

Real time dynamics of β -catenin expression during *Hydra* development, regeneration and Wnt signalling activation

ROBERTA IACHETTA¹, ALFREDO AMBROSONE^{1, #}, ALEXANDER KLIMOVICH², JÖRG WITTLIEB²,
GIADA ONORATO¹, ALESSIA CANDEO³, COSIMO D'ANDREA^{3, 5}, DANIELA INTARTAGLIA^{1, ##}, NUNZIA SCOTTI⁶,
MARTINA BLASIO¹, ANGELA TINO¹, ANDREA BASSI^{3, 4} and CLAUDIA TORTIGLIONE^{*, 1}

¹Istituto di Scienze Applicate e Sistemi Intelligenti "E. Caianiello", Consiglio Nazionale delle Ricerche, Pozzuoli, Italy,

²Zoological Institute, Christian-Albrechts-University of Kiel, Kiel, Germany, ³Dipartimento di Fisica, Politecnico di Milano, Milano, Italy, ⁴Istituto di Fotonica e Nanotecnologie, Consiglio Nazionale delle Ricerche, Milano, Italy, ⁵Center for Nano Science and Technology, Italian Institute of Technology, Milano, Italy and ⁶Istituto di Bioscienze e Biorisorse, Consiglio Nazionale delle ricerche, Portici, Italy

ABSTRACT Understanding the dynamic cellular behaviours driving morphogenesis and regeneration is a long-standing challenge in biology. Live imaging, together with genetically encoded reporters, may provide the necessary tool to address this issue, permitting the *in vivo* monitoring of the spatial and temporal expression dynamics of a gene of interest during a variety of developmental processes. Canonical Wnt/ β -catenin signalling controls a plethora of cellular activities during development, regeneration and adulthood throughout the animal kingdom. Several reporters have been produced in animal models to reveal sites of active Wnt signalling. In order to monitor *in vivo* Wnt/ β -catenin signalling activity in the freshwater polyp *Hydra vulgaris*, we generated a β -cat-eGFP transgenic *Hydra*, in which eGFP is driven by the *Hydra* β -catenin promoter. We characterized the expression dynamics during budding, regeneration and chemical activation of the Wnt/ β -cat signalling pathway using light sheet fluorescence microscopy. Live imaging of the β -cat-eGFP lines recapitulated the previously reported endogenous expression pattern of β -catenin and revealed the dynamic appearance of novel sites of Wnt/ β -catenin signalling, that earlier evaded detection by mean of *in situ* hybridization. By combining the Wnt activity read-out efficiency of the β -catenin promoter with advanced imaging, we have created a novel model system to monitor in real time the activity of *Hydra* β -cat regulatory sequences *in vivo*, and open the path to reveal β -catenin modulation in many other physiological contexts.

KEY WORDS: *Hydra*, live imaging, Wnt, β -catenin reporter, light sheet fluorescence microscopy

Wnt signalling is a highly conserved signalling pathway that plays crucial role in the animals by controlling the embryonic development, tissue homeostasis and stem cell maintenance in all metazoans (Lim and Nusse, 2013). As expected, disruption or aberrant activation at any level of this complex pathway can lead to developmental abnormalities and diseases, including cancer (Anastas and Moon, 2013, Barker and Clevers, 2006). Since the initial discovery, over the last 30 years, biochemical and genetic analyses performed on vertebrate model organisms and cell cultures dissected the pathway in its single components, and identified large families of

Wnt ligands, receptors, co-receptors and modulators (<http://www.stanford.edu/group/nusselab/cgi-bin/Wnt/>). Wnt signals are classified as canonical or non-canonical based on the capacity of the former to induce ectopic axis in *Xenopus* embryos (McMahon and Moon, 1989) and to transform mouse mammary cells (Wong *et al.*, 1994). The canonical pathway is also named the Wnt/ β catenin pathway, due to the central role played by the effector β -catenin

Abbreviations used in this paper: ALP, alsterpaullone; GFP, green fluorescent protein; LSFM, light sheet fluorescence microscopy.

*Address correspondence to: Claudia Tortiglione. Via Campi Flegrei 34, 80078 Pozzuoli, Italy. Tel: +39 0818675306. Fax: +39 0818675126.
E-mail: claudia.tortiglione@cnr.it - web: <http://www.tortiglione.com/nanobiomoleculargroup/Sito/Home.html> -  <https://orcid.org/0000-0003-1447-7611>

#Current address: Department of Pharmacy, University of Salerno, Fisciano, Italy. ##Current address: Telethon Institute of Genetics and Medicine, Pozzuoli, Italy.

Supplementary Material (fifteen figures) for this paper is available at: <https://doi.org/10.1387/ijdb.180092ct>

Submitted: 13 March, 2018; Accepted: 23 April, 2018.

(β -cat) and a destruction complex that controls its stability and activity. Binding of Wnt ligand to receptors of the frizzled family initiates a cascade of events finally resulting in the inhibition of the activity of the destruction complex and β -cat stabilization. Stabilized β -cat accumulates in the cytoplasm and then enters the nucleus where it interacts with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor family, ensuring efficient activation of Wnt signalling target genes (Clevers, 2006, MacDonald et al., 2009).

While biochemical and genetic players of canonical Wnt pathway are well characterized during animal development, their temporal and spatial activation in differentiated cells of adult organisms is strictly controlled by mechanisms that are yet not well understood. The fundamental core of the Wnt pathway (Wnt, Frizzled, and downstream effectors) is evolutionarily ancient and is extant in the early-emerging multicellular animals including Cnidaria, in which it mediates axial patterning (Guder et al., 2006, Holstein, 2012, Lengfeld et al., 2009). In the freshwater polyp *Hydra vulgaris*, the expression of Wnt genes is localized to a small region at the apical tip of the hypostome. This localized expression is critical for setting up and maintaining the primary body axis in intact polyps, and during budding and regeneration processes (Guder et al., 2006, Hobmayer et al., 2000, Lengfeld et al., 2009). In particular, during very early head regeneration, its enhanced expression labels the new developing head structures. Remarkably, other transcriptional components of Wnt/ β -catenin signalling such as Tcf and β -cat are transcriptionally upregulated earlier than Wnt during budding and regeneration (Gee et al., 2010, Hobmayer et al., 2000, Petersen et al., 2015) indicating their involvement in establishing a localized signalling centre. The fundamental role played by β -cat in the gene regulatory network underlying head regeneration has been thoroughly dissected over the last decade (Chera et al., 2009,

Guder et al., 2006, Hobmayer et al., 2000, Lengfeld et al., 2009, Petersen et al., 2015). Within the first 30-60 min after dissection the loss of endodermal cell integrity and the movement of ectodermal cells determinate the wound closure. During this pre-patterning phase, up to 12 h, specific gene networks are activated (Gufler et al., 2018). In particular, nuclear β -cat up-regulates a set of target genes in a position independent manner. Later on, β -cat takes action in the transition from pre-patterning to differentiation of terminal head- and foot-specific structures, controlling at translational level specific transcription factors. The mechanisms underlying activation of pathway components in head regenerates during the pre-patterning phase are still unclear, and may possibly rely on Wnt ligands released by amputated cells (Chera et al., 2009), which activate the pathway or may depend on the modulation of casein kinase which in turn upregulates β -cat and enhances Wnt transcription (Nakamura et al., 2011, Petersen et al., 2015).

