Pre-bilaterian origin of the blastoporal axial organizer

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The startling capacity of the amphibian Spemann organizer to induce naïve cells to form a Siamese twin embryo with a second set of body axes is one of the hallmarks of developmental biology. However, the axis-inducing potential of the blastopore-associated tissue is commonly regarded as a chordate feature. Here we show that the blastopore lip of a non-bilaterian metazoan, the anthozoan cnidarian Nematostella vectensis, possesses the same capacity and uses the same molecular mechanism for inducing extra axes as chordates: Wnt/β-catenin signaling. We also demonstrate that the establishment of the secondary, directive axis in Nematostella by BMP signaling is sensitive to an initial Wnt signal, but once established the directive axis becomes Wnt-independent. By combining molecular analysis with experimental embryology, we provide evidence that the emergence of the Wnt/β-catenin driven blastopore-associated axial organizer predated the cnidarian-bilaterian split over 600 million years ago.

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The ability of the amphibian dorsal blastopore lip to induce ectopic body axes upon transplantation has fascinated biologists for nearly a century. Although axis-forming blastoporal organizers were long thought to be a chordate-specific feature, transplantation of a fragment of the mid-gastrula blastopore lip in the sea anemone *Nematostella vectensis*, a member of the early branching non-bilaterian phylum Cnidaria (which, in addition to sea anemones, includes corals, hydroids and jellyfish), also resulted in the formation of an ectopic body axis, just like the Mangold-Spemann organizer in amphibians. Whether this similarity reflects the homologous or convergent origin of the cnidarian and vertebrate blastoporal axial organizers remained unclear, since the molecular nature of the signal conveying axial organizer properties to the embryonic tissue has not been determined outside deuterostomes.

In the frog *Xenopus*, the Spemann organizer expresses Wnt and BMP antagonists, whose microinjection in single vegetal blastomeres can induce ectopic rostral or dorsal structures correspondingly. However, complete ectopic body axes, like the ones seen during transplantation of the dorsal blastopore lip, or the Nieuwkoop center cells, nearly never form. In contrast, complete ectopic body axes can be induced by single blastomere injection of Wnt-1, Xwnt-8, dishevelled, β-catenin and dominant negative GS3Kβ mRNA, showing that Wnt/β-catenin signaling is crucial for setting up the organizer. Pharmacological activation of Wnt/β-catenin signaling in *Nematostella* larvae affects the oral-aboral patterning, as detected by changes in the expression of Wnt2, Wnt4, brachyury (Bra) and several other genes. Moreover, it also results in the formation of double-headed polyps, making canonical Wnt signaling the prime candidate for the role of the early axial inducer in *Nematostella*, similar to the situation in frog. We set out to test whether Wnt signaling is conveying inductive capacity to the blastoporal organizer of *Nematostella*.

In this paper, using a combination of experimental embryology and molecular analyses, we show that two *Nematostella* Wnt ligands are capable of conveying axial organizing capacity to any region of the gastrula ectoderm, where they are co-expressed. Our data suggest an ancient origin of the Wnt/β-catenin dependent blastopore-associated axial organizer, predating the cnidian-bilaterian split.

**Results**

Wnt1 and Wnt3 convey axial organizer capacity. We addressed the role of Wnt signaling in axis formation in *Nematostella* by performing CRISPR/Cas9-mediated knockout of the putative intracellular Wnt/β-catenin signaling inhibitor APC. Primary polyps mosaic for the AFC mutation formed multiple ectopic tentacles and oral openings in F0 (Fig. 1a, Supplementary Fig. 1) suggesting that ectopic activation of Wnt/β-catenin signaling in discrete cell patches is sufficient for the induction of head structures. The *Nematostella* genome harbors 13 Wnt genes, which are expressed in staggered domains along the oral-aboral axis of the embryo and larva. Since only the blastopore lip is capable of inducing a second axis upon transplantation, our search for a candidate Wnt was restricted to Wnt genes, which are expressed in the blastopore lip of the *Nematostella* mid-gastrula. Five Wnt genes are expressed in concentric, partially overlapping ectodermal rings around the blastopore at mid-gastrula: Wnt1, Wnt2, Wnt3, Wnt4 and WntA. To further narrow down the list of candidates, we subdivided the donor blastopore lips into four minute fragments along the oral-aboral axis and tested whether a certain part of the lip is particularly efficient in inducing secondary axes. We found that fragments immediately next to the bend of the lip were the most potent inducers (Fig. 1c).

