EHF has critical roles in airway epithelial dysfunction

Ets Homologous Factor (EHF) has critical roles in epithelial dysfunction in airway disease

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ABSTRACT

The airway epithelium forms a barrier between the internal and external environments. Epithelial dysfunction is critical in the pathology of many respiratory diseases including cystic fibrosis (CF). Ets homologous factor (EHF) is a key member of the transcription factor (TF) network that regulates gene expression in the airway epithelium in response to endogenous and exogenous stimuli. *EHF,* which has altered expression in inflammatory states, maps to the 5' end of an intergenic region on Chr11p13 that was implicated as a modifier of CF airway disease. Here we determine the functions of EHF in primary human bronchial epithelial (HBE) cells and relevant airway cell lines. Using EHF chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) and RNA-seq after EHF depletion, we show that EHF targets in HBE cells are enriched for genes involved in inflammation and wound repair. Furthermore, changes in gene expression impact cell phenotype, since EHF depletion alters epithelial secretion of a neutrophil chemokine and slows wound closure in HBE cells. EHF activates expression of the SAM pointed domain-containing ETS transcription factor (SPDEF), which contributes to goblet cell hyperplasia. Our data reveal a critical role for EHF in regulating epithelial function in lung disease.

The airway epithelium plays a critical role in lung function in both normal and pathological states. It forms an active barrier between the external and internal environments, responding to a wide range of stimuli. Because this cell layer is constantly exposed to the outside atmosphere, it has developed mechanisms to respond rapidly to external insults. Following mechanical damage, the epithelial cells proliferate and migrate to close the resulting wound (1). In response to foreign pathogens and particles, the cells release immune factors (2). The airway epithelium also drives the mucociliary escalator, which physically removes debris from the lungs (3,4). In the presence of chronic damage or infection, these pathways can malfunction and contribute to lung pathology in diseases such as cystic fibrosis (CF) (3,5,6).

CF is a common autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The main cause of morbidity and mortality in CF is lung disease (7). Airway dysfunction in CF patients is complex, but altered ion transport leads to a decreased ability to clear microorganisms, chronic infection, and inflammation. Neutrophil-dominant inflammation is present early in the disease (8). There is

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increased secretion of cytokines including interleukin-13 (IL-13) and interleukin-17a (IL-17a) in the lungs, which is exacerbated in patients infected with *Pseudomonas aeruginosa,* the predominant pathogen in the CF airway (11-13). IL-13 augments mucin production by the lung epithelium and triggers goblet cell hyperplasia (14). IL-17a induces neutrophil chemokine production by the epithelial cells (15). The amplified mucus and cytokine levels cause destruction of lung tissue by initiating remodeling and fibrosis, which correlate with pulmonary dysfunction (16-19). Similar pathways are activated in other airway diseases, including chronic obstructive pulmonary disease (COPD) and asthma (20,21). Modulation of gene expression underlies the response of the lung epithelium to the environment, both in normal and disease states, so it is critical to understand the causative mechanisms. Pivotal to these processes is the transcription factor (TF) network that regulates development, normal function, and response to disease in respiratory epithelial cells. TFs respond to normal and aberrant stimuli by binding to the genome at regulatory sites, thereby altering gene expression. Ets homologous factor (EHF) is a member of the epithelial-specific Ets transcription factor subfamily that is expressed in the bronchial epithelium (22-24). In response to inflammatory mediators including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), bronchial EHF expression is increased in an NF-κB-dependent manner, suggesting a role for EHF in the epithelial response to inflammation (25). In prostate cancer, loss of EHF leads to epithelial-to-mesenchymal transition (EMT) and increased metastatic potential (26). In EMT, epithelial cells lose contact with adjacent cells and become more motile (27). Similar pathways may be involved in lung fibrosis (28).

Recent genome-wide association studies (GWAS) led to an interest in EHF as a potentially important factor in CF lung pathology. These studies identified intergenic single nucleotide polymorphisms (SNPs) at chr11p13 that strongly associate with lung disease severity in CF patients carrying the most common disease-causing mutation, F508del (29,30). These SNPs are likely within or close to *cis*-regulatory elements for nearby genes. *EHF* maps immediately adjacent (5') to this intergenic region, suggesting a role as a

modifier of lung disease in CF patients. We hypothesize that EHF regulates genes involved in cellular processes that impact the severity of epithelial pathology in airway disease, including inflammation and wound repair.

This study investigates the role of EHF in primary human bronchial epithelial (HBE) cells. Using chromatin immunoprecipitation followed by sequencing (ChIP-seq) and RNA-sequencing (RNA-seq) after EHF depletion, we identified putative direct targets of EHF in HBE cells. These genes are enriched in processes that are involved in lung pathology. Cellular assays determined that EHF-modulated changes in gene expression alter epithelial phenotypes. Our results suggest that EHF is involved in multiple processes that influence lung function in disease. Of particular relevance to CF are the release of neutrophil chemokines and wound repair.

RESULTS

ChIP-seq generates a genome-wide binding signature for EHF in primary HBE cells− To identify genome-wide binding sites for EHF, ChIP-seq was performed on cultures from two tissue donors. The antibody specific for EHF was used previously for ChIP-seq in Calu-3 lung adenocarcinoma cells (31). The HOMER irreproducible discovery rate pipeline (32) was used to call a total of 11,326 peaks with an irreproducible discovery rate (IDR) < 0.05 (Table S1). Sonicated input DNA was used as background. The normalized tag counts at each called peak were significantly correlated between the two biological replicates (r=0.29, p<0.0001).

The Cis-regulatory Element Annotation System (CEAS) software (33) was used to annotate peaks based on genomic location (Fig 1A) and determined that EHF sites were enriched at promoters (-1 kb relative to the transcription start site) and 5' untranslated regions (UTRs) of genes (Fig 1B). Consistent with its role as a TF controlling cell-type specific gene expression, peaks of EHF occupancy were also found in intergenic and intronic regions, which may represent *cis*-regulatory elements such as enhancers. These regulatory sites are marked by specific histone modifications. Active or poised promoters and enhancers are enriched for histone 3 lysine 4 trimethylation (H3K4me3) and H3K4me1, respectively, while active regions are

specifically marked by histone 3 lysine 27 acetylation (H3K27ac) (34,35). To determine whether EHF peaks are found in regions with these histone modifications, as predicted by their genomic locations, ChIP-seq was performed using antibodies against H3K4me1, H3K4me3 and H3K27ac in HBE cells. EHF sites were enriched for all marks with a bimodal distribution around the center of the peak, in accordance with a nucleosome depleted center region where transcription factors bind (Fig 1C). Consistent with an over-representation of EHF sites at promoters, the H3K4me3 mark was the most enriched.

De novo motif analysis was performed on the significant EHF ChIP-seq peaks, which identified the epithelial-specific Ets motif as the most enriched, found in 84.25% of sites within 200bp of the center of the peak (Fig 2A). The motif analysis also identified secondary enriched motifs. The binding motifs for activator protein-1 (AP-1), Forkhead domain and Krüppel-like factor (KLF) transcription factor families were among the most enriched secondary motifs, found in 20.94%, 15%, and 13.54% of peaks, respectively (Fig 2A).

To confirm that EHF and AP-1 co-localize in the genome, ChIP-seq for c-Jun and JunD, members of the AP-1 protein complex, was performed in Calu-3 lung adenocarcinoma cells. ENCODE consortium-validated antibodies were used to perform 2 biological replicates for each protein, and the HOMER IDR method was used to call significant peaks of transcription factor binding. A total of 11,517 and 7,524 sites were identified for c-Jun and JunD, respectively. In addition, the HOMER IDR protocol was used to call peaks in 2 biological replicates of EHF ChIP performed in Calu-3 cells, which we published previously (31). These three data sets were then intersected (Fig 2B). Twenty-five percent of the EHF peaks overlapped at least one c-Jun or JunD peak, with 59% of these containing both c-Jun and JunD sites, 32% having only c-Jun and 9% containing only JunD. EHF sites that overlap JunD binding sites are enriched at the promoters (Fig 2C). The nearest gene annotation method was used to determine potential genes regulated by the overlapping binding sites, and these were subjected to a gene ontology process enrichment analysis using MetaCore from Thomson Reuters

version 6.26 (Fig 2D). The overlapping sites are found near loci involved in differentiation, development and wound repair, suggesting they may have a combined role in regulating phenotypes important for lung pathology.

EHF regulates genes involved in lung pathology −To detect genes that are directly and indirectly regulated by EHF, RNA-seq was performed on EHF-depleted (using a targeted siRNA) and negative control siRNA-treated HBE cells (Fig 3A). Cufflinks (36) was used to identify a total of 1,145 genes that were differentially regulated (DEGs) following EHF depletion (FDR <0.05), with 625 increasing and 520 decreasing in expression (Fig 3B, Table S2). A multi-dimensional scaling (MDS) analysis was performed, which confirmed that 4 of 5 samples within each treatment clustered together and away from the second group (Fig S1).

To identify putative direct targets of EHF, the EHF ChIP-seq data and the DEGs identified by RNA-seq after EHF depletion were intersected using Binding and Expression Target Analysis (BETA) (37). EHF was found to have significant activating and repressive function (Fig 3C), consistent with gene expression changes in the positive and negative direction following EHF knockdown. This analysis revealed 425 putative direct targets that are repressed by EHF, and 434 that are activated (Tables S3 and S4). The forkhead box A1 (FOXA1) motif was enriched in EHF sites near repressed genes as compared with non-regulated genes (p=2.85e-13). The EHF-repressed genes included the following that encode transcription factors: GATA binding protein 6 (*GATA6*), v-myc avian myelocytomatosis viral oncogene homolog (*MYC*), CCAAT/enhancer binding protein (C/EBP) gamma and beta (*CEBPG and CEBPB*), HOP homeobox (*HOPX*), and *KLF5*. Genes activated by EHF include the SAM pointed domain-containing ETS transcription factor (*SPDEF*), which is involved in airway goblet cell development (38,39), and the inflammatory S100 calcium binding proteins *S100A8, S100A9,* and *S100A12*.

