The Bilaterian Head Patterning Gene *six3/6* Controls Aboral Domain Development in a Cnidarian

Chiara Sinigaglia¹, Henriette Busengdal¹, Lucas Leclère¹, Ulrich Technau², Fabian Rentzsch¹*

¹ Sars Centre for Marine Molecular Biology, University of Bergen, Bergen, Norway; ² Department of Molecular Evolution and Development, Centre of Organismal Systems Biology, Faculty of Life Sciences, University of Vienna, Vienna, Austria

Abstract

The origin of the bilaterian head is a fundamental question for the evolution of animal body plans. The head of bilaterians develops at the anterior end of their primary body axis and is the site where the brain is located. Cnidarians, the sister group to bilaterians, lack brain-like structures and it is not clear whether the oral, the aboral, or none of the ends of the cnidarian primary body axis corresponds to the anterior domain of bilaterians. In order to understand the evolutionary origin of head development, we analysed the function of conserved genetic regulators of bilaterian anterior development in the sea anemone *Nematostella vectensis*. We show that orthologs of the bilaterian anterior developmental genes *six3/6, foxQ2*, and *irx* have dynamic expression patterns in the aboral region of *Nematostella*. Functional analyses reveal that *NvSix3/6* acts upstream of *NvFoxQ2a* as a key regulator of the development of a aboral broad territory in *Nematostella*. *NvSix3/6* initiates an autoregulatory feedback loop involving positive and negative regulators of FGF signalling, which subsequently results in the downregulation of *NvSix3/6* and *NvFoxQ2a* in a small domain at the aboral pole, from which the apical organ develops. We show that signalling by *NvFGFa1* is specifically required for the development of the apical organ, whereas *NvSix3/6* has an earlier and broader function in the specification of the aboral territory. Our functional and gene expression data suggest that the head-forming region of bilaterians is derived from the aboral domain of the cnidarian-bilaterian ancestor.

Introduction

The head of bilaterians develops at the anterior end of the anterior-posterior body axis and is characterized by an accumulation of interconnected nerve cells, the brain. The polarity of the anterior-posterior (A-P) axis is in most bilaterians prefigured by the animal–vegetal axis of the oocyte, with the head forming anterior end developing from the animal hemisphere and the site of gastrulation from the vegetal hemisphere [1–3].

Comparative work in bilaterians has revealed highly conserved molecular mechanisms involved in the patterning of the anterior-posterior axis. Orthologous *hox* genes have been shown to have staggered anterior expression borders, providing a unique signature of *hox* gene expression for consecutive domains along the A-P axis. However, while *hox* genes are essential regulators of axial patterning, their expression in most bilaterians does not extend to the anterior-most part of the embryo, where the head will form [e.g., (4–8)].

The anterior, *hox*-free region of bilaterian embryos gives rise to head and brain structures and an important regulator of its development is the conserved homeodomain transcription factor *six3*. The expression pattern of *six3* is conserved in the three clades of bilaterians: deuterostomes, ecdysozoans, and lophotrochozoans [9–18]. Knockdown of *six3* function in sea urchin results in loss of the anterior/apical plate, *six3* RNAi in the beetle *Tricholium castaneum* leads to failure of anterior head development [11,19], and *six3* knockout mice lack almost the entire forebrain [20], suggesting that *six3* regulated anterior development already in the last common ancestor of all bilaterians.

A second well-conserved marker for anterior-most territories is the forkhead domain transcription factor *foxQ2*, which functions downstream of *six3* in the development of the anterior/apical domain of sea urchin, *Tetrahyridium castaneum* leads to failure of anterior head development [11,19], and *six3* knockout mice lack almost the entire forebrain [20], suggesting that *six3* regulated anterior development already in the last common ancestor of all bilaterians.

The posterior pole of a wide range of bilaterians is characterised by the expression of secreted signalling molecules of the *wnt* family. The posterior expression of canonical *wnt* genes results in the nuclear accumulation of β-catenin and determines the site of gastrulation [23–32].

Despite the advanced understanding of the mechanisms that pattern the anterior-posterior axis and position the head of bilaterians, the evolutionary origins of axial patterning and head formation are not clear. As the sister group to Bilateria, cnidarians are an essential outgroup to understand the origin of the bilaterian head.
Author Summary

The evolutionary origin of head development is a fundamental question for understanding the evolution of animal body plans. Bilaterally symmetrical animals (Bilaterians) have an anterior-posterior (head-to-tail) axis, whose anterior end is usually characterized by a nervous system centralization, the brain. This region is often associated with a distinct structure, the head, and its development is regulated by a set of conserved transcription factors and signalling molecules. Bilaterians evolved from an ancestor shared with cnidarians (corals, sea anemones, jellyfish), but brain-like structures are absent in cnidarians, although they have an obvious oral-aboral axis. Cnidarian larvae move with the aboral pole forward, but as adult polyps this pole is anchored to the ground, while the oral end is used for feeding. It is unclear whether one of the termini of cnidarians corresponds to the bilaterian head-forming region. We show here that in the sea anemone Nematostella vectensis genes regulating bilaterian head development are expressed at the larval aboral pole and that a key anterior developmental gene, \textit{six3/6}, controls the development of the aboral pole. These findings support the hypothesis that the anterior, head-forming, region of bilaterians and the aboral region of cnidarians derived from the same domain of their last common ancestor and are therefore homologues.

anterior-posterior axis. Cnidarians (sea anemones, corals, jellyfish) do not have a brain-like centralization of the nervous system, but they possess a clear oral-aboral axis [33]. This axis is commonly assumed to be related to the anterior-posterior axis of bilaterians, but there is no consensus as to whether one of the poles of the cnidian axis corresponds to the anterior, head-forming pole of bilaterians. Competing hypotheses about the relation of the cnidian primary body axis to the anterior pole of bilaterians suggest that either the oral or the aboral pole of cnidarians is homologous to the bilaterian anterior pole or that there is no homologous region in cnidarians. Similar to many ciliated bilaterian larvae, some anchozooan cnidian planulae swim with an apical tuft, located opposite to the gastrulation site, pointing forward. These observations have led to the interpretation that the aboral pole of cnidarians corresponds to the anterior pole of bilaterians (e.g., [34–36]). In direct contrast, observations from comparative embryology motivated the hypothesis that the oral pole of cnidarians corresponds to the anterior pole of bilaterians [3]. In cnidarians, gastrulation occurs in the domain that is derived from the animal pole of the fertilized egg and the blastopore becomes the only opening of the organism, whereas bilaterians gastrulate from the vegetal pole, but their mouth and anterior nervous system form from the animal domain [3,37–42]. This fundamental embryological difference lead to the hypothesis that in stem bilaterians, determinants of the gastrulation site changed from the animal to the vegetal pole, while determinants of mouth and brain development remained at the animal pole, meaning that the oral pole of cnidarians corresponds to the anterior pole of bilaterians [3]. In a third hypothesis, the absence of nervous system centralization and the incongruent expression of orthologs of some bilaterian anterior patterning genes have been interpreted to suggest that the anterior region of bilaterians has no equivalent in cnidarians [43] (see also [34,44] for reviews).

The expression patterns of homologs of bilaterian axial patterning genes in cnidarians have not provided support for any of the above-mentioned scenarios. \textit{Hox} genes have frequently been used to understand axial patterning in cnidarians; however, since these genes are not expressed at the anterior-most end of bilaterians, they do not allow the identification of a putative equivalent of the head-forming region in cnidarians. Moreover, anterior group \textit{Hox} genes have been found to be expressed either at the aboral pole (\textit{Clytia}, \textit{Eulithora}, \textit{Podocoryne}; [45–47]) or at the oral pole (\textit{Nematostella}; [48]) of cnidian planulae.

Expression of cnidian \textit{Wnt} ligands is strongly correlated with the gastrulation site, both in species that gastrulate by invagination and by unipolar ingestion [49–51]. Functional studies have shown that canonical \textit{Wnt}/\textit{\beta}-catenin signalling is required for proper axial patterning by promoting oral fates in \textit{Hydractinia}, \textit{Clytia}, and \textit{Hydra} [50–53], and it has been shown to promote endoderm formation in \textit{Nematostella} [54,55]. These data suggest that a \textit{Wnt}-expressing signalling centre was already present at the gastrulation site of the last common ancestor of cnidarians and bilaterians. This observation is often taken as evidence that the site of gastrulation in cnidarians identifies their posterior pole, as in bilaterians. However, even if this holds true, it remains unclear whether the opposite, aboral pole is developmentally equivalent to the bilaterian anterior pole (see above).

