Haploinsufficiency at the *Nkx3.1* locus: A paradigm for stochastic, dosage-sensitive gene regulation during tumor initiation

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Summary

Tumorigenesis requires sequential accumulation of multiple genetic lesions. In the prostate, tumor initiation is often linked to loss of heterozygosity at the *Nkx3.1* locus. In mice, loss of even one Nkx3.1 allele causes prostatic epithelial hyperplasia and eventual prostatic intraepithelial neoplasia (PIN) formation. Here we demonstrate that Nkx3.1 allelic loss extends the proliferative stage of regenerating luminal cells, leading to epithelial hyperplasia. Microarray analysis identified Nkx3.1 target genes, many of which show exquisite dosage sensitivity. The number of *Nkx3.1* alleles determines the relative probabilities of stochastic activation or inactivation of a given target gene. Thus, loss of a single *Nkx3.1* allele likely results in hyperplasia and PIN by increasing the probability of completely inactivating select Nkx3.1-regulated pathways within a subset of affected cells.

Introduction

Cancer arises via selective accumulation of genetic and epigenetic changes that confer a growth advantage on affected cells. Mechanistic studies of tumorigenesis have identified two distinct classes of loci that are frequent targets of genetic change (Fearon and Vogelstein, 1990). These include conventional oncogenes, which act in a dominant, gain-of-function manner and often encode proteins that promote cell proliferation. The second class consists of tumor suppressor genes, which elicit a loss-of-function phenotype and encode proteins that limit cell growth or survival. As originally outlined by Knudson, both alleles of prototypical tumor suppressor genes must be affected to exact the pro-tumorigenic phenotype (Knudson, 1985).

The kinetics of tumor development suggest that tumor formation should be an almost impossibly difficult task. For instance, by conservative estimate, a single cell requires four genetic mutations in order to proliferate ad infinitum and evade pro-apoptotic stimuli (Renan, 1993). Therefore, at normal rates of somatic mutation, the likelihood of tumor formation becomes vanishingly small (Seshadri et al., 1987; Quon and Berns, 2001). However, if the initial genetic lesion provides a selective growth advantage to the affected cell and its clonally expanding daughter cells, then these statistical arguments become less compelling. It has been proposed that haploinsufficiency at tumor suppressor loci is a likely mechanism for clonal expansion following an initial genetic hit (Quon and Berns, 2001). Thus, in contrast to the "two-hit" requirement originally proposed by Knudson, haploinsufficiency at tumor suppressor loci would allow for manifestation of pre-neoplastic or neoplastic phenotypes following mutation of only a single allele of a tumor suppressor. Indeed, recent work on tumor suppressor genes has demonstrated a role for haploinsufficiency at several loci, including *p27*, *p53*, *Dmp1*, and *Nkx3.1* (Cook and McCaw, 2000; Fero et al., 1998; Venkatachalam et al., 1998; Inoue et al., 2001; Abdulkadir et al., 2002). In these cases, mice lacking one allele of these tumor suppressors develop cancerous or pre-cancerous lesions despite expression of the remaining wild-type allele. The relevance of these findings in mouse models is supported by data from patients in which loss of heterozygosity (LOH) is frequently observed at the tumor suppressor locus, yet a single wild-type allele is retained in the tumor (e.g., *p27*, *p53*, and *Nkx3.1*) (Pietenpol et al., 1995; Davidoff et al., 1991; Voeller et al., 1997).

Haploid deletion of one tumor suppressor gene, Nkx3.1, is frequently associated with prostate cancer and prostatic intraepithelial neoplasia (PIN) (Macoska et al., 1995; Bova et al.,

SIGNIFICANCE

Recent studies have demonstrated that haploinsufficiency at tumor suppressor loci contributes to somatic tumorigenesis. Haploinsufficient loss of one such tumor suppressor, Nkx3.1, is associated with prostate cancer. Here we describe the effects of *Nkx3.1* gene dosage on target gene expression. These analyses establish a model for haploinsufficient tumorigenesis wherein loss of a single *Nkx3.1* allele results in major alterations in the expression of specific target genes. Such dramatic effects in gene regulation occur because Nkx3.1 dosage modulates the probability of whether a target gene exists in the "on" versus the "off" state. These results may account for some of the earliest genetic changes of prostate tumorigenesis and may reflect changes that occur in many types of somatic tumor initiation.

1993; He et al., 1997). *Nkx3.1* encodes a homeodomain protein that is expressed specifically in the luminal epithelia of the prostate (He et al., 1997; Bieberich et al., 1996). Consistent with the observations of LOH in human PIN and prostate cancer, mice in which a single Nkx3.1 allele is conditionally deleted in adulthood develop prostatic hyperplasia and PIN (Abdulkadir et al., 2002). The hyperplastic lesions of these mice, as well as conventional heterozygous mice, retain Nkx3.1 protein expression from the remaining wild-type allele (Bhatia-Gaur et al., 1999; Abdulkadir et al., 2002). These results imply the existence of Nkx3.1-regulated genetic circuits that modulate cell proliferation and are exquisitely sensitive to Nkx3.1 dosage. In light of these observations, we have utilized Nkx3.1$^{+/-}$ and Nkx3.1$^{-/-}$ mice to characterize the underlying mechanisms of gene dosage-dependent tumor suppression. Here, we show that Nkx3.1 regulates the rate at which proliferating luminal epithelial cells exit the cell cycle and that deletion of one or both alleles of Nkx3.1 extends the transient proliferative phase of luminal cells during prostate regeneration. Using gene expression profiling, we have identified discrete haploinsufficient and nonhaploinsufficient clusters of Nkx3.1 target genes. Intriguingly, haploinsufficient Nkx3.1 targets exhibit a varied response to the loss of one Nkx3.1 allele, as the expression of some targets is completely lost in the Nkx3.1$^{+/-}$ prostate, whereas the expression of others is less affected. The quantitative effects observed at the level of the entire prostate, in fact, reflect qualitative changes in target gene expression at the cellular level. Thus, a reduction in Nkx3.1 dosage predominantly affects the *number* of cells expressing a given target gene. These findings have been integrated into a molecular model of haploinsufficiency and tumor initiation.

Results

Characterization of Nkx3.1 effects on regenerating prostate epithelia

Current models of epithelial turnover in the prostate hold that terminally differentiated luminal epithelial cells are derived from an androgen-responsive, transiently proliferating population of luminal amplifying cells (English et al., 1987; Isaacs, 1987). Amplifying cells, in turn, are believed to arise from prostatic stem cells. Evidence for the existence of luminal amplifying cells comes, primarily, from the castration-testosterone replacement (TR) model of prostate regeneration, where the implantation of testosterone pellets into castrated mice promotes a well-characterized series of events in the prostate (summarized in Figure 1A) (English et al., 1987; Isaacs, 1987). A dramatic burst of luminal cell proliferation is observed at 3 days post-TR, but by 7 days post-TR, these amplifying cells have terminally differentiated into secretory luminal epithelia. Given the hyperplastic phenotype of Nkx3.1$^{+/-}$ and Nkx3.1$^{-/-}$ mice, we hypothesized that Nkx3.1 might regulate the proliferation and/or subsequent differentiation of prostate epithelia in a dosage-sensitive manner. To test this hypothesis, we castrated wild-type, Nkx3.1$^{+/-}$, and Nkx3.1$^{-/-}$ mice at 8 weeks of age. At 14 days post-castration, sustained-release testosterone pellets were implanted, and tissues were collected at specific time points post-TR ranging from 0–14 days (Figure 1A). In wild-type mice, Nkx3.1 mRNA was rapidly induced within 24 hr of TR (Figure 1B). Nkx3.1$^{+/-}$ prostates experienced a similar pattern of Nkx3.1 induction post-TR, though the level of Nkx3.1 transcript was quantitatively reduced, a finding consistent with the haploinsufficient Nkx3.1 phenotype.

