1   **The PP2A/4/6 subfamily of phosphoprotein phosphatases regulates DAF-16**

2   **during aging and confers resistance to environmental stress in postreproductive**

3   **adult *C. elegans***

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8   **Short title:** Age-dependent function of PP2A/4/6 phosphatases

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23   **Abstract**

24   Insulin and insulin-like growth factors are longevity determinants that negatively

25   regulate Forkhead box class O (FoxO) transcription factors. In *C. elegans* mutations

26   that constitutively activate DAF-16, the ortholog of mammalian FoxO3a, extend lifespan

27   by two-fold. While environmental insults induce DAF-16 activity in younger animals, it

28   also becomes activated in an age-dependent manner in the absence of stress,

29   modulating gene expression well into late adulthood. The mechanism by which DAF-16

30   activity is regulated during aging has not been defined. Since phosphorylation of DAF-

31   16 generally leads to its inhibition, we asked whether phosphatases might be necessary

32   for its increased transcriptional activity in adult *C. elegans*. We focused on the PP2A/4/6

33   subfamily of phosphoprotein phosphatases, members of which had been implicated to

34   regulate DAF-16 under low insulin signaling conditions but had not been investigated

35   during aging in wildtype animals. Using reverse genetics, we functionally characterized

36   all *C. elegans* orthologs of human catalytic, regulatory, and scaffolding subunits of

37   PP2A/4/6 holoenzymes in postreproductive adults. We found that PP2A complex

38   constituents PAA-1 and PPTR-1 regulate DAF-16 during aging and that they cooperate

39   with the catalytic subunit LET-92 to protect adult animals from ultraviolet radiation. PP4

40   complex members PPH-4.1/4.2, SMK-1, and PPFR-2 also appear to regulate DAF-16 in

41   an age-dependent manner, and they contribute to innate immunity. Interestingly, SUR-6

42   but no other subunit of the PP2A complex was necessary for the survival of pathogen-

43   infected animals, suggesting that a heterotypic PP4 complex functions during aging.

44   Finally, we found that PP6 complex constituents PPH-6 and SAPS-1 contribute to host

45   defense during aging, apparently without affecting DAF-16 transcriptional activity. Our

46   studies indicate that a set of PP2A/4/6 complexes protect adult *C. elegans* from

47   environmental stress, thus preserving healthspan. Therefore, along with their functions

48   in cell division and development, the PP2A/4/6 phosphatases also appear to play critical

49   roles later in life.

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67   **Introduction**

68   Perhaps more than any other, the Insulin and IGF-signaling (IIS) pathway has

69   been unequivocally established as a primary modulator of lifespan across evolutionarily

diverse species (1). Genetic and behavioral changes that modify IIS pathway activity change the quality and length of life dramatically. Loss-of-function mutations in the insulin receptor correlate with a 2- to 3-fold extension in the lifespan in some species (2). Moreover, insulin signaling mutants appear more youthful and are more resistant to a variety of environmental insults, demonstrating a connection between stress resistance and lifespan (3,4). Thus, the ability of an organism to withstand encounters with stressful stimuli or to otherwise neutralize the consequent damage associated with acute stress is a primary determinant of its overall health during aging and, ultimately, how long it lives. The IIS pathway, and its ultimate target, the Forkhead box family (FoxO) transcription factor, have a profound influence on lifespan and stress resistance. Consequently, understanding their regulation over time could suggest the basis for the milestones of aging and reveal potential avenues for manipulating the rate of aging.

In *C. elegans* the FoxO protein DAF-16 is a transcriptional regulator that determines lifespan in part by conferring resistance to environmental insults that have the potential to cause catastrophic cellular and molecular damage. As the ultimate downstream target of the insulin and IIS pathway, the transcriptional activity of DAF-16 is inhibited when insulin-like ligands are bound to the insulin receptor, DAF-2. In the absence of these soluble ligands, DAF-16 is free to translocate to the nucleus where it upregulates the expression of genes that promote longevity, and it is because of this function of DAF-16 that *daf-2* mutants live twice as long as their wildtype counterparts (5). DAF-16 activity is also triggered even if the IIS pathway is intact when animals encounter harmful stimuli including high temperature and ultraviolet radiation (6). Under these stress-inducing conditions, DAF-16 targets may include genes that encode

93 detoxifying enzymes, immune effectors, and chaperones that restore homeostasis,

94 preserve cellular health and contribute to host defense (7). Recently reported evidence

95 suggests that DAF-16 may be activated in an age-dependent manner, despite the

96 absence of acute stress. During adulthood, the expression levels of the *daf-16a* isoform,

97 and to an even greater extent the *daf-16d* isoform progressively increase (8). This is

98 coupled to increased expression of a subset of DAF-16 transcriptional targets in adults

99 as compared to in larvae, suggesting that DAF-16 is activated as *C. elegans* age (9).

100 How DAF-16 activity might be regulated in an age-dependent manner and whether that

101 regulatory mechanism is unique to aging is not known.

102 Studies of larval stage worms and especially of *daf-2* mutants have revealed that

103 the transcriptional activity of DAF-16 is regulated on two primary levels. First, inside the

104 nucleus DAF-16 associates with other proteins that modulate its transcriptional output.

105 For example, HCF-1 binds to DAF-16 and interferes with its ability to bind to the

106 promoters of certain target genes (10). Conversely, the basic helix-loop-helix

107 transcription factor HLH-30 forms a complex with DAF-16, and the two proteins together

108 then co-regulate a subset of targets enriched for genes that promote longevity. The very

109 presence of DAF-16 inside the nucleus is subject to control from multiple inputs,

110 representing a second level of regulation. The nuclear localization of DAF-16 is

111 inhibited predominantly through the IIS pathway. Specifically, IIS signaling activates the

112 evolutionarily conserved kinase AKT-1 which phosphorylates DAF-16. Assuming that

113 this modification of DAF-16 has the same effect that it does when FoxO transcription

114 factors in other species are phosphorylated, a binding site for 14-3-3 proteins is created

115 and the nuclear export signal of DAF-16 is exposed, leading to its expulsion from the

116 nucleus and sequestration in the cytosol (11). Supporting this possibility, DAF-16

117 accumulates in the nuclei of larval worms that lack the 14-3-3 proteins PAR-5 and FTT-

118 2 (12,13). The inhibitory phosphorylation imparted to FoxO transcription factors by AKT

119 is opposed by the action of phosphoprotein phosphatase PP2A. In mammals, both

120 FoxO1 and FoxO3a are substrates of PP2A, and biochemical evidence indicates that

121 PP2A selectively dephosphorylates the AKT phosphorylation sites of FoxO family

122 members (14–17). Moreover, PP2A also dephosphorylates AKT itself, thereby

123 inactivating it (18–20). This particular mechanism of PP2A functioning as an indirect

124 positive regulator of FoxO transcription factors appears to be conserved in *C. elegans*.

125 In *daf-2* mutants, the constitutive activation of DAF-16 requires PPTR-1, a conserved

126 regulatory subunit of PP2A complexes (21). PPTR-1 associates with AKT-1 but not

127 DAF-16 in worms, and overexpression of PPTR-1 reduces the levels of phosho-AKT-1.

128 Since as a whole the expression levels of insulin-like peptides considered to be agonists

129 of DAF-2 either remain constant or increase during adulthood, the presence of a

130 sustained inhibitory signal through the IIS pathway that persists in adult animals seems

131 likely (9). A regulator that can counteract this signal is therefore an attractive candidate

132 for functioning to modulate the activity of DAF-16 during aging, and this is what led us to

133 develop the hypothesis that phosphoprotein phosphatases modulate DAF-16

134 transcriptional activity in an age-dependent manner.

135   In mammals, PP2A may generally be considered to play an anti-aging role, in

136 part because it protects organisms from age-related disease. For example, PP2A is the

137 primary phosphatase that dephosphorylates Tau protein in the brain and therefore helps

138 to prevent the neurodegeneration that leads to Alzheimer's disease (22). The level and

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139   activity of PP2A detected in postmortem brains of Alzheimer's patients are, in fact,

140   reduced compared to healthy controls (23). Similarly, low PP2A activity in monkey

141   brains is associated with elevated levels of phosphorylated alpha-synuclein, a hallmark

142   of Parkinson's disease (24,25). Outside of the context of acute disease, PP2A activity in

143   the liver of SAMP8 mice is reduced and is correlated with increased levels of inactive,

144   phosphorylated FoxO1 which may be responsible for the animals' shortened lifespan

145   (26).

146   PP2A is part of a subfamily of phosphoprotein phosphatases that also includes

147   PP4 and PP6. Phosphoprotein phosphatases (PPPs) comprise a large class of

148   evolutionarily conserved enzymes, that antagonize kinases by removing phosphate

149   groups from a broad repertoire of substrates. The PPP class enzymes are structurally

150   distinct from the PPM Ser/Thr phosphatases in that members of the PPM family are

151   Mg^{2+}-dependent, unlike members of the PPP family(27). PPPs can be divided into two

152   evolutionarily distinct branches composed of PP1/PP2A/PP2B and PP5/PPEF/PP7

153   based on divergent regulatory and catalytic domains, with the PP2A subgroup

154   containing the PP2A/PP4/PP6 subfamily(27). PPP family phosphatase active sites are

155   similar, but differ in sensitivity and response to certain small molecule inhibitors(28).

156   While PP3/5/7 contain N- or C-terminal domains that act as built in regulatory factors,

157   the PP1/2A/4/6 catalytic subunits all have a highly conserved globular domain that acts

158   in concert with one (PP1/6) or two (PP2A/4/6) binding partners. Catalytic subunits of the

159   PP2A/4/6 subfamily associate with regulatory proteins and, in some cases, a scaffolding

160   protein to comprise a functional holocomplex. Multiple versions of the PP2A/4/6

161   complexes are possible because there are several genes whose products bind and

162   regulate the same catalytic subunit(29). For example, in humans there are 18 different

163   regulatory proteins capable of associating with the PP2A enzyme, allowing for the

164   generation of nearly 100 holoenzymes (30,31). The combinatorial diversity of these

165   complexes is believed to contribute to the subcellular localization, substrate specificity,

166   and thus the functional capacity of the phosphoprotein phosphatases, depending on

167   their subunit composition(32).

168   In evolutionarily diverse species, the PP2A/4/6 subfamily regulates a wide array

169   of processes, including cell cycle progression, meiosis, cellular differentiation, and the

170   activity of multiple signaling pathways including the IIS pathway (30,33–42).

171   Interestingly, in *C. elegans* PP2A is not the only member of the PP2A/4/6 family with a

172   connection to the insulin signaling pathway. Just as the PP2A subunit PPTR-1 is

173   required for the extended lifespan of *daf-2* mutants, so too is the regulatory subunit of

174   the PP4 complex SMK-1 (43). SMK-1 is orthologous to human PP4R3, *D. melanogaster*

175   flfl, and *S. cerevisiae* Psy2p. During embryonic development in worms, SMK-1

176   associates with replicating chromatin, triggering the recruitment PPH-4, the catalytic

177   subunit of the PP4 complex, and silencing the CHK-1-mediated response to DNA

178   damage, allowing cell cycle progression(44). Outside of the context of embryonic

179   development, SMK-1 appears to be constitutively expressed in the nucleus of several

180   tissues where it plays a role in longevity and stress resistance. Reminiscent of

181   phenotypes associated with inhibiting PPTR-1, when *smk-1* expression is blocked by

182   RNAi, *daf-2* animals are no longer resistant to a variety of stresses (43). Furthermore,

183   the expression of a subset of DAF-16 transcriptional targets is reduced following RNAi

184   against *smk-1* because of a deficiency in transcription initiation (45). This apparent

185 functional overlap between PP2A and PP4 in regulating DAF-16 in a genetic

186 background where DAF-16 is hyper-activated raises the question as to which member is

187 principally responsible for activating DAF-16 during the aging process in wildtype

188 animals. Moreover, considering that different combinations of subunits allow for the

189 possibility of multiple versions of the PP2A/4/6 complexes, a more nuanced version of

190 this question would ask about the specific identity of the individual constituents of the

191 complex(es) responsible for regulating DAF-16 during adulthood. This is what our study

192 sought to address.

193   Here we present the first systematic investigation of the role of the PP2A/4/6

194 subfamily of phosphoprotein phosphatases during aging in wildtype *C. elegans*. We

195 used a reverse genetic approach to functionally characterize all *C. elegans* orthologs of

196 human PP2A/4/6 catalytic, scaffold, and regulatory subunits in juvenile and

197 postreproductive adult animals. Our data suggest that the PP2A and PP4 complexes

198 regulate the age-dependent activity of DAF-16. Moreover, we present evidence to

199 support the existence of multiple versions of PP2A, PP4, and PP6 complexes with

200 apparent specialized functions to protect adult *C. elegans* from different environmental

201 stresses and thus as a group contribute to preserving healthspan.

202 **Methods**

203 C. elegans *growth and maintenance*

204   Bristol N2 and VIL001 *mjyIs001 [Plys7::gfp] C. elegans* strains were grown and

205 maintained under standard laboratory conditions (46). To generate strain VIL001,

206 stable transgenic animals harboring an extrachromosomal *lys-7* promoter::gfp fusion

207 construct (47) were subjected to gamma irradiation to yield a chromosomal integration

208   of *Plys7::gfp*. F_2 segregants of the irradiated P_0 animals yielding 100% GFP-expressing

209   progeny were selected for further analysis. One of these lines was backcrossed to the

210   N2 wildtype strain seven times and then designated VIL001.

C. elegans synchronization

212   Worms were synchronized via sodium hypochlorite treatment (48). After hatching

213   overnight in sterile M9 buffer, approximately 2000 L1 stage worms were dropped to fresh,

214   pre-seeded RNAi plates or to NGM plates seeded with *E. coli* OP50 (see below).

