundant in controls and largely absent in *Eed* mutants. Dysgenic tubules are present in *Eed* mutants (green arrows). (D) H3K27me3 staining (pink) showing areas of absent staining in *Eed* mutants (yellow arrows). (E) WT1 (green) and laminin (red) immunostaining of P0 kidneys. All images are representative of at least three kidneys analyzed. Scale bars in A: 500 μ m.

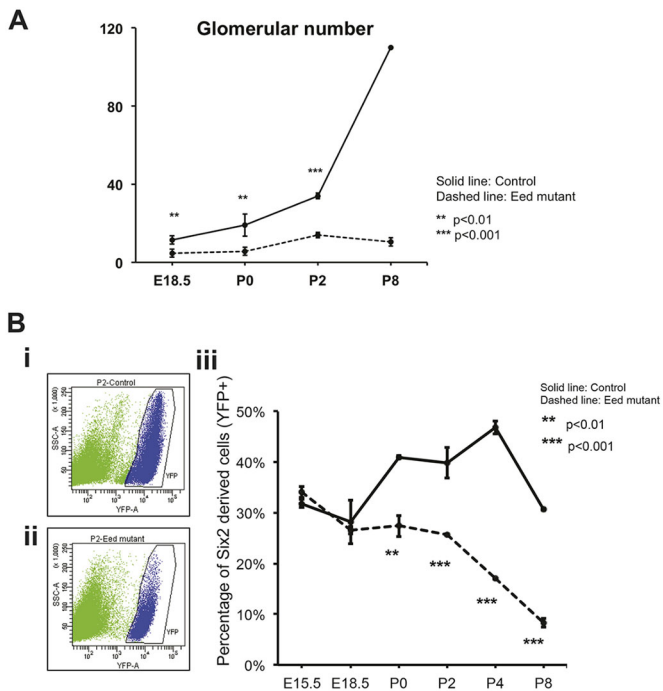


Fig. 2. Quantification of kidney growth. (A) Glomerular counts at E18.5, P0, P2 and P8 kidneys. See Materials and Methods for details of counting. Data are presented as mean±s.d. ** $P < 0.05$, *** $P < 0.01$, Student's *t*-test. (B) FACS analysis of cells derived from P2 (i) *Six2-TGC; Eed fl/+ R26R-EYFP+* kidneys; (ii) *Six2-TGC; Eed fl/fl R26R-EYFP+* kidneys. (iii) Cell counts from time points indicated on the x-axis are plotted as YFP-expressing cells as a percentage of the total cells sorted. The YFP+ cells were designated as shown in blue for the P2 time point in (i,ii). *P*-values in A and Biii refer to differences between controls and mutants at the specific time points. At least four kidneys per time point/genotype were used. Data are presented as mean±s.d. ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

(eYFP) reporter allele from *B6.129X1-Gt(ROSA)26Sor^{tm1(EYFP)Cos/J}* mice (henceforth referred to as *R26R-EYFP* mice) with *Eed* mutants or control (*Eed fl/+*, *Six2-TGC*) mice (Fig. 2B). At embryonic day (E)18.5, approximately similar proportions of sorted cells expressed the eYFP reporter. However, by postnatal day (P)0 the percentage of eYFP-expressing cells was diminished in *Eed* mutants and was greatly decreased (10% versus 30%) by P8. Glomerular counts (as a measure of nephron number) also indicated that the numbers of induced and differentiated nephrons leveled off after E18.5 in *Eed* mutants, whereas these increased nearly sixfold in control *Six2-TGC; Eed fl/+* mice between E18.5 and P8 (P8) (Fig. 2A), as previously described for mouse kidneys (Cebrián et al., 2004; Short et al., 2014).

Nephron differentiation in *Eed* mutant mice

Few differentiated tubules resembling mature nephrons were present based on the histological appearance of kidneys of *Eed* mutant mice. The tubules [defined by the presence of a laminin-containing basement membrane (Fig. 1E)] that were present did not have the histological appearance of normally differentiated nephrons, such as the presence of abundant eosinophilic proximal tubules (Fig. 1C and Fig. S2, for a larger version of Fig. 1C). Immunostaining for H3K27me3 revealed that areas containing undifferentiated tubules corresponded to the absence of H3K27me3 deposition (Fig. 1D). The growth of the kidney results from both the continual induction of new nephrons (between E12.5 and P2 in mouse) and the expansion of each individual nephron from a few hundred cells into an extensive

epithelial unit (Short et al., 2014). We first examined nephron differentiation in *Eed* mutant mice. After MET, newly induced PTAs develop into an immature form known as the S-shaped tubule, where segmentation into the various parts of the nephron begins to become apparent. S-shaped tubules were present in *Eed* mutants (Fig. 1C and Fig. S2), but the reduced number of differentiated tubules indicated that PRC2 activity is required to form mature nephrons. Consistent with this appearance, *Lotus tetragonolobus* lectin (LTL)-lectin staining showed reduced numbers of differentiated proximal tubules in these kidneys at all time points examined, including E18.5, P0, P2 and P8 (Fig. 1B). Distal tubules, assessed by staining for SLC12A3, were present in proportion to the number of proximal tubules in *Eed* mutants (Fig. 3A,B). Staining for SLC12A1 was also used to assess the presence of loops of Henle (Fig. 3C,D). Although a few SLC12A1-containing tubules were present in *Eed* mutants, these tubules did not have the morphological appearance of loops of Henle, suggesting that this nephron segment involves additional differentiation steps requiring polycomb activity.

