Coordinated spatial and temporal expression of Hox genes during embryogenesis in the acoel Convolutriloba longifissura
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Abstract

Background: Hox genes are critical for patterning the bilaterian anterior-posterior axis. The evolution of their clustered genomic arrangement and ancestral function has been debated since their discovery. As acoels appear to represent the sister group to the remaining Bilateria (Nephrozoa), investigating Hox gene expression will provide an insight into the ancestral features of the Hox genes in metazoan evolution.

Results: We describe the expression of anterior, central and posterior class Hox genes and the ParaHox ortholog Cdx in the acoel Convolutriloba longifissura. Expression of all three Hox genes begins contemporaneously after gastrulation and then resolves into staggered domains along the anterior-posterior axis, suggesting that the spatial coordination of Hox gene expression was present in the bilaterian ancestor. After early surface ectodermal expression, the anterior and central class genes are expressed in small domains of putative neural precursor cells co-expressing ClSoxB1, suggesting an evolutionary early function of Hox genes in patterning parts of the nervous system. In contrast, the expression of the posterior Hox gene is found in all three germ layers in a much broader posterior region of the embryo.

Conclusion: Our results suggest that the ancestral set of Hox genes was involved in the anterior-posterior patterning of the nervous system of the last common bilaterian ancestor and were later co-opted for patterning in diverse tissues in the bilaterian radiation. The lack of temporal colinearity of Hox expression in acoels may be due to a loss of genomic clustering in this clade or, alternatively, temporal colinearity may have arisen in conjunction with the expansion of the Hox cluster in the Nephrozoa.

Background

Hox genes encode transcription factors that contain a characteristic helix-turn-helix DNA binding domain - the homeodomain. Hox genes regulate the expression of other genes during development and their molecular characterization and expression patterns indicate that these genes are involved in specifying regional identities along the anterior posterior (A-P) axis in a diverse range of bilaterian animals [1,2]. Orthologs of the Hox genes are subdivided into anterior, central and posterior classes, according to their suspected evolutionary ancestry and their corresponding expression along the A-P axis. Surprisingly, in most, but not all, bilaterians whose genomes have been sequenced, the Hox genes are organized in a
This suggests that the related to a corresponding ParaHox prior to the separation of the cnidarian and bilaterian linear arose by the duplication of a single proto-Hox cluster be paralogs of the genomes (for example, group of genes that are also clustered in some animal Similar attention has been given to the before its expansion or how these genes were indeed organized in a cluster in the last common ancestor of protostomes and deuterostomes. However, the emerging consensus is that the last common cnidarian-bilaterian ancestor had one or two anterior and one posterior class gene that are clear orthologs to the bilaterian Hox genes and two ParaHox genes, namely a Gsx and a proto-Xlox/Cdx gene [22,28].

Since the cnidarian Hox complement appears to be much smaller than that of protostomes and deuterostomes, and also shows intra-taxon variation in the gene expression along the main body axes, bilaterian Hox cluster expansion remains unclear. Similarly, it is not clear when these genes first became involved in axial patterning and which structures first utilized Hox genes for their patterning. In order to gain more insight into the nature of the early bilaterian Hox genes and their role in body patterning, we investigated the expression patterns of the Hox gene complement in the acelos - a clade of relatively simple marine worms that posses an orthogonal nervous system and one mouth opening but lack an anus to the syncitial gut [30-33]. Morphological analyses [34] and molecular phylogenies using different genes suggest that acelos form an early branch in the Bilateria [35-41]. Phylogenomic studies had difficulty in placing acelos in the animal tree of life [27,42]. However, a recent phylogenomic study that uses 1487 genes places Acoela and Nemertodermatida - which together form a clade called Acoelomorpha [43] - as the sister group to all remaining Bilateria with high statistical support [44]. Morphological similarities between the stem cell system of acelos and platyhelminthes [36] are thus either ancestral traits of their last common ancestor or convergence [44].

Congruent with their phylogenetic position, acelos appear to possess only a subset of the Hox genes found in protostomes and deuterostomes, namely one anterior, one central and one posterior class gene, as well as an ortholog of the posterior ParaHox gene Cdx [45]. Nemertodermatida, the sister group of acelos, seem to have a similar subset of Hox genes belonging to the same three Hox classes [46]. A homeodomain survey on nemertodermatids revealed

Hox and ParaHox Evolution - Insight from Cnidarians and Acoels

The diversity of contexts in which Hox genes are utilized in bilaterian development illustrates the need to examine the deployment of these genes in broader evolutionary lineages. For example, it is still not known whether these genes were used to provide positional specific patterns of differentiation to all cells or if they were used to pattern specific compartments of organ systems such as the nervous system, axial mesoderm or the digestive tract. Furthermore, it is necessary to determine which tissue the ancestral Hox genes were patterning, since bilaterian Hox genes are involved in patterning different germ layers in different lineages [23,24]. In vertebrates, the same Hox genes are expressed in the neural tube and in paraxial mesoderm and have different anterior boundaries. This indicates that these genes were potentially co-opted independently for their roles in different tissues somewhere in the vertebrate lineage [25,26].

