

1 Revisiting the Hayflick Limit: 2 Insights from an Integrated Analysis 3 of Changing Transcripts, Proteins, 4 Metabolites and Chromatin

5 Michelle Chan¹, Han Yuan^{1†}, Ilya Soifer^{1†}, Tobias M. Maile¹, Rebecca Y. Wang¹,
6 Andrea Ireland¹, Jonathon O'Brien¹, Jérôme Goudeau¹, Leanne Chan¹, Twaritha
7 Vijay¹, Adam Freund¹, Cynthia Kenyon¹, Bryson Bennett¹, Fiona McAllister¹,
8 David R. Kelley¹, Margaret Roy¹, Robert L. Cohen¹, Arthur D. Levinson¹, David
9 Botstein^{1‡}, David G. Hendrickson^{1‡}

*For correspondence:

botstein@calicolabs.com (FS);
dgh@calicolabs.com (FS)

10 ¹ Calico Life Sciences, LLC

11 †These authors contributed
equally to this work

‡These are corresponding authors.

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

24 Introduction

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

40 Beyond growth arrest and telomere shortening, phenotypic changes exhibited in replicatively
41 senescent cells include the senescence associated secretory phenotype (SASP). Proteins of the
42 SASP include proinflammatory cytokines, growth factors, angiogenic factors, and proteases. The
43 SASP has been shown to play a role in paracrine signaling whereby senescent cells can promote
44 local wound healing and/or drive healthy neighboring cells into senescence (*Acosta et al., 2013*;
45 *Coppé et al., 2006*; *Demaria et al., 2014*; *Coppé et al., 2008*). Replicatively senescent cells also accu-
46 mulate DNA and protein damage, accumulate lipids, and lose regulatory control of mitochondria
47 and lysosomes (*Gorgoulis et al., 2019*).

48 The phenotypic similarity (telomere attrition, epigenetic alterations, mitochondrial dysfunction
49 and loss of proteostasis) between the cell autonomous aging hallmarks and *in vitro* senescence has
50 led to the hypothesis that senescent cells *in vivo* play a causal role in organismal aging and aging-
51 related diseases (*Hernandez-Segura et al., 2018*; *Campisi and Di Fagagna, 2007*; *Sedelnikova et al.,*
52 *2004*; *Jeyapalan and Sedivy, 2008*; *Herbig et al., 2006*; *Childs et al., 2015*; *López-Otín et al., 2013b*).

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

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

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

83 Results

84 Transcriptomic profiles of replicative senescence (RS), radiation induced senescence 85 and increasing cellular density.

86 To capture the replicative senescence process with high resolution we designed an experiment to
87 continuously grow and intermittently sample cells from a starter batch of WI-38 cells that had un-
88 dergone only 20 population doublings (PDL 20) (Supplemental methods). To distinguish between

89 replicative senescence dependent changes and those arising from altered cell density and growth
90 rate, we performed a cell density control study (Supplemental methods). Briefly, early PDL cells
91 were sampled at increasing levels of cell density to measure gene expression changes associated
92 with cellular density and decreasing cell proliferation independent of replicative senescence (Fig-
93 ure 1A). In addition to the cell density control, we also included TERT immortalized WI-38 cells
94 (hTERT) grown in parallel and sampled alongside WT cells as a control for long term culturing and
95 day-to-day sampling batch effects (*Bodnar et al., 1998*). The hTERT cell line was generated in ad-
96 vance of the experiment. Frozen aliquots of the hTERT line were thawed alongside and sampled
97 in parallel with WT WI-38 cells. As expected, hTERT immortalized cells grew at a constant rate and
98 did not slow or cease growth (Figure 1B).

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

104 We first examined the bulk RNA-seq data to compare and contrast replicative senescence with
105 radiation induced senescence, cell density, and hTERT cells (quality control metrics located in Figure
106 1 source data 1). Differential gene expression analysis using RNA-seq revealed 6,955 genes change
107 with increasing PDL, 7,065 genes change in response to ionizing radiation, and 9,958 genes vary
108 with increasing cell density (FDR adjusted p-value < 0.01, Figure 1 source data 2). Notably, the tran-
109 scriptional changes we observed were consistent with previous studies. Figure 1D shows the strong
110 correlation of the pattern of induced transcription established in our PDL 50 cultures and the fibrob-
111 last derived senescence-associated signature compiled across multiple fibroblast senescence gene
112 expression experiments from cell lines derived from different tissue depots (*Hernandez-Segura
113 et al., 2017*). We also observed induction of senescence-associated- β galactosidase activity (Figure
114 1C) as well as p16 and p21 in both our RNA-seq, proteomics data, and via western blot (Figure 1
115 supplement 2, Figure 1 supplement 3). These data reveal that the cells in our time course display
116 classic features of senescent cells and exhibit transcriptomic changes that are highly correlated
117 with previous studies of replicative senescence. Together, these data strongly suggest that our
118 WI-38 cells successfully reached replicative senescence.

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

125 To facilitate a biological interpretation of these shifting transcriptomic landscapes, we applied
126 Gene Set Enrichment Analysis (GSEA) using the MSigDB Hallmark annotation sets to learn which
127 general processes are shared and distinct between replicative senescence, radiation induced senes-
128 cence, and cell density (Figure 1F, Figure 1 source data 3) (*Liberzon et al., 2015; Subramanian et al.,
129 2005; Mootha et al., 2003*). All three perturbations (and not the immortalized cells), exhibited dra-
130 matic reductions in expression of genes belonging to S, G2M, and M cell cycle phases, consistent
131 with cessation of cell division (Figure 1F - top cluster). Perhaps driven by this shift, we also found
132 significant overlap in additional enriched annotations among genes induced in replicative senes-
133 cence, radiation induced senescence and cell density, albeit with some variation in significance.
134 Many of the shared enriched annotations can be categorized as stress responses, e.g. apoptosis,
135 p53 pathway, inflammatory and interferon responses, and STAT3/5 signaling. In addition, we also
136 observed enrichment of several development/differentiation gene sets, including myogenesis,
137 angiogenesis, and adipogenesis.

138 We observed relatively few gene sets with discordant patterns across the replicative senes-
139 cence, radiation induced senescence, and cell density experiments (Figure 1E). However, the gene

140 set for Epithelial to Mesenchymal Transition (EMT) was significantly enriched in genes that increased
141 with replicative senescence as opposed to radiation induced senescence (Benjamini-Hochberg cor-
142 rected $p=5.5e-6$ vs. Benjamini-Hochberg corrected $p=0.12$) or cell density wherein the term was ac-
143 tually underrepresented, but not significantly so (Benjamini-Hochberg corrected $p=0.4$). Although
144 replicative senescence, radiation induced senescence and cell density are highly similar at the ab-
145 stracted level of enriched gene sets, it is clear in Figure 1E that there are many induced genes
146 specific to replicative senescence. In addition, we applied GSEA to each individual time point for
147 each condition (Figure 1 supplement 4, Figure 1 source data 4) and found the EMT gene set is
148 enriched early and robustly during replicative senescence. Thus the EMT gene set appears to rep-
149 resent a particularly important aspect of replicative senescence biology.

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

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

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

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

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

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

189 It has previously been reported and observed here (Figure 2 supplement 5) that cell size in-

190 creases with PDL in primary cell lines (*Ogrodnik et al., 2019*). We hypothesized that perhaps the
191 higher PDL cells by nature of their larger size, might reach contact inhibition faster than early PDL
192 cells. In this scenario, given the high correlation between the gene expression signatures of senes-
193 cence and cell density, the increase in senescence signature could be an artifact of cell culture
194 arising from changing morphology. To test this possibility, we scored our single cells again with
195 modified signatures for senescence and cell density composed only of genes uniquely induced in
196 either perturbation (Figure 2 supplement 4). We found that the specific senescent signature in-
197 creases with PDL in WT WI-38 cells in contrast with the specific cell density signature which stays
198 relatively flat. These data provide more evidence supporting a gradual increase in the senescent
199 gene expression program that is independent from changes in cell density.

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

212 **Proteomic landscape of replicative senescence WI-38 cells suggests large metabolic** 213 **alterations**

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

223 Multiple enriched sets pointed to replicative senescence dependent shifts in cellular energy uti-
224 lization (Figure 3A). Of note, we found that although under-enriched in our replicative senescence
225 transcriptome (Figure 1F), The oxidative phosphorylation hallmark was highly enriched in the pro-
226 teomics data. We plotted the oxidative phosphorylation genes driving the set enrichment in Figure
227 3 supplement 2. This projection revealed that many genes in this set fall into a quadrant (positive
228 proteomic, negative transcriptomic) suggesting discordant regulation of these genes.

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

236 Next, we examined the annotations for the highest enriched hallmark sets from Figure 3A and
237 found strong enrichment of multiple KEGG metabolic pathways. (Figure 3B). In addition, both the
238 glucose transporter SLC2A1/GLUT1 and the fatty acid scavenger CD36 transcripts exhibited strong,
239 early, and replicative senescence specific up-regulation (Figure 3C). Together these results point to

240 a drastically altered replicative senescence metabolic landscape.

241 **RS WI-38 cells exhibit increased utilization of fatty acid metabolism and glycolytic** 242 **shunts**

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

252 Following the glucose, we looked at the central stem of glycolysis as a potential source of energy
253 supplying the observed increases in the TCA cycle and oxidative phosphorylation (Figure 3D,E). We
254 found that metabolites and enzymes dedicated to pyruvate generation and/or lactate production
255 did not change concordantly. We instead found that most of the glycolytic shunts exhibited up-
256 regulation at both the metabolite and protein level (Figure 3E). These results suggest an increase
257 in allocation of glucose for manufacture of various biomolecules and their precursors (glycogen,
258 hexosamines, phospholipids) (Figure 3E).

259 In contrast, metabolites and enzymes involved in fatty acid import and oxidation for the pur-
260 pose of energy generation appear strongly up-regulated (Figure 3E). We also saw that metabo-
261 lites involved in the *de novo* production of phospholipids via the Kennedy pathway are highly up-
262 regulated, specifically phosphotidylethanolamines precursors (Figure 3 supplement 5).

263 From these data, it is clear that replicative senescence WI-38 cells undergo drastic shifts in
264 metabolism. Specifically, we see increased glucose utilization in glycolytic shunts coupled with
265 an increase in fatty acid import and oxidation. It is possible that replicative senescence cells are
266 switching to fatty acid oxidation to fuel increased TCA cycling and oxidative phosphorylation as
267 glucose is diverted to macromolecule production. Indeed, metabolomic data collected from vari-
268 ous types of senescence models has shown increased fatty acid oxidation, lipid accumulation, TCA
269 up-regulation, and glycolytic alterations (*Zwerschke et al., 2003; Ogrodnik et al., 2017; Flor et al.,*
270 *2017; Unterluggauer et al., 2008; Johmura et al., 2021*). Likewise, consensus metabolomic findings
271 in EMT models report increased TCA cycle products, altered lipid metabolism, and activated hex-
272 osamine pathway (*Hua et al., 2020; Corbet et al., 2020; Lucena et al., 2016*). These data provide
273 functional metabolic evidence supporting a connection between replicative senescence and the
274 EMT hallmark enrichment observed in RNA and protein expression.

275 **Nicotinamide N-methyltransferase (NNMT) links nicotinamide adenine dinucleotide** 276 **(NAD) and methionine metabolism as a putative heterochromatin regulator**

277 Metabolic regulation of epigenetic state is an increasingly recognized mechanism through which
278 nutrient availability influences cellular function by regulating the abundance of co-factors required
279 for histone modifications (*Lu and Thompson, 2012*). Specifically, Nicotinamide adenine dinucleotide
280 (NAD⁺) and methionine metabolism power the deacetylation and methylation required for main-
281 taining repressive DNA conformations (*Lu and Thompson, 2012*). In a compelling intersection, per-
282 turbation of NAD, methionine, and heterochromatin levels have all previously been reported in
283 multiple aging contexts including replicative senescence (*James et al., 2016; López-Otín et al., 2016;*
284 *Kozieł et al., 2014; Yousefzadeh et al., 2020; Benayoun et al., 2015*). In compiling our replicative
285 senescence data for the metabolic and proteomic components of the NAD and methionine path-
286 ways, we found that one of the largest and earliest changes for any protein (or transcript) was the
287 increased expression of Nicotinamide N-methyltransferase (NNMT) (Figure 4A,B).

288 DNA and histone methylation require abundant levels of the universal methyl donor S-adenosyl
289 methionine (SAM). NNMT not only depletes SAM by catalyzing the removal of the SAM methyl
290 group, it does so by fusing it to the NAD⁺ precursor nicotinamide (NAM) resulting in the production
291 of methyl nicotinamide (MNA) (*Ulanovskaya et al., 2013*). Thus, NNMT effectively acts as a sink for
292 the two primary metabolites a cell requires for silencing chromatin and regulating gene expression
293 (*Komatsu et al., 2018*). We found that MNA levels mirror those of NNMT; increasing early and ro-
294 bustly during replicative senescence progression consistent with high NNMT activity. SAM and its
295 sans-methyl version (S-adenosyl homocysteine (SAH)) were depleted with replicative senescence
296 albeit to a lesser extent than the observed increase in MNA. NAMPT and NMNAT1, enzymes in the
297 NAD salvage pathway, were weakly down-regulated at the protein level (Figure 4A,B).

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

305 **Increased DNA accessibility and transcription from nucleolar and lamin associated** 306 **domains is a dominant feature of the replicative senescence epigenome**

307 To study genome-wide changes in the epigenetic landscape of senescent cells, we collected ATAC-
308 seq data during the replicative senescence time course. We size selected our ATAC-seq libraries for
309 smaller fragments (< 300 base pairs) to enrich for nucleosome free regions (Figure 5 supplement 1
310 and Figure 5 supplement 2). Samples from all PDLs and hTERT PDL controls exhibited similar ATAC-
311 seq fragment size distribution emblematic of nucleosomal patterning, high alignment rates, very
312 low mitochondrial read percentages, and strong enrichment around transcriptional start sites (Fig-
313 ure 5 supplement 1, Figure 5 supplement 3, Figure 1 source data 1). These QC metrics are indicative
314 of high quality ATAC-seq libraries at all sampling time points and PDLs. We then quantified the read
315 distribution across chromatin states previously annotated for another human fetal lung fibroblast
316 cell line (IMR-90, Figure 5 source data 1) (*Ernst and Kellis, 2015*). We categorized 25 distinct states
317 into 4 broad categories: promoters, enhancers, transcription, and miscellaneous, the last of which
318 is composed largely of heterochromatic and undefined states, e.g. H3K9me3 and H3K27me3 rich
319 regions, zinc-finger repeats, and quiescent (defined by the absence of histone marks, accessibility,
320 or gene features) which we refer to as "undefined" (*Ernst and Kellis, 2015*).

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

328 To determine if this shift in accessibility to undefined and heterochromatic states was indicative
329 of increasing noise versus coherent changes in accessibility, we used the ATAC-seq reads to call
330 peaks of localized accessibility. From the WT and hTERT ATAC-seq data from all time points we
331 identified 363,470 ATAC-seq peaks (*Zhang et al., 2008*) (Figure 5 source data 2 and 3). We found
332 a slight ~5% but significant (p=0.014) linear PDL-dependent decrease in the fraction of reads in
333 peaks during replicative senescence (Figure 5 supplement 4A, Figure 5 source data 4). We tested
334 experimentally whether the increased heterochromatic accessibility was an artifact of increasing
335 numbers of dead cells in later PDL samples by repeating the experiment using late (PDL 45) cells
336 with a cross-linking agent (propidium monoazide-PMA) that renders DNA from dead cells inert as
337 previously described (*Hendrickson et al., 2018*). No significant change in read distribution across

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

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

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

365 Digging deeper into NADs, we next asked if chromatin states within NADs might respond dif-
366 ferently than the rest of the genome to increasing PDL given the large PDL-dependent increase
367 in accessibility we observed in Figure 5D. We repeated the analysis shown in Figure 5B with two
368 subsets; peaks excluded from NADs or peaks overlapping NADs. (Figure 5 supplement 5). Divid-
369 ing these two sets of peaks into the 25 chromatin states revealed that as expected, NAD excluded
370 peaks mirror the pattern observed for significantly changing peaks across all chromatin states. Con-
371 versely peaks overlapping NADs were found to increase in accessibility in a variety of chromatin
372 states (e.g. poised promoters and many classes of enhancers) that exhibit reduced accessibility
373 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).

384 Together these results highlight a striking increase in accessibility within nucleolar associated
385 DNA that connects changes in the transcription with a global shift in the epigenome.

386 **Transcriptional regulators of the replicative senescence transcriptome and epigenome**

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

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

400 Out of the FOX family transcription factors, FOXE1 is unique in that it exhibits one of the most
401 specific (no change in hTERT, cell density or radiation) and largest increases in replicative senes-
402 cence gene expression (40-50x) (Figure 6 supplement 1). FOXE1 is annotated as a thyroid specific
403 transcription factor with putative roles in thyroid development and cancer (*Kallel et al., 2011*;
404 *Ding et al., 2019*). It has also been reported to regulate two signaling molecules up-regulated in
405 the replicative senescence time course; TGF- β 3 and WNT5A *Venza et al. (2011)*; *Pereira et al. (2015)*.
406 Furthermore, it has been reported that many of the FOX family transcription factors are relatively
407 promiscuous binders of each others canonical motifs. Thus it is possible that the increased ac-
408 cessibility in peaks with FOX motifs during replicative senescence may be driven by FOXE1 activity
409 *Rogers et al. (2019)*. Despite the specificity and magnitude of replicative senescence induction,
410 FOXE1's function in replicative senescence is unclear and warrants further investigation.

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

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

432 Lastly, given that YAP1/TEAD1 activity appears to increase during replicative senescence against
433 the backdrop of an altered epigenomic context, we tested for an interaction between TEAD1 motifs
434 and the increasingly accessible NADs and LADs. We found that not only are TEAD1 sites significantly
435 enriched within NADs and LADs ($p < 7.17e-07$, and $p < 6.12e-03$ respectively hypergeometric), but

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

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

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

457 **scRNA-seq trajectory analysis resolves WI-38 cells' approach to replicative senes-** 458 **cence**

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

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

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

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

486 comb group complex). Also in the transition region of pseudotime, the earliest enrichments appear
487 for transcription factors related to EMT (SNAI2), TGF- β signaling (SMAD3), YAP1/TEAD1 activity, and
488 the AP1 complex (JUN, FOS, FOSL2). We observed continued enrichment for these transcription
489 factors and processes throughout the rest of the transition phase and into late pseudotime.

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

499 **Replicatively senescent WI-38 fibroblasts express canonical myofibroblast markers** 500 **and metabolic features**

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

510 Previous work has demonstrated that there exists mechanistic and functional association be-
511 tween telomerase inhibition, senescence, and myofibroblasts. Senescence is an integral part of
512 the wound healing processes; upon injury resolution, activation of a senescence-like phenotype
513 prevents unchecked collagen secretion and fibrosis by preventing myofibroblast proliferation and
514 earmarking them for subsequent immune clearance (*Demaria et al., 2014; Krizhanovsky et al.,*
515 *2008; Jun and Lau, 2010; Mellone et al., 2016; Razdan et al., 2018; Liu et al., 2002, 2006*).

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

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

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

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

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

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

557 **Expression of YAP1/TEAD1 targets during RS**

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

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

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

575 **Inhibiting the YAP1/TEAD1 interaction in WI-38 cells suppresses expression of YAP1/TEAD1 576 targets and the replicative senescence gene signature**

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

582 Recently *Mascharak et al. (2021)* showed verteporfin treatment of wounds in mice alleviated the
583 fibrotic state during wound healing, reverted the profibrotic transcriptional program, and reduced
584 the myofibroblast population. Verteporfin is also known to be an inhibitor to the formation of the

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

598 We applied GSEA analysis using the MSigDB Hallmark annotation to understand what pathways
599 are affected by verteporfin treatment of cells using the RNA-seq data from the verteporfin treated
600 samples relative to non-treated control. Consistent with our hypothesis that YAP1/TEAD1 plays a
601 role in regulating the FMT-like transition of cells undergoing replicative senescence, treatment of
602 late passage cells with verteporfin resulted in negative enrichment of pathways relevant to FMT
603 and TGF- β signaling (Figure 8 supplement 1B). We also took genes driving the Hallmarks EMT
604 annotation enrichment in replicative senescence cells to see if inhibition of YAP1/TEAD1 complex
605 formation aids in reducing EMT pathway gene expression. Compared to the no treatment control,
606 the majority of genes induced during replicative senescence that drive the GSEA Hallmarks EMT
607 enrichment have strikingly decreased in expression after 2 hrs of treatment. These data suggest
608 that the use of verteporfin to inhibit the YAP1/TEAD1 interaction can impede the FMT-like transition
609 observed in replicative senescent cells (Figure 8 supplement 1D).

