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1 # Pervasive positive and negative feedback regulation of insulin-like signaling in *Caenorhabditis*
2 # *elegans*

3

4 Colin S. Maxwell[2#], Rebecca E. W. Kaplan[1#], Nicole Kurhanewicz Codd[3] and L. Ryan Baugh[1*]

5

6 [1]Department of Biology, Duke University, Durham, North Carolina, United States of America

7 [2]Current address: Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill,
8 North Carolina, United States of America

9 [3]Current address: Department of Biology, University of Oregon, Eugene, Oregon, United States
10 of America

11

12 [#]These authors contributed equally to this work.

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18 *Corresponding author, L. Ryan Baugh
19 Department of Biology
20 Duke University
21 Box 90338
22 Durham, NC 27708-0338
23 Phone: 919-613-8179
24 E-mail: ryan.baugh@duke.edu

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25 **Abstract** (175 words)

26

27 The *C. elegans* insulin-like signaling network supports homeostasis and developmental
28 plasticity. The genome encodes 40 insulin-like peptides and one receptor. Feedback regulation
29 has been reported, but the extent of feedback and its effect on signaling dynamics during a
30 state transition has not been determined. We measured mRNA expression for each insulin-like
31 peptide, the receptor *daf-2*, components of the PI3K pathway, and its transcriptional effectors
32 *daf-16*/FoxO and *skn-1*/Nrf at high temporal resolution during transition from a starved,
33 quiescent state to a fed, growing state in wild type and mutants affecting *daf-2*/InsR and *daf-*
34 *16*/FoxO. We also analyzed the effect of temperature on insulin-like gene expression. We found
35 that numerous PI3K pathway components and insulin-like peptides are affected by signaling
36 activity, revealing pervasive positive and negative feedback regulation. Reporter gene analysis
37 demonstrated that the *daf-2*/InsR agonist *daf-28* positively regulates its own expression and that
38 other agonists cross-regulate *daf-28* transcription through feedback. Our results show that
39 feedback regulation of insulin-like signaling is widespread, suggesting a critical role of feedback
40 in signaling dynamics in this endocrine network and likely others.

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43 **Introduction**

44 Insulin-like signaling maintains homeostasis by responding to fluctuations in nutrient
45 availability and altering gene expression. Work in *C. elegans* has shown that insulin-like
46 signaling also allows developmental plasticity. For example, insulin-like signaling regulates
47 whether larvae become reproductive or arrest as dauer larvae, a developmental diapause that
48 occurs in unfavorable conditions (Hu, 2007). Insulin-like signaling also contributes to continuous
49 variations in phenotype, for example in regulation of aging and growth rate (Murphy and Hu,
50 2013). However, it is unclear how signaling dynamics are regulated such that the pathway can
51 maintain a phenotypic steady-state (homeostasis) or promote developmental plasticity,
52 depending on conditions.

53 Insulin-like signaling is regulated by feedback in diverse animals. Pancreatic β-cell-
54 specific insulin receptor-knockout mice are poor at glucose sensing, have a diminished insulin
55 secretory response, and tend to develop age-dependent diabetes (Otani *et al*, 2004). In addition,
56 the full effect of glucose on pancreatic β-cells grown *in vitro* requires the insulin receptor
57 (Assmann *et al*, 2009). FoxO transcription factors, effectors of insulin signaling, activate
58 transcription of insulin receptors in Drosophila and mammalian cells (Puig and Tjian, 2005),
59 suggesting a relatively direct, cell-autonomous mechanism for feedback regulation. However,
60 evidence for such direct feedback regulation has not been found in *C. elegans* (Kimura *et al*,
61 2011).

62 Insulin-like signaling regulates the expression of insulin-like peptides in *C. elegans*,
63 suggesting a relatively indirect, cell-nonautonomous mechanism for feedback regulation. The *C.*
64 *elegans* genome encodes a family of 40 insulin-like peptides that can function as either agonists
65 or antagonists of the sole insulin-like receptor *daf-2* (Pierce *et al*, 2001). Systematic analyses of
66 insulin-like peptide expression and function suggest substantial functional specificity rather than
67 global redundancy (Fernandes de Abreu *et al*, 2014; Ritter *et al*, 2013). *daf-2*/InsR signals

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68 through a conserved phosphoinositide 3-kinase (PI3K) pathway to antagonize the FoxO
69 transcription factor *daf-16* (Fig. 1A; Murphy and Hu, 2013). *daf-16*/FoxO represses transcription
70 of the *daf-2* agonist *ins-7*, creating positive feedback (Murphy *et al*, 2003). This positive
71 feedback results in "FoxO-to-FoxO" signaling, which has been proposed to coordinate the
72 physiological state of different tissues in the animal (Alic *et al*, 2014; Murphy *et al*, 2007; Zhang
73 *et al*, 2013). *daf-16* also activates transcription of the *daf-2* antagonist *ins-18*, again producing
74 positive feedback (Matsunaga *et al*, 2012a; Murphy *et al*, 2003). Insulin-like peptide function has
75 been reported to affect insulin-like peptide expression (Fernandes de Abreu *et al*, 2014; Ritter *et*
76 *al*, 2013), consistent with feedback regulation. To the best of our knowledge, negative feedback
77 regulation has not been reported, despite the fact that homeostasis generally relies on it
78 (Cannon, 1929). Furthermore, the extent of feedback regulation, and whether it is positive or
79 negative with respect to pathway activity, is unknown.

80 We sought to determine the extent of feedback regulation in insulin-like signaling in *C.*
81 *elegans*. *C. elegans* larvae that hatch in the absence of food arrest development in the first
82 larval stage ("L1 arrest" or "L1 diapause"), and insulin-like signaling regulates L1 arrest and
83 development (Baugh, 2013). We performed a genetic analysis of gene expression, measuring
84 expression of all 40 insulin-like peptides as well as components of the PI3K pathway in *daf-*
85 *2*/InsR and *daf-16*/FoxO mutants, which have perturbed signaling activity. We analyzed larvae
86 in L1 arrest and over time after feeding, as they transition from quiescence to growth. The
87 rationale is that by identifying genes whose expression is affected by insulin-like signaling that
88 themselves affect signaling activity we can infer feedback regulation. We report extensive
89 feedback, both positive and negative, acting relatively directly at the level of the PI3K pathway
90 and also indirectly via regulation of peptide expression. This work suggests that feedback
91 regulation of insulin-like signaling is pervasive and that this feedback functions to stabilize
92 signaling activity during constant conditions while allowing rapid responses to new conditions.

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93 **Results**

94 *daf-2*/InsR acts through *daf-16*/FoxO to affect gene expression

95 We used the NanoString nCounter platform to measure expression of genes related to
96 insulin-like signaling in fed and starved L1 larvae at high temporal resolution during the
97 transition between developmental arrest and growth (Malkov *et al*, 2009). Total RNA was
98 prepared from whole worms and hybridized to a codeset containing probes for all 40 insulin-like
99 genes as well as components of the PI3K pathway and *sod-3*, a known DAF-16/FoxO target. In
100 addition to wild type (WT), we analyzed mutations affecting *daf-2*/InsR and *daf-16*/FoxO to
101 ascertain the effects of insulin-like signaling activity on expression. We used the reference allele
102 of *daf-2*, e1370, as well as a stronger allele, e979 (Gems *et al*, 1998). We used a null allele of
103 *daf-16*, mgDf47, as well as a *daf-16(mgDf47); daf-2(e1370)* double mutant to analyze epistasis.
104 Mutations affecting *daf-2* are generally temperature sensitive, and insulin-like signaling
105 responds to temperature. We therefore measured expression during L1 starvation at three
106 different temperatures. We also fed bacteria to starved L1 larvae of each of the five genotypes
107 and measured gene expression over time during recovery from arrest in a highly synchronous
108 population (Fig. 1B). This experimental design enabled us to measure the effects of temperature,
109 nutrient availability, and insulin-like signaling activity on genes related to insulin-like signaling
110 itself during a critical physiological state transition.

