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TGF-β signaling molecules in *Hydra*: role of BMP and BMP inhibitors during pattern formation

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ABSTRACT Understanding the evolution of body plans has been one of the major areas of investigation in developmental and evolutionary biology. Cnidaria, the sister group to bilaterians, provides an opportunity to elucidate the origin and evolution of body axes. *Hydra*, a freshwater cnidarian, is a useful model to study signaling pathways governing pattern formation, which are conserved up to vertebrates including humans. The transforming growth factor β (TGF-β) signaling pathway is one of the fundamental pathways that regulate axis formation and organogenesis during embryonic development. In this article, we discuss the TGF-β pathway members identified in *Hydra* along with other cnidarians with an emphasis on bone morphogenetic proteins (BMPs) and their inhibitors. TGF-β members, especially those involved in BMP signaling pathway, are mainly involved in maintaining the Organizer region and patterning the body axis in *Hydra*. Identification of other members of this pathway in *Hydra* and fellow cnidarians would provide insights into the evolution of body axes and pattern formation in more complex metazoans.

KEYWORDS: Hydra, evolution, pattern formation, BMP, BMP inhibitors, TGF-β

Introduction

Seven conserved pathways, Wnt, transforming growth factor-β (TGF-β), Receptor Tyrosine Kinases, Hedgehog, Janus kinase/signal transducer and activator of transcription (JAK/STAT), Notch and nuclear receptor pathways drive cell signaling during the embryonic development and are used repeatedly during later developmental processes in metazoans (Gerhart, 1999; Barolo and Posakony, 2002). Many of these signaling pathways act by ligand binding to their respective receptors, activating transcription factors leading to regulation of gene expression (Trompouki *et al*., 2011). Alterations in spatio-temporal expression patterns of transcription factors and signaling components not only increase the extent of cellular response but also affect the complexity of cross talk between different pathways (Itasaki and Hoppler, 2010). Interestingly, several components of these seven signaling pathways have been identified in basal phyla such as Porifera (Liongue and Ward, 2013; Borisenko *et al*., 2016, 2019) and Cnidaria (Hobmayer *et al*., 2000; Miller *et al*., 2000; Reinhardt *et al*., 2004; Technau *et al*., 2005; Kasbauer *et al*., 2007; Putnam *et al*., 2007) and are seen to be conserved up to humans. This clearly shows that many of these signaling pathways had already evolved to a greater or lesser extent in the common ancestors of the bilaterians.

The TGF-β signaling pathway is one of the fundamental signal transduction machineries crucial for early embryogenesis, axis formation, organogenesis, and development. This pathway plays an important role in providing positional information during embryogenesis in both invertebrates and vertebrates (Hobmayer *et al*., 2001). The pathway is known to regulate diverse cellular processes including cell proliferation, differentiation, migration, apoptosis, and adult tissue homeostasis in vertebrates. Deregulation of the pathway leads to several pathological conditions including cancer (Massagué *et al*., 2000). The entire TGF-β superfamily can be divided into two groups: (1) bone morphogenetic proteins (BMPs), the most prominent TGF-β ligands, which induce signaling by the canonical small mothers against decapentaplegic (Smad)-dependent pathway mediated by two types of serine/threonine kinase receptors, BMP receptor (BMPR) -I and -II, through activation of Smad 1, 5 and 8 (Chen and Shen, 2004) or by the Smad-independent pathway, which involves TGF-β Activated Tyrosine Kinase 1 (TAK1) and Mitogen Activated Protein Kinase (MAPK) (Zhang and Li, 2005) and (2) the

Abbreviations used in this paper: BMP, Bone morphogenetic protein; HyBMP, Hydra BMP; HySmad, Hydra Smad; Smad, Suppressor of mothers against decapentaplegic; TGF-β, Transforming growth factor-β; WISH, Whole mount in situ hybridization.

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TGFs, activin, nodal and myostatin, which act through Smads 2 and 3 (Wharton and Derynck, 2009). Signals are mediated through a heterotetrameric receptor unit composed of two transmembrane type I and type II receptors. Ligand binding to the type II receptors causes activation of serine-threonine kinase domain of the type I receptors and results in receptor Smad (R-Smad) phosphorylation. Phosphorylated R-Smads form hetero-oligomeric pairing with common/collaborating Smads (Co-Smads), enter the nucleus and regulate gene expression. Two inhibitory Smads (I-Smads), Smads 6 and 7 also exist, which regulate TGF-β signaling by inhibiting the R-Smads and Co-Smads. It is evident from the sequence similarities that these Smads arose due to gene duplication; their evolutionary origin and functions, however, remain unclear. In this review article, we discuss key molecules involved in establishment of the axis in *Hydra* and other cnidarians since this can potentially provide insights into axial patterning during metazoan evolution.

Axis formation in cnidarians

Cnidarians, the sister group of bilateria, possess an oral-aboral axis with a radial symmetry as opposed to the distinct anterior-posterior, dorsal-ventral and left-right axes found in bilaterians, which are regulated by Wnt, TGF-β/BMP and Nodal signaling pathways, respectively (Watanabe *et al*., 2014a). In bilaterians, axial patterning is predominantly controlled by the Wnt pathway, which also helps in the establishment of the Organizer, specification of dorsal-ventral and anterior- posterior axes and imparts positional information during vertebrate and invertebrate embryonic development. Owing to their unique phylogenetic position and relatively simple body plan, molecular mechanisms governing formation of body axes can be better understood by studying cnidarians. *Nematostella vectensis*, *Clytia hemisphaerica* and *Hydra* are some of the best studied cnidarian models to understand axial patterning. In *Nematostella*, an anthozoan cnidarian, 13 Wnt genes are expressed along the oral-aboral axis in the developing embryo and larval forms, while only Wnt1 and Wnt3 are shown to possess the secondary axis inductive capacity (Kraus *et al*., 2016). In the hydrozoan *Clytia hemisphaerica*, role of two Frizzled receptors and the maternally deposited Wnt3 ligand in the establishment of oral/aboral axis has been reported (Momose and Houliston, 2007; Momose *et al*., 2008). Similar role for Wnt signaling in the establishment of oral axis in the colonial Hydrozoan *Hydractinia* (Duffy *et al*., 2010) and in the anthozoan cnidarian *Acropora millepora* (Hayward *et al*., 2015) has been reported. This suggests that the dependency on Wnt pathway members for establishing axial patterning predates the cnidarian-bilaterian split.

