

Human dermal fibroblast senescence drives increased protein synthesis, ribosome biogenesis, and adaptations of translation and metabolism

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Article

Keywords: cellular senescence, protein synthesis, ribosome biogenesis, senescence-associated secretory phenotype (SASP), skin aging

Posted Date: October 15th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11241215/v1>

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Additional Declarations: Competing interest reported. AT, FM, GG, NA, and YBK are full-time employees of CHANEL Fragrance & Beauty, 8 rue du Cheval Blanc 93500 Pantin, France. The remaining authors declare no conflicts of interest.

Abstract

Cellular senescence is characterized by permanent cell cycle arrest and a proinflammatory secretory phenotype called the SASP. Although senescent cells have traditionally been linked to reduced protein synthesis and ribosome biogenesis, the adaptations required to sustain the production and secretion of the SASP remain poorly understood. Herein, we compared translational dynamics among stress-induced senescent, contact-inhibited quiescent, and proliferating human dermal fibroblasts. Based on O-propargyl-puromycin labeling, nucleolar morphology assessment, polysome profiling, and metabolic assays, senescent cells exhibited increased global protein synthesis, supported by enhanced ribosome biogenesis *via* rDNA promoter derepression and nucleolar expansion. Polysome profiling revealed selective translation of transcripts related to secretion, autophagy, and energy metabolism. Metabolic analyses highlighted a shift toward mitochondrial oxidative phosphorylation to meet elevated ATP demands. These findings challenge the prevailing view that senescence is associated with translational decline and reveal that senescent cells undergo coordinated translational and metabolic adaptations to sustain their secretory functions, providing insights into senescence biology and identifying potential avenues for therapeutic intervention.

INTRODUCTION

Senescence is a cellular response to damage and stress that locks mitotically active cells into permanent proliferation arrest. This process is accompanied by a shift in cellular morphology toward an enlarged, flattened shape^{1,2}. Cellular senescence is widely considered a tumor-suppressing mechanism that prevents the propagation of cells with damaged genomes. Although senescent cells are nonproliferating, they are resistant to apoptosis³. This, alongside an imbalance between the generation and clearance of senescent cells by the immune system, leads to their accumulation with age^{4–10}. Many independent studies have observed senescent cell accumulation with aging across species, including mice, rats, baboons, rhesus macaques, and humans^{11–17}. Senescent cell accumulation in tissues is associated with various aging-related diseases, which are collectively termed “senopathies”¹⁸. For example, senescent cells are present within human atherosclerotic lesions¹⁹. In diabetes, these cells appear to contribute to tissue dysfunction²⁰, whereas in osteoarthritis, senescent chondrocytes have been found to accumulate in cartilage^{21,22}.

A defining feature of senescent cells is the secretion of factors comprising the senescence-associated secretory phenotype (SASP), which encompasses inflammatory cytokines, chemokines, growth factors, and metalloproteases^{23,24}. The SASP contributes to a microenvironment that supports tumor cell growth and migration²⁵. Additionally, SASP factors can affect adjacent nonsenescent cells and extracellular matrix, thereby triggering inflammation or fibrosis. Damage to adjacent healthy cells can be sufficiently severe to induce apoptosis or bystander senescence²⁶, further increasing the senescent burden of the tissue^{27,28}. Collectively, these processes result in a persistent proinflammatory environment that

contributes to the detrimental role of persistent senescent cells in aging and favors the development of age-related pathologies.

Therefore, cellular senescence has become an attractive target for therapeutic interventions in recent years. Experiments have revealed that the selective elimination of senescent cells can ameliorate certain age-related functional declines and extend lifespan and/or healthspan²⁹. Senotherapeutics, broadly categorized as either “senolytics,” which selectively kill senescent cells, or “senomorphics,” which modulate functions such as SASP and morphology of senescent cells to match those of young cells, have been evaluated in numerous studies in the context of different cell types and pathologies³⁰. To effectively identify new senotherapeutics, the differences between senescent cells and their nonsenescent counterparts must be clarified, and this task is complicated by the high degree of heterogeneity in senescence, also referred to as “senotypes”³¹.

Senescent cells are often described as dormant or “zombie” cells, giving the impression that they are inactive. Indeed, previous studies have revealed that protein production rates and ribosome biogenesis are decreased in several senescent cell types and across different senescent inducers compared with proliferating cells^{32,33}. Subsequent studies have further demonstrated that free ribosomal proteins can induce cellular senescence. For instance, accumulation of RPS14 (uS11) in the soluble nonribosomal fraction can inhibit CDK4, preventing Rb phosphorylation and thereby inducing cell cycle arrest and senescence in a p16^{INK4A}-like manner³⁴. Together, these findings have contributed to the prevailing model that senescence is accompanied by attenuated translational capacity and reduced metabolic activity. However, several observations challenge this paradigm. Senescent cells possess an enlarged cell volume and a hyperactive secretory phenotype, which would be expected to require high levels of *de novo* protein synthesis. Notably, proliferating cells, to which senescent cells are most often compared, have a highly active metabolism because of their constant demand for cell growth and subsequent division. Still, one report recently observed elevated global protein synthesis in oncogene-induced senescent IMR-90 lung fibroblasts over proliferating control cells³⁵. Whether phenotypic shifts in senescence are driven by transcriptional abundance or by selective regulation at the translational level still needs investigation.

In this study, we addressed these contradictions by comparing translational dynamics between stress-induced senescent primary human dermal fibroblasts (HDFs) and their contact-inhibited quiescent counterparts, a physiologically relevant non-proliferative state. Analyzing quiescent cells enabled us to investigate differences in total and specific protein synthesis, ribosome biogenesis, possible translational regulation, and shifts in global metabolic pathways attributable to cellular senescence, without the confounding metabolic demands of cell proliferation. Contact-inhibited HDFs are ideal for comparison, as these cells undergo a transient, fully reversible cell-cycle arrest³⁶ without being starved. As most somatic cells *in vivo* reside in non-proliferative states, this comparison provides a framework for understanding how senescence reshapes ribosome biogenesis, protein synthesis, and cellular metabolism independently of cell division³⁷.

RESULTS

Establishment and validation of a robust stress-induced senescence model in primary HDFs

To investigate changes in translational dynamics in cellular senescence, we first established a reliable model of stress-induced premature senescence (SIPS) in primary HDFs using the chemotherapeutic doxorubicin, which induces DNA double-strand breaks and oxidative stress. We treated the cells twice for 3 days each, followed by a 2-week recovery period to allow the cells to fully transition to the senescent phenotype. We tailored the concentration to each HDF strain derived from 3 individual tissue donors to account for interindividual variability. In addition to proliferating cells, we included contact-inhibited quiescent cells in our experiments to compare two distinct nonproliferating cellular states within the same donor strain (Fig. 1A).

To confirm the effectiveness and reliability of our protocol, we assessed whether a large proportion of cells had entered senescence. After the treatment, cells exhibited the enlarged, flattened morphology characteristic of cellular senescence. Although cells remained subconfluent throughout the 3-week treatment, we did not observe any morphologically mitotic cells in the culture. Conversely, proliferating HDFs exhibited the typical spindle-like morphology, and a large proportion of mitotic cells was detected. Contact-inhibited quiescent HDFs featured a similar morphology to proliferating cells. These cells appeared more tightly packed with an elongated shape, and no mitotic cells were observed (Fig. 1B).

Consistent with these observations, measurement of the incorporation of the thymidine analog bromodeoxyuridine (BrdU) revealed a marked reduction in DNA synthesis in both senescent and quiescent cells. Only 10% of senescent cells and 3% of quiescent cells incorporated BrdU, compared with 58% of proliferating cells, confirming that senescent cells and quiescent cells had stopped active proliferation (Fig. 1C).

Staining for senescence-associated β -galactosidase (SA- β -gal) activity, the most frequently used senescence marker¹², revealed intense blue staining in doxorubicin-treated cells, contradicting the findings in proliferating controls. Quiescent cells were also positive for SA- β -gal, consistent with previous findings that β -galactosidase activity is not entirely specific for cellular senescence, as it can also be induced by stressors such as prolonged confluence in culture (Fig. 1D)³⁸.

To confirm the expected mode of action of doxorubicin-induced senescence, we investigated DNA damage levels across different cell states. We stained for phosphorylated histone H2A.X (γ H2A.X), a DNA damage marker that has also been used as a senescence biomarker in various cell types^{38–43}. Indeed, senescent cells consistently included a significantly higher number of γ H2A.X foci per nucleus than quiescent and proliferating cells, suggesting that senescent cells retained elevated levels of unrepaired DNA damage (Fig. 1E, F). Our previous study using the same senescence model reported elevated p21 and reduced lamin B1 mRNA expression in senescent HDFs compared with both quiescent and proliferating cells⁴⁴, further validating the senescent phenotype.

Collectively, these results indicate that our doxorubicin treatment protocol reproducibly and uniformly induces cellular senescence in HDFs. The comparison with contact-inhibited quiescent cells provides a non-proliferative control, allowing us to distinguish senescence-associated changes in protein synthesis, ribosome biogenesis, and cellular metabolism from the general consequences of cell-cycle arrest.

Global protein synthesis is increased in cellular senescence

Upon entering senescence, cells must adapt their metabolism accordingly. Although stable proliferation arrest reduces the metabolic demand associated with continuous cell growth and division, senescent cells produce and secrete SASP components. Because SASP production increases metabolic demand, especially regarding *de novo* protein synthesis, we compared global translation rates in senescent, quiescent, and proliferating cells. Additionally, because senescent cells exhibit high heterogeneity^{45,46}, we investigated subpopulations with differences in translational behavior.

