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## Development and regeneration of Sox2+ endoderm progenitors is regulated by a HDAC1/2-Bmp4/Rb1 regulatory pathway

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### Abstract

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 de-repression 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

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progenitor cells during both development and adult homeostasis. Ablation of Sox2-expressing cells in the adult disrupts tissues homeostasis leading to multi-organ failure and lethality (Arnold et al., 2011). However, the mechanisms regulating maintenance and differentiation of Sox2<sup>+</sup> 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 (Hdacs) 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; Ito et al., 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 development of the proximal airways of the lung. The loss of Sox2 expression is caused, in part, by the de-repression of Bmp4 expression, a direct target gene of Hdac1/2. Increased Bmp4 expression leads to decreased Sox2<sup>+</sup> proximal progenitors and an expansion of Sox9<sup>+</sup>/Id2<sup>+</sup> 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 de-repression of Rb1 and inhibition of cell cycle progression. In the postnatal lung, airway epithelial loss of Hdac1/2 expression does not lead to Bmp4 de-repression or changes in homeostatic Sox2 expression. However, in a model of airway injury and regeneration, loss of Hdac1/2 expression de-represses Rb1 expression, along with p21/Cdkn1a, and p16/Ink4a, leading to a persistent loss of Sox2<sup>+</sup> epithelial cell regeneration after injury. These data show that Sox2<sup>+</sup> progenitors in the lung are regulated by Hdac1/2 during development and regeneration through de-repression of Bmp4 and Rb1 in a stage specific fashion, providing a differential role for chromatin remodeling factors in endoderm development and regeneration.

## 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 (Fig. 1A and D). Hdac1 continues to be broadly expressed at later stages in both the endoderm and mesenchyme of the lung (Fig. 1B and C). In contrast, Hdac2 expression decreases in the mesenchyme after E12.5 and is expressed primarily in proximal airway epithelium by E17.5 (Fig. 1E and F). Hdac2 expression was detected in both secretory cells and ciliated cells in the adult lungs (Supplemental Fig. S1M-P). 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.

## 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 Hdac1<sup>flox/flox</sup> and Hdac2<sup>flox/flox</sup> alleles and the Shh<sup>cre</sup> 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 Hdac1/2<sup>ShhcreDKO</sup>. Hdac1 and Hdac2 were efficiently deleted in the mutant lung epithelial cells from E12.5 and later (Fig. 1G-L). Hdac1/2<sup>ShhcreDKO</sup> mutants all died at birth due to respiratory distress (data not shown) and examination of lungs from E17.5 Hdac1/2<sup>ShhcreDKO</sup> mutants showed a severe loss of branching morphogenesis resulting in large dilated sac like structures instead of well-formed lung lobes (Fig. 1R and S). To assess the chronological onset of these defects, we examined Hdac1/2<sup>ShhcreDKO</sup> lungs and controls at E10.5 and E12.5. At E10.5, the trachea of Hdac1/2<sup>ShhcreDKO</sup> mutants had separated from the esophagus and the first branching event that generates the main stem bronchi had occurred normally (Supplemental Fig. S1A-F). However, while control lungs had begun to branch extensively by E12.5, Hdac1/2<sup>ShhcreDKO</sup> mutant lungs exhibited a severe inhibition of branching (Fig. 1M and P). This defect in branching morphogenesis was also obvious at E14.5 (Fig. 1N and Q). 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 Hdac1/2<sup>ShhcreDKO</sup> 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 Hdac1/2<sup>ShhcreDKO</sup> mutants expressed Nkx2.1 while the esophagus in both control and Hdac1/2<sup>ShhcreDKO</sup> mutants expressed p63 (Fig. 1T and U). Nkx2.1 continued to be expressed in the abnormal lung epithelium of Hdac1/2<sup>ShhcreDKO</sup> mutants at E17.5 (Fig. 1V and W) 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 Hdac1/2<sup>ShhcreDKO</sup> mutant lungs at E12.5. 149 genes were down-regulated and 70 genes were up-regulated more than 1.4-fold in Hdac1/2<sup>ShhcreDKO</sup> 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 Hdac1/2<sup>ShhcreDKO</sup> mutant lungs (Fig. 2A). The developing airways of the lung are patterned in a distinct proximal-distal manner with Sox2 expression marking the proximal airway progenitor population which 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., 2011; Tompkins et al., 2009). Conversely, Sox9 and Id2 mark distal endoderm progenitors in the early lung (Rawlins et al., 2009). Although Sox9+/Id2+ distal progenitors are multi-potent prior to E13.5, their progenitor capacity is restricted to generating distal alveolar epithelial lineages after this time point (Rawlins et al., 2009) (Fig. 2B).

Immunostaining of a series of histological sections from the anterior to posterior regions of both control and Hdac1/2<sup>ShhcreDKO</sup> lungs showed a significant loss of Sox2 protein expression at E12.5 (Fig. 2C). This was confirmed by Q-PCR (Fig. 2D). At E10.5, a small

number of Sox2<sup>+</sup> cells were observed in the dorsal aspect of the developing trachea in both control and Hdac1/2<sup>ShhcreDKO</sup> mutant lungs, although the number of Sox2<sup>+</sup> cells appeared slightly reduced in the Hdac1/2<sup>ShhcreDKO</sup> mutants (Supplemental Fig. S1C and F). This is likely due to the incomplete loss of Hdac1/2 protein prior to E11.5 (Supplemental Fig. S1A, B, D, E), despite the fact that Shh<sup>cre</sup> driven 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 Shh<sup>cre</sup> line (Supplemental Fig. S1G-J), yet Sox2 expression did not appear to be affected in the esophagus (Supplemental Fig. S1K and L). 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 Hdac1/2<sup>ShhcreDKO</sup> mutant lungs, we examined the expression of Sox9 by immunostaining. Sox9 expression was expanded throughout both the proximal and distal regions of Hdac1/2<sup>ShhcreDKO</sup> mutant lungs (Fig. 2E). Q-PCR showed that levels of Sox9 expression in the whole lung were not significantly altered in Hdac1/2<sup>ShhcreDKO</sup> mutant lungs (Fig. 2F), possibly due to decreased Sox9 expression on a per cell basis in Sox9<sup>+</sup> 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 Hdac1/2<sup>ShhcreDKO</sup> mutant lungs as shown by both *in situ* and Q-PCR (Fig. 2F and Supplemental Fig. 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., 2009; Que et al., 2007; Tompkins et al., 2011; Tompkins et al., 2009). To assess whether Hdac1/2<sup>ShhcreDKO</sup> 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 Hdac1/2<sup>ShhcreDKO</sup> mutant lungs (Fig. 2G-J). 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 Hdac1/2<sup>ShhcreDKO</sup> mutant lungs (Fig. 2K-N and Supplemental Fig. S2D-G). Expression of the goblet cell marker gene Clca3 was not changed in Hdac1/2<sup>ShhcreDKO</sup> mutant lungs (Supplemental Fig. S2H and I). In contrast, distal lineage markers including T1alpha (alveolar type I cells) and Sftpc (alveolar type II cells) were still present in the mutant lungs (Fig. 2M and Supplemental Fig. 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<sup>+</sup> 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<sup>+</sup> 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., 2000; Weaver et al., 1999; Yin et al., 2008). These studies revealed that Bmp4 expression was significantly up-regulated in Hdac1/2<sup>ShhcreDKO</sup> mutants (Fig. 3A). *In situ* hybridization showed that Bmp4 expression was expanded throughout the early endoderm of Hdac1/2<sup>ShhcreDKO</sup> mutants (Fig. 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., 2000; Weaver et al., 1999). Expression of members of the Wnt and Fgf pathways as well as downstream effectors of these pathways were not significantly altered in Hdac1/2<sup>ShhcreDKO</sup> mutants. Expression of Notch1 was slightly up-regulated whereas expression of the Notch effector Hes1 was slightly down-regulated. However, such minor and inconsistent alterations in expression of Notch signaling components suggests that Notch is not significantly disrupted in Hdac1/2<sup>ShhcreDKO</sup> 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 over-expression 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 hours. Exogenous Bmp4 caused a decrease in both the expression and number of Sox2+ cells in the lung (Fig. 3C). This decrease resulted in inhibition of proximal airway endoderm development as noted by decreased Scgb3a2 and SSEA1 expression (Fig. 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 (Fig. 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 (Fig. 3C). Of note, these changes in gene expression and spatial localization of Sox9+ cells were all similar to what was observed in Hdac1/2<sup>ShhcreDKO</sup> mutants (Fig. 2).