Similarly, components of TGF-β/BMP signaling pathway, which are involved in dorsoventral axis formation in vertebrates and correspond to establishing oral-aboral axis in cnidarians were investigated and resulted in the identification of several components of the TGF-β/BMP pathway and their inhibitors. It is interesting to note, however, that the regulation of directive axis formation in cnidarians is quite different from that observed in the bilaterians. In *Nematostella*, overlapping expression patterns of decapentaplegic (dpp) and its inhibitor Chordin are seen during the development of the embryo on one side of the blastopore, while in *Drosophila* and *Xenopus*, dpp and its antagonists, short gastrulation gene (*sog*) and its ortholog Chordin are expressed in opposing domains (Hayward *et al*., 2002; Matus *et al*., 2006; Rentzsch *et al.,* 2006). A gradient of BMPs and asymmetric expression of its inhibitors, dpp and Chordin on one side of the oral end of the developing embryo also suggest presence of a secondary axis that acts perpendicular to the oral-aboral axis and their role in secondary axis patterning in *Nematostella* (Finnerty *et al*., 2004; Matus *et al*., 2006; Saina *et al*., 2009; Genikhovich and Technau, 2017). One of the BMP-interacting proteins, repulsive guidance molecule (RGM) is also shown to be co-expressed along with BMP2/4 and Chordin and has been shown to play a role in developing bilateral symmetry in *Nematostella* (Leclère and Rentzsch, 2014). A similar expression of dpp is also seen in the anthozoan coral, *Acropora*, suggesting a role in establishing the directive axis (Hayward *et al*., 2002). This suggests that the molecular toolkit of major signaling pathways for the formation of dorsal-ventral and left-right axes in bilaterians is already present in the cnidarians.

Axial patterning in *Hydra*

Hydra, a freshwater hydrozoan, is one of the most favorite models to study axis formation due to its simple body plan, oral-aboral axis and radial symmetry (Ghaskadbi, 2020a, Ghaskadbi, 2020b; Wang *et al*., 2020). It exhibits a simple, yet defined body plan with a single oral-aboral axis comprising of a hypostome that serves as mouth surrounded by a few tentacles at the oral end, and a basal disc at the aboral end using which it attaches to the substratum. The cnidarian oral opening is often compared to the posterior end of bilaterians, where *wnt* signaling is active and is required for posterior growth (Meinhardt, 2002; Martin and Kimelman, 2009; Petersen and Reddien, 2009). Bilaterians are broadly classified into protostomes and deuterostomes based on the site of gastrulation that forms endodermal tissue during blastopore formation and gives rise to either mouth or anus. In both protostomes and deuterostomes, the vegetal pole cells and animal pole cells form endoderm and oral/anterior structures, respectively. While the formation of anus from the site of gastrulation in deuterostome is not questioned, development of mouth from the site of gastrulation in protostomes is disputed (Martindale, 2013). For example, in priapulid, an ecdysozoan worm, the gastrulation pattern and the molecular markers expressed are similar to those seen in deuterostomes (Martín-Durán *et al*., 2012) suggesting a need to study gastrulation patterns in protostomes.

Establishment and maintenance of the polarity of anterior-posterior axis is often controlled by Wnt signaling along with TGF pathway members which are conserved across different animal phyla. In case of cnidarians, the oral structure is developed from the animal pole cells as seen in bilaterians (Martindale, 2013) and shows expression of Wnt pathway members, thus conferring cnidarian oral axis similarity to that of posterior end of bilaterians. In deuterostomes, including echinoderms and hemichordates, Wnt signaling aids in posteriorization of neural ectoderm, while it induces oral nervous system in another anthozoan cnidarian, *Nematostella* suggesting that the bilaterian posterior end is similar to the cnidarian oral side (Watanabe *et al*., 2014b). This is also supported by the fact that *Hydra* hypostome shows expression of *wnt* and *Brachyury* (Technau *et al*., 1999; Hobmayer *et al*., 2000), the posterior expressing genes in vertebrates suggesting that the marginal zone of vertebrates and the *Hydra* head Organizer are similar and the absence of head specific genes such as *Nkx* in the hypostome further supports the close similarity between oral-aboral axis of *Hydra* and posterior-anterior axis of vertebrates (Grens *et al*., 1996; Meinhardt, 2012; Holstein, 2022, 2024). Also, the expression of Wnt-3 in the posterior tip of *Hydractinia* larvae that develops into the oral pole confirms