111 *daf-16*/FoxO mediates the effects of *daf-2*/InsR on expression of genes involved in
112 insulin-like signaling. *daf-16* is required for canonical effects of *daf-2*, such as dauer formation
113 and lifespan extension (Hu, 2007; Murphy and Hu, 2013) . However, *daf-2* also acts through
114 other effector genes of the PI3K pathway, such as *skn-1*/Nrf (Tullet *et al*, 2008), as well as other
115 signaling pathways, such as RAS (Nanji *et al*, 2005). In addition, genome-wide expression
116 analyses of *daf-16* have mostly been performed in a *daf-2* mutant background (*daf-2* vs. *daf-16*;
117 *daf-2*) without analysis of WT and/or *daf-16* single mutants (Tepper *et al*, 2013), making
118 analysis of epistasis between *daf-2* and *daf-16* with gene expression as a phenotype impossible.

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119 Since epistasis was not analyzed, these studies could not determine whether *daf-16* mediated
120 all of the effects of *daf-2* on gene expression or if other effectors made a significant contribution.
121 A correlation matrix between genotypes over all conditions tested indicates that mutating *daf-2*
122 affected expression, with a stronger effect of the e979 allele than e1370, as expected (Fig. 1C).
123 *daf-16* also had a clear effect, and it was epistatic to *daf-2.* That is, the expression profile of the
124 double mutant is similar to that of the *daf-16* single mutant but not *daf-2*. Statistical analysis of
125 individual genes together with examination of expression patterns across genotypes
126 corroborated the results of correlation analysis, failing to identify genes with significant effects of
127 *daf-2* not meditated by *daf-16.* These results show that *daf-2* affects expression of genes
128 involved in insulin-like signaling and that these effects are mediated exclusively by *daf-16*,
129 consistent with feedback regulation.
130

131 *daf-16*/FoxO affects expression of multiple PI3K pathway genes

132 We analyzed expression of several components of the PI3K pathway, as well as *daf-*
133 *2*/InsR and its transcriptional effectors *daf-16*/FoxO and *skn-1*/Nrf (Lin *et al*, 1997; Ogg *et al*,
134 1997; Tullet *et al*, 2008). The known direct target of DAF-16, *sod-3*/SOD (Oh *et al*, 2006), was
135 up-regulated in *daf-2* mutants and down-regulated in the *daf-16* mutant, with *daf-16* epistatic to
136 *daf-2*, in both starved and fed larvae (Fig. 2, S1 and Table 1). The exemplary behavior of this
137 positive control demonstrates the power of our experimental design. Notably, *daf-16* expression
138 drops to background levels in the *daf-16* deletion mutant (Fig. 2 and S1), as expected. We
139 previously reported that *daf-2* is up-regulated during L1 arrest (Chen and Baugh, 2014). We see
140 here that *daf-2* is actually repressed by *daf-16* (Fig. 2 and S1). Given that *daf-2* is up-regulated
141 during starvation, when *daf-16* is active, this result may be considered paradoxical. Our
142 interpretation is that *daf-2* expression is independently regulated by nutrient availability and *daf-*
143 *16* in opposing ways, illustrating regulatory complexity of the system. Nonetheless, since DAF-2
144 antagonizes DAF-16 activity via the PI3K pathway, these results indicate positive feedback

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145 between the sole insulin-like receptor and its FoxO transcriptional effector (Table 1). Likewise,
146 *age-1*/PI3K, which transduces *daf-2* signaling activity, was repressed by *daf-16*, also suggesting
147 positive feedback. However, *pdk-1*/PDK, *akt-1*/Akt and *akt-2*/Akt, downstream components of
148 the PI3K pathway, were each activated by *daf-16*, albeit with relatively complex dynamics,
149 suggesting negative feedback. Likewise, *daf-16* expression is reduced in *daf-2* mutants (Fig. 2),
150 where its activity is increased, suggesting it represses its own transcription to produce negative
151 feedback (Table 1). *skn-1*/Nrf expression was also reduced in *daf-2* mutants and increased in
152 *daf-16* mutants, suggesting that insulin-like signaling positively regulates expression of both of
153 its transcriptional effectors. Notably, the effects described here for each gene were consistent
154 for fed and starved larvae (Fig. 2, S1 and Supp. Data File 2). In summary, insulin-like signaling
155 acts through *daf-16*/FoxO to regulate multiple critical components of the pathway itself,
156 consistent with a combination of positive and negative cell-autonomous feedback regulation.
157

158 *daf-16*/FoxO affects expression of most insulin-like peptides

159 Insulin-like genes display complex dynamics in response to different levels of insulin-like
160 signaling activity. Our codeset contained probes for all 40 insulin-like genes, and we reliably
161 detected expression for 28 of them. Similar to what we saw with components of the PI3K
162 pathway (Fig. 2, S1 and Table 1), *daf-16* appears to function as an activator in some cases and
163 a repressor in others (Fig. 3, S2 and Table 1), but its function with respect to each gene affected
164 was again consistent between fed and starved conditions (Fig. 3, S2 and Supp. Data File 2). For
165 example, expression of *daf-28*, perhaps the most studied insulin-like peptide in *C. elegans*
166 (Chen and Baugh, 2014; Cornils *et al*, 2011; Fernandes de Abreu *et al*, 2014; Hung *et al*, 2014;
167 Li *et al*, 2003; Patel *et al*, 2008), was up-regulated in *daf-16* mutants and down-regulated in *daf-*
168 *2* in starved and fed larvae (Fig. 3, S2 and Table 1), suggesting it is repressed by *daf-16.*
169 Remarkably, all but three of the 28 reliably detected insulin-like genes were significantly affected
170 by *daf-16* (Table 1). Mutation of *daf-16* caused up-regulation of twelve insulin-like genes and

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171 down-regulation of thirteen, suggesting that *daf-16* directly or indirectly regulates transcription of
172 most insulin-like genes.
173 Inference of feedback as positive or negative is complicated by the fact that individual
174 insulin-like peptides function as either agonists or antagonists of *daf-2*/InsR (Pierce *et al*, 2001).
175 Biochemical data and structural modeling suggest that function as an agonist or antagonist is a
176 property of the peptide (Matsunaga *et al*, 2018), as opposed to the context in which it is
177 expressed. To infer whether the net effect of feedback regulation is positive or negative with
178 respect to insulin-like signaling activity (*daf-2*/InsR activity), we took into account whether *daf-16*
179 appears to activate or repress the insulin-like gene and whether that gene encodes a putative
180 agonist or antagonist. DAF-2 antagonizes DAF-16 activity, and so *daf-16* repression or
181 activation of an agonist or antagonist, respectively, would hypothetically result in positive
182 feedback. *daf-16* repression or activation of an antagonist or agonist, respectively, would
183 hypothetically result in negative feedback. For example, *daf-28* was originally identified on the
184 basis of its constitutive dauer-formation phenotype. *daf-28* is up-regulated in rich conditions and
185 it promotes dauer bypass (reproductive development), similar to *daf-2*/InsR, consistent with
186 function as an agonist of *daf-2* (Li *et al*, 2003). *daf-16* repression of *daf-28* expression therefore
187 suggests positive feedback in this case (Table 1).
188 A number of studies have performed genetic analysis of insulin-like peptide function,
189 determining whether individual insulin-like genes have similar or opposite loss-of-function
190 phenotypes to *daf-2*, and thus whether they presumably function as agonists or antagonists,
191 respectively (Chen and Baugh, 2014; Cornils *et al*, 2011; Fernandes de Abreu *et al*, 2014; Hung
192 *et al*, 2014; Kawano *et al*, 2006; Li *et al*, 2003; Matsunaga *et al*, 2012a; Matsunaga *et al*, 2012b;
193 Michaelson *et al*, 2010; Patel *et al*, 2008; Pierce *et al*, 2001). When we previously analyzed
194 expression of insulin-like peptides in starved and fed L1 larvae, we found remarkable
195 concordance between function (agonist or antagonist) and expression (positive or negative
196 effect of food, respectively) (Chen and Baugh, 2014). Out of thirteen insulin-like peptides

