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

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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 that in turn binds with other protein cofactors (Bartel
49 2018). The protein factors in miRISC inhibit translation and cause destabilization of the
50 transcript, thus silencing the expression of the target (Jonas and Izaurralde 2015; Bartel
51 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 diverse animal species, including
57 insects, killifish, and nematodes (Alvarez-Saavedra and Horvitz 2010; Karp *et al.* 2011;
58 Meuti *et al.* 2018, Reynolds 2019). Although changes in miRNA levels have been
59 documented before, during, and after diapause, little is known about changes in miRISC
60 activity and how they may impact development in the 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

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

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

ALG-1 and ALG-2 are the best characterized somatic miRNA-specific Argonaute proteins in *C. elegans* and act partially redundantly (Grishok *et al.* 2001; Tops *et al.* 2006; Vasquez-Rifo *et al.* 2012). In addition, other Argonautes including ALG-5 and RDE-1 can also bind miRNAs (Seroussi *et al.* 2023). While loss of both *alg-1* and *alg-2* results in embryonic lethality, loss of *alg-1* alone *(alg-1(0))* causes a reiterative phenotype, suggesting ALG-2 and the other Argonautes are not sufficient to mediate

87 the activity of the heterochronic miRNAs (Grishok *et al.* 2001; Zinovyeva *et al.* 2014).
88 Strikingly, this phenotype is strongly suppressed in post-dauer *alg-1(0)* mutant animals
89 (Karp and Ambros 2012). This finding suggests that miRNA activity is enhanced after
90 dauer, such that *alg-2* activity becomes sufficient. Prior work has demonstrated that
91 levels of heterochronic miRNAs are the same or reduced in post-dauer larvae compared
92 to continuously developing larvae, suggesting that the enhanced miRNA activity may
93 arise from modulation of miRISC (Karp *et al.* 2011; Karp and Ambros 2012).
94 Kinases are good candidates to mediate miRISC enhancement after dauer
95 because they are known modulators of miRISC activity. In *C. elegans* and other
96 animals, kinases phosphorylate Argonaute and other miRISC components, thereby
97 altering miRISC activity (Wilczynska and Bushell 2015; Frédérick and Simard 2022). To
98 identify conserved kinases that may enhance miRNA activity after dauer, we carried out
99 an RNAi screen for conserved kinase-encoding genes that are necessary for the post-
100 dauer suppression of *alg-1(0)* reiterative phenotypes. We found that *air-2, bub-1, chk-1,*
101 and *nekl-3* are required to prevent vulval defects and reiterative phenotypes in *alg-1(0)*
102 mutants after dauer. Furthermore, RNAi of these genes causes more penetrant
103 phenotypes in an *alg-1* background than in wild-type, suggesting that the kinases they
104 encode may enhance miRNA function after dauer.
105
106 **Methods:**
107 ***C. elegans* strain and maintenance.** All strains were maintained on Nematode Growth
108 Medium (NGM) plates seeded with *E. coli* strain OP50 at 15˚C or 20˚C (Brenner 1974).
109 Strains used in the study were: XV88 *daf-7(e1372); maIs105[col-19p::gfp]; alg-*

110 *1(gk214),* VT1777 *daf-7(e1372); maIs105,* XV85 *alg-2(ok302); daf-7(e1372); maIs105,*
111 and VT1274 *alg-2(ok302); maIs105.*
112
113 **RNAi.** A list of genes encoding kinases conserved between *C. elegans* and humans
114 was obtained from (Deng *et al.* 2019). Their list was generated using the Ortholist tool
115 (Shaye and Greenwald 2011; Kim *et al.* 2018). A complete list of RNAi clones used, and
116 their sources is provided in Table S1. RNAi bacteria were grown in LB media containing
117 50µL/mL carbenicillin shaking overnight at 37˚C. RNAi bacteria were then seeded on
118 NGM plates containing 200µg/mL IPTG and 50µg/mL carbenicillin ("RNAi plates"). For
119 the primary screen, 12-well plates were used, and for all other experiments, 60mm
120 plates were used. The primary screen was conducted in duplicate. RNAi clones that
121 caused reiterative alae defects in post-dauer adults were verified by Sanger sequencing
122 using the M13F primer (Eurofins Genomics).
123
124 **Embryo Isolation.** Gravid adult hermaphrodites were treated with a bleach solution
125 (0.4% sodium hypochlorite dissolved in 1M NaOH) for two 2-minute incubations at room
126 temperature. The embryos were washed with sterile water and then added to RNAi
127 plates.
128
129 **Dauer Induction.** The *daf-7(e1372)* allele was used to induce dauer formation (Vowels
130 and Thomas 1992; Karp 2018). Embryos were incubated on RNAi plates at 24°C for 48
131 – 50 hours, corresponding to the time just after the molt into dauer (Karp 2018).
132

