© 2015. Published by The Company of Biologists Ltd | Development (2015) 142, 2081 doi:10.1242/dev.126235

CORRECTION

# A small molecule screen identifies a novel compound that induces a homeotic transformation in *Hydra*

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There was an error published in *Development* **140**, 4788-4796.

On p. 4794, the incorrect plasmid was referenced. The plasmid used was pHyVec16 (GenBank Accession Number KP145000).

The authors apologise to readers for this mistake.

Development (2013) doi:10.1242/dev.094490

**Fig. 4. DAC-2-25 targets ectodermal epithelial cells in a cell-autonomous manner.** (A) A schematic representation of the method used to produce chimeric *Hydra* (see Materials and methods for more details). (B) The chimera described in A was exposed to DAC-2-25 and ectopic tentacles arose in a manner similar to that seen in AEP animals. (C) A chimera with Zürich ectoderm and AEP endoderm did not respond to DAC-2-25. (D,E) Ectopic tentacles did not form in an animal with AEP i-cells and Zürich epithelial layers. (F,G) Mosaic *Hydra* were produced when chimeras (shown in D,E) budded. These mosaics were exposed to DAC-2-25 and ectopic tentacles were seen only in epithelial tissue derived from the AEP donor.

sufficient, in the numbers seen in our experiments, to induce the ectopic tentacle phenotype in Zürich epithelial tissue.

In some cases, buds formed during the course of DAC-2-25 treatment on animals in which both AEP and Zürich epithelium occupied the budding zone, yielding buds composed, to varying degrees, of both AEP and Zürich epithelium. These buds gave us an opportunity to examine the response of mosaic animals with varying compositions of source tissues. We obtained longitudinally mosaic animals, animals where a 'finger' of AEP tissue extended into Zürich epithelium, and animals in which the hypostome was Zürich and the rest of the animal AEP (Fig. 4F,G). We found that ectopic tentacles formed only in regions containing AEP ectoderm. These results indicate that the phenotype produced by DAC-2-25 is cell-autonomous.

To further examine the cell autonomy of the response to DAC-2-25, we grafted the upper half of a Zürich animal to the lower half of a transgenic AEP animal. When exposed to DAC-2-25, ectopic tentacles were not formed in the Zürich region of the animal and did not appear in the AEP region of the body column of the mosaics until ectopic tentacles began to appear in the corresponding region in the control animal. These results indicate that the temporal component of the DAC response is an autonomous feature of the AEP tissue.

### DAC-2-25 transforms the body column into tentacle zone

We considered four explanations for the origin of ectopic tentacles in DAC-2-25-treated animals: (1) formation of tentacles on tissue that retains its body column identity; (2) conversion of body column tissue into tentacle zone tissue (i.e. a homeotic transformation); (3) conversion of body column into a combination of body column and tentacle zone; or (4) expansion of the hypostome with concomitant accumulation of displaced tentacles (Fig. 5A). To distinguish among these possibilities, we used genes whose expression patterns define tissue identity along the oral/aboral axis. These include: (1) a marker of the organizer, the *Hydra* Wnt3 ortholog (*HyWnt3*), which is expressed at the tip of the hypostome (Hobmayer et al., 2000); (2) a marker of hypostome, the *Hydra* T-box gene *HyBra2*, which is expressed throughout the hypostome, but not in the tentacle zone (Bielen et al., 2007); (3) a marker of tentacles, *HyAlx*, an *aristaless*-related homeobox gene that is expressed in rings at the bases of the tentacles (Smith et al., 2000); (4) a marker for tentacle zone, *Hym-301* (Hobmayer et al., 2000; Smith et al., 2000; Takahashi et al., 2005); and (5) a marker for body column tissue, a gene for a novel secreted protein (GenBank Accession Number XP_002165635) (Hwang et al., 2007). This gene was described as being expressed in body column endodermal epithelial cells by Hwang et al. (Hwang et al., 2007), but we have found that it is expressed in gland cells in the body column (Fig. 5E).

