The longevity response to warm temperature is neurally controlled via the regulation of collagen genes

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Abstract

Studies in diverse species have associated higher temperatures with shorter lifespan and lower temperatures with longer lifespan. However, the mechanisms behind these inverse effects of temperature on longevity are not well understood. Here, we demonstrate that in *Caenorhabditis elegans*, functional loss of NPR-8, a G protein-coupled receptor related to mammalian neuropeptide Y receptors, increases worm lifespan at 25°C but not at 20°C or 15°C, and that the lifespan increase at 25°C is regulated by the NPR-8-expressing AWB and AWC chemosensory neurons as well as AFD thermosensory neurons. RNA sequencing revealed that both warm temperature and old age profoundly alter gene expression. Further investigation uncovered that the NPR-8-dependent longevity response to warm temperature is achieved by regulating the expression of a subset of collagen genes. As elevated collagen expression is a common feature of

many lifespan-extending interventions and enhanced stress resistance, collagen expression could be critical for healthy aging.

Introduction

Studies in diverse species have associated lower temperatures with longer lifespan and higher temperatures with shorter lifespan, aside from extreme temperatures that could endanger organism survival (*1*). These inverse effects of temperature on longevity were first recorded in poikilotherms, such as the fruit fly *Drosophila melanogaster* (*2*), the cladoceran crustacean *Daphnia magna* (*3*), the nematode *Caenorhabditis elegans* (*4*), as well as in poikilothermic vertebrates such as the South American fish *Cynolebias* (*5*). This phenomenon was also observed in homeotherms such as mice (*6*) and humans (*7*), although with some obscurity (e.g., females have higher body temperatures than males and yet females live longer (*1*)). Nonetheless, there is a clear trend for higher temperatures being associated with shorter lifespan across species.

Most early studies on the longevity response to temperature attempted to interpret the inverse relationship using the rate of living theory, which posits that higher temperatures increase chemical reaction rates, thus speeding up the aging process, whereas lower temperatures do just the opposite (*8*). At the core of this theory is applying the second law of thermodynamics to living organisms, speculating that aging results from increased molecular disorder, and that higher temperatures enhance thermodynamic entropy, thus inducing a faster rate of aging (*8*). Although temperatures could alter the accumulation of molecular damage, recent studies have identified specific cells, cellular molecules, and signaling pathways that are involved in the

47 longevity response to temperature, indicating that this response is a regulated process and not
48 simply due to temperature-induced changes in thermodynamics (*1, 9*). In *C. elegans*, the cold-
49 sensitive TRP channel TRPA-1 can detect temperature drops in the environment and extend
50 lifespan by inducing calcium influx into the cell and activating protein kinase C, which, in turn,
51 activates the pro-longevity transcription factor DAF-16/FOXO (*10*). Interestingly, TRPA-1 also
52 mediates lifespan shortening when worms are subjected to low-temperature treatment during the
53 larval stage (*11*). Moreover, thermosensory AFD neurons and AIY interneurons were found to be
54 required for maintaining *C. elegans* lifespan at warm temperature (25°C) through the DAF-
55 9/DAF-12 steroid-signaling pathway (*12*). Such neural regulation was controlled by the worm
56 cyclic AMP-responsive element-binding protein (CREB) transcription factor (*13*). DAF-41, the
57 *C. elegans* homolog of co-chaperone P23, which has a possible role in protein folding, is also
58 required for the normal longevity responses to both cold and warm temperatures, indicating a
59 potential role of proteostasis in these responses (*14*). Several factors involved in lipid metabolism
60 pathways, including MDT-15/Mediator 15 (*15*), PAQR-2 (*16*), and prostaglandin (*17*), also
61 modulate longevity in response to low temperatures. Measuring the lifespans of a diverse array
62 of short- or long-lived mutant worms at different temperatures revealed that temperature-induced
63 lifespan changes are genetically controlled by temperature-specific gene regulation (*18*). These
64 studies suggest that the longevity response to temperature is a complex and highly regulated
65 process, and that integrative research is needed to define the relationships between temperature,
66 aging mechanisms, and longevity.
67
68 Here, using the *C. elegans* model, we demonstrate that functional loss of NPR-8, a neuronal G
69 protein-coupled receptor (GPCR) related to mammalian neuropeptide Y receptors, extends worm

70 lifespan at 25°C but not at 20°C or 15°C, and that the extension in lifespan at 25°C is regulated
71 by the NPR-8-expressing amphid chemosensory neurons AWB and AWC as well as AFD
72 thermosensory neurons, indicating that the nervous system governs longevity in response to
73 higher temperature. Integrative RNA sequencing analysis to examine the relationships between
74 temperature, aging, and NPR-8 revealed that both warm temperature and old age profoundly
75 alter gene expression. Genetic manipulation and functional assays further uncovered that NPR-8-
76 regulated collagen expression contributes to the extended lifespan of *npr-8* mutant animals at
77 25°C and is also required for maintaining the lifespan of wild-type animals. Since elevated
78 collagen expression is a common feature of many lifespan-extending interventions and enhanced
79 stress resistance to various environmental stimuli (*19, 20*), collagen expression could promote
80 healthy aging and may hold the key to our quest for long and healthy life.
81
82 **Results**
83
84 **Functional loss of NPR-8 extends *C. elegans* lifespan at warm temperature**
85 Three temperatures (15°C, 20°C, and 25°C) are routinely used for *C. elegans* propagation in the
86 laboratory, and their inverse effects on lifespan have been well documented, (i.e., an
87 approximately 75% increase in lifespan occurs with a 5°C drop in temperature) (*4*). During our
88 work on neural regulation of *C. elegans* defense against pathogen infection (*20*), we
89 unexpectedly observed that under noninfectious conditions, *C. elegans* lacking functional NPR-8
90 (*npr-8(ok1439)* animals) lived longer than wild-type *N2* animals at 25°C, whereas the mutant
91 and wild-type animals had the same lifespan at 20°C or 15°C (Fig. 1A, 1B and 1C). To
92 determine whether the observed longevity phenotype at the warm temperature of 25°C was due

93 to the deletion mutation in *npr-8*, we performed lifespan assays at 25°C using the transgenic
94 strain JRS17 in which *npr-8* is re-expressed under its native promoter in *npr-8(ok1439)* animals
95 (*20*). Re-expression of NPR-8 significantly decreased the extended lifespan of the *npr-8* deletion
96 mutants (Fig. 1D), confirming that the mutation in *npr-8* is indeed responsible for the extended
97 longevity phenotype at warm temperature. To investigate whether this phenotype is allele-
98 specific, we performed lifespan assays using another *npr-8* null strain (*npr-8(ok1446)* animals)
99 that contains a deletion mutation in the *npr-8* gene at a different location than the one in *npr-*
100 *8(ok1439)* animals (*21*). Our results showed that, like *npr-8(ok1439)* animals, *npr-8(ok1446)*
101 animals also lived longer than wild-type animals at 25°C but had wild-type lifespan at 20°C and
102 15°C (Fig. S1), indicating that the extended lifespan phenotype is not allele-specific but is
103 caused by the general lack of NPR-8 function. These results suggest that NPR-8 suppresses
104 lifespan at warm temperature, contributing to the inhibitory effect of warm temperature on
105 longevity.
106
107 **The NPR-8-dependent longevity response to warm temperature is neurally regulated**
108 NPR-8 is a neuronal GPCR expressed in the amphid sensory neurons AWB, ASJ and AWC (*20*).
109 Our previous study demonstrated that NPR-8 functions in these neurons to regulate *C. elegans*
110 defense against pathogen infection (*20*). To determine whether the NPR-8-dependent longevity
111 response to warm temperature is also neurally controlled, we took advantage of our established
112 rescue strains that specifically express NPR-8 in the individual neurons under cell-specific
113 promoters in *npr-8(ok1439)* animals (*20*). Lifespan assays at 25°C with these animals showed
114 that re-expressing NPR-8 in AWB or AWC neurons rescued the extended lifespan phenotype of
115 the mutants, while re-expression of NPR-8 in ASJ neurons had no effect on the mutants' lifespan

116 (Fig. 2A). Like *npr-8(ok1439)* animals, all rescue animals exhibited lifespan similar to that of
117 wild-type animals at 20°C (except the ASJ rescue animals, which displayed slightly longer
118 lifespan than wild type) (Fig. S2). These results indicate that the lack of functional NPR-8 in
119 AWB and AWC neurons is responsible for the mutants' extended lifespan at 25°C. In other
120 words, NPR-8 functions in AWB and AWC neurons to regulate the longevity response to warm
121 temperature.
122
123 In *C. elegans*, AFD thermosensory neurons are required for maintaining lifespan at warm
124 temperature (25°C), and ablation or inactivation of these neurons shortens the worm's lifespan
125 (*12, 13*). To test whether AFD neurons also function in the NPR-8-dependent longevity response
126 to warm temperature, we examined how a loss-of-function mutation in *ttx-1*, a gene critical for
127 AFD differentiation and function (*22*), affected the lifespan of *npr-8(ok1439)* animals at warm
128 temperature. To this end, we crossed *npr-8(ok1439)* and *ttx-1(p767)* animals to generate *ttx-*
129 *1;npr-8* double mutant animals, then subjected the single and double mutant animals, along with
130 wild-type controls, to lifespan assays at 25°C. Consistent with previous reports (*12, 13*), the lives
131 of thermosensory-defective *ttx-1(p767)* animals were shorter than those of wild-type animals
132 (Fig. 2B). Interestingly, *ttx-1;npr-8* double mutant animals displayed longer lifespan than *ttx-*
133 *1(p767)* animals but shorter lifespan than *npr-8(ok1439)* animals (Fig. 2B), indicating that the
134 *ttx-1* and *npr-8* mutations reduce each other's effect on longevity at warm temperature. These
135 results suggest that the warm temperature-induced longevity response is regulated by a circuit of
136 thermosensory and chemosensory neurons working in an antagonistic manner.
137
138 **Both warm temperature and old age broadly alter gene expression in *C. elegans***

