Haploinsufficient Prostate Tumor Suppression by Nkx3.1

A ROLE FOR CHROMATIN ACCESSIBILITY IN DOSAGE-SENSITIVE GENE REGULATION

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Transcription factor haploinsufficiency plays a role in the pathogenesis of many diseases, including cancer. In a mouse model of prostate tumor initiation, loss of a single allele of the tumor suppressor Nkx3.1 stochastically inactivates the expression of a class of dosage-sensitive target genes. Here we show that dosage sensitivity is associated with the differential histone H3/H4 acetylation states of Nkx3.1 target genes. When histone acetylation is induced in Nkx3.1+/− mouse prostates with the histone deacetylase inhibitor Trichostatin A, Nkx3.1 can bind to and reactivate the expression of dosage-sensitive target genes. We incorporated our findings into a mathematical model that entails the association of Nkx3.1 with histone acetyltransferase activity. Subsequent experiments indicate that Nkx3.1 associates with and recruits the histone acetyltransferase p300/CREB-binding protein-associated factor to chromatin. Finally, we demonstrate a role for the dosage-sensitive target gene intelectin/omentin in suppressing prostate tumorigenicity. Our results reveal how the interplay between transcription factor dosage and chromatin affects target gene expression in tumor initiation.

Haploinsufficiency, in which one allele of a gene is unable to support normal cellular function, has been associated with many human disorders, including cancer and developmental defects (1, 2). In tumorigenesis, haploinsufficiency may allow the clonal expansion of cells carrying a single mutated copy of a tumor suppressor gene, thereby increasing the size of the target cell population available for subsequent mutations during the course of tumor progression (2). Haploinsufficiency can be detrimental to various regulatory processes in cell biology such as cell cycle regulation, apoptosis, and DNA synthesis or repair (3). Several tumor suppressor genes, including Trp53, Dmp1, and Nkx3.1, encode transcription factors that demonstrate haploinsufficiency where loss of one allele is sufficient for the development of pre-neoplastic and neoplastic lesions (4–9). Increased stochasticity or noise in gene expression may contribute to haploinsufficiency by increasing the variability in cellular behavior or phenotype (3, 10). Nevertheless, at the molecular level, the mechanisms involved in haploinsufficient tumor suppression have remained unclear.

Nkx3.1 is an androgen-regulated prostate-specific homeodomain-containing transcription factor. Prostate tumor initiation is often linked to the loss of heterozygosity at the Nkx3.1 locus, and deletion of Nkx3.1 in mice leads to prostatic epithelial hyperplasia and prostatic intraepithelial neoplasia (8, 9). Gene expression profiling of the prostates of Nkx3.1-mutant mice has identified a class of genes that shows exquisite dosage sensitivity, i.e. the expression of these genes in Nkx3.1+/− cells was similar to that in Nkx3.1−/− cells (11). In addition, Nkx3.1 dosage appeared to regulate target gene activation in a stochastic manner. These observations are consistent with a model of haploinsufficient tumor suppression in which reduction in Nkx3.1 dosage increases the probabilities of inactivation of select target genes important for tumorigenesis (11, 12). However, none of these dosage-sensitive target genes has been shown to functionally affect prostate cell growth or tumorigenicity. In addition, the mechanisms involved in dosage-sensitive gene regulation by Nkx3.1 were unknown.

Previous transgenic studies in mice and Drosophila suggest that stochastic expression of transgenes in response to alterations in transcription factor dosage is related to changes in higher order chromatin structure (13, 14). In mice bearing a heterochromatic A5 transgene, reduction of dosage of an activating transcription factor significantly reduced the frequency of localization of the transgene to euchromatin with associated reduction in expression of the transgene (13). Ahmad and Henikoff (14) utilized a green fluorescent protein reporter subject to position-effect varigation in Drosophila to show a correlation between fluctuations of chromatin states and stochastic activation or inactivation of gene expression. More recent studies in budding yeast indicate a role for chromatin remodeling and interconversion between inactive to active promoter states in the control of stochasticity in gene expression (10, 15).