133 **Dauer recovery**. For the primary screen, dauer recovery was induced by washing
134 dauer larvae off the RNAi plates with sterile water, adding the dauer larvae to fresh
135 RNAi plates, and shifting the worms to 20ºC. For all other experiments, dauer larvae
136 were selected by incubating worms with 1% (w/v) SDS (sodium dodecyl sulfate) for 30
137 minutes at room temperature (Cassada and Russell 1975; Karp 2018). The dauer
138 larvae were then washed with sterile water, added to fresh RNAi plates, and shifted to
139 20ºC to induce dauer recovery. Post-dauer larvae were incubated at 20˚C for 48 hours
140 to obtain young post-dauer adults.
141
142 **New RNAi clones**. RNAi clones for *air-2, chk-1,* and *bub-1* were created based on the
143 protocol described in (Kamath and Ahringer 2003). Briefly, primers were designed to
144 amplify regions of these genes that were distinct from those contained in the Ahringer
145 RNAi clones. The amplified sequences were cloned into the L4440 vector by TA
146 cloning. The resulting plasmids were sequenced, then transformed into HT115 bacteria.
147 Primers used for cloning are listed below.
148 *air-2* forward: 5'-TACTCCACAGAAGGGAGGGT-3'
149 *air-2* reverse: 5'-ATGTTGGCCACTAAGCTGAAATC-3'
150 *chk-1* forward: 5'-GGCGGAGAGACAGAATGCTT-3'
151 *chk-1* reverse: 5'-CCGAGTGCTCCACATTGACT-3'
152 *bub-1* forward: 5' CCGTCGACATGTGGTCTTGA-3'
153 *bub-1* reverse: 5'-GAGGTTTGCGTCACTGGAGA-3'
154

155 **Microscopy.** For the primary screen, bursting and/or protrusion of the vulva (Rup or Pvl
156 phenotypes) of the post-dauer adults were assessed using a dissecting microscope
157 (Zeiss Stereo V12 fitted with M2 Bio for fluorescence). In the secondary screen, the *alg-*
158 *1(0)* mutant phenotypes were assessed using a Zeiss AxioImager D2 compound
159 microscope. Post-dauer animals were immobilized using 0.1M levamisole on 2%
160 agarose pads. DIC and fluorescence images were taken using an AxioCam MRm Rev 3
161 camera and ZEN 3.2 software. GFP was visualized with a high-efficiency GFP shift-free
162 filter at 63x with an exposure time of 9ms.
163
164 **Results and discussion.**
165 **RNAi screen of the conserved kinome to identify miRISC regulators acting after**
166 **dauer.**
167 If *C. elegans* larvae lacking *alg-1* develop continuously from embryo to adult, the
168 adults display reiterative phenotypes (Grishok *et al.* 2001; Zinovyeva *et al.* 2014). In
169 contrast, these phenotypes are suppressed if *alg-1(0)* mutant larvae develop through
170 the dauer diapause stage (Karp and Ambros 2012). We hypothesized that modulation of
171 miRISC activity in post-dauer animals could account for this difference. Since kinases
172 are known to modulate miRISC in various physiological and environmental contexts
173 (Wu *et al.* 2011; Alessi *et al.* 2015; Olejniczak *et al.* 2018), we designed an RNAi screen
174 of all of the conserved kinases in *C. elegans* (Kim *et al.* 2018; Deng *et al.* 2019). If post-
175 dauer *alg-1(0)* mutants display reiterative phenotypes after depletion of a particular
176 kinase, that kinase is a candidate for modulating miRISC function in post-dauer animals.

