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Hydra, a model system to trace the emergence of boundaries in developing eumetazoans

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ABSTRACT In developing embryos, boundary formation between neighbouring groups of cells is essential to establish compartments which later fulfil specialized functions. The ability to form such boundaries has likely developed early in animal evolution - due to functional requirements imposed by the necessity to separate tissues which protect the animal, take up food or ensure propagation. Essential for boundary formation are local cues which may be provided by the intersection of diffusible molecules or set locally by activation of membrane-bound receptors and transcription factors. In the simple diploblastic *Hydra*, a representative of the basally branching metazoan Cnidaria, tissue boundaries are morphologically detectable between the body column and terminally differentiated head and foot structures. In adult polyps, these borders correspond to sharp lines of differential gene expression. They form *de novo* during regeneration and budding of a young polyp. Functional studies strongly suggest the involvement of FGFR/Notch signalling in the establishment of the parent-bud boundary, and it is very likely that these pathways interact with the WNT and BMP systems. How boundaries in the head and foot regions are generated is still unclear. Expression patterns of transcription factors like Cngsc, HyAlx, HyBra, HyOtx, Prdl-a, CnNK2 and Manacle show strong position dependency and may be involved in regulating gene expression on either side of the boundaries, by interpreting positional information during their formation and maintenance. Due to its simplicity, the easy accessibility to pharmacological interference and, recently, transgenesis, *Hydra* is an interesting prebilaterian model system to study the emergence of boundary-forming mechanisms during evolution.

KEY WORDS: *boundary, evolution, Hydra, Notch, FGFR*

Introduction

All multicellular animals with their diverse body plans develop from one fertilised egg. During embryonic development cells multiply and differentiate, and specialized structures emerge from fields or layers of cells, which are initially identical. Differential gene expression induced by signalling from within these fields and from the surrounding stabilises compartments necessary for the emergence of cells with different and mutually exclusive properties. The separation process requires formation of molecular and morphological boundaries, which prevent intermingling of cells with different destinies, allow stable expression of transcription factors in adjacent fields and often themselves function as new signalling centres to establish refined patterns within the respective domains (Sánchez-Camacho *et al.*, 2005; Dahmann *et al.*, 2011). Eventually adult animals are formed with their cells organised in tissues and organs physically separated from each other.

Boundary formation in multicellular animals has been described in a manifold of developmental contexts. Although the targets for signalling at boundaries and during their formation are diverse depending on the characteristics of the developing tissues, a number of signalling pathways have been recurrently found to be involved. In the following we will shortly introduce boundary-forming signal systems in vertebrates and insects, for which orthologues have been identified and partially characterised in Cnidaria and then summarize what is known in *Hydra*.

Abbreviations used in this paper: BMP, bone morphogenetic protein; FGF, fibroblast growth factor.

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Signalling between adjacent cells

Of particular importance for the establishment of perfectly separated developmental compartments are the Notch and ephrin signalling pathways. Receptors and ligands of both pathways are membrane proteins. They either contain transmembrane domains as in the case of Notch, ephrin B and ephrin receptors (Eph), or are attached to the cell membrane, e.g. by GPI anchors, like the A-type ephrins (Bray 2006; Himanen *et al.,* 2007). Signalling through both pathways therefore occurs between cells that are in direct contact, and it results in differential responses in adjacent cells. Best-known examples are the mutually exclusive differentiation programs started by Notch signalling in the nervous system or at tissue boundaries and the adhesive-anti-adhesive cycles controlled by Eph/ephrin signalling, which are essential for pathfinding of neurons and neural crest cells, for angiogenesis (Himanen *et al.,* 2007; Pasquale 2008) or for the specification of cell layers during gastrulation (Rohani *et al.,* 2011). Since Eph-ephrin signalling is bidirectional, both, signal sending and signal receiving cells, are instructed and thus able to respond differentially.

Our knowledge concerning the molecular mechanisms involved in boundary formation comes from investigations in bilaterian animals, especially in insects and in vertebrates. Very well described examples include the establishment of the dorsal/ventral boundary in the *Drosophila* wing (Kim *et al.,* 1996) or segment boundaries of the *Drosophila* leg, which are established in imaginal discs of the larvae (de Celis *et al.,* 1998). In vertebrates a lot of research has been invested into studying boundary formation between rhombomers of the developing brain and the establishment of segment boundaries (reviewed in Dubrulle and Pourquie, 2002).

In most of these instances Notch signalling has been shown to be involved. The molecular mechanisms are just emerging: Recently, in an elegant tissue culture model, Sprinzak and coworkers elucidated how the propagation of modest differences in the amount of Notch receptors and their ligands at membranes of adjacent cells by use of the intrinsic kinetic of cis- and trans-signalling responses can lead to amplification of the signals and thus the formation of defined regions of differential gene expression (Sprinzak *et al.,* 2009; Sprinzak *et al.,* 2011).

Signalling involving diffusible molecules

Directly cooperating with the immediate cell-to-cell signalling of Notch- and ephrin pathways, are FGF- and Wnt-signalling pathways (Takahashi *et al.,* 2005). Both pathways depend on diffusible ligands and membrane-bound receptors and are thus able to integrate signals, which move across longer distances.

