Zebrafish Cxcr4a determines the proliferative response to Hedgehog signalling

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SUMMARY
The Hedgehog (Hh) pathway plays dual roles in proliferation and patterning during embryonic development, but the mechanism(s) that distinguish the mitogenic and patterning activities of Hh signalling are not fully understood. An additional level of complexity is provided by the observation that Hh signalling can both promote and inhibit cell proliferation. One model to account for this apparent paradox is that Hh signalling primarily regulates cell cycle kinetics, such that activation of Hh signalling promotes fast cycling and an earlier cell cycle exit. Here we report that activation of Hh signalling promotes endodermal cell proliferation but inhibits proliferation in neighbouring non-endodermal cells, suggesting that the cell cycle kinetics model is insufficient to account for the opposing proliferative responses to Hh signalling. We show that expression of the chemokine receptor Cxcr4a is a critical parameter that determines the proliferative response to Hh signalling, and that loss of Cxcr4a function attenuates the transcription of cell cycle regulator targets of Hh signalling without affecting general transcriptional targets. We show that Cxcr4a inhibits PKA activity independently of Hh signalling, and propose that Cxcr4a enhances Hh-dependent proliferation by promoting the activity of Gli1. Our results indicate that Cxcr4a is required for Hh-dependent cell proliferation but not for Hh-dependent patterning, and suggest that the parallel activation of Cxcr4a is required to modulate the Hh pathway to distinguish between patterning and proliferation.

KEY WORDS: Cxcr4a, Hedgehog, Endoderm, Proliferation, Zebrafish

INTRODUCTION
During embryonic development, cell proliferation must be closely coordinated with patterning and differentiation. The Hedgehog (Hh) pathway controls both patterning and proliferation, and misregulation of Hh signalling has been implicated in the development and progression of several types of cancer (Ingham and McMahon, 2001; Pasca di Magliano and Hebrot, 2003; Jiang and Hui, 2008). However, the mechanisms that enable cells to distinguish between the mitogenic and patterning activities of Hh signalling are not fully understood. The secreted ligand Hh can act as a morphogen and specifies distinct transcriptional states depending on the concentration of the ligand. Hh binds its receptor, the 12-transmembrane domain protein Patched (Ptc), and by doing so overcomes the inhibition by Ptc of the pathway transducer protein Smoothened (Smo), a seven-transmembrane domain protein (Ingham and McMahon, 2001; Hooper and Scott, 2005; Ingham et al., 2011). Activation of Smo leads to responses mediated by the Gli family of transcription factors, which function both as repressors and activators of transcription (Ingham and McMahon, 2001; Hooper and Scott, 2005; Hui and Angers, 2011). The transcriptional outcome of Hh signalling is thought to depend on the balance between the activator and repressor forms.

cAMP-dependent protein kinase A (PKA) acts as a negative regulator of Hh signalling (Li et al., 1995; Jiang and Struhl, 1995; Hammerschmidt et al., 1996). In Drosophila, PKA induces hyperphosphorylation of the Gli homologue Cubitus interruptus (Ci), and targets Ci for proteolytic processing to yield the Ci repressor form (Aza-Blanc et al., 1997; Price and Kalderon, 1999; Hooper and Scott, 2005). As PKA also restricts the transcriptional activity of the full-length Ci protein (Wang et al., 1999), inhibition of PKA is required both to block the formation of Ci repressors and for the formation of the transcriptional activator form of Ci. In its inactive state the PKA holoenzyme consists of two catalytic (PKAc) and two regulatory (R) subunits. cAMP induces the dissociation of the inhibitory R subunits from the PKAc subunits, leading to activation of PKA phosphotransferase activity.

