HvNotch coordinates two independent pattern forming systems during head regeneration in Hydra by supporting a lateral inhibition process restricting the tentacle system.

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Abstract

Wnt- and Notch signaling pathways are involved in regulating *Hydra* head regeneration. The Notch-inhibitor DAPT blocks expression of transcriptional repressor genes including *CnGsc* and *HyHes* and attenuates hypostomal *HyWnt3*- and *HyBMP2/4* expression in regenerating *Hydra* heads while increasing expression of *HyBMP5/8b* and the c-fos-related gene *HyKayak*. Inhibition of the transcriptional activity of β-catenin blocks expression of *HyAlx* during *Hydra* head regeneration and prevents formation of tentacles, however hypostome regeneration and *Wnt3* expression patterns remain intact. This indicates competing pathways for hypostome and tentacle regeneration. We tested this hypothesis on fresh water polyps of *Craspedacusta,* which do not have tentacles and thus after head removal only regenerate a hypostome with a crescent of nematocytes around the mouth opening. We found that head regeneration in *Craspedacusta* was inhibited by the β-catenin-inhibitor iCRT14, but not by the Notch-inhibitor DAPT, indirectly confirming that in *Hydra* Notch-signaling maybe needed to balance separate signaling modules for hypostome and tentacle formation, involving *Wnt3/BMP2/4* or *HyAlx/HyBMP5/8b* expression*,* respectively. We suggest that during head regeneration, Notch works by inhibiting *HyBMP5/8b* expression and *HyKayak* mediated repression of *HyWnt3*, rather than directly activating transcription at the *HyWnt3 promoter*. We thus conclude that *HvNotch* mediates between two pattern forming systems in *Hydra* by coordinating a lateral inhibition process restricting the tentacle system.

Introduction

The small freshwater polyp *Hydra* is a simple metazoan. It belongs to the pre-bilaterian phylum of cnidaria and consists of a foot, a body column and a head with a hypostome and a ring of tentacles. Asexual reproduction occurs by budding. Sexual reproduction takes place from fertilized eggs when male and female gametes are formed on the *Hydra* body column (reviewed in (Steele 2012)).

Hydra polyps have the capacity for complete regeneration. After being cut into small tissue parts they will regenerate a head and a foot accurately and at the same position as before. This indicates that whole-body pattern information is conserved in the body column during adult life of *Hydra* polyps (reviewed in (Bode 2003)). Moreover, as observed in 1908 by Ethel Browne, specific parts of *Hydra* tissue have organizer capacity. When head tissue (according to Ethel Browne "peri-tentacular" tissue), regenerating tips or early buds are transplanted anywhere along the body column of a host animal, they recruit the host tissue to form an ectopic head growing out into a whole new hydranth (Browne 1909, MacWilliams 1983). *Hydra* organizer tissue as defined by Ethel Browne's experiments shares this head organizer property with embryonic organizers, e.g. the dorsal lip of frog blastopores known as the Spemann organizer (Hamburger 1969). There is an intriguing similarity in gene expression at the amphibian Spemann organizer and at the *Hydra* head organizer (for recent analysis (Ding, Ploper et al. 2017). The transcriptional repressor *goosecoid*, for instance is expressed in the dorsal blastopore lip cells of frog embryos. In the *Hydra* head, *CnGsc*, a *goosecoid*-homolog is

prominently (not solely) expressed in head cells between the hypostome and the tentacle zone and thus in the organizer cells as defined by Ethel Browne (Broun, Sokol et al. 1999).
The molecular components of the *Hydra* head organizer and their functional interactions are not very well understood. *HyWnt3* has been suggested in setting up the organizer, which is supposed to be composed of a short-range self-activating head activator, a long-distance travelling head inhibitor and a gradient of competence to form a new head at positions along the body column (Hobmayer, Rentzsch et al. 2000, Broun, Gee et al. 2005). These ideas follow the Gierer-Meinhardt model of pattern formation by "local self-activation and lateral inhibition" (Meinhardt and Gierer 1974, Meinhardt and Gierer 2000). They have been supported by a remarkable series of transplantation experiments (MacWilliams 1983, Broun, Gee et al. 2005). Moreover, *Hydra* has 11 *Wnt* genes, all of which are expressed in the head and/or tentacles. Of those, most are suggested to induce canonical Wnt-signaling through nuclear translocation of β-catenin, while *HyWnt5* and *HyWnt8* have been shown to be associated with non-canonical Wnt-signaling in the planar cell polarity pathway (Hobmayer, Rentzsch et al. 2001, Lengfeld, Watanabe et al. 2009, Philipp, Aufschnaiter et al. 2009). Recently, a Wnt3/β-catenin/Sp5 feedback loop was suggested to be involved in *Hydra* head patterning (Münder, Käsbauer et al. 2010, Vogg, Beccari et al. 2019, Moneer, Siebert et al. 2021). Most known mammalian BMP-pathway genes have homologs in *Hydra*. These include *Smad*, *HyBMP5/8b* and *HyBMP2/4* (Hobmayer, Rentzsch et al. 2001, Reinhardt, Broun et al. 2004, Lengfeld, Watanabe et al. 2009, Philipp, Aufschnaiter et al. 2009, Watanabe, Schmidt et al. 2014).

In previous investigations we had found that Notch was instrumental for head regeneration and organizer formation and necessary to establish a strong *HyWnt3*-signal in regenerating head tissue. Notch inhibition with the presenilin inhibitor DAPT or the NICD-inhibitor SAHM-1 prevented head regeneration and blocked *HyWnt3* expression in regenerates. In contrast, expression of the tentacle boundary gene *HyAlx* and the tentacle metalloprotease gene *HMMP* were not strongly inhibited by DAPT or SAHM1 in regenerating head tissue. However, both genes did not obtain their correct expression patterns and thus proper tentacles were not formed. Similar experiments using a transgenic *Hydra* strain expressing an NICD directed hairpin dsRNA have confirmed the regeneration phenotypes seen with pharmacological inhibitors (Pan Qin, bioRxiv 2024, reviewed by Scientific Reports). Strikingly, transplantation experiments revealed that DAPT-treated regenerating head tissue had lost the capacity to form an organizer (Münder, Käsbauer et al. 2010, Münder, Tischer et al. 2013).

Here we have sought to understand how Notch-signaling functions in expression of HyWnt3 and establishing organizer tissue during head regeneration. To exclude the possibility that Notch inhibition had a direct effect on β-catenin transcriptional activity we compared the effects of the Notch-inhibitor DAPT with the effect of the β-catenin inhibitor iCRT14 (Gufler, Artes et al. 2018). While, similar to DAPT, complete heads were not regenerated in the presence of iCRT14 we observed regeneration of hypostomes. Accordingly, and also in contrast to DAPT, the expression of *HyWnt3* at the regenerated hypostome was not blocked

by iCRT14 and iCRT14 treated regenerating head tissue retained the ability to form a second axis when transplanted into the body column of a host animal. These results indicated that Notch was not directly connected to β-catenin function. We then investigated the effect of the Notch-inhibitor DAPT and iCRT14 on the gene expression dynamics of *HyWnt*- and *HyBMP*-genes and of transcriptional regulators *Hydra Sp5*, *HyAlx* and *CnGsc* during *Hydra* head regeneration by RT-qPCR (Käsbauer, Towb et al. 2007). Our results clearly revealed that *HyWnt3* was controlled by Notch-signaling and *HyAlx* by β-catenin activity. Additionally, we noted that Notch-inhibition increased the expression of *HyBMP5/8b* while blocking the expression of *HyBMP2/4* in the regenerating head.

From this we conclude that hypostome- and tentacle regeneration are regulated by two independent patterning systems. We suggest that Notch activity is required to block the tentacle system. This process involves Notch-mediated inhibition of *HyBMP5/8b* and of negative regulators of *HyWnt3* and *HyBMP2/4* expression. We present the cFos-related transcription factor HyKayak as a likely candidate for negative regulation of *HyWnt3*.

Thus, Notch-signaling mediates a lateral inhibition process necessary to coordinate head and tentacle regeneration. In order to strengthen this idea we compared the properties of head regeneration between *Hydra* polyps and polyps of *Craspedacusta sowerbii*, the latter does not have tentacles. Supporting our model, Notch was not needed to regenerate *Craspedacusta* heads. iCRT14, in contrast, inhibited head regeneration of *Craspedacusta* polyps indicating a requirement for β-catenin dependent gene expression for this process.

Methods

Animal treatment

Hydra polyps were cultured in *Hydra* Medium (HM) (0.29mM $CaCl_2$, 0.59 mM $MgSO_4$, 0.5 mM $NaHCO_3$, 0.08 mM K_2CO_3 dissolved in MiliQ water) at a constant temperature of 18°C. They were fed with freshly hatched *Artemia nauplii* 2-3 times per week, with the exception of two days prior to conducting the experiments. *Craspedacusta sowerbii* polyps were grown in modified HM (0.29mM $CaCl_2$, 0.59 mM $MgSO_4$, 0.5 mM $NaHCO_3$, 0.08 mM K_2CO_3 dissolved in MiliQ water) at 19°C. They were fed with *Brachionus calyciflorus* twice a week. For regeneration experiments, all animals were decapitated at 80% of their body length and left to regenerate for 3-4 days in HM containing the respective inhibitors dissolved in 1% DMSO. Control animals were left to regenerate in HM with 1% DMSO. Treatments included 35 µM DAPT/1% DMSO or 5 µM iCRT14/1% DMSO for 8 hrs, 24 hrs, 36 hrs, 48 hrs, 72 hrs or 96 hrs after head removal. Time point 0 refers to animals immediately after the head was cut off. The inhibitor/DMSO containing medium was renewed every 12-14 hrs.

