

# Inhibition of nucleotide synthesis promotes replicative senescence of human mammary epithelial cells

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Cellular senescence is a mechanism by which cells permanently withdraw from the cell cycle in response to stresses including telomere shortening, DNA damage, or oncogenic signaling. Senescent cells contribute to both age-related degeneration and hyperplastic pathologies, including cancer. In culture, normal human epithelial cells enter senescence after a limited number of cell divisions, known as replicative senescence. Here, to investigate how metabolic pathways regulate replicative senescence, we used LC-MS-based metabolomics to analyze senescent primary human mammary epithelial cells (HMECs). We did not observe significant changes in glucose uptake or lactate secretion in senescent HMECs. However, analysis of intracellular metabolite pool sizes indicated that senescent cells exhibit depletion of metabolites from nucleotide synthesis pathways. Furthermore, stable isotope tracing with <sup>13</sup>C-labeled glucose or glutamine revealed a dramatic blockage of flux of these two metabolites into nucleotide synthesis pathways in senescent HMECs. To test whether cellular immortalization would reverse these observations, we expressed telomerase in HMECs. In addition to preventing senescence, telomerase expression maintained metabolic flux from glucose into nucleotide synthesis pathways. Finally, we investigated whether inhibition of nucleotide synthesis in proliferating HMECs is sufficient to induce senescence. In proliferating HMECs, both pharmacological and genetic inhibition of ribonucleotide reductase regulatory subunit M2 (RRM2), a rate-limiting enzyme in dNTP synthesis, induced premature senescence with concomitantly decreased metabolic flux from glucose into nucleotide synthesis. Taken together, our results suggest that nucleotide synthesis inhibition plays a causative role in the establishment of replicative senescence in HMECs.

Cellular senescence is an irreversible growth arrest that occurs in response to a variety of cellular stresses including DNA damage, oncogenic signaling, and oxidative stress (1). Senescent cells accumulate in aging tissues and contribute to age-related decline in tissue function (2-4). In culture, replicative senescence occurs in normal cells after a limited number of population doublings, the so-called Hayflick limit (5). Following activation of oncogenes including KRAS and BRAF, senescence acts to prevent proliferation, thus preventing against cancer (6-9). In addition to its role in aging and tumor suppression, senescent cells can contribute to hyperplastic pathologies including cancer through secretion of numerous pro-inflammatory signals (*i.e.* senescence-associated secretory phenotype) (10).

Although senescent cells are permanently arrested, they are highly metabolically active and demonstrate significant metabolic differences compared with proliferating, nonsenescent cells (11). Studies of human fibroblasts in culture have shown that replicative senescence is accompanied by increased glycolysis (12-14). In oncogene-induced senescence, increased glucose consumption is shunted away from the pentose phosphate pathway, leading to decreased nucleotide synthesis (6, 15, 16). Alterations in mitochondrial function can also induce senescence through 5' AMP-activated protein kinase- and p53-dependent pathways (17, 18). Importantly, it has also been demonstrated that metabolic genes, including phosphoglycerate mutase, pyruvate dehydrogenase, and malic enzymes, can regulate entry into and escape from senescence (8, 19, 20). Therefore, identifying the mechanisms by which metabolism regulates senescence is essential to understanding the senescence program during aging and tumor suppression.

Primary human mammary epithelial cells (HMECs)<sup>2</sup> have been shown to exhibit two mechanistically distinct senescence barriers to immortalization: stasis and agonescence (21). Stasis is a retinoblastoma-mediated growth arrest that occurs in the absence of DNA damage and is independent of p53 (22). Agonescence, or telomere dysfunction-associated senescence, is driven by critically shortened telomeres that trigger both a p53-dependent cell cycle arrest and a DNA damage response



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<sup>&</sup>lt;sup>2</sup> The abbreviations used are: HMEC, human mammary epithelial cell; CRISPRi, CRISPR-dCas9 interference; EdU, 5-ethynyl-2'-deoxyuridine; FDR, false discovery rate; GSEA, gene set expression analysis; PD, population doubling(s); RRM2, ribonucleotide reductase regulatory subunit M2; SA-β-gal, senescence-associated β-galactosidase; hTERT, human telomerase; SAHF, senescence-associated heterochromatic foci; TCA, tricarboxylic acid; PRPP, phosphoribosyl pyrophosphate; NA, numerical aperture; PAI-1, plasminogen activator inhibitor-1.

(23, 24). Because properties associated with senescence in mesenchymal cell types such as fibroblasts may not accurately reflect senescence in epithelial cells (22), the study of primary HMECs is required to understand how these senescence barriers are involved in normal HMEC biology, including aging and oncogenesis. How these senescence barriers are regulated by cellular metabolism in this primary cell type has not been investigated previously.

Here, we report that replicative senescence in primary HMECs is accompanied by a dramatic inhibition of nucleotide synthesis, including reduced flux of both glucose- and glutamine-derived carbon into nucleotide synthesis pathways. Expression of human telomerase (hTERT) in HMECs both induced immortalization and maintained flux into nucleotide synthesis. In addition, treatment of proliferating HMECs with an inhibitor of ribonucleotide reductase regulatory subunit M2 (RRM2), a key enzyme in dNTP biosynthesis, induced senescence and recapitulated the metabolomic state of replicative senescence. Taken together, our results indicate that nucleotide metabolism is a key regulator of replicative senescence in HMECs.