Attempts to identify a conserved role of bilaterian head development genes in cnidarian development have so far been unsuccessful. These attempts were based on a limited number of genes, and their inconsistent expression patterns [43,56–58] do not rule out that a core set of bilaterian anterior patterning genes with a conserved function in axial patterning exists in cnidarians.

In the present study we show that such a conserved set of bilaterian anterior development genes regulates aboral pole development in the sea anemone \textit{Nematostella vectensis}. \textit{Nematostella} gastrulates by invagination, forming a free swimming planula that carries a ciliated apical organ at the aboral pole. The planula settles on the aboral pole and develops into a sessile polyp [40,59–61]. We analysed the role of orthologs of two conserved bilaterian anterior development genes, \textit{NvSix3/6} and \textit{NvFoxQ2a}, and show that they are involved in the development of the aboral territory in \textit{Nematostella} by establishing an autoregulatory FGF feedback loop that leads to the patterning of the aboral territory into distinct domains. Our functional and expression data suggest that the bilaterian head is derived from the aboral region of the last common ancestor of cnidarians and bilaterians.

Results

Bilaterian Head Genes Are Expressed in the Aboral Domain of Developing \textit{Nematostella}

Recent gene expression analyses in bilaterian embryos have identified \textit{six3} and \textit{foxQ2} as highly conserved developmental markers of anterior/apical territories (Figure S1 and Introduction). Moreover, functional studies revealed an essential role for \textit{six3} in anterior development in sea urchins, vertebrates, and the arthropod \textit{Tribolium} [11,19,20], indicating that \textit{six3} likely played this role in the common ancestor to all Bilateria.

The \textit{Nematostella} genome contains a single gene of the \textit{six3/6} group and four \textit{foxQ2} genes [62]. In situ hybridization revealed that \textit{NvSix3/6} and one of the \textit{Nematostella} \textit{foxQ2} genes, \textit{NvFoxQ2a}, display regionally restricted expression patterns: from the blastula stage on, they are expressed in a broad domain on one side of the embryo (Figure 1A1 and A2, 1B1 and B2), which can be identified as the aboral side following the onset of gastrulation (Figure 1A3 and A4, 1B3 and 4). From the mid-planula stage, the expression is excluded from a small spot-like domain at the aboral pole, preceding the appearance of the long cilia of the apical tuft (Figure 1A5–7, 1B5–7). Similar expression dynamics have been described for \textit{NvFoxD1} [63], a gene that is also expressed in the
anterior/apical domain of the sea urchin larva and in the mouse forebrain \[21,64\]. In addition, \(NvSix3/6\) is expressed in a small number of scattered cells in both endo- and ectoderm of the planula, which by morphology resemble sensory cells (Figure S2). Like \(NvSix3/6\) and \(NvFoxQ2a\), the single \textit{Nematostella} ortholog of the bilaterian anterior marker gene \(iroquois/irx\) \[9,16,62,65–70\] is expressed in a broad aboral domain at the blastula and gastrula stages. However, after gastrulation, the expression of \(NvIrx\) becomes restricted to a small spot at the aboral pole, matching the site from which \(NvSix3/6\) and \(NvFoxQ2a\) are excluded (Figure 1C1–C7). The expression dynamics of \(NvIrx\) are very similar to that of several previously described genes that have been described previously are shown in gray. Note that \(NvSoxB(1)\), \(NvHoxF/Anthox1\), and \(NvFGF8a\) have additional expression domains not indicated in the cartoon.

doi:10.1371/journal.pbio.1001488.g001

Evolutionary Origin of Head Development

Figure 1. Bilaterian anterior marker genes are expressed at the aboral pole of \textit{Nematostella}. (A1–E7) In situ hybridizations, probes are indicated on the left; developmental stages above the images. (A–E2, A–E3, A–E5, and A–E6) are lateral views, from planula stage on with the aboral pole to the left. At the blastula stage, embryos are oriented with the assumption that the staining is continuous between the blastula and gastrula stage. Note that the future oral and aboral sides cannot be distinguished morphologically prior to gastrulation. (A–E4 and A–E7) are aboral views. Expression domains of \(NvSix3/6\), \(NvFoxQ2a\), \(NvIrx\), and \(NvFGFa1\) are highly similar until the gastrula stage and then segregate into mutually exclusive domains. \(NvFoxJ1\) expression becomes detectable only after gastrulation. (F1 and F2) Double in situ hybridization with \(NvSix3/6\) (red) and \(NvFGFa2\) (blue) probes, showing the early restriction of the \(NvFGFa2\) expression from the gastrula stage on. (G–I) Schematic representation of the three types of aboral expression patterns: (G) "ring genes," (H) "spot genes," and (I) "late genes." Synexpression groups are indicated on the right side: genes that have been described previously are shown in gray. Note that \(NvSoxB(1)\), \(NvHoxF/Anthox1\), and \(NvFGF8a\) have additional expression domains not indicated in the cartoon.

Single and double in situ hybridizations show that the restriction of \(NvFGFa1\) and \(NvFGFa2\) expression precedes the occurrence of the gap in the expression of \(NvSix3/6\) and \(NvFoxQ2a\) (Figure 1F1–F2 and unpublished data).

The apical sense organ, which develops at the aboral pole, consists of a heterogenous group of cells, most of which carry the long cilia that constitute the apical tuft. \(FoxJ1\) is an important regulator of cilial formation in vertebrates \[74\], and it has been shown to be expressed in the apical tuft of the sea urchin larvae \[21\]. Similarly, we found that expression of \(NvFoxJ1\) \[62\] is first detectable at the midplanula stage as a spot at the aboral pole, coincident with the development of the apical organ (Figure 1E1–E7).

From these data it is evident that a subset of bilaterian head development genes and components of the FGF signalling pathway are expressed at the aboral pole of \textit{Nematostella} with distinct dynamics. At the blastula and gastrula stages, \(NvSix3/6\),
*NvFoxQ2a, NvIrx, NvFGFa1, NvFGFa2, and NvFGFRa are broadly expressed at the aboral pole, but after gastrulation they segregate into mutually exclusive domains: *NvSix3/6, NvFoxQ2a, and NvFoxD1* [63] are expressed in a broad ring surrounding the apical organ (“ring genes,” Figure 1G), whereas the expression of *NvIrx, NvFGFa1, NvFGFa2, and FGFra* becomes restricted to the apical organ (“spot genes,” which include also *NvSosB(1) and NvHoxF/Anthox1*, Figure 1H). *NvFoxJ1* exemplifies a third type of aboral expression pattern, with an onset of expression as a spot only after gastrulation (“late genes,” Figure 1I), probably indicating a function in the differentiation of the apical organ cells.

**FGF Signalling Is Required to Exclude *NvSix3/6* from the Apical Organ Domain**

In order to understand how the observed dynamics of the expression patterns relate to the development of the aboral region, we conducted a series of morpholino (MO)-mediated gene knockdown experiments. Injection of two different control morpholinos had no effect on the development or on gene expression patterns. The expression of EGFP from specific reporter mRNAs was blocked when co-injected with the gene specific, but not with the control morpholinos (Figure S4 and Materials and Methods).