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

616 Discussion

617 The study of replicative senescence in human tissue culture has proven to be an informative model
618 for learning how genetic and environmental factors impact cellular senescence. However, the field
619 has not fully taken advantage of the 'omics' revolution. Rekindled by the advent of senolytics, inter-
620 est in the molecular underpinnings of senescence has burgeoned in recent years as researchers
621 seek to design therapeutic strategies for ablating senescent cells (*Amor et al., 2020; Elmore et al.,*
622 *2018; Wagner and Gil, 2020; Aghajanian et al., 2019*). However, many such studies span only one
623 or two systematic data modalities. Here, to fully leverage the power of recent advances in high-
624 dimensional profiling, we revisit the original Hayflick limit in WI-38 lung fibroblasts cells with a
625 battery of assays including RNA-seq, ATAC-seq, scRNA-seq, proteomics, and metabolomics in an
626 effort to capture the defining features of replicative senescence at every step of the central dogma
627 and beyond. Our results are summarized graphically in Figure 9.

628 One important feature of our study is the number and type of control conditions. From our
629 pilot studies, it was clear that studying replicative senescence in isolation would preclude our ability
630 to know what features of change were specific to replicative senescence versus high cell density
631 and/or DNA damage induced growth arrest. Thus, we included cellular density as an alternate
632 method for arresting growth and exposure to ionizing radiation as an alternate methodology for
633 senescence induction and DNA damage response. Lastly, all of our modalities were paired with
634 samples collected from an hTERT immortalized cell line grown in parallel. Importantly, very little

635 changed across all modalities in the hTERT cell line consistent with the changes we highlight in 9
636 as specific to replicative senescence.

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

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

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

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

684 Further metabolic changes (hexoamine/collagen synthesis and fatty acid oxidation) then sup-
685 port the new pro-fibrotic state. Finally, end point senescent cells reinforce the senescent state

686 and contribute to neighboring cell progression towards senescence via secretion of inflammatory
687 factors and SASP.

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

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

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

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

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

725 In general, the FMT hypothesis provides a conceptual framework and integrative model for
726 linking the multi-modal senescent phenotypes we observed to multiple human age-related dis-
727 eases. Given the observation that fibrosis and senescence markers correlate with increasing age
728 in multiple tissues, it is possible that FMT might be a widespread phenomenon underlying many
729 age-related pathologies (*Yousefzadeh et al., 2020; Idda et al., 2020*). Future work harnessing multi-
730 modal single cell technology coupled with relevant *in vivo* models will aid greatly in determining
731 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		
Transfected construct	pCDH-CMV-hTERT-EF1a-puro	This paper	N/A	Lentiviral plasmid
Commercial assay	Senescence <i>beta</i> -Galactosidase	Cell Signaling Tech.	9860	
Commercial assay	MycoAlert Mycoplasma Detection	Lonza	LT07-218	
Commercial assay	Direct-zol RNA Miniprep Plus	Zymo Research	R2072	
Commercial assay	Chromium Single Cell 3' v2	10x Genomics	120237	
Commercial assay	Chromium Single Cell A Chip	10x Genomics	1000009	
Commercial assay	Tagment DNA Enzyme and Buffer	illumina	20034197	
Commercial assay	Clean and Concentrator-5	Zymo Research	D4014	
Commercial assay	NEBNext® High-Fidelity 2X PCR	NEB	M0541L	
Commercial assay	TruSeq Stranded mRNA Library	illumina	20020595	
Commercial assay	Bioanalyzer High Sensitivity DNA	Agilent	5067-4626	
Commercial assay	Pierce BCA Protein Assay	Thermo Fisher	23227	
Commercial antibody	Mouse anti-human p16 antibody	BD Biosciences	554079	
Commercial antibody	Mouse anti-human p21 antibody	BD Biosciences	556430	
Commercial assay	Pippin Prep 2% 100-600 bp	Sage Science	CDF2010	
Commercial Media	TrypLE Express Enzyme	Gibco	12604013	
Commercial Media	DMEM	Gibco	11885084	
Chemical compound	Verteporfin	RD Systems	1243926	
Other	Zorbax Extend C18 column	Aglient	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	
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		

732 **Methods and Materials**

733 **Cell Culture**

734 WI-38 maintenance and subculturing for replicative senescence time course-WT and hTERT
735 WI-38 cells were obtained from the Coriell Institute (AG06814-N) at PDL 15. The cells were grown in
736 Dulbecco's modified Eagle's medium (DMEM, Gibco, 11885084) supplemented with 10% dialyzed
737 fetal bovine serum (FBS, Sigma, F0392) and maintained in an incubator set to 37° C, 5% CO₂, and
738 20% O₂. 0.3-0.5 E6 WI-38 cells were seeded and maintained on 10 cm collagen coated plates (Corn-
739 ing, 354450) and split when cells reached about 70% confluence. For WI-38 cells PDL 15 to PDL 40,
740 it took about 4 days to reach 70% confluence. WI-38s >PDL 40 were slower growing, and therefore
741 split every 5-7 days instead; media was replenished every 3-4 days (SText 1). Cells were passaged
742 by washing the cell monolayer with PBS, followed by incubating cells with TrypLE Express (Gibco,
743 12604013) for 5 minutes. Media was added to neutralize the TrypLE and the cell suspension was
744 collected into conical tubes. The cell suspension was spun at 200 x g for 5 minutes and the super-
745 natant was aspirated. The resulting cell pellet was resuspended in media and cells were counted
746 with viability measurements on a ViCell XR Cell Analyzer (Beckman Coulter). Cell numbers taken
747 from the ViCell were used to calculate population doublings (PDLs). The following formula was
748 used to calculate PDLs:

$$749 \text{ PDL} = \log_2(\text{number of cells harvested}) - \log_2(\text{number of cells seeded})$$

750 All cells collected for assays were sampled in triplicate, at 2.5-3 days after seeding, targeting
751 60-70% confluence to avoid the confounding effects of confluence. Confluence levels were deter-
752 mined with a phase contrast EVOS microscope (ThermoFisher). Slower growing cells (PDL > 40)
753 were sampled at 3.5-4 days after seeding. Cells were tested every month for mycoplasma con-
754 tamination using the MycoAlert Mycoplasma Detection Kit (Lonza). A smaller subset of three PDL
755 timepoints (PDL 45, PDL 55, PDL 56) were generated in a secondary time course to ensure "deep"
756 senescence (2-4 months after cell cycle cessation) was maintained as previously reported (*De Cecco*
757 *et al., 2019*).

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

760 **WI-38 irradiation time course**

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

766 **WI-38 cell density time course**

767 0.3 E6 WI-38 cells at PDL 23 were seeded and grown on 10 cm collagen coated plates in DMEM
768 supplemented with 10% FBS. Cells were grown and sampled intermittently between 1 day and 10
769 days for transcriptome profiling. Samples at each time point were taken in triplicate. RNA was
770 extracted according to methods below.

771 **SA-βGAL staining**

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

774 **WI-38 hTERT cells and lentiviral transduction**

775 293T cells were transfected with a lentiviral target plasmid expressing hTERT (pCDH-CMV-hTERT-
776 EF1a-puro) and lentiviral packaging constructs overnight; 48 hours later, viral supernatant was
777 collected. WI-38 cells were transduced with viral supernatant in the presence of 5 μg/mL polybrene
778 and selected for 7 days with 1 μg/ml of puromycin.

779 **WI-38 + verteporfin treatment**

780 200,000 WI-38 cells at PDL 38 were seeded onto each well of a 6-well collagen coated plate and
781 grown with 2 ml of DMEM supplemented with 10% FBS. After 4 days of growth, cells were treated
782 with 5 or 10 μ M of verteporfin (RD Systems 5305/10) for 2 hours or DMSO. RNA was extracted after
783 2 hours of treatment according to methods described below.

784 **Bulk RNA-seq methods**

785 RNA collection and library preparation

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

795 Read processing and quantification

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

804 Differential expression analysis

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

810 Batch correction and hierarchical clustering

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

817 Gene set enrichment analysis

818 For gene set enrichment analysis, we downloaded the MSigDB Hallmark gene sets from misgDB
819 website (version 7, gene symbols) *Liberzon et al. (2015)*. For each time course (replicative senes-
820 cence, radiation induced senescence, and cell density) we we ranked all genes by the log₂ fold
821 change across time generated by DESeq2. Then GSEA was performed on the ranked gene set us-
822 ing the R package "fgsea" version 1.16.0 which is an implementation of GSEA in R (*Subramanian*
823 *et al., 2005; Korotkevich et al., 2019; Mootha et al., 2003*). By default, GSEA tests for enrichment
824 of each gene set in each condition in both directions. We report the -log₁₀ Benjamini-Hochberg

825 corrected p-value in Figure 1 and use the normalized enrichment score (NES) to assign direction of
826 the change.

827 **Single Cell 3' RNASeq Methods**

828 Cell collection

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

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

840 The raw single cell reads were demultiplexed by sample using bcl2fastq. Alignment, cell barcode
841 demultiplexing, transcript quantification and sample merging were carried out with CellRanger
842 3.0 using the hg38 CellRanger 3.0 gene annotation ("refdata-cellranger-GRCh38-3.0.0", cellRanger)
843 with default options and parameters. Filtered cell by gene matrices were normalized using Seurat
844 (version = 4.0.1.9005) and SCTransform (version = 0.3.2) (*Hafemeister and Satija, 2019; Stuart*
845 *et al., 2019*). Dimension reductionality was carried out with PCA (n = 50). Clusters were defined
846 using the louvain algorithm resolution= and cells were visualized with UMAP projection *McInnes*
847 *et al. (2018); Becht et al. (2019); Blondel et al. (2008)*. For cell cycle scoring and phase determination
848 as well as senescence scoring we applied the Seurat implementation of the scoring function as
849 previously described (*Nestorowa et al., 2016*).

850 DESeq2 was used for differential gene expression analysis to identify significantly changing
851 genes within individual clusters as a function of increasing PDL (PDL by mitotic phase) (Fig. 3D)
852 using a an input matrix of gene counts by cells per PDL by mitotic phase. Gene counts were sum-
853 marized across cells for each mitotic phase (PDL 50 removed). To expedite computation, we re-
854 stricted analysis to highest expressed 8,000 genes. For each PDL by cell cycle phase grouping, we
855 required that >15 representative cells must exist to be considered in analysis. To visualize differ-
856 entially expressed genes, we converted single cell counts to counts per million (+1 pseudocount)
857 and averaged across PDL by cell cycle phase and calculated the log₂ fold change at each PDL by
858 cell cycle phase against the earliest PDL for that cell cycle phase using a concatenated list of all
859 significantly changing genes generated by DESeq2 from each cell cycle phase.

860 Pseudotime analysis

861 For pseudotime analysis of the single cell data, we used the R package monocle3 (version=1.0.0)
862 which implements PCA, Leiden clustering, and UMAP prior to partitioning and trajectory analy-
863 sis. We first isolated WT cells by removing hTERT cells and those not belonging to the primary
864 grouping. To focus trajectory analysis on the replicative senescence progression, we used only
865 the top genes found to significantly change with replicative senescence in bulk RNA-seq for PCA
866 (padj < 0.01) and log₂ fold change > 3 or log₂ fold change < -3). In applying the Monocle trajec-
867 tory analysis: (learn_graph(cds,close_loop = FALSE,learn_graph_control = list(minimal_branch_len
868 = 35, geodesic_distance_ratio = .5, euclidean_distance_ratio=1), we designated cycling cells at the
869 opposite end of UMAP project from the senescent cells as the start point manually. (*Trapnell et al.,*
870 *2014; Qiu et al., 2017; Cao et al., 2019; Traag et al., 2019; Levine et al., 2015; McInnes et al., 2018*).
871 After establishing a trajectory, we then employed the Monocle3 "graph test" function to isolate
872 genes that significantly change as a function of pseudotime. Pseudotime estimation was output

873 from monocle3 using the learn graph function for building a trajectory. Smoothed pseudotime tra-
874 jectories use for Figure 7C were calculated for significantly changing genes (with pseudotime) by
875 binning cells across pseudotime into 60 bins and using a cubic spline to estimate expression at each
876 bin. For each gene, the smoothed trajectory was set from 0 (minimal) to (1) maximal expression.
877 Genes were organized with K-median clustering (k=25) using cosine similarity. For visualization the
878 median expression value for each pseudotime bin and cluster was calculated. Genes from each
879 cluster were fed into LISA TF analysis (below)

880 ATAC-SEQ methods

881 ATAC-seq Library Preparation and Sequencing

882 Freshly harvested cells were used for all reactions. Briefly, the cell monolayer was washed with PBS,
883 trypsinized with TrypLE Express (Gibco 12604013), resuspended in media, and cells were pelleted.
884 Cells were counted and 100,000 cells were used in each reaction. Cell lysis, DNA transposition, and
885 library construction was followed from the Omni-ATAC protocol (Corces et al. 2017). Libraries were
886 amplified for 13 total cycles. Sample purification and size selection were performed on the Pippin
887 high throughput size selection platform using 2% agarose cassettes to isolate fragments < 300
888 base pairs (Sage Science). Quality of ATAC-seq libraries were assessed with the Agilent Bioanalyzer
889 2100 with DNA high sensitivity chips (Agilent Technologies 5067-4626). Libraries were sequenced
890 on the HiSeq4000 with paired end sequencing using 2 x 150 bp reads (Illumina).

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

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

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

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

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

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

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

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

917 Limma-voom (Limma version = 3.46.0) was used for differential accessibility analysis (*Ritchie
918 et al., 2015*). Fold change and fdr adjusted p-values were estimated using moderated t-test statistic
919 based on the model: time + condition + time:condition. We performed separate tests for time point
920 1 versus each of the other time points.

921 ATAC-seq chromatin state and NAD/LAD analysis

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

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

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

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

943 **Metabolomics**

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

945 Twenty-four hours before metabolite extraction, the medium was aspirated, cells were washed
946 with unconditioned medium and then the medium was replaced. For metabolite extraction, cells
947 were washed once in 37°C warm PBS-buffer immediately followed by the addition of 3.5 mL of
948 freezer-cooled (-20°C) LC-MS grade 80:20 MeOH/H₂O (Sigma Aldrich). The plates were then held
949 at -20°C for 2 hours, then harvested with a sterile cell scraper while at -20°C and transferred to
950 -20°C cold 5 mL centrifuge tubes (Eppendorf Lo-Bind). After centrifuging the cell-extracts in a 4°C
951 centrifuge for 5 min at 2000 x g, the supernatants were transferred into new cold centrifuge tubes
952 and dried under nitrogen at 4°C. Dried extracts were stored at -20°C.

953 LC-MS/MS analysis of cell culture extracts

954 Dried supernatants were resuspended in 200 µL of water containing 1 µg/mL of deuterated lysine
955 and deuterated phenylalanine and 250 ng/mL of deuterated succinate (Sigma Aldrich) as internal
956 standards. For negative ion mode, the resuspended samples were diluted 1:4 in water, for positive
957 ion mode, they were diluted 1:4 in acetonitrile. Samples were then centrifuged at 18000 x g for
958 5 minutes, the supernatant was moved to HPLC vials and 5 µL was injected for analysis by LC-MS
959 on Vanquish UPLCs (Thermo Scientific) coupled to Q Exactive Plus mass spectrometers (Thermo
960 Scientific).

961 For analysis in negative ion mode, separation of compounds was achieved by reverse-phase
962 liquid chromatography on a Zorbax Extend C18 column (150 x 2.1 mm, 1.8 µm particle size (Ag-
963 ilent 759700-902). Mobile phase A was 10 mM Tributylamine and 15 mM Acetic Acid in 97:3 wa-
964 ter:methanol at pH 4.95 and mobile phase B was methanol. Prior to injection, the column was
965 equilibrated in 0% B for 4 minutes. The gradient eluted isocratically in 0% B for 2.5 minutes, in-
966 creased to 20% B over 2.5 min, held at 20% B for 2.5 min, increased to 55% B over 5.5 min, increased
967 to 95% B over 2.5 minutes, maintained at 95% B for 3 min, then decreased to 0% B over 0.5 min,
968 where it was held for 3 min, resulting in a total run time of 26 min. The LC separation was carried
969 out at 30°C column temperature using a 200 µL/min flow rate. For MS-analysis, parameters on

970 MS1 were set to 70,000 resolution with an AGC target of 1e6 at a maximum IT of 100 ms. The scan
971 range was 70 to 1050 m/z. MS2 parameters were set to 17,500 resolution at loop count 6, an AGC
972 target of 1e5 at a maximum IT of 50 ms, an isolation window of 1 m/z and an underfill ratio of 1%.
973 Dynamic exclusion was set at 20s with an apex trigger from 3 to 12s. Stepped collision energies
974 were set to 20, 50 and 100% NCE.

975 For analysis in positive ion mode, compounds were separated via hydrophilic liquid interaction
976 chromatography (HILIC), using a SeQuant ZIC-pHILIC column (150 x 2.1 mm, 5 μ m particle size)
977 Millipore (150460). Mobile phase A consisted of 20 mM ammonium bicarbonate at pH 9.2 in H₂O,
978 and mobile phase B was acetonitrile. Prior to injection, the column was equilibrated for 6 minutes
979 in 80% B. The gradient then decreased to 20% B over 20 min, then to 15% B over 2 min, returned
980 to 80% B over 0.5 min and held there for 1.5 min for a total run time of 30 min. The column
981 temperature was 35 °C with a flow rate of 150 μ L/min. For MS-analysis the MS1 parameters were
982 as described for negative ion mode except the AGC target was 3e6. MS2 parameters were the
983 same with following exceptions: dynamic exclusion was set to 25s with an apex trigger from 3 to
984 10s. Stepped collision energies were set to 20, 40 and 80% NCE.

985 Metabolomics batch correction and differential analysis

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

998 Finally, the normalized log₂ ratios were fit to a linear model to test for a linear trend across the
999 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
1000 replicates for each time point and $t=(1, \dots, 4)$ indexes the cell passage vector $T=(33, 37, 46, 50)$.

1001 The model was fit with the `lm()` function in R Version 4.1.2. P-values from the two-sided hy-
1002 pothesis test $H_0 : \beta_1 = 0$ were FDR adjusted (across all proteins) using the `qvalue` function from the
1003 `qvalue` R package (version =2.26.0). If present, asterisks in figures indicate that the FDR adjusted
1004 p-value from this test was < .05.

1005 Proteomics methods

1006 Materials and sample preparation, extraction and digestion

1007 LC-MS grade organic solvents, water, and tandem mass tag (TMT) isobaric reagents were pur-
1008 chased from Thermo Fisher Scientific (Waltham, MA). Trypsin was ordered from Promega Corpo-
1009 ration (Madison, WI) and Lys-C from Wako Chemicals USA (Richmond, VA). Sep-Pak C18 cartridges
1010 were from Waters Corporation (Milford, MA). Unless otherwise stated, all other chemicals were
1011 purchased from Sigma-Aldrich (St. Louis, MO).

1012 At time of sample collection, cells were trypsinized off the monolayer with TrypLE Express and
1013 media was used to neutralize the reaction. Cells were pelleted at 200 x g for 5 minutes and the
1014 supernatant was aspirated. Cell pellets were flash frozen in liquid nitrogen and stored at -80C until
1015 all samples were collected and ready for proteomics analysis. Cell pellets were resuspended in 450
1016 μ L of lysis buffer (75mM NaCl, 3% SDS, 50 mM HEPES, pH 8.5) and lysed by passage through a BD
1017 PrecisionGlide 21-gauge needle (20X). The lysate was sonicated for 5 minutes and then centrifuged
1018 (5 min, 16,000 x g) to remove cellular debris and the supernatant was collected.

1019 Proteins were reduced with 5 mM dithiothreitol (DTT) for 30 minutes at 56 °C with shaking. Pro-
1020 teins were then alkylated with 15 mM iodoacetamide (IAM) for 30 minutes at room temperature
1021 (RT) in the dark, and excess IAM was quenched with 5 mM DTT for 20 minutes at RT in the dark.
1022 Protein purification was accomplished using a methanol-chloroform precipitation. Briefly, 800 µL
1023 methanol, 200 µL chloroform and 600 µL water were sequentially added to 200 µL of cell lysate,
1024 with 5 second intervals of vortexing between each addition. The samples were centrifuged for 30
1025 minutes (16,000 x g at 4°C) to induce phase separation and both the top and bottom layers were
1026 removed. The precipitated protein pellet was washed with 600 µL methanol, vortexed briefly, then
1027 centrifuged for 10 minutes (16,000 x g at 4 °C). The methanol layer was removed and protein pellets
1028 were dried at RT for 10 minutes. Protein pellets were resuspended in digestion buffer (8 M urea,
1029 50 mM HEPES, pH 8.5). The urea concentration was diluted to 4 M, then proteins were digested
1030 with Lys-C overnight (10 ng/µL, 25 °C, 16 h). The urea concentration was further diluted to 1 M and
1031 samples were digested with trypsin (5 ng/µL) for 6 hours at 37 °C.

1032 Following digestion, peptides were acidified with trifluoroacetic acid (TFA) to a final concentra-
1033 tion of 0.5% TFA. Peptides were desalted using Sep-Pak C18 solid-phase extraction (SPE) columns
1034 and samples were eluted sequentially, first with 40% acetonitrile (ACN)/0.5% acetic acid and then
1035 80% ACN/0.5% acetic acid. Eluted peptides were dried in a CentriVap Benchtop Vacuum Concen-
1036 trator (Labconco, Kansas City, MO) running at 30 °C. Peptide concentrations were measured using
1037 the Pierce BCA Protein Assay Kit, then 50 µg aliquots of each samples were dried in the CentriVap
1038 for further processing.

