1   **Nutrient sensing pathways regulating adult reproductive diapause in *C. elegans***

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25   **ABSTRACT**

26   Genetic and environmental manipulations, such as dietary restriction (DR), can improve both

27   health span and lifespan in a wide range of organisms, including humans. Changes in nutrient

28   intake trigger often overlapping metabolic pathways that can generate distinct or even opposite

29   outputs depending on several factors, such as when DR occurs in the lifecycle of the organism or

30   the nature of the changes in nutrients. Due to the complexity of metabolic pathways and the

31   diversity in outputs, the underlying mechanisms regulating diet-associated pro-longevity are not

32   yet well understood. Adult reproductive diapause (ARD) in the model organism *Caenorhabditis*

33   *elegans* is a DR model that is associated with lengthened lifespan and reproductive potential

34   (Angelo and Van Gilst 2009). As the metabolic pathways regulating ARD have not yet been

35   explored in depth, we performed a candidate-based genetic screen analyzing select nutrient-

36   sensing pathways to determine their contribution to the regulation of ARD. Focusing on the three

37   phases of ARD (initiation, maintenance, and recovery), we find that ARD initiation is regulated

38   by fatty acid metabolism, sirtuins, AMPK, and the O-linked N-acetyl glucosamine (O-GlcNAc)

39   pathway. Although ARD maintenance was not significantly influenced by the nutrient sensors in

40   our screen, we found that ARD recovery was modulated by energy sensing, stress response,

41   insulin-like signaling, and the TOR pathway. We also discovered that fatty acid β-oxidation

42   regulates ARD initiation through a pathway involving the O-GlcNAc cycling enzyme, OGT-1,

43   acting with the nuclear hormone receptor NHR-49. Consistent with these findings, our analysis

44   revealed a change in levels of neutral lipids associated with ARD entry defects. Our findings

45   thus identify novel conserved genetic pathways required for ARD entry and recovery and

46   identify new genetic interactions that provide insight into the role of OGT and OGA.

47

INTRODUCTION

Aging is a leading risk factor for chronic human diseases such as diabetes, cancer, and neurodegeneration (Niccoli and Partridge 2012). Our understanding of aging has undergone a paradigm shift in the past 30 years as research indicates that aging may not be just an inevitable decline in health accompanied by increased disease incidence (Gems and Partridge 2013). Indeed, manipulation of genetic pathways and environmental conditions can modulate healthy aging (health span) and overall lifespan (Kenyon *et al.* 1993; Kenyon 2010). Thus, the aging process is now seen as a potential therapeutic target to improve health span in a rapidly growing elderly population (de Magalhães *et al.* 2012).

Dietary restriction (DR) is one of the most well-studied methods for increasing longevity in model organisms and has also been observed to improve overall markers of health in humans (Stein *et al.* 2012; Tinsley and La Bounty 2015). Specific changes in nutrient intake and the subsequent alteration to key metabolic processes is thought to contribute to the improvements in health and lifespan observed in a plethora of organisms (Fontana and Partridge 2015; López-Otín *et al.* 2016). However, identifying the underlying mechanisms and their role in longevity remains an intense and controversial area of research (Kennedy *et al.* 2007; Mair and Dillin 2008). While different forms of DR can trigger divergent pro-longevity pathways with distinct outcomes (Greer and Brunet 2009), they often share common features including involvement of overlapping nutrient-sensing factors and changes to metabolism, particularly fat and carbohydrate stores (Houtkooper *et al.* 2010; Lapierre and Hansen 2012; Finkel 2015; Solon-Biet *et al.* 2015).

DR-dependent pro-longevity pathways are highly conserved, suggesting research in model organisms, such as the nematode *Caenorhabditis elegans,* can inform dietary

71   interventions and therapeutic targets relevant for improving human health span. For any

72   organism, there is a delicate balance between nutrient acquisition, development, aging, and

73   reproduction to ensure the health and propagation of the species. *C. elegans* is an exceptional

74   model that has been used extensively to examine the intersection of longevity and these

75   biological processes because it has a short life cycle, is highly fecund, and is genetically

76   amendable with signaling pathways that are evolutionarily conserved.

77   Changes in nutrient status affect the life cycle of *C. elegans* at several key points during

78   development. Extended periods with limited or no nutrients can result in a diapause state, after

79   which the animal has a full or extended lifespan upon re-feeding (Hu 2007). In the more recently

80   identified adult reproductive diapause (ARD) (Angelo and Van Gilst 2009), when the nematode

81   undergoes DR in the mid-L4 larval stage, approximately 1/3 of worms remain in L4 (do not

82   reach adulthood), 1/3 undergo bagging (matricide), and 1/3 enter the ARD state. ARD is

83   characterized by worms that enter into adulthood with the retention of one to two "frozen"

84   embryos, which are maintained throughout the starvation period, and the shrinkage of the

85   germline to a small pool of germ cells. This pool of germ cells are capable of regenerating the

86   entire germline after re-feeding and exit from ARD. A related study defined a process called the

87   oogenic germline starvation response, in which germline shrinkage is linked to maintenance of

88   the oocyte pool (Seidel and Kimble 2011). More recent findings also suggest that the

89   transgenerational sterility observed in Piwi mutants defective in piRNAs may represent a

90   dynamic form of ARD (Heestand *et al.* 2018). Thus, ARD represents a powerful system to

91   analyze the interplay between DR, nutrient-sensing, germline dynamics, and lifespan extension.

92   A number of nutrient sensors have been found to play important roles under various DR

93   conditions, including key factors in the target of rapamycin (TOR) pathway, AMP-activated

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94   protein kinase (AMPK) pathway, insulin signaling, hexosamine signaling, and fat metabolism

95   pathway. Teasing apart the regulation of pro-longevity pathways has proven to be complicated,

96   due in part to the complex web of interactions inherent in metabolic processes. For example, the

97   transcription factor DAF-16/FoxO, which has been extensively researched for its role in pro-

98   longevity, can be regulated by insulin-like signaling, TOR pathway, and sirtuin activity and, in

99   turn, regulates transcription of components of the TOR pathway and the energy-sensing AMPK,

100   among many others (Berdichevsky *et al.* 2006; Lant *et al.* 2010; Lapierre and Hansen 2012). In

101   addition, numerous proteins required for pro-longevity pathways, such as various components of

102   the insulin receptor signaling pathway, including FoxO (Housley *et al.* 2008; Yang *et al*. 2008;

103   Klein *et al.* 2009), and AMPK (Bullen *et al.* 2014) are substrates of the enzyme O-GlcNAc

104   transferase (OGT) and are post-translationally modified by O-GlcNAc, a downstream component

105   of the hexosamine signaling pathway (Hanover *et al.* 2009). Further, other nutrient-sensing

106   cellular components, including many known to play important roles in longevity, also interact

107   physically and/or genetically with OGT and O-GlcNAcase (OGA), the enzyme responsible for

108   O-GlcNAc removal (Ruan *et al.* 2013; Hardivillé and Hart 2014). While OGT and OGA are at

109   the hub of many of the metabolic pathways important to pro-longevity they have not yet been

110   analyzed extensively for their interactions and functions in longevity, in part due to their being

111   essential in most organisms. However, OGT-1 and OGA-1 are not essential in *C. elegans*

112   (Hanover *et al.* 2005; Forsythe *et al*. 2006)*,* offering a unique opportunity to explore genetic

113   interactions and analyze their role in longevity.

114   We sought to determine how nutrient status is sensed and transduced, what metabolic

115   changes occur, and what genes are essential during the three distinct ARD phases. To explore the

116   contribution of select nutrient sensors during ARD, we examined the ability of *C. elegans* strains

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117   to enter, maintain, and recover from ARD using mutants in key pathways that have well-

118   characterized roles in longevity. Using a candidate gene approach, we examined key nodes in fat

119   metabolism, insulin signaling, TOR, AMPK, sirtuin, and O-GlcNAc cycling (hexosamine)

120   pathways. Additional epistasis studies revealed an important role the *nhr-49*-dependent fatty acid

121   β-oxidation pathway for regulation of ARD entry in concert with O-GlcNAc cycling. These

122   results have important implications for understanding the dynamic activity of nutrient sensors in

123   longevity as well as furthering our understanding of the important functions and genetic

124   interactions for OGT and OGA.

125

126   **MATERIALS AND METHODS**

127   **Data Availability Statement:**

128   Supplemental figures, tables, methods and datasets have been uploaded via the GSA Figshare

129   portal. These files include all of the supplemental figures (Supplmental Figures) mentioned in

130   the text and table of raw data and statistical analysis (Supplemental Tables). Methods for

131   quantification are also listed: https://gsajournals.figshare.com/s/d16c34f3167aac98f9e2

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133   **Strains**

134   *C. elegans* strains were maintained and cultured under standard laboratory conditions at 20°C

135   with the food source *E. coli* OP50 as described (Brenner 1974). Specific alleles of *ogt-1* and *oga-*

136   *1* used in this study have previously been characterized, including *ogt-1(ok1474), ogt-1(ok430)*

137   (Hanover *et al.* 2005), *oga-1(tm3642)* (Bond *et al.* 2014), and *oga-1(ok1207)* (Forsythe *et al.*

138   2006). The alleles *ogt-1(jah01), oga-1(av82),* and *oga-1(av83)* were generated during the course

139   of this study using CRISPR/Cas9, as described below. The strain *daf-16(mu86)* was a gift from

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140   Dr. Michael Krause. The allele *nhr-49(nr2041)* was kindly gifted to us by Dr. Mark Van Gilst.

