nature portfolio

https://doi.org/10.1038/s41586-024-08285-0

Supplementary information

Ageing limits stemness and tumorigenesis by reprogramming iron homeostasis

In the format provided by the authors and unedited

Supplementary Information Guide

Supplementary information of this manuscript includes Supplementary Figures 1-11, Supplementary Discussion and Source Data.

Source Data. Provided in a separate spreadsheet.

Supplementary Table 1: Differential gene expression analysis of aged vs. young AT2 cells transformed ex vivo controlled for passage or diffusion pseudotime (dpt).

Supplementary Table 2: Aging-associated differential gene expression shared between AT2 cells and LUAD (FDR < 0.05).

Supplementary Table 3: Gene set enrichment analysis using genes in the aged vs. young differential gene expression signatures shared between AT2 cells and LUAD.

Supplementary Table 4: Differential gene expression analysis of aged and young autochthonous KP-Cas9 lung tumors in the context of Nupr1 knockout or control sgRNA.

Supplementary Table 5: Differentially methylated cytosines in aged vs. young AT2 cells.

Supplementary Table 6: Differentially methylated cytosines in aged vs. young KP LUAD cells.

Supplementary Table 7: Aging-associated differentially methylated cytosines in AT2 cells at aging-associated differentially expressed genes annotated based on histone marks in AT2 cells.

Supplementary Table 8: Aging-associated differentially methylated cytosines in the proximity of transcription starting sites (TSS) of Nupr1 and Lcn2, annotated based on histone marks in AT2 cells.

Supplementary Table 9: Differential gene expression following DNMT1 inhibition in young AT2 cells.

Supplementary Table 10: Differential gene expression results following shDnmt1expression in young AT2 cells.

Supplementary Table 11: Information of selected Nupr1 enhancers.

Supplementary Table 12: Sequences of short hairpin RNAs (including knockdown efficiency), single guide RNAs, and PCR primers used in the study.

Supplementary Table 13: Details of pyrosequencing assay.

Supplementary Table 14: List of archived normal human lung resections.

Supplementary Table 15: List of archived human lung cancer sections and freshly resected normal human lung tissues.

Supplementary Table 16: Correlation of aged vs. young KP LUAD gene expression with human patients with KRAS mutant LUAD (TCGA data).

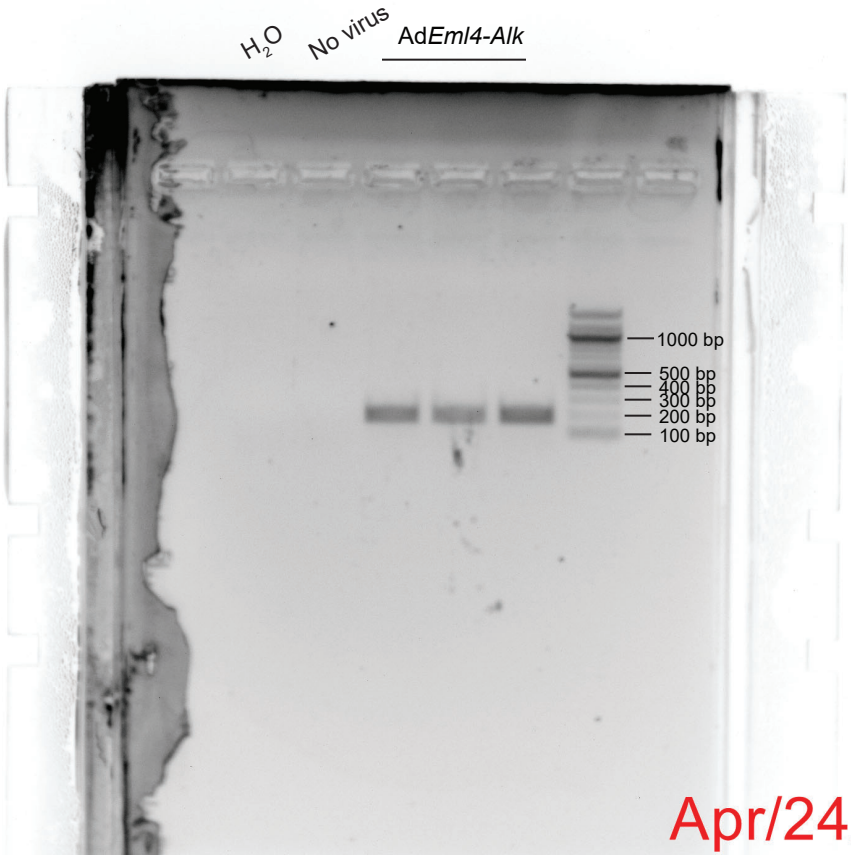
Supplementary Table 17: List of reagents.

Supplementary Table 18: Numerical p values that are not shown in the figures.

Supplementary Table 19: Conversion rate of methylated cytosines in the EM-Seq assay.

