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A heat shock protein and Wnt signaling crosstalk during axial patterning and stem cell proliferation

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Running head: Hsp and Wnt link in a cnidarian

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SUMMARY

Both Wnt signaling and heat shock proteins play important roles in development and disease. As such, they have been widely, though separately, studied. Here we show a link between a heat shock protein and Wnt signaling in a member of the basal phylum, Cnidaria. A heat shock at late gastrulation in the clonal marine hydrozoan, *Hydractinia*, interferes with axis development, specifically inhibiting head development, while aboral structures remain unaffected. The heat treatment upregulated *Hsc71*, a constitutive *Hsp70* related gene, followed by a transient upregulation, and long-term downregulation, of Wnt signaling components. Downregulating *Hsc71* by RNAi in heat-shocked animals rescued these defects, resulting in normal head development. Transgenic animals, ectopically expressing *Hsc71*, had similar developmental abnormalities as heat-shocked animals in terms of both morphology and *Wnt3* expression. We also found that *Hsc71* is upregulated in response to ectopic Wnt activation, but only in the context of stem cell proliferation and not in head development. *Hsc71*'s normal expression is consistent with a conserved role in mitosis and apoptosis inhibition. Our results demonstrate a hitherto unknown crosstalk between heat shock proteins and Wnt/ β -catenin signaling. This link likely has important implications in understanding normal development, congenital defects and cancer biology.

INTRODUCTION

Heat shock protein (Hsp) 70 family members have been extensively studied for their ability to protect cells from proliferative and environmental stress factors. These proteins function as constitutive and stress-inducible molecular chaperones. The expression patterns of Hsps are regulated accordingly, with constitutive and stress-induced genes. Due to their ability to chaperone a broad array of proteins, Hsps have been implicated in diverse biological processes, from antigen presentation and immune stimulation, to virus replication, cancer survival, neurological diseases, and stem cell self-renewal (Ahmad, 2010; Chapman et al., 2010; Karapanagiotou et al., 2009; Milani et al., 2002; Nirde et al., 2009). Hsps also function in conjunction with various signaling pathways, e.g. Ras-PKA (Shapiro et al., 2009), JAK/STAT3 (Longshaw et al., 2009), and IRE1a-XBP1 (Gupta et al., 2010). Hsps' mode of action is complex and not well understood (Prinsloo et al., 2009).

Despite being phylogenetically highly conserved in sequence, at least some of the tasks fulfilled by Hsps might have changed during animal evolution. To address this issue it is necessary to study animals that belong to early diverging phyla. Furthermore, due to the complexity of the Hsps' roles, studying Hsps in lower animals may not only reveal ancestral Hsp functions, but also ones that are cryptic in higher animals. Cnidarians are good candidates for such studies, as they form the sister group to all bilaterians, and are morphologically simple. Furthermore, cnidarian model organisms are amenable to functional studies including gene expression analyses, gene knockdown and transgenesis.

Hydractinia echinata (Fig. S2) is a cnidarian model organism (Frank et al., 2001). This animal forms clonal colonies, but also reproduces sexually on a daily basis. The clonal nature of *Hydractinia* is thought to be dependent on a population of stem cells, called interstitial cells or i-cells, present in the animal life-long and continuously giving rise to all somatic cell types and to gametes (Frank et al., 2009; Künzel et al., 2010; Müller et al., 2004). Like other cnidarians, *Hydractinia* has a relatively simple morphology, consisting of two epithelial sheets (epidermis and gastrodermis) that are separated by a basement membrane, the mesoglea. *Hydractinia* polyps are radial symmetric and possess an oral-aboral axis (Fig. S2). This polarity is generated and maintained throughout the animal's life cycle by the canonical Wnt pathway, which specifies the position of the head (Duffy et al., 2010; Müller et al., 2007; Plickert et al., 2006), but Wnt also controls stem cell fate (Teo et al., 2006).

A screen for Wnt target genes in *Hydractinia* revealed a member of the *Hsp70* family that was upregulated following global Wnt activation. Here we show that this gene (*Hsc71*) is associated not only with stem cell proliferation and apoptosis inhibition, like Hsps from other organisms (Gupta et al., 2010; Samali and Cotter, 1996), but also with axis development, forming hitherto undescribed links with the canonical Wnt pathway.

MATERIALS AND METHODS

Animals

Hydractinia echinata colonies were collected from Galway Bay, Ireland by SCUBA diving. They were cultured in natural seawater at 18°C under 14/10 light-dark regimes and were fed brine shrimp nauplii (*Artemia salina*) six times a week. This diet was

supplemented by ground fish once a week. Fertilized eggs were collected daily about an hour after the light-induced spawning and the embryos kept in Petri dishes for three days until embryonic development was completed, and metamorphosis-competent planula larvae stopped autonomous development. Metamorphosis was induced by a three-hour pulse treatment of 116 mM CsCl in seawater as previously described (Frank et al., 2001; Müller and Buchal, 1973) and the animals were allowed to settle and metamorphose on glass coverslips.

Suppressive subtractive hybridization (SSH)

Polyps were cut away from colonies at their base and incubated in seawater containing 28 mM LiCl for 24 hours. Control polyps were kept in seawater only. One hour fifteen minutes after the treatment, total RNA from LiCl-treated and control polyps was extracted and suppressive subtractive hybridization (SSH) was performed as has previously been reported (Duffy and Frank, 2011). Clones from the subtracted library were sequenced and the differential expression of selected ones was verified by qPCR following LiCl or azakenpaullone treatment. Azakenpaullone, known to be a more specific blocker of GSK3 (Kunick et al., 2004), was used in comparison to Lithium ions. Thirty-one-hour old embryos were incubated in seawater containing 28mM LiCl or 1 μ M azakenpaullone for 18.5 hours. Stock solutions of azakenpaullone were prepared with DMSO and stored at -20° C. Working solutions were prepared immediately before use as described (Müller et al., 2007; Plickert et al., 2006) and incubation of the specimens was carried out in the dark.

Obtaining *Hsc71* sequence

A 795 bp fragment of the *Hsc71* gene was obtained from the SSH for genes upregulated by mimicking global Wnt signaling with LiCl (see above).

To obtain full length sequences, the SMART RACE protocol (Clontech) was used for 3' RACE, and the *Hydractinia* trans-spliced leader 5'ACTCACACTATTTCTAAGTCCCTGAG3' was used for isolating the 5' sequence. *Hsc71* specific primers were designed from the cloned sequence obtained from the SSH. The gene specific primers used were (1) 3' *Hsc71* obtained with the forward primer 5'ACAACCTATTCATGTGTTGGCGTTTTCC3'; (2) 5' *Hsc71* obtained with the reverse primer 5'GCAACTTGATTTTTAGCAGCATCACCC3'. The *Hsc71* nucleotide sequence has been deposited in GenBank under the accession number JN400918.

Quantitative, real-time PCR (qPCR)

Total RNA was extracted from samples to be analyzed by acid guanidinium/phenol:chloroform extraction, digested with RQ1 RNase free DNase (Promega), and phenol:chloroform purified. RNA was reverse transcribed to cDNA using Omniscript Reverse Transcriptase (Qiagen). qPCR was performed on a StepOne Plus Real Time PCR System (Applied Biosystems) with SYBR reagents (Roche) according to the manufacturer's recommended protocol. Gene expression was normalized to the expression of *GAPDH*. Generated PCR products were analyzed by melt curve analysis, by gel electrophoresis and by sequencing. The following primer sets were used: *GAPDH* nested forward 5'TGCTACAACCTGCCACACAGAAAA3' and reverse 5'CACCACGACCATCTCTCCATTT3'.

Hsc71 forward 5' CCAACCGGCACCACTTAAA3' and reverse 5'TTCTGTGTCTGTGAATCCAACATAGC 3'.

Wnt3 forward 5'CCAACACCAACGCGAAGTATG3' and reverse 5'ACCTTCCCCGACACTTCTGA3'.

Brachyury forward 5'CCAACCGGCACCACTTAAA3' and reverse 5'CGAGCTAACGGCGACACTTT3'.

Tcf forward 5' AAATAGCTGCAGAGTTTACGTTAAAAGA3' and reverse 5'TGCCACCTTTTCCCAAGAAT3'. *Tcf*, *Brachyury*, *Wnt3* and *GAPDH* primers were designed from the GenBank accession numbers GU145278, AF312733.1, AM279678 and DT622622, respectively.

RNAi experiments

RNAi was performed as previously described (Duffy et al., 2010; Millane et al., 2011). Essentially, animals were incubated in 0.5 ml of seawater in which dsRNA has been added to a final concentration of 80 ng/μl. Templates for RNA synthesis were made by PCR. Two templates were made for each dsRNA, one with a T7 promoter site on the forward primer and a second with a T7 on the reverse primer. Primer pairs for *Hsc71* were as follows:

T7 forward 5'GATCATAATACGACTCACTATAGGG TTCGCTTTTGATCAGCCTCT3' and reverse 5'GTTGGAAAACGCCAACACAT3' or forward 5'TTCGCTTTTGATCAGCCTCT3' and T7 reverse 5'GATCATAATACGACTCACTATAGGG GTTGGAAAACGCCAACACAT3'

The resulting template of 171 bp for *Hsc71* was used to make single stranded RNA as previously described (Duffy et al., 2010; Millane et al., 2011).

Heat treatments

Four groups of embryos were prepared:

(1) Heat treatment: Twenty eight to thirty hours after fertilization embryos were exposed to a 45-minute temperature shock of 28°C, after which they were returned to the normal culturing temperature of 18°C.

(2) Control: Embryos were maintained at normal culturing conditions.

