

1           **Conserved nuclear receptors controlling a novel trait**  
2           **target fast-evolving genes expressed in a single cell**

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16 **Abstract**

17

18 Environment shapes development through a phenomenon called developmental plasticity.  
19 Deciphering its genetic basis has implications for understanding evolution and adaptation to  
20 novel environments, yet molecular studies are scarce. Here, we expanded the gene regulatory  
21 network controlling predatory vs. non-predatory morphology in the nematode *Pristionchus*  
22 *pacificus*. First, we isolated a mutant in the nuclear hormone receptor *nhr-1* with a previously  
23 unseen phenotypic effect. It disrupts mouth-form determination and results in animals  
24 combining features of both wild-type morphs. Further, we identified common targets of NHR-  
25 1 and the previously identified nuclear hormone receptor NHR-40 through transcriptomics.  
26 Unlike their highly conserved regulators, the target genes have no orthologs in *Caenorhabditis*  
27 *elegans* and likely result from lineage-specific expansions. An array of transcriptional reporters  
28 revealed co-expression of all tested targets in the same pharyngeal gland cell. The  
29 morphological remodeling of this cell accompanied the evolution of teeth and predation, linking  
30 rapid gene turnover with morphological innovations.

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34 **Keywords**

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36 Developmental plasticity, nuclear hormone receptors, *Pristionchus pacificus*, gene regulatory  
37 networks, switch genes, Astacins, evolutionary novelty.

## 38 **Introduction**

39

40           Developmental plasticity is the ability to generate different phenotypes in response to  
41 environmental input<sup>1</sup>. As a result, even genetically identical individuals may develop distinct  
42 phenotypes, the most extreme example being castes in social insects<sup>2</sup>. Developmental  
43 plasticity is attracting considerable attention in the context of adaptation to climate change<sup>3-6</sup>  
44 and as a facilitator of evolutionary novelty<sup>7-11</sup>. However, the role of plasticity in evolution has  
45 been contentious<sup>6,12</sup> because the genetic and epigenetic underpinnings of plastic traits have  
46 long remained elusive. Nonetheless, recent studies have begun to elucidate associated  
47 molecular mechanisms in insects and nematodes<sup>13-16</sup>. Ultimately, the identification of gene  
48 regulatory networks (GRN) controlling plasticity will provide an understanding of development  
49 in novel environments and enable testing theories about the long-term evolutionary  
50 significance of plasticity.

51           The free-living nematode *Pristionchus pacificus* has recently been established as a  
52 model to study plasticity<sup>13</sup>. These worms can develop two alternative mouth forms, called  
53 eury stomatous (Eu) and stenostomatous (St) mouth forms, respectively. Eu morphs have a  
54 wide buccal cavity and two large opposed teeth enabling predation on other nematodes, while  
55 St morphs have a narrow buccal cavity and one tooth limiting their diet to microbial sources<sup>17,18</sup>  
56 (Fig. 1A-C, SFig. 1A). The wild-type *P. pacificus* strain PS312 preferentially forms Eu morphs  
57 in standard culture conditions on agar plates, but becomes predominantly St in liquid culture<sup>19</sup>.  
58 Additionally, nematode-derived modular metabolites excreted by adult animals induce the  
59 predatory Eu morph<sup>20,21</sup>. A forward genetic screen identified the sulfatase gene *eud-1* as a  
60 developmental switch confirming long-standing predictions that plastic traits are regulated by  
61 binary switches<sup>18</sup>. Subsequent studies implicated several other enzyme-encoding genes, such  
62 as *nag-1*, *nag-2*, and *sult-1/seud-1* in regulating mouth-form plasticity<sup>22-25</sup>. Additionally, the  
63 chromatin modifier genes *lsy-12* and *mbd-2* influence *eud-1* expression<sup>26</sup>. In contrast, only  
64 one transcription factor, the nuclear hormone receptor (NHR) NHR-40, was so far found to  
65 regulate mouth-form fate<sup>27</sup>, and no downstream targets have been identified (Fig. 1D).

66           Here, we leveraged the power of suppressor screen genetics to identify the conserved  
67 nuclear hormone receptor NHR-1 as a second transcription factor controlling mouth-form  
68 development. It differs from *nhr-40* and all the other genes identified to date in that *nhr-1*  
69 mutants develop a morphology that combines features of the two morphs, consistent with  
70 disrupted mouth-form determination. Furthermore, transcriptomic profiling revealed that NHR-  
71 40 and NHR-1 share transcriptional targets, which exhibit functional redundancy and are  
72 expressed in a single pharyngeal gland cell, g1D. This cell has undergone extreme  
73 morphological remodeling in nematode evolution, which is associated with the emergence of  
74 teeth and predatory feeding. Interestingly, *nhr-1* and *nhr-40* are well conserved, whereas all  
75 target genes are rapidly evolving and have no orthologs in *C. elegans*. This study enhances  
76 the understanding of the GRN regulating mouth-form plasticity, elucidates the evolutionary  
77 dynamics of underlying genes and links morphological innovations with rapid gene evolution.

78 **Results and Discussion**

79

80 **Suppressor screen in *nhr-40* identifies another NHR gene regulating mouth-form**  
81 **development**

82 While our previous studies have identified various components involved in the  
83 regulation of mouth form plasticity, most of these genes are expressed in neurons responsible  
84 for environmental sensing and we are yet to find factors acting in the tissues forming the mouth  
85 structure. Therefore, we looked for more downstream factors by conducting a suppressor  
86 screen in the mutant background of *nhr-40*. This is the most downstream gene in the current  
87 GRN controlling *P. pacificus* mouth-form plasticity and it encodes a transcription factor<sup>27</sup>. We  
88 mutagenized *nhr-40(tu505)* worms, which are all-Eu, and isolated one allele, *tu515*, that had  
89 a no-Eu phenotype (Fig 1E, Table 1). The phenotype was fully penetrant, both in the presence  
90 of *nhr-40(tu505)* and after outcrossing, *i.e.* Eu animals were never observed under any culture  
91 condition. Thus, *tu515* represents a novel factor influencing the mouth-form ratio. Interestingly,  
92 however, *tu515* mutants also exhibited a non-canonical mouth morphology (Fig. 1A, SFig. 1A,  
93 SFig. 3). In contrast to all previously isolated mutants, which either display altered mouth-form  
94 frequencies or an aberrant morphology, *tu515* individuals develop a morphology that  
95 combines normal features of the two morphs with no apparent dimorphism. Specifically, *tu515*  
96 mutants closely resemble the St morph in that they have a flattened dorsal tooth, lack a fully  
97 developed right ventrosublateral tooth, and the anterior tip of the promesostegostom aligns  
98 with the anterior tip of the gymnostom plate. However, the width of the mouth and the curvature  
99 of the dorsal tooth appear intermediate between Eu and St, and the right ventrosublateral ridge  
100 is frequently enlarged and resembles an underdeveloped tooth of the Eu morph (Fig. 1A, SFig.  
101 1A). Therefore, while other known mutants affect mouth-form determination by changing the  
102 preferred developmental trajectory, *tu515* is the first mutant that disrupts determination,  
103 resulting in non-canonical morphology that resembles the St morph but combines features of  
104 both morphs.

105 To map *tu515*, we performed bulk segregant analysis. We examined the list of non-  
106 synonymous and nonsense mutations within the candidate region on the X chromosome  
107 (SFig. 2B, STable 1) and discovered a non-synonymous mutation in another NHR-encoding  
108 gene, *nhr-1*. The substitution changed the sequence of a highly conserved FFRR motif within  
109 the DNA recognition helix<sup>28</sup> to FFRW, which may cause the loss of DNA-binding activity. We  
110 performed the following experiments to verify that *nhr-1* is the suppressor of *nhr-40(tu505)*.  
111 First, we created *nhr-1* mutants using CRISPR/Cas9 by generating frameshift mutations at the  
112 beginning of the ligand-binding domain (LBD). The resulting alleles *tu1163* and *tu1164*  
113 exhibited a no-Eu phenotype and the same morphological abnormalities as *tu515* (Fig. 1A,  
114 SFig. 1A, Table 1). Second, we crossed the *tu1163* and *tu515* mutants and established that  
115 *tu1163/tu515* trans-heterozygotes were no-Eu showing that the two mutants do not  
116 complement each other (Table 1). Third, we overexpressed the complementary DNA (cDNA)  
117 of *nhr-1* driven by the *nhr-1* promoter region in the *nhr-1(tu1163)* mutant background and  
118 obtained an almost complete rescue (Table 1). Fourth, we crossed *nhr-1(tu1163)* with *nhr-*  
119 *40(tu505)* and observed a highly penetrant no-Eu phenotype in double mutant animals, similar  
120 to the phenotype of *tu515 nhr-40(tu505)* mutants (Table 1). Taken together, frameshift alleles  
121 of *nhr-1* and the original suppressor allele *tu515* exhibit the same phenotype, do not  
122 complement each other, and have identical epistatic interactions with *nhr-40(tu505)*.  
123 Therefore, we conclude that *nhr-1* is the suppressor of *nhr-40(tu505)*.

124

### 125 **Reverse genetic analysis of *nhr-40* results in all-stenostomatous mutants**

126 The available alleles of *nhr-1* and *nhr-40* have different phenotypes with regard to  
127 mouth-form frequency and morphology. This is surprising because NHRs often form  
128 heterodimers<sup>29</sup>, in which case *loss-of-function* phenotypes of interacting partners are identical.  
129 Two different hypotheses could explain our observations. First, *nhr-1* and *nhr-40* may indeed  
130 have different functions. Second, the three available alleles of *nhr-40* (*tu505*, *iub6*, *iub5*), all of  
131 which are non-synonymous substitutions outside of the DNA-binding domain (DBD)<sup>27</sup>, may  
132 represent *gain-of-function* alleles. Our previous analysis had suggested that these alleles are

133 *loss-of-function* based on the phenotype of *nhr-40* overexpression, which resulted in all-St  
134 animals<sup>27</sup>. However, we recently realized that in *C. elegans*, overexpression of *Cel-nhr-40* and  
135 *loss-of-function* of *Cel-nhr-40* induced by RNAi and a deletion mutation all cause similar  
136 developmental defects<sup>30</sup>. This may occur if NHR-40 inhibits its own transcription<sup>31</sup> or if the  
137 concatenated coding sequence of the rescue construct acts as a substrate to induce RNAi<sup>30</sup>.  
138 Therefore, we investigated *nhr-40* in *P. pacificus* further, and generated nonsense alleles  
139 using CRISPR/Cas9.

