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A bipolar role of the transcription factor ERG for cnidarian germ layer formation and apical domain patterning

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Abstract

Germ layer formation and axial patterning are biological processes that are tightly **linked** during embryonic development of most metazoans. In addition to canonical WNT, it has been proposed that ERK-MAPK signaling is involved in specifying oral as well as aboral territories in cnidarians. However, the effector and the molecular mechanism underlying latter phenomenon is unknown. By screening for potential effectors of ERK-MAPK signaling in both domains, we identified a member of the ETS family of transcription factors, *Nverg* that is bi-polarly expressed prior to gastrulation. We further describe the crucial role of NvERG for gastrulation, endomesoderm as well as apical domain formation. The molecular characterization of the obtained NvERG knock-down phenotype using previously described as well as novel potential downstream targets, provides evidence that a single transcription factor, NvERG, simultaneously controls expression of two different sets of downstream targets, leading to two different embryonic gene regulatory networks (GRNs) in opposite poles of the developing embryo. We also highlight the molecular

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Author contributions

ER, MJL and MQM – Conceived, designed and performed experiments. ARA, HJ, TC, PD, MI – generated, collected and analyzed data. ER, MQM - Contributed reagents/materials/analysis tools. ER, ARA, HJ, MQM - Drafted the manuscript. All authors read and approved the final manuscript

Competing interests

The author(s) declare(s) that they have no competing interests.

interaction of cWNT and MEK/ERK/ERG signaling that provides novel insight into the embryonic axial organization of *Nematostella*, and show a cWNT repressive role of MEK/ERK/ERG signaling in segregating the endomesoderm in two sub-domains, while a common input of both pathways is required for proper apical domain formation. Taking together, we build the first blueprint for a global cnidarian embryonic GRN that is the foundation for additional gene specific studies addressing the evolution of embryonic and larval development.

Keywords

ERK signaling; embryonic development; gastrulation; endomesoderm; apical organ; ERG; ETS transcription factor; gene regulatory network; *Nematostella vectensis*; sea anemone; cnidarian; gene expression; evolution

Introduction

Fibroblast Growth Factor (FGF) induced ERK signaling plays a crucial role in various aspects of mesoderm formation and coordinating cell movements in bilaterian animals [1–11]. FGFs bind to FGF Receptors (FGFRs) that are part of the RTK (Receptor Tyrosine Kinase) family, in order to activate an intracellular MAP Kinase (RAS/MEK/ERK) signaling cascade leading to the phosphorylation of transcription factors and thus the repression or activation of downstream targets [12]. Well known transcriptional regulators whose activity can be controlled by MEK/ERK signaling, belong to the ETS domain containing family of transcription factors [13].

Cnidarians are the extant sister group to all bilaterians and their phylogenetic position makes them very interesting for understanding the evolution of biological novelties [14–16]. One intensely used cnidarian model is the anthozoan sea anemone *Nematostella vectensis* that can easily be cultured and manipulated under laboratory conditions and for which functional genomic tools are well established [16–21]. Based on the sequenced *Nematostella* genome [19], 15 putative FGF ligands and three potential receptors have been identified [22,23] with the spatial expression patterns reported for three ligands and two receptors [22–24]. Two ligands and one receptor (*NvfgfA1*, *NvfgfA2*, *NvfgfrA*) are expressed in the vegetal hemisphere/apical domain, the ligand *Nvfgf8* is expressed in the animal hemisphere and its descendants, and the receptor *NvfgfrB* in derivatives of both poles. Interestingly, *Nvsprouty*, a well described downstream target and modulator of FGF signaling [25–27], is also expressed in both extremities of the developing embryo/larvae [22]. Only based on these expression patterns, FGF signaling has been suggested to play a role in gastrulation and neural development [22]. However, the importance of the FGF pathway has so far only been analyzed at late stages of development and shown to be crucial for apical organ formation and metamorphosis [23,28,29].

The vegetal pole of cnidarian embryos gives rise to the apical organ, characterized by an apical tuft, a group of long cilia, at the aboral most part of the planula larvae [17,23,30]. Recent studies have shown that a gene regulatory module involving the transcription factor Six3/6, FGF signaling as well as Frizzled 5/8, that potentially signals through β -catenin, is required to specify and pattern the apical domain, form the apical tuft and subsequently

allow the process of metamorphosis into a sessile juvenile [23,28,29,31]. Unfortunately, little is known about the role of MEK/ERK signaling in the specification of the apical domain prior to the onset of gastrulation.

Cnidarians are so-called diploblastic animals that, although they possess the genetic toolkit involved in bilaterian mesoderm formation, lack a true mesodermal germ layer [24,30,32]. A precise embryonic cell lineage analysis has yet to be performed in cnidarians due to the lack of a stereotyped cleavage program but existing labeling experiments clearly indicate that derivatives of cells from the animal hemisphere in *Nematostella* gives rise to the epitheliomuscular gut, the pharynx and the mouth of the planula larva [33,34]. In a previous study, we have defined three gene expression domains within the animal hemisphere of the blastula prior to the onset of gastrulation; the central domain, the central ring and the external ring that appears to give rise to the gut (bodywall endomesoderm), pharynx and mouth respectively [24]. This work also showed that canonical WNT signaling (cWnt) is required for proper gene expression within all three domains, in particular for genes expressed within the central ring domain and normal pharynx formation [24]. Interestingly, cWnt/TCF represses expression of the potential FGF ligand, *fgf8A*, in the central ring (animal hemisphere) restricting its expression to the central domain, suggesting a role of FGF induced ERK/MAPK signaling in endomesoderm formation [24]. However, a recent study that focuses on the role of ERK/MAPK signaling in the initiation of the neurogenic program in *Nematostella* development, suggest that FGFR might not be the (sole) activator of this pathway in the presumptive endomesoderm [35]. The same authors have also shown that pharmacologically inhibition of ERK/MAPK signaling using U0126, a potent inhibitor of the ERK activating kinase MEK [36,37], after fertilization blocks gastrulation and endomesoderm formation [35]. As this treatment perturbs gene expression within the animal hemisphere as well as the apical domain, this further suggests a dual role of this pathway in germ layer formation and axial patterning [35]. In addition, by using a genome wide expression array approach, the authors have identified a large set of putative downstream targets of this pathway of which only the genes potentially involved in neurogenesis have been reported [35].

In this study, we present the spatio-temporal expression of NvERG, a member of the ETS family of transcription factors that is expressed in both, the central domain of the animal hemisphere as well as in the apical domain of the vegetal hemisphere. Inhibition of NvERG phenocopies the effects of disrupting MEK/ERK signaling, causing the failure of gastrulation and endomesoderm formation as well as the perturbation of apical tuft development. Fine scale temporal and spatial gene expression analysis of genes identified in a differential genome wide expression array comparing DMSO (control) and U0126 treated embryos [35] enabled us to describe 39 potential downstream targets of this pathway that are expressed in the presumptive endomesoderm as well as in the apical domain. Finally, molecular analysis of the resulting phenotype in NvERG morphants, highlights its crucial role for setting up the gene regulatory networks (GRNs) underlying endomesoderm forming within the central domain as well as apical domain patterning. Interestingly, we functionally confirmed a computational prediction [38] that NvERG negatively regulates *Nvbra* expression in the central domain, in order to restrict its expression in the central ring. This work enables us today to draw a global blueprint of genetic interactions governing

specification, patterning and morphogenic events underlying embryonic development of *Nematostella*.

Materials and Methods

Culture and spawning of *Nematostella vectensis*

Adult *Nematostella* were cultivated either at the Kewalo Marine Laboratory/PBRC of the University of Hawaii (USA), the Whitney Laboratory for Marine Bioscience of the University of Florida (USA) or the Institute for Research on Cancer and Aging of the University of Nice-Sophia-Antipolis (FRA). Culture and spawning/fertilization was performed according to the protocol described in [24]. Fertilized eggs were kept in dark in filtered 1/3 seawater at 16°C until the desired stage.

RNA Extraction and quantitative PCR (qPCR)

RNA Extraction and quantitative PCR (qPCR) was performed following protocols described in [24]: For the fine scale temporal analysis total RNA was extracted from the following stages (in hours post fertilization, hpf): 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, 28, 32, 40, 48. For the molecular phenotype analysis, total RNA was extracted 24hpf. Samples were obtained from three biological replicates and performed in three technical replicates. qPCR analysis using a LightCycler 480 (Roche) utilizing LightCycler 480 SYBR Green 1 Master mix (Roche, #04887352001) was carried out as described previously [39]. The full list of qPCR primer pairs and their efficiency used in this study can be found in Table S2 or [35]. The housekeeping genes *Nvactin* and/or *Nvgadph* were used to normalize relative fold changes between control and manipulated embryos and each qPCR analysis was repeated on independent biological replicates.

In situ hybridization, actin and nuclear staining

Previously described gene sequences were used to sub-clone into pGemT (Promega, #A3600) from mixed stage cDNA. All other sequences used in this study were isolated in the course of a microarray analysis [35]. Genome predictions as well as EST sequence information were combined to design primer pairs (Table S3, [35]) that allow the amplification and cloning of genes between 0.5 kb and 2 kb. PCR amplified cDNA fragments, corresponding to partial or full-length sequences of the gene of interest, have been cloned into pGEMT vectors (Promega, #1360). T7 and SP6 primers have been used to amplify the insert and subsequently used for anti-sense probe synthesis using either the T7 or SP6 promoters (Ambion, #AM1330, #AM1333). Probe integrity was validated by RNA electrophoresis and presented expression patterns observed in at least three independent experiments. Accession numbers for all analyzed genes in this study can be found in [35] as well as in Table S4, S5). Embryo fixation, probe synthesis and *in situ* hybridization were performed as previously described [24,30]. To analyze embryonic and larval morphology, we used Biodipy FL Phalloidin (Molecular Probes/Invitrogen, #B607), propidium iodide (Sigma, #81845) and an anti-acetylated tubulin antibody (Sigma, #T6793), to stain f-actin, the cell nuclei and the apical tuft respectively following the protocols described previously [40].