141   The strains RB754 *aak-2(ok524),* RB1988 *acs-2(ok2457),* TJ1052 *age-1(hx546),* BX153 *fat-*

142   *7(wa36),* RB1206 *rsks-1(ok1255),* VC199 *sir-2.1(ok434),* and QV225 *skn-1(zj15)* were obtained

143   from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, Minneapolis, MN)

144   strain bank, which is funded by the NIH Office of Research Infrastructure Programs (P40

145   OD010440). All of these strains were backcrossed to our wild-type N2 Bristol laboratory strain a

146   minimum of four times prior to analysis to ensure homogenous genetic background.

147   Genotyping was conducted using nested PCR, as described by the CGC, or using

148   sequencing (Eurofins Genomics, Louisville, KY) in the case of point mutants. Primers for *ogt-*

149   *1(ok1474), ogt-1(ok430), oga-1(tm3642),* and *oga-1(ok1207)* are described elsewhere (Bond *et*

150   *al.* 2014, Forsythe *et al.* 2006) and are available upon request. Primers for *aak-2(ok524), acs-*

151   *2(ok2457), rsks-1(ok1255),* and *sir-2.1(ok434)* were derived from the sequences available on

152   wormbase.org and CGC. Primers for *daf-16(mu86), nhr-49(nr2041),* and the point mutants *age-*

153   *1(hx546), fat-7(wa36),* and *skn-1(zj15)* were generated using Primer3 (Rozen and Skaletsky

154   2000) and are available on request.

155   Double mutants were generated by crossing, with the presence of deletion alleles verified

156   either using nested PCR or by sequencing, in the case of point mutants. Double mutants were

157   backcrossed a minimum of four times to our wild-type N2 Bristol strain before use in analysis.

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159   **Generation of CRISPR/Cas9 whole gene knockouts of *ogt-1* and *oga-1: ogt-1(jah01), oga-**

160   ***1(av82),* and *oga-1(av83)***

161   The alleles *ogt-1(jah01)*, *oga-1(av82),* and *oga-1(av83)* were generated during the course of

162   this study using CRISPR/Cas9 genome editing. These alleles are full gene deletions of the ORF

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163   of *ogt-1* and *oga-1,* respectively. We used the direct-delivery protocol developed by the

164   Seydoux lab (Paix *et al.* 2017) and screened for edits using the co-conversion *dpy-10* method

165   (Arribere *et al.* 2014). The crRNAs and repair template sequences are listed below. The repair

166   template contains the deletion and perturbs the PAM sites to prevent Cas9 from re-cutting. From

167   15 injected P0s, we identified 3 "jackpots" and picked a total of 40 rollers from them. Using

168   PCR genotyping, we identified and isolated three heterozygous lines from each. Homozygous

169   animals were identified in the F2 generation and subjected to sequencing of the relevant *ogt-1* or

170   *oga-1* region. For *ogt-1,* two lines contained the expected full deletion, which we subsequently

171   named *ogt-1(jah01).* With *oga-1,* two of the three lines contained the expected deletion, while

172   the third contained an additional ~17bp deletion. The two expected *oga-1* deletions were named

173   *oga-1(av82),* which were the strains used in our analysis. We also kept the larger *oga-1* deletion

174   and named it *oga-1(av83).*

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176   *ogt-1* **sequences:**

177   Targeted gene crRNA1 (*ogt-1* deletion N-terminal)

178   5' − AAUUUCAGAAUUAUAUCGAAGUUUUAGAGCUAUGCUGUUUUG − 3'

179   Targeted gene crRNA2 (*ogt-1* deletion C-terminal)

180   5' − GCUUGUGAAUAGAUUUUCGAGUUUUAGAGCUAUGCUGUUUUG − 3'

181   Repair template:

182   5' − CCAATAGTTCAATTTCCAAATTTCAGAATTATATCACACGGCTTGTGAATAGATTTTCGAAGGATTTTTA − 3'

183

184   *oga-1* **sequences:**

185   crRNA1 (*oga-1* deletion N-terminal):

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186   5' – GTTCACAGTCAATACTTAAT – 3'

187   crRNA2 (*oga-1* deletion C-terminal:

188   5' – CAAAATCGTTGAATTTGACT – 3'

189   Repair template:

190   5' – TTTTATTAAGAGTGAAAGTTGATGACAACATTTCCTGATTCAAATTCAACGATTTTGTTCCAACCGGGATAT – 3'

191

192   **Adult reproductive diapause**

193   Adult reproductive diapause experiments were performed as described previously (Angelo and

194   Van Gilst 2009), with minor adjustments. The overall experimental approach for initiation of

195   ARD is illustrated in **Supplemental Figure 1**. Briefly, nematodes were cultured at 20°C on

196   nematode growth medium (NGM) with *E. coli* OP50 as the food source at a density of 3,000 per

197   60 mm tissue culture dish. Worms were synchronized using hypochlorite bleaching of gravid

198   worms with a dilute alkaline hypochlorite solution, as previously described (Porta-de-la-Riva *et*

199   *al.* 2012). Eggs harvested from bleaching of gravid worms were then left with gentle rocking

200   overnight at room temperature in M9 solution. Hatched L4s were then plated on NGM plus

201   OP50 plates to complete synchronization. This synchronization process was repeated three times.

202   Following the third synchronization, 10,000 L1 worms (plate counting visually by

203   sectors) were grown on a single 100 mm NGM + OP50 plate and visually inspected at intervals

204   to ensure that over 90% of worms were in mid-L4 (approximately 48 hr, depending on growth of

205   strain). Mid-L4 worms were then washed off the plate with M9 and collected in a 15 ml conical

206   tube. The tubes were spun down at 20°C for 2 min at 1500 rpm. This washing was repeated 5

207   times with 5 ml M9 to ensure all bacteria was removed. Prior to the fifth and final spin, worms

208   were incubated in 5 ml M9 with gentle rocking at room temperature for 30 min to remove

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209   bacteria from gut. Following the 30 min incubation, all media was aspirated and the nematodes

210   were suspended in 1 ml of M9, counted, and plated on high agarose tissue culture plates without

211   bacteria/food source (ARD plates). ARD plates were prepared and poured as follows: 1 L: 3 g

212   NaCl and 15 g agarose autoclaved in 1 L water. After autoclave sterilization, filter sterilized

213   solutions of the following were added: 1 ml of 1M CaCl$_2$, 1 ml of 1M MgSO$_4$, 25 ml potassium

214   phosphate buffer (pH 6.0), and 1 ml cholesterol (8 mg/ml) (Angelo and Van Gilst 2009). Using

215   this solution, 60 mm plates were filled to two-thirds full in the hood to ensure sterility. After

216   plates were cooled and equalized to 20°C worms were added at a density of 3,000 worms per 60

217   mm plate. As a control, 3,000 of the synchronized and washed mid-L4s were plated separately

218   from the worms that were put on the ARD plates and were instead plated on NGM plates with

219   OP50 to allow analysis of lifespan and brood size in parallel to starved worms used to assess the

220   ARD phenotype.

221   Worms were visually inspected every 24 hours (up to 72 hours, depending on strain) after

222   plating and characterized as either in ARD, in L4, or bagged. At day 5, day 10, and day 30 after

223   mid-L4 worms were plated on ARD plates, worms were subjected to Oil Red O (ORO) staining,

224   carminic acid staining, lifespan analysis, and brood size counts as described below. Each

225   experiment was repeated a minimum of three times from start to finish.

226

227   **Lifespan, germline, and brood analysis**

228   At least 10 worms from each ARD plate were collected individually at day 5, day 10, and day 30

229   post-plating on ARD plates. The worms were visually inspected to ensure ARD status and placed

230   singly onto individual NGM plus OP50 plates and incubated at 20°C to monitor for restoration of

231   the germline, analysis of overall number of progeny following ARD (brood size), and post-ARD

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232   lifespan. Restoration of the germline was followed visually at day 5, day 10, and day 30 using

233   standard microscopy techniques on a Zeiss LSM 700 confocal microscope (Carl Zeiss

234   Microscopy, LLC, Thornwood, NY) with a Plan-Apochromat 20x/0.8 objective lens. Brood size

235   was monitored by carefully transferring the individually recovered worms every 24 hr to fresh

236   NGM plus OP50 plates until the end of their reproductive phase, marked by the absence of eggs.

237   Progeny of the post-ARD worms were counted after each transfer and added to total brood size

238   reported for each animal. For all experiments, any ARD worm that failed to thrive in the first 48

239   hr or disappeared was censored.

240   The post-ARD (recovered) worms were then inspected daily after the reproductive period

241   to determine lifespan. When worms no longer responded to gentle poking with a platinum pick,

242   they were scored as dead. Lifespan counts for post-ARD animals includes the days in ARD, such

243   that the lifespan post-re-feeding was added to the number of days the animals were starved.