#### Results

# Establishment of a human mammary epithelial cell model of senescence

To study the metabolic alterations that accompany replicative senescence, we used normal diploid HMECs. These cells have been shown previously to accurately represent the molecular changes that occur during replicative senescence in vivo (21). We observed linear growth for  $\sim 15$  population doublings (PD), after which cell growth slowed until cells ceased proliferation at  $\sim$ 40 PD (Fig. 1A). HMECs at  $\sim$ 40 PD were viable and showed little to no cell death (Fig. 1B). However, these cells showed an enlarged, flattened, and irregular morphology that is typical of senescent cells (25) (Fig. 1C). To confirm that HMECs at  $\sim$ 40 PD were senescent, we first measured senescence-associated  $\beta$ -gal (SA- $\beta$ -gal) activity using C<sub>12</sub>FDG, a fluorogenic substrate for SA- $\beta$ -gal activity in live cells (26), and observed increased SA- $\beta$ -gal activity at PD 40 (Fig. 1D). Next, we confirmed that senescent HMECs exhibited a lack of DNA synthesis by measuring incorporation of the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) (Fig. 1*E*). Notably, Hoechst staining revealed that senescent HMECs did not exhibit senescenceassociated heterochromatic foci (SAHF), a frequent but not obligatory marker of senescence (27). However, we observed that a significant fraction of senescent HMECs were multinuclear (Fig. 1F and Fig. S1), which has been observed in senescent human melanocyte systems (28) and can result from aberrant mitotic progression in oncogene-induced senescence (29). Next, we tested for expression of the senescence markers p16 and p21. Western blotting revealed that senescent HMECs exhibited increased expression of p21 but not p16 (Fig. 1G). Because SAHF formation is driven by p16 but not p21, this observation is consistent with our finding that senescent HMECs do not exhibit SAHF (27, 30). We also tested for expression of the senescence marker PAI-1 (plasminogen activator inhibitor-1) (31-33) by Western blotting and

observed increased expression in senescent HMECs (Fig. 1*G*). Finally, to investigate whether senescent HMECs experienced DNA damage, we tested for expression of the DNA damage marker  $\gamma$ -H2AX by Western blotting. Interestingly, expression of  $\gamma$ -H2AX was slightly decreased as cells entered senescence (Fig. 1*H*), suggesting that senescent HMECs do not exhibit dsDNA breaks (22). Taken together, these data demonstrate that HMECs enter senescence around 35–40 PD.

#### Senescent HMECs do not exhibit a glycolytic shift

Having established a model system for replicative senescence, we analyzed proliferating and senescent HMECs using LC-MS-based metabolomics (34, 35). First, we measured the consumption and secretion rates of glucose and lactate, respectively (Fig. 2A and Table S1). Neither glucose consumption, lactate secretion, nor the ratio of glucose consumption to lactate secretion was significantly altered, suggesting that senescent HMECs do not exhibit an overall glycolytic shift. Because senescent fibroblasts increase secretion of citrate (13), we next examined the secretion of TCA cycle metabolites. Senescent HMECs exhibited decreased secretion of most TCA cycle metabolites, although only aconitate and malate demonstrated significant reductions (Fig. S2A). Finally, we examined the consumption and secretion of amino acids in proliferating and senescent cells. Although consumption and secretion of many amino acids was reduced in senescent HMECs, only aspartate secretion was significantly reduced compared with proliferating HMECs (Fig. S2B). Notably, glutamine consumption, a significant carbon source for the TCA cycle, was not significantly reduced. Taken together, these data demonstrate that senescent HMECs are highly metabolically active, without significant changes in glycolytic ratio or in exchange of TCA cycle intermediates and amino acids with the extracellular medium.

# *Replicative senescence in HMECs is accompanied by significant alterations in nucleotide and nucleoside pool sizes*

Next, we quantified the intracellular metabolite pool sizes in both proliferating and senescent HMECs. In five independent experiments, we measured 111 metabolites in at least two independent experiments (Table S2). Of the measured metabolites, 11 were significantly increased and 31 were significantly decreased in senescent cells (false discovery rate-corrected p value < 0.01). Notably, we did not observe significant change in the AMP/ATP ratio of senescent HMECs (Fig. S2C), in contrast to previous findings in senescent human fibroblasts (14, 36). Visualization of these data on a volcano plot revealed that several deoxyribonucleoside di- and triphosphates (dNDPs and dNTPs), including dCDP, dTDP, dADP, dATP, and dUTP, were significantly down-regulated in senescent cells (Fig. 2, B and C). Conversely, intracellular levels of guanine and uridine were significantly up-regulated. Increased intracellular levels of uridine were accompanied by increased secretion of uridine and the corresponding nucleobase uracil (Fig. 2D). Visualization of the log<sub>2</sub> -fold changes in intracellular pool size on a metabolic pathway map also revealed decreased metabolite pool sizes for most of glycolysis, the TCA cycle, the pentose





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**Figure 1. Establishment of an HMEC model of senescence.** *A*, HMECs ceased proliferation at 35–40 population doublings. HMECs were cultured in M87A medium with medium replacement every 2 days (every day after 50% confluence) and passaged at 80–90% confluence (21). Four independent cultures are shown. *B*, HMEC viability was unaffected by senescence arrest. Viability of proliferating (<15 population doublings) and senescent (>35 population doublings) cells is shown. HMEC viability was measured by trypan blue staining. *C*, HMECs acquired enlarged, flattened, and irregular morphology at PD 40. Phase-contrast images and cell sizes are represented at PD 10 and 40. *Scale bar*, 100  $\mu$ m.\*\*, *p* < 2 × 10<sup>-16</sup> by Mann–Whitney *U* test. *D*, HMECs showed increased activity of SA-*β*-gal at PD 35 measured by fluorescence signal of C<sub>12</sub>EDG. SA-*β*-gal measurements are shown at PD 10 and 35. SA-*β*-gal activity was calculated as follows: mean of samples labeled with C<sub>12</sub>EDG – mean of samples without C<sub>12</sub>EDG.\*\*, *p* value less than 0.0001 by Student's *t* test. *E*, measurement of DNA synthesis by EdU incorporation showed decreased DNA synthesis in senescent cells.\*\*, *p* < 0.001 by Student's *t* test. *F*, immunofluorescence imaging of proliferating and senescent HMECs using Hoechst and membrane dye. HMECs at PD 37 showed an increased number of multinucleated cells (denoted by *arrows*). *Scale bar*, 50  $\mu$ m.\*, *p* < 0.002 by Student's t test. *G*, immunoblot for p16, p21, PAI-1, and actin with lysates from proliferating and senescent HMECs. Recombinant human p16 protein (40 ng) was used as a positive control in the p16 Western blotting. *H*, immunoblot for  $\gamma$ -H2AX and actin with lysates from the 293T cell line treated with the DNA-damaging agent doxorubicin (*Dox;* 1  $\mu$ M for 24 h) or control was used as a positive control. Actin was used as an equal loading control. *Error bars*, S.D.

phosphate pathway, and nucleotide synthesis metabolites (Fig. 2*E*). Metabolite set enrichment set analysis demonstrated that nucleoside mono-/di-/triphosphates were the most significantly down-regulated metabolic pathway in senescent cells (Fig. S2*D*). Taken together, these data suggest that replicative senescence in HMECs induces dramatic changes in global metabolism with a particularly strong decrease in nucleotide metabolic pool sizes.