177 To perform our RNAi screen, we used an *alg-1(0)* strain that contained the
178 temperature-sensitive *daf-7(e1372)* allele to induce dauer formation at 24˚C and allow
179 recovery at lower temperatures (Vowels and Thomas 1992; Karp 2018). A *col-19p::gfp*
180 transgene was also included to allow analysis of that aspect of adult cell fate (Liu et al.
181 1995). Moving forward, unless otherwise specified, it should be presumed that all
182 strains described here contain *daf-7(e1372); maIs105[col-19p::gfp]* in the background.
183 Embryos were added to RNAi plates and incubated at 24˚C for 2 days, or until just after
184 dauer formation. Dauer larvae were washed off with water, transferred to fresh RNAi
185 plates, and shifted to 20˚C to promote recovery. Post-dauer *alg-1(0)* adults were then
186 screened for the vulval defects associated with compromised miRISC function (Figure
187 1A). We used *lacZ* RNAi as a negative control and *kin-3* RNAi as a positive control
188 because we previously showed that RNAi of *kin-3* caused reiterative phenotypes in
189 post-dauer *alg-1(0)* mutants (Alessi *et al.* 2015). *kin-3* encodes the catalytic subunit of
190 Casein Kinase II (CK2), and CK2 phosphorylates the miRISC proteins ALG-1 and CGH-
191 1 to promote miRISC function (Alessi *et al.* 2015; Shah *et al.* 2023). We used a
192 previously published list of the 247 *C. elegans* kinases with human orthologs (Kim *et al.*
193 2018; Deng *et al.* 2019). Some kinases are represented by more than one RNAi clone,
194 giving us 286 RNAi clones in total (Table S1).
195 In our primary screen, performed in duplicate, we used a dissecting microscope
196 to look for vulval defects in *alg-1(0)* post-dauer adults. Vulval defects included both
197 bursting (Rup) and protruding vulva (Pvl) phenotypes. We focused on vulval defects
198 because these phenotypes are easily visible in the dissecting microscope. As expected,
199 we saw almost no vulval defects after treatment with *lacZ* RNAi (Figure 1B). By

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

201 12 additional conserved kinases that caused similar phenotypes. We observed a broad