Standardising conditions for RTqPCR

For quantitative estimates of gene expression dynamics during *Hydra* head regeneration over time we performed real-time quantitative reverse transcription (RT)/PCR (qPCR) experiments. We used a fluorescence based qPCR-method and adhered to the quality standards of the MIQE guidelines (Bustin, Benes et al. 2009). After *in silico* primer design, each primer pair was empirically validated for (1) specificity defined by a single melt peak corresponding to a unique band of expected size, (2) efficiency defined by doubling of the signal in every cycle and (3) sensitivity defined by a broad linear range, and reproducibility. Primers and gene accession numbers are listed in table S1. Total RNA was isolated from *Hydra* polyps and RNA quality was tested with the Agilent bioanalyzer. Only RNA with an integrity number higher than 8 was used for cDNA synthesis. During head regeneration, mRNA for RT-qPCR was isolated from whole regenerates collected after 8, 24, 36 and 48 hrs (t= 8, 24, 36, 48). Immediately after head removal the sample for t=0 was obtained. All experiments included three biological replicates with three technical replicates each. Quantitative gene expression for each gene was calculated as the ratio of target gene expression to housekeeping gene average (relative normalized gene expression). We plotted the relative normalized gene expression of analysed genes against the regeneration time points. Housekeeping genes included *GAPDH, PPIB, RPL13*.

Regression analysis of comparative expression levels

To visualise temporal changes in expression levels of different genes, we used appropriate regression methods. In particular, we used generalised additive models (GAMs) (Hastie & Tibshirani, 1990; Wood, 2017) enabling the visualisation of nonlinear dependencies on the time-dependent variables based on appropriate regression splines (Wood, 2017). Here, we used the Tweedie probability distribution (Kokonendji, Demetrio, et al., 2004; Kokonendji, Dossou-Gbete, et al., 2004) which is known to describe non-negative (possibly overdispersed) data well – in particular if mean values are close to zero. Temporal autocorrelation of model residuals has been investigated based on pacf-plots (Field et al., 2012; Wood, 2017) and was not apparent. The optimal amount of smoothness of regression splines has been estimated separately for each temporal expression pattern based on generalized cross-validation methods (Wood, 2017). For the analysis of expression patterns relative to the control (DMSO) type, the response variable in regression analysis has been defined by dividing separately for each experiment/time point the mean value of the repeated measurements of the treatment of interest (DAPT respectively iCRT) by the mean value of the repeated measurements of the corresponding DMSO treatment from the same experiment/time point.

Semithin sections with Richardson staining

Animals were fixed with 4% PFA and prepared for semithin sectioning by re-fixation in 1% osmium tetroxide solution for 2 hours. Samples were washed with water and dehydrated 4 times with serial acetone dilutions (30%, 50%, 70%, 90%, four times 100%). Finally, they were embedded in Spurr low viscosity embedding medium standard mix, which was exchanged 4 times, and dried after each exchange for 24 hours at 60°C in a cuboid shape. The resin embedded probes were sectioned with a semi-diamond and stained after Richardson on a

microscope slide. One drop of colour solution (1% Azur in H_2O and 1% methylene blue in 1% $Na_2B_4O_2$ in H_2O mixed 1:1) covering the semithin sections was heated to 80°C for 30 seconds and cleansed with water. After drying, the slides were analysed with a brightfield microscope.

Histochemistry of polyps

Polyps were relaxed in 2% urethane and fixed with 4% PFA in HM for 1 hr. They were permeabilized with ice-cold 100% ethanol and blocked in 0.1% Triton/1% BSA in PBS. For phallodin staining they were incubated with Phalloidin-iFluor 488 (abcam, ab176753) (1:500) for 1 hr, followed by DAPI (1:1000) staining before mounting on slides with Vectashield. Slides were analysed with a Leica SP5 point scanning laser confocal microscope equipped with oil immersion HCX PL APO Lambda Blue 20x 0.7 and 63x 1.4 objective lenses. Alexa-488 fluorochromes were visualized with an argon laser at an excitation wavelength of 488 nm and emission filters of 520-540 nm and a diode laser at an excitation wavelength of 405 nm and emission filter at 450-470 nm was used for DAPI. The produced light optical serial sections were stacked with the ImageJ plugin StackGroom to produce 3D images of the treated polyps. DAPI staining of nematocyte capsules was done according to (Szczepanek, Cikala et al. 2002).

Transplantation experiments

Non-budding *Hydra* polyps were pre-treated with 5 µM iCRT14/1% DMSO in HM for 24 hrs. After that they were bisected at 80% of the body column underneath the head and left to regenerate in iCRT14 treated HM for another 24 hrs. The newly regenerated head region (top20%) was grafted onto a blue host animal (treated with Ewan's Blue for two weeks) at about 50% of the body column. After 3 hrs the rod was removed and the animals left in HM for another 48 hrs. Finally, the animals were classified for the presence of newly formed secondary axes displaying a clear hypostome and tentacles. Tissue recruitment was recognized by the blue/white colour distribution within the new axes.

ShRNA knockdown

shRNA design and production were done according to Karabulut's protocol (Karabulut, He et al. 2019). For electroporation, 30 budless Hydra polyps were washed 5 times with MiliQ water and incubated for 45 min in MiliQ water. Then, excess water was removed and replaced with 200 µl of a 10 mM HEPES solution at pH 7.0. The suspended animals were then transferred into a 4mM gap electroporation cuvette, and 4 µM of purified shRNA or scramble shRNA was added to the cuvette. The mixture was mixed by gently tapping the cuvette for 5 times and incubated for 5 min to let animals relax before electroporation. The electroporation was carried out using the BTX Electro Cell Manipulator 600 by setting up the condition to 250 V, 25 ms, 1 pluse, 125 µF capacitance. 500 µl of restoration medium (80 % HM and 20% dissociation medium (3.6 mM KCl; 6 mM $CaCl_2$; 1.2 mM $MgSO_4$; 6 mM sodium citrate; 6 mM sodium pyruvate; 6 mM glucose; 12.5 mM TES; and 50 mg/ml rifampicin, pH6.9)) was added into the cuvette immediately after electroporation. The entire volume of eletroporated animals were then transferred into a petri dish. In our experiment, three times of

electroporation were done every two days to achieve a significant knockdown of HyKayak. And two hairpins of Kayak were used for electroporation at 1:1.

Multiple sequence alignment and phylogenetic analysis

The multiple sequence alignment was done using Clustal Omega. The conserved bZiP domain was identified by PROSITE. The phylogenetic tree was produced by MEGA. The involved protein sequences were retrieved from Uniprot and NCBI.

Subcellular fractionation and Western blot

500 Hydras were dissociated into single cells with 10 mL dissociation medium by pipetting. After centrifuge at 2000 g for 10 min, the cellular pellet was resuspended in 500 µl RIPA buffer (25 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 % NP40, 1 % sodium deoxycholate, 0.1 % SDS, 10 ng/ml Pepstatin A, 10 ng/ml Aprotinin, 10 ng/ml Leupeptin, 0,5 mg/ml Pefablock) and incubated for 20 min on ice. Subsequently, the mixture was homogenized with a Dounce homogenizer for 30 times and then centrifuged for 10 min at 1000 g. The resulting supernatant including cytoplasmic proteins was collected and labelled as CP. The pellet was treated with 500 µl RIPA buffer, and then sonicated at 180 watts for 3 min (in rounds of 10 sec sonication and 50 sec rest for each cycle). After centrifuging at 14000 g for 30 min, the supernatant was collected and labelled as nuclear proteins (NP), the pellet was resuspended with the same volume of RIPA buffer and kept for SDS-PAGE gel analysis.

For DNase treatment, the pellet from the second centrifuge was resuspended with 500 µl RIPA buffer supplemented with 200 units/mL DNase, 10 mM $CaCl_2$ and 10mM $MgCl_2$, and incubated at room temperature for 15 min. After centrifuging at 1000 g for 10 min, the supernatant was collected, while the pellet was resuspended in 500 µl RIPA buffer with 2 M NaCl and incubated on ice for 10 min. Then the same centrifuge was done and the supernatant and pellet was collected for gel analysis.

Western blots were stained with the inhouse mouse anti-Kayak monoclonal antibody.

Co-immunoprecipitation

HEK293T cells were transferred with C-terminal HA-tagged Kayak and N-terminal GFP-tagged Jun-epi or Kayak using Lipofectamine 2000 (11668030, Thermo Fisher). The GFP-trap agarose beads (ABIN509397, ChromoTek) were used for immunoprecipitation as described previously (Webby, Wolf et al. 2009, Heim, Grimm et al. 2014). Western blot was stained with the following primary antibodies: mouse anti-GFP antibody (11814460001, Roche) and rabbit anti-HA antibody (H6908, Sigma Aldrich).

