

# 1 **Spatial transcriptomics reveals a conserved segment polarity program** 2 **that governs muscle patterning in *Nematostella vectensis***

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## 21 **Highlights**

- 22 • *Nematostella* endomesodermal tissue forms metameric segments and displays a  
23 transcriptomic profile similar to that observed in bilaterian mesoderm
- 24 • Construction of a comprehensive 3-D gene expression atlas enables systematic dissection  
25 of segmental identity in endomesoderm
- 26 • *Lbx* and *Uncx*, two conserved homeobox-containing genes, establish segment polarity in  
27 *Nematostella*
- 28 • The Cnidarian-Bilaterian common ancestor likely possessed the genetic toolkit to  
29 generate polarized metameric structures

30

31 **Summary**

32 During early animal evolution, the emergence of axially-polarized segments was central to the  
33 diversification of complex bilaterian body plans. Nevertheless, precisely how and when segment  
34 polarity pathways arose remains obscure. Here we demonstrate the molecular basis for segment  
35 polarization in developing larvae of the pre-bilaterian sea anemone *Nematostella vectensis*.  
36 Utilizing spatial transcriptomics, we first constructed a 3-D gene expression atlas of developing  
37 larval segments. Capitalizing on accurate *in silico* predictions, we identified Lbx and Uncx,  
38 conserved homeodomain-containing genes that occupy opposing subsegmental domains under  
39 the control of both BMP signaling and the Hox-Gbx cascade. Functionally, *Lbx* mutagenesis  
40 eliminated all molecular evidence of segment polarization at larval stage and caused an aberrant  
41 mirror-symmetric pattern of retractor muscles in primary polyps. These results demonstrate the  
42 molecular basis for segment polarity in a pre-bilaterian animal, suggesting that polarized  
43 metameric structures were present in the Cnidaria-Bilateria common ancestor over 600 million  
44 years ago.

45

46 **Keywords**

47 Cnidaria, segment polarity, spatial transcriptomics, germ layer evolution, Lbx, Uncx

## 48 **Introduction**

49 Bilaterian body plans are commonly constructed from a linear series of metameric segments  
50 along the anterior-posterior (AP) axis (Bateson, 1894; Christ et al., 1998; Diaz-Cuadros et al.,  
51 2021). A paraxial polarity program further subdivides each of these segments into opposing  
52 compartments with asymmetric developmental potential that will give rise to distinct parts of  
53 the nervous system, musculature and skeleton (Benazeraf and Pourquie, 2013; Onai et al., 2014).  
54 Segment polarization is thus a prominent feature observed in nearly all segmented animals and  
55 enables the formation of complex metameric structures with regionalized functions (Pourquie,  
56 2000; Scott and Carroll, 1987). Still, despite superficial similarities, the genetic programs that  
57 establish segment polarity vary considerably, bringing into question the existence of an ancestral  
58 segment polarity program in the Urbilaterian common ancestor (Onai *et al.*, 2014; Seaver, 2003;  
59 Tautz, 2004).

60 Cnidarians (jellyfish, hydroids, corals and sea anemones) are the sister group to bilaterians and  
61 are traditionally considered simple organisms with radial symmetry and an unsegmented body  
62 (Hyman, 1940). Contrary to this view, it has been recognized for over a century that many  
63 cnidarian species, especially in the basal class Anthozoa, display internal bilateral symmetry by  
64 forming metameric structures called gastric pouches along their directive axis (Pax, 1913). For  
65 instance, during larval development in the starlet sea anemone *Nematostella vectensis*, the  
66 endomesoderm undergoes tissue segregation to form eight bi-radially positioned segments (S1-  
67 S8, numbered clockwise with S1 being the largest), each constituting part of the future nerve net,  
68 musculature and gastrodermis (He et al., 2018; Leclere and Rentzsch, 2014; Steinmetz et al.,  
69 2017). During this process, pairs of nascent segment boundaries arise paraxially following a  
70 stereotypic sequence, regulated by a cnidarian Hox-Gbx code (He *et al.*, 2018). On top of the  
71 metameric larval body plan, adult structures such as the retractor muscles are patterned in a  
72 segmentally-polarized manner, suggesting compartments with distinct developmental potential  
73 exist within each morphologically homogenous segment. Despite sharing similar design  
74 principles with bilaterian segments, little is known about the cellular and molecular basis for  
75 segment polarity establishment in cnidarians, hindering our ability to draw meaningful  
76 conclusions regarding the evolutionary origin of the metameric body plan.

77 The rapid development of spatial transcriptomics allows unbiased characterization of gene  
78 expression patterns in diverse biological contexts (Dries et al., 2021; Lohoff et al., 2022; Marx,  
79 2021; van den Brink et al., 2020). However, widely-used spatial transcriptomic approaches such  
80 as Slide-seq are restricted to 2-dimensional tissue sections, offer limited resolution, and are  
81 difficult to employ in small 3-dimensional samples such as the embryos of marine invertebrates  
82 (Rodrigues et al., 2019; Stickels et al., 2021). Fortunately, several computational approaches have  
83 been developed to circumvent these challenges, mostly relying on high-resolution landmark gene  
84 expression patterns to infer the likely coordinates of a given cell from conventional single cell  
85 RNA-seq (scRNA-seq) datasets (Achim et al., 2015; Deng et al., 2019; Karaiskos et al., 2017; Moriel  
86 et al., 2021; Nitzan et al., 2019; Satija et al., 2015).

87 Here we employed an *in silico* spatial transcriptomic approach to explore the molecular basis for  
88 segment polarity establishment in *Nematostella*. By generating a tissue-enriched scRNA-seq  
89 dataset, we first profiled the transcriptomic landscape of major endomesodermal cell clusters at  
90 mid-planula stage (72hpf), a time point at which the segmentation process has just completed.  
91 Next, guided by a set of 34 landmark genes, we constructed a 3-D spatial gene expression atlas  
92 (EndoAtlas) using the novoSpaRc algorithm, which predicts the spatial patterns for 15,542 genes  
93 that were expressed in the developing endomesoderm (Moriel *et al.*, 2021; Nitzan *et al.*, 2019).  
94 We validated our *in silico* predictions using fluorescent *in situ* hybridization (FISH) and  
95 systematically characterized segment identity markers downstream of the cnidarian Hox-Gbx  
96 hierarchy. Intriguingly, we also identified two conserved homeobox-containing genes, *Lbx* and  
97 *Uncx*, that establish opposing subsegmental domains concurrently with the segmentation  
98 process. Functional perturbations using short hairpin RNA (shRNA) knockdown and CRISPR/Cas9  
99 mutagenesis demonstrated the requirements of the Bone Morphogenetic Protein (BMP)  
100 signaling pathway and the Hox-Gbx cascade in setting up the paraxial *Lbx-Uncx* polarity. In  
101 addition, *Lbx* loss of function abolished the molecular polarity and led to duplication of the  
102 retractor muscles in the resultant primary polyps. Lastly, phylogenetic analysis suggested that  
103 *Lbx* and *Uncx* first emerged in the Cnidaria-Bilateria common ancestor, consistent with an ancient  
104 origin for segment polarization in animals.

## 105 Results

### 106 Cellular and molecular profiling of developing endomesoderm in *Nematostella*

107 In *Nematostella*, the segmented larval body plan serves as a blueprint for the development of  
108 the adult anatomy. During metamorphosis, each segment takes on a different identity (tentacle-  
109 bearing or non-tentacle-bearing) while fusing with the neighboring segments at the boundaries  
110 to form the eight mesenteries which compartmentalize the gastric cavity of polyps (**Figure 1A**).  
111 Molecular markers for segmentally-polarized adult structures, such as the retractor muscles,  
112 start to exhibit asymmetric distribution at the planula stage, suggesting that distinct  
113 developmental identities are established early during the segmentation process (**Figure 1B**).  
114 These observations prompted us to focus on mid-planula stage larvae and investigate the  
115 molecular mechanisms for segment polarity.

116 To analyze the cellular and molecular composition of larval segments on a global scale, we first  
117 employed scRNA-seq. At 72hpf, the endomesoderm constitutes a small proportion (<9%) of larval  
118 cells comparing to the ectoderm (Sebe-Pedros et al., 2018). We therefore combined osmolarity  
119 shock and chemical digestion to develop a tissue isolation method that specifically removes the  
120 ectoderm from developing larvae (**Figure 1C and D**, see also STAR Methods). Close examination  
121 of tissue isolates generated using this approach revealed the retention of part of the pharyngeal  
122 ectoderm adjacent to the intact segmented endomesoderm. By performing scRNA-seq on these  
123 isolates, we obtained 10,408 cells that passed a UMI cutoff of 500 (**Figure 1E**, see also STAR  
124 Methods). Subsequent UMAP projection identified 11 transcriptionally-distinct cell populations.  
125 Annotation of cluster identities was achieved using a set of published germ layer markers in  
126 *Nematostella*, such as *FoxA* (Pharyngeal ectoderm, PE) and *NvSnail1* (Endomesoderm, EN)  
127 (Fritzenwanker et al., 2004), as well as novel cluster markers identified by Seurat (**Figure S1**).  
128 Importantly, genes specifically expressed in the body wall ectoderm such as *Dlx* (Ryan et al., 2007)  
129 and *Anthox1* (Kamm et al., 2006; Ryan et al., 2007) were detected at minimal levels in this dataset,  
130 confirming the successful depletion of ectoderm during the isolation procedure (**Figure S1E**). As  
131 illustrated by the UMAP, EN is largely divided between the myogenic (ME) and neurogenic (NE)  
132 lineages, with the ladder further differentiating into two distinct larval neuron populations

133 marked by the cnidarian neuropeptides *RWamide* (N1) and *PRGamide* (N2), respectively (**Figure**  
134 **S1G** and **H**) (Koch and Grimmelikhuijzen, 2019; Takahashi, 2020).

135 Interestingly, in *Nematostella* endomesoderm we observed enriched expression of genes whose  
136 homologs are typically associated with bilaterian mesoderm specification and differentiation,  
137 such as *FoxC1*, *Six1/2*, *Six4/5*, *Meox* and *Csrp2* (**Figure 1F**) (Chen et al., 2005; Mankoo et al., 2003;  
138 Miyasaka et al., 2007; Wilm et al., 2004). In addition, markers of bilaterian mesodermal cell types,  
139 including *MHC-st* (striated muscle), *Mef2a* (myocyte) and *Aif1* (macrophage/phagocyte) were  
140 enriched in the ME lineage (**Figure 1F, Figure S1I**) (Donovan et al., 2018; Nguyen et al., 1994;  
141 Noden et al., 1999). Conversely, pharyngeal ectodermal clusters were highly enriched for genes  
142 whose bilaterian homologs are involved in the induction of definitive endoderm, such as *FoxA2*,  
143 *Hhex* and *Nkx6* (**Figure 1F, Figure S1D**) (Burtscher and Lickert, 2009; Martinez Barbera et al., 2000;  
144 Pedersen et al., 2005). Taken together, these results demonstrate the complex cellular and  
145 molecular nature of the segmented *Nematostella* endomesoderm and argue for a revised view  
146 of germ-layer homology between cnidarians and bilaterians, consistent with previous candidate-  
147 centered gene expression studies (Martindale et al., 2004; Steinmetz, 2019; Steinmetz *et al.*,  
148 2017; Technau, 2020).

#### 149 ***In silico* construction of a 3-dimensional gene expression atlas**

150 To visualize endomesodermal gene expression on a global scale, we leveraged our single cell  
151 transcriptomic data to construct an *in silico* gene expression atlas. To achieve this, we applied  
152 novoSpaRc, a computational approach built on the premise that within a developing tissue cells  
153 with physical proximity tend to share similar transcription profiles, and thus one can infer the  
154 probable spatial distribution of individual cells within a defined space by solving an optimal-  
155 transport problem (Nitzan *et al.*, 2019). Within the framework of novoSpaRc, we first generated  
156 a three-dimensional (3-D) model based on the morphological features of the actual  
157 endomesoderm (**Figure S2**) and projected cell clusters of endomesodermal origin (*Neuron1*,  
158 *Neuron2*, *Neuronal Precursor*, *Bulk Endomesoderm*, and *Myogenic Endomesoderm*, a total of  
159 5,451 cells) onto 8,791 vertices located within this 3-D space (**Figure 2A**). In the initial  
160 unsupervised projection, novoSpaRc inferred the spatial position of cells based only on the  
161 internal structure of the data, and the prediction outcomes failed to recapitulate known gene

162 expression patterns (exemplified by *Gdf5*, *Tbx15* and *Arp6*). This is likely because the default  
163 optimal arrangement of cells did not take into consideration the complex, metameric nature of  
164 the tissue, and indicated that spatial landmark genes were needed to guide the algorithm (Moriel  
165 *et al.*, 2021). We therefore performed a fluorescent *in situ* hybridization (FISH) screen and  
166 identified 34 landmark genes that exhibited spatially distinct expression domains within the  
167 endomesoderm (**Figure S3A**). Binarized spatial expression matrices of these genes were then  
168 provided as additional input for novoSpaRc. The landmark-guided prediction successfully  
169 recapitulated the expression patterns of all 34 marker genes, with a mean Pearson correlation of  
170 0.81 (**Figure S3B**).

171 We next validated the predictive power of Endo-atlas by analyzing the expression of 16 novel  
172 genes using FISH (**Figure 2B**). Among these we identified territorial genes expressed along the  
173 oral-aboral axis (e.g. *Notum1*, *Wntless*, *Nkx2.8*) as well as cell type-specific genes that displayed  
174 polarized expression in certain segments (*Atoh1* and *FoxQ1*). In all cases, the FISH results were in  
175 close accordance with our predictions, further supporting the accuracy of this approach. Lastly,  
176 we systematically predicted expression patterns for 15,542 genes that passed the minimum  
177 detection threshold in the *Nematostella* endomesoderm and constructed an online database  
178 named Endo-atlas (<http://endoatlastest-env.eba-qtpfn7qz.us-east-1.elasticbeanstalk.com/>).