TABLE 1

EXPRESSION DOMAIN AND POSSIBLE FUNCTIONS OF TGF-β SIGNALING PATHWAY MEMBERS IN *HYDRA*

Gene name	Expression domain in adult polyps	Possible functions	References
Hysmad1	Body column except basal disc	Nematocyte and germ cell specification, oogenesis	Hobmayer *et al.*, 2001
HyBMP5-8a	Body column except basal disc	?	Reinhardt *et al.*, 2004
HyBMP5-8b	Base of the tentacles, lower 1/3 of body column	Tentacle formation, patterning of lower body column	Reinhardt *et al.*, 2004
chordin	Head region	Organizer formation	Rentzsch *et al.*, 2006, 2007
nodal	Presumptive budding zone	Determination of biradial symmetry	Watanabe *et al.*, 2014a
activin	Ubiquitous expression towards oral side	?	Watanabe *et al.*, 2014a
noggin	Hypostome, base of the tentacles,lower body column, basal disc	Organizer maintenance, tentacle formation	Chandramore *et al.*, 2010; Krishnapati *et al.*, 2020
gremlin	Budding zone	Budding	Krishnapati *et al.*, 2020

that the oral side of cnidarians is equivalent to the posterior pole of bilaterians (Duffy *et al*., 2010). In *Hydra*, *HyWnt*, an orthologue of *wnt3A* is expressed in the hypostome, which acts as the head organizer and plays a role in controlling axial polarity and patterning (Hobmayer *et al*., 2000; Broun and Bode, 2002). In addition to the Wnt/β-catenin pathway, recent findings suggest involvement of Hippo/YAP pathway in establishing new axis during budding by regulating YAP (Yorkie in *Drosophila*), that acts upstream of the Wnt pathway (Brooun *et al*., 2022). Presence of the first YAP was reported from *Trichoplax adhaerens*, a placozoan. All components of the core machinery of the modern Hippo/YAP pathway are seen in *Nematostella* (Hilman *et al*., 2011). During regeneration on the oral side, expression of Hippo/YAP pathway components is regulated followed by activation of Wnt-PCP pathway members. This suggests interaction between these two pathways during regeneration and in determining anterior-posterior polarity (Schaffer *et al*., 2016). Furthermore, Wnt has been shown to act directly on different components of TGF-β pathway during secondary axis patterning in *Hydra*. Work from our laboratory has shown the roles of two BMP antagonists Noggin and Gremlin in axis formation and tentacle patterning in *Hydra* (Chandramore *et al*., 2010; Krishnapati *et al*., 2020). Over the past few years, quite a few players from the TGF-β pathway have been identified in *Hydra* and have been shown to play key roles in axial patterning (Table 1). These studies suggest presence of coordinated signaling among Hippo-Wnt-TGF-β pathways in determining axial patterning in *Hydra*.

TGF-β signaling pathway in *Hydra*

In addition to the Wnt pathway, another highly conserved pathway in defining the three axes during development and patterning in both invertebrates and vertebrates is the TGF-β pathway. Several members of TGF-β signaling pathway are involved in dorsal-ventral axis patterning in bilaterians, such as, *Drosophila* (invertebrate) and *Xenopus* (vertebrate). Though initially thought to have evolved in bilaterians, discovery of several components of TGF-β pathway in the basal phyla, such as, cnidarians (Samuel *et al*., 2001 Hayward *et al*., 2002; Matus *et al*., 2006) and poriferans (Suga *et al*., 1999) revealed their conserved role in development and patterning different tissues and their early evolution.

The initial discovery of TGF-β pathway in *Hydra* came from the identification of *Hysmad1*, an orthologue of receptor Smads (Hobmayer *et al*., 2001). Structurally, the protein Hysmad1 shows similarity to both R-Smads and Co-Smads of higher metazoans and belongs to the Smad 1-5-9 subgroup. Thirteen out of the 18 conserved amino acid residues that help in the Smad2-Smad anchor for receptor activation (SARA) -interaction and a phosphorylation motif at the C-terminus are present in Hysmad1 indicating the presence of an active BMP/Smad signaling in *Hydra* (Hobmayer *et al*., 2001). This has been further confirmed by the identification of two *BMP5-8* orthologues, *HyBMP5-8a* and *HyBMP5-8b* in *Hydra* (Reinhardt *et al*., 2004). Expression of *HySmad1* transcripts in the interstitial oocyte precursor cells and nurse cells during oocyte differentiation indicates its role in oogenesis in *Hydra*, which confirms conservation of similar functions in higher metazoans such as in *Drosophila* (Xie and Spradling, 1998). While *Hysmad1* appears to have a uniform expression along the body axis during budding and regeneration with increased levels during oogenesis, expression of the ligands *HyBMP5-8a* can be detected throughout the body column and *HyBMP5-8b* at the base of the tentacles and in the lower one third of the body column in adult *Hydra* (Reinhardt *et al*., 2004). Graded expression of *HyBMP5-8b* along the body axis in *Hydra* suggests its role in axial patterning that confers positional information during budding and regeneration.

Analysis of proteomic and transcriptomic data revealed altered levels of several components of Wnt and TGF pathway, which showed differentially upregulated Wnt ligands and their downstream target genes. Members of TGF signaling pathway, Activin, *BMP2/4* and *BMP5/8c* are downregulated, while the BMP ligands, Noggin and Chordin are strongly upregulated during head regeneration (Petersen *et al.*, 2015). Interestingly, members of Cerberus-Gremlin-DAN family are downregulated during head regeneration (Reddy *et al*., 2019). This suggests that both Wnt and TGF signaling pathways are used during patterning of the opposite ends of the axis in *Hydra*. Expression of *wnt3A* and *HyBMP5-8b* at the oral and aboral ends in *Hydra,* thus is analogous to the expression of Wnt and BMP pathway members during dorsoventral patterning in *Xenopus* (Cho *et al*., 1991; Hobmayer *et al*., 2000). It is also interesting to note that BMPs and other genes including Nodal, Brachyury, GATA, Forkhead and Snail, needed for mesodermal specification and patterning in vertebrates are identified in *Hydra,* which lacks mesoderm (Technau and Bode, 1999; Technau and Scholz, 2003; Nakamura *et al*., 2011; Watanabe *et al*., 2014a). Many of these vertebrate mesodermal marker genes are expressed in the endoderm of *Hydra*; similarly, expression of these several mesodermal marker genes is also seen in basal endoderm in *Nematostella,* and are often referred to as mesendodermal markers. This suggests that the bilaterian mesoderm could have descended from the endoderm of their diploblastic ancestors (Technau, 2020).