Results

1. Hypostome formation in iCRT treated, but not in DAPT treated regenerates

Hydra polyps treated either with iCRT14 as described in (Gufler, Artes et al. 2018), or with the Notch-inhibitor DAPT, as described in (Münder, Tischer et al. 2013), fail to regenerate a

complete head after decapitation. DAPT blocks regulated intramembrane proteolysis by presenilin and thus prevents NICD-translocation to the nucleus in several organisms including *Hydra* (Dovey, John et al. 2001, Käsbauer, Towb et al. 2007). DAPT in *Drosophila* and zebrafish and recently in *Hydra* have been shown to closely phenocopy loss of Notch function (Geling, Steiner et al. 2002, Micchelli, Esler et al. 2003), (Pan Qin, bioRxiv 2024, reviewed by Scientific Reports). iCRT14 inhibits the interaction of nuclear β-catenin with TCF in mammalian cell lines and in *Hydra* (Gonsalves, Klein et al. 2011, Gufler, Artes et al. 2018).

First, we treated Hydra polyps with 5 µM iCRT14 for 48 hrs after head removal, and observed that they did not regenerate their heads during the time of treatment, while control animals treated with DMSO (the solvent for iCRT14) clearly showed several regularly spaced tentacle buds at this time point (Fig. 1A). iCRT and DMSO were then replaced with normal *Hydra* medium. Control animals showed long tentacles 24 hrs later, iCRT treated animals, however only recovered normal head structures at the 120 hr time point, 48 hrs later than control polyps.

To inspect the morphology of DAPT and iCRT14 treated head regenerates more closely, after the 48hrs treatment they were sliced and semithin sections were histologically stained with Richardson tissue stain. Amongst other structures, this dye stained the mesoglea dark blue. Fig. 1B shows middle sections of polyps. The mesoglea is emphasized by red lines. A 'gap' in the mesoglea at the hypostome of an uncut animal is apparent, which becomes visible again after head regeneration in *Hydra* medium, DMSO and iCRT14, but not in DAPT treated animals. In addition, we analyzed regeneration in 'water melon' AEP-vulgaris hydra polyps. These polyps express green fluorescent protein (GFP) in the whole of the ectoderm and red fluorescent protein (RFP, dsRed) in the whole of the endoderm (polyps were a kind gift from Rob Steele, UC Irvine). Fig. 1C shows laser scanning microscope optical middle sections allowing detection of the mouth opening. Again, hypostome morphology is observed in animals after regeneration in DMSO or iCRT14, but not in DAPT. We also quantified regeneration of hypostomes and tentacles in DAPT- and iCRT14 treated regenerates in comparison with control animals (Fig. 1E). 70 % of iCRT14 treated animals regenerated an intact hypostome with a detectable mouth opening, but tentacles were not formed. DAPT treated animals did not regenerate a mouth opening, and in 25% of regenerates aberrant tentacles were found at the tip, as we had described previously (Münder, Tischer et al. 2013). The presence of a hypostomal mouth opening in iCRT14 treated polyps prompted us to perform in situ-hybridisation for *HyWnt3* in such regenerates. As shown in Fig. 1D, *HyWnt3* expression at the hypostome was evident in control regenerates and in such that had been treated with iCRT14. This is different from DAPT treated regenerates, which do not express *HyWnt3* (see (Münder, Tischer et al. 2013).

2. Organizer formation in iCRT14 treated, but not in DAPT treated regenerates

In previous work we had shown that DAPT-treated regenerating *Hydra* heads lacked organizer activity, as they did not induce formation of ectopic heads and hydranths when transplanted

into the body column of a host animal (Münder, Tischer et al. 2013). This was in accordance with the loss of *HyWnt3* expression in Notch-inhibited regenerates. We now asked the question whether iCRT14 treated head regenerates would retain organizer capacity, as they do express *HyWnt3*. We therefore transplanted regenerating *Hydra* heads (upper 20% of polyps) after 24 hrs treatment with iCRT14 or DMSO (for control) into the body column of Ewan's blue stained host animals. Fig. 2 shows that 80 % of control regenerates formed ectopic hydranths after transplantation into the body column of the host. 80 % of iCRT14 treated regenerates were also able to form ectopic hydranths and most of them recruited host tissue, indicating that they had re-acquired organizer activity. This is in accordance with their expression of *HyWnt3*. These data suggest two things. (1) Up-regulation of *HyWnt3* during the regeneration process is not dependent on β-catenin transcriptional activity and (2) If *HyWnt3* directed hypostome formation, it would have to be via a non-canonical Wnt-signaling pathway.

3. Comparison of gene expression dynamics during *Hydra* head regeneration in control animals, DAPT-treated and iCRT14 treated animals

3.1. Effect of Notch inhibition on gene expression dynamics during head regeneration in *Hydra*

As indicated above DAPT-treated *Hydra*-polyps completely fail to regenerate a new head after decapitation, they do not form a hypostome and, although sometimes producing tentacle tissue, they will not regenerate a properly patterned crescent of tentacles (Münder, Tischer et al. 2013). In a previous transcriptome analysis of DAPT treated *Hydra* polyps, beside *HyHes*, the tentacle boundary gene *HyAlx,* the "organizer" gene *CnGsc* and the *HySp5* gene had been suggested to be potential direct Notch-target genes in *Hydra* (Moneer, Siebert et al. 2021). The same analysis had revealed that the fos-related transcription factor *kayak* was up-regulated when Notch-signaling was blocked.

We now performed RT-qPCR analysis to compare gene expression dynamics of these genes during head regeneration after 0, 8, 24, 36 and 48 hrs. Animals were either treated with 30 µM DAPT or 1% DMSO as control. Timepoint 0 was measured immediately after head removal. This revealed that *HyHes* expression was partially inhibited by DAPT, thus confirming previously published data indicating this repressor gene as a direct target for NICD (Münder, Käsbauer et al. 2010). *HyAlx* expression levels were not significantly influenced by DAPT. *CnGsc* expression under DAPT treatment initially (8hrs) was comparable to control levels, but then it was strongly inhibited. This goes along with the observed absence of organizer activity in regenerating *Hydra* tips (Münder, Tischer et al. 2013). *HySp5* expression, while also normal at 8 hrs, was similarly inhibited by DAPT at later time points (Fig. 3A). *Kayak*, on the other hand, was overexpressed during regeneration in DAPT in comparison to control regenerates (Fig. 3A).

In addition, we tested the expression dynamics of the two *BMP*-homologs described in *Hydra*, *HyBMP5/8b* and *HyBMP2/4* (Reinhardt, Broun et al. 2004, Watanabe, Schmidt et al. 2014). Interestingly, they were reciprocally affected by Notch inhibition. *HyBMP2/4* expression was blocked with DAPT over the course of regeneration (Fig. 3A). In contrast, *HyBMP5/8b* expression was drastically increased.

We had previously shown by in-situ hybridisation that *HyWnt-3* is not expressed in DAPT treated head regenerates (Münder, Tischer et al. 2013). This was confirmed now by RT-qPCR measurements, which revealed that *HyWnt-3* was expressed well above control levels 8 hrs after head removal, however after this time point it was strongly inhibited by DAPT and almost completely lost after 48 hrs (Fig. 3B). Eventually we analyzed most of the *Wnt*-genes suggested to engage in canonical Wnt-signalling including *HyWnt 1*, 7, 9/10c, 11 and 16 (Lengfeld, Watanabe et al. 2009). In DAPT they all were expressed at control levels 8 hrs after head removal, however, then expression was declining to almost zero (Fig. 3B).

3.2. Effect of β-catenin inhibition on gene expression dynamics during head regeneration in *Hydra*

Next, following the same procedure as described for DAPT, we compared the gene expression dynamics of iCRT14 treated regenerates with control regenerates in 1 % DMSO, which is the solvent for iCRT14. It had been suggested that *HyWnt3* expression is controlled by β-catenin mediated transcription and therefore subject to self-activation (Nakamura, Tsiairis et al. 2011). In contrast to this idea, we found that expression of *HyWnt3* was not inhibited by iCRT14, it even appeared to be slightly up-regulated during the first half of the regeneration period (Fig. 4B). This is in accordance with in-situ hybridization for *HyWnt3* as shown in Fig. 1D confirming that 48 hrs after head removal, not only *HyWnt3* expression patterns in ecto- and endodermal cells of the hypostome were faithfully regenerated, but *HyWnt3* expression levels were also restored (Fig. 4B). In contrast, *HyAlx* expression was completely abolished by iCRT14 (Fig. 4A) in accordance with the observation that iCRT14 treated head regenerates did not regenerate any tentacles (Fig. 1A). Moreover, as *HySp5* had been implicated in regulating *HyWnt3* transcription (Vogg, Beccari et al. 2019) we tested the effect of iCRT14 on its expression dynamics during head regeneration and found that Hy*Sp5* expression was not significantly affected by iCRT14 treatment (Fig. 4A).

For further comparison of the effects of Notch- and β-catenin inhibition on *Hydra* head regeneration, we tested the expression of the "organizer" gene *CnGsc.* With iCRT14, *CnGsc* levels remained similar to control regenerates, and were mildly decreased at the 48 hrs time point (Fig. 4A). No significant changes in expression dynamics of iCRT14 treated regenerates in comparison with controls were found for both, *HyBMP2/4* and *HyBMP5/8b* (Fig. 4A).

Expression dynamics of canonical *HyWnt* genes during head regeneration was differentially affected by iCRT14. iCRT14 inhibits *HyWnt1*, *HyWnt16* and to a lesser extent *HyWnt7* over the

whole course of regeneration. For *HyWnt11* and *HyWnt9/10c* inhibition up to 36 hrs is less pronounced, and at 48 hrs the same expression levels as found in control animals are reached.