The ectopic tentacles in DAC-2-25-treated animals show rings of *HyAlx* expression at their bases, as is seen with normal tentacles (Fig. 6B), indicating that *HyAlx* functions in formation of ectopic tentacles as it does in normal tentacles (Smith et al., 2000). We produced a transgenic line in which the *Hym-301* promoter drives GFP expression (H301p::GFP::H301t), such that GFP expression is activated after cells are displaced from the body column into the head (Fig. 6C), similar to endogenous Hym-301 expression (Takahashi et al., 2005). The presence of GFP in the tentacles and the upper region of the hypostome of the transgenic animals, where *Hym-301* gene expression is not detected by *in situ* hybridization, is probably due to the long half-life of GFP, allowing it to persist in cells that have been displaced into the tentacles and upper hypostome. In H301p::GFP::H301t animals chronically exposed to DAC-2-25, the aboral boundary of GFP expression moved down the animal in synchrony with the formation of ectopic tentacles (Fig. 6D). This result is consistent with at least a partial transformation of body column tissue into tentacle zone and rules out ectopic tentacle formation from what is otherwise body column tissue.

When expression of the body column marker was examined using animals that had been chronically treated with DAC-2-25, we found that the oral expression boundary of the marker moved down the animal in synchrony with ectopic tentacle formation (Fig. 6F). This result indicates that the tissue containing ectopic tentacles has lost features of body column tissue and, together with the *Hym-301* result, supports the hypothesis that the body column is transformed into tentacle zone.

We investigated the expression pattern of *HyWnt3* in DAC-2-25-treated animals using two different transgenic *HyWnt3* promoter::GFP transgenic lines obtained from the Holstein lab: in one, expression of the transgene is restricted to the ectoderm [ec(HyWnt3FL::GFP)]; and in the other, expression is restricted to the endoderm [en(HyWnt3FL::GFP)] (Nakamura et al., 2011).

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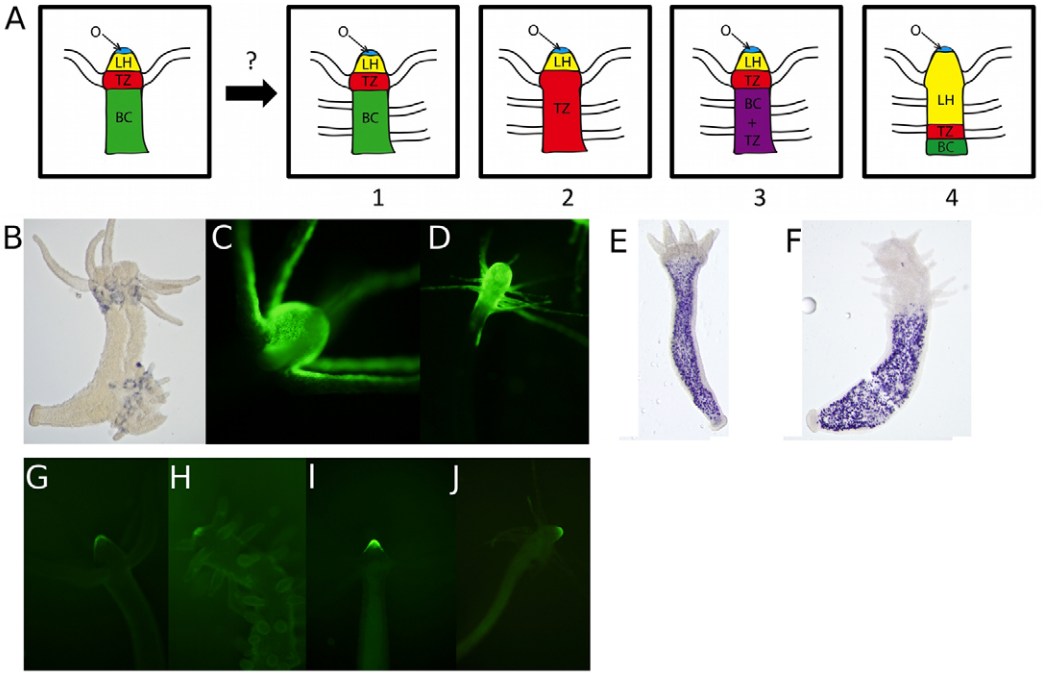