244   Lifespan was graphed, and statistics were performed as previously described using GraphPad

245   and one-way ANOVA (Bond *et al*., 2014). A minimum of 40 ARD animals from four

246   independent experiments were used for analysis of each strain.

247   The controls also underwent the same analysis but without ever being placed on the ARD

248   plates and, instead, were grown on standard NGM plus OP50 plates at 20°C.

249

250   **Oil Red O staining**

251   The protocol for Oil Red O (ORO) staining was adapted from previously described methods

252   (O'Rourke *et al.* 2009) with minor adjustments. Prior to starting experiment, a stock solution of

253   0.5 g/100 ml ORO in isopropanol was allowed to equilibrate for at least two days at room

254   temperature with gentle rocking and light protection. On the day of the experiment, a fresh

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255   dilution of ORO stock solution was diluted to 60% with water and rocked for at least 1 hr. Dilute

256   ORO was then filter sterilized with a 0.22 μm filter.

257   Nematodes were washed off NGM plates with OP50 (control) or high agarose ARD

258   plates with 1 ml of 1x PBS + 0.01% Triton X-100 (PBST). Worms were washed 3 times with 1

259   ml of PBST and allowed to settle by gravity after each washing. Cuticles were permeabilized by

260   resuspending worms in 120 μl of PBST and 120 μl of 2x MRWB buffer (160 mM KCl, 40 mM

261   NaCl, 14 mM Na$_2$EGTA, 1 mM spermidine-HCl, 0.4 mM spermine, 30 mM Na-PIPES pH 7.4,

262   and 0.2% β-mercaptoethanol) with 2% paraformaldehyde. Worms were gently rocked for 1 hr at

263   room temperature. Animals were allowed to settle by gravity, buffer was aspirated, and animals

264   were washed with 1x PBST twice. Worms were then resuspended in 60% isopropanol and

265   incubated 15 min with gentle rocking at room temperature to dehydrate. Worms were allowed to

266   settle by gravity and remaining isopropanol was removed. 1 ml of 60% ORO was added and the

267   worms were incubated overnight with gentle rocking. After the overnight incubation, ORO was

268   removed and worms were resuspended in 100 μl of 1x PBST. 1 μl 1x DAPI was then added to

269   the 20 μl PBST, as an internal control for permeabilization, and flicked to mix. Worms were then

270   mounted on slides on an agarose pad and visualized with Zeiss LSM 700 confocal microscope

271   using either a Plan-Apochromat 10x/0.45 or Plan-Apochromat 20x/0.8 objective lens, as

272   described below.

273

274   **Carminic acid staining**

275   Carmine dyes such as carminic acid are used to complex with glycogen and short glucose

276   polymers to produce a fluorescent signal responsive to the levels of glucose polymer. Thus, it is

277   a useful tool to analyze glycogen storage (Hanover *et al.* 2005). Carminic acid was introduced to

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278 worms through feeding as previously described (Hanover *et al*. 2005). Briefly, OP50 was

279 inoculated into LB broth with 1 mg/ml carminic acid and grown overnight at room temperature

280 in a light protected vial. OP50 with carminic acid was then used to seed standard NGM plates.

281 Animals were plated on carminic acid containing plates following initial mid-L4 harvest

282 (controls) or post-ARD at day 5, day 10, and day 30 and were visualized with Zeiss LSM 700

283 confocal microscope using a Plan-Apochromat 20x/0.8 objective lens, as detailed below.

284

285 **Microscopy & Image Analysis**

286   Microscope images were acquired on a Zeiss LSM 700 confocal microscope using either

287 Plan-Apochromat 10x/0.45 or a Plan-Apochromat 20x/0.8 objective lens. In all cases:

288 photomultiplier tube (PMT) offset was adjusted so that zero photons corresponded to a pixel

289 value of zero; and PMT gain was adjusted to fully utilize the available 8-bit dynamic range

290 while avoiding saturating the images. The same settings were used across all images being

291 compared.

292   When acquiring the (non-fluorescence) ORO images, sequential scans were performed

293 with the 488nm, 555nm, and 639nm lasers while using the transmitted light detector to produce

294 an RGB image like what one would acquire using a color camera under widefield illumination

295 from white light. Differential Interference Contrast (DIC) optics were also in place while

296 imaging to provide contrast sufficient to distinguish sample anatomy at the same time as

297 recording ORO. Either the 10x/0.45 or the 20x/.8 objective lens was used for ORO imaging—

298 there was no difference in quantitation because the numerical aperture of the condenser was

299 limited to <0.45, and kept constant across experiments.

14

300   When acquiring fluorescence images of carminic acid, a 555nm laser was used for

301   excitation. The emission was passed through a laser blocking filter and a wide open pinhole to

302   produce a non-confocal image. The emission was further filtered through a SP640 (640 nm short

303   pass) emission filter, to produce a widefield fluorescence image appropriate for quantitating total

304   signal from the sample volume. Simultaneously with the fluorescence channel, a DIC image was

305   also acquired. Only images acquired with the 20x/0.8 lens were used for quantitation.

306   For quantitative image analysis, Fiji software (Schindelin *et al*. 2012) was used,

307   employing in-house macros customized to streamline the processing for the user. For both ORO

308   and carminic acid, regions of interest (ROIs) were manually traced very roughly around the

309   edges of each worm, further refined by automatic segmentation, then the average intensity per

310   pixel was calculated. This mean pixel value was divided by the diameter of the worm, measured

311   manually by the user, to provide a relative value for quantitating mean carminic acid

312   fluorescence per unit volume. For the ORO (RGB) images, a more in-depth analysis was

313   performed, converting the intensities to relative absorbance (pseudo-fluorescence) at each pixel

314   location, then combining with automated physical measurements of the worm from the ROI, to

315   calculate an average ORO density per unit volume. Quantitative comparisons could then be

316   made (see **Supplementary Methods**). For presentation, all images had their intensities re-scaled

317   to maximize the dynamic range and visual appeal of the images. Images of ORO and

318   fluorescence that were being compared were rescaled by the same amount in those channels.

319

320   **RESULTS**

321   **Diverse nutrient sensors are required for ARD entry**

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322   To date, only the *nhr-49(nr2041)* mutant allele of NHR-49, a protein involved in fatty

323   acid metabolism, has been characterized as having a significant role in ARD entry (Angelo and

324   Van Gilst 2009). We therefore sought to identify additional regulators of ARD dynamics,

325   focusing on the three distinct phases of ARD; initiation (number of animals entering ARD vs

326   bagging or L4 fate), maintenance (germline shrinkage and embryo arrest), and recovery (post-

327   ARD lifespan, germline regrowth, and brood size) (Angelo and Van Gilst 2009) (**Figure 1A**).

328   Using a candidate gene approach, we focused on key nutrient-responsive pathways. The

329   pathways profiled included AMP/energy signaling (AAK-2/AMPK), insulin-like signaling

330   (AGE-1/PI(3)K, DAF-16/FoxO), TOR (RSKS-1/S6K), stress signaling (SKN-1/Nrf),

331   carbohydrate metabolism/hexosamine signaling (OGT-1/OGT and OGA-1/OGA), and sirtuins

332   (SIR-2.1/Sirt1). The alleles analyzed were chosen based on their viability under the experimental

333   conditions and that they have been previously characterized for their roles in longevity. Note that

334   the raw data for all experiments described herein is available in the **Supplementary Tables.**

335   ARD initiation is clearly discernible by 48 to 72 hours after mid-L4 worms are placed on

336   ARD plates and can be distinguished from the bagging or L4 fates by the morphology of the

337   worms, particularly the presence of one to two "frozen" embryos in adult worms (Angelo and

338   Van Gilst 2009) (**Supplemental Figure 1**). We found that *age-1(hx546), daf-16(mu86), rsks-*

339   *1(ok1255),* and *skn-1(zj15)* did not significantly alter ARD initiation, suggesting that insulin-like

340   signaling, the TOR pathway, and stress response are not major contributors to entry (**Figure 1B**).

341   In contrast, *aak-2(ok524), oga-1(ok1207), ogt-1(ok430), ogt-1(ok1474)* (including additional

342   alleles of *oga-1* and *ogt-1*, discussed below), *sir-2.1(ok434),* and our positive control *nhr-*

343   *49(nr2041)* all contribute to the initial sensing and response to starvation during ARD entry, as

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344   these mutants displayed a significant reduction in the ability of nematodes to successfully initiate

345   ARD (**Figure 1B, Supplemental Figure 2**).

346   Associated with ARD initiation, we noted significant differences between the mutant

347   strains in the distribution of animals adopting either the L4 or bagging fate. Both L4 and bagging

348   had a higher degree of variability than ARD entry, in general, suggesting that the ARD entry

349   may be more tightly regulated than the other two fates (**Supplemental Figure 2; Supplemental**

350   **Table 1**). Interestingly, some of the mutant alleles showing reduced ARD entry (*nhr-*

351   *49(nr2041), ogt-1(ok1474),* and *oga-1(ok1207)*) did so while producing sizable numbers of

352   animals adopting both the L4 fate and bagging. The other strong entry defective alleles, *aak-*

353   *2(ok524)* and *sir-2.1(ok434),* produced predominantly L4 arrested larvae with few bagging or in

354   ARD (**Supplemental Figure 2; Supplemental Table 1**), suggesting there may be alternative

355   mechanisms at play in the regulation of entry between mutants and, therefore, distinct pathways.

