An enhancer from the 8q24 prostate cancer risk region is sufficient to direct reporter gene expression to a subset of prostate stem-like epithelial cells in transgenic mice

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Ting, Man-Chun; Liao, Chun-Peng; Yan, Chunli; Jia, Li; Groshen, Susan; Frenkel, Baruch; Roy-Burman, Pradip; Coetze, Gerhard A.; and Maxson, Robert, "An enhancer from the 8q24 prostate cancer risk region is sufficient to direct reporter gene expression to a subset of prostate stem-like epithelial cells in transgenic mice." Disease Models & Mechanisms. 5, 3. 366-374. (2012).  
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An enhancer from the 8q24 prostate cancer risk region is sufficient to direct reporter gene expression to a subset of prostate stem-like epithelial cells in transgenic mice

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SUMMARY

Regions in the 8q24 gene desert contribute significantly to the risk of prostate cancer and other adult cancers. This region contains several DNA regions with enhancer activity in cultured cells. One such segment, histone acetylation peak 10 (AcP10), contains a risk single nucleotide polymorphism (SNP) that is significantly associated with the pathogenesis of colorectal, prostate and other cancers. The mechanism by which AcP10 influences cancer risk remains unknown. Here we show that AcP10 contains a sequence that is highly conserved across terrestrial vertebrates and is capable in transgenic mice of directing reporter gene expression to a subset of prostate luminal epithelial cells. These cells include a small population of Nkx3.1-positive cells that persist even after androgen ablation. Castration-resistant Nkx3.1-positive (CARN) cells were shown by others to function both as stem cells and cells of origin of prostate cancer. Our results thus provide a mechanism by which AcP10 could influence prostate cancer risk.

INTRODUCTION

Independent genome-wide association studies (GWAS) have identified multiple risk regions for many epithelial cancers in the gene-poor region of chromosome 8q24 (Ghoussaini et al., 2008; Haiman et al., 2007; Tomlinson et al., 2008; Zanke et al., 2007). We (Jia et al., 2009; Pomerantz et al., 2009a) and others (Tuupanen et al., 2009; Wasserman et al., 2010; Wright et al., 2010) have identified enhancers in this region that loop to the potent oncogene Myc, located 200–400 kb telomeric (Ahmadiyeh et al., 2010; Wright et al., 2010). Apart from this intriguing but still unproven association with Myc expression, the cellular and molecular mechanisms underlying the increased prostate cancer risk mediated by AcP10 remain unclear. Recently, Wasserman et al. carried out a bacterial artificial chromosome (BAC) transgenic survey of the 8q24 region in which they identified a 5 kb genomic fragment spanning rs6983267 that was active in the mouse prostate (Wasserman et al., 2010). Stains of whole prostates from postnatal day 21 (P21) mice suggested overlap in Myc and transgene expression; however, no histological analysis was performed, leaving identification of the cell type in which the transgene was expressed unclear. Here we document a link between AcP10 activity and a subpopulation of prostate cells that is both a stem population and a potential cell of origin of prostate cancer.

The prostate contains three epithelial cell types: luminal secretory cells, basal cells and neuroendocrine cells (Marker et al., 2003). Prostate stem cell populations have been initially identified within the basal cell population (Goldstein et al., 2010b), but androgen depletion and replenishment experiments have demonstrated the presence of stem cells within the luminal compartment (Wang et al., 2009). The growth and maintenance of the prostate are strongly dependent on androgens (Marker et al., 2003). When androgens are depleted by castration, the prostate undergoes involution. During this process, most luminal cells, and a smaller percentage of basal cells, are lost. Shen and colleagues (Wang et al., 2009) have identified a population of luminal epithelial cells that persists after androgen ablation. These cells are characterized by their expression...
of the homeobox gene *Nkx3.1*, whose expression is regulated by androgens and in turn regulates the development of the prostate epithelium. A functional variant in Nkx3.1 is also associated with prostate cancer susceptibility and downregulates its expression (Akamatsu et al., 2010). In normal adult mice, Nkx3.1 is expressed in most or all luminal cells. Despite its expression-dependence on androgens, a small population of Nkx3.1-positive cells remains after androgen depletion. These cells, designated castration-resistant Nkx3.1-positive (CARN) cells, are capable of giving rise to all three prostate epithelial lineages (Wang et al., 2009). Moreover, these cells can serve as a cell of origin in PTEN-mediated prostate cancer development in mice (Wang et al., 2009).