Cnidarians play an important role in unraveling the evolutionary origins of the Hox and ParaHox clusters, since they form the sister-group to the Bilateria [27] and do not appear to possess all Hox classes found in protostomes and deuterostomes. Recent investigations of the Hox gene complement in cnidarians (for example, corals, sea anemones, and jellyfish) have led to dramatically different interpretations of Hox/ParaHox gene evolution in the cnidarian-bilaterian ancestor [17,21,22,28,29]. One reason for this is the difficulty in establishing clear orthology assignments of cnidarian Hox and ParaHox genes to those of the Bilateria. In addition, lineage specific gene duplications have complicated attempts at reconstructing Hox gene evolution. Furthermore, studies of the expression patterns in representatives of the two branches of the Cnidaria - the anthozoans and medusozoans - suggest that their developmental function differs in both lineages [22,28]. However, the emerging consensus is that the last common cnidarian-bilaterian ancestor had one or two anterior and one posterior class gene that are clear orthologs to the bilaterian Hox genes and two ParaHox genes, namely a Gsx and a proto-Xlox/Cdx gene [22,28].

contiguous cluster in which the order of genes along the chromosome is reflected in their expression domains along the A-P axis, a phenomenon called 'spatial colinearity' [3-7]. Additionally, in some cases the developmental timing of expression of sequential Hox genes also corresponds to their relative positions in the genome - a phenomenon that has been described as 'temporal colinearity' [8-10]. Both patterns suggest that spatial and temporal colinearity might have been present in the organism that possessed the ancestral Hox cluster [11].

Similar attention has been given to the ParaHox genes, a group of genes that are also clustered in some animal genomes (for example, Branchiostoma) and are thought to be paralogs of the Hox genes [12]. Phylogenetic evidence from bilaterian taxa suggests that each of the three known ParaHox gene classes (Gsx, Xlox and Cdx) is more closely related to a corresponding Hox class than to each other. This suggests that the Hox cluster and ParaHox cluster arose by the duplication of a single proto-Hox cluster prior to the separation of the cnidarian and bilaterian lineages [12-14]. Organized clusters of Hox genes, composed of anterior class (paralog groups Hox1, Hox2), Hox3, central class (Hox4 - 8) and posterior class (Hox9-15) genes, are present in the annelid Capitella teleta [5], vertebrates and cephalochordates [15,16], which suggests that Hox genes were indeed organized in a cluster in the last common ancestor of protostomes and deuterostomes. However, it is still unclear how this cluster evolved, how many Hox genes were present in the ‘core’-cluster of the ancestor before its expansion or how these Hox genes are related to their evolutionary sisters, the ParaHox genes [11,17-22].

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that, in addition to the Cdx gene present in acoels, a fragment of an Hox ortholog has also been recovered [46]. These data suggest that the acoelomorph Hox complement is close to the predictions that have been made about the composition of the ancestral Hox cluster before its expansion in the remaining Bilateria [13,20]. As cnidarians, the sister group to the Bilateria, have no central class Hox gene, acoels are the pivotal group for reconstructing the origin and expansion of the central class genes and the possible roles of Hox genes in the evolution of bilaterian complexity.

We cloned orthologs of the three Hox gene classes and an ortholog of the ParaHox gene Cdx from the acoel Convolutriloba longifissura and investigated the embryonic and juvenile expression. We describe their deployment in relation to temporal and spatial collinear patterns and reconstruct their possible function in germ layer specification in the stem species of the Bilateria by comparing them with expression data from other bilaterian lineages.

Results
Gene orthology and composition of the acoel Hox genes

Gene orthology assignments of Hox genes are notoriously difficult to analyse because of a lack of phylogenetic signal contained in the 60 amino acids that compose the conserved homeodomain. The limited information results in extremely low support values for the basal branches and a high sensitivity of the tree topology to the methods and outgroups used in the analysis [17,21,22,28,47]. The inclusion of the cnidian Hox and ParaHox gene sequences has led to distinctly different hypotheses about Hox and ParaHox gene evolution. Based on their phylogenetic position as a sister to protostomes and deuterostomes, acoels can provide an insight into the evolution of the Hox and ParaHox genes. We performed phylogenetic analyses of the Hox genes, using the homeodomain and flanking regions, and analysed the Hox gene composition of specific motifs that regulate the binding specificity of Hox genes in other metazoans (Figure 1, Additional file 1: Figure S1 and Table S1). Our primary goal was to establish the gene orthology to acoel Hox genes and we found that maximum likelihood (ML) analyses provided the most stable results. Tree topology was tested by conducting ML analyses with and without cnidian sequences (Figure 1, Additional file 1: Figure S1).

The results from both ML analyses of the acoel Hox genes were consistent with: one C. longifissura Hox gene grouping with the anterior (Hox1/labial); one with central (Hox4/5); and one with posterior Hox classes (Figure 1, Additional file 1: Figure S1). We have named these ClantHox, ClintHox, and CpostHox, respectively. The Cdx ortholog of C. longifissura ClCdx groups with the Cdx ortholog of the acoel Symasagittifera roscoffensis and other bilaterian Cdx orthologs (Figure 1, Additional file 1: Figure S1).

Orthology assignments are consistent in ML analyses, with or without cnidian sequences. However, the topology of the branching of the individual paralog groups differs dramatically between both analyses. As in previous analyses, the basal branches receive no, or minor, support [17,21,22,28,47]. In the analysis that includes several hydrozoan and anhozoan Hox genes, the earliest branch is formed by the ParaHox gene Gsx (Figure 1). The exclusion of these genes led to an early branch of the Hox1 class genes (Additional file 1: Figure S1). These conflicting results have also been discovered in previous Hox gene analyses [22,28].

In neither analyses was the previously observed orthology assignments of the ParaHox genes Gsx, Xlox and Cdx to the Hox gene classes Hox1, Hox3 and postHox [12,19], respectively, have not been confirmed. If both gene clusters arose by a duplication of a proto-Hox cluster, the ParaHox genes would group as sisters to the corresponding Hox ortholog. However, this is not the case, since ParaHox genes either form separate branches (Gsx and Xlox in Figure 1) or their grouping with other Hox genes differs between the analyses. The cnidian Hox and ParaHox orthologs form either the sister group to their bilaterian orthologs or form separate branches (Cdx and postHox, see Figure 1). In our analysis, the scyphozoan Hox gene which was previously assigned to the posterior Hox class (CheHox9-14B) groups with the even-skipped orthologs. We, therefore, cannot confirm that this is a true Hox ortholog as has previously been stated [28]. The differences between these and previous analyses demonstrate the difficulties of Hox gene orthology assignment of cnidian sequences and leave the question of the early origin of Hox and ParaHox genes unsolved. They do, however, indicate the significance of the cnidian lineage for reconstructing early Hox gene evolution.