356   We were intrigued to find that *oga-1* and *ogt-1* mutants exhibited severe ARD entry

357   defects similar to *nhr-49(nr2041)* (**Figure 1B; Supplemental Figure 2; Supplemental Table**

358   **1**). We verified this novel finding by generating and analyzing targeted full gene deletions, *oga-*

359   *1(av82)* and *ogt-1(jah01)*. These new CRISPR/Cas9 alleles were confirmed to have similar

360   phenotypes as the previously characterized *ogt-1* and *oga-1* alleles, including lifespan and brood

361   size under normal laboratory conditions (**Figure 3A; Supplemental Table 2**). As with the other

362   two *ogt-1* alleles, we observed a highly significant ARD entry defect with *ogt-1(jah01)* (**Figure**

363   **1B**). The *oga-1* alleles tested all also showed significant reduction in ARD entry, but were more

364   variable and showed a milder phenotype than the *ogt-1* alleles (**Figure 1B, Supplemental**

365   **Figure 2**), suggesting the removal of O-GlcNAc does not have as strong of an influence as

366   addition of O-GlcNAc.

367

Genes encoding *O*-GlcNAc cycling enzymes work in concert with *NHR-49* to modulate

ARD initiation

370   Based on the similarity of phenotypes for the single mutants of *nhr-49, oga-1,* and *ogt-1,*

371   as well as their known overlapping roles in metabolism, we next chose to explore the genetic

372   interaction between these mutants by analyzing the ARD initiation phenotypes in double

373   mutants. Our epistasis analysis suggested that these three genes act in the same pathway. Based

374   on action of the gene products, we place *ogt-1* upstream of both *nhr-49* and *oga-1,* and *oga-1*

375   acting upstream of *nhr-49*. Double mutants with an *ogt-1* allele and either an *nhr-49* or *oga-1*

376   allele had entry defects roughly equivelant to the *ogt-1* allele on its own. Compared to the strong

377   defects in entry observed for *nhr-49* and *ogt-1* mutants, *oga-1* mutants had a more intermediate

378   entry defect. Analysis of double mutants showed *oga-1* alleles behaved differently when with

379   *nhr-49*, as *nhr-49(nr2041); oga-1(av82)* had a severe entry defect like *nhr-49*, while *nhr-*

380   *49(nr2041); oga-1(ok1207)* had a more modest phenotype similar to other *oga-1* alleles (**Figure**

381   **1B**). Taken together, our data suggests that *nhr-49, oga-1,* and *ogt-1* function in the same

382   pathway to regulate ARD entry.

383

Distinct nutrient sensors are required for ARD recovery

385   Successful maintenance of ARD can be determined by examining germline shrinkage and

386   embryo preservation that occurs after entry into ARD. Characteristics of the recovery stage of

387   ARD is marked by the dynamic germline regrowth, production of progeny (brood size), and

388   post-ARD lifespan (Angelo and Van Gilst 2009). Thus, to further define which nutrient sensors

18

389   regulate each of these stages of ARD, we next looked at the dynamics of the germline, brood

390   size, and lifespan.

391   Despite significant changes in ARD entry, we observed that all mutant strains exhibited

392   robust germline shrinkage once ARD was established, analogous to wild-type worms and the

393   frozen embryos were retained throughout the starvation period (**Figure 2, left panel**). Further,

394   post-ARD, where worms were re-fed for two days following 30 days of starvation, a robust

395   regrowth of the germline was observed in all strains (**Figure 2, right panel**). Thus, in this set of

396   nutrient sensors, dynamic germline shrinkage and regrowth is a general feature of ARD once it is

397   established, as is the maintenance of the "frozen" embryos. Notably, only the apoptosis factor

398   CED-3 has been found to disrupt maintenance phenotypes to date (Angelo and Van Gilst 2009),

399   suggesting that the maintenance of ARD may be independent of nutrient sensing and supporting

400   the hypothesis that the distinct phases of ARD may be genetically separable (Angelo and Van

401   Gilst 2009; see also **Discussion**).

402   As noted previously, post-ARD animals retain their ability to reproduce, albeit to a

403   significantly diminished degree. However, this was found to be largely dependent on the amount

404   of sperm retained in the hermaphrodites, with selfing producing significantly less offspring than

405   mating post-ARD (Angelo and Van Gilst 2009). In our analysis of brood size following ARD,

406   we found that there was a high level of variability in the number of progeny, even between

407   replicates of the same strains within the same experiment (**Supplemental Table 2**). Surprisingly,

408   reduced brood size did not correlate with reduced sperm counts (**Supplemental Figure 3**) nor

409   with the germline dynamics we observed (**Figure 2**). This suggests that the relationship between

410   ARD recovery as measured by brood size is complex and may not correlate to sensing and

411   responding to nutrient changes but may involve other processes (see **Discussion**). We therefore

412   chose to focus on post-ARD lifespan as a measure of ARD recovery.

413   Post-ARD lifespan is a means to quantify the survivability of ARD, or the ability to

414   recover and thrive following re-feeding after time spent in ARD (Angelo and Van Gilst 2009).

415   Thus, we analyzed the lifespan of worms recovered after 30 days of ARD using standard lifespan

416   assays. We found that *aak-2(ok524), daf-16(mu86), rsks-1(ok1255),* and, to a lesser extent, *skn-*

417   *1(zj15)* mutants had significantly reduced post-ARD lifespans (**Figure 3A**). Based on the

418   phenotypes of the different mutants during the various stages of ARD, we suggest that recovery

419   may largely be regulated by the TOR pathway, as opposed to insulin-like signaling or stress

420   response (see **Discussion**).

421   Overall, we found that whereas *nhr-49, oga-1*, *ogt-1,* and *sir-2.1* play a role in ARD

422   initiation, *daf-16, rsks-1,* and *skn-1* function primarily in recovery from ARD, with *aak-2* playing

423   a role in the regulation of both states. Conversely, none of the alleles that we explored

424   significantly impacted maintenance of the ARD (summarized in **Figure 3B**).

425

426   **Mitochondrial β-oxidation but not fatty acid desaturation is required for ARD entry**

427   Due to the strong phenotypes we observed for ARD entry, the novel role for O-GlcNAc

428   signaling, and the similarity in phenotypes for *nhr-49* and *ogt-1* mutants, we next explored this

429   entry pathway in more detail. We chose to focus of *ogt-1* and not *oga-1* for the rest of the

430   experiments due to the variability of *oga-1* alleles and the weaker entry phenotype.

431   NHR-49 transcriptionally regulates both fatty acid desaturation, via FAT-7, and

432   mitochondrial β-oxidation, via ACS-2 (Van Gilst *et al.* 2005a) (**Figure 4A**). To determine which

433   of these NHR-49-dependent pathways is relevant for ARD entry we analyzed *acs-2(ok2457)*

20

434   (mitochondrial beta-oxidation) and *fat-7(wa36)* (fatty acid desaturation) mutants for ARD

435   phenotypes. Whereas the *acs-2* mutant had a significant defect in ARD entry, mutant *fat-7* entry

436   was comparable to wild-type. Further, the double mutant *acs-2(ok2457);ogt-1(ok1474)* was most

437   similar to the *ogt-1(ok1474)* single mutant phenotype for percentage of each fate adopted

438   (**Figure 4B; Supplemental Figure 2**). Thus, our findings demonstrate that *acs-2* plays a role in

439   the initiation of ARD entry downstream of *ogt-1*. As we had observed with the *nhr-49, oga-1,*

440   and *ogt-1* single mutants, we did not observe a significant difference in lifespan of the *fat-7* or

441   *acs-2* mutants post-ARD, suggesting that *acs-2* function in ARD entry is also separable from

442   recovery (**Figure 4C**).

443

444   **Neutral lipid changes associated with ARD entry**

445   Changes in lipid metabolism play a prominent role in multiple pro-longevity pathways

446   (Schroeder and Brunet 2015). NHR-49 and the O-GlcNAc cycling enzymes have well-

447   established roles in the regulation of fatty acid metabolism (Van Gilst *et al.* 2005a; Van Gilst *et*

448   *al.* 2005b; Bond and Hanover 2014); however, lipid storage in ARD has not yet been directly

449   analyzed.

450   To determine if the genetic interaction we observed between *nhr-49* and *ogt-1* during

451   ARD initiation was relevant to lipid metabolism, we used the lysochrome dye Oil Red O (ORO),

452   which is widely used to stain triglycerides (TAGs) (O'Rourke *et al.* 2009). We examined the

453   various strains for ORO staining in control (fed conditions) and after 30 days in ARD (prolonged

454   exposure). Fed *ogt-1(ok1474)* worms showed significantly lower ORO staining compared to N2s

455   (**Figure 5A,** quantified **Figure 5B**), though the distribution of signal was not noticeable different.

