

1 Gene Cascade Finder: A tool for identification of gene cascades
2 and its application in *Caenorhabditis elegans*

3 Short title: GCF for gene cascade identification

4

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15

16 **Abstract**

17 Obtaining a comprehensive understanding of the gene regulatory networks, or gene cascades,
18 involved in cell fate determination and cell lineage segregation in *Caenorhabditis elegans* is a long-
19 standing challenge. Although RNA-sequencing (RNA-Seq) is a promising technique to resolve these
20 questions, the bioinformatics tools to identify associated gene cascades from RNA-Seq data remain
21 inadequate. To overcome these limitations, we developed Gene Cascade Finder (GCF) as a novel
22 tool for building gene cascades by comparison of mutant and wild-type RNA-Seq data along with
23 integrated information of protein-protein interactions, expression timing, and domains. Application
24 of GCF to RNA-Seq data confirmed that SPN-4 and MEX-3 regulate the canonical Wnt pathway
25 during embryonic development. Moreover, *lin-35*, *hsp-3*, and *gpa-12* were found to be involved in
26 MEX-1-dependent neurogenesis, and MEX-3 was found to control the gene cascade promoting
27 neurogenesis through *lin-35* and *apl-1*. Thus, GCF could be a useful tool for building gene cascades
28 from RNA-Seq data.

29

30 **Introduction**

31 Spatially and temporally regulated gene expression is essential to precisely modulate cellular
32 behaviors during development in multicellular organisms. Elucidating gene cascades during early
33 embryonic development may improve our understanding of mechanisms of cell fate determination
34 and lineage segregation [1-3]. The nematode *Caenorhabditis elegans*, a model organism of
35 development research, comprises 959 cells in adult hermaphrodites with robustness and
36 reproducibility of the cell lineage [3]. Additionally, over 80% of the *C. elegans* proteome shows
37 homology with human proteins [4], providing a particularly valuable model organism for studies of
38 the developmental system.

39 PAR proteins, which are expressed immediately after fertilization, are associated with
40 formation of the anterior-posterior polarity of P0 cells and control the localization of polarity
41 mediators such as SPN-4, MEX-1, and MEX-3 in *C. elegans* [5]. Aberrant expression of the genes
42 encoding these proteins affects cellular and developmental regulation, leading to embryonic lethality
43 in early embryogenesis [6-9]. Specifically, SPN-4 is localized in all blastomeres at the four-cell stage
44 and plays essential roles in axial rotation [8, 10-12]; MEX-1 is expressed in the P1 blastomere, and
45 loss of its function leads to excessive muscle formation [7, 13]; and MEX-3 is expressed in the AB
46 blastomere, and causes excessive muscle formation and hatching failure in mutants [14-16].
47 Although polarity mediators regulate protein synthesis by binding to the 3'-untranslated region of the
48 target mRNA, it is difficult to directly identify their associated gene cascades.

49 Conventional genetic and molecular biological approaches have focused on the target gene to
50 be identified and have clarified functions and identified related genes, representing a bottom-up
51 approach. Both functional analysis of individual genes and comprehensive analysis of the genome
52 are indispensable for identification of gene cascades. After determination of the whole genome
53 sequence of *C. elegans* in 1998, genome-wide analysis via a top-down approach was made possible

54 [17], representing the beginning of the post-genome sequencing era. Transcriptomics via DNA
55 microarray analysis [18], proteomics via mass spectrometry analysis [19], and phenomenon analysis
56 by RNA interference [20] have been extensively reported. Furthermore, several methods for
57 comprehensive analyses have been developed, including protein-protein interaction analysis using a
58 yeast two-hybrid system and phage display [21, 22] and multiple mutation analysis using knockdown
59 mutants. At the same time, the WormBase database was constructed to integrate the vast quantities of
60 data obtained from these genome-wide analyses [23]. Accordingly, the development of new
61 technologies and methodologies has enabled the accumulation of detailed genome-wide data.

62 Next-generation sequencing (NGS) has now replaced conventional Sanger sequencing [24].
63 Conventional Sanger sequencing can simultaneously analyze 8–96 sequencing reactions, whereas
64 NGS can simultaneously run millions to billions of sequencing reactions in parallel. This technique
65 can dramatically and quickly determine the gene sequences in organisms whose whole-genome
66 sequences have already been determined. Even at the laboratory level, genomic sequencing results
67 can be produced in only a few days, enabling researchers to obtain genome-wide information rapidly.
68 Furthermore, RNA sequencing (RNA-Seq) has recently been developed to measure gene expression
69 levels by counting the number of sequence reads obtained from converting RNA into cDNA [25].
70 Existing RNA-Seq data analysis tools include RSeQC [26], which measures the quality control of the
71 obtained data; Cufflinks [27], which involves genome mapping; and IsoEM [28], which identifies
72 isoforms within a dataset. These tools can be used to identify gene expression variations from RNA-
73 Seq data. TopGO (Alexa et al., 2006) is an analytical tool used to identify gene function based on
74 RNA-Seq data and can confirm the functions of genes with varying expression levels. In addition,
75 Cascade R was established to identify the gene cascade of a query gene [29]. Cascade R constructs
76 an intergenic network of knockout genes from the results of DNA microarray analysis. However, it
77 requires multiple timeline datasets from microarray analyses.

