Clearance of senescent macrophages ameliorates tumorigenesis in KRAS-driven lung cancer

Graphical abstract

1. Identification/isolation of p16+ senescent cells
2. scRNAseq transcriptomic profiles
3. p16+ cell ablation and macrophage depletion
4. Human validation and translational relevance

For a Figure360 author presentation of this figure, see https://doi.org/10.1016/j.ccell.2023.05.004.

Highlights

- The p16-FDR mouse model can be used to isolate, trace, and ablate senescent cells
- Senescent macrophages in lung tumors and naturally aged lungs are molecularly similar
- Senescent macrophage ablation decreases tumor burden by promoting immunosurveillance
- Human pre-malignant lung tumors contain macrophages expressing senescent markers

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In brief
Haston et al. identify a population of senescent macrophages with pro-tumorigenic activities in KRAS-driven murine models of lung adenocarcinoma, which show a unique molecular signature that is conserved in the non-tumorigenic, normal aged lung. They also uncover the presence of macrophages expressing senescent markers in human pre-malignant lung tumors.
Clearance of senescent macrophages ameliorates tumorigenesis in KRAS-driven lung cancer


SUMMARY

The accumulation of senescent cells in the tumor microenvironment can drive tumorigenesis in a paracrine manner through the senescence-associated secretory phenotype (SASP). Using a new p16-FDR mouse line, we show that macrophages and endothelial cells are the predominant senescent cell types in murine KRAS-driven lung tumors. Through single cell transcriptomics, we identify a population of tumor-associated macrophages that express a unique array of pro-tumorigenic SASP factors and surface proteins and are also present in normal aged lungs. Genetic or senolytic ablation of senescent cells, or macrophage depletion, result in a significant decrease in tumor burden and increased survival in KRAS-driven lung cancer models. Moreover, we reveal the presence of macrophages with senescent features in human lung pre-malignant lesions, but not in adenocarcinomas. Taken together, our results have uncovered the important role of senescent macrophages in the initiation and progression of lung cancer, highlighting potential therapeutic avenues and cancer preventative strategies.

INTRODUCTION

The strong association of cellular senescence with important pathologies such as cancer and aging has led to significant interest in understanding the incidence and role of this cellular state in vivo. Cellular senescence is characterized as a stable arrest of the cell cycle with an altered state of gene expression, while viability and metabolic functions are maintained. It has been established that cellular senescence is the result of various persistent cellular stresses, such as the accumulation of unresolved DNA damage, telomere attrition during cellular replication, oncogene activation, loss of tumor suppressors, exposure to ionizing radiation, reactive oxygen species, and cytotoxic compounds. Molecularly, the senescent state is generally identified by the expression of cyclin-dependent kinase inhibitors (CDKIs), especially p16 (encoded by the Cdkn2a gene), as well as expansion of the lysosomal compartment, manifested by increased senescence-associated-β-galactosidase (SA-β-Gal) activity. Furthermore, a hallmark of senescent cells is their secretion of an array of soluble and insoluble factors that include pro-inflammatory mediators, growth factors, and extracellular tissue remodeling components, collectively termed the senescence-associated secretory phenotype (SASP). The SASP is known to mediate profound pleiotropic paracrine and autocrine interactions with both senescent and non-senescent cells. Cellular senescence was initially thought to function primarily as a potent tumor suppressive mechanism that limits the ability...
Figure 1. mCherry-positive cells express p16\textsuperscript{INK4a} and show limited co-localization with KRAS-G12D transformed cells during lung tumorigenesis

(A) Targeting strategy to generate the p16\textsuperscript{FDR} allele. Exons 1–3 of Cdkn2a are shown. P2A, 2A self-cleaving peptide; FLPo, mammalian optimized FLP-recombinase; HA, homology arm. Red arrows show primers used to screen for correct insertion of the FDR cassette by PCR and Sanger sequencing.

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of damaged cells to proliferate.\textsuperscript{13} Paradoxically, cellular senescence can also exert a variety of tumor-promoting effects, depending on the cellular context and composition of the SASP.\textsuperscript{2,16,18,19} Recent research has indicated that cellular senescence may also be a potent driver of organisinal aging and the progression of age-associated pathologies, such as the development of cancer, likely mediated cell non-autonomously by SASP components.\textsuperscript{3,4,15,16}

Regrettably, studying cellular senescence in vivo has been hampered by the lack of specific markers. This difficulty is compounded by the mounting evidence regarding the heterogeneity in the senescent state, which depends on the cell type undergoing senescence and the nature of the inducing stimulus.\textsuperscript{17} However, the CDK inhibitor p16\textsuperscript{INK4a} is widely regarded as the most robust surrogate marker of cellular senescence in vivo.\textsuperscript{18} Importantly, cells expressing p16\textsuperscript{INK4a} have been observed to accumulate during the aging process as well as in numerous models of spontaneous, genetically-induced and transplanted cancer development, underlying their potential importance.\textsuperscript{19} Evidencing this, the ablation of p16 INK4a-expressing cells in vivo resulted in a decrease of age-associated defects and delays spontaneous tumorigenesis with concomitant improvements in both health span and lifespan.\textsuperscript{15,16,20,21} Further investigation into the role of senescence in the tumor microenvironment (TME) and the specific mechanisms by which a decrease in p16\textsuperscript{INK4a}-expressing cells is able to delay cancer development is warranted to advance our understanding of cellular transformation.

Here, we have generated a new mouse model, termed p16-FDR, and used it in combination with oncogenic KRAS-driven non-small cell lung carcinoma (NSCLC) murine models to investigate the role that p16\textsuperscript{INK4a}-expressing cells play in influencing lung tumor development.

**RESULTS**

**Generation of the p16-FDR mouse model**

The p16-FDR mouse model was generated by targeting the bicistronic FDR cassette, encoding the optimized flippase-recombinase (FLPo) and a diphtheria toxin receptor (DTR)/mCherry fusion protein into the 3’ end of the Cdkn2a (p16) locus in embryonic stem cells (Figure 1A).

To determine if the targeted p16-FDR allele is activated in response to cellular senescence, p16\textsuperscript{+/+} and p16\textsuperscript{FDR/+} MEFs were induced to become senescent by serial passage.\textsuperscript{22} The majority of cells were proliferative at passage 2, but ceased to proliferate by passage 6. SA-β-Gal staining, a readout of cellular senescence,\textsuperscript{23} was almost completely absent at passage 2, but revealed strong staining in passage 6 (Figure S1A). In agreement, EdU incorporation revealed a statistically significant decrease in the number of S-phase dividing cells at passage 6 when compared with passage 2 (Figures S1B and S1C). Immunocytochemical staining did not reveal mCherry expression in p16\textsuperscript{FDR/+} MEFs at both passages 2 and 6, as well as in p16\textsuperscript{FDR/+} MEFs at passage 2. In contrast, mCherry expression was significantly elevated at passage 6 in p16\textsuperscript{FDR/+} cells (Figures S1B and S1C). Providing further evidence for senescence induction and activation of Cdkn2a locus, qRT-PCR revealed a significant increase in mRNA abundance of Cdkn2a (both p16\textsuperscript{INK4a} and p16\textsuperscript{FDR}) and Cdkn1a (p21\textsuperscript{CIP1}) at passage 6 relative to passage 2 (Figure S1D). mCherry mRNA was also significantly elevated in p16\textsuperscript{FDR/+} MEFs at passage 6 relative to passage 2 (Figure S1D). Fluorescence-activated cell sorting (FACS) isolation and qRT-PCR analysis of mCherry-positive and mCherry-negative mouse embryonic fibroblasts (MEFs) at passage 6 revealed that mCherry-positive cells had a significantly increased abundance of Cdkn2a (p16\textsuperscript{INK4a}) (a 51-fold increase, p = 0.0001) and mCherry (a 186-fold increase, p = 0.0001) mRNA relative to mCherry-negative cells (Figure S1E). Furthermore, bulk RNA-sequencing analysis of mCherry-positive and mCherry-negative MEFs at passage 6 demonstrated an enrichment for a signature of cellular senescence and absence of proliferation-associated genes in mCherry-positive cells by gene set enrichment analysis (GSEA) (Figure S1F).

We next tested whether p16\textsuperscript{FDR/+} MEFs could be traced and ablated. Senescent p16\textsuperscript{+/+} and p16\textsuperscript{FDR/+} MEFs (passage 6) were transfected with a frt-STOP-frt-GFP reporter plasmid, which drives the expression of GFP on flippase-mediated recombination of a transcriptional stop signal. GFP expression was observed only in p16\textsuperscript{FDR/+}, but not in p16\textsuperscript{+/+}-transfected MEFs (Figure S1G). Finally, to assess the capacity of the DTR allele to mediate cell death in vitro, we cultured MEFs in the presence and absence of diphtheria toxin (DT). At passage 6, but not at passage 2, treatment of p16\textsuperscript{FDR/+} MEFs with DT resulted in a statistically significant decrease in cell numbers (>70% decrease) relative to p16\textsuperscript{+/+} MEFs (Figures S1H and S1I).

