Novel Insights from a Multiomics Dissection of the Hayflick Limit

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Abstract The process wherein dividing cells exhaust proliferative capacity and enter into replicative senescence has become a prominent model for cellular aging *in vitro*. Despite decades of study, this cellular state is not fully understood in culture and even much less so during aging. Here, we revisit Leonard Hayflick's original observation of replicative senescence in WI-38 human lung fibroblasts equipped with a battery of modern techniques including RNA-seq, single cell RNA-seq, proteomics, metabolomics, and ATAC-seq. We find evidence that the transition to a senescent state manifests early, increases gradually, and corresponds to a concomitant global increase in DNA accessibility in nucleolar and lamin associated domains. Furthermore, we demonstrate that senescent WI-38 cells acquire a striking resemblance to myofibroblasts in a process similar to the epithelial to mesenchymal transition (EMT) that is regulated by the transcription factors YAP1/TEAD1 and TGF- β 2. Lastly, we show that verteporfin inhibition of YAP1/TEAD1 activity in aged WI-38 cells robustly attenuates this gene expression program.

Introduction

Replicative senescence in animal cells growing *in vitro* was first discovered by Leonard Hayflick. He found that primary human diploid fibroblast cell lines ceased to proliferate after an extended number of serial passages (Hayflick, 1965). Since then, considerable work has been done to describe this phenomenon. A major causal feature of replicative senescence is telomere erosion, a process in which the telomeres gradually shorten with increasing cellular divisions. Eventually, the telomeres become uncapped which triggers a DNA damage response that results in cell cycle exit (Harley *et al.*, 1990). It is understood that this is due to the absence of the telomerase reverse transcriptase (hTERT), the catalytic component of human telomerase which adds telomeric sequences to the ends of chromosomes to maintain telomere length in germ cells and stem cells. As hTERT activity is undetectable in normal human somatic cells, telomere attrition is a common aging phenotype hypothesized to underlie cellular senescence at the organismal level (López-Otín *et al.*, 2013a; Meyerson *et al.*, 1997). Replicative senescence of human somatic cell lines *in vitro* can be avoided by overexpression of hTERT which prevents telomere shortening and confers apparently unlimited replicative capacity (Bodnar *et al.*, 1998).

Beyond growth arrest and telomere shortening, phenotypic changes exhibited in replicatively senescent cells include the senescence associated secretory phenotype (SASP). Proteins of the SASP include proinflammatory cytokines, growth factors, angiogenic factors, and proteases. The SASP has been shown to play a role in paracrine signaling whereby senescent cells can promote local wound healing and/or drive healthy neighboring cells into senescence (Acosta *et al.*, 2013;

43 *Coppé et al., 2006; Demaria et al., 2014; Coppé et al., 2008*). Replicatively senescent cells also accu-
44 mulate DNA and protein damage, accumulate lipids, and lose regulatory control of mitochondria
45 and lysosomes (*Gorgoulis et al., 2019*).

46 The phenotypic similarity (telomere attrition, epigenetic alterations, mitochondrial dysfunction
47 and loss of proteostasis) between the cell autonomous aging hallmarks and *in vitro* senescence has
48 led to the hypothesis that senescent cells *in vivo* play a causal role in organismal aging and aging-
49 related diseases (*Hernandez-Segura et al., 2018; Campisi and Di Fagagna, 2007; Sedelnikova et al.,*
50 *2004; Jeyapalan and Sedivy, 2008; Herbig et al., 2006; Childs et al., 2015; López-Otín et al., 2013b*).

51 Consistent with this model, several age-related disease states can be directly linked to telomere
52 length or telomerase activity. For example, up to 15% of familial idiopathic pulmonary fibrosis
53 (IPF) cases arise from mutations in telomerase and up to 25% of sporadic cases occur in people
54 with telomere lengths less than the 10th percentile. (*Tsakiri et al., 2007; Armanios et al., 2007;*
55 *Cronkhite et al., 2008; Alder et al., 2008; Stuart et al., 2015; Raghu et al., 2006; Duckworth et al.,*
56 *2021; Stuart et al., 2014; Dai et al., 2015*). Furthermore, the elimination of senescent cells in a
57 number of age-related diseases, such as cardiac fibrosis, pulmonary fibrosis, neurodegenerative
58 diseases, osteoporosis, and metabolic disorders have been argued to alleviate the disease state
59 (*Pignolo et al., 2020*). Clinical trials for senolytics targeting fibrotic diseases, osteoporosis, frailty,
60 and metabolic syndromes are currently underway (*Borghesan et al., 2020*).

61 The mechanism by which senescent cells might contribute to aging phenotypes is currently
62 still unclear (*Hernandez-Segura et al. (2018, 2017)*). In an effort to bring clarity to the replicative
63 senescence process *in vitro* that in turn could elucidate *in vivo* function, we revisited and redesigned
64 the original Hayflick experiment. Making use of recent advances in technology we tracked changes
65 in cell state throughout the replicative lifespan of the original Hayflick WI-38 cell line using bulk
66 RNA-seq, single cell RNA-seq (scRNA-seq), ATAC-seq, metabolomics and proteomics.

67 Overall, our data recapitulate many known features of the *in vitro* senescence process while
68 simultaneously providing novel insight. First, the time resolution of our experiment coupled with
69 single cell trajectory analysis reveals that senescence is a gradual process that shares transcrip-
70 tional, proteomic, and metabolomic features with epithelial-mesenchymal transition (EMT). Sec-
71 ond, our metabolomic data identifies Nicotinamide N-methyltransferase (NNMT) activity as a po-
72 tential initiating event in replicative senescence dependent loss of epigenetic silencing. Third, we
73 show that these genomic regions that exhibit increased accessibility with increasing cellular age
74 are concentrated in nucleolar/lamin associated domains and correspond with observed changes
75 in the replicative senescence transcriptome. Lastly, integration across data modalities reveals that
76 senescent WI-38 cells bear a strong resemblance to myofibroblasts. We provide bioinformatic and
77 experimental evidence that the YAP1/TEAD1 transcription factor complex and TGF- β 2 signaling are
78 putative regulators of the transition to this state. Together our data suggests that a process sim-
79 ilar to fibroblast to myofibroblast transition (FMT; analogous to EMT) is an intrinsic aspect of the
80 replicative senescence phenotype in WI-38 fibroblasts.

81 Results

82 Transcriptomic profiles of replicative senescence, radiation induced senescence, 83 and increasing cellular density.

84 To capture the replicative senescence process with high resolution we designed an experiment
85 to continuously grow and intermittently sample cells from a starter batch of WI-38 cells that had
86 undergone only 20 population doublings (PDL 20) (methods). To distinguish between replicative
87 senescence dependent changes and those arising from altered cell density and growth rate, we
88 performed a cell density control study (methods). Briefly, early PDL cells were sampled at increas-
89 ing levels of cell density to measure gene expression changes associated with cellular density and
90 decreasing cell proliferation independent of replicative senescence (Figure 1A). In addition to the
91 cell density control, we also included TERT immortalized WI-38 cells (hTERT) grown in parallel and

102 sampled alongside wild type (WT) cells as a control for long term culturing and day-to-day sampling
103 batch effects (*Bodnar et al., 1998*). The hTERT cell line was generated in advance of the experiment.
104 Frozen aliquots of the hTERT line were thawed alongside and sampled in parallel with WT WI-38
105 cells. As expected, hTERT immortalized cells grew at a constant rate and did not slow or cease
106 growth (Figure 1B).

107 We also included a radiation induced senescence condition to test for differences between repli-
108 cation and radiation induced senescence and to isolate changes arising from acute DNA damage
109 response. Finally, we sampled proliferating WT and hTERT WI-38 cells at multiple PDLs for RNA-
110 seq, scRNA-seq, proteomics, metabolomics and ATAC-seq until the WT WI-38 cells had reached
111 senescence as measured by the cessation of growth (Figure 1B, Figure 1 supplement 1).

112 We first examined the bulk RNA-seq data to compare and contrast replicative senescence with
113 radiation induced senescence, cell density, and hTERT cells (quality control metrics located in Figure
114 1 source data 1). Differential gene expression analysis using RNA-seq revealed 6,955 genes change
115 with increasing PDL, 7,065 genes change in response to ionizing radiation, and 9,958 genes vary
116 with increasing cell density (FDR adjusted p-value < 0.01, Figure 1 source data 2). Notably, the tran-
117 scriptional changes we observed were consistent with previous studies. Figure 1D shows the strong
118 correlation of the pattern of induced transcription established in our PDL 50 cultures and the fibrob-
119 last derived senescence-associated signature compiled across multiple fibroblast senescence gene
120 expression experiments from cell lines derived from different tissue depots (*Hernandez-Segura
121 et al., 2017*). We also observed induction of senescence-associated- β galactosidase activity (Figure
122 1C) as well as p16 and p21 in both our RNA-seq, proteomics data, and via western blot (Figure 1
123 supplement 2, Figure 1 supplement 3). These data reveal that the cells in our time course display
124 classic features of senescent cells and exhibit transcriptomic changes that are highly correlated
125 with previous studies of replicative senescence. Together, these data strongly suggest that our
126 WI-38 cells successfully reached replicative senescence.

127 Hierarchical clustering of all significantly changing genes across the four conditions highlighted
128 several important features of our experiment. First, as expected, gene expression in the hTERT-
129 immortalized WI-38 cells remained largely stable. Second, replicative senescence, radiation in-
130 duced senescence, and cell density exhibited many shared, but also unique, gene expression
131 changes with respect to both the identity of differentially expressed genes and the magnitude
132 of their changes (Figure 1E).

133 To facilitate a biological interpretation of these shifting transcriptomic landscapes, we applied
134 gene set enrichment analysis (GSEA) using the MSigDB Hallmark annotation sets to learn which gen-
135 eral processes are shared and distinct between replicative senescence, radiation induced senes-
136 cence, and cell density (Figure 1F, Figure 1 source data 3) (*Liberzon et al., 2015; Subramanian et al.,
137 2005; Mootha et al., 2003*). All three perturbations (and not the immortalized cells), exhibited dra-
138 matic reductions in expression of genes belonging to S, G2M, and M cell cycle phases, consistent
139 with cessation of cell division (Figure 1F - top cluster). Perhaps driven by this shift, we also found
140 significant overlap in additional enriched annotations among genes induced in replicative senes-
141 cence, radiation induced senescence and cell density, albeit with some variation in significance.
142 Many of the shared enriched annotations can be categorized as stress responses, e.g. apoptosis,
p53 pathway, inflammatory and interferon responses, and STAT3/5 signaling. In addition, we also
observed enrichment of several developmental gene sets including myogenesis, angiogenesis, and
adipogenesis.

We observed relatively few gene sets with discordant patterns across the replicative senes-
cence, radiation induced senescence, and cell density experiments (Figure 1E). However, the gene
set for epithelial to mesenchymal transition (EMT) was significantly enriched in genes that increased
with replicative senescence as opposed to radiation induced senescence (Benjamini-Hochberg cor-
rected p=5.5e-6 vs. Benjamini-Hochberg corrected p=0.12) or cell density wherein the term was ac-
tually underrepresented, but not significantly so (Benjamini-Hochberg corrected p=0.4). Although
replicative senescence, radiation induced senescence, and cell density are highly similar at the ab-

143 stracted level of enriched gene sets, it is clear in Figure 1E that there are many induced genes spe-
144 cific to replicative senescence. In addition, we applied GSEA to each individual time point for each
145 condition (Figure 1 supplement 4, Figure 1 source data 4) and found the EMT gene set is enriched
146 early and robustly during replicative senescence. Thus the EMT gene set appears to represent a
147 particularly important aspect of replicative senescence biology.

148 **Single-cell RNA-seq reveals that replicative senescence is a gradual process.**

149 Interestingly, the vast majority of gene expression changes evident by PDL 50 begin to manifest
150 at much earlier PDLs. This observation is consistent with two distinct possibilities: (A) The senes-
151 cence expression changes accrue early and gradually in the majority of cells without respect to
152 proliferation status, or (B) the bulk RNA-seq profiles are a changing admixture of transcriptionally
153 distinct mitotic, G1, and senescent cells. Both models have substantial support in the literature
154 (*Tang et al., 2019; Wiley et al., 2017; Nassrally et al., 2019; Whitney et al., 1980; Passos et al., 2007;*
155 *Smith and Whitney, 1980*). To discriminate between these possibilities, we employed single-cell
156 RNA-seq (scRNA-seq) to directly measure percentages of senescent and cycling cells.

157 Briefly, we collected between 1,000-2,000 cells at increasing PDLs of WI-38s and matched hTERT
158 time points at beginning and end of time course. We applied a modified (see methods) 10X Ge-
159 nomics protocol to capture and preserve the more fragile senescent cells (Figure 2 supplement 1).
160 We profiled a total of 11,000 cells (9,000 WT and 2,000 hTERT). When aggregated into pseudo-bulk
161 expression profiles (i.e the summation of all cells), the single cell results are highly correlated with
162 expression changes observed in bulk RNA-seq ($r=0.81$), Figure 2 supplement 2).

163 We first classified cells as either S, G2M, or G1 phase using canonical markers for the S and
164 G2M phases as previously described *Nestorowa et al. (2016)*. As expected, based on bulk tran-
165 scriptomics, the number of cells in either S or G2M decreases with increasing PDL whereas the
166 cycling cell proportions are stable in the hTERT timepoints (Figure 2A,B).

167 Next, we projected all WT cells and two hTERT time points together to identify broad patterns
168 in replicative aging PDL cells versus cycling hTERT cells. To do this, we employed UMAP projection
169 as opposed to t-SNE as the former is faster and preserves local structure more so than the latter
170 (*McInnes et al., 2018*). Cells from the two cell lines organize separately, however share a similar
171 geometry composed of a S/G2M phase roundabout and a large G1 lobe (Figure 2C). Although the
172 overall pattern of WI-38 cells for both cultures was highly similar, we observed progression of cell
173 grouping with PDL that had no concordance with increasing hTERT time points (Figure 2D - red
174 to blue). Specifically, PDL 50 cells (but not the temporally paired hTERT PDL ctrl 50 time point)
175 organize apart from all other cells within the UMAP projection. To test if these cells were senescent,
176 we scored all cells with a senescent gene expression signature derived from the bulk RNA-seq and
177 found that the highest scoring cells reside within this grouping composed of PDL 50 cells (methods)
178 (Figure 2 supplement 4).

179 To test model A vs. B, we compared the pseudo-bulk gene expression profiles across the cell
180 cycle at each PDL. We reasoned that if replicative senescence were gradual, we would observe
181 the replicative senescence gene expression pattern even in young cycling cells without respect
182 to G1/S/G2M. Indeed, the replicative senescence signature is present in all cell cycle phases even
183 in early PDLs (Figure 2E- left panels vs. far right panel). In addition, bioinformatic segregation
184 and UMAP projection of cells scored as S/G2M phase cells revealed that the dominant source of
185 variance in cycling cells is the PDL (Figure 2 supplement 3). Lastly, we did not observe any cells <
186 PDL 50 in close association with the senescent cells in the UMAP projection PDLs (Figure 2C,D).

187 It has previously been reported and observed here (Figure 2 supplement 5) that cell size in-
188 creases with PDL in primary cell lines (*Ogrodnik et al., 2019*). We hypothesized that perhaps the
189 higher PDL cells by nature of their larger size, might reach contact inhibition faster than early PDL
190 cells. In this scenario, given the high correlation between the gene expression signatures of senes-
191 cence and cell density, the increase in senescence signature could be an artifact of cell culture
192 arising from changing morphology. To test this possibility, we scored our single cells again with

193 modified signatures for senescence and cell density composed only of genes uniquely induced in
194 either perturbation (Figure 2 supplement 4). We found that the specific senescent signature in-
195 creases with PDL in WT WI-38 cells in contrast with the specific cell density signature which stays
196 relatively flat. These data provide more evidence supporting a gradual increase in the senescent
197 gene expression program that is independent from changes in cell density.