In summary, Notch-signaling appears to be responsible for continued expression of all canonical *HyWnt* genes during head regeneration after their initial uprise 8 hrs after head removal, including *HyWnt3*. This also applies for the suggested *Hydra* organizer gene *CnGsc,* and *BMP2/4 (Broun, Sokol et al. 1999)*. These data support our earlier investigations, which had revealed that regenerating head tissue loses its organizer function when it is treated with DAPT (Münder, Tischer et al. 2013). Moreover, Notch activity is required for inhibiting *HyKayak* and *HyBMP5/8b* gene expression during regeneration, which coincides with DAPT causing downregulation of the transcriptional repressor and Notch-target gene *HyHes*. On the other hand, *Hydra* head regeneration does not require β-catenin transcriptional activity to express *HyWnt3* and to form a new hypostome. In contrast, β-catenin dependent transcription is required to express *HyAlx* and form tentacles.

Thus, organizer activity is found in regenerating head tissue expressing *HyWnt3* (iCRT14-treated) but not in such without *HyWnt3* (DAPT treated). Moreover, β-catenin transcriptional activity is essential during head regeneration for the expression of *HyAlx* and tentacle formation. This again suggests that β-catenin transcriptional activity is not required for regenerating *Hydra* tissue to express *HyWnt3* and regenerate a mouth opening/ hypostome. It is also not needed for the regenerating head tissue to acquire organizer activity. In contrast, Notch-activity is required for increasing *HyWnt3* expression during head regeneration, forming a new hypostome and regeneration of organizer tissue.

In summary, these RT-qPCT analyzes showed that Notch-signaling during *Hydra* head regeneration is necessary for activating all *HyWnt*-genes expressed in the *Hydra* head region and implicated in canonical Wnt-signalling. It is also necessary for activation of *BMP2/4* expression, a gene expressed in the *Hydra* head and body column. Moreover, Notch is required to express the transcriptional repressor genes *HyHes* and *CnGsc*. Genes expressed in *Hydra* tentacles and at tentacle boundaries, including *HyAlx*, are not so strongly dependent on Notch-signaling, *HyBMP5/8b* appears to be inhibited by Notch. This is also the case for the *Hydra*-homolog of *c-fos*, *HyKayak.*

4. *HyKayak*

HyWnt3, albeit inhibited by DAPT specifically during head regeneration, had so far not been indicated as a potential target for Notch-mediated gene activation in *Hydra* (Münder, Tischer et al. 2013, Moneer, Siebert et al. 2021). It is therefore possible, that the absence of *HyWnt3* expression in Notch-inhibited regenerating heads is due to the presence of a repressor, rather than the absence of transcriptional activation.

According to our previous report, the *Hydra* fos-homolog kayak (t5966aep) was up-regulated after Notch-inhibition with DAPT (Moneer, Siebert et al. 2021). The up-regulation of *HyKayak* by DAPT suggests *HyKayak* as a potential target gene for Notch-regulated repressors including *HyHes* and *CnGsc,* and as such a potential repressor of *HyWnt3* gene transcription. Domain structure and sequence comparison of *HyKayak* with *fos*-and *jun*-sequences from *Caenorhabditis, Drosophila*, chicken, mouse and human revealed strong conservation of the bZiP-domain (basic leucine zipper domain), which is responsible for DNA-binding and dimerisation (Fig. S1A, B). Phylogenetic analysis showed that *HyKayak* is related to *c-fos*-sequences of various species including *Hydra* (Fig. S1C). Fos-proteins interact with Jun-proteins (also bZIP domain proteins) to form the transcriptional regulation complex AP-1 (activator protein 1) (Karin, Liu et al. 1997). In *Hydra* we found two transcripts encoding Jun-related proteins, *HyJun_nem* (t17964aep) expressed in nematoblasts, and *HyJun-epi* (t19405aep) expressed in all cells, with especially high levels in epithelial cells (Siebert, Farrell et al. 2019). *HyKayak* is expressed mainly in ectodermal cells (Siebert, Farrell et al. 2019). By SDS-PAGE of *Hydra* lysates and staining with anti-*HyKayak* antibody (see Fig. S1-A), we found that *HyKayak* protein remained in the pellet fraction and only a small percentage could be solubilised after treatment with DNAse, suggesting that *HyKayak* is strongly associated with DNA (Fig. S1-B), supporting its role as a DNA-binding protein. We then performed immunoprecipitation of *HyKayak* and HyJun_epi-proteins expressed in HEK293T-cells revealing that *HyKayak* did not interact with itsself, but strongly interacted with HyJun_epi protein (Fig. S2). This is similar to human cFos, which does not form homodimers, but heterodimerises with Jun-proteins (Kouzarides and Ziff 1988). Assuming that *HyKayak* may be forming dimers with HyJun_epi we used the Fos/jun-inhibitor T5224 to block DNA-binding of Fos/Jun-complexes (Xiong, Ouyang et al. 2022) and analyzed resulting gene expression and phenotypes during *Hydra* head regeneration. This revealed a slight inhibition of *HyKayak* expression in contrast to strong up-regulation of *HyJun* (Fig. 5A, B), indicating that T5224 induces a severe change in the complex composition of these two transcription factors. This is in accordance with previously published observations in human prostate cell lines where *fos*-loss-of-function had resulted in up-regulation of *jun*-expression (Riedel, Cai et al. 2021). We found here, that *HyWnt3* expression was also up-regulated by T5224 (Fig. 5C). Polyps treated with T5224 regenerated shorter and less tentacles than control animals within 3 days after head removal (Fig. S3). To confirm that *HyKayak* was the source of *HyWnt3* overexpression we knocked down *HyKayak* using shRNA directed against *HyKayak*. We achieved *HyKayak* knockdown by ca. 80% in comparison with mock treated control polyps or polyps treated with scrambled control shRNA. Importantly, knockdown of *HyKayak* induced up-regulation of *HyWnt3* (Fig. 5F). From these data we conclude that (1) *HyKayak* can inhibit expression of *HyWnt3* and (2) it may work within the AP-1 complex together with Jun-epi. We hypothesize that *HvNotch* blocks the *HyWnt3* inhibitory activity of *HyKayak* by activating *HyHes* and *CnGsc,* which may repress *HyKayak.* This is necessary to reactivate expression of *HyWnt3* during head regeneration, which is the preposition for hypostome- and organizer formation.

5. Regeneration of *Craspedacusta* polyps

Our data dissect the regeneration of *Hydra* heads into two processes, formation of the hypostome and head, and formation of tentacles. Notch-signaling then appears to be responsible for "organizing" two independent morphogenetic processes. In order to test this hypothesis, we asked, how inhibition of Notch-signaling and β-catenin activity might affect regeneration of polyps with a simpler, one-component, head. We used polyps of the fresh water hydrozoan *Craspedacusta sowerbii.* They have a mouth opening that is surrounded by epithelial cells carrying nematocytes and they don't possess tentacles (Ramos, Peronti et al. 2017).

Craspedacusta polyps are shown in Figure 6A. They often occur as mini-colonies with one foot carrying two polyps. Actin fibres are running along the polyp's body column and form a ring where the two polyps separate just above the foot. Actin cushions carrying nematocysts are visible and indicate the positions of capsules along the body column and in a ring surrounding the mouth opening (Fig. 6A, B). Additional capsule staining with DAPI (Szczepanek, Cikala et al. 2002) very clearly reveals the pattern of nematocysts in the head (Fig. 6B). When we removed the heads of the polyps, most of them fully regenerated within 96 hrs (Fig. 6B). Some retracted into a podocyst (the 'dauerstadium') (Fig. S4). Polyps treated with DMSO or DAPT also completed head regeneration after 96 hrs. However, treatment with iCRT14 prevented head regeneration (Fig. 6C, quantification Fig. S4). This indicates that β-catenin activity is required for head regeneration in *Craspedacusta* polyps, whereas Notch-signaling is dispensable.

Discussion

Here we show that inhibition of β-catenin transcriptional activity with iCRT14 during *Hydra* head regeneration inhibits the re-formation of tentacles while still allowing the re-formation of a hypostomal mouth opening. This was in accordance with the undisturbed re-establishment of the expression pattern of *HyWnt3* in iCRT14 treated head regenerates and with the formation of organizer tissue revealed by transplantation experiments. In contrast, when Notch-signalling was inhibited with DAPT hypostomes were not regenerated. Sometimes irregular tentacles appeared. Moreover, such regenerates did not form organizer tissue (Münder, Tischer et al. 2013). Therefore, we investigated the dynamics of gene expression for genes of the Notch, Wnt- and BMP-signaling pathways and the transcription factor genes *HyAlx, HyKayak* and *CnGsc* over 48 hrs of *Hydra* head regeneration. Some of these genes are strongly or exclusively expressed in the head or at the tentacle boundaries including *HyAlx* and *HyBMP5/8b* at the tentacle boundaries, and *HyWnt 1*, *-3, -7, -9/10c, -11* and *-16* and BMP2/4 in the hypostome and head (Reinhardt, Broun et al. 2004, Lengfeld, Watanabe et al. 2009, Watanabe, Schmidt et al. 2014). When heads are removed, *Wnt*-gene expression is low in the residual polyp. However, it increases to maximum levels within 48 hrs, in accordance with restoration of the complete head during this time. The major results of this analysis are schematically displayed in Table 1. We distinguish two phases of regeneration, the first 8 hrs and the time thereafter. When Notch signaling was inhibited with DAPT, we

observed that *HyWnt*-1, 3, 7, 1/10, 11 and 16, all suggested in canonical Wnt-signalling, were re-expressed in regenerating head tissue 8 hrs after head removal. After this time point, their expression levels declined to almost zero. *HyAlx* expression was not so strongly inhibited and sometimes irregular tentacles arose. This was completely in accordance with our previous study (Münder, Tischer et al. 2013). In addition, expression of the *cFos*-homolog *HyKayak* was strongly increased and expression of the organizer gene *CnGsc* was abolished. When we studied the effect of β-catenin inhibition on gene expression dynamics we found that inhibition of β-catenin dependent transcriptional activation did neither affect the expression dynamics of *HyWnt3*, nor the formation of a hypostome or the organizer properties of regenerating head tissue. Very clearly, however, expression of *HyAlx* and formation of tentacles was inhibited by iCRT14. In contrast to *HyWnt3*, re-expression of *HyWnt1, 7* and *16* after head removal seemed to strongly require β-catenin dependent transcription. According to previously published in-situ hybridisation data, *HyWnt7* and *16* have a broader expression zone in the head in comparison with *HyWnt3* and partially cover the peri-tentacle region (Reinhardt, Broun et al. 2004).