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197 consistently found to function as putative agonists or antagonists based on genetic analysis, we
198 classified all thirteen the same way based on expression, while classifying eight additional
199 peptides as well. This classification relied on separate time-series analyses of starved and fed
200 larvae (Chen and Baugh, 2014), and inspection of the fed time series here did not reveal
201 discrepancies between the two studies. We therefore included our previous putative functional
202 classifications based on nutrient-dependent expression in Table 1, which tentatively assigns
203 function to all but two of the 25 genes affected by *daf-16*. As explained above, putative agonists
204 repressed by *daf-16*, like *daf-28*, hypothetically result in positive feedback, since *daf-2* signaling
205 antagonizes *daf-16*. We identified seven genes like this in addition to *daf-28.* Conversely,
206 activation of a putative antagonist should also produce positive feedback, which we infer in five
207 cases, while activation of an agonist should produce negative feedback, which we infer in six
208 cases. Finally, repression of a putative antagonist should produce negative feedback, which we
209 infer in four cases. In summary, activation and repression of putative agonists and antagonists
210 by *daf-16* is common, with positive and negative feedback hypothetically resulting from each
211 different regulatory combination in multiple instances.
212

213 Temperature affects insulin-like gene expression

214 We analyzed expression of insulin-like genes at 15, 20 and 25°C during L1 starvation.
215 *daf-2* mutants are generally temperature-sensitive (Gems *et al*, 1998), *daf-16* is localized to the
216 nucleus at high temperatures (Henderson and Johnson, 2001), and *daf-2* mutants are heat-
217 resistant (Munoz and Riddle, 2003). These observations suggest that insulin-like signaling
218 responds to temperature. We hypothesized that temperature sensitivity results from
219 temperature-dependent regulation of insulin-like peptide expression. Consistent with *daf-16*
220 being active at elevated temperature, expression of its direct target *sod-3* was positively
221 affected by temperature (Fig. S2 and Table S1). In support of our hypothesis, temperature
222 affected mRNA expression of 21 out of 28 reliably detected insulin-like genes (Fig. S2 and

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223 Table S1). *daf-28* expression was lower at higher temperatures, consistent with its role in
224 promoting dauer bypass (Li *et al*, 2003), and confirmed in a recent publication (O'Donnell *et al*,
225 2018). Expression of twelve insulin-like genes was lower at higher temperatures and nine were
226 expressed higher at higher temperatures. However, there is no apparent correlation between
227 putative function as agonist or antagonist and positive or negative regulation in response to
228 higher temperature. Notably, although most insulin-like genes displayed significant temperature-
229 dependent expression, the effect of temperature on expression was minor compared to nutrient
230 availability.
231
232 Feedback mediates cross-regulation among insulin-like genes
233 Reporter gene analysis validated the effect of *daf-16*/FoxO on *daf-28* expression. We
234 previously used quantitative RT-PCR to validate the nCounter approach to measuring insulin-
235 like gene expression in *C. elegans* (Baugh *et al*, 2011), and we used transcriptional reporter
236 genes to confirm positive regulation of several putative agonists in fed larvae, including *daf-28*
237 (Chen and Baugh, 2014). A P*daf-28*::GFP transcriptional reporter gene again confirmed up-
238 regulation in response to feeding (Fig 4A). Expression was evident but faint in anterior neurons
239 and posterior intestine of starved L1 larvae, and it was brighter after being fed for 6 hr.
240 Quantification of whole-animal fluorescence with the COPAS BioSorter provided robust
241 statistical support for qualitative observations (Fig. 4B). Note that the statistics for this analysis
242 were performed on the means of individual biological replicates, as opposed to each individual
243 in a replicate. Thus, statistical significance is due to reproducibility despite relatively small effect
244 sizes. Critically, expression appeared elevated in *daf-16* mutants compared to WT, in both
245 starved and fed larvae (Fig. 4A). However, we did not observe a difference in the anatomical
246 expression pattern in *daf-16* compared to WT. Quantification showed that the effect of *daf-16* is
247 statistically significant (Fig. 4B). Notably, the effect of food was larger than that of *daf-16*, as
248 expected based on nCounter results (Fig. 3). In addition, the effects of food and *daf-16* are

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249 independent, suggesting that up-regulation of *daf-28* in response to feeding is not simply due to
250 inhibition of *daf-16* leading to de-repression of *daf-28*. These results support the conclusion that
251 *daf-16* represses *daf-28* transcription, consistent with feedback regulation.

252 Widespread feedback regulation of insulin-like signaling via transcriptional control of
253 insulin-like peptides suggests that activity of individual insulin-like genes should affect
254 expression of themselves and others. We analyzed expression of P*daf-28*::GFP in insulin
255 mutants to test this hypothesis. P*daf-28*::GFP transgene expression was significantly reduced in
256 a *daf-28* mutant (Fig. 4A,B). This result suggests that positive feedback mediated by *daf-16*
257 repression of *daf-28*, *daf-28* agonism of *daf-2*/InsR, and *daf-2* inhibition of *daf-16* results in *daf-*
258 *28* effectively promoting its own expression. *daf-28*, *ins-4* and *ins-6* coordinately regulate dauer
259 entry and exit (Cornils *et al*, 2011), and they redundantly promote L1 development in response
260 to feeding (Chen and Baugh, 2014). *ins-4, 5* and *6* are in a chromosomal cluster, so we
261 analyzed a deletion allele that removes all three (Hung *et al*, 2014). P*daf-28*::GFP expression
262 was significantly reduced in fed larvae of the *ins-4, 5, 6* mutant compared to WT (Fig. 4A,B).
263 This result suggests that feedback regulation results in cross-regulation among insulin-like
264 peptides such that the function of one peptide affects the expression of others. Compound
265 mutants affecting *ins-4, 5, 6* and *daf-28* grow slowly as fed L1 larvae and display starvation
266 resistance during L1 arrest (Chen and Baugh, 2014), and P*daf-28*::GFP expression was also
267 reduced consistent with these phenotypes (Fig. 4B). In summary, reporter gene analysis
268 suggests physiological significance of feedback regulation, consistent with function of individual
269 insulin-like peptides affecting expression of others.
270
271 **Discussion**

272 We determined the extent of feedback regulation of insulin-like signaling in *C. elegans* in
273 starved and fed L1 larvae. We show that mRNA expression of nearly all detectable insulin-like
274 genes is affected by insulin-like signaling activity, revealing pervasive feedback regulation. We

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275 also show that several components of the PI3K pathway, including *daf-2*/InsR and *daf-16*/FoxO,
276 are affected by signaling activity. Together these results suggest that feedback occurs inter- and
277 intra-cellularly (Fig. 4C). Furthermore, we show that feedback is positive and negative at both
278 levels of regulation. Finally, we demonstrate that feedback regulation results in auto- and cross-
279 regulation of insulin-like gene expression.

280 We detected substantially more regulation of insulin-like genes by *daf-16*/FoxO than
281 previously reported in genome-wide expression analyses. We also detected extensive effects of
282 temperature on insulin-like gene expression. In contrast to other expression analyses, our
283 analysis employed highly synchronous populations of larvae, improving sensitivity. Sensitivity
284 was also likely improved by focusing on proximal effects of nutrient availability, which has robust
285 effects on insulin-like signaling. In addition, the nCounter assay conditions used are optimized
286 for sensitivity and precision (Baugh *et al*, 2011), improving power to detect differential
287 expression. We also analyzed the effects of *daf-16* mutation in a WT background as well as a
288 *daf-2* mutant background, in fed and starved larvae, producing four independent opportunities to
289 detect an effect of *daf-16.* Finally, we sampled extensively, not only with biological replicates,
290 but also with three different temperatures during L1 arrest as well as nine time points after
291 feeding. Taken together, these features likely explain why we detected such extensive effects.

292 Other nutrient-dependent pathways also regulate expression of insulin-like genes and
293 PI3K pathway components. That is, insulin-like signaling does not account for all of the
294 observed effects of nutrient availability on gene expression (Fig. 4C). For example, we show
295 that *daf-28* expression is up-regulated in response to feeding and that it is repressed by *daf-*
296 *16*/FoxO. Since DAF-16 is nuclear and active during starvation and is excluded from the
297 nucleus in response to feeding (Henderson and Johnson, 2001), it is conceivable that up-
298 regulation of *daf-28* in response to feeding is due to inactivation of DAF-16 and de-repression of
299 *daf-28.* However, this model predicts that *daf-28* expression should be equivalent in starved and
300 fed *daf-16* mutant larvae, but it is not. To the contrary, induction of *daf-28* in fed larvae occurs

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301 with similar magnitude in each genotype tested. This was true with mRNA expression analysis
302 by nCounter as well as transcriptional reporter gene analysis. Despite numerous examples of
303 *daf-2* and *daf-16* affecting expression, the effects of nutrient availability are generally evident in
304 all genotypes, indicating the influence of other nutrient-dependent pathways (Fig. 4C).

