Development and Regeneration of Sox2+ Endoderm Progenitors Are Regulated by a HDAC1/2-Bmp4/Rb1 Regulatory Pathway

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SUMMARY

The mechanisms that govern the maintenance and differentiation of tissue-specific progenitors in development and tissue regeneration are poorly understood. We show that development of Sox2+ progenitors in the lung endoderm is regulated by histone deacetylases 1 and 2 (Hdac1/2). Hdac1/2 deficiency leads to a loss of Sox2 expression and a block in proximal airway development. This is mediated in part by derepression of Bmp4 and the tumor suppressor Rb1, which are direct transcriptional targets of Hdac1/2. In contrast to development, postnatal loss of Hdac1/2 in airway epithelium does not affect the expression of Sox2 or Bmp4. However, postnatal loss of Hdac1/2 leads to increased expression of the cell-cycle regulators Rb1, p21/Cdkn1a, and p16/Ink4a, resulting in a loss of cell-cycle progression and defective regeneration of Sox2+ lung epithelium. Thus, Hdac1/2 have both common and unique targets that differentially regulate tissue-specific progenitor activity during development and regeneration.

INTRODUCTION

Many tissues contain resident progenitor populations that are critical for both development and postnatal repair and regeneration. The foregut endoderm is a multipotent tissue that generates multiple organs during development, including the lung, thyroid, liver, and pancreas. Sox2 is expressed in the developing foregut endoderm, and lineage-tracing experiments have demonstrated that Sox2+ cells can act as tissue-specific progenitors in several tissues, including the lung, stomach, and testes (Arnold et al., 2011). Accumulating evidence also points to the functional importance of Sox2 in regulating tissue-specific progenitor cells during both development and adult homeostasis. Ablation of Sox2-expressing cells in the adult disrupts tissues homeostasis leading to multiorgan failure and lethality (Arnold et al., 2011). However, the mechanisms regulating maintenance and differentiation of Sox2+ progenitors during development and tissue regeneration is poorly understood.

In addition to direct control of cell fate decisions by DNA binding transcription factors, epigenetic regulation of gene expression is also important for integrating signaling input and transcriptional output during development (Xu et al., 2011). Histone deacetylases (Hdac) play an important role in regulating chromatin compaction through deacetylation of histones, which counterbalances the action of histone acetyltransferases (HATs). Although many Hdacs are widely expressed, little is known about their direct transcriptional targets and how they regulate tissue-specific gene expression. In the lung, decreased expression of HDAC2 has been associated with chronic obstructive pulmonary disease (COPD), a broad disease spectrum that leads to the irreversible loss of airway and alveolar structure and function and is thought to be due to a chronically defective injury-regeneration cycle caused by environmental insults (Ito et al., 2005, 2006). However, little is known about the roles of chromatin remodeling complexes, including Hdac1/2, in either lung development or postnatal homeostasis and regeneration.

In this report, we show that Hdac1/2 are necessary for Sox2 gene expression, which in turn, is required for the development of the proximal airways of the lung. The loss of Sox2 expression is caused, in part, by the derepression of Bmp4 expression, a direct target gene of Hdac1/2. Increased Bmp4 expression leads to decreased Sox2+ proximal progenitors and an expansion of Sox9+/Id2+ distal progenitors in the developing lung, resulting in a failure to form proximal airways in the lung. Importantly, reduction of Bmp4 expression partially rescues Sox2 expression in vivo during development. We also show that Rb1 is a direct target of Hdac1/2-mediated repression and loss of Hdac1/2 leads to derepression of Rb1 and inhibition of cell-cycle progression. In the postnatal lung, airway epithelial loss of Hdac1/2 expression does not lead to Bmp4 derepression or changes in homeostatic Sox2 expression. However, in a model of airway injury and regeneration, loss of Hdac1/2 expression derepresses Rb1 expression, along with p21/Cdkn1a and...
In comparison to control littermates (M–O), Hdac1/2ShhcreDKO mutants developing airway epithelium. Hdac2 from E12.5 through E17.5 (arrowheads). Dotted lines outline the overall smaller lung (Q–S, arrowheads). Of note, individual loss of either Hdac1 or Hdac2 did not result in obvious defects in lung morphogenesis, and these mutants were viable (data not shown), which indicates that Hdac1 and 2 are redundantly required for lung development.

To determine whether normal tracheal specification had occurred in Hdac1/2ShhcreDKO mutants, expression of Nkx2.1, a marker of the early respiratory endoderm, including the trachea and p63, which is expressed preferentially in the esophagus at this stage, was examined by immunohistochemistry at E11.5. Lung endoderm identity is maintained through development as noted by continuous expression of Nkx2.1 and p63, which is expressed preferentially in the esophagus at (Figures 1E and 1F). Hdac2 expression was detected in both secretory cells and ciliated cells in the adult lungs (Figures S1M–S1P available online). These data indicate that the expression patterns of Hdac1 and Hdac2 overlap during lung development and suggest that the both factors may play an important role in regulating proximal airway endoderm development.