Previous studies have shown that increased Bmp4 expression increases apoptosis in multiple tissues including the lung (Bellusci et al., 1996). Hdac1/2<sup>ShhcreDKO</sup> mutant lungs exhibited an increase in apoptosis from E11.5 through E14.5 as noted by increased activated caspase-3 immunostaining (Supplemental Fig. S3A and B). Treatment of wild-type lung explants with exogenous Bmp4 also increased apoptosis (Supplemental Fig. S3C and D). As in Hdac1/2<sup>ShhcreDKO</sup> 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 (Fig. 3D and E, and 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 (Fig. 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 (Fig. 3F) (Wikenheiser et al., 1993). Loss of Hdac1/2 expression led to increased H3K9 acetylation on the Bmp4 promoter (Fig. 3G), while acetylation was unaffected in an unrelated intergenic region upstream of Bmp4 (Fig. 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 (Fig. 3I), without altering the acetylation level at the intergenic region (Fig. 3J). Together, these data indicate that Bmp4 is a direct target of Hdac1/2 during lung endoderm development and its de-repression leads to down-regulation of Sox2 expression, expansion of Sox9+/Id2+ progenitors, and increased endoderm apoptosis.

To determine if reducing the dose of Bmp4 would partially rescue the loss of Sox2 expression in Hdac1/2<sup>ShhCreDKO</sup> mutants, we deleted one copy of *Bmp4* in the



Hdac1/2<sup>ShhCreDKO</sup> mutants using a floxed allele of Bmp4 (Kulesa and Hogan, 2002). We then performed IHC for Sox2 in the Hdac1/2<sup>ShhCreDKO:Bmp4 $\Delta$ /+</sup> lungs at E12.5 to assess the expression of Sox2. While Sox2 expression was not detectable in any of the Hdac1/2<sup>ShhCreDKO</sup> lungs we have examined (n=7), we found that 50% of Hdac1/2<sup>ShhCreDKO:Bmp4 $\Delta$ /+</sup> lungs (n=6) showed partial restoration of Sox2 expression in the bronchi and airway epithelium upon deletion of one copy of Bmp4 (Fig 3K-3P). Epithelial apoptosis was also reduced in the Hdac1/2<sup>ShhCreDKO:Bmp4 $\Delta$ /+</sup> lungs (Supplemental Fig. S3E and F). Overall lung structure, however, was not fully restored although additional branch points were evident in a subset of the compound mutant lungs (Supplemental Fig. S3G-K). 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 Hdac1/2<sup>ShhCreDKO</sup> 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 Hdac1/2<sup>ShhcreDKO</sup> mutant lungs as shown by phospho-histone H3 staining and the decrease in proliferation was uniform throughout the mutant lungs (Fig. 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., 2003; Lagger et al., 2002; 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 Hdac1/2<sup>ShhcreDKO</sup> mutants. In addition to increased expression of p21/Cdkn1a, we observed increased expression of the tumor suppressor retinoblastoma-1 (Rb1) in Hdac1/2<sup>ShhcreDKO</sup> mutant lungs (Fig. 4C). Q-PCR and immunostaining showed that Rb1 as well as p16/Ink4a and p21/Cdkn1a were all increased in Hdac1/2<sup>ShhcreDKO</sup> mutant lungs (Fig. 4D-J). p57, another cell cycle regulator that has been reported to be a direct target of Hdac1/2, was not up-regulated in the Hdac1/2<sup>ShhcreDKO</sup> mutant lungs and was not expressed at significant levels in the control lungs (data not shown and (Yamaguchi et al., 2010).

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 (Fig. 4K and L). siRNA mediated knock-down of Hdac1/2 in MLE12 cells or TSA treatment of lung bud explants resulted in increased H3K9 acetylation at the Rb1 promoter (Fig. 4M and N). 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 regulation 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 Hdac1/2<sup>ScgblalcreDKO</sup> mutants using an Scgblal<sup>cre</sup> line which is active in the proximal airway secretory epithelium beginning at birth (Li et al., 2012). Hdac1/2 were efficiently deleted in Hdac1/2<sup>ScgblalcreDKO</sup> lungs as

shown by immunostaining of eight week old mutant lungs (Fig. 5A-D). 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 Hdac1/2<sup>Scgbl1creDKO</sup> mutants (Fig. 5E-M). Proliferation and apoptosis was also not affected in Hdac1/2<sup>Scgbl1creDKO</sup> mutants (data not shown). Importantly, expression of Sox2, which is expressed in the postnatal secretory lineage, was not altered in the lungs of Hdac1/2<sup>Scgbl1creDKO</sup> mutants (Fig. 5G, K, M). 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) (Fig. 6A). Hdac1/2<sup>Scgbl1creDKO</sup> mutant and Scgbl1<sup>cre</sup> control mice were treated with naphthalene and the epithelial regeneration process was assessed at multiple time points using immunostaining and Q-PCR for Scgbl1 (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 Hdac1/2<sup>Scgbl1creDKO</sup> and control lungs were depleted as shown by the dramatic decrease in Scgbl1 and Sox2 expression (Fig. 6B-E). Ten days post-injury, while a significant recovery in the number of secretory cells was observed in Scgbl1<sup>cre</sup> control lungs, Hdac1/2<sup>Scgbl1creDKO</sup> mutants displayed a dramatic inhibition in regeneration of Scgbl1<sup>+</sup>/Sox2<sup>+</sup> secretory cells after injury (Fig. 6F and G). Examination of Hdac1/2<sup>Scgbl1creDKO</sup> mutant lung airways using H+E staining showed that they lacked extensive repopulation of secretory cells ten days after injury, in contrast to control airways which had begun to repopulate with secretory epithelium. These alterations in Scgbl1<sup>+</sup>/Sox2<sup>+</sup> cells were also observed at one month after injury suggesting that the loss of regeneration is persistent and not transient (Fig. 6H and I). Beta-tubulin IV expression showed a slight decrease in ciliated cells in the mutant lungs by Q-PCR at ten days post-injury and normal expression level at 1 month, consistent with the fact that naphthalene specifically depletes the secretory lineage (Fig. 6F-I). Sox2 expression in ciliated cells was decreased in the Hdac1/2<sup>Scgbl1creDKO</sup> mutants both at ten days and 1 month after injury (Supplemental Fig. S4A-F), further exacerbating the overall decrease in Sox2 expression in these mutants. Goblet cell markers Mucin5ac and Agr2 were not changed in the Hdac1/2<sup>Scgbl1creDKO</sup> mutant lungs prior to or during the regeneration process indicating a lack of an effect on goblet cell differentiation in these mutants (Supplemental Fig. S4G-M).

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. Q-PCR and *in situ* hybridization did not reveal changes in Bmp4 expression in Hdac1/2<sup>Scgbl1creDKO</sup> lungs (Supplemental Fig. S5 and data not shown). In contrast, proliferation of regenerating airway epithelium of Hdac1/2<sup>Scgbl1creDKO</sup> mutant lungs was significantly compromised during the course of regeneration, especially between day 4 and day 10 post-injury when the naphthalene resistant facultative progenitors expand to regenerate the airway epithelium (Fig. 7A and B). 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 (Fig. 7C-H). Of note, expression of these proteins was not altered in Hdac1/2<sup>Scgbl1creDKO</sup> lungs prior to injury (Supplemental Fig. S5), suggesting that Hdac1/2 regulation of Rb1, p21/Cdkn1a, and p16/

Ink4a is only re-activated during the regeneration process. These data indicate that Hdac1/2 regulate lung endoderm development 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. De-repression 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 signaling activity inhibits Sox2 expression (Domany 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 de-repression of p16/Ink4a and p21/Cdkn1a (Wilting 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 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 de-repression is associated with decreased



proliferation. Unlike Bmp4, Rb1 is also up-regulated 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; Ito et al., 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; Nyunoya et al., 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 while 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.

## METHODS AND MATERIALS

Additional details on methods and materials can be found in Supplemental Information.

