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β-Catenin acts in a position-independent regeneration response in the simple eumetazoan *Hydra*

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ABSTRACT

Wnt/β-Catenin signaling plays crucial roles in regenerative processes in eumetazoans. It also acts in regeneration and axial patterning in the simple freshwater polyp *Hydra*, whose morphallactic regenerative capacity is unparalleled in the animal kingdom. Previous studies have identified *β-catenin* as an early response gene activated within the first 30 min in *Hydra* head regeneration. Here, we have studied the role of β-Catenin in more detail. First, we show that nuclear β-Catenin signaling is required for head and foot regeneration. Loss of nuclear β-Catenin function blocks head and foot regeneration. Transgenic *Hydra* tissue, in which β-Catenin is over-expressed, regenerates more heads and feet. In addition, we have identified a set of putative β-Catenin target genes by transcriptional profiling, and these genes exhibit distinct expression patterns in the hypostome, in the tentacles, or in an apical gradient in the body column. All of them are transcriptionally up-regulated in the tips of early head and foot regenerates. In foot regenerates, this is a transient response, and expression starts to disappear after 12–36 h. ChIP experiments using an anti-*Hydra*Tcf antibody show Tcf binding at promoters of these targets. We propose that gene regulatory β-Catenin activity in the pre-patterning phase is generally required as an early regeneration response. When regenerates are blocked with iCRT14, initial local transcriptional activation of *β-catenin* and the target genes occurs, and all these genes remain upregulated at the site of both head and foot regeneration for the following 2–3 days. This indicates that the initial regulatory network is followed by position-specific programs that inactivate fractions of this network in order to proceed to differentiation of head or foot structures. *brachyury1* (*hybra1*) has previously been described as early response gene in head and foot regeneration. The HyBra1 protein, however, appears in head regenerating tips not earlier than about twelve hours after decapitation, and HyBra1 translation does not occur in iCRT14-treated regenerates. Foot regenerates never show detectable levels of HyBra1 protein at all. These results suggest that translational control mechanisms may play a decisive role in the head- and foot-specific differentiation phase, and HyBra1 is an excellent candidate for such a key regulator of head specification.

1. Introduction

The capacity to rebuild lost body parts by regeneration is present in many metazoan phyla, particularly in ancestral taxa including cnidarians (Brokes and Kumar, 2008; Layden et al., 2016; Bradshaw et al., 2015). Regeneration was first described in the cnidarian freshwater polyp *Hydra* by Abraham Trembley (1744), and since then *Hydra* has been one of the classic regeneration models. The capacity of regeneration in *Hydra* is almost unlimited. Tiny pieces of excised tissue regenerate complete polyps, and even dissociated cell suspensions self-organize and rebuild normal individuals (Gierer et al., 1972). Head or foot removal results in regeneration of the missing structure along the original axial polarity. Several comprehensive reviews discuss these features in detail (Bode, 2003; Holstein et al., 2003; Fujisawa, 2003; Bosch, 2007; Galliot, 2013). A recent report provides striking evidence that the spatial organiza-

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tion of the polyp´s actin cytoskeleton provides polarity information during regeneration (Livshits et al., 2017).

The basis for regeneration in *Hydra* is the developmental capacity and stemness of epitheliomuscular cells that build the polyp's double layered body wall (Marcum and Campbell, 1978; Sugiyama and Fujisawa, 1978). These cells continuously undergo self-renewal by mitotic division and differentiate head- or foot-specific epithelial cells as required during regular tissue turnover and regeneration. Within the first hour of *Hydra* regeneration, injury signals initiate wound healing and diverse cellular responses (Kobatake and Sugiyama, 1989). Endodermal epithelial cells at the cutting site partially lose their epithelial configuration and first seal the wound, while ectodermal epithelial cells flatten and slide over the endodermal tissue to rebuild the epidermal bilayer (Mattes, 1925). This re-organization of tissues at the wound site involves rapid development of septate and gap junctions (Bibb and Campbell, 1973; Wood and Kuda, 1980a, 1980b; Seybold et al., 2016) as well as re-synthesis of mesoglea, that instantly retracts from sites of cutting (Sarras, 2012). While regeneration can occur without cell division ("morphallaxis"; Cummings and Bode, 1984), head removal results in an immediate block and later up-regulation of mitosis (Holstein et al., 1991). Another wounding response proposed is a rapid release of head or foot inhibition at the amputated site (MacWilliams, 1983a; MacWilliams et al., 1970). In head regenerates, head activation and axis inducing capacity then rises gradually to reach maximal levels at about 12 h (MacWilliams, 1983b). A molecular pre-patterning program emerges in the regenerating tissue in this phase of rising activation potential, and this program sets the stage for the subsequent differentiation of head and foot structures. Complete regeneration into fully intact polyps is finished after about 4–5 days.

In comparison to *Hydra* foot regenerates, head regenerates have been comparatively well studied. Candidate gene and recent systematic transcriptome and proteome studies have led to accumulating data on the molecular dynamics of *Hydra* head regeneration. Unknown injury signals first stimulate a wound healing program including immune and stress response factors (Manuel et al., 2006; Wenger et al., 2014; DuBuc et al., 2014; Petersen et al., 2015). Then, the pre-patterning program is established within the period between 0.5 and 12 h by activating signaling cascades, protein kinases, and transcription factors (Arvizu et al., 2006; Petersen et al., 2015). The roles of most of the signaling pathways is not well understood, and they need to be functionally analyzed in more detail in order to decipher their detailed functions and interactions in the pre-patterning gene regulatory network.

