Lifespan Extension by Preserving Proliferative Homeostasis in *Drosophila*

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Abstract

Regenerative processes are critical to maintain tissue homeostasis in high-turnover tissues. At the same time, proliferation of stem and progenitor cells has to be carefully controlled to prevent hyper-proliferative diseases. Mechanisms that ensure this balance, thus promoting proliferative homeostasis, are expected to be critical for longevity in metazoans. The intestinal epithelium of *Drosophila* provides an accessible model in which to test this prediction. In aging flies, the intestinal epithelium degenerates due to over-proliferation of intestinal stem cells (ISCs) and mis-differentiation of ISC daughter cells, resulting in intestinal dysplasia. Here we show that conditions that impair tissue renewal lead to lifespan shortening, whereas genetic manipulations that improve proliferative homeostasis extend lifespan. These include reduced Insulin/IGF or Jun-N-terminal Kinase (JNK) signaling activities, as well as over-expression of stress-protective genes in somatic stem cell lineages. Interestingly, proliferative activity in aging intestinal epithelia correlates with longevity over a range of genotypes, with maximal lifespan when intestinal proliferation is reduced but not completely inhibited. Our results highlight the importance of the balance between regenerative processes and strategies to prevent hyperproliferative disorders and demonstrate that promoting proliferative homeostasis in aging metazoans is a viable strategy to extend lifespan.

Introduction

Lifespan of many organisms can be increased by optimizing both genetic and environmental conditions, including reducing caloric intake [1–3], increasing oxidative stress protection [4,5] and reducing Insulin/IGF1 signaling (IIS) [6–8]. These different interventions are likely to be acting through related mechanisms, notably by increasing stress-protective gene expression in differentiated somatic cells, prolonging their functional lifespan and delaying tissue degeneration [7,9–12]. In addition to such stress-protective mechanisms, metazoans also maintain tissue homeostasis through regenerative processes that rely on the long-term maintenance of a functional population of somatic stem and progenitor cells. For these cells, a similar, and perhaps more significant, relationship between stress protection and lifespan is expected, as their long-term maintenance is critical to conserve regenerative capacity. This relationship is complicated, however, by the fact that such cells are mitotically active, and their deregulation thus has the potential to promote dysplasia and increase the incidence of cancer [13,14]. Accordingly, mammalian stem cells generally exhibit a robust intrinsic ability to limit and repair intracellular damage [15–19], yet also employ strong anti-proliferative mechanisms that prevent cancer, but limit the regenerative capacity of stem cells in old age [18,20–22]. The regenerative decline of many tissues is thus caused by oxidative stress and DNA damage in stem and progenitor cells, as well as by cell-autonomous up-regulation of cell cycle inhibitors like p16, and by changes in the systemic environment [13,20,23–27]. Accordingly, processes that maintain the regenerative capacity of stem and progenitor cell populations, but prevent hyper-proliferation and cancer (i.e. processes that promote proliferative homeostasis), are expected to significantly influence longevity of the organism [28].

Recent studies in mouse hematopoietic stem cells (HSCs) indicate that the IIS pathway and its downstream transcription factor Foxo constitute an important regulatory system that controls stem cell stress protection while also influencing proliferation [8,29–32]. Foxo (Daf-16 in *Caenorhabditis elegans*) is repressed by IIS and is required for the lifespan extension observed when IIS activity is reduced either systemically, or specifically in adipose tissue [6–8]. Foxo induces the expression of genes involved in scavenging reactive oxygen species (ROS) and repairing damage to DNA and proteins, while also inducing cell cycle inhibitors [33–37]. Loss of Foxo in HSCs therefore results in increased proliferation of the HSC population, while boosting ROS levels and increasing apoptosis. As a consequence, the long term repopulating ability of HSCs is reduced [18,29,30,32].

*Drosophila* is emerging as a genetically tractable model to assess the importance of regeneration in lifespan and aging [38–43].
Recently identified somatic stem cells in *Drosophila* include intestinal stem cells (ISCs) in the posterior midgut epithelium, as well as stem cells in malpighian tubules [44] and the hindgut [45,46]. ISCs are critical for regeneration and maintenance of the midgut epithelium [47–50]. These cells are characterized by the expression of the marker genes escargot and Delta, and divide asymmetrically to give rise to a new ISC and an Enteroblast (EB) that differentiates into one of two cell types: Enterocytes (ECs) and Enteroendocrine cells (EEs). In contrast to the mammalian lineage, no transit amplifying cell population exists in *Drosophila*; ISC being the only dividing cell type in the midgut epithelium [47–49]. In young animals, ISCs divide rarely, as less than 5 mitoses can be observed at any given timepoint in the intestine [42,47–49]. In response to stressful challenges, however, ISC proliferation is strongly increased, a regenerative response that allows restoring large parts of the intestinal epithelium in response to damaging agents, such as pathogens, genotoxins, or ROS inducing compounds [42,43,50–53]. Interestingly, this regenerative function of ISCs can have deleterious consequences for the organism, as excessive proliferation of ISCs in response to stress is accompanied by the accumulation of mis-differentiated cells in the intestine, which ultimately disrupts epithelial integrity with a dysplastic phenotype [43]. In the aging gut, such dysplasia is accelerated and can be visualized by their continuous expression of the ISC/EB marker escargot (esg; [42,43,54]). This dysplastic phenotype is readily observed in old flies expressing GFP under the control of the esgGal4 driver, and can be classified into four distinct categories that correlate with the frequency of pH3+ cells per gut and thus serve as an accessible quantitative criterion for intestinal dysplasia within a fly population (Figure 1B and Text S1). Importantly, age-related dysplasia is not accompanied by aberrant proliferation of ISC daughter cells, as confirmed by

**Results**

**Intestinal regeneration influences lifespan**

Recent studies suggest a significant influence of intestinal regeneration on fly viability. Flies in which intestinal dysplasia is accelerated are short lived [43], while animals with impaired ISC proliferation or daughter cell differentiation die faster when infected by enteropathogenic bacteria than wild-type flies [50,54]. These observations indicated that genetic conditions in which intestinal homeostasis is preserved might result in increased lifespan.

To start testing this hypothesis, we first tested the requirement of ISC-mediated tissue renewal for optimal lifespan. Ectopic activation of Notch signaling in ISCs was previously shown to irreversibly impair their function by promoting differentiation [43,49]. In order to abolish ISC function, we thus transiently expressed an activated form of Notch (IntraCellular Domain; NICD) in ISCs and EBs using the esgGal4 driver. In young adult esgGal4 heterozygous flies, Gal4 activity is restricted to ISCs and EBs in the intestine, to malpighian tubule stem cells, as well as to the testis and salivary glands, and is not detected in other tissues (Figure S1). To prevent developmental effects of the expression of UAS-driven transgenes, we used a heat-inducible system in which esgGal4 is combined with a temperature-sensitive Gal80 (TARGET system, [55]; Figure S2D). Transient expression of NICD for 7 days in young flies, significantly shortens lifespan (Figure S2A), supporting the notion that maintaining somatic stem cell function is critical for optimal lifespan. Importantly, longevity is not significantly affected when these flies are kept at a permissive temperature throughout life, confirming that lifespan shortening is caused by transient adult expression of NICD, and not by ectopic expression of the protein during development (Figure S2B; further confirming the selective inducibility of the employed TARGET system, UAS-linked transgene expression is detectable in esgG4, tubGal800′ flies only at the restrictive temperature, 29°C, Figure S2D, S2E).