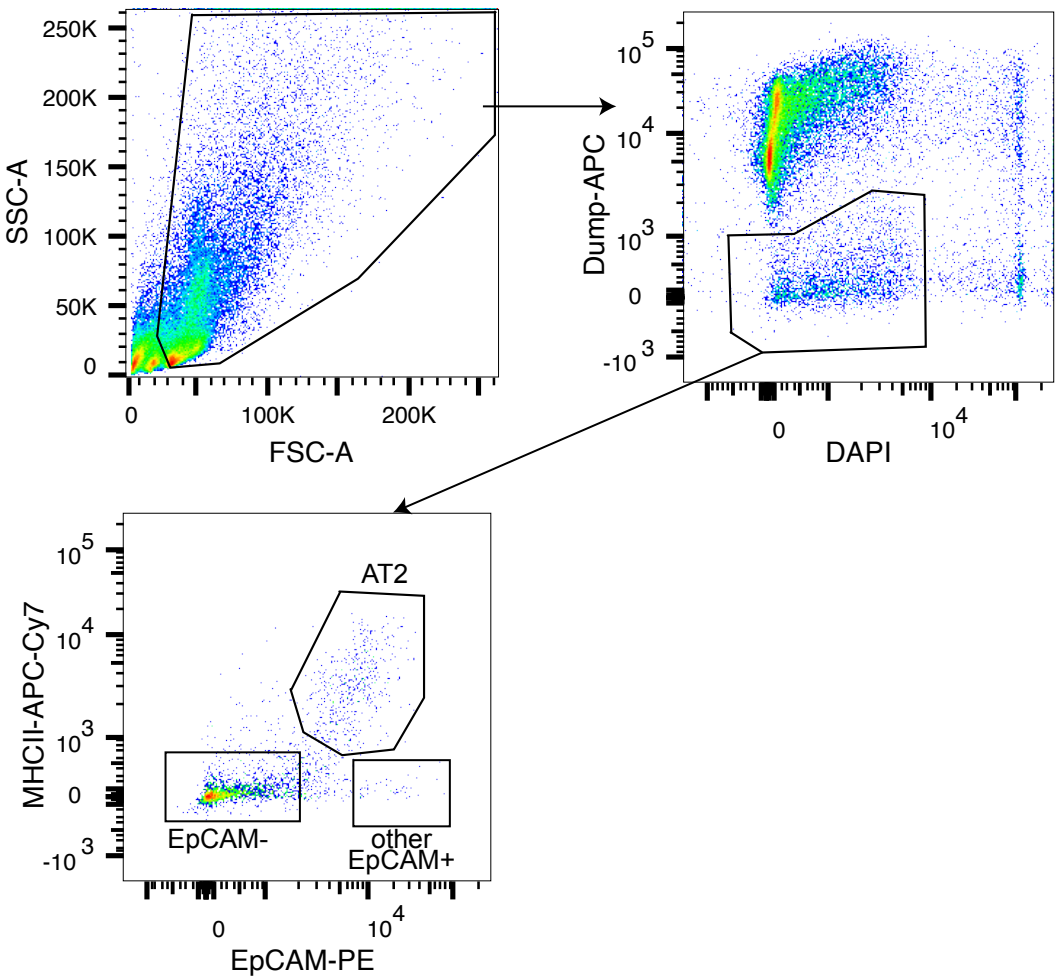
Supplementary Table 20: Veterinary pathology review of aged and young lung tissues 28 days after hyperoxia-induced lung injury by S. E. Carrasco, DVM, MPVM, MSc, PhD.

Supplementary Figure 1. Raw Data for Extended Data Figure 1i



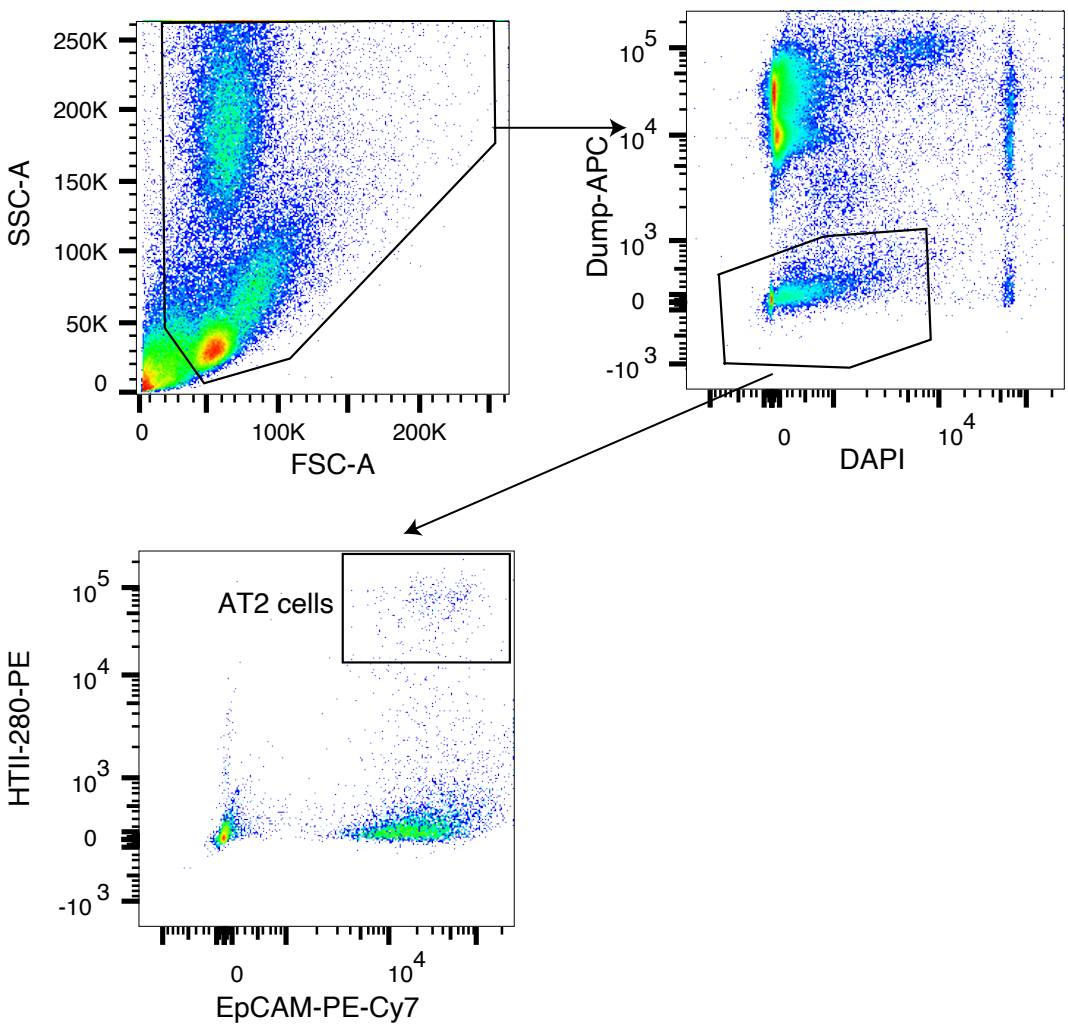
Raw Data (Gel)

