Genetic Identification of Novel Separase regulators in *Caenorhabditis elegans*

Michael Melesse†, Dillon E. Sloan†, Joseph T. Benthal†, Quincey Caylor†, Krishen Gosine†, Xiaofei Bai† and Joshua N. Bembenek†

AUTHOR AFFILIATIONS

†Department of Biochemistry, Cellular and Molecular Biology, University of Tennessee, Knoxville, TN

Running title: Novel regulators of *C. elegans* Separase

Keywords: Separase, PPH-5, HSP-90, Suppressors, C. elegans

Corresponding Author:

Joshua N. Bembenek

1414 Cumberland Ave.

C211 Walters Life Sciences Building

Knoxville, TN 37996

(865)-974-4085

bembenek@utk.edu

ABSTRACT

Separase is a highly conserved protease required for chromosome segregation. Although observations that separase also regulates membrane trafficking events have been made, it is still not clear how separase achieves this function. Here we present an extensive ENU mutagenesis suppressor screen aimed at identifying suppressors of *sep-1(e2406)*, a temperature sensitive maternal effect embryonic lethal separase mutant. We screened nearly a million haploid genomes, and isolated sixty-eight suppressed lines. We identified fourteen independent intragenic *sep-1(e2406)* suppressed lines. These intragenic alleles map to seven SEP-1 residues within the N-terminus, compensating for the original mutation within the poorly conserved N-terminal domain. Interestingly, 47 of the suppressed lines have novel mutations throughout the entire coding region of the *pph-5* phosphatase, indicating that this is an important regulator of separase. We also found that a mutation near the MEEVD motif of HSP-90, which binds and activates PPH-5, also rescues *sep-1(e2406)* mutants. Finally, we identified six potentially novel suppressor lines that fall into five complementation groups. These new alleles provide the opportunity to more exhaustively investigate the regulation and function of separase.

34 ## INTRODUCTION

35 Separase is a highly conserved cysteine protease required for proper chromosome
36 segregation during anaphase of both meiotic and mitotic stages of cell division (Peters et al.
37 2008). Separase proteolytic activity is inhibited during interphase and early mitosis by its
38 pseudosubstrate inhibitor, securin (Nasmyth, K. A., 2002). The protease activity of separase is
39 critical for the cleavage of kleisin subunits of the cohesin complex (Uhlmann et al. 2000, Hauf et
40 al. 2001). Cohesin holds sister chromatids together prior to their proper attachment to spindles
41 and alignment on the metaphase plate preceding anaphase (Nasmyth and Haering, 2009).
42 Separase has also been implicated in various cell cycle regulatory functions. In budding yeast,
43 separase stabilizes the anaphase spindle by cleaving the spindle and kinetochore associated
44 protein, Slk19 (Sullivan et al. 2001). It is also involved in the release of the essential mitotic
45 phosphatase Cdc14 in budding yeast (Sullivan and Uhlmann 2003). In mammalian cells,
46 separase licenses centriole duplication (Baskerville et al. 2008) and a domain within its N-
47 terminus binds and inhibits the Cyclin B-Cdk1 complex (Gorr et al. 2005). In mammalian cells,
48 separase has also been shown to associate with membranes and its depletion is associated with
49 swelling of the trans-golgi network and decreased constitutive protein secretion (Bacac et al.
50 2011). In *Arabidopsis*, separase mutant cells display mitotic failure due to defects in vesicle
51 trafficking along microtubules, which is critical for synthesis of a cell plate during cytokinesis
52 (Moschou et al. 2016). Therefore, there are numerous functions of separase during the cell cycle,
53 and how each are regulated has not been fully elucidated.

54 In *Caenorhabditis elegans*, separase is known to regulate multiple cell cycle events
55 beyond its chromosome segregation functions (Bembenek et al. 2007, 2010). It has been
56 demonstrated to regulate cell cycle related membrane transport events critical for both

57 cytokinesis and embryonic development. During meiosis, the *C. elegans* eggshell is formed
58 around a fertilized embryo to prevent polyspermy and provide both mechanical as well as
59 osmotic protection for the developing embryo (Olson et al. 2012; Stein and Golden 2015).
60 Formation of the eggshell is dependent on the progression of the embryonic cell cycle and
61 requires cargo released via cortical granule exocytosis (CGE), which occurs during anaphase I
62 (Bembenek et al. 2007). Importantly, separase localizes to cortical granules and is required for
63 their exocytosis during anaphase, independently of chromosome segregation.

64 Various separase mutants have been identified in budding yeast, mouse and human cells.
65 Many of these mutants compromise the protease function of separase and directly affect its role
66 during chromosome segregation. Interestingly, the hypomorphic separase mutant (*sep-1(e2406)*),
67 originally isolated by David Livingstone in a screen for temperature sensitive mutants defective
68 in cell division (Siomos et al. 2001), is a partial separation of function allele. *sep-1(e2406)* is a
69 C450Y missense mutation in the N-terminal region of separase and has minimal effect on the
70 chromosomal segregation role of separase but significantly diminishes cortical granule
71 exocytosis. In embryos, SEP-1(e2406) can be observed on the spindle, but shows reduced
72 localization to cortical granules and results in a lower number of exocytic events. Another
73 separase mutant (*sep-1(ax110)*) is a non-conditional allele that also results in minimal
74 chromosome segregation defects and leads to cytokinesis failure (Richie et al. 2011). This allele
75 is a missense mutation (H738P) in the protease domain of SEP-1 that is maternal effect
76 embryonic lethal. These alleles potentially provide a unique opportunity to learn more about the
77 membrane trafficking functions of separase.

78 Previous attempts to learn more about separase regulation used *sep-1(e2406)* to identify
79 the PPH-5 phosphatase as a suppressor of separase (Richie et al. 2011). This effort screened 1.0

80 X 10^5 genomes and identified three suppressors including one *pph-5* allele, *pph-5(av101)*, an
81 intragenic *sep-1* (L556F) mutant and another mutant that maps to LG III. Mutations in *pph-5* as
82 well as RNAi mediated knockdown rescues *sep-1(e2406)* (Richie et al. 2011), suggesting that
83 *pph-5* is a negative regulator of separase function. The *pph-5(av101)* suppressor allele, which is a
84 missense mutation (P375Q), does not suppress *sep-1(e2406)* at 24° but was effective in
85 suppressing *sep-1(ax110)* at all tested temperatures (Richie et al. 2011). This observation
86 suggests that there might be underlying differences in the effects of these SEP-1 mutations on
87 separase function.

88 PPH-5 is a widely conserved phosphatase that contains N-terminal tetratricopeptide
89 repeats (TPRs) and a C-terminal phosphatase domain. PP5 (human PPH-5), originally identified
90 as a regulator of a variety of cellular signaling pathways including glucocorticoid receptor
91 signaling, displays low phosphatase activity when purified due to the autoinhibitory role of its
92 TPR domain (Chen et al. 1996). Interestingly, PP5 binds CDC16 and CDC27, components of the
93 Anaphase Promoting Complex/Cyclosome (APC/C) (Ollendorff and Donoghue 1997). The
94 APC/C is an E3 ubiquitin ligase required for activation of separase at the metaphase to anaphase
95 transition and is regulated by phosphorylation (Kraft et al. 2003; Chang and Barford 2014;
96 Musacchio 2015). The precise mechanism by which PPH-5 regulates separase is unknown, but
97 these findings suggest that it may be an important regulator of the metaphase to anaphase
98 transition.