We next assessed the relationship between ISC proliferation rates, intestinal dysplasia and lifespan (Figure 1). In wild-type flies, the number of dividing ISCs detectable at a given timepoint (as measured by the number of pH3+ cells) increases 10-fold between 3 days and 30 days of age when reared at 25°C or between 3 days and 18 days of age when reared at 29°C (Figure 1A, [42]). This increase is accompanied by a progressive accumulation of polyploid, mis-differentiated cells that accumulate at the basal membrane of the epithelium and can be visualized by their continuous expression of the ISC/EB marker escargot (esg; [42,43,54]). This dysplastic phenotype is readily observed in old flies expressing GFP under the control of the esgGal4 driver, and can be classified into four distinct categories that correlate with the frequency of pH3+ cells per gut and thus serve as an accessible quantitative criterion for intestinal dysplasia within a fly population (Figure 1B and Text S1). Importantly, age-related dysplasia is not accompanied by aberrant proliferation of ISC daughter cells, as confirmed by
analysis of pH3+ cell frequencies and clonal growth rates in individual marked ISC lineages in old flies or in stress conditions (Figure S3). This is consistent with previous ISC lineage analysis demonstrating that no transit amplifying population of cells exist in the Drosophila midgut [48]. The frequency of pH3+ cell numbers in the gut is thus a direct measure of ISC proliferation rates.

To influence intestinal proliferation rates in aging flies, we modulated the activities of the JNK or IIS pathways in ISCs and EBs using the esgGal4 driver. Activation of both pathways with this driver increases ISC proliferation [43,53,54]. We activated or inhibited JNK by expressing the JNK Kinase Hemipterous (Hep) or dsRNA against the JNK Bsk (BskRNAi), respectively, or activated...
Reduced age-associated dysplasia in long-lived IIS loss-of-function conditions

To test this idea, we first evaluated proliferative homeostasis in long-lived fly populations (Figure 2). Intestinal dysplasia in non-labeled intestines can be quantified by defining categories based on the extent of BrdU incorporation in the intestinal epithelium (reflected both ISC divisions and endoreplication of daughter cells), and the loss of tissue architecture observed when staining with the membrane marker armadillo (arm, Figure 2A, see Text S1). Since reduced IIS activity extends lifespan in flies, we assessed whether long-lived fly lines with reduced IIS activity would exhibit delayed dysplasia. Indeed, limiting IIS activity systemically reduces the age-associated increase in the frequency of pH3+ cells, as well as the increase in intestinal BrdU incorporation and the loss of epithelial architecture in the gut (Figure 2B and 2C). IIS activity was reduced by ablating insulin-producing cells (IPCs) through expression of the pro-apoptotic gene reaper (rpr) under the control of dilp2Gal4, by reducing the gendose of the insulin receptor substrate-homologue Chico, or in trans-heterozygotes for the dilp2Gal4, by reducing the genedose of the insulin receptor +/− (Figure 2A, see Text S1). Flies with all three genetic conditions are robustly long-lived [56–59], suggesting that the reduction in intestinal dysplasia observed here is associated with longevity.

Interestingly, in these long-lived lines, IIS activity is reduced, but not absent, since the insulin receptor can signal directly to PI3K, bypassing the requirement for Chico [60], and ablation of IPCs results in loss of selected insulin-like peptides, whereas insulin-like peptides expressed in other tissues, such as the fatbody and germline, are retained [61–64]. Accordingly, the average number of pH3+ cells decreased significantly, but moderately, indicating that in these long-lived animals, proliferative homeostasis is preserved without negatively impacting regenerative capacity (Figure 2C and 2D).

Repression of IIS in the ISC lineage inhibits ISC proliferation and shortens lifespan

The Insulin signaling pathway has wide-ranging functions in growth, metabolism, and reproduction [6–8,65–68]. The observed correlation between the extent of age-related dysplasia and lifespan in the genetic conditions tested above could thus be a secondary consequence of other physiological changes. To test more directly whether impairing IIS activity in the ISC lineage would influence age-related dysplasia and affect lifespan, we over-expressed a dominant-negative Insulin receptor (InRDN; [69]), dominant-negative PI3K (PI3KDN) [70]), a dsRNA targeting the IIS downstream kinase Akt [AktRNAi, Figure S4], as well as wild-type Foxo under the control of heat-inducible esgGal4 (esgGal4, tubGal80°). In all four cases, we observed strongly reduced age-
related dysplasia of the intestinal epithelium, confirming that IIS activity in ISCs is required for the age-related over-proliferation of these cells (Figure 3A). However, this reduction was as strong as when JNK was repressed by expression of BskRNAi, suggesting that impaired regeneration in these guts might also limit viability and reduce lifespan. We tested this prediction and found indeed that expression of Foxo, AktRNAi, or DPP110DN under the control of esgGal4, Gal80R (at 29°C) or of Foxo under the control of esgGal4 (25°C) caused significant lifespan shortening (Figure 3B–3D, Figure S3, Table S2, Table S3).

Reduced IIS activity in the ISC lineage thus shortens lifespan most likely by preventing regeneration. Supporting this interpretation, we found that ISC clones (induced by somatic recombination using the MARCM system; [71,72]) homozygous for InR<sup>Es19</sup> or InR<sup>Es25</sup>, or over-expressing Foxo, have a strongly reduced ability to grow (and thus to generate newly differentiated ECs and EEs, Figure 3E). The extent of this growth repression was significantly more severe than in chico<sup>Es1</sup> homozygous mutant clones (Figure 3E). Interestingly, InR<sup>Es19</sup> homozygous mutant flies are short-lived (as opposed to InR<sup>Es19</sup>/InR<sup>Es25</sup> transheterozygotes; [56]), further strengthening the notion that impaired regeneration of the intestinal epithelium of these flies is associated with shorter lifespan.

**Lifespan extension by limiting IIS and JNK signaling in somatic stem cells**

Taken together, the results described above support the notion of a critical relationship between proliferative homeostasis, regeneration and lifespan: reduced ISC proliferation, and thus limited age-related dysplasia (as in dli<sup>1-2pr</sup>, chico<sup>Es1</sup> homozygotes and InR<sup>Es19</sup>, InR<sup>Es25</sup> transheterozygotes), is beneficial, while impaired ISC proliferation, and thus reduced regenerative capacity (as in InR<sup>Es19</sup> homozygotes or in flies over-expressing Foxo in the ISC lineage), shortens lifespan. This relationship can be illustrated by comparing relative lifespan with the fraction of flies with low, intermediate or high frequencies of ISC proliferation at 30 days (reared at 25°C) for the genotypes discussed above (Figure 3F).

