Nematode germ granule assembly is linked to mRNA repression

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Summary

RNA-protein (RNP) granules are non-membrane bound organelles with enigmatic roles in RNA metabolism. Metazoa contain RNP “germ granules” specialized for germline development. *Caenorhabditis elegans* P-granules are liquid droplet germ granules that require PGL proteins for assembly. Here we investigate PGL proteins to understand the relationship between P-granule assembly and germline function. We determine the crystal structure of a PGL N-terminal domain (NTD) and find that it dimerizes. From the structure, we identify mutations that disrupt PGL dimerization *in vitro* and prevent PGL granule formation in mammalian cells in culture. These same mutations in nematodes prevent assembly of PGL into P-granules and cause sterility. Using a protein-mRNA tethering assay, we show that mRNAs recruited to PGL-1 are repressed, while mRNAs recruited to PGL-1 mutants defective for granule assembly are expressed. Therefore, the effects of PGL on mRNA repression and fertility are tightly linked to its formation of higher-ordered assemblies.

Introduction

Subcellular localization can be critical for RNA control. The locations of RNAs and RNA regulatory proteins within a cell can dictate whether RNAs are translated or repressed (Singh et al., 2015). RNA-protein (RNP) granules are non-membrane bound "organelles" found ubiquitously in cells. These granules can be relatively inert, with little exchange of components between the granules and their environment, or they can behave as liquid droplets, with components capable of freely diffusing between the granule and cytoplasm (Hyman et al., 2014; Wu and Fuxreiter, 2016). RNP granules contain factors that regulate mRNA turnover, sequestration and translation and have been proposed to function in mRNA regulation (Buchan, 2014). Yet despite intense interest, the connection between granule assembly and biological function is poorly understood.

52 Metazoan germ cells contain specialized RNP germ granules with functions in RNA metabolism
53 and small regulatory RNA biogenesis (Voronina et al., 2011). *Caenorhabditis elegans* germ
54 granules, called P-granules, are necessary for adult germ cell maintenance and totipotency
55 (Strome and Updike, 2015). P-granules display liquid droplet behavior (Brangwynne et al.,
56 2009) and have similarities in subcellular location and composition to germ granules in
57 *Drosophila* and vertebrates (Voronina et al., 2011). In adult germ cells (**Figure 1A**), P-granules
58 localize to the cytoplasmic face of nuclear pores and contain mRNAs and proteins involved in
59 RNA metabolism (Updike and Strome, 2010). Yet the molecular function of P-granules is poorly
60 defined. One suggested role has been mRNA repression. This idea is based on localization of
61 repressed mRNAs to P-granules (Schisa et al., 2001) and upregulation of aberrant transcript
62 expression when P-granules are lost (Campbell and Updike, 2015; Knutson et al., 2017; Updike
63 et al., 2014). Because these lines of evidence are indirect and based on gene knockouts of
64 major P-granule assembly proteins, it remains unclear whether transcript repression depends
65 on P-granule components or their assembly into granules.
66
67 P-granule formation relies on key assembly proteins. Crucially important are the PGL-1 and
68 PGL-3 proteins (Kawasaki et al., 2004; Kawasaki et al., 1998), close paralogs we henceforth
69 refer to as PGL. PGL protein contains a central dimerization domain (DD) and C-terminal low
70 complexity RGG repeats (**Figure 1B**) (Aoki et al., 2016; Kawasaki et al., 2004; Kawasaki et al.,
71 1998). Genetic removal of PGL causes mislocalization of P-granule proteins (Amiri et al., 2001),
72 aberrant expression of spermatogenic and somatic mRNAs (Campbell and Updike, 2015;
73 Knutson et al., 2017; Updike et al., 2014), and temperature-dependent sterility (Kawasaki et al.,
74 2004; Kawasaki et al., 1998). PGL proteins self assemble into granules, both *in vitro* using
75 purified recombinant protein (Saha et al., 2016), and in intestinal nematode cells or mammalian
76 cells in culture when expressed on their own (Hanazawa et al., 2011; Updike et al., 2011).
77 These artificial PGL granules display liquid droplet behavior (Saha et al., 2016), indicating that

PGL protein alone is sufficient to recapitulate the biophysical properties of P-granules in nematode cells (Brangwynne et al., 2009). Many RNP granule assembly proteins rely on low complexity sequences for low affinity, multivalent interactions (Banani et al., 2017). PGL lacks large regions of low complexity, with the exception of its C-terminal RGG repeats that are not necessary for granule formation (Hanazawa et al., 2011; Saha et al., 2016). The central DD domain can dimerize (Aoki et al., 2016), but higher ordered assembly demands additional protein contacts.

P-granules serve as a paradigm for RNP granules with liquid droplet properties. We have investigated the PGL assembly protein to understand PGL multimerization into a granule and to probe the relationship between granule formation and its biological function. We determined the structure of the PGL N-terminal domain (NTD), found that it dimerizes and identified amino acids required for NTD dimerization that are also required for PGL assembly into granules *in vitro*, in mammalian culture cells and germ cells in living nematodes. Indeed, PGL multimerization is critical for nematode fertility. Using a protein-mRNA tethering assay in living nematodes, we show that reporter mRNAs recruited to PGL are repressed, and that their repression requires PGL assembly into granules. This study therefore provides direct *in vivo* evidence that RNP granule formation is linked to mRNA repression.

Results

Structure determination of a second PGL dimerization domain

Granule formation is driven by multimer-multimer interactions (Bergeron-Sandoval et al., 2016). The central PGL DD provides one multimerization site (Aoki et al., 2016), but since PGL protein alone can form granules (Hanazawa et al., 2011; Saha et al., 2016), we postulated the existence of another PGL multimerization region critical for granule assembly. The region N-terminal to DD (**Figure 1B**) had high sequence conservation (**Figure S1A**), implying a critical

104 role in PGL function. Our initial efforts to express trypsin-mapped recombinant protein fragments
105 of this N-terminal region proved unfruitful. However, we had noticed that the original DD N-
106 termini were disordered in crystal structures (Aoki et al., 2016). When the N-terminal
107 recombinant protein was extended to include these disordered residues (**Figure S1A**), we
108 obtained robust expression sufficient for biochemical and structural characterization (**Figure**
109 **S2A-B**). Henceforth, we refer to this stable protein fragment as the N-terminal domain (NTD)
110 (**Figure 1B**).
111
112 We determined the *C. japonica* PGL-1 NTD crystal structure to 1.5 Å (**Figure 1C-D**, see **Table**
113 **S1** for statistics, see Methods for further details on crystallization and structure determination).
114 The NTD had a novel fold consisting of 11 alpha helices and a single N-terminal beta strand
115 (**Figure 1D**). The crystal asymmetric unit (ASU) was composed of four NTD domains (**Figure**
116 **1C**). These four NTDs were structurally similar (RMSD 0.219 - 0.254, chains B-D aligned to A),
117 except for minor differences in their termini and internal loops. More relevant, they possessed
118 two pairs of identical interfaces (**Figure 1C**). One of these interface pairs consisted of a network
119 of conserved amino acid side chains making extensive salt bridges and hydrogen bonds
120 (**Figures 2A-C and S2C-E**). The complexity and conservation of these interactions suggested
121 biological relevance. We first tested for dimerization *in vitro*. Recombinant PGL-3 NTD formed a
122 dimer on a sizing column combined with multi-angle light scattering (SEC-MALS, **Figure 2D-E**).
123 We postulated that the conserved interface in the NTD crystal structure might be its dimerization
124 interface. To test that idea, we used our structural model and *in silico* prediction (Kortemme et
125 al., 2004) to design missense mutations that disrupt the interface. These analyses yielded two
126 distinct mutants: K126E K129E with two mutated residues and R123E with a single mutated
127 residue. Both NTD mutants formed monomers rather than dimers in solution (**Figure 2D-E**). We
128 conclude that the dimers observed in the crystal structure represent the NTD dimer detected in
129 solution.

130

131 **PGL NTD dimerization is critical for granule formation**

132 To assess the role of NTD dimerization in PGL granule self-assembly, we first turned to an
133 assay in mammalian cells where PGL expressed alone assembles into granules (Hanazawa et
134 al., 2011). Similar to that report, GFP-tagged PGL-1 formed large cytoplasmic granules in cells
135 (**Figure 2F-G**), while GFP alone was diffuse (**Figure 2H**). However, if we mutated the GFP-
136 tagged PGL-1 to either K126E K129E or R123E, PGL-1 no longer formed granules (**Figure 2I-**
137 **J**). We conclude that NTD dimerization is essential for self-assembly of PGL granules in
138 mammalian cells.

139

140 We next asked whether NTD dimerization was essential for PGL function and granule assembly
141 in the nematode germline. We inserted a SNAP tag (Keppler et al., 2003) using CRISPR gene
142 editing (Paix et al., 2015) at the endogenous *pgl-1* locus (**Figure 3A and S1B**). Wild-type PGL-1
143 (N2) and PGL-1::SNAP were similarly fertile at 20°C and 25°C (**Figure 3B**); in contrast, a *pgl-1*
144 null mutant was mostly sterile at 25°C (**Figure 3B**), as reported previously (Kawasaki et al.,
145 2004; Kawasaki et al., 1998). SNAP-tagged PGL-1 permitted visualization of the protein with
146 essentially no background (**Figure 3C-D**). PGL-1::SNAP assembled into cytoplasmic granules
147 at the nuclear periphery (**Figure 3D**), similar to those seen with antibody staining to untagged
148 PGL-1 and PGL-3 (Kawasaki et al., 2004; Kawasaki et al., 1998). The SNAP-tagged protein
149 therefore provides a simple way to evaluate PGL assembly into granules.