β-catenin signaling defines the location of the organizer. To get insight into the molecular response of the embryonic tissue and, particularly, the organizer to the elevated Wnt/β-catenin signaling in *Nematostella*, we modulated the Wnt/β-catenin signaling (Fig. 2a–c) by inhibiting GS3Kβ kinase with 1-Azakanepulone (AZK) at different times during embryonic development. The treatment with 2.5 μM AZK from early cleavage to mid-gastrula (hereafter referred to as early treatment) resulted in the formation of gastrulae with smaller blastopores and pre-endodermal plates (Fig. 2d–g, Supplementary Fig. 3); however, the process of gastrulation was not perturbed. By day 2 (a stage corresponding to the early planula in controls), these AZK treated embryos addi-

These fragments express Wnt1, Wnt3, WntA and, possibly, Wnt4, but not Wnt2, making the first four molecules the candidates for the role of head inducers. We then injected plasmids, where each of the four candidate Wnt genes was placed downstream of the EF1α promoter, into single blastomeres of the embryos at 8- or 16-cell stage. Dextran-Alexa488 fluorescence shows (Fig. 1d) that such injections result in the formation of a randomly located coherent patch of Wnt-expressing cells. While injections of 20 ng μl−1 solution of Wnt4 and WntA plasmids were unable to induce the formation of the secondary axis, Wnt1 and Wnt3 plasmid injections resulted in the formation of incomplete secondary axes in 9–14%, respectively (Fig. 1e, Supplementary Fig. 2). An incomplete secondary axis represents a lateral outgrowth with tentacles and, sometimes, pharynx, but not connected with the mesentery system of the polyp. Interestingly, co-injection of Wnt1 and Wnt3 (both, at 20 and 10 ng μl−1 each) led to a much stronger induction, increasing the rate of ectopic axis formation to about 50%, including a large number of complete ectopic axes with tentacles, pharynx and contractile mesenteries connected with the mesenterial system of the host polyp (Fig. 1e, Supplementary Fig. 2). Moreover, transplantation of fluorescent fragments of the aboral tissue ectopically expressing Wnt1 and/or Wnt3 was able to induce formation of the secondary axes (in 30% of the cases), while this part of the embryo is absolutely devoid of inductive capacity in the uninjected control embryos (Fig. 1e). Similar to the frog embryo, the blastoporal lip of the *Nematostella* early and mid-gastrula also expresses BMP antagonist chordin (Chd)17. However, ectopic overexpression of Chd by single blastomere injection does not induce ectopic axes (n = 0/120) (Fig. 1e). We conclude that Wnt/β-catenin signaling acts as head inducer in *Nematostella*, and Wnt1 and Wnt3 appear to act synergistically in conveying the inductive capacity to the blastoporal organizer tissue. Unfortunately, fluorescent dextran used as injection tracer is not detectable in the primary polyps. In order to test whether the progeny of the EF1α::Wnt1/EF1α::Wnt3 injected blastomere becomes part of the induced ectopic axis, we added the EF1α::mOrange2 plasmid to the injection mix and showed that the mOrange2-expressing tissue contributes to the pharynx and the hypostome of the induced secondary head (Fig. 1f).
were upregulated throughout the embryo (Fig. 3a, Supplementary Fig. 4, Supplementary Table 1), suggesting that these genes are activated by medium as well as high level of Wnt/β-catenin signaling (Fig. 3b). In contrast, in increasing AZK concentrations, Wnt1, Wnt2 and Wnt4 started to be upregulated in more and more aboral positions, while vacating the oral domain and finally disappearing from the embryo (Fig. 3a, Supplementary Fig. 4, Supplementary Table 1). This finding suggests that Wnt1, Wnt2 and Wnt4 are activated by medium levels but repressed, either directly or indirectly, by high levels of Wnt/β-catenin signaling (Fig. 3b). To control for the possibility that the observed changes in the expression were due to the described off-target effect of high concentrations of AZK on cyclin dependent kinase 1 (CDK1) rather than on GSK3β18, we treated the embryos with the CDK1/CDK2/CDK5 inhibitor Aminopurvalanol A (AMPV) in the same time window as the early AZK treatment, i.e., from early cleavage to mid-gastrula. As expected, higher concentrations of AMPV arrested the development, either immediately after