While in the hypostome of an adult *Hydra* β -cat is located in the cell nuclei (Broun et al., 2005) a predominantly cytoplasmic β -cat localization is observed in other body regions. It suggests that in contrast to its role as a transcriptional co-factor in the hypostome, β -cat may be involved in other non transcriptional functions, such as in mediating cell-cell adhesion via adherents junction contacts (Hobmayer et al., 2000, Hobmayer et al., 1996).

The dynamic regulation of the β -cat gene expression has been tackled by manipulation techniques, such as treatment with alsterpaullone (ALP), a specific inhibitor of GSK-3 β , (Leost et al., 2000). Treatment of *Hydra* with ALP caused elevated level of nuclear β -cat throughout the body column, whose cells acquired head organizer inducing capacity providing evidence of β -cat role in the head organizer (Broun et al., 2005). These observations, made on fixed samples, provide only "snapshots", from which the entire dynamic processes of regulation can be deduced. This inferior lack

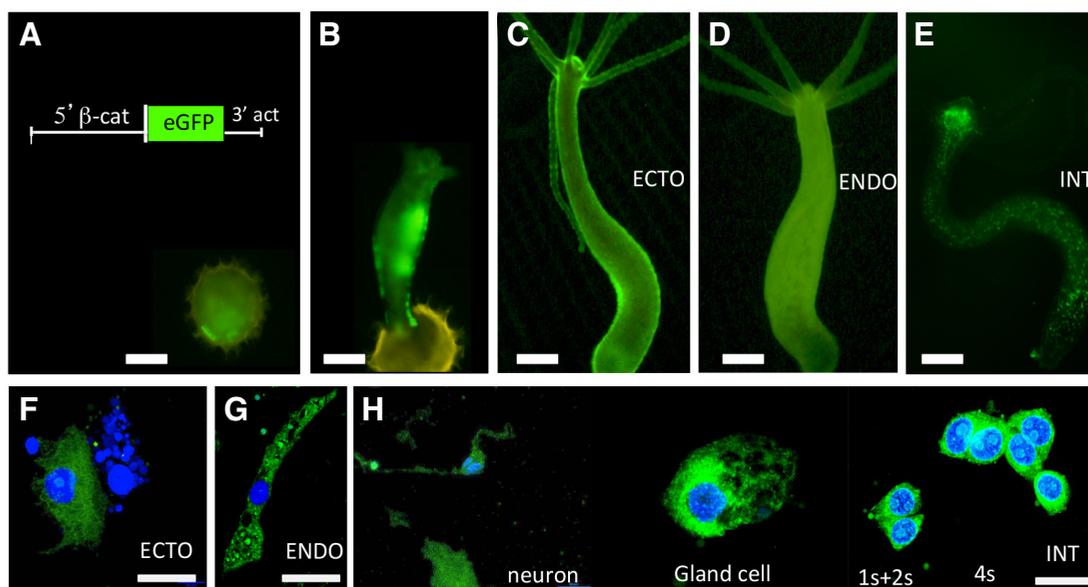


Fig. 1. β -catenin promoter is active during development and in all adult cell lineage. (A) A *Hydra* embryo 2 weeks post injection of the recombinant plasmid shown in the upper part shows diffuse reporter expression, indicating activation of the β -cat promoter during embryo development. Reporter expression continues in the hatched polyp (B), and after clonal propagation founder polyps were selected in which eGFP was restricted in the ectoderm (C), in the endoderm (D), or in the interstitial cells (E). Homogeneous expression of β -cat driven eGFP in single cells obtained from ECTO (F), ENDO (G) and INT (H) lines was analysed by immunostaining on *Hydra* single cell suspensions, using anti-GFP antibody. Fluorescence images were acquired by laser scanning confocal microscopy. Scale bars, 500 μ m, A-E; 20 μ m in F-G; 10 μ m in H.

of temporal resolution urges development of new tools, intended to *in vivo* monitoring the dynamic regulation of gene expression. Transgenic polyps expressing a β -cat fused to GFP under the actin promoter were developed in this direction (Gee *et al.*, 2010). Although constitutive and ubiquitous expression of β -cat under a strong promoter contributed to deciphering β -cat role in the continuous maintenance and activity of the head organizer, it does not provide realistic information on β -cat physiological expression. For this reason here we produced a transgenic reporter *Hydra* expressing the eGFP reporter under the native β -cat promoter, containing all cis-elements sequences to drive expression in different physiological contexts, and exploited it to gain knowledge on β -cat controlled dynamics in living *Hydra* polyps. In order to characterize whole dynamic expression *in vivo*, we used Light Sheet Fluorescence Microscopy (LSFM), a technique that is particularly suitable for the reconstruction of an entire biological sample in 3D with single cell resolution (Santi, 2011). Taking advantage of the high acquisition speed and reduced photo-toxicity of LSFM we could record developmental and regeneration processes in *Hydra* during long-term time-lapse experiments. By monitoring adult homeostasis, budding, regeneration and forced activation of Wnt/ β -cat pathway by chemical compounds, we add new clues on known processes triggered by Wnt/ β -catenin signalling, and open the path to detect β -catenin modulation in many other physiological contexts.

Results

Generation of Wnt/ β -cat reporter *Hydra*

To generate Wnt/ β -cat reporter *Hydra*, a DNA fragment of 1143 bp upstream of the transcriptional start of the β -cat gene, including 21 nucleotides of β -cat coding sequence (Fig. 1A), was cloned into the *pHyVec13* vector (kind gift from R. Steele), upstream and in-frame with the eGFP coding sequence, replacing the actin promoter. Promoter scanning by bioinformatics tool predicts multiple putative transcription factor binding sites within this regulative region (Fig. S1), as recently described in detail (Gufler *et al.*, 2018). Other elements of the construct, including the 3'-flanking region downstream of the GFP coding sequence, remained intact. Before stable transformation, the DNA construct was tested by transient expression using a modified biolistic method (details are in the Supporting Information). eGFP fluorescence (Fig. S2) was detectable 96 h