305 We provide evidence that *daf-16*/FoxO activity leads to activation and repression of
306 genes involved in insulin-like signaling. Both modes of regulation were observed for putative
307 *daf-2*/InsR agonists and antagonists, supporting the conclusion agonists and antagonists both
308 contribute to positive and negative feedback regulation. However, we used genetic and not
309 biochemical analysis, so we do not know if DAF-16 regulation is direct or indirect. DAF-16 is
310 thought to function primarily as an activator (Riedel *et al*, 2013; Schuster *et al*, 2010), with
311 repression ("class II" targets) occurring indirectly via its antagonism of the transcriptional
312 activator PQM-1 (Tepper *et al*, 2013). However, a role of *pqm-1* in L1 arrest and recovery has
313 not been investigated. Nonetheless, *akt-1*/Akt, *akt-2*/Akt, *skn-1*/Nrf and *daf-16*/FoxO were each
314 included on a list of 65 high-confidence direct DAF-16 targets (Schuster *et al*, 2010). We found
315 each of these to be regulated by *daf-16*, with *skn-1* and *daf-16* being repressed, consistent with
316 direct repression independent of PQM-1. Mechanistic details aside, this work reveals extensive
317 positive and negative feedback regulation of insulin-like signaling.

318 Insulin-like peptide function regulates expression of insulin-like genes. We used reporter
319 gene analysis to show that function of *daf-28*, a *daf-2* agonist repressed by *daf-16*, affects its
320 own transcription. Furthermore, we showed that function of other agonists cross-regulate *daf-28*
321 transcription. These results are consistent with reports of insulin-like peptides affecting
322 expression of insulin-like genes (Fernandes de Abreu *et al*, 2014; Ritter *et al*, 2013), though in
323 this case we demonstrate an intermediary effect of *daf-16*/FoxO. Given that we found most
324 insulin-like genes to be regulated by insulin-like signaling, cross regulation among insulin-like
325 peptides is likely common.

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326 We believe the physiological significance of feedback regulation is to stabilize signaling
327 activity in variable environments. Negative feedback supports homeostasis, returning the
328 system to a stable steady state (Cannon, 1929). In contrast, positive feedback supports rapid
329 responses and switch-like behavior (Ingolia and Murray, 2007). We speculate that by combining
330 negative and positive feedback, the insulin-like signaling system is able to maintain homeostasis
331 at different set points of signaling activity. That is, in constant conditions negative feedback
332 stabilizes signaling activity, but when conditions change (e.g., differences in nutrient availability)
333 positive feedback allows signaling activity to respond rapidly and negative feedback helps it
334 settle to a new steady state rather than displaying runaway dynamics. In addition, signaling
335 occurs in the context of a multicellular animal, with tissues and organs that presumably vary in
336 their energetic and metabolic demands. Consequently, FoxO-to-FoxO signaling resulting from
337 feedback may be relatively positive or negative in different anatomical regions, governed by the
338 peptides involved, serving to coordinate the animal's physiology appropriately (Kaplan and
339 Baugh, 2016; McMillen *et al*, 2002). In any case, the extent of feedback suggests that it is a very
340 important means of regulation. We imagine that insulin-like signaling in other animals and other
341 endocrine signaling systems are also rife with feedback, and that it is critical to system
342 dynamics.
343
344

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345 **Materials and Methods**

346 Nematode culture and sample collection

347 The following *C. elegans* strains were used for gene expression analysis on the
348 NanoString nCounter platform: N2 (wild type), PS5150 (*daf-16(mgDf47)*), CB1370 (*daf-*
349 *2(e1370)*), DR1942 (*daf-2(e979)*), GR1309 (*daf-16(mgDf47); daf-2(e1370)*). Strains were
350 maintained on NGM agar plates with *E. coli* OP50 as food at 15°C (DR1942) or 20°C (all others).
351 Liquid culture was used to obtain sufficiently large populations for time-series analysis with
352 microgram-quantities of total RNA. Larvae were washed from clean, starved plates with S-
353 complete and used to inoculate liquid cultures (Lewis, 1995). A single 6 cm plate was typically
354 used, except with CB1370 and DR1942, for which two and three plates were used, respectively.
355 Liquid cultures were comprised of S-complete and 40 mg/ml *E. coli* HB101. These cultures were
356 incubated at 180 rpm and 15°C for four days (with the exception of DR1942, which was
357 incubated for five days), and eggs were prepared by standard hypochlorite treatment, yielding in
358 excess of 100,000 eggs each. These eggs were used to set up another liquid culture again
359 consisting of S-complete and 40 mg/ml HB101 but with a defined density of 5,000 eggs/ml.
360 These cultures were incubated at 180 rpm and 15°C for five days (N2, PS5150 and GR1309),
361 six days (CB1370) or seven days (DR1942), and eggs were prepared by hypochlorite treatment
362 with yields in excess of one million eggs per culture. These eggs were cultured in S-complete
363 without food at a density of 5,000 eggs/ml at 180 rpm so they hatch and enter L1 arrest. For
364 starved samples at 20°C and 25°C, they were cultured for 24 hr and collected, and for 15°C
365 they were cultured for 48 hr. Fed samples were cultured for 24 hr at 20°C, and then 25 mg/ml
366 HB101 was added to initiate recovery by feeding. Fed samples were collected at the time points
367 indicated. Upon collection, larvae were quickly pelleted by spinning at 3,000 rpm for 10 sec,
368 washed with S-basal and spun three times, transferred by Pasteur pipet to a 1.5 ml plastic tube
369 in 100 μl or less, and flash frozen in liquid nitrogen. Samples were collected in at least two but

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370 typically three independent biological replicates where the entire culture and collection process
371 was repeated.
372
373 RNA preparation and hybridization
374 Total RNA was prepared using 1 ml TRIzol (Invitrogen) according to the manufacturer's
375 instructions. 3 μg total RNA was used for hybridization by NanoString, Inc (Seattle, WA USA),
376 as described (Chen and Baugh, 2014). The codeset used included the same probes for all
377 insulin-like genes as in Chen, 2014 with the exception of *ins-13*, which was replaced here. The
378 codeset also included probes for additional genes not included in Chen, 2014 (for a complete
379 list of genes targeted see Supplementary Data File 1) as well as standard positive and negative
380 control probes.
381
382 Data analysis
383 nCounter results were normalized in a two-step procedure. First, counts for positive
384 control probes (for which transcripts were spiked into the hybridization at known copy numbers)
385 were used to normalize the total number of counts across all samples. Second, the total number
386 of counts for all targeted genes except *daf-16* (the deletion mutant used did not produce signal
387 above background) was normalized across all samples. Insulin genes with a normalized count
388 of less than 5,000 were excluded from further analysis because they displayed a cross-
389 hybridization pattern indicating that they were not reliably detected. The complete normalized
390 data set is available in Supplementary Data File 1.
391 Statistical analysis was used to assess the effects of *daf-16* (in fed and starved samples)
392 and temperature (starved samples only). For the effect of *daf-16* in fed samples, two tests were
393 used: a non-parametric ANCOVA with the null hypothesis that loess lines connecting the points
394 of the *daf-16* single mutant (or the *daf-16; daf-2* double mutant) and wild type (or *daf-2(e1370)*)
395 are overlapping. This test was implemented using the R package "sm" (Bowman and Azzalini,

