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Bachelor thesis:

Wnt signaling in determination and patterning of cnidarian primary body axis

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Abstract

Among the emerging animal models in molecular biology, cnidarians are of a special interest. Because they are an outgroup of bilateralians, their detailed investigation at cellular and molecular level could give us insight into evolution of many novelties apparently specific for bilateralians. Previous ideas about cnidarian radial symmetry as an ancestral trait have been challanged by discovery of mechanisms responsible for bilateralian dorsoventral patterning in anthozoans. Extensive work has already revealed that ancestral genome was much richer in known gene families and subfamilies and that substantial gene loss occured in metazoan evolution. Recently, many new data about wnt signaling in cnidarians have appeared. Wnts play essential role in cnidarian primary body axis establishment and patterning. In oocytes of many cnidarian species one or more components of wnt/beta-catenin pathway serve as asymmetrically deposited maternal factors and activation of the pathway determines future oral pole. Larvae of a new anthozoan model *Nematostella vectensis* express at least ten different wnts. Many of them are concentrated around blastopore, the future mouth, while others are expressed in more distant body parts. Their precise function is still enigmatic but they probably participate in body patterning, especially of body parts around mouth, which are the most complex when nervous system and nematocyte diversity are considered. Ability of polyp hypostome, the mouth adjacent tissue, to organize surrounding cells was explored in *Hydra* quite extensively. Hypostome is a source of Wnt molecules that instruct cells along the primary axis to adapt a specific morphological and physiological state according to their position. During hydrozoan vegetative reproduction processes like budding and growth of colonies new hypostomes form and organize surrounding tissues to form new individuals. At least some of the responses involve activation of noncanonical wnt pathway known to act in cellular morphogenesis via cytoskeleton reorganization in some bilateralians. Thus, wnt signaling is diversified in cnidarians, both in the terms of ligand representation and responding pathways, and so it apparently was in the last common cnidarian-bilateralian ancestor.

Key words: cnidarians, primary body axis, Wnt, *Nematostella*, *Hydra*, organizer,

Abstrakt

V současné molekulární biologii přibývá hodně živočišných modelových organismů. Mezi nimi jsou i někteří žahavci. Výzkum žahavců pomáhá odhalit klíčové procesy které nastaly v průběhu evoluce mnohobuněčných živočichů a odlišit evoluční novinky dvoustranně symetrických živočichů („bilateralia") od znaků které byly přítomny již u jejich společného předka s žahavci. Paprsčitá symetrie žahavců, dříve považována za samozřejmou, je pravděpodobně odvozeným znakem a bazální žahavci jako sasanka *Nematostella vectensis* jsou ve skutečnosti dvoustranně symetričtí. Geny které se účastní diferenciace dorsoventrální osy u živočichů s dvoustrannou tělní symetrií jsou přítomny i u sasanky *Nematostella.* Průzkum žahavčích genomů poukazuje na komplexitu původních metazoálních genomů a značnou ztrátu genů v evoluci jednotlivých živočišných linií. Jednou z bohatě zastoupených prastarých genových rodin jsou signální proteiny Wnt. Doposud nashromážděná data poukazují na jejich značný význam při určení polohy primární tělní osy ve vývoji mnoha druhů žahavců a následné diversifikaci tkání podél této osy. U embryí a larev sasanky *Nematostella vectensis* hraje významnou roli více než deset paralogů Wnt. Mnoho z nich je exprimováno poblíž blastopóru (budoucího ústního otvoru) a má patrně význam pro určování povahy okolních tkání (okolí ústního otvoru je zřejmě nejrůznorodější oblastí polypů sasanky *Nematostella*, alespoň co do přítomnosti různých typů neuronů a žahavých buněk), ale některé působí v jiných tělních úsecích. Vlastnosti tkáně blízko ústního otvoru polypa fungující jako „organizátor" jsou dobře známy u nezmarů. Z této oblasti proudí do ostatních tkání molekuly morfogenu Wnt a v okolních tkáních vyvolávají specifickou odpověď podle jejich vzdálenosti od zdroje. Pro vegetativní množení polypů pučením a rozrůstání jejich kolonií je důležité vznikání nových organizátorů. Tyto stimulují okolní buňky k seskupení do nových polypů. Některé z mechanismů podílejících se na organizaci tkání jsou závislé na „nekanonických Wnty stimulovaných drahách". Tyto dráhy i diverzita na úrovni Wntových signálních molekul jsou tedy vlastní i žahavcům, což poukazuje na jejich pravděpodobnou přítomnost u společných předků žahavců a „bilaterálií.

Klíčová slova: žahavci, Wnt, tělní osy, *Hydra*, *Nematostella,* organizátor,

List of abbreviations

beta-TrCP	beta-transducin repeat-containing protein
AP	anteroposterior
APC	adenomatous polyposis coli
BMP	bone morphogenetic protein
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CER	cerberus
CK	casein kinase
CRD	cysteine-rich domain
DAG	diacylglycerol
Dkk	dickkopf
dpp	decapentaplegic
Drho	*Drosophila* Rho
Drok	*Drosophila* Rho kinase
Dsh	dishevelled
DV	dorsoventral
Fzd(Fz)	frizzled
Hybra	*Hydra* brachyury
HyDkk	*Hydra* dickkopf
HyTCF	*Hydra* T-cell factor
HyWnt	*Hydra* Wnt
GBP/Frat	GSK3 binding protein/frequently rearranged in advanced T-cell lymphomas
GSK3beta	glycogen synthase kinase 3beta
Hh	hedgehog
IP_3	inositol triphosphate
JAK/STAT	Janus kinase/signal transducers and activator of Transcription
Krm	kremen
LiCl	lithium chloride
LR	left-right
LRP	lipoprotein receptor-related protein

NvTCF	*Nematostella vectensis* T-cell factor
NvWnt	*Nematostella vectensis* Wnt
PCP	planar cell polarity
PKC	protein kinase C
Ror	receptor tyrosin kinase-like orphan receptor
Ryk	related to receptor tyrosine kinase
RTK	receptor tyrosine kinase
sFRP	secreted frizzled related protein
sog	short gastrulation
TCF/LEF	T cell factor/lymphoid enhancer factor
TF	transcription factor
TGF-beta	transforming growth factor beta
WIF	wnt inhibitory factor

Contents

1. Introduction

One of the fascinating things on multicellular organisms is organization of their form. Thousands to billions of cells execute their specific functions and populate specific places to create an ordered body with elaborate axial coordinate system. This system is established during early embryogenesis by precise communication and mutual instruction of individual cells. There are several signaling pathways reused in metazoan development over and over: Wnt, Hh, Notch, RTK, JAK/STAT, and TGF-beta superfamily pathway. Besides some taxon-specific morphogenetic events driven by these pathways, there are many conserved functions they are responsible for in animal development. Although originally described in "higher animals" most of them later turned out to be also present in basal metazoan clades. On the other hand, most of them occur scarcely or not at all outside metazoans. Thus, evolution and expansion of these pathways have played key role in such major transitions as the origin of animal multicellularity, tissue systems, germ layers and body axes. Studying degree of diversification and developmental functions of these pathways in groups like cnidarians and sponges can provide us deeper understanding of correlation between genetic and morphological complexity, ancestral state, key events that led to evolution of particular traits and perhaps help us uncover phenomena which are present but not distinctive in model species investigated so far. Particularly interesting panmetazoan cell-communication molecules are Wnt morphogens. They are responsible for many events in animal development, some of which are conserved across distant taxa. They play a part in cell proliferation, migration, polarity regulation and fate decision processes including apoptosis. Recent research of several cnidarian species has added a lot of evidence for key role of Wnt and members of its pathways in axes specification and patterning in these animals. In this thesis, I am providing a small summary of what is currently known about such processes in cnidarians.

