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**Chemical signaling regulates axon regeneration
via the GPCR–Gq α pathway in *Caenorhabditis
elegans***

Running Title: Chemical signaling in axon regeneration

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K.S., and Y.S. performed genetic and biological experiments. R.A.B. and A.R.D.
produced ascr#5. T.S. and Y.S. performed all of the other experiments. K.M. and
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48 **Abstract**

49

50 **Chemical communication controls a wide range of behaviors via**
51 **conserved signaling networks. Axon regeneration in response to injury is**
52 **determined by the interaction between the extracellular environment and**
53 **intrinsic growth potential. In this study, we investigated the role of**
54 **chemical signaling in axon regeneration in *Caenorhabditis elegans*. We**
55 **find that the enzymes involved in ascaroside pheromone biosynthesis,**
56 **ACOX-1.1, ACOX-1.2, and DAF-22, participate in axon regeneration by**
57 **producing a dauer-inducing ascaroside, ascr#5. We demonstrate that the**
58 **chemoreceptor genes, *srg-36* and *srg-37*, which encode G protein-coupled**
59 **receptors (GPCRs) for ascr#5, are required for adult-specific axon**
60 **regeneration. Furthermore, the activating mutation in *egl-30* encoding Gq α**
61 **suppresses axon regeneration defective phenotype in *acox-1.1* and *srg-36***
62 ***srg-37* mutants. Therefore, the ascaroside signaling system provides a**
63 **unique example of a signaling molecule that regulates the regenerative**
64 **pathway in the nervous system.**

65 **Significance Statement**

66

67 **In *C. elegans*, axon regeneration is positively regulated by the EGL-30**
68 **Gq α –JNK MAP kinase cascade. However, it remains unclear what signals**
69 **activate the EGL-30 pathway in axon regeneration. Here, we show that**
70 **SRG-36 and SRG-37 act as upstream GPCRs that activate EGL-30. *C.***
71 ***elegans* secretes a family of small-molecule pheromones called**
72 **ascarosides, which serve various functions in chemical signaling. SRG-36**
73 **and SRG-37 are GPCRs for the dauer-inducing ascaroside, ascr#5.**
74 **Consistent with this, we found that ascr#5 activates the axon regeneration**
75 **pathway via SRG-36/SRG-37 and EGL-30. Thus, ascaroside signaling**
76 **promotes axon regeneration by activating the GPCR–Gq α pathway.**

77 Introduction

78

79 The ability of axons to regenerate after an injury is a fundamental and conserved
80 property of neurons, which is influenced by the balance between extrinsic factors
81 that inhibit or promote axon outgrowth and intrinsic growth capacity of neurons
82 (Kaplan et al., 2015). In adult mammals, regeneration following nerve injury
83 occurs efficiently in the peripheral nervous system, whereas the central nervous
84 system does not successfully regenerate after injury (Mahar and Cavalli, 2018).
85 This difference in regeneration potential has been attributed to the combined
86 effects of extrinsic signals and intrinsic growth capacity (Tedeschi and Bradke,
87 2017). However, the mechanisms underlying the regulation of regeneration by
88 the extracellular environment in the adult nervous system remain unclear.

89 Recent studies on axon regeneration in the genetic model animal,
90 *Caenorhabditis elegans*, have revealed that the JNK MAP kinase (MAPK)
91 cascade is a key intrinsic regulator of axon regeneration and may act to sense
92 axonal damage (Fig. 1A; Hisamoto and Matsumoto, 2017). The JNK pathway
93 consists of MLK-1 MAPKKK, MEK-1 MAPKK, and KGB-1 JNK; it is inactivated at
94 the KGB-1 activation step by VHP-1, a member of the MAPK phosphatase family
95 (Mizuno et al., 2004). The *vhp-1* loss-of-function mutation leads to
96 hyperactivation of the JNK pathway, resulting in developmental arrest at an early
97 larval stage. We had previously conducted a genome-wide RNAi screening for
98 suppressors of *vhp-1* lethality and isolated 92 *svh* genes (Li et al., 2012; Shimizu
99 et al., 2021). Analysis of these *svh* genes shed new light on the regulation of
100 axon regeneration. Moreover, two distinct protein kinases act as MAP4Ks for
101 MLK-1 MAPKKK in a life stage-dependent manner (Pastuhov et al., 2012).
102 Ste20-related kinase, MAX-2, phosphorylates and activates MLK-1 primarily at
103 the L4 stage to promote axon regeneration. In contrast, the protein kinase C

(PKC) homolog, TPA-1, can activate MLK-1 at the young adult stage but not at the L4 stage. The Gq α protein, EGL-30, acts as an upstream component of TPA-1. EGL-30 activates a phospholipase C β (PLC β), EGL-8, which in turn generates diacyl-glycerol (DAG), an activator of TPA-1 (Fig. 1A). Endocannabinoid anandamide (AEA) inhibits axon regeneration via the Go α protein, GOA-1, which antagonizes EGL-30. NPR-19 and NPR-32 function as G protein-coupled receptors (GPCRs) for AEA (Pastuhov et al., 2012). Therefore, increased signaling from the AEA pathway suppresses the EGL-30–EGL-8–TPA-1 signaling cascade, which inhibits the activation of the JNK pathway, thereby reducing axon regeneration. However, it remains unclear what signals activate the EGL-30 pathway in the regulation of axon regeneration.

In many organisms, the extracellular environment is interpreted through chemical signaling systems mediated by small molecules (Peso et al., 2015). *C. elegans* secretes a family of small-molecule pheromones called ascarosides, which participate in diverse chemical signaling functions (Park et al., 2019). In particular, ascarosides facilitate the transition to the non-feeding, long-lived, and highly stress-tolerant dauer stage (Butcher et al., 2007). Ascarosides also have a number of other important effects on behaviors, such as aggregation, avoidance, and mating attraction. Therefore, the animals optimize the extent of these developmental or behavioral decisions by assessing the environmental conditions that affect their survival strategies (Butcher, 2017). Ascarosides are initially synthesized as glycolipids with extremely long side chains that are subsequently shortened by the peroxisomal fatty acid (FA) β -oxidation pathway (Butcher, 2017). This pathway is composed of four enzymes: acyl-CoA oxidases (ACOXs), enoyl-CoA hydratase (MAOC-1), hydroxyacyl-CoA dehydrogenase (DHS-28), and β -ketoacyl-CoA thiolase (DAF-22). More than 200 ascaroside-like

130 compounds have been identified by metabolomics and they are divided into two
131 main classes: ω -ascarosides and (ω -1)-ascarosides (von Reuss et al., 2017).

132 In this study, we found that one dauer-inducing ω -ascaroside, *ascr#5*
133 (*asc- ω C3*; C3), acts on the EGL-30 Gq signaling pathway to promote axon
134 regeneration. Furthermore, we revealed that the *srg-36* and *srg-37* genes, which
135 encode GPCRs for *ascr#5*, are required for axon regeneration by functioning
136 upstream of EGL-30. These findings strengthen the link between chemical
137 inputs and a conserved regulatory mechanism for axon regeneration.

138

139 **Materials and Methods**

140

141 ***C. elegans* strains**

142 The *C. elegans* strains used in this study are listed in Table 1. The strains,
 143 KU501, KU456, and KU457 have been previously reported (Pastuhov et al.,
 144 2012). All strains were maintained on nematode growth medium plates and fed
 145 with bacteria of the OP50 strain of *Escherichia coli* using the standard method
 146 (Brenner, 1974).

147

148 **Plasmids**

149 *Pacox-1.1::acox-1.1* was constructed by inserting a genomic DNA, which
 150 includes a 1.3-kb region of the *acox-1.1* promoter, the *acox-1.1* coding region,
 151 and a 187 bp of the 3' untranslated region (3'UTR), into pCR2.1.
 152 *Pacox-1.1::nls::gfp::3'UTR (acox-1.1)* was constructed by replacing the
 153 *acox-1.1* coding region of the *Pacox-1.1::acox-1.1* plasmid with NLS-GFP region
 154 in pPD95.67. The cDNAs used in this study were isolated from the pACT cDNA
 155 library (Sakamoto et al., 2005). *Punc-25::acox-1.1* (cDNA), *Punc-25::acox-1.2*
 156 (cDNA), and *Punc-25::daf-22* (cDNA) plasmids were constructed by inserting
 157 each cDNA into the pSC325 vector. pPD95.75-*Pges-1* (Inoue et al., 2005),
 158 pPD52.102, and pPD95.75 vectors were used to construct *Pges-1::acox-1.1*,
 159 *Pmec-7::acox-1.1*, *Pmec-7::acox-1.2*, and *Pmec-7::gfp*, respectively.
 160 *Psrg-36::srg-36::sl2::gfp* and *Psrg-37::srg-37::sl2::gfp* plasmids (McGrath et al.,
 161 2011) are a generous gift from Dr. Cori Bargmann. *Punc-25::srg-36::sl2::gfp* and
 162 *Punc-25::srg-37::sl2::gfp* were constructed by replacing the *srg-36* promoter
 163 region of *Psrg-36::srg-36::sl2::gfp* and the *srg-37* promoter region of
 164 *Psrg-37::srg-37::sl2::gfp* with the *unc-25* promoter of pSC325.
 165 *Punc-25::nes::tdTomato* was constructed by replacing the CFP coding region of

166 *Punc-25::nes::cfp* (Hisamoto et al., 2018) with *tdTomato* cDNA (Clontech).
 167 *Pmyo-2::dsred-monomer* have been previously described (Li et al., 2012).
 168 Promoter regions for the analysis of gene expression patterns were determined
 169 by confirming their ability to rescue the phenotype of the corresponding mutant
 170 when combined with protein-coding sequences.