# Glucose-derived carbon fuels nucleotide synthesis in proliferating but not senescent HMECs

Because changes in metabolite pool size do not always accurately reflect changes in metabolic flux (37), we next analyzed metabolic flux in proliferating and senescent HMECs by stable isotope labeling. We cultured proliferating and senescent HMECs with [U-<sup>13</sup>C]glucose followed by LC-MS metabolomics. To gain a





global picture of the contribution of glucose-derived carbon to intermediary metabolites, we calculated glucose fractional contribution for each metabolite (38) (Table S3). Plotting the glucose fractional contribution on a volcano plot revealed that metabolites from the pyrimidine synthesis, including UMP, UDP, UTP, dCDP, dCTP, dTDP, and dTTP, showed significantly decreased incorporation of glucose-derived carbon (Fig. 3, A and B). Notably, whereas phosphoribosyl pyrophosphate (PRPP), one of the precursors for pyrimidine synthesis, demonstrated only a small change in glucose-derived carbon incorporation, UMP and the downstream products of pyrimidine synthesis (e.g. dCDP) exhibited almost complete loss of glucose-derived carbon incorporation (Fig. 3, B and C). Visualization of the  $[U^{-13}C]$ glucose fractional incorporation on a metabolic pathway map confirmed that the pyrimidine synthesis pathway demonstrated the largest decrease in glucose fractional incorporation (Fig. 3D). In contrast, metabolites from the TCA cycle showed little to no change in [U-<sup>13</sup>C]glucose fractional incorporation or isotopomer distributions (Fig. S3).

We next used [1,2-13C]glucose to investigate possible alterations in the ratio of glucose that enters the pentose phosphate pathway versus glycolysis (39). However, we observed no significant difference in the percentages of M1 and M2 lactate when comparing proliferating and senescent cells (Fig. S4A). There was, however, a significant decrease of <sup>13</sup>C incorporation into UMP and downstream pyrimidines, consistent with our observation using U- $^{13}$ C-labeled glucose (Fig. S4 (B and C) and Table S4). Next, we analyzed proliferating and senescent HMECs labeled with [U-<sup>13</sup>C]glutamine to test for changes in TCA cycle flux. We observed a decreased ratio of reductive to oxidative flux in the TCA cycle for senescent HMECs; however, the -fold change was small (Fig. S4D). Visualization of the [U-<sup>13</sup>C]glutamine fractional contribution to metabolites revealed significant changes in the contribution of glutamine-derived carbon to pyrimidine synthesis (Fig. S4 (E and F) and Table S5). Taken together, these data suggest that senescent cells strongly downregulate the flux of carbon into pyrimidine synthesis while leaving glycolysis and the TCA cycle unaffected.

We next queried published gene expression data to ask whether inhibition of nucleotide synthesis is broadly reflected at the transcriptional level in senescent cells. We analyzed (i) microarray data from HMECs in replicative senescence (*i.e.* stasis) (22) and (ii) RNA-Seq data from IMR90 fibroblasts induced to senesce by ionizing radiation (2). Gene set expression analysis (GSEA) (40) across all KEGG metabolic pathways (n = 78) (41) demonstrated a robust suppression of both pyrimidine metabolism (hsa00240) and purine (hsa00230) pathways in senescent cells (Fig. S5 (A and B) and Table S6). In both data sets, expression of RRM2, which catalyzes the biosynthesis of deoxyribonucleotides from ribonucleotides, was strongly decreased in senescent cells (not shown). Consistent with these data, Western blotting in our HMECs demonstrated down-regulation of RRM2 in senescent cells (Fig. 3*E* and Fig. S5*C*). Taken together, these data suggest that senescent HMECs exhibit reduced nucleotide synthesis.

# HMEC immortalization with telomerase restores nucleotide pools and fluxes

Because transduction of HMECs with exogenous hTERT can immortalize pre-stasis HMECs (42), we next tested the effects of hTERT-mediated immortalization on the metabolic profiles of HMECs. We expressed either hTERT or a firefly luciferase control in proliferating HMECs (PD 13). hTERT-expressing HMECs continued to grow logarithmically in culture, whereas HMECs expressing luciferase ceased to proliferate at PD  $\sim$ 33 (Fig. 4A). 60 days after transduction with luciferase and hTERT (PD 35 and 85 for luciferase and hTERT, respectively), we observed cuboidal cell morphology in hTERT-expressing cells, similar to proliferating, nonimmortalized HMECs (Fig. 4B). In contrast, luciferase-expressing cells acquired a senescent morphology and exhibited increased SA- $\beta$ -gal activity (Fig. 4, B and C). Next, we confirmed that senescent luciferase-expressing HMECs exhibited a lack of DNA synthesis (Fig. 4D) and increased expression of the senescence markers p21 and PAI-1 (Fig. 4*E*). Taken together, these data demonstrate that hTERT expression efficiently immortalized HMECs.

We then profiled luciferase- and hTERT-expressing HMECs using LC-MS metabolomics. Consistent with unmodified HMECs, medium footprint analysis revealed that glucose consumption and lactate secretion rates were not significantly different in proliferating, hTERT-expressing and senescent, luciferase-expressing cells (Fig. S6 and Table S7). Secretion of guanine and uridine was increased in senescent, luciferase-expressing HMECs. We next examined intracellular metabolite pool sizes in luciferase- and hTERT-expressing cells at days  $\sim$ 15 and  $\sim$ 60 following viral transduction and drug selection. At day  $\sim$ 15, both luciferase- and hTERT-expressing cells were proliferating, whereas at day ~60, luciferase- but not hTERTexpressing cells were senescent. Metabolomic profiling demonstrated that hTERT-expressing cells experienced no significant changes in dNDP and dNTP pool sizes between days  $\sim$ 15 and  $\sim$ 60 (Fig. 4F and Table S8). In contrast, luciferase-expressing cells exhibited significantly decreased dNDP and dNTP metabolite pool sizes, closely mirroring observations in untransduced proliferating and senescent HMECs.



