ORIGINAL RESEARCH

Connecting COPD GWAS Genes
FAM13A Controls TGFβ2 Secretion by Modulating AP-3 Transport

Lu Gong[1,2], Samuel Bates[1,2], Jian Li[2,3], Dandi Qiao[1,2], Kimberly Glass[1,2], Wenyi Wei[2,4], Victor W. Hsu[2,3*], Xiaobo Zhou[1,2*], and Edwin K. Silverman[1,2*]

[1]Channing Division of Network Medicine, and [3]Division of Rheumatology, Inflammation and Immunity, Brigham and Women's Hospital, Boston, Massachusetts; [2]Harvard Medical School, Harvard University, Boston, Massachusetts; and [4]Department of Pathology, Beth Israel Deaconess Medical Center, Boston, Massachusetts

ORCID IDs: 0000-0002-6763-4636 (V.W.H.); 0000-0002-7127-2869 (X.Z.); 0000-0002-3641-3822 (E.K.S.).

Abstract

Chronic obstructive pulmonary disease (COPD) is a common, complex disease and a major cause of morbidity and mortality. Although multiple genetic determinants of COPD have been implicated by genome-wide association studies (GWASs), the pathophysiological significance of these associations remains largely unknown. From a COPD protein–protein interaction network module, we selected a network path between two COPD GWAS genes for validation studies: FAM13A (family with sequence similarity 13 member A)–AP3D1–CTGF–TGFβ2. We find that TGFβ2, FAM13A, and AP3D1 (but not CTGF) form a cellular protein complex. Functional characterization suggests that this complex mediates the secretion of TGFβ2 through an AP-3 (adaptor protein 3)–dependent pathway, with FAM13A acting as a negative regulator by targeting a late stage of this transport that involves the dissociation of coat–cargo interaction. Moreover, we find that TGFβ2 is a transmembrane protein that engages the AP-3 complex for delivery to the late endosomal compartments for subsequent secretion through exosomes. These results identify a pathophysiological context that unifies the biological network role of two COPD GWAS proteins and reveal novel mechanisms of cargo transport through an intracellular pathway.

Keywords: chronic obstructive pulmonary disease; network medicine; network validation; genome-wide association study; cell trafficking

Clinical Relevance

After validating the network connection among FAM13A (family with sequence similarity 13 member A), AP3D1, and TGFβ2, we identified TGFβ2 as a novel cargo of AP3 (adaptor protein 3)–mediated cellular trafficking that is secreted in exosomes. We also identified a unique mechanism by which FAM13A negatively regulates this cellular trafficking process. Our work not only provides new understanding of TGFβ2's regulation but also reveals a molecular mechanism linking two genome-wide association study genes involved in chronic obstructive pulmonary disease susceptibility.

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*These senior authors contributed equally to this work

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Data Availability: All data generated during or analyzed during the current study are available from the corresponding author on reasonable request. Uncropped images of experimental blots are provided in Figure E6 in the data supplement.

Correspondence and requests for reprints should be addressed to Edwin K. Silverman, M.D., Ph.D., Channing Division of Network Medicine, Brigham and Women's Hospital, 181 Longwood Avenue, Boston, MA 02115. E-mail: ed.silverman@channing.harvard.edu.

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Chronic obstructive pulmonary disease (COPD) is a common, chronic, progressive lung disease; it is the fourth leading cause of death in the United States and is a major cause of morbidity and mortality around the world (1). The development of COPD results from both long-term environmental exposures (typically cigarette smoking) and genetic factors. Recent genome-wide association studies (GWASs) have identified 82 genetic loci that are significantly associated with COPD (2); however, the key genes and functional variants within most of these GWAS loci are unknown (3). In addition, identifying biological connections among the known COPD GWAS genes has been challenging, thus limiting their scientific impact.

We previously identified a set of proteins hypothesized to be connected by protein–protein interactions, known as a disease network module. To build this disease network module, we started with a set of well-established COPD genes (from COPD GWASs and Mendelian syndromes that include COPD) as initial input genes for building the network (referred to as "seed genes") (4). The COPD GWAS genes used as seed genes for this network analysis were located in genomic regions that were significantly associated with COPD and often had strong biological support for involvement in COPD pathogenesis and/or nicotine addiction; they included *IREB2* (5), *MMP12* (6), *HHIP* (7), *RIN3* (8), *CHRNA3* (9), *CHRNA5* (10), *TGFB2* (8), and *FAM13A* (family with sequence similarity 13 member A) (11). All of the gene products of these COPD GWAS seed genes had publicly available protein–protein interaction data except for FAM13A; therefore, we included affinity purification/mass spectrometry data from FAM13A and identified a disease network module of 163 connected proteins (in addition to FAM13A) (11).

Although molecular network models of complex diseases can provide important biological insights, functional validation studies are required to confirm these molecular relationships and to investigate their pathobiological mechanisms (12). We postulated that closely connected proteins in the protein–protein interaction network for COPD would be more likely to have related biological functions in COPD pathogenesis. In this study, we selected the network path FAM13A–AP3D1–CTGF–TGFβ2 for further analysis. Like *FAM13A*, *TGFB2* is another GWAS gene implicated in COPD (8, 13).

Substantial evidence links the TGFβ family of proteins (consisting of TGFβ1, TGFβ2, and TGFβ3) with COPD pathogenesis, as they are involved in multiple cellular events relevant to lung biology, including extracellular matrix production, cellular proliferation, and immune modulation (14). Although most COPD research has focused on TGFβ1, elevated levels of TGFβ2 have been found in lungs of spontaneously hypertensive rats exposed to cigarette smoke (14). Moreover, TGFβ2 has been found to impact rat lung branching during morphogenesis and mucin production in human bronchial epithelial (HBE) cells (15, 16).

Although the *FAM13A* genomic region has consistently been a top association in COPD GWASs, there has been limited understanding of its biological roles. We hypothesized that the postulated protein–protein interactions connecting FAM13A and TGFβ2 would implicate important biological mechanisms involving these COPD GWAS genes that would provide novel insights into COPD pathogenesis.

Methods

Network Visualization and Analysis

The COPD disease network module was visualized by using Cytoscape 3.8.2 (17) using a forced directed layout. COPD GWAS nodes were then repositioned for visualization purposes. Shortest path analysis was performed by using PesCa 3.0 (18).

Cell Lines and Cell Culture

16HBE cell line was purchased from Sigma (SCC150), and human embryonic kidney 293T cells were purchased from the American Type Culture Collection. These cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS, penicillin (50 U/ml), and streptomycin (50 μg/ml). Normal human bronchial epithelial cells (NHBE cells) were purchased from Lonza (CC-2540) and propagated by using a BEGM Bullet Kit (CC-3170, Lonza) as per the manufacturer's instructions. Cells were cultured in humidified incubators (Thermo Fisher Scientific) set at 37°C and 5% CO_2. Whether *Mycoplasma* was present was tested routinely by using the Mycoalert Detection Kit from Lonza (LT07-218).

Fractionation of the Secreted Proteins in Cell Culture Medium

16HBE cells were seeded in 10-cm cell culture dishes (Corning). Secreted proteins were collected after culturing in 10 ml of OptiMEM (31985070, Gibco) for 12 hours. Two hundred microliters of the collected medium was subjected to ultracentrifuge at 100,000 × *g* for 45 minutes, resulting in the pellet fraction containing all membrane-bound proteins and the supernatant fraction containing all soluble proteins. Meanwhile, cells were washed with PBS and lysed with 10 ml of lysis buffer containing 1% Nonidet P-40 to generate the total intracellular fraction. All fractions were then subjected to ELISA to quantify the TGFβ1 and TGFβ2 protein concentrations.

ELISA

ELISAs were performed by using a Human TGF-β2 Quantikine ELISA Kit (DB250, R&D Systems) and a Human TGF-β1 ELISA Kit (ELH-TGFβ1, RayBiotech) according to the manufacturers' protocols.

