The NANCI–Nkx2.1 gene duplex buffers Nkx2.1 expression to maintain lung development and homeostasis

Michael J. Herriges,1 David J. Tischfield,2,3 Zheng Cui,4 Michael P. Morley,4 Yumiao Han,5 Apoorva Babu,4 Su Li,4 MinMin Lu,4 Isis Cendan,3 Benjamin A. Garcia,5 Stewart A. Anderson,2,3 and Edward E. Morrissey4,6,7,8

1Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA; 2Neuroscience Graduate Group, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA; 3Department of Psychiatry, Children’s Hospital of Philadelphia, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA; 4Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA; 5Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA; 6Penn Center for Pulmonary Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA; 7Penn Cardiovascular Institute, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA; 8Penn Institute for Regenerative Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

A subset of long noncoding RNAs (lncRNAs) is spatially correlated with transcription factors (TFs) across the genome, but how these lncRNA–TF gene duplexes regulate tissue development and homeostasis is unclear. We identified a feedback loop within the NANCI [Nkx2.1-associated noncoding intergenic RNA]–Nkx2.1 gene duplex that is essential for buffering Nkx2.1 expression, lung epithelial cell identity, and tissue homeostasis. Within this locus, Nkx2.1 directly inhibits NANCI, while NANCI acts in cis to promote Nkx2.1 transcription. Although loss of NANCI alone does not adversely affect lung development, concurrent heterozygous mutations in both NANCI and Nkx2.1 leads to persistent Nkx2.1 deficiency and reprogramming of lung epithelial cells to a posterior endoderm fate. This disruption in the NANCI–Nkx2.1 gene duplex results in a defective perinatal innate immune response, tissue damage, and progressive degeneration of the adult lung. These data point to a mechanism in which lncRNAs act as rheostats within lncRNA–TF gene duplex loci that buffer TF expression, thereby maintaining tissue-specific cellular identity during development and postnatal homeostasis.

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Long noncoding RNAs [lncRNAs], RNA transcripts ≥200 nucleotides that do not encode a functional protein, have been shown to regulate a wide variety of biological processes [Wapinski and Chang 2011; Li and Chang 2014; Meller et al. 2015; Li et al. 2016]. Despite growing evidence that lncRNAs play important roles in regulating gene expression, their role in tissue development and homeostasis remains unclear [Li and Chang 2014; Feyder and Goff 2016; Perry and Ulitsky 2016]. Recent work from our laboratory and others has identified a class of lncRNA that are spatially located near critical transcription factor [TF] genes [Guttman et al. 2009; Ulitsky et al. 2011; Herriges et al. 2014]. These TF–lncRNA duplexes can be further divided into those that reside upstream of and embedded within the regulatory region of TFs or those that reside downstream from and outside the proximal promoter and regulatory elements of the TF gene. This spatial localization is conserved across species, with mouse and human TF–lncRNA duplexes exhibiting common syntenic locations in the genome, which suggests a critical regulatory function for regulating gene expression.

In both mice and humans, the lncRNA NANC [Nkx2.1-associated noncoding intergenic RNA] is located downstream from the TF Nkx2.1, a major regulator of pulmonary development and homeostasis [Herriges et al. 2014]. Inactivation of Nkx2.1 during development leads to dramatic loss in branching morphogenesis along with defective lung epithelial lineage differentiation [Minoo...
et al. 1995, 1997), while postnatal loss of Nkx2.1 expression results in defects associated with a loss of lung epithelial homeostasis and respiratory failure (Snyder et al. 2013). Despite the importance of maintaining Nkx2.1 expression in the lung, little is known about how its expression is regulated (Herriges and Morrisey 2014).

Here we show that within the NANCI-Nkx2.1 duplex, NANCI plays an essential role in regulating tissue identity by acting as a transcriptional rheostat to buffer Nkx2.1 expression. During lung development and in adult lung homeostasis, NANCI acts in cis to positively regulate Nkx2.1, and, in turn, Nkx2.1 directly inhibits NANCI expression. Together, this generates a negative feedback loop between NANCI and Nkx2.1, which buffers against dramatic reductions in Nkx2.1 expression. NANCI expression is also controlled through interactions with Hnrnpab and Hnrnpd, which promote turnover of the NANCI transcript to modulate NANCI expression and, in turn, Nkx2.1 expression. Surprisingly, loss of NANCI expression by itself has a minimal impact on lung development and homeostasis. However, concurrent in trans mutations of both NANCI and Nkx2.1 disrupt the buffering loop, resulting in persistent Nkx2.1 deficiency. This persistent loss of Nkx2.1 expression leads to defects in the perinatal innate immune system and progressive lung degeneration due to a loss of lung epithelial cell identity and cellular reprogramming to a posterior endoderm fate. Together, these findings establish a new paradigm for TF-lncRNA duplexes in which lncRNAs act as rheostats to buffer expression of critical TFs to maintain cell fate and normal tissue homeostasis.

Results

Expression of NANCI in a subset of Nkx2.1+ cells during lung, brain, and thyroid development

To fully examine the expression of NANCI in a cell lineage-specific fashion as well as the consequences of loss of NANCI function, we generated a NANCI reporter line (NANCIcreERT2:RFP) that also disrupts expression of NANCI (Fig. 1A). The NANCIcreERT2:RFP allele contains a polyadenylated cassette encoding a tamoxifen-inducible cre recombinase (creERT2) linked to a TdTomato red fluorescent protein (RFP) that replaces much of exon 1 in the NANCI locus, including the splice donor site. This allows for the isolation and characterization of NANCI-expressing cells, including tamoxifen-inducible lineage tracing.
The reporter construct was inserted into a region lacking H3K4me1 or DNase hypersensitivity peaks, suggesting that the insertion is not directly influencing a genomic enhancer region (Supplemental Fig. 1A; The ENCODE Project Consortium 2012). The Nanci<sup>creERT2:RFP</sup> reporter line exhibits broad expression of RFP throughout the lung epithelium, consistent with previous Nanci in situ hybridizations expression patterns (Fig. 1B; Herriges et al. 2014). However, in both embryonic and adult lungs, we identified a subset of Nkx2.1<sup>+</sup> cells that expressed significantly lower or no detectable RFP (Fig. 1C,D white arrowheads). In contrast, we were unable to find any RFP/<sup>Nkx2.1</sup><sup>+</sup> cells, suggesting that Nanci is not expressed in cells lacking Nkx2.1 expression.