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**Figure Legends**

(a) Schematic representations of the experiments showing the evolution of the expression of the genes over time. (b) Bar graphs showing the percentage of embryos with expression of the different genes at different stages. (c) Graphical representation of the number of embryos with expression of the genes at different stages. (d) Photographs of embryos showing the expression patterns of the genes at different stages. (e) Graphical representation of the number of embryos with expression of the genes at different stages. (f) Photographs of embryos showing the expression patterns of the genes at different stages.
addition of the drug (5 or 10 μM AMPV) or several cell cycles later (2 μM AMPV), and resulted in the death of the embryos by the time gastrulation had to start. We then used a sub-lethal concentration of AMPV (1 μM), in which the embryos were able to gastrulate, and performed α-phosphohistone H3 antibody staining with 1 μM AMPV treated embryos, 15 μM AZK treated embryos and DMSO control embryos at the gastrula stage. Metaphase plates were observed in all three treatments (Supplementary Fig. 5a), however no shifts in the expression domains of Wnt2, Wnt4 and Bra could be detected in the AMPV treated embryos (Supplementary Fig. 5b), suggesting that the observed effects of the AZK treatment on gene expression (Fig. 3a) were due to the inhibition of GSK3β.

To test whether the change of expression domains of Wnt1 and Wnt3 in AZK treated embryos correlated with the changes in the inductive capacity of the gastrula tissue, we performed transplantations using donor embryos subjected to the early treatment with 2.5 μM AZK. Strikingly, and concomitant with the loss of Wnt1 expression in the blastopore lip in the AZK treated embryos, transplanted blastopore lips of the AZK treated embryos were completely unable to induce secondary axes in the recipients (Fig. 3c,d). However, the normally non-inductive aboral tissue, which in the AZK-treated embryos represents the tissue co-expressing Wnt1 and Wnt3 (Fig. 3a), could induce ectopic axis formation in the recipients just like the wild type blastopore lips (Fig. 3c,d), suggesting a shift of the organizer capacity from the blastopore lip to an aboral location. Further supporting the role of Wnt/β-catenin signaling in organizer formation, morpholino knockdown of Tcf, the conserved transcriptional co-factor of β-catenin, abolished the inductive capacity in the morphant blastopore lip (Fig. 3c,d).

**Establishment of the inductive territory.** Our data clearly show that axial organizer formation in *Nematostella* is a Wnt/β-catenin signaling dependent process. It has been previously demonstrated that nuclear β-catenin starts to accumulate at the future oral side of the *Nematostella* embryo already during early clevage and that Dishevelled protein is localized at the animal pole of the unfertilized egg. To get further insight into the establishment of the future inductive territory during development, we analyzed the expression of Wnt1, Wnt2, Wnt3, Wnt4, WntA, axin1-like, Tcf and Bra from early blastula to pre-gastrula in the wild type embryos (Fig. 4). Tcf, is strongly expressed in the 6 h post fertilization (hpf) blastula (although we did not see an accumulation at the future oral pole as previously suggested), and axin1-like, is weakly expressed at 6hpf. In contrast, the ectodermal Wnt genes (except Wnt4) become detectable in 10 hpf blastulae, together with Bra (Fig. 4). Interestingly, Wnt1, Wnt3 and WntA are first expressed in a patch of cells and only later...
**Figure 3 | Organizer capacity shifts to follow changes in Wnt1 and Wnt3 expression.** (a) Expression of Wnt1, Wnt2, Wnt4, Chd and Dpp expands aborally while clearing from the oral domain and disappears with the increasing AZK concentrations; expression of Wnt3, axin1-like, Tcf and Bra expands globally and reaches saturation. Blastopores point up; scale bar: 50 μm. (b) Model of expression of a gene activated at medium Wnt/β-catenin signaling levels (upper row; blue highlight represents the range of Wnt/β-catenin signaling levels when the gene can be expressed) versus a gene activated at medium and high Wnt/β-catenin signaling levels (lower row; green highlight represents the range of Wnt/β-catenin signaling levels when the gene can be expressed) in the AZK treatments. Blue curve: wild type level of Wnt/β-catenin signaling along the oral-aboral axis. Lilac curve: Wnt/β-catenin signaling level in a low concentration of AZK. Orange curve: Wnt/β-catenin signaling level in a high concentration of AZK. Red dashed lines mark the Wnt/β-catenin signaling thresholds and the corresponding positions on the oral-aboral axis where these thresholds are reached. (c,d) Scheme (c) and developmental outcomes (d) of transplantation of wild type (wt) and 2.5 μM AZK treated (AZK) blastopore lips and aboral ectoderm fragments as well as blastopore lips of the CtrlMO and TcfMO injected gastrulae into wild type recipient gastrulae. Coloured rectangles on (c) correspond to the colours of the bars on (d). Only untreated or CtrlMO blastopore lips and AZK treated aboral ectoderm fragments are inductive.
refine into a ring, while Wnt2 and Bra are detected as a ring from the start. The early expression patterns of Wnt1, Wnt3 and WntA suggest that they are either involved in the activation of the genetic programme defining the pre-endodermal plate or co-regulated by the same early β-catenin input that activates the endoderm determination programme throughout Eumetazoa.19–25. Once the endodermal territory is determined, Wnt1, Wnt3 and WntA expression becomes displaced out of the prospective endodermal cells, likely by a yet unknown negative feedback mechanism, and becomes confined to the ectodermal tissue surrounding the endoderm. These ectodermal cells will become the axial organizer.