**Figure 2. Replicative senescence in HMECs is accompanied by significant alterations in metabolite pool sizes.** *A*, glucose consumption, lactate secretion, and ratio of glucose consumption to lactate secretion are unchanged in senescent HMECs. Metabolite extracts from blank and conditioned media were analyzed by LC-MS. Secretion or uptake is calculated as follows: conditioned medium – blank medium, normalized to integrated cell number. Secreted metabolites have positive values, and consumed metabolites have negative values. *B*, *volcano plot* of intracellular pool sizes for all measured metabolites. Data represent average weighted  $log_2$ -fold change (senescent/proliferating) and FDR-corrected Fisher's combined *p* value from five independent experiments. Depicted metabolites were measured in at least two independent experiments. *C*, depletion of dNDPs and dNTPs and accumulation of nucleobases and nucleosides in senescent HMECs (same data as in *B* for selected dNDPs, dNTPs, guanine, and uridine). \* and \*\*, FDR-corrected Student's *t* test *p* values less than 0.02 and 0.002, respectively. *D*, extracellular secretion and consumption measured by LC-MS as in *A* for the nucleobases adenine, guanine, and uracil and the nucleoside uridine. Secreted metabolites have positive values, and consumed metabolites have negative values. \*, FDR-corrected Student's *t* test *p* < 0.001. *E*, metabolic pathway map depicting the log<sub>2</sub>-fold change (senescent/proliferating) intracellular pool sizes of metabolites in glycolysis, pentose phosphate pathway, nucleotide synthesis, and TCA cycle using the indicated *color scale*. Metabolites that were not measured are shown as *small circles* with *gray color*. Isomer that were not resolved with LC-MS are shown as *diamonds*. *Error bars*, S.D.



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**Figure 3. Glucose-derived carbon fuels nucleotide synthesis in young but not senescent HMECs.** *A*, purine and pyrimidine metabolites show significantly reduced [ $U^{-13}$ C]glucose carbon labeling in senescent HMECs. The *volcano plot* represents the average log<sub>2</sub>-fold change (senescent/proliferating) for fractional contribution (38) of  $U^{-13}$ C-labeled glucose and FDR-corrected Fisher's combined *p* value from two independent experiments. *B*, glucose flux into nucleotide synthesis pathways is globally reduced in senescent HMECs. Shown is the fractional contribution of  $U^{-13}$ C-labeled glucose for selected pyrimidines. \* and \*\*, FDR-corrected Student's *t* test *p* values less than 0.01 and 0.0001, respectively. *C*, [ $U^{-13}$ C]glucose isotopomer distributions for dCDP but not PRPP are significantly different in senescent HMECs. *D*, metabolic pathway may depicting the average log<sub>2</sub>-fold change of fractional contribution from [ $U^{-13}$ C]glucose is not possible to and the TCA cycle. Metabolites that were not measured are shown as *small circles* with *gray color*. Metabolites with isomers that were not the solved on LC-MS are shown as *diamonds*. *E*, immunoblot for RRM2 and actin with lysates from proliferating and senescent HMECs. A protein lysate from the 293T cell line was used as a positive control, and actin was used as an equal loading control. The RRM2 antibody was obtained from GeneTex. *Error bars*, S.D.

Next, we used U-<sup>13</sup>C-labeled glucose to assess the fractional contribution of glucose to the metabolism of HMECs expressing hTERT and luciferase. This analysis revealed that  $[U^{-13}C]$ glucose fractional incorporation in hTERTexpressing cells was unchanged between days ~15 and ~60 for pyrimidines and purines, including UMP, UDP, UTP, dADP, dATP, dTDP, and dCDP (Fig. 4*G* and Fig. S7 and Table S9). In contrast, luciferase-expressing HMECs exhibited significantly reduced fractional incorporation of  $[U^{-13}C]$ glucose into these pyrimidines and purines, similar to observations in untransduced proliferating and senescent HMECs.





Visualization of the glucose fractional contribution on a metabolic pathway map confirmed a global inhibition of carbon flux to nucleotides downstream of PRPP (Fig. 4*H*). In addition, metabolite set enrichment set analysis confirmed that nucleoside mono-/di-/triphosphates exhibited significantly decreased [U-<sup>13</sup>C]glucose-derived label in senescent, luciferase-expressing HMECs (Fig. S8). Notably, hTERT also induced moderate down-regulation of glucose-derived carbon to TCA cycle metabolites, a difference that was not observed comparing unmodified proliferating and senescent HMECs. Finally, we measured protein expression of RRM2 using Western blotting and observed significant decrease in high-PD senescent luciferaseexpressing HMECs (Fig. 4*I*). Taken together, these results demonstrate that hTERT immortalization maintains nucleotide pools and fluxes in HMECs.

#### Inhibiting ribonucleotide reductase induces senescence

Based on the metabolomic profile of senescent and proliferating HMECs, we hypothesized that reduced nucleotide synthesis plays a causative role in induction of senescence. To test this hypothesis, we first treated proliferating HMECs with triapine, an inhibitor of RRM2 (43). Indeed, treatment of proliferating HMECs (PD 10) with triapine effectively blocked cell proliferation (Fig. 5A). Following triapine treatment, cells acquired a typical senescent morphology and were significantly increased in size (Fig. 5B). Additionally, triapine-treated HMECs exhibited increased SA- $\beta$ -gal activity (Fig. 5C), reduced DNA synthesis (Fig. 5D), and increased expression of p21 and PAI-1 (Fig. 5E and Fig. S9A). To confirm the role of RRM2 in senescence, we next used CRISPRi-mediated knockdown of RRM2 expression (CRISPR-dCas9-KRAB) (44). Expression of a sgRNA against RRM2 but not a nontargeting sgRNA control efficiently knocked down RRM2 expression and inhibited cell growth in proliferating HMECs (Fig. 5F). We then confirmed that RRM2 knockdown increased SA-β-gal activity (Fig. 5G), reduced DNA synthesis (Fig. 5H), and increased expression of p21 and PAI-1 (Fig. 51 and Fig. S9B). Taken together, these data demonstrate that inhibition of RRM2 is sufficient to induce senescence in primary HMECs.

