Slug/Snail2 is involved in the repression of proliferation genes by TGF-β in bronchial epithelial progenitor cells and is deregulated in abnormal epithelium

Chamseddine Ben Brahim[1,2], Charlotte Courageux[1,2], Ariane Jolly[3], Bérengère Ouine[4], Aurélie Cartier[4], Pierre de la Grange[3], Leanne de Koning[4], Pascale Leroy[1,2*]

[1] INSERM UMR1152, Physiopathology and Epidemiology of Respiratory Diseases, Paris, France

[2] Faculty of Medicine, Paris Diderot University, Bichat Campus, Paris, France.

[3] GenoSplice, Paris, France

[4] Institut Curie, PSL Research University, Department of Translational Research, RPPA platform, Paris, France

*Corresponding author

E-mail: pascale.leroy@inserm.fr (PL)

Abstract

Slug/Snail2 belongs to the Epithelial-Mesenchymal Transition (EMT)-inducing transcription factors that are involved in development and diseases. Slug is also highly expressed in normal adult stem/progenitor cells of several epitheliums, and in such is unique among these transcription factors. By comparing primary bronchial basal cells from normal subjects to those from subjects with Chronic Obstructive Pulmonary Disease (COPD), a respiratory disease in which subjects present many anomalies of their bronchial epithelium and higher levels of Transforming Growth Factor (TGF)-β in their lungs, in an air-liquid interface culture system that allows regenerating a bronchial epithelium similar to the one *in vivo*, we reveal that Slug has higher expression levels in basal/progenitor cells from COPD when in presence of TGF-β but that it does not repress the epithelial marker E-cadherin either in normal or in COPD cells, even when treated with TGF-β. To investigate Slug role in human primary bronchial basal/progenitor cells we performed loss of function experiments to determine Slug downstream genes and we characterized the impact of TGF-β on these genes. We show that Slug downstream genes are different in normal and COPD subjects. In particular, we identified a set of proliferation-related genes whose expression is decreased by TGF-β when cells are induced to differentiate, and that are among the genes repressed downstream of Slug in normal but not in COPD cells. In COPD the levels of expression of these genes are higher than in normal cells in presence of TGF-β □□□□

that present many anomalies in their bronchial epithelium this function of Slug is lost and Slug and proliferation genes are simultaneously but independently regulated by TGF-β.

Introduction

Slug (Snail2) belongs with Snail (Snail1) and Smuc (Snail3) to the Snail family of zinc finger transcription factors and has been studied essentially for its function as an inducer of Epithelial-Mesenchymal Transition (EMT) (1). Like other EMT-inducing transcription factors it is expressed in a wide variety of tissues and cell types, during embryogenesis and organ formation and in adult normal and pathological, and it is involved in EMT-related processes and overexpressed in numerous carcinoma. However, in contrast to other EMT-inducing transcription factors, Slug is expressed in adult stem/progenitor cells in normal epitheliums. Recently, it has been shown to control stem/progenitor cell growth during mammary gland morphogenesis (2) and to repress differentiation in epidermal progenitor cells (3). In the lung, Slug is expressed in spheroids of stem/progenitor cells isolated from human adult airway epithelia (4) and, by microarray analysis, it was found enriched in both mouse and human airway basal cells (5, 6).

Basal cells are the adult stem/progenitor cells of the airway epithelium: they can self-renew and/or differentiate to repair the epithelium after injury (7). These cells can be isolated from bronchial tissue and, when grown in an air-liquid interface (ALI) culture system, reconstitute the pseudostratified bronchial epithelium (8). In this system, basal cells keep the memory of the characteristics of their source tissue up to passage 3 to 4; e.g. when isolated from patients with Chronic Obstructive Pulmonary Disease (COPD), they reconstitute an epithelium with anomalies characteristic of COPD (9).

66 COPD is a respiratory disease characterized by a progressive and irreversible loss of
67 respiratory capacity, with subjects at stage GOLD (Global initiative for chronic Obstructive Lung
68 Disease) 1/A having mild symptoms and at GOLD 4/D having severe symptoms. One of the
69 characteristics of COPD subjects is a remodeling of the airways with, in particular, many
70 anomalies of the epithelium lining the airways such as basal cells hyperplasia, goblet cell
71 metaplasia or squamous metaplasia (10, 11). Cigarette smoke is the main cause of COPD with
72 80-90% of patient developing COPD being smokers. Several studies have shown that cells
73 exposed to cigarette smoke keep the memory of the exposure and establish a "field of injury" all
74 along the airway epithelium (12). The hypothesis is that the anomalies of the epithelium would
75 result from an imbalance in the basal/progenitor cell fate, with this imbalance progressively
76 worsening with the disease stage and leading to an increase of the extent of the anomalies. These
77 deregulations are thought to be irreversible, since the anomalies are maintained even after
78 subjects quit smoking (13). Noticeably, COPD subjects have an increased risk of lung cancer
79 compared to non-COPD smokers, in particular of squamous carcinoma in the proximal bronchus
80 (14, 15).

81 Our goal was to better understand Slug function in bronchial epithelium basal/progenitor
82 cells. We hypothesized that Slug is involved in basal/progenitor cell fate i.e. self-renewal and
83 differentiation, and, that its deregulation therefore leads to anomalies in the bronchial epithelium.
84 Slug is a transcription factor and we focused on determining the downstream genes that it,
85 directly or indirectly, regulates. The ALI cell culture system allows regenerating, from primary
86 basal cells, a bronchial epithelium with characteristics close to the in vivo source tissue, providing
87 a good model to characterize the genetic program that takes place during differentiation.

We used COPD subjects to compare with normal subjects as they present many anomalies in their bronchial epithelium that can be reproduced in the ALI cell culture system. Moreover, features of EMT have been reported in COPD airways (9, 16) and Transforming Growth Factor (TGF)-β is found at higher levels in COPD lung tissues (17). We also wanted to characterize the effects of TGF-β, since it has been shown to regulate Slug expression (18), to be, in certain conditions, an EMT-inducing factor (19), and to play a role in stem/progenitor cell fate (20), we also wanted to characterize its effects. We thus determines Slug expression and characterized its downstream genes in bronchial epithelial progenitor cells, comparing cells from normal and COPD subjects at the onset of differentiation in presence or absence of TGF-β.

Materials and methods

Study subjects and cell isolation

Human lung tissues, non-COPD (non-smokers (n=6) and smokers (n=6)) and COPD (smokers (n=6) and Ex-smokers (n=6)) (see S1 Appendix for characteristics), were obtained from patients undergoing lung lobectomy for peripheral lung carcinoma for removal of a primary lung tumor and who gave informed consent. The study was approved by the ethics committee of Paris Nord, IRB 00006477 Paris 7 University, France. COPD patients were diagnosed according to the GOLD guidelines. Lung tissues used in this study were dissected as far away as possible from the tumor. Primary human bronchial epithelial cells were isolated from a piece of large bronchus according to standard protocol.

Cell culture and ALI differentiation system

110 Primary human bronchial epithelial cells (Passage 1 or 2) were expanded on flasks coated
111 with collagen I (BD Biocoat) in bronchial epithelial growth medium (BEGM), composed of
112 bronchial epithelial basal medium (BEBM) supplemented with the SingleQuots kit (Lonza) and
113 incubated at 37°C in 5% de CO2. Cells were expanded and differentiated in ALI (Air Liquid
114 Interface condition) according to Fulcher et al (8) with the following modifications: Cells ≤90%
115 confluent were plated in BEGM at a density of 1.5 x 10^5 / cm2 on Transwell cell culture inserts
116 with a polyethylene (PET) membrane and 0.4 μm pores (Corning Costar) coated with Collagen
117 IV (Sigma). The next day, medium was replaced with ALI medium. Undifferentiated cells
118 correspond to cells submerged and at day 3 post-plating. Cells at the onset of differentiation
119 correspond to cells put in ALI condition (removal of the medium at the top of the culture) at day 3
120 post-plating and further cultured for 6 days. TGF-β1 (Peprotech) was added at a concentration of
121 1 ng/ml 2 days before the switch to ALI condition.
122

123 shRNA lentivirus transduction

124 Protocol for transduction was obtained from D. Bryant (21) and adapted as following:
125 Cells were plated at a density of 10^5/cm2 in BEGM on Transwell cell culture inserts with a PET
126 membrane and 0.4μm pores (Corning Costar) coated with Collagen IV (Sigma), and allow to
127 adhere 3-4 h at 37˚C, 5% CO2. shRNA lentiviral transduction particles from Mission RNAi
128 TRC2 corresponding to SNAI2 specific or control non-targeting sequences (see S2 Appendix for
129 sequence) were added on top of the cells at a MOI of 10. Inserts were swirled and placed O.N. at
130 37˚C, 5% CO2. Medium was diluted by half with BEGM and insert placed back at 37˚C, 5% CO2
131 for 1 day. Medium was replaced with ALI medium. At day 4 post-transduction, cells were

132 changed to ALI conditions. Cells were lysed for RNA or protein analysis at day 6 post-
133 transduction.
134

135 Immunofluorescence staining of cell layers

136 All steps were done on a rocking table. Cells on inserts were rinsed with phosphate buffer
137 saline (PBS)+ 1x, fixed with cold 4% paraformaldehyde in PBS+ for 10 min at 4°C, then
138 permeabilized with 0.2% Triton-X100 in PBS for 10 min. For staining, blocking, incubations and
139 washing were done with PFS (0.7% fish gelatin (Sigma) and 0.025% saponin in PBS 1x). Cells
140 were first blocked with PFS for 30 min at room temperature, then incubated with primary
141 antibodies at 4°C overnight. After extensive wash with PFS, cells were incubated at R.T. for 1 h
142 with the corresponding Alexa Fluor conjugated secondary antibody at a dilution of 1:1000, then
143 washed extensively. Cells were quickly rinsed twice with PBS 1x, adding Hoescht 33342 at 1
144 μg/ml in the first wash. Filters with the cells were cut out and mounted between a glass slide and
145 a coverslip using ProLong gold (Invitrogen). Slides were left a minimum of 48 h at room
146 temperature before observation under the microscope. Slides were frozen at - 20˚C for long term
147 storage. (see S3 Appendix for antibody references).
148

149 RNA extraction

150 Cells were rinsed with phosphate buffer saline (PBS) before to be homogenized in RNA
151 lysis buffer (NucleoSpin RNA Kit, Macherey-Nagel), supplemented with 1% □-
152 Mercaptoethanol. Lysates were vortexed and proceeded immediately or snapped Frozen and
153 stored at -80˚C. Total RNA was extracted on column using the NucleoSpin RNA Kit (Macherey-

154 Nagel). DNase I treatment was done directly on the column. RNA concentration was determined
155 using a NanoDrop.
156

157 cDNA synthesis

158 For each sample, 0.3 μg of total RNA was reverse transcribed as previously described
159 (22) with the following modifications: Briefly, total RNA was annealed with 0.1 mg/ml
160 oligo(dT)15 primer (Promega) and cDNA synthesis was performed with M-MLV Reverse
161 Transcriptase (Promega) for 1 h at 42˚C. A control without reverse transcriptase was done for
162 each series of cDNAs.
163

