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Evolution of Developmental Control Mechanisms

# Notch signalling defines critical boundary during budding in *Hydra*

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ABSTRACT

Boundary formation is an important mechanism of development and has been studied in a number of bilaterian model organisms where it is often controlled by Notch, FGF and Wnt signalling. Tissue boundaries are also formed in simple pre-bilaterian animals. The boundary between parent and bud during asexual reproduction in the fresh water polyp *Hydra vulgaris* is an example. The *Hydra* homolog of the FGF-receptor FGFR (*kringelchen*) and some components of the Wnt signalling pathway are expressed at this boundary, but their precise functions are unknown. In this work we have discovered an important role for Notch signalling at this boundary. Notch signalling is needed to sharpen the *kringelchen* expression zone during the final budding stages from an initially broad band into a clear line demarcating the boundary between bud and parent. Expression of the Notch target gene *HyHes* and the putative matrix metalloprotease MMP-A3 was observed at the boundary shortly before the bud began to constrict and differentiate foot cells. When Notch signalling was inhibited with the presenilin inhibitor DAPT the expression pattern for *kringelchen* changed dramatically into a diffused pattern. The expression of both *HyHes* and *MMP-A3* was abolished. Moreover, morphogenesis of the bud was not completed and buds did not constrict, failed to form a foot and never detached from the parent. This resulted in the formation of two-headed animals. We suggest that the function of Notch signalling during budding in *Hydra* is in promoting the formation of two stripes of differing gene expression, which are needed to differentiate the foot of the bud and a progressing narrowing of the mesoglea on the side of the parent.



## Introduction

Development in metazoans from the fertilised egg into highly patterned structures with differentiated tissues and organs is governed by a relatively small number of signalling pathways, which regulate the expression of defined master transcription factors that govern embryonic development. In recent years and especially advanced through analyzing EST and whole genome sequencing projects, it has become clear that the major developmental signalling pathways arose in metazoans before the appearance of bilaterians. One of these pathways is the Notch signalling pathway. Notch signalling is involved in many developmental processes where it regulates cell fates and pattern formation. The latter often involves the establishment of boundaries between developing tissues (reviewed by Lai, 2004). Notch signalling appears especially well suited to direct boundary formation because both Notch ligands (Delta, Jagged and Serrate) and Notch receptors are transmembrane proteins. Therefore signalling occurs when the signalling cell is in physical contact with the responder cell.

We have recently begun to analyse Notch signalling in the fresh water hydrozoan *Hydra vulgaris* (Käsbauer et al., 2007). *Hydra is* a member of the pre-bilaterian phylum Cnidaria. Its body plan is simple with only one body axis. It has a hypostome with a ring of tentacles at the apical end and a peduncle with a basal disc at the basal end. The entire body of a hydra is formed from two epithelial cell layers, the ectoderm and the endoderm. Epithelial cells in the hydra body column proliferate and get displaced into the tentacles and into the foot, where they differentiate into battery or foot cells. Spaces between the epithelial cells are populated by cells of the interstitial cell lineage. These include pluripotent stem cells and their differentiation products, nerve cells, gland cells, nematocytes and, in sexually reproducing animals, germ cells (David and Murphy, 1977).

The two epithelial cell layers are separated by the acellular mesoglea, an extracellular matrix (ECM) containing collagen and laminin (Fowler et al., 2000; Sarras and Deutzmann, 2001; Sarras et al., 1994). The mesoglea is a self supporting structure, which reflects accurately the shape of a hydra even after removal of the epithelial

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cells (Day and Lenhoff, 1981; Shimizu et al., 2008). It has recently been characterised by fluorescence and electron microscopy (Shimizu et al., 2008). Less understood is what shapes the mesoglea during morphogenesis. A single *Hydra* matrix metalloprotease (HMMP) has been described previously. It is ubiquitously expressed in hydra tissue (endoderm) and was shown to be able to digest hydra ECM components *in vitro* (Leontovich et al., 2000). It was hypothesized that this enzyme could facilitate morphogenesis by acting on the ECM. Here we have identified a novel matrix metalloprotease, MMP-A3, with 52% sequence identity to HMMP and an identical domain structure. We show that it is specifically expressed during budding at late stages when the bud constricts.

Budding is the predominant way of reproduction in *Hydra*. During this process parent tissue is displaced directly into the bud. Budding is initiated in the lower third of the parent body column, the budding zone (Otto and Campbell, 1977). Cells in concentric rings around the tip of the bud are recruited from the parent animal until the bud has reached a certain extension. Then morphogenesis begins. First tentacle rudiments appear at the tip of the bud. Shortly after that the body column constricts at the base of the bud. Between the constricted region and the parent tissue, the bud then differentiates basal disc cells (Otto and Campbell, 1977). After completion of the basal disc the bud detaches from the parent animal.

Notch signalling involves regulated intracellular proteolysis, mediated by the membrane protease presenilin (RIP; Kopan et al., 1996), which is part of the γ-secretase complex. Components of this complex and the Notch signalling mode are conserved in *Hydra* (Gazave et al., 2009; Käsbauer et al., 2007). Notch signalling can be inhibited with the presenilin inhibitor DAPT. Such inhibition studies have revealed that *Hydra* Notch signalling is needed for post-mitotic differentiation of nematocytes and female germ cells (David and Murphy, 1977; Käsbauer et al., 2007).

Here we are reporting that Notch signalling is also necessary for the formation of a boundary between bud and parent animal in *Hydra*. The sole *Hydra* homolog of *hairy/enhancer of split* (hes) genes is expressed at this boundary. *Hyhes* belongs to the hairy/enhancer of split (Hes) gene family, which includes prominent target genes for Notch signalling in bilaterian model organisms. Members of this family encode nuclear repressor proteins that belong to the group E of the bHLH superfamily, an ancient class of eukaryotic transcription factors (Simionato et al., 2007). We have only found one *Hes* gene in *H. vulgaris*, which is consistent with previous analysis of the bHLH gene family in *Hydra magnipapillata* (Simionato et al., 2007). Promoter analysis in the upstream region of this gene showed the presence of binding sites for the canonical repressors of Notch target genes, human CBF1, *Drosophila* Su(H) and *Caenorhabditis* Lag1 (CSL binding sites (Ehebauer et al., 2006)).

The *Hydra* FGF-receptor homolog Kringelchen is also important for budding. It is expressed early at the tip of the bud. At the end of parental tissue recruitment, *kringelchen* is expressed in a circular zone surrounding the buds base and stays there until the bud detaches. After detachment, a small ring of epithelial cells continue expressing it on the parent tissue (this is the name giving feature of this gene, "*kringelchen*" means "small ring" in German; Sudhop et al., 2004). In the present study we report that inhibition of Notch signalling prevents *HyHes* expression and changes the expression of *kringelchen* at the bud's base into a diffused pattern. This in turn prevents the expression of the novel matrix metalloprotease MMP-A3 and thus constriction of the buds base and its detachment from the parent animal.

## Materials and methods

### *Hydra culture*

*H. vulgaris* strain Basel were grown in mass culture at a constant temperature of 18 °C in hydra medium (0.1 mM KCl, 1 mM NaCl, 0.1 mM $MgSO_4$, 1 mM Tris and 1 mM $CaCl_2$). The animals were fed regularly with freshly hatched *Artemia nauplii*.

### *HyHes reporter cloning*

HvNICD and $HvN^{\Delta E}$ (amino acids 25-516 deleted) were cloned into the HoTG(reen) expression vector (HoTG(reen) for the EGFP expression vector) using *Sma*I and *Eco*RI sites for HvNICD and *Nhe*I and *Eco*RI restriction sites for $HvN^{\Delta E}$ (Böttger et al., 2002). The GFP coding sequence was replaced by Notch sequences for overexpression of untagged proteins from the *Hydra* actin promoter yielding plasmids HoTHvNICD and $HoTHvN^{\Delta E}$. The 600-bp sequence upstream of the spliced leader acceptor site of the *HyHes* genomic region was amplified via PCR using primers 5′-CATTGCACCCA AGTGTTTGAGGAAAA-3′ and 5′GGATGTCTTTCTGTGTCCGT CAT-3′ and cloned into the pCR-bluntII-TOPO vector (Invitrogen). They were then cloned into the *Pst*I sites of the HoTG expression vector replacing the actin promoter sequence yielding the vector HePG(reen). For analyzing the S1/S2 mutation the sequence at −206 to −200 was changed from CACGCTT to GAGGGTT.

### *HyHes cloning*

A *Hairy/Enhancer of split* homolog was identified after a Blast Search of the *Hydra* genome with the amino acid sequences of mouse HES1 and 2. Based on the gene model Hma2.221888 (Contig38052: 56016…57542), primers were designed: 5′-ATGACGGACACAGAAA-GACATCC-3′ and 5′-TTATTCGTACGGTCGCCAAACTA-3′. These were used for PCR amplification from *Hydra* cDNA. The resulting 519-bp cDNA was named *HyHes* and cloned into the vector pBluescriptII KS (Fermentas) and used as DNA template for in situ RNA probe synthesis.

### *Hydra MMP-A3 cloning*

A 1401-bp fragment encoding a putative MMP was amplified by PCR from *Hydra* cDNA using primers 5′-TGAGAGTTTTCAAGAC-GAACCA-3′ and 5′- AGCAGTGGAGGAAATCGTTC-3′ and cloned into the vector pCR II-TOPO (Invitrogen). The primers were designed based on the gene model Hma2.206710 (Contig33069: 27149… 23842), encoding a putative new *Hydra* MMP, which was found after blasting the *Hydra* genome with the nucleotide sequence of HMMP (Leontovich et al., 2000). The novel gene was termed MMP-A3 as one of several genes found in the Blast search. The 1401-bp fragment was used as template for in situ probe synthesis.

### *Transfection of hydra cells*

Gold particles (1.0 μm, BioRad) were coated with plasmid DNA according to instructions of the manufacturer. They were introduced into hydra cells with the Helios gene gun system (BioRad) as previously described (Böttger et al., 2002).

### *Whole-mount in situ hybridisation*

Single and double whole-mount in situ hybridisation experiments with either digoxigenin or fluorescein labeled RNA probes were carried out as described (Grens et al., 1995; Hansen et al., 2000; Lindgens et al., 2004).

### *DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) treatment*

A 10-mM stock of DAPT (Calbiochem) in DMSO was diluted in hydra medium and applied at a concentration of 40 μM. The final DMSO concentration was always 1%. The DAPT medium was renewed

every 12 h over a period of 48 h. Control animals were treated with hydra medium containing 1% DMSO.