Next, we tested whether inhibition of RRM2 could induce senescence in the immortalized but nontransformed mammary

epithelial cell line MCF10A. Indeed, triapine treatment slowed cell growth, induced SA- $\beta$ -gal activity, reduced DNA synthesis, and increased expression of p21 and PAI-1 (Fig. S9, *C*–*G*). Together with data from primary HMECs, these data demonstrate that inhibition of RRM2 activity can induce senescence in immortalized mammary epithelial cells.

To investigate metabolomic alterations upon triapine-induced senescence, we again performed untargeted LC-MS metabolomics. HMECs treated with triapine exhibited depleted dNDP and dNTP pool sizes and increased intracellular levels of the nucleoside uridine (Fig. 5J). In contrast, we observed no significant change in the intracellular pool sizes of glucose, ribose 5-phosphate, and PRPP (Fig. 5J and Table S10). Tracing U-13C-labeled glucose in triapine-treated HMECs highlighted the down-regulation of glucose flux into nucleotide biosynthesis (Fig. 5K). Examination of the  $U^{-13}C$ -labeled glucose incorporation levels in triapine-treated cells showed that fractional contribution of glucose to PRPP was not significantly changed, similar to replicative senescence (Fig. 5 (K and L) and Table S11). However, there was a significant inhibition of glucose flux into pyrimidines in triapine-induced senescent HMECs (Fig. 5, K and L). In contrast, glucose flux into the TCA cycle did not significantly change upon triapine treatment (Fig. 5L). Metabolite set enrichment set analysis demonstrated that nucleoside and deoxynucleoside mono-/di-/triphosphates were the most significantly decreased metabolic pathways in triapine-induced senescent cells (Fig. S10). Taken together, these results indicate that inhibition of nucleotide synthesis is sufficient to trigger senescence in nonimmortalized HMECs.

# Integrative analysis of glucose contribution to metabolites in proliferating and senescent HMECs

To identify the most consistent alterations in senescent cells across our metabolomic experiments, we conducted unsupervised hierarchical clustering of the glucose fractional contribution to each metabolite across experiments. Proliferating HMEC cultures (low PD, hTERT expression, and DMSO treatment) and senescent HMEC cultures (high PD, luciferase expression, and triapine treatment) were separated into two well-defined clusters (Fig. 6). In addition, nucleotides were tightly clustered together to reveal decreased levels of glucose-

Figure 4. hTERT immortalization restores nucleotide pools and fluxes. A, expression of hTERT in proliferating HMECs prevented occurrence of senescence growth arrest. Shown is a representative growth curve demonstrating that hTERT-expressing HMECs continued to grow logarithmically in culture, whereas HMECs expressing luciferase ceased to proliferate at PD ~33. Filled triangles and squares indicate the PD at which cells were extracted for metabolomics. The x axis represents days since completion of drug selection. hTERT and luciferase expression were confirmed by Western blotting. Actin was used as an equal loading control. B, phase-contrast images of luciferase- and hTERT-expressing HMECs at PD 35 and 85, respectively. Luciferase-expressing HMECs acquired enlarged, flattened, and irregular morphology typical of senescent cells. hTERT-expressing HMECs maintained their epithelial cell morphology. Scale bar, 100  $\mu$ m. C, luciferase-expressing HMECs showed increased activity of SA- $\beta$ -gal measured by fluorescence signal of C<sub>12</sub>FDG at SA- $\beta$ -gal measurements are shown at day  $\sim$  60 following drug selection. SA- $\beta$ -gal activity was calculated as follows: mean of samples labeled with C<sub>12</sub>FDG – mean of samples without C<sub>12</sub>FDG.\*\*, p < 0.0001 by Student's t test. D, measurement of DNA synthesis by EdU incorporation showed decreased DNA synthesis in senescent, luciferase-expressing cells at day  $\sim$ 45 following drug selection. \*\*, p < 0.00004 by Student's t test. E, immunoblot for p21, PAI-1, and actin with lysates from luciferase- and hTERT-expressing HMECs at day ~45 following drug selection. F, hTERT-expressing HMECs maintain purine and pyrimidine pools. Luciferase- and hTERTexpressing cells were profiled by LC-MS metabolomics at days  $\sim$  15 and  $\sim$  45 following drug selection. Luciferase-expressing cells were senescent at day  $\sim$  45, but all other samples were still proliferating. Data from proliferating (PD 9) and senescent primary HMECs (PD 37) is shown for comparison. \* and \*\*, p < 0.04and 0.008, respectively. G, hTERT-expressing HMECs maintain glucose flux to purines and pyrimidines as measured by fractional contribution of U-13C-labeled glucose for selected purines and pyrimidines, as well as the nucleotide synthesis precursor PRPP. The number of days following drug selection for hTERT- and Juciferase-expressing HMECs was the same as in *F*. Data from proliferating and sensecent primary HMECs are shown for comparison. \* and \*\*, *p* value less than 0.04 and 0.002, respectively. *H*, metabolic pathway map depicting the average  $\log_2$ -fold change of  $[U^{-13}C]$ glucose fractional contribution for senescent, luciferase-expressing (day ~45) compared with proliferating, hTERT-expressing cells (day ~45) on the indicated *color scale* for metabolites in glycolysis, pentose phosphate pathway, nucleotide synthesis, and the TCA cycle. Metabolites that were not measured are shown as *small circles* with *gray color*. Isomers that were not resolved with LC-MS are shown as diamonds. I, immunoblot for RRM2 and actin with lysates from senescent, luciferase-expressing and proliferating, hTERT-expressing HMECs. Error bars, S.D.





derived carbon incorporation in senescent cells (*red cluster*). This *heat map* also highlighted the moderate down-regulation of glucose contribution to TCA cycle metabolites in hTERT-expressing HMECs (*blue cluster*). Taken together, this confirms that inhibited nucleotide synthesis is a hallmark of senescent HMECs.

