Reviewed Preprint
v1 • May 29, 2026
Not revised

For correspondence:
jon.houseley@babraham.ac.uk

Competing interests: HHM and JH have filed a patent relating to this work, PCT/GB2024/051407

Funding: See page 26

Reviewing editor: Weiwei Dang, Baylor College of Medicine, United States

Decoupling AMPK from fatty acid synthesis allows maintenance of fitness late in life

Hanane Hadj-Moussa[1], Megan Ulusan[1], Dorottya Horkai[1,2], Mohammed Kamran Afzal Mirza[1], Jonathan Houseley[1]

[1]Epigenetics Programme, Babraham Institute, Cambridge, United Kingdom • [2]ZOMP, Cavendish Laboratory, Cambridge, United Kingdom

eLife Assessment

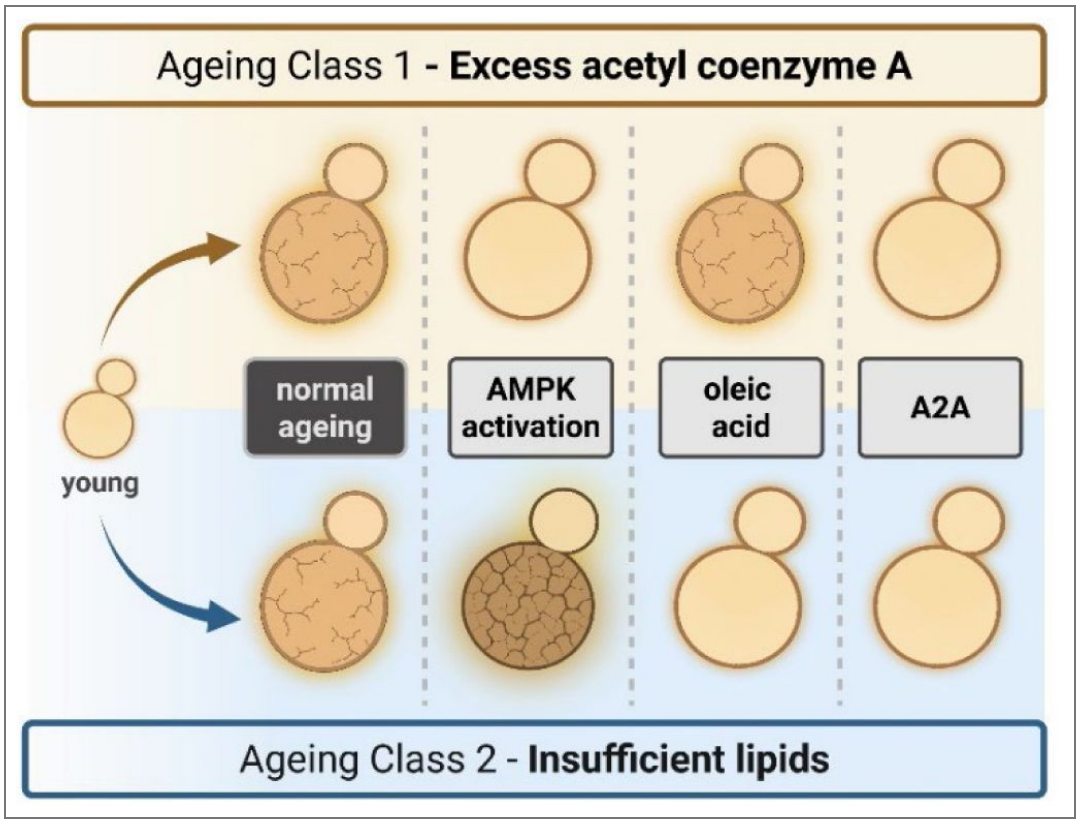
This study addresses an **important** question in aging biology by combining metabolic, genetic, and functional approaches to examine how cytosolic acetyl-CoA metabolism influences late-life fitness in replicatively aging yeast. The evidence supporting the roles of AMPK activation, mitochondrial acetyl-CoA utilization, and fatty acid synthesis in shaping distinct aging-associated phenotypes is **convincing** overall, with the engineered A2A strain providing a particularly elegant demonstration of coordinated metabolic regulation. However, several conclusions would benefit from clarification or moderation, particularly regarding the relationship between late-life fitness and replicative lifespan, the interpretation of "senescence," the proposed existence of distinct aging subpopulations, and the extent to which the data support mechanistic claims about lipid starvation, acetyl-CoA excess, and chromatin-based aging pathways.

https://doi.org/10.7554/eLife.111611.1.sa4

Abstract

Although lifespan has long been the focus of ageing research, preventing functional decline late in life is a more pressing societal need. Here, we investigate the basis of senescence and declining fitness during replicative ageing in budding yeast, and describe a metabolic perturbation that preserves late-life fitness even on an unrestricted glucose diet. We show that senescence can be prevented by constitutive activation of AMPK, though only for approximately half the ageing population, and use genetic and functional assays to link this heterogeneous response with differences in cytosolic acetyl coenzyme A (Acetyl-CoA) metabolism. In one class of ageing cell, AMPK activity maintains fitness late-in-life through pathways that transport cytosolic Acetyl-CoA into mitochondria, but AMPK also inhibits fatty acid synthesis which leads to lipid starvation in the other class of ageing cell. Therefore, AMPK activity has both positive and negative effects, but we show that constitutive AMPK activity uncoupled from fatty acid synthesis inhibition (the A2A mutant) suppresses senescence and maintains fitness in both classes of ageing cell. We further implicate lipid starvation and excess acetyl coenzyme A availability as major drivers of senescence in replicatively aged wild-type yeast. Our work shows that ageing is not intrinsically associated with declining fitness, at least in yeast, and that re-engineering highly conserved metabolic pathways allows fitness to be preserved very late in life.

Graphical abstract



Introduction

Traditional conceptions of ageing implicate a progressive accumulation of damage leading to systemic degradation in performance until death, with evolutionary pressures acting to maximise early life fitness and fecundity at the expense of ageing health (1–3). The healthspan problem – the societal need to increase the fraction of human life spent in good health – is intractable under such models, since poor health late in life is an inevitable intermediate stage in the damage accumulation leading to death. However, ageing is not uniform across populations and from yeast to humans some members of any population maintain excellent fitness late in life (4–6); increasing the population fraction of such low-senescence individuals is a primary aim of ageing research. Interventions such as caloric restriction and mTOR inhibition can improve late-life fitness (7–11), but caloric restriction is an unrealistic societal intervention and mTOR inhibition has side effects, so new interventions are required.

Replicative ageing of the budding yeast *Saccharomyces cerevisiae* is a widely used model of ageing. Budding yeast cells divide asymmetrically into a mother cell and a daughter cell, with the mother cell undergoing only a limited number of divisions before permanently exiting the cell cycle (12). Mother cells maintain a rapid and uniform cell cycle for most of their replicative lifespan but the cell cycle usually slows dramatically in the last few divisions indicating loss of fitness (12–14), an effect known as 'senescence' following the classical definition of the term 'senescence' to denote a period of declining fitness for the organism at the end of life, which should not be equated with cellular senescence, the permanent cell cycle arrest caused by irreparable damage in mammalian cells. A sharp transition termed the senescence entry point (SEP) marks the change from rapid to slow, heterogeneous cycling and coincides with the appearance of apparent pathological markers (14,15). Not all cells senesce, and populations are extremely heterogeneous in some experimental systems, with individual cells following either a short-lived trajectory marked by an early SEP and accumulated foci of a mitochondrial Tom70-GFP marker when present, or a long-lived trajectory

with little evidence of senescence (6,16,17). We recently reported that ageing yeast on galactose instead of glucose prevented senescence without extending lifespan across the whole population, providing a massive improvement in late-life fitness without caloric restriction (18).

The relevant metabolic differences caused by ageing on galactose are unclear, but suppression of senescence required both respiration and AMPK (18), a highly conserved kinase that reconfigures energy utilisation in response to poor nutrient availability by increasing respiration, mobilising internal energy stores, inducing autophagy and moderating fatty acid biosynthesis (reviewed in (19,20)). Due to its pivotal role in the response to low energy, AMPK has long been a focus of efforts to understand caloric restriction (21,22) and is required for the benefits of caloric restriction in yeast, worms and flies (23–25). Overexpression of the AMPK catalytic subunit extends lifespan in *C. elegans* and *D. melanogaster*, and high doses of the indirect AMPK activator metformin have been reported to extend lifespan and healthspan of mice (26–30). However, the mechanism underlying these lifespan effects is unclear, and the generality of these effects is uncertain, as other studies of metformin have reported no lifespan improvement in flies, mice or humans (31,32).

Caloric restriction robustly extends lifespan of budding yeast (33–35), but AMPK activity has different effects on yeast lifespan depending on the ageing paradigm employed, shortening replicative lifespan but extending chronological lifespan (36,37). Yeast AMPK is a central regulator of energy metabolism and shares close mechanistic and functional parallels with its counterpart in higher eukaryotes. Snf1, the catalytic subunit of AMPK in yeast, is activated by phosphorylation at a structurally conserved site, Thr210, by kinases Sak1, Elm1 and Tos3, and is regulated in response to glucose availability through Snf1 dephosphorylation, pH and subunit binding (38–40).

Suppression of fatty acid synthesis through inhibitory phosphorylation of acetyl coenzyme A carboxylase (ACC) is a highly conserved function of AMPK; this has been described for yeast Acc1, *Drosophila* ACC and mammalian ACC1/ACC2 (41–44). Fatty acids are synthesised from cytosolic Acetyl-CoA, which ultimately derives from glycolysis either through respiration in mammals or through oxidation of fermentation intermediates to acetate in yeast. Regardless of the source, fatty acid synthesis above immediate requirements acts as a sink of energy and carbon that can be mobilised during future scarcity by AMPK through suppression of fatty acid synthesis and promotion of fatty acid catabolism.

Here, we determine the basis of senescence and fitness loss in replicatively ageing yeast and demonstrate that senescence can be almost completely avoided by rewiring the conserved AMPK-fatty acid metabolic regulatory system.

Results

AMPK suppresses senescence through mitochondrial Acetyl-CoA import pathways

AMPK is active and necessary for growth on galactose but not on glucose (45,46) and is required for cells ageing on galactose to avoid senescence (18). We therefore asked whether activation of AMPK during ageing on glucose can recapitulate the benefits of ageing on galactose. For this, we compared log phase populations of *S. cerevisiae* (average age 0-1 generation) to purified cohorts aged on rich glucose media for 24 or 48 hours (average replicative ages ~12 and ~18 generations, at which points cells are ~95% and ~30% viable, respectively). Experiments were performed in batch culture with diploid cells using the mother enrichment program (MEP) to enrich aged cells, a well-characterised system that recapitulates lifespan effects of short- and long-lived mutants (47). Cell wall biotin labelling was used to purify aged mother cells, with density gradient separation to remove dead cells and debris (48,49). Cells carried Tom70-GFP as a marker of senescence (6,14), which we have previously shown to be strongly associated with other marks of senescence in the MEP system including vacuolar size, transcriptional dysregulation and chromosomal aberrations (18,50). Cells were co-stained for bud scars using WGA to quantify replicative age (51–53), and an Rpl13a-mCherry control marker that is unaffected by senescence was included in most experiments (54) (Figure 1A).

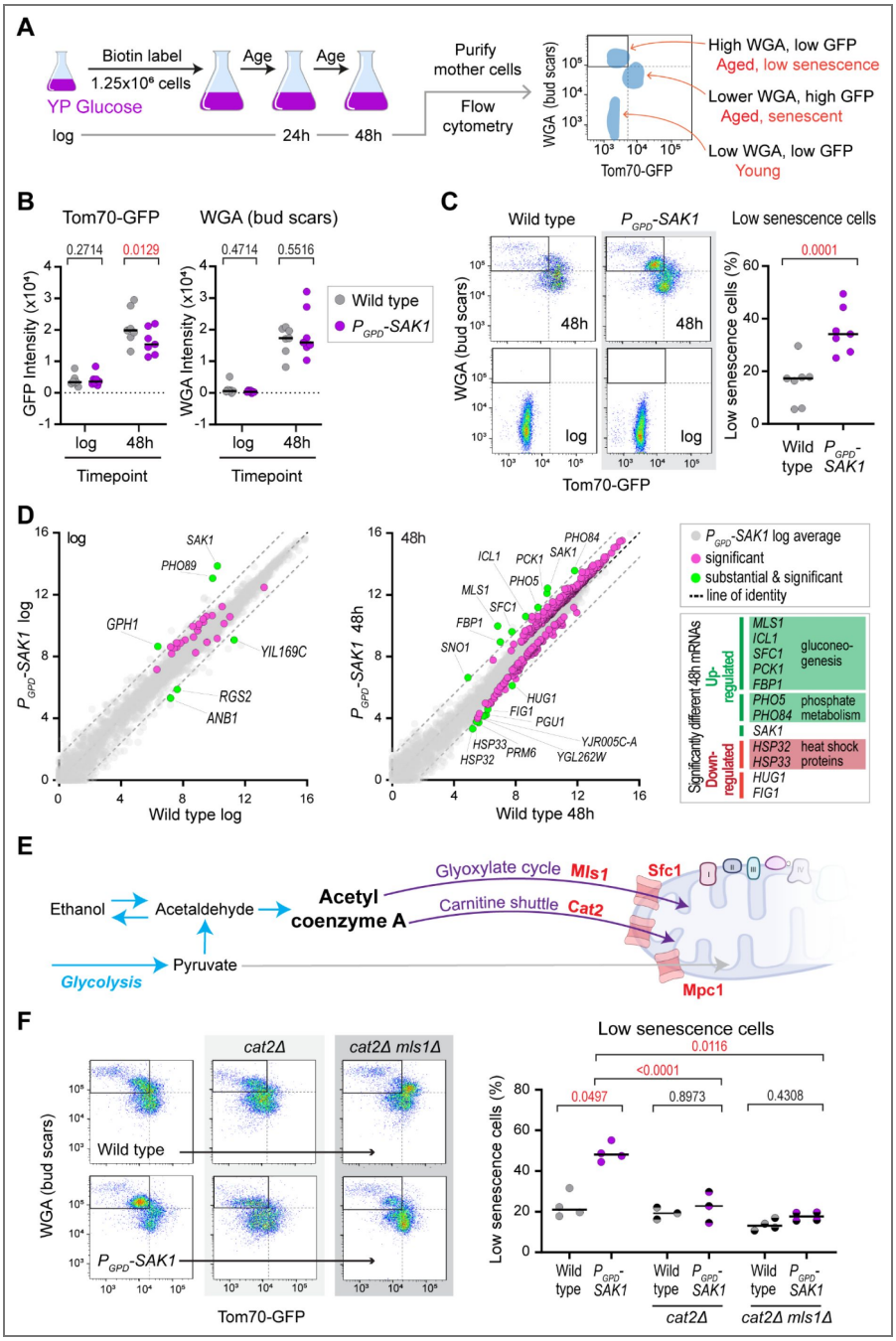


Figure 1. AMPK suppresses senescence through mitochondrial Acetyl-CoA import pathways