RNAi treatment

216   Animals were treated with RNAi through their food source as described by Fraser

217   *et al.* with modifications as described below (49). All RNAi sequences were confirmed by

218   sequencing prior to use. Bacteria from the Source BioScience library were streaked onto

219   LB plates containing ampicillin (amp, 5µg/mL) and tetracycline (tet, 1.25µg/mL) and

220   allowed to grow overnight at 37ºC. One colony from each plate was used to inoculate

221   200mL of LB with amp (1ng/mL) and grown overnight in a 37ºC shaker. Bacteria were

222   centrifuged at 5,000g for 10 minutes, resuspended in 20mL of LB containing amp, and

223   1mL was dropped onto individual RNAi plates (standard NGM plates with 1mg/mL

224   carbenicillin, 2µM IPTG). Lawns of *E. coli* RNAi clones were allowed to grow for at least

225   two days before worms were introduced to the plates. Except in the cases where either

226   *let-92* or *paa-1* were the targets of the treatment, synchronized cohorts of L1 larvae were

227   dropped onto seeded RNAi plates and maintained at 20ºC. When these animals reached

228   the L4 stage they were transferred to pre-seeded RNAi plates containing 25 µg/mL FUdR

229   (5-fluorodeoxyuridine). Since initiating knockdown of *let-92* and *paa-1* at L1 prevented

230   maturation past L4, RNAi treatment targeting these genes was delayed until animals had

231   reached L4 on NGM plates seeded with OP50. Worms subjected to heat stress or UV

232   irradiation remained on RNAi plates until they died. For bacterial infection assays, worms

233   were transferred from the RNAi plates at either the L4 stage or at Day 6 of adulthood to

234   plates containing *Pseudomonas aeruginosa* where they remained throughout the

235   duration of the assay.

236   Pseudomonas aeruginosa (PA14) *infection assays*

237   Infection assays were carried out as described in Tan *et al.* with the following

238   modifications (50). For each infection assay replicate worms were cultured on RNAi plates

239   as described above and approximately 30 animals were transferred to each of three slow

240   kill assay 3.5 cm plates at L4 and Day 6 where they were maintained at 25ºC for the

241   duration of the experiment. These plates contained 25 µg/mL FUdR, were seeded with 6

242   µl of an overnight culture of PA14, and then incubated for one night at 37ºC and a second

243   night at room temperature prior to the start of the infection assay. Worm survival was

244   scored in the morning and evening every day by counting the number of animals that

245   responded to gentle prodding with a wire pick. Worms that failed to move were scored as

246   dead and removed from the plates.

247   *Thermotolerance assay*

248   After being synchronized by sodium hypochlorite treatment as described above,

249   L1 larvae were dropped on to RNAi plates seeded with a clone expressing dsRNA to

250   target a given gene of interest. Worms were maintained on those plates until the L4 larval

251   stage when they were split into two groups. The first group of worms was subjected to

252   thermal stress as described previously with some modifications (43). Briefly,

253   approximately 100 L4 worms were distributed evenly between three pre-seeded 3.5 cm

254   RNAi food plates containing FUdR and then incubated at 35ºC for the duration of the heat

255   treatment and scored every few hours for survival with animals being counted as alive if

256   they responded to gentle prodding with a wire pick. The second group of worms,

257   consisting of ~2000 animals, was divided among three 6 cm pre-seeded RNAi + FUdR

258   plates by chunking and were maintained at 20˚C. At the sixth day of adulthood, ~100 of

259   these animals were subjected to heat stress according to the same procedure as outlined

260   above for L4 thermal stress assays.

261   *UV irradiation assay*

262   Synchronized groups of worms were cultured on RNAi plates beginning at the L1

263   larval stage. Worms were maintained on those plates until the L4 larval stage when they

264   were split into two groups. The first group of approximately 100 worms was distributed

265   among three 3.5 cm pre-seeded RNAi + FUdR plates for UV treatment. Plates were

266   placed in an irradiator (UV Stratalinker® 1800, Stratagene) with lids removed and were

267   irradiated with 1mJ/m/s^2 UV. Following treatment worms were maintained at 20ºC and

268   scored for survival daily until all animals had died. Worms were scored as alive if they

269   responded to gentle prodding with a wire pick. The remaining second group of L4 larval

270   worms were transferred to RNAi + FUdR plates by chunking and were maintained at

271   20ºC until they reached Day 6 of adulthood when they were subjected to the same UV

272   treatment and scoring regimen.

273   *Fluorescence microscopy*

274   Images of Plys-::GFP expression in *C. elegans* were taken at Day 6 on a Nikon

275   Eclipse E800 equipped with a Jenoptik digital camera. Worms were immobilized on agar

276   pads using polybead® polystyrene 0.10μm microspheres (Polysciences, Inc.), and

277　pictures were taken quickly thereafter to avoid undo stress. Transgenic animals

278　expressing *Plys7::gfp* were assigned to one of three categories based on the relative GFP

279　expression levels (low, medium, and high) according to the following criteria: low—overall

280　weak expression of GFP in the intestine or GFP expression limited to anterior of intestine,

281　just behind the pharynx; medium—bright GFP expression from the anterior intestine to

282　the midbody but little to no expression in posterior intestine; high—robust GFP expression

283　throughout the entire length of intestine. The percentage GFP expression for each sample

284　was calculated using a minimum of 200 animals and then the percentages were averaged

285　across at least 3 biological replicates.

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287　*Statistical analyses*

288　　　To calculate median lifespans (LT_{50}s) a three parameter sigmoidal curve was fit

289　to plots of survival (fraction of worms alive versus time) according to the general

290　equation $y=a/(1+e^{(-(x-x0)/b)})$ using SigmaPlot version 14 (Systat Software, San Jose, CA).

291　This equation was used to determine the point at which 50% of the animals in the assay

292　had died. The average fold difference between the LT_{50} of mutant strains or

293　experimental RNAi treatments and control animals was calculated, and the statistical

294　significance of that difference was assessed across all three replicates using a two-

295　tailed Student's t-test.

296　**Results**

297　In silico *analysis of the* C. elegans *PP2A/4/6 phosphoprotein phosphatases*

298　　　In both yeast and mammals significant structural and functional similarities

299　between members of the PP2A/4/6 family of phosphoprotein phosphatases exist. In

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300 addition, proteomic and smaller scale biochemical studies have revealed non-canonical

301 associations between subunits of the holoenzyme complexes (32,51,52). To ask how

302 the PP2A/4/6 proteins in *C. elegans* compare to their human counterparts we first took

303 an *in silico* approach. Alignments of the sequences of the three human catalytic

304 subunits PP2Ac, PP4c and PP6c with their *C. elegans* orthologs revealed strong

305 homology between them (Fig. S1, Table S1). For example, LET-92 and PPH-4.1 are

306 both greater than 80% identical to PP2Ac and PP4c, respectively. Key structural motifs

307 and regulatory residues are especially well-conserved both within catalytic subunits of

308 the PP2A/4/6 family and across species. Based on these similarities, we anticipated that

309 our studies would demonstrate significant parallels between these phosphatases in

310 worms and humans.

311   We next asked whether *C. elegans* PP2A/4/6 proteins were expected to

312 associate with the same binding partners as their human orthologs *in vivo*. To answer

313 this question we performed database searches, using SMK-1 and PPH-4.1 as

314 representative subunits for our analyses. We first used Wormbase to compile a list of

315 PPH-4.1 interactors (Table S2). Drawing mainly from biochemically verified

316 associations, high throughput yeast two-hybrid data, and interaction networks generated

317 *in silico*, this list captures a broad spectrum of potential interactors. Included among

318 them are not only proteins that could be members of a trimeric complex along with PPH-

319 4.1 but also regulators, functional analogs, and possible substrates that may be

320 dephosphorylated by PPH-4.1. In addition to SMK-1, two other worm homologs of PP4

321 regulatory subunits, PPFR-1 and PPFR-2, were included as potential PPH-4.1

322 interactors. The only other worm orthologue of a PP4 regulatory subunit, F46C5.6, was

323 absent from the list of PPH-4.1 interactors found on Wormbase. Both orthologs of the

324 yeast Tip41p-Tap42p regulatory system that modulates all members of the PP2A/4/6

325 family were identified as PPH-4.1 interactors, namely PPFR-4 and ZK688.9,

326 orthologous to mammalian α4 and TIPRL, respectively. One other notable potential

327 PPH-4.1 interactor is PPH-6, which encodes the *C. elegans* orthologue of PP6c, the

328 catalytic subunit of the related but distinct PP6 complex. When we compared the list of

329 potential PPH-4.1 interactors with the list of proteins predicted by Wormbase to interact

330 with SMK-1, we expected to find significant overlap, especially among orthologues of

331 the PP4 complex. Instead, although PPH-4.1 was listed as an interactor of SMK-1 the

332 only other potential PP4 complex member that was in common to the list of interactors

333 for both PPH-4.1 and SMK-1 was the regulatory subunit PPFR-1 (Table S3).

334 To gain further insight into the possible relationships between SMK-1, PPH-4.1,

335 and their potential binding partners, we performed interaction network analysis using

336 Genemania. All homologues of the PP2A/4/6 family earmarked as possible interactors

337 of PPH-4.1 or SMK-1 on Wormbase along with the modulator PPFR-4 were included.

338 This analysis yielded additional putative PPH-4.1 interactors (Fig S2). Among these

339 were PAA-1 and Y71H2AM.20, *C. elegans* orthologues of human PR65α/PR65β and

340 PTPA, respectively. This was surprising because in human cells these proteins

341 associate with PP2c, the catalytic subunit of the PP2 complex, and not the PP4

342 complex. Specifically, PR65α/PR65β are scaffolding units of the PP2 complex, and

343 PTPA has been shown to modify the catalytic site of the PP2c enzyme (53–55). This

344 raises the intriguing possibility that certain subunits may associate with both a PP4c-

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345   containing complex and with PP2A complexes in *C. elegans*, a scenario that is

346   supported by biochemical evidence in mammalian systems (32,51).

347   Taken together, our database queries led to the unexpected finding that *C.*

348   *elegans* orthologs of proteins not only of the PP4 complex but also of canonical subunits

349   of the PP2A and PP6 holoenzyme complexes could potentially associate with SMK-1

350   and PPH-4.1. This lent credence to our experimental strategy of considering all *C.*

351   *elegans* orthologs of the entire PP2A/4/6 family so that evidence supporting putative

352   novel interactions between subunits in the context of aging would not be missed. Our

353   database searches also revealed that evolutionarily conserved proteins that act to

354   regulate the PP2A/4/6 holoenzymes by affecting the assembly of the complexes or by

355   manipulating the active site of the catalytic subunit may also be binding partners of

356   PPH-4.1.

357   Separate from posttranslational modifications of the PP2A/4/6 family catalytic

358   subunits that influence their affinity for specific regulatory subunits, the catalytic activity

359   of the enzymes is in parallel regulated by the evolutionarily conserved Tip41-Tap42

360   system. Constituents of this regulatory module may form transient or stable associations

361   with PP2A/4/6 proteins. Originally identified in *Saccharomyces cerevisiae*, both the Tip

362   41 and Tap 42 proteins function in the TORC pathway (56,57). Specifically, Tip41p

363   antagonizes TORC by inhibiting Tap42, a negative regulator of the PP2A-related

364   phosphatase Sit4p that dephosphorylates TORC substrates. In this manner Tip41p

365   functions to activate PP2A-like catalysis by opposing Tap42. The mammalian ortholog

366   of Tip41p, called TIPRL, on the other hand, cooperates with α4 (mammalian Tap42p) to

367   promote mTORC activity by inhibiting the PP2A/4/6 phosphatases (58). In particular

368   TIPRL and α4 together bind to holoenzymes and displace metal ions from the active site

369   of the catalytic subunit to produce a latent yet stable complex. Catalytic activity of such

370   decommissioned enzymes can be restored by PTPA, which reloads the metal ions back

371   into position (59). *C. elegans* orthologs of all three of these proteins came up in our

372   database searches for PPH-4.1 interactors. Since one of the objectives of our study was

373   to uncover functional evidence that might implicate physical associations between

374   proteins we therefore elected to include them as part of our functional analyses. In total

375   we characterized 20 genes including each *C. elegans* ortholog of human PP2A/4/6

376   subunits along with orthologs of the regulatory proteins TIPRL, α4 and PPTA (Table 1).

377   **Table 1.** *C. elegans* orthologs of human PP2A/4/6 holoenzyme subunits and regulatory

378   proteins.

