

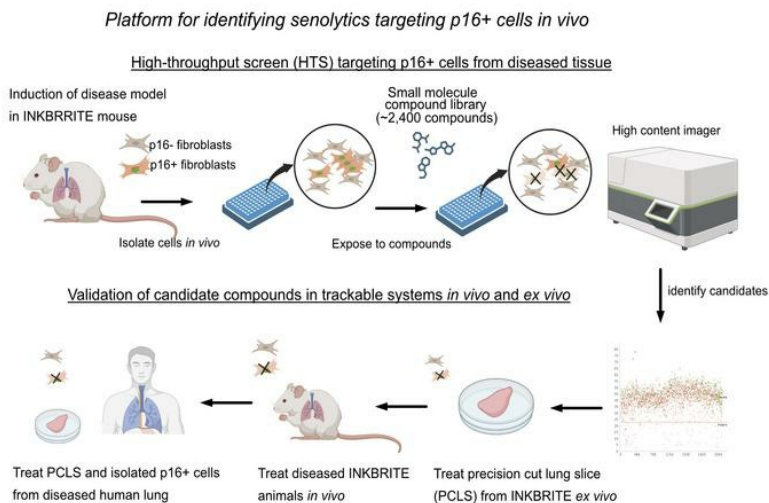
## An *in vivo* screening platform identifies senolytic compounds that target $p16^{INK4a+}$ fibroblasts in lung fibrosis

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1 **Title**

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3 **An *in vivo* screening platform identifies senolytic compounds that target *p16<sup>INK4a+</sup>***  
4 **fibroblasts in lung fibrosis**

5

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39 **Conflict of interest statement:**

40 The authors have declared that no conflict of interest exists.

1 **Abstract**

2 The appearance of senescent cells in age-related diseases has spurred the search for  
3 compounds that can target senescent cells in tissues (“senolytics”). However, a major  
4 caveat with current senolytic screens is the use of cell lines as targets where  
5 senescence is induced *in vitro*, which does not necessarily reflect the identity and  
6 function of pathogenic senescent cells *in vivo*. Here, we developed a new pipeline  
7 leveraging a fluorescent murine reporter that allows for isolation and quantification of  
8  $p16^{Ink4a+}$  cells in diseased tissues. By high-throughput screening *in vitro*, precision cut  
9 lung slice (PCLS) screening *ex vivo*, and phenotypic screening *in vivo*, we identified a  
10 HSP90 inhibitor (XL888) as a potent senolytic in tissue fibrosis. XL888 treatment  
11 eliminated pathogenic  $p16^{Ink4a+}$  fibroblasts in a murine model of lung fibrosis and  
12 reduced fibrotic burden. Finally, XL888 preferentially targeted  $p16^{INK4a}$ -high human lung  
13 fibroblasts isolated from patients with idiopathic pulmonary fibrosis (IPF), and reduced  
14  $p16^{INK4a+}$  fibroblasts from IPF PCLS *ex vivo*. This study provides proof of concept for a  
15 platform where  $p16^{INK4a+}$  cells are directly isolated from diseased tissues to identify  
16 compounds with *in vivo* and *ex vivo* efficacy in mouse and human respectively and  
17 provides a senolytic screening platform for other age-related diseases.

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## 1 **Introduction**

2 Hayflick's original description of a stereotyped proliferative arrest for primary cells in  
3 culture as "senescence at the cellular level (1)" gave way to geroscience that seeks to  
4 unravel the relationship between cellular aging and age-related diseases (2). While  
5 initial studies of senescence mostly occurred in cell culture, the identification of  
6 biomarkers *in vitro* was extrapolated to study *in vivo* phenomena in aging tissues,  
7 exemplified by the use of  $p16^{INK4a}$  expression as a biomarker of senescent cells in  
8 tissues (3). The construction of mouse models where  $p16^{INK4a+}$  cells can be genetically  
9 depleted to ameliorate a host of age-related phenotypes validated this approach (4-9),  
10 and identified senescent cells in tissues as potential targets for pharmacotherapy (10).  
11 The pipeline to identify compounds targeting senescent cells ("senolytics") involves  
12 screening cytotoxicity on cell lines where senescence is induced *in vitro*, followed by  
13 validation in preclinical animal models where senescent cells are thought to be present  
14 (11). However, this approach assumes that senescent cells derived from cell lines in  
15 culture are identical to senescent cells *in vivo* that reside in far more diverse cellular  
16 environments.

17         Single cell transcriptome analyses have demonstrated that senescent  
18 phenotypes *in vitro* are heterogeneous and dependent on senescence inducers and cell  
19 types (12, 13). Based on these studies, senescent cells *in vivo* would be expected to  
20 display even more functional heterogeneity given the diverse tissue microenvironment  
21 they would encounter. Leveraging an ultrasensitive GFP reporter of  $p16^{INK4a}$  (Ink4a-H2B-  
22 GFP reporter-in-tandem, or INKBRITE), we recently demonstrated that  $p16^{INK4a+}$   
23 fibroblasts *in vivo* exhibit a spectrum of senescent phenotypes that directly correlated

1 with the range of  $p16^{Ink4a}$  transcript levels (14). The recognition that senescent cells are  
2 functionally heterogeneous has profound implications for senolytic screening pipelines,  
3 as target cell type selection could significantly influence the candidates identified. This  
4 has already become apparent as different senolytic screens have identified compounds  
5 that have non-overlapping efficacy against different cell types and preclinical models  
6 (15). To improve the ability to identify senolytics with *in vivo* efficacy against the disease  
7 of interest, we set out to develop a screening platform that 1) utilizes senescent cells  
8 directly isolated from diseased tissues as screening targets, and 2) provides an *in vivo*  
9 platform for direct validation of senolytic activity in the tissue of origin.

10 Idiopathic pulmonary fibrosis (IPF) is an age-related lung disease where telomere  
11 shortening and senescence have been implicated in the pathogenesis (16, 17).  
12 Histologic examination of IPF lungs have demonstrated the presence of biomarkers  
13 associated with senescence in alveolar type 2 (AT2) cells and lung fibroblasts (18),  
14 which was confirmed in single cell atlases of IPF lungs showing enrichment of *CDKN2A*  
15 (encoding  $p16^{INK4a}$ ) expression in epithelial and fibroblast subsets (19, 20). Despite  
16 histologic evidence of  $p16^{INK4a+}$  cells in IPF and animal models of lung fibrosis, their  
17 pathogenic role is less clear. Genetic ablation of  $p16^{Ink4a+}$  cells in an animal model of  
18 lung fibrosis improved lung function, but there was no evidence that this animal model  
19 improved standard fibrotic endpoints such as hydroxyproline content (21). Furthermore,  
20 genetic depletion studies preclude the ability to identify  $p16^{Ink4a+}$  cells in the fibrotic  
21 tissue and study their behavior in a prospective manner. To determine whether  $p16^{Ink4a+}$   
22 cells are a viable target for therapeutic intervention in lung fibrosis, we need to isolate  
23 and characterize these cells *in vivo*. In this study, we characterized the identity of

1 fibrotic  $p16^{Ink4a+}$  fibroblasts *in vivo* and outlined a senolytic screening platform that  
2 provides scalability and validation by leveraging the ability to isolate and track  $p16^{Ink4a+}$   
3 cells from diseased tissues. Utilizing our new platform, we identified a novel senolytic  
4 compound that deleted  $p16^{Ink4a+}$  cells and reduced fibrotic burden in a murine model of  
5 lung fibrosis, and preferentially targeted  $p16^{INK4a+}$  fibroblasts from human IPF lung  
6 samples.

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## 1 **Results**

### 2 **Murine $p16^{Ink4a+}$ lung fibroblasts display profibrotic identities and phenotypes *in*** 3 ***vivo*.**

4 Our previous study showed that  $p16^{Ink4a+}$  fibroblasts dynamically respond to tissue  
5 injury in the lung (14). To define the potential contribution of  $p16^{Ink4a+}$  fibroblasts to  
6 fibrogenesis, we performed single cell RNAseq (scRNAseq) on fibrotic INKBRITE lungs.  
7 Leveraging our capacity to isolate  $p16^{Ink4a+}$  cells by fluorescent sorting, we isolated  
8 GFP+ fibroblasts (CD45- EpCAM- CD31-) from lungs of INKBRITE mice treated with  
9 bleomycin (14 days post injury, or dpi). We profiled 7,846 cells and identified 6 clusters  
10 with distinct marker gene expressions that corresponded with previously annotated  
11 fibroblast subsets identified in fibrotic murine lungs (22, 23) (Figure 1A, Supplemental  
12 Figure 1, A and B). In addition to the adventitial, alveolar, and peribronchial fibroblast  
13 subsets that were previously identified in the uninjured lungs, we observed significant  
14 fractions of “pathologic” (expressing numerous pro-fibrotic genes) and stress-activated  
15 fibroblasts that were found to arise *de novo* in the fibrotic lung (Figure 1, A-C) (22, 23).  
16 These pathologic fibroblasts were mostly absent from our prior single cell dataset of  
17  $p16^{Ink4a+}$  fibroblasts isolated from naphthalene injured INKBRITE lungs (14)  
18 (Supplemental Figure 1, A-E), showing that the lineage fate of  $p16^{Ink4a+}$  fibroblasts  
19 changes with the injury context. Immunohistochemistry (IHC) of injured INKBRITE lungs  
20 demonstrated infiltration of GFP+ fibroblasts within dense, fibrotic regions (Figure 1D,  
21 dashed circles). IHC analysis validated the scRNAseq results, demonstrating the  
22 presence of  $p16^{Ink4a+}/Cthrc1+$  fibroblasts within the fibrotic regions that also contain  
23 other fibrotic markers such as ACTA2, COL1A1, and TAGLN (Figure 1D).

1 To determine phenotypic differences between  $p16^{Ink4a-}$  and  $p16^{Ink4a+}$  fibroblasts  
2 in fibrotic tissue, we FACSsorted GFP+ and GFP- fibroblasts from bleomycin-injured  
3 INKBRITE lungs for downstream analyses. Quantitative PCR (qPCR) confirmed the  
4 upregulation of  $p16^{Ink4a}$  as well as p21 in GFP+ fibroblasts, along with phenotypic  
5 markers associated with senescence such as cell size, DNA damage, and proliferative  
6 arrest (Supplemental Figure 2, A-E). Furthermore,  $p16^{Ink4a+}$  fibroblasts demonstrated  
7 significant upregulation for genes enriched in pathologic fibroblasts compared to  
8  $p16^{Ink4a-}$  fibroblasts (Figure 1E), which was confirmed on immunocytochemistry of  
9 sorted GFP+ and GFP- fibroblasts from fibrotic INKBRITE lungs (Supplemental Figure  
10 3, A-C). To investigate potential differences in response to fibrotic stimuli between  
11  $p16^{Ink4a+}$  and  $p16^{Ink4a-}$  cells, we isolated GFP+ and GFP- lung fibroblasts from uninjured  
12 INKBRITE lungs and stimulated them with recombinant TGF- $\beta$ 1.  $p16^{Ink4a+}$  fibroblasts  
13 exhibit elevated fibrotic gene expression both before and after TGF- $\beta$ 1 stimulation  
14 compared to  $p16^{Ink4a-}$  fibroblasts (Figure 1F). These data indicate that  $p16^{Ink4a+}$  lung  
15 fibroblasts preferentially give rise to pathologic fibroblasts in lung fibrosis. More  
16 importantly, our data suggest that different types of  $p16^{Ink4a+}$  fibroblasts arise in different  
17 pathologic contexts, which could also dictate divergent susceptibility of  $p16^{Ink4a+}$  cells to  
18 senolytics under different tissue conditions.

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### 20 **$p16^{Ink4a}$ expression in lung fibroblasts augments the fibrotic response.**

21 To determine the role of  $p16^{INK4a}$  expression in the fibrotic response, we first generated  
22 a dual lentiviral system (Lenti-tTS/rtTA + Lenti-TRE-p16-T2A-dTomato) to overexpress  
23  $p16^{INK4a}$  in a doxycycline-dependent manner (14). Primary human lung fibroblasts



1 isolated from control cadaveric donors were transduced with our  $p16^{INK4a}$   
2 overexpression (OE) vectors, followed by doxycycline and fibrotic induction *in vitro*  
3 (addition of TGF- $\beta$ 1).  $p16^{INK4a}$  OE significantly augmented the expression of pathologic  
4 fibroblast genes in response to TGF- $\beta$ 1 compared to control fibroblasts (Figure 2A).  
5 Interestingly,  $p16^{INK4a}$  OE in the absence of TGF- $\beta$ 1 did not induce pro-fibrotic gene  
6 expression (Figure 2A), suggesting the  $p16^{INK4a}$  alone does not independently drive the  
7 fibrotic response, but rather primes the fibroblast response to a fibrotic stimulus.