We have previously shown that the development of the apical organ in *Nematostella* is controlled by the opposing activities of two FGF ligands, with *NvFGFa1* being essential for apical organ development and *NvFGFa2* counteracting FGF receptor activity to limit the size of the apical organ ([72]; Figure 2A–C). We used the morpholinos (MOs) against *NvFGFa1* and *NvFGFa2* in order to understand the dependence of the different aborally expressed genes on FGF signalling. Injection of *NvFGFa1* MO does not affect the width of the *NvSix3/6, NvFoxQ2a*, and *NvFoxD1* expression domains at the planula stage, however the spot-like gap at the aboral pole is not present any longer (Figure 2D, E, G, H and Figure S5). Expression of the “spot-genes” *NvIrx, NvSosB(1), and NvHoxF/Anthox1* and of the “late gene” *NvFoxJ1* is suppressed (Figure 2J, K, M, N, P, Q, S, T) in a specific manner, as

![Figure 2. FGF signalling specifically controls gene expression in the apical organ territory.](https://doi.org/10.1371/journal.pbio.1001488.g002)
demonstrated by unaffected expression of NvSoxB(1) (Figure 2N) and NvHoxF/Anthox1 (Figure 2Q) in domains outside the aboral pole. In contrast, injection of NvFGFa2 MO, which results in an expansion of the apical organ [72], leads to the opposite effect. For NvSix3/6 and NvFoxQ2a, the gap in the expression domains at the aboral pole is expanded (Figure 2F and I), whereas for the “spot genes” and “late genes” the aboral expression domain is enlarged (Figure 2L, O, R, U). These results suggest that the broad aboral expression of NvSix3/6 and NvFoxQ2a is independent of FGF signalling but that their suppression at the aboral pole after gastrulation requires FGF activity. Furthermore, the expression of “spot” and “late” genes at the planula stage requires FGF signalling, suggesting an apical organ-specific function of the FGF pathway.

NvSix3/6 Is Required for the Development of the Aboral Region

As the broad aboral expression of NvSix3/6 and NvFoxQ2a is unaffected by the loss of FGF signalling, we tested whether these genes act upstream of FGF signalling. Injection of a translation-blocking NvSix3/6 MO resulted in planulae that do not extend normally along the oral-aboral axis and that lack the apical tuft (Figure 3A and B). The expression of NvFGFa1, NvFGFa2, NvFGFrA, NvFoxQ2a and NvFoxD1, NvFoxJ1, and the other spot genes is absent or strongly reduced in NvSix3/6 MO-injected planulae (Figure 3D and E, G and H, M and N, P and Q, S and T, V and W, Y and Z, and Figure S6), whereas the expression of NvSix3/6 itself is maintained, except for the absence of the gap corresponding to the apical tuft region (Figure 3J and K). Injection of two different translation-blocking morpholinos against NvFoxQ2a has a comparably small effect, since the overall morphology of the planula is unaffected, and the apical organ, although smaller, is still present. In situ hybridization analysis shows slightly reduced expression of the “spot genes” (Figure 3F, I, R, U, X, AA) and a smaller gap of the “ring genes” NvSix3/6 and of NvFoxQ2a itself (Figure 3L, O).

Thus, at the planula stage, NvSix3/6 acts upstream of the other analysed genes, suggesting that it represents a key regulator for the aboral region.

Overactivation of FGF Receptor Signalling Rescues Apical Organ Formation in NvSix3/6 Morphants

The injections of NvSix3/6 and NvFGFa1 morpholinos indicated that NvSix3/6 may act at an earlier stage than (and probably upstream of) FGF signalling in the development of the aboral pole. To test more precisely the epistatic relation between NvSix3/6 and NvFGF signalling, we simultaneously blocked NvSix3/6 and overactivated FGFR signalling. To achieve overactivation of FGFR signalling, we suppressed the translation of NvFGFa2, since we had previously shown that the expansion of the apical organ after NvFGFa2 MO injection is caused by excessive FGFR signalling [72]. While injection of NvSix3/6 MO leads to a loss and NvFGFa2 MO to an expansion of the apical organ and corresponding changes in marker gene expression (Figure 4A–C, E–G, I–K, M–O), co-injection of NvSix3/6 MO and NvFGFa2 MO restores the development of the apical organ, resulting in a moderately expanded structure (Figure 4D, H, L, P).

To confirm that this effect is indeed caused by FGFR activation, we treated the NvSix3/6 MO + NvFGFa2 MO injected animals with the FGFR inhibitor SU5402 in DMSO [72,75] or with only DMSO as a control. In order to avoid defects in gastrulation, we started the treatment at the end of this phase. SU5402 treatment of the double-injected animals results in planulae lacking the apical organ, and in situ hybridization shows suppression of aboral NvFGFa1 expression and a lack of the gap in the NvSix3/6 expression (Figure 4Q–X).

Thus, activation of FGF receptor signalling is necessary and sufficient for apical organ formation even when translation of NvSix3/6 is blocked, suggesting that NvSix3/6 is acting early in aboral domain development, upstream of FGFR signalling. Furthermore, since the SU5402 treatment was started after gastrulation, FGFR signalling appears to have a distinct function at a late stage of aboral domain development.

NvHoxF/Anthox1 and NvSoxB(1) Are Required for Apical Organ Formation

We have shown above that the apical organ expression of NvHoxF/Anthox1 and NvSoxB(1) is absent in NvFGFa1 morpholino-injected animals. Moreover, at the planula stage, NvFGFa1 is required for the expression of NvFGFa2 and NvFGFa1 itself [72]. Surprisingly, we found that morpholinos targeting NvHoxF/Anthox1 or NvSoxB(1) have differential effects on the expression of the two FGFs. While injection of NvHoxF/Anthox1 MO and NvSoxB(1) MO both result in planulae lacking the apical tuft (Figure 5A–C), only the expression of NvFGFa1 becomes undetectable (Figure 5D–F), whereas the expression of NvFGFa2 is slightly reduced (Figure 5G–I). Likewise, in NvHoxF/Anthox1 MO-injected planulae, only the gap in the expression of NvSix3/6 but not of NvFoxQ2a is absent (Figure 5J, K, M, N), while NvSoxB(1) MO leads to a loss of the gap in both NvSix3/6 and NvFoxQ2a expression domains (Figure 5L, O).

These results show that, although NvFGFa1 is required for the expression of NvFGFa1 and NvFGFa2 and for the suppression of NvSix3/6 and NvFoxQ2a in the apical organ, the regulation of these genes involves at least partially different regulatory inputs. Whether these differences are of quantitative or qualitative nature remains to be determined.

Regulatory Interactions Change between the Gastrula and Planula Stages

Until the midgastrula stage, “ring” and “spot” genes share a broad aboral expression domain that only at the late gastrula stage starts to segregate into mutually exclusive domains (Figure 1). This suggests that the regulatory interactions between these genes may change after gastrulation. To test this scenario, we analysed the effect of morpholino injections at the midgastrula stage by in situ hybridization to detect potential spatial changes in expression and by quantitative RT-PCR to detect changes in expression levels that may not affect the extension of the expression domains. Since morpholinos can affect the stability of the targeted mRNA, we excluded the respective target genes from the qPCR analysis. Consistent with the observed effect at the planula stage, injection of NvSix3/6 morpholino has no negative effect on the expression of NvSix3/6 itself (Figure 6A, E), but substantially reduces the expression of NvFoxQ2a, NvFGFa1, and NvFGFa2 (Figure 6B–D, F–H, U).

Injection of NvFGFa1 morpholino reveals differential regulation at the gastrula and planula stages, respectively. In contrast to the planula stage, the expression of NvFGFa1 itself is rather up-regulated at the gastrula stage (Figure 6K), whereas NvFGFa2 is downregulated (Figure 6L, V), as it is at the planula stage. Interestingly, injection of a morpholino-targeting NvFGFa2 strongly reduces the expression of NvFGFa1 and NvFGFa2 at the gastrula stage (Figure 6S, T), while it has no effect on the expression of NvSix3/6 and NvFoxQ2a (Figure 6Q, R). This suggests that an as yet unidentified FGF is required for the early
expression of \( N\text{v}FGFa1 \) and \( N\text{v}FGFa2 \). This idea is supported by the increase in expression of these two genes upon knockdown of the inhibitory \( N\text{v}FGFa2 \) (Figure 6O, P, W).

Thus, the main difference in the interactions of these genes in the gastrula stage compared to the planula stage is a lack of positive feedback of \( N\text{v}FGFa1 \) on its own expression at the gastrula stage, suggesting that the autoregulatory FGF signalling loop becomes fully established only after gastrulation.

The Aboral Domain Acquires a More Central Fate in \( N\text{v}Six3/6 \)-Depleted Planulæ

The analysis of \( N\text{v}Six3/6 \) MO-injected animals has shown that \( N\text{v}Six3/6 \) is required for the proper expression of all aboral marker genes except for \( N\text{v}Six3/6 \) itself, suggesting that \( N\text{v}Six3/6 \) is acting at a high level in the regulatory network that controls the specification of the aboral domain. To support this hypothesis we
studied the expression of a marker gene for the central domain of the *Nematostella* planula, *NvWnt2*. *NvWnt2* is expressed in a belt-like domain midway between the oral and aboral poles (Figure 7A–C; [49]), and it is essentially unaffected by loss of *NvFGFRa* signalling [72].