456   ORO staining is present throughout the body of worms in fed conditions, but is greatly

457   diminished and becomes largely restricted to arrested oocytes after 30 days in ARD (**Figure 5A,**

458   quantified **Figure 5B**). This restriction of ORO staining to the frozen embryos was significantly

459   stronger in the single mutant worms *nhr-49(nr2041)* and *ogt-1(ok1474)* worms, which also

460   showed the lowest amounts of ORO staining overall (**Figure 5A**). Between the fed and ARD

461   conditions, *ogt-1(ok1474)* animals did not show a significant decrease in quantified ORO

462   staining, likely due to the already low lipid stores in this strain. Additionally, the quantification

463   was normalized by volume, which may underestimate the effect of ARD on total triglycerides

464   per animal, as worms in ARD are significantly smaller than fed worms.

465   Notably, we observed the strong depletion of lipid stores as early as 24 hours of

466   starvation (**Supplemental Figure 4A**), suggesting it is an early response. As a control, we also

467   monitored cuticle penetrance of a small molecule dye using DAPI which revealed comparable

468   penetrance between strains (**Supplemental Figure 4B**). We further observed that in other strains

469   analyzed, those without major effects on ARD entry (*age-1(hx546)*, *fat-7(wa36)*, and *skn-*

470   *1(zj15)*) showed higher levels of ORO staining throughout the animal in ARD than wild-type

471   worms in ARD (**Supplemental Figure 5, Figure 5**). Strains with significant ARD entry defects

472   (*acs-2(ok2457), sir-2.1(ok424),* and *oga-1(tm3642)*) showed reduced ORO staining that is more

473   highly localized to the arrected embryo while in ARD (**Supplemental Figure 5**). This

474   correlation suggests lipid stores play an important role in the initiation of this form of diapause.

475   Based on the interplay between carbohydrate and fatty acid metabolism, as well as the

476   role of O-GlcNAc in carbohydrate metabolism, we looked next at glycogen storage using

477   carminic acid staining. Under control husbandry conditions, our analysis suggested a change in

478   the pattern of glycogen storage in *nhr-49(nr2041)* and *ogt-1(ok1474)* worms, though

479   quantification of the fluorescent signal showed no significant difference between strains

480   (**Supplemental Figure 6**). After 30 days of ARD, these stains all showed higher carminic acid

481   signal normalized by volume. This may be patially attributable to the reduction in size

482   associated with ARD, and suggests glycogen stores are relatively unaffected or increased by

483   ARD. Interestingly, *nhr-49(nr2041)* worms in ARD showed dramaticly higher carminic acid

484   staining (a 3-fold increase compared to N2) than did non-ARD worms, to an extent we did not

485   observe with the other strains assessed (**Supplemental Figure 6**). With our findings of lipid

486   reduction in ARD, this suggests glycogen stores may be maintained or increased at the expense

487   of lipid stores while in ARD. These results suggest ARD has interesting effects on glycogen

488   storage, but do not correlate with the entry defects we see in *nhr-49* and *ogt-1* mutant strains.

489

490   **DISCUSSION**

491   **Diverse roles for nutrient sensors at different phases of ARD**

492   Building on the previously discovered role of *nhr-49* in ARD entry (Angelo and Van

493   Gilst 2009), we demonstrate that *aak-2, oga-1, ogt-1,* and *sir-2.1* also regulate ARD entry, thus

494   implicating fat metabolism, the hexosamine pathway, and energy status in the initial sensing and

495   dissemination of the ARD entry signal. Further, based on our genetic epistasis analysis and

496   phenotypic similarities, we suggest that *nhr-49, oga-1,* and *ogt-1* act in the same pathway to

497   regulate entry, with *ogt-1* acting upstream of the other genes (**Figure 1B; Figure 6**). On the other

498   hand, while *sir-2.1* and *aak-2* also had defective entry, their phenotypes were distinct from the

499   *ogt-1* pathway, particularly in relation to percentage of each fate adopted (**Supplemental Figure**

500   **2**) and fat storage (**Supplemental Figure 5**). Based on these distinct phenotypes, we postulate

501   that *sir-2.1* and *aak-2* function in a distinct pathway to modulate ARD entry (**Figure 6**), and

502   suggest that alternative metabolic mechanisms can act to regulate ARD entry.

23

503   ARD maintenance, which was characterized by the shrinkage of the germline and

504   retention of embryo(s) during ARD, was not significantly altered in any of the strains analyzed

505   (**Figure 2**). Previous research found that apoptosis (CED-3) plays a role in maintenance (Angelo

506   and Van Gilst 2009). Research with CED-3 and our results herein suggest that the maintenance

507   of ARD may be independent of nutrient sensing but, rather, may rely more homeostatic

508   processes coming into play during this extended period.

509   ARD recovery, as assessed by post-ARD lifespan, was disrupted in *aak-2, daf-16, rsks-1,*

510   and, to a minor extent, *skn-1* mutants (**Figure 3**). While *daf-16* is most often associated with

511   insulin-like signaling, it is also known to regulate and be regulated by the AMPK and TOR

512   pathways (Antikainen *et al.* 2017). Based on the phenotypic similarity between *rsks-1* (TOR) and

513   *daf-16,* we speculate that the TOR pathway is primarily responsible for recovery from ARD,

514   particularly as loss of *age-1*, which acts upstream of *daf-16* in insulin-like signaling, did not have

515   a significant impact on any of the ARD phases (**Figure 1B; Figure 2; Figure 3**). Further, while

516   *aak-2* (AMPK) can also influence *daf-16* we find that *aak-2* had significant impact on both entry

517   and exit, a distinct phenotype from *daf-16* (**Figure 1B; Figure 3**).

518   Overall, our findings support the previous assertion that ARD phases may be largely

519   genetically separable (Angelo and Van Gilst 2009), with a general role for sensing changes in

520   energy status both to enter and exit ARD. This complex constellation of regulators and distinct

521   stages of ARD offers a promising model for future exploration of the contribution of these

522   pathways in the regulation of longevity and may shed light on some of their dynamic roles under

523   various pro-longevity regimens.

524

525   **O-GlcNAc cycling enzymes are required for initiation of ARD in concert with NHR-49**

24

526   O-GlcNAc cycling is a highly dynamic nutrient-sensing post-translational modification (PTM)

527   that allows for a rapid and reversible response to metabolic fluctuations. This PTM is uniquely

528   situated at the node of multiple pro-longevity pathways and, thus, represents a promising target

529   for therapeutic intervention (Love *et al.* 2010; Bond and Hanover 2014) . However, the role of

530   O-GlcNAc in longevity has yet to be fully explored, as it is essential in most organisms. Our

531   results demonstrate that *ogt-1* and *oga-1* function in the sensing and dissemination of specific

532   signals to enable ARD entry. Further, *ogt-1* and *oga-1* act in concert with the gene encoding the

533   transcription factor NHR-49 to modulate ARD initiation (**Figure 1B**). Analysis of multiple

534   alleles suggest that both *ogt-1* and *oga-1* are required for ARD entry but *oga-1* mutants are more

535   variable in their effect on ARD entry. Biochemically, OGA-1 is known to act downstream of

536   OGT-1 by dynamically removing the O-GlcNAc modification from proteins (Vocadlo 2012).

537   Depending upon nutrient status, O-GlcNAc levels can vary widely with variable levels

538   accumulating on proteins upon blocking O-GlcNAc cycling. It is also possible that OGA-1,

539   while still functioning in the same pathway as OGT-1, may also have additional functions

540   independent of O-GlcNAc removal. For example, it has been suggested that the *lin-4* microRNA

541   controls the levels of both *sbp-1* and *oga-1* to regulate fat metabolism, longevity, ROS

542   production and locomotion (Zhu *et al.* 2010).

543   OGA-1 has an evolutionarily conserved acetyltransferase-like domain whose precise

544   function is not yet known (Toleman *et al.* 2004; Alonso *et al.* 2014). Both OGT-1 and OGA-1

545   are known to physically interact with several proteins and can form complexes that alter the

546   localization and function of other proteins (Yang *et al.* 2002; Chen *et al.* 2013; Resto *et al.* 2016;

547   Zhang *et al.* 2016; Eustice *et al.* 2017). While most of this information is derived from studies

548   on mammalian O-GlcNAcase, the protein is highly conserved. Among the proteins that O-

25

549   GlcNAcase binds are the fatty acid synthetases under conditions of stress (Groves *et al.* 2017)

550   and OGA accumulates on lipid droplets in a manner suggesting a role in their assembly and

551   mobilization (Keembiyehetty *et al.* 2011). Thus, OGA-1 may have other non-O-GlcNAc cycling

552   functions, such as physically interacting with proteins in a way that suppresses, or otherwise

553   interferes with, their activity. Indeed, our epistasis analysis of the role of *oga-1* in ARD entry

554   suggests that OGA-1 may still functions biochemically in concert with OGT-1 to regulate NHR-

555   49, but the regulation of fat metabolism may be more complex leading to a less penetrant defect

556   in ARD entry.