From this we conclude for the process of *Hydra* head regeneration: (1) *HyWnt3* re-expression during head regeneration is independent of β-catenin transcriptional activity suggesting that in regenerating heads HyWnt3 does not signal through the canonical pathway to form the hypostome and organizer tissue. (2) *HyWnt3* is not up-regulated via β-catenin dependent autoactivation as had been suggested previously to occur in un-disturbed polyps (Nakamura, Tsiairis et al. 2011). (3) *HyAlx* and tentacle gene expression is directed by β-catenin, but not by *HvNotch*.

This left us with two apparently independent pathways for head- and tentacle regeneration, one directed by Notch-signaling and involving *HyWnt3* and all canonical *HyWnts*, and the other involving β-catenin transcriptional activity and *HyAlx,* but not Notch-signaling. According to the Notch paradigm for lateral inhibition, Notch activates transcriptional repressors, including *HyHes* genes and thereby suppresses specific cell fates in signal receiving cells, whereby allowing those fates in the signaling cells (Bray 2006). Our data suggest, that DAPT inhibited the expression of two established transcriptional repressor genes, *HyHes* and *CnGsc*. This poses the question for targets of these repressors, which should be up-regulated when Notch-signaling is inhibited.

Indeed, we describe two genes with this behavior. *HyBMP5/8b* expression was strongly increased in DAPT treated regenerates. The same was true for the *Hydra* Fos-homolog *HyKayak*, encoding a potential component of the AP1 transcriptional complex. In addition, Fos is suggested to be a negative regulator of its own promoter (Sassone-Corsi, Sisson et al. 1988), and Fos can function as a repressor on cellular immediate-early genes, like Egr genes (Gius, Cao et al. 1990). Both repressions are mediated by the C-terminus of Fos and are independent of Jun (Gius, Cao et al. 1990, Ofir, Dwarki et al. 1990). However, The C-terminus of Fos is not required for the repression of cardiac transcription and muscle creatine kinase

(MCK) enhancer (Lassar, Thayer et al. 1989, Li, Chambard et al. 1992, McBride, Robitaille et al. 1993). The general idea that *HyKayak* could repress the *HyWnt3* gene was confirmed by shRNA mediated *HyKayak*-knockdown, which resulted in up-regulation of *HyWnt3* expression. In addition *HyJun-epi* was also up-regulated. Moreover, pharmacological inhibition of the AP1-complex with T5224 during head regeneration also showed that *HyWnt3* and *HyJun-epi* were strongly up-regulated. We therefore suggest that the *Hydra* Fos-homolog *HyKayak* inhibits *HyWnt3* expression and is itself target for a Notch-induced transcriptional repressor (like *HyHes*) in the regenerating *Hydra* head.

On the basis of these investigations, we offer a new hypothesis about the function of Notch-signaling for *Hydra* head regeneration. It involves dissecting the head regeneration process into two parts that we consider to be directed by separate signaling modules. Hypostome and head formation would require *HyWnt3* and *HyBMP2/4*, whereas tentacle tissue formation requires β-catenin dependent transcription to express *HyAlx and HyBMP5/8b*. Notch is needed to mediate a lateral inhibition process. One possibility is that the Notch-target genes *HyHes* and *CnGsc* can repress genes that inhibit *HyWnt3* after its initial up-rise 8 hrs after head removal. Our initial data strongly suggest *HyKayak* as part of the AP1-complex as candidate gene for such a function. Moreover, the requirement for HvNotch activity is dependent on the regeneration time. At early time points it is apparently not needed, but between 8 and 48 hrs after head removal loss of HvNotch activity severely impairs the regeneration process (Fig. 7A, B).

By antibody staining with an antibody binding tentacle proteins and another one labelling nerve cells, it had previously been shown that after head removal, tentacle tissue is formed prior to hypostomal tissue (Bode, Awad et al. 1988). This was confirmed by the discovery of the *HyAlx*-gene and the study of its expression pattern during head regeneration and budding (Smith, Gee et al. 2000). When heads are cut off just underneath the tentacle ring, the tentacle signal appears at the most oral position and then becomes shifted to a more distant part from the head due to an inhibitory influence of the regenerating head system. This inhibition appears to depend on Notch-signaling. Without Notch-signaling, the tentacle system takes over and prevents establishment of the head system altogether (Fig. 7B). Here we suggest that *HyBMP5/8b* and an inhibitor of *HyBMP2/4* and *HyWnt3* (e.g. *HyKayak*) are crucial parts of the tentacle system that have to be repressed in order to allow the head system to be established.

We furthermore suggest that the HvNotch effect initially is localized to the most oral part of the regenerating tip. A similar lateral inhibition is required in the tentacle zone to allow the formation of tentacle boundaries. Here we have previously suggested that Notch could act by activating *HySp5* to repress *HyWnt3* expression in the tentacle zone (Moneer, Siebert et al. 2021). It would then appear that the function of HvNotch-signaling during head regeneration is to locally de-repress *HyWnt3* at the tip of the head tissue. By directly inducing the potential *HyWnt3* inhibitor *HySp5* in the peri-tentacle zone HvNotch might block *HyWnt3* transcription in the tentacle zone at a later time when tentacle boundaries are formed.

The described model would predict that Notch is not required in a regeneration system, which only establishes one new structure or axis. This is the case in *Craspedacusta* polyps, which do not have tentacles. We show that head regeneration of *Craspedacusta* polyps requires the activity of β-catenin. However, DAPT did not have an effect on this regeneration process, corroborating the idea of Notch mediating between two pattern forming processes during head regeneration in *Hydra* polyps. One could even speculate that in evolutionary terms, the *Hydra* tentacle system may correspond to the *Craspedacusta* head.

We conclude that *HvNotch* mediates between two independent pattern forming systems during regeneration in *Hydra* by inducing a lateral inhibition process restricting the tentacle system.

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Data availability
All data presented in the main manuscript and supplementary files will be provided by the corresponding author (Angelika Böttger) upon requests.

Author contribution statement
A.B. and M.J. conceived this study. M.S., L.S. A.dP.,J.M. carried out quantitative RT-PCR and in situ hybridisation, L.S. provided all work on Craspedacusta, Q.P provided all work on Hykayak. M. M. provided GAM-based visualisation of gene expression, M.S.,J.M. and A.B. drafted the manuscript. All authors revised and approved the manuscript.

Additional information

Competing interests:
The authors declare no competing interests.