Nomenclature

Nomenclature of the newly identified genes follows the approach used in (24). To distinguish between previously published genes, and newly identified putative TFs and signaling molecules, we used the best Blast Hit identification, followed by “- like” to designate the newly identified gene sequences. While “Blast hit” approaches can be used to provide a general idea of the protein family, a detailed phylogenetic analysis is required to better resolve these gene orthologies, especially when paralogy issues or when multiple gene predictions are present for one gene family.

cDNA construction, MO-NvERG design and microinjection

cDNA constructs encoding the wild type ORF (NvERG) and a dominant negative form (NvERG-DB1) of NvERG, were generated by PCR using the following primers:

NvERG_FWD 5' ATGTATGGTTTAAGTTCAGAATC-3'

NvERG-DB1_FWD 5'-ATGTTCAATGCCAGCCCGATG-3'

NvERG_REV 5'GGCGTAGTAGGTCATACTGGC-3'

The reverse primer used in combination with both forward primers was lacking the stop codon for fusion with a C-terminal Venus fluorescent tag. All cDNA constructs were cloned, linearized and transcribed according to [20,24]. mRNAs were injected in zygotes at final concentrations of 0.2–0.5 mg/ml. A splice blocking morpholino antisense oligonucleotide (Gene Tools) was designed (MO-NvERG 5'-CTTACTTTTTCTCAAGACGCACAGA-3') to target the exon3-intron3 boundary of NvERG and used from 0.3 – 0.9mM without noticeable toxicity. A control MO (MO-CTRL 5' -AGAGGAAGAATAACATACCCTGTCC-3' (35)) was also injected at a concentration of 0,9 mM. Animals were sorted after injection to eliminate the un-injected animals as indicated by the lack of fluorescence. Microinjections were carried out as described in [20,24] and the following primers used to test the splice-blocking efficiency of the MO (Figure 1):

NvERG_Mossplice_FWD 5'-ACCAAAGAACACGTTCCGCCAGTGGA-3',

NvEtsA_Mossplice_REV 5' ATCGCAAACCCCGAGGCTCTCC-3'

Imaging—*in situ* hybridization images were taken on either a Zeiss AxioScop 2 or a Zeiss Axio Imager A2 mounted with an AxioCam camera triggered by Axiovision software (Carl Zeiss). All expression patterns described here have been submitted to Kahi Kai, a comparative invertebrate gene expression database [41] hosted at <http://www.kahikai.org/index.php?content=genes>. Scoring of treatment phenotypes was performed on either a Zeiss Z-1 Axio imager or a Zeiss Axio Imager A2 microscope and confocal imaging was conducted on either a Zeiss LSM710 or Zeiss LSM Exciter microscope running the LSM ZEN software (Carl Zeiss). Fluorescent images were false-colored, the fluorescent channels merged using ImageJ (<http://rsbweb.nih.gov/ij/>) and cropped to final size in Photoshop Cs6 (Adobe Inc.).

Results

Nverg is expressed in the central and the apical domains

In recent reports from *Nematostella*, genes belonging to the family of ETS transcription factors have been identified and reported [24,35]. However, the entire complement of this transcription factor family in cnidarians is currently unknown. We have identified 12 genes that encode proteins predicted to contain an ETS DNA binding domain defining this family of transcription factors. A phylogenetic analysis of those factors has revealed that *Nematostella* possess members of 8 out of 11 ETS sub-families (Fig. 1Aa, Fig. S1) [42,43]. We have further analyzed their spatio-temporal expression at late blastula stages and observed clear expression patterns for 3 genes (*Nvets-likeA* (previously called *NvelkA-like* [24]), *Nvpea3* (previously called *Nvpea3-like* [35]) and *Nverg*. *Nvets-likeA* was identified to respond to over-activated canonical Wnt signaling (1-azakenpaullone treatments) and is expressed in the central domain [24]. *Nvpea3*, identified to be downstream of ERK/MAPK signaling (U0126 treatments) is expressed in individual cells of a circumferential territory within the ectodermal body wall [35]. One gene that retained our particular attention was *Nverg*, whose transcripts were detected in a bi-polar manner within the central as well as the apical domains of the late blastula/very early gastrula (Fig. 1Ad,Ae). While it is only detected in the central domain at blastula stages (Fig. 1Ad, inset), the apical expression appears progressively when the animal regions flattens prior to its invagination (Fig. 1Ad, Ae). At later stages this gene was expressed within the forming mouth opening, the gastrodermis as well as the apical pole (Fig. 1Af). Interestingly, this gene has not been previously identified in any of the microarray studies analyzing the effects of blocking FGF signaling in the apical domain [29] or general ERK/MAPK inhibition [35], suggesting that it may have been missed or that its activity might be regulated by post-transcriptional modifications. Fine-scale qPCR analysis of *Nverg* temporal gene expression revealed that it is maternally expressed and that the onset of zygotic expression occurs between 12–14 hours post fertilization (hpf, at 17C, Fig1Ab).

Inhibition of NvERG prevents formation of the gut and perturbs the genesis of the apical tuft

In order to block activity of NvERG during *Nematostella* development, we used a splice blocking morpholino (MO-NvERG) targeting exon three of this gene and thus creating a truncated version of the protein that is lacking the DNA binding domain (Fig. 1Ba). Microinjecting increasing concentrations of MO-NvERG into oocytes followed up by PCR revealed that MO-NvERG injection at 0.9mM causes drastic splice defects (Fig. 1Bb). We further analyzed the morphological phenotypes induced by the disruption of NvERG function and observed that MO-NvERG morphants entirely failed to gastrulate and form a gut (Fig. 1Cf–h) compared to control embryos (Fig. 1Cb–d). This resulting phenotype can be the direct consequence of the absence of morphogenetic movements required for gastrulation or indirectly, caused by the lack of bodywall endomesoderm that becomes the future gut. In addition, MO-NvERG also perturbs the formation of the apical tuft (Fig. 1Ch) as revealed by acetylated tubulin staining [23]. In order to confirm the specificity of MO-NvERG, we performed a rescue experiment by overexpressing mRNA encoding a wild-type, Venus tagged version of NvERG after MO-NvERG injection, and observed that gastrulation

movements are restored in the majority of analyzed embryos (Fig. S2A–G). A control rescue experiment using *TomatoNLS* mRNA instead of *NvERG:Venus*, failed to rescue the phenotype (Fig. S2D,H), showing that the rescued phenotype is NvERG specific. We also microinjected a dominant negative form of NvERG (NvERG-DB) that is lacking the transactivation domain in the N-terminus of the protein [44]. While not as efficient as MO-NvERG, injection of NvERG-DB caused i) severe perturbations of gut formation and gastrulation movements (that appear to recover partially during later embryonic development) and ii) the perturbation of apical tuft development (Fig. 1i–l). All together these experiments show that NvERG is required for gut formation and participates in apical tuft genesis. Interestingly, this loss of NvERG function in *Nematostella* causes a phenotype that is strikingly similar to the one described from blocking ERK/MAPK signaling using U0126 [35], supporting the idea that NvERG might be one of the effectors of ERK/MAPK signaling in the animal as well as the vegetal hemispheres.

Differential expression of ERK/MAPK targets along the animal-vegetal axis

A previous study has reported the differentially expressed genes identified in a genome-wide expression array comparing DMSO treated controls with U0126 treated blastula stages [35]. The spatial expression pattern presented in this study were focused on genes that were expressed in individual cells and that might be involved in early neurogenesis in *Nematostella* [35]. However, our *in situ* hybridization screen also revealed genes that were specifically expressed in the animal or vegetal hemispheres at blastula stages (24hpf) and at the end of gastrulation (48hpf) (Fig. 2, 3). While we focused on newly identified genes, we included previously published genes in our analysis to either obtain additional spatial information (e.g. *Nvgata* [30]) or because their expression domains had not been characterized during the initial period of our analysis (e.g. *Nvsix3/6*, *Nvfoxq2a* [28], *Nvsfip1* [29] and *Nvfz5/8* [31]). All original publications corresponding to a given gene (sequence identification and/or gene expression pattern) can be found in [35], Tables S4 and S5.

