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2 **Reduced expression of C/EBP β -LIP extends health- and lifespan in mice**

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4 Christine Müller^{1,2§}, Laura M. Zidek^{2§}, Tobias Ackermann¹, Tristan de Jong¹, Peng Liu³,
5 Verena Kliche², Mohamad Amr Zaini¹, Gertrud Kortman¹, Liesbeth Harkema⁴, Dineke S.
6 Verbeek⁵, Jan P. Tuckermann³, Julia von Maltzahn², Alain de Bruin^{4,5}, Victor Guryev¹, Zhao-
7 Qi Wang² and Cornelis F. Calkhoven^{1,2*}

8

9 ¹ European Research Institute for the Biology of Ageing, University Medical Centre
10 Groningen, University of Groningen, Antonius Deusinglaan 1, NL-9700 AD Groningen, the
11 Netherlands.

12 ² Leibniz Institute on Aging - Fritz Lipmann Institute, Beutenbergstrasse 11, D-07745 Jena,
13 Germany.

14 ³ Institute for Comparative Molecular Endocrinology, University of Ulm, Helmholtzstraße 8/1 D-
15 89081 Ulm, Germany.

16 ⁴ Dutch Molecular Pathology Centre, Faculty of Veterinary Medicine, Utrecht University,
17 Yalelaan 1, NL-3584 CL Utrecht, the Netherlands.

18 ⁵ Department of Genetics, University Medical Center Groningen, University of Groningen,
19 Antonius Deusinglaan 1, NL-9700 RB Groningen, the Netherlands.

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21 [§] These authors contributed equally to this work.

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24 * Corresponding author: c.f.calkhoven@umcg.nl

Abstract

Ageing is associated with physical decline and the development of age-related diseases such as metabolic disorders and cancer. Few conditions are known that attenuate the adverse effects of ageing, including calorie restriction (CR) and reduced signalling through the mechanistic target of rapamycin complex 1 (mTORC1) pathway. Synthesis of the metabolic transcription factor C/EBP β -LIP is stimulated by mTORC1, which critically depends on a short upstream open reading frame (uORF) in the *Cebpb*-mRNA. Here we describe that reduced C/EBP β -LIP expression due to genetic ablation of the uORF delays the development of age-associated phenotypes in mice. Moreover, female C/EBP $\beta^{\Delta uORF}$ mice display an extended lifespan. Since LIP levels increase upon aging in wild type mice, our data reveal an important role for C/EBP β in the aging process and suggest that restriction of LIP expression sustains health and fitness. Thus, therapeutic strategies targeting C/EBP β -LIP may offer new possibilities to treat age-related diseases and to prolong healthspan.

Introduction

Delaying the occurrence of age related-diseases and frailty (disabilities) and thus prolonging healthspan, would substantially increase the quality of life of the ageing population and could help to reduce healthcare costs. Calorie restriction (CR) or pharmacological inhibition of the mTORC1 pathway by rapamycin are considered as potential effective interventions to delay aging and to increase healthspan in different species (Kaeberlein, Rabinovitch, & Martin, 2015). However, for humans CR is a difficult practice to maintain and may have pleiotropic effects depending on genetic constitution, environmental factors and stage of life. Likewise, the long-term use of rapamycin is limited by the risk of side effects, including disturbed glucose homeostasis, impaired wound healing, gastrointestinal discomfort and others (Augustine, Bodziak, & Hricik, 2007; de Oliveira et al., 2011; Lamming et al., 2012; Wilkinson et al., 2012). Therefore, there is a need to investigate alternative targets that are part of the CR/mTORC1 pathway that can be manipulated to reach similar beneficial effects. Our work suggests that the transcription factor C/EBP β may provide such a target.

C/EBP β regulates the expression of metabolic genes in liver and adipose tissue (Desvergne, Michalik, & Wahli, 2006; Roesler, 2001). From its mRNA three protein isoforms are synthesized through the usage of different translation initiation sites: two isoforms acting as transcriptional activators, Liver-enriched Activator Protein (LAP) -1 and -2, and a transcriptional inhibitory isoform called Liver-enriched Inhibitory Protein (LIP) (Descombes & Schibler, 1991). We showed earlier that translation into LIP depends on a *cis*-regulatory uORF (Figure 1A) and is stimulated by mTORC1 signalling (Calkhoven, Müller, & Leutz, 2000; Jundt et al., 2005; Zidek et al., 2015). Pharmacological or CR-induced inhibition of mTORC1 in mice selectively reduces LIP-protein synthesis and thereby increases the LAP/LIP ratio in different tissues (Zidek et al., 2015). Experimental reduction of LIP expression by genetic ablation of the uORF in C/EBP $\beta^{\Delta uORF}$ knockin mice is associated with a CR-type improved metabolic profile, including enhanced fatty acid oxidation and reduction of steatosis, improved insulin sensitivity and glucose tolerance, and higher adiponectin levels. Notably, these metabolic improvements are achieved without reducing calorie intake (Albert & Hall, 2015; Zidek et al., 2015). Because of the similarities between C/EBP $\beta^{\Delta uORF}$ mutation and CR we investigated lifespan and age-associated phenotypes in C/EBP $\beta^{\Delta uORF}$ mice.

Here we show that the C/EBP $\beta^{\Delta uORF}$ mutation is associated with an increase in lifespan and reduced tumour incidence in female mice. In addition we show an improvement in a broad spectrum of age-associated phenotypes to varying degrees in males and females.

Results

Others showed that LIP levels increase during aging in liver and white adipose tissue (WAT) (Hsieh et al., 1998; Karagiannides et al., 2001; Timchenko et al., 2006). Similarly, in our cohorts of wt C57BL/6J mice LIP levels are significantly higher in livers of old (20-22 months) versus young (5 months) mice, resulting in a decrease in the LAP/LIP ratio during ageing (Figure 1B,C and figure 1-supplement 1A). In contrast, in C/EBP $\beta^{\Delta uORF}$ mice LIP levels are low and stay low in old mice. LAP levels in C/EBP $\beta^{\Delta uORF}$ males and to a lesser extend in females are increased, which is probably due to additional initiation events at the LAP-AUG by ribosomes that normally would have initiated at the uORF (Calkhoven et al., 2000). The

Cebpb-mRNA levels are comparable at different ages and in the different genotypes (Figure 1D,E). Similarly, LIP expression is higher in white adipose tissue (WAT) of old female mice (WAT from males is not available) (Figure1-supplement 1B). Since translation into LIP is stimulated by mTORC1 through phosphorylation of 4E-binding protein (4E-BP) (Zidek et al., 2015), we reasoned that the higher LIP levels in aged livers and WAT might correlate with increased mTORC1 signalling with age. While the analysis of mTORC1-downstream phosphorylation of 4E-BP1, p70 ribosomal protein S6 kinase 1 (S6K1) did not reveal a significant difference between young versus old or wt versus *C/EBPβ^{ΔuORF}* mice in liver (Figure1-supplement 1C,D), 4E-BP1 phosphorylation was significantly higher in old compared to young WAT samples from both wt and *C/EBPβ^{ΔuORF}* females (Figure1-supplement 1E,F). In contrast phosphorylation of ribosomal S6 protein in WAT was not significantly altered upon ageing. Thus, LIP levels increase with age and this increase is dependent on the uORF in the *Cebpb*-mRNA and seems to correlate with mTORC1/4E-BP1 signalling in WAT but not in the liver.

We hypothesised that the *C/EBPβ^{ΔuORF}* mutation may have positive effects on healthspan and lifespan based on the CR-like metabolic improvements in *C/EBPβ^{ΔuORF}* mice (Zidek et al., 2015). A lifespan experiment was set up comparing *C/EBPβ^{ΔuORF}* mice with wt littermates (C57BL/6J) in cohorts of 50 mice of each genotype and gender. The survival curves revealed an increase in median survival of 20.6% (difference in overall survival $p=0.0014$ log-rank test, $n=50$) for the female *C/EBPβ^{ΔuORF}* mice compared to wt littermates (Figure 2A). From the 10% longest-lived females, nine out of ten were *C/EBPβ^{ΔuORF}* mice (Supplementary file 1), showing that the maximum lifespan of *C/EBPβ^{ΔuORF}* females is significantly increased ($p=0.0157$ Fisher's exact test). If maximum lifespan is determined by the mean survival of the longest-lived 10% of each cohort, *C/EBPβ^{ΔuORF}* females show an increase of 9.14% ($p\text{-value}=0.00105$ Student's t-test.). For the male cohort we observed a modest increase in median survival of 5.2%, however the overall survival was not significantly increased ($p=0.4647$ log-rank test, $n=50$) (Figure 2B). The increase in median survival of the combined cohort of *C/EBPβ^{ΔuORF}* mice (males & females) was 10.5% (with a significant increase in overall survival $p=0.0323$ log-rank test, $n=100$) (Figure 2-supplement 1A and supplementary file 1). The observed median survival for wt females (623 days) is lower than

what most other labs have reported for C57BL/6J females. We reasoned that this was due to a high incidence of ulcerative dermatitis (UD) we observed particularly in our female cohort (females: 19 mice or 38% for wt and 26 mice or 52% for C/EBP $\beta^{\Delta uORF}$; males: 15 mice or 30% for wt and 10 mice or 20% for C/EBP $\beta^{\Delta uORF}$). UD is a common and spontaneous condition in mice with a C57BL/6J background that progress to a severity that euthanasia is inevitable (Hampton et al., 2012). Therefore, survival curves were also calculated separately for UD-free mice and for mice that were euthanized because of serious UD (Figure 2C-F, Figure 2-supplement 1B,C and supplementary file 1 for complete overview). These data show that median lifespan of UD-free wt females are in a more normal range (740 days) and that the C/EBP $\beta^{\Delta uORF}$ mutation results in a significant increase of median survival specifically in females irrespective of the condition of UD. Moreover, the median survival of the C/EBP $\beta^{\Delta uORF}$ UD-free females (860.5 days) is higher compared to both wt females and wt males (829 days). The survival curves show an increase in early mortality for the male C/EBP $\beta^{\Delta uORF}$ mice in the complete and UD-free cohorts (Figure 2B,D). For these cohorts we performed a daily chi-square test to access differences between wt and C/EBP $\beta^{\Delta uORF}$ males on each day of the lifespan and found a significant ($p < 0.05$) reduction in survival only for the UD-free C/EBP $\beta^{\Delta uORF}$ males spanning the period 582-637 days, including four mortalities (Figure 2-supplement 1D,E). Taken together, these data show that a significant lifespan extension can be concluded only for female C/EBP $\beta^{\Delta uORF}$ mice.

Aging is the most important risk factor for development of cancer. A reduction in cancer incidence is recurrently observed upon CR, rapamycin-treatment or manipulation of other pathways that increase longevity in several animal models (Anisimov et al., 2011; Colman et al., 2009; Komarova et al., 2012; Mattison et al., 2012; Neff et al., 2013; Serrano, 2016; Weindruch & Walford, 1982). Mice in the lifespan cohorts that died or were sacrificed according to humane endpoint criteria underwent necropsy and tumours were analysed by a board certified veterinary pathologists of the Dutch Molecular Pathology Centre (DMPC). The incidence of neoplasms was markedly reduced in female C/EBP $\beta^{\Delta uORF}$ mice compared to female wt mice (68% \rightarrow 45,8%, $p=0.025$ Fisher's exact test) (Figure 3A). Furthermore, tumours were detected on necropsy at a higher age in female C/EBP $\beta^{\Delta uORF}$ mice compared to wt mice indicating a delay in tumour development (Figure 3C). The increase in median

145 survival of the tumour bearing C/EBP $\beta^{\Delta uORF}$ females was 25.49% compared to that of tumour
146 bearing wt females ($p=0.0217$ log-rank test) (Figure 3-supplement 1A). Also the tumour load
147 (number of different tumour types per mouse) and the tumour spread (total number of
148 differently located tumours per mouse irrespective of the tumour type) were lower in female
149 C/EBP $\beta^{\Delta uORF}$ mice (Figure 3-supplement 1B). For males no significant reduction in tumour
150 incidence was detected in C/EBP $\beta^{\Delta uORF}$ mice (Figure 3B,D). The survival of tumour bearing
151 mice and the tumour load was similar in wt and C/EBP $\beta^{\Delta uORF}$ males, while the tumour spread
152 seems to be even slightly increased in C/EBP $\beta^{\Delta uORF}$ male mice (Figure 3-supplement 1C,D).
153 The main tumour types found in female mice were lymphoma, hepatocellular carcinoma and
154 histiocytic sarcoma. The occurrence of all three types was reduced in C/EBP $\beta^{\Delta uORF}$ females
155 (Supplementary file 2). For other tumour types the single numbers are too small to make a
156 clear statement about a change in frequency. In male mice hepatocellular carcinoma and
157 histiocytic sarcoma were the most frequent tumour types observed. Although the overall
158 tumour incidence was similar in C/EBP $\beta^{\Delta uORF}$ and wt males, the frequency of hepatocellular
159 carcinoma was reduced in the C/EBP $\beta^{\Delta uORF}$ males (Supplementary file 2).

160 Apart from the reduced tumour incidence and the increase in survival of tumour-
161 bearing C/EBP $\beta^{\Delta uORF}$ females, also the survival of tumour-free female C/EBP $\beta^{\Delta uORF}$ mice was
162 significantly extended by 25.13% ($p=0.0467$ log-rank test) compared to wt tumour-free
163 females (Figure 3-supplement 1E). This suggests that both the tumour incidence and
164 additional unrelated factors contribute to the increased survival of C/EBP $\beta^{\Delta uORF}$ females. The
165 observed increase in median lifespan of tumour-free C/EBP $\beta^{\Delta uORF}$ males of 19.71% does not
166 correlate with a statistically significant increase in the overall survival ($p=0.4647$ log-rank test)
167 (Figure 3-supplement 1F). However, the survival curve points to a possible health
168 improvement in the median phase of the male lifespan. Taken together, the C/EBP $\beta^{\Delta uORF}$
169 mutation in mice restricting the expression of LIP results in a significant lifespan extension
170 and decreased tumour incidence in females but not in males.

171 Typically, CR-mediated, genetic or pharmacological suppression of mTORC1
172 signalling is accompanied by the attenuation of an age-associated decline of health
173 parameters (Johnson, Rabinovitch, & Kaeberlein, 2013). We examined the selected health
174 parameters of body weight and composition, glucose tolerance, naïve/memory T-cell ratio,

motor coordination and muscle strength in separate ageing cohorts of young (3-5 months) and old (18-20 months for females and 20-22 months for males) mice. In addition, we compared the histological appearance of selected tissues (liver, muscle, pancreas, skin, spleen and bone) between old (20/22 months) wt and C/EBP $\beta^{\Delta uORF}$ mice. Body weight was significantly increased in all old mice (Figure 4A,B). The increase for the old female C/EBP $\beta^{\Delta uORF}$ mice was significantly smaller compared to old wt littermates, while for the males there was no significant difference between the genotypes (Figure 4A,B). The slightly lower body weight for the young C/EBP $\beta^{\Delta uORF}$ males was also observed in our previous study (Zidek et al., 2015). A similar pattern was observed regarding the fat content that was measured by abdominal computed tomography (CT) analysis (Figure 4C,D and Figure 4-supplement 1C). The volumes of total fat increased strongly in old mice both in visceral and subcutaneous fat depots (Figure 4-supplement 1A,B). Old female C/EBP $\beta^{\Delta uORF}$ mice accumulated significantly less fat in the visceral and subcutaneous fat depots than wt females, while there was no difference for male mice (Figure 4-supplement 1A, B). The lean body mass was slightly lower in old female C/EBP $\beta^{\Delta uORF}$ mice and increased in male wt mice compared to young mice (Figure 4-supplement 1A, B). Thus, female C/EBP $\beta^{\Delta uORF}$ mice gain less fat upon aging similar to mice under CR or upon prolonged rapamycin treatment (Fang et al., 2013). In contrast, although male C/EBP $\beta^{\Delta uORF}$ mice had a lower body weight and subcutaneous fat content at a young age compared to wt mice they were not able to maintain this difference during the aging process, which correlates with the lack in lifespan extension. In addition, we found an increase in mRNA expression of the macrophage marker *Cd68* as a measure for age-related macrophage infiltration in visceral WAT of old mice, which was attenuated in female but not in male C/EBP $\beta^{\Delta uORF}$ mice (Figure 4-supplement 1D).

Impaired glucose tolerance is a hallmark of the aging process, which is improved by CR (Barzilai, Banerjee, Hawkins, Chen, & Rossetti, 1998; Mitchell et al., 2016). The intraperitoneal glucose tolerance test (IPGTT) showed that glucose clearance, calculated as the area under the curve (AUC), is significantly less efficient in old wt compared to young wt mice (Figure 4E,F). Old C/EBP $\beta^{\Delta uORF}$ females and males perform significantly better in the IPGTT test than old wt littermates, which is reflected by the lower AUC value. Therefore, the C/EBP $\beta^{\Delta uORF}$ mutation protects against age-related decline of glucose tolerance in males and

females.

The ageing associated increase in memory/naïve T-cell ratio is a robust indicator for the progression of the immunological ageing progress. At a young age naïve T cells predominate and memory T cells are relatively scarce. Upon ageing the naïve T cell population is strongly reduced with a concomitant increase in the memory T cell population, resulting in an increased ratio of memory to naïve T cells (Hakim, Flomerfelt, Boyiadzis, & Gress, 2004). The ratio of memory (Cd44^{high}) to naïve (Cd44^{low}/Cd62L^{high}) cytotoxic T (Cd8⁺) cells or memory (Cd44^{high}) to naïve (Cd44^{low}/Cd62L^{high}) helper T (Cd4⁺) cells was analysed by flow cytometric analysis. Both increased upon aging in the blood of males and females of both genotypes (Figure 5A-D). However in C/EBP $\beta^{\Delta uORF}$ mice of both genders this increase was significantly attenuated compared to wt mice (Figure 5A-D and figure 5-supplement 1A-D). These data suggest that the C/EBP $\beta^{\Delta uORF}$ mutation preserves a more juvenile immunological phenotype during ageing.

Aging is associated with a significant decline in motor coordination and muscle strength (Barreto, Huang, & Giffard, 2010; Demontis, Piccirillo, Goldberg, & Perrimon, 2013). In the rotarod test the time is measured that mice endure on a turning and accelerating rod as an indication for their motor-coordination. As expected, rotarod performance decreased with age both for wt female and male mice (Figure 6A). Remarkably, rotarod performance was completely preserved in old C/EBP $\beta^{\Delta uORF}$ females but not in C/EBP $\beta^{\Delta uORF}$ males. In the beam walking test the required crossing time and number of paw slips of mice traversing a narrow beam are measured. Old mice needed more time to cross the beam reflecting loss of motor coordination upon ageing (Figure 6B). The aging-associated increase of the crossing time was less severe in C/EBP $\beta^{\Delta uORF}$ males and females, although statistically significant only in males (Figure 6B). Nevertheless, the strong increase in the number of paw slips in old wt mice is almost completely attenuated in C/EBP $\beta^{\Delta uORF}$ males and females (Figure 6C). Note that the number of paw slips by young C/EBP $\beta^{\Delta uORF}$ males is already significantly lower compared to young wt males. During the wire hang test the time is measured that mice endure to hang from an elevated wire which serves as an indication for limb skeletal muscle strength (Brooks & Dunnett, 2009). Similar to the rotarod test, the decline in wire hang performance that is seen in old wt mice is completely restored for the female but not for the

male C/EBP $\beta^{\Delta uORF}$ mice (Figure 6D).

