Drosophila as a model for ageing

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Abbreviations: 4EBP – translation initiation factor 4E binding protein; AMPK – AMP activated protein kinase; AOP – anterior open; ATF4 – activating transcription factor 4; CHOP – C/EBP homologous protein; CR – calorie restriction; DR – dietary restriction; eIF – eukaryotic translation initiation factor; ERK – extracellular signal-regulated kinase; ETS – E-twenty six; FKH – forkhead; FOXO – forkhead BoxO; GCN – general control non-derepressible; IIS – insulin and IGF-1 like signaling; ilp – insulin-like peptide; PI3K – phosphoinositide 3-kinase; ROS – reactive oxygen species; mTOR – mechanistic target of rapamycin; ORF – open reading frame; REPTOR – repressed by TOR; S6K – S6 protein kinase; TSC – tuberous sclerosis complex

Abstract

Drosophila melanogaster has been a key model in developing our current understanding of the molecular mechanisms of ageing. Of particular note is its role in establishing the evolutionary conservation of reduced insulin and IGF-1-like signaling in promoting healthy ageing. Capitalizing on its many advantages
for experimentation, more recent work has revealed how precise nutritional and genetic interventions can improve fly lifespan without obvious detrimental side effects. We give a brief summary of these recent findings as well as examples of how they may modify ageing via actions in the gut and muscle. These discoveries highlight how expanding our understanding of metabolic and signaling interconnections will provide even greater insight into how these benefits may be harnessed for anti-ageing interventions.
Introduction

*Drosophila melanogaster* has been used as a model organism for ageing research for more than 100 years. Possibly the first quantitative account of *Drosophila* lifespan under lab conditions was reported by Roscoe Hyde in 1913, in which he observed, and correctly outlined, how hybrid vigour could account for apparent lifespan extension when crossing two inbred lines (Hyde, 1913). A more systematic programme of work on *Drosophila* ageing, which established many of the conditions we still employ today, was initiated in 1921 by Raymond Pearl & Silvia Parker. In their first article (Pearl and Parker, 1921), they report a catastrophic air conditioning failure that destroyed their colony of mice, which were intended for ageing studies and, after consultation with Prof Morgan and Dr Loeb, they took the decision to use *Drosophila* as their model for lifespan research. In this and 13 subsequent articles during the course of 14 years, Raymond Pearl and co-authors outline the basic dietary requirements of *Drosophila* for survival, as well as the effects of repeated anaesthesia (with ether), inbreeding depression, adult housing density and life-stage-specific temperature variations on adult lifespan.

At a similar time, the effect of temperature on the duration of life was reported (Loeb and Northrop, 1916), and established that a 10°C reduction in temperature resulted in an approximate doubling of lifespan, thus conforming to the same temperature coefficient as enzyme reactions in solution. Since flies are ectotherms, these findings promoted the concept that lifespan could be determined by an organism’s rate of living (Pearl, 1928) as if it was dictated by the consumption of a substrate which was available in a predetermined quantity, or was related to the products/by-products of metabolic biochemistry.

The rate of living can dictate lifespan if the damaging by-products of aerobic metabolism as well as mechanical damage accumulate to the point of being overwhelming (Harman, 1956). Biological organisms fight back against such damage by enzyme systems and small molecules to mop up and/or repair chemical damage, as well as by the replacement of damaged cells with new cells derived from stem cells. Whether or not this damage is the fundamental cause of ageing, or if inappropriate
and uncontrolled continuation of growth is key (Blagosklonny, 2006), the incentive to maintain biological systems in working order and thus ensure longevity is provided by the imperative to reproduce. Thus, in an organism like *Drosophila* where there is no recognised advantage of parental or grand-parental care, evolutionary selection pressure drops after the age of first reproduction and physiological systems become freer to decline (Medawar, 1952; Rose et al., 2008; Williams, 1957), which defines ageing.

**Flies show physiological signs of ageing**

Ageing can be described as the decline in function over time that leads to reduced fertility and eventual death. The characteristics of population survival for *Drosophila* can reveal information about the progression of ageing as well as any additional, life-shortening effects from non-ageing related deaths due to things such as poor genetic stock and/or environmental factors (Pearl and Parker, 1921; Piper and Partridge, 2016). A typical, healthy and well-maintained outbred *Drosophila* population will have a median lifespan of approximately 70 days and maximum of approximately 90 days at 25°C (Ziehm and Thornton, 2013; Ziehm et al., 2013).

At a more detailed level of physiological decline, numerous markers of ageing-related loss of function can be observed. These include changes to metabolism (reduced resting metabolic rate, decreased protein and fat synthesis), behavior (reduced feeding, courtship and exploration, and increased sleep fragmentation), reduced stress resistance, reduced reproductive capacity (reduced egg laying and hatching success, decreased sperm and accessory fluid production and sperm competitive success), altered neuronal function (impaired learning and memory), modified physical activity (impaired negative geotaxis, reduced voluntary flying and walking), reduced immune capacity, progressive dysplasia and reduced barrier function in the gut, and compromised cardiac function (Figure 1 (Gargano et al., 2005; Grotewiel et al., 2005; Iliadi et al., 2012; Tamura et al., 2003). These features are important to study for two reasons: first, to understand how any lifespan-modifying intervention may affect the progression of these metrics of “health” over time, and; secondly, ascertaining if any of these changes play a causal role in the demographic ageing of
the fly and thus could be targeted by interventions to slow ageing.

