The ERK MAPK pathway modulates Gq-dependent locomotion in *Caenorhabditis elegans*

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Running Title: The ERK pathway modulates Gq signaling

Keywords: Gq signaling, ERK MAPK pathway, Rho small GTPase, *C. elegans*, G protein

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Abstract

The heterotrimeric G protein Gq regulates neuronal activity through distinct downstream effector pathways. In addition to the canonical Gq effector phospholipase Cβ, the small GTPase Rho was recently identified as a conserved effector of Gq. To identify additional molecules important for Gq signaling in neurons, we performed a forward genetic screen in the nematode *Caenorhabditis elegans* for suppressors of the hyperactivity and exaggerated waveform of an activated Gq mutant. We isolated two mutations affecting the MAP kinase scaffold protein KSR-1 and found that KSR-1 modulates locomotion downstream of or in parallel to the Gq-Rho pathway. Through epistasis experiments, we found that the core ERK MAPK cascade is required for Gq-Rho regulation of locomotion, but that the canonical ERK activator LET-60/Ras may not be required. Through neuron-specific rescue experiments, we found that the ERK pathway functions in head acetylcholine neurons to control Gq-dependent locomotion. Additionally, expression of activated LIN-45/Raf in head acetylcholine neurons is sufficient to cause an exaggerated waveform phenotype and hypersensitivity to the acetylcholinesterase inhibitor aldicarb, similar to an activated Gq mutant. Taken together, our results suggest that the ERK MAPK pathway modulates the output of Gq-Rho signaling to control locomotion behavior in *C. elegans.*

49 **Introduction**

50 The heterotrimeric G protein Gq is a conserved regulator of neurotransmission in
51 metazoans. Gq is highly expressed in neurons in mammals and in the nematode *C.*
52 *elegans* (Wilkie *et al.* 1991; Lackner *et al.* 1999). In its canonical signaling pathway, Gq
53 activates phospholipase Cβ (PLCβ) to cleave the membrane lipid phosphatidylinositol
54 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG)
55 (Rhee 2001). An increased DAG concentration at the synapse helps trigger synaptic
56 vesicle release (Miller *et al.* 1999; Lackner *et al.* 1999).

57 In addition to activating PLCβ, Gq directly binds and activates the Rho guanine
58 nucleotide exchange factor (GEF) Trio, which in turn activates the small GTPase Rho
59 (Lutz *et al.* 2005, 2007; Williams *et al.* 2007). In mature *C. elegans* neurons, the Rho
60 ortholog RHO-1 regulates synaptic activity through multiple G protein-dependent
61 mechanisms. First, RHO-1 acts downstream of the G_{12}-class G protein GPA-12 by
62 binding to and inhibiting the diacylglycerol kinase DGK-1. Inhibition of DGK-1 allows
63 DAG to accumulate at the synapse, thereby increasing synaptic vesicle release
64 (McMullan *et al.* 2006; Hiley *et al.* 2006). Second, Gq-Rho signaling promotes
65 neurotransmitter release by recruiting the sphingosine kinase SPHK-1 to presynaptic
66 terminals (Chan *et al.* 2012). Third, Gq-Rho signaling positively regulates the NCA-
67 1/NALCN cation channel to regulate locomotion (Topalidou *et al.* 2017a). Here we
68 identify the extracellular signal-related kinase mitogen-activated protein kinase (ERK
69 MAPK) pathway as a positive regulator of neuronal activity acting downstream of or in
70 parallel to Gq and Rho in acetylcholine neurons.

ERK MAPK signaling acts extensively in animal development, cellular proliferation, and cancer signaling (Yoon and Seger 2006; Karnoub and Weinberg 2008; Sun *et al.* 2015). ERKs are highly expressed in mammalian neurons (Boulton *et al.* 1991; Ortiz *et al.* 1995) and act in both the nucleus and at the synapse to regulate synaptic activity and plasticity (Thomas and Huganir 2004; Sweatt 2004; Mao and Wang 2016b). In *C. elegans*, the ERK pathway is required for multiple developmental events including specification of the vulva (Sternberg 2005; Sundaram 2013), and also acts in several types of neurons to control behavior. ERK signaling is activated in response to odorants in the AWC sensory neuron to regulate chemotaxis to volatile odorants and in AIY interneurons to mediate odor adaptation (Hirotsu *et al.* 2000; Hirotsu and Iino 2005; Chen *et al.* 2011; Uozumi *et al.* 2012). ERK is also activated in the ASER sensory neuron to regulate chemotaxis to salt (Tomioka *et al.* 2006; Tomida *et al.* 2012). ERK signaling regulates foraging behavior by acting in the IL1, OLQ, and RMD neurons (Hamakawa *et al.* 2015). Finally, the ERK pathway has been shown to act in interneurons to regulate the nose touch response, a mechanosensory behavior (Hyde *et al.* 2011).

In the canonical ERK MAPK pathway, extracellular ligand binding activates transmembrane receptor tyrosine kinases (RTKs), and adaptor proteins recruit a GEF to activate the small GTPase Ras (LET-60 in *C. elegans*). Upon Ras activation, LIN-45/Raf translocates to the plasma membrane where it interacts with Ras and the scaffold protein KSR-1. KSR-1 facilitates the activation of LIN-45/Raf and the subsequent phosphorylation of the MAPK cascade consisting of LIN-45/Raf, MEK-2/MEK, and MPK-1/ERK (Sundaram 2013). In this study, we found that the ERK MAPK pathway

consisting of KSR-1, LIN-45/Raf, MEK-2/MEK and MPK-1/ERK modulates Gq-Rho signaling in acetylcholine neurons, but that surprisingly LET-60/Ras may not be required.

98 **Materials and Methods**

99

100 ***C. elegans strains***

101 All strains were cultured using standard methods and were maintained at 20°C.
102 Table S1 contains all the strains used in this study.

103

104 ***Isolation and mapping of the ksr-1(ox314) and ksr-1(yak10) mutations***

105 The *ox314* and *yak10* mutants were isolated from an ENU mutagenesis
106 suppressor screen of the activated Gq mutant *egl-30(tg26)* (Ailion *et al.* 2014). We
107 mapped the *ox314* mutation by its activated Gq suppression phenotype using single
108 nucleotide polymorphisms (SNPs) in the Hawaiian strain CB4856 as described (Davis *et*
109 *al.* 2005).The *ox314* mutation was mapped to an approximately 709 kb region in the
110 middle of the X chromosome between SNPs on cosmids F45E1 and F53A9 (SNPs
111 F45E1[1] and pkP6158). This region included 159 predicted protein-coding genes. A
112 complementation test of *ox314* and *yak10* in the *egl-30(tg26)* background showed these
113 to be alleles of the same gene. Whole genome sequencing (see below) identified these
114 as mutations in *ksr-1*, and we confirmed this by performing a complementation test with
115 the deletion allele *ksr-1(ok786).*

116

117 ***Whole genome sequencing***

118 Strains EG4198 *egl-30(tg26); ox314* and XZ1340 *egl-30(tg26); yak10* were
119 sequenced to identify candidate mutations. DNA was purified according to the Hobert
120 Lab protocol (http://hobertlab.org/whole-genome-sequencing/). Ion Torrent sequencing

121 was performed at the University of Utah DNA Sequencing Core Facility. Each data set
122 contained roughly 18,400,000 reads of a mean read length of 160 bases, resulting in
123 about 30X average coverage of the *C. elegans* genome. The sequencing data were
124 processed on the Galaxy server at usegalaxy.org (Afgan *et al.* 2016). SNPs and indels
125 were identified and annotated using the Unified Genotyper and SnpEff tools (DePristo *et*
126 *al.* 2011; Cingolani *et al.* 2012). After filtering for mutations in open reading frames, we
127 found each strain to have unique stop mutations in *ksr-1*, in the middle of the interval
128 where we mapped *ox314*. *ox314* is a G to A transition that causes a stop codon at
129 amino acid K463, and *yak10* is an A to T transversion that causes a stop codon at
130 W254.
131
132 ***Locomotion assays***
133 Track waveform and radial locomotion assays were performed on 10 cm
134 nematode growth medium (NGM) plates seeded with 400 µl of OP50 *E. coli* culture and
135 spread with sterile glass beads. Bacterial lawns were grown at 37°C for 16 hrs and the
136 plates were stored at 4°C until needed. For track waveform measurements, five first day
137 adult animals were placed on a plate and allowed to roam for 2-5 min. We then
138 recorded each animal's tracks following forward locomotion. Track pictures were taken
139 at 40X on a Nikon SMZ18 microscope with the DS-L3 camera control system. Pictures
140 of worm tracks were processed using ImageJ. Period and 2X amplitude were measured
141 freehand using the line tool. For each worm, we calculated the average
142 period/amplitude ratio of five individual track bends (Figure 1C). For assays with the
143 temperature sensitive allele *sos-1(cs41)*, all strains were grown at 20°C and shifted to

144 the non-permissive temperature of 25°C for 24 hours before being assayed. For radial
145 locomotion assays, ten to fifteen first day adult animals were picked to the center of a
146 plate and were then allowed to move freely for 40 minutes. The positions of worms were
147 marked and the distances of the worms from the starting point were measured. For all
148 waveform and radial locomotion assays, the experimenter was blind to the genotypes of
149 the strains assayed.
150
151 ***Microscopy***
152 Photographs of moving worms were taken at 60X on a Nikon SMZ18 microscope
153 with the DS-L3 camera control system. The worms were age-matched as first day
154 adults grown at 20°C.
155
156 ***Constructs and transgenes***
157 Plasmids were constructed using the three-slot multisite Gateway cloning system
158 (Invitrogen). Plasmids and primers used are found in Tables S2 and S3. The *ksr-1*, *lin-*
159 *45*, *let-60*, and *egl-30* cDNAs were amplified by PCR from a worm cDNA library and
160 cloned into [1-2] Gateway entry vectors. Activating Raf mutations T626E/T629D were
161 introduced into the *lin-45* cDNA vector by two sequential site-directed mutagenesis
162 reactions (Q5 kit, NEB) with primers oBC094/095 and oBC096/097, respectively, and
163 then confirmed by sequencing. The dominant Ras mutation S17N was introduced into
164 the *let-60* cDNA by site-directed mutagenesis with primers oBC109/110. The *tg26*
165 activating Gq mutation R243Q was introduced into the *egl-30* cDNA by site-directed
166 mutagenesis with primers oET254/255. cDNAs were cloned into expression constructs

167 under different neuronal promoters using the multisite Gateway system. Specifics of
168 promoter fragments used are given in Table S2. For expression in different subclasses
169 of acetylcholine neurons, we used the *Punc-17H* promoter that is expressed in head
170 acetylcholine neurons (Hammarlund *et al.* 2007; Topalidou *et al.* 2017a) and the *Punc-*
171 *17β* promoter that is expressed in ventral cord acetylcholine motor neurons (Charlie *et*
172 *al.* 2006; Topalidou *et al.* 2017a). Proper expression of *ksr-1*, *lin-45* and *let-60* was
173 confirmed by including an operon GFP::H2B in the [2-3] slot of the expression
174 constructs. The operon GFP template *tbb-2 3'utr::gpd-2 operon::GFP::H2B:cye-1 3'utr*
175 (Frøkjær-Jensen *et al.* 2012) results in untagged proteins whose expression can be
176 monitored by GFP expression.
177
178 ***Injections and chromosomal integrations***
179 Worms carrying the activated *lin-45* transgenes *Punc-17::lin-45** and *Punc-*
180 *17H::lin-45** as extrachromosomal arrays were generated by injecting pBC37 or pBC44
181 at 20 ng/μL or 10 ng/μL respectively along with co-injection markers pCFJ104 (*Pmyo-*
182 *3::mCherry*) at 5 ng/μL, pCFJ90 (*Pmyo-2::mCherry*) at 2.5 ng/μL, and the carrier DNA
183 Litmus 38i to a final concentration of 100 ng/μL DNA (Mello *et al.* 1991). Worms carrying
184 the dominant negative *let-60* transgene *Prab-3::let-60(S17N)* were generated by
185 injecting pBC50 at 20 ng/ul with the same co-injection markers. Worms carrying the
186 activated Gq *egl-30(tg26)* transgene *Punc-17H::egl-30(tg26)* were generated by
187 injecting pET102 at 10 ng/ul with the co-injection marker pCFJ90 (*Pmyo-2::mCherry*) at
188 1 ng/μL. MosSCI lines were generated as described (Frøkjær-Jensen *et al.* 2012) using
189 an injection mix containing 10-15 ng/μL targeting vector, 50 ng/μL pCFJ601 (*Peft-*