Genetic deletion of the Cdkn2a locus can lead to premature death and tumorigenesis in vivo.\textsuperscript{24} However, long-term follow-up of p16\textsuperscript{FDR/+} (n = 26) and p16\textsuperscript{+/+} (n = 19) wild-type mice up to 600 days showed no significant differences in terms of survival or evidence of spontaneous tumorigenesis in major organs (Figures S1J and S1K). Moreover, when oncogenic KRAS-G12D was conditionally expressed in the lungs of 6- to 8-week-old p16\textsuperscript{+/+} and p16\textsuperscript{FDR/+} mice through intranasal instillation of adenoviral Cre (AdCre) to induce NSCLC,\textsuperscript{25} both genotypes showed analogous survival (Figure S1J). Immunohistochemistry against TTF1, a marker of lung adenomas and adenocarcinomas,\textsuperscript{26} and Ki67 expression were comparable...
To investigate the role of p16 INK4a-expressing cells during mouse lung development, we generated mostly present in the lung TME with spontaneous tumorigenesis, or induced lung tumorigenesis when compared with p16<sup>FDR/+</sup> control mice.

p16<sup>INK4a</sup> expression is known to increase in almost all mouse tissues during aging. We quantified the number of mCherry-expressing cells in young (1–2 months of age, n = 5) and aged (20–22 months of age, n = 5) p16<sup>FDR/+</sup> mice by FACS (Figure 1B). This analysis revealed a significant increase in number of mCherry cells in the brain, lung, kidney, heart, adipose tissue, and adrenal gland of aged relative to young mice. Additionally, a non-significant trend toward increased numbers of mCherry-positive cells was observed in the spinal cord, liver, skeletal muscle, esophagus, and bone marrow. Together, these data demonstrate that the p16<sup>FDR/+</sup> mice are suitable to study senescence in vivo in the context of tumorigenesis and aging.

**p16<sup>INK4a</sup>-expressing cells with senescent features are mostly present in the lung TME**

To investigate the role of p16<sup>INK4a</sup>-expressing cells during mouse NSCLC development, we generated Kras<sup>G12D/+;p16<sup>INK4a</sup>+</sup>, Rosa26<sup>loxP-STOP-loxP-YFP/+</sup> triple heterozygous mice (hereafter KY-FDR). In these mice, Cre-mediated recombination of both the Kras-G12D and the Rosa26 alleles results in the simultaneous expression of oncogenic Kras-G12D and YFP, the latter allowing genetic tracing of Kras-G12D-transformed tumor cells. Therefore, Kras-transformed tumor cells are labeled with YFP, whereas p16<sup>INK4a</sup>-expressing cells are labeled with mCherry (Figure 1C).

Analysis of mCherry-expression in tumor-bearing KY-FDR lungs, 8 weeks after AdCre induction, revealed that mCherry-positive cells were detectable, with the majority being immunoreactive for CDKi p16<sup>INK4a</sup> (79% ± 7.4%) and p21<sup>Cip1</sup> (75% ± 5.5%) (Figures 1D and 1E). Importantly, almost all p16<sup>INK4a</sup>-positive cells (98% ± 3.2%) colocalized with mCherry expression (Figures 1D and 1F). mCherry-positive cells showed a low degree of co-localization with the proliferation markers Ki67 (7% ± 1.9%) and phosphorylated-Rb (14% ± 2.5%), which would both be expected to be low or absent in non-proliferative or senescent cells (Figures 1D and 1E). Furthermore, FACS analysis of lungs from KY-FDR mice revealed that these mCherry-positive cells were almost completely mutually exclusive from the YFP-positive tumor cell population at 8 weeks after tumor induction (Figure S2B).

Quantification of the degree of co-localization between mCherry- and YFP-expressing cells by immunofluorescence also revealed that YFP-positive lung adenoma cells displayed little co-localization with mCherry (8 weeks after AdCre: 5.8% ± 1.8%), suggesting a lack of proliferative arrest or senescence induction in the lung tumors (Figures 1G and S2A). In contrast, a high degree of co-localization was observed between YFP-positive tumor cells and mCherry-expressing cells at 2 weeks after tumor induction (69.9% ± 25.5%, p = 0.0367) (Figures 1G and S2A). A significant proportion of these early YFP-positive transformed cells at 2 weeks after tumor induction showed expression of p16<sup>INK4a</sup> (50% ± 2.3%) and p21<sup>Cip1</sup> (39% ± 8.6%), in contrast with YFP-positive tumor cells at 8 weeks after induction, which showed little co-localization (p16<sup>INK4a</sup>, 1% ± 0.8%, p = 0.0001; p21<sup>Cip1</sup>, 14% ± 2.9%, p = 0.0483) (Figures 1G and S2A). Supporting these observations, expression of the proliferative markers Ki67 and phosphorylated-Rb was significantly reduced in YFP-positive cells at 2 weeks relative to 8 weeks after induction (2 weeks: Ki67, 7.6% ± 0.9%; phosphorylated-Rb, 7.6% ± 2%; 8 weeks: Ki67, 22.9% ± 4%, p = 0.0433; phosphorylated-Rb, 83.5% ± 3.5%, p = 0.0001) (Figures 1G and S2A).

Next, we traced the p16-expressing cells using the flippase gene present in the FDR allele in Kras<sup>G12D/+;p16<sup>FDR/+</sup>, Rosa26<sup>loxP-STOP-loxP-YFP/+</sup> mice at 8 weeks after AdCre induction. Strikingly, patches of EGFP-positive cells were observed throughout the lung tumors, potentially indicating a clonal origin for these traced cells (Figures S2C–S2F). Moreover, these EGFP-positive cells lacked expression of p16<sup>INK4a</sup> and were enriched for the proliferation marker Ki67, indicating the absence of a senescent state (Figure S2G).

Finally, we sought to characterize molecularly the distinct cell populations identified in tumor-bearing lungs. Three cell populations were FACS isolated from KY-FDR lungs at 8 weeks after tumor induction and subjected to bulk RNA-sequencing (n = 4 mice): (i) mCherry (p16<sup>INK4a</sup>)-positive cells; (ii) YFP (KRAS-G12D)-positive cells; and (iii) YFP- and mCherry-double-negative (YFP/mCherry-negative) cells (Figure 2A). Initial validation by qRT-PCR and differential expression analyses of RNA datasets revealed the increased expression of mCherry, cell cycle inhibitors, SASP, and lysosomal components in mCherry-positive compared with YFP/mCherry-negative cells or YFP-positive sorted cells (Figures 2B and 3A). Furthermore, reduced expression of genes promoting cell cycle progression (such as E2f1 and Cdc genes) was observed in mCherry-positive cells (Figure 2B). GSEA furthered these observations by showing that mCherry-expressing cells are positively enriched for signatures of cellular senescence, SASP, and lysosomal pathways, and negatively enriched for DNA replication pathways that promote proliferation (Figures S3B–S3E). The YFP-positive population lacked expression of cellular senescence or SASP gene expression, relative to mCherry-positive cells (Figure S3C). Instead, YFP-positive cells exhibited a signature of Kras-transformed lung epithelial cells with expression of proliferation-associated genes (Figures S3D and S3E). YFP/mCherry-double negative cells predominantly showed a signature of immune cell types (Figure S3E).

Together these data indicate that the p16-FDR allele is able to identify mCherry (p16<sup>INK4a</sup>)-expressing cells in the context of lung tumorgenesis in vivo. The majority of the identified p16<sup>INK4a</sup>-expressing cells are found to be non-tumor cells within the TME showing a signature of cellular senescence and SASP.

**Macrophages and endothelial cells are the predominant senescent cell types found in KRAS-driven tumor-bearing lungs**

To define cellular identities, we used xCell to interrogate the RNA-sequencing datasets previously described (Figures S3F and S3G). YFP-positive tumor cells showed an almost exclusive enrichment for epithelial cells and complete absence of enrichment for the majority of stromal and lymphoid cell types. As expected for a mixture of non-tumor lung cell types, the YFP/mCherry-double negative cell fraction seemed to comprise a wide variety of lymphoid, endothelial, and stromal cell types. Curiously, the YFP/mCherry-double negative cell fraction demonstrated low scores for myeloid derivatives such as monocytes and macrophages, which would be expected to be abundant in
Figure 2. mCherry (p16INK4a)-expressing cells from Kras<sup>G12D/+; p16<sup>DR/+/YFP<sup>+/ (KY-FDR) tumor-bearing lungs show a signature of cellular senescence and SASP

(A) Schematic showing the three FACS isolated cell fractions used for bulk RNA-seq.

(B) Heatmap of differentially expressed genes.

