An RNAi screen for conserved kinases that enhance microRNA activity after dauer in *C. elegans*

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Running title: Post-dauer kinase screen

Keywords: Diapause, dauer, *C. elegans,* microRNA, Argonaute, kinase, chromosome segregation, molting, checkpoint-kinase

Abstract:

Gene regulation in changing environments is critical for maintaining homeostasis. Some animals undergo a stress-resistant diapause stage to withstand harsh environmental conditions encountered during development. MicroRNAs (miRNAs) are one mechanism for regulating gene expression during and after diapause. MicroRNAs downregulate target genes post-transcriptionally through the activity of the miRNA-Induced Silencing Complex (miRISC). Argonaute is the core miRISC protein that binds to both the miRNA and to other miRISC proteins. The two major miRNA Argonautes in the *C. elegans* soma are ALG-1 and ALG-2, which function partially redundantly. Loss of *alg-1 (alg-1(0))* causes penetrant developmental phenotypes including vulval defects and the reiteration of larval cell programs in hypodermal cells. However, these phenotypes are essentially absent if *alg-1(0)* animals undergo a diapause stage called dauer. Levels of the relevant miRNAs are not higher during or after dauer, suggesting that activity of the miRISC may be enhanced in this context. To identify genes that are required for *alg-1(0)* mutants to develop without vulval defects after dauer, we performed an RNAi screen of genes encoding conserved kinases. We focused on kinases because of their known role in modulating miRISC activity. We found RNAi knockdown of four kinase-encoding genes, *air-2*, *bub-1*, *chk-1,* and *nekl-3*, caused vulval defects and reiterative phenotypes in *alg-1(0)* mutants after dauer, and that these defects were more penetrant in an *alg-1(0)* background than in wild type. Our results implicate these kinases as potential regulators of miRISC activity during post-dauer development in *C. elegans*.

41 **Introduction:**

42 The regulation of gene expression in response to changing environments is
43 critical to maintaining homeostasis. One mechanism for modulating gene expression in
44 different contexts is microRNA-mediated gene silencing. MicroRNAs (miRNAs) are ~22
45 nucleotide non-coding RNAs that regulate mRNA transcripts post-transcriptionally via
46 the miRNA Induced Silencing Complex (miRISC). MiRNAs bind to the 3'UTR of the
47 target mRNA via imperfect complementarity, thereby recruiting miRISC. Argonaute is
48 the core miRNA-interacting protein which in turn binds with other protein cofactors
49 (Bartel 2018). The protein factors in miRISC inhibit translation and cause destabilization
50 of the transcript, thus silencing the expression of the target (Jonas and Izaurralde 2015;
51 Bartel 2018).

52 Changes in miRNA activity can influence gene expression in a variety of
53 physiological or environmental contexts (Leung and Sharp 2010; Galagali and Kim
54 2020). When confronted with adverse environments, some animals can enter a stress-
55 resistant and developmentally arrested diapause stage (Hand *et al.* 2016). MicroRNAs
56 have been implicated in the regulation of diapause in insects and nematodes (Alvarez-
57 Saavedra and Horvitz 2010; Karp *et al.* 2011; Meuti *et al.* 2018). Although changes in
58 miRNA levels have been documented before, during, and after diapause, little is known
59 about changes in miRISC activity and how they may impact development in the
60 diapause life history.

61 *C. elegans* larval development is a useful model system to explore the
62 modulation of miRISC activity during diapause. *C. elegans* larvae develop through four
63 larval stages (L1-L4) before becoming adults (Byerly *et al.* 1976). Larval development

64 occurs continuously when the environment is favorable for reproduction. In unfavorable
65 environments, larvae can pause their development after the second larval molt in the
66 stress-resistant dauer diapause stage (Cassada and Russell 1975). If dauer larvae
67 encounter favorable environmental conditions, they can recover and resume
68 development through post-dauer larval stages that are developmentally equivalent to
69 those occurring in continuously developing larvae (Liu and Ambros 1991; Euling and
70 Ambros 1996).

71 One developmental system regulated by miRNAs is the hypodermal stem-cell-
72 like seam cells. Seam cells divide in a particular pattern and sequence at each larval
73 stage. At adulthood, seam cells cease dividing and differentiate (Sulston and Horvitz
74 1977; Ambros and Horvitz 1984). Larval vs. adult seam programs are regulated by
75 heterochronic genes that encode miRNAs and their targets (Ambros and Horvitz 1984;
76 Rougvie and Moss 2013; Galagali and Kim 2020). The loss of heterochronic miRNAs or
77 impaired function of miRISC proteins causes a reiterative phenotype whereby the
78 pattern and sequence of seam cell divisions appropriate to earlier developmental stages
79 is reiterated in later stages (Ambros and Horvitz 1984; Reinhart *et al.* 2000; Grishok *et*
80 *al.* 2001; Abbott *et al.* 2005).

81 ALG-1 and ALG-2 are the two major somatic miRNA-specific Argonaute proteins
82 in *C. elegans* and act partially redundantly (Grishok *et al.* 2001; Tops *et al.* 2006;
83 Vasquez-Rifo *et al.* 2012). While loss of both *alg-1* and *alg-2* results in embryonic
84 lethality, loss of *alg-1* alone *(alg-1(0))* causes a reiterative phenotype, suggesting *alg-2*
85 is not sufficient to mediate the activity of the heterochronic miRNAs (Grishok *et al.* 2001;
86 Zinovyeva *et al.* 2014). Strikingly, this phenotype is strongly suppressed in post-dauer

87 *alg-1(0)* mutant animals (Karp and Ambros 2012). This finding suggests that miRNA
88 activity is enhanced after dauer, such that *alg-2* activity becomes sufficient. Prior work
89 has demonstrated that levels of heterochronic miRNAs are the same or reduced in post-
90 dauer larvae compared to continuously developing larvae, suggesting that the
91 enhanced miRNA activity may arise from modulation of miRISC (Karp *et al.* 2011; Karp
92 and Ambros 2012).