190 *3::Mos1* transposase), negative selection markers pGH8 (*Prab-3::mCherry*) at 10 ng/μL,
191 pCFJ104 (*Pmyo-3::mCherry*) at 5 ng/μL, pCFJ90 (*Pmyo-2::mCherry*) at 2.5 ng/μL,
192 pMA122 (*Phsp16.41::peel-1*) at 10 ng/μL, and carrier DNA Litmus 38i to a final
193 concentration of 100 ng/μL DNA.
194 Extrachromosomal arrays were integrated into the genome by exposure to 4000
195 rads of gamma irradiation. Irradiated young adult hermaphrodites were transferred to 10
196 cm OP50 plates (5 worms/plate) and grown to starvation. The plates were chunked and
197 grown to starvation twice more to enrich for stably expressing lines. When nearly
198 starved, 8 animals per plate were picked to individual plates. The progeny were then
199 screened for 100% stable transmission, indicating integration into the genome.
200 Integration was confirmed by mapping the transgene to a chromosome.
201
202 ***Aldicarb assays***
203 35 mm aldicarb assay plates were poured with NGM supplemented with 1 mM
204 aldicarb. The plates were seeded with 5 uL OP50 and dried at room temperature
205 overnight. Animals were picked onto the OP50 lawn to begin the assay (time 0) and
206 then kept at room temperature. Every 15 minutes, animals were scored for paralysis by
207 lightly touching the nose of the animal with an eyebrow hair. Animals were scored as
208 paralyzed if the worm displayed no locomotor response to three nose touches and had
209 no pharyngeal pumping. Animals that left the OP50 lawn were picked back onto the
210 food.
211
212 ***Statistical analysis***

213 P values were determined using GraphPad Prism 5. Normally distributed data
214 sets were analyzed with a one-way ANOVA and Bonferroni's *post hoc* test when group
215 size was unequal, or with Tukey's *post hoc* test when group size was equal. Data sets
216 with non-normal distribution (using the Shapiro-Wilk normality test) were analyzed with
217 a Kruskal-Wallis test and Dunn's *post hoc* test. Data sets with multiple independent
218 variables were analyzed by two-way ANOVA and Bonferroni's *post hoc* test.
219
220 ***Reagent and data availability***
221 Strains and plasmids are listed in Tables S1 and S2 and are available upon
222 request. Primers are listed in Table S3. The authors state that all data necessary for
223 confirming the conclusions presented in the article are represented fully within the
224 article and Supplemental Material.
225

226 **Results**

227

228 **KSR-1 regulates locomotion downstream of or in parallel to Gq**

229 In *C. elegans,* the heterotrimeric G protein Gq regulates synaptic vesicle release
230 (Hu *et al.* 2015). Gq is a key regulator of neuromuscular activity, as loss-of-function
231 mutants in *egl-30* are nearly paralyzed (Brundage *et al.* 1996) whereas the gain-of-
232 function mutant *egl-30(tg26)* has hyperactive locomotion with an exaggerated loopy
233 waveform (Doi and Iwasaki 2002; Bastiani *et al.* 2003) (Figure 1, A, C, and D). To
234 identify pathways required for Gq signaling, we performed a forward genetic screen in
235 *C. elegans* for suppressors of the activated Gq mutant *egl-30*(*tg26)*. This screen has
236 been used to identify new components of the Gq signal transduction pathway and
237 proteins important for dense-core vesicle function (Williams *et al.* 2007; Ailion *et al.*
238 2014; Topalidou *et al.* 2016; Topalidou *et al.* 2017a; b). Two previously uncharacterized
239 suppressors identified in this screen, *ox314* and *yak10*, showed similar suppression of
240 the loopy waveform and hyperactivity of *egl-30(tg26)* animals (Figure 1D). When
241 crossed away from the *egl-30(tg26*) background, both mutants moved with wild-type
242 waveform (Figure 1D), but at a slightly slower rate. We mapped the *ox314* allele near
243 the center of the X chromosome (see Materials and Methods), and a complementation
244 test showed that *ox314* and *yak10* are mutations in the same gene since they fail to
245 complement in an *egl-30(tg26)* background.

246 We used whole genome sequencing to identify *ox314* and *yak10* as nonsense
247 mutations in *ksr-1* (Figure 1B, see Materials and Methods). KSR-1 is a scaffold protein
248 that facilitates the localization and interactions required for the Ras-mitogen activated

249 protein kinase (MAPK) cascade consisting of Raf, MEK, and ERK (Kornfeld *et al.*
250 1995b; Sundaram and Han 1995; Nguyen *et al.* 2002; Zhang *et al.* 2013). The deletion
251 allele *ksr-1(ok786)* also suppressed the loopy waveform of the activated Gq mutant
252 identically to *ox314* and *yak10.* These results suggest that KSR-1 activity is required for
253 regulation of locomotion rate and waveform by Gq.

254

255 **The ERK MAPK cascade acts to promote Gq signaling**

256 Because the loss of the MAPK scaffold *ksr-1* suppresses the activated Gq
257 mutant *egl-30(tg26)*, we asked whether other components of the Ras-ERK pathway
258 would also suppress. Since the core components of the Ras-ERK pathway are required
259 for viability, we built double mutants of activated Gq with reduction-of-function mutations
260 in genes at each step of the ERK cascade. Mutations in Raf (*lin-45(sy96)*), MEK (*mek-*
261 *2(n1989)*, *mek-2(ku114)*), and ERK (*mpk-1(ga117)*, *mpk-1(oz140)*) all suppressed the
262 loopy waveform of activated Gq animals similarly to *ksr-1(ok786)* (Figure 2, A and B;
263 Figure S1, A and B). However, mutations in Ras (*let-60(n2021)*) and the upstream
264 pathway activators EGF (*lin-3(e1417)*) and the EGF receptor (*let-23(sy12)*) did not
265 suppress activated Gq (Figure 2C). Because *let-60* is required for viability, most *let-60*
266 alleles including *n2021* are partial loss-of-function (Beitel *et al.* 1990). We also analyzed
267 the dominant negative D119N allele *let-60(sy93)* that disrupts Ras binding to guanine
268 nucleotides and thus prevents Ras activation (Han and Sternberg 1991). We found that
269 *let-60(sy93)* also did not suppress the loopy waveform of activated Gq (Figure 2D).
270 Additionally, we expressed the dominant negative *let-60* mutation S17N specifically in
271 neurons and did not observe suppression of the loopy waveform of activated Gq (Figure

272 S2D). These results support the possibility that ERK activation in this pathway occurs
273 through a Ras-independent mechanism.

274 Because partial loss-of-function mutations in the ERK MAPK pathway genes
275 downstream of Ras showed clear suppression of activated Gq, we were surprised to
276 find that partial loss-of-function mutations in Ras did not suppress. If LET-60/Ras is
277 indeed not required, Gq might instead activate the ERK pathway via other Ras-
278 subfamily proteins. To test this possibility, we made double mutants of activated Gq with
279 putative null alleles of R-Ras/*ras-1,* M-Ras/*ras-2,* and Rap1/*rap-1* and found that they
280 also did not suppress activated Gq (Figure S2A). To further investigate whether this
281 pathway acts independently of Ras, we tested mutations in GEFs that activate Ras. The
282 temperature-sensitive RasGEF mutant *sos-1(cs41)* did not suppress activated Gq when
283 shifted to the non-permissive temperature (Figure S2B). Additionally, a null mutation in
284 the neuronal RasGEF *rgef-1* also did not suppress activated Gq (Figure S2C). In
285 genetic screens for vulval induction mutants, additional factors such as the PP2A
286 subunit *sur-6* (Sieburth *et al.* 1999) and ion transporter *sur-7* (Yoder *et al.* 2004) were
287 identified as positive regulators of Ras-ERK activity. However, the *sur-6(sv30)* and *sur-*
288 *7(ku119)* mutations did not suppress activated Gq locomotion (data not shown). These
289 data suggest either that only a low level of Ras activity is needed to properly activate
290 ERK signaling downstream of Gq, or that ERK signaling acts independently of LET-
291 60/Ras and other known *C. elegans* Ras family proteins to regulate locomotion
292 downstream of Gq.

293

294 **KSR-1 and the ERK MAPK cascade modulate Rho signaling**

295 Three classes of suppressor mutations were isolated in our forward genetic
296 screen of activated Gq, as characterized by their molecular roles and unique
297 suppression phenotypes (Topalidou *et al.* 2017a; b). We grouped together a class of
298 suppressor mutations including *ox314, yak10*, and the RhoGEF Trio (*unc-73* in *C.*
299 *elegans*) by their characteristic strong suppression of the loopy waveform of activated
300 Gq (Topalidou *et al.* 2017a; b), suggesting that *ksr-1* might act in the same pathway as
301 *unc-73*.

302 We have shown that Gq regulates locomotion via the small GTPase Rho (RHO-1
303 in *C. elegans*) (Topalidou *et al.* 2017a). Transgenic expression of an activated RHO-1
304 mutant (G14V) in acetylcholine neurons (here called "*rho-1(gf)*") causes worms to have
305 a loopy waveform and impaired locomotion (McMullan *et al.* 2006) (Figure 3A). To
306 examine whether *ksr-1* acts in the Gq-Rho pathway we tested whether mutations in *ksr-*
307 *1* suppress the phenotypes of *rho-1(gf)* worms. We found that the *ksr-1* alleles *ok786,*
308 *ox314,* and *yak10* all suppressed the loopy waveform of *rho-1(gf)* worms (Figure 3, A,
309 B, and D). Because *rho-1(gf)* worms have a slow locomotion rate and loopy waveform,
310 these mutants do not efficiently travel long distances. We used radial locomotion assays
311 (see Materials and Methods) to quantify the locomotion phenotype of *rho-1(gf)* worms.
312 *rho-1(gf) ksr-1* double mutants had an increased radial distance traveled compared to
313 *rho-1(gf)* alone (Figure 3B). These data suggest that KSR-1 acts downstream of or in
314 parallel to the Gq-Rho pathway to regulate locomotion.

315 Since *ksr-1* mutants suppress the exaggerated waveform of both activated Gq
316 and activated Rho animals, we expected that loss of other ERK pathway components
317 would also suppress activated Rho. We made double mutants of activated Rho (*rho-*

318 *1(gf)*) with reduction-of-function alleles of the Ras-ERK pathway and found that
319 mutations in Raf, MEK, and ERK suppressed the *rho-1(gf)* loopy waveform phenotype
320 (Figure 3, A, C, and D). However, the *let-60(n2021)* and *let-60(sy93)* Ras mutations did
321 not suppress the loopy waveform of *rho-1(gf)* worms (Figure 3, A, C, and D). These
322 data suggest that the ERK pathway acts downstream of or in parallel to the Gq-Rho
323 pathway to regulate locomotion, possibly in a Ras-independent manner.

324

325 **The ERK MAPK cascade acts in acetylcholine neurons to control locomotion**

326 Members of the ERK pathway are expressed in neurons in *C. elegans* (Dent and
327 Han 1998; Hunt-Newbury *et al.* 2007), and Gq and Rho act in acetylcholine neurons to
328 promote synaptic release and regulate locomotion (Lackner *et al.* 1999; McMullan *et al.*
329 2006). To determine whether the ERK pathway also acts in neurons to modulate Gq
330 signaling, we expressed the *ksr-1* cDNA under promoters driving expression in specific
331 types of neurons. Single-copy transgenic expression of *ksr-1* under an acetylcholine
332 neuron promoter (*Punc-17*) or under a head acetylcholine neuron promoter (*Punc-17H)*
333 fully reversed the *ksr-1* suppression of the loopy waveform of activated Gq worms
334 (Figure 4). *ksr-1* expression in ventral cord acetylcholine motor neurons (*Punc-17β*) or
335 GABA neurons (*Punc-47)* did not significantly reverse the *ksr-1* suppression of activated
336 Gq (Figure 4). This suggests that ERK signaling primarily functions in the acetylcholine
337 interneurons or motor neurons of the head to modulate Gq-dependent locomotion.