2. Background information

2.1 Wnt signaling pathways

Wnt is a secreted glycoprotein and a notoriously known morphogen - molecule providing positional information for cells surrounding the "organizer"- a source of the signal. Experiments suggest that Wnts are hydrophobic, probably due to palmitoylation by endoplasmic reticulum resident enzyme porcupine. Such modification would enhance membrane-binding properties and short-range effects but make long-range signaling difficult. Several mechanisms that could bypass this obstacle have been suggested. Most probably, Wnts travel to more distant targets as components of large lipoprotein particles called argosomes (Bartscherer and Boutros, 2008). Transcytosis is also considered to help Wnt spreading across tissues (Mikels and Nusse, 2006). Several signaling pathways that involve Wnts are known. Each of them intersects with many other intracellular signaling pathways (van Amerongen, 2008). Distinct sets of Wnt ligands and their receptors can activate these pathways to a different extent, but pairing properties of different ligands and receptors are not well known due to difficult Wnt purification (Mikels and Nusse, 2006). Individual Wnt paralogs are highly conserved, so their functions must be very specific. However, relationship between particular Wnt-receptor pair and pathway is not fixed and is context dependent. Presence of different coreceptors might play an important role in response specification (Cadigan and Liu, 2005).

In the so-called canonical pathway, also known as Wnt/beta-catenin pathway, the effector beta-catenin is normally either tightly bound to cytoskeleton at cell-junctions or targeted for proteasomal degradation by a multiprotein complex containing APC, axin and GSK3beta. When GSK3beta phosphorylates beta-catenin this is bound by an F-box protein called beta-TrCP. beta-TrCP is a component of E3 ubiquitin ligase. beta-catenin is ubiquitinated and degraded in proteasome. Binding of Wnt to its receptor Frizzled (a seven pass transmembrane protein resembling G-protein coupled receptors which binds Wnt by a cysteine-rich domain) and the coreceptor LRP5/6, that can form a ternary complex with Wnt and Fzd, causes intracellular Disheveled stabilization. LRP5/6 probably binds to axin with its cytoplasmic tail. In vertebrates, GBP/Frat binds to both GSK3beta and Dsh thus could lead to dissociation of GSK3beta from the destruction complex (Miller, 2002). Intracellular part of LRP5/6 contains casein kinase target sites, which have to be phosphorylated for proper signaling. This is

stimulated by Wnt binding (Cadigan, 2006). Activated Dsh prevents GSK3beta from phosphorylating beta-catenin, which remains stable and relocalizes to nucleus. There it forms a complex with TFs of TCF/LEF family, which results in transcription changes at regulated promoters. Major role of the Wnt/beta-catenin pathway is cell determination during development (Miller, 2002).

In Wnt/planar cell polarity pathway (or simply Wnt/polarity pathway), signaling affects cytoskeleton movements. F Wnt zd receptors and cytoplasmic scaffold Dsh are the only known molecules involved in Wnt/PCP signaling in both vertebrates and invertebrates. Genetic studies on *Drosophila* have revealed several components of PCP, including small GTPase DrhoA, *Drosophila* rho-associated kinase (Drok), Jun N-terminal kinase (JNK), myosin II, myosin VIIA and products of the novel genes *flamingo/starry night*, *fuzzy*, *inturned*, and *strabismus/van gogh*. The Wnt/Ca^{2+} pathway involves activation of heterotrimeric G-protein, an increase in intracellular Ca^{2+} and activation of PKC and CamKII (some clues suggest than Fzd might regulate Ca^{2+} release in the absence of Wnt as well). It can be activated by a distinct group of Wnt ligands and Fzd receptors from those that activate other pathways. Downstream targets are currently unknown (Miller, 2002).

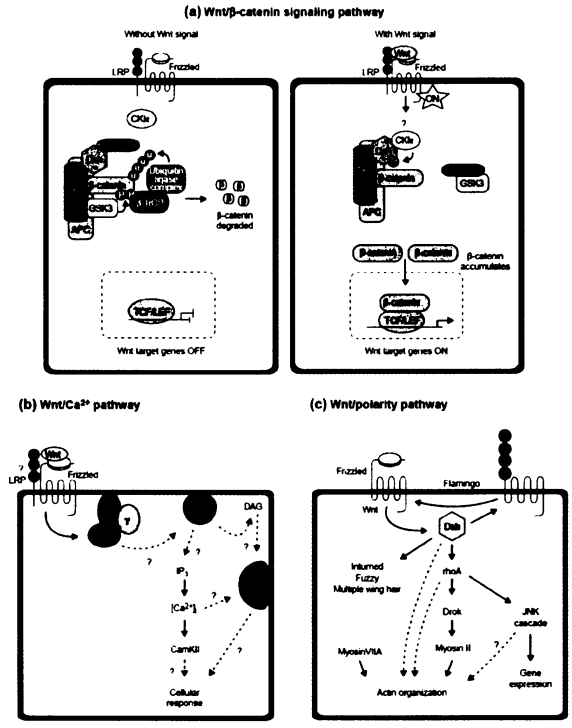

Fig. 1: Current models of Wnt signaling pathways (Miller, 2002).

Recent works have accumulated evidence for existence of other Wnt receptors: Atypical tyrosine kinase Ryk (shown to be important for the noncanonical Wnt pathway during axon guidance and salivary gland migration in *Drosophila*), which contains WIF domain known to bind Wnts and Ror2 with CRD. Ryk probably plays some role in Wnt/PCP and inhibitins the Wnt/beta-catenin pathway. Ror2 and Ryk have also been found to interact with Fzd, so they might act as coreceptors (van Amerongen et al., 2008).

Complexity of Wnt signaling is enhanced by presence of at least two classes of secreted modulators: sFRP class includes sFRP family (secreted frizzled-related proteins), WIF-1 and cerberus, while Dickkopf class consists of certain Dickkopf family proteins. sFRPs might be localized at membrates and released as a response to some factor or directly secreted. It should be emphasized that under some circumstances sFRPs potentiate rather than inhibit Wnt activity. Cerberus is a multivalent protein able to inhibit both Wnt and BMP pathway. WIF-1 is also a sectreted inhibitor directly binding to Wnts. Members of the Dkk family act via LRP5/6 binding with coreceptor kremen facilitating the interaction (Kawano and Krypta, 2003). Wnt spreading is also influenced by proteoglycans of glypican family. They are necessary for a proper incorporation into argosomes in *Drosophila* and might be important for concentrating ligands and providing them to receptors (Miller, 2002).

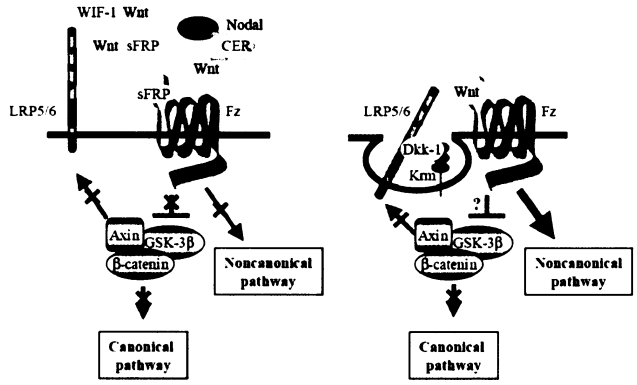

Fig.2: Wnt inhibitors. Left: WIF-1, sFRP and CER inhibit both canonical and noncanonical Wnt patway, because they bind directly to Wnts. sFRP may also bind to Fzd and prevent it from interaction with Wnt. Right: Dkk forms comlex with Krm and LRP5/6. This leads to LRP5/6 endocytosis, so it cannot facilitate Wnt/beta-catenin pathway activation. Wnt can still activate noncanonical pathway(s) (Kawano and Kypta, 2003).

