



# The rise of the starlet sea anemone *Nematostella vectensis* as a model system to investigate development and regeneration

Michael J. Layden,<sup>1\*</sup> Fabian Rentzsch<sup>2</sup> and Eric Röttinger<sup>3</sup>

Reverse genetics and next-generation sequencing unlocked a new era in biology. It is now possible to identify an animal(s) with the unique biology most relevant to a particular question and rapidly generate tools to functionally dissect that biology. This review highlights the rise of one such novel model system, the starlet sea anemone *Nematostella vectensis*. *Nematostella* is a cnidarian (corals, jellyfish, hydras, sea anemones, etc.) animal that was originally targeted by EvoDevo researchers looking to identify a cnidarian animal to which the development of bilaterians (insects, worms, echinoderms, vertebrates, mollusks, etc.) could be compared. Studies in *Nematostella* have accomplished this goal and informed our understanding of the evolution of key bilaterian features. However, *Nematostella* is now going beyond its intended utility with potential as a model to better understand other areas such as regenerative biology, EcoDevo, or stress response. This review intends to highlight key EvoDevo insights from *Nematostella* that guide our understanding about the evolution of axial patterning mechanisms, mesoderm, and nervous systems in bilaterians, as well as to discuss briefly the potential of *Nematostella* as a model to better understand the relationship between development and regeneration. Lastly, the sum of research to date in *Nematostella* has generated a variety of tools that aided the rise of *Nematostella* to a viable model system. We provide a catalogue of current resources and techniques available to facilitate investigators interested in incorporating *Nematostella* into their research.

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\*Correspondence to: layden@lehigh.edu

<sup>1</sup>Department of Biological Sciences, Lehigh University, Bethlehem, PA, USA

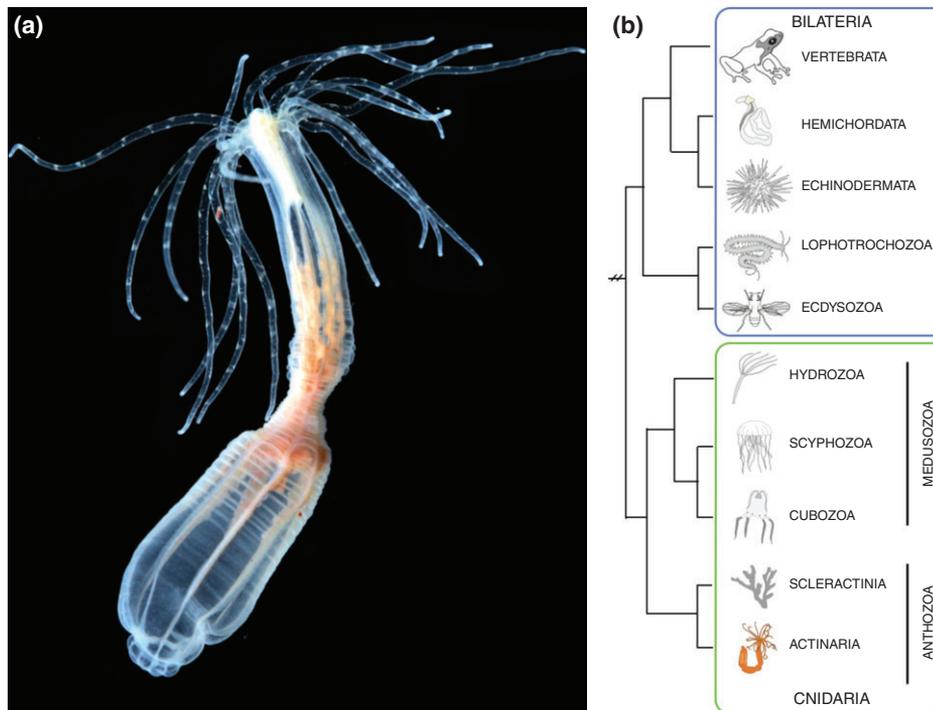
<sup>2</sup>Sars Centre for Marine Molecular Biology, University of Bergen, Bergen, Norway

<sup>3</sup>Institute for Research on Cancer and Aging (IRCAN), CNRS UMR 7284, INSERM U1081, Université de Nice-Sophia-Antipolis, Nice, France

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## INTRODUCTION

Many EvoDevo researchers wished to understand how evolutionary radiation gave rise to the impressive array of biodiversity present in a group of animals called the bilaterians. To answer this question, biologists sought to infer the bilaterian molecular and morphological ‘starting point’ by identifying ancestral traits likely present in the ancestor of all bilaterians (the urbilaterian) through comparisons to non-bilaterian species. Cnidarian animals (hydras, jellyfish, corals, siphonophores, and sea anemones) are widely accepted to be the sister taxon to



**FIGURE 1** | *Nematostella vectensis* is an anthozoan cnidarian sea anemone. (a) Image of adult *Nematostella* polyp. Image taken by Eric Röttinger. (b) Phylogeny showing the sister relationship of cnidarians to bilaterians and *Nematostella*'s position within the cnidarians.

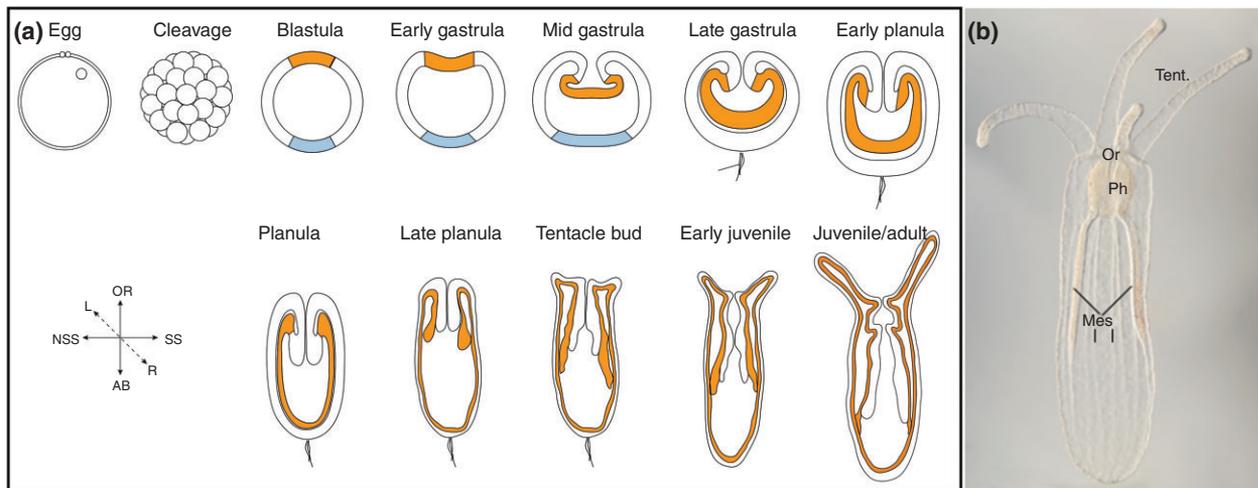
bilaterians<sup>1–3</sup> (Figure 1(b)). The sister relationship makes cnidarians the most closely related non-bilaterians and the obvious group to target for developing a model animal. The Cnidaria have two main clades, the Medusozoa (hydras, jellyfish, and siphonophores) and the Anthozoa (e.g., corals, sea anemones) (Figure 1(b)). *Hydra* had long been used as an animal model to better understand regeneration and principles of patterning; however, they produce small numbers of embryos in culture, and those embryos are poorly accessible during most of their development.<sup>4,5</sup> In 1992, Hand and Uhlinger reported a protocol to maintain the complete life cycle of *Nematostella* in culture, which allowed for robust investigation of cnidarian development.<sup>4</sup> As *Nematostella*, a number of other cnidarian species such as *Clytia* and *Hydractinia* have been developed as models.<sup>5,6</sup> However, *Nematostella* is the most developed and commonly used anthozoan cnidarian model. Together investigations in multiple cnidarian systems will complement and strengthen our understanding of cnidarian biology, and thus provide insight into the ancestral tool kit that gave rise to the complex bilaterian body plans. Here we focus on *Nematostella* and provide brief summaries of the current state of research on three well-studied aspects of *Nematostella* development in regards to bilaterians

(axial patterning, emergence of mesoderm, and nervous system evolution). We also provide references to the tools available for researchers interested in introducing *Nematostella* into their research program, and briefly comment on additional areas, such as regeneration, where *Nematostella* offers a novel model system to better understand animal biology.