1039 Tandem mass tag (TMT) labeling

1040 Dried peptides were resuspended in 50 µL 200 mM HEPES/30% anhydrous ACN, then 200 µg of
1041 each TMT tag was added to 50 µg peptides. TMT 131c was used as the 'bridge sample' while the
1042 other tags (126, 127n, 127c, 128n, 128c, 129n, 129c, 130n, 130c, 131n) were used to label the in-
1043 dividual samples. The TMT reaction was incubated for 1 hour at room temperature with gentle
1044 shaking, then quenched with 11 µL 5% hydroxylamine/200mM HEPES. All samples were acidified
1045 to a final concentration of 0.5% TFA. A small amount (4 µL) of each labeled sample was combined
1046 and desalted using StageTips to check TMT ratios and labeling efficiency. The TMT-labeled sam-
1047 ples were then combined at a 1:1:1:1:1:1:1:1:1:1 peptide ratio into 11-plex samples (*Rappsilber*
1048 *et al., 2007*). The combined samples were desalted using Sep-Pak C18 cartridges and dried under
1049 vacuum.

1050 High pH Reversed-Phase (HPRP) fractionation

1051 The pooled TMT-labeled peptides were fractionated using high pH reversed-phase liquid chro-
1052 matography on an Agilent 1260 Infinity HPLC equipped with a diode array detector set at 215, 220
1053 and 254 nm wavelengths (Agilent Technologies, Santa Clara, CA). Peptides were separated on an
1054 Agilent ZORBAX Extend-C18 column (4.6 mm x 250 mm, 5 µm particle size) running at 500 µL/min
1055 at 25°C. Peptides were eluted with a gradient with initial starting condition of 100% buffer A (5%
1056 ACN, 10 mM ammonium bicarbonate) and 0% buffer B (95% ACN, 10 mM ammonium bicarbonate).
1057 Buffer B was increased to 35% over 60 minutes, then ramped up to 100% B in 6 seconds where it
1058 was held for 5 minutes. Buffer B was then decreased to 0% over 6 seconds and held for 10 min-
1059 utes to re-equilibrate the column to original conditions. The samples were fractionated into 96
1060 fractions, then pooled into 12 fractions as previously described (*Huttlin et al., 2010*). The fractions
1061 were dried under vacuum and resuspended in 5% ACN/5% formic acid (FA) for LC-MS/MS analysis.

1062 Proteomics Data Acquisition and Analysis

1063 LC-MS/MS Data Acquisition All samples were analyzed by an Orbitrap Fusion Lumos Tribrid mass
1064 spectrometer coupled to an EASY-nLC 1200 (Thermo Fisher Scientific). Peptides were separated us-
1065 ing a microcapillary column (100 µm x 250 mm long, filled in-house with Maccel C18 AQ resin, 1.8
1066 µm, 120 Å; Sepax Technologies, Newark, DE) operating at 60 °C with a flow rate of 300 nL/min. Pep-

1067 tides were eluted into the mass spectrometer using a 180 min method, with acetonitrile increasing
1068 from 6 to 30% over a 165 min linear gradient in 0.125% formic acid. Mass spectrometry data was
1069 collected in data-dependent acquisition (DDA) mode. A high resolution MS1 scan (500-1200 m/z
1070 range, 60,000 resolution, AGC 5 x 10⁵, 100 ms max. injection time, RF for S-lens 30) was collected
1071 in the Orbitrap, and the top 10 precursors were selected for MS2 and MS3 analysis. Ions were iso-
1072 lated using a 0.5 m/z window for MS2 spectra. The MS2 scan was performed in the quadrupole ion
1073 trap (CID, AGC 1 x 10⁴, 30% normalized collision energy, 35 ms max. injection time) and the MS3
1074 scan was analyzed in the Orbitrap (HCD, 60,000 resolution, max. AGC 5 x 10⁴, 250 ms max. injec-
1075 tion time, 50% normalized collision energy). The max. cycle time was set at 5 s. For TMT reporter
1076 ion quantification, up to 6 fragment ions from each MS2 spectra were selected for MS3 analysis
1077 using synchronous precursor selection (SPS).

1078 Proteomics data analysis

1079 The *Ting et al. (2011)* software pipeline and methods developed in the the Haas and Gygi labs
1080 was used to process all proteomics data (*Ting et al., 2011*). Raw files were converted to mzXML
1081 files and searched against a composite human UniProt database containing forward and reverse
1082 sequences using the Sequest algorithm. MS/MS spectra were matched with fully tryptic peptides
1083 from this composite dataset using a precursor ion tolerance of 20 ppm and a product ion toler-
1084 ance of 0.6 Da. TMT modification of peptide N-termini and lysine residues (+229.162932 Da) and
1085 carbamidomethylation of cysteine residues (+57.02146 Da) were set as static modifications. Oxi-
1086 dation of methionine residues (+15.99492 Da) was set as a variable modification. Peptide spectral
1087 matches were filtered to a 1% false discovery rate (FDR) using linear discriminant analysis (LDA) as
1088 previously described (*Ting et al., 2011*). Non-unique peptides that matched to multiple proteins
1089 were assigned to proteins that contained the largest number of matched redundant peptides se-
1090 quences using the principle of Occam's razor (*Ting et al., 2011*).

1091 Quantification of TMT reporter ion intensities was performed by extracting the most intense
1092 ion within a 0.003 m/z window at the predicted m/z value for each reporter ion. TMT spectra were
1093 used for quantification when the sum of the signal-to-noise for all the reporter ions was greater
1094 than 200 and the isolation specificity was greater than 0.75.

1095 Peptide level intensities were converted to log₂ ratios by dividing each scan by the intensity in
1096 the bridge channel. Relative protein abundance, for each sample, was estimated with the posterior
1097 mean from a previously described Bayesian model using methods as described by *O'Brien et al.*
1098 (*2018*) with code located at <https://github.com/ColtoCaro/compositionalMS>.

1099 Finally, the relative abundance estimates were fit to a linear model to test for a linear trend
1100 across the passages as described by *Gaun et al. (2021)*. For each protein, we fit the model $y_{it} = \beta_0 +$
1101 $\beta_1 T_i + \epsilon_{it}$, where $i=(1, \dots, 3)$ indexes the three replicates for each time point and $t=(1, \dots, 7)$ indexes the
1102 cell passage vector $T=(18, 25, 32, 33, 37, 46, 50)$.

1103 The model was fit with the `lm()` function in R Version 3.6.0. P-values from the two-sided hypoth-
1104 esis test $H_0 : \beta_1=0$ were FDR adjusted (across all proteins) using the `p.adjust()` function. If present,
1105 asterisks in all figures indicate that the FDR adjusted p-value from this test was $< .01$.

1106 Regulatory analysis

1107 LISA TF analysis

1108 Identification of putative regulators of the gene expression changes observed in the bulk RNA-seq
1109 experiments and the pseudotime analysis was carried out using (*Qin et al., 2020*); a recent algo-
1110 rithm built to leverage the vast amount of protein-DNA interactions catalogued via ENCODE. We
1111 used the GUI hosted at <http://lisa.cistrome.org/> to enter lists of genes derived from K-medians
1112 pseudotime clustering clustering. The online GUI has a max input of 500 genes. Accordingly, for
1113 clusters containing >500 genes, each gene was ranked by correlation with the cluster median and
1114 the top 500 genes were used. LISA output consists of a ranked file of transcription factors and chro-
1115 matin modifiers with enrichment p-values associated with specific ENCODE experiments. Given

1116 that the calculated p-values were derived from gene sets of different sizes and from different num-
1117 ber of supporting experiments, we used the ranks as the input parameter for the LISA analysis
1118 shown in Figures 6D and 7D. A universe of top factors across clusters was compiled by concate-
1119 nating the top 5 TFs from each individual LISA output. The rank matrix was then centered across
1120 clusters to identify TFs with the highest rank in specific clusters.

1121 ATAC-seq peak motif enrichment

1122 We built a binary peak-by-motif matrix where each row is a binary vector representing the presence
1123 of 405 motifs we used from CisBP (Catalog of Inferred Sequence Binding Preferences) which is a
1124 freely available online database of transcription factor (TF) binding specificities: <http://cisbp.cabr.utoronto.ca>
1125 (*Weirauch et al., 2014*). We only used motifs for TFs with measurable RNA-seq in WI-38 cells. In or-
1126 der to characterize transcription factor activity changes during senescence, we implemented both
1127 a gene-centric and a gene-independent approach.

1128 In the gene-centric approach, we tested enrichment of each of the 405 motifs in peaks that are 1)
1129 associated with a gene set of interest (within 50kb), and 2) significantly associated with senescence
1130 (adjusted p-value < 0.001 in PDL 50 vs PDL 20) as compared to all the other peaks using binomial
1131 test.

1132 Alternatively, we trained a ridge logistic regression model using the binary motif matrix as fea-
1133 tures to distinguish peaks of significantly increased accessibility during senescence from peaks of
1134 significantly reduced accessibility in PDL 50 vs PDL 20. A model trained on two thirds of the data
1135 distinguishes the two sets of peaks with AUC=0.67 on held-out peaks. We then trained 10 inde-
1136 pendent models using all the data to evaluate the coefficients to identify features (motifs) that are
1137 most predictive of senescence.

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1143 Author Contributions

1144 MR, DB, ADL, and RC conceived the project. MC and DGH planned the experiment. MC, RW, AI,
1145 JG, LC, TM, and TV performed the experiments. DGH led the data analysis. DGH, HY, IS, and JO
1146 performed data analysis. DGH, MC, DB and DRK wrote and edited the manuscript. MR, DB, ADL,
1147 RC, AF, CK, BB, FM, DRK, DGH provided support and oversight for the project.

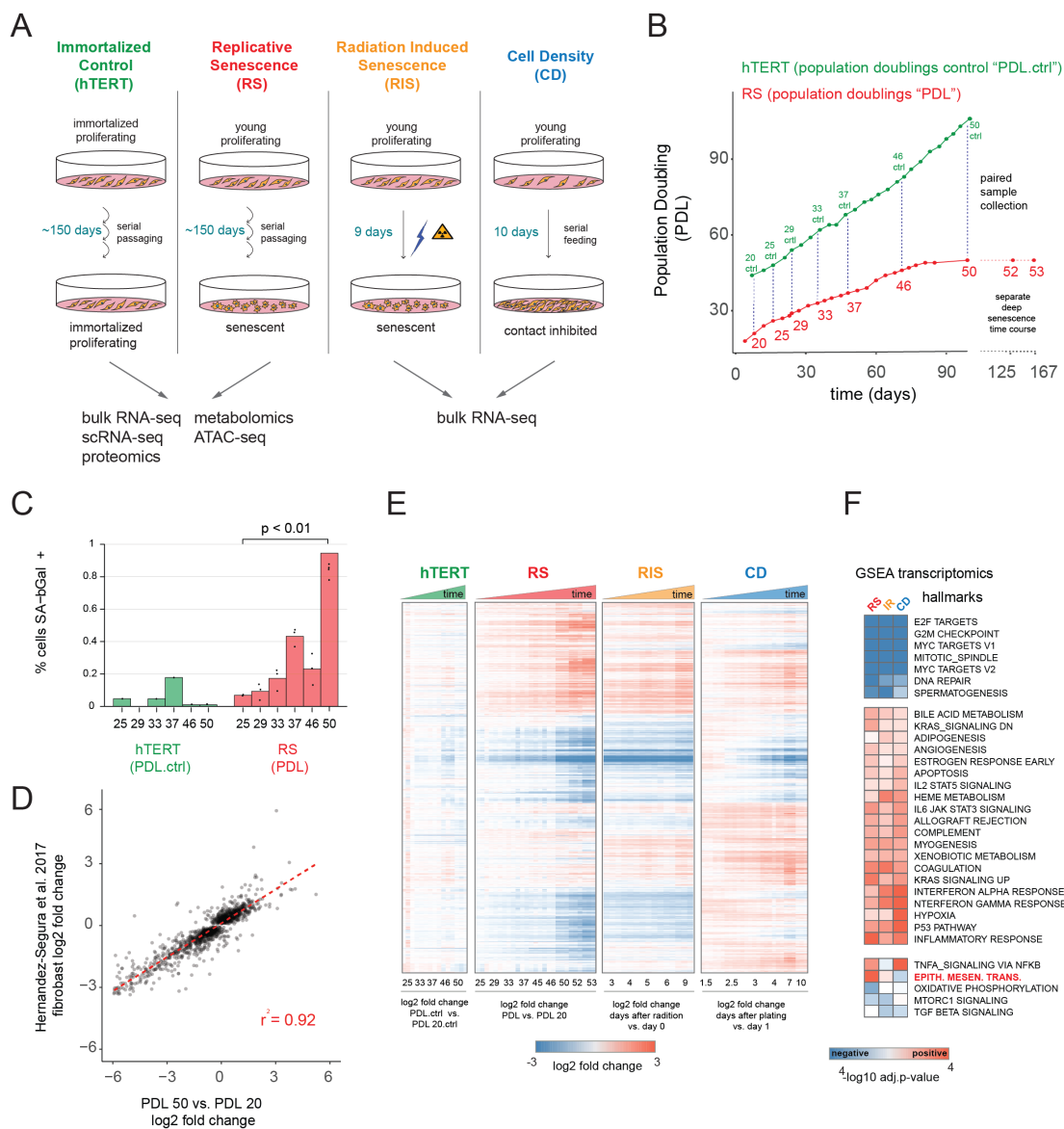


Figure 1: Expression dynamics of replicative senescence (RS), radiation induced senescence (RIS), increasing cell density (CD) and hTERT WI-38 cells. **(A)** Experimental design for the RS, RIS, CD and hTERT experiments. **(B)** Days in culture vs. population doublings (PDL) for WT WI-38 cells (red) 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 (*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

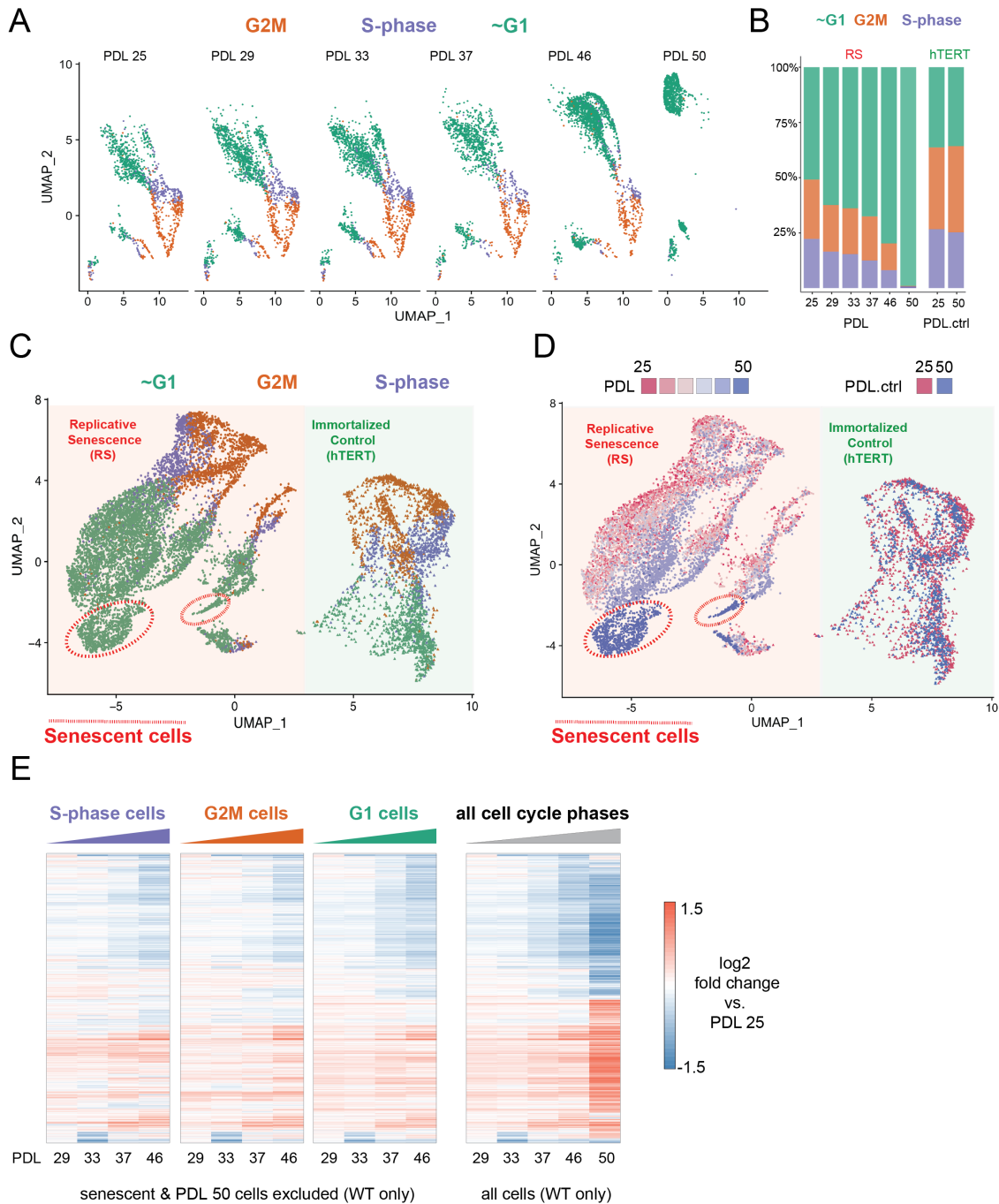


Figure 2: Cell cycle exit and distribution on approach to replicative senescence (RS) does not explain gradual increase in the replicative senescence transcriptome. **(A)** Individual UMAP projections of WT WI-38 cells; PDL colored by phases of the cell cycle (G1 = green, G2/M = orange, S-phase = purple). **(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 from increasing PDLs (RS) or time point (hTERT)-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

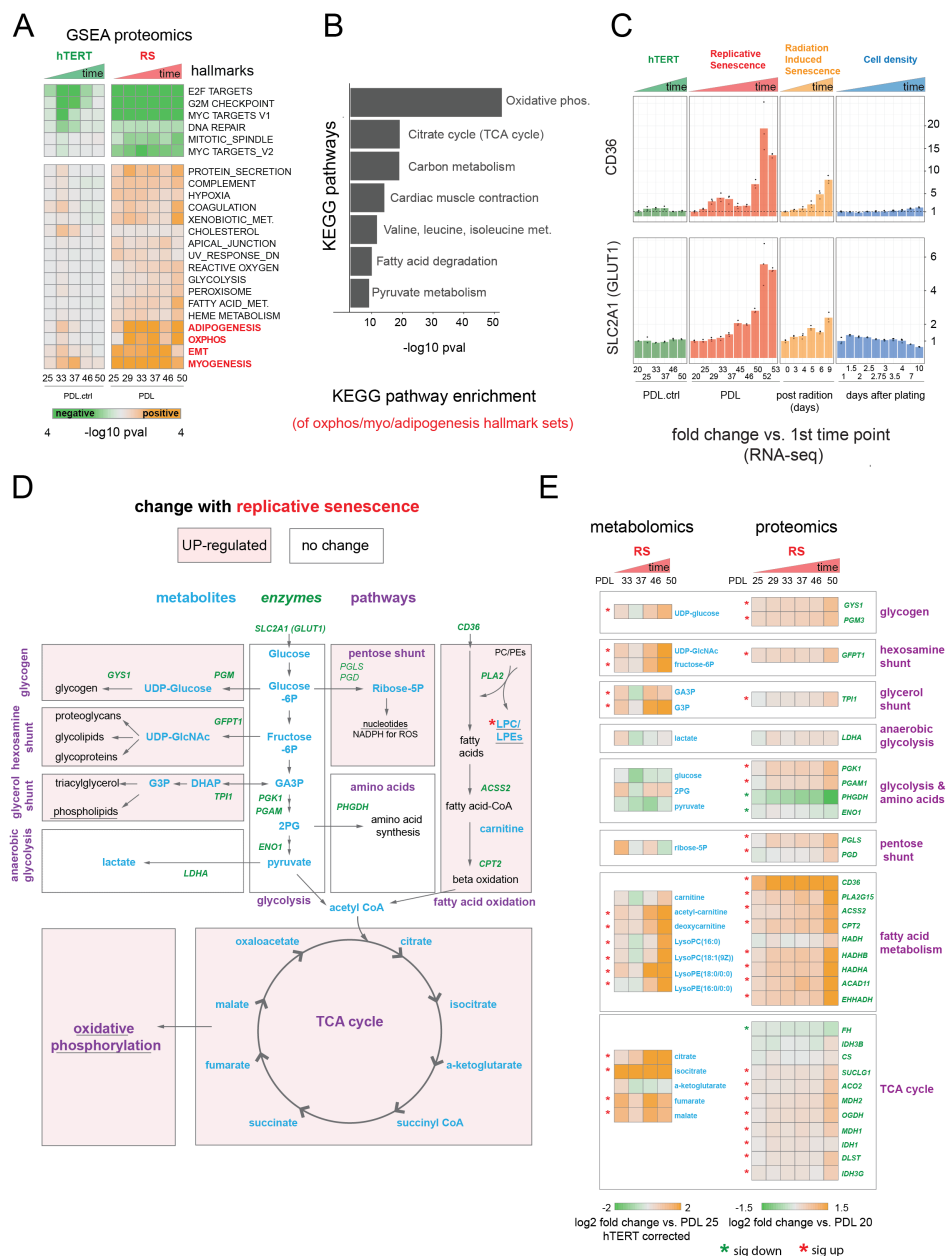


Figure 3: Proteomic and metabolomic changes during replicative senescence. **(A)** Significant (Benjamini-Hochberg adjusted p -value < 0.01) GSEA results for protein changes ($n=3$) using the MSigDB Hallmarks. Reference time point not shown. Values are $-\log_{10}$ p -value and are colored by the direction (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 italicized in green. **(E)** Metabolite ($n=4$) and protein changes ($n=3$) with senescence from D. Average hTERT 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 $p < 0.05$ 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