### Microarray Analysis

RNA was isolated from E12.5 lungs from *Shh<sup>cre</sup>* control and *Hdac1/2<sup>ShhCreDKO</sup>* 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 ([www.bioconductor.org](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 has been deposited into the Gene Expression Omnibus database and the accession number is GSE39946.

### Quantitative PCR

Total RNA was isolated from lungs at indicated time points using RNeasy Mini Kit (Qiagen). cDNA was synthesized from total RNA by using SuperScript Strand Synthesis

System (Invitrogen). Quantitative PCR was performed using the SYBR green system (Applied Biosystems) with primers listed in the Supplementary Experimental Procedures. GAPDH expression values were used to control for RNA quality and quantity. 5-6 lungs were used for each embryonic lung Q-PCR experiment and 3-5 lungs were used for each adult lung Q-PCR experiment and data shown are the average  $\pm$  standard error of mean (S.E.M.).

### ChIP Assays

For ChIP assay on Hdac1/2 antibodies, at least 15 E12.5 lung buds were dissected and cross-linked by 3.7% formaldehyde. Cross-linked tissue was sonicated to obtain genomic DNA fragments between 200-400bp. Chromatin was prepared using a ChIP Assay Kit (Millipore). ChIP antibodies (Hdac1 and Hdac2, Abcam) were used to precipitate the Hdac1/2 bound regions, which was then detected and quantified by PCR and Q-PCR with primers listed in Supplementary 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 while 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) and harvested after 48 hours to generate chromatin. E11.5 lung buds were cultured with or without 50nM TSA for 24 hours 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 500ng/ $\mu$ L recombinant Bmp4 or BSA medium for 48 hours. The lung buds were then fixed in 2% PFA for histology or stored for RNA extraction.

### Naphthalene Injury

Adult mice of 8-10 weeks old were injected intraperitoneally with 250mg/kg body weight of naphthalene dissolved in corn oil as previously described (Li et al., 2012). At indicated time points after injection, the right lung lobe was collected for RNA extraction and left lobes were inflation fixed with 10% formalin for histological sections. Scgb1a1<sup>cre</sup> mice were used as control mice.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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### REFERENCES

- Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, Seandel M, Geijsen N, Hochedlinger K. Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. *Cell Stem Cell*. 2011; 9:317–329. [PubMed: 21982232]
- Bellusci S, Henderson R, Winnier G, Oikawa T, Hogan BL. Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development*. 1996; 122:1693–1702. [PubMed: 8674409]

- Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T. Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*. 1998; 391:597–601. [PubMed: 9468139]
- Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, Sougnez C, Greulich H, Muzny DM, Morgan MB, et al. Somatic mutations affect key pathways in lung adenocarcinoma. *Nature*. 2008; 455:1069–1075. [PubMed: 18948947]
- Domyan ET, Ferretti E, Throckmorton K, Mishina Y, Nicolis SK, Sun X. Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2. *Development*. 2011; 138:971–981. [PubMed: 21303850]
- Giangreco A, Reynolds SD, Stripp BR. Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. *Am J Pathol*. 2002; 161:173–182. [PubMed: 12107102]
- Goss AM, Tian Y, Cheng L, Yang J, Zhou D, Cohen ED, Morrissey EE. Wnt2 signaling is necessary and sufficient to activate the airway smooth muscle program in the lung by regulating myocardin/Mrtf-B and Fgf10 expression. *Dev Biol*. 2011; 356:541–552. [PubMed: 21704027]
- Goss AM, Tian Y, Tsukiyama T, Cohen ED, Zhou D, Lu MM, Yamaguchi TP, Morrissey EE. Wnt2/2b and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. *Dev Cell*. 2009; 17:290–298. [PubMed: 19686689]
- Harfe BD, Scherz PJ, Nissim S, Tian H, McMahon AP, Tabin CJ. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell*. 2004; 118:517–528. [PubMed: 15315763]
- Ito K, Ito M, Elliott WM, Cosio B, Caramori G, Kon OM, Barczyk A, Hayashi S, Adcock IM, Hogg JC, et al. Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *N Engl J Med*. 2005; 352:1967–1976. [PubMed: 15888697]
- Ito K, Yamamura S, Essilfie-Quaye S, Cosio B, Ito M, Barnes PJ, Adcock IM. Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF-kappaB suppression. *J Exp Med*. 2006; 203:7–13. [PubMed: 16380507]
- Izvolosky KI, Shoykhet D, Yang Y, Yu Q, Nugent MA, Cardoso WV. Heparan sulfate-FGF10 interactions during lung morphogenesis. *Dev Biol*. 2003; 258:185–200. [PubMed: 12781692]
- Kulesa H, Hogan BL. Generation of a loxP flanked bmp4loxP-lacZ allele marked by conditional lacZ expression. *Genesis*. 2002; 32:66–68. [PubMed: 11857779]
- Lagger G, Doetzelhofer A, Schuettengruber B, Haidweger E, Simboeck E, Tischler J, Chiocca S, Suske G, Rotheneder H, Wintersberger E, et al. The tumor suppressor p53 and histone deacetylase 1 are antagonistic regulators of the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 gene. *Mol Cell Biol*. 2003; 23:2669–2679. [PubMed: 12665570]
- Lagger G, O'Carroll D, Rembold M, Khier H, Tischler J, Weitzer G, Schuettengruber B, Hauser C, Brunmeir R, Jenuwein T, et al. Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. *EMBO J*. 2002; 21:2672–2681. [PubMed: 12032080]
- LeBoeuf M, Terrell A, Trivedi S, Sinha S, Epstein JA, Olson EN, Morrissey EE, Millar SE. Hdac1 and Hdac2 act redundantly to control p63 and p53 functions in epidermal progenitor cells. *Dev Cell*. 2010; 19:807–818. [PubMed: 21093383]
- Li S, Wang Y, Zhang Y, Lu MM, Demayo FJ, Dekker JD, Tucker PW, Morrissey EE. Foxp1/4 control epithelial cell fate during lung development and regeneration through regulation of anterior gradient 2. *Development*. 2012; 139:2500–2509. [PubMed: 22675208]
- Linker C, Stern CD. Neural induction requires BMP inhibition only as a late step, and involves signals other than FGF and Wnt antagonists. *Development*. 2004; 131:5671–5681. [PubMed: 15509767]
- Lu MM, Yang H, Zhang L, Shu W, Blair DG, Morrissey EE. The bone morphogenic protein antagonist gremlin regulates proximal-distal patterning of the lung. *Dev Dyn*. 2001; 222:667–680. [PubMed: 11748835]
- Ma P, Pan H, Montgomery RL, Olson EN, Schultz RM. Compensatory functions of histone deacetylase 1 (HDAC1) and HDAC2 regulate transcription and apoptosis during mouse oocyte development. *Proc Natl Acad Sci U S A*. 2012; 109:E481–489. [PubMed: 22223663]