99 One of the well-studied regulatory pathways of PPH-5 is its interaction with the
100 molecular chaperone HSP-90. The crystal structure of auto-inhibited human phosphatase 5 (PP5)
101 shows that access to the enzyme active site is blocked by a combination of the TPR domain and a
102 C-terminal *a*J-helix (Yang et al. 2005). HSP-90 binds the TPR domain of PPH-5 to release auto-

103 inhibition and promote phosphatase activity towards protein substrates (Haslbeck et al. 2015).
104 HSP-90 consists of three highly conserved domains and binds its client proteins via its middle
105 domain (MD), while it binds co-chaperones via its C-terminal domain (Schopf et al. 2017). The
106 very C-terminal MEEVD motif is critical for HSP90 interaction with TPR domain containing co-
107 chaperones like PP5. As a major protein chaperone, HSP-90 is known to bind multiple proteins
108 (Haslbeck et al. 2013). Available HSP-90 mutants as well as RNAi in *C. elegans* cause penetrant
109 pleiotropic phenotypes (Inoue et al. 2006; Gillan et al. 2009; Gaiser et al. 2011). To our
110 knowledge, there is no evidence linking HSP-90 to regulation of separase in any system.
111 In this paper, we present the results of a genetic suppressor screen aimed at uncovering
112 regulators of separase. We identified intragenic suppressors, *pph-5* mutants, a novel *hsp-90* allele
113 and unknown alleles that fall into five complementation groups. These suppressors may provide
114 important insight into separase regulation and function.

115 ## MATERIALS AND METHODS

116 **Mutagenesis and Selection**

117 Strains were maintained as described (Brenner 1974). *sep-1(ax110)* screen: *sep-1(ax110)*/hT2
118 [*bli-4(e937) let-?(q782) qIs48 (Pmyo-2::gfp; Ppes-10:: gfp; Pges-1::gfp)*] (I,III) worms were
119 synchronized by bleaching with hypochlorite and grown to L4. Mutagenesis was performed by
120 incubating worms with 0.5mM ENU for 4 hours at 25° and recovering in 50ml of M9 overnight
121 at 15°. 30 P_0s were plated to 81 100mm plates, transferred to 25° and incubated. After one
122 generation, 50 unbalanced (non-green, should be homozygous for *sep-1(ax110)*) F_2 progeny
123 from each of the 81 100mm plates were moved onto 60mm OP50 plates and checked for fertility.
124 From each non-green F_3 producing plate, at least 6 plates of non-green animals were cloned and
125 genotyped. Candidate suppressed lines were confirmed to be homozygous for *sep-1(ax110)* and
126 sequenced for mutations at the *pph-5* locus.

127 *sep-1(e2406)* screen: homozygous *sep-1(e2406)* worms were synchronized by bleaching with
128 hypochlorite and grown to L4. Worms were mutagenized with 0.5mM ENU in M9 for 4 hours
129 and recovered in 50ml of M9 for 1 hour at 15°. 100 mutagenized worms were moved to each of
130 60 MYOB plates and incubated at 15°. P_0s were moved to new plates daily. The number of F_1
131 worms on each plate were estimated and plates were grown for multiple generations at 15°.
132 These plates were then chunked and incubated at 20° and allowed to produce offspring. Plates
133 that yielded embryos were cloned and backcrossed to *sep-1(e2406)* for multiple generations.

134 **Identification of suppressor mutations**

135 Genotyping: *sep-1(ax110);* primers (oASP-UTK-3 and oASP-UTK-4) were used to amplify a
136 *sep-1* fragment by PCR. The PCR product was then digested with a restriction enzyme (SacII),
137 which is introduced by the *sep-1(ax110)* mutation. The *sep-1(e2406)* allele was genotyped by

138 sequencing a PCR fragment amplified using a pair of primers (oASP-UTK-34 and oASP-UTK-
139 29) and sequenced with oASP-UTK-7.

140 PCR and sequencing: PCR primers were used to amplify the locus of interest from worm
141 lysates. PCR products were then gel purified and sequenced. Three PCR fragments of *sep-1*, five
142 of *pph-5* and two of *hsp-90* were amplified, spanning across each gene. Primers used for PCR
143 and sanger sequencing of *sep-1*, *pph-5* and *hsp-90* loci are listed in supplementary tables (Tables
144 S2, S3 and S4).

145 **Characterization of suppressed lines**

146 Hatching assay: four P_0 L4 larvae were placed in each of 35mm OP50 NGM plates and
147 allowed to lay embryos for 24 hours at the experimental temperature (15°, 20° or room
148 temperature). Worms were then moved to new plates and returned to temperature to continue
149 laying embryos. The number of embryos and hatched animals on overnight plates was counted
150 on each plate and plates were incubated for 24 hours. The following day, the number of
151 unhatched embryos or hatched larvae was counted and % hatching was quantified.

152 RNAi feeding: Worms were moved onto NGM plates with ampicillin and isopropyl-b- D-
153 thiogalactopyranoside which were seeded with HT115(DE3) bacteria carrying RNAi feeding
154 constructs for 24 hours. Worms were then moved onto new RNAi feeding plates daily and
155 hatching embryos were counted. Unless otherwise stated, five L1 stage worms per strain were
156 fed at 20°. Animals were moved to new RNAi feeding plates after reaching the L4 stage, and
157 hatching was quantified daily for 48 hours.

158 Western blot analysis: Worms were grown at 20° on 100mm OP50 seeded plates for one
159 generation and collected by washing in M9 buffer. Each worm pellet was resuspended in 1xSDS
160 loading buffer (2μl/mg of pellet) and heated in a microwave (4x20 sec with 1 min cooling).

161 Lysates were then centrifuged (15,000 x RCF, 10 min) and supernatant was transferred into new
162 tubes. 10 μl of worm lysate was then loaded per well and analyzed by standard western blot.
163 SEP-1 was detected by using a polyclonal rabbit antibody (Richie et al. 2011) at a dilution of
164 1:750. Secondary antibody used was anti-rabbit 700 from Li-Cor and quantified using the Image
165 Studio software. Non-specific bands were used to normalize signals between lanes. All antibody
166 incubations were done in the presence of 5% (w/v) non-fat milk.

167 Complementation tests: Twenty-five males generated using *him-5 RNAi* bacterial feeding for
168 each strain were mated with five hermaphrodites on unseeded NGM plates and incubated at 20°
169 for 24 hours. Mated worms were then moved to OP50 seeded 60mm plates and allowed to lay F_1
170 embryos at 15°. Once F_1 worms reach L4 stage and the presence of ~50% male animals was
171 observed, indicative of successful mating, four L4 hermaphrodites in triplicate were moved to
172 OP50 seeded 35mm NGM plates and incubated at 20°. Viability of F_2 embryos was determined.

173 ## RESULTS AND DISCUSSION

174 **Identification of suppressors**

175 To identify genes that regulate separase function, we performed mutagenesis screens for
176 suppressors of two separase mutants, *sep-1(ax110)* and *sep-1(e2406)* (Figure 1A). We first
177 screened for suppressors of the non-conditional *sep-1(ax110)* mutant (Figure S1A) which
178 introduces a point mutation in the protease domain of SEP-1(H738P) and is maternal effect
179 embryonic lethal. We postulated that this separase allele might be differentially impaired relative
180 to the temperature sensitive *sep-1(e2406)* allele, which introduces a mutation in the TPR-like
181 domain (C450Y) and might be suppressed by a different set of mutations. This suppressor screen
182 identified four independent suppressors of *sep-1(ax110)*, all of which were *pph-5* mutants
183 (*erb1(S229L)*, *erb2(M380T), erb3(L77P)* and *erb4(L77P)*) from 56,404 genomes screened
184 (Figure 1B and Figure S1B). This is consistent with a previous finding that *sep-1(ax110)* is
185 completely rescued by loss of *pph-5* (Richie et al. 2011). Therefore, we focused our efforts
186 towards identifying suppressors of *sep-1(e2406)*.