198 Together, these results argue in favor of a gradual model (A) of replicative senescence wherein
199 cells ramp up expression of the replicative senescence program with increasing PDL even when
200 still proliferative. We cannot rule out there are a small minority of cells that enter senescence early
201 in the experiment as previously reported (*Smith and Whitney, 1980; Passos et al., 2007*). However,
202 for the cells that made it to PDL 50, we observed that the senescent gene expression program man-
203 ifests in their primogenitors. These data are also consistent with the previously reported changes
204 in cellular phenotype (larger cell size and increased cycling time) with increasing PDL (*Macieira-*
205 *Coelho and Azzarone, 1982; Ogrodnik et al., 2019; Neurohr et al., 2019; Absher et al., 1974*). Import-
206 tantly, this observation suggests that aspects of cellular senescence are present in non-senescent
207 cells. This result raises the intriguing possibility that the reported pathological features of senes-
208 cent or senescent-like cells *in vivo* might also manifest in cells that are not classically senescent.

209 **Proteomic landscape of replicative senescence WI-38 cells suggests large metabolic** 210 **alterations.**

211 We next turned to our proteomics data generated from the same cultures and time points as above.
212 We obtained high confidence measurements for ~8500 proteins (Figure 3 supplement 1, Figure
213 3 source data 1). Similar to our transcriptional results, the hTERT samples did not exhibit large
214 changes in the proteome with experimental progression (Figure 3 supplement 1). Overall, we ob-
215 served high concordance between transcript and protein levels with very few outliers ($r=0.71$ for
216 PDL 50 vs. PDL 20, Figure 3 supplement 2). We employed GSEA using hallmark annotations and
217 observed a sharp depletion of proliferation and mitosis associated gene sets supporting our pre-
218 vious findings (Figure 3A, Figure 3 source data 1). Likewise, the proteomics data recapitulated
219 enrichment of the EMT annotation set along with the adipogenesis and myogenesis.

220 Multiple enriched sets pointed to replicative senescence dependent shifts in cellular energy uti-
221 lization (Figure 3A). Of note, we found that although under-enriched in our replicative senescence
222 transcriptome (Figure 1F), The oxidative phosphorylation hallmark was highly enriched in the pro-
223 teomics data. We plotted the oxidative phosphorylation genes driving the set enrichment in Figure
224 3 supplement 2. This projection revealed that many genes in this set fall into a quadrant (positive
225 proteomic, negative transcriptomic) suggesting discordant regulation of these genes.

226 The oxidative phosphorylation hallmark set is composed of multiple closely linked mitochon-
227 drial complexes and functions that regulate cellular energy flux, e.g. TCA cycle, fatty acid oxida-
228 tion, pyruvate metabolism, ATP synthase etc. We divided the oxidative phosphorylation gene set
229 into into these constituent parts and visualized the changes in proteins with increasing PDL. We
230 observed PDL-dependent increased in proteins from all categories except for mitochondrial as-
231 sembly and structures. These results are consistent with altered mitochondrial function (Figure 3
232 supplement 3).

233 Next, we examined the annotations for the highest enriched hallmark sets from Figure 3A and
234 found strong enrichment of multiple KEGG metabolic pathways. (Figure 3B). In addition, both the
235 glucose transporter *SLC2A1* and the fatty acid scavenger *CD36* transcripts exhibited strong, early,
236 and replicative senescence specific up-regulation suggesting altered cellular energy requirements
237 (Figure 3C). Together these results point to a drastically altered replicative senescence metabolic
238 landscape.

Senescent WI-38 cells exhibit increased utilization of fatty acid metabolism and glycolytic shunts.

To generate a metabolic profile for replicative senescence in WI-38 cells, we harvested samples at increasing PDLs alongside input material for all other data types (methods). From our metabolomics data, we identified 285 compounds (Figure 3 supplement 4, Figure 3 source data 2). To remove batch effects that were observed in several PDL and the matched hTERT samples, we corrected each PDL with its paired hTERT sample. We then calculated significant changes over time for each metabolite (FDR adjusted $p < 0.05$, Figure 3 source data 2). To further guide our metabolic analysis, we focused our queries based on the enriched metabolic pathways found in the proteomic analysis. Specifically we studied the changes to glycolysis, oxidative phosphorylation and fatty acid metabolism (Figure 3D).

Following the glucose and the increased expression of the glucose importer *SLC2A1*, we looked at the central stem of glycolysis as a potential source of energy fueling the altered senescent metabolism suggested by our proteomics data. (Figure 3D,E). We found that metabolites and enzymes dedicated to pyruvate generation and/or lactate production did not change in a concordant manner. We instead found that most of the glycolytic shunts exhibited up-regulation at both the metabolite and protein level (Figure 3E). These results suggest an increase in allocation of glucose for manufacture of various biomolecules and their precursors (glycogen, hexosamines, phospholipids) alongside or in lieu of pyruvate or lactate production (Figure 3E).

Metabolites and enzymes involved in fatty acid import (e.g. *CD36*) and oxidation for the purpose of energy generation appear strongly up-regulated (Figure 3E). We also saw that metabolites involved in the *de novo* production of phospholipids via the Kennedy pathway are highly up-regulated, specifically phosphatidylethanolamines precursors (Figure 3 supplement 5).

From these data, it is clear that WI-38 cells approaching replicative senescence undergo drastic shifts in metabolism. Specifically, we observed increased glucose utilization in glycolytic shunts coupled with an increase in fatty acid import and oxidation. It is possible that the senescent cells are switching to fatty acid oxidation to fuel increased TCA cycling and oxidative phosphorylation as glucose is diverted to macromolecule production. Indeed, metabolomic data collected from various types of senescence models has shown increased fatty acid oxidation, lipid accumulation, TCA up-regulation, and glycolytic alterations (Zwerschke et al., 2003; Ogrodnik et al., 2017; Flor et al., 2017; Unterluggauer et al., 2008; Johmura et al., 2021). Likewise, consensus metabolomic findings in EMT models report increased TCA cycle products, altered lipid metabolism, and activated hexosamine pathway (Hua et al., 2020; Corbet et al., 2020; Lucena et al., 2016). These data provide functional metabolic evidence supporting a connection between replicative senescence, metabolic rewiring, and the increased expression of EMT related genes.

Nicotinamide N-methyltransferase (NNMT) links nicotinamide adenine dinucleotide (NAD) and methionine metabolism as a putative heterochromatin regulator.

The interplay between metabolism and the epigenome is an increasingly recognized mechanism through which nutrient availability shapes chromatin and regulates cell state. (Lu and Thompson, 2012). The metabolites nicotinamide adenine dinucleotide (NAD⁺) and methionine metabolism power the deacetylation and methylation of histone tails and DNA required for maintaining repressive DNA conformations (Lu and Thompson, 2012). In a compelling intersection, perturbation of NAD, methionine, and heterochromatin levels have all previously been reported in multiple aging contexts including replicative senescence (James et al., 2016; López-Otín et al., 2016; Koziel et al., 2014; Yousefzadeh et al., 2020; Benayoun et al., 2015). In review of our replicative senescence data for the metabolic and proteomic components of the NAD and methionine pathways, we found that one of the largest and earliest changes for any protein (or transcript) was the increased expression of Nicotinamide N-methyltransferase (NNMT) (Figure 4A,B).

DNA and histone methylation require abundant levels of the universal methyl donor S-adenosyl

methionine (SAM). NNMT not only depletes SAM by catalyzing the removal of its methyl group, it does so by fusing the methyl group to the NAD⁺ precursor nicotinamide (NAM) resulting in the generation of methyl nicotinamide (MNA) (Ulanovskaya et al., 2013). Thus, NNMT effectively acts as a sink for two of the primary metabolites a cell requires for silencing chromatin and regulating gene expression (Komatsu et al., 2018). We found that MNA levels mirror those of NNMT; increasing early and robustly during replicative senescence progression consistent with high NNMT activity. Both SAM and its methyl-depleted counterpart, S-adenosyl homocysteine (SAH), were depleted with replicative senescence albeit to a lesser extent than the observed increase in MNA. NAMPT and NMNAT1, enzymes in the NAD salvage pathway, were weakly down-regulated at the protein level (Figure 4A,B).

High NNMT expression has been implicated in SAM depletion in embryonic stem cells and cancer-associated fibroblasts (CAFs) (Eckert et al., 2019; Sperber et al., 2015). In both cases the functional consequences were similar; DNA hypomethylation and decreased capacity to form or maintain silenced loci and heterochromatin. The important point is that the loss of silencing is actively promoted through NNMT activity. One intriguing hypothesis is that increased NNMT activity with replicative senescence promotes loss of silencing and heterochromatin via SAM depletion (Figure 4C).

Increased DNA accessibility and transcription from nucleolar and lamin associated domains is a dominant feature of the replicative senescence epigenome.

To study genome-wide changes in the epigenetic landscape of senescent cells, we collected ATAC-seq data during the replicative senescence time course. ATAC-seq libraries from all PDLs and hTERT PDL controls exhibited similar ATAC-seq fragment size distribution emblematic of nucleosomal patterning, high alignment rates, very low mitochondrial read percentages, and strong enrichment around transcriptional start sites. To enrich for nucleosome free regions of DNA, we applied size selection to our ATAC-seq libraries prior to sequencing (methods, Figure 5 supplement 1, Figure 5 supplement 2, supplement 3 and Figure 1 source data 1). These quality control metrics are indicative of high quality ATAC-seq libraries at all sampling time points and PDLs.

We next quantified the read distribution across chromatin states previously annotated for another human fetal lung fibroblast cell line (IMR-90, Figure 5 source data 1) (Ernst and Kellis, 2015). We categorized 25 distinct states into 4 broad categories: promoters, enhancers, transcription, and miscellaneous, the last of which is composed largely of heterochromatic and undefined states, e.g. H3K9me3 and H3K27me3 rich regions, zinc-finger repeats, and quiescent (defined by the absence of histone marks, accessibility, or gene features) which we refer to as "undefined" (Ernst and Kellis, 2015).

In comparing the read distribution across chromatin states from hTERT immortalized cells and WT WI-38 cells, we observed an increase in ATAC-seq reads falling into the undefined chromatin regions at the relative cost to all other states (Figure 5A-left panel). We next plotted the read distribution across chromatin states as a function of increasing time for both cell lines and found these shifts to be associated with increasing PDL in the WT WI-38 cells; hTERT ATAC-seq read proportions across chromatin states remained stable over time (Figure 5A-right panel).

To determine if this shift in accessibility to undefined and heterochromatic states was indicative of increasing noise versus coherent changes in accessibility, we used the ATAC-seq reads to call peaks of accessibility. From the WT and hTERT ATAC-seq data from all time points, we identified 363,470 ATAC-seq peaks (Figure 5 source data 2 and 3). We found a slight but significant (~5%, $p=0.014$, linear regression) linear PDL-dependent decrease in the fraction of reads in peaks during replicative senescence (Figure 5 supplement 4A, Figure 5 source data 4). We tested experimentally whether the increased heterochromatic accessibility was an artifact of increasing numbers of dead cells in later PDL samples by repeating the experiment using late (PDL 45) cells with a cross-linking agent (propidium monoazide-PMA) that renders DNA from dead cells inert as previously described (Hendrickson et al., 2018). No significant change in read distribution across chromatin states was

338 observed with addition of PMA (Figure 5 supplement 4B).

339 We then divided the ATAC-seq peaks into each of the 25 discrete chromatin states and calcu-
340 lated the fold change in accessibility for each state and PDL compared to the first time point. We
341 observed that changes in peak accessibility mirrors that for all reads. Again we observed a clear
342 increase in accessibility with PDL across the miscellaneous category with a concomitant decrease
343 in all other states in WT WI-38 cells and not in immortalized hTERTs (Figure 5B).

344 The undefined state from the miscellaneous category piqued our interest for two reasons, one
345 being that it alone accounted for 20-40% of all ATAC-seq reads and a large 10% increase with
346 senescence. The second point of interest was the observed early onset of change (Figure 5A,B). In
347 looking to define the undefined state, we found that Dillinger et. al. previously reported that the
348 undefined state is largely overlapping with both lamin associated domains (LADs) and nucleolar
349 associated domains (NADs) (Dillinger et al., 2017). In support of this observation, we too found
350 that the undefined chromatin state is markedly gene poor and overwhelmingly overlapping with
351 both empirically defined LADs and NADs (Z-score 90 and 150) that had previously reported in IMR-
352 90 fibroblasts (Figure 5 supplement 4C, Figure 5 source data 5 and 6) (Dillinger et al., 2017; Sadaie
353 et al., 2013). For reference, Figure 5 supplement 4E provides a chromosome level view of the
354 significant overlap between replicative senescence accessible ATAC-seq peaks in the undefined
355 state with NADs and LADs compared to all peaks annotated in WI-38 cells.

356 To control for the fact that the chromatin state annotations we used were derived from a differ-
357 ent, albeit closely related cell line, we performed the same analysis using gene annotations which
358 are cell line independent. We found that peaks in intergenic regions increase in accessibility with
359 PDL on average which is consistent with the undefined (and gene poor) chromatin state annota-
360 tion from IMR-90 cells. These results suggest that the chromatin states defined in IMR-90 cells are
361 capturing the chromatin states in WI-38 cells (Figure 5 supplement 4D).

362 We tested and found that ATAC-seq peaks falling within NADs and LADs exhibit increased ac-
363 cessibility with replicative senescence ($p < 10^{-16}$ for both: Wilcoxon rank-sum test). Notably, this
364 trend was much greater in magnitude for peaks in NADs versus peaks in LADs, with a median log2
365 fold change of 0.98 for NADs vs. 0.24 for LADs (Figure 5D).

366 Digging deeper into NADs, we next asked the chromatin residing within NADs might respond
367 differently than the rest of the genome to increasing PDL. We repeated the analysis shown in Fig-
368 ure 5B with two subsets; peaks excluded from NADs or peaks overlapping NADs. (Figure 5 supple-
369 ment 5). Dividing these two sets of peaks into the 25 chromatin states revealed that as expected,
370 NAD excluded peaks mirror the pattern observed for significantly changing peaks across the whole
371 genome in the original analysis. Conversely peaks overlapping NADs were found to increase in ac-
372 cessibility in a variety of chromatin states (e.g. poised promoters and many classes of enhancers)
373 that exhibit reduced accessibility across the rest of the genome with increasing PDL.

374 One interpretation is that although the aggregate peak accessibility data appear to suggest that
375 transcription is globally shutting down, the NAD specific view reveals rather that transcription is
376 induced in a subset of loci. Although widespread, the reduced accessibility in euchromatin we
377 observed in aggregate is not reflective of a pervasive shutdown of transcription across the entire
378 genome. To formally test this hypothesis, we next asked if the shifts in NAD/LAD accessibility corre-
379 late with productive transcription and found that indeed, senescence induced genes significantly
380 overlap NAD domains. Radiation and cell density induced genes exhibited weaker (cell density) or
381 nonexistent (radiation) overlap with NADs and LADs. These data suggest that increasing accessibil-
382 ity in LADs and specifically NADs is a prominent feature of the replicative senescence epigenome
383 that is directly connected to the senescent gene expression program (Figure 5C). Together these
384 results highlight a striking increase in accessibility within nucleolar associated DNA that connects
385 changes in the transcription with a global shift in the epigenome.

Transcriptional regulators of the replicative senescence transcriptome and epigenome.

