Ageing, TOR and amino acid restriction: a cross-tissue transcriptional network connects GATA factors to *Drosophila* longevity

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Abstract

Animal lifespan can be extended by dietary restriction (DR), but at a cost to fitness. This phenomenon depends on essential amino acids (EAAs) and TOR signalling, but roles of specific tissues and downstream transcriptional regulators are poorly characterised. Manipulating relevant transcription factors (TFs) specifically in lifespan-limiting tissues may ameliorate ageing without costs of DR. Here we identify TFs which regulate the DR phenotype in *Drosophila*, analysing organs as an interacting system and reducing its transcriptional complexity by two orders of magnitude. Evolutionarily conserved GATA TFs are predicted to regulate the overlapping effects of DR and TOR on organs, and genetic analyses confirmed that these TFs interact with diet to determine lifespan. Importantly, *Srp* knockdown insulated fly lifespan from the pernicious effects of EAAs, but tissue-specific knockdown reduced the corrolary costs. These results provide the first indication that benefits of EAAs for early-life fitness can be decoupled from longevity by tissue-specific transcriptional reprogramming.

Background

How can lifelong health be maximised? Answering this question is a major goal, as ever-increasing human lifespans outpace advances in gerontology, at great social, personal and financial cost [1]. Dietary restriction (DR) has the evolutionarily conserved capacity to improve lifelong health by reducing nutrient intake, but at a cost of reduced biological fitness and vigour in youth [2]. Despite having been discovered 80 years ago [3], the molecular mechanisms underpinning lifespan extension by DR remain elusive. Defining these mechanisms could ameliorate the burden of ageing without the costs of DR.

Calories do not fully account for the benefits of DR: specific nutrients and their relative ratios are key [4–6]. In *Drosophila*, the ratio of dietary sugar to yeast modulates lifespan, which is explained by essential amino acids (EAAs) from the yeast [7], and importantly the same mechanism is conserved in mice [8,9]. Recent evidence indicates that the phenotype of EAA-restricted *Drosophila* is recapitulated by pharmacologically suppressing the Target of Rapamycin (TOR) pathway [10,11], consistent with molecular evidence that EAAs positively regulate TOR [12]. Understanding of how TOR curtails lifespan is incomplete, although maintenance of proteome quality likely plays a role [13–15]. TOR also affects transcription [16–18], but to date this effect has been relatively poorly studied.

In *Drosophila*, transcriptomic responses to DR and TOR have been characterised at the cellular and organismal levels [18], but information on tissue-specific and organ-specific transcription is a requisite advance for many reasons. Primarily, an animal's overall phenotype is determined by tissues coordinating to match their collective functions to the environment. Consequently, to fully understand organismal phenotypes we must account for tissues as an interacting and integrated system. By quantifying the changes to that system in low-TOR or DR states, we gain a complete view of how tissues collectively mediate the DR phenotype. Such an understanding is likely to help identify manipulations of regulators of the DR transcriptional state, which may impart longevity even under *ad libitum* feeding. Minimising the number of tissues in which these regulators are manipulated may ameliorate associated costs, if costs and benefits of DR are mediated by distinct tissues. This approach recognises the findings of studies of insulin signalling, which indicate that tissue-restricted manipula-

tions are sufficient to extend lifespan, and therefore that transcriptional mechanisms affecting longevity are incompletely described by organismal analyses [19,20]. Altogether, these observations suggest that understanding longevity via DR requires (1) modelling of transcription across systems of interacting tissues, (2) identification of changes to that system under DR, (3) quantification of the TOR-dependence of these changes, (4) prediction and testing of tissue-specific regulators of DR-like gene expression.

Recent analytical advances facilitate prediction of transcriptional regulators from patterns of gene expression. Regulatory elements, such as transcription factors, can be predicted by enrichment analysis amongst groups of genes [21,22]. Transcriptional network analysis, also known as coexpression analysis, identifies transcripts which are expressed together [23,24]. The patterns in a transcriptional network therefore reflect the tendency of genes to be switched on and off together, which can be considered a reflection of the underlying gene-regulatory machinery. Consequently, transcriptional network analyses provide a strong basis from which to predict regulatory elements. Network analysis can also answer the need to understand tissues as a complete system, by identifying how patterns of expression in one tissue which correspond to that in others. Therefore, by defining transcriptional networks across tissues and how they change under DR, we can distill the complex interactions between transcripts and tissues that define the phenotype of the DR animal, and systematically reflect the status of the underlying gene-regulatory machinery.

Here, we address a key question: By studying transcription across tissues, can we identify manipulations of transcriptional regulators to recapitulate the DR phenotype? This study is facilitated by dietary manipulations that offer a precise toolset to dissect DR. Previously, we developed a semi-defined *Drosophila* diet, which is optimal for early-life egg laying, in which 50% of available EAAs are provided as a supplement to yeast-based medium [7,10]. Against this “fully fed” control, lifespan can be extended by two interventions, both with correlated fecundity costs to egg laying in early life: either omission of the EAA supplement (i.e. DR); or the addition of rapamycin (EAA+rapamycin), which extends lifespan in the presence of EAAs by suppressing TOR pharmacologically. These tools allow us to establish the effects of EAA dilution, and the TOR-dependence of these effects. Taking these diets and capitalising on orthology between *Drosophila* and vertebrate organs, we studied transcriptomes in the brain, fat body (the analogue of the vertebrate liver and adipose), gut, ovary

and thorax (which largely comprises muscle). We then used transcriptional network analysis to analyse these tissues integratively, reflecting underlying regulatory machinery. Promoter analysis was then applied to identify candidate transcription factor regulators. Crucially, when we tested these transcription factors genetically, we were able to validate their role as mediators of dietary effects on lifespan, and also demonstrate that costs of lifespan extension can be mitigated by targeting interventions to specific tissues.

Results

The effects of DR on ageing are thought to be mediated by the TOR pathway. Here, by characterising the organ-specific transcriptional changes caused by EAA restriction (DR) and pharmacological TOR suppression, we isolate changes that are associated with longevity in both experimental conditions, and address whether the effects of DR mimic the effects of low TOR. We also associate *cis*-regulatory elements with these changes, to predict relevant transcription factors. The study design is presented in Figure 1. We term DR as a diet-induced longevity condition, and rapamycin supplementation as a drug-induced longevity condition.

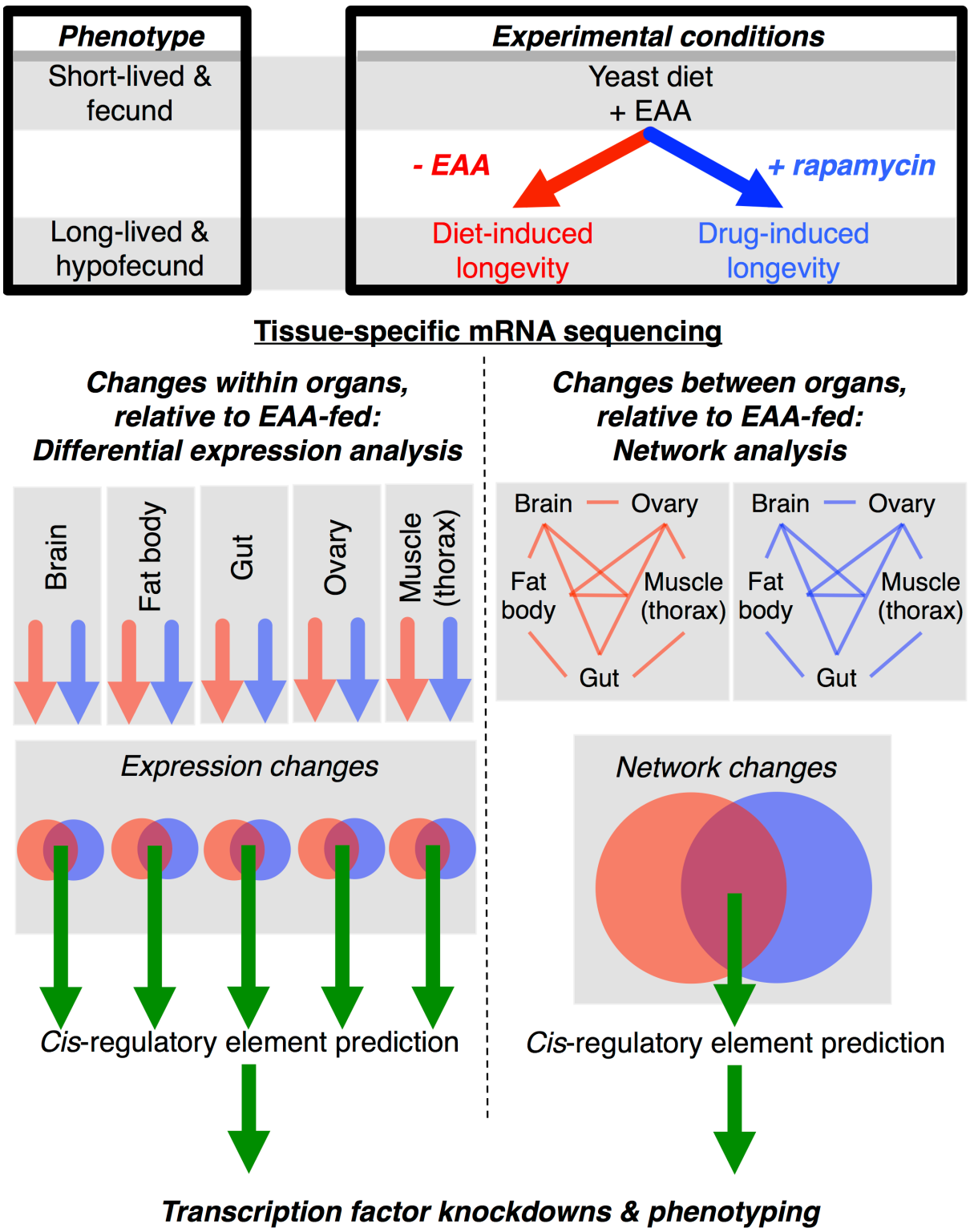

Figure 1. Study design. Against a control in which short lifespan is determined by enrichment of essential amino acids (EAAs), lifespan is extended by DR of the EAAs (diet-induced longevity), or pharmacologically, by administration of the TOR-suppressive drug rapamycin (drug-induced longevity). This lifespan extension comes at a biological cost of reduced early-life fitness (i.e. fecundity). Tissue-specific transcriptional changes associated with both conditions are therefore longevity-

associated, EAA-dependent and TOR-dependent. Identifying *Cis*-regulatory elements associated with these transcriptional networks predicts mechanisms by which DR affects transcription via TOR.

