Krüppel-like factor 5 regulates wound repair and the innate immune response in human airway epithelial cells

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A complex network of transcription factors regulates genes involved in establishing and maintaining key biological properties of the human airway epithelium. However, detailed knowledge of the contributing factors is incomplete. Here we characterize the role of Krüppel-like factor 5 (KLF5), in controlling essential pathways of epithelial cell identity and function in the human lung. RNA-seq following siRNA-mediated depletion of KLF5 in the Calu-3 lung epithelial cell line identified significant enrichment of genes encoding chemokines and cytokines involved in the proinflammatory response and also components of the junctional complexes mediating cell adhesion. To determine direct gene targets of KLF5, we defined the cistrome of KLF5 using ChIP-seq in both Calu-3 and 16HBE14o- lung epithelial cell lines. Occupancy site concordance analysis revealed that KLF5 colocalized with the active histone modification H3K27ac and also with binding sites for the transcription factor CCAAT enhancer-binding protein beta (C/EBPβ). Depletion of KLF5 increased both the expression and secretion of cytokines including IL-1β, a response that was enhanced following exposure to Pseudomonas aeruginosa lipopolysaccharide. Calu-3 cells exhibited faster rates of repair after KLF5 depletion compared with negative controls in wound scratch assays. Similarly, CRISPR-mediated KLF5-null 16HBE14o- cells also showed enhanced wound closure. These data reveal a pivotal role for KLF5 in coordinating epithelial functions relevant to human lung disease.

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on the differentially expressed genes. The cistrome of KLF5 was defined by chromatin immunoprecipitation followed by deep sequencing (ChIP-seq). In addition to determining the genome-wide occupancy of KLF5, we used gene annotation to identify its direct targets. Intersection of the differentially expressed genes with the direct target datasets showed that KLF5 regulates genes involved in several critical functions of the airway epithelium, including cell adhesion and the proinflammatory response. Next, we used functional assays in airway epithelial cells to validate the genomic predictions. Using KLF5 depletion by siRNA and KLF5-null (CRISPR-generated) cell lines, we showed that KLF5 deficiency dysregulated key proinflammatory cytokines and enhanced recovery from wounding in comparison to negative controls or clonal wild-type respectively.

Results

KLF5 regulates key biological processes in the human airway epithelium

To determine the target genes of KLF5 in human lung epithelial cells, we performed RNA-seq on triplicate samples of Calu-3 cells treated with either negative control (NC) siRNA or siRNA specific for KLF5. Using a fold change of ±1.5 and an adjusted p-value threshold of 0.05, we identified 533 upregulated genes and 443 genes that were downregulated (Fig. 1A). Among the most significantly upregulated genes were those encoding several chemokines and cytokines such as C-X-C Motif Chemokine Ligand 6 (CXCL6), C-C Motif Chemokine Ligand 2 (CCL2), and C-X-C Motif Chemokine Ligand 8 (CXCL8), also known as IL-8. Also, several genes encoding proteins involved in cell adhesion and tight junction complexes were significantly upregulated, for example, intercellular adhesion molecule 1 (ICAM1) and claudin 2 (CLDN2). The efficacy of the siRNA treatment was confirmed by a consistent, greater than threefold reduction in KLF5 transcript abundance (Fig. 1B). This result was confirmed at the KLF5 protein level by western blot (Fig. S1). A 2-fold increase in CFTR transcript levels (Fig. 1B) also confirmed our early observations (31) on the repressive role of KLF5 on the gene in airway epithelial cells. KLF5 depletion also caused a significant downregulation of SPDEF and upregulation of FOXA1 transcripts, two TFs that are well characterized in the transcriptional network of these cells. To further examine the effect of KLF5 depletion on the TF expression landscape, the RNA-seq data were filtered for known human TFs using the v1.01 human TF database (32) (Fig. 1C). Twenty-seven TFs were found to be upregulated and 19 were downregulated, with genes encoding major developmental factors such as NOTCH1, RUNXI, and RUNX3 among the most significantly downregulated. Next, GO process enrichment analysis was performed for all upregulated DEGs (Fig. 1D). Recurrent in both the biological process (BP) and molecular function (MF) ontology terms were enrichments of genes involved in cell adhesion and the inflammatory response. Furthermore, the top cellular compartment (CC) terms were involved in the cell surface and extracellular matrix. A significant proportion of upregulated DEGs were also involved in the extracellular signal-related protein kinase 1 and 2 (ERK1/ERK2) cascade. Gene set enrichment analysis was then performed using the Hallmark database to further classify all genes that were differentially expressed on KLF5 depletion (Fig. 1E). When both up- and downregulated genes were assessed simultaneously, significant enrichment for pathways involved in (a) tumor necrosis factor alpha (TNFα) signaling via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and (b) EMT was identified, with some evidence for the enrichment of genes involved in the inflammatory response.