In this study we have explored the mechanisms by which reduced Nkx3.1 dosage has dramatically different effects on the stochastic expression of individual endogenous target genes and the relevance of this form of gene regulation to tumorigenesis. Our results show a role for differential histone H3/H4 acetylation states and Nkx3.1 occupancy at target gene loci in dosage-sensitive gene regulation. We also show a role for a dosage-sensitive Nkx3.1 target gene in prostate tumorigenesis, providing the first functional evidence in support of the stochastic, dosage-sensitive gene regulation model of haploinsufficient tumor suppression.
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EXPERIMENTAL PROCEDURES

ChIP Assays, Two-step ChIP Assays—Chromatin immunoprecipitation (ChIP)2 assays were performed using the ChIP assay kit from Upstate Biotechnology (Lake Placid, NY) with the following modifications. Anterior prostates from Nkx3.1 mutant mice littermates aged between 12 to 16 weeks (9) were excised, snap-frozen, and ground into a powder under liquid nitrogen. The tissue was fixed in 1% formaldehyde for 15 min at room temperature. Fixation was stopped by the addition of glycine to a final concentration of 125 mM. After several washes in ice-cold phosphate-buffered saline, the tissue pellet was resuspended in NEBA buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA), dounce-homogenized, and incubated for 15 min on ice. Cell nuclei were collected and lysed with a lysis buffer. Chromatin was sheared to a size of ~500–1000 base pairs and diluted 1:10 with ChIP dilution buffer. Samples were precleared and precipitated overnight at 4 °C with the following antibodies: pan-acetylated H3 (Upstate; 06–599), pan-acetylated H4 (Upstate; 06–598), Nkx3.1 (Santa Cruz Biotechnology; T-19X), PCAF (Santa Cruz; H-369X), and rabbit or mouse IgG (Santa Cruz). Immune complexes were collected with salmon sperm DNA-saturated protein-A/G-Sepharose for 3 h and washed extensively following the manufacturer’s protocol. Samples were reverse cross-linked at 65 °C overnight with 0.3 M NaCl and 30 μg of RNase. Input and bound DNA were extracted with PCR purification kit (Qiagen) and analyzed by quantitative PCR (Applied Biosystems 7300) using SYBR Green. ChIP values are presented as -fold enrichment of antibody immunoprecipitation (normalized to antibody input) to isotype IgG control immunoprecipitation (normalized to IgG input), as described earlier (16, 17). Two-step ChIP assays were performed as described earlier (18). In brief, components were eluted from the first immunoprecipitation reaction by incubation with 10 mM dithiothreitol at 37 °C for 30 min and diluted 1:50 in ChIP dilution buffer followed by reimmunoprecipitation with the second antibodies.

Mouse Prostate Explants, TSA Treatment, and Quantitative RT-PCR Analysis—Mouse prostate explants were performed as described previously (19). Briefly, anterior prostates were excised, minced, and treated with either vehicle (ethanol), Trichostatin A (TSA) (Sigma) at a concentration of 500 ng/ml for 8 h or 5-acazycytidine 1 μM for 24 h. Prostate tissue was harvested in complete Dulbecco’s modified Eagle’s medium/F12 50/50 medium. RNA was extracted with TRizol reagent, and cDNA was synthesized and analyzed by quantitative RT-PCR (Applied Biosystems 7300) using the SYBR Green method as described (19). The explant experiments were independently repeated under the same conditions.

Western Blot Analysis—For Western blot analysis, lysates from anterior prostate tissue were prepared and probed with acetylated H3 antibody (Upstate). The membranes were stripped and reprobed with total H3 antibody as a loading control (Upstate) and Nkx3.1 antibody (Santa Cruz; T19). Lysates from stable knockdown LNCaP cells were prepared and probed with Nkx3.1, intelectin (Phoenix Pharmaceuticals, Inc.) and actin (Santa Cruz) antibodies.

Co-immunoprecipitation—Lysates were prepared from LNCaP cells and wild type mouse prostate tissue. The lysates (500 μg of protein) were precleared with protein-A/G Plus-agarose beads and incubated overnight at 4 °C with 2 μg each of rabbit IgG, goat IgG, Nkx3.1, or PCAF antibodies (Santa Cruz). Immune complexes were collected and washed three to five times with ice-cold extraction buffer. The precipitated protein complexes were analyzed by Western blotting.

RNA Interference—Lentiviral Nkx3.1 short hairpin RNA (shRNA) construct (RHS4186) and retroviral intelectin shRNA construct (RHS1764) were purchased from Open Biosystems. Intelectin shRNA was cloned into the same lentiviral vector for a valid comparison with shNKK3.1. The constructs were transfected into LNCaP cells with Superfect reagent (Qiagen). Stable pooled clones were selected in complete medium supplemented with 500 μg/ml of G418 for 2 weeks and knockdown efficiency was confirmed by Western blotting.