171

172 **Transgenic animals**

173 Transgenic animals were obtained using the standard *C. elegans* microinjection
 174 method (Table 1; Mello et al., 1991). *Pmyo-2::dsred-monomer*,
 175 *Pacox-1.1::acox-1.1*, *Pges-1::acox-1.1*, *Punc-25::acox-1.1*, *Pmec-7::acox-1.1*,
 176 *Punc-25::acox-1.2*, *Pmec-7::acox-1.2*, *Pmec-7::gfp*, *Pacox-1.1::nls::gfp*,
 177 *Punc-25::daf-22*, *Psrg-36::srg-36::sl2::gfp*, *Psrg-37::srg-37::sl2::gfp*,
 178 *Punc-25::srg-36::sl2::gfp*, *Punc-25::srg-37::sl2::gfp*, and *Punc-25::nes::tdTomato*
 179 plasmids were used in *kmEx1551* [*Pacox-1.1::acox-1.1* (25 ng/μl) +
 180 *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1552* [*Pges-1::acox-1.1* (25 ng/μl) +
 181 *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1553* [*Punc-25::acox-1.1* (25 ng/μl) +
 182 *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1554* [*Pmec-7::acox-1.1* (25 ng/μl) +
 183 *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1556* [*Punc-25::acox-1.2* (25 ng/μl) +
 184 *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1557* [*Pmec-7::acox-1.2* (25 ng/μl) +
 185 *Pmec-7::gfp* (25 ng/μl) + *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1560*
 186 [*Pacox-1.1::nls::gfp* (25 ng/μl) + *Punc-25::nes::tdTomato* (25 ng/μl) +
 187 *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1576* [*Pacox-1.1::nls::gfp* (25 ng/μl) +
 188 *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1562* [*Punc-25::daf-22* (25 ng/μl) +
 189 *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1564* [*Psrg-36::srg-36::sl2::gfp* (25
 190 ng/μl) + *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1565* [*Psrg-37::srg-37::sl2::gfp*
 191 (25 ng/μl) + *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1566*
 192 [*Psrg-36::srg-36::sl2::gfp* (25 ng/μl) + *Psrg-37::srg-37::sl2::gfp* (25 ng/μl) +

193 *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1569* [*Punc-25::srg-36::sl2::gfp* (25
 194 ng/μl) + *Psrg-37::srg-37::sl2::gfp* (25 ng/μl) + *Pmyo-2::dsred-monomer* (5 ng/μl)],
 195 *kmEx1570* [*Psrg-36::srg-36::sl2::gfp* (25 ng/μl) + *Punc-25::nes::tdTomato* (25
 196 ng/μl) + *Pmyo-2::dsred-monomer* (5 ng/μl)], *kmEx1573*
 197 [*Punc-25::srg-36::sl2::gfp* (25 ng/μl) + *Pmyo-2::dsred-monomer* (5 ng/μl)], and
 198 *kmEx1574* [*Punc-25::srg-36::sl2::gfp* (25 ng/μl) + *Punc-25::srg-37::sl2::gfp* (25
 199 ng/μl) + *Pmyo-2::dsred-monomer* (5 ng/μl)], respectively.

200

201 **Generation of the *acox-1.4(km92)* mutation using CRISPR–Cas9**

202 The *acox-1.4(km92)* mutation was generated using the previously described
 203 CRISPR–Cas9 system (Dokshin et al., 2018). The CRISPR guide RNA
 204 [5'-CCCGUCCUCGGUGAGAUCCGUUUUAGAGCUAUGCU-3'] was
 205 synthesized (Integrated DNA Technologies: IDT) and co-injected with the
 206 trans-activating CRISPR RNA (IDT), *Streptococcus pyogenes* Cas9 3NLS (IDT)
 207 protein, and the pRF4(*rol-6d*) plasmid into the KU501 strain. Subsequently, each
 208 F1 organism carrying the transgene was transferred onto a new dish and used
 209 for single-worm PCR, followed by DNA sequencing to detect the mutations. The
 210 *acox-1.4(km92)* mutation is a 5-bp deletion in exon 1 of the *acox-1.4* gene,
 211 causing a frameshift and premature stop codon in exon 1.

212

213 **Microscopy**

214 Standard fluorescent images of transgenic animals were observed under a 100x
 215 objective using a Nikon ECLIPSE E800 fluorescent microscope and
 216 photographed using a Zyla CCD camera. Confocal fluorescent images were
 217 taken using a Zeiss LSM-800 confocal laser scanning microscope. For analyzing
 218 the expression of *acox-1.1* or *srg-36* in GABAergic neurons, more than ten
 219 axons were analyzed and gene expression was examined every 30 min for 5 h

220 after injury.

221

222 **Axotomy**

223 Axotomy and microscopy were performed as previously described (Li et al.,
224 2012). Animals were subjected to axotomy at the L4 or young adult stage. The
225 imaged commissures that had growth cones or small branches present on the
226 proximal fragment were counted as “regenerated.” Proximal fragments that
227 showed no change after 24 h were counted as “no regeneration.” A minimum of
228 15 individuals with 1–3 axotomized commissures were observed for most
229 experiments. Most of the animals with the same genotype regenerated a similar
230 number of axons.

231

232 **Pheromone treatment**

233 *Ascr#5*, synthesized as described previously (Butcher et al., 2008), was
234 dissolved in DMSO and diluted in M9 media to a final concentration of 1 μ M.
235 After incubating young adult stage animals in this solution for 6 h, axons were
236 cut with a laser and incubated on NGM plates containing *ascr#5* (1 μ M) for 24 h
237 before microscopic observation. For the dauer assay, embryos were incubated
238 on NGM plates containing *ascr#5* (1 μ M) and the OP50 strain for 3 days at 25°C,
239 and then the numbers of dauer and non-dauer larvae were counted. The rate of
240 dauer formation was calculated by dividing the number of dauer larvae by the
241 total number of dauer and non-dauer larvae.

242

243 **Phylogenetic analysis**

244 Evolutionary relationships among candidate genes were determined by
245 constructing neighbor-joining phylogenetic trees using the MEGAX software
246 (Stecher et al., 2020). Evolutionary distances were calculated using the Poisson

247 correction method (Zuckerkindl and Pauling, 1965).

248

249 **Experimental design and statistical analyses**

250 None of the experiments were randomized, and researchers were not blinded to
251 the group assignments during the experiments and evaluation of results. The
252 sample size was determined based on previous studies that assayed axon
253 regeneration in the GABAergic neurons of *C. elegans* (Yanik et al., 2004;
254 Hammarlund et al, 2009). Approximately 50 axons per animal per group were
255 scored. With this sample size, a 30% difference in axon regeneration was
256 detected with an 80% probability of detection calculated by the Fisher's exact
257 test based on the sample size. However, due to issues (small body size, thin
258 axons, and weak GFP expression for unknown reasons) originating from specific
259 mutants, it was sometimes challenging to excise the same number of axons in
260 all groups. Statistical analysis was conducted as described in a previous study
261 (Pastuhov et al., 2012). Confidence intervals (95%) were calculated using the
262 modified Wald test, and two-tailed *P*-values were calculated using Fisher's exact
263 test on GraphPad QuickCalcs
264 (<https://www.graphpad.com/quickcalcs/contingency1/>). Values with $P < 0.05$
265 were considered statistically significant.

266 **Results**

267

268 **Identification of SVH-18/SRX-16**

269 In *C. elegans*, axon regeneration is regulated by the EGL-30 Gq α –JNK pathway
 270 (Fig. 1A; Sakai et al., 2021). However, the GPCRs that act upstream of EGL-30
 271 remain unknown. To identify additional components functioning in the JNK
 272 pathway, we previously conducted a genome-wide RNAi screening for
 273 suppressors of *vhp-1* lethality and isolated 92 *svh* genes (Li et al., 2012; Shimizu
 274 et al., 2021). Indeed, we isolated the *egl-30* gene as *svh-12* (Shimizu et al.,
 275 2021). To identify GPCRs involved in the EGL-30 signaling pathway, we
 276 examined whether *svh* genes encode GPCRs. We identified the *svh-18* gene,
 277 which encodes SRX-16, a predicted GPCR chemoreceptor in the *srx* gene
 278 family (Fig. 1B,C). To determine the effect of *srx-16* on axon regeneration, we
 279 assayed regrowth after laser axotomy in γ -aminobutyric acid (GABA)-releasing
 280 D-type motor neurons, which extend their axons from the ventral to the dorsal
 281 nerve cord (Yanik et al., 2004; Hammarlund et al., 2009). In young adult
 282 wild-type animals, about 62% of laser-severed axons could initiate regeneration
 283 within 24 h (Fig. 1D; Table 2). We found that the *srx-16(tm7585)* deletion
 284 mutation (Fig. 1B) did not affect axon regeneration (Fig. 1D; Table 2). Therefore,
 285 the function of SRX-16 in the JNK pathway is to regulate larval growth but not
 286 axon regeneration.