139 To gain molecular insights into the NPR-8-dependent longevity response to temperature, we
140 employed RNA-seq to compare gene expression in young and old wild-type and *npr-8(ok1439)*
141 animals propagated at different temperatures. To this end, we collected five replicates of eight
142 groups of RNA samples (young (0-day-old) and 9-day-old adult wild-type and *npr-8(ok1439)*
143 animals propagated at 20°C and 25°C) (Table 1). These samples were then submitted to the
144 Washington State University Genomics Core for RNA-seq analysis. The resulting sequence data
145 (FASTQ files) were deposited into the National Center for Biotechnology Information (NCBI)
146 Sequence Read Archive (SRA) database through the Gene Expression Omnibus (GEO). The
147 processed gene quantification files and differential expression files were deposited in the GEO.
148 All of these data can be accessed through the GEO with the accession number GSE186202. A
149 summary of our RNA-seq analysis is listed in Table 2.
150
151 Using the RNA-seq data, we first assessed how propagation temperature affects gene expression
152 by comparing the expression profiles of wild-type young-adult (0-day-old) animals grown at
153 25°C with those from animals grown at 20°C (Group 3 vs. Group 1 in Table 1). In total, 16,135
154 genes were identified and quantified with a false discovery rate (FDR) of 5%. Among these
155 genes, 2,265 were upregulated and 5,069 were downregulated at least two-fold in animals grown
156 at 25°C relative to those grown at 20°C. The changes in expression of almost half of the total
157 identified genes (45%) indicate that growth temperature profoundly affects gene expression in *C.*
158 *elegans*. This might explain, at the molecular level, why temperature is so crucial for life in that
159 even modest variations in temperature can alter many aspects of behavior and physiology
160 including aging (*1*). Gene ontology (GO) analysis of the 2,265 upregulated genes identified 340
161 significantly enriched biological processes, which can be loosely divided into seven categories:

162 cell cycle, reproduction, metabolic/biosynthetic processes, organelle organization, development,
163 DNA replication/repair/transcription/recombination, and other processes (Table S1). GO analysis
164 of the 5,069 downregulated genes identified 68 significantly attenuated biological processes,
165 which can be loosely divided into five categories: signaling, phosphorylation/dephosphorylation,
166 immune response, ion/transmembrane transport, and other processes (Table S2). It is not clear
167 which regulated biological processes are responsible for the shortened lifespan of *C. elegans* at
168 warm temperature. The enrichment of genes involved in metabolic/biosynthetic processes may
169 reflect an increased rate of metabolism at 25°C relative to 20°C (Table S1), which could provide
170 a molecular basis for the rate of living theory. It is worth noting that among all of the
171 downregulated biological processes (Table S2), signaling is the most significantly reduced and
172 includes reduction in GPCR signaling pathway (GO term GO:0007186), neuropeptide signaling
173 pathway (GO:0007218), synaptic signaling (GO:0099536), nervous system process
174 (GO:0050877), and other signaling processes. A total of 523 genes encoding signaling molecules
175 such as GPCRs and neuropeptides as well as enzymes involved in the metabolism of
176 neurotransmitters or neurohormones contribute to the downregulation of signaling processes
177 (Table S3). These data demonstrate that signaling processes, especially those controlled by the
178 nervous system, are reduced in animals propagated at 25°C compared to those propagated at
179 20°C, indicating a potential connection between neural signaling and longevity in *C. elegans*.
180 Indeed, signaling from AFD thermosensory neurons and their downstream AIY interneurons are
181 required for maintaining lifespan at 25°C (*12, 13*). However, detailed information about neural
182 circuits that control the longevity response to temperature is lacking.
183

184 We next examined how aging affects gene expression by comparing the expression profiles of 9-
185 day-old wild-type adult animals with those of 0-day-old adult animals grown at 20°C (Group 2
186 vs. Group 1 in Table 1). We found that 3,792 genes were upregulated, and 4,937 genes were
187 downregulated at least 2-fold in old animals relative to young adults. The changes in expression
188 of more than half of the total identified genes indicate that, like temperature, age also has
189 profound effects on *C. elegans* behavior and physiology. GO analysis of the 3,792 upregulated
190 genes identified 428 significantly enriched biological processes, which can be loosely divided
191 into six categories: cell cycle, reproduction/development, metabolism, organelle organization,
192 DNA replication/repair/transcription, and other processes (Table S4). GO analysis of the 4,937
193 downregulated genes identified 56 significantly attenuated biological processes, which can be
194 loosely divided into five categories: ion/transmembrane transport,
195 phosphorylation/dephosphorylation, signaling, immune response, and other processes (Table S5).
196 These data show that similar biological processes are regulated by old age and warm
197 temperature, suggesting that both conditions could be stressful to *C. elegans*. Interestingly and
198 importantly, GO analysis of the downregulated genes also revealed 65 attenuated molecular
199 functions, with "structural constituent of cuticle" being the most significantly reduced (Table
200 S6A). One hundred and seven downregulated genes contribute to the reduction in cuticle
201 structure activity, among which 88 encode cuticular collagens (Table S6B), indicating that
202 cuticular collagens could be essential for aging and longevity. Indeed, previous studies have
203 shown that the expression levels of collagen genes decline with age and that increased collagen
204 expression is a common feature of multiple conserved longevity pathways and essentially every
205 longevity intervention (*19, 23*). Cuticular collagens have also been implicated in lengthening *C.*
206 *elegans* lifespan under stress such as pathogen infection and oxidative stress (*19, 20*).

207

208 **NPR-8 differentially regulates collagen genes in an age- and temperature-dependent**

209 **manner**

210 To assess the role of NPR-8 in the temperature-induced longevity response, we compared the
211 expression profiles of young and old *npr-8(ok1439)* animals with those of wild-type animals
212 maintained at different propagation temperatures. For 0-day-old young adults grown at 20°C,
213 643 genes were upregulated and 272 genes were downregulated at least 2-fold in *npr-8(ok1439)*
214 animals relative to wild-type controls. While GO analysis of the 272 downregulated genes did
215 not yield significantly attenuated biological processes or molecular functions, a similar analysis
216 of the 643 upregulated genes identified seven significantly enriched molecular functions, with
217 the GO term "structural constituent of cuticle" being the most significantly enriched (Table
218 S7A). Sixteen upregulated genes were related to cuticle structure activity, nine of which encoded
219 cuticular collagens (Table S7B). These data indicate that at the normal growth temperature of
220 20°C, NPR-8 suppresses the expression of a subset of cuticular collagen genes in young-adult
221 wild-type animals. This result is consistent with our previous study showing that NPR-8
222 suppresses the basal expression of collagen genes (*20*). For 9-day-old adult animals grown at
223 20°C, 1,487 genes were upregulated and 1,305 genes were downregulated at least 2-fold in *npr-*
224 *8(ok1439)* animals relative to wild-type controls. The total number of regulated genes tripled at
225 old age compared to young age, indicating that the effects of NPR-8 on *C. elegans* physiology
226 are amplified by aging. GO analysis of the 1,487 upregulated genes identified 33 significantly
227 enriched biological processes, which can be divided into three categories:
228 phosphorylation/dephosphorylation, metabolic process, and other processes (Table S8). GO
229 analysis of the 1,305 downregulated genes revealed 13 attenuated molecular functions, with the

230 GO term “structural constituent of cuticle” being the most significantly reduced (Table 3A).
231 Seventy-three downregulated genes contributed to the decrease in cuticle structure activity, 60 of
232 which encoded cuticular collagens (Table 3B). These data indicate that at old age, NPR-8
233 upregulates the expression of cuticular collagens in wild-type animals, which is surprising and is
234 in direct contrast with NPR-8’s function at young age when it suppresses cuticular collagens
235 (Table S7). More surprisingly, eight of the nine collagen genes (except *col-98*) that changed
236 expression at young age were also regulated at old age (compare Table 3B to Table S7B). These
237 results are unexpected and raised a perplexing question, that is, how does NPR-8, a single type of
238 GPCR, exerts opposite effects on collagen expression at different stages of the *C. elegans* life
239 cycle? Does *npr-8* influence both fitness and aging similar to genes described in George
240 Williams’ antagonistic pleiotropic theory that have beneficial effects on fitness in early life but
241 are detrimental in later life (*24*)?
242
243 We next examined the gene expression profiles of young and old *npr-8(ok1439)* and wild-type
244 animals grown at 25°C. For 0-day-old young adults, 1,482 genes were upregulated and 419
245 genes were downregulated at least 2-fold in *npr-8(ok1439)* animals relative to wild-type animals.
246 GO analysis of the 1,482 upregulated genes identified 17 enriched molecular functions, with the
247 GO term “structural constituent of cuticle” being the most significantly enriched (Table S9A).
248 Seventy upregulated genes were related to cuticle structure activity, 56 of which encoded
249 cuticular collagens (Table S9B). These data indicate that, similar to 0-day-old young adults
250 grown at 20°C (Table S7), NPR-8 also suppresses the expression of cuticular collagen genes in
251 young adults grown at 25°C. However, there are many more collagen genes suppressed at 25°C
252 than at 20°C (56 genes vs. 9 genes, compare Table S9B with Table S7B), indicating that warm