As the phylogenetic analyses of the homeodomain alone did not provide robust insight onto Hox gene orthology, we also compared motifs in N- and C-terminal flanking regions including the PBX-binding domain and its distance from the homeodomain in Hox and ParaHox orthologs (Additional file 1: Table S1). These motifs have been shown to play an important role in DNA-binding specificity and protein-protein interaction of Hox genes [23,48-51]. The PBX-motif is also present in non-Hox family genes (for example, in NK and LIM domain genes) [52] and is thus plesiomorphic for Hox genes. However, its presence, diagnostic motifs and distance (length of the ‘linker’) from the homeodomain has changed during the evolution of metazoan Hox genes and can thus provide
Figure 1
Maximum likelihood phylogenetic analysis with cnidarian Hox orthologs. Ingroup cnidarian sequences indicated in blue; Hox and ParaHox genes of acoels are indicated in red. The arrowhead points out the Turritopsis 'Xlox' ortholog. Bootstraps above 60 are indicated in percentages. The alignment includes the flanking regions of Additional Table 1. Cl = Convolutriloba; Sr = Symasgittacea; Ip = Isodiametra; Nv = Nematostella; Cly = Clythia; Hyd = Hydra; Cap = Capitella; Tc = Tribolium; Fen = Flaccisagitta; Sk = Saccoglossus; Pf = Psychodera; Bra = Branchiostoma; Chae = Chaetopterus; Tr = Trichoplax; Hr = Haliotis; Es = Endeis; Cup = Cupiennius; Xl = Xenopus; Pc = Podocoryna; Xen = Xenoturbella; Sp = Strongylocentrotus.
phylogenetic information for the identification of Hox family information [52-57].

Overall, the acelo Hox sequences fit the expected pattern predicted in a previous study that analysed Hox gene structure [55]. The anterior Hox orthologs of Isodiametra pulchra, Symagastitiera roscoffensis and Convolutriloba longifissura contain a PBX-motif, which is a similar distance to the homeodomain (28/37/38 amino acids) compared to other bilaterian anterior class Hox genes (17-55 amino acids; Additional file 1: Table S1). The N-motif of the anterior Hox gene is similar to other bilaterian Hox1 orthologs (both share the Na-signature; see Additional file 1: Table S1). However, the C-motif of the anterior Hox ortholog in acelo is shorter than in all other Hox genes, with a stop-codon present close to the homeodomain (Additional file 1: Table S1).

The central class ortholog of acelo contains a PBX-motif that is close to the homeodomain (2 amino acids), as it is in the bilaterian orthologs of Hox6/7 (5-8 amino acids), but its C-motif has greater similarity to the bilaterian Hox4/5 orthologs. The acelo central class ortholog also shares similarities with the N-motif of bilaterian Hox2, Hox3, central class, Gsx and Xlox genes (all have the Nb motif). The posterior Hox ortholog of C. longifissura shares the PBX binding domain of ambulacrarian (hemichordates + echinoderms) and cephalochordate posterior Hox orthologs. The PBX-motif of posterior class Hox genes is located close to the N-terminal of the homeodomain and thus lacks the linker sequence found in the more anterior class Hox genes [52]. The N-motif of acelo posterior Hox genes also has posterior Hox specific residues (Nb-x-motif; Additional file 1: Table S1).

The cnidarian putative Hox and ParaHox orthologs also share specific residues with their bilaterian orthologs. In the cases of the cnidarian anterior Hox and ParaHox (Gsx) orthologs, the motif pattern is conserved in relation to that of the bilaterian orthologs. A PBX-motif is present in the posterior Hox genes Che9-14A and Che9-14C of Clytia and in the Nematostella Anthox1a, which is located directly or close (a single amino acid) to the homeodomain which is similar to the bilaterian posterior Hox class genes. This supplies additional evidence that, contrary to previous suggestions, these genes are indeed posterior Hox genes [17,47]. The recently reported clear Xlox ortholog in the hydrozoan Turritopsis dohrnii [21] does not exhibit either the characteristic PBX binding domain or the similarities in the N-motif of bilaterian Xlox genes. This could be due to a secondary loss of these motifs in the hydrozoan lineage. However, an orthology of the gene with Xlox is not supported in our phylogenetic analysis, where it groups clearly with the previous described Xlox/Cdx ortholog (NvHD065) of Nematostella (Figure 1).

Although our phylogenetic analysis clearly assigns the acelo Hox genes to the anterior, central and posterior class, the internal grouping remains unclear. The lack of resolution and the lack of additional Hox genes in acelo cannot exclude that possibility that the three Hox genes found in acelo represent ancestral precursors of the bilaterian Hox cluster as has been suggested [20]. For this reason, we name the Hox genes according to their class (Clant-, Clcent-, ClpostHox) and do not make premature assignments to a specific sub-class of an extended bilaterian Hox complement. Our analysis suggests that a true anterior and posterior gene has been present in the bilaterian-cnidarian ancestor and that the central class genes are a novelty of the Bilateria. A Hox3 ortholog has not been found in cnidarians or acelo, so it appears to have evolved in the lineage to the common ancestor of protostome-deuterostome.

