A microRNA network regulates expression and biosynthesis of wild-type and ΔF508 mutant cystic fibrosis transmembrane conductance regulator

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Production of functional proteins requires multiple steps, including gene transcription and posttranslational processing. MicroRNAs (miRNAs) can regulate individual stages of these processes. Despite the importance of the cystic fibrosis transmembrane conductance regulator (CFTR) channel for epithelial anion transport, how its expression is regulated remains uncertain. We discovered that miRNA-138 regulates *CFTR* expression through its interactions with the transcriptional regulatory protein SIN3A. Treating airway epithelia with an miR-138 mimic increased *CFTR* mRNA and also enhanced CFTR abundance and transepithelial Cl^- permeability independent of elevated mRNA levels. An miR-138 anti-miR had the opposite effects. Importantly, miR-138 altered the expression of many genes encoding proteins that associate with CFTR and may influence its biosynthesis. The most common *CFTR* mutation, ΔF508, causes protein misfolding, protein degradation, and cystic fibrosis. Remarkably, manipulating the miR-138 regulatory network also improved biosynthesis of CFTR-ΔF508 and restored Cl^- transport to cystic fibrosis airway epithelia. This miRNA-regulated network directs gene expression from the chromosome to the cell membrane, indicating that an individual miRNA can control a cellular process more broadly than recognized previously. This discovery also provides therapeutic avenues for restoring CFTR function to cells affected by the most common cystic fibrosis mutation.

ATP-binding cassette transporter | epithelial ion transport | protein biosynthesis

MicroRNAs (miRNAs) are an evolutionarily conserved class of small (~21–24 nt) noncoding RNAs that play key roles in the transcriptional and posttranscriptional regulation of gene expression (1, 2). The effector functions of miRNAs in several facets of pulmonary biology have been identified (3–5), but their actions in airway epithelia are just emerging (6–8). Although there is no established role for miRNAs in the regulation of fluid and electrolyte transport in the airways, this process is vital to homeostasis and is often perturbed in disease states.

CFTR encodes an anion channel that is regulated by ATP hydrolysis and phosphorylation and is expressed in epithelia and other cell types (9, 10). CFTR conducts Cl^-, HCO_3^-, and other anions (11, 12) and through these activities plays a critical role in regulating the volume and composition of airway surface liquid. Mutations in *CFTR* cause cystic fibrosis (CF) (9, 13), an autosomal recessive disease involving the airways, sweat glands, intestines, pancreas, liver, and reproductive tract. The majority of CF-associated morbidity and mortality arises from progressive pulmonary infection and inflammation (14). The most common *CFTR* mutation, ΔF508, is present on ~70% of mutant alleles (13) and causes protein misfolding, degradation, and CF (9, 15). If CFTR-ΔF508 trafficks to the cell membrane, as occurs with low-temperature (16) or chemical chaperone treatment (17), then the mutant protein retains channel function, albeit with reduced residency and open-state probability (18, 19). Because the majority of people with CF have one or two ΔF508 alleles, there is intense interest by academic and industry laboratories in identifying interventions that might restore function to this misprocessed protein.

CFTR is a low-abundance mRNA in airway epithelia (20), and its temporal and spatial expression are tightly regulated (21, 22). Although the *CFTR* promoter has been studied extensively, its complex regulation remains incompletely understood (23, 24). Because miRNAs play key roles in the transcriptional and posttranscriptional regulation of at least 60% of human genes (1, 2), we hypothesized that they may provide a previously unidentified mechanism for regulating CFTR abundance and thereby its function (20).

Results

MiRNA Profiling Identifies a Candidate Regulator of *CFTR*. We profiled global miRNA expression in well-differentiated primary cultures of human airway epithelia by quantitative PCR. Of the 115 miRNAs identified, 31 were highly expressed (C_q <25) (*SI Appendix*, Table S1). We interrogated these expressed miRNAs for possible direct or indirect interactions with CFTR and found no miRNAs with conserved target sites in the CFTR 3′ UTR. However, Targetscan, Pictar, and Miranda software-based analyses of these 31 miRNAs identified the *SIN3A* (*SIN3 homolog A*) gene as a highly conserved candidate miR-138 target. SIN3A is a transcriptional regulator belonging to the Sin3–histone deacetylase core complex (25, 26). Notably, SIN3A protein has conserved motifs that bind to the chromatin insulator protein CCCTC-binding factor (CTCF), a ubiquitously expressed, highly conserved transcriptional repressor that recruits SIN3A and other proteins to the promoters of target genes (27, 28). Importantly, the *CFTR* locus contains functional CTCF-binding sites (29). We thus hypothesized that miR-138 and SIN3A regulate *CFTR*.