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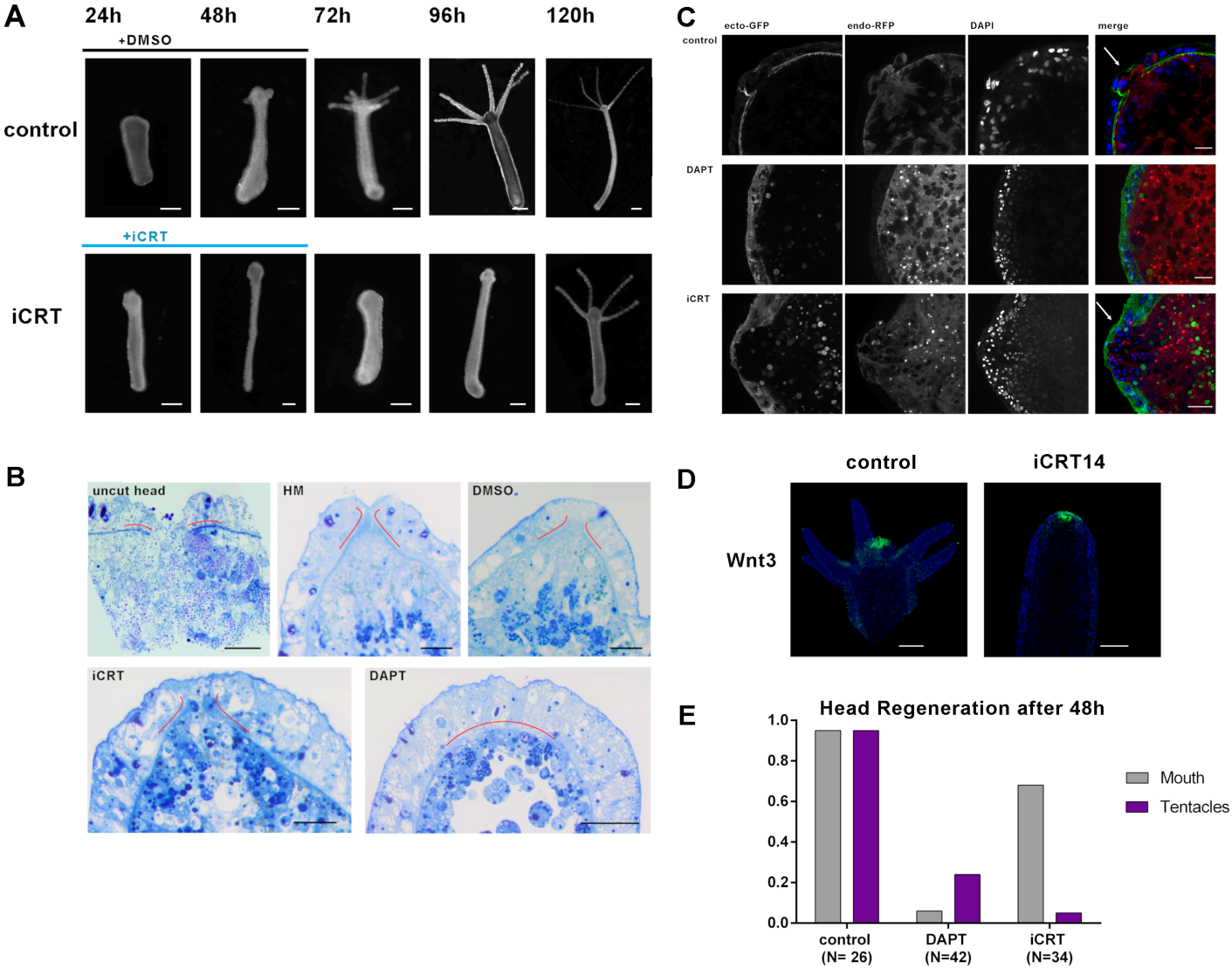

Figure 1: Regeneration of *Hydra* head structures

(A) Head regeneration progress of *Hydra* polyps at indicated time points after head removal in *hydra* medium with DMSO or iCRT14 for inhibiting β-catenin. **(B)** Semi-thin sections covering the hypostome region of *Hydra* polyps after Richardson-staining: untreated polyp (uncut head); polyps after 48 hrs of head regeneration in *Hydra* medium (HM), DMSO control, DAPT and iCRT14. Mesoglea appears as dark blue line, red lines are added to highlight the mesoglea at the hypostome region. Scale bar: 20 µm. **(C)** Confocal stack images covering the regenerated hypostome region of *Hydra* strain AEP 'Water melon' polyps 48 hrs after head removal in DMSO, DAPT and iCRT14. GFP present in ectodermal cells, dsRed present in endodermal cells and DAPI-DNA-stain are imaged as indicated. Right hand panels show merged images. White arrows indicate hypostomal opening in iCRT14-treated and control animals, but not in DAPT treated polyps. Scale bar: 10 µm. **(D)** Fluorescent In-situ hybridization for *HyWnt3* expression in polyps 48 hrs after head removal in iCRT14 or in DMSO control, as indicated. Scale bar: 100 µm. **(E)** Quantification of regeneration of *Hydra* head structures (hypostome and tentacles) 48 hrs after head removal and regeneration DMSO control, DAPT and iCRT14.

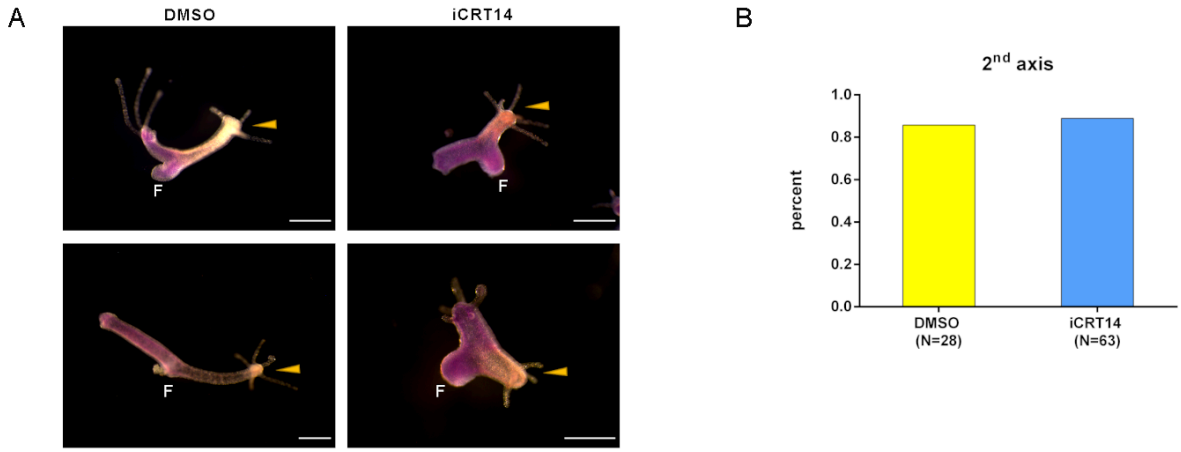

Figure 2: Organizer activity of regenerating *Hydra* head tissue
Tissue from regenerating tip of polyps 24 hrs after head removal in DMSO control, or iCRT14 was transplanted into the middle of blue host polyps stained with Ewan's blue. **(A)** Microscopical images were taken 48 hrs after transplantation. Newly formed second axes are indicated by yellow arrows. The transplanted tissue appears orange, the host tissue appears blue, feet are indicated by F. **(B)** Percentage of transplants forming new axes in DMSO control, or in iCRT14.

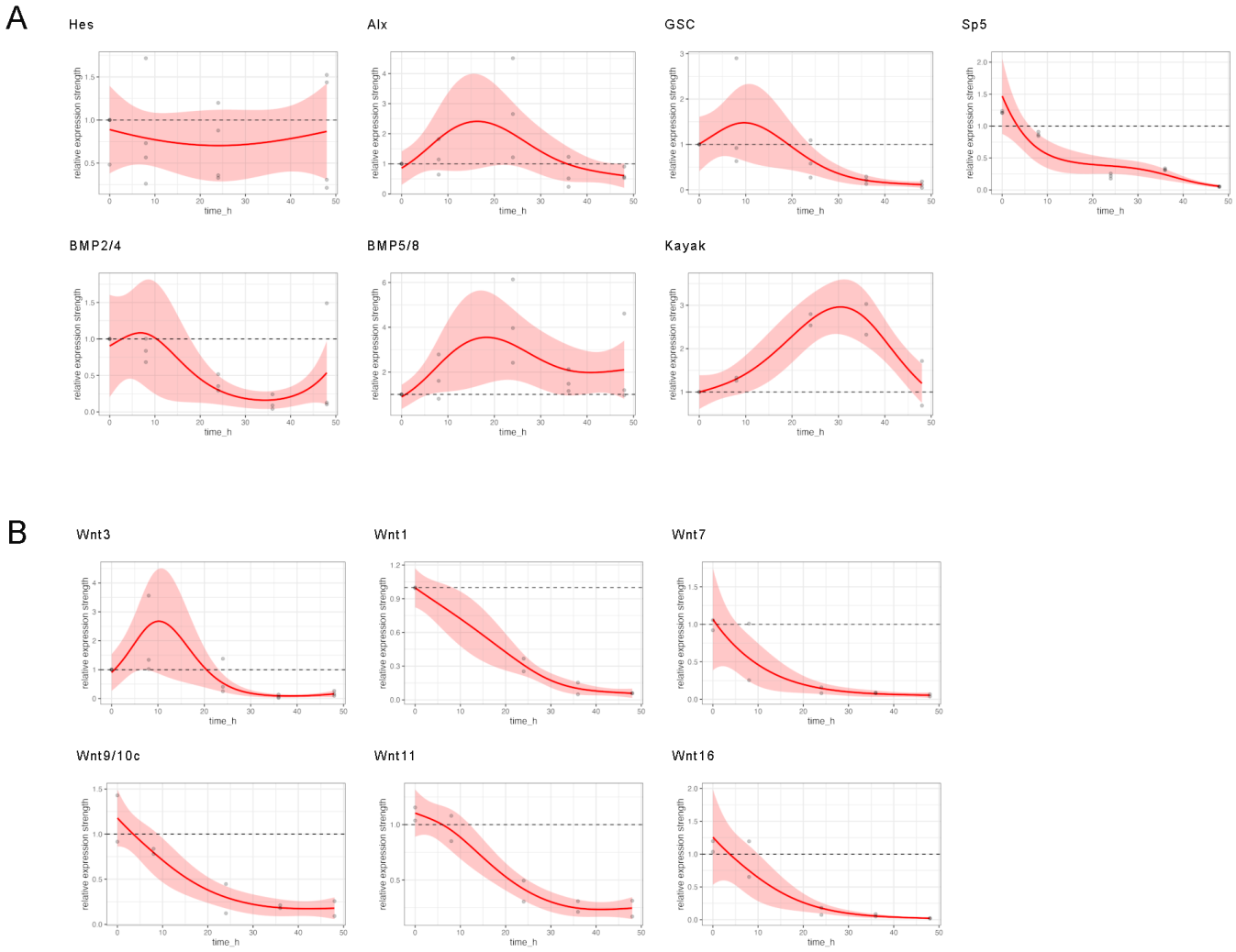

Figure 3: Gene expression dynamics of selected genes in DAPT inhibited regenerates. GAM-based visualisation of gene expression as measured by RT-qPCR in DAPT-treated animals relative to control animals (y-axis) depending on the time after head removal (x-axis). Grey points show raw data (quotients of mean values of DAPT-treated relative to DMSO-treated animals), the coloured lines show the smooth GAM-based estimates and colour-shaded areas are 95% confidence bands. Gene expression was followed for 48 hrs after head removal in DMSO control and DAPT. For time point 0 polyps were used immediately after head removal without any exposure to inhibitor or control medium. **(A)** *HyHes, HyAlx, CnGsc, HySp5, HyKayak, HyBMP2/4* and *HyBMP5/8.* **(B)** *HyWnt3* and *1, 7, 9/10c, 11, 16* during 48 hrs. Relative normalized expression was related to the housekeeping genes GAPDH, RPL13, EF1α and PPIB.