Strikingly, in *NvSix3/6* MO-injected animals, the expression of *NvWnt2* extends progressively to the aboral pole, while the oral expression boundary is unaffected (Figure 7D–F). This is consistent with the hypothesis that at least part of the aboral domain acquires...
a more central fate. However, NvSix3/6 MO-injected planulae appear shortened compared to control planulae, which could indicate that instead of being respecified, the cells of the aboral domain are not generated at all or undergo apoptosis [76,77]. To distinguish between these possibilities, we quantified the number of nuclei at different time points. We could not detect a significant difference in the number of nuclei in NvSix3/6 MO versus control planulae (Figure 7G).

This suggests that the observed aboral expansion of the NvWnt2 expression domain in NvSix3/6 MO-injected animals reflects a reprogramming of the aboral domain to a more oral fate. Mutual repression of six3 and canonical Wnt signalling has been observed in vertebrates and sea urchins [11,20,78,79], and the aboral expression of foxQ2 in the hydrozoan Clytia expands orally when Wnt3 is inactivated [51]. To test whether a similar situation is present during Nematostella development, we overactivated canonical Wnt signalling by chemically inhibiting Glycogen Synthase Kinase 3 (GSK3), a negative regulator of the Wnt pathway. Incubation with 2 mM of the GSK3 inhibitor Azaken-paullone [80] leads to a complete suppression of aboral NvSix3/6 and NvFoxQ2a expression at gastrulation (Figure 7H, I, L, M) and a shift of the expression of NvWnt2 and the oral marker NvFkh [81,82] towards the aboral pole (Figure 7J, K, N, O). At higher concentrations (5 mM), NvWnt2 expression is lost and NvFkh is expressed throughout the embryos (L.I., and F.R., unpublished observation). Thus, similar to some bilaterians, NvSix3/6 is required to prevent ectopic wnt expression and can itself be repressed by ectopic canonical Wnt activity.

**NvSix3/6 Is Dispensable for Neurogenesis But Required for Morphogenesis and Cell-Type Specification in the Aboral Area**

In vertebrates, sea urchin, and Tribolium, suppression of six3 function impairs neural development [11,19,20,77]. To test whether this is also the case in Nematostella, we used two neural markers. NvRFa encodes a neuropeptide and is expressed in individual cells along the entire oral-aboral axis at the gastrula stage (Figure 8A and [83]). Injection of NvSix3/6 MO does not have a significant effect on the NvRFa expression (Figure 8B). NvDmrtB is a transcription factor that is expressed in individual cells in the aboral half of the gastrula and that is involved in neural development (Figure 8C; [84]). Individual cells expressing NvDmrtB are still present at the aboral pole of NvSix3/6 MO-injected gastrulae, but their number is reduced and they are restricted to a smaller aboral territory (Figure 8D). These observations indicate that NvSix3/6 is not required for neurogenesis in general, but is involved in the specification of aboral neural cell types, presumably as a consequence of a more general role in aboral development.

Planulae injected with NvFGFa1 or NvSix3/6 morpholinos both lack an apical organ, but their overall morphology is strikingly different. To better understand the role of these two genes, we stained injected animals with the nuclear stain DAPI and the F-actin stain Phalloidin to visualize their tissue morphology. In control MO-injected planulae, the ectodermal cells in the aboral region acquire a columnar morphology after gastrulation, whereas ectodermal cells in more central areas have a cuboidal morphology (Figure 8E). The nuclei of the cells in the centre of the aboral
region migrate to a more basal position before these cells form the long cilia of the apical tuft (Figure 8E; [85]). In NvFGFa1 MO-injected planulae, the ectodermal cells in the aboral region still become columnar, but the basal migration of nuclei does not occur (Figure 8F). In contrast, in NvSix3/6 MO-injected planulae, the cells of the aboral region retain a cuboidal morphology and nuclei do not migrate to a more basal position (Figure 8G). Thus, consistent with the effect on the expression of NvWnt2, the aboral ectoderm of planulae injected with NvSix3/6 displays a morphology resembling that of more central ectoderm, while NvFGFa1 morphants specifically fail to initiate apical organ formation at the centre of the aboral region. Interestingly, in planulae injected with NvSix3/6 MO, basal migration of nuclei still occurs, but the outgrowth of long cilia is blocked, suggesting that NvHoxF/Anthox1 acts at a late stage of apical organ formation (Figure 8H).

The broader requirement of NvSix3/6 for the development of the apical organ is also reflected in the expression of NvHoxF/Anthox1. In addition to the expression of NvFoxQ2a, the apical organ expression is absent and the expression in individual cells is strongly reduced (Figure 3W). In contrast, in NvFGFa1 morphants, only the apical organ expression is absent, but expression in the scattered cells persists (Figure 2Q).

Taken together, NvSix3/6 is required for the development of the whole aboral territory, whereas NvFGFa1 has a specific function in the development of the apical organ.

Discussion

Control of Aboral Domain Development in Nematostella by NvSix3/6 and FGF Signalling

Our functional analyses lead us to a two-step model in which NvSix3/6 acts as a key regulator of the development of the aboral domain of Nematostella vectensis. First, the loss of NvFGFa1 and NvFoxQ2a expression in NvSix3/6 morphants already at the gastrula stage suggests that NvSix3/6 is required during early stages to determine the identity of the whole aboral region. In a second phase, after gastrulation, NvFGFa1 signalling via NvFGFRa is required to suppress the expression of NvSix3/6, NvFoxQ2a, and NvFoxD1 at the aboral pole. This local suppression of the “ring genes” is necessary to allow the

---

**Figure 6.** NvSix3/6 and NvFGFRa are required for the initiation of a FGF signalling feedback loop at gastrulation. (A–T) In situ hybridizations with probes indicated on top and injected morpholinos on the left. All animals are at the midgastrula stage (24 hpf); lateral views with aboral pole to the left are shown next to aboral views. In NvSix3/6 morphants, the expression of NvFGFa1 and NvFGFa2 is reduced (C, D, G, H). In contrast to the situation at the planula stage, the expression of NvFGFa2 but not NvFGFa1 is reduced in NvFGFa1 morphants (K, L). NvFGFRa is required for the expression of NvFGFa1 and NvFGFa2, but not for NvSix3/6 and NvFoxQ2a (Q–T). (U–W) Quantitative RT-PCR of (U) NvSix3/6 MO-, (V) NvFGFa1 MO-, and (W) NvFGFa2 MO-injected embryos at the midgastrula stage (24 hpf). Fold changes of the relative expression levels of the indicated genes are shown; values between [−1, +1] mean no change, and +2 corresponds to 100% increase. Error bars represent the standard deviation of three biological replicates.

doi:10.1371/journal.pbio.1001488.g006
differentiation of the apical organ cells, with the activation of a specific cassette of genes that includes the presumably ciliary gene NvFoxJ1.

Regarding the development of the apical organ, the key function of NvSix3/6 appears to be the initiation of an autoregulatory loop involving NvFGFa1 and NvFGFa2, which uses positive (auto-activation of NvFGFa1) and negative (activation of the inhibitory NvFGFa2) feedback. This system is probably autonomously able to control the expression of FGF ligands and restrict their expression to the most aboral part of the larva, where the apical organ will form, after the down-regulation of NvSix3/6 itself (Figure 9A,B). Thus, although inhibition of NvSix3/6 or NvFGFa1 both leads to a lack of the apical organ, this phenotype reflects two different developmental roles: NvSix3/6 has a broad and early role in determining the identity of the aboral region, while NvFGFa1 has a later, apical organ-specific function.

However, in contrast to knockdown of NvFGFa1, morpholinos targeting NvFGFRa suppress the expression of NvFGFa1 and NvFGFa2 already at the gastrula stage, suggesting that an as yet unidentified NvFGF ligand might be involved in the initiation of the NvFGFa1–NvFGFa2 feedback loop. The Nematostella genome contains 15 FGFs, of which 10 have not been characterized yet [71,72] and may play a role in this process.

