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Identification of an NKX3.1-G9a-UTY transcriptional regulatory network that controls prostate differentiation

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Abstract

The *NKX3.1* homeobox gene plays essential roles in prostate differentiation and prostate cancer. We now show that loss-of-function of *Nkx3.1* in mouse prostate results in down-regulation of genes that are essential for prostate differentiation, as well as up-regulation of genes that are not normally expressed in prostate. Conversely, gain-of-function of *Nkx3.1* in an otherwise fully-differentiated non-prostatic mouse epithelium is sufficient for re-specification to prostate in renal grafts in vivo. In human prostate cells, these activities require the interaction of NKX3.1 with the G9a histone methyltransferase via the homeodomain, and are mediated by activation of target genes such as UTY (KDM6c), the male-specific paralog of UTX (KDM6a). We propose that an NKX3.1-G9a-UTY transcriptional regulatory network is essential for prostate differentiation, and we speculate that disruption of such network predisposes to prostate cancer.

Main text

Among the tissues of the male urogenital system, the prostate and seminal vesicle are secretory organs that develop in close proximity under the influence of androgens (Fig. S1A) (1, 2). However, the prostate develops from the urogenital sinus, an endodermal derivative,

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whereas the seminal vesicle from the Wolffian duct, a mesodermal derivative. Among genes that distinguish prostate and seminal vesicle, the *Nkx3.1* homeobox gene is among the earliest expressed in the presumptive prostatic epithelium during development, and its expression in adults is primarily restricted to prostatic luminal cells (3, 4), which are secretory cells that are the major target of prostate neoplasia (4, 5). Accordingly, loss-of-function of *Nkx3.1* results in impaired prostate differentiation and defects in luminal stem cells, as well as predisposes to prostate cancer (3, 4).

Analyses of expression profiles from *Nkx3.1* wild-type (*Nkx3.1*$^{+/+}$) and *Nkx3.1* mutant (*Nkx3.1*$^{-/-}$) prostates revealed down-regulation of genes associated with prostate differentiation, such as *FoxA1* (*Forkhead Box A1*), *Pbsn* (*Probasin*), *HoxB13*, and *Tmprss2* (*Transmembrane Protease, Serine 2*), as well as luminal cells (*cytokeratins 8 and 18*), and up-regulation of basal cell markers (*cytokeratins 5 and p63*) (Figs. 1A, S2A, Dataset S1, (6)). Surprisingly, *Nkx3.1*$^{-/-}$ versus *Nkx3.1*$^{+/+}$ prostates display up-regulation of genes that are expressed, albeit not exclusively, in seminal vesicle, namely *Svs6*, *Sva*, *Svs4*, and *Svs5* (Fig. 1A, S2A) (7). These differentially expressed genes were significantly enriched in a gene signature comparing seminal vesicle versus normal prostate, and conserved in both mouse and humans (Fig. 1B, Fig. S1B, S2B). At the cellular level, we observed reduced expression of Probasin and a corresponding up-regulation of Svp2 in *Nkx3.1*$^{-/-}$ versus *Nkx3.1*$^{+/+}$ prostates (Fig. S1C–E, S2C; Tables S1–3) (8).

Considering that loss-of-function of *Nkx3.1* leads to aberrant prostate epithelial differentiation, we asked whether its gain-of-function in a non-prostatic epithelium is sufficient to induce prostate differentiation. Toward this end, we performed tissue recombination assays, in which relevant epithelial and mesenchymal tissues are recombined in vitro followed by growth under the kidney capsule of host mice in vivo (Fig. 1C) (2). It is well-established that prostate formation requires both epithelial and mesenchymal tissues, as well as an appropriate source of androgens (Fig. S3), and that non-endodermal epithelium, even those that are androgen-regulated, such as seminal vesicle, do not form prostate in this assay (2).

Therefore, we infected seminal vesicle epithelium with a lentivirus expressing *Nkx3.1* (or the control vector) to ask whether *Nkx3.1* expression can induce prostate differentiation in tissue recombinants (Fig. 1C). As expected, tissue recombinants made with prostate epithelium (PE) generate prostate-like grafts, which are distinguished by their prostate-like ductal structures, histological appearance resembling prostate epithelium, and expression of markers of prostate differentiation, including Nkx3.1, probasin, FoxA1, and HoxB13 (N=19) (Fig. 1D,E, S4A; Table S4). Also as expected, tissue recombinants made with seminal vesicle epithelium (SVE) lacking *Nkx3.1* generate seminal vesicle-like grafts, which are distinguished by their lack of discernable ductal morphology, histological appearance resembling seminal vesicle, and the absence of expression of markers of prostate differentiation (N=20) (Fig. 1D,E; S4A; Table S4).