- Magnaghi-Jaulin L, Groisman R, Naguibneva I, Robin P, Lorain S, Le Villain JP, Troalen F, Trouche D, Harel-Bellan A. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature*. 1998; 391:601–605. [PubMed: 9468140]
- Montgomery RL, Davis CA, Potthoff MJ, Haberland M, Fielitz J, Qi X, Hill JA, Richardson JA, Olson EN. Histone deacetylases 1 and 2 redundantly regulate cardiac morphogenesis, growth, and contractility. *Genes Dev*. 2007; 21:1790–1802. [PubMed: 17639084]
- Nyunoya T, Monick MM, Klingelhutz A, Yarovinsky TO, Cagley JR, Hunninghake GW. Cigarette smoke induces cellular senescence. *Am J Respir Cell Mol Biol*. 2006; 35:681–688. [PubMed: 16840774]
- Nyunoya T, Monick MM, Klingelhutz AL, Glaser H, Cagley JR, Brown CO, Matsumoto E, Aykin-Burns N, Spitz DR, Oshima J, et al. Cigarette smoke induces cellular senescence via Werner's syndrome protein down-regulation. *Am J Respir Crit Care Med*. 2009; 179:279–287. [PubMed: 19011155]
- Park WY, Miranda B, Lebeche D, Hashimoto G, Cardoso WV. FGF-10 is a chemotactic factor for distal epithelial buds during lung development. *Dev Biol*. 1998; 201:125–134. [PubMed: 9740653]
- Que J, Luo X, Schwartz RJ, Hogan BL. Multiple roles for Sox2 in the developing and adult mouse trachea. *Development*. 2009; 136:1899–1907. [PubMed: 19403656]
- Que J, Okubo T, Goldenring JR, Nam KT, Kurotani R, Morrissey EE, Taranova O, Pevny LH, Hogan BL. Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development*. 2007; 134:2521–2531. [PubMed: 17522155]
- Rawlins EL, Clark CP, Xue Y, Hogan BL. The Id2+ distal tip lung epithelium contains individual multipotent embryonic progenitor cells. *Development*. 2009; 136:3741–3745. [PubMed: 19855016]
- Shu W, Guttentag S, Wang Z, Andl T, Ballard P, Lu MM, Piccolo S, Birchmeier W, Whitsett JA, Millar SE, et al. Wnt/beta-catenin signaling acts upstream of N-myc, BMP4, and FGF signaling to regulate proximal-distal patterning in the lung. *Dev Biol*. 2005; 283:226–239. [PubMed: 15907834]
- Sommer CA, Stadtfeld M, Murphy GJ, Hochedlinger K, Kotton DN, Mostoslavsky G. Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells*. 2009; 27:543–549. [PubMed: 19096035]
- Sutherland KD, Proost N, Brouns I, Adriaensen D, Song JY, Berns A. Cell of origin of small cell lung cancer: inactivation of Trp53 and Rb1 in distinct cell types of adult mouse lung. *Cancer Cell*. 2011; 19:754–764. [PubMed: 21665149]
- Tompkins DH, Besnard V, Lange AW, Keiser AR, Wert SE, Bruno MD, Whitsett JA. Sox2 activates cell proliferation and differentiation in the respiratory epithelium. *Am J Respir Cell Mol Biol*. 2011; 45:101–110. [PubMed: 20855650]
- Tompkins DH, Besnard V, Lange AW, Wert SE, Keiser AR, Smith AN, Lang R, Whitsett JA. Sox2 is required for maintenance and differentiation of bronchiolar Clara, ciliated, and goblet cells. *PLoS One*. 2009; 4:e8248. [PubMed: 20011520]
- Tsao PN, Vasconcelos M, Izvolsky KI, Qian J, Lu J, Cardoso WV. Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. *Development*. 2009; 136:2297–2307. [PubMed: 19502490]
- Weaver M, Dunn NR, Hogan BL. Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. *Development*. 2000; 127:2695–2704. [PubMed: 10821767]
- Weaver M, Yingling JM, Dunn NR, Bellusci S, Hogan BL. Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. *Development*. 1999; 126:4005–4015. [PubMed: 10457010]
- Wikenheiser KA, Vorbroker DK, Rice WR, Clark JC, Bachurski CJ, Oie HK, Whitsett JA. Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. *Proc Natl Acad Sci U S A*. 1993; 90:11029–11033. [PubMed: 8248207]
- Wilting RH, Yanover E, Heideman MR, Jacobs H, Horner J, van der Torre J, DePinho RA, Dannenberg JH. Overlapping functions of Hdac1 and Hdac2 in cell cycle regulation and haematopoiesis. *EMBO J*. 2010; 29:2586–2597. [PubMed: 20571512]

- Xu CR, Cole PA, Meyers DJ, Kormish J, Dent S, Zaret KS. Chromatin “prepattern” and histone modifiers in a fate choice for liver and pancreas. *Science*. 2011; 332:963–966. [PubMed: 21596989]
- Yamaguchi T, Cubizolles F, Zhang Y, Reichert N, Kohler H, Seiser C, Matthias P. Histone deacetylases 1 and 2 act in concert to promote the G1-to-S progression. *Genes Dev*. 2010; 24:455–469. [PubMed: 20194438]
- Yin Y, White AC, Huh SH, Hilton MJ, Kanazawa H, Long F, Ornitz DM. An FGF-WNT gene regulatory network controls lung mesenchyme development. *Dev Biol*. 2008; 319:426–436. [PubMed: 18533146]
- Zhao W, Dai F, Bonafede A, Schafer S, Jung M, Yusuf F, Gamel AJ, Wang J, Brand-Saberi B. Histone deacetylase inhibitor, trichostatin A, affects gene expression patterns during morphogenesis of chicken limb buds in vivo. *Cells Tissues Organs*. 2009; 190:121–134. [PubMed: 19147985]