Supplementary Figure 2



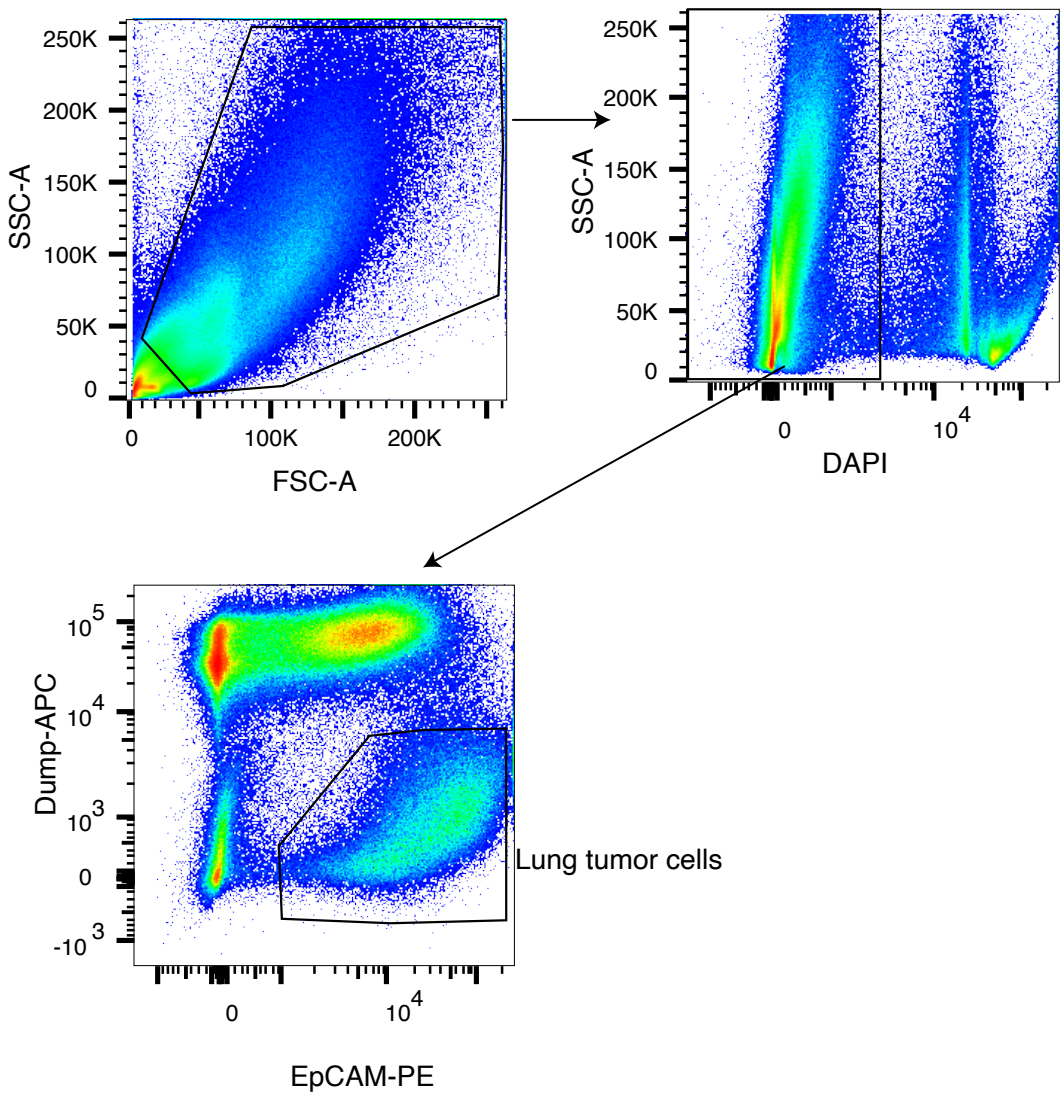
Example of gating strategy for flow cytometry analysis and sorting of mouse AT2 cells.

Supplementary Figure 3



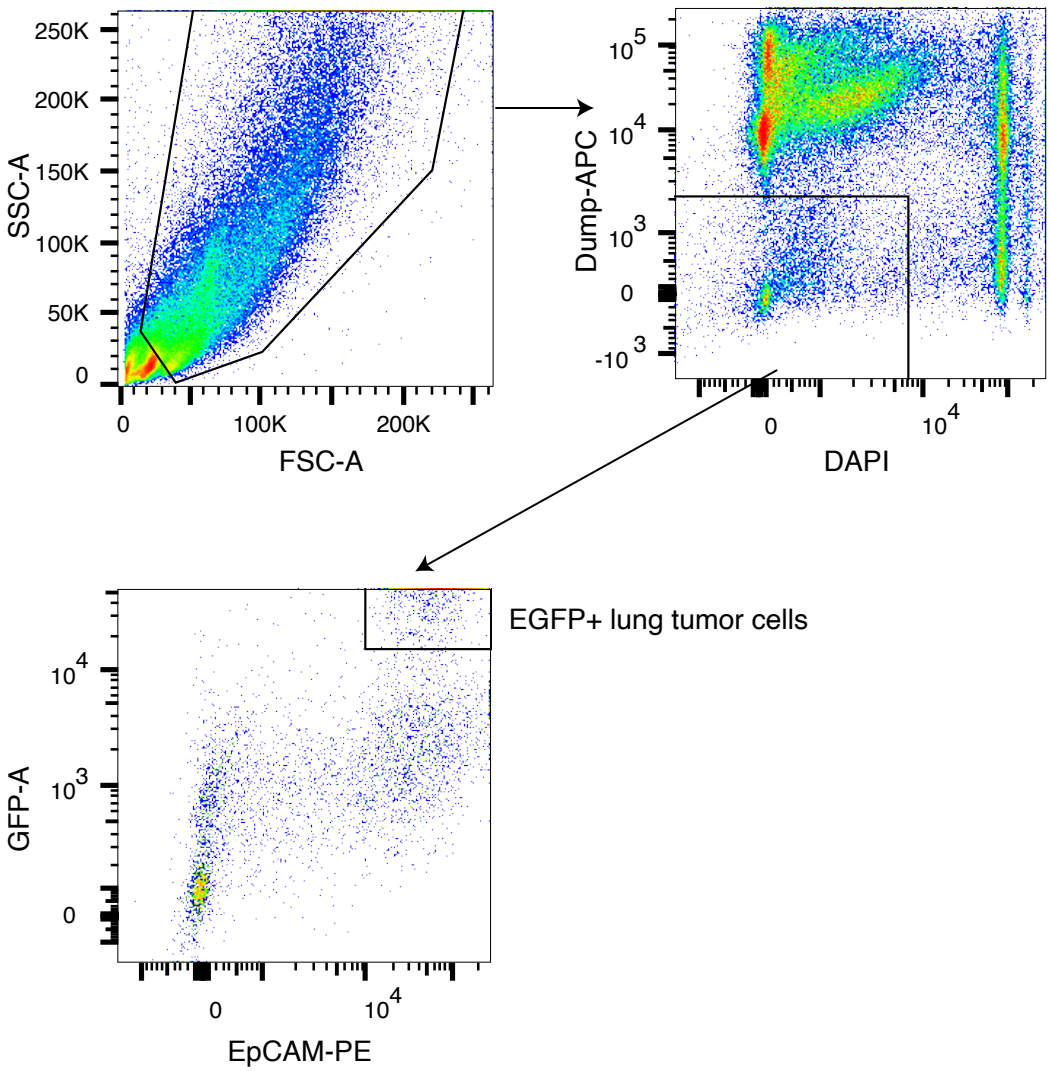
Example of gating strategy for fluorescence-activated cell sorting of human AT2 cells.