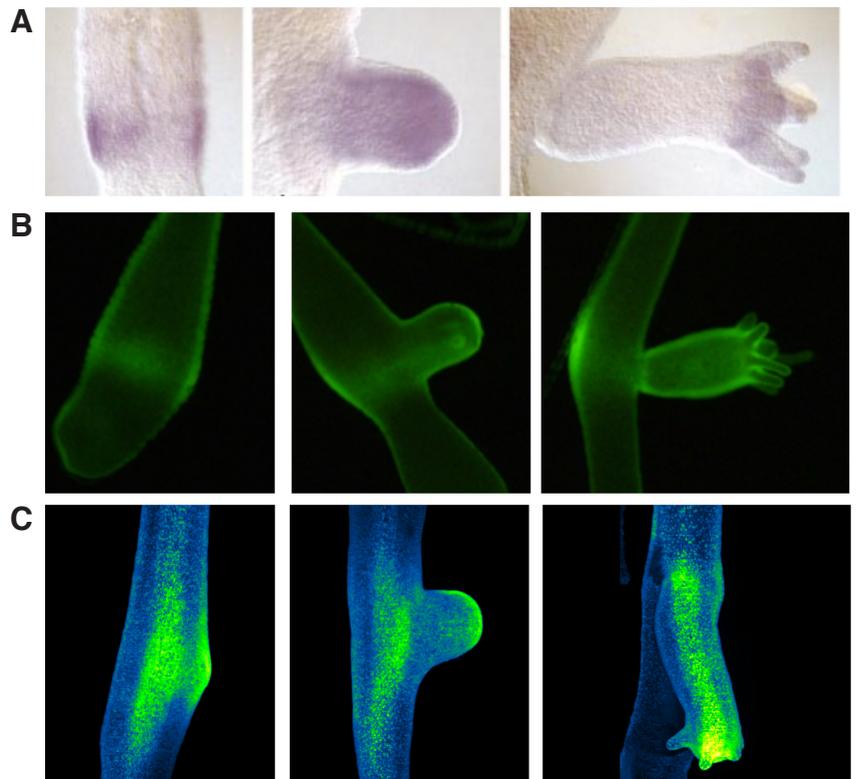
post transfection, restricted to the hypostome, providing functional evidences of the engineered reporter construct in *Hydra* and confirming high Wnt/ β -cat signalling activity in this region.

Genetic transformation of *Hydra vulgaris* strain AEP embryos by microinjection was performed as previously described (Wittlieb *et al.*, 2006). All embryos transfected showed a diffuse fluorescence, reflecting previously detected endogenous β -cat expression throughout embryogenesis (Froebus *et al.*, 2003)(Fig. 1A). Out of 31 injected embryos 11 hatched, were initially mosaic, with patches of eGFP signal observed along the body column (Fig. 1B, Fig. S3, S4). Further, these founder polyps were clonally propagated and selected for enrichment of transgenic cells to form clonal fully-transgenic *Hydra* lines. All polyps demonstrated normal morphology and behaviour, indicating no adverse effects of embryo manipulation or eGFP expression. For further analysis three lines derived from a single founder (#C3) were selected (Fig. 1C-E), expressing eGFP in ectoderm (ECTO), endoderm (ENDO) or interstitial lineage (INT). PCR analysis performed on polyps not expressing eGFP, derived from mosaic founders, showed the absence of exogenous eGFP DNA sequence (data not shown), indicating that the absence of fluorescence was due to the absence of the transgene and not to a position-dependent suppression of the transgene expression.

In fully transgenic ECTO and ENDO polyps reporter gene expression was detected throughout the polyp (Fig. 1C, D), recapitulating the endogenous expression of *Hydra* β -cat (Gee *et al.*, 2010, Hobmayer *et al.*, 2000). Single-cell suspensions obtained by maceration and immunostained with anti-GFP antibody showed homogeneous composition of ECTO transgenic polyps by only eGFP+ ectodermal epitheliomuscular cells, and ENDO polyps by only eGFP+ endodermal cells (Fig. 1F-G). In animals expressing β -cat-eGFP as a weak punctuated pattern (by *in vivo* whole imag-

Fig. 2. β -cat driven eGFP expression *in vivo* recapitulates β -cat expression analyzed by *in situ* hybridization.

In situ hybridization (A) showing β cat expression during budding (Images with permission of (Hobmayer *et al.*, 2000)). In the final stage of budding expression is detected in the apical hypostome. *In vivo* fluorescence imaging (B) of β -cat eGFP-ECTO transgenic polyp-showing β -cat reporter upregulation in the prospective budding zone, and then in the newly formed bud. A circular fluorescent pattern depicts the tentacle emerging domains. Upregulation persists in the new bud, throughout budding process. (C) LSFM Maximum Intensity Projections of stacks acquired during a time-lapse of the budding event. False colours (blue-green) are used to highlight the fluorescence increase in the budding region.