8 To determine the necessity of  $p16^{INK4a}$  expression in the fibroblast response to  
9 fibrotic stimuli *in vivo*, we deleted  $p16^{INK4a}$  in fibroblasts with a mesenchymal-specific  
10 Cre-driver ( $Dermo1^{Cre/+}$ ). As we had previously reported, fibroblast-specific deletion of  
11  $p16^{INK4a}$  ( $Dermo1^{Cre/+};p16^{flox/flox}$ , referred to as  $D1^{p16CKO}$ ) did not alter the gross  
12 morphology in the uninjured lung(14). Induction of fibrotic injury with bleomycin  
13 demonstrated that  $D1^{p16CKO}$  animals exhibited attenuated fibrotic response on histology  
14 and collagen deposition (as measured by hydroxyproline) compared to controls (Figure  
15 2, B and C). IHC analysis demonstrated that fibroblast-specific deletion of  $p16^{INK4a}$   
16 reduced the number of fibroblasts expressing pro-fibrotic markers in the bleomycin-  
17 injured lungs (Figure 2, D and E). These experiments demonstrated that  $p16^{INK4a}$   
18 expression primes the fibroblasts to augment the fibrotic response but is not sufficient to  
19 drive fibrosis in the absence of a fibrotic stimulus.

20

21 **High throughput screen (HTS) identified compounds targeting  $p16^{INK4a}$ + lung**  
22 **fibroblasts isolated from fibrotic tissue.**

1 Proper target selection is one of the most important factors for successful compound  
2 screens. To identify compounds that will specifically target  $p16^{Ink4a+}$  fibroblasts from  
3 fibrotic lungs, we leveraged the ability to isolate  $p16^{Ink4a+}$  fibroblasts *in vivo* from  
4 diseased tissues utilizing the INKBRITE reporter. We purified  $p16^{Ink4a+}$  (GFP+) and  
5  $p16^{Ink4a-}$  (GFP-) fibroblasts *in vivo* directly from the fibrotic lungs of INKBRITE animals  
6 injured with bleomycin (14 dpi). The fluorescent tag allowed us to mix GFP+ and GFP-  
7 fibroblasts at a 1:1 ratio into 384-well plates, so that each well can serve as an internal  
8 control when comparing the cell viability of  $p16^{Ink4a+}$  and  $p16^{Ink4a-}$  fibroblasts. The ability  
9 to combine the target ( $p16^{Ink4a+}$ ) and bystander ( $p16^{Ink4a-}$ ) cells together in the same  
10 well also allowed us to scale up the screen to a chemical library of roughly 2,000 small  
11 molecules with annotated biologic activity (Figure 3A). The goal of the primary screen  
12 was to identify the most potent compounds that killed off GFP+ fibroblasts while sparing  
13 GFP- fibroblasts as determined by the percentage of GFP+ fibroblasts (%GFP+) in each  
14 well. The fluorescent intensity of the INKBRITE reporter and nuclear localization of H2B-  
15 GFP allowed image segmentation of GFP+ and GFP- nuclei in over 2,600 wells with  
16 high content imaging, and we identified 37 compounds that exceeded the statistical  
17 threshold of 3 sigma (<20%GFP+) from the mean (45%GFP+ in negative control/vehicle  
18 wells) (Figure 3, B and C). Analysis of the annotated biological pathways demonstrated  
19 numerous pathways previously implicated in senolysis (24) (eg. HSP90, BCL-2, PI3K  
20 inhibitors) as well as potentially novel ones (eg. HDAC, proteasome inhibitor) (Figure  
21 3D). Of note, previously identified senolytics such as dasatinib, quercetin, and fisetin all  
22 reduced %GFP+ below the mean, but none exceeded the 3-sigma threshold for  
23 secondary validation (Supplemental Table 1).

1 To define the potency of these compounds to delete  $p16^{Ink4a}$ + cells, we selected  
2 32 compounds with the lowest %GFP+ for secondary validation with dose-response  
3 curves to determine the half-maximal inhibitory concentration (IC50) for reduction of  
4 %GFP+ fibroblasts (Figure 3E). Each compound was tested over 10 concentrations to a  
5 maximum of 20 mM. The validation screen yielded 8 compounds that had an IC50  
6 (%GFP) below 2  $\mu$ M, which was composed mostly of HSP90 and HDAC inhibitors  
7 (Supplemental Table 2). The top candidates typically had a maximal effect ( $E_{max}$ ) of  
8 reducing GFP+ fibroblast viability <10% (Supplemental Figure 4). The top 3 candidates  
9 had IC50 (reduction of %GFP) below 1  $\mu$ M, and they were Trichostatin A (TSA, HDAC  
10 inhibitor), XL888 (heat shock protein 90, or HSP90 inhibitor), and Ganetesipib (HSP90  
11 inhibitor) (Figure 3F).

12

### 13 **An *ex vivo* model using precision cut lung slices (PCLS) to validate senolytic** 14 **candidates.**

15 A bottleneck to the compound screening pipeline is narrowing the top hits that are most  
16 likely to be efficacious in animal disease models, which can be expensive and time-  
17 consuming to perform. To predict *in vivo* efficacy to streamline our screen, we  
18 incorporated an *ex vivo* platform to test the top hits in our screen in a more physiologic  
19 setting that is easily scalable. *Ex vivo* culture of precision cut lung slices (PCLS) has  
20 been utilized to model lung diseases such as IPF as well as drug testing for lung fibrosis  
21 (25). We first generated PCLS cultures from bleomycin-injured INKBRITE animals  
22 (Figure 4, A and B). Flow cytometry analysis demonstrated high viability of the cells in  
23 the PCLS at the end of the 5-day culture period (Supplemental Figure 5A). We tested

1 the top candidates identified in the secondary validation in the fibrotic INKBRITE PCLS  
2 *ex vivo* using the same standard drug concentration as the primary screen. Again, we  
3 wanted to examine the effect of the compound on  $p16^{Ink4a+}$  fibroblasts relative to  
4  $p16^{Ink4a-}$  fibroblasts, so we examined the %GFP in the fibroblast population (CD45-  
5 /EpCAM-/CD31-, Figure 4C) on flow analysis of the treated PCLS. Flow analysis  
6 demonstrated that both HSP90 inhibitors (XL888 and ganetespib) reduced the %GFP+  
7 fibroblasts in the INKBRITE PCLS, while TSA had no effect (Figure 4D and  
8 Supplemental Figure 5B). Histology of INKBRITE PCLS demonstrated preserved  
9 architecture and continued presence of ACTA2+/ $p16^{Ink4a+}$  and COL1+/ $p16^{Ink4a+}$   
10 fibroblasts in the fibrotic regions of the lung (Figure 4E). IHC analysis of XL888-treated  
11 PCLS demonstrated a reduction of GFP+ACTA2+ and GFP+COL1+ fibroblasts (Figure  
12 4, E and F). We also screened two other HDAC inhibitors with low IC50 in the dose-  
13 response validation *in vitro* (fimepinostat and dacinostat), but neither compound  
14 reduced %GFP+ fibroblasts *ex vivo* in our PCLS assay (Supplemental Figure 5C).  
15 Finally, we tested other previously described senolytics in our PCLS model  
16 (dasatinib+quercetin or D&Q, fisetin, ABT-263 and ABT-737) in comparison with XL888,  
17 and only XL888 reduced %GFP+ fibroblasts compared to vehicles (Supplemental  
18 Figure 5D). GFP over-expression in normal lung fibroblasts did not enhance  
19 susceptibility to XL888-mediated killing (Supplemental Figure 5E). Finally, we tested  
20 XL888 on PCLS isolated from INKBRITE animals induced with a non-fibrotic injury  
21 using naphthalene (airway injury followed by full repair). In contrast to fibrotic injured  
22 PCLS, XL888 did not significantly reduce the % of GFP+ fibroblasts from naphthalene  
23 injured PCLS (Supplemental Figure 5F). These experiments suggest that HSP90

1 inhibitors are the most promising candidates to target  $p16^{Ink4a+}$  fibroblasts *in vivo* in  
2 preclinical animal models of lung fibrosis.

3

4 **XL888 deletes  $p16^{Ink4a+}$  lung fibroblasts *in vivo* and attenuates fibrotic**  
5 **remodeling in mice.**

6 To determine whether the PCLS screen correlated with *in vivo* activity against  $p16^{Ink4a+}$   
7 fibroblasts, we set up an *in vivo* validation step where each of the candidate compounds  
8 tested in PCLS (XL888, ganetespib, TSA, fimepinostat, and dacinostat) was  
9 administered in our preclinical model of lung fibrosis and compared against vehicle-  
10 treated control animals (each compound required a different vehicle cohort because  
11 each is formulated differently with different routes of administration). INKBRITE animals  
12 were first injured with bleomycin to establish fibrosis, followed by administration of the  
13 candidate senolytic daily for 2 weeks (Figure 5A). For each compound, we determined  
14 the maximum effective dose based on literature search of previous use in other pre-  
15 clinical models. At the end of the treatment period, we determined the selective impact  
16 of the compound on  $p16^{Ink4a+}$  fibroblasts using an identical flow cytometry strategy as  
17 described for PCLS (Figure 4C). Flow analysis of single cell lung suspension at the end  
18 of the study period demonstrated that only XL888 treatment resulted in a significant  
19 reduction in the %GFP+ fibroblasts *in vivo* compared to vehicle controls (Figure 5, B  
20 and C, Supplemental Figure 6, A-D).

21 To determine whether XL888 targeted pathologic  $p16^{Ink4a+}$  subsets described in  
22 our scRNAseq analysis, we repeated the XL888 treatment in fibrotic INKBRITE animals  
23 for histologic analysis. IHC showed that treatment of XL888 reduced the GFP+/ACTA2+

1 fibroblasts within fibrotic regions of the lung (Figure 5, D and E). To determine whether  
2 XL888 reduced the overall fibrotic burden in the lung, we performed trichrome staining  
3 and hydroxyproline quantification in another cohort of XL888 treated animals compared  
4 with vehicles, which showed reduction in fibrotic remodeling in the lung as well as  
5 collagen content as measured by hydroxyproline (Figure 5, F and G). Finally, we  
6 administered D&Q in our preclinical model using the same dosing schedule as  
7 previously reported (21) (3 times for 3 weeks treatment period) in the INKBRITE mice,  
8 and we could not observe a difference in the %GFP+ fibroblasts at the end of the study  
9 period, although there was a trend towards reduction in %GFP+ immune and  
10 endothelial cells (Supplemental Figure 6, E-G). D&Q did not change the total collagen  
11 content by hydroxyproline assay (Supplemental Figure 6H). These results showed that  
12 XL888, identified from our HTS platform, effectively eliminated  $p16^{Ink4a}$ + fibroblasts *in*  
13 *vivo* and attenuated pulmonary fibrosis in animal model. The results demonstrated that  
14  $p16^{Ink4a}$ + fibroblasts play a functional role in the development of lung fibrosis and  
15 indicate their potential as therapeutic targets for diseases involved in the accumulation  
16 of those cells.

17

### 18 **Human $p16^{INK4a}$ + fibroblasts contribute to pathologic fibroblast subsets in IPF.**

19 While IPF fibroblasts cultured *in vitro* have been reported to demonstrate senescent  
20 characteristics (26), we sought to determine the expression pattern of *CDKN2A*  
21 (encoding both  $p16^{INK4a}$  and  $p19^{ARF}$ ) in our previous scRNAseq of IPF lung fibroblasts *in*  
22 *vivo* (22) (Figure 6A, Supplemental Figure 7A). Our previous analysis of IPF fibroblasts  
23 demonstrated distinct clusters of alveolar and adventitial fibroblasts comparable to

1 those defined in mice, along with the emergence of a *CTHRC1+/COL1A1<sup>hi</sup>/ACTA2<sup>hi</sup>*  
2 pathologic fibroblast subset similar to the one that arose in murine lungs after bleomycin  
3 injury (22). Analysis of *CDKN2A* expression showed significant enrichment in the  
4 *CTHRC1+/COL1A1<sup>hi</sup>/ACTA2<sup>hi</sup>* fibroblast subset that had been found to form fibroblastic  
5 foci in the IPF lungs (Figure 6, B-D). qPCR of IPF fibroblasts demonstrated significantly  
6 higher *p16<sup>INK4a</sup>* expression compared to lung fibroblasts isolated from normal controls  
7 (Supplemental Figure 7B, patient demographics data in Supplemental Table 3). IHC  
8 analysis of IPF lungs demonstrated the presence of *p16<sup>INK4a</sup>+ /ACTA2+* and  
9 *p16<sup>INK4a</sup>+ /CTHRC1+* cells within areas of dense fibrotic remodeling, and the absence of  
10 these cells in the normal control lungs (Figure 6E, Supplemental Figure 7C).

11 We previously reported a technique to isolate senescent *p16<sup>INK4a</sup>-hi* fibroblasts  
12 from the human lung by pulsing human lung fibroblasts with CellTrace Far Red (CT<sup>FR</sup>),  
13 a fluorescent dye that is diluted with cell division, and isolating CT<sup>FR</sup>-retaining (CT<sup>FR</sup>-hi)  
14 cells that developed proliferative arrest in culture (14). We applied this technique to  
15 segregate CT<sup>FR</sup>-hi and lo fibroblasts from IPF lungs (from patients undergoing lung  
16 transplantation), and qPCR demonstrated significant enrichment of *p16<sup>INK4a</sup>* expression  
17 in the CT<sup>FR</sup>-hi population (but not *p14<sup>ARF</sup>* encoded in the same locus) along with other  
18 pathologic fibroblast markers such as *p21*, *CTHRC1*, *HAS1*, and *POSTN* (Figure 6F,  
19 Supplemental Figure 7D). These results demonstrate that profibrotic IPF lung fibroblasts  
20 are enriched for *p16<sup>INK4a</sup>* expression, and they can be isolated for drug testing.