Genes expressed within the animal hemisphere (endomesoderm)

We observed 24 localized expression patterns within the animal hemisphere (Nvmae-like, Nvbmp1-like, Nvmeis-like, Nvsix4/5, Nvrunt, Nvperlecan-like, Nvkielin-like, NvfosB-like, Nvtbx1, Nvfox1, Nvpdgr-like, Nvret-like2, Nvfgfr-like, Nvpou-like1, Nvpou-like2, Nvk50-5, Nvgata, NveHand-like, Nvfz1-like, Nvtbx20-like, Nvmusk-like, NvephrinB-like, Nvhd058, Nvhes-like2, Fig. 2). While some genes were only faintly detected (e.g. Nvtbx1, Nvfox1 (Fig. 2O,P), only one gene, Nvk50-5 was not detected at the blastula stage (but was by the gastrula stage)(Fig. 2Zb). However, among the 23 genes that displayed localized gene expression within the animal hemisphere at the blastula stage (24hpf @17°C), only Nvhes-like2 was detected in the central ring (Fig. 2Zp). All other analyzed genes were expressed in the central domain prior to the onset of gastrulation (Nvmae-like, Nvbmp1-like, Nvmeis-like, Nvsix4/5, Nvrunt, Nvperlecan-like, Nvkielin-like, NvfosB-like, Nvtbx1, Nvfox1, Nvpdgr-like, Nvret-like2, Nvfgfr-like, Nvpou-like1, Nvpou-like2, Nvgata, NveHand-like, Nvfz1-like, Nvtbx20-like2, Nvmusk-like, NvephrinB-like, Nvhd058, Fig. 2A–F,M,O,Q,R,Y,Za,Zc,Zd,Zk–Zo).

However, at the end of gastrulation (48hpf at 17°C), the expression domains of the same set of genes are not only restricted to a single domain as seen at the blastula stage, but are expressed in at least five distinct territories (Fig. 2G–L,S–X,Ze,Zj,Zq–Zv). Only *Nvgata* is expressed in individual cells within the ectoderm (Fig. 2Zi) confirming a previous description [30]. Six genes, *NvfosB-like*, *Nvhes-like2*, *Nvmae-like*, *runt*, *Nvk50-5* and *NvephrinB-like* are expressed only within the oral ectoderm (Fig. 2T,Zh,K,Zh,Zt). *Nvmeis* is expressed in the oral ectoderm, pharyngeal ectoderm, as well as pharyngeal and body wall endomesoderm (Fig. 2I). Transcripts of *Nvmusk-like* are only detected in the pharyngeal ectoderm (Fig. 2Zs) and expression of *Nvtbx20-like2* only in the pharyngeal endomesoderm (Fig. 2Zr). *Nvbmp1-like*, *Nvsix4/5*, *Nvperlecan-like*, *Nvkielin-like*, *Nvtbx1-like*, *Nvfox1*, *Nvpdgr-like*, *Nvret-like2*, *Nvfgfr-like*, *Nvpou-like1*, *Nvpou-like2*, *NveHand-like*, *Nvfz1-like*, *Nvhd058*, transcripts are clearly detected in body wall endomesoderm and potentially also in the pharyngeal endomesoderm (Fig. 2H,J,L,S,U,V,W,X,Ze,Zf,Zg,Zj,Zq,Zu).

Genes expressed within the ectoderm/apical domain

Our *in situ* hybridization screen also revealed expression patterns of 15 genes in continuous territories within the ectoderm/apical domain (*Nvdkk124*, *Nvsftp1/5*, *Nvc-myc-like*, *Nvtolloid-like*, *Nvlhx6*, *Nvhd146*, *NvfoxD1*, *Nvsix3/6*, *Nvhmx3-like*, *Nvfoxq2a*, *Nvfz5/8*, *Nvax1*, *Nvr3-like*, *Nvsp8/9-like*, *Nvwnt7B*, Fig. 3). At the blastula stage, we observed very restricted expression of *Nvdkk124*, *Nvc-myc-like* and *Nvlhx6* towards the vegetal most part of the embryo (Fig. 3A,C,E) and broader expression within the apical domain of *Nvsftp1/5*, *Nvtolloid-like*, *Nvhd146*, *NvfoxD1*, *Nvsix3/6*, *Nvhmx3*, *Nvfoxq2*, *Nvfz5/8* and *Nvax1* (Fig. 3B,D,F,M–R). While we did not observe localized expression prior to gastrulation for *Nvr3-like* (Fig. 3Y) transcripts of *Nvsp8/9-like* were detected in a circumferential ring (Fig. 3Z) and those of *NvWnt7B* faintly throughout the entire blastula but lacking a territory that appears to correspond to the central domain (Fig. 3Za).

Of the twelve genes that were exclusively expressed within the apical domain of the blastula stage, one can distinguish two groups of genes based on their expression domains at the end of gastrulation (48hpf). While transcripts of *Nvlhx6*, *NvfoxD1*, *Nvfoxq2a* and *Nvfz5/8* are detected in a broader domain that appears to correspond to the sub-apical as well as the apical pole domains (Fig. 3K,S,V,W) expression of *Nvdkk124*, *Nvsftp1/5*, *Nvcmyc-like*, *Nvtolloid-like*, *Nvhd146*, *Nvsix3/6*, *Nvhmx3-like* and *Nvax1* seem more restricted to only the apical pole (Fig. 3G,H,I,J,L,T,U,X). *Nvr3-like* is restricted to a region of the gastrula stage that corresponds to the sub-apical pole domain (Fig. 3Zb), while *Nvsp8/9-like* expression is localized in a broader territory that spans the sub-apical pole and the body wall ectoderm (Fig. 3Zc) and *NvWnt7B* within the bodywall ectoderm, the sub- as well as the apical pole domains (Fig. 3Zd).

Of all gene expression patterns described here only *NvfosB-like* (Fig. 2N,T) and *Nvret-like2* (Fig. 2R,X) have been identified from the set of genes that were up regulated after U0126 treatments [35]. Thus, taken together these data strongly suggest that functional ERK/ MAPK signaling is crucial for specification and patterning events throughout the entire embryo by the blastula stage. Additional double *in situ* hybridization experiments are

required to fine-tune the precise boundaries of expression domains and the relationships that may exist with neighboring domains.

Temporal gene expression of endomesodermal and ectodermal genes

Spatial expression data, providing information about the presence of maternal transcripts or zygotic upregulation of a given gene, is crucial for the design of functional studies, to predict potential genetic interactions, and build gene regulatory networks [45]. We thus performed a fine scale RT-qPCR analysis (0–48hpf, every two hours) of 23 endomesodermal and 18 apical domain genes (Fig. 4C,D, Fig. S3). This set of analyzed genes contains most of the genes characterized above (Fig. 2, 3) but for the sake of enhancing the current view of the endomesodermal and ectodermal GRNs we also included genes whose ectodermal expression patterns were previously reported (see Fig. 4C,D and Tables S4 and S5 in [35] for references).

From the 41 analyzed genes, the vast majority (68%; n=28/41) were not expressed maternally (Cp value, corresponding to the cycle number at detection threshold > 34.00), only one gene did not show significant zygotic upregulation, while all remaining analyzed genes were zygotically expressed before the onset of gastrulation (20–24hpf, Fig. 4C,D). Within the endomesodermal genes (Fig. 4C), the first gene zygotically upregulated was *NvfosB-like* (8–10hpf), followed a few hours later first by *Nvhes-like2*, *Nvmusk-like*, *Nvsix4/5* (12–14hpf), then by *NveHand-like*, *Nvpdgfr-like*, *Nvmae-like* and *Nvtbx20-like2* (16–18hpf). The vast majority (14/23) within this group of genes, are zygotically upregulated either 18–20hpf (*Nvfz1-like*, *Nvgata*, *Nvkielin-like*, *Nvmeis*, *Nvpou-like1*, *Nvpou-like2*, *Nvrunt*) or 20–24hpf (*Nvbmp1-like*, *Nvfgfr-like*, *Nvfox1*, *Nvhhd058*, *Nvk50-5*, *Nvperlecan-like*, *Nvtbx1-like*). Interestingly, the general zygotic activation pattern of the 18 genes expressed in the ectoderm (Fig. 4D) was earlier than the other group of genes. In fact, *Nvlhx6* was zygotically upregulated 4–6hpf, followed by *Nvsp8/9-like*, *NvfgfA1*, *NvfgfA* (8–10hpf), *Nvax1*, *Nvfz5/8* (10–12hpf), *Nvfoxq2a* (12–14hpf), *Nvhmx3-like*, *NvfgfA2*, *Nvrx3-like* and *Nvsfp1/5* (14–16hpf). While *Nvdkk124*, *Nvhhd146* and *Nvsix3/6* are upregulated at 16–18hpf the last set of genes, *Nvc-myc-like*, *Nvtolloid-like*, *NvfoxD1* and *NvWnt7b* are zygotically regulated between 18–20hpf and 20–24hpf respectively.

NvErg is required to specify the central domain within the animal hemisphere

In order to identify downstream targets of NvERG required for gastrulation, endomesoderm as well as apical domain formation, we injected MO-NvERG into the fertilized egg and performed qPCR analysis on genes expressed in distinct domains along the animal-vegetal axis of the blastula stage (see Fig. 4A). Of the 47 genes expressed in the central domain, 18 genes (*Nvadmp-related*, *Nvfox1*, *Nvrunt*, *Nvmae-like*, *Nvpou-like2*, *NvotxA*, *Nvret-like2*, *Nvgli*, *Nvhhd050*, *Nvtolloid*, *NvelkA-like*, *Nvkielin-like*, *Nvbmp1-like*, *Nvgsc*, *NvotxB*, *Nvtbx1*, *Nvperlecan-like* and *NvotxC*) were significantly down-regulated (Fig. 5A). The only gene that is up-regulated in this context is *Nverg* which might be caused by a stabilizing effect of the morpholino targeting *Nverg* [28]. In order to confirm that NvERG-DB causes a similar phenotype than Mo-NvERG even at the molecular levels, we performed qPCR analysis on *NvERG-DB* injected embryos at 24hpf. In line with the milder phenotype observed in latter animals at later developmental stages, the downregulation of gene

expression shows a similar trend but not the same amplitude (Fig. S4). Genes that are expressed in both, the central domain as well as the central ring, or only restricted to the central, external or circumferential rings were not significantly affected by NvERG down-regulation (Fig. 5A).