In the present study, we used a transgenic approach to assess the cell-type specificity of AcP10 activity. We show that a 1.5 kb fragment, spanning the rs6983267 ancestral and risk G allele, contains a DNA sequence with exceptionally highly conservation in mammals, birds and reptiles. We further show that, in mice, this sequence directs reporter gene expression to a small subset of epithelial cells of the prostate, colon, breast and skin, but not to cells of the lung or liver. Androgen ablation experiments suggest, strikingly, that AcP10 is active in CARN cells. It is also active in a subset of Myc-expressing cells. Our results provide a plausible mechanism as to how AcP10 can influence cancer risk: by affecting the Myc pathway in a set of cells crucial for prostate growth and tumorigenesis.

RESULTS

The AcP10 enhancer region is highly conserved among vertebrates

To identify conserved and thus potentially functional DNA elements in the AcP10 region, we compared syntenic DNA

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**Fig. 1. Conservation of the AcP10 enhancer in terrestrial vertebrates and geographical distribution of the rs6983267 SNP.** (A) We used software available on the University of California Santa Cruz (UCSC) genome browser to assess the degree of DNA sequence conservation in the region of the AcP10 enhancer. Shown is the Placental Mammal Basewise Conservation assessed by PhyloP (blue vertical lines), and vertebrate Multiz alignment of sequences from rhesus monkey, mouse, dog, elephant, opossum, platypus, chicken, lizard, *Xenopus tropicalis* and stickelback (black vertical lines). The 1.5 kb fragment containing the AcP10 enhancer is indicated by the horizontal line above. The nucleotide sequence of the region surrounding the rs6983267 functional SNP is shown below. Note that all species shown have the G allele. (B) Global distribution of the high-risk G allele and lower risk T allele of rs6983267 as assessed by the UCSC genome browser. Note the prevalence of the G allele in Africa, suggesting that it might have originated there.
segments across diverse vertebrate groups (Fig. 1). We identified a sequence of 1750 nucleotides that was highly conserved among placental and marsupial mammals. This sequence contained a core of 450 nucleotides that was significantly conserved in mammals, reptiles and birds. The functional SNP rs6983267, shown previously to be causally associated with risk of prostate cancer (Haiman et al., 2007), lay just outside this core sequence, within the region exhibiting conservation among mammals (Fig. 1A). Interestingly, the ancestral G allele imposes prostate cancer risk in multiple populations and might be related to the known higher risk of prostate cancer among recent descendants of Africans such as African-Americans (Fig. 1B).

**A conserved AcP10 sequence is sufficient to direct lacZ reporter expression to prostate, mammary gland, colon and hair follicle**

To investigate the mechanism by which AcP10 might influence cancer risk, we asked whether a fragment bearing the conserved region is capable of driving reporter gene expression to specific locations in mouse tissues. We produced transgenic mouse lines carrying the AcP10-hsp68-lacZ construct. We examined three such lines for transgene expression in mouse tissues (A-H). Tissues were sectioned and stained for β-gal activity (blue) and counterstained with nuclear fast red. (A) Sections of prostate from a control, non-transgenic mouse. (B) Sections of prostate from lines 1, 2 and 3. Note β-gal-positive cells in lumenal epithelium in each line. Line 2 was further examined for β-galactosidase expression in the colon (C), mammary gland (D), skin (E), liver (F), lung (G) and spleen (H). Note expression in colon, mammary gland and skin. AP, anterior prostate; DLP, dorsolateral prostate; VP, ventral prostate. Arrows indicate lacZ-expressing cells.

![Fig. 2. AcP10 directs reporter gene expression to cells of the prostate, mammary gland, colon and skin.](image-url)
tissues in transgenic mice. We produced a transgene in which the 1.5 kb fragment was inserted in a vector containing the hsp68 minimal promoter and lacZ [encoding β-galactosidase (β-gal)] reporter gene. This vector has been used extensively for assessing enhancer activity in vivo (Brugger et al., 2004).

We injected the construct into mouse zygotes and produced three independent transgenic lines. We examined 8-week-old animals of each line for β-gal expression in a variety of tissues. Representative examples are shown in Fig. 2. Staining was apparent in prostate, colon, mammary gland and hair follicle. No staining was detected in liver or lung. The tissue distribution of β-gal activity was similar in the three independent transgenic lines (Fig. 2 and data not shown), suggesting that position effects were not the cause of tissue-specific transgene expression.