**Fig. 5. Markers of positional identity show conversion of body column to tentacle zone upon chronic treatment with DAC-2-25.** (A) Four models to explain the identity of the tissue containing ectopic tentacles produced in response to DAC-2-25. (Far left) An untreated *Hydra* has four tissue identities associated with the head and upper body column: O, organizer; LH, lower hypostome; TZ, tentacle zone; BC, body column. We hypothesized that: (1) ectopic tentacles could form within tissue that has retained body column identity; (2) ectopic tentacles could arise within tissue that has been transformed into tentacle zone; (3) ectopic tentacles could arise in tissue that has both body column and tentacle zone identities; or (4) that the lower hypostome expands, pushing the tentacle zone down the body column, while previously formed tentacles are maintained. (B) *In situ* hybridization for *HyAlx* in a polyp treated for 11 days; ectopic tentacles have rings of *HyAlx* expression in their ectoderm, as seen in untreated controls [not shown, described elsewhere (Smith et al., 2000)]. H301p::GFP::H301t, a transgenic line in which the Hym-301 promoter drives GFP expression in the ectoderm (C), after 27 days of exposure to DAC-2-25 (D), the zone of GFP expression has expanded. (E) An *in situ* hybridization for a body column-specific gene in an untreated animal and after 23 days of exposure to DAC-2-25 (F). The transgenic line Ec(HyWnt3FL::GFP) created by Nakamura et al. (Nakamura et al., 2011), in which the Wnt3 promoter drives expression of GFP in the ectoderm (G). (H) The region of GFP expression in ec(HyWnt3FL::GFP) did not expand upon exposure to DAC-2-25. The expression pattern of GFP in a transgenic reporter for *HyBra2* in the ectoderm (I) does not change in response to DAC-2-25 (J).

Chronic exposure to DAC-2-25 did not alter the GFP expression pattern in either line (Fig. 6G,H and data not shown). If DAC-2-25 causes expansion of the hypostome at the expense of body column, such elongation would be accompanied by continuous displacement of the tentacle zone downwards. Existing tentacles would then be displaced upwards into the elongating hypostome and new tentacles would emerge at the front of the downward moving tentacle zone. To test this possibility, we produced a transgenic line in which the *HyBra2* promoter drives GFP expression, [ec(*HyBra2::GFP*]. This line faithfully recapitulates *HyBra2* expression as described by Bielen et al. (Bielen et al., 2007) (Fig. 6I). When this line was exposed to DAC-2-25, the transgene expression domain was not altered (Fig. 6J). Taken together, the data from the various transgenic lines lead to the conclusion that DAC-2-25 treatment causes the body column to be transformed into tentacle zone.

### Structure-activity relationship (SAR) studies identify features of DAC-2-25 required for biological activity

The ultimate goal of these studies is to identify the protein target of DAC-2-25, which could potentially be achieved by affinity chromatography coupled with mass spectrometry. This approach requires structure-activity relationship (SAR) studies to identify appropriate sites for immobilization of the molecule on a solid support and to identify the most active derivative of the molecule. Forty-three derivatives of DAC-2-25 were synthesized (supplementary material Appendix S1) and one was purchased (Sigma-Aldrich), and subsequently tested on the AEP strain (the strain used in the original screen) using the chronic exposure assay. The studies described above were carried out at a DAC-2-25 concentration of 5 µM. All derivatives were first tested at 5 µM, and if no phenotype was produced within 3 weeks of exposure, the derivative was tested at 10 and 20 µM. If ectopic tentacles were not seen at these higher concentrations, the compound was considered inactive. If a derivative induced ectopic tentacles at 5 µM, it was re-tested at 1 µM, 500 nM, 100 nM and 50 nM.

