Antagonistic BMP–cWNT signaling in the cnidarian *Nematostella vectensis* reveals insight into the evolution of mesoderm

Naveen Wijesena[a,1], David K. Simmons[a], and Mark Q. Martindale[a,2]

[a]Whitney Laboratory for Marine Bioscience, University of Florida, Saint Augustine, FL 32080

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Gastrulation was arguably the key evolutionary innovation that enabled metazoan diversification, leading to the formation of distinct germ layers and specialized tissues. Differential gene expression specifying cell fate is governed by the inputs of intracellular and/or extracellular signals. Beta-catenin/Tcf and the TGF-beta bone morphogenetic protein (BMP) provide critical molecular signaling inputs during germ layer specification in bilaterian metazoans, but there has been no direct experimental evidence for a specific role for BMP signaling during endomesoderm specification in the early branching metazoan *Nematostella vectensis* (an anthozoan cnidarian). Using forward transcriptomics, we show that beta-catenin/Tcf signaling and BMP2/4 signaling provide differential inputs into the cnidarian endomesodermal gene regulatory network (GRN) at the onset of gastrulation (24 h postfertilization) in *N. vectensis*. Surprisingly, beta-catenin/Tcf signaling and BMP2/4 signaling regulate a subset of common downstream target genes in the GRN in opposite ways, leading to the spatial and temporal differentiation of fields of cells in the developing embryo. Thus, we show that regulatory interactions between beta-catenin/Tcf signaling and BMP2/4 signaling are required for the specification and determination of different embryonic regions and the patterning of the oral–aboral axis in *Nematostella*. We also show functionally that the conserved "kernel" of the bilaterian heart mesoderm GRN is operational in *N. vectensis*, which reinforces the hypothesis that the endoderm and mesoderm in triploblastic bilaterians evolved from the bifunctional endomesoderm (gastrodermis) of a diploblastic ancestor, and that slow rhythmic contractions might have been one of the earliest functions of mesodermal tissue.

cell signaling | cell fate | evodevo | heart kernel | gene regulatory network

Gastrulation is the morphogenetic event in metazoan embryonic development that leads to germ layer specification and organismal axial patterning (1). The complex cell signaling events that regulate both morphogenetic and cell-type specification is controlled temporally and spatially by complex networks of hierarchical regulatory inputs and outputs referred to as gene regulatory networks (GRNs) (1–5). During germ layer specification in triplobloblastic bilaterians with three germ layers (ectoderm, mesoderm, and endoderm), early endomesoderm (the embryonic precursor of both endoderm and mesoderm) induction is followed by the segregation of endodermal (e.g., lining of the gut and its derivatives) from mesodermal (e.g., muscle, connective tissue, kidney, somatic gonad, and coelomic lining) derivatives. Generally accepted to have evolved at the base of the bilaterian lineage, the presence of a mesoderm has played a crucial role in the evolution of complex animal body plans (5, 6). GRNs regulating initial embryonic endomesoderm specification are relatively well known in a variety of model bilaterian systems (7–12). Canonical Wnt (cWnt) signaling (13–17) and BMP signaling (18–21) play crucial regulatory roles during axis patterning and endomesoderm specification in bilaterians.

To characterize the molecular basis for the evolution of distinct mesodermal and endodermal tissues from an ancestral bifunctional endomesoderm, we studied the development of the diploblastic anthozoan cnidarian, *Nematostella vectensis*. Cnidarians are the sister group to the triploblastic bilaterians (Fig. 1*B*) and although anthozoans possess clear signs of bilaterality (22), their adult body plan contains only an outer epidermis and an inner bifunctional gastrodermis that lines the gastric cavity and has both absorptive and contractile properties (Fig. 1*A*). As in bilaterians, cWnt signaling plays a critical role in regulating gastrulation and germ layer specification in *N. vectensis* (Fig. 1*A*). A protein called beta-catenin becomes localized to the nucleus in the blastomeres of the animal hemisphere, the location of presumptive endomesoderm formation, through the function of Disheveled, a key cytoplasmic component of the Wnt signaling pathway. Stabilization of beta-catenin, along with its coactivator TCF activates downstream target genes of the endomesodermal GRN (23–26) (Fig. 1*A*). Based on the expression of downstream target genes of cWnt signaling, the animal hemisphere contains at least four different domains: the central domain, the central ring, the central domain + ring, and the external ring before the onset of gastrulation (23–25) (Fig. 1*C*). Although BMP2/4 signaling in *Nematostella* has been studied during the formation of the directive axis (26–29) and implicated in a role in endomesoderm specification (26–28) (Fig. 1*A*), there has been no direct experimental evidence for a specific role played by BMP signaling during endomesoderm specification or for interactions with cWnt signaling.

Significance

Definitive mesoderm (e.g., muscle cells) evolved in the ancestor of the bilateria but is not present in their sister group, Cnidaria. Forward transcriptomics and gene knockdown in the anthozoan *Nematostella vectensis* show that both cWnt and BMP 2/4 signaling pathways reciprocally regulate components of the endomesodermal gene regulatory network (GRN) during development to set up distinct regional territories prior to the onset of gastrulation. Furthermore, the conserved "kernel" of the bilaterian heart mesoderm GRN is operational in the endomesoderm of the anthozoan *N. vectensis*. This GRN fails to have a "lockdown" feedback loop found in bilaterian GRNs that may help explain the highly regenerative potential of adult cnidarian endomesoderm, suggesting that the endoderm and mesoderm arose from the bifunctional endomesoderm.