150

151 To probe the role of PGL NTD dimerization in the nematode germline, we introduced the
152 assembly mutants into PGL-1::SNAP (**Figure S1B**) and assayed effects on fertility and granule
153 formation. For both K126E K129E and R123E mutants, many were sterile at 20°C and nearly all
154 were sterile at 25°C (**Figure 3B**). Indeed, the percentage of sterile animals was higher than
155 seen in a *pgl-1* null mutant (**Figure 3B**), suggesting that abolishing NTD dimerization had a

156 dominant-negative effect. Both interface mutants had smaller than normal germlines and many
157 lacked oocytes (**Figure S3B-D**), similar to *pgl-1* and *pgl-1 pgl-3* null mutant germlines
158 (Kawasaki et al., 2004; Kawasaki et al., 1998). We next examined expression and localization of
159 mutant SNAP-tagged PGL-1 proteins, in both fertile (**Figure 3**) and sterile germlines (**Figure**
160 **S3**). Both K126E K129E and R123E mutant proteins were expressed, but their distribution was
161 largely diffuse (**Figures 3E-F and S3F-G**). The mutant proteins did form small perinuclear
162 granules in some germ cells of all gonads imaged (**Figure 3E-F and S3F-G**), and for each
163 mutant we found a single germline (1/59 for K126E K129E; 1/54 for R123E) with small PGL-1
164 perinuclear granules in all germ cells (**Figure S3H-I**). Therefore, both PGL-1 mutant proteins are
165 capable of incorporating into P-granules, but do so much more weakly than their wild-type
166 counterparts (**Figure 3D**). We conclude that PGL NTD dimerization is critical for fertility and
167 efficient PGL granule formation.
168
169 We wondered why sterility of PGL-1 NTD dimerization mutants was more severe than that of a
170 *pgl-1* null mutant. One plausible explanation was interference with assembly of other P-granule
171 components into granules, which might yield dominant-negative effects. Normally, PGL-1
172 interacts with PGL-3 (Kawasaki et al., 2004), and both PGL-1 and PGL-3 rely on GLH-1 or GLH-
173 4 Vasa helicases to localize to the nuclear periphery (Spike et al., 2008; Updike et al., 2011). In
174 contrast, GLH proteins assemble at the nuclear pore independently of PGLs (Kawasaki et al.,
175 2004; Kawasaki et al., 1998; Kuznicki et al., 2000). We postulated that PGL-1 assembly mutants
176 might interfere with assembly of PGL-3 into granules but not affect GLH-1 localization. To test
177 this idea, we epitope-tagged endogenous *pgl-3* and *glh-1* (see Methods) and compared
178 localization of PGL-3::V5 and GLH-1::Myc in germ cells expressing wild type PGL-1::SNAP or
179 mutant PGL-1::SNAP K126E K129E. With wild-type PGL-1::SNAP, all three proteins, PGL-1,
180 PGL-3 and GLH-1, co-localized in granules at the nuclear periphery (**Figure 3G-K**), as
181 previously observed for untagged proteins (Kawasaki et al., 2004; Kuznicki et al., 2000).

182 However, with mutant PGL-1::SNAP, the wild-type PGL-3 protein became diffuse with
183 occasional small perinuclear granules, a distribution similar to PGL-1 mutant protein (**Figure 3L-**
184 **N**). By contrast, GLH-1 localized at the nuclear periphery independent of PGL-1 or PGL-3
185 (**Figure 3O-P**). Therefore, the PGL-1 assembly mutant affected PGL-3 assembly and
186 incorporation into P-granules, but it did not abolish GLH-1 localization. Because the percent
187 sterility of PGL-1 K126E K129E mutants was similar to that of *pgl-1 pgl-3* double null mutants
188 (Kawasaki et al., 2004), we suggest that the severe sterility of PGL-1 NTD mutants results from
189 effects on both PGL-1 and PGL-3 assembly into granules.
190
191 **Granular PGL represses mRNAs *in vivo***
192 Prior studies have suggested that P-granules regulate mRNA expression (see Introduction). To
193 directly test whether P-granules can regulate mRNAs, we relied on a protein-mRNA tethering
194 assay, widely used to investigate RNA regulatory proteins (Baron-Benhamou et al., 2004; Coller
195 and Wickens, 2002). Our assay examined the fate of mRNAs to which PGL-1 was tethered via
196 λN22, a short peptide that binds with high affinity and sequence specificity to the boxB RNA
197 hairpin (Baron-Benhamou et al., 2004). Versions of this method were used previously in worms
198 and other organisms (Baron-Benhamou et al., 2004; Wedeles et al., 2013). For the reporter, we
199 inserted three boxB sites into the 3'UTR of a ubiquitously-expressed, germline GFP-histone
200 reporter (**Figure 4A**, Methods) (Zeiser et al., 2011). To tether PGL to the GFP reporter mRNA,
201 we inserted the λN22 peptide sequence into our PGL-1::SNAP protein (**Figures 4A** and **S1B,**
202 Methods). Addition of λN22 to PGL-1 rendered homozygous worms sterile (0% fertile, n=94),
203 but the λN22-tagged *pgl-1* gene could be maintained and tested as a fertile heterozygote (PGL-
204 1::SNAP::λN22/+). The logic of our strategy is simple: if tethered wild-type PGL-1 represses
205 GFP expression, we can then test assembly-defective PGL-1 to ask if repression relies on
206 granule formation (**Figure 4A**).
207

208 We assayed reporter expression in both living animals and fixed, extruded gonads. In living
209 animals harboring both reporter and PGL-1::SNAP without λN22, GFP was expressed robustly
210 (**Figures 4B**), but in those with both reporter and PGL-1::SNAP::λN22, GFP was absent (**Figure**
211 **4C**). When fixed, control germlines expressed GFP robustly (**Figure 4E**), but those with PGL-
212 SNAP::λN22 had either no detectable GFP (**Figure 4F**) or extremely faint GFP (2/39 germline).
213 The wild-type PGL-1::SNAP::λN22 formed perinuclear granules (**Figure 4E-F**), though the
214 SNAP signal was lower, perhaps because animals were heterozygous. Regardless, the key
215 conclusion is that PGL-1 tethering dramatically decreased GFP expression from the mRNA
216 reporter.
217
218 One possible explanation for loss of GFP expression might have been germline silencing, a
219 phenomenon common for genes expressing foreign proteins and thought to prevent deleterious
220 mRNAs from entering the cytoplasm (Hoogstrate et al., 2014). To ask if the reporter had been
221 silenced, we used single molecule fluorescence *in situ* hybridization (smFISH) to detect *gfp*
222 RNAs. Control germ cells harboring PGL-1::SNAP without λN22 (**Figure S4A-D**) possessed
223 nuclear and cytoplasmic puncta (**Figure S4D**). We interpret the nuclear puncta as active
224 transcription sites and cytoplasmic puncta as mRNAs, based on a previous study (Lee et al.,
225 2016). In these control germ cells, GFP protein fluorescence was robust (**Figure S4B**), and
226 PGL-1::SNAP localized to perinuclear granules (**Figure S4C**). Germ cells harboring PGL-
227 1::SNAP::λN22 (**Figure S4E-H,M**) also possessed nuclear and cytoplasmic puncta (**Figure**
228 **S4H,M**), but cytoplasmic puncta were fewer and frequently colocalized with P-granules (**Figure**
229 **S4G-H,M**). These germ cells possessed *gfp* reporter transcripts and thus were not subject to
230 germline silencing. However, they had no GFP protein expression, indicating that reporter
231 expression was repressed by PGL-1 tethering.
232

233 Our structural insight into PGL provided an opportunity to test how PGL granule formation
234 affected its ability to repress mRNAs. We introduced K126E K129E into PGL-1::SNAP::λN22 to
235 prevent robust granule assembly (**Figure S1B**). Mutant homozygotes had modest fertility (21%
236 fertile, n=96) that was comparable to other NTD mutant worms (**Figure 3B**). Dimerization-
237 defective PGL-1 failed to repress the reporter RNA to which it was tethered: most germ cells
238 expressed GFP, both in living worms (**Figure 4D**) and fixed gonads (**Figure 4G**). The PGL-1
239 mutant protein was diffuse and non-granular (**Figure 4G**), as expected. By smFISH for gfp
240 reporter RNA, germ cells with mutant PGL-1::SNAP::λN22 had large puncta in their nuclei and
241 small puncta throughout their cytoplasm (**Figure S4I-L**), similar to gonads expressing PGL-
242 1::SNAP without λN22 (**Figure S4A-D**). Formally, the PGL-1 interface residue mutations might
243 affect granule assembly and mRNA repression independently. However, the simplest
244 explanation is that PGL-1 must assemble into granules for mRNA repression.
245

246 **Discussion**

247 P-granules are paradigmatic liquid droplet RNP granules and have been predicted to be sites of
248 mRNA repression. This idea was based on several observations. Repressed mRNAs appear
249 trapped in P-granules (Schisa et al., 2001); P-granules are necessary to repress aberrant
250 expression of spermatogenic and somatic transcripts (Campbell and Updike, 2015; Knutson et
251 al., 2017; Updike et al., 2014); and P-granule components include inhibitory RNA binding
252 proteins, like the Pumilio homolog, FBF-2 (Voronina et al., 2012) and RNA regulatory enzymes,
253 like the Argonaut/Piwi PRG-1 (Batista et al., 2008) and the deadenylase PARN-1 (Tang et al.,
254 2016). In this work, we report the discovery of PGL NTD dimerization and demonstrate that PGL
255 NTD dimerization is critical for granule formation, fertility and mRNA repression *in vivo*. Based
256 on these results, we propose that mRNAs in P-granules are repressed and that this repression
257 requires PGL assembly into granules (**Figure 4H)**. PGL contains two dimerization domains (this
258 work; Aoki et al., 2016), but mutations disrupting DD dimerization have been elusive. A critical

259 future direction is to investigate how each dimerization domain contributes to higher order and
260 likely oligomeric assembly.
261
262 Granule assembly proteins form a structural network that relies on multivalency and low affinity
263 interactions for plasticity (Bergeron-Sandoval et al., 2016). We have discovered that PGL uses
264 at least one dimerization domain to form granules, but our results do not address the low affinity
265 interactions that must be present to drive liquid droplet behavior. Critical granule assembly
266 proteins have been identified for several RNP granules. Examples include Oskar and Vasa for
267 *Drosophila* polar granules (Breitwieser et al., 1996; Markussen et al., 1995; Vanzo and
268 Ephrussi, 2002), EDC3 and LSM4 for P-bodies (Decker et al., 2007), and MEG-3 and MEG-4
269 for embryonic P-granules (Wang et al., 2014). These examples rely on a combination of
270 multimerization domains and low complexity, intrinsically disordered sequences to facilitate
271 granule formation (Decker et al., 2007; Jeske et al., 2015; Ling et al., 2008; Nott et al., 2015;
272 Wang et al., 2014). We suggest that PGL also makes low affinity contacts that work with its
273 dimerization interfaces to facilitate granule formation. Recombinant PGL proteins make granules
274 on their own *in vitro* (Saha et al., 2016), suggesting that PGL has low affinity contacts in the full-
275 length protein. RGG repeats facilitate granule formation in other assembly proteins (Nott et al.,
276 2015), but PGL did not require its RGG repeats to form granules in mammalian cell culture
277 (Hanazawa et al., 2011). The RGG repeats may instead be needed to trigger robust granule
278 assembly with RNA (Saha et al., 2016) or impart liquid droplet properties associated with PGL in
279 nematodes (Brangwynne et al., 2009).
280
281 Our study provides direct evidence that recruiting mRNAs to a liquid droplet RNP granule
282 represses their expression. The mechanism of repression remains unclear. P-granule
283 components include RNA turnover enzymes and translation inhibitory proteins (Updike and
284 Strome, 2010) that may repress PGL-tethered mRNAs directly. Alternatively, tethered mRNAs