In contrast to Notch and ephrins, diffusible signals like FGF and Wnt, but also TGFβ/BMP, Shh or Dpp, define, at least initially, regions instead of sharp boundaries and they provide positional and local information. The intersections of diffusible signals may generate Cartesian coordinates and provide positional information for morphogenetic boundaries (Niehrs 2010). In this context, it is important to note that diffusible molecules may act as morphogens. Their concentration profile provides precise thresholds for transcription factor activity, which defines sharp boundaries within a graded morphogen distribution (Sánchez-Camacho *et al.,* 2005). In the very complex organ systems of higher developed animals, FGF, transforming growth factor-β (TGF-β), Wnt and Shh were identified as potential morphogens and work in concert with Notch locally to define boundaries.

Although in principle freely diffusible, most known growth factors and morphogens underly restricted diffusion. Posttranslational modifications like lipidation limit the range of e.g. Wnt ligands. Moreover, Wnt, Shh and several of the 22 vertebrate FGFs, which are secreted in the interstitial space, are scavenged by heparan sulfate proteoglycans (HSPGs) of the extracellular matrix (ECM) (Häcker *et al.,* 2005; Yu *et al.,* 2009). Their controlled release from the ECM, is a regulatory option. HSPGs thus restrict the range of action of secreted growth factors and thereby may sharpen boundaries.

As exemplified by FGFs, growth factors may also be removed from a diffusion gradient by binding to their receptors and subsequent endocytosis of ligand-receptor complexes: following stoechiometric binding of FGF, two FGFR dimerize and autophosphorylate to achieve the activation of downstream signalling pathways. The receptor-ligand complex is subsequently internalized and either targeted to the lysosomal compartment for degradation – or to the recycling compartment. Provided FGF is available in limited amounts, its binding to the receptor generates a local "sink". In vertebrates, such a source - sink mechanism (= FGF release – establishment of a gradient - capture and removal of FGF) has been demonstrated and discussed in the context of establishing the midbrain-hindbrain boundary (MHB)(Yu *et al.,* 2009).

This boundary is a well-known example for interaction of FGF and WNT signalling systems. It is established and maintained by a combination of WNT1, FGF8 and transcription factor signalling: at the anterior margin *Wnt1* and *Otx2* expression domains are located, the posterior margin is precisely defined by *FGF8* and *Gbx2* domains (Acampora *et al.,* 2001). Any change in their expression pattern or the diffusion range of Wnt1 or FGF8 ligands causes fatal effects in the developing brain. During evolution, a similar boundary seems to have existed already in the urbilaterian brain: *Gbx2* and *Otx2* are expressed at the interface between the *Drosophila* Deuto- and Tritocerebrum in a mutually exclusive manner. In the flour beetle *Tribolium,* besides *gbx* and *otx* also *Wnt1* and *Tc-fgf8* are expressed locally in the developing brain (Bolognesi *et al.,* 2008). Thus, the boundary in the tripartite insect brain might correspond to the MHB in vertebrates (Hirth *et al.,* 2003), but functional data are not yet available.

In summary, boundary formation can be achieved by local interaction, but also at the intersections of diffusible molecules or set by morphogen thresholds. This positional information will then be refined by secondary processes like local activation of growth factor receptors or transcription factors.

Boundaries in prebilaterian animals

Since boundaries are prerequisite to establish compartments specialized for different functions, and also to establish regionalization along one or more body axes, they occur already in prebilaterian animals like *Hydra*. Elucidation of the molecular mechanisms involved in their formation and maintenance is only at its beginning, but became easier with genome projects and the establishment of transgenic approaches (Steele 2002; Wittlieb *et al.,* 2006; Chapman *et al.,* 2010).

Tissue composition and dynamics in *Hydra*

The cnidarian *Hydra* is a well-characterized representative of a prebilaterian. As detailed in other reports of this review series, its simple body plan comprises a head with tentacles and a mouth opening at the tip of a cone-shaped hypostome, a gastric column and a foot, consisting of a peduncle with a basal disc at its end (Fig. 1). Two single-celled epithelial layers surround the body and form the outer ectoderm (or epidermis) and the inner endoderm (or gastrodermis, see other reviews of this series). These epithelial cells are self-renewing. They constitute epithelio-muscular cells, with an apical epithelium-forming cell body and basal, bidirectionally oriented processes containing contractile fibres. Epithelio-muscular cells of the endoderm thus function as circular muscles, ectodermal ones as longitudinal muscles. Cells of both layers secrete an acellular, collageneous mesoglea, which corresponds to the extracellular matrix (ECM) of higher animals (Sarras 2012). The epithelial cells are anchored in and separated by this ECM (Zhang *et al.,* 2007). In the interstitial spaces of both layers so-called interstitial cells reside, which harbour continuously self-renewing pluripotent stem cells and their differentiation products, such as nerve cells, nematocytes, gland cells and germ cells (David 2012; Hobmayer *et al.,* 2012). All cells of the interstitial and the two epithelial cell lineages are constantly renewed by proliferation in the gastric column. In consequence, tissue is constantly displaced towards the terminally differentiated, non-proliferating tentacles and the basal disc (Fig.1). Additionally, mass tissue movement transfers gastric tissue into buds. These form regularly perpendicular to the parent body axis in well fed animals and constitute the means of asexual reproduction (reviewed in (Bode 1996). During budding, longitudinal and circular epithelial cell processes undergo massive rearrangement to match the new tissue orientation. The young polyp differentiates apical and basal structures and finally separates its epithelia from the parent having formed its own basal disc. Several marker genes for regional specification during growth and budding in *Hydra* have been described (Steele 2012). Tissue properties and gene expression patterns have been used to establish mathematical models for *Hydra* patterning (Meinhardt 2012).