Conserved Regulation of Anterior Development in Bilateria and Aboral Development in Cnidaria
Six3, foxQ2, and FGF genes have been shown to be expressed at the anterior/apical pole of protostome and deuterostome embryos and larvae (summarized for sea urchin in Figure 9; see also Figure S1 and [9,10,15,17,18,21,22,86–89]), suggesting that these genes are part of a conserved regulatory module for anterior development. The role of six3 has been analysed in detail in the sea urchin Strongylocentrotus purpuratus [11]. As in Nematostella, sea urchin six3 is a key regulator of the apical domain—that is, the domain opposite to the gastrulation site. The sea urchin apical pole domain is characterized by an initial thickening of the ectodermal epithelium, which subsequently gives rise to the long cilia-bearing cells of the apical organ, and various surrounding cells, including serotonergic neurons (reviewed in [90]). In embryos injected with a six3 morpholino, the ectodermal thickening does not occur and neither the apical organ nor the neurons develop [11]. Microarray analysis revealed that in sea urchin six3 is a positive regulator of the expression of foxQ2, foxJ1, and frizzled 5/8 [11], genes that are also expressed at the aboral pole of Nematostella downstream of NvSix3/6 (Figures 1 and 3; [55] and unpublished data).

However, despite the well-conserved overlapping expression in anterior/apical domains, there are also differences between the Nematostella and bilaterian expression domains. For example, while the expression patterns of NvSix3/6 and NvFoxQ2a are indistinguishable throughout development, their expression in sea urchin is highly similar only until the midblastula stage, when six3 expression becomes excluded from the centre of the apical plate, while foxQ2 remains expressed in this central apical domain of the sea urchin embryo (Figure 8B,C). Similarly, the anterior expression domain of six3 in the hemichordate Saccoglossus kowalevskii and the brachiopod Terebratalia transversa is slightly wider than that of...
Evolutionary Origin of Head Development

Figure 8. Reduction of aboral neural markers and loss of aboral morphology in NvSix3/6 morphants. (A–D) Lateral views of in situ hybridizations at the gastrula stage, with aboral pole to the left. Probes are indicated on the left, with morpholinos on top. Injection of NvSix3/6 MO leads to a reduction of aboral NvDmrB positive neurons, but not to a general loss of neurons. (E–H) Lateral views of midplanula (72 hpf) animals labelled with DAPI (blue), Phalloidin (green), and anti-acetylated tubulin antibody (red). Aboral pole is to the left, and injected morpholinos are indicated on top. Dashed boxes mark the apical organ region; NvFGFa1 morphants lack basally positioned nuclei (F); in NvSix3/6 morphants (G), no difference between aboral and central ectoderm is visible. In NvHoxFAnthox1 morphants, the aboral indentation with basal migration of aboral pole nuclei occurs, but no apical tuft develops (H). doi:10.1371/journal.pbio.1001488.g008

foxQ2 [9,18,87]. This suggests that although six3 and foxQ2 have an ancient function in anterior development, there have been modifications in their exact interactions, indicating that even very ancient developmental modules retain some degree of plasticity.

The Relation of the Cnidarian Oral-Aboral Axis to the Anterior-Posterior Axis of Bilaterians

Integration of the data presented here with studies in other cnidarians and in bilaterians shows that the aboral domain of cnidarians and the head-forming anterior domain of bilaterians share the expression of several transcription factors and signalling molecules (Figures 9 and S1). This suggests that in the last common ancestor of cnidarians and bilaterians, aboral (i.e., contrablastoporal) development was controlled by six3/6 and the aboral domain was additionally characterized by the expression of foxQ2, frizzled 5/8, rx, irx, and probably FGF signalling genes and foxD1. The most parsimonious explanation of these observations is that the anterior part and thus the head of bilaterians is derived from the aboral domain of the cnidarian-bilaterian ancestor.

The alternative hypothesis that cnidarians do not have a region homologous to the bilaterian head is based on the inconsistent expression of orthologs of some bilaterian anterior markers in cnidarians. However, the notion of a conserved aboral/anterior pattern mechanism does not require that all bilaterian anterior genes are already involved in cnidarian aboral development. Rather, we conjecture that additional genes became integrated into a more elaborate bilaterian anterior patterning system. This is likely for some genes that do not have clear orthologs in cnidarians (e.g., fox6 or irx2). It could also be the case for orthodenticle/otx genes whose ancestral function might have been in endoderm development [43, 58, 91–96] or for orthopedia/otg genes, which mark restricted anterior neural domains and cell types (e.g., dopaminergic neurons) in bilaterians [9, 18, 97–99] and which consistent with the lack of a brain is not aborally expressed in Nematostella [100]. Analysis of additional bilaterian anterior markers (e.g., fez, foxG1, tailless) in cnidarians will help to define a putative ancient aboral/anterior patterning system.

Our data are not consistent with the hypothesis in which the molecular determinants of the gastrulation site and those for bilaterian anterior neural development both localize to the animal pole in cnidarians and became spatially decoupled only after the site of β-catenin nuclearization moved to the vegetal pole in the ancestor of bilaterians [3]. This hypothesis predicts that conserved bilaterian anterior development genes should be expressed, and function on the oral side of cnidarians, which is not consistent with the data presented here. Instead, conserved blastoporal and anterior patterning genes localise to and function at opposite poles in cnidarians, although with reversed orientation when related to the animal-vegetal axis of the oocyte.

Evidence from several cnidarian and many bilaterian species suggests that the gastrulation site of the cnidarian-bilaterian ancestor was characterized by the activity of Wnt/β-catenin signalling. Thus, the development of the two poles of cnidarian gastrulae appears to be regulated by two conserved molecular systems. Furthermore, our data suggest that antagonism between Wnt signalling genes and canonical Wnt signalling in axial patterning may therefore is a zygotic event. In contrast, Wnt3 [24], which is negatively regulated by orally expressed orthodenticle/otx genes whose ancestral function might have been in endoderm (e.g., pax6 or nk2.1). It is likely for some genes that do not have clear orthologs in cnidarians. Rather, we conjecture that additional genes became integrated into a more elaborate bilaterian anterior patterning system. This is likely for some genes that do not have clear orthologs in cnidarians (e.g., fox6 or irx2). It could also be the case for orthodenticle/otx genes whose ancestral function might have been in endoderm development [43, 58, 91–96] or for orthopedia/otg genes, which mark restricted anterior neural domains and cell types (e.g., dopaminergic neurons) in bilaterians [9, 18, 97–99] and which consistent with the lack of a brain is not aborally expressed in Nematostella [100]. Analysis of additional bilaterian anterior markers (e.g., fez, foxG1, tailless) in cnidarians will help to define a putative ancient aboral/anterior patterning system.

foxQ2 [9,18,87]. This suggests that although six3 and foxQ2 have an ancient function in anterior development, there have been modifications in their exact interactions, indicating that even very ancient developmental modules retain some degree of plasticity.

It is worth noting that the suggested homology of the domains at the two poles of the cnidarian and bilaterian primary body axes does not necessarily mean that the complete patterning of their axes is homologous. For example, it remains to be shown that a region corresponding to the trunk region of bilaterians exists in cnidarians. Similarly, the conservation of regulators for the specification of the aboral region does not necessarily mean that the apical organs of anthozoans and bilaterians are homologues. Further studies addressing the development and function of the different cell types constituting the apical organ are necessary to resolve this question.
Early Evolution of Head Development

The head of most bilaterians is characterized by a centralization of the nervous system, the brain. In contrast, despite regional differences in neuron density, neither Nematostella nor other cnidarian planulae or polyps display a comparable brain-like centralization of the nervous system at the oral or aboral end [83,85,101,102]. In sea urchin and Tribolium, six3 is required for anterior neural and epidermal development [11,19], whereas in vertebrates the expression and function of six3 is restricted to the anterior nervous system [20,77]. Our data indicate that Nvsix3/6 is not required for neurogenesis per se, although it influences the development of the aboral NvDmrtB positive neurons. We cannot rule out that Nvsix3/6 has an additional, direct function in the specification of aboral neurons, but based on the effects on general aboral markers and morphology, we favour the idea that the effect on neural specification is a secondary consequence of the broader anterior/aboral patterning predates the evolution of any anterior nervous system [86]. This suggests that patterning systems with tissue-specific functions may have evolved earlier than the corresponding structures [86]. In any case, the function of NvSix3/6 in anterior neural and epidermal development [11,19] may become restricted to the nervous system in the chordate lineage. Similarly, patterning systems controlling the development of secondary brain signalling centres in vertebrates may have evolved earlier than the corresponding structures [86].