164 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

165 Quantitative PCR was done on the QuantStudio 6 Flex with the QuantStudio Real-Time
166 PCR software using SYBR green PCR master mix (Applied BioSystems). Primers (see S2
167 Appendix for primer sequences) were designed to be used with an annealing temperature of 60°C
168 and an elongation time of 1 min. For a given gene target, cDNA volume used was chosen to be in
169 a Ct range from 20 to 30, using 0.125 μM each forward and reverse primer. Glyceraldehyde-3-
170 Phosphate Dehydrogenase (GAPDH) was used to normalize for cDNA amounts between
171 samples.
172

173 Protein extraction

174 Cells were rinsed with cold Tris 20 mM pH 7.5 and lysed directly on inserts with RIPA
175 buffer (Tris pH 7.5, 50 mM; NaCl, 150 mM, NP-40 (leg10) or Triton X-100, 1%: Sodium
176 deoxycholate, 1%; SDS 0.1%). Cells were scrapped and lysates were placed on a wheel for 30
177 min at 4˚C. DNA was sheared with a needle and lysates spun down at 14K for 10 min at 4˚C.
178 Protein concentration in the lysates was determined using BCA protein assay (Pierce). Lysates
179 were proceed immediately or snapped frozen and stored at -80˚C.
180

181 **Reverse Phase Protein Array (RPPA)**

182 RPPA was done by the RPPA platform at Curie Institute on samples prepared in RIPA
183 buffer (as described in protein extraction) and stored at -80°C. RPPA was done as following:
184 Protein concentration was determined (Pierce BCA reducing agent compatible kit, ref 23252).
185 Samples were printed onto nitrocellulose covered slides (Supernova, Grace Biolabs) using a
186 dedicated arrayer (2470 arrayer, Aushon Biosystems). Five serial dilutions, ranging from 1000 to
187 62.5 µg/ml, and four technical replicates per dilution were printed for each sample. Arrays were
188 labeled with 32 specific antibodies, of which 8 were described in this study (see S3 Appendix for
189 antibody references) or without primary antibody (negative control), using an Autostainer Plus
190 (Dako). Briefly, slides were incubated with avidin, biotin and peroxydase blocking reagents
191 (Dako) before saturation with TBS containing 0.1% Tween-20 and 5% BSA (TBST-BSA). Slides
192 were then probed overnight at 4°C with primary antibodies diluted in TBST-BSA. After washes
193 with TBST, arrays were probed with horseradish peroxidase-coupled secondary antibodies
194 (Jackson ImmunoResearch Laboratories, Newmarket, UK) diluted in TBST-BSA for 1 h at RT.
195 To amplify the signal, slides were incubated with Bio-Rad Amplification Reagent for 15 min at
196 RT. The arrays were washed with TBST, probed with IRDye 800CW Streptavidin (LiCOR)

197 diluted in TBST-BSA for 1 h at RT and washed again in TBST. For staining of total protein,
198 arrays were incubated 30 min Super G blocking buffer (Grace Biolabs), rinsed in water, incubated
199 5 min in 0,000005% Fast green FCF (Sigma) and rinsed again in water. The processed slides
200 were dried by centrifugation and scanned using a Innoscan 710-AL microarray scanner
201 (Innopsys). Spot intensity was determined with MicroVigene software (VigeneTech Inc). All
202 primary antibodies used in RPPA have been previously tested by Western Blotting to assess their
203 specificity for the protein of interest. Raw data were normalized using Normacurve (23), which
204 normalizes for fluorescent background per spot and a total protein stain.

205

206 Gene expression profiling

207 Microarray analysis was performed on biological triplicate samples. Total RNA were
208 amplified and labeled before hybridization onto Affymetrix human Gene 2.1 ST GeneChip
209 according the manufacturer, by the Genomics platform at Curie Institute, Paris (24). Array
210 datasets were controlled using Expression console (Affymetrix) and further analyses and
211 visualization were made using EASANA (GenoSplice, www.genosplice.com), which is based on
212 the FAST DB annotations (25, 26) Gene Array data were normalized using quantile
213 normalization. Background corrections were made with antigenomic probes and probes were
214 selected as described previously (27). Only probes targeting exons annotated from FAST DB
215 transcripts were selected to focus on well-annotated genes whose mRNA sequences are in public
216 databases (25, 26). Bad-quality selected probes (e.g., probes labeled by Affymetrix as 'cross-
217 hybridizing') and probes whose intensity signal was too low compared to antigenomic
218 background probes with the same GC content were removed from the analysis. Only probes with
219 a DABG P value ≤ 0.05 in at least half of the arrays were considered for statistical analysis (27).

220 Only genes expressed in at least one compared condition were analyzed. To be considered
221 expressed, the DABG P value had to be ≤ 0.05 for at least half of the gene probes. We performed
222 an unpaired Student's t-test to compare gene intensities in the different biological replicates.
223 Genes were considered significantly regulated when fold-change was ≥ 1.2 and P value ≤ 0.05.
224 Significant KEGG pathways (28), REACTOME pathways (29) and GO terms were retrieved
225 using DAVID (30) from union of results of all, up- and down-regulated genes separately. Data set
226 GEO ID numbers are GSE122957 and GSE123129.
227

228 **Image capture and analysis**

229 Images of cell layers stained by immunofluorescence were captured using a SP8 Leica
230 confocal microscope equipped with a X 40 objective. The following excitation sources where
231 used: a diode 405-nm, an argon laser 488-nm, a diode 561-nm, a diode 633-nm. Detectors were
232 PMT and HyD. Digital images were analyzed using ImageJ software.
233

234 **Statistical Analysis**

235 Biological replicates were n ≥ 11 and data generated by at least 2 independent
236 experiments. For RNA or protein levels, mean is ±SD and statistical analysis was carried out by
237 parametric paired or unpaired two-sided t-test as appropriate. For fold-change, mean is ±SEM and
238 statistical analysis was carried out by a one sample two-sided t-test. Correlations were computed
239 as Pearson correlation coefficients and P value determined by two-sided test. Significance was
240 accepted when P value < 0.05.
241

Results

Slug/Snail2 is the only EMT-inducing transcription factor highly expressed in basal cells and it co-expresses with epithelial and mesenchymal markers

Slug/Snai2 gene has been found by microarray analysis to be highly expressed in basal cells of the human airway epithelium (5, 6). To understand the role of Slug in these stem/progenitor cells, we used primary basal cells isolated from human bronchial epithelium and first characterized these cells for the expression of Slug.

We confirmed by immunocytochemistry that primary basal cells grown at confluence and maintained undifferentiated are all progenitor cells as shown by the expression of the marker p63, and that they all express Slug that co-localizes with p63 in their nuclei **(Fig 1A).** Similar expression profiles were obtained with cells from COPD subjects **(S1 Fig).** Slug is an EMT-inducing transcription factor and we also determined the expression of other transcription factors with this property. While Slug is highly expressed, the expression of Snail1 and Zeb1 is close to background and Twist1 does not exceed 10% of Slug levels, and this in both normal and COPD cells **(Fig 1B).** To determine the epithelial status of these progenitor cells, we studied the expression of epithelial and mesenchymal EMT-related markers. We found that among the genes coding for junction proteins, the epithelial marker E-cadherin (E-cad/CDH1) is expressed while N-cadherin (N-cad/CDH2), an EMT-related mesenchymal marker, is not expressed **(Fig 1C).** We also observed a high level of expression of the genes coding for the cytoskeletal proteins cytokeratin 5 (KRT5) and vimentin (Vim), respectively an epithelial and a mesenchymal marker,

263 as well as expression of ACTA2, the gene coding for the mesenchymal marker α-smooth muscle
264 actin (a-sma). (**Fig 1D**). In conclusion, both COPD and normal epithelial progenitors co-express
265 epithelial and mesenchymal markers.

266 **Fig 1. Bronchial epithelial progenitors express Slug in their nuclei and co-express epithelial and mesenchymal**
267 **markers.**

268 Primary bronchial epithelial basal cells were grown on filters and analyzed undifferentiated at confluence, either by
269 fluorescent immunocytochemistry (A) or by RT-qPCR (B-D). (A) Normal cells were fixed and labeled
270 simultaneously with progenitor cell marker p63 (white) and Slug (green) antibodies and with Hoechst as a marker of
271 nuclei (blue). Bars are 20 µm. (B-D) RNA were extracted from normal and COPD cells and analyzed by RT-qPCR.
272 GAPDH was used to normalize cDNA amounts between samples and results were calculated as a ratio on GAPDH.
273 Data are for n ≥ 11 and experiments were done at least in duplicate. (B) Expression of the EMT-inducing
274 transcription factors Snai1, Twist1 and Zeb1 shown as a percentage of Slug mRNA, with mean ±SEM (C)
275 Expression of the mesenchymal marker N-cad/CDH2 shown as a percentage of the epithelial marker E-cad/CDH1
276 mRNA, with mean ±SEM (D) mRNA levels of KRT5 gene, an epithelial cytoskeletal marker and ACTA 2 and Vim
277 genes, mesenchymal cytoskeletal markers. Results shown are log2 (ratio on GAPDH) and are presented as a scatter
278 plot with the mean ±SD. ns: non significant.

279

280 Slug/Snail2 expression is increased in COPD cells when compared to
281 normal cells at the onset of differentiation in presence of TGF-β.

282 We then determined the expression of Slug and the EMT-related markers in cells induced
283 to differentiate using the ALI cell culture system that allows basal/progenitor cells to differentiate
284 into a characteristic bronchial pseudostratified epithelium. The complete differentiation takes 3 to
285 4 weeks in this system. However, we were interested in the onset of the genetic differentiation
286 program and therefore studied the early timepoints. TGF-β can play a role in stem/progenitor cell
287 fate (20); it also regulates Slug expression, is a potential EMT-inducing factor and is expressed at

288 higher levels in COPD airways compared to normal (17, 18). We thus compared the expression of
289 Slug and the EMT-related markers at the onset of differentiation in absence or presence of TGF-β
290 to determine if progenitor cells from normal and COPD responded differently to TGF-β. We used
291 low concentration of TGF-β (1ng/ml) to be in conditions similar to the physiological range.