Supplementary Figure 4



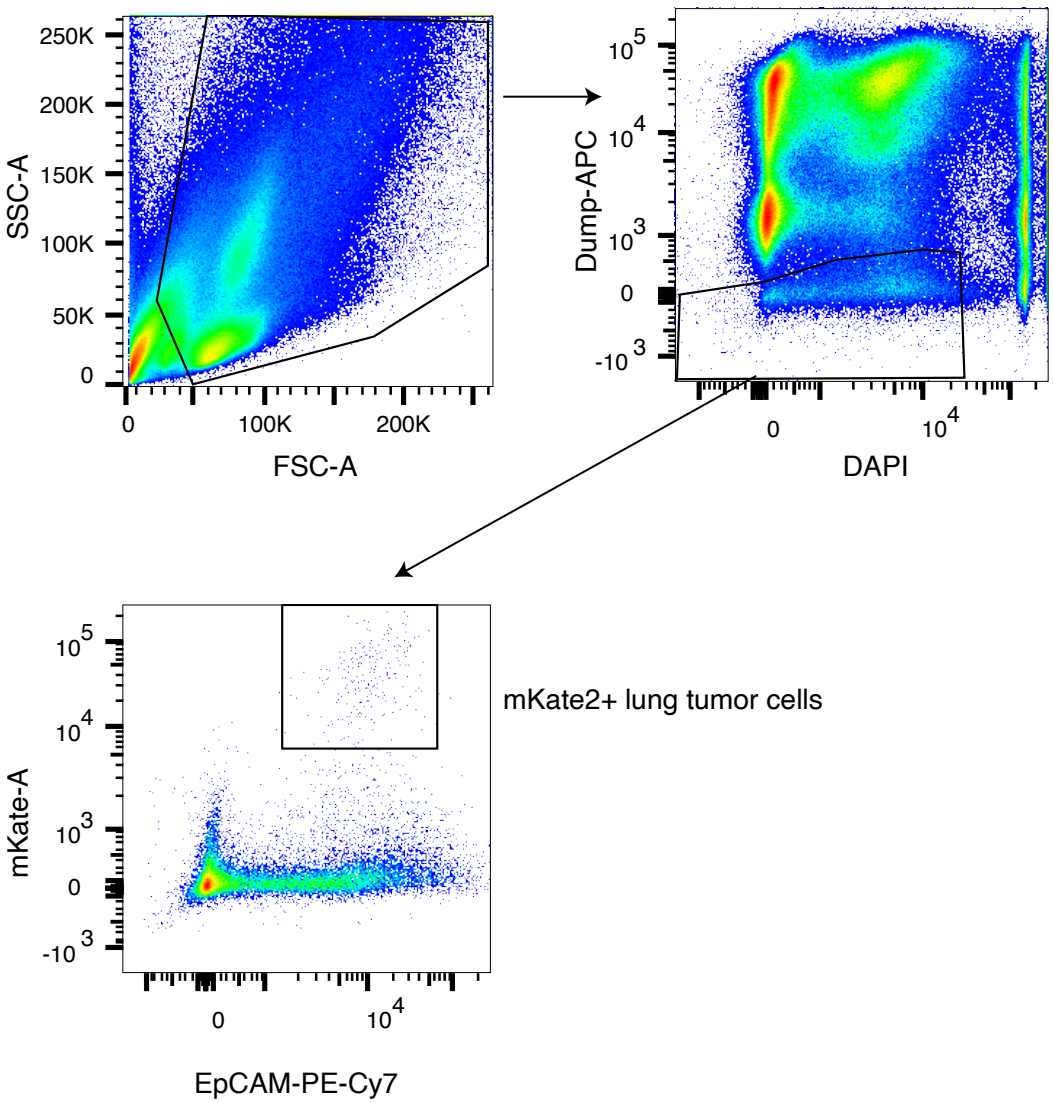
Example of gating strategy for fluorescence-activated cell sorting of mouse *Kras*$^{G12D/+}$; *Trp53*$^{fl/fl}$ lung tumor cells without fluorescent reporter.