253 temperature broadens NPR-8's inhibitory effect on collagen gene expression in young animals.
254 For 9-day-old adults, 1,307 genes were upregulated and 631 genes were downregulated at least
255 2-fold in *npr-8(ok1439)* animals relative to wild-type animals. GO analysis of the 1,307
256 upregulated genes revealed 29 significantly enriched biological processes, which can be divided
257 into three categories: phosphorylation/dephosphorylation, metabolic process, and other processes
258 (Table S10). These data demonstrate that in old animals, the biological processes suppressed by
259 NPR-8 at 25°C are similar to those suppressed by NPR-8 at 20°C (compare Table S10 with
260 Table S8), indicating that these specific NPR-8 effects are age-related, not temperature-
261 dependent. GO analysis of the 631 downregulated genes did not yield any significantly enriched
262 biological processes or molecular functions. This is in striking contrast with old worms grown at
263 20°C in which a large number of cuticular collagen genes (60 genes) were downregulated in *npr-*
264 *8(ok1439)* animals relative to wild-type animals (Table 3B). Because collagen expression
265 promotes longevity (*19, 23*), this lack of downregulation of collagen expression at 25°C may
266 explain the extended lifespan of *npr-8(ok1439)* animals at warm temperature.
267

268 **NPR-8-regulated collagen genes contribute to the extended lifespan of *npr-8(ok1439)***
269 **animals at 25°C**

270 To determine whether collagen expression plays a role in *npr-8(ok1439)* animals' extended
271 lifespan at warm temperature, we inactivated NPR-8-regulated collagen genes using RNA
272 interference (RNAi) or deletion mutation in *npr-8(ok1439)* and wild-type animals and then
273 performed lifespan assays at 25°C on these animals. For the five most-regulated collagen genes
274 tested (downregulated most in *npr-8(ok1439)* animals at 20°C but lacking such downregulation
275 at 25°C, Table 3B), inactivation of each of them (*rol-1*, *col-49*, *co-77*, *col-139*, or *col-179*)

276 significantly suppressed the extended lifespan of *npr-8* mutant animals at 25°C (Fig. 3A and 3C).
277 Inactivation of *col-49* and *col-179* also suppressed the lifespan of wild-type animals (Fig. 3B).
278 These results indicate that the expression of NPR-8-regulated collagen genes contributes to the
279 extended lifespan of *npr-8(ok1439)* animals at 25°C and is also essential for maintaining the
280 lifespan of wild-type animals at warm temperature. This is consistent with the longevity-
281 promoting role of collagens in the aging process (*19, 23*).
282

283 ***npr-8(ok1439)* animals have a less-wrinkled cuticle and appear younger than age-matched**
284 **wild-type animals**

285 During our study, we observed that *npr-8* mutant animals appeared categorically younger than
286 age-matched wild-type animals by exhibiting fewer wrinkles in the cuticle. To investigate this
287 phenomenon in detail, we performed scanning electron microscopy (SEM) to examine the cuticle
288 of wild-type and *npr-8(ok1439)* animals at various ages (3-, 6-, and 9-day old adults) under the
289 two different propagation temperatures of 20°C and 25°C. Characteristic structural features of
290 the *C. elegans* cuticle include small circumferential furrows separated by broader ridges called
291 annuli and longitudinally oriented ridges called alae (Fig. 4A) (*25*). Distinct alae are considered
292 an indicator of worm adulthood (*26*). Indeed, we observed that 3-day-old and 6-day-old wild-
293 type adult animals propagated at 20°C exhibited smooth cuticle with distinct alae structures (Fig.
294 4A and 4B). At the same temperature, 9-day-old wild-type adults displayed a wrinkled cuticle
295 with distorted alae (Fig. 4C), whereas 9-day-old *npr-8(ok1439)* animals maintained distinct alae
296 structures on a smooth surface (Fig. 4I). These results demonstrated that there are NPR-8-
297 dependent differences in the cuticles of mutant and wild-type animals. In contrast to what was
298 observed at 20°C, wrinkled cuticles and disintegrated alae were observed among 6-day-old wild-

299 type adults at 25°C (Fig. 4E), and by day 9, alae were disrupted (Fig. 4F), suggesting that warm
300 temperature speeds up aging. Compared to wild-type adults, both 6-day-old and 9-day-old *npr-*
301 *8(ok1439)* animals displayed a smooth cuticle with distinct structural morphology at 25°C (Fig.
302 4K and 4L), indicating younger-than-wild-type physiological age and perhaps better overall
303 health. Why would the functional loss of a neuronal GPCR lead to a less-wrinkled cuticle? Since
304 the 1930s, researchers have known that in humans, a finger with nerve damage will not wrinkle
305 under water (*27*). In fact, a medical test, the Wrinkling Test, has been developed to check the
306 wrinkling of patients to assess their possible peripheral nerve damage (*28*). However, the
307 molecular connections between wrinkles and the nerve damage remain unknown. Previously, we
308 have shown that NPR-8-regulated collagen genes are expressed in the cuticle as well as in
309 hypodermal and rectal cells (*20*). The difference in cuticle appearance between *npr-8* mutants
310 and wild-type animals could be due to the differential expression of cuticular collagen genes in
311 these animals, as revealed by the above-described RNA-seq data. Different collagen
312 compositions in the skin controlled by the nerve could also explain the Wrinkling Test.
313

314 **Discussion**

315

316 As in many other invertebrate and vertebrate animals, temperature has inverse effects on
317 longevity in *C. elegans*. Mechanistically, this inverse relationship is not well understood. In the
318 current study, we have demonstrated that functional loss of NPR-8 increases *C. elegans* lifespan
319 at 25°C but not at 20°C and 15°C, indicating that NPR-8 is involved in the longevity response to
320 warm temperature. Further investigation revealed that such regulation is achieved by altering the
321 expression of a subset of collagen genes, and that this regulation is controlled by the NPR-8-

322 expressing amphid chemosensory neurons AWB and AWC as well as AFD thermosensory
323 neurons. These two types of sensory neurons work in an antagonistic manner to maintain
324 longevity at 25°C. Therefore, an NPR-8-dependent neural-longevity regulatory circuit has
325 emerged whereby the nervous system controls the longevity response to warm temperature by
326 regulating collagen expression. However, many details of this regulatory circuit are lacking. For
327 example, which neurons form a network with AWB, AWC and AFD neurons to control
328 longevity at warm temperature? What neuroendocrine signaling pathways are used to relay
329 neural signals to non-neural tissues, since we have previously found that NPR-8-regulated
330 collagens are primarily expressed in the cuticle and hypodermal and rectal cells (*20*)? How does
331 collagen expression influence aging? Addressing these questions will provide important
332 mechanistic insights into the temperature-induced longevity response and define the role of the
333 nervous system in this process.
334
335 The inverse relationship between longevity and temperature has long been explained using the
336 rate of living theory, which posits that higher temperatures increase chemical reaction rates, thus
337 speeding up the aging process (*8*). However, recent research has identified specific molecules,
338 cells, and signaling pathways that are involved in the longevity response to temperature,
339 indicating that this response is a regulated process and not simply due to changes in
340 thermodynamics (*1, 9*). The actual mechanisms behind the inverse relationship seem not quite
341 that clear. On the one hand, our study provides further evidence supporting that the temperature-
342 induced longevity response is indeed a regulated process. We have identified the neuronal GPCR
343 NPR-8 and the AWB, AWC and AFD neurons that function in the *C. elegans* longevity response
344 to warm temperature, indicating that this response is controlled by the nervous system. On the

345 other hand, our RNA-seq data revealed the enrichment of many genes involved in
346 metabolic/biosynthetic processes in wild-type animals at 25°C relative to 20°C (Table S1),
347 reflecting increased metabolism at warm temperature. Thus, our data provide a partial molecular
348 basis for the rate of living theory, supporting the idea that there is some scientific truth in this
349 theory. As the rate of living theory and the view of the temperature-induced longevity response
350 being a regulated process are not mutually exclusive, we propose a hybrid hypothesis in which
351 higher temperatures speed up chemical reactions and the aging process in both thermodynamic
352 and neurally regulated manners in living organisms.
353
354 An important finding of our current study is that the expression of NPR-8-regulated collagen
355 genes is required for maintaining the lifespan of wild-type animals at 25°C, and that the
356 expression of these genes contributes to the extended lifespan of *npr-8(ok1439)* animals at warm
357 temperature (Fig. 3). Collagen expression has also been implicated in enhanced resistance to
358 pathogen infection (*20*) and oxidative stress (*19*). In fact, elevated collagen expression is a
359 common feature of multiple conserved longevity pathways and essentially every longevity
360 intervention (*19, 29-31*). These findings indicate a general role of collagen in aging. As stress
361 resistance is a critical parameter for assessing healthy lifespan, or healthspan (*32, 33*), collagen
362 expression likely promotes healthy aging. Indeed, we observed that *npr-8* mutant animals with
363 increased collagen expression had a less-wrinkled cuticle and appeared younger than age-
364 matched wild-type animals (Fig. 4). Remarkably, mammalian studies have shown that placing
365 senescent cells or aged stem cells in "young extracellular matrix" (ECM; collagen is the
366 predominant component of ECM) rejuvenates them (*34-36*). How such ECM-cell interactions
367 reverse the cellular aging process remains a mystery. It is reasonable to speculate that the