H. sapiens		*C. elegans*	
Gene	**Protein**	**Gene**	**Protein**
PP2A catalytic subunits			
PPP2CA	Ppp2cα/PP2Acα	*let-92*	LET-92
PPP2CB	Ppp2cβ/PP2Acβ	*let-92*	LET-92
PP2A scaffolding subunits			
PPP2R1A	Aα/PR65α	*paa-1*	PAA-1
PPP2R1B	Aβ/PR65β	*paa-1*	PAA-1
PP2A B/B55 regulatory subunits			
PPP2R2A	PR55α/Bα	*sur-6*	SUR-6
PPP2R2B	PR55β/Bβ	*sur-6*	SUR-6
PPP2R2C	PR55ɣ/Bɣ	*sur-6*	SUR-6
PPP2R2D	PR55δ/Bδ	*sur-6*	SUR-6
PP2A B'/B56 regulatory subunits			
PPP2R5A	B'α/B56α/PR61α	*pptr-1*	PPTR-1

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PPP2R5B	B'β/B56β/PR61β	*pptr-1*	PPTR-1
PPP2R5C	B'γ/B56γ/PR61γ	*pptr-2*	PPTR-2
PPP2R5D	B'δ/B56δ/PR61δ	*pptr-2*	PPTR-2
PPP2R5E	B'ε/B56ε/PR61ε	*pptr-1*	PPTR-1
PP2A B" regulatory subunits			
PPP2R3A	B"α/PR72	T22D1.5	T22D1.5
PPP2R3B	B"β/PR70/PR48	F43B10.1	F43B10.1
PPP2R3C	B"γ/G5PR	*rsa-1*	RSA-1
PP2A B'''/striatin regulatory subunits			
STRN/PPP2R6A	B'''α/STRN	cash-1	CASH-1
STRN3/PPP2R6B	B'''β/STRN/SG2NA	cash-1	CASH-1
STRN4/PPP2R6C	B'''γ/STRN4	n/a	n/a
PP4 catalytic subunits			
PPP4C	Ppp4c/PP4/PPX	*pph-4.1*	PPH-4.1
		pph-4.2	PPH-4.2
PP4 regulatory subunits			
PPP4R1	PP4R1	*ppfr-1*	PPFR-1
PPP4R2	PP4R2	*ppfr-2*	PPFR-2
PPP4R3α	Ppp4R3α	*smk-1*	SMK-1
PPP4R3β	Ppp4R3β	n/a	n/a
PPP4R4	PP4R4	F46C5.6	F46C5.6
PP6 catalytic subunits			
PPP6C	Ppp6c/PP6	*pph-6*	PPH-6
PP6 regulatory subunits			
PPP6R1	Ppp6R1/PP6R1	*saps-1*	SAPS-1
PPP6R2	Ppp6R2/PP6R2	*saps-1*	SAPS-1
PPP6R3	Ppp6R3/PP6R3	*saps-1*	SAPS-1
ANKRD28	PP6-ARS-A/PPP1R65	n/a	n/a
ANKRD44	PP6-ARS-B	n/a	n/a
ANKRD52	PP6-ARS-C	n/a	n/a
PP2A/4/6 Activating/inactivating proteins			
α4/IGBP	α4/IGBP	*ppfr-4*	PPFR-4
TIPRL	TIPRL	ZK688.9	ZK688.9

379   *PPP2R4/PTPA*   PTPA/PR53 | Y71H2AM.20   Y71H2AM.20

380   The names of all human genes encoding catalytic, regulatory, or scaffolding

381   constituents of PP2A/4/6 subfamily complexes are listed along with their orthologs in *C.*

382   *elegans*. Genes encoding proteins that regulate the phosphatases through physical

383   associations are also included. Corresponding protein names are provided.

384

385   *Functional characterization of SMK-1 during aging*

386   To investigate the PP2A/4/6 family of protein phosphatases during aging in *C.*

387   *elegans* we began by studying the PP4 complex. We chose to focus on the SMK-1

388   regulatory subunit in particular as a test case to validate our experimental approach

389   based on reverse genetics because direct binding between SMK-1 and the catalytic

390   subunit PPH-4.1 had been demonstrated *in vitro* with recombinant proteins (44).

391   In light of this connection, we expected that if SMK-1 functions as part of a PP4 complex

392   in adult animals, RNAi knockdown of *smk-1*, *pph-4.1*, and potentially other constituent

393   subunits of the complex should all result in similar if not identical phenotypes. Moreover,

394   if a SMK-1/PPH-4.1-containing PP4 complex regulates DAF-16 during aging then RNAi

395   targeting subunits of that complex should phenocopy RNAi targeting *daf-16*. Our

396   strategy was to first compile a set of phenotypes attributable to *smk-1* inhibition in adult

397   animals. These phenotypes would then generate a metric to which other genes

398   encoding *C. elegans* orthologs of human PP4 complex members could be compared to

399   determine the likelihood of them cooperating to produce the same output, including

400   regulating the age-dependent increase in transcriptional activity of DAF-16. Further,

401   functional evidence indicating that particular subunits act together would imply that they

402 associate with each other *in vivo*. We anticipated that the same comparative analysis

403 could be applied to putative constituents of the PP2A and PP6 complexes to determine

404 whether they might also function during aging in *C. elegans*. Because we were

405 interested in characterizing the SMK-1/PPH-4.1-containing complex in the context of

406 aging, we conducted all of our studies on adult worms at Day 6 of adulthood when DAF-

407 16 is transcriptionally active in parallel to control L4 larval stage animals (10). A two-

408 tiered panel of assays was applied to evaluate the function of SMK-1 during aging. First,

409 we asked whether SMK-1 is necessary for the transcriptional activity of DAF-16 in adult

410 *C. elegans* by examining the expression of an *in vivo* reporter of DAF-16 activity, *Plys-*

411 *7::GFP* in animals treated with RNAi targeting *smk-1*. We then functionally

412 characterized *smk-1* by assessing its contribution to the ability of animals to resist acute

413 environmental insults including bacterial infection with *Pseudomonas aeruginosa*

414 (PA14), exposure to elevated temperature, and irradiation with ultraviolet (UV) light. In

415 each stress assay, the phenotype resulting from *smk-1* knockdown was compared to

416 the effect of knocking down *daf-16*.

417   We expected that if SMK-1 is necessary for the function of DAF-16 during

418 adulthood, just as it is in the *daf-2(e1370)* mutant background, then RNAi targeting

419 either *smk-1* or *daf-16* would have similar consequences. With minor exceptions, this is

420 what we found. After initiating RNAi to knockdown *smk-1* or *daf-16* at the L1 larval

421 stage, we first monitored the expression of the *Plys7::gfp* reporter over time by

422 fluorescence microscopy (Fig. 1A). Compared to worms at the L4 larval stage, the

423 expression of GFP driven by the promoter of *lys-7* in the intestines was substantially

424 higher in Day 6 adults, consistent with an age-dependent increase in the transcriptional

425   activity of DAF-16. In examining a population of animals we were able to assign

426   individuals to one of three categories based on their GFP expression pattern (Fig. 1A).

427   In general, we observed robust and uniform GFP expression along the length of the

428   intestine among the majority of individuals in a synchronized population of Day 6 adults.

429   These animals were scored as "high" GFP expressers. However, in a proportion of

430   animals high levels of GFP expression were primarily confined to a few anterior

431   intestinal cells with progressively weaker GFP expression in more posterior cells.

432   Animals with this pattern of GFP expression were classified as "medium" GFP

433   expressers. An even smaller fraction of worms expressed low levels of intestinal GFP

434   that was frequently difficult to detect. RNAi targeting *daf-16* or *smk-1* had no effect on

435   expression levels of the *Plys7::gfp* reporter in L4 larvae. By Day 6 of adulthood,

436   however, *daf-16* knockdown resulted in a significant decrease in GFP transcription (Fig.

437   1B). This suggests that DAF-16 becomes activated in an age-dependent manner,

438   consistent with previous studies (8,9). We found that knocking down *smk-1* also

439   resulted in decreased reporter expression in Day 6 adults (Fig. 1B), and so together our

440   results indicate that the transcriptional activity of DAF-16 during aging in non-stressed

441   wildtype animals may be regulated by SMK-1.

442   **Figure 1. SMK-1 confers resistance to bacterial infection and ultraviolet radiation**

443   **in adult *C. elegans.*** (A) Animals expressing the *Plys7::gfp in vivo* reporter for DAF-16

444   transcriptional activity were examined by fluorescence microscopy at D6 of adulthood.

445   Age-synchronized isogenic animals could be assigned to one of three categories based

446   on the relative expression of GFP observed in the intestine. "Low": little to no GFP

447   expression "Medium": GFP expression most apparent in the anterior portion of the

448   intestine but also at lower levels toward the midbody. "High": robust GFP expression

449   along the entire length of the intestine. DIC, differential interference contrast image

450   corresponding to the fluorescence image (GFP) shown above. (B) Quantification of

451   worms in the Low (orange), Medium (blue), and High (grey) categories of *Plys7::gfp*

452   expression at the L4 stage and D6 of adulthood following RNAi treatment targeting

453   either *daf-16* or *smk-1*. n>200 animals for each RNAi treatment in three biological

454   replicates. The average number of worms in each category is shown with error bars

455   representing standard deviation from the mean. (C and D) Results of *Pseudomonas*

456   *aeruginosa* infection assays. The survival of *C. elegans* subjected to RNAi against

457   indicated genes beginning at L1 and exposed to *P. aeruginosa* continuously beginning

458   at L4 (C) or D6 of adulthood (D) is plotted as the fraction of animals alive as a function

459   of time. (E and F) Results of UV irradiation assays. Survival curves for *C. elegans*

460   subjected to RNAi against the indicated genes from L1 until death and exposed to

461   1000mJ/s^2 UV radiation at L4 (E) or D6 of adulthood (F). Hours represent time elapsed

462   since the end of the irradiation treatment. (G and H) Results of heat stress assays.

463   Survival curves for RNAi-treated *C. elegans* incubated at 35°C beginning at L4 (E) or

464   D6 of adulthood (F) until they died. Hours represent the total time worms were

465   incubated at 35° C. Beginning at the L1 stage, worms were maintained on RNAi plates

466   without interruption for the duration of their lives. n>90 animals for each RNAi treatment

467   in three biological replicates. Purple, L4440; yellow, *daf-16* RNAi; blue, yellow, *smk-1*

468   RNAi.

469

470   Continuing to examine the functional parallels between the function of SMK-1

471   under low IIS conditions and during normal aging, we next asked whether SMK-1 is

472   required to confer resistance to environmental stress in adult *C. elegans*. Consistent

473   with the *Plys7::gfp* results, knocking down either *smk-1* or *daf-16* had no effect on the

474   ability of L4 animals to resist bacterial infection, UV irradiation, or thermal stress (Fig.

475   1C, E, G; Table 2). On the other hand, both *smk-1* and *daf-16* were necessary for Day 6

476   adults to withstand challenge with either *P. aeruginosa* infection (Fig. 1D) or with UV

477   irradiation (Fig. 1F), as knockdown of either gene reduced the median survival of

478   animals exposed to these insults (Table 2). We found that *daf-16* but not *smk-1*

479   contributes to resistance from thermal stress in adult *C. elegans*, consistent with the role

480   of SMK-1 in *daf-2(e1370)* mutants (Fig. 1 G, H) (43,45). Taken together the results of

481   our stress assays demonstrate a cooperation between SMK-1 and DAF-16 in wild type

482   animals. They suggest that during aging SMK-1 modulates the transcriptional activity of

483   DAF-16, thereby regulating DAF-16-mediated resistance to multiple insults including

484   bacterial infection and ultraviolet irradiation.

485   **Table 2.** Summary of phenotypes resulting from knockdown of members of the

486   PP2A/4/6 family and their regulators.

| | | | *P. aeruginosa* Infection | | | | L4 |
| | | | L4 | | Day 6 | | |
			Average relative LT50	p-Value	Average relative LT50	p-Value	Average relative LT50
PP2A		let-92	NA	NA	0.746	2.69E-01	NA
	paa-1	NA	NA	0.897	4.69E-01	NA	
	pptr-1	0.949	2.68E-01	0.809	1.11E-01	0.940	
	sur-6	0.913	4.29E-01	0.784	1.31E-02	0.822	
	cash-1	1.057	4.70E-01	1.054	6.44E-01	1.131	
	pptr-2	0.966	3.30E-01	0.881	4.23E-01	0.949	

	rsa-1	1.014	7.07E-01	1.150	3.85E-01	0.937
	F43B10.1	0.912	1.18E-01	0.906	6.51E-02	0.979
	T22D1.5	1.055	6.33E-01	0.941	6.11E-01	1.053
PP4	*pph-4.1*	0.991	8.54E-01	0.922	2.49E-02	0.850
	pph-4.2	0.898	1.54E-01	0.526	8.63E-03	0.922
	smk-1	0.887	4.73E-04	0.570	1.75E-10	0.985
	ppfr-2	0.987	7.86E-01	0.886	3.98E-02	1.252
	ppfr-1	0.995	9.06E-01	0.950	1.29E-01	1.017
	F46C5.6	0.972	7.92E-01	1.083	6.04E-01	1.270
PP6	*pph-6*	1.009	7.64E-01	0.585	1.47E-02	1.100
	saps-1	1.037	4.11E-01	0.563	2.41E-02	1.024
Regulators	Y71H2AM.20	1.060	2.28E-01	0.771	1.11E-01	1.376
	ppfr-4	1.098	3.79E-02	0.843	2.41E-01	0.989
	ZK688.9	0.990	8.90E-01	0.909	4.92E-01	1.218

Key	
	Reduces *Plys7::gfp* expression
	Significant Enhanced Susceptibility
	Significant Resistance
	>10% Enhanced Susceptibility
	>10% Resistance

487

488 Genes tested in our functional analyses are grouped according to the PP2A/4/6

489 holoenzyme with which they are typically associated. Orthologs of regulators of the

490 PP2A/4/6 family in humans are grouped separately. The table presents aggregated data

491 of phenotypes observed for RNAi-treated animals in the DAF-16 reporter expression

492 assay and in each of the three stress assays. Genes whose names are highlighted in

493 blue are required for the increase in *Plys-7::GFP* expression at Day 6. Each row lists the

494 average relative median lifespan (relative LT_{50}) for L4 and Day 6 adults challenged with

495   the indicated environmental stress upon knockdown of one particular gene. Average

496   relative LT_{50} was calculated by dividing the average LT_{50} of RNAi-treated animals in a

497   given stress assay by the average LT_{50} of age-matched control animals in that same

498   assay. Therefore, it represents the proportional increase or decrease in median lifespan

499   under stressful conditions that are attributable to RNAi inhibition of the gene of interest.

500   Where knockdowns enhanced sensitivity to stress, decreasing median lifespan after

501   exposure to stress by more than 10%, the average relative LT_{50} value is shaded in

502   orange. When RNAi treatment conferred resistance to stress and increased the median

503   lifespan of stressed worms by more than 10%, the average relative LT_{50} value is shaded

504   in pink. Statistical significance was determined by Student's t-test, with a threshold of

505   p<0.05. p-values associated with significant susceptibility and resistance are shaded in

506   green and yellow, respectively.