### 179 ***Nematostella* endomesodermal segments possess distinct molecular identities**

180 *Nematostella* endomesodermal segmentation is regulated by a group of Hox-Gbx genes  
181 expressed in an overlapping nested fashion (**Figure 3A**) (Chourrout *et al.*, 2006; He *et al.*, 2018;  
182 Hudry *et al.*, 2014; Ryan *et al.*, 2007). Guided by the Endo-atlas predictions, we next sought to  
183 identify segment identity genes acting downstream of the Hox-Gbx network to convey distinct  
184 developmental potential to otherwise identical segments. Indeed, we were able to confirm  
185 segment-restricted expression patterns of a large cohort of genes (**Figure 3B to E**). The polar  
186 segment S1 was marked by transmembrane receptors such as *Fgfr-like* and *CaSR*, with a subset  
187 of S1 cells adjacent to the pharyngeal ectoderm collectively expressing ADP-Ribosyltransferase  
188 *Art5*, neuropilin *Nrp2* and D1-dopamine receptor (**Figure 3B**). The enrichment of neuronal genes  
189 in segment S1 correlates with the asymmetric localization of *GLWamide*<sup>+</sup> neurons within the  
190 developing endomesoderm (Watanabe *et al.*, 2014). The tentacle-bearing segment pairs S2/S8

191 and S4/S6 shared several common markers, such as the structural protein *Col6A5*, the  
192 transcription factor *Zic3* as well as an unknown protein *Nv2.2940* (**Figure 3C**). Interestingly, only  
193 the S4/S6 segment pair expressed *Tgfr3*, *F5* and *Cdx* (**Figure 3D**), hinting at a molecular identity  
194 distinct from that of segments S2 and S6. Indeed, segments S2 and S8 give rise to exocoels in  
195 which incomplete mesenteries start to form during juvenile development, whereas S4 and S6 are  
196 endocoels that are incapable of secondary segmentation (Berking, 2007; Ikmi and Gibson, 2010;  
197 Ikmi et al., 2020). Segment S5, the polar segment opposite S1, possessed many unique molecular  
198 markers, such as the extracellular proteins *Efemp1* and *F8* as well as several Cnidaria-specific  
199 genes with unknown functions such as *Nv2.7863* (**Figure 3E**).

200 To test whether Hox genes control the molecular identities of endomesodermal segments, we  
201 next performed RNA-seq in *Anthox1a*, *Anthox8* and *Anthox6a* homozygous mutant backgrounds.  
202 At 72hpf, the expression levels of the segment identity genes described above displayed drastic  
203 changes corresponding to the loss of each Hox gene (**Figure 3F**). For instance, in the absence of  
204 *Anthox1a* the physical boundaries of S5 are abolished, leading to the formation of a single fused  
205 segment S4-6 (He *et al.*, 2018). Accompanying these morphological changes, we found that the  
206 expression of the S4/S6 identity marker *Tgfr3* expanded into the previous S5 territory, consistent  
207 with the expansion of S4/S6 identity across the fused segment (**Figure 3H**). Conversely, in the  
208 absence of *Anthox8* the S1-sided physical boundaries of S4/S6 are abolished, resulting in the  
209 formation of fused segments S3-4 and S6-7. In this case we observed the complete loss of *Tgfr3*  
210 expression (**Figure 3H**). These results are consistent with the altered tentacle patterns reported  
211 in Hox mutant polyps and demonstrate a molecular basis for homeotic transformation in  
212 *Nematostella*.

### 213 ***Lbx*, *Uncx* and the molecular polarity of *Nematostella* endomesodermal segments**

214 Intriguingly, our Endo-atlas predictions identified two homeobox-containing transcription factors  
215 with segmentally polarized expression patterns: the *Ladybird* homolog *Lbx* and the *Unc-4*  
216 homolog *Uncx* (**Figure 4A** and **B**). In 72hpf planulae, *Lbx* expression specifically demarcated the  
217 S1-sided segment boundaries in segment pairs S2/S8, S3/S7, S4/S6 and was ubiquitously  
218 expressed in segment S5. *Uncx* was expressed in a complementary pattern to *Lbx*, where it was  
219 specifically enriched at the S5-sided boundaries in all three segment pairs and uniformly



220 expressed in segment S1. *Lbx* and *Uncx* thus demarcate opposing territories with distinct  
221 molecular identities within all three segment pairs. Temporally, this polarity was established prior  
222 to the segmentation process, as *Lbx* stripes flanking future segment S1 and *Uncx* stripes flanking  
223 future segment S5 are detected before the formation of physical segment boundaries (**Figure S4**).  
224 These observations indicate that the *Lbx-Uncx* polarity program could be directly influenced by  
225 *Nematostella* Hox-Gbx genes, which also turn on prior to boundary establishment.

226 To interrogate the upstream control of segment polarization, we examined the expression of *Lbx*  
227 and *Uncx* in *Anthox1a*, *Anthox8* and *Anthox6a* mutants (**Figure 5A to D**). *Anthox1a* mutants fail  
228 to form the S5 segment boundaries and instead generate an enlarged fusion segment S4-5-6.  
229 Consequently, the *Lbx* expression domain corresponding to segment S5 was absent, resulting in  
230 ubiquitous *Uncx* expressed in the center of the fusion segment (**Figure 5B**). *Anthox8* mutants fail  
231 to form boundaries between segments S3/S4 and S6/S7 and instead generate two enlarged  
232 fusion segments S3-4 and S6-7. Consequently, S1-sided *Lbx* stripes within segments S4 and S6  
233 were abolished (**Figure 5C**). Both fusion segments still retained an *Lbx-Uncx* polarity along the  
234 directive axis, despite the increased segment size. Similarly, *Anthox6a* mutants fail to form  
235 boundaries between segments S2/S3 and S7/S8 and instead generate two enlarged fusion  
236 segments S2-3 and S7-8. Consequently, S1-sided *Lbx* stripes within segments S3 and S7 were  
237 abolished, and a positionally-shifted *Lbx-Uncx* polarity can be observed in both fusion segments  
238 (**Figure 5D**). Taken together, these results suggest that Hox genes provide segment-specific  
239 regulatory inputs towards *Lbx-Uncx* polarization.

240 Importantly, Hox genes are homogenously expressed within developing endomesodermal  
241 segments and their activities are thus unlikely to be sufficient to generate polarized *Lbx-Uncx*  
242 stripes. In *Nematostella*, BMP signaling forms an activity gradient across the endomesoderm  
243 which directly activates Hox-Gbx genes and defines their expression territories (Genikhovich et  
244 al., 2015; Kraus et al., 2016; Leclere and Rentzsch, 2014; Rentzsch et al., 2006; Wijesena et al.,  
245 2017). We therefore suspected the existence of an additional regulatory input from the BMP  
246 pathway upstream of *Lbx-Uncx* polarity. This hypothesis was further strengthened by the  
247 existence of a phosphorylated Smad1/5 (pSmad1/5) binding peak upstream of the *Lbx* locus from  
248 ChIP-seq data (Knabl et al., 2022), indicating that *Lbx* is a direct target of the BMP pathway. To

249 test this experimentally, we first abolished BMP activity by knocking down major BMP pathway  
250 components in *Nematostella* including *Bmp4*, *Bmp5*, *Chordin* and *Smad1/5* (**Fig. 5E, Fig. S5A to**  
251 **C**). In line with previous work, no endomesodermal segments were formed in shRNA-injected  
252 larvae (**Figure S5A**). The resulting unsegmented endomesoderm lacked *Lbx* expression, and  
253 uniformly expressed *Uncx* (**Fig. 5E, Fig. S5B and C**). Furthermore, the segment S1 marker *Arp6*  
254 expanded across the endomesoderm, suggesting that the entire tissue was transformed into an  
255 S1-like ground state in the absence of BMP activity (**Figure S5H**). Next, by knocking down the TGF-  
256  $\beta$  pathway signaling molecule *Gdf5*, we examined the effect of a weakened BMP gradient on  
257 segment polarity. In the absence of *Gdf5*, the flattened BMP activity gradient failed to activate  
258 the high-threshold Hox genes *Anthox1a* and *Anthox8*, while the low-threshold genes *Anthox6a*  
259 and *Gbx* were unperturbed, resulting in the fusion of segments S3-S7 (**Fig. 5F, (Knabl et al., 2022)**).  
260 Contrary to the fused segments observed in different Hox mutants, these S3-S7 fusions displayed  
261 an altered polarity, where *Lbx* became ubiquitous and *Uncx* was undetectable (**Fig. 5F**).  
262 Collectively, these results support a model in which a stepwise decreasing activation signal from  
263 Hox-Gbx genes counteracts the continuously decreasing repressive signal from the BMP pathway  
264 to generate segmentally polarized *Lbx* stripes along the directive axis (**Fig. S5I to K**). *Lbx* is thus  
265 only expressed at the S1-sided borders of each Hox-Gbx expression domain, where repressive  
266 BMP activity is lowest and thus overcome by the activation input.

267 To further validate the model, we examined the expression of polarity markers in larvae injected  
268 with two independent shRNAs targeting the Hox binding partner *Pbx* (**Fig. 5G, Fig. S5D to G**).  
269 Knockdown of *Pbx* disrupted the function of all Hox-Gbx genes acting downstream of the BMP  
270 pathway, leading to the formation of an unsegmented endomesoderm at the planula stage (**Fig.**  
271 **S5D**). Although morphologically resembling the *Bmp4* KD condition (**Fig. S5A**), *Pbx* KD larvae  
272 exhibited a distinct transcriptomic profile (**Fig. S5E to G, Table S3**). *Anthox8*, which requires Pbx-  
273 dependent self-activation, was significantly downregulated, whereas *Anthox6a* and *Gbx* were  
274 upregulated (**Fig. S5G; He et al., 2018**). Moreover, despite the lack of segment boundaries, *Arp6*  
275 expression was still restricted to a polar region corresponding to segment S1, suggesting that a  
276 BMP activity gradient that represses the ground state identity remained functional in the absence  
277 of *Pbx* (**Fig. S5H**). Interestingly, the unsegmented endomesoderm in *Pbx* KD larvae still displayed

278 polarized *Lbx-Uncx* patterns: two *Lbx* stripes flanking the *Arp6* domain were observed, restricting  
279 a high level of *Uncx* expression to the S5-sided endomesoderm (**Fig. 5G**). These results indicate  
280 that *Gbx* likely retains some gene regulatory activity in the absence of *Pbx*, resulting in the  
281 establishment of a modified *Lbx-Uncx* polarity in the unsegmented endomesoderm (**Fig. S5L**).

### 282 ***Lbx* controls polarized positioning of retractor muscles in *Nematostella***

283 To investigate the developmental requirements for segment polarization, we next generated two  
284 mutant alleles that disrupt the major functional domains of the LBX protein (**Figure 6A, S7B and**  
285 **C**). *Lbx* homozygous mutants did not display segmentation defects at 72hpf and metamorphosed  
286 into polyps with four properly positioned tentacle primordia (**Figure S6A and B**). However, these  
287 mutant polyps exhibited abnormal elongation of the oral-aboral axis, possessing a shortened  
288 body column and stubby tentacles (**Figure S6C to F**). Consistent with these defects, *Lbx*  
289 homozygous polyps were unable to feed effectively and were quickly outcompeted by *wild-type*  
290 and *Lbx/+* siblings in mixed cultures (**Figure 6B**).

291 The observations above suggest that *Lbx* mutants may have additional defects and prompted us  
292 to investigate other segmentally polarized features of developing polyps, including the  
293 positioning of the retractor muscles (RMs). In *wild-type* animals, RMs formed on the S1-side of  
294 mesentery pairs m2/m7, m3/m6 and m4/m5 and on the S5-side of mesenteries m1/m8,  
295 indicated by the expression of the marker gene *MelC4* (**Figure 6C**). In *Lbx* mutant polyps, however,  
296 each mesentery displayed symmetric RM patterning. Mesentery pair m1/m8 no longer formed  
297 RMs, while the rest of the mesenteries formed duplicated RMs, specifically on their S5-side  
298 (**Figure 6D**). At the morphological level, the duplicated RMs exhibited inverted polarity, with their  
299 actin-rich basal myonemes facing each other, separated by the mesoglea (**Figure E to G**). Since  
300 each mesentery is formed by joining two compartments from neighboring segments, the RM  
301 patterning defects we observed in *Lbx* mutants are consistent with aberrant segment polarity.  
302 Indeed, the expression of *Lbx*, *Nv2.5420* and other S1-sided genes were diminished in *Lbx*  
303 homozygous mutants (**Figure 6H**). Conversely, *Uncx* became ubiquitously expressed across the  
304 endomesoderm, together with other S5-sided genes such as *Tspear* and *Thsd4*, suggesting that  
305 normal segment polarity collapsed in the absence of *Lbx* (**Figure 6H and I**).

## 306 **An Evolutionarily conserved segmental polarity program in *Nematostella***

307 The identification of the *Lbx-Uncx* segment polarity module in a cnidarian animal prompted us to  
308 further investigate the evolutionary origin of both genes. By performing reciprocal BLAST  
309 searches using the full-length as well as homeobox sequences of *Nematostella* LBX and UNCX  
310 proteins, we confirmed the presence of both genes in the genomes of five additional anthozoan  
311 species, including *Montipora capitata*, *Acropora digitifera*, *Stylophora pistillata*, *Xenia spp.* and  
312 *Scolanthus callimorphus* (**Figure S7C and D, Table S3**). To our surprise, non-anthozoan classes  
313 including Scyphozoa (represented by *Aurelia aurita* and *Nemopilema nomurai*), Cubozoa  
314 (represented by *Tripedalia cystophora* and *Morbakka virulenta*) and Hydrozoa (represented by  
315 *Hydra magnipapillata*, *Hydra viridissima*, *Hydractinia symbiolongicarpus* and *Hydractinia*  
316 *echinata*) do not possess *bona fide* *Lbx* or *Uncx* (**Figure 7A, Table S3**). Furthermore, we were  
317 unable to identify definitive *Lbx-Uncx* homologs in the basal metazoan phyla Porifera  
318 (represented by *Ephydatia muelleri* and *Amphimedon queenslandica*), Ctenophora (represented  
319 by *Hormiphora californensis*, *Pleurobrachia bachei* and *Mnemiopsis leidyi*) and Placozoa  
320 (represented by *Trichoplax adhaerens*) (**Table S3**). In contrast, phylogenetic reconstruction using  
321 full length amino acid sequences strongly supports the homology between cnidarian LBX-UNCX  
322 proteins and their bilaterian counterparts (**Figure S7A and B**). Taken together, these results  
323 indicate that *Lbx* and *Uncx* first emerged in the Cnidaria-Bilateria common ancestor, likely  
324 through a gene duplication event, and were partially or completely lost in several unsegmented  
325 lineages, including non-anthozoan cnidarians, acoels, nematodes as well as bryozoans (**Figure 7A,**  
326 **Table S3**). Interestingly, both *Lbx* and *Uncx* have been implicated to function as polarity genes in  
327 segmented bilaterians including arthropods, annelids and vertebrates (De Graeve et al., 2004;  
328 Dray et al., 2010; Jagla et al., 1997; Mansouri et al., 2000; Saudemont et al., 2008; Treffkorn et  
329 al., 2018). Based on these observations, it is plausible that an *Lbx/Uncx*-like segment polarity  
330 module existed in the cnidarian-bilaterian common ancestor, which was subsequently lost or  
331 modified in diverse animal lineages during body plan evolution.