Protein Transmembrane Structure Prediction and Motif Search

The potential transmembrane structure in TGFβ2 was predicted by using a hidden Markov model (19, 20). The tyrosine-based motif was identified by using the Multiple Expectation Maximizations for Motif Elicitation Suite (University of Nevada, Reno and University of Washington) (21).

Sequence Conservation Analysis

The full protein sequences of TGFβ1 (accession number NP_000651.3) and TGFβ2 (accession number NP_003229.1) were downloaded from National Center for Biotechnology Information reference gene database. The ClustalW algorithm (Desmond G. Higgins, University College Dublin) was used for multisequence alignment.

Statistical Analysis

Data are expressed as the mean ± SD, and the statistical significance as determined by analysis in GraphPad Prism is reported in the figures and figure legends. Statistical differences among multiple groups were calculated by using ANOVA, and then pairwise testing between groups was performed by using a Tukey multiple comparison test. For pairwise comparisons in ELISA experiments, the Welch *t* test, which allows differences in variance between groups, was used instead of a standard two-tailed *t* test. For other pairwise comparisons, standard two-tailed *t* tests were

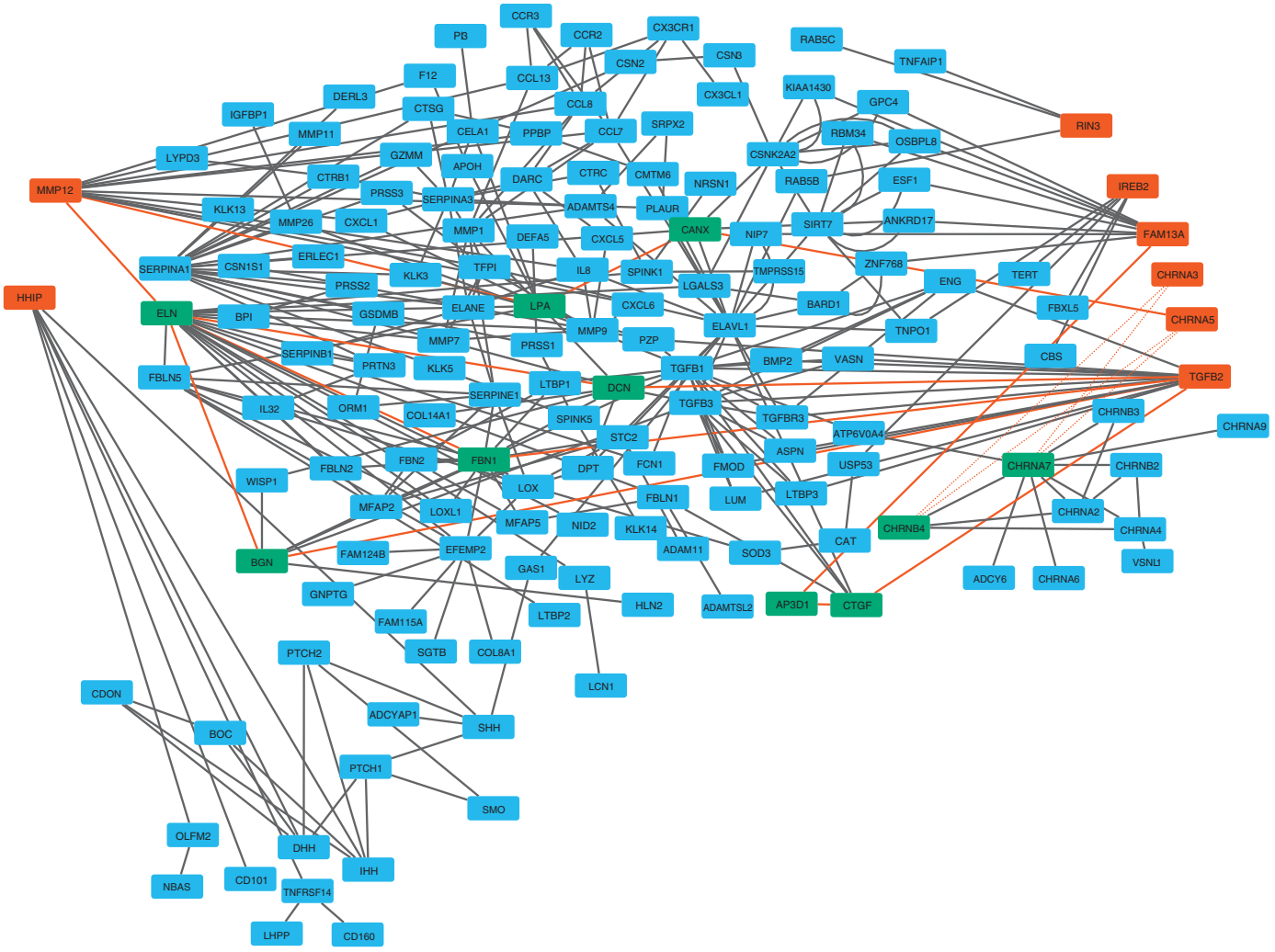

Figure 1. Shortest network paths in the chronic obstructive pulmonary disease (COPD) network module. The COPD network module from Sharma and colleagues (4) is shown, with the eight COPD genome-wide association study genes that were used as seed genes to build the module being denoted by red nodes. The shortest paths among these eight genome-wide association study genes are shown with green nodes and red edges; the one-node connections are shown with a dotted red line, and the two-node connections are shown with a solid red line. FAM13A = family with sequence similarity 13 member A.

used. A P value of <0.05 was considered to indicate statistical significance. In figures, asterisks denote statistical significance ($^{*}P < 0.05$ and $^{**}P < 0.01$).

Results

Selecting a Network Path for Functional Validation Studies

To identify novel biological relationships among COPD GWAS genes, the shortest paths in the protein–protein interaction COPD network module among the eight COPD GWAS genes were identified (4) (Figure 1). There were two shortest paths with one intervening node (CHRNA5–CHRNA7–CHRNA3 and CHRNA5–CHRNB4–CHRNA3), which included components of the nicotinic acetylcholine receptor. There were five shortest paths with two intervening nodes. Three of these two-node paths linked TGFβ2 to components of the lung microfibril and/or elastic fiber (22), a known repository of TGFβ in the lung (23), and to MMP12, an elastolytic proteinase (TGFβ2–BGN–ELN–MMP12, TGFβ2–DCN–ELN–MMP12, and TGFβ2–FBN1–ELN–MMP12). In addition to these known biological relationships, two-node connections between CHRNA5 and MMP12 (CHRNA5–CANX–LPA–MMP12) and between FAM13A and TGFβ2 (FAM13A–AP3D1–CTGF–TGFβ2) were identified, potentially indicating novel biological relationships among COPD GWAS genes. Considering the reported biological effects of TGFβ2 in rat lung development and in response to cigarette smoke (14, 16), the identification of functional genetic variants influencing FAM13A and TGFβ2 in those COPD GWAS regions, and the absence of any known relationship between FAM13A and TGFβ2 (24, 25), we selected the FAM13A–AP3D1–CTGF–TGFβ2 pathway for further validation and mechanistic investigation.

Validating a Network Connection among FAM13A, AP3D1, and TGFβ2

By performing coprecipitation studies initially, we found that FAM13A, TGFβ2, and AP3D1 form a complex in 293T cells (Figure 2A). We also found that TGFβ2 associates with CTGF and with AP3D1 (Figure 2B).