An enrichment analysis was performed on up-regulated and down-regulated predicted direct targets using MetaCore (Fig 3D). EHF-activated genes were found in pathways including regulation of degradation of misfolded proteins, extracellular matrix (ECM) remodeling, and EMT; diseases

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comprising infections and inflammation; and gene ontology processes including response to stress, immune response, and response to wounding. EHF-repressed genes were enriched in gene ontologies involving negative regulation of RNA synthesis, transcription and metabolic processes.

*EHF and FOXA1 may co-regulate transcription factors important for lung epithelial development−*Lung organogenesis and airway epithelial differentiation are complex processes coordinated by a network of transcription factors. Multiple TFs within this network were identified as putative EHF targets in our genome-wide data. These include genes with nearby peaks of EHF occupancy and/or DEGs after EHF depletion, among which are *GATA6, HOPX, KLF5, SPDEF*, retinoic acid receptor beta (*RARB*)*, FOXA1* and *FOXA2*. To validate direct or indirect targets, EHF was depleted in primary HBE cell cultures derived from 4 HBE donors (Fig S2). Expression of key TFs was determined using reverse transcription and quantitative polymerase chain reaction (RT-qPCR) (Fig 4A). *HOPX, KLF5,* and *RARB* abundance significantly increased following EHF depletion, *SPDEF* levels significantly decreased. *CEBPG* and *FOXA1* showed an increase and decrease, respectively, that did not reach statistical significance (*CEBPG* p=0.056, *FOXA1* p=0.08). Because of the substantial variation between primary cultures, these non-significant changes may be biologically relevant, but would require additional validation. *GATA6* showed no change*,* and *FOXA2* levels were variable between donors. Nearby peaks of EHF occupancy evident in the ChIP-seq data suggest that most of these genes may be direct targets (Fig 4C, S3). To confirm this prediction, 4 biological (donor) replicates of EHF ChIP-qPCR were performed in primary HBE cultures (Fig 4B). EHF binding was confirmed at intron 1 of *CEBPG,* intergenic sites 5' of *FOXA1, HOPX, and RARB,* and the promoter of *SPDEF*.

The sites of EHF occupancy near *CEBPG, HOPX, RARB*, and *SPDEF* also contain sequences with the Forkhead domain motif. Since EHF represses all but one of these genes (*SPDEF*), and the FOXA1 motif is enriched amongst EHF binding sites near repressed genes genome-wide, we next asked whether FOXA1 co-regulates gene expression with EHF at these loci. FOXA1 was depleted in HBE primary cultures from 3 donors using a specific siRNA and a non-targeting

control, and gene expression was measured by RT-qPCR (Fig S4, 4D). Depletion of FOXA1 significantly elevated expression of *EHF, FOXA2, HOPX* and *KLF5*, and decreased *SPDEF* levels (p=.008). *RARB* did not change. Furthermore, the EHF sites near *HOPX* and *SPDEF* were bound by FOXA1 as shown by 4 biological replicates of ChIP-qPCR in primary HBE cells from 2 donors (Fig 4E). The FOXA1 enrichment near *RARB* was lower than the other sites, which may explain why FOXA1 KD does not alter *RARB* expression.

*EHF depletion alters expression of cytokines important for neutrophil migration and survival−*Genes involved in inflammation and infection are enriched among putative direct targets of EHF identified by ChIP-seq and RNA-seq. Two EHF binding sites were observed at the chr4q13.3 region encompassing the genes for the neutrophil chemokines *CXCL1, CXCL6,* and *IL8*, one 5' to *IL8* and the other 3' to *CXCL1* (Fig 5A). The sites identified by EHF ChIP-seq were confirmed by ChIP-qPCR in HBE cell chromatin from 4 donors (Fig 5A). The bronchial epithelium releases these cytokines in response to stimuli including IL-17a, a pro-inflammatory cytokine involved in multiple lung pathologies including CF (12,15), and lipopolysaccharide (LPS), a component of the gram-negative cell wall that induces a strong immune response in the lung (40). The role of EHF in regulating these immune mediators was studied in Calu-3 lung adenocarcinoma cells, which express *CXCL1, CXCL6* and *IL8*. To determine whether EHF regulates secretion of the three cytokines encoded by these genes, both at a basal level and in an inflammatory milieu, EHF was depleted in Calu-3 cells using a specific siRNA, with a non-targeting siRNA as control (Fig S5, S6). Cells were then treated with vehicle (bovine serum albumin [BSA]) or IL-17a for 4 hours, or vehicle (PBS) or LPS from *Pseudomonas aeruginosa* for 24 hours, and RNA was extracted. Cytokine expression was then quantified using RT-qPCR (Fig 5B-C). *CXCL6* expression was repressed upon EHF depletion in normal conditions and following LPS and IL-17a treatment. EHF knockdown was associated with increased *IL8* expression following LPS treatment, but there was no change in basal levels. *CXCL1* was not significantly regulated by EHF in either condition. To determine if the changes in *CXCL6* transcript

abundance altered CXCL6 cytokine secretion, colorimetric sandwich ELISAs were utilized to quantify its release into conditioned media. EHF depletion decreased CXCL6 secretion (Fig 5D), but did not significantly alter IL-8 secretion (Fig 5E), consistent with no change in *IL8* expression at basal levels.

Depletion of EHF slows wound closure in HBE cells from wildtype and cystic fibrosis patients−Among genes with nearby peaks of EHF occupancy that are also activated by EHF are many that are involved in EMT and response to wounding. When the lung epithelial barrier is damaged, cells at the edge of the wound migrate to close it. This mechanism may be impaired in airway epithelial cells that carry a *CFTR* mutation, suggesting that it contributes to CF lung pathology (5,41). Wound repair can be measured *in vitro* with a wound scratch assay, which was performed in primary HBE cells from 3 wildtype (WT) and 3 CF donors. EHF was depleted using siRNA, and the cells were grown to a confluent layer on plastic (undifferentiated). The layer was scratched, and the wounds were imaged at 0, 3 and 6 hours post-injury (Fig 6A). By 6 hours, many of the scratches had closed. The cells were lysed and EHF depletion was confirmed by quantification of western blots by gel densitometry (Fig 6B-C). The initial wound size was not significantly different between the negative control (NC) and EHF siRNA treated cells. The area was measured at subsequent time points and the percent of initial injury size was calculated (Fig 6D-E). In WT HBE cells at the 3 hour time point, the scratches in the EHF depleted cells remained significantly larger than those in the NC cells (p<0.01). In CF cells, EHF knockdown significantly delayed the reduction in wound size at both 3 and 6 hours. This corresponds to a slowing of wound closure in the EHF depleted cells compared to NC in both WT and CF cells.

DISCUSSION

Our data show that EHF is a critical regulator of multiple lung epithelial functions that are integral to pulmonary diseases, including cystic fibrosis. Among CF patients there is a wide variation in airway phenotypes, which are under strong genetic control (42). However, lung disease severity does not correlate with specific mutations in *CFTR* (43), implicating other loci (gene

modifiers) in modulating lung disease in this population. Our data suggest *EHF* may be one of those modifiers based on its functions described here and its genomic location at chr11p13, adjacent to SNPs that strongly associate with lung disease severity in CF patients (29,30). Previous genome-wide studies of EHF demonstrated its contribution to maintenance of epithelial barrier integrity and wound repair in Calu-3 lung adenocarcinoma cells (31) and in the mouse cornea (44). Here, we investigated the role of EHF in primary HBE cells, with a focus on understanding how it may contribute to lung pathology. The outcomes not only confirm our previous observations, but also reveal substantial novel findings on how EHF participates in lung epithelial differentiation and inflammation.

The intersection of ChIP-seq data sets gives an overall indication of the function of EHF with respect to gene regulation. EHF sites are over-represented at promoters (1 kb upstream of the TSS). A role for EHF in both activating and repressing gene expression is confirmed by the BETA output, which shows significant activity in both directions. This is consistent with previous studies at specific loci that demonstrate both active and repressive actions for EHF, depending on the cellular context (22,47,48). In our current data, the fraction of genes activated by EHF is greater than the repressed component, consistent with the presence of active histone marks at EHF sites.

Since EHF occupies the relatively common Ets motif, and it can increase or decrease gene expression, its interactions with other TFs are likely critical to specify binding location and mechanisms of action. The motif analysis of EHF binding sites in HBE cells shows that the most enriched secondary motif is for AP-1. These data are consistent with our previous findings that the AP-1 motif is over-represented in sites of EHF occupancy in Calu-3 cells (31). The AP-1 TF complex is made up of hetero- and homo-dimers of Jun, Fos and other family members. Regulation of transcription by AP-1 has an important role in differentiation, cell injury, inflammation and apoptosis in the lung (49,50). AP-1 activation may be elevated in CF tracheal cells as compared to their wildtype counterparts (51). EHF represses genes that contain both EHF and AP-1 motifs at their promoters (22), which suggests that the two factors can co-regulate gene expression. To

determine whether EHF and Jun proteins co-localize genome-wide, we performed ChIP-seq for c-Jun and JunD in Calu-3 cells and intersected these data with our EHF occupancy profile published previously (31). There is significant coincidence between binding sites for EHF and both AP-1 complex proteins. The overlapping sites are found near genes involved in multiple pathways important for lung epithelial function in disease, including differentiation and wound repair. Further investigation of genes co-regulated by EHF and AP-1 could elucidate potential targets to modulate inflammation and wound repair in the airway.