Taken together these data demonstrate that the decline in motor coordination and muscle strength is less severe and partly abrogated in female C/EBP $\beta^{\Delta uORF}$ mice. The results for the old male C/EBP $\beta^{\Delta uORF}$ mice are not that clear since they show an improved performance only in the beam walking test. One possible explanation is that only the beam walking test measures purely motor coordination skills whereas the results from the rotarod and wire hang tests are influenced in addition by muscle strength and endurance. Old C/EBP $\beta^{\Delta uORF}$ males thus might have maintained their motor coordination upon ageing but still suffer from an ageing-dependent loss of muscle strength.

By histological examination of different tissues we observed a reduction in some age-related alterations in C/EBP $\beta^{\Delta uORF}$ mice compared to old wt controls (Supplementary file 3). We observed a reduced severity of hepatocellular vacuolation and cytoplasmic nuclear inclusions in male C/EBP $\beta^{\Delta uORF}$ mice; in the pancreas both male and female C/EBP $\beta^{\Delta uORF}$ mice showed a reduced occurrence and severity of islet cell hyperplasia; in skeletal muscle the number of regenerating muscle fibres was higher in male C/EBP $\beta^{\Delta uORF}$ mice; the incidence of dermal inflammation was lower in female C/EBP $\beta^{\Delta uORF}$ mice. Unexpectedly, a slightly increased level of inflammation was detected in the livers of female C/EBP $\beta^{\Delta uORF}$ mice. The incidence of other potential age-related pathologies like focal acinar cell atrophy and inflammation in the pancreas, liver polyploidy, spleen lymphoid hyperplasia and extramedullary haematopoiesis, intramuscular adipose tissue infiltration, subcutaneous fat atrophy and bone density were not significantly altered between old wt and C/EBP $\beta^{\Delta uORF}$ mice. We found slightly reduced plasma IGF-1 levels in old C/EBP $\beta^{\Delta uORF}$ females compared to old wt females (Supplementary file 3). A reduction in circulating IGF-1 levels was also found in mice under CR and is believed to be an important mediator of health- and lifespan extending effects of CR (Breese, Ingram, & Sonntag, 1991; Mitchell et al., 2016). Taken together our data show that multiple, but not all, ageing associated alterations are attenuated in C/EBP $\beta^{\Delta uORF}$ mice, and to different extends in males and females.

Finally, we performed a comparative transcriptome analysis from livers of 5 and 20 months old wt and C/EBP $\beta^{\Delta uORF}$ female mice (T. de Jong & V. Guryev, 2018; T de Jong & V. Guryev, 2018; Müller, 2018). A principal component analysis revealed that there was a clear

effect of the genotype on gene expression only in the old mice suggesting that the differences in gene expression between wt and C/EBP $\beta^{\Delta uORF}$ mice are aging dependent (Figure 7-supplement 1). This is supported by the finding that in young mice only 42 genes were differentially regulated between wt and C/EBP $\beta^{\Delta uORF}$ mice (FDR < 0.01; 24 genes upregulated and 18 genes down-regulated in C/EBP $\beta^{\Delta uORF}$ mice compared to wt mice) while in old mice we found 152 differentially regulated genes (FDR < 0.01; 127 genes upregulated and 25 genes downregulated in C/EBP $\beta^{\Delta uORF}$ mice compared to wt mice). Gene ontology (GO) analysis using the David database (Huang da, Sherman, & Lempicki, 2009) of the genes upregulated in old C/EBP $\beta^{\Delta uORF}$ mice in comparison to old wt mice revealed GO terms including “External side of plasma membrane”, “Positive regulation of T-cell proliferation”, and “immune response” (see Supplementary file 4 for the complete list of GO-terms) whereas the GO-terms: “Acute phase” and “Extracellular space” were significantly downregulated (Supplementary file 5). Despite the improved metabolic phenotype of C/EBP $\beta^{\Delta uORF}$ mice (Zidek et al., 2015), the analysis did not reveal GO-terms related to metabolism. We reasoned that metabolic genes might not be detected as differentially regulated because they are subject of expression heterogeneity in old mice. Comparison between the coefficient of variation of individual transcripts between young and old mice revealed that inter-individual variation of gene expression increases with age in both genotypes (Figure 7A,B) supporting earlier observations made by others (White et al., 2015). Direct comparison between old wt and C/EBP $\beta^{\Delta uORF}$ mice showed that this effect is less pronounced in C/EBP $\beta^{\Delta uORF}$ mice (Figure 7C). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway and GO-term enrichment analysis of the highly variably expressed genes in the aged livers revealed that in wt mice particularly metabolic genes related to fatty acid metabolism and oxidative phosphorylation were affected which was not observed in C/EBP $\beta^{\Delta uORF}$ mice (Figure 7D and supplementary file 6 and 7). In addition, genes whose de-regulation is connected to ageing-associated diseases like Non-alcoholic fatty liver disease, Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and cancer were affected by high inter-individual variation in expression levels in old wt but not in old C/EBP $\beta^{\Delta uORF}$ mice (Figure 7D). On the other hand genes involved in cell cycle, transcription and RNA biology showed higher inter-individual variation in old C/EBP $\beta^{\Delta uORF}$ mice compared to wt controls (Supplementary file 7). These

findings suggest that expression control of metabolic genes and genes involved in ageing-associated diseases stays more robust upon aging in C/EBP $\beta^{\Delta uORF}$ mice.

Discussion

Taken together, here we show that loss-of-function mutation of a single *cis*-regulatory element - the uORF - in the *Cebpb*-mRNA, which prevents the translation into the transcription factor C/EBP β -LIP, results in a remarkable juvenile phenotype in aged mice including lower cancer incidence, lower body weight and body fat, better glucose tolerance, lower memory/naïve T cell ratios, and better maintenance of motor coordination. However, we observed clear differences between males and females, with only females showing improvements for cancer incidence, body weight, fat content, Rotarod- and wire hang test performance. In addition, a significant lifespan extension was only observed for the female C/EBP $\beta^{\Delta uORF}$ mice.

We do not know what causes the female specific lifespan extension. C/EBP transcription factors are known for their crosstalk with hormone receptors, including estrogen, progesterone and glucocorticoid receptors (Calkhoven, Snippe, & Ab, 1997; Chang, Parra, Centrella, & McCarthy, 2005; Grontved et al., 2013; Rotinen et al., 2009; Seagroves, Lydon, Hovey, Vonderhaar, & Rosen, 2000; Siersbaek, Nielsen, & Mandrup, 2012; J. Zhang et al., 2010). Therefore, obvious differences in hormone receptor regulation between males and females may determine outcome of shifts in LAP/LIP ratios. Notably, the C/EBP $\beta^{\Delta uORF}$ mutation in males results in higher LAP expression in the liver and therefore 1.5 fold higher LAP/LIP ratios compared to females (Figure 1B,C). Possibly, higher LAP levels in males have some adverse effects on health and lifespan, which may neutralize the beneficial effects of LIP deficiency. In line with this assumption is that the C/EBP $\beta^{\Delta uORF}$ males show an increase in early deaths (Figure 2B,D) that is significant in UD-free males (Figure 2-supplement 1E) and is mainly due to early cancer development (Figure 3D). A similar scenario has been described for short-term treatment with a high dose of rapamycin that failed to extend lifespan of female mice due to frequent development of aggressive haematological cancers (Bitto et al., 2016).

The sex dependent differences we found are intriguing in the light of studies investigating the lifespan extending effects of CR, rapamycin and mutations in the mTORC1

pathway. For example, CR by 20% has a greater lifespan extending effect in female C57BL/6J or DBA/2J mice compared to males (Mitchell et al., 2016). In addition, moderate overexpression of the mTORC1-upstream inhibitor TSC1 or deletion of the mTORC1-downstream S6K1 results in lifespan extension only in females (Selman et al., 2009; H. M. Zhang, Diaz, Walsh, & Zhang, 2017). Notably, downregulation of LIP under low mTORC1 signalling is dependent on 4E-BP1/2 function and not on inhibition of S6K1 (Zidek et al., 2015). Thus, the bias towards female lifespan extension upon reduced mTORC1 signalling seems to be a common feature irrespective of whether the S6K1 or 4E-BP branch is affected. Mutations affecting both mTORC1 and mTORC2 show ambiguous effects; lifespan extension is limited to females in mice heterozygous for mTOR and its cofactor mammalian lethal with Sec 13 protein 8 (mLST8) (Lamming et al., 2012), while in a mTOR-hypomorphic mouse model lifespan extension is observed in both males and females (Wu et al., 2013). Similarly, inhibition of mTORC1 with rapamycin results in either a gender biased or a general lifespan extension depending on the study design and rapamycin concentration used. For example, treatment of genetically heterogeneous mice as well as C57BL/6J or C57BL/6Nia mice with a low dose of rapamycin (from 4.7 to 14 ppm) for different time periods has lifespan extending effects that are stronger in females than in males (Fok et al., 2014; Harrison et al., 2009; Miller et al., 2011; Miller et al., 2014; Y. Zhang et al., 2014). In contrast, treatment with higher concentrations of rapamycin (42 ppm) results in a further increase in lifespan and almost completely alleviates the difference between the sexes (Miller et al., 2014). However, injection of an even higher rapamycin dose (8 mg/kg/day, corresponding to 378 ppm dietary rapamycin) extended lifespan only in males and not in females with serious side effects in females as mentioned above (Bitto et al., 2016). These data indicate that rapamycin treatment with low and probably sub-optimal doses creates differences between sexes (Kaeberlein, 2014). Although the mechanisms behind these sex-dependent differences are not known, our study suggests that mTORC1-LIP regulation may be involved. Possibly, lifespan-extending pathways downstream of mTORC1 are differentially affected by different rapamycin concentrations, and in a gender dependent way. Providing LIP expression is downregulated by low concentrations of rapamycin the female-biased effect on lifespan might be determined predominantly by low LIP levels as well as by the regulation of other highly

sensitive targets like for example S6K1 that similarly shows female specific effects (Selman et al., 2009). At higher rapamycin doses, additional pathways might be engaged from which both males and females benefit. Finally, at too high rapamycin concentrations additional adverse (gender specific) effects might counteract the beneficial effects of rapamycin. Therefore, further research on both positive and negative events downstream of mTORC1 is required to be able to tailor treatment and to minimize side effects.

Also in mouse strains with alterations in other pathways like the somatotrophic axis lifespan extension is often, but not always, more pronounced in females (Brown-Borg, 2009). Examples of somatotrophic-related female biased lifespan extension are Ames dwarf mice that are deficient in growth hormone (GH) and prolactin production (Brown-Borg, Borg, Meliska, & Bartke, 1996) and insulin-like growth factor 1 (IGF-1) receptor heterozygous mice (Holzenberger et al., 2003). Also in these mouse models the reason for the female biased lifespan extension is not known.

What contributes to the extended lifespan in the female $C/EBP\beta^{\Delta uORF}$ mice? Our data indicate that reduced tumour incidence is involved. In line with this is that knockin mice with elevated LIP levels show an increased tumour incidence upon ageing that goes along with reduced survival compared to wt controls (Begay et al., 2015). LIP overexpression can stimulate cell proliferation, migration and transformation *in vitro* and high LIP levels have been detected in different human tumour tissues (Anand et al., 2014; Arnal-Estape et al., 2010; Calkhoven et al., 2000; Haas et al., 2010; Jundt et al., 2005; Park et al., 2013; Raught et al., 1996; Zahnow, Younes, Laucirica, & Rosen, 1997). Together, these studies suggest an oncogenic role of LIP and that the reduction of LIP in the $C/EBP\beta^{\Delta uORF}$ mice counteracts tumour development at least partially by cell intrinsic mechanisms. Although the incidence of certain tumours like hepatocellular carcinoma is similarly reduced in male $C/EBP\beta^{\Delta uORF}$ mice (Supplementary file 2) the overall tumour incidence was not different in comparison to the wt males, again indicating gender specific effects of the $C/EBP\beta^{\Delta uORF}$ mutation. Besides tumour development other parameters contribute to the lifespan extension in female $C/EBP\beta^{\Delta uORF}$ mice as revealed by the survival curves of the tumour-free female mice (Figure 3-supplement 1E). Notably, the ageing-associated increase in body weight and body fat was attenuated in female but not in male $C/EBP\beta^{\Delta uORF}$ mice although at younger age also $C/EBP\beta^{\Delta uORF}$ males

show a reduced body weight and fat content (Figure 4). Our earlier data showed that food intake is not reduced in the C/EBP $\beta^{\Delta uORF}$ mice (Zidek et al., 2013) suggesting that the increase in fat catabolism and other features like the observed higher physical activity cause leanness of the C/EBP $\beta^{\Delta uORF}$ mice (Zidek et al 2013). In accordance with the difference in fat content we observed a reduction in macrophage infiltration in white adipose tissue from female but not from male C/EBP $\beta^{\Delta uORF}$ mice (Figure 4-supplement 1D). Inflammation of the visceral adipose tissue is a common feature of the ageing process and is believed to contribute to insulin resistance and other ageing-associated diseases (Mau & Yung, 2017). Therefore, reduced inflammation in adipose tissues could contribute to the extended health and lifespan of the female C/EBP $\beta^{\Delta uORF}$ mice.

Global liver transcriptome analysis revealed an increase in the inter-individual variation of gene expression between individuals from the same genotype. However, there is less variation C/EBP $\beta^{\Delta uORF}$ between old females than old wt females. A similar increase in the inter-individual variation of gene expression was also identified by others (Cellerino & Ori, 2017; White et al., 2015) and might reflect different ageing rates within the same group of individuals. Intriguingly, the inter-individual variation in specific pathways and gene groups is different for C/EBP $\beta^{\Delta uORF}$ compared to wt mice. Particularly genes connected to metabolic pathways and to ageing-associated diseases showed high expression heterogeneity in old wt but not in old C/EBP $\beta^{\Delta uORF}$ females. Whether the increased inter-individual variation of metabolic transcripts in old wt mice is a direct effect of the observed increase of the inhibitory-acting LIP isoform or is due to unknown secondary effects has to be clarified in future studies. It is however conceivable that increased transcriptional robustness in the old C/EBP $\beta^{\Delta uORF}$ mice contributes to the extension in health- and lifespan of the female C/EBP $\beta^{\Delta uORF}$ mice.

Transcriptome and gene ontology (GO) enrichment analysis in liver revealed some involved mechanisms that could contribute to the youthful and long-lived phenotype of the C/EBP $\beta^{\Delta uORF}$ females. We found reduced expression of acute phase response genes in livers from old C/EBP $\beta^{\Delta uORF}$ females. Acute phase response genes are associated with inflammation and their expression in the liver increases upon ageing (Lee et al., 2012). Moreover, expression of acute phase response genes is inhibited by CR or treatment with the CR-mimetic metformin (Martin-Montalvo et al., 2013) suggesting similar protective

mechanisms. In addition we observed the upregulation of several genes connected to lymphocyte biology in the C/EBP $\beta^{\Delta uORF}$ livers. This fits to the increase in lymphoplasmatic inflammation in the liver of old female C/EBP $\beta^{\Delta uORF}$ mice (Supplementary file 3). It is generally believed that ageing associated lymphocyte infiltration rather promotes the ageing process by increasing inflammatory signals (Singh et al., 2008) that abrogate glucose homeostasis. Nevertheless, recently this view was challenged by showing that hepatic inflammation, involving the activation of IKK β , can also be beneficial for maintaining glucose homeostasis (Liu et al., 2016). Furthermore, infiltration lymphocytes can also contribute to the removal of senescent or pro-tumorigenic cells, thereby acting protective (Kang et al., 2011). Further research is required to find out whether in the case of the female C/EBP $\beta^{\Delta uORF}$ mice lymphocyte infiltration in the liver has adverse or beneficial effects.

We showed earlier that a *cis*-regulatory uORF in the *Cebpb*-mRNA leader sequence is required for translation into LIP, which is stimulated by mTORC1-4E-BP1 signalling (Calkhoven, Bouwman, Snippe, & Ab, 1994; Calkhoven et al., 2000; Wethmar et al., 2010; Zidek et al., 2015). Intriguingly, other uORF-dependent translation events are known to be involved in lifespan regulation. In yeast, translation of the *GCN4*-mRNA into the GCN4 transcription factor - a basic leucine zipper (bZIP) domain transcription factor like the C/EBPs - is controlled by four uORFs (Hinnebusch, 2005). Phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2 α) by the GCN2 kinase in response to amino acid deprivation or upon other stressors results in global inhibition of translation initiation while GCN4 translation is stimulated due to the skipping of inhibitory uORFs. GCN4 activates genes involved in amino acid biosynthesis and stress response in order to alleviate nutrient stress (Hinnebusch, 2005). GCN4 expression is elevated under different conditions that extend either replicative or chronological lifespan in yeast like glucose restriction, inhibition of TOR signalling, depletion of 60s ribosomal subunits or deletion of the arginine transporter canavanine resistance 1 (CAN1) gene and was shown to be at least partially required for the lifespan extending effects of these interventions (Beaupere et al., 2017; Cherkasova & Hinnebusch, 2003; Kubota, Obata, Ota, Sasaki, & Ito, 2003; Martin-Marcos, Hinnebusch, & Tamame, 2007; Steffen et al., 2008; Valenzuela, Aranda, & Gonzalez, 2001; Yang, Wek, & Wek, 2000). Furthermore, the overexpression of GCN4 is sufficient to extend replicative

lifespan in yeast suggesting that GCN4 is a major player in the regulation of yeast lifespan (Mittal et al., 2017). In mammals expression of the GCN4 ortholog ATF4 is similarly upregulated in response to stress-induced eIF2 α -phosphorylation through skipping of inhibitory uORFs in the *Atf4*-mRNA (Vattem & Wek, 2004). Although an involvement of ATF4 in lifespan regulation in mammals has not been addressed so far, increased expression of *Atf4* was found in livers of long-lived mouse models and upon treatments that extend lifespan and in fibroblasts from slow-ageing Snell dwarf and *Pappa* KO mice (Li, Li, & Miller, 2014; Li & Miller, 2015). In the fibroblasts, increased *Atf4* expression was accompanied by an increased stress resistance indicating that *Atf4* might play a role also for mammalian lifespan. Notably, C/EBP β and ATF4 pathways are integrated through heterodimers that bind to composite binding sites (Fawcett, Martindale, Guyton, Hai, & Holbrook, 1999) suggesting that C/EBP β -ATF4 dimers are involved in health and lifespan regulation in mammals with C/EBP β -LAP working together with ATF4 in gene activation while C/EBP β -LIP probably counteracting it. In yeast the deletion of 60s ribosomal subunits was shown to result in a general reduction of occupancy of uORFs indicating uORF skipping although an effect on translation efficiency of the main reading frame was not observed for most of the mRNAs (Mittal et al., 2017). Still there might be a subset of uORF containing mRNAs that might be coregulated under low 60s availability and/or other conditions that result in lifespan extension and mediate the lifespan extending effects. In this respect it is intriguing that uORF-mediated translation into the C/EBP β -LIP isoform is reduced upon knockdown or mutation of the Shwachman-Bodian-Diamond Syndrome (SBDS) protein that is required for 60S ribosomal subunit maturation (In et al., 2016). Thus, uORF mediated translation regulation could be a more general mechanism adjusting gene expression during stress response that might play an important role in lifespan extension.