Drosophila Smo has been shown to function as a Gαi-coupled receptor, suggesting that Smo can inhibit PKA through downregulation of adenylyl cyclase and cAMP levels (Ogden et al., 2008). By contrast, the coupling of vertebrate Smo to Gαi is controversial (Ayers and Therond, 2010), although pertussis toxin expression in zebrafish embryos phenocopies at least some aspects of overactivation of Hh signalling (Hammerschmidt and McMahon, 1998). In vertebrates, inhibition of PKA phosphotransferase activity leads to activation of Hh target gene expression, suggesting that the role of PKA in regulating Gli activity is conserved (Hammerschmidt et al., 1996; Concordet et al., 1996; Epstein et al., 1996; Ungar and Moon, 1996; Tuson et al., 2011). PKA controls the proteolytic cleavage of Gli2 and Gli3 to...
yield the repressor forms (Dai et al., 1999; Wang et al., 2000; Pan et al., 2006; Pan et al., 2009; Tuson et al., 2011) and may also regulate the activator forms of Gli (Humke et al., 2010). Although Gli1 lacks the N-terminal repressor domain and acts solely as a transcriptional activator, Gli1 does contain consenssous PKA phosphorylation sites, and overexpression of Gli1 in Drosophila indicates that PKA restricts the transcriptional activity of Gli1 (Marks and Kalderon, 2011).

In cerebellar granule neuron precursors (CGNPs), Hh signalling promotes proliferation through transcriptional activation of cell cycle regulators such as N-myc and cyclin D1 (Kenney and Rowitch, 2000; Kenney et al., 2003; Oliver et al., 2003). However, in the zebrafish retina, Hh signalling has been reported to promote cell cycle exit through transcriptional activation of the CDK inhibitor p57 (Shkumatava and Neumann, 2005). The basis for these opposite proliferative responses to Hh signalling is unclear. One model postulates that Hh signalling primarily influences cell cycle kinetics (Locke et al., 2006), such that Hh signalling promotes fast cycling and an earlier cell cycle exit (Agathocleous et al., 2007). Proliferation assays would consequently give seemingly contradictory results depending on the developmental stage when the analysis is carried out. An alternative model is that proliferative responses to Hh signalling are context dependent. Although Hh can promote cell cycle progression, a mitogenic response may require the parallel activation of additional pathways to promote cell proliferation (Klein et al., 2001; Kenney et al., 2004; Ju et al., 2009).

Here we report a novel requirement for Hh signalling in endodermal cell proliferation in zebrafish gastrula stage embryos. Using a cell proliferation assay based on transplantation, we show that activation of Hh signalling induces opposite proliferative responses in endodermal and non-endodermal cells during gastrulation. We show that the chemokine receptor Cxcr4a, which is expressed in the gastrula stage endoderm, determines the proliferative response to Hh signalling. Epistasis analysis places the function of Cxcr4a downstream of, or in parallel to, Smo, but upstream of Gli1. Although we show that Cxcr4a can enhance the transcriptional activation of gli1 downstream of Smo, Cxcr4a does not affect the general transcriptional outcome of Hh signalling, as assessed by ptc1 expression. Moreover, the upregulation of gli1 by Cxcr4a is dependent on Smo, suggesting that Cxcr4a acts through additional mechanisms to promote Hh-dependent proliferation. We show that Cxcr4a inhibits PKA activity, and propose that a major role of Cxcr4a is to promote the transcriptional activity of Gli1. Our results suggest that the activation of Cxcr4a is required in parallel to Hh signalling specifically to promote the transcriptional activation of Hh pathway target genes required for cell proliferation.

**MATERIALS AND METHODS**

**Animals**

Embryos from wild-type (wt) (AB/Tubingen, SAT), smos154+/- and smos154−/− in crosses were used. Genotyping of smo alleles was performed as described previously (Aanstad et al., 2009).

**Microinjection of mRNA and morpholinos**

Capped mRNA was synthesised using the Ambion mMessage Kit and injected into one-cell stage embryos using: wt and mutant smo mRNA, 250 pg (Aanstad et al., 2009); ccr4a mRNA, 250 pg (Nair and Schilling, 2008); cas mRNA, 400 pg (Dickmeis et al., 2001); dnPKA mRNA, 200 pg (Hammerschmidt et al., 1996); and gli1 mRNA, 0.5 or 10 pg (Karlstrom et al., 2003). For morpholinos see supplementary material Table S1.

**Immunohistochemistry and in situ hybridisation**

Antibody stainings were performed as described previously (Aanstad et al., 2009), using rabbit anti-phospho-Histone H3 (Abcam), mouse anti-GFP (Roche), anti-mouse Alexa 488-conjugated and anti-rabbit Alexa 546-conjugated antibodies (Invitrogen). p57 was amplified using primers p57-F (5′-TGACATCGATGGGGAAGGTGACTTTGTTGA-3′) and p57-R (5′-TGCCTCAGCCGCTCTGACGATACCA-3′), cloned into pcS2+ and verified by sequencing. In situ hybridisation was performed as described previously (Aanstad et al., 2009).