2.2 Cnidarian model organisms

Cnidarians are animals with a complex lifecycle. Generally, a ciliated larva (planula) develops from zygote and after a brief period, it undergoes metamorphosis into a sedentary life-from called the polyp. In medusozoan lifecycle, there is an additional free-swimming stage called medusa, which develops from the polyp. In many species the cycle is further modified, mostly by absence of one or more stages. For further considerations, it is important to note that anterior pole of planula (defined by the direction of swimming and presence of sensory structures) becomes aboral pole of polyp. There are two cnidarian species used predominantly in experimental biology: the traditional model *Hydra* (mostly used species are *H.vulgaris* and *H.magnipapillata*) and the sea anemone *Nematostella vectensis*. Other cnidarians widely used in developmental biology are the colonial hydrozoans *Hydractinia echinata* and the coral *Acropora millepora*. *Hydra* benefits from decades of research and vast amount of information about its biology is available. Due to enormous regeneration capability, grafting experiments can be done easily with *Hydra*. Most of works on its development are about regeneration, asexual reproduction and adult body maintenance. Embryonic development is not well accessible (most of it occurs inside thick cuticle) and largely modified by absence of the planula stage. Although *Nematostella* has joined the main metazoan models only recently, its genome has already been sequenced and many methods have been successfully applied on it. As an anthozoan, it is evolutionary rather distant from hydrozoans (fossil material suggests at least 550 million years of independent evolution (Chen et al., 2002)) and thus knowledge of this species should complement data from *Hydra* to provide more precise picture about cnidarians and help us distinguish ancestral traits of the group (and possibly of the common ancestor with bilateralia) from lineage-specific innovations. It is important to note, that derived character of any trait within cnidarians cannot be distinguished on basis of anthozoan and medusozoan comparison without information about the trait in an outgroup (eg. bilateralia). Protocols for standard methods in molecular biology, including transgenesis, have been developed for both main cnidarian models, thus allowing functional studies. Another useful method applicable on both model and nonmodel species, more or less without taxonomic constrains, is treatment with small organic inhibitors or activators. I mention it here, because many studies investigating functions of Wnt pathways in cnidarians are largely based on such an approach. For example pallones like alsterpaullone or azakenpaullone and LiCl are widely used for GSK3beta inhibition with the same experiments often repeated using subsequently two or three such compounds in order to filter out nonspecific effects (for

example, see Plickert et al., 2006; Muller et al., 2007). More detailed information about model cnidarians is provided in specialized reviews on emerging model organisms (Wilkins 2000; Frank et al.; 2001, Fujisawa 2006; Darling et al., 2005; Miller and Ball, 2000).

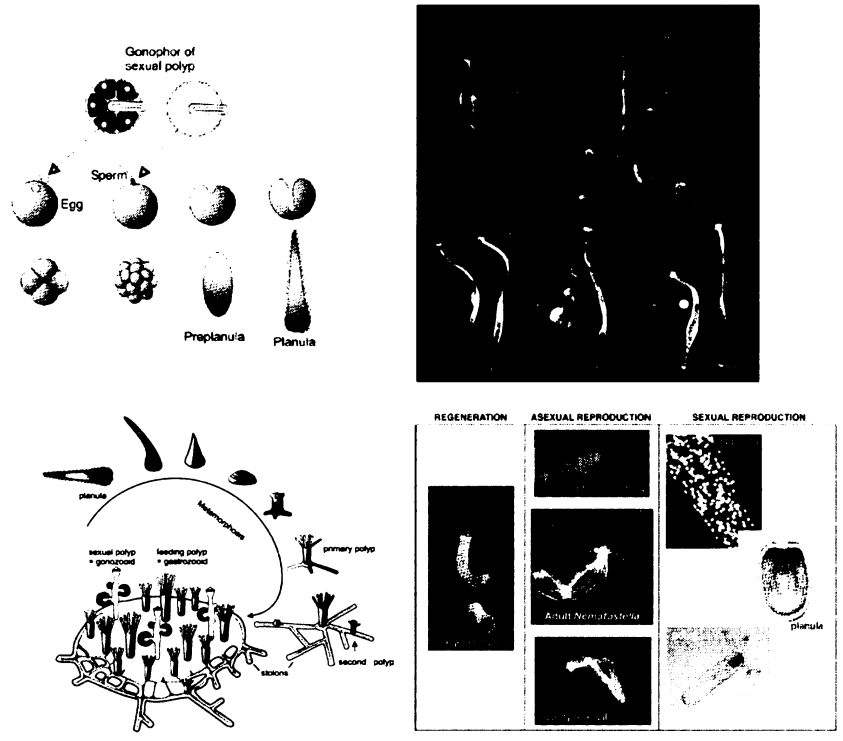

Fig. 3: Life cycles of selected cnidarian species. Upper left: Fertilization and development of *Hydractinia echinata* (Plickert et al., 2006); Lower left: Metamorphosis and colony growth of *Hydractinia echinata* (Muller et al., 2007); Upper right: *Hydra* life cycle with several stages of budding (B1-B4), three stages of male and female germ cell development (M1-M3, F1-F3, respectively), egg (E), hatchling (H) and adult polyp (0) depicted (Bottger and Alexandrova, 2007); Lower right: Life stages, regeneration and two vegetative reproduction modes (physal pinching and polarity reversal) of *Nematostella vectensis* (Darling et al., 2005).

2.3 Members of Wnt pathways in cnidarian genomes

Recent genomic analyses have revealed that ancestral metazoan genomes were relatively complex. First invertebrate model organisms sequenced and most extensively studied (*Drosophila melanogaster*, *Caenorhabditis elegans*) turned out to have been chosen little unfortunately. Both are members of ecdysozoan clade, which was found to have undergone extensive gene loss in the past. Thus, many early comparisons of vertebrate and invertebrate genomes were biased and let to overestimations of old gene families' expansion events during vertebrate phylogeny and number of new families unique for vertebrates (Miller et al., 2005).

Wnt: 14 different Wnt transcripts have been isolated from *Nematostella* with members of most subfamilies known from vertebrates included (*WntA*, *Wnt1-8*, *Wnt10-11*) (Kusserow et al., 2005). There are two paralogs of *Wnt8* and two different splice variants of *Wnt7* in *Nematstella* (Sullivan et al., 2007). Originally only one *Wnt* paralog was isolated from *Hydra* (Hobmayer et al., 2000) and most studies done so far did not include other *Hydra Wnt* genes, often not even considering their existence, but now it seems that no substantial reduction in Wnt family has occurred during hydrozoan evolution. Recently a whole batch of *Wnt* paralogs has been cloned, namely othologs of *Wnt1*, *2*, *5*, *7*, *8*, *11* and *16* found in *Nematostella* and mammalian genomes and 3 genes related to *Wnt9* and/or *Wnt10* with unclear orthology. These were named *Wnt9/10a*, *b*, *c* and they are probably products of two duplications). No orthologs of *Wnt4*, *6* or *A* were identified (Lengfeld et al 2009). *Wnt3* and *Wnt5A* have been found in *Hydractinia echinata* genome (Plickert et al. 2006, unpublished result (GI: 159570494), respectively). Several Wnts are known from *Clytia hemisphaerica*: *Wnt3, 5* and *9* and four divergent sequences (marked as *WntX1A*, *WntX1B*, *WntX2* and *WntX3*) (Momose et al. 2008).

Frizzled receptors: There are ten *Fzd* genes in human genome divided into four families, out of which three have their members in *Nematostella* genome as well. (Two are members of *Fzd1/2/7* subfamily, two of *Fzd4/9/10* subfamily and two of *Fzd5/8* subfamily (Lee et al., 2006)). Single frizzled receptor from *Hydra vulgaris* (most closely related to human *Fzd7*) was originally characterized (Minobe et al., 2000). Recently, *Hydra Fzd2* has been identified and isolated (Philipp 2009). *In silico* analyses suggest presence of *Fzd5/8* and *Fzd4/9/10* in *Hydra magnipapillata* (Guder et al., 2006b). *CheFz1* (homolog of vertebrate *Fzd1/2/3/6/7*) together with *CheFz3* (homolog of vertebrate *Fzd4/9/10*) have been recovered from *Clytia*

hemisphaerica (Momose et al., 2007) and *Fzd1* from *Hydractinia* (Teo et al 2006). *In silico* analyses revealed *LRP5/6* ortholog in *Hydra* and *Nematostella*, but the homology is very low (Guder et al., 2006 b).