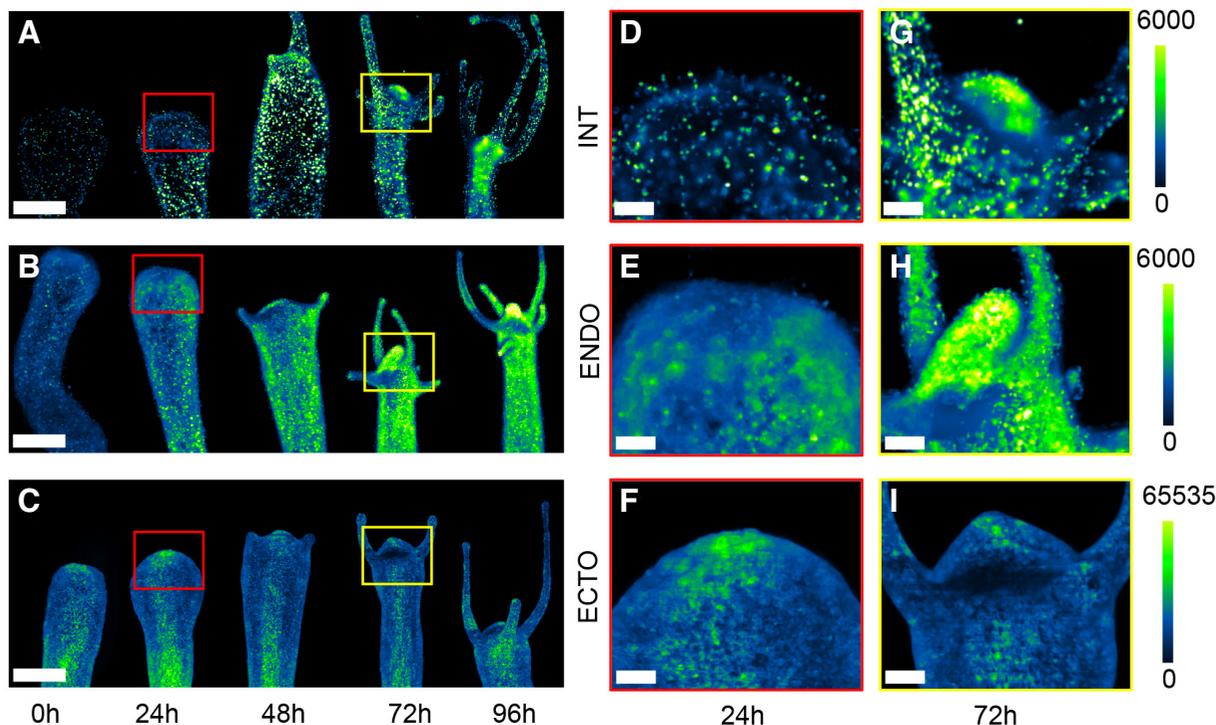


Fig. 3. Time lapse analysis of β -cat driven eGFP expression during regeneration. Activity of β -cat promoter was imaged in each cell lineage by acquiring β -cat-eGFP INT (A), ENDO (B) and ECTO (C) polyp during the whole process of regeneration. Details of the hypostomal region at 24 h and 72 h for INT polyp (D,G), ENDO (E,H), ECTO (F,I). Acquisitions are shown every 24 h from 0 h (right after amputation) to 96 h post amputation. Images are Maximum Intensity Projections of the acquired light sheet microscopy stacks. The images are shown in false colors. The color bar shows the fluorescence intensity in each pixel, indicating that the fluorescence signal emitted by ECTO is an order of magnitude higher than the one emitted by INT and ENDO. Scale bars are 500 μ m in A,B,C and 100 μ m in D,E,F,G,H,I.

ing) (Fig. 1E), immunostaining revealed eGFP expression in all interstitial cell types, i.e. stem cells (1s, 2s) and their proliferating and differentiating derivatives (nematoblast nests, neurons, gland cells (Fig. 1H). In these polyps, referred to as INT, the fluorescence was enhanced in the endodermal hypostomal region (Fig. S5), probably due to the high concentration of gland cells in this region. Supplementary Fig. S6, S7 and S8 show whole animals and single sagittal sections of the acquired stacks of images relative to ECTO, ENDO and INT reporter lines, which provide unequivocal eGFP expression pattern per each cell lineage.

The reporter gene expression pattern, monitored in each line from establishment of transgenesis to date, did not change over time, suggesting the transgene stability and the possibility to study dynamic processes over long periods.

In sum, the created transgenic lines can be used further to monitor *in vivo* the dynamic transcriptional activity of β -cat promoter in different developmental processes independently in three cell lineages.

Wnt/ β -cat signalling during budding

Asexual reproduction in *Hydra* starts with the appearance of an evagination of the body column wall (ca 2/3-rds down the body column length), followed by elongation into a protrusion. After formation of a head with tentacles and foot at the apical and basal ends of the protrusion, the bud detaches. Both canonical and non canonical Wnt signalling participate in the tissue evagination process (Philipp *et al.*, 2009) and β -cat reporter analysis may

reveal important spatiotemporal clues on β -cat driven processes. To this aim, adult transgenic *Hydras* were monitored by fluorescence microscopy throughout the entire budding process. In the ECTO line, enhanced fluorescence expression was detected in a ring-like domain in the forthcoming budding zone, approximately 18-24 h before a tissue evagination becomes evident (Fig. 2B, Fig. S9). The enhanced fluorescence persisted in the emerging bud all along the budding process, and it was maintained until bud detachment, mirroring β -cat expression pattern detected by *in situ* hybridization (Fig. 2A) (Hobmayer *et al.*, 2000). However, reporter gene expression was not limited to the apical region of the bud, but was clearly augmented throughout the body, suggesting extended promoter activity compared to that detected by *in situ* hybridization. A time-lapse acquisition by LSFM was then performed, capturing the whole expression dynamic of the fluorescence signal (Fig. 2C, Fig. S10). The enhancement of the eGFP expression in the gastric region persisted throughout the evagination process, being not limited to the budding region, but extending in the upper and lower regions, and throughout the whole bud length.

In the ENDO line a similar increase in the fluorescence in the budding region was observed, although less pronounced compared to the ECTO line, while in the interstitial line no change in the reporter gene expression was detectable, interstitial cells being thoroughly dispersed uniformly in the growing bud (data not shown). This evidence supports a major role played by β -cat expressing epithelial cells during morphogenesis.

β -cat promoter activity during regeneration

Hydra polyps display a unique developmental plasticity among adult multicellular organisms: after amputation, they regenerate a missing part in few days (3-4 days in optimal culture conditions). The cellular and molecular events displayed by a head-regenerating tip have been exhaustively investigated (Bode, 2003, Bosch, 2007, Buzgariu *et al.*, 2018, Chera *et al.*, 2009, Galliot *et al.*, 2006, Petersen *et al.*, 2015). Here we analysed the full expression dynamic of the β -cat promoter *in vivo*, in regenerating heads of all transgenic lines by LSMF in time lapse experiments. Fig. 3 depicts the β -cat dynamics during regeneration in INT, ENDO and ECTO *Hydra* transgenic lines, from the time of amputation up to 96 h post amputation (p.a). Data were acquired every 40 min and are shown every 24 h. The INT line (expressing reporter gene in interstitial cells and their derivatives) is characterized by single fluorescent cells evenly distributed throughout the body column and numerous fluorescent nerve cells, especially at the foot and peduncle regions (Fig. 3A, Fig. S5). Early stages p.a. show evident changes in the eGFP expressing cells, that are detectable both in intensity and spatial pattern, and are more pronounced in the gastric region (Fig. 3A). At later stages (48 h p.a.) the number of eGFP+ cells greatly increases throughout the whole body column up to the newly patterned hypostome and emerging tentacle buds. The massive contribution of β -cat expressing endodermally-located gland cells in the head regeneration appears evident from 72 h p.a. onwards. Fig. 3A shows enhanced fluorescence in the hypostome at 96 h, progressively decreasing toward the gastric region (Fig. S11). The active involvement of β -cat expressing endodermal cells during regeneration was revealed by the analysis of the ENDO line. The

process of head regeneration of a ENDO polyp is presented in the Fig. 3B. A marked enhancement of an endodermal fluorescence was detectable since the beginning of the process. The signal intensity progressively increases all over the body, reaching the highest level in the hypostomal region, 96 h post amputation.