A: Experimental scheme for analysis of replicatively aged yeast **B:** Impact of *SAK1* overexpression on Tom70-GFP (marker of senescence onset) and WGA (marker of replicative age). p-values calculated by two way ANOVA, n=7 **C:** Separation of wild type and P_{GPD}-*SAK1* low senescence (high WGA low Tom70-GFP) and high senescence (low WGA high Tom70-GFP) populations by flow cytometry, and quantification of low senescence population at 48 h. p-value calculated by t test, n=7 **D:** Comparison of gene expression profiles of wild type and P_{GPD}-*SAK1* cells at log phase and after 48 h ageing by mRNA-seq. Data is an average of 3 biological replicates, significantly different genes (p<0.01 by DEseq2) are shown in purple, genes both substantially (>3x indicated by dotted lines) and significantly different are highlighted in green. Substantially and significantly upregulated genes are annotated. **E:** Simple schematic of the relationship between AMPK, mitochondria and glycolysis pathways. Central glucose processing by glycolysis leading to fermentation is shown by blue arrows, AMPK activated pathways as purple arrows. Key enzymes in this study are shown in red. **F:** Representative flow plots and quantification of the low senescence population at 48 h in wild type and P_{GPD}-*SAK1* mutants lacking the carnitine shuttle (*cat2*Δ) and mutants lacking both the glyoxylate cycle (*mls1*Δ) and *cat2*Δ. p-values calculated by one way ANOVA, n=3-4

AMPK was activated upon glucose by constitutive overexpression of the upstream kinase Sak1 using a P_{GPD} promoter as previously described (55,56) (Figure S1A). After 48 h ageing, only a subtle decrease in the Tom70-GFP senescence mark was detected in P_{GPD}-*SAK1* cells by bulk measurements (Figures 1B, S1B), but a new subpopulation of aged cells with low Tom70-GFP and high WGA emerged (Figures 1A, 1C top left quadrant). This subpopulation has a greater replicative age (marked by WGA) since non-senescent cells continue to cycle at normal speed rather than slowing down after the SEP, and so reach a higher replicative age in 48 h. Low Tom70-GFP and high replicative age therefore mark a sub-population of aged cells with reduced senescence that is specific to the P_{GPD}-*SAK1* mutant (Figure 1C right).

To understand how P_{GPD}-*SAK1* reduces senescence, we performed RNA-seq comparing P_{GPD}-*SAK1* to wild type cells at log and 48 h. Although AMPK is a major regulator of gene expression (57), only 21 genes were significantly differentially expressed at log phase in P_{GPD}-*SAK1* (Figure 1D, left), but a greater effect was observed in aged P_{GPD}-*SAK1* cells: 472 genes were significantly different from wild type ($p<0.01$) amongst which the up-regulated genes were enriched for respiration and cytoplasmic translation functions, while the down-regulated genes were not enriched for any GO terms. However, only 17 genes were substantially different (>3-fold: 8 down, 9 up including *SAK1*, Figure 1D right): the down-regulated genes shared no apparent connection, but the 9 up-regulated genes included all 5 of a co-regulated gene set that directs cytosolic Acetyl-CoA into gluconeogenesis via the glyoxylate cycle - *MLS1*, *ICL1*, *PCK1*, *SFC1* and *FBP1* (58,59). This pathway is not used during normal growth on glucose, though plentiful cytosolic Acetyl-CoA is available, but it is required during growth on carbon sources such as ethanol and acetate when Acetyl-CoA becomes the fuel for respiration as well as the feedstock for gluconeogenesis and associated anabolic pathways.

The glyoxylate cycle converts cytosolic or peroxisomal Acetyl-CoA into succinate for transport into mitochondria by Sfc1. Deletion of *MLS1* or *SFC1* significantly reduced the low senescence sub-population in P_{GPD}-*SAK1* (Figure S1C), however Acetyl-CoA can also be transported from the cytoplasm into mitochondria after conjugation to carnitine by carnitine acetyl transferases (Figure 1E). Although multiple carnitine acetyl transferases exist outside mitochondria, this carnitine shuttle depends on the mitochondrial carnitine acetyltransferase Cat2 and deletion of *CAT2*, either alone or in combination with *mls1*Δ almost completely abrogated the effect of P_{GPD}-*SAK1*, with very few cells achieving a low senescence phenotype at 48 h (Figure 1F).

The transport of cytosolic Acetyl-CoA into mitochondria fuels respiration during growth on acetate and ethanol, and our results indicate that this process also fuels respiration during ageing on glucose. Indeed, the low senescence population in P_{GPD}-*SAK1* is dependent on the electron transport chain and does not form in P_{GPD}-*SAK1 cox9*Δ cells, but is independent of pyruvate derived from glycolysis entering mitochondria directly via the mitochondrial pyruvate carrier Mpc1 (Figure S1D). Therefore, AMPK activation on glucose maintains mitochondrial activity and prevents the decline of mitochondria marked by high Tom70-GFP intensity, which was previously described as the cause of senescence on one yeast ageing trajectory (6,60).

Combining AMPK and Acc1 activity to prevent senescence

AMPK only suppresses senescence in a sub-population of cells, defining two classes of ageing cells – those in which senescence is reduced by AMPK activation and those in which AMPK activation is ineffective. This led us to ask whether AMPK activation also has a negative impact that dominates in the latter class. Having implicated Acetyl-CoA in senescence, we investigated other fates of this metabolite that are also regulated by AMPK. Cytosolic Acetyl-CoA is the building block for fatty acids, being processed into malonyl coenzyme A by ACC to initiate fatty acid synthesis, however ACC is inhibited by AMPK to suspend energy storage in fatty acids under low energy conditions. Yeast ACC (Acc1) is inhibited by AMPK through phosphorylation at two serines, with Ser1157 being the main regulatory site (Figure 2A) (61,62). To test whether Acc1 inhibition drives senescence in the sub-population of cells that do not respond to Sak1 over-expression, we combined P_{GPD}-*SAK1* with an *acc1*^{S1157A} mutation that prevents Acc1 inactivation by AMPK, thereby creating the A2A mutant (AMPK and Acc1 active).

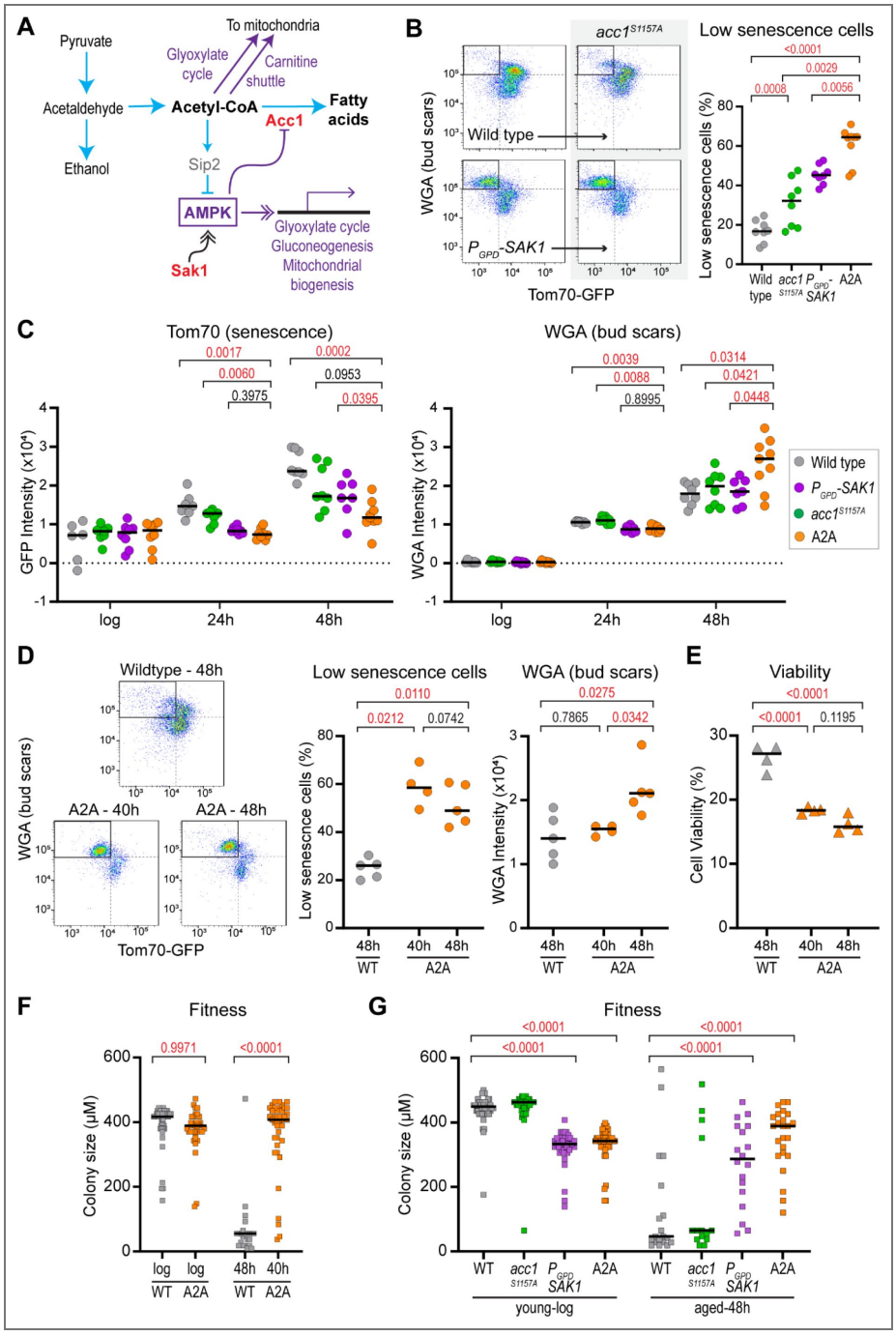


Figure 2. Combining AMPK activity and fatty acid synthesis to avoid senescence

A: Schematic highlighting the regulation of fatty acid synthesis by AMPK. Key proteins for this figure are highlighted in red. **B:** Representative flow plots and quantification of the low senescence population in acc1^{S1157A}, P_{GPD}-*SAK1* and A2A at 48 h. p values calculated by one way ANOVA, n=8-9 **C:** Population medians for Tom70-GFP and WGA in acc1^{S1157A}, P_{GPD}-*SAK1* and A2A at log phase, 24 h and 48 h. p values calculated by two way ANOVA, n=7-9 **D:** Representative flow plots of wild type and A2A at matched average replicative age (48 h for wild type versus 40 h for A2A), with quantification of the low senescence population and of median WGA (bud scars). p values calculated by one way ANOVA, n=4-5 **E:** Percentage cell viability in cultures aged for 40 and 48 h. p value calculated by t test, n=4. **F:** Size of colonies formed in 24 h on a YPD plate by log phase and age-matched wild type and A2A. Only cells that eventually formed colonies within 3 days were included to ensure all tested cells were viable. p values calculated by two way ANOVA, n=14-39 **G:** Size of colonies formed in 24 h on a YPD plate by log phase and 48 h-aged wild type, acc1^{S1157A}, P_{GPD}-*SAK1* and A2A. Only cells that eventually formed colonies within 3 days were included to ensure all tested cells were viable. p values calculated by two way ANOVA, n=15-61

Young A2A cells proliferate equivalently to wild-type cells in liquid culture (Figure S2A), and do not have an extended replicative lifespan (Figure S2B), but do show a significant and substantial increase in the low senescence population relative to P_{GPD}-*SAK1* (Figure 2B). Separate analysis of Tom70-GFP and WGA revealed that the A2A and P_{GPD}-*SAK1* populations were equivalent on average at 24 hours, but A2A consistently presented lower Tom70-GFP and higher WGA by 48 h (Figures 2C, S2C, S2D). Population measurements of Tom70-GFP and WGA do vary between experiments, but the differences were consistent across many experiments (Figure S2D, n>25), and bud scar counting confirmed that A2A cells reach a higher age than wild-types (Figure S2E). The combination of AMPK activation and unfettered Acc1 activity therefore allows the majority of cells to age without senescence. A2A cells cultured for 40 h reached an average replicative age comparable to wild-type at 48 h and, at this point, formed a very clear low senescence population, confirming that SEP onset is delayed or absent in age-matched cells (Figure 2D). This reduction of senescence occurred despite the viability of A2A cells (based on the ability to form a colony on a YPD plate) being lower at 40 h than wildtype at 48 h (Figure 2E). Importantly, at 40 h, >50% of the A2A cells are in the low Tom70-GFP, high age population even though <20% are viable, meaning that many cells reach the end of their replicative lifespan without passing through the SEP.

Tom70-GFP and WGA signals are only indirect indicators of cell fitness, whereas the growth rate of colonies formed by individual young or old cells provides a direct physiological measure of cell fitness. While young A2A cells formed slightly smaller colonies than did wild-type cells, the colonies formed by aged A2A cells were much larger than those formed by wild-type cells of equivalent replicative age (Figure 2F). Indeed, aged A2A cells formed colonies of similar size to young A2A cells, showing equivalent fitness, whereas most aged wild-type cells divided only once or not at all across the 24-hour assay period. Only viable cells that formed colonies within 3 days were included in this analysis, so the non-dividing aged wild-type cells were viable but had a cell division time greater than 24 hours. This demonstrates that A2A cells maintain excellent fitness late in life, whereas the fitness of aged wild-type cells is very low. A comparison of the fitness of the wild type, *acc1*^{S1157A}, P_{GPD}-*SAK1* and A2A strains at a fixed 48 h time point revealed that A2A maintained the best fitness despite also having the highest median age, while P_{GPD}-*SAK1* showed variable fitness, and *acc1*^{S1157A} was similar to wild type (Figure 2G). It is worth noting that colony formation by log phase cells carrying the P_{GPD}-*SAK1* construct was consistently slightly slower than wildtype (5% longer doubling time on average) although this does not manifest in liquid growth assays.

These experiments show that the beneficial effects of AMPK activation during ageing can be increased substantially if Acc1 inhibition is prevented. High AMPK activity combined with normal Acc1 activity results in a robust low senescence ageing phenotype and high late life fitness without decreasing mortality rate.

Different classes of A2A cells depend on respiration and Acc1 activity

We then asked whether all A2A cells avoid senescence through the same mechanism. Given that the required factors (Acc1, the carnitine shuttle, the glyoxylate cycle) each deplete the cytosolic Acetyl-CoA pool, a prime candidate is the negative feedback loop involving the AMPK subunit Sip2, which inhibits AMPK when cytosolic Acetyl-CoA availability is high (63) (Figure 3A). It is not hard to imagine that the metabolic balance emerging from this negative feedback loop varies between cells, with some cells being more dependent than others on cytosolic Acetyl-CoA removal by Acc1 to maintain AMPK activity. Depleting Acetyl-CoA reduces bulk histone acetylation (64), and we observed this effect in A2A cells, indicating that cytosolic-CoA availability is indeed decreased (Figure 3B). However, AMPK activation with P_{GPD}-*SAK1* yielded the same split population in the *sip2*Δ strain as in wildtype (compare Figs. 1C, 3C), and the progressive increase in the low senescence population achieved with P_{GPD}-*SAK1* and P_{GPD}-*SAK1 acc1*^{S1157A} (A2A) was also

observed in a *sip2*^{3R} mutant that does not respond to Acetyl-CoA (63)(Figure S3A). Therefore, the differential responses to AMPK activation in the ageing population cannot be attributed to Sip2-mediated feedback, but depletion of cytosolic Acetyl-CoA may be beneficial independent of Sip2.