Outside of the lung, Nanci and Nkx2.1 are coexpressed in the forebrain and thyroid (Herriges et al. 2014). To characterize Nanci expression in the brain, we compared RFP and Nkx2.1 expression at embryonic day 12.5 (E12.5) and E18.5. At E12.5, Nanci was expressed in the medial ganglionic eminence (MGE), anterior entopeduncular/preoptic area (PoA), hypothalamus, and developing globus pallidus (Supplemental Fig. 1B). This expression pattern overlapped with that of Nkx2.1. At E18.5, Nanci continued to be expressed in most Nkx2.1<sup>+</sup> cells of the hypothalamus and globus pallidus. In the thyroid, Nanci expression was restricted to the Nkx2.1<sup>+</sup> epithelium (Supplemental Fig. 2A). In the lung, Nanci expression was more variable than Nkx2.1 expression.

**Nanici is preferentially expressed in alveolar type 2 (AT2) and secretory epithelial cells in the mature lung**

To further explore the expression of Nanici and how it relates to cell lineage identity in the lung, we used fluorescence-activated cell sorting (FACS) to isolate adult lung epithelial cells from Nanci<sup>creERT2:RFP</sup>/+ mice based on RFP expression (Supplemental Fig. 3A). This sorting generated three populations [RFP-high, RFP-med, and RFP-low], which were further analyzed by quantitative real-time PCR (qPCR) to measure RFP, Nanici, and Nkx2.1 expression (Supplemental Fig. 3B). The expression of Nanici as well as Nkx2.1 correlated with RFP expression from the Nanci<sup>creERT2:RFP</sup>/+ line. This suggests that the Nanci<sup>creERT2:RFP</sup> reporter accurately reflects the variations in Nanici expression, which in turn correlates with Nkx2.1 expression.

Gene expression analysis of the RFP-high population reveals that it is composed primarily of Sftp3<sup>+</sup> AT2 cells and, to a lesser extent, Scgb1a1<sup>+</sup> secretory cells (Fig. 1E). Foxj1<sup>+</sup> ciliated cells and Pdpn<sup>+</sup> AT1 cells are restricted primarily to the RFP-med and RFP-low populations. Analysis of these cell types by immunohistochemistry (IHC) verifies that AT2 and secretory cells [Fig. 1F, yellow arrows] express higher levels of RFP than neighboring epithelial cells [Fig. 1F, white arrowheads]. Likewise, ciliated and AT1 cells [Fig. 1F, yellow arrows; Supplemental Fig. 3E, yellow arrows] exhibit lower expression of RFP than neighboring epithelial cells [Fig. 1F, white arrowheads]. Although they were not detectable by qPCR, neuroendocrine cells also express RFP at higher levels than the surrounding epithelial cells (Supplemental Fig. 3E). The majority of goblet and basal cells, found in mainstem bronchi, has negligible RFP expression; however, there were rare RFP-positive basal cells (Supplemental Fig. 3E) FACS of epithelial cells using an Nkx2.1<sup>GFP</sup> reporter line likewise reveals three distinct populations of Nkx2.1-expressing lung epithelium (Fig. 1G; Supplemental Fig. 3C,D). However, unlike Nanici expression, Nkx2.1 expression does not clearly separate the lung epithelial cell lineages examined by qPCR or IHC [Fig. 1H; Supplemental Fig. 3F]. These data suggest that Nanici expression levels, but not Nkx2.1 expression, can delineate epithelial cell lineages in the lung, with the highest levels of Nanici expression found in AT2 and secretary cells.

**Nanici expression is essential for normal levels of Nkx2.1 expression**

Our previous work suggested that shRNA-mediated knockdown of Nanici leads to decreased Nkx2.1 expression (Herriges et al. 2014). Therefore, we analyzed expression of Nanici and Nkx2.1 in Nanci<sup>creERT2:RFP/creERT2:RFP</sup> homozygous mice. At E18.5, Nanci<sup>creERT2:RFP/creERT2:RFP</sup> mice show an almost complete loss of Nanici expression by qPCR and in situ hybridization (Fig. 2A; Supplemental Fig. 4B). This reduced Nanici expression resulted in decreased expression of Nkx2.1 and known target genes, including Abca3, Sftpa, and Sftpc, while genes not directly regulated by Nanici [Pdpn and Foxj1] were unaffected (Supplemental Fig. 4A). In adult mice, loss of Nanici results in a 24% reduction in Nkx2.1 expression, which did not lead to significant changes in the expression of Nkx2.1 target genes. To determine whether expression of other genes is significantly affected upon loss of Nanici expression, we used RNA sequencing [RNA-seq] to compare gene expression in E18.5 wild-type and Nanci<sup>creERT2:RFP/creERT2:RFP</sup> lung epithelial cells. Surprisingly, Nanici was the only gene identified as having significantly different expression, suggesting that the moderate effect that loss of Nanici has on Nkx2.1 expression does not result in a dramatic effect on overall lung epithelial gene expression.

Despite reduced Nkx2.1 expression, Nanici<sup>creERT2:RFP/creERT2:RFP</sup> mutants display no discernable morphological defects at E18.5 or 10 wk of age (Supplemental Fig. 4C). This is consistent with the fact that Nkx2.1<sup>−/−</sup> mice have similarly reduced Nkx2.1 expression levels but no clear morphological defects at these stages. Thus, while Nanici promotes Nkx2.1 expression during lung development, loss of Nanici alone does not reduce Nkx2.1 expression sufficiently to induce detectable morphological defects.

**Nanici and Nkx2.1 form a negative feedback loop that buffers Nkx2.1 expression**

To test whether Nkx2.1 regulates Nanici expression, we analyzed Nanici expression in Nkx2.1<sup>GFP</sup>/+ mice, in which one Nkx2.1 promoter drives expression of GFP...
instead of the Nkx2.1 transcript [Longmire et al. 2012]. At E18.5, these mice have a 25% increase in expression of NANC1, indicating that Nkx2.1 inhibits NANC1 expression [Fig. 2B]. Likewise, NANC1 expression in Nkx2.1<sup>GFP/+</sup> mice increases twofold and threefold at 10 wk and 6 mo of age, respectively. This increase in NANC1 expression with age correlates with increased relative Nkx2.1 expression such that Nkx2.1<sup>GFP/+</sup> mice express wild-type levels of Nkx2.1 by 6 mo. Analysis of NANC1 and Nkx2.1 expression in the brains of Nkx2.1<sup>GFP/+</sup> mice indicates that a similar feedback loop exists in the brain [Supplemental Fig. 1C]. Furthermore, using chromatin immunoprecipitation (ChIP):qPCR [ChIP: PCR], we found that Nkx2.1 binds to a predicted Nkx2.1-binding site within the NANC1 promoter, suggesting that Nkx2.1 directly inhibits NANC1 expression [Supplemental Fig. 5C,D]. This is consistent with the ability of Nkx2.1 to directly repress expression of genes, including Nrp2 [Nobrega-Pereira et al. 2008]. Together, this indicates that NANC1 and Nkx2.1 form a negative feedback loop that can buffer Nkx2.1 expression, especially in situations of a compromised Nkx2.1 locus, such as in Nkx2.1<sup>GFP/+</sup> mice.