Nkx3.1 protein levels followed a similar expression profile (Figure 1D). At 3 days post-TR, Nkx3.1 is coexpressed with the proliferation marker Ki-67. By 7 days post-TR, these dividing cells exit the cell cycle, as evidenced by the dramatic reduction in Ki-67-positive cells. The differentiated luminal cells retain Nkx3.1 expression. Altogether, these observations confirm the luminal identity of the proliferating cell population, as Nkx3.1 has previously been established as a luminal epithelial marker. Furthermore, the coexpression of Ki-67 and Nkx3.1 at 3 days post-TR indicates that Nkx3.1 does not, by itself, oppose proliferation in regenerating luminal epithelia.

To test the effects of Nkx3.1 on growth arrest in the regenerating prostate, we assayed Ki-67 expression in wild-type, Nkx3.1$^{+/-}$, and Nkx3.1$^{-/-}$ prostates by quantitative RT-PCR (Figure 1C). In wild-type mice, Ki-67 expression peaks at 3 days post-TR and drops precipitously by day 7. This expression pattern correlates with luminal amplifying cell proliferation, which peaks at day 3, and subsequent terminal differentiation, which ensues by day 7. The Ki-67 profiles of Nkx3.1$^{+/-}$ and Nkx3.1$^{-/-}$ mice exhibit an extended proliferative phase. For instance, at 7 days post-TR, Ki-67 levels are approximately 5-fold higher in Nkx3.1$^{+/-}$ prostates and 12-fold higher in Nkx3.1$^{-/-}$ prostates relative to wild-type. To determine whether the increase in Ki-67 mRNA levels reflects an increase in the number of proliferating cells, we counted the number of cells that incorporated Brdu in wild-type, Nkx3.1$^{+/-}$, and Nkx3.1$^{-/-}$ prostates at 3 and 7 days post-TR (Figures 2A–2F). The luminal epithelial marker E-cadherin was used to confirm the luminal identity of dividing and non-dividing cells. At three days post-TR, we observed similar levels of Brdu incorporation in E-cadherin-positive cells of wild-type, Nkx3.1$^{+/-}$, and Nkx3.1$^{-/-}$ prostates. By 7 days post-TR, the fraction of Brdu-positive, E-cadherin-positive, wild-type cells was dramatically reduced relative to 3 days post-TR. However, a much larger population of cells continued to divide in Nkx3.1$^{+/-}$ and Nkx3.1$^{-/-}$ prostates ($p < 0.05$ and $p < 0.01$, respectively). Thus, the loss of one or both alleles of Nkx3.1 precludes timely withdrawal of luminal amplifying cells from the cell cycle. By 14 days post-TR, the extended proliferative phase results in extensive hyperplasia in the Nkx3.1-deficient prostate (Figures 2G and 2H).

Identification of Nkx3.1 target genes

The effects of Nkx3.1 loss on androgen-mediated proliferative changes indicate that Nkx3.1 and androgens act collaboratively to regulate epithelial cell proliferation and differentiation in the regenerating prostate. The realization that these effects can be observed, even after loss of a single Nkx3.1 allele, suggests the presence of a subset of androgen target genes that are regulated in an Nkx3.1 dosage-sensitive manner. To identify Nkx3.1 target genes, we castrated 8-week-old wild-type, Nkx3.1$^{+/-}$, and Nkx3.1$^{-/-}$ mice and implanted sustained-release testosterone capsules at 14 days post-castration. Total RNA was harvested at 0, 1, 3, 7, 14 days after testosterone treatment as well as from uncastrated 8-week-old mice. Two separate pools of RNA from each time point, each containing RNA harvested from three individual mice, were used as templates to synthesize probes for microarray analysis. Genes that differ significantly between wild-type and Nkx3.1$^{-/-}$ prostates throughout the timecourse were identified by significance analysis of microarrays (SAM)

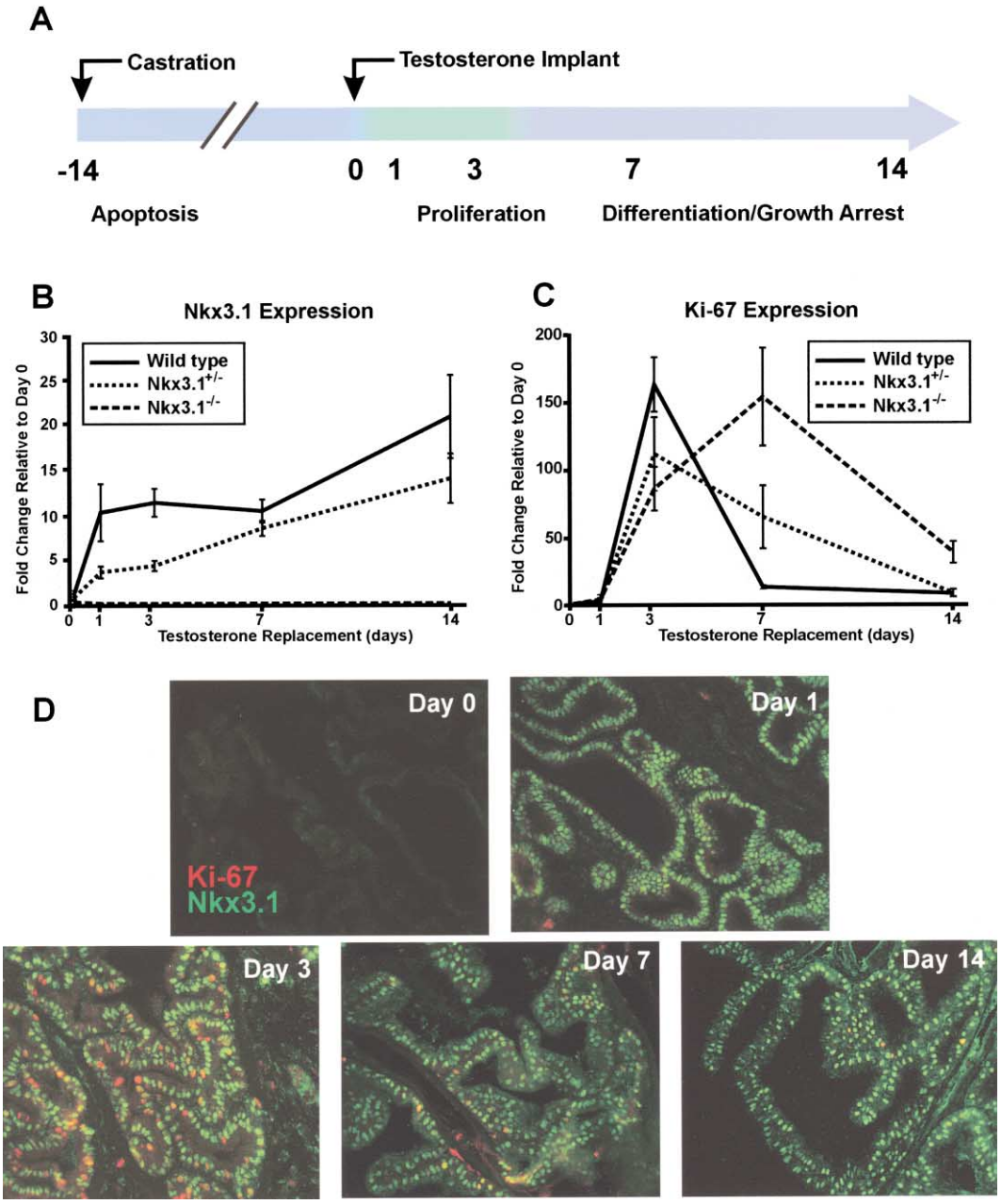

Figure 1. Nkx3.1 regulates the transient proliferative phase of regenerating prostate epithelia

