Foxp1/2/4-NuRD Interactions Regulate Gene Expression and Epithelial Injury Response in the Lung via Regulation of Interleukin-6

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To determine the underlying mechanism of Foxp1/2/4-mediated transcriptional repression, a yeast two-hybrid screen was performed that identified p66β, a transcriptional repressor and component of the NuRD chromatin-remodeling complex. We show that direct interactions between Foxp1/4 and p66β are mediated by the CR2 domain within p66β and the zinc finger/leucine zipper repression domain found in Foxp1/2/4. These direct interactions are functionally relevant as overexpression of p66β in combination with Foxp factors cooperatively represses Foxp target gene expression, whereas loss of p66 and Foxp factors results in de-repression of endogenous Foxp target genes in lung epithelial cells. Moreover, the NuRD components HDAC1/2 associate in a macromolecular complex with Foxp proteins, and loss of expression or inhibition of HDAC1/2 activity leads to de-repression of Foxp target gene expression. Importantly, we show in vivo that Foxp1 and HDAC2 act cooperatively to regulate expression of the cytoprotective cytokine interleukin-6, which results in increased resistance to hyperoxic lung injury in Foxp1/HDAC2 compound mutant animals. These data reveal an important interaction between the Foxp transcription factors and the NuRD chromatin-remodeling complex that modulates transcriptional repression critical for the lung epithelial injury response.

Transcriptional regulation is dependent on the coordinated interaction of multiple protein factors within the chromatin environment. This interaction is orchestrated in a spatial and temporal manner that is critical for organogenesis as well as adult homeostasis. In tissues such as the lung, where the epithelial lining is directly exposed to the external environment, this process is likely to be dynamic, particularly in the adult where external insults occur continuously.

Multiple transcription factor families are involved in regulating lung development as well as lung injury repair and regeneration. One such family is the forkhead box (Fox) family of transcription factors, which has been conserved throughout evolution and currently includes more than 20 different members. All Fox proteins contain a winged-helix or forkhead domain that is responsible for mediating DNA binding. In the lung, several Fox gene subfamilies, including FoxA, -F, -J, -M, and -P, are expressed in either epithelial or mesenchymal cells during development, and these genes have been shown to play an important role in regulating gene expression and cell lineage specification (1–7). We have previously shown that the Foxp1/2/4 family is expressed in an overlapping pattern in the developing airway epithelium and adult (2, 4, 5). Foxp1/2/4 proteins are highly conserved and modular in structure with all three proteins containing long glutamine repeats in the N-terminal region, a central repression domain that contains a leucine zipper and zinc finger, and a forkhead/winged-helix DNA binding domain located in the C-terminal end of the protein. The Foxp proteins differ from other Fox family members in that they require protein dimerization through the leucine zipper region to bind DNA and effectively repress transcription (8). Although Foxp factors are known transcriptional repressors, their mechanism of action is only recently being elucidated and most likely involves interactions with other transcriptional modulators (9).

To characterize the molecular mechanisms underlying Foxp1/2/4 transcriptional repression, we utilized a yeast two-hybrid approach to identify protein partners of Foxp1/2/4 important for their function. From this screen, we isolated p66β, a component of the NuRD/MeCP1 chromatin-remodeling complex (10, 11). We show that p66β interacts with Foxp1/2/4 to repress gene transcription, and this repression involves HDAC1/2 and their associated histone deacetylase activities, demonstrating that HDAC1 and HDAC2 are both important mediators of Foxp mediated transcriptional regulation. We also show in vivo that Foxp1-HDAC2 interactions are important for the airway epithelial response to hyperoxic lung injury in part through direct regulation of the cytoprotective cytokine IL-6. Together, these data reveal an important interaction between the Foxp1/2/4 and NuRD complexes, which mediates the response of lung epithelium to hyperoxic injury.
MATERIALS AND METHODS

Yeast Two-hybrid Screen—The previously described transcriptional repression domain of mouse Foxp2 (aa 250–500) was used as a bait to isolate Foxp1/2/4 protein interaction partners with an adult lung cDNA library (Clontech). Multiple isolates of p66β were identified and sequenced.