(C) Double immunofluorescence staining of mCherry expression in KY-FDR lungs at 8 weeks after tumor induction with specific cell-type markers.

(D and E) Quantification of the degree of co-localization between mCherry and the different lung cell-type markers from (C).

(F) Double immunofluorescence staining of mCherry with CD68 in young (2 months) and aged (20–22 months) p16<sup>DR/+ lungs and tumorigenic KY-FDR lungs (2, 8, and 16 weeks after tumor induction).

(G) Quantification of the proportion of mCherry-positive cells, relative to total DAPI<sup>+</sup> cells from (F).

(H) Quantification of the proportion of CD68-positive macrophages, relative to total DAPI<sup>+</sup> cells from (F).

(I) Quantification of the proportion of CD68-positive macrophages that express mCherry from (F). Quantifications based on analysis of at least three independent lungs. Data are mean ± SEM. Means were statistically compared by one-way ANOVA. Scale bars, 100 μm. (Also see Figure S3).
Figure 3. scRNA-seq reveals unique SASP factors and surface proteins in $p16^{INK4a}$-expressing macrophages

(A) UMAP showing cell type identity of combined mCherry-positive (1,060) and mCherry-negative (7,652) cells isolated from KY-FDR mice, at 8 weeks after AdCre induction.

(B) Proportion of mCherry-positive and mCherry-negative cells according to cellular identity.

(C) UMAP plot showing the re-clustering of only the interstitial macrophage population, demonstrating the separation of mCherry-positive and mCherry-negative cells.

(D) UMAP of the interstitial macrophage population, with the average expression of senescence markers shown.

(E) GSEA comparing mCherry-positive with mCherry-negative interstitial macrophages showing a significant enrichment of senescence-related pathways in mCherry-positive cells.

(F) Bubbleplot showing the expression of putative SASP factors and cell surface markers identified from differentially expressed genes between mCherry-positive and mCherry-negative interstitial macrophages.

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tumorigenic lungs. Of note, mCherry-positive cells showed a marked enrichment for myeloid lineages such as monocytes and macrophages, as well as for endothelial cells. These cells also showed some enrichment for lymphoid cell types, but to a much lesser extent than YFP/mCherry-double negative cells. This was supported by GSEA analysis comparing mCherry-positive against YFP/mCherry-double negative datasets, which revealed positive enrichment for pathways regulating phagocytosis and macrophage identity, as well as endothelial cell development and VEGF receptor activity (Figures S3H and S3I).

To validate this in silico analysis, immunofluorescence was performed using specific cell markers. As predicted, the majority of mCherry-positive cells were found to co-localize with macrophage (CD68⁺, 63% ± 17.8%) and endothelial (platelet endothelial cell adhesion molecule [PECAM⁺], 24% ± 10.7%) cell markers (Figures 2C and 2D). Further analysis of M1 and M2 macrophage subsets by immunofluorescence revealed that the majority of the mCherry-positive cells express the M2 polarization marker CD206, as opposed to the M1 polarization marker iNOS (M1 iNOS⁺, 4.5% ± 0.5%; M2 CD206⁺, 66.7% ± 9.4%) (Figures 2C and 2D). A very low proportion of mCherry-positive cells were found to be immunoreactive for markers of T cells (CD3⁺, 5% ± 1.9%), B cells (CD19⁺, 1.5% ± 2.5%), type I (aquaporin 5⁺, 6.5% ± 1.4%), and type II alveolar cells (pro-surfactant C, 1.1% ± 1.6%) (Figures 2C and 2D). This analysis also revealed that a proportion of CD68-positive (35% ± 9.3%) and PECAM-positive (39% ± 9.8%) cells were immunoreactive for mCherry-expression (Figure 2E). In agreement with these findings, 25.6% (±8.1%) of CD68-positive cells were found to be immunoreactive for p16INK4a and conversely, 80% (±7.01%) of p16INK4a-positive cells expressed CD68 (Figures S3J–S3L). Together, these data suggest that a significant proportion of macrophages and, to a lesser extent, endothelial cells, activate the p16-FDR allele.

Next, we analyzed the dynamics of senescent macrophage appearance during lung tumor development in KY-FDR mice. mCherry-positive cells were significantly increased in tumorigenic lungs at 2, 8, and 16 weeks after tumor induction relative to 2 months old non-tumor-bearing p16⁺FDR/+ control lungs (Figures 2F and 2G). Total CD68⁺ macrophages were not different between control and tumorigenic lungs at 2 and 8 weeks after tumor induction. However, total macrophage numbers were significantly increased in tumors at 16 weeks after induction (Figures 2F and 2H). Importantly, the proportion of CD68⁺ macrophages co-expressing mCherry were significantly increased in tumorigenic lungs at 2 and 8 weeks after tumor induction relative to control lungs (Figures 2F and 2I). In contrast, when compared with 2 weeks after induction, we observed a trend toward a decrease in the proportion of mCherry⁺ macrophages at 8 and 16 weeks, which reached significance at 16 weeks (Figures 2F and 2I).

Finally, we sought to explore potential commonalities between p16INK4a-expressing cells between tumorigenic and aged lungs. Analysis of aged p16⁺FDR/+ lungs (20–22 months old) revealed a significant increase in the proportion of mCherry-positive cells, relative to young control p16⁺FDR/+ lungs (2 months old) (Figures 2F and 2G). The proportion of total macrophages showed a trend toward an increased abundance in aged, relative to young, p16⁺FDR/+ lungs (Figures 2F and 2H). Of note, the numbers of macrophages expressing mCherry were increased in aged p16⁺FDR/+ relative to young control lungs (Figures 2F and 2I). Together, these data indicate that both tumorigenic and aged lungs show a significantly greater proportion of senescent macrophages expressing p16INK4a.

**Tumor- and age-associated p16INK4a-expressing macrophages in the lung share a unique senescence signature**

Next, we performed 10x single cell RNA-sequencing (scRNA-seq) of p16INK4a⁺-expressing and non-expressing cells from KY-FDR lungs, 8 weeks after tumor induction. Cell-type projections against known reference sets of genes coupled with clustering, as well as manual annotations of established marker genes, led to the assignment of cell-type identities; these corresponded with the majority of known lung cell types (Figure 3A). Importantly, the majority of mCherry-positive cells were identified as interstitial/alveolar macrophages and capillary/vascular endothelial cells (Figure 3B), which was predicted from our previous bulk transcriptomic and immunofluorescence analyses. Although not all mCherry-positive cells were found to express Cdkn2a or the FDR cassette (Figures S4A–S4C), this was shown to be caused by a technical transcript dropout limitation of 10x scRNA-seq. Comparisons between FDR-expressing and FDR-non-expressing mCherry-positive cells failed to identify any differentially expressed genes in capillary endothelial cells and just 23 genes in macrophages. Interrogation of these 23 genes was deemed not biological significant (e.g., by gene ontology, GSEA, etc.) (Figures S4D and S4E).

Comparison of mCherry-positive interstitial macrophages and capillary endothelial cells with their mCherry-negative counterparts, revealed significantly differentially expressed genes (interstitial macrophages = 783 genes, capillary endothelial cells = 539 genes) and separate UMAP clustering (Figures 3C and S4F). Using a curating list of senescence-related transcripts (Table S1), we found that mCherry-positive interstitial macrophages and capillary endothelial cells showed a higher association with senescence genes than their mCherry-negative counterparts (Figures 3D and S4G). GSEA analyses revealed enrichment of senescence-related gene sets in both mCherry-positive macrophages and endothelial cells; for instance, negative regulation of proliferation (Cdkn2a and Hpgd), immune response (Irf1 and Parp14), response to stress (Pdia3 and Hspa5), cytokine production/response (Gbp2 and Irf1), and lysosomal compartment expansion (Lamp1 and Laptm4a) (Figures 3E and S4H).