Boundaries in *Hydra* and state-of-the-art of molecular mechanisms involved in their establishment

The *Hydra* body is subdivided by several morphologically distinguishable boundaries, which all correlate to molecular boundaries (Fig. 1). From top to bottom these boundaries are (i) the ecto-endodermal boundary at the hypostome tip (mouth opening), where both tissues are in direct contact. (ii) The hypostome – tentacle boundary, where proliferating hypostomal cells meet the protruding and terminally differentiating ectodermal battery cells and flat endodermal epithelio-muscular cells (Fig.1A, red line). (iii) The tentacle - body column boundaries (grey and blue in Fig.1A) where proliferating ectodermal epithelio-muscular cells differentiate into battery cells which take up mature nematocytes with their cysts by transendocytosis. (iv) The body-peduncle boundary located right

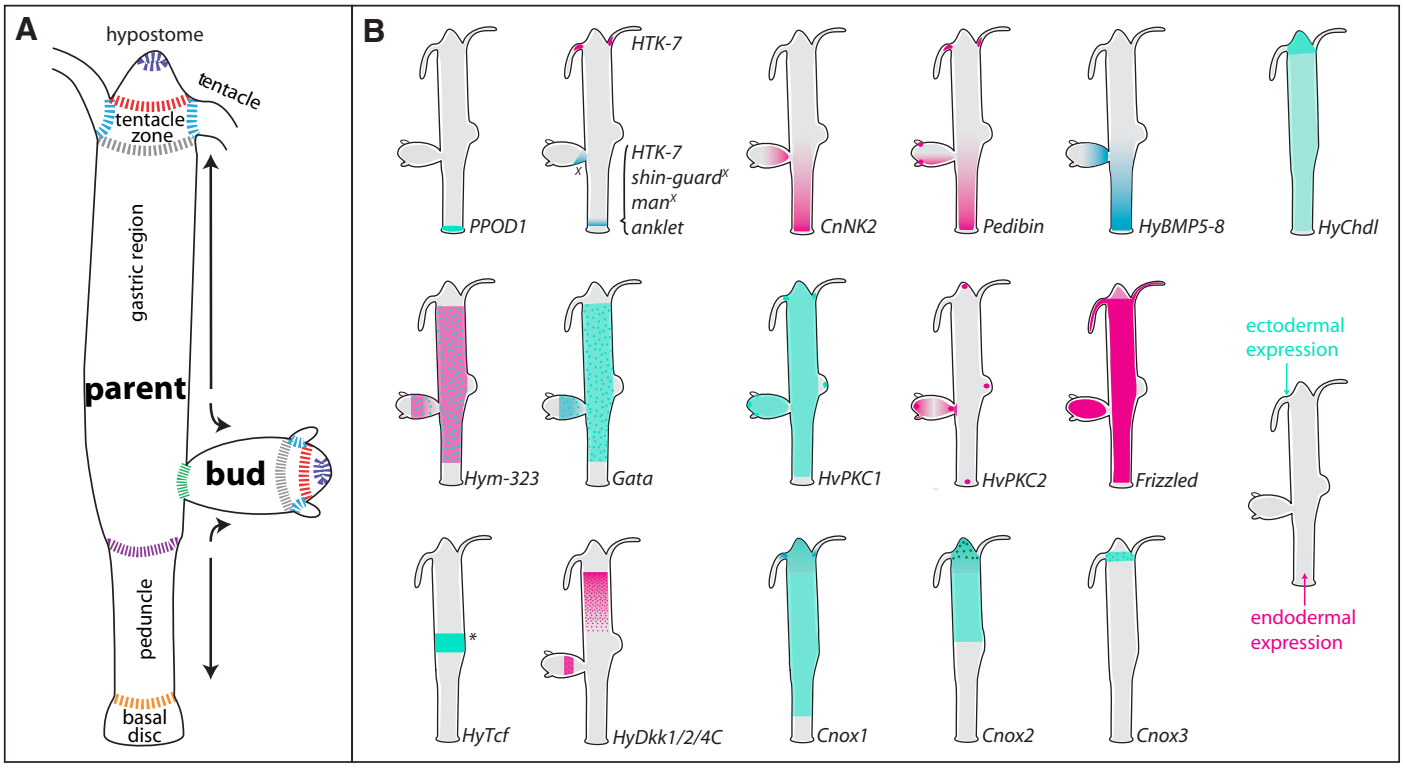

Fig. 1. Schematic overview of morphological boundaries, mass tissue movement and gene expression domains. (A) *Morphological boundaries.* **(B)** *Selected expression boundaries of genes in adult* Hydra. *Dots indicate that only a certain population of epithelial cells (*Hym-323, Gata, Cnox2 *and* Cnox3*) or of gland cells (*HyDkk1/2/4C*) express the gene. The asterisk indicates that* HyTcf *is expressed transiently in the budding competent zone prior to bud evagination.* Shin guard *and* manacle *are expressed asymmetrically in the early developing bud (x).*