285 may be trapped within granules by avidity to high PGL concentrations, blocking their access to
286 translational machinery. Regardless, this work adds to the emerging theme that granules play a
287 general role in repression. Liquid droplet stress granules sequester the mTORC1 protein
288 complex to block activation of mTOR signaling (Wippich et al., 2013). Mammalian cells can trap
289 hormones and melanin in amyloid-like aggregates to prevent signaling (Fowler et al., 2006; Maji
290 et al., 2009). RNP granules have also been proposed to repress mRNAs. mRNA repressors are
291 found in RNP granules (e.g. DDX6/Dhh1, Decker and Parker, 2012) but their repressive
292 activities can function independently of granules (Carroll et al., 2011). Granule formation of a
293 yeast amyloid-like RNA binding protein correlates with translational inhibition of transcripts
294 critical for gametogenesis (Berchowitz et al., 2015), and P-bodies contain mRNAs that are
295 translationally repressed in cells (Hubstenberger et al., 2017). Further studies that pair insights
296 into mechanisms of granule assembly with direct *in vivo* assays of regulation will be pivotal
297 moving forward to decipher the mechanistic function of other RNP granules in their natural
298 biological context.
299

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445
446

447 **Figure Legends**

448 **Figure 1**. Crystal structure of the PGL NTD

449 (A) Left, *C. elegans* adult hermaphrodite possesses two gonadal arms with proliferating germ
450 cells at one end (asterisk) and differentiating gametes at the other. Gonads make sperm (blue)
451 first and then oocytes (pink). Right, P-granules (magenta) reside at the nuclear periphery of all
452 germ cells until late oogenesis. (B) Linear diagram of *C. elegans* PGL-1. (C) Crystal structure of
453 *C. japonica* PGL-1 NTD to 1.5 Å. NTD has four copies per asymmetric unit (ASU). Copies in
454 yellow, gold, tan, and brown. Arrows indicate two pairs of subunit interfaces in the ASU. Red
455 arrows highlight the interface relying on conserved amino acids (see text). (D) Enlarged image
456 of a single NTD.

457

458 **Figure 2**. NTD dimerization and its role in PGL self-assembly

459 (A) Structural model of the NTD dimer. (B-C) Enlargement of dimer interface (red box in A).
460 PGL-1 K126 and K129 (B), and R123 (C) interact with apposing subunit side chains. Residue
461 labels in yellow or gold to indicate their representative subunits. (D-E) Size exclusion
462 chromatography and multi-angle light scattering (SEC-MALS) of recombinant PGL-3 NTD wild
463 type (D-E), K126E K129E (D) and R123E (E) proteins. A280 UV absorbance (left y axis) was
464 normalized to the maximum value. Molecular weight (MW, right y axis) for MALS in daltons
465 (Da). Wild-type protein (blue) measured the approximate size of a dimer, while both mutant
466 proteins (red) measured approximately as monomers. (F) Diagram of *C. elegans* PGL-1 C-
467 terminally tagged with GFP. (G-J). Representative images of GFP-tagged PGL-1 (G), GFP
468 alone (H), and GFP-tagged PGL-1 K126E K129E (I) and R123E (J) mutants expressed in
469 Chinese Hamster Ovary (CHO) cells. Cell cultures were imaged live, and GFP-positive cells
470 counted for the presence or absence of granules. Images show the majority result (percentages
471 noted above image). Scale bar, 10 μm.

472

473 **Figure 3**. NTD dimerization is critical for fertility and P-granule formation in nematodes

474 (A) Site of SNAP tag insertion in *C. elegans* PGL-1. (B) Fertility of SNAP tagged PGL-1 animals.
475 Percentages were obtained after scoring individuals for production of larval progeny after 5 days
476 at either 20°C or 25°C. (C-P) Extruded adult germlines, fixed, stained and imaged in same
477 region of meiotic pachytene (see **Figure S3A**). (C-F) Representative images of SNAP staining
478 to visualize PGL-1 expression and granule formation. All images are partial z-stacks to
479 maximize visualization of P-granules. Images were taken from germlines producing embryos;
480 similar images were obtained from germlines too defective to make embryos (**Figure S3F-G**).
481 (C) Control, wild type animal lacking SNAP tag shows virtually no background staining (n=20).
482 (D) PGL-1::SNAP localizes to granules around nuclei (n=49). (E) PGL-1::SNAP K126E K129E
483 is diffuse (n=38). (F) PGL-1::SNAP R123E is diffuse (n=24). (G-P) Representative images
484 showing localization of three P-granule components in germ cells expressing either PGL-
485 1::SNAP (G-K, n=20) or PGL-1::SNAP K126E K129E (L-P, n=14). (G,L) DNA (DAPI); (H,M)
486 SNAP (PGL-1::SNAP or mutant); (I,N) V5 (PGL-3); (J,O) MYC (GLH-1); (K,P) Merge. Scale bar,
487 10 μm for all images, except two-fold enlargements of nuclei in white boxes that are placed
488 outside main images.

489

490 **Figure 4**. PGL assembly is required for repression of tethered mRNA reporter *in vivo*

491 (A) Tethering assay. The reporter mRNA encodes GFP-histone H2B and harbors three boxb
492 hairpins in its 3'UTR; a ubiquitous germline promoter drives expression (see Methods). λN22
493 peptide (light blue) is inserted into PGL-1::SNAP. Binding of PGL-1::SNAP::λN22 to boxB
494 hairpins recruits PGL-1 to reporter mRNA. (B-D) GFP reporter expression in germ cells of live
495 animals. Above, brightfield image; below GFP fluorescence (green); auto fluorescence (red). n,
496 number of animals scored for GFP expression. Scale bar, 10 μm, in (B) applies to all images.
497 (E-G) Representative images of PGL granule formation, seen by SNAP staining (magenta), and
498 GFP fluorescence (green) in fixed gonads. n, number of germlines scored for GFP expression.

499 Scale bar, 10 μm, in (E) applies to all images. (H) Model of P-granule assembly and function.
500 Left, NTD dimerization allows PGL granule formation, which traps and represses mRNA
501 transcripts. Right, loss of PGL granule formation derepresses granule-localized mRNA
502 transcripts. See text for further Discussion.
503
504

505 **Supplemental Figure Captions**

506

507 **Table S1**. *C. japonica* PGL-1 NTD crystal structure data and model statistics

508

509 **Figure S1**. PGL sequence alignment and locus

510 (A) Sequence alignment of PGL NTD domain in *C. elegans* (*Ce*), *C. japonica* (*Cj*), *C. brenneri*
511 (*Cbn*), *C. briggsae* (*Cbr*), *C. remanei* (*Cr*). Alignment and conservation (cons.) determined by T-
512 Coffee (Magis et al., 2014). Starred residues (*) are identical. Period (.) and colon (:) residues
513 are similar. Residues participating in salt bridges only are in orange. Residues participating in
514 hydrogen bonds only are in yellow. Residues forming both hydrogen bonds and salt bridges are
515 highlighted in red. *C. elegans* PGL-1 missense mutations and their allele numbers are labeled.
516 Dashed lines mark the end of PGL-1 NTD domain and start of PGL-1 DD domain. (B) *pgl-1*
517 (ZK481.4a.1) primary transcript. 5' and 3' UTRs are grey, exons are white, numbered 1-8 and
518 separated by introns. Sites of *pgl-1* mutations are labeled, including location of SNAP tag
519 (magenta) and λN22 fusion (blue).

520

521 **Figure S2**. Supplemental biochemical and structural analyses of PGL NTD

522 (A,B) Coomassie-stained polyacrylamide gel of recombinant PGL NTD wild type and mutant
523 protein. Ladder marker sizes labeled in kilodaltons (kDa) on right. (A) Recombinant *C. japonica*
524 (Cj) PGL-1 NTD protein used for crystallization. Recombinant *C. elegans* PGL-3 NTD protein
525 included for comparison. (B) Wild type and mutant *C. elegans* PGL-3 NTD recombinant proteins
526 used for biochemical characterization. (C) Tables of predicted hydrogen bonds and salt bridges
527 at the NTD dimerization interface. Amino acid numbers correspond to *C. japonica* PGL-1 NTD.
528 (D-E) Surface representation of the NTD dimerization interface. (D) Amino acids colored by
529 identity (red) and similarity (pink). (E) Amino acids at dimerization interface (purple).

530

531 **Figure S3**. Supplemental images of PGL-1 dimerization mutants

532 (A) Schematic of an adult hermaphrodite germline. An asterisk marks proliferating germ cells
533 here and in images B-D. The germline produces oocytes at this stage; sperm were made earlier
534 and stored in the spermatheca (not shown). Red box marks region imaged in F-I. (B-D)
535 Representative brightfield images of extruded gonads from worms grown at 20°C. Scale bar, 10
536 µm. (B) PGL-1::SNAP gonads are of normal size and produce oocytes and embryos. (C)
537 Representative images of PGL-1::SNAP K126E K129E sterile gonads, which are small and
538 produce no gametes. (D) PGL-1::SNAP R123E sterile gonads are also small and produce no
539 gametes. (F-I) Representative partial z-projection stacks of SNAP and DNA stained germlines.
540 Scale bar, 10 µm, applies for all images. (F,G) PGL-1 is expressed in sterile gonads (no
541 embryos observed). (F) n=21 gonads imaged; (G) n=30 gonads imaged. (H-I) In single rare
542 gonads, PGL-1 mutants were seen to assemble into granules in all germ cells. Note the diffuse
543 staining between nuclei, which is not seen in the wild-type PGL-1::SNAP (see **Figure 3D**).

544

545 **Figure S4**. GFP reporter is not subject to germline silencing

546 Gonads were extruded from animals harboring (A-D) PGL-1::SNAP (n=30); (E-H,M) PGL-
547 1::SNAP::λN22 (n=27); (I-L) PGL-1::SNAP::λN22 K126E K129E (n=10). Gonads were fixed and
548 imaged for *gfp* RNA using smFISH (A,E,I), GFP protein fluorescence (B,F,J), DNA (DAPI) and
549 SNAP (C,G,K). The three are merged in D, H, L, and M. White arrows mark examples of
550 intranuclear puncta; black arrows mark examples of cytoplasmic puncta. (I) Six additional
551 examples of germlines harboring PGL-1::SNAP::λN22, imaged for *gfp* RNA, DNA, and SNAP.
552 Scale bar, 5 µm, for all images, except image in inset (white box) enlarged 2.5-fold. For
553 germline location, see **Figure S3A**.