Further analyses revealed the unique expression of several surface makers (Folr2, Cd209g, Cd209f, and Cd163) in mCherry-positive macrophages relative to all other lung cell...
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types included in this analysis (Figures 3F and 3G). Immunofluorescence analysis confirmed the expression of FOLR2 in mCherry-positive cells in both tumorigenic KY-FDR as well as non-tumorigenic aged p16\(^{FDBN/4}\) lungs (Figure S4I). This comparative analysis also uncovered a SASP signature that consisted of growth factors (Bmp2 and Igf1), chemokines and cytokines (Il10, Ccl2, Ccl12, Ccl24, Cxc13, and Fcn4) and extracellular matrix modifiers (Mmp9 and Timp2) (Figure 3F). Of note, we identified a subset of these SASP factors (Bmp2, Il10, Ccl2, Ccl7, Ccl8, Ccl24, Cxc13, and Fcn4) that were uniquely expressed in mCherry-positive interstitial macrophages relative to all other lung cell types in our scRNA-seq analysis (Figure 3G). These factors have been reported to be involved in tumorigenesis including lung cancer.\(^{33-36}\) To test their potential tumor-promoting activity, we cultured FACS-isolated YFP (KRAS-G12D)-positive tumor cells in the presence of each of these factors or vehicle control and assessed total cell counts. This study revealed IL10 and CCL2 are able to increase total tumor cell numbers relative to vehicle controls (IL10, 29% ± 6.8%; CCL2, 25% ± 1.8%) (Figures S4J and S4K).

mCherry-positive capillary endothelial cells showed a measurable but decreased secretory phenotype (i.e., fewer differentially expressed secreted factors with lower levels of increased expression relative to mCherry-negative endothelial cells), which consisted of Dbi, Hilpda, Hmbg1, Igbp7, Itgb1, Pecam1, Bol2, Cst3, and Nampt expression (Figure S4L). However, unlike mCherry-positive macrophages, these secreted factors (other than Hilpda) failed to show the same specificity when their expression was measured across all lung cell types captured by this experiment (Figure S4M). An analysis of unique cell surface markers upregulated in mCherry-positive capillary endothelial cells revealed four genes (Ly6a, Ly6c1, Cdch5, and Cd34) that showed the highest degree of expression in mCherry-positive capillary endothelial cells (and mCherry-positive vascular endothelial cells), relative to all other lung cell types (Figure S4M).

To explore further the similarities between macrophages in tumorigenic and aged lung contexts, we performed scRNA-seq of mCherry-positive cells from 20 to 22 months old p16\(^{FDBN/4}\) mice (Figure S4N). Annotating of cellular identity revealed a similar cellular diversity to that found in mCherry-positive cells from tumorigenic lungs, with capillary and vascular endothelial cells and interstitial macrophages making up the majority (58.04%) of mCherry-positive cells (capillary endothelial cells, 34.23%; vascular endothelial cells, 10.57%; interstitial macrophages, 13.24%) (Figure S4O). Remarkably, the previously identified interstitial macrophage SASP and surface marker signatures from tumorigenic lungs (the eight unique SASP factors and the four surface markers) were found to be completely conserved in the mCherry-positive interstitial macrophages from aged lungs (Figures 3H and S4P). In addition, FN1\(^{+}\) macrophages in the aged lungs also showed conservation of several SASP markers (Figures 3H and S4P). The reasons why FN1\(^{+}\) macrophages were not detected in the tumorigenic lungs are not known.

Together, these data confirm that macrophages and endothelial cells, with specific signatures of cellular senescence, make up the majority of the p16\(^{INK4a}\)-expressing cells in the lung TME. However, only macrophages show a unique SASP signature composed of pro-tumorigenic factors and specific surface proteins, which are conserved in the normal aged lung.

**Pharmacogenetic ablation of mCherry (p16\(^{INK4a}\))-expressing cells or senolytic treatment ameliorates lung tumor burden**

We next tested the consequences of ablating p16\(^{INK4a}\)-expressing cells in the context of developing lung tumors with either DT or the senolytic ABT-737 (Figure 4A).\(^{37,38}\) FACS analysis of tumor bearing lungs revealed a significant reduction in mCherry-positive cells in both DT and ABT-737 treatment groups, relative to vehicle control (Figures 4B and 4G). Concomitant with this, both treatment groups also showed a significant decrease in the proportion of p16\(^{INK4a}\), and CD68-expressing cells in tumor-bearing lung tissue, relative to vehicle controls by immunofluorescence (Figures 4C, 4D, 4H, and 4I). A trend toward decreased mCherry expression in PECAM-positive endothelial cells in tumor-bearing lungs was also observed in both DT and ABT-737 treatment groups relative to vehicle controls (Figures 4E and 4J). Moreover, analysis of Ki67 immunofluorescence revealed a significant decrease in the proliferative index of the lung tumors in the DT- and ABT-737-treated groups, relative to the vehicle treated controls, at 8 weeks after tumor induction (Figures 4F and 4K).

Remarkably, DT or ABT-737 treatment led to a decrease in the number of visible tumors on the surface of the lungs by
wholenumount fluorescence imaging at 8 weeks after tumor induction when compared with vehicle-treated mice (Figure 5A). The proportion of YFP-positive tumor cells (as assessed by FACS) and number of YFP-positive tumor lesions (as assessed histologically) were decreased significantly upon either DT or ABT-737 treatment relative to the vehicle group (Figures 4L–4N). These effects were specific to the KY-FDR mice, as DT treatment of AdCre-induced Kras<sup>G12D</sup>/p16<sup>+/−</sup>;Rosa26<sup>loxP-STOP-loxP-YFP+</sup> control mice, lacking the p16-FDR allele, did not result in a decrease in the numbers of YFP-positive tumor cells (Figures S5B and S5C). Similarly, the proportion of p16<sup>NK4A</sup>− and CD68-positive cells was not significantly different between vehicle and DT-treated groups in Kras<sup>G12D</sup>/p16<sup>−/−</sup>;Rosa26<sup>loxP-STOP-loxP-YFP+</sup> mice (Figures S5D–S5F).

Next, we made use of the Kras-FSG12v model, in which the expression of oncogenic KrasG12V is prevented by a flanking DNA sequence, which allows for lung tumor induction after intratracheal administration of adenovirus-FLP (AdFLP).<sup>39</sup> Histological analysis at 5 months after AdFLP induction revealed that p16<sup>NK4A</sup> expression was observed to a higher degree in lung hyperplasias and adenomas, relative to adenocarcinomas (Figures S6A and S6B). A similar trend was also observed for p21<sup>CIP1</sup> expression between the different stages of tumorigenesis (Figures S6A and S6B). Analysis of Ki67-positive proliferating cells demonstrated an inverse relationship in which lung hyperplasia and adenomas showed a lower degree of proliferation than adenocarcinomas (Figures S6A and S6B). Moreover, histological analysis revealed that 36.9% (±16.0%) of F4/80-positive macrophages in the tumor-bearing lungs of Kras<sup>FSG12v/+</sup> mice express p16<sup>NK4A</sup>, with 45.1% (±25.6%) of p16<sup>NK4A</sup>-positive cells expressing F4/80 (Figures 5C, S6C, and S6E). Therefore, similar to the previous observation in KY-FDR mice, senescence markers are most commonly observed in early, rather than late, stages of tumorigenesis in Kras<sup>FSG12v/+</sup> mice, with the majority of the positive cells being found in the TME in a subset of macrophages.

In agreement with our previous analysis, lung tumor-bearing Kras<sup>FSG12v/+</sup> mice, treated with ABT-737, showed a significant decrease in tumor-associated p16<sup>NK4A</sup>-expressing cells, relative to vehicle control (Figures 5A, 5B, and 5D). The area of lung tumors occupied by PECAM-positive (CD31-positive) endothelial cells regularly co-expressing p16<sup>NK4A</sup> was also significantly decreased in ABT-737-treated mice (Figures 5B, 5D, and 5E). In contrast, apoptosis was increased and proliferation was decreased upon ABT-737 treatment relative to controls, which was associated with a significant decrease of the tumor burden relative to the percent of lung area (Figures 5B and 5D). We also found that senolytic treatment decreased the burden of hyperplasias and, as expected, adenocarcinomas relative to lung area, but not the burden of adenomas (Figure 5E). This result suggests that the ablation of senescent cells in the TME decreases cell proliferation of transformed cells and malignant progression to adenocarcinoma. In accordance to the histology evaluation, microcomputed tomography analysis at 10.5 months after tumor induction revealed that ABT-737 treatment significantly decreased tumor burden relative to vehicle-treated mice, as determined by the total volume occupied by lungs per mouse (vehicle-treated animals, 66.09 mm<sup>3</sup> ± 33.11 average tumor burden per mouse (n = 5 mice); ABT-737-treated animals, 22.53 mm<sup>3</sup> ± 10.38 (n = 7 mice); p = 0.0295) (Figures 5F and 5G). Strikingly, ABT-737-mediated chemical ablation of senescent cells resulted in a significant prolonged survival (Figure 5H).

Collectively, the above data demonstrate that ablation of p16<sup>NK4A</sup>-expressing senescent cells, including populations of macrophage and endothelial cells, significantly decreases tumor burden in two different KRAS-driven mouse models of NSCLC thereby resulting in an increased survival.

