Co-option of the limb patterning program in cephalopod eye development

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Abstract

Background: 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. Cephalopods have evolved a highly acute visual system with a cup-shaped retina and a novel refractive lens in the anterior, important for a number of sophisticated behaviors including predation, mating, and camouflage. Almost nothing is known about the molecular-genetics of lens development in the cephalopod.

Results: Here we identify the co-option of the canonical bilaterian limb patterning program 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 Drosophila limb, and find the regulatory relationship reversed, with ectopic Wnt signaling leading to lens loss.

Conclusion: This regulatory divergence suggests that duplication of SP6-9 in cephalopods may mediate the co-option of the limb patterning program. Thus, our study suggests that this program could perform a more universal developmental function in radial patterning and highlights how canonical genetic programs are repurposed in novel structures.

Keywords: Eye, Cephalopod, Eye evolution, Lens, Vision, Spiralia, Wnt, Limb patterning, Dlx

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Main text
Background
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 [1–3]. 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 [3–9]. This network is necessary in both vertebrate and cephalopod limb development and is expressed in a similar proximal-distal pattern in a diversity of outgrowths [1, 3, 5, 10–25]. 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 do not support an ancestor with appendages and therefore the network’s ancestral function remains unclear [1–3]. Many alternative hypotheses have been proposed, including an ancestral role in the nervous system, body axis formation, and radial patterning [2, 3, 26–30]. 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 [31]. 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 [31]. 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 [32–38]. Cephalopods have a single-chambered eye, morphologically convergent with the vertebrate eye, composed of a cup-shaped retina and a single refractive lens [39]. Here we perform the first in-depth molecular description of lens development in the squid Doryteuthis pealeii, we identify spatiotemporal 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) [40–42]. Lentigenic cells are arranged circumferentially around the developing lens and extend long cellular processes, fusing into plates to form the lens (Fig. 1A) [40, 41, 43–45]. 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) (Figs. 1B and 2A) [43, 44, 46]. 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 [47, 48] (Supplemental Figure 1). The first evidence of expression correlates with changes in nuclear morphology at stage 21 (Fig. 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 [46, 50]. 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) (Fig. 2B, Supplemental Figure 2, Supplemental Figure 3). The proximal central cells lacking DpSix3/6 expression correspond to the LC2 population (Fig. 2A, B”). Asymmetry along the animal anterior-posterior axis in the eye is also apparent, with enrichment on the anterior side of the animal (Fig. 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) (Fig. 2C, Supplemental Figure 2, Supplemental Figure 3). 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) (Fig. 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, Meis, Pbx, and Dac at stages 21 and 23 (Fig. 2, Supplemental Figure 2, Supplemental Figure 3). All genes were clearly expressed in the developing anterior segment and lentigenic cells with the exception of DpDac (Fig. 2E–G, Supplemental Figure 2C–2I, Supplemental Figure 3). We find DpSP6-9a and DpDlx have overlapping expression, in the central cup cells (cc) and all proximal cells (LC2 and pm) (Fig. 2D–E”, Supplemental Figure 3). DpMeis and DpPbx are both broadly expressed in the
anterior segment during lens development, with \textit{DpPbx} excluded from the LC2 cells (Fig. 2F\textsuperscript{G}, Supplemental Figure 3).

It is known that the transcription factor \textit{aristaless} is necessary for the most distal tip of the \textit{Drosophila} limb in the limb program [9]. The evolutionary relationship of Prd-like homologs (Arx/\textit{Aristaless}, Alx/\textit{Aristaless-like}, Rx/\textit{Retinal Homeobox}, and Hbn/\textit{Homeobrain}) is ambiguous across species [51]. We identified three candidate Prd-like genes in \textit{D. pealeii} and performed in situ hybridization for all three homologs, \textit{DpHbn}, \textit{DpPrdl-1}, and \textit{DpPrdl-2} (Supplemental Figure 2K, L) [46]. \textit{DpHbn} is expressed in the anterior segment in the distal central cup cells (cc) while \textit{DpPrdl-1} and \textit{DpPrdl-2} are excluded from the eye (Fig. 2H\textsuperscript{G} and Supplemental Figure 2C, C', K and L, Supplemental Figure 3). \textit{DpHbn}'s central, distal expression recapitulates \textit{aristaless} expression in the developing \textit{Drosophila} limb.