554

Figure 1
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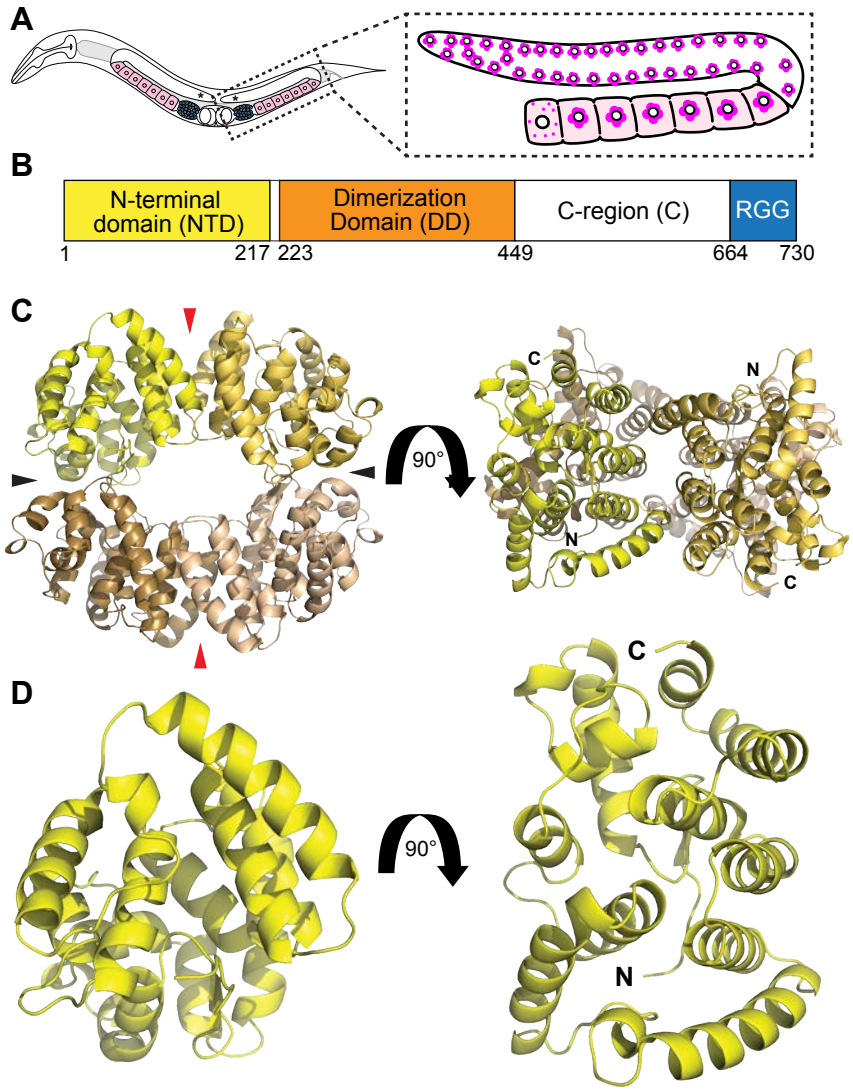

Figure 2
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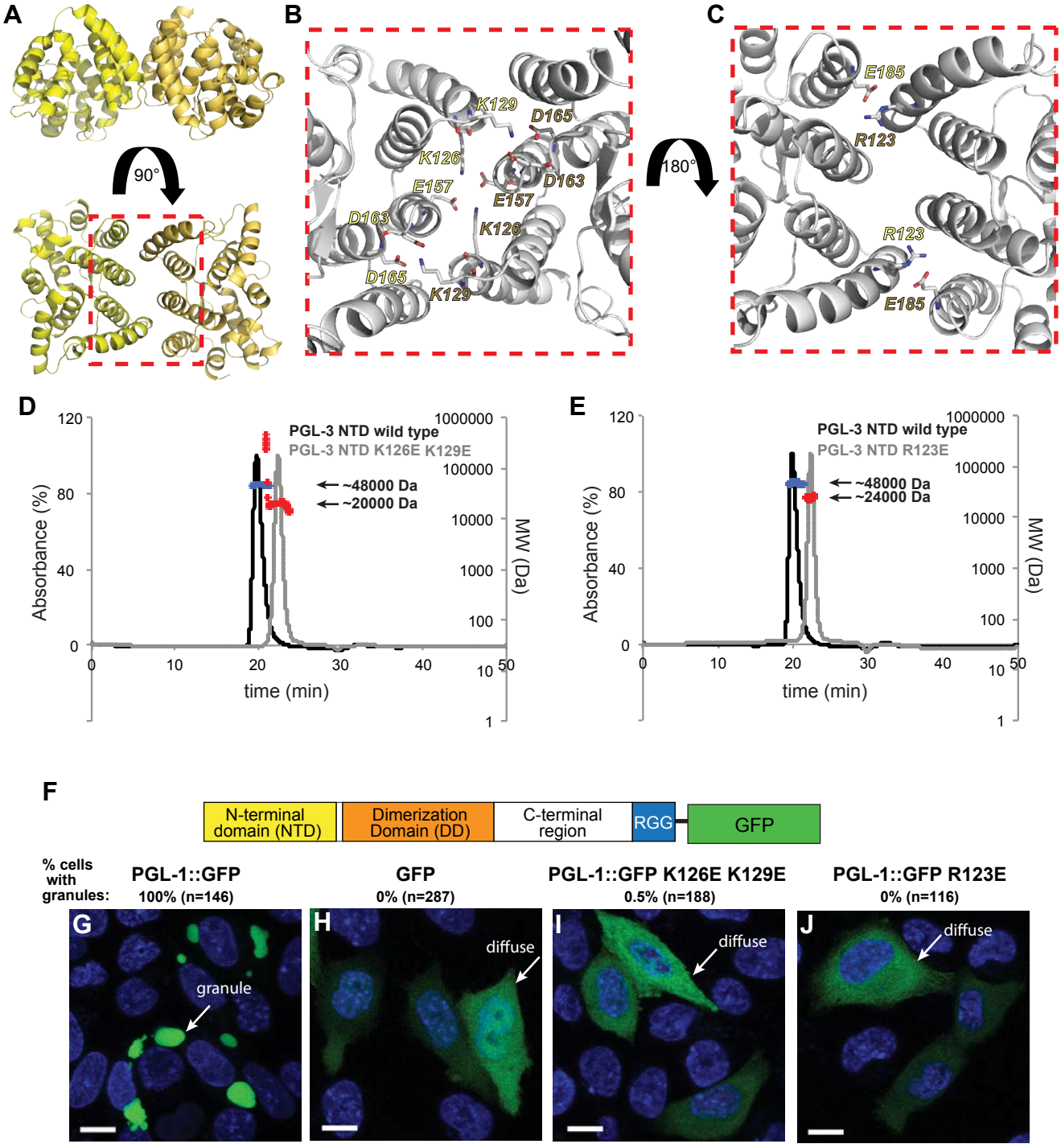

A

B

Protein	allele	20°C % sterile	20°C n	25°C % sterile	25°C n
PGL-1	N2	1.0	96	2.2	93
PGL-1::SNAP	*q894*	1.1	93	8.6	93
PGL-1 null	*bn102*	3.3	92	82.3	96
PGL-1::SNAP K126E K129E	*q960*	72.0	93	100	92
PGL-1::SNAP R123E	*q975*	22.1	95	97.9	96