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[1]Present address: Faculty of Applied Science, Rajarata University of Sri Lanka, Mihintale 50300, Sri Lanka.

[2]To whom correspondence should be addressed. Email: mqmartin@whitney.ufl.edu.

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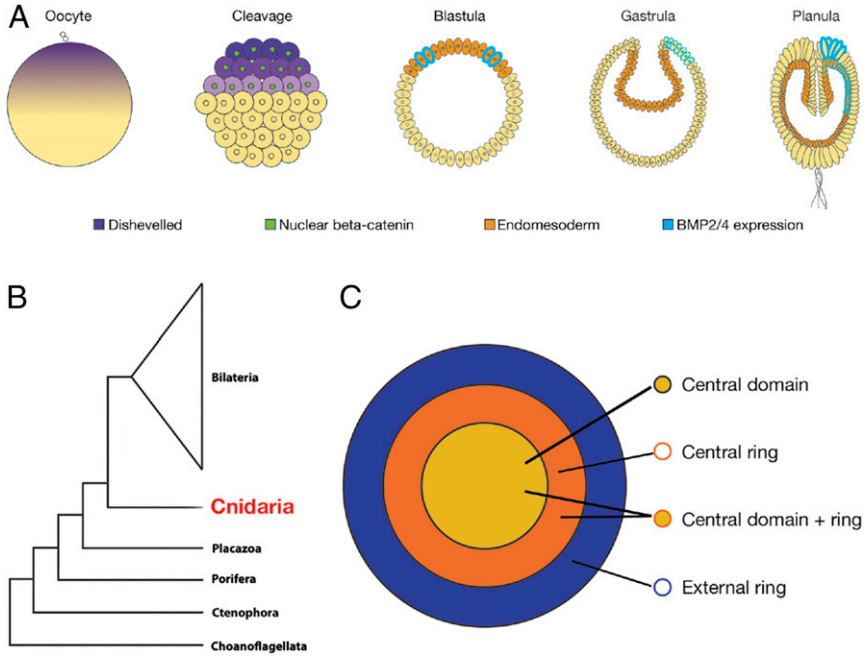

Fig. 1. Gastrulation and germ layer specification in *Nematostella*. (*A*) cWnt signaling provides inputs into the endomesodermal GRN in *Nematostella* to activate endomesodermal gene expression in the animal half blastomeres. BMP2/4 expession during early and later development in *Nematostella* suggests that it might be playing different regulatory roles during development. (*B*) Phylogenetic relationships among major metazoan lineages showing the position of Cnidaria as the sister taxon to all bilaterians (61). (*C*) At 24 hpf (late blastula stage) the animal hemisphere contains at least four domains defined by differential gene expression (25).

Results

NvBMP2/4 Is Required for Normal Gastrulation Movements and Germ Layer Segregation in *Nematostella*. *NvBMP2/4* shows localized expression in the animal hemisphere from early in development, indicating a potential role in gastrulation and germ layer segregation (27, 28). To test this hypothesis, we injected a translation-blocking *NvBMP2/4* antisense morpholino (NvBMP2/4-MO) (26) into uncleaved zygotes to knockdown NvBMP2/4 protein levels in developing *Nematostella* embryos and collected midgastrula-stage embryos [48 h postfertilization (hpf)] and analyzed them using scanning confocal microscopy. Results of this analysis showed that both NvBMP2/4-MO–injected embryos and control-MO–injected embryos had undergone the initial stages of gastrulation morphogenesis normally, but the *NvBMP2/4* morphant embryos were unable to form an organized epithelial endodermal layer (Fig. 2*A*). Both control and morphant embryos showed normal development of the ectoderm (Fig. 2*A*). This phenotype was highly reproducible and was similar to the phenotype seen in embryos overexpressing a dominant negative (dn) form of *NvDishevelled* that inhibits Wnt/beta-catenin signaling (24, 30). This similarity in phenotypes pointed to a potential role for *NvBMP2/4* in germ layer specification in *Nematostella* embryos through an interaction with canonical Wnt signaling.