A: A schematic demonstrating the castration-testosterone replacement model of prostatic growth and differentiation.
B: *Nkx3.1* expression was examined after castration-TR by qRT-PCR (solid lines [——], wild-type; dotted lines [·····], Nkx3.1$^{+/-}$; dashed lines [-------], Nkx3.1$^{-/-}$). Error bars show the SEM from N ≥ 5 prostates.
C: Ki-67 expression was examined after castration-TR by qRT-PCR (solid lines [——], wild-type; dotted lines [·····], Nkx3.1$^{+/-}$; dashed lines [-------], Nkx3.1$^{-/-}$). Error bars show the SEM from N ≥ 5 prostates.
D: Immunohistochemistry was performed on regenerating wild-type prostates for Ki-67 (red) and Nkx3.1. Note the presence of luminal epithelial cells that coexpress Nkx3.1 and Ki-67 (yellow).

(Tusher et al., 2001). This algorithm identifies significantly altered genes from replicate data sets and calculates an expected false discovery rate. From the Nkx3.1 data series, the SAM algorithm identified a list of 57 significantly altered Nkx3.1 target genes represented by 62 independent probe sets. The estimated false discovery rate for this list was 1.1%. Hierarchical clustering of the Nkx3.1 target genes revealed discrete clusters of positively and negatively regulated Nkx3.1 target genes (Figure 3). Quantitative RT-PCR (qRT-PCR) analysis of several Nkx3.1 target genes confirmed the validity of the methods used as all 15 genes analyzed were aberrantly regulated in Nkx3.1-deficient prostates (Figures 4 and 5 and data not shown).

Through this analysis, we found genes that were positively regulated by Nkx3.1 and were therefore much lower in the ab-

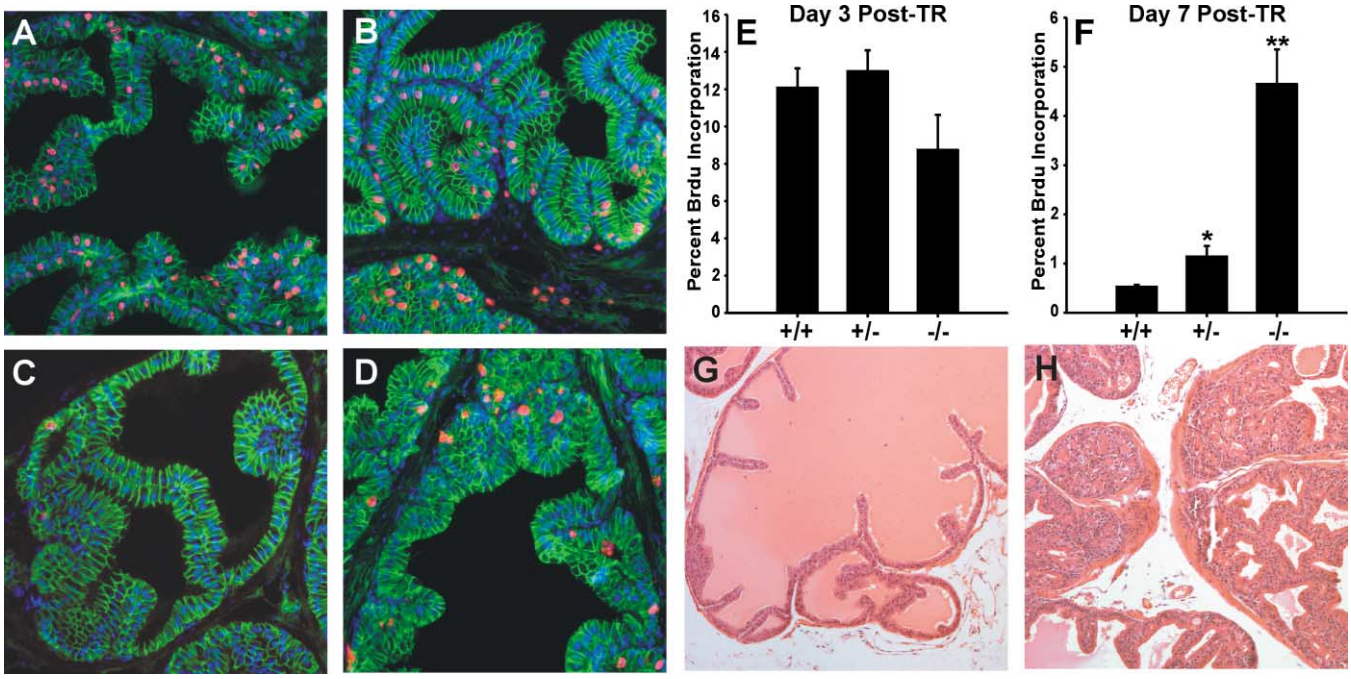

Figure 2. Nkx3.1 deficiency results in extended period of cell proliferation and luminal hyperplasia in the prostate following castration-TR

A–D: Cell proliferation was assayed by Brdu incorporation at 3 (**A** and **B**) and 7 (**C** and **D**) days post-TR. Dividing cells were labeled in wild-type (**A** and **C**) and Nkx3.1$^{-/-}$ (**B** and **D**) prostates during a 3 hr Brdu pulse. Immunohistochemistry was performed for Brdu (red) and the luminal epithelial cell marker E-cadherin (green). Nuclei were counterstained with bisbenzimide (blue).

E and **F:** Wild-type, Nkx3.1$^{+/-}$, and Nkx3.1$^{-/-}$ tissues were labeled with Brdu and stained as described above. Percentages of Brdu-labeled luminal cells were determined by examining ~1000 E-cadherin-positive cells from prostates harvested at 3 and 7 days post-TR (**E** and **F**, respectively). *Nkx3.1* allelic loss resulted in a significant increase in Brdu incorporation at 7 days post-TR. Error bars represent the SEM for each group (N = 2–3; *$p < 0.05$; **$p < 0.01$).

G and **H:** H&E staining revealed dramatic hyperplasia in the Nkx3.1$^{-/-}$ prostate at 14 days post-TR (**H**) relative to wild-type (**G**).

sence of Nkx3.1 (e.g., *intelectin*, *probasin*). Genes were also identified that are normally repressed by Nkx3.1 and were therefore expressed at higher levels in Nkx3.1-deficient mice (e.g., *angiopoietin 2*, *elafin-like II*). Interestingly, the expression of all Nkx3.1-regulated genes identified in this study are influenced by the androgen status of the animal. This is consistent with the observation that the expression of Nkx3.1 itself is regulated by androgens, when gene activation is considered. However, the identification of genes, such as *angiopoietin 2*, that are regulated by androgens only in the absence of Nkx3.1 was unexpected and suggests that Nkx3.1 functions as a selector that modulates expression of potential androgen target genes. Among the positively regulated Nkx3.1 target genes were known markers of prostatic luminal differentiation, including β-*microseminoprotein* (β-*MSP*) and *probasin*. In humans, β-MSP is one of three major prostatic secretory products (Abrahamsson et al., 1988). Human prostate tumors exhibit reduced levels of β-MSP, as might be predicted given the frequent loss of Nkx3.1 protein expression in prostate cancer (Tsurusaki et al., 1998). *Probasin* is a well-studied androgen target in the rodent prostate (Johnson et al., 2000). Nkx3.1 regulation of these genes is consistent with its role as a mediator of luminal differentiation in the prostate.