292 TGF-β significantly increases Slug mRNA expression while its effect on the expression of
293 E-cad/CDH1 is not statistically significant. Slug remains the most highly expressed EMT-
294 inducing transcription factor and among the cell-cell junction markers, E-cad/CDH1 is still the
295 most expressed when compared to the mesenchymal marker N-cad/CDH2. Similar effects are
296 found for both normal and COPD cells **(Fig 2 A-B and D-E)**. Immunocytochemistry shows that
297 while Slug is expressed in the nuclei and E-cad is mainly expressed at the cell-cell junctions in
298 cells at the onset of differentiation, TGF-β leads to delocalization of E-cad to the cytoplasm, with
299 similar profiles of expression being observed in normal and COPD cells **(S2A-D Fig).** Among the
300 cytoskeletal markers, Vim is highly upregulated by TGF-β, while KRT5 and ACTA2 levels do
301 not change significantly. Similar effects are observed in normal and COPD cells **(Fig 2C).**

302 **Fig 2. Effect of TGF-β on Slug and EMT-associated markers in normal and COPD bronchial epithelial**
303 **progenitors.**

304 Primary bronchial epithelial basal cells, normal and COPD, were grown on filters and at confluence changed to ALI
305 culture to induce differentiation, without TGF-β or in presence of 1ng/ml of TGF-β. Cells were analyzed at day 6 of
306 ALI culture for mRNA expression. RNA were extracted from normal and COPD cells and analyzed by RT-qPCR.
307 GAPDH was used to normalize cDNA amounts between samples and results were calculated as a ratio on GAPDH.
308 Data shown represent the mean for n $\geq$ 11, and experiments were done at least in duplicate. (A-C) Results are
309 presented as the fold-change induced by TGF-β on mRNA expression of Slug (A), E-cad/CDH1 (B) and genes
310 coding for cytoskeletal proteins (C) with mean ±SEM, and compare normal and COPD cells. (D) Expression of the
311 EMT-inducing transcription factors Snail1, Twist1 and Zeb1 shown as a percentage of Slug mRNA, with mean
312 ±SEM. (E) Expression of the mesenchymal marker N-cad/CDH2 shown as a percentage of the epithelial marker E-

313 cad/CDH1 mRNA, with mean ±SEM. Statistical significance is at P value < 1.00E-03 *** as indicated. ns: non
314 significant.

315 In response to TGF-β, the resulting levels of Slug mRNA are similar in normal and COPD
316 cells, and this is also the case for E-cad/CDH1 and Vim mRNA (**Fig 3A)**. However, when we
317 determined the expression at the protein level by Reverse Phase Protein Array (RPPA), which
318 allows studying all the samples simultaneously, thus, reducing variability due to technical bias
319 (31), we found that TGF-β leads to significantly higher levels of Slug in COPD cells. E-cad
320 protein levels are also higher in COPD in presence of TGF-β, while Vim levels do not differ
321 significantly between normal and COPD cells **(Fig 3B**). Both in normal and COPD cells, and in
322 absence or presence of TGF-β, a strong positive correlation between Slug and E-cad protein
323 levels is observed (**Fig 3C).** These results indicate that Slug is deregulated in COPD
324 basal/progenitor cells at the onset of differentiation in presence of TGF-β.

325 **Fig 3. Slug, E-cad/CDH1 and Vim expression in normal and COPD bronchial epithelial progenitors.**
326 Primary bronchial epithelial basal cells, normal and COPD, were grown on filters and at confluence changed to ALI
327 culture to induce differentiation, without TGF-β or in presence of 1ng/ml of TGF-β. Cells were analyzed at day 6 of
328 ALI culture either for mRNA expression (A) or for protein expression (B-C). (A) Comparison of the mRNA levels in
329 normal and COPD cells in presence of TGF-β of Slug, E-cad/CDH1 and Vim. RNA were extracted from normal and
330 COPD cells and analyzed by RT-qPCR. GAPDH was used to normalize cDNA amounts between samples and results
331 were calculated as a ratio on GAPDH. Data are for n ≥ 11 with experiments done at least in duplicate and results are
332 shown as log2 (ratio on GAPDH) and presented as a scatter plot with the mean ±SD. (B-C) Comparison of the
333 protein levels in normal and COPD cells in absence or presence of TGF-β, of Slug, E-cad and Vim. Proteins lysates
334 were prepared from normal and COPD cells and analyzed by RPPA. Data shown are for n ≥ 11. (B) Protein levels are
335 shown as log2 (values) and are presented as scatter plots with the mean ±SD. (C) Correlations between Slug and E-
336 cad protein levels in absence or presence of TGF-β for normal and COPD cells. Results are Pearson correlations
337 calculated with log2 (expression levels) and are presented as scatter plots with a regression line. Statistical
338 significance is at P value < 1.00E-02 ** or < 1.00E-03 *** as indicated. ns: non significant.

339

340 **Microarray analysis of Slug knockdown identifies genes involved in**
341 **proliferation that are repressed downstream of Slug in normal but**
342 **not COPD cells**

343 We found that Slug responds differently to TGF-β in normal and COPD bronchial
344 progenitor cells and that it does not repress E-cad in either cell type. To better understand Slug
345 function in these progenitor cells, we wanted to identify the genes downstream of Slug and to
346 determine if these genes were different between normal and COPD cells. It has been reported that
347 the expression level of Slug determines the differentiation status of keratinocytes (3), and we
348 chose a Slug knockdown approach rather than an overexpression that could also change Slug
349 specificity, therefore its downstream genes, in our cell model. We knocked down Slug in normal
350 and COPD cells using shRNA from Mission RNAi that were validated and selected the most
351 efficient shRNA that was within the coding strand in order to limit non-specific downstream
352 genes (32). To determine the genes whose expression was modified by Slug knockdown
353 compared to a control siRNA, we performed a total RNA microarray analysis. We used an
354 Affymetrix human Gene 2.1 ST GeneChip, that allows to test >30,000 coding transcripts and
355 11,000 long intergenic non-coding transcripts.

356 Slug Knockdown resulted in a statistically significant decrease of Slug mRNA with
357 knockdown levels determined by RT-qPCR of 43% (fold-change 1.77, P value <1.00E-04) and
358 54% (fold-change 2.17, P value 4.20E-03), respectively for normal and COPD cells. Slug
359 knockdown level was low, however within the range expected for all Mission RNAi validated
360 Slug sequences. Microarray analysis gave lower fold-change for Slug: 1.48 (P value 1.36E-02)

361 and 1.39 (P value 8.96E-03), respectively for normal and COPD samples, and for this reason we
362 chose to set the minimum fold-change threshold at 1.2, instead of 1.5 usually used, for selecting
363 genes among those with a statistically significant change of expression (P value ≤5.00E-02). We
364 focused on the genes in normal cells with an expression significantly changed according to these
365 criteria. **A**mong the 805 genes identified downstream of Slug in normal cells, there is a majority
366 (514/63.9%) of upregulated, i.e. genes that would be repressed directly or indirectly by Slug. This
367 is coherent with Slug being a repressor (33, 34). Since we found that Slug expression is
368 deregulated in COPD cells at the onset of differentiation in presence of TGF-β, we were
369 particularly interested in genes responding to differentiation and TGF-β. To identify these genes,
370 we also performed a microarray analysis on RNA from basal cells at the onset of differentiation,
371 in presence or absence of TGF-β, using undifferentiated cells as control. **S1 Table** is the list of
372 the 514 genes upregulated in normal cells with their fold change in normal and COPD cells
373 knocked down for Slug as well as their respective response to differentiation or TGF-β. Setting
374 the minimum fold-change at 1.2, we found that 398 of these genes (77.4%, a large majority)
375 respond to differentiation and/or TGF-β and, among these 193 (48.5%) respond to both. We
376 selected the genes responding to both differentiation and TGF-β and classified them in 4 groups
377 according to their combined type of response. **Fig 4A** shows histograms representing the number
378 of genes for each group and small bars indicating the mean fold-increase induced by Slug
379 knockdown for these genes in normal (blue) and COPD (red) cells. The large majority of the
380 genes are repressed by TGF-β and among these, genes that are upregulated during differentiation
381 have a similar mean fold-increase induced by Slug knockdown in normal and COPD cells. In
382 contrast, the 68 genes that are downregulated during differentiation have a much lower mean
383 fold-increase in COPD than in normal cells. These genes are thus repressed by Slug upon

384 differentiation ot TGFbeta in normal cells, but this repression is lost in COPD cells. A search for
385 enriched gene pathways using KEGG, REACTOME and Gene Ontology (GO) databases revealed
386 that 45 out of these 68 genes are involved in processes related to proliferation or cell cycle. **Table**
387 **1** shows that all these 45 genes, except for 3, are much less or not repressed downstream of Slug
388 in COPD compared to normal cells.

389 We further studied the expression of 5 of these proliferation-related genes and first
390 confirmed by RT-qPCR the difference in the fold change between normal and COPD cells; we
391 also studied E-cad/CDH1, cytokeratin 5/KRT5 and Vim/VIM genes that were not among the 514
392 genes significantly increased by Slug knockdown in normal cells. **Fig 4B** shows the correlation
393 between microarray and RT-qPCR values for these 8 genes in both normal and COPD cells. The
394 strong correlation (r^2 values of 0.9318 and 0.8671, respectively for normal and COPD), validates
395 the microarray analysis and also confirms that the 5 proliferation-related genes are upregulated by
396 Slug knockdown in normal but not COPD cells. No significant effect of Slug knockdown is seen
397 on CDH1 and KRT5 expression, while VIM expression decreases significantly and similarly for
398 normal and COPD. This confirms that CDH1/E-cad is not a target of Slug in bronchial progenitor
399 cells from both normal and COPD. KRT5 is also not regulated downstream of Slug, while in
400 contrast, VIM is induced downstream of Slug and similarly in cells from normal and COPD
401 subjects.

402 **Table 1. List of 45 proliferation genes upregulated by Slug knockdown in normal bronchial**
403 **basal/progenitor cells.**

	KD Normal (Mean)		KD COPD (Mean)	
Gene Symbol	**Fold-Change (log2)**	**P-value**	**Fold-Change (log2)**	**P-value**
DTL	0.98	1.06E-02	0.11	3.05E-01
NCAPH	0.93	4.21E-02	-0.06	8.26E-01
CCNA2	0.87	4.62E-03	0.12	4.06E-01
UHRF1	0.87	6.60E-03	-0.04	8.24E-01
ASPM	0.86	1.57E-02	0.12	1.80E-01
FOXM1 // NRIP2	0.83	3.41E-02	0.11	4.89E-01
MCM10	0.77	3.66E-02	0.65	4.10E-02
FAM83D	0.75	1.40E-04	0.21	5.20E-01
CKAP2L	0.75	6.06E-03	-0.11	6.37E-01
FBXO5	0.72	4.98E-02	0.01	8.72E-01
CASC5	0.71	3.04E-02	0.31	1.94E-01
TYMS	0.68	3.04E-02	0.00	9.90E-01
PCNA	0.67	3.02E-03	0.26	3.54E-02
PRC1	0.65	4.02E-03	0.07	6.53E-01
MKI67	0.64	4.47E-02	-0.08	5.01E-01
CEP55	0.61	1.02E-02	-0.16	3.03E-01
CENPE	0.60	3.02E-02	0.18	5.11E-01
MIS18A	0.59	2.23E-02	0.26	4.04E-01
FANCI	0.59	2.26E-02	0.08	6.87E-01
GPSM2	0.54	1.16E-03	0.20	1.45E-01
BORA	0.54	3.05E-02	0.15	5.95E-01
CENPF	0.54	9.86E-03	-0.10	4.00E-01
DLGAP5	0.54	2.38E-02	-0.19	2.71E-01
AURKB	0.51	5.20E-04	0.33	1.15E-01
KIF20A	0.51	2.55E-02	0.11	4.57E-01
KIF20B	0.51	2.56E-02	-0.01	9.41E-01
UBE2C	0.50	2.10E-02	0.32	3.89E-01
CDC20	0.49	3.58E-03	0.07	7.63E-01
CCNB1	0.48	4.94E-02	-0.03	6.89E-01
GINS1	0.48	1.35E-02	-0.08	7.93E-01
FANCD2	0.46	6.88E-03	0.25	2.54E-02
SKA2	0.44	2.55E-02	0.11	5.64E-01
HELLS	0.43	3.01E-02	-0.11	1.53E-01
CKS2	0.43	3.29E-02	-0.15	4.78E-01
NASP	0.41	3.34E-02	0.12	3.31E-02
ALDH3A1	0.40	1.53E-02	1.12	1.46E-03
FEN1	0.38	2.94E-02	0.37	1.06E-01
RFC4	0.37	4.18E-02	0.11	6.10E-01
HIST2H4A // HIST2H4B	0.34	1.02E-03	0.49	2.03E-01
HJURP	0.32	1.72E-02	0.10	7.84E-01
CDKN3	0.32	3.37E-02	-0.04	5.49E-01
PIR // VEGFD	0.30	4.13E-02	0.10	5.06E-01
PRIM1	0.30	3.66E-03	-0.10	4.67E-01
RNASEH2A	0.29	1.09E-02	0.04	5.42E-01
CDCA7L	0.28	1.14E-02	-0.06	6.18E-01