Therefore, we used O-propargyl-puromycin (OPP) pulse labeling coupled to single-cell flow cytometry to quantify total protein synthesis⁴⁷. We observed that protein production was on average 44.2% higher in senescent cells than in quiescent cells in three independent HDF donors (Fig. 2A). Compared with senescent cells, proliferating cells exhibited higher protein production, consistent with the elevated biosynthetic requirements associated with continuous growth and division. To account for heterogeneity across senescence models, we repeated the experiment using epithelial kidney cells and used alternative senescence induction methods. Specifically, we used doxorubicin to induce senescence in human renal proximal tubular epithelial cells (RPTECs; Fig. 2B). We also included HDFs driven into senescence by either serial passaging until they reached replicative senescence or repeated sublethal H₂O₂ stress (Fig. 2C, D). Compared with quiescent cells, protein synthesis was increased by 500% in senescent RPTECs, by 37% in H₂O₂-treated senescent HDFs, and by 12% in replicative senescent HDFs. Thus, a consistent increase in global protein production versus that in quiescent cells was observed for all tested cell types and senescence induction methods, indicating that increased protein production might be a universal feature of the senescence phenotype.

This increase in global protein synthesis could at least in part be attributable to the production and secretion of SASP factors. Several known senotherapeutic interventions modulate SASP factor secretion. Thus, we tested the senomorphics metformin and rapamycin, as well as mild methionine restriction^{48–52}. All three treatments failed to reverse the senescence-associated increase in protein production at the tested concentrations (Supplementary Fig. 1A). Global protein synthesis is also linked to cell size⁵³, and senescent cells are typically enlarged. To test whether cell size drives the senescence-associated increase in protein translation, we normalized the OPP signal to forward scatter, a proxy for cell size, and still observed a consistent increase in the protein production rate (Supplementary Fig. 1B). We speculated that the observed increase in global protein production might have resulted from decreased translational fidelity in cellular senescence⁵⁴. As a consequence of increased levels of misfolded, and thereby dysfunctional, proteins, senescent cells might increase global protein synthesis as a compensatory mechanism. We therefore measured protein aggregation in senescent and quiescent

HDFs using the dye ProteoStat⁵⁵, but detected no difference in the amount of misfolded proteins (Supplementary Fig. 1C). Taken together, these results indicated that SASP factor production, cell enlargement, or elevated protein misfolding alone cannot explain the senescence-associated increase in protein synthesis, and suggest broader senescence-associated translational adaptations.

We next asked when enhanced translational activity emerges during the transition in senescence. To examine this, we collected samples at eight time points during the 3-week senescence induction period and measured global protein synthesis using OPP incorporation. The samples displayed a sharp increase in protein production at the start of doxorubicin treatment (day 1), which flattened toward the end of doxorubicin incubation (day 8). Protein production progressively increased during the subsequent recovery period, until reaching its highest level at day 21. Conversely, protein production progressively decreased as confluence increased in quiescent cells (Fig. 2E). Additionally, we collected supernatant from quiescent and SIPS cells at three time points to measure the levels of the SASP factors MMP1 and IL-6⁵⁶. The secretion of these factors also increased during the senescence induction period (Supplementary Fig. 1D). In summary, senescence-associated increases in total protein synthesis begin early as a direct response to cellular stress. Increased protein synthesis continues, accompanied by SASP secretion and increasing cell size, throughout the transition into “deep senescence”.

Ribosome biogenesis increases in cellular senescence through ribosomal DNA (rDNA) promoter derepression

Having established that senescent cells exhibit elevated global protein synthesis, we hypothesized that they either require more productive ribosomes or increase ribosome biogenesis to expand the pool of active ribosomes. Ribosome biogenesis occurs in the nucleoli, and changes in nucleolar morphology have often been linked to alterations in their activity^{57–60}. Additionally, nucleolar size has been studied in the context of cellular senescence. Multiple studies have reported that senescent cells display only a single enlarged nucleolus per nucleus^{34,61,62}, prompting us to investigate whether senescence induction affects nucleolar morphology.

We stained senescent cells from three HDF donors for fibrillarin as a nucleolar marker. Compared with quiescent cells, the mean nucleolar area was enlarged by a factor of approximately 2.4–5.9 in senescent cells. We observed no significant difference in nucleolar size between quiescent and proliferating cells, indicating that the increase in nucleolar size was specific to senescence rather than the cell cycle state. We also counted the number of distinct nucleoli per nucleus. When combining the results for all three donors, the number of nucleoli was significantly lower in senescent cells than in quiescent ($p = 0.0015$) and proliferating cells ($p = 0.02$; Fig. 3A, Supplementary Fig. 2A).

The structure and morphology of nucleoli closely reflect their transcriptional activity and function^{59,62–64}. Nucleoli comprise three compartments: the fibrillar center (FC), on the edge of which RNA polymerase I transcribes the precursor of three of the four ribosomal RNAs (rRNAs); the dense fibrillar component (DFC), in which pre-rRNAs are subsequently processed and extensively modified; and the granular

component (GC), in which pre-rRNAs associate with ribosomal proteins before being released into the nucleoplasm. Using antibodies against UBF-1 (FC), fibrillarin (DFC), and B-23 (GC), we visualized the nucleolar structure in senescent, proliferating, and quiescent cells and observed apparent differences. Most strikingly, UBF-1 changed from a diffuse distribution throughout the nucleus in proliferating cells to a more condensed signal confined to the nucleoli in senescent cells. These changes were consistent with nucleolar reorganization toward a more transcriptionally active state (Fig. 3B).

To determine whether nucleolar remodeling translated into increased ribosome production, we quantified global RNA synthesis and rRNA abundance. In mammalian cells, Pol-I– and Pol-III–mediated RNA transcription accounts for 80% of all RNA production⁶⁵. Through pulse-labeling for 24 h using the uridine analog 5-ethynyl-uridine (5-EU), which labels nascent RNA but not DNA⁶⁶, we quantified overall RNA synthesis *via* flow cytometry. Senescent cells displayed a 2.1–2.4-fold stronger signal than quiescent cells (Fig. 3C). To test whether this increase is attributable to increased rRNA synthesis, we isolated RNA from the same number of cells in each cellular state. We then loaded this total RNA onto a denaturing agarose gel and quantified 18S and 28S rRNA abundance through densitometry. Senescent cells contained significantly higher amounts of both rRNAs than quiescent cells, indicating a higher number of mature ribosomes per senescent cell (Fig. 3D, Supplementary Fig. 2C). We also quantified total mRNA using MeTaFlow⁶⁷. We observed an increase in total mRNA content in senescent cells that was not rescued by exposure to the senomorphics metformin and rapamycin or mild methionine restriction (Supplementary Fig. 2B).

To investigate the mechanism underlying increased rRNA transcription in cellular senescence, we examined epigenetic silencing *via* methylation of the rDNA promoter. We isolated genomic DNA from cells under three different states, followed by digestion with a methylation-insensitive (MspI) or methylation-sensitive (HpaII) restriction enzyme⁶⁸. qPCR revealed that quiescent cells exhibited higher rDNA promoter methylation than proliferating or senescent cells (Fig. 3E). This finding, alongside the observed nucleolar expansion, suggests that rDNA promoters are derepressed in senescent cells, leading to more open chromatin at rDNA loci and increased rRNA synthesis.

To determine whether ribosomes are both more abundant and more translationally active in senescent cells, we performed an *in vitro* translation assay using equal amounts of ribosomes isolated from proliferating, quiescent, and SIPS HDFs and spiked into a ribosome-depleted HeLa cell extract. Quantification of the radioactive signal revealed no significant differences in translational activity among the three cellular states, although a slight, nonsignificant trend toward increased translation in senescent cells versus quiescence was observed in three of the four donors. These results indicate that ribosome productivity does not significantly differ among proliferating, quiescent, and SIPS cells (Fig. 3F), further suggesting that the senescence-associated increase in global protein synthesis is primarily attributable to increased ribosome abundance rather than increased specific activity.

Taken together, our results indicate that compared with quiescent cells, senescent HDFs exhibit extensive nucleolar remodeling, increased ribosome biogenesis, and derepression of rDNA promoters,

resulting in an expanded pool of translationally competent ribosomes that supports elevated protein synthesis.

Polysome profiling demonstrates selective translational remodeling supporting secretory and metabolic programs in senescent cells

Having established that senescent cells increase global protein synthesis through enhanced ribosome biogenesis independently of cell proliferation, we next investigated whether this expanded translational capacity preferentially supports specific cellular programs, such as SASP. We therefore performed polysome profiling to isolate and deep-sequence mRNAs associated with polysomes⁶⁹. For comparison with the whole transcriptome, we also isolated and sequenced total RNA from the same lysates. We reasoned that mRNAs associated with polysomes are translated efficiently, leading to high intracellular protein levels and influencing the cellular phenotype.

When comparing the polysome-enriched mRNAs to the transcriptomes of quiescent and senescent HDFs from two donors (HDF76 and HDF85) using Adata2seq⁷⁰, we identified 2258 differentially expressed mRNAs (1159 upregulated and 1099 downregulated, adjusted p-value < 0.05). Of these transcripts, 913 were upregulated and 823 downregulated exclusively at the transcriptomic level, whereas 222 were upregulated and 269 downregulated exclusively at the translational level. Conversely, only 24 mRNAs were significantly upregulated, and 7 mRNAs were downregulated when examining buffered transcripts. The abundance of these mRNAs changes exclusively at the transcriptomic level (Fig. 4A). The complete list of differentially expressed mRNAs is provided in the Supplemental Data. Thus, cellular senescence reshapes gene expression at both the transcriptional and translational levels, with a distinct subset of mRNAs regulated specifically through differential polysome recruitment.