To further test this buffering feedback loop, we generated mice with concurrent in <i>trans</i> mutations of NANC1 and Nkx2.1 [NANC1<sup>creERT2:RFP/+:Nkx2.1<sup>GFP/+</sup></sup>]. If NANC1 acts in <i>cis</i>, NANC1<sup>creERT2:RFP/+:Nkx2.1<sup>GFP/+</sup></sup> mice should display a further reduction in Nkx2.1 expression. Indeed, at E18.5 and 10 wk and 6 mo of age, NANC1<sup>creERT2:RFP/+:Nkx2.1<sup>GFP/+</sup></sup> mice had a significant reduction in Nkx2.1 expression relative to the other genotypes [Fig. 2B]. Analysis of AT2-enriched and AT2-depleted epithelial cell populations in adult NANC1<sup>creERT2:RFP/+:Nkx2.1<sup>GFP/+</sup></sup> mice indicates that Nkx2.1 is decreased in both cell populations [Fig. 5A,B]. This suggests that reduced Nkx2.1 expression reflects decreased gene expression in multiple epithelial cell types and not just a loss of Nkx2.1-high AT2 cells. At 6 mo, NANC1<sup>creERT2:RFP/+:Nkx2.1<sup>GFP/+</sup></sup> mice also have increased expression of GFP, which is expressed from the Nkx2.1 locus in <i>cis</i> to the remaining

Figure 2. NANC1 and Nkx2.1 form a lncRNA–TF duplex that buffers Nkx2.1 expression. (A) NANC1<sub>creERT2:RFP/+:Nkx2.1<sub>GFP/+</sub></sub> mice have reduced expression of NANC1 and Nkx2.1 at E18.5 and 10 wk of age. (B) In contrast, Nkx2.1<sup>GFP/+</sup> mice have increased NANC1 expression, indicating that Nkx2.1 inhibits NANC1. Thus, NANC1 and Nkx2.1 form a negative feedback loop that buffers Nkx2.1 expression. (C) Increased expression of GFP and RFP in 6-mo-old NANC1<sub>creERT2:RFP/+:Nkx2.1<sub>GFP/+</sub></sub> mice. (D) These data suggest a model in which NANC1 acts in <i>cis</i> to regulate Nkx2.1 expression. (E) Spatial classification of lncRNA–TF based on genomic orientation of the lncRNA and TF identifies other duplexes with the lncRNA located downstream from the TF, similar to the NANC1–Nkx2.1 duplex. (F,G) Knockdown of Hnrnpab and Hnrnpd, but not Hnrnpa2b1, results in increased expression of NANC1 and Nkx2.1, suggesting that Hnrnpab and Hnrnpd inhibit NANC1 stability. E18.5 RNA was extracted from EpCAM<sup>+</sup> lung cells, while 10-wk and 6-mo samples were collected from whole lungs. For A, box and whisker plots for E18.5 and 10 wk were derived from <i>n</i> = (5,6) and <i>n</i> = (5,5) biological replicates, respectively. For B and C, box and whisker plots for E18.5, 10 wk, and 6 mo were derived from <i>n</i> = (5,11,7,5), <i>n</i> = (6,9,5,8), and <i>n</i> = (9,5,8,8) biological replicates, respectively. For F, means ± SEM were derived from <i>n</i> = (6,6,5,6) biological replicates. *<i>P</i> < 0.05; **<i>P</i> < 0.01; ***<i>P</i> < 0.001; ****<i>P</i> < 0.0001, two-tailed Student’s t-test.
To determine the effects of disrupting the NANCI-Nkx2.1 buffering loop during development, we analyzed pulmonary morphology and gene expression at E18.5 and postnatal day 4 (P4). At E18.5, both Nkx2.1<sup>1<sup> GFP/</sup> and NANCI<sup>creERT2:RFP/</sup>-Nkx2.1<sup>1<sup> GFP/</sup> mutants have mild morphological defects in the developing alveolar space, as indicated by extended distal airspaces that lacked much of the alveolar septation found in wild-type mice (Fig. 3A). Moreover, at this time point, both Nkx2.1<sup>1<sup> GFP/</sup> and NANCI<sup>creERT2:RFP/</sup>-Nkx2.1<sup>1<sup> GFP/</sup> mutants have reduced expression of markers for several lung epithelial cell lineages, indicative of delayed pulmonary development (Fig. 3B,C). Nkx2.1<sup>1<sup> GFP/</sup> and NANCI<sup>creERT2:RFP/</sup>-Nkx2.1<sup>1<sup> GFP/</sup> mutants exhibit ectopic expression of Hnf4a, a TF expressed in gastric epithelium, consistent with previous work suggesting that Nkx2.1 represses a gastric transcriptional program (Snyder et al. 2013).

By P4, Nkx2.1<sup>1<sup> GFP/</sup> mice express normal levels of many lung epithelial markers except Sfpc, suggesting compensatory mechanisms for maintaining Nkx2.1 expression in the perinatal period (Fig. 3D–F). In contrast, the NANCI<sup>creERT2:RFP/</sup>-Nkx2.1<sup>1<sup> GFP/</sup> mutants exhibit alveolar simplification and decreased expression of lineage markers for AT2, secretory, multiciliated, and AT1 cells (Fig. 3F). At P4, Hnf4a expression had increased dramatically in NANCI<sup>creERT2:RFP/</sup>-Nkx2.1<sup>1<sup> GFP/</sup> mutants, suggesting increased activation of ectopic posterior endoderm transcriptional pathways in these mutants due to persistent Nkx2.1 deficiency. These data suggest that without NANCI in cis, the functional Nkx2.1 allele is unable to normalize perinatal Nkx2.1 expression.