404 Genes statistically significant upregulated by Slug knockdown in normal cells and downregulated by both
405 differentiation and TGF-β.
406 Genes were selected for a log2 (fold-Change) ≥ 0.26. Statistical significance is at P value < 5.00E-02
407 Comparison with fold-change for Slug knockdown in COPD cells.
408

409 **Fig 4. Microarray analysis of Slug knockdown identifies proliferation genes repressed in normal**

410 **bronchial epithelial progenitors.**

411 Knockdown with shRNA specific of Slug were performed on primary bronchial epithelial basal cells, normal and
412 COPD, Knockdown cells were grown on filters and, at confluence, changed to ALI culture to induce differentiation.
413 Cells were analyzed at day 2 of ALI culture for mRNA expression. RNA were extracted from normal and COPD
414 cells and analyzed by microarray analysis on an Affymetrix chip. Data are for n $\geq$ 3 each, normal and COPD. (A)
415 Genes significantly upregulated by Slug knockdown in normal cells and responding to both differentiation and TGF-
416 β were classified in 4 groups according to their response to differentiation and TGF-β. Histograms represent the
417 number of genes in each group with genes upregulated by TGF-β in red and genes downregulated by TGF-β in green,
418 and genes upregulated during differentiation as checkboard and genes downregulated during differentiation as
419 oblique lines. Horizontal bars represent the mean fold-increase for each group of genes, in blue for normal cells and
420 in red for COPD cells. (B) Validation of microarray by RT-qPCR and comparison of Slug knockdown effect in
421 normal (blue) and COPD (red) cells for CDH1, KRT5 and VIM genes as well as proliferation-related genes. Results
422 are Pearson correlations calculated with log2 (fold-change) and are presented as scatter plots with regression line and
423 R-square values.
424
425

426 TGF-β represses the expression of proliferation-related genes in 427 progenitor cells at the onset of differentiation and Slug is involved in 428 this repression in normal but not in COPD cells.

429 We found a set of proliferation-related genes that have an increased expression following
430 Slug knockdown in normal but not in COPD cells; this suggests that these genes are directly or
431 indirectly repressed by Slug in normal cells but that this regulation does not take place in COPD.
432 This is supported by the strong and statistically significant inverse correlation between Slug and
433 the mRNA levels of these genes that is observed in undifferentiated normal but not COPD cells
434 (**Table 2**). Moreover, in normal cells, in addition to Ki67 mRNA, a strong inverse correlation also

435 exists between Slug and Ki67 protein, a widely used marker strictly related to proliferation (35)
436 **(Fig 5A).** These correlations are found with Slug mRNA and not with the protein.

437 **Table 2. Correlation between proliferation genes and Slug in normal and COPD**
438 **undifferentiated basal cells**

	Normal		COPD	
	Slug mRNA	**Slug Protein**	**Slug mRNA**	**Slug Protein**
PRC1 mRNA	**- 0.7331** (*6.70E-03*)	0.3264 (*3.27E-01*)	- 0.2567 (*4.46E-01*)	0.3178 (*3.41E-01*)
FOXM1 mRNA	**- 0.7680** (*3.50E-03*)	0.3295 (*3.23E-01*)	- 0.3640 (*2.71E-01*)	0.2530 (*4.53E-01*)
CCNA2 mRNA	**- 0.7615** (*4.00E-03*)	0.3514 (*2.89E-01*)	- 0.1772 (*6.02E-01*)	0.2739 (*4.15E-01*)
PCNA mRNA	**- 0.7365** (*6.30E-03*)	0.3437 (*3.01E-01*)	0.0286 (*9.33E-01*)	0.0519 (*8.80E-01*)
KI67 mRNA	**- 0.7322** (*6.80E-03*)	0.3329 (*3.17E-01*)	- 0.1092 (*7.49E-01*)	0.0833 (*8.08E-01*)

439 Pearson correlation coefficients and associated P value between expression levels.
440 Computation was performed on log2 of values.
441 Statistical significance was determined with a two-sided t-test. Correlations are significant at P value <
442 5.00E-02.
443
444 At the expression level in undifferentiated cells, only Ki67 mRNA is statistically
445 significant higher in COPD when compared to normal cells, and other proliferation-related genes
446 show only a tendency in the same direction (**Fig 5B)**. However, in cells at the onset of
447 differentiation in presence of TGF-β, significant lower levels are found in normal cells including
448 for Ki67 protein, with the exception of PCNA that shows only a weak not statistically significant
449 tendency (**Fig 5C)**. TGF-β decreases the expression of these proliferation-related genes and its
450 mean effect is in accordance with the mean difference of their expression levels between normal
451 and COPD cells (**Fig 5D)**.
452 To determine the implication of Slug in the repression of the proliferation-related gene
453 expression by TGF-β in normal cells, we analyzed the correlation between Slug and these genes.
454 **Table 3 and Fig 5E** show that in normal cells, there are significant inverse correlations between
455 the combined effects of differentiation and TGF-β on Slug and the proliferation-related gene
456 mRNA levels, except for PCNA. A good tendency for an inverse correlation is also found for
457 Ki67 protein. No such inverse correlations are found in COPD cells, showing that Slug is

458 involved in the repression of the proliferation-related gene expression by TGF-β in normal cells
459 but that this function of Slug is lost in COPD cells.

460 **Table 3. Correlation between the effect of differentiation and TGF-β on proliferation genes and on**
461 **Slug in normal and COPD basal/progenitor cells**
462

	Normal		COPD	
	Slug mRNA	**Slug Protein**	**Slug mRNA**	**Slug Protein**
PRC1 mRNA	**- 0.6983 (*1.15E-02*)**	- 0.4189 (*2.00E-01*)	0.4013 (*2.21E-01*)	**0.6755 (*2.25E-02*)**
FOXM1 mRNA	**- 0.6003 (*3.91E-02*)**	- 0.3884 (2.38E-01)	0.5383 (*8.76E-02*)	**0.8093 (*2.50E-03*)**
CCNA2 mRNA	**- 0.7056 (*1.04E-02*)**	- 0.3021 (3.67E-01)	0.3527 (*2.87E-01*)	0.5576 (*7.47E-02*)
PCNA mRNA	- 0.2238 (*4.84E-01*)	- 0.1194 (7.27E-01)	0.2448 (*4.68E-01*)	0.1919 (*5.72E-01*)
KI67 mRNA	**- 0.5840 (*4.62E-02*)**	- 0.4359 (1.80E-01)	0.2768 (*4.10E-01*)	0.5275 (*9.54E-02*)

463 Pearson correlation coefficients and associated P value between the effect of differentiation and TGF-β.
464 Statistical significance was determined with a two-sided t-test. Correlations are significant at P value <
465 5.00E-02
466

467 **Fig 5. Proliferation genes are more repressed by TGF-β in normal bronchial epithelial progenitors than in**
468 **COPD.**

469 Primary bronchial epithelial basal cells, normal and COPD, were grown on filters and analyzed undifferentiated at
470 confluence or grown on filters and at confluence changed to ALI culture to induce differentiation, without TGF-β or
471 in presence of 1 ng/ml of TGF-β. Cells were analyzed undifferentiated or at day 6 of ALI culture either for mRNA or
472 for protein expression. RNA or proteins lysates were prepared from normal and COPD cells and analyzed
473 respectively by RT-qPCR and RPPA. For RT-qPCR, GAPDH was used to normalize cDNA amounts between
474 samples and results were calculated as a ratio on GAPDH. Data shown are for n ≥ 11. □□□□Correlations between
475 Slug mRNA and Ki67 mRNA and protein levels in undifferentiated normal cells.□Results are Pearson correlations
476 calculated with log2 (expression levels) and are presented as scatter plots with a regression line. (B, C) Comparison
477 of the mRNA levels of proliferation-related genes and protein levels of Ki67 between normal and COPD cells,
478 undifferentiated (B), at the onset of differentiation without TGF-β or in presence of 1 ng/ml of TGF-β (C). Results
479 shown are log2 (ratio on GAPDH) for mRNA or log2 (values) for protein and are presented as a scatter plot with the
480 mean ±SD. (D) Comparison of TGF-β effect on the expression of proliferation-related genes and proliferation marker
481 Ki67 protein between normal and COPD cells. Results are presented as the fold-change induced by TGF-β on mRNA
482 expression with mean ±SEM. (E, F) Correlations between Ki67, mRNA or protein, and Slug mRNA in normal cells

483 (E) or Slug protein in COPD cells (F) for the effect of differentiation and TGF-β. Results are Pearson correlations
484 calculated with the level of effect and are presented as scatter plots with a regression line. Statistical significance is at
485 P value < 5.00E-02 *, < 1.00E-02 ** or < 1.00E-03 *** as indicated. ns: non significant.
486

487 The higher levels of Slug protein and of proliferation-related genes 488 observed in COPD progenitor cells are differently induced by TGF-β

489 Slug protein and proliferation-related gene levels are higher in COPD cells in presence of
490 TGF-β (**Fig 3B and Fig 5C**) and, in contrast to normal cells, positive correlations, significant or
491 with a good tendency, are observed between the combined effects of differentiation and TGF-β
492 on Slug protein levels and that on the proliferation-related gene levels, with the exception of
493 PCNA. A strong positive correlation is also found between Slug and Ki67 protein (**Table 3 and**
494 **Fig 5F**). To understand the link between the higher expression of Slug protein and of the
495 proliferation-related gene in COPD progenitor cells in presence of TGF-β, we determined the
496 expression of signaling pathways downstream of TGF-β, using RPPA. In addition to the
497 canonical TGF-β signaling pathway Smad3-dependent, we also considered β-catenin (β-cat)
498 signaling pathway known to crosstalk with Smad signaling and to be involved in proliferation
499 (36). While TGF-β increases the level of phosphorylation of Smad3, i.e. activates Smad3-
500 dependent pathway, both in normal and COPD cells and at similar levels, it turns on β-cat
501 signaling (i.e. decreases phosphorylation of β-cat) only in COPD. In addition, TGF-β increases
502 the levels of total Smad3 and β-cat also only in COPD (**Fig 6 A and B**).
503 Slug protein levels correlate positively with both total Smad3 and β-cat levels, but do not
504 correlate with the phosphorylation levels of Smad3 or β-cat (**Fig 6C and D**). In contrast, a
505 positive correlation, significant or as a strong tendency, is found between the activation of β-cat

506 (i.e. decrease of β-cat phosphorylation) and the levels of Ki67 protein and of the proliferation-
507 related genes, with the exception of PCNA (**Table 4, Fig 6E).** These results show that, in COPD
508 cells, the higher levels of Slug protein and of the proliferation-related gene expression found in
509 presence of TGF-β, are induced by TGF-β through different downstream pathways.