In contrast, tissue recombinants made with SVE expressing exogenous *Nkx3.1* more closely resemble prostate than seminal vesicle. In particular, the Nkx3.1-expressing SVE grafts are distinguished by their appearance of prostate-like ductal structures, histological appearance

resembling prostate, and expression of markers of prostate differentiation, including probasin, FoxA1, and HoxB13 (N=26) (Fig. 1D,E; S4A; Table S4); this was not the case for tissue recombinants made with SVE expressing an unrelated homeobox gene, *Msx1* (N=3) (Fig. 1D,E; Table S4). Moreover, expression profiling analyses showed that tissue recombinants made from SVE expressing *Nkx3.1* were significantly enriched with a signature of prostate versus seminal vesicle (Fig. S4B,C). Therefore, Nkx3.1 is sufficient to re-specify a fully differentiated non-prostate epithelium to form prostate in vivo.

To study the underlying mechanisms, we established a cell-based assay using an immortalized human prostate cell line, RWPE1, which expresses low levels of NKX3.1, low levels of luminal cytokeratins, high levels of basal cell markers, and low levels of markers of prostate differentiation, such as androgen receptor (AR), FOXA1, PSA, TMPRSS2, and HOXB13 (Fig. 2A–D). Infection of RWPE1 cells with a lentivirus expressing *NKX3.1* resulted in high levels of NKX3.1 protein and robust DNA binding (Fig. 2B,C). Expression profiling and western blot analyses of the NKX3.1-expressing versus control RWPE1 cells revealed up-regulation of luminal cell markers (cytokeratins 8 and 18), down-regulation of basal cell markers (cytokeratin 5 and p63), and up-regulation of prostate differentiation markers (AR, FOXA1, PSA, TMPRSS2, and HOXB13) (Fig. 2B,D). Furthermore, when combined with embryonic mesenchyme and grown under the renal capsule, NKX3.1-expressing RWPE1 cells generate prostate-like grafts that morphologically and histologically resemble prostate, including having basal and luminal cell layers and expressing markers of prostate differentiation (N=15), whereas the control RWPE1 cells failed to grow in this assay (N=12) (Fig. 2E, S5; Table S4). In contrast, a mutated protein, NKX3.1(T164A), which has a mutation in the homeodomain that renders it impaired for DNA binding (Fig. 2C) (9), did not induce prostate differentiation in vitro nor did it promote prostate growth in tissue recombinants in vivo (N=7) (Fig. 2B–E, S5, Table S4). Therefore, the ability for NKX3.1 to induce prostate differentiation requires a functional DNA binding domain.

Many of the functions of homeoproteins are mediated by protein-protein interactions that often occur through the homeodomain (10). Among NKX3.1-interacting proteins identified by mass spectrometry (Figs. 3A, S6) (8) was G9a (also called EHMT2, euchromatic histone lysine N-methyltransferase 2), a histone methytransferase that forms a complex with a related histone methyltransferase, GLP, to promote di-methylation of lysine 9 on histone 3 (H3K9me2) (11). G9a is essential for embryonic development (12) and interacts with other homeoproteins to mediate differentiation (13, 14). In co-immunoprecipitation assays, NKX3.1 interacted with endogenous G9a, as well as GLP, which requires the homeodomain and is directly correlated with DNA binding by NKX3.1 (Fig. 3B, S7A–C). In contrast, NKX3.1 did not interact with other histone methyltransferases, including SUV39H1 (Suppressor of Variegation 3–9 Homolog 1), which promotes tri-methylation of lysine 9 on histone 3 (H3K9me3), and EZH2 (Enhancer of Zeste 2), promotes tri-methylation of lysine 27 on histone 3 (H3K27me3) (Fig. S7A,B).