#### Discussion

Cellular senescence is a state of irreversible cell cycle arrest that contributes to degenerative and hyperplastic phenotypes in aging and cancer. Although senescent cells are withdrawn from the cell cycle, they remain highly metabolically active (11). Here, we show that inhibition of nucleotide synthesis regulates replicative senescence in primary HMECs. The inhibition of nucleotide synthesis in senescent HMECs was reflected both in reduced pool sizes (Fig. 2E) and in reduced metabolic flux into nucleotide synthesis (Fig. 3D and Fig. S4). In contrast, fluxes from glucose and glutamine into glycolysis, the pentose phosphate pathway, and the TCA cycle were not significantly changed. Importantly, treatment of proliferating HMECs with an inhibitor of RRM2, a key enzyme in dNTP synthesis, demonstrated that inhibition of nucleotide synthesis was sufficient to induce senescence and recapitulate the metabolomic signature of HMEC replicative senescence. Taken together, these findings demonstrate the crucial role of nucleotide synthesis in cellular senescence of a primary epithelial cell type, a finding with implications for both aging and tumor development.

The study of metabolism during replicative senescence has relied heavily on fibroblasts, a mesenchymal cell type. These studies have demonstrated that human fibroblasts gradually exhibit a more glycolytic metabolism as they become senescent (12-14). Similarly, senescence induced by oncogene activation also leads to increased glycolysis (8, 16). Here, using a primary epithelial cell type (HMEC), we did not observe significant alterations in either glucose uptake or lactate secretion in senescent HMECs (Fig. 2A). Additionally, the fractional contribution of glucose and glutamine to glycolysis and the TCA cycle was unchanged in senescent HMECs. In contrast to senescent fibroblasts (14), HMEC senescence was also not accompanied by an energy crisis (Fig. S2C). Taken together, these results suggest that epithelial and fibroblast cells exhibit distinct metabolic alterations during senescence. These metabolic differences may reflect the fact that senescent epithelial and fibroblast cells exhibit distinct transcriptional profiles (22).

Although replicative senescence in our HMEC system does not involve oncogene activation, the inhibition of nucleotide synthesis we observed resembles the metabolic alterations found in oncogene-induced senescence. Notably, in fibroblasts, oncogenic RAS induces senescence by suppressing nucleotide metabolism, leading to the depletion of dNTP pools (6, 9). This depletion of dNTP pools is mediated by RAS-induced repression of RRM2 mRNA and protein levels. Similarly, senescent HMECs showed a significant down-regulation of RRM2 protein expression (Fig. 3E and Fig. S5C). Notably, in HMECs, chemical inhibition of RRM2 was sufficient to induce senescence and recapitulated the metabolomic signature of senescent HMECs (Figs. 5 and 6). Together, our findings extend the relevance of nucleotide metabolism in senescence to a nontransformed, primary cell type in the absence of oncogene activation.

In the current study, we observed depletion of dNTPs in the absence of  $\gamma$ -H2AX induction, suggesting that senescent HMECs do not experience DNA double-strand breaks during the time course of our experiments (Fig. 1E). However, because the maintenance of sufficient and balanced dNTP pools is essential for maintaining genomic integrity (45, 46), the inability to synthesize dNTPs may eventually lead to replication stress, genomic instability, and mutagenesis (47, 48). Indeed, genomic instability in the early stages of tumorigenesis induced by cyclin E oncogenes is coupled to insufficient nucleotide availability (49). In addition, we observed a significantly increased number of multinucleated cells in the senescent HMEC culture, which has previously been linked to genomic instability (50), telomere dysfunction (51), polyploidy (52), and tumor initiation (28). Additionally, the extent of senescence experienced by mouse embryonic fibroblasts during immortalization correlates with the amount of genomic instability in immortalized cell lines (34). Interestingly, in RAS-expressing fibroblasts, senescence-associated nucleotide deficiencies can be bypassed by inactivation of the serine/threonine kinase ATM, which is activated by DNA damage (53). However, because senescent HMECs in this study are not experiencing DNA double-strand breaks, they may more closely resemble hypoxic cells, which experience replication stress but not DNA damage (54). Similar to hypoxic cells, senescent cells up-regulate RRM2B (55), a subunit of the ribonucleotide reductase complex that may pre-

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**Figure 5. Inhibiting ribonucleotide reductase induces senescence.** *A*, treating proliferating HMECs with the RRM2 inhibitor triapine inhibits cell growth. Cells at PD ~8 were treated with 2  $\mu$ M triapine or DMSO for 72 h. *B*, phase-contrast images and cell sizes of HMECs treated with triapine or DMSO. HMECs acquire enlarged, flattened, and irregular morphology typical of senescent cells within 3 days of treatment with triapine. *Scale bar*, 100  $\mu$ m. \*\*,  $p < 2 \times 10^{-16}$  by Mann–Whitney *U* test. *C*, triapine-treated HMECs showed increased activity of SA- $\beta$ -gal measured by fluorescence signal of C<sub>12</sub>EDG. SA- $\beta$ -gal activity was calculated as follows: mean of samples labeled with C<sub>12</sub>EDG — mean of samples without C<sub>12</sub>EDG). \*\*, p < 0.0001 by Student's *t* test. *D*, measurement of DNA synthesis by EdU incorporation showed decreased DNA synthesis in triapine-treated cells. \*, p < 0.003 by Student's *t* test. *E*, Western blotting of p21, PAI-1, and actin for triapine-treated cells. *F*, genetic knockdown of RRM2 expression inhibits HMEC growth. Cells at PD ~10 were infected with sgRNA against RRM2 or nontargeting control. Western blotting of RRM2 and actin for HMECs infected with sgRNA against RRM2 or nontargeting control. *G*, knockdown of RRM2 expression induces SA- $\beta$ -gal activity was calculated as follows: mean of samples without C12FDG. \*\*, p < 0.0001 by Student's *t* test. *H*, measurement of DNA synthesis by EdU incorporation showed decreased DNA synthesis in RRM2 knockdown cells. \*, p < 0.0001 by Student's *t* test. *H*, measurement of DNA synthesis by EdU incorporation showed decreased py Student's *t* test. *I*, Western blotting of p21, PAI-1, and actin for HMECs infected with sgRNA against RRM2 or nontargeting control. *J*, triapine treatment results in depletion of dNDPs and dNTPs and induces uridine accumulation. \*, p < 0.003 by Fisher's combined *t* test. *K*, fractional contribution of U-<sup>13</sup>C-labeled glucose for selected pyrimidines. \* and \*\*, p values less than 0.03 and 0.