510 **Table 4. Correlation between proliferation genes and Smad3 or β-cat in COPD basal/progenitor**
511 **cells in presence of TGF-β.**

	Smad3		β-catenin	
	Phospho/Total	**Total**	**Phospho/Total**	**Total**
PRC1 mRNA	- 0.1911 *(5.74E-01*)	0.3377 (*3.10E-01*)	**- 0.6551 (***2.87E-02* **)**	**0.7683 (***5.70E-03* **)**
FOXM1 mRNA	- 0.2244 (*5.07E-01*)	0.291 (*3.85E-01*)	**- 0.6511 (***3.00E-02* **)**	**0.6877 (***1.94E-02* **)**
CCNA2 mRNA	- 0.2726 (4.17E-01)	0.3709 (*2.62E-01*)	**- 0.5391 (***8.70E-02* **)**	**0.7667 (***5.90E-03* **)**
PCNA mRNA	- 0.1091 (7.49E-01)	0.314 (*3.47E-01*)	- 0.3975 (*2.26E-01*)	**0.6769 (***2.22E-02* **)**
KI67 mRNA	- 0.2256 (5.05E-01)	0.2769 (*4.10E-01*)	**- 0.7155 (***1.33E-02* **)**	**0.7872 (***4.00E-03* **)**

512 Pearson correlation coefficients and associated P value between protein levels (phosphorylated/Total or
513 Total). Computation was performed on log2 of values.
514 Statistical significance was determined with a two-sided t-test. Correlations are significant at P value <
515 5.00E-02
516

517 **Fig 6. In COPD, TGF-β induced higher levels of Slug protein and proliferation genes through different**
518 **pathways.**

519 Primary bronchial epithelial basal cells, normal and COPD, were grown on filters and at confluence changed to ALI
520 culture to induce differentiation, without TGF-β or in presence of 1 ng/ml of TGF-β. Cells were analyzed at day 6 of
521 ALI culture for protein expression. Proteins lysates were prepared from normal and COPD cells and analyzed by
522 RPPA. Data shown are for n ≥ 11. (A-B) Comparison of TGF-β effect on the ratio Phospho/Total or total protein
523 expression levels of Smad3 (A) and β-cat □B□ between normal and COPD cells. Results are presented as the fold-
524 change induced by TGF-β with mean ±SEM. □C-E□□ Correlations between Smad3 or β-cat and Slug or Ki67
525 protein levels in COPD cells in presence of TGF-β. Smad3 (Total protein level or ratio Phospho/Total) and Slug
526 protein levels (C), β-cat (Total protein level or ratio Phospho/Total) and Slug protein levels (D) or Ki67 protein levels
527 (E). Results are Pearson correlations calculated with log2 (expression levels) or log2 (Ratio Phospho/Total) and are
528 presented as scatter plots with a regression line. Statistical significance is at P value < 5.00E-02 *, < 1.00E-02 ** or
529 < 1.00E-03 *** as indicated. ns: non significant.
530

Discussion

In this work, we show that in bronchial basal/progenitor cells, Slug is co-expressed with epithelial and mesenchymal markers and that, despite being highly expressed in the cell nuclei, it does not repress E-cad, even in presence of TGF-β, and this in normal cells as well as COPD cells that have been described to reconstitute a bronchial epithelium with anomalies and features of EMT (9). Using a Slug knockdown approach and comparing between normal and COPD cells the genes downstream of Slug that respond to both differentiation and TGF-β, we identified a large set of genes involved in cell cycle and proliferation that are directly or indirectly repressed targets of Slug in normal but not in COPD basal/progenitor cells. By comparing the expression of several of these genes between normal and COPD cells, we found that their mRNA levels as well as Ki67 protein levels are lower in normal cells at the onset of differentiation when in presence of TGF-β. We also found a negative correlation between the effect of TGF-β on their expression and that on Slug at the onset of differentiation in normal cells, while in contrast, a mild positive correlation was observed in COPD. This shows that normal and COPD basal/progenitor cells respond differently to TGF-β, with Slug being involved in the repression of proliferation genes by TGF-β in normal cells, while this function of Slug is lost in COPD.

Bronchial basal/progenitor cells express both epithelial and mesenchymal markers simultaneously, both in normal and COPD cells and similarly in presence of TGF-β. In mammary stem cells a co-expression of epithelial and mesenchymal markers has also been reported leading to the concept of hybrid phenotype (37, 38). In our studies, apart from Slug, only the mesenchymal marker VIM is highly induced by TGF-β in normal and COPD cells, resulting in similar levels in both cell type. This induction of VIM expression is coherent with our finding that it belongs to the genes that are induced downstream of Slug and that it is induced at similar

554 levels in normal and COPD cells. These results are in agreement with a report showing that Slug
555 is important for the up-regulation of Vim by TGF-β in epithelial cells (18). On the contrary, E-
556 cad/ CDH1 gene is not among the genes whose expression is modified by Slug knockdown,
557 confirming that it is not a target of Slug in bronchial basal/progenitor cells. Co-expression of Slug
558 and E-cad have also been reported in mammary stem cells (39). In our study, we observe a
559 positive correlation between Slug and E-cad proteins in all conditions tested, indicating that Slug
560 does not repress E-cad in these cells even in COPD and in presence of TGF-β. Several studies
561 have reported features of EMT in COPD bronchial epithelium. However, this is based on studies
562 only revealing cells that double stain for epithelial and mesenchymal markers or on studies
563 showing only a slight lower expression of E-cad in COPD. Also theses studies concern cells from
564 *ex-vivo* epithelium or fully differentiated epithelium *in vitro* ALI culture, and suggest that COPD
565 cells may be rather imprinted for EMT, and in such, have a higher potential to enter EMT in
566 permissive conditions (9, 17, 40).

567 In search of Slug function in basal/progenitor cells, we identified genes involved in
568 proliferation that are repressed downstream of Slug in normal but not COPD cells. Among the
569 genes studied, PCNA is apart: It is the gene with the least difference between normal and COPD
570 of Slug KD effect, and this is coherent with the fact that there is no significant difference of
571 expression between normal and COPD and no correlation with Slug for the effect of TGF-β. In
572 addition to its role in DNA replication, PCNA has also a role in DNA repair, making it a poor
573 proliferation marker. In normal cells, Slug mRNA, but not protein, correlates with these
574 proliferation-related genes. We cannot exclude that, by an unknown mechanism, Slug mRNA is
575 involved in the repression of these genes; alternatively, Slug being regulated at the post-
576 translational level (41) they could be repressed by a form of Slug protein not recognized by the

577 antibody used, and this form would be absent in COPD. In contrast to normal cells, a positive
578 correlation is found between the effect of TGF-β on these proliferation genes and on Slug. Our
579 results confirm the involvement of Slug in proliferation previously reported and can provide an
580 explanation to the fact that that some studies report Slug to be a repressor of proliferation (42-44),
581 while others report a positive link with proliferation (2, 45, 46). Normal and COPD basal cells
582 may represent two cell states with a different regulation of proliferation genes by Slug and TGF-β
583 and this could be similar to what is observed in the different studies that use cell lines from
584 different origin.

585 Our work reveals that genes downstream of Slug are different between normal and COPD
586 cells and that they respond differently to TGF-β. In normal cells, Slug is involved in the response
587 of proliferation genes to TGF-β while in COPD TGF-β regulates Slug and the proliferation genes
588 through different mechanisms, with proliferation gene expression being linked to β-cat activation
589 by TGF-β. In fact, Slug, β-cat and Smad3 protein have all been reported to be stabilized
590 following GSK-3β inactivation (41, 47, 48) and we can speculate that in COPD cells, the
591 increased levels of these proteins results from such a mechanism induced by TGF-β.

592 TGF-β function is complex and has a wide spectrum of effects depending on cell state: it
593 is a cell cycle inhibitor in normal cells and a tumor promoter in malignant cells (49, 50). The
594 difference between normal and COPD cells could reflect these antagonistic effects of TGF-β,
595 COPD being in a premalignant state. Moreover, the higher expression of Slug protein seen in
596 COPD basal cells in presence of TGF-β could increase during repeated injury. Slug levels of
597 expression define its function (3) and overexpression of Slug induces EMT in epithelial cells
598 (33). We can also speculate that such deregulations of the progenitor cells could ultimately lead to

599 a shift in Slug function, becoming an EMT-inducing factor. That could explain the higher EMT
600 features found in COPD epithelium and the increase risks for COPD to develop lung carcinomas.
601

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612

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765

766 # Supporting information

767 **S1 Fig. Bronchial epithelial progenitors from COPD express Slug in their nuclei.**
768 Primary bronchial epithelial basal cells from COPD were grown on filters and analyzed undifferentiated at
769 confluence by fluorescent immunocytochemistry. Cells were fixed and labeled simultaneously with progenitor cell
770 marker p63 (white) and Slug (green) antibodies and with Hoechst as a marker of nuclei (blue). Bars are 20 μm.
771

772 **S2A-D Fig. Bronchial epithelial progenitors express Slug in their nuclei and co-express E-cadherin with or**
773 **without TGF-β.** Normal (A-B) or COPD (C-D) primary bronchial epithelial basal cells were grown on filters and at
774 confluence changed to ALI culture to induce differentiation, without TGF-β (A, C) or in presence of 1 ng/ml of TGF-
775 β (B, D). Cells were analyzed at day 6 of ALI culture by fluorescent immunocytochemistry. Cells were fixed and
776 labeled simultaneously with Slug (green) and E-cad (red) antibodies and with Hoechst as a marker of nuclei (blue).
777 Bars are 20 μm.
778

S1 Table. List of the 514 genes upregulated by Slug knockdown in normal bronchial basal/progenitor cells.

Genes statistically significant upregulated by Slug knockdown in normal bronchial basal/progenitor cells: Table shows the response of the genes to differentiation and TGFβ and fold-change for Slug knockdown in COPD cells. Genes were selected for a log2 (fold-Change) ≥0.26. Statistical significance is at p-val < 5.00E-02

S1 Appendix: Characteristics of the study subjects.