In addition to alveolar simplification and developmental delay, NANCI<sup>creERT2:RFP/</sup>-Nkx2.1<sup>1<sup> GFP/</sup> mutants at P4 display a localized severe perinatal inflammatory response. This response is exemplified by engorged distal airspaces filled with a mixture of proteinaceous debris and immune cells (six out of 13 by flow or histological analysis) (Fig. 4A). Flow analysis indicates a dramatic influx of neutrophils in NANCI<sup>creERT2:RFP/</sup>-Nkx2.1<sup>1<sup> GFP/</sup> mutants at P4 (Fig. 4B). These mutants display gaps in the epithelial barrier in both the proximal and distal airways (Fig. 4C, white arrowheads) as well as E-cadherin-positive cells in the airway lumen (Fig. 4C, yellow arrows). This suggests a loss of epithelial barrier integrity, which likely contributes to the severe inflammatory response.

To better characterize the causes of this perinatal phenotype, we used RNA-seq to compare gene expression in wild-type, Nkx2.1<sup>1<sup> GFP/</sup> and NANCI<sup>creERT2:RFP/</sup>-Nkx2.1<sup>1<sup> GFP/</sup> lung epithelium at E18.5, prior to evidence of an immunological response (Supplemental Fig. 6A). By gene ontology (GO) analysis, NANCI<sup>creERT2:RFP/</sup>-Nkx2.1<sup>1<sup> GFP/</sup> mutants displayed altered expression of several gene categories, including increased expression of genes involved in mitosis and decreased expression of genes involved in regulation of immune response (Fig. 4D, Supplemental Table 3). Increased mitosis was verified by Ki67 staining, which indicated increased proliferation of AT2 and secretory cells but not AT1 or ciliated cells (Supplemental Fig. 6B). Combined with the reduced expression of lung epithelial lineage markers, these data suggest that the lung epithelium in NANCI<sup>creERT2:RFP/</sup>-Nkx2.1<sup>1<sup> GFP/</sup> mutants is more proliferative but immature compared with wild-type controls or Nkx2.1<sup>1<sup> GFP/</sup> mutants.

In the immune response categories, expression of colony-stimulating factor 2 (Csf2) was significantly
decreased in \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mutants. Cs2 is a key regulator of epithelial barrier function and a cytokine that promotes granulocyte and monocyte maturation (Overgaard et al. 2015; Whitsett and Alenghat 2015). Cs2 deficiency leads to pulmonary alveolar proteinosis (PAP), in which the pulmonary airspace is filled with noncellular debris, similar to the phenotype observed in \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mutants at P4. qPCR verified that Cs2 is significantly down-regulated in Nkx2.1GFP/+ and \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mutants at E18.5 but only in \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mice at P4 (Fig. 4E). Nkx2.1 binds to the Cs2 promoter, suggesting that Nkx2.1 directly activates Cs2 expression in the maturing pulmonary epithelium [Fig. 4G,H].

Despite reduced expression of Cs2, perinatal \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mutant lungs contain a large population of neutrophils, suggesting up-regulation of other cytokines that recruit neutrophils. Using our RNA-seq data as well as qPCR, we found that the cytokine secreted phosphoprotein 1 (Spp1), also known as osteopontin, was up-regulated in \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mutants at E18.5 (Fig. 4F). Spp1 promotes recruitment of neutrophils, consistent with the increased neutrophil population in \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mutant lungs (Zissler et al. 2016). However, we did not observe Nkx2.1 binding to the Spp1 promoter, suggesting that up-regulation of Spp1 is a secondary response to the barrier defects in these lungs [Fig. 4G,H, Supplemental Table 4]. This is consistent with previous Nkx2.1 ChIP-seq (ChIP combined with high-throughput sequencing) data showing that Nkx2.1 binds to the Cs2 promoter but not the Spp1 promoter [Snyder et al. 2013]. Taken together, these data suggest that the perinatal phenotype observed in \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mutants results in part from multiple defects, including Cs2 deficiency, elevated Spp1 expression, and a loss of epithelial barrier integrity.

Despite the profound perinatal phenotype seen in \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mutants, these animals survive to at least 4 wk of age, when the lung reaches maturity. Unlike the earlier time points, at 4 wk of age, Nkx2.1 expression is not significantly different between

\begin{figure}
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\caption{Disruption of the \textit{NANCI}–Nkx2.1 buffering loop leads to persistent developmental delay. (A) At E18.5, \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mice have a mild morphological defect in the developing alveolar space, as noted by thickened septae in hematoxylin and eosin (HE)-stained tissue. (B) IHC for cell type-specific markers showing reduced expression of secretory (Sgb1a1) and AT2 (Sftpc) cell markers. (C) At E18.5, both Nkx2.1GFP/+ and \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mice exhibit decreased expression of several cell type-specific markers and ectopic expression of the posterior endoderm marker Hnf4a. (D) By P4, only \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ mice exhibit significant alveolar simplification in HE-stained tissue. (E) IHC for cell type-specific markers showing reduced expression of secretory (Sgb1a1) and AT2 (Sftpc) cell markers at P4. (F) At P4, only \textit{NANCI}creERT2.RFP/+:Nkx2.1GFP/+ lungs exhibit reduced expression of the pulmonary cell type markers and ectopic expression of Hnf4a. In contrast, Nkx2.1GFP/+ mice have largely normalized development. Bars: A, D, 200 µm; B, E, 25 µm. RNA was extracted from EpCAM+ lung cells. For C and F, box and whisker plots were derived from n = (5,11,7,5) and n = (6,5,7,5) biological replicates, respectively. [*] P < 0.05; [**] P < 0.01; [***] P < 0.001; [****] P < 0.0001, two-tailed Student’s t-test.}
\end{figure}
Nkx2.1GFP/+ and NANCICreERT2:RFP/+:Nkx2.1GFP/+ lungs (Supplemental Fig. 7A). This increase in Nkx2.1 expression is not accompanied by increased expression in NANC1, suggesting that a NANC1-independent mechanism is responsible for the normalization of Nkx2.1 expression. By 4 wk of age, the exogenous immune cells and PAP phenotype have also resolved, and lung morphology is similar to that of wild-type lungs (Supplemental Fig. 7B). Furthermore, expression of pulmonary cell-type markers has largely normalized to wild-type levels in these mutants (Supplemental Fig. 7C). At this stage, Csf2 expression has increased and Spp1 expression has decreased in NANCICreERT2:RFP/+:Nkx2.1GFP/+ lungs, countervailing the earlier altered expression of these cytokines and correlating with a resolution of the innate immune response observed in the perinatal period.

