Supplemental Figure 1

Replicate lifespan curves demonstrating lack of senescence in cultures treated with a combination of rapamycin and methionine restriction. Cultures maintained under identical conditions to those presented in Figure 1 show similar patterns of growth and a lack of senescence related arrest at high passages in methionine restricted cultures treated with 1nM rapamycin. Panels A and B contain the growth curves from HCF cells while Panel C contains the growth curve for WI-38 fibroblast cells. Conditions are as follows: Ctrl-Standard MEM with 30mg/L methionine, MethR 3mg/L-Standard MEM with 1 mg/L methionine, MethR 1mg/L-Standard MEM with 1 mg/L methionine. Cultures which are also treated with 1nM rapamycin are indicated by Rapa +. All cultures were initiated from the division of a single culture strain at time 0. Time 0 relates to a culture at population doubling 27 in Panel A, population doubling 40 in Panel B, and population doubling 24 in Panel C.
Supplemental Figure 2

Methionine restriction and rapamycin treatment alter cell cycle kinetics in human cardiac fibroblasts.

Cell cycle analysis of human cardiac fibroblasts cultured as described in Fig 1 at Week 4 of lifespan. Panel A) Overlay of cell cycle profiles of cells cultured without rapamycin (left panel) or with rapamycin (right panel) under control conditions (30mg/L methionine, gray), methionine restriction (3mg/L, green and 1 mg/L, red), or no methionine added (purple). Compare the 8N population (3rd peak) under the methionine-restricted conditions without and with rapamycin. Panel B) Quantified data from Panel A, comparing the percentage of cells in each phase of the cell cycle (G1, S, and G2) as well as the percentage of cells that contain a greater than G2 DNA content, which represents the 8N population in panel A. Bars marked with an asterisk represent values that are significantly different from relative control values (with or without rapamycin) at p<0.05 and bars marked with a pound sign represent values that are significantly different between cells with or without rapamycin within
the same methionine group (ie, control or methionine restriction). Results are representative of three independent experiments.
Supplemental Figure 3

Telomere length is decreased in the absence of telomerase activity under methionine restriction and rapamycin combination treatment. Telomere length assessments as performed by the method of Cawthorn (Cawthon, 2002) are presented in Panel A. Population doublings (PD) for each sample is provided. The senescent sample was isolated from a control culture at PD 54 when the cultures had undergone proliferative arrest as assessed by cell number. The results of an analysis of telomerase activity are presented in Panel B. Human foreskin fibroblasts harboring an exogenous gene construct containing the telomerase cDNA (BJ Tert) serve as a positive control.
Supplemental Figure 4

Enhanced autophagy with methionine restriction and rapamycin treatment.

Panel A) Protein levels of endogenous LC3BI and LC3BII determined by immunoblot analysis. Panel B) Autophagic flux determined by densitometry of protein bands from Panel A. The ratio of LC3BII to LC3BI was normalized to actin with and without 0.1 nM leupeptin/20 nMNH₄. The ratio of each condition with or without leupeptin/NH₄ was determined. Data are presented relative to controls. Results are representative of at least three experiments. Panel C) Autophagic flux evaluated as in (A) using cells expressing GFP-LC3B and an antibody specific for the GFP protein. The cleaved GFP fragment at the bottom of the blot represents processed autophagosomal GFP-LC3BII. Results are representative of at least two independent
experiments. Panels D and E) GFP-LC3B-expressing cells were treated under the indicated conditions and GFP-containing puncta were quantified by fluorescence microscopy. Bar labels are as follows: standard conditions (30 mg/L methionine, Ctrl); methionine restriction (MetR); 1nM rapamycin treatment (Rapa); combination methionine restriction and rapamycin treatment (Rapa+MetR), and methionine deficient (0 mg/L added, MetDEF). Values for bars marked with an asterisk are significantly different from untreated control cultures at $P<0.05$ and values for bars marked with a pound sign represent values that are significantly different between the combination of methionine restriction and rapamycin treatment exposed to leupeptin/NH$_4$ and other conditions exposed to leupeptin/NH$_4$. Panel F) Proliferation following the introduction of an shRNA construct targeting ATG5. The top panel contains an immunoblot demonstrating knockdown of ATG5 following lentiviral transduction of shATG5. A replicative lifespan curve measuring cPDLs up to 10 weeks is shown below the immunoblot. Control values for scrambled RNA vector controls are labeled shScrm.
Supplemental Figure 5

Activation of GCN2 and ATF4 signaling by methionine restriction and rapamycin treatment.