cWnt and BMP2/4 Signaling Provides Reciprocal Inputs to the Cnidarian Endomesodermal GRN. We then specifically investigated the roles of both cWnt signaling and BMP2/4 signaling in endomesodermal patterning at the onset of gastrulation in *Nematostella* (24 hpf) to determine whether these two pathways are providing differential inputs to the cnidarian endomesodermal GRN, and if so, whether the interaction between these two signaling pathways provides any insight into the evolutionary origins of distinct mesodermal and endodermal tissues. We used RNA-sequencing (RNA-seq) to identify differential inputs into the GRN through beta-catenin/TCF (cWnt) signaling and BMP2/4 signaling in the developing *Nematostella* embryo. The dataset consisted of three different transcriptomes with three biological replicates: one from NvTcf-deficient embryos (by injection of a dominant negative mRNA form of NvTcf that lacks the transactivation domain, one from BMP2/4-deficient embryos (by injection of antisense morpholino-injected embryos), and one from dextran-injected control embryos. This analysis identified a set of 271 genes that were up-regulated in embryos in which beta-catenin/TCF signaling was inhibited (Table S1) and another set of 227 genes that were down-regulated in the same embryos (Table S2). In embryos with reduced BMP2/4 signaling, 147 genes showed increased expression levels (Table S3), whereas 133 genes were down-regulated, compared with control embryos (Table S4). Interestingly, there was no overlap between genes being up-regulated or down-regulated, between embryos with decreased cWnt signaling compared with decreased BMP2/4 signaling. Instead, a subset of genes was differentially regulated reciprocally by Tcf signaling and BMP2/4 signaling (up-regulated by Tcf and down-regulated by BMP2/4 and vice versa) (Fig. 2*C*). Eighty-six genes showed increased expression levels in cWnt signaling-deficient embryos and showed decreased expression levels in BMP2/4 signaling-deficient embryos (Table S5). Another 57 genes were down-regulated in cWnt signaling-deficient embryos while being up-regulated in BMP2/4 signaling-deficient embryos (Table S6). Furthermore, some of the differentially regulated genes are already known to play a critical role in specifying the oral–aboral axis, highlighting a crucial early function of BMP2/4 signaling before a role in the specification of the directive axis during later development (26, 31, 32) and confirming potential roles suggested by previous in situ expression patterns (26–28). Thus, these results demonstrate that beta-catenin/TCF and BMP2/4 signaling interact to provide additional spatial patterning of the endomesoderm (Fig. 2).

Integration of Inputs from Beta-Catenin/Tcf Signaling and BMP2/4 Signaling Plays a Role in Defining Specialized Domains in the Developing *Nematostella* Embryo. Unlike the case for bilaterians, endomesoderm is specified at the animal pole of the developing *Nematostella* embryos and beta-catenin/TCF signaling plays a