The KLF5 cistrome reveals indirect regulation

The genome-wide occupancy of KLF5 in airway epithelial cells is currently uncharacterized though identification of its direct targets, based upon binding site location within or near gene loci, would be valuable. To address this KLF5 ChIP-seq was performed in replica in both the Calu-3 and 16HBE14o cell lines. Peaks were filtered for read enrichment passing an irreproducible discovery rate threshold of 0.05 and a q value ≥3. We identified a total of 2105 and 3520 significant peaks in Calu-3 and 16HBE14o respectively. Consistent with the biological processes that were impacted upon KLF5 depletion, coincidence of KLF5 sites of occupancy was found in both cell lines at multiple genes involved in cell adhesion, for example, the catenin beta 1 (CTNNB1) and epithelial cell adhesion molecule (EPCAM) gene loci (Fig. 2A, i and ii). KLF5 was bound primarily at promoters, intergenic regions, and within genes. To determine which of the genes that were differentially expressed on KLF5 depletion were direct targets, we intersected the Calu-3 ChIP-seq dataset with the DEGs identified by RNA-seq (Fig. 2B). Among 1221 genes with at least one KLF5 peak within 20kb of the locus, only 64 were differentially downregulated and 52 upregulated (Table S1). GO process enrichment analysis of the overlapping gene sets identified response to external stimulus to be associated with genes upregulated following KLF5 depletion. These results suggest that the extensive alterations in the transcriptome, which were evident upon KLF5 depletion, were largely indirect. Next, the KLF5 IDR peak sets from Calu-3 and 16HBE14o were intersected to generate a consensus airway cell ChIP-seq dataset. Although KLF5 was found to bind primarily at promoters or distal intergenic regions, over half of the consensus sites were within 1kb of the gene start site (Fig. S2). Gene annotations for consensus KLF5 peaks were filtered for transcription factors and stratified by location of the KLF5 peak (Fig. 2C). In contrast to the relatively equal distribution of KLF5 occupancy between gene promoters and nonpromoter elements, 30 of the 35 transcription factor genes had a KLF5 peak within the 3kb promoter region. Furthermore, the only one of these TFs differentially expressed in the RNA-seq was NF-kappa-B inhibitor zeta (NFKBIZ).

To confirm that KLF5 occupancy directly impacts gene expression in vitro, we evaluated the mucin 1 (MUC1) gene promoter. MUC1 is significantly downregulated upon KLF5 depletion and shows a robust peak of KLF5 occupancy within...
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1kb of the transcription start site in the Calu-3 ChIP-seq data. We showed earlier that elements within the MUC1 promoter controlled gene expression and recruited DNA-binding protein complexes (33). Matrix-scan using Regulatory Sequence Analysis Tools (RSAT) (34) identified a putative KLF5-binding motif in the MUC1 promoter, corresponding to the ChIP-seq peak at –533 bp to –539 bp with respect to the transcription start site. We performed site-directed mutagenesis to abolish this KLF5 motif in a luciferase reporter gene vector driven by the MUC1 promoter. The WT and mutant promoter plasmids were transiently transfected into 16HBE14o− cells and lysates subjected to a dual-luciferase assay (Fig. S3). Mutation of the
**Figure 2.** The KLF5 cistrome in Calu-3 and 16HBE14o− cells. Analysis of ChIP-seq data. A, IGV Genome browser graphic shows tracks of KLF5 and H3K27ac ChIP-seq tag counts identified by IDR in Calu-3 (maroon) and 16HBE14o− (blue) within 70kb of the CTNNB1 gene (i) and 46kb of the EPCAM gene (ii). B, UpSet plot comparing genes with at least one significant KLF5 Calu-3 peak within 20 kb of the gene body to DEGs identified in the Calu-3 RNA-seq following KLF5 depletion. C, table of transcription factors with at least one KLF5 peak shared in Calu-3 and 16HBE14o− datasets within 20 kb of the gene body. Annotated genes were stratified by the location of the peak. D, Scatterplot of the incidence of the top 20 transcription factor binding motifs found under KLF5 peaks shared in Calu-3 and 16HBE14o−. Red points indicate motifs for Krüppel-like factors. E, gene ontology analysis for all genes with a consensus KLF5 peak within 20kb of the gene body.
KLF5-binding motif significantly reduced the relative luciferase expression compared with WT.