507   *Putative subunits of the PP4 and PP2A complexes influence the transcriptional activity*

508   *of DAF-16 in adult* C. elegans

509   To expand our analyses to include all putative subunits of the PP4 complex and,

510   in fact, to the entire PP2A/4/6 family, we first surveyed the effect of knocking down each

511   gene individually on the age-dependent increase in *Plys7::gfp* expression (Fig. 2). RNAi

512   treatments that resulted in greater than 25% of worms being assigned to the low GFP

513   expression category were considered to function in a similar manner to SMK-1 to

514   regulate the activity of DAF-16. None of the genes that we tested had a significant effect

515   on the expression of the *Plys7::gfp* reporter in L4 larvae (Fig. 2A). At Day 6, knockdown

516   of the PP4 catalytic subunit *pph-4.1* and its paralog *pph-4.2* had some of the strongest

517   effects on GFP expression, resulting in a higher proportion of worms in the low

518   expressing category, similar to the effect of knocking down *smk-1* (Fig 2B). This result

519   was expected since SMK-1 and PPH-4.1/PPH-4.2 associate *in vivo* (44,45). RNAi

520   targeting F46C5.6 and *ppfr-2*, *C. elegans* homologues of two other PP4 regulatory

521   subunits, also modestly increased the proportion of worms expressing low levels of

522   GFP, yet this effect was not statistically significant. Notably, inhibiting the expression of

523   several worm homologues of PP2 complex members also suppressed the age-

524   dependent increase in *plys7::gfp* expression. Inhibition of *pptr-1*, *sur-6*, T22D1.5, *paa-1*,

525   and F43B10.1 all resulted in an increase in low levels of GFP expression at Day 6

526   relative to control animals. In addition, Y71H2AM.20, the *C. elegans* ortholog of the

527   regulatory protein PTPA was also found to be necessary for the age-dependent

528   increase in *plys-7::GFP*. These results suggest that SMK-1 and PPH-4.1 function

529   together in adult *C. elegans,* and they implicate roles for both the PP4 and PP2A

530   complexes in regulating the transcriptional activity of DAF-16 during aging.

531   **Figure 2. Genes encoding putative members of the PP2A, PP4, and PP6**

532   **complexes are required for the age-dependent increase in DAF-16 transcriptional**

533   **activity.** Quantification of worms in the Low (orange), Medium (blue), and High (grey)

534   categories of *Plys7::gfp* expression at the L4 stage (A) and D6 of adulthood (B)

535   following RNAi treatment targeting the indicated gene. n>200 animals for each RNAi

536   treatment in three biological replicates. The average number of worms in each category

537   is shown with error bars representing standard deviation from the mean.

538

539

540

2

541   *Members of the PP2A/4/6 subfamily contribute to innate immunity during aging*

542   We found that *smk-1* is necessary to confer resistance to bacterial infection in

543   Day 6 adults, as is DAF-16 (Fig 1D). This result leads to the prediction that *pph-4.1*,

544   encoding the catalytic subunit of the PP4 complex, is also required for host defense

545   during aging. Further, based on the results of our *in vivo* reporter assay for DAF-16

546   transcriptional activity, we wondered whether PP2A subunits may also function in innate

547   immunity later in life. We addressed these possibilities as part of our characterization of

548   the PP2A/4/6 family in adults challenged with *P. aeruginosa*. In a phenotype reminiscent

549   of the *smk-1* knockdown, RNAi targeting homologues of the PP4 catalytic subunit *pph-*

550   *4.1* or its paralog *pph-4.2* reduced the ability of Day 6 adults to resist bacterial infection

551   but had no effect on the susceptibility of L4 larvae to pathogen (Fig. 3A, B, G, H). The

552   magnitude of the resulting decrease in median lifespan (LT_{50}) of infected animals

553   caused by inhibiting *pph-4.2* expression was comparable to the effect of *smk-1*

554   knockdown, but *RNAi* targeting *pph-4.1* had a considerably milder effect across our

555   replicates (Table 2). Coupled with the results of our *in vivo* reporter assay, this result

556   represents a second independent circumstance in which RNAi inhibition of *smk-1*

557   phenocopied the knockdown of *pph-4.1/4.2*. We also considered it as providing

558   validation to our experimental approach since our functional analyses confirmed

559   biochemically verified interactions between SMK-1 and PPH-4.1/4.2.

560   **Figure 3. Members of putative PP4 and PP6 holoenzymes along with the PP2A**

561   **regulatory subunit SUR-6 are required for innate immunity during adulthood in *C.***

562   ***elegans*.** Following RNAi treatment beginning at the L1 stage to target *C. elegans*

563   homologs of catalytic and regulatory subunits of the PP4 (A and B), PP2A (C and D),

564   and PP6 (E and F) complexes, worms were infected with *P. aeruginosa* at the L4 larval

565   stage (A,C,E) or at D6 of adulthood (B,D,F). The fraction of worms alive at each time

566   point after infection was initiated is plotted as a function of time in hours. In all cases

567   RNAi targeting *daf-16* or *smk-1* and the empty RNAi vector L4440 were included as

568   controls. Only RNAi knockdowns that produced statistically significant phenotypes are

569   shown. (G and H) The average median survival (LT_{50}) of animals treated with RNAi

570   targeting the indicated genes following infection with *P. aeruginosa* at L4 (G) or D6 (H)

571   is shown as a fraction of the average median survival of L4440 controls. Bars, standard

572   error of the mean (SEM). Bar colors correspond to the protein phosphatase complex to

573   which products of the indicated genes belong or to controls. Dark blue: L4440, *daf-16*,

574   and *smk-1*; light blue: PP2A; dark purple: PP4; light purple: PP6. Asterisks indicate

575   RNAi treatments producing statistically significant differences in median survival

576   (p<0.05). Horizontal lines are drawn at a relative median survival of 1 and, for a

577   reference in (H) the relative median survival of adult worms treated with RNAi against

578   *pph-4.1*. Red arrowheads are beneath the names of genes encoding catalytic subunits

579   of the PP2A, 4 and 6 complexes.

580   Along with *pph-4.1/4.2* and *smk-1*, RNAi inhibition of another PP4 regulatory

581   subunit homologue, *ppfr-2* (PP4R2 in humans), also reduced the median lifespan of

582   worms infected at Day 6 of adulthood but had no effect on the ability of L4 larvae to

583   resist infection (Fig. 3A, B, G, H). No other PP4 regulatory subunits were found to be

584   required for innate immunity in Day 6 adults (Fig. 3H; Fig S3). These results imply that

585   PPFR-2 may act in conjunction with SMK-1 and PPH-4.1 to protect adult *C. elegans*

586   from bacterial pathogens.

587   We found genes encoding putative subunits of other protein phosphatase

588   complexes to also be important for host defense in adult *C. elegans*. In addition to the

589   effect of inhibiting the expression of PP4 subunits, one of the ten RNAi treatments

590   directed against *C. elegans* orthologs of PP2A subunits enhanced the susceptibility of

591   adult animals to *P. aeruginosa* infection. Knockdown of *sur-6*, the sole worm homolog of

592   human PP2A B/B55 regulatory subunits, significantly reduced the median lifespan of

593   infected Day 6 adults but had no effect on larvae (Fig. 3C, D, G, H; Table 2). We found

594   no evidence of a role for other orthologs of PP2A complex subunits in adult or larval

595   innate immunity (Fig. 3H; Fig. S4). In an unexpected result, inhibiting the expression of

596   two orthologues of PP6 complex members *pph-6*, the catalytic subunit, and *saps-1*, a

597   regulatory subunit, accelerated the rate of death from infection when worms were

598   challenged with *P. aeruginosa* at Day 6 of adulthood but not at the L4 larval stage (Fig.

599   3E, F, G, H). This was surprising because none of the PP6 members appear to be

600   required for the increased expression of *plys7::gfp* in adults, a departure from *smk-1*-like

601   phenotypes (Fig. 2B). Taken together, our results indicate that the entire PP2A/4/6

602   family plays a role in innate immunity during aging and that the PP6 complex may do so

603   without influencing the transcriptional output of DAF-16.

604   *SMK-1 and the PP2A complex confer UV resistance in postreproductive* C. elegans

605   We found SMK-1 to be important not only for host defense in Day 6 animals, but

606   also for resistance to ultraviolet irradiation, consistent with its role in *daf-2(e1370)*

607   mutants (Fig 1F; Table 2) (43,60). We therefore expected that other components of the

608   PP4 complex would be required for survival upon exposure to UV light during aging.

609   This was not the case. No canonical members of the PP4 holoenzyme, including the

610   catalytic subunits, seem to contribute to UV tolerance in *C. elegans* as knocking them

611   down did not affect the survival of irradiated L4 larvae or Day 6 adults (Fig. 4A, B, G, H;

612   Fig. S5). On the other hand, inhibiting expression of the PP2A catalytic subunit, *let-92*,

613   the scaffolding protein *paa-1* and the regulatory subunit *pptr-1* caused Day 6 adult

614   animals exposed to UV light to die more rapidly, leading to significant reductions in their

615   median lifespans (Fig. 4 C, D, G, H; Table 2). Other orthologs of PP2A subunits do not

616   appear to play a role in conferring resistance to UV light (Fig. S6). Targeting PP6

617   complex subunits by RNAi was also inconsequential to the ability of adults to resist UV

618   irradiation (Fig. 4E, F, G, H; Fig. S7). Our data therefore suggest that a complete PP2A

619   holoenzyme is involved in the ability of post-reproductive animals to resist UV exposure

620   and that it may act in conjunction with SMK-1.

621   **Figure 4. Resistance to UV irradiation in adult *C. elegans* is conferred by the**

622   **PP2A complex and SMK-1.** RNAi treatment was initiated at the L1 stage to target *C.*

623   *elegans* homologs of catalytic and regulatory subunits of the PP4 (A and B), PP2A (C

624   and D), and PP6 (E and F) complexes and was continued for the duration of the assay.

625   Worms were exposed to UV radiation at the L4 larval stage (A,C,E) or at D6 of

626   adulthood (B,D,F) after which their survival under standard culturing conditions was

627   monitored. The fraction of worms alive at each time point following the UV treatment is

628   plotted as a function of time. In all cases RNAi targeting *daf-16* or *smk-1* and the empty

629   RNAi vector L4440 were included as controls. Only RNAi knockdowns that produced

630   statistically significant phenotypes are shown. (G and H) The average median survival

631   (LT_{50}) of animals treated with RNAi targeting the indicated genes following exposure to

632   UV light at L4 (G) or D6 (H) is shown as a fraction of the average median survival of

633   L4440 controls. Bars, standard error of the mean (SEM). Bar colors correspond to the

634   protein phosphatase complex to which products of the indicated genes belong or to

635   controls. Dark blue: L4440, *daf-16*, and *smk-1*; light blue: PP2A; dark purple: PP4; light

636   purple: PP6. Asterisks indicate RNAi treatments producing statistically significant

637   differences in median survival (p<0.05). Horizontal lines are drawn at a relative median

638   survival of 1. Red arrowheads are beneath the names of genes encoding catalytic

639   subunits of the PP2A, 4 and 6 complexes.

640

641   *The PP2A regulatory subunit SUR-6 promotes resistance to heat stress in adult worms*

642   Despite its roles in conferring resistance to bacterial pathogens and ultraviolet

643   irradiation, SMK-1 does not contribute to thermotolerance in adult *C. elegans* even

644   though DAF-16 does (Fig. 1G, H). In like manner, we found no evidence to suggest that

645   the PP4 catalytic subunits PPH-4.1 or PPH-4.2 function to protect animals from thermal

646   stress during aging (Fig. 5C, D, G. H). However, RNAi targeting a putative regulatory

647   subunit F46C5.6, the ortholog of PP4R4, resulted in a surprising modest but significant

648   increase in resistance to high temperatures in Day 6 adult worms but not larvae (Fig.

649   5A, B, G, H). Neither of the other two *C. elegans* orthologs of PP4 regulatory subunits

650   PPFR-1 and PPFR-2 appear to influence survival of heat stressed animals at the larval

651   or adult stages (Fig. 5G, H; Fig. S8). On its own this result suggests that in contrast to

652   the roles of other PP4 regulatory subunits, F46C5.6 could function to inhibit the catalytic

653   activity of the PP4 complex in adult animals. Yet considering that there is no

654   corresponding enhanced sensitivity to heat stress upon knockdown of *pph-4.1* or *pph-*

655   *4.2*, this scenario seems unlikely.

656 **Figure 5. The PP2A subunit SUR-6 contributes to thermotolerance in adult worms.**

657 RNAi treatment targeting *C. elegans* homologs of catalytic and regulatory subunits of

658 the PP4 (A-D), PP6 (C and D), and PP2A (E and F) complexes was initiated at the L1

659 stage and continued for the duration of the assay. To induce thermal stress at the L4

660 larval stage (A,C,E) or at D6 of adulthood (B,D,F) worms were shifted from 20° C to 35°

661 C until all of the animals had died. The fraction of worms alive at each time point during

662 the incubation at high temperature is plotted as a function of time in hours. In all cases

663 RNAi targeting *daf-16* or *smk-1* and the empty RNAi vector L4440 were included as

664 controls. Only RNAi knockdowns that produced statistically significant phenotypes are

665 shown. (G and H) The average median survival (LT_{50}) of animals treated with RNAi

666 targeting the indicated genes following the shift to 35° C at L4 (G) or D6 (H) is shown as

667 a fraction of the average median survival of L4440 controls. Bars, standard error of the

668 mean (SEM). Bar colors correspond to the protein phosphatase complex to which

669 products of the indicated genes belong or to controls. Dark blue: L4440, *daf-16*, and

670 *smk-1*; light blue: PP2A; dark purple: PP4; light purple: PP6. Asterisks indicate RNAi

671 treatments producing statistically significant differences in median survival (p<0.05).

672 Horizontal lines are drawn at a relative median survival of 1. Red arrowheads are

673 beneath the names of genes encoding catalytic subunits of the PP2A, 4 and 6

674 complexes.

675

676 Out of all of the other members of the PP2A/4/6 family that we tested, only RNAi

677 targeting *sur-6*, encoding a regulatory subunit of PP2A, reduced the survival of Day 6

678 adult *C. elegans* maintained at elevated temperature (Fig 5E, F, G, H). Knockdown of

679 orthologs of the catalytic, regulatory, or scaffolding subunits of PP2A did not affect the

680 survival of adults challenged by thermal stress (Fig. 5H; Fig. S9). Although inhibiting

681 *pph-6* expression increased the resistance of L4 worms the thermal stress (Fig. 5C),

682 this effect did not persist into adulthood (Fig. 5D). Upon exposure to high temperature,

683 the survival of Day 6 animals treated with RNAi targeting *pph-6* or *saps-1* was

684 comparable to the control group, (Fig. 5H; Fig. S10). Our do not implicate a complete

685 PP2A/4/6 holoenzyme as functioning to confer resistance to heat stress during

686 adulthood, However, individual members of the PP2A/4/6 family may influence the

687 response to thermal stress as part of other pathways in an age-specific manner.