To parse out the regulatory logic of the senescence gene expression program, we leveraged our ATAC-seq data to gain insight into which transcription factors regulate replicative senescence accessibility via transcription factor motif analysis. Having cataloged a universe of ATAC-seq peaks with significant changes in accessibility, we next assigned peaks to neighboring genes. Taking the top replicative senescence differentially expressed genes, we searched the proximal ATAC-seq peaks for enriched transcription factor motifs (Figure 6A). We found enriched motifs for TEAD1, CEBP family, SMAD family, AP1 complex, and FOX family transcription factors.

We also applied an orthogonal gene-independent methodology for determining which motifs are predictive of replicative senescence induced increases in ATAC-seq peak accessibility. Consistently, we again found TEAD1 motifs to be the most predictive feature (mean coefficient = 0.25 across 10 models-methods). In addition, we also found evidence for FOXE1 and SMAD1 regulation as well as other senescence related transcription factors, e.g. CEBPB and TP53 (Figure 6B).

Out of the FOX family transcription factors, *FOXE1* is unique in that its transcript exhibits one of the most specific (no change in hTERT, cell density or radiation) and largest increases in replicative senescence gene expression (40-50x) (Figure 6 supplement 1). *FOXE1* is annotated as a thyroid specific transcription factor with putative roles in thyroid development and cancer (Zannini et al., 1997; Kallel et al., 2011; Ding et al., 2019). It has also been reported to regulate two signaling molecules up-regulated in the replicative senescence time course; *TGFB3* and *WNT5A* (Figure 1, source data 2) Venza et al. (2011); Pereira et al. (2015). Furthermore, it has been reported that many of the FOX family transcription factors are relatively promiscuous binders of each others canonical motifs. Thus it is possible that the increased accessibility in peaks with FOX motifs during replicative senescence may be driven by *FOXE1* activity Rogers et al. (2019). Despite the specificity and magnitude of replicative senescence induction, *FOXE1*'s function in replicative senescence is unclear and warrants further investigation.

We returned to our bulk RNA-seq to test for enrichment of protein-DNA binding events (mapped by ENCODE) in regulatory elements proximal to our replicative senescence differentially expressed genes by using Landscape *in silico* deletion Analysis (LISA) (Qin et al., 2020). Plotting transcription factor enrichment for genes depleted with replicative senescence against genes induced during replicative senescence revealed 3 broad cohorts of transcription factors; proliferating cell transcription factors, replicative senescence transcription factors, and transcription factors whose binding was enriched around both sets of genes (Figure 6C). As expected, proliferation specific transcription factors are replete with cell cycle specific transcription factors e.g. E2F and RBP family transcription factors. A large portion of the transcription factors exhibiting replicative senescence specificity belong to 4 categories; inflammation transcription factors (NFKB, CEBPB), AP1 subunits (JUN, JUND, FOSL2), YAP1-TEAD1 components (TEAD1/4, YAP1, WWTR1), and EMT transcription factors (SNAI2, TCF21).

TEAD1 is a member of the TEA domain transcription factors whose functions range across a wide swath of biology depending on context and binding partner. TEAD transcription factors cannot induce gene expression without a cofactor, which is most often YAP1 (yes-associated protein 1) a key downstream effector of Hippo signaling (Azakie et al., 2005; Chen et al., 1994; Benhadou et al., 2012; Yu et al., 2015; Landin Malt et al., 2012; Vassilev et al., 2001; Zhao et al., 2008; Ma et al., 2019; Piccolo et al., 2014). Consistent with our identification of TEAD1 and YAP1 as replicative senescence regulators, YAP1 activation has been tied to EMT, anti-apoptosis, telomere dysfunction, inflammation, and positive regulation of fatty acid oxidation (Kurppa et al., 2020; Lee et al., 2019; Peng et al., 2017; Zhang et al., 2017; Chakravarti et al., 2020).

Lastly, given that YAP1/TEAD1 activity appears to increase during replicative senescence against the backdrop of an altered epigenomic context, we tested for an interaction between TEAD1 motifs and the increasingly accessible NADs and LADs. We found that not only are TEAD1 sites significantly enriched within NADs and LADs ($p < 7.17 \times 10^{-7}$, and $p < 6.12 \times 10^{-3}$ respectively hypergeometric), but

we also discovered a greater-than-additive increase in accessibility with replicative senescence for TEAD1 motifs that occur within a NAD ($p < 3.12 \times 10^{-7}$). These results suggest that there is a functional connection between TEAD1 activity and NAD domains during replicative senescence. Thus, The changing epigenetic context of replicative senescence may alter TEAD1 binding and target expression induction with increasing PDL.

Collectively, these analyses uncover a common theme amongst putative regulatory transcription factors; Hippo signaling (YAP1/TEAD1), EMT transcription factors, and TGF- β signaling (SNAI2, SMAD activity). SNAI2 has been shown to work in tandem with the YAP1/TEAD1 complex and these pathways often work towards similar biological ends (Tang et al., 2016; Kurppa et al., 2020). Together, these transcription factors are reported as highly involved with proliferation, EMT, ECM production, fibrosis, and apoptosis avoidance (Kim et al., 2019a).

Lastly, given that the LISA results are based on binding events collected from a vast multitude of cell lines, we wondered if the same transcription factors might be found to regulate senescence in a completely different cellular context. To test this we took significantly induced genes from a senescence model using astrocytes and oxidative stress as the senescence trigger (Crowe et al., 2016). Plotting transcription factor enrichment for genes induced in astrocyte senescence against transcription factor enrichment for WI-38 replicative senescence genes revealed that although there were a substantial number of discordant transcription factors, there was a clear population of transcription factors highly enriched in both senescence models (Figure 6D). Notably, the top concordant transcription factors ranked at the top in both contexts and recapitulate all the previous results e.g. YAP1/TEAD1, SNAI2, CEBP family transcription factors and AP1 subunits.

scRNA-seq trajectory analysis resolves WI-38 cells' approach to replicative senescence.

A wealth of recent work in single cell transcriptomics has demonstrated that ordering single cells in a process-specific trajectory often reveals nuanced timing and dynamics of gene expression that bulk assays cannot capture (Qiu et al., 2017; Trapnell et al., 2014). Mapping this trajectory is frequently referred to as "pseudotime analysis". We employed pseudotime analysis to arrange single WI-38 cells along a pathway to senescence setting proliferating cells as the trajectory "root". As expected, early and late PDL cells concentrated at the beginning and end of the pseudotime trajectory respectively (Figure 7A). We next performed differential expression analysis to identify genes that change significantly over pseudotime. We plotted examples of genes changing early (CENPK—an S-phase cell-cycle-regulated gene), midway (SNAI2—a master regulator of EMT), or late (PAPPA—a prominent SASP factor) in pseudotime in Figure 7B.

We next generated gene expression trajectories for the top (by significance p -value < 0.001) 5,000 differentially expressed genes across pseudotime (Figure 7 supplement 1, Figure 7 source data 1). To identify the temporal relationship between the biological processes and transcription factors that compose replicative senescence in WI-38 cells, we clustered and ordered pseudotime trajectories (Figure 7 supplement 2). Broadly, we classified the pseudotime expression pattern as early, transition, or late based on the maximum median value of all constituent genes for each cluster (Figure 7C). We performed LISA transcription factor and GO enrichment analysis on each cluster and used our findings to assign putative functional labels across pseudotime (Figure 7D, Figure 7 supplement 2, Figure 7 source data 1 and 2).

Early pseudotime is dominated by the transcription factors (E2F) and GO terms associated with cell cycle progression through the G2M and S phases. Moving down the y-axis deeper into pseudotime, we observe that the next primary cluster of transcription factors and functional annotations exhibited widespread enrichment across all of pseudotime. Furthermore, transcription factors enriched in this cluster are involved with basic cellular functions (e.g. euchromatin maintenance, transcription, growth) and are likely representative of normal WI-38 function in G1 phase.

Moving forward into the transition phase of pseudotime, we observed an enrichment of transcription factors regulating higher order chromatin structure (CTCF) and epigenetic silencing (poly-

comb group complex). Also in the transition region of pseudotime, the earliest enrichments appear for transcription factors related to EMT (SNAIL2), TGF- β signaling (SMAD3), YAP1/TEAD1 activity, and the AP1 complex (JUN, FOS, FOSL2). We observed continued enrichment for these transcription factors and processes throughout the rest of the transition phase and into late pseudotime.

Lastly, late in pseudotime, we observed enrichment of transcription factors and functional annotations related to regulation of inflammatory processes (NFKB1, RELA, ERG1, CEBPB) and changes in cellular morphology (Figure 7D, Figure 7 supplement 2). These observations are consistent with observations made with bulk RNA-seq that replicative senescence WI-38 cells exhibit transcriptomic features similar to that observed in TGF- β signaling, EMT, and with YAP1/TEAD1 activity. Collectively, these results present a possible order of operations for replicative senescence progression that highlights an initial cessation of active mitotic cycling, followed by an epigenetic shift that precedes a strong EMT/TGF- β signal before segueing into a pro-inflammatory secretory state.

Senescent WI-38 fibroblasts express canonical myofibroblast markers and metabolic features.

Given the repeated observations linking replicative senescence with EMT, (Figure 1, Figure 3), TGF- β and YAP1/TEAD1 activity (Figure 6, Figure 7), we considered the possibility that these processes are connected through the fibroblast to myofibroblast transition (FMT), a subtype of EMT in which stressed or injured fibroblasts differentiate into myofibroblasts (Phan, 2008; Piersma et al., 2015; Hinz, 2007). Upon receiving cues mediated by injury or stress (e.g. activated TGF- β), fibroblasts can trans-differentiate into myofibroblasts, whose functions as “professional repair cells” include increased proliferation, migration, apoptosis avoidance, cell and tissue contraction, and ECM/collagen deposition to promote tissue repair and wound closure (Hinz and Lagares, 2020; Gibb et al., 2020).

Previous work has demonstrated that there exists mechanistic and functional association between telomerase inhibition, senescence, and myofibroblasts. Senescence is an integral part of the wound healing processes; upon injury resolution, activation of a senescence-like phenotype prevents unchecked collagen secretion and fibrosis by preventing myofibroblast proliferation and earmarking them for subsequent immune clearance (Demaria et al., 2014; Krizhanovsky et al., 2008; Jun and Lau, 2010; Mellone et al., 2016; Razdan et al., 2018; Liu et al., 2002, 2006).

To further explore this proposition, we retrieved canonical myofibroblast marker genes and direct transcriptional targets of the YAP1/TEAD1 complex to determine to what extent these genes are expressed in WI-38 replicative senescence cells at both the RNA and protein level. First, we examined canonical myofibroblast markers (Hinz and Lagares, 2020), collagen produced by myofibroblasts (Zhang et al., 1994), genes upregulated in myofibroblasts derived from idiopathic pulmonary fibrosis (IPF) patients (Strunz et al., 2020), and effectors and targets of TGF- β signaling in two data modalities: bulk RNA-seq and bulk proteomics (Figure 8A).

For the majority of genes in the curated myofibroblast panel, expression of transcripts and protein increased with PDL. Importantly, expression of smooth-muscle actin (ACTA2), a classic myofibroblast marker, increases strongly in replicative senescence. The expression of follistatin-like protein (FSTL1), also known to be strongly expressed in smooth muscle, shows a similar pattern, as does fibrillin (FBN1). All three of these are associated with smooth muscle and TGF- β family regulation. In addition we observed an increase in both fibrillar and basal lamina collagens in our data at both the RNA and protein levels.

Collagen processing is a multi-step process requiring the coordination of multiple enzymes and metabolites (Lodish and Zipursky, 2001). Review of the collagen synthesis pathway alongside our metabolomic and proteomic data provide further confirmation that WI-38 replicative senescence cells exhibit altered collagen metabolism (Figure 8B). We observed up-regulation of multiple pathway enzymes as well as increased abundance of hydroxyproline, a primary constituent amino acid of collagen protein. In addition, we observe a striking depletion of ascorbate (vitamin C) which is

required for proline hydroxylation and is an essential vitamin. It is possible that our observations underestimate the collagen production potential of replicative senescent WI-38 cells as they appear limited in terms of collagen production by the amount of supplemented vitamin C (*Boyer et al., 1998*).

Mellone et al. (2016) recently reported that although senescent fibroblasts share features with myofibroblasts, this resemblance does not extend to fibrogenic ECM components, e.g. collagen (*Mellone et al., 2016*). However, it is important to note that *Mellone et al. (2016)* focused on radiation induced senescence rather than replicative senescence, and we similarly observed less or no induction of many of these same genes in our radiation induced senescence experiment. In addition, it is possible that with more time (> 10 days), fibrogenic ECM expression could initiate in the irradiated WI-38 cells.

Moving forward in our myofibroblast panel, we observed that expression of TGF- β cytokine, TGF- β 1, decreased significantly with replicative senescence in both RNA and protein. However, we see robust induction of the TGF- β isotype 2 (TGF- β 2) cytokine with replicative senescence (Figure 8A) as TGF- β 1 abundance drops, which indicates a switch in TGF- β isotypes with replicative senescence. It has been shown TGF- β 2 is a more potent inducer of the endothelial to mesenchymal transition (EndMT) *in vitro* compared to TGF- β 1 and TGF- β 3 in human microvascular endothelial cells, and TGF- β 2 may be playing a similar role here in inducing FMT and replicative senescence in WI-38 cells (*Sabbineni et al., 2018*). The distinct functional roles of different TGF- β isotypes are largely unknown, although both are known to activate the SMAD transcription factors. On the basis of our data, it seems likely that the TGF- β paralog relevant here might not be TGF- β 1, but TGF- β 2.

Expression of YAP1/TEAD1 targets during RS.

Next, we retrieved a gene set of YAP1/TEAD1 targets assembled by Kurppa et al. from five separate studies (*Kurppa et al., 2020*). Taking the intersection of the five YAP1/TEAD1 gene target lists, we only kept genes present in at least 2 of the studies and plotted the remainder in Figure 8C across the two data types. We arranged the YAP1/TEAD1 targets by the two predominant expression patterns—decrease with replicative senescence (top) and increase with replicative senescence (bottom). As with myofibroblast markers, the data from both modalities is largely concordant.

Interestingly, we noted a striking bifurcation whereby YAP1 targets tend towards either strong down-regulation or strong up-regulation. The down regulated partition is heavily enriched for classic cell cycle regulators such as TOP2A, CDC20, BIRC5, and CDK9 suggesting that this dichotomy in YAP1 activity is heavily influenced by the cell cycle and consistent with recent work (*Kim et al., 2019b; de Sousa et al., 2018*).

Excitingly, TGF- β 2 was one of the two YAP1 targets found in four out of five collected YAP1/TEAD1 data sets, supporting the inference that the TGF- β 2 and not TGF- β 1 is the relevant paralog and potentially regulated by YAP1/TEAD1 in the replicative senescence context. In addition, another YAP1 target, thrombospondin-1 (THBS1), is also upregulated at both the protein and transcript level with RS; THBS1 is known to act as an activator of TGF- β signaling, and specifically TGF- β 2, through proteolytic cleavage of latent TGF- β (*Ribeiro et al., 1999*).

Inhibiting the YAP1/TEAD1 interaction in WI-38 cells suppresses expression of YAP1/TEAD1 targets and the replicative senescence gene signature.

In all, the data reveal that replicative senescence WI-38 cells share multiple defining transcriptomic and proteomic features with myofibroblasts. Furthermore, we found that a subset of YAP1/TEAD1 targets are both induced with replicative senescence, and are principal components of TGF- β signaling (TGF- β 2, THBS1). Thus the YAP1/TEAD1 complex may be acting in convergence with TGF- β signaling with increasing PDL to enact a myofibroblast-like state that we recognize as replicative senescence.