## OVERVIEW OF NEMATOSTELLA VECTENSIS BIOLOGY

### Morphology and Life Cycle of *Nematostella*

*Nematostella vectensis* lives in estuarine environments like salt marshes or brackish water pools, and is thus normally exposed to significant fluctuations in temperature and salinity. The physiological tolerance required for life in an unsteady environment may contribute to the relative ease with which *Nematostella* can be cultured. The life cycle of *Nematostella* takes approximately 12 weeks in culture.<sup>4</sup> They are dioecious, and fertilization occurs in the external environment after spawning. Zygotes initially undergo a series of reductive cleavages to form a blastula. Gastrulation occurs via invagination and initiates at the animal pole (Figure 2(a)). The gastrula



**FIGURE 2** | Development and morphology of *Nematostella*. (a) Schematic of *Nematostella* developmental stages. Modified from Ormestad et al. Ref 90. Orange represents presumptive endoderm in blastula and the endoderm in all subsequent stages. The outer ectoderm is white, and the aboral ectodermal domain is in light blue. (b) DIC image taken by Aldine Amiel of juvenile polyp. The mouth (oral opening) is indicated by (Or), the tentacles by (tent), the pharynx by (Ph), and the mesenteries by (mes). Oral is up in all images.

stage is followed by the planula stage, which lasts from approximately 24 h post fertilization (hpf) to 96 hpf (temperature dependent, unpublished observations). Planulae are free swimming and during this stage the developing anemone undergoes a slight elongation and formation of pharynx and mesenteries occurs.<sup>7</sup> The planula undergoes a subtle type of metamorphosis, which is first apparent by the arrest of swimming movement as well as the formation of tentacle buds, and results in formation of the four tentacle juvenile polyp. At this point growth and maturation of the polyp occurs in a nutrient dependent manner until they reach sexual maturity. Development from egg to juvenile polyp occurs over roughly 6 days.<sup>4,7–10</sup>

The morphology of *Nematostella* is superficially simple. They are diploblastic animals, meaning that they consist of two germ layers, a bifunctional internal endoderm also referred to as the entoderm, endomesoderm, or gastrodermis as well as an outer ectoderm (Figure 2(a)). They possess a sac-like gut with a single oral opening that corresponds to the site of gastrulation<sup>7,8</sup> (Figure 2(b)). The adult mouth is surrounded by as many as 16 tentacles<sup>11</sup> (Figure 1(a)). They have a noticeable pharynx and eight radially repeated mesentery structures that run along the long oral–aboral axis<sup>12</sup> (Figure 2(b)). The mesenteries are comprised of gonads, cnidocytes (stinging cells), and myoepithelial cells that allow the animal to quickly contract along the long axis.<sup>12–14</sup> The two earliest forming mesenteries are termed the primary (previously termed directive) mesenteries; they are situated ca 160° from one another.<sup>15</sup> There are two main axes to the anthozoan body plan, the oral–aboral axis, which is the primary

axis and runs from the oral opening to the opposite aboral side, and the directive axis. The directive axis is oriented orthogonal to the oral–aboral axis. Although, cnidarians have been described as radially symmetric around the long oral–aboral axis of the animal, it has long been known that this is not the case for anthozoans. The arrangement of retractor muscles on the mesenteries and the presence of a ciliated groove (the siphonoglyph) on one side of the pharynx establish a clear, although subtle bilateral symmetry (summarized in Ref 16). The exact relationship between oral–aboral and directive axes and the anterior–posterior and dorsal–ventral axes are still unclear, but numerous investigations have shed a great deal of light on this issue, and they are discussed below.

The ectoderm and endoderm have differentiated epithelial cells and specialized cells (neurons, gland cells, myoepithelial cells, etc.) intermixed with each other. This is in contrast to most bilaterians, which organize specialized cell types into organs with a dedicated function. However, it should be noted that distinct regions of the animal are enriched for particular types of differentiated cells. For example, tentacles, which serve to capture and move prey to the mouth, are highly enriched for offensive cnidocyte stinging cells, which envenomate prey.<sup>17</sup> In addition, regional patterning is observed for neural markers.<sup>18–20</sup> The ectoderm serves as a protective barrier between the animal and environment. Differentiated cell types found in the ectoderm include sensory neurons, cnidocytes stinging cells, gland cells, and interneurons with as of yet unknown function. The endoderm contains absorptive cells that serve in

digestion and nutrient uptake. The non-mesentery endoderm contains neurons, myoepithelial cells (musculature), and gland cells.<sup>12,14,18</sup> Between the endoderm and ectoderm lies a mostly acellular mesoglea. The mesoglea contains extracellular matrix as well as some neurites extending from both the endodermal and ectodermal neurons.<sup>14,21</sup> The mesoglea also contains amoebocyte cells that appear to be migratory, but as of yet their function is unknown.<sup>14</sup>

## *Nematostella* Genome and Experimental Tools

In 2007, the genome of *Nematostella* was released. Based on early comparisons between protostome and deuterostome genomes, it was believed that the cnidarian genome would be relatively simple (e.g., lacking many genes or gene families present in bilaterians). Surprisingly, the *Nematostella* genome is incredibly complex. Nearly all of the gene families present in bilaterians were found in *Nematostella*. Of note, is a nearly complete catalog of the Wnt ligands present in deuterostome bilaterians.<sup>22,23</sup> In addition, there was considerable syntenic conservation between stretches of cnidarian and vertebrate genomes and high conservation of intron-exon boundaries with vertebrate genomes.<sup>23</sup> All told at the genomic level, vertebrates and *Nematostella* are more similar to each other than either group is to ecdysozoan model animals (*Drosophila* and nematodes).<sup>24</sup> Thus, the first insight about evolution of bilaterian complexity was that complex molecular architecture predated the emergence of bilaterians and that there is no simple correlation between organismal and genomic complexity. The quest for such a correlation is still attracting attention and has driven the adaptation of new experimental tools for *Nematostella*: microRNAs and other smallRNAs have been isolated systematically.<sup>25,26</sup> ChIP-seq has been used to identify epigenetic marks and predict regulatory elements,<sup>27</sup> and improved transcriptome resources can provide insight into the prevalence of alternative splicing and long non-coding RNAs.<sup>28–30</sup>

Because of the sequencing of the *Nematostella* genome, tool development has occurred on a relatively rapid scale providing new investigators an array of mechanisms for incorporating *Nematostella* into their research. Table 1 highlights all the tools and resources available for *Nematostella* and provides references and hyperlinks to resource pages for protocols and genomic tools. First, the sequenced annotated genome offers the ability to rapidly identify gene homologs.<sup>23</sup> Although a second generation genome is still in the works, transcriptome studies provide refined gene models.<sup>28,29</sup> Tools for

visualizing gene expression and protein localization using transgenics, mRNA *in situ* hybridization, injection of mRNA fusion constructs, and antibody staining have been developed.<sup>12,31,38,42</sup> The ability to knockdown gene function using morpholinos or knockout gene function using CRISPR/Cas9 or TALEN-Fok approaches is now available.<sup>35–38</sup> Overexpression via mRNA injection allows for misexpression in early embryos.<sup>19,30,38,39</sup> Recently, a heat-shock responsive promoter was identified paving the way for more complex inducible constructs and the ability to test gain of function phenotypes at later stages of development.<sup>35</sup> Technology improving conditional gene regulation would strengthen *Nematostella*'s utility as a model system. Protocols for ChIP-seq and RNA-seq have also been developed.<sup>27–29</sup> To date *Nematostella* has proven amenable for the adaptation of methods deployed in other animal systems to disrupt gene function and analyze phenotypes in the sea anemone, and with advances like CRISPR/Cas9 technology it seems reasonable that tool development in *Nematostella* will continue to be relatively straight-forward.

## OVERVIEW OF AXIAL PATTERNING

### Formation and Patterning of the Oral–Aboral Axis

How the oral–aboral primary body axis of cnidarians relates to the anterior–posterior axis of bilaterians is a longstanding question. Cnidarian polyps lack a brain-like concentration of the nervous system at one pole of the axis (see below) and a through gut in which the mouth would be located anterior to the anus, which are key morphological features that correlate with the orientation of the anterior–posterior axis in most bilaterians. The lack of these features is usually considered to represent an evolutionarily early body plan, and accordingly studies on extant cnidarian polyps can provide insights into the ground state from which traits like cephalization and a through gut evolved. Despite the dual function as mouth and anus, the single body opening of cnidarian polyps is by convention called oral opening and the opposite end of the primary body axis is called the aboral pole or physa (Figure 2). Labeling experiments in *Nematostella* demonstrate that the blastopore and thus the body opening derive from the animal pole of the egg, i.e., the site where the polar bodies are extruded and where the first cleavage initiates.<sup>7,8</sup> This had previously been observed in hydrozoans,<sup>43</sup> which suggests that the relation between oocyte polarity and the oral–aboral axis is conserved in different cnidarians clades. Gastrulation from the