(3) Rescue: Embryos to be rescued from the heat treatment were incubated in *Hsc71* dsRNA in seawater (80 ng/μl) for 25.5 hours, 3.5 hours of which were before the start of the heat treatment. The embryos were then exposed to the same heat treatment as the heat only embryos, while remaining in the RNAi solution.

(4) RNAi only: Embryos were incubated in dsRNA solution for the same duration as the rescue animals but were not exposed to the heat treatment.

One day after the heat treatment the embryos from all four groups had reached the planula stage and were induced to metamorphose. Stolon and tentacle measurements for each animal were made 1 and 2 days after the start of metamorphosis induction.

In situ hybridization

In situ hybridization was performed as described (Gajewsky et al., 1996). Hybridizations were performed at 55°C. *Hsc71* probes were synthesized from a pGEM-T plasmid containing the 795 bp fragment of the gene obtained from the SSH. *Wnt3* 309 bases probes were synthesized from a plasmid containing a PCR fragment obtained by the following primers:

forward 5'ATGGATTGTTTCCATAAAGTTTTACTGCTG3' and

reverse 5'GGTCCAAAACACTGACCCTTTC3'.

Bromodeoxyuridine (BrdU) proliferation assay

Animals were incubated in seawater containing 200 μ M BrdU for 2 hours and thereafter washed with seawater. Forty minutes after the end of the BrdU incubation they were fixed with paraformaldehyde and subjected to in situ hybridization (see above). BrdU detection was then performed as was described previously (Duffy and Frank, 2011).

TUNEL cell death detection assay

After in situ hybridization was performed, animals were washed with PBST. They were then incubated in TUNEL reaction mixture (Roche 11684795910) at 37°C for 80 minutes. Animals were then washed with PBST.

Ectopic expression of *Hsc71*

Transgenic animals, ectopically expressing *Hsc71* were generated. An expression construct that included the full coding region of *Hsc71*, fused to enhanced green fluorescent protein (EGFP) coding sequence, driven by the *Hydractinia Actin1* promoter was microinjected into one-two cell stage embryos as described previously (Künzel et al., 2010; Millane et al., 2011). About 100 pl linearized vector solution was microinjected to each embryo at 2-5 ng/nl.

RESULTS

Identification and sequence analysis of a *Hydractinia* homologue of *Hsc71*

In a search for Wnt responsive genes in the cnidarian *Hydractinia echinata* we identified a 795 bp fragment of a gene with high sequence similarity to human Hsc71. The search was done by comparing the transcriptome of normal animals to one of LiCl treated colonies by suppressive subtractive hybridization (SSH) (Duffy and Frank, 2011). LiCl is known to block GSK3 (van Noort et al., 2002), thereby stabilizing β -catenin and activating Wnt targets (Fig. S1A). The function of LiCl has been demonstrated in cnidarians previously (Hassel et al., 1993; Teo et al., 2006). qPCR was used to verify the upregulation of the gene as a response to Wnt activation by either LiCl or azakenpaullone, a more specific inhibitor of GSK3 (Kunick et al., 2004). Indeed, azakenpaullone caused a higher upregulation of *Hsc71* compared to LiCl (Fig. S1B). In addition, a putative Tcf binding element was identified in the *Hsc71* promoter region (Fig. S3B), consistent with the gene being a Wnt target.

Primers were designed from the SSH-obtained fragment to amplify the 3' end of the full-length transcript by RACE PCR. The 5' end was obtained using the *Hydractinia* trans-spliced leader. This leader sequence is added to many *Hydractinia* transcripts (Chapman et al., 2010; Duffy et al., 2010), similar to the situation in *Hydra* (Stover and Steele, 2001), and can be used to easily get full 5' ends of *Hydractinia* mRNAs. The full-length mRNA encoded a 653 amino acid long protein with a predicted molecular mass of 71 kDa. The protein had high sequence similarity with the human Heat Shock Cognate 71 kDa (isoform 8, variant 1), also known as HSPA8 (Kampinga et al., 2009) (GenBank accession number BAD96505) with 81% identical amino acid residues (Fig. S3A). The gene was named as *Hydractinia Hsc71-like*, henceforth abbreviated as *Hsc71*. BLAST analysis on the NCBI website revealed that the most closely related protein outside the Cnidaria is the gastropod *Haliotis diversicolor* heat

shock cognate protein 70. The closest cnidarian hit is the *Hydra magnipapillata* heat shock protein 70, GenBank accession numbers ACO36047 and AAA29213, respectively. The *Hydractinia* Hsc71 contains putative GSK3 phosphorylation sites (Fig. S3A) (Cohen and Frame, 2001; Eivers et al., 2009) as is the case in *Xenopus* (De Robertis, personal communication).

Expression pattern of in *Hsc71* normal animals

In situ hybridization was used to study the temporal and spatial expression of *Hsc71* throughout the life cycle of normal animals. The gene was found to be expressed in all life stages of *Hydractinia* (Fig. 1), confirming it to be a constitutively expressed member of the heat shock family. In early embryos, *Hsc71* was expressed ubiquitously (Fig. 1A). As of the late post-gastrula stage (also called pre-planula stage), *Hsc71* mRNA became progressively downregulated in the prospective oral pole, and was predominantly endodermal in the aboral regions. In the planula larva, expression level was generally lower, but the spatial expression remained unchanged (Fig. 1B). *Hsc71* expression was upregulated once metamorphosis was induced by CsCl, but it maintained the same spatial expression pattern with lower expression in the prospective oral pole (Figs 1C, S4). By the end of metamorphosis, *Hsc71* expression became concentrated in an aboral ring of i-cells at the base of the primary polyp (Fig. 1D). During metamorphosis of the planula and emergence of the primary polyp, i-cells proliferate and migrate first from the endodermal cell mass to the interstices of the epidermis, and then from the body column into the newly developing stolonal system, where they reside during adult life (Fig. 1E) (Frank et al., 2009). *Hsc71* mRNA was undetectable in tentacles and was low in epithelial cells of the stolonal epidermis. Expression levels decreased in the polyp during maturation,

reaching undetectable values by in situ hybridization when the polyps reached their final size (not shown). Expression remained strong in stem cells in the stolons. However, weaker expression levels were also detectable in stolon epithelial cells, primarily in the gastrodermal epithelium, while stolon tips remained unlabeled.

As *Hydractinia* stolons elongate, new, clonal polyps bud at regular intervals. At the onset of budding, *Hsc71* was strongly upregulated throughout the early bud (Fig. 1F). As the bud developed, expression became restricted first to the polyp body column, excluding the tentacles (Fig. 1G), and later to the gastrodermis of the lower, aboral part of the developing polyp, being undetectable in the developing head (Fig. 1H). Eventually, *Hsc71* mRNA dissipated from the rest of the body column when the polyp became fully-grown, similar to the expression pattern in primary polyps (not shown).

Strong expression of *Hsc71* was also detected in i-cells in the germinal zone of sexual polyps. This is the location of i-cells that are committed to become gametes (Fig. 1I). *Hsc71* was also upregulated in developing gametes within gonads of female and male colonies (Fig. 1J, K, respectively), but was undetectable in mature gametes (not shown).

***Hsc71* is expressed in Wnt-induced proliferating i-cells**

Since in situ hybridization showed high *Hsc71* expression during stages and in regions of rapid cell proliferation, we aimed at analyzing this feature in more detail.

Ectopic activation of the canonical Wnt pathway in *Hydractinia* by pharmacologically blocking GSK3 causes a proliferation burst of i-cells (Duffy et al., 2010; Teo et al., 2006). Within 24 hours, LiCl treated polyps developed ectopic outgrowths that strongly expressed *Hsc71*. These outgrowths are distinct from those obtained by ectopic *Pln* expression, because they derive from the expansion of existing i-cells, rather than from *Pln*-mediated conversion of epithelial cells to i-cells (Millane et al., 2011). Such animals, double labeled with BrdU and *Hsc71* mRNA in situ hybridization, confirmed that S-phase i-cells strongly expressed *Hsc71*, which was also the case in untreated animals (Fig. 2), suggesting an essential role of *Hsc71* in Wnt mediated stem cell proliferation.

Hsc71 and apoptosis

Members of the Hsp70 family are known to have an anti-apoptotic role (Gupta et al., 2010; Samali and Cotter, 1996). To examine the conservation of this function in cnidarians, we analyzed *Hsc71* expression during early *Hydractinia* metamorphosis. This stage is known to commence with extensive, spatially and temporally predictable apoptotic events (Seipp et al., 2001; Seipp et al., 2010; Wittig et al., 2011). Co-labeling by TUNEL and *Hsc71* mRNA during metamorphosis was performed. Consistent with previous studies, apoptosis began shortly after the induction of metamorphosis and was concentrated in the tail pole (posterior) of the larvae, which corresponds to the oral pole of the developing polyp (Fig. 3). *Hsc71* was complementarily expressed to the regions undergoing apoptosis (Fig. 3). As metamorphosis progressed, the *Hsc71*-negative tissue was removed by apoptosis, while the *Hsc71*-expressing tissues survived (Fig. S4). These data are consistent with

Hsc71 potentially having a conserved anti-apoptotic role in cnidarians, similar to that shown in other metazoans.