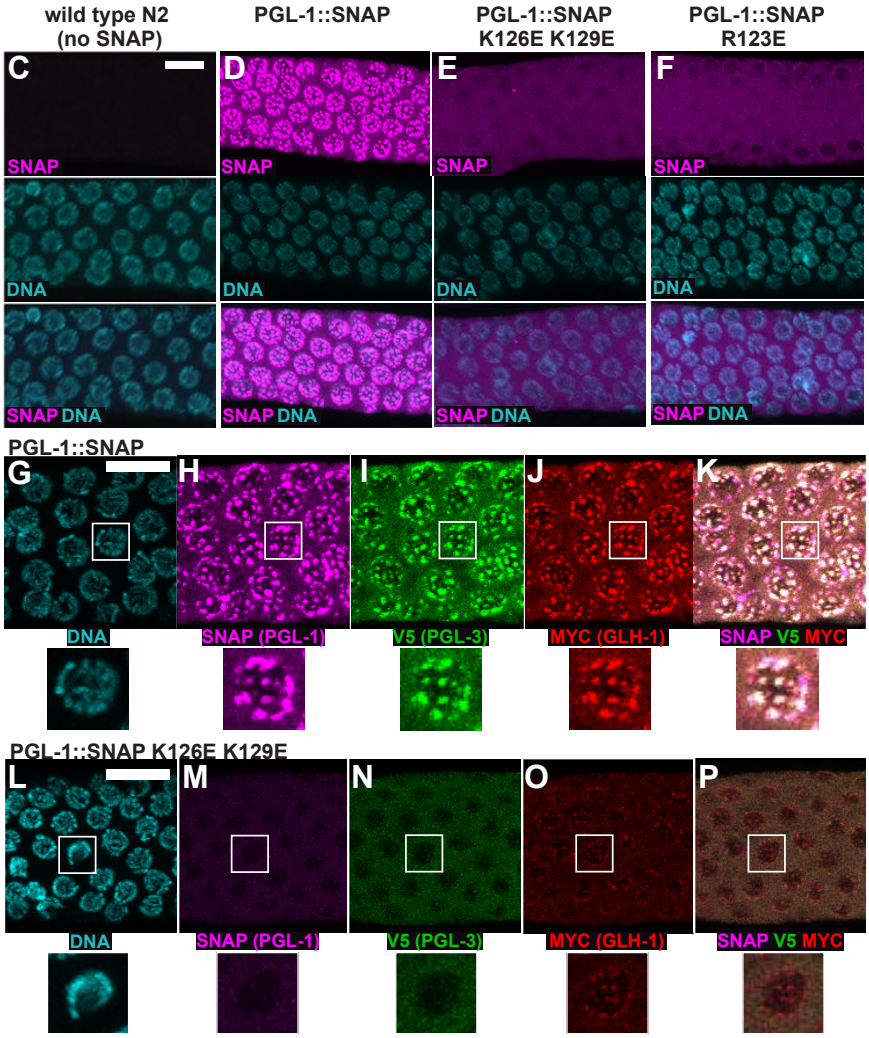

Figure 4
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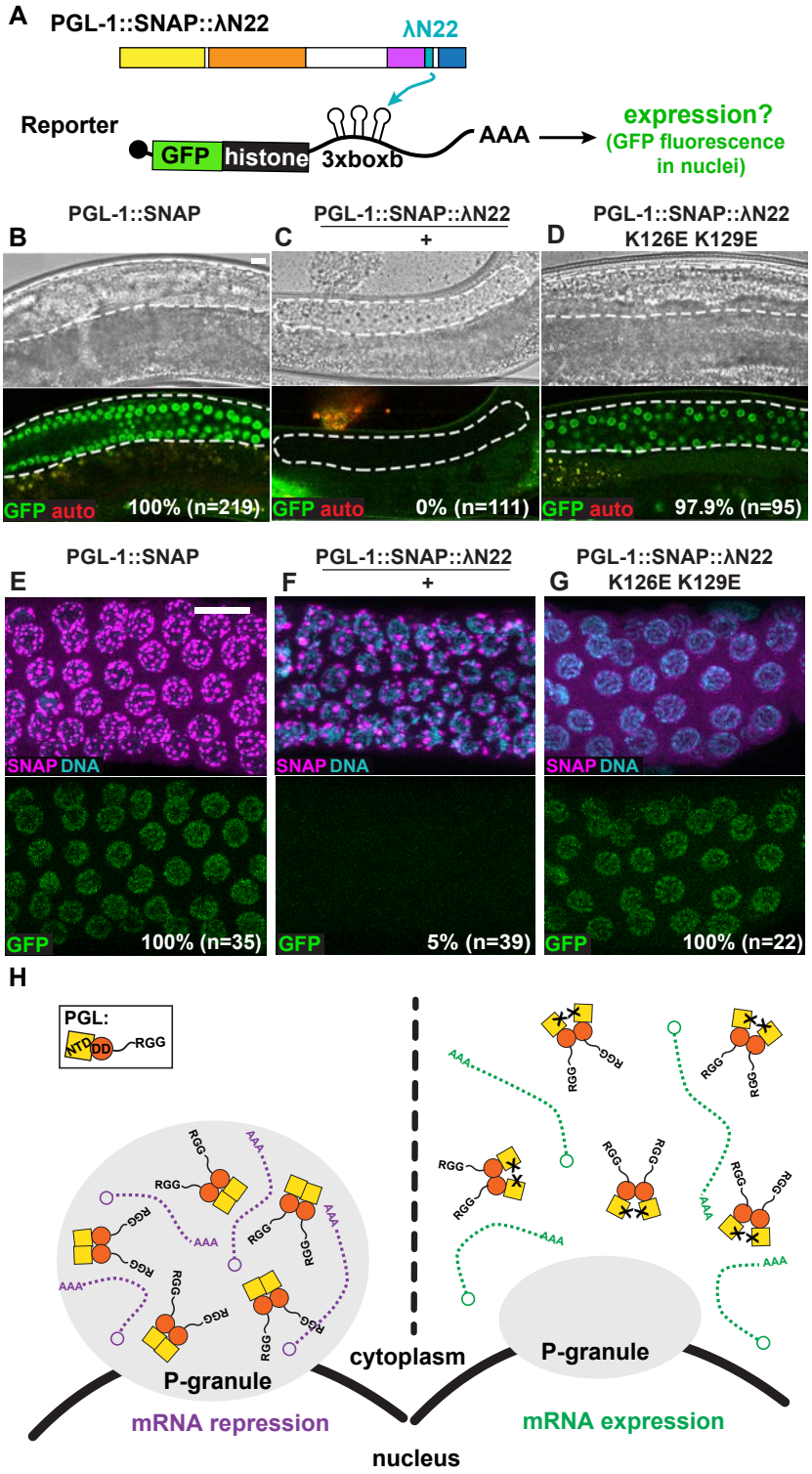

Table S1. Data collection and refinement statistics.

	JaPGL-1N SeMet (5W4D)	JaPGL-1N wt (5W4A)
Wavelength	0.9786	0.984
Resolution range	48.47 - 1.599 (1.656 - 1.599)	30.36 - 1.5 (1.554 - 1.5)
Space group	C 1 2 1	C 1 2 1
Unit cell	133.3 94.8 72.5 90 91.4 90	132.77 94.67 72.95 90 90.756 90
Total reflections	880332 (81451)	2176741 (194944)
Unique reflections	115340 (11239)	143729 (14301)
Multiplicity	7.6 (7.2)	15.1 (13.6)
Completeness (%)	97.06 (95.16)	99.77 (99.33)
Mean I/sigma(I)	24.44 (2.30)	16.91 (1.85)
Wilson B-factor	22.04	22.44
R-merge	0.0469 (0.8775)	0.08108 (1.282)
R-meas	0.05038 (0.945)	0.08353 (1.332)
R-pim	0.01827 (0.348)	0.01984 (0.357)
CC1/2	0.999 (0.74)	0.997 (0.662)
CC*	1 (0.922)	0.999 (0.893)
Reflections used in refinement	115302 (11238)	143649 (14297)
Reflections used for R-free	1424 (129)	1468 (151)
R-work	0.1607 (0.2590)	0.1681 (0.3038)
R-free	0.1925 (0.2707)	0.2039 (0.3232)
CC(work)	0.962 (0.864)	0.967 (0.802)
CC(free)	0.939 (0.819)	0.974 (0.794)
Number of non-hydrogen atoms	7593	7785
macromolecules	6768	6813
ligands	168	136

solvent	657	836
Protein residues	846	853
RMS(bonds)	0.010	0.010
RMS(angles)	1.00	0.99
Ramachandran favored (%)	98.68	98.10
Ramachandran allowed (%)	1.32	1.90
Ramachandran outliers (%)	0.00	0.00
Rotamer outliers (%)	1.33	0.66
Clashscore	2.29	1.86
Average B-factor	30.58	30.21
macromolecules	29.29	28.97
ligands	53.64	51.75
solvent	37.95	36.82
Number of TLS groups	1	1

Statistics for the highest-resolution shell are shown in parentheses.

Figure S1
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A

```
CePGL-1     1 MEANKREIVDFGGLRSYFFPNLAHYITKNDEELFNNTSQANKLAAFVLGASKDAPGDEDILEMILPNDANAAVIAAGMDV  80
CePGL-3     1 MEANKRQIVEVDGIKSYFFPHLAHYLASNDELLVNNIAQANKLAAFVLGATDKRPSNEEIAEMILPNDSSAYVLAAGMDV  80
CjPGL-1     1 MDTNKREIVEFLGIRTYFFPNLALYAVNNDELLVSDPNKANSFAAYVFGASDKKPSVDDIVQILFPSGSDSGTILTSMDT  80
CbnPGL-1    1 MEANKREIVETGGIKSYLFSNLAQYVTKNAELLQKTPKQANSLAAFVIGVSAERPTKDDILEMIIPNGANAAVLAAGMDV  80
CbrPGL-1    1 MELNKREIVEVGGIKSCFFPNLALYASKNSEALLNDPKSTNLFAANVFGALKDQPNENDITEMILPQDANADVLAAGMDA  80
CrPGL-1     1 MENNKRGVVEAKGIKSHYFQTLANYVSNNLELLHNNPKQANSFAASVFGSTAPI-DEKDLLDLLVPSDANADALAAGMDC  80
cons.         *: *** :*:  *:::  *  ** *  .* * * .   .:* :** *:*        .:: :::.*..:.: .: :.**

                                                  R123   K126   K129
CePGL-1    81 CLLLGDKFRPKFDAAAEKLSGLGHAHDLVSVID-----DDKKLGMLARKAKLKKTEDAKILQALLKV-IAIDDAAEKFVE 154
CePGL-3    81 CLILGDDFRPKFDSGAEKLSQLGQAHDLAPIID-----DEKKISMLARKTKLKKSNDAKILQVLLKV-LGAEEAEEKFVE 154
CjPGL-1    81 LLALGPDFLTEFKKRNQDLARFNLTHDLSILAQGDEDAAKKKLNLMGRKAKLQKTEAAKILAILIKT-INSEENYEKFTE 159
CbnPGL-1   81 CFLLGEEYRTNFQTAGEQLAQLNHSHDILAAVD-----DKKKLESLLRKTKIRKTPDAKILQRILTVHLEREEPLEKFEE 155
CbrPGL-1   81 CLLLGKQYHQLFESANERLSVLGRTHDLASIKD-----DEKKLTVLARRTKLKKTEGAKILQILIEA-IAEEDVFEKFMK 154
CrPGL-1    81 CLLLGEKYRPHFDAAVQQLARLGRTHDVATVID-----DEKKFTALSKKTKLKKTDEAKILQAFFKI-HST-EDEEKFEA 153
cons.          : ** .:   *.   : *: :. :**:    :      .**:  : :::*::*:  ****  ::       :  ***

                                                                NTD domain      DD domain
CePGL-1   155 LTELVSQLDLDFDVYVLTKILGLISEETSDEVDIIRDNVVNAFDSCKPLLKQLMLDGPK---SEPADPFISLLMDPL-EE 230
CePGL-3   155 LSELSSALDLDFDVYVLAKLLGFASEELQEEIEIIRDNVTDAFEACKPLLKKLMIEGPK---IDSVDPFTQLLLTPQ-EE 230
CjPGL-1   160 LSELC-GLDLDFDAYVFTKILGLEDEDTADEVEVIRDNFLNRLDQTKPKLADIIRNGPAVVELTPAEQFSRLLEVPVDES 238
CbnPGL-1  156 LSELC-ALDLDFDAYVLIKLLDIENEETAEEIEVVRENIQEIFQKANPLLKQLMEQGPQ---TEPADEFTELLRAPIGDD 231
CbrPGL-1  155 IAELC-SHDLDFDAYVLIKALGLECEEAVEEFKIIRENVLAVLKECNPLLSELLVDGPK---GAPVNEFTQLLLGSLSEE 230
CrPGL-1   154 ISELC-QLDLDFDAYVFIKALTLENEENQELVETIKDNLVEAWNKSNPLLVKLLLEGVKE--QDPVDKFTYLLLQPLTEA 230
cons.         ::**    *****.**: * * :  *:  : .. :::*.    .  :* * .:: :*       ..: *  **  .  :
```