**Evolutionary conservation of the genetic basis of ageing**

Given the discovery that ageing is under genetic control and that different organisms have evolved vastly different lifespans, it is evident that ageing would be modifiable by genetic manipulations. From 1983-1993 the first single gene mutations to extend lifespan were identified and characterized in worms as defective in components of the insulin and IGF-1 like signaling (IIS) pathway (Friedman and Johnson, 1988; Kenyon et al., 1993; Klass, 1983). In 2001, amongst the first fly mutants to extend lifespan two were found (Clancy et al., 2001; Tatar et al., 2001), both of which also acted to reduce IIS. Subsequently, mutations in mouse insulin or IGF-1 signaling were also shown to extend life (Blüher et al., 2003; Holzenberger et al., 2003), and subsequent genome wide association studies link polymorphisms in the insulin pathway transcription factor FOXO with length of life in humans (Anselmi et al., 2009; Flachsbart et al., 2009; Kuningas et al., 2007; Pawlikowska et al., 2009; Willcox et al., 2008). Thus, at least some aspects of the genetic basis of ageing are evolutionarily conserved, establishing small, short-lived invertebrates, such as *Drosophila*, as useful tools for examining the molecular mechanisms of ageing.

**Why use flies in ageing research?**

*Drosophila* have many advantages for use in ageing research and, as highlighted above, when used in conjunction with the nematode worm *Caenorhabditis elegans*, as well as other short-lived invertebrates such as the baker’s yeast *Saccharomyces cerevisiae*, they are an extremely effective tool for studying evolutionarily conserved aspects of ageing (Kennedy et al., 2017). Where yeast can rapidly provide information about aspects of cellular ageing, the additional interactions at play within and between tissues of a multicellular, differentiated, organism (such as IIS) can be modeled in worms and flies. As a general rule, worms live for ~3 weeks and flies for ~3 months. When combined for their individual experimental strengths, the invertebrates can function as an effective pipeline of discovery of evolutionarily conserved
interventions to enhance lifespan, which can be targeted for experiments in the longer lived vertebrate systems, such as killifish (lifespan ~6-8m), mice (~3y) and rats (~3y).

The particular features of Drosophila that make it an effective model for ageing research are: its low cost of rearing and housing, the absence of regulatory oversight for their use in experiments, the ease of generating large populations, its well defined dietary requirements, its easily quantified reproductive output, its distinct tissues that can be dissected and genetically manipulated, and a large collection of readily available genetic tools, including CRISPR reagents for genome editing as well as constructs for over-expressing or knocking down any gene in a tissue and timing specific manner. Fly tissues are equivalent to many of those found in mammals, including the heart and kidney, both absent in C. elegans, and a high proportion (77%) of genes associated with ageing-related diseases in humans are expressed in the equivalent fly tissues (Kennedy et al., 2017). Most importantly, their relatively short lifespan means they can be used in repeated rounds of experimentation to refine conditions that maximise life.

However, some of these advantages double as disadvantages. In particular, the fact that Drosophila melanogaster is so small makes characterizing health with ageing a difficult prospect. What’s more, no one knows what flies die of, although recent studies examining increased gut dysplasia and leakiness with ageing in females may give clues (Rera et al., 2013). Probably the simplest, and most popular, non-destructive assay for ageing-related health is to measure the ability of flies to climb (negative geotaxis) (Gargano et al., 2005). This yields a combined measure of neuronal and muscular function and, by this measure, IIS mutants that are long lived are also healthier for longer (Gargano et al., 2005).

**Delivering healthy ageing without side effects**

A principle goal of biogerontology is to shift the emphasis from understanding and treating the symptoms associated with ageing to comprehending the underlying molecular mechanisms with the hope of using this knowledge to design therapeutic approaches that prevent or slow the appearance of multiple ageing-related symptoms
and thus compress morbidity. A key aspect of this work is to understand the interrelationship between the benefits offered by interventions into ageing and their associated costs, in order to maximize health improvement with fewest side-effects. With increasingly fine-scaled adjustments to experimental conditions, for example dietary composition, and with interventions targeted to specific tissues and cell types at specific times during the life cycle, there is increasing evidence that broad-spectrum amelioration of the effects of ageing can be achieved (Giannakou et al., 2004; Grandison et al., 2009; Mair et al., 2003). In this endeavor, model organisms including *Drosophila* are key, since it is only through many rounds of experimentation with slightly adjusted conditions, that such discoveries have been made.

**Outline and aims**

Recently, nine hallmarks of ageing have been recognized as of widespread occurrence in different organisms (López-Otín et al., 2013), with experimental evidence available to indicate that their occurrence contributes to the progression of ageing related functional decline. These hallmarks are: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication. A central assumption in the field of biogerontology, is that ageing results from a combination of these mechanisms and that the various hallmarks are interconnected, with different types of loss of function interacting to cause the ageing process.