Cell Count—For cell count, 1 × 10⁵ cells were seeded in 10-cm plates in duplicates supplemented with a complete RPMI medium and counted at the indicated times. At least two independent experiments were performed. Results are presented as mean ± S.D.

MTT Assays—LNCaP/shNKX3.1 or DU145 cells were seeded at a density of 10⁴ cells in quadruplicate in a 96-well flat-bottomed tissue culture plate (Fisher) in 100 μl of complete medium. Intelectin expression vector coexpressing green fluorescent protein (20) or green fluorescent protein-only control was transfected. At variable indicated times 10 μl of 3-[4,5-di-methylthiazolyl-2]-2,5-diphenyl-tetrazolium bromide (MTT reagent) (ATCC) was added to each well and incubated for 2–4 h at 37 °C until purple precipitates were visible. The reaction was terminated by addition of 100 μl of detergent reagent in the dark for 2 h, and the absorbance was recorded at 570 nm. Western blot was used to monitor transfected protein expression. At least two independent experiments were performed. Results are presented as mean ± S.D.

Soft Agar Colony Formation Assays—For soft agar colony formation assay, 0.8 × 10⁵ cells were suspended in a complete RPMI medium containing 0.3% of agarose and overlaid onto a bottom layer of solidified 0.5% agar in a complete RPMI medium. The colonies (>0.5-mm size) were counted after 2 weeks under a light microscope. At least two independent experiments were performed. Results are presented as mean ± S.D.

Xenograft Studies—Cells (2 × 10⁶) were suspended in Matrigel (BD Biosciences) for a total volume of 400 μl/injection site and injected subcutaneously in right and left flanks of male athymic mice (8 weeks old) (Jackson Laboratories). Mice were monitored daily, and tumor sizes were measured twice weekly in two dimensions (width, W and length, L) with calipers. Average tumor volume (V) was calculated as V = 0.5 × L × W². At the termination of the experiment, mice were sacrificed; tumors were excised and weighed. All animal care followed approved institutional guidelines of Vanderbilt University. Results are presented as mean ± S.D.

2The abbreviations used are: ChIP, chromatin immunoprecipitation; PCAF, p300/CREB (cAMP-response element-binding protein)-associated factor; TSA, trichostatin A; HAT, histone acetylase transferase; HDAC, histone deacetylase; RT-PCR, reverse transcription PCR; shRNA, short hairpin RNA; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide.
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*Statistical Analysis*—Data were analyzed by Student’s t-test, and results were considered significant at $p \leq 0.05$. Results are presented as mean ± S.D.

**Mathematical Model**—Let $G_a(t)$ denote the level of active genes at time $t$, $G_{in}(t)$ the level of inactive genes, $Nkx(t)$ the level of Nkx3.1, $HAT(t)$ the level of histone acetyltransferase, $NkxHAT(t)$ the level of the Nkx3.1-HAT complex, and $mRNA(t)$ the level of target gene mRNA (either intelectin or probasin). The mRNA pathway is characterized by three constants: $K_1$, $K_2$, and $K_3$. We assume that the total number of genes, $G_0$, is conserved so that $G_a(t) + G_{in}(t) = G_0$. Using compartment analysis, we can write down individual differential equations for a compartment, e.g. the active gene compartment. Individual components in the pathway do not necessarily mean creation or consumption of the substance in the compartment. For example, mRNA dynamics are governed by $G_a(t)$ and $Nkx(t)$, but these components are not consumed in the creation of $mRNA(t)$. Using the above scheme and the diagram in Fig. 4A, we construct a system of ordinary differential equations as shown in Equations 1–4.

\[
\frac{d(NkxHAT)}{dt} = \lambda \cdot Nkx \cdot HAT \tag{Eq. 1}
\]

\[
\frac{d(G_a)}{dt} = -K_2 \cdot G_a + K_1 \cdot NkxHAT \cdot G_{in} \tag{Eq. 2}
\]

\[
\frac{d(G_{in})}{dt} = K_2 \cdot G_a - K_1 \cdot NkxHAT \cdot G_{in} \tag{Eq. 3}
\]

\[
\frac{d(mRNA)}{dt} = K_3 \cdot Nkx \cdot G_a \tag{Eq. 4}
\]

Notice that if we add the second and third differential equations, we find the following as shown in Equation 5.