*Antibody staining*

Anti-Notch antibody was obtained after immunization of chicken with a Notch protein fragment corresponding to the intracellular domain of *Hydra* Notch, which had been expressed in *Escherichia coli* from the plasmid pRSET:HvNotch and purified under denaturing conditions. Immunglobulin (IgY) was purified from egg yolk and diluted according to titer. Part of the IgY fraction was affinity purified on immobilized Notch protein on sepharose and shown to have the same properties towards the antigen as the IgY fraction. IgY was then used in the experiments. For staining, animals were relaxed in 2% urethane in hydra medium and fixed with 2% paraformaldehyde in hydra medium for 1 h at room temperature. They were washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 15 min, blocked with 0.1% Triton X-100, 1% BSA in PBS and incubated with anti-Notch chicken IgY overnight at 4 ° C. After three washes with PBS, animals were incubated for 2 h with anti-chicken-Cy3 or anti-chicken-AMCA-conjugated second antibody (Molecular Probes). They were washed again three times with PBS, counterstained for DNA with TO-PRO-3 (Molecular Probes, 1 μg/ml in PBS) and mounted on slides with Vectashield mounting medium (Alexis Biochemicals). For controls, the first or second antibody was omitted in individual staining reactions. As a result, the specific staining of the respective structures was lost (not shown).

*Confocal imaging*

After treatment of hydra animals with 2% urethane in hydra medium, they were fixed with 2% paraformaldehyde in hydra medium for 1 h at room temperature. Fixed animals were counterstained for DNA with 1 μM TO-PRO-3 (Molecular Probes) in PBS for 5 min and mounted on slides with Vectashield mounting medium (Alexis Biochemicals) prior to scanning. Light optical serial sections were acquired with a Leica (Leica Microsystems, Heidelberg, Germany) TCS SP confocal laser-scanning microscope equipped with an oil immersion Plan-Apochromat 100/1.4 NA objective lens. Fluorochromes were visualized with an argon laser with excitation wavelength of 488 nm and emission filters of 520–540 nm for EGFP. For mRFPmars and Cy3, a krypton laser with excitation wavelength of 568 nm and emission filters of 575–585 nm was used. The helium–neon laser with excitation wavelength of 633 nm and emission filter of 660–760 nm was for TO-PRO-3. Image resolution was 512×512 pixel with a pixel size ranging from 195 to 49 nm depending on the selected zoom factor. The axial distance between optical sections was 300 nm. To obtain an improved signal-to-noise ratio, each section image was averaged from three successive scans. The 8-bit grey scale single-channel images were overlaid to an RGB image assigning a false colour to each channel and then assembled into tables using Adobe Photoshop 8.0 and ImageJ 1.32j software.

*Phylogeny and gene analyses*

Neighbour joining (NJ) trees were calculated from ClustalX multiple alignments of protein sequences (conserved basic helix–loop–helix domain) with 10.000 bootstrap replica. The trees were displayed using NJplot (Thompson et al., 1997). GenBank nucleotide accession numbers of *Hes* genes used for phylogenetic analysis are as follows: *Helobdella robusta Hes*: A144625; *Branchiostoma floridae HairyA*: AY349467; *Ciona intestinalis HairyA*: NM_001078521; *Strongylocentrotus purpuratus Hairy/Enhancer of split*: AY445629; *Caenorhabditis elegans lin-22*: AF020555; *Homo sapiens Hes1/Hairy*: L19314, *Hes2*: AL031848, *Hes3*: AL031847 (chromosome 1), *Hes4*: AB048791, *Hes5*: NT_004350 (chromosome 1), *Hes6*: AB035179, *Hes7*: AB049064; *Drosophila melanogaster Hairy:* X15905, *E(spl)m3*: M96165, *E(spl)m8*: X16553, *E(spl)mβ*: X67047; and *Mus musculus Hes1*: NM_008235, *Hes2*: NM_008236, *Hes6*: AB035178, *Hes7*: AB049065.

*Analysis of exon–intron structure*

For mouse, *Hes* genes data for the exon–intron structure were taken from gene models of the mouse genome informatics (Bult et al., 2008). The genomic data for *HyHes* were taken from the hydra genome website http://www.hydrazome.metazome.net at JGI (Joint Genome Institute) and CIG (Center for Integrative Genomics, UC Berkley).

*Promoter analysis*

Analysis of the *HyHes* promoter sequence was performed manually on the basis of the following consensus sequences: CSL high affinity binding site GTG A/G GAA and its complement CAC T/C CTT; inverted sequence AAG G/A GTG with its complement TTC C/T CAC; CSL low affinity binding sites with R as first, fourth and sixth base of the high affinity site; N-Box: CACNAG; Class C (binding site for bHLH repressors) CACGNG; E- Box (binding sequences for bHLH activation proteins): CANNTG; subgroup class A: CANCTG and class B: CANGTG. For confirmation, the following online programs were used: PROMO (Farre et al., 2003; Messeguer et al., 2002); TFSEARCH (http://www.rwcp.or.jp/papia) and TEES (Jonathan Schug and G. Christian Overton, 1997, URL: http://www.cbil.upenn.edu/tess).

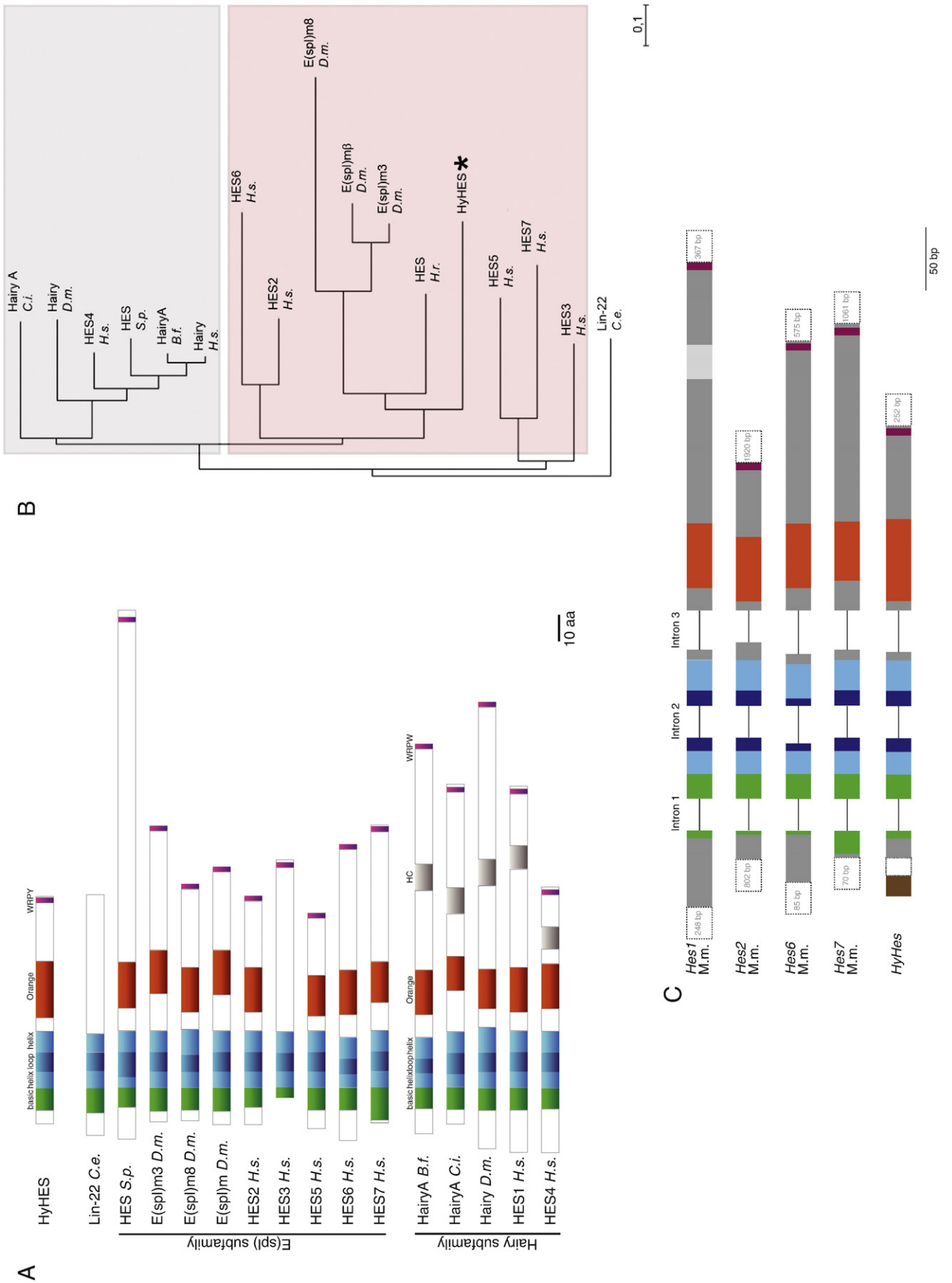
## Results

*The Hydra Hes gene*

The complete HES protein sequence from *Hydra* (HyHES) is shown in Fig. S1 in an alignment with HES5 from mouse. The highly conserved basic helix–loop–helix regions with their conserved proline residues and the Orange domains are indicated. The *Hydra* Orange domain has two putative α-helices (according to PredictProtein) and it is 14 amino acids longer than Orange domains from mouse and *Drosophila* (Dawson et al., 1995). The conserved C-terminal WRPW motif is modified in the Hydra sequence to WRPY. Phylogenetic analysis of HyHES with hairy and "Enhancer of split" proteins from vertebrates and invertebrates reveal that HyHES belongs to the "Enhancer of split" subfamily. This is confirmed by comparison of the domain structures (Figs. 1A, B). Hairy proteins have a C-terminal HC domain, which is not found in the *Hydra* sequence (Fig. 1A). Analysis of the genomic organisation of the *HyHes* gene indicates four exons and three introns, all of which are conserved in mammalian *Hes* genes (shown for four mouse *Hes* genes in Fig. 1C, modified from Sakagami et al., 1994). The first intron interrupts the coding region for the basic domain, the second lies in the region encoding the loop of the HLH domain and the third is in the sequence encoding the part between the HLH and the Orange domain. Splicing occurs at canonical sites (GT- AG).