Protein-Protein Interaction Assays—The Foxp1 RD (aa 251–489), SD1 (aa 251–408), SD2 (aa 407–489), and Foxp4 RD (aa 228–469) GST constructs were made through PCR amplification of the appropriate coding sequence and subcloned into the pGEX4T2 vector, and GST fusion proteins were generated as previously described (8). In vitro transcribed and translated proteins were generated using the Transcend™ non-radioactive translation detection system (Promega, Madison, WI). Proteins were resolved on SDS-PAGE and visualized with the Transcend™ chemiluminescent substrate.

Cell Culture and Transient Transfection Assays—NIH3T3, HEK293, MLE-12, and MLE-15 cells were cultured as previously described (8, 12). All luciferase assays were performed in six-well plates using 5 × 10^5 cells. The full-length Foxp1 and Foxp4 expression plasmids have been described previously (2, 5). The rat T1α promoter luciferase reporter was generously provided by Dr. Maria Ramirez (13). The mouse IL-6 promoter luciferase construct was generated by amplification of wild-type mouse lung cDNA. HDAC1-FLAG was kindly provided by Gerd Blobel, and the HDAC2-FLAG has been previously described (14). Full-length p66β cDNA was subcloned into the pCMVTag3B vector. Cells were transfected with FuGENE 6, and 0.5 g of reporter vector and 1 μg of cDNA expression vector. Cells were treated with Scriptaid, Nullexscript, or DMSO for 48 h at a final concentration of 4 μg/ml siRNA for p66α, p66β, Foxp1, HDAC1, and HDAC2 as well as control siRNA were purchased from Dharmacon and used following the manufacturer’s instructions. Luciferase assays were performed 48 h after the start of transfection using a commercially available kit (Promega). RNA was isolated from cells 48 h after transfection or indicated treatment and quantitative PCR (Q-PCR) was performed using primers listed in supplemental Table S1. Data shown represent the average of three assays ± S.E. compared with β-actin expression.

Co-immunoprecipitation Assays—Co-immunoprecipitation assays were performed by transfecting three 10^7 HEK-293 cells with the indicated expression plasmids and harvesting cell lysates after 24–48 h. Cell lysates were incubated with the indicated antibodies, and immunoprecipitates were isolated using Protein A/G-agarose (Santa Cruz Biotechnology) as described (8, 15). The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with either the anti-Myc polyclonal or the anti-FLAG monoclonal antibody as indicated. Reactive bands were resolved using the SuperSignal West Femto substrate kit (Pierce), and blots were exposed 1–10 min depending on antibodies used.

Animal Studies—The generation and genotyping of the Foxp1 and HDAC2 mutants mouse lines has been previously described (14, 16). Hyperoxic injury was induced by housing mice in a 90% O2 atmosphere in a hyperbaric chamber for 72 h. Animals were euthanized within 1 h of chamber exit and lung tissue harvested for analysis.

Histology—Lungs were inflation-fixed (20 cm of water pressure) in 4% paraformaldehyde then submerged in 4% paraformaldehyde for 24 h. All tissues were dehydrated through a series of ethanol washes and embedded in paraffin and sectioned. The following antibodies and concentrations were used in the histological studies: IL-6 (Santa Cruz #SC-1265, 1:50) and p-ERK (Cell Signaling Technology #4695, 1:100). To evaluate apoptosis, the TUNEL assay was performed. Merged images of TUNEL-positive and nuclear Hoescht-stained sections were evaluated using ImageJ software. TUNEL-positive cells were counted in four different microscopic fields per section in five animals per genotype.