Identification of the Sequences

Gene models for Nvsix3/6, NvIrx, and NvFoxJ1 were identified by Larroux et al. [62]. NvFoxQ2a was first identified by Magie et al. [63] as NvFox2 and then assigned to the Q2 family by Chevalier et al. [24]; here, following the nomenclature of Larroux et al. [62] we named the gene NvFoxQ2a.

The complete sequences were obtained by RACE, with the SMART RACE cDNA Amplification Kit (BD Biosciences). The primer sequences are included in the Table S1. NCBI accession numbers are KC137590 (Nvsix3/6), KC137591 (NvFoxQ2a), KC137592 (NvIrx), and KC137593 (NvFoxJ1).

In Situ Hybridization and Immunostaining

In situ hybridization was performed as described previously [72,104]. Probes were synthesized from full-length cDNA clones with Megascript Kits (Ambion), using either Digoxigenin or FITC-labeled UTP (Roche).
The immunostainings were performed as in [83], DAPI (1:1,000 Molecular Probes) was used as nuclear stain, Alexa Fluor 488-conjugated phalloidin (1:25, Molecular Probes) for filamentous actin, and anti-acetylated tubulin antibody (mouse, 1:500, Sigma T6799) to label cilia.

Morpholino Injection

Microinjections were conducted with a Femtojet (Eppendorf), as previously described [72]. Morpholinos (GeneTools) were tested for appropriate working concentrations and then injected at a concentration of 333 or 666 nM, together with Alexa dye-coupled dextran (final concentration 50 ng/µl). As control we used two morpholinos that did not produce any hit in the Nematostella genome database (Joint Genome Institute): a NvSix3/6 mismatch MO and a generic control MO, used previously. All used morpholinos are translation blocking; the sequences are presented in Table S2. The described phenotypes were observed in 60%–90% of the injected embryos in at least three independent experiments. For control experiments, the morpholino target sequences were added upstream of EGFP by PCR (added nucleotides relative to the start codon: NvSix3/6 1 to +10, and the fragments were cloned into vector pCS2+). Capped mRNA was synthesized in vitro using the sp6 mMessageMachine kit (Ambion) and purified with NucAway spin columns (Ambion). The mRNAs (60 ng/µl) were injected together with the corresponding gene-specific and control morpholinos, respectively. EGFP mRNA without any of the morpholino target sites was used as an additional control in co-injections. Images were acquired at 24 hpf with identical settings.

Inhibitor Treatment

The FGFR inhibitor SU5402 (Calbiochem) was applied at a final concentration of 20 µM in 0.1% DMSO, and the GSK3 inhibitor Azakenpaullone (Sigma) was applied at final concentrations of 2 µM and 5 µM in 0.1% DMSO. Control animals were incubated in 0.1% DMSO only. Solutions were applied after gastrulation and changed after 8 hours for SU5402 and from 4 h after fertilization (8–16 cell embryos) until gastrula stage for Azakenpaullone.

qPCR

RNA of injected embryos was extracted with the RNAqueous kit (Ambion) and genomic contamination removed with TURBO DNA-free kit (Ambion). The RNA quality was analyzed with an Agilent 2100 Bioanalyzer, and the cDNA was synthesized with Super Script III RT, using random hexamers as primers (Invitrogen). The qPCR analysis was performed with a SYBR Green I kit (QIAGEN), on a CFX96 Real-time cycler (BioRad).

The efficiency of all primers was determined with tenfold dilution series: all primer pairs had efficiencies ranging between 94% and 102%. Each qPCR sample was repeated in a double technical replicate, and each analysis repeated with at least 3 biological replicates (independent injections). We tested our control genes for stability using the online tool available at the Cotton EST Database (http://www.leonxie.com/referencgene.php), and we selected ATP synthase (GeneID: 5511629), EF1β (GeneID: 5505225), and Ribosomal Protein L23 (GeneID: 5516837). Primer sequences are provided in Table S3.

The relative changes in expression between control-injected and MO-injected and MO-injected were calculated with the ΔΔCt method, assuming primer efficiency of 100%. In every analysis we used at least two reference genes, whose values were then averaged for normalization. The obtained ratios are displayed as fold change: the down-regulated genes (ratio<1) are represented with the negative reciprocal value.

Imaging

In situ pictures were taken using a Nikon Eclipse E800 and a Nikon AZ100M microscope; the images were adjusted in Photosop CS5. The confocal images were recorded using a Leica SP5 confocal microscope; confocal stacks were processed with the Leica software and then adjusted in Photoshop. Counting of nuclei (stained with DAPI) was performed with the Imaris software (BitPlane), using the Spot algorithm.

Supporting Information

Figure S1 Summary of the expression of bilaterian anterior genes and their cnidarian orthologs. The color code for expression categories during embryonic and larval stages is shown at the bottom, with references at the top right of each circle. The list of references can be found in Text S1. Nd, not determined; na, not applicable (no clear ortholog present in genome). Note that an expression pattern has been published for an Acropora millepora gene termed vnslm/nk2.1 (ref. 90 in Text S1), but and the Acropora gene are orthologs of nk2.2. The maternally localized frizzled genes in Clytia are not orthologous to frizzled3/8.

Figure S2 NvSix3/6 is expressed in some individual cells in the ecto- and endoderm. (A and B) In situ hybridizations with NvSix3/6 probe at the planula stage, lateral views, aboral pole to the left in (A) and to the bottom in (B). Close-ups show expression in individual cells outside the main aboral expression domain (arrowheads). Scale bar, 100 µm.

Figure S3 Expression patterns of NvFGFa2, NvSoxB(1), and NvHoxF/Anthox1. (A1–C4) In situ hybridizations with DIG-labelled probes; developmental stages are indicated on top, with probe on the left side. All three genes are expressed at the presumptive aboral side from the blastula stage on. Only the NvSoxB(1) signal is detectable already at the cleavage stage (A1–C1). NvSoxB(1) is also expressed in the pharynx (B3 and 4; [68]), and NvHoxF/Anthox1 is expressed in scattered ectodermal cells in addition to the aboral pole (C4; [49]). Scale bar, 100 µm.

Figure S4 Morpholino control experiments. Overview images of gastrula embryos injected with the indicated morpholinos and mRNAs. mRNAs were synthesized from reporter constructs in which the morpholino target sites are cloned in front of the EGFP coding sequence. The gene-specific morpholinos block expression of their target (L-O) but not of control mRNAs (A-E). Control morpholino 2 does not affect expression of any mRNA (F-J). All images were acquired with identical settings, and the brightness of the whole figure was enhanced to make the gastrulae in (K-O) visible.

Figure S5 NvFGFa1 represses NvFoxD1 expression in the apical organ domain. In situ hybridizations with NvFoxD1 probe at the planula stage, with lateral views with aboral pole to the left (A and B); aboral views in (A’ and B’); B’ is tilted sideways. NvFGFa1 is a “ring gene,” since it is expressed aborally, with a gap in the apical organ region (A, A’; [63]). Injection of NvFGFa1 MO suppresses the gap formation (B, B’). Scale bar, 100 µm.