368 signaling role of collagens, which is less understood than their structural role (*37*), is likely
369 involved in aging. Indeed, collagens have been shown to bind to diverse families of cell-surface
370 receptors, including integrins, receptor tyrosine kinases, and immunoglobulin-like receptors, thus
371 influencing cellular physiology and behavior (*38, 39*). The integrin-signaling complex, acting as
372 a major link between collagen and the cytoskeleton, has been implicated in modulating longevity
373 and thermotolerance (*40*). These studies suggest that research deciphering how collagen
374 expression promotes healthy aging may hold the key to the human request for long and healthy
375 life.
376

377 **Materials and Methods**

378

379 ***C. elegans* strains**

380 *C. elegans* strains were cultured under standard conditions and fed *E. coli* OP50. Wild-type
381 animals were *C. elegans N2* Bristol. *npr-8 (ok1439)*, *npr-8(ok1446)*, *ttx-1 (p767)* (AFD-defective
382 mutant), and *col-179(ok3010)* animals were obtained from the Caenorhabditis Genetics Center
383 (University of Minnesota, Minneapolis, MN) and backcrossed with wild-type N2 for 3 to 8
384 times. JRS30 [*npr-8(ok1439);col-179(ok3010)*], YYL11 [*ttx-1(p767);npr-8(ok1439)*], JRS17
385 [*npr-8p(2kb)::npr-8 gDNA::gfp*] (native NPR-8 rescue strain), JRS18 [*str-1p(1.3kb)::npr-8*
386 *gDNA::gfp*] (NPR-8 rescue in AWB neurons), JRS19 [*str-2p(2kb)::npr-8 gDNA::gfp*] (NPR-8
387 rescue in AWC neurons), and JRS20 [*trx-1p(1.1kb)::npr-8 gDNA::gfp*] (NPR-8 rescue in ASJ
388 neurons) were generated using standard genetic techniques (*20*).
389

390 **Lifespan assay**

391 Wild-type and mutant worms were maintained at 20°C and fed *E. coli* OP50 on NGM medium.
392 For temperature-specific assays at 15°C and 25°C, worm strains were maintained for at least two
393 generations at the respective temperature before being used for egg synchronization. For egg
394 synchronization, 3x20 gravid adult worms per strain were used. The time window of egg
395 synchronization was set for 1 hr, during which all animals were maintained at the respective
396 assay temperature (15°C, 20°C, or 25°C). *E. coli OP50* lawn was prepared by spreading a 50-µl
397 drop of an overnight culture of bacteria on the NGM medium (3.5-cm petri plates). Plates were
398 incubated at 37°C for 16 hrs and then cooled down at room temperature for at least 1 hr before
399 seeding with synchronized worms. 3x20 synchronized worms for each group were transferred
400 onto the live *E. coli* OP50 plates. Worms were counted and transferred to fresh, live *E. coli* OP50
401 culture daily until death. All assays were performed in triplicates for each strain.
402
403 **RNA isolation**
404 To gain molecular insights into the NPR-8-dependent longevity response to temperature, we
405 employed RNA-seq to compare gene expression in young and old wild-type and *npr-8(ok1439)*
406 animals propagated at different temperatures. To this end, we collected five replicates of eight
407 groups of RNA samples (young (0-day-old) and 9-day-old adult wild-type and *npr-8(ok1439)*
408 animals propagated at 20°C and 25°C) (Table 1). The temperature-specific worm cultivation and
409 synchronization steps were performed as described in the lifespan assay section. A portion of
410 synchronized adult animals were collected at the age of 0-day old, and the remaining animals
411 were grown till day 9 before collection. These animals were washed every day using M9 buffer
412 and the adult animals were filtered using cell strainer (40-µm nylon filter, Falcon) and
413 transferred to *E. coli* OP50-seeded NGM plates. Worms were washed and collected with M9

414 buffer then snap-frozen in the TRIzol reagent (Thermo Fisher Scientific) and stored at -80°C
415 until RNA isolation. RNA was extracted using the QIAzol lysis reagent (Qiagen) and purified
416 with the RNeasy Plus Universal Kit (Qiagen).
417
418 **RNA sequencing**
419 Total RNA samples were obtained as described above and submitted to the WSU Genomics Core
420 for RNA-seq analysis. RNA-seq and related bioinformatic analyses were performed following
421 our published protocols with modifications (*20*). Specifically, after alignment of FASTQ files to
422 the *C. elegans* reference genome (ce10, UCSC) using HISAT2 (*41*), gene expression
423 quantification and differential expression were analyzed using Cuffquant and Cuffdiff,
424 respectively (*42*). The raw sequence data (FASTQ files) were deposited in the National Center
425 for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database through the
426 Gene Expression Omnibus (GEO). The processed gene quantification files and differential
427 expression files were deposited in the GEO. All of these data can be accessed through the GEO
428 with the accession number GSE186202.
429
430 **Scanning electron microscopy (SEM) analysis**
431 To determine age- and temperature-dependent structural changes in the cuticle, we performed
432 SEM to examine wild-type and *npr-8 (ok1439)* animals at different ages (3-, 6-, and 9-day-old
433 adults) propagated at 20°C or 25°C. Temperature-specific worm maintenance and
434 synchronization were the same as described in the lifespan assay. Sample preparation and image
435 acquisition were performed as we previously described (*20*).
436

437 **RNA interference**

438 The collagen genes *col-77, col-49, col-139,* and *rol-1* were individually silenced using RNAi in
439 wild-type and *npr-8 (ok1439)* animals at 25°C. The temperature-specific worm cultivation and
440 synchronization steps were performed as described in the above lifespan assay section. The
441 RNAi protocol was performed as we previously described (*20*). The impact of collagen gene
442 silencing on *C. elegans* lifespan was assessed using lifespan assays.

443

444 **Statistical analysis**

445 For *C. elegans* lifespan assays, animal survival was plotted as a non-linear regression curve using
446 the PRISM computer program (version 9, GraphPad Software, Inc. La Jolla, CA). Lifespan
447 curves were considered different than the appropriate controls when p-values were < 0.05. Prism
448 uses the product limit or Kaplan-Meier method to calculate survival fractions and the logrank test
449 (equivalent to the Mantel-Heanszel test) to compare survival curves. All of the experiments were
450 repeated at least 3 times, unless otherwise indicated.