Supplementary Figure 5



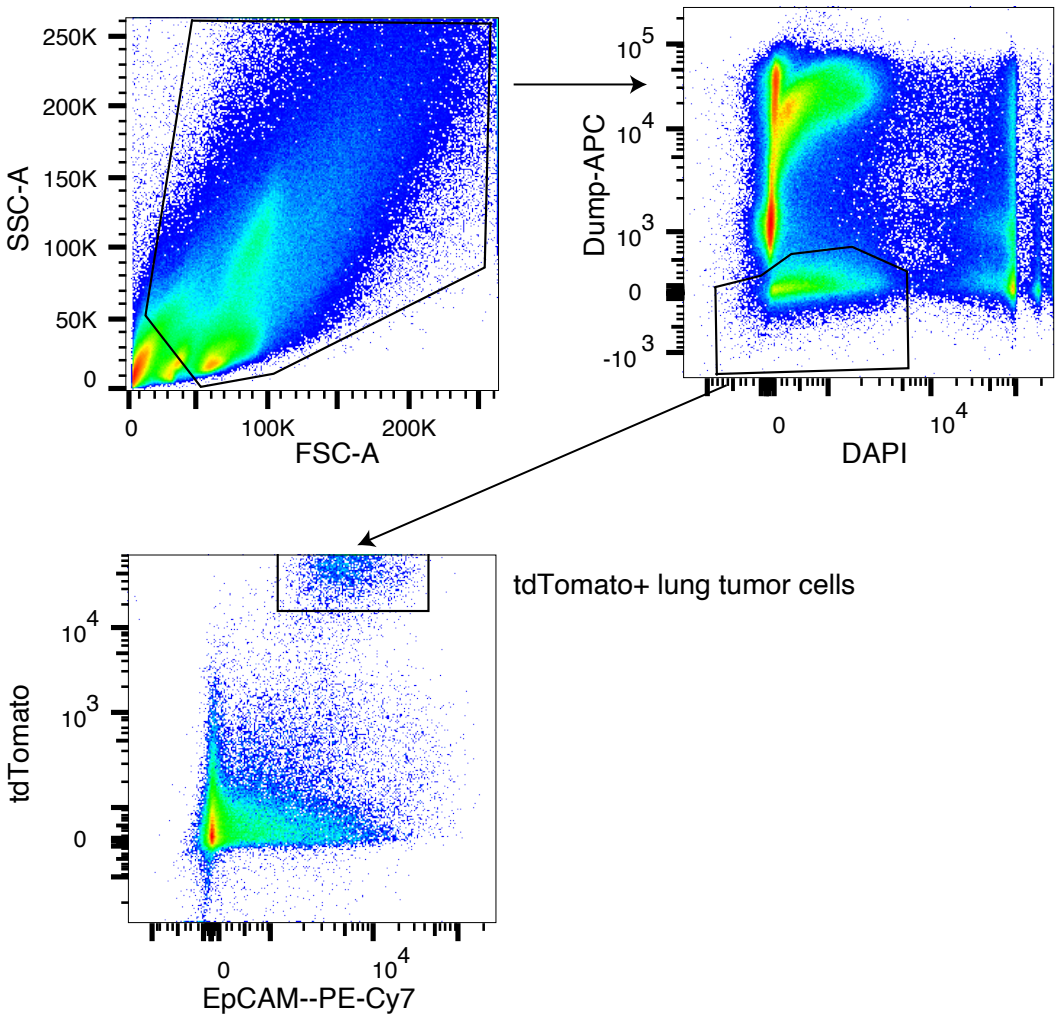
Example of gating strategy for fluorescence-activated cell sorting of *Kras*$^{G12D/+}$; *Trp53*$^{fl/fl}$; *Rosa26*$^{EGFP\text{-}Cas9/+}$ mouse lung tumor cells, which express EGFP fluorescent reporter.

Supplementary Figure 6



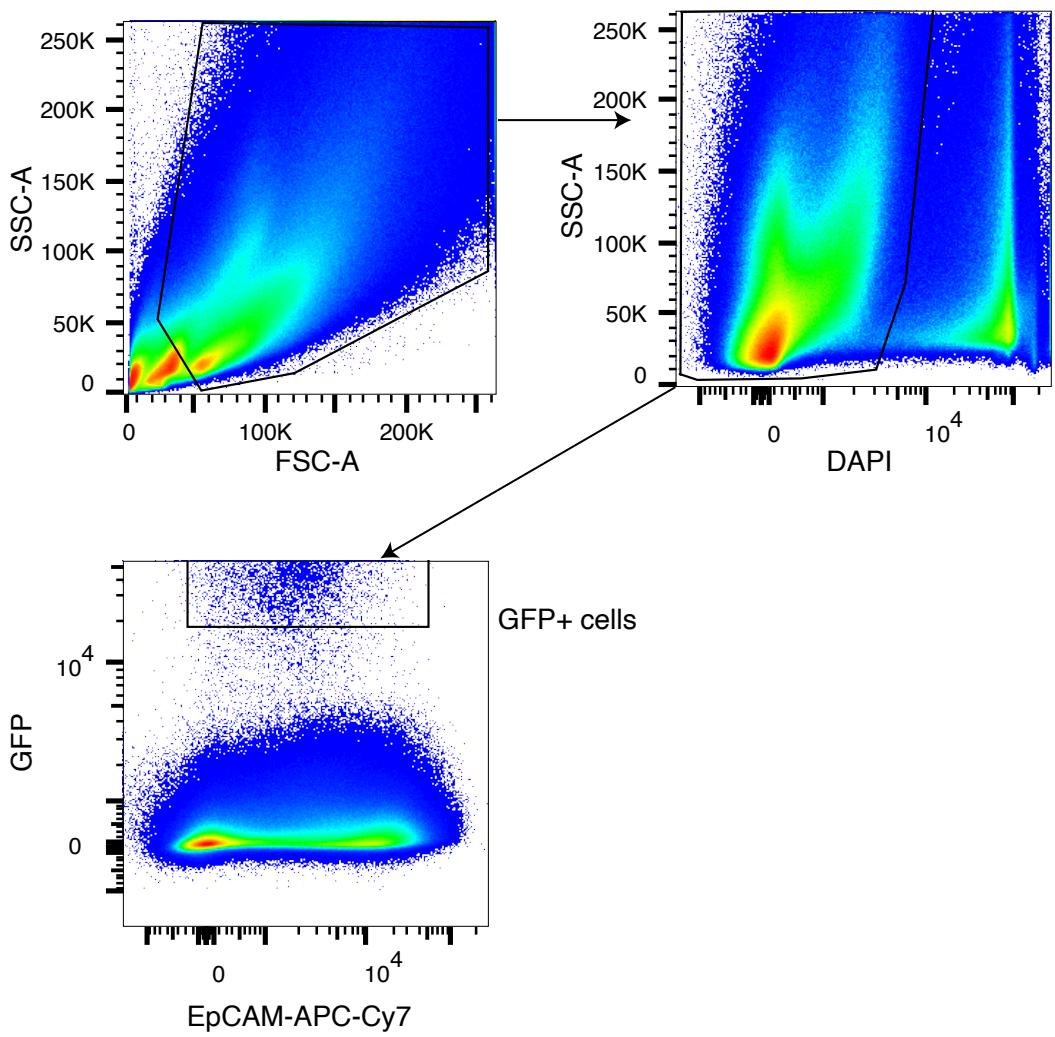
Example of gating strategy for fluorescence-activated cell sorting of *Kras*$^{G12D/+}$; *Trp53*$^{fl/fl}$; *Rosa26*$^{rtTA\text{-}IRES\text{-}mKate2/+}$ mouse lung tumor cells, which express mKate2 fluorescent reporter.