To study the functional relevance of the interaction between NKX3.1 and G9a, we co-infected RWPE1 cells with an NKX3.1-expressing lentivirus together with an shRNA to deplete G9a (shG9a) or SUV39H1 (shSUV39H1) as a control (Fig. 3C–E, S5). These

shRNA reduced expression of G9a or SUV39H1, as well as their respective histone marks, H3K9me2 and H2K9me3, while not affecting expression of NKX3.1 (Fig. 3D). However, depletion of G9a, but not SUV39H1, impaired the ability of NKX3.1 to induce prostate differentiation, as evident by western blot analysis in cell culture, as well as prostate growth in the tissue recombinant assay in vivo (N=9) (Figs. 3D,E, S5, Table S4). These findings demonstrate that the interaction of NKX3.1 with G9a is required for induction of prostate differentiation.

Considering that induction of differentiation by NKX3.1 requires its homeodomain and corresponding DNA binding activity (Fig. S7), we reasoned that these functions are likely to be mediated by gene transcription. Among NKX3.1 target genes that have predicted functions for differentiation and are conserved between mice and humans (Fig. S8) (8), we focused on *UTY*, using *EDEM2* as a control. In particular, G9a binds to the promoter of *UTY*, but not *EDEM2*, which is dependent on NKX3.1 binding and is required for NKX3.1-mediated up-regulation of *UTY* expression (Fig. 4A, S9). Notably, UTY (KDM6c, Ubiquitously Transcribed Tetratricopeptide Repeat Containing, Y-Linked) is the male-specific paralog of UTX (KDM6a), a histone demethylase that is essential for viability and is frequently deregulated in cancer (15–17). Although its functions as a histone demethylase are uncertain (16), UTY is essential for male fertility as well as the development and differentiation of male-specific organs (16), and has been linked to prostate cancer (18).

Therefore, we studied the consequences of depleting UTY for prostate differentiation in NKX3.1-expressing or control RWPE1 cells by co-infection with an shRNA to UTY (shUTY) or EDEM2 (shEDEM2) as a control (Figs. 4B–D, S5). Expression of these shRNA reduced expression of UTY or EDEM2, respectively, while not affecting expression of NKX3.1 (Fig. 4C). Moreover, depletion of UTY, but not EDEM2, impaired the ability of NKX3.1 to induce prostate differentiation in vitro as well as in tissue recombinants in vivo (N=8) (Fig. 4C,D; Table S4).

We asked whether Uty is also required for prostate specification in vivo by performing analogous experiments using the mouse tissue recombinant model. Depletion of Uty (shUty) in *Nkx3.1*-expressing SVE abrogated the ability of *Nkx3.1* to generate prostate, as was evident by the resulting tissue recombinants (SVE + Nkx3.1 + shUty), which more closely resemble seminal vesicle than prostate (N=8) (Fig. 4E). Moreover, expression profiling of tissue recombinants generated from the Uty-depleted *Nkx3.*1-SVE revealed their significant enrichment in a gene signature comparing seminal vesicle versus prostate (Fig. S10).

Cumulatively, our findings support a model in which NKX3.1 regulates the expression of a gene program associated with prostate differentiation, while simultaneously inhibiting the inappropriate expression of non-prostatic genes (Fig. S11A). In particular, *Nkx3.1* can re-specify a fully differentiated mouse tissue to form prostate in vivo, while its expression in basal-like human prostate epithelial cells can promote their differentiation to luminal-like cells that form prostate in vivo. Furthermore, these functions of NKX3.1 are mediated by the coordinate actions of G9a and UTY (Fig. S11A,B). Notably, we find that G9a functions as a co-regulator of NKX3.1, which is associated with activation, as well as repression, of NKX3.1 target genes. This further underscores the complexity of G9a function in

transcriptional control. Indeed, although G9a is a histone methyltransferase for a "repressive" mark, it is active on euchromatin (11), and it has been shown that G9a can repress or activate transcription depending on the context (19, 20). This includes the glucocorticoid receptor (21, 22), which also interacts with NKX3.1 by mass spectrometry (Fig. S6) and has been implicated in drug resistance in prostate cancer (23). Thus, we envision that our findings presage a role for G9a in both prostate differentiation and cancer.

Our findings also shed new light on UTY as an essential downstream mediator of NKX3.1 in prostate differentiation. Given the importance of UTY for male fertility as well as for differentiation of male-specific organs (16, 24), our current study provides an example of how, in addition to the well-known role of androgen signaling, the promotion of "maleness" may be essential for prostate differentiation.