## 332 Discussion

### 333 ***Nematostella* segment identity is determined via a Hox-Gbx hierarchy**

334 We previously proposed that the *Nematostella* Hox-Gbx hierarchy generates a binarized identity  
335 outcome for each segment: either tentacle forming or non-tentacle forming (**Figure 3A**).  
336 However, this simplified conception of segment identity fails to explain other segment-specific  
337 features, such as the development thickened primary mesenteries which only form at the  
338 boundaries between segments S2 and S3 and S7 and S8. In addition, as the primary polyp grows,  
339 new tentacles emerge following a stereotypic pattern, and non-tentacle bearing segments will  
340 eventually permit tentacle growth once the polyp reaches a certain size (Ikmi *et al.*, 2020). The  
341 development of Endo-atlas now permits a deeper understanding of *Nematostella* segment  
342 identity at the molecular level. Polar segment S1 possesses a unique transcriptomic profile, being  
343 the only segment that does not express any Hox-Gbx genes, thus representing the “ground state”  
344 of endomesoderm (**Figure S5I**). Segment S1 is also highly neurogenic and has been shown as the  
345 first segment to differentiate *GLWamide*<sup>+</sup> neurons (Watanabe *et al.*, 2014). The even-numbered  
346 segment pairs S2/S8 and S4/S6, being tentacle forming segments, share somewhat similar  
347 transcriptomic profiles, exemplified by the co-expression of *Dmbx2*, *Zic3*, *Col6A5* and *Nv2.2940*  
348 (**Figure 3C**). However, each of these segment pairs also possess some distinguishing markers.  
349 Segments S2 and S8 co-express the homeobox-containing genes *Dmbx1* and *Q50*, while segments  
350 S4 and S6 co-express *Anthox7*, *Tgfr3*, *F5* and *Cdx* (**Figure 3D**, **Figure S3A**). The remaining non-  
351 tentacle bearing segments S3/S7 and S5 also exhibit drastically different transcriptomic profiles,  
352 with the polar segment S5 possessing many unique segment markers: *Efemp1*, *F8* and *Nv2.7863*  
353 among others (**Figure 3E**, **Figure S3A**). Consequently, each segment possesses a unique molecular  
354 identity along the directive axis. In general, the even-numbered segments are more similar to  
355 each other, while the odd-numbered segment pairs show fewer similarities. These observations  
356 suggest that *Nematostella* Hox-Gbx genes share some common targets but also possess unique  
357 sets of downstream targets, reminiscent of their bilaterian counterparts.

### 358 **Segment polarity in *Nematostella* is cooperatively determined by BMP and Hox-Gbx genes**

359 As demonstrated by previous studies, BMP signaling is instrumental during directive axis  
360 establishment and endomesoderm patterning in *Nematostella* (Leclere and Rentzsch, 2014;  
361 Rentzsch *et al.*, 2006; Wijesena *et al.*, 2017). Prior to segment formation, BMP signaling forms an  
362 activity gradient across the endomesoderm, which peaks at the future segment S5 and gradually  
363 diminishes towards segment S1 (Genikhovich *et al.*, 2015). Hox-Gbx genes are activated along  
364 this gradient at different thresholds. The expression boundaries of each Hox-Gbx genes then  
365 determine the locations of physical boundaries, resulting in the formation of 3 segment pairs  
366 (S2/S8, S3/S7 and S4/S6) as well as two polar segments (S1 and S5) along the directive axis (He  
367 *et al.*, 2018). In the current work, we further demonstrate the existence of a paraxial molecular  
368 polarity consisting of two homeobox containing genes: *Lbx* and *Uncx* (**Fig. 4**). These factors are  
369 expressed in complementary expression territories within each segment pair and convey distinct  
370 developmental potentials (**Fig. 4, Fig. 6**).

371 How are subsegmental *Lbx* and *Ubx* expression domains established? We propose that *Lbx*  
372 receives counteracting regulatory inputs from the BMP pathway and Hox-Gbx genes (**Fig. S5I**). In  
373 this model, BMP signaling represses *Lbx* expression with decreasing strength along its activity  
374 gradient, whereas Hox-Gbx genes are able to activate *Lbx* expression with different strengths. In  
375 this scenario, BMP's capacity to repress *Lbx* is lowest at the S1-side of each segment. In parallel,  
376 we propose that the Hox-Gbx activation strength decreases along the same axis, with *Gbx* having  
377 the weakest capacity to induce *Lbx* near the S1-side. Consequently, within each segment pair,  
378 *Lbx* is only expressed in the S1-sided regions where the decreasing BMP activity can be overcome  
379 by the activation input provided by Hox-Gbx genes. Further, polar segment S1, which lacks Hox-  
380 Gbx expression, does not turn on *Lbx* during normal development, and displays the same "ground  
381 state" molecular profile (*Arp6*<sup>+</sup>, *Uncx*<sup>+</sup>) as the entire unsegmented endomesoderm under BMP  
382 KD conditions (**Fig. 5, Fig. S5H**).

383 Contrary to expectations, we found that knocking down the Hox binding partner *Pbx* does not  
384 recapitulate BMP KD conditions at the molecular level (Knabl *et al.*, 2022). In fact, despite the  
385 lack of all physical boundaries, segment S1 identity, as marked by *Arp6* expression, is still confined  
386 to the S1-sided polar region, and the paraxial segment polarity program remains active in *shPbx*  
387 injected larvae (**Fig. 5G, Fig. S5D and H**). These observations suggest that the axial patterning

388 activity of BMP signaling is not entirely dependent on the proper functioning of Hox-Gbx genes.  
389 Moreover, *Gbx* appears to retain its activation input into the *Lbx* locus in the absence of *Pbx*,  
390 resulting in the formation of two weak *Lbx* stripes flanking the unsegmented S1 territory (**Fig.**  
391 **S5L**). Combined, these observations support the idea that segment polarity establishment in  
392 *Nematostella* is the outcome of combinatorial regulatory interactions between an upstream  
393 signaling gradient (BMP) and its direct downstream compartment identity genes (Hox-Gbx).  
394 Together, they determine polarity gene expression domains prior to and independent of the  
395 establishment of physical segment boundaries.

### 396 **The evolution of the *Lbx-Uncx* segment polarity module**

397 *Lbx* and *Uncx* are both conserved homeobox-containing transcription factors and are known to  
398 exhibit segmentally polarized expression patterns during embryogenesis in various bilaterian  
399 lineages. Originally discovered in *Drosophila* and classified as a segment polarity gene, *Lbx*  
400 encodes a NK class homeobox protein that specifies muscle identity during body segmentation  
401 in arthropods and vertebrates (De Graeve *et al.*, 2004; Jagla *et al.*, 1995; Juarez-Morales *et al.*,  
402 2021; Ochi and Westerfield, 2009; Wotton *et al.*, 2008). In the annelid *Platynereis*, *Lbx*  
403 demarcates future posterior segment boundaries and forms complementary stripes with other  
404 polarity genes including *Engrailed*, *Wnt1* and *Tlx* (Saudemont *et al.*, 2008). This process is  
405 dependent on the upstream Hedgehog signal and occurs prior to the formation of physical  
406 segment boundaries (Dray *et al.*, 2010). A similar *Lbx* expression pattern was reported in  
407 Onychophora (velvet worms), where *Lbx* stripes are detected anterior to the growing segmental  
408 furrows (Treffkorn *et al.*, 2018). Chordates, however, have lost segmentally polarized *Lbx*  
409 expression and instead rely on the gene to specify migratory hypobranchial and appendicular  
410 muscle progenitors (Kusakabe *et al.*, 2020; Ochi and Westerfield, 2009). This transition of the *Lbx*  
411 expression pattern coincides with the emergence of a different mode of body segmentation in  
412 chordates, where somatic musculature does not develop in a segmentally polarized manner.

413 *Uncx* is a member of the PRD class homeobox genes and was initially identified in *C. elegans* as  
414 *Unc-4*. In protostomes, *Uncx* function is largely restricted to the nervous system, where it  
415 specifies motor neuron identity, sometimes in a repeated, segmentally polarized fashion (Cho  
416 and Park, 2008; Lacin *et al.*, 2020; Miller *et al.*, 1992; Walthall, 1995). In contrast, the mammalian

417 *Unc-4* homolog *Uncx4.1* serves as one of the best characterized posterior identity markers of the  
418 developing somites, where it instructs the formation of distal ribs, transverse processes and  
419 pedicles of the neural arches on vertebrae (Leitges et al., 2000; Mansouri *et al.*, 2000; Neidhardt  
420 et al., 1997; Schragle et al., 2004).

421 Given that *bona fide* *Lbx* and *Uncx* homologs cannot be identified in basal metazoan clades  
422 including Ctenophora, Porifera and Placozoa, it is likely that both genes first evolved in the  
423 Cnidaria-Bilateria common ancestor, in concert with the emergence of a bi-radially segmented  
424 body plan (**Fig. S7, Fig. 7, Table S3**). However, despite being implicated as segment polarity genes  
425 in diverse bilaterian systems, *Lbx* and *Uncx* have not been shown to function together during  
426 polarity establishment in animals other than *Nematostella*. One potential explanation is that  
427 there has been convergent evolution in the patterning of somatic musculature and nervous  
428 systems in diverse metamerical animal lineages. Consequently, important cell identity regulators  
429 such as *Lbx* and *Uncx* could be frequently employed in a segmentally polarized manner and thus  
430 exhibit similar expression patterns under the control of distinct upstream signals. Alternatively,  
431 we postulate that the *Lbx-Uncx* pair could represent an ancient and rudimentary segment  
432 polarity module that has been modified and rewired under the control of different signaling  
433 pathways to pattern the incredible diversity of metamerical structures that have arisen during  
434 bilaterian evolution.



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450 available at GEO: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173786>. The  
451 *Nematostella* Endo-atlas can be accessed at: [http://endoatlastest-env.eba-gtqpf7qz.us-east-](http://endoatlastest-env.eba-gtqpf7qz.us-east-1.elasticbeanstalk.com/)  
452 [1.elasticbeanstalk.com/](http://endoatlastest-env.eba-gtqpf7qz.us-east-1.elasticbeanstalk.com/). The NV2 genome and transcriptome used in this study are hosted on  
453 SIMRbase: <https://genomes.stowers.org/>.