451

452

453 **References**

454 1. G. Keil, E. Cummings, J. P. de Magalhaes, Being cool: how body temperature influences ageing
455 and longevity. *Biogerontology* **16**, 383-397 (2015).
456 2. J. Loeb, J. H. Northrop, Is There a Temperature Coefficient for the Duration of Life? *Proceedings*
457 *of the National Academy of Sciences of the United States of America* **2**, 456-457 (1916).
458 3. J. W. Macarthur, W. H. Baillie, Metabolic activity and duration of life. I. Influence of temperature
459 on longevity in Daphnia magna. *Journal of Experimental Zoology* **53**, 221-242 (1929).
460 4. W. A. Van Voorhies, S. Ward, Genetic and environmental conditions that increase longevity in
461 Caenorhabditis elegans decrease metabolic rate. *Proceedings of the National Academy of*
462 *Sciences of the United States of America* **96**, 11399-11403 (1999).
463 5. R. L. Walford, R. K. Liu, Husbandry, life span, and growth rate of the annual fish, Cynolebias
464 adloffi E. Ahl. *Experimental Gerontology* **1**, 161-168 (1965).
465 6. B. Conti, M. Sanchez-Alavez, R. Winsky-Sommerer, M. C. Morale, J. Lucero, S. Brownell, V. Fabre,
466 S. Huitron-Resendiz, S. Henriksen, E. P. Zorrilla, L. de Lecea, T. Bartfai, Transgenic mice with a
467 reduced core body temperature have an increased life span. *Science* **314**, 825-828 (2006).
468 7. B. A. Rikke, T. E. Johnson, Lower body temperature as a potential mechanism of life extension in
469 homeotherms. *Exp Gerontol* **39**, 927-930 (2004).
470 8. L. A. Demetrius, Boltzmann, Darwin and Directionality theory. *Physics Reports* **530**, 1-85 (2013).
471 9. B. Kim, J. Lee, Y. Kim, S. V. Lee, Regulatory systems that mediate the effects of temperature on
472 the lifespan of Caenorhabditis elegans. *J Neurogenet* **34**, 518-526 (2020).
473 10. R. Xiao, B. Zhang, Y. Dong, J. Gong, T. Xu, J. Liu, X. Z. Xu, A genetic program promotes C. elegans
474 longevity at cold temperatures via a thermosensitive TRP channel. *Cell* **152**, 806-817 (2013).
475 11. B. Zhang, R. Xiao, E. A. Ronan, Y. He, A. L. Hsu, J. Liu, X. Z. Xu, Environmental Temperature
476 Differentially Modulates C. elegans Longevity through a Thermosensitive TRP Channel. *Cell*
477 *reports* **11**, 1414-1424 (2015).
478 12. S. J. Lee, C. Kenyon, Regulation of the longevity response to temperature by thermosensory
479 neurons in Caenorhabditis elegans. *Current biology : CB* **19**, 715-722 (2009).
480 13. Y. C. Chen, H. J. Chen, W. C. Tseng, J. M. Hsu, T. T. Huang, C. H. Chen, C. L. Pan, A C. elegans
481 Thermosensory Circuit Regulates Longevity through crh-1/CREB-Dependent flp-6 Neuropeptide
482 Signaling. *Developmental cell* **39**, 209-223 (2016).
483 14. M. Horikawa, S. Sural, A. L. Hsu, A. Antebi, Co-chaperone p23 regulates C. elegans Lifespan in
484 Response to Temperature. *PLoS genetics* **11**, e1005023 (2015).
485 15. D. Lee, S. W. A. An, Y. Jung, Y. Yamaoka, Y. Ryu, G. Y. S. Goh, A. Beigi, J. S. Yang, G. Y. Jung, D. K.
486 Ma, C. M. Ha, S. Taubert, Y. Lee, S. V. Lee, MDT-15/MED15 permits longevity at low temperature
487 via enhancing lipidostasis and proteostasis. *PLoS biology* **17**, e3000415 (2019).
488 16. Y. L. Chen, J. Tao, P. J. Zhao, W. Tang, J. P. Xu, K. Q. Zhang, C. G. Zou, Adiponectin receptor
489 PAQR-2 signaling senses low temperature to promote C. elegans longevity by regulating
490 autophagy. *Nat Commun* **10**, 2602 (2019).
491 17. H. J. Lee, A. Noormohammadi, S. Koyuncu, G. Calculli, M. S. Simic, M. Herholz, A. Trifunovic, D.
492 Vilchez, Prostaglandin signals from adult germ stem cells delay somatic aging of Caenorhabditis
493 elegans. *Nat Metab* **1**, 790-810 (2019).
494 18. H. Miller, M. Fletcher, M. Primitivo, A. Leonard, G. L. Sutphin, N. Rintala, M. Kaeberlein, S. F.
495 Leiser, Genetic interaction with temperature is an important determinant of nematode
496 longevity. *Aging Cell* **16**, 1425-1429 (2017).
497 19. C. Y. Ewald, J. N. Landis, J. Porter Abate, C. T. Murphy, T. K. Blackwell, Dauer-independent
498 insulin/IGF-1-signalling implicates collagen remodelling in longevity. *Nature* **519**, 97-101 (2015).

499 20. D. Sellegounder, Y. Liu, P. Wibisono, C. H. Chen, D. Leap, J. Sun, Neuronal GPCR NPR-8 regulates
500 *C. elegans* defense against pathogen infection. *Sci Adv* **5**, eaaw4717 (2019).
501 21. C. e. D. M. Consortium, large-scale screening for targeted knockouts in the Caenorhabditis
502 elegans genome. *G3 (Bethesda)* **2**, 1415-1425 (2012).
503 22. J. S. Satterlee, H. Sasakura, A. Kuhara, M. Berkeley, I. Mori, P. Sengupta, Specification of
504 thermosensory neuron fate in C. elegans requires ttx-1, a homolog of otd/Otx. *Neuron* **31**, 943-
505 956 (2001).
506 23. Y. V. Budovskaya, K. Wu, L. K. Southworth, M. Jiang, P. Tedesco, T. E. Johnson, S. K. Kim, An elt-
507 3/elt-5/elt-6 GATA transcription circuit guides aging in C. elegans. *Cell* **134**, 291-303 (2008).
508 24. G. C. Williams, Pleiotropy, natural selection, and the evolution of senescence. *Evolution* **11**, 14
509 (1957).
510 25. R. Lints, D. H. Hall, The cuticle. *in WormAtlas*, (2009).
511 26. Z. F. Altun, D. H. Hall, Introduction to *C. elegans* anatomy. *In WormAtlas*, (2009).
512 27. S. O'Riain, New and simple test of nerve function in hand. *Br Med J* **3**, 615-616 (1973).
513 28. T. M. Vasudevan, A. M. van Rij, H. Nukada, P. K. Taylor, Skin wrinkling for the assessment of
514 sympathetic function in the limbs. *Aust N Z J Surg* **70**, 57-59 (2000).
515 29. J. Myllyharju, K. I. Kivirikko, Collagens, modifying enzymes and their mutations in humans, flies
516 and worms. *Trends in genetics : TIG* **20**, 33-43 (2004).
517 30. B. H. Toyama, M. W. Hetzer, Protein homeostasis: live long, won't prosper. *Nature reviews.*
518 *Molecular cell biology* **14**, 55-61 (2013).
519 31. J. Varani, M. K. Dame, L. Rittie, S. E. Fligiel, S. Kang, G. J. Fisher, J. J. Voorhees, Decreased
520 collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast
521 function and defective mechanical stimulation. *Am J Pathol* **168**, 1861-1868 (2006).
522 32. C. Y. Ewald, J. I. Castillo-Quan, T. K. Blackwell, Untangling Longevity, Dauer, and Healthspan in
523 Caenorhabditis elegans Insulin/IGF-1-Signalling. *Gerontology* **64**, 96-104 (2018).
524 33. A. Bansal, L. J. Zhu, K. Yen, H. A. Tissenbaum, Uncoupling lifespan and healthspan in
525 Caenorhabditis elegans longevity mutants. *Proceedings of the National Academy of Sciences of*
526 *the United States of America* **112**, E277-286 (2015).
527 34. H. R. Choi, K. A. Cho, H. T. Kang, J. B. Lee, M. Kaeberlein, Y. Suh, I. K. Chung, S. C. Park,
528 Restoration of senescent human diploid fibroblasts by modulation of the extracellular matrix.
529 *Aging Cell* **10**, 148-157 (2011).
530 35. Y. Sun, W. Li, Z. Lu, R. Chen, J. Ling, Q. Ran, R. L. Jilka, X. D. Chen, Rescuing replication and
531 osteogenesis of aged mesenchymal stem cells by exposure to a young extracellular matrix.
532 *FASEB J* **25**, 1474-1485 (2011).
533 36. C. Y. Ewald, The Matrisome during Aging and Longevity: A Systems-Level Approach toward
534 Defining Matreotypes Promoting Healthy Aging. *Gerontology* **66**, 266-274 (2020).
535 37. A. C. Teuscher, E. Jongsma, M. N. Davis, C. Statzer, J. M. Gebauer, A. Naba, C. Y. Ewald, The in-
536 silico characterization of the Caenorhabditis elegans matrisome and proposal of a novel collagen
537 classification. *Matrix Biol Plus* **1**, 100001 (2019).
538 38. A. Sainio, H. Jarvelainen, Extracellular matrix-cell interactions: Focus on therapeutic applications.
539 *Cell Signal* **66**, 109487 (2020).
540 39. I. Boraschi-Diaz, J. Wang, J. S. Mort, S. V. Komarova, Collagen Type I as a Ligand for Receptor-
541 Mediated Signaling. *Front Phys-Lausanne* **5**, (2017).
542 40. C. Kumsta, T. T. Ching, M. Nishimura, A. E. Davis, S. Gelino, H. H. Catan, X. Yu, C. C. Chu, B. Ong,
543 S. H. Panowski, N. Baird, R. Bodmer, A. L. Hsu, M. Hansen, Integrin-linked kinase modulates
544 longevity and thermotolerance in C. elegans through neuronal control of HSF-1. *Aging Cell* **13**,
545 419-430 (2014).

546 41. D. Kim, B. Langmead, S. L. Salzberg, HISAT: a fast spliced aligner with low memory requirements.
547 *Nature methods* **12**, 357-360 (2015).
548 42. C. Trapnell, A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L. Salzberg, J. L.
549 Rinn, L. Pachter, Differential gene and transcript expression analysis of RNA-seq experiments
550 with TopHat and Cufflinks. *Nature protocols* **7**, 562-578 (2012).

551

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564
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566 analyzed data. S.N.P. and Y.L. wrote the paper.
567
568 **Competing interests:** The authors declare that they have no competing interests.
569
570 **Data and materials availability:** The RNA sequencing data have been deposited in NCBI's
571 Sequence Read Archive (SRA) database through the Gene Expression Omnibus (GEO).
572 Processed gene quantification files and differential expression files have been deposited in GEO.
573 All of these data can be accessed through the GEO with the accession number GSE186202. All
574 data needed to evaluate the conclusions in the paper are present in the paper and/or the
575 Supplementary Materials. Additional data are available from the authors upon request. The *C.*

576 *elegans* strains and plasmids constructed by us and the primer sequences used for their

577 construction are available upon request.