We have further analyzed the molecular effect of perturbing NvERG function by *in situ* hybridization in order to confirm the qPCR data and to gain additional spatial insight. Analyzing the spatial expression patterns of central domain genes are in line with the qPCR data and showed that *nvsprouty*, *nvsix4/5* and *nvmeis* were unaffected in NvERG morphants (Fig. 5B–D,G–I). Further in agreement with the quantitative expression information, *Nvbmp1-like*, *Nvperlecan-like*, *NvotxA*, *NvotxB*, *NvotxC*, *Nvmae-like* and *Nvgli* are no longer/faintly detected in the majority of MO-NvERG injected embryos (Fig. 5E,F,J,K, L–P, Q–U). A report using computational approaches to predict gene interactions in *Nematostella* has suggested that NvERG in the central domain might repress *Nvbra* in order to restrict its expression to the central ring [38]. We therefore analyzed central ring gene expression (Fig. 5V–Y,Z–ZC) in MO-NvERG injected embryos, even though their expression levels didn't vary significantly in our qPCR assays. The *in situ* information obtained for *NvfoxB*, *NvwntA*, *Nvwnt8* (Fig. 5W–Y, ZA–ZC) were in line with the qPCR data and revealed no variations in response to NvERG inhibition. Interestingly though, *Nvbra* transcripts in NvERG morphants were now also detected in the central domain as well as the central ring (Fig. 5V,Z). The expanded expression domain of this gene highlights the importance of NvERG not only to induce expression of central domain genes, but also to repress specific gene expression in that domain. Thus, NvERG is crucial for the segregation of the central domain and central ring territories during early *Nematostella* development.

NvERG is a key player of the apical domain gene regulatory network

In addition to its expression in the central domain, *Nverg* transcripts are also detected in the apical domain (Fig. 1Ad–Af) raising the question about its role in patterning this territory. Quantitative molecular analysis of 15 genes expressed in the apical domain revealed that only five genes (*Nvsix3/6*, *Nvtolloid-like*, *Nvhd146* and *NvfoxD1*, *NvfgfA2*) are significantly downregulated after MO-NvERG injection prior to the onset of gastrulation (24hpf, Fig. 6A). Consistent with this result, spatial expression analysis revealed that no transcripts were detected in the apical domain for *Nvsix3/6*, *NvfoxD1*, *Nvhd146* and *NvfgfA2* for the majority of NvERG morphants (Fig. 6A, G–I,K). Surprisingly, we also observed a visible downregulation of *Nvsftp1/5* by *in situ* hybridization, although there was no striking effect observed by qPCR (Fig. 6A, J). These effects of NvERG down-regulation on genes expressed in the vegetal most domains are in line with the bipolar gene expression of this gene. In addition, these data suggest that the phenotype of MO-NvERG on apical tuft formation (Fig. 2h) is a direct consequence of this knockdown, rather than an indirect effect caused by the failure of gastrulation.

FGF/FGFR signaling and the transcription factor NvSix3/6 have been shown to play a crucial role for apical domain patterning at the end of gastrulation [23,28]. However, no information is available concerning the roles of those genes prior to the onset of gastrulation. In order to gain a better understanding about the relationship between NvFGFRA, NvSix3/6

and NvERG, we inhibited NvFGFRA and NvSix3/6 using previously described morpholinos [23,28] and analyzed gene expression of the same set of apical domain genes (Fig. 6A, Q–U, Y–ZA) as for NvERG morphants. In NvSix3/6 morphant early gastrula, only *NvLhx6* expression is affected (Fig. 6A, Y). However, inhibition of NvFGFRA shows that expression of *NvLhx6*, *NvfgfA1*, *NvfgfA2*, *Nvsix3/6*, *Nvhd146* and *Nvsfrp1/5* is downregulated (Fig. 6A). While *in situ* expression analysis clearly confirmed the loss of *NvfgfA2*, *Nvhd146*, *NvLhx6* and *Nvsfrp1/5* expression, the reduction of *Nvsix3/6* expression after blocking NvERG function seems subtle (Fig. 6Q–U). The clear overlap of NvFGFRA and NvERG downstream targets, strongly suggest that NvERG activity is partly mediated by NvFGFRA/MAPK signaling in the apical domain. In addition, we have assessed whether apical domain expression of *Nverg* is NvSix3/6 or FgfrA dependent. While *erg* expression is not affected in Six3/6 morphants, the apical domain expression of *erg* is not detected anymore in MO-FgfrA injected embryos, suggesting that apical *erg* expression requires functional FgfrA signaling.

Discussion

The ETS gene family in *Nematostella*

The ETS family of genes is evolutionarily conserved [42,43,46,47] and formed by transcriptional regulators involved in various aspects of development, differentiation, hormone responses and tumorigenesis [4,48–55]. Our genome wide survey of ETS transcription factors present in *Nematostella* revealed the presence of 12 members of this gene family (Fig S1). Nine of them belong to eight out of the eleven described subfamilies (SPI, ESE, TEL, TCF, ETS, ERG, ELG, PEA3). While we were not able to identify members of the PDEF, ELF and ERF subfamilies, we identified three additional genes (*Nvets-like-A*, *Nvets-like-B* and *Nvets-like-C*) that do not group in neither of the subfamilies but are predicted to contain an ETS domain (Fig S1). Expression information for *Nvpea3*, *Nvets-likeA* and *Nverg* have been reported ([24,35], this study), however, additional work is needed to gain insight into the spatial and temporal expression dynamics of those transcription factors during cnidarian development.

The PDEF, ESE, TEL, ETS, ERG and ELG subfamilies in bilaterian animals are characterized, in addition to the ETS domain, by the presence of a Pointed domain that is involved in a series of complex interactions with co-factors to modulate gene expression of downstream targets [47,56,57]. To our surprise, the only ETS gene product in *Nematostella* predicted to possess a Pointed domain is NvERG (Fig S1), suggesting a potential modulation of its transcriptional activity by other Pointed domain containing proteins. Interestingly, one of the downstream targets of MEK/ERK signaling and NvERG we identified is NvMae-like, a Pointed domain containing protein described to regulate transcriptional activity of YAN (*Drosophila* ortholog of TEL) and Pointed (*Drosophila* ortholog of ETS-2) [58]. *Nvmae-like* is zygotically expressed in the central domain under the control of NvERG suggesting that both proteins interact to potentiate transcriptional activity in this territory and further enhance segregation of specific domains within the animal domain.

A global gene regulatory network orchestrating specification and patterning events of the early embryo

Our data show that MEK/ERK signaling upstream of the transcription factor ERG is required during early embryogenesis in both hemispheres of the blastula: in the animal pole for specifying endomesoderm that probably lead indirectly to the observed failure of gastrulation and gut formation; in the vegetal pole, for specifying the apical domain and participating in apical tuft development. Thus, the present spatial and temporal expression data combined with the molecular characterization of NvERG specific knock-down experiments enabled us i) to add new genes to the existing endomesoderm GRN [24] in particular within the central domain of the blastula stage (Fig. 7), ii) extend this GRN to genes involved in early apical domain (ectoderm) specification at the same stage (Fig. 7), and iii) draft a global GRN framework for body wall endomesoderm, pharynx (endomesoderm and ectoderm), mouth, body wall ectoderm, sub-apical and apical domains, including components of the *Nematostella* nervous system (Fig. S5). In order to provide an up to date view of the genetic interactions during *Nematostella* development, the present networks (Fig. 7, Fig. S5) also include previously published functional data [24,39], [23,28,35,39,59]. In the current version, no claim about direct genetic interactions is made, and additional experiments such as *cis*-regulatory or CHIP-seq analysis are required.

The present results clearly show that NvERG is one of the main transcription factors involved in this bi-polar activity and might be one of the effectors of MEK/ERK signaling. In fact, MEK/ERK signaling is known to control transcriptional activity (enhancer or repression) of ETS transcription factors by phosphorylating specific residues [4] [60] [13] [61]. *Nverg* appears not to be transcriptionally controlled by MEK/ERK signaling as it was not identified as being one of the MEK/ERK downstream targets [35]. Nonetheless, we identified certain downstream targets of MEK/ERK [35] that are also controlled by NvERG (e.g. *NvotxA*, Fig. 5), suggesting a functional control of NvERG by ERK/MEK signaling. Using a phosphorylation motif prediction software (PhosphoMotif Finder, www.hprd.org), we identified 196 potential **Serine/Threonine Kinase/phosphatase motifs** in NvERG, 15 of which might be more prone to be sensitive to regulation by ERK [61]. However, a precise and systematic approach is required to identify the residue(s) responsible for the activation of ERG in *Nematostella*. Interestingly, we also observed that a few genes (e.g. *Nvsprouty*) downstream of MEK/ERK signaling [35] are not sensitive to NvERG knock-down (Fig. 5), suggesting that other MEK/ERK transcriptional effectors are also involved in initiating the embryonic GRN in *Nematostella*.