78 Genes, especially those expressed in early embryogenesis, function in chronological order
79 rather than having only a single function, and genes responsible for functional expression often exert
80 their effects at the bottom of gene cascades. STRING [30], BIOGRID [31], and WormBase [23] are
81 databases of protein-protein interactions and the gene-dependent regulation of transcription and
82 translation. In order to predict genetic cascades from these databases, researchers currently must
83 perform separate analyses. Moreover, although RNA-Seq can be used to easily acquire large amounts
84 of data via a semi-automatic process, the subsequent analysis must be performed manually and is
85 therefore quite time-consuming. Therefore, the data acquisition capacity currently exceeds the data
86 analysis capacity. Accordingly, automation of the analysis using bioinformatics tools is an important
87 research subject.

88 In this study, we performed RNA-Seq analysis of the polarity mediator mutants *spn-4*, *mex-1*,
89 and *mex-3* in *C. elegans*. Next, we developed a novel tool, Gene Cascade Finder (GCF), to extract
90 genes with a high probability of being directly or indirectly regulated by these polarity mediators.
91 Finally, the gene cascade and its validity were examined.

92

93 **Methods**

94

95 **Strains**

96 *C. elegans* N2, *mex-1* (or286), and *mex-3* (eu149) strains, and *Escherichia coli* OP50 strain were
97 provided by the Caenorhabditis Genetics Center (<https://cgc.umn.edu/>), and the *C. elegans* *spn-4*
98 (tm291) strain was provided by National BioResource Project [32].

99

100 **Culture of *C. elegans* and synchronization at the early embryo stage**

101 All strains except for *mex-1* (or286) were cultured on nematode growth medium agar coated with *E.*
102 *coli* OP50 at 20°C. Because *mex-1* (or286) strain is a temperature-sensitive mutant strain, it was
103 cultured at 15°C to strengthen its phenotype [13]. Furthermore, all strains were transferred to S-Basal
104 solution inoculated with *E. coli* OP50 at 20°C for large culture. To obtain early embryos from the
105 culture medium, when *C. elegans* adults had only 3–5 eggs, they were synchronized using an alkaline
106 bleaching method, and the early embryos were recovered [33]. These *C. elegans* early embryos were
107 used as the samples for RNA-Seq analysis.

108

109 **RNA-Seq analysis**

110 The mRNAs of the synchronized *C. elegans* early embryos were purified using RNeasy Minikit
111 (Qiagen NV, Venlo, the Netherlands). Purified mRNAs were reverse-transcribed into cDNAs,
112 amplified by polymerase chain reaction, and fragmented using a TruSeq RNA Sample Prep Kit
113 (Illumina, Inc.). The amplified cDNAs were sequenced using Hi-Seq2000 (Illumina, Inc.) and the
114 sequenced cDNAs were mapped to the *C. elegans* genome sequence and counted according to

115 WormBase (WS190) [23] using DNAnexus. Using this procedure, the mRNA expression levels were
116 obtained as reads per kilobase of exon per million mapped reads (RPKM) [34]. The gene name and
117 RPKM values of wild-type and mutant genes were filed for input data in GCF (S1 Table).

118

119 **Comparative quantitative gene expression analysis**

120 Expression levels of gene i in the wild-type and mutant were defined as x_i and y_i , respectively, and
121 the change rates in these gene expression levels (R_i) were determined as shown in Equation 1.
122 Because the data obtained by RNA-Seq analysis had a non-normal distribution, the data were
123 subjected to non-parametric tests using Equations 2a and 2b.

$$124 \quad R_i = y_i/x_i \quad (i = 1, \dots, N) \quad (1)$$

$$125 \quad R_i < M_i - Q_i, \quad M_i + Q_i < R_i \quad (2a)$$

$$126 \quad M_i - Q_i < R_i < M_i + Q_i \quad (2b)$$

127 Where N is the number of genes, and M_i and Q_i are the median and quartile deviation, respectively.
128 Genes that satisfied the condition of Equation 2a were assumed to show expression level fluctuations,
129 and genes satisfying the condition of Equation 2b were assumed to not show expression level
130 fluctuations.