In summary, reduced signalling through the mTORC1 pathway is thought to mediate many of the beneficial effects of CR or rapamycin treatment (Johnson et al., 2013), and both conditions restrict mTORC1-controlled translation into LIP (Calkhoven et al., 2000; Zidek et al., 2015). These and other studies firmly place LIP function downstream of mTORC1 at the nexus of nutrient signalling and metabolic gene regulation (Figure 8). However, upon ageing, LIP expression increases (the LAP/LIP ratio decreases) in the liver and WAT whereas

475 significant changes in mTORC1/4E-BP1 signalling were only detected in WAT (Figure 1-
476 supplement 1C-F). Possibly, in the liver other pathways play a role in age-related upregulation
477 of LIP as has been described for the RNA-binding protein CUGBP1 (Karagiannides et al.,
478 2001; Timchenko et al., 2006).

479 Experimental reduction of the transcription factor C/EBP β -LIP in mice recapitulates
480 many of the effects of CR or treatment with rapamycin, including the reduced cancer
481 incidence and the generally more pronounced extension of lifespan in females. We have
482 developed a high-throughput screening strategy that allows for discovery of small molecular
483 compounds that suppress the translation into LIP (Zaini et al., 2017). The identification of
484 such compounds or conditions that reduce LIP translation may reveal new ways of CR-
485 mimetic based therapeutic strategies beyond those using mTORC1 inhibition.

486 **Materials and methods**

487 **Key Resources Table**

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional Information
Genetic reagent (mus musculus)	C/EBP $\beta^{\Delta uORF}$	DOI:10.1101/gad.557910 DOI:10.15252/embr.201439837	NA	mice were further back-crossed to 12 generations into C57BL/6 background
Antibody	CD4-PE-Cy7 conjugated	BD Pharmingen	Cat#: 552775	dilution 1:200
Antibody	CD62L-FITC conjugated	BD Pharmingen	Cat#: 561917	dilution 1:200
Antibody	CD3e-PE conjugated	eBioscience	Cat#: 12-0031	dilution 1:200
Antibody	CD8a-eFluor 450 conjugated	eBioscience	Cat#: 48-0081	dilution 1:200
Antibody	CD44-APC conjugated	eBioscience	Cat#: 17-0441	dilution 1:200
Antibody	C/EBP β (E299) (rabbit monoclonal)	Abcam	Cat#: ab32358	dilution 1:1000
Antibody	β -actin (rabbit polyclonal)	Abcam	Cat#: ab16039	dilution 1:1000
Antibody	β -actin (clone C4) (mouse monoclonal)	MP Biomedicals	Cat#: 69100	dilution 1:10000
Antibody	4E-BP1 (C19) (goat polyclonal)	Santa Cruz	Cat#: sc-6024	dilution 1:400
Antibody	phospho-4E-BP1 (Thr 37/46) (rabbit polyclonal)	Cell Signaling	Cat#: 9459	dilution 1:1000
Antibody	p70S6K	Cell Signaling	Cat#: 9202	dilution 1:1000
Antibody	phospho-p70S6K (Thr389) (108D2) (rabbit monoclonal)	Cell Signaling	Cat#: 9234	dilution 1:1000
Antibody	S6 ribosomal protein (54D2) (mouse monoclonal)	Cell Signaling	Cat#: 2317	dilution 1:1000
Antibody	phospho-S6 ribosomal protein (Ser235/236) (2F9) (rabbit monoclonal)	Cell Signaling	Cat#: 4856	dilution 1:1000
Antibody	HRP-linked anti rabbit IgG	GE Healthcare	Cat#: NA934	dilution 1:5000
Antibody	HRP-linked anti mouse IgG	GE Healthcare	Cat#: NA391	dilution 1:5000
Antibody	HRP-linked anti goat IgG	Santa Cruz	Cat#: sc-2056	dilution 1:5000
Sequence-based reagent	<i>Actb</i> (β -actin) (F)	DOI:10.15252/embr.201439837	NA	5'-AGAGGGGAA ATCGTGCGTG AC-3'

Sequence-based reagent	<i>Actb</i> (β -actin) (R)	DOI:10.15252/embr.201439837	NA	5'-CAATAGTG ATGACCTGGC CGT-3'
Sequence-based reagent	<i>Cebpb</i> (F)	DOI:10.15252/embr.201439837	NA	5'-CTGCGGG GTTGTTGAT GT-3'
Sequence-based reagent	<i>Cebpb</i> (R)	DOI:10.15252/embr.201439837	NA	5'-ATGCTCGA AACGGAAAA GGT-3'
Sequence-based reagent	<i>Cd68</i> (F)	this paper	NA	5'-GCCCACC ACCACCAGT CACG-3'
Sequence-based reagent	<i>Cd68</i> (R)	this paper	NA	5'-GTGGTCC AGGGTGAGG GCCA-3'
Commercial assay or kit	Mouse IGF specific AssayMax ELISA kit	BioCat/Assaypro LLC	Cat#: EMI1001-1-AS	
Commercial assay or kit	Lightning Plus ECL reagent	Perkin Elmer	Cat#: NEL103001EA	
Commercial assay or kit	Rneasy Plus Mini kit	QIAGEN	Cat#: ID:74134	
Commercial assay or kit	Transcriptor First Strand cDNA Synthesis kit	Roche	Cat#: 4379012001	
Commercial assay or kit	Light Cyclor 480 SYBR Green I Master Mix	Roche	Cat#: 04707516001	
Commercial assay or kit	TruSeq Sample Preparation V2 Kit	Illumina	Cat#: RS-122-2002	
Commercial assay or kit	Restore Western Blot Stripping buffer	Thermo Fisher	Cat#: 21063	
Commercial assay or kit	RBC-Lysis buffer	BioLegend	Cat#: 420301	
Commercial assay or kit	QIAzol Lysis reagent	QIAGEN	Cat#: ID:79306	
Software, algorithm	GraphPad Prism 7.0	Graphpad Software, La Jolla, CA		
Software, algorithm	DAVID database 6.8	doi:10.1038/nprot.2008.211		
Software, algorithm	STAR 2.5.2b	doi:10.1093/bioinformatics/bts635		
Software, algorithm	Ensembl gene build 86	http://www.ensembl.org		
Software,	EdgeR package	doi:10.1152/physiolgenomics.00106		

algorithm		.2011		
Software, algorithm	gProfiler tool	doi:10.1093/nar/gkw199		
Software, algorithm	Image Quant LAS 4000 Mini Imager software	GE Healthcare		

Mice

C/EBP $\beta^{\Delta uORF}$ mice described in (Wethmar et al., 2010) were back-crossed for 12 generations into the C57BL/6J genetic background. Mice were kept at a standard 12-h light/dark cycle at 22°C in individually ventilated cages (IVC) in a specific-pathogen-free (SPF) animal facility on a standard mouse diet (Harlan Teklad 2916). Mice of the ageing cohort were analysed between 3 and 5 months of age (young) and between 18 and 20 months (old females) or between 20 and 22 months (old males) and were derived from the same breeding pairs as mice used in the lifespan experiment. The body weight of the ageing cohorts was determined before the start of the experimental analysis. All of the animals were handled according to approved institutional animal care and use committee (IACUC) protocols of the Thüringer Landesamt für Verbraucherschutz (#03-005/13) and University of Groningen (#6996A).

Lifespan experiment

C/EBP $\beta^{\Delta uORF}$ and wt littermates (50 mice from each genotype and gender) derived from mating between heterozygous males and females were subjected to a lifespan experiment. Mice were housed in groups with maximum five female mice or four male mice per cage (separated in genotypes and genders) and did not participate in other experiments. Mice were checked daily and the lifespan of every mouse (days) was recorded. Mice were euthanized when the condition of the animal was judged as moribund and/or to be incompatible with continued survival due to severe discomfort based on the independent assessment of experienced animal caretakers. All mice that were found dead or were euthanized underwent necropsy with a few exceptions when the grade of decomposition of dead animals prevented further examination (number of mice without necropsy: n=0 for wt females; n=2 for C/EBP $\beta^{\Delta uORF}$ females; n=3 for wt males and n=5 for C/EBP $\beta^{\Delta uORF}$ males. Survival curves were calculated with the Kaplan-Meier method. Statistical significance was determined by the log-rank test using GraphPath Prism 7 software. Maximum lifespan was determined by the

number of mice for each genotype that were within the 10% longest-lived mice of the combined (wt and C C/EBP $\beta^{\Delta uORF}$) cohorts. Statistical significance of observed differences was calculated with Fisher's exact test. In addition, the mean lifespan (\pm SEM) of the 10% longest lived mice within one genotype was compared to the mean lifespan of the 10% longest lived mice of the other genotype, and the statistical significance was calculated with the Student's T-test.

Tumour incidence

Suspected tumour tissue found during necropsy of the lifespan cohorts was fixed in 4% paraformaldehyde and Haematoxylin & Eosin stained tissue slices were analysed by experienced board-certified veterinary pathologists of the Dutch Molecular Pathology Centre (Utrecht University) to diagnose the tumour type. Tumour incidence was calculated as percentage of mice with pathologically confirmed tumours in respect to all mice from the same cohort that underwent necropsy. Tumour occurrence was defined as the time of death of an animal in which a pathologically confirmed tumour was found. Tumour load was defined as number of different tumour types found in the same mouse and tumour spread was defined as number of different organs harbouring a tumour within the same mouse irrespective of the tumour type with the exception that in those cases in which different tumour types were found in the same organ a number >1 was rated.

Motor coordination experiments

Rotarod test: Mice were habituated to the test situation by placing them on a rotarod (Ugo Basile) with constant rotation (5 rpm) for 5 min at two consecutive days with two trials per mouse per day separated by an interval of 30 min. In the test phase two trials per mouse were performed with accelerating rotation (2-50 rpm within 4 min) with a maximum trial duration of 5 min in which the time was measured until mice fell off the rod. **Beam walking test:** Mice were trained by using a beam of 3 cm width and 100 cm in length at two consecutive days (one trial per mouse per day). At the test day mice had to pass a 1 cm wide beam, 100 cm in length and beam crossing time and number off paw slips upon crossing was measured during 3 trials per mouse that were separated by an interval of 20 min. To

determine of the number of mistakes the number of paw slips per trial was counted upon examination of recorded videos. Wire Hang test: To measure limb grip strength mice were placed with their four limbs at a grid with wire diameter of 1 mm at 20 cm over the layer of bedding material and the hanging time was measured until mice loosened their grip and fell down. Three trials of maximal 60s per mouse were performed that were separated by an interval of 30 min.

Body composition

The body composition was measured using an Aloka LaTheta Laboratory Computed Tomograph LCT-100A (Zinsser Analytic) as described in (Zidek et al., 2015). Percentage body fat was calculated in relation to the sum of lean mass and fat mass.

Bone measurements

Bones of the hind legs were freed from soft tissue and fixed in 4% paraformaldehyde. For determination of the bone volume, trabecular thickness, trabecular number and trabecular separation femurs were analysed by micro CT (Skyscan 1176, Bruker) equipped with an X-ray tube (50 kV/500µA). The resolution was 9 µm, rotation step was set at 1°C, and a 0.5 mm aluminium filter was used. For reconstruction of femora, the region of interest was defined 0.45 mm (for trabecular bone) or 4.05 mm (for cortical bone) apart from the distal growth plate into the diaphysis spanning 2.7 mm (for trabecular bone) or 1.8 mm (for cortical bone). Trabecular bone volume/tissue volume (%), trabecular number per µm, trabecular thickness (µm) and trabecular separation (intertrabecular distance, µm) was determined according to guidelines by ASBMR Histomorphometry Nomenclature Committee (Dempster et al., 2013).

Glucose tolerance

The intraperitoneal (i.p.) glucose tolerance test (IPGTT) was performed as described in (Zidek et al., 2015). Mice without initial increase in blood glucose concentration were excluded from the analysis.

Flow cytometry

Blood cells from 300 µl blood were incubated in RBC-Lysis buffer (Biolegend) to lyse the red blood cells. Remaining cells were washed and incubated with a cocktail of fluorochrome-conjugated antibodies (Cd4-PE-Cy7 (#552775) and Cd62L-FITC (#561917) from BD Pharmingen; Cd3e-PE (#12-0031), Cd8a-eFluor 450 (#48-0081) and Cd44-APC (#17-0441) from eBioscience.), incubated with propidium iodide for the detection of dead cells and analysed using the FACSCanto II analyser (BD Biosciences). The following T cell subsets were quantified: Cd3⁺, Cd8⁺, Cd44^{high} cytotoxic memory T cells; Cd3⁺, Cd8⁺, Cd44^{low}, Cd62L^{high} cytotoxic naïve T cells, Cd3⁺, Cd4⁺, Cd44^{high} helper memory T cells and Cd3⁺, Cd4⁺, Cd44^{low}, Cd62L^{high} helper naïve T cells.

Histology

Tissue pieces were fixed with 4% paraformaldehyde and embedded in paraffin. Sections were stained with Haematoxylin and Eosin (H&E) and age-related pathologies or tumour types were analysed by experienced board-certified veterinary pathologists of the Dutch Molecular Pathology Centre (Utrecht University). Semi-quantification of muscle regeneration was done by counting the number of myofibers with a row of internalized nuclei (>4) for five 200x fields. Other ageing-associated lesions were scored subjectively and the severity of the lesions was graded on a scale between 0 and 3 with 0 = absent; 1 = mild; 2 = moderate and 3 = severe.

Immunoblotting and quantification

Mouse liver and WAT tissue was homogenized on ice with a glass douncer in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM TRIS pH 8.0 supplemented with protease and phosphatase inhibitors). Liver extracts were sonicated immediately, WAT extracts were incubated for 1 hour on ice, centrifuged for 15 min at 4°C after which the lipid layer was carefully removed using a cotton bud and then sonicated. Equal amounts of total protein were separated by SDS-PAGE, transferred to a PVDF membrane and incubated with the following antibodies: C/EBPβ (E299) from Abcam, β-actin (ab16039) from Abcam or (# 69100, clone C4) from MP Biomedicals; 4E-BP1 (C-19) from Santa Cruz; phospho-p70S6K (Thr389) (108D2), p70S6K (#9202), phospho-S6 ribosomal protein (Ser235/236) (2F9), S6 ribosomal protein (54D2), and phospho-4E-BP1 (Thr 37/46) (#9459)

from Cell Signaling Technology and HRP-linked anti rabbit or mouse IgG from GE Healthcare and HRP-linked anti goat IgG from Santa Cruz. Lightning Plus ECL reagent (Perkin Elmer) was used for detection and for re-probing membranes were incubated in Restore Western Blot Stripping buffer (Thermo Fisher). The detection and quantification of protein bands was performed with the Image Quant LAS 4000 Mini Imager (GE Healthcare) using the supplied software.

Quantitative real-time PCR

Mouse liver or visceral fat tissue was homogenized on ice with a motor driven pellet pestle (Kontes) in the presence of QIAzol reagent (QIAGEN) and total RNA was isolated as described in (Zidek et al., 2015). cDNA synthesis was performed from 1 µg of total RNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche) using random hexamer primers. qRT-was performed with the LightCycler 480 SYBR Green I Master mix (Roche) using the following primers: *Actb* (β-actin): 5'-AGA GGG AAA TCG TGC GTG AC-3' and 5'-CAA TAG TGA TGA CCT GGC CGT-3'; *Cebpb*: 5'-CTG CGG GGT TGT TGA TGT-3' and 5'-ATG CTC GAA ACG GAA AAG GT-3'; *Cd68*: 5'-GCC CAC CAC CAG TCA CG-3' and 5'- GTG GTC CAG GGT GAG GGC CA-3'.

Enzyme-linked immune-sorbent assay (ELISA)

Plasma was prepared as described in (Zidek et al., 2015) and the IGF-1 specific ELISA was performed according to the instructions of the manufacturer (BioCat).

RNA-seq Analysis

Liver tissue from young (5 months) and old (20 months) wt and C/EBPβ^{ΔuORF} mice (from six individuals per group) was homogenized on ice with a motor driven pellet pestle (Kontes) in the presence of QIAzol reagent (Qiagen) and total RNA was isolated as described in (Zidek et al., 2015). Preparation of the sequencing libraries was performed using the TruSeq Sample Preparation V2 Kit (Illumina) according to the manufacturer's instructions. High-throughput single-end sequencing (65 bp) of the libraries was performed with an Illumina HiSeq 2500 instrument. Reads were aligned and quantified using STAR 2.5.2b (Dobin et al., 2013)

against primary assembly GRCm38 using Ensembl gene build 86 (<http://www.ensembl.org>). Genes with average expression level below 1 fragment per million (FPM) were excluded from the analysis. A generalized linear model was used to identify differential gene expression using EdgeR package (McCarthy, Roche, & Forde, 2012; Robinson, McCarthy, & Smyth, 2010). The library normalization was left at the standard setting (trimmed mean of M-values, TMM). The resulting p-values were corrected for multiple testing using the Benjamini-Hochberg procedure. Data visualization, calculation of CV (coefficient of variation) and statistical tests were conducted using custom R scripts (Processed data and R script available at http://www.genomes.nl/CEBPB_delta_uORF/ (T de Jong & V. Guryev, 2018) or https://github.com/Vityay/CEBPB_delta_uORF (T. de Jong & V. Guryev, 2018)). Gene ontology (GO) analysis was performed using the DAVID database version 6.8 (Huang et al., 2009) with default DAVID database setting with medium stringency and *Mus musculus* background. KEGG pathway analysis was performed using gProfiler tool (Reimand et al., 2016). For dataset see (Müller, 2018).