187 The *sep-1(e2406)* mutation results in a temperature sensitive maternal effect embryonic
188 lethality. When L4 animals are shifted to 20°, the lowest temperature at which lethality is fully
189 penetrant, *sep-1(e2406)* hermaphrodites lay 100% dead embryos. *sep-1(e2406)* embryos are
190 unable to perform cortical granule exocytosis and fail to build an eggshell when maintained at
191 25° (Bembenek et al. 2007; Richie et al. 2011). We utilized an ENU mutagenesis approach to
192 isolate suppressors of *sep-1(e2406)* that result in viable F3 progeny at the restrictive temperature
193 of 20° (see Materials and Methods) (Figure 1A). This approach yielded a total of sixty-eight
194 independent suppressor lines from a total of 9.6 X 10^5 haploid genomes (as determined by
195 counting the approximate number of mutagenized F1 progeny). Each suppressor line was cloned

196 and backcrossed with the original *sep-1(e2406)* line to reduce non-suppressing background
197 mutations and homozygotes were isolated. A candidate gene sequencing approach was utilized to
198 identify suppressor mutations within *sep-1*, *pph-5* and *hsp-90* (formerly known as *daf-21*) (see
199 Materials and Methods). We also isolated six lines with novel unknown mutations belonging to
200 at least four complementation groups.

201 **Intragenic suppressors of *sep-1(e2406)* are exclusively N-terminal**

202 There were fourteen independent suppressor lines identified as intragenic *sep-1(e2406)*
203 suppressors. All intragenic *sep-1(e2406)* suppressors resulted in missense mutations within the
204 N-terminal region of SEP-1 and none are found in the catalytic domain of the protein (Figure
205 2A). Some mutations were identified from multiple independent lines. Interestingly, mutation in
206 lysine 556 was identified in six lines.

207 The types of missense mutations observed include ones that increase the size of amino
208 acid side chains while preserving charge (A64V, A392I, A471V and D541E). We also found
209 mutations that remove charged side chains and introduce hydrophobic residues (T357I and
210 N517I). The residue most frequently mutated was L556 and both changes we observed result in
211 the introduction of aromatic side chains (L556F and L556H, Figure 2A, S2). It is also notable
212 that L556F was previously identified as an intragenic *sep-1(e2406)* suppressor (Richie et al.
213 2011). We find that multiple residues in the N-terminus can be changed to restore function to the
214 *sep-1(e2406)* mutant and restore viability (Figure 2 B and C).

215 One possible mechanism of suppression is that these mutations affect the stability of
216 separase. To address this, we performed western blotting analysis of SEP-1 abundance in each of
217 the suppressed lines. The separase protein is detectable in adult worms (Figure 2D) showing that
218 proteins carrying suppressor mutations are expressed. Quantification shows that the original

219 SEP-1(e2406) mutant protein is 40% as abundant as wild type SEP-1. The least effective
220 rescuing mutation, *erb27* (V392I), is expressed at about twice the level of wild type separase.
221 The three most effective rescuing mutations (*erb17* (N517I), *erb10* (L556H) and *erb5* (L556F))
222 have varying levels of expression. SEP-1(erb5) is 2.5-fold as abundant as wild type whereas
223 SEP-1(erb17) and SEP-1(erb10) are expressed at 1.5-fold of wild type. The least abundantly
224 expressed mutant, SEP-1(erb16) *(T357I)*, is not the least effective suppressor. No clear
225 correlation is observed between protein abundance and rescuing ability, suggesting that these
226 mutations do not simply affect protein levels, but may affect separase structure and function.
227 To gain more insight into these mutations, we mapped mutated suppressor residues onto
228 the recently published Cryo-EM structure of SEP-1 in complex with its pseudosubstrate
229 inhibitory chaperone; IFY-1 (securin) (PDB 5MZ6, Boland et al. 2017). This analysis reveals
230 that there is no clustering of mutated residues to any specific surface in the TPR like domain of
231 the N-terminus (Figure 2E). C450, the residue mutated in SEP-1(e2406), is at the edge of helix
232 16 and part of an unstructured loop containing about sixty amino acids between helix 15 and 16
233 of the TPR-like domain. The effects of C450Y mutation on the structure of the TPR-like domain
234 have not been elucidated, but there is a potential that introducing a large aromatic residue on this
235 solvent exposed loop may be unfavorable and could lead to a structural rearrangement of the
236 SEP-1 N-terminal domain. The residues mutated in suppressed lines are found on helices not
237 near C450, facing the interior of the protein and are likely involved in intramolecular interactions
238 (Figure S2). These mutations have the potential of introducing new intramolecular interactions
239 leading to improved structural stability of the SEP-1 TPR-like domain that may be disrupted in
240 SEP-1(e2406). It is important to consider that the separase Cryo-EM structure represents a

241 securin-bound fold of the enzyme, which is inactive. The active conformation of separase might
242 bring these key residues into more obvious functionally relevant orientations.

243 ***pph-5* mutants are most frequently identified *sep-1(e2406)* suppressors**

244 The majority of *sep-1(e2406)* suppressors identified from our analysis are mutations in
245 the protein phosphatase, *pph-5*. The types of mutations identified include premature stop codons
246 (12 alleles), splice site mutations (6 alleles), as well as amino acid substitutions (29 alleles).
247 Missense *pph-5* suppressing mutations span the full length of the protein, altering both the TPR
248 as well as the phosphatase domain. Excluding mutations that introduce a premature stop codon,
249 our screen has identified twenty-five unique amino acid substitutions across the protein (Figure
250 3A). Missense suppressor mutations occur both within the TPR domain and the phosphatase
251 domain of PPH-5, suggesting that both domains are required for PPH-5 regulation of separase.
252 The capacity of these mutations to rescue *sep-1(e2406)* varies, as assayed by the proportion of
253 embryos able to hatch at the restrictive temperature (Figure 3 B and C). Strong RNAi
254 knockdown of *pph-5* (*pph-5 RNAi*) in these suppressed lines also results in improvement of
255 suppression (Table 1) suggesting that they are reduction-of-function mutations.

256 It has been shown that *pph-5* mutants do not suppress *sep-1(e2406)* by bypassing
257 separase requirement (Richie et al. 2011) as RNAi knockdown of *sep-1* still results in lethality in
258 suppressed lines. It is likely that the suppressors we have identified are *pph-5* reduction of
259 function mutants and restore viability in a similar manner as previously identified *pph-5* mutants.
260 Our data do not preclude the possibility that *pph-5* acts in a separase independent pathway to
261 restore viability to *sep-1(e2406)* animals. We favor our proposed model because mutations in
262 *pph-5* have been demonstrated to restore mutant separase localization (Richie et al. 2011). One
263 suppressor mutation in *pph-5* (L77P) was independently identified in both screens as a

264 suppressor of conditional (*sep-1(e2406)*) and non-conditional (*sep-1(ax110)*) separase mutants.
265 This extensive collection of *pph-5* mutants provides a valuable tool for structure-function as well
266 as genetic analysis of this phosphatase.

267 **HSP-90 suppressor reveals novel regulator of separase**

268 The biochemical evidence connecting PPH-5 with HSP-90 (Haslbeck et al. 2015)
269 prompted us to test if any of the suppressors were *hsp-90* mutations. We sequenced the *hsp-90*
270 locus of the remaining suppressed lines that did not carry any suppressing intragenic or *pph-5*
271 mutations. We found that *erb71* has a single missense mutation that changes methionine 661 into
272 lysine (Figure 4A) that has an intermediate ability to restore hatching to 31% (Table 2). When
273 isolated from *sep-1(e2406)*, the *hsp-90*(*erb71*) allele has minimal effect on embryonic survival at
274 20° (84% hatching) which suggests that the essential functions of HSP-90 are minimally affected
275 (Figure 4B). The rescue observed with *pph-5(RNAi)* is greater than the 31% survival observed in
276 *hsp-90*(*erb71)*. This suggests that either the M661L mutation does not completely disrupt the
277 PPH-5 activating functions of HSP-90 or that PPH-5 can still be active without HSP-90.
278 Consistent with this, we observed improved survival (92.9% hatching) when *pph-5(RNAi)* was
279 performed in a *sep-1(e2406); hsp-90(erb71)* animal (Table 2). The identification of a HSP-90
280 allele that can suppress a temperature sensitive separase mutation is consistent with the
281 hypothesis that HSP-90 acts via its regulation of PPH-5. Our data, however, do not exclude the
282 possibility that HSP-90 directly regulates separase independent of PPH-5.

