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

Naveen Wijesena\textsuperscript{a,1}, David K. Simmons\textsuperscript{a}, and Mark Q. Martindale\textsuperscript{a,2}

\textsuperscript{a}Whitney Laboratory for Marine Bioscience, University of Florida, Saint Augustine, FL 32080

Edited by Richard M. Harland, University of California, Berkeley, CA, and approved June 1, 2017 (received for review January 29, 2017)

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 Nemastostella vectensis (an anthozoan cnidarian). Using forward transcriptomics, we show that beta-catenin/Tcf signaling and BMP2/4 signaling provide differential inputs into the cnidian endomesodermal gene regulatory network (GRN) on the onset of gastrulation (24 h postfertilization) in Nemastostella vectensis. Surprisingly, beta-catenin/Tcf signaling and BMP2/4 signaling regulate a subset of common downstream target genes in the cnidian endomesodermal gene regulatory network (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 Nemastostella. We also show functionally that the conserved “kernel” of the bilaterian heart mesoderm GRN is operational in Nemastostella, 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.

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 Nemastostella 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 Nemastostella. This GRN fails to have a “lockdown” feedback loop found in bilaterian GRNs that may help explain the highly regenerative potential of adult cnidian endomesoderm, suggesting that the endoderm and mesoderm arose from the bifunctional endomesoderm.

Author contributions: N.W. and M.Q.M. designed research; N.W., D.K.S., and M.Q.M. performed research; N.W., D.K.S., and M.Q.M. analyzed data; and N.W. and M.Q.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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

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

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1701607114/-/DCSupplemental.
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. 2C). 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). 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
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. 1C): 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. 2B). 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. 2B). 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 Bilateral 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. 3B) 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. 3A). 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 CRISPR/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. 4A). 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 endomesodermal expression is missing, confirms the specificity of the heart kernel to only endomesodermal tissue (Fig. 4A). Thus, by 48 hpf, components of the bilateral 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. 4B). In the bilateral 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. 2B). Therefore, results of this study suggest that BMP2/4 signaling plays a critical role in further patterning endomesoderm in specific specialized tissues in *Nematostella* 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. 2B). 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. 2B 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. 2E). 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 metazoa into two main groups: the triploblasts with three germ layers, an endoderm, an endoderm and a mesoderm; and the diploblasts, with only an endoderm 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 non-bilaterian *Nematostella* (6). These findings support the hypothesis that the mesoderm and endoderm of bilaterians evolved from the bifunctional endoderm 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 bilaterian mesodermal orthologs 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 smooth myoepithelial-like muscle cells in the gastrodermis (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 corresponds 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. 3B to Fig. 4B) 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 mesoderm 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.

**ACKNOWLEDGMENTS.** This work is supported by National Institutes of Health Grant GM093116.


Supporting Information

Wijesena et al. 10.1073/pnas.1701607114

**SI Materials and Methods**

**Culture and Spawning and Microinjection of Nematostella vectensis.** Nematostella embryos were cultured at the Whitney Laboratory for Marine Bioscience, University of Florida. Males and females were kept in separate bowls in one-third (1/3x) seawater, and the adults were spawned by putting them in a light cycle as described by Hand and Uhlinger (62). The gelatinous mass around the eggs was removed using 4% 1-cysteine followed by five washes of 1/3x seawater. Microinjections were carried out as described earlier (63). The fertilized/microinjected eggs were cultured in filtered 1/3x seawater at 17°C.

**Morpholino Antisense Oligonucleotide Sequences.** The NvBMP2/4 morpholino sequences have been reported previously (26): NVNkx2.5 MO (5′–3′): CCATTGACACTGAAAAAGTTACAAGG; NvGata MO (5′–3′): ACTGTTTCCATTGAGATCACAGG; and standard control MO (5′–3′): CCTTTACCTCAGTTACAA- TTATA.

A 2-mM stock solution of each morpholino was diluted in autoclaved miliQ water to a final concentration of 1 mM in the microinjection mix.

**CRISPR/Cas9-Mediated Mutagenesis.** Single-guide RNAs for CRISPR/Cas9-mediated mutagenesis of Nematostella NvNkx2.5 were synthesized using the following oligos as described by Dang et al. (64): NvNkx2.5 GR1, ATGTCAATCAATACCAATT; NvNkx2.5 GR2, GTTAAAGCAACGCGAATA; NvNkx2.5 GR3, GCCGTCCCTTTGTAAGT; NvNkx2.5 GR4, AAAACGACTCTAGCCAG; NvNkx2.5 GR5, AATGCATATCTTGGCTTTC; NvNkx2.5 GR6, GAGAACAAACCGGTGCTG; NvNkx2.5 GR7, AGATGGA-GAGACGTGCTT; and NvNkx2.5 GR8, GACAAGACCTGTACAGCTT.