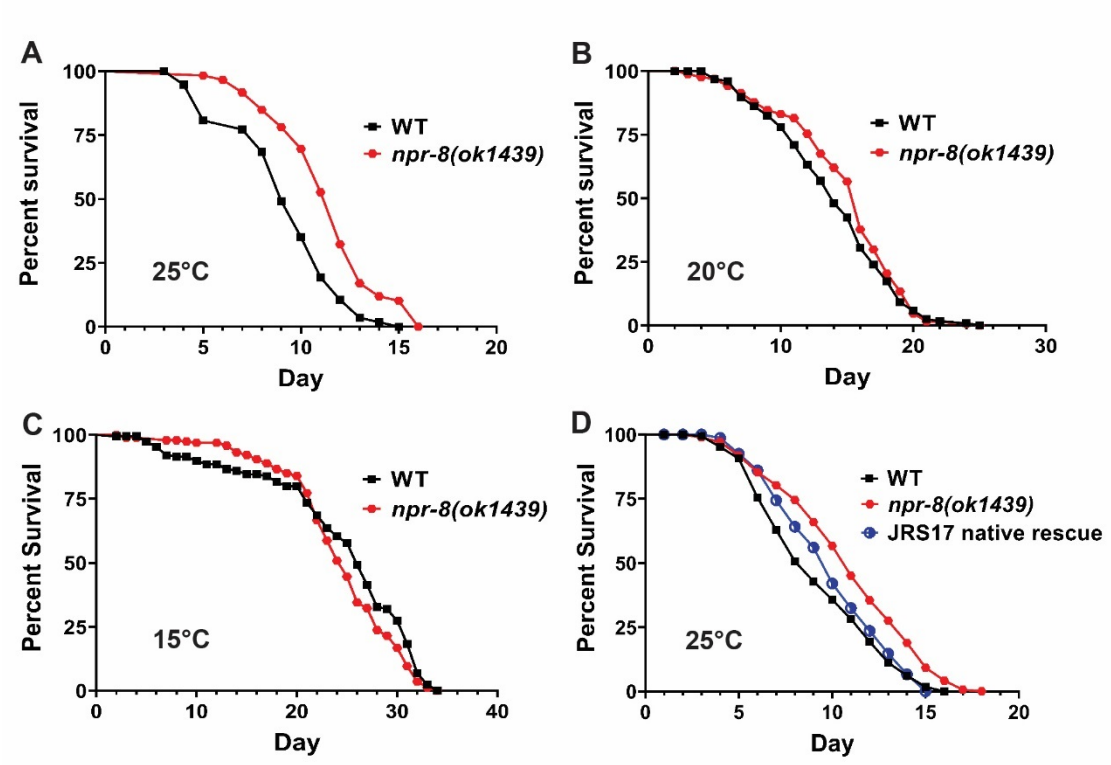
578

579 **Figures and Tables**

580

581

582 **Fig. 1**
583



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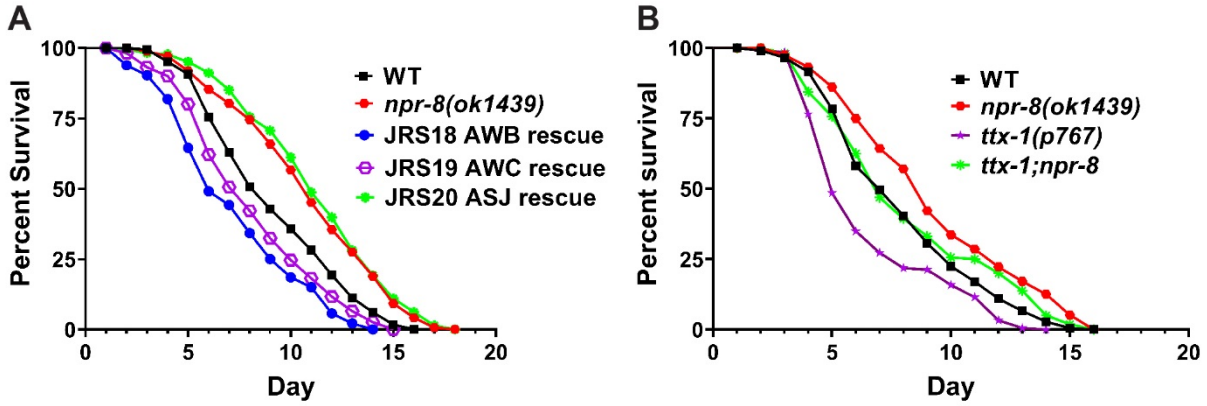
585

586 **Fig. 1. Functional loss of NPR-8 extends lifespan at 25°C but not at 20°C or 15°C.** WT and
587 *npr-8(ok1439)* animals were grown on *E. coli* strain OP50 at 25°C **(A)**, 20°C **(B)**, or 15°C **(C)**,
588 and scored for survival over time. The graphs are the combined results of three independent
589 experiments. Each experiment included $n = 60$ adult animals per strain. p-values represent the
590 significance level of the mutants relative to the WT, $p < 0.0001$ in (A), $p = 0.1640$ in (B), and $p =$
591 0.1126 in (C). **(D)** WT, *npr-8(ok1439)*, JRS17 (*npr-8* expression restored in *npr-8(ok1439)* under
592 its native promoter) animals were grown on *E. coli* strain OP50 at 25°C and scored for survival
593 over time. The graphs are the combined results of three independent experiments. Each
594 experiment included $n = 60$ adult animals per strain. p-values are relative to *npr-8(ok1439)*: WT,
595 $p < 0.0001$; JRS17, $p < 0.0001$.

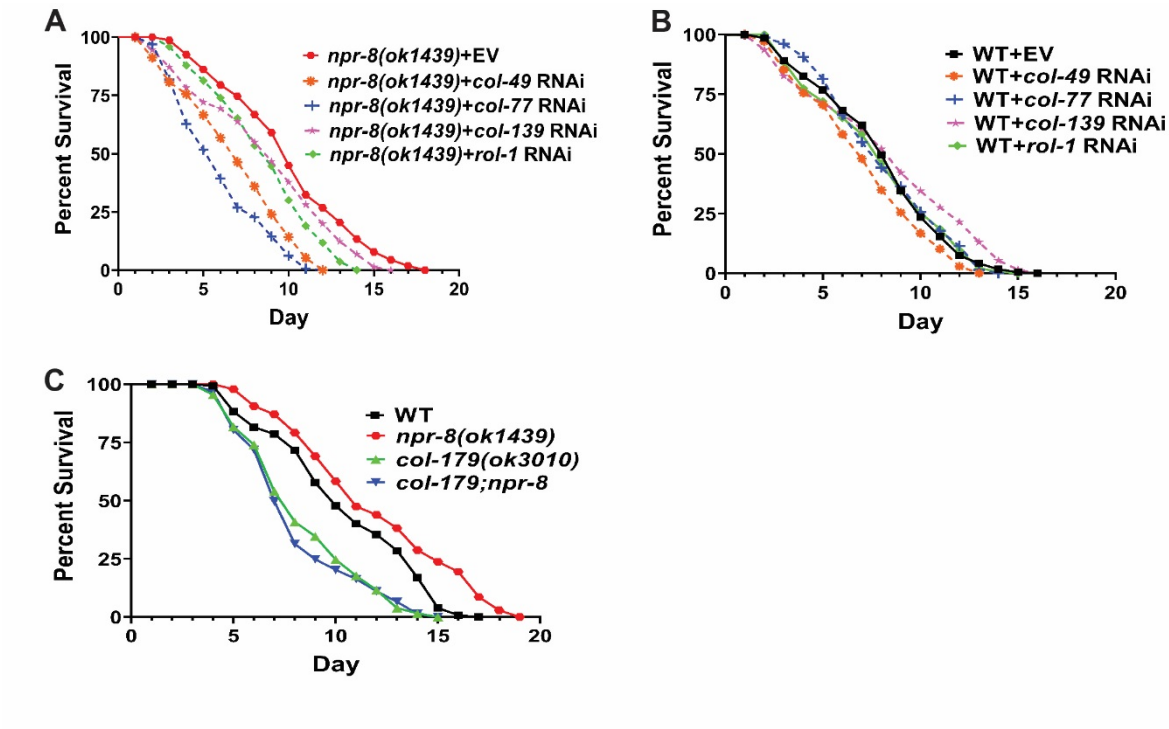
596 **Fig. 2**
597
598



599
600

601 **Fig. 2. The NPR-8-dependent longevity response to warm temperature is neurally**
602 **regulated. (A)** WT, *npr-8 (ok1439)*, and rescue animals were grown at 25°C and scored for
603 survival over time. JRS18, *npr-8* expression rescued in AWB neurons; JRS19, *npr-8* expression
604 rescued in AWC neurons; and JRS20, *npr-8* expression rescued in ASJ neurons. The graphs are
605 the combined results of three independent experiments. Each experiment included $n = 60$ adult
606 animals per strain. p-values are relative to *npr-8 (ok1439)*: WT, $p < 0.0001$; JRS18, $p < 0.0001$;
607 JRS19, $p < 0.0001$; JRS20, $p = 0.3476$. **(B)** WT, *npr-8(ok1439)*, *ttx-1(p767)*, *ttx-1;npr-8* animals
608 were grown at 25°C and scored for survival over time. The graphs are the combined results of
609 three independent experiments. Each experiment included $n = 60$ adult animals per strain. p-
610 values relative to *npr-8(ok1439)*: WT, $p = 0.0001$; *ttx-1(p767)*, $p < 0.0001$; *ttx-1;npr-8*, $p =$
611 0.0101. p-values relative to WT: *ttx-1(p767)*, $p < 0.0001$; *ttx-1;npr-8*, $p = 0.1872$.