To test this model more directly, and to confirm that improved proliferative homeostasis is sufficient to extend lifespan, we repressed IIS and JNK activities in ISCs and EBs using 5961 Geneswitch-Gal4 (5961GS; Figure 4, [73]). This RU486-inducible driver recapitulates the esgGal4 expression pattern in the intestine, albeit at much lower levels, allowing moderate repression of IIS and JNK activities in an RU486-dependent manner ([73], Figure 4A–4C; we assessed GFP expression under the control of 5961GS in various tissues, and observed weak RU486-dependent induction only in the intestine, Figure S6). Female flies expressing InR<sup>DN</sup>, DPP110<sup>DN</sup>, Akt<sup>RNAi</sup>, Bsk<sup>DN</sup>, or Bsk<sup>RNAi</sup> under the control of this driver show moderately, but significantly, reduced intestinal proliferation at old age (Figure 4D–4J). Importantly, these flies are significantly longer lived when exposed to RU486 than isogenic siblings exposed to mock treatment (median lifespan extended at least 10% for all conditions); whereas control flies show almost no RU486-dependent change in longevity (1% change in median lifespan; Figure 4D–4K and Table S4; expression of the same transgenes in males resulted in no significant lifespan effect, not shown). All together, these results strongly support the model outlined above.

**Expression of stress-protective genes in the ISC lineage limits age-associated dysplasia**

IIS and JNK signaling activities thus have to be carefully balanced to maintain intestinal homeostasis and regenerative capacity. This balance will ultimately influence the expression of Foxo target genes, which encode stress-protective proteins as well as cell cycle inhibitors and pro-apoptotic factors that are expected to have antagonistic consequences for stem and progenitor cell maintenance and proliferation. Accordingly, a critical and pleiotropic function of Foxo proteins in stress-protection, proliferation and apoptotic control of stem cells has been described for the hematopoietic system in mice [10,29–32]. Selectively increasing the expression of stress-protective Foxo target genes in the ISC lineage might thus be sufficient to limit age-related dysplasia without impairing regeneration, thus recapitulating the consequences of organism-wide moderate reduction of IIS activity, and potentially extending lifespan.

To test this hypothesis, we used esgGal4 to express Hsp68, a heatshock protein that extends lifespan when expressed in the whole fly [74], and Jafrac1, a peroxiredoxin that detoxifies ROS and can increase lifespan when expressed in the brain [75,76]. Both genes are Foxo targets ([Figure S7; [75]) and strikingly, we found that both caused a significant delay in dysplasia (both in the posterior midgut, as well as when assessing invasion of the proventriculus by GFP-positive cells; Figure 5A and Figure S6), accompanied by moderate reduction in the frequency of pH<sup>3</sup> cells in the gut (Figure 5B). Since dysplasia in aging intestinal epithelia is accompanied by increased expression of Di [43], we further tested the expression of Di in these intestines and found a significant decrease in Di accumulation compared to wild-type animals, confirming that the accumulation of mis-differentiated ISC progeny in these flies is reduced (Figure 5C).

**Improved metabolic homeostasis in flies expressing stress-protective genes in somatic stem cell lineages**

Dysplasia in the aging intestinal epithelium is expected to cause defects in nutrient absorption, resulting in deficient nutrient stores in the organism and disrupting metabolic homeostasis. Since hsp68 and jafrac1 expression in ISCs and their daughter cells significantly delays intestinal dysplasia, we tested whether the maintenance of metabolic homeostasis was improved in these flies. The amount of free glucose, triglycerides and glycogen stored by old wild-type flies is significantly reduced compared to young animals (Figure 5D). When Jafrac1 or hsp68 were expressed under the control of esgGal4, however, high levels of these nutrient stores were maintained in aging flies. This rescue of metabolic homeostasis correlates with increased starvation tolerance (Figure 5E), further supporting the idea that maintenance of intestinal homeostasis by protecting somatic stem cells is critical for metabolic health of aging flies.

**Lifespan extension by stress-protective gene expression in stem cell lineages.**

To assess whether over-expressing stress-protective genes using the esg-Gal4 driver would be sufficient to extend lifespan, we compared demographics of multiple independent populations of flies expressing jafrac1 and hsp68 under the control of esgGal4 to isogenic wild-type controls. Strikingly, we observed consistent and significant lifespan extension in both males and females when jafrac1 and hsp68 were expressed (Figure 6A and 6B; Table S5). To exclude that the esgGal4-driven expression of stress-protective genes in salivary glands is causing the observed lifespan extension, we also tested the lifespan of flies in which hsp68 was expressed using GMR-Gal4, an eye-specific driver that also expresses Gal4 in salivary glands, and found no effect (Figures S1B, S1C, S9). We further confirmed the beneficial consequences of Jafrac1 and Hsp68 expression on lifespan using the weaker 5961GS driver, and found moderate but significant extension of lifespan in flies expressing both transgenes (Figure 6C, Table S6). Evidently, expressing selected
stress-protective Foxo target genes in the ISC lineage is sufficient to recapitulate the effects of reducing IIS or JNK activity in these cells.

**Discussion**

Our results indicate that proliferative homeostasis in high turnover tissues is limiting for *Drosophila* lifespan and highlight the importance of mechanisms that balance pro- and anti-mitotic activities. In the ISC lineage, this balance involves fine-tuning the activities of the pro-mitotic IIS and JNK signaling pathways to ensure appropriate supply of newly formed ISC daughter cells while limiting dysplasia. Accordingly, we observe moderately reduced intestinal proliferation rates in long-lived IIS mutants, as well as lifespan extension when IIS or JNK signaling are moderately reduced in the ISC lineage. This association between proliferative activity in the intestinal epithelium and lifespan is illustrated in Figure 7. Strikingly, intestinal proliferation rates correlate with relative lifespan over a wide range of genotypes.

Our results further show that the stress-protective components of the Foxo-regulated gene expression program are sufficient to maintain proliferative homeostasis, extending lifespan of the organism. Reduction of IIS activity, which extends lifespan in many organisms, is thus accompanied by the preservation of regenerative processes. While reducing IIS activity or activating Foxo in adipose tissue is sufficient to extend lifespan of flies, mice and worms [63–68], our results suggest that the anti-proliferative and stress-protective consequences of Foxo activation in high-turnover tissues also contribute to lifespan extension in IIS loss-of-function conditions. Interestingly, a tumor-suppressing role for Foxo in mice and *C. elegans* has been reported [77–79], while Foxo regulates redox homeostasis in mouse HSCs [18,29,30,32,77]. Reduced IIS activity thus optimizes somatic maintenance, metabolism and regenerative processes in complex metazoans, and all three physiologic consequences of IIS repression seem to contribute to achieve maximum lifespan.

It remains to be tested whether the lifespan extension commonly observed in flies exposed to dietary restriction (DR) is also associated with delayed intestinal dysplasia. Reduced IIS activity contributes to lifespan extension in DR conditions [80–82] suggesting that reduced intestinal dysplasia might contribute to DR-induced lifespan extension.

