amino-acid pair distances\(^2\). Subdivision of rat–mouse–human codon triplets into classes represented in Tables 1–3 easily follows from the genetic code table. All suitable triplets of bacterial genomes were obtained from the NCBI Entrez database and processed analogously. All alignments are available at ftp://ftp.ncbi.nih.gov/pub/kondrashov/

**Fluctuating negative selection**

We assumed that negative selection at a codon switches off and on at random moments. The expected waiting times (in units of time since rat–mouse divergence) for off to on and on to off switches are \(T\) and \(R\), respectively. Thus, negative selection is present with a probability of \(1/(1 + b)\), where \(b = f_r + f_m\). If the total duration of episodes of negative selection in rat and mouse lineages were \(f_r\) and \(f_m\), respectively, at a two-substitution codon \(P_s = 2\), \(P_r = 2(1 - r)\) and \(P_e = 2(1 - f_r + f_m)\). This model was studied by Monte–Carlo simulations. For each pair of values of \(T\) and \(R\) we performed 1,000,000 runs. Initially, negative selection in both rat and mouse lineages was off with probability \(1/(1 + b)\) and on with probability \(b/(1 + b)\). Then, switches of negative selection and accumulation of substitutions in the two lineages occurred independently. For each run, we calculated the probability that at a codon exactly two substitutions took place in rat and mouse lineages, assuming that substitutions occur independently, only when negative selection is off, with the instant rate \(0.2\) (the value of rat–mouse divergence). After this, the probability of pattern 1 was calculated within two substitution codons.

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**Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body**

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In *Drosophila melanogaster*, ageing is slowed when insulin-like signalling is reduced: life expectancy is extended by more than 50% when the insulin-like receptor (*InR*) or its receptor substrate (*chico*) are mutated, or when insulin-producing cells are ablated\(^3,4\). But we have yet to resolve when insulin affects ageing, or whether insulin signals regulate ageing directly or indirectly through secondary hormones. *Caenorhabditis elegans* lifespan is also extended when insulin signalling is inhibited in certain tissues, or when repressed in adult worms\(^5,6\), and this requires the forkhead transcription factor (FOXO) encoded by *daf-16* (ref. 6). The *D. melanogaster* insulin-like receptor mediates phosphorylation of dFOXO, the equivalent of nematode *daf-16* and mammalian FOXO3a\(^7,8\). We demonstrate here that dFOXO regulates *D. melanogaster* ageing when activated in the adult peripheral fat body. We further show that this limited activation of dFOXO reduces expression of the *Drosophila* insulin-like peptide *dilp-2* synthesized in neurons, and represses endogenous insulin-dependent signalling in peripheral fat body. These findings suggest that autonomous and non-autonomous insulin signalling combine to control ageing.

To investigate whether activated dFOXO affects ageing in *D. melanogaster* we conditionally expressed dFOXO in specific adult tissues. Without ligand binding at the insulin-like receptor, dFOXO remains unphosphorylated and is transported to the nucleus where it promotes factors that retard cell growth and proliferation\(^4,7,8\). We transformed *D. melanogaster* with UAS-constructs, containing either a wild-type full-length complementary DNA of *dFOXO* (UAS-*dFOXO*) or dFOXO with the three protein kinase B (PKB) phosphorylation sites mutated to permit insulin-insensitive nuclear transport (UAS-*dFOXO*-TM). Expression of these constructs in the eye disc reduced growth (Supplementary Fig. S1), as has previously been reported for independent transfectants of UAS-*dFOXO* and for a phosphorylation-site mutant of human FOXO3a\(^7,8\). The constitutive expression of UAS-*dFOXO* or UAS-*dFOXO*-TM killed larvae when promoted from *actin-GAL4*, or when expressed from fat body (*adh-GAL4*) or neurons (*ELAV-GAL4*) (Supplementary Table S1). Therefore, conditional expression of *dFOXO* is required to bypass developmental lethality as well as to study its impact on ageing exclusively in the adult stage.