Panel A) Protein levels of phosphorylated GCN2, phosphorylated eIF2α, ATG12, and ATG5 as determined by immunoblot analysis. Panel B) mRNA levels of ATF4 and CHOP determined by qRT-PCR analysis and mRNA levels of ClpP determined by NanoString analysis. Bar labels are as follows: standard conditions (30 mg/L methionine, Ctrl), methionine restriction (1mg/L, MetR); 1nM rapamycin treatment (Rapa), and combination methionine restriction and rapamycin treatment (Rapa+MetR). Values for bars marked with an asterisk represent values that are significantly different from relative control values (with or without rapamycin) at $p<0.05$ and values for bars marked with a pound sign represent values that are significantly different between untreated and rapamycin-treated cells within the same methionine treatment group (30 mg/L or 1mg/L methionine).
Supplemental Figure 6

Differential expression analysis identifying unique genes in cell treated with the combination treatment.

Lists of differentially expressed genes with a minimum of a log 2-fold change in expression and a corrected $p$-value of 0.05 were generated using DeSeq2 and incorporated into the Venn diagram. Differentially expressed genes were derived by comparison of rapamycin-treated versus early passage (RC_EP), methionine-restricted versus early passage (Met_EP), and combination methionine-restricted and rapamycin-treated versus early passage (MR_EP) cultures. Numbers represent the number of genes that are differentially expressed under each condition compared with early-passage cells. Areas of overlap indicate genes that are common to the overlapping treatments and areas with no overlap represent differentially-expressed genes that are unique to that treatment. Differentially-expressed genes unique to the methionine restriction/rapamycin treatment combination were divided into lists based on expression level relative to early-passage cells; those showing increased expression relative to early-passage (UP) and those with decreased expression (DOWN). The top 5 KEGG pathways...
identified using the DAVID analysis tool in the UP gene group are presented in Table 1. No pathways were identified by DAVID analysis in the DOWN gene group.
mRNA levels of pro-inflammatory genes associated with senescence are differentially affected by rapamycin, methionine restriction, and the combination of methionine restriction and rapamycin. A subset of senescence associated inflammatory markers which are increased during senescence in the human cardiac fibroblast cells were evaluated for their pattern of expression under the growth conditions used in this study using the RNA sequencing data (Panel A). Key changes such as IL-6 and MMP3 were also evaluated using quantitative analysis by NanoString, which confirmed the pattern of expression (Panel B).
Supplemental Figure 8

RNASeq Run Validation

R derived dot plot showing the Log10 of the Reads PerKilobase of transcript per Million mapped reads on each axis. A-C) These plots display normalized RNASeq expression values comparing each sample to the others in the triplicate for Run 1, EarlyPass sample D) This plot displays normalized RNASeq expression values comparing an identical RNA sample (EarlyPass_A=EarlyPass_1) run in 2 separate sequencing runs (Run 1 on the X axis and Run 2 on the Y axis). In the upper right corner of the plot the statistics from linear regression analysis show that the correlation of gene expression between the 2 runs is as follows: adjusted R-squared: 0.9994, F-statistic: 4.165e+06 on 1 and 23391 DF, p-value: < 2.2e-16.
Supplemental Figure 9

Nanostring vs RNASEq Validation

A-E) R derived dot plot showing the Log10 of the Reads PerKilobase of transcript per Million mapped reads on each axis. These plots display normalized RNA expression values comparing Nanostring and RNASEq methods of RNA counting (Nanostring on the X axis and RNASEq on the Y axis). Each dot plot shows the comparison of gene expression for each treatment group in the study (EarlyPass, LatePass, RapaCtrl, MethRes and Combo). F) Linear regression analysis statistics for each comparison are listed.