In this review, we highlight recent research with *Drosophila* that has contributed to our current understanding of the molecular mechanisms of ageing, and interventions that can ameliorate its effects. In particular, we focus on some of the ways by which the various hallmarks of ageing in *Drosophila* are shown to be interconnected, with the intention of identifying nodes of control that might be fruitful for future research. In doing so, we have not been exhaustive in our coverage of how *Drosophila* has contributed to our knowledge of the mechanisms of ageing, and nor do we cover the contribution of the fly to our understanding of how ageing has evolved. For additional
information and related topics, we point the reader to several other reviews (Barnes and Partridge, 2003; Gems and Partridge, 2013; Kapahi et al., 2016; Kirkwood, 2002; Kirkwood and Shanley, 2005; Partridge, 2001; Partridge and Gems, 2002; Rose, 1994)

**Dietary manipulations and lifespan**

One of the most important discoveries in the field of ageing research is that mild dietary restriction (DR; often called calorie restriction (CR)), without malnutrition, can extend lifespan. The first reported instance is most usually attributed to Clive McCay for his work on white rats (McCay et al., 1935), and since then DR has been implemented successfully to extend lifespan in yeast (Jiang et al., 2000), worms (Klass, 1977), flies (Chapman and Partridge, 1996) and monkeys (Colman et al., 2009; Mattison et al., 2012) although here the benefits appear to be lessened by increased age-related frailty (Hultström, 2015). Independent of the longevity effects, diet restriction in monkeys is thought to extend healthspan (Mattison et al., 2012, 2017). Interestingly, work from flies also indicates that DR reduces age-specific mortality rapidly, and apparently overturns the effects of dietary history (Mair et al., 2003) – a finding that promises DR benefits at any stage in life. As a result of these collective findings, and because food manipulation is accessible to anyone who chooses, DR in some form has become a relatively mainstream activity for health-aware humans that hold out the long term hope of gaining extra years of healthy life (Rizza et al, 2014).

**Diet Balance**

Recent work with model organisms, in particular *Drosophila* and mice, has begun to reveal that reduced intake of specific nutrients, rather than of overall calories, mediates the health benefits of DR, with dietary protein playing a key role (Grandison et al., 2009; Mair et al., 2005; Miller et al., 2005; Zimmerman et al., 2003). Furthermore, altering the proportions of the macronutrients (protein, lipid, carbohydrate) in a diet that is consumed *ad libitum* can induce an extension of
lifespan equivalent to that seen when restricting access to all dietary nutrients (Lee et al., 2008; Mair et al., 2005; Skorupa et al., 2008; Solon-Biet et al., 2014). It is not clear if altered diet balance extends life by the same or different mechanisms as traditional DR/CR protocols, since the precise molecular and physiological mechanisms involved have not been identified in either case. A further complication is that, at least in mice, DR usually involves a feed/fast cycle in the experimental animals, since they consume all of their restricted diet as soon as it is supplied to them and fast for the remainder of the 24h period (Speakman et al., 2016). Whatever the mechanisms at work, from the point of view of humans, most of whom find it impossible to comply with a DR regime, changing diet balance can be considered a more manageable practice than simply eating less food. Much effort is thus focused on developing diets in which the nutritional components have been manipulated to mimic the effects of fasting for improving long-term health (eg (Cheng et al., 2017)).

For those experiments where the dietary macronutrient balance for flies and mice has been altered, the optimum for lifespan falls at a point where the protein content, as a proportion of total dietary energy, falls below the optimum for reproduction (Jensen et al., 2015; Lee et al., 2008; Mair et al., 2005; Skorupa et al., 2008; Solon-Biet et al., 2015, 2014). This pattern of responses to diet balance has also been observed for ants (Dussutour and Simpson, 2012), crickets (Harrison et al., 2014; Maklakov et al., 2008) and the Queensland fruit fly (Fanson et al., 2009). Given the strength of this trend and its apparent evolutionary conservation, it is interesting to probe the mechanisms by which protein is such a strong determinant of lifespan.

Several studies have manipulated individual amino acids in the diets of flies and mice, and shown that single essential amino acid dilutions can extend lifespan. By far the most consistent is the effect of reducing the essential amino acid methionine which extends life of both flies and mice, but is accompanied by lowered egg laying in flies, and reduced growth rate and enhanced early life mortality in mice (Grandison et al., 2009; Miller et al., 2005). The downstream mechanism mediating the effects of this intervention are not clear, but much attention has been paid to methionine’s critical roles in translation initiation, its degradation to cysteine via the transulfurylation pathway and the subsequent role of its catabolites in protein
methylation and cellular detoxification. Each of these functions could act to modify a hallmark of ageing.

Given the dozens of nutrients involved and their interacting effects on each other in the context of whole organism physiology, there are doubtless additional subtle nutritional interactions that can modify lifespan. While this opens up a vast research opportunity, it also raises an important issue in the field of Drosophila research: there are dozens of “standard” Drosophila diets (Piper and Partridge, 2007) in use by different laboratories and there is little consistency in how these are reported. If extremely small differences in nutrients, such as the dilution of a single amino acid, can modify lifespan, then it is highly likely that each different study using a different diet comprised of natural ingredients will yield some degree of lifespan variation. While holidic diets can be used to get around this problem (Piper et al., 2014), the expense and complexity of their preparation can be off-putting. A more reasonable expectation is that all components of complex diets are made explicit upon publication and basic nutritional analyses of the constituents are made available.

**Nutrient signaling pathways and lifespan**

The long history and vast body of research showing that diet modifies lifespan leads inevitably to the conclusion that nutrient sensors and their downstream signalling pathways mediate these responses. Here we present knowledge about the major nutrient signaling pathway discoveries that have been shown to extend Drosophila lifespan.

**Insulin / IGF-1 like signaling**

Insulin is typically associated with maintenance of glucose homeostasis, a function that is at least partially conserved in Drosophila (Graham and Pick, 2017). It also has important roles in growth, reproduction, adult health and ageing (Hafen, 2004; Nässel and Broeck, 2016). The Drosophila genome encodes 8 insulin-like peptides (ilps 1-8) as well as a single insulin receptor and the intracellular components of the canonical PI3K branch (Nässel and Broeck, 2016). Upon activation, these transduce a signal to phosphorylate and inactivate the transcription factor FOXO by nuclear
exclusion (Puig et al., 2003). Together, this system is thought to fulfill the combined functions of mammalian insulin, IGF-1 and relaxins (Nässel and Broeck, 2016).