We used the mifepristone inducible-GAL4 system (annotated P[Switch\(^\dagger\)] and GeneSwitch\(^\dagger\)) to drive the expression of UAS-constructs in defined adult tissues. Ingested mifepristone strongly induced reporter expression at all ages (Supplementary Fig. S1), and the compound alone had no effect on adult survival (Supplementary Fig. S2). Adult survival was not improved when UAS-*dFOXO*-TM was induced by a pan-neuronal driver (ELAV-GeneSwitch), or in glial cells (P[Switch] MB221) or neurolemna (P[Switch] S\(_{13}\)) (Fig. 1; Supplementary Table S2). Thus, broadly activated dFOXO in neuron-associated cells is not sufficient to slow ageing; however, it may do so if expressed in subsets of cells within these tissues. Similarly, expression of UAS-*dFOXO*-TM or UAS-*dFOXO* did not affect survival when induced with the P[Switch] strain S\(_{106}\), an efficient promoter in the fat body\(^9\). In contrast, survival was significantly increased in both sexes when *dFOXO* was induced
with the P[Switch] strain S1,32, which is also expressed in fat body. Multiple independent inserts of UAS–dFOXO-TM and of wild-type UAS–dFOXO increased median lifespan by as much as 35% when induced with 25 μg ml\(^{-1}\) mifepristone and 56% when induced by 50 μg ml\(^{-1}\) mifepristone; averaged across trials, lifespan was increased by 15.5% in males and 19.4% in females (Fig. 1; Supplementary Table S2). Because the phosphatase and tensin homologue protein (PTEN) antagonizes phosphatidylinositol-3-OH kinase activity, which promotes nuclear localization of endogenous dFOXO and inhibits TOR function, we induced UAS–dPTEN with S1,106 and S1,32. Survival was unaffected when PTEN was expressed from S1,106 but increased by about 20% when expressed from S1,32 (Fig. 1; Supplementary Table S2). Together, these data demonstrate that dFOXO activated in a specific tissue can regulate lifespan in adult D. melanogaster.

To understand how S1,32 but not S1,106 can improve lifespan we compared their patterns of expression in adult tissue (Fig. 2). S1,106 was expressed in fat body of the thorax, abdomen and occasionally in the cavity surrounding the mouthparts. In contrast, S1,32 was found expressed in fat body of the head and not in the abdomen or thorax (Fig. 2; Supplementary Fig. S5); S32 uniquely appears in the pericerebral fat body located above the brain. We mapped the insertion site of S1,32 to the first intron of bunched. Although bunched was identified in egg follicles, S1,32 does not express in

![Figure 1](image-url)
these cells (Fig. 2e), perhaps because it is inserted near two intronic open reading frames. We conclude that specifically activated dFOXO in the adult head fat body is sufficient to slow ageing.

Surprisingly, although UAS–dFOXO-TM induced by S132 increased longevity it did not affect fecundity (Supplementary Fig. S2). On the other hand, when we challenged adults with an acute oxidative stress agent (paraquat), survival was improved when UAS–dFOXO-TM was induced by S132 (in the head fat body) but not by S1106 (Fig. 1), in agreement with long-lived insulin signalling mutants of C. elegans and D. melanogaster that are often stress resistant12. Similarly, lipids are frequently elevated in C. elegans and D. melanogaster insulin-signalling mutants13,14 and as anticipated, when dFOXO-TM was expressed in the head fat body, lipid aggregates appeared in this tissue (Fig. 3h). Remarkably, in the same animals, lipids also accumulated in the peripheral fat tissue even though this construct was not expressed outside the head (Fig. 3p).

To understand how dFOXO-TM that is expressed exclusively in the head fat body can regulate integrated physiological traits such as ageing, stress resistance and lipid metabolism, we followed the cellular location of the dFOXO protein in the head and peripheral fat body (Fig. 3). Without transgene induction, endogenous dFOXO was distributed throughout the cytoplasm in all cells. On expression of UAS–dFOXO-TM, antibody-labelled dFOXO was increased in both the cytoplasm and nuclei of the targeted tissue. Notably, dFOXO-TM induced by S132 in head fat body also increased endogenous dFOXO nuclear localization of peripheral fat body, in agreement with the pattern of lipid accumulation. In contrast, dFOXO-TM expressed in peripheral fat body did not affect endogenous dFOXO in the head (Supplementary Fig. S3). As endogenous dFOXO in peripheral tissue can become localized in the nucleus in response to decreased insulin signalling, these results suggest that dFOXO that is activated in the head fat body retards systemic levels of the insulin ligand.