A dual-luciferase reporter assay revealed that miR-138 repressed SIN3A expression in a dose-dependent manner, by binding to its 3′ UTR (*SI Appendix*, Fig. S1). This effect was site-specific; mutating the two miR-138 binding sites in the *SIN3A* 3′ UTR relieved the repression in vitro. Transfection of polarized

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE38956).

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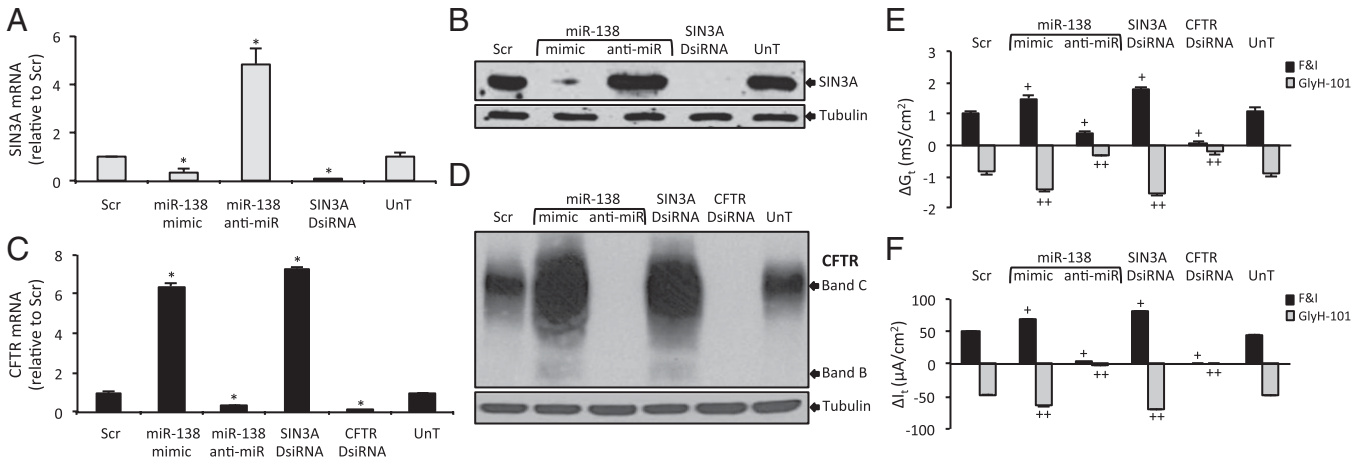


Fig. 1. miR-138 and SIN3A regulate CFTR expression in airway epithelia. (*A*) SIN3A mRNA abundance in human primary airway epithelia at 24 h after the indicated interventions ($n = 6$). Scr, negative control; SIN3A DsiRNA, positive control; UnT, untransfected cells. (*B*) SIN3A protein abundance in primary airway epithelia at 72 h posttransfection. A representative immunoblot is shown. (*C*) *CFTR* mRNA abundance in Calu-3 cells at 24 h after indicated transfections. CFTR DsiRNA, positive control. (*D*) CFTR protein abundance in Calu-3 cells at 72 h posttransfection (R-769 antibody). (*E* and *F*) Changes in conductance (G_t) (*E*) and transepithelial current (I_t) (*F*) with indicated treatments. Basal resistance range, 397–586 ohm*cm^2. Error bars indicate mean ± SE. $^*P < 0.01$ relative to Scr; $^+P < 0.01$, $^{++}P < 0.01$ relative to ΔG_t and ΔI_t in Scr-transfected samples on forskolin and IBMX (F&I) and CFTR inhibitor GlyH-101 treatment, respectively.

primary cultures of human airway epithelia with an miR-138 mimic reduced, and that of an miR-138 anti-miR increased, SIN3A mRNA and protein levels (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S2). These findings validate *SIN3A* as an miR-138 target in airway epithelia.

Mir-138 Regulates CFTR Expression and Function by Relieving SIN3A-Mediated Repression. To test the hypothesis that miR-138 regulates *SIN3A* and thereby *CFTR* expression in airway epithelia, we used the Calu-3 cell line, which expresses CFTR (30). Treatment of Calu-3 cells with an miR-138 mimic or a Dicer-substrate siRNA (DsiRNA) against *SIN3A* increased CFTR mRNA and protein levels (Fig. 1 *C* and *D* and *SI Appendix*, Fig. S3), whereas the miR-138 anti-miR markedly reduced CFTR mRNA and protein abundance (Fig. 1 *C* and *D* and *SI Appendix*, Fig. S3). CFTR creates an ion permeability, and thus its function can be assessed by measuring transepithelial electrical conductance. The miR-138 mimic and SIN3A DsiRNA treatments increased CFTR-mediated Cl^- conductance (G_t) and current (I_t) in polarized Calu-3 epithelia, whereas the miR-138 anti-miR had the opposite effects (Fig. 1 *E* and *F*).