21

22 **XL888 deletes human *p16<sup>INK4a</sup>+ fibroblasts from IPF lungs.***

1 To determine whether XL888 preferentially targets  $p16^{INK4a}$ -hi relative to  $p16^{INK4a}$ -lo  
2 fibroblasts from human lungs, we sorted CT<sup>FR</sup>-hi and lo fibroblasts isolated from  
3 explanted IPF lungs. CT<sup>FR</sup>-hi and lo fibroblasts were sorted into separate wells and  
4 dose-escalation challenge with XL888 was performed along with other previously  
5 identified senolytics (ABT263, ABT737, and dasatinib) to determine their potency in  
6 deleting  $p16^{INK4a}$ -hi vs.  $p16^{INK4a}$  lo fibroblasts. Treatment of XL888 preferentially deleted  
7 CT<sup>FR</sup>-hi fibroblasts compared to CT<sup>FR</sup>-lo IPF fibroblasts (Figure 7A). In contrast,  
8 previously described senolytic compounds did not exhibit selective targeting of CT<sup>FR</sup>-hi  
9 fibroblasts, similar to what we found in the PCLS derived from INKBRITE murine lungs  
10 (Supplemental Figure 5D). To investigate the potential efficacy of XL888 in IPF *ex vivo*,  
11 we utilized PCLS generated from IPF lung tissues (Figure 7B). Flow cytometry analysis  
12 indicated high viability of the cells in the PCLS at the end of the 5-day culture period  
13 (Supplemental Figure 7E). IHC analysis of human PCLS showed that XL888 treatment  
14 decreased pathologic  $p16^{INK4a}$ +ACTA2+ and  $p16^{INK4a}$ +CTHRC1+ fibroblasts (Figure 7,  
15 C and D). Taken together, these results demonstrate that our senolytic HTS identified a  
16 compound that preferentially targets  $p16^{INK4a}$ + fibroblasts from fibrotic lungs, which was  
17 subsequently validated *in vivo* and *ex vivo* respectively in mouse models of lung fibrosis  
18 and human IPF samples.

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## 1 **Discussion**

2 The success of high-throughput screens (HTS) of small molecules, as defined by the  
3 identification of compounds that are effective *in vivo*, is highly dependent on whether the  
4 screening target approximates the physiological target that will ultimately serve to  
5 validate the discovery (27). Molecular and cellular targets are selected based on their  
6 relevance to the disease state, as well as the ease with which these targets can be  
7 obtained. Over the past decade, remarkable progress has been made on the  
8 identification of “senolytics,” or compounds targeting cells with senescent properties that  
9 have been implicated in age-related pathologies. Hypothesis-driven candidate screens  
10 that identified senolytics such as D&Q, navitoclax, and fisetin have been shown to  
11 display *in vivo* efficacy in disease models (10, 28, 29), however, the cellular targets for  
12 these senolytics are not redundant. Utilizing various human cell lines where senescence  
13 is induced *in vitro*, these early studies showed that different senolytics have cell-type  
14 specific efficacy *in vitro*, which accounts for the difference in their efficacy in different  
15 mouse disease models *in vivo*. This suggested a heterogeneity in the mechanisms  
16 adopted by senescent cells to resist apoptosis and highlighted the need for a more  
17 rational approach where the screening target cell type directly approximates the  
18 pathogenic cell type *in vivo* being targeted. An ideal screening platform would utilize  
19 disease and organ-specific senescent cells as the screening target, coupled with a  
20 reporter system to validate whether the drug candidates are targeting the same cell type  
21 *in vivo*. However, a major challenge in the field has been isolating senescent cells *in*  
22 *vivo* for prospective studies, as early mouse reporters of senescence (based on the

1 expression of  $p16^{Ink4a}$ ) have been mostly utilized to delete senescent cells for functional  
2 studies.

3 We recently generated an ultrasensitive fluorescent reporter of  $p16^{Ink4a}$   
4 (INKBRITE) that allowed us to identify cells with senescent characteristics *in vivo* as  
5 well as to isolate them from tissues (14). Utilizing this tool, we wanted to determine  
6 whether we could improve the existing senolytic screening pipeline by increasing the  
7 precision and scalability of HTS. We chose lung fibrosis as our model disease because  
8  $p16^{Ink4a+}$  cells have been previously reported to arise *de novo* in the fibrotic lung (19,  
9 20, 26, 30), but the therapeutic role for existing senolytics is less clear. Utilizing our  
10 INKBRITE model, we identified specific  $p16^{INK4a+}$  cell types present in the fibrotic lungs  
11 of mouse and human, showing the contribution of senescent cells to a pathologic  
12 fibroblast subtype that was recently implicated in driving fibrotic remodeling (22, 23).  
13 More importantly, our single cell analysis demonstrated that the fate of  $p16^{Ink4a+}$   
14 fibroblasts is context dependent, as we did not observe the emergence of pathologic  
15  $p16^{Ink4a+}$  fibroblasts in a different injury model (naphthalene) with a different  
16 regenerative outcome. This supports the emerging view that senescent cells are not  
17 monolithic, but rather represent heterogeneous subpopulations that are capable of  
18 diverse responses to physiologic stimuli that can be harmful or beneficial (31). This is  
19 highlighted by our data that  $p16^{INK4a}$  expression alone is not sufficient to drive the  
20 fibrotic response, but rather serves to prime the fibroblasts for maximal fibrotic induction  
21 in response to TGF- $\beta$ 1.

22 The context dependent nature of  $p16^{Ink4a+}$  fibroblasts would also suggest that  
23 their susceptibility to cell killing could differ across tissue states *in vivo*, thus providing

1 the rationale for a senolytic platform that targets specific pathologic contexts. By using  
2 *p16<sup>Ink4a</sup>*+ fibroblasts isolated from fibrotic lungs as the screening target while  
3 simultaneously using *p16<sup>Ink4a</sup>*- fibroblasts as the control within the same well, we were  
4 able to scale up the HTS to include over 2,000 compounds with known biologic activity.  
5 Furthermore, the ability to track these cells allowed us to utilize fibrotic INKBRITE lungs  
6 to validate the efficacy of candidate senolytics in deleting *p16<sup>Ink4a</sup>*+ fibroblasts in  
7 preclinical models. This pipeline can be easily retrofitted to identify candidate senolytics  
8 in alternative disease models where *p16<sup>Ink4a</sup>*+ cells play a pathogenic role. As part of our  
9 screening pipeline, we also leveraged the PCLS *ex vivo* culture system to validate our  
10 HTS and streamline our discovery platform. Our study highlights the potential utility of  
11 the PCLS for preliminary drug screening prior to *in vivo* experimentation, given its  
12 capacity to maintain fibrotic fibroblasts in their native tissue architecture and preserved  
13 extracellular environment comparable to intact tissues (32). We showed high  
14 concordance in the efficacy of candidate senolytic in deleting *p16<sup>Ink4a</sup>*+ cells when  
15 comparing PCLS *ex vivo* with whole animal *in vivo*. This approach can significantly  
16 streamline the screen and reduce the amount of animal testing, which is the most costly  
17 and time-consuming part of the pipeline. Furthermore, it allows us to extend our studies  
18 into human diseased tissues in parallel with preclinical animal models to strengthen the  
19 target validation process (Supplemental Figure 8).

20 Our screening platform ultimately identified XL888, an HSP90 inhibitor, as the  
21 most promising senolytic in our screen for lung fibrosis. XL888 was one of the top hits in  
22 the initial HTS, and we were able to validate its potency in deleting *p16<sup>Ink4a</sup>*+ fibroblasts  
23 in both preclinical model of murine lung fibrosis as well as PCLS of IPF lung tissue.

1 Importantly, XL888 attenuated multiple fibrotic indicators, including total collagen  
2 content, in the preclinical model of lung fibrosis. XL888 is currently in Phase I clinical  
3 trials as a potential anti-cancer agent in solid tumors (33), but our study would suggest a  
4 potential role in fibrotic diseases. While our screen was hypothesis-free, it was  
5 reassuring that we identified a compound class that has previously been shown to  
6 exhibit senolytic properties. HSP90 inhibitors such as geldamycin and 17-AAG were  
7 identified in a previous screen targeting progeroid fibroblasts in mice (29), although  
8 neither compound achieved our 3-signal threshold for secondary validation.  
9 Together, our findings demonstrated that senescent cells, as identified by our novel  
10 INKBRITE reporter for  $p16^{INK4a}$ , contribute to profibrotic fibroblasts in pulmonary fibrosis.  
11 Furthermore, leveraging HTS with our *in vivo* reporter, we identified a new senolytic  
12 compound and validated its efficacy in fibrosis through direct measurement of  
13 senescent cell reduction *in vivo* along with attenuation of disease phenotype. We also  
14 described a method to isolate  $p16^{INK4a+}$  cells from human tissues to validate candidate  
15 senolytics in patient samples that will strengthen the rationale for clinical trials. A major  
16 caveat of our study is that clearly not all  $p16^{INK4a+}$  cells are functionally senescent. Our  
17 prior work had shown that senescent characteristics correlated with  $p16^{INK4a}$  expression  
18 in the lung fibroblasts *in vivo*, which represented a spectrum of phenotypes rather than  
19 a digital (on and off) property (14). This illustrates the complexity of senescent  
20 phenotypes *in vivo* that is not as well described compared to senescent cells *in vitro*, but  
21 also supports the necessity of screening against cellular targets *in vivo* to increase our  
22 understanding of how senolytics function to target specific subtypes of cells with  
23 senescent properties arising *in vivo*.

## 1 **Methods**

### 2 **Human lung samples**

3 Studies involving human tissue were approved by the UCSF Institutional Review Board.  
4 All subjects provided written informed consent. Peripheral regions of the normal lungs  
5 were obtained to select for the distal regions of the lung from brain-dead donors that  
6 were rejected for lung transplantation. IPF lung specimens were taken from the  
7 periphery of the lung at the time of lung transplant. Age and sex of tissue donors are  
8 listed in Supplementary Table 3.

9

### 10 **Sex as a biological variable**

11 Sex was not considered as a biological variable.

12

### 13 **Animal studies**

14 All mice were housed and treated in accordance with the IACUC protocol approved at  
15 the University of California, San Francisco. Mice between the ages of 8-12 weeks old  
16 were used for the experiments with balance of gender between groups. C57BL/6 mice  
17 were obtained from Jackson Laboratory. Generation and genotyping of INKBRITE and  
18 *Dermo1<sup>Cre/+</sup>·p16<sup>flox/flox</sup>* lines were performed as previously described (14). For bleomycin-  
19 induced injury, mice were given pharmaceutical-grade bleomycin (Hospira) dissolved in  
20 PBS via intranasal instillation (2.5 U per kg body weight). For naphthalene injury, mice  
21 were administered with 300 mg/kg of naphthalene (Sigma) dissolved in corn oil by  
22 intraperitoneal injection. For XL888 treatment, mice were treated with 62.5 mg/kg via  
23 oral delivery 5 days a week for 2 weeks starting 10 days after bleomycin injury. XL888  
24 was dissolved in 10 mM hydrochloric acid (HCL) with the concentration of 15.625

1 mg/ml. After vigorous vortexing, the dissolved XL888 was delivered to the mice using  
2 oral gavage daily. For DQ treatment in mice, C57BL/6 mice received a single dose of  
3 bleomycin (2.5 U/kg, day 1) and received dasatinib (5 mg/kg) and quercetin (50 mg/kg)  
4 at day 5, 11, and 17 following the previous study (21). Whole lung tissues from the mice  
5 were collected at day 23 for hydroxyproline assay.

6

### 7 **Histology and immunohistochemistry**

8 For paraffin embedded mouse lungs, mouse right ventricles were perfused with 1 ml  
9 PBS and the lungs were inflated with 4% PFA, and then fixed in 4% PFA overnight at  
10 4°C. After fixation, the lungs were washed by cold PBS X 4 times in 2 hrs at 4°C and  
11 dehydrated in a series of increasing ethanol concentration washes (30%, 50%, 70%,  
12 95% and 100%). The dehydrated lungs were incubated with Xylene for 1 hr at RT and  
13 with paraffin at 65°C for 90 min X 2 times, and then embedded in paraffin and  
14 sectioned. Human and mouse PCLS samples were fixed in 4% PFA for 30 mins. After  
15 PBS washes, slices were embedded in OCT after 30% sucrose incubations. 6-8 µm  
16 thick cryosections were used for immunohistochemistry. Following antibodies were  
17 used: GFP (1:400, Abcam, ab6673), Alpha smooth muscle actin (1:200, Abcam,  
18 ab5694), Transgelin (1:200, Abcam, ab14106), and Collagen I (1:200, Abcam,  
19 ab21286). Human lung fragments were fixed and processed as the mouse lungs.  
20 Antibodies used for human lung slide staining were ACTA2 (1:200, Abcam, ab5694),  
21 p16<sup>INK4a</sup> (1:200, Santa Cruz, sc-56330), and CTHRC1 (1:200, Abcam, ab85739).  
22 Collagen staining was performed using Trichrome stain kit according to the

1 manufacturer's protocol (Abcam, ab150686). Images were captured using Zeiss Imager  
2 M1 or Leica Stellaris 5.

3

#### 4 **RNA *in situ***

5 Paraffin-embedded lung sections were used for RNA in situ detection of Cthrc1 using a  
6 RNAscope Multiplex Fluorescent Reagent kit (ACD biotechnne) according to the  
7 manufacturer's instructions.