Cells in the pars intercerebralis of the adult brain synthesize insulin...
peptides. To test whether activated dFOXO in the head fat body influences insulin production we measured messenger RNA levels of the seven Drosophila insulin-like peptides (dilp) Complementary DNA was prepared from the heads of S2/TUAS–dFOXO-TM adults fed mifepristone or treated as controls. In a preliminary screen, multiple independent samples were specifically analysed for dilp message abundance using microarrays: dilp-2 alone was reduced in response to activated dFOXO in the head fat body (Supplementary Fig. S4). We used these samples to perform quantitative polymerase chain reaction with reverse transcription (RT–PCR) to determine robustly the relative abundance of dilp message originating in the adult brain: the dilp-2 message decreased nearly threefold whereas dilp-3 and dilp-5 were unchanged (Fig. 4a).

Therefore, insulin signalling within the head fat body influences transcription of one specific dilp of the neuronal insulin-producing cells. Studies across model systems have established that insulin-like signalling can control lifespan non-autonomously from a limited set of cells or a specific tissue. In C. elegans, these cells may occur primarily in the intestine and secondarily in neurons. In mice, a disrupted insulin receptor in the adipose tissue across all life stages altered adult adipose morphology, decreased fasted insulin levels disrupted insulin receptor in the adipose tissue across all life stages altered adult adipose morphology, decreased fasted insulin levels and modestly increased adult survival. Here we show with the fly that activated dFOXO in the head fat body is sufficient to increase both male and female lifespan, to increase resistance to oxidative challenge and to alter whole-animal lipid metabolism. Therefore, in D. melanogaster systemic secondary signals must function downstream of dFOXO, activated in the head fat body. Both juvenile hormone and 20-hydroxyecdysone are reduced in D. melanogaster mutants of InR, and both these hormones have the potential to regulate lifespan. Candidates for secondary hormone signals have yet to be identified in C. elegans but these may involve sterols because daf-9, a cytochrome P450 related to mammalian steriodogenic hydroxylases, functions downstream of daf-2 but upstream of daf-12, which encodes a putative nuclear hormone receptor.

Our data suggest that an insulin peptide itself may function as one secondary messenger of insulin-regulated ageing in D. melanogaster (Fig. 4b). A similar model is emerging for C. elegans: the peptide encoded by ins-7 accelerates ageing and is a systemic agonist of the daf-2 encoded receptor, whereas functional DAF-16 in the intestine non-autonomously activates DAF-16 in distant tissues and is sufficient to increase lifespan. If daf-2 is uniquely reduced when dFOXO is activated in the head fat body of D. melanogaster; this transcriptional change will decrease the amount of circulating insulin peptide released from insulin-producing neurons. Notably, ablation of neuronal cells expressing daf-2 is sufficient to retard demographic ageing and to retard functional decline of the adult heart (R. J. Wessells, E. Fitzgerald, J. R. Cypher, M.T. & R. Bodmer, unpublished data). Ageing of the heart, however, can also be delayed when insulin signalling is inhibited exclusively within the cardiac tissue itself, including heart-specific expression of dFOXO-TM. If senescence of tissue and systems throughout the adult is regulated autonomously by insulin, decreased circulation of this peptide downstream of regulatory insulin action within the head fat body could extend lifespan by reducing the mortality risk associated with degeneration of the soma as a whole.

**Methods**

**Drosophila strains**

ELAV-GeneSwitch-Gal4 and the P(Switch)–Gal4 enhancer trap strains and their expression patterns were provided by G. Roman. UAS–PTEN was w1118 hs-flpU129; UAS–PTEN (III), provided by B. Edgar. With pUAST plasmids containing dFOXO (described in ref. 1) we conducted P-element-mediated germine transformation in an w1118 background and recovered multiple homogenous viable strains with a single insert of UAS–dFOXO-TM (construct of wild-type cDNA). Animals for all described assays were generated from crosses of virgin females from the GT1 strain with males of each dFOXO strain.