Recently *Mascharak et al. (2021)* showed verteporfin treatment of wounds in mice alleviated the fibrotic state during wound healing, reverted the profibrotic transcriptional program, and reduced

the myofibroblast population. Verteporfin is also known to be an inhibitor to the formation of the YAP1/TEAD1 complex (Wang et al., 2016). To test for a direct role for YAP1/TEAD1 in transcriptional regulation of YAP target genes, myofibroblast marker expression and the EMT signature during replicative senescence transition, we treated late passage WI-38 PDL 40 cells with verteporfin or DMSO as an untreated control. We utilized RNA-seq to capture the gene expression changes on cells treated with verteporfin. First, to confirm the verteporfin treatment inhibited expression of canonical YAP target genes in WI-38 cells, we checked the expression of *CTGF*, *CYR61*, and *TGFB2* transcripts (Figure 8 supplement 1A) (Zhao et al., 2008; Zhang et al., 2009; Chen et al., 2001). We found that treatment with 10 uM verteporfin for 2 hours reduced expression of these three YAP target genes by 70% relative to the no treatment control (Figure 8 supplement 1A, Figure 8 source data 1). We expanded the analysis to the myofibroblast markers and YAP targets used in Figure 8 and observed that verteporfin treatment reduces the gene expression of most myofibroblast markers and collagen and many YAP1 target genes that were shown to increase with replicative senescence (Figure 8A, C).

We applied GSEA analysis using the MSigDB Hallmark annotation to understand what pathways are affected by verteporfin treatment of cells using the RNA-seq data from the verteporfin treated samples relative to non-treated control. Consistent with our hypothesis that YAP1/TEAD1 plays a role in regulating the FMT-like transition of cells undergoing replicative senescence, treatment of late passage cells with verteporfin resulted in negative enrichment of pathways relevant to FMT and TGF- β signaling (Figure 8 supplement 1B). We also took genes driving the EMT annotation enrichment during replicative senescence to see if inhibition of YAP1/TEAD1 complex formation aids in reducing EMT pathway gene expression. Compared to the no treatment control, the majority of genes induced during replicative senescence that drive the EMT enrichment exhibit a striking decrease in expression after two hours of treatment. These data suggest that the use of verteporfin to inhibit the YAP1/TEAD1 interaction can impede the FMT-like transition observed in replicative senescent cells (Figure 8 supplement 1D).

To gain a global understanding of how inhibiting the YAP1/TEAD1 interaction affects the transcriptomic landscape of late passage WI-38 cells, we compared the gene expression signature of replicative senescence cells to that of verteporfin treated cells and found that verteporfin treatment globally reverses many of the gene expression changes occurring with replicative senescence (Figure 8 supplement 1C). This results suggest that YAP1/TEAD1 transcriptional activity is a crucial regulator of a large swath of the replicative senescence transcriptome.

Discussion

The study of replicative senescence in human tissue culture has proven to be an informative model for learning how genetic and environmental factors impact cellular senescence. However, the field has not fully taken advantage of the 'omics' revolution. Rekindled by the advent of senolytics, interest in the molecular underpinnings of senescence has burgeoned in recent years as researchers seek to design therapeutic strategies for ablating senescent cells (Amor et al., 2020; Elmore et al., 2018; Wagner and Gil, 2020; Aghajanian et al., 2019). However, many such studies span only one or two systematic data modalities. Here, to fully leverage the power of recent advances in high-dimensional profiling, we revisit the original Hayflick limit in WI-38 lung fibroblasts cells with a battery of assays including RNA-seq, ATAC-seq, scRNA-seq, proteomics, and metabolomics in an effort to capture the defining features of replicative senescence at every step of the central dogma and beyond. Our results are summarized graphically in Figure 9.

One important feature of our study is the number and type of control conditions. From our pilot studies, it was clear that studying replicative senescence in isolation would preclude our ability to know what features of change were specific to replicative senescence versus high cell density and/or DNA damage induced growth arrest. Thus, we included cellular density as an alternate method for arresting growth and exposure to ionizing radiation as an alternate methodology for senescence induction and DNA damage response. Lastly, all of our modalities were paired with

635 samples collected from an hTERT immortalized cell line grown in parallel. Importantly, very little
636 changed across all modalities in the hTERT cell line consistent with the changes we highlight in 9
637 as specific to replicative senescence.

638 The kinetics and precise timing of senescence onset have been obscured by low temporal reso-
639 lution and ensemble measurements that cannot differentiate between global shifts in gene expres-
640 sion versus changing proportions of senescent cells (*Passos et al., 2007; Xu et al., 2013; Victorelli*
641 *and Passos, 2017; Smith and Whitney, 1980; Tang et al., 2019; Wiley et al., 2017; Nassrally et al.,*
642 *2019; Whitney et al., 1980*). Here, with a combination of high time resolution and single cell RNA-
643 seq, we provide evidence that the early manifestation of the senescent gene expression reflects
644 gradual changes on a per cell basis rather than changing cell proportions. In effect, individual cells
645 “show their age” with increasing PDL long before permanently exiting the cell cycle and transiting
646 fully into the senescent state. The implications of this conclusion extend to organismal aging. For
647 example, the percentage of senescent cells calculated from aging organisms varies greatly depend-
648 ing on the marker/phenotype used (*Ogrodnik, 2021*). The reported disparities could be explained
649 in part by the use of early versus end stage markers. Likewise, it is possible the reported increase in
650 fibroblast heterogeneity and altered functionality with age is a direct result of cells slowly moving
651 along a spectrum towards replicative senescence (*Shin et al., 2020; Mahmoudi et al., 2019*). Import-
652 antly, the gradual progression suggests that cells need not reach the endpoint to elicit a pheno-
653 type. For instance, proliferative fibroblasts isolated from IPF patients exhibited multiple senescent
654 features and phenotypes in addition to accelerated senescence progression (*Yanai et al., 2015*).
655 Lastly, this phenomenon is not constrained to fibroblasts as we observe that the salient regulatory
656 features of replicative senescence extend to cell types as distant as astrocytes (Figure 6D).

657 In our data, the pattern of gene expression annotated to EMT as a unique feature of replicative
658 senescence that consistently presents early and robustly at both the RNA, protein, and single cell
659 level. Given the fundamental nature of the EMT transition with respect to cellular function (devel-
660 opment, fibrosis, and wound healing), it is not surprising that this hallmark tracks with multiple
661 proteins and gene sets suggesting drastic metabolic rewiring. In our metabolic data we highlight
662 shifts in carbon and fatty acid utilization that have been reported previously as hallmark metabolic
663 features of EMT. These metabolic changes demonstrate that our observations represent an au-
664 thentic change in cellular state as opposed to a superficial uptick in a few EMT related genes.

665 The data presented above across multiple data modalities to provide a clear connection be-
666 tween senescent cells and myofibroblasts supported by independent observations at the level
667 of DNA, RNA, protein, transcription factor activity and metabolism (Figure 9). In light of these,
668 replicative senescence resembles a specialized subtype of EMT specific to the trans-differentiation
669 of fibroblasts into myofibroblasts (FMT) in response to wound healing (*Lombardi et al., 2019; Gibb*
670 *et al., 2020; Hinz and Lagares, 2020*). We hypothesize that during fibrotic disease states and/or age,
671 fibroblasts migrate to sites of micro-injuries. As these proliferating fibroblasts become replicatively
672 aged, they are triggered (by DNA damage or other insults) to rewire their metabolism to induce FMT
673 via active epigenetic reorganization (NNMT-SAM/NAD sink). It is important to note here that the
674 *in vitro* DNA damage here arises primarily from telomere erosion documented by the observation
675 that our hTERT control cultures do not exhibit the same changes. However, genotoxic stress *in*
676 *vivo* may originate from a variety of endogenous and environmental sources e.g. reactive oxygen
677 species, replication stress, chemical exposure etc.

678 Following increases in DNA accessibility, expression of newly opened TEAD1/YAP1/SMAD target
679 genes cement FMT transition by promoting fibrosis and a myofibroblast-like, ECM-secreting state.
680 This model is supported by the synergy between TEAD1 motifs and NAD domains we report and
681 reconciles conflicting reports that YAP1/TEAD1 inhibition can both prevent and promote senes-
682 cence (*Fu et al., 2019; Kurppa et al., 2020; Xie et al., 2013; Jia et al., 2018*). Basically, the functional
683 consequences of YAP1/TEAD1 inhibition will depend on the epigenetic organization of the cells
684 used.

685 Further metabolic changes (hexoamine/collagen synthesis and fatty acid oxidation) then sup-

port the new pro-fibrotic state. Finally, end point senescent cells reinforce the senescent state and contribute to neighboring cell progression towards senescence via secretion of inflammatory factors and SASP.

We think that this transition is distinct from classic FMT as the endpoint cells are not proliferative, but instead bear striking resemblance to lingering senescent myofibroblasts that can persist long after wound repair is complete (*Hinz and Lagares, 2020*). Rather than a privileged or unique state, perhaps replicative senescence is better categorized as a DNA damage mediated path to a potentially common stress-induced endpoint.

The complex interplay between metabolism and epigenetic regulation preclude easy determination of a causal factor in translating DNA damage into the replicative senescence/EMT program in WI-38 cells. Does EMT regulate metabolism or vice versa? Here we present compelling evidence on the side of metabolism. We observe both an early and sharp rise in NNMT expression and activity in addition to a global increase in heterochromatin accessibility. These results are consistent with NNMT's reported role as global epigenetic regulator through its methylation sink activity (*Komatsu et al., 2018; Eckert et al., 2019; Pissios, 2017; Ulanovskaya et al., 2013*).

Importantly, the observed shifts in repressed chromatin induced by NNMT are functional and may play a central role in fibroblast biology and stress response in multiple contexts. First, *Eckert et al. (2019)* recently demonstrated that NNMT activity and the resulting heterochromatin reorganization initiate the expression program of cancer-associated fibroblasts (CAFs) associated with oncogenic stroma *in vivo* (*Eckert et al., 2019*). Similar to senescent fibroblasts and myofibroblasts, the defining features of CAFs are increased cytokine production, metabolic rewiring, and ECM alteration and production (*Sahai et al., 2020*). Second, NNMT is one the most up-regulated genes in a TGF- β mediated *in vitro* FMT conversion in WI-38 cells (*Walker et al., 2019*). Finally, the changes in silenced chromatin we observed during replicative senescence overlap with induction of gene expression driving the senescent phenotype (Figure 5D). In fact, pseudotime analysis argues that chromatin reorganization may precedes the FMT induction as evidenced by the loss of polycomb activity that appears prior to enrichment of the EMT and YAP1/TEAD1 transcription factors enrichments (Figure 7D).

The path to replicative senescence process has many *in vivo* parallels with implications for aging and pathogenesis. After observing induction in WI-38 cells of IPF myofibroblast markers (Fig. 8A), we expanded our literature search and found a striking overlap (TGF- β signaling, YAP1 activity, and EMT) between our data and scRNA-seq expression profiles from alveolar epithelial cells collected from IPF patients (*Xu et al., 2016*). These studies also report a large induction of TGF- β 2 relative to TGF- β 1 which is consistent with our findings and highlights a clear connection between *in vitro* replicative senescence and an *in vivo* disease state.

Another example of such a connection arises from our LISA analysis: one of the top enriched transcription factors, TCF21, has been implicated in atherosclerotic disease progression. Wirka et. al. found that TCF21 promotes the transition of vascular smooth muscle cells into a novel fibroblast-like cell type they dub "fibromyocytes" owing to their possession of both fibroblast and myocyte phenotypes in atherosclerotic lesions in both mice and humans (*Wirka et al., 2019*).

In general, the FMT hypothesis provides a conceptual framework and integrative model for linking the multi-modal senescent phenotypes we observed to multiple human age-related diseases. Given the observation that fibrosis and senescence markers correlate with increasing age in multiple tissues, it is possible that FMT might be a widespread phenomenon underlying many age-related pathologies (*Yousefzadeh et al., 2020; Idda et al., 2020*). Future work harnessing multi-modal single cell technology coupled with relevant *in vivo* models will aid greatly in determining the exact order of events and physiological import.

Table 1: Key Resources Table.

Reagent type	Designation	Source or reference	Identifiers	Additional info
Cell Line (H. sapiens)	WI-38 fibroblasts	Coriell	AG06814-N	
Cell Line (H. sapiens)	WI-38 hTERT	This paper	immortalized WI-38 human fibroblasts, methods	
Transfected construct (H. sapiens)	pCDH-CMV-hTERT-EF1a-puro	This paper		Lentiviral plasmid, methods
Commercial assay or kit	Senescence- β -Galactosidase	Cell Signaling Tech.	9860	
Commercial assay or kit	MycoAlert Mycoplasma Detection	Lonza	LT07-218	
Commercial assay or kit	Direct-zol RNA Miniprep Plus	Zymo Research	R2072	
Commercial assay or kit	Chromium Single Cell 3' v2	10x Genomics	120237	
Commercial assay or kit	Chromium Single Cell A Chip	10x Genomics	1000009	
Commercial assay or kit	Tagment DNA Enzyme and Buffer	illumina	20034197	
Commercial assay or kit	Clean-and-Concentrator-5	Zymo Research	D4014	
Commercial assay or kit	NEBNext® High-Fidelity 2X PCR	NEB	M0541L	
Commercial assay or kit	TruSeq Stranded mRNA Library	illumina	20020595	
Commercial assay or kit	Bioanalyzer High Sensitivity DNA	Agilent	5067-4626	
Commercial assay or kit	Pierce BCA Protein Assay	Thermo Fisher	23227	
antibody	anti-human p16 antibody (Mouse monoclonal)	BD Biosciences	RRID:AB_395229	(1:250)
antibody	anti-human p21 antibody (Mouse monoclonal)	BD Biosciences	RRID:AB_396414	(1:250)
Commercial assay or kit	Pippin Prep 2% 100-600 bp	Sage Science	CDF2010	
Chemical compound, drug	verteporfin	RD Systems	1243926	
Other	Zorbax Extend C18 column	Agilent	759700-902	
Other	SeQuant ZIC-pHILIC column	EMD Millipore	150460	
Software, algorithm	R (v4.0.3 and 3.6.2)	r-project.org/	RRID:SCR_001905	
Software, algorithm	Salmon (v 0.8.2)	combine-lab.github.io/salmon/	RRID:SCR_017036	
Software, algorithm	DESeq2 (v1.30.1)	bioconductor	RRID:SCR_015687	
Software, algorithm	sva package(v3.38.0)	bioconductor	RRID:SCR_012836)	
Software, algorithm	fgsea 1.16.0	bioconductor	RRID:SCR_020938	
Software, algorithm	CellRanger 3.0	10x Genomics	RRID:SCR_017344	

Table 2: Key Resources Table.