**Transplants**

Donor embryos were injected with mRNA and/or morpholinos and a 0.05% solution of Rhodamine-dextran (MW 10,000, Invitrogen). Transplants were performed as described previously (Westerfield, 2005).

**Fluorescence-activated cell sorting (FACS)**

For each sample, at least 800 embryos were dissociated at 9 hpf as described previously (Covassin et al., 2006) and sorted using a Becton Dickinson FACSVantageSE by means of their scatter signals and fluorescence intensities.

**Quantitative (q) RT-PCR**

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and cDNA generated using the Maxima First-Strand cDNA Synthesis Kit for qRT-PCR (Fermentas). qRT-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems) and 5′+HOT FIREPol Evagreen qPCR Mix Plus (ROX) (Solis BioDyne). For primers see supplementary material Table S1.

**Renilla luciferase (Rluc) protein fragment complementation assay (PCA)**

We have generated a stable HEK293T cell line coexpressing the PCA fusion proteins RII-Rluc-F[1] and PKAc-Rluc-F[2], as described previously (Stefan et al., 2007; Stefan et al., 2011). Cells were grown in DMEM supplemented with 10% foetal bovine serum. Twenty-four hours after seeding, cells were transiently transfected in 24-well plate format with zebrafish Cxcr4a using TransFectin Lipid Reagent (Bio-Rad). Twenty-four or 48 hours post-transfection, cells were treated for 15 minutes with 50 μM forskolin or with the recombinant protein Cxcl12b [100 ng/ml (Boldajipour et al., 2011)]. Following treatment, growth medium was exchanged and cells were resuspended in 150 μL PBS. Cell suspensions were transferred to 96-well plates and subjected to bioluminescence analysis using the LMax II84 luminometer (Molecular Devices). Rluc bioluminescence signals were integrated for 10 seconds following addition of the Rluc substrate benzyl-coelenterazine (5 mM; Nanolight). Decreased bioluminescence signals indicate dissociation of the RII-RlucF[1]-PKAc-RlucF[2] complex and activation of PKA kinase activity.

**Statistics**

Statistical analysis was performed using Student’s t-test or one-way ANOVA analysis. *P<0.05 was considered significant.

**RESULTS**

Hh signalling is required for endodermal proliferation

We have previously described smo154+, an allele that carries a C-to-Y substitution in the extracellular domain of Smo (Aanstad et al., 2009). Further characterisation of smo154+ mutant embryos revealed that it acts as a dominant-negative allele (supplementary material Fig. S1), and uncovered a novel early endoderm phenotype not previously associated with loss of Hh signalling. In smo154+ mutant and smo morphant embryos we observed a reduced number of cells positive for the endodermal marker sox17 at 9 hpf (Fig. 1B,C), suggesting a reduction of endoderm. To investigate whether Hh pathway gain-of-function could increase the number of endodermal cells, we injected morpholinos against ptc1 and ptc2 (ptc MOs)
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Hh and Cxcr4a in proliferation

division undertook in this period. At the end of gastrulation, donor cells were also monitored for expression of Tg(sox17:EGFP) to confirm their endodermal fate (Fig. 2D). The number of transplanted endodermal cells doubled from the beginning to the end of gastrulation (Fig. 2B,C,E), suggesting that, on average, each endodermal cell undergoes one round of cell division during gastrulation (see supplementary material Table S2 for quantification of the transplant experiments).

In contrast to wt cells, smo morphant donor cells did not increase in number after transplantation (Fig. 2E), although proliferation was restored by coinjection of wt mouse smo mRNA (Fig. 2E). To investigate whether the lack of increase in endodermal cell number was due to increased cell death, we used time-lapse imaging to monitor the behaviour of the transplanted smo morphant cells throughout gastrulation. Neither cell division nor cell death was observed in a total of 87 transplanted cells in three embryos in three independent experiments, suggesting that the reduced number of endodermal cells reflects a decrease in cell proliferation. Injection of ptc MOs into donor embryos significantly increased proliferation compared with control cells (Fig. 2E, \( P < 0.001 \)). Transplantation of wt endodermal cells into smo or ptc morphant host embryos did not affect proliferation (Fig. 2F). These results are consistent with the results in whole embryos, indicating that Smo is required cell-autonomously for endoderm proliferation during gastrulation.