From analysis of the ECTO line during head regeneration (Fig. 3C), a moderate increase in eGFP+ expressing cells was detected after amputation in the hypostome and at higher extent at the gastric region level, which has not been reported by *in situ* hybridization. Optical sections of each transgenic line performed at the beginning and at the end of time lapse experiments (Fig. S12) show reporter gene expression being restricted to lineage specific cells. Altogether the time-lapse analysis of β -cat-eGFP expressing polyps, transgenic in single cell lineage, highlights the key role of endodermal cells in driving the regenerative process. This is in line with recent identification of β -cat target genes, all expressed in the endoderm and strongly upregulated during the early phase of head and foot regeneration (Gufer, 2018).

eGFP expression is upregulated in response to GSK-3 β inhibition and correlates with β -cat expression in ectopic tentacles

To determine whether the β -cat-eGFP reporter responds to signals that trigger the Wnt pathway, we activated the Wnt pathway by inhibition of the glycogen synthase kinase-3 β GSK3 using ALP (Leost *et al.*, 2000). β -cat reporter animals were treated with ALP for 24 h, and imaged at 24 h, 48 h and 72 h post treatment. In Fig. 4 images from a β -cat-eGFP ECTO polyp clearly show progressive increase of fluorescence throughout the body length and in the tissue organizing the new tentacles. Localised reporter

expression before tentacle protrusion and throughout morphogenesis confirms the role of β -cat in molecular pre-patterning and differentiation of head specific structures. Sagittal section of the acquired stack for the ECTO polyp (Fig. 4 B,C) confirms that the activity of the β -cat promoter is confined to the ectodermal layer. Similarly, in ENDO polyps, eGFP fluorescence remains restricted to endodermal cells, indicating the lineage specific contribute to ectopic tentacle formation (Fig. S13). Time lapse LSMF performed on β -cat-eGFP INT polyps shows a slight increase of reporter expression both in the hypostome and in the body (Fig. S14).

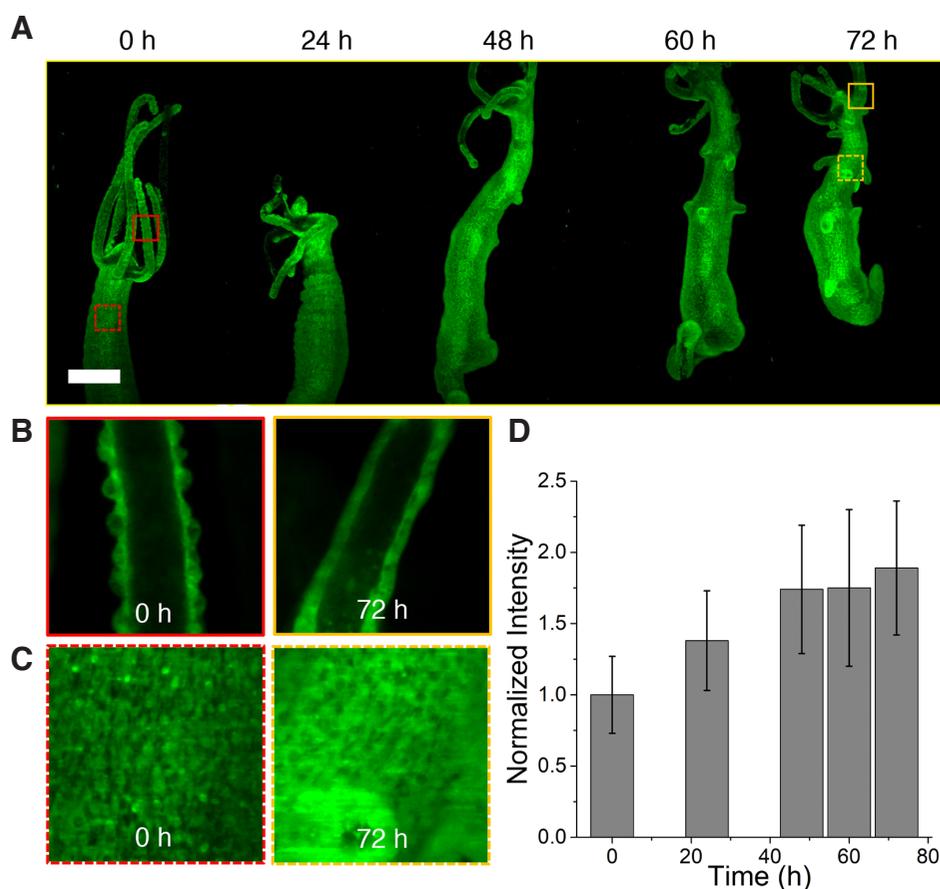


Fig. 4. Temporal dynamics of β -catenin expression during Wnt signalling activation. β -cat-eGFP ECTO transgenic polyps were exposed 24 h to 5 μ M ALP and acquired with LSMF. Images are Maximum Intensity Projections of the acquired light sheet microscopy stacks, at 0 h, 24 h, 48 h, 60 h and 72 h from treatment (A). Details of the tentacle and body regions at the beginning (red squares) and at the end (yellow squares) of the acquisition are shown in (B) and (C), respectively. Fluorescence intensity as a function of time, quantified on the body region of ECTO polyps ($n=3$) after segmentation of the LSMF data (D).

Discussion

We generated fluorescent transgenic β -cat reporter strains of *Hydra* and performed an in depth characterization of its expression in adults during budding, regeneration and pharmacological activation of the Wnt pathway, dissecting the dynamic regulation of β -cat promoter activity in each cell lineage during these events. The reporter construct, comprising a fragment of the β -cat promoter driving eGFP expression, provides spatiotemporal information on endogenous β -cat promoter activity, substantially recapitulating the expression patterns obtained from *in situ* hybridization. eGFP reporter expression was detected in expected sites of β -cat promoter activity, *i.e.* apical tip of emerging buds and regenerating heads and in all cells of lineage-restricted transgenic line (ectodermal, endodermal and interstitial cells, throughout the body) which may account for the second function of β -cat in cell-cell adhesion, described in *Hydra* and other cnidarians (Broun *et al.*, 2005, Hobmayer *et al.*, 2000, Wikramanayake *et al.*, 2003). Additionally, novel sites of β -cat promoter activity were identified depicting the dynamic expression pattern in different physiological contexts. During development of a new bud, β -cat promoter was found active along the whole bud length, gradually extending from the emerging zone up to the bud apical end. During head regeneration the possibility to analyse the three separate lineage expressing lines allowed us to identify the endoderm as the tissue where the β -cat promoter is most active, and culminating at 96 h. These findings agree with those previously obtained in early regenerates by *in situ* hybridization (Gee *et al.*, 2010, Gufler *et al.*, 2018, Hobmayer *et al.*, 2000) showing β -cat up-regulation in the apical hypostome. However, marked differences were found at later stages, when *in situ* hybridization failed to detect β -cat expression, while in the present work β -cat promoter activity is observed up to 96 h, when the differentiation of head structure takes place. The crucial role of endoderm in pre-patterning, but not later phases of regeneration is supported by recent identification of a set of endoderm specific genes (*zebra*, *matrixin*, *cathepsinL 1-like*, *hv100202099*, *bmp-binding like*) controlled by β -cat that may orchestrate the wound healing immediately post-dissection (Gufler *et al.*, 2018). β -cat has been suggested to act also at translational level to dictate specific differentiation programs, although the underlying mechanisms have been not ruled out. Here we show that β -cat promoter activation is not transient, but controls and sustains also late differentiation programs.