Wnt/β-catenin signaling and the directive axis. In the vertebrate organizer, the initial β-catenin signal activates the expression of BMP antagonists such as chordin, thus suppressing ventral fates9. Anthozoan cnidarians, unlike their radially symmetric hydroid and jellyfish relatives, possess not only the oral-aboral body axis but also a second, directive axis, orthogonal to the oral-aboral axis. The key players for the establishment of the directive axis are the bmp4 homologue Dpp and the chordin homologue Chd26. Their initially radial expression starts at 14 hpf (Fig. 4) and shifts to the same side of the directive axis upon BMP signaling-dependent symmetry break in late gastrula17,26. It has been previously shown that Dpp and Chd is negatively affected by suppression of Wnt/β-catenin signaling by iCRT14 (ref. 11), and microarray analysis showed that both genes react to 10 μM AZK13. Interestingly, the expression of Dpp and Chd reacted to the early AZK treatment in a similar way as the expression of Wnt1, Wnt2 and Wnt4, displaying an aboral expansion at lower AZK concentrations and disappearing at higher AZK concentrations, indicative of the lack of directive axis (Fig. 3a, Supplementary Fig. 5). The disappearance of Chd expression was previously described11 for a different GSK3β inhibitor, alsterpaullone (ALP), although only one concentration of ALP was used. Surprisingly, we did not observe the reported upregulation11,13 and ubiquitous expansion11 of Dpp expression in early AZK treatment. The effect of the early AZK treatment is in stark contrast to the effects of the late AZK treatment (24 h incubation in 2.5 or 5 μM AZK starting from late gastrula), which we performed after the time of blastoporal organizer formation and directive axis establishment. Late AZK treatment did not prevent larva development and primary polyp formation, but resulted in the oral transformation of the aboral end of the embryo. Notably, such embryos never developed ectopic axes laterally, but rather re-specified the aboral half of their oral-aboral axis to acquire oral fates (Fig. 5a). Strikingly, late AZK treatment did not affect the asymmetric expression of Dpp and Chd (Fig. 5b, Supplementary Fig. 4, Supplementary Table 2), while the

Figure 4 | Establishment of the oral inductive territory in Nematostella. At 10 hpf Wnt1, Wnt2, Wnt3 and WntA are the first Wnt genes to be expressed. Wnt2 expression starts as a ring excluding the presumptive pre-endodermal plate. Wnt1, Wnt3 and WntA expression starts as a patch in the presumptive pre-endodermal plate and by 14–18 hpf begins to form a ring around it. Brachyury expression is first detectable as a ring at 10 hpf. Wnt4, Dpp and Chd start to be expressed in a ring around the pre-endodermal plate by 14 hpf. On lateral views, animal/oral ends of the embryos point up. Scale bar: 100 μm.
expression of the oral marker FoxA \(^{27}\) clearly demonstrated the transformation of the aboral domain into an oral domain (Fig. 5b). These observations suggest that the initial, radially symmetric, circumblastoporal expression of Dpp and Chd might rely on the correct intensity of β-catenin signaling, however, Dpp and Chd expression becomes self-regulating and does not require Wnt/β-catenin input, once the directive axis is established.