140 We introduced mutations in two different locations in *nhr-40* (Fig. 2A). The alleles  
141 *tu1418* and *tu1419* truncate the DBD. The *tu1420* allele contains a frameshift at the beginning  
142 of the LBD while leaving the DBD intact. We phenotyped the newly obtained mutants in liquid  
143 S-medium, which represses the Eu morph, and on agar plates, which induces it<sup>19</sup>. All  
144 frameshift alleles had a completely penetrant all-St phenotype in both culture conditions, which  
145 is opposite to the original ethyl methanesulfonate (EMS) alleles (Table 1). The newly obtained  
146 *nhr-40* mutants displayed no morphological abnormalities seen in *nhr-1* mutants. Additionally,  
147 we created a *null* allele, *tu1423*, which contains a 13 kb deletion or rearrangement of the locus  
148 (SFig. 2A). This *null* allele again had a completely penetrant all-St phenotype and showed no  
149 morphological abnormalities (Table 1). To eliminate the possibility that the phenotype of the  
150 EMS mutants was caused by random mutations outside *nhr-40*, we introduced a nucleotide  
151 substitution identical to *iub6* via homology-directed repair (Fig. 2A). Indeed, the two resulting  
152 alleles, *tu1421* and *tu1422*, had an all-Eu phenotype, identical to that of *iub6* and other EMS  
153 alleles, and opposite to that of the frameshift alleles (Table 1). Thus, frameshift mutations in  
154 DBD, LBD, and the deletion/rearrangement of the entire gene have an opposite phenotype to  
155 that of the three previously isolated non-synonymous substitutions. We conclude that *tu505*,  
156 *iub6*, *iub5*, *tu1421* and *tu1422* are *gain-of-function* alleles.

157

## 158 **NHR-40 and NHR-1 interact post-transcriptionally**

159 In GRNs, transcription factors may activate or repress each other transcriptionally<sup>32–36</sup>,  
160 or alternatively, they may interact at the post-transcriptional level. The latter includes indirect

161 interactions, such as independent binding to the same promoters<sup>37</sup>, or ligand-mediated  
162 interactions<sup>38</sup>. To distinguish if *nhr-1* and *nhr-40* interact at the transcriptional or post-  
163 transcriptional level, we analyzed the transcriptomes of wild type, *nhr-1 loss-of-function*, *nhr-  
164 40 loss-of-function* and *nhr-40 gain-of-function* mutants at two developmental stages (Fig. 3A).  
165 RNA collected from J2-J4 larvae is enriched with transcripts expressed at the time of mouth-  
166 form determination, as environmental manipulation during this time window affects morph  
167 frequency<sup>39</sup>. RNA collected from J4 larvae and adults is enriched with transcripts expressed  
168 at the time of mouth-form differentiation, because cuticularized mouthparts that distinguish the  
169 two morphs are believed to be secreted during the J4-adult molt<sup>40</sup>. We found that at both time  
170 points, *nhr-40* transcript levels were not affected by *loss-of-function* of *nhr-1*. Similarly, *nhr-1*  
171 transcript levels were not affected by *loss-of-function* of *nhr-40*, although they were slightly,  
172 but not significantly increased by *nhr-40 gain-of-function* (Fig. 2B). Thus, at the transcriptional  
173 level, both *nhr* genes remain unaffected by the *loss-of-function* of the other *nhr* gene.  
174 Therefore, NHR-40 and NHR-1 may interact at the post-transcriptional level, although the  
175 possibility remains that their transcriptional interaction in specific cells is masked in whole-  
176 animal transcriptome data. The lack of linear transcriptional regulation is consistent with  
177 different phenotypic effects of *nhr-40* and *nhr-1*.

178

### 179 ***nhr-40* and *nhr-1* are expressed at the site of polyphenism**

180 Next, we wanted to determine the expression pattern of *nhr-1* and *nhr-40* and test if  
181 they were co-expressed. We took three complementary approaches to establish the  
182 expression pattern of *nhr-1*. First, we created transcriptional reporters comprising the  
183 presumptive promoter region upstream of the potential start site in the second exon fused with  
184 TurboRFP or Venus. The resulting expression pattern was broad with the strongest expression  
185 in the head, including both muscle and gland cells of the pharynx, and what may be the  
186 hypodermal and arcade cells (Fig. 2D, SFig. 1B). Second, we performed antibody staining  
187 against an HA epitope tag in the *nhr-1* rescue line described above. We observed a similar  
188 expression pattern that was predictably localized to the nuclei (Fig. 2C). Finally, we used



189 CRISPR/Cas9 to “knock in” an HA tag in the endogenous *nhr-1* locus at the C-terminus of the  
190 coding sequence. Antibody staining against HA revealed a similar expression pattern but with  
191 a weaker signal due to the lower number of copies of endogenous DNA (SFig. 1C). Together,  
192 these results show that NHR-1 localizes to nuclei of multiple cells in the head region, with  
193 strong expression in pharyngeal muscle cells, which presumably secrete structural  
194 components of the teeth.

195 To explore whether NHR-40 and NHR-1 are expressed in overlapping tissues, we  
196 created a double reporter line, in which the *nhr-40* promoter is fused to TurboRFP and the *nhr-1*  
197 promoter to Venus. We observed a strong and consistent expression of *nhr-40* in the head.  
198 Specifically, it localized to the pharyngeal muscle cells and cells whose cell body position is  
199 consistent with them being arcade or hypodermal cells (Fig. 2D, SFig. 1D). *nhr-40* and *nhr-1*  
200 signals co-localized in a subset of presumptive hypodermal and arcade cells, and in the  
201 pharyngeal muscles. In contrast, only *nhr-1* was expressed in the dorsal pharyngeal gland cell  
202 g1D (Fig. 2D, SFig. 1D,E). In summary, while the expression of *nhr-40* is more restricted than  
203 the expression of *nhr-1*, the two genes display robust co-localization in several cell types.

204

### 205 **Suppressor screen in *nhr-1* failed to identify downstream target genes**

206 The experiments described above established that two NHR-type transcription factors  
207 control mouth-form plasticity in *P. pacificus*. We speculate that NHR-40 and NHR-1 regulate  
208 a set of target genes, which execute the developmental decision and generate alternative  
209 phenotypes. To identify such downstream target genes, we performed genetic and  
210 transcriptomic analyses. In the first attempt, we conducted two suppressor screens in the *nhr-1*  
211 (*tu1163*) mutant background. In total, we screened approximately four times more gametes  
212 than in our first suppressor screen, but we isolated no all-Eu lines. There are three  
213 explanations for this result. First, functional *nhr-1* may be essential for the Eu morph. Second,  
214 the number of downstream targets may be small, and a considerably larger screen is required  
215 to identify them. Third, the downstream targets may be redundant, and multiple genes may

216 need to be inactivated to change the phenotype. Therefore, we took an alternative approach  
217 and identified targets of NHR-40 and NHR-1 through transcriptomic profiling.

218

### 219 **Common transcriptional targets of NHR-40 and NHR-1 encode extracellular proteins** 220 **expressed during mouth-form differentiation**

221 Since NHR-40 and NHR-1 are co-expressed and regulate the same phenotype, we  
222 speculate that they regulate a set of common target genes. We analyzed the full list of genes  
223 differentially expressed between the wild type and mutant samples from the experiments  
224 described above. Given the pleiotropic action of NHR-40 and NHR-1, we applied the following  
225 selection criteria. We only retained genes whose transcript levels at either of the two examined  
226 time points were simultaneously altered in *nhr-1*, *nhr-40 loss-of-function*, and *nhr-40 gain-of-*  
227 *function* mutants (Fig. 3A). Only 28 genes satisfied this criterion, and their expression changed  
228 in the same direction in the *loss-of-function* mutants of *nhr-1* and *nhr-40*. We further retained  
229 those genes whose expression changed in one direction in the *loss-of-function* mutants of *nhr-*  
230 *1* and *nhr-40*, and in the opposite direction in the *gain-of-function* mutants of *nhr-40* (Fig. 3A),  
231 resulting in a list of 24 genes, provided in Table 2. Interestingly, the expression of 23 of them  
232 decreased in the *loss-of-function* mutants (Table 2).

233 We hypothesized that if the making of cuticularized mouthparts involves these genes,  
234 they must encode extracellular proteins, and their expression is likely to be biased towards  
235 the time of mouth-form differentiation. To verify the extracellular function of the target proteins,  
236 we predicted signal peptides and compared the list of targets with the genome-wide pattern.  
237 Indeed, we found that the targets of NHR-40 and NHR-1 are significantly enriched with genes  
238 containing signal peptides (Fig. 3B). To examine a potential temporal expression bias, we  
239 compared the wild-type transcriptomes at the time of mouth-form determination and mouth-  
240 form differentiation. While most genes in the genome (51%) showed uniform expression at the  
241 two time points, 23 of the 24 targets of NHR-40 and NHR-1 were more highly expressed at  
242 the time of mouth-form differentiation (Fig. 3B). Surprisingly, we also observed a third trend in  
243 our data set. While only 12% of all genes in the genome are located on the X chromosome,

244 15 of the 24 targets of NHR-40 and NHR-1 were X-linked (Fig. 3B). Previously identified genes  
245 associated with mouth-form plasticity are frequently situated on the X-chromosome, including  
246 both *nhr-40* and *nhr-1*, and additionally the multigene locus comprising *eud-1*, *nag-1* and *nag-*  
247 *2*. While the exact meaning of this phenomenon remains unclear, the X chromosome in *C.*  
248 *elegans* is enriched with hermaphrodite-biased somatically expressed genes<sup>41</sup>. Accordingly,  
249 the incidence of Eu morphs is higher in *P. pacificus* hermaphrodites than in males<sup>39</sup>, which  
250 may be reflected in the chromosomal distributions of the genes associated with the Eu morph.  
251 In summary, the downstream targets of NHR-40 and NHR-1 are enriched with genes that are  
252 X-linked, encode extracellular proteins, and are more highly expressed at the time of mouth-  
253 form differentiation.

254 To explore the potential functions of the NHR-40 and NHR-1 targets, we used  
255 information about their annotated protein domains. Surprisingly, 12 of the 24 genes contain  
256 an Astacin domain (Table 2). Astacins are secreted or membrane-anchored Zinc-dependent  
257 endopeptidases, first described in the crayfish *Astacus astacus*<sup>42</sup>. Of the 40 genes present in  
258 *C. elegans*, only *dpy-31*, *nas-6* and *nas-7* have known functions, whereby mutations in these  
259 genes result in abnormal cuticle synthesis<sup>43,44</sup>. Another five of the 24 NHR targets encode a  
260 CAP (cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1) domain (Table  
261 2), which is contained in extracellular proteins with diverse functions<sup>45-47</sup>, including the  
262 proteolytic modification of extracellular matrix<sup>48</sup>. Two genes belong to the glycoside hydrolases  
263 family 18 (Table 2), which includes chitinases and chitinase-like proteins<sup>49</sup> that may modify the  
264 cuticle, as chitin is the main component of the cuticle in nematodes<sup>50</sup>. Finally, the NHR target  
265 list includes an unannotated protein, PPA30108 (Table 2), which contains multiple GGX  
266 repeats, where X is F or R. Intriguingly, a similar sequence repeat has been proposed to  
267 facilitate the formation of elastic fibers by structural proteins of spider silk<sup>51,52</sup>. Thus, the  
268 examination of the coding sequences and domain composition of the targets of NHR-40 and  
269 NHR-1 shows that most encode enzymes that may directly modify the cuticle, and one gene  
270 encoding what may be an elastic structural protein.