93 Kinases are good candidates to mediate miRISC enhancement after dauer
94 because they are known modulators of miRISC activity. In *C. elegans* and other
95 animals, kinases phosphorylate Argonaute and other miRISC components, thereby
96 altering miRISC activity (Wilczynska and Bushell 2015; Frédérick and Simard 2022). To
97 identify conserved kinases that may enhance miRNA activity after dauer, we carried out
98 an RNAi screen for conserved kinase-encoding genes that are necessary for the post-
99 dauer suppression of *alg-1(0)* reiterative phenotypes. We found that *air-2, bub-1, chk-1,*
100 and *nekl-3* are required to prevent vulval defects and reiterative phenotypes in *alg-1(0)*
101 mutants after dauer. Furthermore, RNAi of these genes causes more penetrant
102 phenotypes in an *alg-1* background than in wild-type, suggesting that the kinases they
103 encode may enhance miRNA function after dauer.
104

105 **Methods:**

106 ***C. elegans* strain and maintenance.** All strains were maintained on Nematode Growth
107 Medium (NGM) plates seeded with *E. coli* strain OP50 at 15˚C or 20˚C (Brenner 1974).
108 Strains used in the study were: XV88 *daf-7(e1372); maIs105[col-19p::gfp]; alg-*

109 *1(gk214),* VT1777 *daf-7(e1372); maIs105,* XV85 *alg-2(ok302); daf-7(e1372); maIs105,*
110 and VT1274 *alg-2(ok302); maIs105.*
111
112 **RNAi.** A list of genes encoding kinases conserved between *C. elegans* and humans
113 was obtained from (Deng *et al.* 2019). Their list was generated using the Ortholist tool
114 (Shaye and Greenwald 2011; Kim *et al.* 2018). A complete list of RNAi clones used, and
115 their sources is provided in Table S1. RNAi bacteria were grown in LB media containing
116 50µL/mL carbenicillin shaking overnight at 37˚C. RNAi bacteria were then seeded on
117 NGM plates containing 200µg/mL IPTG and 50µg/mL carbenicillin ("RNAi plates"). For
118 the primary screen, 12-well plates were used, and for all other experiments, 60mm
119 plates were used. The primary screen was conducted in duplicate. RNAi clones that
120 caused reiterative alae defects in post-dauer adults were verified by Sanger sequencing
121 using the M13F primer (Eurofins Genomics).
122
123 **Embryo Isolation.** Gravid adult hermaphrodites were treated with a bleach solution
124 (0.4% sodium hypochlorite dissolved in 1M NaOH) for two 2-minute incubations at room
125 temperature. The embryos were washed with sterile water and then added to RNAi
126 plates.
127
128 **Dauer Induction.** The *daf-7(e1372)* allele was used to induce dauer formation (Vowels
129 and Thomas 1992; Karp 2018). Embryos were incubated on RNAi plates at 24°C for 48
130 – 50 hours, corresponding to the time just after the molt into dauer (Karp 2018).
131

132 **Dauer recovery**. For the primary screen, dauer recovery was induced by washing
133 dauer larvae off the RNAi plates with sterile water, adding the dauer larvae to fresh
134 RNAi plates, and shifting the worms to 20ºC. For all other experiments, dauer larvae
135 were selected by incubating worms with 1% (w/v) SDS (sodium dodecyl sulfate) for 30
136 minutes at room temperature (Cassada and Russell 1975; Karp 2018). The dauer
137 larvae were then washed with sterile water, added to fresh RNAi plates, and shifted to
138 20ºC to induce dauer recovery. Post-dauer larvae were incubated at 20˚C for 48 hours
139 to obtain young post-dauer adults.
140
141 **New RNAi clones**. RNAi clones for *air-2, chk-1,* and *bub-1* were created based on the
142 protocol described in (Kamath and Ahringer 2003). Briefly, primers were designed to
143 amplify regions of these genes that were distinct from those contained in the Ahringer
144 RNAi clones. The amplified sequences were cloned into the L4440 vector by TA
145 cloning. The resulting plasmids were sequenced, then transformed into HT115 bacteria.
146 Primers used for cloning are listed below.
147 *air-2* forward: 5'-TACTCCACAGAAGGGAGGGT-3'
148 *air-2* reverse: 5'-ATGTTGGCCACTAAGCTGAAATC-3'
149 *chk-1* forward: 5'-GGCGGAGAGACAGAATGCTT-3'
150 *chk-1* reverse: 5'-CCGAGTGCTCCACATTGACT-3'
151 *bub-1* forward: 5' CCGTCGACATGTGGTCTTGA-3'
152 *bub-1* reverse: 5'-GAGGTTTGCGTCACTGGAGA-3'
153

154 **Microscopy.** For the primary screen, bursting and/or protrusion of the vulva (Rup or Pvl
155 phenotypes) of the post-dauer adults were assessed using a dissecting microscope
156 (Zeiss Stereo V12 fitted with M2 Bio for fluorescence). In the secondary screen, the *alg-*
157 *1(0)* mutant phenotypes were assessed using a Zeiss AxioImager D2 compound
158 microscope. Post-dauer animals were immobilized using 0.1M levamisole on 2%
159 agarose pads. DIC and fluorescence images were taken using an AxioCam MRm Rev 3
160 camera and ZEN 3.2 software. GFP was visualized with a high-efficiency GFP shift-free
161 filter at 63x with an exposure time of 9ms.
162
163 **Data availability.** All *C. elegans* strains and newly made RNAi clones are available
164 upon request. The authors state that all data necessary for confirming the conclusions
165 of the article are present within the article, figures, and tables, including supplementary
166 information.
167
168 **Results and discussion.**
169 **RNAi screen of the conserved kinome to identify miRISC regulators acting after**
170 **dauer.**
171 If *C. elegans* larvae lacking *alg-1* develop continuously from embryo to adult, the
172 adults display reiterative phenotypes. In contrast, these phenotypes are suppressed if
173 *alg-1(0)* mutant larvae develop through the dauer diapause stage (Karp and Ambros
174 2012). We hypothesized that modulation of miRISC activity in post-dauer animals could
175 account for this difference. Since kinases are known to modulate miRISC in various
176 physiological and environmental contexts (Wu *et al.* 2011; Alessi *et al.* 2015; Olejniczak

177 *et al.* 2018), we designed an RNAi screen of all of the conserved kinases in *C. elegans*
178 (Kim *et al.* 2018; Deng *et al.* 2019). If post-dauer *alg-1(0)* mutants display reiterative
179 phenotypes after depletion of a particular kinase, that kinase is a candidate for
180 modulating miRISC function in post-dauer animals.