**TABLE 1** | List of Techniques and Tools Available for Culturing and Experimental Manipulation of *Nematostella*

Culture and Care of <i>Nematostella</i>	
Culture and spawning	Hand and Uhlinger <sup>4</sup> ; Fritzenwanker and Technau <sup>9</sup> ; Genikhovich et al., <sup>31</sup>
Inducing and staging regeneration	Amiel et al., <sup>32</sup> ; Dubuc et al., <sup>33</sup> ; Bossert et al., <sup>34</sup>
Spatiotemporal gene expression	
mRNA <i>in situ</i>	Wolenski et al., <sup>13</sup> ; Genikhovich et al., <sup>31</sup>
Immunolocalization	Wolenski et al., <sup>13</sup>
Transgenic reporters	Renfer et al., <sup>12</sup> ; Ikmi et al., <sup>35</sup>
Gene function	
Morpholino	Magie et al., <sup>36</sup> ; Rentzsch et al., <sup>37</sup> ; Layden et al., <sup>38</sup>
mRNA misexpression	Wikramanayake et al., <sup>39</sup> ; Layden et al., <sup>38</sup>
CRISPR/Cas9, TALEN/Fok1	Ikmi et al., <sup>35</sup>
Inducible promoters	Ikmi et al., <sup>35</sup>
Genome and 'omics' level analysis	
Annotated genome	(Putnam et al., <sup>23</sup> ) <a href="http://genome.jgi-psf.org/Nemve1/Nemve1.home.html">http://genome.jgi-psf.org/Nemve1/Nemve1.home.html</a> , <a href="http://cnidarians.bu.edu/stellabase/index.cgi">http://cnidarians.bu.edu/stellabase/index.cgi</a> , <a href="http://metazoa.ensembl.org/Nematostella_vectensis/Info/Index">http://metazoa.ensembl.org/Nematostella_vectensis/Info/Index</a>
Transcriptomes	Tulin et al., <sup>29</sup> ; Helm et al., <sup>28</sup> <a href="http://figshare.com/articles/Nematostella_vectensis_transcriptome_and_gene_models_v2_0/807696">http://figshare.com/articles/Nematostella_vectensis_transcriptome_and_gene_models_v2_0/807696</a>
ChIP-Seq protocol	Schwaiger et al., <sup>27</sup>
RNA-seq protocol	Helm et al., <sup>28</sup> ; Tulin et al., <sup>29</sup>
Microarray approaches	Röttinger et al., <sup>40</sup> ; Fritz et al., <sup>11</sup> ; Sinigaglia et al., <sup>41</sup>

animal hemisphere is found in cnidarians and ctenophores, a second non-bilaterian clade, but it is strikingly different from bilaterians, which gastrulate from the vegetal hemisphere. Despite this difference a general similarity exists in the molecular systems that control the development of axial polarity and patterning in cnidarian and bilaterian lineages.

### **Hox Genes and Axial Patterning in Cnidarians**

Initially, *Hox* genes were used to understand the relation of the oral–aboral axis and the anterior–posterior axis, but no consensus emerged from these studies. Cnidarians possess anterior *hox* and *parahox* genes, but the presence of central and posterior *hox* genes is controversial. *Hox* genes that do not belong to the anterior group have either been classified as posterior, as central or posterior, or as without affinity to anterior, central, or posterior groups.<sup>44–48</sup> In *Nematostella*, one of the five anterior *hox* genes is expressed in the pharynx (which is derived from the oral domain) at the planula stage,<sup>44</sup> whereas three other anterior *hox* genes are expressed in a unilateral domain in the endoderm.<sup>44,45</sup> Of the two potential posterior *hox* genes, one is expressed at the aboral pole and the other one in a unilateral endodermal

domain of the same oral–aboral extension as the three anterior *hox* genes.<sup>44</sup> Interestingly, the domains of the endodermally expressed genes have clearly different extensions along the secondary body axis (see also below).<sup>45</sup> These expression patterns suggest that *hox* genes are involved in axial patterning in *Nematostella*; however, they also show that they are not a reliable tool to clarify the relation of the cnidarian and bilaterian primary body axes. This is further illustrated by the observation that orthologs of the aborally expressed putative posterior *hox* gene are expressed at the opposite pole in the hydrozoans *Clytia hemispherica* and *Podocoryne carnea*.<sup>47,49</sup> Also, it should be noted that further functional characterization of cnidarian *hox* genes coupled with improved sampling of cnidarian genomes for putative *hox* homologs will likely prove insightful in regards to understanding how *hox* genes became so prominent in patterning the bilaterian anterior–posterior axis.

### **Wnt Signaling and the Formation of Embryonic Polarity**

In most bilaterians, intracellular components of the Wnt/ $\beta$ -catenin signaling pathway are key players for the establishment of axial polarity and consequently, the role of this pathway has been studied extensively

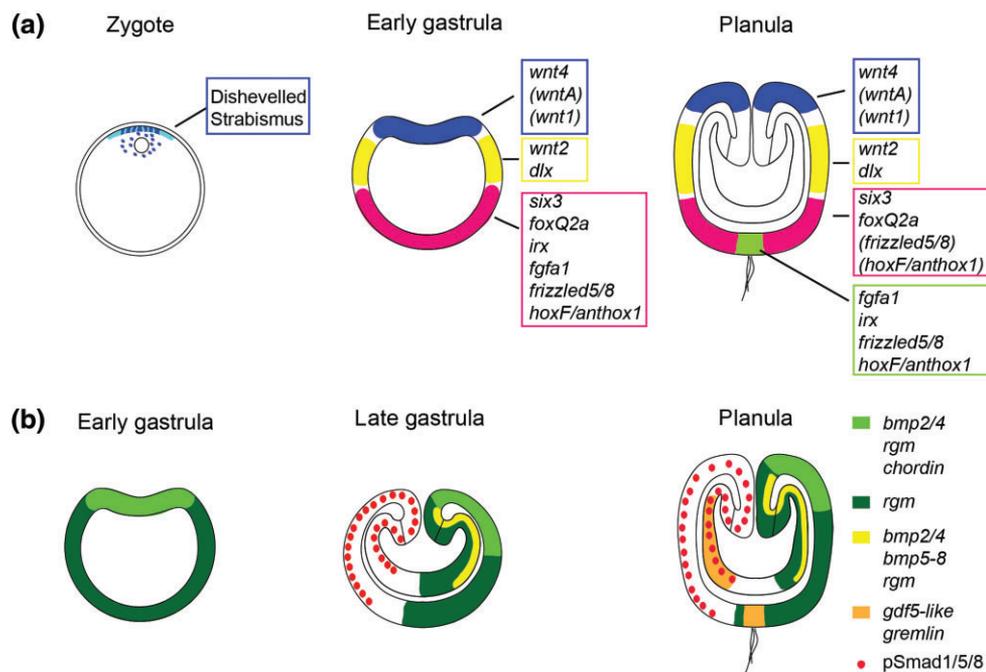
in *Nematostella*. In the unfertilized egg, NvDsh protein localizes predominantly to the cell cortex adjacent to the pronucleus and after fertilization it associates with the zygotic nucleus (Figure 3(a)). NvDsh remains enriched at the first cleavage furrow and on one side (presumably the animal domain) of the blastula.<sup>8</sup> Consistent with an involvement of the  $\beta$ -catenin branch of Wnt signaling (canonical Wnt), an antibody against *Xenopus*  $\beta$ -catenin and injection of mRNA encoding a Nv $\beta$ -catenin-GFP fusion construct revealed preferential stabilization and nuclearization on one side of the embryo from the 32-cell stage on and later in the invaginating part of the gastrula.<sup>39</sup> Interference with the function of Nvdsh, Nvtcf, and Nv $\beta$ -catenin led to conflicting results about their requirement for the development of axial polarity. Depending on the experimental approach, either complete absence of morphological polarity and failure to gastrulate; or only a defect in the specification of endoderm and pharynx was observed.<sup>8,39,40,50</sup> Interestingly, a component of the non-canonical Wnt/PCP signaling pathway, NvStrabismus (NvStbm), is also enriched at the animal pole

from zygote to gastrula stage (Figure 3(a)). Knock-down of *Nvstbm* by morpholino injection prevented gastrulation, but not the localized expression of endodermal markers,<sup>50</sup> indicating that this branch of Wnt signaling is specifically required for the morphogenetic movements at gastrulation.

Wnt signaling appears to be important in the development of early asymmetry, but despite the animal pole localization of NvDsh and Nv $\beta$ cat it is still not clear how the polarity of the oocyte is initially determined. Future work focused on identification of the mechanisms that stabilize NvDsh at the animal pole will likely yield critical cues as to the most upstream cues that regulate oral–aboral patterning in *Nematostella*.

### Wnt Signaling and the Patterning of the Oral–Aboral Axis

While the role of Wnt/ $\beta$ -catenin signaling in the initial formation of the oral–aboral axis remains unclear, several studies support a role for this



**FIGURE 3** | Gene expression during axial patterning. Localization of transcripts (in italic) and proteins involved in the patterning of the oral–aboral (a) and directive (b) axis, respectively. The developmental stages are indicated above each cartoon. Note that not all details of the expression patterns are captured. In (a), the expression domain of *wnt4* is broader than that of *wntA* and *wnt1*. *frizzled5/8* is at planula stage strongly expressed at the aboral pole (green) and weakly in a broader aboral domain (pink). *hoxF/anthox1* is at planula stage expressed in the small aboral pole domain (green) and in individual cells within the broader aboral domain (pink). It is not clear whether the expression domains depicted in blue, yellow, and pink are directly abutting each other. In the zygote, Disheveled protein is localized at the cortex and around the nucleus. Disheveled and Strabismus protein remain preferentially localized to the animal/blastoporal region at least until gastrula stage. In (b), BMP5-8 is at gastrula stage co-expressed in the ectoderm with *bmp2/4*, *chordin*, and *rgm* (i.e., the domain in light green). pSmad1/5/8 staining forms a gradient with highest levels on the side opposite to the *bmp* expressing side and low levels on the *bmp* expressing side. See main text for references.

pathway in the subsequent patterning along this axis. Starting from mid-blastula stage on, the expression of individual Wnt genes demarcates distinct, staggered domains within the oral half of the embryo<sup>22,40,51</sup> (Figure 3(a)). Both in the ecto- and endoderm, these expression domains show little overlap and could in principle determine separate territories along this part of the oral–aboral axis. Over activation of Wnt/ $\beta$ -catenin signaling by GSK3 inhibitors has shown that different levels of  $\beta$ -catenin can indeed elicit shifts in the expression of regional markers, indicating that high levels of Wnt/ $\beta$ -catenin signaling promote oral pole identity.<sup>20,40,52</sup> Consistently, knockdown of *Nutcf* leads to an expansion of the aboral marker gene *Nvfgfa1* and to the loss of the oral ectoderm markers *Nvwnt2* and *Nvchordin*.<sup>40</sup> Surprisingly, there are no reports on the function of individual Wnt ligands in *Nematostella*, but it will be important to analyze these molecules in an endogenous context to understand potential differences in their signaling mechanisms. These studies together with data from other cnidarian species<sup>53–55</sup> clearly suggest that Wnt signaling has ancient functions in the determination of the site of gastrulation and the patterning of the oral–aboral axis.