In our further analyses of *Eed* mutants, we used the *B6;129S6-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J}* reporter allele (henceforth referred to as *R26R-tdTomato* reporter mice) activated by the *Six2-TGC* transgene to define cell populations co-expressing GFP and tdTomato by FACS from kidneys of control *R26R-tdTomato; Eed fl/+* and *Eed* mutant, *R26R-tdTomato* P0 mice. In these reporter mice, expression of the *R26R-tdTomato* reporter is directed by a CAG enhancer-promoter construct (Niwa et al., 1991). We made the fortuitous observation that two distinct populations of cells were present in control P0 kidneys based on lower or higher tdTomato expression along with high green fluorescent protein (GFP) expression from the *Six2-TGC* transgene (Fig. 3A, henceforth these are referred to as 'tdTomato low' and 'tdTomato high' populations). Most interestingly, in kidneys of P0 *Eed* mutant, *R26R-tdTomato* mice, the population expressing high levels of tdTomato was almost missing (Fig. 3B). The physiological basis for the differential expression of tdTomato from this transgene is unknown, but might relate to SRF/MRTF-binding sites in the chick *ACTB* promoter within the CAG construct and the differential activity of these actin level-sensitive transcription factors (Knöll, 2010; Xu et al., 2016) in various cell types of the developing kidney. RNA sequencing (RNA-seq) was used to define these populations obtained from control and *Eed* mutant mice by FACS (Table S1, Fig. S4 for reproducibility of samples). Two comparisons are shown, one emphasizing a kidney development gene set [with Gene Ontology (GO) annotation GO:0001822; Fig. 3C] and the other an ion transport gene set (with GO annotation GO:0006811) (Fig. 3D). GO analysis (Supek et al., 2011) of control tdTomato populations demonstrated that the lower tdTomato population mainly represented NPC and PTA cells, whereas the high tdTomato population was derived from more differentiated nephrons (Fig. 3C,D and Fig. S5A,B). As expected, the GO analysis identified components, including various 'membrane transport' and 'metabolic' processes, in the control high tdTomato-expressing population, and progenitor-related components, such as 'urogenital system development' and 'tube development', in the control low tdTomato-expressing population (Fig. S5A,B).

Comparisons of gene expression in four cell populations (control and *Eed* mutant tdTomato low, and control and *Eed* mutant, tdTomato high) revealed interesting observations suggestive of the role of polycomb in regulating gene expression during organogenesis. Using the kidney development gene set, the control and *Eed* mutant tdTomato low populations displayed a small but significant number of differences (Fig. 3C). Most notably *Lhx1*, which encodes a Lim-homeodomain transcription factor expressed in the renal

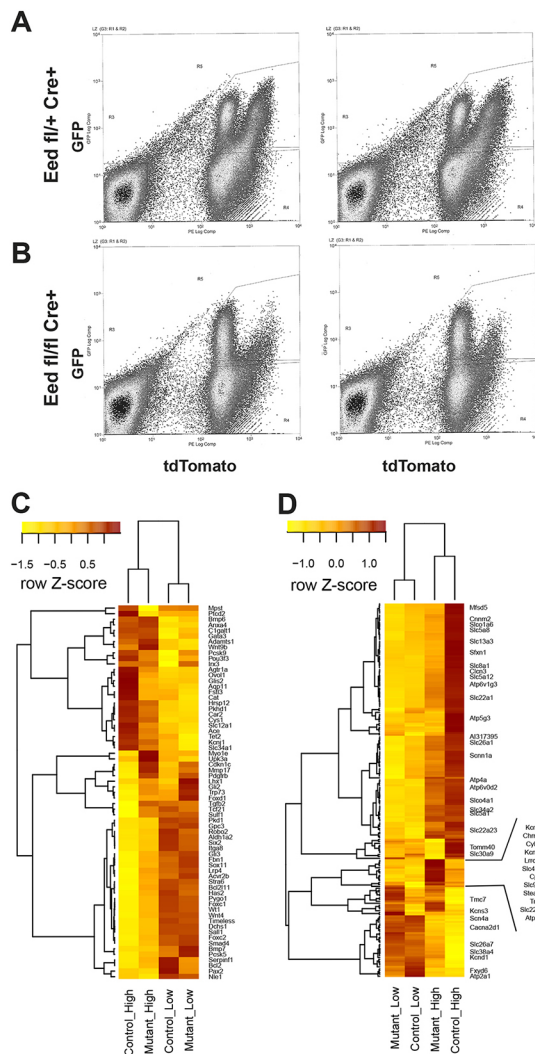


Fig. 3. RNA-seq analysis of tdTomato-expressing cells. (A) FACS of kidney cells from *Six2-TGC, Eed fl/+, R26R-tdTomato* reporter mice. The results from two individual mice are shown, representative of 10 biological replicates. x-axis: tdTomato expression level; y-axis: GFP expression level. (B) FACS of *Six2-TGC, Eed fl/fl, R26R-tdTomato* reporter mice. (C, D) Heatmaps summarizing the expression of (C) kidney development (GO: 0001822) and (D) ion transport (GO:0006811)-specific gene sets identified as being differential. Expression values are normalized along the rows using Z-score transformation. Only selected genes are labeled in (D) for clarity.