Disruption in the NANC1–Nkx2.1 gene duplex leads to a failure of pulmonary homeostasis and reprogramming of lung epithelium to a gastric endoderm fate

While NANCICreERT2:RFP/+:Nkx2.1GFP/+ mutants were relatively normal at 4 wk of age, adult 10-wk-old mutants began to exhibit inflammation of the large airways as well as basal and mucous cell metaplasia (Fig. 5A). This phenotype was accompanied by Nkx2.1 deficiency, consistent with the known role of Nkx2.1 in inhibiting airway inflammation (Maeda et al. 2011). We also observed reduced expression of Scgb1a1, a marker of the airway secretory cell lineage (Fig. 5G). In contrast to the perinatal period, the inflammatory reaction at 10 wk was not associated with significant alterations in Csf2 or Spp1 expression (Fig. 5G).

Alveolar simplification and bronchiolization of the alveoli were observed in NANCICreERT2:RFP/+:Nkx2.1GFP/+ mutants at 10 wk (Fig. 5A, black arrowheads). Bronchiolization of the alveoli is characterized by cells resembling bronchiolar epithelium lining normal or thickened alveolar walls [Wang et al. 2009]. These bronchiolized distal alveoli consist primarily of Sox2+/Scgb1a1+ cells, suggesting that they represent airway secretory cells [Fig. 5B]. While we did not observe any Sox2+/Pdpn+ AT1 cells or Sox2+/Foxj1+ multiciliated epithelial cells, there were rare Sox2+/Sftpc+ and Scgb1a1+/Sftpc+ cells [Fig. 5C–F]. At 10 wk of age, there is also a resurgence of ectopic Hnf4a expression in NANCICreERT2:RFP/+:Nkx2.1GFP/+ mutants.
Pdpn and Scgb1a1 expression is decreased in NANCI\textsuperscript{creERT2:RFP/+::Nkx2.1\textsuperscript{GFP/+}} mutants at 10 wk, indicating disruption in the NANCI–Nkx2.1 regulatory gene duplex and defects in AT1 and secretory cell homeostasis (Fig. 5G).

Six-month-old NANCI\textsuperscript{creERT2:RFP/+::Nkx2.1\textsuperscript{GFP/+}} mutants exhibit further progression of the phenotypes observed at earlier stages. RNA-seq analysis of the lung epithelial cells at 6 mo of age revealed decreased expression of genes involved in respiratory and epithelial development but increased expression of genes involved in digestion, confirming the reprogramming of pulmonary epithelial cells to a posterior endoderm fate (Fig. 6A,B; Supplemental Fig. 8A,B). At this time, airway inflammation has resolved, but there is a dramatic loss of pulmonary secretory cells and ectopic expansion of goblet [Muc5ac\textsuperscript{+}] and basal [Krt5\textsuperscript{*}/Trp63\textsuperscript{*}] cells (Fig. 6C; Supplemental Fig. 8C). The bronchiolization of the alveolar region has progressed concomitantly with a sharp decrease in expression of the AT2 marker Sftpc (Fig. 6C; Supplemental Fig. 8B). The bronchiolized alveolar region contains epithelium bearing markers of secretory and ciliated cells of the proximal airways as well as ectopic Krt5\textsuperscript{*}/Trp63\textsuperscript{*} cells in the distal airspaces, suggesting either loss of alveolar epithelium and extension of airway basal cells into the alveolar space or a reprogramming of alveolar epithelium into a basal cell phenotype [Fig. 6D–F; Supplemental Fig. 8C]. Importantly, this bronchiolized epithelium expresses high levels of the Hnf4a P2 isoform, but not the P1 isoform, characteristic of stomach epithelial cells (Supplemental Fig. 8D; Czaplinski et al. 2005). This ectopic Hnf4a expression is not found in the airways of the lung, suggesting that the gastric reprogramming is specific to the bronchiolized alveoli (Supplemental Fig. 8E). Importantly, these bronchiolized regions contain Sox9\textsuperscript{*}/Hnf4a\textsuperscript{*} cells, resembling gastric stem cells (Fig. 6G,H). Additional markers of gastric endoderm, including Lgr5, Vsig1, Lgals4, and Ctse, are also dramatically increased in NANCI\textsuperscript{creERT2:RFP/+::Nkx2.1\textsuperscript{GFP/+}} mutants at 6 mo (Fig. 6H). The NANCI\textsuperscript{creERT2:RFP/+::Nkx2.1\textsuperscript{GFP/+}} peripheral epithelium is highly apoptotic and degenerative at this stage (Supplemental Fig. 8F). This correlates with increased expression of immune response genes and recruitment of macrophages to clear cellular debris in the airways (Fig. 6B, Supplemental Fig 8G,H). These molecular and cellular changes result in a dramatic loss of alveolar structure in NANCI\textsuperscript{creERT2:RFP/+::Nkx2.1\textsuperscript{GFP/+}} mutants at this time point (Fig. 6C). These phenotypes are in contrast to all of the other genotypes examined, including Nkx2.1\textsuperscript{GFP/+} mice, whose gene expression profile and
morphology are indistinguishable from those of wild-type mice (Fig. 6C; Supplemental Fig. 8B–D,F). At these time points, the adult brain showed no major morphological defects (data not shown). However, the thyroids of both NANCi<sup>creERT2:RFP/−/Nkx2.1<sup>CreERT2,RFP/+</sup> and NANCi<sup>creERT2:RFP/−/Nkx2.1<sup>CreERT2,RFP/+</sup> mice have up-regulation of genes involved in digestion and innate immune response but decreased expression of genes involved in epithelial and respiratory morphogenesis. The second column of this table reflects genes affected out of total genes in each category. (C, top row) By 6 mo, inflammation around the large airways has receded, leaving behind fibrous tissue (HE staining). The airway epithelium has reduced expression of the secretory cell marker Scgb1a1 but expanded expression of basal [p63 and Krt5] and goblet [Muc5ac] cell markers. (Bottom row) Normal alveolar architecture is highly disrupted in NANCi<sup>creERT2:RFP/−/Nkx2.1<sup>CreERT2,RFP/+</sup> mice (HE staining). (D–G) The distal bronchiolized epithelium also includes ciliated cells [D], basal-like cells [E, F], and Sox9<sup>−/−</sup>, Hnf4a<sup>−/−</sup> cells [G]. (H) qPCR for gastric markers. Bars: C, 200 µm; D–G, 25 µm. RNA for RNA-seq was extracted from purified epithelial cells, while RNA for qPCR was extracted from whole-lung samples. For H, box and whisker plots were derived from n = [8,3,5,5] biological replicates. (*) P < 0.05; (**) P < 0.01; (***) P < 0.001; (****) P < 0.0001, two-tailed Student’s t-test.