Supplementary Figure 7



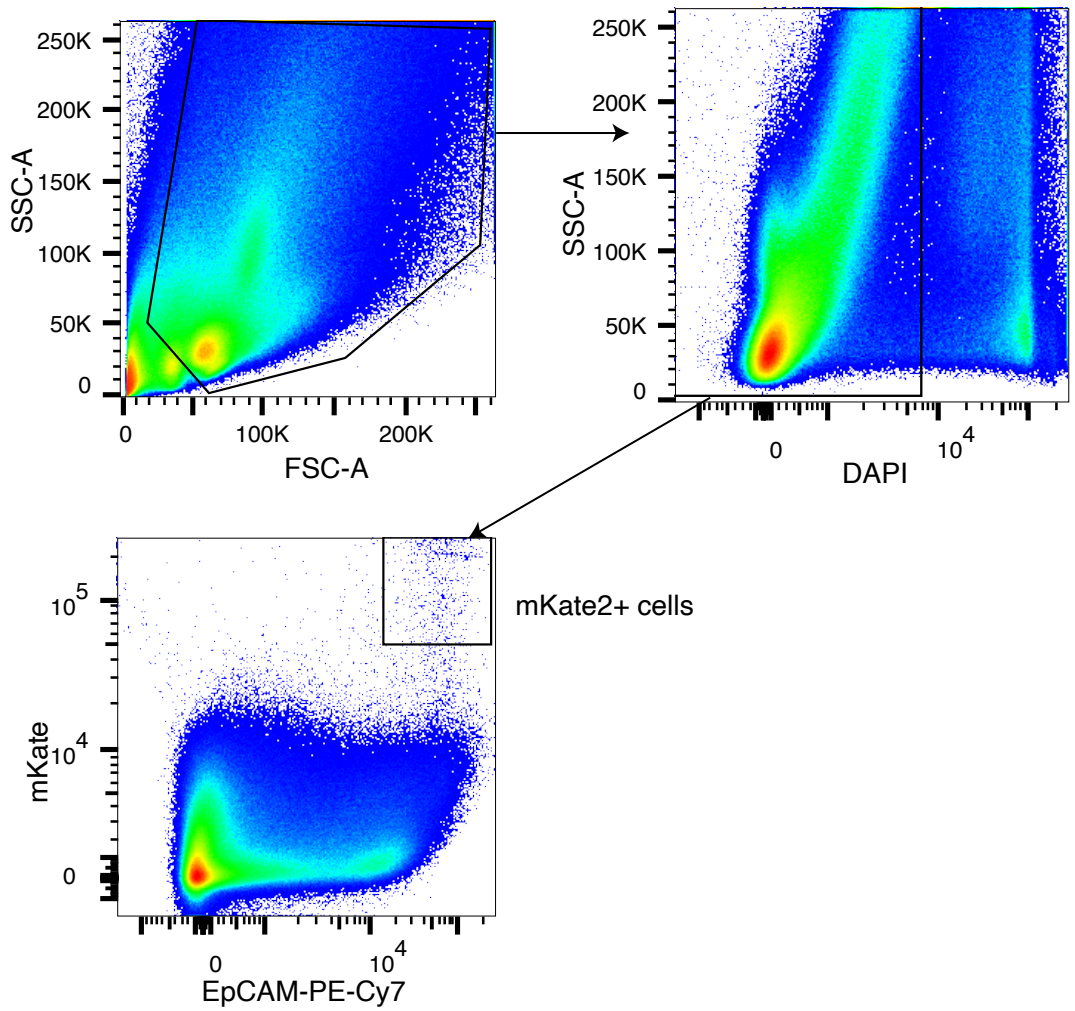
Example of gating strategy for fluorescence-activated cell sorting of *Kras*$^{G12D/+}$; *Trp53*$^{fl/fl}$; *Rosa26*$^{tdTomato/+}$ mouse lung tumor cells, which express tdTomato fluorescent reporter.

