# Co-option of the Limb Patterning Program in Cephalopod Lens Development

Stephanie Neal*, Kyle J. McCulloch*, Francesca Napoli, Christina M. Daly, James H. Coleman, Kristen M. Koenig

*These authors contributed equally to this work

## Affiliations

John Harvard Distinguished Science Fellowship Program, Harvard University, Cambridge, MA 02138 USA.

Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138 USA

## Corresponding author email address

kmkoenig@fas.harvard.edu

**Keywords:** Cephalopod, lens, Distal-less, SP6-9, co-option, Eye evolution, limb outgrowth

**Abstract**

Across the Metazoa, similar genetic programs are found in the development of analogous, independently evolved, morphological features. The functional significance of this reuse and the underlying mechanisms of co-option remain unclear. Here we identify the co-option of the canonical bilaterian limb pattering program redeployed during cephalopod lens development, a functionally unrelated structure. We show radial expression of transcription factors *SP6-9/sp1, Dlx/dll, Pbx/exd, Meis/hth,* and a *Prdl* homolog in the squid *Doryteuthis pealeii*, similar to expression required in *Drosophila* limb development. We assess the role of Wnt signaling in the cephalopod lens, a positive regulator in the developing limb, and find the regulatory relationship reversed, with ectopic Wnt signaling leading to lens loss. This regulatory divergence suggests that duplication of SP6-9 in cephalopods may mediate this co-option. These results suggest that the limb network does not exclusively pattern appendage outgrowth but is performing a more universal developmental function: radial patterning.

## INTRODUCTION

In the Metazoa, homologous networks of transcription factors are necessary for the development of some analogous structures in distantly related taxa. The limb patterning program is an example of this developmental process homology (Shubin et al., 1997; Erwin & Davidson, 2002; Pueyo & Couso, 2005). The limb program was first identified in the development of the proximal-distal axis of the *Drosophila* leg. The transcription factor *SP6-9/sp1* is upstream of other program members, *Dlx/dll, Pbx/exd, Meis/hth, Dac* and *Arx/ar,* each required for patterning specific regions of limb outgrowth (Panganiban et al., 1994; Panganiban et al., 1997; Dong et al., 2001, Dong et al., 2002; Peuyo & Couso, 2005; Estella et al., 2012; Campbell & Tomlinson, 1998). This network is necessary in both vertebrate and cephalopod limb development and is expressed in a similar proximodistal pattern in a diversity of outgrowths (Panganiban et al., 1997; Shubin et al., 1997; Maas & Bei, 1997; Mercader et al., 1999; Panganiban & Rubenstein, 2002; Prpic, 2003; Angelini & Kaufman, 2005; Pueyo & Couso, 2005; Shubin et al., 2009; Moczek & Rose, 2009; Capellini et al. 2011; Lapan & Reddien, 2011; Ibarretxe et al., 2012; Grimmel et al., 2016; Sanz-Navarro et al., 2019; Ramanathan et al. 2018; Setton & Sharma; 2018; Tarazona et al., 2019; Prpic, 2019). This suggests that, although each appendage is not homologous, an outgrowth program may have been present in the ancestor. Current fossil evidence and the prevalence of limbless taxa does not support an ancestor with appendages and therefore the network's ancestral function remains unclear (Shubin et al., 1997; Erwin & Davidson, 2002; Pueyo & Couso, 2005). Many alternative hypotheses have been proposed, including an ancestral role in the nervous system, body axis formation and radial patterning (Minelli, 2000; Pueyo & Couso, 2005; Lemons et al. 2010; McDougall et al., 2011; Plavicki et al., 2016; Carroll et al., 1994; Erwin & Davidson, 2002). To understand the nature of this homology and how these co-option events occur, experiments with better sampling across the phylogeny of animals and greater diversity of developmental context are required.

Recent work identified a duplication of SP6-9 in cephalopods (McCulloch and Koenig, 2020). Both paralogs are expressed in the developing limb in the squid *Doryteuthis pealeii*, while one paralog, *DpSP6-9a*, shows unique expression in the lens-making cells during eye development (McCulloch and Koenig, 2020). With SP6-9 a known regulator in the limb patterning program, this new domain of expression could result in the co-option of the program in the cephalopod eye, providing a useful heterologous developmental context to better understand the network's function.

The image-forming eye is a classic example of biological complexity and the lens is a requisite innovation in all high-resolution visual systems (Darwin, 1859; Arendt, 2009; Dakin, 1928; Walls, 1939; Koenig & Gross, 2020; Nilsson, 2013; Jonasova & Kozmik, 2008). Cephalopods have a single-chambered eye, morphologically convergent with the vertebrate eye, composed of a cup shaped retina and a single refractive lens (Packard, 1972). Here we perform the first in-depth molecular description of lens development in the squid *Doryteuthis pealeii*, we identify spaciotemporal expression of the limb patterning program in the developing eye and lens, and we demonstrate a negative regulatory role of canonical Wnt signaling upstream of the program.

## RESULTS AND DISCUSSION

### Cephalopod Lentigenic Cell Differentiation and Early Anterior Segment Heterogeneity

The anterior of the cephalopod eye, or the anterior segment, is composed primarily of lens generating cells (lentigenic cells) (Williams, 1909; Arnold, 1967; Brahma, 1978). Lentigenic cells are arranged circumferentially around the developing lens and extend long cellular processes, fusing into plates to form the lens (Figure 1A) (Meinertzhagen, 1990; Williams, 1909; Arnold, 1965; Arnold, 1967; West et al., 1995). We identified the first evidence of differentiated lentigenic cells starting at late stage 21, using a previously described nuclear morphology, unique to one of the three lentigenic cell types (LC2) (Figure 1B) (Arnold, 1967; West et al., 1995; Koenig et al., 2016). The number of LC2 cells continues to grow until reaching pre-hatching stage (Stage 29). We performed staged *in situ* hybridization for a homolog of *DpS-Crystallin*, the most abundant family of proteins in the cephalopod lens (Chiou, 1984; West et al., 1994) (Supplemental Figure 1). The first evidence of expression corresponds to changes in nuclear morphology at stage 21 (Figure 1C).

We sought to understand the molecular heterogeneity of cells in the early developing anterior segment, of which nothing is currently known. Using previously published candidates and RNA-seq data, we performed *in situ* hybridization screens at stage 23 to identify unique cell populations (Koenig et al., 2016; Ogura et al., 2013). We find *DpSix3/6* at stage 23 expressed in the anterior segment in the distal cells that make a central cup (*cc*), as well as a marginal population of cells in the most proximal tissue (*pm*) (Figure 2B''). The proximal central cells lacking *DpSix3/6* expression correspond to the LC2 population (Figure 2A'' &B''). Asymmetry along the animal anterior-posterior axis in the eye is also apparent, with enrichment on the anterior side of the animal (Figure 2B''). We also find the gene *DpLhx1/5*, expressed in a distal-marginal population of cells

in the anterior segment (*dm*), and excluded from the distal central cup cells (*cc*) (Figure 2C''). Together these genes show distinct populations of cells present early in development and provide a helpful molecular map of the anterior segment tissue at this time point: central cup cells (*cc*), LC2 cells (*lc2),* proximal-marginal cells (*pm),* and distal-marginal cells (*dm*) (Figure 2) .

**Proximal-Distal Limb Patterning Genes in the Anterior Segment of the Cephalopod**

To assess whether genes involved in appendage patterning may be required for cephalopod lens development, we identified and performed *in situ* hybridization for the genes *Dlx, Pbx, Meis,* and *Dac* at stage 21 and 23 (Figure 2, Supplemental Figure 2). All genes were clearly expressed in the developing anterior segment and lentigenic cells with the exception of *DpDac* (Figure 2E-G, Supplemental Figure 2I-2J'). We find *DpDlx* and *DpSP6-9a* have overlapping expression, in the central cup cells (*cc*) and all proximal cells (LC2 and *pm*) (Figure 2D-E'''). *DpPbx* and *DpMeis* are both broadly expressed in the anterior segment during lens development, with *DpPbx* excluded from the LC2 cells (Figure 2F''& 2G'').

It is known that the transcription factor *aristaless* is necessary for the most distal tip of the *Drosophila* limb in the limb program (Campbell and Tomlinson, 1998). The evolutionary relationship of Prd-like homologs (Arx/Aristaless, Alx/Aristaless-like, Rx/Retinal Homeobox and Hbn/Homeobrain) is ambiguous across species (Schiemann et al., 2017). We identified three candidate Prd-like genes in *D. pealeii* and performed *in situ* hybridization for all three homologs, *DpHbn*, *DpPrdl-1* and *DpPrdl-2* (Supplemental Figure 2K, L) (Koenig et al, 2016). *DpHbn* is expressed in the anterior segment in the distal central cup cells (*cc*) while *DpPrdl-1* and *DpPrdl-2* are excluded from the eye (Figure 2H'' and Supplemental Figure 2C, C', K and L). *DpHbn's* central, distal expression recapitulates *aristaless* expression in the developing *Drosophila* limb.

Our data show that the majority of the proximal-distal patterning genes in the developing limb, including *SP6-9*, *Dlx*, *Meis*, *Pbx*, as well as the Prd-like homolog, *Hbn,* show expression in concentric and overlapping cell populations surrounding the developing lens in the squid (Figure 2). This pattern of expression is strikingly similar to the bullseye-like pattern of expression of these genes in the developing *Drosophila* limb imaginal disc and suggests a co-option of this regulatory program for a new function: patterning the cephalopod anterior segment and lens (Angelini & Kaufman, 2005).

**Canonical Wnt Signaling Genes Expressed During Anterior Segment Development**

The duplication of SP6-9 in cephalopods provides a substrate for the evolution of cis-regulation, which could result in novel expression of the limb patterning program in the cephalopod lens. In appendage outgrowth, active Wnt signaling is upstream of the expression of SP6-9 (Cohen, 1990; Estella et al., 2003). To assess whether Wnt may be acting upstream in the cephalopod anterior segment or whether novel regulatory mechanisms may be at play, we performed *in situ* hybridization for members of the Wnt signaling pathway at stage 21 and stage 23 (Figure 3, Supplemental Figure 3). We were interested in identifying cells in the anterior segment or in adjacent tissue that may be a source of the Wnt morphogen. We performed *in situ* hybridization for seven Wnt homologs, with most *Wnt* genes expressed in the retina (Figure 3A', 3C', 3D-G). *DpWnt8, DpWnt11* and *DpProtostome-specific Wnt* show the most robust retinal expression (3A', 3F & 3G) and *DpWnt7* is the only Wnt expressed in the anterior segment (Figure 3C). *DpWnt6* showed no evidence of expression in the developing eye (data not shown). These data support the hypothesis that Wnt signals emanating from neighboring tissues could regulate anterior segment development.

To identify cells with potential active Wnt signaling, we analyzed the expression of Fz genes, which encode a family of Wnt receptors. We find that *DpFz* receptors are expressed broadly throughout the embryo. A subset of these (e.g. *DpFz1/2/7*, *DpFz4*, and *DpFz5/8*) are expressed in the majority of cells in the anterior segment, while others, like *DpFz9/10*, are excluded from the anterior segment (Figure 3H-K, Supplemental Figure 3). On close examination we find that *DpFz5/8* is excluded asymmetrically in the anterior segment and may be important for anterior-posterior patterning (Figure 3J' & J''). *DpFz1/2/7* is excluded from the distal-marginal cells and central cup cells and interestingly, the central cup cells lacking *DpFz1/2/7* are those that express all the limb patterning program genes (Figure 3K'&K''). These data suggested that the exclusion of active Wnt signaling may be important in the cephalopod anterior segment, supporting a potential negative regulatory role for Wnt signaling.

**Ectopic Wnt Activation Leads to the Loss of the Lens**

To assess the hypothesis that Wnt signaling is playing a negative regulatory role in anterior segment development, we utilized well-characterized pharmacological compounds that act as agonists of the Wnt pathway (Hedgepeth et al. 1997; Klein & Melton; Sato et al., 2004). We empirically determined a working concentration of both LiCl (0.15M) and CHIR99021 (250um). We bathed embryos in the compound or vehicle control for 24 hours at stage 21, the onset of lentigenic cell differentiation, and immediately fixed thereafter. Embryos were sectioned and

assessed for phenotypes. Stage 21 control embryos show a thickened anterior segment, identifiable lentigenic cells, and small lens primordia (Figure 3L). LiCl-treated stage 21 embryos show a complete absence of lens formation: No anterior segment thickening, lentigenic cells, or lens tissue. These data suggest that ectopic Wnt pathway activation inhibits lens and anterior segment development (Figure 3L', Supplemental Figure 4A). CHIR99021 treatment showed similar phenotypes (Supplemental Figure 4A). We assessed LiCl treated and control animals for cell death and find little difference between control and treated eyes suggesting that toxicity is unlikely the reason for these phenotypic changes (Supplemental Figure 4B).

We were interested in the consequence of activating the Wnt pathway after lentigenic cell differentiation. We performed the same 24 hour LiCl exposure at stage 23 and find the lens smaller and the anterior segment less thick than control animals, but lentigenic cells and lens tissue remain identifiable. This suggests that ectopic Wnt signaling does not impact cell identity in differentiated lentigenic cells (Figure 3M & M').

The lack of lens growth in stage 21 treated animals may be a result of an imposed delay in lens formation or it may be a result of the loss of lens potential. To differentiate between these possibilities we allowed treated animals to recover. We bathed experimental and control embryos, at both stage 21 and 23, for 24 hours, washed out the solution and allowed animals to develop for an additional 48 hours. LiCl treated stage 21 embryos never recover a lens (Figure 4N & 4N') while LiCl treated stage 23 embryos do form a small but morphologically abnormal lens (Figure 4O & 4O'). This abnormal lens is larger than the lens found in animals immediately fixed after treatment, suggesting that existing lentigenic cells at stage 23 continue to contribute to lens formation and growth. However, because the stage 23 treated lens is markedly smaller than control, it suggests that further lentigenic cell differentiation is lost in treated animals. These data suggest that ectopic Wnt signaling leads to the disruption of lens potential and the lack of proper lentigenic cell differentiation.

Despite the remarkable loss of the lens, these data do not clearly distinguish between the loss of lentigenic cell fate or proper cell function, such as the growth of the cellular processes that form the lens. To assess if lentigenic cell fate is lost, we performed *in situ* hybridization experiments for *DpS-Crystallin* on LiCl treated animals. We saw two types of expression phenotypes, either a significant decrease (Type I) or a complete loss (Type II) in *DpS-Crystallin* expression as compared to control (Figure 4P, P' & P''). We find all *DpS-Crystallin* expression exclusively dorsal to the site of lens formation suggesting that these cells may differentiate first. These data show that ectopic Wnt signaling results in the loss of lentigenic cell fate and that our

treatment may have interrupted a dorsal-to-ventral wave of differentiation in some embryos (Figure 4A). In addition, we assessed other anterior segment markers, including *DpSix3/6* and *DpLhx1/5,* and these genes show a consistent loss of expression in the most severe phenotypes, (Supplemental Figure 4C).

**Limb Patterning Program Regulatory Evolution**

To address if Wnt signaling is upstream of the limb patterning program, we performed *in situ* hybridization of limb transcription factors after LiCl treatment (Figure 3Q-3S, Supplementary Figure 4C). Similar to *DpS-Crystallin* expression, we again see a mild reduction (Type I) or loss and severe reduction (Type II) of expression. Our milder phenotypes, again, show a dorsal asymmetry, which can be most easily seen in *DpSP6-9A, DpDlx* and *DpHbn* (Figure 3Q, Q', Q'', 3R, R', R'' and 3S, S', S''). Changes are also visible but less obvious in *DpPbx* and *DpMeis* expression, with *DpPbx* only showing a mild phenotype (Supplemental figure 4C). These data support the placement of Wnt signaling upstream of the limb patterning program in a negative regulatory role.