Figure 6. Integrative analysis of glucose contribution to metabolites in proliferating and senescent HMECs. Shown is a *heat map* comparing fractional contribution of glucose with commonly measured metabolites. *Columns* represent individual samples. *Red* indicates up-regulation, and *blue* indicates down-regulation. Nucleotides cluster together and are up-regulated in proliferating samples compared with senescent samples. *Error bars*, S.D.

serve the capacity for sufficient nucleotide synthesis during hypoxia to avoid DNA damage.

In senescent HMECs, we observed that the flux of glucosederived carbon was more severely down-regulated in pyrimidine synthesis than in purine synthesis (Figs. 3D, 4H, and 5L). This difference between pyrimidine and purine synthesis was not reflected in metabolite pool sizes, highlighting the power of stable isotope tracing to reveal metabolic changes not apparent in metabolite pool sizes (37, 56). Pyrimidine synthesis can be specifically controlled through mTOR/S6K1-mediated phosphorylation of CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase), the enzyme that catalyzes the first three steps of *de novo* pyrimidine synthesis (57). Additionally, loss of urea cycle enzymes, including carbamoyl phosphate synthetase-1 (CPS1), can lead to pyrimidine but not purine depletion (58). Although it is not yet



clear why pyrimidines are more affected than purines in senescent HMECs, this observation remains a subject of active investigation.

Taken together, our results indicate that suppression of nucleotide synthesis is a critical metabolomic alteration regulating senescence in primary HMECs. A more detailed understanding of altered metabolism as both a cause and a consequence of senescence will have important implications. In both aging (2) and cancer (59), therapeutic targeting of senescence has shown great promise. Similarly, therapeutics targeting the emerging metabolic differences between senescent and nonsenescent cells may prove useful tools for improving human health.

#### **Experimental procedures**

#### Cell culture

HMECs were purchased from Thermo Scientific and cultured in M87A medium (50% MM4 medium and 50% MCDB170 supplemented with 5 ng/ml epidermal growth factor, 300 ng/ml hydrocortisone, 7.5 µg/ml insulin, 35 µg/ml bovine pituitary extract, 2.5 µg/ml transferrin, 5 µM isoproterenol, 50 µM ethanolamine, 50 µM o-phosphoethanolamine, 0.25% FBS, 5 nM triiodothyronine, 0.5 nM estradiol, 0.5 ng/ml cholera toxin, 0.1 nm oxytocin, 1% antibiotic-antimycotic, no AlbuMax) in atmospheric oxygen. MCF10A cells were obtained from the American Tissue Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium/ F-12 medium supplemented with 20 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, 100 µg/ml insulin, 100 ng/ml cholera toxin, 0.1 nM oxytocin, 5% horse serum, and  $1 \times$  penicillin/streptomycin in atmospheric oxygen. Glucose and glutamine-free Dulbecco's modified Eagle's medium was purchased from Corning (90-113-PB), Ham's F-12 was purchased from US Biological (N8542-12), and MCD170 medium was purchased from Caisson Labs (MBL04). Glucose and glutamine were added to the media at the appropriate concentration for each medium type. Cells were lifted with TrypLE at 80-90% confluence and seeded at a density of 2.3  $\times$   $10^3/cm^2$ . Cell viability and diameter were measured with a trypan blue assay using a TC20 automated cell counter (Bio-Rad).

#### Genetic modification

Proliferating HMECs were infected at PD 14 with pLenti-PGK-hygro (Addgene 19066) encoding either hTERT or firefly luciferase. Following infection, cells were selected with 5  $\mu$ g/ml hygromycin for 7 days. Following selection, cells were maintained in culture with 2  $\mu$ g/ml hygromycin. To knock down expression of RRM2, proliferating HMECs were infected at PD 10 with lentiviral vector expressing dCas9-KRAB and sgRNA against RRM2 (forward, 5'-CACCGACACGGAGGGAGAGC-ATAG-3') or nontargeting control (forward, 5'-CACCGGTA-TTACTGATATTGGTGGG-3'). The lentiviral vector used for CRISPRi, pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro, was a gift from Charles Gersbach (Addgene plasmid 71236)

#### Senescence-associated $\beta$ -gal measurements

HMECs were incubated with 100 nm bafilomycin A1 (Sigma-Aldrich) for 1 h to raise the lysosomal pH to 6.0 (26), followed by

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incubation with 33  $\mu$ M C<sub>12</sub>FDG (Setareh Biotech) for 1 h, lifted with TrypLE, resuspended in ice-cold PBS, and then analyzed on a Miltenyi Biotec MACSQuant flow cytometer to measure fluorescence (26). Data were processed and analyzed with FlowJo 7.6.1 software, and the mean fluorescent signal for each sample was exported. Values for SA- $\beta$ -gal activity were calculated as follows: mean of samples labeled with C12FDG - mean of samples without C12FDG. Data were normalized to the SA- $\beta$ -gal activity of nonsenescent samples.

#### EdU incorporation

EdU staining was performed using the Click-iT Plus EdU Alexa Fluor 594 Imaging kit (Thermo Fisher Scientific, C10639). Briefly, cells were seeded in glass bottom dishes (MatTek part number P35G-1.5-10-C) with appropriate density and then incubated with 10 µM EdU for 6 h at 37 °C. If membrane staining was desired, cells were incubated with 1 imesMemBrite Fix 640/660 (Biotium 30097-T) for 5 min at 37 °C. Next, cells were fixed with 3.7% formaldehyde followed by 0.5% Triton X-100 permeabilization at room temperature. Cells were incubated with Click-iT Plus reaction mixture for 30 min at room temperature protected from light. Finally, for nuclear staining, cells were incubated with  $1 \times$  Hoechst 33342 for 30 min at room temperature protected from light and stored in PBS until imaging.