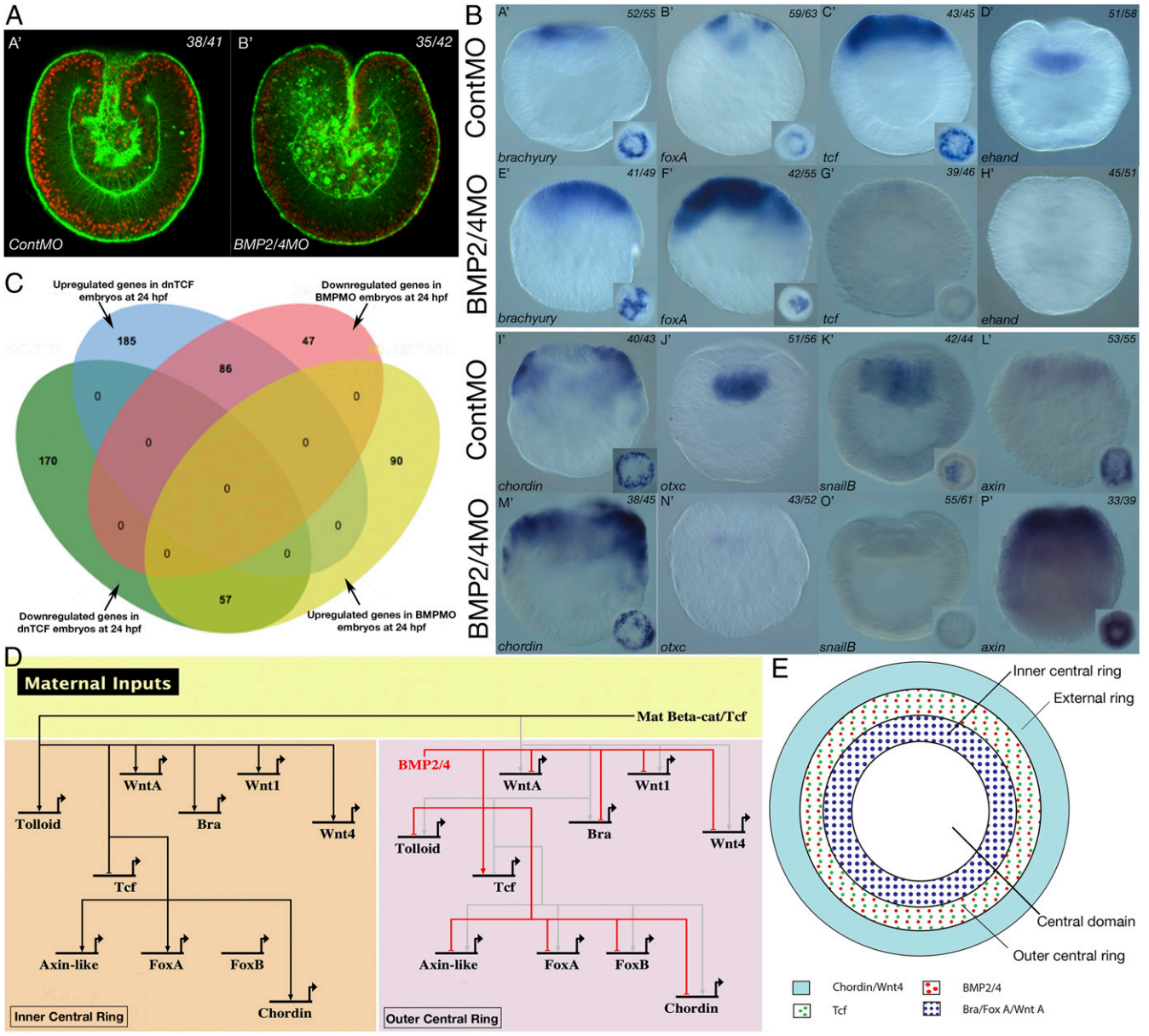

Fig. 2. Differential inputs from cWnt and BMP2/4 signaling. (*A*) Effect of NvBMP2/4 knockdown and NvTcf knockdown on germ layer specification in *Nematostella* embryos. (*A*′) Control-MO–injected embryo at 48 hpf. (*B*′) NvBMP2/4-MO–injected embryo at 48 hpf. Embryos are stained with phalloidin (green) and propidium iodide (red). Note how disorganized the internal issue is and the lack of endomesodermal epithelialization. (*B*) Effects on gene expression after BMP MO injection (*E*′–*H*′ and *M*′–*P*′) compared with control embryos (*A*′–*D*′ and *I*′–*L*′) analyzed by in situ hybridization. *Insets* show oral views of the embryo. (*C*) Venn diagram depicting unique and shared sets of down-regulated and up-regulated genes between dnTCF-injected embryos vs. dextran-injected control embryos and BMP2/4 morpholino-injected embryos vs. dextran-injected control embryos at 24 hpf. (*D*) Model GRN circuit that would potentially result in separating the central ring domain into an inner and an outer ring. (*E*) At 24 hpf, the animal hemisphere contains at least four domains defined by differential gene expression as demonstrated by Röttinger et al. (25): the central domain, the central ring, the central ring + ring domain, and the external ring. Based on the model proposed in this study, integrated inputs from canonical Wnt signaling and BMP2/4 signaling splits the central ring domain into two: an outer central ring coexpressing *NvTcf* and *NvBMP2/4* and an inner central ring expressing *NvBra*, *NvFoxA*, and *NvWntA*.

crucial role in the specification and segregation of germ layers in these embryos (23–25, 30). Based on differential gene expression, the animal hemisphere of the 24-hpf *Nematostella* embryo has been divided into at least four domains (Fig. 1*C*): a central domain, a central ring (where both *NvBMP2/4* and *NvTcf* are expressed), central domain plus central ring, and an external ring (25). Using RNA-seq, we determined that genes were being regulated in opposite ways through beta-catenin/TCF signaling and BMP2/4 signaling in all four expression domains in the animal hemisphere. In the external ring, *NvChordin* and *NvWnt4* were up-regulated in *NvBMP2/4* morphant embryos, whereas they were down-regulated in TCF-deficient embryos. Central ring genes *NvFoxA*, *NvWntA*, and *NvBra* were also up-regulated in BMP2/4 morphant embryos, whereas *NvTcf*, another central ring gene, was down-regulated. In contrast, embryos deficient in beta-catenin/Tcf signaling showed decreased *NvFoxA*, *NvWntA*, and *NvBra* expression and increased *NvTcf* expression. *NvAxin*, which is expressed in the central domain and the central ring, was up-regulated in *NvBMP2/4* morphant embryos but down-regulated in TCF-deficient embryos. Central domain gene

NvEhand showed increased levels of expression in beta-catenin/Tcf signaling-deficient embryos, whereas it was down-regulated in BMP2/4 morphant embryos (Fig. 2*B*). Reduced expression levels of *Chordin*, *NvFoxA*, *NvBra*, *NvAxin*, and *NvWnt 4* were also seen in a separate study (25), which analyzed the expression of these genes in embryos by overexpressing a dominant negative version of the TCF protein using whole mount in situ hybridization (WMISH). Differential gene expression observed in NvBMP2/4 morphant embryos were confirmed with WMISH experiments in this study (Fig. 2*B*). Our results from transcriptome analysis and validated by in situ hybridization experiments indicate a crucial role for combinatorial signaling events resulting from the integration of beta-catenin/Tcf signaling and BMP2/4 signaling in defining specialized domains after the initial, broad specification of endomesoderm in the animal hemisphere by the activation of canonical Wnt signaling.