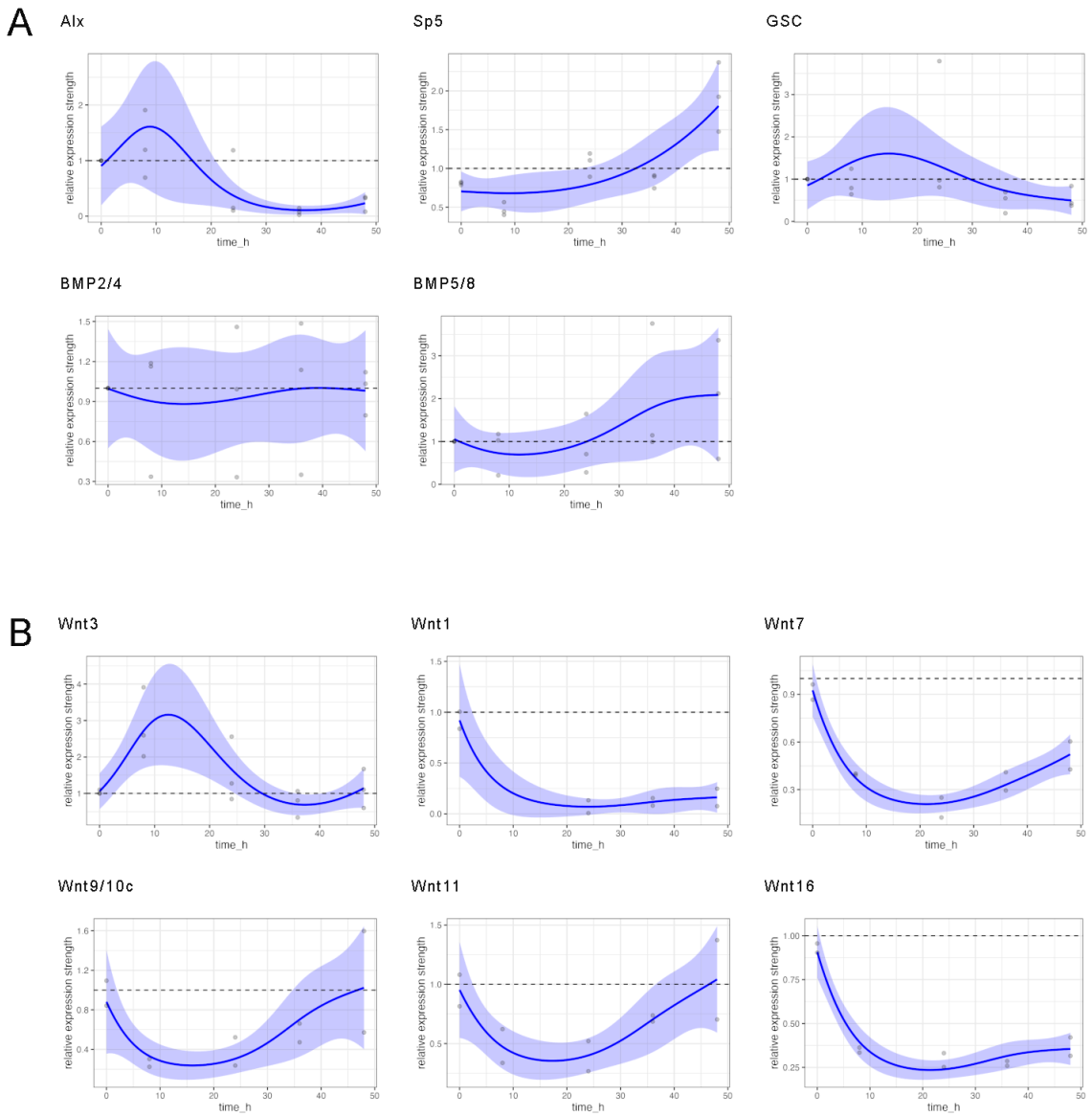

Figure 4: Gene expression dynamics of selected genes in iCRT14 inhibited regenerates. GAM-based visualisation of gene expression as measured by RT-qPCR in iCRT14-treated animals relative to control animals (y-axis) depending on the time after head removal (x-axis). Grey points show raw data (quotients of mean values of DAPT-treated relative to DMSO-treated animals), the coloured lines show the smooth GAM-based estimates and colour-shaded areas are 95% confidence bands. Gene expression was followed for 48 hrs after head removal in DMSO control and iCRT14. For time point 0 polyps were used immediately after head removal without any exposure to inhibitor or control medium. **(A)** *HyAlx, CnGsc, HySp5, HyBMP2/4* and *HyBMP5/8.* **(B)** *HyWnt3, 1, 7, 9/10c, 11* and *16* during 48 hrs of *Hydra* head regeneration in iCRT14 or DMSO control. Relative normalized expression was related to the housekeeping genes GAPDH, RPL13, EF1alpha and PPIB.

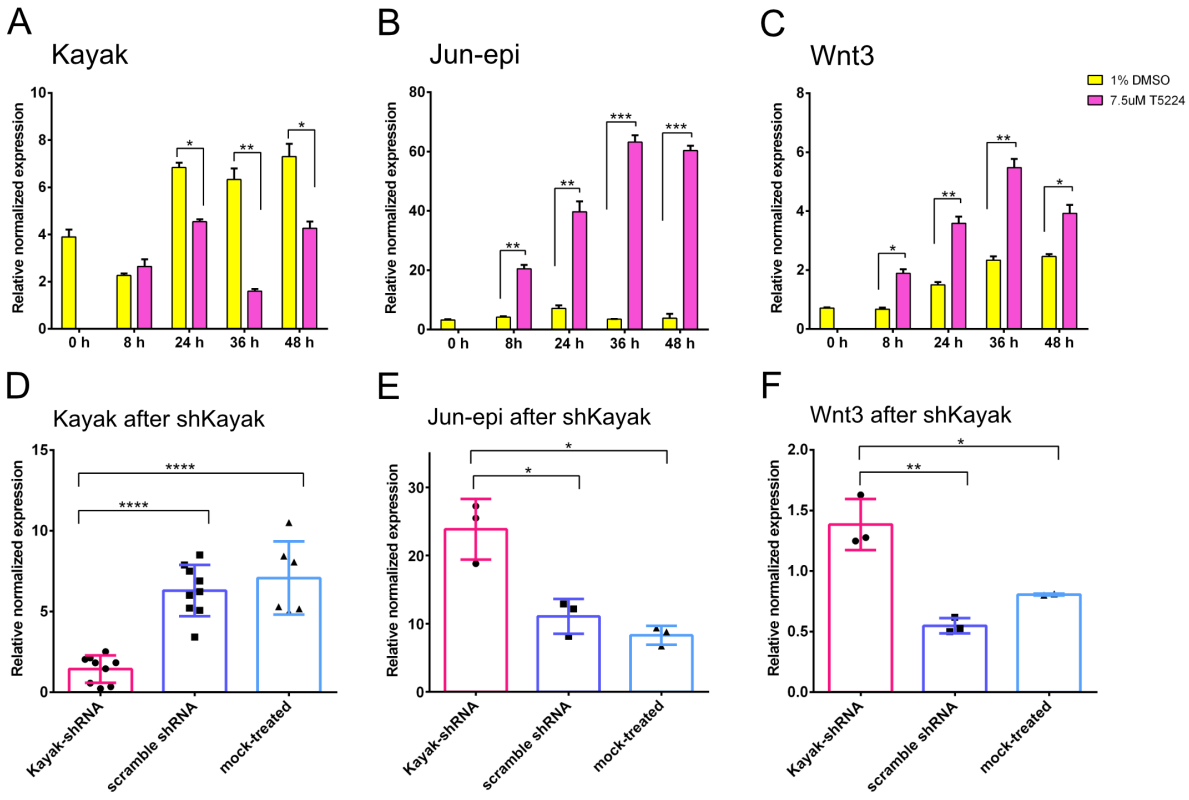

Figure 5: Gene expression dynamics of selected genes in *T5224* treated regenerates corresponds with gene expression in kayak knockdown polyps

RT-qPCR measured the expression of *HyKayak* **(A)** *HyJun_epi* **(B)** and *HyWnt3* **(C)** for 48 hrs after head removal in DMSO control and in 7.5 µM T5224 for inhibition of Fos/AP1 activity. For time point 0 polyps were used immediately after head removal without any exposure to inhibitor or control medium. RT-qPCR measured the expression levels of **(D)** *HyKayak,* **(E)** *HyJun-epi* and **(F)** *HyWnt3* after knockdown *of HyKayak* by shRNA, compared to mock-treated control and control with scramble shRNA. Relative normalized expression was related to the housekeeping genes GAPDH, RPL13, EF1alpha and PPIB.