RESULTS

Identification of p66β as an Interacting Protein of Foxp1/2/4—To investigate the mechanism by which Foxp1/2/4 repress gene transcription, we used the previously identified repression domain in Foxp2 as bait in a yeast two-hybrid protein-protein interaction screen. Of note, this repression domain is highly conserved between Foxp1/2/4 family members (8). From these studies we isolated several clones of p66β, a component of the NuRD chromatin-remodeling complex (10, 11, 17). p66β interacted with the Foxp2 repression domain but not the empty bait vector in the yeast two-hybrid assay (Fig. 1A). p66β and its highly related homologue p66α are components of the NuRD chromatin-remodeling complex and have been shown to interact with chromatin-associated proteins such as histones (10, 11, 17). To verify that p66β interacted with other members of the Foxp1/2/4 family, we performed co-immunoprecipitation assays from transfected HEK-293 cells using epitope-tagged versions of Foxp1 and Foxp2 and p66β. p66β interacted with both Foxp1 and Foxp2 in these assays confirming the results from the yeast two-hybrid assay (Fig. 1, C and D). Moreover, Foxp1 interacted with another NuRD component metastasis-associated 1 protein (MTA) in co-immunoprecipitation assays (Fig. 1E).

p66β and Foxp1 Interact through Multiple Contact Points in the Foxp1 Repression Domain—p66β and its highly related homologue p66α are modular proteins containing several domains thought to regulate interactions with other components of NuRD and chromatin proteins such as histones (10, 11, 17). These include the CR1 and CR2 domains, which are thought to mediate protein-protein interactions with the methyl-CpG binding domain protein (MBD) component of NuRD and histones, respectively (11, 17). In addition, a C2–C2 type zinc finger is located in the C terminus of the protein (Fig. 1B). To determine which regions within Foxp1/2/4 and p66 proteins were important for this interaction, GST pulldown assays were performed using in vitro transcribed and translated p66 constructs with the Foxp repression domain fused to GST. The
FIGURE 1. p66β interacts with Foxp1/2/4. A, yeast two-hybrid screen was performed using the Foxp2 repression domain as bait, and p66β was identified as a protein partner (a and b, quadrant 1). No interaction was seen with the empty bait plasmid (a and b, quadrant 2). B, schematic diagram of the mouse p66β protein showing the MBD/NuRD interaction domain (CR1) as well as the histone interaction domain (CR2). C, co-immunoprecipitation of FLAG-p66β with Myc-Foxp1 and Myc-Foxp2 shows interaction between these factors in HEK-293 cells. Untransfected cells immunoprecipitated with FLAG antibody was used as a negative control. D, total p66β expression in experiment in C as shown by Western blotting with the anti-FLAG antibody. E, the NuRD component MTA associates with Foxp1 in co-immunoprecipitation assays. F, diagram of GST fusion protein constructs subdomain 1 (SD1) and subdomain 2 (SD2) within the Foxp1 repression domain (RD). SD1 contains the leucine zipper and zinc finger, while SD2 contains the CtBP1 binding motif in Foxp1. G, pulldown assays using GST-Foxp1SD1 and GST-Foxp1SD2 with in vitro transcribed and translated full-length p66β. H, diagram of p66β deletion constructs. I, GST pulldown assays show that GST-Foxp1RD and GST-Foxp4RD interacts through the CR2 domain of p66β. GST-Foxp1RD and GST-Foxp4RD interacts with all p66β deletions, except when the CR2 domain is absent (1–191). GST-luciferase control (GST-luc) and reticulocyte minus p66β DNA (no DNA) are used as negative controls.
repression domain in the Foxp subfamily can be further divided into two subdomains based on the presence of several protein-protein interaction motifs, including the leucine zipper, zinc finger, and the CtBP1 binding sequence (Fig. 1F) (8). Surprisingly, p66/H9252 interacted with both of these subdomains indicating that this interaction is mediated by multiple contact points within Foxp proteins (Fig. 1G). To delineate which regions within p66/H9252 were important for this interaction, p66/H9252 deletion constructs were generated based on important domains identified in previous p66α/β structure-function studies (Fig. 1H) (11, 17). These studies revealed that the CR2 domain in p66β is essential for interaction with both the SD1 and SD2 domain in Foxp proteins (Fig. 1I). However, the highly conserved zinc finger was not required for this interaction (Fig. 1J). Together, these data show that the CR2 domain is required for p66β interaction with Foxp proteins, and there are multiple interaction points in the Foxp repression domain responsible for this interaction.