To determine whether reducing the availability of Acetyl-CoA is beneficial in ageing, we deleted *ALD6*, which encodes the primary cytoplasmic acetaldehyde reductase. Loss of Ald6 reduces the supply of acetate from fermentation to be processed into Acetyl-CoA (65,66). Although this did not have a large effect on the low Tom70-GFP, high WGA population as defined in Figure 1, the deletion of *ALD6* was effective in decreasing Tom70-GFP and increasing age at harvest (Figure 3D). These findings showed that *ald6*Δ cells maintain high fitness for a longer portion of life. However, fitness measured at 48 h was very low (Figure 3E) meaning that the fitness gains from reducing cytosolic Acetyl-CoA availability are temporary and are lost by 48 h; therefore, the A2A phenotype cannot be attributed solely to a reduction of Acetyl-CoA availability.

Conversely, the low senescence of A2A populations may have different causes in different cells, with some cells requiring AMPK-driven respiration fuelled by cytosolic Acetyl-CoA and other cells requiring the combination of high AMPK and high Acc1 activity. To test this hypothesis, we deleted C*AT2* and *MLS1* in A2A, which should impair ageing fitness of only those cells requiring AMPK-driven respiration: this resulted in the population dividing between low and high senescence states based on flow cytometry and fitness (Figure 3F,G). Similarly, deleting *COX9* to prevent respiration removed a subset of cells from the low senescence population (Figure S3B). Therefore, cells that achieve a low senescence state through respiration in P_{GPD}-*SAK1* avoid senescence by the same mechanism in A2A, but the cells that avoid senescence due to the *acc1*^{S1157A} mutation in A2A use a mechanism independent of the carnitine shuttle and glyoxylate cycle. We further asked whether respiration itself or reduction in Acetyl-CoA availability is important for the first group of cells by deleting *ALD6* in P_{GPD}-*SAK1*; if respiration is important, this should suppress the benefit of P_{GPD}-*SAK1* by reducing the fuel for respiration, whereas if Acetyl-CoA removal is important then this should be neutral or beneficial. Working in *sip2*Δ to avoid an increase in AMPK activity due to reduced Acetyl-CoA availability, we observed that *ald6*Δ increased the low senescence population through decreasing Tom70-GFP (S3C), and therefore the beneficial impact of P_{GPD}-*SAK1* on this pathway arises from Acetyl-CoA removal rather than a benefit of respiration.

These experiments define two classes of ageing cells with distinct metabolic needs, coherent with the model of two ageing trajectories previously proposed (6). In one class, respiration fuelled by cytosolic Acetyl-CoA is required to suppress senescence, while in the other class respiration is not required but maintaining Acc1 activity is critical. These processes all consume cytosolic Acetyl-CoA, and reducing the availability of this metabolite is beneficial but insufficient to prevent senescence.

Fatty acid supply is limiting in ageing cells that do not respond to AMPK activation

Acc1 converts Acetyl-CoA to Malonyl-CoA as the first step in fatty acid synthesis, a biosynthetic activity that may or may not be important during ageing. We asked whether fatty acids or perhaps the Malonyl-CoA intermediate prevent senescence onset in A2A, against a null hypothesis that Acc1 simply provides an alternative pathway to respiration for moderating cytosolic Acetyl-CoA levels (Figure 4A). To test this hypothesis, we attempted to reproduce the low senescence phenotype of A2A by manipulating fatty acid synthesis in P_{GPD}-*SAK1*, again in the absence of Sip2 to avoid feedback on AMPK activity (Figure 4B).

Malonyl-CoA was recently found to be an endogenous mTOR inhibitor (67), and mTOR inhibition is well known to increase ageing health and lifespan (11). However, a low dose of the fatty acid synthase inhibitor cerulenin, which causes Malonyl-CoA to accumulate (67,68), profoundly decreased the low senescence population (Figure 4B, conditions 2,3). A higher dose of cerulenin was also tested in combination with oleic acid supplementation to complement the complete blockade of fatty acid synthesis, but this combination had very little effect (Figure 4B, condition

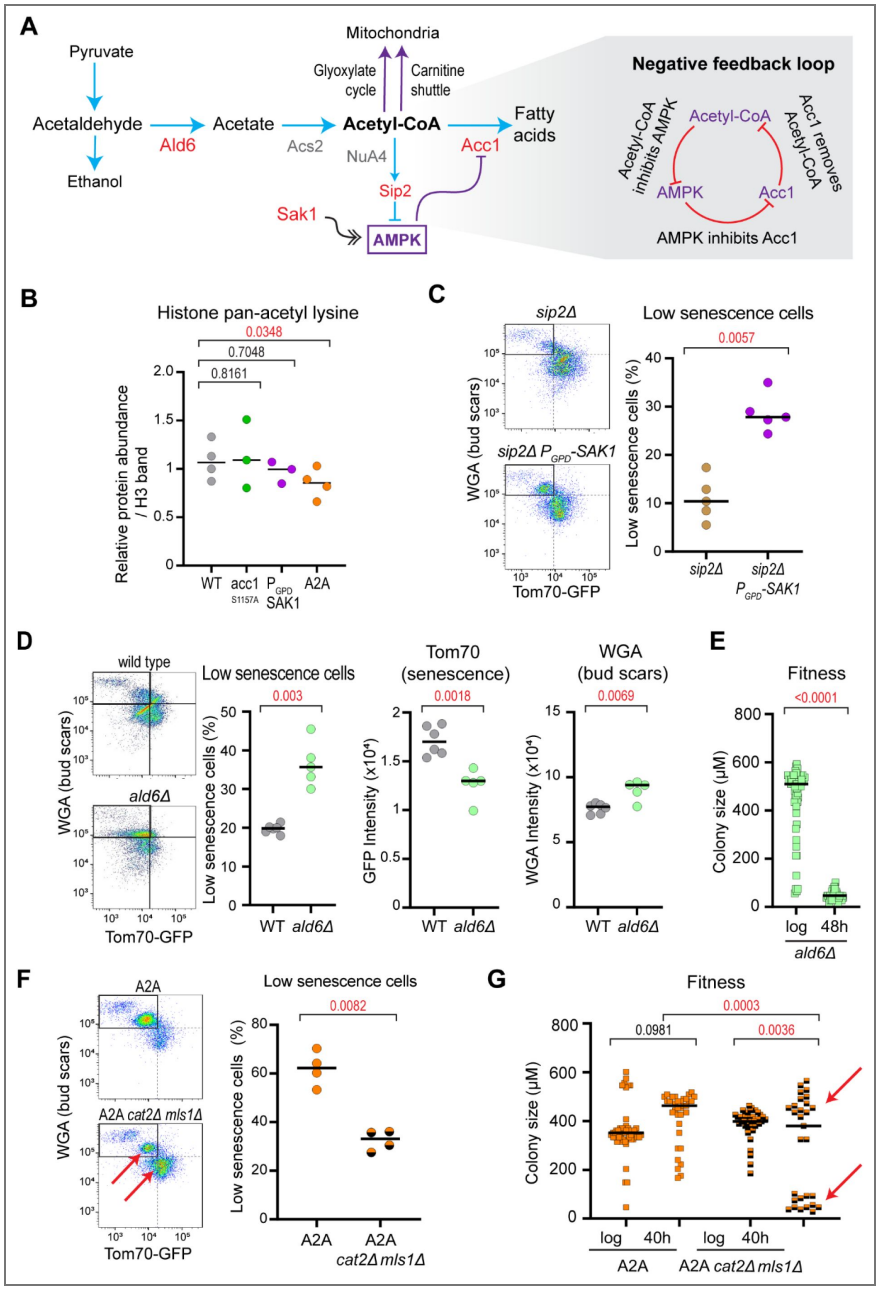


Figure 3. Separate classes of A2A cells depend on respiration and fatty acid availability

A: Schematic of acetyl coenzyme A biosynthesis and the emergence of a negative feedback loop through AMPK regulation by Sip2. Key proteins for this figure are highlighted in red. **B:** Western blot analysis of acetyl lysine on H3 in log phase wild type, *acc1*^{S1157A}, P_{GPD}-*SAK1* and A2A cells. Membranes were probed with mouse anti-H3 and rabbit anti-pan-acetyl lysine using a 2-colour detection system. p values calculated by one-way ANOVA, n=3-4. **C:** Representative flow plots and quantification of low senescence population in *sip2*Δ and *sip2*Δ P_{GPD}-*SAK1* at 48 h. p-values calculated by t-test, n=5 **D:** Representative flow plots and quantification of low senescence population in wild type and *ald6*Δ at 48 h. Population medians are also shown for Tom70-GFP and WGA in wild type and *ald6*Δ at 48 h. p values calculated by t-test, n=5-6 **E:** Size of colonies formed in 24 h on a YPD plate by log phase and 48 h-aged wild type and *ald6*Δ. Only cells that eventually formed colonies within 3 days were included to ensure all tested cells were viable. p values calculated by one-way ANOVA, n=36-57 **F:** Representative flow plots and quantification of low senescence population in A2A mutants lacking both carnitine acetyltransferase activity (*cat2*Δ) and the glyoxylate cycle (*mls1*Δ) at 48 h. Arrows indicate two populations, p-value calculated by t-test, n=4 **G:** Size of colonies formed in 24 h on a YPD plate by log phase and 40 h-aged A2A and A2A *cat2*Δ *mls1*Δ. Only cells that eventually formed colonies within 3 days were included to ensure all tested cells were viable. Arrows indicate two populations, p values calculated by two way ANOVA, n=29-55

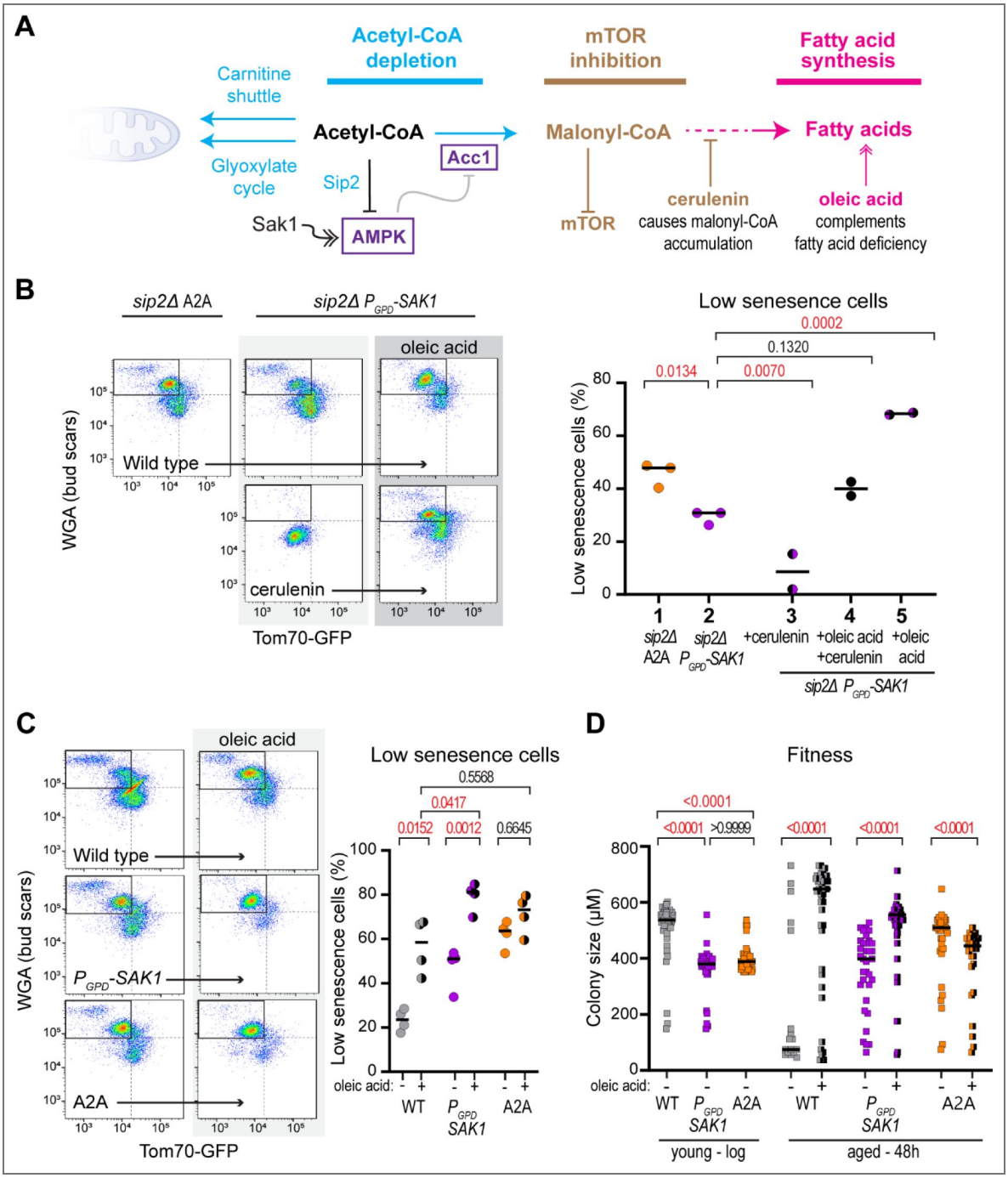


Figure 4. Fatty acids are particularly important in ageing cells that do not respond to AMPK activation

A:Schematic of potential mechanisms for the beneficial effect of ACC1 activity: Inhibition of mTOR mediated by malonyl coenzyme A, the direct product of Acc1, or efficient synthesis of fatty acids, compared to a null hypothesis that Acc1 acts to remove cytosolic acetyl-CoA, for example if these cells are respiratory-deficient. **B:** Representative flow plots and quantification of low senescence population at 48 h in *sip2Δ P$_{GPD}$-SAK1 acc1^{S1157A}* and *sip2Δ P$_{GPD}$-SAK1* supplemented with 2.5 μM cerulenin (condition 3), 10 μM cerulenin + 0.04% oleic acid + 0.04% Tween 80 (condition 4) or 0.04% oleic acid + 0.04% Tween 80 (condition 5). p-values calculated by one-way ANOVA, n=2-3 **C:** Representative flow plots and quantification of low senescence population at 48 h in wild type, *P$_{GPD}$-SAK1* and A2A supplemented with 0.04% oleic acid + 0.04% Tween 80. p-values calculated by one-way ANOVA, n=4 **D:** Size of colonies formed in 24 h on a YPD plate by log phase and 48 h-aged wild type, acc1^{S1157A}, *P$_{GPD}$-SAK1* and A2A supplemented with 0.04% oleic acid + 0.04% Tween 80. Only cells that eventually formed colonies within 3 days were included to ensure all tested cells were viable. p values calculated by one-way ANOVA, n=19-52

4). Therefore, Acc1 activation in A2A cells does not work through increased Malonyl-CoA levels. In contrast, supplementation with only the monounsaturated fatty acid oleic acid increased the low senescence population of P_{GPD}-*SAK1* cells even more than *acc1*^{S1157A}, showing that fatty acid scarcity is the primary driver of senescence in ageing cells that do not respond to AMPK activation alone.