vesicle (Kobayashi et al., 2005), was overexpressed in mutant kidneys (Figs 3C and 4C). Given that pretubular aggregates appear to undergo MET in *Eed* mutant kidneys, but fail to complete differentiation into mature nephrons, this overexpression of *Lhx1* suggests that PRC2-mediated repression of *Lhx1* is a crucial step in nephron differentiation beyond the renal vesicle stage. Additionally, a gene normally expressed in the stromal as opposed to the nephron lineage, *Foxd1*, was overexpressed in *Eed* mutant tdTomato-low cells, suggesting a role for PRC2 in maintaining lineage-specific patterns of gene expression. In contrast to genes that are involved in MET and nephron differentiation, such as *Lhx1*, genes that affect the proliferation and self-renewal of NPCs, such as *Six2, Wt1, Pax2* and *Sall1*, were either expressed at slightly higher levels in control tdTomato-low cells or not differentially expressed, suggesting that PRC2 has a less crucial role in directly repressing the expression of genes required for the self-renewal of

NPCs. A heat map summarizing our overall results is shown in Fig. S6. Consistent with the patterns emerging from the heat maps, GO analysis showed developmental and renal physiological processes to be over-represented in control tdTomato-low cells (Fig. S5D), whereas relatively few developmental processes were overexpressed in mutant control-low cells (Fig. S5C), and those present were representative of nonkidney-specific processes. Furthermore, consistent with the overall appearance of *Eed* mutant versus control kidneys, the ion channel heat map showed that most genes were overexpressed in control compared with *Eed* mutant kidneys (Fig. 3D) and GO analysis revealed that many kidney-specific physiological processes were over-represented in control tdTomato-high cells (Fig. S5F). A small number of ion channel and transport-related genes were overexpressed in *Eed* mutant tdTomato-high cells, including those encoding transferrin, ceruloplasmin and several others (Fig. 3D). At present, there is no apparent commonality or GO terms represented among these genes that distinguishes them from the larger group that is more highly expressed in control kidneys. Accordingly, fewer GO terms were over-represented in *Eed* mutant tdTomato-high cells (Fig. S5E). Nevertheless, several developmental GO terms were represented in the *Eed* mutant tdTomato-high cells, suggesting a general failure to repress developmental pathways in these cells.

Abnormal NPC gene expression in kidneys of *Eed* mutant mice

The smaller size of kidneys in *Eed* mutant mice suggested a premature disappearance of *Six2*-expressing NPCs. Therefore, we sought to determine the molecular basis for decreased progenitor cell endowment in kidneys of *Eed* mutant mice. *Six2* mRNA is tightly restricted to the cap mesenchyme in the nephrogenic zone of developing kidneys (Self et al., 2006). In *Eed* mutant kidneys, *Six2*-expression cap mesenchyme cells were reduced in number, and *Six2* mRNA levels were decreased by quantitative RT-PCR (qPCR) in tdTomato-low cells from *Six2-TGC, R26R-tdTomato* P2 kidneys (Fig. 4). Despite the reduced *Six2* mRNA in the nephrogenic zone, low-level expression of *Six2* mRNA could be detected by *in situ* hybridization in the dysgenic tubules that were present in *Eed* mutant kidneys (Fig. 4A,C). Antibody staining could not detect SIX2 protein in *Eed* mutant kidneys, suggesting that SIX2 protein was either below the limits of detection or not expressed in *Eed* mutants. Together, these results suggest that repression of gene expression by PRC2 is not only required to maintain self-renewal of kidney progenitor cells, particularly during the later stages of kidney development (post E18.5), but might also have a role in repressing *Six2* expression to allow proper nephron differentiation. Furthermore, even though we inactivated a repressive complex, no patterns of increased gene expression were detected that would obviously account for the premature loss of NPCs.

In situ hybridization and antibody staining for PAX2 protein indicated that PTAs and renal vesicles were also decreased by P0 in *Eed* mutants, such that, in some places, only Pax2-expressing ureteric bud derivatives were observed without adjacent PTAs or comma and S-shaped tubules (Fig. 4A,C). *Pax2* mRNA was also greatly reduced, as revealed by qPCR (Fig. 4C).

Expression of WT1, a transcription factor crucial for the maintenance of kidney NPCs, was also decreased in *Eed* mutant kidneys (Fig. 1E), although *Wt1* mRNA levels in tdTomato-low cells did not differ significantly in *Eed* mutants compared with tdTomato-low cells from control kidneys (Fig. 4C). *Wt1* is expressed in differentiating cells and in mature podocytes of the glomerulus. Accordingly, *Wt1* staining showed fewer maturing glomeruli in mutant kidneys (Fig. 1E).