8

#### 9 **Lung digestion and Fluorescence Activated Cell Sorting (FACS)**

10 Dissected mouse lung was tracheally perfused with a digestion cocktail of Collagenase  
11 Type I (225 U/ml, Thermo Fisher), Dispase (15 U/ml, Thermo Fisher) and Dnase (50  
12 U/ml, Sigma) after perfusion with PBS and removed from the chest. The lung was  
13 incubated in a digestion cocktail for 45 mins at 37°C with continuous shaking. The  
14 mixture was then washed with a FACS buffer (2% FBS and 1% Penicillin-Streptomycin  
15 in DMEM). The mixture was passed through a 70 µm cell strainer and resuspended in a  
16 red blood cell (RBC) lysis buffer, then passed through a 40 µm cell strainer. Cell  
17 suspensions were incubated with the appropriate conjugated antibodies in a sorting  
18 buffer for 30 min at 4°C and washed with FACS buffer. Doublets and dead cells were  
19 excluded based on forward and side scatter and SYTOX Blue (Invitrogen, S34857),  
20 respectively.

21 The following antibodies were used for staining: CD45-PE-Cy7 (Invitrogen, 50-  
22 112-9643), CD45-BV421 (BD, 563890), CD31-BV711 (BD, 740680), CD31-BV421  
23 (Invitrogen, 48-0311-82), EpCAM-PE (BD, 563477), EpCAM-BV421 (BD, 563214).

1 Immune (CD45-biotin, Biolgened, 103104), epithelial (CD326-biotin, Biolegend, 118204)  
2 and endothelial (CD31-Biotin, Biolegend, 102404) cells are removed with EasySep  
3 mouse streptavidin RapidShperes (StemCell, 19860A), when applicable. FACS was  
4 performed on a BD FACS Aria using FACSDiva Software. CD45- CD31- EpCAM- cells  
5 were sorted for mesenchymal cells, the GFP- and GFP+ fibroblasts were further  
6 separated and were sorted into FACS buffer. Analysis was performed using FlowJo  
7 software.

8 For the human lung, a distal piece (~10 cm<sup>3</sup>) was dissected from the whole lung  
9 and washed with HBSS X 4 times in 15 min. The piece of lung was further diced with  
10 razor blades and was added into the digestion cocktail of Collagenase Type I (225 U/ml,  
11 Thermo Fisher), Dispase (15 U/ml, Thermo Fisher) and Dnase (100 U/ml, Sigma). The  
12 mixture was incubated for 2 h at 37°C and vortexed intermittently. The mixture was then  
13 liquefied with a blender and passed through 4X4 gauze, a 100 mm and a 70 mm cell  
14 strainer. The mixture was resuspended in RBC lysis buffer, before passing through a 40  
15 mm cell strainer. The cell suspensions were incubated with the antibodies in the FACS  
16 buffer for 30 min at 4°C and washed with the FACS buffer. The following antibodies  
17 were used for staining: CD45-APC-Cy7 (BioLegend, 304014), CD31-APC-Cy7  
18 (BioLegend, 303120), CD11b-APC-Cy7 (BD Biosciences, 557754), EpCAM-PE  
19 (BioLegend, 324206). DAPI (0.2 mg/ml) was used to exclude dead cells. Single cells  
20 were selected and CD45- CD11b- CD31- EpCAM- cells were sorted for mesenchymal  
21 cells. Cells were sorted into FACS buffer. FACS analysis was performed by FACSDiva  
22 (BD).

23



## 1 **Quantitative RT-PCR (qPCR)**

2 Total RNA was obtained from cells using PicoPure RNA Isolation Kit (Applied  
3 Biosystems, KIT0204) or RNeasy mini kit (QIAGEN, 74106), following the  
4 manufacturers' protocols. cDNA was synthesized from total RNA using the SuperScript  
5 Strand Synthesis System (Thermo Fisher, 18080044). Quantitative RT-PCR (qRT-PCR)  
6 was performed using the SYBR Green system (Thermo Fisher, F415L). Relative gene  
7 expression levels after qRT-PCR were defined using the  $\Delta\Delta C_t$  method and normalizing  
8 to the housekeeping genes. The qRT-PCR primers used for mouse are as follows:  
9 Cthrc1-F: CAGTTGTCCGCACCGATCA; Cthrc1-R: GGTCCTTGTAGACACATTCCATT;  
10 Col1a1-F; TGA CTGGAAGAGCGGAGAGT; Col1a1-R: GTTCGGGCTGATGTACCAGT;  
11 Col3a1-F: CTGTAACATGGAAACTGGGGAAA; Col3a1-R:  
12 CCATAGCTGAACTGAAAACCACC; Spp1-F: AGCAAGAACTCTTCCAAGCAA; Spp1-  
13 R: GTGAGATTCGTCAGATTCATCCG; Acta2-F: ACTCTCTTCCAGCCATCTTTCA;  
14 Acta2-R: ATAGGTGGTTTCGTGGATGC; Postn-F: TGGTATCAAGGTGCTATCTGCG;  
15 Postn-R: AATGCCCAGCGTGCCATAA; S100a4-F: TGAGCAACTTGGACAGCAACA;  
16 S100a4-R: CTTCTTCCGGGGCTCCTTATC; Tagln-F:  
17 GGTGGCTCAATTCTTGAAGGC; Tagln-R: TGCTCCTGGGCTTTCTTCATA; p16INK4a-  
18 F: AATCTCCGCGAGGAAAGC; p16INK4a-R: GTCTGCAGCGGACTCCAT; p21-F:  
19 TAAGGACGTCCCCTTTGCC; p21-R: CGTCTCCGTGACGAAGTCAA; Gapdh-F:  
20 GGCCCCTCCTGTTATTATGGGGGT; Gapdh-R:  
21 CCCCAGCAAGGACACTGAGCAAGA. The primers used for human are as follows:  
22 *p16INK4a*-F: GTCGGGTAGAGGAGGTGCG; *p16INK4a*-R:  
23 CATGACCTGGATCGGCCTC; *p21*-F: TTGTACCCTTGTGCCTCGCT; *p21*-R:

1 CGTTTGGAGTGGTAGAAATCTGTC; *CTHRC1*-F: GTGGCTCACTTCGGCTAAAAT;  
2 *CTHRC1*-R: CACTAATCCAGCACCAATTCCTT; *HAS1*-F:  
3 TCAAGGCGCTCGGAGATTC; *HAS1*-R: CTACCCAGTATCGCAGGCT; *SPP1*-F:  
4 GAAGTTTCGCAGACCTGACAT; *SPP1*-R: GTATGCACCATTCAACTCCTCG; *POSTN*-  
5 F: CTCATAGTCGTATCAGGGGTCG; *POSTN*-R: ACACAGTCGTTTTCTGTCCAC;  
6 *ACTA2*-F: AAAAGACAGCTACGTGGGTGA; *ACTA2*-R:  
7 GCCATGTTCTATCGGGTACTTC; *COL1A1*-F: GGGGTAAGTCCCTTTCTGCC;  
8 *COL1A1*-R: ATTGCCTTTGATTGCTGGGC; *RPL19*-F: CCCATCTTTGATGAGCTTCC;  
9 *RPL19*-R: TGCTCAGGCTTCAGAAGAGG.

10

## 11 **Single-cell RNA sequencing and analysis**

12 Single cell sequencing was performed on a 10X Chromium instrument (10X Genomics)  
13 at the Institute of Human Genetics (UCSF, San Francisco, CA). Briefly, live mouse lung  
14 cells were sorted and resuspended in 50  $\mu$ l PBS with 0.04% BSA at 1,000 cells/ $\mu$ l and  
15 loaded onto a single lane into the Chromium Controller to produce gel bead-in  
16 emulsions (GEMs). GEMs underwent reverse transcription for RNA barcoding and  
17 cDNA amplification. The library was prepped with the Chromium Single Cell 3' Reagent  
18 Version 3 kit. The samples were sequenced using the HiSeq2500 (Illumina) in Rapid  
19 Run Mode. We used the Seurat R package along with a gene-barcode matrix provided  
20 by CellRanger for downstream analysis. Following the standard workflow of Seurat, we  
21 generated Seurat objects after using ScaleData, RunPCA, RunUMAP. For human  
22 scRNA-seq data, we used processed scRNA-seq data from normal and IPF lungs from

1 GSE147066. After generating subsets of lung fibroblasts, violin plots and density plots  
2 were generated.

3

#### 4 **Cell Culture**

5 Freshly isolated mesenchymal cells from INKBRITE lungs (GFP- or GFP+) or human  
6 lung fibroblasts were cultured in DMEM/F-12 (Thermo Fisher, 11330032) with 10% FBS  
7 and 1% Pen/Strep. The medium was changed every 2 days and lung fibroblasts were  
8 maintained for no more than 3 passages.

9

#### 10 **TGF- $\beta$ 1 *in vitro* stimulation**

11 Fibroblasts were sorted from fibrotic INKBRITE lungs.  $1 \times 10^5$  cells were seeded into 48  
12 well plates and cultured in DMEM/F12 with 2% FBS and 1% Pen/Strep for 24 h.  
13 Medium was changed to serum free DMEM with 1% Pen/Strep for 24 h. After the serum  
14 starvation, medium was changed to serum-free DMEM/F-12 with 1% Pen/Strep and 1  
15 ng/ml TGF- $\beta$ 1 (Peprotech, 10778-032). After 24-48 h stimulation, RNA was obtained  
16 using RNeasy mini kit (QIAGEN, 74106).

17

#### 18 **Lentivirus infection**

19 Primary human lung fibroblasts were seeded and infected the following day with  
20 lentivirus (Lenti- tTS/rtTA, Lenti-TRE-p16INK4a-T2A-dTomato). On day 1, the fibroblasts  
21 were infected with lentivirus at 5 multiplicity of infection (MOI) in DMEM-F12 with 10%  
22 FBS and polybrene at 5  $\mu$ g/ml. On day 2, cells were washed with 1X PBS 4 times and  
23 placed on regular media (DMEM-F12, 10%FBS, 1% PS). Doxycycline (1  $\mu$ g/ml)

1 treatment began 72 to 96 hours later for Lenti-tTS/rtTA and Lenti-TRE-p16 dual-  
2 transduced cells.

3

#### 4 **Adenovirus infection**

5 Primary mouse lung fibroblasts were seeded and infected the following day with  
6 adenovirus expressing GFP. On day 1, the fibroblasts were infected with adenovirus at  
7 260 MOI in DMEM-F12 with 10% FBS. On day 2, cells were washed with 1X PBS 4  
8 times and placed on regular media (DMEM-F12, 10%FBS, 1% PS). Cells were treated  
9 with XL888 from day 3 to day 6.

10

#### 11 **High-throughput screening**

12 The screen was performed in collaboration with the Small Molecule Discovery Center  
13 (SMDC) at UCSF. INKBRITE mice received a single dose of bleomycin at 2.5 U/kg and  
14 lung tissues were collected 14 days after the injury. Single cell suspension of the lung  
15 was prepared and sorted as described above. A suspension of GFP+ and GFP-  
16 fibroblast cells (1:1 ratio) was plated in 384 well-plates (Greiner Bio-One, 781096) at  
17 2000 cells/well density using WellMate™ liquid dispenser (Matrix). After an overnight  
18 incubation, 2400 test compounds from SelleckChem bioactive and epigenetic library  
19 were added using Biomek FXp (Beckman Coulter) pin tools to a final concentration of 1  
20  $\mu\text{M}$ , followed by a 3-day incubation. On the third day, plates were washed with 1X PBS  
21 and NucView530 dye mix was dispensed at 2  $\mu\text{M}$  final concentration into each well  
22 using EL406 washer dispenser (BioTek), followed by an hour incubation. Plates were  
23 then washed with 1X PBS and fixed with an addition of 4% PFA + 1 $\mu\text{g/ml}$  Hoechst mix

1 into each well. After 15 min incubation, plates were washed with 1X PBS and imaged.  
2 Fluorescence images were captured using IN Cell Analyzer 6500HS (Cytiva) at 20X  
3 magnification, 4 field-of-views per well. Images were processed using IN Cell Developer  
4 Tool Box. Nuclear mask was created from the Hoechst channel and was applied to both  
5 GFP and NucView channels to calculate the number of total cells, GFP+, GFP-, and  
6 NucView+ cells.

7 Compounds that caused GFP+ cell count to be below the 3SD limit of the  
8 negative control (wells with no test compound) were determined as hit candidates.  
9 Thirty-two compounds were selected for a follow up validation screen based on  
10 compounds' identity and targets. Dose response curves and their IC50s were generated  
11 for these 32 compounds with the concentration ranging between 0.005  $\mu$ M to 2.5  $\mu$ M (2-  
12 fold dilution).

13

#### 14 **Generation of precision-cut lung slices (PCLS) culture**

15 For mouse PCLS, INKBRITE mice were injured with 2.5 U/kg of bleomycin and lung  
16 tissues were collected 2 weeks after the injury. The lungs were perfused with PBS  
17 through the right ventricle and inflated with 1 to 2 ml of 2% agarose (Thermo Fisher,  
18 16550100) dissolved in PBS by trachea. After inflation, the trachea was tied with a  
19 suture to prevent agarose leakage. Lungs were dissected from the chest cavity and  
20 submerged in ice-cold PBS to solidify agarose. Lung lobes were sliced at a width of 500  
21  $\mu$ m using a vibratome (Leica, VT 1000S).