181 To perform our RNAi screen, we used a strain that contained the temperature-
182 sensitive *daf-7(e1372)* allele to induce dauer formation at 24˚C and allow recovery at
183 lower temperatures (Vowels and Thomas 1992; Karp 2018). Embryos were added to
184 RNAi plates and incubated at 24˚C for 2 days, or until just after dauer formation. Dauer
185 larvae were washed off with water, transferred to fresh RNAi plates, and shifted to 20˚C
186 to promote recovery. Post-dauer *alg-1(0)* adults were then screened for the vulval
187 defects associated with compromised miRISC function (Figure 1A). We used *lacZ* RNAi
188 as a negative control and *kin-3* RNAi as a positive control because we previously
189 showed that RNAi of *kin-3* caused reiterative phenotypes in post-dauer *alg-1(0)* mutants
190 (Alessi *et al.* 2015). *kin-3* encodes the catalytic subunit of Casein Kinase II (CK2), and
191 CK2 phosphorylates the miRISC proteins ALG-1 and CGH-1 to promote miRISC
192 function (Alessi *et al.* 2015; Shah *et al.* 2023). We used a previously published list of the
193 247 *C. elegans* kinases with human orthologs (Kim *et al.* 2018; Deng *et al.* 2019). Some
194 kinases are represented by more than one RNAi clone, giving us 286 RNAi clones in
195 total (Table S1).

196 In our primary screen, performed in duplicate, we used a dissecting microscope
197 to look for vulval defects in *alg-1(0)* post-dauer adults. Vulval defects included both
198 bursting (Rup) and protruding vulva (Pvl) phenotypes. We focused on vulval defects
199 because these phenotypes are easily visible in the dissecting microscope. As expected,

200 we saw almost no vulval defects after treatment with *lacZ* RNAi (Figure 1B). By

201 contrast, *kin-3* RNAi caused a high penetrance of vulval defects (Figure 1B). We found

202 12 additional conserved kinases that caused similar phenotypes. We observed a broad

203 range of penetrance of vulval defects which could be due to different degrees of

204 requirement for vulval development and/or variable effectiveness of RNAi.

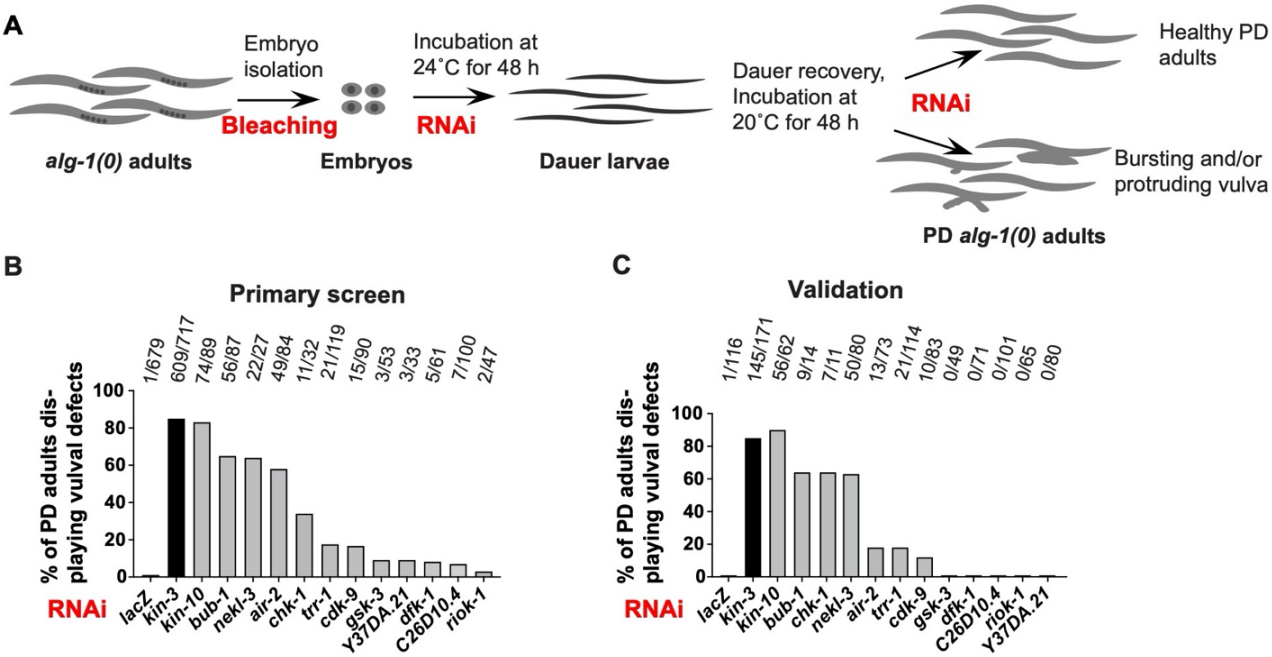

Fig. 1. Kinases identified in a primary screen for enhancement of an *alg-1(0)* phenotype after dauer. A. Strategy for RNAi screening. The *daf-7(e1372)* allele was used to induce dauer formation at 24ºC and allow recovery at lower temperatures. See Methods for details. **B.** RNAi clones that produced vulval defects (Rup and/or Pvl phenotypes) in the primary screen. The screen was performed in duplicate and clones that produced vulval defects in both plates are shown here. Table S1 shows data for all clones that produced vulval defects in at least one well. The remaining clones produced no vulval defects in either well (≥20 adults scored per well). Numbers in B and C indicated the number of post-dauer (PD) adults displaying vulval defects over the total PD adults scored. **C.** The 12 hits from the primary screen were retested for vulval defects. Seven genes caused bursting and/or protrusion of the vulva in post-dauer *alg-1(0)* adults. Among these 7 genes, 6 were novel *alg-1* interactors.