To better understand these changes, we examined the mechanisms underlying differential mRNA recruitment to translating ribosomes in senescent cells. Because 5' UTR architecture is an important determinant of translation initiation^{71,72}, we compared 5' UTR length, nucleotide composition, and upstream open reading-frame content between translationally upregulated and downregulated transcripts. We found that mRNAs translationally downregulated in senescent cells had 5' UTRs that were longer than the average 5' UTR length of all expressed mRNAs. Conversely, translationally upregulated mRNAs displayed the opposite trend (Fig. 4B). We observed no significant differences in nucleotide composition of 5' UTRs, except that the number of cytidines in mRNAs translationally upregulated in senescent cells was significantly higher than the average value (Supplementary Fig. 3C–F). Upstream open reading frames (uORFs) in a strong Kozak context are known to decrease translational efficiency^{73,74}. Indeed, mRNAs translationally repressed in SIPS cells were enriched for uORFs in strong Kozak contexts, whereas translationally upregulated mRNAs exhibited the opposite pattern (Fig. 4C). Thus, both 5' UTR length and uORF presence are potential determinants of selective mRNA translation during cellular senescence.

To investigate the functional consequences of these gene expression changes, we analyzed the enrichment of Gene Ontology (GO) terms in our dataset using the fgsea package⁷⁵. Pathways associated with autophagy and secretion were among the main GO terms translationally upregulated in SIPS cells. These changes were consistent with the increased protein secretion observed in senescent cells. Conversely, pathways associated with cell cycle regulation and RNA processing were among the main downregulated GO terms (Fig. 4D), reflecting the epigenetic changes and stable cell cycle arrest characteristic of senescent cells. At the transcriptomic level, similar GO terms were up- and downregulated (Supplementary Fig. 3A). Lastly, analysis of the small fraction of buffered mRNAs revealed that pathways involved in synaptic signaling, memory, and ion transport were upregulated, whereas those involved in translation and mitochondrial gene expression were downregulated (Supplementary Fig. 3B).

To further investigate if the changes to the translome are associated with senescence, we assessed the enrichment of the SenMayo gene set, a collection of genes associated with cellular senescence⁷⁶. Indeed, we observed significant enrichment of SenMayo genes in the translome [normalized enrichment score (NES) = 1.835], confirming that doxorubicin exposure induces a robust senescence-specific gene expression signature in HDFs (Fig. 4E). In addition, other important senescence-related genes such as MMP3, LAMP1, LAMP2, ITGB3, PAI-2/SERPINB2, and PERP were significantly upregulated at the translome level in SIPS cells.

We further reasoned that the senescence-associated increases in global protein synthesis and ribosome biogenesis would likely require a concomitant increase in ATP production, as approximately half of a cell's total ATP turnover is devoted to protein synthesis⁷⁷. Analysis of the "ATP metabolic process" (NES = 1.615) and "glycolytic process" (NES = 1.925) revealed significant enrichment of these pathways in senescent cells (Fig. 4F, G), whereas "oxidative phosphorylation" showed a positive but nonsignificant trend (NES = 0.884; Fig. 4H). Consistent with these findings, an increase in mitochondrial number has previously been reported in senescent cells⁷⁸. Thus, senescent cells appear to upregulate pathways linked to ATP metabolism and glycolysis to compensate for the increased energy demands associated with elevated protein synthesis.

Senescent cells adapt their metabolism to sustain increased biosynthetic demand

Having identified increased protein synthesis and ribosome biogenesis as defining features of senescence, we next asked how senescent HDFs remodel their energy metabolism to sustain their elevated translational activity. Thus, we used the SCENITH method to further investigate the pathways utilized by senescent cells for ATP production compared with those utilized by proliferating and quiescent cells⁷⁹. By selectively blocking glycolysis (2-deoxy-D-glucose) or oxidative phosphorylation (oligomycin A) and quantifying OPP incorporation, we assessed the relative contribution of distinct metabolic pathways to the energy required for protein production in proliferating, quiescent, and senescent cells. After normalization to untreated cells, we calculated glycolytic capacity and fatty acid/amino acid oxidation (FAO/AAO) capacity. Glycolytic capacity represents the percentage of protein

production that can be maintained when cells are restricted to glycolysis, whereas FAO/AAO capacity represents the percentage that can be maintained when cells rely exclusively on oxidative phosphorylation.

We observed differences in the effects of 2-deoxy-D-glucose, which inhibits glycolysis, and oligomycin A, which inhibits oxidative phosphorylation, across the three cellular states, indicating a senescence-associated shift in metabolic pathway utilization (Fig. 5A). More specifically, senescent cells exhibited lower glycolytic capacity (Fig. 5B) but higher FAO/AAO capacity (Fig. 5C) than quiescent cells, indicating a greater reliance on oxidative phosphorylation. Consistent with these findings, senescent cells exhibited a significantly stronger MitoTracker signal than quiescent cells, as measured *via* flow cytometry (Fig. 5D), potentially indicating greater mitochondrial mass or a higher mitochondrial membrane potential. Conversely, quiescent cells showed a preference for glycolysis for ATP production. Overall, senescent cells maintained a higher rate of protein production than proliferating and quiescent cells regardless of the metabolic pathway inhibited. This finding may indicate greater metabolic flexibility in meeting energy demands during cellular senescence. Collectively, these findings show that senescent cells undergo metabolic remodeling to support the energetic demands of elevated protein synthesis.

DISCUSSION

Although senescent cells have traditionally been considered inactive biological entities that accumulate with age⁸⁰, research by our group and others has shown that these cells are highly active, producing and secreting proinflammatory and tissue-remodeling factors that induce bystander senescence in neighboring cells, promote inflammaging, and actively contribute to age-related pathologies^{23,80–82}. Consistent with this notion, we observed a global increase in protein synthesis in senescent HDFs, which appeared to be linked to SASP production. Remarkably, elevated protein synthesis may represent a common feature of cellular senescence, as we observed a similar phenotype in replicative and H₂O₂-induced senescence, as well as in senescent renal epithelial cells. A deeper understanding of the mechanisms underlying this increase in protein synthesis could facilitate the development of a new generation of senotherapies that specifically target elevated protein production in senescent cells.

Our findings suggest that the prevailing view that cellular senescence is invariably associated with reduced global protein production and impaired ribosome biogenesis should be reconsidered. In the senescence models examined here, senescent cells exhibited increased global protein synthesis, RNA transcription, and ribosome biogenesis. Differences between our findings and those of previous studies may be attributable to the choice of non-senescent controls and the time point of analysis after senescence induction. We included contact-inhibited quiescent cells to enable a more physiologically relevant comparison by reducing the confounding effects of proliferation. In addition, quiescent cells represent the majority of cells in the human body. Conversely, previous studies have predominantly compared senescent cells with proliferating cells. Indeed, although assays in proliferating cells showed some variability, potentially reflecting differences in cell-cycle state, ribosome biogenesis and global protein synthesis were consistently reduced in quiescent cells, irrespective of the donor. Our findings

therefore suggest that comparisons with proliferating cells may have obscured important anabolic features of senescence by confounding senescence-associated adaptations with the biosynthetic demands of cell growth and division.

We further analyzed the cells 2 weeks after senescence induction to ensure that they had entered a stable, uniform senescent state and to minimize the potential influence of acute stress responses or ongoing cellular adaptation. This approach is supported by studies demonstrating that cells undergo extensive changes during the transition from early to late or deep senescence. In deep senescence, autophagy increases, and both the composition and abundance of the SASP change^{45,83}. Accordingly, in our model, we observed a steady increase in protein production during senescence induction, reaching a plateau after 2 weeks. These observations indicate that enhanced protein synthesis is not merely an acute stress response to doxorubicin exposure but rather a progressive feature acquired during the establishment of a deep senescent phenotype.

The senescence-associated increase in global protein production requires enhanced ribosome biogenesis, which is accompanied by changes in nucleolar structure. Conversely, acute nucleolar stress and inhibition of ribosome biogenesis have previously been linked to the induction of oncogene-induced and replicative senescence^{34,62}. Further studies are needed to determine whether senescence induced by nucleolar stress or free ribosomal proteins is also associated with increased protein synthesis despite altered ribosome biogenesis, which mRNAs are preferentially translated under these conditions, and how the composition of the SASP is affected.

Our analysis of mRNAs actively translated by polysomes and total mRNAs showed that most mRNAs encoding SASP factors and other senescence-related genes are already upregulated at the transcript level under senescence. This is consistent with the idea that deep senescence, once established, represents a stable cellular state that can be maintained for extended periods⁸⁴. However, regulation of gene expression by selective mRNA translation is frequently observed as a rapid response to acute stress⁸⁵. The enriched GO terms in the translome were largely consistent with known changes in senescent cells, namely the upregulation of secretion- and autophagy-related genes and downregulation of cell cycle, DNA replication, and RNA processing genes. However, a subset of genes was exclusively regulated at the translation level in senescent cells. These results indicate that senescent cells use their expanded translational capacity unevenly, preferentially directing it toward secretory, autophagic, and stress-adaptation programs. Our findings support the view that the selection of these specific transcripts for translation is based on their 5' UTR composition. Reduced 5' UTR length and abundant uORFs in a strong Kozak context can alter initiation dynamics^{71,74}. Shorter 5' UTRs are easier to scan, allowing ribosomes to reach the start codon more quickly and begin protein synthesis. Conversely, longer 5' UTRs, especially when containing uORFs in a strong Kozak context, might act as a roadblock that traps scanning ribosomes. Consequently, mRNAs lacking these inhibitory features bypass translational bottlenecks and ensure efficient production of some key senescence proteins, such as MMP3, LAMP1, LAMP2, ITGB3, PAI-2/SERPINB2, and PERP. A potential limitation of our study is that cellular senescence

can lead to alternative transcription start site usage, which can alter 5' UTR length and composition^{86,87}. Although our length-matched controls confirmed robust uORF enrichment, future studies should combine polysome profiling with 5' end sequencing to provide deeper insights into condition-specific 5' UTR remodeling.