22 For human PCLS, fresh lung tissues were obtained from IPF patients that  
23 underwent lung transplantation. After washing with PBS, the tissues were inflated with

1 warm 2% agarose and placed in cold PBS. The lung specimens were cut into strips and  
2 sliced into 600  $\mu\text{m}$  thick slices using a vibratome.

3 The slices were cultured in DMEM/F-12 (Thermo Fisher, 11330032) with 1%  
4 Pen/Strep under standard cell culture conditions (37C, 5% CO<sub>2</sub>). ABT263 (2.5  $\mu\text{M}$ ),  
5 ABT737 (2  $\mu\text{M}$ ), Fisetin (10  $\mu\text{M}$ ), DQ (1  $\mu\text{M}$  + 20  $\mu\text{M}$ ), and XL888 (1  $\mu\text{M}$ ) were treated  
6 during the culture. 1  $\mu\text{M}$  of concentration was used for other candidate compounds  
7 (TSA, ganetespib, fimepinostat, and dacinostat). At day 5, cultured PCLSs were  
8 processed for downstream analyses.

9

#### 10 **Flow cytometry analysis of mouse PCLS**

11 The lung slices were placed into 15 ml conical tubes containing 1 ml of digestion  
12 cocktail of Dispase (3 U/ml, Thermo Fisher) and Dnase (50 U/ml, Sigma) after PBS  
13 washes. The slices were incubated in a digestion cocktail for 30 mins at 37°C with  
14 continuous shaking. The mixture was then washed with a FACS buffer (2% FBS and  
15 1% Penicillin-Streptomycin in DMEM). The mixture was passed through a 70  $\mu\text{m}$  cell  
16 strainer. Cells were stained with antibodies and analyzed by flow cytometry as  
17 described above.

18

#### 19 **Hydroxyproline assay**

20 Collagen content in the lungs was assessed by measuring the hydroxyproline level  
21 using the Hydroxyproline Colorimetric Assay Kit (K555-100) from BioVision. Briefly, lung  
22 tissue was homogenized in water and the homogenized samples were hydrolyzed by  
23 incubation with 12N hydrochloric acid at 120°C for 3 hours. The hydrolysates were

1 oxidized using chloramine T, followed by incubation with Ehrlich's perchloric acid  
2 reagent. Absorbance was measured at 560 nm.

3

#### 4 **CellTrace Far Red (CT<sup>FR</sup>)**

5 Isolated fibroblasts were cultured for 3 days and stained with CellTrace Far Red  
6 reagent. The fibroblasts were detached and stained with 1  $\mu$ M of CellTrace for 20  
7 minutes at 37°C (1 million cells per ml) following the manufacturer's protocol. After  
8 staining, the cells were washed with media and cultured for 3 to 4 days. Serum-starved  
9 cells after CT<sup>FR</sup> staining were used to separate CT<sup>FR</sup> high and low cells based on CT<sup>FR</sup>  
10 levels. CT<sup>FR</sup> stained cells within the high intensity range of 95 to 97% encompassing  
11 serum-starved, non-proliferating cells were sorted as CT<sup>FR</sup> high cells, while cells  
12 exhibiting a lower intensity range were sorted as CT<sup>FR</sup> low cells.

13

#### 14 **Statistics**

15 All data are presented as mean  $\pm$  SD. Statistical differences between the groups were  
16 compared using unpaired two-tailed Student's *t* test or one-tailed Student's *t* test for 2  
17 groups or 1-way ANOVA for multiple groups. Statistical significance was defined as \**P* <  
18 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. Statistical details and the number of replicates for each  
19 experiment can be found in the figure legends. The following formula is used to  
20 compute sample size and power:

21

$$n_A = \kappa n_B \text{ and } n_B = \left(1 + \frac{1}{\kappa}\right) \left(\sigma \frac{z_{1-\alpha/2} + z_{1-\beta}}{\mu_A - \mu_B}\right)^2$$

$$1 - \beta = \Phi(z - z_{1-\alpha/2}) + \Phi(-z - z_{1-\alpha/2}), \quad z = \frac{\mu_A - \mu_B}{\sigma \sqrt{\frac{1}{n_A} + \frac{1}{n_B}}}$$

- 1
- 2  $\kappa = n_A/n_B$  is the matching ratio
- 3  $\sigma$  is standard deviation
- 4  $\Phi$  is the standard normal distribution function
- 5  $\Phi^{-1}$  is the standard normal quantile function
- 6  $\alpha$  is Type I error
- 7  $\beta$  is Type II error, meaning  $1 - \beta$  is power
- 8

## 9 **Study approval**

10 Animal husbandry and all experiments were conducted under Institutional Animal Care  
 11 and Use Committee-approved protocols at University of California, San Francisco (No.  
 12 AN191522-01J). Human specimen isolation from explanted lungs of patient undergoing  
 13 lung transplantation at UCSF is approved by Institutional Review Board at UCSF (No.  
 14 13-10738).

15

## 16 **Data availability**

17 Previously published human scRNA-seq data that are re-analyzed in this study are  
 18 available in NCBI Gene Expression Omnibus (GEO) under the accession number  
 19 GSE147066. The sequencing data of the mouse that support the findings of this study  
 20 have been deposited in the accession number GSE235352. The values for all data  
 21 points in graphs are reported in the Supporting Data Values file.

22

## 23 **Author contributions**



1 J.L. and T.P. conceived the experiments and wrote the manuscript. J.L., S.R., M.Z.,  
2 M.K., N.R., C.R., G.P., C.W., K.A., and T.T. performed the experiments. P.W. provided  
3 clinical specimen. D.S. and M.A. provided expertise and feedback.

4

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13 Ireland Program Award for human lung collection.

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1 **Figure Legends**

2 **Figure 1.  $p16^{Ink4a+}$  fibroblasts contribute to pathologic fibroblasts in mouse model**  
3 **of lung fibrosis.**

4 (A) Experimental scheme for scRNAseq of  $p16^{Ink4a+}$  (GFP+) fibroblasts from the  
5 INKBRITE lung after bleomycin-induced fibrosis (14 dpi).

6 (B) Violin plot showing profibrotic gene expressions in the different  $p16^{Ink4a+}$  fibroblast  
7 subsets *in vivo*.

8 (C) Visualization of *Acta2*, *Cthrc1*, *Col1a1* and *Tagln* expression pattern within the  
9 fibroblast subsets.

10 (D) Representative images showing *Cthrc1* (RNAscope in situ), ACTA2, TAGLN, and  
11 COL1A1 (immunostaining) in lung sections of bleomycin-injured INKBRITE mice (14  
12 dpi) co-localized with GFP (arrows:  $p16^{Ink4a+}$  fibroblasts). Scale bars, 100  $\mu$ m.

13 (E) qPCR analysis of purified GFP+ and GFP- fibroblasts from bleomycin-treated  
14 INKBRITE lungs (n = 5-6 biological replicates, experiment repeated 2X).

15 (F) qPCR analysis of cultured GFP+ and GFP- fibroblasts isolated from fibrotic  
16 INKBRITE lungs after treatment of recombinant TGF- $\beta$ 1 or vehicle (n =3 technical  
17 replicates, experiment repeated 2X).

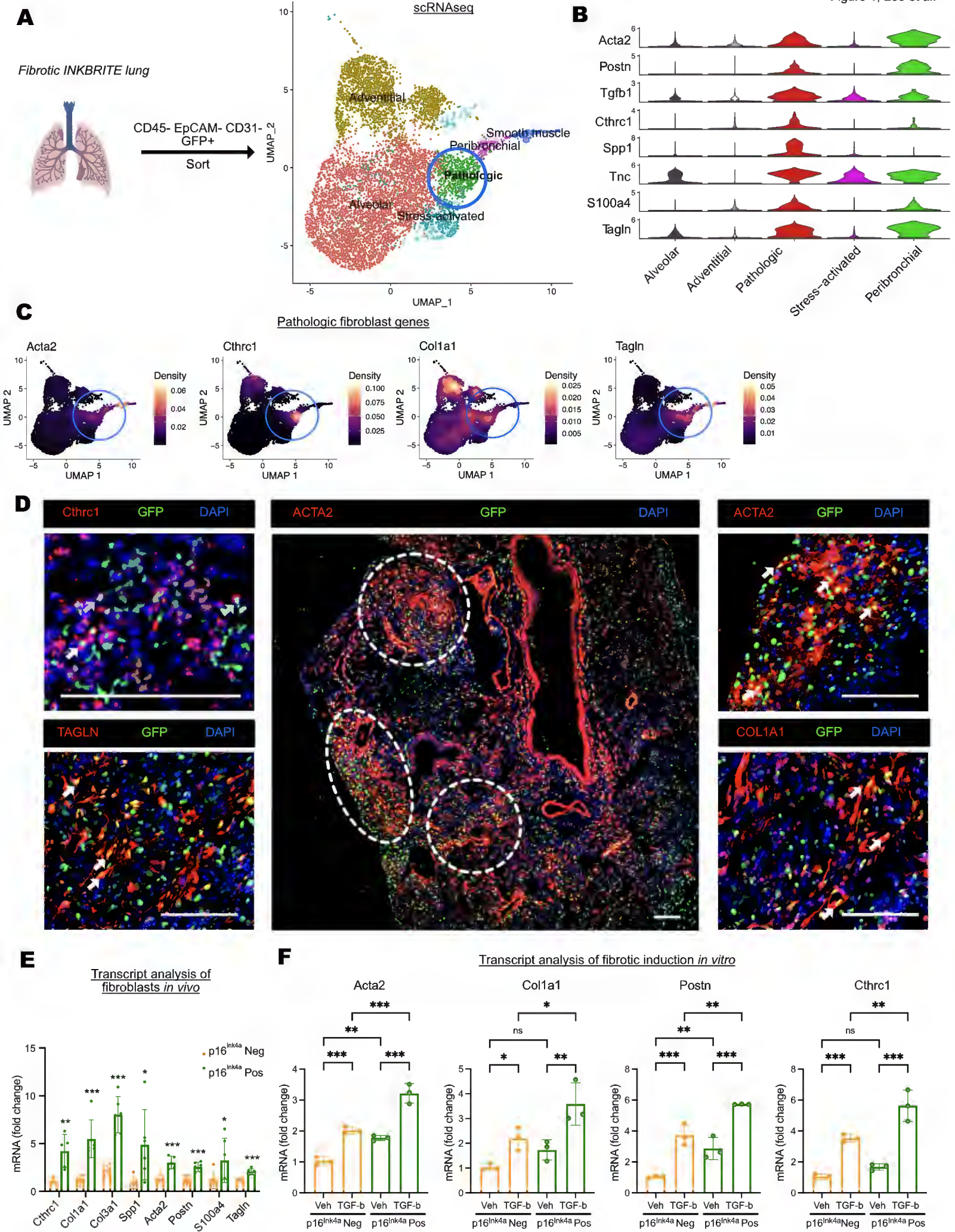
18 Data are represented as mean  $\pm$  SD.; \* $P$  < 0.05; \*\* $P$  < 0.01; \*\*\* $P$  < 0.001; two-tailed  
19 Student's t test (E); or one-way ANOVA (F).

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1 **Figure 2. *p16<sup>INK4a</sup>* expression primes lung fibroblasts to augment the fibrotic**  
2 **response.**

3 **(A)** Transcript analysis of cultured primary human lung fibroblast isolated from control  
4 cadaveric donors transduced with two lentiviral vectors to overexpress (OE) human  
5 *p16<sup>INK4a</sup>* with doxycycline induction followed by addition of TGF- $\beta$ 1. (n =3 technical  
6 replicates, experiment repeated 2X)

7 **(B)** Representative H&E sections of *Dermo1<sup>Cre/+</sup>;*p16<sup>flox/flox</sup>** and control (*p16<sup>flox/flox</sup>*)  
8 animals injured with bleomycin to induce lung fibrosis.

9 **(C)** Hydroxyproline assay to quantify collagen in the left lung of *Dermo1<sup>Cre/+</sup>;*p16<sup>flox/flox</sup>**  
10 and control animals 14 days following bleomycin injury (n = 5 control, 9 mutant  
11 biological replicates).

12 **(D)** Representative IHC showing ACTA2 and COL1 immunostaining in lung sections of  
13 bleomycin-injured *Dermo1<sup>Cre/+</sup>;*p16<sup>flox/flox</sup>** and control (*p16<sup>flox/flox</sup>*) animals (14 dpi).

14 **(E)** IHC quantification of ACTA2+ and COL1+ fibroblasts from (D) (n = 5 control, 9  
15 mutant biological replicates), Scale bars, 200  $\mu$ m.

16 Data are represented as mean  $\pm$  SD.; \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; two-tailed  
17 Student's t test **(A, C, E)**.