Statistical analysis

Biological replication is indicated (n=x). All graphs show average \pm standard error of the mean (s.e.m.). The unpaired, two-tailed Student's t-Test was used to calculate statistical significance of results with * p< 0.05; ** p<0.01; *** p< 0.001. Significance of the differences in survival curves were analysed using the log-rank test using Prism7 (GraphPad Software) and significance of the difference in maximum lifespan (number of mice from one cohort within the 10% longest lived mice calculated from the combined cohort) and tumour incidence was calculated using the Fisher's exact test with *p<0.05. Daily Chi-square test calculations were carried out to examine the significance of parts of the survival curves.

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References

- Albert, V., & Hall, M. N. (2015). Reduced C/EBP β -LIP translation improves metabolic health. *EMBO Rep*, 16(8), 881-882. doi:10.15252/embr.201540757
- Anand, S., Ebner, J., Warren, C. B., Raam, M. S., Piliang, M., Billings, S. D., & Maytin, E. V. (2014). C/EBP transcription factors in human squamous cell carcinoma: selective changes in expression of isoforms correlate with the neoplastic state. *PLoS One*, 9(11), e112073. doi:10.1371/journal.pone.0112073
- Anisimov, V. N., Zabezhinski, M. A., Popovich, I. G., Piskunova, T. S., Semchenko, A. V., Tyndyk, M. L., . . . Blagosklonny, M. V. (2011). Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice. *Cell Cycle*, 10(24), 4230-4236. doi:10.4161/cc.10.24.18486
- Arnal-Estape, A., Tarragona, M., Morales, M., Guiu, M., Nadal, C., Massague, J., & Gomis, R. (2010). HER2 silences tumor suppression in breast cancer cells by switching expression of C/EBP β isoforms. *Cancer Res*, 70(23), 9927-9936. doi:10.1158/0008-5472.CAN-10-0869
- Augustine, J. J., Bodziak, K. A., & Hricik, D. E. (2007). Use of sirolimus in solid organ transplantation. *Drugs*, 67(3), 369-391.
- Barreto, G., Huang, T. T., & Giffard, R. G. (2010). Age-related defects in sensorimotor activity, spatial learning, and memory in C57BL/6 mice. *J Neurosurg Anesthesiol*, 22(3), 214-219. doi:10.1097/ANA.0b013e3181d56c98
- Barzilai, N., Banerjee, S., Hawkins, M., Chen, W., & Rossetti, L. (1998). Caloric restriction reverses hepatic insulin resistance in aging rats by decreasing visceral fat. *J Clin Invest*, 101(7), 1353-1361. doi:10.1172/JCI485
- Beaupere, C., Wasko, B. M., Lorusso, J., Kennedy, B. K., Kaeberlein, M., & Labunskyy, V. M. (2017). CAN1 Arginine Permease Deficiency Extends Yeast Replicative Lifespan via Translational Activation of Stress Response Genes. *Cell Rep*, 18(8), 1884-1892. doi:10.1016/j.celrep.2017.01.077

- Begay, V., Smink, J. J., Loddenkemper, C., Zimmermann, K., Rudolph, C., Scheller, M., . . . Leutz, A. (2015). Deregulation of the endogenous C/EBPbeta LIP isoform predisposes to tumorigenesis. *J Mol Med (Berl)*, 93(1), 39-49. doi:10.1007/s00109-014-1215-5
- Bitto, A., Ito, T. K., Pineda, V. V., LeTexier, N. J., Huang, H. Z., Sutlief, E., . . . Kaeberlein, M. (2016). Transient rapamycin treatment can increase lifespan and healthspan in middle-aged mice. *Elife*, 5. doi:10.7554/eLife.16351
- Breese, C. R., Ingram, R. L., & Sonntag, W. E. (1991). Influence of age and long-term dietary restriction on plasma insulin-like growth factor-1 (IGF-1), IGF-1 gene expression, and IGF-1 binding proteins. *J Gerontol*, 46(5), B180-187.
- Brooks, S. P., & Dunnett, S. B. (2009). Tests to assess motor phenotype in mice: a user's guide. *Nat Rev Neurosci*, 10(7), 519-529. doi:10.1038/nrn2652
- Brown-Borg, H. M. (2009). Hormonal control of aging in rodents: the somatotrophic axis. *Mol Cell Endocrinol*, 299(1), 64-71. doi:S0303-7207(08)00285-2 [pii] 10.1016/j.mce.2008.07.001
- Brown-Borg, H. M., Borg, K. E., Meliska, C. J., & Bartke, A. (1996). Dwarf mice and the ageing process. *Nature*, 384(6604), 33. doi:10.1038/384033a0
- Calkhoven, C. F., Bouwman, P. R., Snippe, L., & Ab, G. (1994). Translation start site multiplicity of the CCAAT/enhancer binding protein alpha mRNA is dictated by a small 5' open reading frame. *Nucleic Acids Res*, 22(25), 5540-5547.
- Calkhoven, C. F., Müller, C., & Leutz, A. (2000). Translational control of C/EBPalpha and C/EBPbeta isoform expression. *Genes Dev*, 14(15), 1920-1932.
- Calkhoven, C. F., Snippe, L., & Ab, G. (1997). Differential stimulation by CCAAT/enhancer-binding protein alpha isoforms of the estrogen-activated promoter of the very-low-density apolipoprotein II gene. *Eur J Biochem*, 249(1), 113-120.
- Cellerino, A., & Ori, A. (2017). What have we learned on aging from omics studies? *Semin Cell Dev Biol*, 70, 177-189. doi:10.1016/j.semcdb.2017.06.012
- Chang, W., Parra, M., Centrella, M., & McCarthy, T. L. (2005). Interactions between CCAAT enhancer binding protein delta and estrogen receptor alpha control insulin-like growth factor I (igf1) and estrogen receptor-dependent gene expression in osteoblasts. *Gene*, 345(2), 225-235. doi:10.1016/j.gene.2004.11.017
- Cherkasova, V. A., & Hinnebusch, A. G. (2003). Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. *Genes Dev*, 17(7), 859-872. doi:10.1101/gad.1069003
- Colman, R. J., Anderson, R. M., Johnson, S. C., Kastman, E. K., Kosmatka, K. J., Beasley, T. M., . . . Weindruch, R. (2009). Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science*, 325(5937), 201-204. doi:10.1126/science.1173635
- de Jong, T., & Guryev, V. (2018). CEBPB_delta_uORF. Github. https://github.com/Vityay/CEBPB_delta_uORF. 9aca665.
- de Jong, T., & Guryev, V. (2018). Processed data and R script for analysis and visualization of transcriptome variability. http://www.genomes.nl/CEBPB_delta_uORF/
- de Oliveira, M. A., Martins, E. M. F., Wang, Q., Sonis, S., Demetri, G., George, S., . . . Treister, N. S. (2011). Clinical presentation and management of mTOR inhibitor-associated stomatitis. *Oral Oncol*, 47(10), 998-1003. doi:10.1016/j.oraloncology.2011.08.009

- Demontis, F., Piccirillo, R., Goldberg, A. L., & Perrimon, N. (2013). Mechanisms of skeletal muscle aging: insights from *Drosophila* and mammalian models. *Dis Model Mech*, 6(6), 1339-1352. doi:10.1242/dmm.012559
- Descombes, P., & Schibler, U. (1991). A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell*, 67(3), 569-579. doi:0092-8674(91)90531-3 [pii]
- Desvergne, B., Michalik, L., & Wahli, W. (2006). Transcriptional regulation of metabolism. *Physiol Rev*, 86(2), 465-514. doi:10.1152/physrev.00025.2005
- Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., . . . Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, 29(1), 15-21. doi:10.1093/bioinformatics/bts635
- Fang, Y., Westbrook, R., Hill, C., Boparai, R. K., Arum, O., Spong, A., . . . Bartke, A. (2013). Duration of rapamycin treatment has differential effects on metabolism in mice. *Cell Metab*, 17(3), 456-462. doi:10.1016/j.cmet.2013.02.008
- Fawcett, T. W., Martindale, J. L., Guyton, K. Z., Hai, T., & Holbrook, N. J. (1999). Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. *Biochem J*, 339 (Pt 1), 135-141.
- Fok, W. C., Chen, Y., Bokov, A., Zhang, Y., Salmon, A. B., Diaz, V., . . . Richardson, A. (2014). Mice fed rapamycin have an increase in lifespan associated with major changes in the liver transcriptome. *PLoS One*, 9(1), e83988. doi:10.1371/journal.pone.0083988
- Grontved, L., John, S., Baek, S., Liu, Y., Buckley, J. R., Vinson, C., . . . Hager, G. L. (2013). C/EBP maintains chromatin accessibility in liver and facilitates glucocorticoid receptor recruitment to steroid response elements. *EMBO J*, 32(11), 1568-1583. doi:10.1038/emboj.2013.106
- Haas, S. C., Huber, R., Gutsch, R., Kandemir, J. D., Cappello, C., Krauter, J., . . . Brand, K. (2010). ITD- and FL-induced FLT3 signal transduction leads to increased C/EBPbeta-LIP expression and LIP/LAP ratio by different signalling modules. *Br J Haematol*, 148(5), 777-790. doi:10.1111/j.1365-2141.2009.08012.x
- Hakim, F. T., Flomerfelt, F. A., Boyiadzis, M., & Gress, R. E. (2004). Aging, immunity and cancer. *Curr Opin Immunol*, 16(2), 151-156. doi:10.1016/j.coi.2004.01.009
- Hampton, A. L., Hish, G. A., Aslam, M. N., Rothman, E. D., Bergin, I. L., Patterson, K. A., . . . Rush, H. G. (2012). Progression of ulcerative dermatitis lesions in C57BL/6Crl mice and the development of a scoring system for dermatitis lesions. *J Am Assoc Lab Anim Sci*, 51(5), 586-593.
- Harrison, D. E., Strong, R., Sharp, Z. D., Nelson, J. F., Astle, C. M., Flurkey, K., . . . Miller, R. A. (2009). Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature*, 460(7253), 392-395. doi:10.1038/nature08221
- Hinnebusch, A. G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol*, 59, 407-450. doi:10.1146/annurev.micro.59.031805.133833
- Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Geloen, A., Even, P. C., . . . Le Bouc, Y. (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature*, 421(6919), 182-187. doi:10.1038/nature01298

- Hsieh, C. C., Xiong, W., Xie, Q., Rabek, J. P., Scott, S. G., An, M. R., . . . Papaconstantinou, J. (1998). Effects of age on the posttranscriptional regulation of CCAAT/enhancer binding protein alpha and CCAAT/enhancer binding protein beta isoform synthesis in control and LPS-treated livers. *Mol Biol Cell*, 9(6), 1479-1494.
- Huang da, W., Sherman, B. T., & Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*, 4(1), 44-57. doi:10.1038/nprot.2008.211
- In, K., Zaini, M. A., Müller, C., Warren, A. J., von Lindern, M., & Calkhoven, C. F. (2016). Shwachman-Bodian-Diamond syndrome (SBDS) protein deficiency impairs translation re-initiation from C/EBPalpha and C/EBPbeta mRNAs. *Nucleic Acids Res*, 44(9), 4134-4146. doi:10.1093/nar/gkw005
- Johnson, S. C., Rabinovitch, P. S., & Kaeberlein, M. (2013). mTOR is a key modulator of ageing and age-related disease. *Nature*, 493(7432), 338-345. doi:10.1038/nature11861
- Jundt, F., Raetzel, N., Müller, C., Calkhoven, C. F., Kley, K., Mathas, S., . . . Dorken, B. (2005). A rapamycin derivative (everolimus) controls proliferation through down-regulation of truncated CCAAT enhancer binding protein {beta} and NF-{kappa}B activity in Hodgkin and anaplastic large cell lymphomas. *Blood*, 106(5), 1801-1807. doi:2004-11-4513 [pii] 10.1182/blood-2004-11-4513
- Kaeberlein, M. (2014). Rapamycin and ageing: when, for how long, and how much? *J Genet Genomics*, 41(9), 459-463. doi:10.1016/j.jgg.2014.06.009
- Kaeberlein, M., Rabinovitch, P. S., & Martin, G. M. (2015). Healthy aging: The ultimate preventative medicine. *Science*, 350(6265), 1191-1193. doi:10.1126/science.aad3267
- Kang, T. W., Yevsa, T., Woller, N., Hoenicke, L., Wuestefeld, T., Dauch, D., . . . Zender, L. (2011). Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature*, 479(7374), 547-551. doi:10.1038/nature10599
- Karagiannides, I., Tchkonja, T., Dobson, D. E., Steppan, C. M., Cummins, P., Chan, G., . . . Kirkland, J. L. (2001). Altered expression of C/EBP family members results in decreased adipogenesis with aging. *Am J Physiol Regul Integr Comp Physiol*, 280(6), R1772-1780.
- Komarova, E. A., Antoch, M. P., Novototskaya, L. R., Chernova, O. B., Paszkiewicz, G., Leontieva, O. V., . . . Gudkov, A. V. (2012). Rapamycin extends lifespan and delays tumorigenesis in heterozygous p53+/- mice. *Aging (Albany NY)*, 4(10), 709-714. doi:10.18632/aging.100498
- Kubota, H., Obata, T., Ota, K., Sasaki, T., & Ito, T. (2003). Rapamycin-induced translational derepression of GCN4 mRNA involves a novel mechanism for activation of the eIF2 alpha kinase GCN2. *J Biol Chem*, 278(23), 20457-20460. doi:10.1074/jbc.C300133200
- Lamming, D. W., Ye, L., Katajisto, P., Goncalves, M. D., Saitoh, M., Stevens, D. M., . . . Baur, J. A. (2012). Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science*, 335(6076), 1638-1643. doi:10.1126/science.1215135
- Lee, J. S., Ward, W. O., Ren, H., Vallanat, B., Darlington, G. J., Han, E. S., . . . Corton, J. C. (2012). Meta-analysis of gene expression in the mouse liver reveals biomarkers associated with inflammation increased early during aging. *Mech Ageing Dev*, 133(7), 467-478. doi:10.1016/j.mad.2012.05.006

- Li, W., Li, X., & Miller, R. A. (2014). ATF4 activity: a common feature shared by many kinds of slow-aging mice. *Aging Cell*, 13(6), 1012-1018. doi:10.1111/accel.12264
- Li, W., & Miller, R. A. (2015). Elevated ATF4 function in fibroblasts and liver of slow-aging mutant mice. *J Gerontol A Biol Sci Med Sci*, 70(3), 263-272. doi:10.1093/gerona/glu040
- Liu, J., Ibi, D., Taniguchi, K., Lee, J., Herrema, H., Akosman, B., . . . Ozcan, U. (2016). Inflammation Improves Glucose Homeostasis through IKKbeta-XBP1s Interaction. *Cell*, 167(4), 1052-1066 e1018. doi:10.1016/j.cell.2016.10.015
- Martin-Marcos, P., Hinnebusch, A. G., & Tamame, M. (2007). Ribosomal protein L33 is required for ribosome biogenesis, subunit joining, and repression of GCN4 translation. *Mol Cell Biol*, 27(17), 5968-5985. doi:10.1128/MCB.00019-07
- Martin-Montalvo, A., Mercken, E. M., Mitchell, S. J., Palacios, H. H., Mote, P. L., Scheibye-Knudsen, M., . . . de Cabo, R. (2013). Metformin improves healthspan and lifespan in mice. *Nat Commun*, 4, 2192. doi:10.1038/ncomms3192
- Mattison, J. A., Roth, G. S., Beasley, T. M., Tilmont, E. M., Handy, A. M., Herbert, R. L., . . . de Cabo, R. (2012). Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. *Nature*, 489(7415), 318-321. doi:10.1038/nature11432
- Mau, T., & Yung, R. (2017). Adipose tissue inflammation in aging. *Exp Gerontol*. doi:10.1016/j.exger.2017.10.014
- McCarthy, S. D., Roche, J. F., & Forde, N. (2012). Temporal changes in endometrial gene expression and protein localization of members of the IGF family in cattle: effects of progesterone and pregnancy. *Physiol Genomics*, 44(2), 130-140. doi:10.1152/physiolgenomics.00106.2011
- Miller, R. A., Harrison, D. E., Astle, C. M., Baur, J. A., Boyd, A. R., de Cabo, R., . . . Strong, R. (2011). Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. *J Gerontol A Biol Sci Med Sci*, 66(2), 191-201. doi:10.1093/gerona/glq178
- Miller, R. A., Harrison, D. E., Astle, C. M., Fernandez, E., Flurkey, K., Han, M., . . . Strong, R. (2014). Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. *Aging Cell*, 13(3), 468-477. doi:10.1111/accel.12194
- Mitchell, S. J., Madrigal-Matute, J., Scheibye-Knudsen, M., Fang, E., Aon, M., Gonzalez-Reyes, J. A., . . . de Cabo, R. (2016). Effects of Sex, Strain, and Energy Intake on Hallmarks of Aging in Mice. *Cell Metab*, 23(6), 1093-1112. doi:10.1016/j.cmet.2016.05.027
- Mittal, N., Guimaraes, J. C., Gross, T., Schmidt, A., Vina-Vilaseca, A., Nedialkova, D. D., . . . Zavalan, M. (2017). The Gcn4 transcription factor reduces protein synthesis capacity and extends yeast lifespan. *Nat Commun*, 8(1), 457. doi:10.1038/s41467-017-00539-y
- Müller, C., de Jong, T., Guryev, V., Calkhoven, CF. (2018). *Transcriptome profiling of liver samples of C/EBPβΔuORF mice*. Retrieved from: <https://www.ebi.ac.uk/arrayexpress/>
- Neff, F., Flores-Dominguez, D., Ryan, D. P., Horsch, M., Schroder, S., Adler, T., . . . Ehninger, D. (2013). Rapamycin extends murine lifespan but has limited effects on aging. *J Clin Invest*, 123(8), 3272-3291. doi:10.1172/JCI67674