205 To ensure our results were reproducible, we re-tested the 12 hits from the

206 primary screen. In addition, in this validation experiment, we selected dauer larvae by

207 treatment with 1% SDS (Cassada and Russell 1975). Treatment with SDS ensured that

208 the adults we screened were indeed post-dauer and not displaying vulval defects
209 because the larvae had escaped dauer formation. Upon retesting, seven genes
210 displayed vulval defects (Figure 1C). One of these genes, *kin-10*, encodes the
211 regulatory subunit of the of CK2 protein and was therefore expected (Alessi *et al.* 2015;
212 Shah *et al.* 2023). We, therefore, focused on the remaining six kinase-encoding genes.
213

214 **Five kinase-encoding genes contribute to adult alae formation in *alg-1(0)* post-**
215 **dauer adults.**

216 In addition to vulval defects, *alg-1(0)* mutant adults display gapped or missing
217 alae after continuous development (Grishok *et al.* 2001; Vasquez-Rifo *et al.* 2012). This
218 reiterative phenotype occurs in heterochronic mutants because the underlying seam
219 cells repeat larval cell programs (Ambros and Horvitz 1984). In addition to gapped or
220 missing alae, *alg-1(0)* animals that develop continuously can display other defects in
221 alae formation, including small breaks and growth that deviates from the straight line
222 observed in wild-type adults (Alessi *et al.* 2015; Shah *et al.* 2023). Furthermore, the loss
223 of *alg-1* can reduce the expression of the adult cell fate marker *col-19p::gfp,* albeit at
224 low penetrance (Zinovyeva *et al.* 2015). If the kinases we identified were important for
225 miRNA function we would expect depletion of these genes to cause alae defects and
226 possibly reduced *col-19p::gfp* in the hypodermis of post-dauer *alg-1(0)* adults.

227 To test the hypothesis that these kinases are required to prevent reiterative
228 phenotypes in post-dauer *alg-1(0)* adults, we performed RNAi on the six hits and looked
229 at adult alae formation and *col-19p::gfp* expression with DIC and fluorescence
230 microscopy. We found that knocking down five of the six genes with RNAi resulted in

231 penetrant alae defects (Figure 2). The most penetrant alae phenotypes were caused by
232 *bub-1* RNAi. RNAi of *cdk-9* produced no reiterative phenotypes and occasional other
233 alae defects. While *lacZ* RNAi produced no alae defects at all, *cdk-9* RNAi was not
234 significantly different from the *lacZ* control. In contrast to the alae defects produced by
235 RNAi, *col-19p::gfp* expression was not reduced by RNAi of any of the six candidate
236 genes and only moderately downregulated in our *kin-3* positive control (Figure S1).
237 Uncoupling of alae defects and *col-19p::gfp* expression has been previously observed in
238 some mutant backgrounds (Hada *et al.* 2010; Hansen *et al.* 2022).
239

240 **RNAi of some kinases produces a stronger phenotype in a miRISC-sensitized**

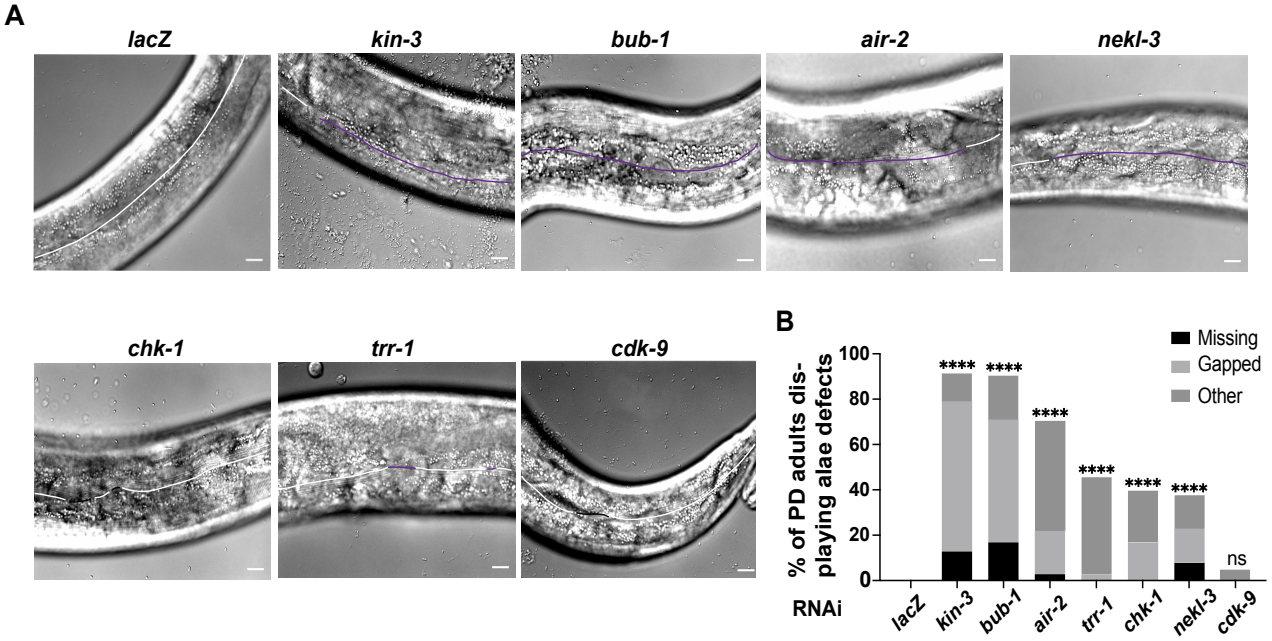

Fig. 2 RNAi of five kinase-encoding genes produces defects in adult alae formation in post-dauer *alg-1(0)* mutants. **A**. Comparison of alae phenotypes in post-dauer *alg-1(0)* adults. A compound microscope with DIC optics was used to visualize the presence of alae in post-dauer adult animals. White lines represent complete alae, purple lines represent gapped alae, and black lines show other alae defects including small breaks or curved or swirly alae. **B**. Quantification of alae defects. **** = $P < 0.0002$; ns = $P > 0.05$, Fisher Exact Test.