In polarized primary cultures of human airway epithelia, transfection with an miR-138 mimic or SIN3A DsiRNA increased, and that of an miR-138 anti-miR reduced, CFTR mRNA and protein levels (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S4). We note that native tissue and primary cultures of airway

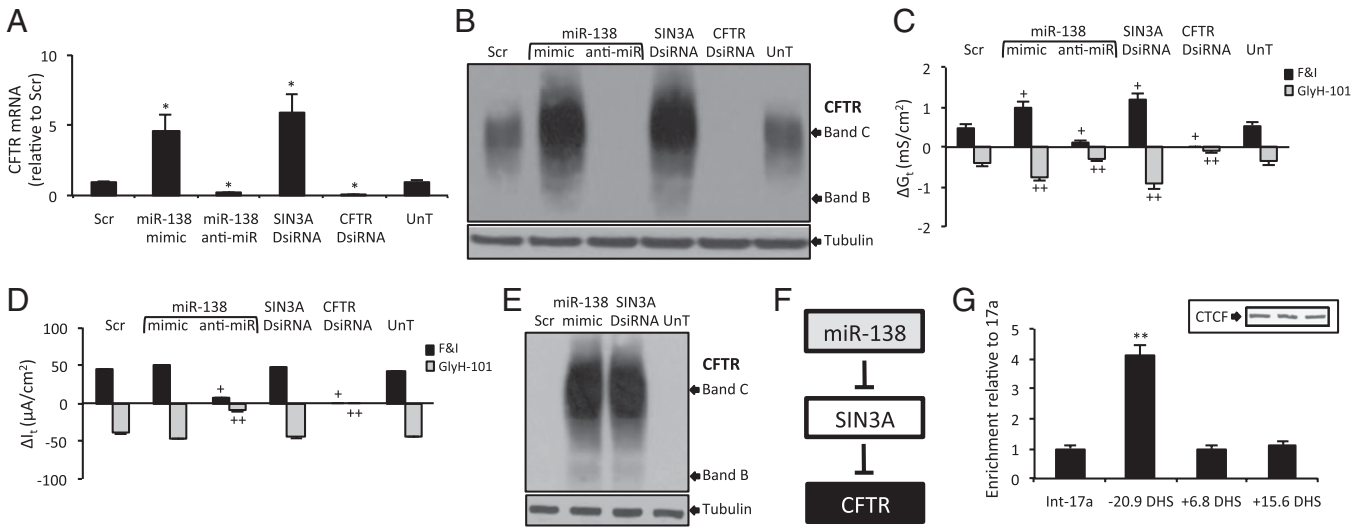


Fig. 2. miR-138 and SIN3A regulate CFTR expression in primary cultures of human airway epithelia and cells with no CFTR expression. (*A*) *CFTR* mRNA abundance in primary airway epithelia at 24 h after interventions ($n = 6$). (*B*) CFTR protein abundance from primary airway epithelia at 72 h posttransfection; R-769 antibody, representative immunoblot. (*C* and *D*) Changes in conductance (G_t) (*C*) and transepithelial current (I_t) (*D*) with indicated treatments. Each bar represents six primary airway epithelial cell cultures each from three donors, pretransfected with the indicated reagents. Basal resistance range, 415–672 ohm*cm^2. (*E*) CFTR protein abundance in HeLa cells; R-769 antibody. (*F*) Schematic representing miR-138– and SIN3A-mediated regulation of *CFTR* expression. (*G*) Fold enrichment of SIN3A, assessed by quantitative PCR after ChIP. Data are normalized to *CFTR* intron 17a DHS. (*Inset*) CTCF immunoblot of lysates from three airway epithelia donors. Error bars indicate mean ± SE; $^*P < 0.01$ relative to Scr; $^{**}P < 0.01$ relative to intron 17a; $^+P < 0.01$ and $^{++}P < 0.01$ relative to ΔG_t and ΔI_t in Scr-transfected samples on F&I and GlyH-101 treatment, respectively.

epithelial cells in which endogenous CFTR is studied have less band B protein than that detected when recombinant CFTR is expressed (31, 32). Treatment with the miR-138 mimic and the SIN3A DsiRNA increased cAMP-stimulated G_t (Fig. 2*C*). There was no change in I_t (Fig. 2*D*), consistent with the presence of other rate-limiting steps for Cl^- secretion in airway epithelia (33). The miR-138 anti-miR reduced both G_t and I_t responses to cAMP-dependent stimulation (Fig. 2 *C* and *D*).