Data are presented as Mean ±SD. Pack-year=1 year smoking 20 cigarettes per day. COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s;

S2 Appendix: Sequences of primers for PCR and shRNA

S3 Appendix: References for antibodies used in immunochemistry and RPPA

CST: Cell Signaling Technology; DBS: Diagnostic BioSystems: BD: Becton Dickinson Biosciences

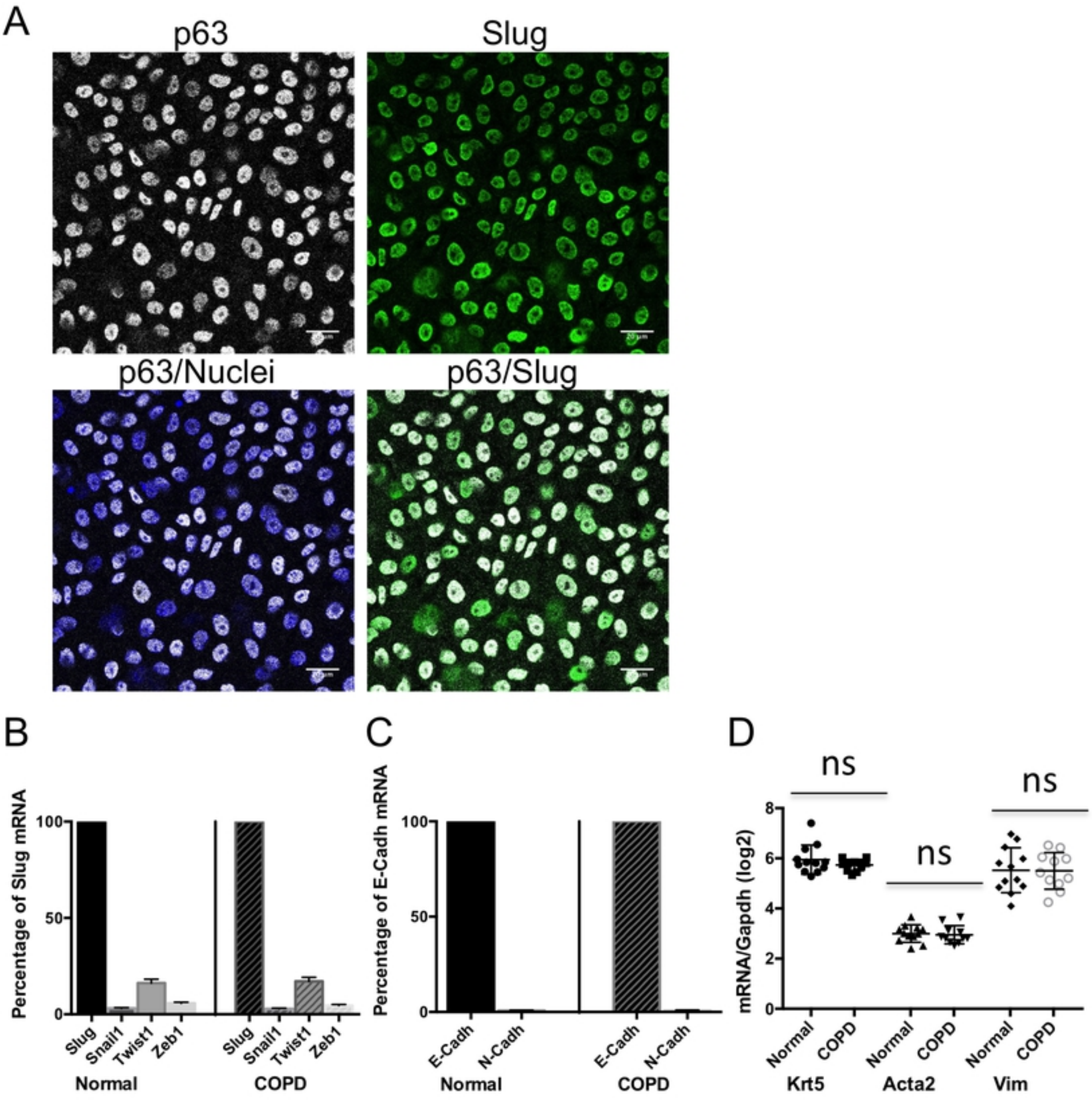

A
p63
Slug
p63/Nuclei
p63/Slug
B
Percentage of Slug mRNA
100
50
0
Slug
Snail1
Twist1
Zeb1
Normal
COPD
C
Percentage of E-Cadh mRNA
100
50
0
E-Cadh
N-Cadh
Normal
COPD
D
mRNA/Gapdh (log2)
8
6
4
2
0
ns
ns
ns
Normal
COPD
Krt5
Acta2
Vim

Figure 1

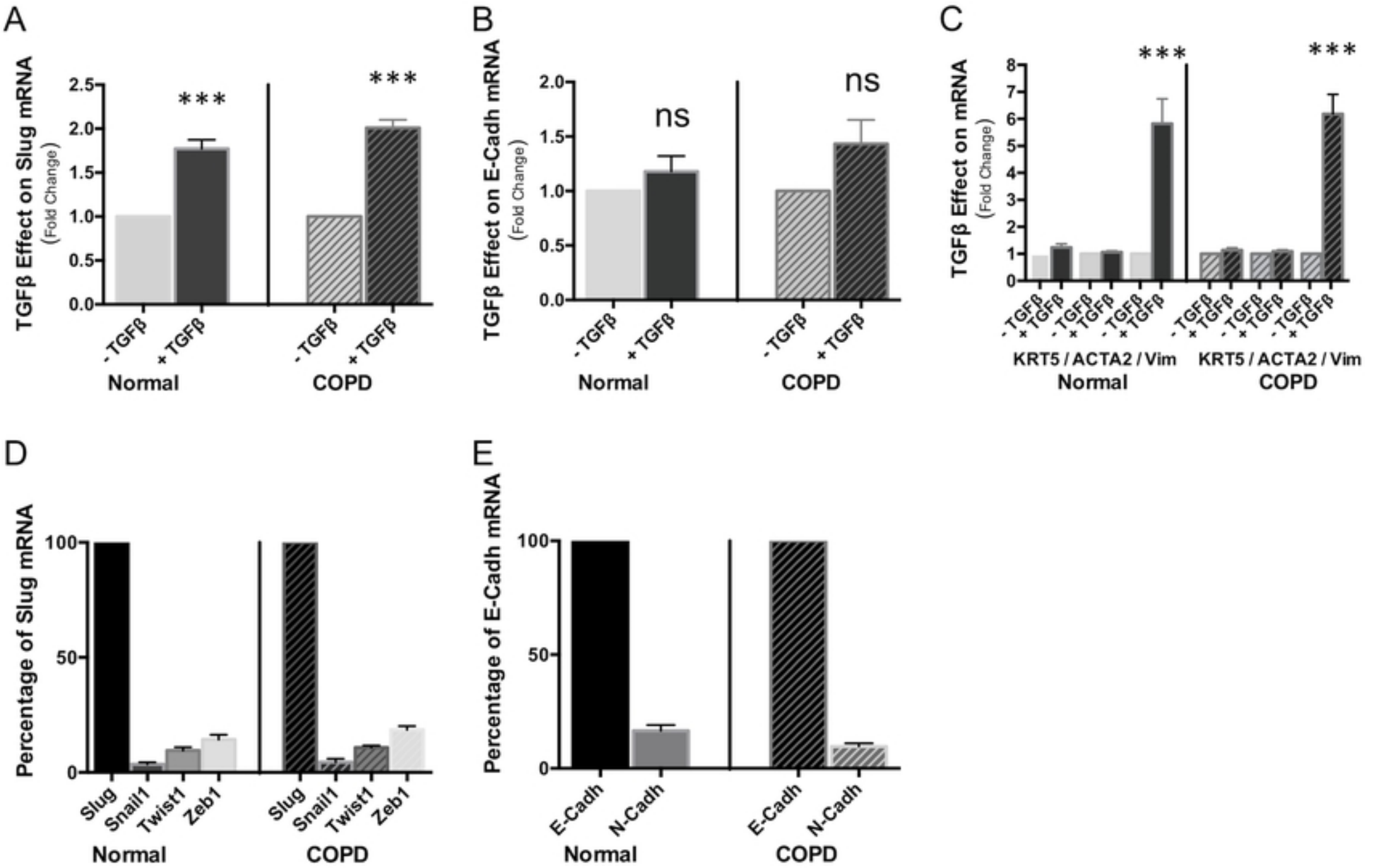

A
TGFβ Effect on Slug mRNA
(Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
- TGFβ
+ TGFβ
- TGFβ
+ TGFβ
Normal
COPD
B
TGFβ Effect on E-Cadh mRNA
(Fold Change)
2.0
1.5
1.0
0.5
0.0
ns
ns
- TGFβ
+ TGFβ
- TGFβ
+ TGFβ
Normal
COPD
C
TGFβ Effect on mRNA
(Fold Change)
8
7
6
5
4
3
2
1
0
***
***
- TGFβ
+ TGFβ
- TGFβ
+ TGFβ
- TGFβ
+ TGFβ
- TGFβ
+ TGFβ
- TGFβ
+ TGFβ
- TGFβ
+ TGFβ
KRT5 / ACTA2 / Vim
KRT5 / ACTA2 / Vim
Normal
COPD
D
Percentage of Slug mRNA
100
50
0
Slug
Snail1
Twist1
Zeb1
Slug
Snail1
Twist1
Zeb1
Normal
COPD
E
Percentage of E-Cadh mRNA
100
50
0
E-Cadh
N-Cadh
E-Cadh
N-Cadh
Normal
COPD