*The HyHes promoter can be regulated by HvNotch*

The *HyHes* genomic region up to 2.2 kb from the transcriptional start site is shown in Fig. 2. The AG-splice acceptor for the spliced leader is indicated (Stover and Steele, 2001). At positions 27 and 40, nucleotides upstream from this sequence TATA box sequences are present (not shown). Two potential CSL binding sites are located at −202 and −206 from the spliced leader acceptor site, three more are found 2035 and 2189–2193 nucleotides upstream. Their arrangement differs from that of the consensus Su(H) binding sites from *Drosophila enhancer of split* genes and CBF binding sites from mouse *Hes1* and *Hes7* genes. The typical "paired sites" consisting of two antiparallel


A
HyHES
Lin-22 C.e.
HES S.p.
E(spl)m3 D.m.
E(spl)m8 D.m.
E(spl)m D.m.
HES2 H.s.
HES3 H.s.
HES5 H.s.
HES6 H.s.
HES7 H.s.
HairyA B.f.
HairyA C.i.
Hairy D.m.
HES1 H.s.
HES4 H.s.
basic helix loop helix
Orange
WRPY
HC
WRPW
E(spl) subfamily
Hairy subfamily
10 aa
B
Hairy A C.i.
Hairy D.m.
HES4 H.s.
HES S.p.
HairyA B.f.
Hairy H.s.
HES6 H.s.
HES2 H.s.
E(spl)m8 D.m.
E(spl)mβ D.m.
E(spl)m3 D.m.
HES H.r.
HyHES *
HES5 H.s.
HES7 H.s.
HES3 H.s.
Lin-22 C.e.
0,1
C
Intron 1
Intron 2
Intron 3
Hes1 M.m.
248 bp
802 bp
85 bp
70 bp
367 bp
Hes2 M.m.
1920 bp
Hes6 M.m.
575 bp
Hes7 M.m.
1061 bp
HyHes
252 bp
50 bp

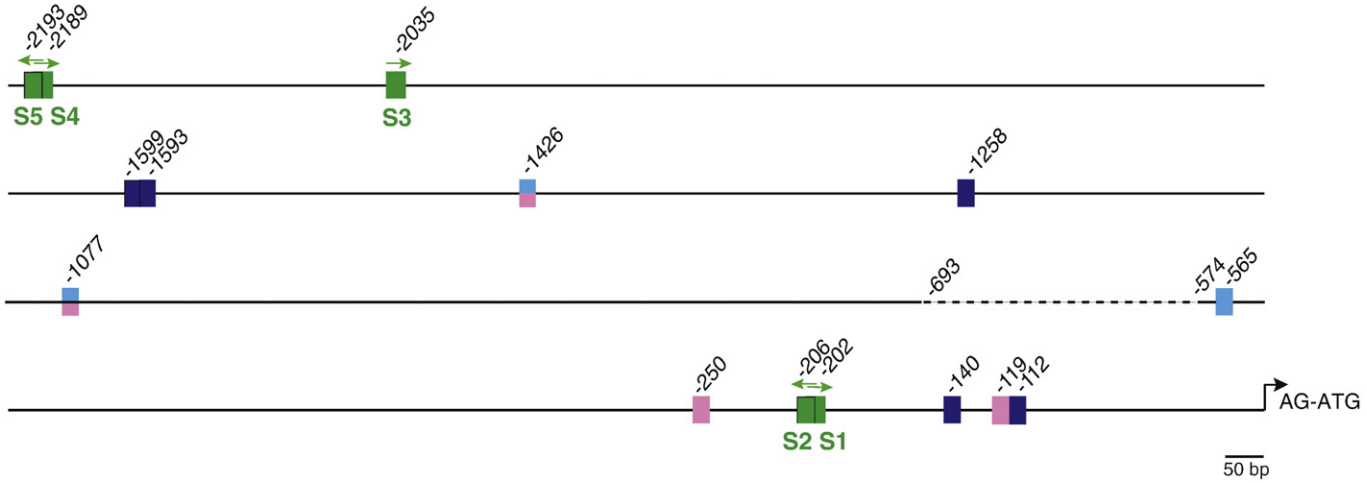



**Fig. 2.** Analysis of the *HyHes* promoter. *Cis*-regulatory sequences in the genomic region 2200 bp upstream of the spliced leader acceptor site for the *HyHes* mRNA: binding sites for CSL (CBF1, Su(H) and **L**ag1) are illustrated as green boxes and are numbered from S1 to S5 (CSL consensus sequence GTG R GAA); arrows indicate the orientation of the binding site on the DNA sense strand; binding sites for bHLH activators (E-boxes, dark blue boxes, consensus sequence CANNTG with subgroup class B consensus CANGTG in light blue) and binding sites for bHLH repressor proteins (N-box in purple, consensus sequence CACGNG, with subgroup class C consensus CACGNG). The dotted line indicates a stretch of highly repetitive DNA. Negative numbers refer to nucleotides upstream of the spliced leader acceptor site AG.

consensus CSL sites separated by 30 nucleotides are not found (Bailey and Posakony, 1995; Nellesen et al., 1999). In the *HyHes* promoter, there are only single sites. However, S2 and S1 as well as S4 and S5 are actually overlapping antiparallel sites (see Fig. 2 and Fig. S2). In addition, putative binding sites for bHLH repressor proteins (class C-site, subtype of N-box) are present in the upstream region of *HyHes*, indicating the potential for autorepression of the *HyHes* promoter by HyHES protein (Kramatschek and Campos-Ortega, 1994). Moreover, bHLH activator sites are present (E-box and subtype of E-box, class B), indicating the potential for synergistic gene activation by the intracellular domain of Notch (NICD) and bHLH transcriptional activators (Cave et al., 2005).

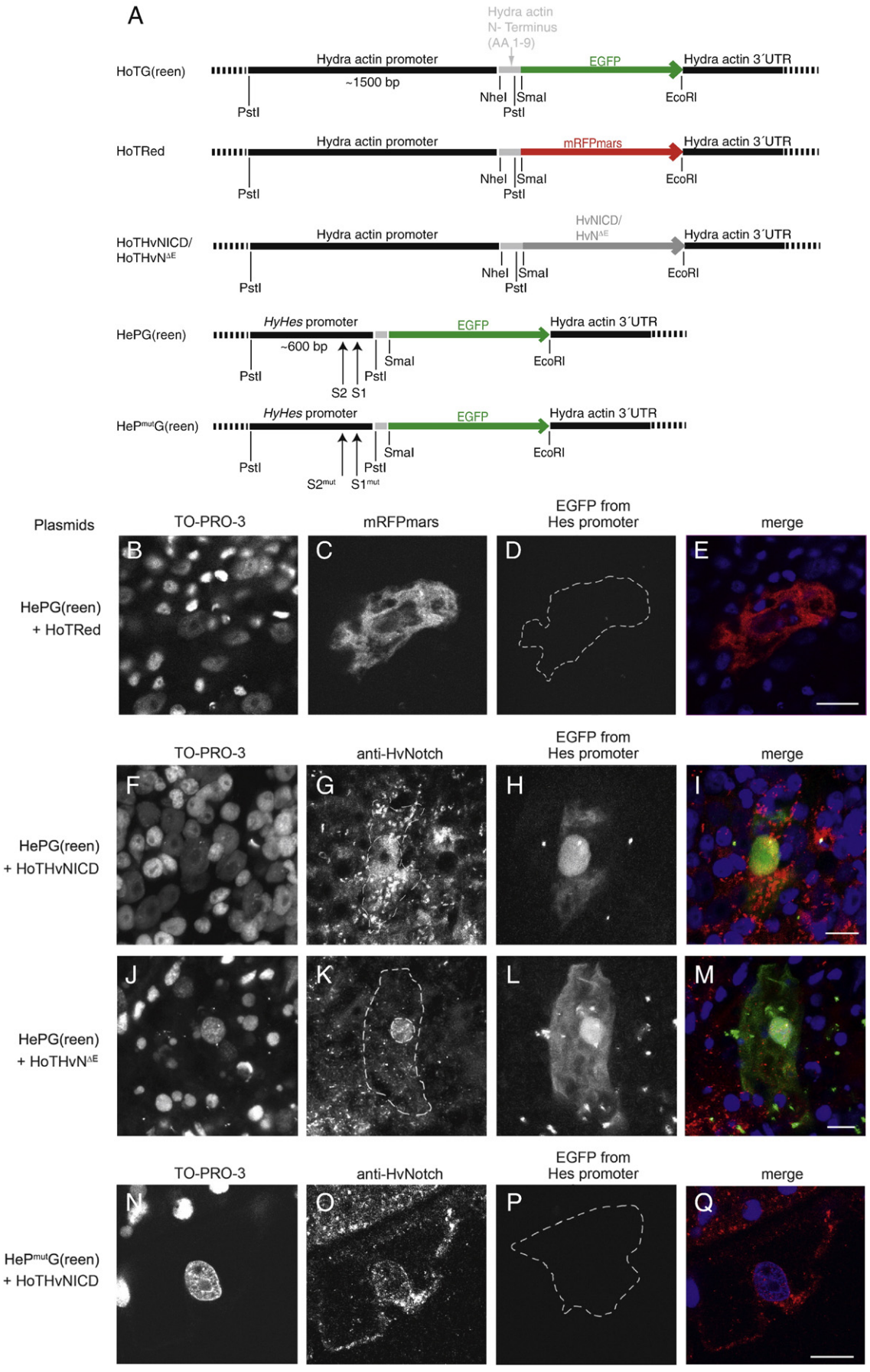
In order to analyse whether the expression of *HyHes* was under the control of NICD, we performed an *in vivo* assay. We used a DNA reporter construct expressing GFP under the control of the *HyHes* promoter. The first 600 nucleotides of the *HyHes* promoter region were cloned into the vector HoTG (EGFP expression vector; Böttger et al., 2002), where they replaced the actin promoter. The new plasmid (HePG(reen)) was biolistically transfected into hydra cells together with the plasmid HoTRed, which allows constitutive expression of mRFPmars from the actin promoter (Fig. 3A). In this assay, it served the identification of transfected cells (Müller-Taubenberger et al., 2006). Transfection resulted in an average of ten cells per animal expressing mRFPmars as is expected for single cell gene gun transfection of hydra cells. However, these cells never expressed EGFP from the *HyHes* promoter (Figs. 3B–E). We then introduced HePG together with HoTHvNICD into the cells (expression of untagged HvNICD from the actin promoter of HoTG; Fig. 3A). Figs. 3F–I show an ectodermal epithelial cell now expressing EGFP from the *HyHes* promoter. Antibody staining with a specific anti-Notch antibody detected HvNICD in the nucleus (Fig. 3G). In this experiment, we also obtained an average of ten cells per animal expressing EGFP from the *HyHes* promoter. Nuclear signals with anti-Notch antibody staining were obtained in every one of them. Finally, we introduced a membrane bound HvNotch mutant, which lacks the ectodomain and is thus expected to be constitutively active, into hydra cells (HvN$^{\Delta E}$; Käsbauer et al., 2007). Again, we found cells with EGFP expression driven by the *HyHes* promoter (Figs. 3A and J–M).