p66α/β Are Necessary and Sufficient for Foxp Repression of Target Genes—p66α/β have been shown to be potent transcriptional repressors (10, 11). To determine what affect p66 proteins had on Foxp transcriptional repression, we performed co-transfection studies using the T1α luciferase promoter as a reporter of Foxp-mediated repression. T1α is a critical cell surface receptor of the mucin class required for proper lung function and a previously identified direct target of Foxp factors (4, 18). Overexpression of Foxp1/2/4 but not p66β represses the −1.4 kb rat T1α luciferase promoter in NIH-3T3 cells. B, overexpression of p66β with Foxp1 and Foxp4 can cooperatively repress the −1.4 kb rat T1α luciferase promoter in NIH-3T3 cells. Control columns (−p66) represent the extent of Foxp-mediated repression as shown in A arbitrarily set to 1 to better show the additional repression mediated by co-expression of p66. C, siRNA knockdown of p66α and/or p66β increases expression of endogenous T1α expression in MLE-15 cells as measured by Q-PCR. D, siRNAs that target p66α do not alter p66β expression, and siRNAs that target p66β do not alter p66α expression as measured by Q-PCR. These data verify the specificity of the siRNAs used. E, siRNA knockdown efficiently reduced expression of Foxp1 in these experiments. F, knockdown of Foxp1 and p66β preferentially increases normal T1α gene repression; siRNA knockdown of Foxp1, Foxp1 and p66α, or Foxp1 and p66β cooperatively increases endogenous T1α expression in MLE-15 cells as measured by Q-PCR, while expression of other epithelial genes, except CC10, is unaltered. G, breakout graph from data in F showing increased expression of CC10 after knockdown of Foxp1 and p66.
pression of Foxp1/2/4 represses the 1.4-kb T1α promoter in NIH-3T3 cells, whereas p66β alone had no affect (Fig. 2A) (13). Co-expression of Foxp1/2/4 and p66β shows that p66β can cooperatively repress the T1α promoter in the presence of Foxp1 and Foxp4 but not Foxp2 (Fig. 2B), suggesting that p66 and the NuRD complex may differentially regulate the Foxp subfamily.

To determine whether p66α/β expression was necessary for regulated expression of Foxp target gene expression, expression of p66α, p66β, or both were inhibited using siRNA-mediated knockdown in the lung epithelial cell line MLE-15. Inhibition of either p66α or p66β increased endogenous T1α expression by ~2-fold (Fig. 2C). Inhibition of both p66α and p66β resulted in an almost 5-fold increase in endogenous T1α expression (Fig. 2C). The siRNAs for p66α or p66β were specific, because they had little effect on expression of the non-targeted p66 gene (Fig. 2D). siRNA inhibition of Foxp1 expression resulted in an approximate 60% knockdown of Foxp1 expression (Fig. 2E). To determine whether Foxp and p66 interactions were important for T1α gene repression, we performed siRNA knockdown assays of both Foxp1 and p66α or p66β and assessed gene expression in MLE-15 cells. Loss of Foxp1, p66α, or both resulted in modest but significant increases in T1α expression as observed above (Fig. 2F). Remarkably, loss of both Foxp1 and p66β leads to a strong cooperative increase in endogenous T1α expression (Fig. 2F). CC10 expression was up-regulated by ~3-fold in agreement with previous reports that it is also a target of Foxp1/2/4 repression (Fig. 2G) (5). In contrast, expression of other lung epithelial-restricted genes, including SP-C and aquaporin-5, was unaffected (Fig. 2H). These data indicate that p66α/β-mediated NuRD activity acts cooperatively with Foxp1 to regulate repression of target genes in lung epithelial cells.