Software, algorithm	SCTransform (v 0.3.2)	satijalab.org/seurat	
Software, algorithm	Seurat (v4.0.1.9005)	satijalab.org/seurat	RRID:SCR_007322
Software, algorithm	monocle3 (v1.0.0)	cole-trapnell-lab.github.io/monocle3	RRID:SCR_018685
Software, algorithm	bowtie2 (v2.3.4.1)	bowtie-bio.sourceforge.net	RRID:SCR_016368
Software, algorithm	samtools (v1.2)	www.htslib.org	RRID:SCR_002105
Software, algorithm	Picard (v2.6.4)	broadinstitute.github.io/picard	RRID:SCR_006525
Software, algorithm	macs2 (v2 2.1.2)	hbctraining.github.io	RRID:SCR_013291
Software, algorithm	GenomicRanges (v1.42.0)	bioconductor	RRID:SCR_000025
Software, algorithm	cutadapt (v2.4)	github.com/marcelm/cutadapt	RRID:SCR_011841
Software, algorithm	bcl2fastq (v2.20)	Illumina	RRID:SCR_015058)
Software, algorithm	regioneR v1.22.0)	bioconductor	
Software, algorithm	ATACseqQC v1.14.4)	bioconductor	
Software, algorithm	LIMMA v3.46.0)	bioconductor	RRID:SCR_010943
Software, algorithm	Qvalue v2.26.0)	combine-lab.github.io/salmonQvalue	RRID:SCR_001073
Software, algorithm	LISA v1	lisa.cistrome.org	

Methods and Materials

Cell Culture

WI-38 maintenance and subculturing for replicative senescence time course-WT and hTERT WI-38 cells were obtained from the Coriell Institute (AG06814-N) at PDL 15. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, 11885084) supplemented with 10% dialyzed fetal bovine serum (FBS, Sigma, F0392) and maintained in an incubator set to 37° C, 5% CO₂, and 20% O₂. 0.3-0.5 E6 WI-38 cells were seeded and maintained on 10 cm collagen coated plates (Corning, 354450) and split when cells reached about 70% confluence. For WI-38 cells PDL 15 to PDL 40, it took about 4 days to reach 70% confluence. WI-38s >PDL 40 were slower growing, and therefore split every 5-7 days instead; media was replenished every 3-4 days (supplementary methods). Cells were passaged by washing the cell monolayer with PBS, followed by incubating cells with TrypLE Express (Gibco, 12604013) for 5 minutes. Media was added to neutralize the TrypLE and the cell suspension was collected into conical tubes. The cell suspension was spun at 200 x g for 5 minutes and the supernatant was aspirated. The resulting cell pellet was resuspended in media and cells were counted with viability measurements on a ViCell XR Cell Analyzer (Beckman Coulter). Cell numbers taken from the ViCell were used to calculate population doublings (PDLs). The following formula was used to calculate PDLs:

$$\text{PDL} = \log_2(\text{number of cells harvested}) - \log_2(\text{number of cells seeded})$$

All cells collected for assays were sampled in triplicate, at 2.5-3 days after seeding, targeting 60-70% confluence to avoid the confounding effects of confluence. Confluence levels were determined with a phase contrast EVOS microscope (ThermoFisher). Slower growing cells (PDL > 40) were sampled at 3.5-4 days after seeding. Cells were tested every month for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). A smaller subset of three PDL timepoints (PDL 45, PDL 55, PDL 56) were generated in a secondary time course to ensure "deep" senescence (2-4 months after cell cycle cessation) was maintained as previously reported (*De Cecco et al., 2019*).

WI-38 hTERT cells were maintained using the same conditions as WT WI-38s, targeting 50-70% confluence for cell splitting and sampling alongside WT cells.

761 Sampling proliferating WI-38 cells at different cell densities confounds assessment of cell
762 cycle state and senescence

763 A limited pilot (performed prior to RS time course) experiment was conducted to determine optimal
764 sampling conditions. Using gene expression as a readout for cellular state, we performed RNA-seq
765 on PDL 20, PDL 29, and PDL 39 WI-38 cells. We plated an equal number of cells in triplicate for the
766 three PDLs and cells were collected for RNA extraction after 4 days of growth. We looked for any
767 significant changes in transcript levels between the three samples.

768 We observed two notable trends. First, we found stronger expression of genes associated with
769 cell cycle and mitosis in the older PDL 39 cells compared to PDL 20 and PDL 29 cells. This was
770 unexpected based on previous observations of slower growth and less active mitosis in cells closer
771 to senescence (*Hayflick, 1965*). Second, the cells at PDL 20 and PDL 29 expressed genes associated
772 with hypoxic stress (Figure 1 supplement 5A).

773 These data, in tandem with the observation that older cells grow at a slower rate, suggest that
774 the highly proliferative younger cells (PDL 20 and 29) grow to higher cell density faster and there-
775 fore encounter earlier in real time, the various normal mitosis-inhibiting stresses, e.g. hypoxia,
776 contact inhibition, and starvation that trigger cell cycle exit (*Dayan et al., 2009*).

777 To understand the effects of growth rates and cell density on gene expression, we performed a
778 cell density study (CD) wherein young PDL cells were sampled at increasing levels of cell density to
779 measure the effects of cell density and proliferation on gene expression (Fig1A). We observed that
780 based on the seeding density we employed (to minimize the stress of sparse plating) the sampling
781 window we had originally targeted (4 days after seeding) was located in a volatile region with re-
782 spect to proliferation rate and hypoxic stress using Cyclin B1 (*CCNB1*) and VegfA (*VEGFA*) expression
783 as proxies for each respectively (Figure 1 supplement 5B).

784 Accordingly, we modified the sampling window by choosing a time interval (2.5-3 days) prior to
785 the onset of VEGF-A induction and *CCNB1* repression (Figure 1 supplement 5B). For older, slower
786 growing cells in the plateauing region of the replicative senescence process (PDL > 40) we shifted
787 the sampling window to 3.5-4 days based on visual inspection and cell counts. Ultimately in the
788 actual extended time course, we tracked cell density with a phase contrast EVOS microscope to
789 ensure cellular confluence was similar across all time points during sampling (Figure 2 supplement
790 5).

791 WI-38 irradiation time course

792 WI-38 cells at PDL 20 were plated in DMEM supplemented with 10% FBS with 50,000 cells per well
793 in a 6-well collagen coated plate. Cells were allowed to settle and adhere to plates. All cells were
794 adhered to the TC plate by 2 hours and were subsequently treated with 10 Gy of X-rays (Faxitron
795 CellRad). Cells were sampled between 1 day and 9 days for transcriptome profiling. Each time
796 point was sampled in triplicate. RNA was extracted according to methods below.

797 WI-38 cell density time course

798 0.3 E6 WI-38 cells at PDL 23 were seeded and grown on 10 cm collagen coated plates in DMEM
799 supplemented with 10% FBS. Cells were grown and sampled intermittently between 1 day and 10
800 days for transcriptome profiling. Samples at each time point were taken in triplicate. RNA was
801 extracted according to methods below.

802 SA- β GAL staining

803 Cells were stained for senescence associated beta-gal using the Senescence β -Galactosidase Stain-
804 ing Kit (Cell Signaling, 9860) by following the manufacturer's published protocols exactly.

805 WI-38 hTERT cells and lentiviral transduction

806 293T cells were transfected with a lentiviral target plasmid expressing hTERT (pCDH-CMV-hTERT-
807 EF1a-puro) and lentiviral packaging constructs overnight; 48 hours later, viral supernatant was

collected. WI-38 cells were transduced with viral supernatant in the presence of 5 μ g/mL polybrene and selected for 7 days with 1 μ g/ml of puromycin.

WI-38 + verteporfin treatment

200,000 WI-38 cells at PDL 38 were seeded onto each well of a 6-well collagen coated plate and grown with 2 ml of DMEM supplemented with 10% FBS. After 4 days of growth, cells were treated with 5 or 10 μ M of verteporfin (RD Systems 5305/10) for 2 hours or DMSO. RNA was extracted after 2 hours of treatment according to methods described below.

Bulk RNA-seq methods

RNA collection and library preparation

Total RNA was extracted from cells using the Direct-zol RNA Miniprep Plus kit (Zymo Research R2072) for all bulk RNA-seq time course experiments and verteporfin treatment experiment. Manufacturer's protocol was followed exactly and in-column DNAase digestion was performed. RNA quality score and concentration was measured using the Fragment Analyzer (Agilent 5200) with the Fragment Analyzer Standard Sense RNA kit (Agilent Technologies DNF-471-0500). All samples required to have a RIN score of >7 for processing. RNA sequencing libraries were prepared as directed using TruSeq® Stranded mRNA Library Prep Kit (Illumina 20020595), with 1000ng of input material. Samples were amplified for 12 cycles of PCR with TruSeq RNA CD Index Plate (Illumina) and pooled. 3nM libraries were loaded across 4 lanes on the HiSeq 4000 (Illumina).

Read processing and quantification

Reads generated from the Illumina HiSeq 4000 were demultiplexed with bcl2fastq (version=2.20) based on the barcode sequence of each sample. Average read depth across samples was 50 million paired-end reads. Reads were pseudo-aligned and quantified using Salmon (version=0.8.2) by deploying the mapping based mode using a Salmon command "index" with default parameters based on 10X genomics hg38 transcriptome annotations optimized for single cell RNA-seq ("refdata-cellranger-GRCh38-3.0.0", cellRanger version 3) to ensure accurate comparison between bulk and single cell RNA-seq (*Patro et al., 2017*). Annotations can be obtained running "wget <https://cf.10xgenomics.com/supp/cell-exp/refdata-cellranger-GRCh38-3.0.0.tar.gz>"

Differential expression analysis

DESeq2 (version=1.30.1) was used for differential analysis of the RNA-seq data *Love et al. (2014)*. Wald test was used to estimate fold change and significance using the model: ~time + batch, where time is a numeric variable representing the fraction of time course complete and batch is a categorical variable used only with replicative senescence with the addition of the "deep" senescence time points. DEGs were defined as having FDR adjusted p-values < 0.01.

Batch correction and hierarchical clustering

For clustering and visualization we corrected the raw replicative senescence count table by batch before converting to transcripts per million (TPM) with a +1 pseudocount and combining with radiation induced senescence and cell density samples using Combat-seq (sva R package version=3.38.0) (*Zhang et al., 2020*). Significant genes from each condition were concatenated to generate a universe of significant change genes used for Figure 1. Each sample was then converted to log2 fold change vs. the mean of the initial time point.

Gene set enrichment analysis

For gene set enrichment analysis, we downloaded the MSigDB Hallmark gene sets from msigDB website (version 7, gene symbols) *Liberzon et al. (2015)*. For each time course (replicative senescence, radiation induced senescence, and cell density) we ranked all genes by the log2 fold change across time generated by DESeq2. Then GSEA was performed on the ranked gene set using the R package "fgsea" version 1.16.0 which is an implementation of GSEA in R (*Subramanian*

854 *et al., 2005; Korotkevich et al., 2019; Mootha et al., 2003*). By default, GSEA tests for enrichment
855 of each gene set in each condition in both directions. We report the $-\log_{10}$ Benjamini-Hochberg
856 corrected p-value in Figure 1 and use the normalized enrichment score (NES) to assign direction of
857 the change.

858 Single Cell 3' RNASeq Methods

859 Cell collection

860 At each time point, singlet hTERT controls and experimental samples were processed with Chromium
861 Single Cell 3' RNAseq kit V2 (10x Genomics 120237) through cDNA amplification cleanup, where
862 they were frozen at -20°C . Once all time points were converted to cDNA, the frozen cDNAs were
863 thawed and batched for library construction. The following modifications were made to the pro-
864 cess: Reverse transcription reactions were brought up to volume with DMEM + 10% FBS instead
865 of water, each emulsion targeted 3000 cells (5200 cells loaded), and cDNA was amplified 12 cycles,
866 with 12 cycles of index PCR. There was no hTERT control for time point 3, and one of the replicates
867 for PDL 25 dropped out during library construction. Remaining samples were pooled at equimolar
868 concentration and sequenced on a HiSeq4000 with the standard 26,8,0,98 run base pairs per read
869 configuration.

870 Single cell data processing, normalization, scoring, clustering and DEG analysis

871 The raw single cell reads were demultiplexed by sample using bcl2fastq. Alignment, cell barcode
872 demultiplexing, transcript quantification and sample merging were carried out with CellRanger
873 3.0 using the hg38 CellRanger 3.0 gene annotation ("refdata-cellranger-GRCh38-3.0.0", cellRanger)
874 with default options and parameters. Filtered cell by gene matrices were normalized using Seurat
875 (version = 4.0.1.9005) and SCTransform (version = 0.3.2) (*Hafemeister and Satija, 2019; Stuart*
876 *et al., 2019*). Dimension reductionality was carried out with PCA ($n = 50$). Clusters were defined
877 using the louvain algorithm resolution= and cells were visualized with UMAP projection *McInnes*
878 *et al. (2018); Becht et al. (2019); Blondel et al. (2008)*. For cell cycle scoring and phase determination
879 as well as senescence scoring we applied the Seurat implementation of the scoring function as
880 previously described (*Nestorowa et al., 2016*).

881 DESeq2 was used for differential gene expression analysis to identify significantly changing
882 genes within individual clusters as a function of increasing PDL (PDL by mitotic phase) (Fig. 3D)
883 using a an input matrix of gene counts by cells per PDL by mitotic phase. Gene counts were sum-
884 marized across cells for each mitotic phase (PDL 50 removed). To expedite computation, we re-
885 stricted analysis to highest expressed 8,000 genes. For each PDL by cell cycle phase grouping, we
886 required that >15 representative cells must exist to be considered in analysis. To visualize differ-
887 entially expressed genes, we converted single cell counts to counts per million (+1 pseudocount)
888 and averaged across PDL by cell cycle phase and calculated the $\log_2$ fold change at each PDL by
889 cell cycle phase against the earliest PDL for that cell cycle phase using a concatenated list of all
890 significantly changing genes generated by DESeq2 from each cell cycle phase.

891 Pseudotime analysis

892 For pseudotime analysis of the single cell data, we used the R package monocle3 (version=1.0.0)
893 which implements PCA, Leiden clustering, and UMAP prior to partitioning and trajectory analy-
894 sis. We first isolated WT cells by removing hTERT cells and those not belonging to the primary
895 grouping. To focus trajectory analysis on the replicative senescence progression, we used only
896 the top genes found to significantly change with replicative senescence in bulk RNA-seq for PCA
897 ($\text{padj} < 0.01$) and $\log_2$ fold change > 3 or $\log_2$ fold change < -3). In applying the Monocle trajec-
898 tory analysis: (`learn_graph(cds,close_loop = FALSE,learn_graph_control = list(minimal_branch_len`
899 `= 35, geodesic_distance_ratio = .5, euclidean_distance_ratio=1)`), we designated cycling cells at the
900 opposite end of UMAP project from the senescent cells as the start point manually. (*Trapnell et al.,*
901 *2014; Qiu et al., 2017; Cao et al., 2019; Traag et al., 2019; Levine et al., 2015; McInnes et al., 2018*).

After establishing a trajectory, we then employed the Monocle3 "graph test" function to isolate genes that significantly change as a function of pseudotime. Pseudotime estimation was output from monocle3 using the learn graph function for building a trajectory. Smoothed pseudotime trajectories use for Figure 7C were calculated for significantly changing genes (with pseudotime) by binning cells across pseudotime into 60 bins and using a cubic spline to estimate expression at each bin. For each gene, the smoothed trajectory was set from 0 (minimal) to (1) maximal expression. Genes were organized with K-median clustering (k=25) using cosine similarity. For visualization the median expression value for each pseudotime bin and cluster was calculated. Genes from each cluster were fed into LISA TF analysis (below)

ATAC-SEQ methods

ATAC-seq Library Preparation and Sequencing

Freshly harvested cells were used for all reactions. Briefly, the cell monolayer was washed with PBS, trypsinized with TrypLE Express (Gibco 12604013), resuspended in media, and cells were pelleted. Cells were counted and 100,000 cells were used in each reaction. Cell lysis, DNA transposition, and library construction was followed from the Omni-ATAC protocol (Corces et al. 2017). Libraries were amplified for 13 total cycles. Sample purification and size selection were performed on the Pippin high throughput size selection platform (Sage Science) using 2% agarose cassettes to isolate nucleosome free fragments that were < 300 base pairs in library fragments and thus corresponding to < 150 base pairs fragments after accounting for sequencing adaptors. Quality of ATAC-seq libraries were assessed with the Agilent Bioanalyzer 2100 with DNA high sensitivity chips (Agilent Technologies 5067-4626). Libraries were sequenced on the HiSeq4000 with paired end sequencing using 2 x 150 bp reads (Illumina).