Since the first report of mutations in IIS that extend fly lifespan (Clancy et al., 2001; Tatar et al., 2001), most other pathway components have also been reported to modulate lifespan (Proshkina et al., 2015) in a manner that requires the FOXO transcription factor (Slack et al., 2011).

Given the goal of biogerontology to find interventions that promote healthy ageing without imposing costs, the large family of Drosophila ilp genes is a tantalizing target for intervention. Although there are clear examples of at least some of the ilps having redundant roles (Grönke et al., 2010) there is evidence that each peptide may elicit different outputs for adaptation to specific conditions: each possesses its own characteristic pattern of expression that varies with developmental time (Brogiolo et al., 2001), tissue (Chintapalli et al., 2007) and in response to complex nutritional stimuli (Post and Tatar, 2016). Genetic manipulation of the ilps has revealed their various roles in growth (Boulan et al., 2015), sugar homeostasis (Graham and Pick, 2017), reproduction (LaFever and Drummond-Barbosa, 2005) and healthy ageing (Grönke et al., 2010; Wessells et al., 2004). Most relevant for this discussion, individually knocking down ilp2 but no other ilp, is sufficient to extend fly lifespan and this effect is augmented by additional knockdown of ilps 3 and 5 (Grönke et al., 2010). These mutants also exhibit enhanced resistance to stressors such as heat, lipophilic toxins and ROS, and also have lowered fecundity and elevated body fat. It is possible to dissociate each of these physiological changes from the longevity phenotype (Broughton et al., 2005; Giannakou et al., 2004; Slack et al., 2011), indicating that precise interventions to alter subsets of change caused by FOXO activation will benefit lifespan without dramatic side effects.

Specific over-expression of FOXO in the gut and fat tissues is sufficient to extend fly lifespan (Giannakou et al., 2004; Hwangbo et al., 2004). By examining the direct transcriptional outputs of activated FOXO in these tissues, Alic et al (2014) identified 5 transcription factors that may mediate the resultant lifespan extension (Alic et al., 2014). One of these, an ETS-family transcriptional repressor called AOP, has a similar set of transcriptional targets as FOXO. Activating AOP in the gut and fat
tissues, either directly by over-expression (Alic et al., 2014) or indirectly by inhibiting RAS/ERK signaling (Slack et al., 2015), revealed that AOP could also extend lifespan. Due to its key function in cancer, RAS/ERK signaling has been the target of a great deal of drug development, and Slack et al also showed that one of the FDA approved drugs to reduce its activity, trametinib, could extend fly lifespan when administered in the food (Slack et al., 2015). This is an important finding as its action may well be relevant to protect higher organisms from ageing and joins a short list of FDA approved pharmaceuticals with demonstrated anti-ageing properties (Roth and Ingram, 2016).

Given that AOP and FOXO work coordinately to extend life when over-expressed in gut and fat tissues, Alic et al characterized the overlap in their transcriptional outputs to seek out changes that could coordinate their roles for longer life (Alic et al., 2014). One of these genes, Obp99b, is a possible humoural factor that could be important for inter-tissue signaling. This gene has also recently been found to be strongly up-regulated in a Drosophila model of slowed ageing through reproductive diapause (Kučerová et al., 2016). This gene awaits further investigation.

**Mechanistic Target of Rapamycin (mTOR) signaling**

mTOR is an amino acid sensitive signaling kinase with an essential role in growth (Saxton and Sabatini, 2017). Genetically reducing mTOR function was first shown to extend life in worms (Vellai et al., 2003), an effect that is evolutionarily conserved to yeast (Kaeberlein et al., 2005), flies (Kapahi et al., 2004) and mice (Lamming et al., 2012).

mTOR is generally regarded as the predominant amino acid sensing and signaling molecule in cells and so it is unsurprising to find that it has been closely linked with longevity in response to changes in dietary macronutrient composition. In particular, for flies, both activation of the mTOR suppressor TSC2 or administration of the mTOR inhibiting drug rapamycin can overcome the lifespan shortening effects of high dietary yeast (the flies’ only source of protein) or elevated dietary amino acids (Bjedov et al., 2010; Emran et al., 2014; Kapahi et al., 2004). A similar association
between dietary protein, mTOR function and lifespan has also been found for mice (Solon-Biet et al., 2014).

Rapamycin, which inhibits TOR activity, can extend lifespan in yeast, (Medvedik et al., 2007), worms (Robida-Stubbs et al., 2012), flies (Bjedov et al., 2010) and is one of most heavily studied drugs to increase lifespan of mice (Miller et al., 2014). In a recent meta-analysis of 29 mouse lifespan experiments using rapamycin, it was found to extend life robustly, but effect size varied with gender (greater effects in females than males) and genetic background (Swindell, 2016). Rapamycin is an FDA approved drug with potential for treating ageing in humans. Indeed pre-treatment with rapamycin can rescue the lowered immune response of elderly subjects to immunization against influenza to youthful levels (Mannick et al., 2014).