Canonical pathway members: Of course, beta-catenin is present in cnidaria (*Nematostella*- Wikramanayake et al., 2005; *Hydra vulgaris*- Hobmayer et al., 1996; *Podocoryne carnea*- Momose et al., 2007). *Nematostella* beta-catenin lacks some of the interaction domains that its bilateralian orthologs contain but both cell-adhesion and cell-signaling domain are present (Wikramanayake et al., 2005). Dishevelled is known from *Hydra vulgaris* (Hobmayer et al., 2000). Critical amino acid residues for its involvement in both canonical and noncanonical pathway are conserved (Philipp et al., 2009). GSK3beta and axin have been found in *Clytia* genome (Momose et al., 2008). *Dsh*, *GSK3beta*, and *TCT/LEF* orthologs have also been identified in *Nematostella* (Lee et al 2006). The latter is known to be present in *Hydra vulgaris* (Hobmayer et al., 2000) and *Hydractinia echinata* (Plickert et al., 2006) as well.

Noncanonical Wnt pathway members: Members of both Wnt/PCP and the calcium pathway are known from *Nematostella* genome*: Flamingo*, *Van Gogh*, *JNK*, *CamKII* and *PKC* (Lee et. al 2006). *Hydra strabismus/van Gogh*, *Rho-kinase* (rok) and *JNK* have also been reported (Philipp et al., 2005; Philipp et al., 2009). Only recently characterized tyrosine kinase receptor Ror has also ortologs in *Hydra* and *Nematostella* (Guder et al., 2006b).

Extracellular regulators: Although Wnt antagonists are absent from *Drosophila* and *C.elegans* genome, they are present in cnidarians. Single *sFRP* known from *Nematostella* most closely aligns with vertebrate *sFRP1/2/5* group (Lee et al., 2006). Two *Dkk3* subfamily members have been found in *Nematostella* (Lee et al., 2006) and one in *Hydra* (Fedders et al., 2004). There is also a *Dkk1/2/4* ortholog in *Nematostella, Hydra magnipapillata* and *Hydra vulgaris* (Guder 2006a). Vertebrates have 4 Dkk genes and a Dkk3 related gene soggy. Gene for kremen, the coreceptor of Wnt-LRP5/6 antagonism, was not identified in cnidarians, but a similar sequence was found in *Acropora* EST, as the only invertebrate putative ortholog identified so far. *WIF-1* inhibitor genes have been recovered from *Nematostella* and *Acropora* ESTs (Technau et al 2005). Recently a *cerberus*-like precursor was annotated in *Hydra magnipapillata* (Guder et al. unpublished, GI: 204517581).

2.4 Body axes in cnidarians

Bilateral animals like mouse or *Drosophila* have 3 body axes: AP, DV, and LR. AP and DV axis are specifically established during embryogenesis by asymmetrical distribution of regulatory molecules and developmental events. Even bilateralian LR axis may display an asymmetry (usually an internal one). Later, an elaborate patterning over embryonic axes divides organism into many domains, creating a coordinate system where every part of embryo can execute its specific response. Traditionally, cnidarians are considered radially symmetric with only one body axis as such, called oral-aboral. However, some anthozoans have internal bilateral symmetry with respect to pharynx morphology and position of ciliary groove (siphonoglyph). The second body axis, perpendicular to OA axis, was named directive. Molecular studies have revealed asymmetric expression of key regulatory molecules over the directive axis. Despite some differences in DV symmetry breaking regulation among bilateralians, antagonistic interaction between TGF-beta superfamily ligands (BMPs, dpp) and chordin/sog proteins is shared by protostomes and deuterostomes. *Dpp*, *chordin* and *BMP 5-8* turned out to be asymmetrically expressed along directive axis in *Nematostella*, at least in some stages (Matus et. al 2006a). Substantially more members of the regulatory network responsible for DV patterning in deuterostomes are known from cnidarians than from ecdyzozoans. Many of them are expressed during *Nematostella* development but it is sometimes difficult to distinguish among roles they play in germ layer segregation, gastrulation processes and AP/DV axis determination with almost nothing known about correlation between molecular asymmetries during early development and morphology of adult polyps. Some of genes (*NvGsc*, *NvBMP5-8*) with clear directive distribution in *Nematostella* have radially symmetric expression pattern in *Hydra*. Thus, some hydrozoans might be true "radiata" after all due to secondary losses (Matus et al., 2006b). In vertebrates, paracrine factor Nodal has a conserved function in determining the left side. *Nodal* also plays role in establishing left-right asymmetry in sea urchin and so it apparently does in tunicates as well (Shen, 2007). There are no clues of left-right asymmetry presence in cnidarians and attempts for detection of *Nodal* orthologs were unsuccessful (Matus et al., 2006b).

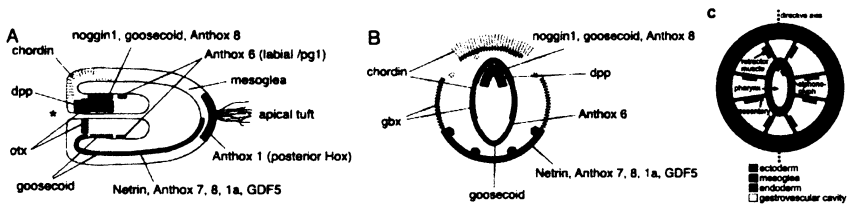

Fig. 4: Schematic demonstration of *Nematostella* body asymmetries. Expression domains of several developmentally important genes, some of them expressed asymmetrically with respect to directive axis, are marked on planula sketch (A-lateral view, B-frontal view) (Matus et al., 2006). C: Model of cross section through polyp pharyngeal region (Finnerty et al., 2004).

3. Wnt signaling in cnidarian axes determination and patterning

3.1 Wnt pathways in polyp hypostome and body column patterning and maintenance

Extensive research of *Hydra* body patterning has been done. This animal is remarkable in the way that most axial patterning and cell differentiation processes continue far beyond embryogenesis and are constantly active. Adult tissue maintenance, developmental mechanisms including differentiation, regeneration, and patterning are tightly coupled. Body column cells are constantly dividing and being displaced towards body extremities, where they are either arrested in G_2 or shed off. Changing of axial position demands permanent axial patterning. Hypostome was long ago shown to have the ability of organizing surrounding tissue and inducing secondary body axis when grafted in the middle of a body column. Therefore, organizer activity was proposed for hypostome and secretion of morphogenetic signals that would influence identity of various body parts by providing so-called positional information was postulated (Steele, 2002). Wnt/beta-catenin pathway was shown to play essential role in these processes with Wnt3a being expressed in a small cluster of ectodermal and endodermal cells at the apical tip of hypostome and TCF in a slightly broader area with graded distribution towards the apex. Dsh, beta-catenin and GSK3beta are expressed uniformly in the polyp with GSK3beta absent from foot and beta-catenin concentration slightly elevated around hypostome (Broun et al., 2005).