below the budding zone. This boundary is not visible equally well in every Hydra strain. It marks a decrease in the diameter of the upper body column and the appearance of big, translucent ecto- and entodermal cells. (v) The peduncle -basal disc boundary, where proliferating ecto- and endodermal epithelio-muscular cells differentiate into mucous-secreting basal disc cells. (vi) In bud-producing animals, the region from which parent tissue is recruited into the bud protrusion is separated from the parent animal by a morphologically hardly visible boundary (green line in Fig. 1A), When the bud has finished its growth, this boundary becomes very sharp and defines the position, where the bud foot forms and complete separation from parent tissue occurs.

Due to the tissue dynamics of *Hydra* and the stem cell properties of its three cell lineages, patterning signals are constantly active in the adults. This obviously includes permanent signalling at the tissue boundaries, and it causes the almost unlimited capacity of *Hydra* for regeneration. During budding and regeneration of adult polyps boundaries have to be formed *de novo*.

In the following, we will summarize which marker genes and signalling components are expressed at the major tissue boundaries (and compartments) in *Hydra* and what is known about boundary-related signalling pathways.

Head and upper body region

The *Hydra* head consists of a hypostome and mouth opening in its center plus a whorl of tentacles below. Specific gene expression domains surrounding the hypostome can be observed in only a couple of cells at the very tip, in broader rings or in the whole region between mouth opening and the lower tentacle border. Fig. 1B and Fig. 2 give an overview of expression zones within the Hydra head and body column for genes expressed in epithelial cells of the ecto- and entodermal layer as well as, in one case (*HyDkk*) in gland cells. These gene expression patterns in adult polyps suggest an astonishingly complex regionalization of the Hydra body, which contrasts the morphological simplicity of the animals.

Since expression in stage 9 of bud development mostly corresponds to the pattern found in adult polyps, the following also referes to patterns shown in Fig. 2 for bud development.

At the very tip of adult polyps and buds, *HvPKC2*, an ε-type PKC encoding gene (Hassel *et al.,* 1998) and several Wnt pathway genes are strongly expressed (Lengfeld *et al.,* 2009). The smallest expression zone is covered by *Wnt3* (Fig. 2), which encompasses only 8-9 cell diameters and is present in both, the ecto- and the endodermal epithelium. Slightly beyond this *Wnt3* zone reaches expression of the Wnt-targeted transcription factor, HyTcf (Hobmayer *et al.,* 2000). An even larger area including the entire hypostome and almost touching the bases of tentacles is covered by *HyBra* expression (Technau and Bode 1999), its expression domain overlaps with *HyWnt7* (Lengfeld *et al.,* 2009) and transcription factors like *budhead* and *prdl-a* (Martinez *et al.,* 1997; Gauchat *et al.,* 1998; Takahashi *et al.,* 2005). The region where hypostome cells proliferate is marked by the expression of *budhead*, which is lacking underneath the proliferative region, but is present again in the space between the tentacles.

Especially striking is the expression of the transcription factor *goosecoid* (*Cngsc*, Fig. 2). It is found only in non-tentacle cells and the apical-most hypostomal cells, thus comprising a two-stripe pattern (Broun *et al.,* 1999). These stripes mark morphologically invisible boundaries and seem to correspond to the expression borders of *Wnt* genes.

At the boundary between tentacles and body column (Fig. 1) proliferating epithelial tissue impinges on tissue made of non-proliferating battery cells. These huge cells have transdifferentiated from body column epithelio-muscular cells and take up mature nematocytes (stinging cells) by transendocytosis.

Genes, which demarcate the basal end of the tentacles and thus the boundary between these two tissues include *HyDsh*, *HvWnt8* and *Hmfz2* (Fig. 2) (Minobe *et al.,* 2000; Philipp *et al.,* 2009). A role for non-canonical Wnt in mediating tentacle evagination has been shown recently. Co-expression of *BMP5-8b* moreover indicates a localized role of BMP and Wnt signalling, which might be complex. Strong expression of a *Hydra* Chordin-like-encoding gene (*HyChdl*, Fig. 1B), a putative antagonist of BMP-signalling, furthermore suggests fine-tuned regulation (Reinhardt *et al.,* 2004; Rentzsch *et al.,* 2007). Potential transcriptional regulators at this boundary are the transcription factors CnOtx, HyAlx, Budhead and Prdl-a (Martinez *et al.,* 1997; Gauchat *et al.,* 1998; Smith *et al.,* 1999; Smith *et al.,* 2000).