ATAC-seq data processing, peak calling, and differential accessibility

We first trimmed the raw fastq files with cutadapt (version=2.4) to remove standard Nextera PE adapters:

```
adapt -a file:$ADAPTER -A file:$ADAPTER -o $SAMPLE.R1.fq -p $SAMPLE.R2.fq -pair-filter=any
-minimum-length=30 $R1 $R2
```

Then we aligned with bowtie2 (version version 2.3.4.1) to align the trimmed reads to hg38: bowtie2 -x \$INDEX -1 \$SAMPLE.R1.fq -2 \$SAMPLE.R2.fq -no-mixed -no-discordant -X 1000

After alignment, we used samtools (version=1.2) flags (-f 0x02 and -q2 0) to filter for only properly paired and high quality reads. PCR duplicates are removed using picard (version=2.6.4) MarkDuplicates. Finally for each bam file, we adjusted the reads ends by Tn5 offset (+4 on + strand, -5 on -strand).

ATAC-seq QC (mitochondrial percent and transcription start site score) and alignment metrics were generated with R package ATACseqQC (version=1.14.4) and multiQC respectively (version=1.11) (Ewels et al., 2016) using TxDb.Hsapiens.UCSC.hg38.knownGene (version = 3.10.0) annotations for transcription start site score calculation.

For peak calling, we created a condition specific peak atlas by pooling all replicates in a specific condition and applied macs2 (version = 2.2.1.2) for peak calling on the pooled bam file with options (-g hs -p 1e-1 -nomodel -shift -37 -extsize 73) (Zhang et al., 2008). In addition, we performed peak calling on each individual replicates as well. Then we performed irreproducible discovery rate analysis on each condition specific peak atlas for each pair of replicates and filter for peaks that are reproducible in at least two replicates (IDR threshold of 0.05). A single accessibility atlas is created by merging condition-specific peak atlas across all conditions (Figure 5 source data 3). Peaks were assigned to nearest gene if it is within 50kb, otherwise it is annotated as intergenic.

Read count was performed using countOverlaps function from R package GenomicRanges (version = 1.42.0) (Lawrence et al., 2013). We performed quantile normalization of the count matrix using normalize.quantiles function of R package preprocessCore (version=1.52.1).

950 Limma-voom (Limma version = 3.46.0) was used for differential accessibility analysis (*Ritchie*
951 *et al., 2015*). Fold change and fdr adjusted p-values were estimated using moderated t-test statistic
952 based on the model: time + condition + time:condition. We performed separate tests for time point
953 1 versus each of the other time points.

954 ATAC-seq chromatin state and NAD/LAD analysis

955 To quantify ATAC-seq reads in chromatin states we retrieved hg38 ENCODE IMR-90 chromatin state
956 labels from (*Ernst and Kellis, 2015*) (Figure 5 source data 1). Next we quantified coverage for each
957 instance of all 25 chromatin states genome wide using countOverlaps function from R package
958 GenomicRanges (*Lawrence et al., 2013*). We performed quantile normalization of the chromatin
959 state count matrix using normalize.quantiles function of R package preprocessCore (Fig. 5A).

960 ATAC-seq peaks were assigned to chromatin states using the findOverlapsOfPeaks function
961 from the R package GenomicRanges (*Lawrence et al., 2013*). We used only significantly ($p < 0.001$)
962 changing peaks (with senescence) from LIMMA-voom analysis. To simplify the overlap of these two
963 sets of genomic intervals, we took only peaks that fell within ("inside") or encompassed a chromatin
964 state annotation ("inside feature"). Peaks encompassing more than one chromatin state interval
965 were discarded (Fig. 5A,B).

966 For analysis of NAD and LAD domain overlap we collected IMR-90 NAD labels from (*Dillinger*
967 *et al., 2017*) and IMR-90 LAD labels from (*Sadaie et al., 2013*) (Figure 5 source data 5 and 6). For
968 calculating overlap Z-score between genomic intervals sets (e.g. NADs/LADs vs. gene annotation
969 (TxDb.Hsapiens.UCSC.hg38.knownGene, version = 3.10.0) for Figure S18C we used the overlap-
970 PermTest function from the R package regioneR (version = 1.22.0) *Gel et al. (2016)*.

971 For testing overlaps between NAD/LAD domains and significantly changing genes, we used the
972 overlapPermTest function from the regioneR package using the top induced genes from each bulk
973 RNA-seq condition (RS, RIS, and CD) against a universe of all genes sampled from the same ex-
974 pression distribution to control NAD and LAD bias across different levels of expression. For gene
975 annotations we used TxDb.Hsapiens.UCSC.hg38.knownGene (version = 3.10.0).

976 Metabolomics

977 Extraction of water-soluble metabolites from lung fibroblast cell culture

978 Twenty-four hours before metabolite extraction, the medium was aspirated, cells were washed
979 with unconditioned medium and then the medium was replaced. For metabolite extraction, cells
980 were washed once in 37°C warm PBS-buffer immediately followed by the addition of 3.5 mL of
981 freezer-cooled (−20 °C) LC-MS grade 80:20 MeOH/H₂O (Sigma Aldrich). The plates were then held
982 at −20 °C for 2 hours, then harvested with a sterile cell scraper while at −20 °C and transferred to
983 −20 °C cold 5 mL centrifuge tubes (Eppendorf Lo-Bind). After centrifuging the cell-extracts in a 4 °C
984 centrifuge for 5 min at 2000 x g, the supernatants were transferred into new cold centrifuge tubes
985 and dried under nitrogen at 4 °C. Dried extracts were stored at −20 °C.

986 LC-MS/MS analysis of cell culture extracts

987 Dried supernatants were resuspended in 200 µL of water containing 1 µg/mL of deuterated lysine
988 and deuterated phenylalanine and 250 ng/mL of deuterated succinate (Sigma Aldrich) as internal
989 standards. For negative ion mode, the resuspended samples were diluted 1:4 in water, for positive
990 ion mode, they were diluted 1:4 in acetonitrile. Samples were then centrifuged at 18000 x g for
991 5 minutes, the supernatant was moved to HPLC vials and 5 µL was injected for analysis by LC-MS
992 on Vanquish UPLCs (Thermo Scientific) coupled to Q Exactive Plus mass spectrometers (Thermo
993 Scientific).

994 For analysis in negative ion mode, separation of compounds was achieved by reverse-phase
995 liquid chromatography on a Zorbax Extend C18 column (150 x 2.1 mm, 1.8 µm particle size (Ag-
996ilent 759700-902). Mobile phase A was 10 mM Tributylamine and 15 mM Acetic Acid in 97:3 wa-
997 ter:methanol at pH 4.95 and mobile phase B was methanol. Prior to injection, the column was

equilibrated in 0% B for 4 minutes. The gradient eluted isocratically in 0% B for 2.5 minutes, increased to 20% B over 2.5 min, held at 20% B for 2.5 min, increased to 55% B over 5.5 min, increased to 95% B over 2.5 minutes, maintained at 95% B for 3 min, then decreased to 0% B over 0.5 min, where it was held for 3 min, resulting in a total run time of 26 min. The LC separation was carried out at 30 °C column temperature using a 200 µL/min flow rate. For MS-analysis, parameters on MS1 were set to 70,000 resolution with an AGC target of 1e6 at a maximum IT of 100 ms. The scan range was 70 to 1050 m/z. MS2 parameters were set to 17,500 resolution at loop count 6, an AGC target of 1e5 at a maximum IT of 50 ms, an isolation window of 1 m/z and an underfill ratio of 1%. Dynamic exclusion was set at 20s with an apex trigger from 3 to 12s. Stepped collision energies were set to 20, 50 and 100% NCE.

For analysis in positive ion mode, compounds were separated via hydrophilic liquid interaction chromatography (HILIC), using a SeQuant ZIC-pHILIC column (150 x 2.1 mm, 5 µm particle size) Millipore (150460). Mobile phase A consisted of 20 mM ammonium bicarbonate at pH 9.2 in H₂O, and mobile phase B was acetonitrile. Prior to injection, the column was equilibrated for 6 minutes in 80% B. The gradient then decreased to 20% B over 20 min, then to 15% B over 2 min, returned to 80% B over 0.5 min and held there for 1.5 min for a total run time of 30 min. The column temperature was 35 °C with a flow rate of 150 µL/min. For MS-analysis the MS1 parameters were as described for negative ion mode except the AGC target was 3e6. MS2 parameters were the same with following exceptions: dynamic exclusion was set to 25s with an apex trigger from 3 to 10s. Stepped collision energies were set to 20, 40 and 80% NCE.

Metabolomics batch correction and differential analysis

Protein lysates derived from same plates used for metabolomics collection were assayed for total protein concentration with Pierce BCA Protein Assay Kit (ThermoFisher 23225) for normalizing raw metabolite values. The median protein concentration for each PDL and PDL.ctrl was calculated and divided by the median protein concentration across all samples for either cell line (WT and hTERT) to derive a protein concentration normalization factor. The raw metabolite values for each PDL and hTERT PDL.ctrl were multiplied by the protein concentration normalization factor for that strain and sample. Protein normalized metabolite values were then converted to log₂ fold change values using the first time point as a reference (Figure 3 source data 2). To remove sample day batch effects, we took the protein normalized metabolite values and divide each WT metabolite value by the corresponding metabolite value in the temporally paired hTERT sample (e.g. PDL 25 values were divided by PDL 25.ctrl values) and converted to log₂ fold change with first sample as reference.

Finally, the normalized log₂ ratios were fit to a linear model to test for a linear trend across the time course. For each metabolite, we fit the model $y_{it} = \beta_0 + \beta_1 T_i + \epsilon_{it}$, where $i=(1, \dots, 3)$ indexes the replicates for each time point and $t=(1, \dots, 4)$ indexes the cell passage vector $T=(33, 37, 46, 50)$.

The model was fit with the `lm()` function in R Version 4.1.2. P-values from the two-sided hypothesis test $H_0 : \beta_1 = 0$ were FDR adjusted (across all proteins) using the `qvalue` function from the `qvalue` R package (version =2.26.0). If present, asterisks in figures indicate that the FDR adjusted p-value from this test was < .05.

Proteomics methods

Materials and sample preparation, extraction and digestion

LC-MS grade organic solvents, water, and tandem mass tag (TMT) isobaric reagents were purchased from Thermo Fisher Scientific (Waltham, MA). Trypsin was ordered from Promega Corporation (Madison, WI) and Lys-C from Wako Chemicals USA (Richmond, VA). Sep-Pak C18 cartridges were from Waters Corporation (Milford, MA). Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

At time of sample collection, cells were trypsinized off the monolayer with TrypLE Express and media was used to neutralize the reaction. Cells were pelleted at 200 x g for 5 minutes and the

1047 supernatant was aspirated. Cell pellets were flash frozen in liquid nitrogen and stored at -80°C until
1048 all samples were collected and ready for proteomics analysis. Cell pellets were resuspended in 450
1049 µL of lysis buffer (75mM NaCl, 3% SDS, 50 mM HEPES, pH 8.5) and lysed by passage through a BD
1050 PrecisionGlide 21-gauge needle (20X). The lysate was sonicated for 5 minutes and then centrifuged
1051 (5 min, 16,000 x g) to remove cellular debris and the supernatant was collected.

1052 Proteins were reduced with 5 mM dithiothreitol (DTT) for 30 minutes at 56 °C with shaking. Pro-
1053 teins were then alkylated with 15 mM iodoacetamide (IAM) for 30 minutes at room temperature
1054 (RT) in the dark, and excess IAM was quenched with 5 mM DTT for 20 minutes at RT in the dark.
1055 Protein purification was accomplished using a methanol-chloroform precipitation. Briefly, 800 µL
1056 methanol, 200 µL chloroform and 600 µL water were sequentially added to 200 µL of cell lysate,
1057 with 5 second intervals of vortexing between each addition. The samples were centrifuged for 30
1058 minutes (16,000 x g at 4°C) to induce phase separation and both the top and bottom layers were
1059 removed. The precipitated protein pellet was washed with 600 µL methanol, vortexed briefly, then
1060 centrifuged for 10 minutes (16,000 x g at 4 °C). The methanol layer was removed and protein pellets
1061 were dried at RT for 10 minutes. Protein pellets were resuspended in digestion buffer (8 M urea,
1062 50 mM HEPES, pH 8.5). The urea concentration was diluted to 4 M, then proteins were digested
1063 with Lys-C overnight (10 ng/µL, 25 °C, 16 h). The urea concentration was further diluted to 1 M and
1064 samples were digested with trypsin (5 ng/µL) for 6 hours at 37 °C.

1065 Following digestion, peptides were acidified with trifluoroacetic acid (TFA) to a final concentra-
1066 tion of 0.5% TFA. Peptides were desalted using Sep-Pak C18 solid-phase extraction (SPE) columns
1067 and samples were eluted sequentially, first with 40% acetonitrile (ACN)/0.5% acetic acid and then
1068 80% ACN/0.5% acetic acid. Eluted peptides were dried in a CentriVap Benchtop Vacuum Concen-
1069 trator (Labconco, Kansas City, MO) running at 30 °C. Peptide concentrations were measured using
1070 the Pierce BCA Protein Assay Kit, then 50 µg aliquots of each samples were dried in the CentriVap
1071 for further processing.

1072 Tandem mass tag (TMT) labeling

1073 Dried peptides were resuspended in 50 µL 200 mM HEPES/30% anhydrous ACN, then 200 µg of
1074 each TMT tag was added to 50 µg peptides. TMT 131c was used as the 'bridge sample' while the
1075 other tags (126, 127n, 127c, 128n, 128c, 129n, 129c, 130n, 130c, 131n) were used to label the in-
1076 dividual samples. The TMT reaction was incubated for 1 hour at room temperature with gentle
1077 shaking, then quenched with 11 µL 5% hydroxylamine/200mM HEPES. All samples were acidified
1078 to a final concentration of 0.5% TFA. A small amount (4 µL) of each labeled sample was combined
1079 and desalted using StageTips to check TMT ratios and labeling efficiency. The TMT-labeled sam-
1080 ples were then combined at a 1:1:1:1:1:1:1:1:1 peptide ratio into 11-plex samples (*Rappsilber*
1081 *et al.*, 2007). The combined samples were desalted using Sep-Pak C18 cartridges and dried under
1082 vacuum.

1083 High pH Reversed-Phase (HPRP) fractionation

1084 The pooled TMT-labeled peptides were fractionated using high pH reversed-phase liquid chro-
1085 matography on an Agilent 1260 Infinity HPLC equipped with a diode array detector set at 215, 220
1086 and 254 nm wavelengths (Agilent Technologies, Santa Clara, CA). Peptides were separated on an
1087 Agilent ZORBAX Extend-C18 column (4.6 mm x 250 mm, 5 µm particle size) running at 500 µL/min
1088 at 25°C. Peptides were eluted with a gradient with initial starting condition of 100% buffer A (5%
1089 ACN, 10 mM ammonium bicarbonate) and 0% buffer B (95% ACN, 10 mM ammonium bicarbonate).
1090 Buffer B was increased to 35% over 60 minutes, then ramped up to 100% B in 6 seconds where it
1091 was held for 5 minutes. Buffer B was then decreased to 0% over 6 seconds and held for 10 min-
1092 utes to re-equilibrate the column to original conditions. The samples were fractionated into 96
1093 fractions, then pooled into 12 fractions as previously described (*Huttlin et al.*, 2010). The fractions
1094 were dried under vacuum and resuspended in 5% ACN/5% formic acid (FA) for LC-MS/MS analysis.