The substantial increase in protein synthesis and ribosome biogenesis observed in senescent cells would be expected to impose considerable energetic demands. Consistent with this notion, we also observed changes in energy metabolism, mainly an enrichment of ATP production pathways. We noted a greater reliance on oxidative phosphorylation than on glycolysis in our senescent HDFs. Accordingly, we observed an elevated MitoTracker signal in senescent cells compared with quiescent cells. Together, these findings suggest that senescent HDFs increase mitochondrial content or membrane potential and shift ATP production toward oxidative phosphorylation. These data support a model in which translational and metabolic reprogramming are tightly coordinated during senescence, ensuring that increased biosynthetic demands can be sustained.

The current study has several limitations, including its primary focus on a single senescence model, namely doxorubicin-induced senescence in HDFs. Given the substantial heterogeneity of cellular senescence⁸⁸, whether elevated ribosome biogenesis and global protein synthesis represent general hallmarks of senescence remains to be systematically investigated across a broader range of senescence inducers, cell types, and *in vivo* contexts. Moreover, it remains unclear whether the overall increase in translation is associated with reduced ribosomal fidelity, which could ultimately induce proteotoxic stress, potentially contribute to the increased autophagy observed in deep senescence⁸³, and further reinforce the senescent state.

Collectively, our findings challenge the long-standing view that cellular senescence is associated with a generalized decline in anabolic activity. Instead, we propose that senescent cells engage a coordinated program of translational, nucleolar, and metabolic remodeling that provides the capacity required to sustain the senescent phenotype. This reprogramming may represent a previously underappreciated opportunity that could be exploited for the development of next generation senotherapeutic interventions.

MATERIALS AND METHODS

Cell culture

HDFs

HDFs were obtained from Evercyte GmbH (Vienna, Austria). The characteristics of the donors are summarized in Table 1, with “PD” referring to population doublings. Cells were cultivated in DMEM/Ham’s F12 1:1 medium (Sigma-Aldrich, St. Louis, MO, USA; #D6421, 500 mL) supplemented with 10% FCS (Sigma-Aldrich; #7524), 4 mM L-glutamine (Sigma-Aldrich; #G7513), and 1 mL of 100 µg/mL Primocine (InvivoGen, San Diego, CA, USA; #ant-pm-2) at 37°C under a 7% CO₂ atmosphere and 90%

humidity. Cells were tested for mycoplasma at regular intervals. Before reaching confluence, proliferating HDFs were detached *via* incubation with StemPro Accutase (Thermo Fisher Scientific, Waltham, MA, USA; #A1110501, 100 mL) at 37°C for 5 min and then passaged at a 1:2 to 1:3 ratio approximately twice per week.

Proliferating cells were seeded at the same time and density as the senescent cells and either passaged every 2–3 days until harvest of all cell types (Fig. 2A) or harvested 24 h after seeding (Figs. 3B, 5A–C). Quiescent cells were seeded at the same time and density as the senescent cells. The medium was changed every 3 days. Cells were harvested together for all cell types.

Table 1
HDF donor details

Donor number	Donor age (years)	Donor sex	PD for seeding (SIPS)
HDF76	65	Female	20–30
HDF85	49	Female	13
HDF161	58	Female	15
HDF164	Unknown	Female	19.5

RPTECs

Primary RPTECs were supplied by Evercyte GmbH. The cells were cultivated in ProxUP medium (Evercyte; #MHT-003) at 37°C under a 7% CO₂ atmosphere and 90% humidity. Cells were tested for mycoplasma contamination at regular intervals. RPTECs were passaged at a 1:2 ratio approximately twice per week.

Induction of SIPS with doxorubicin

We induced senescence using doxorubicin hydrochloride (Sigma-Aldrich; #D1515). Cells were treated with 100–250 nM doxorubicin twice (days 1 and 4) for 3 days each⁴⁴. We adjusted the drug concentration for each donor to achieve stable cell cycle arrest. We examined the cells optically and proceeded to a 2-week recovery period only when no mitotic cells remained. The images shown in Figs. 1B and 2E were acquired using a Nanolive 3D Cell Explorer microscope (Nanolive, Tolochenaz, Switzerland).

Induction of replicative senescence

HDFs were passaged once or twice a week until no proliferation could be detected for 2 weeks.

Induction of SIPS with H₂O₂

SIPS induction with H₂O₂ was performed as previously described⁸⁹.

SA- β -Gal staining

SA- β -gal activity was detected *via* X-Gal staining using standard protocols^{12,90}. Images were captured at $\times 100$ magnification.

Immunofluorescence

Fibroblasts were seeded into a four-well μ -slide (ibidi, Gräfelfing, Germany; #80426) or on coverslips in six-well plates, and SIPS and quiescent cells were prepared as described above. After the recovery phase, proliferating cells were seeded and incubated for 2 days. Cells were then washed with PBS, fixed with 2% formaldehyde in PBS for 10 min at room temperature, and permeabilized with 1% Triton X-100 in PBS for 15 min at room temperature. For fibrillarin and γ H2A.X staining, samples were incubated for 1 h at room temperature with primary antibodies against fibrillarin (Abcam, Cambridge, UK; #ab5821, 1:500, rabbit) and γ H2A.X (Abcam; #ab26350, 1:500, mouse), respectively, both diluted in PBS containing 10% FCS. The cells were then washed three times with PBS and incubated for 1 h at room temperature with secondary antibodies labeled with Alexa Fluor 647 (anti-mouse, Jackson ImmunoResearch, West Grove, PA, USA; #715-605-150) or Alexa Fluor 488 (anti-rabbit, Jackson ImmunoResearch; #715-545-152). DNA was stained with 0.1 μ g/mL DAPI in PBS for 10 min at room temperature. Images were obtained on a wide-field fluorescence microscope [DMI6000B (Leica Microsystems, Wetzlar, Germany) equipped with EL6000 fluorescent lamp (Leica Microsystems) and DFC360 FX CCD camera (Leica Microsystems)] using a $\times 63$ glycerol immersion objective (HCX PL APO 63x/1.30 Gly, Leica Microsystems). Images were then analyzed using ImageJ/Fiji (version 1.54; US National Institutes of Health, Bethesda, MD, USA) and batch-processed using a macro. The DAPI signal was used to localize nuclei *via* thresholding. Subsequently, for each nucleus, the size and number of fibrillarin-positive foci and the number of γ H2A.X-positive foci were determined *via* thresholding.

For nucleolar structure staining, samples were incubated for 1 h at room temperature with primary antibodies against B23 (Sigma-Aldrich; #B0556-100UL, 1:500) and UBF 1 (Thermo Fisher Scientific; #PA5-82245, 1:200), both diluted in PBS containing 10% FCS. Cells were washed again and incubated for one hour at room temperature with secondary antibodies labeled with Alexa Fluor 647 (anti-rabbit, Jackson ImmunoResearch; #711-605-152) or Alexa Fluor 594 (anti-mouse, Jackson ImmunoResearch; #715-585-150). After incubation for 1 h at room temperature with a fluorescently labeled antibody against fibrillarin (Abcam; #ab184817, 1:100), DNA was counterstained with DAPI (0.1 μ g/mL in PBS, 10 min at room temperature). Images were recorded and analyzed as described above.

BrdU staining

BrdU staining was described as previously described⁸².

Quantification of global protein synthesis using OPP

Global protein synthesis was measured as previously described⁴⁷. To quantify OPP labeling, cells were analyzed using a flow cytometer (Beckman Coulter, Brea, CA, USA; Cytoflex S V4-B2-Y4-R3, #B75811). Single cells in the G1 and G2 phases were selected based on forward and side scatter and DAPI staining. Fluorescence intensity was analyzed using Kaluza 1.2 (Beckman Coulter) or FlowJo 10.10 (BD, Franklin Lakes, NJ, USA), and the data were then exported for plotting in R (R Foundation for Statistical Computing, Vienna, Austria) using the ggplot2 package.

Time course of senescence induction

Samples for each time point were seeded on the same day in six-well plates (day 0). Proliferating samples were harvested on day 1. SIPS was induced with doxorubicin as described above. SIPS and quiescent samples were harvested at eight different time points over 3 weeks (days 1, 2, 4, 6, 8, 10, 14, and 21). Protein production was measured using OPP as described above. Additionally, we used a Cy5-labeled mRNA probe (MeTaFlow) to quantify mRNA levels in cells⁶⁷. Supernatant was collected on days 1, 2, 14, and 21 after 24 h of incubation and analyzed using Human MMP1 and Human IL6 ELISA kits (Thermo Fisher Scientific; #EHMMP1X5 and #EH2IL6) according to the manufacturer's protocol.

PROTEOSTAT assay

Samples for each condition were harvested using StemPro, transferred to ice-cold EBC buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.5% NP-40; 1× protease/phosphatase inhibitor cocktail; Cell Signaling Technologies, Danvers, MA, USA; #58725), and lysed using a Qsonica Q700 sonicator (Qsonica, Newtown, CT, USA) (amplitude = 50, process time = 40 s, pulse on time = 10 s, pulse off time = 10 s). Protein levels in all samples were measured using the Bradford Protein Assay Kit (Thermo Fisher Scientific; #23200) according to the manufacturer's protocol. Equal amounts of protein from each experimental condition were used for the PROTEOSTAT protein aggregation assay (Enzo, Farmingdale, NY, USA; #ENZ-51023-KP002). Protein aggregation was measured (excitation = 550 nm, emission = 650 nm) according to the manufacturer's protocol using a Synergy HT plate reader (Agilent, Santa Clara, CA, USA).