In order to see whether the EGFP expression from the *HyHes* promoter depended on the putative CSL binding sites, we created a modified HesP reporter with mutated S1 and S2 sites (from CACGCTT to GAGGGTT; Fig. 3A). After cotransfection of this HeP$^{mut}$G(reen) plasmids with HoTHvNICD in the same experimental setup as described above for HePG(reen) no EGFP expressing cells could be detected (Figs. 3N–Q). From these data, we conclude that *HyHes* expression can be controlled by Notch signalling in *Hydra* and that the S1/S2 sites are critical.

### *HyHes is expressed at the base of the bud shortly before foot formation*

Next we performed *HyHes* in situ hybridisation experiments on whole mounts (Figs. 4A–D). Expression of *HyHes* was seen during budding and was analysed in detail. Budding stages were classified as described by Otto and Campbell (Otto and Campbell, 1977). In order to correlate these stages with the formation of foot tissue whole-mount in situ hybridisation for the foot specific gene *PPOD-1* was performed (Figs. 4M–P; Hoffmeister-Ullerich et al., 2002; Pauly et al., 2003). In early stages of budding, no specific *HyHes* expression could be found (not shown). A clear signal appeared during budding stage 8 when the base of the bud constricted (stage 8, Figs. 4B, C). Expression was restricted to a small ring of ectodermal cells running circumferentially around the base of the bud. The signal disappeared at stage 9, when the bud formed foot tissue (Figs. 4D and P).

**Fig. 1.** *HyHes* gene and protein analysis. (A) Schematic representation of the protein domain structure of different HES proteins. Included are basic domain (green), HLH domain (helix–loop–helix, light and dark blue), Orange domain (orange), WRPW(/Y) tetrapeptide (purple) and the Hairy subfamily specific HC domain (grey). GenBank nucleotide accession numbers: leech (*H.r., Helobdella robusta*) *Hes*: AY144625 Florida lancelet (*B.f., Branchiostoma floridae*) *HairyA*: AY349467; sea squirt (*C.i., Ciona intestinalis*) *HairyA*: NM_001078521; purple sea urchin (*S.p., Strongylocentrotus purpuratus*) *Hairy/enhancer of split*: AY445629; worm (*C.e., Caenorhabditis elegans*) *lin-22*: AF020555; human (*H.s., Homo sapiens*) *Hes1/hairy*: L19314, *Hes2*: AL031848, *Hes3*: AL031847 (chromosome 1), *Hes4*: AB048791, *Hes5*: NT_004350 (chromosome 1), *Hes6*: AB035179, *Hes7*: AB049064; fruit fly (*D. m., Drosophila melanogaster*) *Hairy*: X15905, *E(spl)m3*: M96165, *E(spl)m8*: X16553, *E(spl)mβ*: X67047. (B) Phylogenetic analysis. NJ tree on the basis of a ClustalX alignment of the bHLH domains of HES amino acid sequences and HyHES. *C. elegans* Lin-22 was used as outgroup. The two bHLH subfamilies E(spl) and Hairy are highlighted in red and grey, HyHES is labeled with an asterisk. (C) Schematic representation of the exon–intron structure of *HyHes* in comparison with *Hes1, 2, 6* and *7* from mouse. Introns (numbered 1 to 3) are not to scale (black lines). 5′ and 3′ UTRs are illustrated as dotted rectangles with their length in bp. Coding regions for the different HES protein domains are shown in colour: encoded basic domains in green, HLH domains in light and dark blue, Orange domains in orange, HC domain in light grey, WRPW(/Y) tetrapeptides in purple and hydra spliced leader in brown.


A
Hydra actin
N- Terminus
(AA 1-9)
HoTG(reen)
Hydra actin promoter
~1500 bp
EGFP
Hydra actin 3´UTR
PstI
NheI
SmaI
PstI
EcoRI
HoTRed
Hydra actin promoter
mRFPmars
Hydra actin 3´UTR
PstI
NheI
SmaI
PstI
EcoRI
HoTHvNICD/
HoTHvNΔE
Hydra actin promoter
HvNICD/
HvNΔE
Hydra actin 3´UTR
PstI
NheI
SmaI
PstI
EcoRI
HePG(reen)
HyHes promoter
~600 bp
EGFP
Hydra actin 3´UTR
PstI
SmaI
PstI
EcoRI
S2 S1
HePmutG(reen)
HyHes promoter
EGFP
Hydra actin 3´UTR
PstI
SmaI
PstI
EcoRI
S2mut
S1mut
Plasmids
TO-PRO-3
mRFPmars
EGFP from
Hes promoter
merge
HePG(reen)
+ HoTRed
B
C
D
E
TO-PRO-3
anti-HvNotch
EGFP from
Hes promoter
merge
HePG(reen)
+ HoTHvNICD
F
G
H
I
HePG(reen)
+ HoTHvNΔE
J
K
L
M
TO-PRO-3
anti-HvNotch
EGFP from
Hes promoter
merge
HePmutG(reen)
+ HoTHvNICD
N
O
P
Q

We compared this conspicuous *HyHes* expression pattern with the expression of the *Hydra* FGF-receptor homolog *kringelchen. Kringelchen* is expressed at the boundary between bud and parent in a broad ring-like expression zone from the end of stage 5, when the bud has extended and is ready to make tentacle buds. This zone sharpens when the bud constricts. It remains as a clear boundary between parent and bud. After bud detachment, it can still be found in a ring of epithelial cells on the parent and in a small dot on the bud (Sudhop et al., 2004). Figs. 4E–H shows *kringelchen* expression during budding stages 7–9.

### *Timing of HyHes expression correlates with expression of the novel matrix metalloprotease MMP-A3*

Constriction at the site of the future foot appears as a consistent morphological change prior to bud detachment. It is characterised by a considerable narrowing of the tissue connection between parent and bud, which involves epithelial cell rearrangements and remodelling of the underlying extracellular matrix. In a candidate approach to find putative ECM remodelling enzymes involved in hydra bud formation, we analysed the expression patterns of matrix metalloproteases, which were identified in EST and genomic databases of *Hydra*. We selected *MMP-A3* for further studies because it was specifically expressed at the site of bud constriction (Figs. 4I–L). *MMP-A3* expression appeared approximately at the same time as *HyHes* was expressed. However, in contrast to *HyHes* and similar to *kringelchen*, it remained during foot cell differentiation.

*MMP-A3* was cloned from *Hydra* cDNA. The N-terminus is not complete. However, we found 52% protein sequence identity with HMMP (Fig. 5 and Fig. S3). The partial MMP-A3 cDNA encodes all the typical matrix metalloprotease domains. These include a pro-domain, a catalytic domain, a hinge region and a C-terminal hemopexin domain. The cysteine switch in the propeptide (consensus PRCGxPD) and the catalytic $Zn^{2+}$-binding site (consensus sequence HExxHxxGxxH) are well conserved. A potential convertase cleavage site for activation (consensus RxRR) is present between the propeptide and the catalytic domain. Unlike HMMP, but like most vertebrate MMPs, MMP-A3 has a proline-rich hinge region and two cysteines, which could potentially form a disulfide bond between the hinge region and the hemopexin domain (Fig. 5).

### *Spatial and temporal relationship of HyHes, kringelchen and MMP-A3 expression*

To compare the expression patterns of *kringelchen, HyHes* and *MMP-A3* directly, we carried out double in situ hybridisation experiments (Fig. 6). We found that *HyHes* mRNA was present in bud cells in a ring-like expression zone at the side of the bud adjacent to *kringelchen* expressing cells. There was no overlap (Fig. 6A). Expression of the two genes was seen in cells directly beside each other (Fig. 6A). When the bud narrowed and foot tissue was formed, *kringelchen* expression was confined to the adult tissue below the bud whereas *HyHes* expressed disappeared completely (stage 9, basal disc, see Figs. 4D, P). On the other hand, *MMP-A3* was always expressed in the same cells as *kringelchen* (Fig. 6C) and not in *HyHes* expressing cells (Fig. 6B).

### *DAPT treatment prevents bud detachment, disturbs the expression pattern of kringelchen and prevents expression of MMP-A3*

The transcriptional expression pattern of *HyHes* strongly suggested its involvement in budding at the point of constriction (stage 8). Our promoter analysis had suggested that *HyHes* expression was controlled by Notch signalling. We therefore investigated the effect of Notch inhibition on late budding stages. Previous work in *Hydra* had shown that the presenilin inhibitor DAPT prevents HvNICD translocation to the nucleus and thus inhibits propagation of the Notch signal (Käsbauer et al., 2007). Animals with buds that were just beginning to form (stages 3 to 5; Otto and Campbell, 1977) were incubated with DAPT for 2 days and bud development was followed. We observed that up to 80% of buds did not detach. This resulted in the formation of Y-animals (Figs. 7A and B, 1 week). The percentage of Y-animals depended on the DAPT concentration (Fig. 7A). The Y-animals were observed over a period of 4 weeks after removal of DAPT. We had shown before that Notch signalling was restored 24 h after removal of the inhibitor (Käsbauer et al., 2007). Yet, the animals never formed a foot and remained as Y-animals. The position where the two animals were joined in most cases moved into the direction of the foot within 1 to 2 months, until the two animals remained only joined at the foot (Fig. 7B, 4 weeks). In one case, the position of the joint moved upwards into the direction of the head (not shown).

When Y-animals were fed, they resumed budding. The new buds detached normally from either body column of the Y-animals. However, when we treated fresh buds at stages 3 to 5 again with DAPT for 48 h, these buds could then not detach. They stayed connected to their parent Y-animal and this led to the formation of multi-headed animals (Fig. 7C).

We then looked for *HyHes* expression in DAPT-treated animals. Again animals with buds at stages 3 to 5 were treated with DAPT for 48 h. While control animals started to constrict at this time and exhibited normal *HyHes* expression, DAPT-treated animals did not constrict and *HyHes* expression could not be detected (Figs. 8A, C). With the same experimental design, we analysed the expression of *MMP-A3* and found that the gene was not expressed when Notch signalling was disturbed (Figs. 8B, D). This indicated that Notch signalling was required for *MMP-A3* expression.