**Conclusion**

Our findings indicate that the limb patterning program has been co-opted for anterior segment and lens development in cephalopods and that this co-option does not have a homologous upstream regulatory relationship with Wnt signaling as found in the limb (Estella et al., 2003; Tarazona et al., 2019). This change in signaling and the known duplication of SP6-9 identifies the paralog SP6-9a as a mediator of limb patterning program co-option in the anterior segment. Finally, with little similarity between limb and lens, our work suggests that the function of the limb patterning program in a limbless ancestor was likely a more generic developmental function than outgrowth. Considering present findings, previous work and hypotheses we conclude that the ability to pattern in a radial fashion, as previously proposed, is a more inclusive and likely ancestral function (Figure 4B) (Carroll et al., 1994; Erwin & Davidson, 2002). This work shows the cephalopod lens to be a unique context for future investigation of comparative regulatory changes responsible for co-option, and for identifying the regulatory mechanisms responsible for the emergent radial pattern found in embryos across species.

# Methods

## Animal Husbandry

*Doryteuthis pealeii* egg sacks were obtained from the Marine Biological Labs. Egg sacks were kept at 20 degrees Celsius. Although not required, European guidelines for cephalopod research were followed.

## Histology and TUNEL Staining

Embryos were fixed at 4 degrees Celsius overnight in 4% PFA in filter-sterilized seawater. After fixation embryos were transitioned into 15% and 30% sucrose and embedded in TFM and stored at -80 degrees Celsius. Embryos were cryosectioned in 12 um sections, stained with Sytox Green 1:1000 and Phalloidin 555 1:300 in PBS overnight (Molecular Probes). Tunel stained tissue was processed after sectioning using the Click-iT TUNEL Alexa Fluor 488 kit according to manufacturer's instructions (Invitrogen). Embryos were mounted in VECTASHIELD Hardset antifade mounting medium and imaged on a Zeiss 880 confocal.

## Homolog Identification and Phylogenetics

Genes were preliminarily identified using reciprocal BLAST with *Mus musculus* and *Drosophila melanogaster* sequences as bait with the exception of S-Crystallin where previous *Doryteuthis opalescens* sequences were also used (Altschul et al., 1990). Top hits in the *D. pealeii* transcriptome were trimmed for coding sequence and translated to amino acid sequences. To find related sequences, BLASTp was used, searching only the RefSeq protein database in NCBI filtered for vertebrate and arthropod models, as well as spiralian models for when published annotated sequences could be found. The top hits of each gene name were downloaded and aligned with our *D. pealeii* sequences for each tree using MAFFT in Geneious (Katoh, 2002). To check sequence redundancy and proper outgroups quick trees were made using FastTree. We constructed maximum-likelihood trees on the FASRC Cannon cluster supported by the FAS Division of Science Research Computing Group at Harvard University (Price et al. 2010). Using PTHREADS RAxML v.8.2.10, we ran the option for rapid bootstrapping with search for best maximum likelihood tree, resampling with 1000 bootstrap replicates, the PROTGAMMAAUTO model of amino acid substitution, and otherwise default parameters (Stamatakis, 2014). Fasta alignments, Nexus tree files are found in the Supplemental Data Folder. All PDF versions of the trees are found in Supplemental Figure 1.

**Cloning and Probe Synthesis**

Embryos stg 21-29 were crushed in Trizol reagent. RNA was extracted using standard phenol-chloroform extraction with a clean-up using the Qiagen RNeasy Micro kit. cDNA was synthesized using iScript (Bio-Rad) according to manufacturer protocols. Primers were designed using Primer3 in the Geneious software package from available transcriptomic data (Koenig et al., 2016). PCR products were ligated into the Pgem-T Easy plasmid and isolated using the Qiagen miniprep kit. Plasmids were linearized using restriction enzymes. Sense and anti-sense probes were synthesized using T7 and SP6 polymerase with digoxygenin labelled nucleotides.

***In situ* Hybridization**

Embryos were fixed as previously described (Koenig et al. 2016) and were dehydrated in 100% ethanol and stored at -20 degrees Celsius. Whole-mount *in situ* hybridization was performed as previously described (Koenig et al., 2016). Embryos were imaged using a Zeiss Axio Zoom.V16. Embryos were fixed for sectioning overnight in 4% PFA in artificial seawater and dehydrated in 100% ethanol. Embryos were transitioned into histoclear and embedded in paraffin. Embryos were sectioned on a Leica RM2235 microtome in 5-micron sections. Sections were dewaxed for *in situ* in Histoclear, rehydrated through an EtOH series, and re-fixed for 5 minutes at 4 degrees Celsius in 4% PFA in PBS. Embryos were exposed to Proteinase K for 20 minutes at 37 degrees Celsius and then quenched with glycine. The embryos were then de-acetylated with acetic anhydride. Slides were then pre-hybridized at 65 degrees Celsius for 30-60 minutes and then exposed to probe overnight. Slides were washed in 50% formamide/1x SSC/0.1% Tween-20 hybridization buffer twice, then twice in 1x SSC, .2x SSC and 0.02x SSC, all at 70 degrees Celsius. The slides were then washed at room temperature in MABT three times and blocked in Roche Blocking Buffer for an hour. Slides were incubated in Anti-Dig antibody (Roche) at 1/4000 overnight at 4 degrees Celsius. Slides were washed with MABT and then placed in AP reaction buffer. Slides were then exposed to BCIP/NBT solution until reacted and stopped in PBS. Slides were counterstained with Sytox 1:1000 overnight. Slides mounted in ImmunoHistoMount (Abcam) and imaged on a Zeiss Axioscope. *DpS-Crystallin* embryo *in situs* were transitioned to sucrose and embedded after imaging in whole-mount. Embryos were image on a Zeiss Axioscope.

***Ex ovo* Experimental Culture**

*Ex ovo* culture was performed as previously described in Koenig, 2016. Embryos were bathed in .25 M, .15 M and .07 M LiCl and 100nm, 250nm and 500nm concentration of Wnt Agonist

(CHIR99021) in Pen-Step filter-sterilized seawater to determine a working concentration. Control animals were bathed in equivalent amounts of DMSO or Pen-Strep alone.

## Supplemental Data Files

RAxML Maximum Likelihood trees, 1000 bootstraps.

ANTP_ML_1000bs_final.nex
Axin_ML_1000bs_final.nex
Cry_ML_1000bs_final.nex
Dach_ML_1000bs_final.nex
Dsh_ML_1000bs_final.nex
Fz_ML_1000bs_final.nex
GSK3_ML_1000bs_final.nex
Lhx_ML_1000bs_final.nex
LRP1_ML_1000bs_final.nex
Pangolin_ML_1000bs_final.nex
Prd_domain_ML_1000bs_final.nex
TALE_ML_1000bs_final.nex
Wnt_ML_1000bs_final.nex

MAFFT sequence alignments

ANTP_ML_1000bs_final.fasta
Axin_ML_1000bs_final.fasta
Cry_ML_1000bs_final.fasta
Dach_ML_1000bs_final.fasta
Dsh_ML_1000bs_final.fasta
Fz_ML_1000bs_final.fasta
GSK3_ML_1000bs_final.fasta
Lhx_ML_1000bs_final.fasta
LRP1_ML_1000bs_final.fasta
Pangolin_ML_1000bs_final.fasta
Prd_domain_ML_1000bs_final.fasta
TALE_ML_1000bs_final.fasta
Wnt_ML_1000bs_final.fasta

## Authors' contributions

K.M.K. designed the experiments. S.N., K.J.M., F.N., C.D., J.C., and K.M.K. performed experiments. K.J.M. performed phylogenetic analyses. K.M.K., S.N., and K.J.M. wrote the manuscript with consultation from all authors.

## Competing Interests

Authors declare no competing interests.

**Funding**

This work is supported by the Office of the NIH Director 1DP5OD023111-01, and the John Harvard Distinguished Science Fellowship to K.M.K.

**Acknowledgements**

The authors would like to thank the Koenig and Srivastava lab members for helpful discussions as well as Kevin Woods and the John Harvard Distinguished Science Fellows community for support. We would like to thank Jeffrey Gross, Alex Schier, Mansi Srivastava, and Andrew Murray for comments on the manuscript. We also thank the Marine Biological Labs, the Marine Resources Center, Owen Nichols, Ernie Eldredge, and Shannon Eldredge for assisting in the acquisition of embryos. We would also like to acknowledge the Harvard College undergraduates of LS50: Integrated Science Laboratory Course: Zach Alerte, Vlad Batagui, Eli Burnes, Stephen Casper, Chris Chen, Ahab Chopra, Ralph Estanboulieh, Lily Gao, Pedro Garcia, Saimun Habib, Harry Hager, Maxwell Ho, Charlie Horowitz, Ray Jiang, Prashanth Kumar, Truelian Lee, Arian Mansur, Matthew Mardo, Mark Theodore Meneses, Kendrick Nguyen, Francesco Rolando, Simon Schnabl, Taylor Shirtliff-Hinds, Sorscher Lincoln, William Stainier, Avi Swartz, David Szanto, Sophia Tang, Joey Toker, Analli Torres, Nina Uzoigwe, Rowen VonPlagenhoef, Evelyn Wong, Alexandra Zaloga, Maxwell Zhu.

**References**

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, *215*(3), 403-410. doi: 10.1016/s0022-2836(05)80360-2

Angelini, D. R., & Kaufman, T. C. (2005). Insect appendages and comparative ontogenetics. *Developmental biology*, *286*(1), 57-77. doi: 10.1016/j.ydbio.2005.07.006

Arnold, J. M. (1965). Normal embryonic stages of the squid, *Loligo pealii* (Lesueur). *The Biological Bulletin*, *128*(1), 24-32. doi: 10.2307/1539386

Arnold, J. M. (1966). On the occurrence of microtubules in the developing lens of the squid *Loligo pealii*. *Journal of ultrastructure research*, *14*(5-6), 534-539. doi: 10.1016/s0022-5320(66)80080-1

Arnold, J. M. (1967). Fine structure of the development of the cephalopod lens. *Journal of ultrastructure research*, *17*(5-6), 527-543. doi: 10.1016/s0022-5320(67)80139-4

Arendt, D., Hausen, H., & Purschke, G. (2009). The 'division of labour'model of eye evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1531), 2809-2817. doi: 10.1098/rstb.2009.0104

Brahma, S. K. (1978). Ontogeny of lens crystallins in marine cephalopods. *Development*, *46*(1), 111-118. doi: 10.1016/b978-0-12-483180-3.50009-5

Beldade, P., Brakefield, P. M., & Long, A. D. (2002). Contribution of Distal-less to quantitative variation in butterfly eyespots. *Nature*, *415*(6869), 315. doi: 10.1038/415315a

Brakefield, P. M., Gates, J., Keys, D., Kesbeke, F., Wijngaarden, P. J., Monteiro, A., French, V., & Carroll, S. B. (1996). Development, plasticity and evolution of butterfly eyespot patterns. *Nature*, *384*(6606), 236. doi: 10.1038/384236a0

Campbell, G., & Tomlinson, A. (1998). The roles of the homeobox genes aristaless and Distal-less in patterning the legs and wings of Drosophila. *Development*, *125*(22), 4483-4493.

Capellini, T. D., Zappavigna, V., & Selleri, L. (2011). Pbx homeodomain proteins: TALEnted regulators of limb patterning and outgrowth. *Developmental Dynamics*, *240*(5), 1063-1086. doi: 10.1002/dvdy.22605

Carroll, S. B., Gates, J., Keys, D. N., Paddock, S. W., Panganiban, G. E., Selegue, J. E., & Williams, J. A. (1994). Pattern formation and eyespot determination in butterfly wings. *Science*, *265*(5168), 109-114. doi: 10.1126/science.7912449.

Carroll, S. B., Grenier, J. K., & Weatherbee, S. D. (2013). *From DNA to diversity: molecular genetics and the evolution of animal design*. John Wiley & Sons. doi: 10.1002/ajmg.a.20051

Chiou, S. H. (1984). Physicochemical characterization of a crystallin from the squid lens and its comparison with vertebrate lens crystallins. *The Journal of Biochemistry*, *95*(1), 75-82. doi: 10.1093/oxfordjournals.jbchem.a134605

Chow, R. L., & Lang, R. A. (2001). Early eye development in vertebrates. *Annual review of cell and developmental biology*, *17*(1), 255-296. doi: 10.1146/annurev.cellbio.17.1.255

Cohen, S. M. (1990). Specification of limb development in the *Drosophila* embryo by positional cues from segmentation genes. *Nature*, *343*(6254), 173. doi: doi: 10.1038/343173a0

Cvekl, A., & Ashery-Padan, R. (2014). The cellular and molecular mechanisms of vertebrate lens development. *Development*, *141*(23), 4432-4447. doi: 10.1242/dev.107953

Dakin, W. J. (1928). The eyes of Pecten, Spondylus, Amussium and allied Lamellibranchs, with a short discussion on their evolution. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character*, *103*(725), 355-365. doi:10.1098/rspb.1928.0047

Darwin, C. (2016). *On the origin of species, 1859*. Routledge.

Dong, P. S., Chu, J., & Panganiban, G. (2001). Proximodistal domain specification and interactions in developing *Drosophila* appendages. *Development*, *128*(12), 2365-2372.

Dong, P. S., Dicks, J. S., & Panganiban, G. (2002). Distal-less and homothorax regulate multiple targets to pattern the *Drosophila* antenna. *Development*, *129*(8), 1967-1974.

Estella, C., Rieckhof, G., Calleja, M., & Morata, G. (2003). The role of buttonhead and Sp1 in the development of the ventral imaginal discs of *Drosophila*. *Development*, *130*(24), 5929-5941. doi: 10.1242/dev.00832

Estella, C., Voutev, R., & Mann, R. S. (2012). A dynamic network of morphogens and transcription factors patterns the fly leg. In *Current topics in developmental biology* (Vol. 98, pp. 173-198). Academic Press. doi: 10.1016/B978-0-12-386499-4.00007-0

Erwin, D. H., & Davidson, E. H. (2002). The last common bilaterian ancestor. *Development*, *129*(13), 3021-3032.

Grimmel, J., Dorresteijn, A. W., & Fröbius, A. C. (2016). Formation of body appendages during caudal regeneration in *Platynereis dumerilii*: adaptation of conserved molecular toolsets. *EvoDevo*, *7*(1), 1-14.

Hedgepeth, C. M., Conrad, L. J., Zhang, J., Huang, H. C., Lee, V. M., & Klein, P. S. (1997). Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. *Developmental biology*, *185*(1), 82-91. doi: 10.1006/dbio.1997.8552

Ibarretxe, G., Aurrekoetxea, M., Crende, O., Badiola, I., Jimenez-Rojo, L., Nakamura, T., ... & Unda, F. (2012). Epiprofin/Sp6 regulates Wnt-BMP signaling and the establishment of cellular junctions during the bell stage of tooth development. *Cell and tissue research*, *350*(1), 95-107. doi: 10.1007/s00441-012-1459-8

Jonasova, K., & Kozmik, Z. (2008, April). Eye evolution: lens and cornea as an upgrade of animal visual system. In *Seminars in cell & developmental biology* (Vol. 19, No. 2, pp. 71-81). Academic Press. doi: 10.1016/j.semcdb.2007.10.005

Katoh, K., Misawa, K., Kuma, K. I., & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic acids research*, *30*(14), 3059-3066. doi: 10.1093/nar/gkf436

Klein, P. S., & Melton, D. A. (1996). A molecular mechanism for the effect of lithium on development. *Proceedings of the National Academy of Sciences*, *93*(16), 8455-8459. doi: 10.1073/pnas.93.16.8455

Koenig, K. M., Sun, P., Meyer, E., & Gross, J. M. (2016). Eye development and photoreceptor differentiation in the cephalopod *Doryteuthis pealeii*. *Development*, *143*(17), 3168-3181. doi: 10.1242/dev.134254

Koenig, K. M., & Gross, J. M. (2020) Evolution and development of complex eyes: A celebration of diversity. *Development, 147*(19). doi: 10.1242/dev.182923

Lapan, S. W., & Reddien, P. W. (2011). dlx and sp6-9 Control optic cup regeneration in a prototypic eye. *PLoS Genet*, *7*(8), e1002226. doi: 10.1371/journal.pgen.1002226

Lemons, D., Fritzenwanker, J. H., Gerhart, J., Lowe, C. J., & McGinnis, W. (2010). Co-option of an anteroposterior head axis patterning system for proximodistal patterning of appendages in early bilaterian evolution. *Developmental biology*, *344*(1), 358-362.