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396 1997). For the effect of *daf-16* in starved samples, two tests were used: a bootstrap test was
397 used with the null hypothesis that the *daf-16* single mutant (or the *daf-16; daf-2* double mutant)
398 has the same mean expression level as wild type (or *daf-2(e1370)*) for all temperatures. The
399 effect size of genotype is calculated within each temperature, so it controls for temperature.
400 10,000 permutations of genotype were calculated to get the p-value. For the effect of
401 temperature during starvation, a chi-squared goodness of fit test was used to ask whether
402 temperature explained additional variance in gene expression after controlling for genotype.
403 Benjamani-Hochberg was used to calculate the 'q-value' (Benjamini and Hochberg, 1995), and
404 these q-values were used to identify genes affected by *daf-16* or temperature at a false-
405 discovery rate of 5%. The complete results of statistical analysis is available in Supplementary
406 Data File 2.
407
408 Reporter gene analysis
409 The mgIs40 [P*daf-28*::GFP] reporter (Li *et al*, 2003) was analyzed using the following
410 genetic backgrounds: wild type (N2), *daf-16(mu86)*, *daf-28(tm2308)* and *ins-4, 5, 6(hpDf761)*.
411 Strains were maintained on NGM agar plates with *E. coli* OP50 as food at 20°C. Eggs were
412 prepared by standard hypochlorite treatment. These eggs were used to set up a liquid culture
413 consisting of S-basal without ethanol or cholesterol with a defined density of 1,000 eggs/ml.
414 After 18 hours to allow for hatching, *E. coli* HB101 was added at 25 mg/ml to the fed samples. 6
415 hours post food addition, the samples were washed three times with 10 ml S-basal and then run
416 through the COPAS BioSorter measuring GFP fluorescence. Analysis of the COPAS data was
417 performed in R. Tukey fences were used to remove outliers. Data points were also removed if
418 they were determined to be debris by size or lack of fluorescent signal. This cleanup left a total
419 of almost 165,000 data points. Fluorescence data was normalized by worm density. The Bartlett
420 test of homogeneity of variances rejected the null hypothesis that the samples had equal
421 variance. Therefore, unpaired t-tests with unequal variance were used to determine the

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422 significance of condition and genotype on mean normalized fluorescence. There were three
423 biological replicates for the insulin-like peptide mutants and seven biological replicates for wild
424 type and *daf-16* mutants.
425 For imaging, the samples were prepared in the same way then paralyzed with 3.75 mM
426 sodium azide and placed on an agarose pad on a microscope slide. Images were taken on a
427 compound fluorescent microscope.
428
429 Data Availability
430 The complete normalized data set is available in Supplementary Data File 1. Complete
431 results of statistical analysis is available in Supplementary Data File 2. Raw data and strains
432 used here are available upon request.
433
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439
440 **Author Contributions**
441 LRB conceived of the study and provided funding. LRB, REWK and NKC performed the
442 experiments. CSM and REWK analyzed the data. LRB, CSM and REWK prepared the
443 manuscript.
444
445 **Conflict of Interest**
446 The authors have no conflicts of interest to declare.

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618 **Figure Legends**

619

620 Figure 1. *daf-16*/FoxO is epistatic to *daf-2*/InsR for expression of genes involved in insulin-like
621 signaling. A) A diagram of the *C. elegans* insulin-like signaling pathway. B) A schematic of the
622 experimental design with times and conditions sampled indicated. C) A symmetric matrix of
623 correlation coefficients for pairs of genotypes is presented as a heat map, with scale bar.
624 Examination of individual gene expression patterns confirmed that *daf-16* is epistatic to *daf-2* in
625 each instance without exception.

626

627 Figure 2. Insulin-like signaling regulates expression of genes comprising the insulin-like
628 signaling pathway. Transcript abundance (arbitrary units) is plotted over time during recovery
629 from L1 starvation by feeding in five different genotypes with various levels of insulin-like
630 signaling activity. In addition to *daf-2*/InsR and components of the PI3K pathway, the
631 transcriptional effectors of signaling, *daf-16*/FoxO and *skn-1*/Nrf, are plotted as well as the
632 known DAF-16 target *sod-3*. Note that *daf-16* expression was not detected in *daf-16* mutants.
633 Each gene plotted was significantly affected by *daf-16* (see Tables 1, S1 and supp. data). Error
634 bars reflect the SEM of two or three biological replicates.

635

636 Figure 3. Insulin-like signaling regulates expression of the majority of insulin-like peptides.
637 Transcript abundance (arbitrary units) is plotted over time during recovery from L1 starvation by
638 feeding in five different genotypes with various levels of insulin-like signaling activity. Of 28
639 reliably detected insulin-like genes, 25 were significantly affected by *daf-16* (all but *ins-2, -10*
640 and *-34*; see Tables 1, S1 and supp. data) and are plotted. Error bars reflect the SEM of two or
641 three biological replicates.

642

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643 Figure 4. Insulin-like peptide function affects expression of insulin-like peptides. A)
644 Representative images of a P*daf-28*::GFP transcriptional reporter gene are presented for WT,
645 *daf-16(mu86)*, *daf-28(tm2308)* and *ins-4, 5, 6(hpDf761)* in starved and fed (6 hr) L1 larvae, as
646 indicated. Images were cropped and expression in anterior neurons and posterior gut is shown.
647 B) Quantitative analysis of P*daf-28*::GFP expression using the COPAS BioSorter is presented.
648 The grand average and standard deviation of three to seven biological replicates is plotted for
649 starved (0 hr) and fed (6 hr) L1 larvae. *$p<0.05$, ***$p<0.001$ (unpaired t-test on replicate means,
650 n=3 to 7). C) Pervasive feedback regulation of insulin-like signaling. *daf-2*/InsR antagonizes *daf-*
651 *16*/FoxO via the PI3K pathway. *daf-16* activates expression of three pathway components to
652 produce negative feedback, and it represses expression of two components to produce positive
653 feedback. *daf-16* also appears to activate its own expression, producing positive feedback. *daf-*
654 *16* represses expression of eight putative insulin-like peptide agonists of *daf-2* and activates five
655 putative antagonists, producing positive feedback in each case. *daf-16* also activates six
656 putative agonists and represses four putative antagonists, producing negative feedback. Food
657 activates expression of agonists and represses expression of antagonists, independent of *daf-*
658 *16* and insulin-like signaling. Dashed arrows reflect putative function of insulin-like peptides as
659 agonists or antagonists of *daf-2*, reflecting cell-nonautonomous effects of *daf-16*. Inferred
660 positive feedback is depicted in green and negative feedback in red. Numbers next to colored
661 arrows indicate the number of genes represented by each arrow.
662
663 Figure S1 - complementing Fig. 2. Insulin-like signaling regulates expression of genes
664 comprising the insulin-like signaling pathway during L1 starvation. Transcript abundance
665 (arbitrary units) is plotted during L1 starvation at three different temperatures in five different
666 genotypes with various levels of insulin-like signaling activity. In addition to *daf-2*/InsR and
667 components of the PI3K pathway, the transcriptional effectors of signaling, *daf-16*/FoxO and
668 *skn-1*/Nrf, are plotted as well as the known DAF-16 target *sod-3*. Note that *daf-16* expression

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669 was not detected in *daf-16* mutants. Each gene plotted was significantly affected by *daf-16* (see
670 Tables 1, S1 and supp. data). Error bars reflect the SEM of two or three biological replicates.
671
672 Figure S2 - complementing Fig. 3. Insulin-like signaling and temperature regulate expression of
673 the majority of insulin-like peptides during L1 starvation. Transcript abundance (arbitrary units)
674 is plotted during L1 starvation at three different temperatures in five different genotypes with
675 various levels of insulin-like signaling activity. Of 28 reliably detected insulin-like genes, 25 were
676 significantly affected by *daf-16* and are plotted. 21 were significantly affected by temperature (all
677 but *ins-2, 9, 12, 16, 21, 29, 34*). *ins-10* was affected by temperature but not *daf-16* and is not
678 plotted here (see Tables 1, S1 and supp. data). Error bars reflect the SEM of two or three
679 biological replicates.
680
681 Supp Data File 1. Complete normalized expression data set. Raw data is available upon request.
682
683 Supp Data File 2. Complete statistics for regulation of all genes during starvation and recovery
684 by *daf-16* and temperature.
685