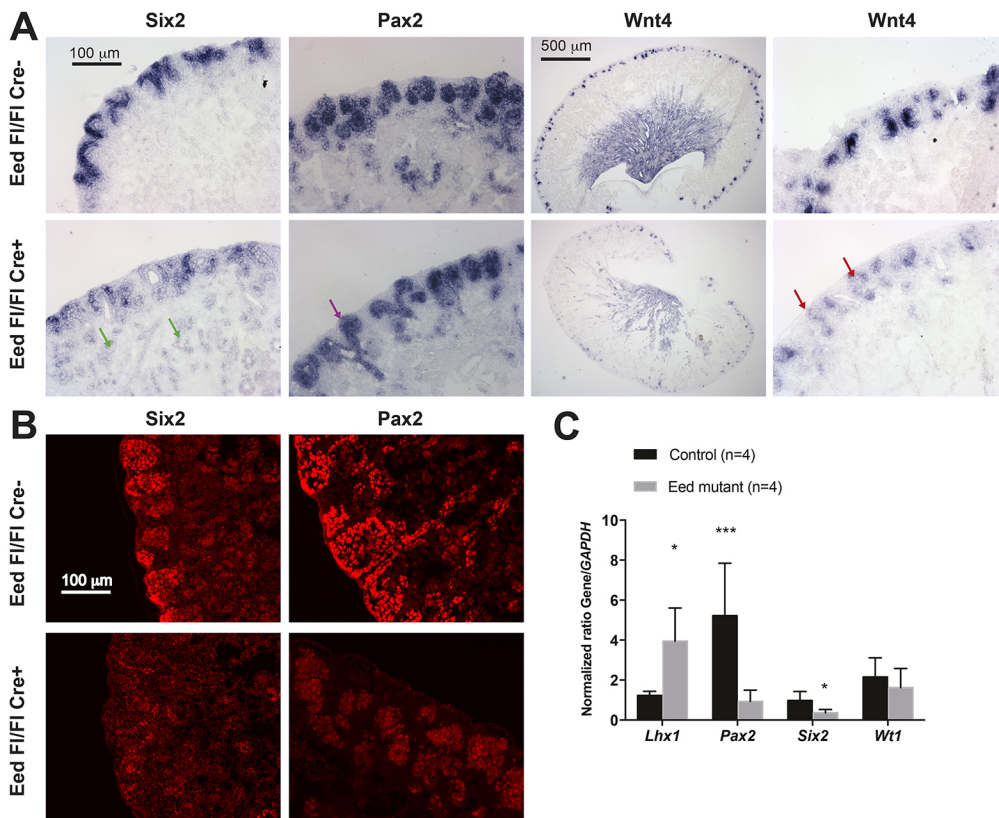


Fig. 4. Gene expression analysis of *Eed* mutant kidneys. (A) *In situ* hybridization analysis of P0 kidneys. Top row: *Eed fl/fl*; bottom row: *Six2-TGC, Eed fl/fl*. The probe used is noted at the top of each column. *Wnt4* is shown as both a low-power image and a higher-power image, whereas all others are at higher power. Green arrows indicate ectopic *Six2* expression; purple arrow indicates *Pax2*-expressing ureteric bud without differentiating nephron structures; and red arrows indicate ectopic *Wnt4* expression in the usual location of the cap mesenchyme adjacent to ureteric bud. The images are representative of three kidneys for each genotype. (B) Immunostaining of SIX2 and PAX2 in P2 kidneys. Top row: *Six2-TGC, Eed fl/+*; bottom row: *Six2-TGC, Eed fl/fl*. The images are representative of three kidneys for each genotype. (C) qPCR analysis of gene expression. * $P < 0.05$, *** $P < 0.01$.

Wnt4 is expressed by PTAs (Stark et al., 1994), which are aggregates of NPCs and the formation of which is induced by *Wnt9b* expressed at the tips of the ureteric bud and its derivative branches (Carroll et al., 2005). *Wnt4* acts in an autocrine fashion to induce MET of the PTA to form the renal vesicle. *Wnt4* staining was less intense in PTAs of *Eed* mutant kidneys (Fig. 4A). In some locations along the nephrogenic zone of *Eed* mutant kidneys, *Wnt4* appeared to be prematurely expressed in small numbers of NPCs adjacent to the ureteric bud derivatives (Fig. 4A), suggesting that PRC2 is involved in repressing *Wnt4* expression in NPCs until they are induced to form PTAs.

***Six2*-expressing cells persist in kidneys of *Eed* mutant mice**

To further determine how PRC2 inactivation affects the maintenance of NPCs during development, FACS was used to detect eGFP-expressing cells using eGFP from the *Six2-TGC* transgene as a reporter of ongoing *Six2* expression (Fig. 5 and Fig. S7). At P2, GFP-expressing cells represented nearly 6% of the total cells in control kidneys, whereas kidneys from *Eed* mutants had <2% GFP-expressing cells. By contrast, a small but significant number of GFP-expressing cells could be detected as late as P8 in *Eed* mutants, a time point at which GFP-expressing cells were absent in control kidneys (0.2% versus undetectable). Thus, it appears that the absence of PRC2-mediated transcriptional repression allows the persistence of low-level *Six2* mRNA expression in the immature dysgenic tubules in kidneys of *Eed* mutant mice.