454

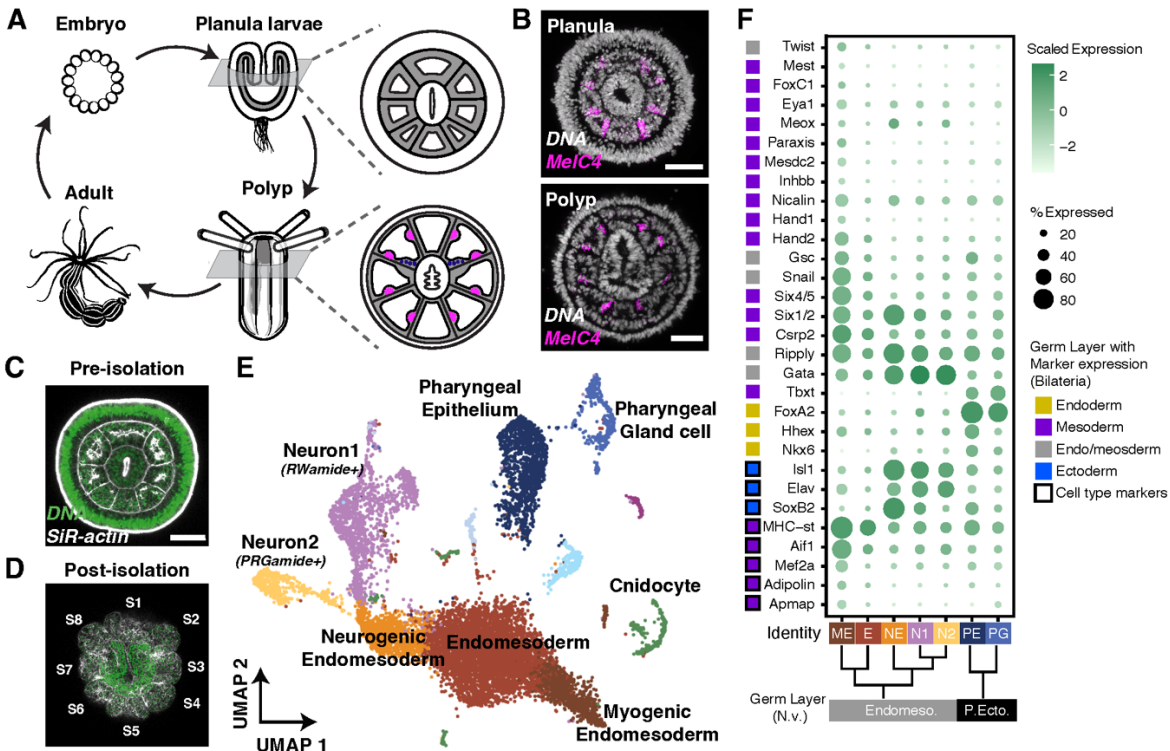
455 **SUPPLEMENTARY MATERIALS**

457 STAR Methods

458 Figure S1-7

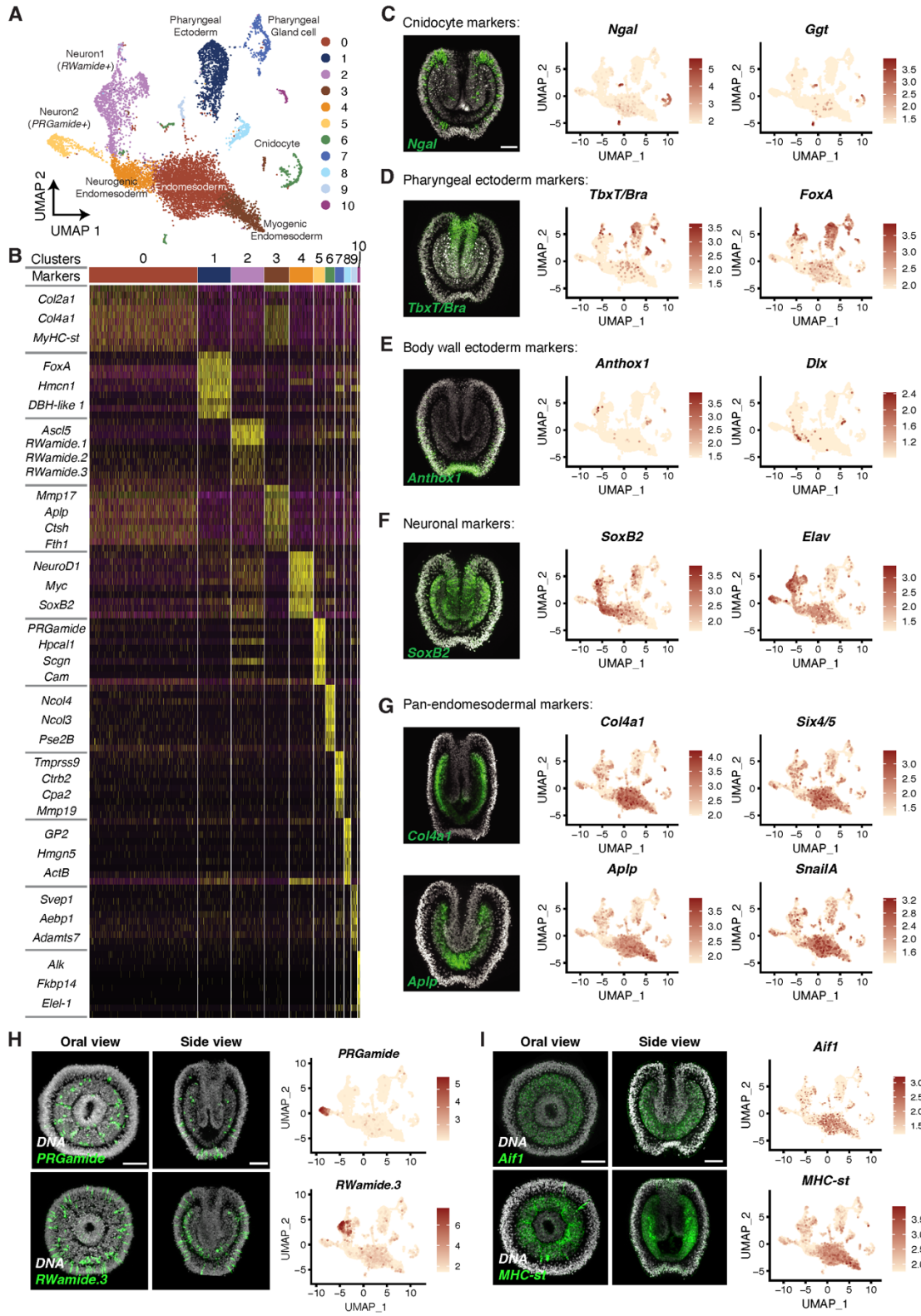
459 Tables S1, S2 and S3

460 **Figures**



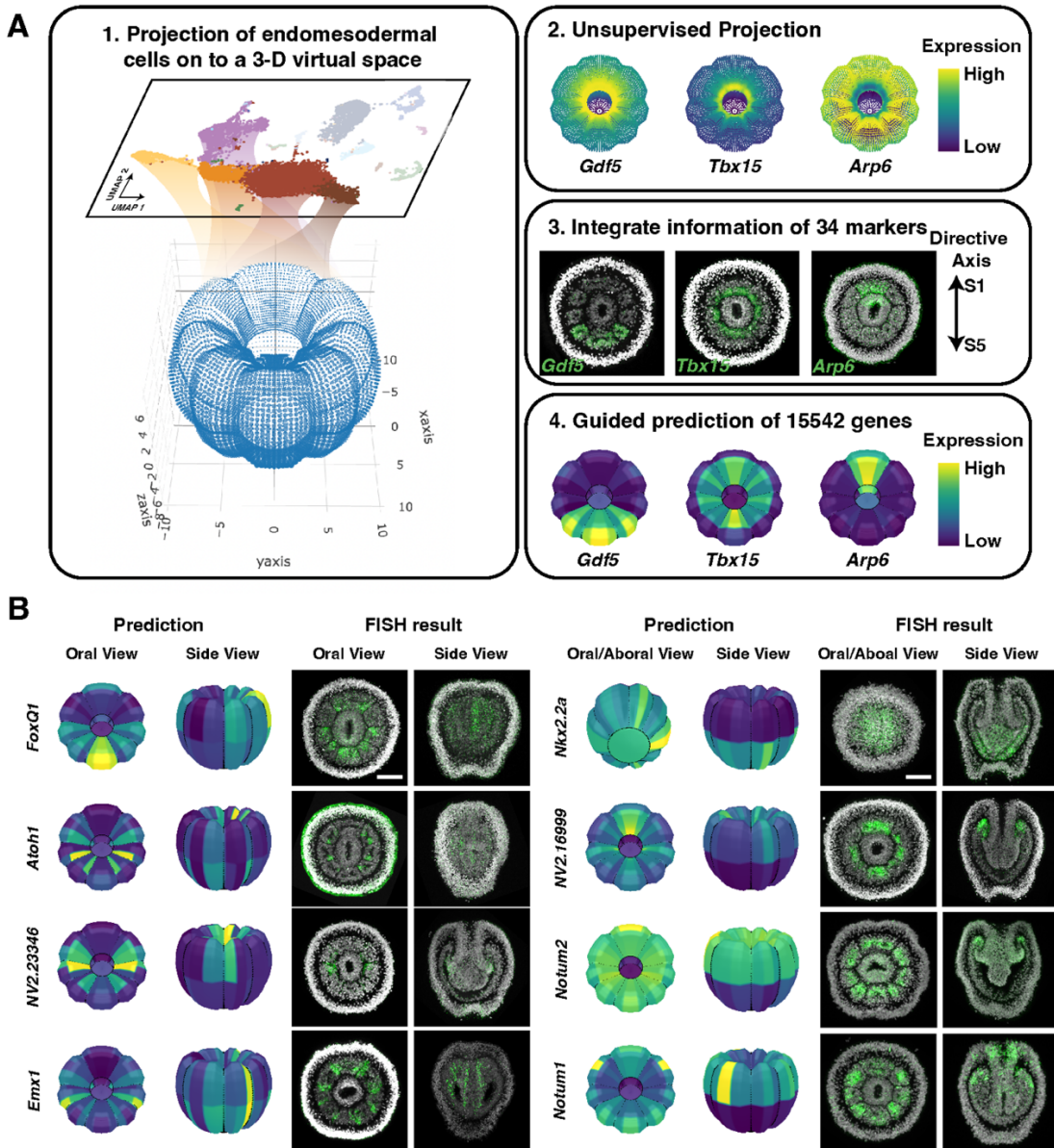
461  
 462 **Figure 1 Molecular characterization of the developing *Nematostella* endomesoderm.** (A)  
 463 Schematic illustration of the *Nematostella* life cycle. At the polyp stage, certain endomesodermal  
 464 cell types such as retractor muscle (*magenta*) and primordial germ cells (*dark blue*) are patterned  
 465 in a segmentally polarized manner along the directive axis. (B) Fluorescent *in situ* hybridization  
 466 showing the expression of *MHC-st*, a molecular marker for retractor muscles, at planula and polyp  
 467 stages. The segmentally polarized expression of *MHC-st* is evident in the planula at 72hpf. Scale  
 468 bars, 50µm. (C and D) F-actin and nuclear staining of 72hpf planula larvae before and after  
 469 removal of the ectoderm. Tissue morphology was preserved during the process as all eight  
 470 segments are clearly visible in endomesodermal isolates (S1 to S8). (E) UMAP projection of single  
 471 cell RNA-seq data from endomesodermal isolates showing the different cell types identified. (F)  
 472 Bubble plot illustrating the expression of homologs of bilaterian germ-layer and cell-type markers  
 473 in different *Nematostella* cell types. Dendrogram in the bottom is based on the transcriptional  
 474 similarities between different clusters. ME, myogenic endomesoderm; E, undifferentiated

475 endomesoderm; NE, neurogenic endomesoderm; N1, neuron cluster 1; N2, neuron cluster 2; PE,  
476 pharyngeal ectoderm; PG, pharyngeal gland cells.



478 **Figure S1 Annotating cell clusters using known and novel marker genes, related to Figure 1. (A)**  
479 UMAP projection showing all cell clusters identified from scRNA-seq of the endomesoderm  
480 isolates. **(B)** Top 10 markers for each cluster, identified by Seurat. Gene names of a subset of  
481 markers was listed on the left. **(C to G)** Fluorescent *in situ* hybridization and single cell feature  
482 plots confirming the expression patterns of different cluster markers. **(C)** Non-pharyngeal  
483 ectodermal markers such as *Anthox1* and *Dlx* were barely detected in the dataset, indicating the  
484 removal of the majority of ectodermal tissue through the isolation process. Scale bar, 50µm. **(H**  
485 **and I)** Fluorescent *in situ* hybridization and single cell feature plots confirming the expression  
486 patterns of different cell type markers in the endomesoderm. Scale bars, 50µm.

487



488

489 **Figure 2 Construction of a 3D gene expression atlas in the developing *Nematostella***

490 **endomesoderm. (A) Workflow of Endo-atlas construction. From scRNA-seq data, cells of**

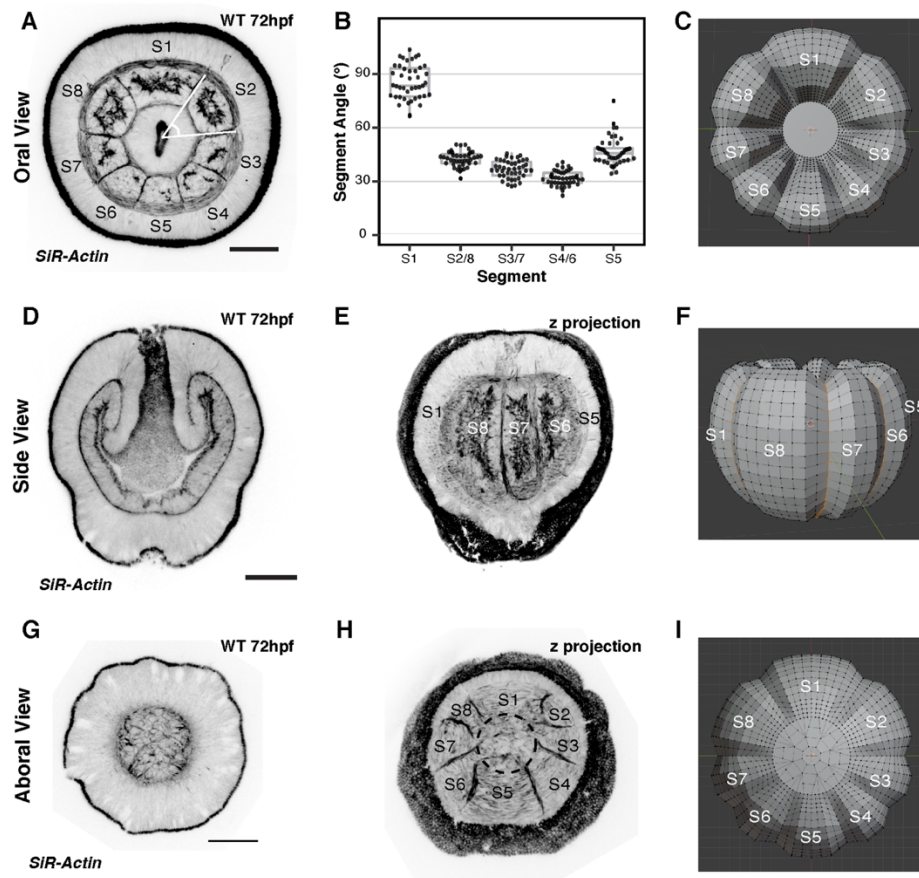
491 **endomesodermal origin were projected on to a 3D virtual space that was based on the**

492 **morphology of 72hpf planulae using novoSpaRc. A total of 34 landmark genes with distinct**

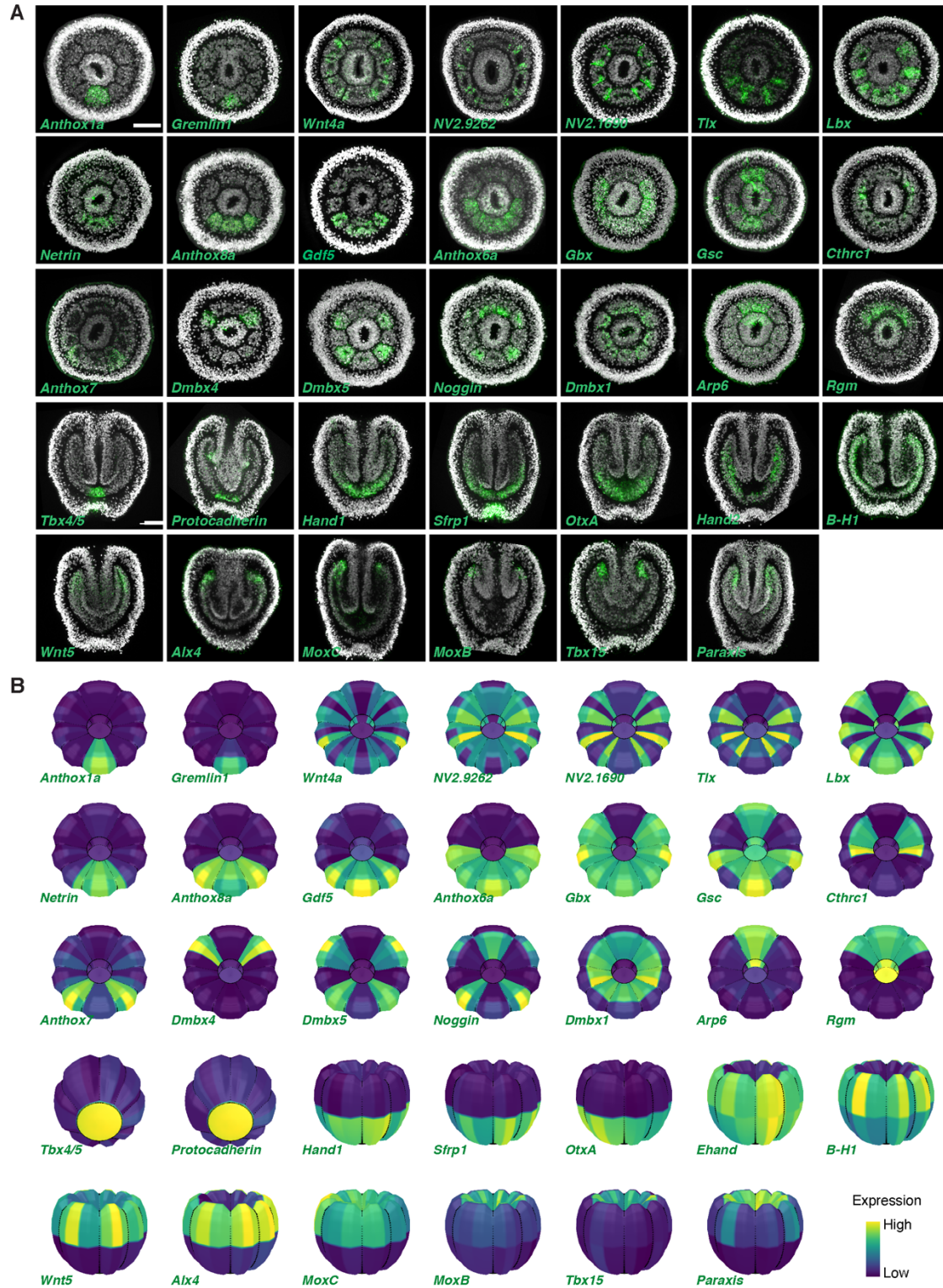
493 **expression patterns were selected based on previous literature. Integrating binarized landmark**