338 We have shown that the ERK pathway is necessary for Gq-dependent effects on
339 locomotion. To determine whether ERK signaling is sufficient to modulate locomotion,

340 we expressed an activated form of *lin-45*/Raf specifically in acetylcholine neurons. Raf
341 kinase activity is regulated via conserved phosphorylation events, and phosphomimetic
342 mutations T626E/T629D in the kinase activation loop of *lin-45* are sufficient to confer
343 constitutive Raf activity (Chong *et al.* 2001). We found that expression of activated Raf
344 in acetylcholine neurons (*Punc-17*) causes a loopy waveform similar to activated Gq
345 and Rho mutants and similar limited dispersal in radial locomotion assays (Figure 5, A
346 and B; Figure S3, A and B). Additionally, expression of activated Raf or activated Gq in
347 head acetylcholine neurons also caused a loopy waveform (Figure S3, A-C).

348 Gq and Rho promote acetylcholine release at the worm neuromuscular junction
349 (Lackner *et al.* 1999; Miller *et al.* 1999; McMullan *et al.* 2006). To determine if Raf
350 activation affects acetylcholine release at the neuromuscular junction, we assayed for
351 sensitivity to the acetylcholinesterase inhibitor aldicarb. Mutants with reduced
352 acetylcholine secretion are resistant to aldicarb, whereas mutants with increased
353 acetylcholine secretion are hypersensitive to aldicarb (Mahoney *et al.* 2006). Activated
354 Gq and Rho mutants have increased rates of paralysis when exposed to aldicarb
355 (Lackner *et al.* 1999; McMullan *et al.* 2006). We found that expression of activated Raf
356 in all acetylcholine neurons or in head acetylcholine neurons also led to aldicarb
357 hypersensitivity, similar to activated Gq and Rho mutants (Figure 5, C and D). However,
358 we found that a *ksr-1* mutation does not suppress the aldicarb hypersensitivity of
359 activated Gq or Rho mutants, and the *ksr-1* mutant on its own has similar aldicarb
360 sensitivity to wild type (Figure 5, E and F). These results suggest that the ERK pathway
361 is not required for synaptic transmission, but is sufficient to stimulate synaptic
362 transmission when constitutively activated. Furthermore, these data suggest that ERK

363 pathway activation in head acetylcholine neurons is sufficient to promote acetylcholine

364 release by motor neurons at the neuromuscular junction.

365

366 **Discussion**

367 In this study we identified KSR-1 and the ERK MAPK cascade as modulators of
368 Gq-Rho signaling. We found that ERK signaling acts in head acetylcholine neurons
369 (interneurons or motor neurons of the head) to modulate Gq-dependent locomotion
370 behavior, especially the waveform of the animal. Our data support the model that Gq-
371 Rho activation of the ERK pathway may be independent of its canonical regulator, the
372 small GTPase Ras/LET-60 (Figure 6).

373 Gq regulates multiple aspects of *C. elegans* locomotion behavior, including the
374 rate of locomotion and waveform. Previous work has shown that these behaviors are at
375 least partially genetically separable, with different pathways downstream of Gq
376 contributing differentially to these behaviors and with Gq likely acting in several classes
377 of neurons including both head acetylcholine neurons and ventral cord acetylcholine
378 motor neurons (Lackner *et al.* 1999; Topalidou *et al.* 2017a). Here we focused on Gq
379 regulation of the waveform, which is strongly dependent on Rho and ERK. The most
380 parsimonious model supported by our findings is that Gq, Rho, and the ERK pathway
381 function cell autonomously in the head acetylcholine neurons to regulate the waveform
382 (Figure 6). Other components of the Gq-Rho pathway have been previously shown to
383 act in head acetylcholine neurons to regulate the waveform (Topalidou *et al.* 2017a; b),
384 and here we show that KSR-1 is required in head acetylcholine neurons to modulate
385 Gq-dependent effects on the waveform. Additionally, expression of activated LIN-45/Raf
386 in head acetylcholine neurons is also sufficient to regulate the waveform and cause
387 loopy locomotion. These data suggest that ERK pathway activity in head acetylcholine
388 neurons is both necessary and sufficient to regulate worm waveform.

389 Though the ERK pathway is required for Gq and Rho-dependent regulation of the
390 waveform, it is not required for the aldicarb hypersensitivity of activated Gq and Rho
391 mutants. This indicates that Gq and Rho regulation of acetylcholine release at the
392 neuromuscular junction is at least partially independent of Gq and Rho regulation of the
393 locomotion waveform. The simplest model is that Gq and Rho signaling regulates the
394 waveform by acting in head acetylcholine neurons through an ERK-dependent pathway,
395 but Gq and Rho signaling regulates synaptic transmission at the neuromuscular junction
396 through an ERK-independent pathway. Overexpression of an activated Gq mutant in
397 ventral cord acetylcholine motor neurons is sufficient to cause an aldicarb
398 hypersensitive phenotype (Lackner *et al.* 1999), suggesting that Gq may act cell
399 autonomously in the ventral cord motor neurons to promote acetylcholine release by
400 these neurons. But as with activated Raf, it is also possible that Gq activity in head
401 acetylcholine neurons may contribute to acetylcholine release by downstream ventral
402 cord motor neurons.
403 The ERK signaling cascade has been well-studied for its regulation of cellular
404 proliferation and differentiation (Sun *et al.* 2015), but also plays important roles in
405 mature neurons and has been associated with synaptic plasticity and memory (Impey *et*
406 *al.* 1999). In addition to activating transcription, ERKs regulate synaptic plasticity both
407 presynaptically and postsynaptically by phosphorylating relevant substrates. ERKs
408 phosphorylate the presynaptic proteins synapsin I and Munc18-1, and postsynaptic
409 proteins such as scaffolds, K_V4.2 potassium channels, and Group I metabotropic
410 glutamate receptors (Jovanovic *et al.* 2000; Thomas and Huganir 2004; Sweatt 2004;
411 Kushner *et al.* 2005; Boggio *et al.* 2007; Vara *et al.* 2009; Mao and Wang 2016a;

412 Schmitz *et al.* 2016). Our findings suggest that the ERK pathway controls Gq-dependent
413 locomotion in *C. elegans* and that activated ERK promotes synaptic transmission.
414 In contrast to many developmental and neuronal roles of Ras-dependent ERK
415 signaling in *C. elegans,* our data suggest that Raf-MEK-ERK signaling may modulate
416 Gq-Rho output independently of Ras/LET-60. One caveat to the conclusion that the
417 ERK pathway acts independently of Ras to control Gq-dependent locomotion is that the
418 alleles of *let-60*/Ras tested here are not null (Han and Sternberg 1990, 1991; Han *et al.*
419 1990; Beitel *et al.* 1990). However, other than *mpk-1(ga117)*, the alleles we used of *lin-*
420 *45*, *mek-2* and *mpk-1* are also not null (Han *et al.* 1993; Kornfeld *et al.* 1995a; Wu *et al.*
421 1995; Lackner and Kim 1998; Hsu *et al.* 2002)**,** yet were able to suppress an activated
422 Gq mutant. Furthermore, the *let*-60/Ras alleles used here have stronger phenotypes in
423 vulval development than the two weak *mek-2*/MEK alleles we used (*n1989* and *ku114*)
424 (Beitel *et al.* 1990; Kornfeld *et al.* 1995a; Wu *et al.* 1995) and the *let-60(n2021)*/Ras
425 mutant has comparable phenotypes to the *lin-45(sy96)*/Raf and *mpk-1(ga117*)/ERK
426 alleles for chemotaxis to odorants and the regulation of foraging behavior (Hirotsu *et al.*
427 2000; Hamakawa *et al.* 2015). Thus, either LET-60/Ras is not required to modulate Gq
428 signaling, or ERK signaling in the locomotor circuit requires a lower threshold of Ras
429 activity and the *let-60* mutants we used have sufficient levels of active Ras to modulate
430 Gq signaling, but not enough for vulval induction or other behaviors.
431 If Ras is not required, how instead might KSR-Raf-MEK-ERK signaling be
432 activated? Normally, active Ras recruits Raf to the plasma membrane but it has been
433 shown that artificial recruitment of Raf to the plasma membrane in the absence of Ras
434 is sufficient to activate Raf signaling (Stokoe *et al.* 1994; Marais *et al.* 1995). Thus,

435 another possible way to activate the ERK pathway would be for Raf to be recruited to
436 the membrane by proteins other than Ras. The most obvious candidates for recruiting
437 Raf are other members of the Ras family such as Rap1 or R-Ras that have a conserved
438 effector-binding domain (Reiner and Lundquist 2016); it has been reported that Rap1
439 can mediate Ras-independent activation of Raf downstream of a Gq-coupled receptor
440 (Guo *et al.* 2001). However, mutations in the worm orthologs of Rap1/RAP-1 and R-
441 Ras/RAS-1 did not suppress the activated Gq mutant, indicating that these Ras-like
442 proteins are not required, at least individually, to activate Raf in this pathway. It is
443 possible that more than one of these Ras family members function redundantly to
444 activate Raf, or that Raf is activated independently of Ras family proteins. There have
445 been other reported cases of Ras-independent activation of the Raf-MEK-ERK pathway
446 (Robbins *et al.* 1992; Honda *et al.* 1994; van Biesen *et al.* 1996; Ueda *et al.* 1996;
447 Drosten *et al.* 2014), some of which involve G protein signaling and protein kinase C,
448 though the precise mechanisms involved are unclear.
449 Gq, Rho, and ERK are also required for the *C. elegans* behavioral and immune
450 response to infection by the bacterium *M. nematophilum* (Nicholas and Hodgkin 2004;
451 McMullan *et al.* 2012). Interestingly, LET-60/Ras was reported to not be required for the
452 immune response (Nicholas and Hodgkin 2004) or only partially required (McMullan *et*
453 *al.* 2012). Additionally, LET-60/Ras was not required for the increased sensitivity to
454 aldicarb caused by bacterial infection (McMullan *et al.* 2012), at least as assayed using
455 the *let-60(n2021*) allele. Thus, the same neuronal Ras-independent ERK pathway we
456 describe here may also modulate Gq signaling in the neuronal response to bacterial
457 infection and possibly the innate immune response as well.

458 Our epistasis analysis suggests that ERK signaling acts genetically downstream
459 of or in parallel to Gq-Rho signaling. How might Gq-Rho signaling lead to ERK
460 activation? ERK activation could occur via a linear pathway downstream of Gq and Rho,
461 or ERK could signal in parallel and converge downstream of Rho to affect neuronal
462 activity (Figure 6). There is precedence for Gq activating ERK via a linear pathway. In
463 the pharyngeal muscle of *C. elegans*, ERK activity is increased by Gq-dependent
464 signaling through protein kinase C (You *et al.* 2006). In the AWC olfactory neuron, ERK
465 activity is increased downstream of Gq signaling via the RasGEF RGEF-1 (Chen *et al.*
466 2011; Uozumi *et al.* 2012). Protein kinase C and RGEF-1 are both activated by DAG,
467 probably produced by the canonical Gq-PLCβ pathway. By contrast, we found that ERK
468 signaling regulates locomotion by modulating the output of the Gq-Rho pathway that
469 acts in parallel to Gq-PLCβ, and does not depend on RGEF-1.
470 How might the ERK pathway modulate neuronal activity downstream of Gq-Rho
471 signaling? Rho regulates neuronal activity and synaptic release through several
472 mechanisms in *C. elegans* neurons, any of which could be targets of ERK signaling.
473 One possible ERK effector is the NCA/NALCN cation channel that acts genetically
474 downstream of Gq-Rho to regulate locomotion rate and waveform (Topalidou *et al.*
475 2017a; b). Though ERK has not been directly connected to NCA/NALCN, mammalian
476 ERK regulates neuronal excitability by directly phosphorylating voltage-gated sodium
477 and potassium channels (Schrader *et al.* 2006; Stamboulian *et al.* 2010) and by
478 regulating channel expression (Yang *et al.* 2015). Given the many possible ways that
479 ERK may regulate excitability or synaptic transmission, *C. elegans* genetics is well
480 suited to determine relevant effectors of Gq-Rho-ERK signaling.
481

482 **Acknowledgements**

483 We thank Brooke Jarvie for the isolation of *ksr-1(yak10)*; Stephen Nurrish for the
484 activated Rho worm strain; Jordan and Jill Hoyt for help with whole-genome sequence
485 analysis; Laura Taylor for help with irradiation of extrachromosomal arrays; Dana Miller
486 for use of her Nikon SMZ18 microscope and camera. Some strains were provided by
487 the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40
488 OD010440). This work was supported by an Ellison Medical Foundation New Scholar
489 Award and by NIH grants R00 MH082109 and R56 NS100843 to M.A.