Our SAR study identified several features of DAC-2-25 that are essential for activity and lack of toxicity. We have found that the aryl ring at the C6 position of the 2-pyridone is required to produce the phenotype and that an alkyl substituent is required in the *para* position of the aryl ring. We have also found that the addition of a methyl group on the pyridone ring at the C3 position increases the activity of the compound 20-fold (Table 1).

To investigate the possibility that inactive derivatives bind to the target molecule and abrogate activity of DAC-2-25 by competition for binding, animals were treated with equimolar concentrations of DAC-2-25 and the inactive derivative DAC-1-217 (Fig. 6, compound II), and twofold excess DAC-1-217. Ectopic tentacles were formed in a time frame similar to that for DAC-2-25 treatment alone in both experiments, indicating that the inactive compound does not compete for binding with DAC-2-25.

One of the derivatives, SKP-IV.9.1 (Fig. 6, compound X), initiated the formation of ectopic tentacles 1 day faster than DAC-

Development (2013) doi:10.1242/dev.094490

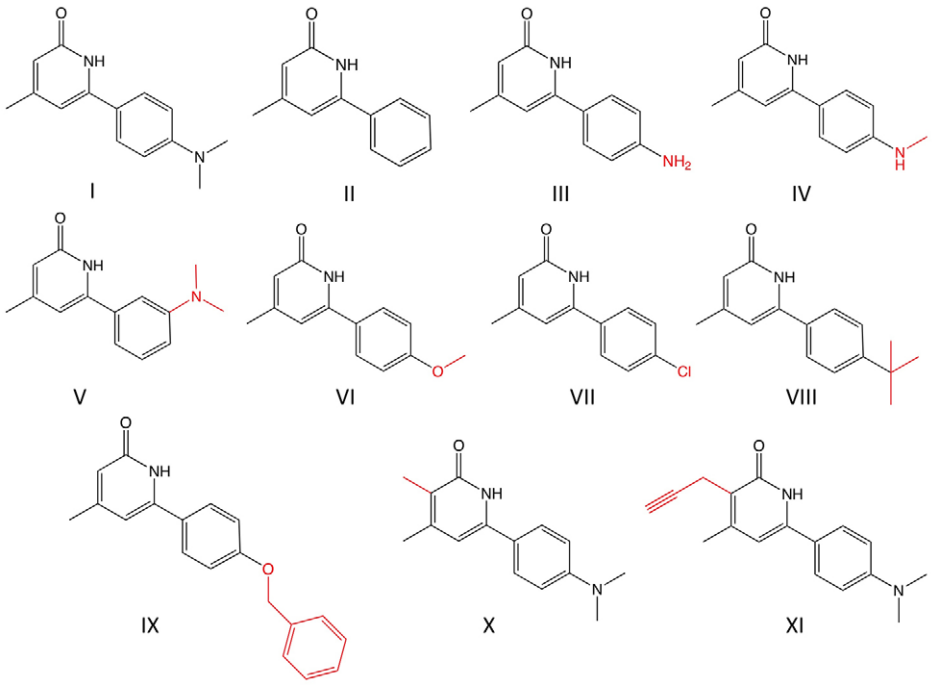


**Fig. 6. DAC-2-25 and derivatives for SAR studies.** A subset of the 44 derivatives of DAC-2-25 (I) that were used for SAR studies is shown. Those that are not shown in the figure are listed in supplementary material Appendix S1. Compounds II, III, V, VII and IX did not induce ectopic tentacles. Compounds IV, VI, VIII, X and XI induced ectopic tentacles.

2-25. An end-point dilution experiment was performed to measure the potency of SKP-IV.9.1 (Table 1). Animals were exposed to either DAC-2-25 or SKP-IV.9.1 at concentrations of 5 µM, 1 µM, 500 nM, 100 nM and 50 nM. Ten animals were exposed for 3 weeks and the number of ectopic tentacles was then counted. DAC-2-25 induces the formation of ectopic tentacles robustly at 5 µM but only one or two ectopic tentacles are produced at 1 µM, and none at lower concentrations. Ectopic tentacles were robustly produced when the animals were treated with SKP-IV.9.1 at all concentrations except 50 nM, where one or two ectopic tentacles were produced. The threshold between inactive and minimal activity (characterized as one or two ectopic tentacles) is between 1 µM and 500 nM for DAC-2-25, and less than 50 nM for SKP-IV.9.1; thus, SKP-IV.9.1 is at least 20-fold more potent than DAC-2-25. Ectopic tentacle formation at the oral end of buds is more sensitive to the compounds than ectopic tentacle formation on the body column. Ectopic tentacles formed at the oral ends of buds at all concentrations of SKP-IV.9.1 tested, but only at 5 µM, 1 µM and 500 nM for DAC-2-25.