Genome-wide motif analysis was then performed on the airway KLF5 consensus peak set with a 50 bp window around the center of the peak (Fig. 2D). The similar DNA-binding motifs for KLFs were the most enriched, as expected in ChIP-seq data for KLF5. Among the top 25 identified, the only motif not belonging to KLFs or members of the AP-1 complex was that of C/EBPβ. The interaction between KLF5 and C/EBPβ has been well characterized in adipocytes (35, 36), though not yet in the airway epithelium. GO analysis was next performed for the closest genes to consensus KLF5 peaks (Fig. 2E). As was seen in the RNA-seq dataset, the most significantly enriched terms were those involved in cell adhesion and the cell–cell junctions. These include both the catenin alpha 1 and beta 1 genes (CTNNA1 and CTNNB1) and keratin 18 (KRT18). Comparison of the annotated genes in the airway peak sets to those of gastric adenocarcinoma cell lines YCC3 and AGS (37) (GEO: GSE51706) revealed an overlap around 5–10% (Fig. S4A). However, GO analysis of annotated genes within 20kb of KLF5 peaks was also significantly enriched for terms related to cadherin binding and cell adhesion. (Fig. S4B).

KLF5 binds to active gene promoters and enhancers

To learn more about the cis regulatory elements (CREs) bound by KLF5 and identify possible cofactors, we intersected the significant peaks of KLF5 in Calu-3 (Fig. 3A, i) and 16HBE14o− (Fig. 3A, ii) with the ChIP-seq data for the active histone modification H3K27ac and RNA Polymerase II (RNAPII) in the same cell lines (38). In both cell lines, KLF5 peaks were consistently found in regions enriched in H3K27ac with a distinct bimodal distribution around the center of the peak, indicating nucleosome depletion at the TF site. A similar coincidence was found between sites of KLF5 and RNAPII occupancy (Fig. 3A, iii and iv), confirming the activity of the factor at sites of transcription initiation. To assess if the enrichment of the C/EBPβ motif under KLF5 peaks seen in Figure 2D coincided with co-occupancy of this factor across the genome, we performed ChIP-seq for C/EBPβ in Calu-3 cells. Direct co-occupancy of these 2 TFs was found at several gene loci. These include differentially expressed genes such as CFTR and NFKBIZ, as well as mucins 5AC and 5B (MUC5AC/MUC5B) and caspase 9 (CASP9) (Fig. S5). We then visualized the signal intensity for C/EBPβ binding around KLF5 peaks and included the H3K27ac and RNAPII occupancy data (Fig. 3B). A distinct subset of KLF5 peaks overlapped with C/EBPβ sites, significantly correlating with the bimodal distribution of the histone mark and concurrent signal of RNAPII at the center of the peak. To further characterize the co-occupancy of KLF5 and C/EBPβ genome-wide, we intersected the two Calu-3 ChIP-seq peak sets. Binding of the two TFs intersected at 775 sites, corresponding to 33.2% of all KLF5 peaks and 8.02% of C/EBPβ peaks (Fig. 3C). Annotation of the closest gene to these co-occupied sites revealed a similar distribution to KLF5 binding alone between promoters and distal intergenic regions.

Cytokine and chemokine expression and secretion are enhanced in KLF5-depleted cells