SIN3A Is a Transcriptional Repressor of *CFTR* Expression. The foregoing data show that miR-138 and SIN3A regulate CFTR expression in epithelia that normally express CFTR. To learn whether they also can control CFTR expression in cells that do not produce CFTR, we studied HeLa and HEK293T cells. The miR-138 mimic and SIN3A DsiRNA markedly increased CFTR mRNA and protein expression (Fig. 2*E* and *SI Appendix*, Figs. S5 and S6). Transfected HeLa cells also exhibited cAMP-dependent anion permeability, as assessed by iodide efflux (*SI Appendix*, Fig. S7). These results implicate SIN3A as a potent regulator of CFTR expression, and further support the notion that miR-138 regulates *CFTR* expression by repressing SIN3A (Fig. 2*F*).

To assess whether SIN3A-mediated CFTR repression involves CTCF-mediated recruitment of SIN3A to the *CFTR* promoter (34), we performed ChIP in primary human airway epithelial cells with a SIN3A antibody. Because SIN3A-mediated transcriptional repression involves recruitment to the promoter of target genes (35), we specifically assessed SIN3A enrichment at the *CFTR* promoter. CTCF has been demonstrated to bind within the −20.9 kb DNase I hypersensitive site (DHS) (i.e, distance from the transcriptional start site) (29) at the *CFTR* promoter. In addition to this site, we also assessed SIN3A enrichment at the +6.8 kb DHS (i.e., distance from the transcriptional stop site), which has been shown to bind CTCF in a tissue-specific manner (29, 36). Indeed, the −20.9 kb DHS was enriched for SIN3A compared with two control regions, *CFTR* intron 17a and +15.6 kb DHS (Fig. 2*G*). The demonstration by ChIP that CTCF and SIN3A interact at the −20.9 kb DHS in primary human airway epithelial cells provides insight into the repressor functions of this important regulatory region, as well as the nature of protein interactions at the *CFTR* promoter.

MiR-138 and SIN3A Regulate Genes Influencing CFTR Protein Maturation. To explore whether miR-138 and SIN3A might have posttranscriptional effects on protein biosynthesis in addition to their direct transcriptional regulation of *CFTR*, we performed additional experiments using HeLa cells stably expressing HA-tagged WT *CFTR* under control of the CMV promoter (18). Cell-based ELISA using an HA antibody revealed an increase in HA-tagged CFTR at the cell surface after treatment with the miR-138 mimic or SIN3A DsiRNA (Fig. 3*A* and *SI Appendix*, Fig. S8*A*), with no changes in transgene mRNA abundance (*SI Appendix*, Fig. S8*B*). This result was further supported by immunoblot analysis (Fig. 3*B* and *SI Appendix*, Fig. S8 *C* and *D*). These data indicate that miR-138 has important posttranscriptional effects on CFTR biosynthesis.

Subsequent global mRNA transcript profiling in Calu-3 epithelia treated with the miR-138 mimic or SIN3A DsiRNA identified a common set of 773 genes whose expression changed in response to these interventions (Fig. 3*C*). Intersecting these gene sets with a curated list of 362 genes with protein products known to associate with CFTR (i.e., CFTR-associated gene network; *SI Appendix*, Table S2) revealed that 34.5% (125 of 362) were in the CFTR-associated gene network, a significant enrichment over random expectations (Fig. 3*C* and *SI Appendix*, Table S3). These 125 genes function in several cellular compartments and many positively influence CFTR protein expression or stability (*SI Appendix*, Table S4). Pathway and Gene Ontology analysis of the 773 differentially expressed genes using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (37) revealed significant enrichment of gene sets in pathways that include chaperones, unfolded protein response, protein ubiquitination and proteosomal catabolic processes, negative regulation of apoptosis, and heat shock (*SI Appendix*, Table S5). These findings further support the conclusion that miR-138 enhances CFTR biogenesis and influences the expression of genes at multiple steps along its biosynthetic pathway.

Manipulating the miR-138–SIN3A Network Rescues Misprocessed ΔF508 CFTR. The most common *CFTR* mutant, ΔF508, generates a protein with an altered structure that is unstable, mislocalized, and rapidly degraded via endoplasmic reticulum-associated degradation (15). Interventions that improve biosynthetic processing, such as low temperature (16), chemical chaperones (17), and small molecules (38, 39), can partially restore CFTR-ΔF508 anion channel function; however, overexpression of the ΔF508 cDNA in heterologous cells or primary airway epithelia does not restore CFTR-dependent anion conductance (40). Because miR-138 increased the biosynthesis of WT CFTR (Fig. 3 *A* and *B*), we hypothesized that it also might improve the biosynthesis of CFTR-ΔF508.