490 **Figure Legends**

491 **Figure 1.** *ksr-1* mutations suppress activated Gq.

492 (A) A *ksr-1* mutation suppresses the coiled posture of activated Gq. The activated Gq
493 mutant *egl-30(tg26)* has small size and deep body bends. *ksr-1(ok786)* suppresses the
494 exaggerated body bends and small size of *egl-30(tg26)* worms.

495 (B) Gene structure of *ksr-1* locus. Locations of the *egl-30(tg26)* suppressor alleles
496 *ox314* and *yak10* are indicated, as well as the position of the *ok786* deletion. The gene
497 structure was drawn using Exon-Intron Graphic Maker (www.wormweb.org/exonintron)

498 (C) A *ksr-1* mutation suppresses the exaggerated waveform of *egl-30(tg26)* mutants.
499 Straightened images of tracks left in bacterial lawns show similar waveform for wild type
500 and *ksr-1(ok786)* worms. *egl-30(tg26)* mutants have an exaggerated waveform, creating
501 tracks with a large amplitude relative to the period. *ksr-1*(*ok786)* suppresses the
502 exaggerated waveform of the *egl-30(tg26)* mutant.

503 (D) *ksr-1* mutations suppress the *egl-30(tg26)* exaggerated waveform. *ksr-1* nonsense
504 alleles *ox314* and *yak10* and the deletion allele *ok786* strongly but incompletely
505 suppress the *egl-30(tg26)* exaggerated waveform. $N \geq 12$, *** $P < 0.001$, one-way
506 ANOVA with Bonferroni's *post hoc* test. All *egl-30(tg26); ksr-1* double mutants were
507 significantly different than *ksr-1* single mutants ($P<0.05$), indicating that suppression is
508 incomplete.

509

510 **Figure 2**. Mutations in the ERK MAPK pathway suppress activated Gq.

511 (A) Mutations in the MAPKKK *lin-45*/Raf and the MAPK *mpk-1*/ERK suppress the coiled
512 posture of *egl-30*(*tg26*) worms. Partial loss of Ras *let-60* activity does not suppress
513 activated Gq posture.
514 (B) Waveform quantification of ERK pathway mutants. Mutations in *lin-45*, *mek-2*, and
515 *mpk-1* suppress activated Gq *egl-30*(*tg26*) similar to *ksr-1* mutations. $N \geq 12$, *** $P <$
516 0.001, one-way ANOVA with Bonferroni's *post hoc* test.
517 (C) Signaling components upstream of Raf do not suppress activated Gq. Mutations in
518 the EGF ligand (*lin-3*) or the EGFR (*let-23*) do not affect the exaggerated waveform of
519 *egl-30*(*tg26*) animals. The Ras partial loss-of-function mutation *let-60(n2021)* does not
520 suppress activated Gq waveform. $N \geq 12$, *** $P < 0.001$, one-way ANOVA with
521 Bonferroni's *post hoc* test.
522 (D) The *let-60(sy93)* dominant negative mutation in Ras does not suppress activated Gq
523 waveform. $N \geq 13$, n.s., not significant, one-way ANOVA with Bonferroni's *post hoc* test.
524
525 **Figure 3.** Mutations in *ksr-1* and the ERK pathway suppress activated Rho.
526 (A) Mutations in ERK pathway genes *ksr-1*, *lin-45*, *mek-2*, and *mpk-1* visibly suppress
527 the coiled posture of animals expressing an activated Rho mutant (G14V) in
528 acetylcholine neurons (*nzIs29[Punc-17::rho-1(gf)]*). Reduction of LET-60/Ras activity
529 does not suppress activated Rho.
530 (B) The *ksr-1*(*ok786*), *ksr-1*(*ox314*), and *ksr-1*(*yak10*) mutations suppress the
531 locomotion of activated Rho animals as shown by radial locomotion assays. $N \geq 38$, ***
532 $P < 0.001$, ** $P < 0.01$, Kruskal-Wallis test with Dunn's *post hoc* test.

533 (C) Mutations in *lin-45* and *mek-2* suppress the locomotion defect of activated Rho
534 animals as shown by radial locomotion assays. The *let-60*(*n2021*) mutation does not
535 significantly suppress activated Rho locomotion. $N \geq 50$, *** $P < 0.001$, Kruskal-Wallis
536 test with Dunn's *post hoc* test.
537 (D) Waveform quantification of ERK pathway mutants. Mutations in *ksr-1*, *lin-45*, *mek-2*,
538 and *mpk-1* show similar suppression of activated Rho, but the Ras reduction-of-function
539 mutant *let-60(n2021)* or dominant negative *let-60(sy93)* do not suppress activated Rho.
540 N ≥ 14, * P < 0.05, n.s., not significant, one-way ANOVA with Bonferroni's *post hoc* test,
541 compared to *rho-1(gf)*.
542
543 **Figure 4.** KSR-1 acts in head acetylcholine neurons to modulate Gq signaling.
544 Single-copy expression of the *ksr-1* cDNA exclusively in acetylcholine neurons (*Punc-*
545 *17*, *yakSi26* transgene) or head acetylcholine neurons (*Punc-17H, yakSi27* transgene)
546 is sufficient to reverse the *ksr-1* suppression of the loopy waveform of activated Gq
547 animals. *ksr-1* expression in ventral cord acetylcholine neurons (*Punc-17β*, *yakSi28*
548 transgene) or GABA neurons (*Punc-47*, *yakSi29* transgene) does not reverse the *ksr-1*
549 suppression of the activated Gq exaggerated waveform. $N \geq 12$, *** $P < 0.001$, ** $P <$
550 0.01, n.s., not significant, Kruskal-Wallis test with Dunn's *post hoc* test.
551
552 **Figure 5.** Increased Raf activity in acetylcholine neurons is sufficient to regulate
553 locomotion and increase acetylcholine release.
554 (A) Transgenic lines expressing an activated form of *lin-45*/Raf (T626E/T629D) in
555 acetylcholine neurons (*yakIs34[Punc-17::lin-45(gf)]*) have exaggerated body bends and

556 coiled posture similar to the activated Gq mutant *egl-30*(*tg26*) and to animals expressing
557 activated Rho in acetylcholine neurons (*nzIs29[Punc-17::rho-1(gf)]*). The wild type and
558 *egl-30(tg26)* photos are the same as shown in Figure 1A, and the *rho-1(gf)* photo is the
559 same as the one in Figure 3A.
560 (B) Expression of activated Rho (*nzIs29[Punc-17::rho-1(gf)]*) or Raf (*yakIs34[Punc-*
561 *17::lin-45(gf)]*) in acetylcholine neurons impairs coordinated locomotion similarly to
562 activated Gq (*egl-30*(*tg26*)). $N \geq 19$, * $P < 0.05$, *** $P < 0.001$, Kruskal-Wallis test with
563 Dunn's *post hoc* test.
564 (C) Animals expressing activated Gq, Rho, or Raf are hypersensitive to the
565 acetylcholinesterase inhibitor aldicarb. Activated Gq (*egl-30*(*tg26*)), activated Rho
566 expressed in acetylcholine neurons (*nzIs29[Punc-17::rho-1(gf)]*), and activated Raf
567 expressed in acetylcholine neurons *yakIs34[Punc-17::lin-45(gf)]*) become paralyzed
568 significantly faster than wild type animals when exposed to 1 mM aldicarb. All strains
569 are significantly different from wild type at t = 60, 75, 90, and 105 minutes. $N \geq 61$, *** P
570 < 0.001, two-way ANOVA with Bonferroni's *post hoc* test.
571 (D) Animals expressing activated Raf in head acetylcholine neurons are hypersensitive
572 to aldicarb. The *yakIs34[Punc-17::lin-45(gf)]* integrated array expressing activated Raf in
573 all acetylcholine neurons and the *yakEx154[Punc-17H::lin-45(gf)]* (#1) and
574 *yakEx168[Punc-17H::lin-45(gf)]* (#2) extrachromosomal arrays expressing activated Raf
575 in head acetylcholine neurons show similar hypersensitivity to aldicarb. All strains are
576 significantly different from wild type at t = 60, 75, 90, and 105 minutes. $N \geq 47$, ** $P <$
577 0.01, two-way ANOVA with Bonferroni's *post hoc* test.

578 (E) *ksr-1* is not required for the aldicarb hypersensitivity of activated Gq. Paralysis of
579 *ksr-1*(*ok786*) animals on 1 mM aldicarb is not significantly different from wild type. The
580 *ksr-1* deletion *ok786* does not suppress the aldicarb hypersensitivity of activated Gq
581 (*egl-30*(*tg26*)). $N \geq 53$.
582 (F) *ksr-1* is not required for the aldicarb hypersensitivity of activated Rho. Paralysis
583 of *ksr-1(ok786)* animals on 1 mM aldicarb is not significantly different from wild type.
584 The *ksr-1* deletion *ok786* does not suppress the aldicarb hypersensitivity of the
585 activated Rho mutant *nzIs29[Punc-17::rho-1(gf)]*. N ≥ 41.
586
587 **Figure 6.** Model for Gq-Rho-ERK signaling. Gq directly activates Trio and Rho (solid
588 arrow) (Lutz et al. 2007). The core ERK cascade acts either downstream of Rho or in
589 parallel (dashed arrows) to modulate locomotion behavior. ERK activation occurs
590 independently of the extracellular growth factor LIN-3, its receptor LET-23, or Ras/LET-
591 60. It is possible that Raf/LIN-45 is activated by a Ras family member other than the
592 canonical worm Ras/LET-60. Our model suggests that Gq-Rho signaling regulates the
593 worm waveform by acting cell autonomously in head acetylcholine neurons in an ERK-
594 dependent pathway. By contrast, we propose that Gq-Rho signaling regulates synaptic
595 transmission at the neuromuscular junction by acting in an ERK-independent pathway,
596 possibly in the ventral cord motorneurons themselves.
597
598 **Figure S1.** Additional mutations in *mek-2* and *mpk-1* suppress activated Gq.
599 (A) The *mek-2(ku114)* mutation suppresses the exaggerated waveform of *egl-30*(*tg26*).
600 $N \geq 14$, *** $P < 0.001$, one-way ANOVA with Bonferroni's *post hoc* test.