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

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

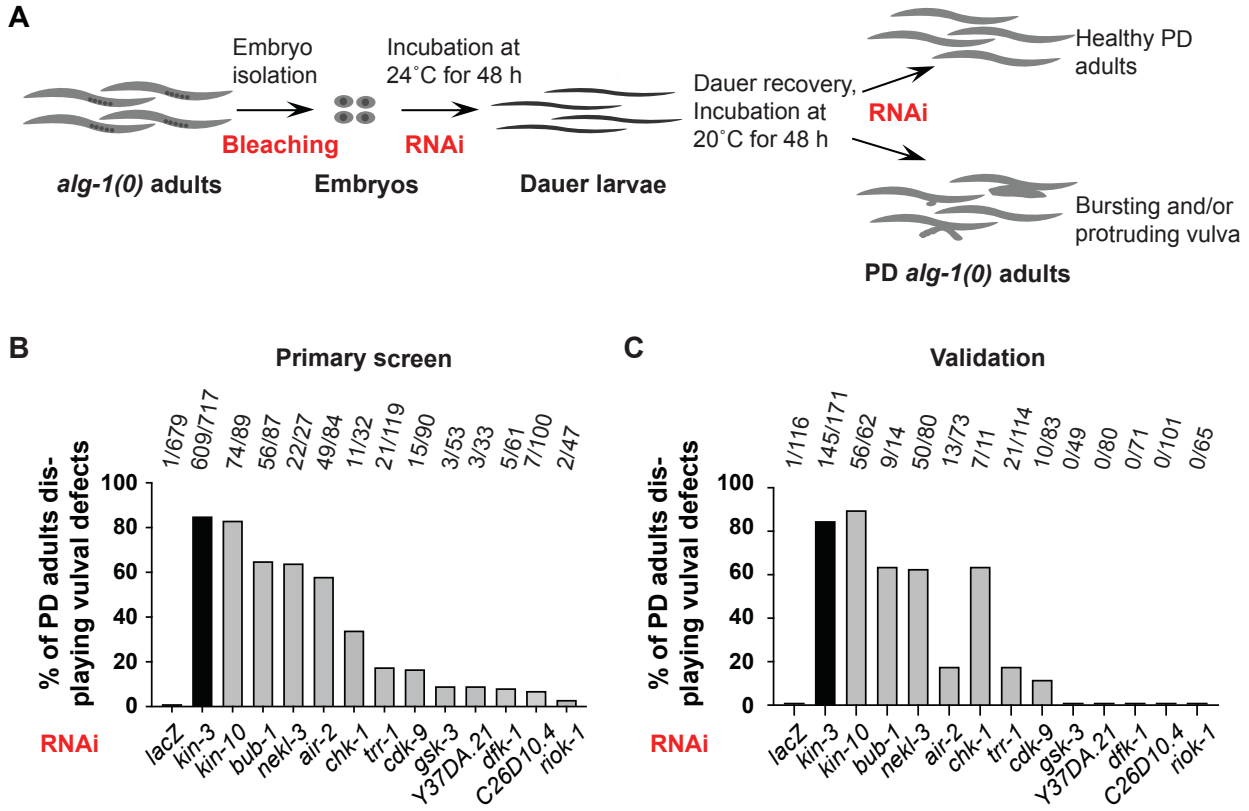

Fig. 1. Kinases identified in a primary screen for enhancement of an *alg-1(0)* phenotype after dauer. A. Strategy for RNAi screening. The full genotype of the strain used for screening was *daf-7(e1372); maIs105[col-19p::gfp]; alg-1(gk214).* The *daf-7(e1372)* allele was used to induce dauer formation 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 indicate 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.

204 To ensure our results were reproducible, we re-tested the 12 hits from the
205 primary screen. In addition, in this validation experiment, we selected dauer larvae by
206 treatment with 1% SDS (Cassada and Russell 1975). Treatment with SDS ensured that
207 the adults we screened were indeed post-dauer and not displaying vulval defects
208 because the larvae had escaped dauer formation. Upon retesting, seven genes
209 displayed vulval defects (Figure 1C). One of these genes, *kin-10*, encodes the
210 regulatory subunit of the of CK2 protein and was therefore expected (Alessi *et al.* 2015;
211 Shah *et al.* 2023). We therefore focused on the remaining six kinase-encoding genes.
212

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

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

226 To test the hypothesis that these kinases are required to prevent reiterative
227 phenotypes in post-dauer *alg-1(0)* adults, we performed RNAi on the six hits and looked
228 at adult alae formation and *col-19p::gfp* expression with DIC and fluorescence
229 microscopy. We found that knocking down five of the six genes with RNAi resulted in
230 penetrant alae defects (Figure 2). The most penetrant alae phenotypes were caused by
231 *bub-1* RNAi. RNAi of *cdk-9* produced no reiterative phenotypes and occasional other
232 alae defects. While *lacZ* RNAi produced no alae defects at all, *cdk-9* RNAi was not
233 significantly different from the *lacZ* control. In contrast to the alae defects produced by
234 RNAi, *col-19p::gfp* expression was not reduced by RNAi of any of the six candidate
235 genes and only moderately downregulated in our *kin-3* positive control (Figure S1).
236 Uncoupling of alae defects and *col-19p::gfp* expression has been previously observed in
237 some mutant backgrounds (Hada *et al.* 2010; Hansen *et al.* 2022).
238
239 **RNAi of some kinases produces a stronger phenotype in a miRISC-sensitized**
240 **background and during post-dauer development.**
241 Vulval defects can be caused by the depletion of genes that regulate processes
242 other than miRISC activity. For example, loss of genes involved in regulation of uterine
243 development can produce similar phenotypes (Seydoux and Greenwald 1989). If the
244 kinases identified in our screen enhance miRISC activity after dauer, we would expect
245 that RNAi of these kinases would produce a stronger phenotype in *alg-1(0)* adults than
246 when *alg-1* was wild type. To test this hypothesis, we performed RNAi on *alg-1(0)* and
247 *alg-1(+)* animals in parallel. RNAi of *bub-1, air-2, nekl-3,* and *chk-1* each displayed a
248 more penetrant phenotype in the miRISC-compromised background (Figure 3A). RNAi