241 **background and during post-dauer development.**

242 Vulval defects can be caused by the depletion of genes that regulate processes
243 other than miRISC activity. For example, loss of genes involved in regulation of uterine
244 development can produce similar phenotypes (Seydoux and Greenwald 1989). If the
245 kinases identified in our screen enhance miRISC activity after dauer, we would expect
246 that RNAi of these kinases would produce a stronger phenotype in *alg-1(0)* adults than
247 when *alg-1* was wild type. To test this hypothesis, we performed RNAi on *alg-1(0)* and
248 *alg-1(+)* animals in parallel. RNAi of *bub-1, air-2, nekl-3,* and *chk-1* each displayed a
249 more penetrant phenotype in the miRISC-compromised background (Figure 3A). RNAi
250 of *trr-1* or *cdk-9* caused a low-penetrance phenotype in both backgrounds, and there
251 was no significant difference between the backgrounds (Figure 3A). While these data do
252 not rule out the involvement of any of these genes in regulating miRISC activity, they
253 provide higher confidence in *bub-1, air-2, nekl-3,* and *chk-1* than *trr-1* or *cdk-9.*
254 Since *alg-1(0)* mutants display vulval defects during continuous development but
255 not after dauer, we next wondered whether the kinases we identified play a greater role
256 in preventing vulval defects in post-dauer hermaphrodites than in those that develop
257 continuously, particularly when miRISC is compromised. We were unable to test this
258 question using *alg-1(0)* mutants because they display penetrant phenotypes during
259 continuous development (Figure 3B). However, animals lacking *alg-2* appear
260 superficially wild-type in both life histories (Grishok *et al.* 2001; Karp and Ambros 2012).
261 We therefore compared phenotypes in each life history following RNAi of *bub-1, nekl-3,*
262 *air-2*, and *chk-1* in an *alg-2(0)* mutant background. We found that RNAi of *bub-1* and
263 *nekl-3* both produced statistically more penetrant phenotypes after dauer (Figure 3C),
264 consistent with these kinases playing a larger role in post-dauer development.

265

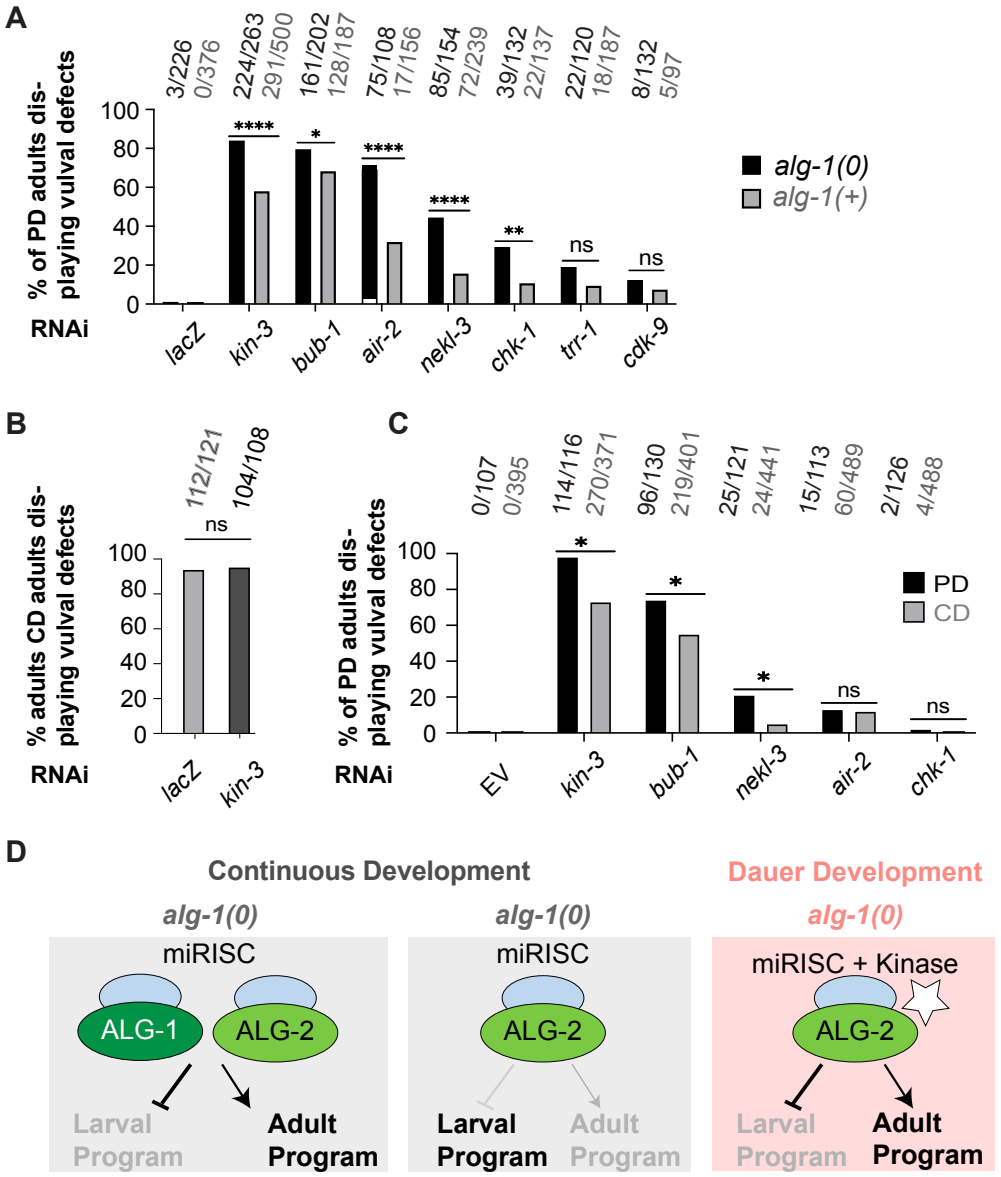

Fig. 3. RNAi of some kinases caused more penetrant phenotypes after dauer and when miRISC was compromised. A. Vulval defects in post-dauer adults with wild-type miRISC were compared to animals with compromised miRISC function *(alg-1(0)* mutants). **B.** During continuous development, the penetrance of vulval defects in *alg-1(0)* adults is high such that no further enhancement is seen when *kin-3* is knocked down. **C.** Vulval defects in *alg-2(0)* young adults after dauer (PD = post-dauer) or after continuous development (CD). Dauer was induced by *daf-7(e1372),* whereas continuously developing animals were wild-type for *daf-7*. *p*-value >0.05 (ns), <0.0332 (*), <0.0021 (**), <0.0002 (****), Fisher exact test. **D)** Model for role of miRISC activity in different developmental contexts to control larval vs. adult seam cell developmental programs.

266

267 By contrast, RNAi of *air-2* and *chk-1* produced low-penetrance phenotypes in both life
268 histories in the *alg-2(0)* background (Figure 3C). While in this latter case there was no
269 statistical difference between post-dauer and continuously developing animals,
270 observing a decrease in penetrance in adults that had developed continuously would be
271 difficult given the low penetrance phenotype in post-dauer adults. Thus, we are unable
272 to draw strong conclusions about the relative role of *air-2* and *chk-1* in post-dauer vs.
273 continuous development.