**HDAC1/2-mediated Repression Is Required for Foxp Repression of Target Genes**—HDAC1 and HDAC2 are core components of the NuRD complex and are required for NuRD-mediated chromatin remodeling and transcriptional repression (19, 20). Therefore, we assessed whether HDAC activity and specifically HDAC1 and HDAC2 were important for Foxp-mediated repression of target genes. MLE-15 cells were treated with either the HDAC inhibitor Scriptaid or its inactive counterpart Nullscript to determine whether inhibition of HDAC activity affected Foxp target gene expression. Nullscript had no affect on expression of the Foxp1/2/4 target gene T1α (Fig. 3A). However, Scriptaid treatment resulted in an almost 5-fold increase in endogenous T1α expression in MLE-15 cells (Fig. 3A). MLEC-15 cells were next transfected with siRNAs targeted to either HDAC1 or HDAC2. Decreased expression of either HDAC1/2 led to a more than 2-fold increase in endogenous T1α expression (Fig. 3B). These data demonstrated that expression of the Foxp target gene was sensitive to HDAC1/2 activity in lung epithelial cells.

To show that Foxp1/2/4 and HDAC1/2 functionally cooperate to repress Foxp1/2/4 target genes, siRNAs targeting Foxp1 and HDAC2 were transfected into MLE-15 cells, and T1α gene expression was assessed. Loss of Foxp1 alone had a minor affect on T1α expression as compared with loss of both Foxp1 and HDAC2, which led to a cooperative increase in T1α expression (Fig. 3C), siRNA knockdown of HDAC1, HDAC2, and Foxp1 effectively inhibited their expression levels (Fig. 3D). Co-immunoprecipitation experiments were performed to show that HDAC1/2 interact with both Foxp4 (Fig. 3E) and Foxp1 (Fig. 3F) further indicating an important functional interaction between these two families of proteins. Together, these data indicate that Foxp target gene expression is regulated in a cooperative manner by interactions between Foxp- and HDAC1/2-containing complexes.

**Foxp-HDAC2 Regulates Hypoxic-induced Lung Epithelial Injury**—To assess the in vivo implications of the Foxp-NuRD interactions, we generated Foxp1+/-:HDAC2+/- compound mutants. Foxp1 null mutants are embryonic lethal at E14.5 and HDAC2 nulls exhibit partial lethality at birth (14, 16). In contrast, Foxp1+/-:HDAC2+/- compound mutants are viable, which allowed us to determine the genetic interaction between these two genes in lung injury studies. Because Foxp1 and HDAC2 are both expressed at high levels in alveolar epithelium (4, 21), we used hypoxic injury, which preferentially injures alveolar type 1 epithelial (AEC1) cells. Loss of AEC1 cells leads to repopulation of the alveolar epithelium through proliferation and differentiation of alveolar epithelial type 2 cells (AEC2) into AEC1 cells (22, 23). Treatment of wild-type mice with 90% oxygen for 3 days leads to a sub-lethal injury as noted by a significant increase in hyaline membrane formation, thickening of the septal wall, and apoptosis (Fig. 4, A, B, E, and G). This injury is also characterized by ruffling of the extended cytoplasmic processes of AEC1 overlaying the endothelial plexus in the alveolar airspace (Fig. 4, K–O). Although wild-type, Foxp1+/-, and HDAC2+/- mice exhibited extensive alveolar epithelial injury, Foxp1+/-:HDAC2+/- reduced levels of apoptosis and AEC1 cell disruption (Fig. 4F–O). Quantitation of the levels of apoptosis shows that Foxp1+/-:HDAC2+/- mice had levels of apoptosis similar to uninjured wild-type animals suggesting that Foxp1+/-:HDAC2+/- mutants were protected against hypoxic lung injury (Fig. 4L).