612 **Fig. 3**
613
614



615
616 **Fig. 3. NPR-8-regulated collagen genes are required for warm temperature-induced**
617 **lifespan extension in *npr-8(ok1439)* animals.** *npr-8(ok1439)* animals **(A)** and WT animals **(B)**
618 were grown on dsRNA for empty vector (EV) or for *rol-1*, *col-49*, *col-77*, or *col-139* at 25°C and
619 scored for survival over time. The graphs are the combined results of three independent
620 experiments. Each experiment included n = 60 adult animals per strain. p-values are relative to
621 *npr-8(ok1439)*+EV in (A): *npr-8(ok1439)+col-49*, p <0.0001; *npr-8(ok1439)+col-77*, p <0.0001;
622 *npr-8(ok1439)+col-139*, p = 0.0009; *npr-8 (ok1439)+rol-1*, p <0.0001. p-values are relative to
623 WT+EV in (B): WT+*col-49*, p = 0.0039; WT+*col-77*, p = 0.9225; WT+*col-139*, p =0.0261;
624 WT+*rol-1*, p = 0.8383. **(C)** WT, *npr-8(ok1439), col-179(ok3010), npr-8;col-179* animals were
625 grown at 25°C and scored for survival over time. The graphs are the combined results of three
626 independent experiments. Each experiment included n = 60 adult animals per strain. p-values
627 relative to *npr-8(ok1439)*: WT, p < 0.0001; *col-179(ok3010)*, p < 0.0001; *npr-8;col-179*, p <
628 0.0001. p-values relative to WT: *col-179(ok3010)*, p < 0.0001; *npr-8;col-179*, p < 0.0001.
629

Fig. 4

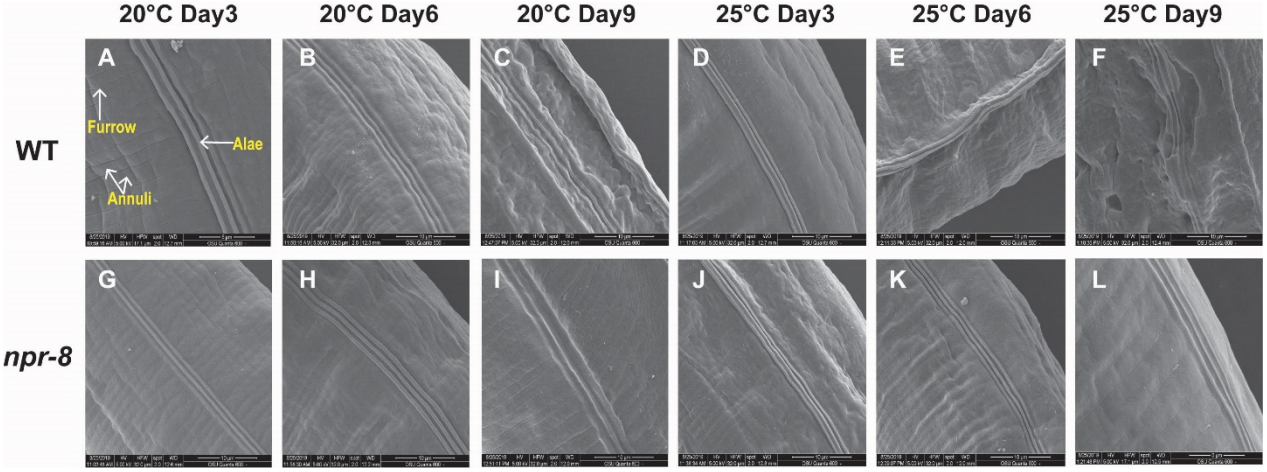


Fig. 4. The cuticle of *npr-8(ok1439)* animals was less wrinkled than that of WT animals at old ages at 20°C and 25°C. Adult animals were collected at days 3, 6 and 9 and imaged using FEI Quanta 600 FEG scanning electron microscope.

638 **Table 1. Grouping of RNA-seq samples**

639

Sample Group	Strain	Propagation Temperature	Adult Age
1	*N2*	20^0C	young adult (0-day)
2	*N2*	20^0C	9-day
3	*N2*	25^0C	0-day
4	*N2*	25^0C	9-day
5	*npr-8(ok1439)*	20^0C	0-day
6	*npr-8(ok1439)*	20^0C	9-day
7	*npr-8(ok1439)*	25^0C	0-day
8	*npr-8(ok1439)*	25^0C	9-day

640

641

642 **Table 2. Summary of RNA-seq analysis**

643

Influencing factor	Comparison	Sample group	Number of regulated genes	Gene ontology (GO) analysis		Data location
Effects of temperature on gene expression	0-day-old wild-type animals at 25^0C relative to 20^0C	Group 3 vs Group 1	2,265 genes upregulated	340 enriched biological processes	cell cycle, reproduction, metabolic/biosynthetic processes, organelle organization, development, DNA replication/repair/transcription/recombination, and other processes	Table S1
			5,069 genes downregulated	68 attenuated biological processes	signaling, phosphorylation/dephosphorylation, immune response, ion/transmembrane transport, and other processes	Table S2 Table S3
Effects of aging on gene expression	wild-type animals at 20^0C day 9 relative to day 0	Group 2 vs Group 1	3,792 genes upregulated	428 enriched biological processes	cell cycle, reproduction/development, metabolism, organelle organization, DNA replication/repair/transcription, and other processes	Table S4
			4,937 genes downregulated	56 attenuated biological processes	ion/transmembrane transport, phosphorylation/dephosphorylation, signaling, immune response, and other processes	Table S5
				65 attenuated molecular functions	"structural constituent of cuticle" is the most significantly reduced	Table S6
Effects of *npr-8* mutation on gene expression	0-day-old *npr-8(ok1439)* animals at 20^0C relative to wild-type controls	Group 5 vs Group 1	643 genes upregulated	7 enriched molecular functions	"structural constituent of cuticle" is the most significantly enriched	Table S7
			272 genes downregulated	No attenuated GO terms		
	9-day-old *npr-8(ok1439)* animals at 20^0C relative to wild-type controls	Group 6 vs Group 2	1,487 genes upregulated	33 enriched biological processes	phosphorylation/dephosphorylation, metabolic process	Table S8
			1,305 genes downregulated	13 attenuated molecular functions	"structural constituent of cuticle" is the most significantly reduced	Table 3
	0-day-old *npr-8(ok1439)* animals at 25^0C relative to wild-type controls	Group 7 vs Group 3	1,482 genes upregulated	17 enriched molecular functions	"structural constituent of cuticle" is the most significantly enriched	Table S9
			419 genes downregulated	No attenuated GO terms		
	9-day-old *npr-8(ok1439)* animals at 25^0C relative to wild-type controls	Group 8 vs Group 4	1,307 genes upregulated	29 enriched biological processes	phosphorylation/dephosphorylation, metabolic process	Table S10
			631 genes downregulated	No attenuated GO terms		

644

645

646

647 **Table 3. GO analysis of downregulated genes in 9-day-old adult *npr-8(ok1439)* animals**
648 **grown at 20°C relative to wild-type control animals**

649 **(A) Attenuated molecular functions**

GO term	Description	P-value[#]	FDR q-value*	Enrichment (N, B, n, b)[§]
GO:0042302	structural constituent of cuticle	4.74E-49	1.22E-45	7.67 (10804,131,785,73)
GO:0005198	structural molecule activity	9.30E-22	1.20E-18	3.19 (10804,349,785,81)
GO:0008239	dipeptidyl-peptidase activity	5.97E-06	5.12E-03	8.03 (10804,12,785,7)
GO:0005201	extracellular matrix structural constituent	3.81E-05	2.45E-02	4.95 (10804,25,785,9)
GO:0000978	RNA polymerase II proximal promoter sequence-specific DNA binding	5.50E-05	2.83E-02	1.94 (10804,269,785,38)
GO:0030246	carbohydrate binding	6.65E-05	2.85E-02	2.23 (10804,167,785,27)
GO:0000987	proximal promoter sequence-specific DNA binding	7.01E-05	2.57E-02	1.92 (10804,272,785,38)
GO:0000977	RNA polymerase II regulatory region sequence-specific DNA binding	7.47E-05	2.40E-02	1.82 (10804,333,785,44)
GO:0001012	RNA polymerase II regulatory region DNA binding	7.47E-05	2.13E-02	1.82 (10804,333,785,44)
GO:0016634	oxidoreductase activity, acting on the CH-CH group of donors, oxygen as acceptor	9.31E-05	2.39E-02	8.60 (10804,8,785,5)
GO:0016491	oxidoreductase activity	1.45E-04	3.39E-02	1.62 (10804,502,785,59)
GO:0097602	cullin family protein binding	1.93E-04	4.14E-02	4.59 (10804,24,785,8)
GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific	2.04E-04	4.03E-02	1.77 (10804,327,785,42)

650 **(B) Downregulated genes related to the reduced cuticle structure activity**