Lastly, there are still relatively few examples in which a single gene can re-specify an otherwise fully-differentiated epithelium to a new fate, as we have observed for *NKX3.1*. Notably, our findings regarding *NKX3.1* in prostate are strikingly concordant with the functions of *NKX2.1* in lung differentiation and lung cancer (25–28). In particular, loss-of function of *NKX2.1* leads to impaired lung differentiation, which is associated with the de-repression of an aberrant gene expression program (28), while conversely *NKX2.1* expression is associated with inhibition of lung metastases (26, 27). Notably, similar to *NKX3.1* in prostate, these actions of *NKX2.1* in lung are dependent upon its level of expression. Thus, these observations suggest that NK-class homeobox genes function as key regulators of tissue-specific differentiation, as well as key gate-keepers whose diminution of expression in specific tissue contexts lead to enhanced susceptibility to cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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One sentence summary

The NKX3.1 homeoprotein re-specifies prostate differentiation in vivo via interaction with the G9a histone methyltransferase and through regulation of target genes, such as UTY.

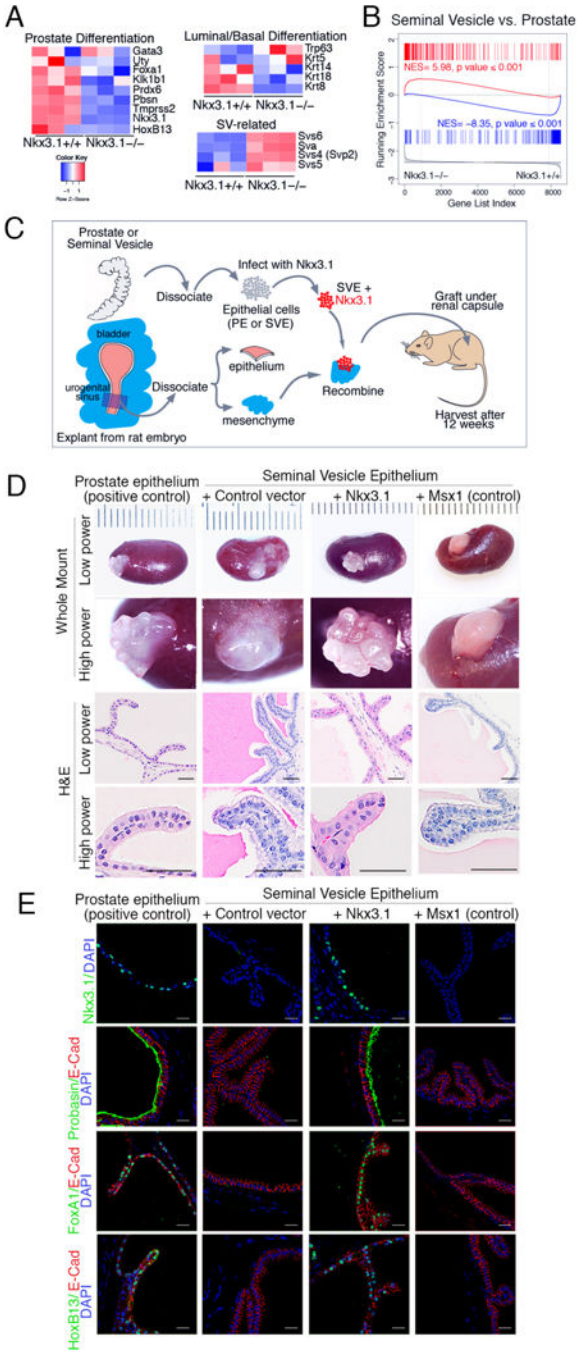

Figure 1. Nkx3.1 re-specifies a non-prostatic epithelium to form prostate in vivo

(A) Heat map representations of differentially expressed genes from *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{-/-}$ prostate (6). (B) GSEA using, as the query gene set, differentially expressed genes from seminal vesicle versus prostate compared with a reference gene signature of *Nkx3.1*$^{-/-}$ versus *Nkx3.1* $^{+/+}$ prostate. (C) Diagram of the tissue recombination assay. Dissociated epithelium from seminal vesicle (SVE) or prostate (PE) is infected with a lentivirus expressing Nkx3.1 (or control). Mesenchyme from rat embryonic urogenital sinus is combined with the epithelium and grown under the renal capsule of host mice. (D,E)

Representative tissue recombinants. (D) (Top) Whole mount images. (Bottom) H&E images. (E) Confocal images of immunofluorescence using the indicated antibodies. Scale bars represent 50 μm. A summary of tissue recombinant data is provided in Table S4.