Wnt/β-Catenin signaling is regarded to play a key role at various stages of head regeneration, but particularly in the pre-patterning phase (Hobmayer et al., 2000; Guder et al., 2006; Chera et al., 2009; Lengfeld et al., 2009; Petersen et al., 2015). It also plays a central role in setting up and maintaining the head organizer in intact polyps, which represents the major inductive signaling center responsible for patterning the polyp´s body axis (Hobmayer et al., 2000; Broun et al., 2005; Gee et al., 2010; Bode, 2011; Petersen et al., 2015). Genes encoding Wnt pathway components are early response genes in head regenerates (Hobmayer et al., 2000; Petersen et al., 2015). Failure to regenerate a head in *Hydra* reg-16 mutants is correlated with a failure to activate Wnt pathway genes (Hobmayer et al., 2000). Two mechanisms have been discussed, how Wnt/β-Catenin signaling may be activated in early head regenerates. First, apoptotic cells in the wound secrete Wnt ligands that activate and stabilize the pathway (Chera et al., 2009; Vriz et al., 2014). Induction of apoptosis results from an injury-induced activation of a MAPK/CREB pathway (Kaloulis et al., 2004; Chera et al., 2011; Galliot, 2013). This mechanism is restricted to head regenerates, and does not occur during early foot regeneration. Second, injury-induced modulation of casein kinases activates *β-catenin* transcription, which in turn activates autocatalytic Wnt production (Nakamura et al., 2011; Petersen et al., 2015).

Injury response and wound healing in *Hydra* regenerates are regarded to rest upon common mechanisms irrespective of the position of the wound. Transcriptome data from *Nematostella* polyps regenerating oral and aboral structures support this view (Schaffer et al., 2016). However, molecular pre-patterning during head and foot regeneration in *Hydra* is generally considered to be orchestrated by different gene regulatory networks. Wnt/β-Catenin signaling is viewed as a head-specific regulator, in spite of a previous observation that β-Catenin is also transcriptionally up-regulated during foot regeneration (Happel, 2000). In this report, we have focused on the role of β-Catenin in *Hydra* regeneration. Both, loss-of-function experiments by using the small molecule inhibitor iCRT14 (inhibitor of Catenin responsive transcription 14) and gain-of-function experiments using a β-Catenin over-activating transgenic strain (β-cat-Tg) demonstrate that β-Catenin is required for both head and foot regeneration. *β-catenin* and several β-Catenin regulated genes are part of a cassette of genes transcriptionally activated in the tip of head and foot regenerates during the pre-patterning phase. Furthermore, our data provide evidence that β-Catenin acts in the transition from pre-patterning to differentiation of terminal head- and foot-specific structures. This involves position-dependent translational regulation of transcription factors, and we propose HyBra1 as one of the key factors for head specification.

2. Results

2.1. β-Catenin is required for head and foot regeneration

We analyzed the effect of β-Catenin on *Hydra* head and foot regeneration by using the small molecule inhibitor iCRT14 known as a potent inhibitor of β-Catenin-Tcf interaction in cnidarians and vertebrates (Suppl. Fig. 1; Gonsalves et al., 2011; Marlow et al., 2013; Watanabe et al., 2014a; Watanabe et al., 2014b). Head and foot regenerates were incubated in various concentrations of iCRT14 immediately after head or foot removal and were then cultured in the presence of inhibitor throughout the entire regeneration process. We found that iCRT14 blocked head regeneration in a dose-dependent manner. Lower doses of up to 2.5 μM caused a substantial reduction of the number of regenerated tentacles as well as their delayed appearance (Fig. 1A). Higher doses from 5 to 20 μM led to an almost total block of tentacle formation at 72 h post amputation (hpa), while control animals had regenerated about five tentacles per polyp by that time (Fig. 1A,B). Notably, all 72 h-regenerates exhibited a rounded cap after head removal. Thus, wound healing had occurred in the early regeneration phase in iCRT14-treated samples.

iCRT14 effects on foot regeneration were analyzed by testing stickiness of the regenerated basal end, by high magnification differential interference contrast (DIC) microscopy, and by peroxidase-staining specific for differentiated basal disc cells. The differentiation time of glue-producing, ectodermal basal disc cells is about 36–48 h in foot regenerates (Hoffmeister and Schaller, 1985). At these time points, we tested iCRT14-treated and control samples for their ability to adhere with their regenerating tip to a glass pipette. Starting with 2.5–5 μM iCRT14, increasing fractions of foot regenerates failed to form adhesive basal discs, and at 10–20 μM iCRT14 almost none of the regenerates could adhere (Fig. 1C). DIC microscopy revealed clear differences in cell morphology in the tip of treated and untreated regenerates. At 48 hpa, iCRT14-treated samples did not exhibit terminally differentiated columnar, vesicle-filled basal disc cells in the ectodermal part of the cap. Their morphology was similar to undifferentiated cells of the body column (Fig. 1D). The non-adhesive phenotype was confirmed by peroxidase-staining, a specific marker of differentiated, glue producing cells of the basal disc. While control regenerates showed a strongly peroxidase-positive basal disc at 48 hpa, there was only faint peroxidase-staining in the cap of treated foot regenerates at this time (Fig. 1E). Taken together, wound healing had occurred in iCRT14-treated foot regenerates, but differentiation of foot structures was obviously blocked by inhibition of nuclear β-Catenin activity.