Our data show that the majority of the proximodistal patterning genes in the developing limb, including \textit{SP6-9}, \textit{Dlx}, \textit{Meis}, \textit{Pbx}, as well as the Prd-like homolog, \textit{Hbn}, show expression in concentric and overlapping cell populations surrounding the developing lens in the squid (Fig. 2). This pattern of expression is similar to the bullseye-like pattern of expression of these genes in the developing \textit{Drosophila} limb imaginal disc and suggests a co-option of this regulatory program for a new function: patterning the cephalopod anterior segment and lens [14].

**Canonical Wnt signaling genes expressed during anterior segment development**

The duplication of SP6-9 in cephalopods may provide a substrate for the evolution of \textit{cis}-regulation, resulting in novel expression of the limb patterning program in the cephalopod lens. In \textit{Drosophila} appendage outgrowth, active Wnt signaling is upstream of the expression of SP6-9 [52, 53]. 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 (Fig. 3, Supplemental Figure 4). 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 (Fig. 3A’, C’, and D–G). DpWnt8, DpWnt11, and DpProtostome-specific Wnt show the most robust retinal expression (Fig. 3A’, F, and G), and DpWnt7 is the only Wnt expressed in the anterior segment (Fig. 3C). DpWnt6 showed no evidence of expression in the developing eye (data not shown). These data support the hypothesis that Wnt signals emanating from the anterior segment or 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 a subset of cells in the anterior segment, while others, like DpFz9/10, are excluded from the anterior segment (Fig. 3H–K, Supplemental Figure 4). On close examination, we find that DpFz5/8 is excluded asymmetrically in the anterior segment and may be important for anterior-posterior patterning (Fig. 3J’, J”, Supplemental Figure 4D). DpFz1/2/7 are 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.
Fig. 3 (See legend on next page.)
similar phenotypes (Supplemental Figure 5A). We observed the lens smaller and the anterior segment less thick than in the beginning of lentigenic cell differentiation. We performed Wnt antagonist treatments (Quercetin) starting at stage 21, treated for 24 h and allowed to recover for 48 h and fixed immediately. M and N control and Wnt agonist (LiCl) treatments started at stage 23, treated for 24 h and allowed to recover for 48 h and fixed. O and O' control and Wnt agonist (LiCl) treatments started at stage 23, treated for 24 h and allowed to recover for 48 h and fixed. Arrowhead highlights the lens. P-S in situ hybridization of anterior segment markers after 24 h 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. 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 μm. Scale for all sectioned images is 50 μm. Anterior is down in all sectioned images. White dotted line in whole mount images identifies the perimeter of the eye.

Control and LiCl agonist treatments started at stage 21, treated for 24 h and fixed immediately. J and K are cartoons of expression in J' and K' respectively. Gradients of expression show intracellular asymmetries in the anterior segment. Black dotted line in sectioned images shows 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 h and fixed immediately. M and M' Control and Wnt agonist (LiCl) treatments started at stage 23 for 24 h and fixed immediately. N and N' Control and Wnt agonist (LiCl) treatments started at stage 21, treated for 24 h and allowed to recover for 48 h and fixed. O and O' Control and Wnt agonist (LiCl) treatments started at stage 23, treated for 24 h and allowed to recover for 48 h and fixed. Arrowhead highlights the lens. P-S in situ hybridization of anterior segment markers after 24 h 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. 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 μm. Scale for all sectioned images is 50 μm. Anterior is down in all sectioned images. White dotted line in whole mount images identifies the perimeter of the eye.
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 (Fig. 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 6A-6C).