#### Confocal microscopy and cell counting

All microscopy was performed on Zeiss 780 or 880 confocal systems. The excitation wavelengths for the stained cells were: 405 nm for Hoechst, 594 nm for EdU, and 633 nm for MemBrite membrane dye (Biotium). All high-magnification images (×40/ 1.1 NA and  $\times 63/1.4$  NA, Zeiss) were sampled at Nyquist with 1-Airy unit pinhole diameters, and all low-magnification images (×10/0.45 NA, Zeiss) were collected with pinhole diameters such that  $40-\mu m$  optical sections were obtained for cell counting. Tiled images were stitched together and analyzed using Imaris (Bitplane) image processing and analysis tools. Images were interrogated for nuclei and the presence of EdU through the Spots module in Imaris.

#### Mass spectrometry-based metabolomics analysis

HMECs were plated onto 6-well plates at a density of  $1-3 \times$ 10<sup>4</sup> cells/cm<sup>2</sup>, depending on the experiment. For flux analysis, after 24 h, medium was replaced by U-13C-labeled glucose, 1,2-13C-labeled glucose, or U-13C-labeled glutamine (Cambridge Isotope Laboratories). Metabolite extraction was performed 24 h after adding labeled medium. For extraction of intracellular metabolites, cells were washed on ice with 1 ml of ice-cold 150 mM ammonium acetate (NH<sub>4</sub>AcO, pH 7.3). 1 ml of -80 °C cold 80% MeOH was added to the wells, samples were incubated at -80 °C for 20 min, and then cells were scraped off and supernatants were transferred into microcentrifuge tubes. Samples were pelleted at 4 °C for 5 min at 15,000 rpm. The supernatants were transferred into a LoBind Eppendorf microcentrifuge tube, the cell pellets were re-extracted with 200  $\mu$ l of ice-cold 80% MeOH and spun down, and the supernatants were combined. Metabolites were dried at room temperature under vacuum and resuspended in water for the

LC-MS run. For extraction of extracellular metabolites, 20  $\mu$ l of cell-free blank and conditioned medium samples were collected from wells. Metabolites were extracted by adding 500  $\mu$ l of  $-80~^\circ\text{C}$  cold 80% MeOH, dried at room temperature under vacuum, and resuspended in water for LC-MS analysis.

Samples were randomized and analyzed on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer coupled to an UltiMate 3000 UHPLC system (Thermo Scientific). The mass spectrometer was run in polarity-switching mode (+3.00/-2.25 kV) with an *m*/*z* window ranging from 65 to 975. Mobile phase A was 5 mM NH<sub>4</sub>AcO, pH 9.9, and mobile phase B was acetonitrile. Metabolites were separated on a Luna 3- $\mu$ m NH<sub>2</sub> 100-Å (150 × 2.0-mm) column (Phenomenex). The flow rate was 300  $\mu$ l/min, and the gradient was from 15% A to 95% A in 18 min, followed by an isocratic step for 9 min and re-equilibration for 7 min. All samples were run in biological triplicate.

Metabolites were detected and quantified as area under the curve based on retention time and accurate mass ( $\leq$ 5 ppm) using TraceFinder 3.3 (Thermo Scientific) software. Raw data were corrected for naturally occurring <sup>13</sup>C abundance (60). Extracellular data were normalized to integrated cell number, which was calculated based on cell counts at the start and end of the time course and an exponential growth equation. Intracellular data were normalized to the cell number and cell volume at the time of extraction. Pathway maps were made with Cytoscape software (61).

#### Gene set and metabolite set enrichment analysis

For gene set expression analysis of RNA data, microarray data from HMECs (22) were compared across pairwise comparisons for stasis and pre-stasis cell cultures. Genes were then ranked by their average rank from individual experiments. RNA-Seq data from IMR90 cells (2) were ranked based on signal/noise ratio of senescent/nonsenescent cells. GSEA was run with the unweighted statistic using the GSEA java applet (40). For metabolite set enrichment analysis of metabolomic data, HMEC intracellular pool sizes or [<sup>13</sup>C]glucose fractional contribution data were ranked based on log<sub>2</sub> -fold change of senescent/proliferating, luciferase/hTERT, or triapine/DMSO. Enrichment analysis was run with unweighted statistic using the GSEA java applet.

#### Statistical analysis

For metabolomics data (n = 3 in each experiment), p value was calculated with a two-tailed Student's t test. To evaluate combined significance from independent experiments, p values were combined with Fisher's method and corrected for false discovery rate using the Benjamini–Hochberg method. For SA- $\beta$ -gal activity, p values were calculated with Student's t test. For cell size analysis, p values were calculated with a Wilcox–Mann–Whitney test because the data were not normally distributed.

#### Immunoblot analysis

Cells were lysed in modified radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM  $\beta$ -glycerophosphate, 0.5 mM Nonidet P-40, 0.25% sodium deoxycholate, 10 mM sodium pyrophosphate, 30 mM sodium fluoride, 2 mM EDTA, 1 mM activated sodium vanadate, 20  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride). Whole-cell lysates were resolved by SDS-PAGE on 4-15% gradient gels and blotted onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 h and then incubated with primary antibodies overnight and secondary antibodies for 2 h. Blots were imaged using the Odyssey IR Imaging System (LI-COR). Primary antibodies used for Western blot analysis were as follows: p16 (10883-1-AP, Proteintech), p21 (2947S, Cell Signaling), PAI-1 (NBP1-19773, Novus Biologicals), y-H2AX (9718S, Cell Signaling), RRM2 (103193, GeneTex; HPAA056994, Millipore Sigma), luciferase (L0159, Sigma-Aldrich), hTERT (600-401-252S, Rockland), and anti-β-actin (66009-1-Ig, Proteintech). Recombinant human p16 protein (Novus H00001029-P01) was used as a positive control for p16 Western blotting.

Author contributions—A. D. and N. G. conceived the study; A. D. performed cell culture and metabolomics experiments. S. P. and A. D. cloned lentiviral vectors for luciferase and hTERT expression. A. D., J. J., and S. F. performed and supervised immunofluorescence microscopy. A. D., J. Y., and F. S. performed Western blotting. A. D., S. L., J. M., and P. W. performed and supervised SA- $\beta$ -gal activity measurements. A. D. and N. G. analyzed all data and wrote the manuscript.

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