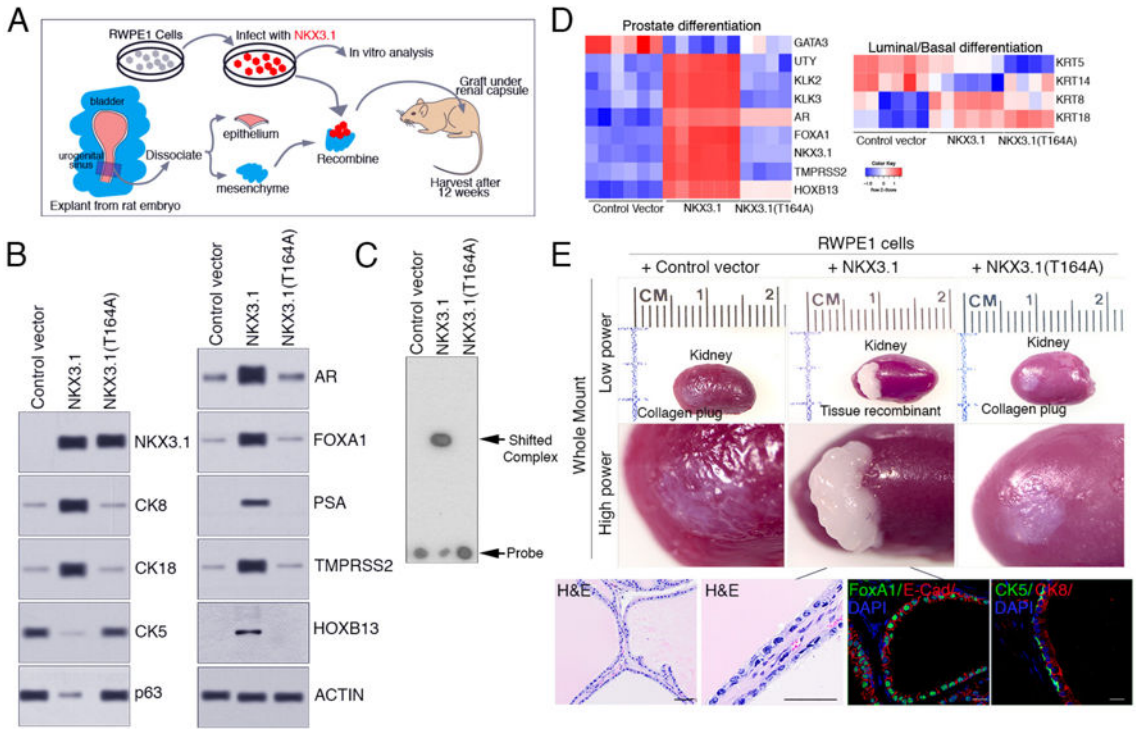

Figure 2. Induction of prostate differentiation by NKX3.1 requires the homeodomain

(A) Diagram of the experimental design. Human RWPE1 prostate epithelial cells are infected with a lentivirus expressing human NKX3.1, NKX3.1(T164A), or a control following by analyses in vitro (B-D) or recombined with mesenchyme and grown under the renal capsule of host mice (E). (B) Western blot analyses. Actin is a control for protein loading. (C) Gel retardation analysis done using nuclear extracts from RWPE1 cells expressing the control vector, NKX3.1, or NKX3.1 (T164A). The arrow indicates the free DNA probe. The experiments in B and C were each performed with 3 independent biological replicates; representative data are shown. (D) Heat map representations of selected differentially expressed genes; a complete list is provided in Dataset S4. (E) Tissue recombinants showing whole mount images, H&E and immunofluorescence staining. The ruler shows cm scale; scale bars represent μm. A summary of all tissue recombinants is provided in Table S4.