494 **gene expression patterns into novoSpaRc significantly increased the prediction accuracy. (B)**

495 **Validation of in silico predictions of novel genes using FISH. Scale bar, 50µm.**

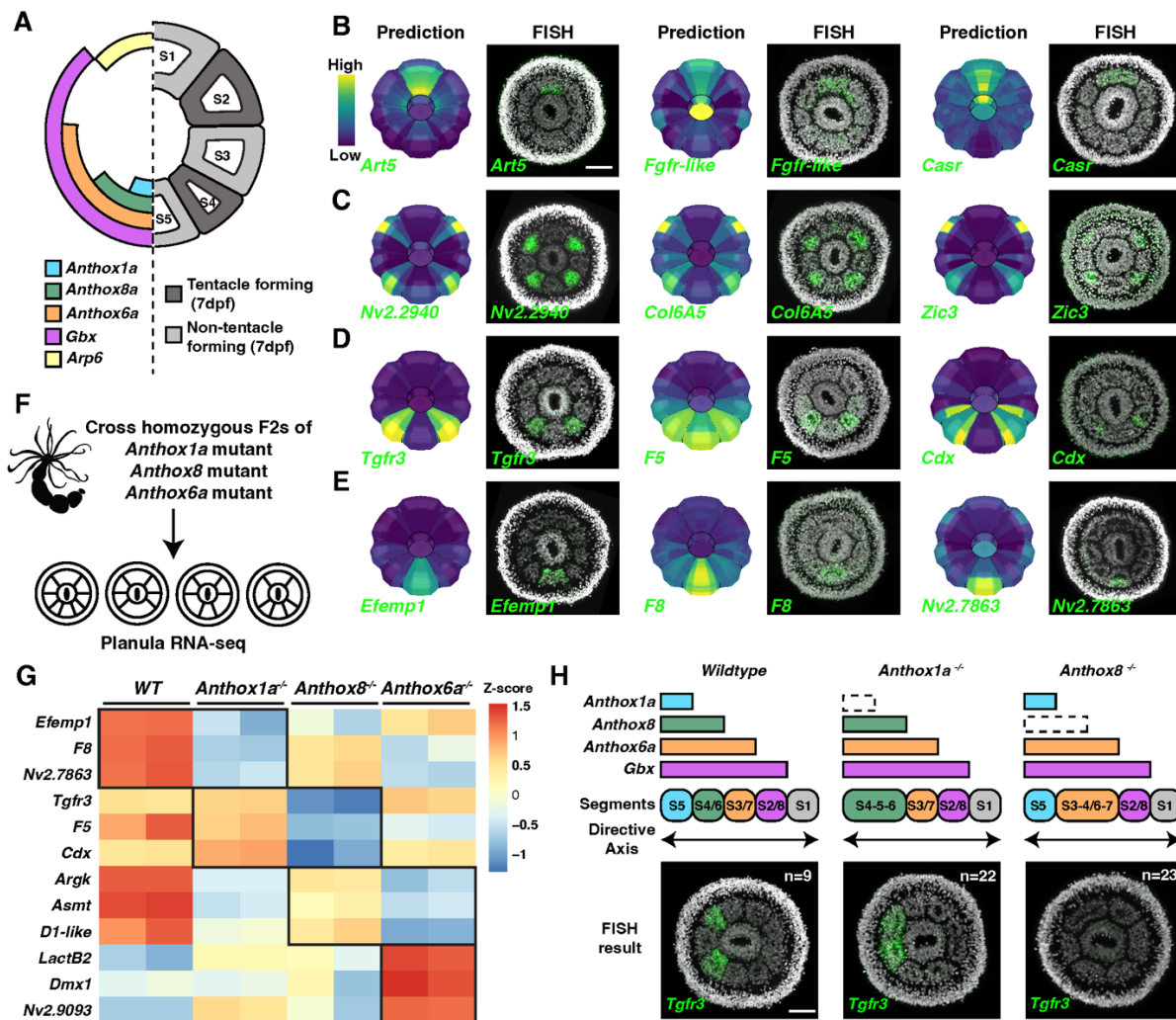


496 **Figure S2 Generating a 3-D model of *Nematostella* endomesoderm based on the morphology**  
497 **and geometry of the tissue, related to Figure 2. (A to C) Oral view comparison of the actual**  
498 **embryo and the model. Size of each segment in the model was determined based on the angle**  
499 **measurements in real larvae. (D to F) Side view comparison of the actual embryo and the model.**  
500 **Most of the endomesoderm as is completely segmented at this time point as segment boundaries**  
501 **are visible along the oral-aboral axis. (G to I) Aboral view comparison of the actual embryo and**  
502 **the model. The aboral most endomesoderm remains unsegmented at 72hpf, which is also**  
503 **depicted in the model. Scale bars, 50µm.**





505 **Figure S3 Expression patterns and Endo-atlas predictions of all 34 landmark genes used in this**  
506 **study, related to Figure 2. (A)** FISH results of landmark genes with distinct patterns in the  
507 developing endomesoderm. Scale bars, 50 $\mu$ m. **(B)** Endo-atlas predictions of the same 34 genes.  
508 Color scale indicates the relative expression value (yellow, high; blue, low).  
509



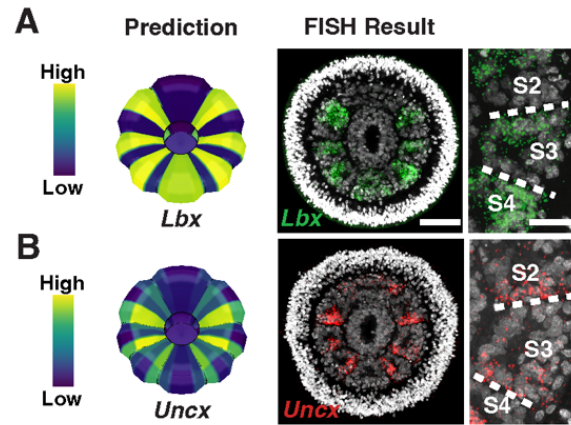
510

511 **Figure 3 Characterization of segment identity genes in *Nematostella*.** (A) Schematic of the  
 512 endomesoderm demonstrating the Hox-Gbx molecular hierarchy underlying segment identity  
 513 establishment. (B-E) Identified segment identity genes downstream of Hox-Gbx genes. (B)  
 514 Segment S1 specific genes; (C) tentacle-bearing segment (S2/S4/S6/S8) specific genes; (D) S4/S6  
 515 specific genes; (E) Segment S5 specific genes. Color scale of *in silico* prediction indicates the  
 516 relative expression level (yellow, high; blue, low). Scale bars, 50µm. (F) Experimental design of  
 517 bulk RNA-seq under *Anthox1a*, *Anthox8a* and *Anthox6a* homozygous mutant backgrounds. (G) z-  
 518 score of identified segmental identity genes in different mutant planulae (72hpf). Boxed up

519 regions indicate expansion/retraction of segmental identities in different mutant backgrounds.

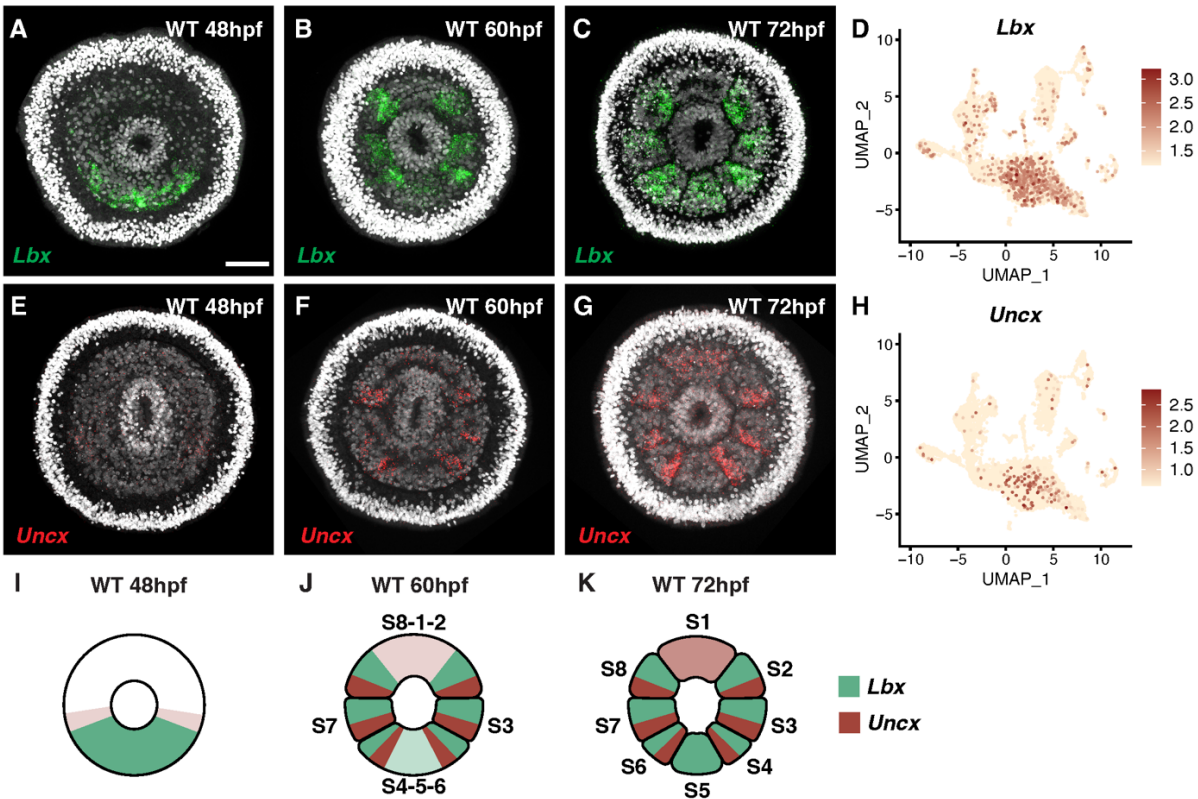
520 **(H)** *Tgfr3* expression in different Hox mutants. Scale bars, 50 $\mu$ m.

521



522 **Figure 4 Identification of a segment polarity program downstream of Hox genes in**  
523 ***Nematostella*.** (A) Endo-atlas prediction and FISH results demonstrating the polarized expression  
524 patterns of homeobox genes *Lbx* in 72hpf planula larvae. Scale bar, 50 $\mu$ m. Right panel shows the  
525 zoom in view of segment S3. Scale bar, 20  $\mu$ m. (B) Endo-atlas prediction and FISH results  
526 demonstrating the polarized expression patterns of homeobox genes *Uncx* in 72hpf planula  
527 larvae. Right panel shows the zoom in view of segment S3.

528



529

530 **Figure S4 The dynamic expression of *Lbx-Uncx* during segment formation, related to Figure 4.**

531 (A to C) *Lbx* expression in 48hpf, 60hpf and 72hpf planula larvae. Scale bar, 50  $\mu$ m. (D) Expression

532 of *Lbx* in the single cell dataset at 72hpf. (E to G) *Uncx* expression in 48hpf, 60hpf and 72hpf

533 planula larvae. (H) Expression of *Uncx* in the single cell dataset at 72hpf. (I to K) Cartoon

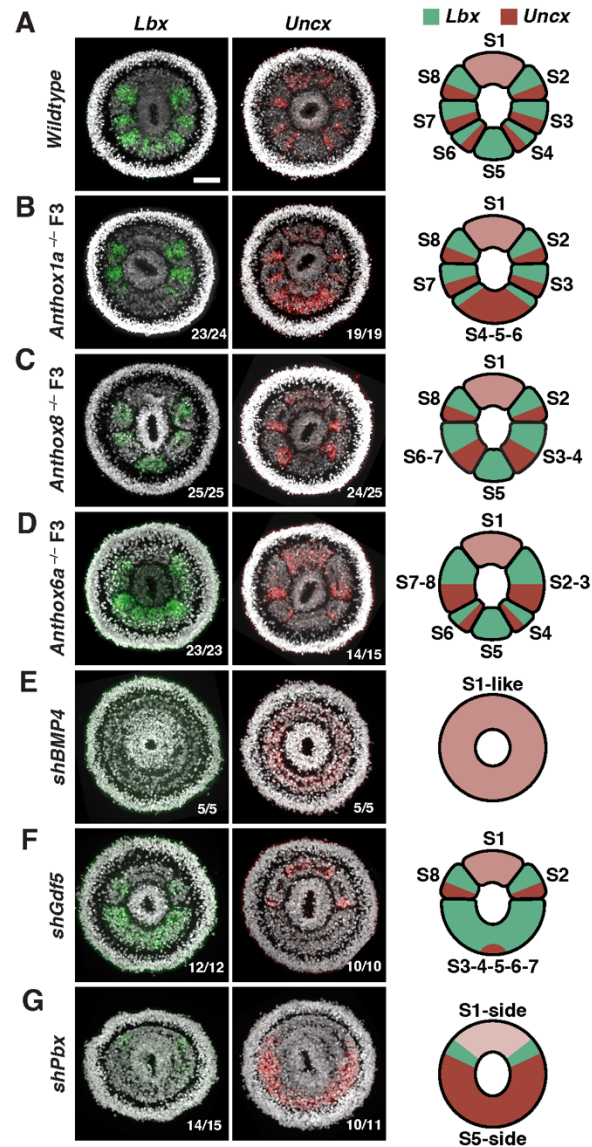
534 illustration of the temporal and spatial expression of *Lbx-Uncx*. As shown in (B and F),

535 subsegmental *Lbx* stripes flanking the future segment S1 appear prior to the formation of physical