274 Finally, we performed RNAi of *bub-1, air-2, nekl-3,* and *chk-1* using independent
275 clones that target different regions of each gene than the region targeted in the original
276 clones. We observed vulval defects using each set of clones, albeit at reduced
277 penetrance (Figure S2). The percent of vulval defects observed for each clone was
278 statistically different from the empty vector control, except the newly created *chk-1* clone
279 (P = 0.088). Both original and newly created *chk-1* clones produced low-penetrance
280 phenotypes, whereas the negative control produced no vulval defects out of 226 adults
281 examined.

282

283 **Kinases identified in this screen**

284 We have identified four kinases that, when depleted, reproducibly enhanced two
285 different *alg-1(0)* phenotypes in post-dauer animals and whose depletion caused more
286 severe defects in a miRISC-compromised background than in wild type (Table 1). The
287 known roles for these kinases are described below.

288 Among the four genes identified as the highest confidence candidates in our
289 screen, *nekl-3* is the only one to have been previously implicated in the regulation of

Table 1: Conservation and function of genes isolated in the screen				
***C. elegans* gene**	**Human ortholog**	**Connection to microRNA function**	**Characterized function**	**Reference**
air-2	AURKB (Aurora kinase B)	None	Chromosome segregation and cytokinesis in mitosis and meiosis	(Schumacher *et al.* 1998; Rogers *et al.* 2002)
bub-1	BUB1 (BUB1 mitotic checkpoint serine/threonine kinase)	None	Chromosome alignment and segregation, spindle assembly, and promotion of anaphase in mitosis and meiosis	(Sharp-Baker and Chen 2001; Kim *et al.* 2015; Edwards *et al.* 2018)
chk-1	CHEK1 (checkpoint kinase 1)	None	DNA damage checkpoint and embryonic S-M checkpoint kinase	(Kalogeropoulos *et al.* 2004)
nekl-3	NEK6/7 (NIMA-related kinase 6/7)	Identified as a weak enhancer of the *let-7(mg279)* Rup phenotype during continuous development.	Clathrin-mediated endocytosis of cargo necessary for molting	(Parry *et al.* 2007; Joseph *et al.* 2020)

miRNA activity. *nekl-3* was isolated in a large-scale RNAi screen for genes that enhance the Rup phenotype of mutants with reduced activity of the *let-7* miRNA (Parry *et al.* 2007). Although *nekl-3* was a relatively weak enhancer and the screen was performed during continuous (non-dauer) development, this observation is consistent with the hypothesis that *nekl-3* can promote miRISC function. This role for *nekl-3* remains unexplored. In contrast, a role for *nekl-3* in the regulation of molting has been

296 well-characterized. *nekl-3* is required for the internalization via clathrin-mediated
297 endocytosis of cargo important for molting (Yochem *et al.* 2015; Joseph *et al.* 2020).
298 Specifically, *nekl*-3 is required for the L1-L2 and L2-L3 molts but not later molts during
299 continuous development. In the dauer life history, *nekl*-3 has a reduced requirement for
300 the L2d-dauer molt and is dispensable for post-dauer molting (Binti *et al.* 2022).
301 In mammals, the *nekl-3* orthologs, NEK family proteins, are best characterized
302 for their role in mitosis and chromosome segregation (Fry *et al.* 2012). Notably, *air-2*
303 and *bub-1* both encode conserved proteins that are critical for chromosome segregation
304 and mitosis and meiosis across species (Schumacher *et al.* 1998; Sharp-Baker and
305 Chen 2001; Rogers *et al.* 2002; Kim *et al.* 2015). Neither of these genes have been
306 previously implicated in miRNA activity. Previous large-scale screens conducted during
307 continuous development found that RNAi knockdown of *bub-1* produced Pvl and
308 bursting phenotypes (Fraser *et al.* 2000). Loss-of-function *bub-1* mutants display a
309 vulvaless (Vul) phenotype and a low-penetrance Pvl phenotype (Wang *et al.* 2009).
310 However, whether these phenotypes are related to the characterized role in
311 chromosome segregation or whether they may indicate a role for *bub-1* in regulating
312 miRNA activity is unknown. Finally, *chk-1* encodes a serine-threonine kinase that plays
313 a role in the checkpoint response to DNA damage and in the S-M checkpoint in
314 embryos (Kalogeropoulos *et al.* 2004).
315

316 **Concluding Remarks**

317 The goal of this study was to identify kinase-encoding genes that may enhance
318 miRISC function in post-dauer development. This study initially found six candidate

genes required to prevent developmental phenotypes in post-dauer *alg-1(0)* animals. Four of these genes, *bub-1, air-2, nekl-3,* and *chk-1*, showed higher penetrance phenotypes in a miRISC-compromised background, making these four genes the most likely to affect miRISC activity after dauer. *bub-1* and *nekl-3* produced more penetrant phenotypes after dauer than during continuous development in an *alg-2(0)* background, whereas *air-2* and *chk-1* produced low-penetrance phenotypes in both life histories in an *alg-2(0)* background. Prior to this work, the only kinase-encoding gene known to enhance *alg-1(0)* phenotypes after dauer was *kin-3,* used as a positive control in this study (Alessi *et al.* 2015). During continuous development, three kinases have been shown to phosphorylate miRISC components to modulate different aspects of miRNA activity: KIN-1/PKA, casein kinase 1 (CK1) and CK2 (Alessi *et al.* 2015; Huberdeau *et al.* 2022; Shah *et al.* 2023). In our screen, only the CK2-encoding genes *kin-3* and *kin-10* were found to be necessary to prevent vulval defects in *alg-1(0)* mutants after dauer, whereas RNAi of *kin-1* and *kin-19,* encoding PKA and CK1 respectively, did not cause vulval defects in our primary screen (*kin-1,* 0/40; *kin-19,* 0/60). While these findings may be explained by incomplete knockdown of *kin-1* and *kin-19,* it is also possible that distinct kinases modulate miRISC in the dauer life history. This study lays the foundation for future studies that can dissect the molecular relationship between these genes and miRISC in *C. elegans* and other species.