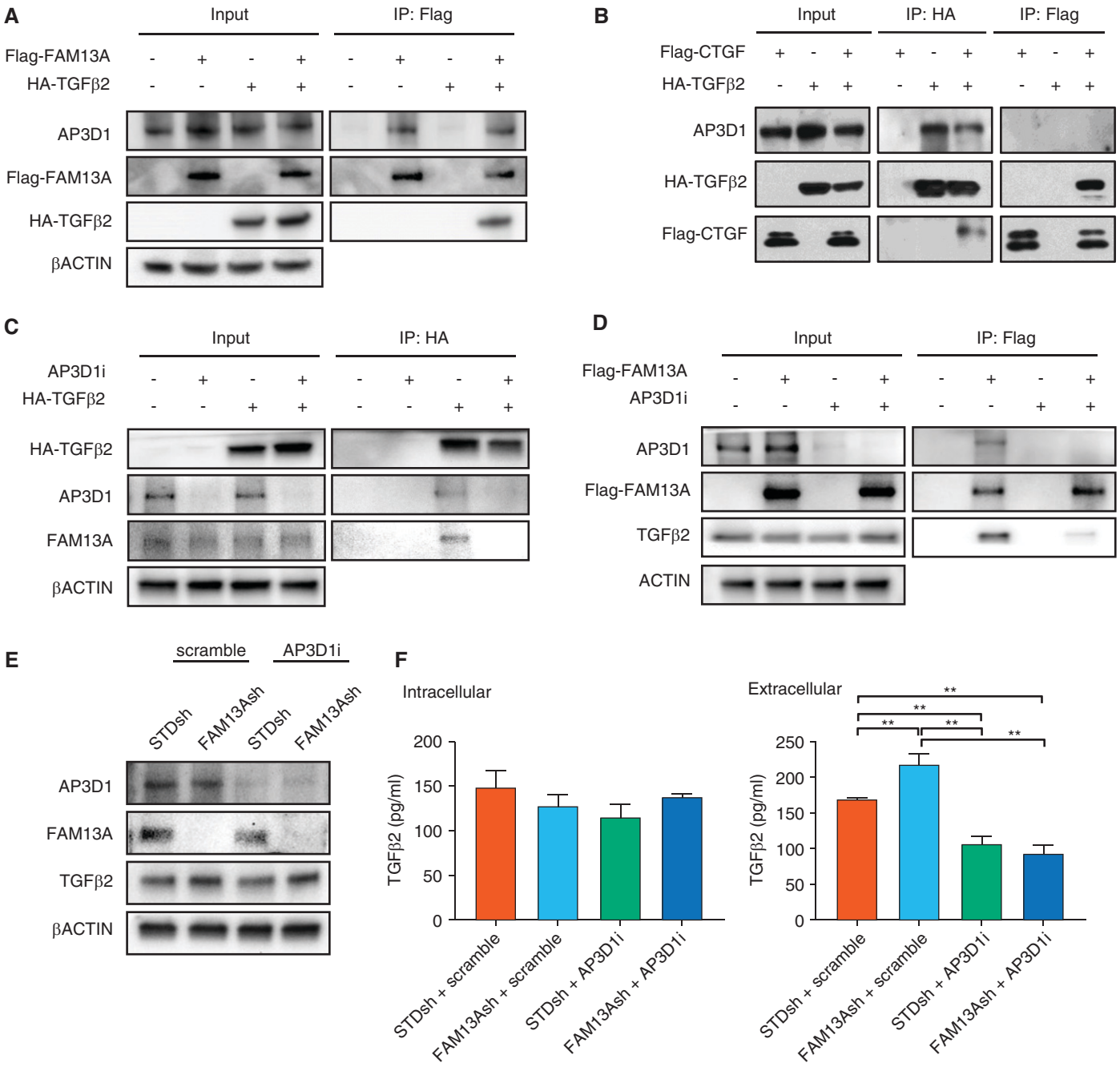

Figure 2. FAM13A binds to TGFβ2 and impedes its secretion. (*A*) 293T cells were transfected with plasmids expressing Flag-FAM13A or HA-TGFβ2. Forty-eight hours later, binding of Flag-FAM13A to HA-TGFβ2 or endogenous AP3D1 was examined by IP. (*B*) 293T cells were transfected with Flag-CTGF or HA-TGFβ2 plasmids as indicated, and binding of HA-TGFβ2 to Flag-CTGF or endogenous AP3D1 was examined by IP of the whole-cell lysate. Cells were treated with 10μM MG132 for 10 hours before harvesting to ascertain that the negative interaction between CTGF and AP3D1 cannot be attributed to the protein degradation of CTGF. (*C*) 16HBE cells were transfected with HA-TGFβ2 plasmids or siRNA targeting *AP3D1* (AP3D1i). Forty-eight hours later, protein binding of TGFβ2 to endogenous FAM13A or AP3D1 was assessed. (*D*) 16HBE cells were transfected with AP3D1i or scramble siRNA, together with a FAM13A plasmid or empty vector. At 2 days after transfection, protein binding of FAM13A to endogenous TGFβ2 and AP3D1 was examined by IP. (*E*) 16HBE cells were infected with lentivirus expressing nontargeting shRNA (STDsh) or shRNA targeting *FAM13A* (FAM13Ash). Three days later, cells were transfected with AP3D1i or scramble siRNA. The indicated protein amount was determined by using immunoblots. (*F*) Intracellular and extracellular TGFβ2 protein amount from assays in *E* were measured by using an ELISA at 2 days after transfection. Data were generated from three repeats. Error bars indicate SDs. $^{**}P < 0.01$. AP3 = adapter protein 3; HBE = human bronchial epithelial.

However, contrary to the connections within the protein–protein interaction module we developed, CTGF failed to bind to AP3D1 in 293T cells. Thus, the connection between the COPD GWAS genes, *FAM13A* and *TGFB2*, was feasible without involvement of CTGF and is even closer than our network analysis had predicted.

Lung epithelial cells likely play a key role in COPD pathogenesis, including airway remodeling (26). Thus, we conducted further studies on an immortalized cell line from the human bronchial epithelium (16HBE cell line). This cell line allowed us to track transfected epitope-tagged FAM13A, as the antibody against FAM13A was inefficient in immunoprecipitating the endogenous protein and thus prevented us from examining interactions by the endogenous FAM13A in primary cells. Intriguingly, knocking down *AP3D1* abolished the binding of FAM13A and TGFβ2 in 16HBE cells (Figures 2C and 2D), indicating that the AP3 (adaptor protein 3) complex may bridge their binding to connect them functionally. Thus, we focused on the FAM13A–AP3D1–TGFβ2 network connection for further functional studies.

AP-3 Mediates the Secretion of TGFβ2 with FAM13A Acting as a Negative Regulator

AP3D1 is a component of the AP-3 coat complex, which generates transport carriers from the trans-Golgi network and the early endosome for delivery to the late endosome and lysosome (27, 28). Besides AP3D1, we previously identified two other components of the AP-3 complex, AP3M1 and AP3B1, as being FAM13A-interacting proteins (11). Thus, we next sought to determine whether TGFβ2 secretion involves transport through an AP-3 pathway in HBE cells (16HBE cells). Indeed, we found that silencing *AP3D1* reduces the extracellular concentration of TGFβ2 (Figures 2E and 2F). Moreover, silencing *FAM13A* increases the level of secreted TGFβ2 (Figures 2E and 2F), which is abolished when *AP3D1* is also silenced (Figures 2E and 2F). Thus, these results suggested not only that TGFβ2 secretion involves an AP-3 pathway but also that FAM13A acts as a negative regulator in this transport.

TGFβ2 Is a Transmembrane Cargo Protein of the AP-3 Pathway

We next addressed a mechanistic question. Coat complexes are recruited to the cytosolic side of intracellular membrane compartments to generate transport vesicles. Thus, as they can only bind cargo proteins that are exposed to the cytosolic side of these membranes, how can the AP-3 complex engage TGFβ2 for its secretion, when considering that the best characterized TGFβ member, TGFβ1, is secreted as a soluble protein, which would involve its transit within the lumen of intracellular membrane compartments?