601 (B) The *mpk-1(oz140)* mutation suppresses the exaggerated waveform of *egl-30*(*tg26*).
602 *N* ≥ 12, *** *P* < 0.001, one-way ANOVA with Bonferroni's *post hoc* test.
603

604 **Figure S2.** The exaggerated waveform of activated Gq does not depend on the
605 RasGEFs *sos-1* and *rgef-1*, or the Ras family members *ras-1*, *ras-2*, and *rap-1*.
606 (A) Deletion mutations *ras-1(gk243)* and *ras-2(ok682),* or the nonsense mutation *rap-*
607 *1(pk2082)* do not suppress the waveform of the activated Gq mutant *egl-30(tg26)*. *N* ≥
608 18, n.s., not significant, one-way ANOVA with Bonferroni's *post hoc* test.
609 (B) Loss of the RasGEF *sos-1* does not suppress activated Gq waveform. Animals
610 carrying the temperature sensitive *sos-1(cs41)* mutation were incubated for 24 hours at
611 the non-permissive temperature of 25° and assayed for their waveform. *N* ≥ 15, n.s., not
612 significant, one-way ANOVA with Tukey's *post hoc* test.
613 (C) The RasGEF deletion mutation *rgef-1(ok675)* does not suppress the exaggerated
614 waveform of activated Gq. *N* = 16, n.s., not significant, one-way ANOVA with Tukey's
615 *post hoc* test.
616 (D) Neuronal expression of the dominant negative Ras mutant S17N (*yakEx158[Prab-*
617 *3::let-60(S17N)]*) does not suppress the coiled posture of the activated Gq mutant *egl-*
618 *30(tg26)*.
619

620 **Figure S3.** Expression of activated LIN-45/Raf or EGL-30/Gq in head acetylcholine
621 neurons is sufficient to cause locomotion with an exaggerated waveform.
622 (A, B) The *yakIs34[Punc-17::lin-45(gf)]* integrated array expressing activated *lin-*
623 *45*(T626E/T629D) in all acetylcholine neurons and the *yakEx154[Punc-17H::lin-45(gf)]*

624 extrachromosomal array expressed in head acetylcholine neurons both cause a coiled
625 posture (A) and exaggerated waveform (B). $N \geq 12$, *** $P < 0.001$, * $P < 0.05$, Kruskal-
626 Wallis test with Dunn's *post hoc* test.
627 (C) Expression of the activated Gq mutant *egl-30(tg26)* R243Q in head acetylcholine
628 neurons (*Punc-17H::egl-30(tg26)*) causes an exaggerated waveform. N ≥ 7, ** P < 0.01,
629 unpaired T test, two-tailed. Overexpression of activated Gq is toxic and these animals
630 were slow growing and had reduced viability and fertility.
631

632 **Table S1. List of strains**

633 N2	Bristol wild strain
634 XZ1151	*egl-30(tg26) I*
635 EG6699	*ttTi5605 II; unc-119(ed3) III*
636 EG6207	*unc-119(ed3) III*
637 RB915	*ksr-1(ok786) X*
638 EG4782	*nzIs29[Punc-17::rho-1(G14V), unc-122::gfp] II*
639 MT4866	*let-60(n2021) IV*
640 PS436	*let-60(sy93) IV*
641 PS427	*lin-45(sy96) IV*
642 MT8666	*mek-2(n1989) I*
643 SD378	*mpk-1(ga117) / dpy-17(e164) unc-79(e1068) III*
644 CB1417	*lin-3(e1417) IV*
645 PS5131	*let-23(sy12) / mIn1[dpy-10(e128) mIs14] II*
646 UP604	*sos-1(cs41) V*
647 VC450	*ras-1(gk243) II*
648 RB852	*ras-2(ok682) III*
649 TZ181	*rap-1(pk2082) IV*
650 RB848	*rgef-1(ok675) V*
651 MH538	*mek-2(ku114) I; let-60(n1046) IV*
652 MT8186	*mpk-1(oz140)/unc-79(e1068) dpy-17(e164) III*

653

654 **The following strains were produced during this study:**

655 EG314	*ksr-1(ox314) X*
656 XZ10	*ksr-1(yak10) X*
657 EG4198	*egl-30(tg26) I ; ksr-1(ox314) X*
658 XZ1340	*egl-30(tg26) I ; ksr-1(yak10) X*
659 XZ1511	*egl-30(tg26) I; ksr-1(ok786) X*

660 XZ1465 *nzIs29[Punc-17::rho-1(G14V), unc-122::gfp] II; ksr-1(ox314) X*

661 XZ1547 *nzIs29[Punc-17::rho-1(G14V), unc-122::gfp] II; ksr-1(yak10) X*

662 XZ2042 *nzIs29[Punc-17::rho-1(G14V), unc-122::gfp] II; ksr-1(ok786) X*

663 XZ1615 *egl-30(tg26) I; let-60(n2021) / nT1[qIs51(Pmyo-2::GFP; Ppes-10::GFP; F22B7.9::GFP)] IV*

664 XZ1626 *egl-30(tg26) I; lin-3(e1417) IV*

665 XZ1630 *egl-30(tg26) I; let-23(sy12) / mIn1[dpy-10(e128) mIs14] II*

666 XZ1677 *nzIs29[Punc-17::rho-1(G14V), unc-122::gfp] II; let-60(n2021) / nT1[qIs51(Pmyo-2::GFP;*

667 *Ppes-10::GFP; F22B7.9::GFP)] IV*

668 XZ1548 *egl-30(tg26) I; lin-45(sy96) IV*

669 XZ1556 *nzIs29[Punc-17::rho-1(G14V), unc-122::gfp] II; lin-45(sy96) IV*

670 XZ1850 *egl-30(tg26) mek-2(n1989) / hT2[bli-4(e937) let(q782) qIs48] I*

671 XZ1851 *mek-2(n1989) / hT2[bli-4(e937) let(q782) qIs48] I; nzIs29[Punc-17::rho-1(G14V), unc-*

672 *122::gfp] II*

673 XZ1700 *mpk-1(ga117) / qC1[dpy-19(e1259) glp-1(q339) qIs26(Plag-2::GFP, rol-6(su1006))] III*

674 XZ1668 *egl-30(tg26) I; mpk-1(ga117) / qC1[dpy-19(e1259) glp-1(q339) qIs26(Plag-2::GFP, rol-*

675 *6(su1006))] III*

676 XZ2043 *nzIs29[Punc-17::rho-1(G14V), unc-122::gfp] II; mpk-1(ga117) / qC1[dpy-19(e1259) glp-*

677 *1(q339) qIs26(Plag-2::GFP, rol-6(su1006))] III*

678 XZ2077 *egl-30(tg26) I; let-60(sy93) IV*

679 XZ2106 *egl-30(tg26) I; let-60(sy93) / nT1[qIs51(Pmyo-2::GFP; Ppes-10::GFP; F22B7.9::GFP)] IV*

680 XZ2150 *nzIs29[Punc-17::rho-1(G14V), unc-122::gfp] II; let-60(sy93) / nT1[qIs51(Pmyo-2::GFP;*

681 *Ppes-10::GFP; F22B7.9::GFP)] IV*

682 XZ2158 *egl-30(tg26) I; yakEx158[Prab-3::let-60(S17N)::tbb-2 3'UTR::OPERON::GFP, Pmyo-*

683 *2::mCherry, Pmyo-3::mCherry]*

684 XZ1855 *egl-30(tg26) I; ras-1(gk243) II*

685 XZ1856 *egl-30(tg26) I; ras-2(ok682) III*

686 XZ2045 *egl-30(tg26) I; rap-1(pk2082) IV*

687 XZ2046 *egl-30(tg26) I; sos-1(cs41) V*

688 XZ2101 *egl-30(tg26) I; rgef-1(ok675) V*

689 XZ1921 *egl-30(tg26) sur-6(sv30) / hT2[bli-4(e937) let(q782) qIs48] I*

690 XZ1857 *egl-30(tg26) I ; sur-7(ku119) X*

691 XZ1854 *nzIs29[Punc-17::rho-1(G14V), unc-122::gfp] II; ras-2(ok682) III*

692 XZ1852 *nzIs29[Punc-17::rho-1(G14V), unc-122::gfp] II; rap-1(pk2082) IV*

693 XZ1880 *yakSi26[Punc-17::ksr-1 cDNA::tbb-2 3'UTR::OPERON::GFP] II*

694 XZ1881 *yakSi27[Punc-17H::ksr-1 cDNA::tbb-2 3'UTR::OPERON::GFP] II*

695 XZ1882 *yakSi28[Punc-17β::ksr-1 cDNA::tbb-2 3'UTR::OPERON::GFP] II*

696 XZ1883 *yakSi29[Punc-47::ksr-1 cDNA::tbb-2 3'UTR::OPERON::GFP] II*

697 XZ1884 *egl-30(tg26) I; yakSi26[Punc-17::ksr-1 cDNA::tbb-2 3'UTR::OPERON::GFP] II; ksr-1(ok786)*

698 *X*

699 XZ1885 *egl-30(tg26) I; yakSi27[Punc-17H::ksr-1 cDNA::tbb-2 3'UTR::OPERON::GFP] II; ksr-*

700 *1(ok786) X*

701 XZ1946 *egl-30(tg26) I; yakSi28[Punc-17β::ksr-1 cDNA::tbb-2 3'UTR::OPERON::GFP] II; ksr-*

702 *1(ok786) X*

703 XZ1947 *egl-30(tg26) I; yakSi29[Punc-47::ksr-1 cDNA::tbb-2 3'UTR::OPERON::GFP] II; ksr-1(ok786)*

704 *X*

705 XZ2015 *unc-119(ed3) III; yakEx122[Punc-17::lin-45* cDNA::tbb-2 3'UTR::OPERON::GFP, Pmyo-*

706 *3::mCherry]*

707 XZ2050 *unc-119(ed3) III; yakIs34[Punc-17::lin-45* cDNA::tbb-2 3'UTR::OPERON::GFP, Pmyo-*

708 *3::mCherry] X*

709 XZ2130 *egl-30(tg26) I; mpk-1(oz140) / oxTi619[Peft-3::TdTomato::H2B cb-unc-119(+)] III*

710 XZ2131 *egl-30(tg26) mek-2(ku114) I / hT2[bli-4(e937) let(q782) qIs48] I;III*

711 XZ2119 *ttTi5605 II; unc-119(ed3) III; yakEx154[Punc-17H::lin-45*::tbb-2 3'UTR::OPERON::GFP,*

712 *Pmyo-2::mCherry, Pmyo-3::mCherry]*

713 XZ2154 *yakEx168[Punc-17H::lin-45*::tbb-2 3'UTR::OPERON::GFP, Pmyo-2::mCherry, Pmyo-*

714 *3::mCherry]*

715 XZxxxx *yakEx[Punc-17H::egl-30(tg26), Pmyo-2::mCherry]* (no strain or allele # given because strain
716 was not viable enough to be frozen)
717
718 **Table S2. List of plasmids**
719 Gateway destination vectors
720 pCFJ150 Gateway destination vector for insertion at chr II Mos site ttTi5605
721
722 Gateway entry clones
723 pEGB05 *Prab-3* [4-1] (pan-neuronal promoter. 1207 bp of the *rab-3* promoter upstream of the
724 ATG)
725 pGH1 *Punc-17* [4-1] (acetylcholine neuron promoter. 3229 bp of the *unc-17* promoter upstream
726 of and including the ATG)
727 pADA180 *Punc-17H* [4-1] (head acetylcholine neuron promoter. derived from the *unc-17* promoter
728 of pGH1, carrying a 127 bp deletion that removes the nerve cord β enhancer)
729 pMA23 *Punc-17β* [4-1] (body acetylcholine neuron promoter. a fusion of the *unc-17* nerve cord β
730 enhancer to the minimal *unc-17* promoter consisting of the 382 bp upstream of and including the ATG)
731 pMH522 *Punc-47* [4-1] (GABA neuron promoter. 1254 bp of the *unc-47* promoter upstream of and
732 including the ATG)
733 pBC11 *ksr-1* cDNA [1-2]
734 pBC26 *lin-45* cDNA [1-2]
735 pBC35 *lin-45** (T626E/T629D) cDNA [1-2]
736 pBC48 *let-60(S17N)* cDNA
737 pET97 *egl-30(tg26)* cDNA [1-2]
738 pADA126 *let-858* 3'UTR [2-3]
739 pCFJ326 *tbb-2* 3'UTR::OPERON::*GFP* [2-3]
740
741 Gateway expression constructs
742 pBC13 *Prab-3::ksr-1* cDNA::*tbb-2* 3'UTR::OPERON::GFP_pCFJ150