Aiden et al. analyzed the genome-wide chromatin state comparing Wilms' tumors (a tumor of the kidney found in infants and young children) to fetal and mature human kidneys (Aiden et al., 2010). Their study found bivalent chromatin (Bernstein et al., 2006) in fetal kidneys at genes known to be important developmental regulators of kidney development. Several of these bivalent sites converted to

repressed chromatin, and were associated with the H3K27me3 mark, in mature kidneys. Among the genes noted by Aiden et al. to express bivalent chromatin was *Six2*, which displayed bivalent chromatin in fetal kidneys and the H3K27me3 mark in mature kidneys (Table S2). Thus, these results are consistent with our own observation that repression of *Six2* expression requires PRC2 function and, consequently, *Six2* is not completely repressed in kidneys of *Eed* mutant mice.

Elevated and abnormal *HeyL* expression in *Eed* mutant kidneys

Notch signaling is required to suppress *Six2* expression and allow nephron differentiation (Chung et al., 2016). The expression of *Notch1* and *Notch2* were not significantly different in *Eed* mutants (Fig. 6A). HeyL, Hey1, Hey2 and Hes1 are basic helix-loop-helix (bHLH) transcriptional repressors that act downstream of Notch signaling (Maier and Gessler, 2000; Fischer et al., 2002). The expression of *HeyL* and *Hey1* (and *Hey2* to a lesser extent) were significantly increased in *Eed* mutants (Fig. 6A). *In situ* identification of *HeyL* mRNA in P2 kidneys demonstrated its expression in renal vesicles and segments of S-shaped tubules (Fig. 6B). By contrast, whereas all *HeyL* expression in controls was in differentiating structures, *HeyL* expression in *Eed* mutants appeared not only in structures resembling poorly formed S-shaped tubules, but also in mature, albeit dysgenic tubules not characteristic of typical nephrons (Fig. 6B). It is not known how Notch signaling represses *Six2* expression (Chung et al., 2016), although HeyL and the other Notch-responsive transcriptional repressors are candidates to mediate this effect. Furthermore, persistence of *Six2* expression despite the high expression of *HeyL* and *Hey1* suggests that PRC2 is required for Notch-mediated repression of *Six2* expression. Additionally, the increased expression of *HeyL* and *Hey1* in *Eed*

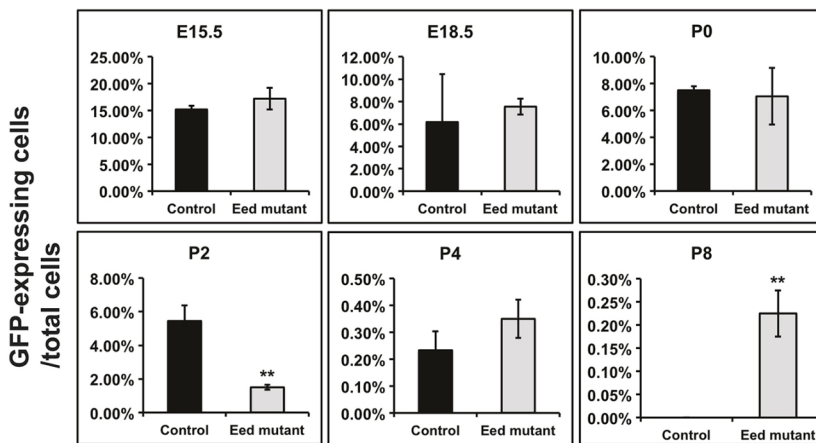


Fig. 5. FACS analysis of *Six2*-expressing cells. The percentage of eGFP-expressing cells as a proportion of total cells is shown. Control: *Six2-TGC, Eed fl/+ , R26R-EYFP +/-*; and *Eed* mutant: *Six2-TGC, Eed fl/fl, R26R-EYFP +/-*. The scales of the y-axis vary at different time points. Data are presented as mean \pm s.d. ** $P < 0.01$, Student's *t*-test. Time points without asterisks were not significantly different. $N =$ at least three for each time point and/or genotype.

mutants suggests that PRC2 is also required to suppress what is usually a transient wave of Notch signaling during the induction of nephron differentiation.

DISCUSSION

We report here the phenotype of mice carrying a targeted inactivation of the polycomb gene *Eed* in kidney NPCs. We observed the earlier than usual disappearance of NPCs, along with a failure of NPCs to properly differentiate into nephrons. A small number of

Six2-expressing cells (based on GFP detection from *Six2-eGFP/Cre* reporter transgenes) persisted in *Eed* mutant mice, suggesting that PRC2 is required to maintain NPCs. *Lhx1*, a transcription factor present in the renal vesicle and S-shaped tubules and required for proper nephron differentiation, was overexpressed in *Eed* mutant kidneys, indicating the PRC2 function is also required for nephron differentiation to proceed past the S-shaped tubule stage, although not specifically for MET.