B ***pgl-1*** **(ZK381.4a.1)**

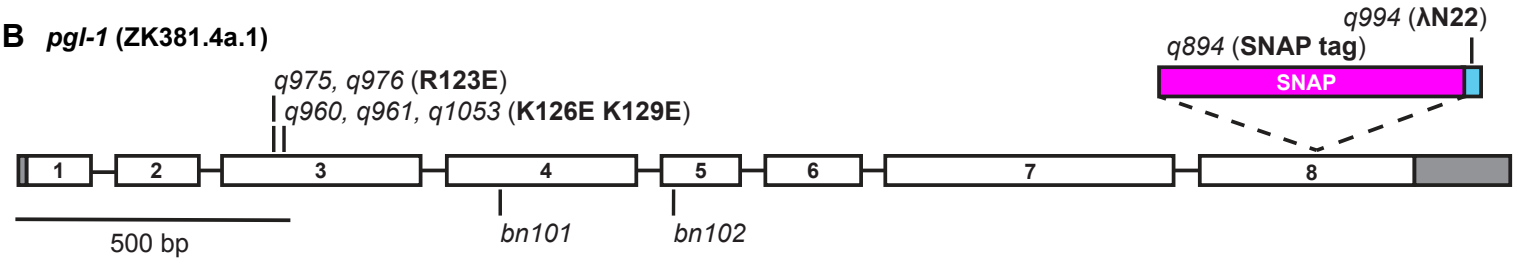
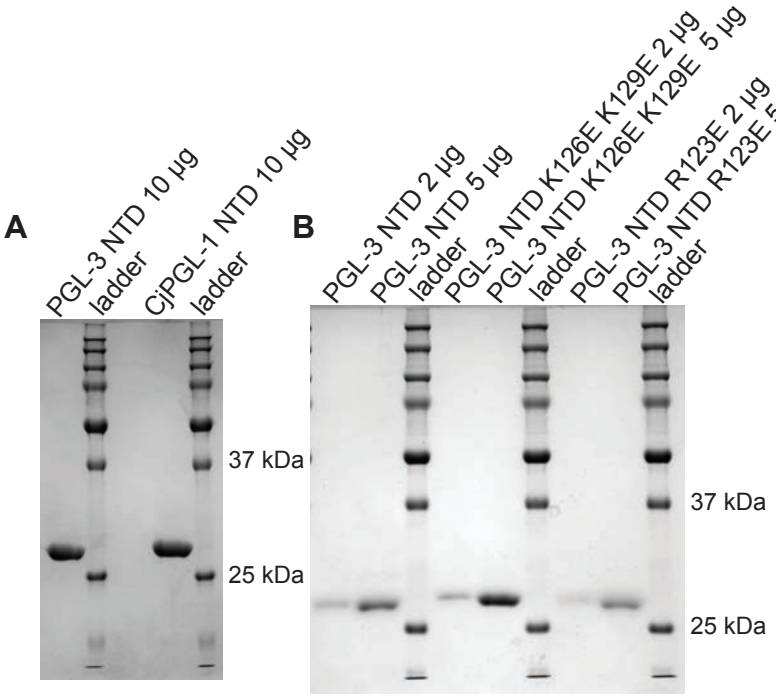


Figure S2
Aoki, et al.



C

H-bonds

subunit 1	subunit 2
A:R128	B:T158
A:R128	B:E189
A:K131	B:E162
A:L132	B:N196
A:K134	B:D167
A:K134	B:D169
A:T158	B:R128
A:E162	B:K131
A:D167	B:K134
A:D169	B:K134
A:E189	B:R128
A:N196	B:L132

salt bridges

subunit 1	subunit 2
A:R128	B:E189
A:K131	B:E162
A:K134	B:D167
A:K134	B:D169
A:E162	B:K131
A:D167	B:K134
A:D169	B:K134
A:E189	B:R128

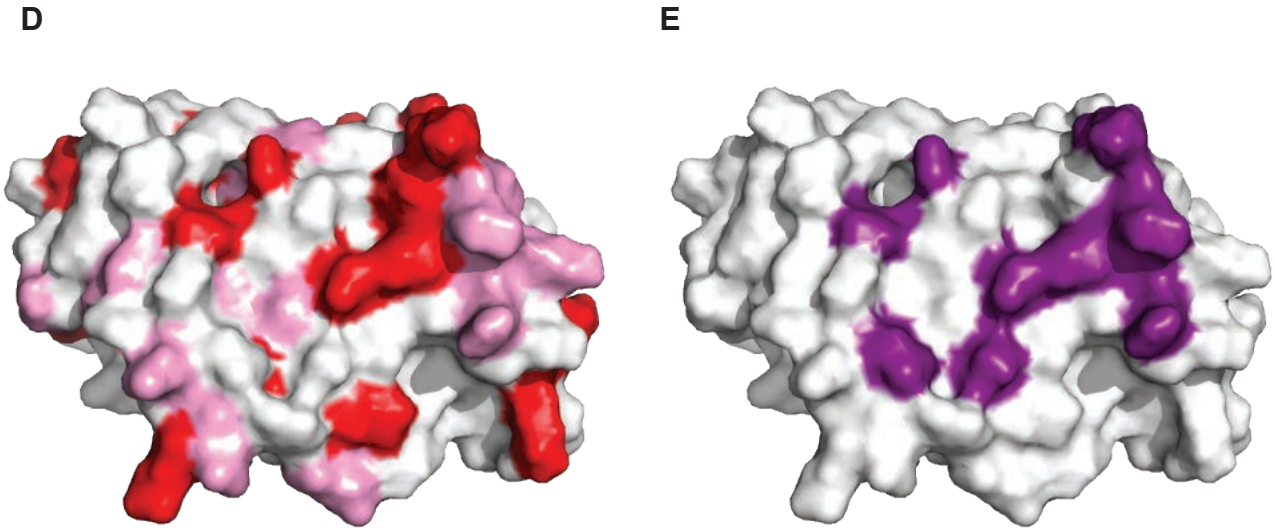


Figure S3
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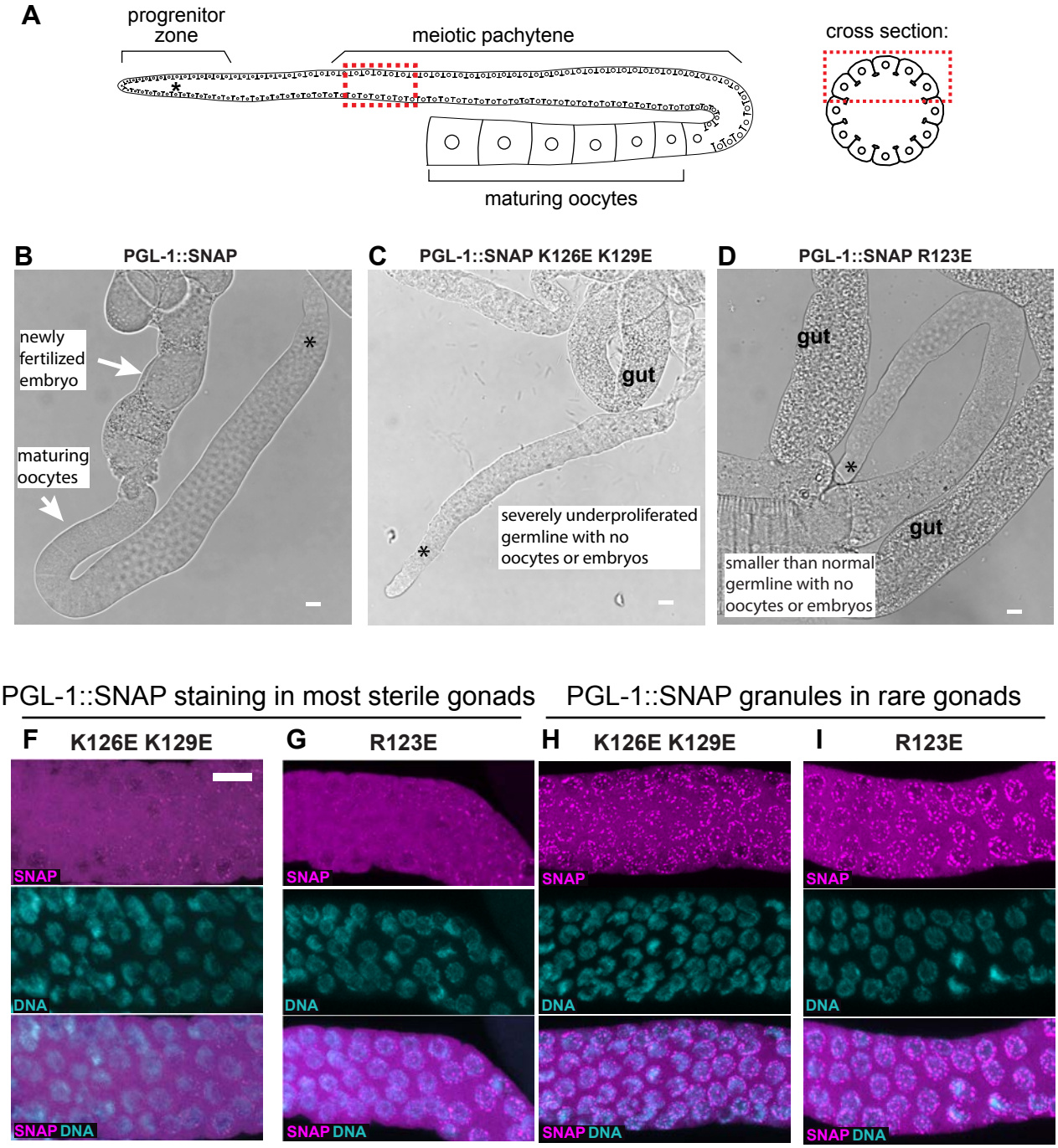