287

288 **Enzymes involved in ascaroside pheromone biosynthesis participate in** 289 **axon regeneration**

290 There are 96 members of the SRX family (Robertson and Thomas, 2006). Of
 291 which, only SRX-43 and SRX-44 act as GPCRs for the indolated ascaroside,
 292 icas#9 (IC-asc-C5; C5) (Greene et al., 2016a, 2016b), whereas the functions of

293 the other members are still unknown. The generated phylogenetic tree shows
 294 that SRX-16 is similar to SRX-43/SRX-44 (Fig. 1C), raising the possibility that
 295 the ascaroside pheromone may function as a signal for activating the EGL-30
 296 pathway, which promotes axon regeneration. To test this possibility, we
 297 examined whether mutants lacking the enzymes involved in ascaroside
 298 production would affect axon regeneration. Ascarosides in *C. elegans* are
 299 synthesized by the FA β -oxidation pathway consisting of ACOXs, MAOC-1,
 300 DHS-28, and DAF-22 (Fig. 2A). We investigated the effects of deletion mutations
 301 in *acox-1.1* and *daf-22* (Fig. 2B) on the regeneration of D-type motor neurons.
 302 The *acox-1.1* gene encodes one of the ACOX enzymes, and the
 303 *acox-1.1(ok2257)* deletion has been verified to abolish the ACOX-1.1 function
 304 (Fig. 2A,B; Joo et al., 2010; Zhang et al., 2016, 2018). The *daf-22* gene encodes
 305 an ortholog of the human sterol carrier protein, SCPx, and the *daf-22(ok693)*
 306 deletion results in the loss of its enzymatic function (Fig. 2A,B; Butcher et al.,
 307 2009; Joo et al., 2009). The *acox-1.1(ok2257)* deletion or the *daf-22(ok693)*
 308 deficiency causes the accumulation of large fat granules in the intestine,
 309 reduced growth rate, and decreased brood size (Joo et al., 2009, 2010). On
 310 comparison with wild-type animals, we found that the frequency of axon
 311 regeneration after laser axotomy was reduced in *acox-1.1(ok2257)* and
 312 *daf-22(ok693)* mutants (Fig. 2C; Table 2). To verify that the *acox-1.1* mutation
 313 causes this defect in axon regeneration, we generated the transgene
 314 *Pacox-1.1::acox-1.1*, which contains the entire genomic *acox-1.1* coding region,
 315 its promoter, and the 3'UTR. Introduction of *Pacox-1.1::acox-1.1* into
 316 *acox-1.1(ok2257)* mutants significantly rescued the regeneration defect (Fig. 2C;
 317 Table 2).
 318 To test whether ACOX-1.1 functions in the EGL-30 signaling pathway, we
 319 examined the genetic interactions between *acox-1.1* and *egl-30*. EGL-30 is the

320 *C. elegans* Gq α , and we employed two *egl-30* alleles, *egl-30(ad805)* and
 321 *egl-30(tg26)* (Fig. 2B). The *egl-30(ad805)* loss-of-function (lf) mutation is a
 322 mutation in the splice acceptor site that reduces the number of copies of
 323 full-length EGL-30 (Brundage et al., 1996). In contrast, the *egl-30(tg26)*
 324 gain-of-function (gf) mutation is a missense mutation that constitutively activates
 325 EGL-30 function by replacing the conserved Arg-243 with glutamine (Doi and
 326 Iwasaki, 2002). We found that the defect in axon regeneration caused by the
 327 *acox-1.1(ok2257)* mutation was not enhanced by introducing the *egl-30(lf)*
 328 mutation (Fig. 2C; Table 2). This result suggests that ACOX-1.1 and EGL-30 act
 329 in the same pathway. Moreover, the activating *egl-30(gf)* mutation could
 330 suppress the *acox-1.1* phenotype (Fig. 2C; Table 2), suggesting that EGL-30
 331 functions downstream of ACOX-1.1. These results support the possibility that
 332 ACOX-1.1 regulates axon regeneration through the EGL-30 pathway.

334 **Expression pattern of *acox-1.1***

335 To investigate the location of ACOX-1.1 when it regulates axon regeneration, we
 336 examined the expression pattern of *acox-1.1*. We constructed a transgene,
 337 *Pacox-1.1::nls::gfp::3'UTR (acox-1.1)*, which consists of the *acox-1.1* promoter,
 338 nuclear localization signal (NLS), fluorescent protein GFP, and *acox-1.1* 3'UTR.
 339 The *acox-1.1* gene functions in the intestine and hypodermis, where it
 340 contributes to the biosynthesis of ascaroside pheromones (Joo et al., 2010).
 341 Consistent with this, the *acox-1.1* gene is exclusively expressed in intestinal
 342 cells but not in D-type neurons. GFP expression was still not observed in D
 343 neurons after axon injury (Fig. 3A).

344 Although *acox-1.1* is expressed in the intestine, we found that the expression
 345 of *acox-1.1* cDNA from the *ges-1* promoter in the intestine could not rescue the
 346 axon regeneration defect in *acox-1.1(ok2257)* mutants (Fig. 3B; Table 2). Recent

347 observations suggest that peroxisomal FA β -oxidation may have an
 348 as-yet-unexplored function in neurons (Park and Paik, 2017). Indeed, we found
 349 that the *acox-1.1* deficiency was rescued by expression of *acox-1.1* cDNA from
 350 the *unc-25* promoter in D-type motor neurons but not from the *mec-7* promoter in
 351 touch neurons (Fig. 3B; Table 2). Similar to *acox-1.1*, the expression of *daf-22*
 352 with the *unc-25* promoter suppressed the *daf-22* defect (Fig. 3B; Table 2). These
 353 results suggest that ACOX-1.1 and DAF-22 promote the regeneration of
 354 damaged neurons in a cell-autonomous manner.

355

356 **Ascaroside ascr#5 promotes axon regeneration**

357 We next evaluated which ascaroside regulates axon regeneration. In *C. elegans*,
 358 ascarosides are grouped into two main classes: ω -ascarosides and
 359 (ω -1)-ascarosides (Fig. 2A; Butcher, 2017). They are biosynthesized via two
 360 parallel β -oxidation pathways, each involving different ACOX enzymes (Fig. 4A;
 361 Zhang et al., 2015, 2016, 2018). The former pathway involves ACOX-1.2, and
 362 the latter depends on ACOX-1.3, ACOX-1.4, and ACOX-3. ACOX-1.1 and
 363 DAF-22 are required in both pathways. The *acox-1.2(gk386052)* and
 364 *acox-3(gk203391)* mutations contain nonsense mutations, and they are
 365 probably null mutations because they result in premature stop codons at Trp-496
 366 and Trp-395, respectively (Fig. 4B). We found that the *acox-1.2(gk386052)*
 367 mutation reduced axon regeneration (Fig. 4C; Table 2). In contrast, the
 368 *acox-1.3(tm5192)* deletion, which disrupts the ACOX-1.3 function (Fig. 4B;
 369 Zhang et al., 2015, 2016) or the *acox-3(gk203391)* mutation had little effect on
 370 axon generation (Fig. 4C; Table 2). Furthermore, a putative null mutation,
 371 *acox-1.4(km92)* (Fig. 4B), did not affect axon regeneration (Fig. 4C; Table 2).
 372 These results suggest that ω -ascarosides are important for axon regeneration.

373 Since ACOX-1.2 influences the production of an ascaroside with a short
 374 ω -side chain, i.e., the dauer pheromone asc- ω C3 (C3; *ascr#5*) (Fig. 2A; Zhang
 375 et al., 2015), we examined the effect of synthetic *ascr#5* on axon regeneration.
 376 First, we supplied *ascr#5* externally to adult-stage *acox-1.2* mutants and then
 377 determined the axon regeneration frequency. We found that the presence of
 378 *ascr#5* was sufficient to induce dauer formation in *acox-1.2(gk386052)* mutant
 379 larvae when added from an embryo (Fig. 5A,B), and it significantly rescued the
 380 axon regeneration defect in *acox-1.2(gk386052)* mutants when introduced at the
 381 young adult stage (Fig. 5C,D; Table 2). These results indicate that *ascr#5* is
 382 involved in axon regeneration.

383

384 ***acox-1.2* expression inside the injured neuron is required for axon** 385 **regeneration**

386 Expression of *acox-1.2* with the *unc-25* promoter in D neurons suppressed the
 387 *acox-1.2* defect (Fig. 4C; Table 2), suggesting that ACOX-1.2 regulates axon
 388 regeneration in a cell-autonomous manner. Therefore, we examined whether
 389 *acox-1.2* expression inside the injured neuron is required for axon regeneration.
 390 To test this possibility, we expressed the *acox-1.2* gene in touch neurons using
 391 the *mec-7* promoter. Touch neuron axons run parallel to the body axis and
 392 intersect perpendicularly to D-type neuron axons (Fig. 6A). Expression of
 393 *acox-1.2* in touch neurons could not rescue the *acox-1.2* deficiency in D-type
 394 motor neuron regeneration (Fig. 6B; Table 2). This result is consistent with the
 395 possibility that ACOX-1.2 functions cell-autonomously in axon regeneration. On
 396 the other hand, when both touch and D neurons were severed simultaneously in
 397 *acox-1.2(gk386052)* mutants expressing *acox-1.2* in touch neurons, the
 398 regeneration defect of D neurons was suppressed (Fig. 6B; Table 2). In wild-type
 399 animals, simultaneous damage to the axons of touch and D neurons did not

400 affect the frequency of D neuron regeneration (Fig. 6B; Table 2). Altogether,
 401 these results suggest that ACOX-1.2 induces *ascr#5* production in axonally
 402 injured touch neurons and the produced and secreted *ascr#5* acts on damaged
 403 D neurons to promote regeneration.