## DISCUSSION

The mechanisms that underlie pattern formation in metazoans are still far from being fully understood. Because of its single axis, its ability to regenerate from pieces of the adult polyp or from aggregates of cells, and its ability to be mathematically modeled (Gierer and Meinhardt, 1972; Meinhardt, 2012), *Hydra* is an important organism for studies of pattern formation. However, the inability to carry out genetic screens has prevented unbiased searches for genes that affect patterning in *Hydra*. Forward genetic screens in *Hydra* are precluded due to the difficulty of obtaining large numbers of embryos and the long and variable period between fertilization and hatching seen in most strains. Although the availability of the *Hydra* genome sequence potentially allows large-scale reverse genetic screens using RNAi, such screens are not feasible using the currently published methods for carrying out RNAi in *Hydra* (Chera et al., 2006; Lohmann et al., 1999; Smith et al., 2000). An unbiased screen for genes involved in *Hydra* patterning using differential display RT-PCR on RNA samples from regenerating aggregates of *Hydra* cells led to identification of a gene encoding a novel peptide expressed during head regeneration (Takahashi et al., 1997). However, there have been no additional reports of genes identified using this approach. We were intrigued by the possibility of applying small molecule screens to *Hydra* owing to the ease with which whole animals can be exposed to chemicals in the culture medium. The sequencing of the *Hydra* genome would facilitate the identification of the targets of small molecules, and the ability to make transgenic animals allows for the production of reporter lines for characterization of the changes in gene expression that result from small molecule exposure.

**Table 1. SKP-IV.9.1 is 20-fold more potent than DAC-2-25**

| | Number of tentacles | |
|---|---|---|
| Concentration | DAC-2-25 | SKP-IV.9.1 |
| 5 µM | >15 | >15 |
| 1 µM | 1-3 ectopic tentacles | >15 |
| 500 nM | 0 ectopic tentacles | >15 |
| 100 nM | 0 ectopic tentacles | >15 |
| 50 nM | 0 ectopic tentacles | 1-3 ectopic tentacles |

Our screen for small molecules that affect patterning in *Hydra* was simple – three bisected adult polyps were placed in each well of a 24-well plate together with 1 ml of medium containing a small molecule. After 7 days of exposure, examination under a dissecting microscope was carried out. Among the first 60 molecules screened, we identified DAC-2-25. The robustness and specificity of the response to DAC-2-25 shows that it is possible to identify small molecules that yield informative patterning phenotypes in *Hydra*.

The initial phenotype we obtained with DAC-2-25, regeneration of extra tentacles, suggested that this molecule was affecting some aspect of the patterning process during head regeneration. Animals chronically exposed to DAC-2-25 were particularly informative in this regard. Chronic treatment gave rise to no visible side effects, indicating that DAC-2-25 has high specificity for its target. The most intriguing aspects of the phenotype seen in chronically

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treated animals are the pace at which extra tentacles were formed and the polarity of their appearance. These features of the phenotype suggest that elaboration of the phenotype might be connected to the graded competence component of *Hydra* patterning described by Meinhardt (Meinhardt, 2012). Tissue competence leads to the polarity of regeneration and is relatively stable, as demonstrated by transplantation experiments in which the probability of polarity reversal of regenerating body columns increases when grafted hypostome and tentacle zone were in contact with the former aboral end of a host body column for more than 96 hours (Wilby and Webster, 1970a; Wilby and Webster, 1970b). And more recently, transplantation experiments have shown that explants from ALP-treated animals retained high competence for head formation for 8 days after removal from ALP, measured by the ability to induce the formation of a secondary axis (Gee et al., 2010). Although phenotypic determinants of organizer tissue remained, the expression levels of *HyWnt3* and of other organizer-associated genes returned to normal within 48 hours of the end of ALP treatment (Gee et al., 2010). The polarity and temporal aspects of the response to DAC-2-25, and its cell autonomy, suggest that DAC-2-25 acts on a pathway within the cell whose ability to respond to the molecule is established by the competence gradient.