**Expression of Convolutriloba longifissura anterior class Hox**

The expression of the anterior class Hox gene in C. longifissura (ClantHox) starts after gastrulation when the embryo is composed of approximately 250 cells. It is expressed in two bilateral regions in the outermost cell layer in the animal hemisphere of the embryo (Figure 2a). Later, these domains of about 10 cells sink below the outermost cell layer (Figure 2b). Double in situ hybridizations with other marker genes allow us to determine the region of ClantHox expression. The acelo ortholog of Six3/6, ClSix3/6 (Figure 2c, d) is expressed in the future anterior region of the juvenile [58] anterior to ClantHox expression. ClantHox expressing cells (Figure 2e) co-express the proneural gene ClSoxB1 which suggests that these cells are neural with defined anterior-posterior boundaries (Figure 2i, j). Previous cell lineage experiments show that this domain is largely generated from the second duet microsmes [59]. In the juvenile, ClantHox is expressed posterior to the statocyst, extending to the end of the body but not at the posterior tip (Figure 2g, h). The strongest expression is found in a bilateral area directly posterior to the statocyst, which is likely to be comprised of descendants of cells expressing ClantHox during their early development (Figure 2c-f).

**Expression of Convolutriloba longifissura central class Hox**

The expression of the C. longifissura central Hox class gene ClcentHox is detected by in situ hybridization shortly after gastrulation, about the same time as ClantHox (Figure 2a, 3a). Expression begins in the posterior part of the embryo (Figure 3a) in ectodermal cells surrounding the site of the closed blastopore (Figure 3b). During later development the expression domain is internalized and expands anteriorly on both sides, forming bilateral bands (Figure 3c). ClcentHox expression becomes refined to two internal
bilateral spots of cells in the area lateral to the future position of the mouth (Figure 3d, e). The expression pattern is very similar to that of ClantHox at this stage (see Figure 2b). However, the anterior border of ClcentHox is more posterior. Like ClantHox, these bilateral cell patches are approximately the same size and are located below the epidermal surface. The identity of these expressing cells could not be determined because the cell types have not been differentiated at this stage. However, an endodermal fate can be excluded since the expression is not in the centre of the embryo, where the digestive syncytium will develop. The location below the outermost cell layer suggests a mesodermal or neural fate. However, these ClcentHox-expressing cells appear to be in the area of ClSoxB1 expression (Figure 2f, 3) which is thought to be involved in neural development in other animals [60]. This correlation with the expression patterns of 'neural' genes suggests that ClcentHox is expressed in neural precursor cells rather than mesodermally derived musculature. The pre-hatched juvenile shows strong expression in two small bilaterally paired domains that could be nerve cell bodies contributing to the orthogonal nervous system (Figure 3f-h).

Expression of Convolutriloba longifissura posterior class Hox
The expression of the ortholog of the posterior class Hox gene in C. longifissura, ClpostHox, starts at the same time as
Figure 3
Expression of ClcentHox in embryos of C. longifissura. (a) Lateral view on the earliest stage of detected ClcentHox expression. Expression is found at the vegetal pole at the site of the closed blastopore (b) Vegetal view at the stage of (a) showing that cells that express ClantHox surround the former blastopore. (c) Lateral view on postgastrula stage. The expression domain is below the ectoderm in two bilateral stripes with a stronger expression in the anterior cells (black arrows). (d) Later stage than (c) shows restriction of the expression domain of ClcentHox to a bilateral cluster of about a dozen cells. (e) Haematoxylin stained histological section of an embryo showing the ClcentHox expression below the outer ectodermal layer. (f) Pre-hatchling with bilateral ClcentHox expression domains that are localized in two separate domains along the anterior-posterior axis. (g) Summary of ClcentHox expression (green) in the juvenile from the dorsal side and left side (h) showing the two separate expression domains of ClcentHox on the left and right side. Asterisk indicates the animal/anterior pole of the embryo. (g, h) Animal to the left.
Expression of ClpostHox in embryos and juveniles of C. longifissura. (a) Vegetal view on the site of gastrulation. ClpostHox expression begins at the vegetal pole of the postgastrula stage of C. longifissura. (b) Lateral view on the later embryo with the ClpostHox expression in the vegetal hemisphere. Asterisk indicates the animal pole. (c) ClpostHox expression is located in the posterior end in all three germ layers of pre-hatchlings. Cellular differentiation is indicated by the appearance of the stato-cyst (st). (d) Dorsal view on the hatched juvenile with fading ClpostHox expression. Localized centers of expression remain visible (black arrowheads). (e) Lateral view of a hatched juvenile of C. longifissura showing strong ClpostHox expression in individual cells in an area of weaker expression, anterior to the left. (f) + (g) Summary expression of ClpostHox (blue) in juveniles. Note that the posterior most tip of the animal is free of expression. Anterior to the left.
the other two Hox genes that is shortly after gastrulation (Figure 4a). Initially ClpostHox expression is detected in a restricted area of about 30 cells in the posterior region of the embryo, in the outermost cell layer. This domain extends over the vegetal hemisphere of the embryo (Figure 4b), which gives rise to the posterior part of the juvenile. The anterior border of the ClpostHox expression domain corresponds to the posterior border of the future mouth and thus overlaps with the posterior ClcentHox expressing domains. This expression of ClpostHox later expands to cells in all three germ layers, including the epidermis. However, during further development, as cell differentiation begins (for example, when the statocyst becomes visible), expression is down-regulated in the epidermis and endoderm (Figure 4c) and becomes restricted to a smaller lateral area below the outer cell layer with a weaker expression extending posteriorly (Figure 4d, e). The expression of ClpostHox persists in the juvenile and is found in four individual neural cells in a bilateral arrangement (Figure 4d-f). Expression in the hatchling is rather diffuse. The expression of ClpostHox is similar to that of the other two Hox orthologs (ClantHox, ClcentHox), suggesting that the gene is involved in neural patterning in the posterior region of the juvenile, but it may also have an early role in posterior patterning of all three germ layers.