131

132 **Dataset for the software**

133 Information on the expression timing and interactions of all genes in *C. elegans* was extracted from
134 WormBase (Version 256) [23] using the application programming interface. The total transcription
135 factors of *C. elegans* were acquired from the gene ontology database Amigo 2
136 (<http://amigo.geneontology.org/amigo>) [35] using the keyword search “GO: 0006351”. Furthermore,

137 all gene IDs, protein IDs, and domain information from Pfam (<https://pfam.xfam.org/>) [36] in *C.*
138 *elegans* required for functional analysis were extracted from UniProt [37].

139

140 **Direct target prediction by GCF**

141 GCF was developed by the algorithm shown in Fig. 1. First, the candidate genes of the target of the
142 query gene were found from transcription factors and genes with no gene expression level
143 fluctuations, as calculated by Equation 2b, with the same cellular localization and phenotype. Query
144 genes bind to target mRNAs to regulate their translation. Thus, the mRNA expression levels of the
145 target genes showed no changes. In addition, the target genes needed to be expressed with the same
146 timing as the query gene because the query gene is directly bound to the target mRNA. Furthermore,
147 the gene cascade of the target genes was mostly consistent with the query gene cascade, suggesting
148 that the target gene may have the same phenotype as the query gene. Therefore, to expand the gene
149 cascade, the target gene should be a transcription factor with downstream genes.

150

151 **Downstream gene identification by GCF**

152 The search for downstream genes was carried out as follows. First, the genes from transcription or
153 protein-protein interactions were extracted as downstream gene candidates of the target gene. Second,
154 the expression timing of the candidate genes was checked. Only candidate genes noted as being
155 expressed in early embryos or in embryos in WormBase were defined as downstream genes of the
156 query's target genes. These first two steps were then repeated to obtain the next downstream genes.
157 Finally, the procedure was repeated until there were no genes left to be extracted to obtain the final
158 gene. Lastly, GCF output the cascade data (S2–S4 Tables). An example showing input obtained from
159 output data from GCF to Cytoscape [38] is provided in Fig. 2.

160

161 **Specific domain search from the constructed gene cascade**

162 Each direct target gene was rooted, and the functions of their bottom genes were investigated using
163 Pfam [36] in UniProt [37]. The P-values of the domains from the bottom gene products were
164 evaluated using the same formula for Gene Ontology in Panther [39]. If the transcription-related
165 domain was extracted from a cascade, the cascade was no longer considered since it would be
166 functioning only after the early embryo stage.

167

168 **Results and Discussion**

169

170 **Development of Gene Cascade Finder**

171 The programming language Ruby was used to construct Gene Cascade Finder (GCF). The web
172 interface of GCF was written in Python. The input data for GCF were data from the wild-type and
173 mutant strains as shown in S1 Table. The output data from GCF were tables of discovered gene
174 cascades (S2–S4 Tables), the data input into Cytoscape (S5 Table), and gene cascade-specific
175 domains and their gene cascades (S6 Table). GCF is available at <http://www.gcf.sk.ritsumei.ac.jp>

176

177 **Analysis of mRNA expression by comparative RNA-Seq**

178 To explore polarity mediator-dependent mechanisms, the effects of deficiencies in polarity mediators
179 were analyzed by performing RNA-Seq analysis in early embryos. From the results of comparative
180 RNA-Seq of the wild-type strain and the *spn-4*, *mex-1*, and *mex-3* mutant strains, 15,288, 15,265, and
181 15,005 genes were identified, respectively (S7 Table). In these gene groups, expression level
182 fluctuations were calculated by examining the median \pm quartile deviations. From this analysis, 6,417
183 genes distributed at $-0.65 < \log_2(\text{RNA expression level ratio}) < 0.69$ in the *spn-4* gene, 6,456 genes
184 distributed at $-0.74 < \log_2(\text{RNA expression level ratio}) < 0.82$ in the *mex-3* gene, and 6,491 genes
185 distributed at $-0.82 < \log_2(\text{RNA expression level ratio}) < 1.10$ in the *mex-1* gene were defined as
186 genes showing no expression level variations (S8 Table).

187

188 **Gene cascade prediction using Gene Cascade Finder**

189 As shown in S1 Table, gene cascade prediction was performed by inputting data obtained by
190 comparative RNA-Seq into GCF. GCF can predict gene cascades by continuously integrating the
191 results from RNA-Seq along with data on gene expression and intermolecular interactions from
192 WormBase. In total, 127, 180, and 226 gene cascades were predicted from 6,418, 6,457, and 8,513
193 genes from the comparative analysis of the *spn-4*, *mex-1*, and *mex-3* mutant strains, respectively (Fig.
194 3 and S6–S8 Tables).

195

196

197 **Extraction of gene cascade-specific domains using Gene Cascade** 198 **Finder**

199 The genes and domains located at the bottom of the gene cascade were extracted for functional
200 analysis of the predicted gene cascade (S9–S11 Tables). Overall, 53, 146, and 143 genes with 32, 34,
201 and 54 specific domains as the bottom gene were extracted from the gene cascades in the *spn-4*, *mex-*
202 *1*, and *mex-3* mutants, respectively.