Heat inducible expression of *Hsc71*

To study a possible heat inducibility of *Hsc71* expression, larvae were exposed to a 28°C heat treatment for 10, 30 or 45 minutes. The larval stage was chosen for this experiment because *Hsc71* expression is relatively low at this stage, and a possible upregulation of the gene should therefore be more easily observed. Control larvae were kept at normal culturing temperatures (18°C). Directly after treatment, the larvae were either fixed and processed for in situ hybridization with the *Hsc71* probe, or RNA was extracted from them for qPCR. These experiments showed a clear increase in *Hsc71* expression following exposure to elevated temperatures, directly proportional to the duration of the treatment (not shown). Moreover, *Hsc71*'s expression became ubiquitous to also include the larval tail (i.e. the prospective polyp head), which normally does not express the gene (Fig. 1B). qPCR showed an over 2 times increase in *Hsc71* mRNA levels in the heat treated animals compared with controls (not shown). The highest increase in heat inducible expression was 3.5 fold in pre-planula (see below), 16hr after heat treatment (Fig. 4). Therefore, despite being constitutively expressed in normal animals, *Hsc71* is also heat responsive, resulting in expression in areas that normally do not express the gene (i.e. the oral pole; Fig. 4B). Consistent with its heat inducibility, analysis of the *Hsc71* promoter region clearly revealed heat shock responsive elements (Fig. S3).

Heat treatment disrupts axial patterning

In order to study the function of *Hsc71* during development we performed heat treatment experiments to increase its expression levels and to ectopically express it at the posterior pole where it is normally absent. Heat treatment experiments were combined with *Hsc71* RNAi to prove the specificity of the resulting phenotypes. Thirty-hour-old embryos (late gastrula, or pre-planula) were exposed to a 45-minute heat treatment at 28°C. As in the later planula larvae stage, this treatment upregulated *Hsc71* expression (Fig. 4; 5G) and rendered it ubiquitous after 30 minutes (Fig. 4B). The heat inducible expression profile as recorded by qPCR, was as follows: 2.75 fold 30 minutes after treatment, 3.5 fold after 16 hours, and 3 fold after 24 hours. Three hours later, after induction of metamorphosis it was reduced to 1.4 fold (Fig. 4). The heat treatment had no visible effect on the anterior development of the embryos, but severely impaired posterior development. The *Hydractinia* larva is patterned along an anterior-posterior (AP) axis with an anterior pole that contains sensory cells and a posterior tapered tail (Fig. 5A) (Plickert et al., 2003). The posterior tail differs in cellular composition from the anterior end by the absence of nerve cells containing neuropeptides of the GLWamide class (Plickert et al., 2003; Schmich et al., 1998a) and by the presence of specific nematocytes (Müller and Leitz, 2002; Weis et al., 1985). The larva's direction of movement is always anterior. The posterior end of the larva expresses *Wnt3* and develops into the polyp head following metamorphosis (the oral pole), while the anterior end attaches to the substratum and develops stolons (the aboral pole). The tapered, posterior (prospective oral) end of the heat-treated embryos did not develop properly and acquired a blunt, anterior-like morphology (Fig. 5B). Initiation of metamorphosis in *Hydractinia* is controlled by anteriorly positioned GLWamide positive neurosensory cells (Plickert et al., 2003; Schmich et al., 1998b). Once stimulated (naturally by bacteria, or experimentally by CsCl), these neurons

secrete GLWamide peptides that diffuse posteriorly and control metamorphosis throughout the animal (Müller and Leitz, 2002). Hence, *Hydractinia's* competence to respond to bacteria or CsCl by entering metamorphosis depends on intact, GLWamide containing neurons at the larval anterior pole. The heat treatment did not affect the larval anterior pole, allowing metamorphosis to commence normally following CsCl treatment, and the animals developed normal-appearing body columns and stolons during metamorphosis. However, the mal-developed posterior pole failed to form proper head structures, resulting instead in headless or head-reduced primary polyps (Fig. 5E). This phenotype is similar to the one obtained when either *Wnt3* or *Tcf* are downregulated by RNAi (Duffy et al., 2010).

To investigate the specific role of *Hsc71* in heat-induced head repression, we performed rescue experiments by downregulating *Hsc71* with RNAi. Animals were heat treated and simultaneously incubated in dsRNA corresponding to the *Hsc71* coding sequence. These experiments resulted in more normally patterned planula larvae (i.e. with normal tapered posterior end: Fig. 5C). When induced to metamorphose, these planulae developed to more normally patterned primary polyps that included both stolons and heads (Fig. 5F). qPCR showed that the net result of the combined heat and RNAi treatments was a *Hsc71* expression level closer to that of untreated animals (Fig. 5G). The morphological results of these experiments were quantified as follows: For each animal, total stolon length and the length of the longest tentacle were measured at 24 and 48 hours after induction of metamorphosis. A head development to stolon development ratio was obtained by dividing the animals' stolon length by the length of their longest developing tentacle. This was taken as a proxy for the level of head development which also accounts for

metamorphosis progression; such ratios were used previously in analyzing the results of experiments which deregulated Wnt signaling (Duffy et al., 2010). At this developmental stage, tentacles do not contract as a response to a mechanical stimulus (as is the case in adults), and measuring the tentacle length therefore accurately reflects the level of tentacle development. The higher the ratio number, the less well developed the head in relation to the stolons. Hence, a general, uniform retardation in development would not affect the ratio. One day after metamorphosis the average ratios were 3.55 for controls, 34.47 for heat-treated, 12.11 for rescue, and 4.11 for RNAi alone (Fig. 5H). In absolute terms, stolon development was not significantly affected by either the heat treatment or by RNAi, while head development was inhibited by embryonic heat treatment, and rescued if *Hsc71* was downregulated (Fig. 5E, F). Interestingly, consistent with previous experience in *Hydractinia*, phenotypic effects can be obtained by less severe RNAi treatments if the animal has also been perturbed in another way (Duffy et al.). Previously a single Tcf RNAi treatment was shown to be sufficient to block head regeneration after decapitation however, a double Tcf RNAi treatment was necessary to block normal head formation in the absence of any other perturbation. Here we show how a single Hsc71 RNAi treatment is sufficient to rescue head development from a heat shock while a single Hsc71 RNAi alone has minimal effect on development.

These results demonstrate that increased Hsc71 expression is sufficient to impede the development of posterior/oral structures, i.e. larval tails and polyp heads.

Wnt genes expression in heat-treated animals

With the role of Wnt signaling in *Hydractinia* axial patterning in mind, we studied the expression of Wnt genes in heat-treated animals. While *Hsc71* expression went up

within an hour following the heat treatment, the expression of *Wnt3* initially remained unchanged in both spatial and quantitative terms. However, 24 hours after heat treatment, *Wnt3* expression became upregulated and its normal posterior polar expression became ubiquitous (Fig. 6A). *Brachyury*, a Wnt target gene, was also upregulated (Fig. 6D). Following metamorphosis induction, *Wnt3* and *Brachyury* expression returned to quantitatively almost normal values but the spatial expression was not completely restored. By 20 hours post metamorphosis induction (44 hours post treatment), *Wnt3* expression was reduced by 60% in heat-treated animals. *Brachyury* and *Tcf* expression were reduced by 25% and 30%, respectively. This is the time at which the polyp head is normally being patterned and requires Wnt signaling. These results suggest that Hsc71 acts in head inhibition by antagonizing *Wnt3* and its targets.

Ectopic expression of Hsc71 in transgenic animals

To further examine the potential of ectopic *Hsc71* expression to affect axial patterning through Wnt modulation, we generated transgenic animals, which ectopically expressed *Hsc71*. The *Hydractinia Actin1* promoter was used to drive *Hsc71* as eGFP (enhanced Green Fluorescent Protein) fusion protein (Fig. 7A). This promoter is epithelial-specific in *Hydractinia* larvae and polyps (Künzel et al., 2010), but is ubiquitous in very early embryos. One or two cell stage embryos were microinjected with 1-5 pl of the construct at 2-5 ng/nl. Control constructs, lacking the *Hsc71* coding sequence, were injected at the same concentrations. Stable expression of the transgene was evident as green fluorescent embryos under blue light within 24-48 hours (Fig. 7).

Similar to the heat-treated embryos, *Hsc71* transgenic embryos developed into embryos and larvae with stunted posterior (i.e. prospective oral) development (Fig. 7B), compared with normal embryos (Fig. 7C). The severity of the phenotype was correlated with the percentage of transgenic cells. First generation transgenic animals are always mosaics (Künzel et al., 2010), because integration of the construct into the genome followed by stable transgene expression is a stochastic process, which also depends on the precise timing and concentration of the injection and construct solution. In this case, animals with high percentages of transgenic cells (evident by high GFP fluorescence) were more severely affected and most of them died without reaching the larval stage. These embryos remain rounded even 48 hours post fertilization, in contrast to their elongated control counterparts (Fig. 7B). Embryos with intermediate levels of fluorescence did reach the larval stage but had impaired posterior poles, strongly resembling heat-treated animals by having two blunt ended poles (Fig. 7D; compare with Fig. 5B).

A small number of transgenic animals (n=8) were successfully induced to metamorphose. Half of the animals metamorphosed only partly, retaining larval structures alongside newly developed polyp features (Fig. 7F; compare with normal metamorphosed polyp on Fig. 7G). This could be explained by the requirement of apoptosis for *Hydractinia* metamorphosis (Seipp et al., 2006); high levels of transgene-driven, potentially anti-apoptotic *Hsc71* possibly prevented removal of larval tissues. The other larvae developed into aberrant polyps with disrupted anterior-posterior polarity that died shortly thereafter (not shown). Hence, *Hsc71* ectopic expression by a transgene also resulted in abnormal anterior-posterior development, similar to the effect of *Hsc71* following heat shock.