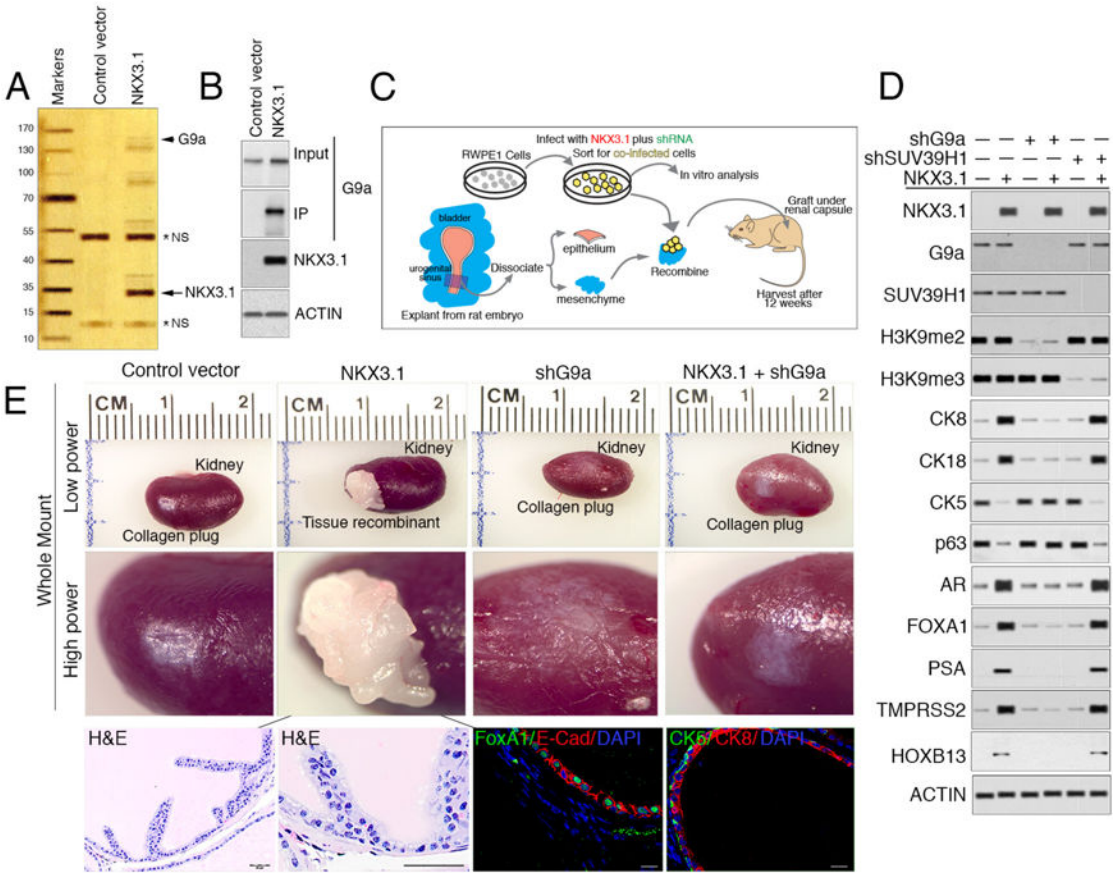

Figure 3. Induction of prostate differentiation by NKX3.1 is mediated via its interaction with G9a

(A,B) Nuclear extracts from RWPE1 cells expressing Flag-HA-NKX3.1 or the control were subjected to immunoprecipitation followed by mass spectrometry (A) or immunoprecipitation (B) (see Fig. S6). (A) Silver stain showing G9a interaction. Markers, as indicated. NS, non-specific bands. (B) Immunoprecipitation followed by Western blot analysis. Input shows 5% of the total protein, and IP shows proteins recovered following immunoprecipitation using an anti-Flag antibody. Experiments were performed with 3 independent biological replicates; representative data are shown. (C) Diagram of experimental design for (D,E). Human RWPE1 prostate epithelial cells were infected with an NKX3.1-expressing lentivirus (expressing RFP), followed by infection with an shRNA-expressing lentivirus (expressing GFP). Co-infected cells were FACS sorted followed by analyses in vitro (D) or generation of tissue recombinants in vivo. (D) Western blot analysis. Experiments were performed with 3 independent biological replicates; representative data are shown. (E) Representative tissue recombinants showing whole mount, H&E and confocal images of immunofluorescence staining. Indicated is the kidney and the collagen plug (for the recombinants that did not grow) or the tissue recombinant. The ruler shows cm scale; scale represent 50 μm. A summary of tissue recombinants is provided in Table S4.