**RESULTS**

Expression of Hdac1/2 during Lung Development

To determine the expression pattern of Hdac1/2 in lung development, we performed immunohistochemistry on embryonic lung sections for Hdac1/2 protein expression at various stages of gestation. Beginning at E12.5, both Hdac1 and Hdac2 are widely expressed in both the endoderm and mesenchyme of the developing lung (Figures 1A and 1D). Hdac1 continues to be broadly expressed at later stages in both the endoderm and mesenchyme of the lung (Figures 1B and 1C). In contrast, Hdac2 expression decreases in the mesenchyme after E12.5 and is expressed primarily in proximal airway epithelium by E17.5 (Figures 1E and 1F). Hdac2 expression was detected in both secretory cells and ciliated cells in the adult lungs (Figures S1M–S1P available online). These data indicate that the expression patterns of Hdac1 and Hdac2 overlap during lung development and suggest that both factors may play an important role in regulating proximal airway endoderm development.

**Loss of Hdac1/2 Expression Leads to Defective Lung Development**

To determine the importance of Hdac1/2 in lung development, we generated a foregut endoderm-specific deletion of these genes using Hdac1flox/flox and Hdac2flox/flox alleles and the Shhcre line, which efficiently drives cre recombination in the early anterior foregut endoderm beginning at approximately E8.75 (Goss et al., 2009; Montgomery et al., 2007). These mutants will now be referred to as Hdac1/2ShhcreDKO. Hdac1 and Hdac2 were efficiently deleted in the mutant lung epithelial cells from E12.5 and later (Figures 1G–1L). Hdac1/2ShhcreDKO mutants all died at birth because of respiratory distress (data not shown), and examination of lungs from E17.5 Hdac1/2ShhcreDKO mutants showed a severe loss of branching morphogenesis, resulting in large dilated sac-like structures instead of well-formed lung lobes (Figures 1R and 1S). To assess the chronological onset of these defects, we examined Hdac1/2ShhcreDKO lungs and controls at E10.5 and E12.5. At E10.5, the trachea of Hdac1/2ShhcreDKO mutants that had separated from the esophagus, and the first branching event that generates the main stem bronchi had occurred normally (Figures S1A–S1F). However, although control lungs had begun to branch extensively by E12.5, Hdac1/2ShhcreDKO mutant lungs exhibited a severe inhibition of branching (Figures 1M and 1P). This defect in branching morphogenesis was also obvious at E14.5 (Figures 1N and 1Q). Of note, individual loss of either Hdac1 or Hdac2 did not result in obvious defects in lung morphogenesis, and these mutants were viable (data not shown), which indicates that Hdac1 and 2 are redundantly required for lung development.

To determine whether normal tracheal specification had occurred in Hdac1/2ShhcreDKO mutants, expression of Nkx2.1, a marker of the early respiratory endoderm, including the trachea and p63, which is expressed preferentially in the esophagus at this stage, was examined by immunohistochemistry at E11.5. Supporting the histological data, the trachea in both control and Hdac1/2ShhcreDKO mutants expressed Nkx2.1, whereas the esophagus in both control and Hdac1/2ShhcreDKO mutants expressed p63 (Figures 1T and 1U). Nkx2.1 continued to be expressed in the abnormal lung epithelium of Hdac1/2ShhcreDKO mutants at E17.5 (Figures 1V and 1W), suggesting that lung epithelial identity is maintained throughout development. These data indicate that loss of Hdac1/2 in the lung endoderm leads to defects in early lung branching morphogenesis but not respiratory specification or separation of the trachea and esophagus.
Hdac1/2 Are Required for Sox2+ Proximal Progenitor Development

To assess the molecular consequences from loss of Hdac1/2 in early lung endoderm, microarray analysis was performed on control and Hdac1/2ShhcreDKO mutant lungs at E12.5. We found that 149 genes were downregulated, and 70 genes were upregulated more than 1.4-fold in Hdac1/2ShhcreDKO mutant lungs (see Table S1). A focused examination of changes in transcription factor gene expression showed that the expression level of Sox2 was decreased in Hdac1/2ShhcreDKO mutant lungs (Figure 2A). The developing airways of the lung are patterned in a distinct proximal-distal manner with Sox2 expression marking the proximal airway progenitor population that will generate most if not all of the epithelial lineages within the trachea and bronchi (Arnold et al., 2011; Que et al., 2007; Sommer et al., 2009; Tompkins et al., 2009, 2011). Conversely, Sox9 and Id2 mark distal endoderm progenitors in the early lung (Rawlins et al., 2009). Although Sox9+Id2+ distal progenitors are multipotent prior to E13.5, their progenitor capacity is restricted to generating distal alveolar epithelial lineages after this time point (Rawlins et al., 2009) (Figure 2B).

Immunostaining of a series of histological sections from the anterior to posterior regions of both control and Hdac1/2ShhcreDKO lungs showed a significant loss of Sox2 protein expression at E12.5 (Figure 2C). This was confirmed by quantitative real-time PCR (Figure 2D). At E10.5, a small number of Sox2+ cells were observed in the dorsal aspect of the developing trachea in both control and Hdac1/2ShhcreDKO mutant lungs, although the number of Sox2+ cells appeared slightly reduced in the Hdac1/2ShhcreDKO mutants (Figures S1C and S1F). This is likely due to the incomplete loss of Hdac1/2 protein prior to E11.5 (Figures S1A, S1B, S1D, and S1E), despite the fact that Shh-smo recombination is observed as early as E8.75 (Goss et al., 2009). Of note, Hdac1/2 were also efficiently deleted in the developing esophageal endoderm using the Shhcre line (Figures S1G–S1J), yet Sox2 expression did not appear to be affected in the esophagus (Figures S1K and S1L). These data show that Hdac1/2 are required for Sox2 expression in the developing lung endoderm but not in the esophagus.