203

204 **Domain analysis of *spn-4*, *mex-1*, and *mex-3* cascades**

205 To predict the functions of the gene cascades, we focused on the functions of genes localized at the
206 bottom of the gene cascade by analyzing the domains of the gene products using the Pfam protein
207 family database. The functions of the 53 SPN-4-mediated genes were obtained as bottom genes to
208 calculate the functional trends in the *spn-4* cascade. Within the 53 bottom genes, 32 domains were
209 classified based on information from the Pfam database (Table 1) (Bateman et al., 2004). When we

210 calculated the numbers of these genes, transcription and signal transduction were obtained at high
211 frequency.

212 Similarly, in the gene cascade of 146 *mex-1*-mediated genes, 27 domains were classified and
213 obtained from the domain analyses to have functions in early embryonic development, cell division,
214 transcription, DNA replication, and signal transduction (Table 1). In contrast, in the gene cascades of
215 the 143 *mex-3*-mediated genes, 54 domains were classified and obtained from the domain analyses to
216 have functions in development, cell cycle, transcription, and signal transduction (Table 1).

217

218 **Evaluation of GCF by assessment of gene cascades in the canonical** 219 **Wnt signaling pathway**

220 Next, we focused on genes involved in SPN-4-mediated signal transduction (Fig. 4A). First, we
221 found that MOM-2, a nematode homolog of the Wnt ligand, is involved in the signal transduction
222 cascade (Table 1). Since both SPN-4 and MOM-2 were previously reported to regulate EMS cell
223 lineage formation and spindle orientation [11, 40], we hypothesized that the SPN-4/MOM-2 gene
224 cascade may have an essential role in early embryogenesis and may regulate the Wnt signaling
225 pathway. Moreover, because OMA-1, MEX-1, and PIE-1, which are known to be essential for
226 MOM-2 expression in embryonic development [40], were also identified in this pathway, the GCF-
227 mediated gene cascade prediction was assumed to be accurate.

228 Similarly, *unc-37*, which encodes a Groucho/TLE homolog that suppresses Wnt signaling,
229 was found as the “bottom gene” in the *spn-4* and *mex-3* cascades (Fig. 4A, B). Thus, we propose that
230 GCF-mediated gene cascade prediction may be useful for identification of gene cascades involved in
231 *C. elegans* embryonic development.

232

233 **Examples of the application of GCF for prediction of new biological**
234 **functions involved in a gene cascade**

235

236 **Endoplasmic reticulum (ER) stress response pathway in a MEX-1-mediated gene**
237 **cascade**

238 Because cell division and DNA replication are regulated by a MEX-1-dependent cascade, we further
239 focused on this signal transduction cascade. In the gene cascade related to signal transduction, *paqr-2*,
240 which encodes an adiponectin receptor, was isolated (Fig. 4C). Interestingly, a stress response
241 pathway is known to regulate stress responses in both mouse and *C. elegans* embryogenesis [40, 41].
242 Thus, an evolutionarily conserved gene cascade against environmental stress may be identified using
243 GCF. Moreover, because adiponectin receptor regulates insulin signaling [42], it is likely that MEX-
244 1/PAQR-2-mediated gene cascades may be involved in ER stress tolerance signaling within or
245 parallel to the insulin signaling pathway during embryogenesis.

246

247 ***lin-35*, *hsp-3*, and *gpa-12* in a MEX-1/DPY-23-mediated gene cascade in neuronal**
248 **development**

249 In a MEX-1/DPY-23-mediated gene cascade, the *mex-1*, *lin-14*, *let-60*, *ces-2*, *unc-13*, and *dpy-23*
250 mutants were shown to exhibit specific phenotypes in neuronal development (Fig. 4D) [12, 43-45].
251 Thus, six of the nine genes of this gene cascade were shown to have essential roles in neuronal
252 development, indicating that MEX-1/DPY-23-mediated gene cascades may regulate neuronal
253 function. Although their roles in neuronal development have not yet been investigated, locomotion
254 defects have been reported in *hsp-3* and *gpa-12* mutants [46, 47]. Similarly, the *lin-35* (n745)
255 mutation has been shown to enhance the neuronal phenotype of the neuronal regulator genes *dpy-13*

256 and *unc-104* [48]. Thus, *lin-35*, *hsp-3*, and *gpa-12* may be involved in a DPY-23-mediated gene
257 cascade in neuronal development in embryos [45]. However, further studies are required to examine
258 this possibility.