\[
\frac{d(G_a)}{dt} + \frac{d(G_{in})}{dt} = 0 \Rightarrow G_a(t) + G_{in}(t) = G_0 = \text{constant} \tag{Eq. 5}
\]

This permits us to replace $G_{in}(t)$ by $G_0 - G_a(t)$. Using this expression in the differential equation for $G_a(t)$, we find the following as shown in Equation 6.

\[
\frac{d(G_a)}{dt} = -K_2 \cdot G_a + K_1 \cdot NkxHAT \cdot (G_0 - G_a) \tag{Eq. 6}
\]

Hence, the model simplifies to the coupled system as shown in Equations 7 and 8.

\[
\frac{d(G_a)}{dt} = -K_2 \cdot G_a + K_1 \cdot NkxHAT \cdot (G_0 - G_a) \tag{Eq. 7}
\]

\[
\frac{d(NkxHAT)}{dt} = \lambda \cdot Nkx \cdot HAT \Rightarrow NkxHAT(t) = NkxHAT(0)
\]

\[
+ \lambda \int_{0}^{t} Nkx(\tau) \cdot HAT(\tau) d\tau \tag{Eq. 8}
\]

where $\lambda$ is a constant that determines the rate of formation of the Nkx3.1-HAT complex, $HAT(t)$ is a prescribed function, and $Nkx(t)$ an experimentally determined function (11), assuming that Nkx3.1 mRNA levels reflect protein levels. The outcome variable for the model is $mRNA(t)$. This can be computed by integrating the differential equation for $mRNA(t)$, in particular, as shown in Equation 9.

\[
mRNA(t) = mRNA(0) + K_3 \int_{0}^{t} Nkx(\tau) \cdot G_a(\tau) d\tau \tag{Eq. 9}
\]

We will take $mRNA(0) = 0$. The $mRNA(t)$ represents target gene levels (intelectin or probasin). The dynamics of the model are dependent on the ratio of initial conditions, $G_a(0)/G_{in}(0)$, which reflects target gene chromatin accessibility and introduces a stochastic component into the model, and the functions $Nkx(t)$ and $HAT(t)$. For the simulations in this report, we have assumed that $HAT(t)$ = constant, $HAT(0)$ and $NkxHAT(0) = 0$.

For modeling the effects of TSA, the kinetic parameter and the chromatin accessibility represented by the initial active/inactive gene ratio $R_0 = G_a(0)/G_{in}(0)$ are assumed to depend on the TSA level, which we assume is constant over the course of the experiment. Our mathematical formulation for the effect of TSA (suitably normalized to be dimensionless) on $K_2$ is given by the relationship $K_2 = \alpha(1 - \mu(TSA)\sigma(R_0))$ as shown in Equation 10.

\[
\mu(TSA) = \frac{TSA}{K_\mu + TSA} \quad \text{and} \quad \sigma(R) = 1 - \frac{R}{K_\sigma + R} \tag{Eq. 10}
\]

where $\alpha$ is a constant.

In the simulations, we have assumed that TSA and $R_0$ are fixed in time. The constants $K_\mu$ and $K_\sigma$ were chosen to fit the experimental data. In our model, the rate constants for intelectin and probasin were kept the same, although in theory they could be different. Remarkably, differentiating between probasin and intelectin by the initial active/inactive gene ratio (i.e. chromatin accessibility) alone, keeping all other parameters constant, was sufficient to simulate experimental findings. All simulations were performed with Mathematica (Wolfram Research, Inc., Champaign, IL).

**RESULTS AND DISCUSSION**

**Differential Histone H3/H4 Acetylation and Nkx3.1 Occupancy at Dosage-sensitive and -insensitive Target Gene Loci**—We reasoned that Nkx3.1 dosage-sensitive and stochastic gene regulation may be mediated by the differential chromatin accessibilities of target gene loci. To investigate this, we examined the histone H3/H4 acetylation states of the promoter regions of two representative target genes in mouse prostate, intelectin and probasin (11). Probasin is a prostate-specific differentiation marker (21) that is insensitive to Nkx3.1 dosage, whereas intelectin is a $\alpha$-galacotyl-specific lectin that is also known as omentin, an adipocytokine (22, 23). Intelectin shows exquisite sensitivity to Nkx3.1 dosage, with its expression in Nkx3.1$^{+/-}$ cells approximating the levels seen in Nkx3.1$^{-/-}$
cells (11). We used ChIP to profile the histone H3 and H4 acetylation patterns in the 2-kb regions of the intelectin and probasin promoters in Nkx3.1 mutant mouse prostate tissues (Fig. 1, A and B, and supplemental Table S1). The intelectin promoter showed higher levels of histone H3 and H4 acetylation in Nkx3.1/H11001 animals compared with Nkx3.1/H11001 or Nkx3.1/H11002 animals, particularly at a region (Int5) that was subsequently found to contain a consensus Nkx3.1 binding site (TAAGTG) (24) by sequence analysis (Fig. 1A). By contrast, on the probasin promoter, H3 and H4 acetylation levels were high in Nkx3.1/H11001 and Nkx3.1/H11002 mice and low in Nkx3.1/H11002 mice (Fig. 1B).