Maas, R., & Bei, M. (1997). The genetic control of early tooth development. *Critical Reviews in Oral Biology & Medicine*, *8*(1), 4-39. doi: 10.1177/10454411970080010101

Mazza, M. E., Pang, K., Reitzel, A. M., Martindale, M. Q., & Finnerty, J. R. (2010). A conserved cluster of three PRD-class homeobox genes (homeobrain, rx and orthopedia) in the Cnidaria and Protostomia. *EvoDevo*, *1*(1), 3.

McCulloch, K. J., & Koenig, K. M. (2020). Krüppel-like factor/specificity protein evolution in the Spiralia and the implications for cephalopod visual system novelties. *Proceedings of the Royal Society B*, *287*(1937), 20202055. doi: 10.1098/rspb.2020.2055

McDougall, C., Korchagina, N., Tobin, J. L., & Ferrier, D. E. (2011). Annelid Distal-less/Dlx duplications reveal varied post-duplication fates. *BMC evolutionary biology*, *11*(1), 1-16.

Meinertzhagen, I. A. (1990). Development of the squid's visual system. In *Squid as experimental animals* (pp. 399-419). Springer, Boston, MA.

Mercader, N., Leonardo, E., Azpiazu, N., Serrano, A., Morata, G., Martínez-A, C., & Torres, M. (1999). Conserved regulation of proximodistal limb axis development by Meis1/Hth. *Nature*, *402*(6760), 425-429. doi: 10.1038/46580

Minelli, A. (2000). Limbs and tail as evolutionarily diverging duplicates of the main body axis. *Evolution & development*, *2*(3), 157-165. doi: 10.1046/j.1525-142x.2000.00054.x

Moczek, A. P., & Rose, D. J. (2009). Differential recruitment of limb patterning genes during development and diversification of beetle horns. *Proceedings of the National Academy of Sciences*, *106*(22), 8992-8997.

Nilsson, D. E. (2013). Eye evolution and its functional basis. *Visual neuroscience*, *30*(1-2), 5-20. doi: 10.1017/S0952523813000035

Ogura, A., Yoshida, Ma., Moritaki, T., Plida,Y., Sese, J., Shimizu, K., Sousounis, K., & Tsonis, P. A. (2013) Loss of the six3/6 controlling pathways might have resulted in pinhole-eye evolution in *Nautilus*. *Scientific Reports* **3,** 1432. doi: 10.1038/srep01432

Packard, A. (1972). Cephalopods and fish: the limits of convergence. *Biological Reviews*, *47*(2), 241-307.

Panganiban G., Irvine S.M., Lowe C., Roehl H., Corley L.S., Sherbon B., Grenier J.K., Fallon J.F., Kimble J., Walker M., Wray G.A., Swalla B.J., Martindale M.Q., & Carroll S.B. (1997). The origin and evolution of animal appendages. *Proceedings of the National Academy of Sciences*. 94(10):5162-6. doi: 10.1073/pnas.94.10.5162.

Panganiban, G., Nagy, L., & Carroll, S. B. (1994). The role of the Distal-less gene in the development and evolution of insect limbs. *Current Biology*, *4*(8), 671-675. doi: 10.1016/s0960-9822(00)00151-2

Panganiban, G., & Rubenstein, J. L. (2002). Developmental functions of the Distal-less/Dlx homeobox genes. *Development*, *129*(19), 4371-4386.

Pueyo, J. I., & Couso, J. P. (2005). Parallels between the proximal–distal development of vertebrate and arthropod appendages: homology without an ancestor?. *Current opinion in genetics & development*, *15*(4), 439-446. doi: 10.1016/j.gde.2005.06.007

Plavicki, J. S., Squirrell, J. M., Eliceiri, K. W., & Boekhoff-Falk, G. (2016). Expression of the *Drosophila* homeobox gene, *Distal-less*, supports an ancestral role in neural development. *Developmental Dynamics*, *245*(1), 87-95. doi: 10.1002/dvdy.24359

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PloS one*, *5*(3), e9490. doi: 10.1371/journal.pone.0009490

Prpic, N. M., & Tautz, D. (2003). The expression of the proximodistal axis patterning genes *Distal-less* and *dachshund* in the appendages of *Glomeris marginata* (Myriapoda: Diplopoda) suggests a special role of these genes in patterning the head appendages. *Developmental biology*, *260*(1), 97-112. doi: 10.1016/s0012-1606(03)00217-3

Prpic, N. M. (2019). Limb Development: A lesson in homology. *eLife*, *8*, e48335. doi: 10.7554/eLife.48335

Ramanathan, A., Srijaya, T. C., Sukumaran, P., Zain, R. B., & Kasim, N. H. A. (2018). Homeobox genes and tooth development: Understanding the biological pathways and applications in regenerative dental science. *Archives of oral biology*, *85*, 23-39. doi: 10.1016/j.archoralbio.2017.09.033

Sanz-Navarro, M., Delgado, I., Torres, M., Mustonen, T., Michon, F., & Rice, D. P. (2019). Dental Epithelial Stem Cells Express the Developmental Regulator Meis1. *Frontiers in physiology*, *10*, 249. doi: 10.3389/fphys.2019.00249

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., & Brivanlou, A. H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nature medicine*, *10*(1), 55-63. doi: 10.1038/nm979

Schiemann, S. M., Martín-Durán, J. M., Børve, A., Vellutini, B. C., Passamaneck, Y. J., & Hejnol, A. (2017). Clustered brachiopod Hox genes are not expressed collinearly and are associated with lophotrochozoan novelties. *Proceedings of the National Academy of Sciences*, *114*(10), E1913-E1922. doi: 10.1073/pnas.1614501114

Setton, E. V., & Sharma, P. P. (2018). Cooption of an appendage-patterning gene cassette in the head segmentation of arachnids. *Proceedings of the National Academy of Sciences*, *115*(15), E3491-E3500. doi: 10.1073/pnas.1720193115

Shubin, N., Tabin, C., & Carroll, S. (1997). Fossils, genes and the evolution of animal limbs. *Nature*, *388*(6643), 639-648. doi: 10.1038/41710

Shubin, N., Tabin, C., & Carroll, S. (2009). Deep homology and the origins of evolutionary novelty. *Nature*, *457*(7231), 818-823. doi: 10.1038/nature07891

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30*(9), 1312-1313. doi: 10.1093/bioinformatics/btu033

577 Tarazona, O. A., Lopez, D. H., Slota, L. A., & Cohn, M. J. (2019). Evolution of limb
578 development in cephalopod mollusks. *eLife*, *8*, e43828. doi: 10.7554/eLife.43828
579 Walls, G. L. (1939). Origin of the vertebrate eye. *Archives of Ophthalmology*, *22*(3), 452-486.
580
581 West, J. A., Sivak, J. G., & Doughty, M. J. (1995). Microscopical evaluation of the crystalline
582 lens of the squid (*Loligo opalescens*) during embryonic development. *Experimental eye*
583 *research*, *60*(1), 19-35. doi: 10.1016/s0014-4835(05)80080-6
584
585 West, J. A., Sivak, J. G., Pasternak, J., & Piatigorsky, J. (1994). Immunolocalization of S-
586 crystallins in the developing squid (*Loligo opalescens*) lens. *Developmental dynamics*, *199*(2),
587 85-92. doi: 10.1002/aja.1001990202
588
589 Williams, L. W. (1909). Anatomy of the Common Squid, *Loligo pealii*, Lesueur. London: E.J.
590 Brill.

## Figure Titles and Legends

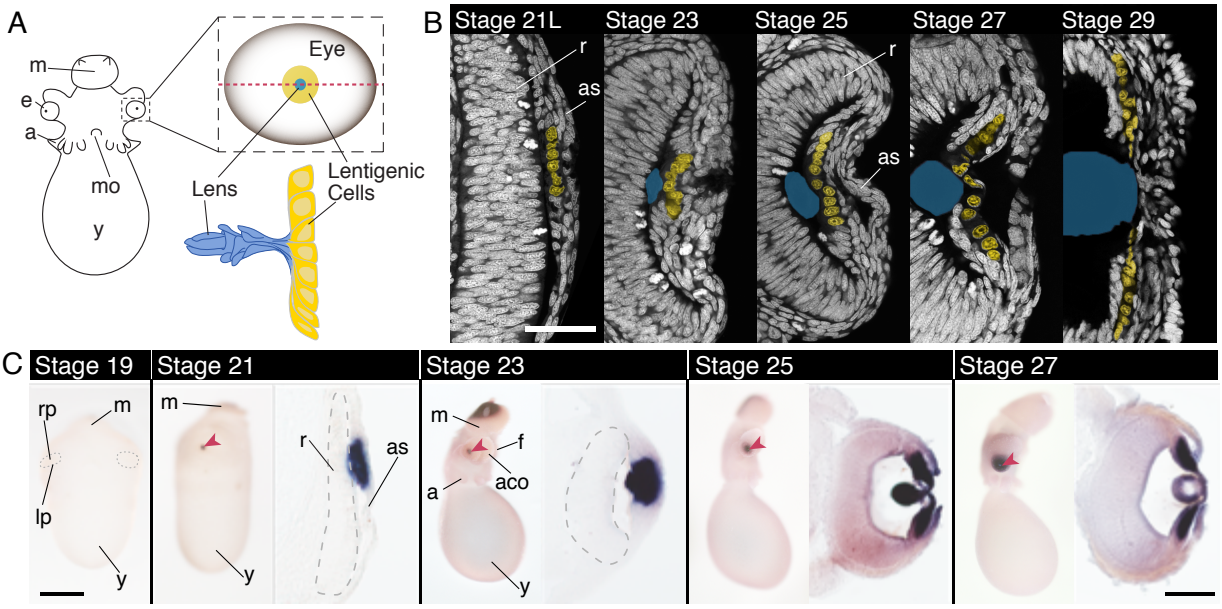



**Figure 1: Lentigenic cell differentiation and *DpS-Crystallin* expression in the squid**

A) Cartoon diagram of a squid embryo (anterior), en face cartoon of the developing eye (red dotted line shows cross-section plane) and developing lentigenic cells and lens. (Cartoon of lens and lentigenic cells based on Arnold, 1967) B) Cross-section of the developing anterior segment at Arnold stages 21 late, 23, 25, 27 and 29 identifying differentiation of lentigenic cells (Arnold, 1968). White: Sytox-Green labeling nuclei, Yellow: False-colored lentigenic cell nuclei corresponding to the LC2 population identified by nuclear morphology (Arnold, 1967; West et al., 1995; Koenig et al., 2016). Blue is the outline of the lens, as identified using phalloidin staining (not shown). First evidence of LC2 cells is late stage 21. Lentigenic cell number multiplies and distribution grows across the anterior segment (*as)* throughout development. Scale is 50 microns. C) *In situ* hybridization of *DpS-Crystallin* in whole-mount and cryo-section. Stage 19 is an anterior view, the boundary between the retina placode and the lip cells is highlighted with a dotted line. No *DpS-Crystallin* expression is apparent at this stage. Stage 21-27 are shown in a lateral view of the embryo on the left and a cross-section of the eye on the right. Anterior of the embyro is down in the sections. The retina is outlined with a dashed grey line in stage 21 and 23. *DpS-Crystallin* expression corresponds with LC2 lentigenic cell population. Scale is 500 microns in whole mount images. Scale is 100 microns in sectioned images. *as*, anterior segment; *a*, arm; *aco*, anterior chamber organ; *e*, eye; *f*, funnel *lp*, lip; *m*, mantle; *mo*, mouth; *rp*, retina placode; *r*, retina; *y*, yolk. Red arrow highlights the lens.

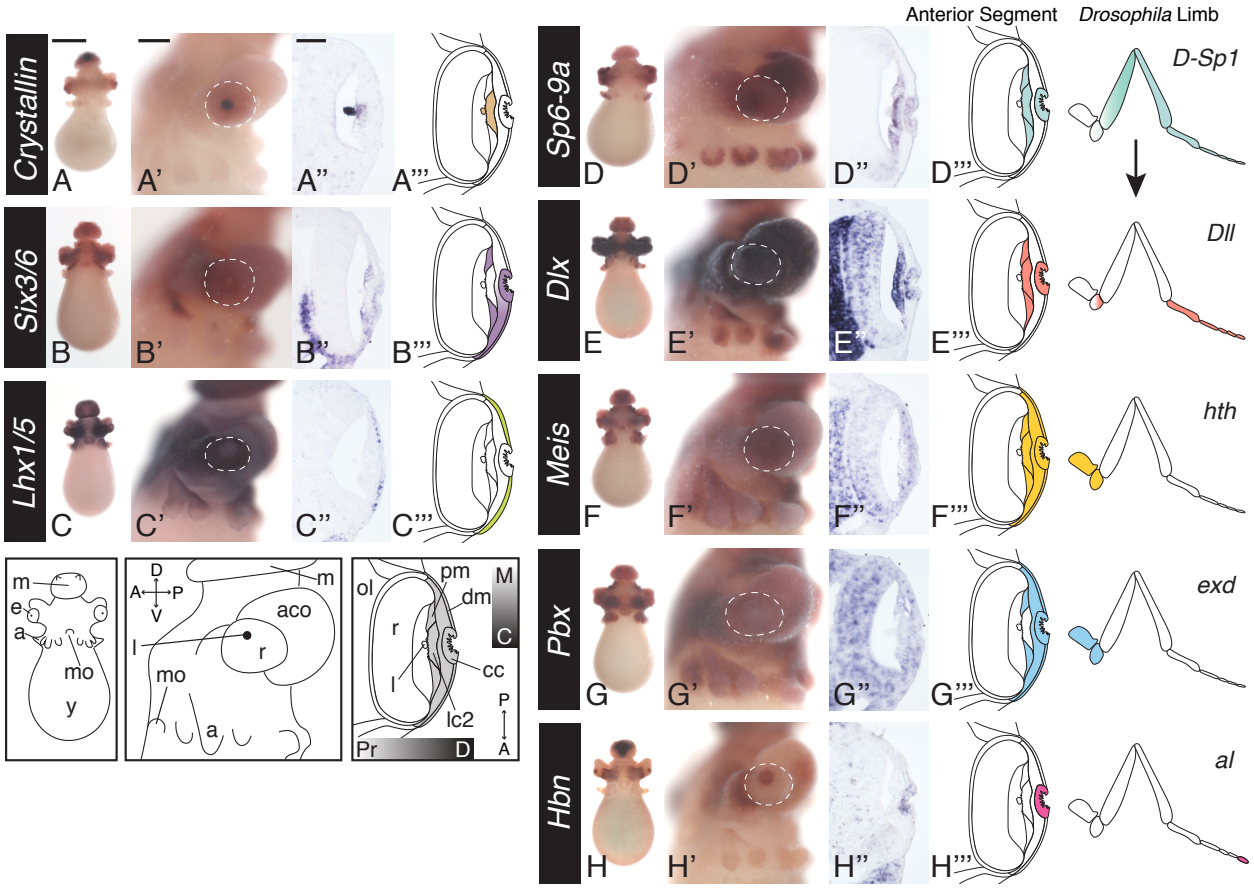



**Figure 2: Limb patterning program expressed in the developing anterior segment**

For each gene: left to right, anterior whole-mount view, lateral whole-mount view (anterior left), cross-section (anterior is down), cartoon summary of anterior segment expression. Dotted white outline in lateral view outlines the perimeter of the eye. A-C) Defining cell populations in the developing anterior segment at stage 23. A, A', A'') *DpS-Crystallin* expression in the anterior segment at stage 23, expressed in the proximal, central cells corresponding with the LC2 cells (*lc2*). Expression is also apparent in the lens. B, B', B'') Expression of *DpSix3/6.* B'') Expression is apparent in the distal, central cup cells (*cc*) and the proximal-marginal (*pm*) anterior segment cells. The proximal-central cells (*lc2*) lack expression of *DpSix3/6*. C, C', C'') *DpLhx1/5* expression. C'') Expression of *DpLhx1/5* is found in the distal-marginal cell (*dm*) population. Expression is excluded from the central cup (*cc*). D-G) Expression of the limb patterning program genes. Summary of the proximodistal expression of each *Drosophila* homolog during proximodistal patterning of the limb is shown on the right H) Prd-like homolog *Homeobrain* (*Hbn*) expression in the distal, central cup cells. *a*, arms; *aco*, anterior chamber organ; *cc*, cup cells; *dm,* distal-marginal cells; *e*, eye; *l*, lens; *lc2,* LC2 cells; *m*, mantle; *mo*, mouth; *pm,* proximal-marginal cells; r, retina; *y*, yolk. Anterior segment highlighted in grey in the cartoon. Orientation abbreviations: M, marginal; C, central; Pr, proximal; D, Distal; A, anterior; P, posterior. Scale for whole-mount anterior view is 500 microns. Scale for lateral whole-mount view 200 microns. Scale for sectioned images 50 microns.