Hh signalling reduces non-endodermal cell proliferation

To investigate whether the role of Hh signalling in promoting cell proliferation is restricted to endodermal cells, we repeated the proliferation transplant assay without cas mRNA injection. In these experiments, Tg(sox17:EGFP) expression was never observed, indicating that our transplanted cells were mesodermal and/or ectodermal (supplementary material Fig. S2). Non-endodermal cells doubled in number during gastrulation (Fig. 2G). Strikingly, in contrast to endodermal cells, non-endodermal smo morphant donor cells showed a significant increase in proliferation compared with controls (Fig. 2G, \( P < 0.001 \)). Activation of Hh signalling by ptc MO injection caused a significant reduction in the proliferation of non-endodermal cells (Fig. 2G, \( P < 0.001 \)), suggesting that Hh signalling promotes the proliferation of endoderm and antagonises proliferation in non-endodermal cells.

N-myc (mycn – Zebrafish Information Network) is a direct transcriptional target of Hh signalling, and is required for the proliferative response downstream of Hh signalling (Kenney et al., 2003; Oliver et al., 2003). N-myc in turn activates the transcription of several cell cycle regulators, including cyclin D1 (cnd1), to promote G1-S phase transition (Kenney and Rowitch, 2000). By contrast, Hh signalling in the zebrafish retina appears to be required for cell cycle exit and cell differentiation through transcriptional activation of p57 (cdkn1c – Zebrafish Information Network) (Shikamata and Neumann, 2005). To investigate the transcriptional profiles of endoderm and mesoderm/ectoderm in smo morphants, we used FACs to sort Tg(sox17:GFP)-positive endodermal cells from GFP-negative non-endodermal cells. smo morphant endodermal cells showed reduced mycn and cnd1 expression (Fig. 2H). mycn transcript levels were also reduced in non-endodermal cells, consistent with mycn being a direct target of Hh signalling, although cnd1 levels were not significantly changed (Fig. 2I). smo morphant endodermal cells showed an upregulation of p57, whereas expression of p57 was strongly reduced in smo morphant non-endodermal cells (supplementary material Table S2).

Fig. 1. smo294 acts as a dominant-negative and reveals a requirement for Hh signalling in endodermal cell proliferation. (A-E) In situ hybridisation for sox17 at 9 hpf showing (A-C) dorsal view and (D,E) animal pole view. smo294 mutant (B) and smo morphant (C) zebrafish embryos showed a reduction in sox17-positive endodermal cells compared with wt siblings (A). Activation of Hh signalling by injection of ptc1 and ptc2 morpholinos (ptc MOs) (E) caused a significant increase in number of sox17-positive cells compared with controls (D). See also supplementary material Fig. S1. (F) Presence of pH3 (red) in Tg(sox17:EGFP)-positive endodermal cells (green) at 9 hpf (dorsal view). Inset illustrates higher magnification view of one pH3-positive endodermal cell. (G,H) Quantification of Tg(sox17:EGFP)-positive (G) and Tg(sox17:EGFP) and pH3 double-positive (H) cells in control and smo morphant embryos. Bars show mean number of cells (G) or mean percentage of double-positive cells (H); error bars indicate s.e.m. n=18 embryos for control and n=23 for smo morphant embryos in three independent experiments.

(Wolff et al., 2003) and found an increased number of endodermal cells at the animal pole (Fig. 1D,E), indicating that Hh signalling promotes endoderm development.

To test whether Hh signalling is required for proliferation of the endoderm, we used an antibody against phosphorylated Histone H3 (pH3) to label cells in M phase. For these experiments we used Tg(sox17:EGFP) embryos, which express EGFP under control of the sox17 promoter (Mizoguchi et al., 2008) (Fig. 1F). smo morphant embryos showed a significant reduction in Tg(sox17:EGFP)-positive cells (Fig. 1G) and in pH3-positive endoderm compared with control embryos (Fig. 1H, \( P < 0.001 \)), suggesting that Hh signalling is required for endodermal cell proliferation in the zebrafish gastrula stage embryo.