The expression pattern of β -cat gene and the combined involvement of β -cat/TCF in the transcriptional activation of Wnt3 in *Hydra* were recently investigated by producing transgenic animals for the eGFP-tagged β -cat gene under the control of the β -cat promoter (Nakamura *et al.*, 2011). In these animals β -cat-eGFP fusion protein was found expressed throughout the body, with nuclear localization in the hypostome. Upon ALP treatment, stabilization of β -cat-eGFP led to increased nuclear accumulation throughout the animal. In the transgenic animals generated in this work, forced activation of Wnt signalling led to the augmented expression of β -cat driven eGFP during pre-patterning and differentiation of ectopic tentacles, confirming the β -catenin central role in Wnt signalling.

It is worth emphasizing that the achievement of additional information was due not only to the possibility offered by transgenic *Hydra* to analyse gene expression in single tissue layers,

but also to the imaging technique adopted. Indeed, using LSMF microscopy, we were able to capture, by continuously imaging the whole samples along time, the dynamic of Wnt signalling activation throughout physiological processes, accurately identifying all major sites of β -cat activity.

Aware of possible misleading interpretations of the observed expression pattern over long periods, due to high stability of eGFP protein, the reporter strains here produced revealed in real time the dynamic expression of β -cat gene in developmental and pharmacological contexts, opening the path to functional studies where β -cat involvement or modulation has not been postulated. Positive and negative regulators acting upstream of Wnt are being identified in vertebrates, and most common functional tests are performed by using luciferase/GFP reporter of β -cat-mediated transcriptional activation, *i.e.* TOPFLASH assays (Veeman *et al.*, 2003), where reporters are driven by multiple copies of TCF binding sites. With the final aim to produce a similar reporter in *Hydra*, we used endogenous β -cat regulatory sequence to drive GFP expression, supported by prediction of regulatory *cis*-elements within this region (Gufler *et al.*, 2018), including the TCF binding site GTTTGAT, *i.e.* TCF-site 3 core previously identified (Nakamura *et al.*, 2011). Considering the central role played by Wnt/ β -cat signalling pathway, including cellular proliferation, differentiation, migration, polarity, and regeneration (van Amerongen and Nusse, 2009), β -cat-GFP reporter lines may be used as ideal genetic background to investigate cross-talk between different signalling pathways, to identify upstream regulators, such as Crypto, Sp5 (Huggins *et al.*, 2017, Morkel *et al.*, 2003) or responsiveness of β -cat transcription to any external chemical or physical stimulation including temperature, feeding regime, presence of microbes. These studies may shed light on how developmental programs are influenced by environment (Bosch *et al.*, 2014), and in which condition these programs are reactivated. This work demonstrated the enhancement of β -cat promoter activity during budding, regeneration and ALP treatment as first functional evaluations. An ongoing study using these lines is aimed to evaluate the action range of Wnt/ β -cat signalling. Indeed, when using ALP for forced activation of Wnt signalling all cells are equally exposed to the drug and ectopic tentacles emerge along the whole body length. What would be an outcome of local delivery of ALP delivery in certain cells? How neighbour cells would respond to Wnt/ β -cat activation and how this effect would be context dependent? In a previous work we produced light responsive microcapsules loaded with ALP and showed their functionality to induce ectopic tentacle formation all over the body (Ambrosone *et al.*, 2016). The reporter lines here produced will provide important clues on systemic effect of Wnt signalling activation in single cells, and on the kinetic of this process, from the time of ALP delivery to GFP appearance and tentacle patterning. Finally, the construct that we engineered may be used as valuable backbone for further genetic manipulations in the context of Wnt/ β -cat -activity.

Materials and Methods

Hydra vulgaris strain AEP polyps were asexually cultured in Hydra medium (1 mM CaCl₂, 0.1 mM NaHCO₃, pH 7) at 18°C and fed three times per week with freshly hatched *Artemia salina* nauplii. For regeneration experiments, 24 h starved polyps were bisected in the upper gastric region and continuously monitored by fluorescence microscopy (see below). Forced activation of Wnt signalling was obtained by treating polyps with

5 μ M ALP solution for 24 hours, as previously described (Broun *et al.*, 2005). Thereafter, polyps were rinsed several times and monitored by fluorescence microscopy.

Generation of transgenic *Hydra*

To generate the β -cat-GFP construct, 1143 bp of β -cat promoter (GenBank no. U38624.1) was amplified from genomic DNA using Platinum High Fidelity Taq polymerase (Invitrogen) and cloned into the BglIII/PstI sites of *pHyVec13* vector (kindly gifted by R. Steel, Irvine), replacing the actin promoter. Primers used for the PCR reaction were: FORW β -CAT: 5'-GGAAGATCTTCTTAGTTATCCGTATATCG-3'; REV β -CAT: 5'-ATGCTGCAGTACCAGTTGAATCCTCCAT-3'. The recombinant construct was sequenced to confirm its structure.

Transient expression of β -cat-eGFP plasmid DNA in *Hydra vulgaris* was performed by biolistic method, as previously described with minor modifications (Scotti and Cardi, 2012). Gold microcarriers (0.6 μ m, BioRad) were coated with plasmid DNA and spread over the center of macrocarrier. DNA delivery was achieved using a PDS-1000/He TM Gene Gun delivery system (Bio-Rad, Hercules, CA, USA). The Petri dish containing the *Hydra* was placed into the target plate holder and then in the third slot (6 cm) from the bottom of PDS chamber. A 1100 psi helium and 27-28 in. of Hg vacuum pressure were used for shooting. After shooting, *Hydra* were maintained in culture and continuously monitored for eGFP expression.

Stable genetic transformation of *Hydra vulgaris* strain AEP embryos was achieved by microinjection as previously described (Wittlieb *et al.*, 2006). Animals were genotyped by PCR. Out of 31 injected embryos 11 hatched, and 7 transgenic founder lines (β -cat-eGFP C3, C2, C6, A1, A2, B3, B2) were established exhibiting similar patterns in each of the cell lineages (ectoderm, endoderm and interstitial cells) but different levels of reporter expression. All lines analysed in this work during budding, regeneration and pharmacological treatment were obtained by clonal propagation of the founder line C3.

Fluorescence immunostaining

To confirm the eGFP cell type composition of each transgenic line immunocytochemistry using anti-GFP antibody was performed on single cells. Fixed *Hydra* cells were first obtained by maceration of β -cat-GFP transgenic polyps as described (David, 1973). Then the macerates were spread on slides, washed and permeabilized with PBS-Tween 0,1% for 1 h. Cells were incubated with a 1:200 dilution of mouse anti-GFP antibody in BSA-T for 3 h at room temperature and after washing incubated with a 1:1000 dilution of Alexa Fluor 488 labelled Goat anti-mouse secondary antibody for 1 h at room temperature. Cells were washed twice in PBS and slides mounted using Vectashield antifade mounting medium with DAPI (Vector Laboratories, Inc. CA).