The AcP10 transgene is expressed in lumenal epithelial cells of the prostate

We examined β-gal expression in greater detail in the prostates of line 2. The mouse prostate has three lobes: anterior, dorsolateral and posterior. As is evident in Fig. 3, β-gal-positive cells were present in all three lobes, but were more abundant in the anterior lobe. Images of sections at higher magnification showed that β-gal-positive cells were located largely, if not exclusively, in the lumenal epithelium (Fig. 3). Co-staining of sections with an antibody against CK8, a marker for lumenal epithelial cells (Xue et al., 1998), and an antibody against β-gal, showed that all β-gal-positive cells are also positive for CK8 (Fig. 4). Staining with an antibody against CK5, which marks basal cells (Xue et al., 1998), showed no overlap with β-gal. These results show that the β-gal-positive cells are lumenal epithelial cells.

The AcP10 transgene is expressed in a subset of cells resistant to androgen ablation and positive for Nkx3.1

Although there is considerable evidence that basal cells contain a stem population (Goldstein et al., 2010a), recent work shows that the lumenal compartment also contains stem cells (Wang et al., 2009). These cells are marked by continued expression of Nkx3.1 after androgen ablation. We asked whether AcP10–β-gal-positive cells also expressed Nkx3.1. We co-stained sections of prostates of AcP10–β-gal transgenic mice with antibodies against β-gal and Nkx3.1. The distribution of AcP10-positive cells in the different lobes of the prostate was unchanged following androgen ablation (data not shown). As reported previously, Nkx3.1 was expressed in all or most lumenal epithelial cells (Wang et al., 2009) (Fig. 5). Following castration, Nkx3.1-positive cells were reduced to small patches of cells and individual cells, consistent with the findings of Wang et al. (Wang et al., 2009). A comparison of the Nkx3.1 expression pattern with that of AcP10–β-gal showed that the two signals overlapped partially (Fig. 5F,I). The fact that the overlap was not greater suggested that there was not a simple gene regulatory relationship between AcP10 and Nkx3.1 in which, for example, one is necessary and sufficient for the expression of the other. It remained possible, however, that there was a non-random association between the two. To test this possibility, we counted foci positive for AcP10-driven β-gal and Nkx3.1 individually, and foci positive for both. We used a chi-square ($\chi^2$) test to investigate whether the number of foci positive for both was greater than expected from chance alone. As is evident from data presented in Table 1, counts performed on four mice consistently showed an association significantly greater than expected, and yielded an overall $P<0.001$ that the association was due to chance (Mantel-
expected, all mutants examined had prostate tumors (n_Pten-probasin-Cre conditional mutants aged 7-9 months). As Cre et al., 2003). We produced mice homozygous for the floxed allele (Wang et al., 2003). In this system, virtually 100% of probasin-Cre mediates efficient recombination of the floxed marker CK8 (B,F; green fluorescence) showed that with the basal cell marker CK5 (A,E; green fluorescence) and the lumenal cell marker CK8 (E,F; green fluorescence) showed that β-gal (C,F; red fluorescence) was found exclusively in lumenal cells (compare overlapped images in E and F).

Counting of β-gal-positive clusters revealed no significant concentrations in any of these three tissue types (data not shown). Marker analysis with the basal marker CK5 and the luminal marker CK8 showed that AcP10-positive tumor cells, like their normal counterparts, co-express CK8 (Fig. 6); thus loss of Pten function did not influence the cell type in which AcP10 was expressed.

**DISCUSSION**

Here we address the mechanism by which highly conserved, non-coding DNA elements within the 8q24 gene desert influence cancer risk. Previously we identified a subregion of 8q24, AcP10, that contains a risk SNP and has enhancer activity in cultured cells (Jia et al., 2009). In the present study, we show that a 1.5 kb fragment, including a highly conserved DNA sequence in which the SNP is embedded, directs reporter gene expression to a subset of lumenal epithelial cells in the prostate. These include some cells that persist after androgen ablation and express the transcription factor Nkx3.1. Shen and colleagues demonstrated that these cells, designated castration-resistant Nkx3.1-positive (CARN) cells, are both stem cells and potential cells of origin of prostate cancer (Wang et al., 2009). We show, moreover, that AcP10 is active in cells of prostate tumors developing in mice in which the Pten gene is inactivated in the prostate. Our results thus provide a plausible mechanism by which the AcP10 enhancer element can influence prostate cancer risk.