### The Development of the Aboral Domain—Clues to the Evolution of Brain Formation?

Brain-like nervous system centralization occurs in most bilaterian clades close to the anterior end of the body,<sup>56</sup> but a readily comparable neural structure is not present in cnidarian polyps. Whether a molecular program that resembles anterior patterning is present in cnidarians, and thus whether they have a body region from which cephalization might have evolved, has been an important question in studies on the molecular control of cnidarian development.<sup>57</sup>

Identification of unambiguous markers to compare the anterior end of the bilaterian body axis to the oral or aboral pole of cnidarians is complicated; however, the transcription factors *six3*, *foxQ2*, and the Wnt regulator *SFRP1* are among the best candidates currently available. In *Nematostella*, these genes are expressed at the aboral pole<sup>20,41</sup> (Figure 3(a)), and *Nvsix3/6* has been shown to be a key regulator of the development of the aboral territory. Knockdown of *Nvsix3/6* leads to a progressive respecification of the aboral area to a more oral identity, as indicated by the expanded expression of *Nvwnt2* toward the aboral pole and the failure of the aboral ectodermal cells to acquire their typical

columnar shape. While the available functional and expression data suggest that the aboral pole of *Nematostella* develops under the control of anterior patterning genes, there are also noticeable differences at a more detailed level, for example in the exact relation of the expression domains, which likely reflect differences in the regulatory interactions between the conserved aboral/anterior genes.<sup>58–62</sup>

Taken together, the development of the oral and aboral domains in *Nematostella* is regulated by conserved patterning genes and signaling pathways with clear similarities of oral to bilaterian posterior, and aboral to bilaterian anterior development. Despite this overall conservation many questions remain: How is Disheveled protein localized to the animal pole and how does this compare to bilaterians? What is the function of the different Wnt genes along the axis and what are their signaling mechanisms? Are other bilaterian anterior genes integrated in the aboral patterning system in *Nematostella*? Addressing these questions will allow a better understanding of the evolution of basic features of animal body plans and they will provide new insights into the logic and the diversity of patterning systems.

### BMP Signaling and the Secondary Body Axis

Traditionally, cnidarians have been described as radially symmetric animals, although bilaterally arranged internal structures have been observed in anthozoans already more than a century ago.<sup>16,63,64</sup> After the discovery of BMP signaling as a unifying mechanism for the patterning of the bilaterian dorso-ventral axis and the re-positioning of the anthozoans as the sister group to the other cnidarian clades, it became conceivable that bilaterality was already present in the last common ancestor of cnidarians and bilaterians. Studies in *Nematostella* and in the coral *Acropora millepora* indeed showed that BMP pathway genes are expressed asymmetrically along the secondary axis, the so-called directive axis.<sup>37,44,65–67</sup> Functional studies in *Nematostella* revealed that the establishment and patterning of the directive axis depend on BMP signaling, but that this pathway is employed in an unconventional manner. *Nvbmp2/4*, *Nvbmp5/8*, and the extracellular BMP regulator *Nvchordin* (*Nvchd*) are expressed radially around the blastopore at early gastrula stage, but their expression then quickly becomes restricted to one side of the mid-gastrula embryo. Surprisingly, all three genes are expressed on the same side, in partially overlapping domains in the ectoderm. The BMPs are in addition

expressed in an elongated endodermal domain on the same side<sup>65,68</sup> and another BMP-related molecule, *Nvgdf5-like*, and a BMP inhibitor, *Nvgremlin*, start being expressed in similar endodermal domains on the opposite side,<sup>44,68</sup> leading to a complex arrangement of BMPs and BMP inhibitors along the directive axis. Knockdown of the *Nvbmps* or of *Nvchd* abolishes all asymmetric gene expression, but in contrast to the situation in many bilaterians, it revealed a negative feedback of the NvBMPs on their own expression, i.e., knockdown led to strong radial expression around the blastopore.<sup>69</sup> This suggested that the domain of *Nvbmp* transcription is actually characterized by low BMP signaling activity, which was later confirmed by immunohistochemical detection of the activated form of the intracellular BMP signal transducer Smad1/5/8.<sup>15,70</sup> Interestingly, *box* genes are expressed along the directive axis in a pattern reminiscent of bilaterian anterior–posterior patterning and their expression is controlled by BMP signaling, the major bilaterian dorso-ventral patterning system.<sup>15,45,70</sup> As for the patterning of the oral–aboral axis, these findings identify an interesting mix of conserved and divergent features. While a role of BMP/Chordin signaling in secondary axis patterning is common to *Nematostella* and bilaterians, the roles of individual players and their positions in this signaling network vary considerably. These studies have revealed a similar molecular basis for the patterning of the secondary body axis; however, because the structures that are arranged in a bilateral manner in anthozoans have no counterpart in bilaterians, it remains unclear whether the directive axis of anthozoans is homologous to the dorso-ventral axis of bilaterians.

Axial patterning in *Nematostella* is directed by conserved developmental regulators with overall similar functions compared to bilaterian model systems, but there are plenty of differences in the exact deployment of these regulators. Expression profiling of animals with manipulated axial patterning and detection of direct targets of key transcription factors by ChIP-seq will likely lead to a detailed picture of the transcriptional control of axial patterning in *Nematostella*, and this will continue to provide insights into the principles of axial patterning systems and their evolution.

## OVERVIEW OF NEMATOSTELLA GERM LAYER SPECIFICATION

Triploblastic animals are characterized by the presence of three distinct germ layers called the endo-

meso-, and the ectoderm, which form during embryogenesis. During bilaterian gastrulation, the ectoderm first separates from the endomesoderm (or mesendoderm), which subsequently segregates into endoderm and mesoderm.<sup>71–73</sup> These tissues are arranged such that ectoderm surrounds the exterior of the animal and interfaces with the environment, endoderm is the innermost germ layer, and the mesoderm lies between the endoderm and ectoderm. The position of each germ layer is critical because each germ layer generates a specific set of specialized tissues during development. For example, the epidermis derives from ectoderm, the gut arises from endoderm, and blood and musculature arise from mesoderm.

Most non-bilaterian animals, with exception to ctenophores and medusa stages of a sub-group of hydrozoan cnidarians are diploblastic animals that possess only two germ layers, the inner endoderm and outer ectoderm. As a result, the evolutionary origin of bilaterian mesoderm and its derivatives is intensely debated and investigated (reviewed in Ref 74–80). As the closest diploblastic relative to the bilaterians, cnidarians can offer key insights as to the origin of the bilaterian mesoderm.

*Nematostella* (and cnidarians in general) generate an epidermis derived from the ectoderm and a gastrodermis derived from the endoderm. There are no mesenchymal muscle cells or other mesodermally derived tissues in cnidarians.<sup>75,81</sup> However, the gastrodermis is bifunctional in that it possesses absorptive cells associated with nutrient uptake and various myoepithelial cells with contractile functions.<sup>82</sup> Thus, the cnidarian endoderm has both properties and cell types that segregate into distinct endodermal and mesodermal tissues in bilaterians. Consistent, with having traditional functions associated with both the bilaterian endoderm and mesoderm, the *Nematostella* endoderm expresses a large number of genes that are historically associated with either bilaterian endodermal or mesodermal formation in triploblastic organisms such as *otx* and *gata*, respectively.<sup>74,83</sup> These observations suggest that the precursor cells of the cnidarian gastrodermis form an endomesodermal germ layer that fails to segregate into formal endoderm and mesoderm. One prediction that arises from this is that the ancestral gene regulatory network (GRN) that describes the development of cnidarian endoderm/gastrodermis gave rise to the specific regulatory network that we associate with specification of endomesoderm in bilaterian animals. Testing a hypothesis as complex as the *Nematostella* endodermal GRN potentially being homologous to the bilaterian endomesodermal GRN would have been more difficult in the pre-‘omics’ era. However, by

exploiting tools developed for studying *Nematostella* development and the ability to rapidly establish novel tools for 'omics' level studies in non-traditional model systems, researchers are building an endodermal GRN in *Nematostella* to determine if and how the cnidarian endodermal GRN gave rise to the endomesodermal GRNs of bilaterians. Addressing this question will allow researchers to better infer the origins and possible mechanisms that gave rise to the bilaterian mesodermal layer.