283 Combining *hsp-90(erb71)* with *pph-5(RNAi)* has little effect on embryonic survival,
284 compared to the effects of *erb71* alone (84% vs 86% hatching) in an otherwise wild type
285 background. No significant changes in hatching were observed when *hsp-90(erb71)* was
286 combined with *pph-5(tm2979)*. *pph-5(tm2979)* is an in-frame deletion that removes 55 amino

287 acids from the PPH-5 TPR domain and potently suppresses *sep-1(e2406)* and *sep-1(ax110)*
288 (Richie et al. 2011). These observations demonstrate that *pph-5* function is not critical, even in a
289 mutant *hsp-90(erb71)* background, for the essential functions of HSP-90. Taken together, these
290 observations support the hypothesis that *hsp-90(erb71)* does not result in a general loss in HSP-
291 90 chaperone activity.

292 It is interesting to note that the mutation in HSP-90(erb71) (M661K) is found just N-
293 terminal to the HSP-90 MEEVD motif, which is critical for HSP-90 to activate PPH-5 (Haslbeck
294 et al. 2015). There is biochemical evidence that the PPH-5/HSP-90 interaction involves
295 additional HSP-90 domains beyond the MEEVD motif. Activation of PPH-5 phosphatase by a
296 peptide containing the MEEVD motif is less than that observed for full-length HSP-90 (Haslbeck
297 et al. 2015). Crosslinking experiments also suggest additional contacts between HSP-90 and
298 PPH-5. The corresponding residue mutated in *HSP-90(erb71)* in human HSP90 (M813) is part of
299 the dimerization interface of two Hsp90 molecules near the site of TPR integrating MEEVD
300 domain; as observed in a cryo-EM structure (PDB 5FWP, Verba et al. 2016). This residue might
301 alter the ability of the MEEVD peptide to bind to the TPR domain of PPH-5 by altering HSP-90
302 C-terminal structure. Therefore, an analogous mutation in other organisms such as human cells
303 may be useful for studies of the HSP90-PP5 pathway. We propose a model for the regulation of
304 separase in which *pph-5* is a negative regulator of separase and PPH-5 activity is positively
305 regulated by interactions with HSP-90 (Figure 4 C). These new alleles of *hsp-90* and *pph-5*
306 provide important tools for future dissection of this pathway.

307 **Novel *sep-1(e2406)* suppressors belong to multiple complementation groups**

308 Our suppressor screen identified six lines without suppressor mutations in the three
309 genes we sequenced (*sep-1*, *pph-5* and *hsp-90*). These suppressed lines have varying degrees of

310 hatching recovery at the restrictive temperature (Figure 5 A). To determine the number of loci
311 represented by this group of alleles, we performed pairwise complementation tests. The hatching
312 efficiency of broods laid by F1 cross progeny between two homozygous suppressed lines was
313 monitored at 20°. As presented in Figure 5 B, these suppressors belong to four, possibly five,
314 complementation groups. Two lines, *sep-1(e2406); erb23* and *sep-1(e2406)*; *erb24* do not
315 complement and their cross progeny demonstrate an intermediate embryonic lethality as
316 compared to the parents. Another mutant, *sep-1(e2406); erb66* mutation appears to be dominant
317 over other suppressors, except *erb67*, and cannot be assigned to a complementation group.
318 Finally, *erb37*, *erb60* and *erb67* do not result in suppression when crossed with other mutants
319 and are likely mutations in three different genes. These observations provide an exciting
320 opportunity to identify novel regulators of separase.

321 **Conclusion**

322 By undertaking this extensive suppressor screen, we set out to identify separase
323 regulators. Our results reveal that the phosphatase, *pph-5*, is a suppressor of *sep-1(e2406)*
324 lethality. The results of our genetic screen highlight the importance of the *pph-5* regulatory
325 pathway. The mechanism by which *pph-5* regulates separase during cytokinesis will be an
326 important focus of future studies. Identifying substrates of PPH-5 that become
327 hyperphosphorylated in a *pph-5* mutant may elucidate this mechanism as well as any additional
328 roles PPH-5 might play during mitosis. We have found that *hsp-90* also functions, likely via its
329 regulation of *pph-5*, as a separase regulator. The sole *hsp-90* suppressor we identified may be a
330 rare hypomorphic mutant whose PPH-5 activating role is selectively reduced without
331 compromising its other critical chaperone functions. Given the high degree of conservation of
332 *pph-5* and *hsp-90*, we expect our observations will be applicable to separase function in other

333 systems as well. We were also able to identify novel intragenic suppressors, all of which are
334 missense mutations in the N-terminal TPR-like domain of SEP-1, providing insight into this
335 poorly characterized domain. TPR domains mediate protein-protein interactions and these
336 residues may be involved in mediating interactions with separase binding partners required for
337 its function. We have additionally isolated lines that carry mutations belonging to at least four
338 complementation groups, giving us the opportunity to more extensively understand separase
339 regulation. We will pursue a whole genome sequencing approach to identify these mutations.
340 This study demonstrates the power of genetics in understanding separase function and regulation.

ACKNOWLEDGMENTS

We would like to thank Aude Peden for performing the ENU screen and isolating suppressors. We would like to thank Chris Turpin for helping with the screen and discussion of the manuscript. We would like to thank Andy Golden from the National Institute of Diabetes & Digestive & Kidney Disease at the National Institutes of Health for his scientific discussion and guidance. We would also like to thank Kevin O'Connell and Bruce McKee for providing critical feedback on the manuscript. We especially thank Wormbase and the Caenorhabditis Genetics Center (CGC). WormBase is supported by grant U41 HG002223 from the National Human Genome Research Institute at the US National Institutes of Health, the UK Medical Research Council and the UK Biotechnology and Biological Sciences Research Council. The CGC (St. Paul, MN), is funded by the National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). This work was funded by startup funds from UT Knoxville and by NIH R01 GM114471.

354 ## LITERATURE CITED

355 Bacac, M., C. Fusco, A. Planche, J. Santodomingo, N. Demaurex, et al., 2011 Securin and
356 Separase Modulate Membrane Traffic by Affecting Endosomal Acidification. Traffic **12**:
357 615–626.

358 Baskerville, C., M. Segal, and S. I. Reed, 2008 The protease activity of yeast separase (Esp1) is
359 required for anaphase spindle elongation independently of its role in cleavage of cohesin.
360 Genetics **178**: 2361–2372.

361 Bembenek, J. N., C. T. Richie, J. M. Squirrell, J. M. Campbell, K. W. Eliceiri, et al., 2007
362 Cortical granule exocytosis in C. elegans is regulated by cell cycle components including
363 separase. Development **134**: 3837–3848.

364 Bembenek, J. N., J. G. White, and Y. Zheng, 2010 A role for separase in the regulation of RAB-
365 11-positive vesicles at the cleavage furrow and midbody. Curr. Biol. **20**: 259–64.

366 Boland, A. et al., 2017 Cryo-EM structure of a metazoan separase–securin complex at near-
367 atomic resolution. Nat. Struct. Mol. Biol. 1–7

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

369 Chang, L. and D. Barford, 2014 Insights into the anaphase promoting complex: a molecular
370 machine that regulates mitosis. Curr. Opin. Struct. Biol. **29**: 1–9.

371 Chen, M. S., A. M. Silverstein,W. B. Pratt, and M. Chinkers, 1996 The tetratricopeptide repeat
372 domain of protein phosphatase 5 mediates binding to glucocorticoid receptor
373 heterocomplexes and acts as a dominant negative mutant. J. Biol. Chem. **271**: 32315–20.

374 Gaiser, A. M., C. J. O. Kaiser, V. Haslbeck, and K. Richter, 2011 Downregulation of the Hsp90
375 System Causes Defects in Muscle Cells of Caenorhabditis Elegans. PLoS One **6**.