***Wnt3* expression in *Hsc71* transgenic animals**

To further analyze the phenotype of the *Hsc71* ectopically expressing transgenic animals, we used in situ hybridization to study the spatial expression of *Wnt3*. One-cell stage embryos were injected with the *Hsc71* ectopic expression construct as described above. About 24 hours later, when the animals reached the late gastrula stage (pre-planula), fluorescent animals were fixed and processed for *Wnt3* in situ hybridization. At this stage, normal animals express *Wnt3* exclusively at the prospective oral pole (Duffy et al., 2010; Plickert et al., 2006) (Fig. 7H) and mRNA is found in epithelial cells and i-cells.

The transgenic animals, however, displayed a very different pattern of *Wnt3* expression (Fig. 7I). Rather than being restricted to epithelial and i-cells at the prospective oral pole, *Wnt3* was expressed only in i-cells throughout the entire embryo (Fig. 7I). Hence, ectopic *Hsc71* expression changed *Wnt3* expression both spatially, being no longer polar, as well as in terms of cell type expressing *Wnt3*, which were restricted to i-cells in *Hsc71* transgenic animals.

DISCUSSION

Hsc71 is expressed in all *Hydractinia* life stages. However, rather than being ubiquitous, the gene is spatially and temporally regulated, having expression levels ranging from very high to undetectable, depending on developmental stage, anatomical location and cell type. In general terms, *Hsc71* mRNA was detected

predominately in proliferating embryonic cells, i-cells and germ cell progenitors, and absent from apoptotic cells, from the head of developing polyps, the whole of mature polyps and from epidermal epithelial cells. Weaker expression levels were recorded in gastrodermal epithelial cells. Interestingly, *Hsc71* mRNA is initially expressed ubiquitously in early embryos and budding polyps being later restricted from posterior/oral regions suggesting that *Hsc71* inhibits the development of posterior/oral structures rather than inhibiting the initial specification of these poles.

Following induction of metamorphosis, *Hsc71* was expressed in the surviving cells, but absent from those undergoing apoptosis. *Hsc71* is also heat responsive. Following heat treatment, the gene's expression increased quantitatively and became ubiquitous. The expression pattern of *Hsc71* is consistent with a role in stem cell proliferation and inhibition of apoptosis. Hence, our data is consistent with functional conservation of the *Hydractinia Hsc71* with its mammalian homologues. As such, the *Hydractinia Hsc71* could be used as a model for these cellular processes, mediated by Hsp/Hsc-related proteins in a simple experimental system that allows genetic manipulation and whole mount gene expression analysis.

Our results provide evidence for links between *Hsc71* and Wnt signaling. First, the gene was discovered in a screen for Wnt responsive genes. Indeed, global activation of Wnt downstream events resulted in a quantitative upregulation in *Hsc71* expression (Fig. S1), as well as the disruption of its spatial regulation, inducing *Hsc71* expressing proliferating stem cells in ectopic tissue outgrowths (Fig. 2). Such Wnt induced neoplasms may prove to be a useful model for the study of early stage Wnt oncogenic potential. It should be noted that forced expression of *Pln*, a POU-domain gene, in

epithelial cells also resulted in stem cell neoplasms (Millane et al., 2011). An important difference between these two types of *Hydractinia* neoplasms is that Pln-induced neoplasms result from the conversion of epithelial cells to stem cells, whereas Wnt activation induced neoplasms result from increased proliferation of existing stem cells.

Second, *Hsc71* also acts upstream of Wnt. Upregulation of *Hsc71* by heat treatment in late gastrulae resulted in deregulation of *Wnt3* and two of its targets, *Brachyury* and *Tcf*. The quantitative expression of these genes initially went up following heat treatment, reaching a peak within 24 hours post treatment. The normal polar expression of *Wnt3* became ubiquitous. About a day later, however, *Wnt3* expression went down to 40% of normal expression and was no longer detectable by in situ hybridization in the oral pole where it is normally expressed. Heat treatment and the resulting change in *Wnt3* expression caused disruption of posterior patterning. Following metamorphosis induction, these heat-treated animals failed to form properly developed heads; some of them were completely lacking any oral features, similar to the phenotype obtained when *Tcf* is downregulated during metamorphosis by RNAi (Duffy et al., 2010). This headless phenotype was rescueable in heat-treated animals by downregulating *Hsc71* with RNAi, showing that the disruption of head development was primarily a specific action of ectopic *Hsc71* rather than being an unspecific effect of the heat treatment. Downregulation of *Wnt3* explains the impaired head development, and suggests that ectopic *Hsc71* acts in *Hydractinia* head inhibition in a Wnt3-dependent fashion. Since the initial response to increased *Hsc71* expression was *Wnt3* upregulation, it is possible that a heat shock given at different

timing would induce ectopic heads. In *Hydra*, a related cnidarian, a localized heat treatment has previously been associated with increased Wnt3 expression in apoptotic i-cells and a proliferation burst of i-cells adjacent to the apoptotic zone, ultimately resulting in de novo head patterning (Chera et al., 2009). The link reported here between Hsc71 and Wnt signaling is a likely intermediary between the heat treatment and head development reported in *Hydra*.

Ectopic expression of *Hsc71* by transgenesis also resulted in anterior-posterior patterning defects, similar to those arising from heat-treated embryos. *Wnt3* expression was also altered in transgenic animals. It was not only spatially deregulated, but expression was restricted to i-cells and no *Wnt3* mRNA was detected in epithelial cells of the oral pole as in normal embryos.

Our data suggest, in addition, that the nature of the links between Hsc71 and Wnt signaling is complex, context-dependent, and might be acting at both protein and mRNA levels. For example, while global Wnt activation by blocking GSK3 caused upregulation and ectopic spatial expression of *Hsc71* mRNA, in normal animals, *Hsc71* and high cell proliferation are absent from the *Wnt3* expressing head. Hence, *Wnt3* does not upregulate *Hsc71* in a head context, nor does extensive proliferation normally occur there. Possibly, Wnt ligands other than Wnt3 are required to upregulate *Hsc71*. Our experiments, which upregulate Wnt downstream events by inhibiting GSK3, are consistent with this scenario.

We propose a model of Hsc71-Wnt crosstalk (Fig. 7J), in which upregulation of *Wnt3* by Hsc71 occurs by Hsc71's role as a chaperone. Extending *Wnt3*'s half-life would also increase its mRNA levels, as *Wnt3* is a target of its own protein (Duffy et al., 2010). The initial upregulation of *Wnt3* could be followed by a later action of Hsc71 on a Wnt antagonist, thereby reducing Wnt activity. These two processes can either be in equilibrium, or one may predominate. At the protein level, Hsc71 includes putative GSK3 phosphorylation sites (Fig. S3), which could lead to Wnt-mediated stabilization of its protein through inhibition of GSK3, but should not necessarily affect its mRNA levels. The promoter region of *Hsc71* also contains putative Tcf binding sites (Fig. S3), suggesting it to be a direct Wnt target. Unraveling the interactions between Hsc71 and Wnt signaling will be key to understanding how this crosstalk may affect development and disease.

The links between HSPs and Wnt, reported here, have yet to be confirmed in vertebrates. However, given the common functioning of Wnts and Hsps during proliferation, their deregulation in cancers (Logan and Nusse, 2004; Nirde et al., 2009), and the ability of a single heat shock to disrupt somitogenesis (Roy et al., 1999; Wargelius et al., 2005) – a process that has been linked to *Wnt3* (Aulehla et al., 2003), it is likely that crosstalk between Wnts and Hsps is evolutionarily conserved, rather than being a cnidarian innovation. Wnt-HSP interactions could have implications for cancer research, where heat shock family members and Wnts are already being investigated as targets for treatment, separately.

While the role of Hsc71 in proliferation, anti-apoptosis and stem cell maintenance is well documented in other organisms, a direct function of a heat shock protein in animal axial development has not been previously reported. Combined with the link between Hsc71 and Wnt, also reported here for the first time, it is plausible to assume that heat shock-related proteins fulfill further, unexpected functions in development and disease.

Acknowledgements

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Figure legends

Figure 1. In situ hybridization of *Hsc71* in normal animals. **(A)** Cleaving embryo, counterstained with DAPI. Dividing nuclei are visible. Inset: 4 cell stage embryo. **(B)** Planula larva. Inset: *Hsc71* positive i-cells. **(C)** Metamorphosing larva. *Hsc71* expression is concentrated in the larval anterior pole that will give rise to stolons (the aboral pole) of the polyp. **(D)** Bottom view of metamorphosed primary polyp. *Hsc71* expressing i-cells are concentrated in a ring-like structure at the base of the polyp and entering the outgrowing stolons. S=stolon; T=tentacle. **(E)** Higher magnification of outgrowing stolons of a primary polyp showing i-cells expressing *Hsc71* (arrow). **(F-H)** Polyp budding. Developing oral pole (head) **(F)** Early stage of polyp budding. *Hsc71* is highly expressed throughout the bud. **(G, H)** Later budding stages. *Hsc71* becomes excluded from the tentacles **(G)** and later, the complete head **(H)**. **(I)** *Hsc71* expressing i-cells in the body column of a sexual polyp (arrow). **(J, K)** *Hsc71* expressing cells (presumably germ cell progenitors) in female **(J)** and male **(K)** gonads (arrows). Scale bars 50 μm (A, G), 100 μm (B, H), 200 μm (C), 150 μm (D), 20 μm (E, K), 200 μm (F), 30 μm (I, J, K). Asterisks in B, C, D, F, G and H point to the oral pole.

Figure 2. *Hsc71* expression in relation to S-phase cells in normal **(A, E, F)** and LiCl treated animals **(B-D, G-I)**. **(A)** *Hsc71* is expressed during wound healing at the aboral pole 24 hours after it was cut. **(B)** LiCl treated polyp 24 hours after cutting. *Hsc71* expression is upregulated throughout the ectopic growths induced by Wnt activation. **(C, D)** Ectopic outgrowths containing strongly expressing *Hsc71* cells **(C)**

with large nuclei which are proliferating, as indicated by BrdU immunoreactivity (**D**).