As shown in Figure 1A, we found significant enrichment of genes involved in the immune response among DEGs following KLF5 depletion in Calu-3 cells, for example, CXCL6 and interleukin 1 beta (IL1B). These cytokines are released by the airway epithelium in response to external stimuli such as lipopolysaccharides (LPS) of the cell wall of Gram-negative bacteria (39, 40). To validate whether KLF5 regulated the expression and secretion of these chemokines/cytokines at the basal level and following external stimulus, Calu-3 cells were treated with nontargeting siRNA or siRNA specific for KLF5, followed by treatment of each group with either vehicle control (PBS) or P. aeruginosa LPS for 4 h. Chemokine/cytokine expression (CXCL1, CXCL6, and IL-1β) was measured by RT-qPCR (Fig. 4A). LPS treatment resulted in a significant increase in the expression of all three cytokines. Consistent with the RNA-seq data, a significant increase in the expression of the CXCL6 and IL1B genes was observed when KLF5 was depleted, with an enhanced increase following LPS treatment. In contrast, CXCL1 levels were unchanged between the control and KLF5-depleted cells. To determine if the change in gene expression coincided with an alteration in IL-1β secretion following KLF5 depletion and LPS treatment, conditioned media was tested using colorimetric sandwich ELISAs (Fig. 4B). IL-1β secretion was significantly increased in the media conditioned by KLF5-depleted cells with a greater increase in the media of cells also treated with LPS, consistent with the gene expression changes under the same conditions. Though IL-1β protein levels in the cell lysate also increased slightly after KLF5 depletion, no evidence for a change in the ratio between the precursor and mature forms of IL-1β was evident (data not shown).

Depletion of KLF5 augments wound repair response

Among the most enriched gene sets identified in both the KLF5 depletion RNA-seq and KLF5 ChIP-seq datasets were those involved in focal adhesion, the extracellular matrix, and cadherin binding. Dysregulation of the wound repair response is pivotal to several airway diseases and is also associated with EMT. To evaluate the contribution of KLF5 to coordinating wound repair processes in vitro, Calu-3 cells treated with negative control or KLF5-targeted siRNA were grown to confluence. The confluent monolayers were scratched/wounded and subsequently imaged every 3 h for 12 h (Fig. 5A). Though no statistically significant difference was observed between treatment groups at the 6-h timepoint, the relative wound size of the KLF5-depleted cells compared with the 3-h timepoint was lower than controls, and this difference reached statistical significance for the 9- and 12-h timepoints (Fig. 5B). Thus, depletion of KLF5 was associated with a significantly enhanced speed of wound repair.

To confirm these observations in an orthogonal model system and in a second airway cell line, 16HBE14o− clonal cell lines without detectable KLF5 protein (KLF5-null) were generated by CRISPR/Cas9 modification. Wound scratch assays were then performed in two wild-type (WT) clonal lines compared with three independent KLF5-null clones (Fig. 3C).
Loss of KLF5 protein in the KLF5-null clones was confirmed using western blot analysis (Fig. 5D). Despite the overall faster rate of wound closure in 16HBE14o− compared with Calu-3 cells, KLF5-null clonal lines recovered from the scratch at a faster rate than the clonal WT lines, and this difference was statistically significant as early as 6 h post-wounding (Fig. 5E).

**Importance of KLF5 in primary human bronchial epithelial cells**

To confirm that the regulatory roles of KLF5 that were observed in experiments in the Calu-3 and 16HBE14o− cell lines are equally relevant to primary human airway epithelial cells, we repeated key experiments in primary HBE cells.
Wound scratch assays were performed in triplicate on HBE cells from two donors after siRNA-mediated depletion of KLF5 compared with an NC siRNA (Fig. 6A). As observed in the cell lines, HBE cells depleted for KLF5 recovered from scratch at a significantly faster rate than controls (Fig. 6B). The slight lag (15 h instead of 9 h) in significant differences in wound closure between KLF5-depleted cells and controls, when compared with the cell lines, is probably due to the slower growth rate of primary epithelial cells. KLF5 expression does not significantly change during scratch induction or recovery in HBE cells (Fig. S6) (GEO:GSE127696) (41). Differential gene expression in KLF5-depleted HBE cells was assayed by RT-qPCR, focusing on the DEGs identified in the Calu-3 RNA-seq data (Fig. 6C). As in Calu-3, the key airway transcription factors SPDEF and FOXA1 were significantly downregulated and upregulated, respectively upon loss of KLF5. We also observed substantial upregulation of ICAM1, CXCL6, and CCL2.

Discussion

The roles of TFs in coordinating the differentiation and development of the lung has been studied extensively (42–45). However, the TF network that maintains the functions of the healthy lung epithelium is less well characterized. Several previous studies focused on members of the Krüppel-like family including KLF4 (46) and KLF15 (47), though examination of the regulatory landscapes of each TF focused primarily on the progression to disease states such as adenocarcinoma. Here, we used genome-wide methods to identify the indirect and potential direct gene targets for KLF5 in these cells. We found that KLF5 regulates genes involved in both the response to external stimuli and cell–cell adhesion, which are two critical functions of the normal lung epithelium with distinct relevance to respiratory health and disease.