271

## 272 **A duodecuple Astacin mutant shows no mouth-form abnormalities**

273           Next, we tested if mutations in the identified genes affected mouth-form frequency or  
274 morphology. We therefore performed systematic CRISPR/Cas9 knockout experiments of the  
275 23 genes downregulated in the *loss-of-function* mutants. To compensate for potential  
276 redundancy between paralogous genes encoding identical domains, we produced lines in  
277 which all such genes are inactivated simultaneously. For example, rather than generating 12  
278 strains with mutations affecting single Astacin-encoding genes, we produced a duodecuple  
279 mutant line, in which we sequentially knocked out all 12 genes (Table 1). We phenotyped the  
280 mutants both on agar plates and in liquid S-medium. However, we detected no significant  
281 change in mouth-form frequencies and no recapitulation of the morphological defects of *nhr-*  
282 *1*. Similarly, we produced a quintuple CAP mutant and double chitinase mutants and observed  
283 no change in mouth-form frequency or morphology (Table 1). We speculate that this may be  
284 caused by the extreme redundancy in the factors involved. For instance, despite mutagenizing  
285 12 Astacin-encoding genes, there are more than 60 such genes in the genome. Consistent  
286 with this, in a phenotypic screen of Astacin genes in *C. elegans*, the majority showed no  
287 detectable phenotypes and the function of one, *nas-7*, was only elucidated due to its  
288 enhancement of a weakly penetrant allele of *nas-6*<sup>44</sup>. Alternatively, it is also possible that some  
289 examined genes function in other tissues unrelated to mouth morphology. Therefore, we next  
290 studied the spatial expression of selected downstream target genes.

291

## 292 **Downstream targets genes are expressed in the same pharyngeal gland cell**

293           We selected six of the 12 Astacin genes, one chitinase gene, one CAP gene, and the  
294 gene bearing similarity to spider silk proteins, and created transcriptional reporters by fusing  
295 their promoters with TurboRFP. Remarkably, all reporter lines showed expression in the same  
296 single cell, the dorsal pharyngeal gland cell g1D (Fig. 3C). In contrast, we found no expression  
297 in the pharyngeal muscles or other expression foci of *nhr-40* and *nhr-1*. Thus, all analyzed  
298 targets are co-expressed with *nhr-1* in g1D (Fig. 3C, SFig. 1E). The recent reconstruction of  
299 the pharyngeal gland cell system of *P. pacificus*<sup>53</sup> revealed that the cell body of g1D is located

300 at the posterior end of the pharynx. It sends a long process through the entire pharynx to the  
301 anterior tip where it connects, via a short duct in the cuticle, to a channel in the dorsal tooth  
302 which opens into the buccal cavity (Fig. 1B, 3C). Importantly, the process of g1D is surrounded  
303 by pharyngeal muscle cells which directly underlie the teeth. Therefore, we hypothesize that  
304 the enzymes excreted from g1D act on the structural components that are themselves  
305 secreted by the pharyngeal muscles.

306

### 307 **Expansion of the pharyngeal gland cells is concomitant with the emergence of teeth**

308 The expression of the targets of NHR-40 and NHR-1 in g1D is remarkable, because  
309 g1D is the site of a major evolutionary innovation in the family Diplogastridae, to which *P.*  
310 *pacificus* belongs. The pharynx in free-living nematodes of the order Rhabditida and the  
311 outgroup<sup>54</sup> family Teratocephalidae is divided into two parts. The anterior part, called the  
312 corpus, is muscular, and in some lineages ends with a dilation, called the median bulb. The  
313 posterior part, called the postcorpus, is divided into a narrow isthmus and a dilation, called the  
314 terminal bulb, which contains muscle cells and three to five gland cells. The terminal bulb  
315 contains muscular valves that form a specialized cuticular structure, the grinder, which helps  
316 fragment food particles<sup>55</sup> (Fig. 4). Phylogenetic reconstruction indicates that the outgroup  
317 Teratocephalidae, and the rhabditid families Cephalobidae and Rhabditidae retained the  
318 ancestral character states, whereby they have a grinder, but no teeth<sup>56-58</sup>. In contrast,  
319 Diplogastridae have no grinder, but they have concomitantly gained teeth at the base of the  
320 family<sup>7,59</sup>. The acquisition of teeth and the loss of the grinder were accompanied by the  
321 reduction of the muscle cells in the postcorpus, and an expansion of three gland cells g1D,  
322 g1VL, and g1VR, one in each sector of the trilaterally symmetrical pharynx<sup>53,59</sup> (Fig. 4). While  
323 the exact role of pharyngeal gland cells in *C. elegans* and other nematodes has remained  
324 elusive<sup>55</sup>, we speculate that the functional remodeling of g1D, in which the target genes of  
325 NHR-40 and NHR-1 are expressed, may be a prerequisite for the formation of teeth and the  
326 evolution of predation. Therefore, we investigated the evolutionary dynamics of the identified  
327 genes expressed in this cell.

## 328 **Conserved transcription factors regulate fast-evolving target genes**

329 To investigate if the morphological lineage-specific evolutionary innovation in *P.*  
330 *pacificus* and Diplogastridae is associated with taxonomically restricted genes, we  
331 reconstructed the phylogeny of NHR genes and their identified targets. This is an important  
332 evolutionary question as recent genomic studies involving deep taxon sampling revealed high  
333 evolutionary dynamics of novel gene families in *Pristionchus*, with only one third of all genes  
334 having 1:1 orthologs between *P. pacificus* and *C. elegans*<sup>60,61</sup>. First, we reconstructed the  
335 phylogeny of NHR genes. We identified similar numbers of NHR genes in the genomes of *P.*  
336 *pacificus* and *C. elegans* - 254 and 266 genes, respectively. In the phylogenetic tree (Fig. 5A),  
337 most clades contained genes from predominantly or exclusively one of the two species. These  
338 genes likely result from lineage-specific duplications and losses, a phenomenon commonly  
339 seen in nematode gene families<sup>62</sup>. *nhr-40* and *nhr-1*, however, belonged to one of the few  
340 clades that contained a mixture of genes from both species, with many genes displaying a 1:1  
341 orthology relationship. Indeed, the *P. pacificus* and *C. elegans* copies of *nhr-40* and *nhr-1*  
342 showed 1:1 orthology with 100% bootstrap support (Fig. 5A). Importantly, *nhr-40* and *nhr-1*  
343 are also extremely closely related to each other (Fig. 5A). Thus, in the overall context of NHR  
344 evolution, *nhr-40* and *nhr-1* are closely related duplicates that have been conserved since the  
345 divergence of *P. pacificus* and *C. elegans*.

346 The conservation of *nhr-40* and *nhr-1* is in stark contrast to the evolutionary history of  
347 their downstream targets. To reconstruct the phylogenies of the Astacin, CAP and chitinase  
348 genes (Fig. 5B-D), we used functional domains rather than complete genes to facilitate the  
349 alignment of genes with different domain architectures. Similar to the case of NHRs, all three  
350 gene families exhibit strong signatures of lineage-specific expansions. Furthermore, all target  
351 genes containing Astacin, CAP and chitinase domains belonged to such lineage-specific  
352 clades (Fig. 5B-D). These findings suggest that the targets of NHR-40 and NHR-1 undergo  
353 rapid turnover. This is further supported by the phylogeny of CAP genes within the genus  
354 *Pristionchus*. Specifically, the five targets identified in *P. pacificus* clustered separately from  
355 the homologs in the early branching species *P. fissidentatus* with 94% bootstrap support (Fig.

356 5E). Thus, two conserved NHRs target rapidly evolving downstream genes of multiple gene  
357 families. We speculate that the striking co-expression of the target genes results from an  
358 ancient regulatory linkage between the NHRs and the promoters of the ancestral target genes.  
359 Such divergent evolutionary dynamics of transcription factors and their downstream targets  
360 might represent general features of GRNs.



## 361 **Conclusions**

362

363 In this study, we expanded the GRN controlling predatory vs. non-predatory plasticity  
364 in *P. pacificus*, thereby enhancing the molecular understanding of plasticity. We uncovered  
365 novel genetic factors and genomic features at two regulatory levels, which allowed linking  
366 rapid gene evolution with morphological innovations associated with plasticity. First, we  
367 identified a mutation in the nuclear receptor gene *nhr-1*, which disrupts mouth-form  
368 determination. Most previously identified genes, such as *eud-1* or *sult-1/seud-1*, influence the  
369 determination process by affecting the preferred developmental trajectory, but the resulting  
370 morphology exhibits no observable differences to the corresponding wild-type  
371 morphology<sup>18,23,24</sup>. On the other hand, interfering with heat shock protein activity, including a  
372 mutation in *daf-21/Hsp90*, produces aberrant morphologies while maintaining the  
373 dimorphism<sup>63</sup>. In contrast to both classes of genetic interventions, mutations in *nhr-1* lead to a  
374 morphology that combines features of normal Eu and St morphs, with no apparent  
375 dimorphism. Therefore, we speculate that NHR-1 is required for mouth-form determination  
376 and the specification of both morphs. On the contrary, we identified that *gain-* and *loss-of-*  
377 *function* mutations in *nhr-40* result in all-Eu and all-St phenotypes, respectively, reminiscent  
378 of the role of *daf-12*, another *nhr* gene, in controlling dauer plasticity in *C. elegans*<sup>64</sup>. Different  
379 phenotypic effects of *nhr-1* and *nhr-40* are also consistent with the lack of evidence of  
380 transcriptional regulation of one factor by the other. Except for DAF-12 in *C. elegans*, no single  
381 nematode NHR has been de-orphanized. Therefore, the identification of the potential ligands  
382 of NHR-1 and NHR-40 may reveal additional layers of regulation and elucidate their cross-  
383 talk. Indeed, recent studies suggested that cytosolic sulfotransferases, including *sult-1/seud-*  
384 *1* in *P. pacificus* and its homolog *ssu-1* in *C. elegans*, may regulate NHRs by modifying their  
385 ligands<sup>23,24,65</sup>.

386 Second, the transcriptomic analysis of *nhr-1* and *nhr-40* mutants revealed an  
387 unexpectedly small number of downstream targets. While cell-specific signals may be masked  
388 in whole-animal transcriptome data, and our selection criteria excluded genes affected by the



389 *gain-of-function* of *nhr-40* in other ways than by exhibiting increased transcript levels, having  
390 a small list of target genes enabled a systematic analysis of their function and expression.  
391 Both the absence of phenotypes in duodecuple and quintuple mutants, and the restricted  
392 expression of all tested genes in the same cell g1D are compatible with extreme redundancy.  
393 Such redundancy might result from features of genome evolution that are common to  
394 nematodes and other animals. Studies over the last decade revealed that nematode genomes  
395 are gene-rich and exhibit high rates of gene birth and death<sup>60,66,67</sup>. In particular, enzyme-  
396 encoding genes are subject to high evolutionary dynamics<sup>62</sup>. Therefore, the position of genes  
397 in GRNs may determine the speed and direction of their evolution. Consistent with this idea,  
398 many genes encoding proteins of signal transduction and their terminal transcription factors  
399 are highly conserved across animals<sup>68-70</sup>. In this study, we complement this knowledge by  
400 showing that the downstream targets of conserved transcription factors are indeed fast  
401 evolving genes. Importantly, their expression focus, the g1D cell, also underwent a major  
402 evolutionary change, whereby its structural and functional remodeling accompanied the  
403 emergence of teeth in the family Diplogastridae. Thus, our study demonstrates that fast-  
404 evolving genes are expressed in a fast-evolving cell, linking morphological innovations with  
405 rapid gene evolution.