The Evolutionarily Conserved "Kernel" of the Bilaterian Heart Mesoderm GRN Is Operational in *Nematostella*. Within the differentially regulated set of genes (Tables S5 and S6), we identified several key genes expressed in bilaterian mesoderm, including key components of the bilaterian heart-field specification GRN kernel (Fig. 3*B*) identified by Davidson and Erwin (4). The transcription factors *NvNkx2.5* and *NvEhand* were up-regulated in embryos deficient in cWnt signaling but were down-regulated in embryos with reduced BMP2/4 signaling (Fig. 3*A*). *Nkx2.5* (a homolog of *Drosophila* gene *Tinman*) (33) is part of the core regulatory network regulating bilaterian heart development and is expressed in cardiac progenitor cells of all vertebrates and functions as a target of inductive signals that initiate cardiogenesis (34–39). *Hand* genes are also part of the GRN kernel regulating heart development and regulate ventricular growth (33, 40, 41). Activation of the bilaterian heart GRN kernel requires signaling input from BMP/Dpp signaling, leading to the subsequent activation of downstream target genes (4, 33, 42–44). Therefore, the down-regulation of *NvNkx2.5*, the *Nematostella* homolog of *Drosophila* Tinman (45) and *NvEhand* in the developing gastrodermis of BMP2/4-deficient *Nematostella* embryos is surprisingly similar to what has been observed during cardiac mesoderm differentiation in bilaterian embryos and suggests the presence of an ancestral heart-field specification GRN kernel in the bifunctional endomesoderm of *Nematostella*. GRN kernels, or subcircuits regulating early specification, such as the heart-field specification GRN kernel, are evolutionarily more constrained compared with subcircuits responsible for subsequent fine-scale patterning and terminal differentiation (4). Therefore, identification of an ancestral heart-field specification GRN kernel in a nonbilaterian diploblastic cnidarian would provide evidence for the conservation of these GRN kernels across eumetazoans.

To determine whether the architecture of the putative *Nematostella* heart-field specification GRN is similar to bilaterians, we investigated the regulatory links within the GRN kernel by systematically knocking down transcription factors in the kernel, using CRSPR/Cas9 genome editing and antisense morpholinos (Figs. S1 and S2). In *NvNkx2.5*-deficient embryos, no *NvEhand* expression was seen at the animal pole at 24 hpf, confirming that *NvEhand* required regulatory input from NvNkx2.5 for its activation (Fig. 4*A*). By 48 hpf, all components of the GRN kernel are expressed in the endomesoderm. *NvGata* expression is pan-endomesodermal (6), whereas the endomesoderm-specific slice variant of *NvMef2* is also expressed pan-endomesodermally at 48 hpf (46). Endomesodermal expression of *NvGata* at 48 hpf is completely lost in *NvNkx2.5*-deficient embryos in both *NvNkx2.5* morpholino injection and CRISPR/Cas9. Interestingly *NvGata* also has a "salt-and-pepper" expression in the ectoderm starting at 24 hpf, and this ectodermal expression remained unchanged following *NvNkx2.5* morpholino injection or CRISPR/Cas9-mediated *NvNkx2.5* knockout. The fact that both morpholino and CRISPR/Cas9 treatments give the same phenotype, and that only endodermal expression is missing, confirms the specificity of the heart kernel to only endomesodermal tissue (Fig. 4*A*). Thus, by 48 hpf, components of the bilaterian heart-field specification GRN kernel are expressed in the endomesodermal gastrodermis of *Nematostella* and, as in bilaterians, the entire circuit is activated by BMP2/4, which activates *NvNkx2.5*, which in turn activates the downstream components *NvEhand*, *NvGata*, and *NvMef2* (Fig. 4*B*). In the bilaterian heart-field specification GRN kernel, in addition to the BMP2/4- and Nkx2.5-dependent activation of downstream components, there are additional feedback loops, including autoregulation of Nkx2.5 by itself and positive feedback from Gata, which activates *Nkx2.5*, *Ehand*, and *Mef2* (4). To test whether there is any feedback from the downstream components in to the GRN, we knocked down NvGata through morpholino-mediated gene knockdown. There were no detectable changes in the expression of any of the component genes in the endoderm (Fig. S3), suggesting that the fine-scale regulation of feedback