Figure S6 Expression of NvFoxD1 and NvFGFa1 regulated by NvSix3/6. In situ hybridizations at the planula stage, probes are
indicated on the left side, with injected morpholinos at the top. (A, B, C, D) are lateral views, with aboral side to the left, and (A’, B’, C’, D’) are aboral views. (A – B’). The high-level expression of NeFGFR at the aboral pole is absent in NsIC3/6 MO-injected animals, but the low-level ectodermal expression persists. (C – D’). NsFox DI expression is strongly reduced upon NsIC3/6 MO injection. Scale bar, 100 μm. (C) and (C’) are the same images as Figure S3A and A’. (TIF)

Table S1 Primer sequences for gene isolation. (DOCX)

Table S2 Morpholino sequences. (DOCX)

Table S3 Primer sequences for qPCR experiments. (DOCX)

References

1. Goldstein B, Freeman G (1997) Axis specification in animal development. Bioessays 19: 105–116.
2. Martin-Medina MQ (2005) The evolution of metazoan axial properties. Nat Rev Genet 6: 591–607.
3. Martin-Medina MQ, Hejnol A (2009) A developmental perspective: changes in the position of the blastopore during bilaterian evolution. Developmental Cell 17: 162–174.
4. Duboule D, Dolle P (1989) The structural and functional organization of the murine HOX gene family resembles that of Drosophila homoeotic genes. EMBO J 8: 1497–1505.
5. Graham A, Papaloizou N, Krumlauf R (1989) The murine and Drosophila homeobox gene complexes have common features of organization and expression. Cell 57: 367–376.
6. Nie W, Stronach B, Panganiban G, Shippy T, Brown S, et al. (2001) Molecular characterization of Telabial and the 3’ end of the Tribolium homoeotic complex. Dev Genes Evol 211: 244–251.
7. Kulakova M, Bakalenko N, Novikova E, Cook CE, Eliseeva E, et al. (2007) Hox gene expression in larval development of the polychaete Nereis virens and Platynereis dumerilii (Annelida, Lophotrochozoa). Dev Genes Evol 217: 39–54.
8. Arowicz J, Lowe CJ (2006) Hox gene expression in the hemichordate Saccoglossus kowalevskii and the evolution of deuterostome nervous systems. Integr Comp Biol 46: 890–901.
9. Lowe CJ, Wu M, Salic A, Evans I, Lander E, et al. (2003) Antero-posterior patterning in hemichordates and the origins of the chordate nervous system. Cell 113: 835–845.
10. Pouzet AJ, Kuhlm A, Groth D, Weise V, Yaguchi S, et al. (2007) A global view of gene expression in lithium and zinc treated sea urchin embryos: new components of gene regulatory networks. Genome Biol 8: R55.
11. Wei Z, Yaguchi J, Yaguchi S, Angerer RC, Angerer LM (2009) The sea urchin animal pole domain is a Six3-dependent neurogenic patterning center. Development 136: 1179–1189.
12. See HC, Drivenes, Ellingsen S, Fjo̧se A (1998) Expression of two zebrafish homologues of the murine Six3 gene demarcates the initial eye primordia. Mech Dev 73: 45–57.
13. Zhou X, Holmman T, Prieter T, Gruß P (2000) Cloning and expression of NsIC3, the Xenopus homologue of murine Six3. Mech Dev 91: 327–330.
14. Oliver G, Mailhos A, Wehr R, Copeland NG, Jenkins NA, et al. (1995) Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development 121: 4045–4055.
15. Kozmik Z, Holland ND, Kreslova J, Oliveri D, Schubert M, et al. (2007) Pax-Six-Eya-Dach network during amphioxus development: conservation in vitro but context specificity in vivo. Dev Biol 306: 143–159.
16. Posnien N, Bashasab F, Bucher G (2009) The insect upper lip (labrum) is a hub of the 3rd instar larval transcriptional program. Nature 457: 760–764.
17. Steinmetz PR, Urbach R, Posnien N, Eriksson J, Kostyuchenko RP, et al. (2007) PaxSIX repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. Genes Dev 17: 368–379.
18. Aronowicz J, Lowe CJ (2006) Hox gene expression in the hemichordate Saccoglossus kowalevskii and the evolution of deuterostome nervous systems. Integr Comp Biol 46: 890–901.
19. Lowe CJ, Wu M, Salic A, Evans I, Lander E, et al. (2003) Antero-posterior patterning in hemichordates and the origins of the chordate nervous system. Cell 113: 835–845.
20. Pouzet AJ, Kuhlm A, Groth D, Weise V, Yaguchi S, et al. (2007) A global view of gene expression in lithium and zinc treated sea urchin embryos: new components of gene regulatory networks. Genome Biol 8: R55.
21. Wei Z, Yaguchi J, Yaguchi S, Angerer RC, Angerer LM (2009) The sea urchin animal pole domain is a Six3-dependent neurogenic patterning center. Development 136: 1179–1189.
22. See HC, Drivenes, Ellingsen S, Fjose A (1998) Expression of two zebrafish homologues of the murine Six3 gene demarcates the initial eye primordia. Mech Dev 73: 45–57.
23. Zhou X, Holmman T, Prieter T, Gross P (2000) Cloning and expression of NsIC3, the Xenopus homologue of murine Six3. Mech Dev 91: 327-330.
24. Oliver G, Mailhos A, Wehr R, Copeland NG, Jenkins NA, et al. (1995) Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development 121: 4045-4055.
25. Kozmik Z, Holland ND, Kreslova J, Oliveri D, Schubert M, et al. (2007) Pax-Six-Eya-Dach network during amphioxus development: conservation in vitro but context specificity in vivo. Dev Biol 306: 143-159.
26. Posnien N, Bashasab F, Bucher G (2007) The insect upper lip (labrum) is a hub of the 3rd instar larval transcriptional program. Nature 457: 760-764.
27. Steinmetz PR, Urbach R, Posnien N, Eriksson J, Kostyuchenko RP, et al. (2007) PaxSIX repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. Genes Dev 17: 368-379.

Text S1 References for Figure S1. (DOCX)

Acknowledgments

We thank the members of the Rentzsch lab for discussions, Gemma Richards for critical reading of the manuscript, and Bård Gjezendorner, Tessa Bargmann, and Alina Rey for excellent care for the Nematostella facility. Part of the image analysis was done at the Molecular Imaging Centre at the University of Bergen.

Author Contributions

The author(s) have made the following declarations about their contributions: Commented on the manuscript: LL, UT. Conceived and designed the experiments: CS, LL, FR. Performed the experiments: CS, HB, LL, FR. Analyzed the data: CS, LL, FR. Wrote the paper: CS, FR.

PLOS Biology | www.plosbiology.org 14 February 2013 | Volume 11 | Issue 2 | e1001488
43. de Jong DM, Hilgop NR, Hayward DC, Reece-Hoyes JS, Pontynen PC, et al. (2006) Components of both major axial patterning systems of the Bilateria are differentially expressed along the primary axis of a ‘radiate’ animal, the anthozoan cnidarian Acropora millepora. Dev Biol 298: 632–643.

44. Rieger R, Ladoux P (2001) Searching for the stem species of the Bilateria. Belg J Zool 37: 27–34.

45. Chiori R, Jager M, Denker E, Wincker P, Da Silva C, et al. (2009) Are Hox genes ancestrally involved in axial patterning? Evidence from the hydroidosan Clytia hemisphaerica (Cnidaria). PLoS ONE 4: e7421. doi:10.1371/journal.pone.0007421

46. Kamm K, Schierwater B, Jakob W, Dellaporta SL, Miller DJ (2006) Axial patterning and diversification in the cnidaria predates the hox group. Curr Biol 16: 920–928.

47. Yauze N, Spring J, Schmidl C, Schmid V (2001) Conservation of Hox ParaHox-related genes in the early development of a cnidarian. Dev Biol 236: 89–98.

48. Finnerty JR, Pang K, Burton P, Paulson D, Martindale MQ (2004) Origins of cephalic segmentation: a comparison of Hox and dpp expression in a sea anemone. Science 304: 1333–1337.

49. Kussrewa A, Pang K, Sturm C, Hrouda M, Lentfer J, et al. (2005) Unexpected complexity of the Wnt gene family in a sea anemone. Nature 433: 156–160.

50. Plickert G, Jacykov V, Frank U, Muller WA, Mokady O (2006) Wnt signaling in hydroid development: formation of the primary body axis in embryogenesis and its subsequent patterning. Dev Biol 298: 368–378.

51. Momose T, de la Calle-Mustienes E, Mayor R, Stemple DL (2000) A maternally localized Wnt ligand required for axial patterning in the cnidian Clytia hemisphaerica. Development 127: 2105–2113.

52. Momose T, Houliston E (2007) Two oppositely localised frizzled RNAs as axis organizers in a cnidian embryo. PLoS Biol 5: e70. doi:10.1371/journal.pbio.0050070

53. Broun M, Gee L, Reinhardt B, Bode HR (2005) Formation of the head organizer in hydra involves the canonical Wnt pathway. Development 132: 297–306.