Unclear is the function of the two epitheliopeptides Hym301 and Pedibin (Takahashi *et al.,* 2005), the role of the RTK Lemon in endodermal basal tentacle cells and in a broader region PKC1 (Hassel *et al.,* 1998; Miller and Steele 2000), as well as that of the *Hydra*-specific unusual RTK Sweet Tooth, which is characterised by an extracellular lectin domain (Reidling *et al.,* 2000). The whole tentacle region is demarcated by scattered expression of the transcription factor Cnox3 in epithelial cells (Gauchat *et al.,* 2000). The endodermal tentacle tissue expresses the *Hydra* metalloprotease, HMMP, (Fig. 2), which is only weakly expressed in the endoderm of adjacent non-tentacle tissue (Leontovich *et al.,* 2000; Sarras 2012).

In the tissue below the tentacles towards the body column, cells are proliferating. Whole-mount BrdU-labelling (Holstein *et al.,* 1991) as well as the upper expression border of *Gata*, a transcription factor (Nakamura *et al.,* 2011), mark this boundary.

In summary, the boundaries between tentacles, tentacle zone and hypostome are clearly marked. The apical end of the polyps contains several molecular boundaries surrounding the mouth opening and exemplified by the *Cngsc* stripes of expression (Fig. 2). The target genes for the transcription factors Cngsc, HyAlx, CnOtx and Cnox3, remain to be elucidated.

Upper body – *de novo* establishment

When a new head is formed by regeneration, the hypostome-specific genes *Wnt3a* and *HyBra* are initially expressed together with tentacle-specific and boundary-specific genes like *HMMP, HyAlx* and *Cngsc* in a diffuse cap covering the regenerating tip (Broun *et al.,* 1999; Smith *et al.,* 2000). Only at later stages their expression zones become separated - particularly conspicuous in the case of *Cngsc*. It is interesting to note that the two *Cngsc* expression domains in contrast develop successively during budding (see below). Separation of expression domains from a uniform early field requires mechanisms for *de novo* formation of the gene expression boundaries in the head. Preliminary results suggest that Notch signalling is involved in this process (Münder *et al.,* in preparation).

Lower body region

The basal end of *Hydra* where proliferating peduncle cells and non-proliferating differentiated basal disc cells meet shows a clear morphological and molecular boundary between basal disc and peduncle (Fig. 1B). Expression of the receptor tyrosine kinase *shin-guard*, the paired like homeobox gene *manacle* and the *Hydra* insulin receptor homolog *HTK7* (Steele *et al.,* 1996; Bridge *et al.,* 2000) as well as *anklet*, a gene encoding a novel, secreted perforin-EGF-domain protein (Amimoto *et al.,* 2006) define this boundary by a narrow expression zone.