Aligning the sequences of TGFβ1 and TGFβ2, we noted that they have a high level of overlapping sequence content, with the exception of the N-terminal portion (Figure 3A; *see also* Figure E1 in the data supplement). By using hidden Markov algorithm–based secondary protein structure prediction (19), we found a plausible transmembrane domain near the N terminus of TGFβ2 (Figures 3A and 3B). Moreover, a tyrosine-based motif, YXXΦ (with Y indicating tyrosine, X indicating any amino acid, and Φ indicating a hydrophobic amino acid), is predicted to reside on the cytosolic side of the putative TGFβ2 transmembrane domain (Figures 3A and 3B). As the AP3 complex has been shown to bind tyrosine-based motifs (27, 28), we next pursued the intriguing possibility that TGFβ2 is transported as a transmembrane cargo protein by AP-3 recognizing a tyrosine-based motif in the N terminus of TGFβ2.

Performing mutations to destroy the two key residues in this binding motif in TGFβ2, from YCVL (consistent with the YXXΦ motif structure) to ACVN (Figure 3C), we found that the resulting mutant has markedly reduced association with AP3D1 and FAM13A (Figure 3D) and is also poorly secreted (Figure 3E). Moreover, the mutant TGFβ2 showed increased accumulation in the cell. Thus, these results supported the hypothesis that TGFβ2 is transported as a transmembrane cargo protein through an AP-3 pathway for its secretion.

We also found that TGFβ2 does not affect the expression of FAM13A, neither at the mRNA level nor at the protein level (Figure E2). In contrast, in results similar to those from previous studies (29, 30), we found that TGFβ1 may affect the expression of FAM13A. In the case of 16HBE cells, there is a trend for TGFβ1 reducing the protein level, but not the mRNA level, of FAM13A (Figure E2).

TGFβ2 Is Mainly Secreted through Exosomes

We then addressed another mechanistic question. The AP-3 coat complex is only known to deliver cargoes to the late endosomal compartments (27, 28). Thus, how can this transport result in TGFβ2 being secreted? Besides being degraded by the lysosome, transmembrane cargo proteins that reach the late endosomal compartments are now appreciated as having a second general fate: being secreted through exosomes because of the late endosome fusing with the plasma membrane (31, 32). Thus, we next examined whether such a fate could explain how TGFβ2 ultimately becomes secreted.

Subjecting the culture medium to high-speed ultracentrifugation, which segregates proteins into soluble versus membrane-bound pools, we detected a significant fraction of secreted TGFβ2 residing in the latter pool (Figure 4A), suggesting that the extracellular TGFβ2 exists mostly in exosomes. We also found that FAM13A regulates TGFβ2 secretion mainly by targeting this pool, as FAM13A overexpression or silencing mainly affects the amount of TGFβ2 in the membrane-bound pool while having minimal effects on the level of soluble TGFβ2 (Figure 4A). Furthermore, we found that the amount of TGFβ2 in the extracellular membrane–bound pool is diminished when its tyrosine-based motif is mutated (Figure 4B). In contrast, we did not detect a significant fraction of extracellular TGFβ1 residing in the membrane-bound pool (Figure 4C), which is consistent with TGFβ1 being known to be secreted as a soluble protein (33). Further expanding on these findings, we observed similar effects of FAM13A overexpression and silencing on the secretion of TGFβ2 and TGFβ1 in primary normal human bronchial epithelial cells (NHBE cells) (Figure E3). Thus, these results not only revealed that transport through an AP-3 pathway ultimately leads to TGFβ2 being secreted in exosomes but also provided further support that TGFβ2 is transported as a transmembrane cargo protein.

FAM13A Retains TGFβ2 on AP3 Vesicles

We next sought insight into how FAM13A negatively regulates the AP-3 transport of TGFβ2. Initially, we performed coprecipitation studies and found that knocking down FAM13A reduces the association of TGFβ2 with AP3D1 (Figure 5A), whereas FAM13A overexpression had the opposite effect of enhancing this association (Figure 5B). In results consistent with these findings, we found by using confocal microscopy that knocking down FAM13A reduces the colocalization of TGFβ2 with AP3D1 (Figures 5C and 5D), whereas we

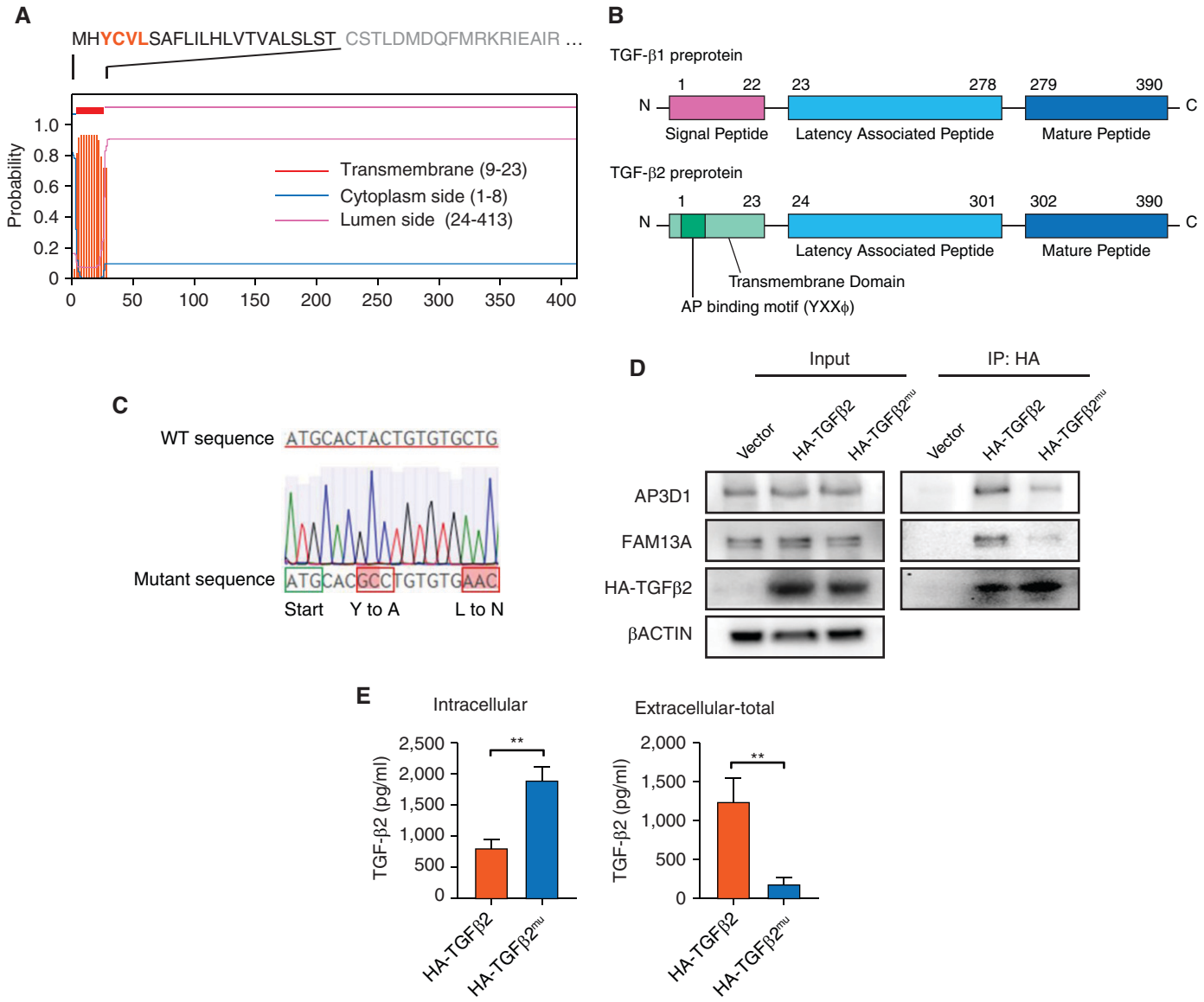

Figure 3. TGFβ2 is a transmembrane cargo protein in AP-3 transport. (*A*) The transmembrane structure was predicted on the basis of the amino acid sequence of TGFβ2. The probability and location of the predicted transmembrane motifs are shown. A tyrosine-based motif (labeled in red) resides near the N terminus of TGFβ2. (*B*) Comparison of domain structures of TGFβ1 versus TGFβ2. (*C*) Mutation of the tyrosine-based motif in TGFβ2. (*D*) 16HBE cells were transfected with HA-tagged WT or mutant TGFβ2, and then the associations of HA-tagged forms of TGFβ2 with endogenous FAM13A and AP3D1 were assessed. (*E*) TGFβ2 levels inside or outside of the cell for assays in *D* were quantified by using an ELISA. Data were generated from three repeats. Error bars indicate SDs. $^{**}P < 0.01$. mu = mutant; WT = wild type.