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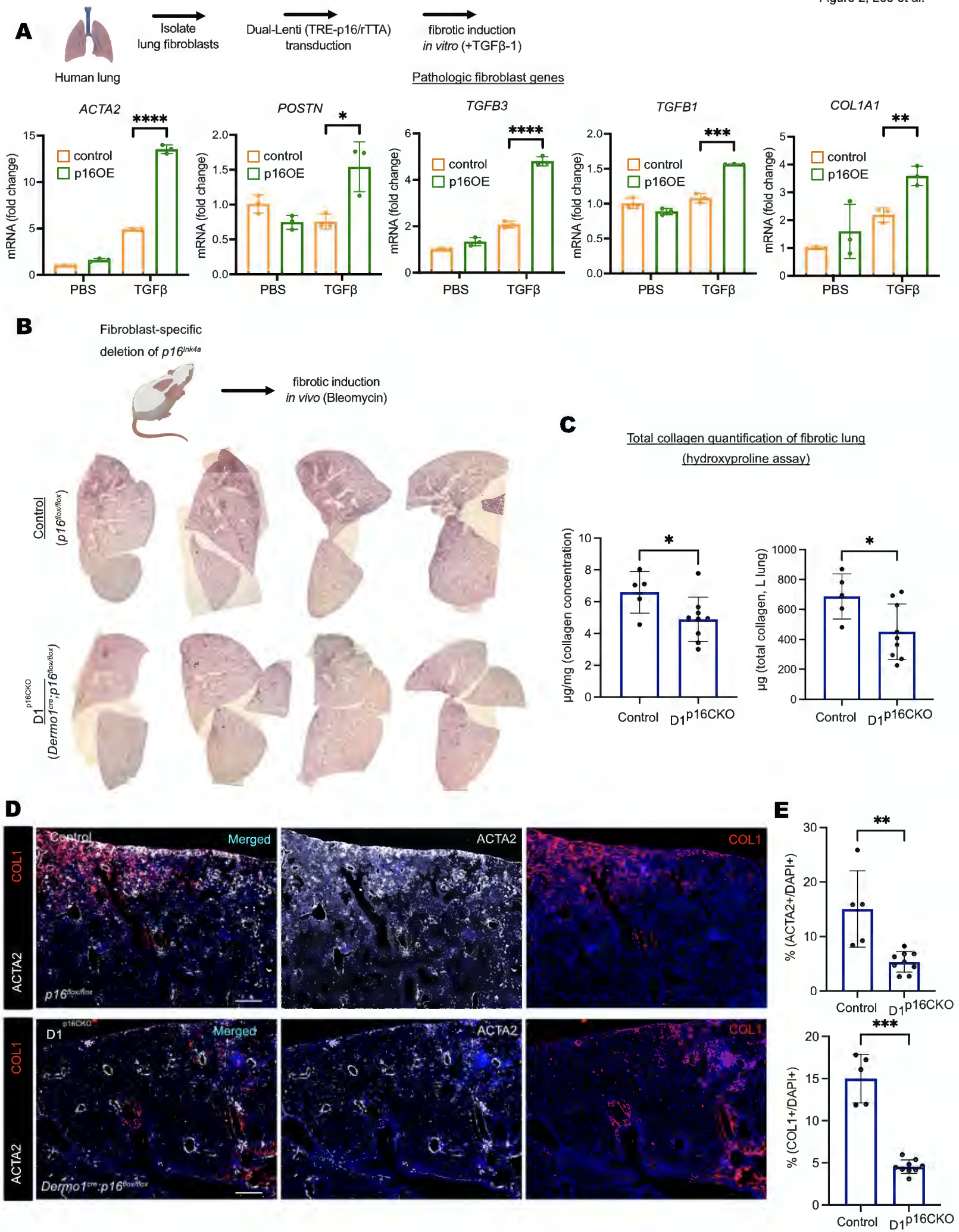
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1 **Figure 3. High-throughput screen (HTS) targeting  $p16^{Ink4a+}$  fibroblasts isolated**  
2 **from fibrotic INKBRITE lungs.**

3 (A) Schematic outline of the HTS to identify compounds targeting  $p16^{Ink4a+}$  (GFP+)  
4 fibroblasts from the fibrotic INKBRITE lungs.

5 (B) Scatter plot showing hit results from each well containing compound (pink) or  
6 vehicle (green). Y-axis indicate %GFP+ cells in each well after compound exposure.  
7 Compounds exceeding 3-sigma for lowest %GFP were selected for validation.

8 (C) Cell count GFP+ and GFP- fibroblasts of the top senolytic candidates.

9 (D) Biologic pathways targeted by the top senolytic candidates.

10 (E) Schematic outline of dose-response analysis of the top senolytic candidate from the  
11 primary screen.

12 (F) Top candidates emerging from the secondary validation using dose-response with  
13 lowest IC50 values, including trichostatin A, XL888, and ganetespib.

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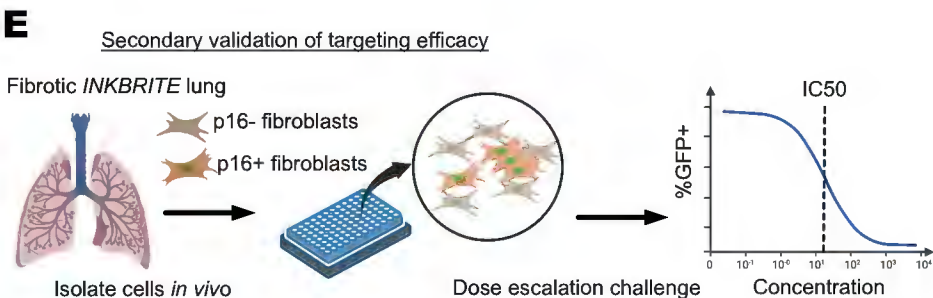
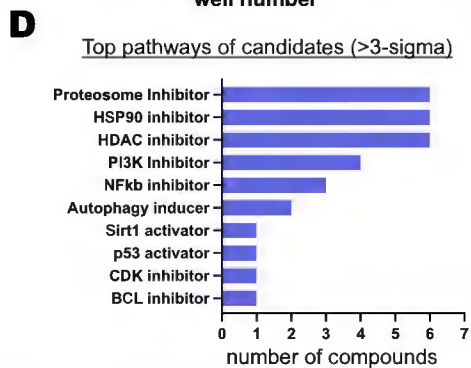
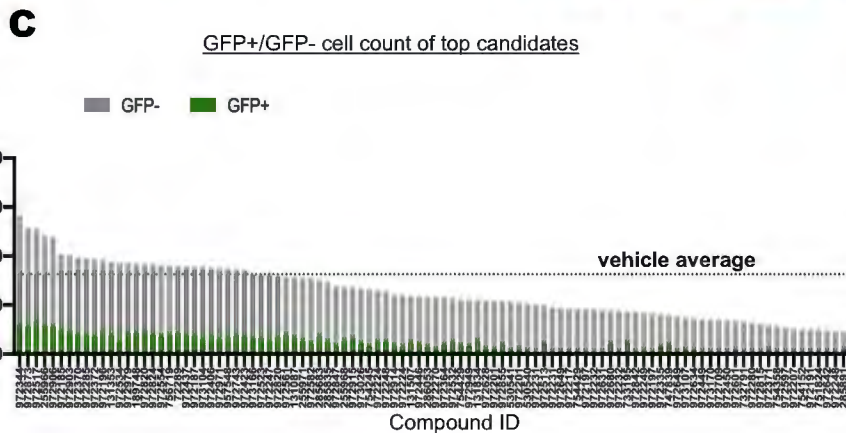
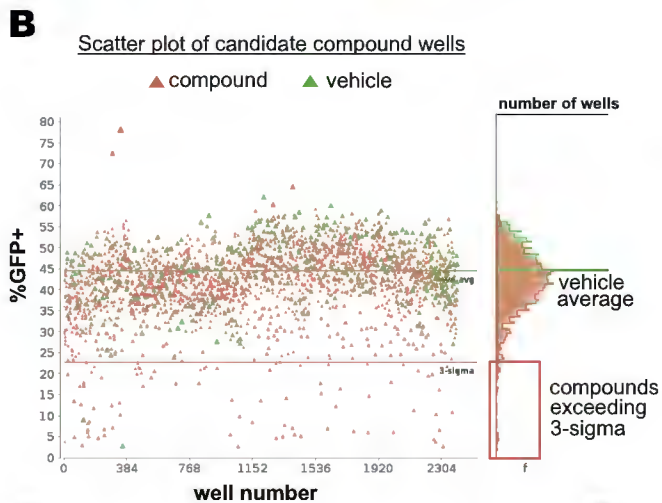
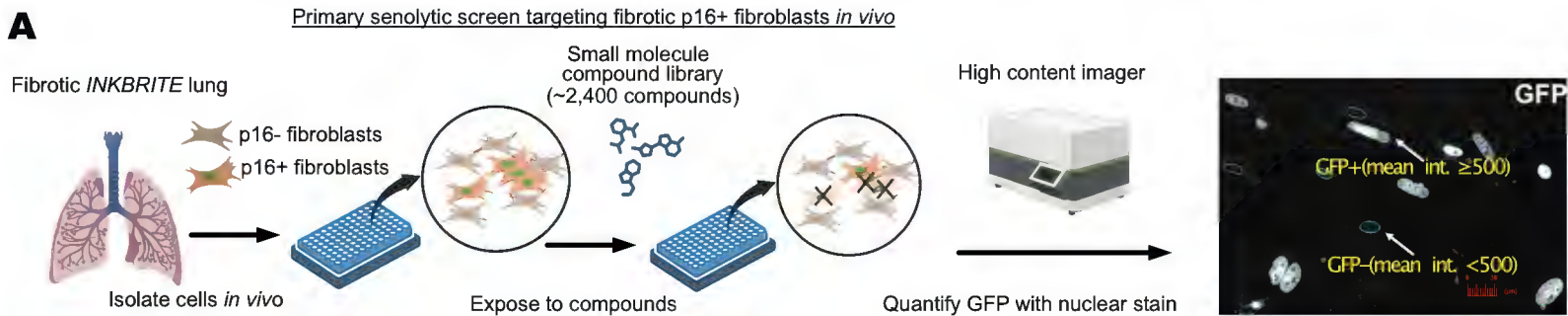
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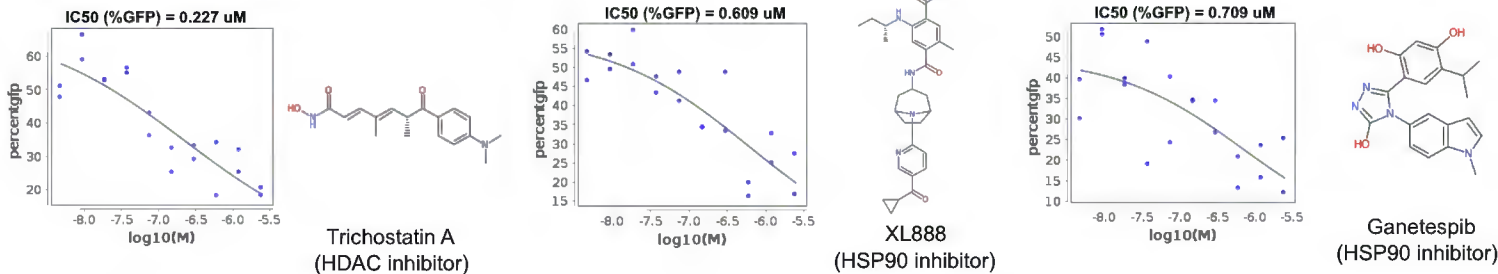
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**F** Top candidate compounds (lowest IC50 for %GFP)



1 **Figure 4. Validation of candidate senolytic compounds using mouse precision-cut**  
2 **lung slices (PCLS) derived from fibrotic INKBRITE lungs.**

3 (A) Experimental scheme for *ex vivo* culture of mouse PCLS derived from fibrotic  
4 INKBRITE mouse to test senolytic candidates.

5 (B) Bright field and GFP images of cultured PCLS. Scale bars= 2000  $\mu\text{m}$ .

6 (C) Gating strategy to analyze GFP+ fibroblasts from mouse PCLS by flow cytometry.

7 (D) Quantification of GFP+ fibroblasts in the PCLS cultured with vehicle or XL888 (1  
8  $\mu\text{M}$ ) for 5 days (n=10 technical replicates, experiment repeated 2X).

9 (E-F) Immunofluorescence analysis (E) and quantification (F) of ACTA2, COL1A1, and  
10 GFP in mouse PCLS treated with vehicle or XL888 (1  $\mu\text{M}$ ). (n=9 technical replicates,  
11 experiment repeated 2X). Scale bars, 50  $\mu\text{m}$ .

12 Data are represented as mean  $\pm$  SD.; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; two-tailed  
13 Student's t test (D, F).