Nodal and Activin

An important feature of the vertebrate body plans is the presence of a left-right (L/R) axis that is distinctive and is conserved in almost all vertebrates. The molecular asymmetry that establishes

symmetry in external structures is directly under the control of several morphogens (Boutet *et al*., 2017). For example, L/R asymmetry at the molecular level is mainly controlled by the expression of *nodal-lefty-pitx* cassette during embryogenesis (Boutet *et al*., 2017). Nodal, a member of the TGF-β superfamily originally discovered in the genetic studies in mouse embryos, mediates signaling through activin receptors followed by phosphorylation and nuclear localization of Smad2 transcription factor (Conlon *et al*., 1991; Schier *et al*., 2003). Though originally discovered for its role in mesoderm induction, role of *nodal* as endoderm inducer in vertebrates strengthened the idea of mesendoderm induction (Schier *et al*., 2003). Further, its instructive role in developing L/R axis is also evident by its expression on the left-hand side of lateral plate mesoderm which is well conserved in vertebrates (Levin *et al*., 1995). This was further confirmed by *nodal* misexpression on the right-hand side, which resulted in abnormalities in determining the organ systems along the axis (Levin *et al*., 1997). Another BMP inhibitor, *cerberus,* expressed on the right-hand side of the embryonic node also helps in maintaining L/R asymmetry by binding directly to the Nodal protein thereby repressing Nodal signaling on the right-hand side (Vandenberg *et al*., 2013; Li *et al*., 2017). Yet another gene of Cerberus/Dan family, *caronte* (*car*), expressed on the left-hand side of the lateral plate mesoderm, maintains L/R asymmetry by inducing *nodal* expression and antagonizes the BMP pathway (Yokouchi *et al*., 1999). This mechanism of *nodal* induction by *car* is also mimicked by *noggin* suggesting that BMP pathway plays a crucial role in repressing *nodal* expression (Capdevila *et al*., 2000). It is also to be noted that the inhibition of Nodal by BMPs in establishing the L/R axis is a conserved phenomenon in vertebrates (Capdevila *et al*., 2000).

Though the left-hand sided activity of Nodal signaling is conserved in vertebrates, right-hand side expression is seen in echinoderms and hemichordates, suggesting an axis inversion in these organisms (Duboc *et al*., 2005). In *Hydra*, *nodal* is expressed asymmetrically in the budding region of the main body axis and in the early buds when the secondary axis perpendicular to the main axis is generated and controls biradial asymmetry (Watanabe *et al*., 2014a). The downstream target gene, *pitx* is also expressed in the same region suggesting that both belong to the same molecular cassette in *Hydra* (Watanabe *et al*., 2014a). Hydra *nodal* has been shown to play a role in breaking the symmetry during bud formation and may act in co-ordination with Wnt pathway members during bud formation (Watanabe *et al*., 2014a). The role of *β-catenin* in inducing *nodal* and *pitx* was shown during bud evagination suggesting that *β-catenin-nodal-pitx* act as the core signaling cassette in controlling axial patterning in *Hydra* (Watanabe *et al*., 2014a). Overexpression of *nodal* upon the removal of a pharmacological inhibitor A-83-01, a specific inhibitor for Activin-receptor-like kinase 4/5/7 in *Hydra* perturbs the biradial symmetry and confirms its role in controlling the symmetry in *Hydra* (Watanabe *et al*., 2014a). BMP inhibitors such as members of the Cerberus/Dan family have also been shown to inhibit *nodal* signaling in vertebrates including humans (Hsu *et al*., 1998; Piccolo *et al*., 1999). In *Hydra*, *gremlin*, one of the BMP antagonists that belongs to DAN family, has been reported by us (Krishnapati *et al*., 2020). It is interesting to see that the expression of *gremlin* during initial stages of budding persists even as the buds develop (Krishnapati *et al*., 2020), while the expression of *nodal,* that is expressed at the presumptive budding zone, disappears during bud development (Watanabe *et al*., 2014a). It is known that Gremlin2 blocks interaction of Nodal to its type II receptor thereby inhibiting *nodal* signaling (Nolan *et al*., 2016). This suggests existence of a possible antagonism between *gremlin* and *nodal* even in *Hydra*.

Activin, originally identified from the ovarian fluid for its ability to release follicle stimulating hormone from the pituitary was later shown to have diverse roles in growth and development (Ying *et al*., 1997). Activin is a polypeptide belonging to the TGF-β family that binds to four transmembrane activin receptors (type I, IB, II, IIB) and follistatin, one of the inhibitors of the BMP pathway. Binding of activin to its type II receptor leads to phosphorylation of type I receptor followed by phosphorylation of Smad2 and Smad3 (Xia *et al*., 2009). Binding of follistatin to activin neutralizes its activity by preventing it from interacting with its own receptors (Thompson *et al*., 2005). Both activin and follistatin have been shown to play roles in mesoderm induction in amphibians. They act as potent inducers and result in the formation of a secondary axis upon ectopic expression in the *Xenopus* embryos (Dohrmann *et al*., 1993). This was further confirmed by injecting activin receptor mRNA, which led to defects in axis formation and mesoderm induction suggesting that activin-activin receptor system is highly regulated during embryogenesis (Hemmati-Brivanlou *et al*., 1992). In *Hydra*, activin-like genes (*ActL1, 3, 4*) are expressed in the endoderm in upper body column excluding the hypostome, while *ActL2* shows expression in the overall body column in *Hydra*. Similarly, in *Nematostella*, expression of *activin* in the endoderm of tentacle buds was shown to have a role in morphogenesis (Matus *et al*., 2006). However, their role in axis asymmetry during bud formation similar to *nodal* was not identified (Watanabe *et al*., 2014a).