Removal of CSF1R-expressing macrophages with senescent features decreases lung tumor burden

We next sought to ascertain the direct implication of macrophages in the observed lung pro-tumorigenic activities. Lung interstitial macrophages are primarily considered to derive from circulating monocytes, which are produced in the bone marrow, whereas alveolar macrophages can be both tissue resident or monocyte-derived macrophages.<sup>40</sup>–<sup>43</sup> CSF1R is a single-pass type I membrane protein and member of the platelet-derived growth factor receptor family that in mice is expressed by the monocyte/macrophage lineage regulating their proliferation and differentiation to lung-infiltrating macrophages.<sup>40</sup> We performed a macrophage depletion experiment through blocking CSF1R signaling<sup>40</sup>,<sup>42</sup>,<sup>44</sup>,<sup>45</sup> (Figure 6A). Administration of 300 μg anti-CSF1R antibody twice a week for 7 weeks significantly decreased the numbers of CD68-positive macrophages and YFP-positive tumor lesions in the lungs of KY-FDR mice at 8 weeks after AdCre instillation (Figures 6B, 6C, 6E, and 6F). Moreover, this decrease in total macrophages correlated with a significant decrease in both the proportion of mCherry-positive cells and the proportion of CD68-positive macrophages that expressed mCherry in

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**Figure 5. Senolytic treatment in Kras<sup>FSG12v/+</sup> mice decreases lung tumorigenesis and promotes survival**

(A) Experimental schematic for the chemical ablation of senescent cells with ABT-737 during lung tumorigenesis in Kras<sup>FSG12v/+</sup> mice. (B) Immunohistochemistry of lung tumors formed in Kras<sup>FSG12v/+</sup> mice, at 10.5 months after tumor induction, subjected to an SA-β-gal activity assay and stained for specific markers. Scale bars, 50 μm. (C) Double immunohistochemical staining assessing the presence of F4/80-positive macrophages and CD31-positive endothelial cells in association with the senescence marker p16<sup>NK4A</sup> in Kras<sup>FSG12v/+</sup> lung adenomas subjected to an SA-β-gal activity assay. Black arrowheads correspond with p16<sup>NK4A</sup>-positive macrophages or CD31-positive endothelial cells, whereas white arrowheads correspond with p16<sup>NK4A</sup>-negative macrophages or endothelial cells. Scale bars, 10 μm. (D and E) Quantification of the immunohistochemistry data from (B) (n = 15 mice, unpaired two-tailed t test). Data are mean ± SD. (F) Tumor burden in Kras<sup>FSG12v/+</sup> mice at month 10.5 after tumor induction, plotted as the total volume occupied by tumors in the lungs per mouse (vehicle, n = 5; ABT-737, n = 7, unpaired two-tailed t test). Data are mean ± SD. (G) Transversal and sagittal micro-computed tomography (CT) scan images and three-dimensional reconstruction of representative lungs scanned at month 10.5 after AdFlipl intratracheal administration. Tumors are delineated and reconstructed in red color. (H) Survival curve of Kras<sup>FSG12v/+</sup> mice. (Also see Figure S6).
anti-CSF1R antibody-treated KY-FDR mice (Figures 6C, 6G, and 6H). As an internal control, the proportion of PECAM-positive endothelial cells that express mCherry is unchanged in KY-FDR mice after anti-CSF1R antibody treatment (Figures 6D and 6I). Together, our results show that CSF1R-expressing macrophages, including mCherry+/CD68+ macrophages, are key players of the TME by directly contributing to tumor progression.

Senescent cell ablation in the TME promotes immunoregulation and reduces tumor vasculature

Depletion of tissue-resident macrophages has been recently shown to result in reduced numbers of regulatory T cells (Tregs) and accumulation of CD8+ T cells, both of these factors contributing to reduced tumor invasiveness and growth in mouse models of NSCLC.56 Immunostaining against FOXP3, a transcription factor expressed in Tregs, revealed a significant

Figure 6. Specific depletion of macrophages ameliorates lung tumor burden in KY-FDR mice

(A) Experimental schematic for pharmacological depletion of macrophages ablation using an anti-CSF1R blocking antibody.
(B) Immunofluorescence staining against YFP from representative histological lung sections from KY-FDR mice treated with either vehicle control or anti-CSF1R blocking antibody treatment, showing a decrease in YFP-positive tumors. Scale bar, 1 mm.
(C and D) Co-immunostaining of mCherry and either CD68 (C) or PECAM (D) in tumor-bearing KY-FDR lungs after either vehicle or anti-CSF1R blocking antibody treatment. Scale bars, 100 μm.
(E–I) Quantification of the proportion of YFP+ tumor cells (E), CD68+ macrophages (F), mCherry+ senescent cells (G), CD68-positive macrophages co-expressing mCherry (H), and PECAM-positive endothelial cells co-expressing mCherry (I) in KY-FDR mice, treated with either vehicle control or anti-CSF1R blocking antibody treatment. All data are mean ± SEM. Means were statistically compared by one-tailed Student’s t test.
decrease in positive cells in lung tumor lesions from DT and ABT-737 treated KY-FDR mice relative to controls (Figures 7A, 7B, and 7F). Concomitantly, in both treatment groups, we also observed a significant increase in the proportion of CD4+ and CD8+ lymphocytes in tumors (Figures 7A, 7C, 7D, and 7E). Of note, after macrophage depletion by blocking CSF1R signaling in tumorigenic KY-FDR mice, we also observed an increased abundance of CD8+ T cells in tumor lesions, implicating macrophages as a critical driver of tumor immunomodulatory effects (Figure S7). Finally, immunostaining against Endomucin, a marker of endothelial cells, revealed that staining was significantly decreased within the tumor lesions in both DT and ABT-737 treatment groups, suggesting a disruption of normal intra-tumoral vascularization upon senescent cell ablation (Figures 7G and 7H). Together, we show that the clearance of senescent cells results in a switch of the T-cell phenotype toward an immunostimulatory TME and a decrease in the tumor vascular network.

Human pre-cancerous lung lesions contain a cell population of macrophages exhibiting senescence features

To ascertain if our results obtained from murine models are relevant to human lung tumors, we sought to investigate the presence of senescent cells in a series of human NSCLC lung cancer specimens corresponding with atypical adenomatous hyperplasia (AAH) (n = 16), adenocarcinoma in situ (AIS) (n = 4), and adenocarcinoma (ADC) (n = 3) (Figure 8A, Table S2). In agreement with the murine data, we observed p16INK4a and p21Cip1 to have the highest level of expression in the earlier AAH and AIS stages of lung tumorigenesis, relative to the later ADCs (Figures 8B and S8A). Ki67 was expressed at low levels in AAH and AIS, but highly expressed in ADC (Figures 8B and S8A). Numbers of CD68-positive macrophages were similar across the different stages of lung tumorigenesis (Figures 8B and S8A). However, we identified a high degree of co-expression of either p16INK4a or p21Cip1 in CD68-positive macrophages only...
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(legend on next page)
in AAH and AIS, including interstitial, alveolar, and bronchial macrophages (Figures 8C, 8D, S8B, and S8C). In contrast, very few ADC-associated CD68-positive macrophages co-expressed these cell cycle inhibitors (Figures 8G and 8D). Finally, we analyzed in human lung lesions the expression of the unique surface markers previously identified in the mCherry+ senescent murine macrophages. Immunofluorescence staining revealed the expression of CD163 and FOLR2 in both p16INK4A-positive and p21Cip1-positive macrophages in human AAH and AIS, but not in ADCs (Figures 8E, 8F, S8D, and S8E). Overall, these data indicate, in agreement with our mouse experiments, that senescence marker expression is correlated with the incipient (pre-cancerous) stages of lung tumorigenesis.

**DISCUSSION**

The role and involvement of cellular senescence during NSCLC initiation and progression remains incompletely understood. While cellular senescence has been reported to occur in oncogenic KRAS-induced lung adenomas, others have not observed senescent phenotypes. Here, we aimed to characterize both oncogene-induced senescence in tumor cells and senescence in the TME of the tumorigenic lung. Using two KRAS-driven lung ADC models, we show that the majority of the senescent cells in the tumors are macrophages and to a lesser extent endothelial cells. Of translational relevance, we report the expression of senescence markers in interstitial, bronchial, and alveolar tissue resident macrophages only at early stages of human lung tumorigenesis (e.g., AAH and AIS), but not in advanced lesions (e.g., ADC). M2-polarized macrophages are widely regarded as being tumor promoting during lung carcinogenesis and are observed to correlate with decreased patient survival, increased metastasis, and increased tumor proliferation. Therefore, although pro-tumorigenic effects of macrophages in lung cancer have been documented, a link to senescence has not been established.

p16INK4A+ Expressing macrophages express many pro-tumorigenic SASP factors that appeared unique in the tumorigenic lung (i.e., Bmp2, Ccl2, Ccl7, Ccl24, Cxcl13, and Il10) and may represent important mediators of paracrine pro-tumorigenic effects (Figure 3). The chemokines CCL7 and CCL24 are known to promote invasiveness, metastasis and stemness of cancer cells. IL10 can exhibit pro-tumorigenic effects, such as creating an immunosuppressive microenvironment that promotes angiogenesis, proliferation, and metastasis. CXCL13 is observed to be secreted by macrophages, promoting renal cancer cell migration, invasion, and epithelial-to-mesenchymal transition. Moreover, CXCL13 is reported to be elevated in human NSCLC patients and is also found to be a predictive risk factor for early stage lung ADC. BMP2 is known to have pro-tumorigenic effects in a variety of cancers, including lung, breast, and prostate. Moreover, our single-cell analysis has also provided evidence for the expression of cell-surface markers that are unique to the mCherry-expressing macrophages, some of which (FOLR2 and CD163) are found to be expressed in pre-cancerous stages of human lung cancer, suggesting the conservation of senescence programs in this cell type in response to tumorigenesis in both mice and human lung tumors (Figures 3, 8, S4, and S8). These markers allow for the possibility of more refined studies in human aged and pre-cancerous lung tissue to better understand the role of the cells. Furthermore, they may also be of usefulness in designing future immunotherapeutic approaches.