Normal homeostasis in the lung epithelium depends on appropriate responses to external stimuli. An abnormal or exaggerated reaction can initiate or exacerbate lung disease. The pivotal role of several TFs in airway development and differentiation is well documented, particularly through studies in rodents. Nkx2-1 is the first marker of anterior foregut cells that will become respiratory epithelial progenitors (52). FoxA1 and FoxA2 are pioneer transcription factors required for branching morphogenesis early in lung development and epithelial differentiation at later stages (53,54). Retinoic acid receptor (RAR) signaling is also necessary for proper branching morphogenesis (55). GATA6 is involved in lung epithelial differentiation throughout development, but is also active in post-natal epithelial regeneration (56-58). Hopx acts downstream of Nkx2-1 to repress expression of GATA6/Nkx2-1-induced expression of type II pneumocyte genes, and is critical for alveolar development (59), while Klf5 is required for maturation of the alveolar respiratory epithelium (60). SPDEF is involved in airway goblet cell differentiation and mucus production and antagonizes the action of FoxA2 (61). While these TFs are best studied in airway epithelial development, they are also likely to be involved in epithelial regeneration and dysplasia during disease. Here, we demonstrate that EHF contributes to the transcriptional network controlling pulmonary gene expression.

By integrating the results of EHF ChIP-seq and its depletion followed by RNA-seq, we identified putative direct targets of the protein. TFs involved in lung development are among these identified targets. EHF represses *HOPX,*

KLF5, and *RARB,* likely through binding to the promoter or a nearby *cis*-regulatory element. EHF binding sites are enriched for the Forkhead motif, which is bound by both FOXA1 and FOXA2. Interestingly, FOXA1 and EHF regulate each other and impact expression of downstream TF targets similarly. Hence, these two factors may cooperate, and perhaps participate together in a feedback loop.

Although it represses most TFs involved in lung development, EHF enhances the expression of *SPDEF,* which participates in goblet cell differentiation in the airway, and is induced by IL-13 (38,39). Given the known role of *SPDEF* in goblet cells (38,39,62), these data implicate EHF in activating goblet cell differentiation, and perhaps perturbing that of other lineages. This suggests that EHF contributes to an IL-13-mediated cell differentiation pathway by activating SPDEF and repressing other transcription factors. IL-13 is a Th2 cytokine that induces goblet cell hyperplasia in the airway through SPDEF, with known roles in CF, asthma, and COPD (11,13,38,39). Previously, immunohistochemical analysis showed the most intense staining for EHF in the airway was in mucous gland epithelial cells, with a smaller fraction of bronchial epithelial cells being positive for EHF (22,23). The pivotal role of submucosal glands in CF airway pathology (63) is particularly relevant to these observations.

In airway diseases such as CF, both IL-13 and IL-17a play a prominent role in promoting inflammation. Having observed that EHF influences expression of genes downstream of IL-13, we chose also to investigate a potential function downstream of IL-17a. Il-17a induces bronchial epithelial cells to release neutrophil chemokines, including CXCL1, CXCL6 and IL-8 (15). These cytokines promote migration and survival of neutrophils. This is critical for CF lung disease, as a neutrophil-dominant inflammation is present early in the illness (8-10). Release of proteases by neutrophils contributes to lung damage (64,65). EHF depletion in lung adenocarcinoma cells increases *IL8* expression and decreases *CXCL6* expression, This correlates with changes in secretion of CXCL6. By increasing CXCL6 secretion, EHF could increase neutrophil recruitment and survival, thereby exacerbating inflammation in the CF lung.

Another important function of lung epithelial cells that may be impaired in CF is response to wounding (5,41). This is problematic in a disease characterized by inflammation and resulting damage to the lung epithelium. We previously showed that EHF depletion slows scratch wound closure in Calu-3 lung adenocarcinoma cells (31). Consistent with these earlier observations are the current genome-wide studies in HBE cells, which show an enrichment of EHF-activated genes in EMT and response to wounding. Moreover, we demonstrate here that EHF depletion slows wound scratch closure in both WT and CF HBE cells. By further impairing a phenotype that may already compromised in *CFTR* mutant epithelial cells, EHF could modify lung disease severity in CF patients. There are certain limitations to the scratch assays performed here because the cells are not differentiated on permeable supports. Hence, some epithelial cells types are absent and cannot contribute to normal wound closure. Differences in the transcriptome of differentiated and undifferentiated cells may also alter their interactions with the environment.

Overall, this study makes a strong case for EHF as a potential modifier of lung pathology in cystic fibrosis and other diseases in which the immune system and epithelial repair mechanisms play a critical role, including COPD and asthma. Although we have not yet established a correlation between the SNPs associated with lung disease severity in CF and EHF transcription, we predict based on our EHF functional data in HBE cells that regulatory variants may impact its expression. A detailed investigation of control mechanisms for *EHF* is required to reveal their contribution to airway disease.

EXPERIMENTAL PROCEDURES

Cell culture−Calu-3 cells were obtained from ATCC and cultured by standard methods. Passage 1 (P1) primary bronchial epithelial cells were obtained under protocol #03−1396 approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board. Informed consent was obtained from authorized representatives of all organ donors. Cells were grown on collagen-coated plastic in bronchial epithelial growth medium (BEGM) (66). Passage 2 or 3 primary cells were used for the study.

ChIP-seq and ChIP-qPCR−Antibodies used in ChIP-seq were against EHF (Clone 5A.5) (22), H3K4me1 (ab8895, Abcam (Ab)), H3K4me3 (07-0473, Millipore), H3K27ac (ab4729), c-Jun (sc-1694, Santa Cruz (SC)), and JunD (sc-74). ChIP-seq was performed as described previously (31) (ChIP-seq experiment information is shown in Table S5). For EHF ChIP-seq, replicates were performed on HBE cells from 2 donors. One donor was used for histone modification ChIP-seq. For c-Jun and JunD ChIP-seq, two biological replicates were performed on Calu-3 cells. Peaks were called using the HOMER-IDR method (IDR<0.05) (32). Histone tag directories were generated using HOMER (68). Downstream analysis was performed using HOMER and Cis-regulatory Element Annotation System (CEAS) (33). To confirm EHF results, ChIP was performed as described followed by qPCR using primer pairs specific for each target of interest and SYBR green (Life Technologies, LT). FoxA1 ChIP was performed as described previously (69) with antibody against FoxA1 (ab23738). For primers see Table S6. All data are deposited at GEO (GSE: GSE85403).

RNA-seq−HBE cells were treated with 30nM of either Silencer Select negative control siRNA #2 (4390846) or EHF siRNA (s25399, both from Ambion) using RNAiMax transfection reagent (LT). 48 hours after transfection, RNA was isolated from 5 samples of each treatment using TRIzol (LT). RNA-seq was performed as described previously (31) (see Table S7 for replicate information). Differentially expressed genes were identified using TopHat and Cufflinks (36). Direct targets of EHF were identified using Binding and Expression Target Analysis (BETA) (37). This software assigns transcription factor binding peaks to genes using a model that decreases the regulatory potential of the TF binding site as the distance to the TSS increases, then sums the contribution of multiple sites near a gene (in this analysis, within 10 kb or a CTCF site, whichever is closer). It also takes into account the significance of gene expression changes following modulation of the transcription factor (with an FDR <0.1). All data are deposited at GEO (GSE: GSE85403).

EHF and FoxA1 knockdown followed by RT-qPCR−TF depletion was done in 3 (FoxA1) or 4 (EHF) HBE donor cultures. EHF depletion was

as described above. For FOXA1, HBE cells were treated with 20nM of control siRNA-A (sc-37007, SC) or HNF-3α siRNA (sc-37930, SC) using RNAiMax. RNA was isolated 48 (FOXA1) or 72 (EHF) hours as above. RT-qPCR was performed using TaqMan Reverse Transcription Reagents kit (LT), primer pairs specific to each gene and SYBR green (for primers see Table S6).

Western Blot−HBE and Calu-3 cells were lysed in NET buffer and proteins analyzed by standard methods. Antibodies used were specific for EHF (Clone 5A.5), β-tubulin (T4026, Sigma-Aldrich), GAPDH (Cell Signaling).

LPS and IL-17a treatment of Calu-3 cells−Cells were forward transfected with NC or EHF siRNA as above. Forty-eight hours later, cells were serum starved for 24 hours, then treated with PBS or 1μg/mL *Pseudomonas aeruginosa* LPS (Sigma, L9134) for 4 hours; or BSA or

10ng/mL IL-17a (R&D Systems) for 24 hours. RNA was isolated using TRIzol, and RT-qPCR (SYBR green) was performed as above (For primers see Table S6).

ELISAs−Cells were forward transfected with NC or EHF siRNA as described. 48 hours post-transfection, media was replaced with serum-free media and conditioned for 24 hours. Media was removed and diluted. Quantikine colorimetric sandwich ELISA for IL-8 (D800C) and CXCL6 (DGC00, both from R&D) was performed according to the manufacturer's protocol.

Wound repair assay−Wound closure assay was performed as previously described (31) on 3 donor wildtype HBE cultures and 3 donor CF HBE cultures. Images were obtained at room temperature using a Leica DMi1 microscope with a PH1 10x objective using an Olympus DP22 camera and cellSens Entry software.