Chordin

Embryonic pattern formation is regulated by the interactions between BMPs and their antagonists, Chordin, Noggin and Follistatin. While BMPs determine ventral cell fate, the inhibitors, Chordin, Noggin and Follistatin act as dorsalizing factors and are expressed in the Organizer. Though all these genes encoding BMP antagonists are expressed simultaneously in the amphibian embryo during gastrulation, they show distinct spatio-temporal expression patterns in other organisms (Streit and Stern, 1999). Among these, *chordin*, the homolog to *Drosophila sog*, was first identified in a differential screen of zygotic dorsal-specific cDNAs and is secreted from the Organizer in amphibian embryos (Sasai *et al*., 1994). Chordin shows characteristic four cysteine-rich repeats, each containing 10 cysteines. The first two cysteine-rich domains bind to BMP2 and BMP4 and form a transient complex that prevents their interaction with cell surface receptors, which is disassociated by the BMP1/metalloproteinases of the Tolloid family (Piccolo *et al*., 1996). This mechanism of binding and dissociation of chordin-BMP complex results in varying concentrations of BMPs and is well conserved in establishing dorsal-ventral axis in invertebrates and vertebrates (Piccolo *et al*., 1996; Marqués *et al*., 1997).

The ability to induce a secondary axis on the ventral side of the host upon transplantation is the best example of the Organizer activity. In amphibian embryos, the dorsal lip of blastopore acts as the Spemann-Mangold Organizer and has the ability to generate cell polarity and specific cell fate in the neighboring tissues (Spemann *et al.*, 1924; Gerhart *et al*., 2001). It is interesting to note several similarities between vertebrate and *Hydra* Organizers in

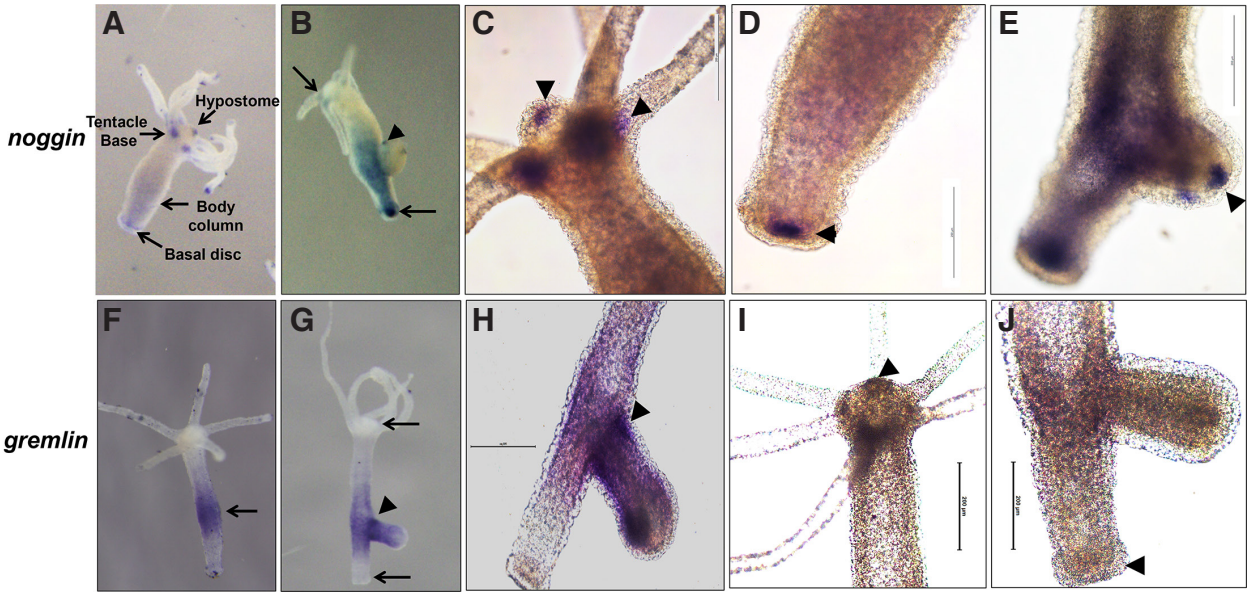

Fig. 1. Localization of *noggin* and *gremlin* in nonbudding and budding *Hydra*. Expression of *noggin* is predominant in the hypostome, base of the tentacles, lower body column and in the basal region in non-budding polyp (A). During bud development, *noggin* is localized at the sites of tentacle emergence (arrowhead in B). High magnification images showing *noggin* expression in the hypostome and base of the tentacles (arrowhead in C), basal disc (arrowhead in D) and at tentacle emergence region (arrowhead in E). *Gremlin* is expressed mainly in the budding region and in the upper body column in the non-budding polyps (arrow in F) with increased expression during bud development (arrowhead in G) with no expression in the basal disc and hypostome (arrows in G). Magnified images showing *gremlin* expression in the budding *Hydra* (arrowhead in H). Absence of *gremlin* expression in the hypostome (arrowhead in I) and basal disc (arrowhead in J).

terms of expression of many genes. For example, marker genes such as *β-catenin*, *noggin* and *chordin* expressed in the vertebrate Organizer are also seen in *Hydra* and overexpression of these *Hydra* genes result in secondary axis formation in vertebrate embryos (Hobmayer *et al*., 2000, Rentzsch *et al*., 2007; Chandramore *et al*., 2010). Similarly, Hensen's node, the avian equivalent to Spemann-Mangold Organizer also secretes molecules such as Noggin, Chordin and Nodal (Chapman *et al*., 2002; Katsu *et al*., 2012). This suggests that the signals responsible for maintaining the Organizer activity are similar and are conserved across phyla in both invertebrates and vertebrates.