In zebrafish, Smo is required for endocrine pancreas specification during gastrulation (Roy et al., 2001; dilio et al., 2002), although this is not a cell-autonomous requirement (Chung and Stainier, 2008). To investigate whether the requirement for Smo in endodermal cell proliferation is cell-autonomous, we designed a proliferation assay based on cell transplantation (Fig. 2A). Donor Tg(sox17:EGFP) embryos were injected with Rhodamine-dextran as a lineage marker and with casanova (cas; sox32 – Zebrafish Information Network) mRNA to convert cells to endoderm. Donor cells were transplanted into wt host embryos and counted at the beginning of gastrulation (Fig. 2B) and again at the end of gastrulation (Fig. 2C) to estimate the number of cell divisions undertaken in this period. At the end of gastrulation, donor cells were also monitored for expression of Tg(sox17:EGFP) to confirm their endodermal fate (Fig. 2D). The number of transplanted endodermal cells doubled from the beginning to the end of gastrulation (Fig. 2B,C,E), suggesting that, on average, each endodermal cell undergoes one round of cell division during gastrulation (see supplementary material Table S2 for quantification of the transplant experiments).
required for the Hh-induced proliferative response of endodermal proliferation (Fig. 3D). We next asked whether Cxcr4a is injection of P proliferation (Fig. 3D, endodermal donor cells showed a significant reduction in mitotic endoderm (Fig. 3C). Furthermore, cxcr4a with this, we found a 26% reduction of endodermal cells in consistent (Mizoguchi et al., 2008), suggesting that Cxcr4a signalling is Cxcr4a resulted in a 24% reduction in endodermal cell numbers Schilling, 2008; Boldajipour et al., 2011). MO knockdown of gastrulation, whereas the ligand Cxcl12b is ubiquitously receptor Cxcr4a is expressed in the zebrafish endoderm during Hh-induced cell proliferation (Klein et al., 2001). The SDF-1 intermingled with mesodermal cells, suggesting that distance from the Hh source. Furthermore, endodermal cells at this stage are intermingled with mesodermal cells, suggesting that distance from the Hh source is not sufficient to explain the different transcriptional responses between endodermal and non-endodermal cells in smo morphants. Although a role for Hh signalling primarily in regulating cell cycle kinetics has been proposed to account for seemingly opposing proliferative outcomes (Locke et al., 2006), our results suggest that additional factors regulate the proliferative response to Hh signalling.

**Cxcr4a determines the proliferative response to Hh signalling**

In CGNPs, the chemokine SDF-1α has been reported to enhance Hh-induced cell proliferation (Klein et al., 2001). The SDF-1 receptor Cxcr4a is expressed in the zebrafish endoderm during gastrulation, whereas the ligand Cxcl12b is ubiquitously expressed in the mesoderm (Mizoguchi et al., 2008; Nair and Schilling, 2008; Boldajipour et al., 2011). MO knockdown of Cxcr4a resulted in a 24% reduction in endodermal cell numbers (Mizoguchi et al., 2008), suggesting that Cxcr4a signalling is required for endodermal proliferation in zebrafish. Consistent with this, we found a 26% reduction of endodermal cells in cxcr4a morphants (Fig. 3A, B), and a reduced percentage of mitotic endoderm (Fig. 3C). Furthermore, cxcr4a morphant endodermal donor cells showed a significant reduction in proliferation (Fig. 3D, P<0.001). Consistent with the idea that the level of the receptor Cxcr4a is not limited in this context, injection of cxcr4a mRNA did not alter endodermal donor cell proliferation (Fig. 3D). We next asked whether Cxcr4a is required for the Hh-induced proliferative response of endodermal cells. Donor cells injected with ptc MOs showed increased proliferation compared with control cells, and co-injection of cxcr4a MO with ptc MOs abolished this response (Fig. 3D).