**Discussion**

In this paper, we showed that the key molecules conveying the inductive capacity to the Nematostella organizer are Wnt1 and Wnt3. Their co-expression in any region of the ectoderm of the gastrula following the single blastomere injection appears to be sufficient for the tissue to acquire axial organizer properties. If expression domains of Wnt/β-catenin signaling dependent genes in the mid-gastrula are modulated by AZK treatment, the inductive capacity is lost in the blastopore lip and becomes confined to the aboral ectoderm, correlated with the shifts in the Wnt1 and Wnt3 expression. Our analysis of the onset of Wnt1 and Wnt3 expression in the wild type Nematostella embryos showed that their early expression is first observed not in a ring, but in a patch of cells in the 10 hpf blastula, and only later it starts to refine into a ring surrounding the presumptive pre-endodermal plate. It seems reasonable to predict that transplantation of a patch of Wnt1 and Wnt3 expressing cells from a 10 hpf blastula, and only later it starts to refine into a ring surrounding the presumptive pre-endodermal plate. It seems reasonable to predict that transplantation of a patch of Wnt1 and Wnt3 expressing cells from a 10 hpf blastula, and only later it starts to refine into a ring surrounding the presumptive pre-endodermal plate. It seems reasonable to predict that transplantation of a patch of Wnt1 and Wnt3 expressing cells from a 10 hpf blastula, and only later it starts to refine into a ring surrounding the presumptive pre-endodermal plate.
showed that the 2d somatoblast emits a signal required for trunk formation and the D-V axis organization in the head, although the authors did not perform transplantations of the 2d cell, and the molecular nature of the signal remains unknown. In general, the D-quadrand and, particularly, the 4d cell and its descendants in Spiralia are the site of nuclear β-catenin stabilization. In the invaginating gastrulae of the species with homoquadrant cleavage these cells are located at the dorsal margin of the blastopore. However, the possible role of β-catenin in the formation of axial organizers in protostome Bilateria requires further thorough analysis. Taken together, our study strongly suggests an ancient origin of the blastopore-associated axial organizer (Fig. 6) based on Wnt/β-catenin signaling, which was maintained in the cnidian, deuterostome and, possibly, in several protostome lineages.

Methods

Animal culture and plasmid microinjection. Animal husbandry was described as described. Briefly, adult polyps were kept in Nematostella medium (10% artificial sea water) at 18°C in the dark. Induction of spawning was performed by placing the animals into 25°C illuminated incubator for 10h. Developing embryos were kept at 21°C. Plasmids carrying different untagged Nematostella Wnt’s and mCherry2 driven by EF1α promoter were injected into individual blastomeres at 8–16 cell stage. Fluorescent Dextran-Alexa488 (Life Technologies) was co-injected as tracer.

CRISPR-Cas9 mediated mutagenesis. Single guide RNAs for CRISPR-Cas9 mediated mutagenesis of Nematostella APC (Genbank KT381584) were synthesized as described using the following oligos: 5’TAGGCACAGGTATGAGGGCCAC, 5’AAACGTGGCCTCTATAGTGTT, nls-Cas9 protein was obtained from PNA Technologies Inc. Control embryos were injected with 500 ng/ml single guide RNA alone. DNA was prepared from individual polyps and a 248 bp long fragment containing the recognition sequence of the guide RNA was amplified using the following oligos: 5’GAATTCCTGGAGAAGATGACA, 5’ CCTGGCATACAAAGGTGACA. For the controls, 10 animals were pooled prior to DNA preparation. PCR products harboring the mutation site were directly sequenced by Microsynth (Balgach, Switzerland), and sequencing chromatograms were analyzed to assess the presence of the discrepancies in comparison to the wild type sequence.