Despite the presence of similarities in gene expression patterns, the Hydra Organizer is not viewed as a direct equivalent to the vertebrate Organizer. In *Hydra*, the Organizer primarily contributes to establishing the oral-aboral axis, mainly controlled by Wnt signaling. In vertebrates, even though, Wnt from the Organizer aids in anterior-posterior axis establishment, its formation from the blastopore remains unaffected even in the absence of the Spemann-Organizer and suggests differences in axis formation between the *Hydra* Organizer and the Spemann-Organizer. This is comprehensively reviewed by Meinhardt (2006), wherein the differences and similarities between *Hydra* Organizer and vertebrate Organizer in axis formation and patterning the tissues are discussed.

In *Hydra*, the hypostome and basal disc act as Organizers with an ability to induce secondary axis upon transplantation (Browne 1909, Hicklin and Wolpert, 1973; Müller, 1996). However, the hypostome tissue alone, anterior to the tentacles and without the tentacle base is absorbed and does not give rise to formation of new Hydranths (Browne, 1909). Experiments conducted by Hicklin and associates showed for the first time that the lateral grafts from the foot region are also able to induce new axis suggesting Organizer activity of the basal disc (Hicklin and Wolpert 1973, Hicklin *et al*., 1973). However, the new axis always forms when the grafting is performed only at the distal end and not at the proximal end. This is possibly due to the inhibitory effects of head inducing gradients at the proximal end resulting in inhibiting the development of new hydranth from foot grafts. On the other hand, reported work from our laboratory has shown that both homoplastic and heteroplastic transplantations, using a piece of hypostome or a complete hypostome with tentacle base and without any conspicuous tentacles on upper, middle and basal regions resulted in successful grafting with more than 90 % accepted grafts (Kadu *et al*., 2012). Similar results are also seen by grafting a complete foot in the upper (with/without hypostome and tentacles), middle and basal regions during homoplastic and heteroplastic transplantations (Kadu *et al*., 2012). The ability to induce secondary axis either by the head or foot Organizers in *Hydra* is due to the presence of molecules that provide instructive signals for initiating fate determination and morphogenesis. Few such molecules like Wnt, Chordin have been identified and shown to have Organizer properties in *Hydra*. Role of *Hydra* chordin-like (*hychdl*) gene in Organizer formation was identified by its endodermal expression pattern during early stages of budding and regeneration, during which the new head Organizer is established. Similarly, role of Chordin in secondary axis formation perpendicular to the oral/aboral axis, comparable to budding in *Hydra*, was shown in *Nematostella* (Saina *et al*., 2009). Heterologous expression of *hychdl* mRNA in *Zebrafish* embryos resulted in inhibition of BMP signaling in a dose dependent manner (Rentzsch *et al*., 2007). Such inhibition of BMP signaling is possibly due to the presence of chordin-, follistatin- and insulin-like growth factor binding protein (IGFBP)- domains in Hychdl. Though follistatin has not been identified in *Hydra* so far, a single domain corresponding to three follistatin domains of human

follistatin has been identified in *Hydra* Chordin. Follistatin is a known BMP inhibitor, while IGFBP inhibits TGF signaling indirectly due to its structural similarity to Twisted gastrulation (Tsg) that interacts with BMPs. This suggests that these two domains in Hychdl may also contribute to inhibition of BMP signaling in *zebrafish* embryos and that the BMP-chordin antagonism may have potential role in establishing axial polarity in *Hydra*.

Noggin

One of the important mechanisms of BMP pathway regulation is by binding of secretory molecules to BMP ligands, which prevent them from interacting with their cell surface receptors. So far, *chordin*, *noggin* and *gremlin* have been identified from *Hydra* and are shown to have role in axial and tentacle patterning (Rentzsch *et al*., 2007; Chandramore *et al*., 2010; Krishnapati *et al*., 2020). Identification of *noggin* in *Hydra* came from our own studies. We found that it is expressed in the hypostome, base of the tentacles and foot region and as distinct spots before the emergence of tentacles during budding (Chatterjee and Ghaskadbi 2001). Recent work from our laboratory further confirmed its endodermal expression in the hypostome, lower body column, base of the tentacles and basal disc (Krishnapati *et al*., 2020). To confirm the specific expression of *noggin* at the hypostome, basal disc and tentacle base, magnified images were taken (Fig. 1 C,D,E, current manuscript). Expression of *noggin1* in the endoderm of tentacle base, tips and at the tentacle emergence zone in the developing buds has also been reported in *Nematostella* (Fig. 2C) suggesting that Noggin plays a role in patterning tentacles in cnidarians. Interestingly, *BMP5-8b* is also localized at the base of the tentacles in *Hydra*, which suggests possible interactions between Noggin and BMP during tentacle patterning. Expression of *noggin* in the head region comprising hypostome and base of the tentacles (Fig. 1C) and basal disc/foot (Fig. 1D) in *Hydra* is particularly interesting as both act as Organizers and induce secondary axis on transplantation (Browne, 1909; Hicklin and Wolpert, 1973; Hicklin *et al*., 1973; Müller, 1996; Kadu *et al*., 2012).

Structurally, *Hydra* Noggin shows characteristic Cysteine ring motif as seen in human Noggin that provides conformational rigidity to the protein (Groppe *et al*., 2002; Krishnapati *et al*., 2020). Although structural conservation does not fully imply functional conservation in higher metazoans, injection of *Hydra noggin* mRNA in two cell stage *Xenopus* embryos resulted in partial duplication of axis in 100 % embryos. When injected in UV-ventralized *Xenopus* embryos, *Hydra* Noggin partially rescued the embryos from the effects of UV irradiation. This dorsalizing effect of *Hydra* Noggin in *Xenopus* embryos was indeed due to inhibition of BMP signaling and its components as confirmed with the animal cap assay (Chandramore *et al*., 2010). These results demonstrate that *Hydra* Noggin functionally mimics a vertebrate Noggin and is conserved in higher metazoans.