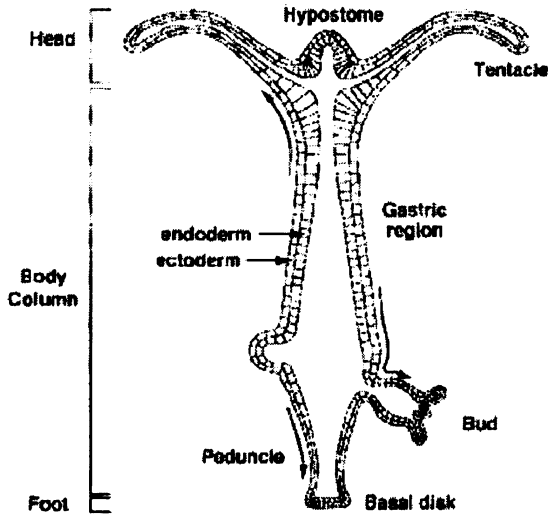

Fig. 5: Schematic representation of *Hydra* body column section. Cell proliferation occurs in a special region of body column. Following migration of cells in marked by arrows. Cells are ultimately shed off at body extremities or used in bud formation (Bode, 2003).

Receptor frizzled (human Fzd7 homolog) is expressed ubiquitously throughout the endoderm and rather rarely in tentacles, what lead to a hypothesis that Wnt activity could serve for endoderm-ectoderm identity regulation (Minobe et al., 2000). This, however, does not seem to be plausible, because endoderm and ectoderm are believed to be separate lineages in *Hydra* and thus permanent signalization is not necessary to keep the layers distinct. This fact is well supported by experiments involving grafting tissue from a *Hydra* with stable GFP expression in endodermal layer into the gastric region of a wild-type host. After the grafting, extensive cell migration occurred but none of the marked endodermal cells populated the ectoderm (Wittlieb et al., 2006). Also, *in silico* analyses predict existence of more *Hydra Fzd* paralogs, which suggests more complex signaling including responses in ectodermal epithelium (Guder et al., 2006b). Two paralogs of *brachyury* (a T-box TF that is member of a positive regulatory feedback loop that involves Wnt) *Hybra1* and *Hybra2* were detected in hypostome (Bielen et al., 2007; Technau et al., 1999). Similar Wnt-positive domain was described in *Hydractinia* with additional Wnt3 and TCF positive cells in a ring in the middle of polyp body, where differentiation of one stem-cell subset occurs (Muller et al., 2007).

Current idea of *Hydra* body patterning is based on autocatalytic environment of signals in which cells are moving and constantly acquiring state typical for their current place. I-cells, the precursors for nematocytes, neurons, gametes and secretory cells, constitute a self-renewing population present exclusively in gastric region. A subset of newly produced I-cells moves towards extremities and changes differentiation status on the way. Experimental change of positional value by ectopic beta-catenin stabilization results in I-cell behavior typical for cells of regions closer to hypostome in untreated polyps (Khalturin et al., 2007). Epithelial cells similarly interpret the positional value. Most drastic changes occur at body extremities (tentacles, body disc) and include alternation of cell shape, cytoskeleton rearrangement and cytoplasmic content conversion. Ectopic stabilization of beta-catenin causes changes of actin cytoskeleton in body column epithelial cells leading into a state typical for head region epithelium. Intermediate pathway components between actin and beta-catenin are not known in this case (Anton-Erxleben et al., 2009). Even neurons are known to change their morphology and function, ultimately being shed-off at body tips. The only exception from the constant movement seems to be oral ring tissue (Koizumi et al., 1992). Other example of positional information interpretation is tentacle maintenance and formation. Cells at bases and tips of tentacles are specifically shaped. These processes seem to be driven

by changes in actin cytoskeleton, which are triggered by activation of Wnt5 (tentacle tip) and Wnt8 (tentacle base) signaling. Neither of these Wnts is expressed in hypostome region and they most probably do not participate in positional gradient maintenance but rather only in its interpretation. Tissue evagination also involves intercalation of the cells. Pharmacological experiments and Fzd2 staining indicate, that they signal via the non-canonical Wnt pathway with Fzd2 as receptor and JNK kinase as a crucial cascade component. The non-canonical pathway is known to govern processes involving epithelial intercalation in vertebrate embryos. Members of the pathway *Hvstbm* and *Hvrok* are expressed ubiquitously in *Hydra* polyps (Philipp et al., 2009).

Two *HyDkk1/2/4* paralogs are expressed in gland cells across *Hydra* body column (*HyDkk1/2/4A* was identified by Guder et al., 2006a; Augustin et al., 2006 described both paralogs) with sharp apical border of expression just below hypostome. *HyDkk1/2/4C* displays graded decline of expression towards the foot (It was shown by grafting experiments, that foot inhibits *HyDkk1/2/4C*). *HyDkk1/2/4A* is expressed at roughly same level throughout the body column. Mutual repression between *Wnt3a* and *HyDkk1/2/4C* was shown experimentally (Augustin et al., 2006). *Dkk3* also exists in *Hydra*, but it is member of a highly divergent group and plays role in terminal nematocyte differentiation rather than being part of the positional value gradient system (Fedders et al., 2003). What we have here is a system of a morphogen and its inhibitor with positive and negative feedback loops. This assembly assures homeostasis of body plan and provides ability of self-renewal after damage. When either mouth or foot pole is lost, it regenerates restoring both original morphology and underlying molecular gradients. Even if both ends are cut off, they regrow with the original polarity. After apical part dissection, wound healing occurs at first. Than, the organizer is renewed with TCF and beta-catenin appearing prior to Wnt3a. Domain of Wnt3a is broader at first and gets subsequently restricted (Hobmayer et al., 2000). In the third phase, tentacles are regenerated with redeployment of the morphogenesis-driving Wnts (Wnt5, Wnt8) that appear prior to tentacle growth but well after hypostome regeneration, suggesting that substantial positional value increase must be achieved for their activation (Philipp et al., 2009). HyDkk1/2/4A is detectable by the onset of wound healing. Experiments involving animal wounding without head removal and head removal without wounding showed that HyDkk1/2/4A responds rather to an injury than to a positional value change. Its expression declines after hypostome reestablishment and disappears before new tentacles start to grow. No changes in HyDkk1/2/4C were reported but the image of *in situ* hybridization provided by Augustin et al.

(2006) shows that while it is expressed to the very tip of remaining body column during early stages of regeneration, later it obtains its usual sharp upper boundary, which results into HyDkk1/2/4C-free hypostome region. Remarkably, the organizer is able to arise spontaneously from a suspension of *Hydra* epithelia cells. The cells begin to adhere until aggregates with organizer activity form, which generate fields of lateral inhibition and eventually reassemble into functional polyps (Technau et al., 2000).

Most recent data show that organizer activity is even more complex. Several *Wnt* paralogs, previously considered lost in hydrozoans, were found in *Hydra*. *HyWnt3, 11,7,16,1,9/10a* and *c* are involved in adult hypostome maintenance and new hypostome formation during budding and regeneration with specific proportions of ectodermal to endodermal expression for particular paralogs. Their activity appears in specific order during hypostome formation. Authors of the study suggest that *HyWnt3* serves as a "master" Wnt on the top of a cascade, because it is the first one expressed during hypostome regeneration and its ectopic application can partially rescue inability of *reg-16 Hydra* strain to regenerate head (application of recombinant mouse Wnt3A gives similar resuls, pointing at high degree of conservation of the protein). Unfortunately, other Wnts were not tested for similar function (Lengfeld et al., 2009). On the other hand, *Wnt3* also seems to be expressed very early in *Nematostella* gastrulation, possibly as the first one of Wnts present around blastopore (Lee et al., 2006) and it is *Wnt3* that plays important role in early development of two hydrozoans: *Clytia* (Amiel et al., 2009) and *Hydractinia* (Plickert et al., 2006).

Unfortunately, almost nothing is known about hypostome organizer properties, Wnt signaling and tissue turnover with ongoing differentiation in adult *Nematostella*. It is able to regenerate mouth and pharynx after a transverse bisection but its regeneration capabilities are reduced when compared to those of *Hydra*. For example, longitudinal bisection is lethal. Interestingly, several *Hox* genes expressed asymmetrically with respect to directional axis during embryogenesis and metamorphosis display symmetrical expression in regenerating *Nematostella*. These results suggest that regenerating polyp uses the already established asymmetry in the intact body part and is not able to create a new one (Burton and Finnerty, 2009). To get any idea about potential Wnt pathway function in *Nematostella* regeneration, asexual reproduction or tissue maintenance and patterning, more data are required. Not even simple experiments with LiCl or paullone treatment have been done yet.