104 *Tissue-specific transcriptomic effects of DR are recapitulated by TOR sup-*
105 *pression*

106 To assess whether the conditions of diet-induced longevity and drug-induced
107 longevity have equivalent transcriptional effects, we first studied the equivalence of
108 these treatments within tissues. We compared the changes in expression of all genes
109 in the transcriptome under these two conditions, with the prediction that the induced
110 changes would be positively correlated. As predicted, in whole flies, brains, guts, ova-
111 ries and thoraces, the diet-induced and drug-induced longevity conditions had posi-
112 tively correlated effects on gene expression (Figure 2). The transcriptional changes
113 observed in these organs therefore mirror the associated changes to lifespan [10].
114 However, the two long-lived conditions did not have equivalent effects in the fat body,
115 indicating that the coordination of function is largely but not obligately coupled across
116 organs. To test explicitly the overlapping effects of DR and TOR suppression at the
117 level of individual transcripts, differential expression analysis was performed. Surpris-
118 ingly, no genes were differentially expressed in the ovary under DR, despite the ro-
119 bust decrease in egg laying in this condition [10]. However, in each other tissue, both
120 lifespan-enhancing treatments had overlapping transcriptional signatures (Table 1).
121 Overall, these analyses demonstrate that the transcriptional effects of DR largely mir-
122 ror those of pharmacological TOR suppression.
123

124

Table I. Overlapping effects of the diet-induced longevity (DR) and drug-induced longevity (rapamycin) conditions on changes in gene expression within specific tissues.

Gene set		Number of genes†			
Tissue	**Expression change***	**DR**	**Rapamycin**	**Overlap**	**P-value§**
Whole fly	Up	254	2239	203	5.55e-121
	Down	40	2159	17	3.99e-06
Brain	Up	154	22	1	0.02
	Down	439	82	63	8.44e-82
Fat body	Up	221	160	6	0.02
	Down	408	156	56	2.75e-46
Gut	Up	8	49	6	7.60e-17
	Down	72	52	6	1.78e-08
Ovary	Up	0	30	NA	NA
	Down	0	47	NA	NA
Thorax	Up	141	39	10	1.32e-13
	Down	153	25	6	8.11e-09

* Relative to EAA diet

† Number of genes showing given change in expression

§ Hypergeometric test

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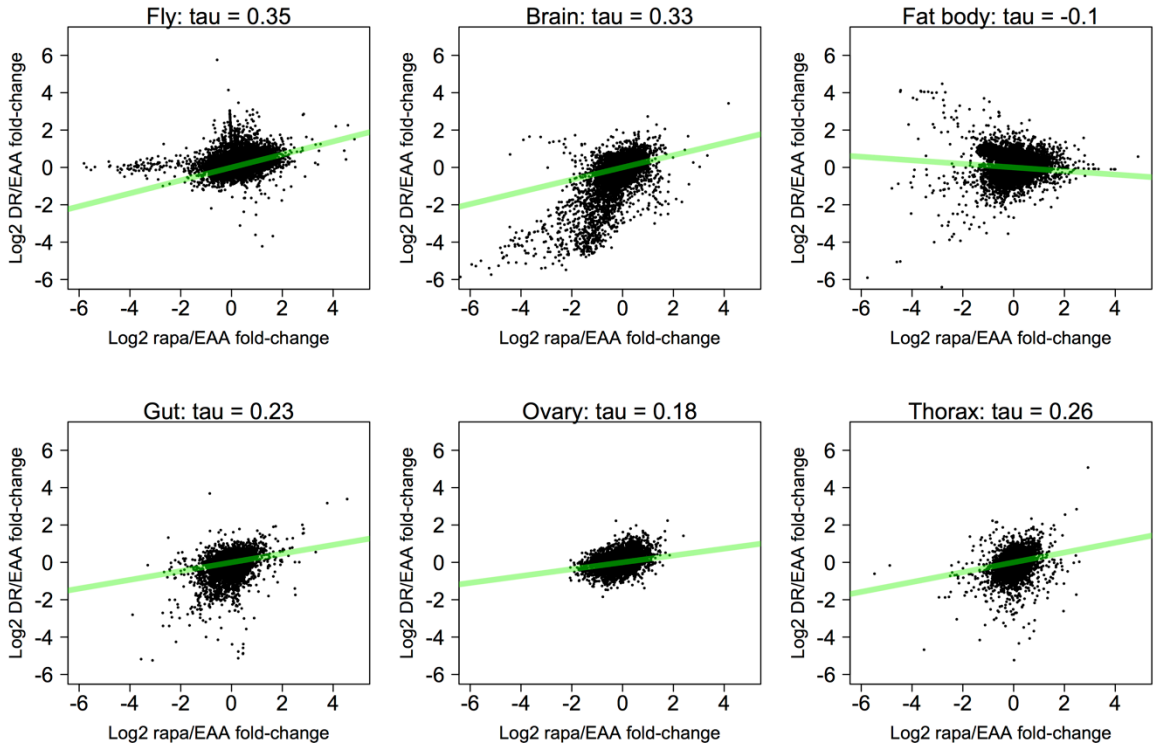
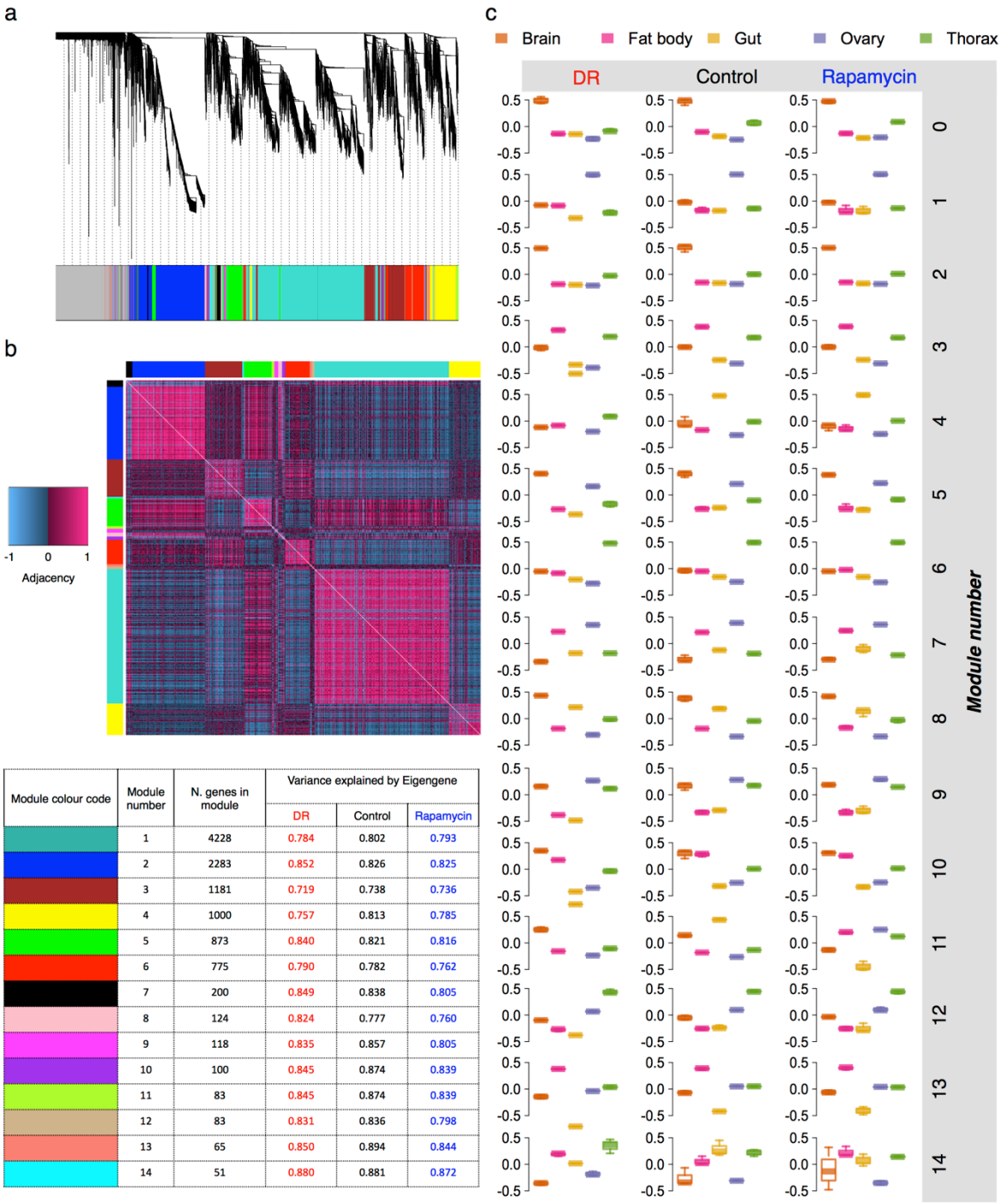


Figure 2. Changes in expression induced by DR correlate those induced by TOR suppression. Log_2 fold-changes in expression in the diet-induced longevity and drug-induced longevity conditions, relative to the EAA-enriched control (DR/EAA fold-change and rapa/EAA fold-change, respectively), were calculated for every gene in the transcriptome. Fold-changes were calculated by DESeq2. Text above panels indicates correlations (Kendall's Tau) between fold-changes induced by the two long-lived conditions. All p-values $< 2.2e^{-16}$

To look for ubiquitous molecular signatures of lifespan extension, we examined overlaps between the transcriptional changes observed between different tissues. Surprisingly, no one transcript responded to DR/TOR in all organs, and the whole-fly samples captured only a small portion of the organ-specific changes associated with lifespan extension (Figure S1). Therefore, DR cannot be understood in terms of any one tissue. To address whether this was functionally relevant, we analysed enrichment of GO terms amongst the transcripts associated with longevity. Most GO terms (83%) were associated with specific organs, showing that there is also no ubiquitous functional signature of DR/TOR (Figure S2). Together these analyses show that the DR regulon can largely be accounted for by TOR (excepting the ovary), but that the identity of the responsive transcripts is tissue-dependent. The organ-specificity of longevity-associated changes indicates parallel responses amongst organs to EAAs and rapamycin, suggesting the coordination of organ-specific functions changes under DR and low systemic TOR.