405 **The SRG-36/SRG-37 GPCRs of *ascr#5* are involved in axon regeneration**

406 *srg-36* and *srg-37* genes are two members of the nematode-specific GPCR
 407 family that encode receptors for *ascr#5* (McGrath et al., 2011). We therefore
 408 determined whether these GPCRs are involved in axon regeneration. Because
 409 the *srg-36* and *srg-37* genes are adjacent to each other in the genome, the
 410 *kyIR95* allele deletes both the genes (Fig. 7A). We found that at the young adult
 411 stage, the frequency of axon regeneration was reduced in *srg-36 srg-37(kyIR95)*
 412 (Fig. 7B; Table 2). SRG-36 and SRG-37 function redundantly to support dauer
 413 formation in response to *ascr#5* (McGrath et al., 2011). To investigate whether
 414 SRG-36 and SRG-37 also show redundancy in regulating axon regeneration or
 415 whether both are required for *ascr#5* signaling, the reporter transgenes
 416 *Psrg-36::srg-36::sl2::gfp* and *Psrg-37::srg-37::sl2::gfp* (Fig. 7A) were introduced
 417 into *srg-36 srg-37(kyIR95)* mutants and we measured the axon regeneration
 418 frequency. These transgenes contain bicistronic fusion genes and are functional
 419 (McGrath et al., 2011). Transgenic animals expressing either of the two
 420 transgenes were defective in axon regeneration, but introducing both transgenes
 421 together rescued the *srg-36 srg-37 (kyIR95)* phenotype (Fig. 7C; Table 2). These
 422 results indicate that both SRG-36 and SRG-37 are required for axon
 423 regeneration after laser axotomy.

424 To confirm that SRG-36/SRG-37 act as receptors for *ascr#5* to promote axon
 425 regeneration, we examined the effect of *ascr#5* addition on axon regeneration in
 426 *srg-36 srg-37 (kyIR95)* mutants. As observed previously (McGrath et al., 2011),

427 *srg-36 srg-37 (kylR95)* mutants were resistant to dauer formation induced by
 428 *ascr#5* (Fig. 5A,B). This result is in parallel with the fact that SRG-36/SRG-37
 429 are GPCRs of *ascr#5*. Similarly, we found that the presence of *ascr#5* could not
 430 rescue the axon regeneration defect in *srg-36 srg-37 (kylR95)* mutants (Fig.
 431 5C,D; Table 2). These results indicate that *ascr#5* promotes axon regeneration
 432 via SRG-36/SRG-37.

433 To determine whether SRG-36 functions in D-type motor neurons, we
 434 examined the expression pattern of *srg-36* using the *Psrg-36::srg-36::sl2::gfp*
 435 reporter gene (McGrath et al., 2011). Previous studies have shown that *srg-36* is
 436 strongly expressed in ASI neurons but are weakly or inconsistently expressed in
 437 several other neurons (McGrath et al., 2011). At the young adult stage, animals
 438 carrying *Psrg-36::srg-36::sl2::gfp* did not show GFP expression in D-type motor
 439 neurons. In addition, no GFP expression was observed in D neurons after axon
 440 injury (Fig. 8). Therefore, to confirm that SRG-36 acts in D-type motor neurons,
 441 we expressed *srg-36::sl2::gfp* from the *unc-25* promoter (Fig. 7A) in *srg-36*
 442 *srg-37 (kylR95)* mutants carrying *Psrg-37::srg-37::sl2::gfp*. We found that the
 443 *srg-36* defect in axon regeneration was rescued by the expression of *srg-36* by
 444 the *unc-25* promoter in D-type motor neurons (Fig. 7D; Table 2). However, the
 445 expression of *Punc-25::srg-36::sl2::gfp* alone could not rescue the *srg-36 srg-37*
 446 *(kylR95)* mutant phenotype (Fig. 7D; Table 2). This is consistent with the idea
 447 that both SRG-36 and SRG-37 are required for axon regeneration. Furthermore,
 448 the expression of *srg-37::sl2::gfp* from the *unc-25* promoter (Fig. 7A) rescued the
 449 *srg-36 srg-37 (kylR95)* mutant phenotype with the *Punc-25::srg-36::sl2::gfp*
 450 construct (Fig. 7D; Table 2). These results demonstrate that SRG-36 and
 451 SRG-37 regulate axon regeneration in injured D-type motor neurons after laser
 452 axotomy in a cell-autonomous manner.

453

454 **SRG-36 and SRG-37 function in the EGL-30 pathway to promote axon**

455 **regeneration**

456 Finally, we investigated whether SRG-36/SRG-37 GPCRs function in the
 457 EGL-30-mediated pathway to promote axon regeneration. We have previously
 458 demonstrated that the CED-10 Rac type GTPase–MAX-2 and EGL-30 Gq α –
 459 TPA-1 PKC pathways regulate axon regeneration mainly at the L4 and young
 460 adult stages, respectively (Pastuhov et al., 2012). Furthermore, it has been
 461 shown that *max-2* is expressed during early development, but not at the young
 462 adult stage (Lucanic et al., 2006). This suggests that TPA-1 replaces MAX-2 to
 463 activate MLK-1 during axon regeneration at the adult stage (Fig. 1A). Therefore,
 464 we examined the relationship between life stage and axon regeneration in
 465 *srg-36 srg-37(kyIR95)* mutants. We found that axon regeneration in *srg-36*
 466 *srg-37(kyIR95)* mutants was reduced only at young adult animals and not at L4
 467 larvae, a phenotype similar to that observed in *egl-30(lf)* mutants (Fig. 7B,E;
 468 Table 2). Thus, SRG-36 and SRG-37 participate in axon regeneration
 469 specifically at the adult stage.

470 We also examined the genetic interactions of *srg-36 srg-37* with *egl-30*. We
 471 found that the regeneration defect in *srg-36 srg-37(kyIR95); egl-30(lf)* triple
 472 mutants was not greater than the regeneration defect in *srg-36 srg-37(kyIR95)*
 473 or *egl-30(lf)* mutants (Fig. 7B; Table 2). This result supports the possibility that
 474 SRG-36/SRG-37 function in the EGL-30 signaling pathway. Furthermore, the
 475 *srg-36 srg-37* phenotype was suppressed by the *egl-30(gf)* mutation (Fig. 7B;
 476 Table 2), suggesting that SRG-36/SRG-37 function upstream of EGL-30. Thus,
 477 SRG-36/SRG-37 GPCRs promote axon regeneration by activating the EGL-30
 478 Gq α pathway.

479 Discussion

480

481 Pheromones are molecules secreted by individuals that can induce changes in
482 the behavior and development of different animals of the same species. *C.*
483 *elegans* secretes ascarosides, which constitute a conserved family of signaling
484 molecules, as pheromones to communicate with other animals and to coordinate
485 population development and behavior (Peso et al., 2015). Originally,
486 ascarosides were identified as components of the dauer pheromone, which is
487 the population density signal. High population density result in ascaroside
488 accumulation, which in combination with additional environmental stimuli, such
489 as limited food availability and temperature stress, promotes larval arrest in the
490 dauer stage (Golden and Riddle, 1984). In this study, we found that ascaroside
491 signaling regulates neural processes in *C. elegans*. In particular, we show that
492 the loss of ascaroside production impairs axon regeneration. Furthermore,
493 GPCR sensing of ascaroside regulates axon regeneration via the EGL-30 Gq α
494 signaling pathway (Fig. 9).

495 The primary site of ascaroside biosynthesis appears to be the intestine, and
496 ascaroside is also likely to be excreted via the intestine (Butcher et al., 2009).
497 However, the expression of the *acox-1.1* gene in the intestine fails to rescue the
498 axon regeneration defect in *acox-1.1* mutants, whereas its expression in injured
499 neurons can restore the *acox-1.1* deficiency. Thus, ACOX-1.1 promotes the
500 regeneration of damaged neurons in a cell-autonomous manner. Furthermore,
501 we demonstrated that *acox-1.2* expression in injured neurons has an important
502 function in regulating axon regeneration. Expression of *acox-1.2* in touch
503 neurons does not rescue the *acox-1.2* deficiency in D-type motor neuron
504 regeneration. However, in *acox-1.2* mutants expressing *acox-1.2* in touch
505 neurons, simultaneous laser ablation of axons of D and touch neurons rescues

the regeneration defect of D neurons. These results suggest that ACOX-1.2 expressed in the damaged touch neuron induces the production of ascaroside, which in turn acts on the nearby damaged D neuron to induce regeneration. Thus, ascaroside is synthesized in axotomized neurons and is required for axon regeneration, suggesting that ascaroside regulates axon regeneration as a non-pheromone signal. Since axotomized neurons gain the ability to synthesize ascaroside in response to axon injury, it appears that transcriptional regulation is necessary to ensure that sufficient amounts of ascaroside are available when axons are damaged. Therefore, it is important to identify the transcription factors that regulate the transcription of genes for ascaroside synthesis after axon injury. Since our *svh* screening revealed genes that encode transcription factors (Shimizu et al., 2021), analysis of these *svh* genes will shed new light into the mechanism underlying the transcription of ascaroside synthesis genes regulated in response to axon injury.