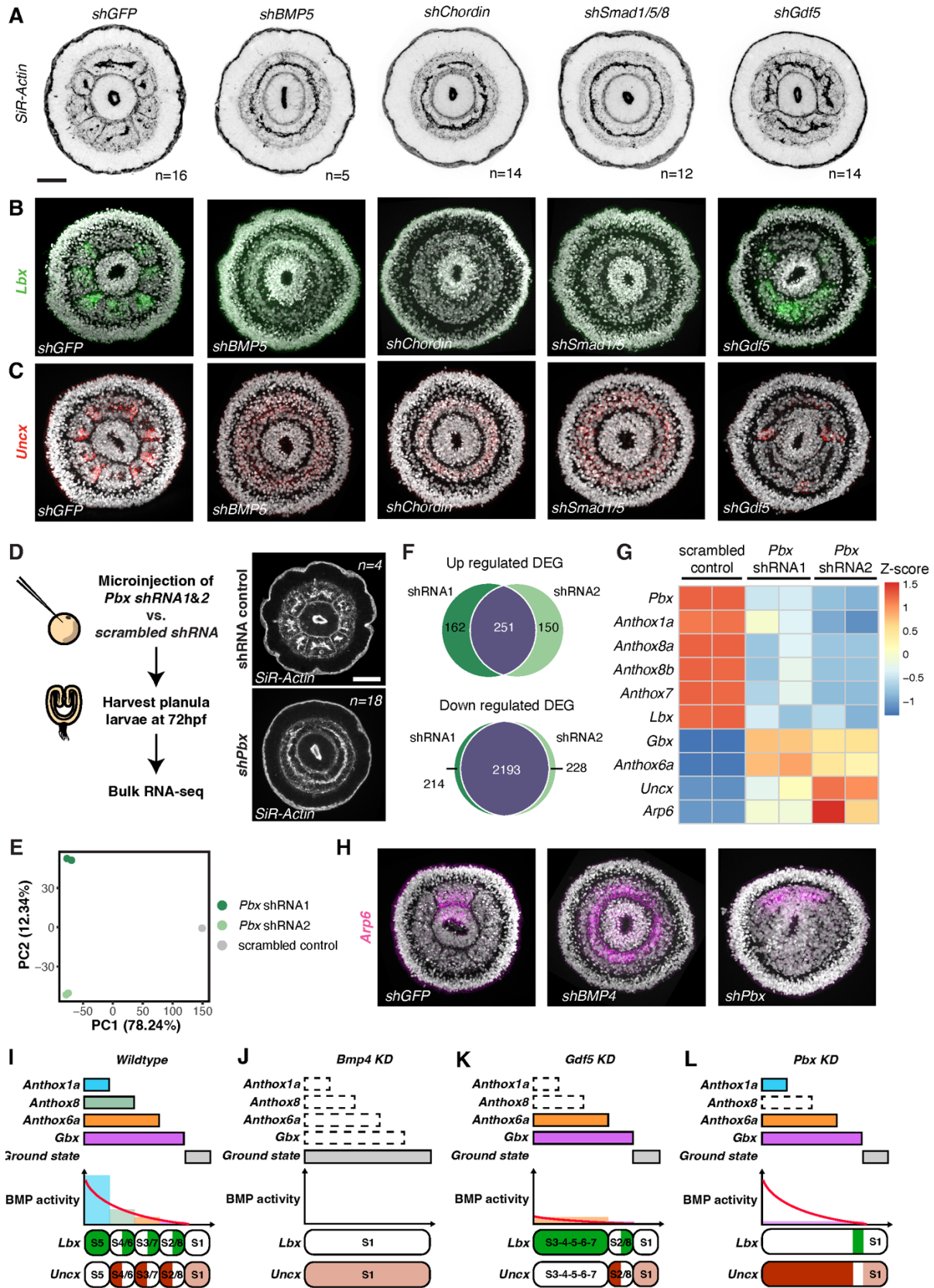
536 segment boundaries between S1/S2 and S1/S8, whereas *Uncx* stripes flanking segment S5 appear

537 prior to the formation of segment S5.

538



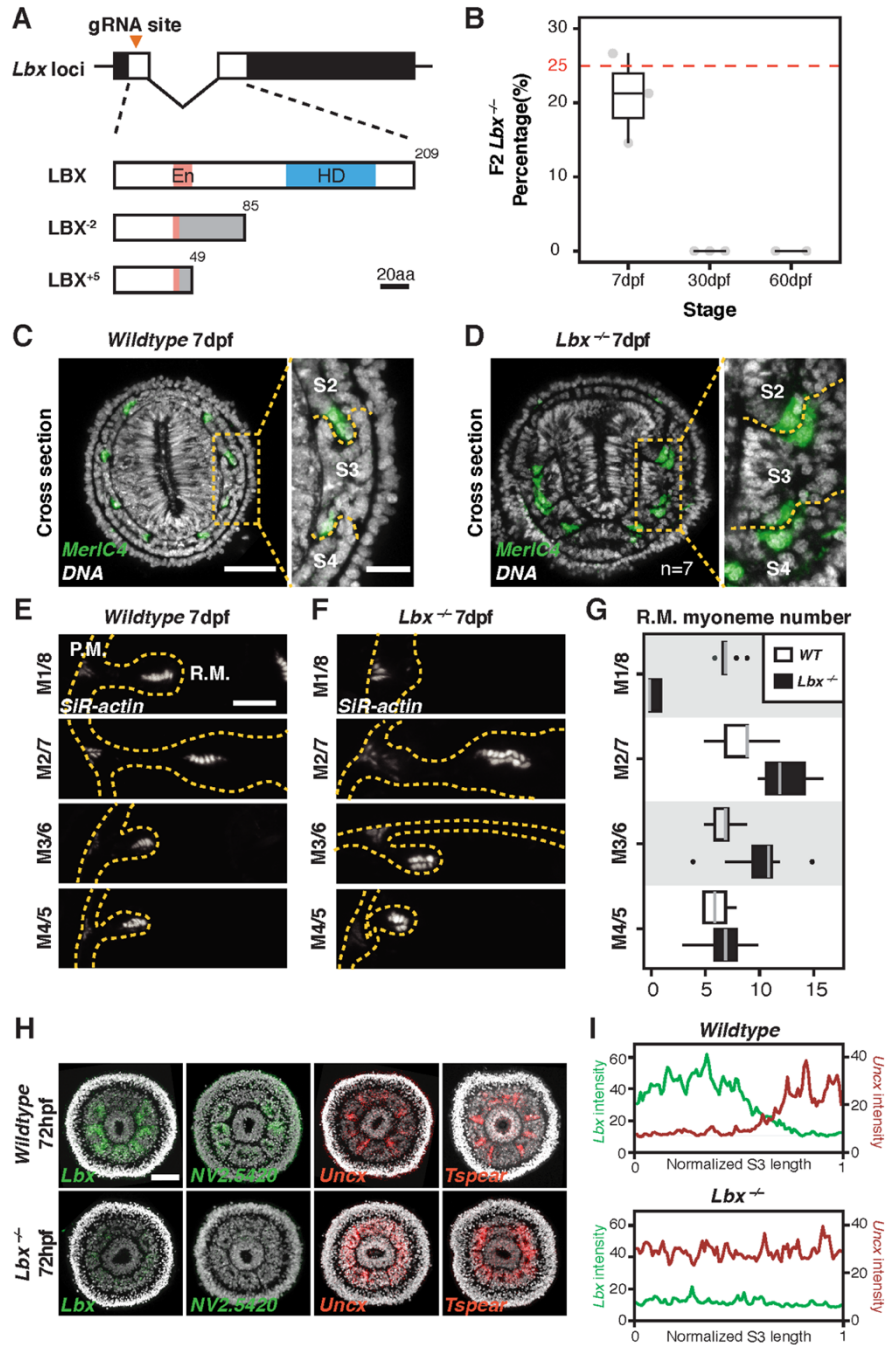
539  
 540 **Figure 5 The *Nematostella* segment polarity program is under the cooperative regulation of**  
 541 **BMP signal and Hox-Gbx genes. (A to D) *Lbx-Uncx* expression patterns under *wildtype* (A),**  
 542 ***Anthox1a<sup>-/-</sup>* (B), *Anthox8<sup>-/-</sup>* (C) and *Anthox6a<sup>-/-</sup>* (D) genetic backgrounds. (E to G) *Lbx-Uncx***  
 543 **expression patterns in animals injected with *shBMP4* (E), *shGdf5* (F) and *shPbx* (G). Embryos were**  
 544 **collected at 72hpf, and the oral views were displayed. Cartoon diagrams on the right depicts the**  
 545 **polarity patterns observed and illustrate the segment names. Numbers on the lower right corner**  
 546 **indicate the observed expression patterns in the total number of animals imaged. Scale bar,**  
 547 **50 $\mu$ m.**  
 548



550 **Figure S5 The upstream regulation of *Lbx-Uncx* polarity, related to Figure 5. (A)** The oral view of  
551 *Nematostella* larvae 72 hours after injection of *shGFP*, *shBMP5*, *shChordin*, *shSmad1/5* and  
552 *shGdf5*. Scale bar, 50µm. **(B and C)** *Lbx-Uncx* expression in animals injected with different shRNAs.  
553 **(D)** Experimental design to evaluate the global transcriptomic changes induced by *Pbx*  
554 knockdown. Two independent shRNAs targeting *Pbx* were used. Side panels show F-actin staining  
555 of 72hpf planulae injected with scrambled control shRNA and *shPbx*, respectively. Scale bar,  
556 50µm. **(E)** Principal Component Analysis of the RNA-seq results. **(F)** Venn diagrams comparing  
557 significantly up and down regulated genes for each shRNA targeting *Pbx*. **(G)** Heatmap of Z-score  
558 of homeobox containing genes under different shRNA injection groups. *Lbx* is down regulated  
559 after *Pbx* knockdown while *Uncx* is up regulated. **(H)** FISH results showing the expression of  
560 segment S1 marker *Arp6* in control, *shBMP4* and *shPbx* injected planulae. Scale bars, 50µm. **(I to**  
561 **L)** Cartoon illustrations of the regulatory logic upstream of the segment polarity program in  
562 *wildtype*, *BMP4* KD, *Gdf5* KD and *Pbx* KD conditions.

563



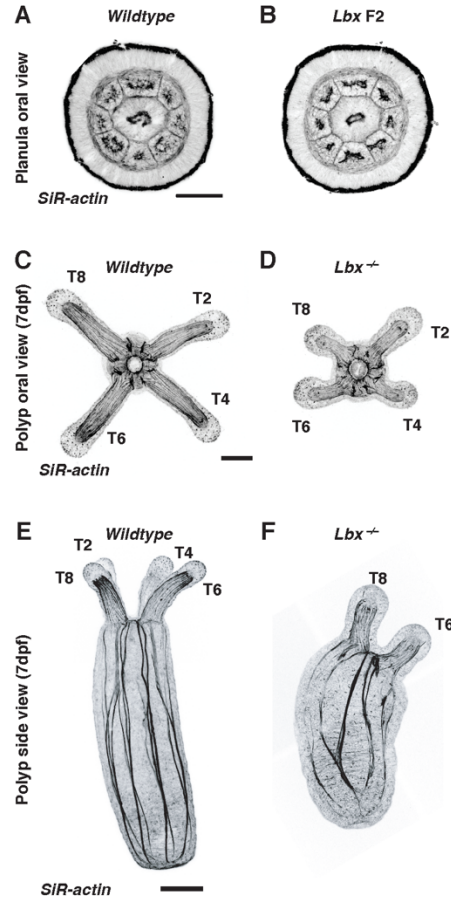


564 **Figure 6** *Lbx* Loss of function resulted in disrupted segment polarity. (A) A single guide RNA was  
 565 selected for CRISPR mutagenesis to generate truncated forms of LBX in two different mutant  
 566 alleles. (B) Quantification of genotypes at 7dpf, 30dpf and 60dpf from F1 heterozygous crosses.  
 567 (C and D) Cross section view of *wildtype* (C) and *Lbx* mutant (D) polyps at 7dpf. Retractor muscle  
 568 cells were labeled with probes against *myosin essential light chain 4* (*Melc4*). Zoomed in images  
 569 showing retractor muscles at the junctions between segment S2, S3 and S4. Scale bars, 50µm for

570 cross section, 15 $\mu$ m for zoomed in images. **(E and F)** F-actin staining showing myoneme  
571 arrangement of retractor muscles residing in different mesenteries between *wildtype* (E) and *Lbx*  
572 mutant (F) polyps at 7dpf. Scale bar, 10 $\mu$ m. **(G)** Quantification of retractor muscle myoneme  
573 numbers between *Lbx* mutant and *wildtype* polyps. **(H)** Expression of segment polarity genes in  
574 *wildtype* and *Lbx* mutant planula larvae at 72hpf. Scale bar, 50 $\mu$ m. **(I)** Quantification of *Lbx-Uncx*  
575 FISH signal along the normalized length of segment S3 in *wildtype* and *Lbx* mutant planula larvae  
576 at 72hpf.