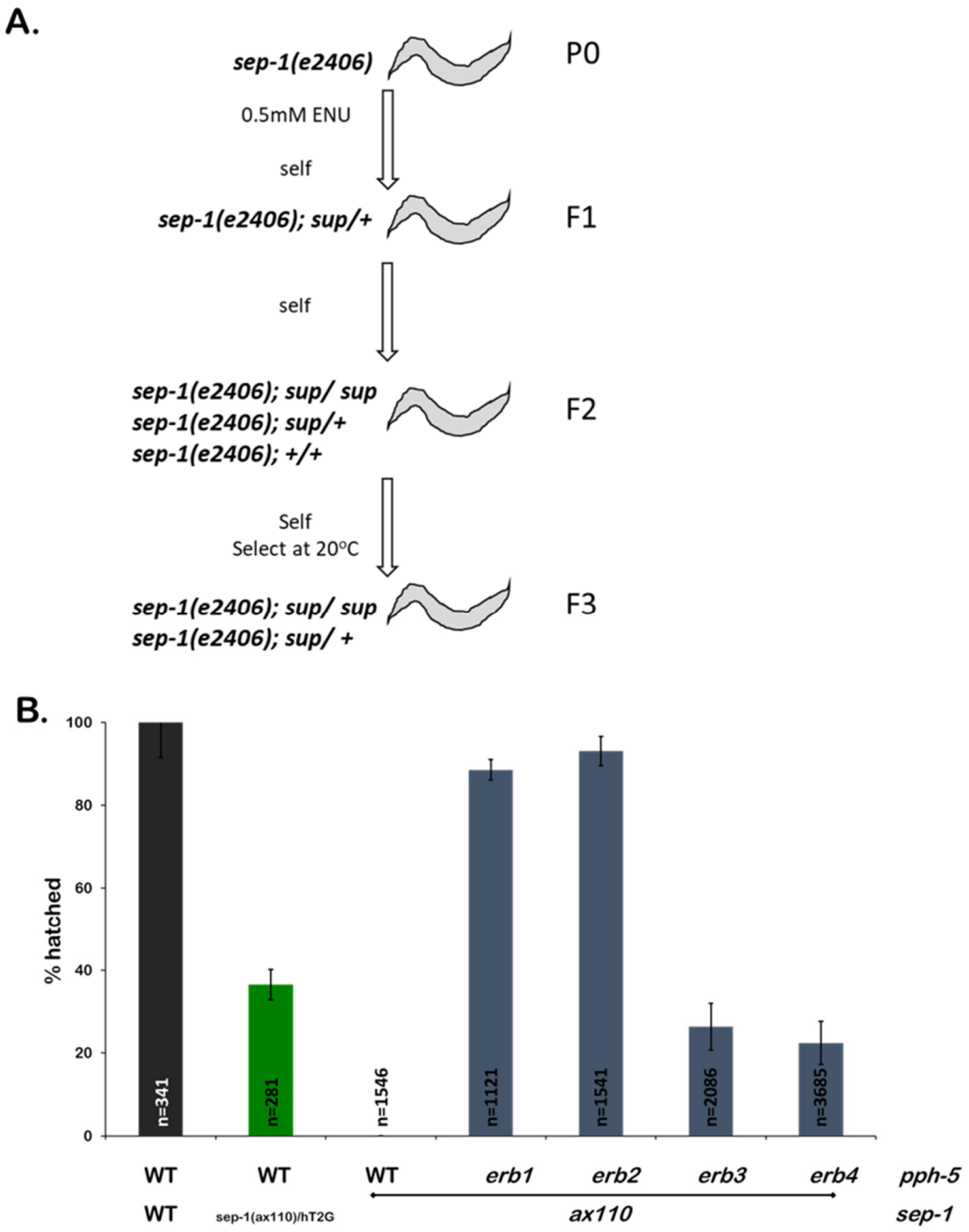
376 Gillan, V., K. Maitland, G. Mccormack, N. A. I. I. N. Him, and E. Devaney, 2009 Functional
377 genomics of hsp-90 in parasitic and free-living nematodes. Int. J. Parasitol. **39**: 1071–1081.
378 Gorr, I. H., D. Boos, and O. Stemmann, 2005 Mutual inhibition of separase and Cdk1 by two-
379 step complex formation. Mol. Cell **19**: 135–141.
380 Haslbeck, V., J. M. Eckl, A. Drazic, D. a. Rutz, O. R. Lorenz, et al., 2015 The activity of protein
381 phosphatase 5 towards native clients is modulated by the middle- and C-terminal domains of
382 Hsp90. Sci. Rep. **5**: 17058.
383 Haslbeck, V., J. M. Eckl, C. J. O. Kaiser, K. Papsdorf, M. Hessling, et al., 2013 Chaperone-
384 interacting TPR proteins in Caenorhabditis elegans. J. Mol. Biol. **425**: 2922–2939.
385 Hauf, S., Waizenegger, I. C. & Peters, J. M. Cohesin cleavage by separase required for anaphase
386 and cytokinesis in human cells. *Science* **293,** 1320–3 (2001).
387 Inoue, T., K. Hirata, Y. Kuwana, M. Fujita, J. Miwa, et al., 2006 Cell cycle control by daf-
388 21/Hsp90 at the first meiotic prophase/metaphase boundary during oogenesis in
389 Caenorhabditis elegans. Dev. Growth Differ. **48**: 25–32.
390 Kraft, C., F. Herzog, C. Gieffers, K. Mechtler, A. Hagting, et al., 2003 Mitotic regulation of the
391 human anaphase-promoting complex by phosphorylation. EMBO J. **22**: 6598–609.
392 Moschou, P. N., E. I. Savenkov, E. A. Minina, K. Fukada, S. H. Reza, et al., 2016 EXTRA
393 SPINDLE POLES (Separase) controls anisotropic cell expansion in Norway spruce (Picea
394 abies) embryos independently of its role in anaphase progression. New Phytol. **212**: 232–243.
395 Musacchio, A., 2015 The Molecular Biology of Spindle Assembly Checkpoint Signaling
396 Dynamics. Curr. Biol. **25**: R1002–R1018.
397 Nasmyth, K. A. Segregating sister genomes: the molecular biology of chromosome separation.
398 Science **297,** 559–65 (2002).

399 Nasmyth, K. & Haering, C. H. Cohesin: Its Roles and Mechanisms. Annu. Rev. Genet. 43, 525–
400 558 (2009).
401 Ollendorff, V. and D. J. Donoghue, 1997 The serine/threonine phosphatase PP5 interacts with
402 CDC16 and CDC27, two tetratricopeptide repeat-containing subunits of the anaphase
403 promoting complex. J. Biol. Chem. **272**: 32011–32018.
404 Olson, S. K., G. Greenan, A. Desai, T. Müller-Reichert, and K. Oegema, 2012 Hierarchical
405 assembly of the eggshell and permeability barrier in C. Elegans. J. Cell Biol. **198**: 731–748.
406 Peters, J. M., A. Tedeschi, and J. Schmitz, 2008 The cohesin complex and its roles in
407 chromosome biology. Genes Dev. **22**: 3089–3114.
408 Richie, C. T., J. N. Bembenek, B. Chestnut, T. Furuta, J. M. Schumacher, et al., 2011 Protein
409 phosphatase 5 is a negative regulator of separase function during cortical granule exocytosis
410 in C. elegans. J. Cell Sci. **124**: 2903–13.
411 Schopf, F. H., Biebl, M. M. & Buchner, J. The HSP90 chaperone machinery. Nat. Publ. Gr. **18,**
412 345–360 (2017).
413 Siomos, M. F., A. Badrinath, P. Pasierbek, D. Livingstone, J. White, et al., 2001 Separase is
414 required for chromosome segregation during meiosis I in Caenorhabditis elegans. Curr. Biol.
415 **11**: 1825– 1835.
416 Stein, K. K. and A. Golden, 2015 The C. elegans eggshell. Wormbook pp. 1–35.
417 Sullivan, M., C. Lehane, and F. Uhlmann, 2001 Orchestrating anaphase and mitotic exit:
418 separase cleavage and localization of Slk19. Nat. Cell Biol. **3**: 771–7.
419 Sullivan, M. and F. Uhlmann, 2003 A non-proteolytic function of separase links the onset of
420 anaphase to mitotic exit. Nat. Cell Biol. **5**: 249–54.