Suppressing mTOR has the effect of generally reducing translation as well as activating autophagy, two heavily studied phenomena thought to lead to longer life by enhancing proteostasis (Taylor and Dillin, 2011). This increasingly popular concept captures the beneficial effects of many longevity interventions across different model organisms and thus qualifies as one of the nine hallmarks of ageing (López-Otín et al., 2013). The most commonly studied molecular targets of mTOR phosphorylation are the translational activator S6 kinase and the translational repressor 4E BP. Knocking down S6K has been reported to be sufficient to extend life in both flies (Kapahi et al., 2004) and mice (Selman et al., 2009) and its suppression is required for rapamycin to extend fly lifespan (Bjedov et al., 2010). 4E BP was also identified as involved in the lifespan response to rapamycin (Bjedov et al., 2010) and DR (Zid et al., 2009), but in a condition-specific manner (Partridge et al., 2011; Tatar, 2011). Finally, functional autophagy is required for lifespan extension by rapamycin treatment (Bjedov et al., 2010), and overexpression of autophagy components ATG1 (Ulgherait et al., 2014) or ATG8a (Simonsen et al., 2007) is sufficient to prolong fly lifespan. Thus, dietary and/or drug treatments to enhance autophagy have become an attractive prospect for healthy ageing.

The polyamine spermidine can enhance lifespan in an autophagy dependent manner in yeast, worms and flies (Eisenberg et al., 2009), and two drugs identified in a screen
for molecules to enhance autophagy, AUTEN-67 and AUTEN-99, can also extend fly lifespan (Kovács et al., 2017; Papp et al., 2015). In all three studies, the lifespan of control flies was extremely short and displayed signs of non-ageing-related deaths, indicating that follow up work is warranted. In the case of spermidine, the evidence for its anti-ageing properties is strengthened by the evolutionary conservation of its effects, which have recently been expanded to include enhanced healthy ageing and lifespan of mice (Eisenberg et al., 2016). This same study also notes that high spermidine intake in humans is associated with lowered risk of cardiovascular disease (Eisenberg et al., 2016). Together, these data promote the case that reduced activity of mTOR lengthens life via enhanced proteostasis, and especially via enhanced autophagy.

In addition to its effects on translation and autophagy, mTOR signals to control other cellular functions, including transcription, through all three RNA polymerases (Ghosh et al., 2014; Iadevaia et al., 2014; Marshall et al., 2012). For the Pol II-related transcription factors, Bülow et al, (2010) identified the forkhead transcription factor FKH (Bülow et al., 2010) and Tiebe et al (2015) identified 2 transcription factors REPTOR and REPTOR-BP as important for larval growth and interacting with FOXO to regulate overlapping sets of genes (Tiebe et al., 2015). None of these interactions have yet been tested in the context of mTOR-mediated longevity. Of the transcription factors noted above that are targeted by mTOR for altered activity, FKH is a candidate for lifespan responses to nutrition since its worm orthologue pha4 is required for DR to prolong life (Panowski et al., 2007). Additionally, a recent study in flies has shown that knocking down the GATA transcription factors serpent or GATAe can suppress the lifespan shortening effects of high dietary amino acids (Dobson et al., 2016). The GATA family of transcriptional regulators modify amino-acid-sensitive transcription in a TOR-dependent manner in yeast and mosquitoes (Cooper, 2002; Park et al., 2006) and have also been associated with longevity in worms (Budovskaya et al., 2008; Zhang et al., 2013). Thus, the various transcriptional outputs of mTOR signaling are worthy of additional investigation for their impact on longevity.
Another important amino acid sensor for adaptation to nutritionally imbalanced diets is the evolutionarily conserved protein kinase GCN2. Its function was first characterized in yeast, where it was shown to be activated by uncharged tRNAs and to phosphorylate and inactivate the translation initiation factor eIF2 (Dever et al., 1992; Wek et al., 1989), thus reducing general translation. At the same time, small ORFs in the 5’ UTR of some genes cause their expression to be selectively up-regulated (Abastado et al., 1991). The GCN4 transcription factor is the product of one such up-regulated gene and it functions to up-regulate dozens of genes involved in amino acid and purine biosynthesis (Hinnebusch, 1988). ATF4 is the fly and mammalian orthologue of GCN4 and its expression is also enhanced by translational control following phosphorylation of eIF2alpha by one of a family of stress responsive kinases that includes a GCN2 orthologue (Vattem and Wek, 2004).

Initial studies on GCN2 in mice indicated that it was required for rapid rejection of amino acid imbalanced diets (Hao et al., 2005; Maurin et al., 2005), but more recent work has shown these effects are likely to act via a non-GCN2-dependent route (Leib and Knight, 2015). A similar role for fly GCN2 has also been reported for larval feeding in *Drosophila* (Bjordal et al., 2014), but this remains to be investigated in adults.

In flies, GCN2 was recently found to be required for longevity in response to yeast restriction, and under these conditions, it phosphorylated eIF2alpha, which resulted in up-regulation of 4EBP (Kang et al., 2017). These conditions were associated with repression of global translation, with the exception of selected proteins whose expression was enhanced (Kang et al., 2017). It will be interesting in future to understand how these up-regulated proteins might function to enhance lifespan. An attractive target is Sestrin2, which in mammals is induced by ATF4 in response to amino acid stress and acts to repress mTOR function (Ye et al., 2015). In flies, sestrin has been implicated in ageing-related physiological decline via its feedback inhibition of mTOR (Lee et al., 2010). It will also be interesting to examine the tissue requirements of this pathway’s protective effects as well as any humoral signals it
may trigger, especially in light of the recent finding that fat-specific amino acid reduction acts via GCN2 to control germline stem cell maintenance in the ovary (Armstrong et al., 2014).