Next we examined in vivo binding of Nkx3.1 at the intelectin and probasin promoters in Nkx3.1 mutant mice by ChIP. Nkx3.1 was bound at the Int5 region of the intelectin promoter in Nkx3.1/+/+ but not in Nkx3.1/+− mice, whereas Nkx3.1 binding to the Pbn1 region of the probasin promoter (containing a consensus Nkx3.1 site) was observed in both Nkx3.1/+− and Nkx3.1/+− mice (Fig. 1C). We used a mouse androgen receptor promoter region containing a consensus Nkx3.1 binding site (AR) as a positive control while Int2 and Pbn4 regions served as negative controls. Thus, expression of intelectin and probasin correlated with the overall histone H3/H4 acetylation states of their promoter regions and Nkx3.1 occupancy in mice of various genotypes.

**Induction of Histone Hyperacetylation with a HDAC Inhibitor Allows Nkx3.1 Binding and Reactivation of Dosage-sensitive Target Gene Expression in Nkx3.1/+− Mouse Prostates**—Our results thus far indicate that in Nkx3.1/H11002 prostate cells, the probasin promoter is hyperacetylated at histones H3/H4, resulting in an “open” chromatin configuration that allows Nkx3.1 binding and activation of gene expression. The intelectin promoter on the other hand is in a hypoacetylated, “closed” chromatin state that precludes Nkx3.1 binding and gene activation. If so, then increasing chromatin accessibility by inducing histone H3/H4 hyperacetylation might allow Nkx3.1 bind-
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ing to the intelectin promoter and gene activation in Nkx3.1 heterozygous cells. To ascertain this, we treated mouse prostate tissue explants with the histone deacetylase inhibitor TSA (26).

The efficacy of our treatment regimen was evident from results of Western blot analyses for acetylated histone H3 (Fig. 2A, upper panel). TSA treatment did not have a discernible effect on Nkx3.1 protein expression (Fig. 2A, lower panel). S-Azacytidine (Azacyt), a DNA hypomethylating agent, was used as additional control (n = 3/genotype). Results are presented as mean ± S.D. **, p < 0.05.

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FIGURE 3. TSA promotes Nkx3.1 binding and reactivation of dosage-sensitive target gene expression in Nkx3.1+/− mouse prostate explants. A, ChIP assays on the intelectin promoter show Nkx3.1 binding to Int5 site but not the Int2 control region following TSA treatment in Nkx3.1+/− mice. Results are presented as mean ± S.D. from n = 4. B–E, quantitative RT-PCR analysis for Nkx3.1 target gene expression following TSA treatment. Dosage-sensitive mRNAs like intelectin (B) and Pdzk1 (C) were significantly up-regulated in TSA-treated Nkx3.1+/− mice prostate explants compared with dosage-insensitive targets like probasin (D) and Sel1L (E). **, p < 0.05. vehicle. Results are presented as mean ± S.D. **, p < 0.05.
on Nkx3.1 protein expression (lower panel). ChIP analysis of the intelectin promoter indicates that TSA treatment led to a significant increase in H3/H4 acetylation in Nkx3.1/H11001/H11002 mouse prostates, particularly in the Int5 region (Fig. 2B and 2D, middle panels). Notably, the levels of histone H3 acetylation observed at the Int5 region in Nkx3.1/H11001/H11002 mice after TSA treatment approximate the baseline levels seen in Nkx3.1/H11001/H11001 mice (Fig. 2B). By contrast, TSA treatment had modest or minimal effects on histone H3/H4 acetylation at the intelectin promoter in Nkx3.1/H11001/H11002 and Nkx3.1/H11001/H11001 mice or the probasin promoter in all genotypes (Fig. 2B–E). These results support the notion that acetylation is a dynamic process and there may be a limit to the maximum acetylation achievable at any promoter. They also suggest that Nkx3.1 may be involved in setting the histone H3/H4 acetylation states of its target genes.