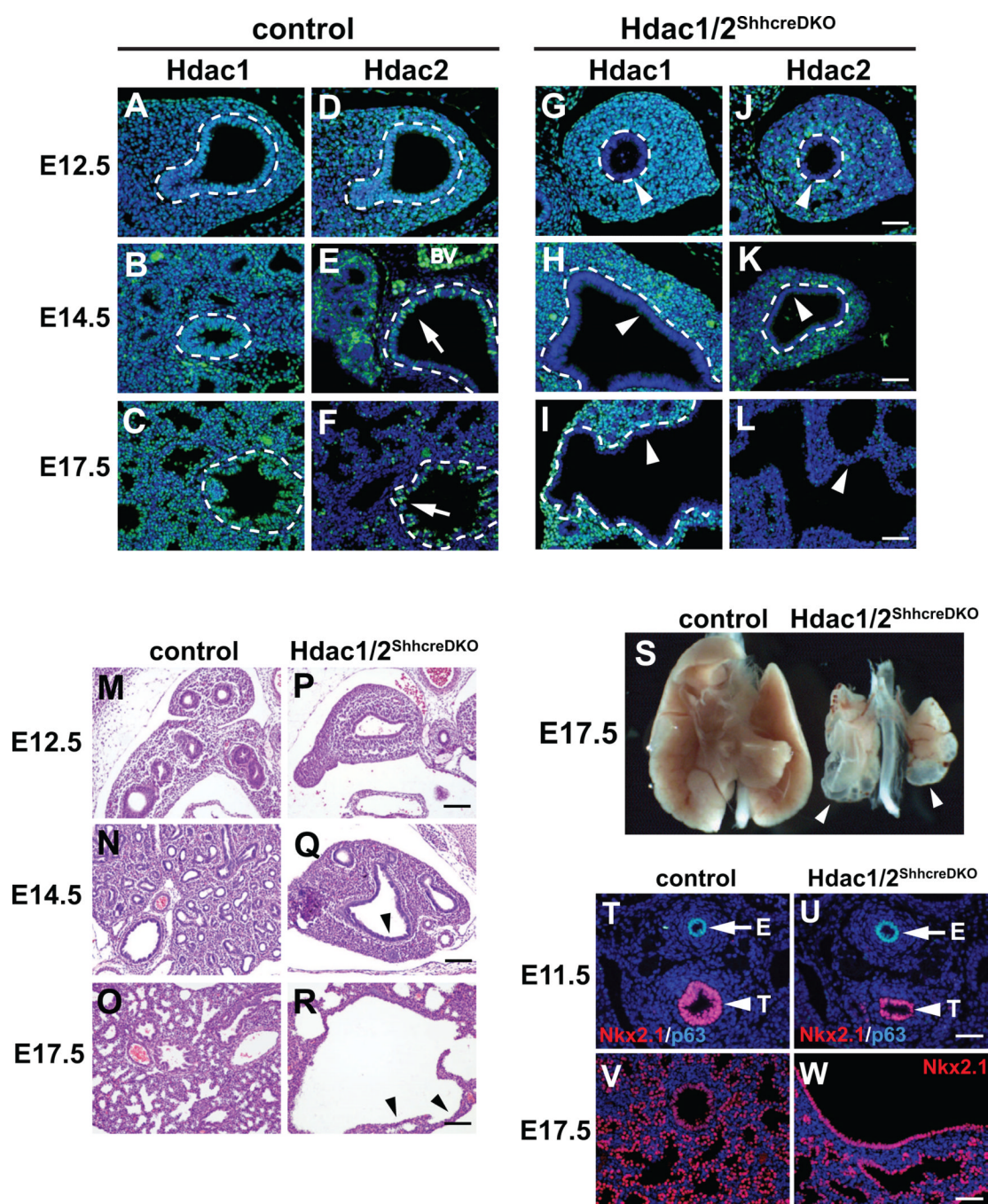
**Highlights**

Hdac1/2 regulate development of Sox2+ endoderm progenitors

Bmp4, Rb1, p21, and p16 are direct targets of Hdac1/2 in lung endoderm

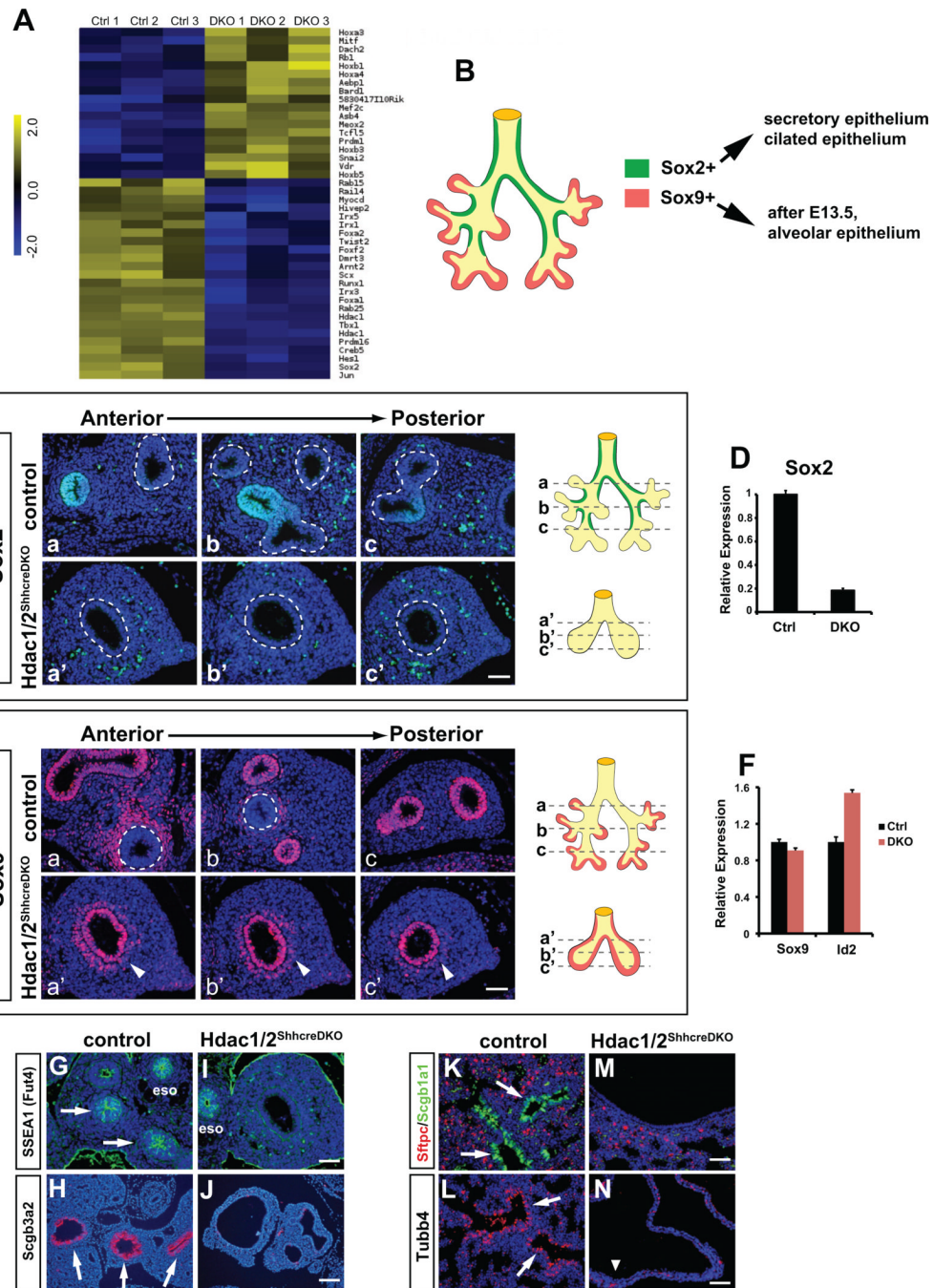
Increased Bmp4 suppresses Sox2 expression in lung endoderm

Hdac1/2 are required for lung secretory epithelial regeneration



**Figure 1. Loss of Hdac1/2 disrupts lung endoderm development but not endoderm identity**  
Hdac1 (A-C) is expressed broadly throughout the lung endoderm and mesenchyme from E12.5 through E17.5. Hdac2 is initially expressed in both lung endoderm and mesenchyme at E12.5 (D) but becomes progressively restricted to proximal airway epithelium by E17.5 (arrows in E and F). Hdac1/2<sup>ShhcreDKO</sup> mutants lack epithelial expression of Hdac1 and Hdac2 from E12.5 through E17.5 (arrowheads in G-L). Dotted lines outline the developing airway epithelium. In comparison to control littermates (M-O), Hdac1/2<sup>ShhcreDKO</sup> mutants display defects in airway branching as exhibited by formation of cysts in the lung at E12.5 (P) and these cysts expand in later development resulting in an overall smaller lung (Q-S, arrowheads). Hdac1/2<sup>ShhcreDKO</sup> mutants maintain proper lung and esophagus specification

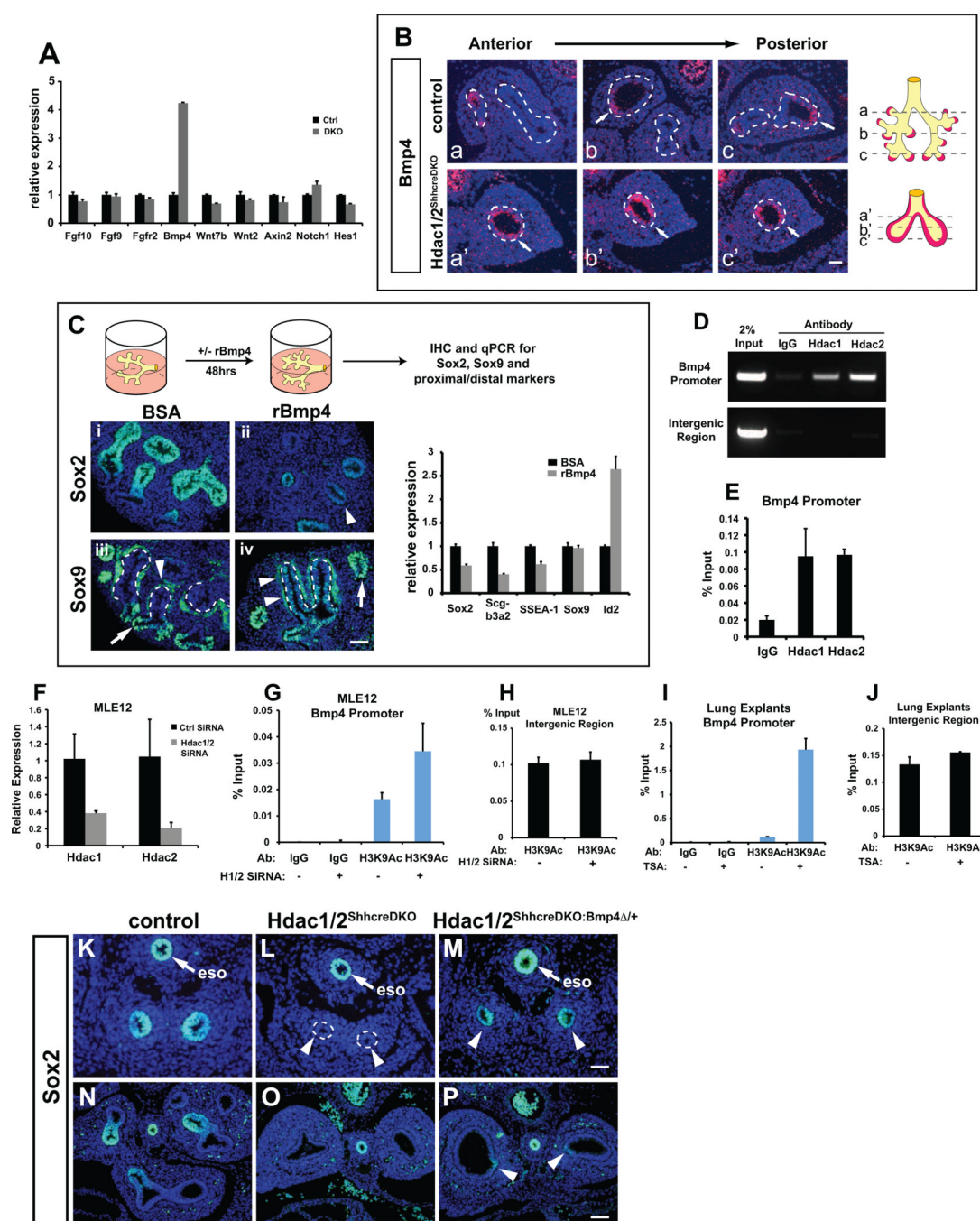
as noted by Nkx2.1 and p63 immunostaining at E11.5 (T and U). Lung endoderm identity is maintained through development as noted by continuous expression of Nkx2.1 (V and W). BV=blood vessel; E=esophagus; T=trachea. Scale bars: J, K, L, U and W =20 $\mu$ m; P, Q and R=40 $\mu$ m. See also Figure S1.