Variable dosage sensitivity among Nkx3.1 target genes

The Nkx3.1 target genes described above provide a unique opportunity to track the effects of haploinsufficiency at the molecular level. To identify the effects of *Nkx3.1* gene dosage on target gene expression, we analyzed their mRNA levels by qRT-PCR. This methodology afforded a highly quantitative measurement of target gene expression that could be performed on a large number of animals in both the uncastrated and castration-TR paradigms ($N \geq 5$ for each time point and genotype). For the uncastrated paradigm, we computed gene expression levels in the Nkx3.1$^{+/-}$ prostates as a percentage of wild-type levels, with the wild-type level representing 100% expression and the level in Nkx3.1$^{-/-}$ representing 0% expression (Figure 4).

These analyses revealed a spectrum of dosage sensitivity among the Nkx3.1-activated genes. Genes such as *probasin* and the *Riken clone 2210008A03* were relatively insensitive to Nkx3.1 dosage, as expression levels in heterozygotes were approximately 72% and 87% of wild-type expression, respectively. At the other end of the spectrum, intelectin expression was essentially lost in the prostates from both Nkx3.1 heterozygotes and homozygotes (Figure 4). These extremes flanked a spectrum of dosage-sensitive responses that included *Riken clone 3300002C04*, *fucosyltransferase 8*, and *PDZ domain containing protein 1*, which were expressed in the Nkx3.1-heterozygous prostate at 55%, 31%, and 16% of wild-type levels, respectively. In general, relative responses in the castration/TR paradigm correlated well with those observed in the uncastrated paradigm (Figure 4). Furthermore, the sensitivity of a given gene to Nkx3.1 gene dosage did not appear to correlate with its wild-type expression level. For example, *intelectin* is highly dosage

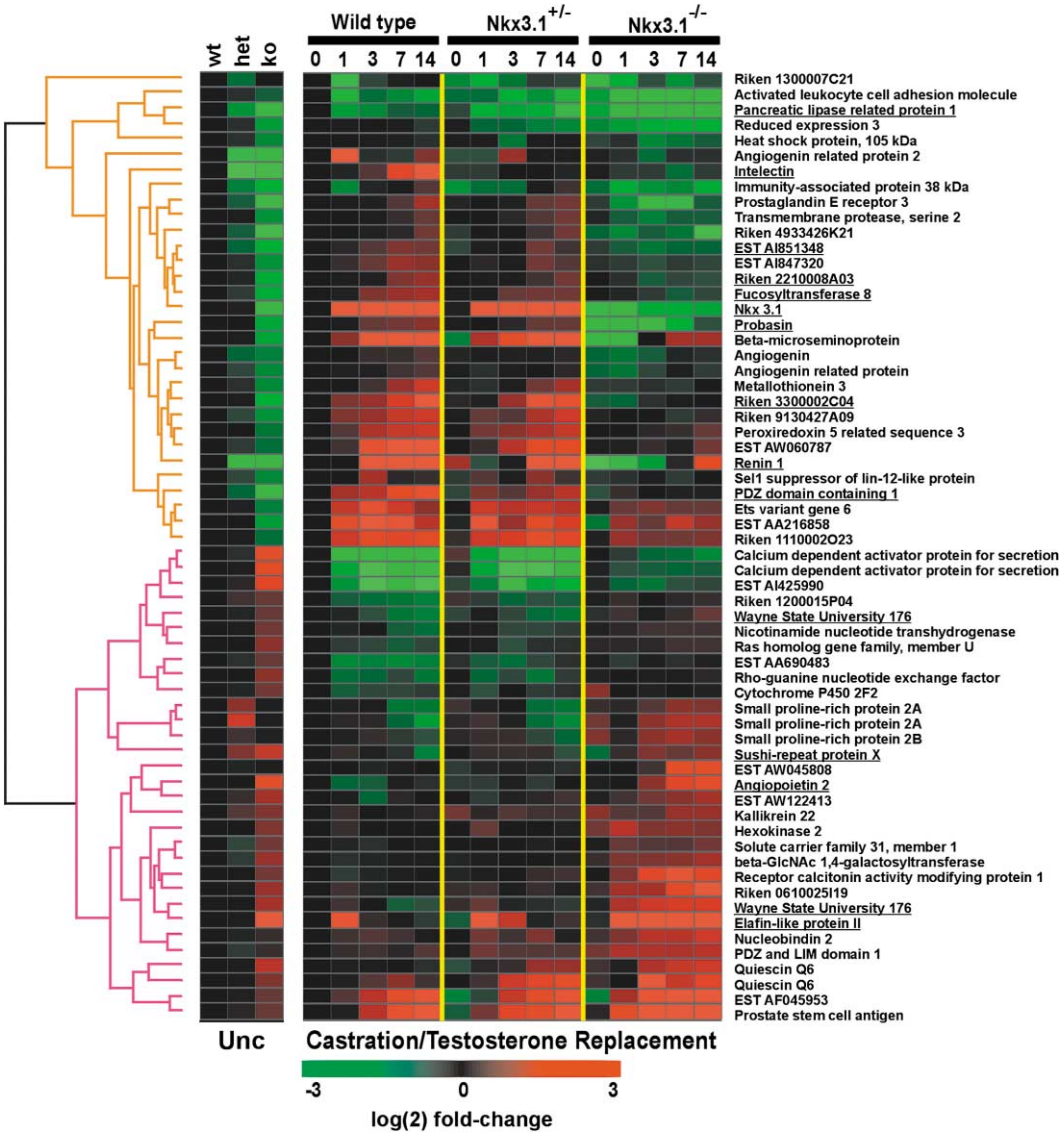

Figure 3. Expression profiles of genes that were significantly altered in Nkx3.1-deficient prostates

Nkx3.1 target genes were identified by SAM analysis of prostate expression profiles from wild-type, Nkx3.1$^{+/-}$, and Nkx3.1$^{-/-}$ mice. For each of the 62 significantly changed probe sets (representing 57 independent genes), the average expression profiles from replicate experiments using prostates from wild-type, Nkx3.1$^{+/-}$, and Nkx3.1$^{-/-}$ mice were hierarchically clustered. Heat map colors reflect log(2) fold-change values relative to expression in the wild-type (uncastrated) or wild-type day 0 (castration-TR) measurements. Distinct groups of positively (orange) and negatively (pink) regulated Nkx3.1 target genes are apparent from the cluster dendogram shown to the left of the heat map. Underlined gene names denote expression profiles that were subsequently examined, and in each case confirmed, by qRT-PCR ($N \geq 5$).

sensitive, yet its expression in wild-type prostate is higher than *Riken 221008A03* but lower than *probasin*. Intriguingly, the negatively regulated Nkx3.1 target genes *WSU-176*, *sushi-repeat protein X*, *angiopoietin 2*, and *elafin-like II* did not display a similar spectrum of dosage sensitivity (Figure 5). In fact, the expression of these genes in the Nkx3.1$^{+/-}$ prostate closely resembled that found in wild-type prostate, with target gene activation observed only in Nkx3.1$^{-/-}$ prostates.