743 pBC31 *Punc-17::ksr-1* cDNA*::tbb-2* 3'UTR::OPERON::GFP_pCFJ150

744 pBC32 *Punc-17H*::*ksr-1* cDNA::*tbb-2* 3'UTR::OPERON::GFP_pCFJ150

745 pBC33 *Punc-17β*::*ksr-1* cDNA::*tbb-2* 3'UTR::OPERON::GFP_pCFJ150

746 pBC34 *Punc-47*::*ksr-1* cDNA::*tbb-2* 3'UTR::OPERON::GFP_pCFJ150

747 pBC37 *Punc-17::lin-45** cDNA*::tbb-2* 3'UTR::OPERON::GFP_pCFJ150

748 pBC44 *Punc-17H::lin-45** cDNA*::tbb-2* 3'UTR::OPERON::GFP_pCFJ150

749 pBC50 *Prab-3::let-60(S17N)* cDNA::*tbb-2* 3'UTR::OPERON::GFP_pCFJ150

750 pET102 *Punc-17H::egl-30(tg26)* cDNA*::let-858* 3'UTR_pCFJ150

751

752 **Table S3. List of primers**

753 oBC011 Forward *ksr-1* cDNA for Gateway [1-2] ENTR vector

754 GGGGACAAGTTTGTACAAAAAAGCAGGCTCAatgatgcaaacccaagttgc

755 oBC012 Reverse *ksr-1* cDNA for Gateway [1-2] ENTR vector

756 GGGGACCACTTTGTACAAGAAAGCTGGGTGaaatgtcgactcgtaacttttcatc

757 oBC085 Forward *lin-45* cDNA for Gateway [1-2] ENTR vector

758 GGGGACAAGTTTGTACAAAAAAGCAGGCTcaATGAGTCGGATTAATTTCAAAAAG

759 oBC086 Reverse *lin-45* cDNA for Gateway [1-2] ENTR vector

760 GGGGACCACTTTGTACAAGAAAGCTGGGTgCTAAATGAGACCATAGACATTGTAGTATG

761 oBC094 Reverse *lin-45* T626E mutagenesis

762 gttcactgtccatttcgttttgacctctgccaagccgaaatctccaatttt

763 oBC095 Forward *lin-45* T626E mutagenesis

764 aaaattggagatttcggcttggcagaggtcaaaacgaaatggacagtgaac

765 oBC096 Reverse *lin-45* T629D mutagenesis

766 cctccgttcactgtccatttatctttgacctctgccaagccga

767 oBC097 Forward *lin-45* T629D mutagenesis

768 tcggcttggcagaggtcaaagataaatggacagtgaacggagg

769 oBC105 Forward *let-60* cDNA for Gateway [1-2] ENTR vector

770 GGGGACAAGTTTGTACAAAAAAGCAGGCTCAatgacggagtacaagcttgtgg

771 oBC106 Reverse *let-60* cDNA for Gateway [1-2] ENTR vector

772 GGGGACCACTTTGTACAAGAAAGCTGGGTGtcacattatttgacacttcttcttcttttg

773 oBC109 Reverse *let-60* S17N mutagenesis

774 ttgaatggtgagtgcatttttaccaactcctccatctccaactacca

775 oBC110 Forward *let-60* S17N mutagenesis

776 tggtagttggagatggaggagttggtaaaaatgcactcaccattcaa

777 oET252 Forward *egl-30* cDNA for Gateway [1-2] ENTR vector

778 GGGGACAAGTTTGTACAAAAAAGCAGGCTcaATGGCCTGCTGTTTATCCG

779 oET253 Reverse *egl-30* cDNA for Gateway [1-2] ENTR vector

780 GGGGACCACTTTGTACAAGAAAGCTGGGTgTTACACCAAGTTGTACTCCTTCAG

781 oET254 Forward *egl-30(tg26)* R243Q mutagenesis

782 TGTGACAACGAGAACCGAATGGAAGAATCGAAAGC

783 oET255 Reverse *egl-30(tg26)* R243Q mutagenesis

784 GCTTTCGATTCTTCCATTCGGTTCTCGTTGTCACA

785

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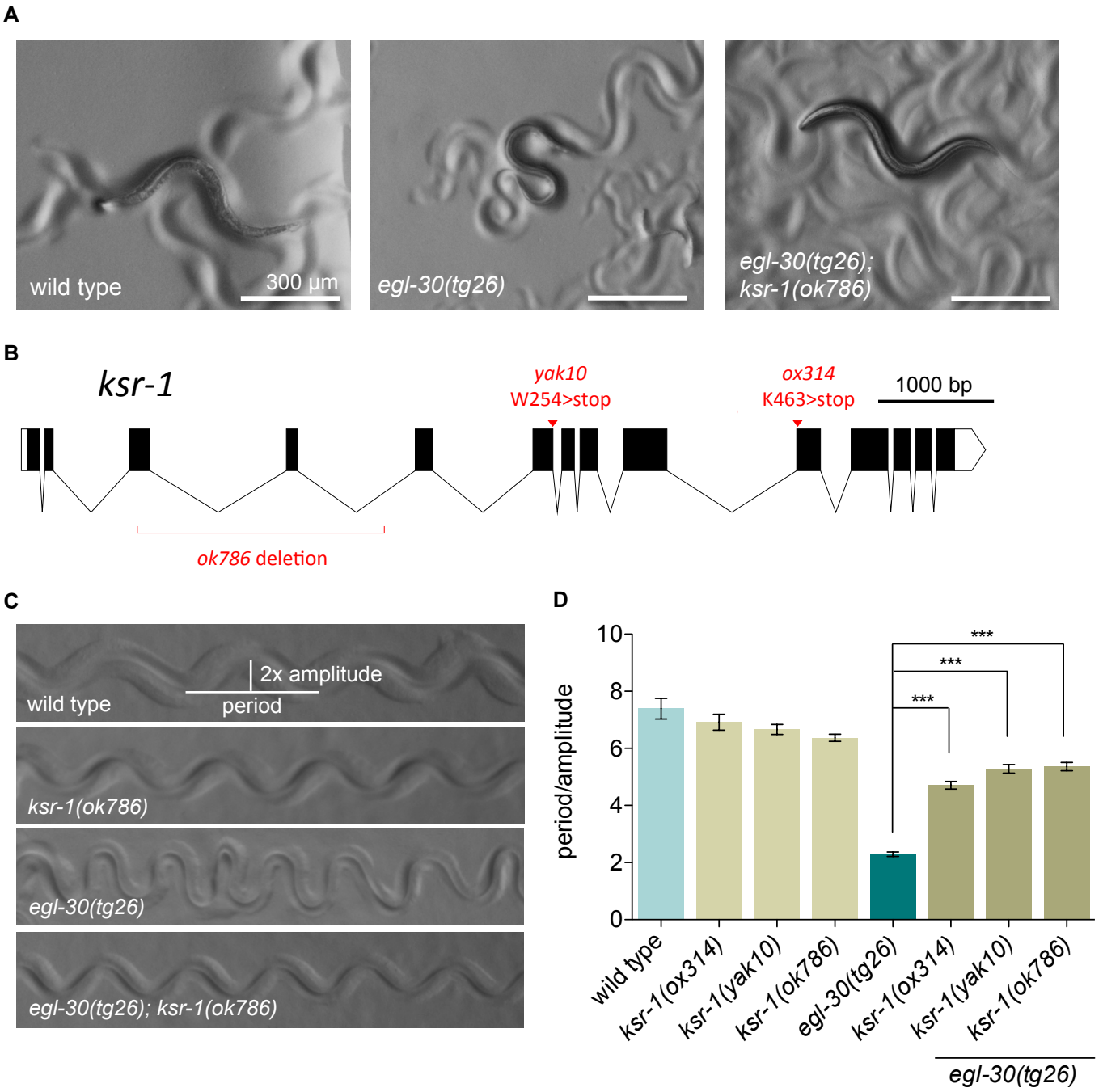
1020

Figure 1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yak10
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ox314
K463>stop
1000 bp
ok786 deletion
C
2x amplitude
period
wild type
ksr-1(ok786)
egl-30(tg26)
egl-30(tg26); ksr-1(ok786)
D
period/amplitude
0
2
4
6
8
10
***
***
***
wild type
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ksr-1(yak10)
ksr-1(ok786)
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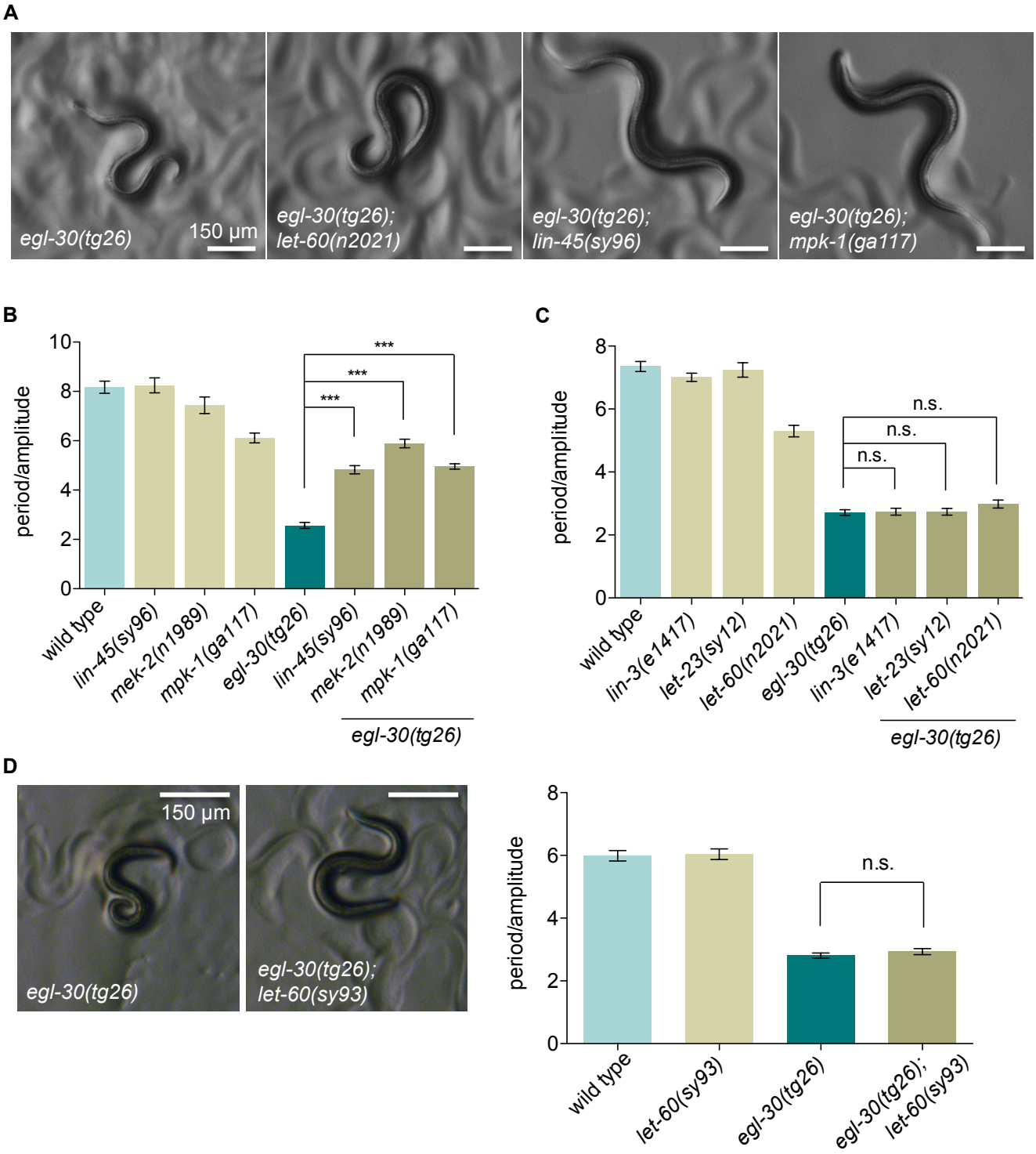
egl-30(tg26)