**Figure 2. Hdac1/2 are required for development of Sox2+ proximal lung endoderm progenitors**  
The heatmap generated from microarray results shows differential expression of 42 transcription factors in the Hdac1/2<sup>ShhcreDKO</sup> mutants compared to Shh<sup>cre</sup> controls including Sox2 (A). The relative spatial distribution of Sox2+ proximal lung endoderm progenitors (green) and Sox9+Id2+ distal lung progenitors (red) is schematized in B. Immunostaining of sections throughout the anterior to posterior regions of the lung shows that Hdac1/2<sup>ShhcreDKO</sup> mutants have a dramatic loss of Sox2 expression at E12.5 (C). The schemes on the rightmost panel of C demonstrate 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<sup>ShhcreDKO</sup> mutants. Q-PCR result confirm the loss

of Sox2 expression in Hdac1/2<sup>ShhcreDKO</sup> mutants at E12.5 (D). Hdac1/2<sup>ShhcreDKO</sup> mutants have expanded Sox9 expression throughout the dysmorphic airways (E, arrowheads). Q-PCR shows that while overall expression levels of Sox9 are unchanged, expression of the distal progenitor marker Id2 is increased in Hdac1/2<sup>ShhcreDKO</sup> mutants (F). Expression of the early proximal epithelium marker SSEA1 (Fut4), secretory epithelium lineage markers including Scgb3a2 and Scgb1a1, and markers of the ciliated epithelial lineage including Tubb4 (arrows in controls) are lost or severely decreased in Hdac1/2<sup>ShhcreDKO</sup> mutants (G-N). In contrast, expression of the alveolar type 2 cell marker Sftpc is still noted in Hdac1/2<sup>ShhcreDKO</sup> mutants (M). White dotted lines mark the boundary between epithelium and mesenchyme. Q-PCR data shown are the average of 5-6 assays  $\pm$  S.E.M.. Eso=esophagus. Scale bars: C, E, I, M and N=20 $\mu$ m; J=40 $\mu$ m. See also Figure S2.

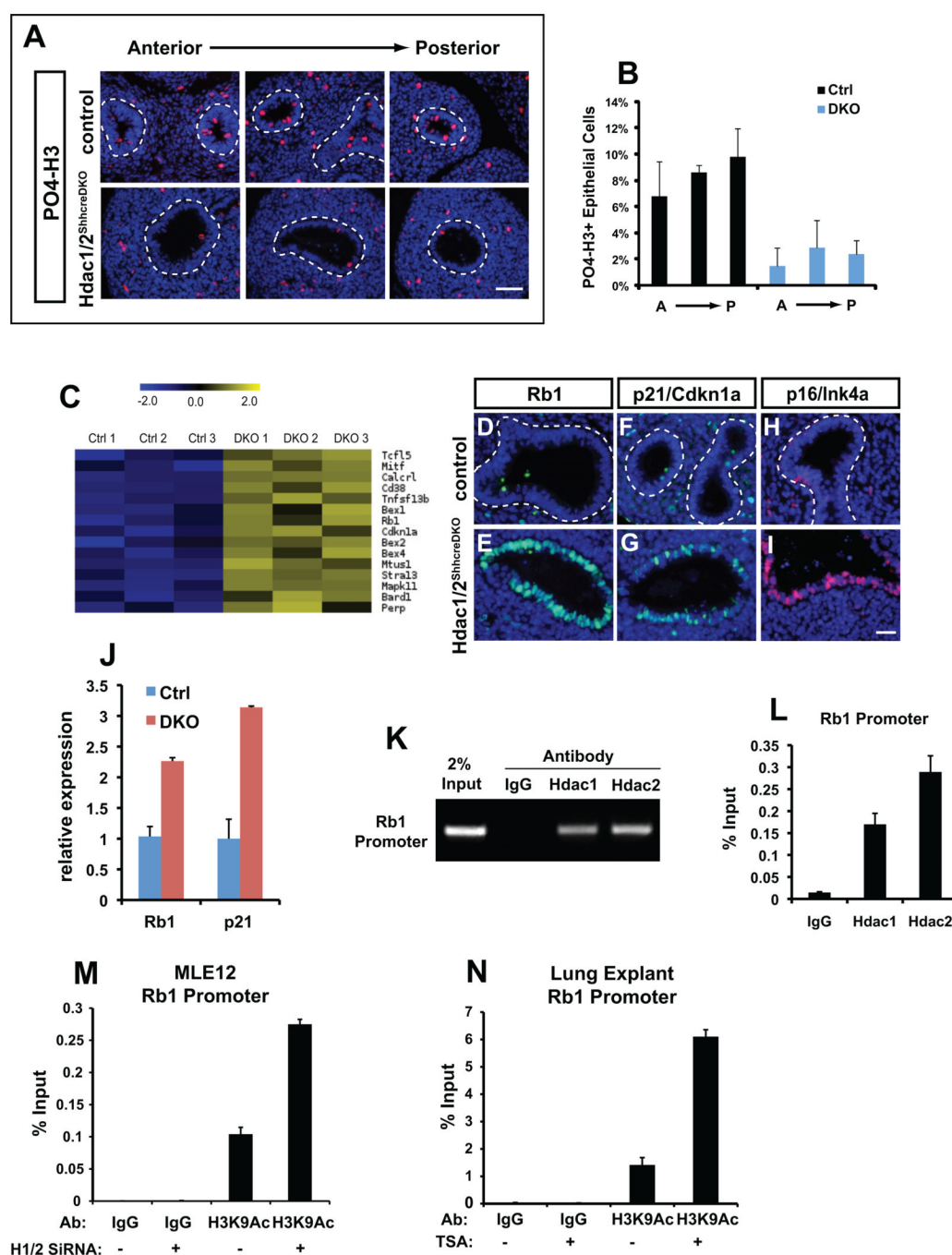




**Figure 3. Loss of Hdac1/2 results in increased Bmp4 expression which, in turn, represses Sox2 expression in the lung**

Q-PCR analysis of major signaling pathway components in Hdac1/2<sup>ShhcreDKO</sup> mutants shows a significant increase in Bmp4 expression (A). *In situ* hybridization shows increased and expanded Bmp4 expression throughout the entire dysmorphic lung endoderm of Hdac1/2<sup>ShhcreDKO</sup> mutants (B, arrows). Treatment of lung explant cultures at E11.5 with recombinant Bmp4 (rBmp4) for 48 hours suppresses Sox2 expression (C-ii, arrowhead) and expands Sox9+ cells in lung endoderm (C-iv, arrowheads). Note that Sox9 normally marks the distal lung epithelial progenitor cells (C-iii and C-iv, arrows) as well as proximal mesenchyme (C-iii, arrowhead). In rBmp4 treated explants, Sox9 expression is expanded