688 *Characterization of putative regulators of the PP2A/4/6 family that are not incorporated*

689 *into the complexes as canonical subunits*

690 In mammals one group of regulatory proteins modulates the function of the entire

691 PP2A/4/6 subfamily of protein phosphatases through mechanisms involving physical

692 interactions and not post-translational modifications. An example of such a protein is $\alpha 4$,

693 which protects the catalytic subunits of the PP2, PP4, and PP6 complexes in humans

694 by inhibiting their ubiquitin-mediated degradation (61). At the same time, $\alpha 4$ can act as

695 a negative regulator by functioning together with TIPRL. For example, $\alpha 4$ and TIPRL

696 bind noncompetitively to PP2Ac and inactivate it by both expelling metal ions from the

697 active site and displacing the scaffold and regulatory subunits (58). Conversely, PTPA

698 can reactivate PP2A/4/6 phosphatases by reloading metal ions back in to the active site

699 (59). Although they are not considered to be constituents of the PP4 holoenzyme,

700 orthologs of all three of these regulators were identified in our database queries as

701 potential interactors of SMK-1/PPH-4.1 (Fig. S2; Table S2). We therefore asked

3

702   whether they might modulate the PP2A/4/6 family during aging in *C. elegans*. Since our

703   data indicate that the PP2A/4/6 family is important for resistance to environmental

704   stress in adult animals, we expected that if PPFR-4 and ZK688.9 cooperate to inhibit

705   the phosphatases in *C. elegans* as they do in mammals, then knocking them down

706   would increase the survival of Day 6 adults challenged with acute insults. Further, since

707   PTPA acts antagonistically to α4 and TIPRL in mammals, we anticipated that RNAi

708   targeting its ortholog Y71H2AM.20 would cause Day 6 adults to become more sensitive

709   to stress. While knocking down Y71H2AM.20 did increase the susceptibility of Day 6

710   adults to bacterial infection and their sensitivity to UV light, neither effect was

711   statistically significant (Table 2; Fig. 6E, F; Fig. S11; Fig. S12). We found no evidence of

712   a role for Y71H2AM.20 in protecting animals from thermal stress (Fig. 6G; Fig. S13).

713   Surprisingly, instead of making adult animals more resistant, when *ppfr-4* was targeted

714   by RNAi, Day 6 *C. elegans* were more sensitive to UV irradiation, yet the effect on the

715   relative median survival of these animals fell just short of the significance threshold (p=

716   0.0548) (Fig. 6A, B, E; Table 2). The only resistance phenotype that we found to be

717   associated with *ppfr-4* knockdown was when L4 animals were challenged with *P.*

718   *aeruginosa* but not when adults were infected (Fig. 6 C, D, F). Our observations suggest

719   that there may be some differences between the roles of this class of PP2A/4/6

720   regulators in *C. elegans* and mammals, but the lack of statistical significance in our data

721   prevents us from drawing definitive conclusions.

722   **Figure 6. PPFR-4, the *C. elegans* ortholog of the α4 regulatory protein, may be**

723   **required for resistance to UV radiation during adulthood.** (A-D) Beginning at the L1

724   larval stage worms were treated with RNAi to knockdown *ppfr-4*. At the L4 stage (A) or

725   Day 6 of adulthood (B) worms were irradiated with UV light and then returned to

726   standard culture conditions where their survival was monitored over time. The fraction of

727   worms alive at each time point following the UV treatment is plotted as a function of

728   time. RNAi targeting *daf-16* or *smk-1* and the empty RNAi vector L4440 were included

729   as controls. At the L4 stage (C) or Day 6 of adulthood (D) worms were transferred to

730   plates seeded with *P. aeruginosa* where their survival was monitored over time. The

731   fraction of worms alive at each time point following the UV treatment is plotted as a

732   function of time. RNAi targeting *daf-16* or *smk-1* and the empty RNAi vector L4440 were

733   included as controls. (E-G) The average median survival (LT_{50}) of L4 and D6 animals

734   treated with RNAi targeting the indicated genes following exposure to UV light (E),

735   infection with *P. aeruginosa* (F) or incubation at 35˚ C (G) is shown as a fraction of the

736   average median survival of L4440 controls. Bars, standard error of the mean (SEM).

737   Asterisks indicate RNAi treatments producing statistically significant differences in

738   median survival ($p<0.05$). Horizontal lines are drawn at a relative median survival of 1.

739   UV, ultraviolet light; SKA, slow kill assay (*P. aeruginosa* infection); HS, heat stress (35˚

740   C).

741

742   **Discussion**

743   The FoxO transcription factor DAF-16 is a major longevity determinant in *C.*

744   *elegans*. Animals with constitutively active DAF-16 live longer and are more youthful

745   than their wildtype counterparts, suggesting that DAF-16 contributes not only to lifespan

746   but also to healthspan (62). Accordingly, one category of DAF-16 transcriptional targets

747   are genes that confer resistance to stress, thus shielding animals from long-lasting

748   cellular damage (7). While exposure to environmental insults induces DAF-16 to

749   upregulate these genes, recent evidence suggests that DAF-16 becomes activated in

750   age-dependent manner in animals that have not been challenged with a stressor (8,9).

751   Here we investigated the mechanism by which DAF-16 is activated during aging in *C.*

752   *elegans*. We focused our studies on the PP2A/4/6 family of phosphoprotein

753   phosphatases, which have been implicated in regulating FoxO transcription factors in

754   evolutionarily diverse species (14–17,19,21,45). Using a reverse genetics approach, we

755   performed functional analyses to survey the complete set of *C. elegans* orthologs of

756   human PP2A/4/6 regulatory and catalytic subunits in the context of aging. Our results

757   suggest that the transcriptional activity of DAF-16 in adult animals is regulated by both

758   PP2A and PP4 complexes. The specific effect of these complexes on DAF-16 appears

759   to be non-overlapping, as each is important for a particular facet of DAF-16-mediated

760   stress resistance. In the course of our studies, we also uncovered evidence of an

761   apparent DAF-16-independent role for the PP6 phosphatase in host defense in post-

762   reproductive adult *C. elegans*, thus highlighting the importance of the entire PP2A/4/6

763   family during aging.

764   The PP2A/4/6 family of phosphoprotein phosphatases regulates a broad

765   spectrum of processes essential to the survival and normal physiology of a variety of

766   cell types. This is achieved in part by controlling pathways that are foundational to

767   higher order cellular function. For example, both the biogenesis and the mRNA splicing

768   function of small nuclear ribonucleoproteins (snRNPs) are regulated by PP4 and PP6,

769   while PP2A contributes to mRNA surveillance through nonsense-mediated decay

770   (NMD) (63–65). PP2A/4/6 phosphatases also regulate membrane trafficking and

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771 influence the dynamics and metabolism of mitochondria (66,67). Further, the activity of

772 key components of major signaling pathways, including NF-KB and JNK, is subject to

773 regulation by PP2A/4/6 (35,68–76). This particular group of phosphatases is perhaps

774 best known, however, for the key roles that its members play with regard to the cell

775 cycle. One of the most critical functions of the PP2A/4/6 family is to negatively regulate

776 the kinases that drive progression of the cell cycle (30,37,38,40,42,76,77). Across

777 evolutionarily diverse species, when cells are permitted to divide the PP2A/4/6

778 phosphatases ensure the faithful transmission of genetic material to daughter cells by

779 regulating the assembly and positioning of the spindle, the cohesion of chromatin, and

780 the attachment of microtubules to kinetochores (30,78–86). Both in the context of

781 mitosis and during interphase PP4 and PP6 in particular further contribute to genome

782 stability by promoting DNA repair and by resolving changes in chromatin structure

783 associated with the response to DNA damage (87–97). Given these functions, it is

784 perhaps not surprising that members of the PP2A/4/6 family have been identified as

785 tumor suppressors (98–102). At the whole organism level, the PP2A/4/6 family is

786 especially important at the very early stages of life, ensuring the survival and

787 developmental progression of embryos (36,103,104). In addition, these phosphatases

788 orchestrate the development of multiple tissues including bone, adipose, innate immune

789 cells, and neurons (34–36,105,106). Certain other functions of PP2A/4/6 indicate that

790 later in life they contribute to longevity. For example, PP6 suppresses inflammation and

791 can promote autophagy, both of which would preserve normal lifespan (107,108).

792 Regulation of IIS components by PP2A is an example of an even more direct

793 connection between the PP2A/4/6 family and aging, since insulin signaling is a

3

794   predominant longevity determinant in invertebrates and other animals (1). Considering

795   these observations and in light of the breadth and magnitude of their impact on cellular

796   function, we were particularly interested in investigating the function of the PP2A/4/6

797   phosphatases during aging, a context in which they have not yet been extensively

798   studied. We asked whether the PP2A/4/6 phosphatases might function in adult *C.*

799   *elegans* to regulate the age-dependent increase in DAF-16 transcriptional activity and

800   contribute to stress resistance.

801   To investigate the potential role of the PP2A/4/6 phosphatases in regulating

802   DAF-16 during aging in *C. elegans*, we used a two-pronged approach where in each

803   branch the phenotype resulting from RNAi knockdown of candidate genes was always

804   compared to the phenotype associated with reduced DAF-16 expression. First we

805   asked whether RNAi-mediated knockdown of genes encoding worm orthologs of human

806   PP2A/4/6 subunits reduced the age-dependent increase in the expression of *plys-*

807   *7::GFP*, an in vivo reporter of DAF-16 transcriptional activity. We then challenged RNAi-

808   treated adult animals with three different types of stress to which DAF-16 is known to

809   confer resistance—bacterial infection, heat, and ultraviolet irradiation. Based on this

810   analysis, we identified 9 members of the PP2A/4/6 family that recapitulated *daf-16*

811   knockdown phenotypes in at least one of the functional assays. We found that some

812   groups of candidate genes encoding putative subunits of the same phosphoprotein

813   phosphatase complex also phenocopied each other, with RNAi knockdown resulting in

814   statistically significant enhanced susceptibility to the same environmental stressor. This

815   result was interpreted as evidence of a putative physical interaction between the gene

816   products, and it is the basis for the identity of the subunits that we propose as

817   constituents of the phosphoprotein phosphatase complexes that regulate DAF-16 during

818   aging.

819   Our data support the possibility of multiple versions of the PP2A/4/6 complexes

820   that are co-expressed during aging in worms that have specialized and partially

821   overlapping functions in preserving health by conferring resistance to environmental

822   insults (Fig. 7). Our results indicate that a PP2A complex comprised of the catalytic

823   subunit LET-92, the scaffolding subunit PAA-1, and the regulatory subunit PPTR-1 is

824   important for DAF-16-mediated resistance to ultraviolet irradiation in adult animals. In

825   like manner a PP4 complex consisting of the catalytic subunit PPH-4.1/4.2, and the

826   regulatory subunits SMK-1 and PPFR-2 appears to be necessary for DAF-16-mediated

827   innate immunity later in life. The PP6 complex PPH-6/SAPS-1 also contributes to host

828   defense in adult animals, but it appears to do so without affecting the transcriptional

829   activity of DAF-16. The genetic and functional evidence that form the basis of our claims

830   regarding these complexes is further substantiated by the fact that in each instance, the

831   constituent subunits are known to be expressed together in the same tissues in *C.*

832   *elegans*, and colocalization to the same subcellular compartment has been

833   experimentally verified for several (Table S4). Different versions of the PP2/4/6

834   complexes may exist in the same cells simultaneously. Alternatively, the composition of

835   the complexes may be dynamic such that individual regulatory subunits associate and

836   dissociate according to cellular demands or in response to external stimuli. Future

837   biochemical studies involving isolation of the complexes from wildtype postreproductive

838   adult *C. elegans* and *in vitro* binding assays will validate the conclusions regarding the

839   molecular composition of PP2A/4/6 complexes based on data from our genetic

840   approach.

841   **Figure 7. Proposed identity of PP2/4/6 complexes in *C. elegans* and their**

842   **functions during aging.** Our data support the existence of at least three central

843   phosphoprotein phosphatase complexes during aging in *C. elegans*, but different

844   combinations of subunits allow for more. The subunit composition of each complex of

845   the PP2/4/6 family in adult *C. elegans* is indicated, according to the following color

846   scheme: purple, PP2A subunits; green, PP4 subunits; red, PP6 subunits. Proteins

847   whose names are labeled in yellow text are required for the age-dependent increase in

848   DAF-16 transcriptional activity as measured in our *in vivo* reporter assay. While the

849   PP2A complex is important for resistance to UV light, both the PP4 and PP6 complexes

850   function in innate immunity in adult worms. Subunit exchange (indicated by dashed

851   arrows) may take place between constituents of the PP2A and PP4 complexes such

852   that SMK-1 associates with a version of the PP2A complex to confer resistance to UV

853   irradiation and SUR-6 associates with PP4 to contribute to host defense. SUR-6 was

854   the only PP2A/4/6 family member found to play a role in thermotolerance during

855   adulthood. Protein structures depicted in this cartoon are for illustrative purposes only.