Acknowledgments

We thank Karp lab members, Jennifer Schisa, and Steven Gorsich (Central Michigan University) for their helpful advice throughout this project. We are grateful to BIO 546

342 students (Central Michigan University) who helped with pilot experiments. We thank
343 WormBase. Some strains were provided by the CGC, which is funded by NIH Office of
344 Research Infrastructure Programs (P40 OD010440). This project was funded by
345 CAREER 1652283 to XK from the National Science Foundation.

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351 References:
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354 Abbott, A. L., E. Alvarez-Saavedra, E. A. Miska, N. C. Lau, D. P. Bartel *et al.*, 2005 The
355 *let-7* microRNA family members *mir-48, mir-84*, and *mir-241* function together to
356 regulate developmental timing in *Caenorhabditis elegans*. Developmental Cell 9:
357 403–414.

358 Alessi, A. F., V. Khivansara, T. Han, M. A. Freeberg, J. J. Moresco *et al.*, 2015 *Casein*
359 *kinase II* promotes target silencing by miRISC through direct phosphorylation of the
360 DEAD-box RNA helicase CGH-1. Proceedings of the National Academy of Sciences
361 201509499.

362 Alvarez-Saavedra, E., and H. R. Horvitz, 2010 Many families of *C. elegans* microRNAs
363 are not essential for development or viability. Current Biology 1–7.

364 Ambros, V., and H. R. Horvitz, 1984 Heterochronic mutants of the nematode
365 *Caenorhabditis elegans*. Science 226: 409–416.

366 Bartel, D. P., 2018 Metazoan MicroRNAs. Cell 173: 20–51.

367 Binti, S., R. V. Melinda, B. B. Joseph, P. T. Edeen, S. D. Miller *et al.*, 2022 A life cycle
368 alteration can correct molting defects in *Caenorhabditis elegans*. Dev Biol.

369 Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics 77: 71–94.

370 Byerly, L., R. C. Cassada, and R. L. Russell, 1976 The life cycle of the nematode
371 *Caenorhabditis elegans*. I. Wild-type growth and reproduction. Developmental
372 Biology 51: 23–33.

373 Cassada, R. C., and R. L. Russell, 1975 The dauerlarva, a post-embryonic
374 developmental variant of the nematode *Caenorhabditis elegans*. Developmental
375 Biology 46: 326–342.

376 Deng, Y., K. L. Luo, D. D. Shaye, and I. Greenwald, 2019 A screen of the conserved
377 kinome for negative regulators of LIN-12 negative regulatory region (“NRR”)-
378 missense activity in *Caenorhabditis elegans*. G3 Genes Genomes Genetics 9: 3567–
379 3574.

380 Edwards, F., G. Maton, N. Gareil, J. C. Canman, and J. Dumont, 2018 BUB-1 promotes
381 amphitelic chromosome biorientation via multiple activities at the kinetochore. Elife 7:
382 e40690.

383 Euling, S., and V. Ambros, 1996 Reversal of cell fate determination in *Caenorhabditis*
384 *elegans* vulval development. Development 122: 2507–2515.

385 Frédérick, P., and M. J. Simard, 2022 Regulation and different functions of the animal
386 microRNA-induced silencing complex. Wiley Interdiscip Rev RNA 13: e1701.

387 Fraser, A. G., R. S. Kamath, P. Zipperlen, M. Martinez-Campos, M. Sohrmann *et al.*,
388 2000 Functional genomic analysis of *C. elegans* chromosome I by systematic RNA
389 interference. Nature 408: 325–330.

390 Fry, A. M., L. O'Regan, S. R. Sabir, and R. Bayliss, 2012 Cell cycle regulation by the
391 NEK family of protein kinases. J. Cell Sci. 125: 4423–4433.

392 Galagali, H., and J. K. Kim, 2020 The multifaceted roles of microRNAs in differentiation.
393 Curr. Opin. Cell Biol. 67: 118–140.

394 Grishok, A., A. E. Pasquinelli, D. Conte, N. Li, S. Parrish *et al.*, 2001 Genes and
395 mechanisms related to RNA interference regulate expression of the small temporal
396 RNAs that control *C. elegans* developmental timing. Cell 106: 23–34.

397 Hada, K., M. Asahina, H. Hasegawa, Y. Kanaho, F. J. Slack *et al.*, 2010 The nuclear
398 receptor gene nhr-25 plays multiple roles in the *Caenorhabditis elegans*
399 heterochronic gene network to control the larva-to-adult transition. Developmental
400 Biology 344: 1100–1109.

401 Hand, S. C., D. L. Denlinger, J. E. Podrabsky, and R. Roy, 2016 Mechanisms of animal
402 diapause: recent developments from nematodes, crustaceans, insects, and fish.
403 American journal of physiology. Regulatory, integrative and comparative physiology
404 ajpregu.00250.2015.

405 Hansen, M. A., A. Dahal, T. A. Bernstein, C. Kohtz, S. Ali *et al.*, 2022 ztf-16 is a novel
406 heterochronic modulator that opposes adult cell fate in dauer and continuous life
407 histories in *Caenorhabditis elegans*. bioRxiv 2022.06.20.496913.

408 Huberdeau, M. Q., V. N. Shah, S. Nahar, J. Neumeier, F. Houle *et al.*, 2022 A specific
409 type of Argonaute phosphorylation regulates binding to microRNAs during *C. elegans*
410 development. Cell Rep. 41: 111822.

411 Jonas, S., and E. Izaurralde, 2015 Towards a molecular understanding of microRNA-
412 mediated gene silencing. Nat Rev Genet 16: 421–433.

413 Joseph, B. B., Y. Wang, P. Edeen, V. Lažetić, B. D. Grant *et al.*, 2020 Control of
414 clathrin-mediated endocytosis by NIMA family kinases. Plos Genet 16: e1008633.

415 Kalogeropoulos, N., C. Christoforou, A. J. Green, S. Gill, and N. R. Ashcroft, 2004 *chk-1*
416 is an essential gene and is required for an S-M checkpoint during early
417 embryogenesis. Cell Cycle 3: 1194–1198.

418 Kamath, R. S., and J. Ahringer, 2003 Genome-wide RNAi screening in *Caenorhabditis*
419 *elegans*. Methods 30: 313–321.

420 Karp, X., 2018 Working with dauer larvae. WormBook 2018: 1–19.

421 Karp, X., and V. Ambros, 2012 Dauer larva quiescence alters the circuitry of microRNA
422 pathways regulating cell fate progression in *C. elegans*. Development 139: 2177–
423 2186.