- Park, B. H., Kook, S., Lee, S., Jeong, J. H., Brufsky, A., & Lee, B. C. (2013). An isoform of C/EBPbeta, LIP, regulates expression of the chemokine receptor CXCR4 and modulates breast cancer cell migration. *J Biol Chem*, 288(40), 28656-28667. doi:10.1074/jbc.M113.509505
- Raught, B., Gingras, A. C., James, A., Medina, D., Sonenberg, N., & Rosen, J. M. (1996). Expression of a translationally regulated, dominant-negative CCAAT/enhancer-binding protein beta isoform and up-regulation of the eukaryotic translation initiation factor 2alpha are correlated with neoplastic transformation of mammary epithelial cells. *Cancer Res*, 56(19), 4382-4386.
- Reimand, J., Arak, T., Adler, P., Kolberg, L., Reisberg, S., Peterson, H., & Vilo, J. (2016). g:Profiler-a web server for functional interpretation of gene lists (2016 update). *Nucleic Acids Res*, 44(W1), W83-89. doi:10.1093/nar/gkw199
- Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26(1), 139-140. doi:10.1093/bioinformatics/btp616
- Roesler, W. J. (2001). The role of C/EBP in nutrient and hormonal regulation of gene expression. *Annu Rev Nutr*, 21, 141-165. doi:10.1146/annurev.nutr.21.1.141
- 21/1/141 [pii]
- Rotinen, M., Celay, J., Alonso, M. M., Arrazola, A., Encio, I., & Villar, J. (2009). Estradiol induces type 8 17beta-hydroxysteroid dehydrogenase expression: crosstalk between estrogen receptor alpha and C/EBPbeta. *J Endocrinol*, 200(1), 85-92. doi:10.1677/JOE-08-0134
- Seagroves, T. N., Lydon, J. P., Hovey, R. C., Vonderhaar, B. K., & Rosen, J. M. (2000). C/EBPbeta (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland development. *Mol Endocrinol*, 14(3), 359-368. doi:10.1210/mend.14.3.0434
- Selman, C., Tullet, J. M., Wieser, D., Irvine, E., Lingard, S. J., Choudhury, A. I., . . . Withers, D. J. (2009). Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science*, 326(5949), 140-144. doi:326/5949/140 [pii]
- 10.1126/science.1177221
- Serrano, M. (2016). Unraveling the links between cancer and aging. *Carcinogenesis*, 37(2), 107. doi:10.1093/carcin/bgv100
- Siersbaek, R., Nielsen, R., & Mandrup, S. (2012). Transcriptional networks and chromatin remodeling controlling adipogenesis. *Trends Endocrinol Metab*, 23(2), 56-64. doi:10.1016/j.tem.2011.10.001
- Singh, P., Coskun, Z. Z., Goode, C., Dean, A., Thompson-Snipes, L., & Darlington, G. (2008). Lymphoid neogenesis and immune infiltration in aged liver. *Hepatology*, 47(5), 1680-1690. doi:10.1002/hep.22224
- Steffen, K. K., MacKay, V. L., Kerr, E. O., Tsuchiya, M., Hu, D., Fox, L. A., . . . Kaeberlein, M. (2008). Yeast life span extension by depletion of 60s ribosomal subunits is mediated by Gcn4. *Cell*, 133(2), 292-302. doi:10.1016/j.cell.2008.02.037
- Timchenko, L. T., Salisbury, E., Wang, G. L., Nguyen, H., Albrecht, J. H., Hershey, J. W., & Timchenko, N. A. (2006). Age-specific CUGBP1-eIF2 complex increases translation of CCAAT/enhancer-binding protein beta in old liver. *J Biol Chem*, 281(43), 32806-32819. doi:10.1074/jbc.M605701200

- Valenzuela, L., Aranda, C., & Gonzalez, A. (2001). TOR modulates GCN4-dependent expression of genes turned on by nitrogen limitation. *J Bacteriol*, 183(7), 2331-2334. doi:10.1128/JB.183.7.2331-2334.2001
- Vattem, K. M., & Wek, R. C. (2004). Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc Natl Acad Sci U S A*, 101(31), 11269-11274. doi:10.1073/pnas.0400541101
- Weindruch, R., & Walford, R. L. (1982). Dietary restriction in mice beginning at 1 year of age: effect on life-span and spontaneous cancer incidence. *Science*, 215(4538), 1415-1418.
- Wethmar, K., Begay, V., Smink, J. J., Zaragoza, K., Wiesenthal, V., Dorken, B., . . . Leutz, A. (2010). C/EBPbetaDeltauORF mice--a genetic model for uORF-mediated translational control in mammals. *Genes Dev*, 24(1), 15-20. doi:24/1/15 [pii] 10.1101/gad.557910
- White, R. R., Milholland, B., MacRae, S. L., Lin, M., Zheng, D., & Vijg, J. (2015). Comprehensive transcriptional landscape of aging mouse liver. *BMC Genomics*, 16, 899. doi:10.1186/s12864-015-2061-8
- Wilkinson, J. E., Burmeister, L., Brooks, S. V., Chan, C. C., Friedline, S., Harrison, D. E., . . . Miller, R. A. (2012). Rapamycin slows aging in mice. *Aging Cell*, 11(4), 675-682. doi:10.1111/j.1474-9726.2012.00832.x
- Wu, J. J., Liu, J., Chen, E. B., Wang, J. J., Cao, L., Narayan, N., . . . Finkel, T. (2013). Increased Mammalian Lifespan and a Segmental and Tissue-Specific Slowing of Aging after Genetic Reduction of mTOR Expression. *Cell Rep*, 4(5), 913-920. doi:10.1016/j.celrep.2013.07.030
- Yang, R., Wek, S. A., & Wek, R. C. (2000). Glucose limitation induces GCN4 translation by activation of Gcn2 protein kinase. *Mol Cell Biol*, 20(8), 2706-2717.
- Zahnow, C. A., Younes, P., Laucirica, R., & Rosen, J. M. (1997). Overexpression of C/EBPbeta-LIP, a naturally occurring, dominant-negative transcription factor, in human breast cancer. *J Natl Cancer Inst*, 89(24), 1887-1891.
- Zaini, M. A., Müller, C., Ackermann, T., Reinshagen, J., Kortman, G., Pless, O., & Calkhoven, C. F. (2017). A screening strategy for the discovery of drugs that reduce C/EBPbeta-LIP translation with potential calorie restriction mimetic properties. *Sci Rep*, 7, 42603. doi:10.1038/srep42603
- Zhang, H. M., Diaz, V., Walsh, M. E., & Zhang, Y. (2017). Moderate lifelong overexpression of tuberous sclerosis complex 1 (TSC1) improves health and survival in mice. *Sci Rep*, 7(1), 834. doi:10.1038/s41598-017-00970-7
- Zhang, J., Gonit, M., Salazar, M. D., Shatnawi, A., Shemshedini, L., Trumbly, R., & Ratnam, M. (2010). C/EBPalpha redirects androgen receptor signaling through a unique bimodal interaction. *Oncogene*, 29(5), 723-738. doi:10.1038/onc.2009.373
- Zhang, Y., Bokov, A., Gelfond, J., Soto, V., Ikeno, Y., Hubbard, G., . . . Fischer, K. (2014). Rapamycin extends life and health in C57BL/6 mice. *J Gerontol A Biol Sci Med Sci*, 69(2), 119-130. doi:10.1093/gerona/glt056
- Zidek, L. M., Ackermann, T., Hartleben, G., Eichwald, S., Kortman, G., Kiehnopf, M., . . . Calkhoven, C. F. (2015). Deficiency in mTORC1-controlled C/EBPbeta-mRNA translation improves metabolic health in mice. *EMBO Rep*, 16(8), 1022-1036. doi:10.15252/embr.201439837

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Figure legends

Figure 1 with 1 supplement

C/EBP β LAP/LIP isoform ratio increases upon ageing.

(A) The graph at the left shows that wt C/EBP β -mRNA is translated into LAP1 and LAP2 through regular translation initiation, while translation into LIP involves a primary translation of the uORF followed by translation re-initiation at the downstream LIP-AUG by post-uORF-translation ribosomes. The graph at the right shows that genetic ablation of the uORF abolishes translation into LIP, but leaves translation into LAP1 and LAP2 unaffected (for detailed description see (Calkhoven et al., 2000; Zidek et al., 2015). (B and C) Immunoblots of liver samples from young (5 months) and old (female 20 months, male 22 months) wt and C/EBP $\beta^{\Delta uORF}$ (B) males and (C) females showing LAP and LIP isoform expression. β -actin expression served as loading control. The LAP/LIP isoform ratio as calculated from quantification by chemiluminescence digital imaging of immunoblots is shown at the right (wt males n=9 young, n=10 old; C/EBP $\beta^{\Delta uORF}$ males, n=11 young, n=10 old; wt females, n=9 young, n=8 old; C/EBP $\beta^{\Delta uORF}$ females, n=10 young, n=10 old). (D and E) C/EBP β mRNA levels as determined by quantitative real-time PCR in (D) males (wt, n=11 young, n=11 old; C/EBP $\beta^{\Delta uORF}$, n=11 young, n=9 old) and in (E) females (wt, n=9 young, n=11 old; C/EBP $\beta^{\Delta uORF}$, n=9 young, n=11 old). P-values were determined by Student's t-test, *p<0.05; **p<0.01; ***p<0.001.

Figure 2 with 1 supplement

Increased survival of female C/EBP $\beta^{\Delta uORF}$ mice.

Survival curves of (A) the complete female cohorts, (B) complete male cohorts, (C) the UD-free female cohorts, (D) UD-free male cohorts, (E) female mice with UD and (F) male mice with UD with the survival curves of wt or C/EBP $\beta^{\Delta uORF}$ mice indicated. The increase in median survival (%) of C/EBP $\beta^{\Delta uORF}$ compared to wt littermates and statistical significance of the increase in the overall survival as determined by the log-rank test is indicated in the figure.

Figure 3 with 1 supplement

Reduced incidence and delayed occurrence of tumours in female C/EBP $\beta^{\Delta uORF}$ mice.

(A) Tumour incidence of females as determined by pathological examination of neoplasms found upon necropsy of mice from the lifespan cohorts (wt, n= 50; C/EBP $\beta^{\Delta uORF}$, n=48). Statistical significance was calculated using Fisher's exact test with *p<0.05. (B) Tumour incidence of males as determined by pathological examination of neoplasms found upon necropsy (wt, n=47; C/EBP $\beta^{\Delta uORF}$, n=45. (C) Tumour occurrence in the female lifespan cohorts upon necropsy is shown for wt (black lines) and C/EBP $\beta^{\Delta uORF}$ mice (red lines). (D) Tumour occurrence in the male lifespan cohorts upon necropsy is shown for wt (black lines) and C/EBP $\beta^{\Delta uORF}$ mice (blue lines).

Figure 4 with 1 supplement

Ageing-associated increase in body weight, fat content and glucose tolerance is attenuated in female C/EBP $\beta^{\Delta uORF}$ mice.

(A) Body weight (g) of young (4 months) and old (19 months) female mice (wt, n=11 young, n=12 old; C/EBP $\beta^{\Delta uORF}$, n=11 young, n=12 old). (B) Body weight of young (4 months) and old (21 months) male mice (wt, n=12 young and old; C/EBP $\beta^{\Delta uORF}$, n=12 young, n=11 old). (C) Body fat content (cm³) as determined by CT analysis of young (4 months) and old (19 months) female mice (wt, n=11 young and old; C/EBP $\beta^{\Delta uORF}$, n=9 young, n=11 old). (D) Body fat content of young (4 months) and old (21 months) male mice (wt, n=12 young and old wt; C/EBP $\beta^{\Delta uORF}$, n=11 young, n=9 old). (E and F) i.p.-Glucose Tolerance Test (IPGTT) was performed with young (4 months) and old (female 19 months, male 21 months) wt and C/EBP $\beta^{\Delta uORF}$ (E) females and (F) males. The area under the curve (AUC) at the right shows the quantification (wt females, n=9 young, n=10 old; C/EBP $\beta^{\Delta uORF}$ females, n=10 young, n=11 old; wt males, n=12 young, n=11 old; C/EBP $\beta^{\Delta uORF}$ males, n=11 young, n=10 old). P-values were determined by Student's t-test, *p<0.05; **p<0.01; ***p<0.001.

Figure 5 with 1 supplement

Ageing-associated increase of the memory / naïve T-cell ratio is attenuated in C/EBP $\beta^{\Delta uORF}$ mice.

The ratio between Cd44^{high} memory T cells and Cd44^{low}/Cd62L^{high} naïve T cells in blood is shown for young (5 months) and old (female 20 months, male 22 months) (**A, B**) females and (**C, D**) males for both (**A, C**) Cd8⁺ cytotoxic and (**B, D**) Cd4⁺ helper T cells as was determined by flow cytometry (wt females, n=10 young, n=12 old; wt males n=12 young and old; C/EBPβ^{ΔuORF} females, n=10 young, n=12 old; C/EBPβ^{ΔuORF} males, n=12 young and old). P-values were determined by Student's t-test, *p<0.05; ***p<0.001.

Figure 6

Ageing associated loss of motor coordination and grip strength is attenuated in C/EBPβ^{ΔuORF} mice.

(**A**) Rotarod performance (time in sec of stay on the rotarod) of young (4 months) and old (female 19 months, male 21 months) wt and C/EBPβ^{ΔuORF} mice is shown separately for females (left) and males (right) (wt females, n=11 young, n=12 old; wt males, n=12 young and old; C/EBPβ^{ΔuORF} females, n=11 young, n=12 old; C/EBPβ^{ΔuORF} males, n=12 young, n=11 old). (**B**) The crossing time (sec) of the beam walking test of young and old wt and C/EBPβ^{ΔuORF} mice, and (**C**) the number of mistakes (paw slips) made while crossing the beam is shown separately for females and males (wt females, n=11 young, n=12 old; wt males, n=12 young and old; C/EBPβ^{ΔuORF} females, n=11 young, n=12 old; C/EBPβ^{ΔuORF} males, n=12 young, n=10 old). (**D**) Grip strength as determined with the wire hang test as hanging time (sec) of young and old wt and C/EBPβ^{ΔuORF} mice for females and males separately. N=11 for young wt and C/EBPβ^{ΔuORF} females and for old C/EBPβ^{ΔuORF} females and n= 12 for old wt females; n=11 for young and old wt males; n=12 for young C/EBPβ^{ΔuORF} males and n=10 for old C/EBPβ^{ΔuORF} males. P-values were determined by Student's t-test, *p<0.05; **p<0.01; ***p<0.001.

Figure 7 with 1 supplement

Ageing-associated increase of inter-individual variation of gene expression affects different genes in livers from wt and C/EBPβ^{ΔuORF} mice.

(**A-C**) Inter-individual variability of liver transcripts compared between (**A**) young (5 months) versus old (20 months) female wt mice, (**B**) young (5 months) versus old (20 months)

C/EBP $\beta^{\Delta uORF}$ female mice and **(C)** old wt (20 months) versus old C/EBP $\beta^{\Delta uORF}$ (20 months) female mice (n=6 for young and old wt and C/EBP $\beta^{\Delta uORF}$ for A,B,C). Coefficient of variation of transcripts with mean expression >1 FPM is plotted against the coefficient of variation of the other group as indicated. Dashed red line represents linear regression and is shifted towards the side that shows higher inter-individual variability. **(D)** KEGG pathway enrichment analysis of genes that show increased inter-individual variability in livers from old wt females compared to old C/EBP $\beta^{\Delta uORF}$ females (Coefficient of variation of wt genes is more than twice as the coefficient of variation of the same gene in C/EBP $\beta^{\Delta uORF}$ females) as indicated by the black bars or of genes that show increased inter-individual variability in livers from old C/EBP $\beta^{\Delta uORF}$ females compared to old wt C/EBP $\beta^{\Delta uORF}$ females (Coefficient of variation of C/EBP $\beta^{\Delta uORF}$ genes is more than twice as the coefficient of variation of the same gene in wt females) as indicated by the red bars. The x-axis indicates the p-value. Only pathways that show significant enrichment (FDR < 0.05) are shown.

Figure 8

Model explaining regulation of LIP under control of mTORC1.

(A) In wt mice C/EBP β -mRNA translation into LIP is modulated by calorie/nutrient availability through mTORC1 signalling, while expression of LAP is not affected. Together with other mTORC1-controlled pathways (Pathway X and Y) LAP/LIP expression ratio determines healthspan and survival. The different pathways may either be, modulated (C/EBP β), activated (Pathway X) or inhibited (Pathway Y) by mTORC1 and may have different sensitivities to mTORC1 modulators (e.g. rapamycin or nutrients), creating diversity in response (e.g. based on gender, genetic background or age). In addition LIP is upregulated by mechanisms during aging that are not well understood. **(B)** Genetic ablation of the C/EBP β -uORF prevents the mTORC1-dependent and/or age-associated upregulation of LIP and results in C/EBP β -dependent health- and lifespan extension. The C/EBP $\beta^{\Delta uORF}$ mutation mimics reduced mTORC1 signalling only at the level of LIP expression, leaving mTORC1 control of pathway X and Y unaffected.

1191 **Supplemental Table legends**

1192

1193 **Supplementary file 1 - Table 1**

1194 **Lifespan experiment summary of results.**

1195 ¹ Number of animals in the cohort

1196 ² Median survival of the cohort (days)

1197 ³ Increase of the median survival (percent)

1198 ⁴ P-value of the increased survival (log-rank test)

1199 ⁵ Mean lifespan of the cohort (days)

1200 ⁶ Standard error of the mean

1201 ⁷ Number of mice in the cohort in the longest-lived decile of the combined cohort (wt and

1202 C/EBP $\beta^{\Delta uORF}$)

1203 ⁸ P-value of increased N_{max}⁷ (Fisher's exact test).

1204

1205 **Supplementary file 2 - Table 2**

1206 **Tumour spectrum in wt and C/EBP $\beta^{\Delta uORF}$ mice.**

1207 *incl. lymphoid leukaemia

1208 **incl. malignant round cell neoplasms

1209 ***tumour type could not be unequivocally determined due to inadequate quality of the fixed

1210 tumour tissue

1211 Absolute numbers of mice with the indicated tumour type of tumours found during necropsy

1212 for wt and C/EBP $\beta^{\Delta uORF}$ males and females are shown. Note that the total number of tumours

1213 is higher than the number of tumour-bearing mice due to the eventual occurrence of different

1214 tumour types in the same mouse.

1215

1216 **Supplementary file 3 -Table 3**

1217 **Occurrence of ageing-associated pathologies in wt and C/EBP $\beta^{\Delta uORF}$ mice.**

1218 Mice were part of the ageing cohort and the age at analysis was 20 months for females and

1219 22 months for males.

1220 ¹ Number of animals showing the pathology (out of the total number of animals analyzed).

1221 ² mean grade of the pathology as calculated from the total number of animals analyzed with 0
1222 = absent, 1 = mild, 2 = moderate and 3 = severe.

1223 ³ Statistical significance of difference found between wt and C/EBP $\beta^{\Delta uORF}$ mice from the same
1224 gender as calculated using the Student's t-test (ns = not significant).

1225 ⁴ Mean number of regenerating muscle fibers found in five histological tissue slices per
1226 mouse. Note that a lower number is an indication for a more progressed ageing phenotype.

1227 ⁵ mean surface area of intramuscular adipose tissue in percent of the total area of analyzed
1228 skeletal muscle tissue as calculated from the total number of animals analyzed.

1229 ⁶ Trabecular bone parameters (percent bone volume/tissue volume; Trabecular number per
1230 mm; trabecular thickness and trabecular separation) measured by micro-CT analysis.

1231

1232 **Supplementary file 4 - Table 4**

1233 **GO-term analysis of genes upregulated in livers of old C/EBP $\beta^{\Delta uORF}$ mice.**

1234 Functional annotation of genes upregulated in livers of old C/EBP $\beta^{\Delta uORF}$ female mice
1235 compared to livers of old wt female mice (FDR < 0.01; 103 from 127 genes; 24 unknown IDs)
1236 using the DAVID database (Huang et al., 2009)

1237

1238 **Supplementary file 5 - Table 5**

1239 **GO-term analysis of genes downregulated in livers of old C/EBP $\beta^{\Delta uORF}$ mice.**

1240 Functional annotation of genes downregulated in livers of old C/EBP $\beta^{\Delta uORF}$ female mice
1241 compared to livers of old wt female mice (FDR < 0.01; 23 from 25 genes, 2 unknown IDs)
1242 using the DAVID database (Huang et al., 2009).