Relation between cWnt and ERK pathway in endomesoderm specification

We have previously shown that cWNT controls expression of genes in different domains within the animal hemisphere, with a predominant role for proper gene expression within the central ring domain [24]. In the present study, focusing only at the animal hemisphere, we have identified and characterized genes downstream of MEK/ERK signaling that are primarily expressed in the central domain at the blastula stage (Fig. 2). Among the genes down-regulated by U0126 treatments for which previous expression patterns have been reported, we can find genes expressed in the central domain (*Nvgli*, *Nvgsc*, *Nvhd50*, *Nvfix-like*, *NvotxA*, *NvotxB*, *NvotxC*, *Nvsprouty*, [22,24,62–67]), in the central ring (*Nvbmp2/4*,

NvfoxA, *Nvlmx*, *NvwntA*, *Nvwnt3*, [24,30,66,68–73]), in both central ring and central domain (*Nvnkd1-like*, [24]) as well as those just in the external ring (*Nvlhx1*, [24,74]). NvERG specific knockdown experiments show that a large part of MEK/ERK downstream targets within the central domain are also NvERG targets suggesting that within the central domain, MEK/ERK signaling might be mediated via NvERG at the transcriptional level. This in turn also suggests that in the other domains of the animal hemisphere, MEK/ERK signaling is mediated by other transcription factors that are yet to be identified.

Temporal qPCR data show that massive zygotic up-regulation of genes within the animal hemisphere identified from the UO126 array [35] begins at about 16hpf (Fig. 4). Interestingly, a temporal qPCR analysis obtained from genes downstream of cWNT signaling show a similar massive zygotic up-regulation of genes but at an earlier stage of development (prior to 14hpf) [24]. Thus, it appears that cWNT specifies a broad “endomesodermal” domain within the animal hemisphere early during embryonic development, and subsequently MEK/ERK signaling is activated to specify a sub-domain (the central domain) to restrict cWNT activity to the central ring domain. The fact that MEK/ERK and NvERG inhibition expand expression of the cWNT target and central ring gene *Nvbra* towards the central domain ([35], Fig. 5) support the idea that MEK/ERK/ERG signaling has a major impact on specifying the central domain (the future gut) and preventing cWNT activity in this domain.

Within the animal hemisphere at blastula stages, perturbing cWNT signaling has a major impact on central ring gene expression [24], while inhibiting MEK/ERK/ERG activity blocks expression of mainly central domain genes (Fig. 5). The role of cWNT signaling on gastrulation movements has been addressed using different approaches obtaining various degrees of phenotypes [24,31,33,75,76]. Nonetheless, the latest study showed that by performing a morpholino-mediated inhibition of Nv β catenin, gastrulation was blocked [31]. The present study shows that inhibition of ERG (Fig. 1) phenocopies inhibition of MEK/ERK signaling [35] by also blocking (directly or indirectly) the invagination of the endomesoderm. It would be of importance to decipher the precise mechanisms and the molecular interplay between cWNT and MEK/ERK/ERG signaling in governing morphogenetic movements of gastrulation in *Nematostella*.

Endomesoderm GRN evolution

Comparative gene regulatory analyses in echinoderm embryos, has suggested the presence of an evolutionarily conserved network “kernel” required for endomesoderm formation [77]. This Kernel is composed of five transcription factors (*bra*, *foxA*, *otx*, *blimp1* and *gataE*) that tightly interact via feedback loops and that severely affect endomesoderm formation when individually knocked-down [77]. Based on the observation that *Nvblimp* orthologs appear not to be expressed prior to the end of gastrulation (Martindale, unpublished) and that *Nvgata* transcripts were only described in individual cells of the ectoderm [30], we have previously proposed that the cnidarians endomesodermal kernel is only composed of *NvfoxA*, *Nvbra* and *NvOtx* (*A*, *B* and *C*) [24]. The present study, clearly shows that in addition to its ectodermal expression during gastrulation [30] *Nvgata* transcripts are detected in the animal hemisphere at the blastula stage, strongly suggesting that this gene could be

part of the cnidarian endomesoderm kernel. At the blastula stage *Nvbra* and *NvfoxA* (both downstream of cWNT signaling [24]) are expressed in the central ring while *Nvotx* (*A,B* and *C*) and *Nvgata* are expressed in the central domain. As NvERG is required to repress *Nvbra* expression in the central domain ([38], this study), these observations foster the idea that rather than being a general endomesoderm kernel connected by feedback loops, those genes are required for the segregation of a central domain and a central ring prior to the onset of gastrulation. A careful analysis of their spatial expression during earlier developmental stages and importantly, functional studies to decipher the precise genetic interactions between those genes is required to better understand a evolutionary conservation of the endomesoderm kernel.

In sea urchins, MEK/ERK signaling, activated during later stages by FGF/FGFR and VEGF/VEGFR [4,7,78,79] is required for mesoderm formation. Interestingly, ETS1 is activated in the primary mesenchyme cells (PMC, mesoderm) by MEK/ERK and crucial for ERG expression (<http://sugp.caltech.edu/endomes/#Veg-6-18-NetworkDiagram>), PMC ingression and differentiation into the specific mesodermal lineage [4]. On the other hand echinoderm cWNT signaling is required to initiate the general endomesoderm GRN and drive endoderm specification once the mesoderm specification program has been launched [80,81]. Our observations about i) cWNT initiating a broad endomesoderm GRN with a particular emphasis on central ring domain expression [24] and ii) MEK/ERK/ERG signaling being required to repress central ring fate (*Nvbra* expression) and specify the central domain illustrate the strong evolutionary conservation of this mechanism between cnidarians and echinoderms. Cnidarians are described not to form a true mesodermal germ layer. However, the expression of the classical mesodermal marker *brachyury* [82] in the central ring, as well as the activation of a MEK/ERK/ERG pathway (required for mesoderm formation in bilaterians [83]) in the central domain, provide additional compelling evidence that both domains together form sub-regionalized territories of the cnidarian endomesoderm. Tissue tracking experiments using photo-convertible fluorescent proteins [84] are required to determine the fate of the central domain and ring. It might also be important to carry out a precise physiological analysis of the differentiated tissues that originated from the endomesodermal territory, to gain more insight in the evolutionary origin of mesoderm in bilaterians.

Relationship between cWnt and MEK/ERK/ERG signaling in patterning the apical domain

Several studies suggested a role of cWNT signaling in ectoderm patterning, as ectopically activating or inhibiting cWNT [24,33,75,76,85], perturbs gene expression within the body wall ectoderm and apical domain. In particular, inhibiting β cat function causes the loss of *Nvfgfa1*, *NvfoxQ2* and *Nvsix3/6* expression in the apical domain [31]. In a previous study, we predominantly reported genes that were up-regulated following ectopic cWnt activation [24]. However, about 30 genes were also downregulated under those conditions (Table S1). Interestingly, 14 out of the 30 identified genes are also down regulated after MEK/ERK inhibition [35]. Among these 14 genes downregulated following MEK/ERK inhibition, seven are expressed in the apical domain (*Nvtolloid-like*, *Nvsfrp1/5*, *Nvlhx6*, *Nvhmx3*, *Nvhd146*, *NvfoxD1*, *Nvax1* (Fig. 3)), four in individual cells throughout the ectoderm (*Nvhd145*, *Nvgfi-like*, *Nvgcm*, *Nvcoup-like2* [35]) and two in derivatives of the animal pole

(*Nvhes-like2*, *Nvgsc* (Fig 2, [24])). Inhibition of NvERG has no detectable effect on genes expressed in the individual cells of the ectoderm (data not shown) or *Nvhes-like2* of the central ring (Fig. 5). However, perturbing NvERG function clearly blocks *Nvgsc* expression in the central domain (Fig. 5) and expression of *Nvtolloid-like*, *Nvsfp1/5*, *Nvhmx3*, *Nvhd146* and *NvfoxD1* (Fig. 6) within the apical domain. These observations strongly support the idea of an antagonistic effect of cWnt and MEK/ERK/ERG signaling in a specific set of genes and that this signaling pathway interplay is crucial for patterning the apical domain. Importantly though, *Nvsix3/6* expression (a key regulator of apical domain formation (28)) requires the combined activity of cWnt (31) as well as MEK/ERK/ERG signaling further underlining the complex interplay of these two pathways to pattern the cnidarian embryonic body axis. Additional gene specific experiments as well as *cis*-regulatory analysis of the above mentioned genes would shed further light on the direct or indirect antagonistic effects of these two major developmental signaling pathways for cnidarian embryogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Characterization of NvERG, a bi-polarly expressed transcription factor in cnidarians
- NvERG is required for endomesoderm and apical domain formation
- Identification of ERG downstream targets, distinctly expressed in both poles
- NvERG simultaneously controls two different GRNs in opposite poles