The transcriptome in Calu-3 cells showed significant enrichment of genes involved in intercellular junctions and the inflammatory response that were repressed by KLF5. These results are consistent with our previous findings for other members of the TF network such as EHF (14) and FOXA1 (15), where both repress expression of KLF5 (14). The regulatory relationship is further supported by the finding that 46 TFs are differentially expressed upon KLF5 depletion and 37 of these have a peak of KLF5 occupancy close to the gene body. Interestingly, only one of these differentially expressed TFs (NFKBIZ) had KLF5 enrichment at the promoter (Fig. 2C), suggesting that KLF5 may have a potent yet indirect role within the TF network. The indirect regulatory potential of KLF5 is further supported by the remarkably low overlap between the direct binding targets identified from our ChIP-seq data in airway epithelial cell lines and the DEG dataset in Calu-3. It is unlikely that this lack of correlation is due poor antibody specificity, as this was validated by western blots of both depletion and CRISPR-null experiments.

Further, the reagent has been used elsewhere in ChIP (48, 49).

Direct interaction between KLF5 and other transcription factors plays important roles in other cell types such as with histone deacetylase 1 (HDAC1) in HeLa cells (50) or cyclin-dependent kinase 2 interacting protein (CINP) in the TSU-Pr1 bladder cancer cell line (51). TFs that bind together at cis-regulatory elements such as enhancers may also act as cofactors with KLF5. To identify these, we examined the motif enrichment under Calu-3 KLF5 peaks. In addition to the observation of similar motifs for other members of the KLF family, we found enrichment of motifs for the AP-1 family of TFs including c-Jun and JunD. The only other highly enriched motif was that of C/EBPβ, a TF with an important role in the cascade of adipocyte development where it coregulates the expression of genes involved in differentiation, in combination with KLF5 and KLF4 (36). ChIP-seq for C/EBPβ in Calu-3 cells confirmed co-occupancy of this factor with KLF5 at several genes with important roles in airway epithelial biology. As C/EBPβ is recruited to substantially more sites in the genome than KLF5, it may have a more general role at enhancers genome-wide. Nevertheless, comparable enrichment of C/EBPβ and AP-1 motifs under KLF5 peaks supports a possible coregulatory role for C/EBPβ in the epithelium.
The consensus KLF5 peaks integrating the cistrome in Calu-3 and 16HBE14o\(^{-}\) were found near genes involved in the cell periphery and cadherin binding as was observed from the transcriptome. The genomic results emphasize the complexity of the regulatory role of KLF5 in the airway epithelium. We found substantial changes in gene expression following depletion of the factor, including genes encoding TFs, yet an almost completely different subset of genes and TFs have KLF5 occupancy near the gene body. Of note, KLF5 binds primarily in regions marked by H3K27ac and occupied by RNAPII. One possibility is that the regulatory role of KLF5 on genes near its binding sites is part of a cascade altering expression of downstream genes. The lack of overlap between TFs with nearby KLF5 peaks and those that are differentially expressed may be due to two or more distinct pathways of regulation. Alternatively, it is possible that KLF5 depletion in Calu-3 does

Figure 5. Wound closure rates are enhanced in cells depleted for KLF5 and KLF5-null cells. A, representative images of NC and KLF5 siRNA-treated WT Calu-3 cells 3, 6, 9, and 12 h after initial scratch. Outline of wound is traced with a white line. Size bar = 400 μm for all panels. B, quantification of scratch width in Calu-3 (n = 9 per group). **p < 0.001, NS, not significant. C, representative images (Lionheart FX) of clonal WT and KLF5-null 16HBE14o\(^{-}\) cells 3, 6, 9, and 12 h after initial scratch. Size bar 1000 μm for all panels. D, Western blot showing loss of KLF5 protein in KLF5-null CRISPR/cas9 clones compared to clonal wild-type with β-tubulin as a loading control. E, quantification of scratch width in 16HBE14o\(^{-}\) clones (n = 12 per group). *p < 0.01, **p < 0.001. All comparisons were performed using a two-way analysis of variance plus multiple comparisons test.
not completely eliminate functional protein. The minimal amounts remaining may be sufficient to occupy sites at some key genes. KLF5 may also act in a tightly monitored feedback loop critical to the airway epithelial cell identity, with the detectable functional consequence of its loss being a shift in the expression of those genes involved in the response to external stimuli and the cell–cell boundary.