Supplementary Figure 8



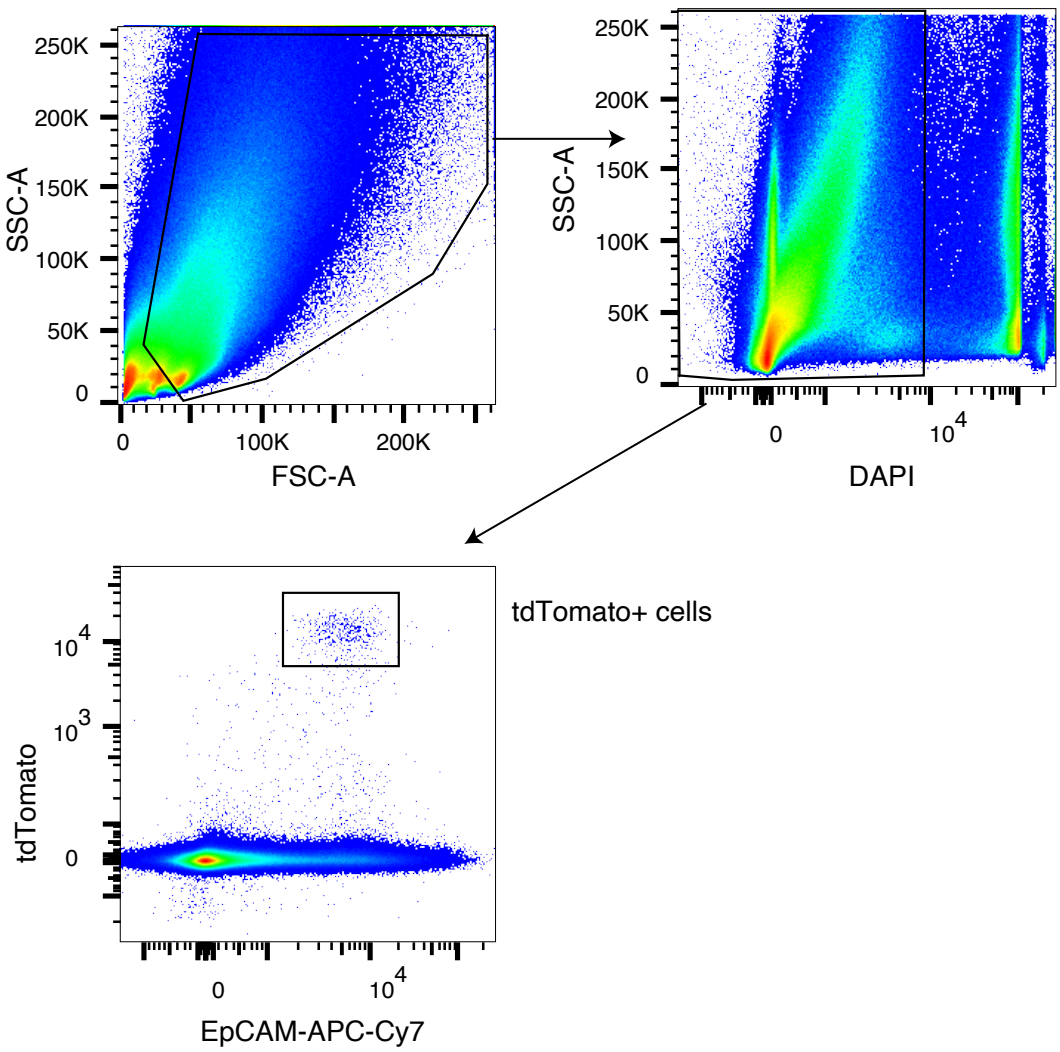
Example of gating strategy for fluorescence-activated cell sorting of GFP+ mouse lung cells from *Rosa26*$^{mTmG/+}$ mice. Fluorescence shifting from membrane-bound tdTomato to membrane-bound GFP suggests the cells were tranduced by lentiviral Cre and shRNAs. AT2 cell identity were further confirmed by single-cell mRNA sequencing.

Supplementary Figure 9



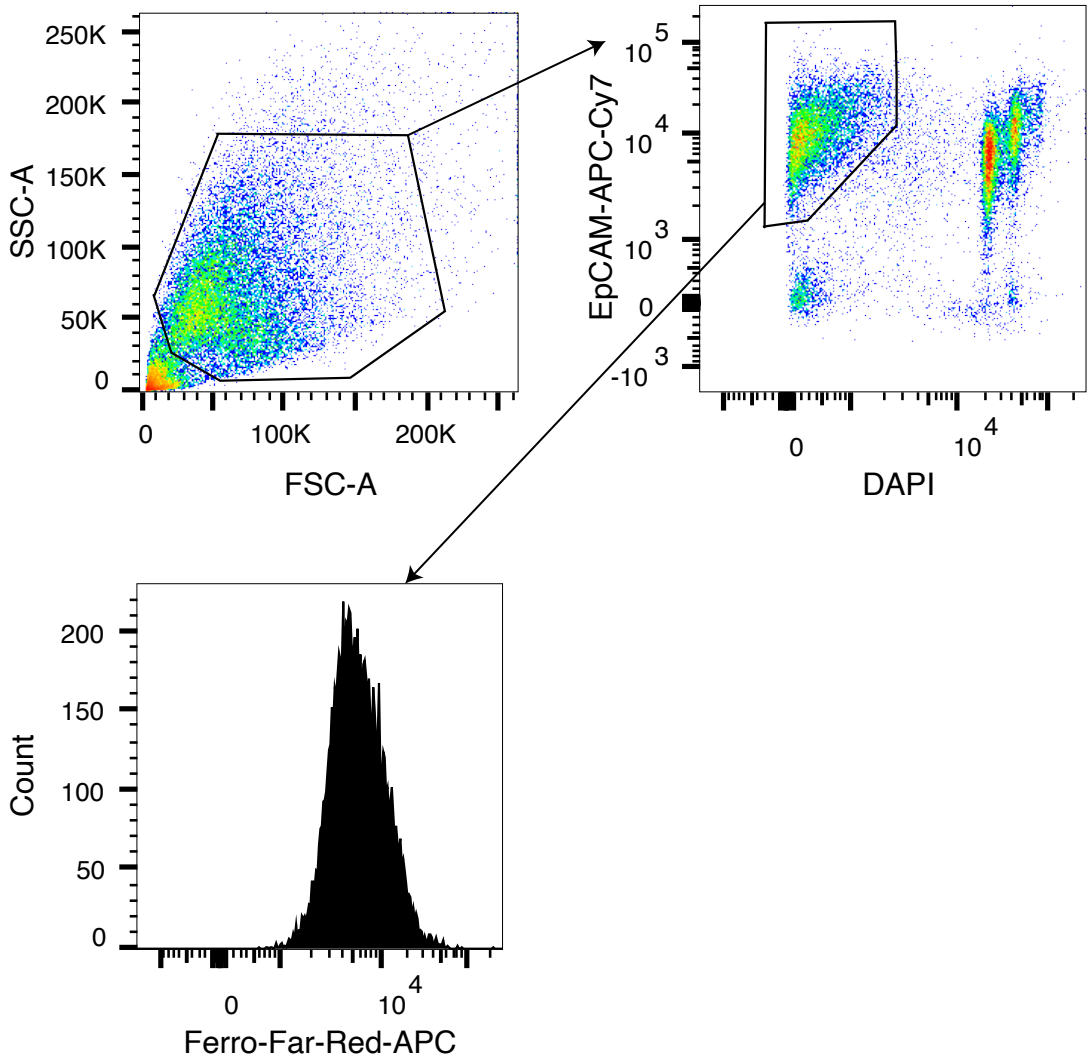
Example of gating strategy for flow cytometry analysis of mKate2+ epithelial cells from *Trp53*$^{fl/fl}$; *Rosa26*$^{rtTA\text{-}IRES\text{-}mKated2/+}$ mice for quantification of transduction efficiency.