406 **Methods**

407

408 **Maintenance of worm cultures and genetic crosses**

409 Stock cultures of all strains used in this study were reared at room temperature (20-  
410 25°C) on nematode growth medium (NGM) (1.7% agar, 2.5 g/L tryptone, 3 g/L NaCl, 1 mM  
411 CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 5 mg/L cholesterol, 25 mM KPO<sub>4</sub> buffer at pH 6.0) in 6 cm Petri dishes,  
412 as outlined in the *C. elegans* maintenance protocol<sup>71</sup>. *Escherichia coli* OP50 was used as food  
413 source. Bacteria were grown overnight at 37°C in L Broth (10 g/L tryptone, 5 g/L yeast extract,  
414 5 g/L NaCl, pH adjusted to 7.0), and 400 µL of the overnight culture was pipetted on NGM  
415 agar plates and left for several days at room temperature to grow bacterial lawns. *P. pacificus*  
416 were passed on these lawns and propagated by passing various numbers of mixed  
417 developmental stages. To cross worms, agar plates were spotted with 10 µL of the *E. coli*  
418 culture, and five to six males and one or two hermaphrodites were transferred to the plate and  
419 allowed to mate. Males were removed after two days of mating.

420

421 **Mouth form phenotyping**

422 We phenotyped worms in two culture conditions. Rearing *P. pacificus* on solid NGM  
423 induces the Eu morph and facilitates identification of Eu-deficient (all-St) phenotypes.  
424 Conversely, growing worms in liquid S-medium (5.85 g/L NaCl, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 6 g/L KH<sub>2</sub>PO<sub>4</sub>,  
425 5 mg/L cholesterol, 3 mM CaCl<sub>2</sub>, 3 mM MgSO<sub>4</sub>, 18.6 mg/L disodium EDTA, 6.9 mg/L  
426 FeSO<sub>4</sub>•7H<sub>2</sub>O, 2 mg/L MnCl<sub>2</sub>•4H<sub>2</sub>O, 2.9 mg/L ZnSO<sub>4</sub>•7H<sub>2</sub>O, 0.25 mg/L CuSO<sub>4</sub>•5H<sub>2</sub>O and 10  
427 mM Potassium citrate buffer at pH 6.0) represses the Eu morph and facilitates identification of  
428 Eu-constitutive (all-Eu) phenotypes<sup>19,71</sup>. As food source, S-medium contained *E. coli* OP50 in  
429 the amount corresponding to 100 mL of an overnight culture with OD<sub>600</sub> 0.5 per 10 mL of  
430 medium. We started phenotyping by isolating eggs from stock culture plates, which contained  
431 large numbers of gravid hermaphrodites and eggs deposited on the agar surface<sup>71</sup>. To isolate  
432 eggs, we washed worms and eggs from plates with water, and incubated them in a mixture of  
433 0.5 M NaOH and household bleach at 1:5 final dilution for 10 min with regular vortexing to

434 disintegrate vermiform stages. Remaining eggs were pelleted at 1,300 g for 30 sec, washed  
435 with 5 mL of water, pelleted again, resuspended in water and pipetted on agar plates or into  
436 S-medium. Agar plates were left at room temperature (20-25°C) for 3-5 days and 25 mL  
437 Erlenmeyer flasks with liquid medium were shaken at 22°C, 180 rpm for 4-6 days. Adult  
438 hermaphrodites were immobilized on 5% Noble Agar pads with 0.3% NaN<sub>3</sub> added as an  
439 anesthetic, and examined using differential interference contrast (DIC) microscopy. Animals  
440 that had a large right ventrosublateral tooth, curved dorsal tooth, and the anterior tip of the  
441 promesostegostom posterior to the anterior tip of the gymnostom plate were classified as Eu  
442 morphs. Animals that did not exhibit these three characters simultaneously were classified as  
443 St morphs, although there was a distinction between the morphology of *nhr-1* mutants and of  
444 other all-St mutants (SFig. 1A).

445

#### 446 **Geometric morphometric analysis**

447 We reused the published<sup>63</sup> landmark data for the wild-type strain RS2333 and the *daf-*  
448 *21(tu519)* mutant. We complemented this data set with newly collected data for the *nhr-*  
449 *1(tu1163)* mutant, whereby we imaged young adults mounted on microscope slides on 5%  
450 Noble agar pads containing 0.3% NaN<sub>3</sub> as an anesthetic. Only individuals with their right body  
451 side facing upwards were imaged. We took stack images of the anterior tip of the head, and  
452 recorded X and Y coordinates of 20 landmarks identical to the ones used in the previous  
453 study<sup>63</sup> using FIJI<sup>72</sup>. Procrustes alignment and PCA were done in R (ver. 3.4.4)<sup>73</sup> using  
454 geomorph package<sup>74</sup>.

455

#### 456 **CRISPR/Cas9 mutagenesis**

457 We followed the previously published protocol for *P. pacificus*<sup>75</sup> with subsequently  
458 introduced modifications<sup>76</sup>. All target-specific CRISPR RNAs (crRNAs) were designed to target  
459 20 bp upstream of the protospacer adjacent motifs (PAMs). We purchased crRNAs and  
460 universal trans-activating CRISPR RNA (tracrRNA) from Integrated DNA Technologies (Alt-R  
461 product line). 10 µL of the 100 uM stock of crRNA was combined with 10 µL of the 100 uM

462 stock of tracrRNA, denatured at 95°C for 5 min, and allowed to cool down to room temperature  
463 and anneal. The hybridization product was combined with Cas9 protein (purchased from New  
464 England Biolabs or Integrated DNA Technologies) and incubated at room temperature for 5  
465 min. The mix was diluted with Tris-EDTA buffer to a final concentration of 18.1  $\mu$ M for the RNA  
466 hybrid and 2.5  $\mu$ M for Cas9. When site-directed mutations were introduced via homology-  
467 directed repair, a ssDNA oligo template designed on the same strand as the gRNA was  
468 included in the mix at a final concentration of 4  $\mu$ M. The diluted mixture was injected in the  
469 gonad rachis of approximately one day old adult hermaphrodites. Eggs laid by injected animals  
470 within a 12-16 h period post injection were recovered, and the F1 progeny were singled out  
471 upon reaching maturity. After F1 animals have laid eggs, they were placed in 10  $\mu$ L of single  
472 worm lysis buffer (10 mM Tris-HCl at pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.45% NP-40, 0.45%  
473 Tween 20, 120  $\mu$ g/ml Proteinase K), frozen and thawed once, and incubated in a thermocycler  
474 at 65°C for 1 h, followed by heat deactivation of the proteinase at 95°C for 10 min. The resulting  
475 lysate was used as a template in subsequent PCR steps. Where possible, molecular lesions  
476 at the crRNA target sites were detected by melting curve analysis on a LightCycler 480  
477 Instrument II (Roche) of PCR amplicons obtained using LightCycler 480 High Resolution  
478 Melting Master (Roche). Presence of mutations in candidate amplicons was further verified by  
479 Sanger sequencing. Alternatively, PCR was done using Taq PCR Master Mix (Qiagen) and all  
480 the F1 were Sanger sequenced. To detect large rearrangements, we conducted whole  
481 genome re-sequencing of lines for which no PCR amplicon containing the crRNA target site  
482 could be obtained. For most such lines, we extracted genomic DNA using GenElute  
483 Mammalian Genomic DNA Miniprep Kit (Merck), whereby we modified the tissue digestion  
484 step by raising the Proteinase K concentration to 2 mg/mL, and prepared next-generation  
485 sequencing (NGS) libraries using Nextera DNA Flex Library Prep Kit (Illumina). For the *nhr-*  
486 *40* null mutant line, we followed a recently introduced cost-effective alternative procedure<sup>77</sup>  
487 with several modifications. Single worms were placed in 10  $\mu$ L water, and frozen and thawed  
488 3 times in liquid nitrogen. Then, we added 10  $\mu$ L 2x single worm lysis buffer (20 mM Tris-HCl  
489 at pH 8.3, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 0.9% NP-40, 0.9% Tween 20, 240  $\mu$ g/ml Proteinase K)

490 and incubated the tubes in a thermocycler at 65°C for 1 h. After a clean-up using HighPrep  
491 beads (MagBio Genomics), DNA was eluted in 7 µL Tris buffer at pH 8.0. Then, 100 pg of  
492 DNA was diluted with water to the total volume of 9 µL, mixed with 2 µL 5X TAPS-DMF buffer  
493 (50 mM TAPS at pH 8.5, 25 mM MgCl<sub>2</sub>, 50% DMF) and 1 µL Tn5 transposase from Nextera  
494 DNA Library Prep Kit (Illumina) diluted beforehand 1:25 in dialysis buffer (100 mM HEPES at  
495 pH 7.2, 0.2 M NaCl, 0.2 mM EDTA, 0.2% Triton X-100, 20% glycerol). The mixture was  
496 incubated for 14 min at 55°C. Tagmented DNA was amplified using Q5 HotStart High-Fidelity  
497 DNA Polymerase (New England Biolabs) for 14 cycles, whereby adapters and indices were  
498 added as primer overhangs, and size-selected for 250–550 bp fragments using HighPrep  
499 beads (MagBio Genomics). NGS libraries prepared using both methods were sequenced in a  
500 paired-end run of a HiSeq 3000 machine (Illumina). Reads were mapped to the El Paco  
501 assembly of the *P. pacificus* genome<sup>78</sup> using Bowtie 2 (ver. 2.3.4.1)<sup>79</sup>. We visually inspected  
502 read coverage in the loci of interest using IGV<sup>80</sup> to identify the precise regions in which  
503 coverage was close to zero.

504

### 505 **EMS mutagenesis**

506 To induce heritable mutations in *P. pacificus*, we incubated a mixture of J4 larvae and  
507 young adults in M9 buffer (3 g/L KH<sub>2</sub>PO<sub>4</sub>, 6 g/L Na<sub>2</sub>HPO<sub>4</sub>, 5 g/L NaCl, 1 mM MgSO<sub>4</sub>) with 47  
508 mM ethyl methanesulfonate (EMS) for 4 h<sup>81</sup>. Subsequently, the worms were allowed to recover  
509 on agar plates with bacteria (see above), and 40-120 actively moving J4 larvae were singled  
510 out. After the animals have laid approximately 20 eggs, they were killed, and F1 progeny were  
511 allowed to develop and reach maturity. F1 animals (which contained heterozygous mutants)  
512 were then singled out, and F2 progeny (which contained a mixture of genotypes, including  
513 homozygous mutants) were allowed to develop until adulthood. In each F1 plate, we  
514 determined the mouth form in 5-10 F2 individuals using Discovery V20 stereomicroscope  
515 (Zeiss). If at least one individual appeared to have a mouth form different from that of the  
516 background strain, such an animal was transferred to a fresh plate and its progeny was  
517 screened again using DIC until we gained confidence that a homozygous line was isolated. In

518 the screen for suppressors of *nhr-40*, we mutagenized *nhr-40(tu505)* worms, which are all-Eu,  
519 screened approximately 1,000 F1 plates, and isolated one no-Eu allele, *tu515*. In an attempt  
520 to identify further downstream target genes, we conducted two suppressor screens in the *nhr-*  
521 *1(tu1163)* mutant background and screened approximately 3,800 F1 plates in total, but found  
522 no Eu individuals.