Cas9 protein was obtained from PNA Bio. A total of 1.25 μg/μL single-guide RNA mix and 1 μg/μL Cas9 protein were injected into fertilized eggs. Control embryos were injected with Cas9 protein only. DNA was prepared from individual polyps and PCR was carried out using the following oligo sequences to identify deletions in the genome: NvBMP2_5 gDNA F1AATGTTTCTGTCTTG-CCTTGCCAG, NvNkx2_5 gDNA F2 GTGTCGGTGCTCTAACAGCTGTG.

**Whole Mount RNA in Situ Hybridization, Actin, and Nuclear Staining.** Whole mount in situ hybridization of Nematostella eggs and embryos was carried out as previously described (6). Digoxigenin-labeled RNA probes were synthesized using the MegaScript Transcription Kit (Ambion). Hybridization of the DIG-labeled RNA probe (1 ng/L) was carried out at 60 °C. Visualization of the labeled probe was carried out using NBT/BCIP as substrate for the alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Bioscience). In situ hybridizations were performed as previously described (6). Bodipy FL Phallacidin (Molecular Probes/Invitrogen, B607) and propidium iodide (Sigma, 81845) were used to stain F-actin and cell nuclei, respectively, as described previously (65) to analyze embryonic morphology.

In situ hybridization images were taken on a Zeiss Axioscop 2 mounted with an Axiovision camera triggered by Axiovision software (Carl Zeiss). Confocal images of stained embryos were taken using a Zeiss LSM710 microscope running the LSM ZEN software (Carl Zeiss).

**RNA-Seq and Reference Mapping.** All nine samples were sequenced in a single lane on the Illumina HiSeq 2500 at the University of Texas Austin Genomic Sequencing and Analysis Facility. Reads were single-end 50 bp. Read quality was checked with Fastqc (https://www.bioinformatics.babraham.ac.uk/projects/), and the first 12 bases were trimmed using the FASTX toolkit along with the removal of any sequences with phred scores below 33. FastqMCF was used to remove any adaptor contamination and homopolymers. The reads were mapped to the edited Nematostella reference transcriptome from ref. 66, which is the genomic filtered model set with the exclusion of mitochondrial and ribosomal RNA sequences. Using Bowtie 2.2.0 beta3 with –very-sensitive-local and -a flags. Counts were generated using the custom script from ref. 66. Differential gene expression was found using EdgeR version edgeR_3.8.5 (67). One count per million in three or more samples was kept to filter low number reads, and the reads were normalized to the trimmed mean of M-values. Testing for differential expression was done using the generalized linear model approach and genes with adjusted P values below 0.05 were considered differentially expressed.

**Fig. S1.** Morpholino mediated knock down and CRISPR/Cas9 mediated knock out of NvNkx2.5 in Nematostella. (A) A splice blocking anti-sense morpholino oligo was designed targeting the splice site between intron1 and exon2. (B) PCR showing an amplified DNA fragment of 880bp from cDNA from control embryos but absent in cDNA from NvNkx2.5 MO injected embryos. NvNkx2.5 gene deletion efficiency was determined by amplifying the NvNkx2.5 gene fragment.
Fig. S2. CRISPR/Cas9 mediated knockout of NvNkx2.5 in Nematostella. (A) gRNA sequences and primers used for the PCR assay are shown on the genomic sequence of NvNkx2.5. (B) Note the truncated fragments of NvNkx2.5 with a smaller size than the wild type resulting from CRISPR/Cas9 mediated mutagenesis. (C) NvNkx2.5 knockout embryos (Cas9 and gRNAs) lack NvNkx2.5 expression compared with control embryos (Cas9 only) embryos at 24 hpf.

Fig. S3. NvGata does not provide regulatory inputs into the heart-field specification GRN kernel in Nematostella. NvNkx2.5 and NvEhand expression is not affected in NvGata MO-injected embryos compared with control MO-injected embryos at 24 hpf.
Table S1. Genes down-regulated by dnTCF vs. dextran controls at 24 hpf

Table S2. Genes up-regulated by dnTCF vs. dextran controls at 24 hpf

Table S3. Genes down-regulated by BMP2/4 morpholino vs. dextran controls at 24 hpf

Table S4. Genes up-regulated by BMP2/4 morpholino vs. dextran controls at 24 hpf

Table S5. Genes up-regulated in dnTCF-injected embryos and down-regulated in BMP MO-injected embryos at 24 hpf

Table S6. Genes down-regulated in dnTCF-injected embryos and up-regulated in BMP MO-injected embryos at 24 hpf

Genes are ordered by the level of up- or down-regulation.