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 (Fig. 3Q–S, Supplementary Figure 6A-6C). Similar to DpS-Crystallin expression, we again see a mild reduction (Type I) or loss and severe reduction (Type II) in region of expression. Our milder phenotypes, again, show a dorsal asymmetry, which can be most easily seen in DpSP6-9A, DpDlx, and DpHbn (Fig. 3Q, Q’, Q”; R, R’, R”; and S, S’, S”). Changes are also visible but less obvious in DpPbx and DpMeis expression, with DpPbx only showing a mild phenotype (Supplemental Figure 6A-6C). These data are consistent with 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 the 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 [24, 53]. This change in signaling and the known duplication of SP6-9 and the novel expression of the SP6-9a paralog in the anterior segment suggests that this duplication may be a mediator of limb patterning program co-option in the anterior segment. In vertebrates, although the limb patterning transcription factors are expressed during central nervous system development, including in the vertebrate retina, they do not have a role in lens development. Our gene expression data also suggest a role for the limb patterning program in the cephalopod nervous system, including the retina. It is known that SP6-9 and Dlx are required for proper regeneration of the lens-less eye in the Planarian Schmidtea mediterranea, supporting an ancestral role in the Lophotrochozoa for these genes in eye formation [18]. The co-option of this network in the cephalopod lens may suggest an elaboration of the ancestral nervous system or retinal tissue [58]. This is also supported by lineage tracing data, where, early in squid development, anterior segment tissue is derived from epithelial cells contiguous with the neighboring retinal primordia [46]. In the vertebrate case, cranial ectodermal...
placodes are the developmental origin of the lens [59]. The vertebrate retina is derived from evagination of forebrain neurectoderm making it unlikely that the lens evolved as an elaboration of retinal tissue [60]. Together this suggests that the convergent evolution of complex phenotypes relies on a diversity of developmental origins.

Finally, with little similarity between limb and lens, our work suggests that the function of the limb patterning program in a limbless ancestor may have been 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 hypothesis, we conclude that the ability to pattern in a radial fashion, as previously proposed, is a more inclusive hypothesis, we conclude that the ability to pattern in a radial fashion, as previously proposed, is a more inclusive hypothesis, we conclude that the ability to pattern in a radial fashion, as previously proposed, is a more inclusive hypothesis, we conclude that the ability to pattern in a radial fashion, as previously proposed, is a more inclusive hypothesis, we conclude that the ability to pattern in a radial fashion, as previously proposed, is a more inclusive hypothesis, we conclude that the ability to pattern in a radial fashion, as previously proposed, is a more inclusive hypothesis, we conclude that the ability to pattern in a radial fashion, as previously proposed, is a more inclusive...
SSC, 0.2x SSC, and 0.02x SSC, all at 70 °C. 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 °C. 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-Green 1:1000 overnight. Slides mounted in ImmunohistoMount (Abcam) and imaged on a Zeiss Axioscope. Dps-Crystallin embryo in situ was transitioned to sucrose and embedded after imaging in whole-mount. Embryos were on a Zeiss Axioscope.

Ex ovo experimental culture
Ex ovo culture was performed as previously described [46]. Embryos were bathed in 0.25 M, 0.15 M, and 0.07 M LiCl; 100 nm, 250 nm, and 500 nm concentration of Wnt Agonist (CHIR99021); and 25 μM, 50 μM, and 100 μM Quercetin in Pen-Step filter-sterilized seawater to determine a working concentration. Control animals were bathed in equivalent amounts of DMSO or Pen-Strep alone.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12195-021-01182-2.

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Authors’ contributions
KMK designed the experiments. S.N., KJM, F.N., C.D., J.C., and KMK performed experiments. KJM performed phylogenetic analyses. KMK, S.N., and K.J.M. wrote the manuscript with consultation from all authors. All authors read and approved the final manuscript.

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Availability of data and materials
All sequences generated and analyzed in this study have been deposited in NCBI’s GenBank database under accession numbers MR202516-MR202549. All multiple sequence alignments and phylogenetic trees are available at doi:10.5061/dryad.vhhmgqpnv [65].

Declarations
Ethics approval and consent to participate
No ethics approvals or consent is required to work with cephalopod embryos.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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