Inhibitor treatments and morpholino experiments. 1-Azakenpaullone (Sigma) and Aminopurvalanol A (abcam) treatments were performed in the time windows and with concentrations specified in the main text. Tcf7 knockdown was performed by injecting a previously published antisense translation blocking morpholino (nls-Cas9; 5’TCAAGAGGCTTACATGCTGTA). Control embryos were injected with 500 ng/ml single guide RNA alone. DNA was prepared from individual polyps and a 248 bp long fragment containing the recognition sequence of the guide RNA was amplified using the following oligos: 5’GAATTCCTGGAGAAGATGACA, 5’ CCTGGCATACAAAGGTGACA. For the controls, 10 animals were pooled prior to DNA preparation. PCR products harboring the mutation site were directly sequenced by Microsynth (Balgach, Switzerland), and sequencing chromatograms were analyzed to assess the presence of the discrepancies in comparison to the wild type sequence.

In situ hybridization and morphological analysis. In situ hybridization was performed as described. Briefly, embryos from cleavage stage till gastrula were fixed for 1 min in 2% glutaraldehyde/3.7% formaldehyde in Nematostella medium (NM) and then for an additional hour in 3.7% formaldehyde/NM. Older embryos and larvae were fixed for 1 min in 0.2% glutaraldehyde/3.7% formaldehyde in Nematostella medium and then for an additional hour in 3.7% formaldehyde/NM. After 5 PTW (1x PBS with 0.1% Tween 20) washes, the embryos were transferred into methanol, then rehydrated with PTW and treated for 3 min with 80 µg/ml proteinase K (Ambion). After stopping the digest with two 4 mg/ml Glycin/PTW washes, the samples were post-fixed for 1 h in 2% glutaraldehyde/3.7% formaldehyde in 0.1 M cacodylate buffer. Then they were washed with 0.1 M cacodylate buffer, post-fixed for 1 h in 1% OsO4/0.1 M cacodylate buffer, washed, dehydrated in ethanol and acetone and finally dried by critical point technique and sputter coated for SEM or embedded into a standard mixture of Araldite and Epon (Electron Microscopy Sciences) for semithin sectioning. 1 µm semithin sections were stained with 1% toluidine blue and imaged under the Nikon 80i compound microscope. SEM was performed with the JEOL JSM-6380LA or CamScan S2 microscopes.

Antibody staining. Rabbit anti-phospho-Histone H3 (Ser10) antibody (Merck Millipore) was used in 1:400 dilution using the antibody staining protocol from. Briefly, the embryos were fixed in 4% paraformaldehyde/PBS-TT (1xPBS, 0.2% Triton X100, 0.2% Tween 20), washed five times in PBS-TT, blocked in 20% heat inactivated sheep serum (Sigma)/1% BSA in PBS-TT, and incubated with the primary antibody overnight at 4°C. After eight 15 min PBS-TT washes, the embryos were blocked again and incubated with the goat anti-rabbit Alexa568 (Life Technologies) at 1:1000 dilution overnight. DAPI was added at the final concentration of 300 nM together with the secondary antibody to counterstain chromatin. The samples were then washed again eight times 15 min with PBS-TT, embedded in Vectashield (Vectorlabs) and imaged under a Leica SP5X CLSM.

Data availability. The data supporting the findings of this study are available within the article and its Supplementary Information. Further details concerning the techniques used in the paper are available on request.

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Author contributions
G.G., Y.K. and U.T. designed the experiments, G.G., Y.K. and A.A. performed the experiments. G.G., Y.K. and P26962 (to G.G.). Patrick Steinmetz cloned and tested the EF1α promoter. Y.K. was supported by the Lise Meitner FWF fellowship (MI140-B17). A.A. was supported by an HFSP postdoctoral fellowship (LT000809/2012-L). We are grateful to the Electron Microscopy Laboratory of the Research Centers of the Lomonosov Moscow State University for the possibility to perform SEM and to the Core Facility for Cell Imaging and Ultrastructure Research of the University of Vienna for the access to the confocal microscope. We thank Claude Desplan, Ira Blitz and Alexandra Schauer for valuable comments on the manuscript.

Additional information
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