421 Uhlmann, F., Wernic, D., Poupart, M. a, Koonin, E. V & Nasmyth, K. A. Cleavage of cohesin by
422 the CD clan protease separin triggers anaphase in yeast. *Cell* **103,** 375–86 (2000).
423 Verba, K. A. et al. Atomic structure of Hsp90-Cdc37-Cdk4 reveals that Hsp90 traps and
424 stabilizes an unfolded kinase. Science (80). **352,** 1542–1547 (2016).
425 Williams, B. D., B. Schrank, C. Huynh, R. Shownkeent, and R. H. Waterston, 1992 A Genetic
426 Mapping System in Caenorhabditis elegans Based on Polymorphic Sequence-Tagged Sites.
427 Genetics **131**: 609–624.
428 Yang, J., S. M. Roe, M. J. Cliff, M. a.Williams, J. E. Ladbury, et al., 2005 Molecular basis for
429 TPR domain-mediated regulation of protein phosphatase 5. EMBO J. **24**: 1–10.

430 **FIGURES**

431



432

433 **Figure 1 Isolation of suppressors of *sep-1(ax110)* and *sep-1(e2406)***

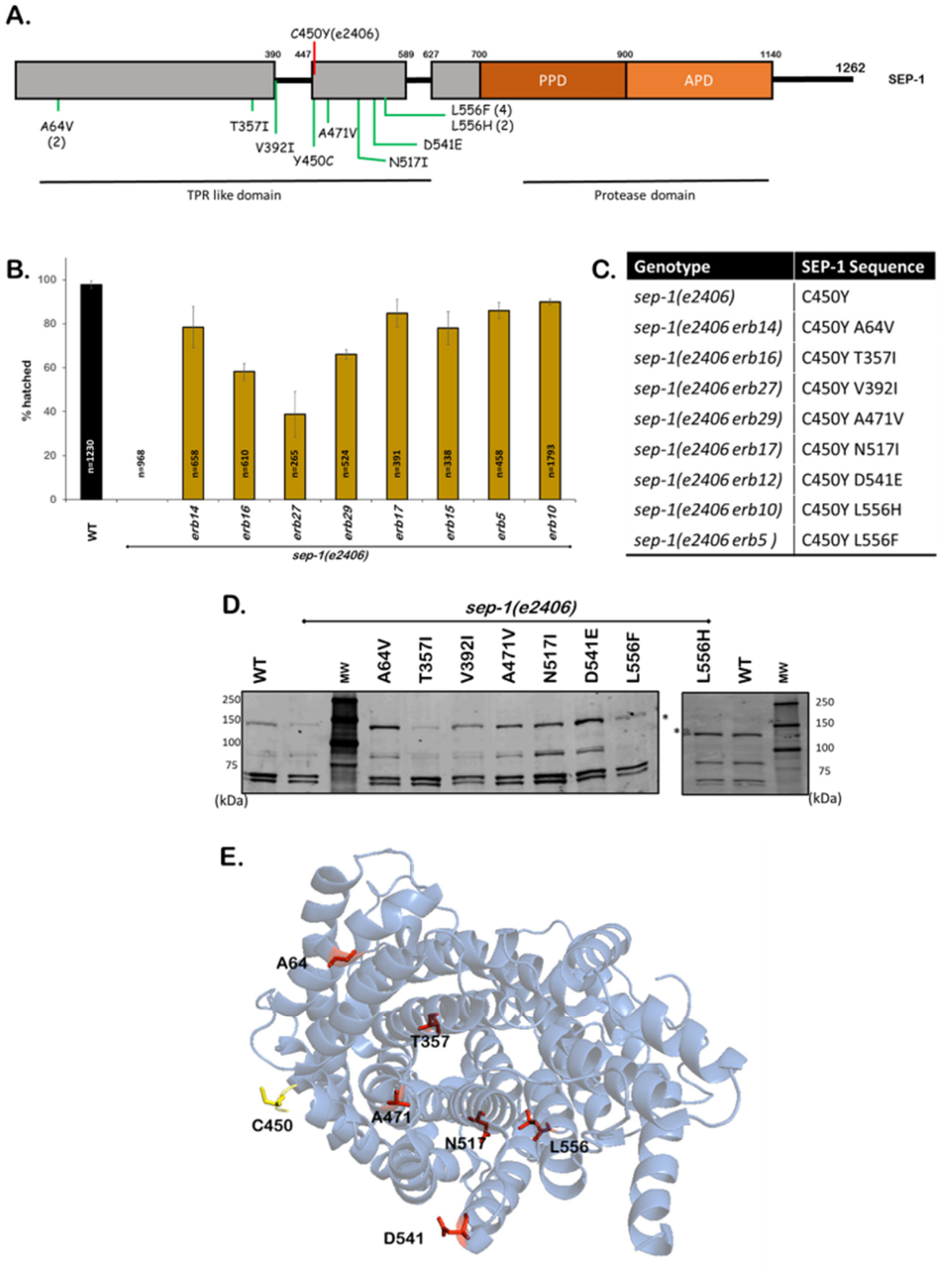
434 **A.** Schematic for the isolation of lethality suppressing mutants in the temperature sensitive *sep-*

435 *1(e2406)* background via ENU mutagenesis. **B.** Mutations in *pph-5* rescue non-conditional *sep-*

436 *1(ax110)* mutants. *sep-1(ax110)* homozygotes carrying mutations in the phosphatase domain,

437 *erb1* (S229L) and *erb2* (M380T), of PPH-5 have lower embryonic lethality relative to those

438 carrying mutations in the TPR domain *erb3/4* (L77P) (n=number of embryos).


A.
C450Y(e2406)
390
447
589
627
700
900
1140
1262
SEP-1
PPD
APD
A64V (2)
T357I
V392I
A471V
Y450C
N517I
D541E
L556F (4)
L556H (2)
TPR like domain
Protease domain
B.
% hatched
n=1230
n=968
n=658
n=610
n=265
n=524
n=391
n=338
n=458
n=1793
WT
erb14
erb16
erb27
erb29
erb17
erb15
erb5
erb10
sep-1(e2406)
C.
Genotype | SEP-1 Sequence
sep-1(e2406) | C450Y
sep-1(e2406 erb14) | C450Y A64V
sep-1(e2406 erb16) | C450Y T357I
sep-1(e2406 erb27) | C450Y V392I
sep-1(e2406 erb29) | C450Y A471V
sep-1(e2406 erb17) | C450Y N517I
sep-1(e2406 erb12) | C450Y D541E
sep-1(e2406 erb10) | C450Y L556H
sep-1(e2406 erb5) | C450Y L556F
D.
sep-1(e2406)
WT
MW
A64V
T357I
V392I
A471V
N517I
D541E
L556F
L556H
250
150
100
75
(kDa)
E.
A64
T357
C450
A471
N517
L556
D541

439

440 **Figure 2 Intragenic mutations suppress *sep-1(e2406)***

441 **A.** Protein diagram illustrating suppressing alleles of SEP-1. The causative *e2406* mutation
442 (C450Y) is depicted with a red line. Novel suppressor mutations are in green on the protein
443 diagram and are exclusively in the N-terminal TPR-like domain of the protein. Numbers in
444 parentheses following a mutation indicate the number of times each suppressor was identified. **B.**
445 Embryonic lethality assays demonstrate that each suppressing mutation restores viability to *sep-*
446 *1(e2406)* worms at 20° (n=number of embryos). **C.** Table listing gene mutations and the
447 resulting missense mutations in SEP-1. **D.** SEP-1 is detectable by western blot in animals
448 carrying *sep-1(e2406)* suppressing mutations. Asterisk indicates SEP-1 (144KDa). **E.** Cryo-EM
449 structure (PDB 5MZ6) illustrating the N-terminal TPR like domain of SEP-1. The residue
450 mutated in *sep-1(e2406)* (C450) is shown in yellow and suppressor mutations are illustrated in
451 red. Mapping of mutated residues onto the structure illustrates that they are distributed
452 throughout the N-terminus.