SCENITH analysis of energy metabolism

Samples were seeded in six-well plates. Proliferating cells were treated, harvested, and fixed with 70% EtOH at – 20°C on day 1. SIPS and quiescence were induced as described above. After 3 weeks, the samples were treated with inhibitors, harvested, and fixed. Treatment was performed as described previously⁷⁹ with an incubation time of 1 h for each inhibitor. Specifically, 100 mM 2-deoxy-D-glucose (Sigma-Aldrich; #D6134) and 1 µM oligomycin A (Sigma-Aldrich; #75351), both alone and in combination, were used. OPP was added to the samples during the last 30 min of incubation. Samples were harvested, and protein production was analyzed by Click labeling of OPP as described above. Relative protein production rates and glycolytic and FAO/AAO capacities were calculated as described previously⁷⁹ using Prism 10.6.1 (GraphPad, Boston, MA, USA).

Quantification of 18S and 28S rRNA

Cells were washed twice with PBS and detached with trypsin. Medium (containing FCS) was added to inactivate trypsin, and the number of cells per milliliter of medium was determined using a TC-10 cell counter (Bio-Rad, Hercules, CA, USA). In total, 100,000 cells from each condition were transferred to 1.5-mL tubes, pelleted ($200 \times g$, 5 min), washed once with PBS, lysed in 1 mL of TRI Reagent (Zymo Research, Irving, CA, USA), incubated at room temperature for 5 min, and stored at -80°C .

One milliliter of cell lysate in TRI reagent was slowly thawed on ice. Then, 200 μL of chloroform were added, the samples were mixed, and the phases were separated by centrifugation ($15,000 \times g$, 15 min, 4°C). The upper phase (approximately 400 μL) was transferred to a new tube containing 500 μL of isopropanol, 10 μL of 3 M NaOAc, and 1 μL of Glycoblu (Thermo Fisher Scientific; #AM9515), and the RNA was precipitated at -20°C overnight. The next day, RNA was pelleted ($15,000 \times g$, 1 h, 4°C); isopropanol was removed; and the pellet was washed once with 75% EtOH, pelleted again, dried at room temperature for 5 min, and then dissolved in 6 μL of sterile nuclease-free water. Five microliters of total RNA were mixed with 0.5 μL of ethidium bromide (10 mg/mL stock solution) and 10 μL of loading dye (1.5 $\times$ MOPS, 55% formamide, 9.6% formaldehyde, bromophenol blue, xylene cyanol, H_2O) and heated to 95°C for 1 min to degrade secondary structures. Then, the mixture was kept on ice and immediately loaded onto the gel (1% agarose, 0.05% formaldehyde in 20 mM MOPS free acid, 8 mM sodium acetate, 1 mM EDTA, pH 7.0). RiboRuler HR (5 μL , Thermo Fisher Scientific; #SM1821) was used as a size marker. After electrophoresis, images were captured using a GBox (Syngene, Bengaluru, India), and the intensities of the 28S and 18S rRNA bands were quantified using ImageJ/Fiji (version 1.54).

Quantification of nascent RNA synthesis using 5-EU

Proliferating, quiescent, and senescent HDFs were prepared as described above in six-well plates. One day after seeding the proliferating cells, the medium was removed and replaced with medium containing 1 mM 5-EU (Jena Biosciences, Jena, Germany; #CLK-N002-10) to label newly synthesized RNA⁶⁵. Cells were incubated for 24 h in the presence of 5-EU, washed, detached using trypsin, pelleted ($200 \times g$, 5 min), fixed with ice-cold 70% EtOH, and stored at 4°C overnight. Click chemistry was performed on the following day. Cell pellets were resuspended in 1 mL of Click chemistry buffer (115 mM Tris/HCl, pH 8.5, 0.1% Triton X-100), resuspended in 500 μL of freshly prepared Click chemistry mix (500 μL of Click chemistry buffer, 10.6 μL of 100 mM CuSO_4 solution, 53.3 μL of 20 mg/mL ascorbic acid solution, and 1 μL of 10 mM Alexa Fluor 647 azide), and incubated in the dark at room temperature for 30 min. Cells were washed with wash buffer (100 mM Tris/HCl, pH 7.4, 2 mM MgCl_2 , 0.1% Triton X-100), DNA was stained with DAPI (0.1 $\mu\text{g}/\text{mL}$ in wash buffer, 10 min at room temperature), and cells were washed a final time. To quantify 5-EU incorporation, cells were analyzed on a CytoFLEX S flow cytometer using a 638-nm (red) laser and 660/10 nm bandpass filter for Alexa Fluor 647 (5-EU), a 405-nm (violet) laser and 450/45 nm bandpass filter for DAPI, and a 488-nm (blue) laser for forward and side scatter. Using Kaluza 1.2 (Beckman Coulter), cells were gated on forward and side scatter, and DAPI staining was used to

exclude events below the G1 peak and above the G2 peak, thereby removing cellular debris and cell aggregates. The remaining events were then exported to CSV files and analyzed using R (version 4.5.0).

Two specificity controls were included: cells not labeled with 5-EU and cells incubated in the presence of 50 µg/mL α -amanitin (Sigma-Aldrich; #06422) and 50 ng/mL actinomycin D (Sigma-Aldrich #A9415) during incubation with 5-EU to block RNA polymerase.

In vitro translation assay

We performed an *in vitro* translation assay as previously described⁹¹. Briefly, we depleted the cell lysates of endogenous methionine and other amino acids using a Sephadex G25 (Sigma-Aldrich; #G2580) spin column before loading onto the sucrose cushion. Ultracentrifugation was performed using 500 µL of sucrose cushion (3.766 g of sucrose, 50 µL of 1 M DTT, 100 µL of 100 mM DMSF in isopropanol, 30 mM HEPES 7.6, 150 mM KAc, 3.9 mM MgAc₂, and deionized water to 10 mL) at 200,000 × *g* for 90 min. To enable direct comparison between gels, densitometry-derived intensity values were normalized. Specifically, the sum of all measured intensities for a given gel was set to 100%, and the relative contributions of the different samples were calculated accordingly. All samples comprising a single technical replicate were loaded onto the same gel.

rDNA promoter methylation assay

Genomic DNA was isolated from proliferating, quiescent, and SIPS HDFs (HDF76) using the DNeasy Blood and Tissue Kit for DNA Isolation (QIAGEN, Hilden, Germany; #69504), and the concentrations were measured using a NanoPhotometer NP80 (Implen, Munich, Germany). Thirty nanograms of DNA were digested for 15 min at 37°C with 10 units/ng DNA MspI and HpaII (New England Biolabs, Ipswich, MA, USA; #R0106S and #R0171S). Subsequent qPCR on a QuantStudio 3 (Thermo Fisher Scientific) was performed using primers for the rDNA promoter: forward, CTGCGATGGTGGCGTTTTTG; and reverse, ACAGCGTGTGCAATAACCC.

MitoTracker assay

Proliferating, quiescent, and SIPS HDFs (HDF76) were prepared as described above. Mitochondrial labeling was performed using MitoTracker Deep Red FM (Thermo Fisher Scientific; #M22426) at 300 nM in serum-free medium for 15 min at 37°C. To quantify, cells were analyzed using a CytoFLEX S flow cytometer. Single cells in the G1 and G2 phases were selected based on forward and side scatter and DAPI staining. Fluorescence intensity analysis was performed with FlowJo 10.10, and the data were then exported for plotting in R using the ggplot2 package.

Polysome profiles and RNA-seq

Library preparation and sequencing

Cells were harvested for polysome profiling from quiescent and senescent cells (HDF76 and HDF85) after the 3-week protocol. To generate the gradients for ultracentrifugation, 10% and 50% sucrose solutions were prepared in gradient buffer (30 mM HEPES–KOH, pH 7.6, 150 mM KOAc, 5 mM MgOAc₂, 4 mM DTT, 1 mM PMSF in isopropanol, 100 µg/mL cycloheximide), and a linear gradient was prepared using the Gradient Master 108 system (Biocomp Instruments, Tatamagouche, Canada; program “long Sucr 10–50% WV 1st”) in ultracentrifugation tubes (Beckman Coulter; #331372). Lysates were layered onto the gradients and centrifuged for 165 min at 39,000 rpm and 4°C in a SW41 rotor (Beckman Coulter) using an Optima XPN-80 ultracentrifuge (Beckman Coulter). Subsequently, the gradients were fractionated using a density gradient fractionation system (Brandel, Gaithersburg, MD, USA; #BR-188). Polysome profiles were plotted from continuous absorbance measurements at 254 nm and used to collect fractions containing polysomes. After fractionation, TRI Reagent was immediately added to polysome-containing fractions, and samples were stored at – 80°C. After thawing, all polysome-containing fractions from the same replicate were pooled and then subjected to RNA isolation. Sequencing was performed on a NovaSeq 6000 (Illumina, San Diego, CA, USA) using the SMART-Seq stranded RNA kit (TaKaRa, Tokyo, Japan). Low-input/degraded mammalian RNA was processed at the Next Generation Sequencing Platform of the University of Bern (Bern, Switzerland).

Bioinformatic analysis

FastQ Trimmer by column (Galaxy version 1.1.1) was used to remove the first seven bases from the 5'-end and the first two bases from the 3'-end of each read. Reads were filtered by quality (Galaxy version 0.69). RNA Star Gapped-read mapper for RNA-Seq data (Galaxy version 2.6.0b-1) was used to align reads to the hg38 (09.02.2020) reference genome using the default options. Aligned reads were counted using featureCounts. Gene expression was measured by RNA-Seq experiments from SAM or BAM (Galaxy version 1.6.4 + galaxy1) using the following parameters: stranded (reverse); count fragments instead of reads, enabled; check paired-end distance, no; only allow fragments with both reads aligned, yes; and exclude chimeric fragments, yes.