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Figure 1

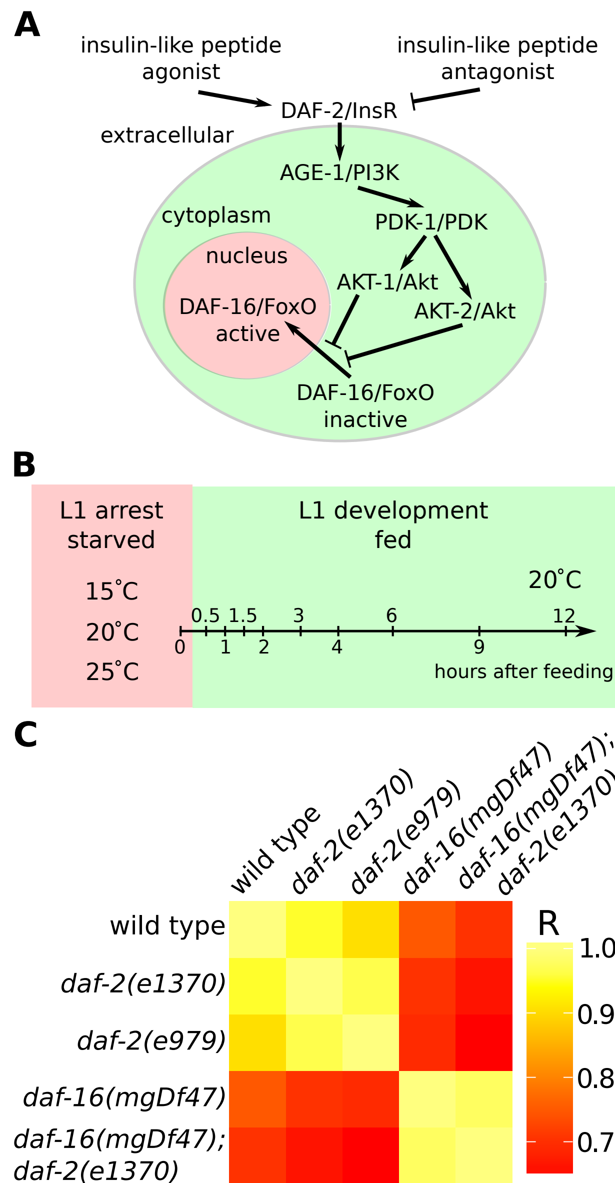

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Figure 2

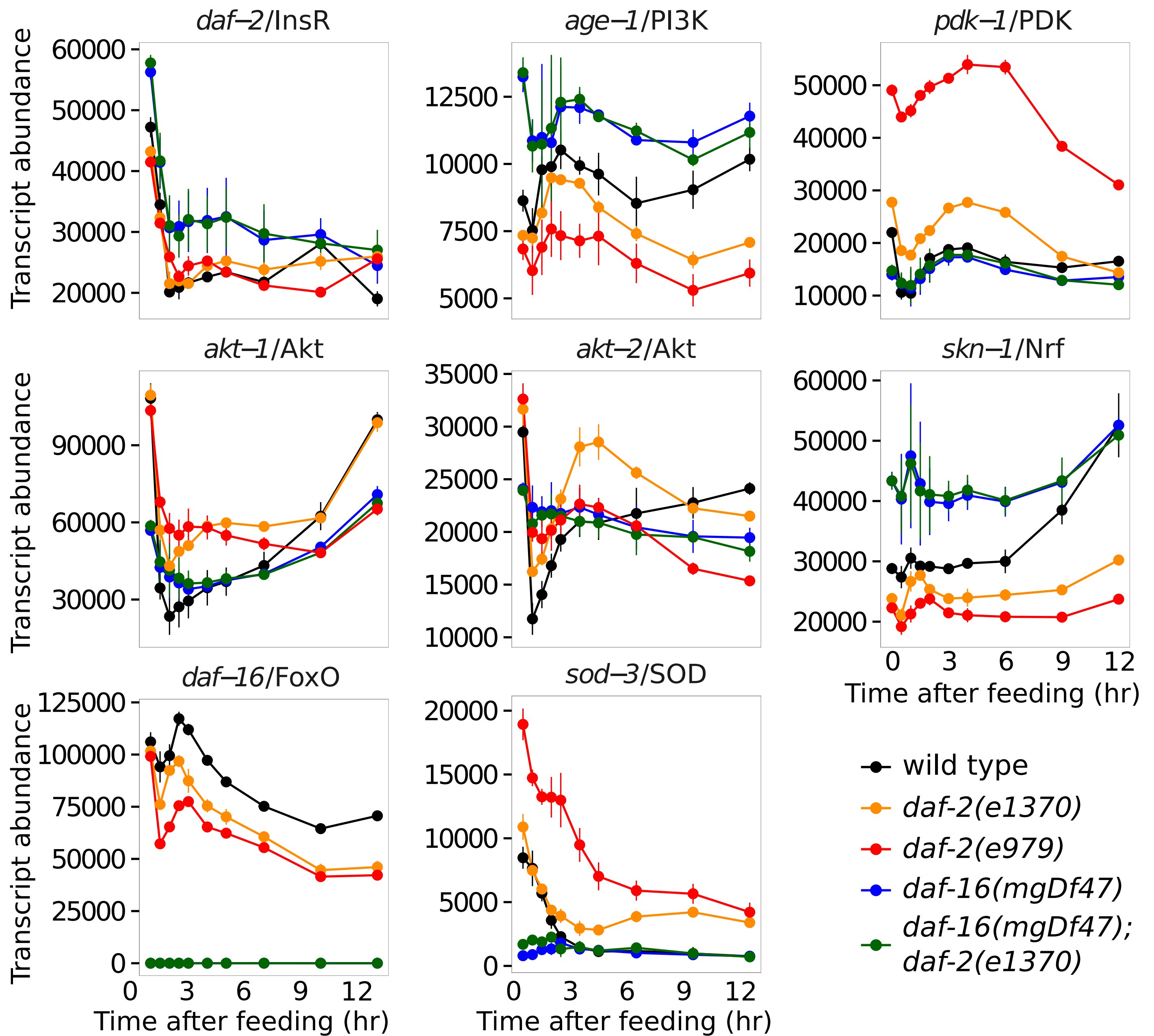

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Figure 3

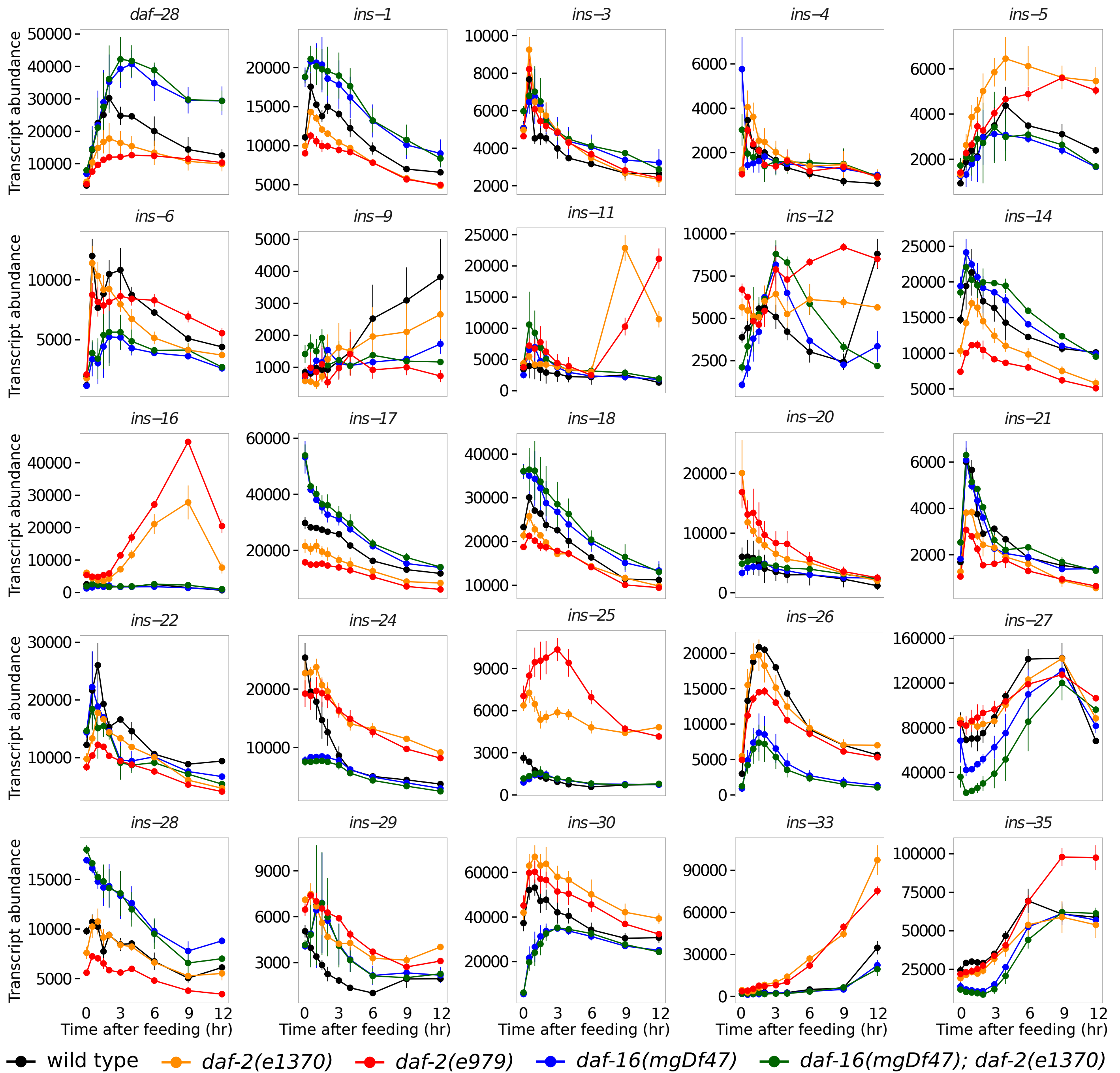

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Figure 4

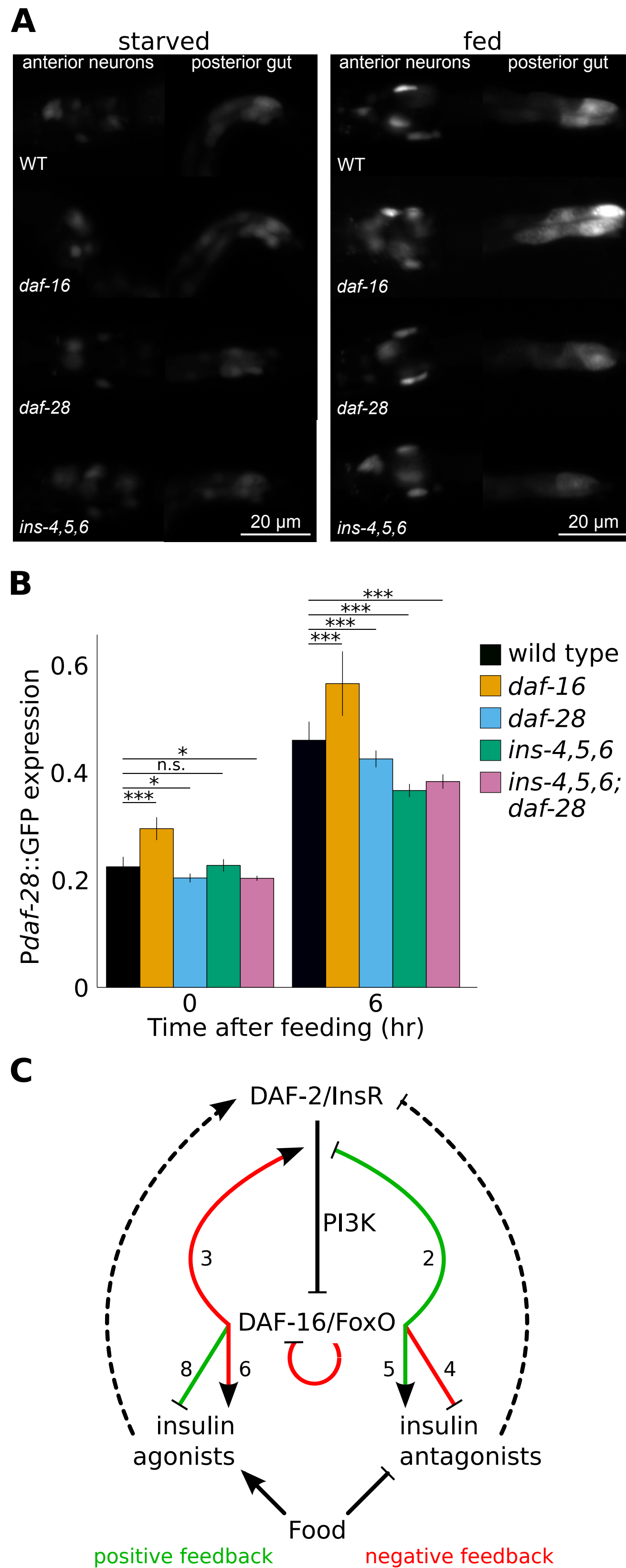

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Figure S1

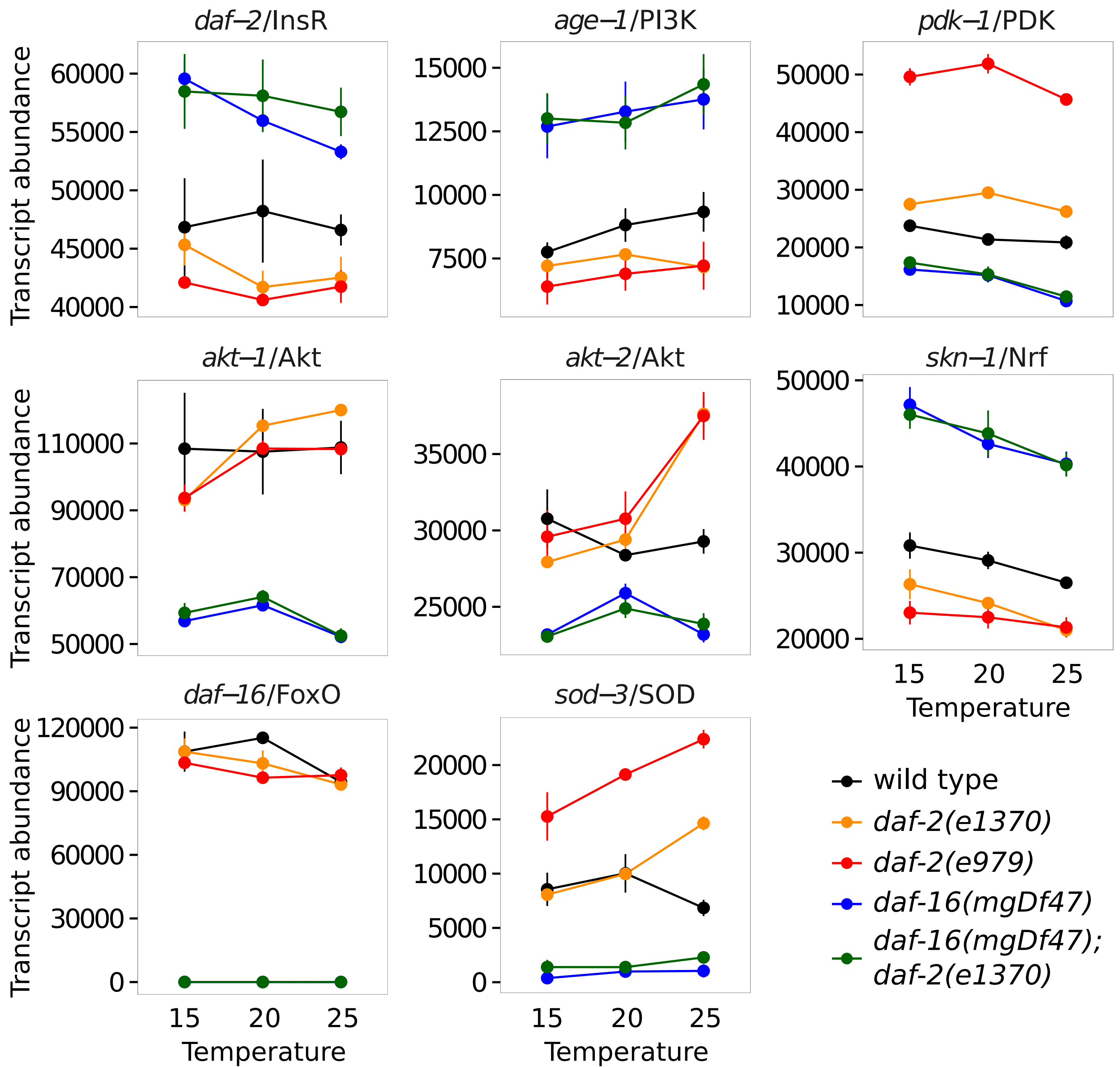

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Figure S2

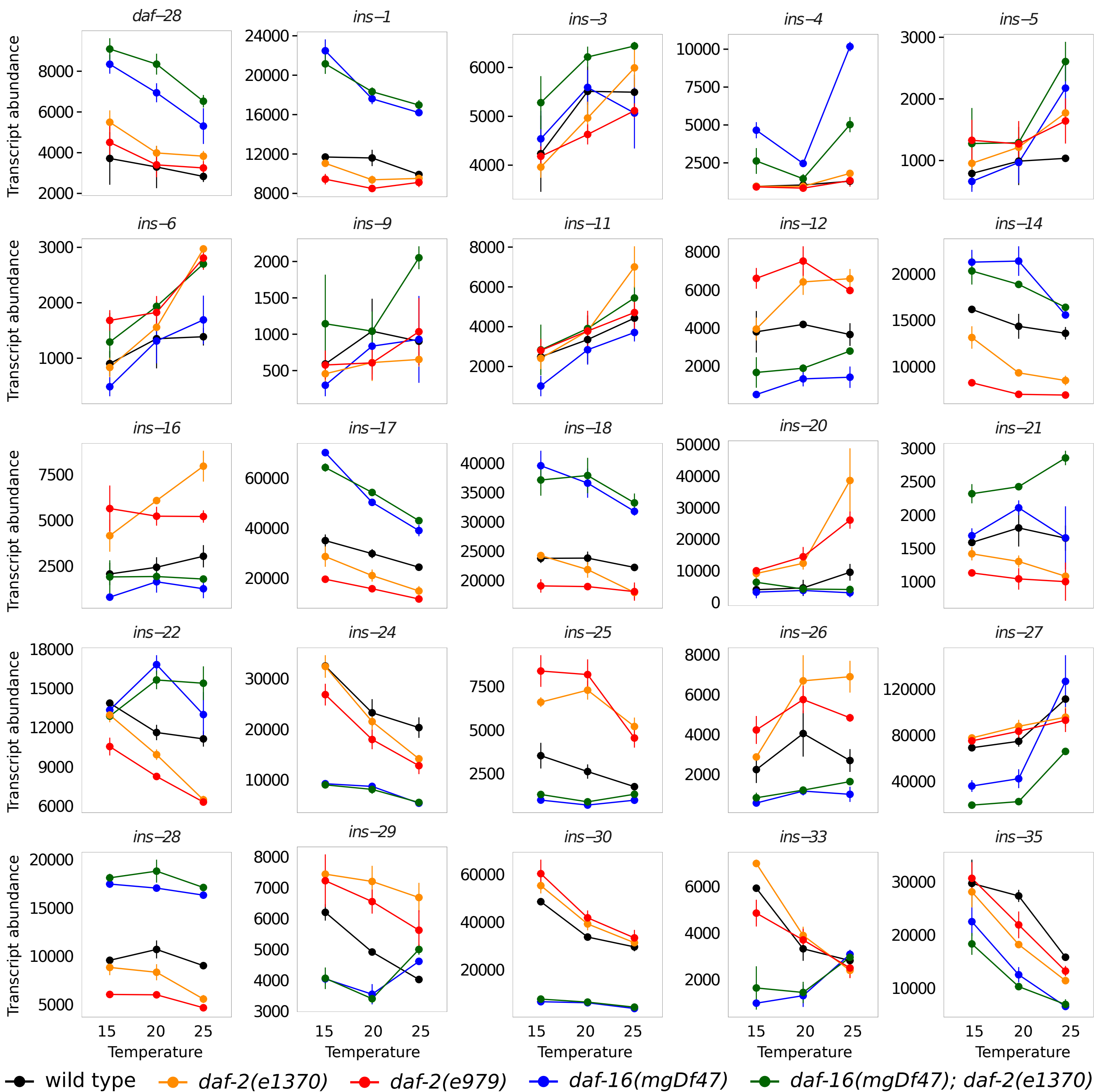

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Table 1. Summary of genes regulated by *daf-16*/FoxO. The gene, whether it is activated or repressed by *daf-16*, putative function of the gene, and whether regulation is predicted to result in positive or negative feedback is presented. Four total tests for regulation were considered (during L1 starvation at three different temperatures and during recovery over time after feeding, comparing *daf-16(mgDf47)* to WT and also *daf-16(mgDf47); daf-2(e1370)* to *daf-2(e1370)*). Results are considered significant if the p-value is below 0.05 in any one test after correction for multiple testing. See supplementary information for complete statistical analysis. Insulin-like peptides are predicted to function as agonists or antagonists of *daf-2*/InsR based on published genetic or expression analysis (Chen et al. 2014 is cited separately for