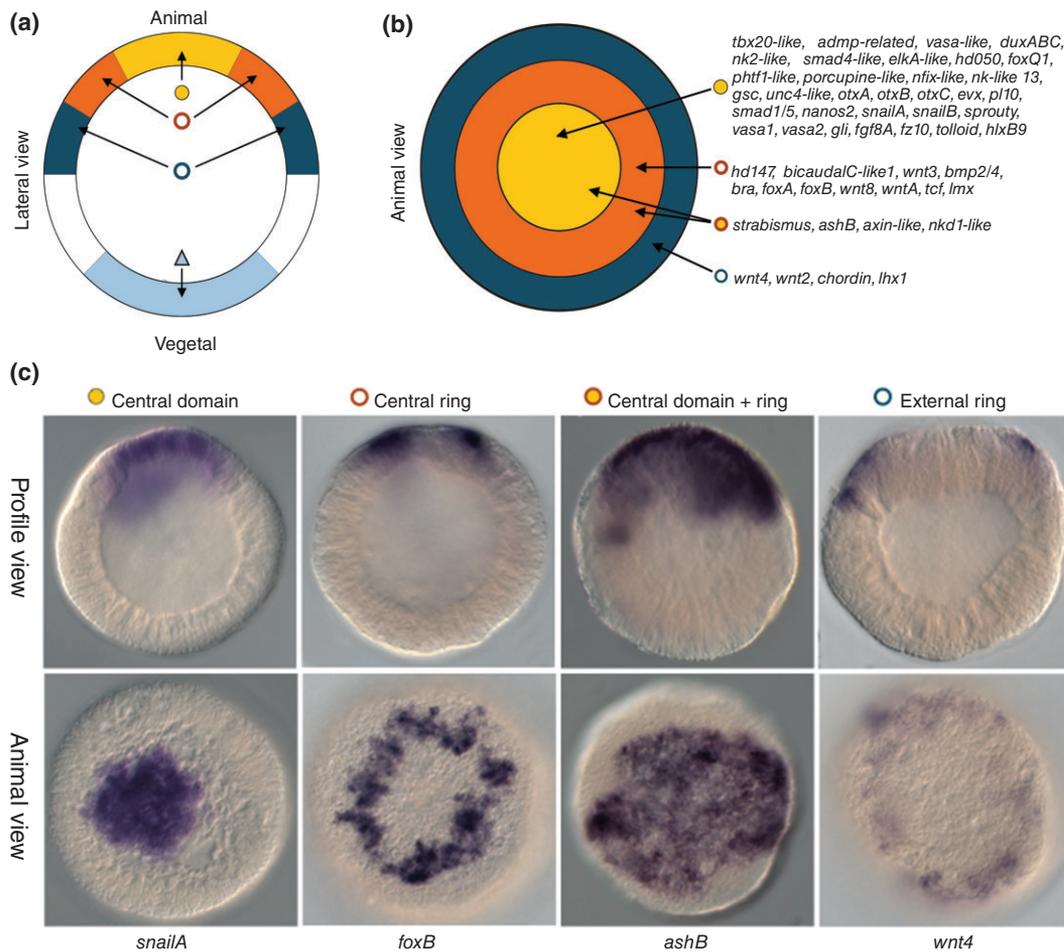
### The Endodermal GRN of *Nematostella*

Bilaterian presumptive endomesoderm cells are demarcated by nuclear  $\beta$ -catenin, which is in cooperation with the transcription factor TCF, the effector of canonical Wnt signaling.<sup>84–88</sup> Because previous reports implicated canonical Wnt (cWnt) signaling and nuclear  $\beta$ -catenin in *Nematostella* endoderm formation,<sup>8,39</sup> researchers sought to identify components of the *Nematostella* endodermal specification pathway by pharmacologically expanding nuclear  $\beta$ -catenin and thus the presumptive endoderm. Animals with increased nuclear  $\beta$ -catenin were subjected to microarray analysis to identify likely targets of  $\beta$ -catenin.<sup>40</sup> This study identified over 100 transcription factors and signaling molecules potentially downstream of nuclear  $\beta$ -catenin.<sup>40</sup> Many key genes associated with bilaterian endoderm and mesoderm formation that were previously shown to be expressed in presumptive endoderm and/or bodywall endoderm in *Nematostella* such as *snailA,B*, *otx*, *foxA,B*, *twist*, *wnts*, *tcf*, and *bmp* were identified in this array.<sup>22,36,68,74,83,89,90</sup> Identified targets were further analyzed by an mRNA *in situ* screen to characterize their wild type spatiotemporal expression patterns. The genes were expressed in one of three distinct expression domains within the animal hemisphere prior to the onset of gastrulation (Figure 4(a)–(c)). The majority of the analyzed genes were expressed in the 'central domain' that corresponds to cells around the animal pole of the blastula (Figure 4). Surrounding this central domain were two rings of expression, an inner 'central ring' that borders or partially overlaps with edges of the central ring, and the 'external ring' surrounding the central ring.<sup>40</sup> The gathered spatial expression patterns are deposited in an online database<sup>91</sup> where they are mined to predict elements of the gene regulatory network that govern endoderm formation and gastrulation movements.<sup>92,93</sup> Current fate mapping experiments clearly demonstrate that the animal pole derivatives are internalized during gastrulation.<sup>8</sup> However, it is not yet clear if cells in the central and

external ring domains are also internalized. Regardless, the majority of genes identified in the microarray represent genes expressed in presumptive endoderm. At first pass it is clear that (1) cells that internalize during gastrulation (cnidarian endoderm and bilaterian endomesoderm) share characteristic nuclear  $\beta$ -catenin, which is often linked to cWnt signaling and (2) the cnidarian endoderm and bilaterian mesoderm both express a highly overlapping suite of genes during development.

By coupling the spatiotemporal gene expression analysis with known and additional functional experiments with the 100 genes identified above researchers were able to generate a preliminary GRN describing endodermal specification at the blastula stage<sup>40</sup> for *Nematostella*. Ectopic activation of cWnt signaling induced the formation of an excess of endoderm and exogastrulation, the knockdown of NvTcf, the potential effector of this pathway, showed that NvTcf is not required for gastrulation movements but for pharynx formation.<sup>40</sup> The molecular analysis of the obtained phenotypes at the blastula stage, revealed that cWnt signaling is required for proper gene expression in all three domains of the animal hemisphere and to restrict expression of aboral gene expression.<sup>40</sup> These results suggest that the canonical Wnt pathway in *Nematostella* plays a crucial role in launching a GRN in a broad domain within the animal hemisphere (endodermal + presumptive ectodermal tissues). This is similar to what is observed in both echinoderms and *Xenopus*.<sup>94</sup> At later developmental stages, other pathways may be required to segregate ectodermal from endodermal fates within the animal hemisphere. Interestingly, the Fgf/Erk and Bmp/Smad pathways are expressed in the central domain and central ring respectively and could play a role in this process.<sup>40</sup>

While there are tremendous similarities between the *Nematostella* endoderm and bilaterian mesoderm in terms of expression patterns and upstream regulators, key differences exist that need to be considered. Most of our understanding about bilaterian endomesodermal GRNs comes from work in echinoderms, where *blimp1*, *otx*, *bra*, *foxA*, and *gataE* form a so-called 'kernel' that is critical for the endomesoderm GRN.<sup>84,95–97</sup> The 'kernel' genes are not only expressed within the endomesodermal domain of the developing sea urchin embryo, but also crucial for specification of the endo and mesodermal germ layer precursors.<sup>84,95–97</sup> Thus, we would expect these genes to be expressed in the central domain in *Nematostella*. The current assumption is that the central domain will mainly give rise to endoderm derivatives /gastrodermis, while all other domains will give rise



**FIGURE 4 |** (GRN) Co-expression domains within the animal hemisphere of the *Nematostella* blastula. (a, b) Diagram illustrating four co-expression domains within the animal hemisphere defined by differential spatial expression of the genes indicated in (b). (c) Examples of genes expression in either the central ring (*NvsnailA*), the central domain (*NvfoxB*), the central domain + ring (*NvashB*), or the external ring (*Nvwnt4*). In situ hybridization expression profiles are shown in either lateral view (a, top row of c) or animal view (b, bottom row of c) to distinguish expression domains. (Reprinted with permission from Ref 40. Copyright 2012 PLoS Genetics)

to ectodermal derivatives, such as the pharynx.<sup>36</sup> Two genes, *Nvotx* and *Nvsnail*, are expressed in the central domain. However, *Nvwnt8*, *NvfoxA*, and *Nvbrachyury* are expressed in the central ring at the blastula stage. In *Nematostella*, a *blimp-like* gene expressed in the animal hemisphere prior to gastrulation has yet to be identified, and *Nvgata* is expressed in individual cells throughout the ectoderm<sup>74</sup> suggesting that neither of these critical ‘kernel’ genes are involved in cnidarian endodermal specification. Based on expression, the only ‘kernel’ gene definitively expressed in the correct region to be specifying the endoderm in *Nematostella* is *Nvotx*, but the central ring expression of *NvfoxA* and *Nvbrachyury* at the blastula stage does not exclude them as possible endodermal GRN regulators during earlier developmental steps. Are these genes also part of the

ancestral ‘endomesodermal’ GRN or do they belong to a GRN that specifies ectodermal domains in the future mouth/pharynx? Functional studies in conjunction with modern cell or tissue tracking techniques (photoconversion, tissue-specific inducible GFP expression) are required to determine the fate of the above described expression domains and to link them to specific GRNs.