Finally, ATF4 and its target gene CHOP have been found in mouse livers to have higher expression in five different treatments known to extend lifespan (caloric restriction, rapamycin treatment, methionine restriction, litter crowding and acarbose treatment (inhibitor of carbohydrate absorption)) (Li et al., 2014). Thus, GCN2 and other stress responsive kinases that activate ATF4 are candidate mechanisms for a convergence of protective effects across the different longevity models.

**AMPK**

The AMP activated protein kinase (AMPK) senses cellular energy status by monitoring intracellular AMP+ADP:ATP levels and is critical in balancing the use and storage of energy generating molecules. In flies, reduced AMPK function results in starvation sensitivity, hyperactivity, hyperphagia and abnormal lipid accumulation – all proposed to be signs that the flies experience symptoms of mild starvation due to inappropriate use of energy stores (Johnson et al., 2010).

Over-expression of AMPK is sufficient to extend lifespan in worms and flies (Apfeld et al., 2004; Greer et al., 2007; Mair et al., 2011; Stenesen et al., 2013; Ulgherait et al., 2014). In flies, up-regulating the AMPK alpha subunit in the fat body, muscle (Stenesen et al., 2013), neurons or intestine (Ulgherait et al., 2014) has been reported to extend lifespan. Interestingly, neuronal-specific over-expression of AMPK results in, and requires, enhanced neuronal autophagy for prolonged life, and also enhanced autophagy and delayed barrier dysfunction in the ageing gut (Ulgherait et al., 2014). This is linked to decreased *ilp2* levels, suggesting that reduced systemic insulin is responsible for coordinating inter-tissue activation of autophagy. In worms, neuronal
AMPK also appears to regulate a systemic signal to promote longevity in peripheral tissues by AMPK activation (Burkewitz et al., 2015). If activation of autophagy is the key lifespan preserving mechanism downstream of activated AMPK, this could occur via its inhibitory action on mTOR (Howell et al., 2017) or via the direct action of AMPK to phosphorylate and activate ATG proteins, which has been observed in mammals (Egan et al., 2011; Kim et al., 2011). Recently, in worms, both AMPK and DR have also been implicated in suppressing ageing-related loss of splicing fidelity, which is also sufficient to enhance lifespan (Heintz et al., 2016).

Metformin is a widely prescribed antidiabetic drug with a range of molecular activities including a reduction in signaling through insulin, IGF-1, mTOR, and AMPK, inhibition of the mitochondrial ETC, as well as a reduction in both ROS production and DNA damage (Barzilai et al., 2016). Given this hit list of longevity assurance mechanisms, it is unsurprising to find numerous reports from both worms (Cabreiro et al., 2013; Haes et al., 2014; Onken and Driscoll, 2010) and mice (Anisimov et al., 2008, 2011) that metformin administration can extend healthy lifespan and delay several markers of ageing related decline (Martin-Montalvo et al., 2013). Indeed, there is strong interest in advancing this drug as a treatment for ageing in humans, with planning and fundraising underway to establish a trial of metformin treatment on 3,000 65-79 year olds, following up for incidences of cardiovascular disease, cancer, dementia and mortality (Barzilai et al., 2016).

Surprisingly, the only report on metformin from *Drosophila* indicates no benefits to lifespan for a range of doses that were shown to be absorbed and stimulate AMPK (Slack et al., 2012). Possible explanations could include the absence of a metformin-sensitive microbiota, which was a requirement for lifespan extension in worms (Cabreiro et al., 2013), or because the experiments were done with outbred flies under dietary conditions already optimized for lifespan, and hence there was no additional effect of the drug to further extend their already long lifespan.
Tissues setting a limit on lifespan

In simple terms, ageing is characterized by varying failure rates across different tissues. If this is translatable to the invertebrate models, then targeting a single organ for protective genetic changes could extend life in one of two ways. Either it improves only the function of the organ to which it is targeted, and if that organ is lifespan-limiting, it will extend life until the next most limiting organ fails. Alternatively, if the target organ can coordinate general physiology to function better for longer, an extension of life and healthspan should be observed. While these two alternatives serve as a useful starting point, there is undoubtedly a more complex interplay between tissues in ageing. Obviously, organism-wide rather than a tissue-specific maintenance of function is more desirable, since it should protect against the intrinsic heterogeneity with which ageing manifests between individuals. Here, we present the findings from two tissue systems in flies, one capable of regeneration and the other post-mitotic, and how targeting them for anti-ageing interventions can extend lifespan. It is clear from both that we are only beginning to understand how each might coordinate whole-organism ageing.

The fly gut

The repeated observation that the gut is a key organ in which genetic modifications can regulate lifespan has highlighted this organ as an interesting target for further investigations. In particular, reducing gut IIS ((Giannakou et al., 2004; Hwangbo et al., 2004); gut and fat tissues), RAS/ERK signalling ((Slack et al., 2015); gut and fat), mTOR signalling ((Kapahi et al., 2004); muscle, gut and nervous system), or activating AMPK and the associated enhancement of autophagy (Ulgherait et al., 2014) is sufficient to extend Drosophila lifespan.

The gut is an important homeostatic organ that must balance apparently conflicting roles: to facilitate digestion and absorption as well as providing an accommodating environment for microbes that aid digestion, while at the same time acting as a barrier in the first line of defense against ingested toxins and
pathogens (Lemaitre and Miguel-Aliaga, 2013). The gut is also interesting because it is one of the few sites in Drosophila that houses a reservoir of active stem cells, making it a focal tissue for understanding the evolutionarily conserved role of the division of stem cells as a key player in ageing (López-Otín et al., 2013). Finally, the gut is also an important endocrine organ, being the production site for numerous signaling peptides involved in metabolic homeostasis (Lemaitre and Miguel-Aliaga, 2013). Thus, there are many reasons why maintaining healthy gut function can serve as a potent enhancer of fitness, and dysfunction can have profound consequences for organismal health. It is entirely possible that maintaining gut function for longer could enhance organismal lifespan either directly by enhancing its digestive and barrier function and/or indirectly via its role in regulating systemic metabolic homeostasis.