Similar to other organs that arise from populations of stem-like progenitors, the nephrons of the kidney arise from a population of stem cells. This stem-like population disappears after a sufficient number of epithelial units, or nephrons, has been induced (McMahon, 2016). *Six2* was originally characterized as a 'gatekeeper' gene that maintains the self-renewal of NPCs (Self et al., 2006). Recent studies indicate that *Six2* activates the expression of target genes required for self-renewal, including *Six2* itself, in NPCs, while at the same time in these NPCs, a complex that includes *Six2*, *Osr1* and TCF factors might attract the Groucho-related co-repressor to repress *Wnt4* and other differentiation-related genes (Park et al., 2012; Xu et al., 2014). Once canonical Wnt signaling is activated by *Wnt9b*, β -catenin might replace Groucho-related proteins on T cell factor (TCF) factors and convert this repressive complex into an activating complex that turns on the expression of genes involved in forming the PTA and initiating formation of the nephron (Park et al., 2012; Xu et al., 2014). Notably, in response to β -catenin, *Wnt4* expression is turned on in the pretubular aggregate and is crucial to inducing MET. *Wnt4* expression is then turned off after the renal vesicle and/or early S-shaped tubule stage.

Therefore, it is of particular interest that we observed premature *Wnt4* expression in kidney NPCs (at least defined by their location in aggregates at the periphery of the kidney and in contact with the ureteric bud). We speculate that *Wnt4* expression is turned on prematurely not only because the SIX2-associated repressive complex is unable to repress *Wnt4* in the absence of PRC2, but also because WT1, the transcription factor that would typically activate *Wnt4* expression in pretubular aggregates (Essafi et al., 2011), is already present in kidney NPCs. Thus, during normal development, when PRC2 is present, SIX2-mediated repression of *Wnt4* in self-renewing NPCs predominates over WT1 activation of *Wnt4*. However, in the absence of PRC2, WT1 activation of *Wnt4* might predominate, leading to an imbalance with decreased self-renewal and increased differentiation of NPCs. By contrast, *Wnt4* spatial expression in PTAs and the renal vesicles themselves is reduced in *Eed* mutant kidneys, possibly because the premature depletion of NPCs results in smaller numbers of

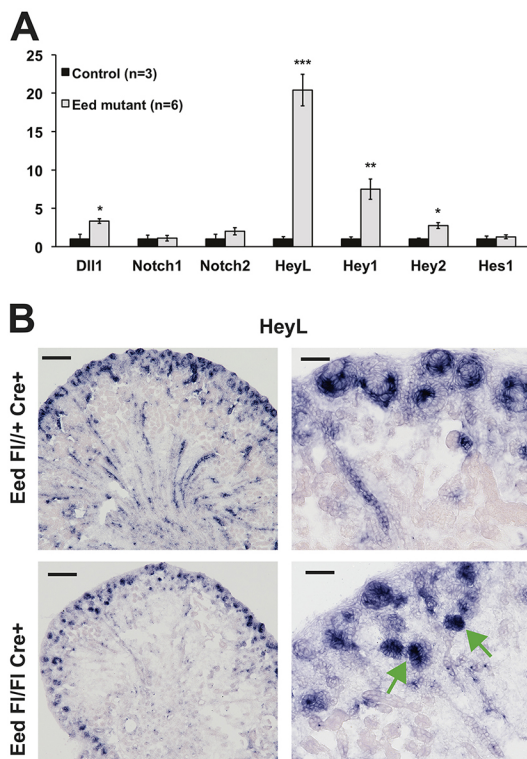


Fig. 6. Notch signaling in *Eed* mutant kidneys. (A) qPCR analysis of gene expression of eYFP-FACS-sorted cells from Control: *Six2-TGC, Eed fl/+ , R26R-EYFP +/-*; and *Eed* mutant: *Six2-TGC, Eed fl/fl, R26R-EYFP +/-*. $N = 3$ controls and 6 mutants. Controls for each gene were set at '1'. Data are presented as mean \pm s.d. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. All results were first normalized to GAPDH. (B) *HeyL* *in situ* hybridization (middle panels), merged images (right panels). *HeyL* is not present in tubules of maturing nephrons but is present in dysgenic tubules of *Eed* mutant (green arrows, lower right panel). Scale bars: 500 μ m, left panels; 50 μ m, right panels. Images are representative of three experiments.

cells contributing to PTAs and subsequent renal vesicles and S-shaped tubules.

In contrast to *Six2*, we did not observe continued *Wnt4* expression in the dysgenic tubules in *Eed* mutant kidneys. This might reflect that WT1 itself does not continue to be expressed, such that there is not a transcriptional activating complex at the *Wnt4* gene. In addition, these observations might provide an indication that some genes, including *Six2*, require the H3K27me3 mark to terminate their expression, whereas other genes, such as *Wnt4*, do not strictly require the H3K27me3 mark for repression. Consistent with this possibility, relatively few genes in the kidney development set were elevated in *Eed* mutant tdTomato-low cells compared with controls (Fig. 3C), *Lhx1* being a notable exception. This is also consistent with our analysis of the data of Aiden et al. (2010), showing that *Six2* was one of only a few genes transiting from a bivalent to H3K27me3 state in fetal compared with mature kidneys. In this regard, it is also notable that overall, our RNA-seq analyses revealed most genes to be unchanged in all control-*Eed* mutant comparisons (Fig. S8). Therefore, we suggest that the PRC2 complex has specific targets during organogenesis and that there are other pathways for inactivating gene expression in the case of non-PRC2 target genes.