Differential expression was analyzed using the Anot2Seq package⁷⁰ in R. “Donor” was included as an independent variable. The results were filtered in R to include only protein-coding genes (according to ENSEMBL annotation). Genes with fewer than 20 counts were removed, and an adjusted p-value of < 0.05 was used as the significance threshold. We used the lengthAnalysis function of the anota2seqUtils package to compare 5' UTR lengths. uORFs starting with canonical ATG codons in a strong Kozak context [(A/G)NNATGG, requiring a purine at – 3 and G at + 4] were quantified using the uorf_analysis function of the anota2seqUtils package. To control for potential confounding by transcript length, comparisons were evaluated against both random and length-matched background gene sets. Gene annotations were mapped to representative curated human NCBI RefSeq coding transcripts.

Gene set enrichment analysis (GSEA)

Ranked lists of the transcriptome/translatome/buffered genes were generated according to their normalized expression values in Anot2Seq⁷⁰. These ranked gene lists were then subjected to GSEA using the fgsea package in R⁷⁵. Enrichment was tested using the following parameters: organism database, 3.22.0; ontology, biological process; minimum gene set size for analysis, 10; maximum number of genes annotated for testing, 500; and p-value cutoff, 0.05. The resulting list of GO terms with adjusted p-value < 0.05 was then visualized using the ggplot2 and enrichplot packages in R.

Declarations

DATA AVAILABILITY

High-throughput sequencing data generated in this study are deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB124424 and are publicly available as of the publication date.

ACKNOWLEDGMENTS

This research was funded in whole or in part by the Austrian Science Fund (FWF) [10.55776/FG24] to MS and [10.55776/I2514] to JG; and the Swiss National Science Foundation [310030E-162559/1 and 10.001.322] to NP. For open access purposes, the author has applied a CC BY public copyright license to any author accepted manuscript version arising from this submission. The financial support provided by the Austrian Federal Ministry of Economy, Energy and Tourism (BMWET), the National Foundation for Research, Technology, and Development of Austria, and CHANEL Fragrance & Beauty to the Christian Doppler Laboratory for Skin Multimodal Imaging of Aging and Senescence is gratefully acknowledged. This project was also supported by EQ-BOKU VIBT GmbH and the BOKU Core Facility Biomolecular & Cellular Analysis. We thank Pamela Nicholson and her team at the next-generation sequencing (NGS) platform of the University of Bern for their valuable contributions.

AUTHOR CONTRIBUTIONS

MSS: Conceptualization, Investigation, Formal analysis, Writing – original draft, Visualization. **FN:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Visualization as an integral part of his PhD thesis at BOKU University (Austria). **YG:** Conceptualization, Investigation. **AB, RLG, KT, CH, LLX:** Investigation. **AT, FM, GG, NA, YBK, MH, FG:** Resources, Writing – review & editing. **NP:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **JG:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **MS:** Conceptualization, Formal analysis, Data curation, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition.

All authors except FN read and approved the final manuscript. Despite our best efforts, we were unable to contact co-author FN, who left the Institute of Molecular Biotechnology at BOKU University upon

completion of his PhD. As a result, he was not able to approve the final accepted version of the manuscript and cannot be held accountable for the work following publication.

CONFLICTS OF INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: AT, FM, GG, NA, and YBK are full-time employees of CHANEL Fragrance & Beauty, 8 rue du Cheval Blanc 93500 Pantin, France. The remaining authors declare no conflicts of interest.

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Figures

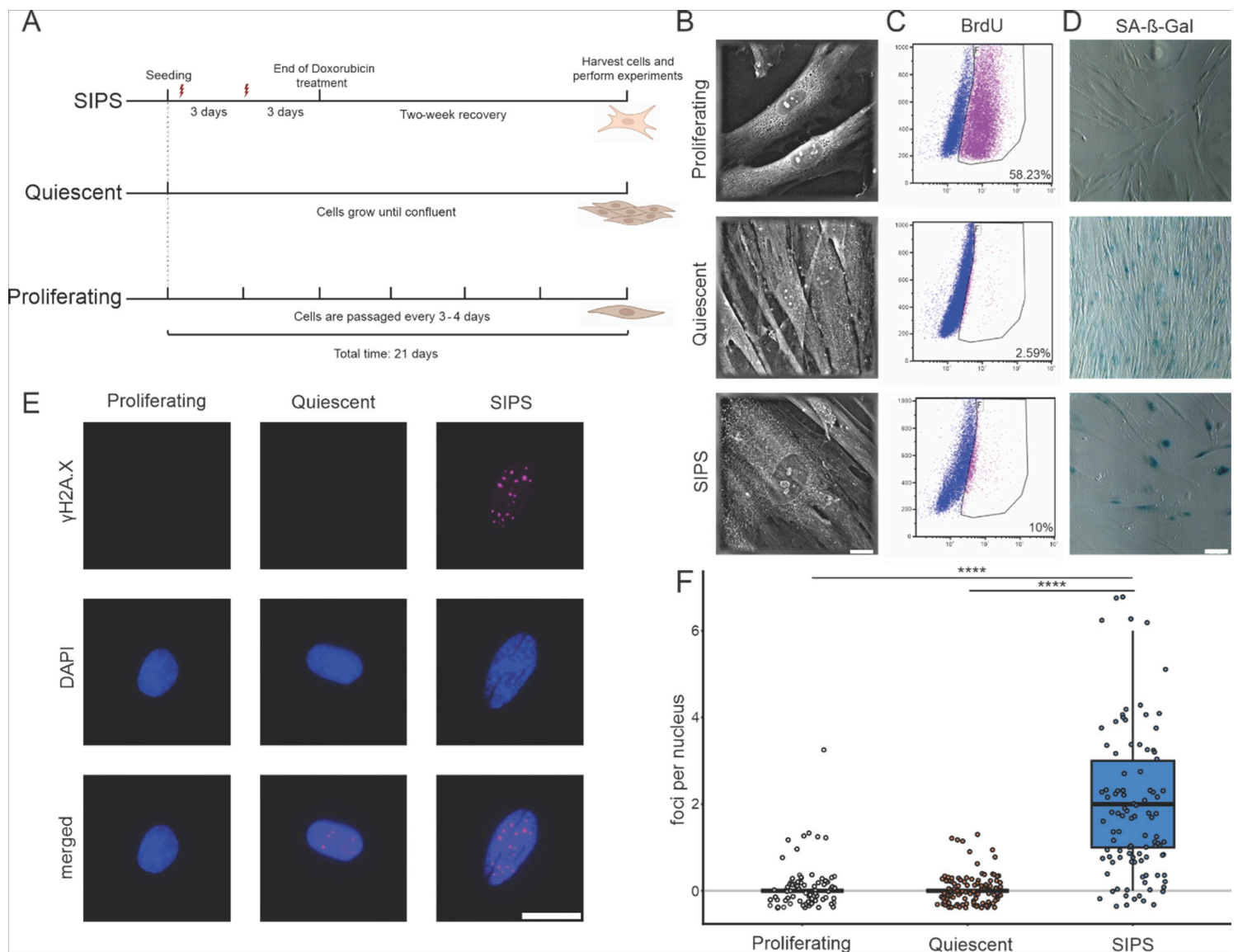


Figure 1

Establishing and validating senescence induction using doxorubicin. **A)** Schematic representation of the generation of stress-induced premature senescent (SIPS), quiescent, and proliferating cells. **B)** Nanolive images of SIPS, quiescent, and proliferating cells exhibiting characteristic changes in cell morphology. Scale bar = 20 μ m. **C)** Proliferating cells displayed high levels of BrdU incorporation, whereas quiescent and SIPS cells exhibited minimal BrdU incorporation, confirming the absence of DNA replication. **D)** SIPS and quiescent cells were positive for senescence-associated β -galactosidase (SA- β -gal) activity, whereas proliferating cells were negative. The scale bar represents 200 μ m. **E)** Representative images of phosphorylated histone H2A.X (γ H2A.X) immunofluorescence in proliferating, quiescent, and SIPS cells. Scale bar = 20 μ m. **F)** Quantification of γ H2A.X foci per nucleus confirmed increased DNA damage in SIPS cells. The data represent cells from three different donors (HDF76, HDF85, and HDF161). One-way ANOVA + Tukey's post hoc test: **** $p < 0.0001$.