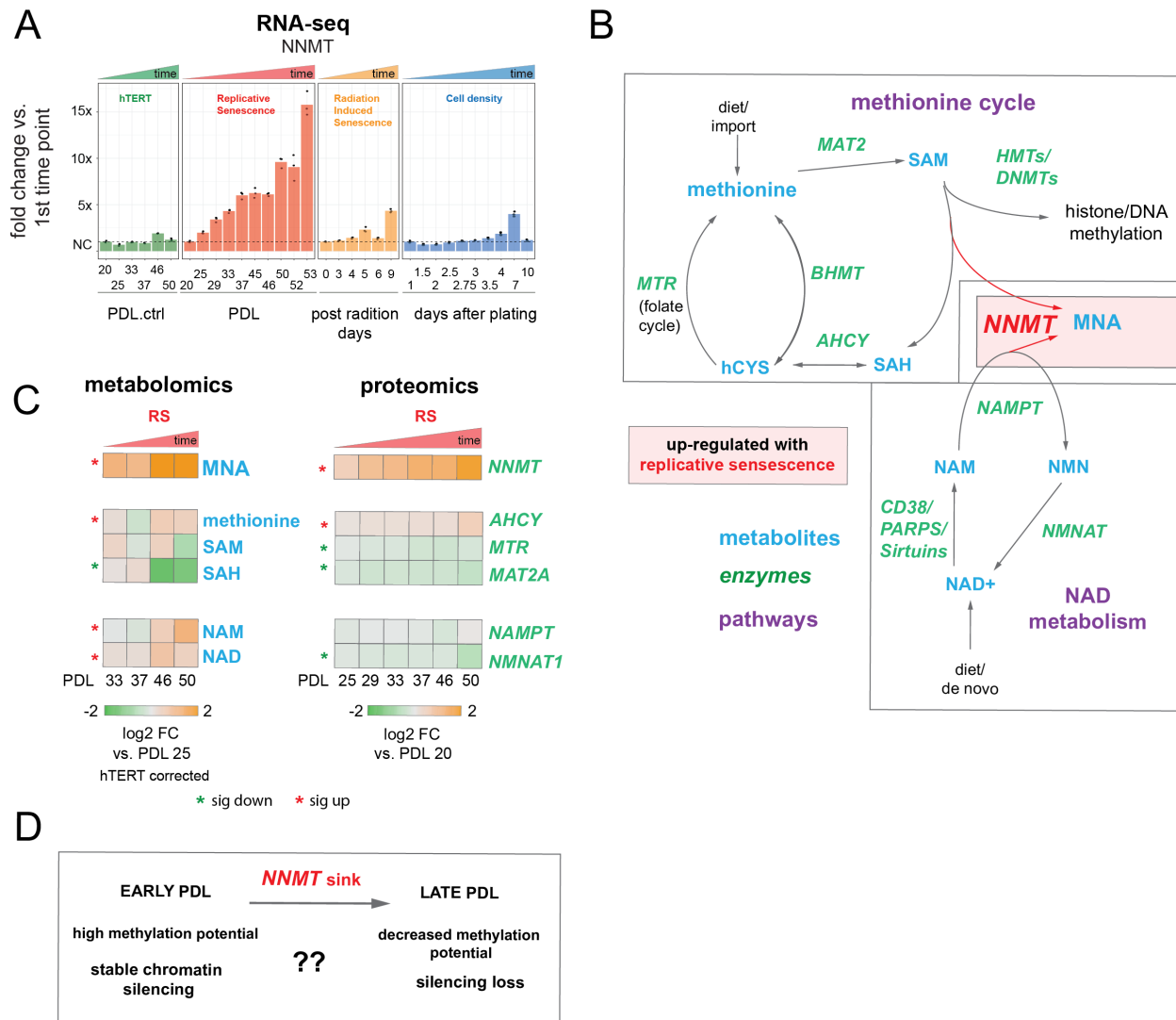


Figure 4: Nicotinamide n-methyltransferase (NNMT) links nicotinamide adenine dinucleotide (NAD) and methionine metabolism during replicative senescence. **(A)** Gene expression fold changes for the NNMT (top) in the hTERT, replicative senescence (RS), radiation induced senescence (RIS), and cell density (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 italicized in green. Shading indicates inferred pathway direction during replicative senescence based on metabolite/protein changes in A. **(C)** Heatmaps of metabolite and protein changes with replicative senescence from B (4 replicate average). 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 $p < 0.01$ proteomics) during replicative senescence are denoted with asterisks. **(D)** Potential model for NNMT and metabolic regulation of chromatin state during replicative senescence.

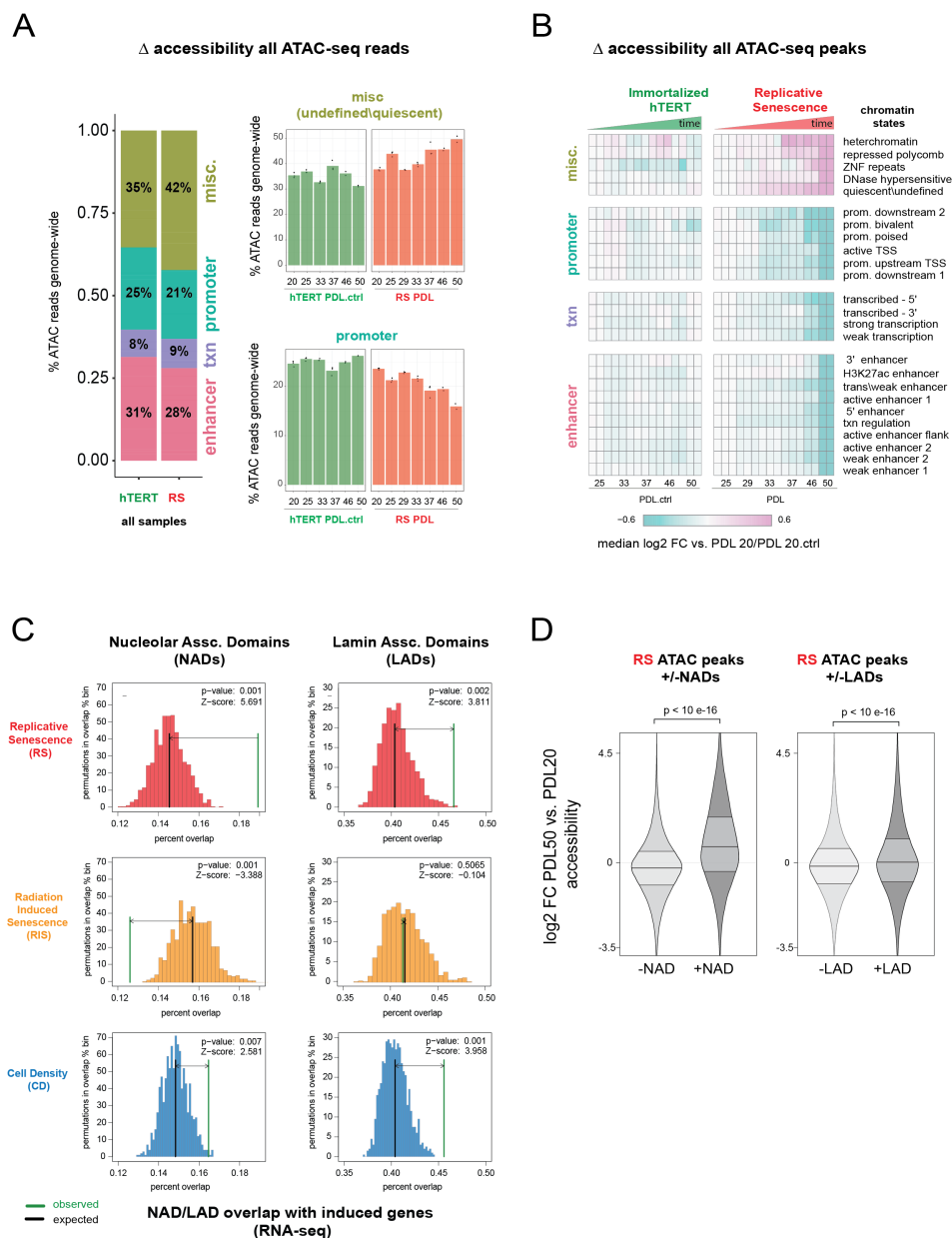


Figure 5: Increased accessibility within heterochromatin and nucleolar associated domains (NADs) is a dominant feature of the replicative senescence (RS) epigenome. **(A)** Percent of ATAC-seq reads falling into 4 broad chromatin states compiled from the ENCODE IMR-90 25 chromatin state prediction for 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 distribution of expected overlap 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

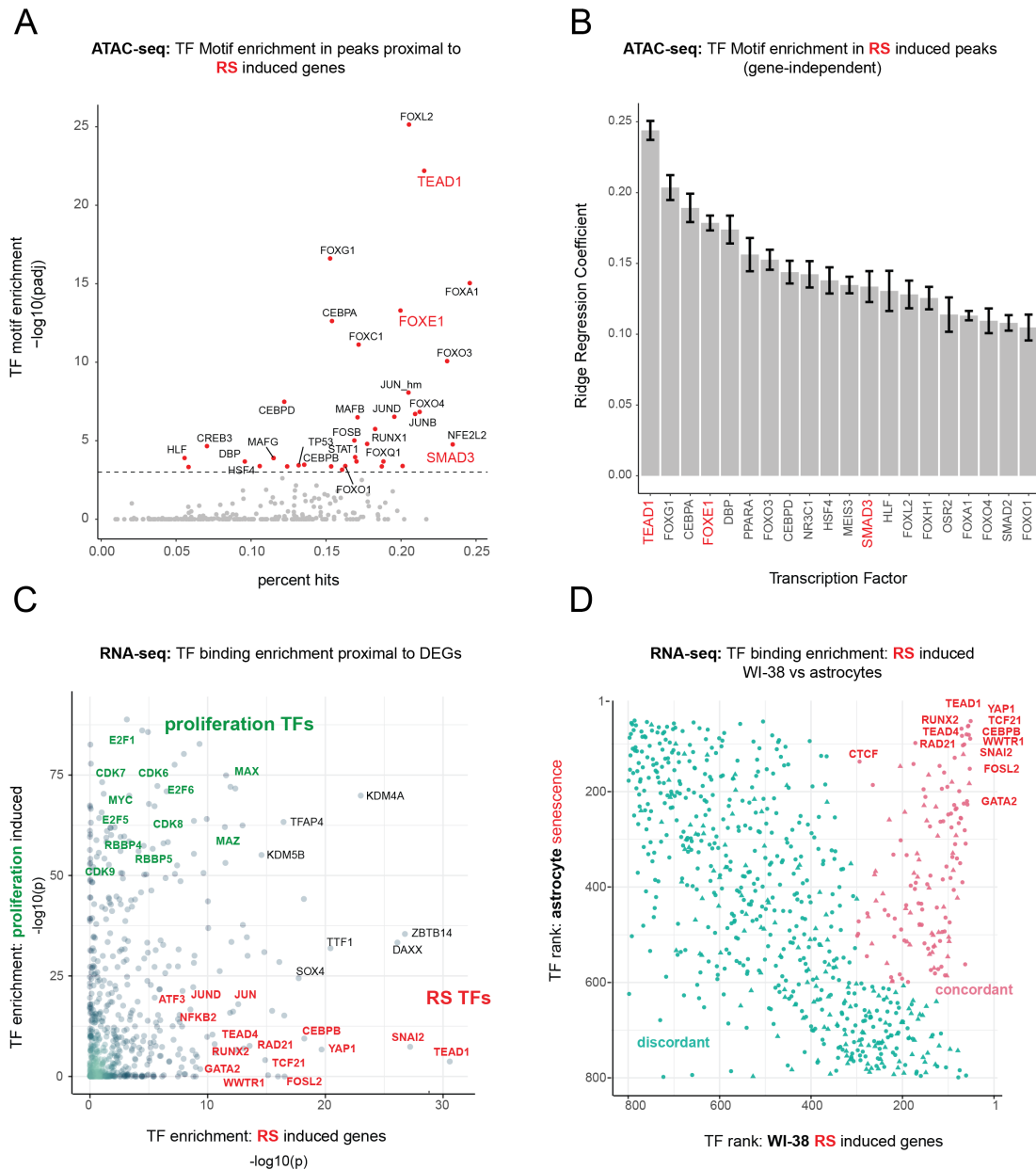


Figure 6: Master transcriptional regulators of replicative senescence **(A)** Scatter plot of transcription factor motif enrichment in ATAC-seq peaks surrounding significantly induced genes (FDR adjusted $p < 0.01$, $\log_2 \text{FC} > 0.5$) during replicative senescence. The Y-axis is the $-\log_{10}$ 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 $p < 0.01$, $\log_2 \text{FC} < -0.5$) vs. replicative senescence induced genes (x-axis, FDR adjusted $p < 0.01$, $\log_2 \text{FC} < -0.5$). 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.

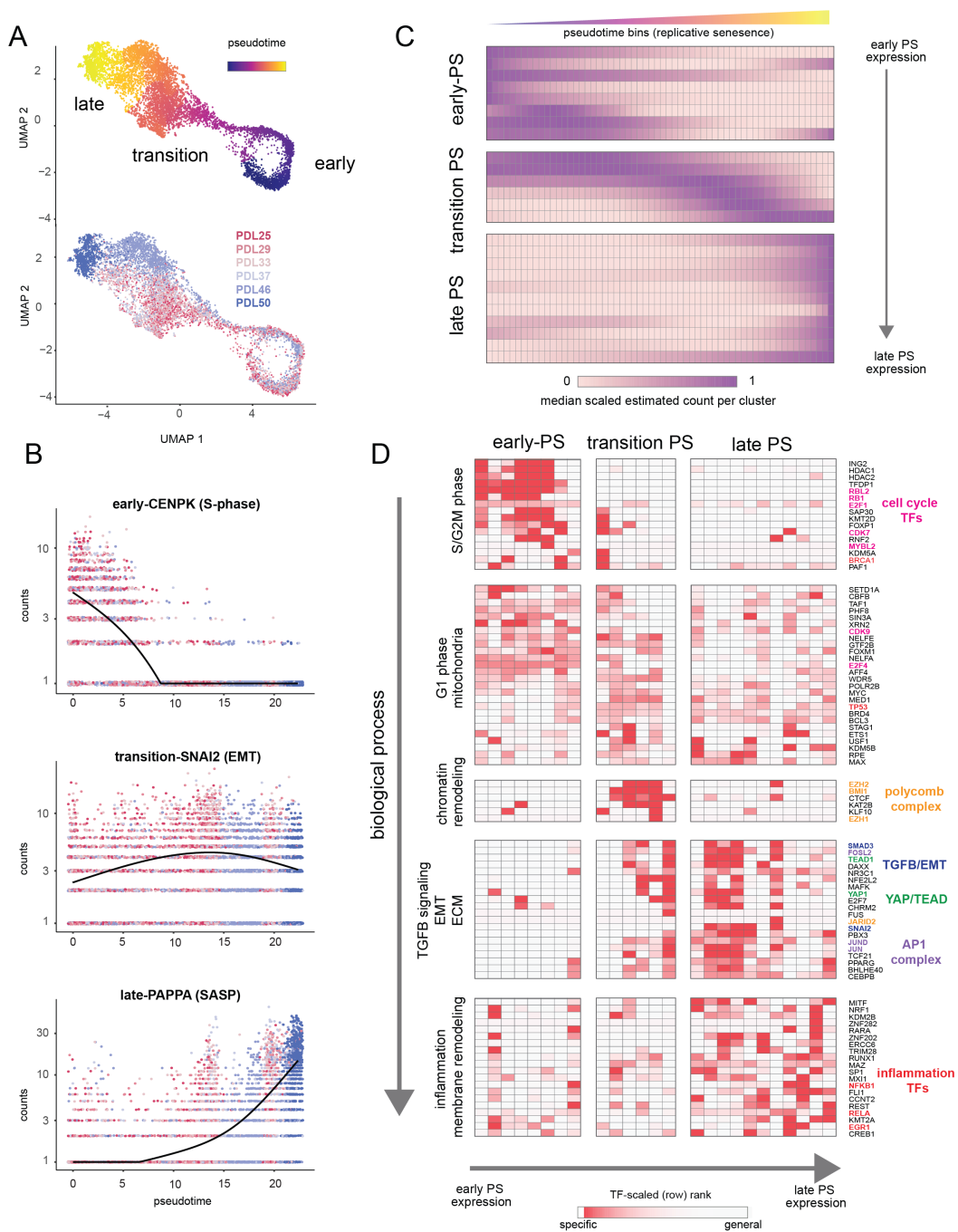


Figure 7: Pseudotime (PS) analysis of WI-38 approach to replicative senescence using single cell RNA-seq. **(A)** UMAP projection of single WI-38 cells collected at increasing PDLs (PDL25-red) to (PDL50-blue) and colored by pseudotime (top). **(B)** Scatterplot of single cell gene expression across pseudotime 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