Using ChIP, we next showed that TSA treatment promotes the binding of Nkx3.1 to the Int5 region but not a control region in Nkx3.1/H11001/H11002 mice (Fig. 3A). Importantly, TSA treatment also significantly induced intelectin mRNA expression in Nkx3.1/H11001/H11002 mice as assessed by quantitative RT-PCR (Fig. 3B). The expression of another dosage-sensitive Nkx3.1 target gene, Pdzk1, was also induced by TSA treatment in Nkx3.1/H11001/H11002 mice (Fig. 3C), whereas the expression of the dosage-insensitive genes probasin and Sel1L (11) remained largely unaffected (Fig. 3D and E). Notably, none of these genes was reactivated by TSA treatment in Nkx3.1/H11002/H11002 mice, emphasizing the requirement for Nkx3.1 in gene induction. We note that both intelectin and Pdzk1 were induced by TSA in Nkx3.1/H11001/H11002 mice to levels 3- to 4-fold above their respective expression levels in wild type mice and that TSA treatment did not lead to a further increase in the expression of any of the genes tested in Nkx3.1/H11001/H11001 prostates.

Apart from the histone modifications, DNA methylation is another common epigenetic event important for regulating gene expression (27). Hence, we investigated the possible involvement of DNA methylation in Nkx3.1 target gene regulation by using the DNA methyltransferase inhibitor 5-azacytidine (28). Unlike TSA, treatment with 5-azacytidine did not have consistent effects on the expression of dosage-sensitive versus dosage-insensitive target genes (Fig. 3B–E). Longer periods of 5-azacytidine treatment could not be tested due to toxicity. Reactivation of dosage-sensitive target genes by TSA emphasizes the role of histone acetylation in the regulation of these Nkx3.1 target genes. These results point to the chromatin accessibility of target gene loci as a major determining factor in the response of target genes to reduced dosages of activating transcription factors. The ability to restore the expression of dosage-sensitive genes with HDAC inhibitors may have important therapeutic implications for disorders due to haploinsufficiency of transcription factors.
A Mathematical Model of Gene Activation by Nkx3.1—To gain further insights into the interactions between Nkx3.1 dosage and chromatin remodeling in target gene regulation, we incorporated our current results and earlier findings (11) into a mathematical model of gene activation by Nkx3.1 (Fig. 4A). According to our model, a target gene can exist in an inactive/closed chromatin/hypoacetylated state or an active/open chromatin/hyperacetylated state competent for transcription. Due to the fact that we observed reduced histone H3/H4 acetylation in Nkx3.1 null cells and focal hyperacetylation in a region containing the Nkx3.1 binding site on the intelectin promoter, we assumed in the model that Nkx3.1 interacts with a histone acetyltransferase (8) and influences the active to inactive gene ratio. TSA also affects this ratio by inhibiting the transition from the active to the inactive state due to its known ability to inhibit HDACs. In addition, we assumed that Nkx3.1 directly affects the rate of transcription from the active gene. Input values for levels of Nkx3.1 in Nkx3.1+/+ and Nkx3.1+/− mice were...
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expression of intelectin and probasin in the presence of one or two Nkx3.1 alleles (Fig. 4, B and C) closely paralleled experimental findings for probasin and intelectin mRNA expression in a castration/testosterone replacement paradigm in Nkx3.1+/+ and Nkx3.1−/− mice (11). We also modeled the effects of TSA. The mathematical relationship between gene inactivation rate constant, \( K_2 \), TSA, and the initial ratio of active to inactive genes is shown in supplemental Fig. S1. Simulations for the effects of TSA showed the most significant induction over baseline in the case of intelectin in the presence of one Nkx3.1 allele (Fig. 4, D–G), similar to the experimental observations for Nkx3.1+/− mice (see Fig. 3B), although the -fold induction observed in the simulation is not as high as that observed experimentally. Overall, the modeling results closely simulated experimental observations regarding dosage-sensitive target gene regulation. This supports the conclusion that chromatin accessibility is an important factor in dosage sensitivity. We have not modeled stochastic variations in Nkx3.1 itself as done by others (29); rather, in our model the initial ratio of active to inactive genes that reflects chromatin accessibility/acetylation introduces a stochastic component into the model and is a major determinant of the dynamics of the model.