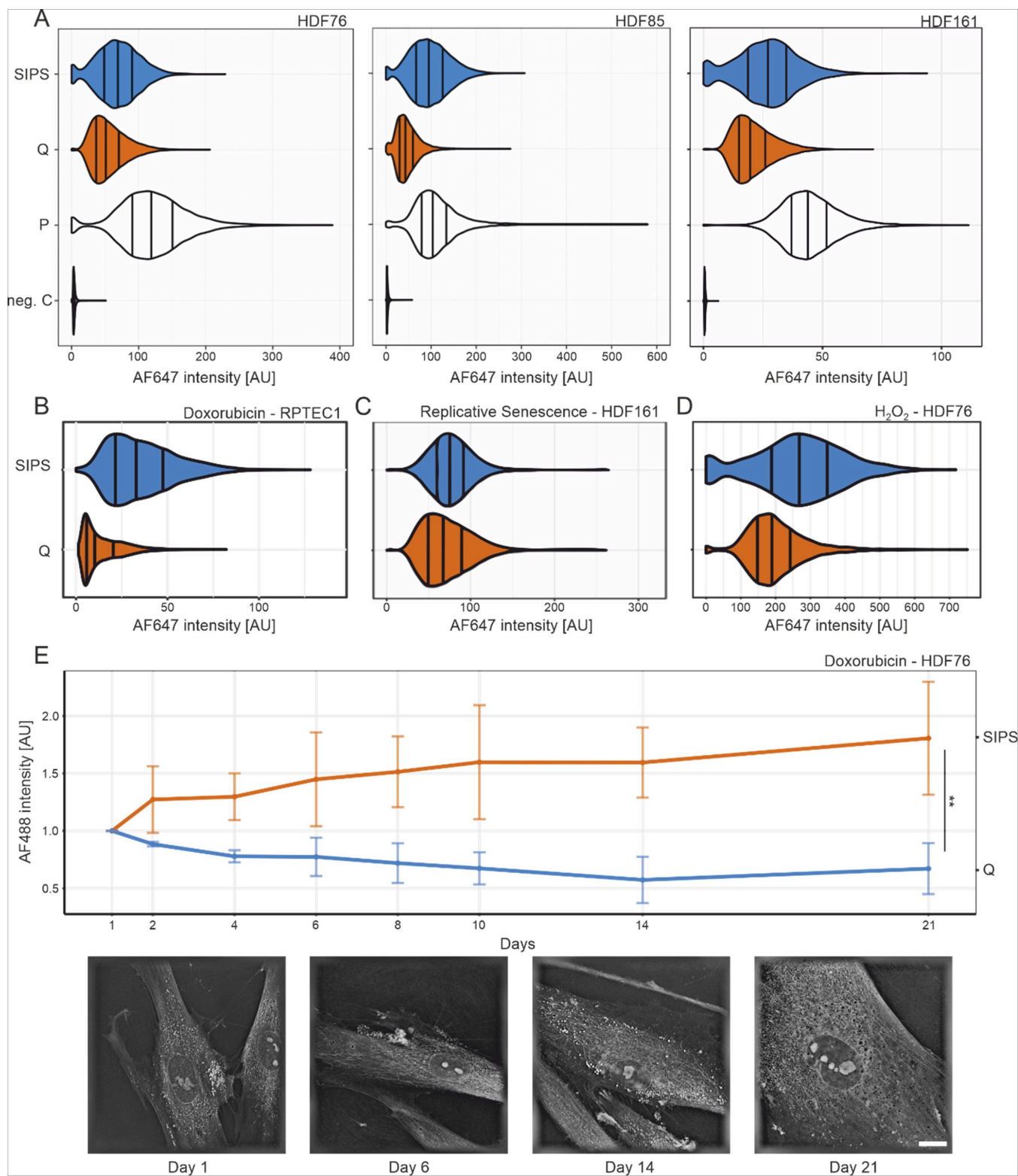


Figure 2

Global protein synthesis increases in cellular senescence. A) SIPS cells exhibited increased signal after O-propargyl-puromycin (OPP) pulse labeling compared to quiescent cells for all three human dermal fibroblast (HDF) donors. **B)** OPP signal measured in renal proximal tubular epithelial cells (RPTEC1) treated with doxorubicin compared with contact-inhibited quiescent controls. **C)** OPP signal measured in HDFs in replicative senescence compared with quiescent controls. **D)** OPP signal measured in cells from

HDF76 in H₂O₂-induced senescence compared with quiescent controls. **E)** OPP signal measured on different days during the 3-week senescence induction period revealed a consistent increase in global protein synthesis compared with quiescent controls. Representative Nanolive images for days 1, 6, 14, and 21 illustrate morphological changes in SIPS cells. Scale bar = 20 μ m. Unpaired *t*-test for day 21, *p* = 0.0056, *N* = 3. Error bars represent the standard deviation. P = proliferating, Q = quiescent, SIPS = stress-induced premature senescence, neg. C = unstained control.

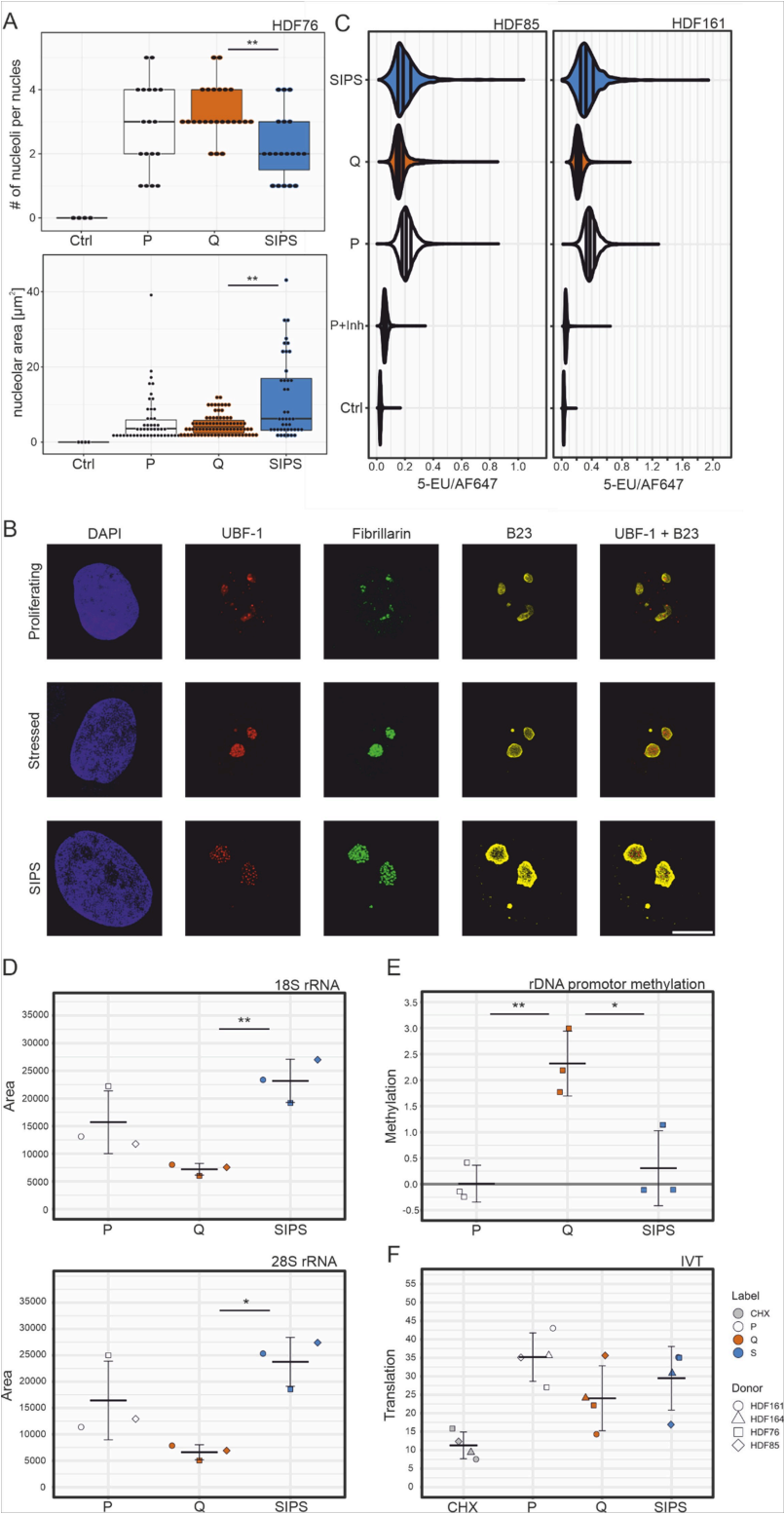


Figure 3

Ribosome biogenesis increases during cellular senescence. A) Quantification of nucleolar number and size after fluorescence staining revealed a significant decrease in number and an increase in size in SIPS human dermal fibroblasts (HDFs) from HDF76 compared with quiescent controls. Nucleolar number, one-way ANOVA + Tukey's post hoc test, $p = 0.0049$; nucleolar area, one-way ANOVA + Games–Howell test, $p = 0.001$. **B)** Representative images of the fluorescent staining of nucleolar compartments. Fibrillar center (UBF-1, red), dense fibrillar component (fibrillarin, green), and granular component (B23, yellow). The nucleolar structure differed among proliferating, acutely stressed (immediately after doxorubicin treatment), and SIPS HDFs. The scale bar represents 10 μm . **C)** Overall RNA synthesis measured *via* 5-ethynyl-uridine (5-EU) incorporation revealed an increase in SIPS HDFs compared with that in quiescent HDFs. $N = 2$ donors. **D)** Quantification of total 18S and 28S rRNA by densitometry after gel electrophoresis of SIPS, quiescent, and proliferating HDFs revealed an increase in SIPS cells compared with that in the quiescent controls. One-way ANOVA + Tukey's post hoc test: 18S, $p = 0.006$; 28S, $p = 0.014$; $N = 3$ donors. **E)** Measurement of rDNA promoter methylation in HDFs from HDF76 with methylation-sensitive digestion highlighted increased methylation in quiescent cells compared with that in SIPS and proliferating cells. One-way ANOVA + Tukey's post hoc test: P vs. Q, $p = 0.0071$; SIPS vs. Q, $p = 0.0135$; $N = 3$ independent experiments. **F)** *In vitro* translation with ribosomes from SIPS, quiescent, and proliferating HDFs revealed no difference in translational efficiency ($*p < 0.05$, $**p < 0.01$). $N = 4$ donors. Error bars represent the standard deviation. P = proliferating, Q = quiescent, SIPS = stress-induced premature senescence, P + Inh = proliferating cells treated with 50 ng/mL actinomycin D and 50 $\mu\text{g/mL}$ α -amanitin, Ctrl = unstained control.