None of these treatments had a strong impact on Tom70-GFP however, with increases in the low Tom70-GFP, high WGA population being driven almost entirely by increased WGA (Figure S4A), indicating that the strongest effect of fatty acid supplementation was to overcome the known short lifespan of the *sip2*Δ background (37). After validating the effect of oleic acid by bud scar counting (Figure S4B), we therefore asked whether oleic acid supplementation also reduces senescence in a wild-type background. The effects were dramatic, with the low senescence population increasing 2.5-fold through decreased population average Tom70-GFP and increased WGA (Figures 4C, S4C). The low senescence population in P_{GPD}-*SAK1* also almost doubled with oleic acid supplementation, but only a small, non-significant effect on A2A was observed (Figure 4C). Notably, oleic acid supplementation and P_{GPD}-*SAK1* each allow approximately half of the population to age with low senescence, while the combination allows most cells to avoid senescence - this is as expected if P_{GPD}-*SAK1* and oleic acid supplementation suppress senescence in different classes of ageing cells.

At 48 h the fitness of wildtype cells supplemented with oleic acid also increased dramatically, P_{GPD}-*SAK1* cells improved somewhat while fitness was significantly but not substantially decreased in A2A (Figure 4D). In fact, the oleic acid-supplemented wild-type cells were fitter than the mutants, which likely reflects the decrease in baseline fitness caused by P_{GPD}-*SAK1* in young cells as noted above. The results for P_{GPD}-*SAK1* confirmed that a subset of cells in which AMPK is activated still undergo senescence because of fatty acid starvation, which can be compensated by the *acc1*^{S1157A} mutation or by oleic acid supplementation.

However, the results for the wild type are more complicated as ~40% of oleic acid-supplemented cells undergo senescence according to flow cytometry whereas all oleic acid supplemented wild-type cells are extremely fit (compare Figures 4C and 4D). This difference arises because fitness assays exclude inviable cells, such as the cells that senesce and lose viability before 48 h due to lack of respiration, which are exactly the cells for which senescence is rescued by AMPK activation. We further noted that the low fitness of aged *ald6*Δ cells was rescued by oleic acid to wild-type levels, showing that the observed fitness defect simply arose from fatty acid starvation rather than from any other function of Acetyl-CoA (Figure S4D). Overall, in wild-type cells, as in P_{GPD}-*SAK1 cells*, high Acc1 activity or oleic acid supplementation prevents senescence in one class of ageing cells while AMPK activation prevents senescence in the other class.

Discussion

Our findings indicate a major role for Acetyl-CoA metabolism in the yeast replicative ageing process. Combining transport of cytosolic Acetyl-CoA into mitochondria with ongoing cytosolic fatty acid synthesis together ensures that cells rarely enter senescence. This is achieved without reducing age-linked mortality and with little effect on the fitness of cells when young, showing that lifespan and fitness decline are separable in the yeast replicative ageing system, and that high fitness can be maintained to the end of life.

Here we have characterised two classes of ageing cells seemingly differentiated by high and low availability of cytosolic Acetyl-CoA, consistent with a previous demonstration that ageing follows two trajectories in yeast (6). Acetyl-CoA is the feedstock for fatty acid synthesis, which is necessary both for membrane production and for energy storage in lipid droplets. However, cytosolic Acetyl-CoA production consumes as much ATP as is generated in glycolysis and thus competes directly with growth. The existence of two classes of cell that follow different ageing trajectories makes sense because yeast cells do not know when food availability will change and so the population employs a bet hedging strategy (69–71): part of the population synthesises excess cytosolic Acetyl-CoA to store as much energy as possible in lipids against future scarcity, while other cells maximise growth from the current food supply by synthesising the bare minimum of cytosolic

Acetyl-CoA needed for membrane formation (Figure 5). Our findings indicate that though both strategies are useful they eventually decrease fitness as cells age, forming a classic antagonistic pleiotropy (3).

Multiple pathways can promote senescence during yeast ageing; which pathway dominates is dependent on the experimental system and therefore the set of stresses experienced by cells (6,15,17,72,73). The SEP in yeast was originally characterised in microfluidic assays, where it is attributed to the accumulation of extrachromosomal rDNA circles (ERCs) (14,15,73). In contrast, ERC accumulation is completely unrelated to senescence during ageing in the batch culture system employed here (18,48,50), which is sensitive to metabolic changes such as a galactose diet or Acetyl-CoA processing; the galactose diet has no effect on cells in microfluidic systems and the impact of caloric restriction has remained controversial, so we do not necessarily expect A2A to prevent senescence in microfluidic systems (74,75). However, our findings parallel studies of yeast chronological ageing which have highlighted the importance of respiration and lipid synthesis, though chronological ageing measurements are performed in glucose-depleted conditions and it has been difficult to translate these findings to ageing under normal nutrient availability (23,64,76). Whatever the root of the difference, ageing in batch culture allows the exploration of ageing biology with little impact from ERC accumulation, a phenomenon that does not occur during ageing in animals.

AMPK is broadly considered to have a pro-longevity effect, however the benefits of AMPK activation under normal diets are limited, and some studies in budding yeast conversely found that AMPK activity is negative for lifespan (77,78). Our work shows that both positions can be true, with AMPK having both beneficial and detrimental impacts under nutrient replete conditions. It is not too surprising that issues arise when AMPK is ectopically activated on high glucose given that AMPK is a central energy regulator that evolved to manage metabolism under low nutrient conditions, and it is perhaps more surprising that a single point mutation largely corrects these issues.

The AMPK-Acc1 regulatory system is conserved from yeast to humans, but the source and fate of cytosolic Acetyl-CoA are different so the combination of AMPK activation with lipid supplementation is unlikely to be universally effective. In human cells, the only major route for removal of excess cytosolic Acetyl-CoA is lipid synthesis to palmitoyl coenzyme A followed by carnitine-dependent transport into mitochondria for oxidation. Ectopic activation of AMPK under high nutrient availability would promote lipid catabolism but inhibits lipid synthesis through ACC1/2 phosphorylation. Preventing inhibition of ACC1/2 by AMPK is ineffective as ACC2 also inhibits fatty acid catabolism so fatty acid synthesis and storage increase, resulting in non-alcoholic fatty liver disease (79). However, we speculate that activating AMPK while selectively preventing AMPK inhibition of ACC1 alone may be effective.

Materials and Methods

Detailed and updated protocols are available at *https://www.babraham.ac.uk/our-research/epigenetics/jon-houseley/protocols*.

Yeast strain construction, culture and labelling

Yeast deletion mutants and fusion constructs were constructed by standard methods and are listed in Table S1, oligonucleotide sequences are given in Table S2. Plasmid templates were pFA6a-GFP-KanMX4, pYM-N14, pAW8-mCherry, pFA6a-HIS3 and pFA6a-URA3 (80–82). To create the *acc1*^{S1157A} point mutation, *ACC1* was amplified with ACC1 F3/R3 and cloned in pRS314, then the S1157A mutation introduced by site directed mutagenesis. MEP **a** ±Kan-P_{GPD}-*SAK1* cells carrying this plasmid were transformed with pGSKU (83) amplified with ACC1 CORE UP45 1 and ACC1 CORE DN45 2 to replace the chromosomal ACC1 S1157 codon with a CORE cassette flanked on each side by I-*Sce*I sites. Cells were plated on YPGal to induce I-*Sce*I then FOA to select for loss of the CORE cassette, then TRP negative cells lacking the pRS314-*acc1*^{S1157A} plasmid were verified by sequencing. *sip2*^{3R} was constructed by a simpler method as *SIP2* is not an essential gene: a CORE cassette flanked by I-*Sce*I sites was amplified from pGSKU using SIP2 3Q-R CORE UP45 and SIP2 3Q-

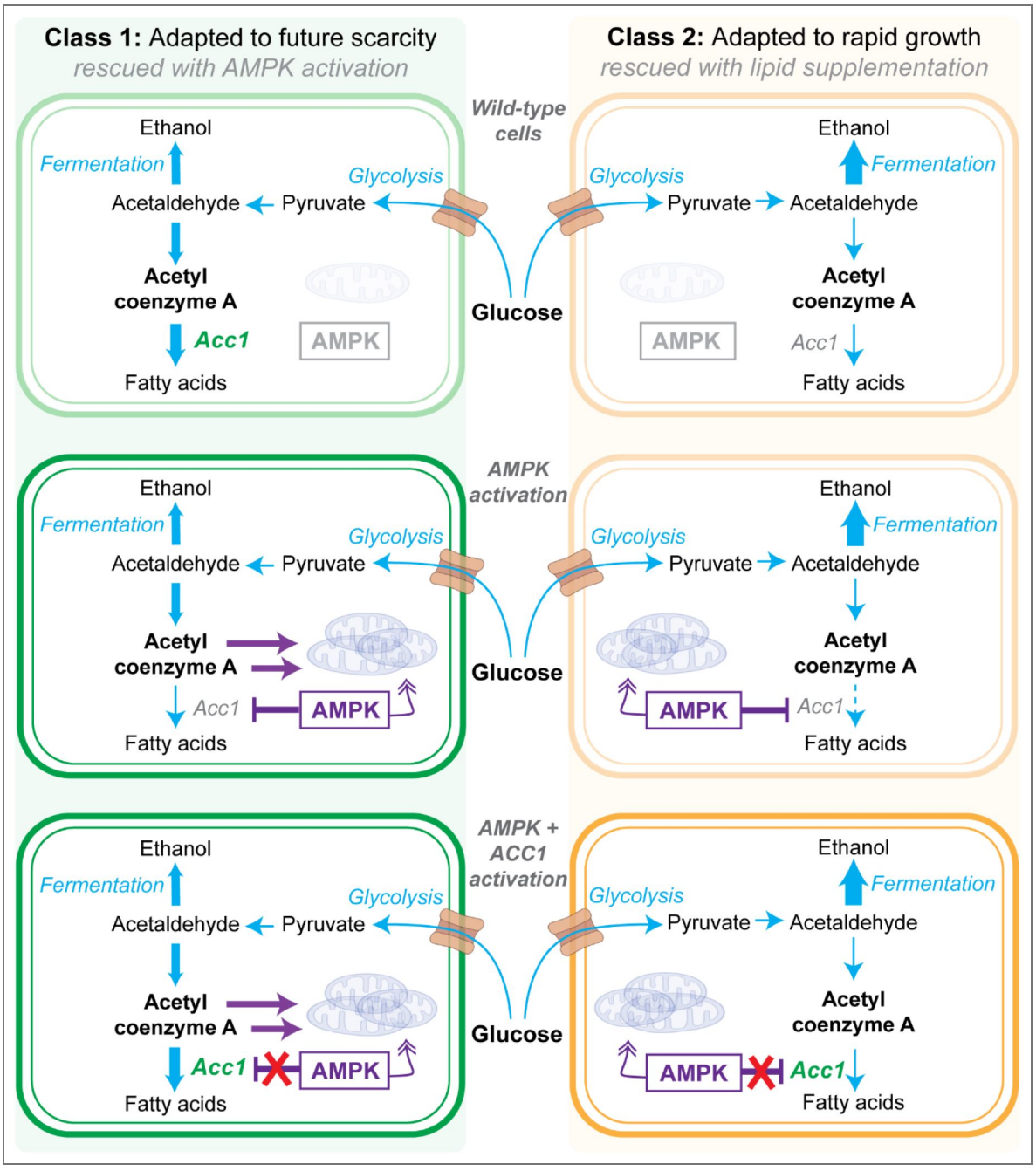


Figure 5. Model of different classes of ageing mediated by Acetyl-CoA and lipid metabolism

Schematic demonstrating the two wild type senescence fates. (i) Adaptation to future scarcity by synthesising excess cytosolic Acetyl-CoA to store as much energy as possible in lipids against future scarcity, and (ii) Adaptation to rapid growth by maximising growth from the current food supply by synthesising the bare minimum of cytosolic Acetyl-CoA.

R CORE DN45 v2 and transformed in destination strains. These were transformed with pRS413 containing an *Eag*I-*Sac*I fragment of *SIP2* with the *sip2*^{3R} sequences, then cells plated on YPGal then FOA.

All cells were grown in YPD media (2% peptone, 1% yeast extract, 2% glucose) at 30°C with shaking at 200 rpm. Media components were purchased from Formedium and media was sterilised by filtration. MEP experiments were performed as described with modifications (84): cells were inoculated in 4 ml YPD and grown for 6-8 hours then diluted in 25 ml YPD and grown for 16-18 hours to 0.2-0.6×10^7 cells/ml in 50 ml Erlenmeyer flasks. 0.125×10^7 cells per aged culture were harvested by centrifugation (15 s at 13,000 g), washed twice with 125 µl PBS and re-suspended in 125 µl of PBS containing ~3 mg/ml Biotin-NHS (Pierce 10538723). Cells were incubated for 30 min on a wheel at room temperature, washed once with 125 µl PBS and re-suspended in 125 µl YPD then inoculated in 125 ml YPD at 1×10^4 cells/ml in a 250 ml Erlenmeyer flask (FisherBrand FB33132) sealed with Parafilm. 1 µM β-estradiol (from stock of 1 mM Sigma E2758 in ethanol) was added after 1.5h. An additional 0.125×10^7 cells were harvested from the log phase culture while biotin labelling reactions were incubating at room temperature. Cells were harvested by centrifugation for 1 min, 4600 rpm, immediately fixed by resuspension in 70% ethanol and stored at −80°C. To minimise fluorophore bleaching in culture, the window of the incubator was covered with aluminium foil, lights on the laminar flow hood were not used during labelling and tubes were covered with aluminium foil during biotin incubation. Lifespan assays in the MEP background were performed as previously described (47,84).