Figure 2

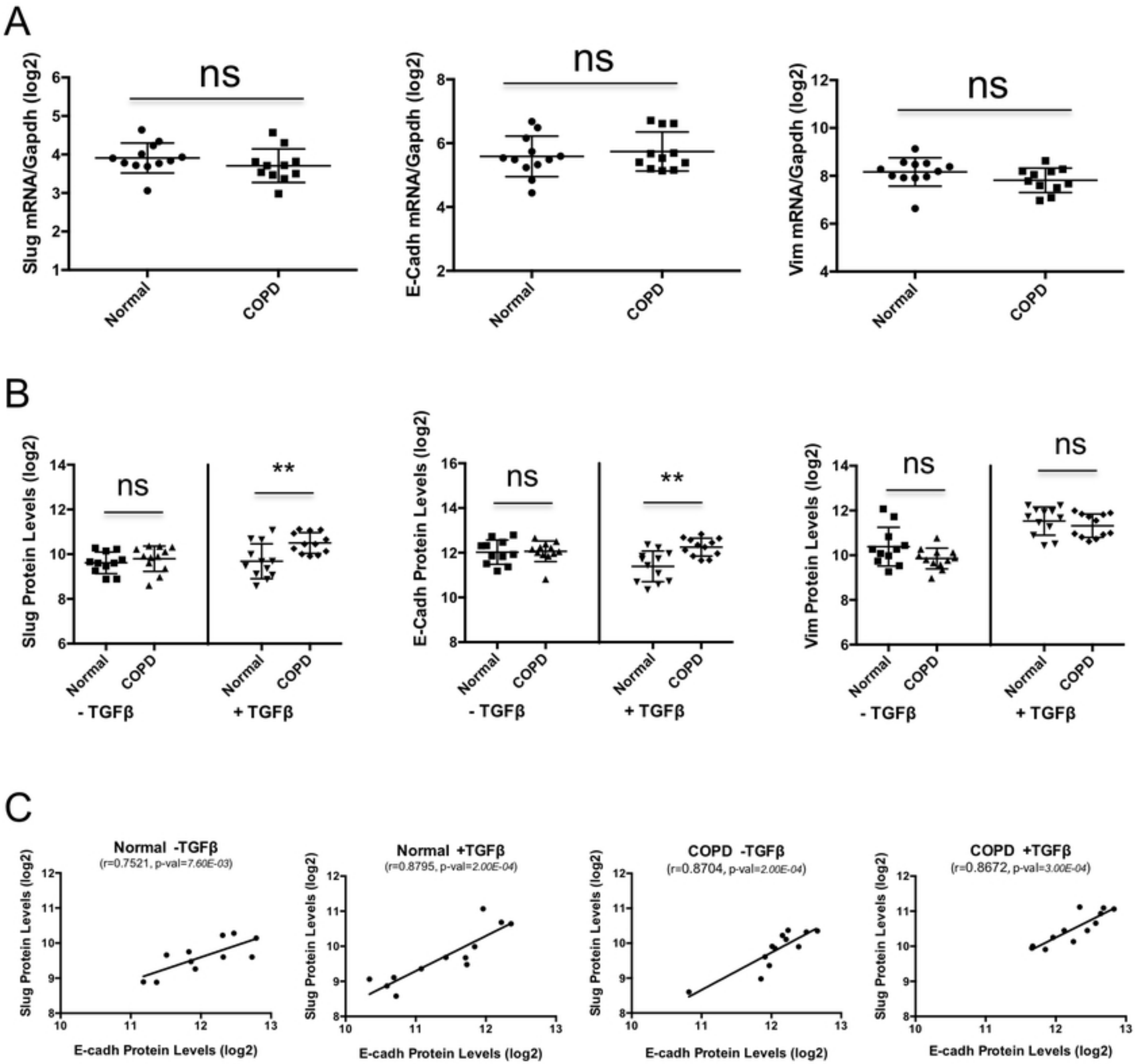
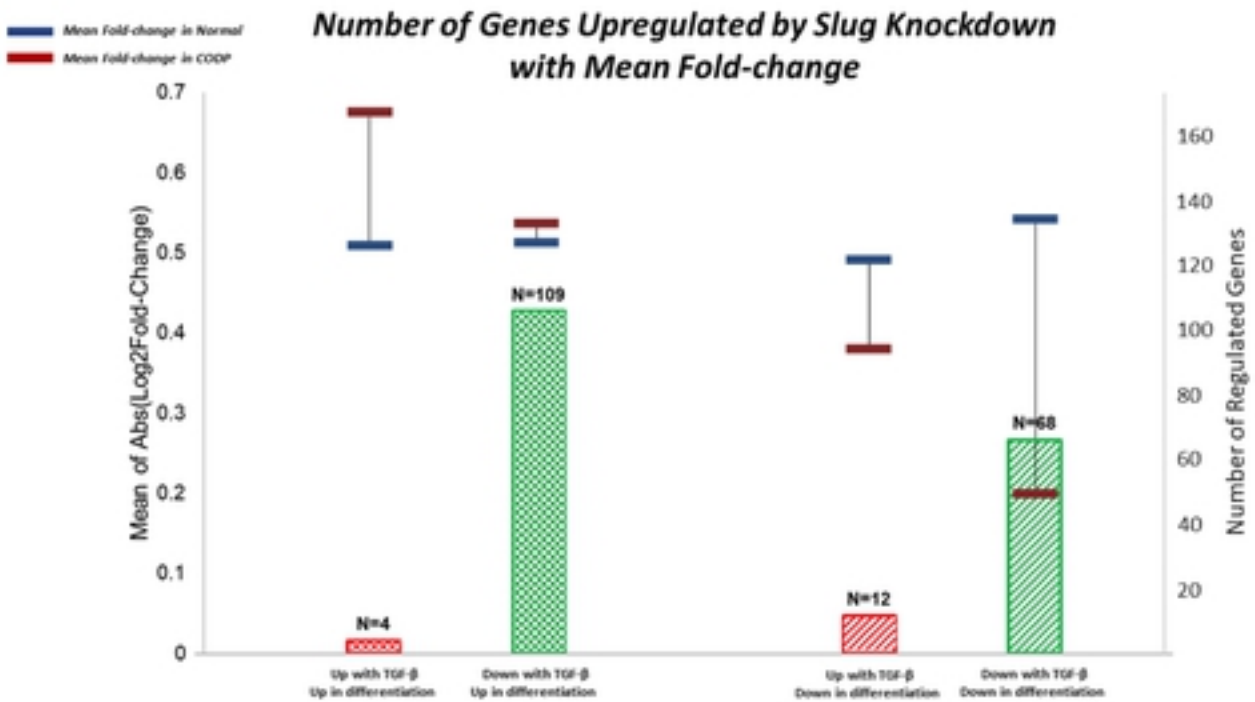

A
Slug mRNA/Gapdh (log2)
ns
Normal
COPD
E-Cadh mRNA/Gapdh (log2)
ns
Normal
COPD
Vim mRNA/Gapdh (log2)
ns
Normal
COPD
B
Slug Protein Levels (log2)
ns
**
Normal
COPD
- TGFβ
+ TGFβ
E-Cadh Protein Levels (log2)
ns
**
Normal
COPD
- TGFβ
+ TGFβ
Vim Protein Levels (log2)
ns
ns
Normal
COPD
- TGFβ
+ TGFβ
C
Normal -TGFβ
(r=0.7521, p-val=7.60E-03)
Normal +TGFβ
(r=0.8795, p-val=2.00E-04)
COPD -TGFβ
(r=0.8704, p-val=2.00E-04)
COPD +TGFβ
(r=0.8672, p-val=3.00E-04)
Slug Protein Levels (log2)
E-cadh Protein Levels (log2)

Figure 3

A


Mean Fold-change in Normal
Mean Fold-change in COOP
Number of Genes Upregulated by Slug Knockdown with Mean Fold-change
Mean of Abs(Log2Fold-Change)
Number of Regulated Genes
N=4
N=109
N=12
N=68
Up with TGF-β Up in differentiation
Down with TGF-β Up in differentiation
Up with TGF-β Down in differentiation
Down with TGF-β Down in differentiation

B

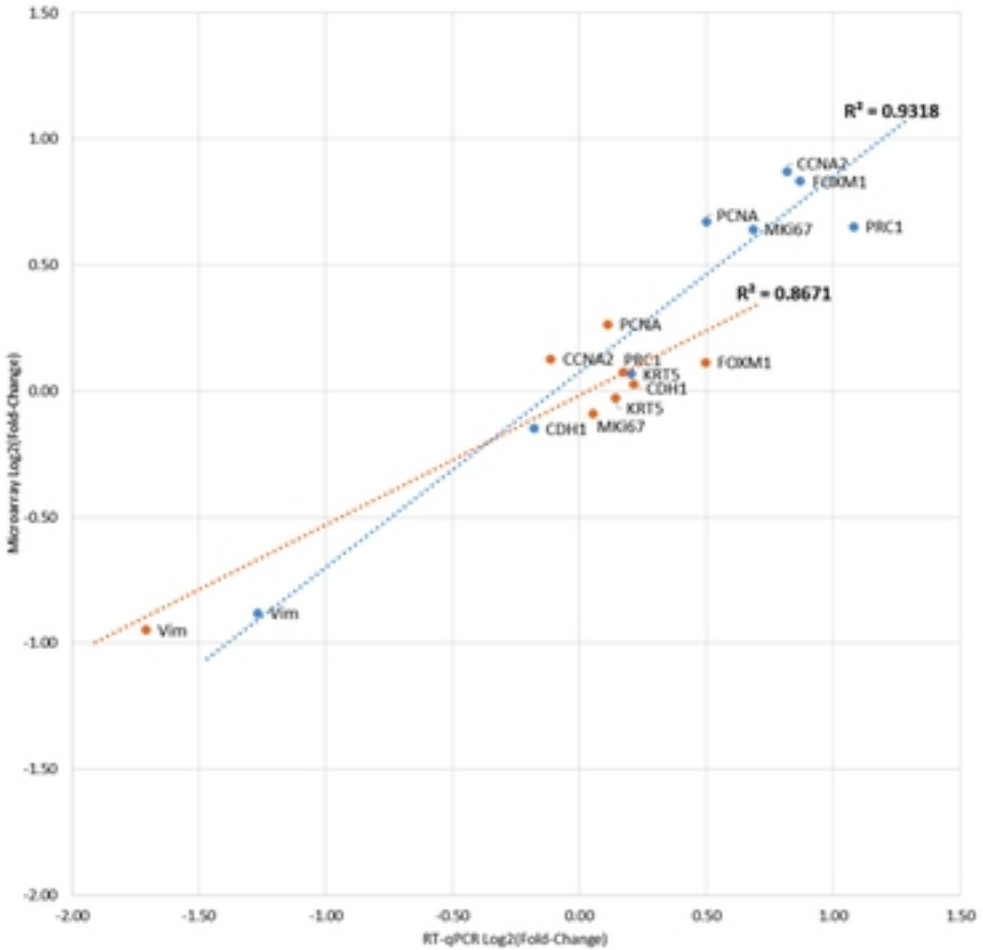

R² = 0.9318
R² = 0.8671
CCNA2
FOXM1
PCNA
MKI67
PRC1
KRT5
CDH1
Vim
Microarray Log2(Fold-Change)
RT-qPCR Log2(Fold-Change)