Nkx3.1 Associates with and Recruits the Histone Acetyltransferase PCAF to Chromatin—The modeling results coupled with our results showing reduced histone acetylation at the probasin and intelectin promoters in Nkx3.1-deficient mice prompted us to examine the possibility that Nkx3.1 is associated with histone acetyltransferase activity. Using co-immunoprecipitation analyses, we found evidence of interaction between Nkx3.1 and PCAF in human prostate cancer cells (LNCaP) and mouse prostate tissue (Fig. 5A). Next we assessed whether Nkx3.1 recruits PCAF to target gene promoters. ChIP assays with mouse prostate tissues showed PCAF binding to the Nkx3.1 consensus sites of both the intelectin (Int5 in Nkx3.1+/+) and probasin (Pbn1 in Nkx3.1+/+ and Nkx3.1−/−) promoters (Fig. 5, B and C). These results are consistent with the “gene-specific targeting model” of histone acetylation (30) that implies recruitment of HATs to particular promoter regions by sequence-specific DNA-binding proteins. We also observed PCAF binding at the Pbn4 control region that does not contain an Nkx3.1 consensus in Nkx3.1+/+ and Nkx3.1−/− mice, presumably due to the ability of
PCAF to bind to acetylated histones through its bromodomain (31). As we observed Nkx3.1 binding to the intelectin promoter in Nkx3.1+/− mice following TSA treatment (see Fig. 3A), we sought to determine whether Nkx3.1 can recruit PCAF to the intelectin promoter under these conditions as well. ChIP assays established that PCAF was recruited to the Int5 region in Nkx3.1+/− mice after TSA treatment (Fig. 5D). Hyperacetylation in the Int5 region after TSA treatment could have also contributed to PCAF recruitment to this site, in addition to the interaction with Nkx3.1. Nevertheless, failure to observe recruitment of PCAF at a control region (Int2) after TSA treatment suggests that Nkx3.1 is involved in the targeted recruit-
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down in LNCaP cells (Fig. 7, A and B). Consistent with it being a dosage-sensitive Nkx3.1 target, the expression of intelectin was dramatically reduced in Nkx3.1 knockout clones (Fig. 7A). By contrast, Nkx3.1 protein levels remained unaffected in intelectin knockout clones (Fig. 7B).

To determine the effects of intelectin on prostate cell growth, we performed a series of in vitro proliferation assays. By cell counting, the proliferation rates of both the intelectin and Nkx3.1 stable knockout clones were significantly increased compared with control cells (Fig. 7C). Consistent with the knockout data, expression of exogenous intelectin in DU145 cells that express low endogenous levels of intelectin and Nkx3.1 resulted in a significant decrease in cell viability as measured by the vital dye MTT (Fig. 7D). Furthermore, expression of exogenous intelectin in Nkx3.1 stable knockout cells led to a dramatic decrease in cell viability by MTT compared with cells transfected with control vector (Fig. 7D and supplemental Fig. S3), indicating rescue of the growth phenotype of Nkx3.1 knockdown cells by intelectin.

We next examined the tumorigenicity of the intelectin and Nkx3.1 knockout cells by performing soft agar colony-formation assays. Consistent with the in vitro proliferation assays, increased colony formation was observed for both the intelectin and Nkx3.1 knockout clones compared with control cells (Fig. 8, A and B), suggesting that the intelectin has tumor suppressor-like activity in prostate cells. To establish the tumor suppressor function of intelectin in vivo, we performed xenograft studies in immunocompromised mice (Fig. 8, C–E). We found that the tumors in mice injected with intelectin and Nkx3.1 knockout cells grew faster and to greater volumes than the tumors in mice injected with control cells (Fig. 8C). Mean tumor weight was also significantly greater in the intelectin and Nkx3.1 knockout group than the control group (Fig. 8B). Consistent with it being a key concentration-dependent event cannot be ruled out at this point. Nevertheless, our results provide an example of how a genetic lesion (haploid loss of the Nkx3.1 tumor suppressor) can engender epigenetic changes (alterations in histone H3/H4 acetylation) that selectively inactivate a dosage-sensitive target gene important for suppressing tumorigenicity. This mechanism may be applicable to other disorders caused by transcription factor haploinsufficiency. The reduced chromatin accessibility of some target genes can lead to their selective inactivation in heterozygous cells, with attendant pathology.

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