results based on genetic or expression analysis; all other citations are for genetic analysis), and positive or negative feedback is predicted based on putative function (agonist or antagonist) and whether the gene is positively or negatively regulated by *daf-16*.

Gene	Regulation by *daf-16*	Putative function	Predicted feedback
daf-2/InsR	repressed	insulin receptor	positive
age-1/PI3K	repressed	PI3K pathway	positive
pdk-1/PDK	activated	PI3K pathway	negative
akt-1/Akt	activated	PI3K pathway	negative
akt-2/Akt	activated	PI3K pathway	negative
skn-1/Nrf	repressed	insulin signaling effector	NA
daf-16/FoxO	repressed	insulin signaling effector	negative
sod-3/SOD	activated	known *daf-16* target	NA
daf-28	repressed	agonist[a,b,c,i,j,k,l]	positive
ins-1	repressed	antagonist[b,d,i,j]	negative
ins-3	repressed	agonist[e,i,j,l]	positive
ins-4	repressed	agonist[c,i,j,k,l]	positive
ins-5	activated	agonist[j,l]	negative
ins-6	activated	agonist[b,c,i,j,k,l]	negative
ins-9	repressed	agonist[k]	positive
ins-11	repressed	antagonist[f,j,k]	negative
ins-12	activated	antagonist[j]	positive
ins-14	repressed	agonist[j]	positive
ins-16	activated	antagonist[l]	positive
ins-17	repressed	antagonist[g,l]	negative
ins-18	repressed	antagonist[d,h,i,j,l]	negative
ins-20	activated	antagonist[j,l]	positive
ins-21	repressed	agonist[j,l]	positive
ins-22	repressed	agonist[j,l]	positive
ins-24	activated	unknown	unknown
ins-25	activated	antagonist[l]	positive
ins-26	activated	agonist[j,l]	negative
ins-27	activated	agonist[j,l]	negative
