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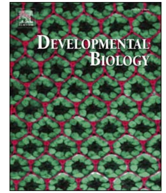

Evolution of developmental control mechanisms

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 bipolarily 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 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.

1. 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 (Schulte-Merker and Smith, 1995; Burdine et al., 1997; Draper et al., 2003; Röttinger et al., 2004, 2008, 2015; Stathopoulos et al., 2004; Yasuo and Hudson, 2007; Ota et al., 2009; Bertrand et al., 2011; Green et al., 2013). 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 (Bertrand et al., 2014). Well known transcriptional regulators whose activity can be controlled by MEK/ERK signaling, belong to the ETS domain containing family of transcription factors (Selvaraj et al., 2015).

Cnidarians are the extant sister group to all bilaterians and their phylogenetic position makes them very interesting for understanding the evolution of biological novelties (Martindale and Hejnol, 2009; Technau and Steele, 2011; Layden et al., 2016a). 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 (Layden et al., 2016a, 2013; Hand and Uhlinger, 1992; Darling et al., 2005; Putnam et al., 2007; Ikmi et al., 2014). Based on the sequenced *Nematostella* genome (Putnam et al., 2007), 15 putative FGF ligands

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and three potential receptors have been identified (Matus et al., 2007; Rentzsch et al., 2008) with the spatial expression patterns reported for three ligands and two receptors (Matus et al., 2007; Rentzsch et al., 2008; Röttinger et al., 2012). 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 (Hacohen et al., 1998; Casci et al., 1999; Kramer et al., 1999), is also expressed in both extremities of the developing embryo/larvae (Matus et al., 2007). Only based on these expression patterns, FGF signaling has been suggested to play a role in gastrulation and neural development (Matus et al., 2007). 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 (Rentzsch et al., 2008; Sinigaglia et al., 2013, 2014).

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 (Hand and Uhlinger, 1992; Rentzsch et al., 2008; Martindale et al., 2004). 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 ß-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 (Rentzsch et al., 2008; Sinigaglia et al., 2013, 2014; Leclère et al., 2016). 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 (Röttinger et al., 2012; Martindale et al., 2004; Technau and Scholz, 2003). 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 (Lee et al., 2007; Fritzenwanker et al., 2007). 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 (Röttinger et al., 2012). 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 (Röttinger et al., 2012). 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 (Röttinger et al., 2012). 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 (Layden et al., 2016b). The same authors have also shown that pharmacologically inhibition of ERK/MAPK signaling using U0126, a potent inhibitor of the ERK activating kinase MEK (Davies et al., 2000; DeSilva et al., 1998), after fertilization blocks gastrulation and endomesoderm formation (Layden et al., 2016b). 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 (Layden et al., 2016b). 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 (Layden et al., 2016b).

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 (Layden et al., 2016b) 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 (Abdol et al., 2017) 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*.

2. Materials and methods

2.1. 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 (Röttinger et al., 2012). Fertilized eggs were kept in dark in filtered 1/3 seawater at 16 °C until the desired stage.

2.2. RNA Extraction and quantitative PCR (qPCR)

RNA Extraction and quantitative PCR (qPCR) was performed following protocols described in (Röttinger et al., 2012): 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 (Layden et al., 2012). The full list of qPCR primer pairs and their efficiency used in this study can be found in Table S2 or (Layden et al., 2016b). The houskeeping 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.

2.3. 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 (Layden et al., 2016b). Genome predictions as well as EST

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