**Discussion**

Several reports have shown that IncRNAs regulate protein-coding genes in cis; however, few have revealed the in vivo consequences of this regulation [Feyder and Goff 2016; Kotzin et al. 2016; Quinn and Chang 2016]. Genetic inactivation of IncRNAs often resulted in no apparent phenotype, leading to the conclusion that many IncRNAs have little impact on gene regulation and tissue development or that their loss is compensated for by other mechanisms [Eissmann et al. 2012; Zhang et al. 2012; Sauvageau et al. 2013; Kohtz 2014]. The present study reveals that NANCi acts as an important rheostat of Nkx2.1 expression that is necessary for maintaining lung epithelial cell identity and function.
shows that while the loss of the lncRNA NANCI alone
does not affect overall lung development or homeostasis
in a significant manner, NANCI is required to maintain
Nkx2.1 expression. Loss of NANCI’s rheostat function
in situations of compromised Nkx2.1 expression leads to
profound innate immune defects and a reprogramming
of lung epithelium toward a posterior endoderm pheno-
type. The overall effect on adult tissue homeostasis is dra-
matic and provides critical insight into how lncRNAs
function as transcriptional rheostats, which are essential
for buffering neighboring protein-coding genes, including
master TFs such as Nkx2.1.

Previous studies have revealed that lncRNAs are found
near TFs more often than other categories of protein-coding
genes [Guttman et al. 2009; Ulitsky et al. 2011; Herr-
riges et al. 2014]. Interestingly, mutations in TFs
underlie many human congenital haploinsufficient dis-
eases [Dang et al. 2008]. In particular, Nkx2.1 haploinsuf-
ciency causes a rare but often lethal disorder called
brain–lung–thyroid syndrome [BLTS] [Hamvas et al.
2013]. Symptoms in BLTS patients include respiratory
failure along with serious thyroid and neural dysfunction.
However, there is a wide variation in the phenotypes ob-
served in BLTS patients, with many lacking a notable phe-
ton type in any one of the tissues that express Nkx2.1. In
addition to BLTS, alterations in Nkx2.1 have been associ-
ated with different forms of lung cancer [Yang et al. 2012;
Yamaguchi et al. 2013]. Given the critical role for TFs
such as Nkx2.1 in both development and tissue homeo-
stasis, exquisite control of their expression levels is essen-
tial. This fine control likely requires multiple modes of
regulation, but the role of TF-associated lncRNAs in this
system is largely unknown.

While lncRNA expression has been shown to be highly
tissue-specific, characterization of in vivo expression pat-
tterns has been hampered by the lack of reporters and the
inability to use standard antibody protein detection tech-
niques [Cabili et al. 2011]. We overcame these limitations
by using a fluorescent and lineage tracing reporter system
to characterize in detail the expression of NANCI and
compare it at cellular resolution with its neighboring TF
and gene duplex partner, Nkx2.1. We found that NANCI
expression, while highly similar to Nkx2.1, shows cell
lineage-specific differences of expression in embryonic and
adult lungs. The highest levels of NANCI expression are
restricted to AT2 and secretory lineages, suggesting that
NANCI plays a preferential role in the development and
homeostatic maintenance of these epithelial lineages.
NANCI-null mice have reduced expression of Nkx2.1,
verifying previous studies indicating that NANCI pro-
motes Nkx2.1 expression [Herriges et al. 2014]. Surpris-
ingly, Nkx2.1 appears to repress NANCI expression
through directly binding to the NANCI promoter, similar
to how Nkx2.1 directly represses expression of Nrp2, sug-
gest a feedback loop within the NANCI–Nkx2.1 gene
duplex (Fig. 7A; Nobrega-Pereira et al. 2008). The net ef-
effect of this loop is to buffer against drastic changes in
Nkx2.1 expression, as illustrated by the normalization
of Nkx2.1 expression in aging Nkx2.1[1GF/−] mice. Previous
studies suggest that NANCI may regulate Nkx2.1 expres-
sion through the recruitment of Wdr5 and the Trithorax
complex; however, further work is needed to verify these
findings [Herriges et al. 2014]. In addition to the NANCI–
Nkx2.1 feedback loop itself, the proteins Hnrnpab and
Hnrnpd inhibit expression of NANCI, adding an addition-
al check on the expression of NANCI and Nkx2.1. Thus,

**Figure 7.** Disruption of the NANCI–Nkx2.1 feed-
back loop leads to three stages of phenotypic prog-
ression in NANCI(CreERT2:RFP/−);Nkx2.1[1GF/−] mice. (A)
NANCI and Nkx2.1 form a negative feedback loop
that buffers Nkx2.1 expression. In addition, the pro-
teins Hnrnpab and Hnrnpd interact with NANCI
and inhibit its expression, potentially acting as anoth-
er check on NANCI and Nkx2.1 expression. Previous
work also suggests that Wdr5 and the Trithorax com-
plex may mediate NANCI’s ability to regulate
Nkx2.1. (B) In this study, we saw three phenotypic
stages in the NANCI(CreERT2:RFP/−);Nkx2.1[1GF/−] mice,
all of which are dependent on Nkx2.1 expression.
First, persistent Nkx2.1 deficiency leads to a perinatal
developmental delay and ectopic expression of Hnf4a,
leading to an innate immune response [stage I]. Over
the next 4 wk, Nkx2.1 expression is up-regulated,
inducing a normalization of development [stage II]. Fi-
nally, in the adult lung, persistent Nkx2.1 deficiency
leads to degeneration of pulmonary epithelial
lineages and a resurgence of a gastric transcription-
program [stage III].
our studies point to a new model for lncRNA regulatory function in which lncRNAs act as rheostats that buffer expression of neighboring TFs. This model suggests that lncRNAs in such lncRNA–TF duplexes are not an absolute requirement for expression of neighboring TFs but instead act as fine-tuning rheostats that ensure proper TF expression in response to genetic or environmental stress.