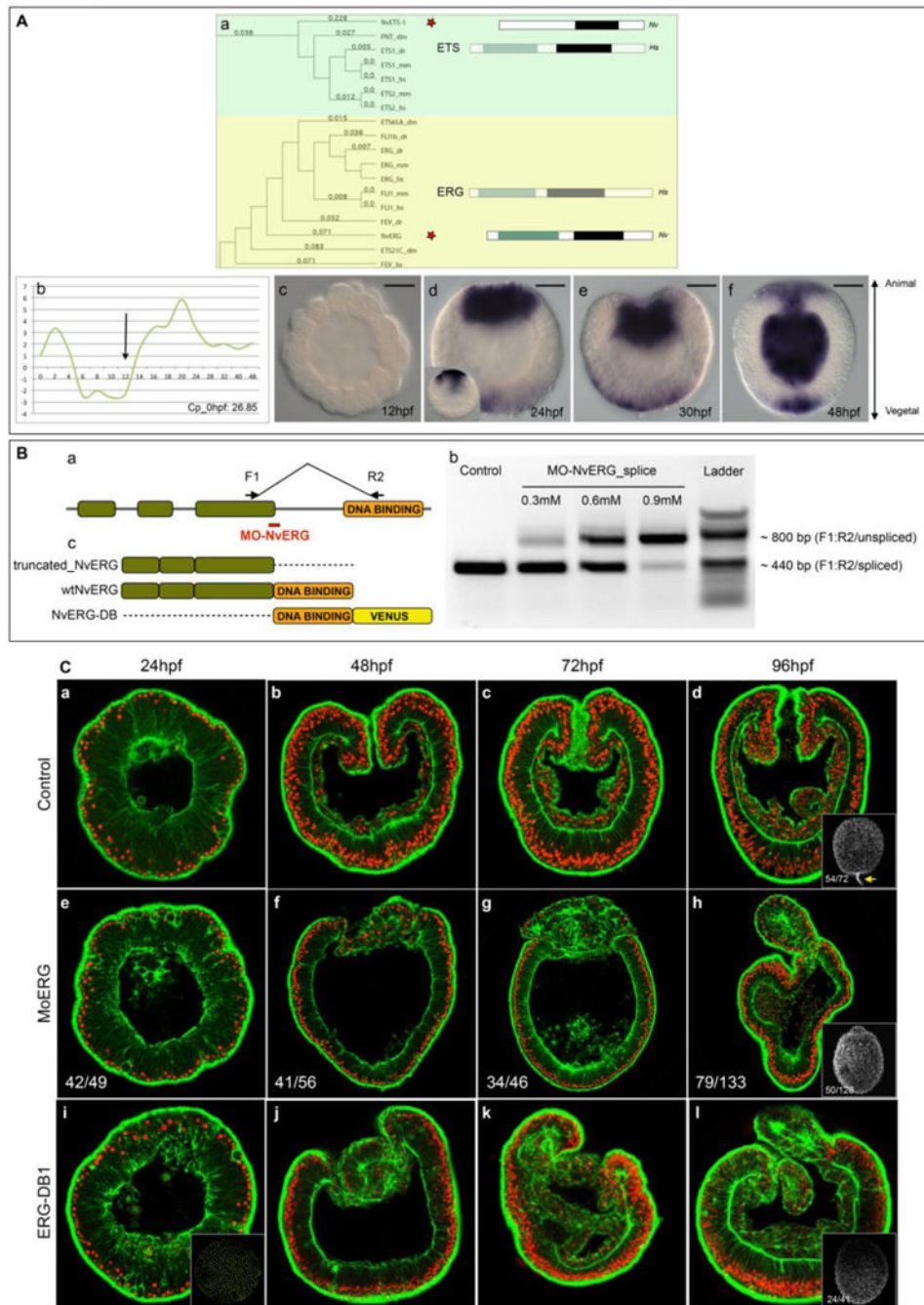


Figure 1. NvErg is required for endomesoderm formation, gastrulation and participates in apical tuft development

(A) Identification of NvErg and analysis of its spatiotemporal expression. (Aa) Excerpt of the phylogenetic analysis of the ETS transcription factor complement in *Nematostella*, indicating the existence of NvERG and NvETS1 orthologs in cnidarians. The full analysis can be found in Fig. S1. To the right of the tree, the bars indicated the protein domain organization of either the human representative (*Hs*, greyed out rectangles) of the subfamily as well as the protein domain organization of the *Nematostella* ortholog (*Nv*). Green

rectangles indicate the Pointed domain, black rectangles the ETS domain. **(Ab)** Temporal expression of *Nverg* analyzed by qPCR during embryonic development of the first 48 hours post fertilization. The y-axis indicates relative fold changes compared to fertilized eggs. **(Ac–Af)** Spatial (*in situ* hybridization) expression of *Nverg* at early (Ac) and late (Ad) blastula, mid (Ae) and late (Af) gastrula stages. **Orientation of blastula stages was determined by the thickening of the animal pole prior to its invagination that was observable in certain embryos of a given batch at the analyzed time point.** Animal pole to the top and vegetal pole to the bottom. **The black bar in the upper right corner of Ac–Af indicates the scale bars: 50µm** **(Ba)** Schematic representation of the genomic organization of NvERG, and the recognition site of MO-NvERG and the position of the PCR primers to verify the efficiency of the splice blocking morpholino. **(Bb)** Splice blocking efficiency of MO-NvERG analyzed by RT-PCR at increasing concentrations of MO-NvERG injected embryos. **(Bc)** Schematic representation of the protein structure of the various tools used in Fig. 1C and Fig. S2. **(C)** Morphological effects of inhibiting NvERG function during early *Nematostella* development. **(Ca–Cd)** Control embryos injected with MO-CTRL, **(Ce–Ch)** embryos injected with MO-NvERG or **(Ci–Cl)** mRNA encoding a dominant negative version of NvERG (NvERG-DB1) at 24 (Ca,Ce,Ci, late blastula), 48 (Cb,Cf,Cj, late gastrula), 72 (Cc,Cg,Ck, early planula) and 96 (Cd,Ch,Cl, late planula) hpf. All images are lateral views with the animal/oral pole to the top and confocal z-sections using phalloidin (green) to show f-actin filaments and propidium iodide (red) to visualize the nuclei. The insets in (Cd, Ch, Ci) correspond to lateral views of embryos stained with acetylated tubulin to visualize the presence or absence of the apical tuft (yellow arrow in Cd). The numbers in the insets indicate the number of embryos with the represented phenotype/total amount of analyzed animals.

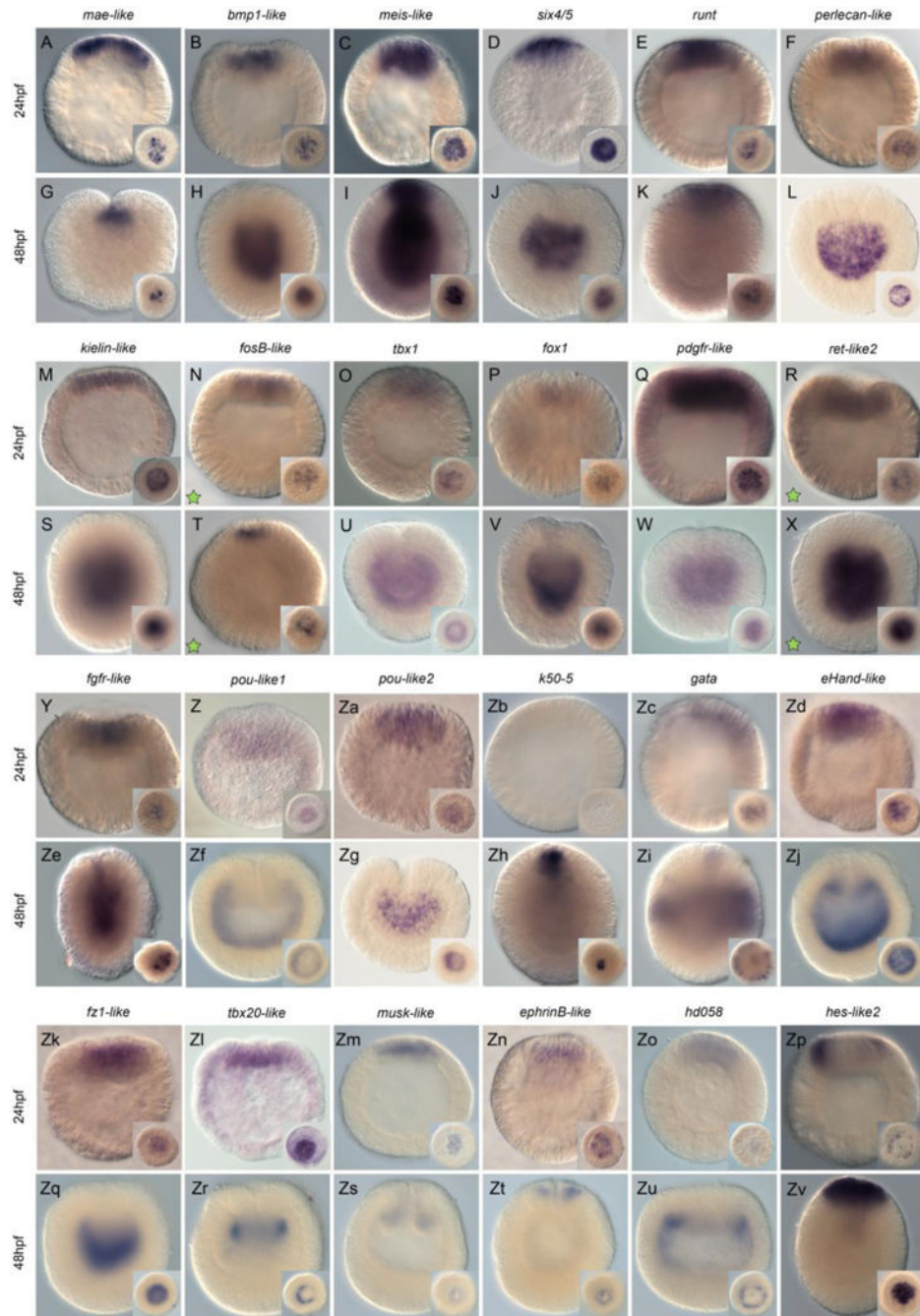


Figure 2. MEK/ERK signaling targets expressed in the presumptive endomesoderm
 Wild-type endomesodermal gene expression analysis by *in situ* hybridization of genes differentially regulated by U0126 treatments. All animals are either blastula (24hpf - A-F, M-R, Y-Zd, Zk-Zp) or gastrula (48hpf - G-L, S-X, Ze-Zj, Zq-Zv) stages. All images are lateral views with the animal pole (presumptive endomesoderm) to the top. The insets correspond to animal pole views (A-G, I, K, M-R, T, Y-Zd, Zh, Zv, Zk-Zp) or optical cross-sections. Antisense probes used as indicated at the top of each pair of embryos. Green

stars in N, T and R, X indicate that these genes were upregulated under U0126 conditions. All other genes were downregulated.