Accumulating evidence shows that highly conserved, non-coding elements (CNEs) have gene regulatory functions (Bulger and Groudine, 2011). The human genome contains many thousands of such elements, which are likely to have originated as retroposons and in some instances have acquired a function in the host genome, a process known as exaptation (Santangelo et al., 2007). Some CNEs have been shown to function as tissue-specific enhancers. In most but not all cases they are also defined by their extremely high degree of DNA sequence conservation across vertebrates (Birney et al., 2007). At least some CNEs are under negative selection, implying that the extremely high degree of sequence conservation is required for function.

The AcP10 element has a central region approximately 450 bp in length that is conserved across terrestrial vertebrates, exhibiting approximately 67% identity to a sequence in lizards. This high degree of conservation suggests a fundamental and slowly evolving function in vertebrates. Such a function might include controlling gene expression in the prostate or in tissues from which the prostate evolved, such as the Mullerian duct (Wake, 1981).

We note that the AcP10 element drives expression in epithelial tissues of the breast, colon and skin, but not in epithelia generally. We have not yet identified the specific cell types in these tissues in which the AcP10 transgene is expressed. This is likely to be of interest at least in the colon because AcP10 is associated with risk of colon cancer as well as prostate cancer.

The fact that the high-risk (G) allele of the SNP rs6983267 is the ancestral allele is evident in comparisons across vertebrates showing the G allele in all groups. It is also apparent in the geographic distribution of the two alleles in humans, which shows the high risk G allele in African populations and the low-risk T allele outside Africa, consistent with the T allele emerging as ancestral humans migrated out of Africa. The T allele might have conferred a selective advantage, perhaps reducing the risk of cancer. Grandparent effects (Hawkes, 2010) on allele frequency – i.e. selection acting at the level of extended family or group – could explain fixation of an allele that does not provide a direct advantage to individuals of reproductive age.
An approach that has been used by several groups to identify stem cells in the mouse prostate is to reduce androgen levels by physical or chemical castration and examine the cells remaining after this treatment (Wang et al., 2009). When androgens are withdrawn, the prostate involutes to a small size. Resupplying androgens causes the gland to regenerate. All the cells required to regenerate the gland are most likely present in the involuted gland following androgen ablation. Using this approach, Shen and colleagues (Wang et al., 2009) showed that a subset of lumenal epithelial cells remain in androgen-depleted prostates. These CARN cells are capable of giving rise to an entire prostate, and are also a cell of origin of prostate cancer.

As we noted previously (Jia et al., 2009), AcP10 has chromatin marks consistent with enhancer activity. Moreover, it can function as an enhancer in cultured cells. Thus, we expected AcP10 to drive reporter gene expression in transgenic mice. We were surprised, however, at the remarkable specificity of transgene expression in a subset of prostate epithelial cells. Marker analysis showed that the AcP10–β-gal-positive cells are lumenal. Positive cells tend to be clustered, implying that such cells are clonally related.

Until recently, only basal epithelial cells were thought to contain stem populations and to serve as cells of origin of prostate cancer (Goldstein et al., 2010a). New findings by the Shen group (Wang et al., 2009) that the lumenal compartment might also have stem cells prompted us to investigate whether the AcP10–β-gal-positive cells exhibit molecular characteristics of lumenal epithelial stem cells. Shen and colleagues showed that the transcription factor Nkx3.1 is expressed in all or most lumenal epithelial cells in control mice (Wang et al., 2009). Upon androgen depletion by castration, the prostate is greatly reduced in size, and Nkx3.1 expression is

**Table 1. Co-expression of AcP10–β-gal and Nkx3.1**

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Total no. of foci</th>
<th>Foci expressing AcP10–β-gal (%)</th>
<th>AcP10–β-gal-positive foci also expressing Nkx3.1 (%)</th>
<th>AcP10–β-gal-negative foci that express Nkx3.1 (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6699</td>
<td>1.8</td>
<td>9.1</td>
<td>3.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>7134</td>
<td>1.5</td>
<td>9.2</td>
<td>5.8</td>
<td>0.14</td>
</tr>
<tr>
<td>3</td>
<td>7297</td>
<td>2.2</td>
<td>8.8</td>
<td>4.0</td>
<td>0.003</td>
</tr>
<tr>
<td>4</td>
<td>3102</td>
<td>1.4</td>
<td>6.7</td>
<td>1.6</td>
<td>0.008</td>
</tr>
<tr>
<td>Overall/Average</td>
<td>–</td>
<td>1.7</td>
<td>8.5</td>
<td>3.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