Using in vivo loss-of-function models for NANCI and Nkx2.1, we show that loss of NANCI expression leads to reduced Nkx2.1 expression but not major morphological defects. This suggests as yet unknown compensatory mechanisms for regulating Nkx2.1 expression in the absence of NANCI. However, by the end of gestation, both Nkx2.1GFP/+ and NANCIcreERT2:RFP/+:Nkx2.1GFP/+ mice show signs of pulmonary developmental delay (Fig. 7B). Postnatal Nkx2.1GFP/+ mice up-regulate NANCI in a compensatory fashion, which increases Nkx2.1 expression back to wild-type levels and restores normal pulmonary maturation. In contrast, concurrent in trans heterozygous loss of both NANCI and Nkx2.1 leads to persistent Nkx2.1 deficiency at all stages of life. At P4, this deficiency begins to reprogram lung epithelial cell identity, causing ectopic expression of a gastric transcriptional program and delayed development of pulmonary epithelial cells necessary for proper lung homeostasis. The persistent Nkx2.1 deficiency in NANCICreERT2:RFP/+;Nkx2.1GFP/+ aged mutants leads to a severe pulmonary degenerative phenotype by 6 mo, as the normal alveolar structure is replaced by ectopically reprogrammed airway and gastric-like cells. These reprogrammed lung epithelial cells cannot maintain lung alveolar epithelial cell identity and are unlikely to be able to efficiently perform gas exchange. This progressive and degenerative phenotype indicates that while a complete loss of NANCI can be compensated for in the presence of two intact Nkx2.1 alleles, further decrease in Nkx2.1 expression due to deletion of one allele results in a failure to maintain expression of this critical TF. This in turn results in a loss of adult lung epithelial cell fate.

There have been several lncRNAs identified near important TFs, including Gata6, Foxa2, Foxf1, and eHand (Guttman et al. 2009; Sauvageau et al. 2013; Herriges et al. 2014, Anderson et al. 2016). These syntenically conserved lncRNAs are found either upstream of or downstream from their neighboring TFs. However, it has been unclear whether the relative localizations or orientations reflect unique functions. The lncRNA Upperhand, which is located 150 base pairs upstream of and antisense to the TF Hand2, has been shown to play an almost essential role for expression of the TF Hand2 (Anderson et al. 2016). The lncRNA Fendrr similarly shares a divergent promoter region with the TF Foxf1 but, like NANCI, is not absolutely necessary for expression of its neighboring TF (Sauvageau et al. 2013). In contrast, NANCI is located downstream from and sense to Nkx2.1. This orientation is observed in a significant subset of lncRNAs associated with crucial TFs such as Foxa2 and Neurog1 (Supplemental Table.1; Herriges et al. 2014). The downstream orientation of these lncRNAs away from the upstream regulatory regions of neighboring coding genes suggests a mode of action different from those that share divergent promoters with the neighboring TF. The NANCINkx2.1 duplex provides a novel model for how this subset of lncRNAs functions as rheostats to buffer expression of neighboring TF genes. This category of lncRNAs, including NANCI, is likely critical for maintaining TF expression in response to genetic or environmental stress. Thus, it will be important to assess their function in trans to their neighboring TFs using compound mutants such as described here. Given the diverse functions identified thus far for lncRNAs, it is essential that their functions be assessed fully and carefully in vivo to define their contribution to tissue development and homeostasis. Analysis and comparison of these lncRNAs will help to reveal the common and unique mechanisms necessary for their ability to act as buffering rheostats for TF expression.
and Dynabeads [Invitrogen, sheep anti-rat IgG] following the manufacturer’s instructions. Adult lungs (4 wk, 10 wk, and 6 mo) were harvested, and remaining forebrains were placed in TRIzol reagent [Invitrogen]. RNA was extracted using the PureLink RNA minikit [Invitrogen]. cDNA was synthesized using the VILO cDNA synthesis kit [Invitrogen]. qPCR was conducted using TaqMan gene expression assays [Applied Biosystems]. Each sample was run in triplicate on the same plate on a Stratagene MX3005P real-time PCR machine following the manufacturer’s recommended protocol.

RNA-seq analysis

E18.5 lung epithelial cells from wild-type, NANCIcreERT2::RFP/+ Nkx2.1GFP/+, and NANCIcreERT2::RFP/++; Nkx2.1GFP/+ lungs [three samples per genotype] were collected as described for qPCR, and RNA was isolated using Trizol. The University of Pennsylvania Next-Generation Sequencing Core performed library preparation and RNA-seq for these samples. Six-month epithelial cells were sorted into Trizol as described for FACS, however, epithelial cells were not sorted into distinct populations based on RFP or GFP expression. Samples were collected from three wild-type, three Nkx2.1GFP/+, and four NANCIfcreERT2::RFP/++; Nkx2.1GFP/+ lungs. Library preparation and sequencing for these samples were performed at GeneWiz, LLC.

Fastq files were assessed for quality control using the FastQC program. Fastq files were aligned against the mouse reference genome (mm9) using the STAR aligner [Dobin et al. 2013]. Duplicate reads were flagged using the MarkDuplicates program from Picard tools. Per-gene read counts for Ensembl (version 67) gene annotations were computed and filtered as TFs using the AnimalTFDB 2.0 database (Zhang et al. 2015). Genes annotated with transcript_type of “lincRNA” and “bidirectional_promoter_lincRNA” were used to define lncRNAs. The distance between each protein-coding gene and the closest nonoverlapping lncRNA was computed using the closest function in the BedTools suite. Protein-coding genes were annotated and filtered as TFs using the AnimalTFDB 2.0 database [Zhang et al. 2015].

Flow cytometric analysis of immune populations

Flow cytometry was used to identify immune cells in P4 lungs. P4 lungs were harvested and processed into a single-cell suspension as described for FACS. Cells were stained with Aqua Live/Dead viability stain to exclude dead cells. Cells were then stained with one of two standard panels of antibodies listed in Supplemental Table 5. After staining for 30 min on ice, cells were washed in PBS and run for data acquisition with a BD LSR11 cytometer using the FACS Diva software [BD Biosciences]. Data were analyzed using FlowJo. For identification of neutrophils and Ly6c+ monocytes, we used the following strategy. After exclusion of dead cells, all other cells expressing the common leukocyte marker CD45 were identified. Cd11b+ myeloid cells were then divided into ly6ghigh neutrophils and Ly6c+ monocytes. For identification of B cells [B220+] and CD4+ T cells, we used the following strategy. After exclusion of dead cells, all other cells expressing the common leukocyte marker CD45 were identified, and lymphocytes were selected using their side scatter and forward scatter profiles. For identification of CD4+ T cells, we gated the population CD4+ cells. B cells were identified as those cells that were CD4+ /CD8− as well as B220−.