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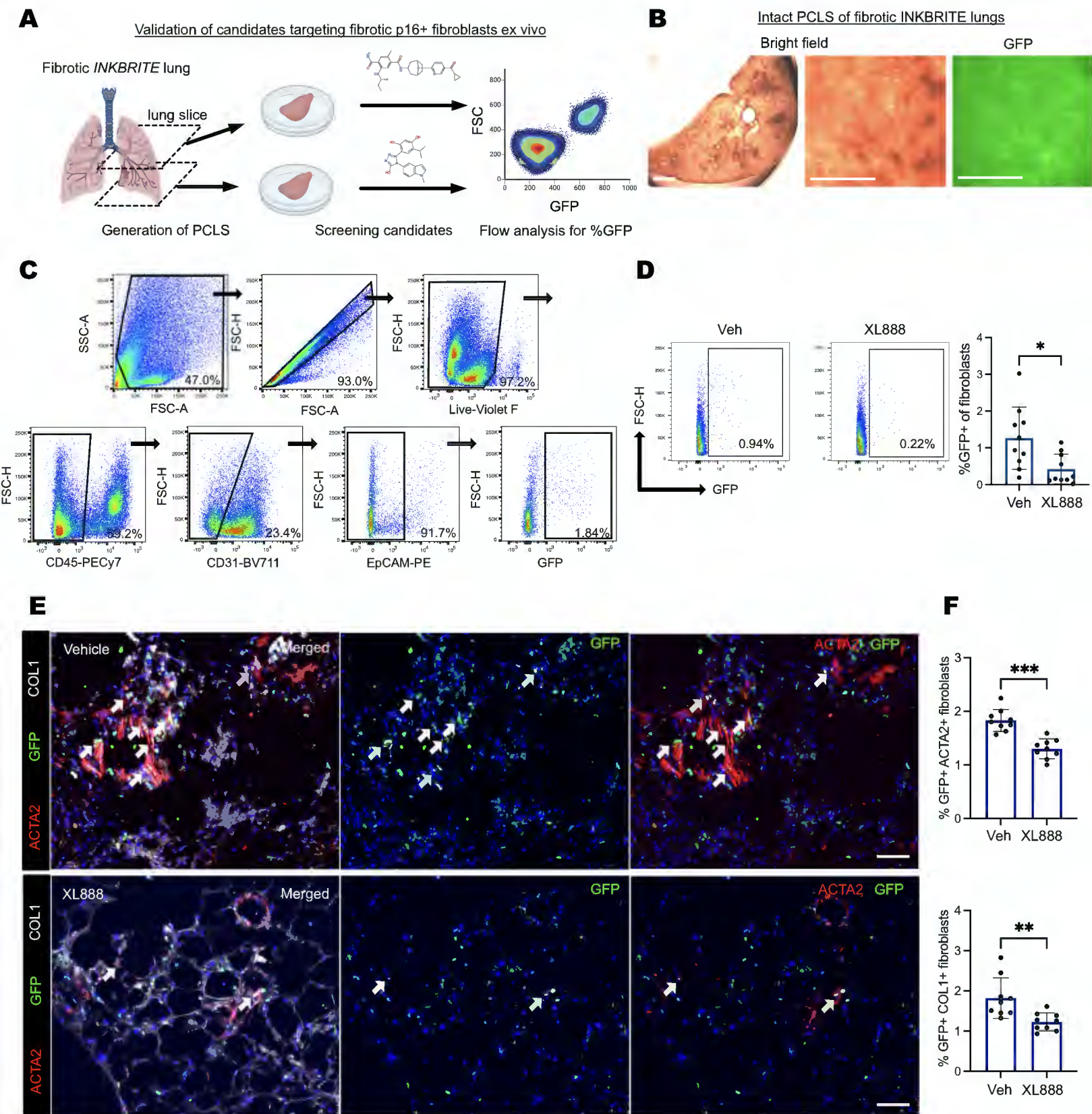
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1 **Figure 5. XL888 deletes  $p16^{Ink4a}$ + fibroblasts and attenuates fibrotic remodeling *in***  
2 ***vivo*.**

3 (A) Schematic outline of animal experiments to validate *in vivo* efficacy of candidate  
4 senolytics.

5 (B-C) Flow cytometry analysis of GFP+ fibroblasts (% of fibroblasts that are GFP+) in  
6 bleomycin-injured lungs of vehicle or XL888 delivered INKBRITE animals (n = 11-12  
7 biological replicates, experiment repeated 2X).

8 (D-E) Immunofluorescence analysis (D) and quantification (E) of GFP+ cells among  
9 ACTA2+ fibroblasts in the lungs of vehicle or XL888-treated INKBRITE mice (n = 4  
10 biological replicates, experiment repeated 2X). Scale bars, 100  $\mu$ m.

11 (F) Representative images (left) and quantification of Masson's trichrome staining of  
12 lung sections from indicated group of mice after bleomycin injury (n = 4 biological  
13 replicates). Scale bars, 1000  $\mu$ m.

14 (G) Quantitative analysis of collagen in lung homogenates from vehicle or XL888  
15 treated animals injured with bleomycin (n = 19-20 biological replicates, experiment  
16 repeated 2X).

17 Data are represented as mean  $\pm$  SD.; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; two-tailed  
18 Student's t test (C); or one-tailed Student's t test (E-G).

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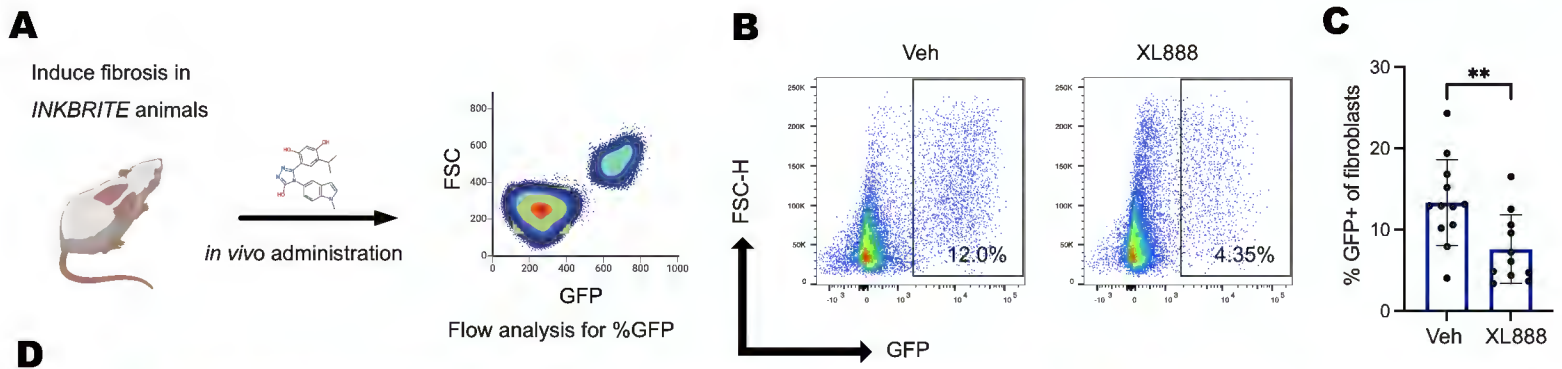
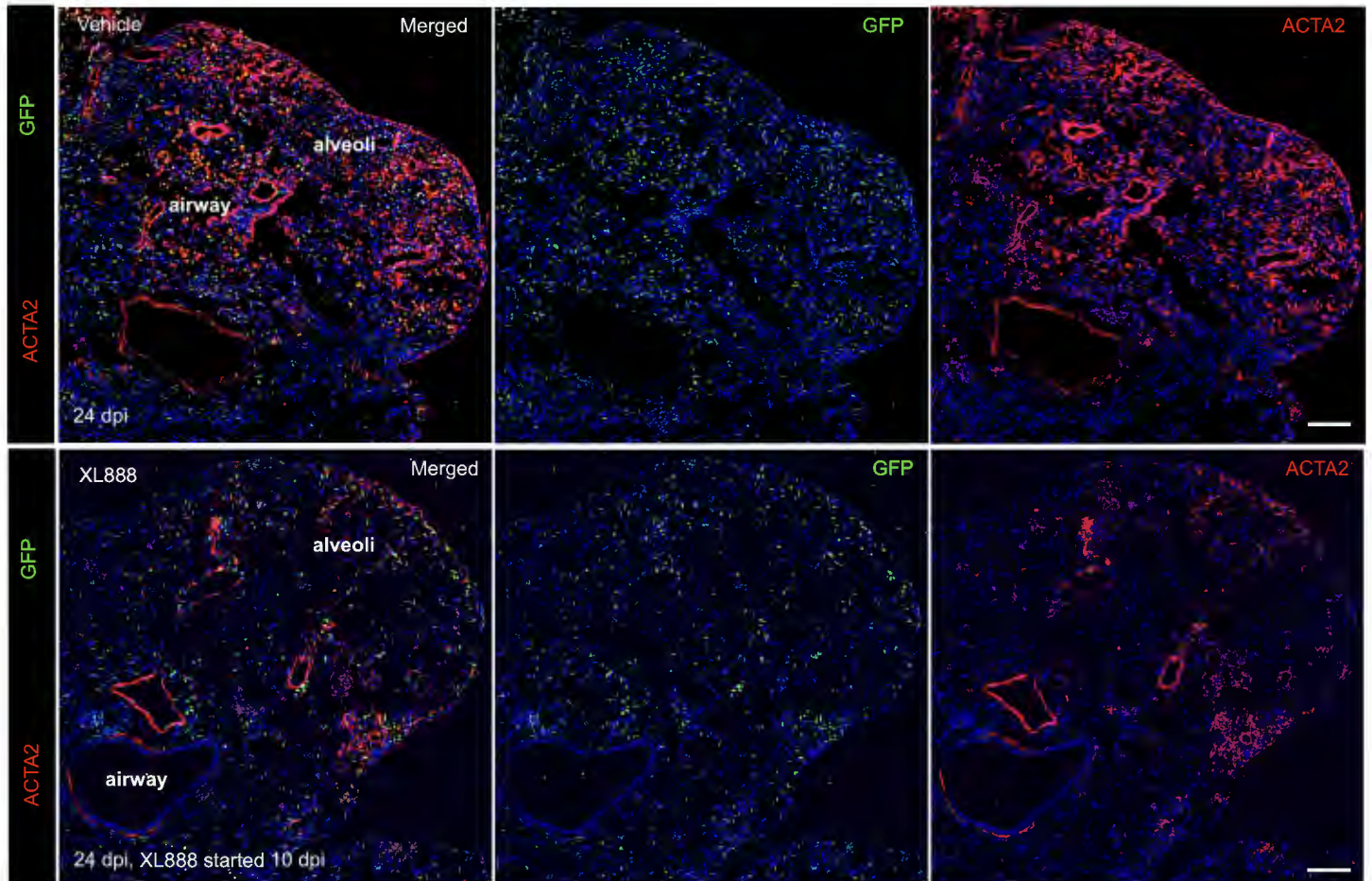
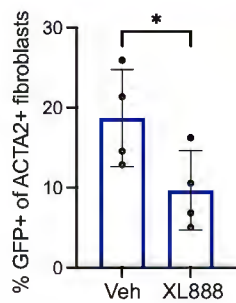
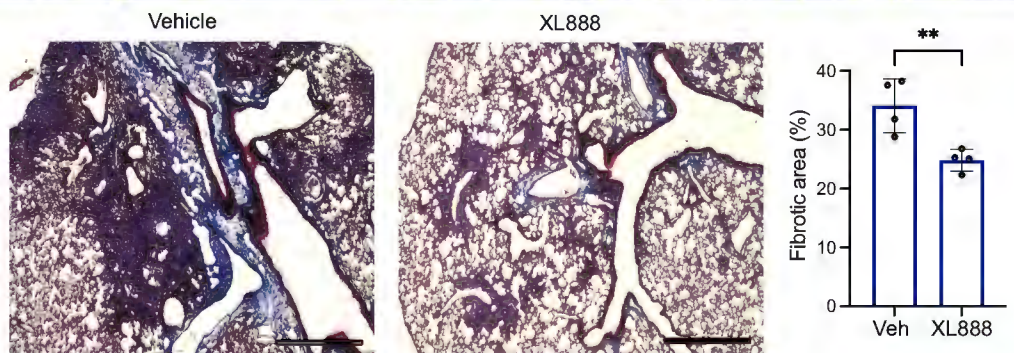
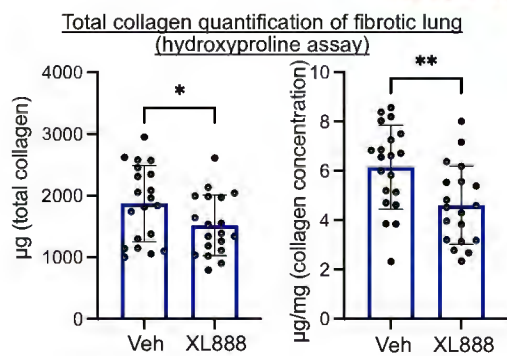
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**D****E****F****G**

1 **Figure 6. Human  $p16^{INK4a}$ + fibroblasts contribute to pathologic fibroblasts in IPF.**  
2 (A) UMAP plot of fibroblast subsets seen in normal human and IPF lungs.  
3 (B) Violin plot showing *CDKN2A* expression in the pathologic fibroblast cluster of IPF  
4 fibroblasts.  
5 (C) Violin plots showing the profibrotic genes in the different fibroblast subsets.  
6 (D) Visualization of *CDKN2A*, *CTHRC1*, *COL1A1*, and *ACTA2* expression patterns in  
7 human lung fibroblasts in IPF and control donor lungs.  
8 (E) Representative images showing *ACTA2*+ $p16^{INK4a}$ + pathologic fibroblasts (arrows) in  
9 lung sections of controls and subjects with IPF. Scale bars, 100  $\mu$ m.  
10 (F) qPCR analysis of genes enriched in pathologic fibroblasts in  $p16^{INK4a}$  high and low  
11 fibroblasts isolated from lungs of IPF patients (n = 9 technical replicates, experiments  
12 repeated with separate IPF donor fibroblasts at least 3 times).  
13 Data are represented as mean  $\pm$  SD.; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; two-tailed  
14 Student's t test (F).