What is the ascaroside that regulates axon regeneration? We show that mutations in *acox-1.1*, *daf-22*, and *acox-1.2* are defective in axon regeneration, whereas *acox-3*, *acox-1.3*, or *acox-1.4* mutations have little effect on axon regeneration. ACOX-1.1, DAF-22, and ACOX-1.2 participate in the β -oxidation cycles that shorten ω -ascarosides, with ACOX-1.2 specifically participating in the last of these β -oxidation cycles, which generates *ascr#5* (Zhang et al., 2018). An *acox-1.2* mutant only shows defects in the production of *ascr#5* and not in that of other ascarosides. Thus, *ascr#5* is a candidate ascaroside responsible for promoting axon regeneration after axon injury. Indeed, *ascr#5* recovers the ability of the *acox-1.2* mutant to regenerate axons. The ascaroside signal is sensed by GPCRs in specific chemosensory neurons (Butcher, 2017). *srg-36* and *srg-37* genes encode GPCRs for *ascr#5* (McGrath et al., 2011). We found that the *srg-36 srg-37* double mutation decreases axon regeneration ability. This

533 indicates that *C. elegans* utilizes SRG-36/SRG-37 GPCRs to recognize ascr#5
534 for initiating axon regeneration after axon injury. The expression of GPCRs for
535 sensing ascarosides is clearly predominant in sensory neurons, whereas
536 *srg-36/srg-37* function in injured motor neurons. In most cases, each GPCR is
537 strictly expressed in different cell types, contributing to their distinct and cell
538 type-specific responses to internal signals (Rohrer and Kobilka, 1998).
539 Furthermore, recent studies have revealed that the expression of several
540 chemoreceptor genes, such as *srh-234* and *odr-10*, is regulated by
541 environmental stimuli (Gruner et al., 2014; Ryan et al., 2014). These results
542 raise the possibility that SRG-36 and SRG-37 are produced in response to nerve
543 injury, activating ascr#5 signaling via the autocrine system and inducing axon
544 regeneration.

545 The ascr#5-specific SRG-36 and SRG-37 provide an example for highly
546 structure-specific ascaroside receptors (McGrath et al., 2011). Our finding that
547 SRG-36 and SRG-37 do not function redundantly in axon regeneration suggests
548 that heterodimerization of SRG-36 and SRG-37 may be necessary to form a
549 functional complex for signal transduction. Recent studies have suggested that
550 GPCRs associate as dimers or higher-order oligomers (Lause, 2010). For
551 example, SRBC-64/SRBC-66 function as part of receptor GPCR dimers or
552 higher-order oligomers, including more specific receptors, such as DAF-37 (Park
553 et al., 2012). Thus, the complex ascaroside signaling properties may partly be
554 due the interaction of several different ascaroside receptors that bind directly to
555 ascarosides.

556 Although ascarosides are specific to nematodes, other similar lipid
557 molecules may contribute to promoting axon regeneration in mammals.
558 Peroxisome proliferator-activated receptor α (PPAR α) induces ACOXs gene
559 expression in mammals (Marcus et al., 1993). In rat dorsal root ganglion neurons,

560 axonal damage increases PPAR protein levels, causing PPAR transport from the
561 distal axons to the nucleus and promoting neuronal regeneration (Lezana et al.,
562 2016). Furthermore, thiazolidinedione, a PPAR agonist, promotes axonal growth
563 in rat hippocampal neurons by activating the JNK pathway in a
564 PPAR α -dependent manner (Quintanilla et al., 2013). Therefore, it is possible
565 that lipid metabolites produced by ACOX enzymes in the peroxisome may
566 induce JNK activation and promote axon regeneration.

567 Each GPCR couples preferentially with a functionally distinct class of G α
568 proteins (Möller et al., 2001). In this study, we found that SRG-36/SRG-37
569 GPCRs activate EGL-30 Gq α and promote axon regeneration. We have recently
570 demonstrated that AEA modulates the axon regeneration response of
571 GABAergic motor neurons after laser axotomy (Pastuhov et al., 2012). AEA
572 functions as an inhibitory signal for axon regeneration, which is transmitted
573 through the NPR-19/NPR-32 GPCRs–GOA-1 Go α pathway and antagonizes
574 EGL-30 Gq α . Therefore, axon regeneration in *C. elegans* appears to be
575 determined by the balance of stimulatory and inhibitory signals, such as
576 ascaroside and AEA, which are transduced by G α protein signal transduction
577 pathways (Fig. 9). Thus, axon regeneration after axonal injury in *C. elegans* is
578 regulated by the Go α –Gq α signaling pathway.

579 **References**

580

581 Brenner S (1974) The genetics of *Caenorhabditis elegans*. Genetics 77:71–94.

582 Brundage L, Avery L, Katz A, Kim UJ, Mendel JE, Sternberg PW, Simon MI

583 (1996) Mutations in a *C. elegans* Gq α gene disrupt movement, egg laying,

584 and viability. Neuron 16:999–1009.

585 Butcher RA, Fujita M, Schroeder FC, Clardy J (2007) Small-molecule

586 pheromones that control dauer development in *Caenorhabditis elegans*. Nat

587 Chem Biol 3:420–422.

588 Butcher RA, Ragains JR, Kim E, Clardy J (2008) A potent dauer pheromone

589 component in *Caenorhabditis elegans* that acts synergistically with other

590 components. Proc Natl Acad Sci USA 105:14288–14292.

591 Butcher RA, Ragains JR, Li W, Ruvkun G, Mak HY (2009) Biosynthesis of the

592 *Caenorhabditis elegans* dauer pheromone. Proc Natl Acad Sci USA

593 106:1875–1879.

594 Butcher RA (2017) Small-molecule pheromones and hormones controlling

595 nematode development. Nat Chem Biol 13:577–586.

596 Doi M, Iwasaki K (2002) Regulation of retrograde signaling at neuromuscular

597 junctions by the novel C2 domain protein AEX-1. Neuron 33:249–259.

598 Dokshin GA, Ghanta KS, Piscopo KM, Mello CC (2018) Robust genome editing

599 with short single-stranded and long, partially single-stranded DNA donors in

600 *Caenorhabditis elegans*. Genetics 210:781–787.

601 Golden JW, Riddle DL (1984) The *Caenorhabditis* dauer larva: developmental

602 effects of pheromone, food, and temperature. Dev Biol 102:368–378.

603 Greene JS, Brown M, Dobosiewicz M, Ishida IG, Macosko EZ, Zhang X, Butcher

604 RA, Cline DJ, McGrath PT, Bargmann CI (2016a) Balancing selection shapes

605 density-dependent foraging behaviour. Nature 539:254–258.

- 606 Greene JS, Dobosiewicz M, Butcher RA, McGrath PT, Bargmann CI (2016b)
 607 Regulatory changes in two chemoreceptor genes contribute to a
 608 *Caenorhabditis elegans* QTL for foraging behavior. *eLife* 5:e21454.
- 609 Gruner M, Nelson D, Winbush A, Hintz R, Ryu L, Chung SH, Kim K, Gabel CV,
 610 van der Linden AM (2014) Feeding state, insulin and NPR-1 modulate
 611 chemoreceptor gene expression via integration of sensory and circuit inputs.
 612 *PLoS Genet* 10:e1004707.
- 613 Hammarlund M, Nix P, Hauth L, Jorgensen EM, Bastiani M (2009) Axon
 614 regeneration requires a conserved MAP kinase pathway. *Science* 323:802–
 615 806.
- 616 Hisamoto N, Matsumoto K (2017) Signal transduction cascades in axon
 617 regeneration: insights from *C. elegans*. *Curr Opin Genet Dev* 44:54–60.
- 618 Hisamoto N, Tsuge A, Pastuhov SI, Shimizu T, Hanafusa H, Matsumoto K (2018)
 619 Phosphatidylserine exposure mediated by ABC transporter activates the
 620 integrin signaling pathway promoting axon regeneration. *Nat Commun*
 621 9:3099.
- 622 Inoue H, Hisamoto N, An JH, Oliveira RP, Nishida E, Blackwell TK, Matsumoto K
 623 (2005) The *C. elegans* p38 MAPK pathway regulates nuclear localization of
 624 the transcription factor SKN-1 in oxidative stress response. *Genes Dev*
 625 19:2278–2283.
- 626 Joo HJ, Yim YH, Jeong PY, Jin YX, Lee JE, Kim H, Jeong SK, Chitwood DJ,
 627 Paik YK (2009) *Caenorhabditis elegans* utilizes dauer pheromone
 628 biosynthesis to dispose of toxic peroxisomal fatty acids for cellular
 629 homeostasis. *Biochem J* 422:61–71.
- 630 Joo HJ, Kim KY, Yim YH, Jin YX, Kim H, Kim MY, Paik YK (2010) Contribution of
 631 the peroxisomal *acox* gene to the dynamic balance of daumone production in
 632 *Caenorhabditis elegans*. *J Biol Chem* 285:29319–29325.