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Figure 3. MEK/ERK signaling targets expressed in broad ectodermal domains

Wild type ectoderm gene expression analysis by *in situ* hybridization of genes downregulated by U0126 treatments. All animals are either blastula (24hpf - A–F, M–R, Y–Za) or gastrula (48hpf – G–L, S–X, Zb–Zd) stages. All images are lateral views with the animal pole to the top. All insets correspond to vegetal pole/aboral views. Antisense probes used as indicated at the top of each pair of embryos.

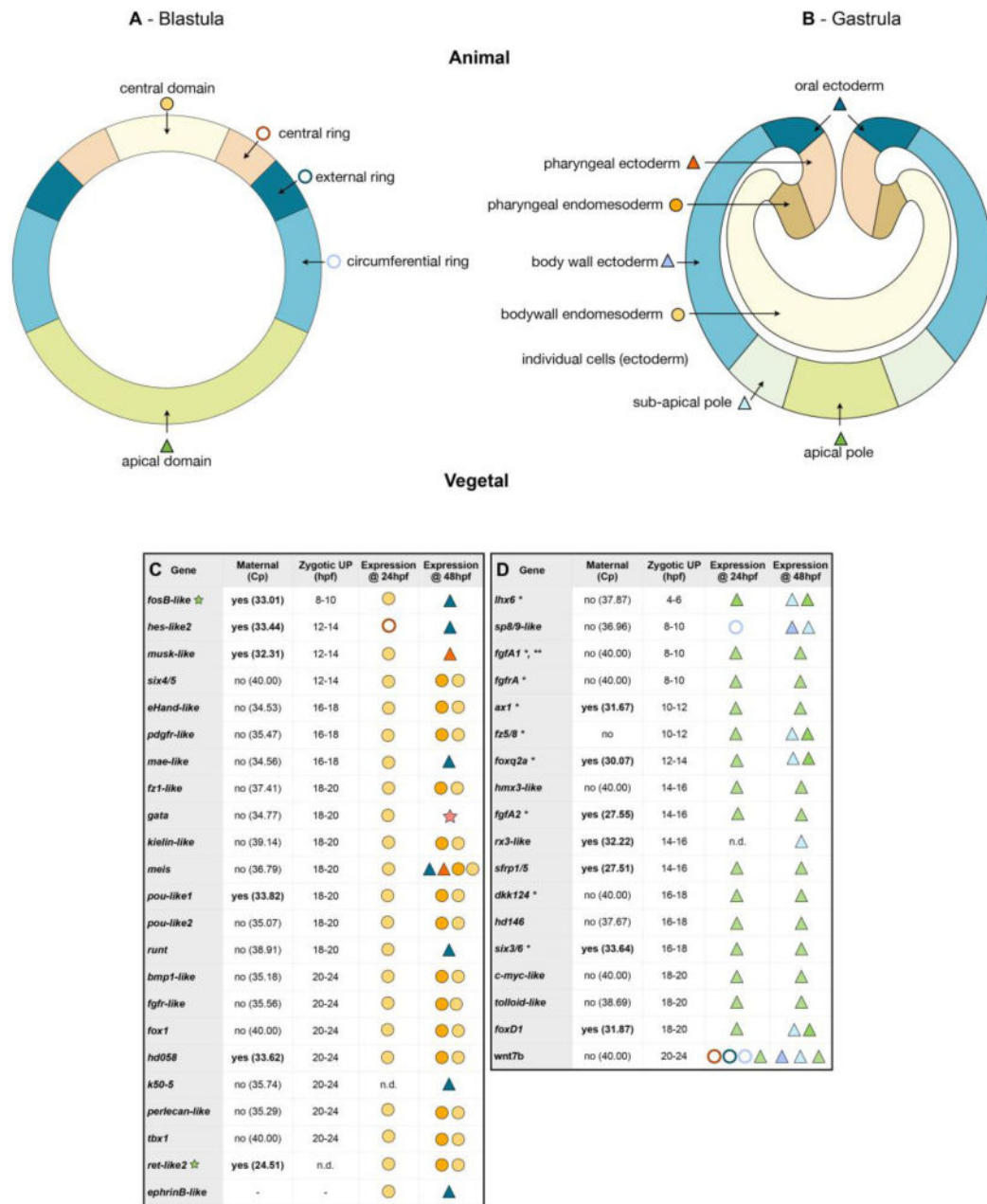


Figure 4. High density gene expression profiling

(A, B) Schematic representation of stage-specific expression domains prior to, and after gastrulation movements. (C, D) Summarized results of the temporal high density profiling (qPCR) used to determine the presence of maternal transcripts and significant zygotic up-regulation of a given gene expressed in (C) endomesodermal, or (D) broad ectodermal domains (see Fig. S3 for details). The Cp value corresponds to the cycle number at detection threshold (crossing point). (hpf) hours post fertilization. Visual keys used to describe the spatial expression domain determined by *in situ* hybridization at 24hpf or 48hpf same as in A,B. (n.d.) Not determined. (*) Indicate genes that have been identified and their spatial blastula and gastrula expression patterns characterized elsewhere (see Tables S4 and S5 in

[35] for references). However, to include them into our GRNs we performed qPCRs also for these genes (i.e. *fgfA1*, *fgfA2*, *ax1* etc...). (**) qPCR value from [24].

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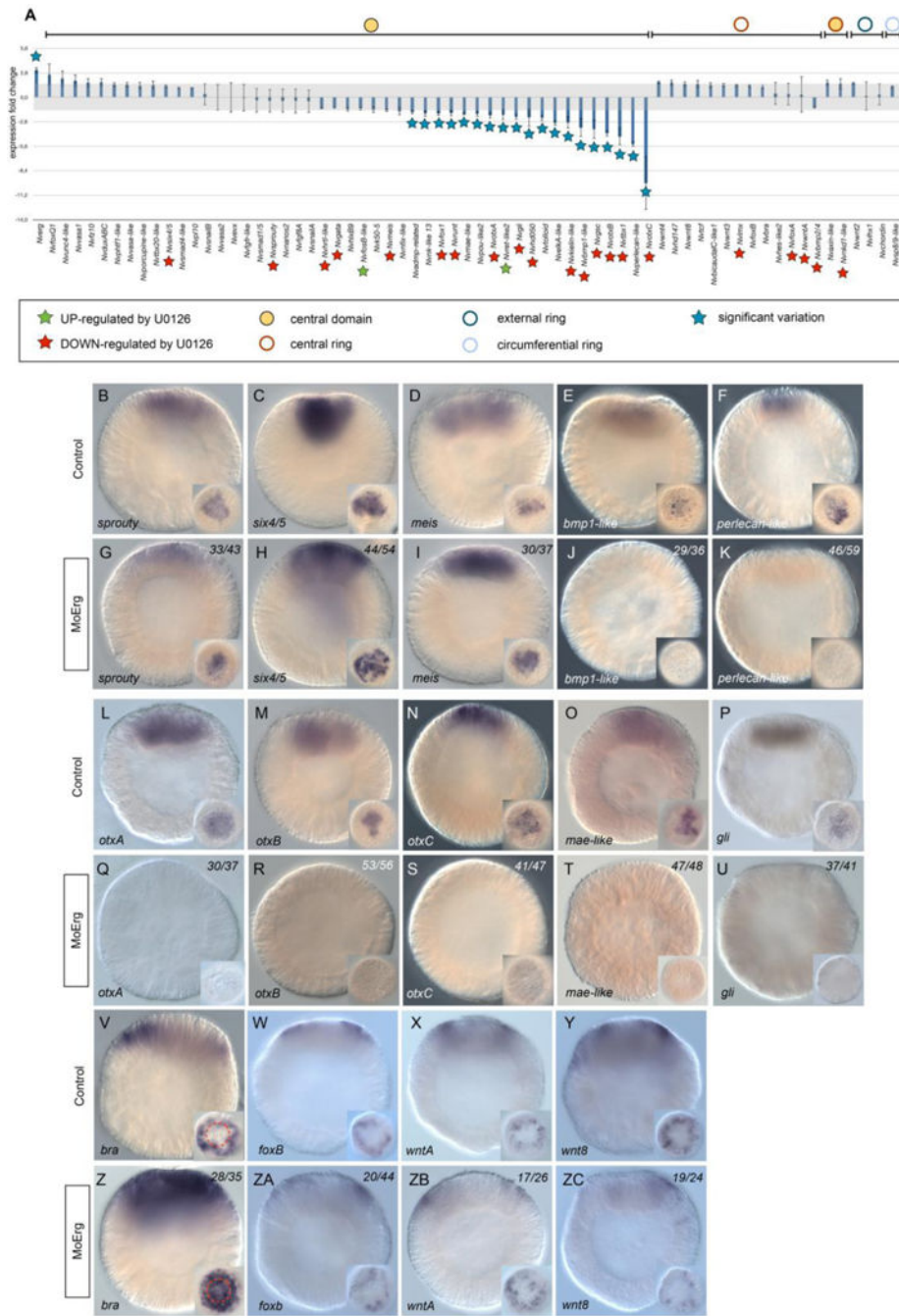