856

857   Our observations both confirm and extend previous reports of either physical or

858   regulatory interactions between DAF-16 and the PP2A or PP4 complexes in *C. elegans*

859   and in mammals. For example, PPTR-1 promotes the dephosphorylation of AKT-1 and

860   is required for the extended lifespan and stress resistance phenotypes of *daf-2* mutants

861   (21). These data strongly implicate PP2A as an indirect DAF-16 regulator in *C. elegans*,

862   but no other PP2A complex members were isolated in the screen for *daf-2* suppressors

863   that yielded *pptr-1*. Our data indicate that LET-92 and PAA-1 complete that tripartite

864   PP2A complex that regulates DAF-16. Supporting this possibility, mammalian

865   orthologues of the same three PP2A subunits that we identified were found to be

866   binding partners of FoxO3a in HEK293 cells (109). Therefore, although the specific

867   substrates may differ between *C. elegans* and mammals, regulation of FoxO

868   transcription factors by PP2A appears to be evolutionarily conserved. The PP2A

869   complex was not the first member of the PP2A/4/6 family to be identified as a regulator

870   of DAF-16 activity. That distinction belongs to the PP4 complex and to SMK-1 in

871   particular. Similar to the effect of inhibiting PPTR-1, loss-of-function mutations in *smk-1*

872   abrogate *daf-2* phenotypes in larvae and young adults, including the elevated

873   expression of DAF-16 transcriptional targets (43). More recently, RNAi inhibition of the

874   PP4 catalytic subunits *pph-4.1/4.2* were also found to partially suppress *daf-2* (45).

875   These subunits along with the regulatory subunit PPFR-2 copurify with SMK-1 from wild

876   type, *daf-2* (e1370) and *daf-18* (mg198) animals, strongly suggesting that they form a

877   complex *in vivo* (45). Our functional analysis provides an independent line of evidence

878   to support this association between PPH-4.1/4.2, SMK-1 and PPFR-2.. Further, our

879   data indicate that this particular version of the PP4 complex plays an important role

880   during aging in wild type animals to stimulate the transcriptional activity of DAF-16.

881   Consistent with characterization of the PP4 catalytic subunits PPH-4.1 and PPH-

882   4.2 in *daf-2* mutants, we found that both are required to confer stress resistance and to

883   activate DAF-16 transcriptional activity in adult animals. RNAi inhibition of either *pph-4.1*

884   or *pph-4.2* resulted in two significant phenotypes in Day 6 adults: reduced expression of

885 the *plys-7::GFP* reporter and enhanced susceptibility to *P. aeruginosa* infection. While

886 knockdown of each gene had a similar effect on *plys-7::GFP* expression levels, RNAi

887 targeting *pph-4.2* caused a more severe pathogen susceptibility phenotype. We also

888 observed that knocking down *pph-4.2* but not *pph-4.1* resulted in a mild but statistically

889 insignificant susceptibility to ultraviolet irradiation (Table 2; Fig S5). This may indicate

890 that the two proteins have only partially overlapping functions. Taken together, our

891 studies indicate that PPH-4.1 and its paralog PPH-4.2 are important during aging and

892 are likely to be coexpressed in adult *C. elegans*.

893 Interestingly, in *C. elegans* the PP2A scaffold PAA-1 seems to associate with the

894 PP4 subunit SMK-1 (45). Whether *paa-1* suppresses *daf-2* as does *smk-1* inhibition is

895 unknown, yet in light of the biochemical evidence, one model is that PAA-1 could be

896 included as a fourth subunit of the PP4 complex in *C. elegans*. Alternatively, there may

897 be hybrid tripartite holoenzymes where two members are canonical subunits of one

898 complex (e.g. PP2A) and the third member is considered to be a canonical subunit of a

899 different complex (e.g. PP4). This would require certain regulatory or scaffolding

900 subunits, perhaps including PAA-1, to have the ability to be incorporated into both PP2A

901 and PP4 complexes. Regardless of their specific stoichiometry, biochemical evidence

902 from mammalian systems supports the possibility of interchange of subunits between

903 complexes of the PP2A/4/6 family. In affinity purification or immunoprecipitation

904 experiments HEK293 cells, PP4c has been isolated as part of complexes with PP2A

905 subunits, including orthologs of PAA-1, SUR-6, and PPTR-2 (51,52,110). Such

906 heterotypic associations between members of the PP2A and PP4 complexes could

907 explain two scenarios that we encountered in our studies where a singular

908   representative of one PP complex shares a susceptibility phenotype with three

909   members that are all part of a second PP complex. In particular, we found Day 6 adults

910   to be susceptible to ultraviolet irradiation upon treatment with RNAi targeting not only

911   the PP2A complex members *let-92*, *pptr-1*, and *paa-1* but also the PP4 subunit *smk-1*.

912   Similarly, *sur-6* was the only PP2A subunit whose knockdown resulted in enhanced

913   susceptibility to bacterial infection, as did RNAi targeting the PP4 complex members

914   *pph-4.1/4.2*, *smk-1*, and *ppfr-2*. In each case a regulatory subunit seems to be operating

915   separately from its cognate catalytic subunit. These results suggest that there may be

916   alternative forms of the PP2A and PP4 complexes that include at least one regulatory

917   subunit of the family that is not typically associated with that complex's eponymous

918   catalytic subunit.

919   In some cases we found that a gene's requirement for the age-dependent

920   increase in *plys-7::GFP* expression was apparently uncoupled from any role that it may

921   have in conferring resistance to stress during adulthood. For example, neither *let-92* nor

922   *ppfr-2* were required for the increase in *plys-7::GFP* expression in adult animals, but

923   each conferred resistance to stress. Since LET-92 appears to mediate resistance to

924   ultraviolet irradiation and PPFR-2 functions in host defense, in this case the functional

925   data do not seem to support the possibility of a subunit exchange to yield a LET-

926   92/PPFR-2 complex. While the requirement for LET-92 in conferring resistance to UV

927   irradiation in adult worms without an accompanying effect on *plys-7::GFP* expression

928   could suggest a DAF-16-independent function, all other PP2A subunit orthologs in our

929   study whose knockdown caused adults to be more sensitive to stress were also

930   necessary for the age-dependent increase in *plys-7::GFP* expression. Considering

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931 these results and in light of the aforementioned evidence from mammalian systems

932 establishing a regulatory interaction between PP2A and FoxO transcription factors, we

933 favor a model in which LET-92 is the catalytic subunit of one or more PP2A complexes

934 that regulate the function of DAF-16 during aging, including its role in protecting animals

935 from environmental insults. In another instance of incongruent phenotypes, knockdown

936 of two putative PP2A B'' regulatory subunits, F43B10.1 and T22D1.5, decreased

937 expression levels of *plys-7::GFP* in Day 6 adults without enhancing the sensitivity of

938 adult animals to environmental stress. Perhaps PP2A complexes that include these two

939 subunits regulate DAF-16 in such a manner as to affect its transcriptional output. While

940 *lys-7* would still be among the transcriptional targets of DAF-16 regulated by such a

941 PP2A complex, other transcriptional targets including genes that are critical for

942 mediating stress resistance would not be. Another possible explanation of this

943 observation is that T22D1.5 and F43B10.1 may have redundant roles in directing DAF-

944 16 to partially overlapping subsets of its transcriptional targets that include *lys-7*. A loss-

945 of-function in either T22D1.5 or F43B10.1 alone would affect the transcriptional output

946 of DAF-16 without completely abrogating DAF-16-mediated stress resistance. Only

947 when the function of both regulators is diminished at the same time would DAF-16-

948 mediated stress resistance be compromised because a critical combination of effectors

949 fails to be upregulated. Testing this possibility would require further functional analyses

950 of double mutants along with transcriptomic studies to determine how the transcriptional

951 targets of DAF-16 during aging might change in the absence of PP2A complex

952 members.

953   In the course of characterizing the entire PP2A/4/6 subfamily in our studies, we

954   uncovered a new role for the PP6 complex in innate immunity during aging in *C.*

955   *elegans*. Prior to our work on PPH-6, the *C. elegans* ortholog of PP6c, it had only been

956   described in the context of development. In worm embryos PPH-6 is required for spindle

957   positioning, similar to the role of its counterpart PP6c in mammals that controls spindle

958   formation and the condensation, alignment, and segregation of chromosomes during

959   mitosis (79,81,82,111). Based on the other functions of PP6c, we expected that PPH-6

960   might be necessary to protect adult *C. elegans* from ultraviolet irradiation. PP6c

961   functions in both non-homologous end joining (NHEJ) and homology-directed repair of

962   DNA, and without it cells are more sensitive to radiation (95,100). We found no

963   evidence to suggest that either PPH-6 or its regulatory subunit SAPS-1 confer

964   resistance to UV in Day 6 animals. Instead, both PPH-6 and SAPS-1 were required for

965   the survival of animals challenged with *P. aeruginosa*. Since the expression of immune

966   effectors is the principle means of host defense in *C. elegans*, we speculate that PP6

967   regulates some aspect of that process either at an upstream step in immune signaling

968   pathways or further downstream to influence the transcription of genes encoding

969   antimicrobial products.

970   As the predominant antagonists to a slew of kinases, the catalytic activity of the

971   PP2A/4/6 family is tightly controlled. Regulators of PP2A/4/6 may influence the

972   composition of the holoenzyme, directly modify the active site of the catalytic subunit, or

973   modulate the stability of the complexes. In some cases, the same regulatory protein can

974   act at multiple levels. Since the substrate specificity of the PP2A/4/6 complexes is

975   determined by the regulatory (B) subunits incorporated into the holoenzyme, one

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976   common mechanism of regulation involves modulating the assembly or the composition

977   of the complexes. For example, some regulators of the PP2A/4/6 family such as CIP2A

978   or Greatwall directly bind to the B or the A (scaffolding) subunits to prevent their

979   incorporation into the multiprotein complexes (77,112). Other regulators add or remove

980   posttranslational modifications to the catalytic subunit that influence its affinity for

981   particular regulatory subunits. While phosphorylation tends to disrupt the interaction

982   between the catalytic and regulatory subunits, methylation enhances the binding of the

983   catalytic subunit to B and B' subunits (113,114). The catalytic subunit is susceptible to

984   additional regulation specifically at the active site. In particular, during its biogenesis,

985   PP2Ac is bound by PTPA which helps to configure the active site for serine/threonine

986   phosphatase activity (53,115). Later, PP2Ac and PP4c may be inactivated by PME1 or

987   by the coordinated function of TIPRL and α4 (homologues of *S. cerevisiae* Tip41p and

988   Tap42p, respectively) that expel metal ions from the active site (58,59,116). When

989   bound by these negative regulators, catalytic subunits or holoenzymes are maintained

990   in a stable latent state. Our studies of the *C. elegans* orthologs of TIPRL (ZK688.9) and

991   α4 (*ppfr-4*) during aging were inconclusive since RNAi treatments targeting them failed

992   to produce statistically significant phenotypes in our stress assays. We did, however,

993   observe a nearly significant increase in the sensitivity of Day 6 adults to UV radiation

994   when *ppfr-4* was knocked down. Taken together with our other data, this could indicate

995   that PPFR-4 plays a role in activating the LET-92/PAA-1/PPTR-1 PP2A complex during

996   aging in *C. elegans*. Indeed, there is some evidence to suggest that α4 is capable of

997   increasing the activity of PP2Ac in mammals (117,118). This may be at least partly

998   attributable to the function of α4 in protecting PP2Ac from ubiquitin-mediated

999   proteasomal degradation (119,120). Since knocking down ZK688.9 did not recapitulate

1000   phenotypes associated with RNAi inhibition of *ppfr-4* in our hands, we are unable to say

1001   whether their gene products are likely to cooperate as they do in mammals. One

1002   possibility, though, is that the interplay between these proteins in *C. elegans* is more

1003   similar to the situation in *Saccharomyces cerevisiae* where Tip41p and Tap42p function

1004   independently and antagonistically (121). Further experiments will be necessary to

1005   investigate the regulatory mechanism governing the activity of the PP2A/4/6 family

1006   during aging in worms and other species.

1007   Our work establishes roles for the PP2A, PP4, and PP6 phosphatases in

1008   contributing to the healthspan of postreproductive adult *C. elegans* through conferring

1009   resistance to environmental insults at least in part by modulating transcription. The

1010   mechanistic basis for this function will be revealed when the substrates of the

1011   complexes in aging animals are uncovered. In light of evidence from mammalian

1012   systems, during aging in *C. elegans* the PP2A and PP4 complexes may act to

1013   dephosphorylate DAF-16 itself or its upstream inhibitory kinases. Yet if this were true

1014   and the phosphatases regulate an early step in DAF-16 activation to counteract

1015   inhibition through IIS, we may have expected to find significant functional overlap

1016   between the PP2A and PP4 complexes such that both would have conferred resistance

1017   to the same stresses. Instead, we found that each complex seems to specialize in

1018   contributing to the resistance to particular insults. This suggests that PP2A and PP4

1019   may regulate DAF-16 at a more downstream step, possibly functioning to tailor the

1020   transcriptional output of DAF-16 to include only discrete subsets of its complete

1021   repertoire of targets. Consistent with this possibility, the transcription elongation factor

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1022   SPT-5 was recently identified as a substrate of PP4 in *daf-2 (e1370)* mutants where it

1023   plays a role in recruiting RNA PolII to the promoters of some but not all of the genes

1024   regulated by DAF-16 (45). Should the PP2A/4/6 family act at a similar level in adult

1025   wildtype animals, there are other means by which its members are known to influence

1026   gene expression that may be of particular relevance to aging. Across evolutionarily

1027   diverse species, these protein phosphatases regulate histone modification, chromatin

1028   organization, and mRNA processing which all undergo significant changes over time

1029   (63,64,89,106,122–125). Since we and others find that the PP2A/4/6 phosphatases are

1030   important for promoting healthspan, it may be by acting through these pathways that the

1031   PP2A/4/6 family modulates the changes in gene expression that are necessary to

1032   uphold vitality.

1033

Acknowledgments

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1478 **Supporting Information**

1479 **Figure S1. The catalytic subunits of the PP2A/4/6 subfamily of phosphoprotein**

1480 **phosphatases are highly conserved between *C. elegans* and humans.** The amino

1481 acid sequences of *C. elegans* LET-92, PPH-4.1, PPH-4.2 isoform a, and PPH-6 were

1482 aligned to their human orthologs PP2Ac, PP4c, and PP6c using Clustal Omega.

1483 Residue numbers are indicated on the right. The degree of conservation of individual

1484 amino acids across all seven proteins is denoted by symbols where asterisks (*)

1485 indicate identical residues, two dots (:) indicate highly similar residues, and one dot (.)