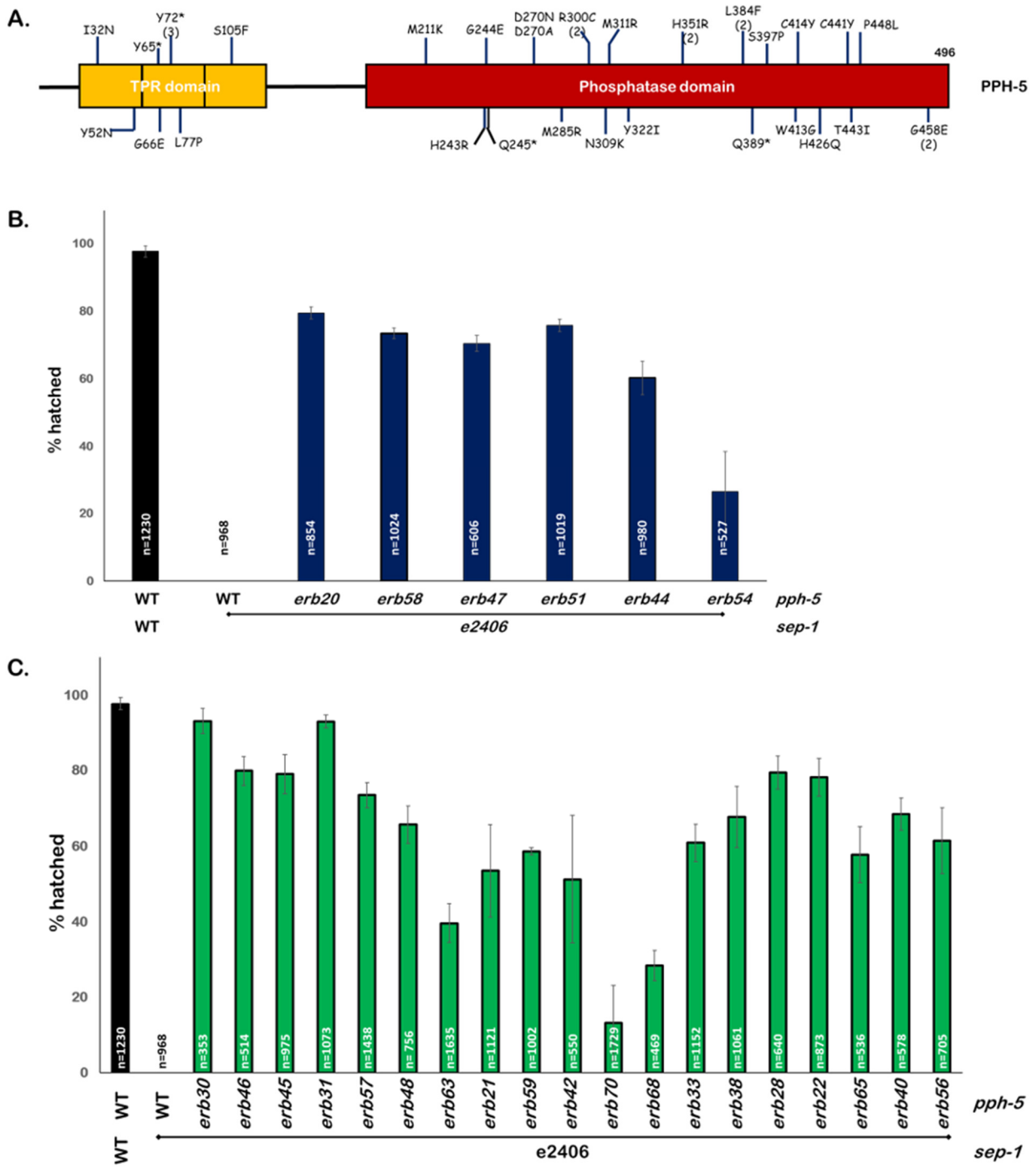


Figure 3 Mutations in *pph-5* are the most frequently identified *sep-1(e2406)* suppressors. **A.** Protein diagram illustrating *pph-5* alleles suppressing *sep-1(e2406)*. These missense mutations span across PPH-5. Numbers in parentheses following a mutation indicate the number of times each suppressor was identified. This diagram does not depict splice site variants or

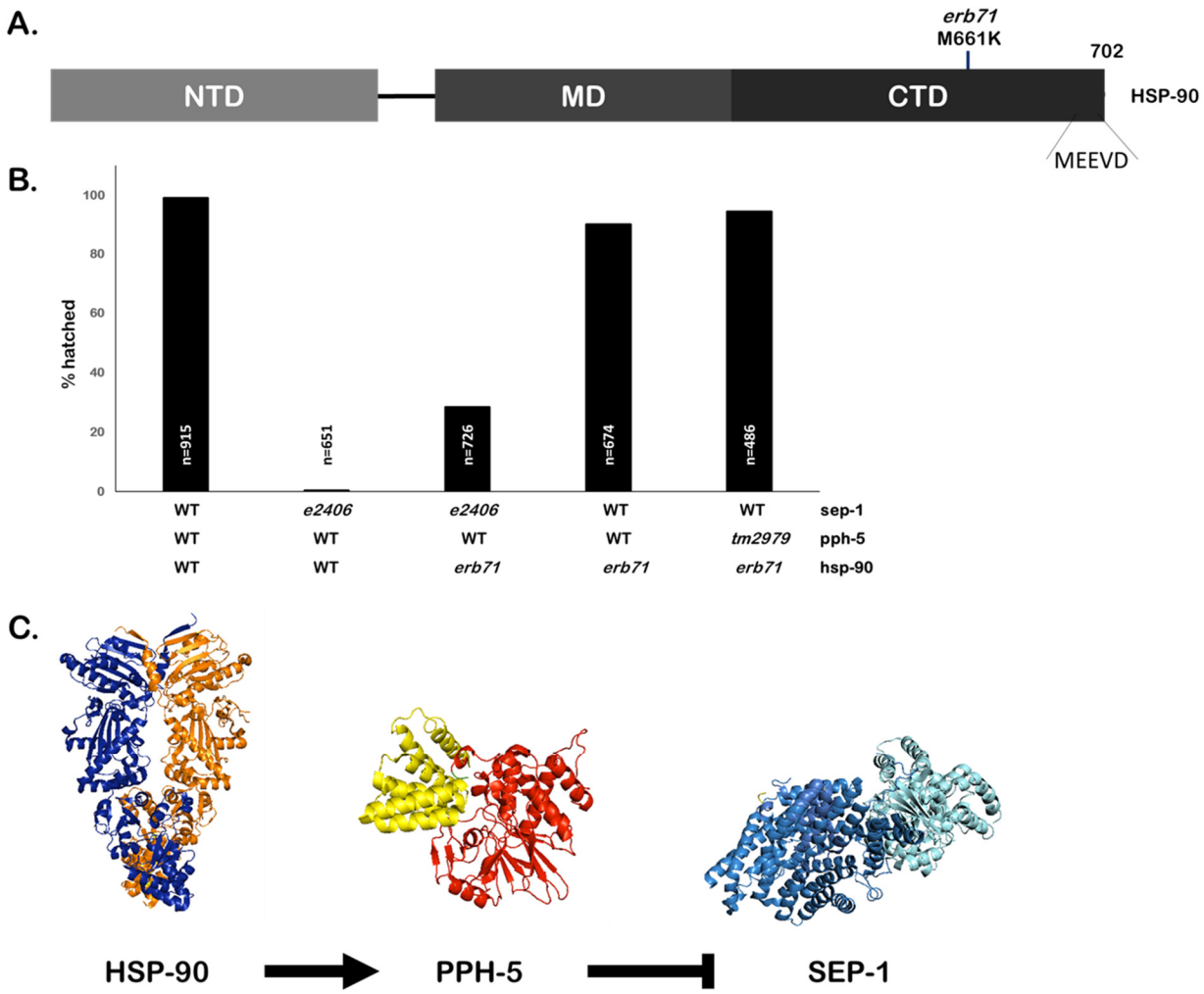
458 frameshift mutations. **B.** Embryonic lethality of *sep-1(e2406)* is rescued by missense mutations

459 in the TPR domain of PPH-5, which might affect substrate binding. **C.** Embryonic lethality of

460 *sep-1(e2406)* is rescued by missense mutations in the phosphatase domain of PPH-5, which

461 might affect catalytic activity (n=number of embryos).