found that FAM13A overexpression had the opposite effect (Figures 5C and 5D). These results were surprising, when considering that a factor that enhances the association of a cargo protein with a coat complex is typically expected to promote cargo transport by promoting the sorting of the cargo into the pathway mediated by the coat complex. However, because FAM13A inhibits the transport of TGFβ2 while enhancing its association with the AP3 complex, we were left to conclude that FAM13A is more likely to inhibit a late stage of a transport pathway, which involves cargo–coat dissociation so that the cargo can be transferred from a transport carrier to its target compartment.

A key prediction of such a role is that FAM13A should also enhance localization of both TGFβ2 and AP3 at the target compartment of AP3 vesicles, which would be the late endosomal compartments. Indeed, we found by using confocal microscopy that FAM13A overexpression enhances the colocalization of TGFβ2 with LAMP1, which marks both the late endosome and the lysosome (34), whereas we found that FAM13A silencing has the opposite effect (Figures 6A and 6B). FAM13A overexpression also promotes the localization of AP3 at these compartments, whereas FAM13A silencing has the opposite effect (Figures E4B and E4C). Moreover, we confirmed that LAMP1 marks not only the lysosome but also the late endosome in 16HBE cells, as it shows appreciable colocalization with Rab7, a late endosome marker (Figure E4A). Thus, the

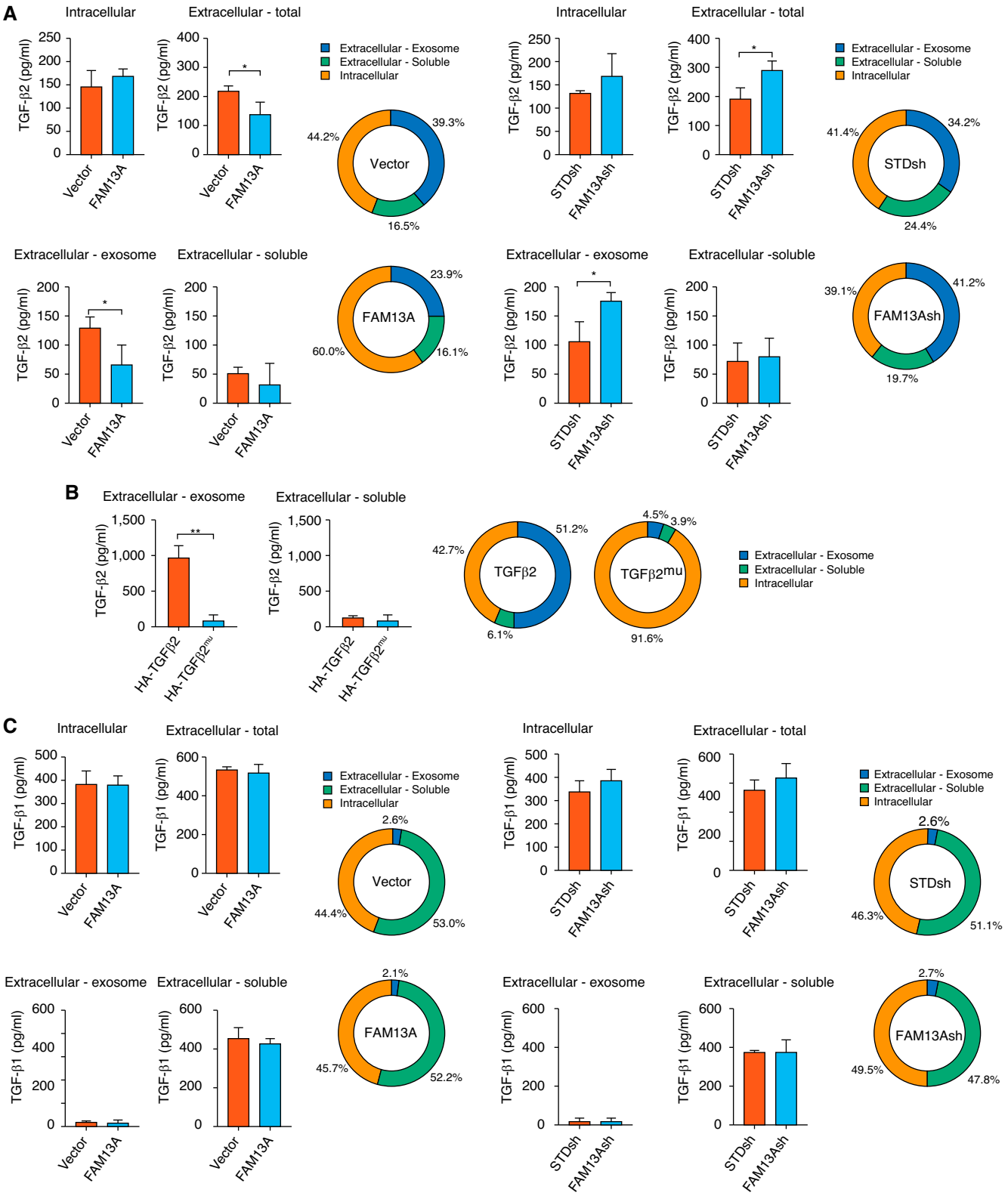

Figure 4. TGFβ2 is secreted in exosomes. (*A*) 16HBE cells were transfected with an empty vector/FAM13A-expressing plasmid or were stably transfected with FAM13Ash/STDsh lentivirally as indicated. TGFβ2 protein levels, either intracellular or extracellular, were quantified by using an ELISA. The total extracellular TGFβ2 protein level was also further subdivided into membrane-bound versus soluble fractions, with exosomes being included in the membrane-bound fraction. (*B*) 16HBE cells were transfected with HA-tagged WT or mutant TGFβ2. TGFβ2 protein levels, either in

collective results further supported the conclusion that FAM13A acts as a negative regulator of TGFβ2 transport by preventing a late stage of AP-3 transport, cargo–coat dissociation, a process that would be needed for TGFβ2 to be transferred from AP3 vesicles to the late endosomal compartments for subsequent secretion through exosomes.

Discussion

GWASs have identified thousands of significant associations in complex diseases, but the impact of these discoveries on our understanding of disease pathogenesis has been relatively limited thus far. We investigated network interactions among proteins expressed by GWAS genes, reasoning that this approach could lead to important insights into mechanisms of complex diseases like COPD. In this study, we have focused on two of the most well-established COPD GWAS genes, *FAM13A* and *TGFB2*. In addition to consistent, genome-wide significant evidence for a genetic association with COPD, potentially functional genetic variants that influence both of these genes have been identified (24, 25). Thus, the *FAM13A* and *TGFB2* genetic loci are clearly associated with COPD, and *FAM13A* and *TGFB2* are key genes within those GWAS loci.