We counted foci expressing β-gal and Nkx3.1 and estimated the total number of foci (Nkx3.1-expressing foci + AcP10-expressing foci + non-expressing foci) as described in the Methods. The percent of cells/foci expressing Nkx3.1 was compared between foci that were positive vs negative for β-gal. The chi-square test (based on the 2×2 table) was used to measure the strength of this difference.
retained in a small subset of luminal cells. Our results show that about 8.5% of AcP10-positive cells are also positive for Nkx3.1, compared with 3.6% of AcP10-negative cells (Table 1). Chi-square analysis suggests that this overlap in expression patterns is significant ($P<0.001$).

That AcP10 is not expressed in all Nkx3.1-positive cells suggests that there is not a simple relationship between the enhancer activity of AcP10 and the identity of such cells or the regulation of Nkx3.1. Thus it is not likely that the main function of AcP10 is to regulate the expression of Nkx3.1 in luminal epithelial cells; nor is it probable that Nkx3.1 is a main determinant of the activity of AcP10 in such cells. Rather, the increased likelihood that AcP10 is expressed in Nkx3.1-positive cells suggests that AcP10 and Nkx3.1 are linked in a regulatory network, although perhaps distantly. Even a distant link, however, could be part of the explanation of how AcP10 increases the risk of prostate cancer. Shen and colleagues (Wang et al., 2009) showed that Nkx3.1-positive cells are also potential cells of origin of prostate cancer in a Pten precursor cell, an idea also suggested by Wasserman and colleagues (Wasserman et al., 2010). We carried out immunostaining with an anti-Myc antibody and found that Myc protein was expressed widely in the prostate epithelium (data not shown), consistent with the findings of Wasserman and colleagues (Wasserman et al., 2010). Although there was some overlap with AcP10 expression, the broad distribution of Myc made it impossible to determine whether this overlap occurred by chance. Clearly, because AcP10 is expressed in few Myc-positive cells, it is not likely to be a principal determinant of Myc expression in the prostate. Nevertheless, AcP10 might have a quantitative effect on Myc expression in a subset of cells. Such an effect could be sufficient to predispose such cells to oncogenic transformation and thus explain the elevated risk of prostate cancer associated with the rs6983267 genotype.

Highly conserved, non-coding elements such as AcP10 are thought to be derived from retroposons (Bejerano et al., 2006). Although the function of such elements is unclear, at least some undergo exaptation, a process by which they are co-opted as participants in a cellular regulatory process that is beneficial to the host organism (Bejerano et al., 2006). Such a process might include the acquisition of an ability to regulate gene expression in a tissue-specific manner and thus control cellular identity. AcP10 could be such an element, and AcP10-expressing cells could have a distinct identity and set of properties, perhaps related to prostate function or development. A key unanswered question is whether the association of AcP10 with oncogenesis is related to such a function in normal prostate development, or is a consequence of a fortuitous ability of AcP10 to direct gene expression to the prostate luminal epithelium.

**METHODS**

**Generation of DNA constructs for microinjection**

An AcP10 (chr8:128482318-128483856; 1538 bp) fragment was released from the AcP10/tk-luc plasmid (Jia et al., 2009) by SacII restriction endonuclease digestion, blunt-filled with Quick Blunting Kit, followed by subcloning into the Smal site of pKSBlue-hsp-lacZ vector. Sequencing revealed the proper insertion in the forward direction of the pKSBlue-hsp-lacZ vector.

**Production and genotyping of transgenic mice**

AcP10-pKSBlue-hsp-lacZ transgenic mouse embryos and lines were generated in the Norris Cancer Center Transgenic/Knockout Mouse Core by pronuclear injection as described by Liu et al. (Liu et al., 1994). DNA was prepared from mouse tails or embryo yolk sacs and used for genotyping of the lacZ allele by PCR as described (Kwang et al., 2002).