To confirm the role of KLF5 on the biological processes identified by genomic methods, we assayed a change in phenotype following depletion of KLF5. As we previously observed with EHF (14), we first assayed the expression and secretion of cytokines and chemokines following KLF5 loss. Subsequent addition of LPS allowed us to further determine if KLF5 plays a role in the epithelial response to stimulus. Other major TFs within the airway epithelial network were shown to play essential roles in innate immunity such as AP-1 (52) as discussed previously and the possible KLF5 cofactor C/EBPβ (53). The dramatic increase in CXCL6 and IL-1β observed in KLF5-depleted cells compared with negative control upon LPS exposure may indicate that in addition to the generally repression of the proinflammatory genes, KLF5 is involved in properly modulating the response to bacterial stimulus. Controlled expression and secretion of IL-1β are critical in the development of the lung (54) and are involved in lung diseases such as chronic obstructive pulmonary disease (COPD) (55).

The substantial increase in both expression and secretion of IL-1β highlights the importance of KLF5 in the proinflammatory response of the airway epithelium. Upregulation of IL1B upon KLF5 depletion is not accompanied by a change in the relative ratio of the precursor or mature forms of the protein; thus the factor is unlikely to be involved in NLRP3-inflammasome-mediated activation of the cytokine and subsequent proinflammatory response. KLF5 may instead only regulate the NFKB-mediated transcription of IL1B. NLRP3 is not expressed in the airway cells utilized here, but another inflammasome gene, NLRC4, is expressed. This may be more relevant to the proinflammatory response of the airway epithelium, since NLRC4 was found to be essential in the response to P. aeruginosa (56, 57). Although we did not find differential expression of IL1B in primary HBE, both CXCL6 and CCL2 were still significantly upregulated. These results support the role of KLF5 in the regulation of cytokine gene expression in airway epithelium.

Interestingly, IL-1β is also known to promote cell migration in the cancer microenvironment (58); thus in addition to the proinflammatory response effect, the secretion of IL-1β may be involved in the processes of cellular adhesion and the response to wounding. The role of KLF5 in regulating the expression of genes involved in cell adhesion was previously shown in the intestinal epithelium (59), but our observations on the airway epithelium are novel. The integrity of intercellular junctions is closely related to the processes of EMT, and their dysregulation may cause a shift to a mesenchymal cellular identity. In both Calu-3 cells depleted for KLF5 and 16HBE14o~KLF5-
null cells, we observed significantly increased rates of wound closure. Maintenance of the cell-to-cell contacts and normal barrier function are critical for ion transport and for the integrity of the airway. As genes involved in cell adhesion were both differentially expressed following KLF5 depletion and had peaks of KLF5 occupancy within 20kb of the gene body, our results indicate that this factor may have a pivotal role in maintaining the integrity and health of the airway epithelium. The same response was found in primary HBE, indicating that the response is not unique to cell lines. Primary bronchial epithelial cells better capture the heterogeneity of the human lung surface epithelium and are among the best models for this tissue. The significantly increased rate of scratch closure following loss of KLF5 implicates this factor in the regulation of genes involved in the intercellular matrix, such as differentially expressed ICAM1. Dysregulation of genes involved in this pathway contributes to the defective response to wounding in the context of disease, as observed in cystic fibrosis (60). Significant upregulation of the cytokines CXCL6 and CCL2 in the cell lines and the primary HBE support the role of KLF5 in the proinflammatory response in the context of both healthy epithelium and in disease.

It is of interest to integrate our data into previous studies on the role of KLF5 in other tissues. Although Klf5−/− mouse embryos died before embryonic day 8.5, Klf5+/− mice survived but exhibited abnormal phenotypes in both the vasculature and gastrointestinal track (25). Significant anatomical alterations in these Klf5 deficient mice were accompanied by changes in extracellular matrix components, consistent with our findings of alterations in wound response rate and the expression of genes encoding proteins involved in the cell periphery of human lung epithelium. A role for Klf5 in the response to injury was also found in mouse biliary duct epithelial cells activated by cholestasis (61). Here, Klf5 regulates genes involved in cellular proliferation following cholestatic injury as well as focal adhesion including Laminin A3 (LAMA3), which was also significantly downregulated following KLF5 depletion in Calu-3 cells. Of note, earlier work also found KLF5 to have an important role in the LPS-induced proinflammatory response in a human intestinal epithelial cell line (IEC6) (62) and in regulation of ICAM1 expression, as we observed here in airway epithelial cells. Intersection of the two gastric adenocarcinoma cell lines with the two airway epithelial cell lines identified many shared direct targets for KLF5, though the majority were specific to one cell lineage or the other. GO analysis of the two gastric adenocarcinoma cell lines showed a similar enrichment for those genes at the cell-to-cell periphery. In combination with these observations on other epithelial cells types, our data in human lung epithelial cells provide strong evidence that KLF5 has a critical role in coordinating the establishment and maintenance of the barrier functions of epithelial tissues. The factor regulates multiple genes within the cadherin and laminin families as well as key components of the canonical Wnt signaling pathway including β-Catenin.