We transfected HeLa cells stably expressing HA-tagged CFTR-ΔF508 cDNA under the control of the CMV promoter (18) with the miR-138 mimic or SIN3A DsiRNA. Suprisingly, we found that mutant CFTR, as detected by ELISA using an HA-specific antibody, reached the cell surface (Fig. 3*D* and *SI Appendix*, Fig. S9*A*) with no change in transgene mRNA abundance (*SI Appendix*, Fig. S9*B*). Immunoblot analysis with an HA-antibody detecting only the transgene CFTR-ΔF508 protein product demonstrated that both interventions increased the abundance of the mature, fully glycosylated CFTR band C (Fig. 3*E* and *SI Appendix*, Fig. S9 *C* and *D*).

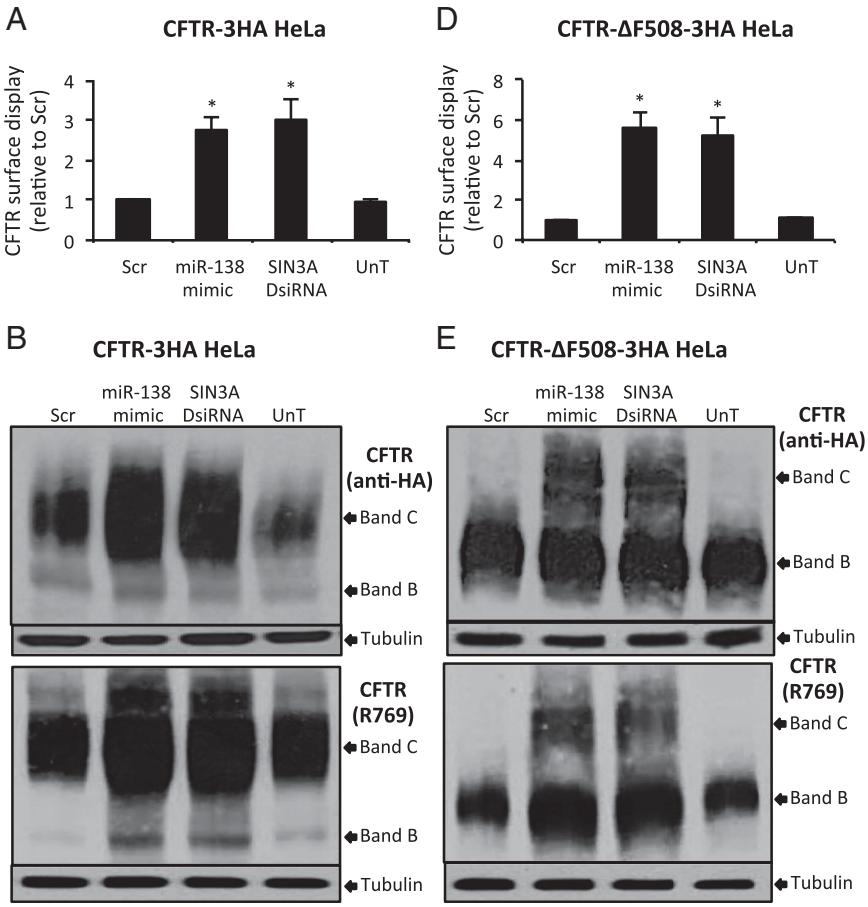
We also expressed a recombinant CMV promoter-driven CFTR-ΔF508 cDNA in primary human *CFTR* null airway epithelia (*CFTR* Q493X/S912X) using an adenovirus (Ad) vector (41). In this setting, CFTR-mediated Cl^- current was restored only in epithelia pretreated with the miR-138 mimic or SIN3A DsiRNA (Fig. 4*A* and *SI Appendix*, Fig. S10). These results further indicate that miR-138– and SIN3A-regulated genes influence the posttranscriptional processing of CFTR.

Next, we tested whether manipulating the miR-138–SIN3A network rescued CFTR-ΔF508–mediated Cl^- transport in CF primary airway epithelial cells. Expression of the miR-138 mimic or SIN3A DsiRNA increased CFTR-ΔF508 mRNA and protein even in primary cultures of CF airway epithelia (Fig. 4*B* and *SI Appendix*, Figs. S11 and S12). Both interventions also restored CFTR-ΔF508–mediated Cl^- transport in these epithelia (Fig. 4*C* and *SI Appendix*, Fig. S12 *B* and *C*). Significant restoration of CFTR-ΔF508–mediated Cl^- transport to varying levels was observed in primary CF epithelia from multiple human donors (Fig. 4*D*), and similar results were obtained in a cell line homozygous for the ΔF508 mutation (*SI Appendix*, Fig. S13). Measurement of lactate dehydrogenase release over a 2-wk period from Calu-3 and CFBE cells transfected with oligonucleotides used in this study (*SI Appendix*, Fig. S14) revealed no cytotoxicity.