Interestingly, the effects of JNK signaling on lifespan are more complex. JNK can extend lifespan when activated in the brain by repressing the expression of insulin-like peptides [37,74,83,84], thus systemically repressing IIS activity [37,74]. Our findings reported here, however, show that JNK activation in the ISC lineage can have deleterious effects and needs to be limited to ensure longevity. Such pleiotropic consequences of JNK have also been reported in other contexts and have significant implications for the development of therapies targeting this pathway [85–87].

The importance of anti-oxidant Foxo target genes in regulating proliferative homeostasis highlights the challenging environment to which the intestinal epithelium is exposed. Apart from extraneous toxins and oxidants, the intestinal epithelium also mounts strong oxidative responses to inflammation, potentially exposing ISCs and daughter cells to high levels of oxidative stress [50,52,88-91]. Our results show that over-expressing stress-protective proteins in the ISC lineage is sufficient to limit and optimize cellular responses to these challenges, thus preserving intestinal homeostasis longer (but not indefinitely, as a significant fraction of these animals do develop dysplasia at older ages).

Intestinal dysplasia is caused by over-proliferation of ISCs in concert with mis-differentiation of ISC progeny, and in long-lived animals both processes are prevented. Due to technical limitations of the Gal4 drivers used, however, we cannot exclude that the expression of stress-protective genes, or of IIS or JNK repressors with esgGal4 or 5961GS affects primarily the differentiation process of EBs rather than the ISC itself. While the inability of IIS mutant clones to grow, and the significant reduction in the number of pH3+ cells in IIS and JNK loss-of-function conditions and in Hsp68 and Jafrac1 over-expressing flies, demonstrates that ISC proliferation is indeed influenced by these manipulations, it is conceivable that this effect might be mediated by indirect, non-cell-autonomous limitation of ISC proliferation by EBs in these conditions. Such a feedback control of ISC division would be interesting, and further studies are needed to test this possibility.

The pattern of esgGal4 and of 5961GS expression further requires considering effects of IIS and JNK activities in other tissues on lifespan: While we can exclude the testes and salivary glands as sources of the observed effects (lifespan effects are observed in both males and females using esgGal4, expression of Jafrac1 and Hsp68 in salivary glands has no effect on lifespan, and 5961GS is not expressed in salivary glands), we cannot currently exclude a contribution of malpighian tubule stem cells. These cells also appear to respond to proliferative signals such as Hep or InR over-expression, and over-proliferate in stressed flies (GFP-labeled cells accumulate in malpighian tubules in these flies; Biteau, unpublished), but the exact mechanism of regeneration and a potential age-related dysplastic phenotype in this tissue remain unexplored. Importantly, a contribution of this somatic stem cell population to the lifespan effects reported here would further support our model of the importance of proliferative homeostasis in high-turnover tissues for *Drosophila* lifespan.
Figure 4. Moderate inhibition of IIS and JNK pathways in somatic stem cell lineages extends lifespan. A. The 5961GS driver is expressed in the intestine and responsive to RU486. GFP can be detected in the posterior midgut of 5961GS-GFP flies after RU486 exposure, no GFP is detected when flies are kept on control food. B. In the intestine, the activity of the 5961GS driver is restricted to ISC and EB. Only LacZ-positive cells express GFP in 5961GS-GFP/egf-LacZ flies. The expression of the reporter egf-LacZ identifies ISC and EB, immunostaining against prospero identifies EE. C. Western-blot analysis of total extract from dissected guts shows that GFP can be detected in the intestine of 5961GS-GFP flies after RU486 exposure. However, the expression level remains much lower than in the intestine of egfGal4-GFP flies. D–J. Moderate reduction of the IIS (F, G, I) and JNK (H, J) pathways using 5961GS extends lifespan. The mortality of sibling flies of the indicated genotypes placed on control food (-RU486) or food supplemented with RU486 (+RU486) was compared at 25°C. The treatment has minimal effect on the longevity of control flies (5961GS,UAS-GFP,+ in w1118 and OreR background), but causes significant increase in longevity of flies with reduced IIS and JNK pathways (5961GS,UAS-GFP,+UAS-InRDN, 5961GS,UAS-GFP,+UAS-Dp110DN, 5961GS,UAS-GFP,+UAS-AktRNAi, 5961GS,UAS-GFP,+UAS-BskDN). The relative extension of the median lifespan is shown for each genetic condition. For each condition, the reduction of ISC proliferation by the treatment was confirmed, as measured by the number of pH3+ cells in the intestinal epithelium, in 50 to 70 days old females (n=12 guts; Averages and SEM; p-values from Student’s t-test * p<0.05, ** p<0.01). K. Summary of lifespan statistics including mean and median lifespan (days) for all conditions. Detailed lifespan analysis is shown in Table S4. doi:10.1371/journal.pgen.1001159.g004
It is interesting that using the weaker 5961GS driver, lifespan extension in IIS and JNK loss-of-function conditions is only observed in females. This sexual dimorphism might be a consequence of a slight difference in driver activity between the sexes (no significant difference in driver activity can be observed, however), or might indicate selective sensitivity of females to intestinal dysplasia. Interestingly, intestinal turnover rates in females are higher than in males [50], indicating a potential reason for such a selective sensitivity. Accordingly, lifespan extension by esg-mediated expression of Hsp68 and Jafra1 is also stronger in females than in males.

Based on the highly conserved regulation of regenerative processes in flies and vertebrates [13,14,25,47–49], our findings suggest that interventions that focus on maintaining regenerative capacity by improving stem and progenitor cell stress-protection...
hold significant promise for slowing aging in higher organisms, including humans. Interestingly, vertebrates seem to have evolved more efficient and extensive cell autonomous anti-proliferative mechanisms in stem cells than flies \[20–22\], resulting in longer-lasting maintenance of homeostasis in high-turnover tissues. The rapid decay of intestinal homeostasis in flies indicates that such control mechanisms have not been acquired in these short-lived animals, yet our data also suggest the potential for active control of proliferation rates in the intestinal epithelium by systemic insulin-like peptide levels. Interestingly, the regulation of stem cell proliferation by IIS and Foxo is conserved in mammalian systems, suggesting that similar systemic control of stem cell proliferation could be harnessed to regulate regenerative capacity and lifespan in vertebrates \[18,31\]. How the maintenance of intestinal homeostasis is influenced by environmental parameters that affect systemic IIS activity is an interesting subject of further studies.