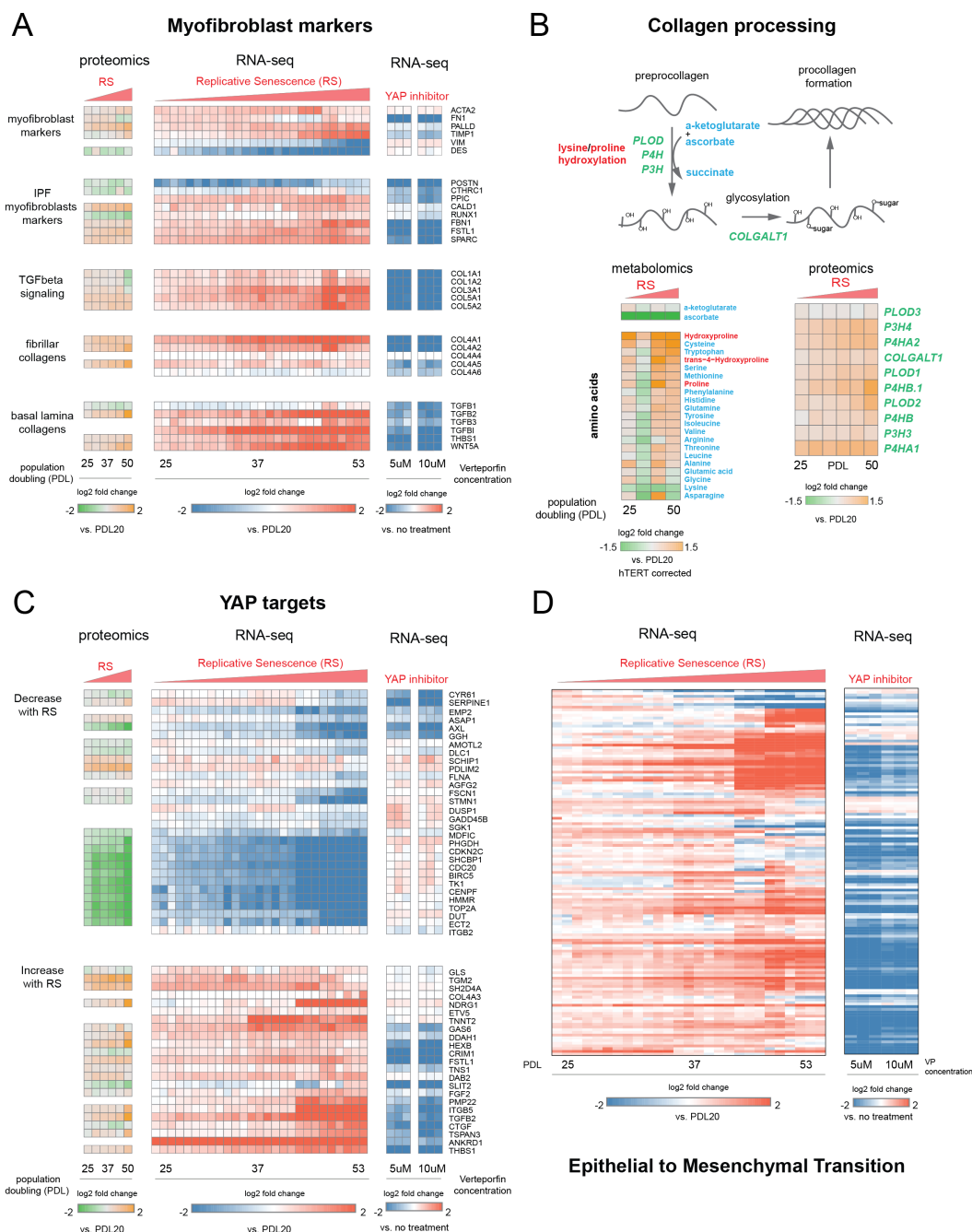
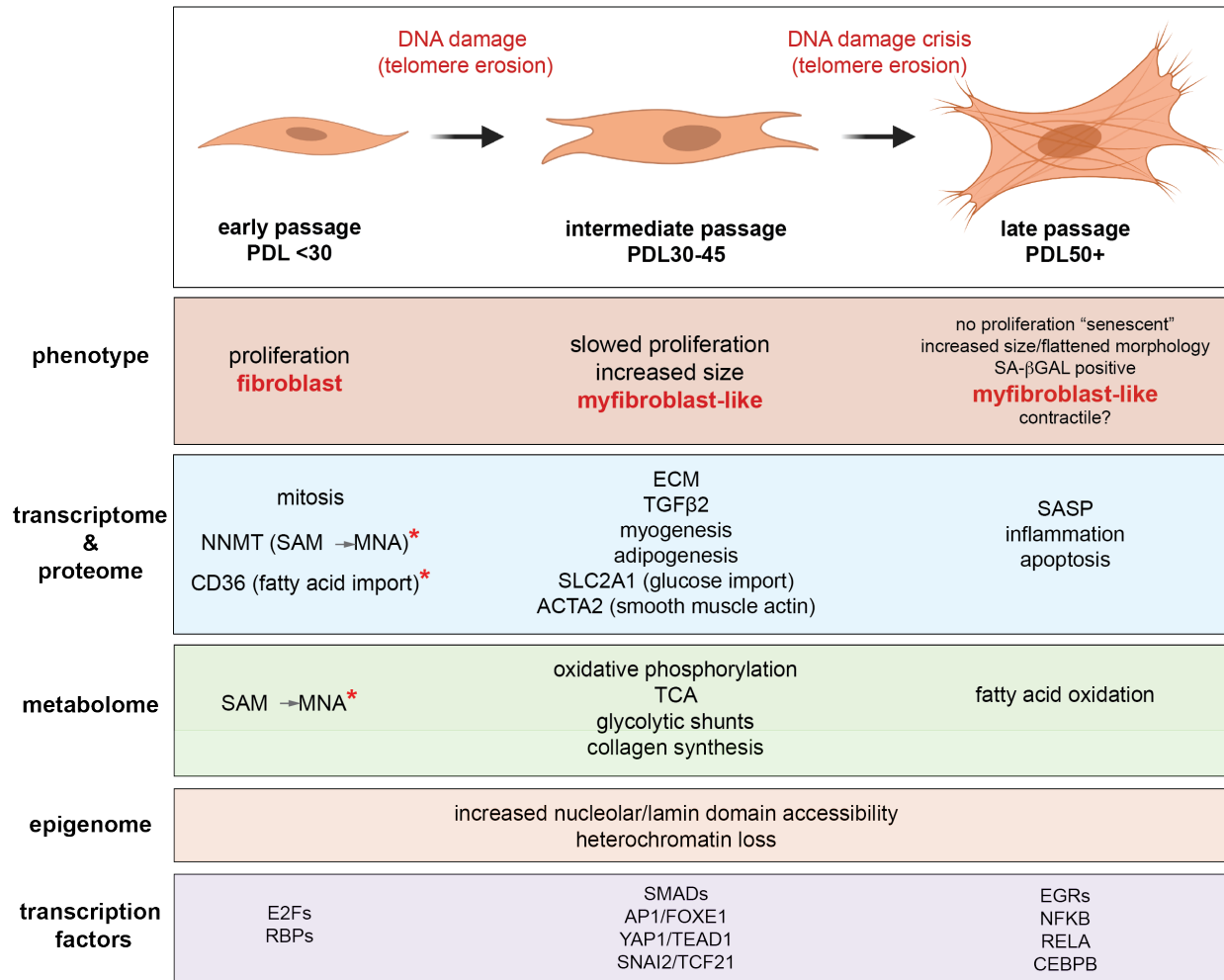


Figure 8: YAP regulation of myofibroblast markers and YAP1/TEAD1 targets during replicative senescence. **(A)** RNA-seq and proteomics heatmaps of selected genes based on myofibroblast markers and biology. Values plotted are log₂ fold change of each PDL vs PDL 20 for replicative senescence timecourse 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 italicized 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 Hallmarks 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 myfibroblast transtion (FMT)?



*early changes during RS

Figure 9: Replicative senescence fibroblast to myfibroblast 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 myfibroblasts.

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		BATCH A						BATCH B			
		PDL20	PDL25	PDL29	PDL33	PDL37	PDL46	PDL50	PDL45	PDL52	PDL53
1528	bulk RNA seq	X	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.

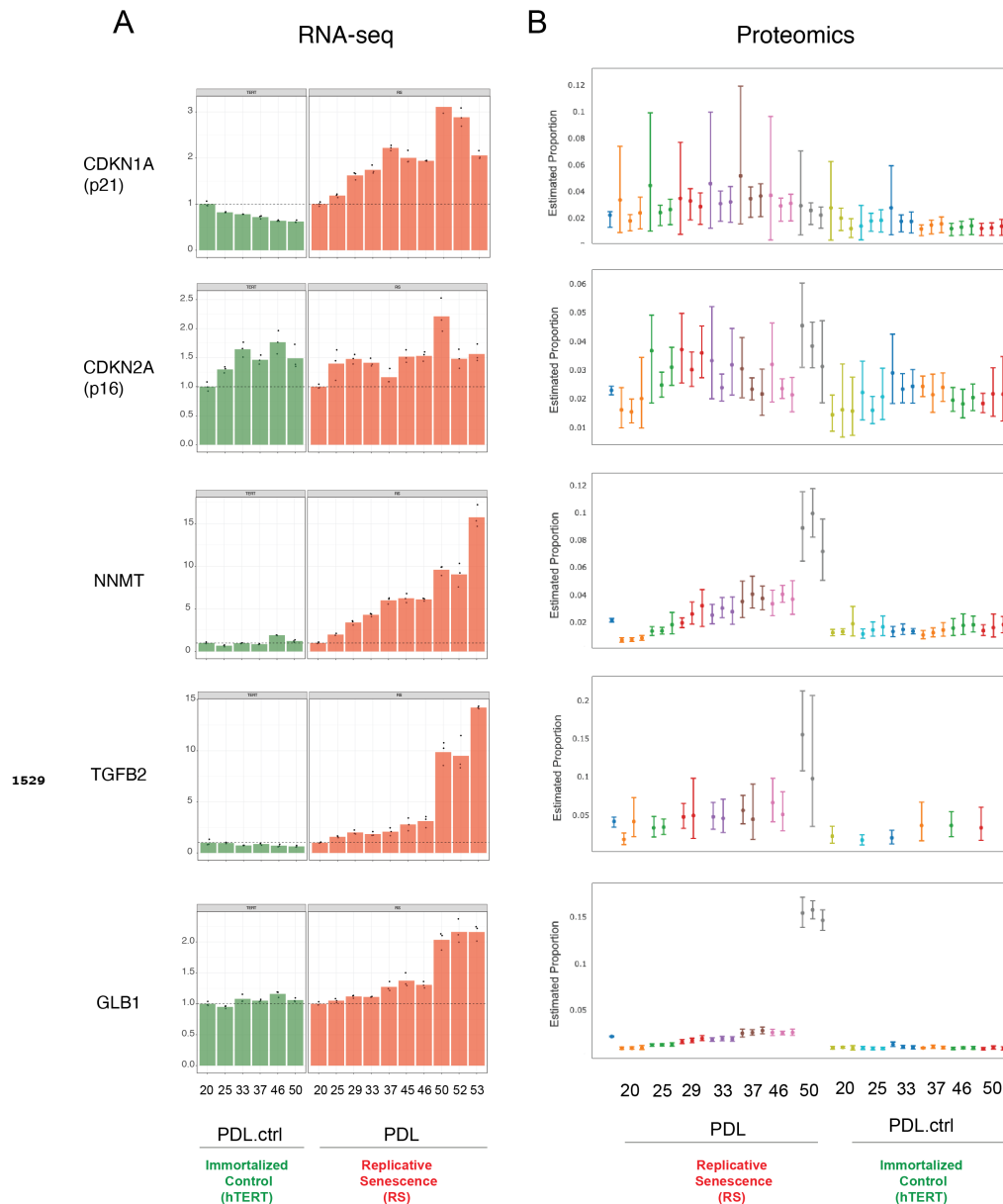


Figure 1-Figure supplement 2. Expression of canonical and novel senescence induced genes **(A)** RNA-seq quantification for p21, p16, NNMT, TGFB2, and GLB1 (beta-galactosidase) transcripts expressed as fold change relative to reference sample: PDL 20 for WT replicative senescence and PDL.ctrl 20 for 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.

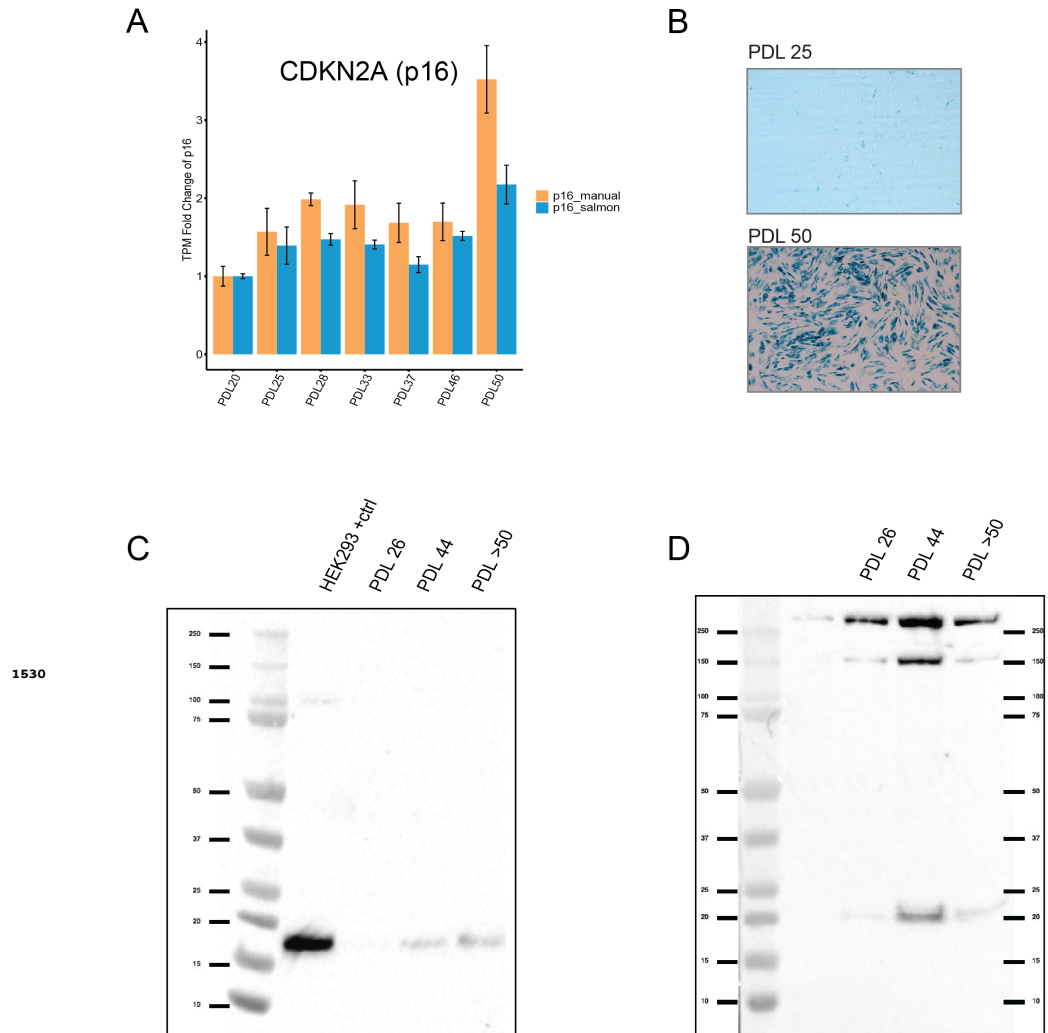


Figure 1-Figure supplement 3. Senescent WI-38 cells express classic senescence markers. **(A)** Quantification of p16 and p14 gene products from the CDKN1A locus. Values plotted are fold change levels relative to PDL 20. TPM values generated by Salmon using the gencode v29 annotation set that includes 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.

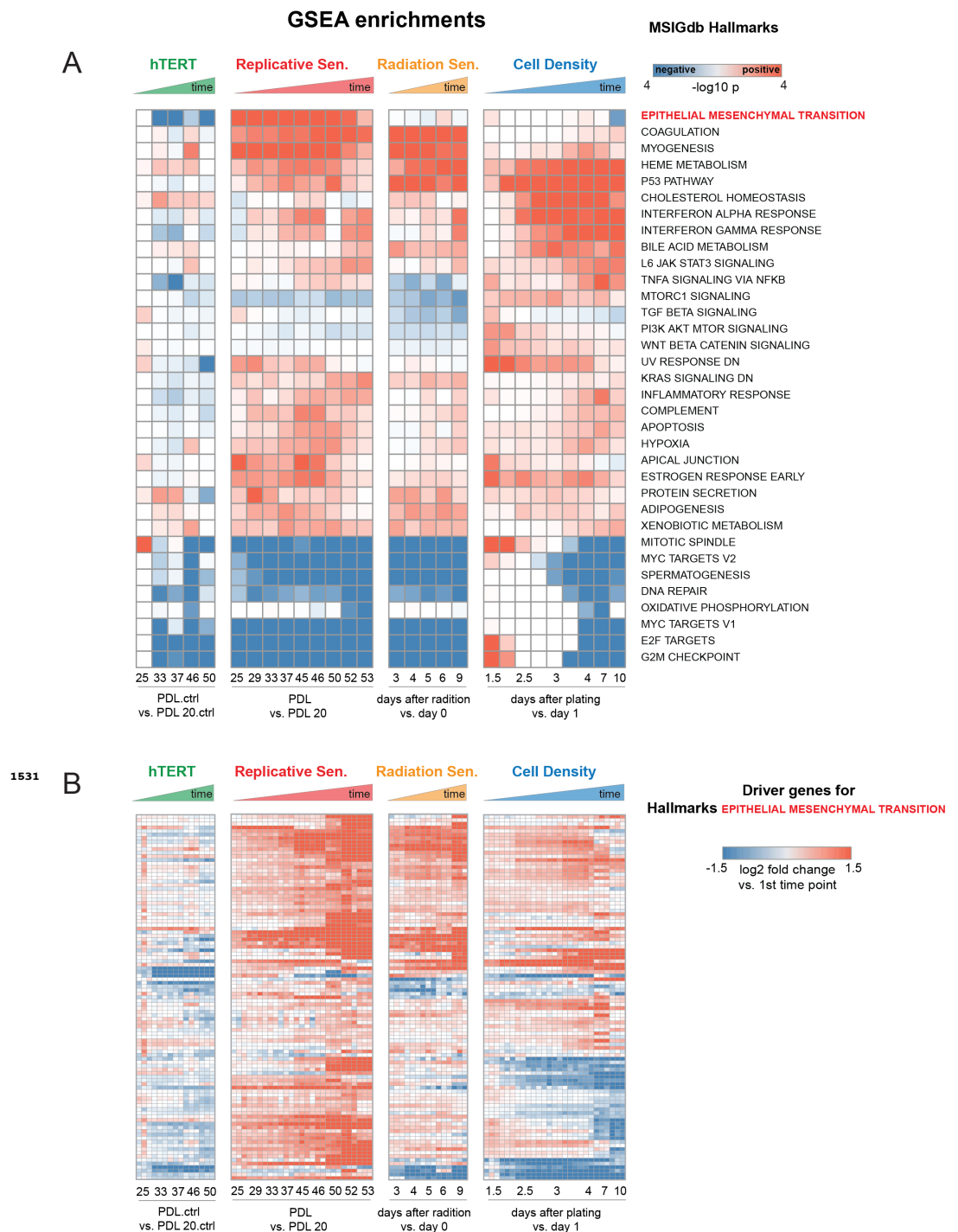


Figure 1-Figure supplement 4. Enrichment and induction of Epithelial to Mesenchymal Transition gene set during approach to replicative senescence. **(A)** GSEA was run using MSigDB Hallmark gene sets. Data used for ranked list was the \log_2 fold changes at each time point vs. first the initial time 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.

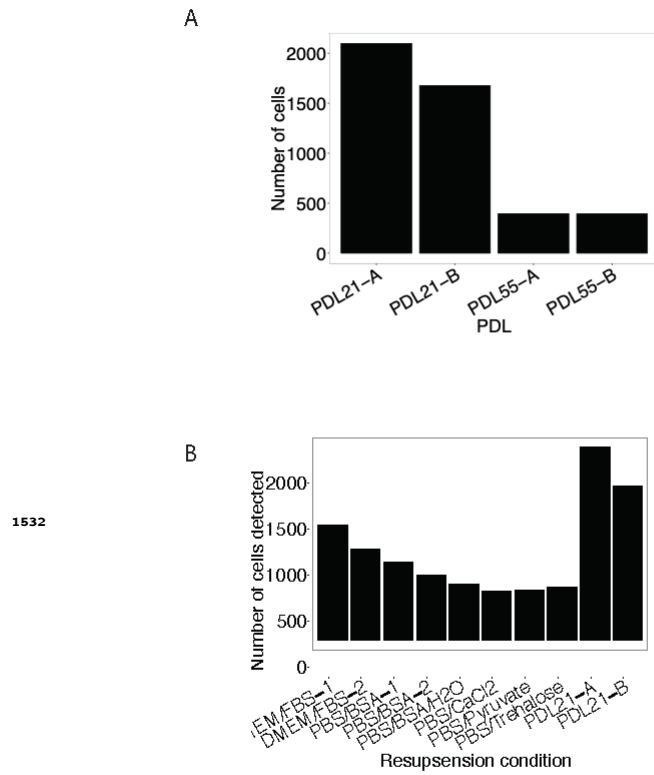


Figure 2-Figure supplement 1. Using a modified resuspension buffer of DMEM+FBS increases detection of senescent cells with the 10x Genomics 3' single cell RNA-seq protocol. **(A)** Barplot comparing the number of cells detected (y-axis) as a function of the age of cells (x-axis). **(B)** Barplot representing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

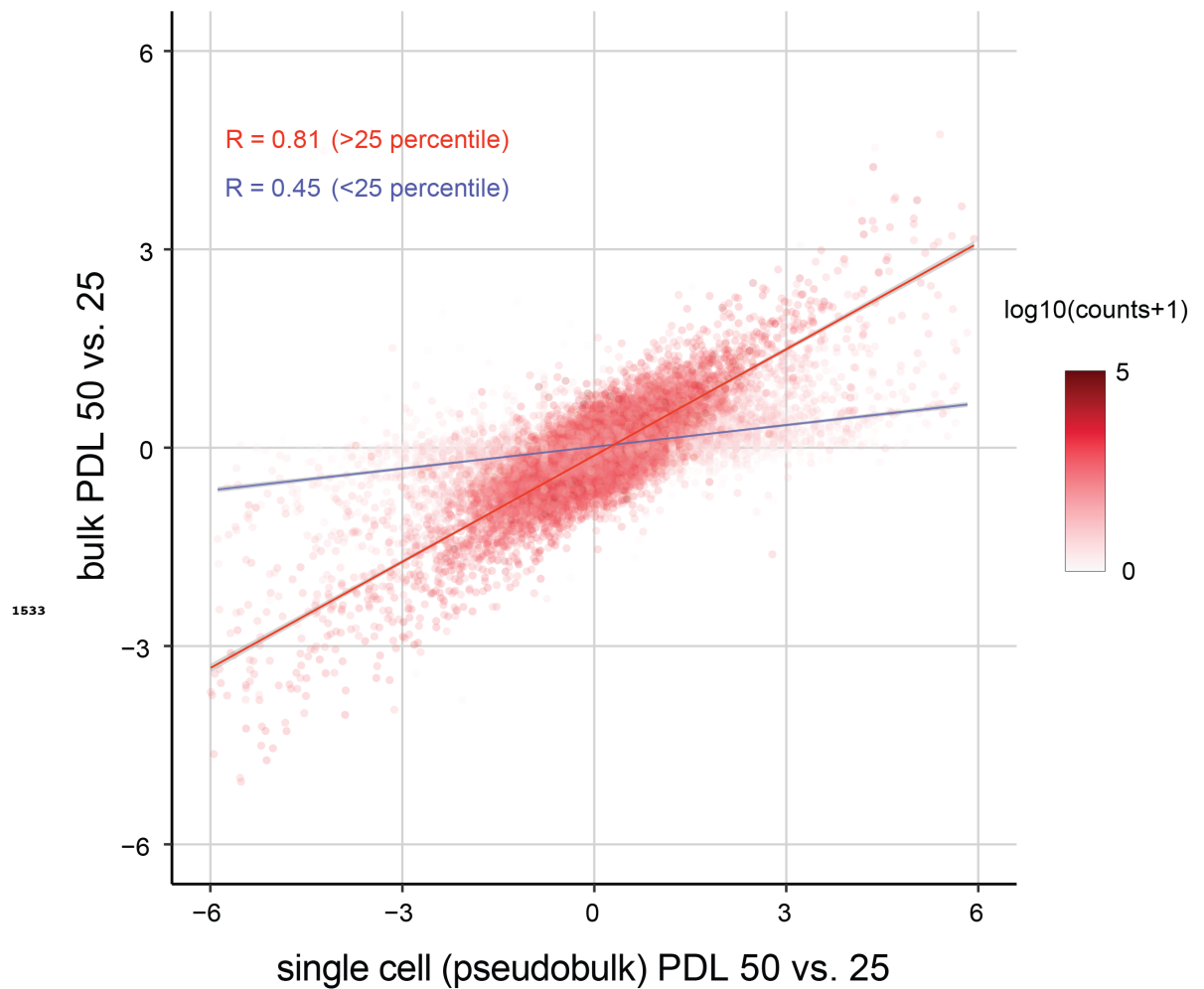


Figure 2-Figure supplement 2. Replicative senescence dependent gene expression changes measured by Bulk RNA-seq and single cell RNA-seq are highly concordant. Scatterplot comparing the log2 fold changes of PDL 50 vs PDL 25 measured using single cell pseudo-bulk count summations (x-axis) and actual bulk 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).