523

#### 524 **Mapping of *tu515***

525 We crossed the *tu515* mutant, produced in the background of the RS2333 strain (a  
526 derivative of the PS312 strain), to a highly-Eu wild type strain PS1843. The resulting males  
527 were crossed to a strain RS2089, which is a derivative of PS1843 containing a morphological  
528 marker mutation causing the Dumpy phenotype. The progeny were allowed to segregate and  
529 100 no-Eu lines were established. Four individuals from each line were pooled and genomic  
530 DNA was extracted from the pool using the MasterPure Complete DNA and RNA Purification  
531 Kit (Epicentre). Additionally, genomic DNA was extracted from the *tu515* line. NGS libraries  
532 were prepared using Low Input Library Prep kit (Clontech) and sequenced on Illumina  
533 HiSeq3000. Raw Illumina reads of the *tu515* mutant and of a mapping panel were aligned to  
534 the El Paco assembly of the *P. pacificus* genome (strain PS312)<sup>78</sup> by the *aln* and *sampe*  
535 programs of the BWA software package (ver. 0.7.17-r1188)<sup>82</sup>. Initial mutations were called  
536 with the *samtools* (ver. 1.7) *mpileup* command<sup>83</sup>. The same program was used to measure  
537 PS312 allele frequencies in the mapping panel at variant positions with regard to whole  
538 genome sequencing data of the PS1843 strain<sup>78</sup>. SFig. 2B shows that large regions between  
539 the positions 5 Mb and 16 Mb of the *P. pacificus* chromosome X exhibit high frequency of the  
540 PS312 alleles (the mutant background) in the mapping panel. In total, 28 non-  
541 synonymous/nonsense mutations (STable 1) in annotated genes (El Paco gene annotations  
542 v1, Wormbase release WS268) were identified in the candidate interval by a previously  
543 described custom variant classification software<sup>84</sup>.

544

545

## 546 Transgenesis

547 To identify putative promoter regions, which included 5' untranslated regions (UTR)  
548 and may have included the beginning of coding sequences, we manually re-annotated the 5'  
549 ends of predicted genes of interest using RNA-seq data and the information about predicted  
550 signal peptides. The ATG codon preceding the signal peptide or the last ATG codon in the  
551 second exon was designated as the putative start codon. As a general rule, the promoter  
552 region included a sequence spanning from the 3' end of the closest upstream gene on the  
553 same strand to the start codon, but if the upstream neighbor gene was located further than 2  
554 kb away, a 1.5-2 kb region upstream of the identified start codon was designated as the  
555 putative promoter. In the case of inverted tandem duplicates in the head-to-head orientation,  
556 the 5' end of the promoter region was approximately in the middle between the start codons  
557 of the two genes. For the reporter constructs, we used the previously published coding  
558 sequences of TurboRFP<sup>85</sup> and Venus<sup>27</sup> fused with the 3' UTR of the ribosomal gene *rpl-23*<sup>85</sup>.  
559 For the *nhr-1* rescue construct, we used the native coding sequence, in which we replaced  
560 native introns with synthetic introns, fused with the native 3' UTR. As the latter fragment could  
561 not be amplified from genomic or complementary DNA in one piece, we purchased a  
562 corresponding gBlocks fragments (Integrated DNA Technologies). FASTA sequences of all  
563 promoter regions, coding sequences and 3' UTRs are provided in SData 1.

564 Plasmids carrying reporter and rescue constructs, listed in STable 2, were created by  
565 Gibson assembly using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) or  
566 a homemade master mix<sup>86</sup>. Small modifications, such as deletions and insertions under 70 bp,  
567 were introduced using Q5 Site-Directed Mutagenesis kit (New England Biolabs). Injection mix  
568 for transformation was created by digesting the plasmid of interest, the marker plasmid  
569 carrying a tail-bound reporter *egl-20p::TurboRFP* (if applicable), and genomic DNA with  
570 FastDigest restriction enzymes (Thermo Fisher Scientific), whereby genomic DNA was cut  
571 with an enzyme(s) that had the same cutting site(s) as the enzyme(s) used to digest the  
572 plasmids. Digested DNA was purified using Wizard SV Gel and PCR Clean-Up system  
573 (Promega), and the components were mixed in the following ratios. Injection mixes with rescue



574 constructs contained 1 ng/ $\mu$ L rescue construct, 10 ng/ $\mu$ L marker, and 50 ng/ $\mu$ L genomic DNA.  
575 Injection mixes with reporter constructs contained 10 ng/ $\mu$ L reporter construct, 10 ng/ $\mu$ L  
576 marker, and 60 ng/ $\mu$ L genomic DNA. The mix was injected in the gonad rachis of  
577 approximately 1 day old hermaphrodites, and their progeny was screened for fluorescent  
578 animals<sup>85</sup>.

579

## 580 **Antibody staining**

581 We followed a previously published protocol<sup>87</sup> with minor modifications. Animals were  
582 washed from mature plates with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl,  
583 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> at pH 7.4), passed over a 5-20  $\mu$ m nylon filter, concentrated  
584 at the bottom of a 2 mL tube and chilled on ice. We then added chilled fixative (15 mM Na-  
585 PIPES at pH 7.4, 80 mM KCl, 20 mM NaCl, 10 mM Na<sub>2</sub>EGTA, 5 mM Spermidine-HCl, 2%  
586 paraformaldehyde, 40% MeOH), froze the worms in liquid nitrogen and thawed them on ice  
587 for 1-2 h with occasional inversion. Subsequently, the animals were washed twice with Tris-  
588 Triton buffer (100 mM Tris-HCl at pH 7.4, 1 mM EDTA, 1% Triton X-100), incubated in Tris-  
589 Triton buffer with 1%  $\beta$ -mercaptoethanol in a thermomixer at 600 rpm for 2 h at 37°C, washed  
590 once in borate buffer (25 mM H<sub>3</sub>BO<sub>3</sub>, 12.5 mM NaOH), incubated in borate buffer with 10 mM  
591 dithiothreitol in a thermomixer at 600 rpm for 15 min at room temperature, washed once in  
592 borate buffer, incubated in borate buffer with ~0.3% H<sub>2</sub>O<sub>2</sub> in a thermomixer at 600 rpm for 15  
593 min at room temperature, and washed once more in borate buffer. Next, the worms were  
594 washed three times with antibody buffer B (0.1% bovine serum albumin, 0.5% Triton X-100,  
595 0.05% NaN<sub>3</sub>, 1 mM EDTA in PBS) on a rocking wheel, incubated with a dye-conjugated  
596 antibody (Thermo Fisher Scientific, cat. # 26183-D550 and cat. # 26183-D488) diluted 1:25 in  
597 antibody buffer A (1% bovine serum albumin, 0.5% Triton X-100, 0.05% NaN<sub>3</sub>, 1 mM EDTA in  
598 PBS) on a rocking wheel in the dark for 3 h at room temperature or overnight at 4°C, washed  
599 three times with antibody buffer B and mounted on slides in a 1:1 mixture of PBS and  
600 Vectashield (Vector Laboratories) with 1  $\mu$ g/mL DAPI added. Slides were imaged using a Leica  
601 SP8 confocal microscope.



## 602 RNA-seq analysis

603 To obtain a sufficient number of eggs, we passed young adult hermaphrodites to new  
604 agar plates with 5-10 animals per plate. After their F1 progeny have laid eggs (5-6 days), they  
605 were bleached (see above), then resuspended in 400  $\mu$ L water per starting plate, pipetted  
606 onto multiple fresh plates with 100  $\mu$ L suspension per fresh plate and placed at 20°C. Animals  
607 were collected at 24 h (corresponding to J2 and J3 larvae), 48 h (J3 and J4 larvae) and 68 h  
608 (J4 instar larvae and young adults) post-bleaching by adding some water to the plates,  
609 scraping off the bacterial lawns with worms in them using disposable cell spreaders and  
610 passing the resulting suspension through a 5  $\mu$ m nylon filter, which efficiently separated worms  
611 from bacteria. Worms were washed from the filter into 1.5 mL tubes, pelleted in a table-top  
612 centrifuge at the maximum speed setting, after which the supernatant was removed and 1 mL  
613 TRIzol (Invitrogen) was added to the worm pellets. Tubes were flash-frozen in liquid nitrogen  
614 and stored at -80°C for up to a month. To extract RNA, worms suspended in TRIzol were  
615 frozen and thawed three times in liquid nitrogen, debris were pelleted for 10-15 min at 14,000  
616 rpm at 4°C, and 200  $\mu$ L of chloroform was added to the supernatant. After vigorous vortexing  
617 and incubation at room temperature (20-25°C) for 5 min, tubes were rotated for 15 min at  
618 14,000 rpm at 4°C. The aqueous phase was combined with an equal volume of 100% ethanol,  
619 RNA was purified using RNA Clean & Concentrator Kit (Zymo Research) and its integrity was  
620 verified using RNA Nano chips on the Bioanalyzer 2100 instrument (Agilent). To analyze the  
621 transcriptome at the time of mouth form determination, we combined 500 ng RNA isolated at  
622 24 h with 500 ng RNA isolated at 48 h post-bleaching, and proceeded to make libraries using  
623 NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs). To  
624 analyze the transcriptome at the time of mouth form differentiation, we prepared libraries from  
625 1  $\mu$ g of RNA isolated at 68 h post-bleaching. For wild type strain PS312, four biological  
626 replicates were collected at different time points. For the mutants, two replicates of two  
627 independent alleles were collected at two different time points, and these were treated as four  
628 biological replicates. Specifically, we sequenced the following alleles: *nhr-1(tu1163) loss-of-*

629 *function, nhr-1(tu1164) loss-of-function, nhr-40(tu505) gain-of-function, nhr-40(iub6) gain-of-*  
630 *function, nhr-40(tu1418) loss-of-function, nhr-40(tu1423) null.*

631 Libraries were sequenced in two paired-end runs of a HiSeq 3000 machine, whereby  
632 we aimed at 10-20 mln reads per library. Raw sequences have been deposited in the  
633 European Nucleotide Archive with the study accession number PRJEB34615, and will be  
634 made available upon acceptance. The fourth biological replicate of wild-type PS312 and all  
635 replicates of the *nhr-40 loss-of-function/null* mutants were sequenced in a different run than  
636 the other samples. To ensure that batch effects were negligible, we additionally re-sequenced  
637 the first three replicates of wild-type PS312 in the same run and verified that coordinates in  
638 PCA conducted using complete transcriptomes were minimally altered when comparing the  
639 same samples sequenced in the two runs. Reads were mapped to the El Paco assembly of  
640 the *P. pacificus* genome<sup>78</sup> using STAR (ver. 020201)<sup>88</sup>. Differential expression analysis was  
641 carried out in R (ver. 3.4.4)<sup>73</sup> using Bioconductor (ver. 3.6)<sup>89</sup> and DESeq2 (ver. 1.18.1)<sup>90</sup>,  
642 whereby we counted reads mapping to El Paco v1 gene predictions<sup>78</sup>. We applied an adjusted  
643 p-value cutoff of 0.05 and no fold change cutoff. Alignments and coverage were visualized in  
644 IGV<sup>80</sup>. To examine the transcript levels of *nhr-1* and *nhr-40*, we repeated differential  
645 expression analysis, whereby we counted reads mapping to Trinity-assembled transcripts  
646 generated from previously published RNA-seq data<sup>26</sup> because the El Paco v1 gene prediction  
647 for *nhr-1* was incorrect in that it was a fusion of multiple neighboring genes. To test the  
648 differences in FPKM values for *nhr-1* and *nhr-40* in different mutants at each of the two time  
649 points, we performed t-test as implemented in the `t.test` function in R (ver. 3.4.4)<sup>73</sup> and applied  
650 false discovery rate (FDR) correction to the p-values obtained. Prior to conducting the t-test,  
651 we verified the assumptions for parametric statistics by performing Shapiro-Wilk test for  
652 normality (`shapiro.test` function) and Levene test for homoscedasticity (`levene.test` function of  
653 the `car` package<sup>91</sup>). Signal peptides were predicted using SignalP (ver. 4.1)<sup>92</sup>. To compare  
654 relative numbers of genes in different categories listed in Fig. 3B, we used chi-squared test as  
655 implemented in the `chisq.test` function in R (ver. 3.4.4)<sup>73</sup>.