- 633 Kaplan A, Tone SO, Fournier A (2015) Extrinsic and intrinsic regulation of axon
634 regeneration at a crossroads. *Front Mol Neurosci* 8:27.
- 635 Lause MJ (2010) Dimerization in GPCR mobility and signaling. *Curr Opin*
636 *Pharmacol* 10:53–58.
- 637 Lezana JP, Dagan SY, Robinson A, Goldstein RS, Fainzulber M, Bronfman FC,
638 Bronfman M (2016) Axonal PPAR γ promotes neuronal regeneration after
639 injury. *Dev Neurobiol* 76:688–701.
- 640 Li C, Hisamoto N, Nix P, Kanao S, Mizuno T, Bastiani M, Matsumoto K (2012)
641 The growth factor SVH-1 regulates axon regeneration in *C. elegans* via the
642 JNK MAPK cascade. *Nat Neurosci* 15:551–557.
- 643 Lucanic M, Kiley M, Ashcroft N, L'etoile N, Cheng HJ (2006) The *Caenorhabditis*
644 *elegans* P21-activated kinases are differentially required for
645 UNC-6/netrin-mediated commissural motor axon guidance. *Development*
646 133:4549–4559.
- 647 Mahar M, Cavalli V (2018) Intrinsic mechanisms of neuronal axon regeneration.
648 *Nat Rev Neurosci* 19:323–337.
- 649 Marcus SL, Miyata KS, Zhang B, Subramani S, Rachubinski RA, Capone JP
650 (1993) Diverse peroxisome proliferator-activated receptors bind to the
651 peroxisome proliferator-responsive elements of the rat
652 hydratase/dehydratase and fatty acid acyl-CoA oxidase genes but
653 differentially induce expression. *Proc Natl Acad Sci USA* 90:5723–5727.
- 654 McGrath PT, Xu Y, Ailion M, Garrison JL, Butcher RA, Bargmann CI (2011)
655 Parallel evolution of domesticated *Caenorhabditis* species targets pheromone
656 receptor genes. *Nature* 477:321–325.
- 657 Mello CC, Kramer JM, Stinchcomb D, Ambros V (1991) Efficient gene transfer in
658 *C. elegans*: extrachromosomal maintenance and integration of transforming
659 sequences. *EMBO J* 10:3959–3970.

- 660 Mizuno T, Hisamoto N, Terada T, Kondo T, Adachi M, Nishida E, Kim DH,
 661 Ausubel FM, Matsumoto K (2004) The *Caenorhabditis elegans* MAPK
 662 phosphatase VHP-1 mediates a novel JNK-like signaling pathway in stress
 663 response. EMBO J 23:2226–2234.
- 664 Möller S, Vilo J, Croning MDR (2001) Prediction of the coupling specificity of G
 665 protein coupled receptors to their G proteins. Bioinformatics 17:S174-S181.
- 666 Park D, O'Doherty I, Somvanshi RK, Bethke A, Schroeder FC, Kumar U, Riddle
 667 DL (2012) Interaction of structure-specific and promiscuous G-protein coupled
 668 receptors mediates small-molecule signaling in *Caenorhabditis elegans*. Proc
 669 Natl Acad Sci USA 109:9917–9922.
- 670 Park JY, Joo HJ, Park S, Paik YK (2019) Ascaroside pheromones: chemical
 671 biology and pleiotropic neuronal functions. Int J Mol Sci 20:3898.
- 672 Park S, Paik YK (2017) Genetic deficiency in neuronal peroxisomal fatty acid
 673 β -oxidation causes the interruption of dauer development in *Caenorhabditis*
 674 *elegans*. Sci Rep 7:9358.
- 675 Pastuhov SI, Fujiki K, Nix P, Kanao S, Bastiani M, Matsumoto K, Hisamoto N
 676 (2012) Endocannabinoid-Go α signalling inhibits axon regeneration in
 677 *Caenorhabditis elegans* by antagonizing Gq α -PKC-JNK signalling. Nat
 678 Commun 3:1136.
- 679 Peso M, Elgar MA, Barron AB (2015) Pheromonal control: reconciling
 680 physiological mechanism and signaling theory. Biol Rev 90:542-559.
- 681 Quintanilla RA, Godoy JA, Alfaro I, Cabezas D, von Bernhardi R, Bronfman M,
 682 Inestrosa NC (2013) Thiazolidinediones promote axonal growth through the
 683 activation of the JNK pathway. PLoS One 8:e65140.
- 684 Robertson HM, Thomas JH (2006) The putative chemoreceptor families of *C.*
 685 *elegans*. Wormbook Jan 6:1–12.
- 686 Rohrer DK, Kobilka BK (1998) G protein-coupled receptors: functional and

- 687 mechanistic insights through altered gene expression. *Phys Rev* 78:1–35.
- 688 Ryan DA, Miller RM, Lee K, Neal SJ, Fagan KA, Sengupta P, Portman DS
 689 (2014) Sex, age, and hunger regulate behavioral prioritization through
 690 dynamic modulation of chemoreceptor expression. *Curr Biol* 24:2509–2517.
- 691 Sakai Y, Hanafusa H, Shimizu T, Pastuhov SI, Hisamoto N, Matsumoto K (2021)
 692 BRCA1–BARD1 regulates axon regeneration in concert with the Gq α –DAG
 693 signaling network. *J Neurosci* 41:2842–2853.
- 694 Sakamoto R, Byrd DT, Brown HM, Hisamoto N, Matsumoto K, Jin Y (2005) The
 695 *Caenorhabditis elegans* UNC-14 RUN domain protein binds to the kinesin-1
 696 and UNC-16 complex and regulates synaptic vesicle localization. *Mol Biol*
 697 *Cell* 16:483–496.
- 698 Shimizu T, Pastuhov SI, Hanafusa H, Sakai Y, Todoroki Y, Hisamoto N,
 699 Matsumoto K (2021) *Caenorhabditis elegans* F-box protein promotes axon
 700 regeneration by inducing degradation of the Mad transcription factor. *J*
 701 *Neurosci* 41:2373–2381.
- 702 Stecher G, Tamura K, Kumar S (2020) Molecular evolutionary genetics analysis
 703 (MEGA) for MacOS. *Mol Biol Evol* 37:1237–1239.
- 704 Tedeshi A, Bradke F (2017) Spatial and temporal arrangement of neuronal
 705 intrinsic and extrinsic mechanisms controlling axon regeneration. *Curr Opin*
 706 *Neurobiol* 42:118–127.
- 707 von Reuss SH, Dolke F, Dong C (2017) Ascaroside profiling of *Caenorhabditis*
 708 *elegans* using gas chromatography-electron ionization mass spectrometry.
 709 *Anal Chem* 89:10570–10577.
- 710 Yanik MF, Cinar H, Cinar HN, Chisholm AD, Jin Y, Ben-Yakar A (2004)
 711 Functional regeneration after laser axotomy. *Nature* 432:822.
- 712 Zhang X, Feng L, Chinta S, Singh P, Wang Y, Nunnery JK, Butcher RA (2015)
 713 Acyl-CoA oxidase complexes control the chemical message produced by

- 714 *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 112, 3955–3960.
- 715 Zhang X, Li K, Jones RA, Steven DB, Butcher RA (2016) Structural
716 characterization of acyl-CoA oxidases reveals a direct link between
717 pheromone biosynthesis and metabolic state in *Caenorhabditis elegans*. Proc
718 Natl Acad Sci USA 113:10055–10060.
- 719 Zhang X, Wang Y, Perez DH, Jones Lipinski RA, Butcher RA (2018) Acyl-CoA
720 oxidases fine-tune the production of ascaroside pheromones with specific
721 side chain lengths. ACS Chem Biol 13:1048–1056.
- 722 Zuckerkandl E, Pauling L (1965) Evolutionary divergence and convergence in
723 proteins. Edited in Evolving Genes and Proteins by Bryson V, Vogel HJ 97–
724 166. Academic Press, New York.

725 **Figure Legends**

726

727 **Figure 1. Characterization of SVH-18/SRX-16.**

728 **A**, The EGL-30 pathway regulating axon regeneration. EGL-30 Gq α activates
 729 EGL-8 PLC β , which in turn generates DAG from phosphatidylinositol
 730 bisphosphate [PI(4, 5)P₂]. DAG activates TPA-1 PKC, resulting in activation of
 731 the JNK pathway to promote axon regeneration mainly at the young adult stage.
 732 The MAPK phosphatase VHP-1 inactivates KGB-1 JNK.

733 **B**, Structure of SRX-16. The black box indicates the transmembrane regions.
 734 The bold line underneath indicates the extent of the deleted region in the *tm7585*
 735 mutant.

736 **C**, Phylogenetic tree depicting the genetic relationships among the GPCRs of *C.*
 737 *elegans*. The phylogenetic tree was constructed using the MEGAX software for
 738 Mac. SRX-16 was compared with chemoreceptors of the SRG superfamily and
 739 other GPCRs whose ligands have been identified. The scale bar represents the
 740 evolutionary distance calculated using the Poisson correction method based on
 741 the number of amino acid substitutions per site. The similarity (%) of amino acids
 742 between SRX-16 and other GPCRs is shown.

743 **D**, Percentages of axons that initiated regeneration 24 h after laser surgery at
 744 the young adult stage. The number of axons examined is shown. Error bars
 745 indicate 95% confidence intervals. NS, not significant.

746

747 **Figure 2. ACOX-1.1 and DAF-22 are required for axon regeneration.**

748 **A**, Ascaroside biosynthesis pathway. Ascarosides fall into two
 749 classes—(ω -1)-ascarosides in which the side chain is attached to the ascarylose
 750 sugar at the penultimate (ω -1) position and ω -ascarosides in which the side
 751 chain is attached at the terminal (ω) position. These two ascaroside classes are

shortened by two β -oxidation pathways, one involving ACOX-1.1, ACOX-3, ACOX-1.4, and ACOX-1.3 to produce (ω -1)-ascaroside pheromones (such as those shown in yellow boxes) and another involving ACOX-1.1 and ACOX-1.2 to produce ω -ascaroside pheromones (such as the one shown in the yellow box). Ascarosides are named based on their structure—asc-(ω)(Δ)C#, with C# indicating the number of carbons in the side chain, ω indicating ω -side chain, and Δ indicating α - β unsaturation.

B, Structures of ACOX-1.1, DAF-22, and EGL-30. Schematic domain diagrams of *C. elegans* ACOX-1.1, DAF-22, and EGL-30 and their human counterparts are shown. The regions deleted in *ok2257* and *ok693* are indicated by black bars. The *egl-30* loss-of-function (*ad805*) and gain-of-function (*tg26*) mutations are shown.