Diet and TOR suppression have overlapping effects on the orchestration of expression across organs

Discrete tissues compartmentalise functions, and an individual's fitness depends on correctly orchestrating these tissue-specific functions to match the nutritional environment. It is therefore possible that the lifespan benefits of DR and TOR suppression may be mediated by certain tissues, and the biological costs by others. Therefore we investigated how DR changes the orchestration of gene expression across organs, studied as an integrated system. To identify interdependent gene expression (i.e. gene coexpression) amongst organs, we employed Weighted Gene Coexpression Network Analysis (WGCNA; [23]). This approach can identify codependent gene expression and reduce dimensionality of complex data: therefore, applying it in our study allowed us to identify codependent gene expression across organs, and changes in those codependencies in the long-lived conditions. After applying quality controls and removing genes with zero variance, 11164 of 13442 analysed genes clustered into 14 modules (Figure 3a, Figure 3b, Supplementary Materials), representing groups of genes that share expression patterns across organs. By describing modularity in our data, this network provides a complete description of the structure of the transcriptome across all organs and diets under study.



Module colour code	Module number	N. genes in module	Variance explained by Eigengene		
			DR	Control	Rapamycin
	1	4228	0.784	0.802	0.793
	2	2283	0.852	0.826	0.825
	3	1181	0.719	0.738	0.736
	4	1000	0.757	0.813	0.785
	5	873	0.840	0.821	0.816
	6	775	0.790	0.782	0.762
	7	200	0.849	0.838	0.805
	8	124	0.824	0.777	0.760
	9	118	0.835	0.857	0.805
	10	100	0.845	0.874	0.839
	11	83	0.845	0.874	0.839
	12	83	0.831	0.836	0.798
	13	65	0.850	0.894	0.844
	14	51	0.880	0.881	0.872

Figure 3. A network description of transcription across *Drosophila* organs and long-lived conditions. (a) Consensus transcriptional modules found across organs in all three experimental conditions. Genes were clustered according to their expression ($\log_2$ RPKM) across organs by Weighted Gene Coexpression Network Analysis (WGCNA), revealing modules of genes showing similar coexpression across organs. Leaves of the tree indicate genes. The tree was cut by hybrid tree cutting to define transcriptional modules (clusters). Tree cutting assigned genes to one of 14 consensus transcriptional modules, indicated by colour-coded vertical lines that together form the horizontal bar beneath the dendrogram (see Supplementary Spreadsheets

for assignments of genes to modules). Genes not assigned to any module are colour-coded grey. **(b)** Module assignments identify clear structure in the transcriptional network, using the EAA-fed control as an example. Modules are indicated by coloured side bars. The heatmap indicates gene-gene adjacency (i.e. signed suared correlations) in expression. **(c)** Representative expression (Eigengenes) of all modules (y-axis showing Eigengene values, A.U.). These data summarise changes in transcriptional networks across organs and experimental conditions. Data are plotted by experimental condition in columns ("DR" = diet-induced longevity, "Rapamycin" = drug-induced longevity, short-lived control = "EAA"), and transcriptional module in rows. Within each plot, boxes are colour-coded by tissue (see key). Boxplots show medians (horizontal midline), 1st and 3rd quartiles (hinges), and range of data points.

We built on the transcriptional network analysis to isolate significant changes in network structure in the two long-lived conditions, by identifying pairs of modules exhibiting significantly changed coregulation. Quantifying module coregulation required reduction of the complexity of the data. Therefore, we summarised gene expression in each module with a single "Eigengene" vector per module (Figure 3c), calculated as the first principal component of expression of the genes in each module [25]. These Eigengenes accounted for between 72% and 89% of the variance in each module (weighted average = 79.86%, Figure 3b), thus simplifying the description of the organ system by two orders of magnitude, from $11.1e^3$ transcripts to 14 Eigengenes, whilst retaining ~80% of total information. We then used these Eigengenes to quantify changes in module-module coregulation. For all pairs of Eigengenes, correlations were calculated in each dietary condition then, for each long-lived condition, the correlation coefficients were subtracted from the corresponding coefficient in the EAA-fed control. This quantified changes in module-module coregulation induced in the two long-lived conditions. Repeating the same procedure on random permutations of the data generated null distributions for each pair of modules. Pairs of modules which showed significantly altered coregulation were identified by comparing the observed changes in correlation coefficients to the null distributions. This analysis thereby systematically identified pairs of modules whose coregulation was significantly altered by the conditions causing longer life (Figure 4). The diet-induced longevity condition significantly changed the coregulation of three pairs of modules (4 & 13, 4 & 3, 14 & 13). All significant changes in the drug-induced longevity condition were paired with module 11 (relative to modules 1, 4, 7, 8, 9, 12, 13). This correlation-permutation approach identified changes in coregulation of pairs of modules, but did not reveal whether those changes were driven by one or both members of each pair. Therefore, by three separate methods, we asked which individual modules' Eigengenes were most strongly perturbed across the experimental conditions (Figure S3). These three analyses identified modules 4, 11 and 14, which were all identified by the correlation-permutation analysis. Therefore, the Eigengene analyses collectively indicated that altered regulation of modules 4, 11 and 14 changes the coordination of functions across organs in the long-lived conditions. Module 11 was associated specifically with drug-induced longevity, module 14 was associated specifically with diet-induced longevity, and module 4 was associated with both diet-induced and drug-induced longevity. These results indicate that the coexpression of

module 4 with other modules may be associated generally with longevity by TOR suppression, whilst modules 11 and 14 exhibit intervention-specific changes in coexpression.

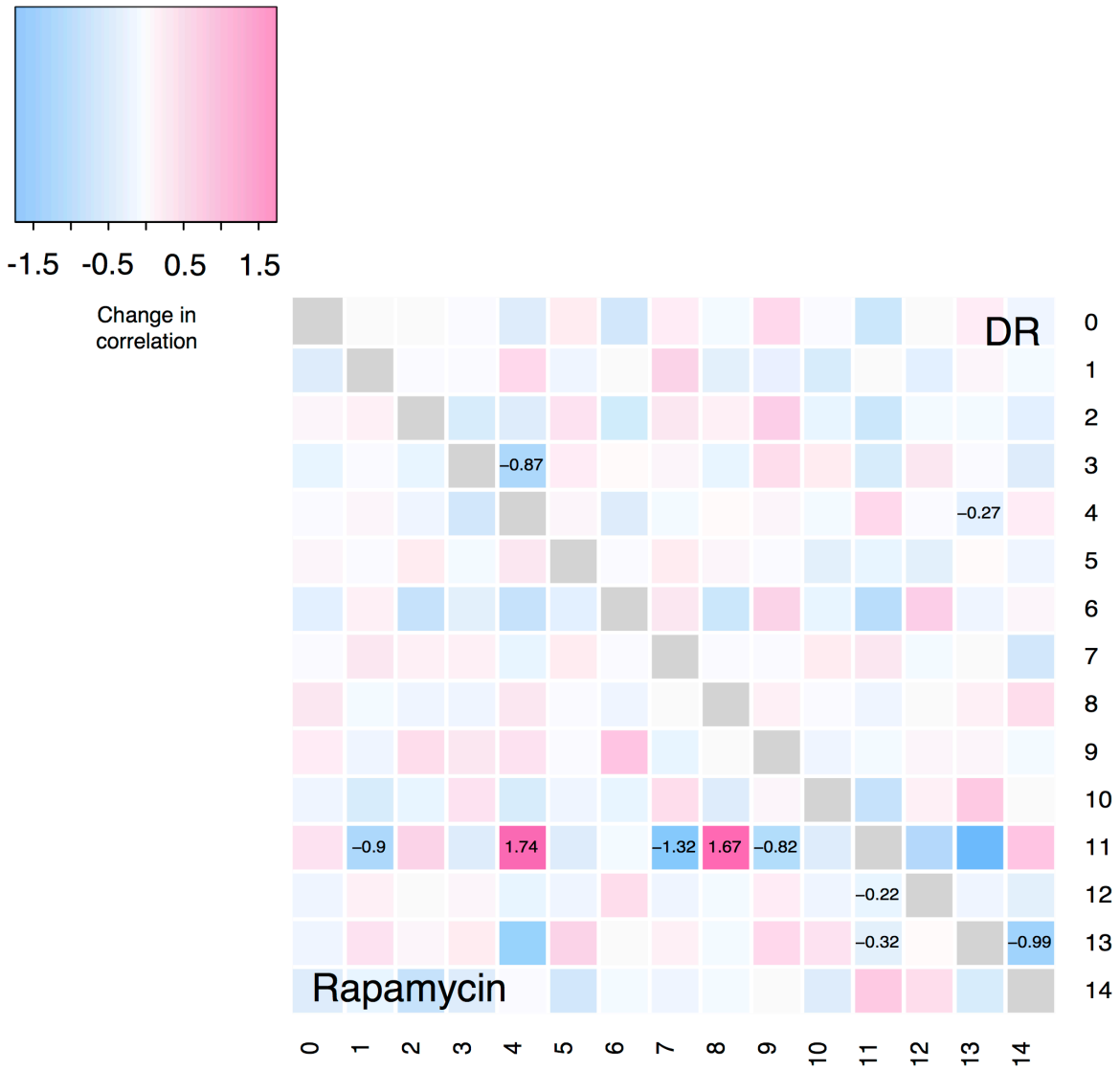


Figure 4. Structure of longevity-associated changes in the transcriptional network. The heatmap shows the observed change in correlation (Spearman's rho) between pairs of modules in response to DR and in response to TOR suppression (Rapamycin), relative to the fully EAA-fed condition. Row and column labels represent transcriptional modules. Values indicate the difference in correlation between the given condition and the EAA-fed condition, and are given when the change was significant ($p \leq 0.05$) according to permutation testing.