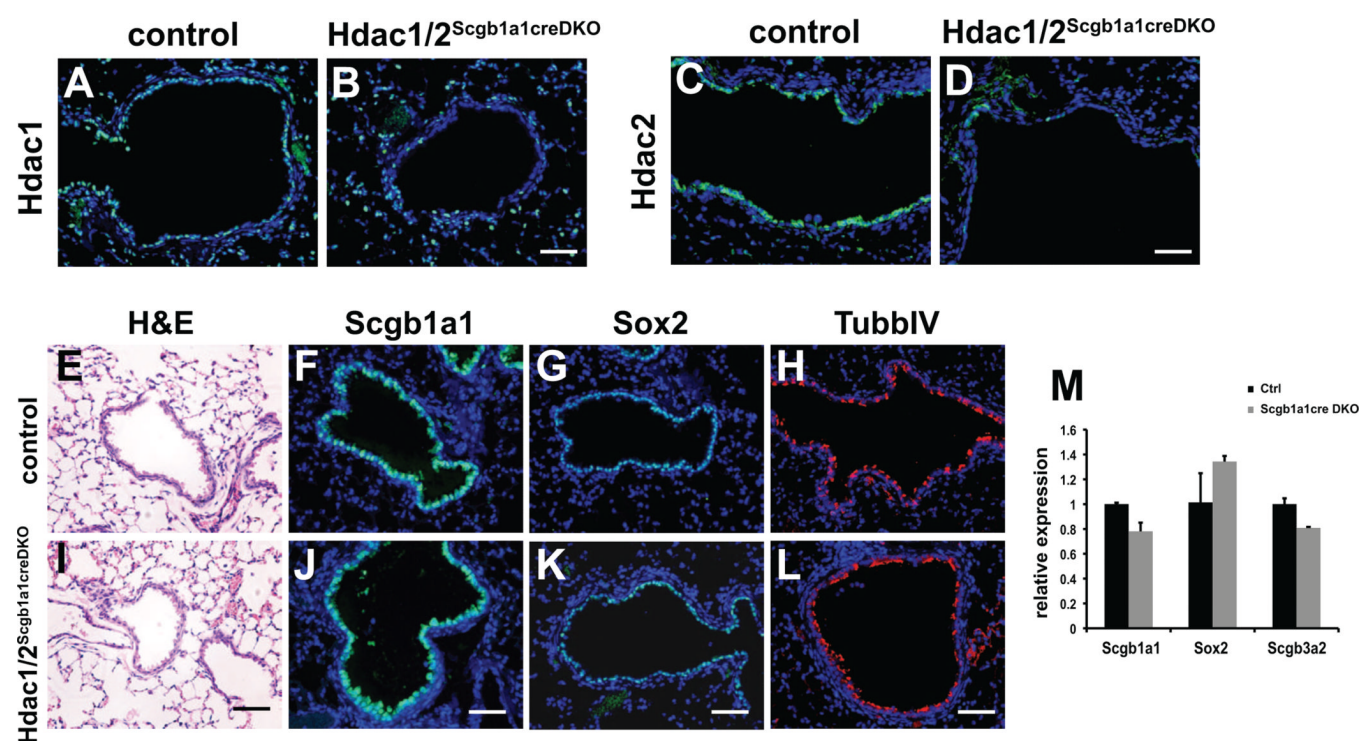
into the proximal airway epithelium of the lung explants (C-iv, arrowheads). Q-PCR shows decreased Sox2 expression along with a decrease in other early proximal epithelial markers including Scgb3a2 and SSEA1 (Fut4) in rBmp4 treated explants (C). Q-PCR also shows increased expression of Id2 in rBmp4 treated lung explants (C). ChIP assays using gel electrophoresis (D) and Q-PCR (E) show that Hdac1/2 directly bind to the Bmp4 promoter in the E12.5 lung. MLE12 cells were transfected with Hdac1/2 or control siRNAs to effectively and specifically inhibit Hdac1/2 expression (F). ChIP assays show increased H3K9 acetylation level at Bmp4 promoter after Hdac1/2 knock-down (G), while an intergenic region upstream of Bmp4 is not affected (H). TSA treated of E11.5 lung explants results in increased level of H3K9 acetylation at the Bmp4 promoter but not the intergenic region (I and J). Loss of one copy of Bmp4 partially rescues Sox2 expression in the Hdac1/2<sup>ShhcreDKO</sup> mutants (K-P). Q-PCR and ChIP PCR data shown are the average of 3-5 assays  $\pm$  S.E.M. Arrowheads in M and P indicate the partial restoration of Sox2 expression. Scale bars: B, C and H=20 $\mu$ m; P=40 $\mu$ m. See also Figure S3.



#### Figure 4. Rb1 is a direct target of Hdac1/2 repression

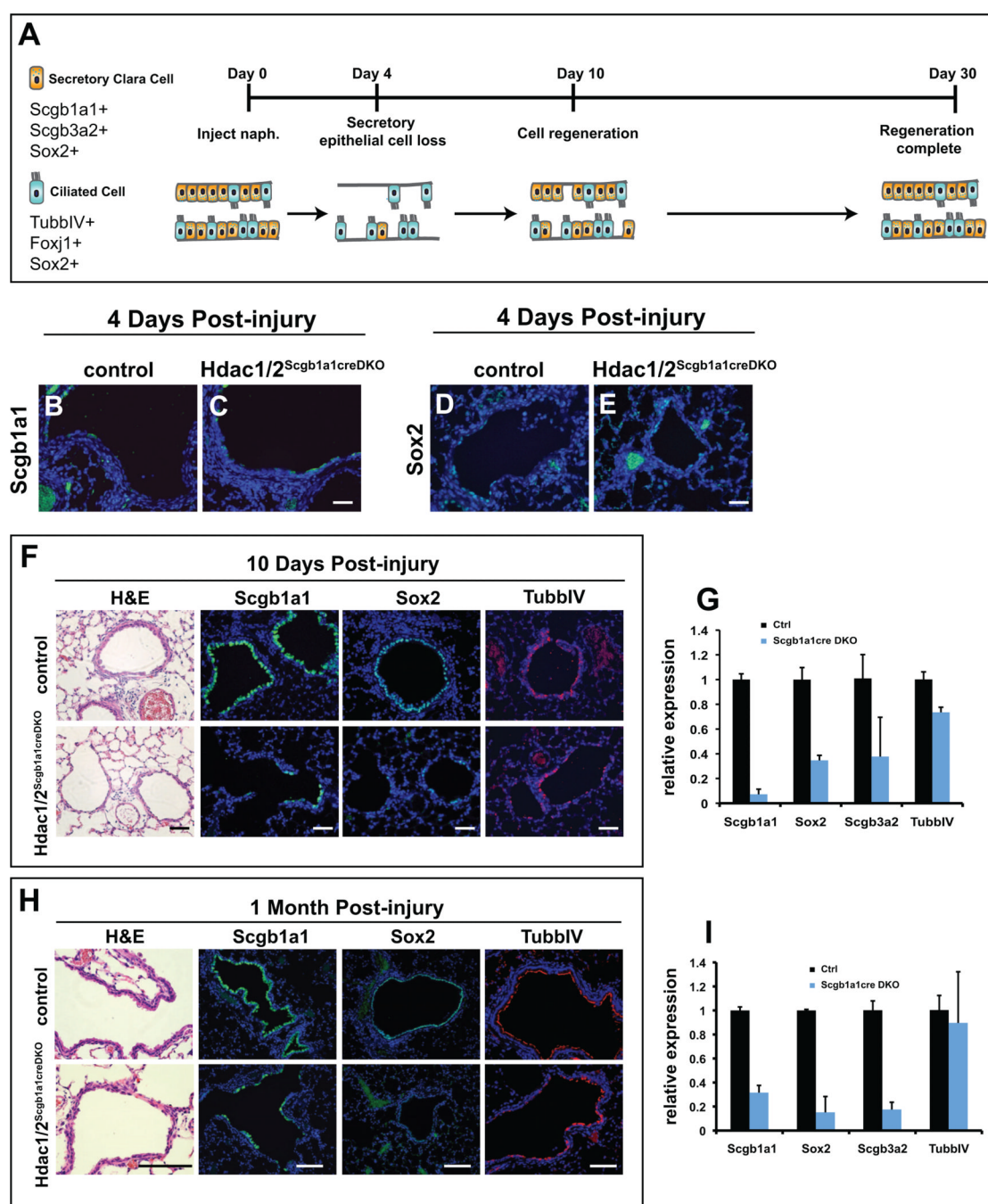
Phospho-histone H3 (PO4-H3) immunostaining shows that proliferation is decreased throughout Hdac1/2<sup>ShcreDKO</sup> mutant lung endoderm (A and B). Heatmap of genes associated with cell proliferation from Hdac1/2<sup>ShcreDKO</sup> mutants shows increased expression of Rb1 and p21/Cdkn1a (C). Immunostaining 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>ShcreDKO</sup> mutants (D-I). Q-PCR confirms the de-repression of Rb1 and p21/Cdkn1a transcripts at E12.5 (J). ChIP assays using gel electrophoresis and Q-PCR show that Hdac1/2 binds robustly to the Rb1 proximal promoter in E12.5 lungs (K and L). Hdac1/2 knock-down in MLE12 cells or treating lung bud

explants with TSA increases the H3K9 acetylation level at the Rb1 promoter (M and N). Q-PCR and ChIP PCR data shown are the average of 3-5 assays  $\pm$  S.E.M. Scale bar: A=20 $\mu$ m; D-I=10 $\mu$ m.