Previous studies have shown that the mitogen-activated protein kinase pathway regulates hypoxic-induced apoptosis and injury in the lung resulting in increased phospho-ERK (p-ERK) expression (24, 25). Immunostaining showed that hypoxia-injured Foxp1+/-:HDAC2+/- mutants had similar levels of p-ERK staining as uninjured wild-type animals, whereas injured wild-type and single heterozygous animals exhibited elevated levels of p-ERK (Fig. 4, P–T). Together, these data suggest that Foxp1+/-:HDAC2+/- mutants are protected from hypoxic-induced lung epithelial injury.

In addition to NuRD, HDAC2 is a component of other chromatin-modifying complexes in the cell, including the sin3a complex (26, 27). Our data showing a direct interaction between the NuRD component p66α/β with Foxp1/2/4 suggests that the NuRD complex is important for the response to hypoxic lung injury in cooperation with Foxp factors. We performed Q-PCR analysis to determine expression of NuRD and sin3a components in hypoxic injury in the lung. Expression of important components of NuRD but not sin3A were up-regulated by hypoxic lung injury (supplemental Fig. S1). These data suggest that the NuRD chromatin-remodeling complex responds preferentially to hypoxic injury, which sup-
ports our hypothesis that the Foxp1-HDAC2 compound mutant hyperoxic phenotype reflects a functionally important Foxp1-NuRD interaction.

Foxp1-NuRD Directly Regulate Expression of the Cytoprotective Cytokine IL-6 in the Lung—The data above suggest that reduced levels of Foxp1-NuRD activity leads to a cytoprotective advantage in the lung epithelium after hyperoxic injury. IL-6 has been shown to be cytoprotective in hyperoxic lung injury (28–30). IL-6 is expressed in both lung epithelium and in immune cells. In lung epithelium, expression begins in early development and persists into adulthood where it is expressed in alveolar epithelium (31). Because Foxp factors are potent transcriptional repressors, we assessed whether IL-6 expression was increased in hyperoxia-treated Foxp1<sup>+/−</sup>:HDAC2<sup>+/−</sup> mutants compared with wild-type injured animals. As previously reported, IL-6 expression is up-regulated in wild-type animals after hyperoxic treatment (Fig. 5A) (32). Hyperoxic-injured Foxp1<sup>+/−</sup> and HDAC2<sup>+/−</sup> single heterozygous mutants exhibited a further increase in IL-6 expression compared with wild-type animals (Fig. 5A). In contrast, Foxp1<sup>+/−</sup>:HDAC2<sup>+/−</sup>

FIGURE 3. HDAC activity involvement with Foxp1-regulated target gene expression. A, the HDAC inhibitor Scriptaid increases expression of endogenous T1α in MLE-15 cells when compared with the negative controls, DMSO (vehicle) and Nullscript as measured by Q-PCR. B, siRNA knockdown of HDAC1 or HDAC2 increases endogenous expression of T1α in MLE-15 cells by Q-PCR. C, siRNA knockdown of Foxp1 and HDAC2 in combination in MLE-15 cells demonstrate a cooperative increase in expression of endogenous T1α as compared with knockdown of each factor alone as measured by Q-PCR. D, siRNA knockdown of Foxp1, HDAC1, and HDAC2 effectively inhibits expression of these factors. E, co-immunoprecipitation of FLAG-HDAC1 and FLAG-HDAC2 with Myc-Foxp4 from 3 x 10<sup>5</sup> HEK-293 cells demonstrates interaction of these proteins in a complex. The arrowhead indicates immunoprecipitated Foxp4 protein. F, co-immunoprecipitation of FLAG-HDAC1 and FLAG-HDAC2 with Myc-Foxp1 in a parallel experiment in HEK-293 cells demonstrates interaction of these proteins in a complex. The arrowhead indicates immunoprecipitated HDAC protein.
Foxp1/2/4-NuRD Regulates Injury Response in the Lung