259

260 **MEX-3/APL-1-mediated neuronal patterning and MEX-3/CDC-14-mediated cell** 261 **fate determination in the MEX-3-mediated gene cascade**

262 Because MEX-3 is specifically expressed in AB cells at the four-cell stage, spatiotemporal-regulated
263 synaptic formation defects in *hbl-1* mutants and *apl-1*-dependent embryonic neuronal patterning may
264 be elucidated by identifying MEX-3/APL-1-mediated gene cascades (Fig. 4E) [49-51]. In parallel,
265 when we focused on the MEX-3/CDC-14-mediated gene cascade (Fig. 4E), CDC-14B, a zebrafish
266 homolog of CDC-14, was shown to be involved in formation of the cilium in sensory neurons [50].
267 Because sensory neurons have cilia in *C. elegans* [52], CDC-14 may be involved in an evolutionarily
268 conserved signaling pathway. Similarly, the *lin-35* (n745) mutation was shown to enhance the
269 neuronal phenotype of neuronal regulator genes [48]. Thus, *lin-35* may be involved in a MEX-
270 3/CDC-14-mediated gene cascade in sensory neuron development. Accordingly, our findings
271 suggested that GCF may be useful for predicting the comprehensive functions of query genes and for
272 identification of new genes involved in known gene cascades.

273

274 **Conclusion**

275 In this study, we created a software program called GCF, which could comprehensively identify
276 genes downstream of the query genes by integrating RNA-Seq data and previously characterized data
277 from WormBase. Using GCF, we analyzed gene cascades of the polarity mediator proteins SPN-4,
278 MEX-1, and MEX-3, and identified 127, 180, and 226 putative gene cascades, respectively. By

279 analyzing the functions of these gene cascades, we confirmed that SPN-4 and MEX-3 regulate the
280 canonical Wnt pathway during embryonic development. Furthermore, we found that the ER stress
281 response and motor neuron development are regulated by MEX-1-dependent cascades, and that
282 neural development is regulated by MEX-3-dependent cascades. Although we used GCF only to
283 evaluate SPN-4, MEX-1, and MEX-3 functions in this study, the method is applicable for other
284 translation or transcription factors involved in early embryogenesis. In addition, GCF provides a
285 general method for predicting the functions of genes involved in a gene cascade during *C. elegans*
286 embryonic development. Taken together, we propose that our strategy using the GCF tool offers a
287 reliable approach for comprehensively identifying networks of embryo-specific gene cascades in *C.*
288 *elegans*. Importantly, GCF can also be applied to humans and other model organisms such as mice
289 and *Drosophila*.

290 In the future, by expanding the algorithm to fit the cell lineage-segregation of *C. elegans* [3],
291 we will be able to predict the precise gene cascades reflecting four-dimensional (spatial and temporal)
292 regulation [53]. Combinational analysis of GCF and molecular biology techniques such as RNA-pull
293 down assays, fluorescent *in situ* hybridization, and phenotypic characterization of the mutants may be
294 required to build a more reliable regulatory network for these gene cascades [54, 55].

295

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301 **References**

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513

514

515 **Table 1.** Scores of the functional characterization of *spn-1*-, *mex-1*-, and *mex-3*-mediated gene
516 cascades by domain analysis.

517

	<i>spn-4</i>	<i>mex-1</i>	<i>mex-3</i>
Transcription	25	6	4
Signal transduction	5	3	12
Development	-	2	7
Cell cycle	-	3	5
Cell division	-	-	-
DNA replication	-	2	3
Transport	-	3	-
Others	2	12	18
Unknown	-	3	4

518

519 Properties of the gene product of the bottom genes were calculated by domain analysis. The sum of
520 the characteristic features of each cascade ($P < 0.05$) was then calculated. Domains with a score less
521 than 1 were classified as “Others”.

522

523 **Figure legends**

524 **Fig. 1.** Schematic for prediction of the gene cascade. Application protocol for GCF. Genes in the
525 predicted cascade are indicated by red frames. To identify the entire gene network, we repeatedly
526 identified the downstream genes. The genes surrounded by black frames were required to identify the
527 genes surrounded by red frames. The information of the labels with asterisks was extracted from
528 WormBase.

529

530 **Fig. 2.** Representative example of the graphic output from GCF. Graphical examples from the GCF
531 software were further processed using Cytoscape, allowing for identification of an output without
532 adding a new input.

533

534 **Fig. 3.** Schematic illustrations of polarity mediator-dependent gene cascades during *C. elegans*
535 embryogenesis. Rendering of each gene cascade was performed using Cytoscape. Nodes indicate
536 each gene in the cascade. Edges indicate the interactions between two genes. Large and intermediate
537 nodes indicate the query genes and the direct target of the query genes, respectively. Other nodes
538 indicate downstream genes. Green nodes indicate genes that are expressed during the early
539 embryonic stage. Purple nodes indicate presumptive early embryonic genes. Red, blue, and black
540 edges indicate positive regulation, negative regulation, and genetic interactions, respectively. Dotted
541 lines indicate protein-protein interactions. (A) *spn-4*-mediated gene cascade. (B) *mex-1*-mediated
542 gene cascade. (C) *mex-3*-mediated gene cascade.