To determine whether distal progenitor development was disrupted in the Hdac1/2ShhcreDKO mutant lungs, we examined the expression of Sox9 by immunostaining. Sox9 expression was expanded throughout both the proximal and distal regions of Hdac1/2ShhcreDKO mutant lungs (Figure 2E). Quantitative real-time PCR showed that levels of Sox9 expression in the whole lung were not significantly altered in Hdac1/2ShhcreDKO mutant lungs (Figure 2F), possibly because of decreased Sox9 expression on a per cell basis in Sox9+ cells or decreased mesenchymal Sox9 expression, which masked the increased Sox9 expression in the epithelium. In contrast, expression of the distal progenitor marker Id2 was both expanded and increased in Hdac1/2ShhcreDKO mutant lungs as shown by both in situ and quantitative real-time PCR (Figures 2F and S2A). These data suggest a critical role for Hdac1/2 in regulating the balance between proximal and distal progenitors during early lung development.

Previous studies have shown that Sox2 is a crucial regulator of proximal endoderm lineage identity and differentiation in the lung (Que et al., 2007, 2009; Tompkins et al., 2009, 2011). To assess whether Hdac1/2ShhcreDKO mutant lungs exhibited defects in proximal cell fate development and differentiation, we examined the expression of early lung proximal progenitor markers SSEA1 (also known as Fut4) and Scgb3a2. Expression of both of these markers was decreased in Hdac1/2ShhcreDKO mutant lungs (Figures 2G–2J). Additional differentiation markers of proximal cell lineages, including Scgb1a1 (secretory Clara cells), beta-Tubulin IV (ciliated epithelial cells), and Ascl1 (neuroendocrine cells), were all greatly reduced or undetectable in Hdac1/2ShhcreDKO mutant lungs (Figures 2K–2N and S2D–S2G). Expression of the goblet cell marker gene Clca3 was not changed in Hdac1/2ShhcreDKO mutant lungs (Figures S2H and S2I). In contrast, distal lineage markers, including T1alpha (alveolar type I cells) and SftpC (alveolar type II cells), were still present in the mutant lungs (Figures 2M and S2C), suggesting that distal epithelial lineage differentiation is not dramatically affected in these mutants.

Hdac1/2 Directly Regulates Bmp4 Expression to Control Proximal Lung Endoderm Progenitor Development

The loss of Sox2+ progenitors in the lung and not in the esophagus suggested a specific role for Hdac1/2 in regulating Sox2 expression in the proximal airways of the lung. Little is understood about the molecular pathways essential for development and differentiation of Sox2+ progenitors. Therefore, we examined expression of multiple pathways known to regulate early lung progenitor specification and differentiation, with a focus on those that are regulators of proximal-distal progenitor differentiation (Domyan et al., 2011; Goss et al., 2009; Izvolsky et al., 2003; Park et al., 1998; Shu et al., 2005; Tsao et al., 2009; Weaver et al., 1999, 2000; Yin et al., 2008). These studies revealed that Bmp4 expression was significantly upregulated in Hdac1/2ShhcreDKO mutants (Figure 3A). In situ hybridization showed that Bmp4 expression was expanded throughout the early endoderm of Hdac1/2ShhcreDKO mutants (Figure 3B) instead of being confined to the endoderm at the distal tip of the branching airways as observed in the control lungs and as has been previously reported (Weaver et al., 1999, 2000). Expression of members of the Wnt and Fgf pathways and the downstream effectors of these pathways were not significantly altered in Hdac1/2ShhcreDKO mutants. Expression of Notch1 was slightly upregulated, whereas expression of the Notch effector Hes1 was slightly downregulated. However, such minor and inconsistent alterations in expression of Notch signaling components suggests that Notch is not significantly disrupted in Hdac1/2ShhcreDKO mutants.

Bmp signaling is an important regulator of anterior foregut endoderm development as well as a regulator of proximal-distal patterning of the developing lung. Inhibition of Bmp signaling through overexpression of the Bmp antagonists noggin or gremlin inhibits distal lung development and expands proximal lung development (Lu et al., 2001; Weaver et al., 1999). How Bmp signaling regulates the balance between proximal and distal lung development is unclear, and whether it affects early progenitor specification, maintenance, or differentiation has not been assessed. To determine whether increased Bmp4 expression could alter the balance between proximal (Sox2+) and distal (Sox9+) lung progenitors, E11.5 lung explants were treated with exogenous Bmp4 for 48 hr. Exogenous Bmp4...
Figure 2. Hdac1/2 Are Required for Development of Sox2+ Proximal Lung Endoderm Progenitors
(A) The heatmap generated from microarray results shows differential expression of 42 transcription factors in the Hdac1/2\textsuperscript{ShhcreDKO} mutants compared to Shh\textsuperscript{cre} controls, including Sox2.
(B) This scheme shows the relative spatial distribution of Sox2+ proximal lung endoderm progenitors (green) and Sox9+/Id2+ distal lung progenitors (red).
(C) Immunostaining of sections throughout the anterior to posterior regions of the lung shows that Hdac1/2\textsuperscript{ShhcreDKO} mutants have a dramatic loss of Sox2 expression at E12.5. The scheme on the right demonstrates the approximate positions of cross-sections along the anterior-posterior axis of the lung buds. The green color outlines the Sox2 expression pattern in control and Hdac1/2\textsuperscript{ShhcreDKO} mutants.
(D) Quantitative real-time PCR results confirm the loss of Sox2 expression in Hdac1/2\textsuperscript{ShhcreDKO} mutants at E12.5.