Our observations also lead us to question why, in the presence of ectopic *Six2* mRNA expression, we observed the early demise of the progenitor population, especially given the inability to turn off *Six2* expression. Furthermore, the inability to turn off *Six2* expression could lead not only to persistent progenitors, but also to excessive nephrogenic blastema or even to Wilms' tumors, a blastemal tumor of the kidney found in young children. Even though there appeared to be very low-level persistence of *Six2* mRNA in the cortex of *Eed* mutant kidneys, the *Six2* mRNA expression present in NPCs of those kidneys appeared to be lower than normal and the SIX2 protein level was below the limits of detection. These observations suggest that, although PRC2 is required to completely turn off *Six2* expression, there are factors required to maintain high levels of *Six2* expression, likely to be in addition to *Six2* itself, the expression of which is not maintained in *Eed* mutant kidneys. These factors are unknown [although we previously demonstrated that SIX2 is not regulated by WT1 (Hartwig et al., 2010)].

An additional answer to this question might be found in previous studies where defective nephron differentiation is accompanied by early loss of progenitors, an example being when we previously inactivated the *Bmp7* gene in podocytes (Kazama et al., 2008), or when *FGF8*, expressed by PTAs, was inactivated in embryonic kidneys (Grieshammer et al., 2005; Perantoni et al., 2005). This, and other observations, led us to suggest that the self-renewal of NPCs requires ongoing nephrogenesis. Indeed, there are few, if any, examples of phenotypes where self-renewal and proliferation of NPCs is maintained in the absence of ongoing nephrogenesis.

As mentioned above, *Six2* is thought to positively regulate its own expression (Park et al., 2012). FGF9 and -20 (Barak et al., 2012), as well as BMP7 (Dudley et al., 1995; Luo et al., 1995; Blank et al., 2009), promote the self-renewal and proliferation of NPCs, suggesting that *Six2* transcription is also downstream of receptor tyrosine kinase signaling and BMP receptor signaling, the latter through JNK MAP kinase (Blank et al., 2009). It remains unclear why these signals might be attenuated in the absence of a functional PRC2 complex. We recently demonstrated that the WT1 target gene *Gas1*, which encodes a GPI-linked membrane protein, amplifies FGF signaling in NPCs (Kann et al., 2015). In *Eed* mutants, WT1 protein levels were significantly reduced in NPCs, suggesting that FGF signaling mediated by GAS1 was reduced in *Eed* mutants,

providing an additional explanation for reduced levels of *Six2*, and consequently decreased self-renewal of NPCs.

Recent studies demonstrate that Notch-mediated cessation of *Six2* expression is required for the differentiation of renal vesicles into S-shaped tubules and more differentiated nephrons (Chung et al., 2016). Therefore, it is possible that the persistent *Six2* expression we observed in *Eed* mutants was partially responsible for the failure of nephron differentiation. Arguing against this explanation is our failure to detect SIX2 by immunostaining in *Eed* mutants, suggesting that, if SIX2 is present, it is likely at low levels. If ectopic expression of *Six2* is partially responsible for the defect in nephron differentiation, the block is probably post MET and at the point of the appearance of the renal vesicle or early S-shaped tubule. Given that Notch signaling is required to stop the expression of *Six2* (Chung et al., 2016), the increased expression of *HeyL* and *Hey1* in *Eed* mutant kidneys was surprising, because this suggests that Notch signaling is hyperactivated in *Eed* mutants. Together, these observations suggest either that HeyL or Hey1 are not the relevant mediators of Notch signaling involved in repressing *Six2* expression, or that HeyL or Hey1 utilize PRC2 to repress *Six2*, and that this complex is absent in induced NPCs of *Eed* mutant kidneys. Further studies will require anti-HeyL or anti-Hey1 antibodies suitable for chromatin immunoprecipitation (ChIP) to test these hypotheses.

MATERIALS AND METHODS

Mice

All animal studies were carried out in accordance with the guidance of the Institutional Animal Care and Use Committee at Boston Children's Hospital. *Eed* floxed allele mice (Xie et al., 2014) and *R26R-EYFP* mice (Jackson Labs 006148) were obtained from Drs Huafeng Xie and Stuart Orkin (Boston Children's Hospital). *R26R-tdTomato* mice (Jackson Labs 007909) were obtained from Dr Joseph Majzoub (Boston Children's Hospital). BAC transgenic *Six2-TGC* mice (Kobayashi et al., 2008) were obtained from Andrew McMahon (formerly Harvard University).

Antibodies and reagents

The antibodies and reagents used were as follows: anti-Pax2, obtained from Greg Dressler (University of Michigan); anti-Six2 (ProteinTech 11562); anti-WT1 C-19 (Santa Cruz sc-192); anti-H3K27me3 (Abcam #6002); anti-laminin (Millipore 4E10); LTL-fluorescein (Vector Laboratories FL-1321); anti-SLC12A1 (Proteintech #18970-1-AP); and anti-SLC12A3 (Sigma #HPA028748).