Sites of KLF5 occupancy genome-wide in airway epithelial cells coincide with regions marked by active histone marks (H3K27ac) and RNAPII recruitment, thus identifying active promoters and enhancers. However, the factor likely exerts its effects in combination with other TFs at the same elements. In gastric epithelial cells, the key cofactors are probably GATA4/6 and in adipocytes or lung epithelium C/EBPβ is implicated as an important interacting factor. Co-occupancy of regulatory elements could identify a subset of genes that drive the downstream effects seen in RNA-seq, but are not coincident with ChIP-seq targets. The underlying mechanisms may involve chromatin reorganization and three-dimensional structure alterations around coincident binding sites with other TFs and RNAPII, as was found recently for the closely related KLF4 (63). Furthermore, the incidence of this regulatory process at different subsets of gene loci in diverse cell types may underlie the similarity between the pathways, yet discordance of gene targets in epithelia from various tissues.

Experimental procedures

Cell culture

Calu-3 (64) and 16HBE14o− (65) cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS).

Primary human bronchial epithelial cell culture

Donor-derived primary human bronchial epithelial (HBE) cells were obtained from the Marsico Lung Institute CF Center Tissue Procurement and Cell Culture Core (University of North Carolina [UNC]) and cultured according to the published protocols (66) in accordance with relevant guidelines. The cells were obtained under protocol #03-1396 approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board. All donors or their authorized representatives provided informed consent for research use of explanted lungs. This work was also approved by the Case Western Reserve University Institutional Review Board.

KLF5 depletion and RNA-seq

Calu-3 cells were treated with either negative control #2 siRNA (Dharmacon, D-001206-14-05), or KLF5 siRNA (Dharmacon, M-013571-01-0005), each at 30 nM using RNAiMax transfection reagent (Life Technologies). Seventy-two hours after transfection, RNA was isolated from three samples of each treatment using TRIzol (Life Technologies). RNA-seq (SR 50 bp) was performed as described previously (67).

Raw reads were aligned with STAR 2.6 (https://github.com/alexdobin/STAR) (68). Aligned reads were then assigned to genomic features with featureCounts version 1.6.3 in the Subread package (http://subread.sourceforge.net/) (69), and differential gene expression was analyzed using DEseq2 version 1.22.1. (https://www.bioc conductor.org/packages/release/bioc/html/DESeq2.html) (70).

Gene ontology and gene set enrichment analyses

Differentially expressed genes were filtered to enrich for genes with a fold change ≥1.5 and Benjamini–Hochberg
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adj usted p-value ≤ 0.01. RNA-seq gene lists were read into the gProfiler GO program and database (71). Dot plots for clustered results of each GO term were generated using the genome-wide annotation for human database and mapped using Entrez gene identifiers (72). Statistically significant results were filtered for categories passing a p-value of 0.001 with the Bonferroni correction for multiple testing. Gene set enrichment analysis was performed using the Hallmark human v6.2 database.

ChIP-seq

ChIP-seq was performed using two antibodies against KLF5 (Santa Cruz sc-398470 and rabbit anti-KLF5 kindly donated by Dr Jonathan Katz (73)) or CCAAT enhancer-binding protein beta (C/EBPβ) (Santa Cruz sc-7962) as described previously (13). Raw reads were processed using the ENCODE Transcription Factor and Histone ChIP-seq processing pipeline (https://github.com/ENCODE-DCC/chip-seq-pipeline2) according to the ENCODE (phase-3) guidelines on the hg19 reference genome. This includes mapping using BWA (74) and peak calling with MACS2 (75). Peak data were filtered using and processed for motif distribution using HOMER (4.7.2q) (http://homer.ucsd.edu/homer/index.html) (76). Consensus peak data were generated using Bedtools intersect (2.29.2) (77), and subsequent annotation was performed using the ChIPseeker package (v3.10) (78). These packages were hosted on the GALAXY platform (79).