TSH test

Serum was collected from 10-wk-old mice and analyzed for TSH levels by ELISA following the manufacturer’s instructions [Thyroid-Stimulating Hormone kit, Cloud-Clone Corp., ABIN415519].

ChIP-PCR

ChIP:PCR was performed as described previously [Snyder et al. 2013]. Adult lung epithelial cells were isolated from a single-cell suspension using an EpCAM antibody [Bioscience, 17-5791-82] in conjunction with anti-APC microbeads [Miltenyi Biotec, 130-090-855] and LS columns [Miltenyi Biotec, 130-042-401]. These cells were sonicated using a Bioruptor for 8 min (30 sec on, 30 sec off), and Nkx2.1-bound DNA was purified using an Nkx2.1 antibody [Millipore, 07-601] and the MAGnify ChIP kit following the manufacturer’s instructions. qPCR was performed on the isolated samples using primers listed in Supplemental Table 4 and normalized to samples isolated using a rabbit IgG antibody. The mouse genome [mm9, human hg19] was scanned for the occurrence of Nkx2-1 motifs using the FIMO program from the MEME suite [Grant et al. 2011].

Sample preparation for mass spectrometry

The MLE12 cell line was obtained from American Type Culture Collection and cultured in the HITES medium [Ham’s F12 [Mediatech], 0.005 mg/mL insulin, 0.01 mg/mL transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-estradiol, 10 mM HEPES, 2 mM L-glutamine] with 2% FBS and 1% penicillin/streptomycin. One week before collection, half of the MLE12 cells were switched to SILAC (stable isotope labeling by amino acids in cell culture) medium containing labeled lysine and arginine. RNA pull-down assay was performed as described [Harrow et al. 2012]. Genes annotated with transcript_type of “lincRNA” and “bidirectional_promoter_lincRNA” were used to define lncRNAs. The distance between each protein-coding gene and the closest nonoverlapping lincRNA was computed using the closest function in the BedTools suite. Protein-coding genes were annotated and filtered as TFs using the AnimalTFDB 2.0 database [Zhang et al. 2015].
Proteomics data analysis

MS data were analyzed using Proteome Discoverer 1.4 [Thermo Scientific] for protein identification/quantification. The mouse UniProt database was used for Mascot searches for assigning peptide sequences and identification of proteins. The search was performed for precursor ion mass tolerance of 10 ppm, fragment ion mass tolerance of 0.6 Da, trypsin digestion, and two missed cleavages. Dynamic modification sets were carbamidomethylation of cysteine and oxidation of methionine. Common contaminants such as keratin and trypsin were eliminated, and only peptides with a false discovery rate <1% were considered.

Western blot

The MS results were verified by Western blot analysis of proteins isolated using NANCI, NANC1 antisense, and IRES-GFP-Puro transcripts. Antibodies were used to test the enrichment of Hnrmab (rabbit, 1:100; Sigma Aldrich), Hnrmab2b1 (mouse, 1:200; Santa Cruz Biotechnology), and Hnrmmd (rabbit, 1:1000; Millipore).

In vitro lentiviral shRNA knockdown

Lentiviral work was performed as described previously [Herriges et al. 2014] with the exception that shRNA oligos [Supplemental Table 5] were annealed into the pLKO.1-Neo vector generously provided by the Winslow laboratory. MLE12 cells were cultured for 6 d in medium containing 50 μg/mL geneticin to select for infected cells before collection and isolation of total RNA.

Pulmonary and thyroid histology

Postnatal lungs were inflated with 2% paraformaldehyde under constant pressure of 25 cm [P4] or 30 cm [4 wk, 10 wk, or 6 mo] water and allowed to fix overnight. E18.5 lungs and dissected thyroids were fixed overnight in 2% paraformaldehyde. Tissue was embedded in paraffin and sectioned. Hematoxylin and eosin staining was performed to examine tissue morphology. In situ hybridization was performed as described previously [Herriges et al. 2014]. IHC was used to detect protein expression using the following antibodies on paraffin sections: cleaved Caspase 3 [rabbit, 1:25; Biocare], E-cadherin [rabbit, 1:100; Cell Signaling], F4/80 [rat, 1:50; Biologiclend], GFP [goat, 1:100; Abcam], Hnf4a [goat, 1:40; Santa Cruz Biotechnology], Hnf4a [rabbit, 1:50; Cell Signaling], Ki67 [rabbit, 1:50; Abcam], Krt5 [rabbit, 1:1500; Covance], Muc5ac [mouse, 1:100; Abcam], Nkx2.1 [rabbit, 1:50; Santa Cruz Biotechnology], Pgp9.5 [mouse, 1:100; RD1], Pdpn [mouse, 1:50; Hybridoma Bank], P63 [mouse, 1:50; Santa Cruz Biotechnology], RFP [rabbit, 1:250; Rockland], Scgb1a1 [goat, 1:20; Santa Cruz Biotechnology], Sftpce [goat, 1:50; Santa Cruz Biotechnology], and Sox9 [rabbit, 1:100; Santa Cruz Biotechnology].

Forebrain histology

Embryonic mice were dissected on ice-cold PBS and fixed overnight at 4°C in RNase-free 4% PFA in 1× PBS. Embryos were then washed twice in 1× PBS and cryoprotected by immersion through sucrose (15% and 30% sucrose in 1× PBS; solutions were changed once embryos had sunk). Afterward, they were embedded in freezing compound, sectioned coronally at 12 μm on a cryostat (Leica), and collected onto slides (Superfrost slides). After allowing the sections to dry for 30 min, brain sections were preincubated for 1 h in blocking buffer (5% bovine serum albumin with 0.1% Triton X-100). Sections were then incubated overnight at 4°C with the following primary antibodies: mouse Nkx2.1 [mouse, 1:200; Abcam] and RFP [rabbit, 1:1000; Rockland].

Statistical analysis

Statistical analysis was performed on the data using Prism 7. Two-tailed Student’s t-test was used for comparison of two experimental groups. Statistical data were considered significant if \( P < 0.05 \).
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