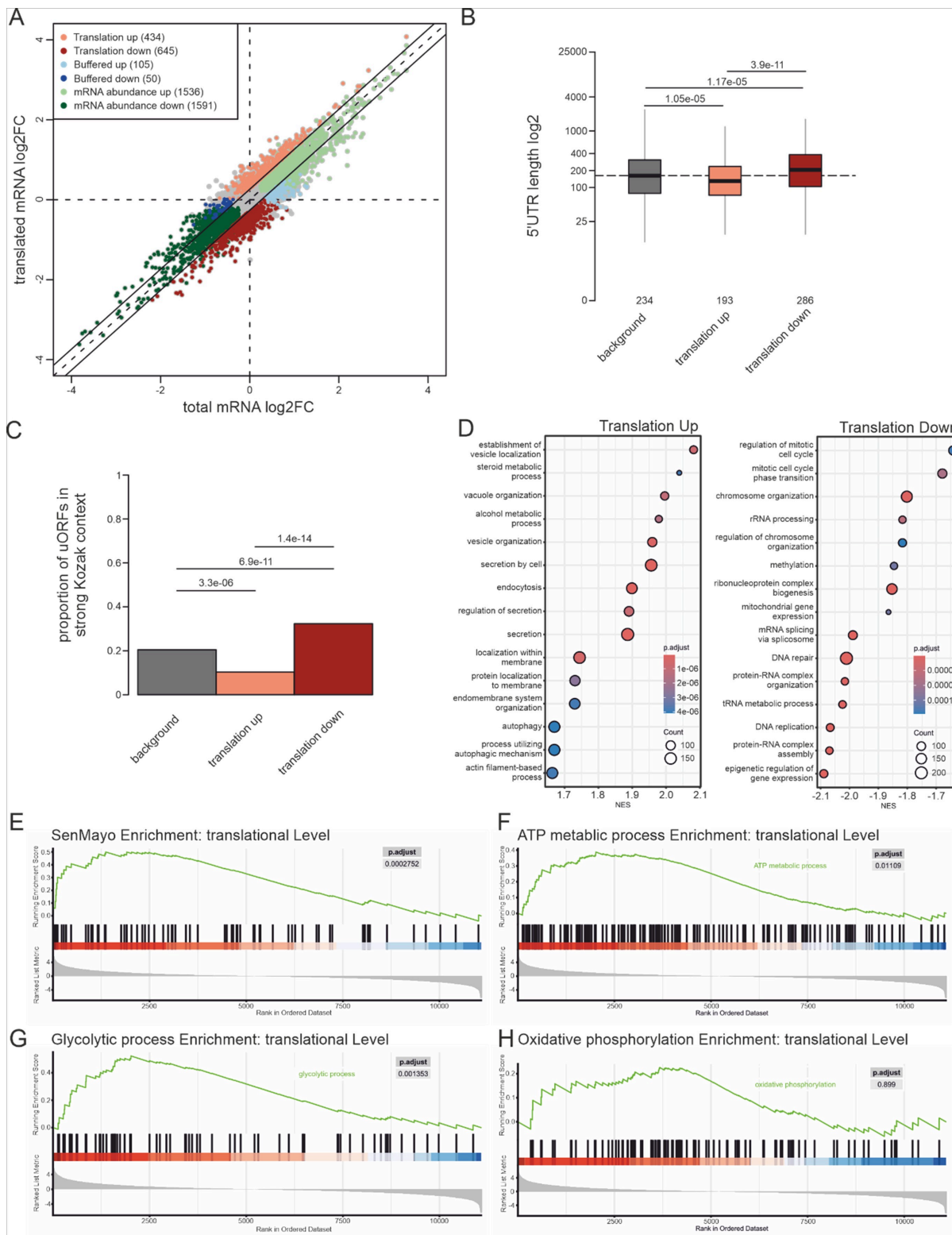


Figure 4

Length of the 5' untranslated region (5' UTR) and the presence of upstream open-reading frames (uORFs) underlie differences in mRNA recruitment between stress-induced premature senescent (SIPS) and quiescent cells. **A)** Correlation of log2 fold changes of total (x) to translated (y) mRNAs of SIPS vs. quiescent human dermal fibroblasts (HDF). "Translated mRNAs" represent mRNAs isolated from polysomes of the same lysates as the total mRNAs. We analyzed two donors in two technical

replicates. **B)** The length of 5' UTRs was increased in mRNAs that were translationally downregulated in SIPS versus the background, whereas upregulated mRNAs exhibited the opposite trend. Wilcoxon's rank-sum test + Bonferroni's correction: P-values are plotted. **C)** The fraction of uORFs in a strong Kozak context was increased in translationally downregulated mRNAs, whereas it was decreased in translationally upregulated mRNAs in SIPS. Wilcoxon's rank-sum test + Bonferroni's correction: P-values are plotted. **D)** Top 15 enriched Gene Ontology pathways in SIPS cells compared with quiescent cells at the translome level. **E)** The "SenMayo" gene set was enriched in SIPS cells compared with quiescent cells. Normalized enrichment score (NES) = 1.835. **F)** The "ATP metabolic process" gene set was enriched in SIPS cells compared with quiescent cells. NES = 1.615. **G)** The "glycolytic process" gene set was enriched in SIPS cells compared with quiescent cells. NES = 1.925. **H)** The "oxidative phosphorylation" gene set was not significantly enriched in SIPS cells compared with quiescent cells. NES = 0.884; Error bars represent the standard deviation.

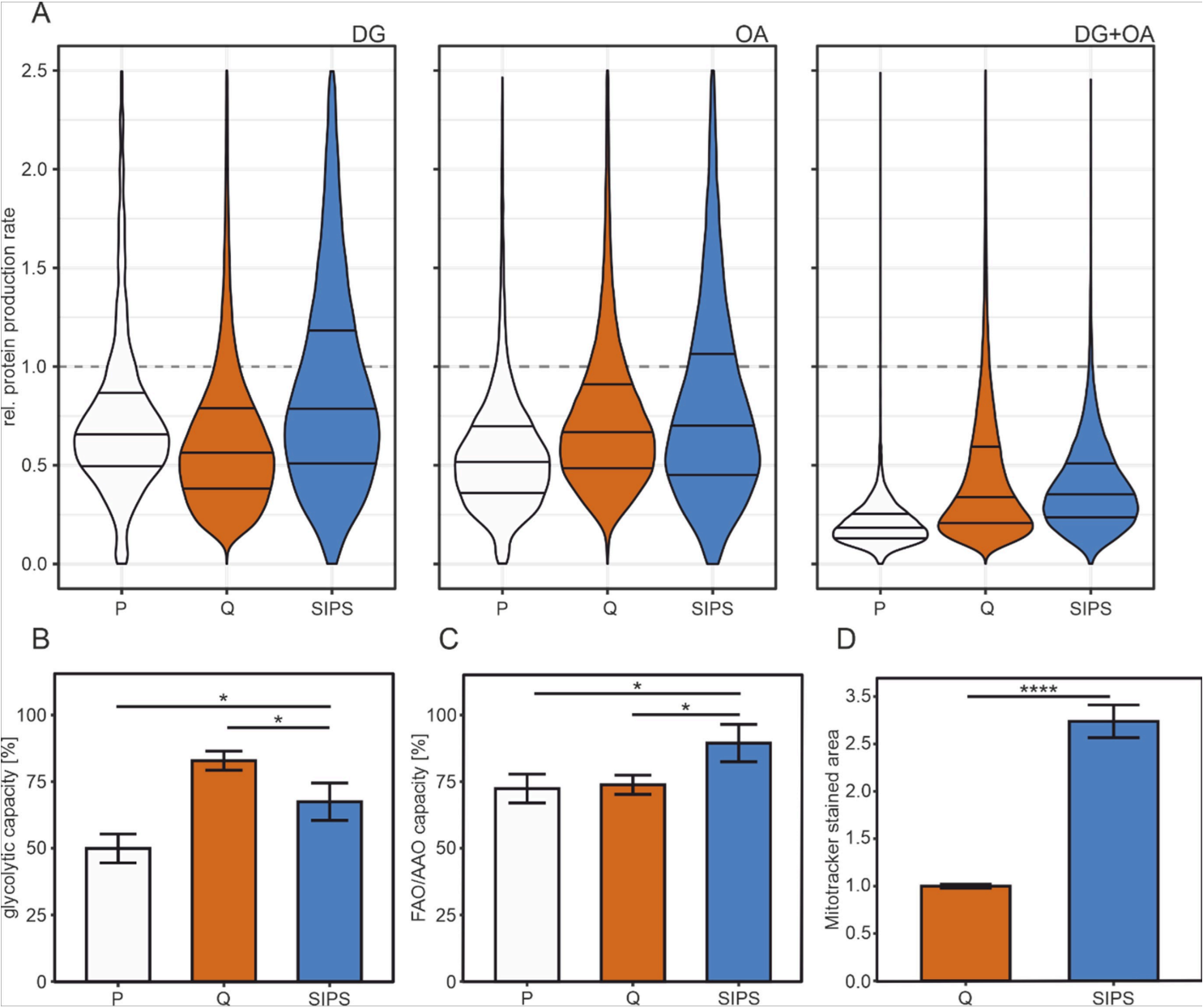


Figure 5

Changes in energy metabolism during cellular senescence. A) Inhibition of different ATP synthesis pathways exerted differing effects on protein production rates (measured using O-propargyl-puromycin pulse labeling) in proliferating, quiescent, and SIPS human dermal fibroblasts (HDFs) from HDF76. **B)** Glycolytic capacity denotes the percentage of protein production that cells could sustain when mitochondrial oxidative phosphorylation was inhibited. SIPS cells exhibited lower capacity than quiescent cells but greater capacity than proliferating controls. **C)** FAO/AAO capacity denotes the percentage of protein production that cells could sustain when glucose oxidation was inhibited. SIPS cells exhibited significantly higher capacity than either quiescent or proliferating cells. One-way ANOVA + Tukey's post hoc test, N = 4. **D)** MitoTracker staining of SIPS HDFs from HDF76 revealed an increase in staining intensity. Unpaired t-test, N = 3; *p < 0.05, ***p < 0.0001. Error bars denote the standard deviation; DG = 2-deoxy-D-glucose, OA = oligomycin A, P = proliferating, Q = quiescent, SIPS = stress-induced premature senescence.

Supplementary Files

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