1243

1244 **Supplementary file 6 - Table 6**

1245 **GO-term analysis of genes showing high inter-individual variation between livers of old**
1246 **wt female mice.**

1247 Functional annotation of genes showing a high inter-individual variation between livers from
1248 old wt female mice. Coefficient of variation of transcript levels of the corresponding gene in
1249 the livers of wt mice is at least twice as big as the coefficient of variation of the transcript

levels of the same gene in the livers from C/EBP $\beta^{\Delta uORF}$ mice; 1386 from 1414 genes, 28 unknown IDs; using the DAVID database (Huang et al., 2009).

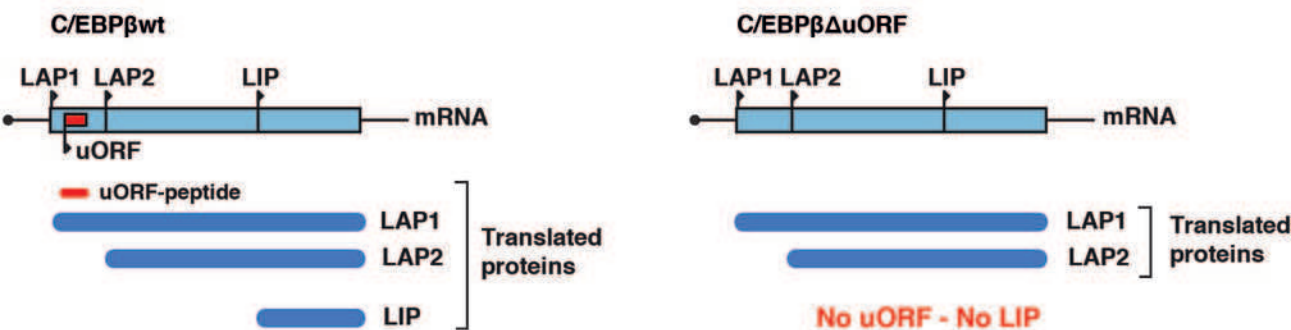
Supplementary file 7 - Table 7

GO-term analysis of genes showing high inter-individual variation between livers of old C/EBP $\beta^{\Delta uORF}$ female mice.

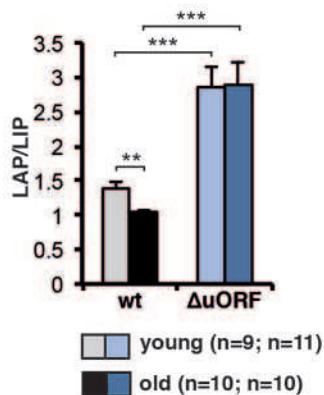
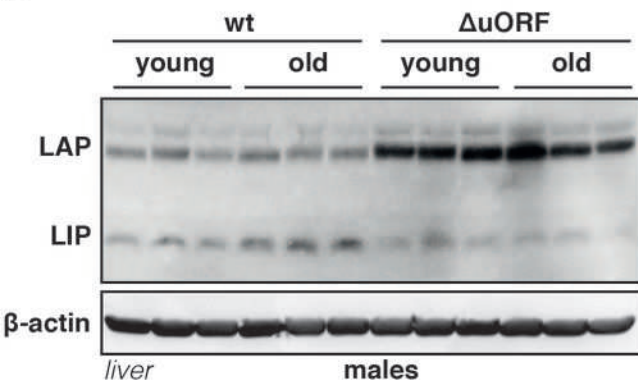
Functional annotation of genes showing a high inter-individual variation between livers from old wt female mice. Coefficient of variation of transcript levels of the corresponding gene in the livers of C/EBP $\beta^{\Delta uORF}$ mice is at least twice as big as the coefficient of variation of the transcript levels of the same gene in the livers from wt mice; 1354 from 1375 genes, 21 unknown IDs; using the DAVID database (Huang et al., 2009).

Figure 1

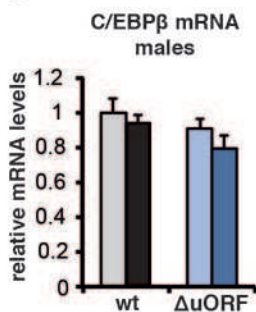
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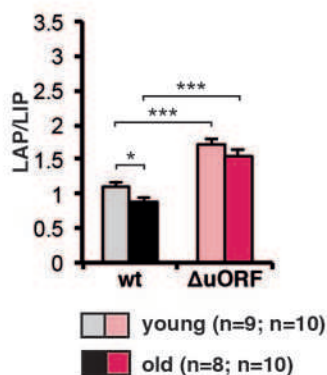
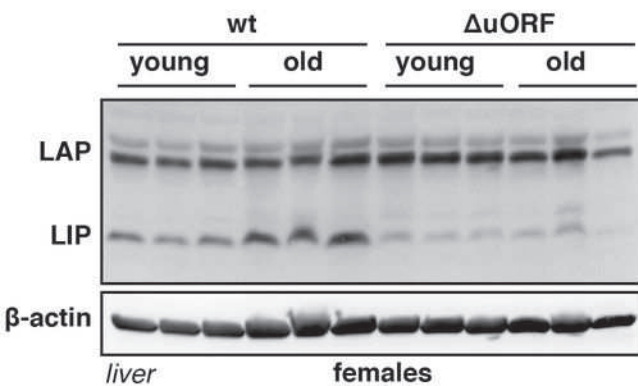
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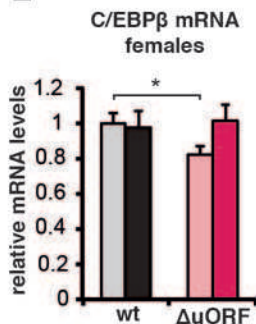


Figure 2

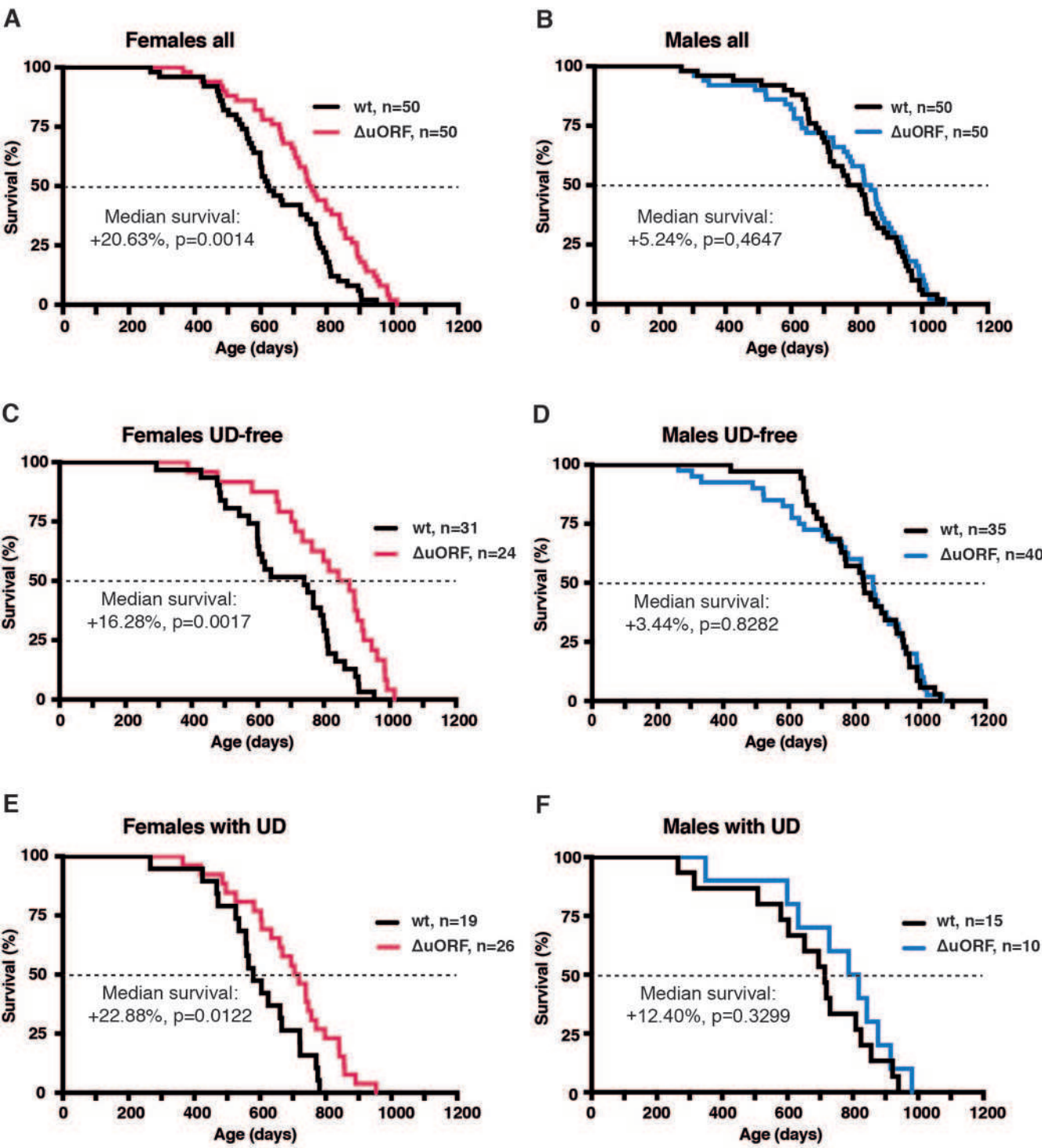
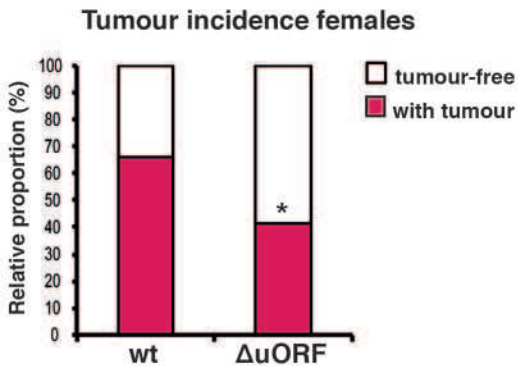
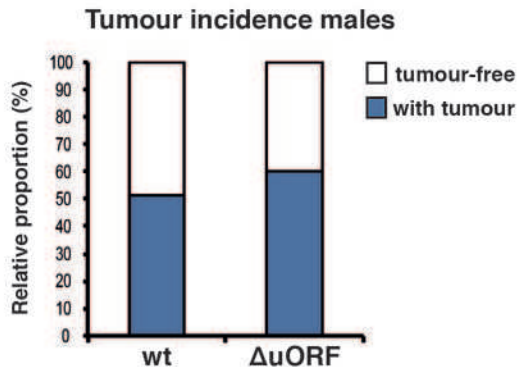


Figure 3

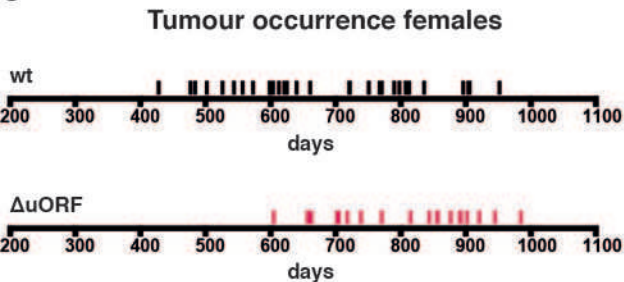
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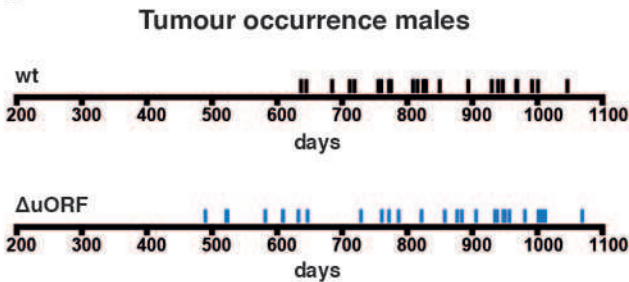


Figure 4

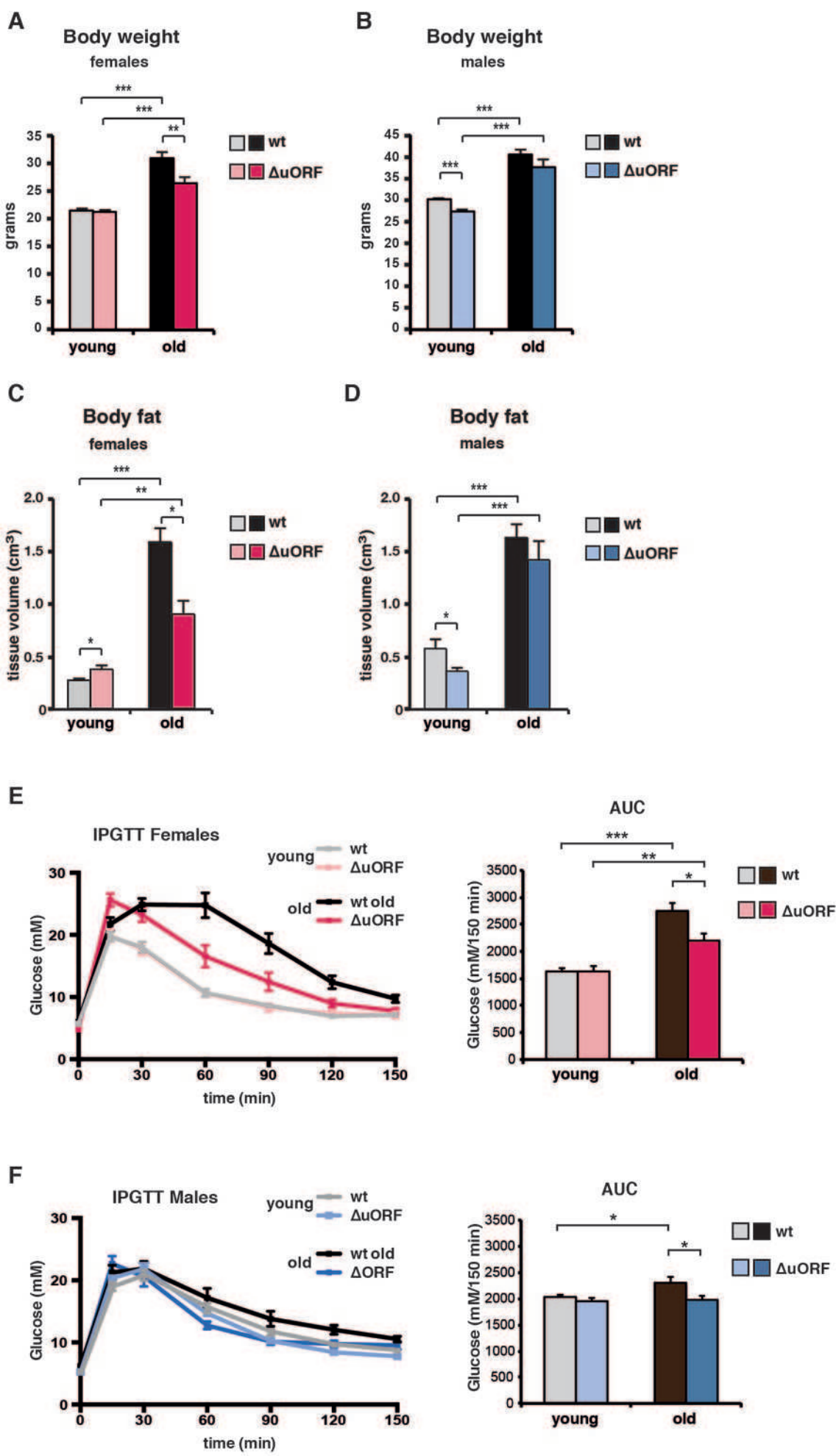
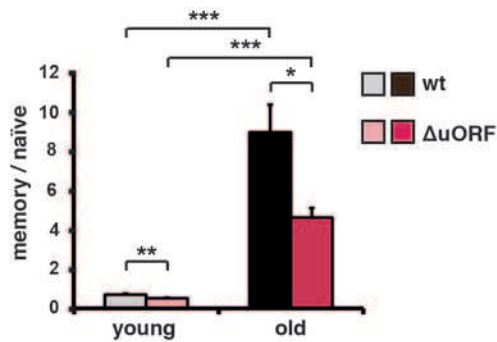


Figure 5

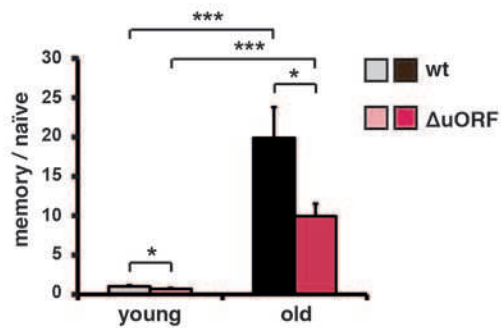
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Cytotoxic T cells
females