Materials and Methods

Drosophila stocks and culture

The following strains were obtained from the Bloomington Drosophila Stock Center: \(w^{1118}\), \(y^{506}\), \(y^{1}\text{w}\), UAS-InR\ textarea{DN}, UAS-Dp110\ textarea{DN}, UAS-rpr, and tub-Gal80\textarea{b}. UAS-Ak\textarea{RNAi} and UAS-Bsk\textarea{RNAi} were obtained from the Vienna Drosophila RNAi Center (transformant ID 2902 and 34138). esg-Gal4 was kindly provided by S. Hayashi; chico\textarea and UAS-FoxoTM by M. Tatar; dilp2-Gal4 by E. Rulifson; UAS-Hep and sep-Gal4 by M. Mlodzik; UAS-NICD by N. Perrimon. MARCM stocks were gifts from N. Perrimon. MARCM stocks were gifts from N. Perrimon. MARCM stocks were gifts from N. Perrimon. MARCM stocks were gifts from N. Perrimon. MARCM stocks were gifts from N. Perrimon. MARCM stocks were gifts from N. Perrimon. MARCM stocks were gifts from N. Perrimon. MARCM stocks were gifts from N. Perrimon. MARCM stocks were gifts from N. Perrimon. MARCM stocks were gifts from N. Perrimon. MARCM stocks were gifts from N. Perrimon. 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Immunostaining and microscopy

Intact guts were fixed at room temperature for 45 minutes in 100 mM glutamic acid, 25 mM KCl, 20 mM MgSO4, 4 mM Sodium Phosphate, 1 mM MgCl2, 4% formaldehyde. All subsequent incubations were done in PBS, 0.5% BSA, 0.1% Triton-X-100 at 4°C.

The following primary antibodies were used: mouse anti-BrdU (Becton Dickson) 1:200; mouse anti-Prospero and anti-Armadillo (Developmental Studies Hybridoma Bank) 1:250 and 1:100; rabbit anti-pH3 (Upstate) 1:1000. Fluorescent secondary antibodies were obtained from Jackson Immunoresearch. Hoechst was used to stain DNA.

Confocal images were collected using a Leica SP5 confocal system and processed using the Leica software and Adobe Photoshop.

BrdU incorporation

Flies were cultured on standard food supplemented with BrdU (final concentration 0.2 mg/ml) for 2 days. Intact guts were fixed as previously described and DNA was denatured by incubating tissue in 3M HCl for 30 minutes. Samples were then processed for immunostaining as described above.

Metabolite measurements

4 to 5 females (without the head) were homogenized in 150 μl of buffer (10 mM KH2PO4, 1 mM EDTA, pH 7.4). 10 μl of cleared extract was used to measure triglycerides, glucose and glycogen concentrations according to the manufacturer’s instructions (Triglyceride Liquidcolor, Stanbio; Glucose and Starch Assay Kits, Sigma).

Lifespan analysis

For lifespan experiments at 29°C using the TARGET system, virgin females (esgGal4, UAS-GFP, tubGal80Δ) were crossed to the following UAS transgenes or the respective wild-type controls: UAS-Hepwt, UAS-BSkRNAi, and UAS-AkRNAi (back-crossed at least 10 generations into w1118); UAS-Foxowt and UAS-Dp110ΔN (in y;w background); and UAS-InR (in w1118 background). Crosses were kept at room temperature. After collection and sorting (60–100 flies/cage), flies were placed at 29°C to age.

The UAS-Jafrac1 and UAS-Hsp68 transgenes were backcrossed 10 times into the w1118 background and kept as an unbalanced stock. Of this stock, 10 to 15 homozygous males (+/+) or UAS/UAS) were independently crossed to 40 yw; esgGal4,UASGFP/CyO virgin females. To control for the effect of over-expressing Hsp68 in salivary glands, males (+/+) or UAS/UAS) from the same backcrossed UAS-Hsp68 stock were independently crossed to w; GMR-Gal4,UAS-GFP homozygous virgins. Crosses and progeny were kept at all times at 25°C. The progeny of these crosses was collected 2 days after hatching and allowed to mate in bottles for 3 days. Flies were finally separated according to their sex and genotype into cages (50–100 flies/cage).

To test the effect of Foxo over-expression using the esgGal4 driver on lifespan, the driver was backcrossed 10 times into the w1118 background and kept as an unbalanced stock. 40 w1118; esgGal4/+ virgin females were crossed to 10–15 yw; UAS-Foxo homozygous males. Crosses were kept at 18°C to minimize driver expression and potential developmental defects associated with Foxo expression. The progeny of these crosses was collected 4 to 5 days after the first fly hatched. Flies were allowed to mate in bottles for 2 days at room temperature. Siblings were finally separated according to their sex and genotype into cages (20–50 flies/cage) and transferred at 25°C.
For RU486 food supplementation, 100 μl of a 5 mg/ml solution of RU460 or vehicle (ethanol 90%) were deposited on top of a food vial and dried for at least 16 hours to ensure complete evaporation, resulting in a 0.2 mg/ml concentration of RU460 in the food accessible to flies (determined using a dye control as previously described for drug treatments [92]).

For all populations, plastic cages (175 ml volume, 5 cm diameter from Greiner bio-one) were used for lifespan experiments. Food, changed every 2 days, was provided in vials inserted into a foam plug (4.9 cm in diameter, 3 cm thick from Greiner bio-one), dead flies were visually identified (flies not moving, not responding to mechanical stimulation and laying on their side or back were deemed dead), and the number of dead flies was recorded. Cages were replaced after 20 days (flies were transferred into new cages without anesthesia). Survival of the different populations was analyzed using the SAS JMP7 statistical software.

The driver lines (esgGal4 and 5961) were used to collect females for all lifespan studies are Wolbachia negative, the lifespan effects observed in flies with reduced JNK or IIS activity in somatic stem cells are thus Wolbachia independent.

Analysis of GFP expression by western blot

Analysis of esgGal4, 5961GS and GMR-Gal4 expression pattern by western blot 5 females (esgGal4>UAS-GFP, 5961GS>UAS-GFP, GMRGal4>UAS-GFP or OregonR) were dissected into heads, guts, salivary glands, ovaries and the carcasses of the thorax and abdomen. Tissues were homogenized in protein sample buffer; proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane using standard procedures. GFP was detected using rabbit anti-GFP antibody (Invitrogen; 1:5000), HRP-conjugated anti-rabbit and chemiluminescence, according to manufacturer instructions. Total proteins, detected using Ponceau staining, or Heterochromatin Protein 1 (detected by immune-staining, anti-H1P1, DSHB; 1:5000) are used as loading controls.

Analysis of gene expression

Total RNA from 5 guts, from embryos, or from dissected 3rd instar larval eye imaginal discs, was extracted using Trizol and cDNA synthesized using Superscript II (Invitrogen). Real time PCR was performed using SYBR Green, a Biorad IQ5 apparatus and the following primers pairs (Delta: 5′-TCA TCG AGC ACT CCA TAG TC-3′; Jafrac1: 5′-CAC GTT GAG CGA GAA CT-3′; Akt: 5′-AAC CGT TTG GGA GTG GGA AAG GAT-3′ and 5′-TCA ACT CCA CAC TCT CTC CGG TAA-3′; Actin: 5′-CTC GCC ACT TGC GTT TAC AGT-3′ and 5′-TCA ATA TCG TCG CAG TTG GTG TGC-3′; Jafrac1: 5′-CAA GTT GAG CGA CTA CAA GG-3′ and 5′-TCA TCG AGC ACT CCA TAG TC-3′). Data was calculated using the ΔCt method and normalized to actin levels. Results are average +/- standard deviation of at least 3 independent biological samples run in triplicate.