Discussion

Here we show that relieving transcriptional repression through the expression of an miR-138 mimic or knockdown of SIN3A profoundly influenced CFTR expression and anion channel activity in airway epithelia, as well as in cells with no basal CFTR expression. Thus, miR-138, acting via SIN3A and other target genes, orchestrates a cellular program that influences WT and mutant CFTR, increasing the biogenesis and cell surface delivery of both (Fig. 4*E* and *SI Appendix*, Tables S3–S5). Through these interactions, the fate of the CFTR-ΔF508 protein in airway epithelia was redirected from proteosomal degradation to a functional anion channel. These findings provide insight into the regulation of CFTR expression and identify a gene network with therapeutic promise.

The present study reveals a previously unrecognized role for SIN3A in the repression of *CFTR* expression. Despite more



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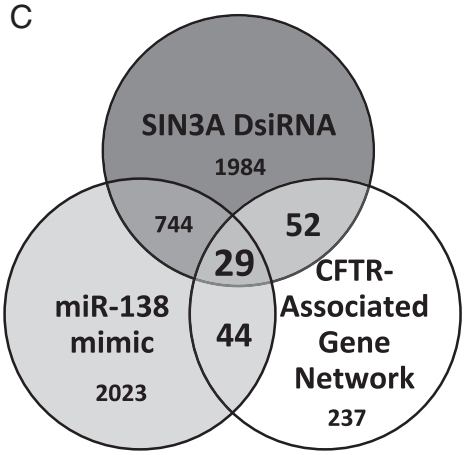

Differentially expressed genes	Enrichment *P*-value
Shared by miR-138 mimic and CFTR-associated gene network: **73 (29+44)**	<2.2E-16
Shared by SIN3A DsiRNA and CFTR-associated gene network: **81 (29+52)**	<2.2E-16
Shared by miR-138 mimic, SIN3A DsiRNA, and CFTR-associated gene network: **29**	1.26E-08

Fig. 3. miR-138 regulates CFTR processing. (*A*) Surface display, as detected by ELISA, of epitope-tagged CFTR in CFTR-3HA HeLa cells transfected with indicated reagents. (*B*) CFTR protein abundance in CFTR-3HA HeLa cells at 24 h post-transfection. (*Upper*) Anti-HA antibody. (*Lower*) R769 antibody. (*C*) Schematic showing regions of intersection of SIN3A DsiRNA, miRNA-mimic, and CFTR-associated genes data sets; $P < 0.05$ (*SI Appendix*, Tables S2–S4). (*D*) Surface display of epitope-tagged CFTR in CFTR-ΔF508-3HA HeLa cells transfected with indicated reagents. (*E*) CFTR protein abundance in CFTR-ΔF508-3HA HeLa cells at 24 h posttransfection. (Upper) Anti-HA antibody. (*Lower*) R769 antibody. Error bars indicate mean ± SE; $*P < 0.01$ relative to Scr.

than 20 y of study (42), the mechanisms governing *CFTR* transcription remain incompletely understood. The *CFTR* promoter has features of a "housekeeping" gene (43, 44), and several transactivating factors and regulatory elements have been characterized (43, 45–47). Given that SIN3A-mediated transcriptional silencing involves associated histone deacetylases (35), identifying an interaction between SIN3A and CTCF on the *CFTR* promoter at the −20.9 kb DHS improves our understanding of how *CFTR* is transcriptionally regulated. However, although SIN3A recruitment by CTCF has been reported previously (28), other DNA-binding proteins also could recruit SIN3A to other sites on the *CFTR* promoter, thereby regulating *CFTR* transcription. A ChIP-seq approach could be used to further identify SIN3A recruitment sites on the *CFTR* promoter. The reciprocal effects of the miR-138 anti-miR in decreasing CFTR mRNA, protein, and transepithelial Cl^- permeability emphasize the role of miR-138 in regulating CFTR expression.