ins-28	repressed	agonist[j,l]	positive
ins-29	activated	antagonist[l]	positive
ins-30	activated	unknown	unknown
ins-33	activated	agonist[e,j,l]	negative
ins-35	activated	agonist[j,l]	negative

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[a] Patel et al. (2008).
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[h] Matsunaga et al. (2012b)
[i] Hung et al. (2014).
[j] Fernandes de Abreu et al. (2014).
[k] Chen et al. (2014) - genetics.
[l] Chen et al. (2014) - expression.

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Table S1. Summary of insulin-like peptides whose expression is affected by temperature during L1 starvation. The gene, the nominal p-value for the effect of temperature after controlling for genotype (not corrected for multiple testing), and whether the gene's expression is positively or negatively correlated with temperature is presented. Only those genes with a p-value below 0.05 after correction for multiple testing are included.

Gene	p-value	Correlation with temperature
daf-28	2.2E-04	negative
ins-1	6.3E-05	negative
ins-3	4.0E-05	positive
ins-4	6.8E-04	positive
ins-5	6.3E-04	positive
ins-6	1.9E-07	positive
ins-10	1.7E-05	positive
ins-11	8.6E-06	positive
ins-14	1.0E-05	negative
ins-17	1.6E-08	negative
ins-18	7.0E-04	negative
ins-20	2.2E-03	positive
ins-22	2.2E-03	negative
ins-24	6.6E-08	negative
ins-25	2.3E-03	negative
ins-26	2.3E-03	positive
ins-27	1.3E-05	positive
ins-28	6.1E-05	negative
ins-30	1.0E-06	negative
ins-33	7.1E-03	negative
ins-35	8.8E-12	negative
sod-3	3.2E-03	positive