By examining expression of several marker genes in animals chronically exposed to DAC-2-25, we found that the body column was converted into tentacle zone. The phenotype is not the result of expansion of the tentacle zone while maintaining the existing body column, i.e. the animal is not longer. Nor is the hypostome noticeably larger in the treated animals. Rather, the enlarged tentacle zone is produced at the expense of body column tissue. Thus, we describe this phenotype as a homeotic transformation because it meets Bateson's definition (Bateson, 1894), i.e. that 'something has been changed into the likeness of something else'. Because of its dynamic patterning, *Hydra* is capable of reversing homeotic transformations, as is seen when animals are removed from DAC-2-25.

Animals treated chronically with DAC-2-25 and animals treated with a pulse of ALP share the feature of forming ectopic tentacles. This similarity suggested that the two molecules might target the same pathway, the canonical Wnt signaling pathway, and perhaps the same molecule, GSK3-β. Sequences of GSK3-β cDNAs from the AEP and Zürich strains rule out the possibility that GSK3-β is the direct target of DAC-2-25 as they encode proteins with identical amino acid sequences. We cannot, however, rule out the possibility that DAC-2-25 targets some other component of the canonical Wnt signaling pathway or a target of this pathway.

To understand the mechanism by which DAC-2-25 affects patterning, we obviously need to know the identity of its target, which we assume to be a protein. A standard approach for identifying the protein target of a small molecule is to immobilize the small molecule and use it to affinity purify the target, which is then identified by mass spectrometry. We have carried out SAR studies to identify sites on DAC-2-25 that could be used for immobilization. As part of the SAR study, we explored the possibility of using click chemistry (Best, 2009) to facilitate the identification of the target of DAC-2-25. In particular, we wanted to determine whether we could produce a bioactive alkyene-containing derivative of DAC-2-25 onto which we could click a biotin-tagged azide molecule to allow affinity purification of the target using streptavidin beads. To this end, a derivative was produced in which a propargyl group was added at the C3 position (supplementary material Appendix S1; Fig. 6, compound XI). This compound is active at 5 μM in the chronic exposure assay. Thus, using this derivative and click chemistry in an attempt to purify the DAC-2-25 target seems reasonable.

We are intrigued by the possibility of using genomic approaches to aid in the identification of the protein targeted by DAC-2-25. By looking for non-synonymous changes in the exons of the genomes of the AEP and Zürich strains, we can potentially identify candidate target proteins for DAC-2-25.

## MATERIALS AND METHODS

### *Hydra* strains and culture

Experiments were carried out using *H. vulgaris* AEP (Martin et al., 1997; Technau et al., 2003), *H. vulgaris* Zürich (provided by Dr Monika Hassel, Philipps-Universität Marburg), *H. vulgaris* 950f (Dr Richard Campbell, University of California, Irvine), *H. viridissima* 1695c (Dr Daniel Martínez, Pomona College) and *H. magnipapillata* strain 105 (Chapman et al., 2010). Animals were cultured as previously described (Lenhoff, 1983).

### Small molecule screening

Small molecules from the UC Irvine Department of Chemistry library were supplied frozen in 10 μl aliquots in 96-well deep-well plates at a concentration of 10 mg/ml in DMSO. Stock solutions were dissolved in 1 ml of *Hydra* medium (HM) and the resulting solutions were then transferred to a 24-well plate. Each well contained the pieces from three bisected adult AEP polyps. The plate was incubated for 1 week at 18°C in the dark. The animals were not fed nor was the medium changed during the incubation period. The plates were then examined under a dissecting microscope.