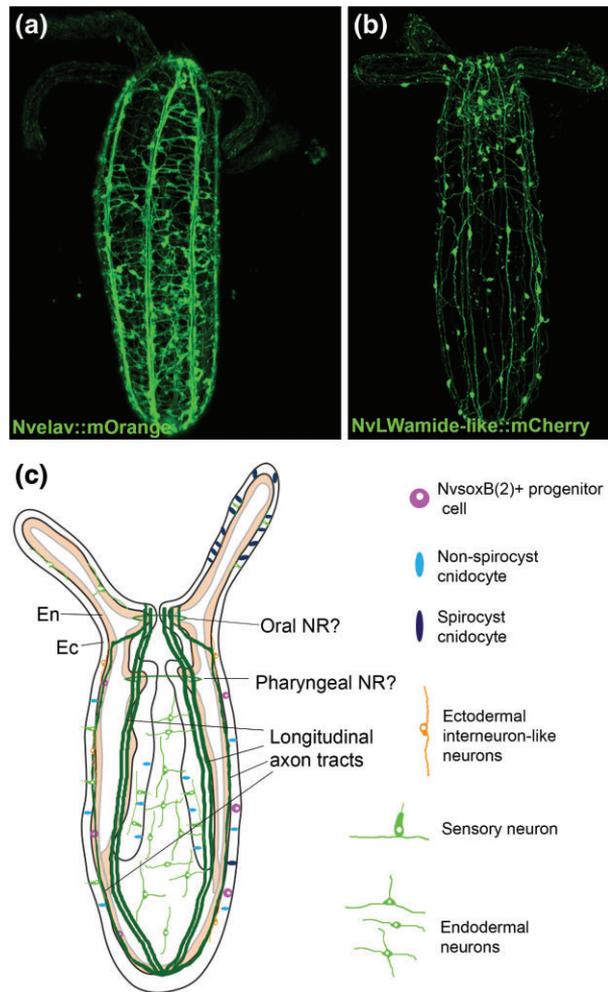
Taken together, it is reasonable to conclude that the bilaterian endomesodermal GRN is derived from an ancestral GRN that also gave rise to the cnidarian endodermal GRN. Nuclear  $\beta$ -catenin and/or canonical Wnt activity is a critical upstream regulator in both GRNs, and they have a highly overlapping suite of genes that define or are functionally required for formation of the endoderm or endomesodermal tissue. It is not yet clear if differences between the

bilaterian endomesodermal GRN and cnidarian preliminary endodermal GRN are going to be sufficient to explain why endomesoderm separates into two distinct tissues and the cnidarian endoderm does not. To address this question in more detail, gene-specific functional studies of these pathways as well as of other transcription factors such as *Nvbrachury* and *NvfoxA* are required to better understand the architecture and the molecular mechanism underlying this cnidarian endodermal GRN and its developmental outputs. For instance do subsets of the later forming bilaterian mesodermal GRN regulate myoepithelial cells. While we begin to get a basic understanding of the wiring of this GRN, future work needs to take into account the dynamics of the regulatory state over time as well as the cis-regulation that underlies this process. This will enable the community to compare these findings with described GRNs from echinoderms and *Xenopus* and determine the mechanistic (i.e., presence of positive/negative feedback loops, lock-down mechanisms of given regulatory states, etc.) similarities or differences that led to the evolution of distinct endodermal and mesodermal germ layers in bilaterians.

## OVERVIEW OF *NEMATOSTELLA* NERVOUS SYSTEM

Most agree that the nervous systems of bilaterians (the central and peripheral nervous systems) are derived from a nerve net-like ancestral nervous system.<sup>98,99</sup> Thus, better understanding development of nerve nets in *Nematostella* and other cnidarians will inform us about the structure and molecular make up of the ancestral nervous system that gave rise to the cnidarian nerve net and the bilaterian nervous systems. Here we will discuss the architecture and development of the *Nematostella* nervous system (Figure 5).

Neurons in *Nematostella* are born in both ectoderm and endoderm,<sup>19,21</sup> and neural soma are intermixed with other cell types. Although, cnidarian nervous systems are described as being a nerve net, there are clear examples of neural condensations and neuropil-like structures in multiple cnidarian species. For example, longitudinal tracts of neurites run along the mesenteries in *Nematostella*<sup>18,21</sup> (Figure 5(a)–(c)). In addition, oral and pharyngeal nerve rings have been reported<sup>18</sup> (Figure 5(c)). It should be noted that while cross reactive antibodies used to identify the pharyngeal and oral nerve rings showed a slightly higher concentration of neurons in a location consistent with that of a putative oral and pharyngeal nerve



**FIGURE 5** | Morphology of *Nematostella* nervous system in juvenile polyps. (a, b) 3D reconstruction of confocal Z-series acquired for two *Nematostella* transgenic lines that label the nervous system (a) NvElav::mOrange and (b) NvLWamide-like::mCherry. (c) Schematic of juvenile polyp nervous system highlights key structures and provides examples of neuronal morphologies observed in transgenic animals.

ring<sup>18</sup> recent data with transgenic lines, such as *Nvelav::morange*, find relatively uniform neural concentrations along the length of the oral–aboral axis, and thus it is not yet definitive that oral and pharyngeal nerve rings are present. *Nematostella* possesses sensory and interneurons as well as myoepithelial cells<sup>18,21</sup> suggesting that dedicated inter-, sensory, and muscle innervating neurons are present in the *Nematostella* nerve net. In addition, *Nematostella* possesses neurosecretory-like gland cells,<sup>100</sup> and a cnidarian-specific neural cell type the cnidocyte.<sup>17</sup> Cnidocytes are considered neural cells because they display mechanosensory and calcium dependent ultrafast exocytosis neural-like properties.<sup>101</sup> Cnidocytes are unique to cnidarians and offer an opportunity to

investigate the evolution novel cell types (Box 1). Identification and characterization of the neuronal classes in *Nematostella* will provide better understanding as to which neurons are interneurons, sensory, motoneurons, and what distinctive properties each class has (neurotransmitter type, receptors, molecular markers, etc.).

## Mechanisms of Neurogenesis

### Neural Induction

Understanding neural induction in *Nematostella* is challenging, because no distinct neural structure like a central nervous system (CNS) exists, and we do not know what the earliest definitively neural gene expression is yet. As a result, how to assay induction phenotypes is unclear. Current data argue that *Nematostella* and bilaterian neural induction occurs via distinct mechanisms. Inhibition of *bmp2/4* (also called Dpp) in the neural ectoderm via Nog and Chd is the most conserved neural induction mechanism in bilaterians. Thus *bmp2/4* homologs suppress central nervous system formation in bilaterians. *Nvbmp2/4* patterns the directive axis and together with *Nvbmp5/8* patterns endoderm. However, loss or gain of *Nvbmp2/4* doesn't disrupt embryonic neural expression in the ectoderm.<sup>69,102</sup> On the other hand, loss or gain of *Nvbmp2/4* activity results in loss of at least a subset of endodermal neurons in planulae.<sup>69,102</sup> In *Nematostella*, it appears NvBmp2/4 is required for endodermal neural development, but can act as an inhibitor at high doses. One caveat to these studies is that loss of later neural expression is more likely due to broader defects in patterning resulting from loss of *Nvbmp2/4* at earlier stages. Truly understanding neural induction will require identification of the earliest markers that distinguish committed neural cell types followed by a systematic assessment of potential upstream inputs that regulate their expression.

### Embryonic Neurogenesis

Many of the core neurogenic programs acting downstream of induction in bilaterians function during *Nematostella* neurogenesis. Neurons arise in the ectoderm at early gastrula stages.<sup>18,19,21</sup> *NvashA* and *NvsoxB(2)*, homologs of the bilaterian neural transcription factors, promote development of embryonic ectodermal neurogenesis.<sup>19,103</sup> *NvashA* is a homolog of *achaete-scute* family bHLH proneural transcription factors. bHLH proneural genes encompass both *atonal* and *achaete-scute* family transcription factors. Bilaterian proneural genes are necessary and

## BOX 1

### CNIDOCYTES AND THE EVOLUTION OF NOVEL CELL TYPES

Cnidocytes are stinging cells found only within the cnidarians. They represent one of the few examples of an unequivocal evolutionary novelty, and thus investigating their composition and development can inform us about the mechanisms by which novel cell types arise. Cnidocytes possess a unique organelle called a cnidocyst that is comprised of a harpoon wound up inside of a capsule. The capsule is generated by a number of cnidarian-specific minicollagen genes. To date functional dissection of the molecular programs that generate cnidocytes is limited. Disruption of Notch signaling inhibits proper differentiation of cnidocytes in *Hydra*<sup>126</sup> and reduces the number of mature cnidocytes in *Nematostella*.<sup>111</sup> In *Nematostella* the neural transcription factors *NvsoxB(2)* and a unique splice isoform of the mesodermal transcription factor *Nvmef2* are required for proper specification of cnidocytes.<sup>30,103</sup> From these limited studies, we can infer that the evolution of this novel cell type likely required the emergence of a novel gene family (the minicollagens) and co-option of pre-existing developmental transcription factors. Interestingly, there are three classes of cnidocytes and some studies suggest species-specific cnidocyte molecular components. This cell type is ripe for further studies focused on understanding the make up and development of cnidocytes which will provide insight into the how novel proteins emerge, and how pre-existing developmental programs interact with new genes to generate novel cell types.