Soon after intestinal stem cells were first described in Drosophila (Micchelli and Perrimon, 2005; Ohlstein and Spradling, 2005), their over-proliferation and mis-differentiation was reported to increase in occurrence with ageing (Biteau et al., 2008; Choi et al., 2008). This has the consequence of compromising barrier function (Rera et al., 2012), with the implication that the gut may limit lifespan by allowing the infiltration of infection. Indeed, there is a strong correlation between the degree of gut proliferation and organismal lifespan (Biteau et al., 2010) as well as between the onset of gut microbial dysbiosis and lifespan (Biteau et al., 2010; Clark et al., 2015; Guo et al., 2014). Protecting flies against gut dysplasia or microbial dysbiosis, either by modifying local inflammatory signaling (Li et al., 2016) or by maintaining innate immunity in a youthful state for longer (Chen et al., 2014; Guo et al., 2014), has beneficial effects for gut microbial homeostasis and can enhance fly lifespan.

Given these important functions, it is not surprising to find that the gut is also critical in mediating the longevity effects of diet. The necessity to maintain dynamic gut growth differs markedly between male and female flies due to their differing nutritional requirements for gamete production. In contrast to
males, females resize their gut in response to nutrient quality and mating status, and thus can balance the benefits of a large gut for better nutrient extraction against the high metabolic costs of maintaining a large gut when nutritional resources do not warrant it. However, this resizing capacity comes at the cost of greater susceptibility to later life overgrowth (Hudry et al., 2016; Regan et al., 2016). Male and female flies also respond differently to DR: male lifespan is extended much less than that of females (Magwere et al., 2004). This may be because the protective effect of low nutrient diets against over-proliferation is more potent in females than in males, an argument that is supported by the finding that males with feminized guts have greater lifespan extension upon DR than non-modified males (Regan et al., 2016).

The degree to which gut dysfunction is generally limiting for lifespan is not clear, and it is possible to find situations where less age-related gut dysplasia and reduced barrier dysfunction do not correlate with longer life (eg male guts are visibly less overgrown and provide a better barrier with age than those of females, and yet males are the shorter-lived sex; (Regan et al., 2016)). But in the cases where gut function does appear to limit lifespan, it is yet to be clarified if prolonged maintenance fundamentally slows systemic ageing, or if it simply prolongs life until other organ failure(s) causes death. For this, a detailed understanding of gut derived signals (eg Obp99b in response to modified FOXO / AOP function) will be key for future work. Whatever the case, the gut is an important place to understand the details of how ageing alters stem cell function, as well as the more complex association with the ageing microbiota and immune system.

Muscle

Loss of muscle mass and function is a major contributor to the physical decline that occurs with ageing in humans. In flies, muscles contribute a large percentage of body mass and they have many structural similarities with those of mammalian muscles. One major difference, however, is the lack of regenerative capacity due to the absence of stem cells, thus rendering the fly
muscle a model of only some aspects of muscle ageing. However, this presents an opportunity to understand how manipulating ageing related dysfunction in non-replacing muscle cells can mediate systemic changes in healthy ageing (Demontis et al., 2013).

One of the first indications that Drosophila muscle can play an important role to slow ageing was uncovered with the discovery that adult, muscle-specific over-expression of dFOXO could extend fly lifespan (Demontis and Perrimon, 2010). This, at least in part, was proposed to be due to enhanced autophagy acting to improve proteostasis with ageing. Critically, this muscle-specific intervention triggered protective effects across the whole organism (eg enhanced autophagy) via an unknown signal that reduced feeding and thus promoted the benefits of DR. Another unknown, muscle-derived signal has been shown to extend lifespan in response to mild muscle-specific mitochondrial dysfunction (Owusu-Ansah et al., 2013) and, although the signals’ identities are not known, in both cases they are thought to act indirectly to reduce systemic insulin signaling. Muscle tissue is also thought to coordinate organismal health by secreting the myokine myoglianin (Demontis et al., 2014). Muscle-specific over-expression of myoglianin or its transcriptional regulator Mnt was shown to be associated with reduced global rRNA, enhanced climbing ability with age and longer lifespan. These data indicate autocrine and endocrine effects of muscle to preserve body function. It will be interesting in future studies to understand exactly how these anti-ageing signaling signals work throughout the organism.

**Understanding the metabolic constraints on improving ageing**

The summaries above place the mechanisms that operate to enhance longevity into a hierarchy, with environmental factors and nutrient sensing higher up, and their effect on a broad network of metabolic and inter-tissue interactions lower
down. It is not surprising that the majority of discoveries have been made at the higher levels of this hierarchy, given the complexity involved at the lower levels, where disparate areas of physiology must coordinate to shape the long-lived phenotype.