We show that the ablation of p16INK4A-expressing cells results in a striking decrease in macrophages (but to a lesser extent endothelial cells) and tumor burden (Figures 4 and 5). Recently, a subset of tissue-resident lung macrophages was found to cell non-autonomously promote lung tumor growth by establishing an immunosuppressive TME. Depletion of tissue-resident macrophages in this study led to decreased tumor growth and invasiveness. Our results are in agreement with this observation. We reveal that senescent cells contribute to generate an immunosuppressive TME (rich in Tregs and low in CD4+ and CD8+ T cells), which can be switched toward an immunostimulatory TME (low in Tregs and high CD4+ and CD8+ T cells) by clearance of senescent cells (Figure 7). Moreover, CSF1R-signalling blockade and macrophage depletion results in a similar phenotype to that observed upon senescent cell ablation experiments (Figure 6). Finally, our experiments also reveal that senescent cell ablation causes disruption of the intra-tumoral vascular network, hence potentially decreasing the delivery of nutrients and O2 to the tumor cells (Figure 7).

Our analysis of aged mouse lungs has found a similar composition of cell types expressing p16INK4A with that of tumorigenic lungs in young mice, with a prominent signature of macrophages and endothelial cells (Figure S4). Remarkably, the unique array of cell surface markers and SASP composition identified in tumor-associated p16INK4A-expressing macrophages in lung tumors from young mice was conserved in age-associated p16INK4A-expressing macrophages in normal lungs (Figure 3). This striking
molecular commonality warrants further investigation to understand the potential critical role these SASP factors from age-associated macrophages may play in driving the increased tumor incidence observed with age.

There are some limitations to this study. As specific depletion of only senescent macrophages was not technically feasible, we cannot rule out the potential contribution of other macrophage or senescent cell populations to tumorigenesis. More specific depletion approaches, based on FOLR2 or CD163 expression, may be able to overcome this limitation. The mechanisms by which developing lung tumors are able to induce cell senescence in macrophages and endothelial cells has not been addressed in this study, but warrants further investigations. At early stages of tumorigenesis (i.e., at 2 weeks after AdCre induction), we have identified KRAS-transformed cells expressing p16INK4a/mCherry, which may suggest a senescent state or at least a sustained cell-cycle arrest. Early tumor cells, if senescent, may be capable of inducing paracrine senescence in TME cells.61 Inflammation brought about by tumor growth may be able to induce senescence in macrophages and endothelial cells. In agreement, a senescent-like state has been described in macrophages in response to a pro-inflammatory microenvironment.62,63

In conclusion, we show that the p16-FDR mouse model represents a powerful new tool to elucidate the identity, characteristics, fate and biological significance of Cdkn2a (p16INK4a)-expressing senescent cells in vivo. We present novel insights into the significance of senescent cells and their cell autonomous and non-autonomous functions during the early stages of lung tumorigenesis and in aged lungs. These novel results suggest that senolytic therapy may represent a treatment modality to interrupt KRAS-driven NSCLC at early and intermediate tumor stages and, in particular, a preventative strategy for patients bearing multifocal pre-cancerous lesions.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Experimental mouse models
  - Human biopsies and ethical regulations
- **METHOD DETAILS**
  - Tissue processing, immunofluorescence and histological staining
  - Viral lung tumour induction
  - Pharmacogenetic and chemical ablation of senescent cells
  - CSF1R-expressing macrophage blockade
  - Micro-CT imaging of lung tumour bearing mice
  - Flow cytometry analysis
  - qRT-PCR analysis
  - Cell culture

- **QUANTIFICATION AND STATISTICAL ANALYSIS**
  - Imaging and cell counting
  - Single cell RNA sequencing data analysis
  - Statistics

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ccell.2023.05.004.

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AUTHORS CONTRIBUTIONS


DECLARATION OF INTERESTS

J.G. has acted as a consultant for Unity Biotechnology, Geras Bio, Myricx Pharma, and Merck KGaA. J.G. owns equity in Geras Bio. J.G. is a named inventor in an MRC patent and a named inventor in another Imperial College patents, both related to senolytic therapies (the patents are not related to the work presented here). Unity Biotechnology, Myricx Pharma, and Pfizer have funded research in J.G.’s laboratory (unrelated to the work presented here).

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects. One or more of the authors of this paper self-identifies as an under-represented...
 ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a member of the LGBTQIA+ community. One or more of the authors of this paper self-identifies as living with a disability.

REFERENCES


mice is associated with p16(INK4a)- and β-galactosidase-positive macrophage accumulation that can be induced in young mice by senescent cells. Aging, 12, 1294–1315. https://doi.org/10.18632/aging.100991.


## KEY RESOURCES TABLE

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**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Juan Pedro Martinez-Barbera (j.martinez-barbera@ucl.ac.uk).

**Materials availability**

There are restrictions to the availability of the p16-FDR mouse line due to the lack of an external centralized repository for its distribution and our need to maintain the stock. We are glad to share this line with reasonable compensation by requestor for its processing and shipping.

**Data and code availability**

- RNA-sequencing datasets have been deposited in the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Experimental mouse models**

The *Kras*<sup>GT2D<sub>25</sub></sup>, *Kras*<sup>GT12V<sub>39</sub></sup>, *Rosa26<sub>Cre-loxP-STOP-loxP-YFP<sub>4</sub></sub>* and *Rosa26<sub>loxP-STOP-loxP-YFP<sub>4</sub></sub>* mice have been previously described. To generate the p16-FDR mouse model a bicistronic targeting vector was first generated containing a mammalian optimised flippase (FLP) recombinase along with a diphtheria toxin receptor (DTR) that was fused with an mCherry fluorescent reporter. The cassette was termed FDR (Flippase, Diphtheria toxin receptor and Reporter) and was synthesised by GenScript (United Kingdom) into a pcDNA3.1(+) plasmid backbone. Both upstream and downstream of the FLP-recombinase, P2A sequences were added to promote ribosomal “skipping” over these regions during translation of the mRNA to produce multiple protein products (i.e., p16<sup>INK4a</sup>, FLP and DTR-mCherry). This cassette was flanked by two sequences homologous to regions 2.5 kb upstream and 1.9 kb downstream of the *p16INK4A* protospacer adjacent motif (PAM) site (Figure 1A). The sequence for the DTR (human membrane-anchored heparin-binding EGF-like growth factor with I117V and L148V mutations to repress the growth factor activities) was kindly provided by Kenji Kohno. The 2.5 kb (5′- homology arm) and 1.9 kb (3′- homology arm) were generated by high-fidelity PCR amplification (Phusion, TermoFisher cat# F553). The sense and antisense primers used for the generation of the 5′ homology arm contained a NotI restriction site at their 5′ end, preceded by a TTAAT cap sequence to improve the efficiency of the digestion process (Table S3). The sense and antisense primers used for the generation of the 3′ homology arm contained a XbaI restriction site at their 5′ end, preceded by a GCG...
cap sequence to guarantee the digestion process (Table S3). Subsequently, the two homology arms were cloned, respectively, upstream and downstream the FDR cassette in the pcDNA3.1(+) plasmid through the NotI and XbaI restriction sites. The final donor plasmid (pcDNA3.1(+)-FDR) was subjected to diagnostic digestion to confirm insert orientation, Sanger sequencing to confirm absence of mutations before being purified for transfection into ES cells. A guide RNA (gRNA) (Table S3) targeting a region 12 nt upstream the p16^{INK4A} stop codon was designed and cloned into the px330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (Addgene, plasmid #42230) to generate the px330-p16-gRNA vector. Briefly, two oligos corresponding to the sense and antisense gRNA in which a BbsI restriction site was added at the 5' end were synthesized and cloned into the backbone vector (px330, linearized with BbsI). To validate insertion in the gRNA scaffold, the modified px330 plasmid was sent for Sanger sequencing. Genome editing of wild type CCE ES cells was carried out by transfection with Lipofectamine 2000. 1x10^6 ES cells were co- transfected with 1 μg of purified px330-p16-gRNA vector, 1 μg of purified pcDNA3.1(+)-FDR donor vector and 125 ng (1:8) of a KO-Puro plasmid (containing a puromycin resistance gene). Following transfection, ESCs were plated at clonal densities (100,000, 50,000 and 25,000 cells) in 10 cm tissue-culture plates on a layer of mitotically inactivated SNH feeder cells in ESC cell medium (15% fetal calf serum (PAA, A15-151), 2mM L-glutamine (Gibco, cat# 25030-024), non-essential amino acids (Gibco, cat# 11140-035), 50mM 2-mercaptoethanol (Gibco, cat# 31350010) in Knockout-DMEM (Gibco, cat# 10829018)). In parallel, a mock transfection, in which the same amount of ES cells was transfected with only the px330-p16-gRNA vector, was performed as a selection control. On the next day, puromycin selection at 2 μg/ml was performed for the following 48 hours. At the end of the selection process, no colonies were detected in the control plate, confirming selection efficacy. The rest of the plates were left to expand for a further 5 to 6 days in normal ES cell medium. Colonies were picked and DNA was extracted. Proper targeting of the FDR cassette into the 3’ end of the p16^{INK4A} gene was screened through PCR using primers which generated a 2915 bp fragment which spans the 5’ homology arm and a 2288 bp fragment which spans the 3’ homology arm (i.e., a pair of primers which are external to either the 5’ or 3’ homology region and inside the FDR cassette) (Figure 1A). PCR-amplified DNA fragments encompassing the targeted Cdkn2a locus were Sanger sequenced to confirm proper integration of the FDR cassette. To determine if only one or both of the Cdkn2a alleles were targeted, amplification of a 312 bp PCR product using the p16-WT-SEQ-FWD and p16-WT-SEQ-REV primers was performed (Table S3). Two ES cells clones were properly targeted and used for blastocyst injections. Germline transmission was confirmed and p16^{FDR/+} mice were crossed with C57BL/6. All mouse procedures were performed with the appropriate ethics and animal usage regulations in the United Kingdom Home Office (Scientific Procedures Act 1986) and the relevant institutional guidelines (UCL ethical review committee) and ARRIVE guidelines. For all studies both male and female mice were used and numbers equalised in experimental and control groups. For tumour induction studies all mice were between 6-8 weeks of age before procedures were initiated.