**Figure 5. Loss of Hdac1/2 expression does not disrupt postnatal airway epithelial homeostasis**  
Hdac1 (A and B) and Hdac2 (C and D) expression are significantly reduced in the airway epithelium of Hdac1/2<sup>Scgb1a1creDKO</sup> mutants compared to Scgb1a1<sup>cre</sup> controls. Loss of Hdac1/2 expression in Hdac1/2<sup>Scgb1a1creDKO</sup> mutants does not disrupt airway epithelial morphology by H+E staining (E and I). Loss of Hdac1/2 expression in Hdac1/2<sup>Scgb1a1creDKO</sup> mutants does not disrupt expression of secretory cell markers Scgb1a1 (F and J) and Sox2 (G and K). Loss of Hdac1/2 expression does not disrupt expression of the ciliated epithelial marker TubbIV (H and L). Q-PCR shows only a minor loss of expression for the secretory marker genes Scgb1a1 and Scgb3a2 and no significant change in Sox2 expression in Hdac1/2<sup>Scgb1a1creDKO</sup> mutants. Q-PCR data shown are the average of 3-5 assays  $\pm$  S.E.M. Scale bars=20 $\mu$ m.

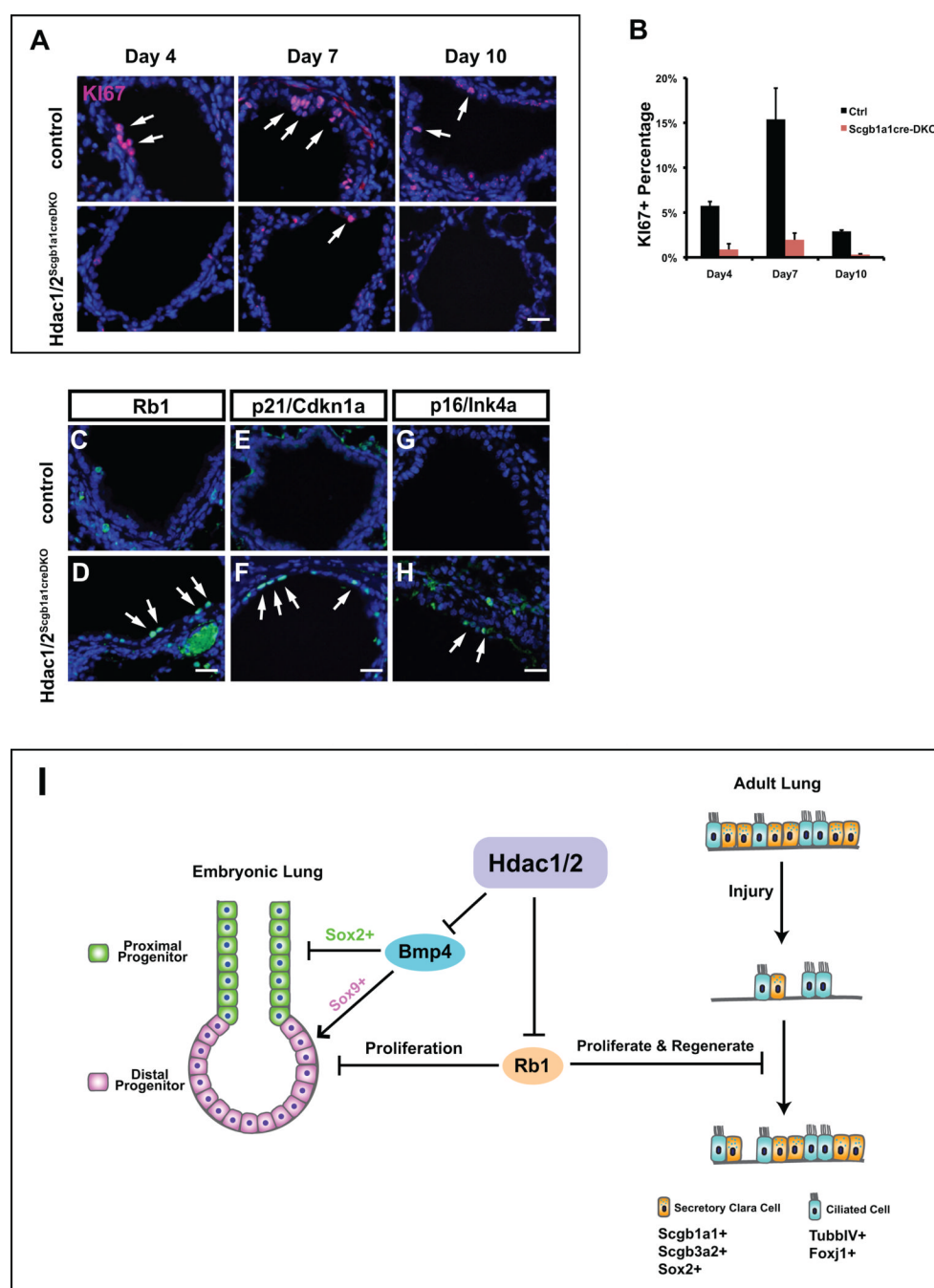




**Figure 6. Hdac1/2 are necessary for regeneration of Sox2+ airway epithelium**

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 one month after injury (A). Immunostaining for Scgb1a1 shows the severe loss in secretory epithelial cells four days post-injury in both Scgb1a1<sup>cre</sup> and Hdac1/2<sup>Scgb1a1cre</sup>DKO mutants (B and C). Ten days post-injury, Scgb1a1+/Sox2+ secretory cells in the airways of the lung have not regenerated in Hdac1/2<sup>Scgb1a1cre</sup>DKO mutants, while Scgb1a1<sup>cre</sup> controls have regenerated normally (F). Q-PCR shows a dramatic loss in Scgb1a1, Sox2, and Scgb3a2 expression with a much smaller loss in TubbIV expression 10 days post-injury (G). At one month post-injury, Hdac1/2<sup>Scgb1a1cre</sup>DKO mutants continue to lack regeneration of

Scgb1a1+/Sox2+ secretory epithelium (H). Q-PCR at one month post-injury confirms decreased Scgb1a1, Sox2, and Scgb3a2 expression while TubbIV expression is unchanged (I). Q-PCR data shown are the average of 3-5 assays  $\pm$  S.E.M. Scale bars: C, E and F= 20 $\mu$ m; H=40 $\mu$ m. See also Figure S4.



**Figure 7. Hdac1/2 promote airway epithelial regeneration through regulation of cell cycle regulators Rb1, p21/Cdkn1a and p16/Ink4a**

Cell proliferation as measured by Ki67 immunostaining is reduced by more than 80% during the airway epithelial regeneration process from days 4-10 in Hdac1/2<sup>Scgbl1creDKO</sup> mutants (A). Quantification of Ki67+ Cells against total bronchiolar epithelial cells in both control and Hdac1/2<sup>Scgbl1creDKO</sup> lungs (B). Rb1, p21/Cdkn1a and p16/Ink4a expression levels are up-regulated during the process of airway regeneration (C-F, arrows). (I) A model for Hdac1/2 action during both lung development and postnatal airway epithelial regeneration. Hdac1/2 inhibits Bmp4, which in turn inhibits Sox2+ proximal lung progenitor and promotes Sox9+ distal progenitor development. Hdac1/2 also regulate Rb1 which inhibits

endoderm proliferation during development and during lung regeneration. Cell counting data shown are the average of 3-4 samples  $\pm$  S.E.M. Scale bars=10 $\mu$ m. See also Figure S5.