656

## 657 **Phylogenetic reconstructions**

658 To identify NHR, CAP, and chitinase genes in the *C. elegans* genome, we retrieved  
659 the current version (PRJNA13758) of predicted proteins and domains from the  
660 <http://wormbase.org> website and selected genes that contained “IPR001628”, “CAP domain”,  
661 and “IPR001223” as predicted InterPro domains, respectively. The list of Astacin genes was  
662 taken from an earlier study<sup>93</sup> and the corresponding gene predictions were manually retrieved  
663 from the <http://wormbase.org> website. To identify NHR, Astacin, CAP, and chitinase genes in  
664 the *P. pacificus* genome, we predicted domains in the El Paco v1 version of gene predictions<sup>78</sup>  
665 using HMMER (ver. 3.1b2) software in conjunction with the PFAM profile database<sup>94</sup> and  
666 selected genes that contained “PF00105”, “Astacin”, “CAP”, and “PF00704” as predicted  
667 PFAM domains, respectively. Manual inspection of the retrieved NHR genes in *P. pacificus*  
668 revealed that many of the gene predictions represent fusion of multiple neighboring genes.  
669 Therefore, we used the information about the predicted domains, RNA-seq data generated in  
670 this study, and Illumina and PacBio RNA-seq datasets generated earlier<sup>26,95,96</sup> to manually  
671 reannotate the NHR gene predictions in *P. pacificus*. We submitted the improved annotations  
672 to <http://wormbase.org> and they will be released in due course. For the tree of CAP domains  
673 in *P. pacificus* and *P. fissidentatus*, we predicted domains in the Pinocchio versions of gene  
674 predictions for both genomes<sup>60</sup> and selected genes that contained “PF00188” as a predicted  
675 PFAM domain. In the case of NHR genes, complete sequences were aligned, while in the  
676 case of other gene families, functional domains extracted using HMMER (ver. 3.1b2) were  
677 aligned to facilitate the alignment of genes with divergent domain architecture. Alignments  
678 were done in MAFFT (ver. 7.310)<sup>97</sup> and maximum likelihood trees were built using RAxML  
679 (ver. 8.2.11)<sup>98</sup>. Protein-based trees were generated with the following parameters: -f a -m  
680 PROTGAMMAAUTO -N 100. In the case of CAP domains in *P. pacificus* and *P. fissidentatus*,  
681 we first generated a protein-based tree and identified a poorly resolved subtree containing the  
682 genes of interest. To increase the number of informative sites, we extracted corresponding  
683 nucleotide sequences, aligned them in MAFFT and built a tree in RAxML with the following  
684 parameters: -f a -m GTRCAT -N 100. Obtained phylogenetic trees were visualized using

685 FigTree (ver. 1.4.2). All phylogenetic trees and corresponding alignments are provided in  
686 SData 2.

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933

934 **Author contributions**

935 B.S. designed and performed all experiments with help from other authors, analyzed  
936 the data, and wrote the manuscript together with R.J.S. and J.W.L.; S.S. designed and created  
937 reporter lines for Astacin genes, performed a suppressor screen in the *nhr-1* mutant  
938 background, and participated in mouth-form phenotyping; J.W.L. and E.M. generated *nhr-40*  
939 mutations using CRISPR/Cas9, J.W.L. additionally generated mutations in the downstream  
940 targets of NHR-1 and NHR-40 and wrote the manuscript together with B.S. and R.J.S.; C.R.  
941 performed bulk segregant analysis; H.W. generated CRISPR/Cas9 mutants and transgenes;  
942 R.J.S. designed and supervised the study, and wrote the manuscript together with B.S. and  
943 J.W.L.

944

945 **Competing interests**

946 The authors are not aware of any competing interests.

947

948 **Materials & Correspondence**

949 Correspondence and requests for materials should be addressed to R.J.S.

950 **Figure legends**

951

952 **Fig. 1.** Mouth-form plasticity in *P. pacificus*. **(A)** Mouth structure of wild-type eurystomatous  
953 (Eu) morph, wild-type stenostomatous (St) morph, *nhr-1* mutant, and *nhr-40* mutant.  
954 Unlabeled images in two focal planes are shown in SFig. 1A. **(B)** Scanning electron  
955 microscopy image of the mouth opening of the Eu morph. **(C)** The Eu morph devouring its  
956 prey. **(D)** Putative gene regulatory network controlling mouth-form plasticity in *P. pacificus*.  
957 **(E)** Design of the suppressor screen. D = dorsal, V= ventral, A = anterior, P = posterior, DT =  
958 dorsal tooth, RVSLT = right ventrosublateral tooth, RVSLR = right ventrosublateral ridge,  
959 EMS = ethyl methanesulfonate.

960

961 **Fig. 2.** Reverse genetics, transcriptomics and expression patterns of *nhr-40* and *nhr-1*. **(A)**  
962 Protein structure of NHR-40 in wild-type and mutant animals. **(B)** Expression levels of *nhr-40*  
963 and *nhr-1* in wild type and mutants as revealed by transcriptomic profiling. **(C)** Antibody  
964 staining against the HA epitope in an *nhr-1* rescue line. **(D)** Expression patterns of *nhr-40*  
965 and *nhr-1* transcriptional reporters in a double reporter line. TurboRFP (magenta) and Venus  
966 (green) channels are presented as maximum intensity projections. Co-expression results in  
967 white color. D = dorsal, V= ventral, A = anterior, P = posterior, N.S. = not significant.

968

969 **Fig. 3.** Target genes of NHR-40 and NHR-1. **(A)** Experimental setup of transcriptomics  
970 experiment and selection criteria to identify target genes. **(B)** Trends among target genes  
971 compared to genome-wide pattern. **(C)** Transmission electron microscopy reconstruction of  
972 the dorsal pharyngeal gland cell (g1D)<sup>53</sup> and expression patterns of transcriptional reporters  
973 for nine selected targets of NHR-40 and NHR-1. TurboRFP channel is presented as  
974 standard deviation projections. *lof* = loss-of-function, *gof* = gain-of-function, \*\*\* = p<0.001, D  
975 = dorsal, V= ventral, A = anterior, P = posterior.

976

977 **Fig. 4.** Evolution of pharynx morphology in the order Rhabditida.

978 **Fig. 5.** Evolution of *nhr-40*, *nhr-1*, and their target genes. Arrowheads point at the genes of  
979 interest. Protein-based trees of NHR genes (**A**), Astacin domains (**B**), chitinase domains (**C**),  
980 and CAP domains (**D**) in *P. pacificus* and *C. elegans*. (**E**) Nucleotide-based tree of the CAP  
981 domains from a poorly-resolved protein-based subtree of all predicted CAP domains in *P.*  
982 *pacificus* and *P. fissidentatus*.

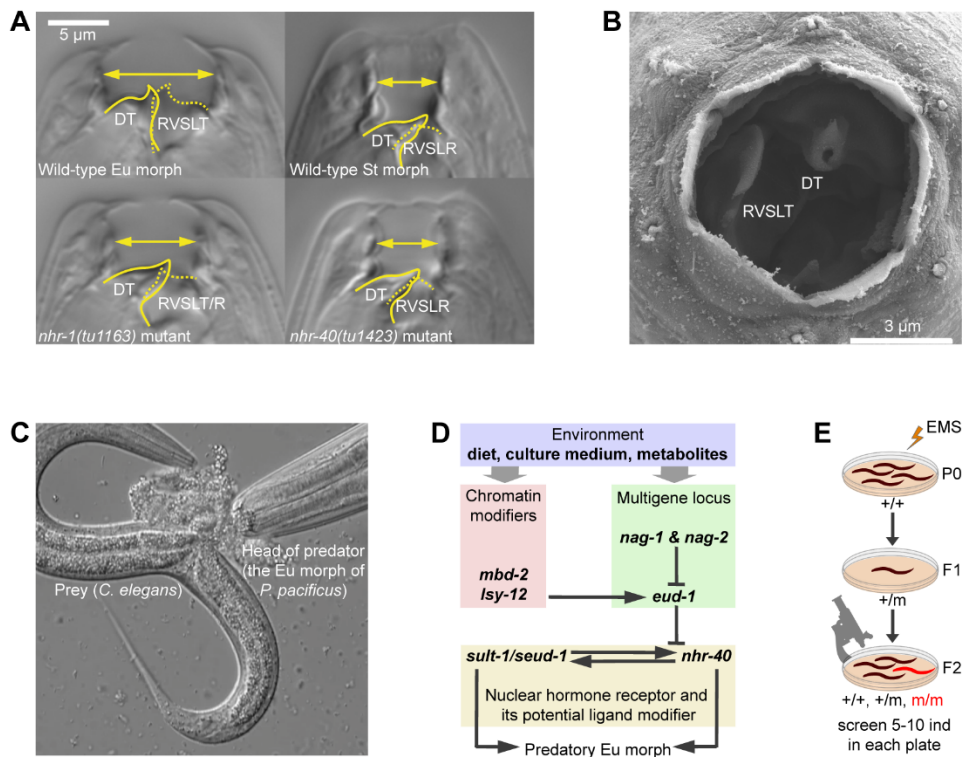
983

984 **Table 1.** Mouth-form frequencies in wild type and mutant lines. The genotype of the  
985 duodecuple Astacin mutant is *PPA03932(tu1259) PPA32730(tu1503);PPA05669(tu1316)*  
986 *PPA05618(tu1317) PPA21987(tu1329) PPA16331(tu1339) PPA27985(tu1340)*  
987 *PPA34430(tu1341) PPA20266(tu1385) PPA42924(tu1386);PPA05955(tu1481)*  
988 *PPA42525(tu1482)*. The genotype of the quintuple CAP mutant is *tuDf6[PPA21912*  
989 *PPA29522 PPA21910] tuDf7[PPA05611 PPA39470] tuDf8[PPA13058 PPA39735]*.