1486 indicates somewhat similar residues at a particular position. Characteristic domains of

1487 the subfamily are indicated, including the helix switch, loop switch, and TPDYFL motif

1488 (31). Specific residues associated with metal ion coordination and catalysis as well as

1489 those that interact with regulatory proteins are denoted by colored symbols according to

1490 the legend (115,116,126,127). In cases where multiple annotations apply to the same

1491   residue, corresponding symbols are vertically stacked and squares may be split

1492   diagonally.

1493   **Figure S2**. **Interaction network for putative subunits of the *C. elegans* PP4**

1494   **complex reveals possible connections to other members of the PP2A/4/6**

1495   **subfamily.** A list of potential subunits of the *C. elegans* PP4 complex including PPFR-1,

1496   PPFR-2, PPH-4.1, and SMK-1 in addition to the regulatory protein PPFR-4 was

1497   submitted for analysis to Genemania (genemania.org). The resulting network diagram

1498   depicts multiple types of interactions including physical interactions (red lines), predicted

1499   interactions (orange lines), or genetic interactions (green lines). Other similarities

1500   between proteins including co-expression (purple lines) or shared protein domains

1501   (yellow lines) are also indicated. In cases where only a Wormbase gene identifier or a

1502   CDS identifier was listed in the network diagram, the *C. elegans* gene name and/or the

1503   human ortholog (in parentheses) is provided in an adjacent grey box. Colored outlines

1504   surrounding nodes indicate that a particular protein is either an ortholog of a human

1505   PP2A/4/6 complex subunit or is an ortholog of a protein that regulates the activity of one

1506   or more members of the PP2A/4/6 family. Since including F46C5.6 as part of the query

1507   resulted in a second node that was not part of the larger network it was eliminated from

1508   the analysis.

1509

1510   **Figure S3. PP4 subunit homologues PPFR-1 and F46C5.6 do not function in**

1511   **innate immunity in *C. elegans*.** RNAi treatment targeting *C. elegans* homologs of

1512   regulatory subunits of the PP4 complex was initiated at the L1 stage. After knockdown

1513   of F46C5.6 (A,C) or *ppfr-1* (B,D), worms were infected at the L4 larval stage (A, B) or at

7

1514 Day 6 of adulthood (C, D). A representative plot of the fraction of worms alive at each

1515 time point after the infection was initiated is shown. In all cases RNAi targeting *daf-16* or

1516 *smk-1* and the empty RNAi vector L4440 were included as controls. Statistical analyses

1517 indicate that neither of the RNAi treatments had a significant effect on the survival of

1518 worms following bacterial infection.

1519

1520 **Figure S4.** *C. elegans* **homologues of human PP2A complex subunits with no**

1521 **apparent role in innate immunity during aging.** Representative survival curves for

1522 worms treated with RNAi to knockdown the indicated gene beginning at L1 and then

1523 infected with *P. aeruginosa* at the L4 larval stage (A-F) or at Day 6 of adulthood (G-N)

1524 are shown. Since RNAi inhibition of *let-92* and *paa-1* arrested larval development,

1525 knockdown of those genes was initiated at the L4 stage and worms were infected only

1526 at Day 6 (M,N). A representative plot of the fraction of worms alive at each time point

1527 after the infection began is shown. All plots include data for animals treated with the

1528 empty RNAi vector L4440 and for RNAi knockdown of *daf-16* and *smk-1*. Statistical

1529 analyses indicate that none of the RNAi treatments had a significant effect on the

1530 survival of worms following bacterial infection.

1531

1532 **Figure S5. Three regulatory subunits of the PP4 complex do not appear to**

1533 **function in conferring resistance to UV irradiation.** RNAi treatment targeting *C.*

1534 *elegans* homologs of regulatory subunits of the PP4 complex F46C5.6 (A, D), *ppfr-1* (B,

1535 E), and *ppfr-2* (C, F) was initiated at the L1 stage and continued for the duration of the

1536 assay. Worms were exposed to UV irradiation at the L4 larval stage (A-C) or at D6 of

7

1537   adulthood (D-F) after which their survival under standard culturing conditions was

1538   monitored. A representative plot of the fraction of worms alive at each time point

1539   following exposure to UV radiation is shown. All plots include data for animals treated

1540   with the empty RNAi vector L4440 and for RNAi knockdown of *daf-16* and *smk-1*.

1541   Statistical analyses indicate that none of the RNAi treatments had a significant effect on

1542   the survival of worms following UV irradiation.

1543

1544   **Figure S6. *C. elegans* homologues of human PP2A complex subunits with no**

1545   **apparent role in conferring resistance to UV irradiation.** Representative survival

1546   curves for worms treated with RNAi to knockdown the indicated gene beginning at L1

1547   and irradiated with ultraviolet light at the L4 larval stage (A-F) or at Day 6 of adulthood

1548   (G-L) are shown. Following irradiation, worms were returned to standard culture

1549   conditions and their survival was monitored over time. A representative plot of the

1550   fraction of worms alive at each time point following exposure to UV radiation is shown.

1551   All plots include data for animals treated with the empty RNAi vector L4440 and for

1552   RNAi knockdown of *daf-16* and *smk-1*. Statistical analyses indicate that none of the

1553   RNAi treatments had a significant effect on the survival of worms following UV

1554   irradiation.

1555

1556   **Figure S7. The PP6 regulatory subunit SAPS-1 plays no role in protecting *C.***

1557   ***elegans* from UV irradiation.** RNAi treatment targeting *C. elegans* homolog of the PP6

1558   regulatory subunit *saps-1* was initiated at the L1 stage and continued for the duration of

1559   the assay. Worms were exposed to UV irradiation at the L4 larval stage (A) or at D6 of

7

1560   adulthood (B) after which their survival under standard culturing conditions was

1561   monitored. A representative plot of the fraction of worms alive at each time point

1562   following exposure to UV radiation is shown. All plots include data for animals treated

1563   with the empty RNAi vector L4440 and for RNAi knockdown of *daf-16* and *smk-1*.

1564   Statistical analyses indicate that RNAi targeting *saps-1* had no significant effect on the

1565   survival of worms following UV irradiation.

1566

1567   **Figure S8. PP4 subunit homologues PPFR-1 and PPFR-2 do not confer**

1568   **thermotolerance to *C. elegans*.** From L1 until death *C. elegans* were treated with

1569   RNAi targeting putative regulatory subunits of the PP4 complex *ppfr-1* (A, C) or *ppfr-2*

1570   (B,D). Worms were shifted from 20° C to 35° C at larval stage L4 (A, B) or D6 (C, D) and

1571   maintained at the high temperature until death. A representative plot of the fraction of

1572   worms alive at each time point during the incubation at 35° C is shown. In all cases

1573   RNAi targeting *daf-16* or *smk-1* and the empty RNAi vector L4440 were included as

1574   controls. Statistical analyses indicate that neither of the RNAi treatments had a

1575   significant effect on the survival of worms under heat stress.

1576

1577   **Figure S9. *C. elegans* homologues of human PP2A complex subunits with no**

1578   **apparent role in thermotolerance during adulthood.** From L1 until death worms were

1579   treated with RNAi targeting *C. elegans* orthologs of catalytic and regulatory subunits of

1580   the human PP2A complex. At either the L4 stage (A-F) or at Day 6 of adulthood (G-N)

1581   worms were shifted from the standard incubation temperature of 20° C to 35° C and

1582   were maintained at the high temperature until death. Since RNAi inhibition of *let-92* and

1583   *paa-1* arrested larval development, knockdown of those genes was initiated at the L4

1584   stage and worms were shifted to high temperature only at Day 6 (M,N). A representative

1585   plot of the fraction of worms alive at each time point during the incubation at 35˚ C is

1586   shown. In all cases RNAi targeting *daf-16* or *smk-1* and the empty RNAi vector L4440

1587   were included as controls. Statistical analyses indicate that none of the RNAi treatments

1588   had a significant effect on the survival of worms under heat stress.

1589

1590   **Figure S10. The PP6 regulatory subunit SAPS-1 plays no role in the response to**

1591   **heat stress.** From L1 until death *C. elegans* were treated with RNAi targeting *saps-1*, a

1592   subunit of the PP6 complex in *C. elegans*. Worms were shifted from 20˚ C to 35˚ C at

1593   larval stage L4 (A) or D6 (B) and maintained at the high temperature until death. A

1594   representative plot of the fraction of worms alive at each time point during the incubation

1595   at 35˚ C is shown. In all cases RNAi targeting *daf-16* or *smk-1* and the empty RNAi

1596   vector L4440 were included as controls. Statistical analyses indicate that RNAi targeting

1597   *saps-1* did not have a significant effect on the survival of worms under heat stress.

1598

1599   **Figure S11.** *C. elegans* **orthologs of regulators of the PP2/4/6 family do not**

1600   **contribute to host defense during adulthood.** Beginning at the L1 stage the *C.*

1601   *elegans* homologs of human PTPA (Y71H2AM.20; A, C) and TIPRL (ZK688.9; B, D)

1602   were targeted with RNAi. Worms were infected with *P. aeruginosa* at the L4 (A, B) larval

1603   stage or at D6 (C, D) of adulthood. A representative plot of the fraction of worms alive at

1604   each time point after the infection was initiated is shown. In all cases RNAi targeting

1605   *daf-16* or *smk-1* and the empty RNAi vector L4440 were included as controls. Statistical

7

1606 analyses indicate that none of the RNAi treatments had a significant effect on the

1607 survival of worms following bacterial infection.

1608

1609 **Figure S12.** *C. elegans* **orthologs of PTPA and TIPRL have no apparent function in**

1610 **conferring resistance to UV irradiation.** RNAi treatment targeting the PTPA ortholog

1611 Y71H2AM.20 (A, B) or the TIPRL ortholog ZK688.9 (C, D) was initiated at the L1 stage

1612 and continued for the duration of the assay. Worms were exposed to UV irradiation at

1613 the L4 larval stage (A, C) or at D6 of adulthood (B, D) after which their survival under

1614 standard culturing conditions was monitored. A representative plot of the fraction of

1615 worms alive at each time point following exposure to UV radiation is shown. All plots

1616 include data for animals treated with the empty RNAi vector L4440 and for RNAi

1617 knockdown of *daf-16* and *smk-1*. Statistical analyses indicate that RNAi targeting

1618 Y71H2AM.20 or ZK688.9 had no significant effect on the survival of worms following UV

1619 irradiation.

1620

1621 **Figure S13.** *C. elegans* **orthologs of regulators of the PP2/4/6 family do not affect**

1622 **susceptibility to heat stress.** Beginning at the L1 stage the *C. elegans* homologs of

1623 human α4 (*ppfr-4*; A, D), PTPA (Y71H2AM.20; B, E), and TIPRL (ZK688.9; C, F) were

1624 targeted with RNAi. Worms were shifted from 20˚ C to 35˚ C at larval stage L4 (A-C) or

1625 D6 (D-F) and maintained at the high temperature until death. A representative plot of

1626 the fraction of worms alive at each time point during the incubation at 35˚ C is shown. In

1627 all cases RNAi targeting *daf-16* or *smk-1* and the empty RNAi vector L4440 were

7

1628   included as controls. Statistical analyses indicate that none of the RNAi treatments had

1629   a significant effect on the survival of worms under heat stress.

1630

1631   **Supplemental Table 1. Percent identity matrix for *C. elegans* and human PP2A/4/6**

1632   **catalytic subunits.** Output from Clustal Omega alignment of *C. elegans* and human

1633   catalytic subunits of the PP2A/4/6 subfamily showing percent identity between each

1634   pairwise comparison. The best matching ortholog in each column (highest percent

1635   identity) is highlighted in green.

1636

1637   **Supplemental Table 2. PPH-4.1 interactors.**

1638   Predicted and experimentally validated interactors of PPH-4.1 as listed in the

1639   Wormbase protein interaction database are shown.

1640

1641   **Supplemental Table 3. SMK-1 interactors.**

1642   Predicted and experimentally validated interactors of SMK-1 as listed in the Wormbase

1643   protein interaction database are shown.