These results indicate that Cxcr4a signalling is required for Hh-induced endoderm proliferation. We hypothesised that Cxcr4a might be a determining factor for the proliferative response of
endodermal cells to Hh signalling, and asked whether the presence of Cxcr4a in non-endodermal cells could alter the response of these cells to overactivation of Hh signalling. Injection of donor embryos with cxcr4a mRNA alone had no effect on cell proliferation (Fig. 3E, \( P>0.9 \)). However, co-injection of ptc MOs and cxcr4a mRNA resulted in a significant increase in proliferation (Fig. 3E) as compared with control cells (\( P<0.001 \)) or with cells injected with ptc MOs alone (\( P<0.001 \)). Together, these results indicate that Cxcr4a is required and sufficient to promote a positive proliferative response to activation of Hh signalling, and that Cxcr4a can convert the non-endodermal proliferative response to Hh pathway activation from a reduction to a stimulation of cell proliferation.

**Cxcr4a modulates Hh signalling**

The block of Hh-induced proliferation in the absence of Cxcr4a suggests that Cxcr4a acts either parallel to, or downstream of, Smo in promoting endoderm proliferation. To prove this, we examined whether cxcr4a mRNA could rescue the proliferation defects exhibited by smo morphant endodermal cells. Co-injection of cxcr4a mRNA with smo MO restored proliferation (Fig. 4A), suggesting that Cxcr4a acts downstream of, or parallel to, Smo in promoting endoderm cell proliferation. qRT-PCR measurements revealed no significant downregulation of cxcr4a mRNA levels in smo morphants compared with controls (supplementary material Fig. S4), suggesting that Hh signalling is not required for cxcr4a mRNA expression in the zebrafish gastrula stage embryo.

d\(_{50}\) (gli1 mutant) embryos showed a reduced number of sox17-positive cells, similar to smo\(_{294}\) mutants (supplementary material Fig. S1), suggesting that Smo acts through Gli1 to promote endoderm cell proliferation. gli1 morphant endodermal donor cells showed reduced proliferation compared with control cells (Fig. 4A). Co-injection of gli1 MO and cxcr4a mRNA did not significantly rescue gli1 morphant endodermal cell proliferation (Fig. 4A, \( P>0.8 \)), indicating that the proliferative effect of Cxcr4a requires Gli1. Co-injection of 10 pg gli1 mRNA fully restored cxcr4a morphant endodermal cell proliferation (Fig. 4A). These results suggest that Cxcr4a acts upstream of Gli1 to promote endoderm proliferation.

To determine whether loss of Cxcr4a signalling affects Hh-dependent transcriptional regulation involved in endoderm proliferation downstream of Gli1, we investigated the transcriptional profile of mycn, ccd1 and p57 in cxcr4a morphant endoderm. Bars show mean fold change of three experiments; error bars indicate s.e.m.

![Fig. 4. Cxcr4a acts downstream of Smo, but upstream of Hh target genes, to promote Hh-dependent endodermal cell proliferation.](image)

\( **P<0.001; \) ns, not significant; one-way ANOVA analysis.

**Fig. 5. Cxcr4a promotes gli1 expression.** (A) Quantification of gli1 mRNA levels in smo and cxcr4a morphant endoderm relative to control. (B) Quantification of gli1 mRNA levels in whole zebrafish embryos relative to controls. (C) In situ hybridisation for ptc1 in control and cxcr4a morphant embryos at 10 hpf. (D) Quantification of ptc1 mRNA levels in whole embryos (10 hpf) injected with cxcr4a mRNA, relative to control. Bars indicate mean fold change; error bars indicate s.e.m. ***\( P<0.001; \) ns, not significant; one-way ANOVA analysis.