**Pten prostate cancer model**

cPten$^{-/-}$; AcP10-pKSBlue-hsp-lacZ mice were generated by crossing of the prostate-specific Pten homozygous-deletion mice with AcP10-pKSBlue-hsp-lacZ transgenic mice. The parental male
DMM

Disease Models & Mechanisms

TRANSLATIONAL IMPACT

Clinical issue
The genetic risk factors for prostate cancer remain poorly characterized. 8q24 is a genomic region that has recently been found to have a highly significant association with prostate cancer risk. 8q24 is a gene desert, and the molecular mechanisms by which it might increase cancer risk are unclear. Previous work by this group showed that a risk-associated single nucleotide polymorphism (SNP) within 8q24 (rs6983267) lies within a genomic region with chromatin marks that are associated with active enhancers. This region, designated AcP10, was found to have enhancer activity in cultured cells.

Results
Now, the authors report on the function of AcP10 in vivo to further elucidate how this region confers prostate cancer risk. They show that a 1.5 kb fragment spanning the risk-associated SNP drives reporter gene expression in a subset of luminal epithelial cells in the mouse prostate. These cells have characteristics of a recently identified population of stem cells, in that they remain after castration and express the marker gene Nkx3.1. Other work has shown that castration-resistant Nkx3.1-expressing cells have stem-like properties and are a cell of origin for prostate tumors in a Pten knockout mouse model.

Implications and future directions
This work documents that AcP10 is an enhancer that can drive reporter expression in a population of putative stem cells in the prostate. Identifying the genes and processes regulated by AcP10 might help to explain how prostate tumors develop, as well as aid in the development of interventions that reduce prostate cancer risk or treat the disease in patients.

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8q24 enhancer and prostate stem cells

mouse harboring the probasin-Cre (PB-Cre4) transgene and floxed Pten (I Pten) allele was described previously (Wang et al., 2003).

Histology and immunostaining
Mouse prostatest were embedded in OCT medium (Histoprep; Fisher Scientific) before sectioning. Analysis of β-gal activity on frozen sections was carried out as described (Ishii et al., 2003). Immunofluorescence was performed using chicken anti-E. coli β-gal (ab9361; Abcam), rabbit anti-mouse CK5 (AF 138; Covance), rat anti-mouse CK8 (TROMA-1 antibody; Developmental Studies Hybridoma Bank, University of Iowa), and rabbit anti-mouse Nkx3.1 (a kind gift from Cory Abate-Shen, Columbia University, NY) diluted in goat serum at 4°C overnight. Detection of primary antibody of β-gal, CK5 and CK8 was performed by incubating rhodamine/FITC-labeled goat anti-chicken/rabbit/rat IgY/G (1:100) for 1 hour at room temperature followed by DAPI counterstaining. Detection of Nkx3.1 signal using TSA Plus Fluorescence Systems (PerkinElmer) was carried out as described (Wang et al., 2009).

Statistics
We counted the number of foci that expressed AcP10–β-gal and/or Nkx3.1. Foci contained an average of 15 cells. The total number of ‘foci’, including expressing and non-expressing cells, was determined by counting the total cell number in a given area and extrapolating to the entire slide. Total cell number was divided by the average size of a focus to give the total number of foci. At least 20 sections were counted for each slide, which was derived from one mouse. A total of four mice were counted. The percent of cells/foci expressing Nkx3.1 was compared between foci, which were positive vs negative for AcP10–β-gal. The Pearson chi-square test (based on the 2×2 table) was used to measure the strength of this difference within each slide. To obtain an ‘overall’ P-value across the four mice, the Mantel-Haenszel chi-square test was used.

ACKNOWLEDGEMENTS
We thank Nancy Wu and Youzhen Yan of the USC/Norris Transgenic/Knockout Mouse Core for their expert assistance.

COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
B.F., P.R.B., G.A.C. and R.M. conceived the approach and prepared or edited the manuscript prior to submission. S.G. performed statistical analysis. M.-C.T., C.-P.L., C.Y. and L.J. performed experiments or contributed reagents.

FUNDING
This work was supported by grants from the Whittier Foundation (’Enhancers at 8q24 and Cancer Risk’ to R.M. and G.A.C.); and the National Institutes of Health [R01DE016320 and R01DE019650 to R.M.; R01CA136924, R01CA109147 and U19CA148537 to G.A.C.; and R01CA59705 and P30CA14089 to P.R.B.].

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