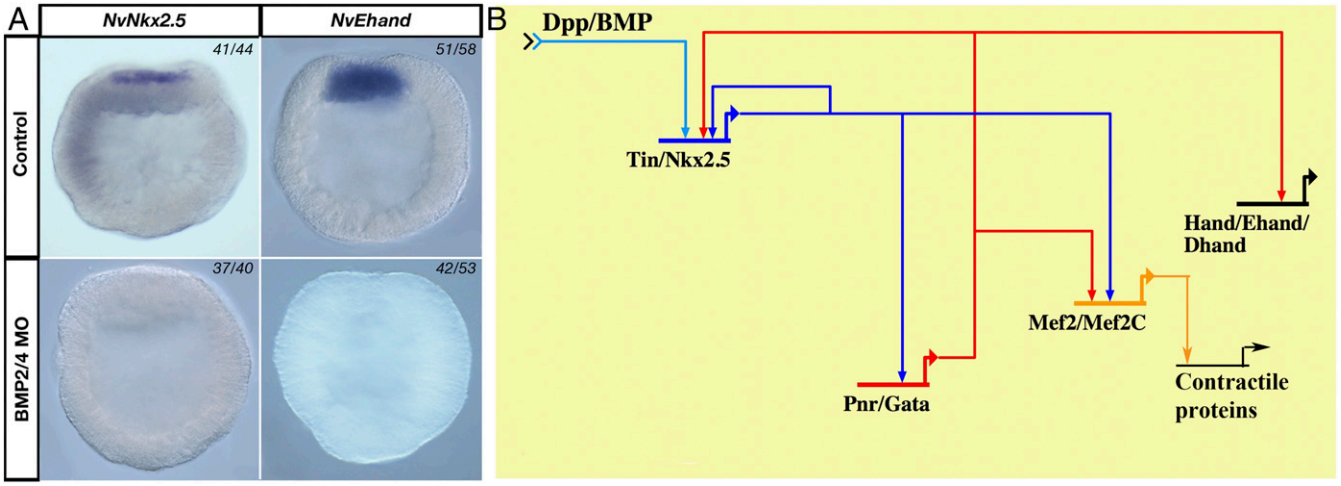

Fig. 3. BMP2/4-regulated components of heart-field specification GRN kernel. (*A*) BMP2/4-deficient embryos lack *NvNkx2.5* and *NvEhand* expression compared with control-MO–injected embryos at 24 hpf. (*B*) Potential heart-field specification GRN kernel assembled from experimental data from *Drosophila* and vertebrates [adapted from Davidson and Erwin (4)].

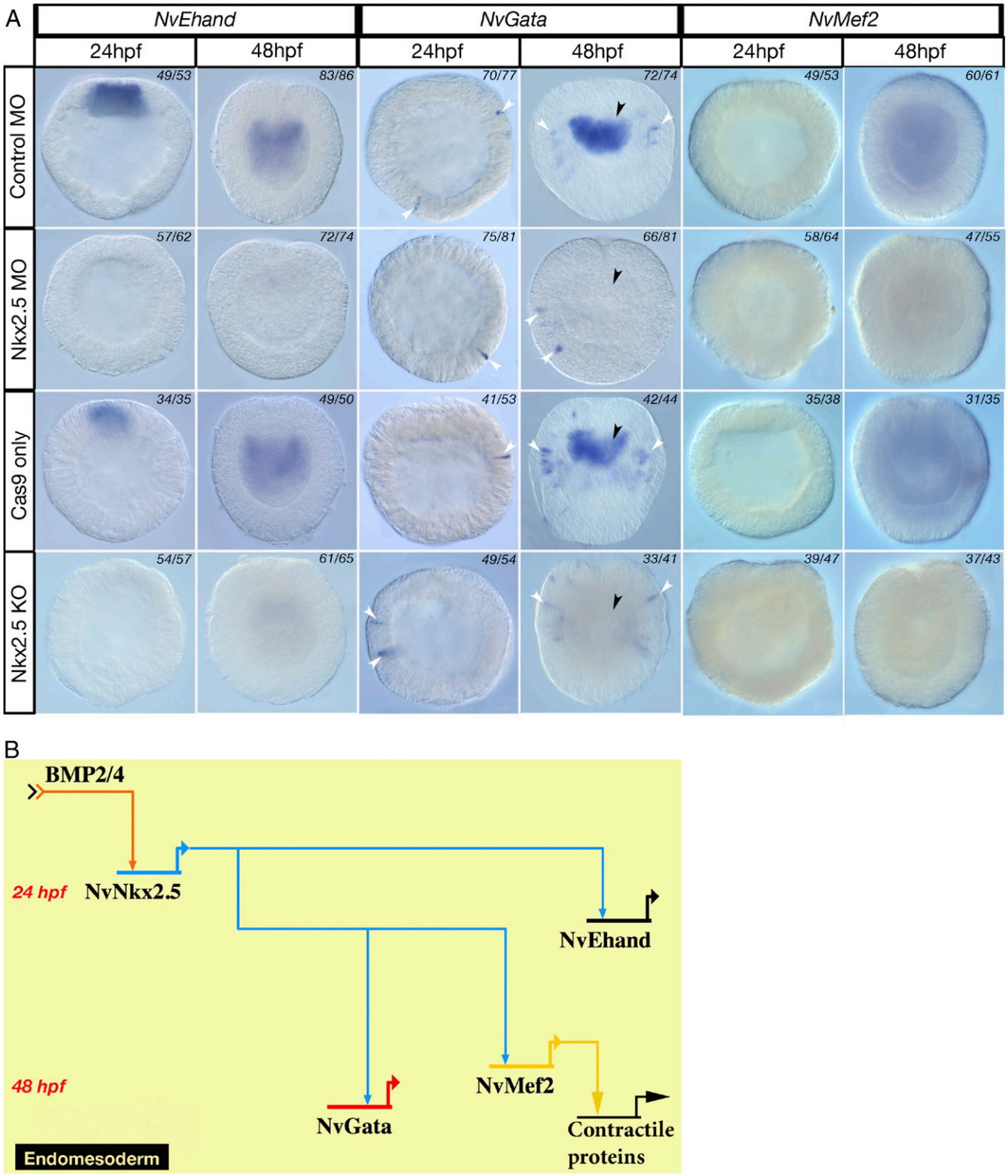

Fig. 4. Ancestral heart-field specification GRN kernel is present in *Nematostella* endoderm. (*A*) Components of the bilaterian heart-field specification GRN, *NvEhand*, and *NvGata* are lost in the endoderm (black arrowheads) and *NvMef2* of NvNkx2.5-deficient embryos compared with their normal expression in control-MO–injected embryos. Ectodermal expression of NvGata is not affected by knockdown of NvNkx2.5 (white arrowheads). (*B*) An ancestral heart-field specification GRN kernel is present in *Nematostella* where NvNkx2.5 is activated by BMP2/4, which in turn activates *NvEhand*, *NvGata*, and *NvMef2* in the endoderm. Note the absence of the Gata lockdown feedback loop.

mechanisms seen in bilaterians is not yet present, or has been lost, in the *Nematostella* GRN kernel.