### Treatment of *Hydra* with DAC-2-25 and its derivatives

Free base forms of DAC-2-25 and its derivatives were dissolved in DMSO at a concentration of 5 mM and diluted in HM to the desired concentration immediately before use. Hydrochloride salts of DAC-2-25 and its derivatives were dissolved in Nanopure water at a concentration of 5 mM and diluted in HM immediately before use. Chronically treated animals were fed three times/week and transferred to medium containing freshly diluted compound following feeding.

### Construction of a transgenic *Hydra* line expressing green fluorescent protein in the endoderm and red fluorescent protein in the ectoderm

This line was made by grafting two lines that we had previously produced, in which GFP was expressed in the endoderm of one line and DsRed2 was expressed in the ectoderm of the other line. In both lines, expression of the reporter gene was under the control of an actin gene promoter (Böttger et al., 2002; Wittlieb et al., 2006). Animals from these two lines were bisected and grafted according to standard methods. During the tissue displacement that occurs in *Hydra*, the two epithelial layers can move at slightly different rates, leading to the two layers getting out of register at the graft boundary. When this occurred, we excised the region of the grafted animal in which the DsRed2-expressing tissue had come to overlie GFP-expressing tissue. Regeneration from the excised piece of tissue led to an animal in which the endoderm was green and the ectoderm was red. This line was named (ecto[β-act::RFP]/endo[β-act::GFP]).

### Construction of a transgenic *Hydra* line expressing red fluorescent protein in all tissue lineages

We constructed a transgenic line in which DsRed2 is expressed in all three lineages. This was carried out by injecting embryos of the AEP strain with the pHyVec5 plasmid, which contains the gene for DsRed2 driven by an actin gene promoter (Dana et al., 2012). A transgenic line was obtained in which only the i-cells expressed DsRed2. This line was clonally propagated and then self-crossed to produce lines that are homozygous diploid for the transgene and express DsRed2 in all three lineages. Genome sequencing has confirmed that these lines contain a single integrated copy of the transgene (R.E.S., unpublished).

### Construction and treatment of chimeras and mosaics

For each transgenic chimera, a single founder animal was produced and propagated clonally and representative animals were treated and observed. A chimera with AEP ectoderm and Zürich endoderm was constructed by grafting of halves of ecto[β-act::RFP]/endo[β-act::GFP] polyps with halves of Zürich strain polyps. As described in the above section, when the two epithelial layers got out of register, part of the body column was cut out and allowed to regenerate. The presence of a completely red ectoderm and the absence of GFP expression in the endoderm indicated a fully chimeric animal.

A chimera with AEP i-cells within Zürich epithelium (AEP i-cell chimera) was constructed by grafting the oral half from a Zürich polyp and the aboral half from a DsRed2 (all-red) polyp. DsRed2-expressing nematocytes and nerve cells could be seen in the upper (Zürich) region of the chimera within 48 hours.

The chimera with AEP endoderm and Zürich ectoderm was obtained from a bud generated by the AEP i-cell/Zürich ectoderm chimera described in the previous paragraph. This animal was propagated clonally. Mosaic animals were obtained in the same manner.

### Production of transgenic reporter *Hydra* lines

Transgenic *Hydra* lines were generated as previously described (Wittlieb et al., 2006). Transgenic *Hydra* are initially mosaic, with only some of the cells containing the transgene. To produce reporter *Hydra* that were fully transgenic, hatchlings were monitored for GFP expression, and clonally propagated until all buds that were produced showed GFP expression in the expected locations for Hym-301 and *HyBra2*.

Construction of H301p::GFP::H301t was carried out using standard recombinant DNA methods, the actin gene promoter and 3′ UTR in the plasmid HyEGFP described by Böttger et al. (Böttger et al., 2002) were replaced respectively with a 1212 bp fragment containing the Hym-301 promoter (ending at −9 relative to the Hym-301 translation start codon) and an 840 bp fragment containing the Hym-301 3′ UTR and polyadenylation site (both amplified from *Hydra magnipapillata* strain 105 genomic DNA).