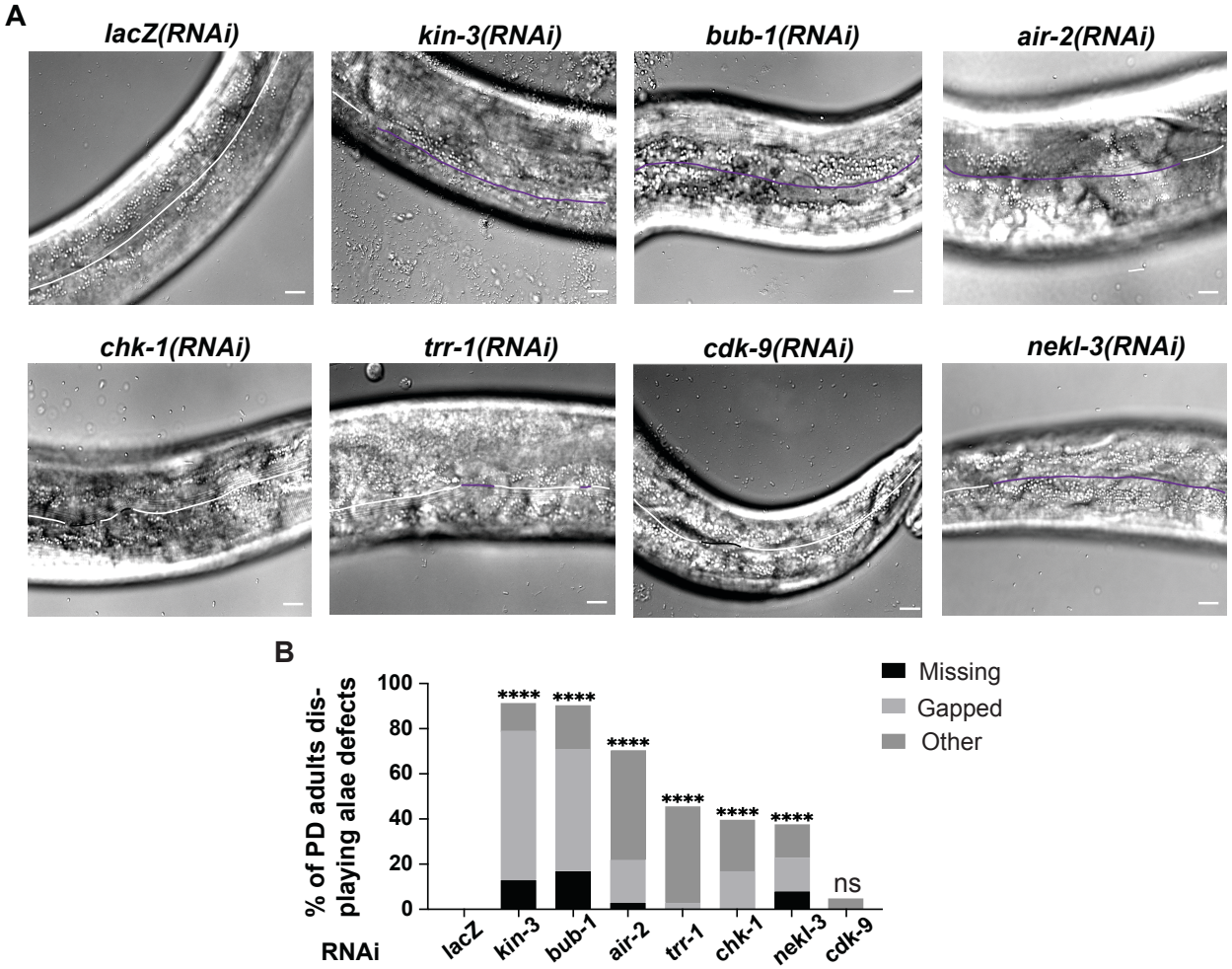

Fig. 2 RNAi of five kinase-encoding genes produces defects in adult alae formation in post-dauer *alg-1(0)* mutants. **A**. Comparison of alae phenotypes in post-dauer *daf-7(e1372); col-19p::gfp; alg-1(0)* adults. A compound microscope with DIC optics was used to visualize the presence of alae in post-dauer 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.