Gremlin

Gremlin is yet another BMP inhibitor that shows dorsalizing effects like Noggin and Chordin. Gremlin, Cerberus and the tumor suppressor, DAN are structurally and functionally related proteins and belong to the same subfamily of BMP inhibitors (Ozaki *et al*., 1993; Bouwmeester *et al*., 1996). This is evident from the fact that all three binds to BMP2 and compete with Noggin suggesting structural/topological similarity, although no sequence similarity to Noggin and Chordin is observed (Hsu *et al*., 1998). It is interesting to note that *gremlin* is not expressed in the Organizer region during gastrulation in *Xenopus* embryos but shows axial patterning activities (Hsu *et al*., 1998). *Gremlin* transcripts form a concentration gradient from aboral to oral side along the oral-aboral axis in the anthozoan cnidarian, *Nematostella vectensis*. Expression of *NvGrm* is seen on the opposite side of *Nvdpp* expression in mid-late planula stages of *Nematostella* (Fig 2C; Rentzsch *et al*., 2006) and in the endoderm

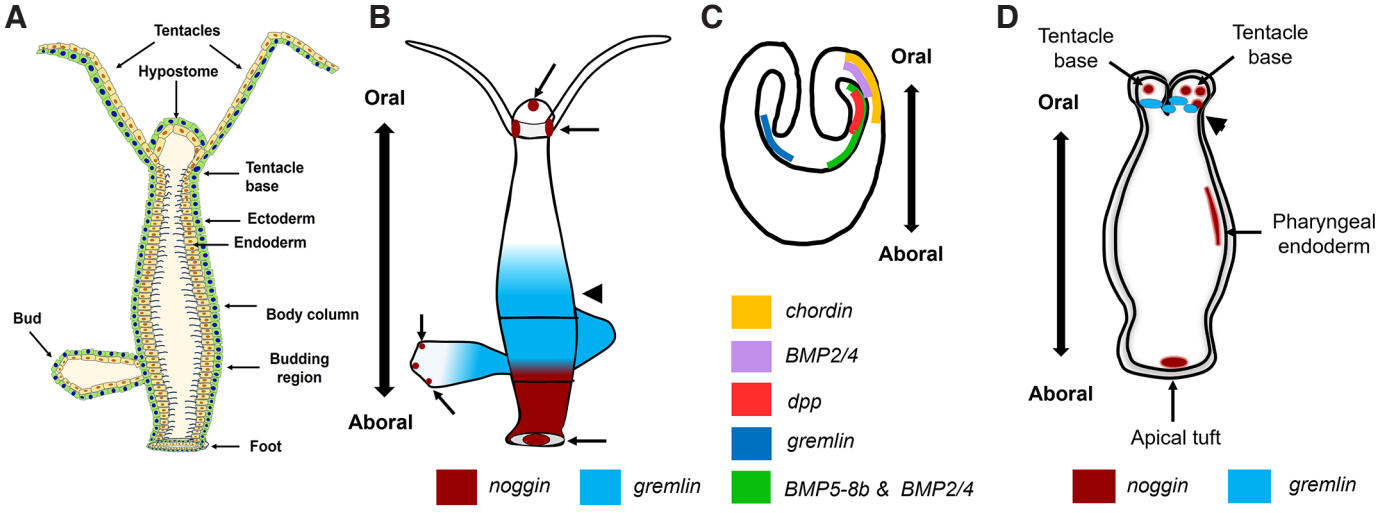

Fig. 2. Expression patterns of BMPs and BMP inhibitors in *Hydra* and *Nematostella*. Schematic representation of different morphological regions in adult *Hydra* (A). Representation of *noggin* and *gremlin* expression in *Hydra* (B) based on data from Krishnapati *et al.*, 2020 and early (C) and late (D) planula stages of *Nematostella vectensis* (lateral views) based on data from Matus *et al.*, 2006; Rentzsch *et al.*, 2006; Saina *et al.*, 2009. Endodermal expression of *noggin* is seen at the base of the tentacles, hypostome and in the basal disc in *Hydra* (arrows in A). Expression of *chordin*, *gremlin* and the ligands *dpp, BMP2/4* and *BMP5-8b* on the oral side of early planula stage of *Nematostella* is shown (C). Expression of *noggin* is also seen at the base of the tentacles in the late planula stage of *Nematostella,* (arrows in D) and in the endoderm of apical tuft (D) that lie on the aboral side. Graded expression of *gremlin* is seen in the endoderm of budding region in *Hydra* (arrowhead in B) while *gremlin* is localized in the region surrounding mouth on the oral side of late planula stage of *Nematostella* (arrowhead in D).

tissue surrounding the mouth in the late planula stages during polyp formation (Fig. 2D; Matus *et al*., 2006), while *NvChd* and *NvBMP5-8* are expressed on the same side along the oral aboral axis (Fig 2D) suggesting the presence of complex interactions within the BMP signaling pathway in cnidarians.

Identification of *gremlin* in a fellow cnidarian prompted us to look for its presence in *Hydra*. We identified a putative *gremlin-like 1* mRNA sequence of 483 bp from *Hydra vulgaris*. Using whole mount *in situ* hybridization (WISH), endodermal expression of *gremlin* in the budding region was reported with clean hypostome and foot regions (Krishnapati *et al*., 2020). The expression pattern of *gremlin* was further confirmed by taking magnified images showing no signal in the hypostome and foot regions (Fig. 1 I,J shown by arrowhead) and strong signal at the base of the bud (Fig. 1 G,H shown by arrowhead). An earlier study has reported increased neuronal cell density at the site of bud formation (Bode *et al*., 1973). A positive correlation between neuronal cell density and rate of budding in *Hydra* has also been demonstrated (Browne *et al*., 1978). Significant levels of *gremlin* transcripts in *Xenopus* neural crest cells and their role in neural crest induction and patterning has been reported (Hsu *et al*., 1998). Expression of *gremlin* during early stages of budding in *Hydra*, where the neuronal cell density is high, suggests its possible expression in the neuronal cell lineage and role in patterning the tissue during bud initiation and development. Interestingly, *gremlin* expression was undetectable in the hypostome (Fig. 1I) and basal region (Fig. 1J) that act as organizing centers in *Hydra*. This lack of expression in the Organizer regions also agrees with the expression pattern of *gremlin* in the *Xenopus* Organizer (Hsu *et al*., 1998) suggesting its primary role in tissue patterning during development.