C, Percentages of axons that initiated regeneration 24 h after laser surgery at the young adult stage. The number of axons examined is shown. Error bars indicate 95% confidence intervals. * $P < 0.05$, ** $P < 0.01$, as determined by Fisher's exact test. NS, not significant.

Figure 3. ACOX-1.1 and DAF-22 regulate axon regeneration in a cell-autonomous manner.

A, Expression pattern of the *acox-1.1* gene. Fluorescent and DIC images of animals carrying *Pacox-1.1::nls::gfp::acox-1.1 3'UTR* and *Punc-25::nes::tdTomato* 1h after excision are shown. D-type motor neurons are visualized using tdTomato fused to a nuclear export signal (NES) under control of the *unc-25* promoter. Blue, yellow, and white arrowheads indicate a severed axon, the corresponding cell body of the injured neuron, and cell nuclei of gut epithelial cells, respectively. The green signal in D-type motor neurons is absent in the cell nucleus, which may be due to channel bleeding from the strong

779 tdTomato signal. Fluorescent and DIC images of animals carrying only
 780 *Pacox-1.1::nls::gfp::acox-1.1 3'UTR* are also shown. The green signal is not
 781 seen around the ventral nerve cord where the cell body of GABAergic neurons
 782 resides. V, ventral side; D, dorsal side. Boxed regions are magnified in the insets.
 783 Scale bar = 100 μ m.

784 **B**, Percentages of axons that initiated regeneration 24 h after laser surgery at
 785 the young adult stage. The number of axons examined is shown. Error bars
 786 indicate 95% confidence intervals. * $P < 0.05$, as determined by Fisher's exact
 787 test. NS, not significant.

788

789 **Figure 4. ACOX-1.2 is required for axon regeneration.**

790 **A**, Phylogenetic tree depicting the genetic relationships among ACOX enzymes
 791 in *C. elegans* and humans. The phylogenetic tree was constructed using
 792 MEGAX software for Mac. The scale bar represents the evolutionary distance
 793 calculated using the Poisson correction method based on the number of amino
 794 acid substitutions per site.

795 **B**, Structures of ACOX-1.2, ACOX-1.3, ACOX-1.4, and ACOX-3. Schematic
 796 domain diagrams of *C. elegans* ACOX-1.2, ACOX-1.3, ACOX-1.4, and ACOX-3
 797 and human ACOX3 are shown. Additionally, the *gk386052* and *gk203391*
 798 mutation sites are indicated. The regions deleted in *tm5192* and *km92* are
 799 shown as black bars.

800 **C**, Percentages of axons that initiated regeneration 24 h after laser surgery at
 801 the young adult stage. The number of axons examined is shown. Error bars
 802 indicate 95% confidence intervals. * $P < 0.05$, *** $P < 0.001$, as determined by
 803 Fisher's exact test. NS, not significant.

804

805 **Figure 5. Effects of ascaroside ascr#5 on dauer formation and axon**

806 **regeneration.**

807 **A**, Dauer induction by *ascr#5*. Arrows indicate animals entering the dauer stage.

808 Scale bar = 500 μ m.

809 **B**, Percentages of dauer formation. Error bars indicate 95% confidence intervals.

810 *** $P < 0.001$, as determined by Fisher's exact test. NS, not significant.

811 **C**, Representative D-type motor neurons in animals 24 h after laser surgery. In

812 *acox-1.2* mutants (when *ascr#5* was present), severed axons exhibited

813 regenerated growth cones (yellow arrowheads). In *acox-1.2* (when *ascr#5* was

814 absent) and *srg-36 srg-37* mutants (when *ascr#5* was present), the proximal

815 ends of axons failed to regenerate (red arrowheads). Scale bar = 10 μ m.

816 **D**, Percentages of axons that initiated regeneration 24 h after laser surgery at

817 the young adult stage. The number of axons examined is shown. Error bars

818 indicate 95% confidence intervals. ** $P < 0.01$, as determined by Fisher's exact

819 test. NS, not significant.

820

821 **Figure 6. The effect of *acox-1.2* expression in touch neurons on the**

822 **regeneration of the D-type motor axon.**

823 **A**, Schematic representation of motor and touch neurons. The D-type motor

824 neurons (magenta) have cell bodies on the ventral side and extend axonal

825 commissures dorsally. The touch neuron (gray) extends a long axon parallel to

826 the long body axis and crosses almost perpendicular to the axons of D-type

827 motor neurons.

828 **B**, Percentages of D-type motor axons that initiated regeneration 24 h after laser

829 surgery in the young adult stage. A schematic representation of motor and touch

830 neurons is shown in the left part. Blue indicates the touch neuron of *acox-1.2*

831 mutants expressing the *acox-1.2* gene. The number of axons examined is

832 shown. Error bars indicate 95% confidence intervals. ** $P < 0.01$, as determined

833 by Fisher's exact test. NS, not significant.

834

835 **Figure 7. SRG-36 and SRG-37 are involved in axon regeneration.**

836 **A**, Genomic region surrounding *srg-36* and *srg-37*, deletion breakpoints in the
837 *kyIR95* allele, and bicistronic fusion genes. Diagrams for
838 *Psrg-36::srg-36::sl2::gfp*, *Psrg-37::srg-37::sl2::gfp*, *Punc-25::srg-36::sl2::gfp*,
839 and *Punc-25::srg-37::sl2::gfp* are shown in the lower part.

840 **B–D**, Percentages of axons that initiated regeneration 24 h after laser surgery at
841 the young adult (YA) stage. The number of axons examined is shown. Error bars
842 indicate 95% confidence intervals. * $P < 0.05$, ** $P < 0.01$, as determined by
843 Fisher's exact test. NS, not significant.

844 **E**, Percentages of axons that initiated regeneration 24 h after laser surgery at
845 the L4 stage. The number of axons examined is shown. Error bars indicate 95%
846 confidence intervals. NS, not significant.

847

848 **Figure 8. Expression pattern of the *Psrg-36::srg-36::sl2::gfp* gene.**

849 Fluorescent and DIC images of animals carrying *Psrg-36::srg-36::sl2::gfp* and
850 *Punc-25::tdTomato* 1h after excision are shown. D-type motor neurons are
851 visualized using tdTomato fused to a nuclear export signal (NES) under control
852 of the *unc-25* promoter. Blue, yellow, and white arrowheads indicate a severed
853 axon, the corresponding cell body of the injured neuron, and the head neuron
854 ASI, respectively. V, ventral side; D, dorsal side. Boxed regions are shown
855 magnified in the insets. Scale bar = 100 μm .

856

857 **Figure 9. A schematic model for regulating axon regeneration by chemical**
858 **signaling pathways.**

859 *Ascr#5* activates the EGL-30–EGL-8–TPA-1 signaling cascade via

860 SRG-36/SRG-37 GPCRs to promote axon regeneration. AEA activates the $G\alpha$
861 protein GOA-1 via NPR-19/NPR-32 GPCRs, which antagonizes EGL-30,
862 inhibiting axon regeneration. Axon regeneration in *C. elegans* is determined by
863 the balance of stimulatory (*ascr#5*) and inhibitory (AEA) chemical signals
864 transduced by $G\alpha$ protein signaling pathways.

Table 1. Strains used in this study.

Strain	Genotype
KU92	<i>acox-1.4(km92) I; juls76 II</i>
KU501	<i>juls76 II</i>
KU456	<i>egl-30(ad805) I; juls76 II</i>
KU457	<i>egl-30(tg26) I; juls76 II</i>
KU1549	<i>juls76 II; srx-16(tm7585) V</i>
KU1550	<i>acox-1.1(ok2257) I; juls76 II</i>
KU1551	<i>acox-1.1(ok2257) I; juls76 II; kmEx1551[P_{acox-1.1}::<i>acox-1.1</i> + P_{myo-2}::<i>dsRedmonomer</i>]</i>
KU1552	<i>acox-1.1(ok2257) I; juls76 II; kmEx1552[P_{ges-1}::<i>acox-1.1</i> + P_{myo-2}::<i>dsRedmonomer</i>]</i>
KU1553	<i>acox-1.1(ok2257) I; juls76 II; kmEx1553[P_{unc-25}::<i>acox-1.1</i> + P_{myo-2}::<i>dsRedmonomer</i>]</i>
KU1554	<i>acox-1.1(ok2257) I; juls76 II; kmEx1554[P_{mec-7}::<i>acox-1.1</i> + P_{myo-2}::<i>dsRedmonomer</i>]</i>
KU1555	<i>acox-1.2(gk386052) I; juls76 II</i>
KU1556	<i>acox-1.2(gk386052) I; juls76 II; kmEx1556[P_{unc-25}::<i>acox-1.2</i> + P_{myo-2}::<i>dsRedmonomer</i>]</i>
KU1557	<i>acox-1.2(gk386052) I; juls76 II; kmEx1557[P_{mec-7}::<i>acox-1.2</i> + P_{mec-7}::<i>gfp</i> + P_{myo-2}::<i>dsRedmonomer</i>]</i>
KU1558	<i>acox-1.3(tm5192) I; juls76 II</i>

KU1559 *juls76 II; acox-3 (gk203391) IV*

KU1560 *kmEx1560[Pacox-1.1::nls::gfp::acox-1.1 3'UTR + Punc-25::nes::tdTomato + Pmyo-2::dsRedmonomer]*