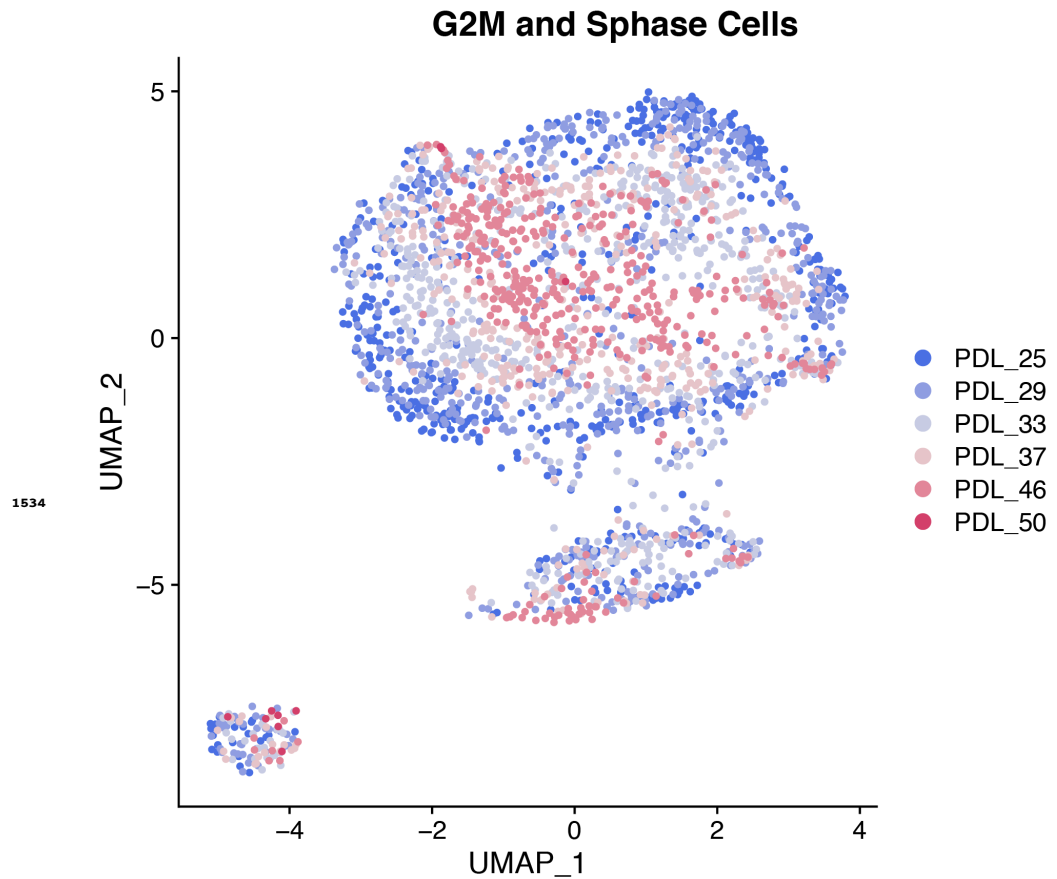


Figure 2-Figure supplement 3. Single cell UMAPs of high mitotic scoring (gene set) WI-38 cells. S phase and G2M scored cells were separated and reprocessed and visualized with a UMAP projection. In mitotic cells, PDL is the primary source of variance across all PDLs.

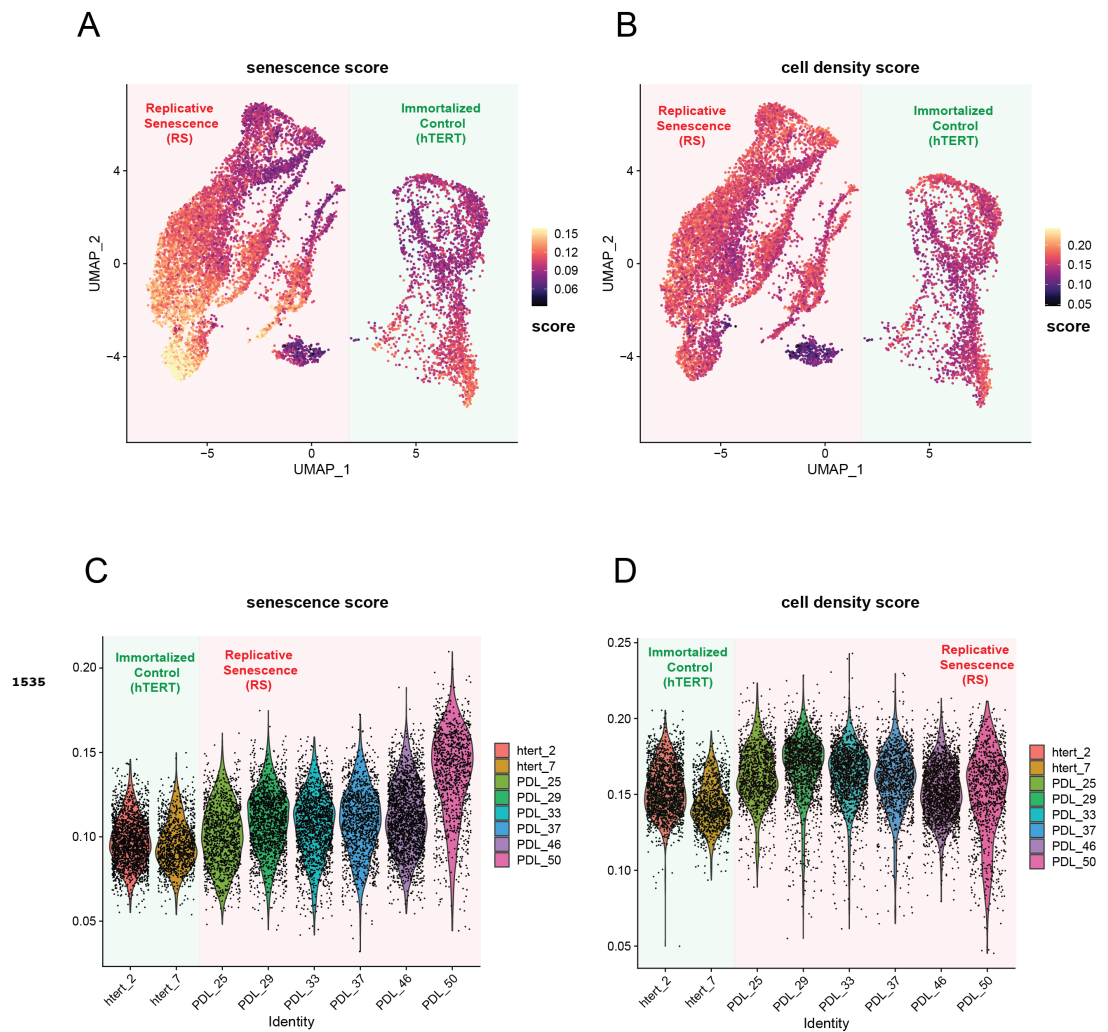


Figure 2-Figure supplement 4. WT and hTERT WI-38 cells scored for senescence and cell density signatures. **(A)** UMAP projection of WT and hTERT single cell RNA-seq scored with a signature composed of significantly increasing genes with replicative senescence that are not significant in cell density 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 are on x-axis. Cell density remains flat with increasing PDL.

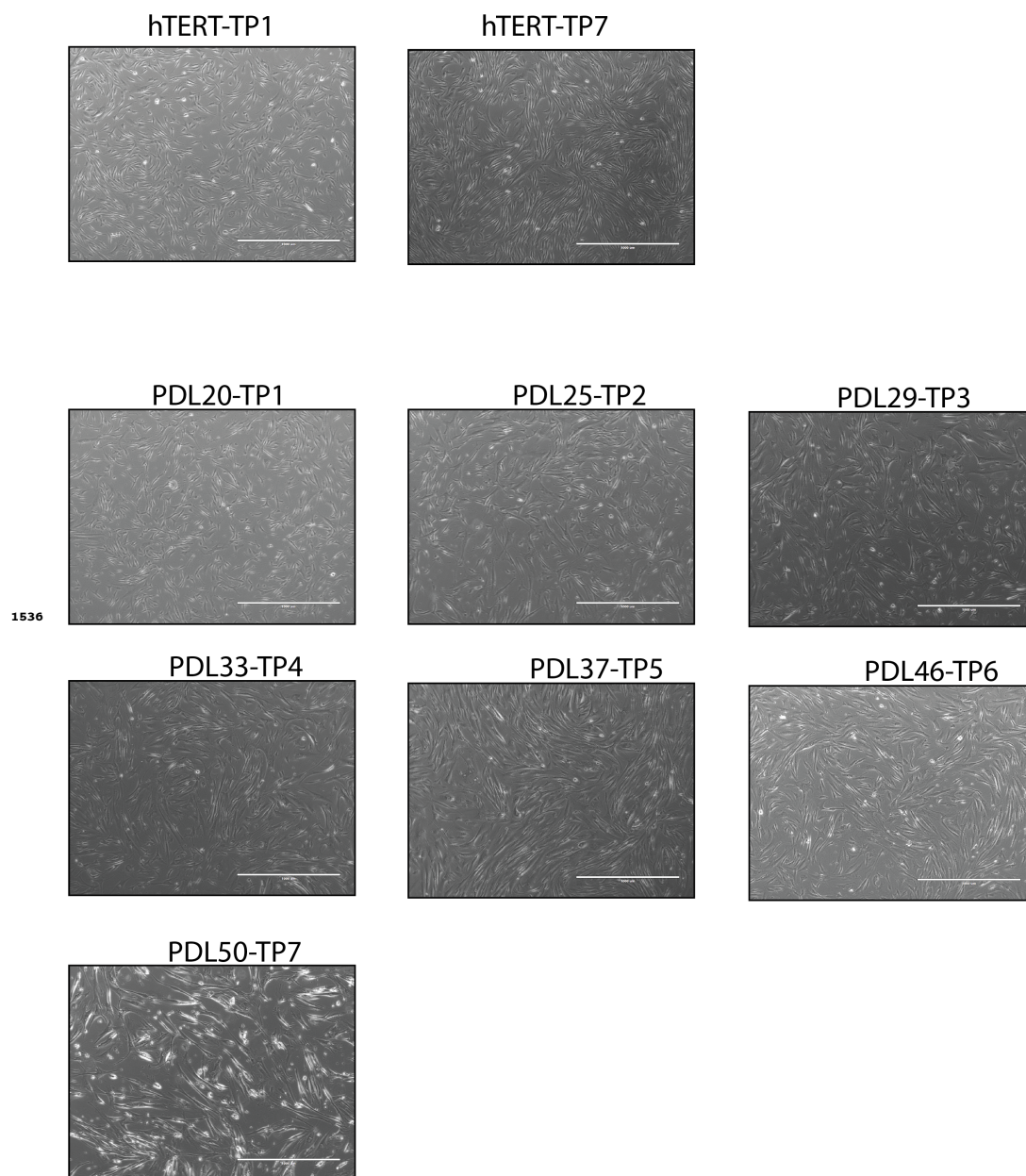


Figure 2-Figure supplement 5. Representative images of WI-38 cells at collection time points.

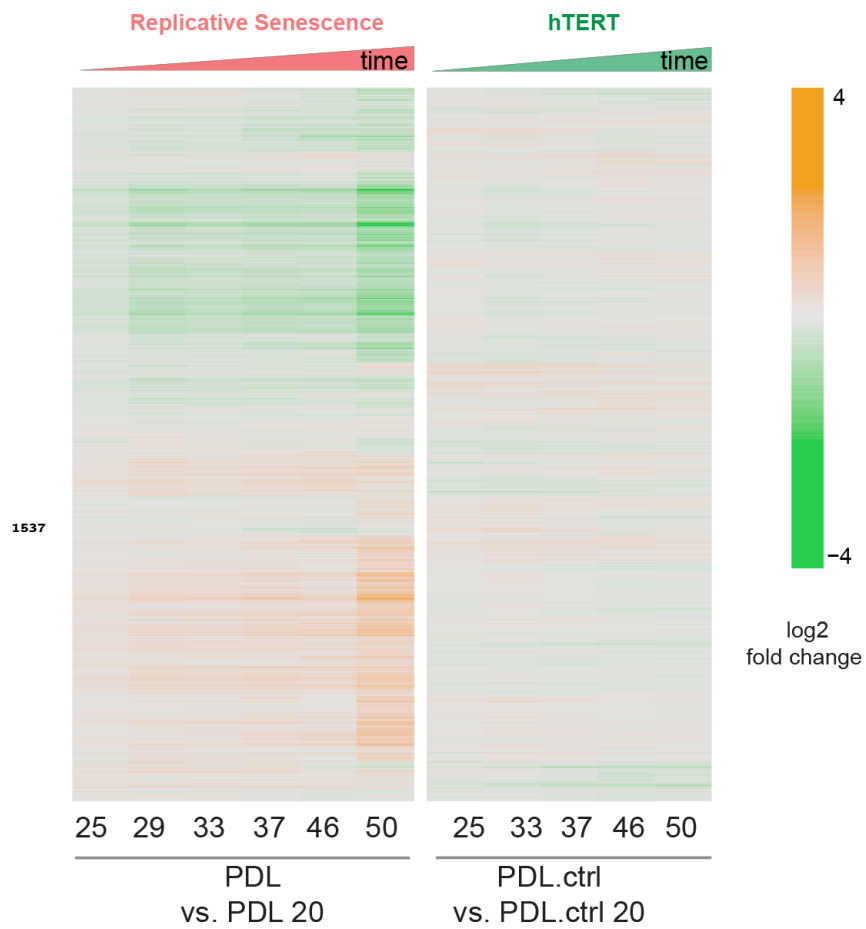


Figure 3-Figure supplement 1. Heatmap of hierarchical clustering of 8000 protein log₂ fold changes at each time point/PDL versus first (not shown) for RS WT WI-38 (left) and hTERT WI-38 cells (right) from high induction (orange) to depletion (green).

RNA vs. protein

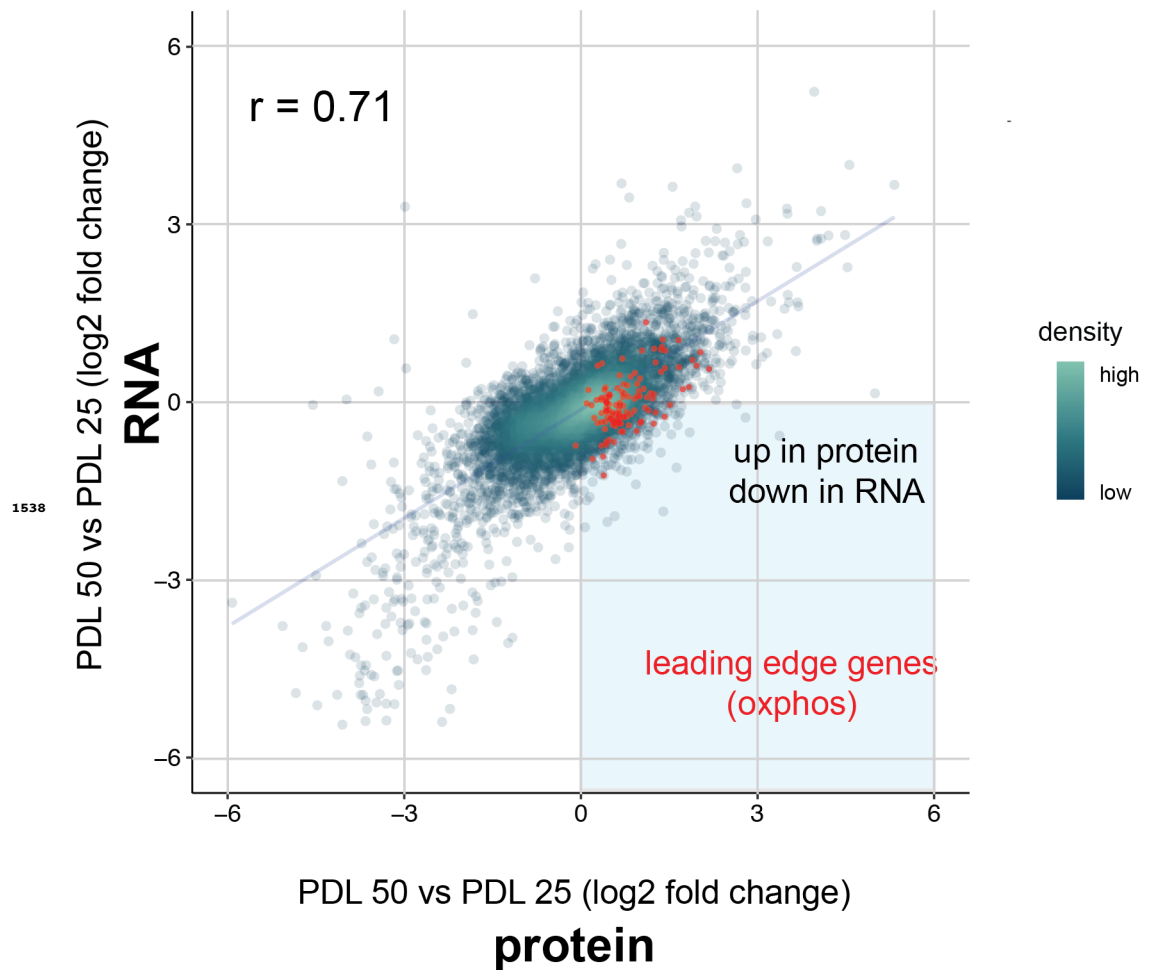


Figure 3-Figure supplement 2. Scatterplot comparing the log2 fold change expression of genes (x-axis) vs. protein (y-axis) in PDL 50 cells relative to PDL 20 cells. Oxidative phosphorylation leading edge genes from GSEA plotted in red. Point density colored from high (white) to low (green).