(**E, F**) *Hsc71* expressing (**E**) S-phase cells (**F**) (BrdU staining) in untreated developing male gonad, arrows indicate two such cells. (**G-I**) LiCl treated polyp 24 hours after cutting. (**G**) Ectopic outgrowths are visible at the aboral pole which contain *Hsc71* expressing cells. (**H, I**) Higher magnification images of the aboral pole showing *Hsc71* expressing proliferating cells. (**H**) Cells strongly expressing *Hsc71* in their cytoplasm are visible; two examples are marked by arrows. (**I**) Proliferating (S-phase) cells labelled by Brd-U (green nuclei) and also expressing *Hsc71* in their cytoplasm (dark circle around nuclei); two examples are marked by arrows. Scale bars 250 μm (A, B), 20 μm (C), 10 μm (D), 25 μm (E, F), 200 μm (G), 50 μm (H, I)

Figure 3. *Hsc71* expression in relation to apoptotic cells during metamorphosis. (**A**) Metamorphosing animal showing *Hsc71* expression in the prospective aboral pole. (**B, C**) Higher magnification of the TUNEL stained prospective oral pole in bright field microscopy showing absence of *Hsc71* expression at this pole (**B**) and blue light (**C**) showing apoptotic nuclei. Scale bars 100 μm (A), 50 μm (B, C)

Figure 4. *Hsc71* expression following heat treatment. (**A-C**) In situ hybridization of control (**A**), heat treated (**B**) heat treated, sense control (**C**) gastrulae, fixed immediately after heat treatment. Heat treatment results in ectopic *Hsc71* expression at the prospective oral pole. Asterisks mark the prospective oral pole. (**D-F**) *Hsc71* expression levels in control and heat treated animals, analyzed by qPCR. Expression levels normalized to GAPDH. (**C**) 16 hours post treatment. (**D**) 24 hours

post treatment. **(E)** 27 hours post treatment and 3 hours post metamorphosis induction.

Figure 5. Phenotypes of heat treated and *Hsc71* RNAi rescued animals. **(A)** Control larva. **(B)** Heat treated larvae. **(C)** Heat treated and RNAi rescued larvae. **(D)** Control metamorphosed primary polyp with tentacles and stolons. **(E)** Heat treated animal with stolons but without tentacles. **(F)** Heat treated and RNAi rescued animals with tentacles and stolons. Asterisks mark the prospective oral pole, T indicates a tentacle and S indicates a stolon. **(G)** *Hsc71* expression levels in control and heat treated, *Hsc71* RNAi and rescue (heat treated and *Hsc71* RNAi) animals, immediately after heat treatment, analyzed by qPCR. Expression levels normalized to GAPDH. **(H)** Average stolon/tentacle length for each of the 4 treatment groups (Control, heat, *Hsc71* RNAi and rescue) 24 hours post metamorphosis (2 days after the heat treatment). Scale bars 100 μ m.

Figure 6. Heat induced *Wnt* gene expression followed by repression. **(A, B)** In situ hybridization of *Wnt3* in animals following heat treatment. Asterisks mark the prospective oral pole. Heat-treated and control animals came from the same batch of embryos and were processed for in situ hybridization in parallel. The colorimetric reaction was stopped at the same time in treated and control animals. **(A)** Control (left) and heat treated (right) planula larvae 24 hours post heat treatment. *Wnt3* expression has been depolarized and is ubiquitously expressed throughout the animal. **(B)** Control (left) and heat treated (right) animal 20 hours post metamorphosis

induction. Expression of *Wnt3* is downregulated. **(C-F)** q PCR of *Wnt3*, *Tcf* and *Brachyury* in cDNA from heat treated animals. Expression levels normalized to GAPDH. **(C)** 16 hours post treatment, **(D)** 24 hours post treatment, **(E)** 27 hours post heat treatment and 3 hours post metamorphosis induction (PI), **(F)** 44 hours post heat treatment and 20 hours post metamorphosis induction. Scale bars 100 μ m.

Figure 7. Ectopic expression of *Hsc71* in transgenic animals. **(A)** Structure of the ectopic expression construct. **(B)** Transgenic embryo, 24 hours post fertilization. Left image, bright light; right image, blue light. **(C)** Normal, 24 hour embryo. **(D)** Transgenic larva, 3 days post fertilization. Arrow points to abnormal, blunt prospective oral pole. **(E)** Three day old larva injected with control construct (only GFP) has a normal tapered oral pole (arrow). Left image, bright light; right image, blue light. **(F)** Metamorphosed transgenic animal showing larval structure but with tentacles (t). **(G)** Normal metamorphosed animal of the same age, expressing the control construct (only GFP) and having stolons (s) and tentacles (t). Upper image, bright light; lower image blue light. **(H)** and **(I)** In situ hybridization of *Wnt3* in wild type **(H)** and *Hsc71* transgenic embryos **(I)**. Transgenic animals express *Wnt3* only in i-cells that are scattered along the A-P axis, in contrast to polarized expression in epithelial and i-cells in normal animals. Oral pole points upwards in all images. **(J)** A model of Hsc71-Wnt interactions. Increased Hsc71 expression results in an increase in Wnt expression by chaperoning the Wnt3 protein (or a component of the Wnt pathway), which is known to upregulate its own mRNA. Hsc71 protein contains putative GSK3 phosphorylation sites and is therefore stabilized by Wnt signaling. Activation of Wnt signaling also upregulates Hsc71 expression through the Tcf

binding sites in the Hsc71 promoter. Increased Hsc71 expression is also capable of downregulating Wnt expression by chaperoning a Wnt antagonist, thus inhibiting the Wnt positive feedback loop. Dashed line: uncertain or indirect interaction. Solid line: confirmed interaction. Black line: protein-protein interaction. Red line: protein-promoter interaction. Arrowhead: activating effect. Flat-headed line: inhibitory effect.

Figure S1. Gene expression levels analyzed by qPCR. Expression levels normalized to GAPDH. **(A)** The relative quantity (RQ) of expression of *Tcf*, *Wnt3* and *Brachyury* in control animals were compared to expression levels in 18.5 hour LiCl treated animals. **(B)** The relative quantity (RQ) of expression of *Hsc71* in control animals was compared to expression levels in LiCl and azakenpaullone treated animals.

Figure S2. The life cycle of *Hydractinia*, the structure of the colony, and the polarity of the larvae and polyp. **(A)** Life cycle. **(B)** Schematic illustration of the structure of a colony. **(C)** The relationship between the larval and polyp polarities.

Figure S3. *Hsc71* sequence data **(A)** Alignment of *Hydractinia* Hsc71 with its human homologue (GenBank Acc. No. BAD96505). Putative GSK3 Ser and Thr phosphorylation sites are shaded. The Ser/Thr residues located four positions C-terminally to other Ser/Thr residues are putative GSK3 priming sites, which need to be phosphorylated by other kinases (Cohen and Frame, 2001; Fuentealba et al., 2007).

All but one site (position 415) are conserved in the human sequence. **(B)** Promoter of *Hsc71*. The heat shock responsive element is shaded grey, putative Tcf binding sites are boxed, the ATG translation start site is in boldface, and the TATA box is underlined.

Figure S4. *Hsc71* expression throughout metamorphosis. Expression is absent from the posterior of metamorphosing animals, which undergoes apoptosis. *Hsc71* is expressed in more anterior tissue which survives metamorphosis. Posterior pole located at top and anterior pole is located at bottom of each image. Axis labels: P; larval posterior pole. An; larval anterior pole. O; polyp oral pole. Ab; polyp aboral pole. **(A)** Un-induced larvae. **(B)** 3 hours post induction **(C)** 4 hours post induction. **(D)** 7 hours post induction **(E)** 20 hours post induction. Tentacle and stolon buds are visible. *Hsc71* expression is now strongest in the i-cell population at the base of the developing polyp. All scale bars 200 μm , except (D) 100 μm