**FIGURE 4. Foxp1+/−:HDAC2+/− mutants are protected against hyperoxic lung injury.** Wild-type, Foxp1+/−, HDAC2+/−, and Foxp1+/−:HDAC2+/− mutants were exposed to 90% O2 for 72 h. A–E, H+E staining; F–J, TUNEL staining; K–O, transmission electron microscopy; and P–T, p-ERK immunostaining were performed. U, quantitation of apoptosis in Foxp1+/−:HDAC2+/− mutants showing decreased numbers of apoptotic cells as compared with single heterozygote mice. Apoptosis was quantitated as described under “Experimental Procedures.” Arrows (K–O) indicate plasma membrane ruffling due to AEC1 cell injury from hyperoxia. Scale bars: A–E = 50 μm; F–J, and P–T = 75 μm; and K–O = 1 μm.

mutants exhibited a substantial cooperative increase in IL-6 expression of >100-fold over wild-type untreated animals and >5-fold over wild-type hyperoxic-treated animals (Fig. 5A). Immunostaining confirmed that this increase in IL-6 mRNA expression results in increased protein expression in lung epithelium (Fig. 5, B and C).

The above data suggest that IL-6 may be a direct target of Foxp1-NuRD-mediated repression in the lung. Two conserved Fox DNA binding sites were found in the mouse IL-6 promoter (Fig. 5D). To determine whether Foxp1 was associated with these sites in the lung in vivo, we performed chromatin immunoprecipitation assays using chromatin from embryonic mouse lungs. These data showed that Foxp1 is associated with both of these sites in the lung (Fig. 5, E and F). To determine whether Foxp1 and other components of NuRD, including p66, HDAC1, HDAC2, MTA2, and MBD3, could repress gene transcription from the IL-6 proximal promoter, we generated a 1.7-kb mouse IL-6 proximal promoter luciferase reporter containing one of these Fox DNA binding sites and performed co-transfection assays in MLE-12 cells. These data show that Foxp1 is capable of repressing gene transcription from the proximal IL-6 promoter (Fig. 5G). Moreover, p66β, MTA2, HDAC1, HDAC2, MTA2, and MBD3 also repressed the mouse IL-6 promoter, and the combined overexpression of Foxp1 and these NuRD components acted cooperatively to further repress the IL-6 promoter (Fig. 5G). Together, these data show that Foxp1, in part through its association with the HDAC2-NuRD complex, represses IL-6 expression in the lung. Decreased expression of Foxp1-HDAC2 results in increased IL-6 expression and associated cytoprotection of alveolar epithelium.

**DISCUSSION**

Interactions between chromatin-remodeling complexes and specific transcription factors play a key role in the regulation of cell type-specific gene expression. In this report, we show that Foxp-NuRD interactions mediate critical lung epithelial gene expression programs required for the hyperoxic injury response. We demonstrate that Foxp factors interact with the NuRD chromatin-remodeling complex through direct interactions with the p66β component of NuRD. These interactions play an important role in regulation of lung epithelial gene expression, and loss of Foxp1 and HDAC2 leads to increased IL-6 expression and decreased apoptosis, which protects Foxp1-HDAC2 compound mutants against hyperoxic lung injury. These data highlight an important molecular control over lung gene expression by Foxp-NuRD interactions. Moreover, they illustrate the role that Foxp1/2/4 and HDACs plays in adult lung remodeling after injury.