Conflict of Interest Statement: The authors have no conflicts of interest to disclose.

Author contributions: SLF and AH conceived and coordinated the study and wrote the manuscript. MJM optimized and performed the EHF ChIP-seq experiments shown in Figure 1. SG performed the H3K4me3 ChIP-seq experiments shown in Figures 1 and 2. LCJ generated the RNA-sequencing libraries for experiments shown in Figure 3. SHR isolated and provided the primary HBE cells. S-HL grew the primary HBE cells, provided expert advice on these cells, and assisted with the wound closure experiments shown in Figure 6. SLF performed and analyzed the remaining experiments shown. AT generated and provided the EHF antibody necessary to complete these studies and gave valuable insights into the biology of EHF. All authors reviewed the results and approved the final version of the manuscript.

REFERENCES

1. Crosby, L., and Waters, C. M. (2010) Epithelial repair mechanisms in the lung. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* **298**, L715-L731

2. Hallstrand, T. S., Hackett, T. L., Altemeier, W. A., Matute-Bello, G., Hansbro, P. M., and Knight, D. A. (2014) Airway epithelial regulation of pulmonary immune homeostasis and inflammation. *Clin. Immunol.* **151**, 1-15

3. Knowles, M. R., and Boucher, R. C. (2002) Mucus clearance as a primary innate defense mechanism for mammalian airways. *J. Clin. Invest.* **109**, 571-577

4. Fahy, J. V., and Dickey, B. F. (2010) Airway mucus funciton and dysfunction. *N. Engl. J. Med.* **363**, 2233-2247

5. Schiller, K. R., Maniak, P. J., and O'Grady, S. M. (2010) Cystic fibrosis transmembrane conductance regulator is involved in airway epithelial wound repair. *Am. J. Physiol. Cell. Physiol.* **299**, C912-C921

6. Cohen, T. S., and Prince, A. (2012) Cystic fibrosis: a mucosal immunodeficiency syndrome. *Nat. Med.* **18**, 509-519

7. Grosse, S. D., Boyle, C. A., Botkin, J. R., Comeau, A. M., Kharrazi, M., Rosenfeld, M., and Wilfond, B. S. (2004) Newborn Screening for Cystic Fibrosis: Evaluation of Benefits and Risks and Recommendations for State Newborn Screening Programs. National Center on Birth Defects and Developmental Disabilities. *C.D.C. M.M.W.R.* **53**, 1-36

8. Khan, T. Z., Wagener, J. S., Martinez, J., Accurso, F. J., and Riches, D. (1995) Early pulmonary inflammation in infants with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **151**, 1075-1082

9. Konstan, M. W., Hilliard, K. A., Norvell, T. M., and Berger, M. (1994) Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. *Am. J. Respir. Crit. Care Med.* **150**, 448-454

10. Rosenfeld, M., Gibson, R. L., McNamara, S., Emerson, J., Burns, J. L., Castile, R., Hiatt, P., McCoy, K., Wilson, C. B., Inglis, A., Smith, A., Martin, T. R., and Ramsey, B. W. (2001) Early Pulmonary Infection, Inflammation, and Clinical Outcomes in Infants With Cystic Fibrosis. *Pediatr. Pulmonol.* **32**, 356-366

11. Hartl, D., Griese, M., Kappler, M., Zissel, G., Reinhardt, D., Rebhan, C., Schendel, D. J., and Krauss-Etschmann, S. (2006) Pulmonary T(H)2 response in Pseudomonas aeruginosa-infected patients with cystic fibrosis. *J. Allergy Clin. Immunol.* **117**, 204-211

12. Tan, H. L., Regamey, N., Brown, S., Bush, A., Lloyd, C. M., and Davies, J. C. (2011) The Th17 pathway in cystic fibrosis lung disease. *Am. J. Respir. Crit. Care Med.* **184**, 252-258

13. Tiringer, K., Treis, A., Fucik, P., Gona, M., Gruber, S., Renner, S., Dehlink, E., Nachbaur, E., Horak, F., Jaksch, P., Doring, G., Crameri, R., Jung, A., Rochat, M. K., Hormann, M., Spittler, A., Klepetko, W., Akdis, C. A., Szepfalusi, Z., Frischer, T., and Eiwegger, T. (2013) A Th17- and Th2-skewed cytokine profile in cystic fibrosis lungs represents a potential risk factor for Pseudomonas aeruginosa infection. *Am. J. Respir. Crit. Care Med.* **187**, 621-629

14. Kuperman, D. A., Huang, X., Koth, L. L., Chang, G. H., Dolganov, G. M., Zhu, Z., Elias, J. A., Sheppard, D., and Erle, D. J. (2002) Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat. Med.* **8**, 885-889

15. Prause, O., Laan, M., Lötvall, J., and Lindén, A. (2003) Pharmacological modulation of interleukin-17-induced GCP-2-, GRO-α- and interleukin-8 release in human bronchial epithelial cells. *Eur. J. Pharmacol.* **462**, 193-198

16. Durieu, I., Peyrol, S., Dominique, G., Bellon, G., Durand, D. V., and Pacheco, Y. (1998) Subepithelial fibrosis and degradation of the bronchial extracellular matrix in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **158**, 580-588

17. Hamutcu, R., Rowland, J. M., Horn, M. V., Kaminsky, C., MacLaughlin, E. F., Starnes, V. A., and Woo, M. S. (2002) Clinical Findings and Lung Pathology in Children with Cystic Fibrosis. *Am. J. Respir. Crit. Care Med.* **165**, 1172-1175

18. Hilliard, T. N., Regamey, N., Shute, J. K., Nicholson, A. G., Alton, E. W., Bush, A., and Davies, J. C. (2007) Airway remodelling in children with cystic fibrosis. *Thorax* **62**, 1074-1080

19. Regamey, N., Jeffery, P. K., Alton, E. W., Bush, A., and Davies, J. C. (2011) Airway remodelling and its relationship to inflammation in cystic fibrosis. *Thorax* **66**, 624-629

20. Kim, V., Rogers, T. J., and Criner, G. J. (2008) New concepts in the pathobiology of chronic obstructive pulmonary disease. *Proc. Am. Thorac. Soc.* **5**, 478-485

21. Holgate, S. T. (2011) The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol. Rev.* **242**, 205-219

22. Tugores, A., Le, J., Sorokina, I., Snijders, A. J., Duyao, M., Reddy, P. S., Carlee, L., Ronshaugen, M., Mushegian, A., Watanaskul, T., Chu, S., Buckler, A., Emtage, S., and McCormick, M. K. (2001) The epithelium-specific ETS protein EHF/ESE-3 is a context-dependent transcriptional repressor downstream of MAPK signaling cascades. *J. Biol. Chem.* **276**, 20397-20406

23. Silverman, E. S., Baron, R. M., Palmer, L. J., Le, L., Hallock, A., Subramaniam, V., Riese, R. J., McKenna, M. D., Gu, X., Libermann, T. A., Tugores, A., Haley, K. J., Shore, S., Drazen, J. M., and Weiss, S. T. (2002) Constitutive and Cytokine-Induced Expression of the ETS Transcription Factor ESE-3 in the Lung. *Am. J. Respir. Cell Mol. Biol.* **27**, 697-704

24. Kas, K., Finger, E., Grall, F., Gu, X., Akbarali, Y., Boltax, J., Weiss, A., Oettgen, P., Kapeller, R., and Libermann, T. A. (2000) ESE-3, a Novel Member of an Epithelium-specific Ets Transcription Factor Subfamily, Demonstrates Different Target Gene Specificity from ESE-1. *J. Biol. Chem.* **275**, 2986-2998

25. Wu, J., Duan, R., Cao, H., Field, D., Newnham, C. M., Koehler, D. R., Zamel, N., Pritchard, M. A., Hertzog, P., Post, M., Tanswell, A. K., and Hu, J. (2008) Regulation of epithelium-specific Ets-like factors ESE-1 and ESE-3 in airway epithelial cells: potential roles in airway inflammation. *Cell. Res.* **18**, 649-663

26. Albino, D., Longoni, N., Curti, L., Mello-Grand, M., Pinton, S., Civenni, G., Thalmann, G., D'Ambrosio, G., Sarti, M., Sessa, F., Chiorino, G., Catapano, C. V., and Carbone, G. M. (2012) ESE3/EHF controls epithelial cell differentiation and its loss leads to prostate tumors with mesenchymal and stem-like features. *Cancer Res.* **72**, 2889-2900

27. Thiery, J. P., and Sleeman, J. P. (2006) Complex networks orchestrate epithelial-mesenchymal transitions. *Nat. Rev. Mol. Cell Biol.* **7**, 131-142

28. Kim, K. K., Kugler, M. C., Wolters, P. J., Robillard, L., Galvez, M. G., Brumwell, A. N., Sheppard, D., and Chapman, H. A. (2006) Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 13180-13185

29. Wright, F. A., Strug, L. J., Doshi, V. K., Commander, C. W., Blackman, S. M., Sun, L., Berthiaume, Y., Cutler, D., Cojocaru, A., Collaco, J. M., Corey, M., Dorfman, R., Goddard, K., Green, D., Kent, J. W., Jr., Lange, E. M., Lee, S., Li, W., Luo, J., Mayhew, G. M., Naughton, K. M., Pace, R. G., Pare, P., Rommens, J. M., Sandford, A., Stonebraker, J. R., Sun, W., Taylor, C., Vanscoy, L. L., Zou, F., Blangero, J., Zielenski, J., O'Neal, W. K., Drumm, M. L., Durie, P. R., Knowles, M. R., and Cutting, G. R. (2011) Genome-wide association and linkage identify modifier loci of lung disease severity in cystic fibrosis at 11p13 and 20q13.2. *Nat. Genet.* **43**, 539-546