543

544 **Fig. 4.** Typical examples of SPN-4, MEX-1, and MEX-3-dependent gene cascades. (A) SPN-4-
545 mediated gene cascade regulates Wnt signaling. (B) MEX-3-mediated gene cascade negatively
546 regulates Wnt signaling. (C) MEX-1-mediated gene cascade regulates endoplasmic reticulum-

547 associated degradation (ERAD) of folding-deficient proteins. (D) MEX-1-mediated gene cascade
548 regulates neuronal development. (E) MEX-3-mediated gene cascade regulates neuronal development.
549

Figure 1

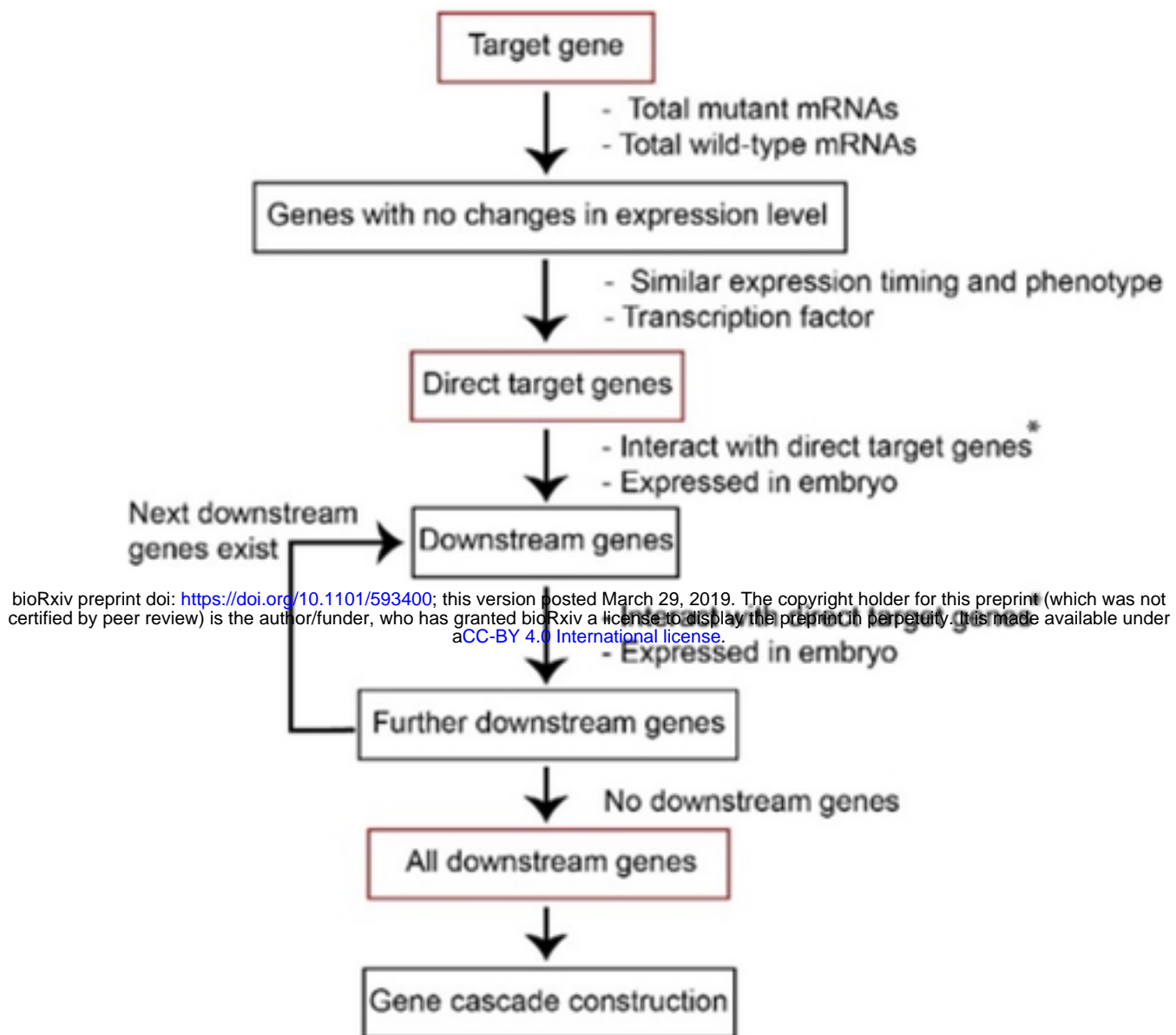
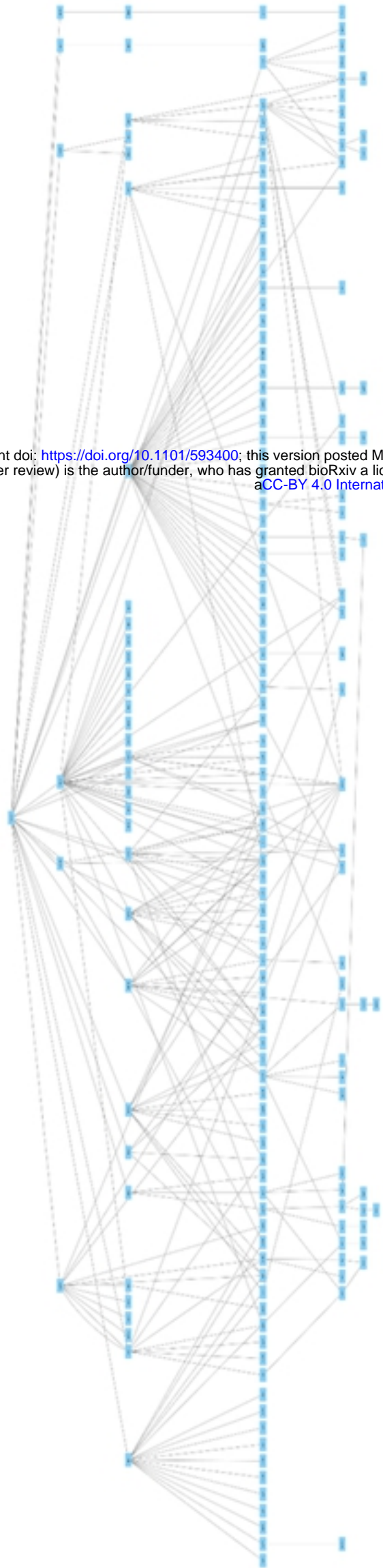