sufficient to induce neural development (summarized in the study of Bertrand, 2002).<sup>104</sup> *Nematostella* has four *achaete-scute* genes and at least seven *atonal-like* genes encoded in its genome.<sup>105</sup> *NvsoxB(2)* is a HMG-box *sox* family transcription factor related to the bilaterian *soxB1* and *soxB2* families. *sox* genes are expressed in developing neurons in bilaterians.<sup>106–108</sup> In particular, *sox* gene expression in neural stem and progenitor cells is well known in neurogenesis of multiple bilaterian species as well as in other cnidarians.<sup>109</sup> There are multiple *sox* gene families that function in neurogenesis as well as development of other tissues. *Nematostella* has 14 *sox* genes, two of which have reported roles in

neural development.<sup>102,103,110</sup> Morpholino knock-down of either *NvashA* or *NvsoxB(2)* results in a reduction of *NvanthoRFamide* and *Nvelav1* expression, and *NvsoxB(2)* morphant animals also show reduced numbers of cnidocytes.<sup>19,103</sup> Global misexpression of *NvashA* induces ectopic expression of neural subtype markers, but interestingly only within their respective domains.<sup>19</sup> Additional work has shown that Notch activity can act to suppress neural development by suppressing *NvashA*, and that Notch activity also suppresses expression of *NvsoxB(2)*.<sup>111,112</sup> These data broadly suggest that neurogenic transcription factors in *Nematostella* function similarly to their bilaterian homologs. Teasing out detailed regulatory networks that promote general neuronal properties and neural subtype development will improve our understanding of the level of conservation between molecular mechanisms governing neurogenesis in cnidarians and bilaterians.

Analysis of *NvsoxB(2)* reporter transgenes determined that as many as 30% *NvsoxB(2)* expressing cells incorporate the thymidine analog EdU in pulse labeling experiments and form clones suggesting that a subset of the *NvsoxB(2)* + cells are proliferative neural progenitor cells.<sup>103</sup> However, it is not entirely clear how widespread deployment of neural progenitor cells is, or how many divisions progenitors are capable of undergoing. Further advances in techniques to label and track individual clones *in vivo* will provide greater detail in future studies. Still missing from our understanding is the origin of the neural progenitor cells. Are neural progenitor cells derived from a dedicated neural stem cell, a multipotent stem cell, or from epithelial cells (like *Drosophila* sensory organ precursors)? Regardless, the presence of progenitor cells in *Nematostella* suggests that not only are the molecular mechanisms of neurogenesis conserved between bilaterians and *Nematostella*, but also the cellular dynamics are also conserved between these groups.

### Larval Neurogenesis

Beginning at late gastrula and early larval stages neural marker gene expression is detected in the endoderm. Throughout larval stages neurogenesis occurs in endoderm and ectoderm. Here too *sox* and bHLH proneural genes have demonstrated roles in neural development. Morpholino mediated knockdown of *NvsoxB(2)* also reduced the number of endodermal neurons labeled by the *Nvelav1* transgenic reporter.<sup>21,103</sup> A more recent study identified an oral population of ectodermal *NvanthoRFamide* labeled neurons and a small population of asymmetrically positioned *NvGLWamide* oral endodermal

neurons.<sup>102</sup> These neurons are patterned by *NvsoxB(2a)* activating expression of *NvashB*, which in turn activates expression of an atonal-like bHLH proneural gene. The *NvGLWamide* neurons specifically require additional positive inputs from *Nvarp6*, which is asymmetrically expressed in the domain that gives rise to the *NvGLWamide* positive cells. Notch activity was also shown to suppress endodermal expression of *NvashA* suggesting that Notch also acts upstream of neurogenic transcription factors during larval endodermal neurogenesis.<sup>112</sup> *NvsoxB(2)* + precursors were identified in the larval endoderm and ectoderm, suggesting that larval neurogenesis occurs via specification of neural progenitor cells.<sup>103</sup> In total the current data argue that molecular mechanisms responsible for neurogenesis are the same in both endoderm and ectoderm. Thus understanding neural development in either tissue will inform about the ancestral neurogenic program that gave rise to the bilaterian nervous systems.

### Mechanisms of Neural Patterning

Neural subtype patterning likely occurs via the combinatorial action of regional patterning and neural differentiation pathways.<sup>18–21,37,41,52</sup> This is most apparent in specification of the apical tuft sensory organ.<sup>20,37,41,113,114</sup> Additional evidence comes from the regional distribution of distinct neural subtypes. Cnidocyte subtypes and molecular markers for ectodermal neurons are differentially distributed along the oral–aboral axis suggesting their identity is tied to regional patterning cues.<sup>17–20,102</sup> This hypothesis is supported by the observation that ubiquitous misexpression of *NvashA* increases the number of neurons born throughout the embryo, but individual neural subtype markers only expand within the limits of their normal domain boundaries.<sup>19</sup> Lastly, patterning of the directive axis restricts *Nvarp6* to one side of the endoderm where it specifies *NvGLWamide* + neurons.<sup>102</sup> Taken together, the current model is that neurogenic programs are induced more or less globally, but that the combinatorial action of regional patterning and neurogenic transcription factors act to produce distinct fates. This is similar to bilaterian CNS development in that a broad neural program is initiated to neuralize a subset of the ectoderm, which is followed by subdivision by regional patterning information to generate distinct domains that produce specific fates. To date our understanding about how regional patterning genes act to generate specific fates is relatively poor in *Nematostella* and further work is needed before we can compare

details of neural patterning between *Nematostella* and bilaterians to each other.

The conserved activity of neurogenic transcription factors between *Nematostella* and bilaterian neural programs is clear, suggesting that the cnidarian nerve net and bilaterian nervous systems are derived from a common ancestor. There is a tremendous amount of speculation about how central nervous systems evolved from something that resembled a cnidarian nerve net. Ideas range from the oral nerve ring in cnidarians being a precursor to bilaterian nerve cords, the idea that the brain originated from the ectodermal nervous system in the aboral region of cnidarian embryos (see above),<sup>115</sup> and possibly the most speculative that bilaterian nervous systems may have a common origin with the endodermal nerve net.<sup>116</sup> To resolve these hypotheses, current research must build on the foundational studies and identify individual neuronal cell types within cnidarians and determine if definitive homology can be drawn between cnidarian and bilaterian neuronal classes. Also, a better characterization of the molecular basis of neural induction in cnidarians must be completed and compared in detail to the bilaterian neural inductive programs. Regardless, of the answers investigating the cnidarian nerve net will improve our understanding of how ancestral nervous systems were patterned and functioned during animal evolution.

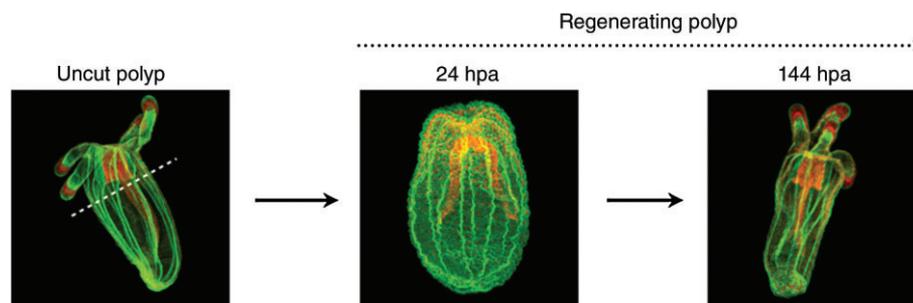
## OVERVIEW OF REGENERATION

*Nematostella* has the potential to distinguish itself as a model to investigate the relationship between development and regeneration. Increasing evidence suggests that regeneration does not faithfully recapitulate development.<sup>117–119</sup> Not to say completely novel molecular programs are deployed during regeneration, but rather subtle differences that likely reflect the unique challenge of regeneration are

involved. Being able to directly investigate and compare development and regeneration in the same species is critical to better understand why and how these processes vary from one another. One reason comparisons between development and regeneration are not more widespread is that most traditional model systems are ideal for studying development or regeneration, but not both. *Hydra*, planarians, and axolotls are the most well known regenerative animal models, but they lack the ability to reliably and cost effectively obtain large numbers of embryos or they lack critical tools to manipulate and/or visualize gene function. Traditional model systems (mouse, *Caenorhabditis elegans*, *Drosophila*, chicks, *Xenopus*, etc.) display little or no regenerative capacity. Zebrafish does offer a powerful developmental system that also displays impressive regenerative capacity.<sup>120</sup> However, most vertebrates are not as regenerative as zebrafish, which suggests that they may have independently derived their regenerative ability. Thus, developing model systems that complement the current regenerative model animals including zebrafish will allow conserved mechanisms of regeneration in animals to be identified. Moreover, by comparing how regeneration and differentiation vary from one another in multiple species, it may be possible to better understand why regeneration does not recapitulate development and use that knowledge to improve design and deployment of regenerative medical therapies. The power of *Nematostella* as a developmental system has been discussed above, but they are also highly regenerative capable of whole body axis regeneration in less than 1 week (Figure 6) suggesting that it is a good choice as a model system to compare development and regeneration.<sup>34,121</sup>