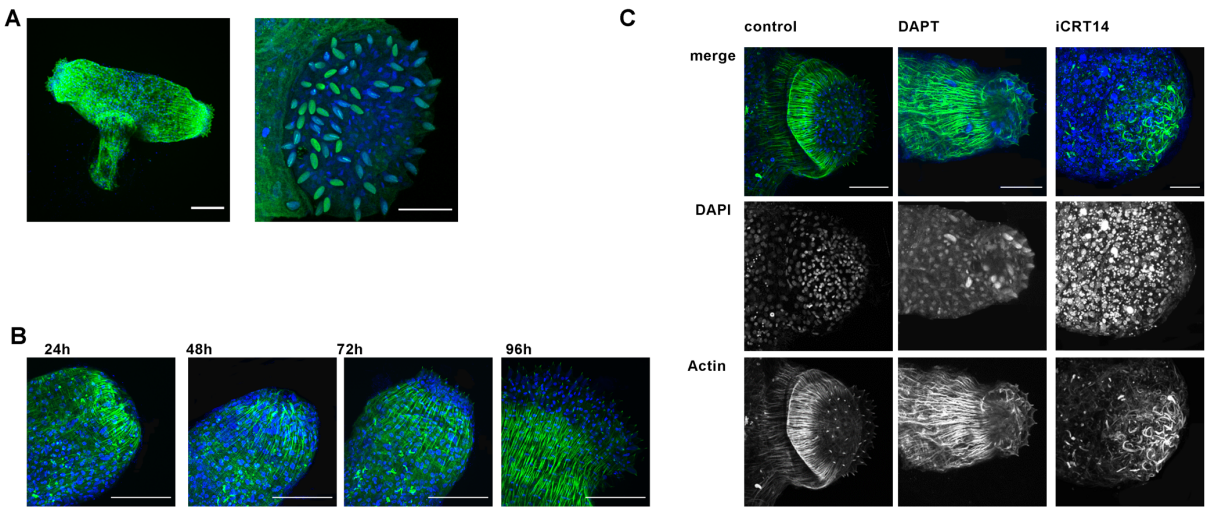

Figure 6: Head regeneration of *Craspedacusta* polyps.
(A) *Craspedacusta* colony with two animals sharing one foot, Scale bar: 100 µm. Magnification of polyp head with nematocytes surrounding the hypostome, Scale bar: 50 µm. Nematocyte capsules stained with DAPI (green). **(B)** Regeneration of *Craspedacusta* polyps at 24, 48, 72 and 96 hrs after head removal with reappearing nematocytes and actin fibres. Scale bar: 50 µm. **(C)** Regenerated heads 96 hrs after head removal in DMSO control, DAPT and iCRT14 as indicated. Scale bar: 50 µm. Actin fibres are shown in green via FITC-phalloidine and nuclei in blue with DAPI.

Table 1: Gene expression results with Notch- and Wnt-signalling inhibition.
RT-qPCR results of investigated genes: *HyHes, CnGsc, HySp5, HyWnt3, 1, 7, 9/10c, 11, 16, HyBMP2/4, HyBMP5/8, HyAlx* and *HyKayak* with inhibition of Notch-signalling by DAPT (red) and inhibition of β-catenin transcriptional activity by iCRT14 (blue). Gene expression is classified within the initial first 8 hrs and between 24-48 hrs of regeneration; up-regulation is indicated by green arrows, downregulation by yellow arrows. Number of arrows refers to strength of the effect.

	groups / genes	DAPT treatment		iCRT14 treatment	
		0-8 hrs	24-48 hrs	0-8 hrs	24-48 hrs
target genes of Notch	Hes	↓↓↓	↓		
	GSC	---	↓↓↓	---	---
	Sp5	---	↓↓↓	---	---
hypostome marker	Wnt3	↑	↓↓↓	↑	---
	Wnt1	---	↓↓↓		↓↓↓
	Wnt7	---	↓↓↓	↓↓↓	↓↓↓
	Wnt9/10c	---	↓↓↓	↓↓↓	↓
	Wnt11	---	↓↓↓	↓↓↓	↓
	Wnt16	---	↓↓↓	↓↓↓	↓↓↓
	BMP2-4	---	↓↓↓	---	---
tentacle marker	Alx	---	---	---	↓↓↓
	BMP5-8	---	↑↑↑	---	---
Inhibitor	Kayak	---	↑↑↑		

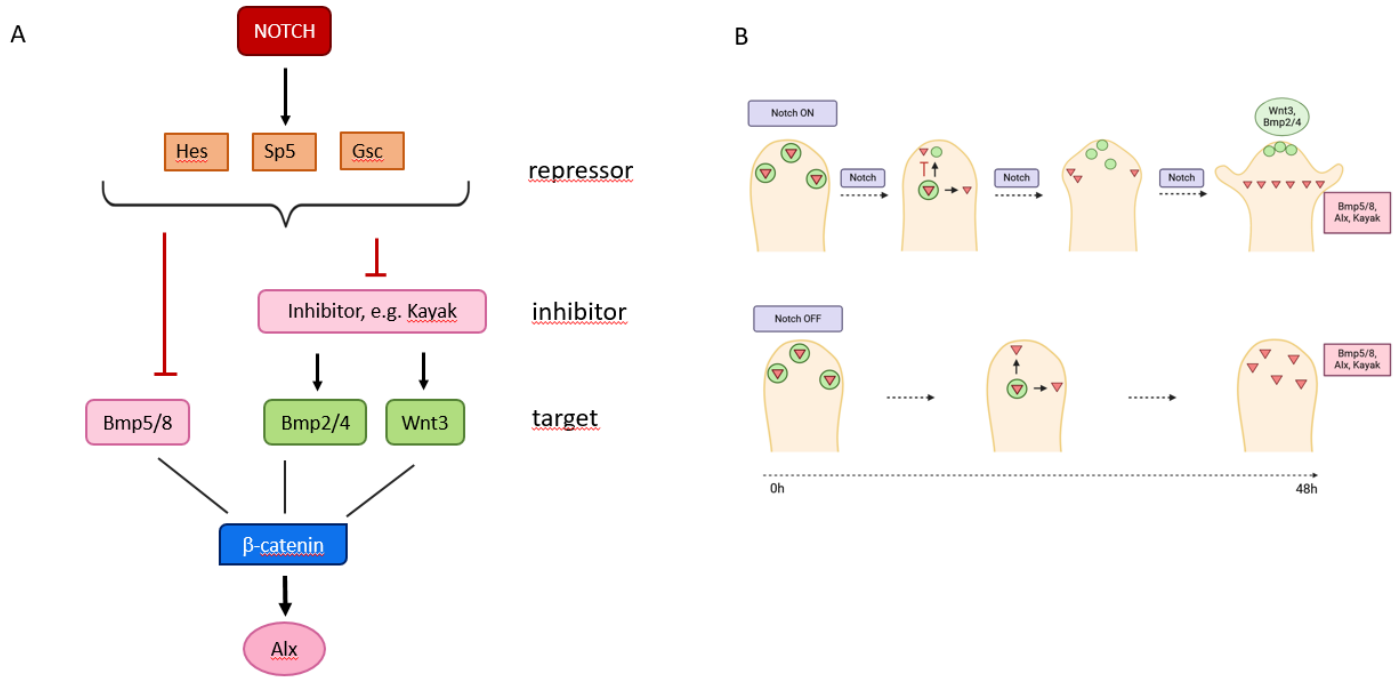

Figure 7: Lateral inhibition model for Notch function during *Hydra* head regeneration
(A) Schematic overview of gene interactions dependent on Notch-signalling during *Hydra* head regeneration. Transcriptional repressors HyHes and CnGsc are activated upon Notch-signalling. Their targets are HyBMP5/8, and inhibitors of HyWnt3 and HyBMP2/4. HyKayak is a strong candidate for a HyWnt3-inhibitor. Notch induced repression of these inhibitors frees the way for expression of HyWnt3 and HyBMP2/4 at the newly forming hypostome and peritentacular tissue, while repression of HyBMP5/8 is maintained in these head regions. β-catenin dependent transcription activates tentacle formation by inducing expression of HyAlx. **(B)** Model for *Hydra* head regeneration in the presence (upper panel) and absence of Notch-signalling (lower panel). After head removal the potential to re-form a hypostome (green circles) and tentacles (red triangles) arises in the regenerating tip of the polyp. Notch-signalling then mediates inhibition of the tentacle fate in the upper part of the regenerate by repressing HyBMP5/8 and inhibitors of HyBMP2/4 and HyWnt3. This allows establishing of the hypostomal axis with formation of the hypostome and peritentacular region, while pushing the tentacle region downwards. With inhibition of Notch (lower panel) repression of hypostome genes (HyWnt3 and HyBMP2/4) is maintained. Therefore only unorganized tentacle fate is possible (red triangles).