990

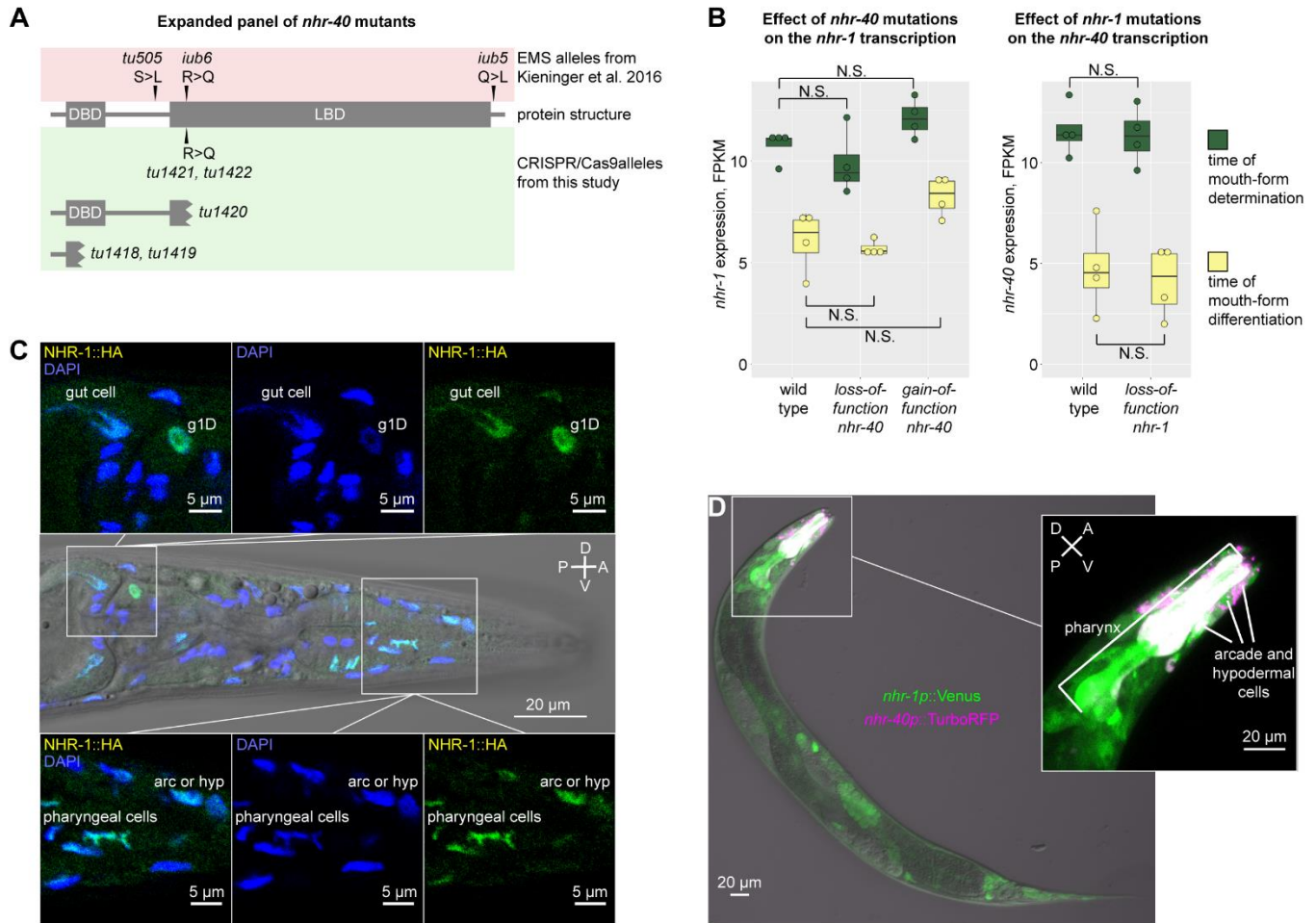
991 **Table 2.** List of targets of NHR-40 and NHR-1.





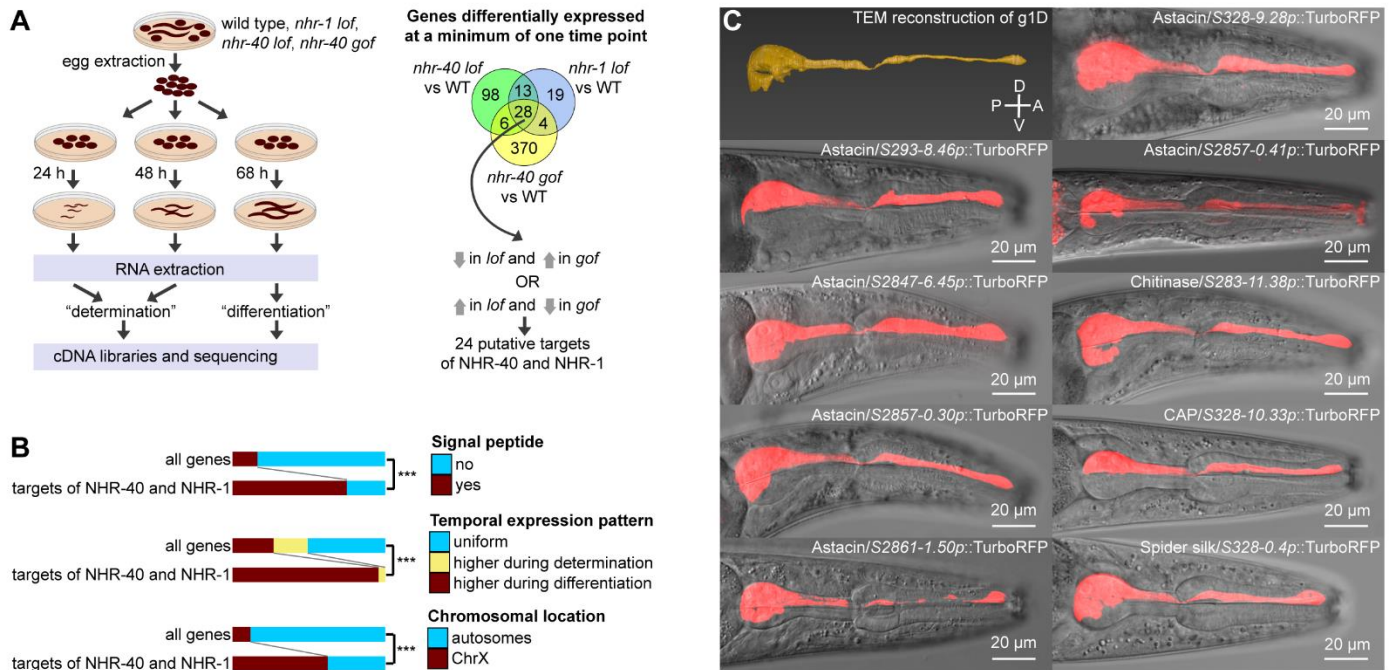
**Fig. 1.** Mouth-form plasticity in *P. pacificus*. **(A)** Mouth structure of wild-type eury stomatous (Eu) morph, wild-type stenostomatous (St) morph, *nhr-1* mutant, and *nhr-40* mutant. Unlabeled images in two focal planes are shown in SFig. 1A. **(B)** Scanning electron microscopy image of the mouth opening of the Eu morph. **(C)** The Eu morph devouring its prey. **(D)** Putative gene regulatory network controlling mouth-form plasticity in *P. pacificus*. **(E)** Design of the suppressor screen.

D = dorsal, V = ventral, A = anterior, P = posterior, DT = dorsal tooth, RVSLT = right ventrosub-lateral tooth, RVSLR = right ventrosublateral ridge, EMS = ethyl methanesulfonate.

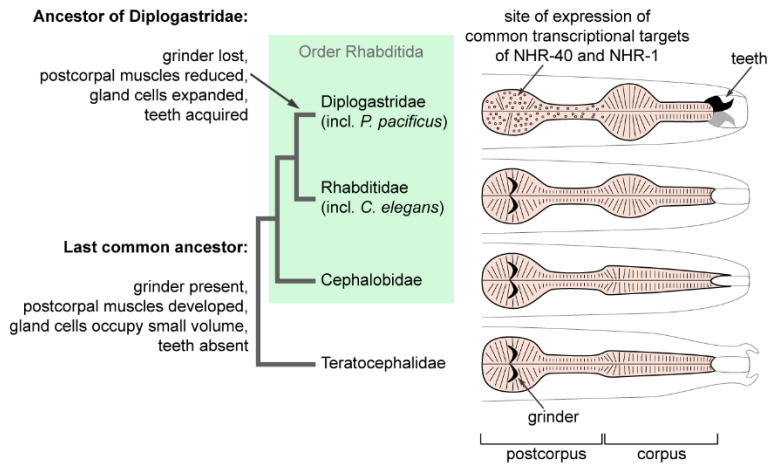


**Fig. 2.** Reverse genetics, transcriptomics and expression patterns of *nhr-40* and *nhr-1*. **(A)** Protein structure of NHR-40 in wild-type and mutant animals. **(B)** Expression levels of *nhr-40* and *nhr-1* in wild type and mutants as revealed by transcriptomic profiling. **(C)** Antibody staining against the HA epitope in an *nhr-1* rescue line. **(D)** Expression patterns of *nhr-40* and *nhr-1* transcriptional reporters in a double reporter line. TurboRFP (magenta) and Venus (green) channels are presented as maximum intensity projections. Co-expression results in white color.