Figure 2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mpk-1(ga117)
B
period/amplitude
0
2
4
6
8
10
***
***
***
wild type
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mek-2(n1989)
mpk-1(ga117)
egl-30(tg26)
lin-45(sy96)
mek-2(n1989)
mpk-1(ga117)
egl-30(tg26)
C
period/amplitude
0
2
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n.s.
n.s.
n.s.
wild type
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let-60(n2021)
egl-30(tg26)
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let-23(sy12)
let-60(n2021)
egl-30(tg26)
D
150 µm
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let-60(sy93)
period/amplitude
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2
4
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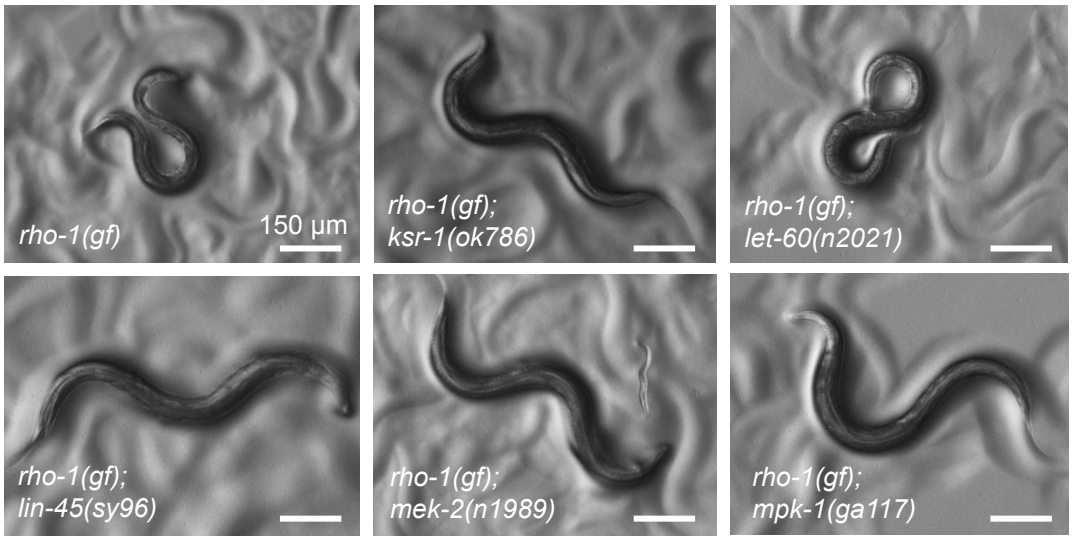
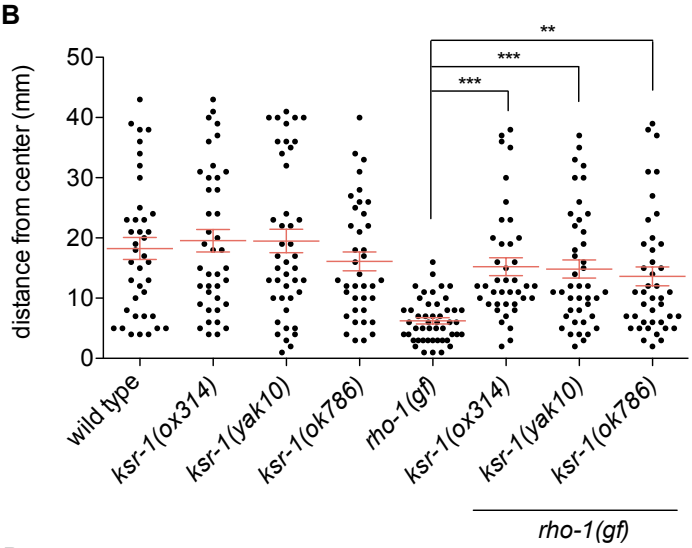
Figure 3

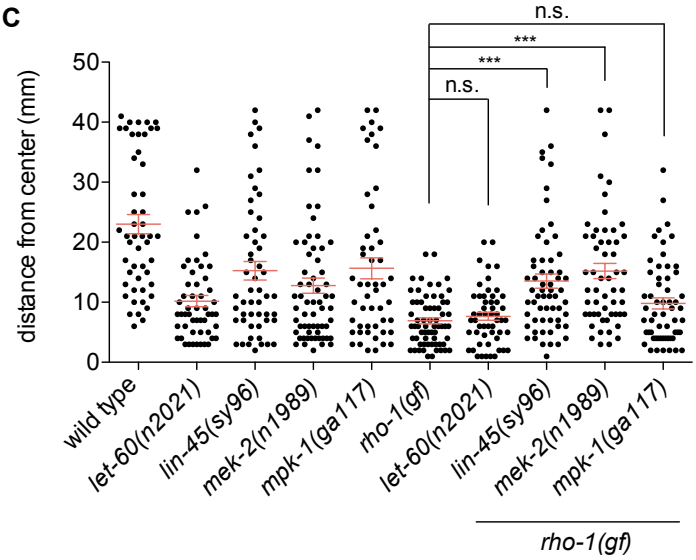
A



B



C



D

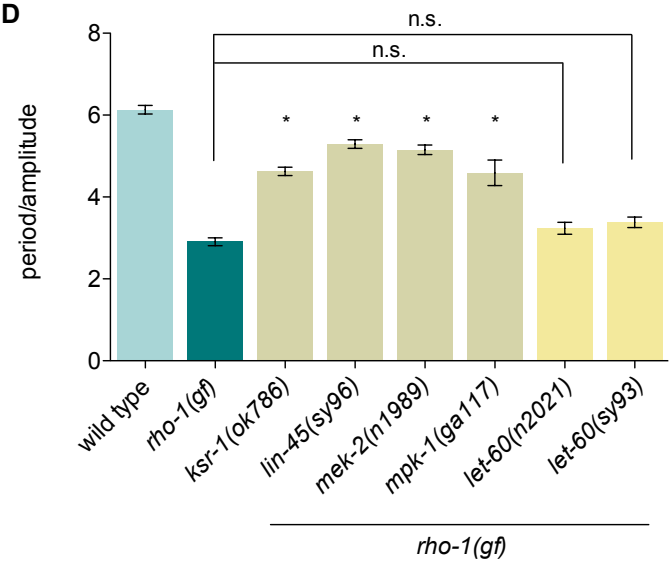


Figure 4

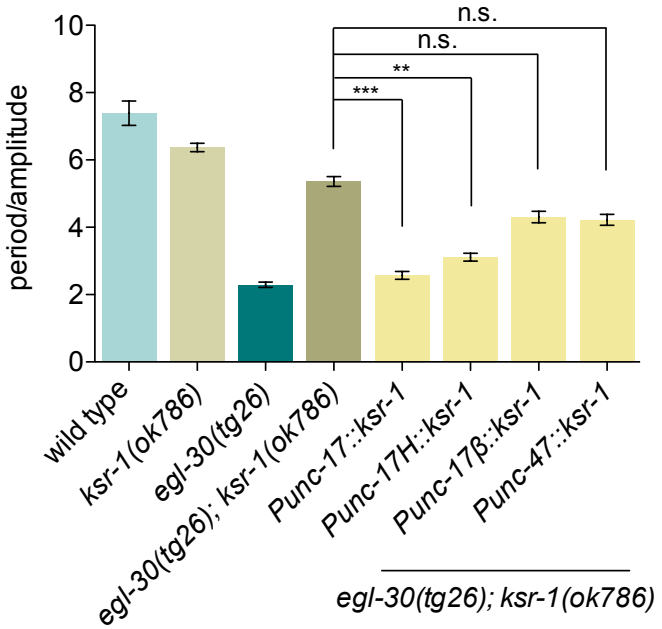

n.s.
n.s.
**
***
10
8
6
4
2
0
period/amplitude
wild type
ksr-1(ok786)
egl-30(tg26)
egl-30(tg26); ksr-1(ok786)
Punc-17::ksr-1
Punc-17H::ksr-1
Punc-17β::ksr-1
Punc-47::ksr-1
egl-30(tg26); ksr-1(ok786)

Figure 5

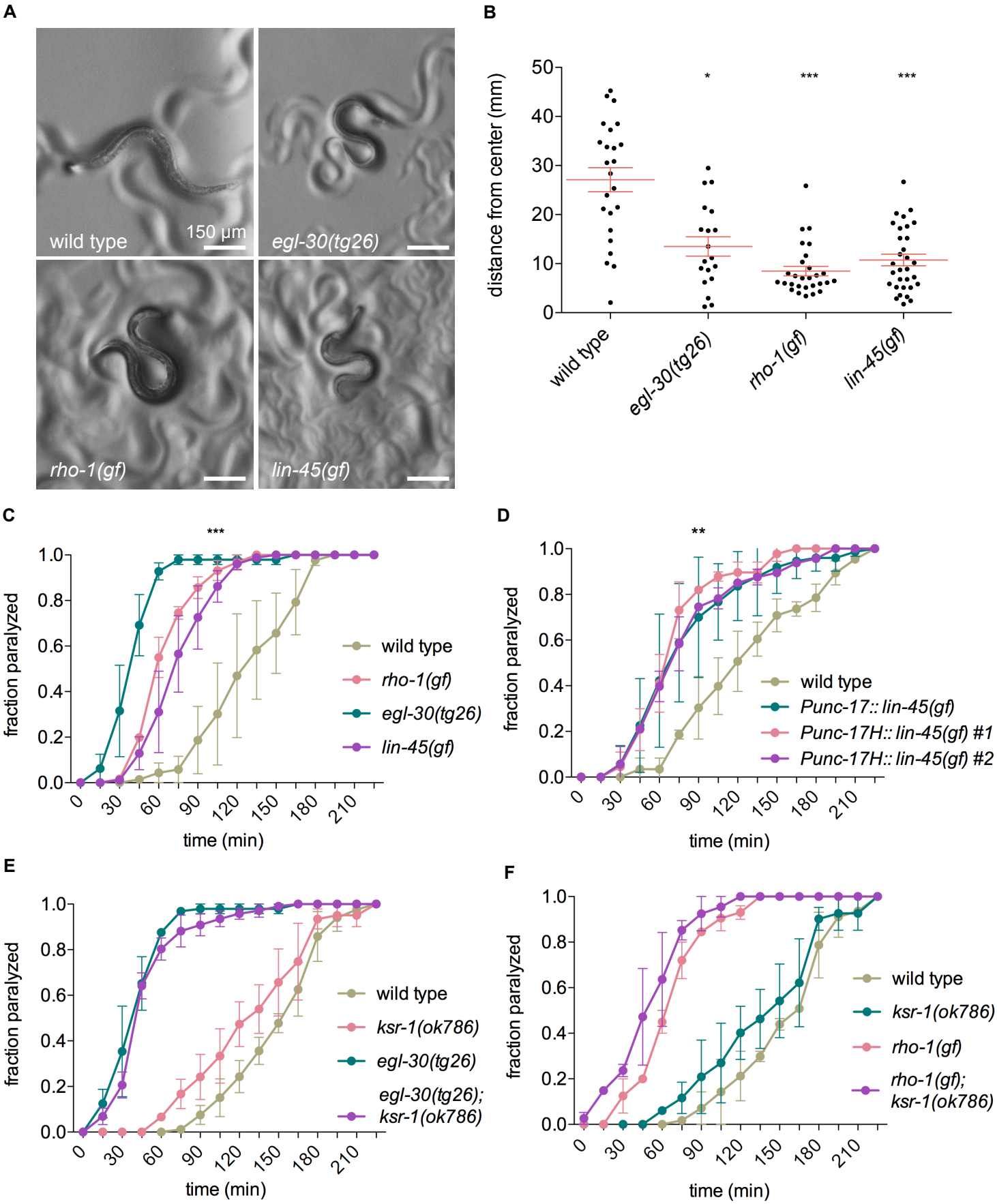

A
wild type
150 µm
egl-30(tg26)
rho-1(gf)
lin-45(gf)
B
distance from center (mm)
50
40
30
20
10
0
*
***
***
wild type
egl-30(tg26)
rho-1(gf)
lin-45(gf)
C
***
fraction paralyzed
1.0
0.8
0.6
0.4
0.2
0.0
0
30
60
90
120
150
180
210
time (min)
wild type
rho-1(gf)
egl-30(tg26)
lin-45(gf)
D
**
fraction paralyzed
time (min)
wild type
Punc-17:: lin-45(gf)
Punc-17H:: lin-45(gf) #1
Punc-17H:: lin-45(gf) #2
E
fraction paralyzed
time (min)
wild type
ksr-1(ok786)
egl-30(tg26)
egl-30(tg26);
ksr-1(ok786)
F
fraction paralyzed
time (min)
wild type
ksr-1(ok786)
rho-1(gf)
rho-1(gf);
ksr-1(ok786)