Supp Fig 4
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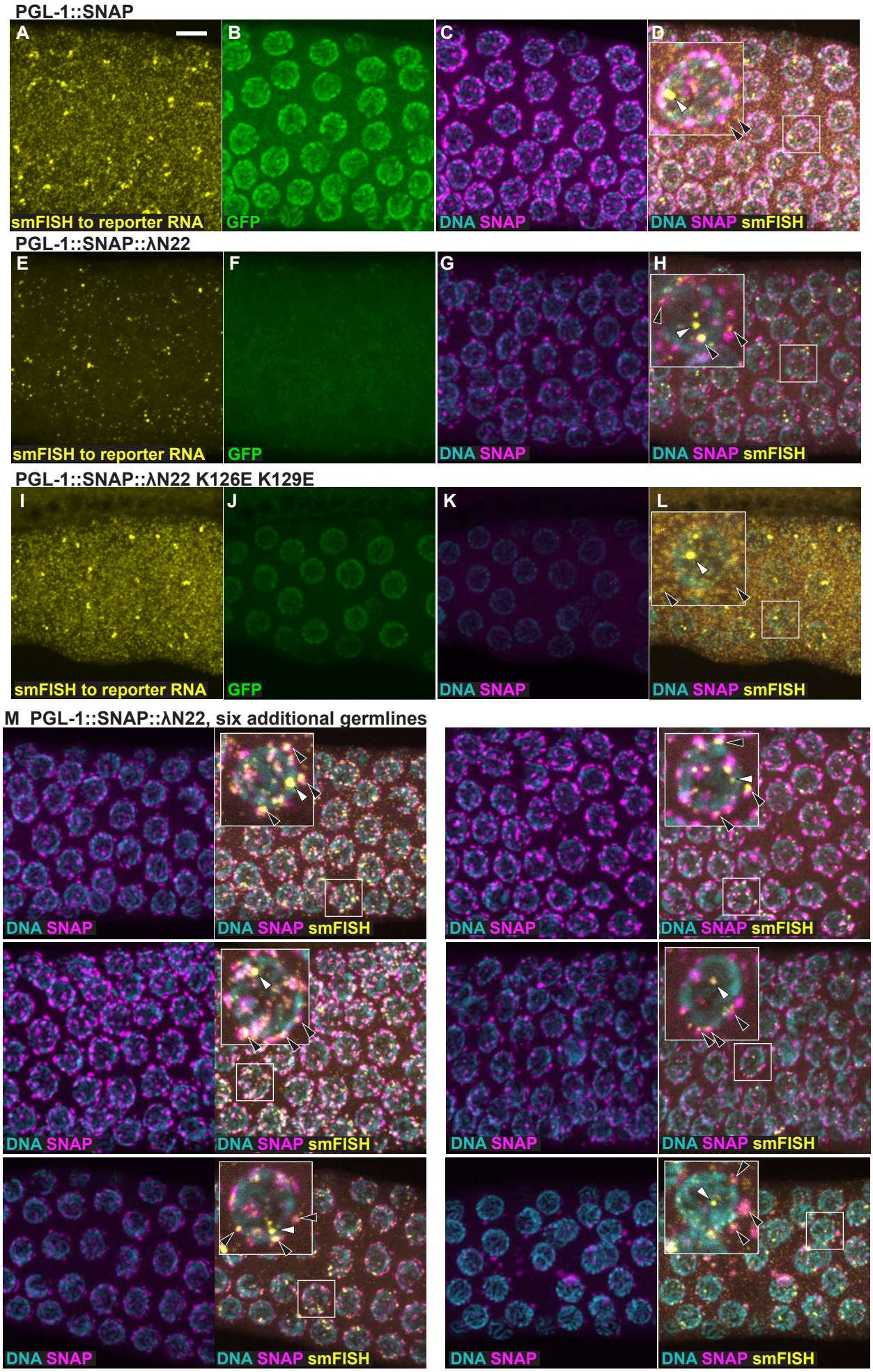

Materials and Methods

Protein expression and purification

We previously used *C. elegans* PGL-3 recombinant protein and limited proteolysis to identify a central dimerization domain (DD) (Aoki et al., 2016). While we could express DD efficiently we could not express recombinant protein that was N-terminal to the cleavage site (PGL-3 amino acid residues 205-206). We tried moving the six histidine purification tag to the N- and C- termini, shortened the protein regions used for expression, and tried several different orthologs with little success. The insight came after aligning protein sequences of several *Caenorhabditid sp.* and studying the DD domain boundary (**Figure S1A**). Protease cleavage occurred in a conserved portion of the N-terminal region and this region was disordered in our DD crystal structures. After inclusion of this region (PGL-3 amino acid residues 205-212), we could express and purify recombinant N-terminal protein from *C. elegans* PGL-1 and its orthologs. We henceforth refer to this region as the N-terminal domain (NTD).

This study used primarily *C. elegans* PGL-3 and *C. japonica* PGL-1 recombinant NTD proteins. The *C. elegans* PGL-3 coding region was PCR amplified from cDNA. A codon-optimized (*E. coli*) version of *C. japonica* PGL-1 NTD was ordered as a gBlock (IDT). We included a six-histidine tag at the C-terminus that was removed later with carboxypeptidase A (Arnau et al., 2006). Constructs were cloned into a pET21a vector (Merck-Millipore) with Gibson Assembly cloning (Gibson, 2011), and plasmids transformed into Rosetta2 cells (EMD-Millipore). Cultures were grown at 37°C with shaking (225 rpm) until ~0.8 OD, cooled for 30-60 minutes, and induced with a final concentration of 0.1 mM IPTG. Cultures were then grown at 16°C with shaking (160 rpm) for 16-18 hours, collected, and bacterial pellets frozen until use. Selenomethionine-incorporated *C. japonica* protein was expressed in SelenoMethionine Medium Complete (Molecular Dimensions), and grown, induced, and collected in a similar manner.

Bacterial pellets were defrosted on ice and reconstituted in lysis buffer (20 mM Sodium Phosphate pH 7.4, 300 mM NaCl, 10 mM imidazole, 5 mM beta-mercaptoethanol (BME)) with protease inhibitors (cOmplete™ EDTA-free, Roche). Lysozyme was added at 50 µg/ml and incubated on ice for 20 minutes prior to lysis in a french press. Samples were spun at low (3220 x g, 4°C, 20 minutes) and high speed

29 (10,000 x g, 20°C, 10 minutes), then incubated with 1.5 ml NiNTA beads (Thermo Scientific) for 1 hour at
30 4°C with rotation. Sample supernatant was separated by gravity flow, washed twice with lysis buffer, and
31 eluted using lysis buffer with increasing imidazole concentrations (20, 40, 60, 80, 100, 250 mM). Eluted
32 samples were checked for protein via Bradford assay (Bio-Rad), and dialyzed overnight in HN buffer (20
33 mM HEPES pH 7.4, 100 mM NaCl). The dialyzed samples were concentrated with a Centriprep 10K
34 concentrator (Millipore), calcium added to 1 mM $CaCl_2$, and the histidine tag removed with
35 carboxypeptidase A bound to agarose (Sigma) at a ratio of 10 protein:1 enzyme (w/w). Samples were
36 incubated at room temperature (~20°C) for 45-90 minutes with rotation prior to supernatant elution by
37 centrifugation in microflow columns (Pierce). Samples were run on a S200 sizing column (GE Healthcare)
38 in HNT buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 0.5 mM TCEP pH 7.4). Fractions containing
39 recombinant protein were collected, concentrated in an Amicon 10K concentrator (Millipore), and protein
40 concentration estimated by A280. Samples were frozen in liquid nitrogen or used immediately.
41
42 **Crystallization and structure determination**
43 *C. elegans* PGL-1, *C. elegans* PGL-3, and *C. japonica* PGL-1 NTD recombinant protein were screened in
44 crystallization conditions using 400 nl hanging and sitting drop 96-well trays set up with the Mosquito
45 (TTP Labtech) in 20°C. Several conditions produced labile crystal plates. Data was collected to 4 Å from
46 *C. elegans* PGL-1 crystal plates, determined to have a very large unit cell (86 Å x 86 Å x 460 Å) and P6
47 point group, and eventually determined to have perfect merohedral twinning. *C. japonica* PGL-1 also
48 crystallized as large (60-150 Å) rhomboid crystals in 40-45% PEG 400 at low (Na Citrate pH 5.5-6.0) and
49 physiologic pH (imidazole pH 7.5-8.0). Crystals grown in citrate or imidazole both diffracted well, but we
50 used imidazole (100 mM imidazole pH 7.5, 45% PEG 400, 1 mM TCEP pH 7.4) due to its higher
51 reproducibility for large crystals and its modestly better resolution. The crystals did not require additional
52 cryo-protection due to the high PEG 400. We eventually collected a full data set to 1.5 Å in space group
53 C2.
54
55 PGL-1 NTD was a novel domain. Novelty and translational pseudosymmetry precluded us from using any
56 model for molecular replacement. Trial heavy atom soaks also proved unfruitful, and the *C. japonica* PGL-

57 1 NTD has just two methonines past the start codon, making selenomethionine phasing challenging. To
58 boost anomalous signal, we mutated two non-conserved isoleucines to methionines (I63M, I212M). This
59 methionine mutant provided phases to 3.6 Å by single anomalous dispersion (SAD) that we used to build
60 a 1.6 Å model of the mutant protein (PDB ID: **5W4D**). We used this model for molecular replacement into
61 the wild-type data set to build a complete 1.5 Å model (PDB ID: **5W4A**). Data and model statistics are in
62 Supplemental Table 1. Model coordinates and data are available at RCSB (www.rcsb.org).
63
64 **Size exclusion chromatography with multi-angle laser light scattering (SEC-MALS)**
65 Molecular weights of *C. elegans* PGL-3 NTD wild type and mutant recombinant protein were determined
66 by conducting SEC-MALS experiments using Agilent Technologies 1260 LC HPLS system (Agilent
67 Technologies) equipped with Dawn® Heleos™II 18-angle MALS light scattering detector, Optilab® T-
68 rEX™ (refractometer with EXtended range) refractive index detector, WyattQELS™ quasi-elastic
69 (dynamic) light scattering (QELS) detector and ASTRA software (all four from Wyatt Technology Europe
70 GmbH). A total of 500 µL (1 mg/mL) of the samples in HNT buffer (20 mM HEPES pH 7.5, 100 mM NaCl,
71 0.5 mM TCEP pH 7.4) were injected and run on a Superdex 75 10/300 GL column (GE Healthcare) pre-
72 equilibrated with the same buffer, at a flow rate of 0.5 mL/min at 20°C. Lysozyme (Sigma-Aldrich Corp.)
73 was used as a control.
74
75 **Mammalian cell culture maintenance, transfection and imaging**
76 Full length PGL-1 was cloned into a pcDNA 3.1 vector (Thermofisher) with a C-terminal eGFP and
77 OLLAS epitope linker. Mutations to PGL-1 were created using Gibson Assembly cloning (Gibson, 2011).
78 Chinese Hamster Ovary (CHO) cells (ATCC) were propagated according to distributor's
79 recommendations. Briefly, cells were grown in F-12K Medium (Gibco) with 10% fetal bovine serum
80 (Gibco), and split with Trypsin 0.25% (Gibco) every 2-3 days. Cells were grown to 70% confluence and
81 transfected with TransIT-CHO Transfection Kit (Mirus Bio). Transfected cells were split the following day
82 and grown in Ibitreat 15 u-Slide 8 well slides (Ibidi) overnight. Hoechst stain (Invitrogen) was added to
83 wells prior to imaging by confocal microscopy for GFP and Hoechst fluorescence, and transmitted light.
84 Well dilutions were chosen based on adequate cell spacing to discern each cell, and 25 fields of view

were taken based on the highest concentration of GFP-positive cells. Experiments were repeated four times with similar results. During image collection, we observed a single example of a granule-like blob in the PGL-1::OLLAS::GFP K126E K129E. The cell appeared unhealthy, and thus the granule may be an artifact of cell death, but we included it in our study for completeness.