HALLMARKS OXIDATIVE PHOSPHORYLATION (proteomics)

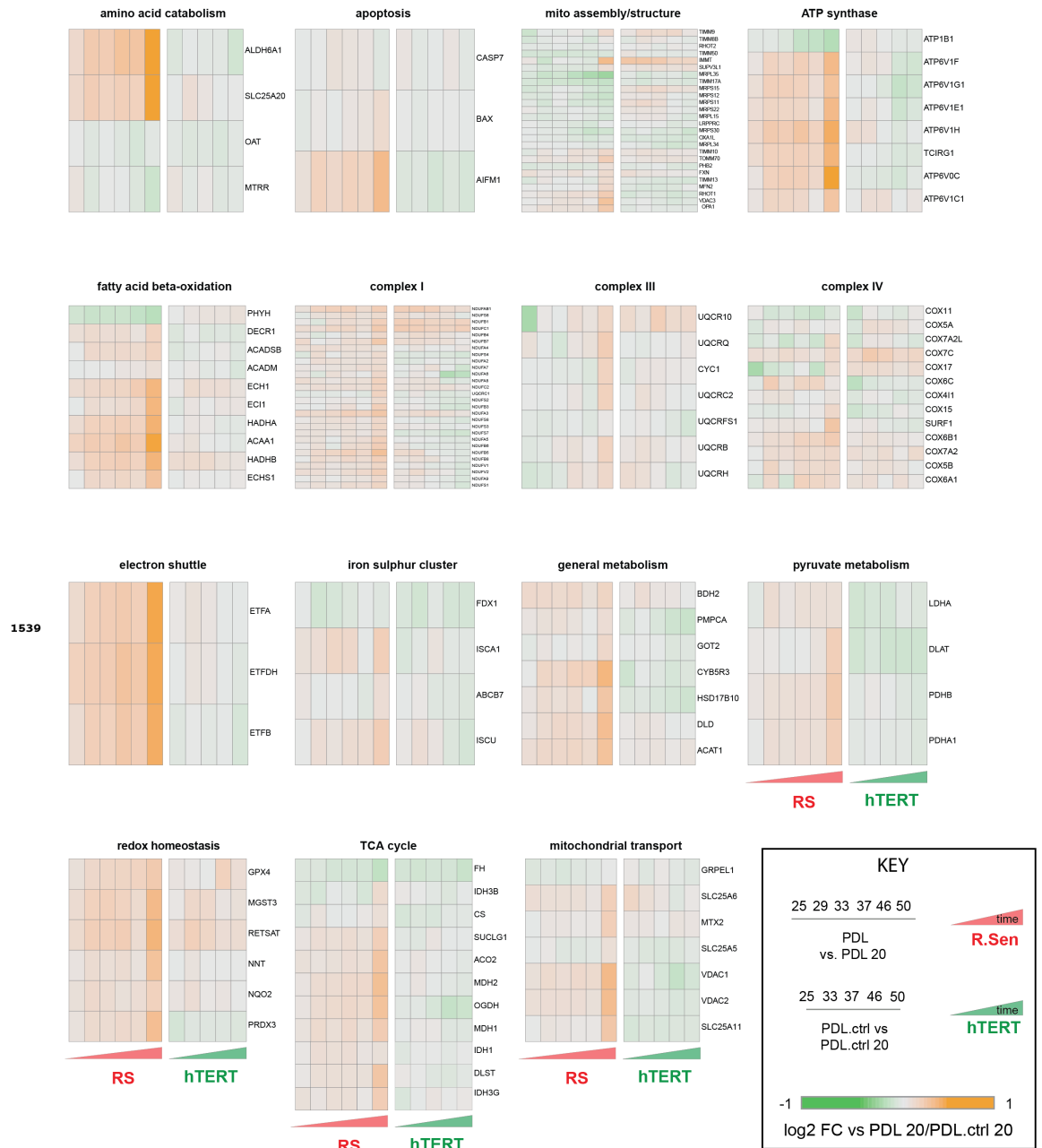


Figure 3-Figure supplement 3. Breakdown of the hallmark oxidative phosphorylation gene set into functional subsets reveals up regulation of most mitochondrial functions during replicative senescence. Heatmaps of log₂ fold change proteomics data for the MSigDB hallmarks oxidative phosphorylation annotations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

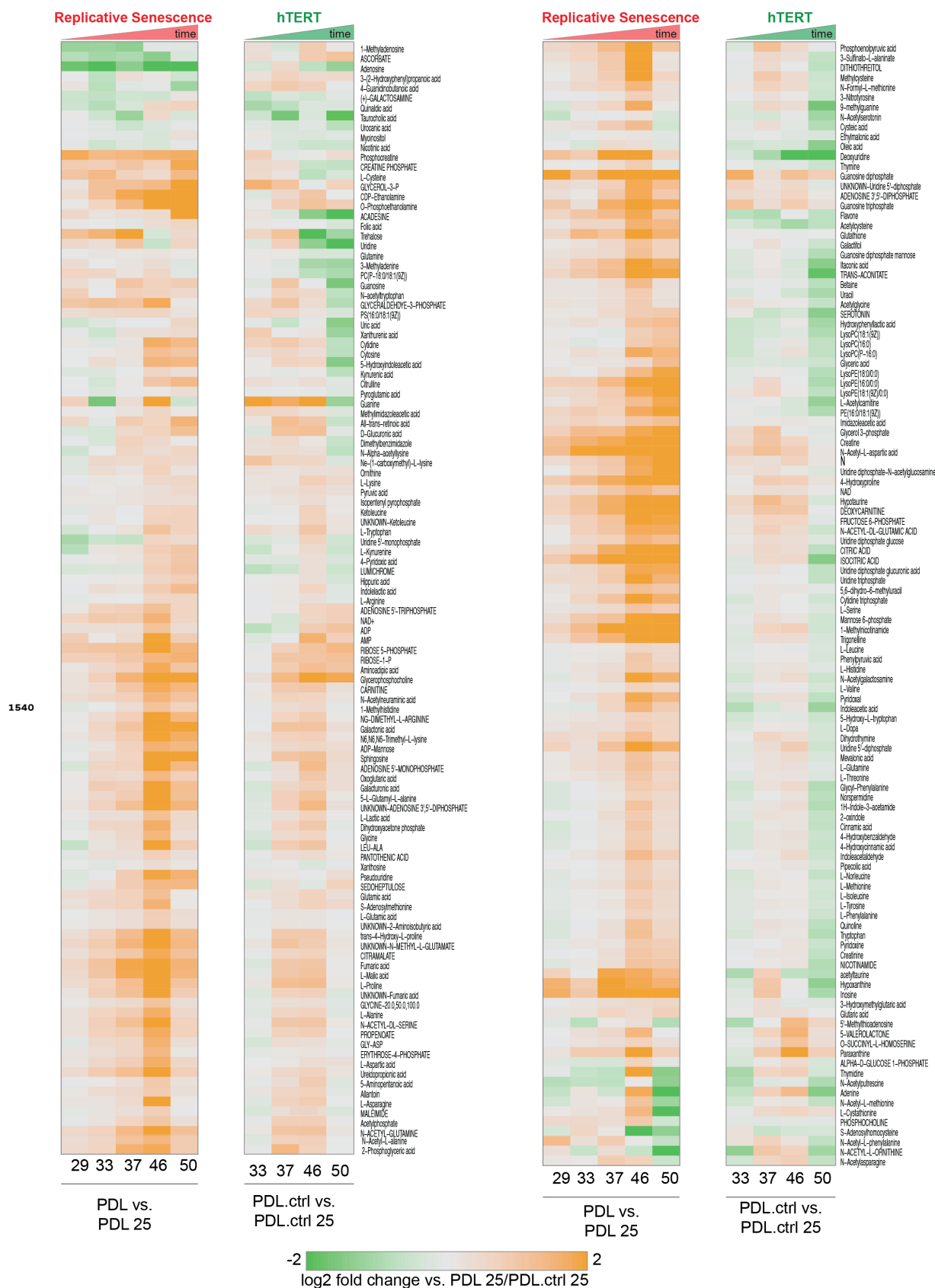


Figure 3-Figure supplement 4. PDL-dependent changes in the senescent metabolome vs hTERT cells. Heatmap of hierarchical clustering of 285 metabolite log₂ fold changes at each PDL or PDL.ctrl versus first (not shown) for RS WT WI-38 (left) and hTERT WI-38 cells (right). Values range from high induction (orange) to depletion (green). Values shown are log₂ Median values for n=4 replicates.

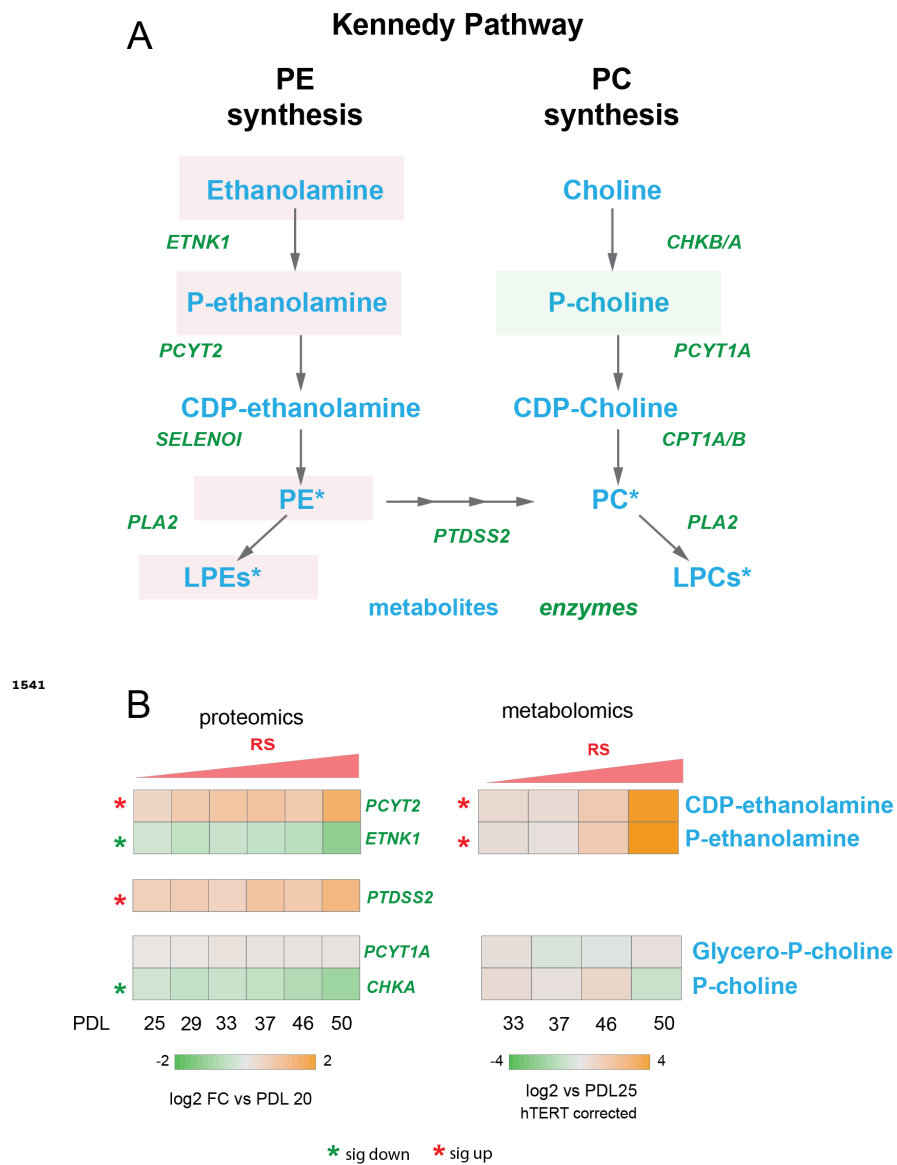


Figure 3-Figure supplement 5. Kennedy Pathway diagram. Metabolites in blue, proteins in italicized green. Heatmaps of log₂ fold changes for metabolites and proteins from A. Median htert corrected values for n=4 replicates are shown. Significant changes (FDR adjusted p<0.05 metabolomics, FDR adjusted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

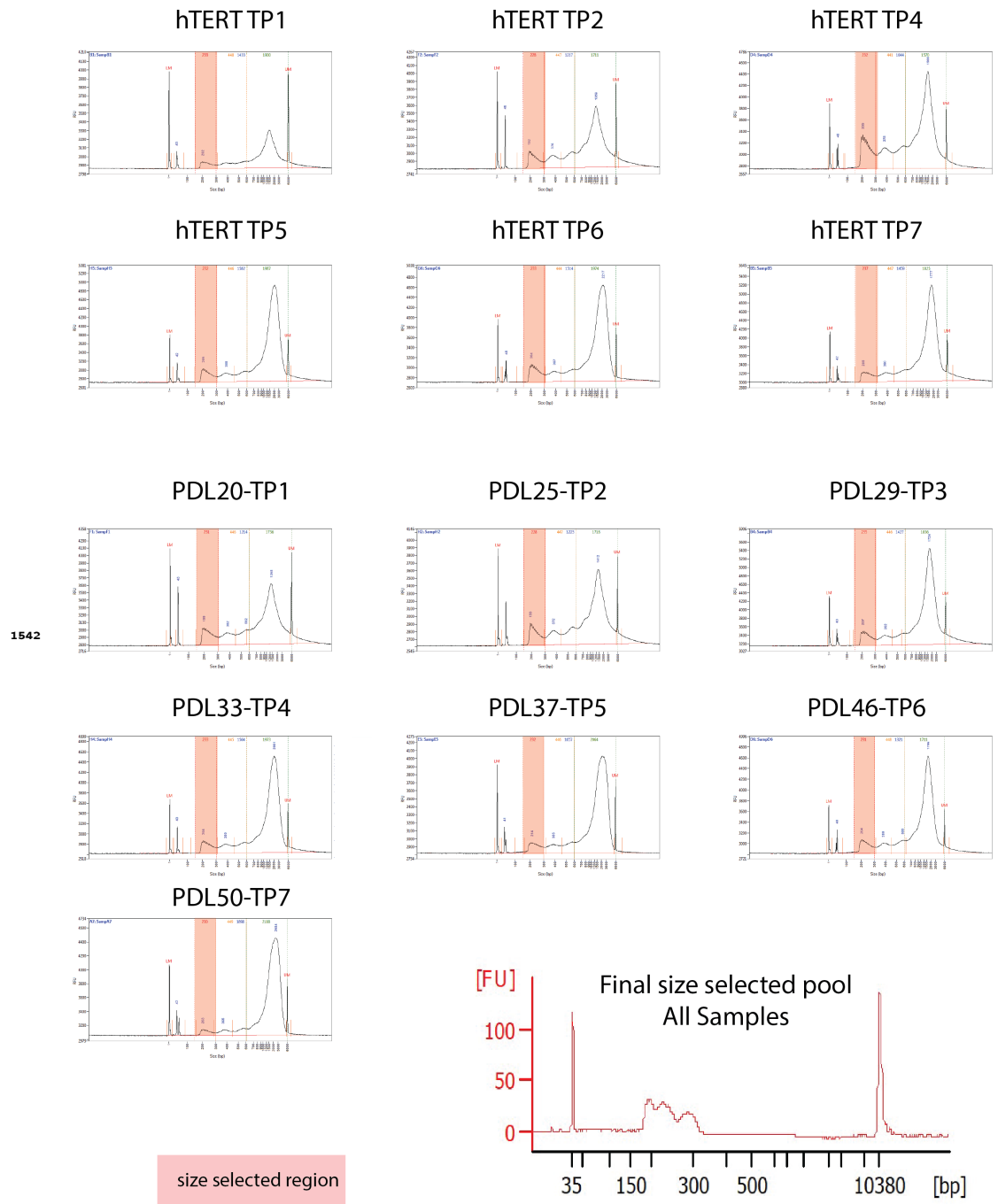
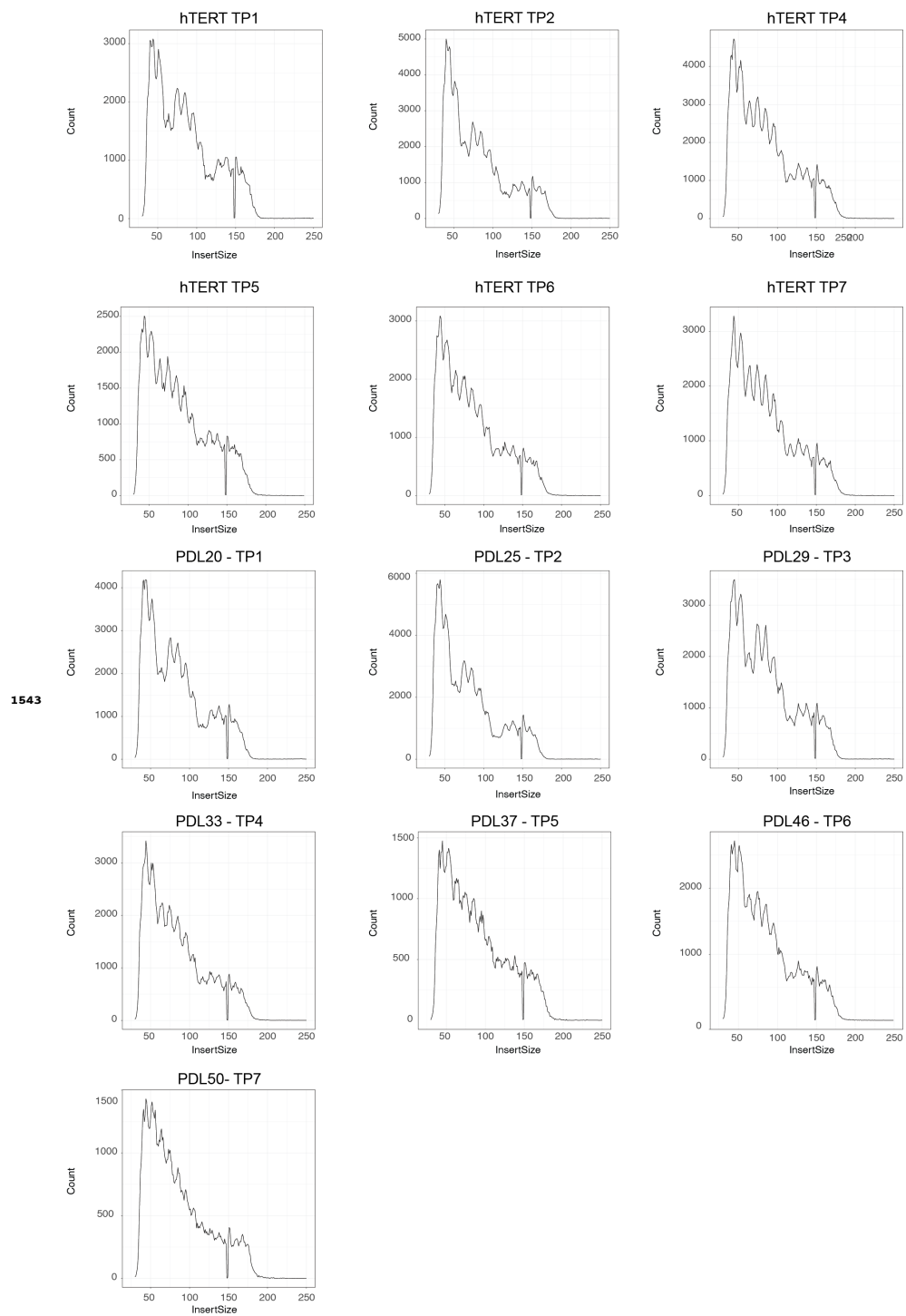
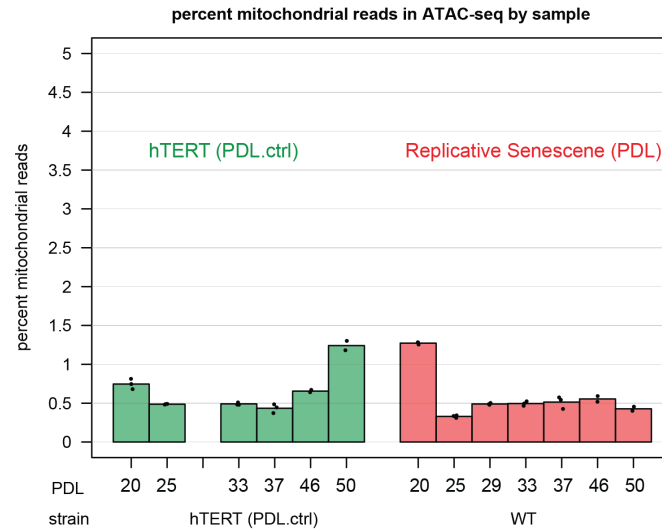


Figure 5-Figure supplement 1. ATAC-seq library fragment distribution and size selection.