Genes	Fold change	Adjusted *P* value[Ψ]	Genes	Fold change	Adjusted *P* value[Ψ]
rol-1	15.3	1.73E-02	col-125	4.7	1.61E-04
col-139	9.3	1.61E-04	col-101	4.5	1.61E-04
col-73	9.2	1.61E-04	col-144	4.2	1.61E-04
col-146	8.9	3.24E-02	col-169	4.2	1.61E-04
col-77	8.3	1.61E-04	col-41	4.2	5.99E-04
col-179	8.1	1.61E-04	sqt-3	4	1.61E-04
col-49	7.9	1.61E-04	col-103	3.9	1.61E-04
dpy-13	7.7	2.89E-02	col-10	3.8	1.61E-04
col-129	7.6	1.61E-04	col-122	3.7	1.61E-04
col-156	7.5	2.86E-02	sqt-2	3.7	1.61E-04
col-20	7.5	1.61E-04	col-161	3.7	1.61E-04
bli-6	7.4	1.61E-04	col-181	3.6	1.61E-04
col-138	7.4	1.61E-04	col-143	3.6	1.61E-04
col-124	7.3	1.61E-04	col-184	3.6	1.61E-04
col-90	7.2	1.61E-04	Y69H2.14	3.5	1.61E-04
dpy-4	7.2	1.61E-04	col-166	3.4	1.61E-04
col-147	7.2	1.61E-04	col-110	3.4	1.08E-02
col-140	7.2	1.61E-04	cut-2	3.4	1.61E-04
col-168	7.1	1.61E-04	col-145	3	1.61E-04

dpy-5	7	1.61E-04	col-12	3	1.61E-04
col-119	7	1.61E-04	col-155	2.9	1.61E-04
col-159	6.9	1.61E-04	col-88	2.8	1.61E-04
col-80	6.7	1.61E-04	col-14	2.8	1.61E-04
col-160	6.6	1.61E-04	col-99	2.8	1.61E-04
col-178	6.4	1.61E-04	col-54	2.6	1.80E-02
rol-6	6.3	1.61E-04	col-65	2.5	5.99E-04
rol-8	6.3	1.61E-04	col-153	2.4	1.61E-04
col-142	6.3	1.61E-04	let-653	2.4	1.61E-04
col-92	6.2	4.70E-02	col-118	2.2	1.61E-04
col-94	6.2	4.42E-02	dpy-8	2.2	1.61E-04
col-19	5.8	1.61E-04	col-173	2.1	4.38E-03
col-133	5.7	1.61E-04	col-176	2	5.44E-03
col-149	5.5	1.61E-04	col-157	2	3.12E-04
col-167	5.2	1.61E-04	col-62	2	8.26E-03
col-170	5.2	9.91E-03	col-130	2	3.07E-02
col-13	5.1	1.02E-02	col-150	2	2.18E-03
col-180	4.8	1.61E-04			

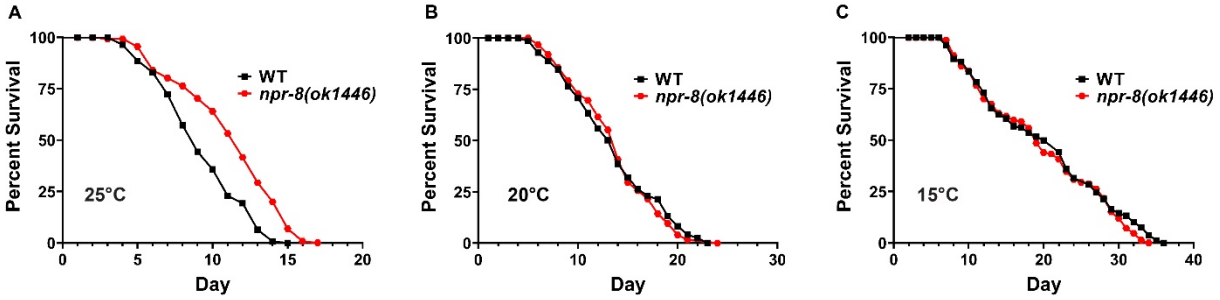
[#] P-value is computed according to the mHG model (Eden *et al.* 2007 PLoS Comp Bio 3(3):e39). * FDR q-value is the correction of the above p-value for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg 1995 J R Statist Soc B 57(1):289-300). [§] Enrichment (N, B, n, b) is defined as follows: N - total number of genes; B - total number of genes associated with a specific GO term; n - number of genes in the target set; b - number of genes in the intersection;Enrichment = (b/n) / (B/N). [Ψ]Adjusted *P* value is the correction of the P value for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg 1995 J R Statist Soc B 57 (1):289–300).

659 **Supplementary Materials**
660
661 **Fig. S1. *npr-8(ok1446)* animals live longer than WT animals at 25^{0}C but not at 20^{0}C or**
662 **15^{0}C.**
663
664 **Fig. S2. NPR-8 rescue animals exhibited lifespan similar to that of wild-type animals at**
665 **20°C.**
666
667 **Table S1. Enriched biological processes in 0-day-old adult wild-type animals at 25°C vs**
668 **20°C**
669
670 **Table S2. Attenuated biological processes in 0-day-old adult wild-type animals at 25°C vs**
671 **20°C**
672
673 **Table S3. Downregulated genes contributing to the attenuated signaling processes in 0-day-**
674 **old adult wild-type animals at 25°C vs 20°C**
675
676 **Table S4. Enriched biological processes in 9-day-old wild-type animals at 20°C vs 0-day-old**
677 **adult animals**
678
679 **Table S5. Attenuated biological processes in 9-day-old wild-type animals at 20°C vs 0-day-**
680 **old adult animals**
681
682 **Table S6. Gene ontology (GO) analysis of downregulated genes in 9-day-old wild-type**
683 **animals grown at 20°C relative to 0-day-old adult animals**
684
685 **Table S7. GO analysis of upregulated genes in 0-day-old adult *npr-8(ok1439)* animals**
686 **grown at 20°C relative to wild-type animals**
687
688 **Table S8. Enriched biological processes in 9-day-old *npr-8(ok1439)* animals grown at 20°C**
689 **vs wild-type animals**
690
691 **Table S9. GO analysis of upregulated genes in 0-day-old adult *npr-8(ok1439)* animals**
692 **grown at 25°C relative to wild-type animals**
693
694 **Table S10. Enriched biological processes in 9-day-old *npr-8(ok1439)* animals grown at 25°C**
695 **vs wild-type animals**
696
697

698 **Fig. S1.**

699

700

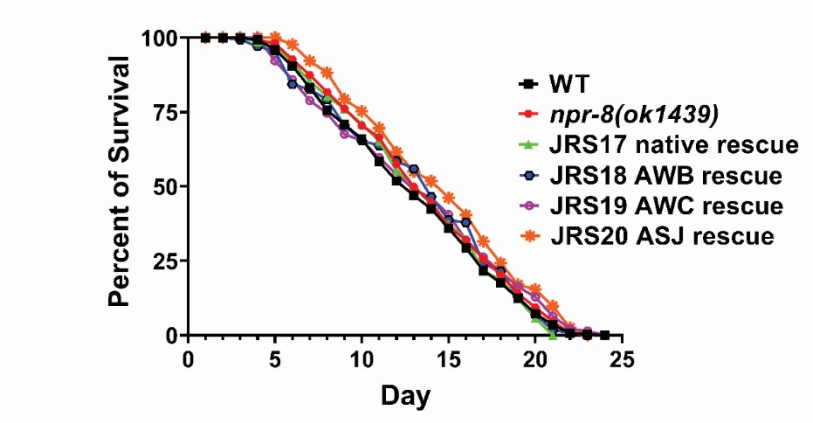


701
702

703 **Fig. S1. *npr-8(ok1446)* animals live longer than WT animals at 25°C but not at 20°C or 15°C.** WT and *npr-*
704 *8(ok1446)* animals were grown on *E. coli* strain OP50 at 25°C **(A)**, 20°C **(B)**, or 15°C **(C)**, and scored for survival
705 over time. The graphs are the combined results of three independent experiments. Each experiment included $n = 60$
706 adult animals per strain. p-values represent the significance level of the mutants relative to the WT, $p < 0.0001$ in
707 (A), $p = 0.8114$ in (B), and $p = 0.2202$ in (C).

708 **Fig. S2.**

709



710
711

712 **Fig. S2. NPR-8 rescue animals exhibited lifespan similar to that of wild-type animals at**
713 **20°C.** WT, *npr-8 (ok1439)*, and rescue animals were grown at 20°C and scored for survival over
714 time. JRS17, *npr-8* expression restored in *npr-8(ok1439)* under its native promoter; JRS18, *npr-8*
715 expression rescued in AWB neurons; JRS19, *npr-8* expression rescued in AWC neurons; and
716 JRS20, *npr-8* expression rescued in ASJ neurons. The graphs are the combined results of three
717 independent experiments. Each experiment included $n = 60$ adult animals per strain. p-values are
718 relative to WT: *npr-8 (ok1439)*, $p = 0.3424$; JRS17, $p = 0.9600$; JRS18, $p = 0.6348$; JRS19, $p =$
719 0.9119; JRS20, $p = 0.0066$.

720
721
722

Table S1 – S10

https://pounce.spokane.wsu.edu/index.php/s/zrDyryXGq32pe85