54. Wikramanyake AH, Hong M, Lee PN, Pang K, Byrum CA, et al. (2003) An ancient role for nuclear beta-catenin in the evolution of axial polarity and germ layer segregation. Nature 426: 446–450.

55. Shumake zadeh VM, Wang Y, No R, Wikramanyake AH (2011) Strahmsinus-mediated primary archenteron invagination is uncoupled from Wnt/beta-catenin-dependent endoderm cell fate specification in Nematostella vectensis (Anthozoa, Cnidaria): implications for the evolution of gastrulation. Evodevo 2: 209–287.

56. Mokady O, Dick MH, Lackschewitz D, Schierwater B, Buss LW (1998) Over one-half billion years of head conservation? Expression of an ems class gene in the sea anemone Nematostella vectensis (phylum, Cnidaria, class, Anthozoa). Development 125: 2463–2474.

57. Finnerty JR, Sainai M, Schmidli C, Schmid V (2004) Conservation of Hox/ParaHox-related genes in the early development of a cnidarian. Dev Genes Evol 214: 119–132.

58. Kim CH, Ota D, Itoh M, Jiang D, Artinger KB, et al. (2000) Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. Nature 407: 913–916.

59. Kamikuch C, Lassenoth K, Leost M, Meijer L, Lemke T (2004) A-Azakapanoodle is a selective inhibitor of glycogen synthase kinase-3beta. Bioorg Med Chem Lett 14: 413–416.

60. Martindale MQ, Pang K, Finnerty JR (2004) Investigating the origins of triploblasty: ‘mesodermal’ gene expression in a diploblastic animal, the sea anemone Nematostella vectensis (phylum, Cnidaria, class, Anthozoa). Development 131: 2463–2474.

61. Lapraz F, Rottinger E, Duboc V, Range R, Duloquin L, et al. (2006) The Xenopus drosophila-related gene Dmr3 is required for olfactory placode neurogenesis. Dev Biol 303:139–52.

62. Nakanishi N, Renfer E, Tecnai U, Rentsch F (2012) Nervous systems of the sea anemone Nematostella vectensis are generated by ectoderm and endoderm and shaped by distinct mechanisms. Development 139: 347–357.

63. Pani AM, Mullarkey EE, Aronowicz J, Asmacopoulos J, Groove EA, et al. (2012) Ancient deuterostome origins of vertebrate brain signalling centres. Nature 483: 289–294.

64. Fritzenwanker JH, Saina M, Technau U (2004) Analysis of forkhead and snail differences in developmental mechanisms within the Anthozoa. BMC Evol Biol 4: 3.

65. Momose T, Houliston E (2007) Two oppositely localised frizzled RNAs as axis organizers in a cnidian embryo. PLoS Biol 5: e70. doi:10.1371/journal.pbio.0050070

66. Parlier D, Moers V, Van Campenhout C, Preill J, Leonardie L, et al. (2012) The Xenopus drosophila-related gene Dmr3 is required for olfactory placode neurogenesis. Dev Biol 357:139–52.

67. Matus DQ, Thomsen GH, Martindale MQ (2007) FGF signaling in gastrulation and neural development in Nematostella vectensis, an anthozoan cnidarian. Dev Genes Evol 217: 137–148.

68. Rentzsch F, Fritzenwanker JH, Scholz CB, Tecnai U (2008) FGF signaling controls formation of the apical sensory organ in the cnidian Nematostella vectensis. Development 135: 1761–1769.

69. Zhimzato C, Iuchi A, Hayward DC, Tecnai U, Ball EE, et al. (2008) Sox genes in the coral Acropora millepora: divergent expression patterns reflect differences in developmental mechanisms within the Anthozoa. BMC Evol Biol 8: 311.

70. Thomas J, Morle L, Soulavie F, Laurencou A, Sangoul S, et al. (2010) Transcriptional control of genes involved in ciliogenesis: a first step in making cilia. Biol Cell 102: 499–515.

71. Mohammadi M, McMahon G, Sun L, Tang C, Hirth P, et al. (1997) Structures of Headless/Tcf3 are required for axial patterning in the cnidian Clytia hemisphaerica. Development 124: 2010–2018.

72. Matus DQ, Thomsen GH, Martindale MQ (2007) FGF signaling in gastrulation and neural development in Nematostella vectensis, an anthozoan cnidarian. Dev Genes Evol 217: 137–148.

73. Lapraz F, Rottinger E, Duboc V, Range R, Duloquin L, et al. (2006) RTK and TGF-beta signaling pathways genes in the sea urchin genome. Dev Biol 300: 132–152.

74. Angerer LM, Yagiuchi S, Angerer RG, Burke RD (2011) The evolution of nervous system patterning: insights from sea urchin development. Development 138: 3613–3623.

75. Bracka A, Okaik A, Taguchi S, Tagawa K, Humphreys T, et al. (2000) Developmental expression of the hemichordate Saccoglossus kowalevskii. Development 126: 959–970.

76. Bertrand S, Camales A, Somorjai I, Belgam CR, Chabod O, et al. (2011) Amphioxus FGF signaling predicts the acquisition of vertebrate morphological traits. Proc Natl Acad Sci U S A 108: 9160–9165.

77. Chaios E, Leonard J, Dickinson K, Baker JC (2005) High-throughput functional screen of mouse gastrula cDNA libraries reveals new components of endoderm and mesoderm specification. Genome Res 15: 44–53.

78. Tseng WF, Jang TH, Huang CB, Yuh CH (2011) An evolutionarily conserved kernel of gata3, gata4, otx2 and prdm1a operates in the formation of endoderm in zebrafish. Dev Biol 357: 541–557.

79. Schubert M, Yu JK, Holland ND, Escriva H, Laudet V, et al. (2005) Retinoic acid signaling acts via Hoxb to establish the posterior limit of the pharynx in the cephalochordate amphioxus. Dev Biol 283: 61–73.

80. Arenas-Mena C, Wong KS (2007) Heart development and the origin of the Zona Limitans Intraembryonica (ZLI) brain organizer. Evodevo 1: 7.

81. Kerner P, Ikmi A, Coen D, Vervoort M (2009) Evolutionary history of the iroquois/irx genes in metazoans. BMC Evol Biol 9: 74.

82. Fullner PQ, Thomsen GH, Martindale MQ (2008) Persistence of headless/Tcf3 is essential for vertebrate head formation. Nature 407: 913–916.
97. Nederbragt AJ, te Welscher P, van den Driesche S, van Loon AE, Dictus WJ (2002) Novel and conserved roles for orthodenticle/otx and orthopedia/opt orthologs in the gastropod mollusc Patella vulgata. Dev Genes Evol 212: 330–337.
98. Ryu S, Mahler J, Acampora D, Holzscheiter J, Erhardt S, et al. (2007) Orthopedia homeodomain protein is essential for diencephalic dopaminergic neuron development. Curr Biol 17: 873–880.
99. Acampora D, Postiglione MP, Avantaggiato V, Di Bonito M, Vaccarino FM, et al. (1999) Progressive impairment of developing neuroendocrine cell lineages in the hypothalamus of mice lacking the Orthopedia gene. Genes Dev 13: 2787–2800.
100. Mazza ME, Pang K, Reitzel AM, Martindale MQ, Finnerty JR (2010) A conserved cluster of three PRD-class homeobox genes (homeobrain, rx and orthopedia) in the Cnidaria and Protostomia. Evodevo 1: 3.

101. Watanabe H, Fujisawa T, Holstein TW (2009) Cnidarians and the evolutionary origin of the nervous system. Dev Growth Differ 51: 167–183.
102. Galliot B, Quaiaud M (2011) A two-step process in the emergence of neurogenesis. Eur J Neurosci 34: 847–862.
103. Fritzenwanker JH, Technau U (2002) Induction of gametogenesis in the basal cnidarian Nematostella vectensis (Anthozoa). Dev Genes Evol 212: 99–103.
104. Rentzsch F, Anton R, Saina M, Hammerschmidt M, Holstein TW, et al. (2006) Asymmetric expression of the BMP antagonists chordin and gremlin in the sea anemone Nematostella vectensis: implications for the evolution of axial patterning. Dev Biol 296: 373–387.