Figure 2



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Figure 3

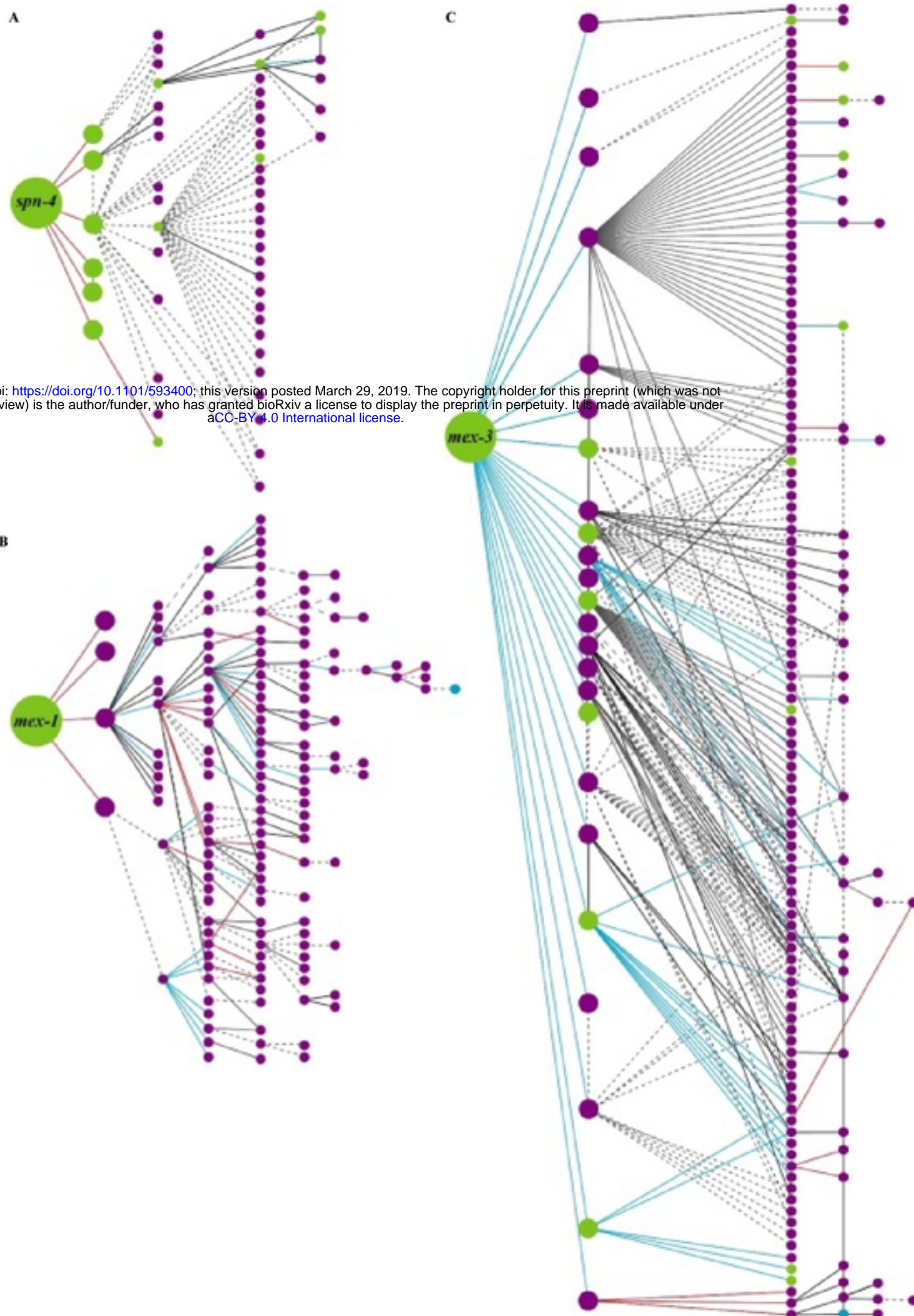