30. Corvol, H., Blackman, S. M., Boelle, P. Y., Gallins, P. J., Pace, R. G., Stonebraker, J. R., Accurso, F. J., Clement, A., Collaco, J. M., Dang, H., Dang, A. T., Franca, A., Gong, J., Guillot, L., Keenan, K., Li, W., Lin, F., Patrone, M. V., Raraigh, K. S., Sun, L., Zhou, Y. H., O'Neal, W. K., Sontag, M. K., Levy, H., Durie, P. R., Rommens, J. M., Drumm, M. L., Wright, F. A., Strug, L. J., Cutting, G. R., and Knowles, M. R. (2015) Genome-wide association meta-analysis identifies five modifier loci of lung disease severity in cystic fibrosis. *Nat. Commun.* **6**, 8382

31. Fossum, S. L., Mutolo, M. J., Yang, R., Dang, H., O'Neal, W. K., Knowles, M. R., Leir, S. H., and Harris, A. (2014) Ets homologous factor regulates pathways controlling response to injury in airway epithelial cells. *Nucleic Acids Res.* **42**, 13588-13598

32.   Karmel, A. (2014) Homer-idr: Second pass updated. DOI: 10.5281/zenodo.11619.

33.   Ji, X., Li, W., Song, J., Wei, L., and Liu, X. S. (2006) CEAS: cis-regulatory element annotation system. *Nucleic Acids Res.* **34**, W551-554

34.   Heintzman, N. D., Stuart, R. K., Hon, G., Fu, Y., Ching, C. W., Hawkins, R. D., Barrera, L. O., Van Calcar, S., Qu, C., Ching, K. A., Wang, W., Weng, Z., Green, R. D., Crawford, G. E., and Ren, B. (2007) Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat. Genet.* **39**, 311-318

35.   Zhou, V. W., Goren, A., and Bernstein, B. E. (2011) Charting histone modifications and the functional organization of mammalian genomes. *Nat. Rev. Genet.* **12**, 7-18

36.   Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L., and Pachter, L. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562-578

37.   Wang, S., Sun, H., Ma, J., Zang, C., Wang, C., Wang, J., Tang, Q., Meyer, C. A., Zhang, Y., and Liu, X. S. (2013) Target analysis by integration of transcriptome and ChIP-seq data with BETA. *Nat. Protoc.* **8**, 2502-2515

38.   Park, K. S., Korfhagen, T. R., Bruno, M. D., Kitzmiller, J., Wan, H., Wert, S., Hershey, G. K. K., Chen, G., and Whitsett, J. A. (2007) SPDEF regulates goblet cell hyperplasia in the airway epithelium. *J. Clin. Invest.* **117**, 978-988

39.   Chen, G., Korfhagen, T. R., Xu, Y., Kitzmiller, J., Wert, S., Maeda, Y., Gregorieff, A., Clevers, H., and Whitsett, J. A. (2009) SPDEF is required for mouse pulmonary goblet cell differentiation and regulates a network of genes associated with mucus production. *J. Clin. Invest.* **119**, 2914-2924

40.   Pace, E., Ferraro, M., Siena, L., Melis, M., Montalbano, A. M., Johnson, M., Bonsignore, M. R., Bonsignore, G., and Gjomarkaj, M. (2008) Cigarette smoke increases Toll-like receptor 4 and modifies lipopolysaccharide-mediated responses in airway epithelial cells. *Immunology* **124**, 401-411

41.   Itokazu, Y., Pagano, R. E., Schroeder, A. S., O'Grady, S. M., Limper, A. H., and Marks, D. L. (2014) Reduced GM1 ganglioside in CFTR-deficient human airway cells results in decreased beta1-integrin signaling and delayed wound repair. *Am. J. Physiol. Cell Physiol.* **306**, C819-830

42.   Vanscoy, L. L., Blackman, S. M., Collaco, J. M., Bowers, A., Lai, T., Naughton, K., Algire, M., McWilliams, R., Beck, S., Hoover-Fong, J., Hamosh, A., Cutler, D., and Cutting, G. R. (2007) Heritability of lung disease severity in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **175**, 1036-1043

43.   The Cystic Fibrosis Genotype-Phenotype Consortium. (1993) Correlation between genotype and phenotype in patients with cystic fibrosis. *N. Engl. J. Med.* **329**, 1308-1313

44.   Stephens, D. N., Herndon Klein, R., Salmans, M. L., Gordon, W., Ho, H., and Andersen, B. (2013) The Ets transcription factor EHF as a regulator of cornea epithelial cell identity. *J. Biol. Chem.* **288**, 34304-34324

45.   Creyghton, M. P., Cheng, A. W., Welstead, G. G., Kooistra, T., Carey, B. W., Steine, E. J., Hanna, J., Lodato, M. A., Frampton, G. M., Sharp, P. A., Boyer, L. A., Young, R. A., and Jaenisch, R. (2010) Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 21931-21936

46.   Encode Project Consortium (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**, 57-74

47.   Taniue, K., Oda, T., Hayashi, T., Okuno, M., and Akiyama, T. (2011) A member of the ETS family, EHF, and the ATPase RUVBL1 inhibit p53-mediated apoptosis. *EMBO Rep.* **12**, 682-689

48.   Kunderfranco, P., Mello-Grand, M., Cangemi, R., Pellini, S., Mensah, A., Albertini, V., Malek, A., Chiorino, G., Catapano, C. V., and Carbone, G. M. (2010) ETS transcription factors control transcription of EZH2 and epigenetic silencing of the tumor suppressor gene Nkx3.1 in prostate cancer. *PloS One* **5**, e10547

49. Reddy, S. P., and Mossman, B. T. (2002) Role and regulation of activator protein-1 in toxicant-induced responses of the lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **283**, L1161-L1178

50. Mossman, B. T., Lounsbury, K. M., and Reddy, S. P. (2006) Oxidants and signaling by mitogen-activated protein kinases in lung epithelium. *Am. J. Respir. Cell Mol. Biol.* **34**, 666-669

51. Verhaeghe, C., Remouchamps, C., Hennuy, B., Vanderplasschen, A., Chariot, A., Tabruyn, S. P., Oury, C., and Bours, V. (2007) Role of IKK and ERK pathways in intrinsic inflammation of cystic fibrosis airways. *Biochem. Pharmacol.* **73**, 1982-1994

52. Herriges, M., and Morrisey, E. E. (2014) Lung development: orchestrating the generation and regeneration of a complex organ. *Development* **141**, 502-513

53. Wan, H., Kaestner, K. H., Ang, S. L., Ikegami, M., Finkelman, F. D., Stahlman, M. T., Fulkerson, P. C., Rothenberg, M. E., and Whitsett, J. A. (2004) Foxa2 regulates alveolarization and goblet cell hyperplasia. *Development* **131**, 953-964

54. Wan, H., Dingle, S., Xu, Y., Besnard, V., Kaestner, K. H., Ang, S. L., Wert, S., Stahlman, M. T., and Whitsett, J. A. (2005) Compensatory roles of Foxa1 and Foxa2 during lung morphogenesis. *J. Biol. Chem.* **280**, 13809-13816

55. Mollard, R., Ghyselinck, N. B., Wendling, O., Chambon, P., and Mark, M. (2000) Stage-dependent responses of the developing lung to retinoic acid signaling. *Int. J. Dev. Biol.* **44**, 457-462

56. Yang, H., Lu, M. M., Zhang, L., Whitsett, J. A., and Morrisey, E. E. (2002) GATA6 regulates differentiation of the distal lung epithelium. *Development* **129**, 2233-2246

57. Zhang, Y., Rath, N., Hannenhalli, S., Wang, Z., Cappola, T., Kimura, S., Atochina-Vasserman, E., Lu, M. M., Beers, M. F., and Morrisey, E. E. (2007) GATA and Nkx factors synergistically regulate tissue-specific gene expression and development in vivo. *Development* **134**, 189-198

58. Zhang, Y., Goss, A. M., Cohen, E. D., Kadzik, R., Lepore, J. J., Muthukumaraswamy, K., Yang, J., DeMayo, F. J., Whitsett, J. A., Parmacek, M. S., and Morrisey, E. E. (2008) A Gata6-Wnt pathway required for epithelial stem cell development and airway regeneration. *Nat. Genet.* **40**, 862-870

59. Yin, Z., Gonzalez, D., Kolla, V., Rath, N., Zhang, Y., Lu, M. M., Kimura, S., Ballard, P. L., Beers, M. F., Epstein, J. A., and Morrisey, E. E. (2006) Hop functions downstream of Nkx2.1 and GATA6 to mediate HDAC-dependent negative regulation of pulmonary gene expression. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **291**, L191-L199

60. Wan, H., Luo, F., Wert, S. E., Zhang, L., Xu, Y., Ikegami, M., Maeda, Y., Bell, S. M., and Whitsett, J. A. (2008) Kruppel-like factor 5 is required for perinatal lung morphogenesis and function. *Development* **135**, 2563-2572

61. Korfhagen, T. R., Kitzmiller, J., Chen, G., Sridharan, A., Haitchi, H.-M., Hegde, R. S., Divanovic, S., Karp, C. L., and Whitsett, J. A. (2012) SAM-pointed domain ETS factor mediates epithelial cell-intrinsic innate immune signaling during airway mucous metaplasia. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 16630-16635