Glomerular counts

Numbers of glomeruli were quantified by counting total glomeruli in a single histological section from the widest diameter of the kidney. For each time point, at least three kidneys from littermate individual mice were analyzed.

Immunohistochemistry

Kidneys were fixed with 4% paraformaldehyde/PBS overnight at 4°C, cyroprotected in 12% sucrose for 12 h and 30% sucrose overnight at 4°C, and then embedded/frozen in optimal cutting temperature (OCT) compound. Immunofluorescence was carried out on kidney cryosections (5 µm, Leica CM3050S) for either native fluorescence of fluorescent proteins or immunostaining of antibodies. For direct imaging of tdTomato red fluorescent protein, sections were coverslipped with Prolong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific P36931). For antibody immunostaining, sections were permeabilized with 0.25% Triton X-100 in PBS for

10 min at room temperature (RT) and blocked with 10% goat serum and 2% BSA for 1 h at RT. Sections were incubated with primary antibodies with 1:200-1:1000 dilution in 5% goat serum and 1% BSA and 0.1 Triton X-100 in PBS at 4°C overnight, then incubated in secondary antibody (Alexa Fluor) with 1:1000 dilution in 5% goat serum and 1% BSA and 0.1 Triton X-100 in PBS at RT for 45 min and mounted with an anti-fade reagent as described above.

RNA *in situ* hybridization

Kidneys were fixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose for 24 h, and then embedded in OCT. 10 µm cryosections were cut by using a Leica CM3050S for tdTomato pre-imaging and then RNA *in situ* hybridization [as previously described (Kann et al., 2015)]. For pre-imaging of native tdTomato fluorescent proteins, sections were removed from -80°C, dried at RT for 20 min, fixed with 4% paraformaldehyde for 10 min, and then covered by RNase-free PBS and a coverslip. Fluorescent images were acquired, and the coverslip then was carefully removed from the section with forceps. Sections were then treated with H₂O₂ (Fischer Scientific, BP2633500), proteinase K (Ambion, AM2542) and acetic acid, prehybridized and subjected to RNA *in situ* hybridization using a standard protocol. Images were captured by the same microscope with bright-field function and merged with pre-imaged fluorescent images at the identical location. The probes were as previously described (Hartwig et al., 2010; Kann et al., 2015).

Fluorescence-activated cell sorting

Kidneys from embryos or mice of different ages (E15.5, E18.5, P0, P2 and P8) were cut into small pieces, trypsinized and dissociated by repetitive pipetting. The trypsinization was terminated by adding complete medium with 10% FBS (Gemini Bio-Products, 100-500). The cells were collected by centrifugation, resuspended in PBS, and filtered three times through 100 µm BD Falcon Cell-Strainer Cap (Falcon, 352235). The single cell population was transferred into a polypropylene tube (Falcon, 352063) and kept on ice until FACS. The GFP+, YFP+ or tdTomato+ (single-positive or double-positive) cells were isolated using a MoFlo (Beckman Coulter) for Figs 3 and 4 or BD FACSAria (BD Biosciences) for Fig. 5 and Fig. S3.

RT-qPCR

qPCR was done using a SuperScript III First strand synthesis system (Thermo Fisher Scientific #18080051) and SYBR Green PCR Master Mix (Thermo Fisher Scientific #4364346) and run on a StepOne Plus RT-PCR system (Applied Biosystems). Primer pairs were as previously described (Hartwig et al., 2010; Kann et al., 2015).

RNA-seq analysis

In total, four replicates, each corresponding to the tdTomato-high and tdTomato-low population, respectively, were sequenced for Control and *Eed* mutant P0 kidneys. However, we excluded four libraries corresponding to two Control High and two Mutant High samples because of the failure of sequencing libraries. Sequenced reads were mapped to the mouse genome (mm9) using bowtie2 with default parameters. HTSeq, DESeq2 and CuffDiff2 packages were used for generating counts and robustly identifying over- and underexpressed genes in High and Low progeny (Langmead and Salzberg, 2012; Trapnell et al., 2013; Love et al., 2014; Anders et al., 2015). GO analysis was summarized using the REVIGO package (revigo.irb.hr). Subsequent customized analyses were done in R. The raw and processed RNA-seq files have been deposited in GEO under accession number GSE102690.

Statistics

All data are presented as mean±s.d. and calculations were performed using Microsoft Excel. Unpaired one-tailed Student's *t*-test was used for glomerular counts and unpaired two-tailed Student's *t*-test was used for all other data to determine statistical significance. *P*-values <0.05 were considered to be statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.Z., J.A.K.; Formal analysis: L.Z., S.E., D.J., J.A.K.; Investigation: L.Z., S.E., M.K., D.J., L.Y., C.S., Y.L., K.N., M.E.T., P.J.P., J.A.K.; Resources: J.A.K.; Data curation: D.J., L.Y., P.J.P., J.A.K.; Writing - original draft: L.Z., J.A.K.; Writing - review & editing: L.Z., J.A.K.; Supervision: J.A.K.; Project administration: J.A.K.; Funding acquisition: J.A.K.

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Data availability

The raw and processed RNA-seq files have been deposited in GEO under accession number GSE102690.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.157149.supplemental>

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