Figure 5. Molecular phenotype analysis of Mo-NvERG injected embryos on genes expressed in the endomesoderm

(A) Changes in gene expression of 66 potential components of the cnidarian endomesoderm GRN within the animal hemisphere after NvERG knock-down compared to control embryos analyzed by qPCR. Changes in gene expression are indicated as relative fold changes compared to MO-CTRL injected embryos (x ± sem, n = 3 per gene). The grey bar indicates no significant change in gene expression (−1.5,1.5). Information on the iconography (stars and circles) are indicated below the graph. Gene expression domains at the blastula stage are

the same as Figure 4A. **(B)** Analysis of the molecular effects of NvERG inhibition (G–K, Q–U, ZA–ZE) compared to control injections (B–F, L–P, V–Z) on endomesodermal gene expression analyzed by *in situ* hybridization. Antisense probes used as indicated in the bottom left corner of each image (also valid for the corresponding inset). The red dashed circle in (V,Z) indicates the central ring expression of *Nvbra* showing extension of its expression domain into the central domain. The numbers in the upper right corner indicates the ratio of embryos with perturbed gene expression to the total number of analyzed embryos. All images are lateral views with the presumptive endomesoderm (animal pole) to the top. Insets are animal pole views.

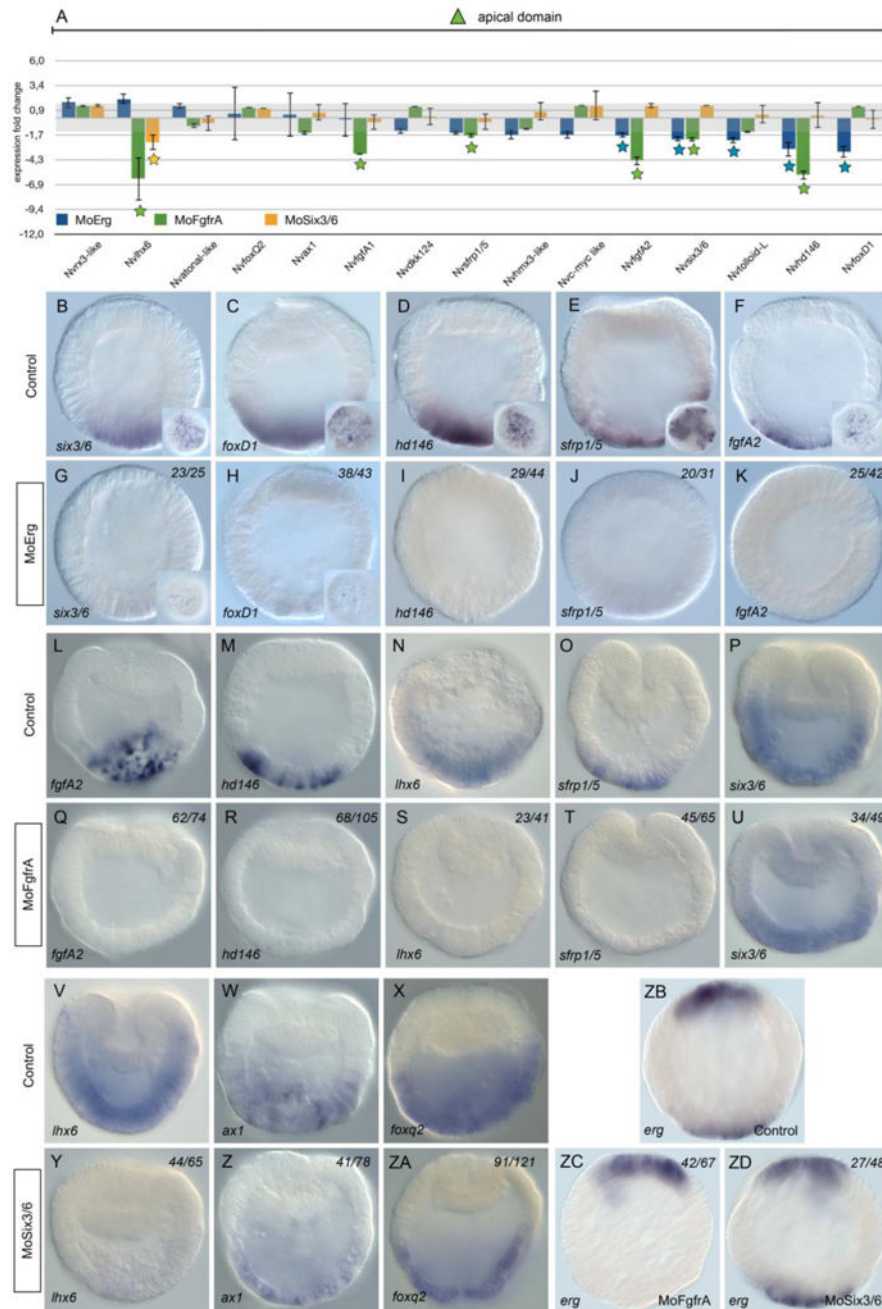


Figure 6. Molecular phenotype analysis of MO-NvERG, MO-NvFGFRA and NvSix3/6 injected embryos on genes expressed in the apical domain

(A) Changes in gene expression of 15 potential components of the cnidarian apical domain GRN within the vegetal hemisphere after NvERG (blue), NvFGFRA (green) or NvSix3/6 (yellow) knock-downs compared to control embryos analyzed by qPCR. Changes in gene expression are indicated as relative fold changes compared to MO-CTRL injected control embryos ($x \pm \text{sem}$, $n = 3$ per gene). The grey bar indicates no significant change in gene expression ($-1.5, 1.5$). Stars below the bars indicate significant variation. Analysis of the molecular effects of NvERG (G–K), NvFGFRA (Q–U) and NvSix3/6 (Y–ZA) inhibition

compared to control injections (B–F, L–P, V–X) on apical domain gene expression analyzed by *in situ* hybridization. Antisense probes used as indicated in the bottom left corner of each image (also valid for the corresponding inset). The numbers in the upper right corner indicates the ratio of embryos with perturbed gene expression to the total number of analyzed embryos. All images are lateral views with the presumptive endomesoderm (animal pole) to the top.

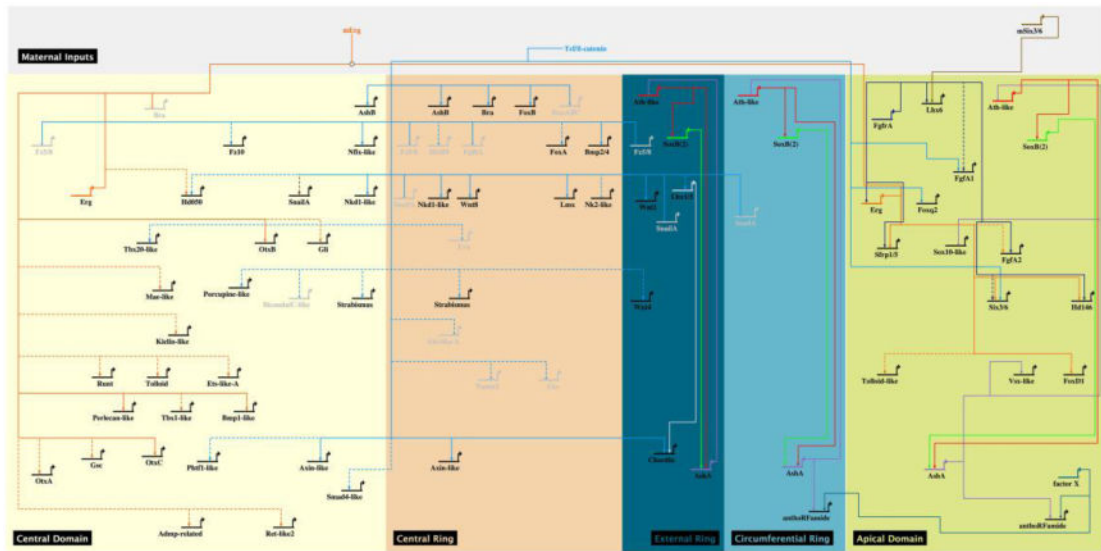


Figure 7. Updated gene regulatory network orchestrating embryonic development in the cnidarian *N. vectensis*

Enhanced Biotapestry diagram [86] of the gene regulatory network describing the gene deployment at 24hpf and regulatory interactions of endomesodermal, ectodermal and neuronal genes identified in previous studies [24,31,35,39,74]. No assumption on whether these interactions are direct or indirect is made. Solid lines indicate functional evidence obtained by qPCR as well as *in situ* hybridization, dashed lines indicate evidence obtained only by qPCR. The colored boxes represent the spatial domains as described in Figure 4A. Genes inactivated by repression in a given territory are represented in light grey. Controversial results [24,76] about the role of cWnt/TCF signaling on *NvsnailA* expression is indicated by a red dashed arrow. The same GRN, including non-connected genes that are expressed within the specific territories is provided in Figure S5. A first draft of the global GRN framework for body wall endomesoderm, pharynx (endomesoderm and ectoderm), mouth, body wall ectoderm, sub-apical and apical domain including components of the *Nematostella* nervous system at the end of gastrulation (48 hpf) is provided in Figure S6.