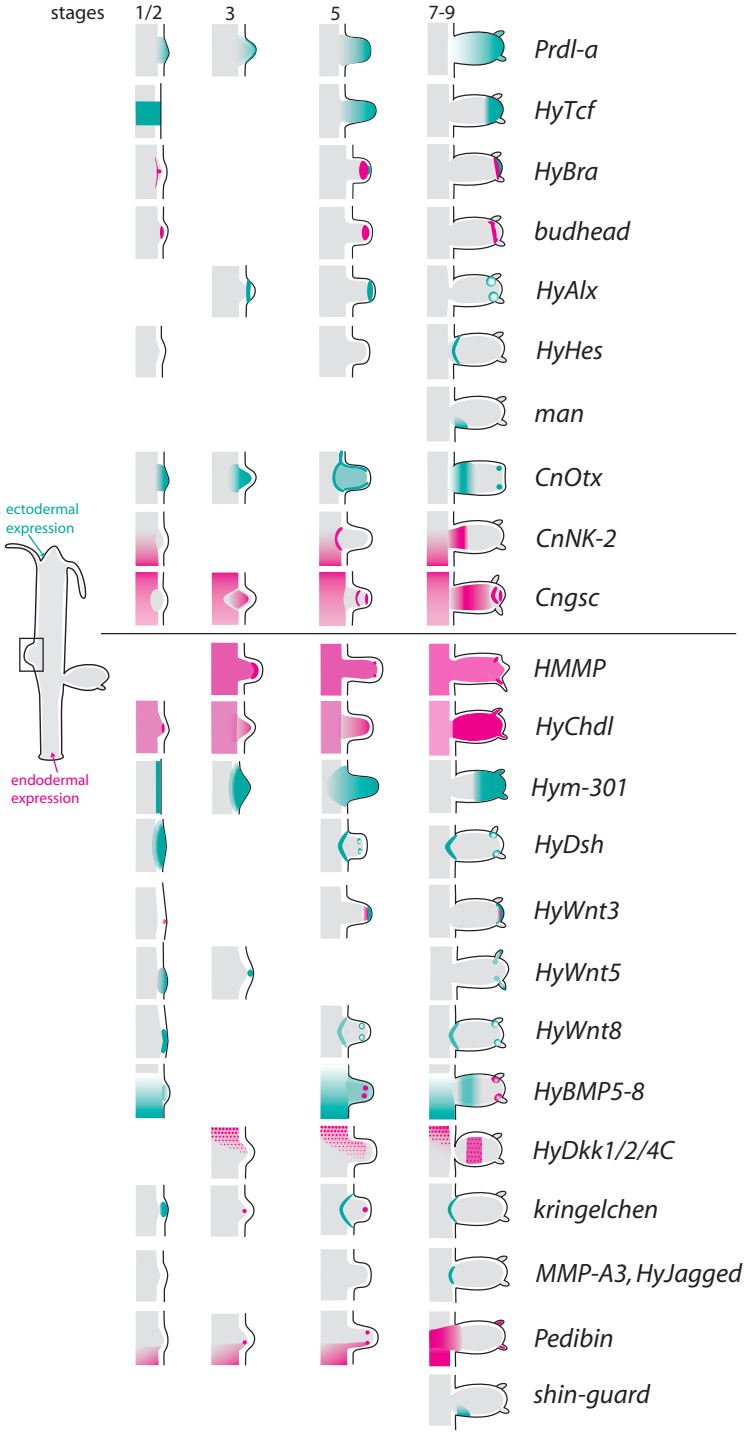

Fig. 2. Schematic representation of expression domains and boundaries in the developing bud. *The upper part summarizes transcription factor expression domains, the lower one those of (diffusible) signalling elements, receptors and proteases.*

As expected for the transition zone towards a terminally differentiated zone, the peduncle - basal disc boundary is marked also by gene expression regions, which are either excluded from the basal disc or expressed exclusively there. *PPOD1*, for example, is restricted to the basal disc (Fig. 1B) and *PPOD2* appears in cells directly adjacent to peduncle cells (Hoffmeister-Ullerich *et al.,* 2002; Thomsen and Bosch 2006). Moreover the basal disc has a very high level of peroxidase activity, which also clearly separates it from adjacent peduncle cells (Hoffmeister and Schaller 1985). Recent results have shown that PPODs encode extracellular lectins and not peroxidases (Pauly *et al.,* 2007) (Böttger *et al.,* in preparation). Like in the hypostome tip, *HvPKC2* expression, is found in a small group of cells in the central basal disc endoderm (Hassel *et al.,* 1998), indicating that this PKC is a good marker for the extreme positional values in the Hydra body (Fig. 1B).

Excluded from the basal disc but expressed in and above the peduncle are the genes *sweet tooth*, encoding an atypical RTK (Reidling *et al.,* 2000), the serine-threonine kinase *HvPKC1* (Hassel *et al.,* 1998), *BMP5-8b* (Reinhardt *et al.,* 2004) and *Frizzled* (Minobe *et al.,* 2000). *pedibin*, encoding a foot-formation-promoting peptide, *CnNK2* and *BMP5-8b* show expression fading out towards the budding region right above the peduncle (Grens *et al.,* 1999; Hoffmeister-Ullerich 2001; Reinhardt *et al.,* 2004).

In short distance above the sharp peduncle-basal disc boundary, a morphologically unrecognizable border is revealed (Fig. 1B) by the expression boundaries of *Gata, Hym323* and *Cnox1* (Gauchat *et al.,* 2000; Takahashi *et al.,* 2005; Takahashi and Fujisawa 2009; Nakamura *et al.,* 2011). This diffuse boundary zone seems to correlate with the lower end of the proliferation zone.

A little higher up the body column in the mid-body region, expression domains of the upper and in the lower body half meet as exemplified by *CnNK2, Pedibin, HyBMP5-8b, prdl-a* and *HyDkk1/2/4C* expression (Grens *et al.,* 1996; Gauchat *et al.,* 1998; Hoffmeister-Ullerich *et al.,* 2002; Reinhardt *et al.,* 2004; Augustin *et al.,* 2006) (Fig. 2). This border is, again, not a sharp one, nevertheless, the region is extremely interesting with respect to morphogenesis: the budding region (see below) is located here. *HyTcf*, the Wnt target transcription factor, marks the budding-competent girdle shortly before a bud begins to evaginate. Increasing expression of the transcription factor *prdl-A* (Gauchat *et al.,* 1998) and the anti-Wnt *HyDkk1/2/4C* (Augustin *et al.,* 2006) demarcate the region as well.

Lower body region – *de novo* establishment

During foot regeneration, the boundary separating basal disc and peduncle has to be formed the novo. *Manacle* is initially expressed in a diffuse cap formed by the regenerating tissue and its expression is only later restricted to the boundary between basal disc and peduncle. At the same time *shin-guard*

expression is initiated precisely at this border region (Bridge *et al.,* 2000). *PPOD1* and *HvPKC2*, which will later be restricted to different regions in the basal disc, are also expressed very early at the tips of foot regenerates. The same is true for *HyBMP5-8b*, which in contrast to the other two genes is excluded from basal disc cells in mature feet. Thus, similar to head regenerates, genes, which belong to different parts of the final foot, are co-expressed in early regenerating feet and separated only later.

Budding

During budding continuously proliferating tissue of the body column is recruited into the bud. The initially observed thickening of the ectoderm can be noticed morphologically as a placode. By ectodermal India ink labelling it became obvious that *Hydra vulgaris* tissue marking a concentric ring on the parent animal is recruited: cells in the centre of this ring were shown to later become hypostome whereas cells at the outer circle end up at the basal end of the bud (Otto and Campbell 1977). Except for the ectodermal cells right above or below the bud, all other cells in this region have to change the orientation of muscle fibres. At final stages of this process the bud base constricts and forms a basal disk. Its connection with the parent becomes increasingly smaller until it detaches.