Cell purification

Percoll gradients (1-2 per sample depending on harvest density) were formed by vortexing 1 ml Percoll (Sigma P1644) with 110 µl 10x PBS in 2 ml tubes and centrifuging 15 min at 15,000 g, 4°C. Ethanol fixed cells were defrosted and washed once with 1 volume of cold PBSE (PBS + 2 mM EDTA) before resuspension in ~100 µl cold PBSE per gradient and layering on top of the pre-formed gradients. Gradients were centrifuged for 4 min at 2,000 g, 4 °C, then the upper phase and brown layer of cell debris removed and discarded. 1 ml PBSE was added, mixed by inversion and centrifuged 1 min at 2,000 g, 4 °C to pellet the cells, which were then re-suspended in 1 ml PBSE per time point (re-uniting samples where split across two gradients). 25 µl Streptavidin microbeads (Miltenyi Biotech 1010007) were added and cells incubated for 5 min on a wheel at room temperature. Meanwhile, 1 LS column per sample (Miltenyi Biotech 1050236) was loaded on a QuadroMACS magnet and equilibrated with cold PBSE in 4 °C room. Cells were loaded on columns and allowed to flow through under gravity, washed with 1 ml cold PBSE and eluted with 1 ml PBSE using plunger. Cells were re-loaded on the same columns after re-equilibration with ~500 µl PBSE, washed and re-eluted, and this process repeated for a total of three successive purifications. After addition of Triton X-100 to 0.01% to aid pelleting, cells were split into 2 fractions in 1.5 ml tubes, pelleted 30 s at 20,000 g, 4 °C, frozen on N2 and stored at −70 °C.

Fitness and viability assays

For live purification, cells were pelleted, washed twice with synthetic complete 2% glucose media, then resuspended in 2 ml final volume of the same media and incubated for 5 min on a rotating wheel with 10 µl MyOne streptavidin magnetic beads (ThermoFisher), isolated using a magnet and washed five times with 1 ml of the same media. Cells were streaked on a YPD plate and individual cells moved to specific locations using a Singer MSM400 micromanipulator. Colony size was measured 24 hours later on the screen of the micromanipulator imaging with a 4x objective, sizes were calibrated using a haemocytometer. Cells that did not form a colony within 72 h were assumed to have been inviable and were not included in the analysis.

For viability assays, log phase cells were inoculated in YPD at 1×10^4 cells/ml and 60 µl spread on a YPD plate. 1 µM β-estradiol was then added to the cultures, and after 40-48 h 1.5 ml of cells were harvested, washed with 100 µl of water and 40 µl spread on a YPD plate. Colonies were counted after 2 d (young) or 3 d (aged).

RNA extraction

Cells were re-suspended in 50 µl Lysis/Binding Buffer (from mirVANA kit, Life Technologies AM1560), and 50 µl 0.5 µm zirconium beads (Thistle Scientific 11079105Z) added. Cells were lysed with 5 cycles of 30 s 6500 ms^{-1} / 30 s on ice in Next Advance Bullet Blender Storm 24 (ThermoFisher) in cold room, then 250 µl Lysis/Binding buffer was added followed by 15 µl miRNA Homogenate Additive and cells were briefly vortexed before incubating for 10 minutes on ice. 300 µl acid phenol : chloroform was added, vortexed and centrifuged 5 min at 13,000 g at room temperature before extraction of the upper phase. 400 µl room temperature ethanol and 2 µl glycogen (Sigma G1767) were added and mixture incubated for 1 hour at −30 °C before centrifugation for 15 minutes at 20,000 g, 4 °C. The pellet was washed with cold 70% ethanol and re-suspended in 10 µl water. 1 µl RNA was glyoxylated and analysed on a BPTE mini-gel, and RNA was quantified using a Qubit RNA HS Assay Kit.

150 ng RNA was used to prepare libraries using the NEBNext Ultra II Directional mRNA-seq kit with poly(A)+ purification module (NEB E7760, E7490) as described with modifications: Reagent volumes for elution from poly(T) beads, reverse transcription, second strand synthesis, tailing and adaptor ligation were reduced by 50%; libraries were amplified for 13 cycles using 2 µl each primer per 50 µl reaction before two rounds of AMPure bead purification at 0.9x and elution in 11 µl 0.1x TE prior to quality control using a Bioanalyzer HS DNA ChIP (Agilent) and quantification using a KAPA Library Quantification Kit (Roche).

Sequencing and bioinformatics

Libraries were sequenced by the Babraham Institute Sequencing Facility using a NextSeq 500 instrument in 75 bp single end mode. After adapter and quality trimming using Trim Galore (v0.6.6), RNA-seq data was mapped to yeast genome R64-1-1 using HISAT2 v2.1.0 (85) by the Babraham Institute Bioinformatics Facility. Mapped data was imported into SeqMonk v1.47.0 (*https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/*) and quantified for $\log_2$ total reads mapping to the antisense strand of annotated open reading frames (opposite strand specific libraries), excluding the mtDNA and the rDNA locus. Read counts were adjusted by Size Factor normalisation for the full set of quantified probes (86).

GO analysis of individual clusters performed using GOrilla (*http://cbl-gorilla.cs.technion.ac.il/*) (87). Quoted p-values for GO analysis are FDR-corrected according to the Benjamini and Hochberg method (q-values from the GOrilla output), for brevity only the order of magnitude rather than the full q-value is given (88).

Flow cytometry

Cell pellets were re-suspended in 240 µl PBS and 9 µl 10% triton X-100 containing 0.3 µl of 1 mg/ml Streptavidin conjugated with Alexa Fluor 647 (Life technologies) and 0.6 µl of 1 mg/ml Wheat Germ Agglutinin (WGA) conjugated with CF405S (Biotium). Cells were stained for 10 min at RT on a rotating mixer while covered with aluminium foil, washed once with 300 µl PBS containing 0.01% Triton X-100, re-suspended in 200 µl PBS and immediately subject to flow cytometry analysis on a Cytek Aurora. Cells for single colour controls were prepared in the same manner as the fully stained samples. On the Aurora, time-matched unmixing reference files were required for different ages as cell size and autofluorescence varied with age. Therefore, numerical differences in fluorescence measurements between different ages are not absolutely quantitative. Cell populations were gated on FSH-H and SSC-H to remove debris, on FSC-A and FSC-H to select for single cells, and streptavidin positive (AF647) cells were selected, all in a hierarchical manner and 10,000 events acquired. Low senescence aged populations were defined using quadrants of low GFP and high WGA, with quadrants being defined on the wild-type and applied uniformly across samples within an experiment. As is visible in the representative flow plots, quadrants are almost invariant across most datasets, with differences arising over time through changes in machine performance.

A subset of samples was acquired on an Amnis ImageStream X Mk II with the following laser power settings: 405 = 25 mW, 488 = 180 mW, 561 = 185 mW, 642 = 5 mW, SSC = 0.3 mW. On the ImageStream, cell populations were gated for; (1) single cells based on area and aspect ratio (>0.8), (2) in-focus cells were gated based on a gradient RMS value (>50), and (3) streptavidin positive (AF647) cells, all in a hierarchical manner and 1,000 events acquired. Before data analysis, compensation was applied according to single-colour controls and auto-generated compensation matrix, see (18) for additional parameters.

As absolute measured fluorescence intensities varied somewhat over time and between machines, median normalisation has been applied across all samples of a given age in certain datasets to allow comparison of Tom70-GFP and WGA intensities between datasets. This is a single transformation applied equally to all samples such that differences between samples are maintained, and is not applied to the quadrant analysis described above.

Statistical analysis

All statistical analysis was performed in GraphPad Prism (v10.4.1), tests and n values are given in Figure legends.

Supplementary information

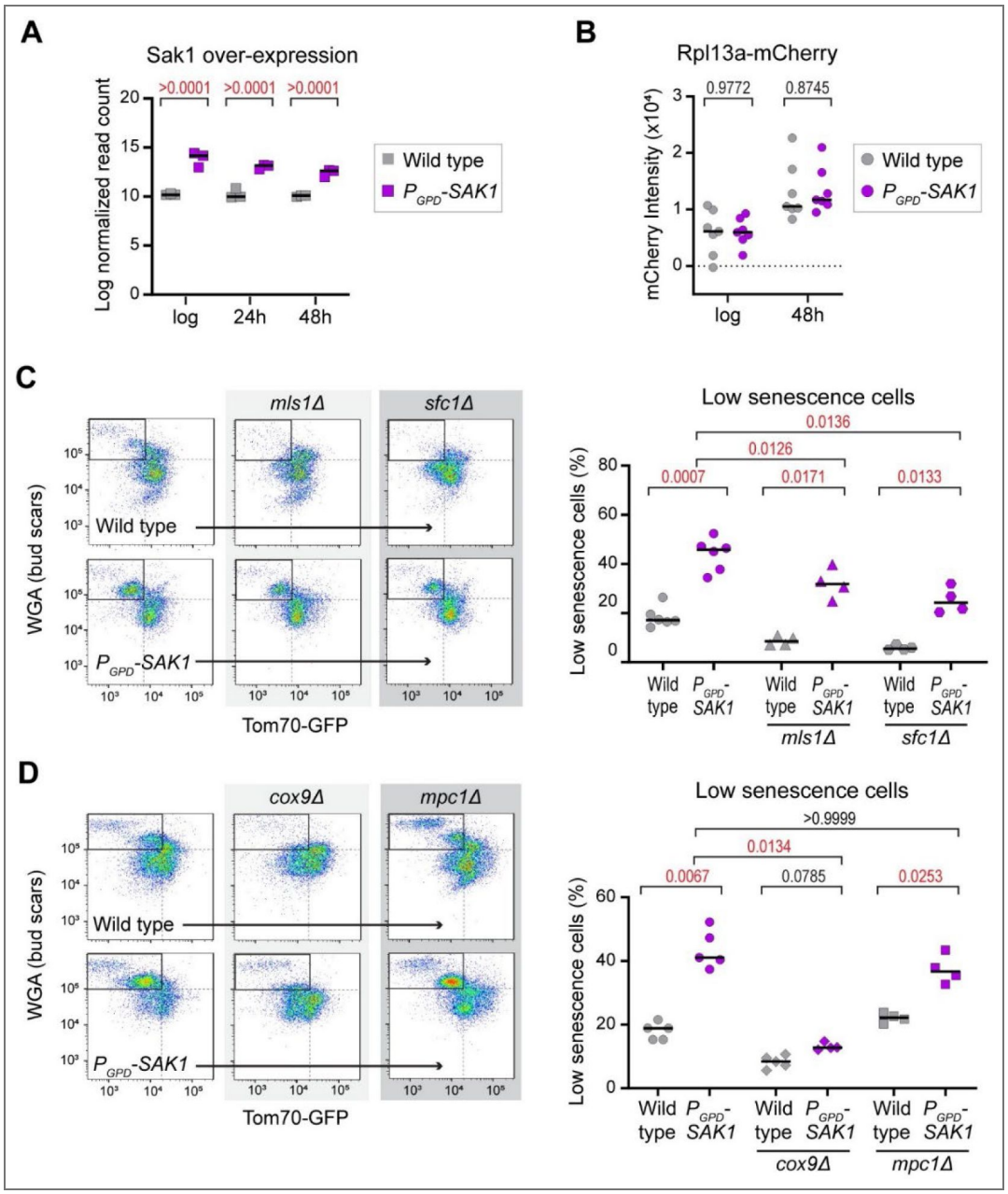


Figure S1. Increased AMPK activity suppresses senescence in a subset of cells A: mRNA expression level of constitutive over-expression of the upstream kinase Sak1 using a P_{GPD} promoter fused to *SAK1* at log phase, 24 h and 48 h. p-values calculated by 1 way ANOVA, n=3 **B:** Impact of *SAK1* overexpression on Rpl13a-mCherry in young and aged cells. p-values calculated by two way ANOVA, n=7 **C:** Representative flow plots and quantification of the low senescence population at 48 h in wild type and P_{GPD}-*SAK1* mutants lacking the glyoxylate cycle (*mls1*Δ) or succinate import (*sfc1*Δ). p-values calculated by one way ANOVA, n=4-6 **D:** Representative flow plots and quantification of wild type and P_{GPD}-*SAK1* low senescence populations at 48 h in mitochondrial mutants lacking a functional electron transport chain (*cox9*Δ) or pyruvate import (*mpc1*Δ). p-values calculated by one way ANOVA, n=4-5

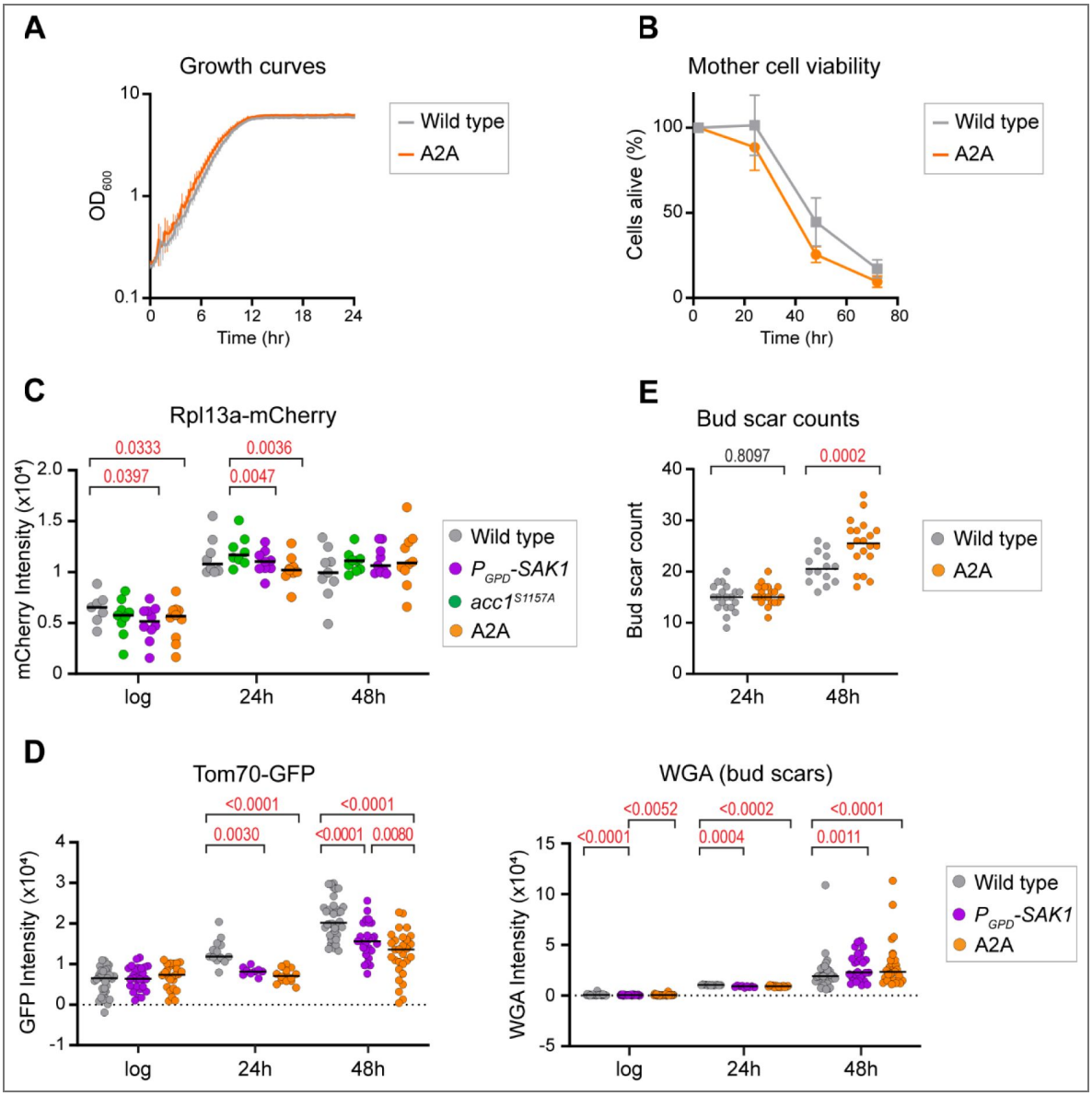


Figure S2. Combining AMPK activity and fatty acid synthesis to avoid senescence

A: Growth curve of wild type and A2A cells in YPD media starting from log-phase pre-cultures. **B:** Lifespan measured in YPD media for wild type and A2A cells based on %age of viable cells remaining at each time point; *n* = 5. **C:** Population medians for Rpl13a-mCherry in wild type, *acc1*^{S1157A}, P_{GPD}-*SAK1* and A2A at log phase, 24 h and 48 h. p values calculated by two way ANOVA, n=7-10. **D:** Population medians for Tom70-GFP and WGA (bud scars) in wild type, P_{GPD}-*SAK1*, and A2A at log phase, 24 h and 48 h made from a compilation of experiments performed during this study. p values calculated by two way ANOVA, n=27-38. **E:** Manual bud scar counts of log phase and 48 h-aged wild type and A2A cells. p values calculated by one way ANOVA, n=14-20