Non-uniform expression patterns of Nkx3.1-activated target genes

Transcriptional regulation is commonly discussed as a graded process with promoter activity increasing proportionally in response to increasing levels of one or more transcription factors. An alternative to the graded model, however, is a stochastic, binary model of transcriptional activation (Fiering et al., 2000). The stochastic model implies that genes exist in either an "on" or an "off" state and that transcription factors regulate the probability of a gene occupying either state. While these models are not mutually exclusive, they raise two possible explanations for the spectrum of Nkx3.1 target gene dosage sensitivity described above. Consistent with the graded model of transcriptional activation, dosage sensitive reductions in target gene levels could be uniform in all luminal cells. Alternatively, the stochastic model would predict a non-uniform reduction in Nkx3.1 target gene levels, with reduced Nkx3.1 gene dosage resulting in a de-

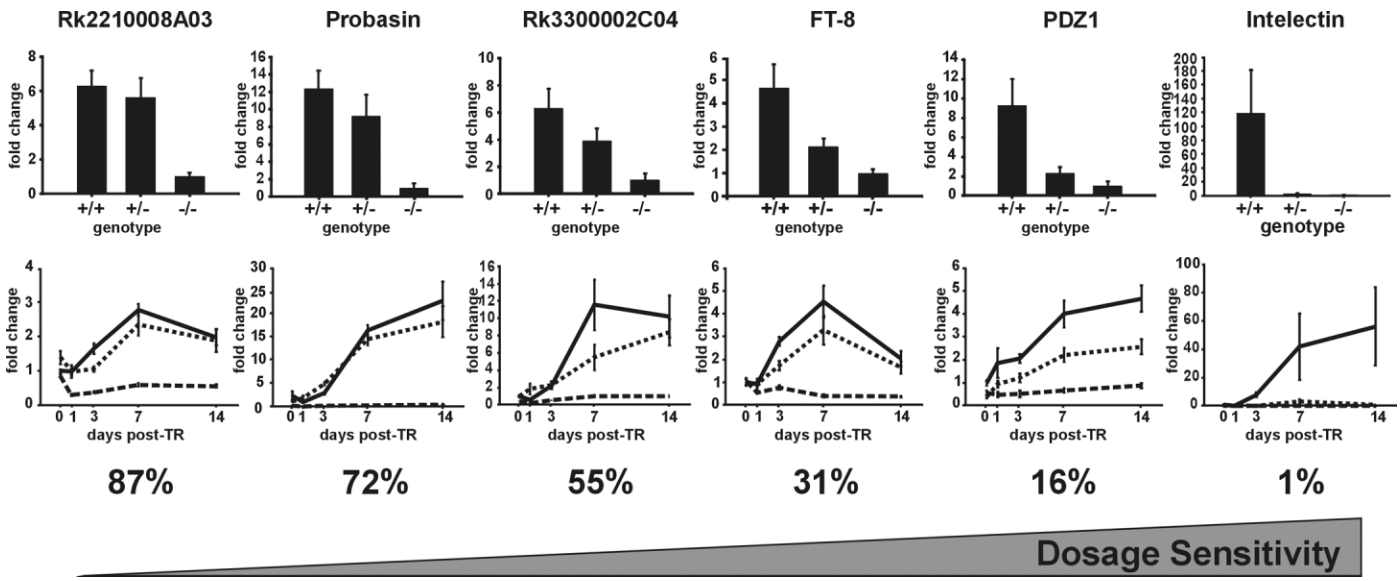

Figure 4. *Nkx3.1* haploinsufficiency affects expression of a subset of positively regulated Nkx3.1 target genes

Nkx3.1 target gene expression was analyzed by qRT-PCR in prostates taken from the uncastrated (top) and castration-TR (bottom) paradigms. For both paradigms, expression changes are presented as fold changes relative to wild-type uncastrated or wild-type, 0 days post-TR values (solid lines [——], wild-type; dotted lines [·····], Nkx3.1$^{+/-}$; dashed lines [--------], Nkx3.1$^{-/-}$). Error bars reflect the SEM from N ≥ 5 mice. Percentages of dosage sensitivity for Nkx3.1$^{+/-}$ prostates, given below each graph, were calculated by setting wild-type expression levels to 100% and Nkx3.1$^{-/-}$ levels to 0%.

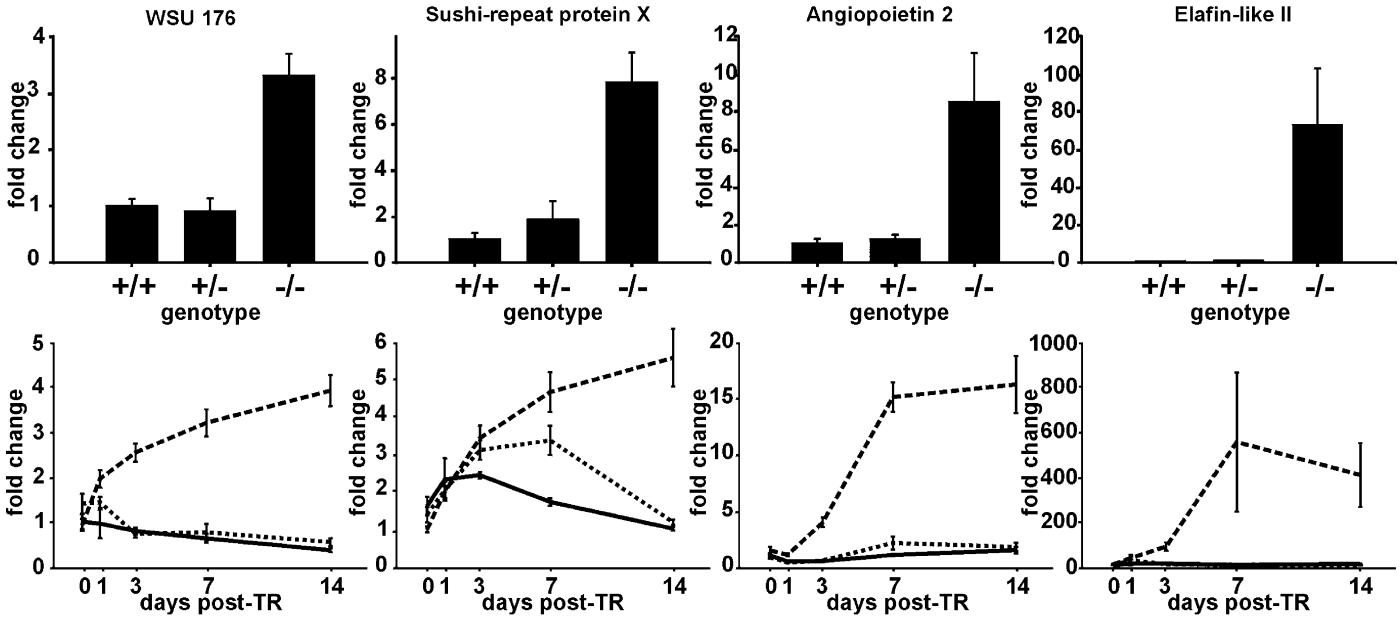

Figure 5. Nkx3.1 represses expression of a subset of androgen-regulated genes

Nkx3.1 target gene expression was analyzed by qRT-PCR as above. Average fold-change values are given relative to wild-type uncastrated (top) or wild-type, 0 days post-TR values (bottom) (solid lines [——], wild-type; dotted lines [·····], Nkx3.1$^{+/-}$; dashed lines [--------], Nkx3.1$^{-/-}$). Error bars reflect the SEM from N ≥ 5 mice.

creased fraction of cells that express a particular target gene (at or near wild-type levels) relative to the fraction of cells that do not express the target gene at all.