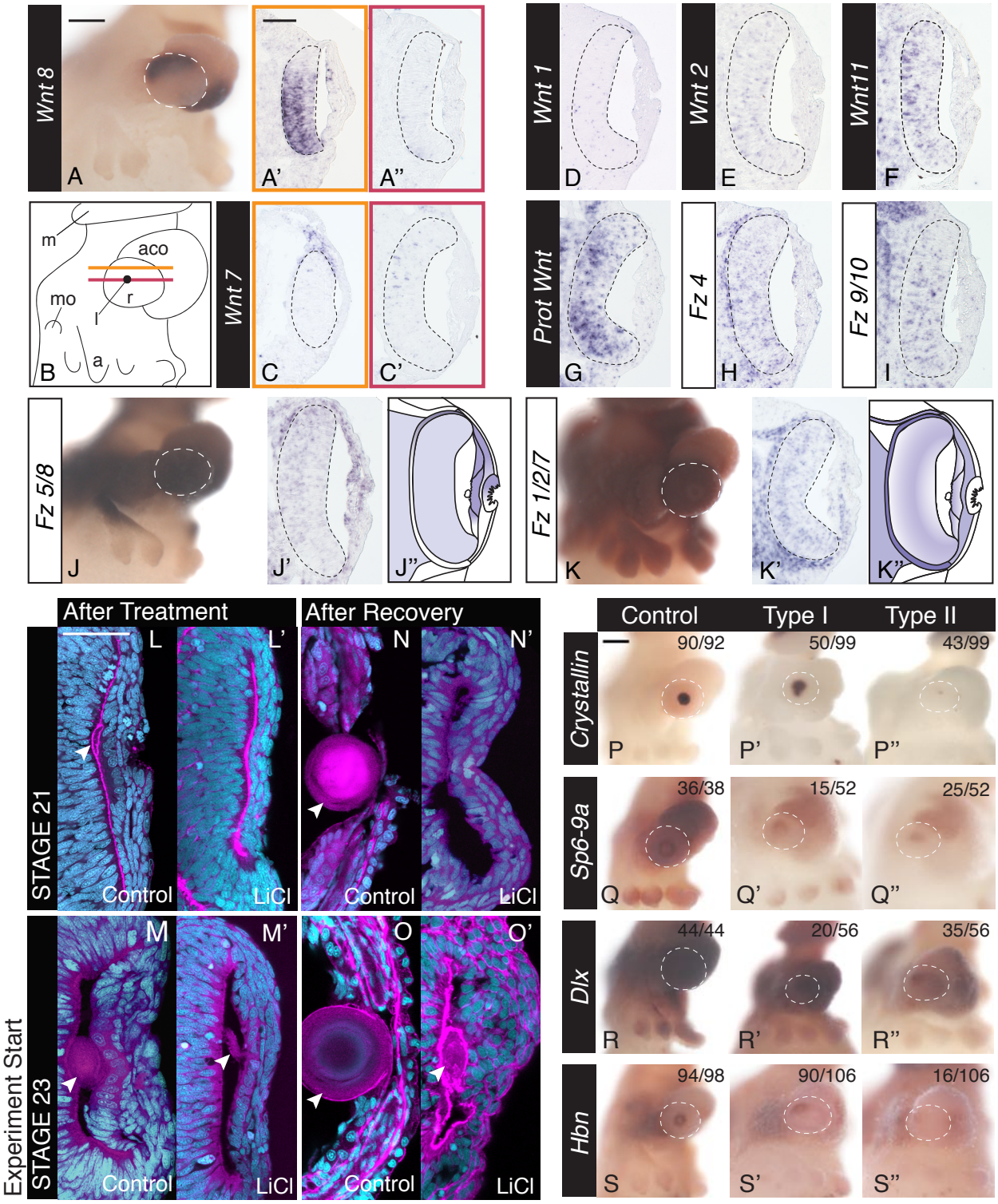



**Figure 3: Wnt signaling pathway expression in the developing cephalopod eye**

A-G) *Wnt* gene expression at stage 23. Based on expression, Wnt7, Wn8, Wnt2, Wnt11 and Prot Wnt are possible candidates to signal the anterior segment. A) Lateral, whole-mount expression of *Wnt8*. A') Dorsal retina expression of *Wnt8*. Location of the section indicated by the orange line in B. A'') Central section lacking retina expression. Location of the section indicated by the red line in B. B) Cartoon of the lateral whole-mount embryo at stage 23. Orange and red lines correspond to the location of the two sections shown in A, A', and C, C'. D-G) Expression of other *Wnt* homologs in central sections. H-K) Expression of Frizzled receptors at stage 23. Fz1/2/7

shows asymmetric expression and Fz5/8 shows specific exclusion from the central cup cells. J and K are lateral view of the whole mount expression. J'' and K'' are cartoons of expression in J' and K' respectively. Black dotted line in sectioned images show the perimeter of the retina. L-O) Anterior segment and lens morphology after Wnt agonist treatment (LiCl). Embryos were cryosectioned and stained with sytox-green (nuclei, cyan) and phalloidin (F-actin, magenta).. L and L') Control and LiCl agonist treatments started at stage 21, treated for 24 hours and fixed immediately. M and M') Control and Wnt agonist (LiCl) treatments started at stage 23 for 24 hours and fixed immediately. N and N') Control and Wnt agonist (LiCl) treatments started at stage 21, treated for 24 hours and allowed to recover for 48 hours and fixed. O and O') Control and Wnt agonist (LiCl) treatments started at stage 23, treated for 24 hours and allowed to recover for 48 hours and fixed. Arrowhead highlights the lens. P-S) *In situ* hybridization of anterior segment markers after 24 hour control and LiCl treatments starting at stage 23. Phenotypes are characterized as Type I (mild) and Type II (severe). The white dotted line outlines the eye in the lateral image and the number of eyes scored in control and the two phenotypes is found in LiCl treated animals in the top right corner. Scale for all lateral whole-mount view images is 200 microns. Scale for all sectioned images is 50 microns. Anterior is down in all sectioned images. White dotted line in whole mount images identify the perimeter of the eye. *m*, mantle; *a*, arms; *aco*, anterior chamber organ; *mo*, mouth; *r*, retina; *l*, lens.

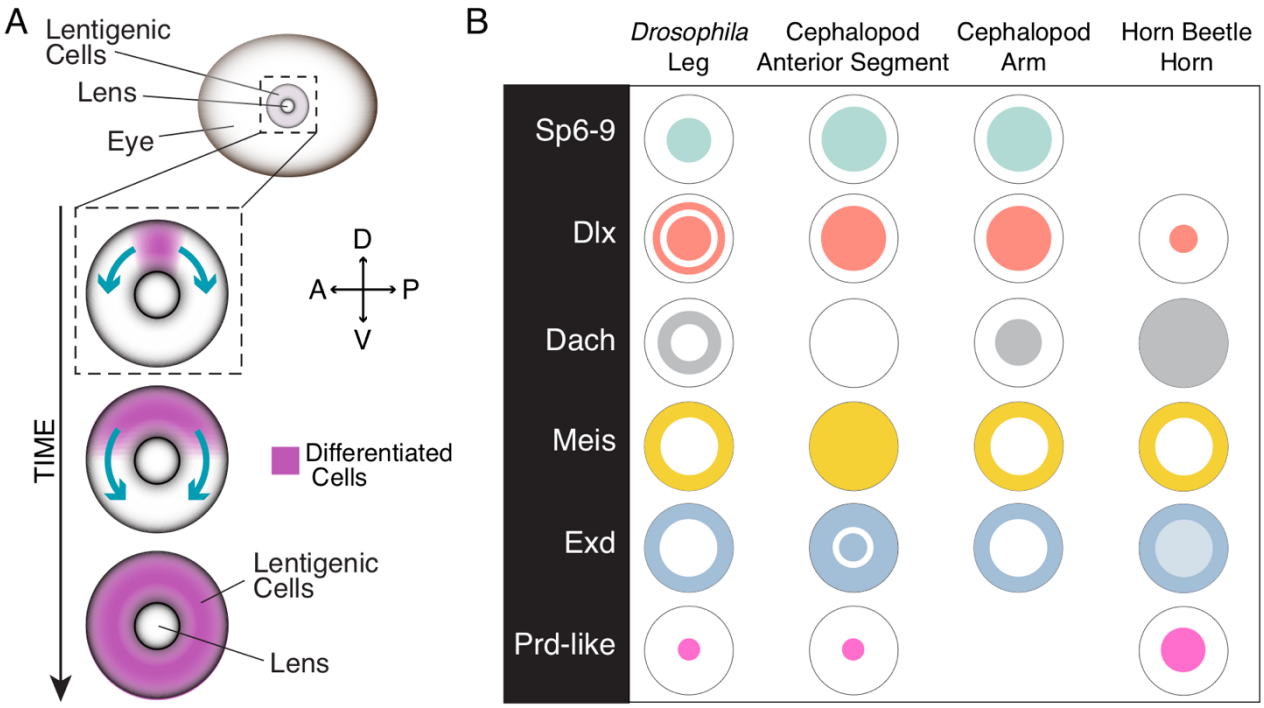



**Figure 4: Ectopic Wnt signaling activation leads to loss of the lens**

A) Model for lentigenic cell differentiation at stage 21. LC2 lentigenic cells differentiate on the dorsal side of the eye first, with a wave moving ventrally. Type I *DpS-Crystallin* embryos have been interrupted in progress. B) En face summary of sample radial expression of the limb patterning program across developmental contexts (Tarazona, 2019; reviewed in Moczek, 2009 and Angelini & Kaufman, 2005).

667 **Supplemental Tables**

668 Supplemental Table 1: All Primer sequences

| | Forward | Reverse |
|---|---|---|
| DpS-Crystallin | GAACATCATGTCGCACCACG | AGTTGCTCGCCTGAGAAGAC |
| DpLhx1/5 | GAAGTGTCTTCGTGCTCCCA | ATTATCGACCGGCGAGGAAC |
| DpDlx | GGCAAGGCTTGGGTAAAACG | GGGGTAGCAGCGATGAGTTT |
| DpMeis | TAGCGTTTCCAAAAGGACCT | CCCCAATACCCGTCATACTC |
| DpPbx | TACTTCGGGAGCAGAGTCGA | TAGCGGTCGTCGTCGTAATG |
| DpHbn | ATACAACGACGACGACCACC | CGCGTGAATACATCCGGGTA |
| DpPrdl-1 | AGAACAACCCAACGTACACA | GCAAACATCGAGTGAATCCC |
| DpPrdl-2 | TCGCATTGAGGATTGATCTT | GGTTGTTGTTGTTGTGTTGTT |
| DpDac | CTGTATGGCTCCAAGTCCTC | GATCTCTGGTCGTCGTTTCA |
| DpGSK3 | GATACGGGTGAACTGGTAGCAATC | CACCAACTGGATAGCCTCTGATG |
| DpLRP1 | TTCCTTGAATAGCCTCATCGGTC | TTCCAAAAAGTGGGTGTGCG |
| DpAxin | CCCTCATTATTCTCCAACCTCCTC | CACAGAGCACTTCAAAAACGGG |
| DpTCF/LEF | GCTTGGGTGGCAAAATGTCG | TGCTGGACTGTTCTGGCAAAC |
| DpDvl | GCAGGCACTTTTTTTAGTAGCGTG | ATGTCCGTTGATGCGAGGTG |
| DpWnt-Prot | GACAGCCTACCTTTATGCCA | TACATTCGCAGTCTTCCGTT |
| DpWnt1 | GTTTGCTTGTATTCGTGCGA | CCCTCCAATCCCAATGAAGT |
| DpWnt2 | GTCGTTTGTGGTCCTTGTTG | GAATGTCAGTTCCAGTTGCG |
| DpWnt7 | GTGCGTTGATGAATCTCCAC | TGTACTCCTCCGTCTTGTTG |
| DpWnt8 | CTGCCAGATACTCCGTGACATTTAC | TTGGTTGGGGAATCGCACTG |
| DpWnt11 | CTTGACATAGCAGCACCACACG | GAACAGTTTGCCAACAGAAGATGG |
| DpFz9/10 | CGTAGTTTCTTGCCCGTAGAC | CGCTGTTTTGTATCAACCCCA |
| DpFz1/2/7 | AAAGCCCCTTAAAGCATCCA | GACCATGCAATTCCACCTTG |
| DpFz4 | TCAGTTCGTCAGCATCAACAT | CCGATATCCTCAACTGCACAA |
| DpFz5/8 | TATTTGCTACCCACGGATCGC | CCGACCACCAAACACATAAAGT |