424 Karp, X., M. Hammell, M. C. Ow, and V. Ambros, 2011 Effect of life history on
425 microRNA expression during *C. elegans* development. RNA 17: 639–651.

426 Kim, T., M. W. Moyle, P. Lara-Gonzalez, C. D. Groot, K. Oegema *et al.*, 2015
427 Kinetochore-localized BUB-1/BUB-3 complex promotes anaphase onset in *C.*
428 *elegans*. J. Cell Biol. 209: 507–517.

429 Kim, W., R. S. Underwood, I. Greenwald, and D. D. Shaye, 2018 OrthoList 2: a new
430 comparative genomic analysis of human and *Caenorhabditis elegans* Genes.
431 Genetics 210: 445–461.

432 Leung, A. K. L., and P. A. Sharp, 2010 MicroRNA functions in stress responses.
433 Molecular Cell 40: 205–215.

434 Liu, Z., and V. Ambros, 1991 Alternative temporal control systems for hypodermal cell
435 differentiation in *Caenorhabditis elegans*. Nature 350: 162–165.

436 Meuti, M. E., R. Bautista-Jimenez, and J. A. Reynolds, 2018 Evidence that microRNAs
437 are part of the molecular toolkit regulating adult reproductive diapause in the
438 mosquito, Culex pipiens. PLoS ONE 13: e0203015.

439 Olejniczak, M., A. Kotowska-Zimmer, and W. Krzyzosiak, 2018 Stress-induced changes
440 in miRNA biogenesis and functioning. Cell Mol Life Sci 75: 177–191.

441 Parry, D. H., J. Xu, and G. Ruvkun, 2007 A Whole-Genome RNAi Screen for *C. elegans*
442 miRNA Pathway Genes. Curr Biol 17: 2013–2022.

443 Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger *et al.*, 2000 The
444 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*.
445 Nature 403: 901–906.

446 Rogers, E., J. D. Bishop, J. A. Waddle, J. M. Schumacher, and R. Lin, 2002 The aurora
447 kinase AIR-2 functions in the release of chromosome cohesion in *Caenorhabditis*
448 *elegans* meiosis. J. Cell Biol. 157: 219–229.

449 Rougvie, A. E., and E. G. Moss, 2013 Developmental transitions in *C. elegans* larval
450 stages. Current topics in developmental biology 105: 153–180.

Schumacher, J. M., A. Golden, and P. J. Donovan, 1998 AIR-2: An Aurora/Ipl1-related protein kinase associated with chromosomes and midbody microtubules is required for polar body extrusion and cytokinesis in *Caenorhabditis elegans* embryos. J. Cell Biol. 143: 1635–1646.

Required for Polar Body Extrusion and Cytokinesis in *Caenorhabditis elegans* Embryos. J Cell Biology 143: 1635–1646.

Rual, J.-F., J. Ceron, J. Koreth, T. Hao, A.-S. Nicot *et al.*, 2004 Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library. Genome Res. 14: 2162–2168.

Seydoux, G., and I. Greenwald, 1989 Cell autonomy of lin-12 function in a cell fate decision in *C. elegans*. Cell 57: 1237–1245.

Shah, V. N., J. Neumeier, M. Q. Huberdeau, D. M. Zeitler, A. Bruckmann *et al.*, 2023 *Casein kinase 1* and *2* phosphorylate Argonaute proteins to regulate miRNA-mediated gene silencing. EMBO Rep.

Sharp-Baker, H., and R.-H. Chen, 2001 Spindle checkpoint protein Bub1 Is required for kinetochore localization of Mad1, Mad2, Bub3, and Cenp-E, independently of its kinase activity. J. Cell Biol. 153: 1239–1250.

Shaye, D. D., and I. Greenwald, 2011 OrthoList: a compendium of *C. elegans* genes with human orthologs. PLoS ONE 6: e20085.

Sulston, J. E., and H. R. Horvitz, 1977 Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. Developmental Biology 56: 110–156.

Tops, B. B. J., R. H. A. Plasterk, and R. F. Ketting, 2006 The *Caenorhabditis elegans* Argonautes ALG-1 and ALG-2: almost identical yet different. Cold Spring Harb. Symp. Quant. Biol. 71: 189–194.

Vasquez-Rifo, A., G. Jannot, J. Armisen, M. Labouesse, S. I. A. Bukhari *et al.*, 2012 Developmental characterization of the microRNA-specific *C. elegans* Argonautes *alg-1* and *alg-2*. PLoS ONE 7: e33750.

Vowels, J. J., and J. H. Thomas, 1992 Genetic analysis of chemosensory control of dauer formation in *Caenorhabditis elegans*. Genetics 130: 105–123.

Wang, X., M. Liu, W. Li, C. D. Suh, Z. Zhu *et al.*, 2009 The function of a spindle checkpoint gene *bub-1* in *C. elegans* development. PLoS ONE 4: e5912.

Wilczynska, A., and M. Bushell, 2015 The complexity of miRNA-mediated repression. Cell death and differentiation 22: 22–33.

484 Wu, C., J. So, B. N. Davis-Dusenbery, H. H. Qi, D. B. Bloch *et al.*, 2011 Hypoxia
485 Potentiates MicroRNA-Mediated Gene Silencing through Posttranslational
486 Modification of Argonaute2. Mol Cell Biol 31: 4760–4774.

487 Yochem, J., V. Lažetić, L. Bell, L. Chen, and D. Fay, 2015 C. elegans NIMA-related
488 kinases NEKL-2 and NEKL-3 are required for the completion of molting. Dev Biol
489 398: 255–266.

490 Zinovyeva, A. Y., S. Bouasker, M. J. Simard, C. M. Hammell, and V. Ambros, 2014
491 Mutations in conserved residues of the *C. elegans* microRNA Argonaute ALG-1
492 identify separable functions in ALG-1 miRISC loading and target repression. PLoS
493 Genetics 10: e1004286.

494 Zinovyeva, A. Y., I. Veksler-Lublinsky, A. A. Vashisht, J. A. Wohlschlegel, and V. R.
495 Ambros, 2015 *Caenorhabditis elegans* ALG-1 antimorphic mutations uncover
496 functions for Argonaute in microRNA guide strand selection and passenger strand
497 disposal. Proceedings of the National Academy of Sciences 112: E5271-80.

498