The situation was different for *kringelchen.* It was still present in DAPT-treated animals. However, its expression pattern was dramatically changed. It appeared in a diffuse pattern of cells around the base of the bud and not as a sharp band anymore. This suggested that a boundary between bud and parent recognisable by the expression of *kringelchen* was disturbed (Fig. 9). The effect depended on the timing of Notch inhibition. When buds were treated with DAPT much later, at stages 7 and 8 around the time of *HyHes* expression, they developed normally and *kringelchen* expression was not disturbed. This is shown in Fig. 9E by comparing two buds from the same animal. The bud that had already been at stages 7 and 8 at the time of treatment has normal *kringelchen* distribution (Fig. 9E′). The second bud that had been DAPT treated at stages 3 to 5 again shows the diffuse *kringelchen* pattern (Fig. 9E″).

### *DAPT does not inhibit foot regeneration*

The most obvious failure of DAPT-treated buds was that they never differentiated foot cells. We therefore asked whether Notch signalling

**Fig. 3.** HvNICD activates the *HyHes* promoter. (A) Schematic overview of the different plasmids used for biolistic transformation of single hydra cells. Name of plasmids on the left hand side, hydra *actin* promoter or *HyHes* promoter combined with EGFP (green arrows), mRFPmars (red arrow) or HvNICD and $HvN^{\Delta E}$ (dark grey arrow). (B–Q) Confocal images of hydra cells expressing EGFP, mRFPmars, HvNICD or $HvN^{\Delta E}$ from the *HyHes* or *Hyactin* promoter as indicated. (B–E) Cotransfection of HePG(reen) with HoTRed; (F–I) cotransfection of HePG(reen) with HoTHvNICD; (J–M) cotransfection of HePG(reen) with $HoTHvN^{\Delta E}$; (N–Q) cotransfection of $HeP^{mut}G$(reen) with HoTHvNICD; (B, F, J, N) DNA staining with TO-PRO-3; (C) mRFPmars-fluorescence; (G,K,O) staining with anti-HvNotch antibody; (D, H, L, P) EGFP-fluorescence (always from *HyHes* promoter); (E, I, M, Q) merged images from all three channels, false colours: DNA (blue), EGFP (green), mRFPmars or anti-HvNotch antibody staining (red). Confocal images are projections, scale bars: 12 μm, dotted lines indicate outline of cells.

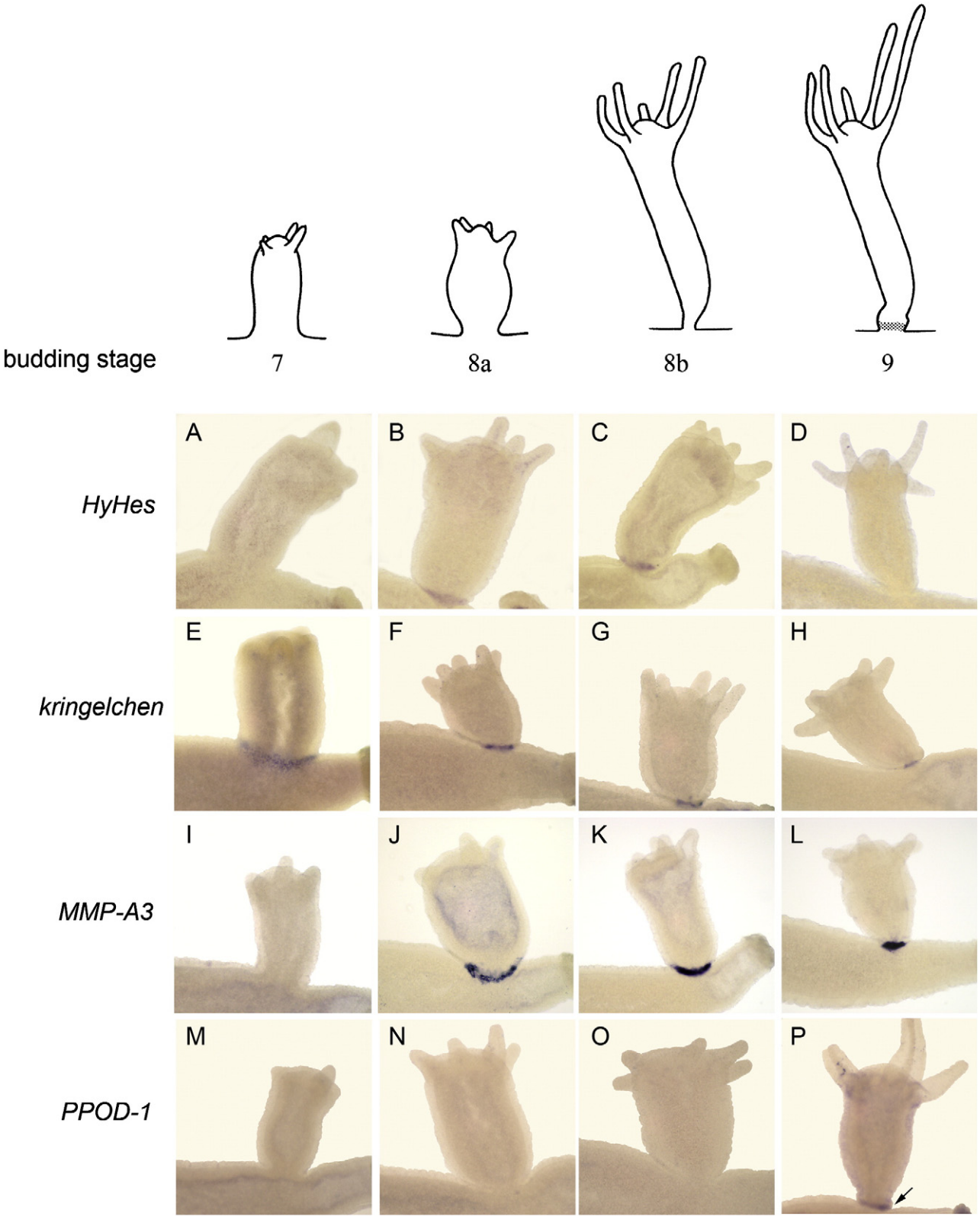



**Fig. 4.** Whole-mount in situ hybridisation for *HyHes*, *kringelchen, MMP-A3* and *PPOD-1* during late budding stages. Description of late budding stages 7 to 9 according to Otto and Campbell (1977) in the upper panel. (A–D) in situ hybridisations for *HyHes*, (E–H) in situ hybridisations for *kringelchen*, (I–L) in situ hybridisations for *MMP-A3* and (M–P) in situ hybridisations for *PPOD-1*. Arrow indicates signal of *PPOD-1* in differentiating basal disc cells of the bud. NBT/BCIP (Roche) was used for staining reactions (blue signals).

was generally required for *de novo* foot formation in *Hydra*. To answer this question, we investigated foot regeneration. After a 5-h pre-incubation period of animals in DAPT or control medium, we cut off the feet of 15 to 33 animals per group and followed regeneration in the continued presence of DAPT or control medium. Regeneration was considered to be complete when the new foot allowed the animals to attach themselves to the plate. The percentage of such animals was observed at different time points. The appearance of regenerated feet

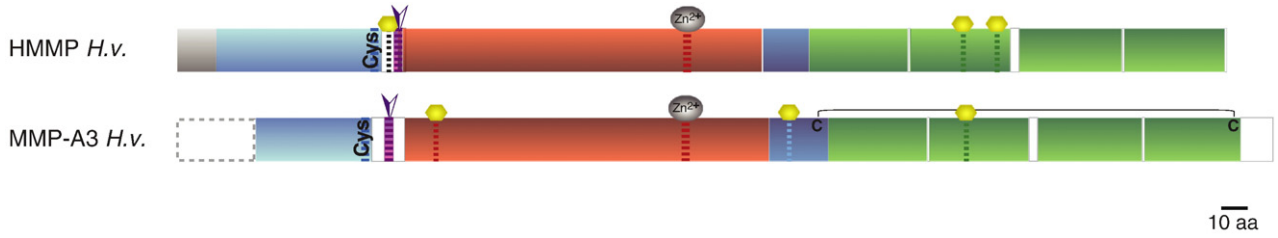



**Fig. 5.** MMP-A3 protein domain analysis. Schematic comparison of the protein domain structure of MMP-A3 and HMMP (GenBank protein accession number: AF162688). The different protein domains are depicted in the following colours: signal peptide (grey), propeptide (light blue) with conserved cysteine switch (Cys), convertase cleavage consensus site (purple triangle), catalytic domain (red) with conserved $Zn^{2+}$-binding region ($Zn^{2+}$), hinge region (dark blue) and hemopexin-like domain (green); N-terminal missing region of MMP-A3 as dotted rectangle; potential *N*-glycosylation sites are indicated with yellow hexagons and the potential disulfide bond between two cysteines (each cysteine illustrated as single capital C) as black line.

showed only a slight delay by 2 to 6 h in the DAPT group (Fig. 10A). Regeneration was completed in all animals 46 h after foot removal. This indicated that Notch signalling was not normally required for foot formation during regeneration.

Since DAPT-treated buds did not form feet and never fell off the parent even after Notch signalling was restored, we specifically investigated foot regeneration in these buds. Buds of DAPT treated and control animals were cut off at budding stage 8. They were then left in the absence of DAPT. Buds in both groups quickly generated feet even slightly faster than foot regeneration in adults (Fig. 10B, compare with Fig. 10A). Previous DAPT treatment had no influence on the capability of these buds to generate a foot.