Supporting Information

Figure S1 Expression pattern of the esgGal4 driver. A. All accessible tissues from flies expressing GFP under the control of the esgGal4 driver were dissected. GFP can exclusively be detected in the Intestinal Stem Cells (ISCs) in the midgut, the Renal Stem Cells (RNSCs) in the malphgian tubules, the testes and the salivary glands. No Fluorescence can be detected in the crop, the hindgut, the ovaries, the brain or the abdominal fat body. B. GMRGal4, used as a control for lifespan experiments, overlaps with esgGal4 expression in the salivary glands. C. Western-blot analysis of dissected tissues from adults in which esgGal4 or GMRGal4 drive expression of GFP. Extracts from OreR (wild-type) flies are shown as controls. Note the overlap of expression of the two drivers in salivary glands, and the exclusive expression of esg-GFP in the intestine and salivary glands. Mobility shift between the GFPs expressed in either line is due to expression of different GFP constructs in esgGal4 or GMRGal4 recombinants. Ponceau Red staining is shown as loading control.

Found at: doi:10.1371/journal.pgen.1001159.s001 (4.01 MB TIF)

Figure S2 ISC function is critical for normal lifespan. A. Inducing differentiation in the ISC lineage (using activated notch intra-cellular domain; esgGal4, UAS-GFP;ubGal80ts/UAS-NICD) results in shortening of lifespan compared to wild-type controls (esgGal4, UAS-GFP;ubGal80ts/+ (j1)w) in both males and females. Flies used in these experiments were reared at a permissive temperature (22 °C, promoting activity of the Gal80 repressor), then transiently shifted to 29 °C at 5 days of age (for 7 days) to deactivate the repressor and irreversibly impair stem cell function through over-expression of NICD. Flies were aged at 25 °C. B. Longevity of (esgGal4, UAS-GFP;ubGal80ts/UAS-NICD) is minimally affected when flies are kept at a permissive temperature (22 °C, promoting activity of the Gal80 repressor) throughout life. This suggests that the TARGET system (ubGal80ts) approach can inhibit developmental effects of transgenes, thus limiting the lifespan effects strictly to changes in the adult. C. Summary of lifespan statistics for all populations including mean and median lifespan (days), as well as XiSquare value and p-Value using log rank test. D. Western blot showing GFP levels in wild-type (no GFP) whole larvae as well as esgGal4, UAS-GFP whole larvae with (esgGal4, UAS-GFP;ubGal80ts) and without the Gal80 repressor. At a permissive temperature (22 °C), no GFP is detected in the presence of ubGal80ts. E. Shifting adult flies (esgGal4, UAS-GFP;ubGal80ts) to 29 °C strongly induces GFP expression in the gut.

Found at: doi:10.1371/journal.pgen.1001159.s002 (0.94 MB TIF)

Figure S3 ISC are the only proliferating cells in the midgut epithelium. A. Confocal image of a representative MARCM clone induced in the intestinal epithelium. The arrowhead indicates the unique pH3+ cell among GFP+ cells. B. Analysis of proliferating cells in the ISC lineage in young (7 days), old (40 days) or stressed flies, as well as ISC over-expressing Hep. For each condition, MARCM clones were observed 7 days after induction. The number of clones containing 1 pH3+ cell is minimal, 2 pH3+ cells or more is indicated. The size of the clones is also reported. No clones with more than 2 pH3+ cells could be detected in any of the conditions tested (>200 clones observed for each), and the clones induced in older or stressed animals are not larger than the clones induced in young animals, suggesting the absence of transient amplifying cells, even in older animals.

Found at: doi:10.1371/journal.pgen.1001159.s003 (1.00 MB TIF)

Figure S4 qRT-PCR confirming the reduction of Akt expression using the UAS-AktRNAi line. RNA was obtained from wild-type and daG4>UAS-AktRNAI embryos, at 25 °C (Daughterless-Gal4 (daG4) is a ubiquitous driver). Actin5C served as internal control. Bars represent the average of 3 independent samples ± standard deviation, and p-value is calculated using Student’s t-test.

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Figure S5 Lifespan analysis of esgGal4 heterozygous flies. A. Survival curves of esgGal4+/ flies compared to their respective wild-type co-isogenic controls. The esgGal4 line was backcrossed 10 generation into w1118 background. esgGal4+/ females were...
crossed to a1 males and survival of the progeny was recorded. These flies represent the isogenic controls for the experiment presented in Figure 3D. B. Summary of the lifespan analysis of esgGal4/+ flies. See Table S3 for complete analysis.

Found at: doi:10.1371/journal.pgen.1001159.s005 (0.33 MB TIF)

**Figure S6** Expression pattern of the 5961GeneSwitch driver. A. Western-blot analysis of dissected tissues from adult 5961GS>GFP females. GFP can exclusively be detected in the intestine after exposure to RU486. Heterochromatin Protein 1 (detected by immuno-staining) and total protein levels (Ponceau Red staining) are shown as loading controls. B. Bright field and fluorescence images of dissected esgGal4>GFP and 5961GS>GFP (fed RU486 for 3 days) females, confirming that 5961-driven transgene expression is much weaker than esg-mediated expression. C. Western-blot analysis of head extracts from adult 5961GS>GFP females, compared to standard neuronal drivers (elavGal4 and elavGeneSwitch), as well as esgGal4. No GFP can be detected in the extracts from 5961GS>GFP flies, further suggesting that the activity of the 5961GS driver is restricted to the intestine and the malpighian tubules.

Found at: doi:10.1371/journal.pgen.1001159.s006 (4.96 MB TIF)

**Figure S7** Induction of Hsp68 and Jafrac1 by Foxo. qRT-PCR demonstrating induction of hsp68 and Jafrac1 in response to constitutively active Foxo (FoxoTM) in third instar eye imaginal discs, using the sepGal4 driver. Average and standard-deviation from 3 independent experiments are shown. p-value is calculated using Student’s t-test. RNA was collected from 10 discs for each experiment. Expression levels are reported as relative to actin5C expression.

Found at: doi:10.1371/journal.pgen.1001159.s007 (0.15 MB TIF)

**Figure S8** Overexpression of stress-protective genes in ISC lineage delays dysplasia in the anterior midgut. A. The age-related loss of tissue can be scored in the anterior midgut. This phenotype can be scored using three distinct categories, based the presence of individual esg+ cells (category 1), the formation of esg+ cell clusters (category 2) or the invasion of the proventriculus by esg+ cells (category 3). The white bars mark the limit between the anterior midgut and the proventriculus (PV). B. Intestinal degeneration in aging (3, 35, and 50 days) control flies (esgGFP>+) and flies overexpressing cytoprotective genes in the ISCs (esgGFP>Jafrac1 and esgGFP>Hsp68) was scored using the method described above. Overexpression of Jafrac1 or Hsp68 delays age-related loss of intestinal architecture in the anterior midgut. p-value from Pearson X2Square test.