Respiratory distress is often treated with oxygen therapy leading to hyperoxic exposure, which, if prolonged, can lead to acute lung injury and potentially progress to acute respiratory distress syndrome (33). Hyperoxic treatment of rodent results in a similar injury as observed in humans with acute lung injury. Adult lung diseases such as chronic obstructive pulmonary disease and emphysema are thought to occur through an iterative injury-repair process, which results in defective epithelial regeneration. Interestingly, previous studies have demonstrated an association between HDAC2 expression and chronic obstructive pulmonary disease. Patients with chronic obstructive pulmonary disease had decreased HDAC2 expression a well as a decrease in overall HDAC activity from lung homoge-
FIGURE 5. IL-6 expression is increased in Foxp1<sup>+/−:HDAC2<sup>+/−</sup> mutants after hyperoxic injury and is a direct target of Foxp1 in the lung. A, expression of IL-6 is increased following hyperoxic injury in wild-type, Foxp1<sup>+/−</sup>, HDAC2<sup>+/−</sup>, and Foxp1<sup>+/−:HDAC2<sup>+/−</sup></sup> mutants as demonstrated by Q-PCR (n = 3). B and C, protein expression of IL-6 is increased in Foxp1<sup>+/−:HDAC2<sup>+/−</sup></sup> mutants in comparison to wild-type littermates following hyperoxic injury. D, the mouse IL-6 promoter contains two Fox DNA binding sites. Chromatin immunoprecipitation assay demonstrating Foxp1 binding to site 1 and site 2 but not intron 1 of the Pdgfrβ gene as assessed by Q-PCR (E) and gel electrophoresis (F). G, overexpression of Foxp1, and NuRD components p66β, HDAC1, HDAC2, MTA2, and MBD3 alone and in combination with Foxp1 cooperatively represses the −1.7-kb mouse IL-6 promoter.
nates (34). At first glance this would appear counterintuitive to our findings that decreased HDAC activity, in particular HDAC2, leads to increased protection from hyperoxic injury. However, IL-6 is a pleiotropic cytokine that has been associated with both positive and negative outcomes depending on the context. It has been shown to be a powerful anti-apoptotic agent, and only recently have the pathways involved in this protective affect been investigated (35). The studies showing protective affects of increased IL-6 expression involve transgenic overexpression and also exhibit a temporal dependence of this protection. Adult mice that overexpress IL-6 specifically in the lung exhibit decreased apoptosis and better survival after hyperoxic injury, whereas neonates exhibit increased mortality associated with increased epithelial apoptosis (30, 36). Our studies focused on the cooperative role between Foxp1 and HDAC2 leading to increased IL-6 expression in the short time frame of hyperoxic injury. Together, these studies show that Foxp1, through the NuRD chromatin-remodeling complex, utilizes the deacetylation activity of HDAC 1/2 to regulate IL-6 expression.

Although the role for chromatin-remodeling complexes and enzymes such as HDACs are poorly understood in lung development and homeostasis, there is increasing evidence that they play key roles in regulating cardiovascular development and cardiac hypertrophy. Loss of HDAC2 results in partial neonatal death, and the surviving animals show resistance to cardiac hypertrophic stimuli (14). Moreover, cardiac-specific loss of both HDAC1 and HDAC2 results in early postnatal lethality associated with a dilated cardiomyopathic phenotype (37). In contrast, increased expression of HDAC2 in transgenic mice leads to an enhanced hypertrophic response in the heart (14). Thus, in the heart HDAC1/2 play important roles not only in development but also in the homeostatic response to injury.

The balance between gene activation and repression through recruitment of co-activator and co-repressors complexes is poorly understood. Foxp1/2/4 have been shown to regulate various aspects of lung, cardiac, and neural development (4, 16, 17). Given the potent repression activity of Foxp1/2/4, it is likely that these factors help to restrict gene expression in tissues where they are expressed to allow for terminal differentiation or for proper adult homeostasis. The presence of multiple protein–protein interaction motifs in Foxp1/2/4 suggests that these factors have the ability to interact with a wide variety of cofactors. Such a large repertoire of interactions would allow for a single factor to modulate gene expression in a spatial and temporal specific fashion depending on the expression of such cofactors. In support of this, Foxp1 has been shown to interact with NCoR1 in cardiac growth (9). Previous reports have shown that NuRD recruitment to DNA via transcription factors appears to be subunit-specific with transcription factors interacting with different subunits of the NuRD complex (42–46). The current study is the first report of DNA binding transcription factors interacting with NuRD via p66. Identification and characterization of additional protein binding partners of Foxp1/2/4 will likely provide important evidence of the multifarious roles that these factors play in development and adult tissue homeostasis.

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