3.2 Wnt signaling in secondary axes induction during asexual reproduction and colony growth

Both budding of polyps during asexual reproduction and growth of colonies are coupled with emergence of a new organizer and turning surrounding tissue into a functional polyp. It is actually budding that serves as a model process of *Hydra* hypostome organization and it is much better explored than *Hydra* embryogenesis is. As in regeneration, TCF and beta-catenin are upregulated prior to Wnt (Hobmayer et al., 2000) with a subsequent restriction of TCF after the initial phase. At the onset of evagination, Wnt3a domain appears and gradually reaches its typical size (Hobmayer et al., 2000). Other *Wnts* (*1, 7,9/10a, 9/10c, 11, 16*) are also expressed from the initial phase and remain at the apex of accumulating tissue during bud enlargement. *HyWnt2* is expressed transiently during the very early stages of budding (Lengfeld et al., 2009). From staining pictures provided, it is not clear whether all paralogs mentioned above start to be expressed simultaneously or in any specific order. All *Wnts* are initially expressed in ectoderm only but they may later undergo a shift to endoderm with final ratio of ectodermal to endodermal expression specific for the particular paralog. It is important to note, that budding (as well as regneration) is essentially a morpholactic process dependent almost solely on recruitment of extant cells in the body column, at least during its earlier stages. In the beginning of budding, some sort of a decision must be done in particular tissue region and cells suddenly execute different behavior than they normally do in the same area of body column-they change direction of movement and are recruited towards the future bud center in a circular pattern. Wnt5 seems to be involved in this response, making endodermal cells adjacent to its source curve towards ectoderm, thus initiating evagination. Its activity disappears when stable organizer forms. Therefore, it is believed that Wnt5 is involved in the initiation of evagination but not in its maintenance (Philipp et al., 2009). Alternatively, apical cells of a larger bud might not need such a sharp curvature as the early-bud epithelium does. *Wnt8* is expressed in a broader region around the forming bud till its detachment. There is no *HyTCF* in that area, which speaks for the non-canonical pathway presence. During the later stages, when organizer of the future polyp arises and deploys, cells along bud obtain a new positional value and adapt to it. This also includes formation of new tentacles (Philipp et al., 2009). We can see a parallel with earlier described events responsible for dynamic body maintenance (Anton-Erxleben et al., 2009; Khalturin et al., 2007).

In many colonial hydrozoans like *Hydractinia*, individual polyps are interconnected by a system of hollow tubes called stolon. Stolon system is increasing its radius and produces new polys by budding at certain distances from the old ones. Buds emerge as local thickenings of the stolon. They express *Wnt* and *TCF* in a small patch and *Fzd* in much broader area. Experimental increase of Wnt/beta-catenin signaling to different degrees by treatment with increasing concentrations of alsterpaullone causes gradual transformations of body parts: first, buds start to form more closely to each other, indicating that inhibitory signal that spreads from a polyp into surrounding stolon tubes can be overcome by ectopic application of stimulatory signals. Higher doses and times of activation result into oversized buds and after very strong increase of positional value, stolon completely turns into a giant clump of polyp tissue that eventually falls apart into biheaded and multiheaded polyps (Muller et al., 2007).

3.3 Members of Wnt pathways in maternal factor deposition and early developmen

Body axes are established very early in development of most animals, often even prior to fertilization. Specifically deposited maternal factors play crucial role in the process. The site of sperm entry end cell division plane(s) at the very beginning of cleavage may also be very important. Cnidarian oocytes are already asymmetric, with two halves called the animal and the vegetal part. It has been shown for some cnidarian species that as early embryos as 8-cell morulas contain blastomeres with defined polarity. When such embryos are dissected to produce animal and vegetal tetrads, the animal parts are capable of developing into a functional planula, whereas vegetal parts are not (Shown for: *Podocoryne carnea* by Momose et al., 2006; *Nematostella* by Lee et al., 2007). On the other hand, both halves of *Phialidium(=Clytia)* embryos develop properly if dissected prior to the beginning of gastrulation. It was suggested that OA axis becomes determined at the time of first cleavage (possibly by a post-fertilization reorganization of cytoplasm) and that beta-catenin plays role in oral end and endoderm specification, based on experiments with *Nematostella* (beta-catenin is selectively stabilized at future oral pole (Wikramanayake et al., 2003); expression of *NvTCF* follows similar spatial and temporal pattern (Lee et al., 2006)). Later, beta-catenin is restricted to the *Wnt*-expressing region (Lee et al., 2006) together with *brachyury*, probably establishing a positive feedback loop (Scholz et al., 2003). Conclusions about postfertilization determination of OA axis in cnidarians were later criticized by Primus and Freeman (2004) who also summarized knowledge on early development and axis specification in hydrozoans: eggs can only be fertilized at the site of polar body extrusion and first cleavage is also initiated at this side marking future posterior of planula (the oral end). Thus, OA axis is either a result of oocyte anisotropy or is set up only upon initiation of the first cleavage. To distinguish between these two models, many experiments with oocyte and zygote mechanical manipulation were performed, sometimes being able to change axis orientation by mechanical interventions into zygote's first plane of division. Later studies carried more light into the conundrum: oocytes of many cnidarian species were shown to have asymmetrically distributed maternal mRNA/protein molecules: animally enriched Dsh in *Nematostella* as a result of its selective degradation at vegetal pole (Lee et al., 2007) and two oppositely positioned Fzd RNAs (CheFz1 at animal and CheFz3 at vegetal pole (Momose et al., 2007)) together with one CheWnt3 (Momose et al., 2008) at animal pole in *Clytia hemisphaerica.* Morpholino-mediated experiments have shown function of CheFz1 in canonical Wnt/beta-

catenin pathway activation and CheFz3 in opposing it resulting into a feedback loop that stabilizes their reciprocal distribution later in development. It seems to be the presence of polarizing molecules at both ends of the oocyte and embryo that allows both halves of dissected blastulae to regenerate, because either of Fzd RNAs can induce secondary body axis if expressed ectopically (Momose et al., 2007). Further experiments even uncovered mechanisms responsible for establishment of these asymmetries. They seem to be different for each of the three maternal factors (Amiel et al., 2009).

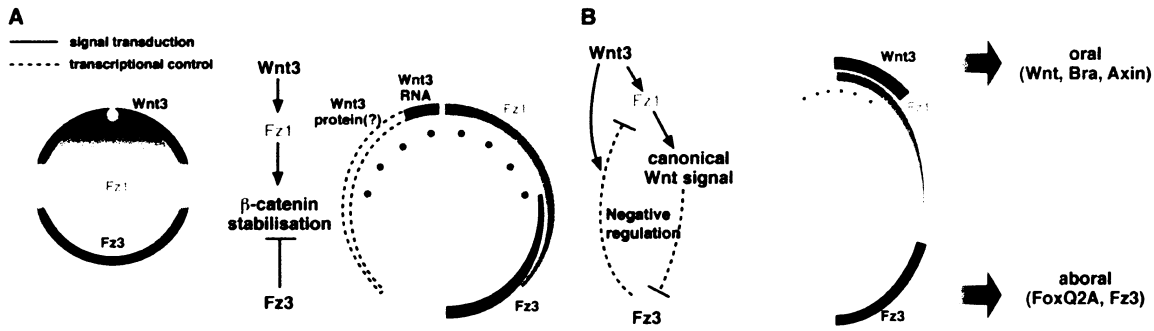

Fig. 6: Clytia hemisphaerica oocyte and embryos as an example of asymetrically distributed maternal factors driving embryonic oral-aboral polarity. A: Distribution in early stages (from egg to early blastula). Fz3 acts as a negative regulator of Wnt/beta-catenin pathway and is probably responsible for restricting of the pathway to the future oral pole. B: Later stages. Arrows and dotted lines represent signaling pathways and transcriptional regulation pathways respectively (Momose et al., 2008).