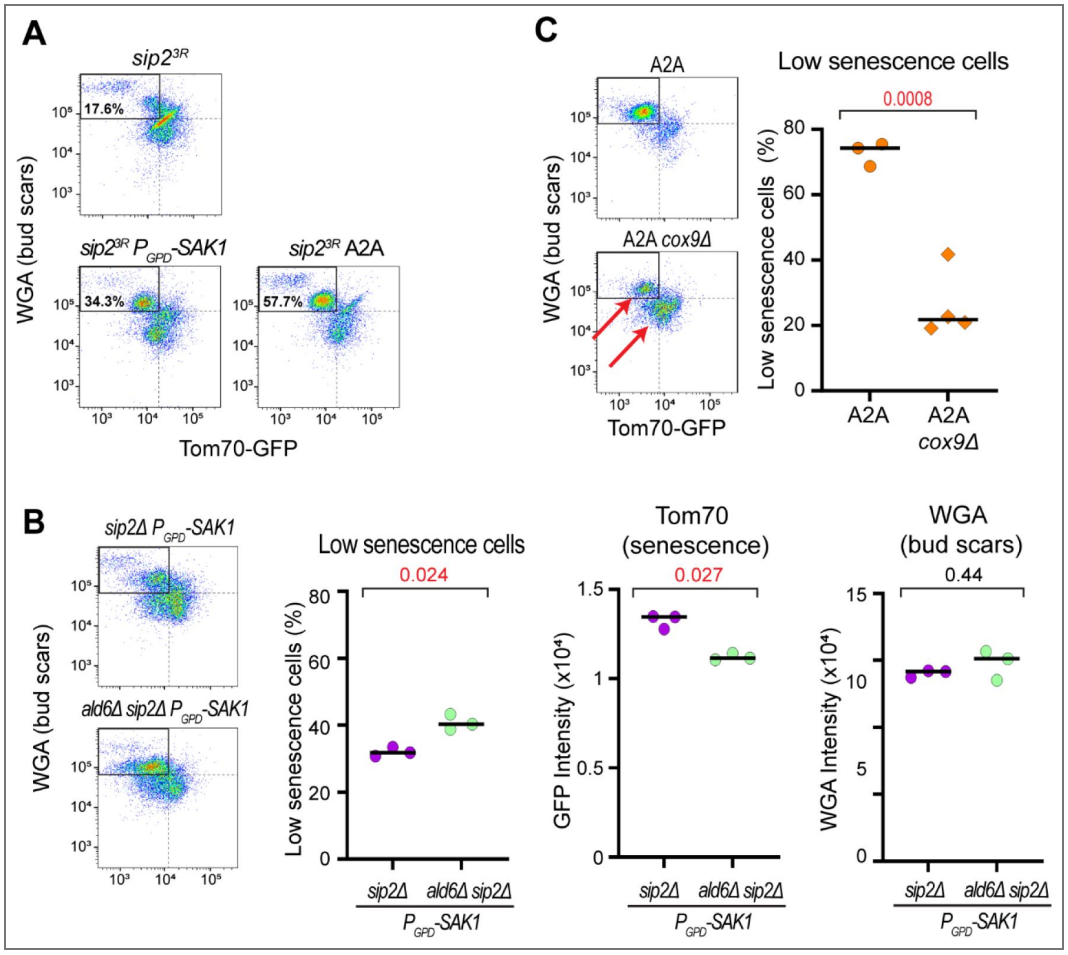


Figure S3. Genetic evidence that cytosolic Acetyl-CoA promotes senescence

A: Representative flow plots of the low senescence population at 48 h in *sip2*^{3R}, *sip2*^{3R} P_{GPD}-*SAK1* and *sip2*^{3R} A2A. **B:** Representative flow plots and quantification of low senescence population in wild type and *sip2*Δ P_{GPD}-*SAK1 ald6*Δ at 48 h. Population medians are also shown for Tom70-GFP and WGA in wild type and *ald6*Δ at 48 h. p values calculated by t-test, n=5-6 **C:** Representative flow plots and quantification of low senescence population at 48 h in A2A mutants lacking the electron transport chain (*cox9*Δ), p-value calculated by t-test, n=3-4.

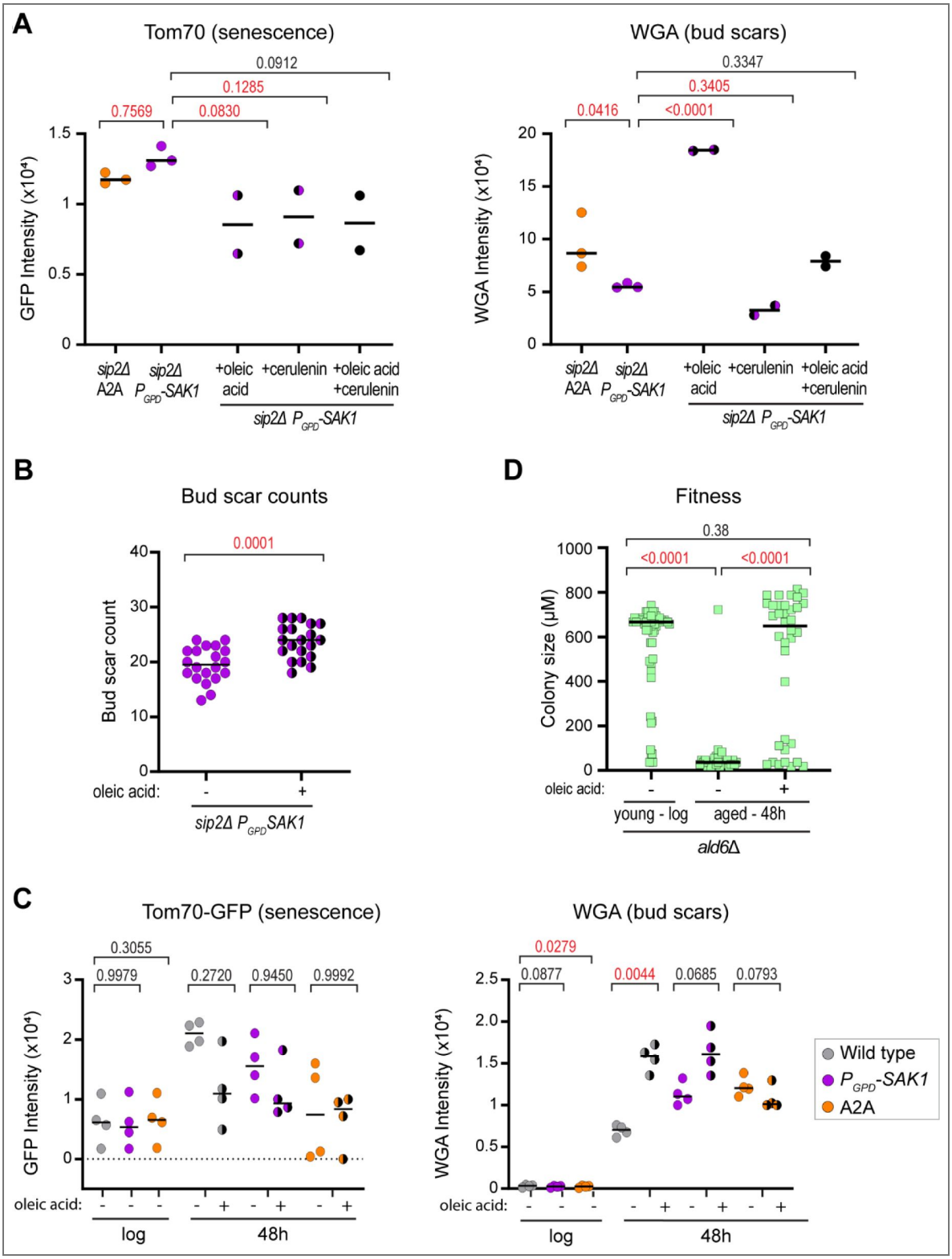


Figure S4. Fatty acids in senescence.

A: Population medians for Tom70-GFP and WGA at 48 h in *sip2*Δ P_{GPD}-*SAK1 acc1*^{S1157A} and *sip2*Δ P_{GPD}-*SAK1* supplemented with cerulenin and/or 0.04% oleic acid + 0.04% Tween 80. p-values calculated by one-way ANOVA, n=2-3 **B:** Manual bud scar counts of 48 h-aged *sip2*Δ P_{GPD}-*SAK1* cells with and without 0.04% oleic acid. p values calculated by one way ANOVA, n=20 **C:** Population medians for Tom70-GFP and WGA at 48 h in wild type, P_{GPD}-*SAK1* and A2A with and without 0.04% oleic acid + 0.04% Tween 80. p-values calculated by one-way ANOVA, n=4 **D:** Size of colonies formed in 24 h on a YPD plate by log phase and 48 h-aged *ald6*Δ cells with 0.04% oleic acid + 0.04% Tween 80. Only cells that eventually formed colonies within 3 days were included to ensure all tested cells were viable. p values calculated by one-way ANOVA, n=28-53

DH18	**WT-RPL13a**	*ade2::hisG his3 leu2 met15D::ADE2/MET15 lys2/LYS2 ura3DO trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1/+ RPL13A-mCherry-Kan/+*
JH1551	**ACC1^{S1157A}-RPL13a** MEP Tom70-GFP Rpl13a-mCherry acc1-S1157A het.	ade2::hisG his3 leu2 met15D::ADE2/+ lys2/+ ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1/+ RPL13A-mCherry-Kan/+ acc1-S1157A/+
DH83	**P_{GPD}-SAK1-RPL13a** MEP diploid TOM70-GFP RPL13A-mCherry SAK1 oe	ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX TOM70-GFP-TRP1 RPL13A-mCherry-KanMX6 KanMX6-Pgdp-SAK1
JH1552	**A2A-RPL13a** MEP Tom70-GFP Rpl13a-mCherry SAK1 oe acc1-S1157A het.	ade2::hisG his3 leu2 met15D::ADE2/+ lys2/+ ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1/+ RPL13A-mCherry-Kan/+ KanMX6-Pgdp-SAK1/+ acc1-S1157A/+
DH192	**COX9Δ** MEP diploid TOM70-GFP RPL13A-mCherry cox9Δ	ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX TOM70-GFP-TRP1 RPL13A-mCherry-KanMX6 cox9::URA3
MU19	**COX9Δ P_{GPD}-SAK1** MEP diploid COX9Δ SAK1oe (TOM70-GFP RPL13A-mCherry)	ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 RPL13A-mCherry-Kan cox9::URA3 KanMX6-Pgdp-SAK1
JH1676	**COX9Δ A2A** cox9Δ/cox9Δ SAK1 oe acc1-S1157A Tom70-GFP Rpl13-mCherry het dip	ade2::hisG his3 leu2 lys2/+ met15::ADE2/+ ura3DO trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX KanMX6-Pgdp-SAK1/+ acc1-S1157A/+ cox9::URA3/cox9::URA3 TOM70-GFP-Kan/+ RPL13a-mCherrry-TRP1/+
MU38	**MPC1Δ** MPC1Δ MEP diploid (TOM70-GFP VPH1-mCherry)	ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 VPH1-mCherry-Kan MPC1::HIS
MU39	**MPC1Δ P_{GPD}-SAK1** MPC1Δ SAK1 oe MEP diploid (TOM70-GFP VPH1-mCherry)	ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 VPH1-mCherry-Kan KanMX6-Pgdp-SAK1 MPC1::HIS
MU40	**MPC1Δ A2A**	ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-

Table S1. Strains used in this work.

All strains are diploid derivatives of the MEP system (47). TOM70-GFP and RPL13a-mCherry markers are heterozygous to avoid growth defect.