62. Rajavelu, P., Chen, G., Xu, Y., Kitzmiller, J. A., Korfhagen, T. R., and Whitsett, J. A. (2015) Airway epithelial SPDEF integrates goblet cell differentiation and pulmonary Th2 inflammation. *J. Clin. Invest.* **125**, 2021-2031

63. Widdicombe, J. H., and Wine, J. J. (2015) Airway Gland Structure and Function. *Physiol. Rev.* **95**, 1241-1319

64. Birrer, P., McElvaney, N. G., Ruderberg, C., Sommer, W., Liechti-Gallati, S., Kraemer, R., Hubbard, R., and Crystal, R. G. (1994) Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **150**, 207-213

65. Voynow, J. A., Fischer, B. M., and Zheng, S. (2008) Proteases and cystic fibrosis. *Int. J. Biochem. Cell Biol.* **40**, 1238-1245

66. Fulcher, M. L., Gabriel, S., Burns, K. A., Yankaskas, J. R., and Randell, S. H. (2005) Well differentiated human airway epithelial cell cultures. *Methods Mol. Med.* **107**, 183-206

67.   Zhen, G., Park, S. W., Nguyenvu, L. T., Rodriguez, M. W., Barbeau, R., Paquet, A. C., and Erle, D. J. (2007) IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. *Am. J. Respir. Cell. Mol. Biol.* **36**, 244-253

68.   Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., Cheng, J. X., Murre, C., Singh, H., and Glass, C. K. (2010) Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell* **38**, 576-589