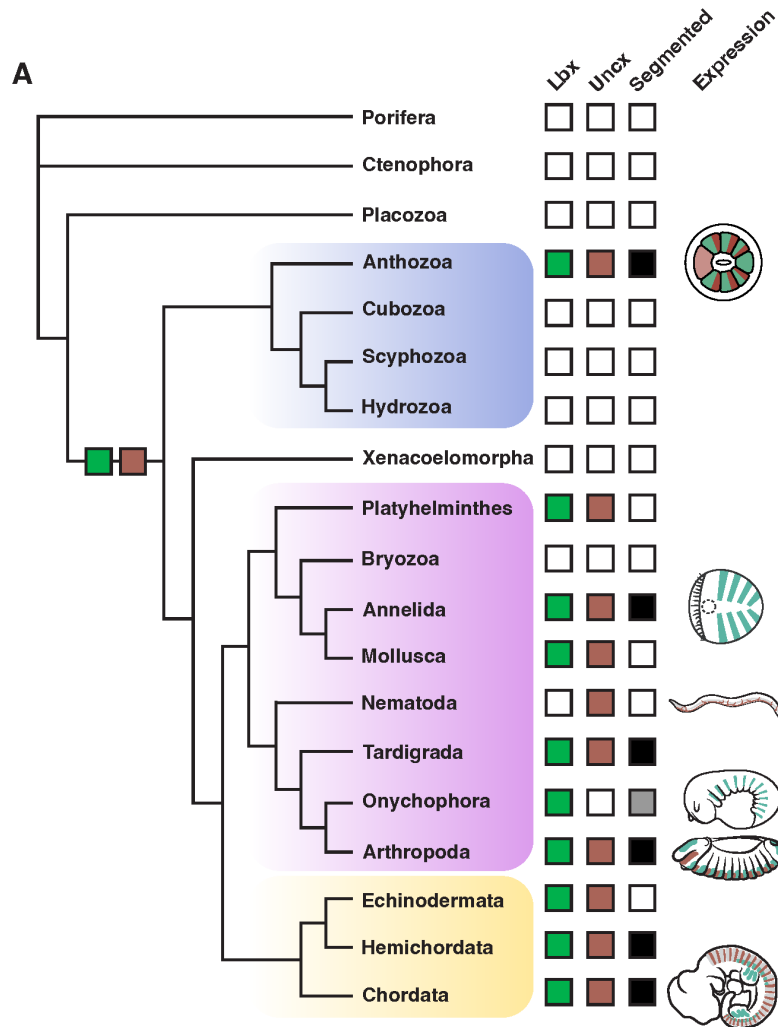
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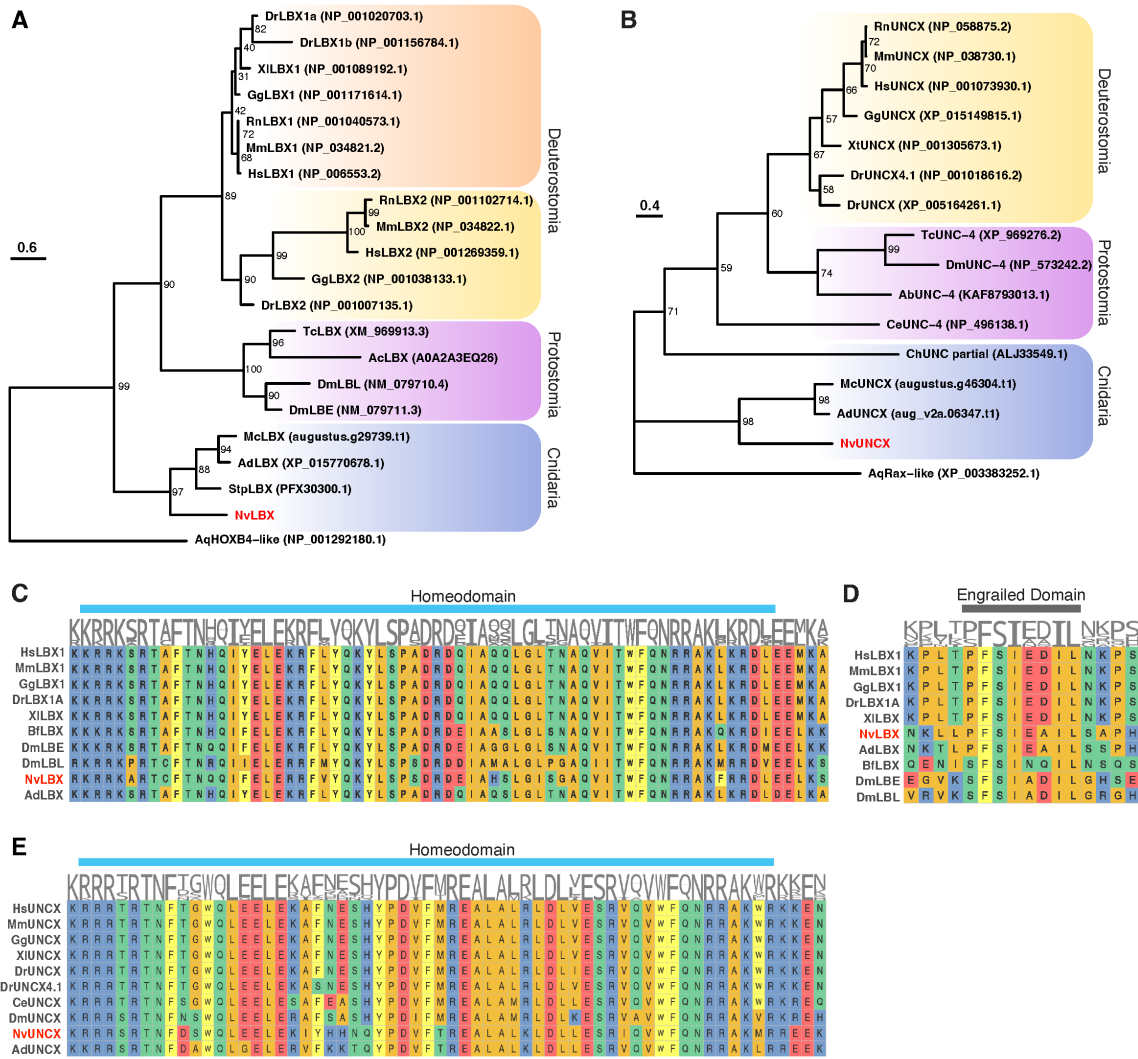


579 **Figure S6 Morphological analysis of *Lbx*<sup>-/-</sup> mutants, related to Figure 6. (A-B)** Planula stage  
580 morphological comparison between *Lbx* homozygous mutants and their *wildtype* siblings. Scale  
581 bars, 50µm. (C-D) Polyp stage morphological comparison between *Lbx* homozygous mutants and  
582 their *wildtype* siblings. Scale bars, 100µm.

583



584 **Figure 7 Evolution of the segmental polarity program.** Phylogenetic tree depicting the  
 585 evolutionary relationships between major metazoan phyla and the presence or absence of  
 586 segmented body plans and *Lbx-Uncx* genes in the respected genomes. The *Lbx-Uncx* expression  
 587 patterns of representative species were adapted from previous publications.



588

589 **Figure S7 *Nematostella* LBX and UNCX are conserved homeodomain-containing proteins,**  
 590 **related to Figure 7. (A)** Maximum-likelihood phylogenetic tree of LBX protein. **(B)** Maximum-  
 591 likelihood phylogenetic tree of UNCX protein. **(C)** Sequence alignment of the homeodomain of LBX  
 592 proteins from different species. **(D)** Sequence alignment of the engrailed domain of LBX proteins  
 593 from different species. **(E)** Sequence alignment of the homeodomain of UNCX proteins from  
 594 different species.

595 **REFERENCES AND NOTES**

- 596 Achim, K., Pettit, J.B., Saraiva, L.R., Gavriouchkina, D., Larsson, T., Arendt, D., and Marioni, J.C.  
597 (2015). High-throughput spatial mapping of single-cell RNA-seq data to tissue of origin. *Nat*  
598 *Biotechnol* **33**, 503-509. 10.1038/nbt.3209.
- 599 Bateson, W. (1894). *Materials for the study of variation treated with especial regard to*  
600 *discontinuity in the origin of species* (Macmillan and Company).
- 601 Benazeraf, B., and Pourquie, O. (2013). Formation and segmentation of the vertebrate body axis.  
602 *Annu Rev Cell Dev Biol* **29**, 1-26. 10.1146/annurev-cellbio-101011-155703.
- 603 Berking, S. (2007). Generation of bilateral symmetry in Anthozoa: a model. *J Theor Biol* **246**, 477-  
604 490. 10.1016/j.jtbi.2007.01.008.
- 605 Burtscher, I., and Lickert, H. (2009). Foxa2 regulates polarity and epithelialization in the  
606 endoderm germ layer of the mouse embryo. *Development* **136**, 1029-1038. 10.1242/dev.028415.
- 607 Chen, J.A., Voigt, J., Gilchrist, M., Papalopulu, N., and Amaya, E. (2005). Identification of novel  
608 genes affecting mesoderm formation and morphogenesis through an enhanced large scale  
609 functional screen in *Xenopus*. *Mech Dev* **122**, 307-331. 10.1016/j.mod.2004.11.008.
- 610 Cho, S.J., and Park, S.C. (2008). Paired-like subclass homeobox genes from the clitellate annelid  
611 *Perionyx excavatus*. *Biochem Genet* **46**, 737-743. 10.1007/s10528-008-9189-z.
- 612 Chourrout, D., Delsuc, F., Chourrout, P., Edvardsen, R.B., Rentzsch, F., Renfer, E., Jensen, M.F.,  
613 Zhu, B., de Jong, P., Steele, R.E., and Technau, U. (2006). Minimal ProtoHox cluster inferred from  
614 bilaterian and cnidarian Hox complements. *Nature* **442**, 684-687. 10.1038/nature04863.
- 615 Christ, B., Schmidt, C., Huang, R., Wilting, J., and Brand-Saberi, B. (1998). Segmentation of the  
616 vertebrate body. *Anat Embryol (Berl)* **197**, 1-8. 10.1007/s004290050116.
- 617 De Graeve, F., Jagla, T., Daponte, J.P., Rickert, C., Dastugue, B., Urban, J., and Jagla, K. (2004). The  
618 ladybird homeobox genes are essential for the specification of a subpopulation of neural cells.  
619 *Dev Biol* **270**, 122-134. 10.1016/j.ydbio.2004.02.014.
- 620 Deng, M., Wang, Y., Zhang, L., Yang, Y., Huang, S., Wang, J., Ge, H., Ishibashi, T., and Yan, Y. (2019).  
621 Single cell transcriptomic landscapes of pattern formation, proliferation and growth in *Drosophila*  
622 wing imaginal discs. *Development* **146**. 10.1242/dev.179754.
- 623 Diaz-Cuadros, M., Pourquie, O., and El-Sherif, E. (2021). Patterning with clocks and genetic  
624 cascades: Segmentation and regionalization of vertebrate versus insect body plans. *PLoS Genet*  
625 **17**, e1009812. 10.1371/journal.pgen.1009812.
- 626 Donovan, K.M., Leidinger, M.R., McQuillen, L.P., Goeken, J.A., Hogan, C.M., Harwani, S.C.,  
627 Flaherty, H.A., and Meyerholz, D.K. (2018). Allograft Inflammatory Factor 1 as an  
628 Immunohistochemical Marker for Macrophages in Multiple Tissues and Laboratory Animal  
629 Species. *Comp Med* **68**, 341-348. 10.30802/AALAS-CM-18-000017.
- 630 Dray, N., Tessmar-Raible, K., Le Gouar, M., Vibert, L., Christodoulou, F., Schipany, K., Guillou, A.,  
631 Zantke, J., Snyman, H., Behague, J., et al. (2010). Hedgehog signaling regulates segment formation  
632 in the annelid *Platynereis*. *Science* **329**, 339-342. 10.1126/science.1188913.

- 633 Dries, R., Chen, J., Del Rossi, N., Khan, M.M., Sistig, A., and Yuan, G.C. (2021). Advances in spatial  
634 transcriptomic data analysis. *Genome Res* 31, 1706-1718. 10.1101/gr.275224.121.
- 635 Fritzenwanker, J.H., Saina, M., and Technau, U. (2004). Analysis of forkhead and snail expression  
636 reveals epithelial-mesenchymal transitions during embryonic and larval development of  
637 *Nematostella vectensis*. *Dev Biol* 275, 389-402. 10.1016/j.ydbio.2004.08.014.
- 638 Genikhovich, G., Fried, P., Prunster, M.M., Schinko, J.B., Gilles, A.F., Fredman, D., Meier, K., Iber,  
639 D., and Technau, U. (2015). Axis Patterning by BMPs: Cnidarian Network Reveals Evolutionary  
640 Constraints. *Cell Rep* 10, 1646-1654. 10.1016/j.celrep.2015.02.035.
- 641 He, S., Del Viso, F., Chen, C.Y., Ikmi, A., Kroesen, A.E., and Gibson, M.C. (2018). An axial Hox code  
642 controls tissue segmentation and body patterning in *Nematostella vectensis*. *Science* 361, 1377-  
643 1380. 10.1126/science.aar8384.
- 644 Hudry, B., Thomas-Chollier, M., Volovik, Y., Duffraisse, M., Dard, A., Frank, D., Technau, U., and  
645 Merabet, S. (2014). Molecular insights into the origin of the Hox-TALE patterning system. *Elife* 3,  
646 e01939. 10.7554/eLife.01939.
- 647 Hyman, L.H. (1940). *The invertebrates: Protozoa through Ctenophora vol. 1 (The McGraw-Hill*  
648 *Companies)*.
- 649 Ikmi, A., and Gibson, M.C. (2010). Identification and in vivo characterization of NvFP-7R, a  
650 developmentally regulated red fluorescent protein of *Nematostella vectensis*. *PLoS One* 5,  
651 e11807. 10.1371/journal.pone.0011807.
- 652 Ikmi, A., Steenbergen, P.J., Anzo, M., McMullen, M.R., Stokkermans, A., Ellington, L.R., and Gibson,  
653 M.C. (2020). Feeding-dependent tentacle development in the sea anemone *Nematostella*  
654 *vectensis*. *Nat Commun* 11, 4399. 10.1038/s41467-020-18133-0.
- 655 Jagla, K., Dolle, P., Mattei, M.G., Jagla, T., Schuhbaur, B., Dretzen, G., Bellard, F., and Bellard, M.  
656 (1995). Mouse *Lbx1* and human *LBX1* define a novel mammalian homeobox gene family related  
657 to the *Drosophila* ladybird genes. *Mech Dev* 53, 345-356. 10.1016/0925-4773(95)00450-5.
- 658 Jagla, K., Jagla, T., Heitzler, P., Dretzen, G., Bellard, F., and Bellard, M. (1997). ladybird, a tandem  
659 of homeobox genes that maintain late wingless expression in terminal and dorsal epidermis of  
660 the *Drosophila* embryo. *Development* 124, 91-100. 10.1242/dev.124.1.91.
- 661 Juarez-Morales, J.L., Weierud, F., England, S.J., Demby, C., Santos, N., Grieb, G., Mazan, S., and  
662 Lewis, K.E. (2021). Evolution of *lbx* spinal cord expression and function. *Evol Dev* 23, 404-422.  
663 10.1111/ede.12387.
- 664 Kamm, K., Schierwater, B., Jakob, W., Dellaporta, S.L., and Miller, D.J. (2006). Axial patterning and  
665 diversification in the cnidaria predate the Hox system. *Curr Biol* 16, 920-926.  
666 10.1016/j.cub.2006.03.036.
- 667 Karaiskos, N., Wahle, P., Alles, J., Boltengagen, A., Ayoub, S., Kipar, C., Kocks, C., Rajewsky, N.,  
668 and Zinzen, R.P. (2017). The *Drosophila* embryo at single-cell transcriptome resolution. *Science*  
669 358, 194-199. 10.1126/science.aan3235.