Expression of the Convolutriloba longifissura ParaHox gene Cdx

The expression pattern of ClCdx has been described previously [58,61] but here we focus on a detailed description of the anterior expression associated with the acoel nervous system and its relationship to other Hox gene expression patterns. ClCdx is expressed in the commissure posterior to the statocyst (Figure 5) and extends anteriorly and ventrally, following the paths of nerve tracks. ClCdx is also expressed in an area surrounding the eyes, which form direct connections to the brain commissure. The expression of ClCdx in the juvenile expands laterally to the posterior end, with a posterior domain in the ventral ectoderm that will form the male gonopore [58].

Discussion

Phylogenetic analyses and evolution of Hox genes

Orthology assignments of Hox genes are notoriously difficult due to the short amino acid sequence of the homeodomain. Previous comparisons of the homeodomains of ParaHox with that of the Hox genes of representative protostomes and deuterostomes revealed sequence similarities between the anterior, Hox3 and posterior Hox classes with that of Gsx, Xlox and Cdx ParaHox classes, respectively [12,19]. These findings led to the hypothesis of a early duplication of a ‘proto-Hox’ cluster composed of three or four proto-Hox genes which gave rise to two separate clusters of three/four Hox and three/four ParaHox genes [11,12,19]. However, recent analyses that included homeodomain genes from cnidarians suggest a rather different evolutionary origin of Hox and ParaHox genes in which a single ‘proto-Hox’ gene gave rise to a Hox and paraHox gene, forming an ancestral Hox1 and Gsx ortholog [22,28]. Tandem duplication events then led to the extension of Hox and ParaHox gene complement we find in the Bilateria [22]. This tandem-duplication hypothesis rejects the previously suggested duplication event of a ‘proto-Hox’ cluster that contained three, or even four, Hox genes (including a Hox3 ortholog) into a Hox and paraHox cluster and challenges the evolutionary relationship of posterior Hox to Cdx (posterior ParaHox) genes of Xlox to paralog group 3 Hox genes.

No study has yet found a bona fide Hox3 ortholog in either cnidarians or acocels and the recent report of an Xlox gene in a hydrozoan [21] seems to be an ortholog of the homeodomain gene NvHD065 in Nematostella (Figure 1). The finding of a clear Gsx ortholog in cnidarians [62] and Trichoplax [63], however, shows that either this ParaHox gene was lost in the acoel lineage or has not yet been found in the homeodomain gene surveys. Only whole genome sequencing can determine how many Hox genes are actually present in acocels and nemertodermatids.

To gain further insight into the phylogenetic relationships of acocel Hox genes we included both N- and C-terminus flanking regions of the homeodomain in our phylogenetic analysis and investigated the presence of conserved domains that have been shown to be responsible for the binding specificity. As in previous phylogenetic analyses of acocel Hox genes [5,45,64], the anterior class Hox orthologs of acocels groups as the sister to all remaining Hox1/labial genes, the central class Hox gene branches with Hox4/5 and the posterior Hox with the posterior class genes Hox9-14. At first glance, this suggests that a Hox6/7 gene has been present in the bilaterian ancestor and been lost in the acocel lineage. However, it does not exclude a tandem duplication event in which a proto central class gene gave rise to Hox4/5 and Hox6/7 and after which the one descendant (Hox6/7) deviated and became more in sequence with it than its sister (Hox4/5). The Hox complement of more basal branching lineages, such as cnidarians, could potentially deliver additional insight for reconstructing the Hox gene complement of the bilaterian ancestor.

The findings of the surveys for Hox and ParaHox genes in cnidarians suggest the presence of two Hox (anterior and posterior) and two clustered [65]ParaHox genes (Gsx and proto-Cdxs) in the last common ancestor of cnidarians and bilaterians. Together with the gene complement in acocels, the results favour a two-gene model of the evolution of the Hox genes from a proto-Hox cluster followed...
by several duplication events that gave rise to the central class and expansion within each class to give rise to the expanded Nephrozoan complement [13].

**The Hox orthologs in Convolutriloba longifissura are expressed in a spatially staggered pattern along the anterior-posterior body axis**

Our results show, that the Hox genes of *C. longifissura* are expressed in a spatially staggered pattern along the anterior-posterior axis in later embryos and juveniles. As in most described bilaterians (except for hexapods and some polychaetes), orthologs of *Hox1/labial* are the most anteriorly-expressed genes of the Hox complement. In chordates this anterior border corresponds to the hindbrain, while in *Drosophila* this border corresponds to the tritocerebrum. *Hox1/labial* expression can be either restricted to a ring-like domain throughout the entire ectoderm (as in *Saccoglossus* [66]) or in a domain with a definite anterior boundary in neuroectodermal and mesodermal tissue that expands to the posterior end of the body (as in *Branchiostoma* [67] and vertebrates). In the protostomes investigated so far, *Hox1/labial* orthologs are expressed in a restricted anterior region [5,68-70], starting in two bilateral domains (in the polychaetes *Capitella, Nereis, Chaetopterus*), which is similar to the pattern we describe for ClantHox in *C. longifissura*. In the juvenile, the most anterior parts of the acoel nervous system anterior to the statocyst are free of ClantHox expression, which is similar to the conditions found in protostomes and deuterostomes (for example, *Haliotis, Platynereis, Capitella, Saccoglossus, Branchiostoma, Drosophila, Chaetopterus*). In both protostomes and deuterostomes, the most anterior part of the acoel nervous system anterior to the statocyst are free of ClantHox expression, which is similar to the conditions found in protostomes and deuterostomes (for example, *Haliotis, Platynereis, Capitella, Saccoglossus, Branchiostoma, Drosophila, Chaetopterus*). In both protostomes and deuterostomes, the most anterior part of the nervous system lacks any Hox gene expression but usually expresses the gene *Six3/6* [71], which is also the case in *C. longifissura* (Figure 3).