In *Podocoryne,* an orphan *Hox* gene *Cnox4-Pc*, (Yanze et al., 2001), and *brachyury* (Spring et al., 2002) are localized at the animal pole and retained there after cleavage. Wnt and TCF are concentrated at the pole of first cleavage and polar body formation in *Hydractinia* and their initial distribution is later reinforced by zygotic transcription at the oral pole and in central endodermal mass. Similar expression kinetics to *Podocoryne brachyury* was observed for *Hydractinia brachyury* (Plickert et al., 2006). Thus, although in somehow different ways, cnidarians seem to establish the oral-aboral axis by asymmetrical distribution of maternal factors leading into asymmetrical activity of beta-catenin signaling. It is possible that beta-catenin played role as a response to a maternal or environmental ancestral polarizing signal and later internal mechanisms for polarity establishment evolved several times independently

with different members of Wnt network as maternally supplied triggers in different clades. Alternatively, members of Wnt network with asymmetric distribution might have been switching during phylogeny of particular clades, supposing that the system was redundant enough and rare events lead to appearance of new maternal factors from time to time followed by loss of the old ones.

Due to its hardly accessible later embryogenesis, only a few studies looked at gene expression during *Hydra* development. Gastrulation occurs by multipolar ingression and leads to formation of internal endodermal cell clumps surrounded by epithelial ectoderm. It antedates cuticle formation, thus all asymmetry on morphological level arises inside the cuticle. However, site of sperm entry corresponds to the future oral pole (Martin et al., 1997) but no functional experiments were performed, so we do not know whether the sperm entry establishes the initial asymmetry or already existing molecular asymmetry predestinates one particular pole for both sperm entry and future hypostome formation. How hypostome development occurs is currently unknown. Fzd, beta-catenin and TCF transcripts detected in oocytes and early embryos (Frobius et al., 2003) might help in starting the event but unfortunately, nothing is known neither about their expression pattern within embryos, nor about potential nuclear beta-catenin activity.

There are some clear parallels between axis specification and gastrulation in bilateralians and cnidarians and it is tempting to suggest how the processes worked in their last common ancestor but one should be careful when making too general statements. Canonical Wnt/beta-catenin signaling and maternal distribution of its components play role in polarization and germ layer specification processes. beta-catenin is recognized as a robust and widely conserved marker of gastrulation and endomesoderm but in detail, its functions differ among various bilateralians (Primus and Freeman, 2004). Genes important in gastrulation processes, axial properties and germ layer segregation show much more uniform expression in examined cnidarians that their orthologs in different bilateralian lineages do (Martindale, 2005). This might point to an ancestral regulatory network with subsequent diversification of functions of its members, but traces of reductive evolution in cnidarians are also evident (Matus et al., 2006b). To make absolute conclusions, we still need data from more species. Comparative approach often helps to answer questions that are too difficult to solve with our current knowledge of single species. For example, beta-catenin was postulated to be used in endodermal specification in *Nematostella* (Wikramanayake et al., 2003) and *Clytia* (Momose

et al., 2007), because proportion of endodermal cells in embryo is much larger after ectopic activation of canonical Wnt pathway. However, both species have unipolar gastrulation situated at future oral pole, so beta-catenin might rather specify oral pole that then contributes to endoderm. This is supported by experimental ectopic beta-catenin stabilization in *Hydractinia* embryos. During its asymmetric multipolar gastrulation, anterior and posterior parts contribute roughly equally to endoderm. Upon ectopic Wnt/beta-catenin pathway activation, multipolar larvae develop but they do not have altered endoderm/ectoderm ratio was not reported (Plickert et al., 2006).

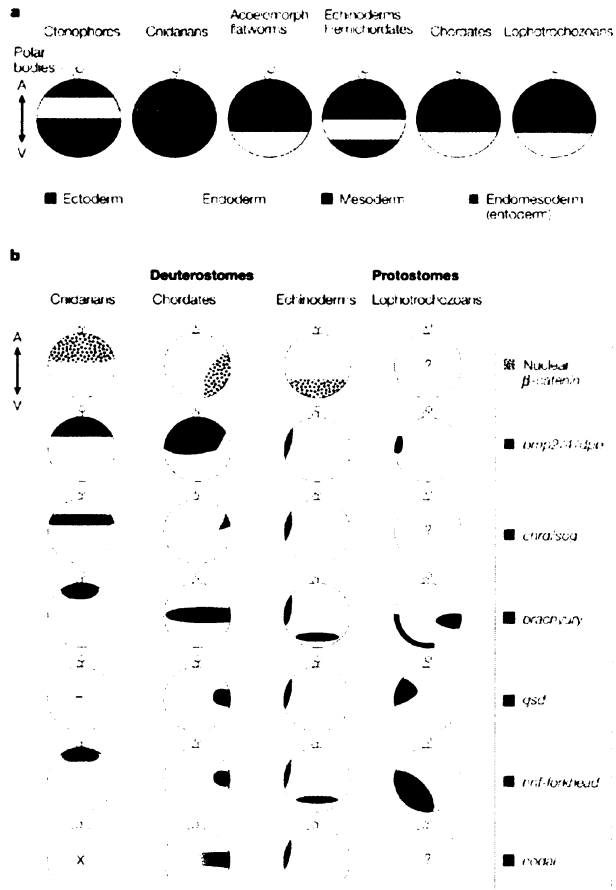

Fig. 7: Axial positions of future germ layers (a) and gastrulation-associated gene expression domains (b) in various metazoan clades mapped onto the animal-vegetal axis of zygotes. Note that some genes (eg. beta-catenin) are quite conservative markers of gastrulation site, while other genes may be expressed in different area than at the future gastrulation site in some lineages (eg. chrd, gsc in echinoderms). Bmp, bone morphogenetic protein; dpp, decapentaplegic; hnf-forkhead, hepatocyte nuclear factor, a forkhead homologue; sog, short gastrulation (Martindale, 2005).

3.4 Wnt signaling in later development (planula and metamorphosis)

The interdependency of asymmetrical Wnt signaling in early development and establishment of hypostome organizer at the same pole of future polyp is sometimes obvious, but not always. In *Hydractinia*, *Wnt* and *TCF* expression become undetectable by *in situ* hybridization in mature planulae. During metamorphosis, waves of concentric *Wnt* and *TCF* expression reappear in settling larvae and result in ultimate mouth opening and formation of localized hypostome area with Wnt and TCF presence (Plickert et al., 2006). It is possible that the event includes features similar to self-organization of hypostome known from *Hydra* with no initial asymmetry necessary. On the other hand, the existing polarity might be utilized for *Wnt* reexpression, using either asymmetrical trigger deposited by embryonic polar Wnt activity or an unknown factor completely independent on maternal TCF/Wnt deposition.