To distinguish between these two models, we performed in situ hybridization on two Nkx3.1 target genes selected from opposite ends of the dosage sensitivity spectrum. *Probasin* is relatively insensitive to the loss of one Nkx3.1 allele. It is highly expressed in both wild-type and Nkx3.1$^{+/-}$ prostates, and even Nkx3.1$^{-/-}$ prostates retain some *probasin* expression (Figure 4 and data not shown). *Intelectin* occupies the opposite end of

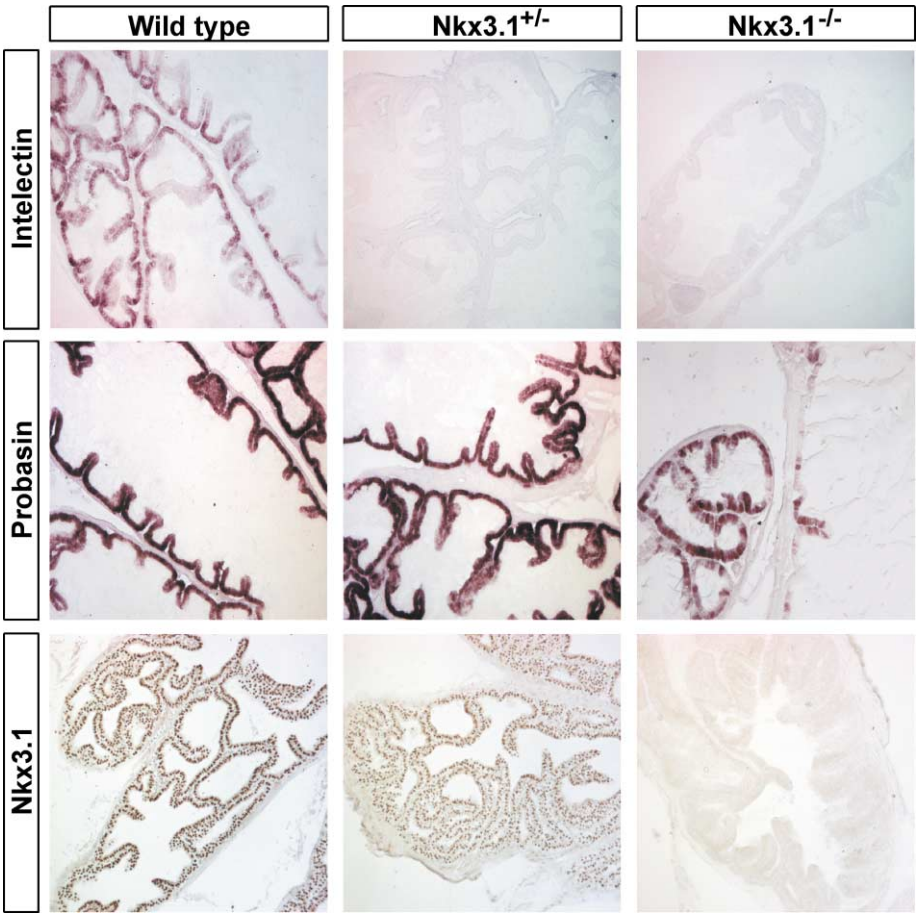

Figure 6. Stochastic, Nkx3.1 gene dosage-sensitive alterations in the expression patterns of Nkx3.1 target genes

The expression patterns of *intelectin* and *probasin* were examined in wild-type, Nkx3.1$^{+/-}$, and Nkx3.1$^{-/-}$ prostates by in situ hybridization. For *intelectin,* note the mosaic pattern of cells that express *intelectin* and cells that do not in the wild-type prostate. A similar mosaic pattern can be seen for *probasin* in the Nkx3.1$^{-/-}$ prostate. Immunohistochemistry with anit-Nkx3.1 antibodies demonstrated a uniform pattern of Nkx3.1 protein expression in wild-type and Nkx3.1$^{+/-}$ prostates (bottom panels).

the dosage sensitivity spectrum, as it is not expressed in either the Nkx3.1$^{+/-}$ or Nkx3.1$^{-/-}$ prostate (Figure 4). In situ hybridization experiments revealed non-uniform expression patterns for both probasin and intelectin (Figure 6). While probasin is expressed uniformly in wild-type and Nkx3.1$^{+/-}$ prostate, the Nkx3.1$^{-/-}$ prostate contains a heterogeneous population of probasin-expressing and nonexpressing luminal epithelia (Figure 6). Interestingly, wild-type prostate presents a similarly heterogeneous pattern of expression with respect to *intelectin*, with only a subset of wild-type luminal epithelia expressing the *intelectin* transcript. We did not observe *intelectin* expression in either Nkx3.1$^{+/-}$ or Nkx3.1$^{-/-}$ prostates by in situ hybridization. Thus, there is a bimodal distribution of expressing and nonexpressing cells for both *probasin* and *intelectin* that contrasts with the relatively uniform distribution of Nkx3.1 protein in both wild-type and Nkx3.1$^{+/-}$ prostates (Figure 6). The probasin expression pattern is particularly supportive of stochastic gene regulation as it is a highly expressed, and therefore easily detected, transcript that is lost entirely in only a subset of Nkx3.1$^{-/-}$ luminal cells. Because these cells lack both copies of the Nkx3.1 gene, the probasin mosaic cannot be attributed to an intercellular variance of Nkx3.1 protein levels. Rather, Nkx3.1 deficiency appears to reduce, but not nullify, the probability of probasin promoter activity. While we cannot rule out graded effects on Nkx3.1 target gene expression, the *intelectin* and *probasin* expression patterns are most consistent with a model for gene induction wherein Nkx3.1 gene dosage predominantly influences the stochastic probability rather than the absolute level of target gene activity in a given cell.

Discussion

The molecular events that occur during the earliest stages of tumorigenesis remain enigmatic. However, it is clear that the first genetic "hit" during tumor development must convey a growth advantage to the affected cell, as the likelihood of accumulating multiple genetic lesions concurrently in a single, normal cell is extremely low (Quon and Berns, 2001). Subsequent proliferation of the affected cells then propagates the initial mutation, thus creating a reservoir of cells that are more susceptible to the damage caused by a second independent mutation (Quon and Berns, 2001). But how, mechanistically, might an initiating lesion be significant enough to enhance proliferation yet subtle enough to avoid triggering the anti-tumorigenic safeguards of well-differentiated cells? Perhaps the more subtle alterations in cell growth emanating from dosage-sensitive loci might play a role in this process. For example, if haploinsufficiency at tumorigenic loci is indeed central to tumor initiation, then small reductions in levels of a tumor suppressor might result in changes in gene expression and increased proliferation. The results of our analysis of Nkx3.1 deficency suggest that loss of a single allele can dramatically influence expression of selected target genes and result in pre-neoplastic lesions.