KU1561 *daf-22(ok693) juls76 II*

KU1562 *daf-22(ok693) juls76 II; kmEx1562[Punc-25::daf-22 + Pmyo-2::dsRedmonomer]*

KU1563 *juls76 II; srg-36 srg-37(kylR95)X*

KU1564 *juls76 II; srg-36 srg-37(kylR95)X; kmEx1564[Psrg-36::srg-36::sl2::gfp + Pmyo-2::dsRedmonomer]*

KU1565 *juls76 II; srg-36 srg-37(kylR95)X; kmEx1565[Psrg-37::srg-37::sl2::gfp + Pmyo-2::dsRedmonomer]*

KU1566 *juls76 II; srg-36 srg-37(kylR95)X;
kmEx1566[Psrg-36::srg-36::sl2::gfp + Psrg-37::srg-37::sl2::gfp + Pmyo-2::dsRedmonomer]*

KU1567 *egl-30(ad805) I; juls76 II; srg-36 srg-37 (kylR95)X*

KU1568 *egl-30(tg26) I; juls76 II; srg-36 srg-37 (kylR95)X*

KU1569 *juls76 II; srg-36 srg-37(kylR95)X;
kmEx1569[Punc-25::srg-36::sl2::gfp + Psrg-37::srg-37::sl2::gfp + Pmyo-2::dsRedmonomer]*

KU1570 *kmEx1570[Psrg-36::srg-36::sl2::gfp + Punc-25::nes::tdTomato + Pmyo-2::dsRedmonomer]*

KU1571 *acox-1.1(ok2257) egl-30(ad805) I; juls76 II*

KU1572 *acox-1.1(ok2257) egl-30(tg26) I; juls76 II*

KU1573 *juls76 II; srg-36 srg-37(kyIR95)X;*
kmEx1573[Punc-25::srg-36::sl2::gfp + Pmyo-2::dsRedmonomer]

KU1574 *juls76 II; srg-36 srg-37(kyIR95)X;*
kmEx1574[Punc-25::srg-36::sl2::gfp + Punc-25::srg-37::sl2::gfp + Pmyo-2::dsRedmonomer]

KU1575 *juls76 II; kmEx1557[Pmec-7::acox-1.2 + Pmec-7::gfp + Pmyo-2::dsRedmonomer]*

KU1576 *kmEx1576[Pacox-1.1::nls::gfp::acox-1.1 3'UTR + Pmyo-2::dsRedmonomer]*

Table 2. Raw data for genotypes tested by axotomy.

Strain	Genotype (<i>juIs76</i> background)	Stage	No. of animals	No. of axons	No. of regenerations (% of total)	P-value	Compared with
KU501 ^a	wild type	YA	24	68	42 (62%)	-	-
KU1549	<i>srx-16(tm7585)</i>	YA	19	53	32 (60%)	0.8493	KU501 ^a
KU501 ^b	wild type	YA	24	64	43 (67%)	-	-
KU1550	<i>acox-1.1(ok2257)</i>	YA	32	53	22 (42%)	0.0086	KU501 ^b
KU1551	<i>acox-1.1(ok2257); Ex[Pacox-1.1::acox-1.1]</i>	YA	23	52	38 (73%)	0.0015	KU1550
KU456	<i>egl-30(lf)</i>	YA	18	50	20 (40%)*	-	-
KU1571	<i>acox-1.1(ok2257); egl-30(lf)</i>	YA	23	56	27 (48%)	0.5645	KU1550
KU457	<i>egl-30(gf)</i>	YA	13	30	21 (70%)*	-	-
KU1572	<i>acox-1.1(ok2257); egl-30(gf)</i>	YA	33	50	34 (68%)	0.0099	KU1550
KU1561	<i>daf-22(ok693)</i>	YA	29	50	22 (44%)	0.0217	KU501 ^b
KU1552	<i>acox-1.1(ok2257); Ex[Pges-1::acox-1.1]</i>	YA	36	52	27 (52%)	0.3307	KU1550
KU1553	<i>acox-1.1(ok2257); Ex[Punc-25::acox-1.1]</i>	YA	35	51	33 (65%)	0.0200	KU1550
KU1554	<i>acox-1.1(ok2257); Ex[Pmec-7::acox-1.1]</i>	YA	19	51	18 (35%)	0.6863	KU1550
KU1562	<i>daf-22(ok693); Ex[Punc-25::daf-22]</i>	YA	20	52	34 (65%)	0.0461	KU1561
KU1555 ^c	<i>acox-1.2(gk386052)</i>	YA	35	51	18 (35%)	0.0008	KU501 ^b

KU1556	<i>acox-1.2(gk386052); Ex[Punc-25::acox-1.2]</i>	YA	22	50	30 (60%)	0.017	KU1555 ^c
KU1558	<i>acox-1.3(tm5192)</i>	YA	35	52	27 (52%)	0.1268	KU501 ^b
KU1559	<i>acox-3(gk203391)</i>	YA	32	50	28 (56%)	0.2470	KU501 ^b
KU92	<i>acox-1.4(km92)</i>	YA	21	53	34 (59%)	1	KU501 ^b
KU1557	<i>acox-1.2(gk386052); Ex[Pmec-7::acox-1.2 + Pmec-7::gfp]</i> only D cut	YA	46	46	14 (30%)	0.669	KU1555 ^c
KU1557	<i>acox-1.2(gk386052); Ex[Pmec-7::acox-1.2 + Pmec-7::gfp]</i> PLM+D cut	YA	46	46	30 (65%)	0.0044	KU1555 ^c
KU1575	<i>Ex[Pmec-7::acox-1.2 + Pmec-7::gfp]</i> only D cut	YA	50	50	35(70%)	0.4349	KU501 ^b
KU1575	<i>Ex[Pmec-7::acox-1.2 + Pmec-7::gfp]</i> PLM+D cut	YA	41	41	25(61%)	1	KU501 ^b
KU1555 ^d	<i>acox-1.2(gk386052) + EtOH</i>	YA	15	40	13 (33%)	-	-
KU1555	<i>acox-1.2(gk386052) + ascr#5 (1 μM)</i>	YA	19	53	34 (64%)	0.0034	KU1555 ^d
KU1563 ^g	<i>srg-36 srg-37(kyIR95) + EtOH</i>	YA	27	68	28(41%)	-	-
KU1563	<i>srg-36 srg-37(kyIR95) + ascr#5 (1 μM)</i>	YA	22	60	28 (47%)	0.5939	KU1563 ^g
KU1563 ⁱ	<i>srg-36 srg-37(kyIR95)</i>	YA	20	50	21 (42%)	0.0083	KU501 ^b
KU456 ^g	<i>egl-30(lf)</i>	YA	21	54	20 (37%)	0.0016	KU501 ^b
KU1567	<i>egl-30(lf) srg-36 srg-37(kyIR95)</i>	YA	20	50	21 (42%)	1 0.6892	KU1563 ⁱ KU456 ^g
KU1568	<i>egl-30(gf) srg-36 srg-37(kyIR95)</i>	YA	28	62	48 (77%)	0.0001	KU1563 ⁱ

KU1564	<i>srg-36 srg-37(kyIR95);</i> <i>Ex[Psg-36::srg-36::sl2::gfp]</i>	YA	35	92	45 (49%)	-	-
KU1564	<i>srg-36 srg-37(kyIR95)</i> Ex(-)	YA	31	85	31 (36%)	0.1283	KU1564
KU1565	<i>srg-36 srg-37(kyIR95);</i> <i>Ex[Psg-37::srg-37::sl2::gfp]</i>	YA	22	63	26 (41%)	-	-
KU1565	<i>srg-36 srg-37(kyIR95)</i> Ex(-)	YA	27	72	24 (33%)	0.3751	KU1565
KU1566	<i>srg-36 srg-37(kyIR95);</i> <i>Ex[Psg-36::srg-36::sl2::gfp + Psg-37::srg-37::sl2::gfp]</i>	YA	25	64	36 (56%)	-	-
KU1566	<i>srg-36 srg-37(kyIR95)</i> Ex(-)	YA	29	75	28 (37%)	0.0281	KU1566
KU1569	<i>srg-36 srg-37(kyIR95);</i> <i>Ex[Punc-25::srg-36::sl2::gfp + Psg-37::srg-37::sl2::gfp]</i>	YA	26	77	42 (55%)	-	-
KU1569	<i>srg-36 srg-37(kyIR95)</i> Ex-	YA	21	63	22 (32%)	0.0281	KU1569
KU1573	<i>srg-36 srg-37(kyIR95);</i> <i>Ex[Punc-25::srg-36::sl2::gfp]</i>	YA	22	57	19(33%)	-	-
KU1573	<i>srg-36 srg-37(kyIR95)</i> Ex(-)	YA	22	57	19(33%)	1	KU1573

KU1574	<i>srg-36 srg-37(kyIR95);</i> <i>Ex[Punc-25::srg-36::sl2::gfp + Punc-25::srg-37::sl2::gfp]</i>	YA	30	81	44 (54%)	-	-
KU1574 Ex(-)	<i>srg-36 srg-37(kyIR95)</i>	YA	31	82	27 (36%)	0.0073	KU1574
KU501 ^h	wild type	L4	24	54	42 (78%)	-	-
KU456	<i>egl-30(lf)</i>	L4	15	41	31 (76%)	0.8112	KU501 ^h
KU1563	<i>srg-36 srg-37(kyIR95)</i>	L4	20	52	39 (75%)	0.8206	KU501 ^h

*: Sakai et al., 2021

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9