462



463

464 **Figure 4 Mutation in the molecular chaperone *hsp-90* suppresses *sep-1(e2406)*.**

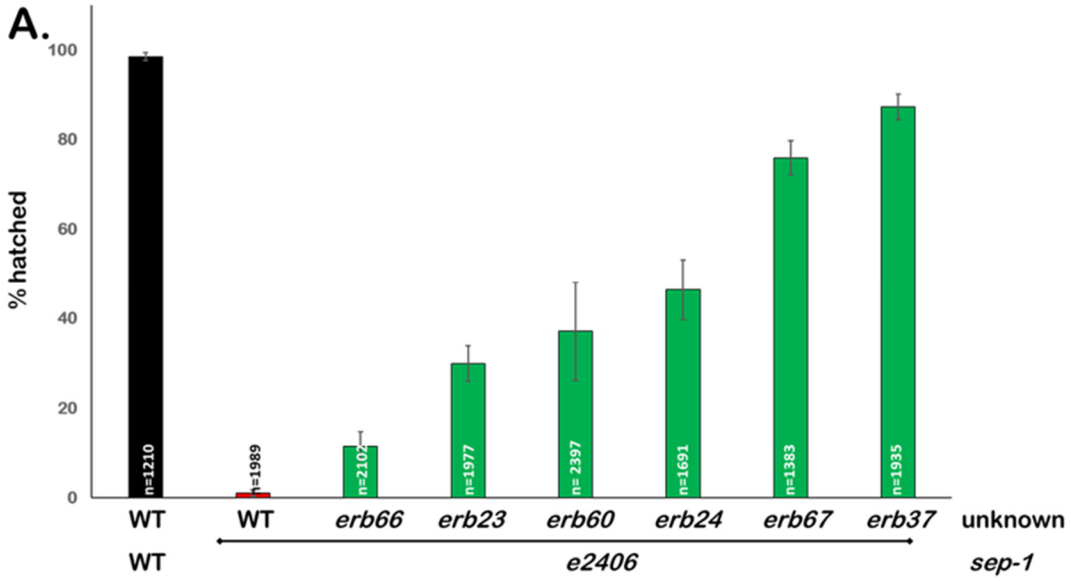
465 **A.** Protein diagram of HSP-90. The *erb71* mutation results in a missense mutation at the C-

466 terminal end of the protein chaperone HSP-90 (M661K), separated by 36 residues from the C-

467 terminal most MEEVD motif. HSP-90 protein domains are also illustrated (NTD, amino-

468 terminal domain; MD, middle domain; CTD, carboxy-terminal domain; MEEVD, Met-Glu-Glu-

469 Val-Asp motif) **B.** The *hsp-90(erb71)* mutant has minimal effect on hatching when present in an
470 otherwise wild type background. Embryonic lethality is not reduced when *hsp-90(erb71)* is
471 combined with a *pph-5* loss of function mutant (n=number of embryos). **C.** Model for separase
472 regulatory pathway: HSP-90 activates PPH-5 to negatively regulate separase function. Loss of
473 this negative regulation suppresses *sep-1(e2406)*. (PDB structures modified from 5MZ6 (SEP-1),
474 4JA9 (PPH-5) and 5FWP (HSP-90)).



B.

		sep-1(e2406)					
		erb23 (29%)	*erb24* (46%)	*erb37* (87%)	*erb60* (37%)	*erb66* (11%)	*erb67* (87%)
sep-1(e2406)	*erb23*		33% (±4%) FTC	C	C	25% (±2%)	C
	erb24			C	C	85% (±1%)	C
	erb37				C	73% (±9%)	C
	erb60					35% (±3%)	C
	erb66						C

Figure 5 Novel suppressors of *sep-1(e2406)* belong to multiple complementation groups

A. Strains carrying novel *sep-1(e2406)* suppressors result in varied rescue of embryonic lethality (n=number of embryos). **B.** Complementation assay based on survival of F2 embryos of a cross

479 between strains carrying novel *sep-1(e2406)* suppressors indicates these suppressors belong to
480 multiple complementation groups. Numbers below each parent strain or in a box representing a
481 cross progeny indicate the percent of embryos that hatch at the restrictive temperature of 20°.
482 The numbers in parenthesis are standard deviations for three replicate hatching assays; C =
483 Complements, FTC = Failure To Complement.

484 ## TABLES

485 **Table 1 Reduction of *pph-5* by RNAi mediated knockdown results in improved hatching**

486 RNAi knockdown of *pph-5* by feeding results in improved hatching efficiency in worms carrying

487 *pph-5* mutations that suppress *sep-1(e2406)* lethality at the restrictive temperature of 20°.

488

		pph-5 RNAi		*No RNAi*
sep-1	***pph-5***	**Total embryo**	**% hatching**	**% hatching**
WT	WT	512	98.1	97.6
e2406	WT	578	72.8	0.0
	erb13 (Y65*)	323	88.9	79.4
	erb58 (I32N)	255	98.0	73.3
	erb47 (Y52H)	235	73.5	70.4
	erb51 (G66E)	589	77.1	75.7
	erb44 (L77P)	226	95.1	60.2
	erb54 (S105F)	298	33.9	26.3
	erb30 (M211K)	113	94.7	92.9
	erb46 (H243R)	215	85.1	79.8
	erb45 (G244E)	604	78.3	78.9
	erb31 (D270A)	320	94.7	92.8
	erb57 (D270N)	434	97	73.4
	erb48 (M285R)	325	91.1	65.6
	erb63 (R300C)	322	75.5	39.6
	erb21 (N309K)	351	95.4	53.4
	erb59 (M311R)	378	65.5	58.6
	erb42 (Y322I)	270	79.3	51.1
	erb72 (H351R)	163	77.6	13.1
	erb68 (S397P)	292	27.3	28.2
	erb33 (W413G)	386	84.5	60.8
	erb38 (C414Y)	255	76.5	67.7
	erb28 (H426Q)	399	95.2	79.3
	erb22 (C441Y)	374	79.1	78.1
	erb65 (T443I)	399	84.4	57.7
	erb40 (P448L)	109	74.3	68.4
	erb52 (G458E)	282	78.0	61.3

489
490

491 **Table 2 RNAi mediated knockdown of *pph-5* in *hsp-90(erb71)* worms**

492 The genetic interaction between *pph-5* and *hsp-90* was investigated by using RNAi mediated
493 knockdown of *pph-5*. Reduction of PPH-5 in a worm carrying a *sep-1(e2406)* rescuing *hsp-90*
494 mutation results in reduced embryonic lethality at the restrictive temperature (20°C). However,
495 *pph-5(RNAi)* has little effect on embryonic lethality of *hsp-90(erb71)*.

496

Strain	Total embryos	% hatching
N2	512	98.4
N2, *pph-5(RNAi)*	352	97.8
sep-1(e2406)	223	0
sep-1(e2406); pph-5(RNAi)	340	71.1
sep-1(e2406); hsp-90(erb71)	181	30.9
sep-1(e2406); hsp-90(erb71); pph-5(RNAi)	157	92.9
hsp-90(erb71)	148	84.4
hsp-90(erb71); pph-5(RNAi)	186	86.5

497