Network analysis of a previously reported protein–protein interaction network module for COPD suggested a protein–protein interaction network connection involving FAM13A–AP3D1–CTGF–TGFβ2 (4). *AP3D1* encodes the δ subunit of the AP-3 coat complex, which mediates intracellular transport from the trans-Golgi network and from the early endosome to the late endosomal compartments that include the late endosome (also known as multivesicular bodies) and lysosomes (27, 28). Mutations in *AP3D1* are associated with Hermansky-Pudlak syndrome (HPS) type 10 (35, 36). Although some forms of HPS are associated with pulmonary fibrosis, whether patients with HPS type 10 can develop pulmonary fibrosis is confounded by the fact that these patients die early in childhood, thereby potentially masking pulmonary fibrosis susceptibility.

Here, we confirmed the protein–protein interactions among FAM13A, AP3D1, and TGFB2, but we found that AP3D1 did not interact directly with CTGF in our model system. The reported interaction between AP3D1 and CTGF in the ConsensusPathDB database (Max Planck Institute for Molecular Genetics) was not validated by our coprecipitation studies. Thus, the protein–protein interaction network modeling pointed toward a novel relationship between COPD GWAS genes, but the observed interaction was even closer than that suggested by the network disease module. Multiple complex diseases have been studied with network analysis methods to identify the disease network module within the molecular interactome of protein–protein interactions, including asthma (37). Our results suggest that follow-up coprecipitation studies can provide valuable validation of these network relationships.

We initially pursued coprecipitation studies to detect a protein complex consisting of FAM13A, TGFβ2, and AP3D1 in cells. To gain insight into the function of this complex, we found that perturbing the level of FAM13A expression affects TGFβ2, specifically its secretion from cells. In contrast, we found no evidence for TGFβ2 affecting FAM13A expression at either the mRNA level or the protein level. We further noted that TGFβ1 has been reported previously to regulate FAM13A levels (29, 30), and we have also observed a trend in the reduction of the FAM13A protein level upon TGFβ1 treatment. In light of this initial set of findings, we focused on understanding how FAM13A regulates the secretion of TGFβ2. This investigation has resulted in multiple noteworthy findings.

First, in contrast to TGFβ1, which is secreted as a soluble protein, we have found that TGFβ2 is secreted as a transmembrane protein. The initial hint came from the consideration that coat complexes are recruited to the cytosolic side of intracellular membrane compartments, which does not allow them to interact with soluble secreted proteins that are transported within the lumen of intracellular compartments. This led us to elucidate that, in contrast to TGFβ1, TGFβ2 is transported as a transmembrane protein by the AP3 pathway, which is mediated by the AP-3 complex recognizing a tyrosine-based motif residing in the cytosolic domain of the transmembrane TGFβ2.

Further support for this conclusion comes from another puzzle that we sought to address. The AP-3 pathway delivers cargoes to the late endosomal compartments (27, 28). Thus, how can transport through an AP-3 pathway result in TGFβ2 being secreted from the cell? As a clue, we noted that the late endosome forms internal vesicles (also known as multivesicular bodies), which can be secreted as exosomes (31, 32). Indeed, in the cell culture medium, most of the secreted TGFβ2 exists in a membrane-bound pool, suggesting its secretion through exosomes. Notably, this finding not only reveals an unanticipated way that transport through the AP-3 pathway can ultimately lead to secretion of TGFβ2 from the cell but also further supports the likelihood that TGFβ2 exists as a transmembrane cargo protein of the AP-3 pathway.

A third notable finding comes from our elucidation of how FAM13A acts as a negative regulator of TGFβ2 transport. Cargo transport through an intracellular pathway requires coat proteins initially binding to cargo proteins for their sorting into transport vesicles. Subsequently, this binding must be released so that the cargo can be transferred from the transport carrier to the target compartment. FAM13A likely inhibits this late stage of cargo transport, as such a role would explain how FAM13A enhances the interaction between TGFβ2 and AP3D1 while also inhibiting TGFβ2 secretion (Figure E5). To our knowledge, a factor that regulates transport at the level of coat–cargo dissociation has not been identified previously. As such, this novel finding contributes to a fundamental understanding of the regulatory mechanisms acting in vesicular transport.

Figure 4. (*Continued*). exosomes or in the soluble extracellular parts, were quantified by using an ELISA. (*C*) 16HBE cells were transfected with an empty vector/FAM13A-expressing plasmid or were stably transfected with FAM13Ash/STDsh lentivirally as indicated. TGFβ1 protein levels, either intracellular or extracellular, were quantified by using an ELISA. The total extracellular TGFβ1 protein level was also further subdivided into membrane-bound versus soluble fractions, with the former being used to track the pool in exosomes. For statistics performed on the studies above, data were generated from three repeats, and error bars indicate SDs. $^{*}P < 0.05$ and $^{**}P < 0.01$.