(legend continued on next page)
caused a decrease in both the expression and number of Sox2+ cells in the lung (Figure 3C). This decrease resulted in inhibition of proximal airway endoderm development as noted by decreased Scgb3a2 and SSEA1 expression (Figure 3C). In contrast, exogenous Bmp4 treatment expanded the number of Sox9+ progenitors such that they were observed in the proximal airways of the treated lungs (Figure 3C). Moreover, Id2 expression was increased by Bmp4 treatment, which is consistent with Id2 as a direct target of Bmp signaling and a marker of distal lung endoderm progenitors (Figure 3C). Of note, these changes in gene expression and spatial localization of Sox9+ cells were all similar to what was observed in Hdac1/2ShhCreDKO mutants (Figure 2).

Previous studies have shown that increased Bmp4 expression increases apoptosis in multiple tissues, including the lung (Bellusci et al., 1996). Hdac1/2ShhCreDKO mutant lungs exhibited an increase in apoptosis from E11.5 through E14.5 as noted by increased activated caspase-3 immunostaining (Figures S3A and S3B). Treatment of wild-type lung explants with exogenous Bmp4 also increased apoptosis (Figures S3C and S3D). As in Hdac1/2ShhCreDKO mutant lungs, this increase in apoptosis was not spatially restricted and was observed throughout the endoderm of the treated explants (data not shown).

To determine whether Bmp4 was a direct target of Hdac1/2 in the developing lung, chromatin immunoprecipitation (ChIP) assays were performed on the Bmp4 promoter region using Hdac1/2 antibodies. These experiments showed robust binding of Hdac1/2 to the proximal promoter region of Bmp4 at E12.5 (Figures 3D and 3E; data not shown). In contrast, Hdac1 and Hdac2 binding was not observed in an unrelated intergenic region 1.5 megabases upstream of the Bmp4 locus (Figure 3D). To assess whether the loss of Hdac1/2 altered the acetylation state of histones on the Bmp4 promoter in lung epithelial cells, Hdac1/2 expression was knocked down in the lung epithelial cell line MLE12 (Figure 3F) (Wikenheiser et al., 1993). Loss of Hdac1/2 expression led to increased H3K9 acetylation on the Bmp4 promoter (Figure 3G), whereas acetylation was unaffected in an unrelated intergenic upstream of Bmp4 (Figure 3H). Similarly, treatment of lung explants with the Hdac inhibitor trichostatin A (TSA) resulted in an elevated level of H3K9 acetylation at the Bmp4 promoter (Figure 3I), without altering the acetylation level at the intergenic region (Figure 3J). Together, these data indicate that Bmp4 is a direct target of Hdac1/2 during lung endoderm development and its derepression leads to downregulation of Sox2 expression, expansion of Sox9+ class2+ progenitors, and increased endoderm apoptosis.

To determine if reducing the dose of Bmp4 would partially rescue the loss of Sox2 expression in Hdac1/2ShhCreDKO mutants, we deleted one copy of Bmp4 in the Hdac1/2ShhCreDKO mutants using a floxed allele of Bmp4 (Kulesza and Hogan, 2002). We then performed immunohistochemistry for Sox2 in the Hdac1/2ShhCreDKO:Bmp4−/+ lungs at E12.5 to assess the expression of Sox2. Although Sox2 expression was not detectable in any of the Hdac1/2ShhCreDKO:Bmp4−/+ lungs we have examined (n = 7), we found that 50% of Hdac1/2ShhCreDKO:Bmp4−/+ lungs (n = 6) showed partial restoration of Sox2 expression in the bronchi and airway epithelium upon deletion of one copy of Bmp4 (Figures 3K–3P). Epithelial apoptosis was also reduced in the Hdac1/2ShhCreDKO:Bmp4−/+ lungs (Figures S3E and S3F). Overall lung structure, however, was not fully restored although additional branch points were evident in a subset of the compound mutant lungs (Figures S3G–S3K). These studies suggest that Hdac1/2 are required to suppress Bmp4 in the proximal lung endoderm to allow for proper airway development. Given the lack of a more robust rescue, these data also suggest that additional molecular pathways are responsible for some aspects of the phenotype observed in Hdac1/2ShhCreDKO mutants.