249 of *trr-1* or *cdk-9* caused a low-penetrance phenotype in both backgrounds, and there

250 was no significant difference between the backgrounds (Figure 3A). While these data do

251 not rule out the involvement of any of these genes in regulating miRISC activity, they

252 provide higher confidence in *bub-1, air-2, nekl-3,* and *chk-1* than *trr-1* or *cdk-9.*

253

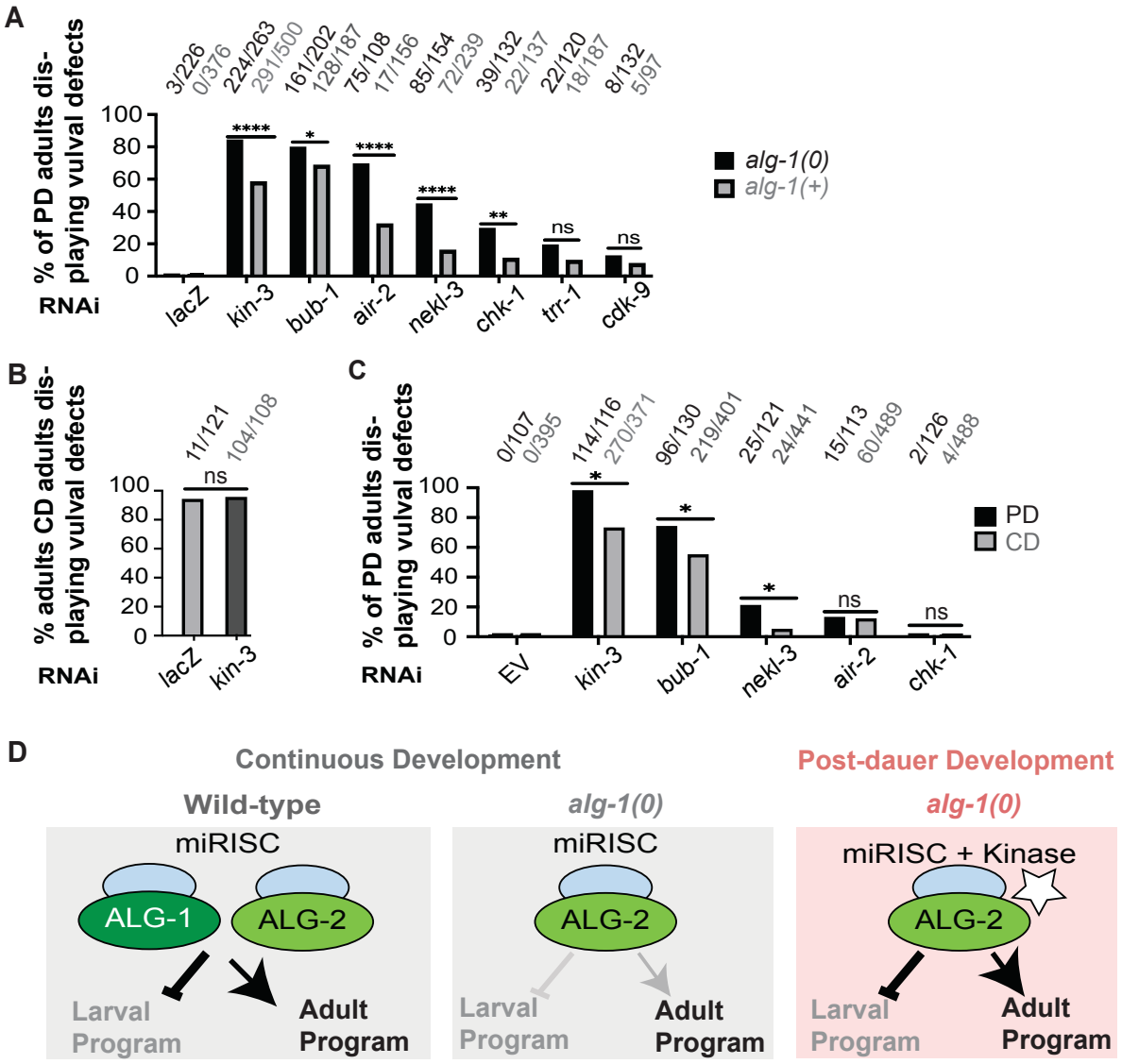

Fig. 3. RNAi of some kinases caused more penetrant phenotypes after dauer and when miRISC was compromised. A. Vulval defects in post-dauer adults with wild-type miRISC were compared to animals with compromised miRISC function *(alg-1(0)* mutants). Both strains contained *daf-7(e1372); col-19p::gfp* in the background. **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). In B and C, 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. Empty vector (EV) was used as a negative control. **D)** Model for role of miRISC activity in different developmental contexts to control larval vs. adult seam cell developmental programs. Note that other Argonautes could act in addition to or in place of ALG-2.

254

255 Since *alg-1(0)* mutants display vulval defects during continuous development but
256 not after dauer, we next wondered whether the kinases we identified play a greater role
257 in preventing vulval defects in post-dauer hermaphrodites than in those that develop
258 continuously, particularly when miRISC is compromised. We were unable to test this
259 question using *alg-1(0)* mutants because they display penetrant phenotypes during
260 continuous development (Figure 3B). However, animals lacking *alg-2* appear
261 superficially wild-type in both life histories (Grishok *et al.* 2001; Karp and Ambros 2012).
262 We therefore compared phenotypes in each life history following RNAi of *bub-1, nekl-3,*
263 *air-2*, and *chk-1* in an *alg-2(0)* mutant background. We found that RNAi of *bub-1* and
264 *nekl-3* both produced statistically more penetrant phenotypes after dauer (Figure 3C),
265 consistent with these kinases playing a larger role in post-dauer development.
266 By contrast, RNAi of *air-2* and *chk-1* produced low-penetrance phenotypes in both life
267 histories in the *alg-2(0)* background (Figure 3C). While in this latter case there was no
268 statistical difference between post-dauer and continuously developing animals,
269 observing a decrease in penetrance in adults that had developed continuously would be