Found at: doi:10.1371/journal.pgen.1001159.s008 (0.67 MB TIF)

**Figure S9** Expression of Hsp68 under the control of the GMRGal4 driver doesn’t affect lifespan. A. Survival curves of GMRGal4> Hsp68 compared to their respective wild type co-isogenic controls. The UAS-Hsp68 line was backcrossed 10 generations into a1118 background. Wild type and UAS siblings were crossed to GMRGal4,UAS-GFP and survival of the progeny was recorded. B. Summary of the lifespan analysis of GMRGal4> Hsp68. No significant difference in longevity was observed between Hsp68 over-expressing flies and their controls.

Found at: doi:10.1371/journal.pgen.1001159.s009 (0.26 MB TIF)

**Table S1** Lifespan analysis of flies with impaired intestinal regeneration. Sex, genotypes, and mean lifespan statistics of the populations used for demographic analysis (Figure 1E) are listed. Experimental and control populations are compared using Log-Rank and Wilcoxon tests (ChiSquare and p-values). All the analysis was performed using the JMP7 statistical software.

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**Table S2** Lifespan analysis of flies with strong reduction of IIS using the esgGal4 driver. Sex, genotypes and mean lifespan statistics of the populations used for demographic analysis (Figure 3B and 3C) are listed. Experimental and control populations are compared using Log-Rank and Wilcoxon tests. All the analysis was performed using the JMP7 statistical software.

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**Table S3** Lifespan analysis of flies overexpressing Foxo using the esgGal4 driver and corresponding controls. Sex, genotypes, and lifespan statistics of individual cohorts used for demographic analysis (Figure 3D) are listed. Mean lifespan and days at which 25% or 75% of the population were dead are shown for each cohort. Flies from the same population are siblings from individual crosses. ChiSquare and p-values are derived from Log-Rank and Wilcoxon Tests. All the analysis was performed using the JMP7 statistical software.

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**Table S4** Lifespan analysis of flies with moderate reduction of IIS and JNK signaling using the 5961GS driver. Genotypes and lifespan statistics of individual cohorts used for demographic analysis in Figure 4 are listed. Mean lifespan, median lifespan and days at which 25% or 75% of the population were dead are shown for each cohort. Flies from the same population (RU486 and +RU486) are siblings from individual crosses. Only females are shown. The significance of the changes observed when the flies are raised on RU486 was tested using Log-Rank and Wilcoxon Tests (ChiSquare and p-values). All the analysis was performed using the JMP7 statistical software.

Found at: doi:10.1371/journal.pgen.1001159.s013 (0.35 MB PDF)

**Table S5** Lifespan analysis of flies with increased stress protection using the esgGal4 driver (esgGal4,GFP> Jafrac1 and esgGal4,GFP> Hsp68). Sex, genotypes, and lifespan statistics of individual cohorts used for demographic analysis (Figure 6A) are listed. Mean and median lifespan and days at which 25% or 75% of the population were dead are shown for each cohort. ChiSquare and p values are derived from Log-Rank and Wilcoxon Tests. All the analysis was performed using the JMP7 statistical software.

Found at: doi:10.1371/journal.pgen.1001159.s014 (0.34 MB PDF)

**Table S6** Lifespan analysis of flies with moderate expression of Jafrac1 and Hsp68 using the 5961GS driver. Genotypes and lifespan statistics of individual cohorts used for demographic analysis (Figure 6C) are listed. Mean and median lifespan and days at which 25% or 75% of the population were dead are shown for each cohort. Flies from the same population (RU486 and +RU486) are siblings from individual crosses. Only females are shown. The significance of the changes observed when the flies are raised on RU486 was tested using Log-Rank and Wilcoxon Tests (ChiSquare and p-values). All the analysis was performed using the JMP7 statistical software.

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**Text S1** Description of scoring methods used to monitor intestinal degeneration in aging flies.

Found at: doi:10.1371/journal.pgen.1001159.s016 (0.03 MB DOC)

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**Author Contributions**

Conceived and designed the experiments: BB JK HJ. Performed the experiments: BB JK SS. Analyzed the data: BB JK HJ. Contributed reagents/materials/analysis tools: MB RL. Wrote the paper: BB JK HJ.
References