## Discussion

*Hydra* buds initially appear as small evaginations of both, the ectodermal and the endodermal cell layers in the lower part of the body column. At the tip of these early buds eight different *Hydra Wnt* genes are expressed. Of those, *Hvwnt2* is specific for the bud and not expressed at the hypostome of the adult (Lengfeld et al., 2009). For the bud to progress, epithelial cells are recruited from circular regions of the parental body column around its tip (Otto and Campbell, 1977). No molecular markers are known, which demarcate the boundary between parent and future bud tissue at this stage. The first evidence of a bud–parent boundary appears at stage 5 (Fig. 11) with the simultaneous expression of both, Wnt-pathway genes connected with non-canonical Wnt signalling (*Hvwnt8*, *Hmfrizzled2* and *Hydishevelled*; Philipp et al., 2009) and the *Hydra* FGF-receptor homolog *kringelchen* (*Sudhop et al., 2004*). *Kringelchen is* initially expressed in a relatively diffuse band with clear cell-to-cell variation extending into both sides of the parent–bud junction (see Figs. 4E and 11, budding stages 5 to 7). At budding stage 8, the *kringelchen* expression zone sharpens considerably; expression in cells away from the boundary disappears while at the boundary now virtually all cells express *kringelchen* in a thin stripe (Fig. 4F). At the same time we can detect mRNA for the matrix metalloprotease MMP-A3. Two-colour in situ hybridisations strongly suggest that *MMP-A3* appears in the same cells that express *kringelchen* (Fig. 6C). Following *MMP-A3* expression, a constriction appears between the parent and the bud. We suggest that MMP-A3 might be directly involved in promoting morphogenesis at the constriction site. Its sequence similarity with HMMP, sequence conservation of the active site and domain structure indicate that it may have the capability to degrade components of the ECM and thus promote remodelling of the mesoglea for morphogenesis to create a progressively narrower tissue connection between bud and parent. In the tissue adjacent to the constriction site, formation of the bud's foot commences. Ultimately the bud detaches from the parent with its foot fully functional (Fig. 11, budding stage 9).

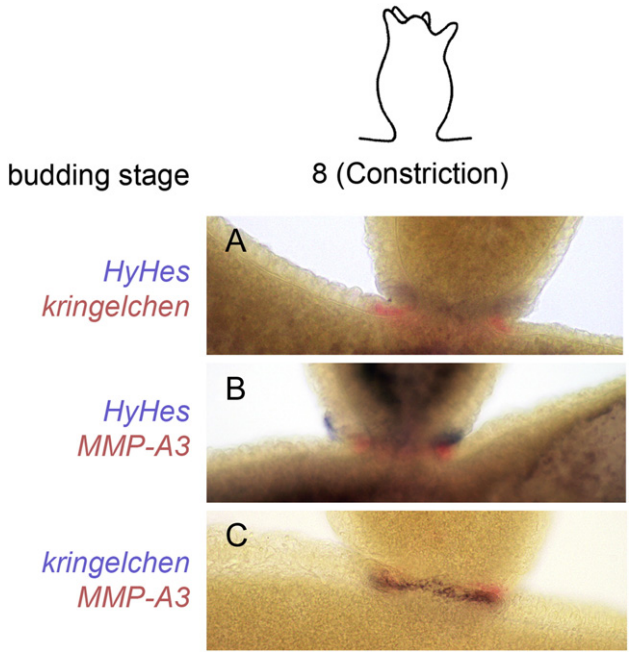



**Fig. 6.** Double whole-mount in situ hybridisation for combinations of *HyHes*, *kringelchen* and *MMP-A3* during late budding stages. Upper panel: schematic representation of budding stage 8 (constriction) according to Otto and Campbell (1977). Double in situ hybridisations at budding stage 8; (A) double in situ hybridisation for *HyHes* (blue) and *kringelchen* (red); (B) double in situ hybridisation for *HyHes* (blue) and *MMP-A3* (red); (C) double in situ hybridisation for *kringelchen* (blue) and *MMP-A3* (red). Staining reactions were carried out using either NBT/BCIP (Roche, blue signals) or FastRed (Sigma, red signals).

Experiments described in this paper suggest that the Notch signalling pathway is important in the development from the diffuse boundary stage to the stage, in which adjacent zones of *MMP-A3* expression and foot gene expression are present. Inhibition of Notch signal transduction with DAPT leads to the loss of both cell differentiation states: neither is *MMP-A3* expressed nor is a foot formed. The *kringelchen* expression zone fails to sharpen. It even appears to become more diffuse than before with the appearance of *kringelchen* expressing cells reaching far into the parent tissue and a small number also into the bud (see Fig. 9D).

Our experiments also suggest that the *hairy/enhancer of split* homolog *HyHes* is the target of Notch signalling in this situation. Like *Hes* genes from other species *HyHes* is regulated by HvNotch through CSL binding sites in its promoter. We have shown that two antiparallel and overlapping CSL binding sites (S1 and S2) within the first 600-bp upstream of the transcription start site are necessary and sufficient to activate a reporter gene in hydra cells in the presence of HvNICD. *HyHes* mRNA *is* expressed transiently in a small ring of cells in the developing bud at budding stage 8 adjacent to the *kringelchen* expression domain in the position where the future foot will be formed. In accordance with the idea that *HyHes* is the target of HvNotch, its expression was prevented when we inhibited Notch signalling.

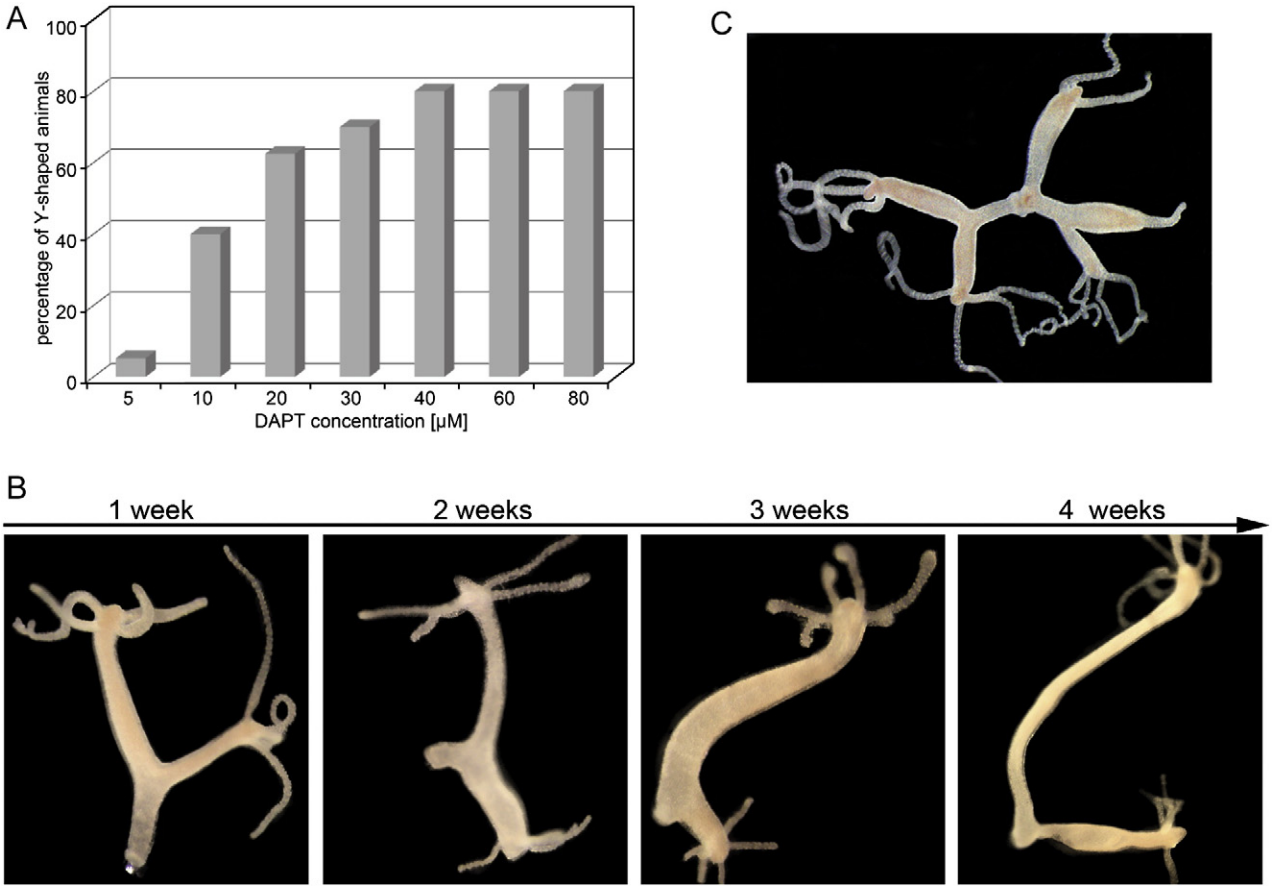



**Fig. 7.** DAPT leads to formation of Y-animals. (A) Percentage of developing Y-animals 7 days after a 48-h period of treatment with different concentrations of DAPT. (B) Fate of Y-animals up to 4 weeks after a 48-h spell of DAPT treatment. From the left to the right images of Y-animals 1, 2, 3 and 4 weeks after DAPT removal. (C) Example of a multi-headed animal after repeated pulses of DAPT treatment.

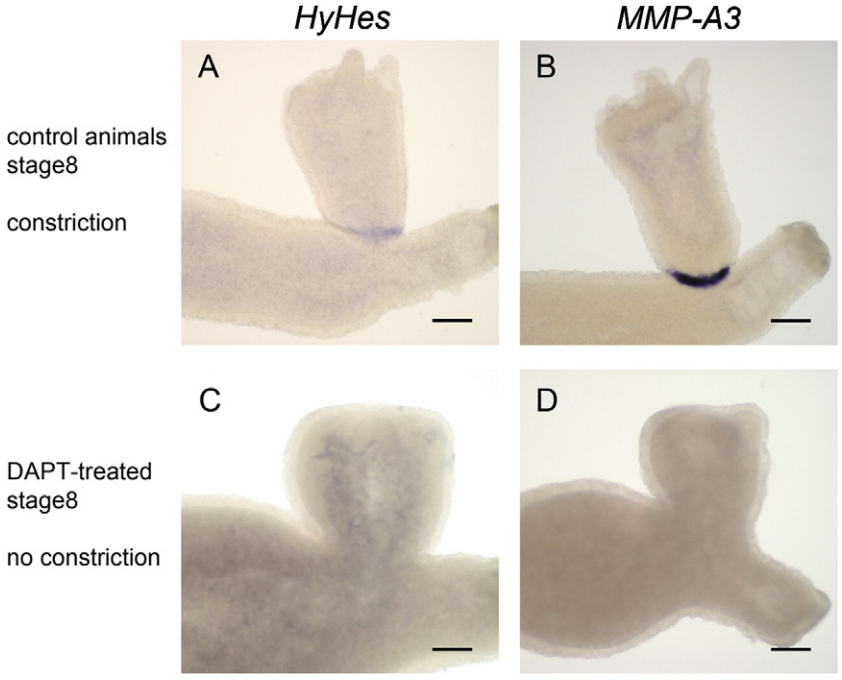



**Fig. 8.** *HyHes* and *MMP-A3* expression are abolished by DAPT. (A, B) In situ hybridisation for *HyHes* and *MMP-A3* of animals at budding stage 8 (constriction), control animals. (C, D) In situ hybridisation for *HyHes* and *MMP-A3* in DAPT-treated animals, animals should be at budding stage 8 like control animals. After DAPT treatment, however, constriction does not occur and strictly speaking stage 8 is not reached; scale bars: 100 µm.