Generation of KLF5-null cell line using CRISPR

A single-guide RNA was designed based on Diaferia et al. (80) targeting exon 2 of KLF5: 5′-CACC CGAA-GAAC TGGTCTAGCAGCTG-3′ and cloned into pBlueScript (pBS) with a modified multiple cloning site. 16HBE14o− cells were transfected after 48 h with pMJ920 (wild-type Cas9 plasmid tagged with GFP) (Addgene, plasmid #42234) and pBS containing the KLF5 exon 2 gRNA using Lipofectamine 3000 (Life Technologies). GFP-positive cells were sorted by manually diluted onto 96-well plates. Clones were expanded (Life Technologies). GFP-positive cells were sorted by

Transient reporter gene (luciferase) assays

Site-directed mutagenesis was performed on the predicted KLF5 motif in the MUC1 promoter driving luciferase reporter gene expression in the pGL3B vector (33), using the Agilent QuikChange Lightning Site-Directed Mutagenesis kit. Cells were cotransfected with empty pGL3B, pGL3B containing the WT MUC1 promoter, or the mutant sequence and a modified pRL Renilla luciferase control vector at a 1:10 ratio using Lipofectamine 3000 (Thermo Fischer Scientific). Cells were lysed after 48 h and assayed on a GloMax Navigator (Promega) for firefly and Renilla luciferase activity using the Dual-Luciferase Reporter Assay Kit (Promega). Transfections were performed in triplicate in two different passages of 16HBE14o− cells.

LPS treatment and RT-qPCR

Cells were serum-starved for 24 h and then treated with PBS or 1 μg/ml Pseudomonas aeruginosa LPS (Sigma L9143). RNA was collected at 4h using TRizol, and qRT-PCR was performed using TaqMan Reverse Transcription Reagents kit (LT), oligo (dT)16, and primer pairs specific to each gene and SYBR green. β-2-microglobulin was the normalizer (For primers see Table S2). Data were transformed using the delta delta Ct method to calculate the difference between experimental and control values. ANOVA was performed on values before (not shown) and after transformation.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatant from siRNA-transfected cells was collected, cleared by centrifugation at 300g for 10 min to remove cell debris, and the supernatant was stored at −80 °C. IL-1β secretion was quantified using the Mini ABTS ELISA Development Kit (PeproTech 900-M95) with no dilution. Standard curves were established using serial dilutions of 1:2 starting with 1000 pg/ml of each target. These assays were performed using the ELISA Buffer Kit (PeproTech 900-K00) according to the manufacturer’s protocol.

Wound repair assay

The wound closure assay was performed as described previously (81) on siRNA-treated Calu-3, using a Leica DMi1 microscope (5× objective) and an MC170 HD camera with Las EZ imaging software v3.0.0.47. Wound width was calculated using ImageJ. Experiments on KLF5-null 16HBE14o− and primary HBE cells were performed using the Lionheart FX automated microscope with a bright field 4× objective. The Biotek scratch assay v1.0 software was used for analysis. All results are from three independent experiments repeated in duplicate for each treatment group, null, or clonal wild-type cell line.

Western blot

KLF5 and clonal wild-type cells were lysed in NET buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1X Sigma Protease Inhibitor), and proteins were analyzed by standard methods. The antibodies used were specific for KLF5 (sc-398470), β-tubulin (T4026, Sigma-Aldrich), or IL-1β (12242S, Cell Signaling).

Data availability

KLF5 and C/EBPβ Genome-wide data are deposited at GEO:GSE164853. H3K27ac and RNAPII Genome-wide data are deposited at GEO:GSE132808.

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Abbreviations—The abbreviations used are: AP-1, activator protein 1; CFTR, cystic fibrosis transmembrane conductance regulator; ChIP-seq, chromatin immunoprecipitation followed by deep sequencing; CINP, cyclin-dependent kinase 2 interacting protein; C/EBPβ, CCAAT/enhancer binding protein beta; ETS, homologous factor; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial–mesenchymal transition; GO, gene ontology; HBE, human bronchial epithelial; HDAC1, histone deacetylase 1; KLF, Krüppel-like factor; LPS, lipopolysaccharide; RSAT, Regulatory Sequence Analysis Tools; SPDEF, SAM-pointed domain ETS factor; TF, transcription factor.

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