Posterior to the bilaterally restricted patches of ClantHox expression, similar domains of the central class Hox gene
(ClcentHox) are expressed in the acoel embryo. Behind these ClcentHox expressing patches of cells, the expression domain of the single posterior class Hox ortholog, ClpostHox begins and it extends to the posterior end of the embryo. This indicates that the spatial colinearity of Hox gene expression was present in the last common ancestor of the Bilateria and that the system that regulates the spatial colinearity was maintained, despite the expansion of the Hox cluster during bilaterian evolution.

The boundaries of expression of the three Hox genes in the un-segmented body of the acoel do not correspond to any overt morphological boundaries as is found in the segments of arthropods, annelids and in the vertebrates of mice. However, the anterior border of the ClantHox expression corresponds to the ‘brain’ commissure behind the statocyst and the patch-like expression of ClcentHox and ClpostHox seem to correspond to specific neural precursors in the orthogonal nervous system (Figure 2, 3, 4). The mouth opening in acoels is formed late in development [72] and its position corresponds to the border just anterior to the expression of the posterior Hox ortholog (Figure 4).

**Expression of Hox orthologs in Convolutriloba longifissura begins simultaneously after gastrulation and shows no temporal colinearity**

Our study of the early expression patterns of the Hox gene complement of C. longifissura shows that all three Hox genes are expressed almost simultaneously at a time after gastrulation when the embryo is composed of about 250 cells (Figure 6). Our observations suggest that there is little temporal colinearity in this acoel species, although we cannot exclude a slightly temporal staggered expression of one or two cell cycles. We interpret our findings as a lack of temporal colinearity of expression, unlike that present in animals which have an intact, organized Hox cluster, such as vertebrates [8], cephalochordates [67], the polychaete Capitella teleta [5], and some insects [73]. Those examples in which the Hox cluster has been dispersed, split or disorganized, such as in Drosophila ([74], C. elegans [75], Ciona [76], Saccoglossus [66], Oikopleura [77] and Strongylocentrotus [78], lack temporal colinearity of Hox expression. Thus, temporal colinearity of Hox expression seems to be associated with a functionally intact Hox cluster [10].

The lack of dramatic temporal staggering of the Hox gene expression in acoels can be interpreted in two ways. Given the consensus view of the presence of a complete Hox cluster with temporal and spatial colinearity at the base of the Bilateria, the lack of temporal colinearity in acoels would suggest that an ancestral Hox cluster has dispersed in the acoel lineage. The reasons for a possible Hox cluster dispersion in acoels are not particularly clear. Explanations used in other taxa (for example, Drosophila, nematodes, Oikopleura) such as rapid development, short generation time or the emergence of a rigid stereotyped cleavage pattern with precocious specification of cell fates [4] do not appear to be applicable in acoels. Although acoel development displays a stereotyped cleavage programme, it is not particularly rapid (occurs in 4-5 days) and the embryos and adults show a high degree of regulative capacity [79]. Most adult acoels are long lived, capable of regeneration and can even reproduce asexually.

Assuming the presence of an integrated Hox cluster in the last common ancestor of these animals, it has been suggested that the concerted mechanism that controls the temporal expression prevents the ancient cluster from its evolutionary dispersion [10,18,80,81]. Recent evidence shows, that the temporal and spatial patterning of Hox expression is controlled by distinct mechanisms [82-84], which might explain why spatial colinearity is maintained even during cluster disintegration [77].

Since the expression of the Hox genes in acoels are turned on simultaneously, another explanation for the lack of temporal colinearity could be that the elements that control a temporal staggered expression has evolved in the nephrozoan lineage in combination with the expansion of the cluster. The temporally staggered regulation of Hox clusters would then be linked by the expansion of the cluster to a larger number of genes and/or a larger genomic region in the course of the Hox cluster evolution of the protostome-deuterostome ancestor [85]. However, a recent analysis using sequencing of bacterial artificial chromosome libraries and fluorescent in situ hybridization in an acoel species suggests a dispersed cluster of Hox genes [86].

**The Hox gene expression in acoels is correlated with neural development in an axial fashion**

Our results suggest, that the Hox genes in the acoel C. longifissura might play a major role in the axial patterning of the nervous system. The simultaneous onset of the Hox genes in the ectoderm followed by the internalization of the expressing cells reflects the internal migration of putative neural precursor cells and is consistent with the results of fate mapping that showed that the nervous system is formed by descendants of the first, second and third duet ectodermal micromeres [59]. The co-expression of ClantHox with the Convolutriloba ortholog of the bilaterian proneural gene Sox1 [87] is consistent with the previously described patterns in other bilaterians and suggests that ClantHox is responsible for the patterning anterior subsets of the nervous system. The overlapping expression of ClcentHox with ClSox1 also suggests a function of ClcentHox in the formation of neural precursor cells in the mid-body region. In contrast, ClpostHox expression is found in a broad posterior domain that includes ectodermal, endodermal and, possibly, mesoder-
mal cells suggesting that it may be involved in patterning more than just the nervous system. However, *CpostHox* expression in the juvenile seems to be more restricted to cells that contribute to the nervous system.

The strong correlation of the acoel *Hox* gene expression with the nervous system development supports the idea of the ancestral role of *Hox* genes in the anterior-posterior patterning and regionalization of the central nervous system. This has been hypothesized since the discovery of *Hox* gene expression in protostomes (leech [88-91], *C. elegans*: [92]*Drosophila*: [93]) and deuterostomes [24,94,95]. Recent studies in the polychaete annelids *Capitella* [5], *Chaetopterus* [69], nereids [70], the molluscs *Halitiis* [68]*Euprymna* [96] and the deuterostomes *Saccoglossus* [66], *Branchiostoma* [67]*Ciona* [97,98] and *Oikopleura* [77] all found that *Hox* genes are expressed in neural tissue [99]. As the nervous system plays such important part in the body plan organization in bilaterians, the ancestral *Hox* gene expression in the central nervous system may have been co-opted for the more complex patterning of additional tissues along the anterior-posterior axis, such as paraxial mesoderm, paired appendages and genitalia [100].