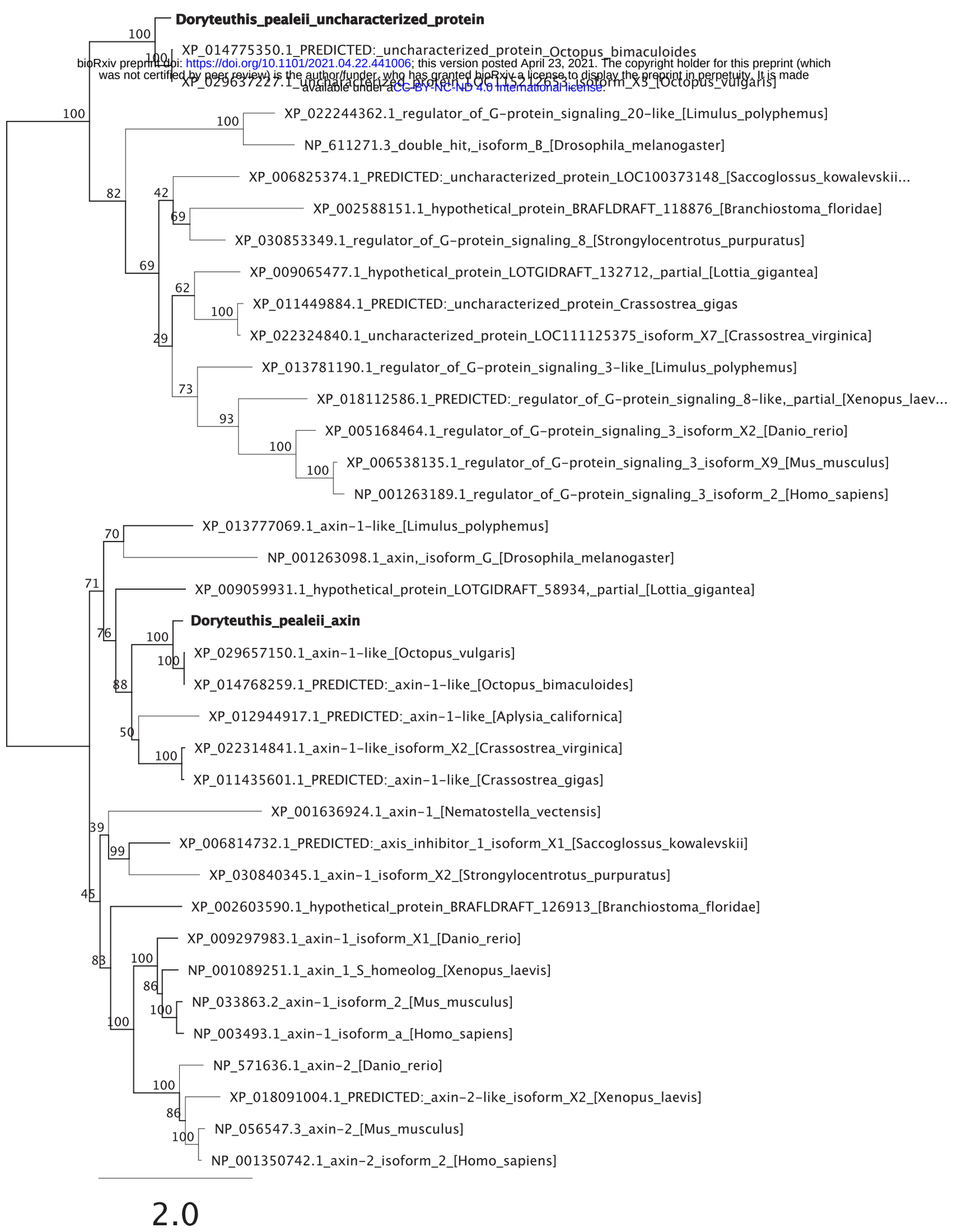
669

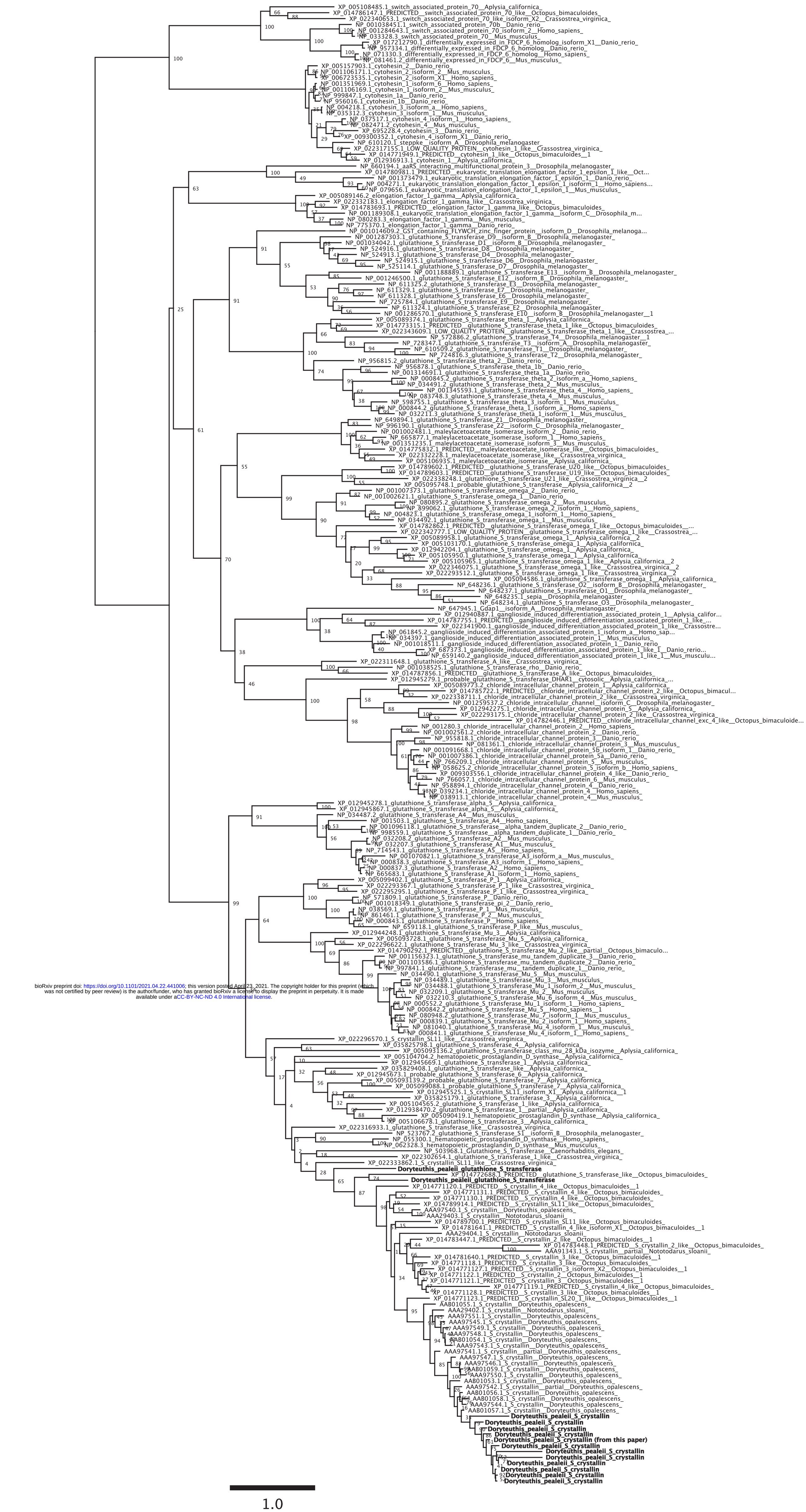
## Supplemental Figure titles

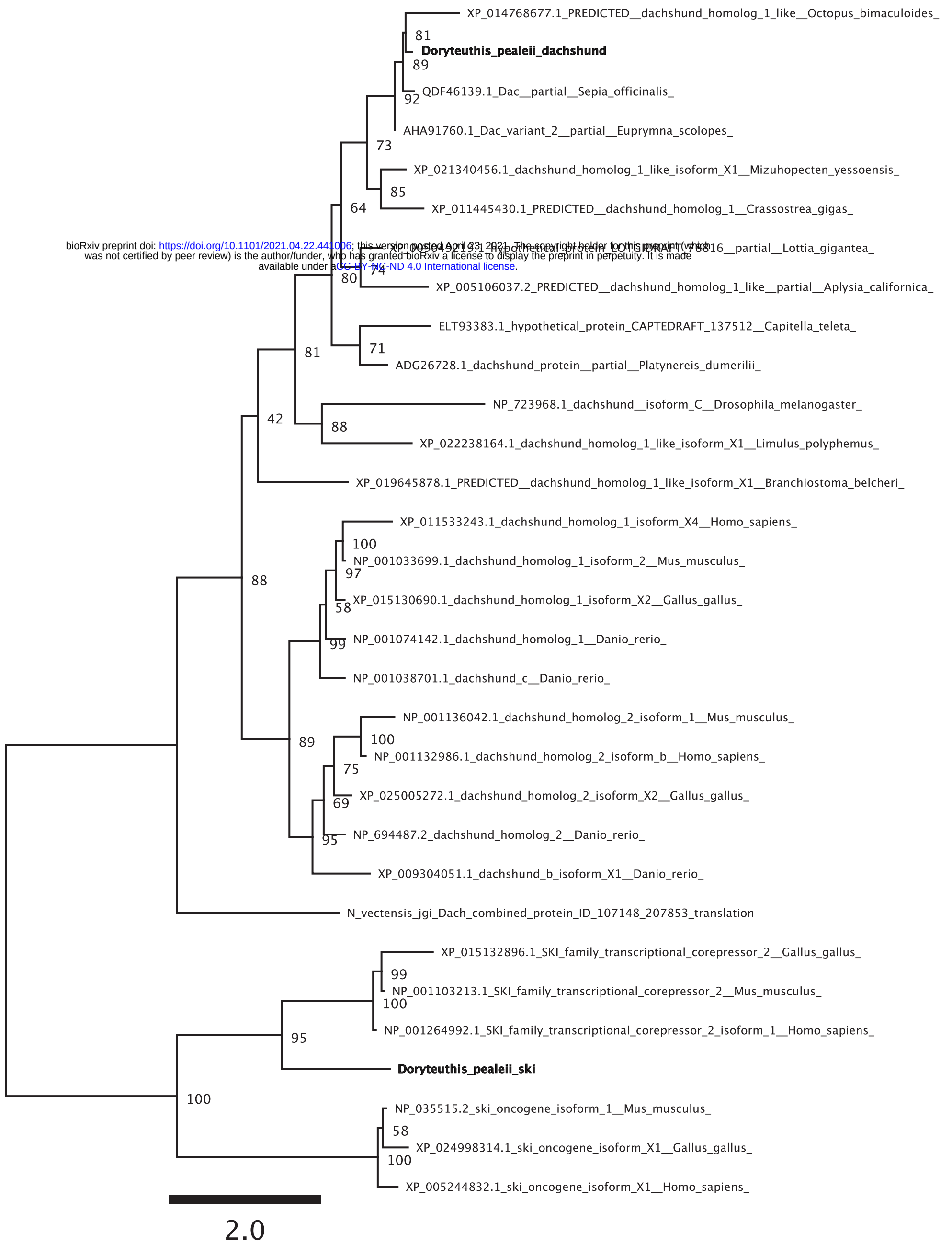
**Sup Figure 1: Maximum-likelihood phylogenetic trees for genes identified in this study**

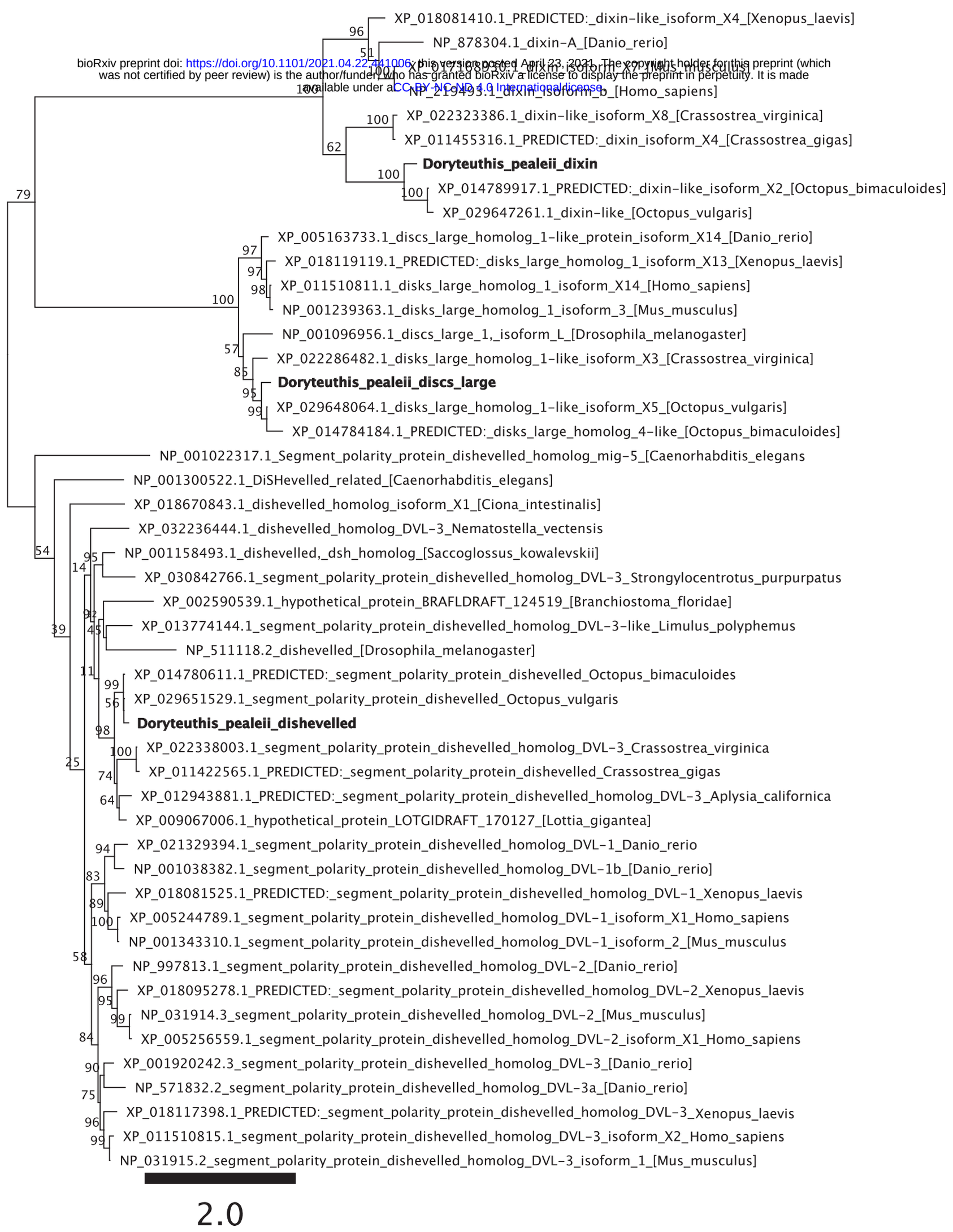
XP_011414574.1_PREDICTED__homeobox_protein_EMX1_isoform_X1__Crassostrea_gigas_
XP_009064180.1_hypothetical_protein_LOTGIDRAFT_58125__partial__Lottia_gigantea_
XP_005109699.1_PREDICTED__uncharacterized_protein_LOC101860203__Aplysia_californica_
**Doryteuthis_pealeii_Emx**
XP_014787377.1_PREDICTED__homeobox_protein_EMX1_like__Octopus_bimaculoides_
XP_011414573.1_PREDICTED__homeobox_protein_EMX2__Crassostrea_gigas_
ADG26729.1_EMX_homeobox_protein__partial__Platynereis_dumerilii_
XP_009064183.1_hypothetical_protein_LOTGIDRAFT_80119__partial__Lottia_gigantea_
ELU15122.1_hypothetical_protein_CAPTEDRAFT_183448__Capitella_teleta_
XP_013777866.1_homeobox_protein_EMX1_like__Limulus_polyphemus_
NP_571355.2_homeobox_protein_EMX2__Danio_rerio_
XP_025007225.1_homeobox_protein_EMX2_isoform_X1__Gallus_gallus_
NP_004039.1_homeobox_protein_EMX2_isoform_1__Homo_sapiens_
NP_034262.2_homeobox_protein_EMX2__Mus_musculus_
NP_571354.1_empty_spiracles_homeobox_3__Danio_rerio_
XP_011530999.1_homeobox_protein_EMX1_isoform_X2__Homo_sapiens_
NP_034261.1_homeobox_protein_EMX1__Mus_musculus_
NP_937787.1_homeobox_protein_EMX1__Danio_rerio_
XP_001232151.3_homeobox_protein_EMX1__Gallus_gallus_
NP_001107793.1_empty_spiracles__Tribolium_castaneum_
EFX74198.1_hypothetical_protein_DAPPUDRAFT_38036__partial__Daphnia_pulex_
NP_731868.1_empty_spiracles__Drosophila_melanogaster_
NP_571381.2_homeobox_protein_Dlx5a__Danio_rerio_
NP_034186.2_homeobox_protein_DLX_5_isoform_1__Mus_musculus_
NP_005212.1_homeobox_protein_DLX_5__Homo_sapiens_
NP_989490.1_homeobox_protein_DLX_5__Gallus_gallus_
NP_571397.2_homeobox_protein_Dlx3b__Danio_rerio_
NP_990135.2_homeobox_protein_DLX_3__Gallus_gallus_
NP_005211.1_homeobox_protein_DLX_3__Homo_sapiens_
NP_034185.1_homeobox_protein_DLX_3__Mus_musculus_
NP_004396.1_homeobox_protein_DLX_2__Homo_sapiens_
NP_034184.1_homeobox_protein_DLX_2__Mus_musculus_
XP_025008373.1_LOW_QUALITY_PROTEIN__homeobox_protein_DLX_2__Gallus_gallus_
NP_571372.1_homeobox_protein_Dlx2b__Danio_rerio_
NP_571386.2_homeobox_protein_Dlx2a__Danio_rerio_
CAJ38799.1_distalless__Dlx_1__Platynereis_dumerilii_
XP_012942952.1_PREDICTED_G0271670_like__partial__Aplysia_californica
ELU07962.1_hypothetical_protein_CAPTEDRAFT_74740__partial__Capitella_teleta_
XP_009046454.1_hypothetical_protein_LOTGIDRAFT_72890__partial__Lottia_gigantea_
ADL14376.1_distal_less_protein__partial__Octopus_bimaculoides_
QDF46136.1_distalless_protein__partial__Sepia_bandensis_
**Doryteuthis_pealeii_Dlx**
XP_013788660.1_homeobox_protein_DLX_6_like__Limulus_polyphemus_
XP_011441569.1_PREDICTED__homeobox_protein_Dlx6a__Crassostrea_gigas_
NP_001034528.2_distal_less__Tribolium_castaneum_
NP_523857.1_Distal_less__isoform_A__Drosophila_melanogaster_
XP_015154954.1_homeobox_protein_DLX_4__Gallus_gallus_
NP_005213.3_homeobox_protein_DLX_6__Homo_sapiens_
NP_034187.1_homeobox_protein_DLX_6__Mus_musculus_
XP_015137078.1_homeobox_protein_DLX_6_isoform_X1__Gallus_gallus_
NP_571398.1_homeobox_protein_Dlx6a__Danio_rerio_
NP_571380.1_homeobox_protein_Dlx1a__Danio_rerio_
NP_001039307.2_homeobox_protein_DLX_1__Gallus_gallus_
NP_835221.2_homeobox_protein_DLX_1_isoform_1__Homo_sapiens_
NP_034183.1_homeobox_protein_DLX_1__Mus_musculus_
NP_031893.3_homeobox_protein_DLX_4__Mus_musculus_
NP_612138.1_homeobox_protein_DLX_4_isoform_a__Homo_sapiens_