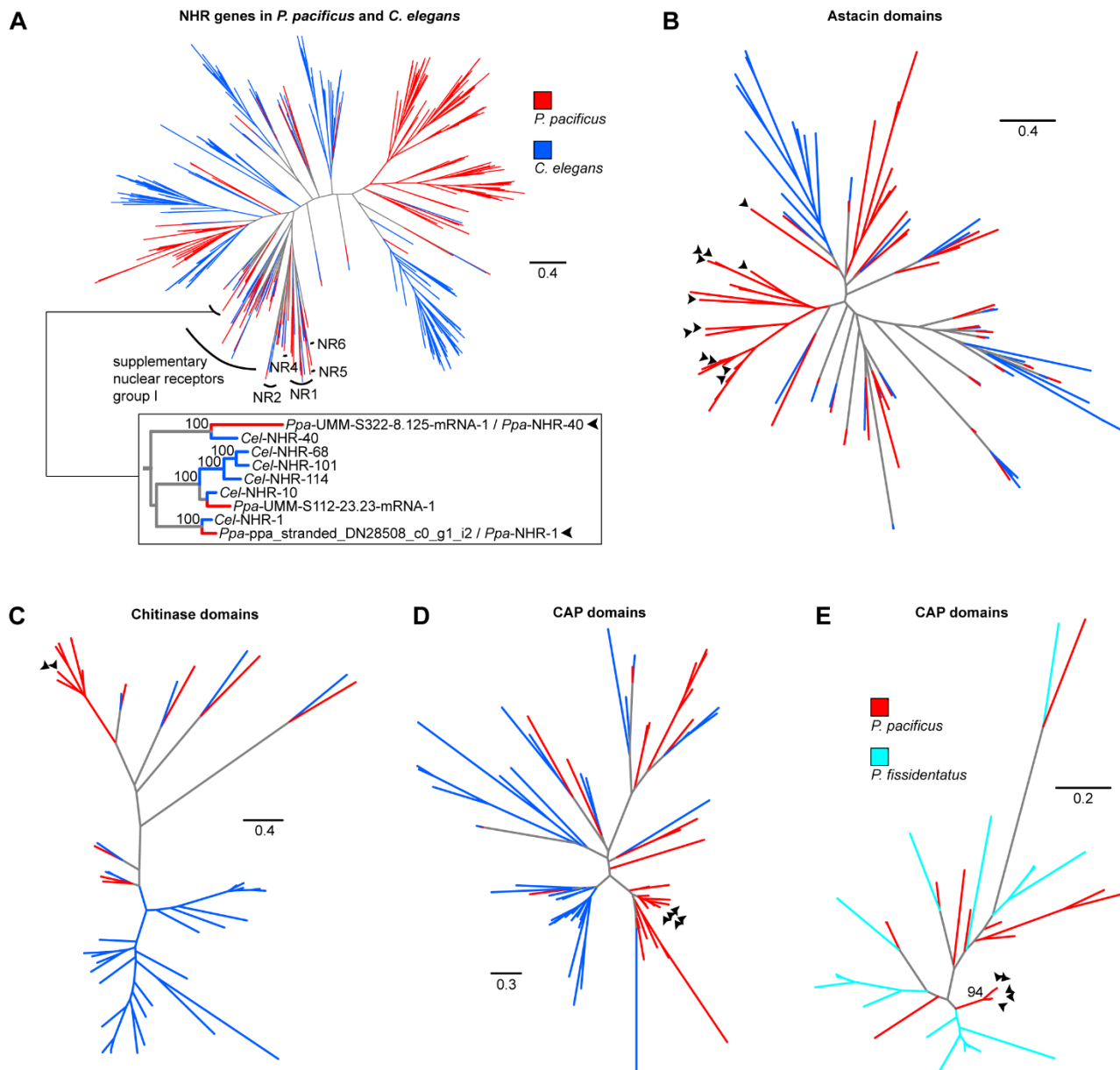
D = dorsal, V = ventral, A = anterior, P = posterior, N.S. = not significant.



**Fig. 3.** Target genes of NHR-40 and NHR-1. **(A)** Experimental setup of transcriptomics experiment and selection criteria to identify target genes. **(B)** Trends among target genes compared to genome-wide pattern. **(C)** Transmission electron microscopy reconstruction of the dorsal pharyngeal gland cell (g1D)<sup>52</sup> and expression patterns of transcriptional reporters for nine selected targets of NHR-40 and NHR-1. TurboRFP channel is presented as standard deviation projections.  
*lof* = loss of function, *gof* = gain of function, \*\*\* =  $p < 0.001$ , D = dorsal, V = ventral, A = anterior, P = posterior.



**Fig. 4.** Evolution of pharynx morphology in the order Rhabditida.



**Fig. 5.** Evolution of *nhr-40*, *nhr-1*, and their target genes. Arrowheads point at the genes of interest. Protein-based trees of NHR genes (A), Astacin domains (B), chitinase domains (C), and CAP domains (D) in *P. pacificus* and *C. elegans*. (E) Nucleotide-based tree of the CAP domains from a poorly-resolved protein-based subtree of all predicted CAP domains in *P. pacificus* and *P. fissidentatus*.

Table 1

Medium	Genotype	Eu, %	N
NGM agar	wild type PS312	98	650
NGM agar	<i>nhr-40(tu505)</i>	100	100
NGM agar	<i>nhr-40(tu505) tu515</i>	0	136
NGM agar	<i>tu515</i>	0	136
NGM agar	<i>nhr-1(tu1163)</i>	0	133
NGM agar	<i>nhr-1(tu1164)</i>	0	140
NGM agar	<i>nhr-1(tu1163)/tu515</i>	0	70
NGM agar	<i>nhr-1(tu1163);tuEx305[nhr-1(+);egl-20p::TurboRFP]</i>	85	110
NGM agar	<i>nhr-1(tu1163);tuEx310[nhr-1(+);egl-20p::TurboRFP]</i>	86	112
NGM agar	<i>nhr-1(tu1163);tuEx328[nhr-1(+);HA;egl-20p::TurboRFP]</i>	86	150
NGM agar	<i>nhr-40(tu505) nhr-1(tu1163)</i>	2	134
NGM agar	<i>nhr-40(tu1418)</i>	0	150
NGM agar	<i>nhr-40(tu1419)</i>	0	150
NGM agar	<i>nhr-40(tu1420)</i>	0	150
NGM agar	<i>nhr-40(tu1423)</i>	0	150
NGM agar	<i>nhr-40(tub6)</i>	100	100
NGM agar	<i>nhr-40(tu1421)</i>	100	150
NGM agar	<i>nhr-40(tu1422)</i>	100	100
NGM agar	duodecuple Astacin mutant	98	55
NGM agar	quintuple CAP mutant	94	50
NGM agar	<i>PPA04200(tu1213) PPA39293(tu1214)</i>	100	50
NGM agar	<i>PPA04200(tu1216) PPA39293(tu1217)</i>	100	50
NGM agar	<i>PPA27560(tu1475)</i>	100	51
NGM agar	<i>PPA27560(tu1476)</i>	100	53
NGM agar	<i>PPA30108(tu1230)</i>	100	50
NGM agar	<i>PPA30108(tu1231)</i>	100	50
NGM agar	<i>PPA30435(tu1477)</i>	100	48
NGM agar	<i>PPA30435(tu1478)</i>	98	54
NGM agar	<i>PPA38892(tu1473)</i>	100	50
NGM agar	<i>PPA38892(tu1474)</i>	100	50
S-medium	wild type PS312	5	850
S-medium	<i>nhr-40(tu505)</i>	100	150
S-medium	<i>nhr-40(tu1418)</i>	0	150
S-medium	<i>nhr-40(tu1419)</i>	0	150
S-medium	<i>nhr-40(tu1420)</i>	0	150
S-medium	<i>nhr-40(tu1423)</i>	0	150
S-medium	<i>nhr-40(tub6)</i>	100	150
S-medium	<i>nhr-40(tu1421)</i>	100	150
S-medium	<i>nhr-40(tu1422)</i>	100	150

Table 2

Chromosome	Wormbase WS268 Identifier	EI Paco annotation v1 Identifier	Predicted PFAM domains	Log fold change, <i>nhr-1</i> vs. wild type, determination time point	Log fold change, <i>nhr-1</i> vs. wild type, differentiation time point	Log fold change, <i>nhr-40</i> loss-of-function vs. wild type, determination time point	Log fold change, <i>nhr-40</i> loss-of-function vs. wild type, differentiation time point	Log fold change, <i>nhr-40</i> gain-of-function vs. wild type, determination time point	Log fold change, <i>nhr-40</i> gain-of-function vs. wild type, differentiation time point
ChrX	PPA05669	UMM-S328-9.28-mRNA-1	Astacin	-6.240745897	-7.986635198	-7.210397982	-7.830279165	2.097757153	1.095985392
ChrIV	PPA42525	UMM-S2847-7.46-mRNA-1	Astacin	-4.841545383	-4.002165721	-7.839896476	-4.679857886	1.438839716	not significant
ChrIV	PPA05955	UMM-S2847-6.45-mRNA-1	Astacin	-4.032019323	-3.717834286	-6.639723524	-5.994683399	1.574571506	1.061560179
ChrX	PPA05618	UMM-S328-7.47-mRNA-1	Astacin	-3.574438844	-2.916374055	-7.745129989	-7.021294279	1.499309832	0.981934683
ChrX	PPA16331	UMA-S293-8.46-mRNA-1	Astacin	-2.698652128	-4.119198644	-4.60891813	-4.200736918	1.709837147	not significant
ChrX	PPA39735	UMM-S328-10.33-mRNA-1	CAP	-2.204596416	-2.494207229	-5.147759463	-5.268476177	1.413344004	not significant
ChrI	PPA32730	UMM-S57-4.91-mRNA-1	Astacin	-2.198833674	-1.648291234	-3.691078965	-3.030762239	1.685906094	0.781158675
ChrX	PPA13058	UMM-S328-10.78-mRNA-1	CAP	-2.072130667	-2.430015473	-4.777644344	-4.798862505	1.417106528	not significant
ChrIV	PPA39293	UMM-S283-11.38-mRNA-1	Glyco_hydro_18	-1.78798789	-1.00008438	-2.965102834	-1.920988766	0.91017037	not significant
ChrX	PPA29522	UMM-S322-3.5-mRNA-1	CAP	-1.6191243845	not significant	-3.144502729	-1.492861278	0.989651252	not significant
ChrX	PPA39470	UMM-S293-11.30-mRNA-1	CAP	-1.536560433	-2.217536886	-4.429166678	-4.946006302	1.368183938	not significant
ChrX	PPA21910	UMA-S322-3.38-mRNA-1	CAP	-1.360420618	not significant	-2.333203285	-1.220283993	0.933478914	not significant
ChrIV	PPA04200	UMM-S283-11.45-mRNA-1	Glyco_hydro_18; MFS_1	-1.260777947	-0.790389646	-2.139628634	-1.317888252	0.858388853	not significant
ChrX	PPA21987	UMA-S322-7.39-mRNA-1	Astacin	-1.088820963	-1.133945969	-1.538105635	not significant	0.872393205	0.857569687
ChrX	PPA27985	UMS-S2861-1.50-mRNA-1	Astacin	-1.024169798	-1.594338612	-1.391792386	-1.82457695	0.856234189	0.920390529
ChrX	PPA30708	UMS-S328-0.4-mRNA-1	none	-0.947256862	-1.972768009	-1.664145426	-3.055668539	1.366224893	0.725689297
ChrII	PPA27560	UMS-S10-46.25-mRNA-1	none	not significant	-2.543527944	not significant	-1.495616763	1.576247265	not significant
ChrI	PPA30435	UMM-S57-36.5-mRNA-1	Lecln_C	not significant	-2.47831085	-6.394747605	-7.474747473	1.742988659	0.834485758
ChrX	PPA34430	UMA-S2861-1.27-mRNA-1	Astacin	not significant	-2.034806495	-1.356487112	-2.16601043	0.954950699	0.920888012
ChrX	PPA38892	UMM-S250-3.76-mRNA-1	SHK	not significant	-1.978289093	-1.766865626	-2.237851004	0.974881875	not significant
ChrX	PPA20266	UMM-S2857-0.30-mRNA-1	Astacin	not significant	-1.886347752	-1.391984972	-2.526477858	1.083601827	not significant
ChrX	PPA42924	UMM-S2857-0.41-mRNA-1	Astacin	not significant	-1.144915887	not significant	-1.729807349	0.848434069	not significant
ChrI	PPA03932	UMM-S7-5.16-mRNA-1	Astacin	not significant	-1.111093666	not significant	-1.621750021	1.106139077	0.751674205
ChrIV	PPA06264	UMA-S2838-46.74-mRNA-1	adh_short; KR; THF_DHG_CYH_C	not significant	2.240401693	not significant	2.955986944	-2.049882222	not significant