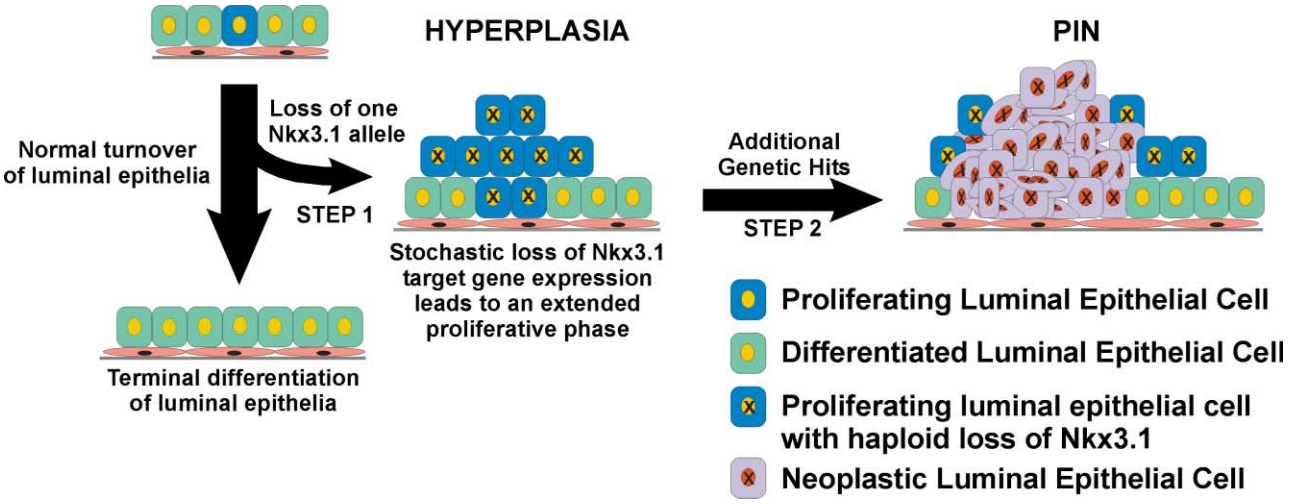

Figure 7. A model depicting the effects of haploinsufficiency on tumor initiation

During normal epithelial turnover, mutation at a single Nkx3.1 allele in the amplifying cell population results in stochastic inactivation of dosage-sensitive Nkx3.1 target genes and a concomitant failure to exit the transient proliferative phase. These defects lead to an expanded population of dividing cells that serve as targets for subsequent genetic change. Additional genetic alterations accompany a transition from hyperplastic cell populations, carrying the initiating "hit" to the Nkx3.1 locus, to PIN lesions that harbor multiple mutations.

Nkx3.1 regulates cell cycle exit during luminal cell regeneration

Nkx3.1-deficient mice develop prostatic hyperplasia as young adults that progresses to PIN over time (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999). Mice lacking only a single allele of Nkx3.1 develop similar, though often less severe phenotypes. Furthermore, mice in which an allele of Nkx3.1 is conditionally deleted in the prostate in adulthood also develop hyperplasia and PIN, consistent with the frequent LOH occurring at the Nkx3.1 locus in human PIN lesions (Abdulkadir et al., 2002). It has therefore been proposed that Nkx3.1 either establishes or maintains luminal epithelia in a growth-arrested state and that the loss of one or both alleles results in excessive luminal cell proliferation (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999). The castration-TR model has allowed for a more thorough analysis of the dosage-sensitive Nkx3.1 phenotype, as it establishes a relatively synchronous population of proliferating and differentiating luminal epithelial cells in the prostate. Analysis of this model indicates that Nkx3.1-deficient luminal cells, and Nkx3.1$^{+/-}$ cells to a lesser extent, fail to appropriately exit the transient proliferating state. Unlike wild-type prostate epithelia, Nkx3.1$^{+/-}$ and Nkx3.1$^{-/-}$ luminal epithelial cells continue proliferating for at least one week after testosterone replacement. This extended proliferation results in luminal hyperplasia that is clearly evident in Nkx3.1$^{-/-}$ prostates by 14 days post-TR.

Despite the delay in terminal differentiation, Nkx3.1-deficient cells do ultimately growth arrest following testosterone replacement, as Ki-67 expression levels in the Nkx3.1$^{-/-}$ prostate approach wild-type levels by 14 days post-TR. How then might *Nkx3.1* allelic loss establish a genetic environment conducive to further transformation? A likely explanation is that the extended transient proliferative state of Nkx3.1$^{+/-}$ and Nkx3.1$^{-/-}$ cells results in an enlarged population of proliferating cells in the prostate, and therefore an enlarged target population for subsequent genetic alterations (Figure 7). This multistep model is consistent with the natural history of PIN formation in the prostate. *Nkx3.1* haploinsufficiency results in global luminal hyperplasia in young adult mice, circa 8 weeks of age. Focal PIN lesions do not become apparent until 6 months of age in Nkx3.1$^{+/-}$ prostates, though compound heterozygosity at the PTEN locus has been shown to expedite the process of PIN formation (Kim et al., 2002). A similar explanation has been offered to describe how the expanded lymphoid compartment of Bcl-2-overexpressing mice might facilitate lymphomagenesis (McDonnell et al., 1989). Transgenic overexpression of Bcl-2 in lymphoid cells results in follicular hyperplasia that, over time, progresses to diffuse large-cell lymphoma (McDonnell and Korsmeyer, 1991). In this model, monoclonal tumors arise from a polyclonal population of pre-neoplastic cells. Ongoing lineage tracing studies should help determine whether PIN lesions in Nkx3.1$^{+/-}$ and Nkx3.1$^{-/-}$ mice are, in fact, derived from polyclonal reservoirs of transient proliferating cells.

Nkx3.1 modulates androgen target gene activity

The extended transient proliferative phase observed in Nkx3.1$^{+/-}$ and Nkx3.1$^{-/-}$ luminal cells suggests that Nkx3.1 and androgens cooperatively regulate gene expression during prostate regeneration. Indeed, both positively and negatively regulated Nkx3.1 target genes are simultaneously regulated by androgens. Preliminary scrutiny of the Nkx3.1 target promoters suggests that Nkx3.1 and androgen receptor (AR) coregulation is direct. We used Transfac 6.2 matrices to screen the non-repeat regions of each target gene promoter for 500 base pair windows that contain both AR and Nkx3.1 consensus sites. In a majority of target promoters, including the intelectin and probasin promoters, this approach identified putative Nkx3.1 and AR binding sites located within 500 base pairs of one another (data not shown). These findings support the hypothesis that Nkx3.1 and AR cooperatively regulate common *cis*-elements, and further molecular characterization of these elements is clearly warranted.

Androgens play a critical role in the growth and maintenance

of the prostate, and androgen signaling is a predominant target for prostate cancer therapeutics. Thus, the androgen-dependent expression profiles of Nkx3.1 target genes further implicate Nkx3.1 as a modulator of crucial mitogenic pathways in the prostate. The disruption of these pathways could account for the haploinsufficient hyperplasia and PIN phenotypes observed in the Nkx3.1 mouse model and the frequent LOH observed at the Nkx3.1 locus in human prostate tumors.

Dosage sensitivity of Nkx3.1 target genes

The proliferative phenotype of Nkx3.1$^{+/-}$ epithelial cells raises the question of how, at the molecular level, heterozygous cells might recapitulate a null phenotype. We have addressed this question by characterizing the effects of Nkx3.1 haploinsufficiency on Nkx3.1 target gene expression using qRT-PCR and in situ hybridization. The sensitivity of Nkx3.1 targets to the loss of one or both Nkx3.1 alleles was variable, with a spectrum of dosage sensitivity observed among the positively regulated Nkx3.1 target genes. Some genes, such as *intelectin* or *PDZ1*, are highly sensitive to mono-allelic loss of *Nkx3.1*, whereas others, such as *Riken 2210008A03* and *probasin*, are relatively refractory to haploinsufficient effects. The expression pattern of Nkx3.1 target genes is also informative, as depending on the Nkx3.1 dosage, both *intelectin* and *probasin* are expressed in a mosaic pattern that includes both expressing and nonexpressing prostate epithelial cells. The simplest and most reasonable explanation for the *probasin* and *intelectin* expression patterns is that they are regulated by stochastic, binary promoters. In this scenario, Nkx3.1 dosage influences the probability of target gene activation rather than the level of expression. The broad spectrum of Nkx3.1 dosage sensitivity observed in the identified subset of Nkx3.1 target genes suggests that the dosage effects vary depending on the architecture of the target gene promoter.