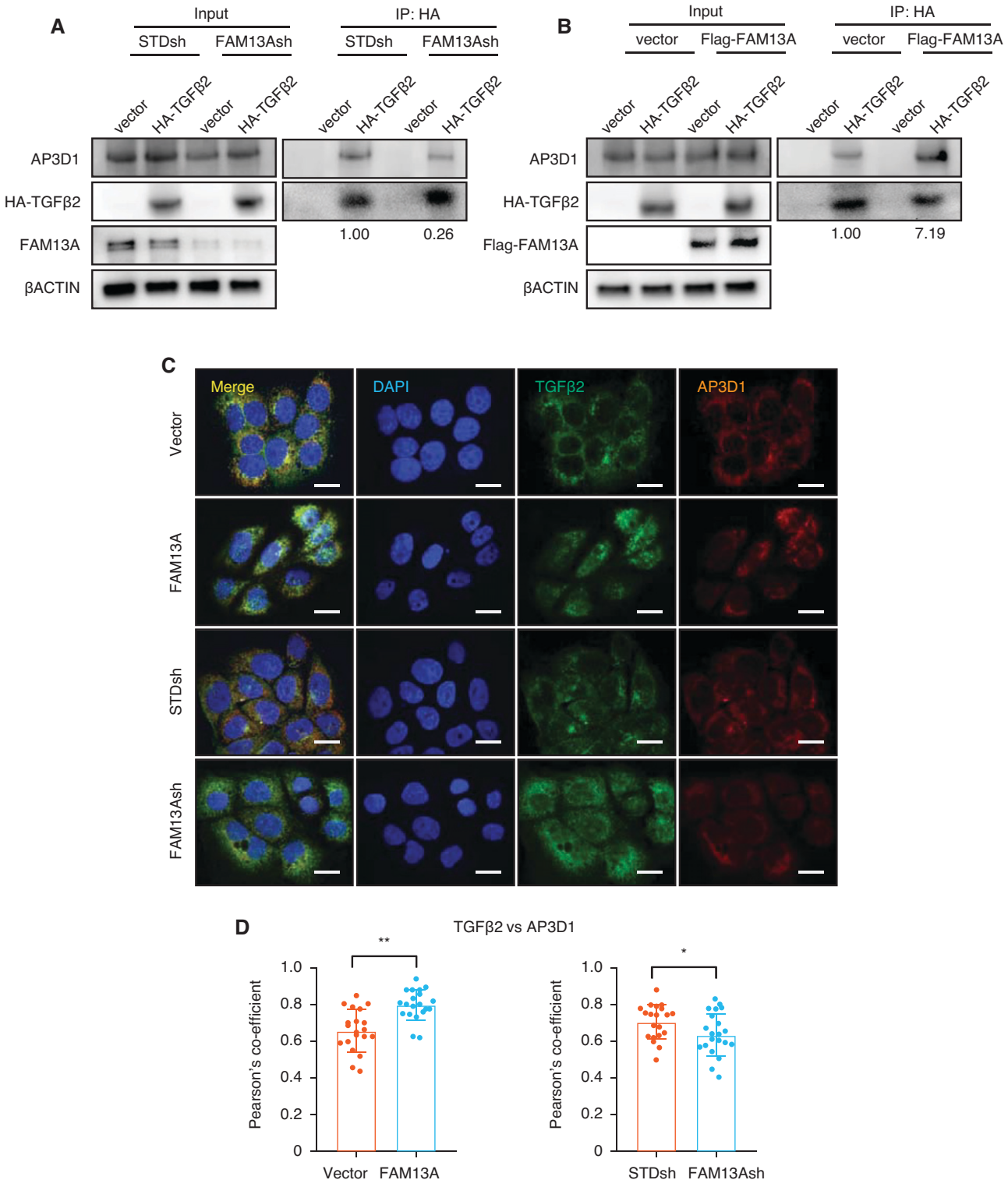

Figure 5. FAM13A stabilizes the interaction between TGFβ2 and the AP-3 complex. (*A*) 16HBE cells were infected with lentivirus expressing FAM13Ash or STDsh. Two days later, protein binding between HA-TGFβ2 and endogenous AP3D1 was assessed by IP. The numbers below the bands indicate the degree of association obtained by normalization to the level of the primary protein immunoprecipitated (HA-TGFβ2). (*B*) 16HBE cells were transfected with empty vector or FAM13A plasmid, together with HA-TGFβ2 plasmid. Two days later, protein binding between HA-TGFβ2 and endogenous AP3D1 was assessed by IP. (*C*) 16HBE cells were transfected with an empty vector/FAM13A or were stably transfected with FAM13Ash/STDsh lentivirally as indicated. Confocal microscopy was then performed to track the nucleus (blue), TGFβ2 (green), and AP3D1 (red). Scale bars, 20 μm. (*D*) The Pearson correlation coefficient was used to quantify the colocalization of TGFβ2 and AP3D1. Twenty cells were randomly picked from three images of each condition for analysis. Error bars indicate SDs. $^{*}P < 0.05$ and $^{**}P < 0.01$.

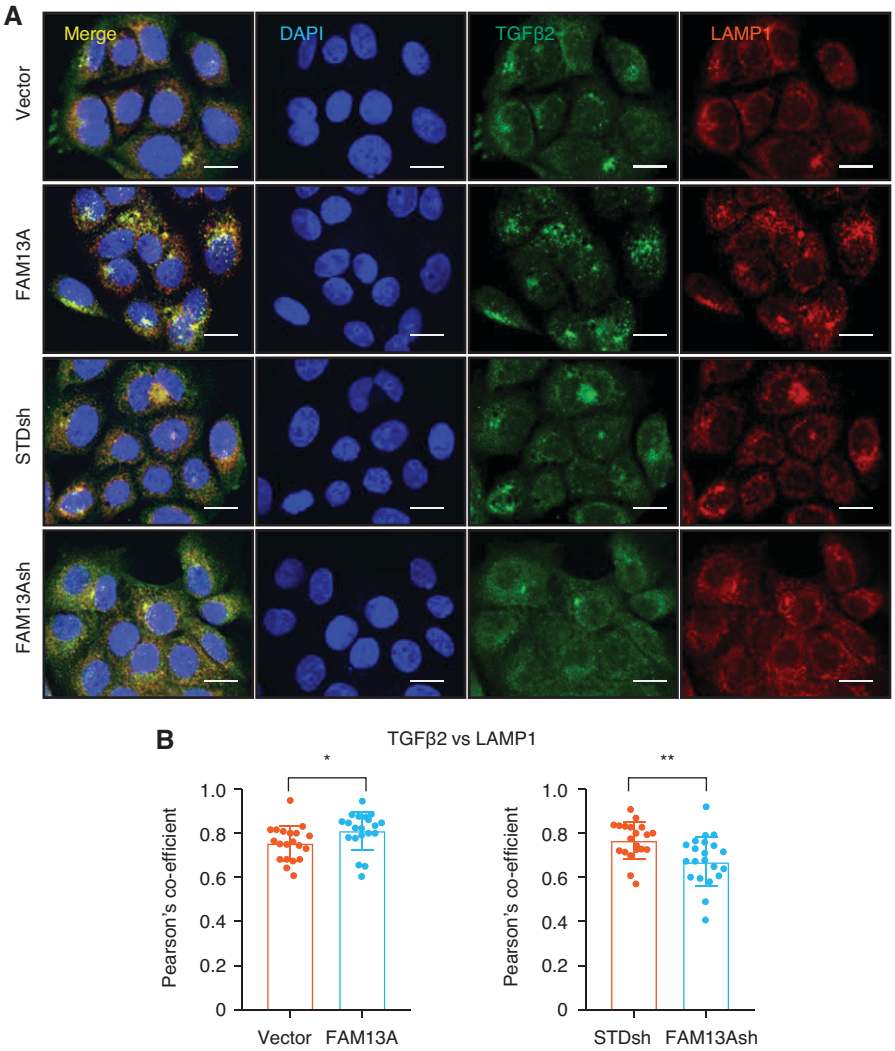

Figure 6. FAM13A enhances the distribution of TGFβ2 at the late endosomal compartments. (*A*) 16HBE cells were transfected with an empty vector/FAM13A-expressing plasmid or were stably transfected with FAM13Ash/STDsh lentivirally as indicated. Confocal microscopy was then performed to track the nucleus (blue), TGFβ2 (green), and LAMP1 (red). Scale bars, 20 μm. (*B*) The Pearson correlation coefficient was used to quantify the colocalization between TGFβ2 and LAMP1. Twenty cells were randomly picked from three images of each condition for analysis. Error bars indicate SDs. $^{*}P < 0.05$ and $^{**}P < 0.01$.

It is further notable that FAM13A was previously reported to be one of the top hits in an unbiased RNA interference screen to identify novel factors regulating the secretory pathway (38). Thus, an intriguing prospect is that the inhibitory role that we have elucidated for FAM13A in TGFβ2 transport could have more widespread relevance. Future pursuit of this possibility may identify additional proteins whose intracellular transport is regulated by FAM13A, thereby providing further insights into mechanisms that contribute to the pathogenesis of COPD.

We had previously found that FAM13A inhibits Wnt/β-catenin signaling (11). In the current study, we identified another function for FAM13A, which unifies the roles of two leading COPD GWAS genes. However, the precise meaning of this biological connection between FAM13A and TGFβ2 for COPD pathogenesis remains to be determined. We speculate that the regulation of exosomal TGFβ2 secretion by lung epithelial cells could play an important role in COPD susceptibility in response to cigarette smoke, potentially by influencing the development of chronic lung inflammation—which can persist for decades after smoking cessation (39). Further research to investigate the impact of the FAM13A–AP3D1–TGFβ2 network connection on lung inflammation, and the relationship of this cell trafficking effect with other FAM13A functions, such as Wnt/β-catenin signaling, will be required.