**Human biopsies and ethical regulations**

Human lung samples from independent patients with differently staged lung cancers were obtained from the Royal Papworth Hospital Research Tissue Bank (Project Number T02722). The retrieval, acquisition, processing and analysis of human biopsies was conducted in accordance with all legal requirements and the principles of good clinical practice. Patients signed the Papworth Hospital Tissue Bank general consent and accepted the use of their biopsy for research purposes. The protocol was approved by the Research Ethics Committee. Information about the human samples used can be found in Table S2. AAH, AIS and AC regions were defined by haematoxylin and eosin-stained paraffin sections. Analysis of p21^{CIP1}, p16^{INK4A}, Ki67 and CD68 immunohistochemical staining was carried out using HALO (v3.3.2541) (Indica Labs, NM USA). An automated analysis method for each staining was established based on Indica Labs’ CytoNuclear v2.0.9 settings. A positive control for each stain was used to establish optimal settings that were then used for all samples, and the % positive stain was determined across the whole lesion of interest.

**METHOD DETAILS**

**Tissue processing, immunofluorescence and histological staining**

Tissue for histological analysis were dissected, fixed, dehydrated and processed for paraffin embedding and sectioning as previously described. Immunoﬂuorescence, immunohistochemistry and haematoxylin and eosin staining were performed as previously described on 3–5 μm paraffin sections. As negative controls, sections were stained with secondary antibody alone for immunofluorescence analysis. For immunohistochemistry, tissue sections were mounted in superfast®plus slides and dried overnight. For double immunohistochemistry, an automated immunostaining platform was used (Autostainer Link, Dako or Bond, Leica). Antigen retrieval was first performed with the appropriate pH buffer and endogenous peroxidase was blocked (peroxide hydrogen at 3%). Slides were then incubated with the appropriate primary antibody as detailed. After the primary antibody, the slides were incubated with the corresponding secondary antibodies and visualization systems when needed (Bond Polymer Refine Detection, Bond, Leica; EnVision FLEX+, Dako) conjugated with horseradish peroxidase. Immunohistochemical reaction was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB) (Dako). Nuclei were counterstained with Harris’s haematoxylin. Finally, the slides were dehydrated, cleared and mounted with a permanent mounting medium for microscopic evaluation. Whole slides were acquired with a slide scanner (AxioScan Z1, Zeiss), and images captured with the Zen Blue Software (Zeiss). A full list of the antibodies and the antigen retrieval methods used is presented in Tables S4 and S5.
Viral lung tumour induction
For lung tumour induction in Kras<sup>G12D+/+</sup> mice, Ad5CMVCre (AdCre) adenoviral particles (University of Iowa, Viral Vector Core) were diluted in DMEM to reach a concentration of 5x10<sup>9</sup> particles/µl and CaCl<sub>2</sub> was added to reach a final concentration of 4mM. 6 to 8 weeks old mice of either sex were anaesthetized by an intra-peritoneal injection of a Domitor/Ketamine solution (0.3 mg/kg Domitor (Vetozoit) and 60 mg/kg Ketamine (Dechra) in 0.9% NaCl). Once anaesthetized, 25 µl of the Ad5CMVCre solution was delivered under each nostril per mouse (i.e., 50 µl of the solution used in total per mouse), for passive inhalation. Following delivery of AdCre, mice were given an intra-peritoneal injection of 1 mg/kg of Antisedan (Zoetis) in 0.9% NaCl to reverse the sedative and analgesic effects. Mice were then allowed to recover on a heated mat. For lung tumour induction in Kras<sup>G12V/+</sup> mice, Ad5CMVFlopo (AdFlopo) adenoviral particles (University of Iowa, Viral Vector Core) were diluted in DMEM to a concentration of 8x10<sup>9</sup> particles/µl and CaCl<sub>2</sub> was added to reach a final concentration of 10 mM. The solution was incubated and used as described above. 6 to 8 weeks old mice were anaesthetized by an intra-peritoneal injection of Ketamine/Xylazine solution (100 mg/kg Ketamine (Vetalar®) and 16 mg/kg Xylazine (Rompun®)). Once anaesthetized, mice were transferred to an incubation platform and an optic fibre light source was placed onto the trachea for illumination. A polyethylene tube (PE10) was inserted in the trachea and a total of 62.5 µl of Ad5CMVFlopo solution was injected into the tube, which was carefully held until the totality of the solution was aspirated by respiration. Following intratracheal delivery, mice were placed onto bedding in an upright position to prevent airway obstruction and allowed to recover in a heated rack.

Pharmacogenetic and chemical ablation of senescent cells
Mice were treated by subcutaneous administration of either DT (10 ng/gram of body weight, twice per week) (Sigma Aldrich, cat# D0564), ABT-737 (25µg/gram of body weight, once per week) (MedChemExpress, cat# HY-50907) or vehicle (either PBS following the DT dosing schedule or a solution of 30% Propylene Glycol, 5% Tween80 and 3.3% Dextrose following the ABT-737 dosing schedule) from the point of AdCre infection until harvesting of the lungs.

CSF1R-expressing macrophage blockade
To deplete CSF1R-expressing monocytes/macrophages in Kras<sup>G12D+/+;p16<sup>−/−</sup>Rosa26<sup>loxP-STOP-loxP-YFP/+</sup></sup> mice, 300 µg of anti-CSF1R (CD115) blocking antibody (Bio X Cell, Clone: AFS98, Catalogue number: BE0213) or isotype control antibody (Bio X Cell, Clone: 2A3, Catalogue number: BE0089) was administered by intraperitoneal injection twice a week from the point of AdCre infection until harvesting of the lungs.

Micro-CT imaging of lung tumour bearing mice
To analyse tumour burden in Kras<sup>G12V/+</sup> mice, animals 10.5 months post-AdFlopo infection were anaesthetized by isoflurane inhalation and scanned using a microPET-CT scanner (Mediso Medical) with an X-ray energy of 35 kVP, an exposure time of 450 ms, a total number of projections of 720 and one projection per step following a semi-circular single field of view scan method. Scans were reconstructed through Butterworth filter and a small voxel size. 3D reconstructions and analyses were performed using Slicer 4.10.2 software (3D Slicer). To determine tumour size, the length (longest diameter, L) and width (diameter perpendicular to the length, W) were measured and the ellipsoid formula was applied ((4/3)xPIx(W/2)<sup>2</sup>x(L/2)).