Existence of so-called Wnt-code has been proposed, based on *Nematostella Wnts'* expression patterns (Kusserow et al., 2005), pointing out its resemblance of bilateralian *Hox* expression pattern across primary body axis. Since then the Wnt-code has been considered to be the ancient body-patterning mechanism and ancestral genomic linkage of Wnts with *Hox*-like colinearity of expression in development was expected. However, only a few Wnts actually exhibit genomic linkage in *Nematostella* (Wnt5-Wnt7/7b and Wnt6-Wnt10) and they lack colinearity during expression (Sullivan et al., 2007). Hypothesis that function of *Hox* genes in recent bilateralians was exhibited by Wnts in the common cnidarian-bilateralian ancestor and that Wnts were substituted by a newly emergent *Hox* code during early bilateralian history should be taken with care. First, *Hox* proteins are TFs, whereas Wnts are signaling molecules. Gradients of morphogens are crucial in the process of different Hox-domain activity establishment. Thus, Hox and Wnt might have had complementary functions in the ancestral body plan formation. Existence of the Hox code in the common ancestor of bilateralians and cnidarians is still a matter of discussions. There are only few data about *Hox* and *Hox*-related genes' expression in *Hydra* polyps. Several works suggest correlation between *cnox-2* expression and specific body regions in *Hydra* and *Hydractinia* but these results are contradictory (Cartwright et al., 1999; Gauchat et al., 2000; Shenk et al., 1993a; Shenk et al., 1993b). After sequencing *Nematostella* genome and performing *Hox*-like gene analysis together with *in situ* hybridizations, it turned out that there were several true *Hox* and *paraHox* genes expressed in distinct domains with respect to primary and secondary body axis (Ryan et al., 2007). Second question is, to what extent do Wnts play different role in

Nematostella body plan organization than they do in *Hydra* patterning, where most of the diversity at tissue level occurs around the hypostome. Recent study concerning *Nematostella* nervous system uncovered considerably more complexity than we know from *Hydra*, with multiple classes of neural cells and cnidocytes. On the other hand, *Nematostella* body column seems to be rather homogenous in composition, similarly to that of *Hydra*, with most of the complexity in the oral area, pharynx and tentacles, with exception of larval apical organ that is situated at the future aboral end and disappears during metamorphosis. Several regions populated with a specific subset of neurons correlate with expression domains of a particular TF or combination of more TFs (Marlow et al., 2009). Wnts might be important during establishment of these domains but there is currently no evidence that it is so and strong influence of other signaling pathways (FGF, TGFbeta) is almost certain. It is unclear to what degree does *Nematostella* nervous system exhibit similar dynamic flux as *Hydra* neurons do, but *Nematostella* lack interstitial stem cells that are responsible for continuous neuron supply in hydrozoans (Marlow et al., 2009). Mechanisms of tissue patterning might be similar but restricted to periods of gastrulation, metamorphosis and regeneration in *Nematostella*. Regions of activity of some *Wnt* paralogs seem to be the same as in *Hydra*, whereas others have clearly unique functions: *NvWnt1*, *NvWnt3* and *NvWnt7* display expression strictly around blastopore and hypostome in development. The same applies to *NvWntA*, which is absent from *Hydra*. *NvWnt10* is expressed in individual cells throughout the body column. Similar expression pattern was reported for *NvWnt11* (Kusslerow et al., 2005) but contradicted by other study that showed staining around blastopore and later in developing tentacles (Lee et al., 2006). On the other hand, *NvWnt2*, *NvWnt4* and *NvWnt6* could have special developmental role in *Nematostella*. *NvWnt4* and *NvWnt6* cease to be expressed after development reaches the primary polyp stage, which might also apply to *NvWnt2* (expression pattern from a primary polyp is not available). *NvWnt2* (ectodermal) and *NvWnt6* (endodermal) are expressed in middle body regions, *NvWnt4* closer to blastopore and forming hypostome and, interestingly, in an asymmetric manner with respect to directive axis. Directive asymmetry was also reported for *NvWntA* expression. *Wnt4* and *Wnt6* have not been detected in *Hydra* so far and HvWnt2 seems to play a special role at the very beginning of bud formation (Philipp et al., 2009). As mentioned earlier, HvWnt5 and HvWnt8 are employed in cell-shaping responses rather than in the positional value establishment (Philipp et al., 2009). Like *HvWnt5*, *NvWnt5* is expressed at tentacle bases in primary polyps. *NvWnt8* is expressed in endodermal area of future mesenteries and then in primary polyp mesenteries. Whether it is necessary for this domain patterning, for tissue morphogenesis during mesentery

formation or for both is not known. *NvDkk1/2/4* is expressed in the posterior of gastrula and planula, possibly excluding Wnt activity from the region and specifying apical organ (Lee et al., 2006).

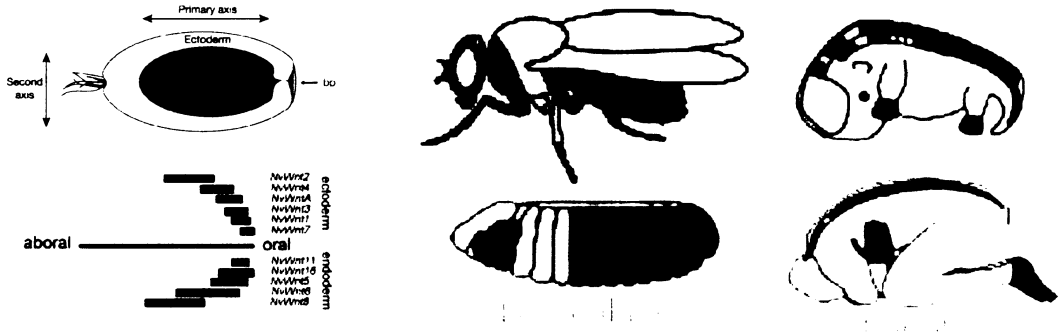

Fig. 8: Left: Schematic representation of all known *Nematostella* Wnt paralogs' expression domains (Kusserow et al, 2005; Lee et al., 2006 upper and lower image respectively). Middle and right: *Hox* expression in *Drosophila* and human respectively (Hueber and Lohmann, 2008). Most of the Wnts in *Nematostella* are staggered around blastopore and their overal arrangement along primary body axis does not resemble bilateralian *Hox* expression pattern very much.

4. Conclusion and outlines

Enough evidence has been gathered for presence of at least two body axes in some cnidarians and last common eumetazoan ancestor. Determination of oral-aboral axis in cnidarians is tightly coupled with activity of beta-catenin signaling network in future oral part initiated by animal-pole localization of specific maternal factors. Almost all *Wnt* subfamilies known from vertebrates exist both in *Hydra* and *Nematostella,* and most of them are part of a regulatory network deployed during hypostome formation. Hypostome is a region with sophisticated signaling activity able to organize surrounding tissue into layers of different cell types across oral-aboral axis, according to their distance from the positional value gradient source. Besides signaling in embryogenesis, well documented in *Nematostella*, it is able to drive patterning in adult hydrozoans, whose tissues undergo constant flux and redifferentiation that needs highly dynamic instructions. Cnidarians are well known for vegetative reproduction, which involves formation of a new organizer at certain distance from the old one and turning surrounding body column or stolon tissue into a new polyp, at least in investigated hydrozoan species. Similarly, after loss of the hypostome, hydrozoan tissues spontaneously regenerate it due to loss of previous high positional value repressing new organizer formation. Details about organizing of *Nematostella* primary axis, particularly about the potential role of Wnts in more distant body region patterning, are still uncertain. Wnts might somehow complement *Hox* genes in axial patterning, but there is no strong evidence that they have a privileged role in the process. As in bilateralians, Wnt signaling may act via the non-canonical pathway in cnidarians. This pathway involves JNK kinase activity and actin cytoskeleton reorganization, which is important in cell intercalation and morphogenetic events. We can clearly see, that in the last common eumetazoan ancestor *Wnt* family was already highly diversified and both the canonical and the non-canonical pathway were utilized in development.

Future research of Wnt signaling in cnidarian models should definitely involve repetition of some previously done experiments on complementary models. For example, quite a lot is known about Wnt signaling during asexual reproduction and regeneration in *Hydra* but almost nothing in *Nematostella.* Another interesting topic in context of Wnts might be cnidarian metamorphosis and medusa body stage organization. It is quite common that well-diversified developmental pathways are reused for several different tasks in one organism, so looking for a potential Wnt role in metamorphosis is promising. The same might apply to patterning during development of more complex structures like gonads, eyes and statocysts.

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