**Survivorship**

Adult survivorship was estimated by the extinct cohort method, with data combined across 3 to replicate demography cages per treatment for each genotype. Each demography cage was initiated with 150 newly eclosed adults, mixed sex; dead flies were removed and scored every two days, at which time fresh food was provided in a vial with 5 ml of cornmeal-sugar-agar media and 0.5 ml of yeast paste (on the vial wall). Yeast paste contained live yeast and water at ratio of 2:1 plus 0.5% acetic acid and 1% ethanol (with or without mifepristone). Mifepristone was at 25 μg ml⁻¹ in the yeast paste, unless otherwise stated. Stress survival was measured over 24 h when adults were exposed to paraquat (30 μM in solution with 1% sucrose and 1% ethanol, with or without mifepristone).

**Immunohistochemistry**

Tissues for cryosection and for whole mount were isolated from the pericerebral fat body of the head, and from peripheral fat body from the posterior abdomen of 4-day-old females. Tissues were incubated with the anti-dFOXO antibody (ref. 7; provided by A. Brunet) we conducted P-element-mediated germine transformation in an w1118 background and recovered multiple homogenous viable strains with a single insert of UAS–dFOXO-TM (construct of wild-type cDNA). Animals for all described assays were generated from crosses of virgin females from the GT1 strain with males of each dFOXO strain.
The transcriptional programme of antibody class switching involves the repressor Bach2

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Activated B cells differentiate to plasma cells to secrete IgM or, after undergoing class switch recombination (CSR), to secrete other classes of immunoglobulins1–4. Diversification of antibody function by CSR is important for humoral immunity. However, it remains unclear how the decision for the bifurcation is made. Bach2 is a B-cell-specific transcription repressor interacting with the small Maf proteins whose expression is high only before the plasma cell stage2–5. Here we show that Bach2 is critical for CSR and somatic hypermutation (SHM)5–8 of immunoglobulin genes. Genetic ablation of Bach2 in mice revealed that Bach2 was required for both T-cell-independent and T-cell-dependent IgG responses and SHM. When stimulated in vitro, Bach2-deficient B cells produced IgM, as did wild-type cells, and abundantly expressed Blimp-1 (refs 9, 10) and XBP-1 (ref. 11), critical regulators of the plasmacytic differentiation5, indicating that Bach2 was not required for the plasmacytic differentiation itself. However, they failed to undergo efficient CSR. These findings define Bach2 as a key regulator of antibody response and provide an insight into the orchestration of CSR and SHM during plasma cell differentiation.

After exposure to antigen in vivo, B-cell responses may involve SHM of the variable (V)-region exons to increase the affinity of antibody, and CSR of the constant (C)-region exons of immunoglobulin heavy chain (IgH) gene to produce antibodies with the same antigen specificity performing unique effector functions1–4,8. Because SHM and CSR change DNA information and are therefore potentially mutagenic, their deployment must be tightly regulated. However, little is known about how transcription factors programme CSR and SHM in B cells.

Antigen-dependent terminal differentiation of B cells takes place within the secondary lymphoid organs such as spleen and lymph nodes14. Using anti-Bach2 antibodies, we performed an immunohistochemical analysis of Bach2 in the mouse spleen. Bach2 was expressed in IgM-positive cells within the lymphoid follicles that were surrounded by marginal sinus expressing mucosal adhesion cell adhesion molecule-1 (MadCAM-1) Fig. 1a). Marginal-zone B cells, which are IgM-positive and located outside MadCAM-1-expressing cells, were negative for Bach2. Bach2 was not detected in the CD3e-positive T cells within the T-cell zone. Such an expression pattern suggests a role for Bach2 in antibody response.

To investigate this possibility, we disrupted the mouse Bach2 gene by replacing the first coding exon with a neo resistance gene cassette (Supplementary Fig. 1). Breeding of Bach2−/− mice resulted in Bach2−/− progeny at the expected mendelian

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(whole mount) anti-rabbit IgG secondary antibody, followed by staining with DAPI. For sections, all images were captured using a standardized exposure.

Quantitative PCR

The cDNA was prepared from 100 heads per sample of 10-day-old females: four independent samples from mifepristone-treated S32/UAS–dFOXO–TM adults (25 g mifepristone), and eight independent samples from controls (four of S32/UAS–dFOXO–TM without mifepristone and four of S32 with mifepristone). Relative message abundance was measured by amplification with SYBR Green I on an ABI Prism 7700 cycler (Applied Biosystems) and standardized against the gapdh message. Differences between treatment and control were tested by nested analysis of variance, with replicate amplifications as a random effect.

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