One of the most interesting events that occur during the development of metazoan embryo is gastrulation, which results in the generation of three germ layers, ectoderm, endoderm and mesoderm. Basal metazoans belonging to phylum Placozoa, Porifera, Cnidaria and Ctenophora, however, show only two germ layers, ectoderm and endoderm and are hence diploblastic. The two germ layers carry out all the necessary functions responsible for morphogenesis, behavior and reproduction. Accumulating evidence shows the importance of TGF-β/BMP pathway in germ layer segregation during early embryonic development and patterning. In *Hydra*, the diploblastic nature is defined by two lineages of epitheliomuscular stem cells, the ectodermal and the endodermal epithelial stem cells. It is interesting to that the majority of BMP ligands and their inhibitors are expressed in the endoderm of *Hydra* and *Nematostella* (Matus *et al*., 2006; Krishnapati *et al*., 2020). Expression of *HyBMP5-8b*, and the inhibitors of BMPs, *chordin*, *noggin* and *gremlin* are observed in *Hydra* endoderm. Interestingly, single cell RNA sequencing using trajectory analysis of ectodermal and endodermal epithelial cells also revealed predominant expression of another BMP antagonist, DAN containing gene, t2758 in the endodermal epithelial cells in the foot and lower body column region (Sieberts *et al*., 2019). Similarly, *BMP5-8, dpp, Smad1/5* and *Smad4* are expressed in the endoderm of *Nematostella*. On the contrary, *Smad1* is expressed in both the germ layers of *Hydra*. However, very little information is available pertaining to the presence of BMP receptors, their expression and roles in axis patterning in *Hydra*. The presence of active signaling of BMPRI was indirectly shown by ectopic expression of BMPRI, which induces NvHoxE and pSmad1/5 expression in the ectoderm (Genikhovich *et al*., 2015). Since the ligands are expressed in the endoderm and the Smads are seen in the ectoderm of *Hydra*, it would be interesting to look at the interlineage interactions to understand the communication between ligands, receptors and the Smad proteins (Hemmrich *et al*., 2012). Another important feature of *Hydra* is the presence of mesoglea between ectoderm and endoderm. Structurally, the acellular matrix is similar to vertebrate extracellular matrix and is rich in fibrillar collagen and laminin (Sarras *et al*., 1994; Zhang *et al*., 2002). Several cell adhesion proteins, ECM proteins and proteoglycans have been identified and shown to play crucial roles in morphogenesis, pattern formation, regeneration and cell-ECM interactions in *Hydra* (Sarras *et al*., 1993; Sarras 2012). Recent evidence suggests the role of Wnt/β-catenin signaling in the remodeling of mesoglea during axis formation in *Hydra* (Veschgini *et al*., 2023). Also, previous results have shown the importance of mesoglea during budding. Since members of the BMP pathway are shown to play key roles in secondary axis formation and patterning in *Hydra*, it would be interesting to look at the remodeling of mesoglea in relation to the TGFβ pathway.

To summarize, the presence of a large number of TGFβ signaling components in *Hydra* suggests that the pathway evolved early in evolution and was recruited for patterning of the body axis. Though Wnt/β-catenin signaling plays a major role in Organizer formation, concomitant expression of BMP inhibitors Chordin and Noggin in the hypostome and basal disc (which exhibit Organizer activity on transplantation) suggests that TGF-β/BMP pathways play crucial role in maintaining the Organizers in *Hydra*. Although, it was initially surprising to identify genes encoding proteins that participate in vertebrate dorsal-ventral and left-right axis in the radially symmetrical cnidarians, identification of a few components of both Wnt and TGF-β pathway members during embryonic development in poriferans (Adamska *et al*., 2007; Windsor Reid *et al*., 2018) also suggests that the molecular tool kit for axis formation evolved long before the cnidarian-bilaterian split. One expects that in the near future, more players in the TGF-β signaling pathway will be identified from *Hydra* and other cnidarians. This will give us a better idea of how cell signaling, crucial for multicellularity, evolved in simple metazoans. This, in turn, may provide us with vital clues regarding evolution of dorsal-ventral, anterior-posterior and left-right axes in more complex metazoans including humans.

Materials and Methods

Whole mount *in situ* hybridization

Whole-mount *in situ* hybridization (WISH) was performed using digoxigenin (DIG)-labeled RNA probes for *gremlin* and *noggin* as described (Krishnapati *et al.,* 2020). Briefly, the complete coding sequences amplified were used for *in vitro* transcription reaction using Dig-RNA labeling kits (Roche) following manufacturer's instructions. WISH was performed as previously described with few modifications. Polyps were relaxed in 2 % urethane for 2 min, proteinase K treatment was performed for 10 min and endogenous alkaline phosphatase was inhibited by heat-inactivation at 70 °C for 15 min in 2 X SSC. Prehybridization with tRNA for 2 h and hybridization with sense and anti-sense riboprobes for 48 h were followed at 60 °C. Post-hybridization washes were carried out with gradients of hybridization buffer and 2X SSC and stringency washes with 0.5X SSC, followed by incubation with anti-digoxigenin antibody. Following color development with Nitro Blue Tetrazolium/5-bromo-

4-chloro 3-indolyl-phosphate p-toluidine salt (NBT/BCIP) and polyps were dehydrated in methanol grades. Whole polyps were imaged using Olympus SZX16 stereo microscope using DP71 camera and high magnification images were taken using Leica microscope DM5500 B using DFC450C camera.

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