To construct a plasmid in which the HyBra2 promoter drives expression of GFP (*HyBra2*::GFP) a DNA segment consisting of 1456 bp upstream of the *HyBra2* start codon (Chapman et al., 2010) fused to the sequence encoding the first nine amino acids of a *Hydra* actin gene (Fisher and Bode, 1989) was commercially synthesized (Genewiz). This DNA segment was cloned upstream of GFP in pHyVec13 (GenBank Accession Number JN982438) to yield ec(HyBra2::GFP).

### *In situ* hybridization

*In situ* hybridization was carried out as previously described (Bode et al., 2008). Some steps were automated using an InsituPro VS *in situ* hybridization instrument (Intavis Bioanalytical Instruments).

### Treatment of *Hydra* with alsterpaullone

ALP treatment was performed as previously described (Broun et al., 2005). ALP (Sigma-Aldrich) was dissolved in DMSO at a stock concentration of 20 mM and aliquots were stored at −80°C. An aliquot of the stock solution was diluted to the desired concentration in HM immediately prior to use.

### Sources of DAC-2-25 derivatives

The initial hit, DAC-2-25, and several related pyridones in the initial screen were provided as part of the UCI Bioactive Fragment Library, a collection of total synthesis intermediates contributed by the UC Irvine Department of Chemistry research groups and compiled by the synthesis facility in the UC Irvine Department of Pharmaceutical Sciences. Additional analogues were synthesized by employing Parra's simple process in which the dienediolate of an α,β-unsaturated carboxylic acid, formed by deprotonation of the corresponding enoic acid, undergoes addition to an aryl- or alkyl-nitrile followed by spontaneous cyclization to form the 2-pyridone ring system in one operation (Brun et al., 1999; Brun et al., 2000). This methodology is well suited to for the preparation of a wide variety of C3-, C4- and C6-substituted pyridones in which the substitution pattern is reliably determined by the respective enoic acid and nitrile starting materials. The sheer simplicity of this one-step procedure is counterbalanced by low to moderate yields (20-80%) in which the rest of the mass balance is unreacted starting materials, but it nonetheless is the method of choice for preparing such pyridone analogues. The experimental details and analytical data for the synthetic derivatives are described in detail in supplementary material Appendix S1. One of the derivatives, 4-(dimethylamino)benzoic acid, was purchased from Sigma-Aldrich (catalog number 275654-25G).

### RT-PCR

RT-PCR of GSK3-β transcripts from the *H. vulgaris* AEP and Zürich strains was performed as previously described (Dana et al., 2012) using the primers GSK3_L (ATGGTTATTTTACGAAACTATATCC) and GSK3_R (TTAA-GAAGCTTCTGTTGAAATATTG).

#### Acknowledgements

We are very grateful to Dr Felix Grün for providing information on the small molecule library and advice on its use. We thank Dr Thomas Holstein for providing the Ec(HyWnt3FL::GFP) and En(HyWnt3FL::GFP) transgenic lines. We thank Dr Peter Fuhrer for his expert technical assistance in the preparation of this manuscript. We greatly appreciate the thoughtful critiques of the manuscript provided by Drs Annalise Nawrocki, Eva-Maria Schoetz Collins and Celina Juliano. We thank Dr Ira Blitz for insightful discussions regarding possible mechanisms of DAC-2-25 action.

#### Competing interests

The authors declare no competing financial interests.

#### Author contributions

K.M.G., C.E.D., S.S.P., D.A.C., Y.N., T.F., A.R.C. and R.E.S. conceived and designed the experiments; K.M.G., C.E.D., S.S.P., D.A.C. and Y.N. performed the experiments; K.M.G., C.E.D., S.S.P., Y.N., T.F., A.R.C. and R.E.S. analyzed the data; K.M.G., A.R.C. and R.E.S. wrote the paper.

#### Funding

This work was supported by grants from the National Institute of General Medical Sciences [R24-GM080537 to R.E.S. and R01-GM057550 to A.R.C.]. Deposited in PMC for release after 12 months.

#### Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.094490/-/DC1

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