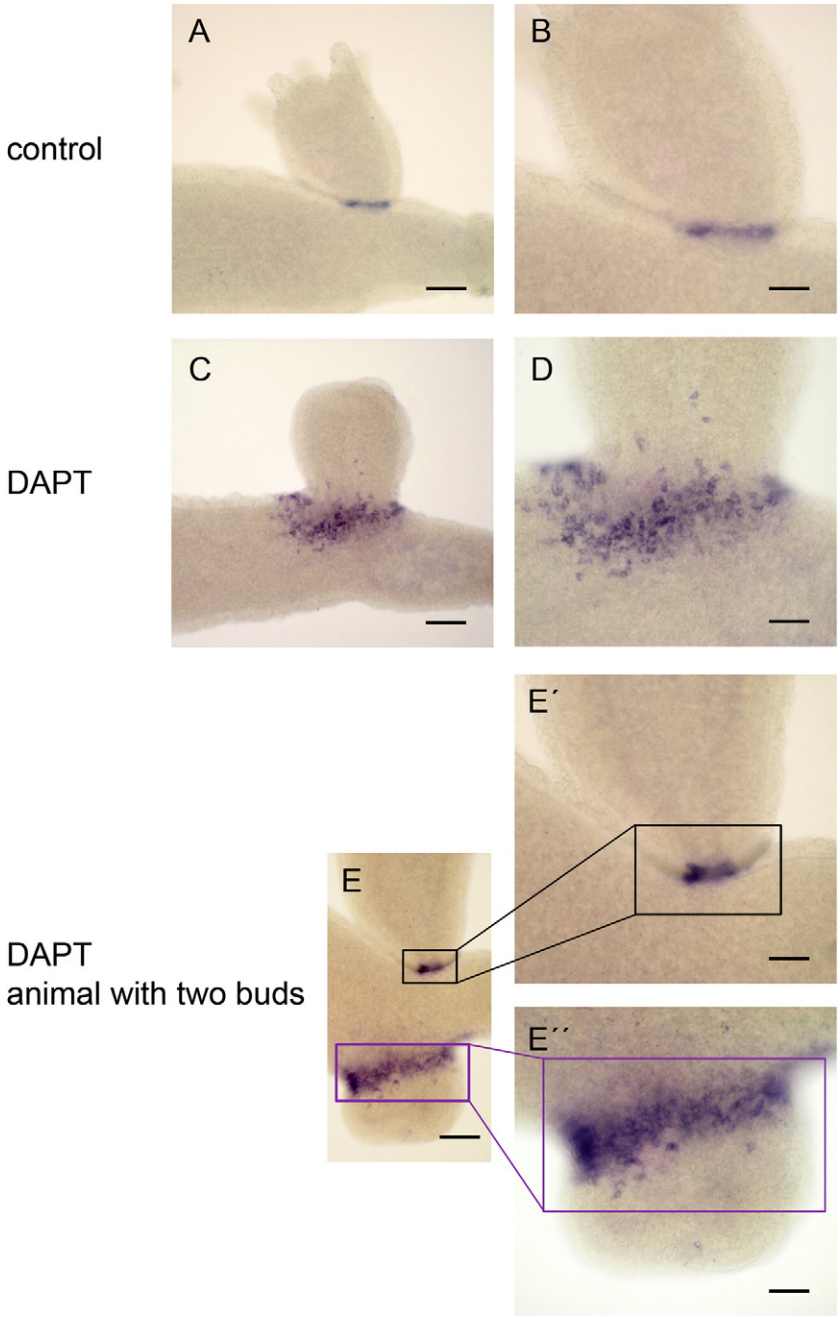
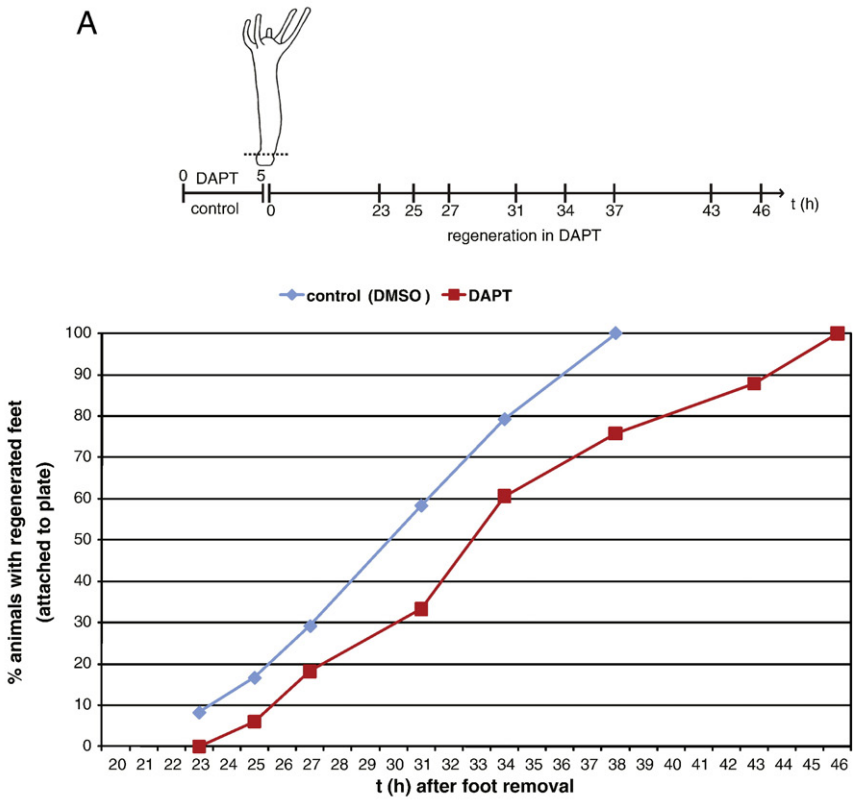


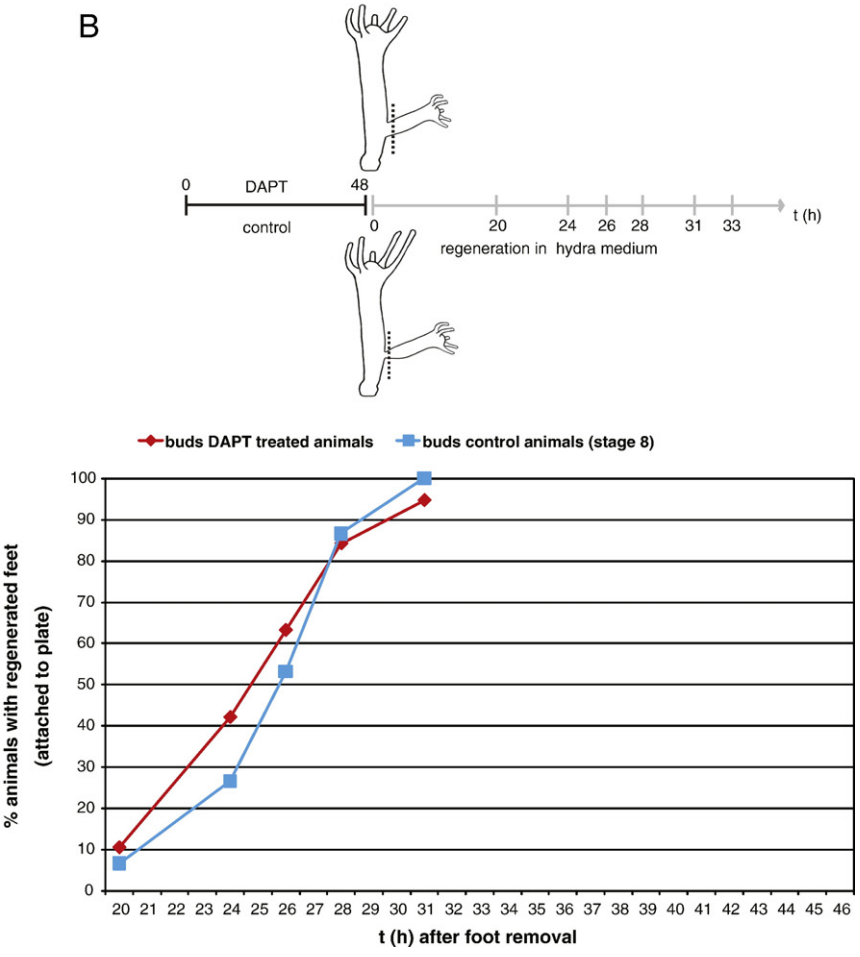

**Fig. 9.** *Kringelchen* expression is disturbed by DAPT. Whole-mount in situ hybridisation for *kringelchen* at budding stage 8 (constriction). (A, B) *kringelchen* expression at budding stage 8 in control animal; (C, D) in situ hybridisation for *kringelchen* at budding stage 8 in DAPT-treated animal; B and D higher magnifications from A and C; (E) DAPT-treated animal with two buds; upper bud: budding stage 9 and 10, DAPT treatment at stage 6 and 7; lower bud: budding stage 8, DAPT treatment at stage 5 (E′, E″) higher magnifications; scale bars: (A, C, E) 100 μm, (B, D, E′,E″) 50 μm.

*MMP-A3* expression also requires Notch signalling. Although *MMP-A3* is expressed in the *kringelchen* positive cells under normal conditions, the inhibition experiments show that the presence of *kringelchen* is not sufficient for *MMP-A3* induction. During DAPT treatment, when the *kringelchen* expression zone was diffuse, *MMP-A3* was not expressed. Thus, gene activity and cell differentiation on both sides of the boundary between the constriction zone (*MMP-A3* expression) and the bud's foot cell differentiation zone (e.g. *PPOD-1* expression) completely depended on Notch signalling.

Although *HyHes* was expressed in cells adjacent to the *kringelchen* expressing cells, it depended on functioning *kringelchen* signalling. Blocking of the FGF-receptor tyrosine kinase activity with SU5402 prevented *HyHes* and *MMP-A3* expression and thus constriction and foot formation of the bud (Sudhop et al., 2004; and data not shown). A plausible explanation for this effect is that *kringelchen* expressing cells respond to FGF-signalling by producing a Notch ligand, for instance Jagged, which we have identified in the *Hydra* genome (HyJagged; Prexl et al., in preparation). Unfortunately, in situ hybridisations have proven to be technically challenging and our results are not conclusive at this point. However, precedence for the induction of Notch ligands by FGF-receptor signalling is found in the literature, e.g., during branch formation in *Drosophila* trachaea (Ghabrial and Krasnow, 2006; Ikeya and Hayashi, 1999).