Figure 1
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Duffy_Fig1

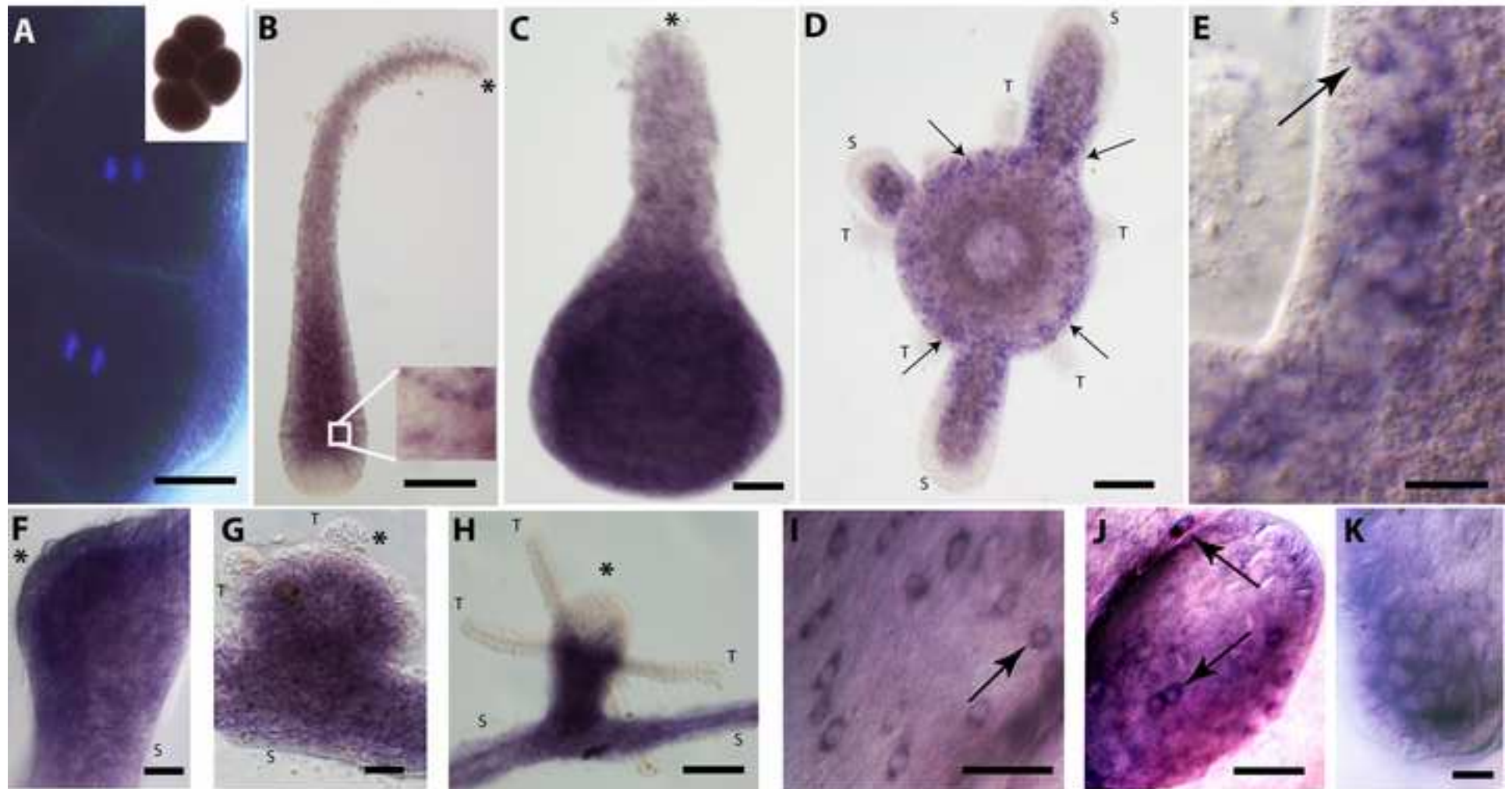


Figure 2
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Duffy_Fig2

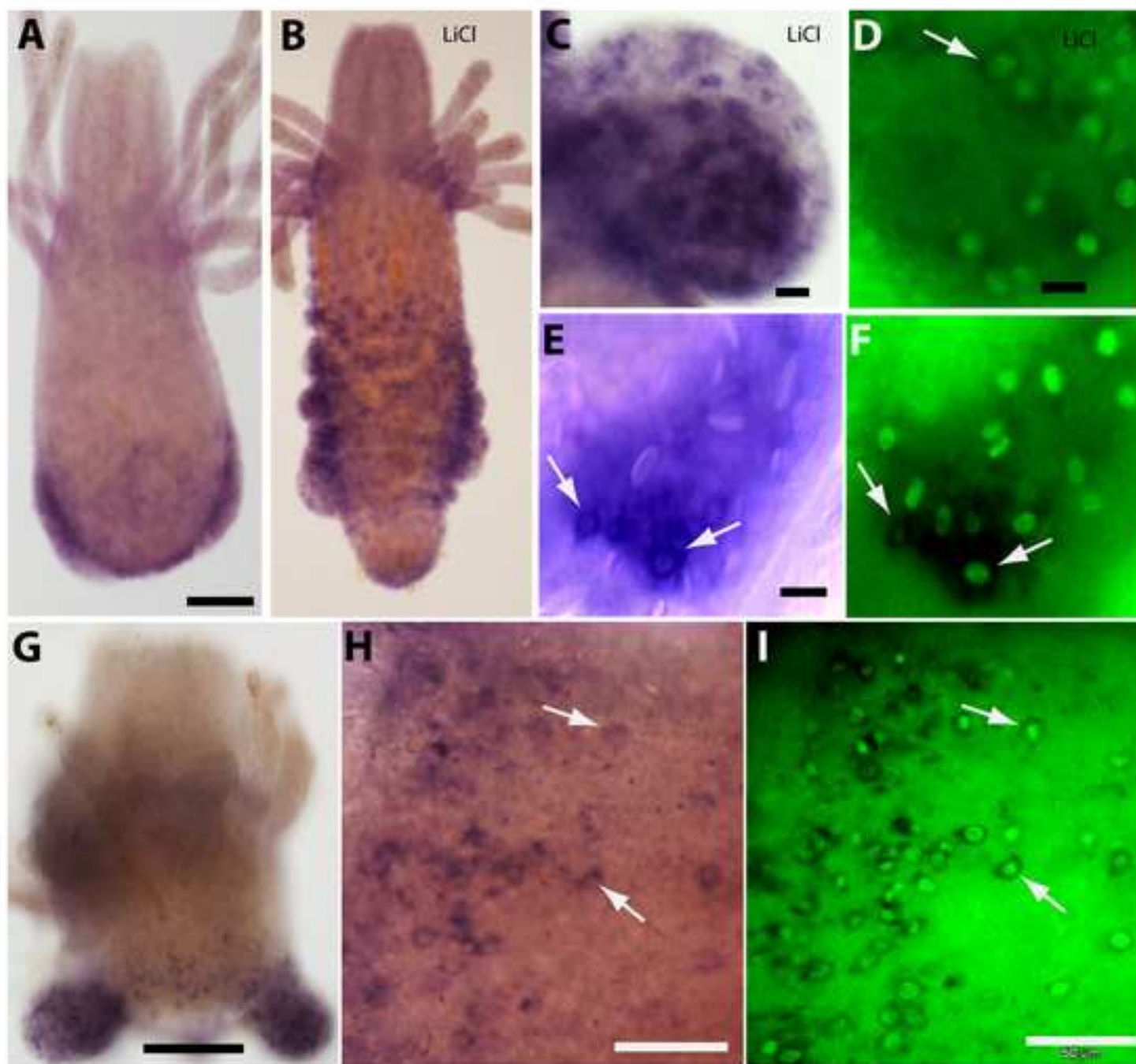


Figure 3
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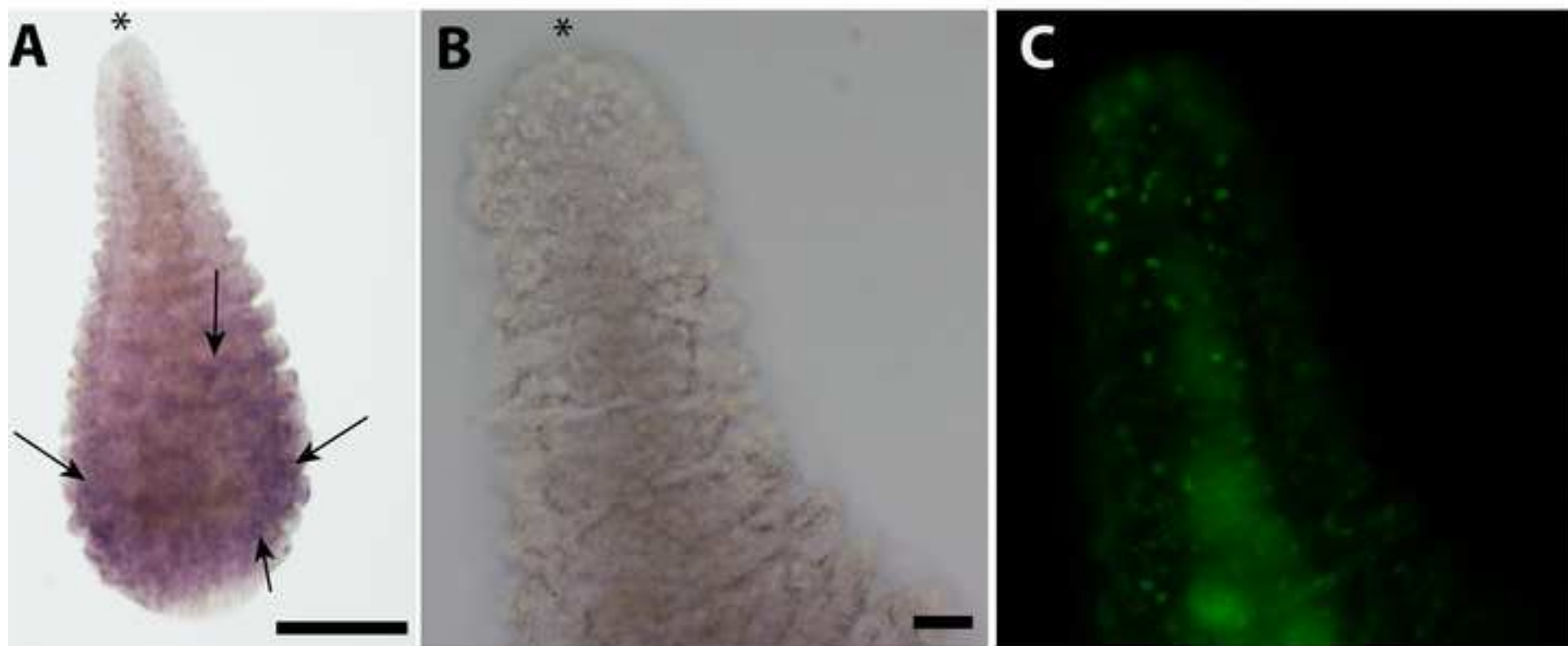