The nine hallmarks of ageing cover a range of changes to the genome and its regulation, alterations to cellular function, and changes in tissues and the systemic environment (López-Otín et al., 2013). It is not known to what extent the molecular events that characterize each hallmark contribute to the overall physiological decline that leads to death, or how one hallmark influences the impact of another. Understanding their interconnection is, however, important for the future of designing therapeutic interventions that seek to slow ageing with minimal side effects. This work involves tracing the lineage of molecular events from the higher signaling levels to the downstream mechanisms. Most discoveries to date are the successes of reductionist approaches to ageing and yet the interconnections and constraints of metabolic and signaling pathways set a broader context to the way ageing hallmarks are expressed. This is illustrated by several recent discoveries showing that substrate availability can be key in dictating how maintenance of the epigenome can function to promote longevity (Berger and Sassone-Corsi, 2015; Brunet and Rando, 2017).

Recent work on Drosophila has shown that longevity is associated with lowered levels of free methionine (Kabil et al., 2011; Laye et al., 2015) to the extent that it is stoichiometrically limiting for translation and egg laying (Kabil et al., 2011; Piper et al., 2017). At the same time, and perhaps partially contributing to intracellular methionine limitation, methionine catabolism via the transulfurylation and associated pathways is enhanced, which results in increased substrates for methylation reactions (Figure 2) (Larson et al., 2012; Parkhitko et al., 2016), as well as the production of glutathione and H2S (Hine et al., 2015; Kabil et al., 2011), each of which can be protective against ageing. Since we know that diet-derived methionine is likely to be in limiting quantities when flies feed on natural diets (Grandison et al., 2009) it follows that the
establishment of methyl-derived epigenetic marks will be highly sensitive to variations in nutrition and metabolic status.

In other work, a metabolic limitation from central carbon metabolism in the form of cytosolic acetyl-CoA availability, has been shown to protect against ageing associated increases in histone acetylation (Peleg et al., 2016). Acetyl-CoA cannot cross the mitochondrial membrane and so must be exported from the mitochondrion as part of citrate shuttles, which also supply the cytosol with reducing power in the form of NADPH (Figure 2). Limiting amounts of acetyl-CoA could have additional effects on ageing-related pathways beyond epigenetic marks, for example by reducing inhibitory acetylation of the pro-longevity transcription factor FOXO (van der Horst and Burgering, 2007) and relieving autophagy from acetylation-mediated repression (Mariño et al., 2014; Schroeder et al., 2014). Indeed, reducing neuronal cystoplasmic acetyl CoA in Drosophila is associated with enhanced autophagy (Eisenberg et al., 2014), and is sufficient to prolong life (Simonsen et al., 2007).

Together, these findings demonstrate how metabolic constraints, which are sensitive to an organism's nutritional status, can dictate the availability of activated C-1 and C-2 units that are required for chromatin maintenance and genomic stability, which in turn protects against ageing.

Metabolic pathways are networked by the shared use of many components, but principally by the relative abundance of ADP/ATP, NAD+/NADH and NADP+/NADPH (Nielsen, 2016). The inevitable consequence is that proper maintenance of these cofactor pairs is important for maintaining general metabolic homeostasis and thus will be important in longevity control (Bonkowski and Sinclair, 2016; Piper et al., 2005). Understanding and manipulating key points in metabolism, derived from genome scale metabolic modeling, may thus be a key future development towards identifying treatments for ageing, similar to the manner in which it has become commonplace in the search for therapeutic targets for cancer (Frezza et al., 2011; Nielsen, 2017; Yun et al., 2015).
Concluding remarks

Studies on the fruitfly *Drosophila* have been critical in developing our current understanding of the molecular basis of ageing. In particular, the combination of the fly's well defined nutritional requirements, its genetically accessible tissue systems and short lifespan make it ideal for developing precision interventions to extend healthy lifespan. Whilst reductionist approaches have been enormously successful in advancing knowledge to date, the work of developing a networked picture of how metabolism, signaling pathways and various tissues combine to produce longevity is only just beginning. For biogerontology to deliver on the promise of benefiting human aging, it will be important to understand how diverse aspects of metabolism are coordinated to achieve greater physiological health. This process will benefit from the development of large-scale metabolic models that pinpoint key nodes of control for intervention. For the same practical reasons that have underpinned its usefulness to date, these studies are particularly well suited to *Drosophila*.

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Figure Legends

Figure 1 Overview of the physiological and genetic factors that dictate *Drosophila* ageing.

(Bottom panel) During their 3-month lifespan, flies exhibit numerous signs of ageing, including changes to metabolism, tissue function, reproductive capacity, physical activity and behaviour. Numerous molecular interactions govern changes in ageing that have been grouped into nine hallmarks (identified in (López-Otín et al., 2013) and illustrated by the nine icons. Note that two are not relevant / have not been studied in *Drosophila* and have thus been moved to the bottom of the figure.) The names of *Drosophila* genes that can be manipulated to extend life are listed, having been categorized as belonging to one of the nine hallmarks. In cases where more categorization could have involved more than one hallmark, the most likely was selected and for those changes not belonging to a hallmark, the extra categories “altered metabolic homeostasis” and “other longevity mutants” were created. These changes can be found at various levels of physiological organization and signaling, reflecting a complex interconnection. There is a growing clarity in the way metabolic networks underpin these interconnections.

Figure 2 Ageing-related maintenance of the epigenome is linked to central metabolic pathways.

Manipulating the availability of activated C1 and C2 units, in the form of S-adenosyl methionine and acetyl-CoA respectively, have been uncovered as important regulators of lifespan in *Drosophila*. Importantly, these beneficial metabolic changes also result in improved maintenance of chromatin structure through modifications to the epigenome. This is thought to protect against transcriptional noise that grows with age. Expanding metabolic network models, and their associated effects on signaling, should reveal other metabolic characteristics of ageing and how critical intervention points may be used to slow their decline.