69.   Kerschner, J. L., Gosalia, N., Leir, S. H., and Harris, A. (2014) Chromatin remodeling mediated by the FOXA1/A2 transcription factors activates CFTR expression in intestinal epithelial cells. *Epigenetics* **9**, 557-565
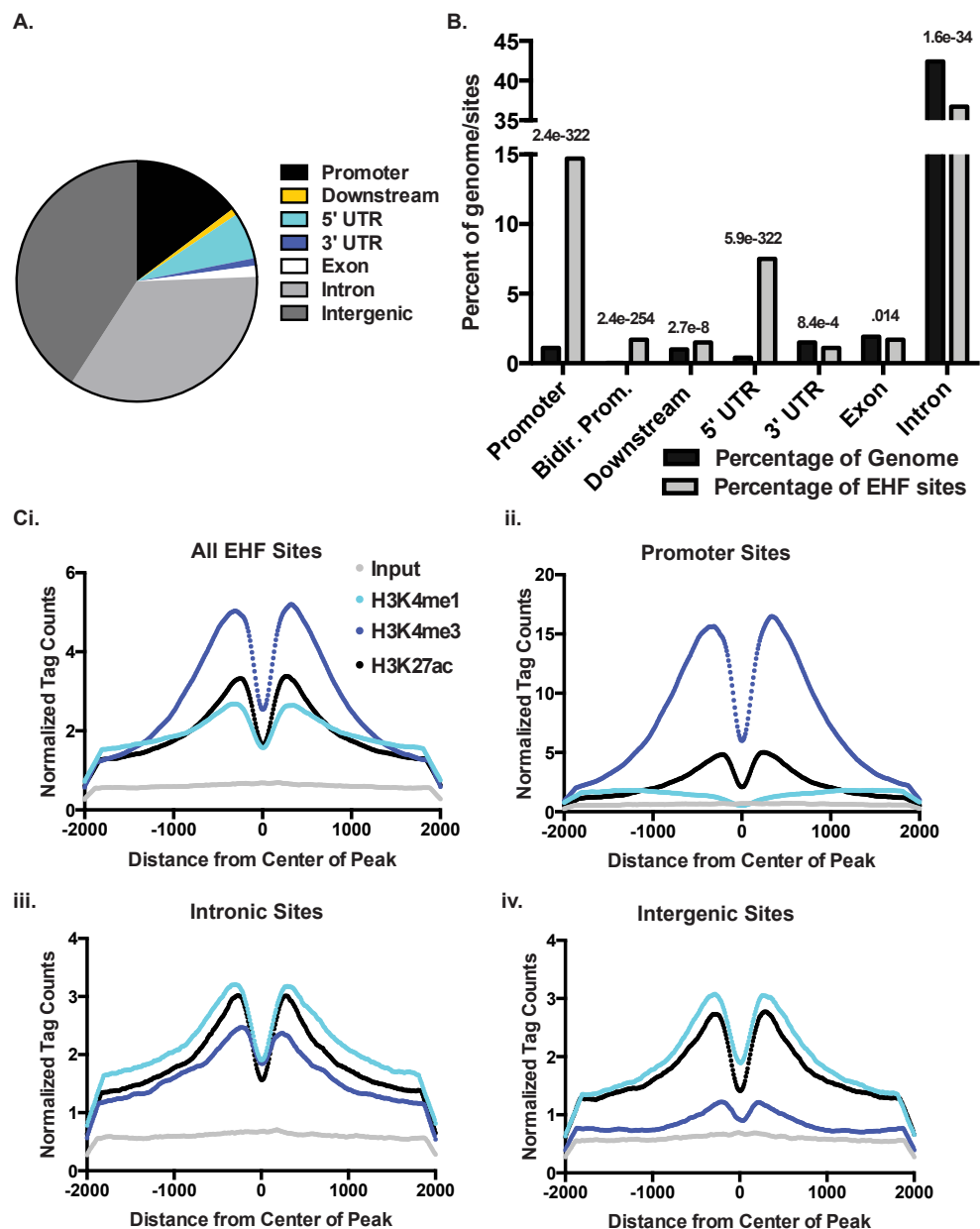
Fig 1. EHF ChIP-seq sites are over-represented at promoters and enriched for activate histone marks. EHF ChIP-seq was performed in undifferentiated HBE cells from 2 donor cultures. A) Percentage of EHF binding sites found at promoters (1 kb upstream of transcription start sites [TSS]), downstream of genes (1 kb), 5' untranslated regions (UTRs), 3' UTRs, exons, introns, and intergenic sites. B) Percentage of EHF ChIP-seq peaks found at specific sites compared to genomic background. Bidirectional promoters (Bidir. prom.) are considered 2.5 kb upstream of TSSs. P-values, derived using CEAS (one-sided binomial test), are shown. C) Normalized tag counts from H3K4me1, H3K4me3, and H3K27ac ChIP-seq in undifferentiated HBE cells, as measured from the center of i) all EHF, ii) promoter-, iii) intronic- and iv) intergenic- binding sites. Tag counts for input sonicated HBE DNA provide a control.
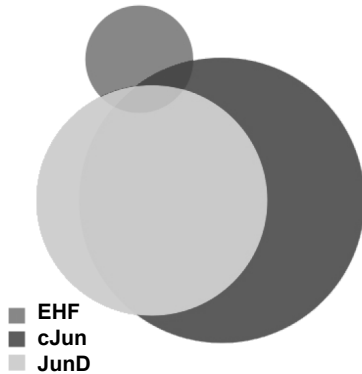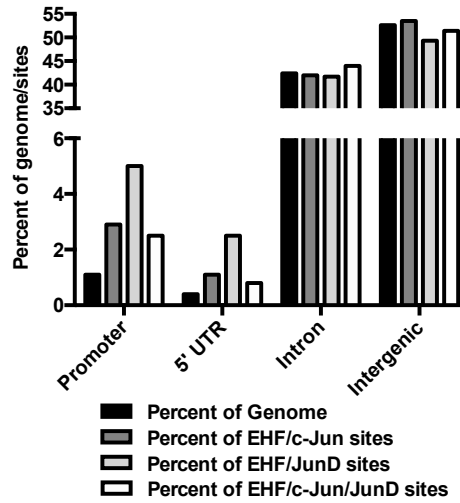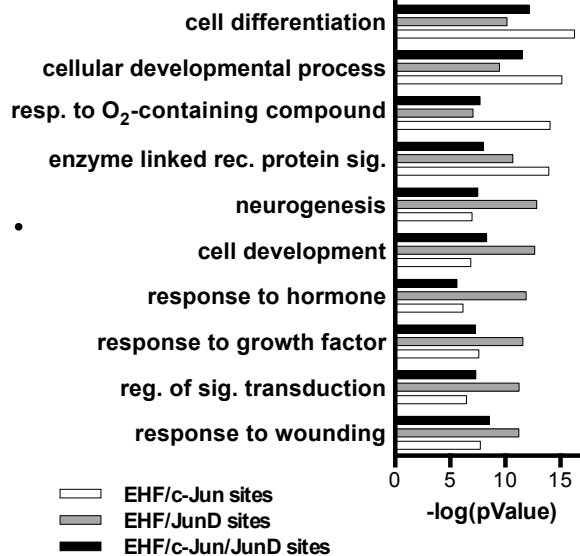
Figure 2. EHF and the activator protein 1 (AP‑1) transcription factor complex bind at overlapping sites genome-wide. A) *De novo* motif analysis within 200 bp of the center of EHF ChIP-seq peaks in undifferentiated HBE cells identified enriched motifs. P-values, derived by HOMER using cumulative binomial distributions, and percent of EHF sites and of background containing each motif are shown below the sequence. B) Intersection of EHF, c-Jun and JunD ChIP-seq binding sites in Calu-3 cells. C) Annotation of EHF/c-Jun, EHF/JunD, and EHF/c-Jun/JunD overlapping sites, compared to the percentage of the genome. P-values derived using CEAS (one-sided binomial test). D) Gene ontology process enrichment analysis was performed on the nearest gene to each EHF/c-Jun, EHF/JunD, and EHF/c-Jun/JunD intersecting region. Shown are the negative logs of the P-values, derived using MetaCore. Abbreviations: Resp. = response, comp. = compound, rec. = receptor, sig. = signaling, reg. = regulation, also used in Figure 3D.
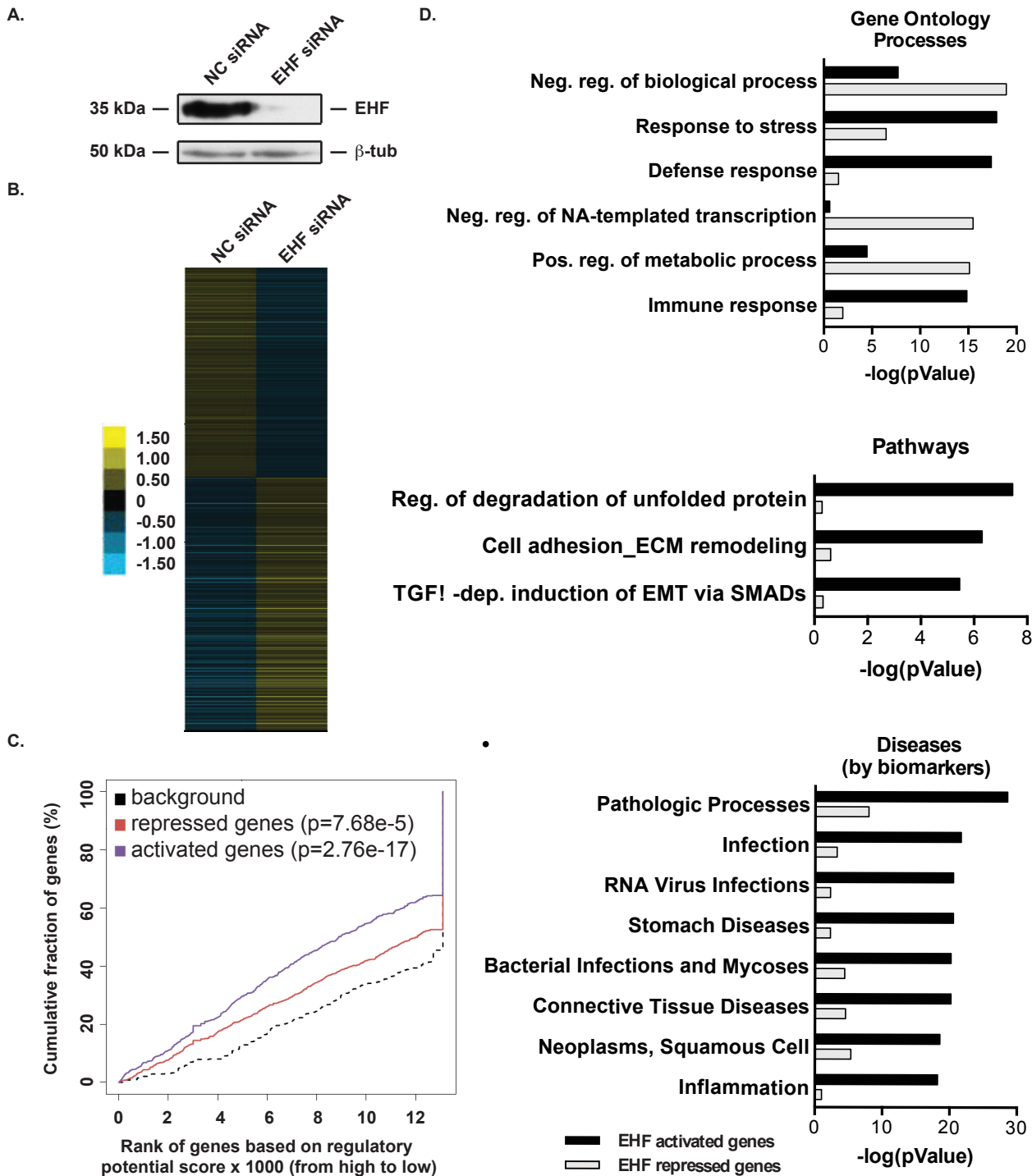
Figure 3. EHF depletion followed by RNA-seq identifies targets involved in the immune response and gene regulation. A) siRNA depletion of EHF in undifferentiated HBE cells was measured by western blot with β-tubulin as a loading control. B) Relative expression of 1,145 genes that show a significant change in expression following EHF depletion in undifferentiated HBE cells (FDR < 0.05). Each row signifies a different gene. The scale is log2 fold change relative to average gene expression. C) Activating and repressive function prediction of EHF in undifferentiated primary HBE cells as determined by BETA software. The red, purple, and dashed lines indicate the down-regulated, up-regulated, and non-differentially expressed genes, respectively. P-values of activated and