198 To identify functions that are likely affected by changes to coexpression
199 across organs, we analysed enrichment of Gene Ontology (GO) terms within each
200 module (Supplementary Materials). Distinct transcriptional modules were associated
201 with non-overlapping functions, indicating that these modules represent functionally
202 distinct suites of genes, and therefore changes to their coexpression is likely physio-
203 logically relevant. Of the modules implicated in responses to DR, Module 4 was en-
204 riched in extracellular metabolic enzymes - particularly peptidases - and also lipases
205 and carbohydrases. Module 14 was enriched in transmembrane sugar transporters
206 and polysaccharide/carbohydrate binding. Module 11 was enriched in ATP-binding
207 cassette (ABC) transporter activity. Taken together, the transcriptional network and
208 GO analyses suggest that longevity is associated with altered orchestration of meta-
209 bolic functions across organs, and not changes to the same metabolic program in all
210 tissues.
211
212 *Predicting transcription factors to regulate longevity-associated transcriptional*
213 *changes*
214 We sought to identify candidate regulators of transcriptional changes associ-
215 ated with diet-induced longevity and drug-induced longevity. We predict that manipu-
216 lating transcription factors (TFs) that regulate DR/TOR-dependent transcriptional
217 regulation will mimic DR by correctly coordinating diverse transcriptional targets. We
218 identified transcription factor binding site motifs (TFBSs) associated with transcrip-
219 tional variation across organs in the long-lived conditions. This process associated
220 each transcriptional module with a potentially unique set of TFs, but clustering mod-
221 ules by these TF sets grouped modules 4, 11 and 14 (Figure 5a), which the transcrip-
222 tional network analyses had implicated in longevity. This clustering therefore sug-
223 gested that these longevity-associated modules share common upstream regulators.
224 Indeed, the sets of TFs identified in the longevity-associated modules overlapped
225 substantially (Figure 5b), and these shared motifs were also the most significantly
226 enriched; for each longevity-associated module, the most strongly enriched motifs
227 bind GATA family TFs (*GATAd, GATAd, grn, pnr, srp*) (Table II). Furthermore, the
228 most significant association observed in the entire analysis was between GATA-
229 binding motifs and Module 4, the only module associated with both diet-induced and
230 drug-induced longevity. Other motifs associated with all three longevity-associated
231 modules were annotated with *Bx* - which physically binds the GATA TF *pnr*; *Ham*,

232 which has roles in cell fate determination [26,27]; and *CG10348*, which has no known
233 regulatory function. However, the association with these latter three transcription fac-
234 tors was less significant than the association with GATA factors. Looking across all
235 modules, all but 2 and 5 were associated with some GATA motifs, however, the
236 strength of enrichment (Escore) for the GATA factors was significantly greater for the
237 longevity-associated modules 14 and 4 (Figure 5c). The final evidence of association
238 between GATA factors and longevity-associated modules was that Module 4 was not
239 only highly enriched in GATA binding sites, but also contained GATAe (Supplemen-
240 tary Files). Experimental studies have shown correlated expression of a TF and its
241 putative targets is a strong predictor of causal relationships [21,22]. Altogether, these
242 findings strongly implicated GATA factors in the tissue-specific transcriptional chang-
243 es observed under low TOR.
244

Table II. Annotations for top five-ranked motifs enriched in association with transcriptional modules. Unannotated motifs are excluded.

Longevity-associated	Module number	TF*	Escore†
Diet-induced & Drug-induced	4	grn srp pnr GATAd GATAe	11.58
		grn srp pnr GATAd GATAe	11.21
		grn srp pnr GATAd GATAe	10.07
		grn srp pnr GATAd GATAe	10.07
		grn srp pnr GATAd GATAe	10.07
Drug-induced	11	grn srp pnr GATAd GATAe	5.95
		grn srp pnr GATAd GATAe	5.88
		CG10348 ham	5.68
		grn srp pnr GATAd GATAe	5.56
		CG10348 ham	5.56
Diet-induced	14	grn GATAd pnr GATAe	10.63
		grn srp pnr GATAd GATAe	9.67
		grn GATAd pnr GATAe	9.05
		grn srp pnr GATAd GATAe	8.69
		grn GATAd pnr GATAe	8.60
No	1	Dref	8.50
		Dref	7.28
		Dref	5.66
		crp	4.78
		CG7928	4.47
No	2	lola	4.57
		lola	4.44
		Trl	4.43
		Trl	4.43
		Trl	4.42
No	3	grh	7.30
		grh	6.64
		GATAe srp pnr GATAd	6.24
		grn srp pnr GATAd GATAe	5.91
		grn srp pnr GATAd GATAe	5.91
No	5	CG9906 CG1924 Cnx99A	4.94
		jim	4.21
		nerfin-1 nerfin-2	4.01
		slp1 slp2 foxo fd19B	3.88
		Dref	3.87
No	6	vri	6.88
		gt	5.73
		Mef2	5.54
		Mef2	5.43
		vri	5.18
No	7	crp	5.08
		pnr	3.82
		HLH106	3.80
		CrebB-17A	3.61
		Hnf4	3.43
No	8	CG10348 ham	5.63
		CG10348 ham	5.44
		scrt	5.35
		scrt	5.00
		CG12605	4.91
No	9	crp	6.92
		Trl	5.44
		Trl	5.44
		Trl	4.96
		Trl CG33260	4.68
		Atf6	5.60
		grn GATAd pnr GATAe	4.37

No	10	CG11071	4.35
		grn GATAd pnr GATAe	4.34
		srp	4.31
No	12	crp	9.27
		CG6792	6.20
		kay Atf3	5.58
		Dref	4.91
		vri	4.74
No	13	HLH106	8.86
		HLH106	7.99
		GATAd	6.58
		srp	6.50
		GATAe	6.37

245 * Genes annotated as associated with the motif
246 † Escore for the motif
247

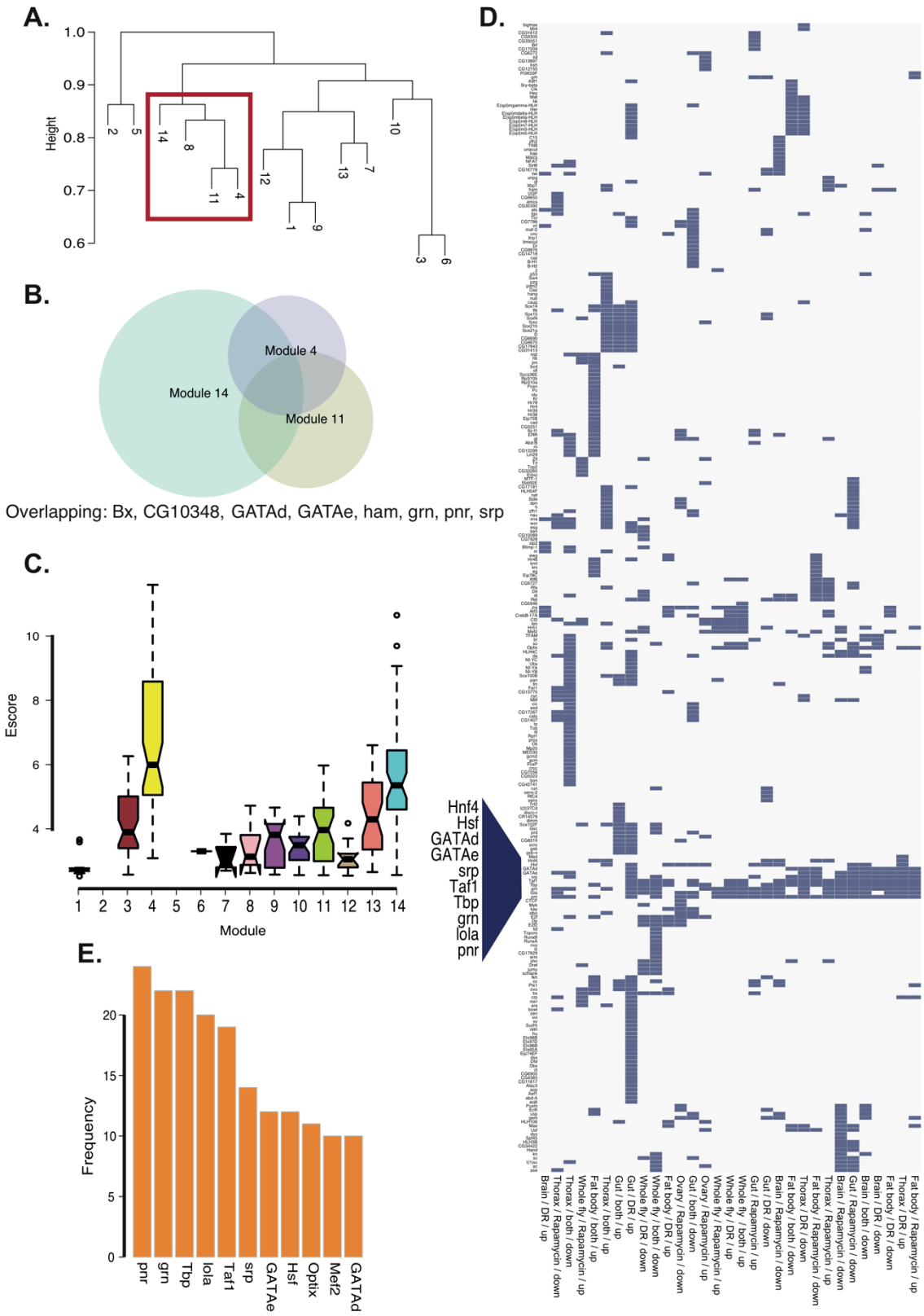


Figure 5. A signature of GATA transcription factors as regulators of the transcriptional response to DR and/or TOR. (a) Lifespan-associated transcriptional modules are grouped by sharing of transcription factor binding site motifs. Each module was tested for enrichment of *cis*-regulatory elements, and clustered by the

presence/absence of associated transcription factor binding site motifs. Dendrogram labels represent module numbers. This analysis clusters together transcriptional modules associated with longevity (4, 11, 14, also clustered with module 8). **(b)** Longevity-associated modules are associated with overlapping sets of transcription factor binding sites. Text below the Venn diagram names the transcription factors associated with binding sites enriched in all three longevity-associated modules. **(c)** Distribution per module of enrichment (Escore) of motifs annotated as binding GATA transcription factors. The box plots show medians (horizontal midline), 1st and 3rd quartiles (hinges), and range of data points. The notches equate to ~95% confidence intervals for the medians [(1.58 * interquartile range) / (square root *n*)]. The plot shows that enrichment of GATA binding sites is particularly strong for modules 4 and 14, both of which are associated with lifespan extension by DR. **(d)** GATA factor binding sites are enriched across multiple gene sets regulated by DR/TOR. Columns represent sets of genes according to tissue and the sign of expression change per treatment. Rows represent TFs associated with the sets of differentially expressed genes by enrichment of binding site motifs in the gene set. The axes are ordered by clustering the presence (blue) and absence (grey) of TF binding sites, using binary distance. Names of transcription factors occurring in a cluster across multiple gene sets have been magnified. **(e)** The bar plot shows the frequency of associations with longevity-associated transcriptional changes within organs, for the most frequently associated TFs. TFs associated with <10 gene sets are excluded.

248 To complement the analysis of transcriptional network regulators, we repeated
249 the same approach on sets of genes which showed changed regulation (differential
250 expression) within organs in one or both of the long-lived conditions (Table III). Con-
251 sistent with the absence of an organ-nonspecific transcriptional signature of DR/TOR,
252 most TFBSs identified by this analysis were associated with specific tissues. Howev-
253 er, one cluster of TFs was associated with multiple gene sets across organs, and that
254 cluster contained the GATA factors (Figure 5d). Across all gene sets, each GATA
255 factor was associated with at least 32% of diet/tissue-specific gene sets (Figure 5e).
256 GATA factors were therefore associated with DR/TOR-dependent transcriptional
257 changess within organs, recapitulating the parallel observations across organs.
258

Table III. Annotations for top five ranked motifs enriched in association with differentially expressed gene sets. Unannotated motifs are excluded.