## *Nematostella* Regeneration

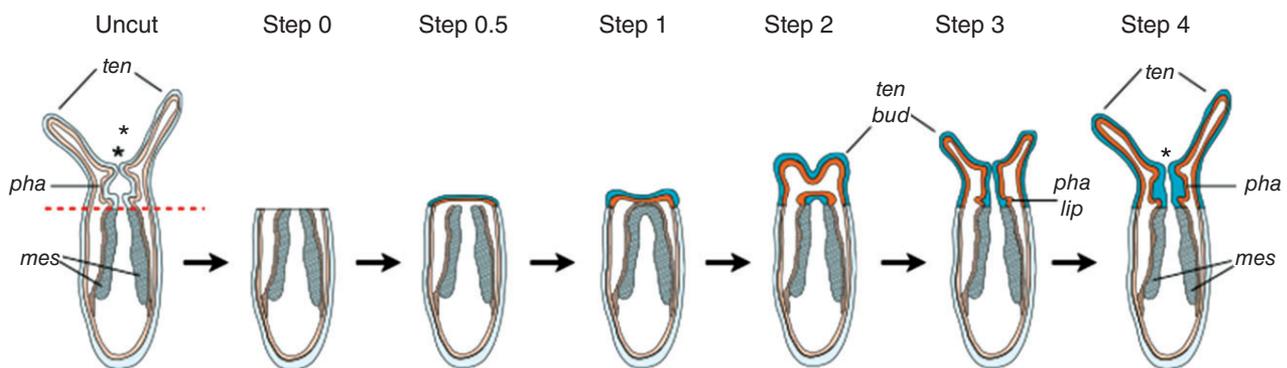
*Nematostella* regeneration is either experimentally or naturally induced by bisection of the animal into two



**FIGURE 6** | Oral regeneration of juvenile *Nematostella* takes place in 6 days. After sub-pharyngeal bisection, missing oral part of the polyps regenerate within 6 days to reform a fully functional organism. Animals were fixed and stained with phalloidin (green) to show f-actin filaments and propidium iodide (red) to visualize the nuclei. All images are lateral views with the oral part to the top.

halves or by physal pinching, respectively. The latter phenomenon is normally initiated by animals undergoing asexual reproduction via transverse fission of the most aboral region (the physa). Unlike *Hydra*, cell proliferation is required for *Nematostella* regeneration,<sup>121</sup> and preliminary studies show that after bisection or physal pinching characteristic events occur during regenerative growth.<sup>34,121</sup> Although recent efforts have focused on characterization of morphological, cellular, and molecular events that occur during *Nematostella* wound-healing and regeneration, these studies remain sparse and are heterogeneous in their experimental approaches (different ages of animals used, different sites of bisections, variable culture temperature, etc.).<sup>33,121–124</sup> A universal protocol or normalized staging series would allow for direct and more accurate comparisons to be drawn between *Nematostella* regeneration, asexual reproduction, and regeneration in other animals. A first attempt to define a normalized staging series for adult *Nematostella* regeneration after supra-physal amputation has been recently presented by Bossert and colleagues.<sup>34</sup> While this study is an important resource, the chosen amputation site is unusual and adult tissue is more difficult to analyze due to the size and opacity of tissue compared to juveniles. In an

effort to have a common foundation for comparing experimental results for scientists working on *Nematostella* regeneration, Amiel and colleagues have compared sub-pharyngeal oral regeneration in juveniles and adults and shown that there are no age but temperature-specific variations in the timing of this process. The reassessment of cell proliferation revealed that mitotic cells are detected as soon as 12 hpa at the amputation site in juveniles and that cellular proliferation is also required for oral regeneration in adults suggesting that the cellular mechanisms underlying this process may be shared between juveniles and adults.<sup>32</sup> In addition, the authors have developed an *in vivo* assay to analyze the wound-healing success and showed that after amputation the wound is completely healed 6 hpa. A detailed morphological analysis of regenerating juveniles revealed a characteristic four-step process (Figure 7).<sup>32</sup> After amputation (1) the mesenteries fuse to each other and enter in contact to the amputation site. When the tentacle buds become visible, (2) the mesenteries are pulled/pushed down to create a gap between the amputation site and the mesenteries where high proliferation is detected and where (3) the pharyngeal lip starts to form, followed by (4) the completion of pharynx formation. Interestingly, the beginning of



Step 0	wound open, mesenteries neither in contact with one another, nor with the amputation site
Step 0.5	wound healed, mesenteries neither in contact with one another, nor with the amputation site
Step 1	mesenteries in contact with one another as well as with the amputation site
Step 2	creation of a gap between mesenteries and oral epithelia, tentacle buds appear
Step 3	appearance of 488+ autofluorescence, pharyngeal lip begins to form, tentacle buds elongate
Step 4	fully formed pharynx, mouth opening, elongated tentacles, regenerated animal

**FIGURE 7** | Diagram summarizing the morphological and cellular events underlying *Nematostella* oral regeneration. The table below the illustration provides definitions for the various regeneration steps. \*, mouth opening; ten, tentacles; ten bud, tentacle bud; pha, pharynx; pha lip, pharyngeal lip; mes, mesenteries. (Reprinted with permission from Ref 32. Copyright 2015 International Journal of Molecular Sciences)

pharynx formation between 60 and 72 hpa is accompanied by the appearance of green autofluorescence that can be used as an *in vivo* assay to determine successful pharynx formation/regeneration. Taken together, this analysis laid down a basic framework to study oral regeneration after sub-pharyngeal amputation in *Nematostella* juveniles by developing assays and defining specific landmarks of this process that can be used to analyze the effects of various perturbation experiments.<sup>32</sup> While both adult or juvenile staging systems allow improved comparison of results obtained from different research groups investigating *Nematostella* regeneration, they are first drafts that need to be improved in the future with additional details (e.g., molecular markers). Importantly, the present information can also serve to define (or to compare with) different regeneration types, such as aboral regeneration that will provide additional information on the conservation/divergence of the overall mechanisms underlying the reformation of missing body parts in *Nematostella* at the molecular, cellular, and tissue level.

### ***Patterning Mechanisms During Nematostella Regeneration***

One of the main questions that can be addressed in *Nematostella* is how patterning during regeneration relates to patterning during development. To date only two papers have compared developmental and regenerative patterning in *Nematostella*. Briefly, *Nematostella* shows that major patterning molecules are similarly deployed during development and regeneration, but that subtle differences in regeneration are observed. In *Nematostella*, high Wnt activity is used to specify oral fates during development (discussed above). Hyperactivation of Wnt activity during regeneration is sufficient to induce complete ectopic oral regeneration in aboral territories.<sup>125</sup> This observation argues that high Wnt activity also specifies oral fates during regeneration. A second study specifically assayed expression of a number of transcription factors with distinct patterns during development.<sup>33,124</sup> The authors of that work identified two classes of genes. One class displayed regenerative expression patterns that more or less resembled the spatial expression pattern each gene displayed during development. A second class, however, showed no expression or distinct expression patterns during regeneration that varied from their pattern during development. Although, studies of *Nematostella* regeneration are still preliminary, they do highlight that a common observation in animals is that

regeneration varies albeit slightly from development. Given the fact that embryonic development is triggered by fertilization of the totipotent oocyte and regeneration is initiated by physical amputation of variable amounts of differentiated and stem cells, it is not surprising that regeneration does not simply recapitulate development. Regeneration requires identification of what is missing and what of the adult body remains, enacting a program to replace the missing cells/tissues, stopping regrowth at the appropriate time, and finally reintegration of new tissues and cell types into an already existing adult body. Identification and characterization of differences between development and regeneration will improve our understanding of what is required for successful regeneration and possibly aid in designing and deploying effective regenerative medical therapies.

## **CONCLUSION**

*Nematostella* was originally developed to better understand the origin and evolution of bilaterian characteristics and animals in general. Those studies yielded an understanding of the mechanisms that pattern the oral–aboral and directive axes of *Nematostella*, a preliminary GRN for formation of the bifunctional endoderm that likely gave rise to the endomesoderm of bilaterians, and the identification and understanding of conserved molecular programs that specify neurons in cnidarians and bilaterians. These three areas of research have been the primary focus of *Nematostella* research, and they provide an initial understanding of how the cnidarian body plan, tissues, and cell types are patterned during development. What we have learned is that while many of the most upstream components necessary to pattern similar structures and cell types in bilaterians are enacted in *Nematostella*, key differences are present. By further investigating these differences, we will not only gain a better understanding of the ancestral state from which bilaterian evolution occurred, but also gain critical insights into the mechanisms by which animals control patterning and cell specification during development to arise at the variety of forms present in extant taxa. In addition, with the emergence of *Nematostella* as a model system, it is now possible to focus on new questions such as understanding the relationship between mechanisms deployed during development and regeneration of identical structures. The future of *Nematostella* is a bright one. Ease of culture and embryo manipulation

make *Nematostella* accessible to essentially any one interested in incorporating this exciting new model into their research program. New perspectives are

likely to open novel avenues into which *Nematostella* can inform us about metazoan biology, evolution, and biomedical applications.

## FURTHER READING

Rigo-Watermeier T et al. Functional conservation of *Nematostella* Wnts in canonical and noncanonical Wnt-signaling. *Biol Open* 2012, 1:43–51.

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