repressed genes compared to non-regulated genes were determined by a Kolmogorov-Smirnov test. D) MetaCore enrichment analysis of direct targets of EHF. Analysis was performed on EHF-activated (black) and –repressed (grey) genes, and divided into gene ontology processes (top), pathways (middle) and diseases (bottom). Shown are the negative logs of the P-values, derived using MetaCore. Abbreviations: Neg. = negative, NA = nucleic acid, pos. = positive, dep. = dependent.
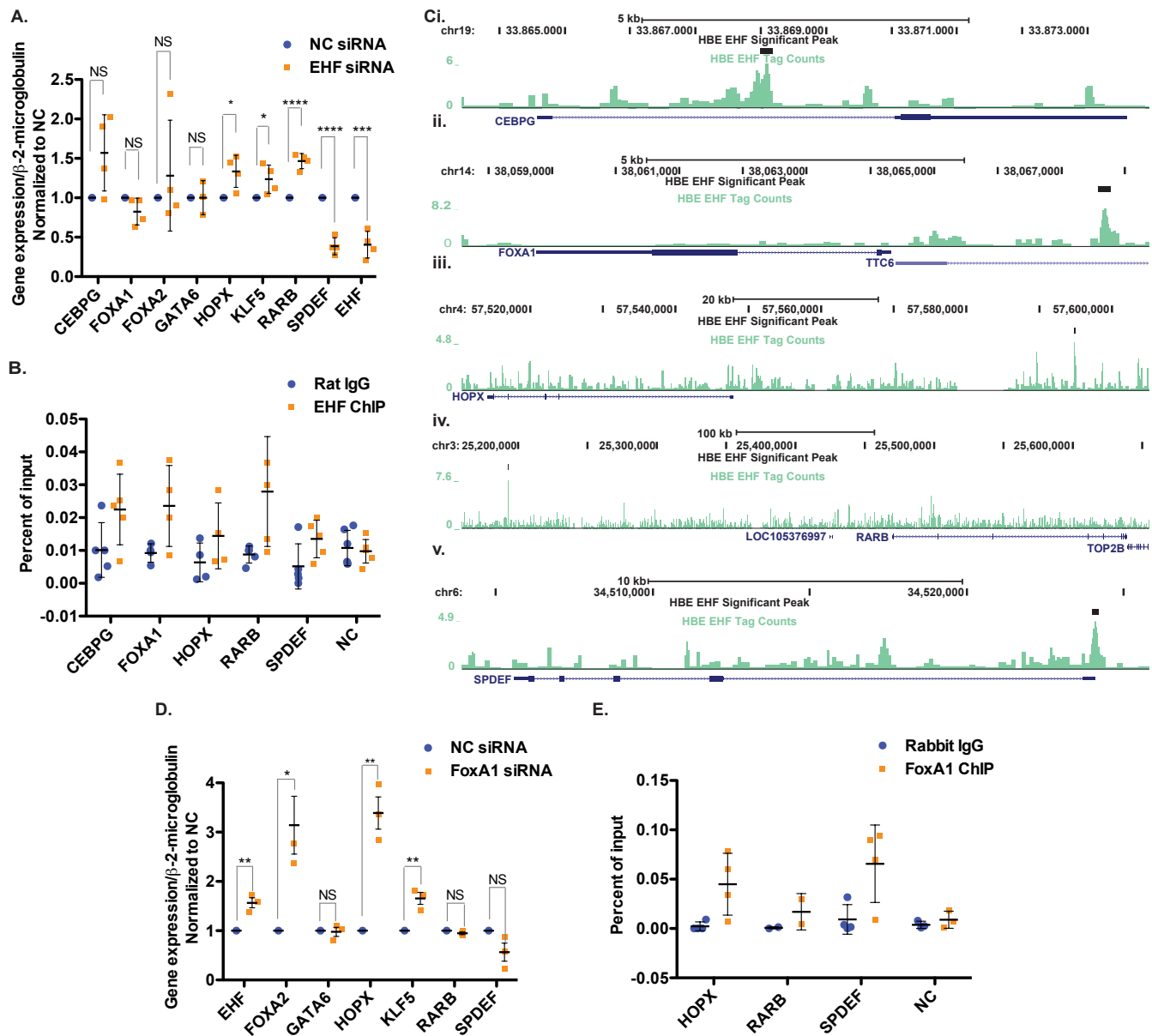
Figure 4. EHF and FOXA1 regulate other transcription factors involved in airway epithelial differentiation in HBE cells. A) EHF depletion by siRNA followed by RT-qPCR identified changes in TF gene expression (n=4). B) EHF ChIP-qPCR confirmed binding at specific sites identified in EHF ChIP-seq (n=4). C) In each panel, Genome Browser tracks show EHF peaks identified by IDR in HBE cells at the top (black), below are the EHF ChIP-seq tag counts (teal) and the gene (blue) for i) CEPBG, ii) FOXA1, iii) HOPX, iv) RARB, and v) SPDEF. D) Alterations in EHF levels and EHF-regulated TF gene expression after FOXA1 siRNA knockdown, shown by RT-qPCR (n=3). E) FOXA1 ChIP-qPCR confirmed its binding to a subset of EHF sites (n=4). Experiments shown in panels A-E were performed in undifferentiated HBE cells. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by an unpaired two-tailed Student's t test. Also used in Figure 6. Bars show average of all experiments, with error bars representing standard deviation (SD).
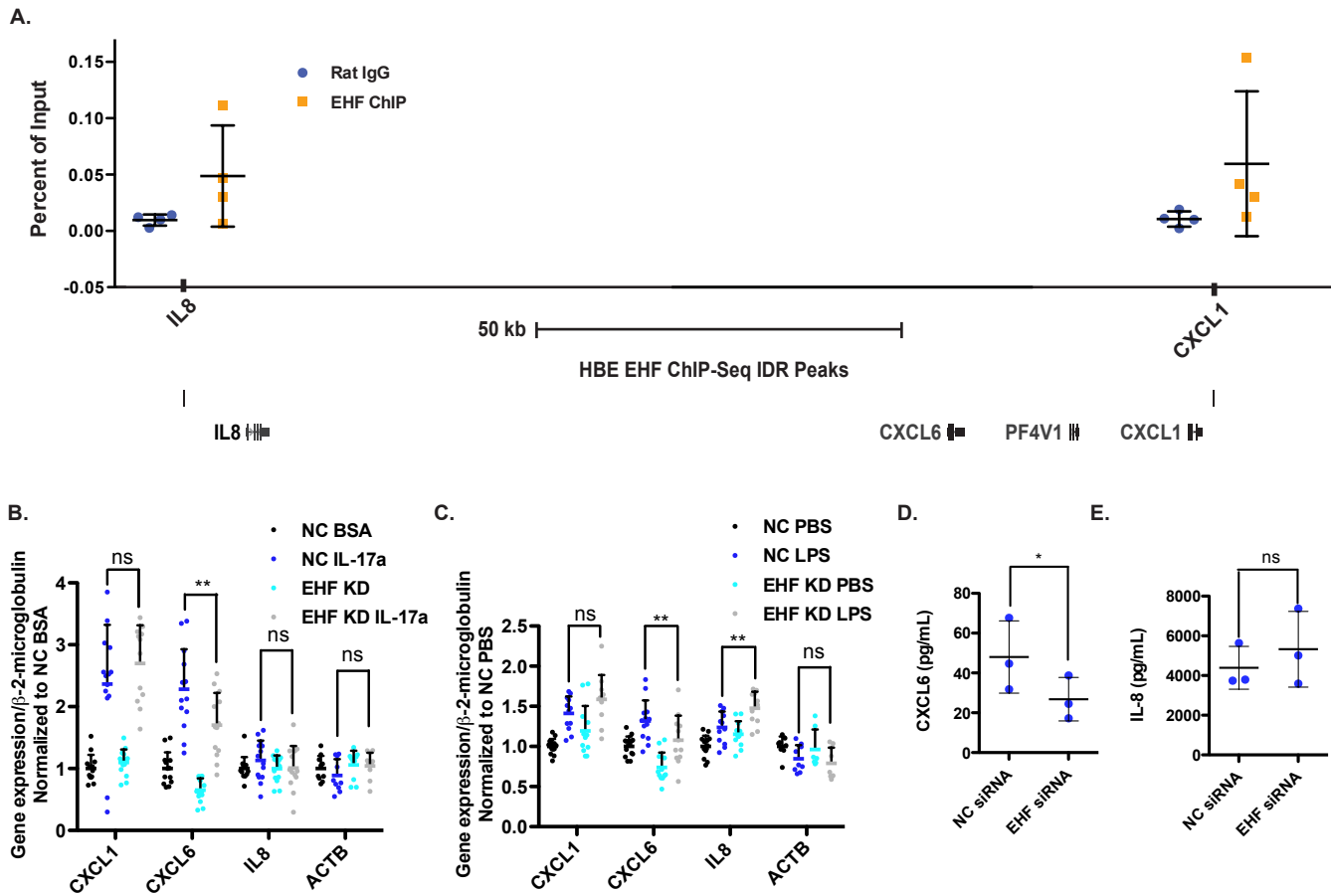
Figure 5. EHF depletion in Calu-3 cells alters secretion of a neutrophil chemokine. A) EHF ChIP-qPCR confirmed its binding to sites near *IL8*, *CXCL6*, and *CXCL1* in HBE cells. Average of all experiments with SD (n=4) (top). Graphic of the chr4q13.3 region with EHF ChIP-seq peaks (bottom). B-C) EHF was depleted (siRNA) in Calu-3 cells followed by their exposure to carrier, IL-17a (B), or LPS (C). Gene expression was measured by RT-qPCR. β-actin (ACTB) was included as a negative control. **P<0.01 by a 2-way ANOVA plus multiple comparisons test. Average with SD. D-E) Secretion of CXCL6 (D) and IL-8 (E) into media conditioned by NC- and EHF-depleted Calu-3 quantified by colorimetric sandwich ELISA (n=3). *P<0.05 by a paired two-tailed Student's t test. Bars show average of all experiments, with error bars representing SD.
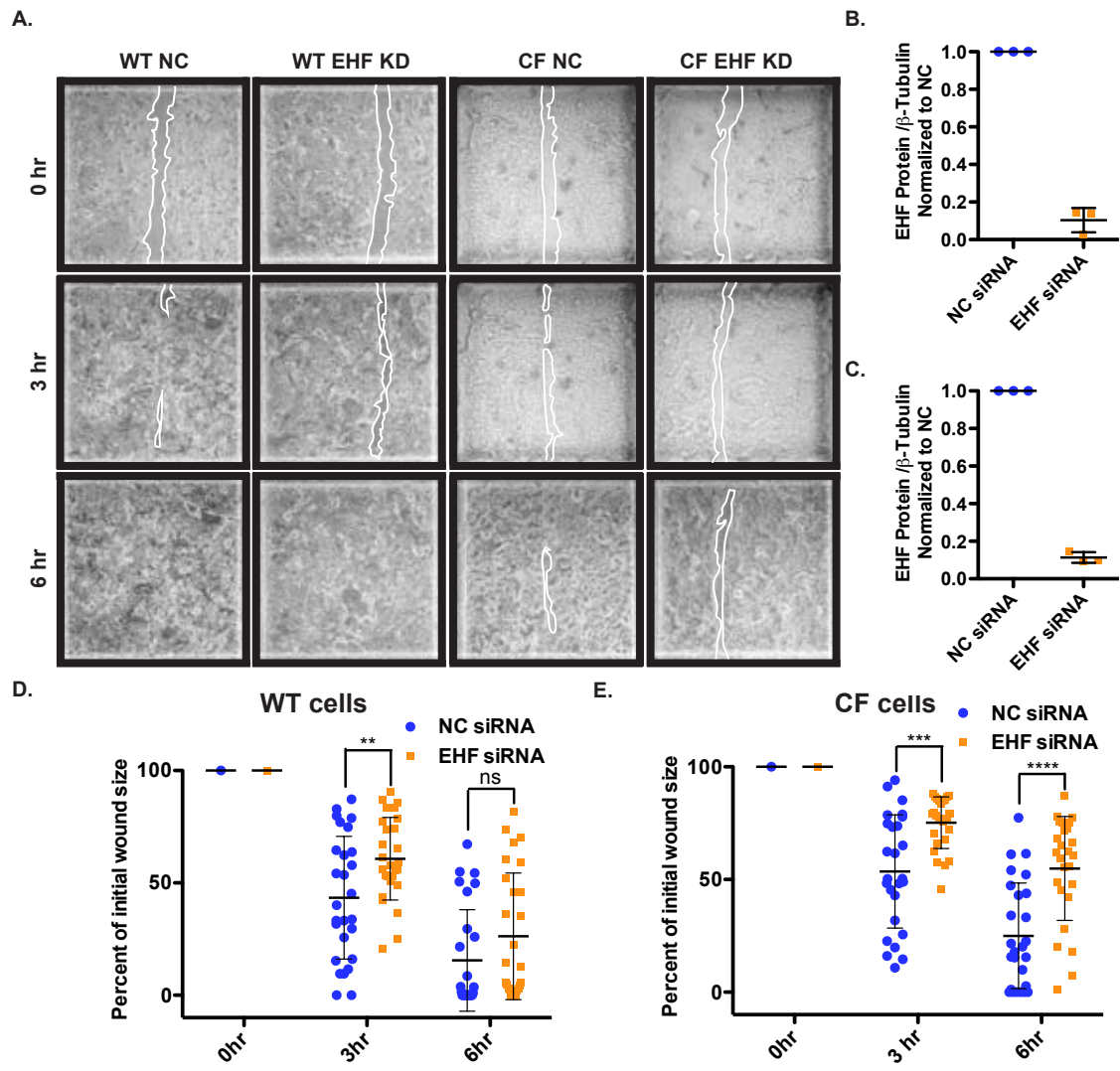
Figure 6. EHF depletion slows wound closure in wildtype (WT) and cystic fibrosis (CF) HBE cells. A) Images of NC and EHF siRNA-treated WT and CF undifferentiated HBE cells at 0, 3, and 6 hours after initial wounding. Outlines of wounds are traced in white. B-C) siRNA depletion of EHF was confirmed by western blot and quantified using gel densitometry in B) WT and C) CF cells (average with SD). D-E) Percent of initial wound size was measured at all time points. Average with SD for D) WT and E) CF HBE cells (3 donor cultures).

Ets Homologous Factor (EHF) has Critical Roles in Epithelial Dysfunction in Airway Disease

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