Figure 4
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Duffy_Fig4

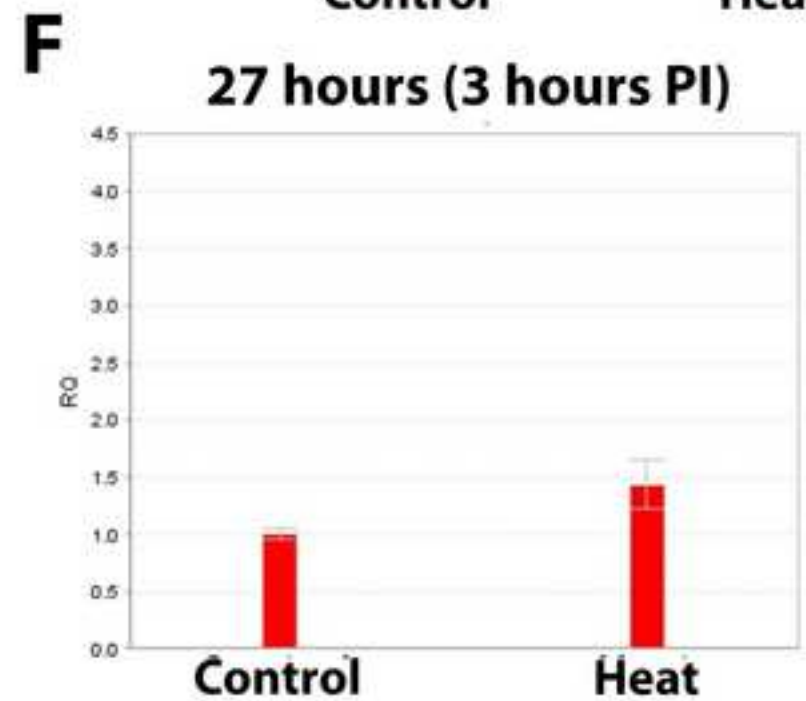
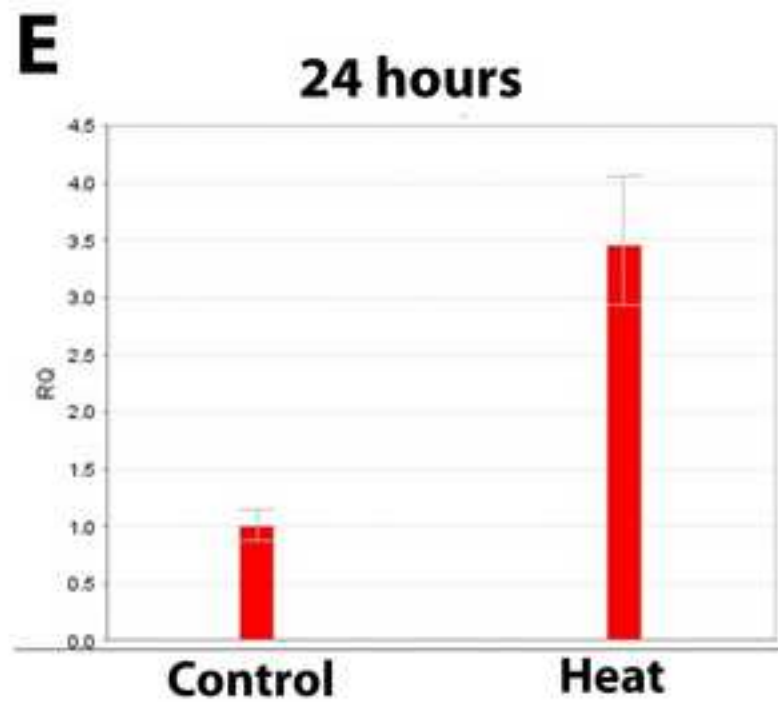
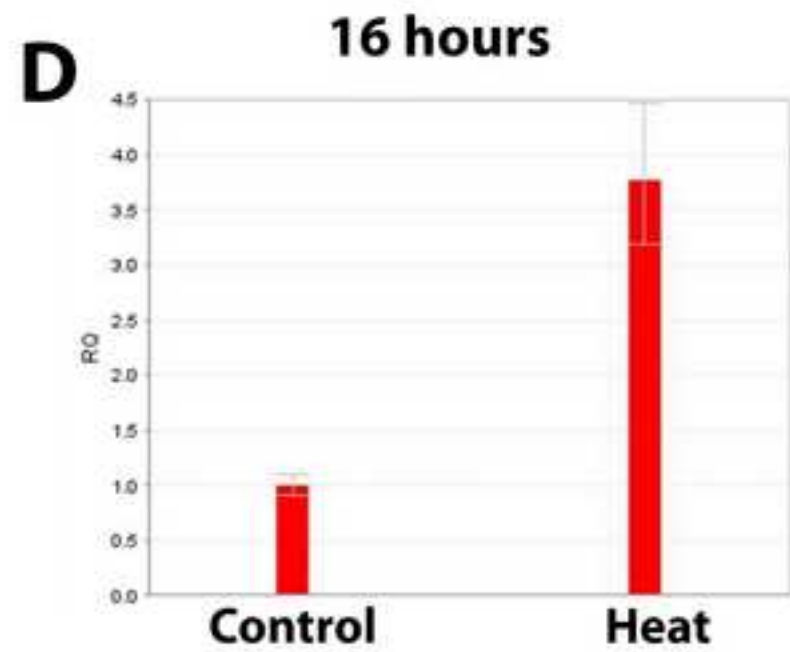
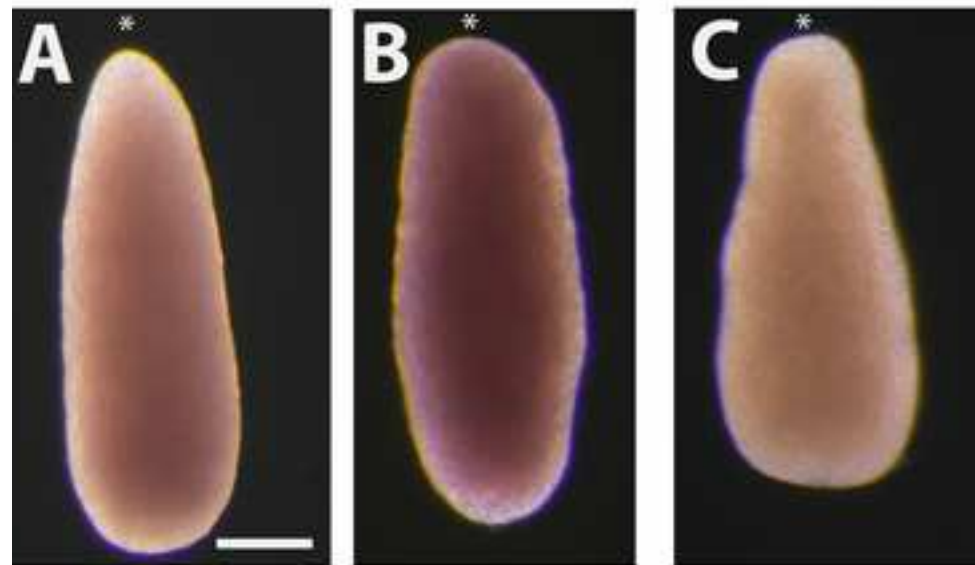
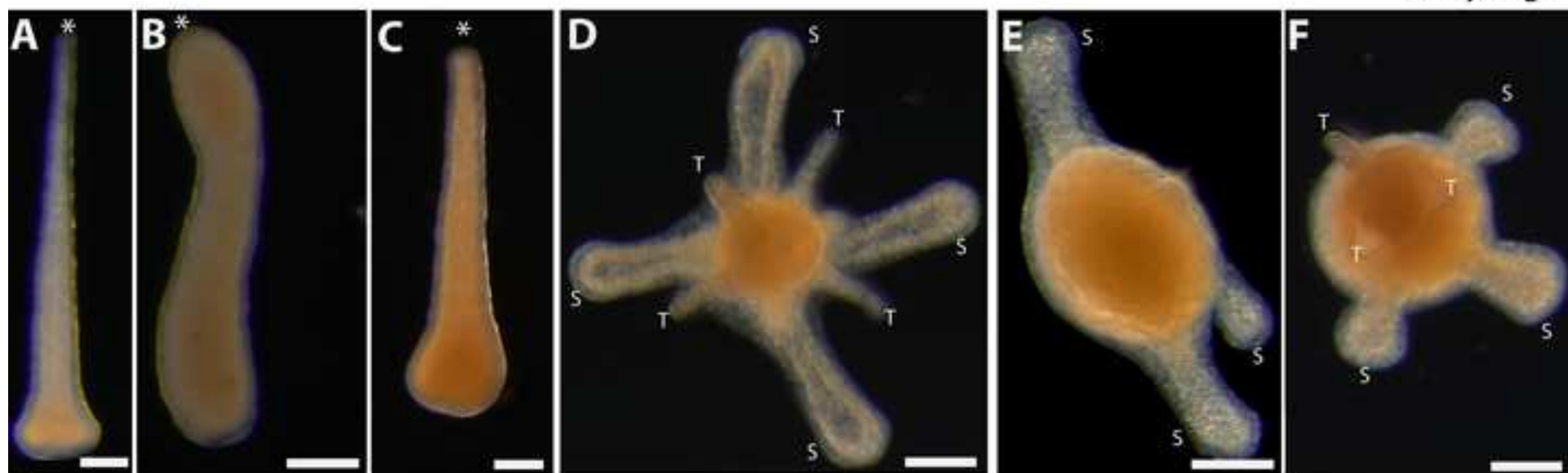
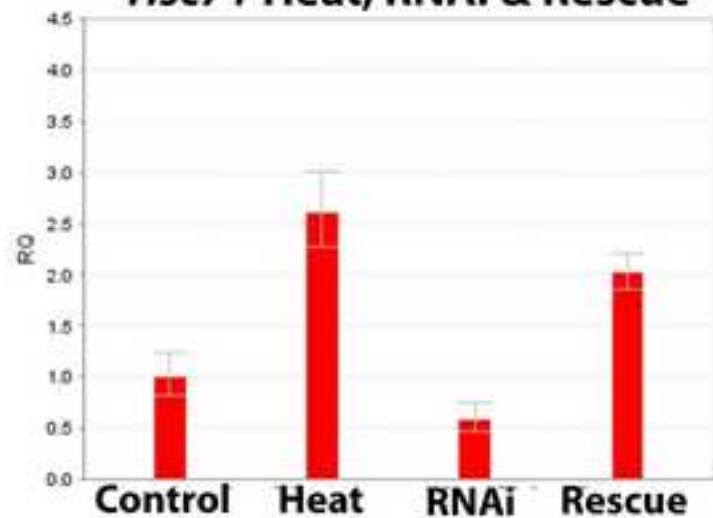