Supplementary Figure 10



Example of gating strategy for flow cytometry analysis of tdTomato+ epithelial cells from *Rosa26*$^{tdTomato/+}$ mice for quantification of transduction efficiency.

Supplementary Figure 11



Example of gating strategy for flow cytometry analysis of labile iron level by Biotracker Ferro Farred dye in lung tumor cells derived from *ex vivo* transformation assays.

Supplementary Discussion

Contrasting aging-associated loss of stemness and cell-intrinsic tumorigenic potential with changes in the microenvironment. Accumulation of somatic mutations and loss of stemness occur in parallel over the course of organismal aging. The significance of this relationship for cancer development has been a subject of debate[1,2], yet experimental studies directly addressing the capacity of aged stem cells or progenitors to transform *in vivo* have been lacking. Although we find aging-associated changes intrinsic in the cell-of-origin are tumor suppressive, our results utilizing heterochronic transplantation suggest the aged lung is a more hospitable microenvironment for tumor growth compared to the young. Similar observations have been made by others utilizing cell line transplantation in other cancer contexts[3]. Mechanisms rendering aged hosts more permissible may include a decline in anti-tumor immunity ("immunosenescence")[4], metabolic changes[5], and alterations in niche factors[3,6].

Reconciling aging-associated increase in absolute iron levels with functional iron insufficiency. Interestingly, like other cell types in aged tissues[7], we found higher baseline levels of total iron in aged AT2 cells than in their young counterparts, suggesting that aged AT2 cells require more iron to maintain stemness and tumorigenic potential. Another possibility is that the aged AT2 cells suffer from inability to mobilize sufficient iron from cellular iron stores, maintained by ferritin and other iron-binding proteins[7], including lipocalin-2. This may be due to aging-associated changes in levels, activity, or subcellular localization of other iron homeostasis factors. Finally, changes in iron-biding carrier molecules may play a role, including the mammalian siderophore 2,5-DHBA that is required for lipocalin-2 to bind iron[8]. Our findings suggest (i) 2,5-DHBA augments labile

iron trapping by lipocalin-2 in the AT2 cells and (ii) its availability in the AT2 cells may be rate-limiting for lipocalin-2 function. It is possible that more of the iron is trapped by 2,5-DHBA in the aged cells compared to the young. Future studies are needed to elucidate the mechanistic underpinnings of functional iron insufficiency in aged AT2 cells despite their higher absolute iron levels when compared to the young.

Possible epigenetic mechanisms responsible for aging-associated loss of differentiation capacity. In addition to its importance for physiologic differentiation, iron has been shown to be essential for epigenetic remodeling in cancer. A recent elegant study found iron is required for the demethylation of repressive histone marks critical to epithelial-mesenchymal transition (EMT) in cancer cells[9]. In line with this work, we found iron insufficiency driven by NUPR1 suppressed progression of lung tumors that managed to initiate in aged mice, suggesting that iron may be required for the cancer cell state transitions that define lung cancer progression[10]. The iron-dependent TET-family methylcytosine dioxygenases and the Jumonji domain-containing histone demethylases (JmjC) are possible candidates responsible for the epigenetic remodeling enabling these cell state transitions, whose activity may be impaired by iron insufficiency[11-13] [14]. Given their common iron dependence[13], it is likely that most or all JmjC enzymes may be impacted by the aging-associated functional iron insufficiency and impart global changes; however future work to definitively identify the key players will be valuable.

Possible other functions of NUPR1 in aging besides regulation of cellular iron. Our results indicate that aging-associated DNA hypomethylation at *Nupr1* enhancers is permissive for upregulation of *Nupr1* expression, which may be induced by various forms of cellular stress[15,16],

including the accumulation of reactive oxygen species, ER stress, DNA damage, inflammation, and increased tumor suppressor activity, all of which occur during aging[17-19]. The combination of stress inputs and the epigenetically permissive hypomethylated state provide a mechanistic explanation for the aberrantly high levels of *Nupr1* in the aged AT2 cells, which deprives the AT2 cells of sufficient iron, leading to loss of stemness and tumorigenic potential. We note that although we identified dysregulation iron homeostasis as the central mechanism downstream of NUPR1 underpinning aging-associated loss of stemness and tumorigenic potential, our results do not rule out that other functions such as mitigation of ROS, ER stress, or DNA damage may be impacted by induction of NUPR1.

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