Discussion

BMP2/4 Signaling Plays a Key Role in Germ Layer Segregation in *Nematostella*. The first provisional GRN-regulating endomesoderm specification in *Nematostella* was developed by Röttinger et al., using inputs from canonical Wnt signaling at the onset of gastrulation at 24 hpf (25). Here we provide evidence that BMP signaling provides additional inputs to the endomesodermal GRN based on *NvBMP2/4* function in the animal hemisphere before gastrulation. These findings are supported by other studies on BMP function during development of *Nematostella*, where it was suggested that BMP signaling might be playing a role in endomesoderm specification based on the gene expression phenotypes seen in embryos with altered BMP signaling (26–28, 32). Two of these studies (26, 32) are mainly focused on BMP function at later stages of development postgastrulation and the findings have provided compelling evidence for a role for BMP signaling in patterning the directive axis in *Nematostella* (26, 32). However, given the highly dynamic temporal and spatial expression patterns of BMP2/4 and other components of the pathway, BMP signaling has the potential of playing multiple roles in regulating different developmental processes (27). The current study confirms that BMP2/4 is involved in endomesoderm specification before its role in patterning the directive axis based on the abnormal endomesoderm development and loss of endomesodermal marker genes (e.g., *NvSnail*, *NvEhand*, and *NvOtxc*) in BMP2/4 morphant embryos (Fig. 2*B*). Therefore, results of this study suggest that BMP2/4 signaling plays a critical role in further patterning endomesoderm in specific specialized tissues in *Nematsotella* after the initial, broad specification of endomesoderm through cWnt signaling. They further indicate a more specific role for BMP2/4 signaling in regulating "mesodermal" genes (*NvSnail*, *NvEhand*, and *NvOtxC*) in the bifunctional endomesoderm. However, the specific mechanisms of BMP signaling-mediated regulation of initial endomesoderm specification in *Nematostella* requires further investigation. Another aspect of this regulation that needs further investigation is the differential regulation of *NvSnail* by cWnt signaling and BMP2/4 signaling. Röttinger et al. (25) showed that knockdown of NvTcf by a dominant negative approach does not affect *NvSnail* expression in the developing embryo but the knockdown of NvBMP2/4 does result in the loss of *NvSnail* expression (Fig. 2*B*). However, *NvSnail* was not detected in the NvBMP2/4 knockdown RNA-seq dataset, likely because it is expressed in a small number of cells or its transcripts are not expressed at high levels in the cells that express it.

Reciprocal Signaling Events Resulting from Integration of cWnt and BMP2/4 Signaling Fine Tune Endomesodermal Patterning of the Early *Nematostella* Embryo. This study shows that canonical Wnt signaling and BMP2/4 signaling differentially regulate a subset of genes at 24 hpf in developing *Nematostella* embryos and these include some key genes that are important in specifying different expression domains in the animal hemisphere. This differential regulation of components of these different domains shows how canonical Wnt signaling and BMP2/4 signaling integrate with one another to define distinct gene expression domains. At 24 hpf, the animal hemisphere of the developing *Nematostella* embryo contains at least four domains defined by differential gene expression (25): the central domain, the central ring, the central ring + central domain, and the external ring. The model proposed in this study suggests that there are at least five domains based on gene expression (at 24 hpf) because the integrated inputs from canonical Wnt signaling and BMP2/4 signaling produce two ring-like domains: an outer central ring coexpressing *NvTcf* and *NvBMP2/4* and an inner central ring expressing *NvBra*, *NvFoxA*, and *NvWntA*. *NvTcf* expression in developing *Nematostella* embryos is dynamic, with uniform expression in early cleavage stages and the expression getting progressively restricted to the animal hemisphere such that by 24 hpf, it is expressed in a ring-like domain (the "central ring") around the blastopore (25, 47). Based on the transcriptome data, canonical Wnt signaling down-regulates *NvTcf* expression and the progressive repression of *NvTcf* expression in the animal hemisphere, leading to the activation of canonical Wnt signaling at around 8 hpf. This initial wave of canonical Wnt signaling activates *NvBMP2/4* in the animal hemisphere (25). However, after this initial activation, *NvBMP2/4* up-regulates *NvTcf* expression and at 24 hpf, both *NvBMP2/4* and *NvTcf* have overlapping ring-like expression domains (Fig. 2 *B* and *E*). The later expression of NvBMP2/4 appears to be independent of canonical Wnt signaling, thus reciprocally regulating only a subset of genes in this domain. This continued expression of *NvTcf* in the outer central ring overlaps with *NvBMP2/4* expression driven by BMP signaling and results in the inhibition of canonical Wnt signaling in this domain (Fig. 2*E*). These interactions result in the inhibition of other target genes of canonical Wnt signaling, including *NvFoxA*, *NvWntA*, and *NvBra*, limiting them to the inner central ring, and *NvChordin* and *NvWnt 4*, limiting these genes to the external central ring. Thus, these two signaling pathways subfunctionalize the outer ring to pharyngeal (inner central ring) and oral (outer central ring) domains. Studies on lineage differentiation potential in mouse embryonic stem cells have shown that canonical Wnt signaling regulates differentiation of stem cells through the down-regulation of Tcf3 (48). A similar mechanism could be operating here to inhibit canonical Wnt signaling in a specific domain in the animal hemisphere; however, further experiments are needed to confirm this idea.