	MPC1Δ + A2A MEP diploid (TOM70-GFP VPH1-mCherry)	CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 VPH1-mCherry-Kan KanMX6-Pgdp-SAK1 acc1-S1157A MPC1::HIS
HH40	**MLS1Δ** MLS1 Δ MEP diploid (Tom70-GFP RPL13A-mCherry)	MAT α ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 RPL13A-mCherry-Kan MLS1::URA3
MU31	**MLS1Δ P_{GPD}-SAK1** MLS1Δ SAK1 oe MEP diploid (Tom70-GFP RPL13A-mCherry)	ade2::hisG his3 leu2 lys2 ura3DO trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX KanMX6-Pgdp-SAK1 MLS1::URA3 Tom70-GFP-TRP1 RPL13A-mCherry-Kan
MU59	**MLS1Δ A2A** MLS1 Δ + Sak1 oe + ACC1 S1157A MEP diploid (Tom70-GFP RPL13A-mCherry)	ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 RPL13A-mCherry-Kan KanMX6-Pgdp-SAK1 acc1-S1157A MLS1::URA3
HH47	**SFC1Δ** SFC1Δ MEP diploid (TOM70-GFP + Rpl13a-mCherry)	MAT α ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 RPL13A-mCherry-Kan SFC1::URA3
MU32	**SFC1Δ P_{GPD}-SAK1** SFC1Δ SAK1 oe MEP diploid (Tom70-GFP RPL13A-mCherry)	ade2::hisG his3 leu2 lys2 ura3DO trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX KanMX6-Pgdp-SAK1 SFC1::URA3 Tom70-GFP-TRP1 RPL13A-mCherry-Kan
MU66	**CAT2Δ MLS1Δ** CAT2Δ + MLS1 Δ MEP diploid (Tom70-GFP RPL13A-mCherry)	ade2::hisG his3 leu2 lys2 ura3DO trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 RPL13A-mCherry-Kan MLS1::URA3 CAT2::HIS
MU45	**CAT2Δ MLS1Δ P_{GPD}-SAK1** CAT2Δ + MLS1Δ + SAK1o/e MEP diploid (Tom70-GFP RPL13A-mCherry)	ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 RPL13A-mCherry-Kan KanMX6-Pgdp-SAK1 MLS1::URA3 CAT2::HIS
MU69	**CAT2Δ MLS1Δ A2A** CAT2Δ MLS1Δ SAK1 oe acc1-S1157A MEP diploid (Tom70-GFP RPL13A-mCherry)	ade2::hisG his3 leu2 lys2 ura3DO trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX KanMX6-Pgdp-SAK1 acc1-S1157A MLS1::URA3 CAT2::HIS Tom70-GFP-TRP1 RPL13A-mCherry-Kan
HH88	**SIP2Δ** Sip2Δ MEP diploid TOM70-GFP VPH1-mCherry	MAT α ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 VPH1-mCherry-Kan Sip2::URA3
HH108	**SIP2Δ P_{GPD}-SAK1** Sip2Δ MEP diploid SAK1 oe (Tom70-GFP + VPH1-mCherry)	MAT a ade2::hisG his3 leu2 lys2 ura3DO trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX KanMX6-Pgdp-SAK1 Sip2::URA3 Tom70-GFP-TRP1 VPH1-mCherry-Kan
HH87	**SIP2Δ A2A** Sip2Δ SAK1 oe acc1-S1157A (Tom70-GFP + VPH1-mCherry)	MAT a ade2::hisG his3 leu2 lys2 ura3DO trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX KanMX6-Pgdp-SAK1 acc1-S1157A Sip2::URA3 Tom70-GFP-TRP1 VPH1-mCherry-Kan

Table S1. (continued)

MU64	**CAT2Δ** MEP diploid (Tom70-GFP RPL13A-mCherry)	ade2::hisG his3 leu2 met15D::ADE2 ura3D0 trp1D63 hoD::SCW11pr-Cre-EBD78-NatMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX Tom70-GFP-TRP1 RPL13A-mCherry-Kan CAT2::HIS

Table S1. (continued)

oJH1520	**TOM70 DN45 1** (GFP tag)	TAGTTTTTGTCTTCTCCTAAAAGTTTTTAAGTTTATGTT TACTGT GAATTCGAGCTCGTTTAAAC
oJH1521	**TOM70 UP45 1** (GFP tag)	AAGATTCAAGAAACTTTAGCTAAATTACGCGAACAGG GTTTAATG CGG ATC CCC GGG TTA ATT AAC
oDH11	**RPL13A-mCherry UP45** (for mCherry tag with pAW8)	AAGAGAGCTAGAGAAAAGGCTGAAGCTGAAGCTGAA AAGAAGAAATGCATGCTTATGGTGAGCAA
oDH12	**RPL13A-mCherry DN45** (for mCherry tag with pAW8)	ATACAAAAATTGTGGATGAAAAATTCTTTGATGAAGT TTTTAGATAAGTTATACTAGTTCGTCGACTGGAT
oJH1777	**ACC1 CORE UP45 1** (for making S1157A mutation in ACC1)	TTCTCCACCTTTCCAACTGTTAAATCTAAAATGGGTAT GAACAGGGCTGT TAGGGATAACAGGGTAAT CCGCGCGTTGGCCGATTCAT
oJH1778	**ACC1 CORE DN45 1** (for making S1157A mutation in ACC1)	TCTTAACGGAGATGACTGACTGTTTGCAACATATGAC AAATCTGAAACAG TTCGTACGCTGCAGGTCGAC
oDH31	**SAK1 over-expression UP45** (pYM-14 GDP promoter insertion 201bp up from start of SAK1)	TATCCAGTCACAATTTTAAAAGCACTTCGTTAACACGT TTGGTTG CGTACGCTGCAGGTCGAC
oDH32	**SAK1 over-expression DN45** (pYM-14 GDP promoter insertion 45bp up from start of SAK1 start codon ATG)	AGGTACATTGACCTCTTCGACGTTAACTTTTTTATCAC TCCTATC CATCGATGAATTCTCTGTCG
oDH113	**COX9 UP45** (COX9 deletion with pFA6a-URA plasmid)	AGCAAGATATTTGCAAACTACTAACTACAAGCGACTT ACACAGAC CGGATCCCCGGGTTAATTAAG
oDH114	**COX9 DN45** (COX9 deletion with pFA6a-URA plasmid)	TAGGAATAAGAATATAATGCGAAAAACAATAGTGGT CAGGTTCGG GAATTCGAGCTCGTTTAAAC
oMU8	**MPC1 UP45** (cloning primers for making MPC1-HIS cassette using pFA6A HisMX6)	TCTACATATATACGTATAGATTTTATTGCACTGTGATC AAAAAGACGGATCCCCGGGTTAATTAAG
oMU9	**MPC1 DN45** (cloning primers for making MPC1-HIS cassette using pFA6a HisMX6)	ATGCATGAACATATCCATCCTCTTAATCCTTGCTTGTTC TTTTTTGAATTCGAGCTCGTTTAAAC
oHH25	**MLS1 UP45** (Forward overlap delete MLS1 [(45bp of left flank) + F1 from pFA6a])	TTTGAACTAAACAAAGTAGTAAAAGCACATAAAAGAA TTAAGAAA CGGATCCCCGGGTTAATTAAG
oHH26	**MLS1 DN45** (Reverse overlap to delete MLS1 [(45bp of right flank) + R1 from pFA6a])	GGCATGAATATATTTTTATATATGTGTACACTGGGGC AAGGGAGA GAATTCGAGCTCGTTTAAAC
oHH33	**SFC1 UP45** (Forward overlap delete SFC1 [(45bp of left flank) + F1 from pFA6a])	TTAGTTTTAGCCGTAAGATAACATAACAAAGAAGAAG AAAGAAAA CGGATCCCCGGGTTAATTAAG
oHH34	**SFC1 DN45**	TTCTATTTTTTCTTTATTTCATTTTGTAGTCCCATTGTTC ATCAT GAATTCGAGCTCGTTTAAAC

Table S2. Oligonucleotides used for making strains.

	(Reverse overlap to delete SFC1 [(45bp of right flank) + R1 from pFA6a])	
oMU13	**CAT2 UP45** (cloning primers for making CAT2-HIS cassette using pFA6A HisMX6)	GTATATATATATATATATCCCTTAAAAACTAAAAAGAA AGCACTCCGGATCCCCGGGTTAATTAAG
oMU14	**CAT2 DN45** (cloning primers or making CAT2-HIS cassette using pFA6a HisMX6)	TAACAAAAATATTCACAAATTAATTGAAGAGGAAAGG TGAAAAATGAATTCGAGCTCGTTTAAAC
oHH96	**Sip2 UP45** (overlap UP45 upstream region for deletion of Sip2 with pFA6a URA3)	AAATAATTGTGCTTTTGGAAGGGTACTGAGAGCAGTG AAGTTTCTCGGATCCCCGGGTTAATTAAG
oHH97	**Sip2 DN45** (overlap DN45 downstream region for deletion of Sip2 with pFA6a URA3)	GCCAAAAATATATACAATACGCCTTTAAAAAGTAGGA ATCCCGGAGAATTCGAGCTCGTTTAAAC

Table S2. (continued)

Data availability

RNA-seq data is deposited at the Gene Expression Omnibus (GEO), Accession GSE308402.

Acknowledgements

We would like to thank Sophie Trefely and Mark Cheong for experimental help and insightful discussions. We also thank Sophie Trefely for critical reading of the manuscript. We would like to thank Rachael Walker, Sam Thompson, and Christopher Hall of the Babraham Institute Flow Facility (Flowcyt04) for extensive experimental advice and troubleshooting. RNA-seq library sequencing and processing were performed by Babraham Institute Genomics (Geno06) and Babraham Institute Bioinformatics (Bioinf01), which receive financial support from the Institute Core Capability Grant (BBSRC CCG).

Additional information

Funding

JH,MU,MKAM - BBSRC (BI Epigenetics ISP; BBS/E/B/000C0523), HHM – UKRI (Horizon Europe Marie Skłodowska-Curie fellowship; EP/Y027892/1), DH - BBSRC (DTP PhD award 1947502).

Funding

Funder	Grant reference number	Author
UKRI | Biotechnology and Biological Sciences Research Council (AFRC)	BBS/E/B/000C0523	Jonathan Houseley
UKRI | Biotechnology and Biological Sciences Research Council (AFRC)	1947502	Jonathan Houseley
UKRI | Engineering and Physical Sciences Research Council (SRC)	EP/Y027892/1	Jonathan Houseley

Author ORCID iDs

Jonathan Houseley: https://orcid.org/0000-0001-8509-1500

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Peer reviews

Reviewer #1 (Public review):

This rigorous and creative study uses an elegant combination of metabolomics, transcriptomics, and budding yeast molecular genetics to discover that (i) activating AMPK to maintain mitochondrial respiration fueled by cytosolic Acetyl CoA and (ii) increasing fatty acid synthesis independent of respiration drive independent pathways that increase the fitness of replicatively-aged budding yeast cells, albeit without increasing their lifespan. This work will be of interest to scientists in the field of aging and metabolism. Some clarifications in the text would address the following concerns, which would increase the impact of the study:

(1) What does activation of AMPK (via PGDP-Sak1 expression) do to the replicative lifespan? How many bud scars, in general, do the subpopulations that are older - yet have less Tom70 (increased mitochondrial fitness) - have, after the 48 hrs time point that they are examining? How many divisions occurred in this 48hr time period - i.e. is it long enough to have all cells reach the end of their replicative lifespan? This information is important to rule out that a subset of the mutant cells just divided faster and hence had more divisions within 48 hrs (growing faster and living longer are different things). Having identical growth curves doesn't indicate per se that they all divide at the same rate, as there may be a subpopulation that divides faster and a subpopulation that doesn't grow so well.

(2) A2A cells do not have an extended replicative lifespan (RLS) but show an increase in the "low senescence" population (Figure 2). If the cells are not becoming senescent, why don't they have longer RLS? Not having a longer lifespan seems inconsistent with the statement that "bud scar counting confirmed that A2A cells reach a higher age than wild type", which comes back to how many times the cells can divide in the 48hr timepoint studied and their rate of cell division? Also, the lifespan curve shown is plotted against time, not cell division number, which does not take into account different division times of cells within the population (described above). It would be much more useful to show standard lifespan curves showing cell division numbers per lifespan per cell.

(3) Increased "fitness" of the old cells is implied from the increased size of the colonies that the old cells can make. However, this is a measure of the fitness of the daughters per se, not the old mother cells. Are the old mothers just passing on healthier mitochondria and more lipids to the daughters, such that they can divide more times? If the aged cells have an "increased fitness", why don't they divide more times themselves (i.e. live longer?).

(4) The statement is made that "these experiments define two classes of aging cells with distinct metabolic needs, coherent with the model of two aging trajectories previously proposed (referencing Nan Hao's work)". However, the big difference here is that in Nan Hao's work, their two aging trajectories influenced the length of lifespan, but that does not appear to be the case here. That distinction should be made clear. Perhaps the authors could also speculate as to why the A2A yeast stops dividing after presumably the same number of cell divisions, even though they have an activated AMPK and activated fatty acid synthesis pathway.

(5) I am a bit confused by the use of the word "senescence" by this lab here and in their previous growth on galactose studies. If yeast don't senesce, which is usually defined as an irreversible arrest of the cell cycle where cells stop dividing, shouldn't the yeast that do not senesce still be dividing and hence have a longer lifespan? Should a different term be used rather than senescence? Such as "fitness late in life". The authors giving their definition of senescence may help reduce this apparent contradiction.

https://doi.org/10.7554/eLife.111611.1.sa3

Reviewer #2 (Public review):

Summary:

In this study, the authors investigate how cytosolic acetyl-CoA metabolism influences replicative aging in budding yeast. They propose that acetyl-CoA regulates aging through three major pathways: (1) mitochondrial transport to support mitochondrial function, (2) fatty acid synthesis, and (3) global protein acetylation. The data show that AMPK activation promotes mitochondrial import of acetyl-CoA and partially mitigates mitochondrial decline in a subset of aging cells.

Furthermore, the engineered A2A strain, which enhances mitochondrial acetyl-CoA utilization while relieving inhibition of fatty acid synthesis, increases the proportion of cells exhibiting a "low senescence" phenotype.

Overall, this is a thoughtful and potentially impactful study that advances our understanding of metabolic control of aging. Addressing the points below, particularly by refining interpretations and, where feasible, incorporating additional analyses, will further strengthen the manuscript and its conclusions.

Strengths:

The study has several notable strengths. It addresses an important question by shifting the focus from lifespan to preservation of late-life fitness, which is highly relevant to aging biology. The work integrates metabolic, genetic, and functional analyses to link cytosolic acetyl-CoA flux with distinct aging outcomes, and the engineering of the A2A strain provides a clear and elegant demonstration of how coordinated pathway modulation can improve cellular fitness.

Weaknesses:

(1) While the manuscript focuses on mitochondrial transport and fatty acid synthesis, cytosolic acetyl-CoA is also a key regulator of histone acetylation and chromatin silencing. It would strengthen the study to consider whether acetyl-CoA depletion contributes to improved fitness through enhanced rDNA silencing. Given the well-established role of rDNA instability in yeast aging, additional experiments examining rDNA silencing and stability would be valuable. For example, monitoring rDNA copy number changes (not necessarily ERCs) under AMPK activation, oleic acid supplementation, and in the A2A strain, similar to approaches used in the authors' prior work, would help clarify whether chromatin regulation contributes to the observed phenotypes.

(2) The current data do not fully distinguish whether AMPK activation and oleic acid supplementation act on distinct subpopulations of aging cells. An alternative explanation is that oleic acid supplementation enhances mitochondrial function and acts additively with AMPK activation, thereby increasing the fraction of cells in the "low senescence" state. Since this distinction is not central to the main conclusions, I suggest softening the language around subpopulation specificity. Emphasizing instead that the A2A strain coordinately modulates multiple branches of acetyl-CoA metabolism to improve late-life fitness would maintain the strength of the central message without overinterpretation.

(3) The manuscript proposes that lipid starvation and excess acetyl-CoA are major drivers of senescence in distinct subpopulations of wild-type aging cells. This conclusion is not yet fully supported by the presented data. Direct measurements of age-dependent divergence in acetyl-CoA and fatty acid levels at the single-cell level would be needed to substantiate this model. Based on the current evidence, a more conservative interpretation would be that aging cells exhibit differential sensitivity to perturbations in acetyl-CoA and lipid metabolism.

Accordingly, I recommend revising the statement in the Abstract ("We further implicate lipid starvation and excess acetyl coenzyme A availability as major drivers of senescence...") and the corresponding discussion text to better align with the data.

https://doi.org/10.7554/eLife.111611.1.sa2

Reviewer #3 (Public review):

Summary:

These findings suggest that PGPD-SAK1 yeast show a subpopulation with lowered TOM70-GFP expression in high bud scar staining aged cells. Deletion of CAT2 or MLS1 reduces this effect. A PGPD-SAK1 acc1S1157A double mutant (called "A2A" here) shows an even larger effect of lowered tom70 expression in high bud scar staining aged cells. Utilization of various additional mutants involved in acetyl-CoA transport, carnitine shuttle, respiration, etc., leads the authors to conclude that these shifts in TOM70-GFP in aged cells are linked to the AMPK-fatty acid metabolic regulatory system.