Gene set				
Tissue	DR*	Rapamycin*	TFs**	Escore†
Whole fly	-	-	Tbp,Taf1	11.11
			E2f,E2f2,Dp	10.22
			Tbp	8.98
			E2f,E2f2,Dp	8.17
			E2f,E2f2,Dp	7.96
Whole fly	+	+	Cf2	6.25
			Mef2	5.41
			bin	5.40
			Cf2	5.37
			Cf2	5.22
Whole fly	-		E2f,E2f2,Dp	6.55
			Tbp,Taf1	6.44
			lola	6.27
			Blimp-1	5.98
			Blimp-1	5.98
Whole fly	+		Cf2	5.84
			Mef2	5.23
			Mef2	5.22
			Optix	5.19
			bin	5.07
Whole fly		-	Top2	4.32
			Dref	4.05
			Trl,CG33260	3.93
			Dref	3.92
			Dref	3.87
Whole fly		+	lola	8.29
			lola	8.27
			lola	7.61
			Optix	4.70
			bin	4.45
Brain	-	-	Tbp	8.40
			Tbp,Taf1	8.35
			grn,pnr	6.85
			pnr	6.21
			lola	6.13
Brain	-		pnr	11.03
			grn	10.04
			ham	10.01
			grn,pnr	9.95
			pnr	9.55
Brain	+		Jra	4.31
			Jra	4.29
			Atf3	4.02
			sna	3.77
			ato	3.68
Brain		-	Tbp,Taf1	9.58
			Tbp	7.63
			srp	5.33
			lola	5.02
			grn,pnr	4.76
			Tbp	8.07
			Tbp,Taf1	7.42

Brain		+	gem	4.89
			pnr	4.63
			tin	4.57
Fat body	-	-	Rel	8.75
			Rel	7.10
			Tbp	6.94
			pnr	5.50
			Tbp,Taf1	4.99
Fat body	+	+	hb	6.89
			hb	6.34
			sqz,rn,Lin29	5.90
			hb	5.83
			sqz,rn,Lin29	5.70
Fat body	-		pnr	5.45
			grn	5.34
			pnr	4.85
			grn	4.77
			GATAe	4.65
Fat body	+		Hsf	6.54
			Hsf	5.75
			Hsf	5.10
			ovo	4.77
			bs	4.49
Fat body		-	GATAd	6.61
			srp	5.77
			pnr	5.53
			GATAe	5.46
			Tbp	5.38
Fat body		+	lola	6.46
			grn	6.45
			lola	6.15
			grn	5.87
			pnr	5.62
Gut	-	-	cic	10.44
			Tbp	8.89
			Tor	7.88
			Tbp,Taf1	6.90
			Tbp	6.81
Gut	+	+	scro	13.34
			oc	12.11
			scro	10.44
			ttk	10.16
			vnd	9.38
Gut	-		gem	6.79
			grn	6.49
			pnr	6.37
			pnr	5.53
			grn,pnr	4.92
Gut	+		oc	8.97
			fru	7.82
			scro	7.61
			fru	7.51
			ken	7.28
Gut		-	zfh1	8.56
			Tbp,Taf1	8.19
			da	8.18
			Tbp	7.93

			da	7.41
Gut		+	Hsf	4.58
			pnr	4.46
			ERR	4.11
			br	3.77
			grh	3.71
Ovary		-	E2f,E2f2,Dp	4.56
			Myb	4.55
			Myb	4.46
			vri	4.41
			vri	4.26
Ovary		+	twi	5.19
			HLH106	4.18
			CG13897	3.89
			bin	3.87
			foxo	3.81
Thorax	-	-	grn	9.63
			grn	8.02
			pnr	7.72
			pnr	7.71
			pnr	7.63
Thorax	+	+	zfh1	6.02
			sna	5.55
			sna	5.41
			ham	5.32
			Rfx	4.24
Thorax	-		Tbp	9.37
			grn,pnr	9.02
			Tbp,Taf1	8.47
			GATAd	7.43
			GATAe	7.07
Thorax	+		Tbp,Taf1	10.54
			Tbp	8.63
			lola	7.43
			grn	6.99
			Hnf4	6.86
Thorax		-	ttk	7.43
			crp	7.19
			ttk	6.93
			Fer1	6.46
			ase	6.34
Thorax		+	Rel	7.19
			Tbp,Taf1	7.02
			Tbp	6.95
			Rel	5.03
			Rfx	4.68

* sign of expression change, relative to EAA diet. The gene set is defined by only the effect of one treatment when only one symbol is given.
** Genes annotated as associated with the motif
† Escore for the motif

260

261

262 The TFBS enrichment analysis associated GATA factors both with transcrip-
263 tional changes within organs and altered functions of the organs as a collective
264 whole. This association was consistent with prior knowledge of the evolutionarily
265 conserved biology of these TFs. GATA factors play known roles in signalling amino
266 acid availability via TOR in evolutionarily diverse eukaryotes (e.g. yeast: [28]; and mos-
267 quitos: [29]), as well as being required for lifespan extension by some tissue-specific
268 genetic lesions in worms [20]. To test for evolutionarily conserved connections between
269 GATA factors and DR-responsive transcripts, we established the association be-
270 tween TFBSs and orthologs of DR-responsive genes across 12 *Drosophila* species.
271 These 12 *Drosophila* species span ~40 of million years of evolution, which due to
272 their rapid development time, equates to a greater evolutionary time than that for the
273 mammalian radiation. The method used for this analysis was independent of that
274 used for *D. melanogaster*, but also showed that the association between GATA mo-
275 tifs and orthologs of DR-responsive genes from *D. melanogaster* was robust across
276 *Drosophila* species (Supplementary Spreadsheets). Together, these data and previ-
277 ous findings strongly implicate GATA factors as evolutionarily-conserved regulators
278 of tissue-specific transcriptional responses to EAAs.

279

280 *Functional roles for GATA factors in mediating dietary effects on lifespan*

281 Of the five *D. melanogaster* GATA TFs, *GATAe* and *srp* were of particular inte-
282 rest for a role in longevity via DR/TOR. *GATAe* was associated more strongly than
283 any other TF with longevity-associated transcriptional changes (see above), and *srp*
284 has known roles in the fat body in regulating oogenesis via yolk proteins [30]. Conse-
285 quently we focussed on testing the roles of *srp* and *GATAe* in dietary regulation of
286 longevity. We knocked down *srp* and *GATAe* with tissue-specific RNAi, and evaluat-
287 ed whether these knockdowns altered the effects of dietary EAAs on egg laying and
288 lifespan. *GATAe* and *srp* were most highly expressed in the gut and fat body, respec-
289 tively (Figure S4), so we targeted *GATAe* in the midgut epithelium (with the TiGS
290 driver: [31]) and *srp* in the fat body (with the S_1106 driver, which drives in both fat body
291 and gut [32], however fat body-specific expression of *srp* (Supplementary Materials)
292 suggests that RNAi phenotypes can be attributed solely to the fat body). These
293 GeneSwitch drivers [33] are activated by feeding flies the RU_{486} inducer (RU).

294

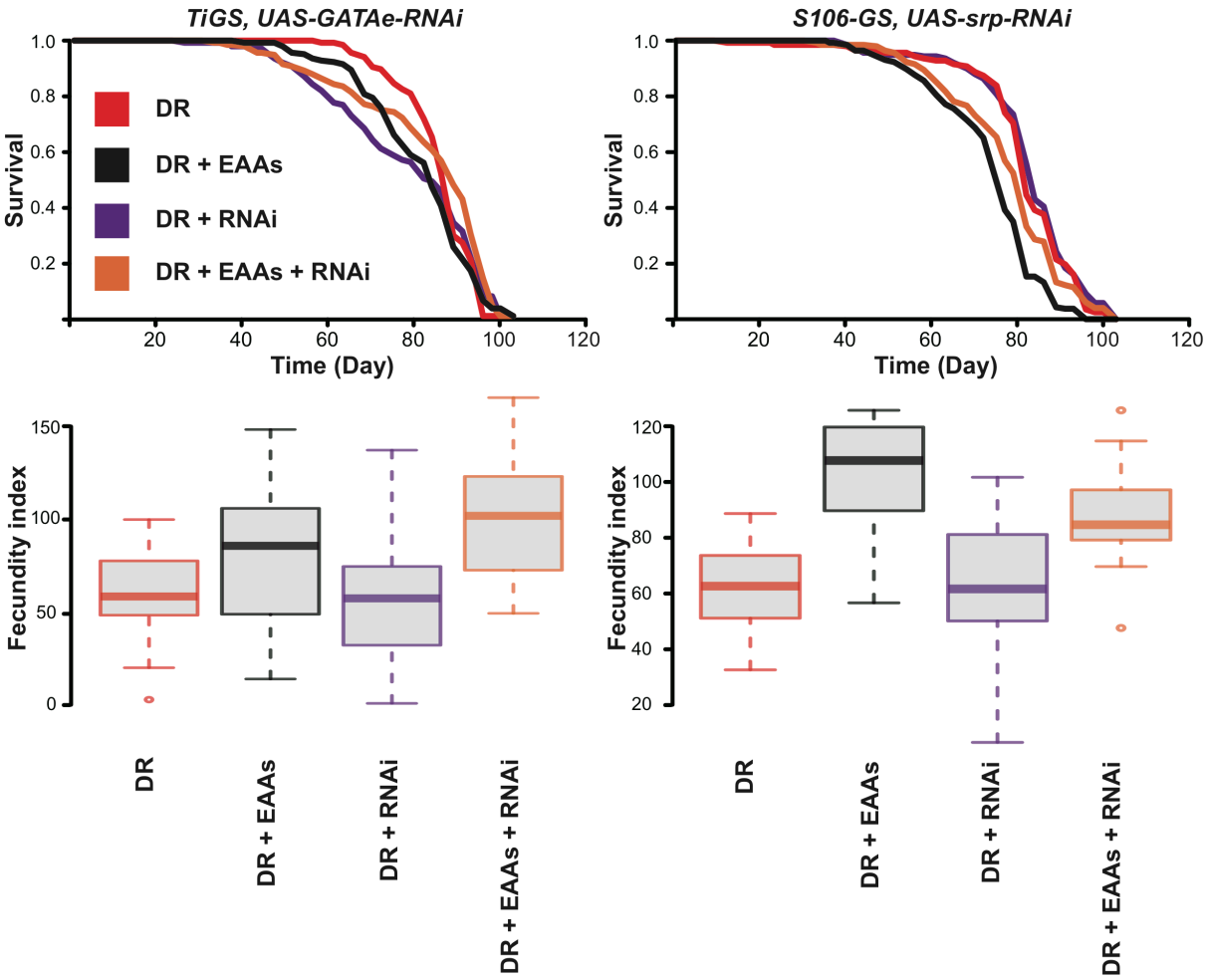


Figure 6. Tissue-restricted knockdown of *GATAe* and *srp* interact with dietary EAAs to determine egg laying and lifespan. Survival curves **(A & C)** and egg laying indices (overnight egg laying by vials of 10 flies; **B & D**) of flies expressing *GATAe*^{RNAi} in the gut (A, B), or *srp*^{RNAi} in the gut and fat body.