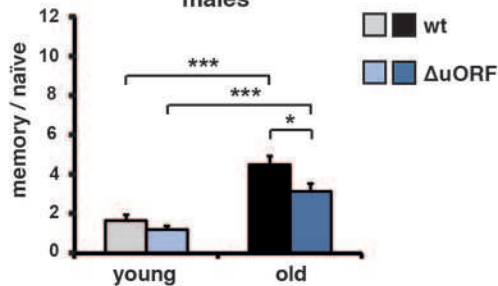
B

Helper T cells
females



C

Cytotoxic T cells
males



D

Helper T cells
males

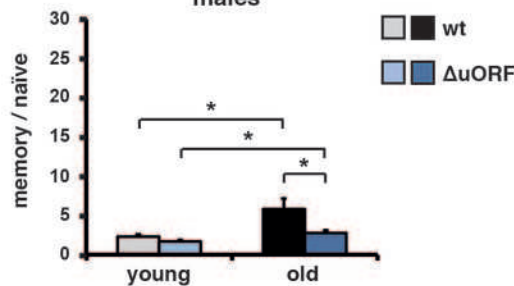


Figure 6

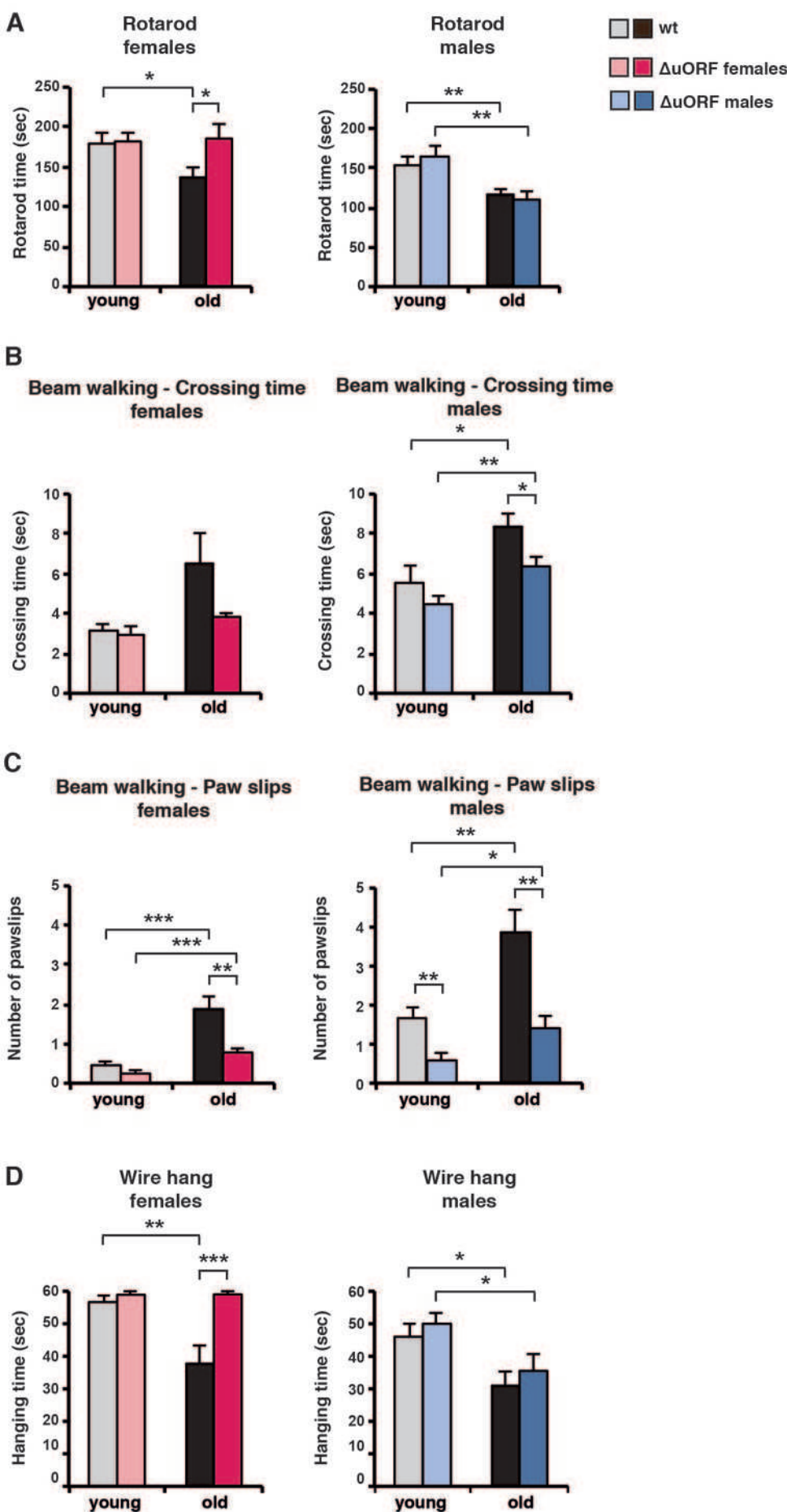
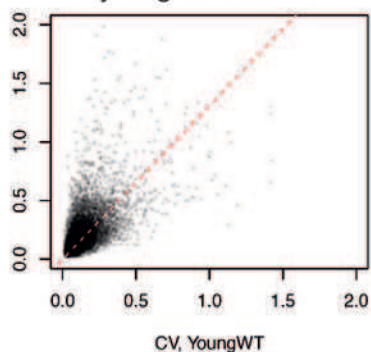
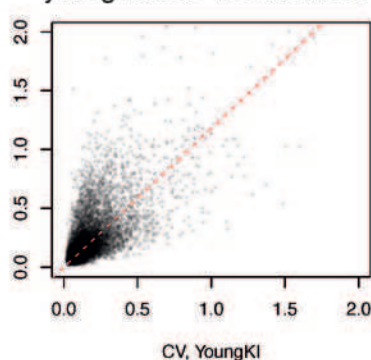


Figure 7

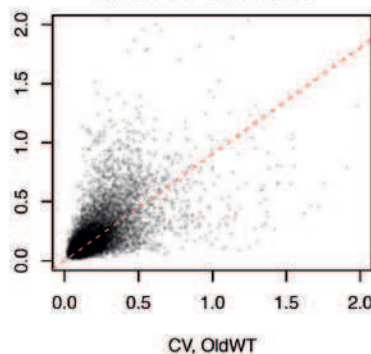
A

Interindividual variation
young wt vs old wt

B

Interindividual variation
young Δ uORF vs old Δ uORF

C

Interindividual variation
old wt vs old Δ uORF

D

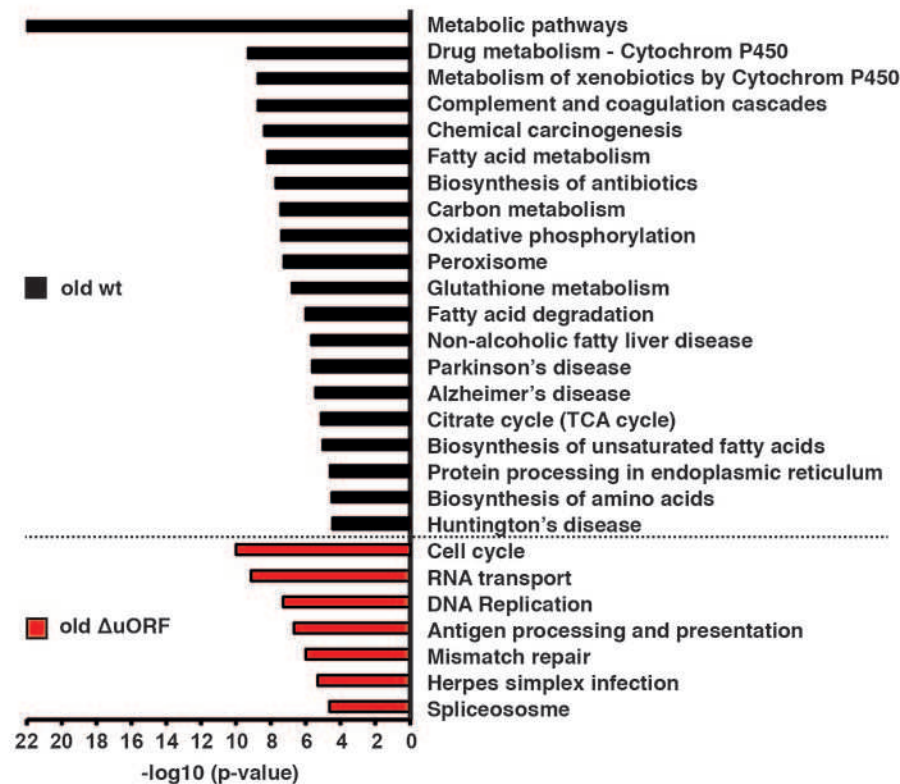
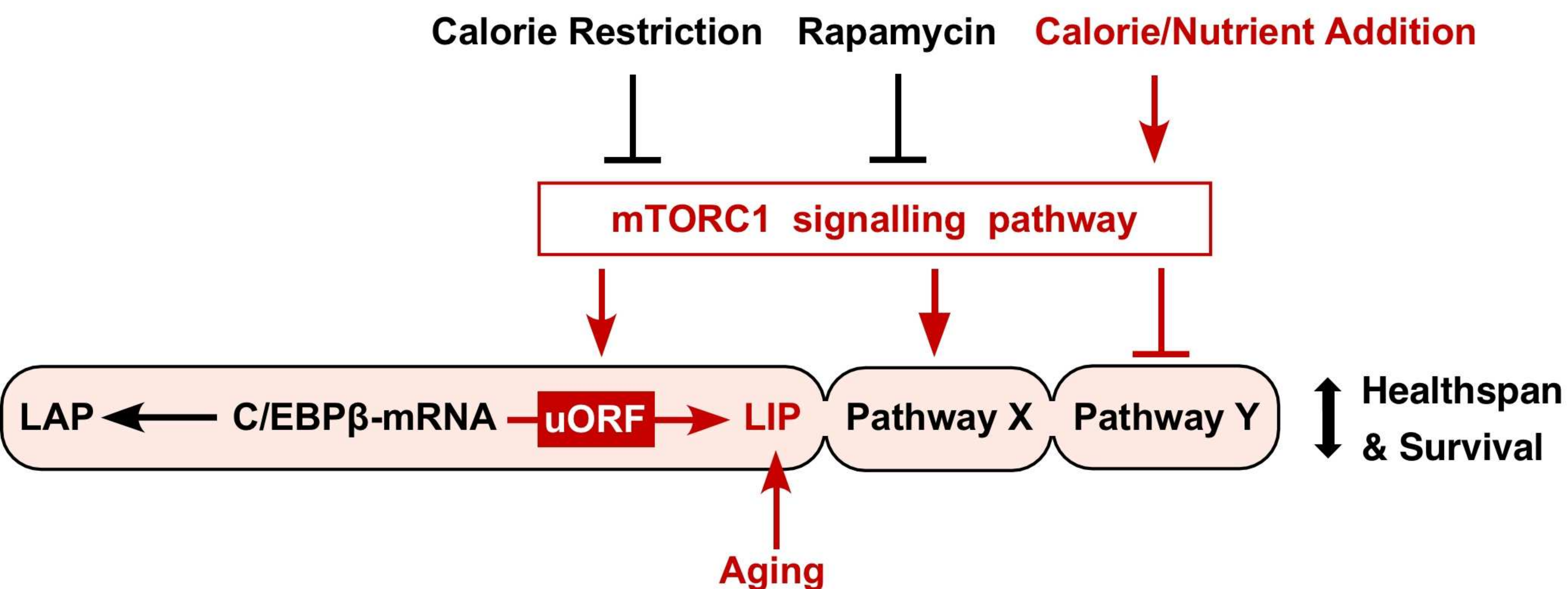
KEGG pathway enrichment
of genes with high inter-individual variation in old females

Figure 8

A. wt mice



B. C/EBPβ^{ΔuORF} mice

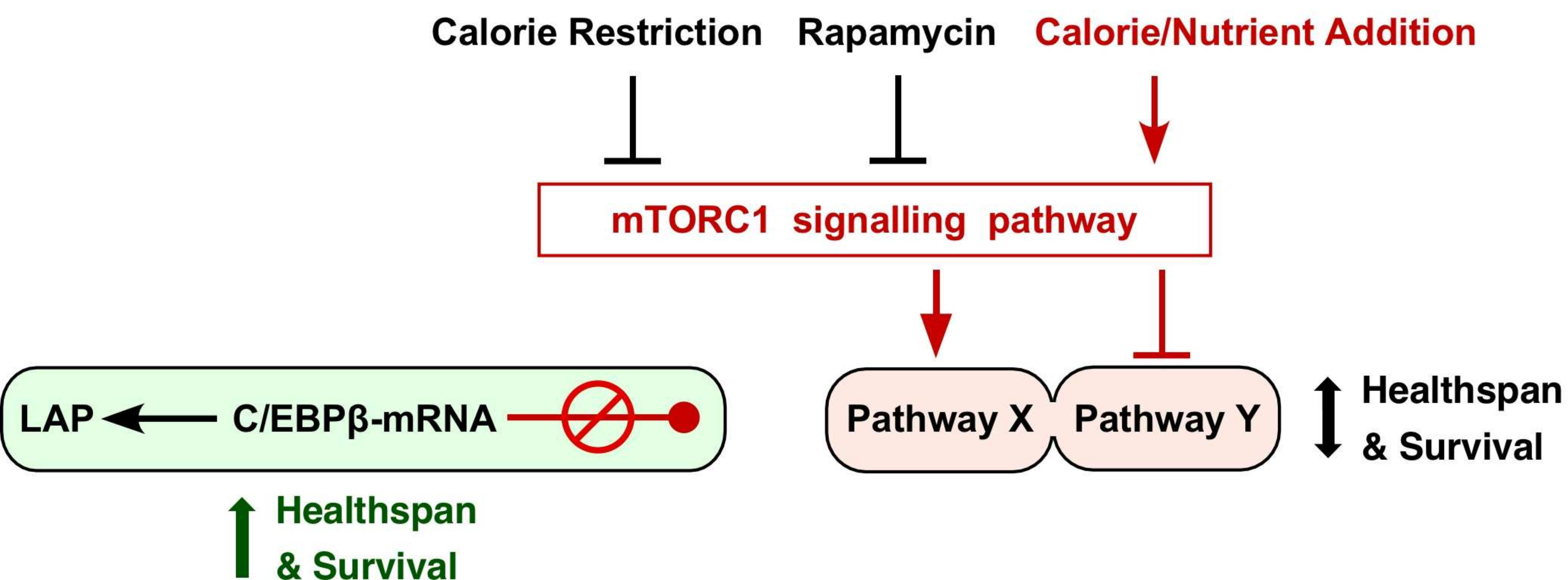
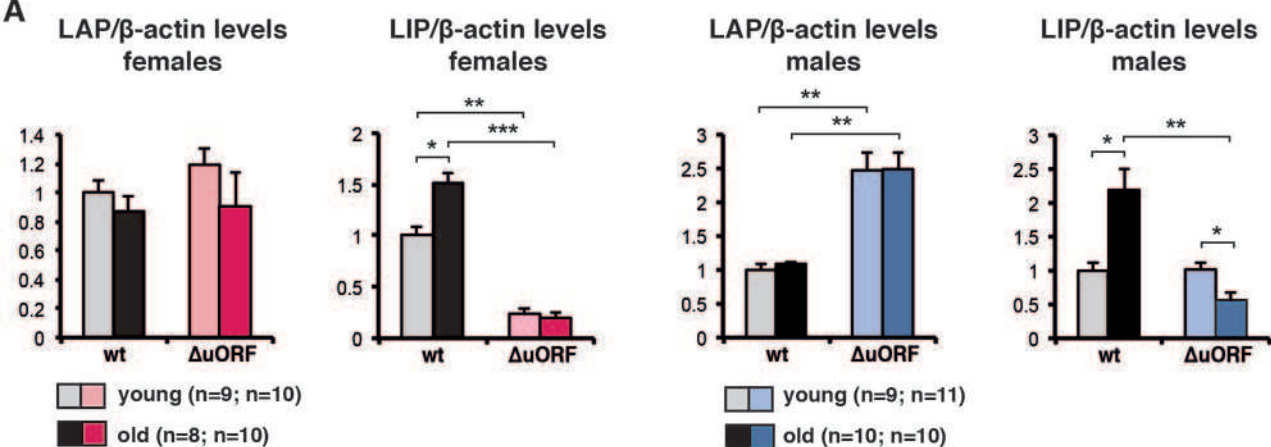
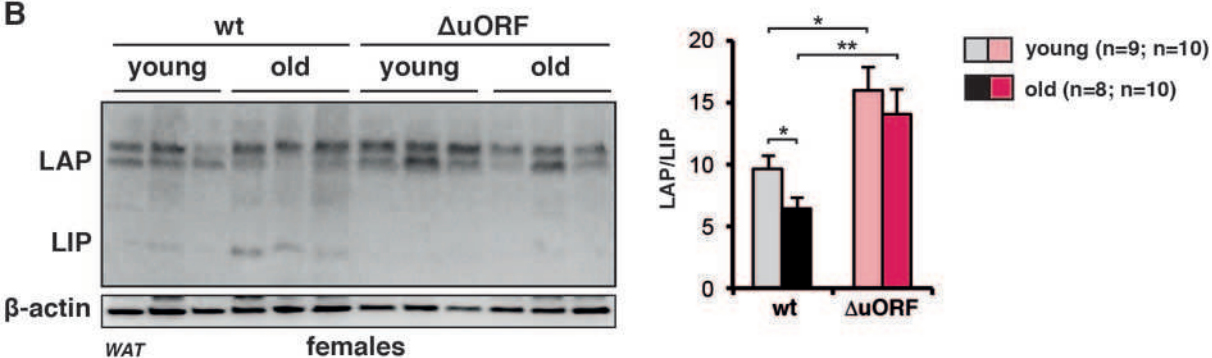


Figure1 - supplement 1

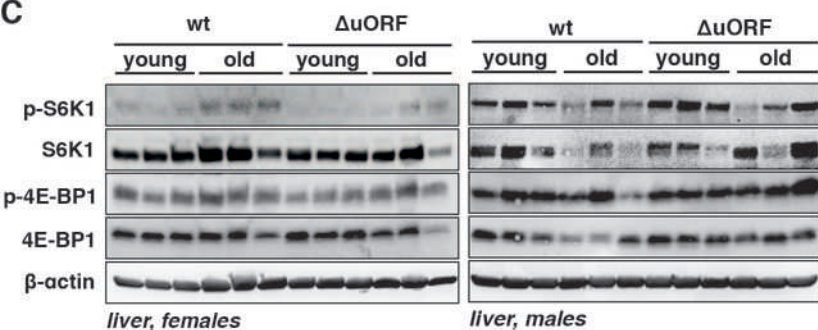
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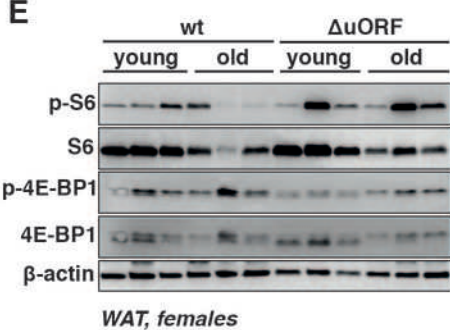
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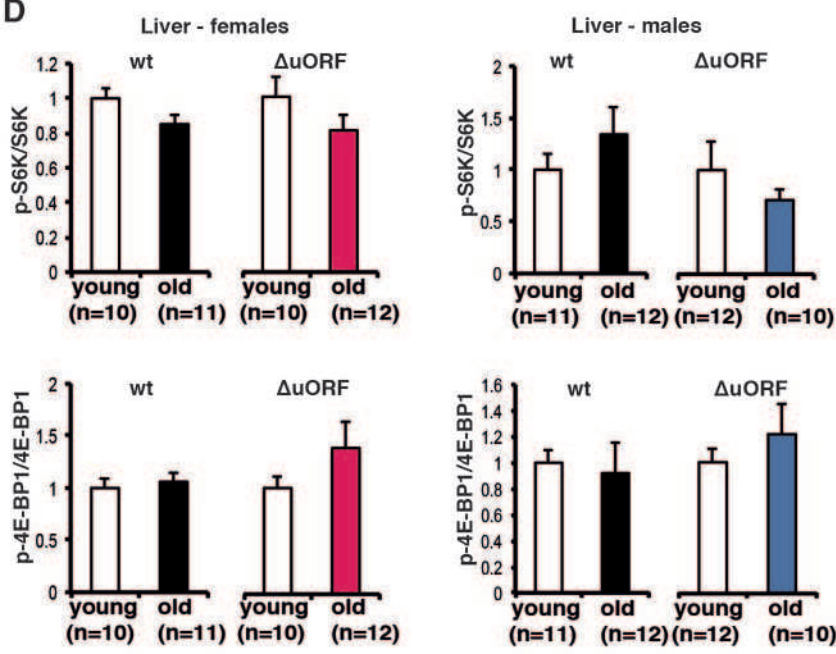
C