The staggered spatial expression of *Hox* genes in protostome and deuterostome centralized nervous systems, has also been used to homologize neuroanatomical structures (for example, the tritocerebrum of *Drosophila* with the hindbrain of chordates) [101,102]. However, our results
from the acoel show that a similar staggered anterior-posterior expression of Hox genes was already present in the nervous system of the last common bilaterian ancestor with a much simpler neural organization. This indicates that metazoan animals appeared to have a general molecular blueprint for axial organization that did not specify the distinct morphological complexity of the final structures. Therefore, the homologization of more complex brain structures between protostomes and deuterostomes on the basis of Hox gene expression is inappropriate. This interpretation is supported by broad comparative studies utilizing the diversity of animal taxa, many of which lack complex brains but possess a similar arrangement of Hox expression, such as the hemichordate Saccoglossus [66,103] and the annelid Capitella [5].

Early blastoporal expression suggests an early role of Hox genes in ectodermal patterning of acoels
Two of the three Hox genes of C. longifissura - the central and the posterior class Hox gene - are expressed at the site of gastrulation. The early onset of ClcentHox at the area of the former blastopore is similar to that found for Hox5 in the hemichordate Saccoglossus [66] and Branchiostoma [67] and for Hox4 and Hox5 in the polychaete Nereis [70] and Chaetopterus [69]. It remains unclear how the specific cell divisions and expression pattern correlates to the spatially very restricted position of ClcentHox expression in two bilateral clusters lateral to the position of the future mouth during later development. Both ClcentHox and ClpostHox are first expressed in the posterior ectoderm in an overlapping manner, before becoming restricted to neural cell fates when the staggered pattern of the expression is established at later stages of development. This restriction is first observed in the expression pattern of ClcentHox followed by the restriction of ClpostHox expression. However, the early expression of these two Hox genes at the blastopore suggests a different function from the later neural expression. A similar biphasic temporal expression is also found in chick development, where the early function of the Hox genes seems to be in axial specification of the gastrulating cells at the primitive streak [104]. Perhaps the Hox genes are involved with chromatin modification, which creates a 'memory' for the future fates, even when the gene is no longer expressed [104]. A similar mechanism could be present in the acoels, polychaetes and hemichordates and needs further investigations.

The expression of the ParaHox Cdx ortholog in C. longifissura
The role of Cdx expression in acoels, in hindgut evolution and the expression along the anterior-posterior axis in different germ layers have already been discussed [58,61]. The expression of CICdx in the anterior brain-commisures and connectives that extend to the ventral fold and the anterior sensory cells is very similar to the expression pattern found in the brain of the polychaete Capitella teleta [105]. Interestingly, Cdx does not seem to be expressed in the brain of nereid polychaetes [106,107] which indicates intra-taxon variability. However, other bilaterians investigated also lack Cdx expression in the anterior neural structures but show Cdx expression in neural and endodermal tissue [108]. Thus, it remains unclear how far the brain expression in Capitella teleta reflects evolutionary ancestry with the acoel neural expression.

The findings that, in several bilaterians, ParaHox genes are expressed associated with the digestive system led to the hypothesis that Gsx, Xlox and Cdx are responsible for the AP patterning of a through gut in the hypothetical Urbilaterian [109]. However, this pattern is not consistent throughout the Bilateria [reviewed in [105]]. The expression of Cdx in the posterior ectoderm in most bilaterian lineages, which often forms the ectodermal hindgut, seems to be highly conserved [110]. In the acoels and nemertodermatids the posterior ectoderm forms the male gonoduct of the adult, which is also composed of at least a part of ectoderm. This suggests that the posterior ectodermal expression was co-opted for hindgut formation in different animal lineages [58,111].

Our survey of Hox and ParaHox genes in the acoel C. longifissura did not reveal any ParaHox genes other than Cdx. This could indicate a loss of additional paralogs or highly divergent homeodomain sequences which could not be recovered with our degenerate polymerase chain reaction (PCR) approach. The fact that an ancestral epithelial digestive system, as it is present in nemertodermatids and cnidarians, has been reduced to the syncytium in the acoel lineage [33] could explain a loss of these genes from the genome. Since the sister group Nemertodermatida possesses an epithelial gut and an Xlox ortholog, expression studies of these genes in these worms may indicate that there was an ancestral role of the other ParaHox genes in bilaterian gut patterning.

Conclusion
Our results of the study of the embryonic expression patterns of the Hox genes in a representative of the likely earliest branch of the Bilateria, the acoelomorphs, show that the small Hox gene complement is expressed in a staggered pattern along the anterior-posterior body axis (Figure 6). This suggests that the spatial expression was regulated by an ancestral regulatory system in the last common ancestor of all bilaterians. However, the simultaneous initiation of Hox gene expression in the acoel shows a lack of the temporal colinearity that is present in some bilaterian lineages, perhaps due to a breakdown of genomic clustering. Our description of the biphasic expression of the Hox genes in the embryo of the acoel C.
**Methods**

**Material**
Gravid adult acoels of the hermaphroditic species *Convolutriloba longifissura* were collected from the sea water tables of the Kewalo Marine Laboratory (Oahu, Hawaii) and reared in glass fingers bowls of filtered seawater in constant light. Egg clusters composed of 25 - 70 zygotes were harvested the morning after collection. The culture was extended with primers against specific genes amplified in this analysis, along with published sequences from the acoels *Symasagit-tifa rosooffensis* and *Isodiametra pulchra*. In addition to the homeodomain, 5' and 3' flanking regions were incorporated in the alignment, including the PBX domain when present (see Additional file 1: Table S1). The software MUSCLE [114] was used for the alignment and corrected manually. The best protein evolution model (RTRev) was determined with the software Protest. Extended *Hox* class genes [115] were used as outgroup. [The nexus files are available on request at andreas.hejnol@sars.uib.no].