295

296 In *GATAe*^{RNAi} flies there was a significant interaction between the effect of
297 feeding the transgene-inducing drug and EAA on the lifespan of GATAe knockdown
298 flies (Cox Proportional Hazards Regression, $P < 0.005$). Specifically, knocking down
299 *GATAe* in the gut accelerated the onset of mortality in early life but, surprisingly, this
300 lifespan shortening was partially rescued by feeding dietary EAAs (Figure 6a). How-
301 ever GATAe^{RNAi} affected neither egg laying nor altered the effect of EAAs on egg lay-
302 ing (Figure 6b), indicating that the regulation of egg laying is independent of the
303 lifespan-limiting process induced by knocking down GATAe in the gut.
304

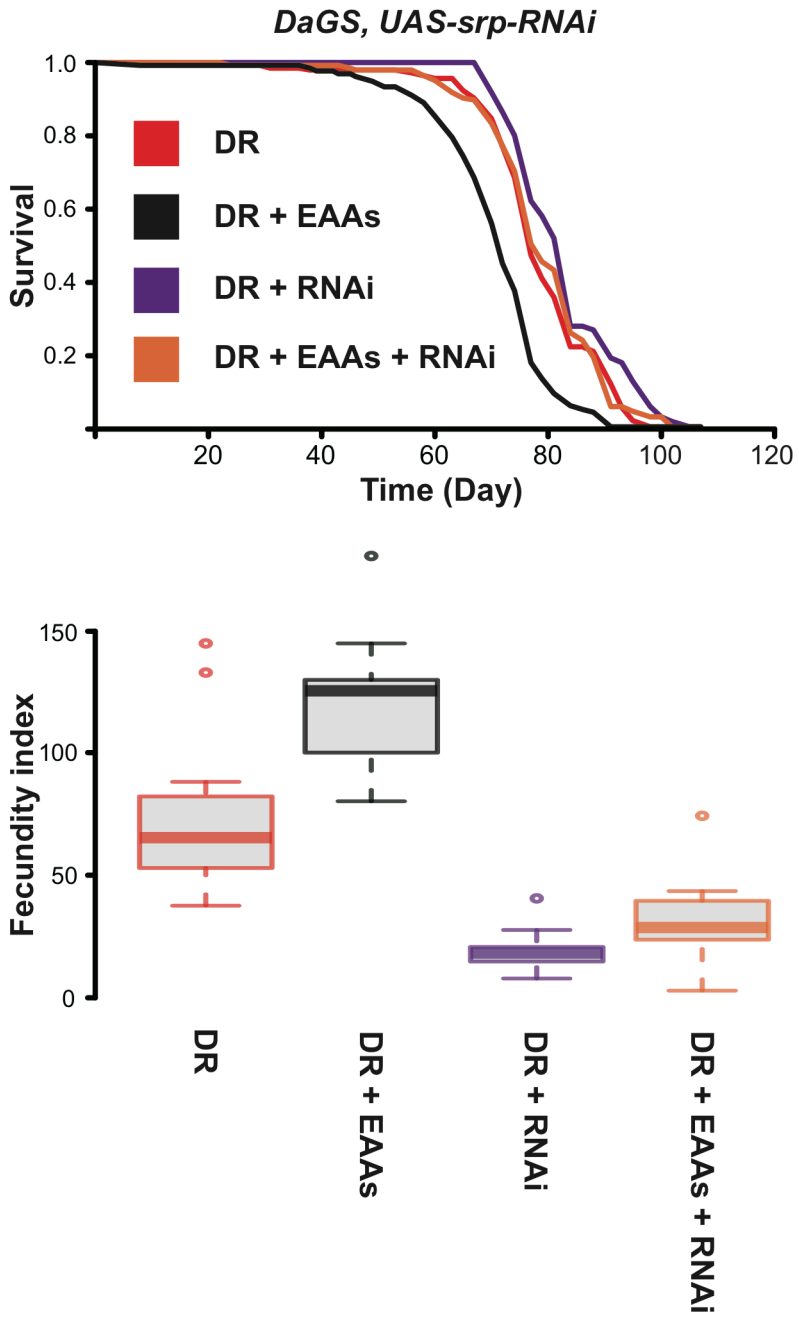


Figure 7. Ubiquitous knockdown of *srp* reverses lifespan-shortening effects of EAAs and desensitises egg laying to EAAs. (A) Survival curves and **(B)** egg laying indices of flies ubiquitously expressing RNAi against *srp*.

305

306 In contrast to *GATAe* knockdown, *srp* knockdown had positive effects on lon-
307 gevity (Figure 6c). Fat body-specific expression of srp^{RNAi} protected flies from the
308 lifespan-shortening effects of EAAs (log-rank test, $p<0.001$), but there was no effect
309 on lifespan in the absence of EAAs ($p=0.57$). Egg laying was not affected by srp
310 knockdown ($p=0.08$). Therefore, knocking down *srp* in the fat body insulates lifespan
311 against the pernicious effects of EAAs, but does not preclude flies from the early-life
312 benefits of EAA feeding for egg laying. Together, the $GATAe^{RNAi}$ and srp^{RNAi} experi-
313 ments validate our bioinformatic analysis, by linking age-dependent physiology with
314 interactions between dietary EAAs and the tissue-specific regulation of GATA factors.
315 Our expression data showed that *srp* is expressed most strongly in the fat
316 body, but is also expressed in other tissues e.g. the ovary. Therefore, we asked
317 whether systemic *srp* knockdown using a *Daughterless* GeneSwitch driver further al-
318 tered fly phenotype. Ubiquitous knockdown of *srp* was sufficient to neutralise the
319 lifespan-shortening effects of EAAs, restoring lifespan to that of flies fed DR food
320 (Figure 7a). Furthermore, ubiquitous *srp* RNAi extended lifespan of flies on DR food,
321 meaning that transgene expression resulted in a median 7 day lifespan extension
322 whether or not the flies were fed high levels of dietary EAAs. Whilst systemic *srp*
323 knockdown extended lifespan more strongly than the tissue-specific knockdown, the
324 tradeoff with egg laying was much greater (Figure 7b). Without induction of srp^{RNAi},
325 the flies laid ~25% more eggs when EAAs were supplemented to the food, but *srp*
326 knockdown arrested egg laying (ANOVA, $p < 2.2^{-16}$) and rendered it insensitive to
327 EAA feeding (EAA * RU interaction, $p<0.005$). The egg laying phenotype thus con-
328 trasted the lifespan phenotype, since egg laying was entirely desensitised to diet,
329 whereas median lifespan remained sensitive. Together, these data indicate that the
330 relationship between lifespan and egg laying is set by an interaction of dietary EAAs
331 and *srp*, and the integration of egg laying and lifespan depends on the tissues in
332 which this interaction occurs.

333

334 # Discussion

335

336 Dietary restriction improves lifelong health and extends lifespan in a range of
337 organisms, from yeast to mammals. A growing body of evidence shows the particular

338 importance of dietary nutrient balance for ageing, particularly lowered pro-
339 tein:carbohydrate ratio [6,34], which implicates amino-acid sensitive TOR signalling [11].
340 This implicit role of TOR was recently validated in *Drosophila*, by the demonstration
341 that active TOR is required for EAA enrichment to shorten lifespan [10]. These results
342 have the important implication that the lifespan effects of EAA restriction are not ac-
343 counted for solely by metabolic effects, but also by cellular signalling. Using the same
344 experimental conditions, our present study predicts lifespan-relevant and EAA-
345 dependent signalling mechanisms, by showing that EAA restriction has transcription-
346 al effects which are congruent - both within and between organs - with those of
347 pharmacological TOR suppression, indicating that the transcriptional effects of DR
348 are indeed mediated in large part by TOR. Furthermore, these overlapping transcrip-
349 tional changes are also associated with overlapping sets of *cis* regulatory elements.
350 Thus, DR and rapamycin have overlapping effects on lifespan, on transcription within
351 organs, on the coordination of functions across organs, and their transcriptional tar-
352 gets share predicted regulators.

353 Our results connect lifespan regulation by DR and low TOR with GATA tran-
354 scription factors. The GATA factor knockdown experiments validate the bioinformatic
355 connections between dietary EAAs, transcription factors and lifelong physiology.
356 Specifically, these experiments show that genetic knockdown of either *GATAe* or *srp*
357 substantially alters the effect of diet on fly survival and, in the case of srp, knockdown
358 both extended lifespan and dictated the influence of diet on egg laying, thus pheno-
359 copying flies treated with rapamycin. Curiously, *GATAe* knockdown in the gut
360 changed the sign of the effect of EAAs on lifespan, suggesting either a different opti-
361 mal dietary balance in these flies, or that elevated EAAs rescued the pathological ef-
362 fects of GATA knockdown. By contrast, *srp* knockdown with two different drivers can-
363 celled the lifespan-shortening effects of EAA enrichment. Furthermore the GATA
364 knockdown experiments show that, whilst lifespan and egg laying normally show
365 coupled responses to diet, they can be decoupled by genetic manipulation of GATA
366 factors, depending on which tissues are targeted. The phenotypic differences be-
367 tween flies in which srp is locally or systemically knocked down provide key evidence
368 that tissue-targeted manipulation of TFs associated with DR can recapitulate the
369 lifespan benefits of DR, and that the associated costs depend on the tissue-
370 specificity of the intervention. This supports the notion that ageing can be ameliorated
371 by specifically targeting pathologies in lifespan-limiting tissues. Whilst there is some

372 existing evidence of a role for GATA factors in lifespan regulation [20], to our
373 knowledge they have not yet been associated explicitly with DR, nor has there been
374 a prior demonstration of any transcriptional intervention blocking the phenotypic ef-
375 fects of EAAs. The indication that costs and benefits of dietary alteration are mediat-
376 ed by distinct tissues has the important biomedical implication that lifelong health
377 may be improved by altering the coordination of function across multiple tissues, and
378 the evolutionary implication that phenotypic tradeoffs evolve due to coupled functions
379 of tissues.