1. Sohal RS, Weinfrucht R (1996) Oxidative stress, caloric restriction, and aging. Science 273: 39–63.
2. Guarente L, Picard F (2005) Calorie restriction—the SIR2 connection. Cell 120: 473–482.
3. Kowaljova J, Guarente L (2003) How does caloric restriction work? Genes Dev 17: 313–321.
4. Stadtman ER (2001) Protein oxidation in aging and age-related diseases. Ann N Y Acad Sci 928: 22–38.
5. Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of aging. Nature 406: 239–247.
6. Tatar M, Bartke A, Antebi A (2003) The endocrine regulation of aging by insulin-like signals. Science 299: 1346–1351.
7. Kenyon C (2005) The plasticity of aging: insights from long-lived mutants. Cell 120: 449–460.
8. Russell SJ, King CR (2007) Endocrine regulation of aging. Nat Rev Mol Cell Biol 8: 681–691.
9. Vigg J, Campisi J (2008) Puzzles, promises, and a cure for aging. Nature 454: 1069–1071.
10. Partridge L, Gems D (2002) Mechanisms of aging: public or private? Nat Rev Genet 3: 165–175.
11. Guarente L, Kenyon C (2000) Genetic pathways that regulate aging in model organisms. Nature 408: 253–262.
12. Finkel T (2002) A conserved regulatory system for aging. Cell 109: 163–165.
13. Radke F, Clevers H (2005) Self-renewal and cancer of the gut: two sides of a coin. Science 307: 1904–1909.
14. Radke F, Clevers H, Riccio O (2006) From gut homeostasis to cancer. Curr Opin Cell Biol 18: 275–289.
15. Van Zant G, Liang Y (2003) The role of stem cells in aging. Exp Hematol 31: 639–672.
16. Rossi DJ, Bryder D, Seita J, Nussenzweig A, Hoeijmakers JH, et al. (2007) FOXO transcription factors and stem cell alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci U S A 102: 9914–9919.
17. Nijjik A, Woodbine I, Marchetti C, Dawson S, Lamber T, et al. (2007) DNA repair is limiting for hematopoietic stem cells during aging. Nature 447: 690–696.
18. Tothova Z, Koliippa R, Huntly BJ, Lee BH, Castrillon DH, et al. (2007) FoxO are critical mediators of hematopoietic stem cell restriction to physiologic oxidative stress. Cell 129: 325–339.
19. Rossi DJ, Bryder D, Zahn JM, Ahlenius H, Sonu R, et al. (2005) Cell intrinsic Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell 1: 470–478.
20. Weissman IL (2000) Stem cells: units of development, units of regeneration, and cancer. Science 290: 686–690.
21. Weissman IL (2001) Stem cells, ageing and the quest for immortality. Nature 441: 659–672.
22. Campisi J, Sedivy J (2009) How does proliferative homeostasis change with age? J Gerontol A Biol Sci Med Sci 64: 164–166.
23. Campisi J, Sedivy J (2009) What causes it and how does it contribute to aging? J Gerontol A Biol Sci Med Sci 64: 164–166.
24. Miyamoto K, Miyamoto T, Kato R, Yoshimura A, Motoyama N, et al. (2008) Foxo3a is required for hematopoietic stem cell self-renewal. Proc Natl Acad Sci U S A 105: 20870–20875.
25. Vigg J, Campisi J (2008) Puzzles, promises, and a cure for aging. Nature 454: 1069–1071.
26. Wagers AJ, Conboy IM (2005) Cellular and molecular signatures of muscle repair is limiting for haematopoietic stem cells during ageing. Nature 447: 690–696.
27. Rossi DJ, Jamieson CH, Weissman IL (2008) Stem cells and the pathways to cancer. Nature 454: 651–655.
28. Tothova Z, Gilliland DG (2007) FoxO transcription factors and stem cell proliferation. Cell Stem Cell 4: 124–127.
29. Miyamoto K, Miyamoto T, Kato R, Yoshimura A, Motoyama N, et al. (2008) FOXO transcription factors and stem cell alterations underlie hematopoietic stem cell aging. Proc Nat Acad Sci U S A 102: 9914–9919.
30. Tatar M, Bartke A, Antebi A (2003) The endocrine regulation of aging by insulin-like signals. Science 299: 1346–1351.
31. Kenyon C (2005) The plasticity of aging: insights from long-lived mutants. Cell 120: 449–460.
32. Russell SJ, King CR (2007) Endocrine regulation of aging. Nat Rev Mol Cell Biol 8: 681–691.
33. Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, et al. (2003) Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature 424: 277–283.
34. Green EL (2005) FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene 24: 7410–7425.
35. Gershman B, Puig O, Hang L, Peitzsch RM, Tatar M, et al. (2007) High-resolution dynamics of the transcriptional response to nutrition in Drosophila: a key role for dFOXO. Physical Genomics 29: 24–34.
36. Junger MA, Rintelen F, Stocker H, Wasserman JD, Vehg M, et al. (2003) The Drosophila Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol 2: 20.
67. Hwangbo DS, Gerstham B, Tu MP, Palmer M, Tatar M (2004) Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature 429: 562–566.
68. Bluher M, Kahn BB, Kahn CR (2003) Extended longevity in mice lacking the insulin receptor in adipose tissue. Science 299: 572–574.
69. Wu Q, Zhang Y, Xu J, Shen P (2003) Regulation of hunger-driven behaviors by neural ribosomal S6 kinase in Drosophila. Proc Natl Acad Sci U S A 102: 13289–13294.
70. Levers SJ, Weinkove D, MacDougall LK, Hafen E, Waterfield MD (1996) The Drosophila phosphoinositide 3-kinase Dpl110 promotes cell growth. Embo J 15: 6334–6359.
71. Lee T, Luo L (2001) Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. Trends Neurosci 24: 251–254.
72. Wu JS, Luo L (2006) A protocol for mosaic analysis with a repressible cell marker (MARCM) in Drosophila. Nat Protoc 1: 2583–2589.
73. Mathur D, Bost A, Driver I, Oldstein B (2010) A transient niche regulates the specification of Drosophila intestinal stem cells. Science 327: 210–213.
74. Wang MC, Bohmamn D, Jasper H (2003) JNK signaling confers tolerance to oxidative stress and extends lifespan in Drosophila. Dev Cell 5: 811–816.
75. Lee RS, Iijima-Ando K, Iijima K, Lee WJ, Lee JH, et al. (2008) JNK/FOXO-mediated neuronal expression of fly homologue of peroxiredoxin II reduces oxidative stress and extends lifespan. J Biol Chem 283: 29454–29461.
76. Radyuk SN, Klichko VI, Spinola B, Sohal RS, Orr WG (2001) The peroxiredoxin gene family in Drosophila melanogaster. Free Radic Biol Med 31: 1090–1100.
77. Paik JH, Kolipara R, Chu G, Ji H, Xiao Y, et al. (2007) FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell 128: 309–323.
78. Pinkston-Gouse J, Kenyon C (2007) DAF-16/FOXO targets genes that regulate tumor growth in Caenorhabditis elegans. Nat Genet 39: 1403–1409.
79. Pinkston JM, Gargian D, Hansen M, Kenyon C (2006) Mutations that increase the life span of C. elegans inhibit tumor growth. Science 313: 971–975.
80. Clancy DJ, Gems D, Hafen E, Levers SJ, Partridge L (2002) Dietary restriction in long-lived dwarf flies. Science 296: 319.
81. Katawa SD, Kapahi P. Dietary restriction and aging, 2009. Aging Cell 9: 103–112.
82. Zul BM, Rogers AN, Katawa SD, Vargas MA, Kolipinski MC, et al. (2009) HE-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila. Cell 139: 149–160.
83. Karpac J, Hui-Thompson J, Falleur M, Jasper H (2009) JNK signaling in insulin-producing cells is required for adaptive responses to stress in Drosophila. Aging Cell 8: 288–295.
84. Karpac J, Jasper H (2009) Insulin and JNK: optimizing metabolic homeostasis and lifespan. Trends Endocrinol Metabol 20: 100–106.
85. Manning AM, Davis RJ (2003) Targeting JNK for therapeutic benefit: from junk to gold? Nat Rev Drug Discov 2: 554–565.
86. Karin M, Gallagher E (2005) From JNK to pay dirt: jun kinases, their biochemistry, physiology and clinical importance. IUBMB Life 57: 283–295.
87. Liu J, Lin A (2005) Role of JNK activation in apoptotic: a double-edged sword. Cell Res 15: 36–42.
88. Ha EM, Lee KA, Seo YY, Kim SH, Lim JH, et al. (2009) Coordination of multiple dual oxidase-regulatory pathways in responses to communal and infectious microbes in drosophila gut. Nat Immunol 10: 949–957.
89. Ha EM, Lee KA, Park SH, Kim SH, Nam HJ, et al. (2009) Regulation of DUOX by the Galphq-phospholipase Cbeta-Ca2+ pathway in Drosophila gut immunity. Dev Cell 16: 386–397.
90. Ryu JH, Ha EM, Oh CT, Seol JH, Brey PT, et al. (2006) An essential complementary role of NF-kappaB pathway to microbicidal oxidants in drosophila gut immunity. EMBO J 25: 3693–3701.
91. Ha EM, Oh CT, Bae YN, Lee WJ (2005) A direct role for dual oxidase in Drosophila gut immunity. Science 310: 847–850.
92. Grover D, Ford D, Brown C, Hoe N, Eremen A, et al. (2009) Hydrogen peroxide stimulates activity and alters behavior in Drosophila melanogaster. PLoS One 4: e7580. doi:10.1371/journal.pone.0007580.