Figure 5
[Click here to download high resolution image](#)

Duffy_Fig5



G *Hsc71* Heat, RNAi & Rescue



H Stolon/tentacle values

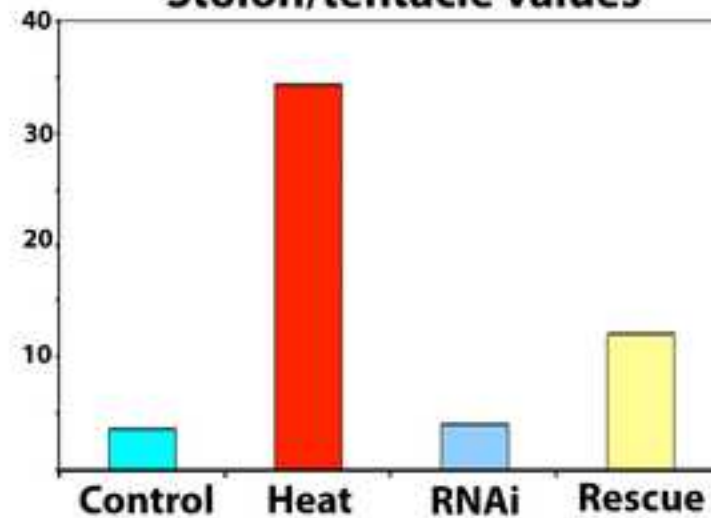


Figure 6
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Duffy_Fig6

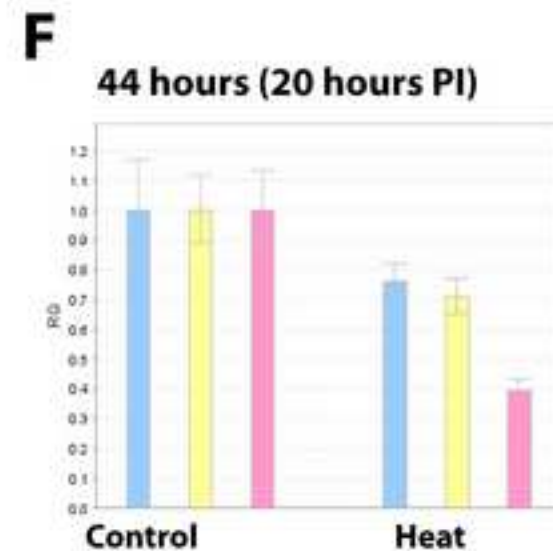
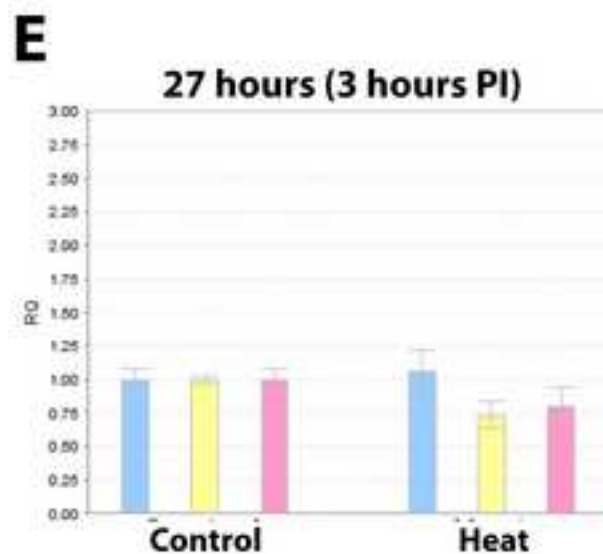
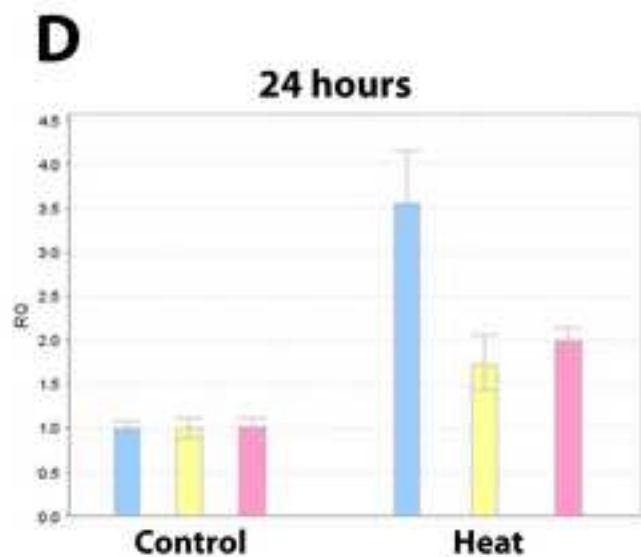
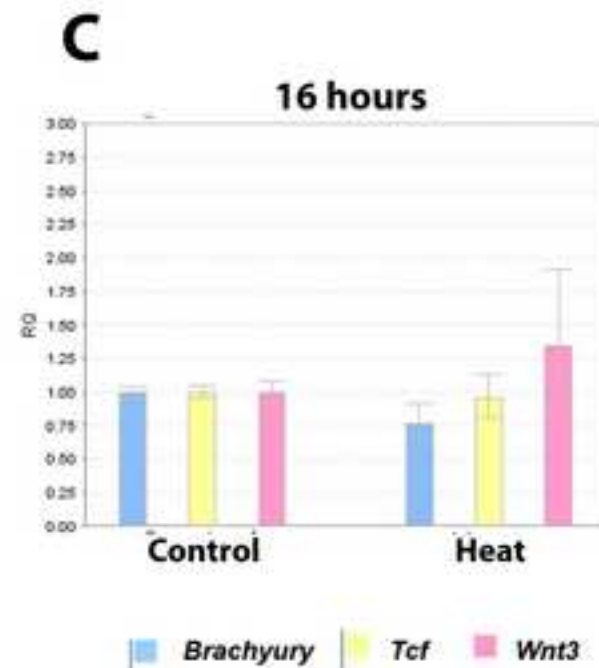
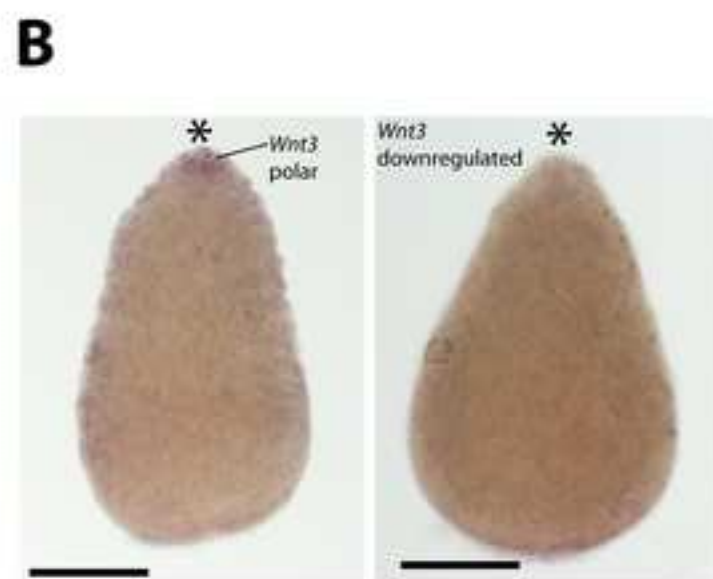
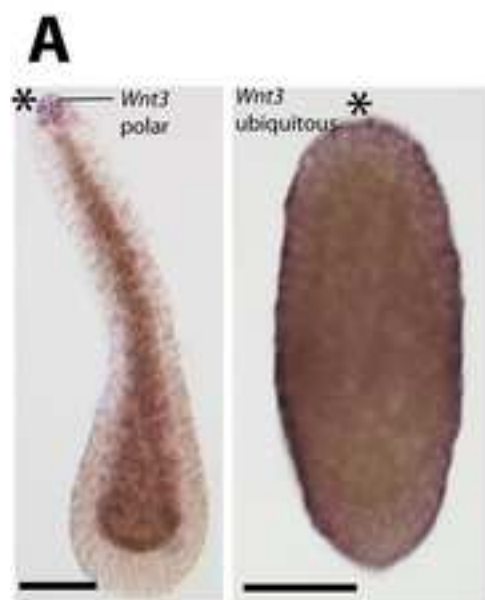


Figure 7
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Duffy_Fig7