Wide field imaging

Dynamics of reporter gene expression were analysed by *in vivo* imaging. Initial observations were performed by fluorescence microscopy using an inverted microscope (Axiovert 100, Zeiss) equipped with a digital color camera (Olympus, DP70) and fluorescence filter sets (BP450-490/FT510/LP515). The software system *CellF* (Olympus) was used for imaging analysis. Laser-scanning confocal data were acquired by using Nikon Eclipse Ti2. Pictures were taken by using Nikon A1R digital cameras.

Light sheet fluorescence microscopy

In order to observe regeneration and budding a LSFM setup (Candéo *et al.*, 2016) was adapted to specifically image *Hydra in vivo*. In LSFM the sample is illuminated with a thin plane of laser light and the fluorescence is collected perpendicularly to the excitation axis through a fast CMOS camera, providing optical sectioning. The sample is rapidly scanned through the illumination plane (light sheet), hence a 3D volume is recorded. In details, a single-mode fiber-coupled laser emitting at 478 nm (MBL-FN-473 CNI), is collimated and then focused in order to form a light sheet on the sample through a cylindrical lens ($f = 75$ mm). The optical power of c.a.

6 mW is split into two beams counter-propagating across the sample (Candéo *et al.*, 2016). The fluorescence emitted by the sample is filtered and collected with a 4X objective lens (Nikon, PlanFluor NA=0.13) and a band pass filter, which in combination with a tube lens create an image at the detector (ORCA Flash 4.0, Hamamatsu). The lateral resolution of the system is approximately 2 μ m and the axial resolution (given by the waist of the focused light sheet) is approximately 15 μ m. The sample is placed in a cuvette filled with water and kept in a 0.8 mm diameter Fluorinated Ethylene Propylene (FEP) similarly to the protocol described in (Bassi *et al.*, 2015). Here *Hydra* are not anesthetized and in order to avoid artefacts induced by the movements of the sample, the acquisition time was set to 30 ms/plane, in order to collect an entire LSFM stack (c.a. 150 planes over a thickness of 0.8 mm) in less than 5 s. During time lapse experiments the measurements were repeated every c.a. 40 min. In these conditions photo-bleaching was negligible.

Fluorescence quantification

Data acquired with LSFM are processed in Matlab and visualized with Fiji. Image stacks are automatically segmented, using a threshold (Fig. S15). Then the software computes the average fluorescence intensity on a 50X50X50um volume selected by the user: the sum of the intensity over the entire volume is calculated and divided by the total volume. The voxel outside the segmented volume are excluded from the calculation automatically.

Acknowledgements

Technical assistance of Mrs. L. Sannino (IBBR-CNR, Portici) with biolistic experiment is gratefully acknowledged. We also thank Giuseppe Marino and Ilaria Panico (ISASI-CNR, Pozzuoli) for technical assistance with animal culture. This work was partially supported by Laserlab-Europe (EU-H2020 654148).

References

- AMBROSONE, A., MARCHESANO, V., CARREGAL-ROMERO, S., INTARTAGLIA, D., PARAK, W.J. and TORTIGLIONE, C. (2016). Control of Wnt/beta-Catenin Signaling Pathway *in vivo* via Light Responsive Capsules. *ACS Nano* 10: 4828-4834.
- ANASTAS, J.N. and MOON, R.T. (2013). WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer* 13: 11-26.
- BARKER, N. and CLEVERS, H. (2006). Mining the Wnt pathway for cancer therapeutics. *Nature reviews. Drug discovery* 5: 997-1014.
- BASSI, A., SCHMID, B. and HUISKEN, J. (2015). Optical tomography complements light sheet microscopy for *in toto* imaging of zebrafish development. *Development* 142: 1016-1020.
- BODE, H.R. (2003). Head regeneration in *Hydra*. *Dev Dyn* 226: 225-236.
- BOSCH, T.C. (2007). Why polyps regenerate and we don't: towards a cellular and molecular framework for *Hydra* regeneration. *Dev Biol* 303: 421-433.
- BOSCH, T.C.G., ADAMSKA, M., AUGUSTIN, R., DOMAZET-LOSO, T., FORET, S., FRAUNE, S., FUNAYAMA, N., GRASIS, J., HAMADA, M., HATTA, M. *et al.*, (2014). How do environmental factors influence life cycles and development? An experimental framework for early-diverging metazoans. *Bioessays* 36: 1185-1194.
- BROUN, M., GEE, L., REINHARDT, B. and BODE, H.R. (2005). Formation of the head organizer in *hydra* involves the canonical Wnt pathway. *Development* 132: 2907-2916.
- BUZGARIU, W., WENGER, Y., TCACIUC, N., CATUNDA-LEMOS, A.P. and GALLIOT, B. (2018). Impact of cycling cells and cell cycle regulation on *Hydra* regeneration. *Dev Biol* 433: 240-253.
- CANDEO, A., SANA, I., FERRARI, E., MAIURI, L., D'ANDREA, C., VALENTINI, G. and BASSI, A. (2016). Virtual unfolding of light sheet fluorescence microscopy dataset for quantitative analysis of the mouse intestine. *J Biomed Opt* 21: 56001.
- CHEHA, S., GHILA, L., DOBRETZ, K., WENGER, Y., BAUER, C., BUZGARIU, W., MARTINO, J.C. and GALLIOT, B. (2009). Apoptotic cells provide an unexpected source of Wnt3 signaling to drive *hydra* head regeneration. *Dev Cell* 17: 279-289.
- CLEVERS, H. (2006). Wnt/beta-catenin signaling in development and disease. *Cell* 127: 469-480.