1095 Proteomics Data Acquisition and Analysis

1096 LC-MS/MS Data Acquisition All samples were analyzed by an Orbitrap Fusion Lumos Tribrid mass
1097 spectrometer coupled to an EASY-nLC 1200 (Thermo Fisher Scientific). Peptides were separated us-
1098 ing a microcapillary column (100 μ m x 250 mm long, filled in-house with Maccel C18 AQ resin, 1.8
1099 μ m, 120 Å; Sepax Technologies, Newark, DE) operating at 60 °C with a flow rate of 300 nL/min. Pep-
1100 tides were eluted into the mass spectrometer using a 180 min method, with acetonitrile increasing
1101 from 6 to 30% over a 165 min linear gradient in 0.125% formic acid. Mass spectrometry data was
1102 collected in data-dependent acquisition (DDA) mode. A high resolution MS1 scan (500-1200 m/z
1103 range, 60,000 resolution, AGC 5 x 10⁵, 100 ms max. injection time, RF for S-lens 30) was collected
1104 in the Orbitrap, and the top 10 precursors were selected for MS2 and MS3 analysis. Ions were iso-
1105 lated using a 0.5 m/z window for MS2 spectra. The MS2 scan was performed in the quadrupole ion
1106 trap (CID, AGC 1 x 10⁴, 30% normalized collision energy, 35 ms max. injection time) and the MS3
1107 scan was analyzed in the Orbitrap (HCD, 60,000 resolution, max. AGC 5 x 10⁴, 250 ms max. injec-
1108 tion time, 50% normalized collision energy). The max. cycle time was set at 5 s. For TMT reporter
1109 ion quantification, up to 6 fragment ions from each MS2 spectra were selected for MS3 analysis
1110 using synchronous precursor selection (SPS).

1111 Proteomics data analysis

1112 The *Ting et al. (2011)* software pipeline and methods developed in the the Haas and Gygi labs
1113 was used to process all proteomics data (*Ting et al., 2011*). Raw files were converted to mzXML
1114 files and searched against a composite human UniProt database containing forward and reverse
1115 sequences using the Sequest algorithm. MS/MS spectra were matched with fully tryptic peptides
1116 from this composite dataset using a precursor ion tolerance of 20 ppm and a product ion toler-
1117 ance of 0.6 Da. TMT modification of peptide N-termini and lysine residues (+229.162932 Da) and
1118 carbamidomethylation of cysteine residues (+57.02146 Da) were set as static modifications. Oxi-
1119 dation of methionine residues (+15.99492 Da) was set as a variable modification. Peptide spectral
1120 matches were filtered to a 1% false discovery rate (FDR) using linear discriminant analysis (LDA) as
1121 previously described (*Ting et al., 2011*). Non-unique peptides that matched to multiple proteins
1122 were assigned to proteins that contained the largest number of matched redundant peptides se-
1123 quences using the principle of Occam's razor (*Ting et al., 2011*).

1124 Quantification of TMT reporter ion intensities was performed by extracting the most intense
1125 ion within a 0.003 m/z window at the predicted m/z value for each reporter ion. TMT spectra were
1126 used for quantification when the sum of the signal-to-noise for all the reporter ions was greater
1127 than 200 and the isolation specificity was greater than 0.75.

1128 Peptide level intensities were converted to log₂ ratios by dividing each scan by the intensity in
1129 the bridge channel. Relative protein abundance, for each sample, was estimated with the posterior
1130 mean from a previously described Bayesian model using methods as described by *O'Brien et al.*
1131 (*2018*) with code located at <https://github.com/ColtoCaro/compositionalMS>.

1132 Finally, the relative abundance estimates were fit to a linear model to test for a linear trend
1133 across the passages as described by *Gaun et al. (2021)*. For each protein, we fit the model $y_{it} = \beta_0 +$
1134 $\beta_1 T_i + \epsilon_{it}$, where $i=(1, ..., 3)$ indexes the three replicates for each time point and $t=(1, ..., 7)$ indexes the
1135 cell passage vector $T=(18, 25, 32, 33, 37, 46, 50)$.

1136 The model was fit with the `lm()` function in R Version 3.6.0. P-values from the two-sided hypoth-
1137 esis test $H_0 : \beta_1 = 0$ were FDR adjusted (across all proteins) using the `p.adjust()` function. If present,
1138 asterisks in all figures indicate that the FDR adjusted p-value from this test was $< .01$.

1139 Regulatory analysis

1140 LISA TF analysis

1141 Identification of putative regulators of the gene expression changes observed in the bulk RNA-seq
1142 experiments and the pseudotime analysis was carried out using (*Qin et al., 2020*); a recent algo-
1143 rithm built to leverage the vast amount of protein-DNA interactions catalogued via ENCODE. We

1144 used the GUI hosted at <http://lisa.cistrome.org/> to enter lists of genes derived from K-medians
1145 pseudotime clustering clustering. The online GUI has a max input of 500 genes. Accordingly, for
1146 clusters containing >500 genes, each gene was ranked by correlation with the cluster median and
1147 the top 500 genes were used. LISA output consists of a ranked file of transcription factors and chro-
1148 matin modifiers with enrichment p-values associated with specific ENCODE experiments. Given
1149 that the calculated p-values were derived from gene sets of different sizes and from different num-
1150 ber of supporting experiments, we used the ranks as the input parameter for the LISA analysis
1151 shown in Figures 6D and 7D. A universe of top factors across clusters was compiled by concate-
1152 nating the top 5 TFs from each individual LISA output. The rank matrix was then centered across
1153 clusters to identify TFs with the highest rank in specific clusters.

1154 ATAC-seq peak motif enrichment

1155 We built a binary peak-by-motif matrix where each row is a binary vector representing the presence
1156 of 405 motifs we used from CisBP (Catalog of Inferred Sequence Binding Preferences) which is a
1157 freely available online database of transcription factor (TF) binding specificities: <http://cisbp.cabr.utoronto.ca>
1158 (*Weirauch et al., 2014*). We only used motifs for TFs with measurable RNA-seq in WI-38 cells. In or-
1159 der to characterize transcription factor activity changes during senescence, we implemented both
1160 a gene-centric and a gene-independent approach.

1161 In the gene-centric approach, we tested enrichment of each of the 405 motifs in peaks that are 1)
1162 associated with a gene set of interest (within 50kb), and 2) significantly associated with senescence
1163 (adjusted p-value < 0.001 in PDL 50 vs. PDL 20) as compared to all the other peaks using binomial
1164 test.

1165 Alternatively, we trained a ridge logistic regression model using the binary motif matrix as fea-
1166 tures to distinguish peaks of significantly increased accessibility during senescence from peaks of
1167 significantly reduced accessibility in PDL 50 vs. PDL 20. A model trained on two thirds of the data
1168 distinguishes the two sets of peaks with AUC=0.67 on held-out peaks. We then trained 10 inde-
1169 pendent models using all the data to evaluate the coefficients to identify features (motifs) that are
1170 most predictive of senescence.

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Figure 1: Expression dynamics of replicative senescence (RS), radiation induced senescence (RIS), increasing cell density (CD) and hTERT WI-38 cells. **(A)** Experimental design for the RS, RIS, CD and hTERT experiments. **(B)** Days in culture vs. population doublings (PDL) for WT WI-38 cells (red) and hTERT immortalized cells (green). Labeled points denote sample collection and are expressed in PDLs for RS time course or PDL controls (PDL.ctrl) for the hTERT time course. Temporally paired samples indicated by vertical dotted lines. PDLs 52 and 53 were collected in a separate "deep" senescence time course. **(C)** Percent cells staining positive for SA-βgal staining (y-axis) at increasing PDLs (x-axis) for WI-38 cells (red) and hTERT (green). Points represent replicate values. **(D)** Scatterplot of log2 fold changes in gene expression for PDL 50 vs. PDL 20 (x-axis) vs. senescence log2 fold change derived from a generalized linear model compiling gene expression changes across multiple fibroblast cell lines during replicative senescence (y-axis) (Hernandez-Segura et al., 2017). **(E)** Hierarchical clustering of significant gene expression changes (FDR adjusted p-value < 0.01) across all conditions (n=3 replicates). Values are log2 fold change vs. the average of the first time point of each condition. Reference time point not shown. **(F)** Significant (FDR adjusted p-value < 0.01) Gene Set Enrichment Analysis (GSEA) results for RS, RIS and CD using the MSigDB Hallmarks annotation set. The -log10 p-value is colored by direction of enrichment (red=up, blue=down).

Figure 1-Figure supplement 1. Sample manifest

Figure 1-Figure supplement 2. Senescence markers

Figure 1-Figure supplement 3. Senescence markers cont'd

Figure 1-Figure supplement 4. Individual time point GSEA

Figure 1-Figure supplement 5. Pilot experiment for WI-38 husbandry.

Figure 2: Cell cycle exit and distribution on approach to replicative senescence (RS) does not explain gradual increase in the replicative senescence transcriptome. **(A)** Individual UMAP projections of WT WI-38 cells; PDL colored by phases of the cell cycle (G1 = green, G2/M = orange, S-phase = purple). **(B)** Bar graph of cell cycle state percentages defined by transcriptomic score (y-axis) by PDL (x-axis) for WT WI-38 cells (left) and hTERT WI-38 cells (right). **(C)** UMAP projections of WT and hTERT WI-38 cells by PDL and PDL.ctrl colored by different phases of the cell cycle defined by transcriptomic score. **(D)** UMAP projection of all WI-38 cells. Increasing PDLs (RS) or PDL control (hTERT) are colored from early (red) to late (blue). **(E)** The replicative senescence transcriptome manifests early in all phases of cell cycle. Heatmap of hierarchical clustering of gene expression values of differentially expressed genes as aggregated transcriptomic profiles for each cell cycle phase and PDL (left) versus all cells (right). Values are log2 fold change of each PDL against the reference PDL 25 (not shown).

Figure 2-Figure supplement 1. Buffer optimization for scRNA-seq

Figure 2-Figure supplement 2. Correlation between bulk and single cell RNA-seq

Figure 2-Figure supplement 3. UMAP of mitotic cells

Figure 2-Figure supplement 4. Senescence and cell density scoring of scRNA-seq cells

Figure 2-Figure supplement 5. Images of cell density at sampling

Figure 3: Proteomic and metabolomic changes during replicative senescence. **(A)** Significant (Benjamini-Hochberg adjusted p -value < 0.01) GSEA results for protein changes ($n=3$) using the MSigDB Hallmarks. Reference time point not shown. Values are $-\log_{10}$ p -value and are colored by the direction (orange=up, green=down). **(B)** KEGG and GO term enrichment of genes from A for selected annotations. **(C)** Gene expression changes ($n=3$) for insulin-independent glucose transporter *SLC2A1* (left) and fatty acid transporter *CD36* (right) in the hTERT, replicative senescence, radiation induced senescence and cell density time courses. **(D)** Diagram of glycolysis, glycolytic shunts, fatty acid import and oxidation, TCA cycle/oxidative phosphorylation. Metabolites are blue, proteins are green. **(E)** Metabolite ($n=4$) and protein changes ($n=3$) with senescence from D. Average corrected metabolite $\log_2$ fold change vs. PDL 25 as reference (not shown) are plotted. Proteomics values are average $\log_2$ fold change vs. PDL 20 as reference (not shown). *:Significant changes (FDR adjusted p -value <0.05 metabolomics, FDR adjusted $p<0.01$ proteomics)

Figure 3-Figure supplement 1. PDL-dependent changes in the senescent proteome vs. hTERT cells

Figure 3-Figure supplement 2. Correlation between bulk RNA-seq and proteomics

Figure 3-Figure supplement 3. Proteomic changes in oxidative phosphorylation annotation

Figure 3-Figure supplement 4. PDL-dependent changes in the senescent metabolome vs. hTERT cells

Figure 3-Figure supplement 5. Kennedy pathway utilization during replicative senescence

Figure 4: Nicotinamide n-methyltransferase (NNMT) links nicotinamide adenine dinucleotide (NAD) and methionine metabolism during replicative senescence. **(A)** Gene expression fold changes for the NNMT (top) in the hTERT, replicative senescence (RS), radiation induced senescence (RIS), and cell density (CD) time courses (3 replicate average, each point a replicate). Heatmaps of metabolite and protein changes during replicative senescence from B (bottom-replicate average). **(B)** Metabolic diagram of the methionine and NAD salvage pathways. Metabolites are blue, proteins are green. Shading indicates inferred pathway direction during replicative senescence based on metabolite/protein changes in A. **(C)** Heatmaps of metabolite (4 replicate average) and protein (3 replicate average) changes with replicative senescence from B. Metabolomics data was batch corrected against hTERT samples and expressed as log₂ fold change vs. PDL 25 as reference (not shown). Proteomics data expressed as log₂ fold change vs. PDL 20 as reference (not shown). Significant changes (FDR adjusted $p < 0.05$ metabolomics, FDR adjusted p -value < 0.01 proteomics) during replicative senescence are denoted with asterisks. **(D)** Potential model for NNMT and metabolic regulation of chromatin state during replicative senescence.

Figure 5: Increased accessibility within heterochromatin and nucleolar associated domains (NADs) is a dominant feature of the replicative senescence (RS) epigenome. **(A)** Percent of ATAC-seq reads falling into 4 broad chromatin states compiled from the ENCODE IMR-90 25 chromatin state prediction for all samples in WT or hTERT WI-38 cells. Percent of all ATAC-seq reads within two specific states (y-axis) vs PDL (x-axis) for WT (red) and hTERT (green) WI-38 cells (bar height denotes average of 3 replicates). **(B)** Median log2 fold change in ATAC-seq counts for significantly changing ATAC-seq peaks binned into ENCODE IMR-90 25 chromatin states (row) for each sample (column, n=2 or 3 replicates). Reference samples are PDL 20 or PDL 20.ctrl for WT and hTERT WI-38 cells respectively (not shown). **(C)** Observed and expected distribution of overlaps of significantly induced genes (from Figure 1, RNA-seq) with NADs and LADs. Replicative senescence (red), radiation induced senescence (RIS) (orange) and cell density (CD) (blue). Median expected number of overlaps and observed number of overlaps shown by black and green lines respectively. **(D)** Average (n=2 or 3 replicates) log2 fold change distribution (PDL 50 vs. PDL 20) for all ATAC-seq peaks that overlap or are excluded from NADs (left) or LADs (right).

Figure 5-Figure supplement 1. ATAC-seq library fragment distribution and size selection

Figure 5-Figure supplement 2. ATAC-seq library fragment distribution after size selection and sequencing

Figure 5-Figure supplement 3. ATAC-seq mitochondrial read percentages and ATAC-seq transcriptional start site enrichment

Figure 5-Figure supplement 4. ATAC-seq QC metrics and extended analysis

Figure 5-Figure supplement 5. Chromatin state profiles of ATAC-seq peaks in Nucleolar Associated Domains

Figure 6: Master transcriptional regulators of replicative senescence **(A)** Scatter plot of transcription factor motif enrichment in ATAC-seq peaks surrounding significantly induced genes (FDR adjusted p-value < 0.01, $\log_2$ FC > 0.5) during replicative senescence. The Y-axis is the $-\log_{10}$ p-value; x-axis is the percent of input genes with the transcription factor motif. All points are transcription factors. Specific transcription factors of interest are called out in red. **(B)** Bar graph of ridge regression coefficient of motif predictive power in model of increasing peak accessibility with replicative senescence. Transcription factors of interest are highlighted in red. **(C)** Scatterplot of transcription factors enriched for binding in regulatory regions around replicative senescence depleted genes (y-axis, FDR adjusted p-value < 0.01, $\log_2$ FC < -0.5) vs. replicative senescence induced genes (x-axis, FDR adjusted p-value < 0.01, $\log_2$ FC < -0.5). Curated cell cycle transcription factors are colored in green; transcription factors of interest e.g. EMT/AP1/YAP1/TEAD1 etc. are colored in red. **(D)** Scatterplot of enriched transcription factors rank for binding enrichment in regulatory regions around senescence induced genes in astrocytes (y-axis) vs. replicative senescence induced genes in WI-38 cells (x-axis). transcription factors with discordant ranks/enrichment are colored in turquoise, transcription factors with concordant ranks/enrichment are colored in red; transcription factors of interest are labeled in red.