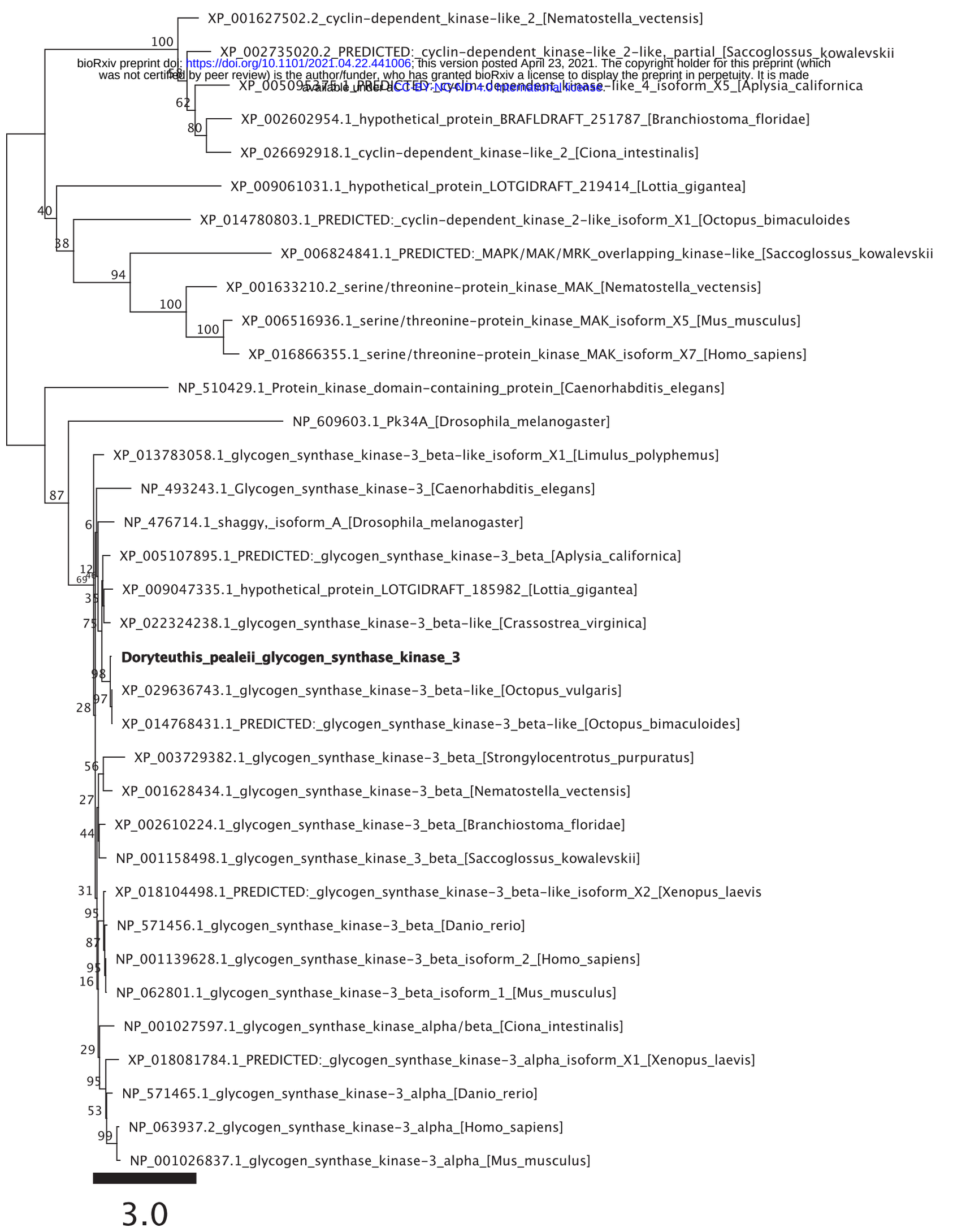
2.0

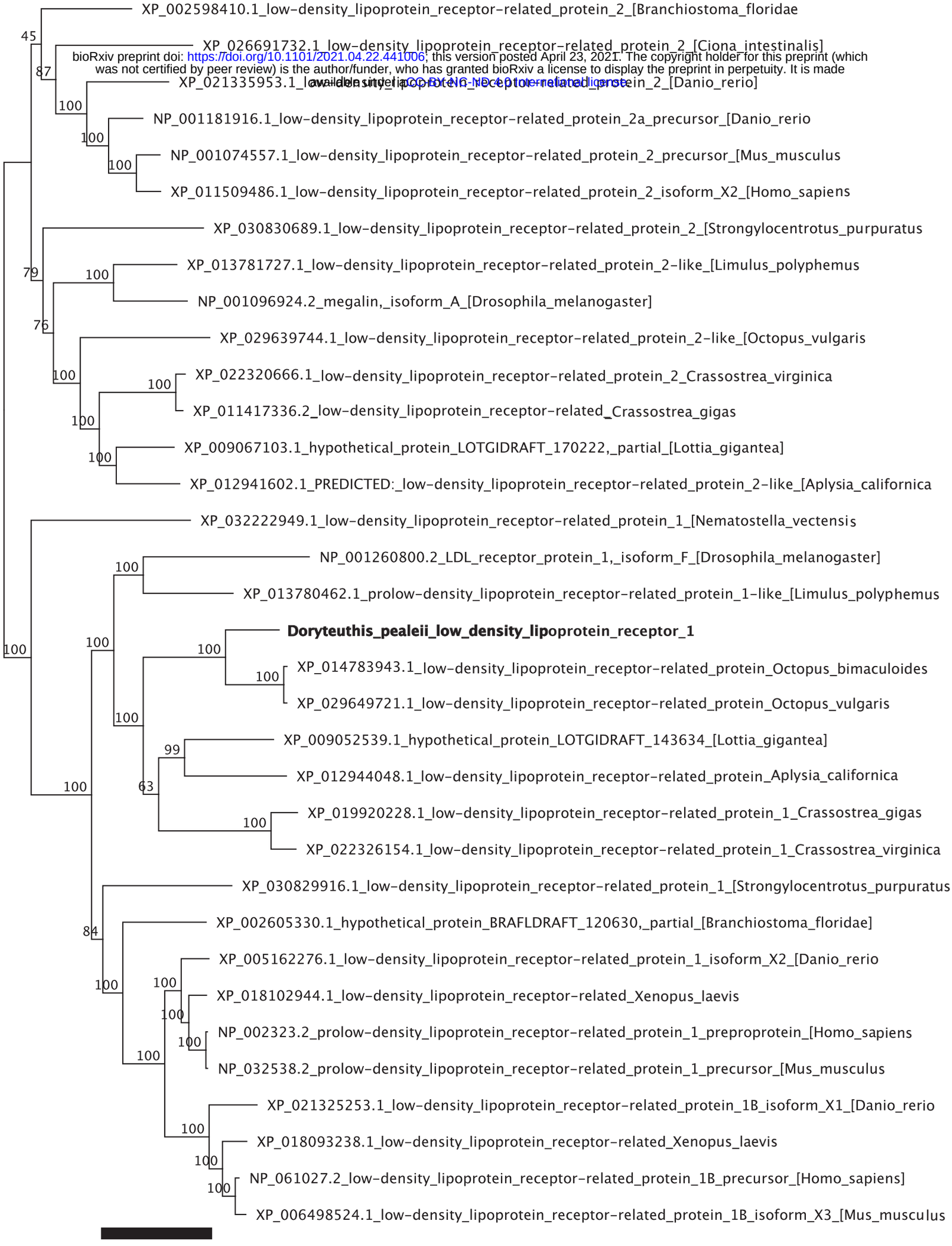


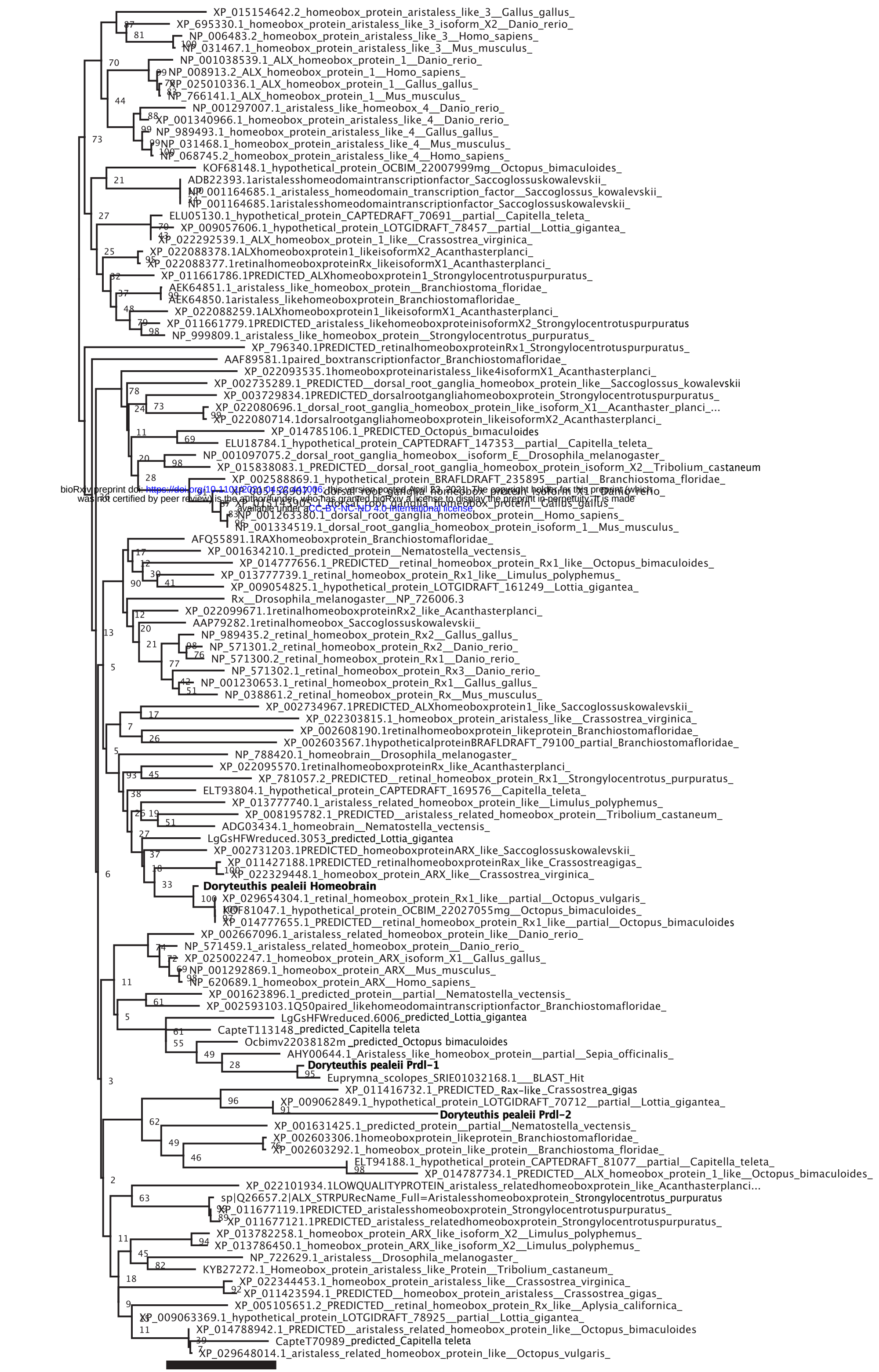






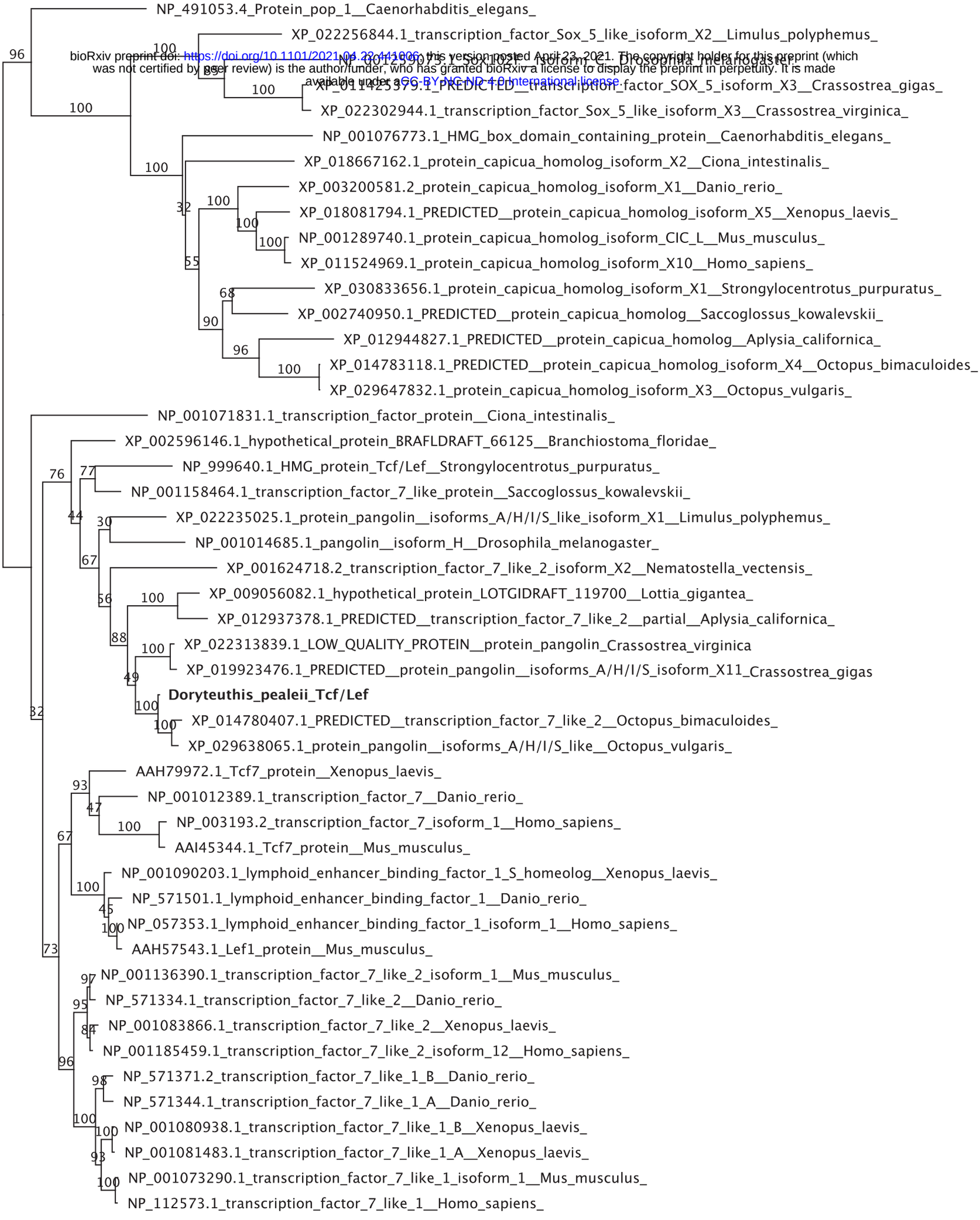






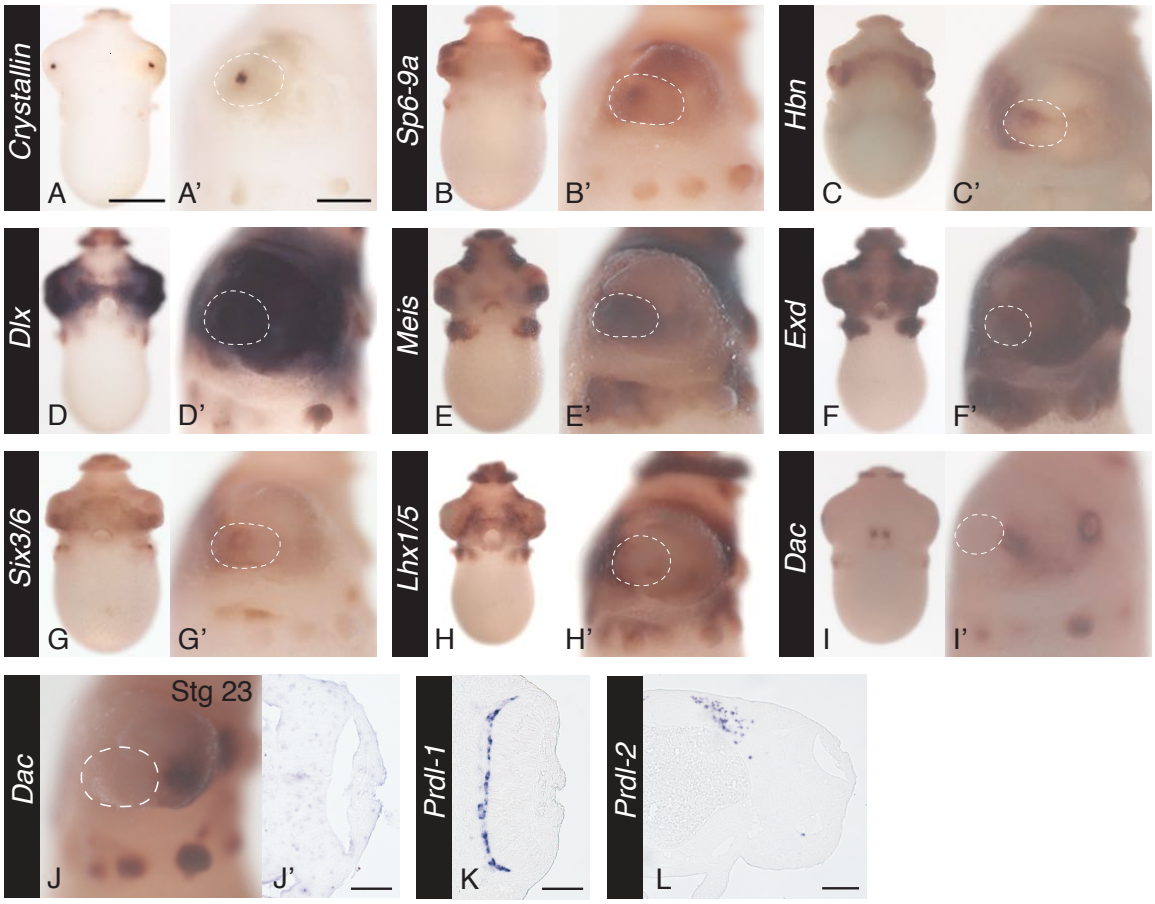
AAH75739.1_Meis3_protein__Danio_rerio_
NP_571853.1_homeobox_protein_Meis3__Danio_rerio_
AAH65977.1_Meis4.1a_protein__Danio_rerio_
NP_571968.1_homeobox_protein_Meis1__Danio_rerio_
XP_015133525.1_homeobox_protein_Meis1_isoform_X1__Gallus_gallus_
XP_011441096.1_PREDICTED__homeobox_protein_Meis1_isoform_X2__Crassostrea_gigas_
XP_014785942.1_PREDICTED__homeobox_protein_Meis1_like_isoform_X3__Octopus_bimaculoides_
**Doryteuthis peale**ii_Meis
XP_009057308.1_hypothetical_protein_LOTGIDRAFT_121570__partial__Lottia_gigantea_
XP_012940532.1_PREDICTED__homeobox_protein_Meis1_like__Aplysia_californica_
AMZ00044.1_transcription_factor_homothorax__partial__Platynereis_dumerilii_
ELT94055.1_hypothetical_protein_CAPTEDRAFT_139538__partial__Capitella_teleta_
XP_022237651.1_homeobox_protein_homothorax_like_isoform_X1__Limulus_polyphemus_
EFX75948.1_putative_transcriptional_factor_Homothorax_protein__Daphnia_pulex_
XP_015837042.1_PREDICTED__homothorax_isoform_X3__Tribolium_castaneum_
AAC47759.1_homothorax__Drosophila_melanogaster_
XP_024305695.1_homeobox_protein_Meis2_isoform_X3__Homo_sapiens_
NP_034955.1_homeobox_protein_Meis2_isoform_2__Mus_musculus_
XP_025006330.1_homeobox_protein_Meis2_isoform_X5__Gallus_gallus_
XP_009292852.1_meis_homeobox_2b_isoform_X1__Danio_rerio_
XP_009291615.1_homeobox_protein_Meis2_isoform_X1__Danio_rerio_
NP_034919.1_homeobox_protein_Meis1_isoform_A__Mus_musculus_
NP_002389.1_homeobox_protein_Meis1__Homo_sapiens_
NP_001333077.1_homeobox_protein_Meis3_isoform_4__Homo_sapiens_
NP_001269401.1_homeobox_protein_Meis3_isoform_a__Mus_musculus_
EFX84465.1_hypothetical_protein_DAPPUDRAFT_314795__Daphnia_pulex_
Homeobox_domain_containing_protein_OS=Tribolium_castaneum_TcasGA2_TC006040
XP_011427482.1_PREDICTED__homeobox_protein_PKNOX2__Crassostrea_gigas_
XP_014768004.1_PREDICTED__homeobox_protein_unc_62_like__Octopus_bimaculoides_
**Doryteuthis pealeii_PKNOX**
XP_009049380.1_hypothetical_protein_LOTGIDRAFT_178004__Lottia_gigantea_
XP_012940812.1_PREDICTED__homeobox_protein_PKNOX1_like__Aplysia_californica_