Mesodermal Gene Expression in *Nematostella* and the Origins of Triploblasty. The formation of mesodermal derivatives was a critical event in metazoan evolution, leading to the evolution of more complex body plans in bilaterians. Generally believed to be a bilaterian invention, presence of a mesodermal germ layer has led to the division of metazoans into two main groups: the triploblasts with three germ layers, an ectoderm, an endoderm and a mesoderm; and the diploblasts, with only an ectoderm and an endoderm (6). However, investigations aimed at understanding the evolutionary origins of triploblasty has led to the discovery of "mesodermal" gene expression in the gastrodermis (endoderm) of the diploblastic nonbilaterian *Nematostella* (6). These findings support the hypothesis that the mesoderm and endoderm of bilaterians evolved from the bifunctional endomesoderm of diploblasts (8). This idea is further supported by the mechanisms by which endoderm and mesoderm are specified in bilaterians, where initial endomesoderm specification is followed by the segregation of distinct endodermal and mesodermal tissues (12, 49). The mechanisms of endomesoderm specification in *Nematostella* show remarkable similarities to bilaterians from the signaling pathways involved to the wiring of the endomesodermal GRN (23–25, 30). However, the mechanisms of segregating endomesoderm to discreet endodermal and mesodermal tissues is understood in only a few systems (12). Segregation of endomesoderm and further differentiation of the endoderm and the mesoderm involve the function of multiple signaling pathways, including cWnt, Notch, FGF, and BMP (12, 18, 50–53). Some of these bilaterian mechanisms are still being characterized but to understand the evolutionary origins of these mechanisms, the function of pathways involved in these processes has to be looked at in diploblastic animals with a bifunctional endomesoderm, and *Nematostella* appears to be an ideal candidate for further study.

GRN Kernels and Terminal Differentiation of Germ Layers. The presence of an ancestral heart-field specification GRN kernel in the diploblastic cnidarian *Nematostella* provides valuable evolutionary insights into the origin of mesoderm. Diploblastic nonbilaterians lack a distinct mesodermal tissue even though the genome possesses

a complex set of bilterian mesodermal orthologs (54) that are expressed in the gastrodermis (6), and cnidarian polyps have myoepithelial cells in the gastrodermis (55, 56). The *Nematostella* muscular system is divided into a body column and a tentacle system. The body column contains three morphologically and functionally distinct muscle groups that are endomesodermal in origin and are used to generate peristaltic movements along the oral–aboral axis to mix the contents of the gut (55, 56). The presence of the heart-field specification GRN kernel in the *Nematostella* gastrodermis suggests that it might be regulating the specification of smooth myoepithelial-like muscle cells in the gastrodermis that function to produce slow contractions, similar to those seen in smooth muscles by calcium-dependent phosphorylation of the myosin regulatory light chain and the phosphorylation of caldesmon on the actin filaments, also present in *Nematostella* (57). *Nematostella* also possess slow rectifying K^+ channels characteristic of slow rhythmic smooth muscle contraction expressed in the gastrodermis (58, 59), which is consistent with the specification of myoepithelial cells with smooth muscle characteristics. These data support the hypothesis that an ancestral heart-field specification GRN kernel as seen in the myoepithelial cells in *Nematostella* regulated the specification of slow contracting smooth muscles that was later co-opted to regulate cardiac muscle in bilaterians. This likely corresponded with the evolution of novel contractile proteins and additional regulatory inputs into the GRN. For example, the absence of troponin in *Nematostella*, a key regulatory protein in powerful cardiac muscle contraction and relaxation in vertebrates (57, 60), suggests that this core kernel was modified to give rise to novel tissue types later in bilaterian evolution. It is also of interest that we detected evolutionary differences in the architecture of the heart kernel GRN. *Nematostella* appears to lack the *Gata* "lockdown" feedback loop (compare Fig. 3*B* to Fig. 4*B*) found in other bilaterian animals studied, which presumably maintains the stability of heart tissue cell fate. One fundamental difference between most bilaterian heart tissue cells and the endomesoderm in cnidarians like *Nematostella* is the dramatic ability of adult cells to regenerate, which appears to have been lost in many bilaterian taxa. It will be of interest to investigate the GRN "logic" in other cell types between regenerating and non-regenerating species to determine whether there are potential patterns in regulatory structure that can help explain the differences in regenerative ability.

There is strong evidence, both embryological and molecular, to suggest that the bifunctional endomesoderm of cnidarians gave rise to both the endoderm and the mesoderm in triploblastic bilaterians (58). Therefore, the discovery of an ancestral mesodermal GRN kernel regulating the expression of mesodermal genes during the specification and differentiation of the gastrodermis of *Nematostella* further reinforces the hypothesis that the endoderm and mesoderm in triploblastic bilaterians evolved from the bifunctional endomesoderm of a diploblastic ancestor.

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