- DAVID, C.N. (1973). A quantitative method for maceration of hydra tissue. *Wilhelm Roux Arch Entwickl Mech Org* 171: 259-268.
- FROBIUS, A.C., GENIKHOVICH, G., KURN, U., ANTON-ERXLEBEN, F. and BOSCH, T.C. (2003). Expression of developmental genes during early embryogenesis of Hydra. *Dev Genes Evol* 213: 445-455.
- GALLIOT, B., MILJKOVIC-LICINA, M., DE ROSA, R. and CHERA, S. (2006). Hydra, a niche for cell and developmental plasticity. *Semin Cell Dev Biol* 17: 492-502.
- GEE, L., HARTIG, J., LAW, L., WITTLIEB, J., KHALTURIN, K., BOSCH, T.C. and BODE, H.R. (2010). beta-catenin plays a central role in setting up the head organizer in hydra. *Dev Biol* 340: 116-124.
- GUDER, C., PHILIPP, I., LENGFELD, T., WATANABE, H., HOBMAYER, B. and HOLSTEIN, T.W. (2006). The Wnt code: cnidarians signal the way. *Oncogene* 25: 7450-7460.
- GUFLER, S., ARTES, B., BIELEN, H., KRAINER, I., EDER, M.K., FALSCHLUNGER, J., BOLLMANN, A., OSTERMANN, T., VALOVKA, T., HARTL, M. *et al.*, (2018). beta-Catenin acts in a position-independent regeneration response in the simple eumetazoan Hydra. *Dev Biol* 433: 310-323.
- HOBMAYER, B., RENTZSCH, F., KUHN, K., HAPPEL, C.M., VON LAUE, C.C., SNYDER, P., ROTHBACHER, U. and HOLSTEIN, T.W. (2000). WNT signalling molecules act in axis formation in the diploblastic metazoan Hydra. *Nature* 407: 186-189.
- HOBMAYER, E., HATTA, M., FISCHER, R., FUJISAWA, T., HOLSTEIN, T.W. and SUGIYAMA, T. (1996). Identification of a Hydra homologue of the beta-catenin/plakoglobin/armadillo gene family. *Gene* 172: 155-159.
- HOLSTEIN, T.W. (2012). The evolution of the Wnt pathway. *Cold Spring Harb Perspect Biol* 4: a007922.
- HUGGINS, I.J., BOS, T., GAYLORD, O., JESSEN, C., LONQUICH, B., PURANEN, A., RICHTER, J., ROSSDAM, C., BRAFMAN, D., GAASTERLAND, T. *et al.*, (2017). The WNT target SP5 negatively regulates WNT transcriptional programs in human pluripotent stem cells. *Nat Commun* 8: 1034.
- LENGFELD, T., WATANABE, H., SIMAKOV, O., LINDGENS, D., GEE, L., LAW, L., SCHMIDT, H.A., OZBEK, S., BODE, H. and HOLSTEIN, T.W. (2009). Multiple Wnts are involved in Hydra organizer formation and regeneration. *Dev Biol* 330: 186-199.
- LEOST, M., SCHULTZ, C., LINK, A., WU, Y., BIERNAT, J., MANDELKOW, E., BIBB, J., SNYDER, G., GREENGARD, P., ZAHAREVITZ, D. *et al.*, (2000). Paullones are potent inhibitors of glycogen synthase kinase-3beta and cyclin-dependent kinase 5/p25. *Eur. J. Biochem* 267: 5983-5994.
- LIM, X. and NUSSE, R. (2013). Wnt signaling in skin development, homeostasis, and disease. *Cold Spring Harb Perspect Biol* 5: a008029.
- MACDONALD, B., TAMAI, K. and HE, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* 17: 9-26.
- MCPAHON, A.P. and MOON, R.T. (1989). Ectopic expression of the proto-oncogene int-1 in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell* 58: 1075-1084.
- MORKEL, M., HUELSKEN, J., WAKAMIYA, M., DING, J., VAN DE WETERING, M., CLEVERS, H., TAKETO, M.M., BEHRINGER, R.R., SHEN, M.M. and BIRCHMEIER, W. (2003). Beta-catenin regulates Cripto- and Wnt3-dependent gene expression programs in mouse axis and mesoderm formation. *Development* 130: 6283-6294.
- NAKAMURA, Y., TSIARIS, C.D., OZBEK, S. and HOLSTEIN, T.W. (2011). Autoregulatory and repressive inputs localize Hydra Wnt3 to the head organizer. *Proc Natl Acad Sci USA* 108: 9137-9142.
- PETERSEN, H.O., HOGER, S.K., LOOSO, M., LENGFELD, T., KUHN, A., WARNKEN, U., NISHIMIYA-FUJISAWA, C., SCHNOLZER, M., KRUGER, M., OZBEK, S. *et al.*, (2015). A Comprehensive Transcriptomic and Proteomic Analysis of Hydra Head Regeneration. *Mol Biol Evol* 32: 1928-1947.
- PHILIPP, I., AUFSCHNAITER, R., OZBEK, S., PONTASCH, S., JENEWEIN, M., WATANABE, H., RENTZSCH, F., HOLSTEIN, T.W. and HOBMAYER, B. (2009). Wnt/beta-catenin and noncanonical Wnt signaling interact in tissue evagination in the simple eumetazoan Hydra. *Proc Natl Acad Sci USA* 106: 4290-4295.
- SANTI, P.A. (2011). Light sheet fluorescence microscopy: a review. *J Histochem Cytochem* 59: 129-138.
- SCOTTI, N. and CARDI, T. (2012). Plastid Transformation as an Expression Tool for Plant-Derived Biopharmaceuticals. *Transgenic Plants: Methods and Protocols, Second Edition* 847: 451-466.
- VAN AMERONGEN, R.E. and NUSSE, R. (2009). Towards an integrated view of Wnt signaling in development. *Development* 136: 3205-3214.
- VEEMAN, M.T., SLUSARSKI, D.C., KAYKAS, A., LOUIE, S.H. and MOON, R.T. (2003). Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr Biol* 13: 680-685.
- WIKRAMANAYAKE, A.H., HONG, M., LEE, P.N., PANG, K., BYRUM, C.A., BINCE, J.M., XU, R. and MARTINDALE, M.Q. (2003). An ancient role for nuclear beta-catenin in the evolution of axial polarity and germ layer segregation. *Nature* 426: 446-450.
- WITTLIEB, J., KHALTURIN, K., LOHMANN, J.U., ANTON-ERXLEBEN, F. and BOSCH, T.C. (2006). Transgenic Hydra allow *in vivo* tracking of individual stem cells during morphogenesis. *Proc Natl Acad Sci USA* 103: 6208-6211.
- WONG, G.T., GAVIN, B.J. and MCPAHON, A.P. (1994). Differential transformation of mammary epithelial cells by Wnt genes. *Mol Cell Biol* 14: 6278-6286.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Stemness in Hydra - a current perspective

Bert Hobmayer, Marcell Jenewein, Dominik Eder, Marie-Kristin Eder, Stella Glasauer, Sabine Gufler, Markus Hartl and Willi Salvenmoser
Int. J. Dev. Biol. (2012) 56: 509-517
<https://doi.org/10.1387/ijdb.113426bh>

Interstitial stem cells in Hydra: multipotency and decision-making

Charles N. David
Int. J. Dev. Biol. (2012) 56: 489-497
<https://doi.org/10.1387/ijdb.113476cd>

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

Angelika Böttger and Monika Hassel
Int. J. Dev. Biol. (2012) 56: 583-591
<https://doi.org/10.1387/ijdb.113454ab>

Frontiers in fluorescence microscopy

José Rino, José Braga, Ricardo Henriques and Maria Carmo-Fonseca
Int. J. Dev. Biol. (2009) 53: 1569-1579
<https://doi.org/10.1387/ijdb.072351jr>

Wnt signaling in hydroid development: ectopic heads and giant buds induced by GSK-3beta inhibitors

Werner Müller, Uri Frank, Regina Teo, Ofer Mokady, Christina Guette and Günter Plickert
Int. J. Dev. Biol. (2007) 51: 211-220
<https://doi.org/10.1387/ijdb.062247wm>

5 yr ISI Impact Factor (2016) = 2.421