270 difficult given the low penetrance phenotype in post-dauer adults. Thus, we are unable
271 to draw strong conclusions about the relative role of *air-2* and *chk-1* in post-dauer vs.
272 continuous development.
273 Finally, we performed RNAi of *bub-1, air-2, nekl-3,* and *chk-1* using independent
274 clones that target different regions of each gene than the region targeted in the original
275 clones. We observed vulval defects using each set of clones, albeit at reduced
276 penetrance (Figure S2). The percent of vulval defects observed for each clone was
277 statistically different from the empty vector control, except the newly created *chk-1* clone

278 (P = 0.088). Both original and newly created *chk-1* clones produced low-penetrance
279 phenotypes, whereas the negative control produced no vulval defects out of 226 adults
280 examined.
281
282 **Kinases identified in this screen**
283 We have identified four kinases that, when depleted, reproducibly enhanced two
284 different *alg-1(0)* phenotypes in post-dauer animals and whose depletion caused more
285 severe defects in a miRISC-compromised background than in wild type (Table 1). The
286 known roles for these kinases are described below.
287 Among the four genes identified as the highest confidence candidates in our
288 screen, *nekl-3* is the only one to have been previously implicated in the regulation of
289 miRNA activity. *nekl-3* was isolated in a large-scale RNAi screen for genes that
290 enhance the Rup phenotype of mutants with reduced activity of the *let-7* miRNA (Parry
291 *et al.* 2007). Although *nekl-3* was a relatively weak enhancer and the screen was
292 performed during continuous (non-dauer) development, this observation is consistent
293 with the hypothesis that *nekl-3* can promote miRISC function. This role for *nekl-3*
294 remains unexplored. In contrast, a role for *nekl-3* in the regulation of molting has been
295 well characterized. *nekl-3* is required for the internalization via clathrin-mediated
296 endocytosis of cargo important for molting (Yochem *et al.* 2015; Joseph *et al.* 2020).
297 Specifically, *nekl*-3 is required for the L1-L2 and L2-L3 molts but not later molts during
298 continuous development. In the dauer life history, *nekl*-3 has a reduced requirement for
299 the L2d-dauer molt and is dispensable for post-dauer molting (Binti *et al.* 2022).
300