1644

1645   **Supplemental Table 4. Location and timing of expression of *C. elegans* PP2A/4/6**

1646   **genes.** Genes listed in this table correspond to the following three groups: 1) those

1647   encoding members of the holoenzyme complexes that we propose in Figure 7; 2)

1648   T22D1.5 and F43B10.1, orthologs of PP2B" regulatory subunits that we found to be

1649   necessary for the age-dependent increase in DAF-16 transcriptional activity; and 3)

1650   orthologs of regulatory proteins. *daf-16* expression information is included as a

1651   reference. Expression of genes in tissues where DAF-16 function has been explicitly

1652   tested in previous studies, including body wall muscle, intestine, and neurons, are

1653   highlighted by coloring the names of those tissues (red, green, and blue, respectively) in
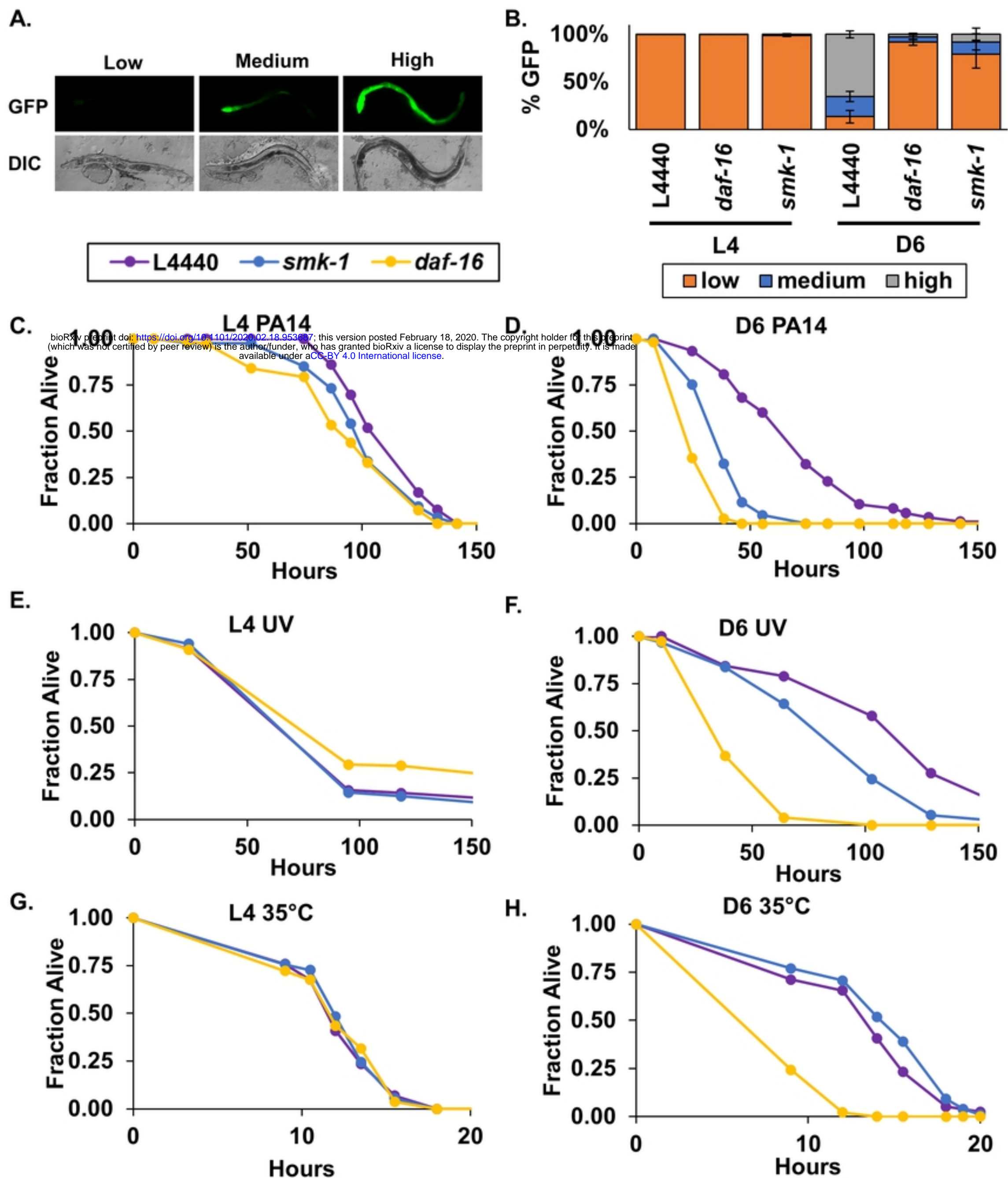
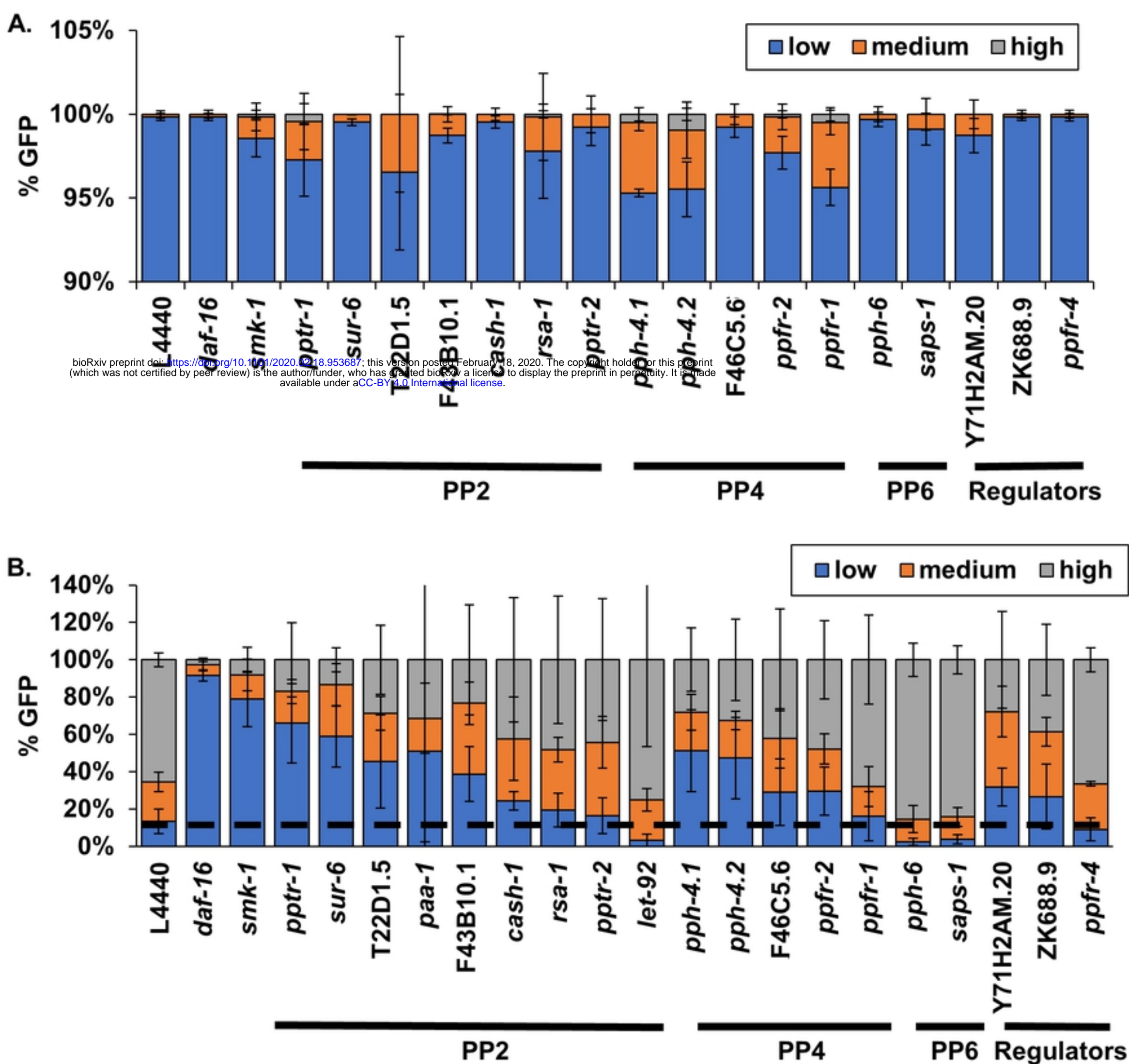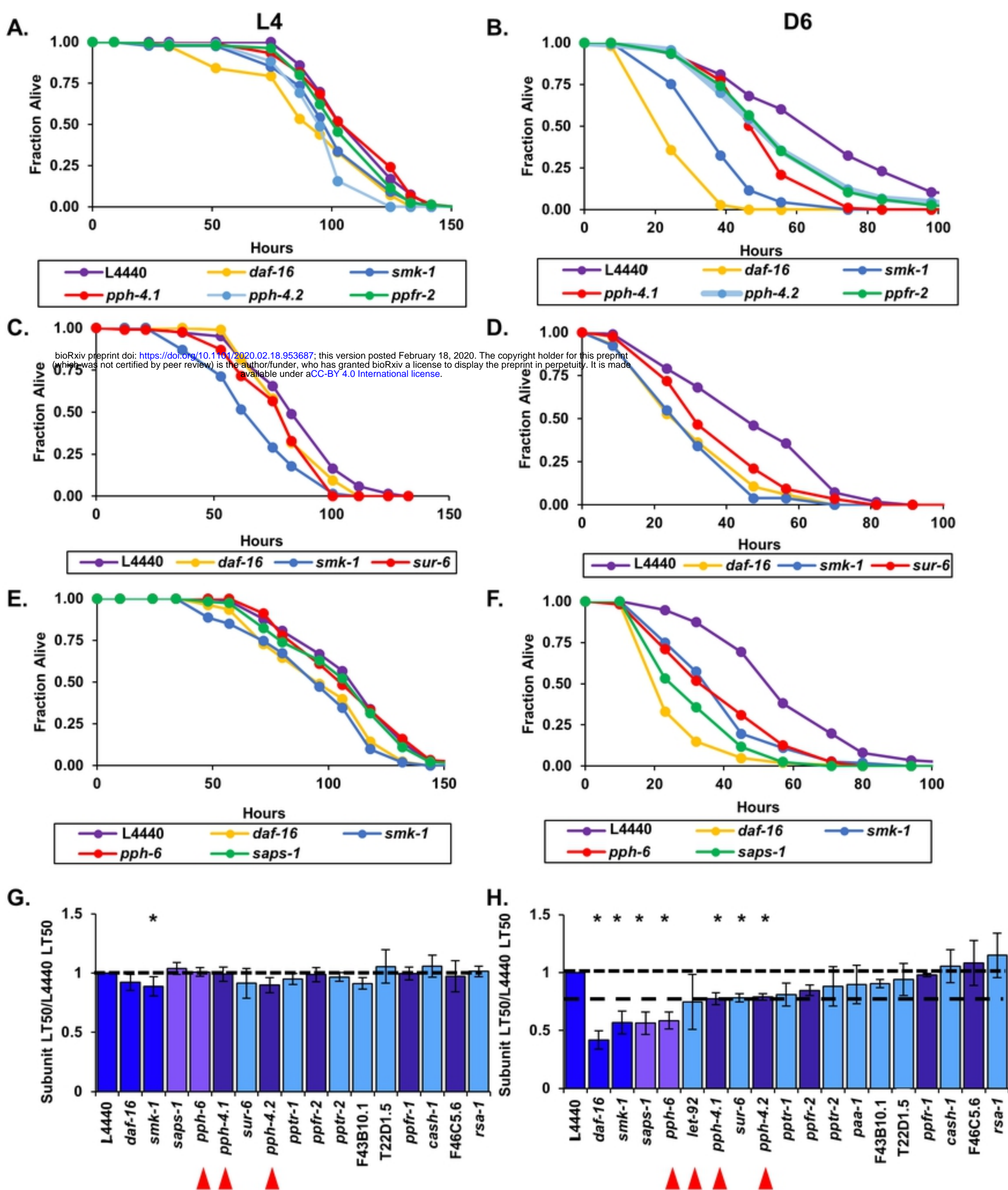1654   the "Anatomical sites of expression" column.
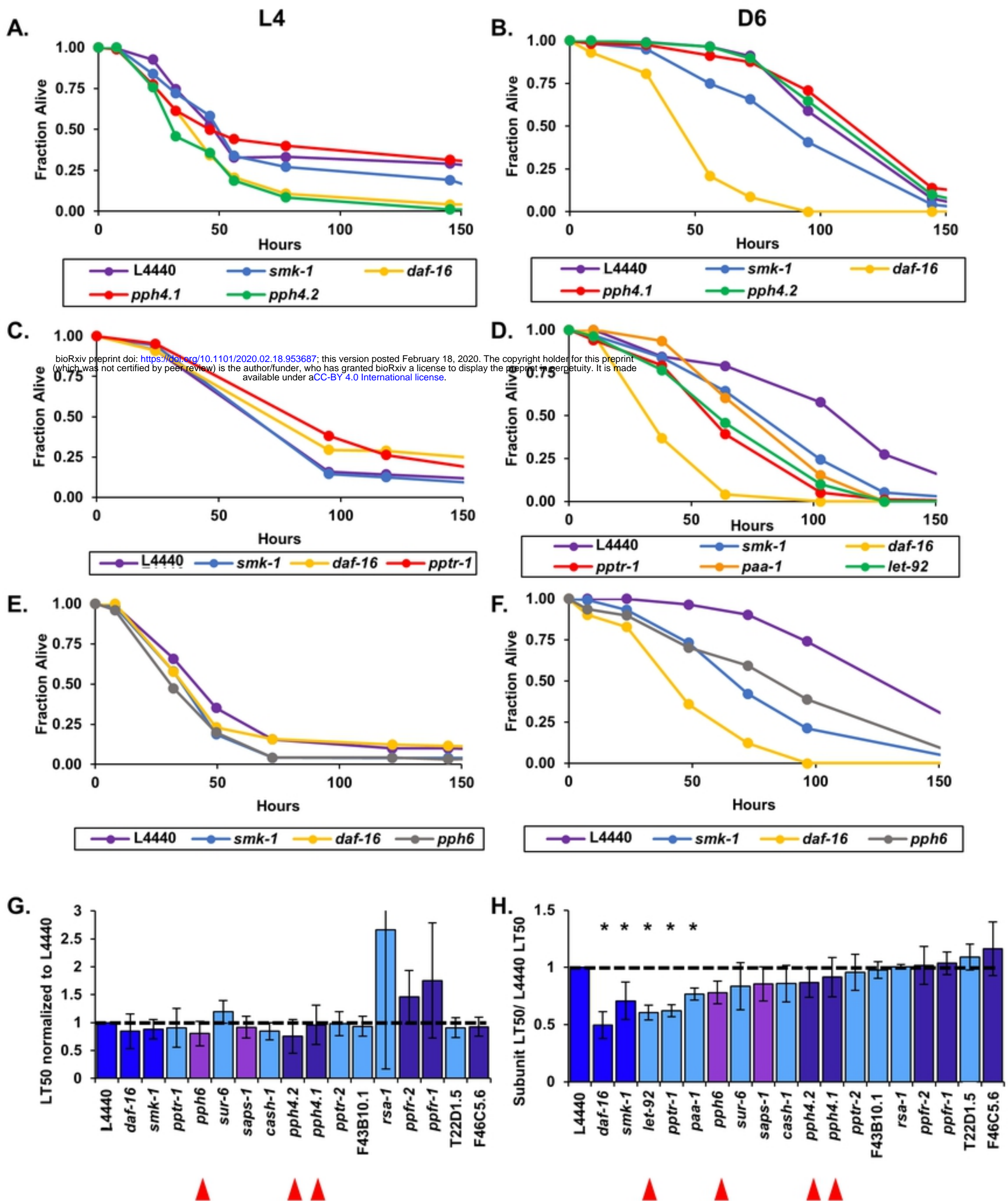
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Figure 1

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