557   Our epistasis analysis using multiple alleles of *ogt-1* and *oga-1*, suggests that *nhr-49, ogt-*

558   *1,* and *oga-1* act in the same pathway (**Figure 1B**). Further, the ARD entry defects of *ogt-1* and

559   *nhr-49* strains correlates with changes in fat stores (**Figure 5A**) but not carbohydrate stores

560   (**Supplemental Figure 6**). Thus, our genetic and staining results suggest that modulation of fat

561   stores is a key factor in ARD entry dynamics in the *ogt-1*-mediated ARD entry pathway.

562   NHR-49 regulates fatty acid metabolism by triggering fatty acid β-oxidation and/or

563   desaturation (Van Gilst *et al.* 2005a). Several desaturases and unsaturated fatty acids are

564   essential under different pro-longevity regimens, with multiple lines of evidence supporting an

565   important role for lipid metabolism in pro-longevity pathways (Wang *et al.* 2008; Lapierre and

566   Hansen 2012; Bustos and Partridge 2017). NHR-49 has a broad role in longevity, such that

567   worms with impaired NHR-49 have decreased longevity under normal, fed conditions. This

568   reduced lifespan under standard conditions is due to decreased fatty acid desaturation (Van Gilst

569   *et al.* 2005a). On the other hand, both desaturation and β-oxidation of fatty acids mediated by

570   NHR-49 is important for the increased longevity related to loss of the germline (Ratnappan *et al.*

571   2014). Our results demonstrate that ARD entry requires the β-oxidation pathway (*acs-2*)

26

572   downstream of *nhr-49*. Overall, the regulation of fatty acid metabolism by NHR-49 has

573   important implications for longevity, the mechanism of which may be context-dependent and

574   dynamic.

575   Our analysis of the genes encoding the O-GlcNAc enzymes and *nhr-49* suggests a

576   complex dynamic is at play. One interpretation of the findings is that O-GlcNAc cycling may act

577   as a metabolic rheostat acting on NHR-49, thereby regulating the switch between fatty acid

578   oxidation and desaturation, depending on environmental conditions. Previous work (Van Gilst *et*

579   *al.* 2005a; Van Gilst *et al*. 2005b) has indicated that during starvation, mitochondrial β-oxidation

580   (*acs-2* and other genes) increases fat consumption at the expense of *fat-7*-dependent fatty acid

581   desaturation. We have also previously examined *fat-7* and *acs*-2 expression in the context of the

582   *ogt-1* and *oga-1* mutants and their role in innate immunity and starvation where deregulation of

583   both of these genes was observed. In this same paper, we also reported no change in the

584   transcription of *nhr-49* for *ogt-1* and *oga-1* in L4 worms (Bond *et al.* 2014). These gene

585   expression findings fit our genetic evidence as lipid stores are depleted in strains defective in

586   ARD entry and *acs-2* seems to be critical for this utilization of stored fats under these conditions,

587   whereas the *fat-7* mutants did not alter ARD initiation (**Figure 4B**).

588

589   **Potential Conservation of NHR-49/PPAR regulation of metabolism in mammalian torpor**

590   **and hibernation**

591   Recent findings suggest that the NHR-49 pathway in *C. elegans* shares many characteristics with

592   PPARα in the mouse (Atherton *et al.* 2008; Taubert *et al.* 2006; Brandstädt *et al.* 2013). In

593   mammals, PPARα is modified by O-GlcNAc which serves to modifiy its transcriptional activity

594   (Ji *et al.* 2012). Intriguingly, PPARα also plays a key role in the control of torpor and FGF-

27

595   21/NPY pathway in animals like hummingbirds, bats and hamsters (Ishida 2009). Torpor is a

596   state of decreased physiological activity in an animal, usually characterized by a reduced body

597   temperature and rate of metabolism. Torpor is often used to help these animals survive during

598   periods of winter season, and it considered as the state in between sleep and hibernation. While

599   there is a clear distinction between hibernation, torpor and diapause, the regulation of these

600   processes may share some genetic determinants. Thus, the genetic findings reported here may

601   potentially highlight a conserved role for O-GlcNAcylation in the NHR-49/PPAR pathway in

602   torpor and hibernation. Indeed, recent findings in ground squirrels suggest that hibernation is

603   associated with elevated O-GlcNAcylation of liver proteins (Jariwala *et al.* 2020).

604

605   **Summary and Conclusions**

606   In this study, we have placed O-GlcNAc cycling in the context of other well established pro-

607   longevity-associated nutrient sensors in modulating adult reproductive diapause. We have

608   identified several novel regulators of ARD initiation including *sir-2.1, aak-2, acs-2, ogt-1* and

609   *oga-1,* in addition to the previously described role for *nhr-49.* Further, we have shown that *skn-*

610   *1*, *rsks-1,* and *daf-16* are important for ARD recovery. Defining key metabolites that are altered

611   will provide additional insight into the central components regulated by the O-GlcNAc node and

612   how these are altered under specific environmental conditions. Along with our novel genetic

613   interactions with *nhr-49*, future exploration of the O-GlcNAc cycling enzymes interactions with

614   nuclear hormone receptors may provide new insight into the role of OGT and OGA as metabolic

615   rheostats. Beyond impaired ARD entry, we found that loss of the O-GlcNAc cycling enzymes

616   converges on the fatty acid metabolic pathways involving mitochondrial fatty acid β-oxidation.

617   The findings described herein indicate that ARD entry and recovery are largely genetically

28

618   separable and that O-GlcNAc cycling acts with other nutrient sensors in ARD initiation. These

619   results have important implications for understanding the dynamic activity of nutrients sensors

620   during DR and for defining the role of these sensors in regulation of mammalian lipid

621   mobilization and metabolism.

622

623   **ACKNOWLEDGMENTS**

630

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848

849   **FIGURE LEGENDS**

850

851   **Figure 1. ARD stages and entry defects in nutrient sensing mutants**

852   (A) ARD stages including initiation, remodeling/maintenance, and recovery. Initiation occurs

853   when worms are removed from a food source during mid-L4. With wild type worms,

854   approximately 1/3 enter ARD, 1/3 have hatched offspring inside (bagging), and 1/3 maintain an

855   L4 state during the first 72 hours of starvation. Once ARD is initiated, worms enter the

856   maintenance phase wherein the germline shrinks and the characteristic arrested embryo(s) are

857   observed. Recovery occurs after starved worms are re-fed. During this stage the germline

858   expands and a normal lifespan commences (adapted from Angelo and Van Gilst 2009).

859   (B) For single mutants ARD initiation was significantly defective in the following strains, as

860   compared to N2: *nhr-49(nr2041*)*, aak-2(ok524), oga-1(av82), oga-(ok1207), ogt-1(jah01), ogt-*

861   *1(ok430)*, *ogt-1(ok1474),* and *sir-2.1(ok424)*. For epistatic analysis, the double mutants *nhr-*

862   *49(nr2041);oga-1(av82)* and *nhr-49(nr2041);oga-1(ok1207)* had phenotypes most similar to

863   *nhr-49(nr2041).* The double mutants *nhr-49(nr2041);ogt-1(ok430)* and *nhr-49(2041);ogt-*

864   *1(ok1474)* were most similar to the single *ogt-1* mutants. The entry defects for double mutants of

865   *ogt-1;oga-1* alleles were similar to *ogt-1* single mutants. A minimum of 300 worms from 3

866   different experiments were counted and statistics were performed in Graph Pad Prism using an

867   Ordinary one-way ANOVA . Statistics are summarized in **Supplemental Table 1**. *P*-value

868   **** = <0.0001, ** = 0.001, * = 0.0265.

869

870   **Figure 2. Germline shrinkage during ARD maintenance and regrowth during recovery.**

871   Representative images (with magnified panels for detail) showing the germline of worms starved

872   (left panel) at day 30 of ARD, with arrested embryos noted with white arrows. The right panel

873   shows worms that have been re-feed for 48 hours after 30 days of ARD, with white dashed lines

874   to indicate the germline, where healthy embryos and/or oocytes can be seen. All strains showed

875   robust shrinkage of the germline during starvation and regrowth after feeding. The 50 μm scale

876   bar shown in the top row applies to all images in each column.

877

878   **Figure 3. Lifespan of mutant strains in recovery after 30 days of ARD and summary model.**

879   (A) Lifespan of wild-type and mutant strains in control husbandry conditions compared to those

880   recovered after 30 days of ARD. Lifespan after recovery from ARD was significantly defective

881   in *aak-2(ok524), daf-16(mu86),* and *rsks-1(ok1255).* The mutant *skn-1(zj15)* also had a

882   significantly reduced lifespan post-ARD but not as strongly as the others. A minimum of 8

883   worms from independent experiments were analyzed for lifespan and statistics were performed

884   in Graph Pad Prism using one-way ANOVA. *P*-value **** = <0.0001, * = 0.0448.

885   (B) Summary of mutants altering ARD stages. While none of the mutants analyzed herein

886   influenced maintenance to a notable degree, *nhr-49, ogt-1,* and *sir-2.1* all strongly influenced

887   ARD entry, with *oga-1* also playing a role but to a lesser degree than the other three. The

888   mutations in *daf-16* and *rsks-1* had a strong impact on recovery whereas *skn-1* had an influence

889   but to a lesser degree. The *aak-2* mutants were the only strain that influenced both ARD

890   initiation and maintanence.