Figure 6

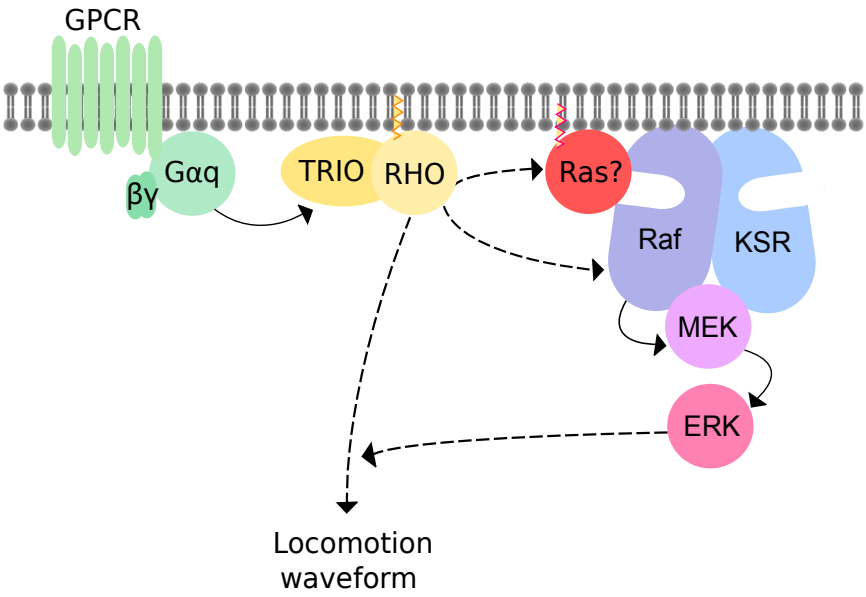


Figure S1

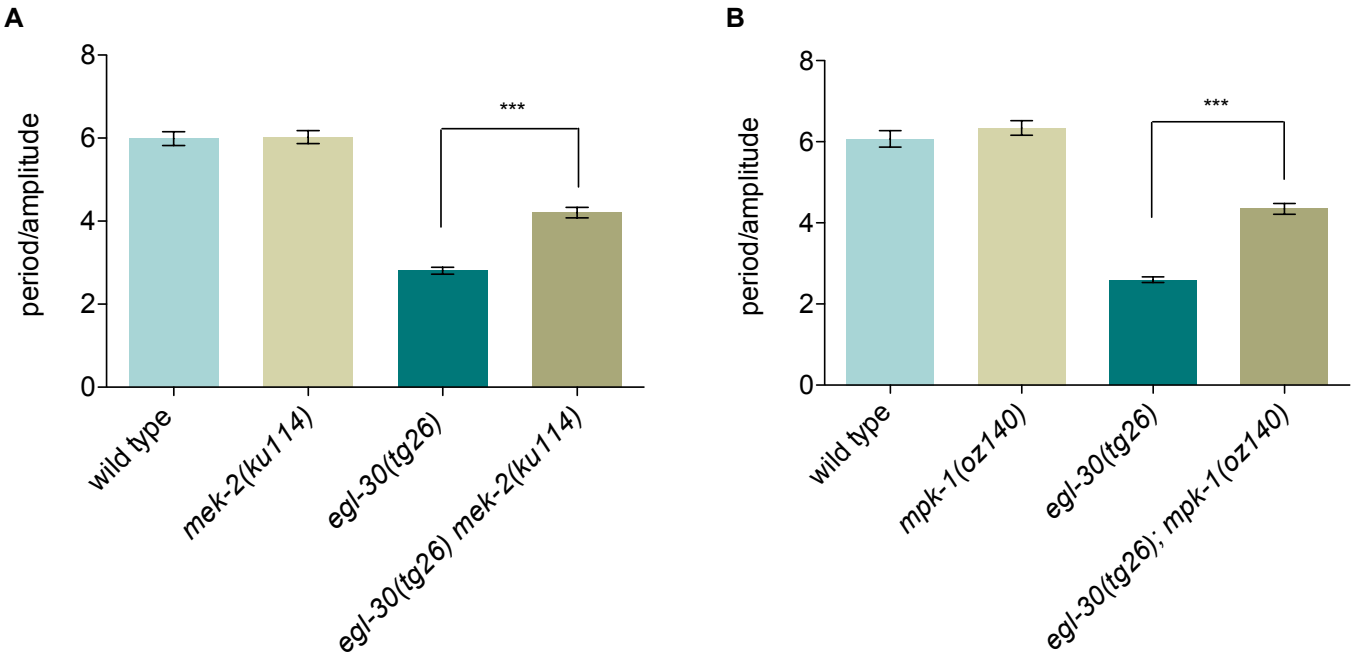

A
period/amplitude
8
6
4
2
0
***
wild type
mek-2(ku114)
egl-30(tg26)
egl-30(tg26) mek-2(ku114)
B
period/amplitude
8
6
4
2
0
***
wild type
mpk-1(oz140)
egl-30(tg26)
egl-30(tg26); mpk-1(oz140)

Figure S2

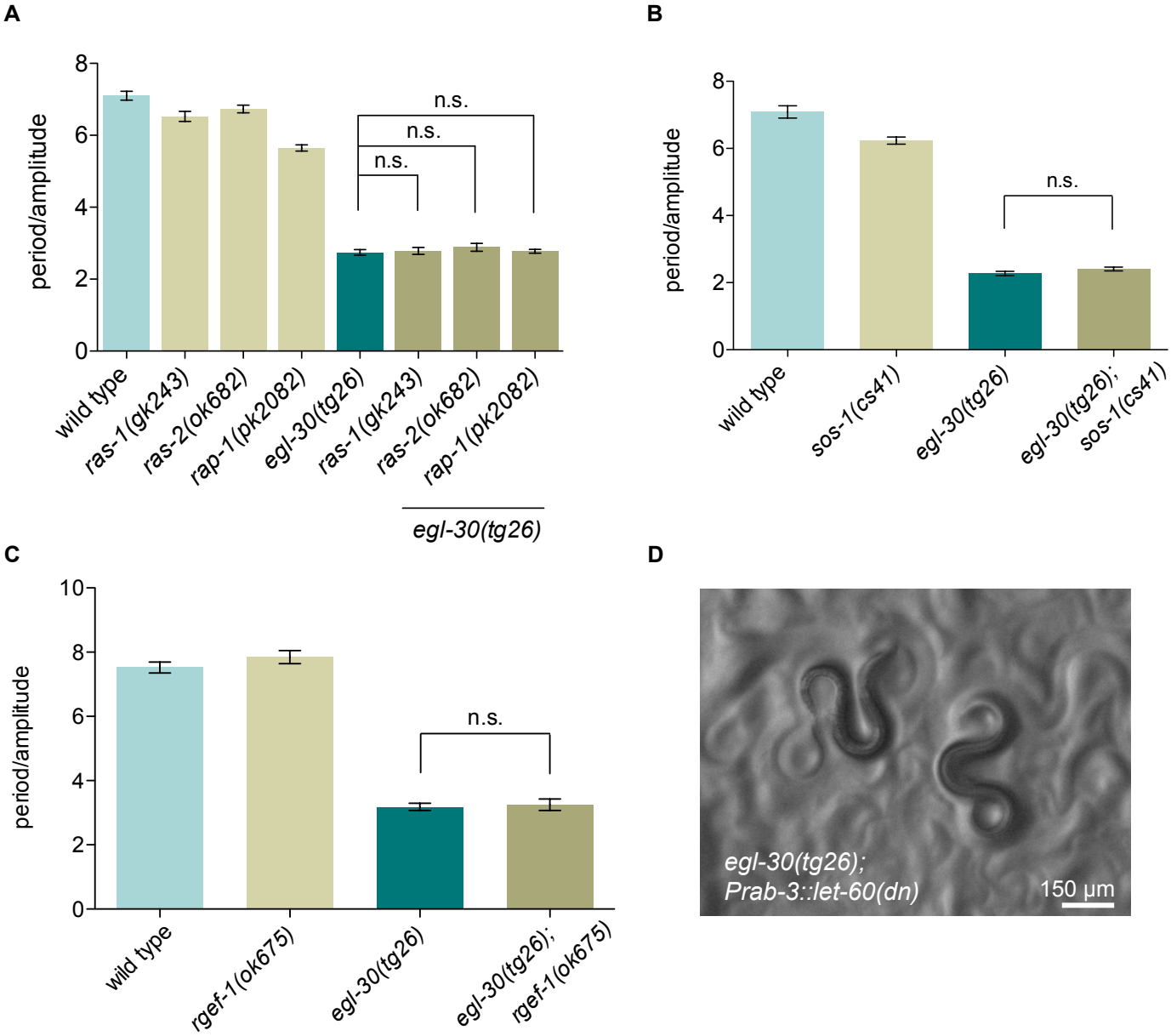
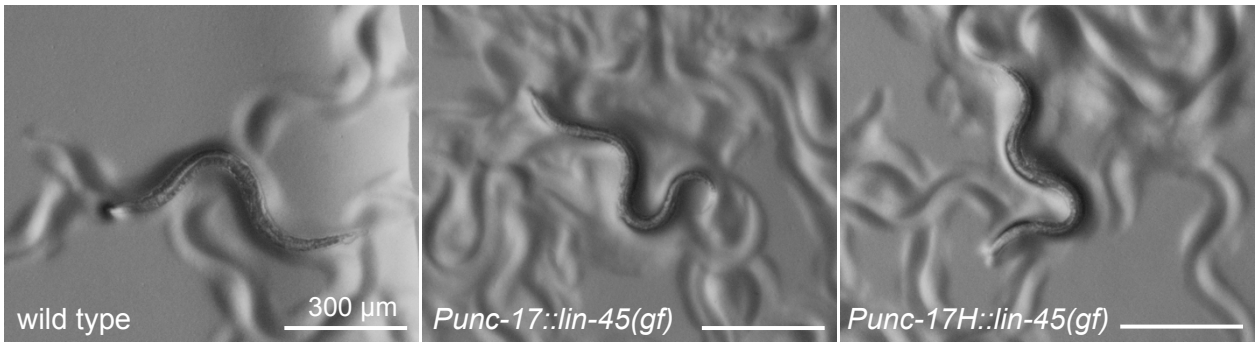
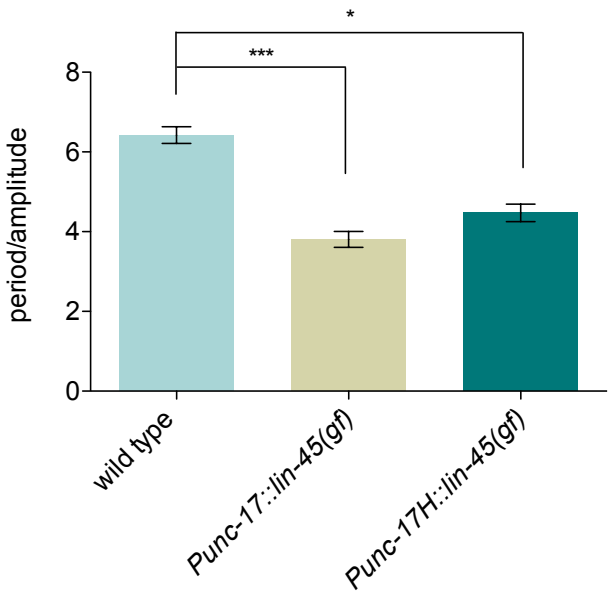
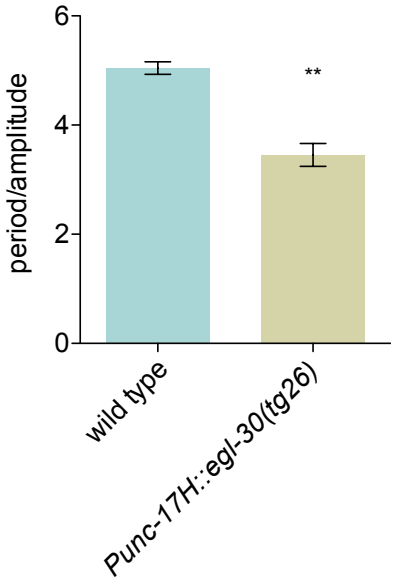

A
period/amplitude
0
2
4
6
8
n.s.
n.s.
n.s.
wild type
ras-1(gk243)
ras-2(ok682)
rap-1(pk2082)
egl-30(tg26)
ras-1(gk243)
ras-2(ok682)
rap-1(pk2082)
egl-30(tg26)
B
period/amplitude
0
2
4
6
8
n.s.
wild type
sos-1(cs41)
egl-30(tg26)
egl-30(tg26); sos-1(cs41)
C
period/amplitude
0
2
4
6
8
10
n.s.
wild type
rgef-1(ok675)
egl-30(tg26)
egl-30(tg26); rgef-1(ok675)
D
egl-30(tg26);
Prab-3::let-60(dn)
150 µm

Figure S3

A



B



C