We have identified a genomic signature associated with the rescue of CFTR-ΔF508 (Fig. 3*C* and *SI Appendix*, Tables S3–S5). Strengths of this study include our use of multiple cell models, including both primary airway epithelia and cells that do not normally express CFTR, to test the effect of miR-138 and SIN3A on CFTR biosynthesis. Our key finding that the miR-138–SIN3A network rescues CFTR-ΔF508 biosynthesis was also replicated in primary airway epithelia from multiple CF donors. The current work also has limitations, primarily the need for additional

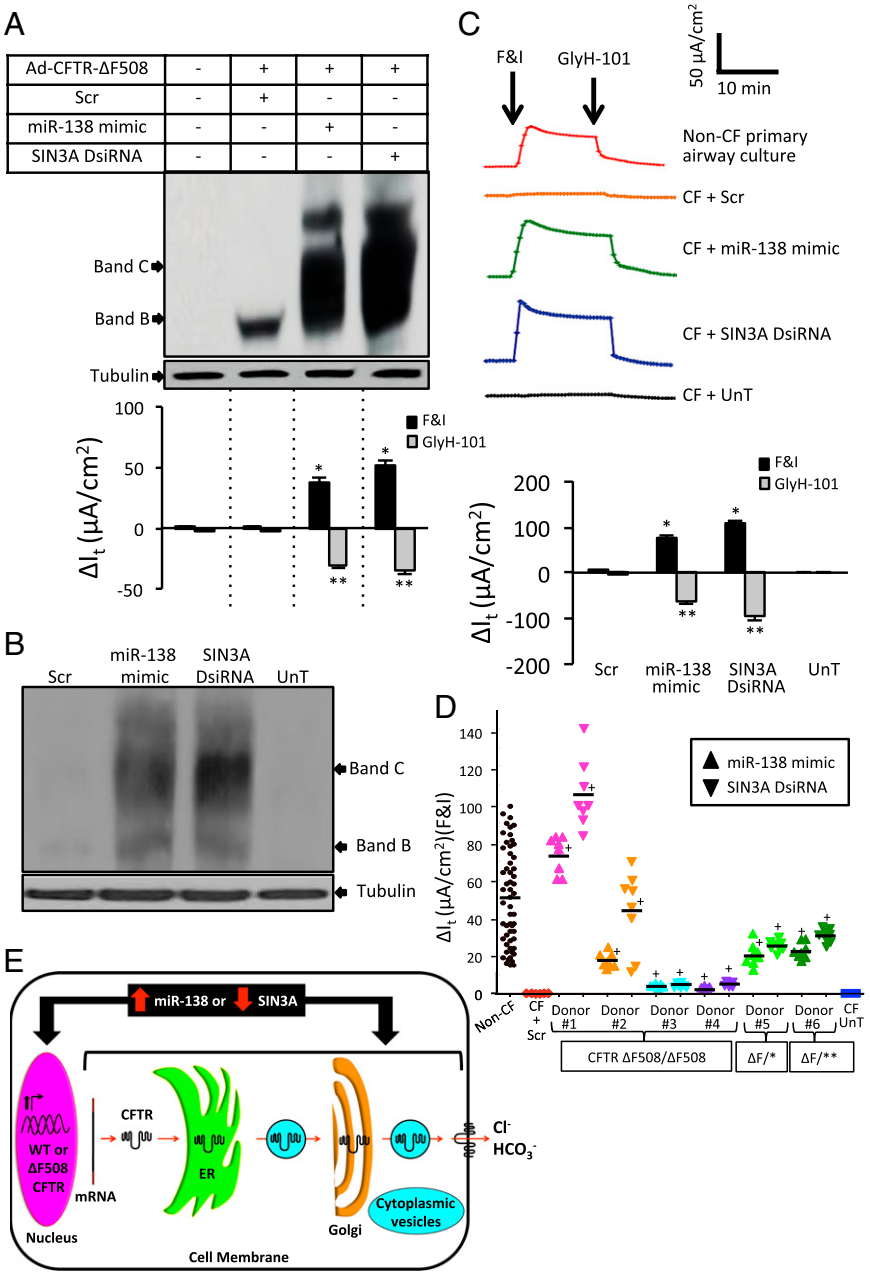

Fig. 4. SIN3A inhibition yields partial rescue of Cl^- transport in CF epithelia. (*A*) (*Upper*) CFTR protein abundance from airway epithelia (*CFTR* Q493X/S912X, 24-1 antibody) after indicated treatments. (*Lower*) Change in I_t values after F&I stimulation and GyH-101 inhibition (one donor, three replicates). Basal resistance range, 279–360 ohm*cm^2. (*B*) Representative CFTR immunoblot from primary epithelia (*CFTR* ΔF508/ΔF508) at 72 h post-transfection; R-769 antibody, donor 2 in *D*. (*C*) Responses of *CFTR* ΔF508/ΔF508 epithelia to indicated interventions (donor 1). (*Upper*) I_t tracings of responses to F&I, followed by GlyH-101 treatment (epithelia pretreated with amiloride and DIDS). (*Lower*) Summary of change in I_t in response to F&I, followed by GlyH-101 treatment (one donor, eight replicates). Basal resistance range, 488–691 ohm*cm^2. Error bars indicate mean ± SE. *$P < 0.01$, **$P < 0.01$ relative to ΔI_t in Scr-transfected samples after F&I and GlyH-101 treatments, respectively; $^+P < 0.01$ relative to Scr. (*D*) Changes in I_t values after F&I treatment of six primary CF airway epithelia cultures transfected with indicated reagents. Six untreated or Scr-treated CF samples served as negative controls; eight non-CF samples served as WT controls. ΔF/* denotes ΔF508/3659delC; ΔF/** denotes ΔF508/R1162X. Horizontal bars indicate means. Basal resistance range, 295–819 ohm*cm^2. (*E*) Working model of steps in CFTR transcription and protein biosynthesis pathway in which miR-138–regulated gene products influence WT and CFTR-ΔF508 (Fig. 3*C* and *SI Appendix*, Tables S2–S5).

studies to better understand the mechanisms through which miR-138 influences CFTR expression and biosynthesis. We do not yet know which miR-138– and SIN3A-regulated genes are ultimately responsible for directing CFTR-ΔF508 processing in airway epithelia. However, SIN3A inhibition alone was sufficient to achieve this result, suggesting that genes directly regulated by SIN3A, or the downstream targets of SIN3A-regulated genes, are key to the effect. A ChIP-seq approach could be used to further identify SIN3A-regulated genes in airway epithelia.