Table 1: Conservation and function of genes isolated in the screen				
***C. elegans* gene**	**Human ortholog**	**Connection to microRNA function**	**Characterized function**	**References**
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)

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 If the kinases we identified do regulate miRISC, this regulation could be direct or
316 indirect. Consistent with direct regulation, *bub-1* and *nekl-3* are expressed in tissues
317 displaying *alg-1(0)* phenotypes, including the vulva and the hypodermis (Yochem *et al.*
318 2015, Tarailo-Graovac *et al.* 2010). Post-embryonic expression data outside of the
319 germline has not been described for *air-2* or *chk-1.* Furthermore, no expression data for
320 any of the four kinases has been reported in post-dauer animals. It will be interesting to
321 determine whether these kinases are expressed in relevant tissues in that context. In
322 addition, core miRISC proteins including ALG-1, ALG-2, AIN-1, and AIN-2, have many
323 phospho-sites identified by phosphoproteomic studies (Huang *et al.* 2020, Li *et al.*
324 2021). It is unclear whether these sites may be phosphorylated by the kinases identified
325 in this study. There are no defined consensus sequences phosphorylated by BUB-1 and
326 NEKL-3. There are loose consensus sequences defined for the human orthologs of
327 AIR-2 ([RK]X[TS]) and CHK-1 (RXX[TS]) that may also apply to *C. elegans* (Sanchez *et*
328 *al.* 1997; Cheeseman *et al.* 2002; Chen *et al.* 2003; Bishop *et al.* 2005; Ferrandiz *et al.*
329 2018). ALG-1, ALG-2, AIN-1, and AIN-2 all contain sequences that match these loose

330 consensus sites; however, these sequences were not found to be phosphorylated in the
331 aforementioned phosphoproteomic studies (Huang *et al*. 2020, Li *et al.* 2021). Notably,
332 these studies were not performed in post-dauer animals. It will be interesting to explore
333 the possibility of direct regulation of miRISC components by these kinases in future
334 work.
335
336 **Concluding Remarks**
337 The goal of this study was to identify kinase-encoding genes that may enhance
338 miRISC function in post-dauer development. This study initially found six candidate
339 genes required to prevent developmental phenotypes in post-dauer *alg-1(0)* animals.
340 Four of these genes, *bub-1, air-2, nekl-3,* and *chk-1*, showed higher penetrance
341 phenotypes in a miRISC-compromised background, making these four genes the most
342 likely to affect miRISC activity after dauer. *bub-1* and *nekl-3* produced more penetrant
343 phenotypes after dauer than during continuous development in an *alg-2(0)* background,
344 whereas *air-2* and *chk-1* produced low-penetrance phenotypes in both life histories in
345 an *alg-2(0)* background.
346 Prior to this work, the only kinase-encoding gene known to enhance *alg-1(0)*
347 phenotypes after dauer was *kin-3,* used as a positive control in this study (Alessi *et al.*
348 2015). During continuous development, three kinases have been shown to
349 phosphorylate miRISC components to modulate different aspects of miRNA activity:
350 KIN-1/PKA, casein kinase 1 (CK1) and CK2 (Alessi *et al.* 2015; Huberdeau *et al.* 2022;
351 Shah *et al.* 2023). In our screen, only the CK2-encoding genes *kin-3* and *kin-10* were
352 found to be necessary to prevent vulval defects in *alg-1(0)* mutants after dauer, whereas

353 RNAi of *kin-1* and *kin-19,* encoding PKA and CK1 respectively, did not cause vulval
354 defects in our primary screen (*kin-1,* 0/40; *kin-19,* 0/60). While these findings may be
355 explained by incomplete knockdown of *kin-1* and *kin-19,* it is also possible that distinct
356 kinases modulate miRISC in the dauer life history. This study lays the foundation for
357 future studies that can dissect the molecular relationship between these genes and
358 miRISC in *C. elegans* and other species.
359
360 **Data availability.** All *C. elegans* strains and newly made RNAi clones are available
361 upon request. The authors state that all data necessary for confirming the conclusions
362 of the article are present within the article, figures, and tables, including supplementary
363 information.
364
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