The observations presented here find precedent in previous investigations of graded versus stochastic promoter activity (Fiering et al., 2000). The liver enzymes carbamoylphosphate synthetase and phosphoenolpyruvate carboxykinase accumulate stochastically in embryonic hepatocytes in response to hormonal stimuli (van Roon et al., 1989). Likewise, cultured mammalian cells carrying a glucocorticoid-responsive *lacZ* reporter gene express β-galactosidase at levels proportional to dexamethasone concentration (Ko et al., 1990). In this study, assays of β-galactosidase activity at the cellular level revealed a heterogeneous population of expressing and nonexpressing cells that resembles the pattern observed for Nkx3.1 target genes in the prostate. This heterogeneity could not be attributed to intrinsic properties of the cells, as colonies derived from single cell clones still exhibited heterogeneous β-galactosidase expression. Thus, the effects on gene expression resulting from loss of a single Nkx3.1 allele are consistent with previous observations of stochastic, dosage-dependent promoter activity.

The relationship between Nkx3.1 dosage and stochastic target gene activity is particularly pertinent when considering how a null molecular phenotype might occur in cells heterozygous at a particular locus. Because Nkx3.1 dosage affects the probability of target gene activation, it is reasonable to predict that the loss of a single Nkx3.1 allele would similarly enhance the probability of complete inactivation of a given target gene in luminal epithelial cells. Inactivation of a given target gene would, at the level of the affected cell, generate a null phenotype for the processes regulated by the protein encoded by that target gene. In the case of tumor suppression, this phenotype might manifest itself as an extended proliferative state that could eventually lead to hyperplasia and neoplasia.

Altogether, the results presented here suggest a mechanism for initiating the multistep process of tumor progression in the prostate (Figure 7). At the point of tumor initiation, the loss of a single Nkx3.1 allele results in stochastic inactivation of dosage-sensitive Nkx3.1 targets that, in turn, extend the proliferative phase of luminal amplifying cells. Clonally expanded populations of these undifferentiated cells could accrue additional genetic lesions that would facilitate progression to PIN and prostate cancer. Further functional characterization of the dosage-sensitive and dosage-insensitive Nkx3.1 targets will help illuminate their specific contributions to the differentiation and immortalization pathways outlined above. Likewise, comprehensive analyses of Nkx3.1 target gene promoters should offer crucial insights into the *cis*-regulatory underpinnings of dosage sensitivity. As haploinsufficiency at tumor suppressor loci is, most likely, a common occurrence during somatic tumorigenesis, the stochastic effects of Nkx3.1 dosage on target gene expression may exemplify a widespread paradigm for tumor initiation. Accordingly, the Nkx3.1 model serves as a platform for further investigating the mechanism of haploinsufficiency and its role in promoting tumorigenesis.

Experimental procedures

Animal procedures

The Nkx3.1 mutant mice used in this study have been described previously (Abdulkadir et al., 2002). For all castration-testosterone replacement experiments, mice were castrated at 8 weeks of age and allowed to recover. At 14 days post-castration, testosterone was replaced by implanting 2 cm silastic capsules (1.5 mm inside diameter, Fisher) filled with 50 mg of testosterone propionate (Sigma). At the indicated times after testosterone replacement, mice were killed by cervical dislocation and their prostates were harvested as described below. All uncastrated mice were sacrificed at 8 weeks of age unless otherwise indicated.

Immunohistochemistry and in situ hybridization

For double labeling immunohistochemistry experiments (Nkx3.1/Ki-67 or E-cadherin/Brdu), paraformaldehyde fixed tissues were stained as described for use of two primary antibodies derived from the same species (Shindler and Roth, 1996). Briefly, tissues were stained with the first primary antibody at concentrations sufficiently low so as to prevent detection without signal amplification (rabbit anti-Nkx3.1, 1:200; or mouse anti-E-cadherin, Transduction Laboratories, 1:10,000). Horseradish peroxidase-conjugated secondary antibodies were used in conjunction with tyramide signal amplification to visualize the first antigen (TSA-plus fluorescein kit, Perkin Elmer). Tissues were subsequently stained with antibodies to the second antigen of interest (rabbit anti-Ki-67, Novacastra, 1:1000; or mouse anti-Brdu, Roche, 1:200) and visualized with Cy3-conjugated secondary antibodies.

In situ hybridization experiments were performed using digoxigenin-labeled riboprobes on paraformaldehyde fixed, cryoprotected frozen sections. In situ hybridization probes corresponded to nt 282–731 of the mouse probasin mRNA and nt 1–506 of the mouse intelectin mRNA.

Brdu incorporation assays

Adult wild-type, Nkx3.1$^{+/-}$, and Nkx3.1$^{-/-}$ mice were castrated and testosterone pellets were implanted as described above. At 3 or 7 days post-TR, mice (n = 2–3 per time point per genotype) were injected intraperitoneally with 6 mg of Brdu and sacrificed 3 hr later. Prostates were fixed overnight in 4% paraformaldehyde and stained for Brdu and E-cadherin as described above. For each prostate, ~1000 E-cadherin-positive cells were counted from multiple sections to determine the fraction of Brdu-positive luminal epithelial cells.

RNA isolation and probe synthesis

For total RNA collection, prostates (anterior, ventral and dorsolateral lobes) were dissected in RNA-later (Ambion) and homogenized immediately in 1 ml Trizol (Invitrogen). RNA was quantified by a Ribogreen fluorometric assay (Molecular Probes). For each microarray probe, equal amounts of total RNA were pooled from three prostates. Replicate experiments were performed for each time point and genotype by pooling RNA collected from two different groups of mice (three mice per group). Probes were synthesized and applied to Affymetrix mouse U74A GeneChips according to the manufacturer's protocol.

Microarray data analysis

We analyzed the microarray gene expression data with the Affymetrix Microarray Suite 5.0 software package. This analysis provides a detection call ("present," "absent," or "moderate") and a numerical signal intensity for each probe set. Genes that were not called present at least once in each data series were excluded from subsequent analyses in order to eliminate any noise that these nonexpressed genes might contribute. We then used the Significance Analysis of Microarrays (SAM) program to identify genes that differ significantly between wild-type and Nkx3.1-deficient tissues within the replicate data series (Tusher et al., 2001). SAM requires two user-set parameters: (1) a minimal fold-change value; and (2) a threshold value (Δ) that can be adjusted to maximize the number of significant genes while minimizing the predicted false discovery rate. We conducted a blocked, two-class unpaired test using a 2-fold-change cutoff and a Δ threshold of 0.5. These parameters yielded a list of 62 significantly changed probe sets representing 57 independent genes. The false discovery rate was 1.1%. The validity of this method was demonstrated by qRT-PCR evaluation of the expression of several Nkx3.1 target genes.

We used average linkage hierarchical clustering to identify groups of positively and negatively regulated Nkx3.1 targets within the list of 62 significant probe sets. Clustering was performed with the Spotfire Decision Site software package using log(2) transformed fold-change values. Clusters of positively and negatively regulated Nkx3.1 target genes are readily apparent by inspection of the cluster dendogram.

Quantitative RT-PCR

Total RNA was isolated from whole prostates as described above. Quantitative RT-PCR was performed using a Model 7700 instrument (Applied Biosystems, Foster City, California). Amplicons were detected using Sybr Green I fluorescence (Molecular Probes) as described elsewhere (Svaren et al., 2000). Target genes were analyzed using standard curves to determine relative levels of gene expression. Individual RNA samples were normalized according to the levels of GAPDH mRNA.

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