**Fixation**
Embryos of known developmental stages were fixed in 3.7% formaldehyde in seawater for 3 hours at 4 °C. Embryos were washed in PTw (PBS + 0.1% Tween 20) with several changes for at least 2 hours to dissolve the red pigment. After a 5-minute wash with distilled water to remove salt, embryos and juveniles were dehydrated by several washes in 100% methanol. Hatchlings and embryos were stored in separate tubes at -20°C for several months and rehydrated for whole mount *in situ* hybridizations.

**Whole mount *in situ* hybridization**

*In vitro* transcribed (Ambion Megascript T7 or SP6 kit) DIG-labelled antisense *in situ* probes were made from PCR amplified gene fragments (*ClCdx* 1155 bp, *ClpostHox* 855 bp, *ClcentHox* 1006 bp, *ClSoxB1* 1450 bp). The whole mount *in situ* hybridization protocol has been previously described and is available online (http://www.natureprotocols.com/2008/09/18/in_situ_protocol_for_embryos_a.php). Double *in situ* hybridizations were carried out using both DIG-labelled and fluorescein-labeled riboprobes. DIG-labelled riboprobes were detected colourimetrically with NBT/BCIP substrates. After the first colour reaction, the alkaline-phosphatase of the anti-DIG antibody was heat-inactivated by incubation for 30 minutes at 60°C, followed by incubation with 0.1 M glycine-HCl (pH 2.2). Fluorescein-labelled riboprobes were detected using only 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). However, this precipitate is more diffuse than the product of the reaction using nitro blue tetrazolium chloride (NBT)/BCIP as a substrate. Additional comparisons of the expression patterns of both genes were made using NBT/BCIP simultaneously for both probes.

Embryos were mounted in 70% glycerol and the expression patterns were documented using an Axiocam high resolution camera on an Axioscope mot2 plus with Axiosvision (Zeiss, Inc, VA, USA) software. The precipitate of the NBT/BCIP reaction reflects infrared light and stacks of expression patterns for three dimensional reconstructions were imaged using NBT/BCIP IR reflection microscopy with a Zeiss LSM 510 confocal microscope [116].

**Gene amplification**
We used standard degenerate primers F: 5'-GARYTNGA-RAARGARIT-3' (ELEKEF) and R: 5'-CKNCKRTTYT-GRAACCA-3' (WFQNRR) for *Hox* gene amplification from *Convolutriloba longifissura* genomic DNA. The search was extended with primers against specific *Hox* and *ParaHox* genes. The gene sequences are deposited in GeneBank with the following accession numbers: *ClSoxB1*: GQ487528, *ClantHox* GQ487529, *ClcentHox* GQ487530, *ClpostHox* GQ487531.

**Phylogenetic analysis**
To determine gene orthology of the *ClSoxB1* gene, phylogenetic analysis using Bayesian inference was conducted (mixed model option and 3,000,000 generations sampled every 1000 generations and 4 chains). A maximum likelihood analysis using the software PhyML [113] with 3000 bootstraps was conducted in order to analyse the gene orthology of the *Hox* genes amplified in this analysis,
Histology

Embryos were embedded in glycol methacrylate (Technovit® 7100) after in situ hybridization according to the manu-
factures (Heraeus Kulzer, Germany) protocol, and sectioned with a microtome with glass knives at the Pacific Biosciences Research Center (University of Hawaii, Honolulu) microscopy facility or with a sliding microtome at the Institute for Biology (Humboldt-University Berlin). Sections were stained with haematoxylin or nile blue using standard histological protocols.

Immunohistochemistry

To label the serotonergic nervous system we used an anti-
body against tyrosinated-tubulin (Sigma, USA) raised in mouse in a concentration 1:200 using a standard neural
antibody staining protocol [117] and visualized using a
Cy3 labelled anti-mouse secondary anti-body (Jackson Laboratories, USA).

Abbreviations

A-P: anterior-posterior; ML: maximum likelihood; PCR:
polymerase chain reaction; BCIP: 5-bromo-4-chloro-3-
indolyl-phosphate; NBT: nitro blue tetrazolium chloride.

Authors’ contributions

AH and MQM wrote the manuscript and designed the
study. AH performed the experiments. Both authors read
and approved the final manuscript.

Additional material

Additional file 1

Additional figures and table. Additional Data Figure S1 - Phylogenetic
analysis of Hox and ParaHox genes that excluded most cidarian
orthologs. Additional Data Figure S2 - Bayesian orthology assignment of
CiSoxB1. Additional Data Table S1 - Motif comparisons of acoel Hox
genes with that of other bilaterians and cnidarians.

Click here for file: [http://www.biomedcentral.com/content/supplementary/1741-7007-7-65-S1.PDF]

Acknowledgements

We thank Jaume Baguñà and Pere Martinez for sharing unpublished data and Eduardo Moreno for his help in the lab during a visit at the Kewalo
Marine Laboratory. Tina Cavallito, from the Microscopic Facility at the
PBRC, and Renate Predhommeau, from the group Vergleichende Zoologie at the Humboldt University Berlin, helped with the fixation and sectioning of the embryos and juveniles for histology. Special thanks go to Tricia Murata for comments that helped to improve the manuscript. The research was funded by NSF Atol grant (EF05-31558), NASA Exobiology and the German Science Foundation (HE5183/2-2).

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