**Endoderm Progenitor Proliferation Is Mediated in Part by Hdac1/2 Repression of Rb1**

Previous studies have shown that a common effect of the combined loss of Hdac1/2 expression is decreased cell proliferation coupled with increased apoptosis (LeBoeuf et al., 2010; Ma et al., 2012; Montgomery et al., 2007). Decreased proliferation was observed in Hdac1/2ShhCreDKO mutant lungs as shown by phospho-histone H3 staining, and the decrease in proliferation was uniform throughout the mutant lungs (Figure 4A). Several important regulators of the cell cycle, including p16/Ink4a and p21/Cdkn1a, have been shown to be direct targets of Hdac1/2-mediated repression (Lagger et al., 2002, 2003; Wilting et al., 2010; Yamaguchi et al., 2010). However, loss of p16/Ink4a or p21/Cdkn1a does not rescue Hdac1/2-mediated inhibition of cell proliferation, suggesting the importance of additional targets of Hdac1/2 in regulating cell-cycle progression (Wilting et al., 2010). Therefore, we examined our microarray data for changes in expression of cell-cycle regulators in Hdac1/2ShhCreDKO mutants. In addition to increased expression of p21/Cdkn1a, we observed increased expression of the tumor suppressor retinoblastoma-1 (Rb1) in Hdac1/2ShhCreDKO mutant lungs (Figure 4C). Quantitative real-time PCR and immunostaining showed that Rb1 as well as p16/Ink4a and p21/Cdkn1a were all increased in Hdac1/2ShhCreDKO mutant lungs (Figures 4D–4J). p57, another cell-cycle regulator that has been reported to be a direct target of Hdac1/2, was not upregulated in the Hdac1/2ShhCreDKO mutant lungs and was not expressed at significant levels in the control lungs (data not shown; Yamaguchi et al., 2010).
Figure 3. Loss of Hdac1/2 Results in Increased Bmp4 Expression, which in Turn Represses Sox2 Expression in the Lung

(A) Quantitative real-time PCR analysis of major signaling pathway components in Hdac1/2<sup>ShhcreDKO</sup> mutants reveals a significant increase in Bmp4 expression.

(B) In situ hybridization shows increased and expanded Bmp4 expression throughout the entire dysmorphic lung endoderm of Hdac1/2<sup>ShhcreDKO</sup> mutants (B, arrows).

(C) Treatment of lung explant cultures at E11.5 with recombinant Bmp4 (rBmp4) for 48 hr suppresses Sox2 expression (ii, arrowhead) and expands Sox9+ cells in lung endoderm (iv, arrowheads). Note that Sox9 normally marks the distal lung epithelial progenitor cells (iii and iv, arrows) as well as proximal mesenchyme (iii, arrowhead). In rBmp4-treated explants, Sox9 expression is expanded into the proximal airway epithelium of the lung explants (iv, arrowheads). Quantitative real-time PCR shows decreased Sox2 expression along with a decrease in other early proximal epithelial markers, including Scgb3a2 and SSEA1 (Fut4), in rBmp4-treated explants. Quantitative real-time PCR also shows increased expression of Id2 in rBmp4-treated lung explants. White dotted lines outline the distal lung endoderm.

(D and E) ChIP assays using gel electrophoresis (D) and quantitative real-time PCR (E) show that Hdac1/2 directly bind to the Bmp4 promoter in the E12.5 lung.
We next tested whether Rb1 was a direct target of Hdac1/2 in the lung. ChIP assays were performed using chromatin extracts from E12.5 lungs. These studies showed that Hdac1/2 bound robustly to the proximal promoter of Rb1 (Figures 4K and 4L). siRNA-mediated knockdown of Hdac1/2 in MLE12 cells or TSA treatment of lung bud explants resulted in increased H3K9 acetylation at the Rb1 promoter (Figures 4M and 4N). Together with the increase in Rb1 expression, these data indicate that Hdac1/2 are required to suppress Rb1 expression to allow for proper proliferation of early lung endoderm. Thus, Hdac1/2 are potent regulators of lung progenitor proliferation by targeting multiple cell-cycle inhibitors in the developing lung, including Rb1, p16/Ink4a, and p21/Cdkn1a.

Hdac1/2 Are Required for Regeneration but Not Homeostasis of Lung Airway Epithelium

The effects of chromatin remodeling complexes and epigenetic regulation on tissue regeneration are poorly understood. Given the potent and specific roles for Hdac1/2 in regulating Sox2+ proximal endoderm progenitor development in the anterior foregut, we assessed whether these factors were important for proximal airway epithelial homeostasis and regeneration in the postnatal lung. We generated Hdac1/2Scgb1a1creDKO mutants using an Scgb1a1 cre line, which is active in the proximal airway secretory epithelium beginning at birth (Li et al., 2012). Hdac1/2 were efficiently deleted in Hdac1/2Scgb1a1creDKO lungs as shown by immunostaining 8-week-old mutant lungs (Figures 5A–5D). In contrast to deletion of Hdac1/2 during development, loss of Hdac1/2 in proximal airway epithelium did not dramatically affect postnatal airway epithelial homeostasis as noted by the normal appearance of the airway epithelium and expression of markers for secretory and ciliated epithelial lineages in Hdac1/2Scgb1a1creDKO mutants (Figures 5E–5M). Proliferation and apoptosis was also not affected in Hdac1/2Scgb1a1creDKO mutants (data not shown). Importantly, expression of Sox2, which is expressed in the postnatal secretory lineage, was not decreased in the Hdac1/2Scgb1a1creDKO mutants (Figures 5G, 5K, and 5M). Thus, Hdac1/2 are not required for postnatal airway epithelial cell homeostasis.