380 The GATA factors are an ancient family of transcription factors, which have
381 well-characterised and essential roles in coordinating development and growth in or-
382 ganisms from yeast to mice. In multicellular differentiated organisms, GATA factors
383 are required in the development of multiple tissue types, which in *Drosophila* includes
384 the heart [35], fat body [36] and gut [37]. GATA factors are also expressed tissue-
385 specifically in adulthood (Figure S4), but their roles in coordinating adult functions are
386 not well characterised. In *Drosophila*, recent studies have shown a role of *GATAe* in
387 maintenance of cell identity and tissue function in the adult gut, but it is not yet clear
388 from which molecular pathways *GATAe* integrates information [38–40]. One of the bet-
389 ter-described roles for GATA factors in adult animals is in nutrient regulation of oo-
390 genesis in mosquitos [29,41–43]. In *Aedes aegypti*, oogenesis requires a blood meal,
391 which contains the mosquito's only source of protein. Egg production is suppressed
392 before feeding, due in part to GATA-mediated repression in the fat body of the major
393 yolk precursor protein gene *Vg* [44]. After the blood meal, *Vg* expression is activated by
394 TOR enhancing expression of the transcriptional activator *AaGATAa* [43]. In *Drosophi-*
395 *la*, there is evidence that this regulatory circuit may be conserved, since *srp* regulates
396 expression of yolk proteins [30]. Our new and recent data show that rapamycin abro-
397 gates higher egg laying caused by EAA enrichment [10], corresponding to changes in
398 the fat body in expression of yolk proteins (Supplementary Materials). The connection
399 in *Drosophila* between dietary nitrogen and GATA transcriptional control is consistent
400 with mechanisms in the yeast *Saccharomyces cerevisiae*, in which selective amino
401 acid catabolism is controlled by a circuit known as Nitrogen Catabolite Repression. In
402 this system, when the available nitrogen sources only support poor growth, TOR-
403 dependent nuclear localisation of a GATA transcription factor triggers the expression
404 of genes involved in the transport and metabolism of less-preferred nitrogen sources

405 [28]. Together, these data point to highly evolutionarily conserved connections between
406 protein uptake, growth and reproduction, TOR signaling and transcriptional control by
407 GATA factors. Theory suggests that ageing results from antagonistic pleiotropic ef-
408 fects of mechanisms that promote growth and reproduction in the young [45]. GATA
409 factors fit these criteria, as nutrient-responsive regulators of growth that we associate
410 with molecular responses to lifespan-extending regimes. Due to the evolutionarily
411 conserved connections between dietary nitrogen and lifespan on one hand, and die-
412 tary nitrogen and GATA factor-regulated transcription on the other, we anticipate new
413 interest in the role that GATA factors play in lifespan regulation.

414 Our experiments demonstrate that the tissue-restricted knockdown of GATA
415 factors is sufficient to modify the lifespan-shortening effect of EAAs. Previous studies
416 have shown that tissue-targeted interventions to lower insulin signalling are sufficient
417 to extend lifespan in worms and flies [46], and GATA factors are required for some
418 such effects. It is well-established that the balance of dietary nutrients has the evolu-
419 tionarily conserved capacity to determine lifelong health [11], and to recognise this bal-
420 anced supply, insulin and TOR signalling must coordinate. Reducing either insulin or
421 TOR signalling is sufficient to extend lifespan, suggesting that the lifespan-extending
422 effects of these two interventions may be mediated by the nexus of their signalling
423 effects. In *C. elegans*, the GATA factor *ELT-2* is required for longevity following die-
424 tary restriction or mutation of the insulin receptor [20], and GATA factor overexpression
425 extends lifespan [47]. Additionally, previous transcriptional studies in both flies and
426 worms have uncovered enrichment of GATA motifs in the insulin regulon [48,49]. To-
427 gether with our data, these results suggest that signals from TOR and other nutrient
428 sensing pathways are mediated at least in part by GATA factors. In light of these da-
429 ta, our demonstration that transcriptional effects of diet are largely tissue-specific
430 suggests that TOR and the GATA factors mediate cell-autonomous interpretations of
431 global signals (e.g. insulins or bioamines), into a local language that dictates physio-
432 logical change appropriate to the tissue in question.

433 Transcription factors known to be downstream of TOR signalling and insulin
434 signalling are candidates for interpretation by GATA factors in *Drosophila. REPTOR*
435 and *REPTOR-BP* have recently been discovered by Tiebe and colleagues as a novel
436 TOR-dependent transcription factor complex [18]. Given the complementary nature of
437 Tiebe *et al*'s cell culture experiments and our study of dissected tissues, an emergent

438 hypothesis is that GATA factors determine organ-specific responses to TOR by modi-
439 fying effects of systemic *REPTOR* signalling. Downstream of insulin signalling, mo-
440 lecular interactions between *FoxO* and TOR signalling are already established: TOR
441 lies in a network of interacting signaling pathways including insulin [50], and *FoxO* is
442 required for reduced insulin signalling to extend lifespan [51]. However, FoxO binds
443 *TOR*'s promoter and is required for normal *TOR* expression, and GATA motifs are
444 associated with genes that are not bound by *FoxO* but show *FoxO*-dependent regula-
445 tion by IIS [49], suggesting a regulatory circuit from IIS to GATA factors via *FoxO* and
446 TOR. Given that *FoxO* is not required for the lifespan-extending effects of DR [52] and
447 that different insulin-like peptides are produced in response to different nutritional
448 stimuli [53], we suggest that signals from IIS via FoxO may be modified in peripheral
449 tissues by TOR-modulated GATA activity, which shapes tissue-specific responses to
450 diet that ultimately modify physiology and longevity (Figure 8).
451

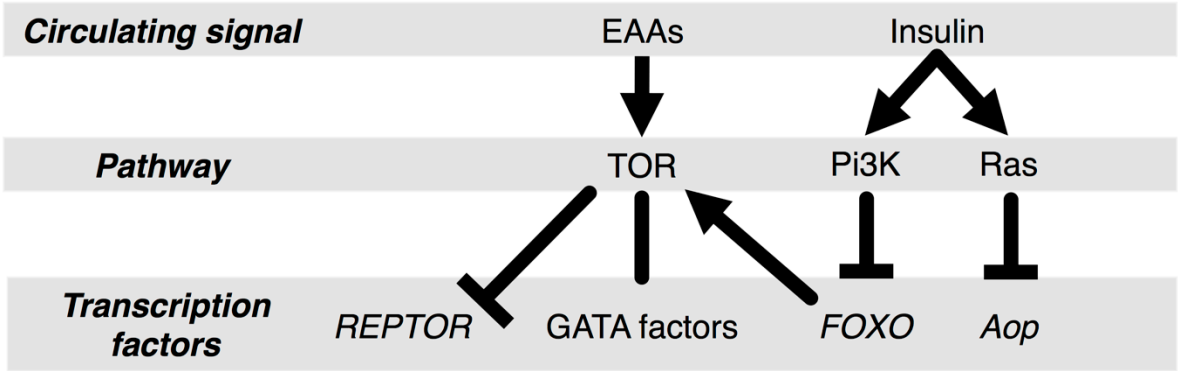


Figure 8. Predicted molecular integration of GATA factors in nutrient-sensing and lifespan-relevant transcriptional networks in *Drosophila*. Our transcriptomic analysis associates transcriptional effects of EAA restriction with TOR, and the TOR-dependent EAA regulon with GATA factors. Previous studies have shown that (1) TOR directly represses *REPTOR*, (2) The insulin pathway bifurcates via Pi3K and Ras to regulate lifespan via *FOXO* and *Aop*, and (3) GATA binding sites are associated with genes that are differentially expressed in *FOXO* mutants but not bound by *FOXO*, that *FOXO* both binds *TOR*'s promoter and *TOR* transcription is reduced in *FOXO* mutants. Together, these results suggest that *TOR* integrates signals from IIS/Pi3K signalling via *FOXO*, that regulons of DR, TOR and *FOXO* are all enriched in GATA factor binding sites, and therefore that GATA factors mediate transcriptional effects of both EAAs and *FOXO* via TOR.

452 The gut appears to be a particularly effective target for interventions to extend
453 lifespan [46]. The adult gut has a critical role in the ongoing health of organisms, bal-
454 ancing the passage of nutrients whilst resisting environmental stresses [54]. In flies,
455 there are complex relationships between age, gut maintenance, diet, metabolism,
456 resident microbiota and expression of antimicrobial genes [55–62]. Recent work sug-
457 gests a central role for the gut in mediating lifespan extension by DR in female flies,
458 revealing sexually dimorphic pathologies in aged female guts [63], dependent on cell-
459 autonomous expression of sex determination pathways [64]. Failure of gut integrity is
460 associated with changes in microbiota and greater antimicrobial peptide expression,
461 and appears to be a marker of imminent death [58,65]. In worms, the GATA factor ELT-2
462 interacts with p38 transcriptional regulators to modify adult gut immunity [66], whilst
463 GATA factors are required for normal gut development and maintenance in *Drosophi-*
464 *la* and mice [38,67]. It is thus tempting to speculate that increased risk of death in late
465 life may be preciptated by a loss of gut integrity enhancing exposure to environmental
466 microbes and toxins. Our results are directly relevant to these issues, because coor-
467 dination across organs of a transcriptional module (module 4) that was perturbed un-
468 der long-lived conditions strongly corresponded to *GATAe* expression, and *GATAe*
469 and paracrine insulin signalling have each been shown to be required for intestinal
470 stem cell proliferation [38,68]. Our lifespan data are consistent with *GATAe* knockdown
471 in the gut increasing mortality, but since this effect appears to be stochastic and not
472 necessarily connected to age, the role of *GATAe* regulation in late-life mortality re-
473 mains to be established. However, fly lifespan is not necessarily extended by en-
474 hancing expression of components of the immune system [69], antibiotic treatment [55] or
475 genetically reducing gut dysplasia [59]. Thus, although compromised gut integrity is
476 clearly a marker of frailty in late life, it may not be obligately linked to death.