A



B

1544

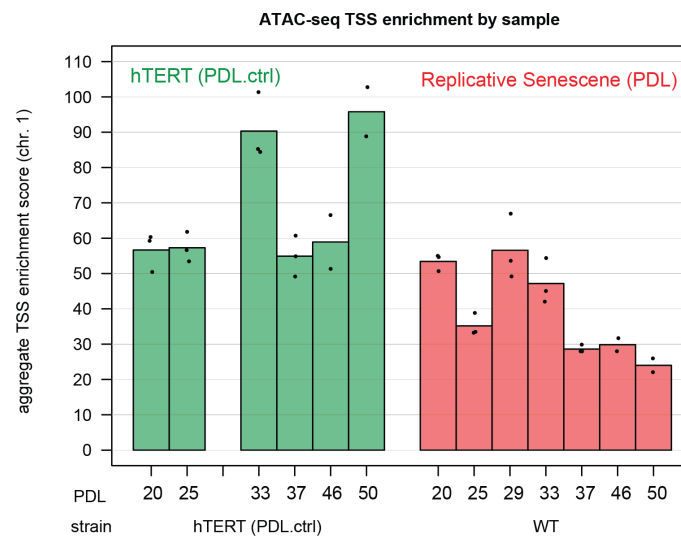


Figure 5–Figure supplement 3. ATAC-seq mitochondrial read percentages and ATAC-seq transcriptional start site enrichment. **(A)** Mitochondrial read percentages in sequenced and aligned ATAC-seq library for WT PDLs (red) hTERT time points (green) in triplicate. **(B)** Transcriptional start site enrichment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

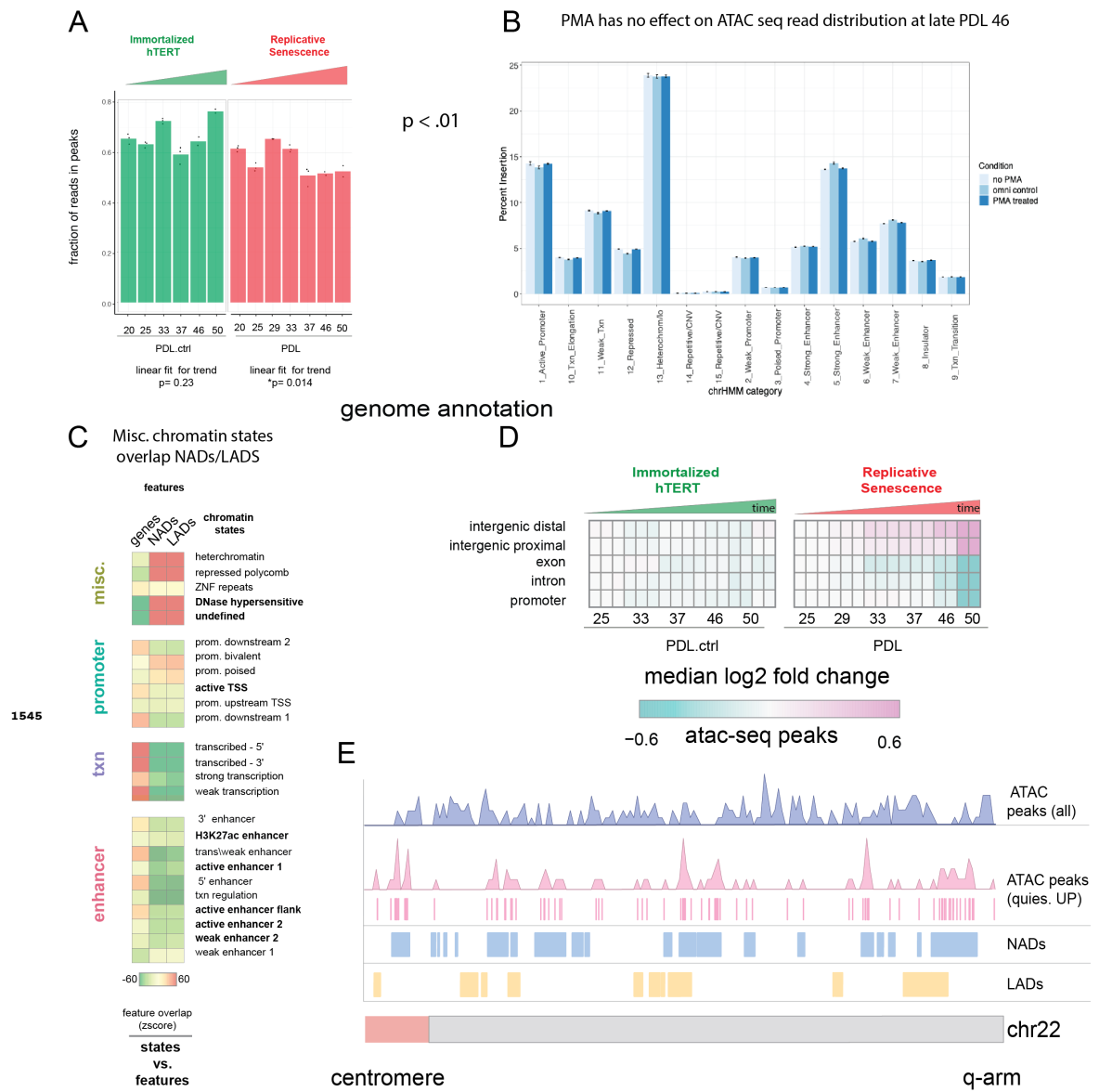


Figure 5-Figure supplement 4. ATAC-seq QC metrics, controls, and NADs/LADs browser shot. **(A)** Fraction of reads in peak by PDL (WT) and PDL.ctrl (hTERT). p values for linear fit over time are shown. WT cells exhibit a slight ~5% but significant reduction of fraction of reads in peaks. **(B)** PDL 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).

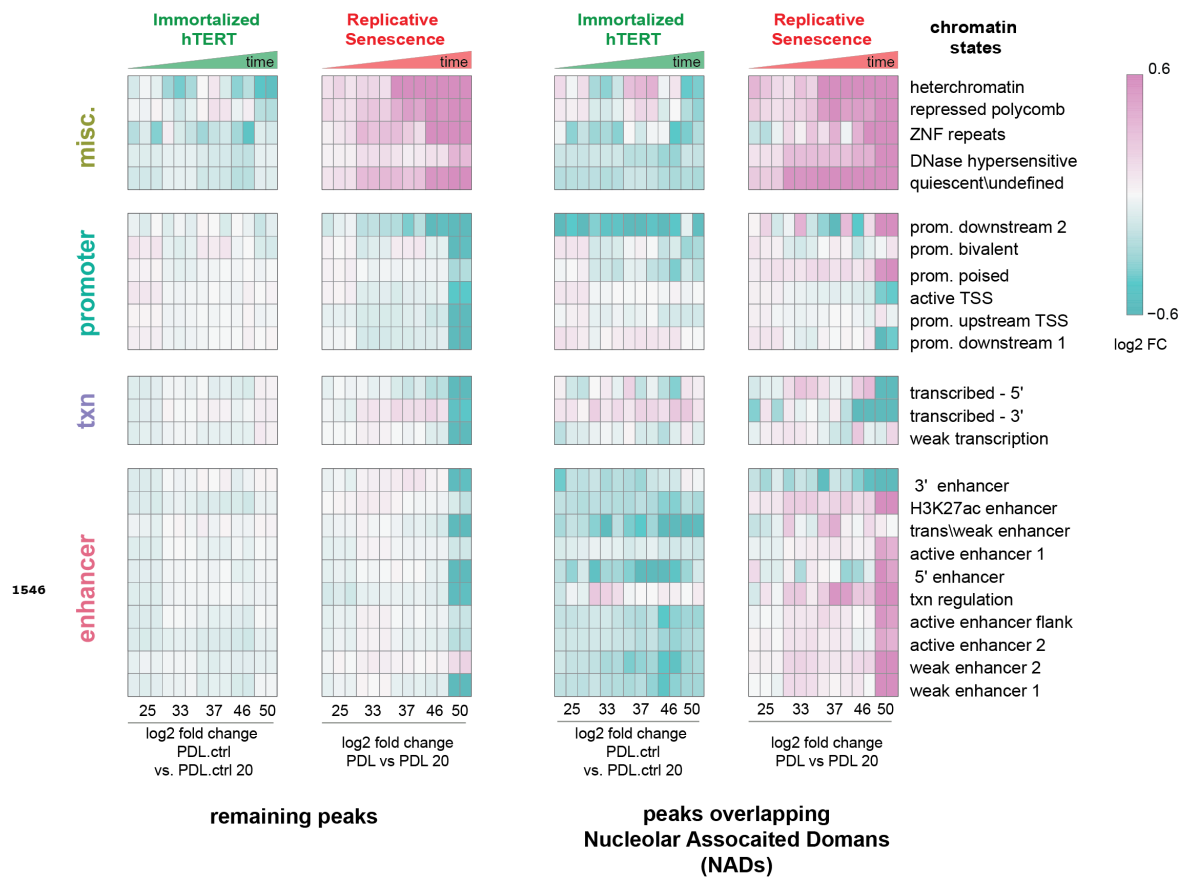


Figure 5-Figure supplement 5. Chromatin state profiles of ATAC-seq peaks in nucleolar associated domains (NADs) vs rest of the genome. Heatmap of all significant ATAC-seq peaks categorized by 25 chromatin states and then divided into peaks overlapping with NADs (right) and peaks excluded from NADs (left) 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.

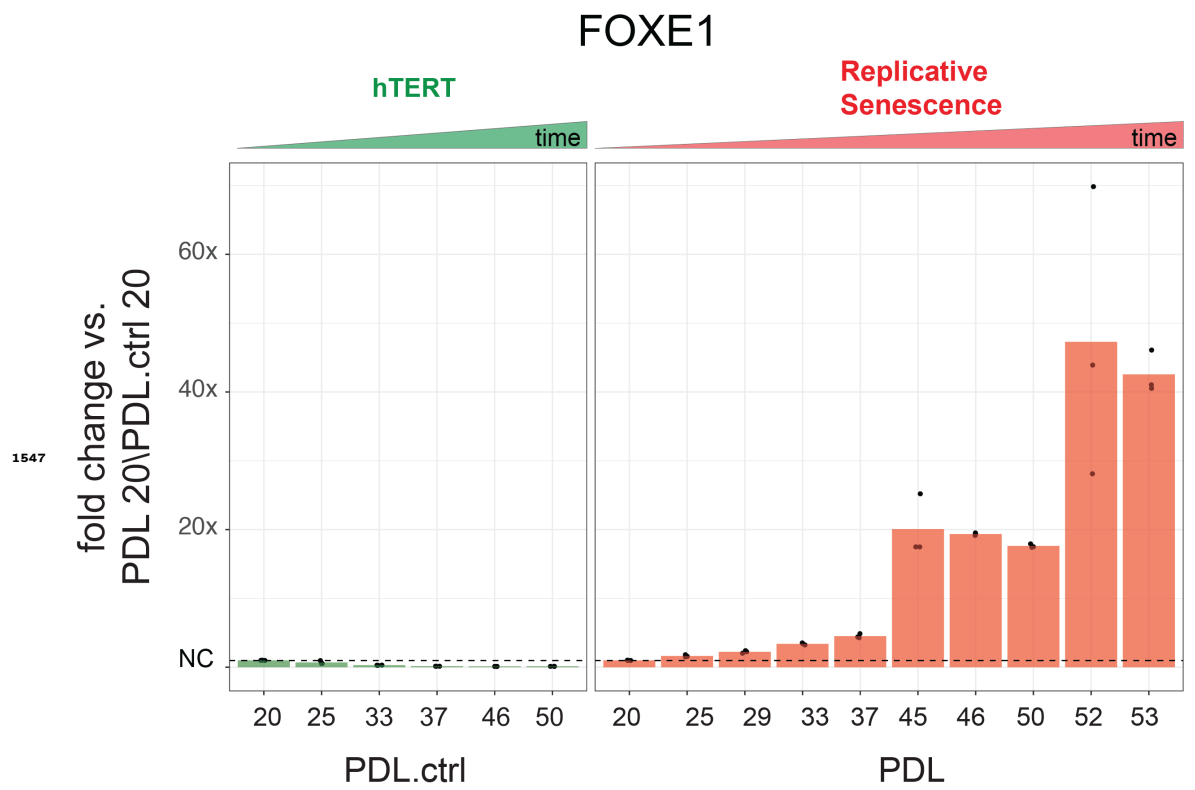


Figure 6–Figure supplement 1. Gene expression fold changes for the transcription factor FOXE1 across conditions. Gene expression fold changes (3 replicate average, each point a replicate) for the transcription factor FOXE1 in the hTERT and replicative senescence (RS) time courses.

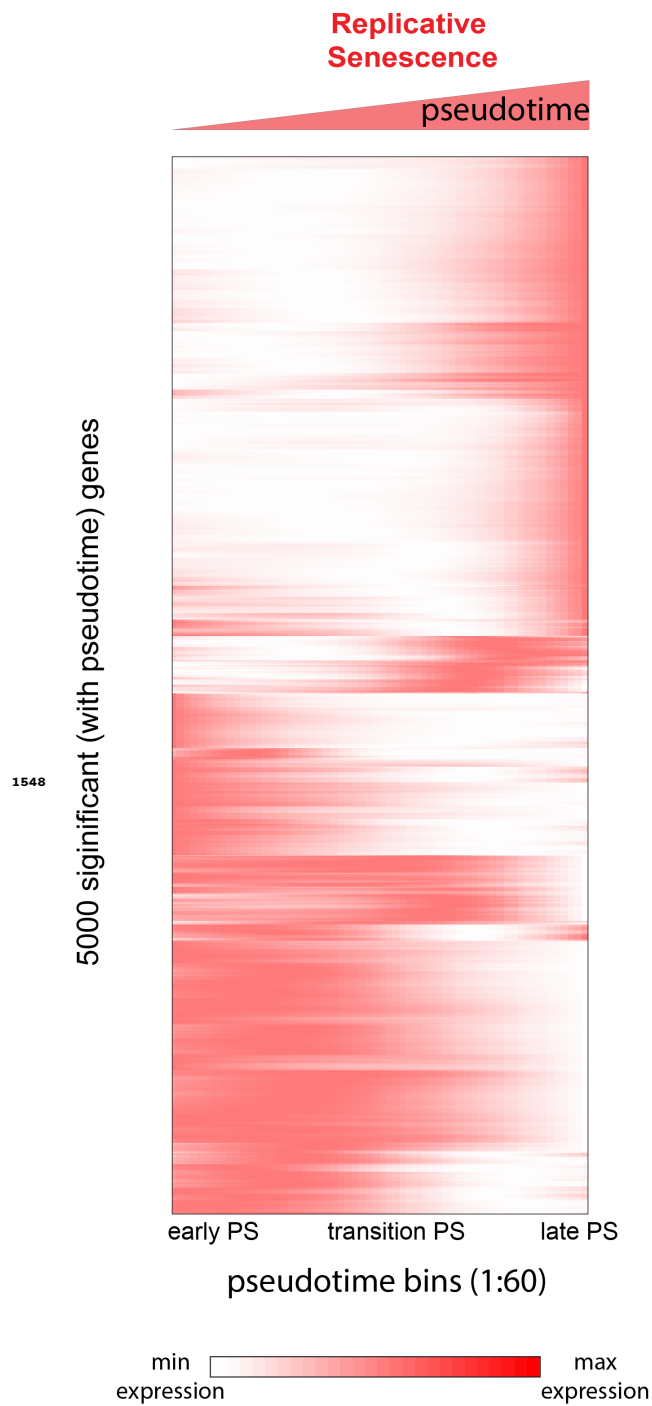


Figure 7-Figure supplement 1. Heatmap of gene expression changes for the top 5,000 significantly changing genes during replicative senescence pseudotime. Genes were hierarchically clustered, gene normalized and scaled (0-1) expression levels are depicted (white = low expression, red = high expression).

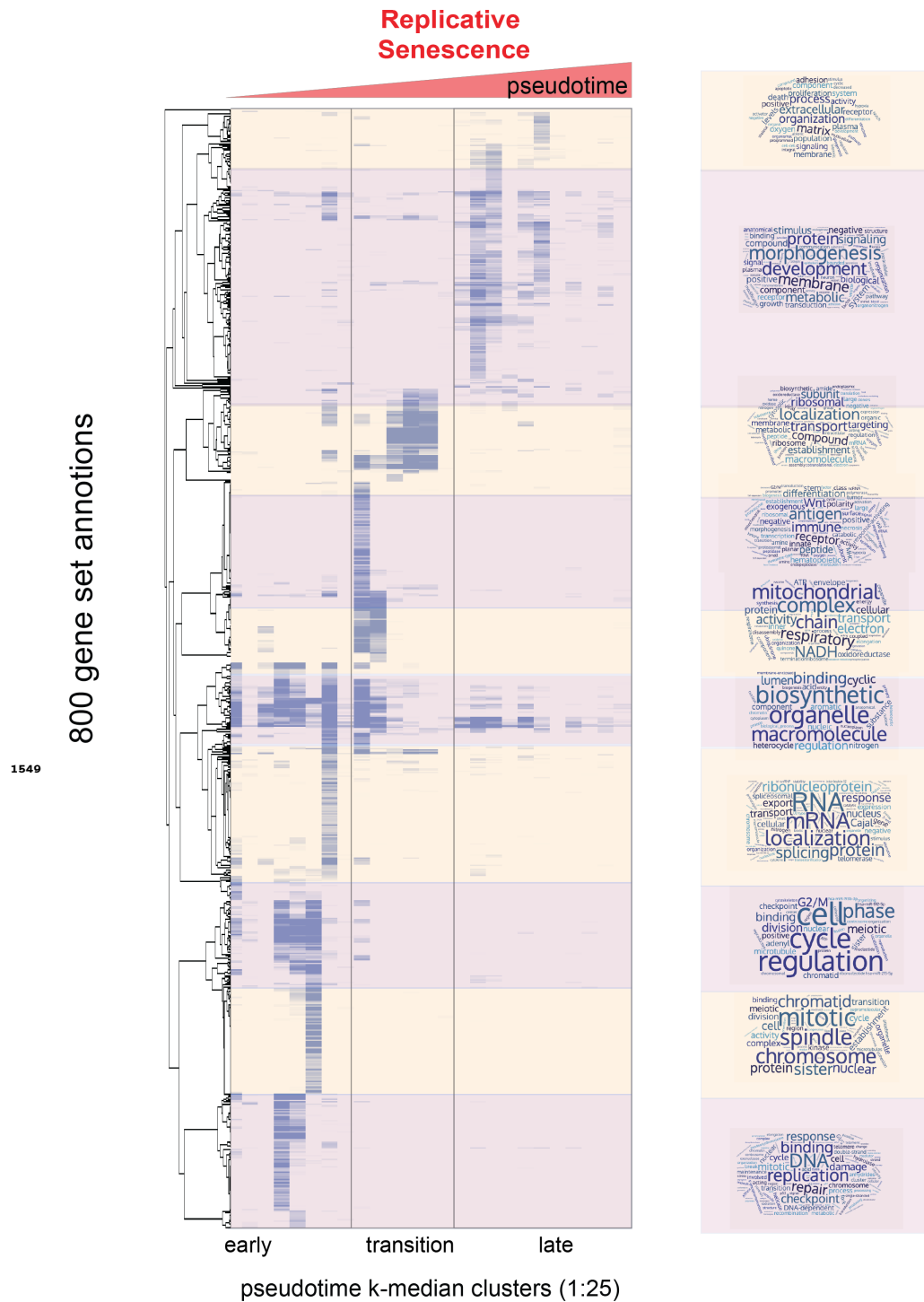
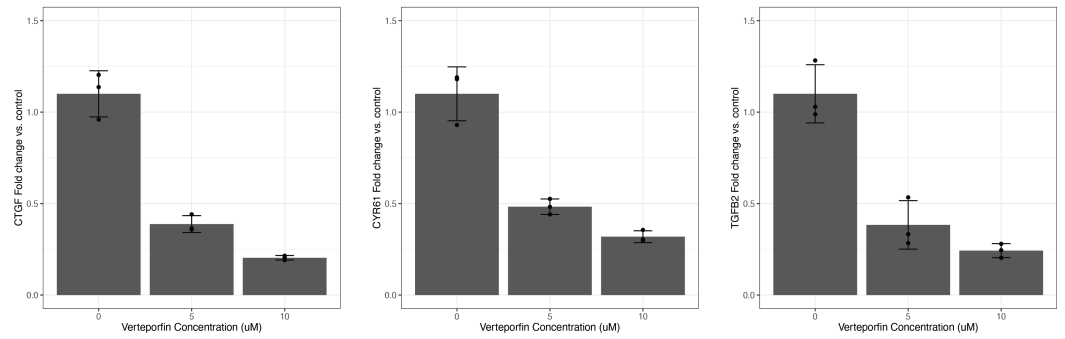
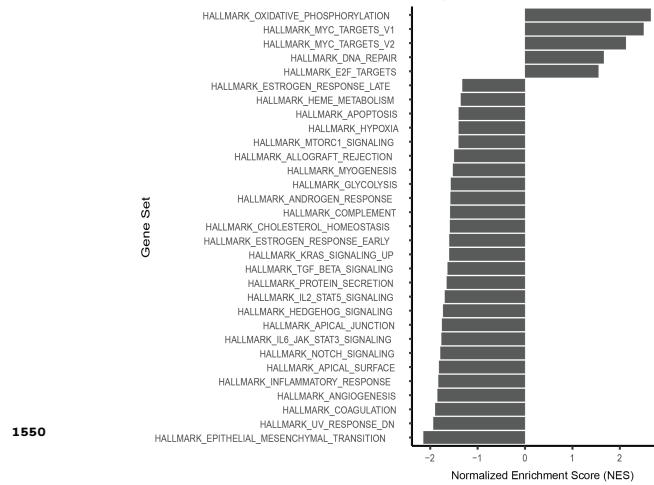


Figure 7-Figure supplement 2. Heatmap of enriched gene sets for replicative senescence pseudotime clusters (k=25). Heatmap of adjusted p-values for GO-term enrichment analysis on individual clusters from 6. Values plotted are row normalized $-\log_{10}$ p-value; light purple to dark purple. The ordered 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



1550

C Gene signature of VP treated cells and RS cells

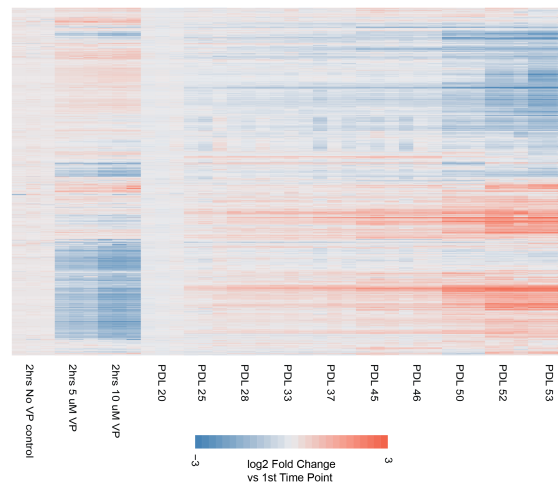


Figure 8-Figure supplement 1. Effect of inhibiting the YAP1/TEAD1 interaction with verteporfin treatment on WI-38 cells. **(A)** Barplots of the gene expression of 3 known YAP1 gene targets in verteporfin treated cells. Values plotted are fold changes relative to the mean of the control samples. Each 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.