The lung has a remarkable capacity to regenerate epithelial lineages, including the secretory cells within the proximal airways after injury. To determine whether expression of Hdac1/2 were important for airway epithelial regeneration, we used a model of airway secretory cell depletion caused by naphthalene exposure. Naphthalene injury depletes the vast majority of airway secretory cells (also called Clara cells) in the postnatal lung, and the airway epithelium will regenerate through a process involving expansion and differentiation of a small subset of naphthalene-resistant secretory cells often referred to as facultative airway progenitors (Giangreco et al., 2002) (Figure 6A). Hdac1/2Scgb1a1creDKO and Scgb1a1cre control mice were treated with naphthalene, and the epithelial regeneration process was assessed at multiple time points using immunostaining and quantitative real-time PCR for Scgb1a1 (secretory cells), beta-Tubulin IV (TubbIV) (ciliated cells), and Sox2, which marks both secretory cells and a significant proportion of ciliated cells in the adult airway. Four days after injury, the majority of secretory cells in both Hdac1/2Scgb1a1creDKO and control lungs were depleted as shown by the dramatic decrease in Scgb1a1 and Sox2 expression (Figures 6B–6E). Ten days postinjury, although a significant recovery in the number of secretory cells was observed in Scgb1a1cre control lungs, Hdac1/2Scgb1a1creDKO mutants displayed a dramatic inhibition in regeneration of Scgb1a1+/Sox2+ secretory cells after injury (Figures 6F and 6G). Examination of Hdac1/2Scgb1a1creDKO mutant lung airways using hematoxylin and eosin staining (H&E) staining showed that they lacked extensive repopulation of secretory cells 10 days after injury, in contrast to control airways, which had begun to repopulate with secretory epithelium. These alterations in Scgb1a1+/Sox2+ cells were also observed at 1 month after injury, suggesting that the loss of regeneration is persistent and not transient (Figures 6H and 6I). Beta-tubulin IV expression showed a slight decrease in ciliated cells in the mutant lungs by quantitative real-time PCR at 10 days postinjury and normal expression level at 1 month, consistent with the fact that naphthalene specifically depletes the secretory lineage (Figures 6F–6I). Sox2 expression in ciliated cells was decreased in the Hdac1/2Scgb1a1creDKO mutants both at 10 days and 1 month after injury (Figures S4A–S4F), further exacerbating the overall decrease in Sox2 expression in these mutants.

Goblet cell markers Mucin5ac and Agr2 were not changed in the Hdac1/2Scgb1a1creDKO mutant lungs prior to or during the regeneration process, indicating a lack of an effect on goblet cell differentiation in these mutants (Figures S4G–S4M).

Given the critical roles of Hdac1/2 in regulating Bmp4 expression and cell-cycle progression during lung development through repression of Rb1, p16/Ink4a, and p21/Cdkn1a, we tested whether Hdac1/2 regulated these cellular pathways during adult regeneration. Quantitative real-time PCR and in situ hybridization did not reveal changes in Bmp4 expression in Hdac1/2Scgb1a1creDKO lungs at 10 days postinjury (Figures S5; data not shown). In contrast, proliferation of regenerating airway epithelium of Hdac1/2Scgb1a1creDKO mutant lungs was significantly compromised during the course of regeneration, especially between day 4 and day 10 postinjury, when the naphthalene-resistant facultative progenitors expand to regenerate the airway epithelium (Figures 7A and 7B). This decrease in proliferation was associated with an increase in the cell-cycle inhibitors Rb1, p21/Cdkn1a, and p16/Ink4a at day 7 after injury (Figures 7C–7H). Of note, expression of these proteins was not altered in Hdac1/2Scgb1a1creDKO lungs prior to injury (Figure S5), suggesting that Hdac1/2 regulation of Rb1, p21/Cdkn1a, and p16/Ink4a is only reactivated during the regeneration process. These data indicate that Hdac1/2 regulate lung endoderm development...
Figure 4. Rb1 Is a Direct Target of Hdac1/2 Repression

(A and B) Phospho-histone H3 (PO4-H3) immunostaining shows that proliferation is decreased throughout Hdac1/2<sup>ShhcreDKO</sup> mutant lung endoderm.

(C) Heatmap of genes associated with cell proliferation from Hdac1/2<sup>ShhcreDKO</sup> mutants shows increased expression of Rb1 and p21/Cdkn1a.

(D–I) Immunostaining for Rb1, p16/Ink4a, and p21/Cdkn1a demonstrates that these cell-cycle inhibitors are undetectable in control lungs at E12.5 but are induced in Hdac1/2<sup>ShhcreDKO</sup> mutants.

(J) Quantitative real-time PCR confirms the derepression of Rb1 and p21/Cdkn1a transcripts at E12.5 in Hdac1/2<sup>ShhcreDKO</sup> mutants.

(K and L) ChIP assays using gel electrophoresis (K) and quantitative real-time PCR (L) show that Hdac1/2 binds robustly to the Rb1 proximal promoter in E12.5 lungs.

(M and N) Hdac1/2 knockdown in MLE12 cells (M) or treating lung bud explants with TSA (N) increases the H3K9 acetylation level at the Rb1 promoter. Quantitative real-time PCR and ChIP PCR data shown are the average of three to five assays ± SEM. Scale bars represent 20 μm in (A) and 10 μm in (D–I).
and regeneration through their differential repression of Bmp4 and the cell-cycle regulators Rb1, p16/Ink4a, and p21/Cdkn1a.

**DISCUSSION**

In these studies, we show that the expression of the critical transcription factor Sox2 is regulated in the anterior foregut through the chromatin remodeling factors Hdac1/2. Development of the proximal Sox2+ endoderm progenitors in the lung requires Hdac1/2 expression via regulation of Bmp4 signaling as well as cell-cycle progression through Rb1, p16/Ink4a, and p21/Cdkn1a. Derepression of Bmp4 expression leads to loss of Sox2+ proximal endoderm progenitors in the lung along with the subsequent failure to form the proximal airways during development. In contrast to development, Hdac1/2 regulate regeneration of Sox2+ airway secretory cells through repression of a cell-cycle program, including Rb1, p16/Ink4a, and p21/Cdkn1a, without affecting Bmp4 expression. These studies show that Sox2+ endoderm progenitors are regulated by both common and distinct Hdac1/2-mediated pathways during development and postnatal tissue regeneration.