477 This study reveals how the fly's tissues interact as a system, and how that
478 system responds to EAA dilution and low TOR. The results showed that the effects of
479 EAA restriction on transcription are likely mediated by TOR via GATA transcription
480 factors. Entirely consistent with this prediction, genetic analysis confirmed that the
481 tissue-specific activity of GATA factors dictate the effect of dietary nutrient balance on
482 phenotype. Importantly, these experiments also suggest that the costs and benefits
483 of dietary variation may be mediated by different tissues, and therefore that benefits
484 may be reaped without fitness tradeoffs by targeting specific tissues. The evolution-
485 ary conservation of GATA factors, of their connection to regulating amino acid me-

486 tabolism, and of the capacity of TOR to mediate lifespan extension, suggests that
487 GATA factors may be relevant to ameliorating ageing by DR in a broad range of or-
488 ganisms, including humans.
489

490 ## Materials & methods

491

492 *Drosophila melanogaster* and diets were prepared according to (10). 1SY me-
493 dium is a dietary restriction (DR: diet-induced longevity) regime that extends lifespan
494 in wild-type and laboratory-maintained flies [71,72], containing 100 g/l autolysed yeast
495 (MP Biomedicals, OH, USA), 50 g/l sucrose (Tate & Lyle, London, UK), 15 g/l agar
496 (Sigma-Aldrich, Dorset, UK), 30 ml/l nipagin (Chemlink Specialities, Dorset, UK), and
497 3 ml/l propionic acid (Sigma-Aldrich, Dorset, UK). EAA food comprised 1SY with the
498 addition of cocktail of EAAs dissolved in pH 4.5 water (final concentrations in fly me-
499 dia: L-arginine 0.43 g/l, L-histidine 0.21 g/l, L-isoleucine 0.34 g/l, L-leucine 0.48 g/l, L-
500 lysine 0.52 g/l, L-methionine 0.1 g/l, L-phenylalanine 0.26 g/l, L-threonine 0.37 g/l, L-
501 tryptophan 0.09 g/l, L-valine 0.4 g/l: all suppled by Sigma). EAA+rapamycin (drug-
502 induced longevity) food consisted of EAA food with the addition of rapamycin (LC la-
503 boratories, MA, USA) dissolved in ethanol, to a final concentration of 200 μM in the
504 diet. For RNAi experiments, RU_{486} (Sigma M8046) dissolved in ethanol was added to
505 1SY or EAA food to a final concentration of 200 μm. Vehicle controls were added to
506 media as appropriate.
507 Outbred wild-type Dahomey flies bearing the endosymbiont *Wolbachia* were
508 cultured on a 12:12 light cycle at 25°C and 60% humidity. For RNAi experiments, the
509 TiGS and $S_1$106 drivers and RNAi constructs (UAS-srp^{RNAi}: Vienna Stock Center
510 #33748; UAS-$GATAe^{TRiP}$: Bloomington Stock Center #33748) were backcrossed into
511 flies bearing the *w* mutation for at least 6 generations (UAS-$GATAe^{TRiP}$ was back-
512 crossed by PCR), and maintained at large population sizes to maintain outbred ge-
513 netic diversity. For all experiments, parents of experimental flies oviposited onto
514 grape juice agar for 18h. Eggs were washed from this agar, added to 1SY and cul-
515 tured to adulthood at standardised density. Newly emerged flies were allowed to ma-
516 te *ad libitum* for 48h before being lightly anaesthetised with CO_2. Males were re-
517 moved, and female flies were allocated to experimental diets. For RNA sequencing,
518 females were maintained on 1SY, EAA or EAA+rapamycin for six days before dissec-

519 tion. The RNA sequencing experiment was independently replicated three times,
520 generating three samples per organ or per whole fly, per diet (with the exception of
521 the gut on 1SY: see Supplementary Text). For egg laying and lifespan experiments,
522 survival was scored three times per week. Egg laying was scored on day 10 after
523 eclosion, after 18h egg laying. Fecundity indices were calculated as number of eggs
524 per female (n=10 females per vial).

525 RNA was collected 6-10h into the flies' light cycle. To prepare RNA for se-
526 quencing, whole flies were flash-frozen. Brains, abdominal fat bodies, ovaries, guts
527 and thoraces were micro-dissected in ice-cold RNAlater solution and frozen at -80°C.
528 RNA was extracted using the QIAGEN total RNA isolation kit and quantified on an
529 Agilent 2100 bioanalyser. Sequencing was performed by the high throughput ge-
530 nomics services center at the Huntsman Cancer Institute (University of Utah). Sam-
531 ple concentration and purity of RNA was measured on a NanoDrop spectrophotome-
532 ter, and RNA integrity was assessed on an Agilent 2200 TapeStation. Illumina
533 TruSeq libraries were prepared from this RNA with the Illumina TruSeq Stranded
534 mRNA Sample Prep kit and sequenced on an Illumina HiSeq2000 101 v3 platform
535 using paired-end sequencing.

536 Reads were aligned to the *D. melanogaster* genome annotation 5.57 using
537 TopHat2 2.0.14 and counted using HTSeq 0.5.4p3 [73,74]. Non-protein coding genes
538 were retained. Unmapped reads were discarded. Enumerated reads were then ana-
539 lysed in R (3.0 & 3.1) using BioConductor. RPKM was calculated from read counts
540 generated by HTSeq, using the EdgeR library.

541 Differential expression across the three experimental conditions was deter-
542 mined with a negative binomial GLM fitted by DESeq2 (1.8.1, [75]), without rejection
543 based on Cook's distance, calculating P-values with a two-sided Wald test, and cal-
544 culating false discovery rate by Storey's method. Intersections between gene sets
545 and enriched GO terms were visualised with the upset package in R [76]. GO term en-
546 richent was analysed using GOrilla (http://cbl-gorilla.cs.technion.ac.il/).

547 Unsigned gene coexpression networks were determined using data from all
548 organs, excluding the whole-fly samples, using the WGCNA package in R [23]. Con-
549 sensus modules were determined automatically using the *blockwiseConsensusMod-*
550 *ules* function with default settings and a power of 26, stipulating a minimum module
551 size of 50 genes. Eigengene determination, variance explained by Eigengenes and
552 clustering were performed using internal WGCNA functions, as per the package tuto-

553 rial
554 (http://labs.genetics.ucla.edu/horvath/CoexpressionNetwork/Rpackages/WGCNA/Tut
555 orials/index.html). Changes in between-module correlations by experimental condi-
556 tion were calculated by custom R functions: correlation matrices (Spearman's rho) for
557 module Eigengenes were calculated in each experimental condition, and changes
558 were calculated by subtracting the DR matrix and the EAA+rapamycin matrix from
559 the EAA matrix, to calculate observed changes in correlations between pairs of
560 Eigengenes under DR and under rapamycin administration, respectively. To generate
561 null distributions for changes in correlations for each pair of modules in each of the
562 two long-lived conditions, the same procedure was repeated 10,000 times, permuting
563 each Eigengene and calculating changes in correlation for each pair. Observed
564 changes were considered significant when they did not fall between the 2.5th and
565 97.5th percentiles of their respective null distribution.
566 The most likely acyclic network between modules was determined by Additive
567 Bayesian Network analysis of module Eigengenes using the R package ABN with
568 10000 iterations. This approach determines the likelihood of inter-dependencies be-
569 tween variables by randomly simulating data and comparing to the observed inter-
570 dependencies. ABN found a consensus structure to the data after only ~1000 itera-
571 tions, indicating that the structure is robust to further simulation. The ABN was plotted
572 in Cytoscape, using hierarchical network ordering. Meta-module analysis and
573 Eigengene perturbation analysis were performed according to [25].
574 Enrichment of cis-regulatory motifs was analysed using i-Cis target [77], exclud-
575 ing genes for which DEseq2 models did not converge. Unannotated CRMs were ex-
576 cluded from further analysis. Samples were hierarchically clustered according to the
577 presence/absence of transcription factor binding sites with the R hclust function, us-
578 ing a binary distance metric. Notches on boxplots, approximating 95% confidence
579 intervals of medians were produced using the "notch" argument to the R boxplot
580 function. Heatmaps of TF:gene-set associations were plotted using the heatmap.2
581 function from the R gplots package, and ordered by heirarchical clustering using bi-
582 nary distance.
583 Analysis of evolutionary conservation of the association between TF binding
584 motifs and differentially expressed genes was conducted per tissue. Groups of differ-
585 entially expressed genes (according to DEseq2 analysis) showing the same signs of
586 fold-change in response to EAA restriction were scanned for significantly over-

587 represented motifs in their promoter regions. 706 motifs were included in the search,
588 from OnTheFly (2014 release), flyreg v2, dmmpmm2009 and idmmpmm2009 data-
589 bases, all provided by MEME 4.10 (http://meme-suite.org). Pscan version 1.2.2 was
590 used to score the motifs for the gene list, using the sets of genes that either didn't
591 show any diet-induced differential expression in any tissue, or were not expressed,
592 as a 'null hypothesis' background. For each gene list, the 706 scores (one per motif)
593 produced by pscan were normalized to *z* scores, and *P* values were computed, cor-
594 rected for multiple testing (Benjamini-Hochberg).

595 The same procedure was repeated for the evolutionarily conserved regulation
596 analysis, except that the genes considered were obtained from the 12 *Drosophila*
597 species (*D.melanogaster* and *D.ananassae*, *D.erecta*, *D.grimshawi*, *D.mojavensis*,
598 *D.persimilis*, *D.pseudoobscura*, *D.sechellia*, *D.simulans*, *D.virilis*, *D.willistoni* &
599 *D.yakuba*) whose genomic sequences are part of FlyBase, taking only 1:1 orthologs
600 of genes which DESeq2 analysis had shown to be differentially expressed in *D. mel-*
601 *anogaster*. Gene selection was done in two steps: for all gene groupings (differential
602 expression and background), genes for which OrthoDB version 7 didn't predict a one-
603 to-one ortholog in all other 11 species were removed. Then, each grouping was ex-
604 panded with all the relevant orthologs predicted by OrthoDB, such that each gene
605 group contained 12 times as many genes at after the first step.

606 Survival data were analysed in Microsoft Excel for log-rank statistics, or in R
607 for Cox Proportional Hazards analysis, using the CoxPH function from the Survival
608 package. Fecundity indices were analysed with ANOVAs fitted with the lm function in
609 R.

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Authors' Contributions

AJD analysed data, performed experiments, designed the study and wrote the manuscript. XH performed experiments. E Blanc analyed data. E Bolukbasi contributed transgenic flies and performed experiments. YF performed experiments. MY performed experiments and designed the study. MDWP designed the study and wrote the paper.

Competing financial interests

The authors declare they have no competing financial interests.

Data availability

RNAseq data have been deposited with NCBI. Custom R scripts have been uploaded to GitHub.