891

892   **Figure 4. Fatty acid beta-oxidation, but not desaturation, plays a role in ARD entry.**

893   (A) NHR-49 regulates both fatty acid beta-oxidation (ACS-2) and desaturation (FAT-7) via

894   genetically separable pathways that are known to generate distinct phenotypic outputs.

895   (B) ARD entry was significantly impaired with *acs-2(ok2457)* but not with the *fat-7(wa86)*

896   mutant. The double mutant *acs-1(ok2457); ogt-1(ok1474)* was also significantly impaired for

897   ARD entry. *P*-value **** = <0.0001 in comparison with N2 as determined using one-way

898   ANOVA.

899   (C) Lifespan under standard conditions and with refeeding at day 30 of ARD was not

900   significantly altered in these mutants as determined by one-way ANOVA.

901

902   **Figure 5. Oil Red O staining reveals redistribution of lipid stores in ARD**.

903   (A) Representative images showing the distribution of Oil Red O in control (top) and starved

904   animals after day 30 of ARD (bottom) in the *ogt-1*-dependent pathway. Retention of TAGs was

905   observed to be strongest in the arrested embryos in all mutant strains (white arrows). Scale bar

906   indicates 50 μm and is consistent across each row.

907   (B) Fiji Image analysis-based quantification of ORO staining (see **Supplemental Methods**) of

908   wildtype and the mutant strains of the *ogt-1*-dependent pathway in normal husbandry (control)

909   and in day 30 of ARD. Because ORO quantification was normalized by volume and ARD worms

41

910   are significantly smaller than well-fed adults, the values shown likely underestimate reductions

911   in lipid stores. Using two-way ANOVA analysis, both variables (genotype and control or ARD

912   condition) were found to have significant effects, and these variables interact significantly. For

913   each strain, mean ORO density decreased after 30 days of ARD: most dramatically for *nhr-*

914   *49(nr2041)* and not significantly for *ogt-1(ok1474).* In normal husbandry conditions, *ogt-1(1474)*

915   showed significantly less ORO signal than did N2 worms. *P*-value **** = <0.0001, *** =

916   <0.001, ** = <0.01.

917

918   **Figure 6. Model of genetic modifiers of ARD phases.**

919   Specific nutrient-sensing pathways contribute to ARD entry and exit. The initial sensing and

920   dissemination of the signal to enter ARD (green pathways) involves genes encoding proteins that

921   regulate fatty acid metabolism (*ogt-1, oga-1, nhr-49*, *acs-2*) and sirtuins (*sir-2.1*). Energy

922   sensing, mediated by the product of *aak-2*, uniquely acts in both ARD initiation and recovery

923   (blue pathway). The two members of the insulin/IGF signaling pathway tested in this study (grey

924   pathways) showed differing results with *age-1* not affecting ARD, while the downstream *daf-16*

925   affected recovery. ARD recovery additionally relies on the TOR pathway (*rsks-1*), which may

926   also interact with *daf-16.* Stress signaling influences ARD recovery as well, with *skn-1* playing a

927   minor role, and another possible pathway interacting with *daf-16*.

928

929   **SUPPLEMENTAL FIGURE AND TABLE LEGENDS**

930

931   **Supplemental Figure 1. Summary of ARD protocol**

932   As described in Materials and Methods, worms were bleached to obtain synchronized L1 larvae.

933   The L1s were then plated at a density of 30,000 per plate, harvested at mid-L4 stage, and grown

934   on plates without food for 48-72 hours. At this stage worms were counted to determine the

935   percentage of animals in arrested L4, bagged adults, or ARD (arrows indicate arrested embryos)

936   as shown in the representative photos.

937

938   **Supplemental Figure 2. Fates adopted by each strain during initiation of ARD**.

939   The L4 and bagging fates tended to have a higher degree of variability than ARD entry, as

940   evidenced by a higher SEM. Select strains had a marked increase in the percentage of worms in

941   L4 including *aak-2(ok524), age-1(hx546), daf-16(mu86), sir-2.1(ok434),* and *skn-1(zj15).* Other

942   mutant strains, *nhr-49(nr2041), oga-1(ok1207),* and *ogt-1(jah01),* were more similar to wildtype

943   in terms of having a more even distribution between L4 and bagging even though this set was

944   defective for ARD entry. It is worth noting that *oga-1(ok1207)* and the full deletion allele *oga-*

945   *1(av82)* displayed a significant defect in ARD entry. However, another allele, *oga-1(tm3642)*

946   exhibited a significantly stronger defect in ARD entry. Considering the heavy mutational load

947   used to create high throuput alleles, the stronger phenotype of *oga-1(tm3642)* may be due to a

948   linked background mutation, as has been recently described for an allele from the same source

949   (Bauer *et al.* 2019). As such, we chose to exclude this allele from further genetic analysis.

950

951   **Supplementary Table 1**. **ARD fates.**

952   Table representation of Supplemental Figure 2. Average percentage of each strain adopting each

953   fate with SEM shown to illustrate variability.

954

Supplemental Table 2. Brood size variability.

The table shows the average brood size under normal husbandry (control) or re-fed animals following 5 or 10 days in ARD. A high degree of variability was observed between and within experimental replicates for the average brood size (SEM). These brood sizes did not correlate to fate adoption of each strain nor with influence on any of stages of ARD. Red indicates a decrease compared to wildtype whereas green indicates an increase vs wildtype.

Supplemental Figure 3. DAPI staining of spermatheca during ARD reveal changes in number of sperm nuclei during ARD.

(A) DAPI staining of worms after 15 days in ARD reveals fewer sperm in the spermatheca of *ogt-1(1474),* and *nhr-49(nr2041)* compared to wild-type N2 worms. In the *oga-1(ok1207)* worms, more sperm were observed. White arrows indicate examples of sperm nuclei. (B) Whereas most of the strains analyzed saw a decrease in the number of sperm nuclei present at day 30 of ARD, *oga-1(ok1207)* had an overall increase compared to wildtype. However, these dynamics did not correlate with changes in brood size among selfed individuals. Green arrows indicate increased brood numbers vs wildtype and red arrows indicate decreased brood vs wildtype.

Supplemental Figure 4. 24 hours of ARD results in noticeable decrease in ORO staining.

(A) Decrease in ORO signal were observable at 24 hr after worms were placed on ARD places, as shown with representative strains.

44

976   (B) Strains were stained with DAPI to demonstrate efficient small-molecule penetrance of

977   cuticles across strains, indicating that changes in ORO staining are not related to differences in

978   cuticle penetrance between strains.

979

980   **Supplemental Figure 5. Additional strains stained for ORO after 30 days of ARD.**

981   In addition to the *ogt-1*-dependent pathway (shown in figure 5) we also looked at strains with

982   diverse phenotypes to see if changes were unique to the *ogt-1* pathway. We observed that outside

983   of the *ogt-1* pathway, strains that did not influence either entry or exit (*age-1(hx546)*), strains

984   that also only influenced entry (*sir-2.1(ok424)*), and strains that influenced only recovery (*skn-*

985   *1(zj15)*) had a marked increase in ORO staining/TAG stores compared to both wildtype and the

986   *ogt-1* pathway. We also observed this same pattern with genes downstream of *nhr-49,* such that

987   *acs-2* (with a defective ARD entry) had reduced ORO staining but *fat-7* (no defect in ARD entry)

988   had a stronger ORO signal. Arrows indicate arrested embryos, which show high ORO staining.

989

990   **Supplemental Figure 6. Changes in glycogen and trehalose levels before and after ARD.**

991   (A) Carminic acid staining (indicative of glycogen and trehalose levels) varied greatly between

992   strains. As we have previously reported (Hanover *et al.* 2005), *ogt-1(1474)* showed slightly

993   higher levels of carminic acid staining than other strains in standard husbandry conditions,

994   though in this study this change did not reach significance. After 30 days of ARD (lower panels)

995   we noted that *nhr-49(nr2041)* had a dramatic increase in staining, while wild type and *ogt-*

996   *1(ok1474)* did not. These results did not correlate with the observed defect in ARD entry for

997   these strains.