At the final stages of budding, we find two clearly defined stripes of gene expression with *HyHes* expressing and *kringelchen* expressing cells corresponding to two mutually exclusive activated states (Fig. 11, constriction stage 8). Meinhardt first showed how such stripes could be patterned. Under the term “lateral activation of


A
0 DAPT 5
control 0
23 25 27 31 34 37 43 46 t (h)
regeneration in DAPT
control (DMSO)
DAPT
% animals with regenerated feet (attached to plate)
t (h) after foot removal


B
0 DAPT 48
control 0
20 24 26 28 31 33 t (h)
regeneration in hydra medium
buds DAPT treated animals
buds control animals (stage 8)
% animals with regenerated feet (attached to plate)
t (h) after foot removal

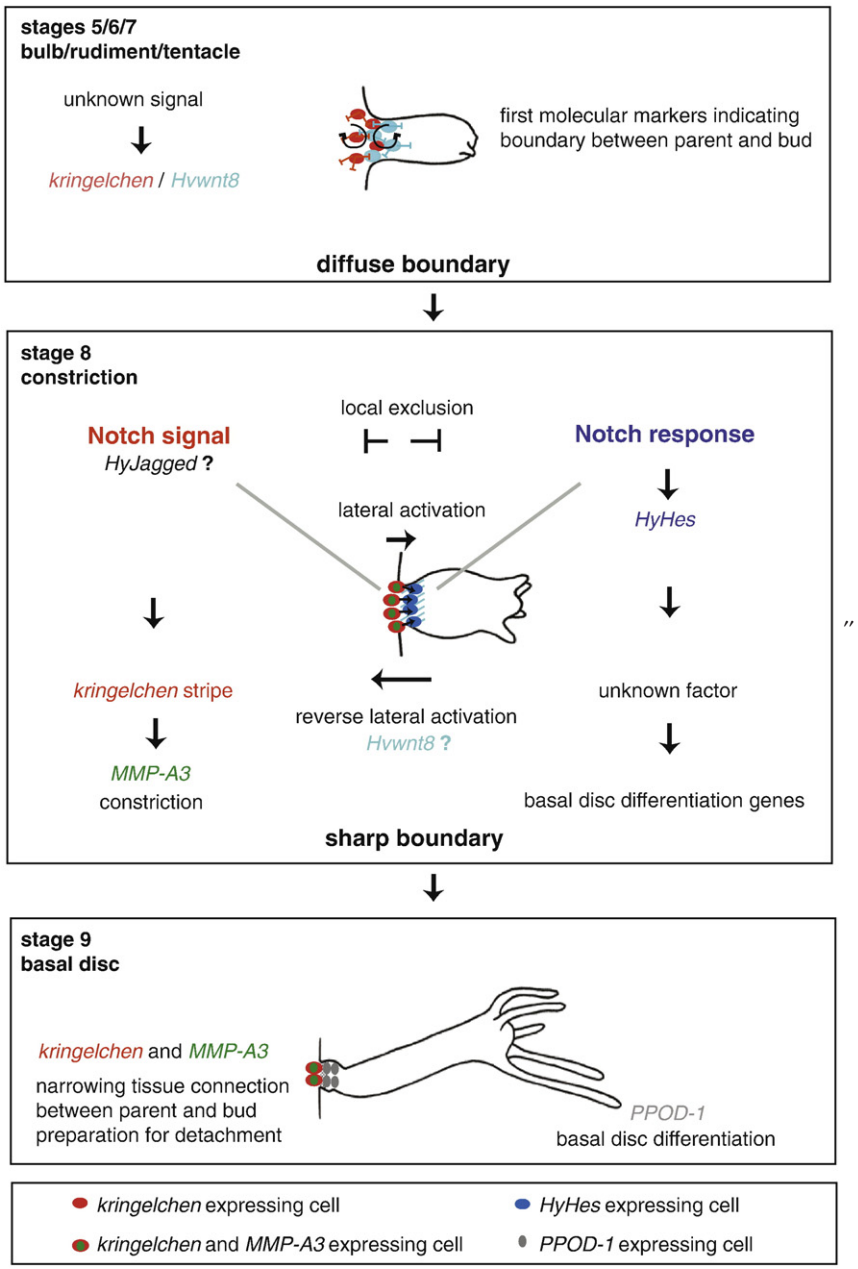



**Fig. 11.** Model for the impact of Notch signalling on final budding stages in *Hydra*. Schematic overview of the interaction of the FGFR and Notch signalling pathways and sequential gene expression of *kringelchen*, *Hvwnt8*, *HyHes*, *MMP-A3* and *PPOD-1* during budding stages 5 to 9 (budding stages according to Otto and Campbell, 1977; *Hvwnt8* expression from Philipp et al., 2009 and Lengfeld et al., 2009).

mutually exclusive states", he described an interaction of two reaction-diffusion systems, each governed by an autocatalytic feedback loop that exclude each other (local exclusion). In addition, each produces a substance, by Meinhardt termed "long ranging help", which increases the autocatalysis of the other system. Individually, each system would tend to produce a salt and pepper pattern. Making these systems mutually dependent on each other can give rise to stripes of alternating gene activity (Meinhardt, 1982).

This concept provides an attractive explanation for the dependence of *kringelchen* expression dynamics on Notch signalling. The initial broad *kringelchen* domain has a pattern expected of an isolated activator–inhibitor system, before mutual exclusion and lateral activation are established (Fig. 11, budding stages 5 to 7); the focussing of this pattern into a compact and uniform line resembles the behaviour expected, when the two autocatalytic systems interact (Fig. 11, constriction stage 8).

**Fig. 10.** Foot regeneration in buds and adults dependent on DAPT. (A) Upper panel: schematic description of the performed experiment: foot regeneration was followed in adult animals pretreated with DAPT or for control with DMSO for 5 h before foot removal; incubation with DAPT or control medium was continued after foot removal; foot regeneration was considered complete when animals attached to the plates; lower panel: diagram showing percentage of animals with regenerated feet dependent on time after foot removal. (B) Upper panel: schematic description of the performed experiment: foot regeneration was followed in buds that were cut off the parent animal at budding stage 8 of DAPT-treated animals (48 h) and control animals; incubation in DAPT was not continued after bud removal, foot regeneration was followed in hydra medium and was considered complete when animals attached to the plates; lower panel: diagram showing percentage of animals with regenerated feet dependent on time after foot removal.

We suggest that Notch signalling provides the basis for such an interaction by facilitating mutual exclusion of cells that participate in constriction and those that differentiate into bud foot cells. It has been shown previously that Notch signalling has the potential to create two adjacent cells that exclude each other (de Celis and Bray, 1997; Miller et al., 2009). This can be achieved by repression of Notch ligand in the Notch-responding cell and inhibition of the Notch-response in the signal-sending cell. By monitoring the combined *cis* and *trans* interactions of Notch ligands and Notch receptors in a tissue culture system through time-lapse microscopy, an elegant new study has provided direct evidence for this. This study presents a mathematical model to show how mutually exclusive signalling states in adjacent cells are generated by mutual inactivation in the Notch-Delta system (Sprinzak et al.).

Clearly, not all components of the system patterning the parent–bud boundary in *Hydra* are known yet. The nature of the postulated activation signals (the "long ranging help") from the *kringelchen* and the *HyHes* expressing cells is unclear at present. When looking at the published expression patterns for *Hvwnt8* at the base of the bud, it is tempting to speculate that HvWnt8 signalling is involved (Fig. 11, stages 5–7 and 8; Philipp et al., 2009).

Strikingly, the need for Notch signalling was transient. DAPT application after stages 7 and 8 when *HyHes* expression had already taken place did not interfere with the completion of budding. On the other hand, when *HyHes* was prevented at stages 7 and 8, budding was never completed even after Notch activity was restored by removal of the inhibitor (Käsbauer et al., 2007). This resulted in the development of stable Y-animals that remained joined at the budding site. As a consequence of normal *Hydra* tissue movement (Campbell, 1967), the joint later moved up or down the animal as it has also been observed in Y-animals that had been obtained after grafting of head tissue to a position above the budding region (Berking, 1977; Harry MacWilliams, personal communication).

A number of modulators of Notch activity are found in the *Hydra* genome, which could be involved in patterning at the parent–bud boundary. We have identified genes encoding the E3 ubiquitin ligase Mindbomb and the negative regulator of Notch signalling, Numb. The second relevant ubiquitin ligase Neuralized has not been found. However, apparently it is present in *Nematostella* (Gazave et al., 2009). Its secondary loss in *Hydra* could well be compensated for by Mindbomb. Another important component of the Notch signalling system is the glycosyl-transferase Fringe, which in higher organisms plays an important role in boundary formation and segmentation (Forsberg et al., 1998; O'Keefe and Thomas, 2001). It possesses an N-terminal hydrophobic region and a Fringe-like/Galactosyl-T superfamily domain (Munro and Freeman, 2000). We have now identified a *fringe* candidate gene model encoding both these domains in the *Hydra* genome. The part that these modulators of Notch signalling might play in boundary formation during budding will have to be investigated in the future.

Boundary formation is an important mechanism to organise the formation of organs during embryonic development in *Bilateria*. Notch and FGFR signalling and the expression of *Hes* genes have been shown in a number of developmental processes involving boundaries. These also include segmentation in vertebrates where boundaries between somites are established (Palmeirim et al., 1997; Pourquie, 1999; Sato et al., 2002). Another example is the formation of the dorsal–ventral boundary in the *Drosophila* wing disc, where the wingless expressing cells that induce the wing margin are defined by Notch signalling (Blair, 2003).

We have now shown that Notch signalling is needed in a non-bilaterian simple cnidarian to establish a boundary between two signalling fields. This suggests a very early origin of this system in animal evolution. Its setup in *Hydra* could be prototypical for many later developmental processes where compartments for the formation of complex differentiated tissues and organs are formed.

## Acknowledgments

We thank Monika Hassel for the *kringelchen* plasmid and Sabine Hoffmeister-Ullerich for the *PPOD-1* plasmid. We especially thank Harry MacWilliams for discussion and help with interpretation of the data. We also thank Charles N. David for helpful advice. This work was sponsored by Deutsche Forschungsgemeinschaft (BO1748-1 and BO1748-5).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.05.517.

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