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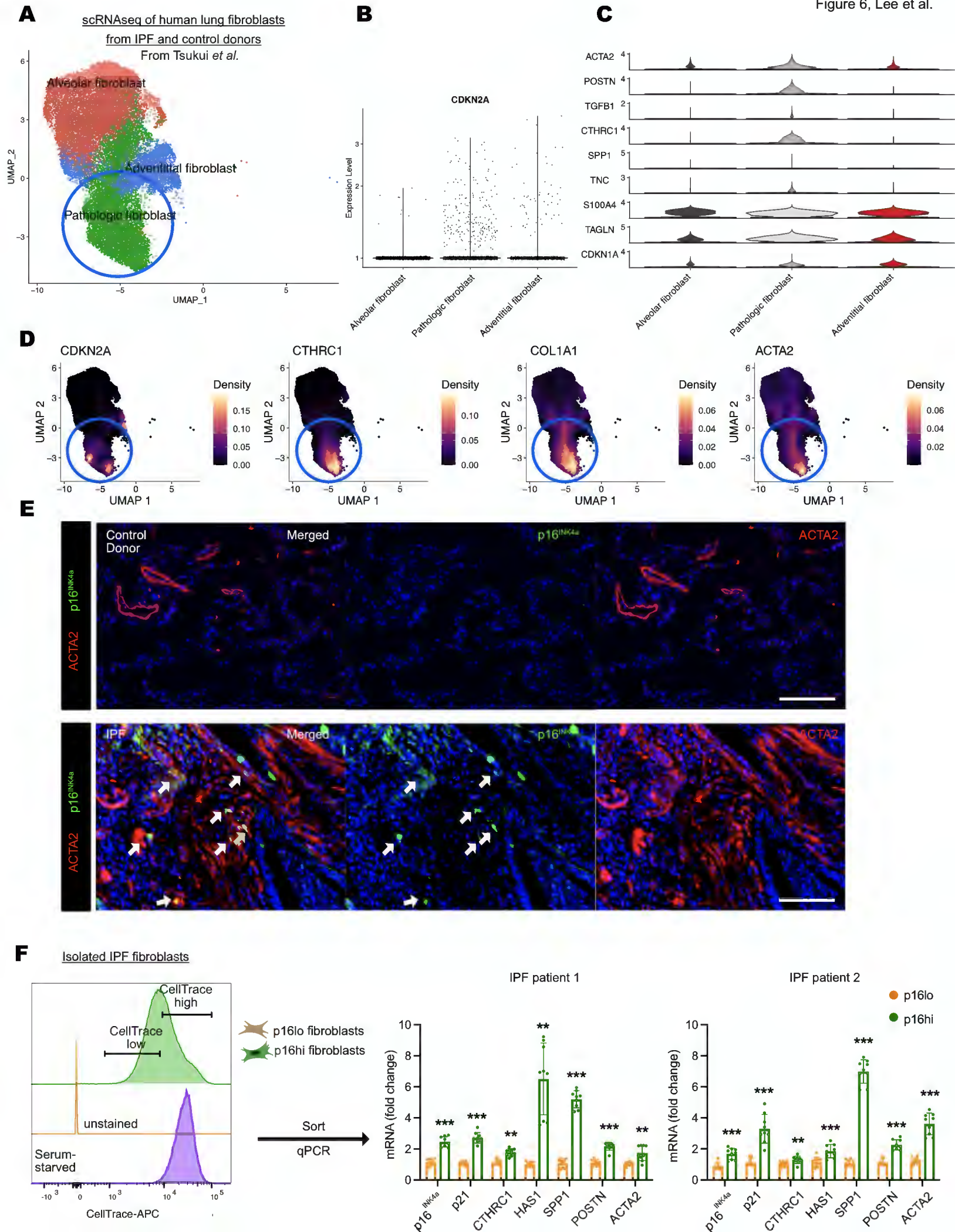
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1 **Figure 7. XL888 targets human  $p16^{INK4a+}$  fibroblasts from IPF lungs *in vitro* and ex**  
2 ***vivo*.**

3 **(A)** (Left) Schematic outline of dose-escalation challenge of candidate senolytics on  
4  $p16^{INK4a}$  high and low fibroblasts isolated from IPF lungs (Right) Ratio of  $p16^{INK4a}$ -hi and  
5  $p16^{INK4a}$ -lo fibroblast's cell count after treatment of senolytics and XL888 with dose-  
6 escalation (n = 3 technical replicates, experiments repeated with separate IPF donor  
7 fibroblasts at least 3 times).

8 **(B)** Schematic diagram depicting *ex vivo* culture of IPF lung with XL888 treatment and  
9 bright field images of cultured human PCLS. Scale bars, 2000  $\mu$ m.

10 **(C)** Immunofluorescence analysis and quantification of ACTA2+  $p16^{INK4a+}$  cells in  
11 vehicle or XL888-treated hPCLS (n=12 slices per condition, sampled from 4 IPF donors  
12 independently, each color represents a different donor). Scale bars, 100  $\mu$ m.

13 **(D)** Immunofluorescence analysis and quantification of CTHRC1+  $p16^{INK4a+}$  cells in  
14 vehicle or XL888-treated hPCLS (n=12 slices per condition, sampled from 4 IPF donors  
15 independently, each color represents a different donor). Scale bars, 100  $\mu$ m.

16 Data are represented as mean  $\pm$  SD.; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; two-tailed  
17 Student's t test **(A, C, D)**.

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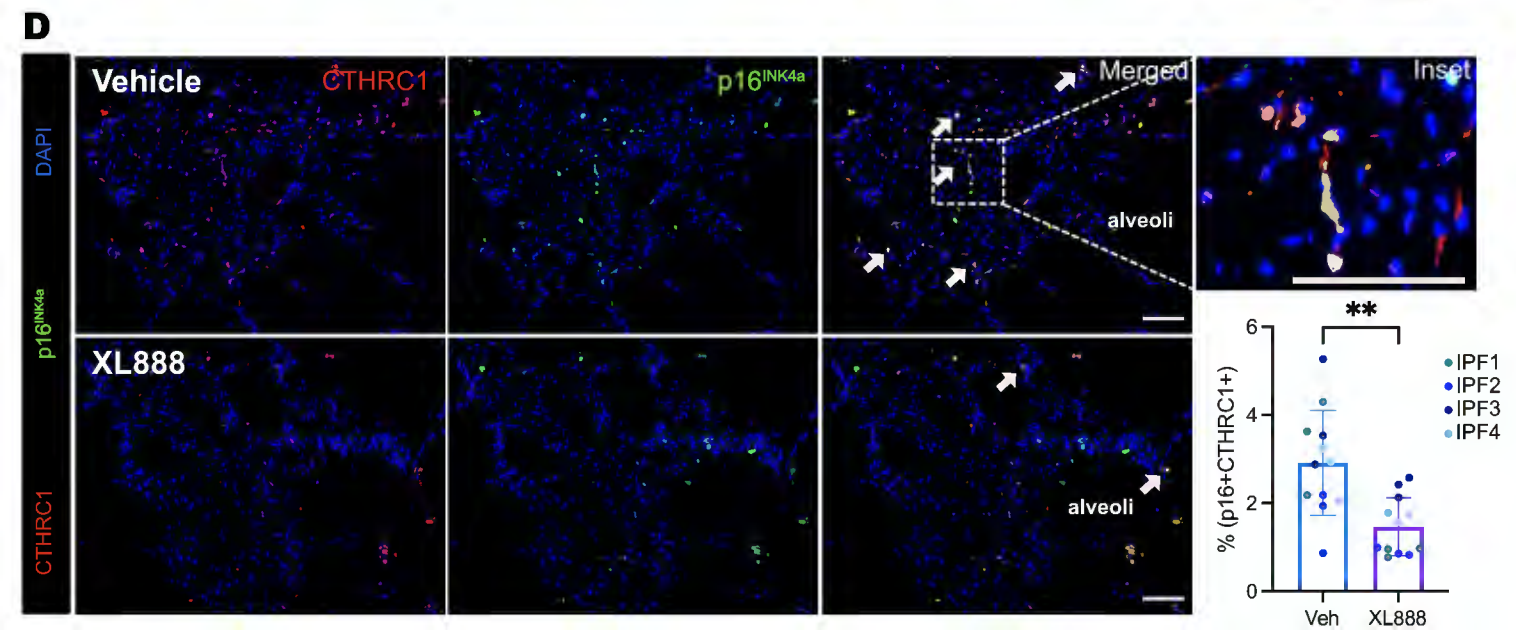
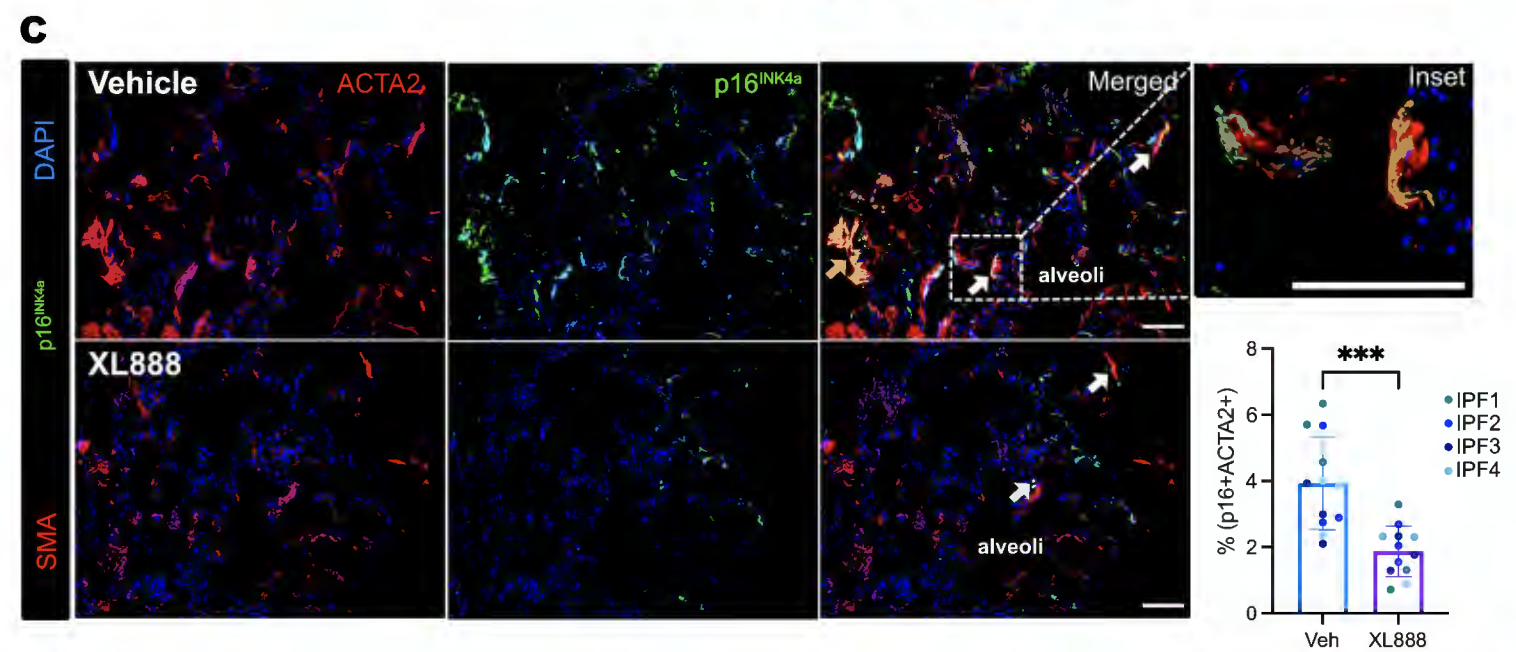
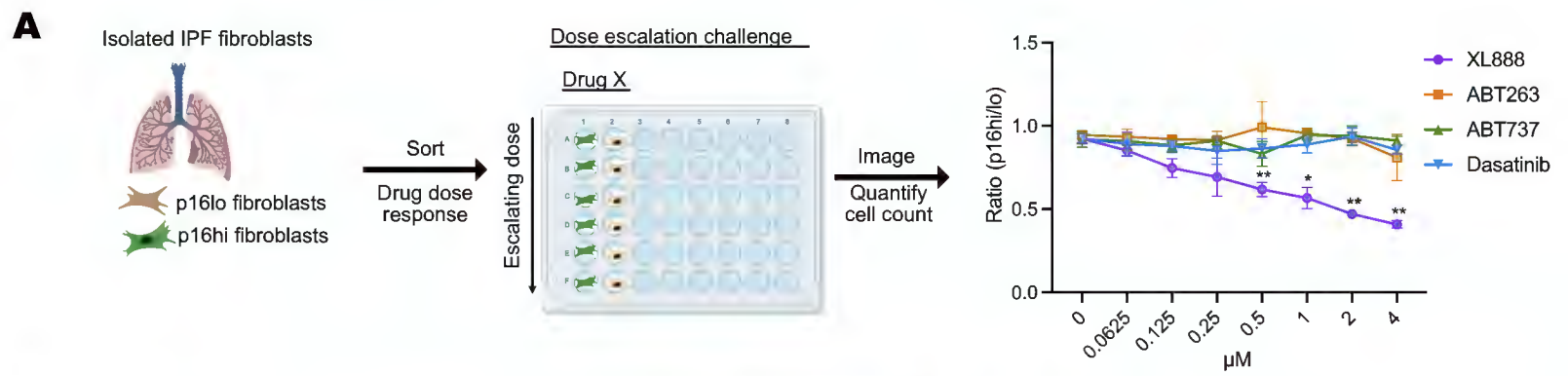
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