45

998   (B) ImageJ based quantification of carminic acid fluorescence by pixel intensity. *P*-value **** =

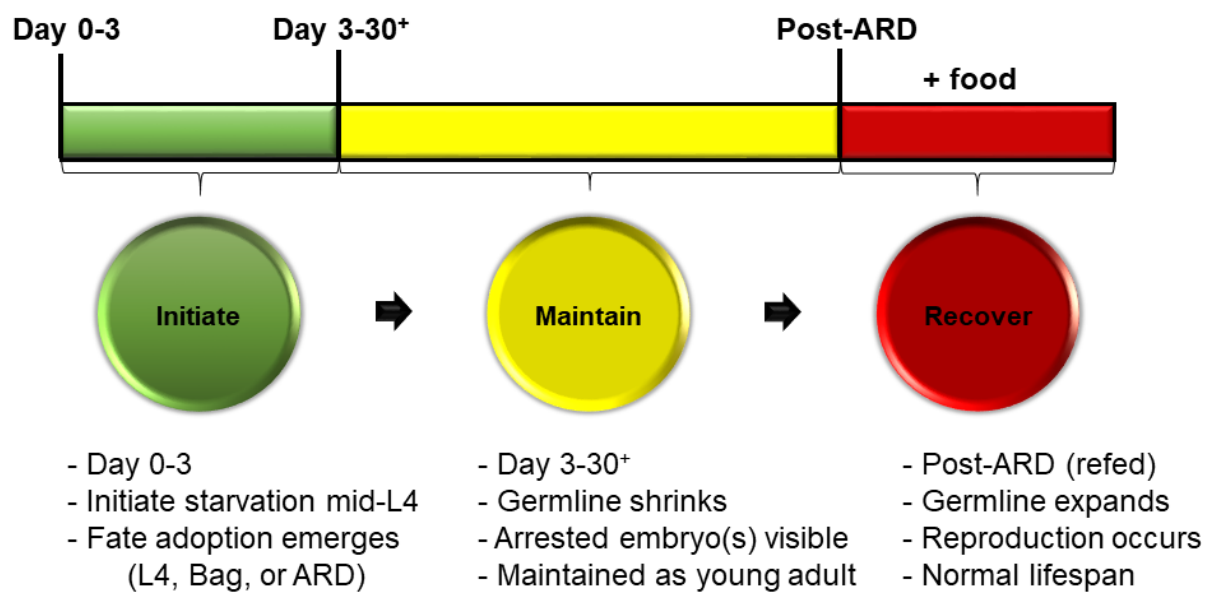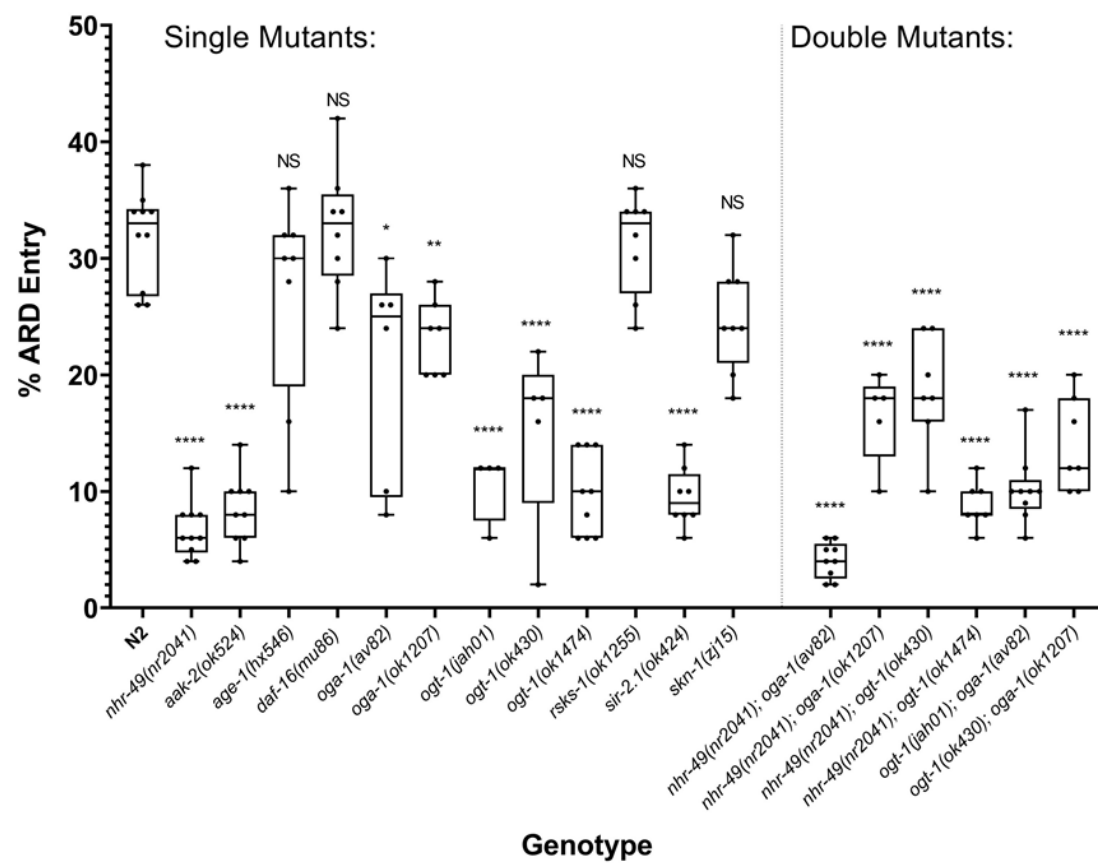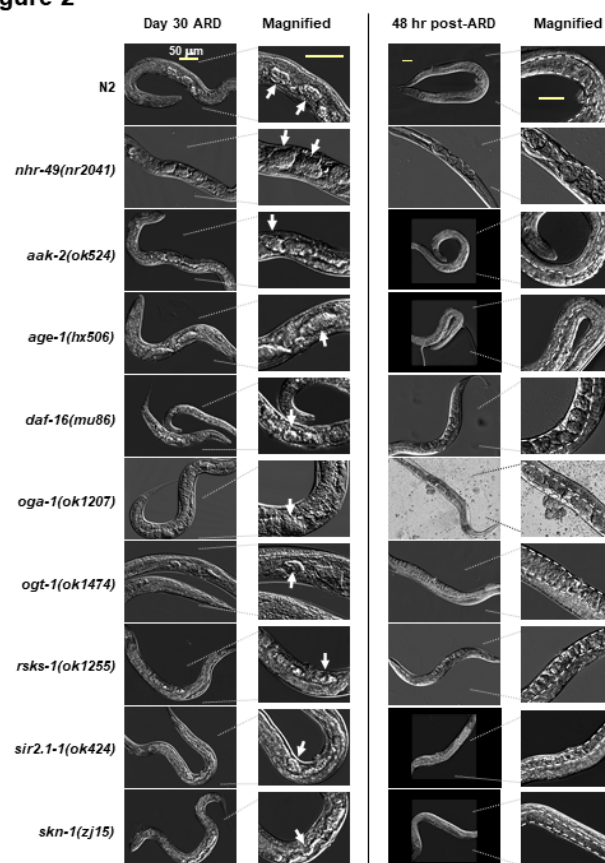999   <0.0001, as determined by two-way ANOVA.

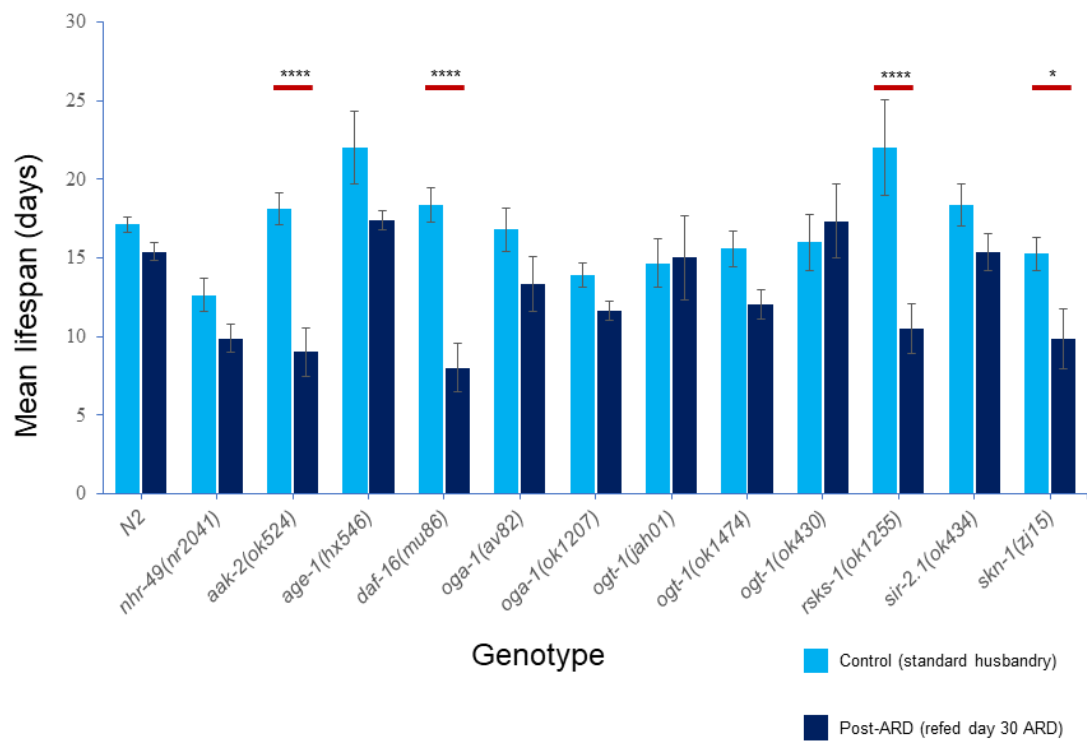**Figure 1**

A

Figure 1

B

Figure 2

Figure 3

A

Mean lifespan (days) vs *Genotype*

Legend:
- Control (standard husbandry)
- Post-ARD (refed day 30 ARD)

Figure 3

B

Figure 4

Figure 5

Figure 6

ARD ENTRY AND RECOVERY PATHWAYS