To determine whether loss of Cxcr4a signalling affects Hh-dependent transcriptional regulation involved in endoderm proliferation downstream of Gli1, we investigated the transcriptional profile of mycn, ccd1 and p57 in cxcr4a morphant endoderm. The transcriptional profile of cxcr4a morphant endoderm with respect to these three genes (Fig. 4B) was highly similar to that of smo morphant endoderm, suggesting that loss of Cxcr4a signalling attenuates the transcriptional outcome of Hh signalling in controlling cell proliferation. Taken together, these results suggest that Cxcr4a is required for the transcriptional activity of Gli1 to promote Hh-dependent cell proliferation. gli1 is a direct transcriptional target of Hh signalling; however, complete loss of Smo function in zebrafish reduces, but does not abolish, expression of gli1 (Karlstrom et al., 2003), suggesting that additional factors contribute to gli1 expression. As Cxcr4a has previously been shown to control transcription in endodermal cells (Nair and Schilling, 2008), we reasoned that Cxcr4a might also regulate gli1 expression, and used qRT-PCR to investigate gli1 mRNA levels in control, smo and cxcr4a morphant embryos. smo morphant endoderm showed reduced gli1 expression levels (Fig. 5A). A 20% reduction of gli1 expression was also found in cxcr4a morphant embryos (Fig. 5A), suggesting that Cxcr4a regulates endodermal gli1 expression. Consistent with this, overexpression of cxcr4a mRNA in whole embryos caused a 1.3-fold increase in gli1 expression (Fig. 5B). Activation of Hh signalling by ptc1 MO injection in whole embryos caused a 1.7-fold increase in gli1 expression, and this increase was further enhanced by co-injection of cxcr4a mRNA (Fig. 5B). In whole embryos, injection of smo MO caused an 80% decrease in gli1 expression levels. Co-injection of cxcr4a mRNA did not restore gli1 expression in smo morphant embryos (Fig. 5B, \( P>0.1 \)), suggesting that the effect of Cxcr4a on gli1 expression requires Smo function. In contrast to gli1 expression, neither gain- nor loss-of-function of Cxcr4a had any significant effect on ptc1 expression (Fig. 5C,D).

Together, these results indicate that Cxcr4a can enhance the transcriptional activation of gli1. However, although Cxcr4a restored proliferation in smo morphant endodermal cells (Fig. 3), Cxcr4a could not compensate for loss of Smo function to restore
mRNA restored proliferation in cells, but not in endodermal cells. However, injection of thus phenocopies suggesting that Cxcr4 acts through G dependent proliferation of CGNPs was pertussis toxin sensitive, supplementary material Table S2. ***PA cxcr4a morphant endoderm.

error bars show s.e.m. n values for each experiment are given in supplementary material Table S2. **P<0.01; one-way ANOVA analysis.

gli1 expression, suggesting that Cxcr4a acts through additional mechanisms to promote Hh-dependent proliferation. Consistent with this model, we did not detect any significant difference in gli1 mRNA expression levels in endodermal and non-endodermal FACS samples (data not shown).

Cxcr4a regulates PKA activity
Klein and colleagues reported that the effect of SDF-1α on Hh-dependent proliferation of CGNPs was pertussis toxin sensitive, suggesting that Cxcr4 acts through Gαi (Klein et al., 2001). Gαi inhibits adenylate cyclase and downregulates the activity of PKA, a negative regulator of Hh signalling. To investigate how PKA activity affects endodermal cell proliferation, we injected mRNA encoding a dominant-negative form of PKA (dnPKA) (Hammerschmidt et al., 1996) into donor embryos, and assessed proliferation using the transplant assay. Interestingly, injection of dnPKA did not significantly increase endodermal cell proliferation compared with controls (Fig. 6A, P<0.9; supplementary material Fig. S5), but significantly decreased the proliferation of non-endodermal cells compared with controls (Fig. 6B, P<0.001). dnPKA thus phenocopies pic MO injection in non-endodermal cells, but not in endodermal cells. However, injection of dnPKA mRNA restored proliferation in smo morphant endodermal donor cells beyond control levels (Fig. 6A). Co-injection of dnPKA also restored proliferation in cxcr4a morphant, but not in gli1 morphant, endodermal donor cells (Fig. 6A), suggesting that inactivation of PKA is required downstream of Smo and Cxcr4a and upstream of Gli1.