E



D



F

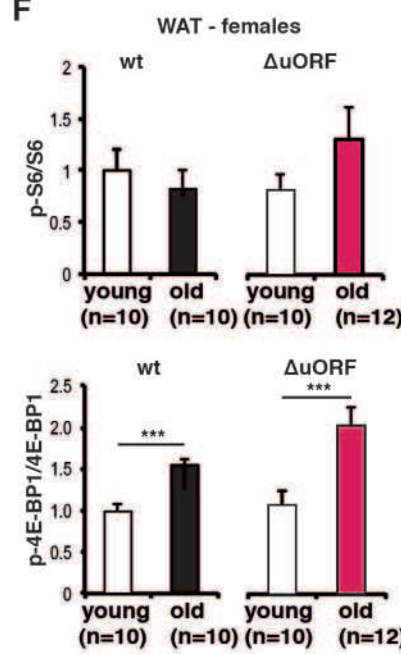


Figure 2 - supplement 1

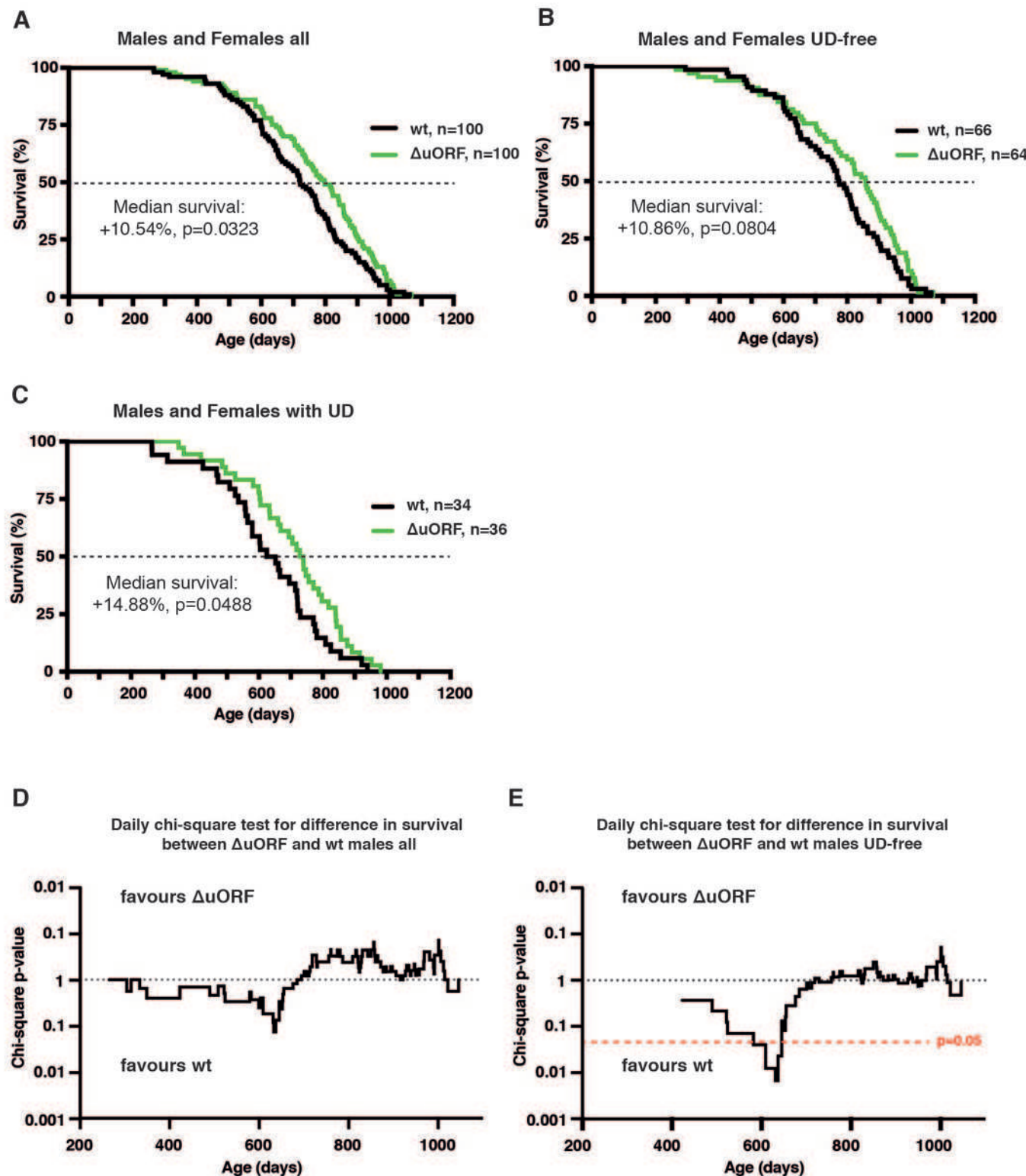


Figure 3 - supplement 1

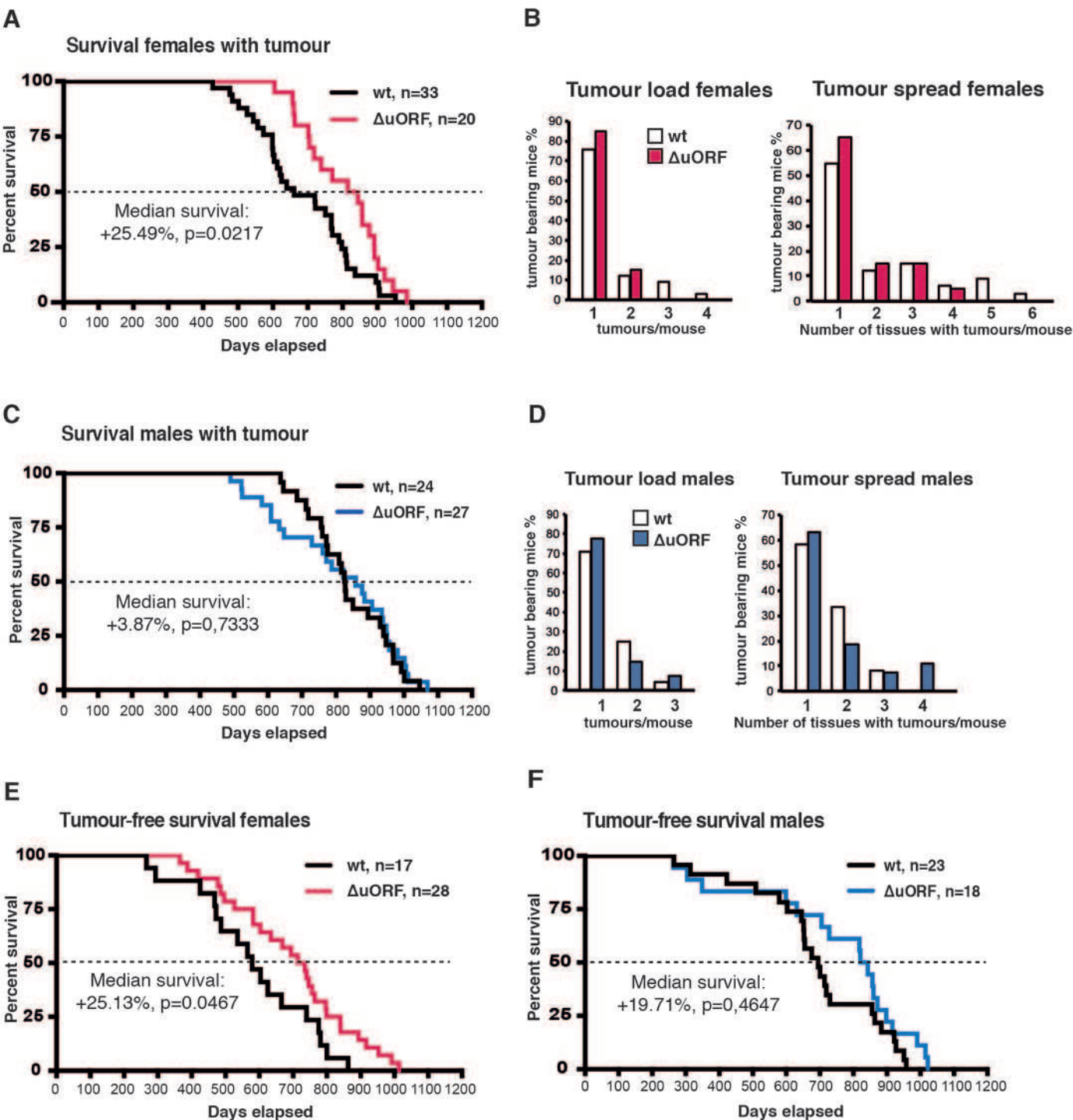
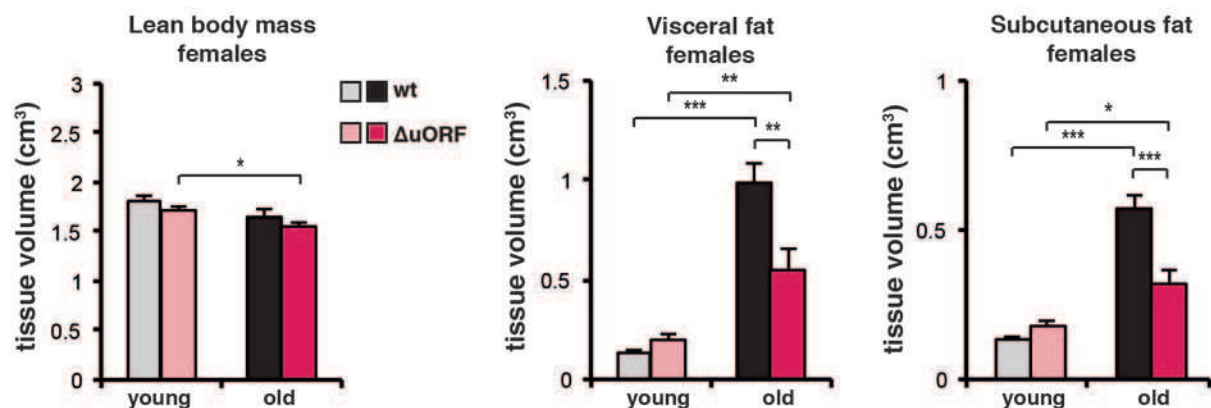
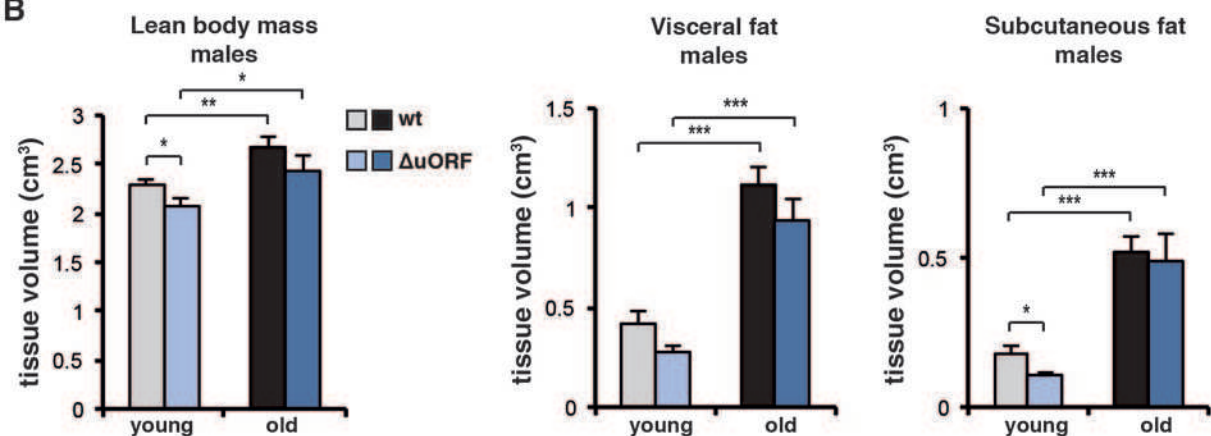


Figure 4 - supplement 1

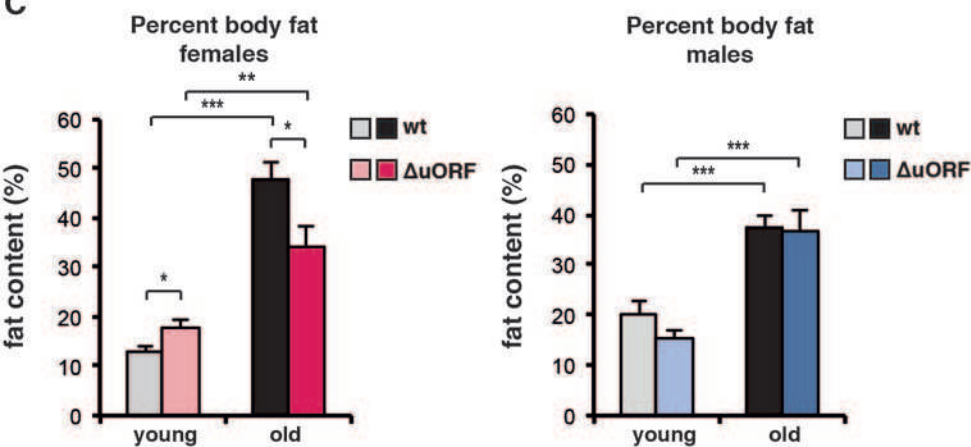
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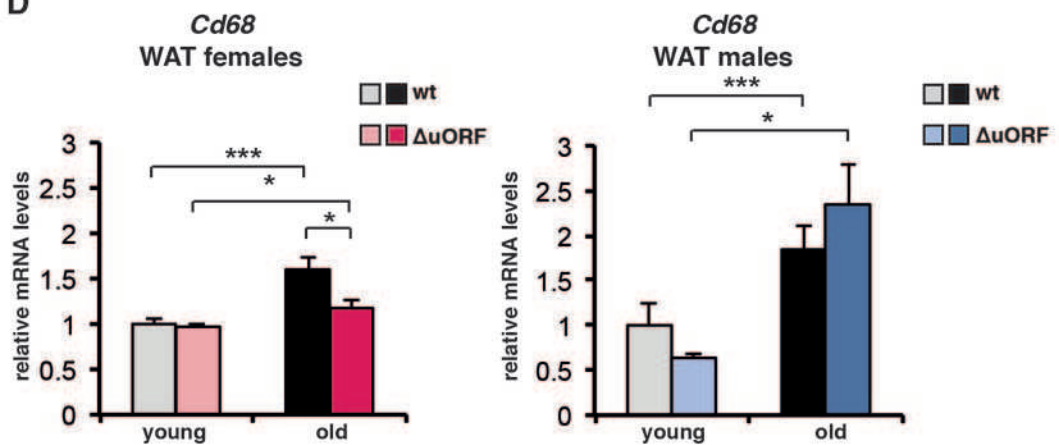
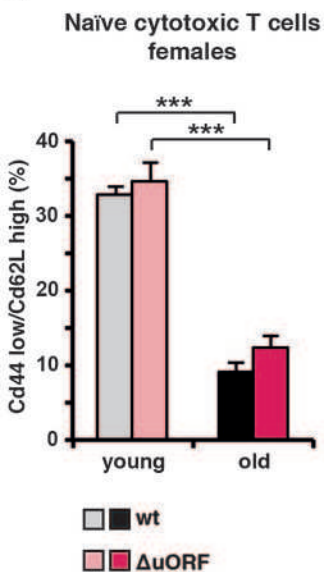
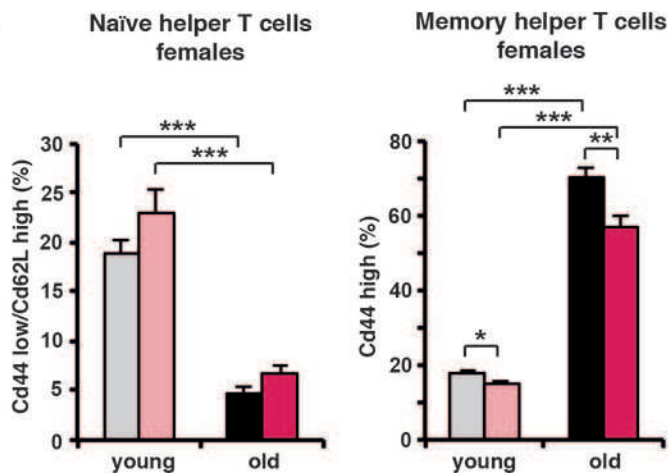


Figure 5 - supplement 1

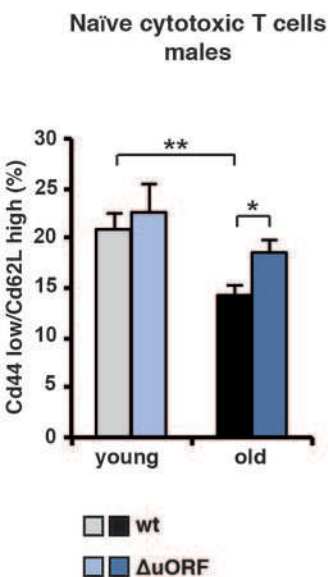
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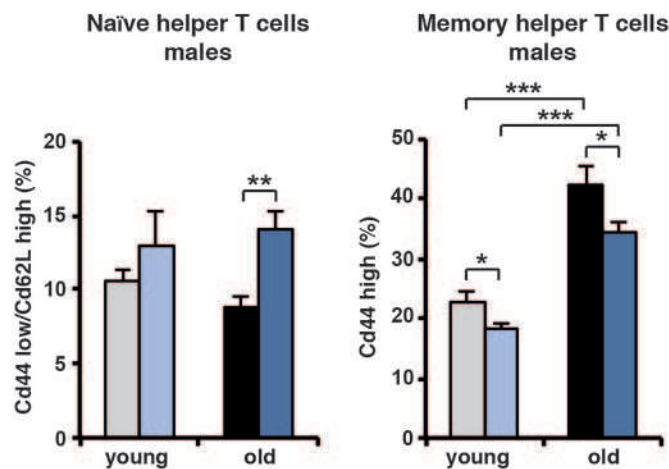


Figure 7 - supplement 1