Worm maintenance, CRISPR mutagenesis, fertility and imaging

Frozen strains:

N2 Bristol

JK5687: *pgl-1(q894)[PGL-1::SNAP] IV*

JK5902: *pgl-1(q975)[PGL-1::SNAP R123E] IV*

JK5898: *glh-1(q858)[GLH-1:: 3xMYC] I; pgl-1(q894)[PGL-1::SNAP] IV; pgl-3(q861)[PGL-3::3xV5] V*

JK5970: *qSi375[(mex-5 promoter::eGFP::linker::his-58::3xboxb::tbb-2 3'UTR) *weSi2] II; pgl-1(q894)[PGL-1::SNAP] IV*

JK5873: *qSi375[(mex-5 promoter::eGFP::linker::his-58::3xboxb::tbb-2 3'UTR) *weSi2] II; pgl-1(q994)[PGL-1:SNAP::λN22]/nT1[qIs51](IV;V)*

JK5874: *qSi375[(mex-5 promoter::eGFP::linker::his-58::3xboxb::tbb-2 3'UTR) *weSi2] II; pgl-1(q994)[PGL-1:SNAP:λN22]/nT1[qIs51](IV;V)*

Worm strains that could not be frozen:

1. *pgl-1(q960)[PGL-1::SNAP K126E K129E] IV*
2. *glh-1(q858)[GLH-1::3xMYC] I; pgl-1(q960)[PGL-1::SNAP K126E K129E] IV; pgl-3(q861)[PGL-3::3xV5] V*
3. *qSi375[(mex-5 promoter::eGFP::linker::his-58::3xboxb::tbb-2 3'UTR) *weSi2] II; pgl-1(q1053)[PGL-1:SNAP:λN22 K126E K129E]/nT1[qIs51](IV;V)*

C. elegans were maintained as previously reported (Brenner, 1974). For CRISPR-Cas9 mutagenesis, a Cas9 protein co-conversion approach was used (Arribere et al., 2014). Briefly, worms were injected with a target CRISPR-Cas9 RNA (crRNA) or a plasmid expressing a Cas9-scaffold with tandem target sequence

113 RNA (sgRNA) to a gene of interest (Arribere et al., 2014), a target crRNA to *dpy-10* or *unc-58*, a
114 scaffolding tracrRNA (IDT), recombinant Cas9 protein (Paix et al., 2015), a *dpy-10/unc-58* repair DNA
115 oligo that inserted a dominant mutation (Arribere et al., 2014), and an epitope tag/missense mutant repair
116 oligo or PCR product. See below for a Table of guide RNAs and repair templates used. F1s with the co-
117 injection marker phenotype were additionally screened by a combination of PCR without or with restriction
118 enzyme digest to identify those with the repair of interest. In JK5687, a SNAP tag (Keppler et al., 2003)
119 was inserted between PGL-1 amino acids G713 and G714 in N2 worms. A 3xMYC tag was added to the
120 N-terminus of GLH-1 between G17 and F18. A 3xV5 tag was added in the C-terminal region of PGL-3
121 between residues G627 and S628. F2s were PCR screened to identify homozygous SNAP alleles and
122 the PCR product sequenced to confirm proper repair. Three worm strains were too infertile to freeze. All
123 worms were outcrossed at least twice with N2, with the exception of (*glh-1(q858)[GLH-1::3xMYC] I; pgl-*
124 *1(q960)[PGL-1::SNAP K126E K129E] IV; pgl-3(q861)[PGL-3::3xV5] V)* that was backcrossed with
125 JK5898.
126
127 Worms were singled into the peripheral wells of a 24-well plate that contained NGM agar and OP50
128 bacteria. Worms were allowed to propagate for 5 days at 20°C or 25°C, and then scored for progeny and
129 gravid progeny. We report the progeny numbers here.
130
131 To analyze GFP reporter expression, L4 larvae were propagated for approximately 24 hours at 20°C,
132 placed in M9 with 0.1 mM levamisole on a glass slide with a cover slip, imaged at 10x magnification on a
133 compound microscope and counted for the presence or absence of GFP fluorescence in its germline.
134 Numbers represent totals from two separate experiments. The reporter images of live worms were taken
135 of worms treated in a similar manner and visualized on a Leica SP8 scanning laser confocal microscope.
136
137 For confocal imaging, germlines were extruded, fixed with 1-2% paraformaldehyde (Electron Microscopy
138 Sciences) and permeabilized with 0.5% Triton-X as previously described (Crittenden et al., 2017).
139 Germlines were incubated with primary antibodies to FLAG (M2® (mouse), Sigma) and GFP (Rabbit anti-
140 GFP, Invitrogen) overnight, stained with fluorophore-labeled secondary antibodies (Alexa 555 Donkey

141 anti-Mouse, Alexa 488 Goat anti-Rabbit; Invitrogen) and DAPI (Invitrogen), washed and mounted in
142 Vectashield (Vector Laboratories).
143
144 For smFISH, gonads were extruded, fixed, and hybridized with single molecule FISH probes as described
145 (Lee et al., 2016). The *gfp* exon probe set contains 38 unique oligonucleotides labeled with CAL Fluor
146 Red 610. Briefly, probes were dissolved in RNase-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
147 to create a 250 μM probe stock. Mid-L4 stage animals were grown on OP50 for 24 hours, then dissected
148 in PBS+0.1% Tween-20 + 0.25 mM levamisole. Animals were fixed in 4% paraformaldehyde for 20
149 minutes, incubated at room temperature in PBS-T (PBS + 0.1% Tween-20) for 10-25 minutes, and
150 equilibrated in smFISH wash buffer (30 mM sodium citrate pH 7.0, 300 mM NaCl, 1% formamide, 0.1%
151 Tween-20, DEPC water) for 10-16 minutes. Samples were then incubated in hybridization buffer (30 mM
152 sodium citrate pH 7.0, 300 mM NaCl, 1% formamide, 10% dextran sulfate w/v, DEPC water) plus 0.5 μM
153 smFISH probe at 37°C for 26-44 hours. 30 nM SNAP 549 ligand was added during the smFISH wash
154 buffer + DAPI wash; samples were washed at 37°C for approximately 60 minutes. Finally, samples were
155 resuspended in 12 μL Antifade Prolong Gold mounting medium (Life Technologies), mounted on glass
156 slides, and cured in a dark drawer for at least 24 hours before imaging.
157
158 Samples were imaged using a Leica SP8 scanning laser confocal microscope, taking 0.3 μm (smFISH
159 experiments) or 1 μm (protein staining) slices in sequence. Maximum intensity partial stack projections
160 were generated and brightness adjusted using ImageJ (Schindelin et al., 2015). All images were treated
161 equally in ImageJ and Photoshop, with the exception of the transmitted light images. Imaging
162 experiments were repeated at least twice with similar results, with the exception of PGL-1:SNAP:λN22
163 K126E K129E worms.

CRISPR-Cas9 guide RNAs and repair oligos

Name	Type	Strain targeted	mutation	Sequence	Enzyme screen
CRISPR-Cas9 guide RNAs:					
glh-1 sgRNA 1	CRISPR-Cas9 sgRNA plasmid	N2	3xmyc	target sequence: TCCACTACCGAATCCAGTTT	
pgl-3 sgRNAin 1	CRISPR-Cas9 sgRNA plasmid	N2	3xV5	target sequence: GCAACGGAACGTCTGGAAG	
pgl-1 crRNA 1	CRISPR-Cas9 RNA	N2	SNAP	target sequence: cccaccagttcagcttatgg	
pgl-1 crRNA 5	CRISPR-Cas9 RNA	JK5687, JK5898, JK5874	K126E K129E	target sequence: gtcttcagtcttcttcagct	
pgl-1 crRNA 8	CRISPR-Cas9 RNA	JK5687	R123E	target sequence: cttcttcagcttggccttac	
SNAP crRNA 1	CRISPR-Cas9 RNA	JK5687	λN22	target sequence: CCTGGGCTGGGTCCTGCAGG	
DNA repair template:					
glh-1 3xmyc repair 1	ssDNA repair oligo	N2	3xmyc	ttccaccggttttattttgattaaaaactttatttcagCgAAAACTGGAAA CGAACAGAAGCTTATTTCCGAGGAAGACCTCGCCG GAGAGCAAAAGCTCATCTCTGAAGAGGATCTTGGAG CCGAACAGAAGCTTATCTCTGAAGAAGACCTCGGAG GATTCGGTAGTGGAGGCGGTTTCGGTGGTGGTAAC AATGGAG	n/a
pgl-3 3xV5 repair 1	ssDNA repair oligo	N2	3xV5	agtttgccagcagcaacggaacCtcCggaCgaggcGGAAAGCCA ATCCCAAACCCACTCCTCGGACTCGACTCCACCGG AGGAAAGCCAATCCCAAACCCACTCCTCGGACTCG ACTCCACCATCGGAAAGCCAATCCCAAACCCACTCC TCGGACTCGACTCCACCGGAtcttatggaggtggtcgcggtgg cgatcgt	BlpI
pgl-1 SNAP	PCR product	JK5687	SNAP	ggattcggtcaatttgctcccaccagttcagcttatggaAGTGGCGGTA TGGACAAAGACTGCGAAATGAAGCGCACCACCCTG GATAGCCCTCTGGGCAAGCTGGAACTGTCTGGGTG CGAACAGGGCCTGCACCGTATCATCTTCCTGGGCAA AGGAACATCTGCCGCCGACGCCGTGGAAGTGCCTG CCCCAGCCGCCGTGCTGGGCGGACCAGAGCCACT GATGCAGGCCACCGCCTGGCTCAACGCCTACTTTC ACCAGCCTGAGGCCATCGAGGAGTTCCCTGTGCCA GCCCTGCACCACCCAGTGTTCCAGCAGGAGAGCTT TACCCGCCAGGTGCTGTGGAAACTGCTGAAAGTGG TGAAGTTCGGAGAGGTCATCAGCTACAGCCACCTG GCCGCCCTGGCCGGCAATCCCGCCGCCACCGCCG CCGTGAAAACCGCCCTGAGCGGAAATCCCGTGCCC ATTCTGATCCCCTGCCACCGGGTGGTGCAGGGCGA CCTGGACGTGGGGGGCTACGAGGGCGGGCTCGCC GTGAAAGAGTGGCTGCTGGCCCACGAGGGCCACA GACTGGGCAAGCCTGGGCTGGGTCCTGCAGGCGG ATCCggaggaggtggtcgcggaggatatggcggtggagaccgtg	n/a
K126E K129E repair	ssDNA repair oligo	JK5687, JK5898, JK5874	K126E K129E	tcgatgacgacaagaagctcggaatgctcgcccgtaaggcTGagctgaa gGagactgaagacgctaagattcttcaagctcttctcaaagt	BlpI
pgl-1 R123E repair	ssDNA repair oligo	JK5687	R123E	tttctgtcatcgatgacgacaagaagctcggaatgctcgcTGAGaaggcc aagctgaagaagactgaagacgctaagattcttc	HpyAV

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