ELU05786.1_hypothetical_protein_CAPTEDRAFT_221385__partial__Capitella_teleta_
XP_005171069.1_homeobox_protein_PKNOX1__Danio_rerio_
XP_025001424.1_homeobox_protein_PKNOX1_isoform_X2__Gallus_gallus_
XP_006523921.1_homeobox_protein_PKNOX1_isoform_X3__Mus_musculus_
NP_001273187.1_homeobox_protein_PKNOX1_isoform_2__Homo_sapiens_
NP_705940.1_homeobox_protein_PKNOX2__Danio_rerio_
XP_989557.1_homeobox_protein_PKNOX2__Gallus_gallus_
NP_001025009.1_homeobox_protein_PKNOX2__Mus_musculus_
NP_071345.2_homeobox_protein_PKNOX2__Homo_sapiens_
XP_022248632.1_homeobox_protein_PKNOX2_like__Limulus_polyphemus_
NP_001278438.1_pre_B_cell_leukemia_transcription_factor_1_isoform_d__Mus_musculus_
XP_016856884.1_pre_B_cell_leukemia_transcription_factor_1_isoform_X4__Homo_sapiens_
QDF46130.1_extradenticle__partial__Sepia_officinalis_
XP_014777430.1_PREDICTED_Pbx_Octopus_bimaculoides
**Doryteuthis pealeii_Pbx**
XP_011452237.1_PREDICTED_Pbx_Crassostrea_gigas
XP_009044535.1_hypothetical_protein_LOTGIDRAFT_92730__partial__Lottia_gigantea_
XP_012941557.1_PREDICTED_Pbx_Aplysia_californica
AMZ00043.1_transcription_factor_extradenticle__partial__Platynereis_dumerilii_
XP_013791623.1_homeobox_protein_extradenticle_like__Limulus_polyphemus_
NP_001034501.1_extradenticle__Tribolium_castaneum_
NP_001259592.1_extradenticle__isoform_D__Drosophila_melanogaster_
NP_571522.1_pre_B_cell_leukemia_transcription_factor_4__Danio_rerio_
XP_015145727.1_pre_B_cell_leukemia_transcription_factor_1_isoform_X3__Gallus_gallus_
XP_423764.1_pre_B_cell_leukemia_transcription_factor_4__Gallus_gallus_
NP_001020125.1_pre_B_cell_leukemia_transcription_factor_4_isoform_1__Mus_musculus_
NP_079521.1_pre_B_cell_leukemia_transcription_factor_4__Homo_sapiens_
NP_571687.1_pre_B_cell_leukemia_homeobox_2__Danio_rerio_
NP_059491.1_pre_B_cell_leukemia_transcription_factor_2__Mus_musculus_
NP_002577.2_pre_B_cell_leukemia_transcription_factor_2__Homo_sapiens_
NP_001277505.1_pre_B_cell_leukemia_transcription_factor_3_isoform_PBX3b__Mus_musculus_
NP_571691.1_pre_B_cell_leukemia_transcription_factor_3_isoform_2__Danio_rerio_
XP_025011808.1_pre_B_cell_leukemia_transcription_factor_3_isoform_X2__Gallus_gallus_
NP_006186.1_pre_B_cell_leukemia_transcription_factor_3_isoform_1__Homo_sapiens_
NP_001077322.2_pre_B_cell_leukemia_homeobox_1__Danio_rerio_