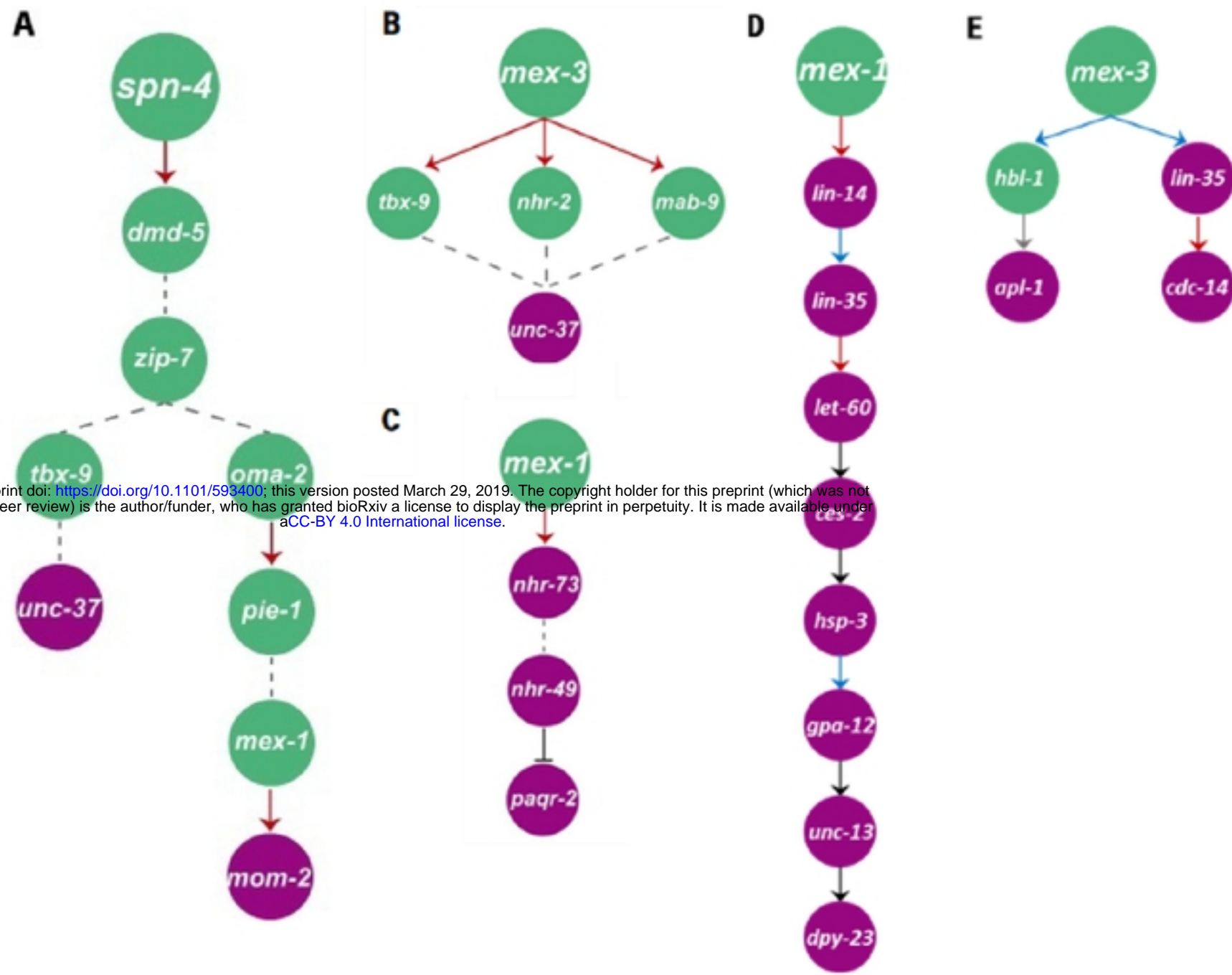


Figure 4



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