Sox2+ is a critical transcription factor important for tissue-specific progenitors in multiple organs. In the lung, Sox2 is essential for development of the entire proximal airway epithelial lineage repertoire. Despite its criticality, little is understood about how Sox2 expression is initiated or maintained during development. Our finding that Hdac1/2 regulates Sox2 expression in the proximal airway epithelium of the developing respiratory system but not the esophagus suggests that its expression is differentially regulated in a tissue-specific fashion. This may be due to the unique requirements of Bmp signaling activity in the anterior foregut endoderm. Previous studies have shown that Bmp signaling is necessary for initial development of the trachea and that its signaling activity inhibits Sox2 expression (Domyan et al., 2011). How this precise activity is initiated and maintained has been unclear, but our data now show that inhibition of Bmp4 expression allows for the proper development of the proximal component of the respiratory system. In this manner, we postulate that Hdac1/2 act as critical repressive chromatin remodeling factors for establishing proximal lung progenitor cell fate, in part, through inhibition of Bmp4.

Additional evidence indicates that Bmp4 may be a nodal point for Hdac-mediated repression and regulation of tissue development through Sox2 expression. Trichostatin A, an inhibitor of class I Hdacs, increases Bmp4 expression in limb bud explants (Zhao et al., 2009). Moreover, increased Bmp4 expression leads to decreased Sox2 expression in the neural tube (Linker and Stern, 2004). The specific effect that loss of Hdac1/2 have on the proximal component of the respiratory system without apparent effects on esophageal development may be due to this unique targeting of Bmp4 signaling.

Hdac1/2 have been shown to regulate cell-cycle progression through derepression of p16/Ink4a and p21/Cdkn1a (Witting et al., 2010; Yamaguchi et al., 2010). However, given the potent inhibition of cell proliferation often observed upon loss of Hdac1/2 in multiple tissues, including the lung, as well as the
**Figure 6. Hdac1/2 Are Necessary for Regeneration of Sox2+ Airway Epithelium**

(A) Naphthalene-induced injury of the airways leads to depletion of the majority of secretory cells in the bronchiolar region of the lung with regeneration of the secretory lineage complete by 1 month after injury.

(B–E) Immunostaining for Scgb1a1 (B and C) and Sox2 (D and E) shows the severe loss in secretory epithelial cells 4 days postinjury in both Scgb1a1cre and Hdac1/2Scgb1a1creDKO mutants.

(F) Ten days postinjury, Scgb1a1+/Sox2+ secretory cells in the airways of the lung have not regenerated in Hdac1/2Scgb1a1creDKO mutants, whereas Scgb1a1cre controls have regenerated normally.

(G) Quantitative real-time PCR shows a dramatic loss in Scgb1a1, Sox2, and Scgb3a2 expression with a much smaller loss in TubbIV expression 10 days postinjury.

(H) At 1 month postinjury, Hdac1/2Scgb1a1creDKO mutants continue to lack regeneration of Scgb1a1+/Sox2+ secretory epithelium.

(I) Quantitative real-time PCR at 1 month postinjury confirms decreased Scgb1a1, Sox2, and Scgb3a2 expression, whereas TubbIV expression is unchanged (I). Quantitative real-time PCR data shown are the average of three to five assays ± SEM. Scale bars represent 20 μm in (C), (E), and (F) and 40 μm in (H). See also Figure S4.
Figure 7. Hdac1/2 Promote Airway Epithelial Regeneration through Regulation of Cell-Cycle Regulators Rb1, p21/Cdkn1a, and p16/Ink4a

(A) Cell proliferation as measured by Ki67 immunostaining is reduced by more than 80% during the airway epithelial regeneration process from days 4–10 in Hdac1/2Scgb1a1creDKO mutants.

(B) Quantification of Ki67+ cells against total bronchiolar epithelial cells in both control and Hdac1/2Scgb1a1creDKO lungs.

(C–H) Rb1 (C and D), p21/Cdkn1a (E and F), and p16/Ink4a (G and H) expression levels are upregulated during the process of airway regeneration in Hdac1/2Scgb1a1creDKO lungs.

(legend continued on next page)
inability of loss of p16/Ink4a and p21/Cdkn1a to reduce Hdac1/2-mediated inhibition of cell proliferation, additional factors are likely involved (Wilting et al., 2010). Our studies have identified Rb1 as a target of Hdac1/2 in the regulation of the cell cycle in lung endoderm. Rb1 is a well-known tumor suppressor whose aberrant silencing is closely associated with lung cancer (Ding et al., 2008; Sutherland et al., 2011). Rb1 and Hdac1 have been reported to physically interact (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998), but whether Rb1 was a direct transcriptional target of Hdac activity was previously not known. Our studies demonstrate that Rb1 is a direct target of Hdac1/2-mediated repression and that its derepression is associated with decreased proliferation. Unlike Bmp4, Rb1 is also upregulated in Hdac1/2-deficient postnatal lung epithelium during the process of lung epithelial regeneration. This increased expression of Rb1 and other cell-cycle inhibitors, including p16/Ink4a and p21/Cdkn1a, leads to a dramatic inhibition of the regenerative capacity of the airway epithelium after naphthalene-induced depletion. Our results suggest that Bmp4 is a unique target of Hdac1/2 during lung development, whereas Rb1-mediated cell-cycle inhibition is a common Hdac1/2-regulated pathway that is shared during both lung development and regeneration.