7.0

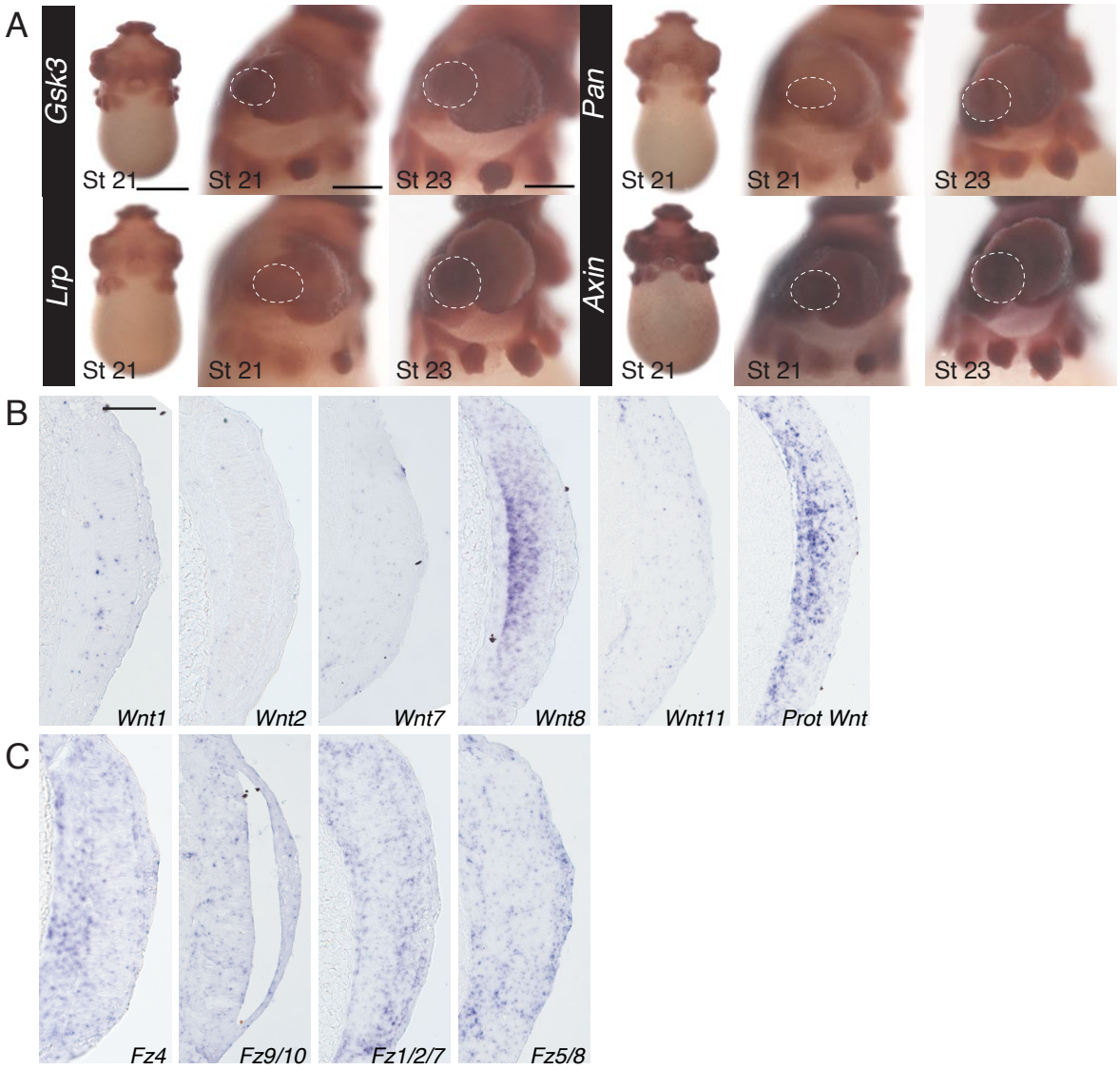


XP_015835532.1_PREDICTED__protein_Wnt_10a_isoform_X2__Tribolium_castaneum_
EFX86388.1_putative_secreted_signaling_factor_WNT10__Daphnia_pulex_
ELT95359.1_hypothetical_protein_CAPTEDRAFT_110385__partial__Capitella_teleta_
**Doryteuthis_pealeii_Wnt10**
XP_014782975.1_PREDICTED__protein_Wnt_10b_like__Octopus_bimaculoides_
XP_009045059.1_hypothetical_protein_LOTGIDRAFT_136550__partial__Lottia_gigantea_
XP_011455876.1_PREDICTED__protein_Wnt_10a_isoform_X2__Crassostrea_gigas_
XP_022327500.1_protein_Wnt_10b_like_isoform_X1__Crassostrea_virginica_
AAO24132.1_Wnt10b__Danio_rerio_
AAB51685.1_Wnt10B__Homo_sapiens_
AAB08086.1_Wnt10b__Mus_musculus_
AAG45153.1_WNT10A__Homo_sapiens_
AAB08085.1_Wnt10a__Mus_musculus_
NP_001006590.1_protein_Wnt_10a_precursor__Gallus_gallus_
AAA03431.1_Wnt10a_protein__Danio_rerio_
EFX86385.1_putative_secreted_signaling_factor_WNT9__Daphnia_pulex_
XP_011455892.2_PREDICTED__protein_Wnt_9a__partial__Crassostrea_gigas_
XP_022327145.1_protein_Wnt_9a_like__Crassostrea_virginica_
ELT95360.1_hypothetical_protein_CAPTEDRAFT_222661__Capitella_teleta_
XP_014788848.1_PREDICTED__protein_Wnt_9a_like__Octopus_bimaculoides_
NP_001131132.1_protein_Wnt_9b_precursor__Danio_rerio_
AAC39950.1_Wnt_15_protein__partial__Mus_musculus_
NP_001307387.1_protein_Wnt_9b_isoform_2_precursor__Homo_sapiens_
NP_001038828.1_protein_Wnt_9a_precursor__Danio_rerio_
NP_990312.2_protein_Wnt_9a_precursor__Gallus_gallus_
NP_003386.1_protein_Wnt_9a_precursor__Homo_sapiens_
NP_647459.1_protein_Wnt_9a_precursor__Mus_musculus_
ELT96282.1_hypothetical_protein_CAPTEDRAFT_90169__partial__Capitella_teleta_
XP_011412295.1_PREDICTED__protein_Wnt_8b__Crassostrea_gigas_
**Doryteuthis_pealeii_Wnt8**
XP_014774798.1_PREDICTED__protein_Wnt_8b_like_isoform_X2__Octopus_bimaculoides_
sp|P51029.1 Protein_Wnt_8b_Danio_rerio
sp|P51028.2 Protein_Wnt_8a_Danio_rerio
XP_016865313.1_protein_Wnt_8a_isoform_X1__Homo_sapiens_
EFX82994.1_putative_secreted_signaling_factor_WNT16__Daphnia_pulex_
XP_022242527.1_protein_Wnt_16_like__Limulus_polyphemus_
NP_001093516.1_protein_Wnt_16_precursor__Danio_rerio_
XP_001233678.4_protein_Wnt_16__Gallus_gallus_
NP_444346.3_protein_Wnt_16_precursor__Mus_musculus_
NP_476509.1_protein_Wnt_16_isoform_1_precursor__Homo_sapiens_
ABD16198.1_Wnt16__partial__Euprymna_scolopes_
XP_014774239.1_PREDICTED__protein_Wnt_16_like__Octopus_bimaculoides_
ELT87681.1_hypothetical_protein_CAPTEDRAFT_149951__Capitella_teleta_
XP_022287541.1_protein_Wnt_16_like__Crassostrea_virginica_
XP_009057928.1_hypothetical_protein_LOTGIDRAFT_105620__partial__Lottia_gigantea_
NP_001020711.1_protein_Wnt_7a__Danio_rerio_
NP_989623.1_protein_Wnt_7a__Gallus_gallus_
NP_004616.2_protein_Wnt_7a_precursor__Homo_sapiens_
NP_033553.2_protein_Wnt_7a_isoform_1_precursor__Mus_musculus_
XP_001920219.1_protein_Wnt_7b_isoform_X1__Danio_rerio_
NP_001165064.1_protein_Wnt_7b_isoform_2__Gallus_gallus_
NP_001157106.1_protein_Wnt_7b_isoform_2__Mus_musculus_
XP_011528668.1_protein_Wnt_7b_isoform_X1__Homo_sapiens_
ELU03126.1_hypothetical_protein_CAPTEDRAFT_112156__Capitella_teleta_
ADR81923.2_wnt_signaling_molecule__Platynereis_dumerilii_
XP_008196351.1_PREDICTED__protein_Wnt_7b_isoform_X1__Tribolium_castaneum_
NP_476810.1_Wnt_oncogene_analog_2__isoform_A__Drosophila_melanogaster_
XP_013778017.1_protein_Wnt_7b_like__Limulus_polyphemus_
EFX66449.1_putative_secreted_signaling_factor_WNT7__Daphnia_pulex_
XP_011440894.1_PREDICTED__protein_Wnt_7b_isoform_X1__Crassostrea_gigas_
XP_009064086.1_hypothetical_protein_LOTGIDRAFT_179503__Lottia_gigantea_
XP_014783133.1_XP_014780612_Wnt_7b_like_Octopus_bimaculoides
ABD16199.1_Wnt7__Euprymna_scolopes_
QDF46142.1_Wnt7__partial__Sepia_officinalis_
**Doryteuthis_pealeii_Wnt7**
EFX87200.1_putative_secreted_signaling_protein_WNT2__partial__Daphnia_pulex_
XP_022257593.1_protein_Wnt_2b_like__Limulus_polyphemus_
XP_416013.3_protein_Wnt_2__Gallus_gallus_
NP_076142.3_protein_Wnt_2_precursor__Mus_musculus_
NP_003382.1_protein_Wnt_2_precursor__Homo_sapiens_
NP_878296.1_protein_Wnt_2__Danio_rerio_
NP_989667.2_protein_Wnt_2b_precursor__Gallus_gallus_
NP_078613.1_protein_Wnt_2b_isoform_WNT_2B2__Homo_sapiens_
NP_033546.2_protein_Wnt_2b__Mus_musculus_
CAD37165.2_Wnt2_protein__Platynereis_dumerilii_
ELU12345.1_hypothetical_protein_CAPTEDRAFT_96953__partial__Capitella_teleta_
XP_009048179.1_hypothetical_protein_LOTGIDRAFT_139906__partial__Lottia_gigantea_
XP_022296940.1_protein_Wnt_2b_A_like_isoform_X2__Crassostrea_virginica_
XP_011444403.1_PREDICTED__protein_Wnt_2b_A_isoform_X4__Crassostrea_gigas_
XP_014786335.1_PREDICTED__protein_Wnt_2b_like__Octopus_bimaculoides_
ABD16195.1_Wnt2/13__Euprymna_scolopes_
QDF46141.1_Wnt2__partial__Sepia_officinalis_
**Doryteuthis_pealeii_Wnt2**
ADK38673.2_Wnt5__Platynereis_dumerilii_
ADB79793.1_WNT5__partial__Capitella_teleta_
XP_022299951.1_protein_Wnt_5b_like__Crassostrea_virginica_
XP_011420711.1_PREDICTED__protein_Wnt_5b__Crassostrea_gigas_
XP_009064088.1_hypothetical_protein_LOTGIDRAFT_130786__partial__Lottia_gigantea_
QDF46137.1_Wnt5__partial__Sepia_bandensis_
XP_014790565.1_PREDICTED__protein_Wnt_5b_like__Octopus_bimaculoides_
**Doryteuthis_pealeii_Wnt5**
ABD16197.1_Wnt5__Euprymna_scolopes_
XP_022249862.1_protein_Wnt_5b_like__Limulus_polyphemus_
EFX66479.1_putative_secreted_signaling_factor_WNT5__partial__Daphnia_pulex_
CAA46002.1_DWnt_3__Drosophila_melanogaster_
XP_974684.1_PREDICTED__protein_Wnt_5b__Tribolium_castaneum_
NP_990218.1_protein_Wnt_5a__Gallus_gallus_
XP_016862616.1_protein_Wnt_5a_isoform_X1__Homo_sapiens_
XP_006518989.1_protein_Wnt_5a_isoform_X2__Mus_musculus_
sp|Q92050.1 Protein_Wnt_5b_Danio_rerio
NP_001032346.1_protein_Wnt_5b_precursor__Gallus_gallus_
NP_116031.1_protein_Wnt_5b_precursor__Homo_sapiens_
NP_033551.2_protein_Wnt_5b_precursor__Mus_musculus_
XP_013783194.2_protein_Wnt_1_like__Limulus_polyphemus_
XP_008195370.1_PREDICTED__protein_Wnt_1__Tribolium_castaneum_
EFX69968.1_secreted_signaling_factor_WNT4_like_protein__partial__Daphnia_pulex_
ELU01968.1_hypothetical_protein_CAPTEDRAFT_216606__Capitella_teleta_
XP_011454083.1_PREDICTED__protein_Wnt_4__Crassostrea_gigas_
XP_022310096.1_protein_Wnt_5b_like__Crassostrea_virginica_
**Doryteuthis_pealeii_Protostome-specific_Wnt**
XP_014771787.1_PREDICTED__protein_Wnt_5b_like__Octopus_bimaculoides_
XP_022238888.1_protein_Wnt_7b_like__Limulus_polyphemus_
NP_001138276.1_protein_Wnt_11_precursor__Danio_rerio_
NP_990115.1_protein_Wnt_11_precursor__Gallus_gallus_
NP_001272721.1_protein_Wnt_11_isoform_a_precursor__Mus_musculus_
ELT92315.1_hypothetical_protein_CAPTEDRAFT_20087__Capitella_teleta_
XP_022341565.1_protein_Wnt_11b_2_like__Crassostrea_virginica_
XP_009044423.1_hypothetical_protein_LOTGIDRAFT_109316__partial__Lottia_gigantea_
KOF62848.1_hypothetical_protein_OCBIM_22021549mg__partial__Octopus_bimaculoides_
**Doryteuthis_pealeii_Wnt11**
ELU08380.1_hypothetical_protein_CAPTEDRAFT_181867__Capitella_teleta_
NP_033549.1_protein_Wnt_4_precursor__Mus_musculus_
NP_110388.2_protein_Wnt_4_precursor__Homo_sapiens_
AAA96518.1_Wnt4__Danio_rerio_
NP_990114.1_protein_Wnt_4_precursor__Gallus_gallus_
EFX72339.1_putative_secreted_signaling_factor_WNT4__partial__Daphnia_pulex_
XP_022249841.1_protein_Wnt_4_like__Limulus_polyphemus_
XP_014777619.1_PREDICTED__protein_Wnt_4_like__Octopus_bimaculoides_
ABD16196.1_Wnt4__Euprymna_scolopes_
**Doryteuthis_pealeii_Wnt4**
XP_009064139.1_hypothetical_protein_LOTGIDRAFT_130946__partial__Lottia_gigantea_
XP_011448637.1_PREDICTED__protein_Wnt_4__Crassostrea_gigas_
XP_022336586.1_protein_Wnt_4_like__Crassostrea_virginica_
NP_001007186.1_protein_Wnt_3a_precursor__Danio_rerio_
NP_149122.1_protein_Wnt_3a_precursor__Homo_sapiens_
NP_033548.1_protein_Wnt_3a_precursor__Mus_musculus_
NP_001165072.1_protein_Wnt_3a_isoform_1_precursor__Gallus_gallus_
NP_001075165.1_proto_oncogene_Wnt_3__Gallus_gallus_
NP_110380.1_proto_oncogene_Wnt_3_precursor__Homo_sapiens_
NP_033547.1_proto_oncogene_Wnt_3_precursor__Mus_musculus_
XP_003199237.2_protein_Wnt_6__Danio_rerio_
XP_015145265.1_protein_Wnt_6_isoform_X1__Gallus_gallus_
NP_006513.1_protein_Wnt_6_precursor__Homo_sapiens_
NP_033552.2_protein_Wnt_6_precursor__Mus_musculus_
EFX86167.1_putative_secreted_signaling_factor_WNT6__Daphnia_pulex_
NP_001260188.1_Wnt_oncogene_analog_6__isoform_C__Drosophila_melanogaster_
NP_001164137.1_Wnt6_protein_precursor__Tribolium_castaneum_
XP_009045057.1_hypothetical_protein_LOTGIDRAFT_136505__Lottia_gigantea_
ELT92804.1_hypothetical_protein_CAPTEDRAFT_182518__partial__Capitella_teleta_
XP_011455879.1_PREDICTED__protein_Wnt_6__Crassostrea_gigas_
XP_022326596.1_protein_Wnt_6_like__Crassostrea_virginica_
**Doryteuthis_pealeii_Wnt6**
XP_014782989.1_PREDICTED__protein_Wnt_6_like__Octopus_bimaculoides_
sp|P24257.1|WNT1_DANRE_RecName__Full=Protein_Wnt_1__Flags__Precursor
AJG06047.1_Wnt1__partial__Danio_rerio_
XP_015128668.1_protein_Wnt_1__Gallus_gallus_
NP_005421.1_proto_oncogene_Wnt_1_precursor__Homo_sapiens_
NP_067254.1_proto_oncogene_Wnt_1_precursor__Mus_musculus_
XP_013788136.1_protein_wingless_like__partial__Limulus_polyphemus_
EFX86386.1_putative_secreted_signaling_factor_WNT1__Daphnia_pulex_
XP_008193162.1_PREDICTED__wingless_isoform_X1__Tribolium_castaneum_
NP_523502.1_wingless__Drosophila_melanogaster_
AAZ08056.1_wingless/Wnt1_protein__Capitella_teleta_
CAD37164.2_Wnt1_protein__Platynereis_dumerilii_
XP_011455886.1_PREDICTED__protein_Wnt_1_isoform_X2__Crassostrea_gigas_
XP_009045056.1_hypothetical_protein_LOTGIDRAFT_170942__Lottia_gigantea_
XP_014782987.1_PREDICTED__protein_Wnt_1_like__Octopus_bimaculoides_
QDF46143.1_Wnt1__partial__Sepia_officinalis_
ABD16194.1_Wnt1__Euprymna_scolopes_
**Doryteuthis_pealeii_Wnt1**

2.0

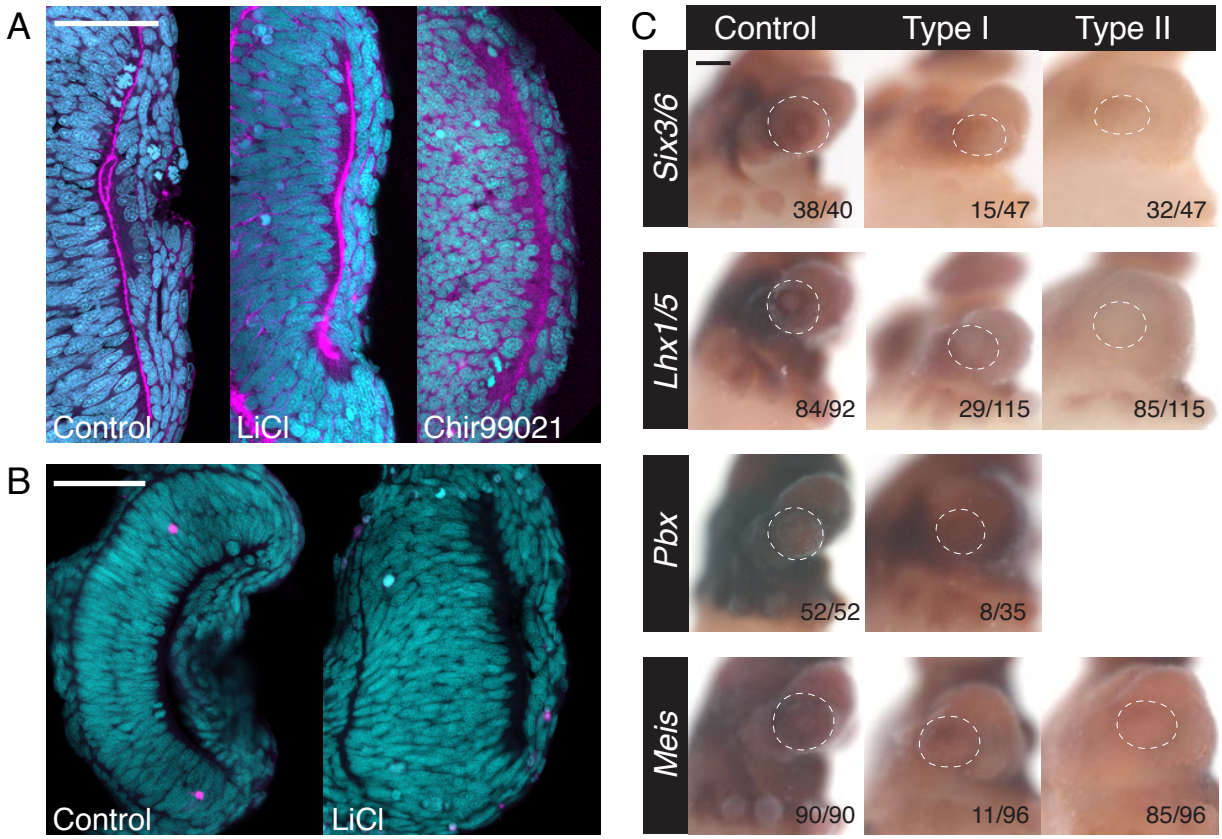



673
674 **Sup Figure 2: Limb network supplemental data**
675 A-I) Gene expression at stage 21 for limb network genes. For all genes from left to right, Anterior
676 whole-mount and lateral whole-mount, anterior to the left. Scale for whole-mount anterior view is
677 500 microns. Scale for lateral whole-mount view 200 microns. J, J') Stage 23 Dac expression. J)
678 Lateral whole mount, anterior to the left. J') Sectioned image of the eye. Anterior is down. K & L)
679 Sectioned image of expression of Prdl-1and Prdl-2. Scale is 50 microns on eye sections, 100
680 microns on brain section (Prdl-2)
681




682

683 **Sup Figure 3: Wnt signaling expression supplemental data**
684 A) Wnt signaling pathway member expression, *Gsk3, Lrp, Pan*, and *Axin*, at stage 21 and 23 in
685 whole-mount. Anterior view of stage 21and lateral views of stage 21 and stage 23 (anterior to the
686 left). B) Wnt gene expression at stage 21 in section. Anterior is down. C) Fz receptor gene
687 expression at stage 21. Anterior is down. Scale for whole-mount anterior view is 500 microns.
688 Scale for lateral whole-mount view 200 microns. Scale for sectioned images 50 microns.
689




690

691 **Sup Figure 4: Wnt agonist experiment supplemental data**

692 A) Wnt agonist experiments starting at stage 21. Embryos were treated for 24 hours and fixed
693 immediately. LiCl and Chir99021 show similar phenotypes: Lack of anterior segment thickness
694 and loss of lens formation. Sytox nuclear stain in cyan, Phalloidin stain in magenta. Scale is 50
695 microns. B) Tunel staining of the eye of Control and LiCl treated embryos. Sytox nuclear stain in
696 cyan, Tunel stain in magenta. Similar amounts of cell death observed in control and treated
697 animals. Scale is 100 microns C) *In situ* hybridization of limb patterning program members and
698 and anterior segment markers after LiCl treatment. Type I (mild) and Type II (severe) phenotype.
699 White dotted line outlines the eye in the lateral image. Number of eyes scored in control and the
700 two phenotypes found in LiCl treated animals in the bottom right corner. Scale for lateral whole-
701 mount view 200 microns.
702