Strengths:

These extensive and clearly described experiments reveal interesting changes in TOM70-GFP intensity in subsets of aged yeast in several mutants eventually identified as linked to the AMPK-fatty acid metabolic regulatory system.

Weaknesses:

(1) 3 biological replicates for mRNASeq is low.

(2) While "Traditional conceptions of ageing implicate a progressive accumulation of damage leading to systemic degradation in performance until death, with evolutionary pressures acting to maximise early life fitness and fecundity at the expense of ageing health." is tangential perhaps to the data and conclusions of the study, both claims of this sentence are at best controversial, and the manuscript is no weaker for their omission.

(3) The statement that "Here, we determine the basis of senescence and fitness loss in replicatively ageing yeast" is a bit strong as a summary of the present careful work presented here. If the authors had created yeast mutants that retained fitness indefinitely, this would be a more appropriate strength of claim to summarize the work.

https://doi.org/10.7554/eLife.111611.1.sa1

Author response:

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *This rigorous and creative study uses an elegant combination of metabolomics, transcriptomics, and budding yeast molecular genetics to discover that (i) activating AMPK to maintain mitochondrial respiration fueled by cytosolic Acetyl CoA and (ii) increasing fatty acid synthesis independent of respiration drive independent pathways that increase the fitness of replicatively-aged budding yeast cells, albeit without increasing their lifespan. This work will be of interest to scientists in the field of aging and metabolism. Some clarifications in the text would address the following concerns, which would increase the impact of the study:*

> *(1) What does activation of AMPK (via PGDP-Sak1 expression) do to the replicative lifespan? How many bud scars, in general, do the subpopulations that are older - yet have less Tom70 (increased mitochondrial fitness) - have, after the 48 hrs time point that they are examining? How many divisions occurred in this 48hr time period - i.e. is it long enough to have all cells reach the end of their replicative lifespan? This information is important to rule out that a subset of the mutant cells just divided faster and hence had more divisions within 48 hrs (growing faster and living longer are different things). Having identical growth curves doesn't indicate per se that they all divide at the same rate, as there may be a subpopulation that divides faster and a subpopulation that doesn't grow so well.*

Increasing AMPK activity increases replicative lifespan [PMID: 25869125], but given our finding that AMPK activation splits the population, such replicative lifespan assays are hard to interpret. Bud scar counts have a similar issue. Hence we restricted the lifespan and bud scar analyses to wt and A2A which are more homogenous (Figures S2 B and E). A2A cells at 48h have ~25% more bud scars than wt cells. Yes, by 48h most of the cells have lost viability (Figure 2E). The reviewer is correct that you can't properly compare the lifespan curves if the cells divide at different rates, hence our follow-up test of wt at 48h vs A2A at 40h viability after we had confirmed that these timepoints captured cells at equivalent replicative ages (Figure 2D,E). This shows that viability of A2A is slightly lower than wt at matched age, indicating a slightly shorter lifespan.

> *(2) A2A cells do not have an extended replicative lifespan (RLS) but show an increase in the "low senescence" population (Figure 2). If the cells are not becoming senescent, why don't they have longer RLS? Not having a longer lifespan seems inconsistent with the statement that "bud scar counting confirmed that A2A cells reach a higher age than wild type", which comes back to how many times the cells can divide in the 48hr timepoint studied and their rate of cell division? Also, the lifespan curve shown is plotted against time, not cell division number, which does not take into account different division times of cells within the population (described above). It would be much more useful to show standard lifespan curves showing cell division numbers per lifespan per cell.*

Our observation that cells can reach the end of life without senescing is consistent with other studies that have studied the life course of individual cells by microscopy [PMID: 31291577, 32675375]. These studies always highlight some proportion of the cells that reach the end of life with no or minimal senescence, though this fraction varies with the experimental system. The question of why cells lose viability without senescing is a complete unknown in the field, but reflects a wider lack of consensus as to why yeast lose viability with replicative age.

We are wary about making strong statements on lifespan for exactly the reason the reviewer picks out. In liquid culture we can only assess viability over time, and it is clear from the comparison of liquid and solid media lifespans performed by the Gottschling lab [PMID: 19652178] that culture system has a huge effect on lifespan, with cells in classical microdissection-based lifespan assays living far longer than they do in liquid. This of course means that classical microdissection assays are not very useful for A2A so we are left with an unsatisfactory approximation. We have therefore restricted our conclusion on lifespan to simply say that lifespan of A2A cells is not extended which our data in Figures 2D,E,S2B does support (see also answer to Q1), and therefore with the majority of A2A cells showing low senescence marks and high fitness at 48h we can conclude that lifespan and fitness loss must be separable.

We will note these limitations of lifespan measurements in the manuscript.

> *(3) Increased "fitness" of the old cells is implied from the increased size of the colonies that the old cells can make. However, this is a measure of the fitness of the daughters per*

> *se, not the old mother cells. Are the old mothers just passing on healthier mitochondria and more lipids to the daughters, such that they can divide more times? If the aged cells have an "increased fitness", why don't they divide more times themselves (i.e. live longer?).*

Yes, colony growth speed is defined by daughter cell replication, and as long as the daughters and subsequent generations divide at the same rate irrespective of whether they come from a young or old mothers then the size of the colony after 24 hours varies based on the time it took the initial mother to produce a daughter. This is what the assay really measures. We note that aged wildtype mothers often do not divide at all in the first 24 hours after being put on an agar plate (hence the tiny reported colony size), even though they do eventually produce a daughter which then forms a colony, whereas A2A cells tend to produce the first daughter rapidly whether young or old. It is known that daughters of aged wildtype mothers also divide slower, which will also contribute to differences in colony size, and this may well result from a lipid and/or mitochondrial contribution, but the primary driver of colony size in 24 hours is the time the mother took to initially divide. We will add this detail to the manuscript.

As noted above, the mechanistic basis of lifespan is unknown, but although senescence can shorten lifespan, our work and that of others shows that lifespan is still limited in the absence of senescence.

> *(4) The statement is made that "these experiments define two classes of aging cells with distinct metabolic needs, coherent with the model of two aging trajectories previously proposed (referencing Nan Hao's work)". However, the big difference here is that in Nan Hao's work, their two aging trajectories influenced the length of lifespan, but that does not appear to be the case here. That distinction should be made clear. Perhaps the authors could also speculate as to why the A2A yeast stops dividing after presumably the same number of cell divisions, even though they have an activated AMPK and activated fatty acid synthesis pathway.*

We will add this distinction. As noted above, we are wary of making strong statements regarding lifespan as the assays we can do in liquid culture are limited. We are therefore similarly wary about speculating about causes for the lack of lifespan difference because in reality all we can do is rule out a big effect. We would love to speculate on why the A2A cells don't have an extended lifespan, but at this point we don't have any good ideas on this point!

> *(5) I am a bit confused by the use of the word "senescence" by this lab here and in their previous growth on galactose studies. If yeast don't senesce, which is usually defined as an irreversible arrest of the cell cycle where cells stop dividing, shouldn't the yeast that do not senesce still be dividing and hence have a longer lifespan? Should a different term be used rather than senescence? Such as "fitness late in life". The authors giving their definition of senescence may help reduce this apparent contradiction.*

We completely agree, this is confusing and noted this distinction in the Introduction. Use of the term senescence to mean a loss of fitness late in life in yeast stems from the classical definition of senescence as applied to whole organisms. However, the term senescence as applied to cells has a more specific meaning in terms of the cell cycle as the reviewer notes. As an individual *S. cerevisiae* is both a cell and an organism, the terminology clashes. However, the marker we largely employ (Tom70-GFP) which in our hands is a very good proxy for fitness was originally defined as marking the senescence entry point (SEP), so overall we feel we can't avoid the term.

> ***Reviewer #2 (Public review):***
>
> *Summary:*

> *In this study, the authors investigate how cytosolic acetyl-CoA metabolism influences replicative aging in budding yeast. They propose that acetyl-CoA regulates aging through three major pathways: (1) mitochondrial transport to support mitochondrial function, (2) fatty acid synthesis, and (3) global protein acetylation. The data show that AMPK activation promotes mitochondrial import of acetyl-CoA and partially mitigates mitochondrial decline in a subset of aging cells.*
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> *Furthermore, the engineered A2A strain, which enhances mitochondrial acetyl-CoA utilization while relieving inhibition of fatty acid synthesis, increases the proportion of cells exhibiting a "low senescence" phenotype.*
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> *Overall, this is a thoughtful and potentially impactful study that advances our understanding of metab to olic control of aging. Addressing the points below, particularly by refining interpretations and, where feasible, incorporating additional analyses, will further strengthen the manuscript and its conclusions.*
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> *Strengths:*
>
> *The study has several notable strengths. It addresses an important question by shifting the focus from lifespan to preservation of late-life fitness, which is highly relevant to aging biology. The work integrates metabolic, genetic, and functional analyses to link cytosolic acetyl-CoA flux with distinct aging outcomes, and the engineering of the A2A strain provides a clear and elegant demonstration of how coordinated pathway modulation can improve cellular fitness.*
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> *(1) While the manuscript focuses on mitochondrial transport and fatty acid synthesis, cytosolic acetyl-CoA is also a key regulator of histone acetylation and chromatin silencing. It would strengthen the study to consider whether acetyl-CoA depletion contributes to improved fitness through enhanced rDNA silencing. Given the well-established role of rDNA instability in yeast aging, additional experiments examining rDNA silencing and stability would be valuable. For example, monitoring rDNA copy number changes (not necessarily ERCs) under AMPK activation, oleic acid supplementation, and in the A2A strain, similar to approaches used in the authors' prior work, would help clarify whether chromatin regulation contributes to the observed phenotypes.*

We have data addressing this point that we will add to the manuscript. In short, we see no difference in gene expression from Sir2-repressed sub-telomeric regions or MAT loci, but the genome-wide gene expression dysregulation associated with age is partially suppressed in *PGPD-SAK1*. However, A2A does not suppress this further, so it is not critical for the suppression of senescence in A2A though we are following this up. ERC accumulation is higher in A2A at 48h, consistent with the cells being older, meaning that ERCs are unlinked to senescence onset as we have previously reported. There is a strong upregulation of transcripts from Sir2-repressed rDNA intergenic spacers with age in all genotypes, but we attribute this simply to the copy number increase of these regions on ERCs rather than a defect in silencing. We have previously looked for heritable changes in rDNA copy number arising during ageing and found (to our surprise) absolutely nothing, so we don't expect any changes under these conditions.

> *(2) The current data do not fully distinguish whether AMPK activation and oleic acid supplementation act on distinct subpopulations of aging cells. An alternative explanation is that oleic acid supplementation enhances mitochondrial function and acts additively with AMPK activation, thereby increasing the fraction of cells in the "low senescence" state. Since this distinction is not central to the main conclusions, I suggest softening the*

> *language around subpopulation specificity. Emphasizing instead that the A2A strain coordinately modulates multiple branches of acetyl-CoA metabolism to improve late-life fitness would maintain the strength of the central message without overinterpretation.*

We agree that oleic acid and the lipids produced downstream of Acc1 in A2A may improve late life fitness via enhanced mitochondrial function, and in support of this Oxygen Consumption Rate is marginally (though significantly) higher in A2A than *PGPD-SAK1*. We will add this data to the manuscript. However, we disagree with the interpretation of an additive effect as we report throughout the study that AMPK activation and lipid biosynthesis/supplementation affect different sub-populations of cells. We do not observe populations of intermediate senescence cells, rather by flow cytometry and fitness assays we observe individual cells in binary low senescence or high senescence states.

> *(3) The manuscript proposes that lipid starvation and excess acetyl-CoA are major drivers of senescence in distinct subpopulations of wild-type aging cells. This conclusion is not yet fully supported by the presented data. Direct measurements of age-dependent divergence in acetyl-CoA and fatty acid levels at the single-cell level would be needed to substantiate this model. Based on the current evidence, a more conservative interpretation would be that aging cells exhibit differential sensitivity to perturbations in acetyl-CoA and lipid metabolism. Accordingly, I recommend revising the statement in the Abstract ("We further implicate lipid starvation and excess acetyl coenzyme A availability as major drivers of senescence...") and the corresponding discussion text to better align with the data.*

We agree and will adjust the abstract to make it clearer that the lipid starvation / excess acetyl coA interpretation is a model.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *These findings suggest that PGPD-SAK1 yeast show a subpopulation with lowered TOM70-GFP expression in high bud scar staining aged cells. Deletion of CAT2 or MLS1 reduces this effect. A PGPD-SAK1 acc1S1157A double mutant (called "A2A" here) shows an even larger effect of lowered tom70 expression in high bud scar staining aged cells. Utilization of various additional mutants involved in acetyl-CoA transport, carnitine shuttle, respiration, etc., leads the authors to conclude that these shifts in TOM70-GFP in aged cells are linked to the AMPK-fatty acid metabolic regulatory system.*
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> *Strengths:*
>
> *These extensive and clearly described experiments reveal interesting changes in TOM70-GFP intensity in subsets of aged yeast in several mutants eventually identified as linked to the AMPK-fatty acid metabolic regulatory system.*
>
> *Weaknesses:*
>
> *(1) 3 biological replicates for mRNASeq is low.*

Thank you for pointing this out. We performed another replicate after posting the initial preprint but didn't update the figure in the eLIFe-reviewed version. We will add this to the scatter plots and analysis in Figure 1, the findings have not changed.

> *(2) While "Traditional conceptions of ageing implicate a progressive accumulation of damage leading to systemic degradation in performance until death, with evolutionary pressures acting to maximise early life fitness and fecundity at the expense of ageing health." is tangential perhaps to the data and conclusions of the study, both claims of*

> *this sentence are at best controversial, and the manuscript is no weaker for their omission.*

We actually feel that this sentence is very important to the message of the manuscript, which is that ageing does not necessarily have to involve a loss of fitness before death. Ageing is often described as the progressive wearing out of components leading to decline and death (with an old car often used as an analogy); in the ageing field this is certainly controversial, but outside the field this remains the normal understanding. We think it is important to state this widely held viewpoint with which our findings are hard to reconcile.

Our interpretation that yeast are bet-hedging as a population growth strategy and this drives ageing in the long term is a classic antagonistic pleiotropy - we will add this term (from the citation that is already in the manuscript) and clarify in the discussion to make it obvious why we are introducing this concept in the introduction.

> *(3) The statement that "Here, we determine the basis of senescence and fitness loss in replicatively ageing yeast" is a bit strong as a summary of the present careful work presented here. If the authors had created yeast mutants that retained fitness indefinitely, this would be a more appropriate strength of claim to summarize the work.*

Indeed - we will refine this sentence.

https://doi.org/10.7554/eLife.111611.1.sa0