A defined morphological boundary between the early bud and parent cells cannot be observed. However, before first signs of evagination are visible, a molecular boundary defines the budding-competent zone (Fig. 1B, Fig. 2): the Wnt pathway genes *HyTcf* and β*-catenin* are expressed in a broad band covering the region were buds will evaginate (Hobmayer *et al.,* 2000). Once bud evagination begins, head specific genes including *Wnt3a* and *HyBra* are expressed in the centre of the protruding placode (Technau and Bode 1999). Tentacle specific genes are also expressed, but they either only appear transiently (*HyAlx*) or extend in concentric zones around the centre (*budhead, HyDsh, Hmfz2, HyWnt8* and *HMMP* (Martinez *et al.,* 1997; Leontovich *et al.,* 2000; Smith *et al.,* 2000; Siebert *et al.,* 2005; Philipp *et al.,* 2009).

Almost the whole region from which cells will later be found in the bud is marked by expression of *CnOtx, prdl-a* and *Hym301* (Fig. 2) at budding stage 3 (Gauchat *et al.,* 1998; Smith *et al.,* 1999; Takahashi *et al.,* 2005). However, the boundary between early *Cnotx-, prdl-a-* and *Hym301*-positive and -negative tissue is not very sharp. These genes roughly mark the region of the bud's body column, which will be defined after completion of bud morphogenesis through sharp boundaries apically towards the tentacles and basally towards the foot tissue. At stage 7-8, when morphogenesis begins, *CnOtx* clearly marks the body-tentacle boundary as well as the basal end of the bud in relatively broad stripes of gene expression.

The homeobox gene *CnNK-2*, is expressed below the budding zone in adult animals and also at the basal end of progressed bud stages (Fig. 1B, Fig. 2). It is conspicuously absent from the early bud. This indicates that the basal tissue of a bud is not defined early on. *CnNK-2* is actually only upregulated in the last set of endodermal cells, which migrates into the bud around stage 6 and ends up in the stalk and basal-most cells of the bud. It appears that a sharp boundary of the bud towards the parent is not clearly determined at the beginning of budding (Siebert *et al.,* 2005). It rather forms late at budding stage 7-8.

The earliest gene demarcating the parent-bud boundary is the *Hydra* FGFR *kringelchen*, which becomes upregulated in a ring, 5-6 cells broad and surrounds the bud base from stage 4 onwards. *kringelchen* marks varying populations of cells while they pass this boundary rather than a certain group of cells. Only late during bud formation, around budding stage 7-8, *kringelchen* expression is refined into a sharp ring which appears to indicate the boundary separating bud and parent tissue. At this point a number of additional genes change their expression zones from diffuse rings into sharp lines. These include *HyDsh and HyWnt8* (Philipp *et al.,* 2009), which are most probably expressed adjacent to each other. Just when these sharp lines of gene expression are produced, *HyHes*, encoding a target transcription factor of the Notch signalling pathway, is expressed transiently in a single-cell band immediately adjacent to *kringelchen.* At the same time the metalloprotease MMP-A3 is expressed in the *kringelchen* positive cells (Münder *et al.,* 2010). From now on, *kringelchen, MMP-A3, HyDsh* and *HyJagged* expression are restricted to parent tissue whereas foot specific genes are expressed in the newly forming bud peduncle (Sudhop *et al.,* 2004; Prexl *et al.,* 2011). Interestingly, *HyWnt*-8 is expressed in cells confining the peduncle at the side of the bud (Philipp *et al.,* 2009).

These data strongly suggests that Notch/Wnt and FGF- signalling pathways are involved in the formation of the parent-bud boundary.

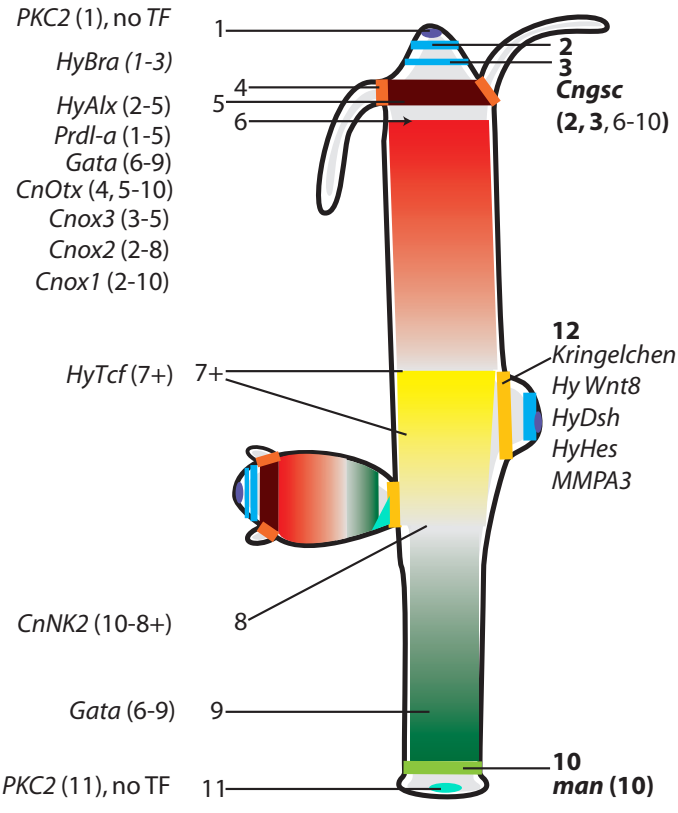

Fig. 3. Summary of epithelial gene expression domains and defined boundaries in *Hydra*. *All boundaries identified by either a sharp line of marker gene expression (right side) or putative boundaries deduced from differential expression zones were numbered. The numbers in brackets indicate, at or between which of these boundaries certain transcription factors are expressed.*