It is possible that changing the expression of only a limited number of genes coregulated by miR-138 and SIN3A would be necessary and sufficient to restore CFTR-ΔF508 function. For example, Sun et al. (48) reported that Derlin-1 (*DERL1*) interacts with both WT and ΔF508 CFTR, reducing their expression. Knockdown of Derlin-1 with siRNA markedly increased the abundance of immature CFTR-ΔF508 protein (48). In addition, siRNA inhibition of *DNAJA1*, *STIP1*, and *HSPA8* is associated with enhanced plasma membrane stability for CFTR-ΔF508 (18). These four genes (*DERL1*, *DNAJA1*, *STIP1*, and *HSPA8*) all showed reduced abundance in response to treatment of epithelia with the miR-138 mimic or SIN3A DsiRNA. Additional selected siRNA and gene addition studies of candidates in the CFTR-associated gene network (Fig. 3*C* and *SI Appendix*, Tables S3–S5) may help identify the subset of genes most influential in CFTR-ΔF508 rescue.

Further steps are needed to carry the findings of the present study toward a therapeutic application. Although transient, partial, and airway-specific delivery of an miR-138 mimic or anti-*SIN3A* siRNA might be therapeutic, the efficient delivery of siRNA or miR mimics to airway epithelia is inefficient with current technology (49, 50). Further advancements in this field will likely lead to new clinical applications. In addition, systemic inhibition of SIN3A function may be undesirable, because the protein is a highly conserved transcriptional repressor that regulates the expression of many genes in a cell- and tissue-specific fashion. An alterative strategy is to focus on the downstream targets of SIN3A that directly mediate the observed CFTR-Δ508

rescue. Drug screening concentrated on identifying small molecules or pharmaceuticals that inhibit SIN3A, or a core subset of gene products responsible for CFTR-ΔF508 rescue, represents another therapeutic approach (51).

Our surprising findings reveal an elegant miRNA-regulated gene network that influences multiple steps in protein biosynthesis. This discovery provides insight into how CFTR expression is regulated, and suggests therapeutic targets for rescuing the function of the mutant CFTR protein most commonly associated with CF. These findings also raise the possibility that manipulating miR-138/SIN3A and their targets might restore function of misprocessed proteins resulting from other genetic diseases.

Materials and Methods

Primary Human Airway Epithelia. Airway epithelia from human trachea and primary bronchus removed from organs donated for research were cultured at the air–liquid interface as described previously (52).

RNA Isolation. Total RNA from human primary airway epithelial cultures and cell lines (Calu-3, HEK293T, HeLa, and CFBE) were isolated using the *mir*Vana miRNA Isolation Kit (Ambion) (53). Total RNA was tested on an Agilent 2100 Bioanalyzer. Only samples with an RNA integrity number >7.0 were selected for downstream processing.

Detailed descriptions of all methods are provided in *SI Appendix, SI Materials and Methods*.

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