Figure 4

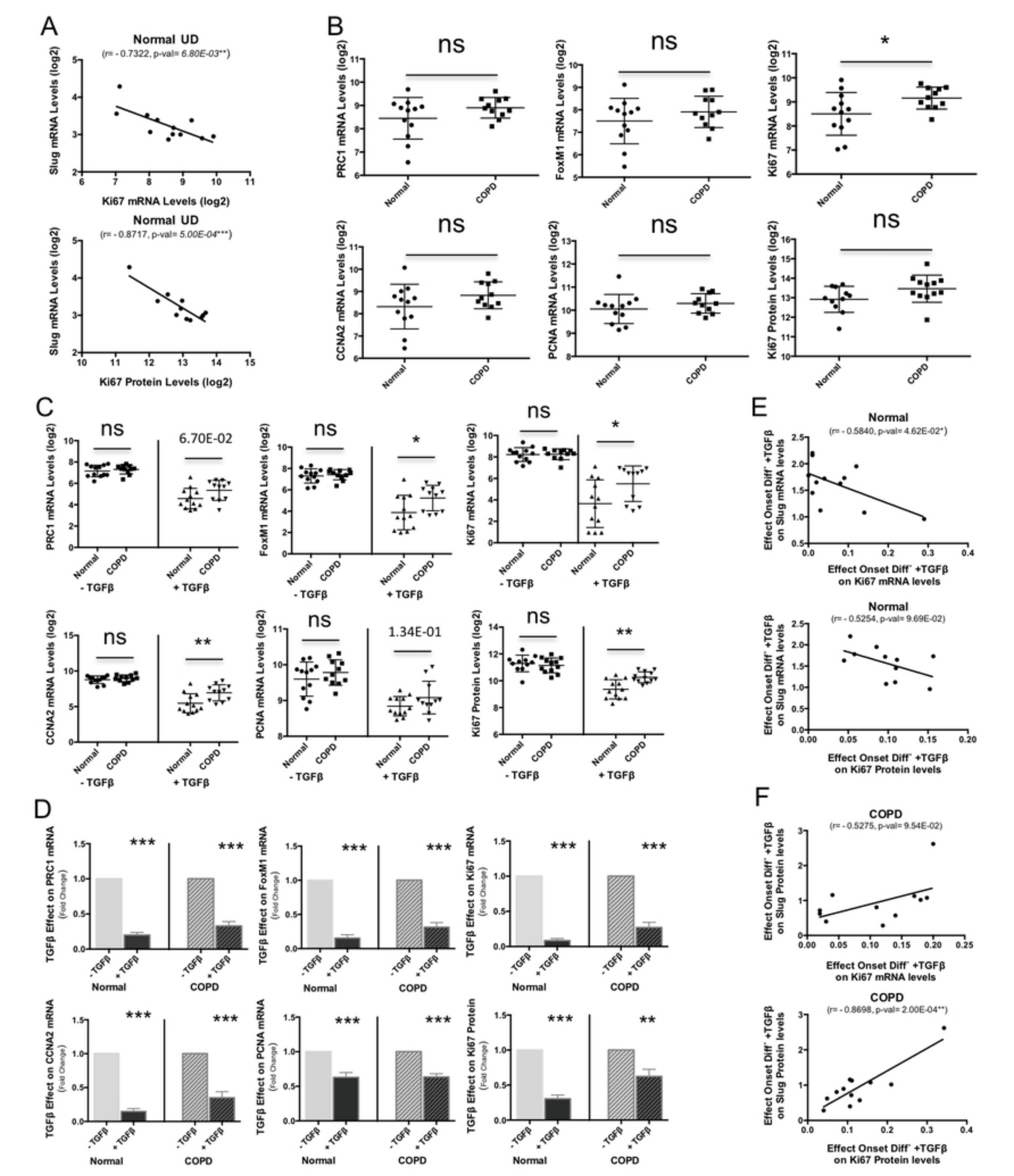

A
Normal UD
(r= - 0.7322, p-val= 6.80E-03**)
Slug mRNA Levels (log2)
Ki67 mRNA Levels (log2)
Normal UD
(r= - 0.8717, p-val= 5.00E-04***)
Slug mRNA Levels (log2)
Ki67 Protein Levels (log2)
B
ns
PRC1 mRNA Levels (log2)
ns
FoxM1 mRNA Levels (log2)
*
Ki67 mRNA Levels (log2)
ns
CCNA2 mRNA Levels (log2)
ns
PCNA mRNA Levels (log2)
ns
Ki67 Protein Levels (log2)
Normal
COPD
C
ns
6.70E-02
PRC1 mRNA Levels (log2)
ns
*
FoxM1 mRNA Levels (log2)
ns
*
Ki67 mRNA Levels (log2)
ns
**
CCNA2 mRNA Levels (log2)
ns
1.34E-01
PCNA mRNA Levels (log2)
ns
**
Ki67 Protein Levels (log2)
Normal
COPD
- TGFβ
+ TGFβ
D
TGFβ Effect on PRC1 mRNA (Fold Change)
TGFβ Effect on FoxM1 mRNA (Fold Change)
TGFβ Effect on Ki67 mRNA (Fold Change)
TGFβ Effect on CCNA2 mRNA (Fold Change)
TGFβ Effect on PCNA mRNA (Fold Change)
TGFβ Effect on Ki67 Protein (Fold Change)
***
**
- TGFβ
+ TGFβ
Normal
COPD
E
Normal
(r= - 0.5840, p-val= 4.62E-02*)
Effect Onset Diff' +TGFβ on Slug mRNA levels
Effect Onset Diff' +TGFβ on Ki67 mRNA levels
Normal
(r= - 0.5254, p-val= 9.69E-02)
Effect Onset Diff' +TGFβ on Slug mRNA levels
Effect Onset Diff' +TGFβ on Ki67 Protein levels
F
COPD
(r= - 0.5275, p-val= 9.54E-02)
Effect Onset Diff' +TGFβ on Slug Protein levels
Effect Onset Diff' +TGFβ on Ki67 mRNA levels
COPD
(r= - 0.8698, p-val= 2.00E-04**)
Effect Onset Diff' +TGFβ on Slug Protein levels
Effect Onset Diff' +TGFβ on Ki67 Protein levels

Figure 5

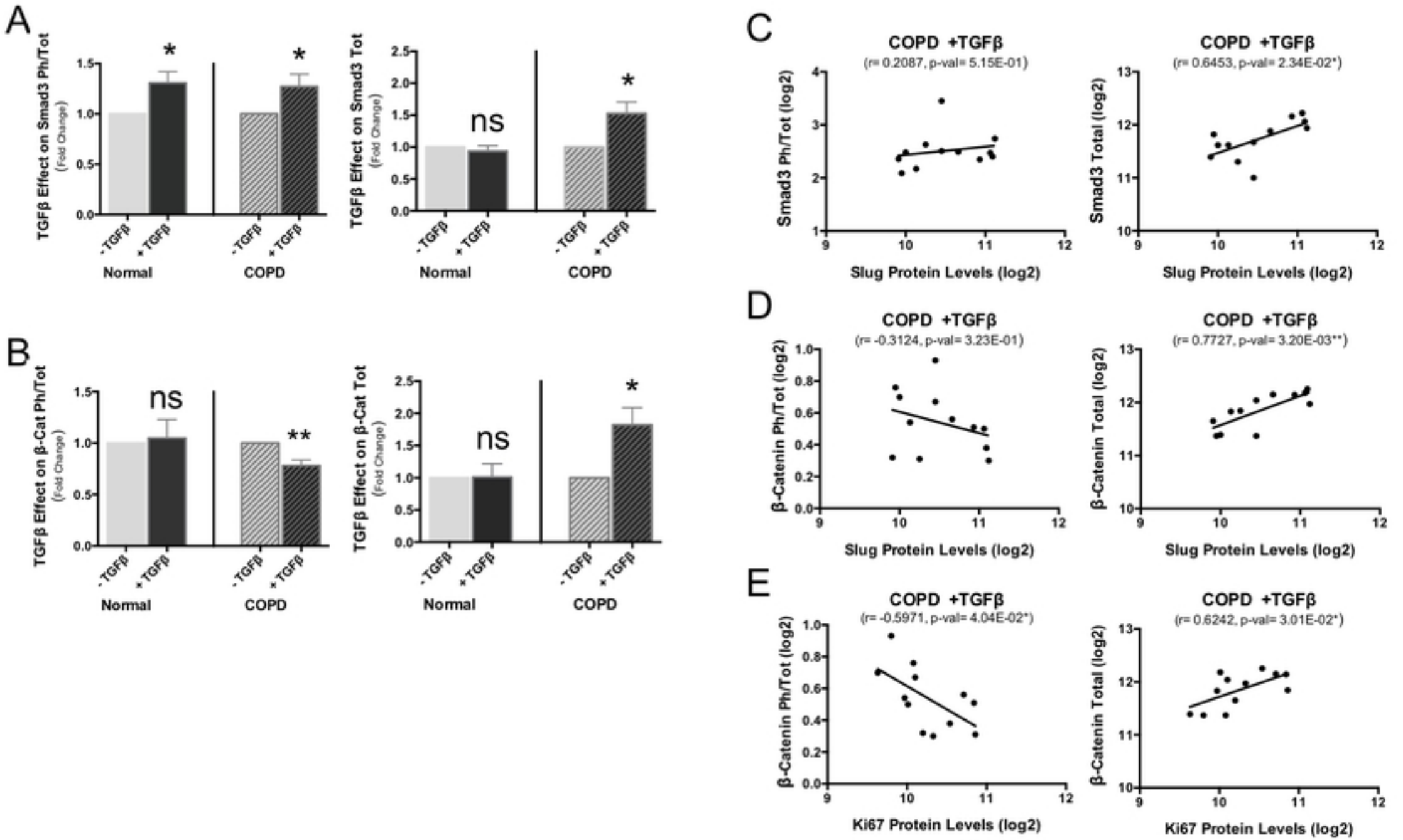

A
TGFβ Effect on Smad3 Ph/Tot
(Fold Change)
1.5
1.0
0.5
0.0
*
*
-TGFβ
+TGFβ
-TGFβ
+TGFβ
Normal
COPD
TGFβ Effect on Smad3 Tot
(Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
ns
*
-TGFβ
+TGFβ
-TGFβ
+TGFβ
Normal
COPD
B
TGFβ Effect on β-Cat Ph/Tot
(Fold Change)
1.5
1.0
0.5
0.0
ns
**
-TGFβ
+TGFβ
-TGFβ
+TGFβ
Normal
COPD
TGFβ Effect on β-Cat Tot
(Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
ns
*
-TGFβ
+TGFβ
-TGFβ
+TGFβ
Normal
COPD
C
COPD +TGFβ
(r= 0.2087, p-val= 5.15E-01)
Smad3 Ph/Tot (log2)
4
3
2
1
9
10
11
12
Slug Protein Levels (log2)
COPD +TGFβ
(r= 0.6453, p-val= 2.34E-02*)
Smad3 Total (log2)
13
12
11
10
9
10
11
12
Slug Protein Levels (log2)
D
COPD +TGFβ
(r= -0.3124, p-val= 3.23E-01)
β-Catenin Ph/Tot (log2)
1.0
0.8
0.6
0.4
0.2
0.0
9
10
11
12
Slug Protein Levels (log2)
COPD +TGFβ
(r= 0.7727, p-val= 3.20E-03**)
β-Catenin Total (log2)
13
12
11
10
9
10
11
12
Slug Protein Levels (log2)
E
COPD +TGFβ
(r= -0.5971, p-val= 4.04E-02*)
β-Catenin Ph/Tot (log2)
1.0
0.8
0.6
0.4
0.2
0.0
9
10
11
12
Ki67 Protein Levels (log2)
COPD +TGFβ
(r= 0.6242, p-val= 3.01E-02*)
β-Catenin Total (log2)
13
12
11
10
9
10
11
12
Ki67 Protein Levels (log2)

Figure 6