- 670 Knabl, P., Schauer, A., Pomreinke, A.P., Zimmermann, B., Rogers, K.W., Müller, P., and  
671 Genikhovich, G. (2022). Analysis of SMAD1/5 target genes in a sea anemone reveals ZSWIM4-6  
672 as a novel BMP signaling modulator. *bioRxiv*, 2022.2006.2003.494682.  
673 10.1101/2022.06.03.494682.
- 674 Koch, T.L., and Grimmelikhuijzen, C.J.P. (2019). Global Neuropeptide Annotations From the  
675 Genomes and Transcriptomes of Cubozoa, Scyphozoa, Staurozoa (Cnidaria: Medusozoa), and  
676 Octocorallia (Cnidaria: Anthozoa). *Front Endocrinol (Lausanne)* 10, 831.  
677 10.3389/fendo.2019.00831.
- 678 Kraus, Y., Aman, A., Technau, U., and Genikhovich, G. (2016). Pre-bilaterian origin of the  
679 blastoporal axial organizer. *Nat Commun* 7, 11694. 10.1038/ncomms11694.
- 680 Kusakabe, R., Higuchi, S., Tanaka, M., Kadota, M., Nishimura, O., and Kuratani, S. (2020). Novel  
681 developmental bases for the evolution of hypobranchial muscles in vertebrates. *BMC Biol* 18, 120.  
682 10.1186/s12915-020-00851-y.
- 683 Lacin, H., Williamson, W.R., Card, G.M., Skeath, J.B., and Truman, J.W. (2020). Unc-4 acts to  
684 promote neuronal identity and development of the take-off circuit in the *Drosophila* CNS. *Elife* 9.  
685 10.7554/eLife.55007.
- 686 Leclere, L., and Rentzsch, F. (2014). RGM regulates BMP-mediated secondary axis formation in  
687 the sea anemone *Nematostella vectensis*. *Cell Rep* 9, 1921-1930. 10.1016/j.celrep.2014.11.009.
- 688 Leitges, M., Neidhardt, L., Haenig, B., Herrmann, B.G., and Kispert, A. (2000). The paired  
689 homeobox gene *Uncx4.1* specifies pedicles, transverse processes and proximal ribs of the  
690 vertebral column. *Development* 127, 2259-2267. 10.1242/dev.127.11.2259.
- 691 Lohoff, T., Ghazanfar, S., Missarova, A., Koulena, N., Pierson, N., Griffiths, J.A., Bardot, E.S., Eng,  
692 C.L., Tyser, R.C.V., Argelaguet, R., et al. (2022). Integration of spatial and single-cell transcriptomic  
693 data elucidates mouse organogenesis. *Nat Biotechnol* 40, 74-85. 10.1038/s41587-021-01006-2.
- 694 Mankoo, B.S., Skuntz, S., Harrigan, I., Grigorieva, E., Candia, A., Wright, C.V., Arnheiter, H., and  
695 Pachnis, V. (2003). The concerted action of *Meox* homeobox genes is required upstream of  
696 genetic pathways essential for the formation, patterning and differentiation of somites.  
697 *Development* 130, 4655-4664. 10.1242/dev.00687.
- 698 Mansouri, A., Voss, A.K., Thomas, T., Yokota, Y., and Gruss, P. (2000). *Uncx4.1* is required for the  
699 formation of the pedicles and proximal ribs and acts upstream of *Pax9*. *Development* 127, 2251-  
700 2258. 10.1242/dev.127.11.2251.
- 701 Martindale, M.Q., Pang, K., and Finnerty, J.R. (2004). Investigating the origins of triploblasty:  
702 'mesodermal' gene expression in a diploblastic animal, the sea anemone *Nematostella vectensis*  
703 (phylum, Cnidaria; class, Anthozoa). *Development* 131, 2463-2474. 10.1242/dev.01119.
- 704 Martinez Barbera, J.P., Clements, M., Thomas, P., Rodriguez, T., Meloy, D., Kioussis, D., and  
705 Beddington, R.S. (2000). The homeobox gene *Hex* is required in definitive endodermal tissues for  
706 normal forebrain, liver and thyroid formation. *Development* 127, 2433-2445.  
707 10.1242/dev.127.11.2433.



- 708 Marx, V. (2021). Method of the Year: spatially resolved transcriptomics. *Nat Methods* 18, 9-14.  
709 10.1038/s41592-020-01033-y.
- 710 Miller, D.M., Shen, M.M., Shamu, C.E., Burglin, T.R., Ruvkun, G., Dubois, M.L., Ghee, M., and  
711 Wilson, L. (1992). *C. elegans unc-4* gene encodes a homeodomain protein that determines the  
712 pattern of synaptic input to specific motor neurons. *Nature* 355, 841-845. 10.1038/355841a0.
- 713 Miyasaka, K.Y., Kida, Y.S., Sato, T., Minami, M., and Ogura, T. (2007). *Csrp1* regulates dynamic cell  
714 movements of the mesendoderm and cardiac mesoderm through interactions with Dishevelled  
715 and Diversin. *Proc Natl Acad Sci U S A* 104, 11274-11279. 10.1073/pnas.0702000104.
- 716 Moriel, N., Senel, E., Friedman, N., Rajewsky, N., Karaikos, N., and Nitzan, M. (2021). NovoSpaRc:  
717 flexible spatial reconstruction of single-cell gene expression with optimal transport. *Nat Protoc*  
718 16, 4177-4200. 10.1038/s41596-021-00573-7.
- 719 Neidhardt, L.M., Kispert, A., and Herrmann, B.G. (1997). A mouse gene of the paired-related  
720 homeobox class expressed in the caudal somite compartment and in the developing vertebral  
721 column, kidney and nervous system. *Dev Genes Evol* 207, 330-339. 10.1007/s004270050120.
- 722 Nguyen, H.T., Bodmer, R., Abmayr, S.M., McDermott, J.C., and Spoerel, N.A. (1994). *D-mef2*: a  
723 *Drosophila* mesoderm-specific MADS box-containing gene with a biphasic expression profile  
724 during embryogenesis. *Proc Natl Acad Sci U S A* 91, 7520-7524. 10.1073/pnas.91.16.7520.
- 725 Nitzan, M., Karaikos, N., Friedman, N., and Rajewsky, N. (2019). Gene expression cartography.  
726 *Nature* 576, 132-137. 10.1038/s41586-019-1773-3.
- 727 Noden, D.M., Marcucio, R., Borycki, A.G., and Emerson, C.P., Jr. (1999). Differentiation of avian  
728 craniofacial muscles: I. Patterns of early regulatory gene expression and myosin heavy chain  
729 synthesis. *Dev Dyn* 216, 96-112. 10.1002/(SICI)1097-0177(199910)216:2<96::AID-  
730 DVDY2>3.0.CO;2-6.
- 731 Ochi, H., and Westerfield, M. (2009). *Lbx2* regulates formation of myofibrils. *BMC Dev Biol* 9, 13.  
732 10.1186/1471-213X-9-13.
- 733 Onai, T., Irie, N., and Kuratani, S. (2014). The evolutionary origin of the vertebrate body plan: the  
734 problem of head segmentation. *Annu Rev Genomics Hum Genet* 15, 443-459. 10.1146/annurev-  
735 genom-091212-153404.
- 736 Pax, F. (1913). *Die Actinien* (G. Plaetzsche Buchdruckerei Lippert & Company).
- 737 Pedersen, J.K., Nelson, S.B., Jorgensen, M.C., Henseleit, K.D., Fujitani, Y., Wright, C.V., Sander, M.,  
738 Serup, P., and Beta Cell Biology, C. (2005). Endodermal expression of *Nkx6* genes depends  
739 differentially on *Pdx1*. *Dev Biol* 288, 487-501. 10.1016/j.ydbio.2005.10.001.
- 740 Pourquoi, O. (2000). Segmentation of the paraxial mesoderm and vertebrate somitogenesis. *Curr*  
741 *Top Dev Biol* 47, 81-105. 10.1016/s0070-2153(08)60722-x.
- 742 Rentsch, F., Anton, R., Saina, M., Hammerschmidt, M., Holstein, T.W., and Technau, U. (2006).  
743 Asymmetric expression of the BMP antagonists *chordin* and *gremlin* in the sea anemone  
744 *Nematostella vectensis*: implications for the evolution of axial patterning. *Dev Biol* 296, 375-387.  
745 10.1016/j.ydbio.2006.06.003.

- 746 Rodriques, S.G., Stickels, R.R., Goeva, A., Martin, C.A., Murray, E., Vanderburg, C.R., Welch, J.,  
747 Chen, L.M., Chen, F., and Macosko, E.Z. (2019). Slide-seq: A scalable technology for measuring  
748 genome-wide expression at high spatial resolution. *Science* 363, 1463-1467.  
749 [10.1126/science.aaw1219](https://doi.org/10.1126/science.aaw1219).
- 750 Ryan, J.F., Mazza, M.E., Pang, K., Matus, D.Q., Baxevanis, A.D., Martindale, M.Q., and Finnerty,  
751 J.R. (2007). Pre-bilaterian origins of the Hox cluster and the Hox code: evidence from the sea  
752 anemone, *Nematostella vectensis*. *PLoS One* 2, e153. [10.1371/journal.pone.0000153](https://doi.org/10.1371/journal.pone.0000153).
- 753 Satija, R., Farrell, J.A., Gennert, D., Schier, A.F., and Regev, A. (2015). Spatial reconstruction of  
754 single-cell gene expression data. *Nat Biotechnol* 33, 495-502. [10.1038/nbt.3192](https://doi.org/10.1038/nbt.3192).
- 755 Saudemont, A., Dray, N., Hudry, B., Le Gouar, M., Vervoort, M., and Balavoine, G. (2008).  
756 Complementary striped expression patterns of NK homeobox genes during segment formation  
757 in the annelid *Platynereis*. *Dev Biol* 317, 430-443. [10.1016/j.ydbio.2008.02.013](https://doi.org/10.1016/j.ydbio.2008.02.013).
- 758 Schragle, J., Huang, R., Christ, B., and Prols, F. (2004). Control of the temporal and spatial *Uncx4.1*  
759 expression in the paraxial mesoderm of avian embryos. *Anat Embryol (Berl)* 208, 323-332.  
760 [10.1007/s00429-004-0404-3](https://doi.org/10.1007/s00429-004-0404-3).
- 761 Scott, M.P., and Carroll, S.B. (1987). The segmentation and homeotic gene network in early  
762 *Drosophila* development. *Cell* 51, 689-698. [10.1016/0092-8674\(87\)90092-4](https://doi.org/10.1016/0092-8674(87)90092-4).
- 763 Seaver, E.C. (2003). Segmentation: mono- or polyphyletic? *Int J Dev Biol* 47, 583-595.
- 764 Sebe-Pedros, A., Saudemont, B., Chomsky, E., Plessier, F., Mailhe, M.P., Renno, J., Loe-Mie, Y.,  
765 Lifshitz, A., Mukamel, Z., Schmutz, S., et al. (2018). Cnidarian Cell Type Diversity and Regulation  
766 Revealed by Whole-Organism Single-Cell RNA-Seq. *Cell* 173, 1520-1534 e1520.  
767 [10.1016/j.cell.2018.05.019](https://doi.org/10.1016/j.cell.2018.05.019).
- 768 Steinmetz, P.R.H. (2019). A non-bilaterian perspective on the development and evolution of  
769 animal digestive systems. *Cell Tissue Res* 377, 321-339. [10.1007/s00441-019-03075-x](https://doi.org/10.1007/s00441-019-03075-x).
- 770 Steinmetz, P.R.H., Aman, A., Kraus, J.E.M., and Technau, U. (2017). Gut-like ectodermal tissue in  
771 a sea anemone challenges germ layer homology. *Nat Ecol Evol* 1, 1535-1542. [10.1038/s41559-017-0285-5](https://doi.org/10.1038/s41559-017-0285-5).
- 772
- 773 Stickels, R.R., Murray, E., Kumar, P., Li, J., Marshall, J.L., Di Bella, D.J., Arlotta, P., Macosko, E.Z.,  
774 and Chen, F. (2021). Highly sensitive spatial transcriptomics at near-cellular resolution with Slide-  
775 seqV2. *Nat Biotechnol* 39, 313-319. [10.1038/s41587-020-0739-1](https://doi.org/10.1038/s41587-020-0739-1).
- 776 Takahashi, T. (2020). Comparative Aspects of Structure and Function of Cnidarian Neuropeptides.  
777 *Front Endocrinol (Lausanne)* 11, 339. [10.3389/fendo.2020.00339](https://doi.org/10.3389/fendo.2020.00339).
- 778 Tautz, D. (2004). Segmentation. *Dev Cell* 7, 301-312. [10.1016/j.devcel.2004.08.008](https://doi.org/10.1016/j.devcel.2004.08.008).
- 779 Technau, U. (2020). Gastrulation and germ layer formation in the sea anemone *Nematostella*  
780 *vectensis* and other cnidarians. *Mech Dev* 163, 103628. [10.1016/j.mod.2020.103628](https://doi.org/10.1016/j.mod.2020.103628).
- 781 Treffkorn, S., Kahnke, L., Hering, L., and Mayer, G. (2018). Expression of NK cluster genes in the  
782 onychophoran *Euperipatoides rowelli*: implications for the evolution of NK family genes in  
783 nephrozoans. *Evodevo* 9, 17. [10.1186/s13227-018-0105-2](https://doi.org/10.1186/s13227-018-0105-2).

784 van den Brink, S.C., Alemany, A., van Batenburg, V., Moris, N., Blotenburg, M., Vivie, J., Baillie-  
785 Johnson, P., Nichols, J., Sonnen, K.F., Martinez Arias, A., and van Oudenaarden, A. (2020). Single-  
786 cell and spatial transcriptomics reveal somitogenesis in gastruloids. *Nature* 582, 405-409.  
787 10.1038/s41586-020-2024-3.

788 Walthall, W.W. (1995). Repeating patterns of motoneurons in nematodes: the origin of  
789 segmentation? *EXS* 72, 61-75. 10.1007/978-3-0348-9219-3\_4.

790 Watanabe, H., Kuhn, A., Fushiki, M., Agata, K., Ozbek, S., Fujisawa, T., and Holstein, T.W. (2014).  
791 Sequential actions of beta-catenin and Bmp pattern the oral nerve net in *Nematostella vectensis*.  
792 *Nat Commun* 5, 5536. 10.1038/ncomms6536.

793 Wijesena, N., Simmons, D.K., and Martindale, M.Q. (2017). Antagonistic BMP-cWNT signaling in  
794 the cnidarian *Nematostella vectensis* reveals insight into the evolution of mesoderm. *Proc Natl*  
795 *Acad Sci U S A* 114, E5608-E5615. 10.1073/pnas.1701607114.

796 Wilm, B., James, R.G., Schultheiss, T.M., and Hogan, B.L. (2004). The forkhead genes, *Foxc1* and  
797 *Foxc2*, regulate paraxial versus intermediate mesoderm cell fate. *Dev Biol* 271, 176-189.  
798 10.1016/j.ydbio.2004.03.034.

799 Wotton, K.R., Weierud, F.K., Dietrich, S., and Lewis, K.E. (2008). Comparative genomics of *Lbx* loci  
800 reveals conservation of identical *Lbx* ohnologs in bony vertebrates. *BMC Evol Biol* 8, 171.  
801 10.1186/1471-2148-8-171.

802