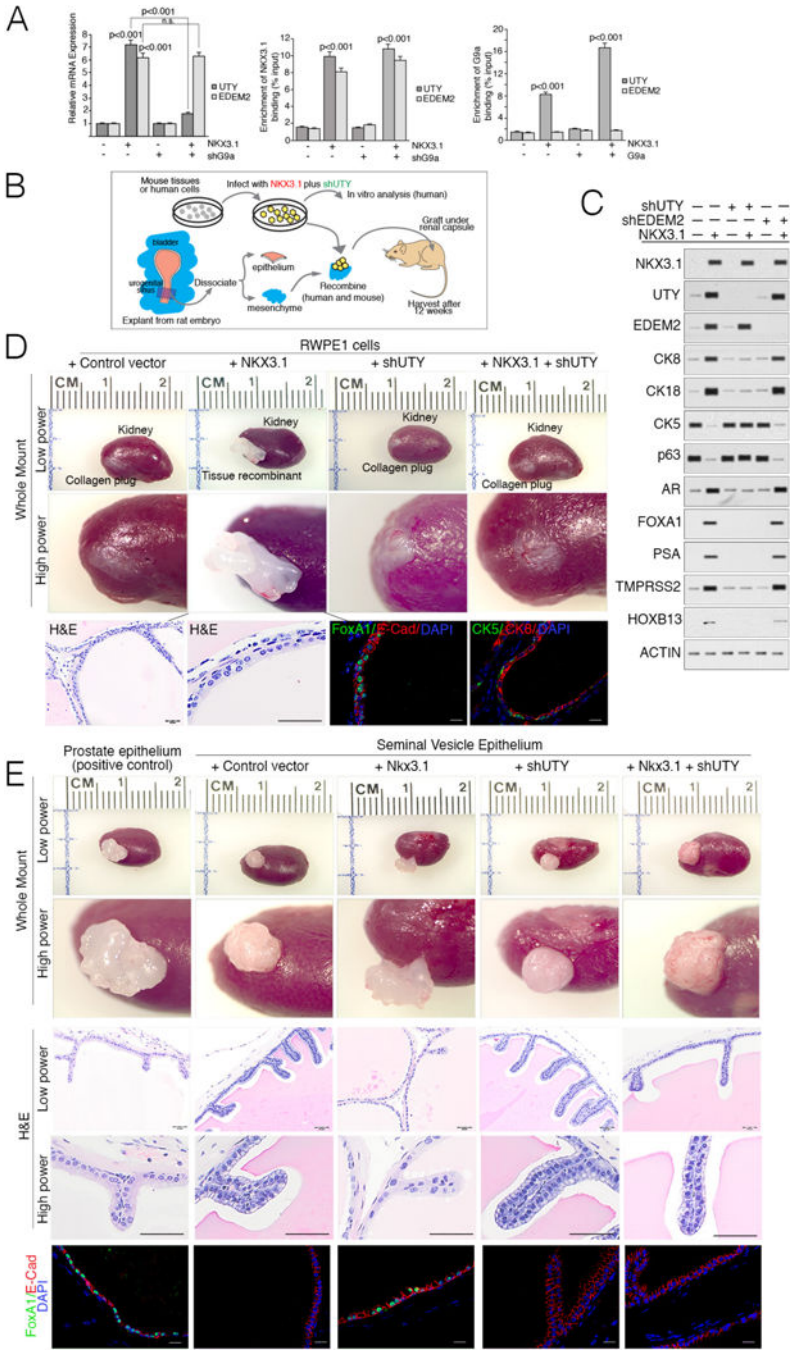

Figure 4. Induction of prostate differentiation by NKX3.1 is mediated by UTY (KDM6c)

(A) Real time PCR showing expression of NKX3.1 target genes, UTY and EDEM2 (left). ChIP-qPCR analysis of NKX3.1 binding (center), and G9a binding (right) to NKX3.1 target genes. Analyses were performed with 3 independent biological replicates. Statistical analysis was done using a 2-tailed *t*-test; data are indicated as mean +/− SD. (B) Diagram of the experimental design for C–E. Human RWPE1 cells (C,D) or mouse tissues (E) were infected with an NKX3.1-expressing lentivirus, followed by infection with an shRNA (or controls). Cells were analyzed in vitro (human, C) or in tissue recombinants (mouse and

human, D and E). (D) Western blot analysis of RPWE1 cells. (D,E) Representative tissue recombinants of human (D) and mouse (E) showing whole mount, H&E and confocal images of immunofluorescence staining. The ruler shows cm scale; scale bars represent 50 μm. A summary of tissue recombinants is provided in Table S4.