In summary, we have identified a potentially important biological connection between COPD GWAS genes via a novel mechanism of cargo transport through the AP3 pathway. Our results also suggest new questions to address for the future. For example, it is uncertain whether cell types other than bronchial epithelial cells have similar regulatory relationships between FAM13A and TGFβ2. Moreover, animal studies will provide an even more

physiological context for understanding our findings. It will also be interesting to determine whether our findings may have relevance to lung diseases other than COPD. In any case, we have demonstrated that protein–protein interaction network analysis can be used to identify potentially important biological relationships among GWAS genes in complex disease. Dissecting these relationships will not only provide new insights into disease mechanisms but will also suggest novel directions in therapeutic intervention. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Kochanek KD, Murphy S, Xu J, Arias E. Mortality in the United States, 2016. *NCHS Data Brief* 2017;(293):1–8.
2. Sakornsakolpat P, Prokopenko D, Lamontagne M, Reeve NF, Guyatt AL, Jackson VE, *et al.*; SpiroMeta Consortium; International COPD Genetics Consortium. Genetic landscape of chronic obstructive pulmonary disease identifies heterogeneous cell-type and phenotype associations. *Nat Genet* 2019;51:494–505.
3. Kheirallah AK, Miller S, Hall IP, Sayers I. Translating lung function genome-wide association study (GWAS) findings: New insights for lung biology. *Adv Genet* 2016;93:57–145.
4. Sharma A, Kitsak M, Cho MH, Ameli A, Zhou X, Jiang Z, *et al.* Integration of molecular interactome and targeted interaction analysis to identify a COPD disease network module. *Sci Rep* 2018;8:14439.
5. Cloonan SM, Glass K, Laucho-Contreras ME, Bhashyam AR, Cervo M, Pabón MA, *et al.* Mitochondrial iron chelation ameliorates cigarette smoke-induced bronchitis and emphysema in mice. *Nat Med* 2016;22:163–174.
6. Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 1997;277:2002–2004.
7. Lao T, Glass K, Qiu W, Polverino F, Gupta K, Morrow J, *et al.* Haploinsufficiency of hedgehog interacting protein causes increased emphysema induced by cigarette smoke through network rewiring. *Genome Med* 2015;7:12.
8. Cho MH, McDonald ML, Zhou X, Mattheisen M, Castaldi PJ, Hersh CP, *et al.*; NETT Genetics, ICGN, ECLIPSE and COPDGene Investigators. Risk loci for chronic obstructive pulmonary disease: a genome-wide association study and meta-analysis. *Lancet Respir Med* 2014;2:214–225.
9. Kaur-Knudsen D, Nordestgaard BG, Bojesen SE. CHRNA3 genotype, nicotine dependence, lung function and disease in the general population. *Eur Respir J* 2012;40:1538–1544.
10. Saccone NL, Culverhouse RC, Schwantes-An TH, Cannon DS, Chen X, Cichon S, *et al.* Multiple independent loci at chromosome 15q25.1 affect smoking quantity: a meta-analysis and comparison with lung cancer and COPD. *PLoS Genet* 2010;6:e1001053.
11. Jiang Z, Lao T, Qiu W, Polverino F, Gupta K, Guo F, *et al.* A chronic obstructive pulmonary disease susceptibility gene, FAM13A, regulates protein stability of β-catenin. *Am J Respir Crit Care Med* 2016;194:185–197.
12. Silverman EK, Schmidt HHHW, Anastasiadou E, Altucci L, Angelini M, Badimon L, *et al.* Molecular networks in network medicine: development and applications. *Wiley Interdiscip Rev Syst Biol Med* 2020;12:e1489.
13. Hobbs BD, de Jong K, Lamontagne M, Bossé Y, Shrine N, Artigas MS, *et al.*; COPDGene Investigators; ECLIPSE Investigators; LifeLines Investigators; SPIROMICS Research Group; International COPD Genetics Network Investigators; UK BiLEVE Investigators; International COPD Genetics Consortium. Genetic loci associated with chronic obstructive pulmonary disease overlap with loci for lung function and pulmonary fibrosis. *Nat Genet* 2017;49:426–432.
14. Hoang LL, Nguyen YP, Aspeé R, Bolton SJ, Shen YH, Wang L, *et al.* Temporal and spatial expression of transforming growth factor-β after airway remodeling to tobacco smoke in rats. *Am J Respir Cell Mol Biol* 2016;54:872–881.
15. Harrop CA, Gore RB, Evans CM, Thornton DJ, Herrick SE. TGF-β_2 decreases baseline and IL-13-stimulated mucin production by primary human bronchial epithelial cells. *Exp Lung Res* 2013;39:39–47.
16. Liu J, Tseu I, Wang J, Tanswell K, Post M. Transforming growth factor β2, but not β1 and β3, is critical for early rat lung branching. *Dev Dyn* 2000; 217:343–360.
17. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, *et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003;13:2498–2504.
18. Scardoni G, Tosadori G, Pratap S, Spoto F, Laudanna C. Finding the shortest path with PesCa: a tool for network reconstruction. *F1000 Res* 2015;4:484.
19. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 2001;305:567–580.
20. Sonnhammer EL, von Heijne G, Krogh A. A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc Int Conf Intell Syst Mol Biol* 1998;6:175–182.
21. Bailey TL, Elkan C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol* 1994;2: 28–36.
22. Baldwin AK, Simpson A, Steer R, Cain SA, Kielty CM. Elastic fibres in health and disease. *Expert Rev Mol Med* 2013;15:e8.
23. Mecham RP, Gibson MA. The microfibril-associated glycoproteins (MAGPs) and the microfibrillar niche. *Matrix Biol* 2015;47:13–33.
24. Castaldi PJ, Guo F, Qiao D, Du F, Naing ZZC, Li Y, *et al.* Identification of functional variants in the FAM13A chronic obstructive pulmonary disease genome-wide association study locus by massively parallel reporter assays. *Am J Respir Crit Care Med* 2019;199:52–61.
25. Parker MM, Hao Y, Guo F, Pham B, Chase R, Platig J, *et al.* Identification of an emphysema-associated genetic variant near *TGFB2* with regulatory effects in lung fibroblasts. *eLife* 2019;8:e42720.
26. Agustí A, Hogg JC. Update on the pathogenesis of chronic obstructive pulmonary disease. *N Engl J Med* 2019;381:1248–1256.
27. Robinson MS, Bonifacino JS. Adaptor-related proteins. *Curr Opin Cell Biol* 2001;13:444–453.
28. Nakatsu F, Ohno H. Adaptor protein complexes as the key regulators of protein sorting in the post-Golgi network. *Cell Struct Funct* 2003;28:419–429.
29. Corvol H, Rousselet N, Thompson KE, Berdah L, Cottin G, Foussigniere T, *et al.* FAM13A is a modifier gene of cystic fibrosis lung phenotype regulating rhoa activity, actin cytoskeleton dynamics and epithelial-mesenchymal transition. *J Cyst Fibros* 2018;17:190–203.
30. Eisenhut F, Heim L, Trump S, Mittler S, Sopel N, Andreev K, *et al.* FAM13A is associated with non-small cell lung cancer (NSCLC) progression and controls tumor cell proliferation and survival. *OncoImmunology* 2016;6: e1256526.
31. Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* 2014;30:255–289.
32. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* 2018;19:213–228.
33. Miyazono K, Olofsson A, Colosetti P, Heldin CH. A role of the latent TGF-beta 1-binding protein in the assembly and secretion of TGF-beta 1. *EMBO J* 1991;10:1091–1101.
34. Cheng XT, Xie YX, Zhou B, Huang N, Farfel-Becker T, Sheng ZH. Characterization of LAMP1-labeled nondegradative lysosomal and endocytic compartments in neurons. *J Cell Biol* 2018;217:3127–3139.
35. Ammann S, Schulz A, Krägeloh-Mann I, Dieckmann NM, Niethammer K, Fuchs S, *et al.* Mutations in AP3D1 associated with immunodeficiency and seizures define a new type of Hermansky-Pudlak syndrome. *Blood* 2016;127:997–1006.
36. Mohammed M, Al-Hashmi N, Al-Rashdi S, Al-Sukaiti N, Al-Adawi K, Al-Riyami M, *et al.* Biallelic mutations in AP3D1 cause Hermansky-Pudlak syndrome type 10 associated with immunodeficiency and seizure disorder. *Eur J Med Genet* 2019;62:103583.

37. Sharma A, Menche J, Huang CC, Ort T, Zhou X, Kitsak M, *et al.* A disease module in the interactome explains disease heterogeneity, drug response and captures novel pathways and genes in asthma. *Hum Mol Genet* 2015;24:3005–3020.
38. Simpson JC, Joggerst B, Laketa V, Verissimo F, Cetin C, Erfle H, *et al.* Genome-wide RNAi screening identifies human proteins with a regulatory function in the early secretory pathway. *Nat Cell Biol* 2012;14: 764–774.
39. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, *et al.* The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 2004;350: 2645–2653.