Flow cytometry analysis
Tissues were dissected into 1 mm³ pieces and dissociated in a solution of 0.5% w/v Collagenase type IV (Gibco, cat# 17104019), 0.1x Trypsin (Gibco), 50 µg/ml Dnasel ( Worthington) and 2.5 µg/ml fungizone (Gibco) in Hank’s Balanced Salt Solution (HBSS (Gibco)) for 30 minutes at 37°C. Tissue fragments were further dissociated into single cells through mechanical trituration using a pipette and filtration through a 70 µm filter. Single cells were washed in HBSS and suspended in FACS media (PBS supplemented with 1% foetal calf serum (PAA), 25 mM HEPES and 10 µg/ml of 4’-6-diamidino-2-phenylindole (DAPI, BD Pharmingen) for live/dead cell discrimination) before being analysed using a BD FACS Aria III Flow Cytometer. Fluorescence was detected using a DAPI 450/50 filter, YFP 530/30 filter and mCherry 610/20 filter, where dead, DAPI positive, cells were excluded. Cells were collected into either RLT buffer (Qiagen) supplemented with 1% 2-mercaptoethanol (Sigma Aldrich) for RNA isolation FACS media at 4°C for 10X single cells RNA-sequencing analysis or in vitro culture.

qRT-PCR analysis
For qRT-PCR analysis of KY-FDR flow-sorted cell populations, 5 µl of eluted RNA was amplified using the Ovation Pico WTA System V2 (Tecnan, cat# 3302-12), followed by purification of the resultant cDNA using the QIAquick PCR purification kit (Qiagen). Quantitative real-time PCR reactions were run in triplicate using the iTaq SYBR Green (BIORAD) and replicated using a minimum of three independent samples for each genotype. Primer sequences are included in Table S6. Results were analysed using the ΔΔCt method.

Cell culture
MEFs were isolated as previously described. 65 To induce cellular senescence MEFs were serially passaged until they were no longer proliferative. SA-β-Gal staining was performed using the senescent cell histochemical staining kit (Cell Signaling Technology, cat# 9860), according to manufacturer’s instructions. Crystal violet staining was performed on methanol fixed MEFs using a solution of 0.5% crystal violet and 25% ethanol in water for 10 minutes. Cells were washed in water and air dried before imaging. For EdU
staining, MEFs were treated with EdU in the media for 30 minutes prior to fixation in 4% PFA. Visualisation of EdU incorporation was conducted using the Click-It EdU imaging kit (Invitrogen, cat# C10337) according to manufacturer’s instructions. Immunocytochemical staining was performed as previously described on MEF cultures fixed in 4% PFA. Transfection was performed using Lipofectamine 2000® (Invitrogen, cat# 11668019) according to manufacturer's instructions. Cells were fixed in 4% PFA and analysed 48 hours following transfection. DT ablution experiment on p16+/+ and p16FDR/+ MEFs was performed at passages 2 and 6. 5,000 cells were plated per well in 96-well plates and DT was added at concentrations of 1, 10 and 100 ng/ml the following day. Three days after the addition of diphtheria toxin the cells were fixed in 4% PFA for 10 minutes on ice and the cells were counted using a haemocytometer. MEFs were processed for total RNA extraction using the RNeasy Micro kit (Qiagen). Approximately 1 µg of total RNA was reverse transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit and random hexamers (Roche, cat# 4379012001). YFP-positive tumour cells from KrasG12D/p16FDR/+;Rosa26RtdTomato-STOP-loxP-lucP-YFP/+ mice were isolated by flow cytometry as described above. 1,500 cells were seeded per well in a 96-well plate and cultured in DMEM + 10% fetal bovine serum and 1X Penstrep supplemented with either DMSO as a vehicle control or 50 ng/ml IL10 (Fisher Scientific Ltd, cat# 16871336), 50 ng/ml Ccl2 (Fisher Scientific Ltd, cat# 17676855), 100 ng/ml Cc7 (BioLegend UK Ltd, cat# 586104), 200 ng/ml Cxcl13 (BioLegend UK Ltd, cat# 583904) and 50ng/ml BMP2 (ABCAM PLC, cat# ab214155) recombinant SASP factors. Cells were cultured for 7 days with 1 media change and re-supplementation of the factors on day 4. Cells were fixed in 10% paraformaldehyde on day 7, washed in PBS and stained with DAPI. Cells were then imaged and the number of DAPI+ cells quantified.

Bulk RNA-sequencing
Flow sorted cells from KY-FDR lungs or MEFs were processed for total RNA extraction using the Rneasy Micro kit (Qiagen). Isolated RNA was processed by the SMARTer V4 low input assay kit (Clontech) to generate amplified cDNA using the strand-switching protocol. Library preparation was performed with 200 pg of amplified cDNA using the Nextera XT preparation kit with 12 cycles of PCR. Sequencing was then performed on an Illumina NextSeq 500 with 43 bp paired end reads. Sequencing data was then aligned in BaseSpace. Differential expression was performed using DESeq2. For GSEA, genes were ranked by the Wald statistic and GSEA performed using the pre-ranked tool in GSEA v2.2 (Broad Institute). Gene sets used in this study were either obtained from public databases (gene ontology (GO), KEGG, REACTOME, molecular signatures database (Broad Institute) or published literature: SASP).

Single cell RNA sequencing
For both single cell RNA-sequencing experiments, flow-sorted mCherry cells were centrifuged and resuspended in complete DMEM to a concentration of 700-1000 cells per microlitre. Cells were partitioned and barcoded using the 10X Genomics Chromium Controller and associated v3 kit components, according to manufacturer’s instructions. Harvested cDNA was assessed and quantified using the Agilent 2100 Bioanalyser and library construction performed using the 10X Single Cell 3’ Library Kit. Libraries were quality controlled by performing a MiSeq Nano PE100 run followed by deeper sequencing on the HiSeq2500 v4 system.

QUANTIFICATION AND STATISTICAL ANALYSIS

Imaging and Cell counting
Whole-mount imaging of lungs was conducted in a Leica MZ FLIII stereomicroscope. Immunofluorescence staining on paraffin sections were visualized with a Leica DMLM widefield microscope. Visualization of immunohistochemical and H&E staining was conducted in a Zeiss Axiosplan2 microscope. Image processing was conducted using Fiji/ImageJ and Zen 3.2 (Carl Zeiss), which included brightness/contrast enhancement and merging of fluorescence channels to produce composite images. Cell counting from microscope images was conducted using either Fiji/ImageJ, with the cell counter plugin, or QuPath software. All in vivo cell counting analysis were performed in three to five non-consecutive histological sections immunostained using the relevant antibodies. 300 to 1000 marker-positive cells were counted for each genotype. DAPI stained YFP-positive tumour cells cultured counting analysis were performed in three to five non-consecutive histological sections immunostained using the relevant antibodies. Cell cycle state was inferred using CellCycleScoring() function; to reduce the effect of cell cycle genes, these were removed prior to further analyses. A pseudogenome was generated from the FDR cassette; the mapping was performed using STAR, with default parameters, on the pseudo-bulk version of each single-cell sample. Next, each mapping read was assigned on the cell barcode; the expression levels of the components of the FDR cassette were calculated as the algebraic sum of mapped reads. The pre-processing of raw count matrices was performed using Seurat (v4.0.3). Gene expression was normalised using sctransform(). Principal component analysis was carried out on the top 3000 highly variable genes; the parameters for the clustering step were refined using ClustAssess. Neighbours were identified using the first 50 PCs and clustering carried out for the clustering step were refined using ClustAssess. Neighbours were identified using the first 50 PCs and clustering carried out.
using the Louvain algorithm with the 20 nearest neighbours for each cell, and cluster resolution=0.1. UMAP projections were calculated using RunUMAP(n.neighbors=20, min.dist=0.3). Cluster-specific marker genes were identified using the Seurat FindMarkers() function. Differentially expressed genes (per cluster and within clusters, across samples) were called on: abs(log₂FC) > 0.25, bonferroni corrected p-value < 0.05, and minimum of 10% of cells expressing the gene in at least one group. Gene set enrichment analysis (GSEA) of DE genes was carried out using gprofiler2 (v0.2.0) using all genes detected (i.e. expression level >0) in the compared cell groups as the background set. Enrichment was tested on the standard GO terms, KEGG and REACTOME pathway databases, and the miRNA and TF regulatory features. The Benjamini-Hochberg correction for multiple testing was applied to GSEA p-values. scMap (v1.6.0) was used to project cells from the scRNA-Seq datasets onto publicly available reference datasets (i.e. ageing lung atlas and the lung dataset from the pre-annotated Tabula Muris Senis consortium). Any further optimisation of cell identity in the different datasets was based on manual annotations of well-known marker genes.

**Statistics**

One-tailed Student’s t test was performed to compare the means of two groups. Groups of three means were compared using a one-way ANOVA. Significance was assumed at \( p < 0.05 \). Sample size varied, but a minimum of three biological replicas were used for analysis. Statistical analyses were performed using GraphPad software. No samples were excluded from analyses. No formal blinding or randomisation was used.