FGFR/Notch signalling controls the establishment of the bud-parent boundary

Support for the hypothesis that FGFR/Wnt/Notch signalling are essential for the establishment of the parent-bud boundary comes from pharmacological inhibition experiments. Inhibition of FGFR-signalling with SU5402 and Notch-signalling with DAPT perturbs bud morphogenesis (Sudhop *et al.,* 2004; Münder *et al.,* 2010). Both inhibitors cause the expression zones of *kringelchen* and *HyDsh* to completely diffuse. As a consequence the buds fail to form feet and never detach, which yields two-headed, so-called Y-animals. Interestingly, such animals can also be obtained when Src (Fabila *et al.,* 2002) and other potential targets for FGFR signalling are inhibited. Moreover, it is also seen as a result of LiCl treatment, which may increase either Wnt-signalling by inhibition of GSK-3β or impair PKC/inositol phosphate/calcium signalling and thereby disturb the boundary by shifting the balance of gene expression (Hassel *et al.,* 1993; Fabila *et al.,* 2002). This hypothesis has not been tested experimentally, yet. The expression data (and pharmacological inhibition) clearly show that boundary formation during budding in *Hydra* relies on Notch and FGFR signalling. Deduced from co-expression data it potentially also involves crosstalk with non-canonical Wnt signalling and HyWnt8.

In summary, a very likely candidate to establish the bud-parent boundary is, like in many other animals, the Notch pathway. Hydra has all major components for bona fide Notch signalling including a well conserved Notch receptor (HvNotch), a putative Notch ligand HyJagged (Prexl *et al.,* 2011), components of the γ-secretase complex including presenilin, metalloproteases of the ADAM family (Kasbauer *et al.,* 2007; Münder *et al.,* 2010). Genes encoding Su(H) (CBF) and the transcriptional repressor HyHes have been identified as well as modulators of Notch signalling such as Fringe and Numb. It was also shown that the signalling mode in the Notch pathway, which is characterised by a regulated intra-membrane proteolysis mediated by metalloproteases of the ADAM family and presenilin, is conserved. Su(H) binding sites have been identified in the HyHes promoter and it was demonstrated that this promoter responds to NotchICD. Finally, the presenilin inhibitor DAPT is able to inhibit Notch-signalling in hydra cells by blocking the release of NICD from the membrane. Using this inhibitor a role for Notch signalling in interstitial stem cell differentiation was demonstrated (Kasbauer *et al.,* 2007).

What about ephrin signalling?

Genes encoding ephrin receptors have only recently been identified in the genome and as ESTs in Hydra (Reddy *et al.,* 2011). Unpublished results suggest that two of these genes are expressed at the base of the Hydra bud. Moreover, two genes encoding ephrins have also been found, but functional data are not available yet (Tischer and Böttger, unpublished). It will be very interesting to see how and where these molecules act in an organism, which lacks a blood circulation system and in which the nerve net is not visibly centralized.

General considerations and perspectives

The simple *Hydra* body is subdivided by a few, morphologically distinguishable, boundaries in the head and foot region. On a molecular level a complex sub-regionalization and additional borders become visible (Fig. 3).

Sharp boundaries in adult *Hydra* are characterised by mutually exclusive gene expression on either side. Examples are *Cngsc* in the hypostome (boundaries 2 and 3 in Fig. 3), *manacle* and *PPOD* at the basal disc (boundary 10) and *kringelchen/HyHes* at the buds base (boundary 12). When the corresponding boundaries are formed de novo, in each case, an initial overlap of gene expression zones, which are later separated, is observed. This sequence of events appears as a particularly simple case of creating tissue boundaries.

Differential expression of signalling molecules including BMP-5-8b, Wnt-8 and receptors for FGF and insulin indicate that these molecules might either define intersections for boundary formation or organise the tissue on either side of the boundaries. In agreement with such a view, the transient expression of *HyTcf* in the budding competent girdle (border region 7+ in Fig. 3) might depend on transiently forming intersections. Notch and FGFR signalling are clearly involved in forming the sharp boundary between bud and parent (Münder *et al.,* 2010; Prexl *et al.*, 2011), a role of ephrin signalling is likely.

In summary, the molecular pathways which are used in all higher animals to form tissue boundaries during development are not only present in prebilaterian animals but have already been recruited for similar tasks. The indicated simplicity of the *Hydra* system provides an ideal background to further elucidate the cellular and molecular mechanisms controlling the separation of initially identical (embryonic) tissue domains, which is the prerequisite to later form structures of high complexity.

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