The potent role for Hdac1/2 in regulating airway epithelial regeneration is interesting in light of recent findings showing that decreased HDAC activity, and in particular HDAC2 expression, is associated with the severity of disease in COPD patients (Ito et al., 2005, 2006). COPD is a spectrum of lung diseases that is represented by progressive lung airway obstructions caused by environmental irritants, such as tobacco smoking. Although cigarette smoke-triggered inflammation has received much attention as a cellular and molecular mechanism of COPD, the inappropriate airway repair and regeneration after repeated insults by smoking is also thought to play an important role. Exposure to cigarette smoke can induce cellular senescence in the mouse lung (Nyunoya et al., 2006, 2009), suggesting the potential role of impaired cell proliferation in response to airway injuries in the progression of the disease. Our findings indicate that Hdac1/2 act to allow for the proper reactivation of cell-cycle progression in the regenerating airway epithelium after injury through repression of Rb1 as well as other cell-cycle regulators, including p16/Ink4a and p21/Cdkn1a. It is important to note that although we did not observe a deficiency in naphthalene-induced regeneration in Hdac2-deficient animals (data not shown), the naphthalene model of secretory cell depletion and regeneration is not a recapitulation of human COPD but rather represents an acute injury and regeneration process. Nonetheless, it is conceivable that the more chronic injury and repair process that underlies human exposure to smoke and other pollutants may lead to a progressive degradation in the ability of HDAC2-deficient airway epithelium to successfully regenerate. Our data suggest that small molecule therapies that alter the balance of histone acetylation may prove useful in treatment of lung diseases such as COPD.

**EXPERIMENTAL PROCEDURES**

Additional details on the Experimental Procedures can be found in the Supplemental Experimental Procedures.

**Microarray Analysis**

RNA was isolated from E12.5 lungs from ShhCre control and Hdac1/2ShhCreDKO embryos. For each sample, six lung buds were collected and pooled. The RNA was then used to generate a biotinylated cRNA probe library for Affymatrix Mouse Gene 1.0ST array. Microarray data were analyzed using the Oligo package available at the Bioconductor website (http://www.bioconductor.org). The raw data were background-corrected by the Robust Multichip Average (RMA) method and then normalized by an invariant set method. Differential gene expression between the control and mutant mice was analyzed by the Limma package available at the Bioconductor website. p values obtained from the multiple comparison tests were corrected by false discovery rates. Heatmap displays were created using the freely available MeV package (http://www.tm4.org/mev/). See Table S1 for the list of genes that were significantly altered. The microarray data have been deposited into the Gene Expression Omnibus database, and the accession number is GSE39946.

**Quantitative Real-Time PCR**

Total RNA was isolated from lungs at indicated time points using an RNaseasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from total RNA by using SuperScript Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using the SYBR green system (Applied Biosystems, Foster City, CA, USA) with primers listed in the Supplemental Experimental Procedures. GAPDH expression values were used to control for RNA quality and quantity. Five to six lungs were used for each embryonic lung quantitative real-time PCR experiment, and three to five lungs were used for each adult lung quantitative real-time PCR experiment, and data shown are the average ± SEM.

**ChIP Assays**

For ChIP assays on Hdac1/2 antibodies, at least 15 E12.5 lung buds were dissected and crosslinked by 3.7% formaldehyde. Crosslinked tissue was sonicated to obtain genomic DNA fragments between 200–400 bp. Chromatin was prepared using a ChIP Assay Kit (Millipore, Billerica, MA, USA); ChIP antibodies (Hdac1 and Hdac2; Abcam, Cambridge, UK) were used to precipitate the Hdac1/2 bound regions, which were then detected and quantified by PCR and quantitative real-time PCR with primers listed in the Supplemental Experimental Procedures. Rabbit IgG was used as a negative control antibody in these assays. The region of the Bmp4 promoter corresponds to approximately 400 bp upstream of the transcriptional start site, whereas the region of the Rb1 promoter corresponds to approximately 700 bp upstream of the Rb1 transcriptional start site. For ChIP assays using H3K9 acetylation antibodies (Abcam), MLE12 cells were transfected with control or Hdac1/2 siRNA (Dharmacon, Lafayette, CO, USA) and harvested after 48 hr to generate chromatin. E11.5 lung buds were cultured with or without 50nM TSA for 24 hr before harvesting to generate chromatin.

**Lung Explant Culture**

E11.5 lung buds were dissected from the embryos and then cultured as previously described (Goss et al., 2011) in the presence of either 500 ng/µl recombinant Bmp4 or BSA medium for 48 hr. The lung buds were then fixed in 2% PFA for histology or stored for RNA extraction.

**Naphthalene Injury**

Adult mice of 8- to 10-weeks-old were injected intraperitoneally with 250 mg/kg body weight of naphthalene dissolved in corn oil as previously described (Li et al., 2012). At indicated time points after injection, the right
Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.01.012.

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REFERENCES