Figure 6–Figure supplement 1. FOXE1 expression during senescence.

Figure 7: Pseudotime (PS) analysis of WI-38 approach to replicative senescence using single cell RNA-seq. **(A)** UMAP projection of single WI-38 cells collected at increasing PDLs (PDL25-red) to (PDL50-blue) and colored by pseudotime (top). **(B)** Scatterplot of single cell gene expression across pseudotime with 3 genes representative of changes that occur early in pseudotime (top), during the transition phase (middle) and in late pseudotime (bottom). Each point is a cell colored by PDL-PDL25 (red) to PDL50 (blue). x-axis is pseudotime, and y-axis is counts for the gene of interest. Black line is a cubic spline. **(C)** Hierarchical clustering and heatmap of smoothed gene expression trajectories over 60 pseudotime bins (x-axis) of 25 K-median gene expression clusters (y-axis). Value plotted is the scaled (min expression to max expression; 0-1) median expression of all genes in the cluster. Clusters are divided into three (early, transition, late) pseudotime categories. **(D)** LISA transcription factor enrichment analysis using the 25 clusters from A divided into the same three (early, transition, late) pseudotime categories. Transcription factors and clusters are further divided on vertical axis into putative groupings based on transcription factor functions and GO term enrichment.

Figure 7-Figure supplement 1. Heatmap of genes changing with senescence pseudotime

Figure 7-Figure supplement 2. Heatmap of enriched gene sets for replicative senescence pseudotime clusters

Figure 8: YAP regulation of myofibroblast markers and YAP1/TEAD1 targets during replicative senescence. (A) RNA-seq and proteomics heatmaps of selected genes based on myofibroblast markers and biology. Values plotted are log2 fold change of each PDL vs. PDL 20 for replicative senescence timecourse and vs. no treatment for late PDL (PDL 40) WI-38 cells treated with the YAP inhibitor verteporfin. Proteomics values are median values for n=3 replicates. Values for individual RNA-seq replicates (n=3) are shown). **(B)** Diagram of collagen processing (left) with metabolites in blue and proteins in green. Collagen specific amino acid derivatives are outlined in red. Heatmap of log2 fold changes for metabolites and proteins involved in collagen processing (right). Proteomics values are median values for n=3 replicates. Metabolomics values are hTERT batch corrected median values for n=4 replicates). **(C)** Heatmaps of YAP1 targets collected by *Kurppa et al. (2020)*. for proteomics and RNA-seq during replicative senescence and in late PDL (PDL 40) WI-38 cells treated with the YAP inhibitor verteporfin. Values plotted are same as in A. **(D)** Heatmap of log2 fold changes relative to PDL 20 or untreated control of leading edge genes from gene set enrichment analysis driving the hallmark EMT signature in verteporfin treated WI-38 cells and during replicative senescence. Values for individual RNA-seq replicates (n=3) are shown.

Figure 8-Figure supplement 1. Effect of inhibiting the YAP1/TEAD1 interaction with verteporfin treatment on WI-38 cells

replicative senescence ~ fibroblast to myofibroblast transtion (FMT)?

*early changes during RS

Figure 9: Replicative senescence fibroblast to myofibroblast transition (FMT) model. We divided Replicative senescence progression into three major categories and summarized our results across all data modalities focusing on features in common with myofibroblasts.

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				BATCH A						BATCH B	
	PDL20	PDL25	PDL29	PDL33	PDL37	PDL20	PDL25	PDL45	PDL52		
bulk RNA seq	X	X	X	X	X	X	X	X	X		
scRNA seq		X	X	X	X	X	X				
proteomics	X	X	X	X	X	X	X				
metabolomics		X	X	X	X	X	X				
ATAC	X	X	X	X	X	X	X				
	TP1	TP2	TP3	TP4	TP5	TP6	TP7				
bulk RNA seq	X	X		X	X	X	X				
scRNA seq		X					X				
proteomics	X	X		X	X	X	X				
metabolomics		X		X	X	X	X				
ATAC	X	X		X	X	X	X				

Figure 1–Figure supplement 1. Sample manifest for WT and hTERT WI-38 cells.

Figure 1-Figure supplement 2. Expression of canonical and novel senescence induced genes **(A)** RNA-seq quantification for *p21*, *p16*, *NNMT*, *TGFB2*, and *GLB1* (beta-galactosidase) transcripts expressed as fold change relative to reference sample: PDL 20 for WT replicative senescence and PDL.ctrl 20 for hTERT. Bar height represents replicate average (n=3) and each point is an individual sample. **(B)** Proteomics quantification for same genes as in A. Estimates of relative protein abundance are provided as proportions of one biological replicate (n=3) relative to the total abundance observed across all other replicates in the experiment. This is obtained across multiple plexes with a Bayesian modeling of the intensities as log2 ratios to a bridge channel (blue sample far left). Protein level estimates from these models are then transformed with the inverse additive log-ratio transformation, resulting in a posterior distribution in proportion space.

Figure 1-Figure supplement 3. Senescent WI-38 cells express classic senescence markers. **(A)** Quantification of *p16* and *p14* gene products from the *CDKN1A* locus. Values plotted are fold change levels relative to PDL 20. TPM values generated by Salmon using the Gencode v29 annotation set that includes *p16* and *p14* in one locus (blue). A manual summation of the *p16* only transcripts in the locus are shown (orange). Error bars represent the standard deviation of three samples. **(B)** Representative images for senescence-associated beta-galactosidase assay. **(C)** Western blot for p16 at three PDLs (26, 44 and >50). Far left lane is p16 positive control derived from HEK293 cells. **(D)** Western blot for p21 at three PDLs (26, 44 and >50). Far left lane is p21 negative control derived from HEK293 cells.

Figure 1-Figure supplement 4. Enrichment and induction of Epithelial to Mesenchymal Transition gene set during approach to replicative senescence. **(A)** GSEA was run using MSigDB Hallmark gene sets. Data used for ranked list was the $\log_2$ fold changes at each time point vs. first the initial time point for each condition as indicated in B (i.e. PDL 20 for replicative senescence). Color indicates $-\log_{10}$ Benjamini-Hochberg p-value and direction of change e.g. red=enriched in up-regulated genes, blue=enriched in down-regulated genes. Epithelial to Mesenchymal Transition is highlighted in red.**(B)** Heatmap of 150 leading edge genes driving GSEA enrichment of EMT annotation compiled from all timepoints. Values are the $\log_2$ fold changes at each time point vs. first the initial time point for each condition as indicated. Values are individual (n=3) replicate samples.

Figure 1-Figure supplement 5. Pilot WI-38 seeding and sampling experiment. A small pilot experiment was used to determine optimal seeding and sampling cell densities in WI-38 cells at 3 different population doublings (PDL). We performed RNA-seq on each PDL 4 days after seeding in triplicate and looked for significant genes and gene set annotations at each PDL. In addition we performed an increasing cell density time course to choose appropriate sampling window and control for cell density effects. **(A)** Heatmap of three highly enriched or under-enriched GO term gene sets. Values are row centered log₂ transcripts per million (TPM). Each column is a sample (n=3 replicates). Red indicates higher than median gene expression, blue is lower than median gene expression across each row. **(B)** Results of cell density experiment on PDL23 cells (Figure 1A). y-axis left: Log₂ fold change (vs. 1 day after plating) plotted against x-axis: day(s) after plating for markers of stress (*VEGFA*-cyan) and markers of mitosis (*CCNB1*-red). y-axis right and dotted line: number of cells present at specified day expressed as percent of maximum collected on day 10. Original sampling window shown in purple interval. Modified sampling window chosen to attenuate reductions in cell cycle arising from cell density shown in green interval.

Figure 2-Figure supplement 1. Using a modified resuspension buffer of DMEM+FBS increases detection of senescent cells with the 10x Genomics 3' single cell RNA-seq protocol. **(A)** Barplot comparing the number of cells detected (y-axis) as a function of the age of cells (x-axis). **(B)** Barplot representing the number of cells detected (y-axis) with various cell resuspension buffers. (y-axis).

Figure 2–Figure supplement 2. Replicative senescence dependent gene expression changes measured by Bulk RNA-seq and single cell RNA-seq are highly concordant. Scatterplot comparing the log2 fold changes of PDL 50 vs. PDL 25 measured using single cell pseudo-bulk count summations (x-axis) and actual bulk RNA-seq (y-axis). The color for each gene is the log10 normalized counts. The r for all genes is 0.77. The r for genes filtered at > 25th percentile is 0.81 (red line) and 0.45 for genes < 25th percentile (blue line) .

Figure 2-Figure supplement 3. Single cell UMAPs of high mitotic scoring (gene set) WI-38 cells. S phase and G2M scored cells were separated and reprocessed and visualized with a UMAP projection. In mitotic cells, PDL is the primary source of variance across all PDLs.

Figure 2-Figure supplement 4. WT and hTERT WI-38 cells scored for senescence and cell density signatures. **(A)** UMAP projection of WT and hTERT single cell RNA-seq scored with a signature composed of significantly increasing genes with replicative senescence that are not significant in cell density experiments derived from bulk RNA-seq. Yellow represents cells scoring high, while black depicts cells scoring low for the senescence score. **(B)** As in A but scored for unique cell density signature. **(C)** Violin plot of cells scored as in A. Y-axis is the score assigned to each cell (point). Each PDL and hTERT PDL.ctrl are on x-axis. Senescence score increases with PDL. **(D)** Violin plot of cells scored as in B for cell density. Y-axis is the score assigned to each cell (point). Each PDL and hTERT PDL.ctrl on x-axis. Cell density remains flat with increasing PDL.

Figure 2-Figure supplement 5. Representative images of WI-38 cells at collection time points.

Figure 3-Figure supplement 1. Heatmap of hierarchical clustering of 8000 protein log2 fold changes at each time point/PDL versus first (not shown) for RS WT WI-38 (left) and hTERT WI-38 cells (right) from high induction (orange) to depletion (green).

RNA vs. protein

Figure 3–Figure supplement 2. Scatterplot comparing the log2 fold change expression of genes (x-axis) vs. protein (y-axis) in PDL 50 cells relative to PDL 20 cells. Oxidative phosphorylation leading edge genes from GSEA plotted in red. Point density colored from high (white) to low (green).

HALLMARKS OXIDATIVE PHOSPHORYLATION (proteomics)

Figure 3–Figure supplement 3. Breakdown of the hallmark oxidative phosphorylation gene set into functional subsets reveals up regulation of most mitochondrial functions during replicative senescence. Heatmaps of log2 fold change proteomics data for the MSigDB hallmark oxidative phosphorylation annotations set broken into constituent functional groups. Key in lower right with x-axis labels and color scale boundaries for all heatmaps.

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Figure 3–Figure supplement 5. Kennedy Pathway diagram. Metabolites in blue, proteins in green. Heatmaps of log2 fold changes for metabolites and proteins from A. Median hTERT corrected values for n=4 replicates are shown. Significant changes (FDR adjusted p<0.05 metabolomics, FDR adjusted p-value<0.01 proteomics) during replicative senescence are denoted with asterisks.

Figure 5-Figure supplement 1. ATAC-seq library fragment distribution and size selection.

Figure 5-Figure supplement 2. ATAC-seq library fragment distribution after size selection and sequencing

A

B

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Figure 5–Figure supplement 3. ATAC-seq mitochondrial read percentages and ATAC-seq transcriptional start site enrichment. **(A)** Mitochondrial read percentages in sequenced and aligned ATAC-seq library for WT PDLs (red) hTERT time points (green) in triplicate. **(B)** Transcriptional start site enrichment in sequenced and aligned ATAC-seq libraries for WT PDLs (red) hTERT time points (green) in triplicate.

Figure 5—Figure supplement 4. ATAC-seq QC metrics, controls, and NADs/LADs browser shot. **(A)** Fraction of reads in peak by PDL (WT) and PDL.ctrl (hTERT). p values for linear fit over time are shown. WT cells exhibit a slight ~5% but significant reduction of fraction of reads in peaks. **(B)** PDL 46 read distribution across chromatin states +/- PMA. **(C)** Chromatin state overlap Z-scores for genes annotations and NADs and gene annotation and LADs. **(D)** Heatmap of median log2 fold change of ATAC-seq peak accessibility binned by genomic annotations (TxDb.Hsapiens.UCSC.hg38.knownGene) for each sample (column, n=2 or 3 replicates). Reference samples are PDL 20 or PDL 20.ctrl for WT and hTERT WI-38 cells respectively (not shown). **(E)** Chromosome level distribution of all ATAC-seq peaks (purple), ATAC-seq peaks increasing in accessibility located within the undefined/quiescent chromatin state annotation (pink), NADs (blue) and LADs (yellow).

Figure 5-Figure supplement 5. Chromatin state profiles of ATAC-seq peaks in nucleolar associated domains (NADs) vs. rest of the genome. Heatmap of all significant ATAC-seq peaks categorized by 25 chromatin states and then divided into peaks overlapping with NADs (right) and peaks excluded from NADs (left) for hTERT and WT replicative senescence time course. Normalized accessibility counts for peaks were aggregated by chromatin state and NAD overlap group. Values shown are log2 fold change of median accessibility for each time point and grouping versus the reference sample. PDL.ctrl 20 for hTERT and PDL 20 for WT replicative senescence time course. n=2 or 3 replicates.

Figure 6–Figure supplement 1. Gene expression fold changes for the transcription factor FOXE1 across conditions. Gene expression fold changes (3 replicate average, each point a replicate) for the transcription factor FOXE1 in the hTERT and replicative senescence (RS) time courses.

Figure 7-Figure supplement 1. Heatmap of gene expression changes for the top 5,000 significantly changing genes during replicative senescence pseudotime. Genes were hierarchically clustered, gene normalized and scaled (0-1) expression levels are depicted (white = low expression, red = high expression).

Figure 7–Figure supplement 2. Heatmap of enriched gene sets for replicative senescence pseudotime clusters (k=25). Heatmap of adjusted p-values for GO-term enrichment analysis on individual clusters from 6. Values plotted are row normalized $-\log_{10}$ p-value; light purple to dark purple. The ordered pseudotime clusters are shown on the x-axis while hierarchically clustered gene sets are shown on the y-axis. Hierarchical clustering of gene set enrichment was used to divide gene sets into 10 major clusters. GO-term names were used to create word clouds reflective of the types and functions of enriched gene sets.

A Expression of YAP1 target genes relative to non-treated control

B GSEA of VP treated samples vs. control

C Gene signature of VP treated cells and RS cells

Figure 8-Figure supplement 1. Effect of inhibiting the YAP1/TEAD1 interaction with verteporfin treatment on WI-38 cells. **(A)** Barplots of the gene expression of 3 known YAP1 gene targets in verteporfin treated cells. Values plotted are fold changes relative to the mean of the control samples. Each condition was sampled at triplicate. Error bars indicate the standard deviation from the mean. **(B)** Barplot representing significant normalized enrichment score for each enrichment term from the MSigDB Hallmarks annotation. FDR adjusted p-values < 0.01. **(C)** Heatmap comparing gene expression changes in replicative senescence and verteporfin treated WI-38 cells. Values plotted are log2 fold change relative to the first time point in each experiment.