To determine whether Cxcr4a regulates PKA activity independently of Smo, we used a cellular PKA biosensor based on the Renilla luciferase (R luc) protein fragment complementation assay (PCA) (Stefan et al., 2007; Stefan et al., 2011). PCA fragments F[1] and F[2] of R luc were fused to PKA regulatory (RII) and catalytic (PKAc) subunits to generate RII-F[1] and PKAc-F[2] (Stefan et al., 2007). Formation of the inactive PKA holoenzyme RII-PKAc triggers the folding and complementation of R luc F[1] and F[2]. The formation of cellular PCA complexes was quantified upon addition of the R luc substrate benzyl-coelenterazine. cAMP binds to PKA R subunits and triggers the dissociation of the inactive PKA RII-PKAc holoenzyme, as indicated by a concomitant loss of bioluminescence (Fig. 7A). Activation of transiently expressed zebrafish Cxcr4a by addition of Cxcl12b ligand resulted in a significant increase in RII-PKAc complex formation, indicating that activation of Cxcr4a leads to inhibition of basal adenyl cyclase activity, thereby downregulating PKA activity (Fig. 7B). The suitability of the PKA biosensor was confirmed by addition of the general and direct adenyl cyclase activator forskolin, which reduces PKA complex formation (Fig. 7B). These results indicate that Cxcr4a acts upstream of adenyl cyclase to regulate PKA activity.

Although PKA negatively regulates Gli2 and Gli3 transcriptional activity, PKA has also been reported to antagonise Gli1 protein activity (Sheng et al., 2006; Marks and Kaideron, 2011). If Cxcr4a acts to promote Gli1 activity, we would expect Cxcr4a and Gli1 to act synergistically to promote cell proliferation. To further investigate the relationship between Gli1, Cxcr4a and PKA in cell proliferation, we overexpressed gli1 mRNA in non-endodermal donor cells and assessed proliferation using the transplant assay. Low levels (0.5 pg) of gli1 mRNA did not significantly affect non-endodermal cell proliferation (Fig. 7C, P>0.9). However, co-injection of cxcr4a mRNA with 0.5 pg gli1 mRNA (Fig. 7C) gave a strong and statistically significant increase in non-endodermal cell proliferation compared with controls (P<0.001) and with cells expressing either cxcr4a (P<0.001) or low levels of gli1 alone (P<0.001). Furthermore, co-injection of dnPKA with 0.5 pg gli1 mRNA phenocopied cxcr4a mRNA injections in giving a statistically significant increase in proliferation (Fig. 7C, P<0.001). Taken together, these results indicate that Cxcr4a acts synergistically with Gli1 to promote cell proliferation, and suggest that, in addition to promoting gli1 expression through Gli2/3 (see supplementary material Fig. S1), Cxcr4a may downregulate PKA activity to promote the transcriptional activity of Gli1 (Fig. 7D).

DISCUSSION
Here we show a novel requirement for Hh signalling and Gli1 in regulating endoderm proliferation in the gastrula stage embryo. Using a proliferation assay based on cell transplantation, we found that Hh signalling increases proliferation in the endoderm but decreases proliferation in non-endodermal cells.

Opposite proliferative responses to Hh signalling in the gastrula stage embryo
The role of Hh signalling in promoting cell cycle exit in the zebrafish retina has been linked to its role in patterning and differentiation (Shkumatava and Neumann, 2005; Masai et al., 2005). However, specification and subsequent differentiation may also influence the proliferative behaviour of cells, such that changes in proliferative responses might constitute secondary effects to a cell fate change. Although we cannot exclude this possibility, the start point of our transplant assay coincides with the onset of Hh signalling in zebrafish embryos (Krauss et al., 1993; Concordet et al., 1996), thus minimising the possibility of secondary effects of Hh signalling in our assay. Whereas Hh signalling promotes proliferation in the endoderm, activation of Hh signalling reduced, and loss of Smo increased, non-endodermal cell proliferation. The transplanted non-endodermal cells in our transplant assay contributed both to mesodermal and ectodermal tissues (data not shown). We cannot exclude the possibility that mesodermal and ectodermal cells have different proliferative responses to Hh signalling that would be obscured by our experimental design. However, an endoderm-like response to activation or inhibition of Hh signalling in individual embryos was never observed in our non-endodermal transplant experiments, supporting the idea of a true difference in the proliferative behaviour of endodermal cells compared with mesodermal and ectodermal cells.
and the absence of Cxcr4a lead to transcriptional activation of the PKA regulatory subunit. We indicate that ligand-activated seven-transmembrane receptors (coupled to G proteins) to quantify the activation status of PKA. AC, adenylate cyclase; pm, plasma membrane; R, PKA regulatory subunit type II; PKAc, PKA catalytic subunit.

Our assay measures cell division events within a strict time window of 4 hours (Fig. 2A). An increase in cycling speed could account for the observed increase in endodermal cell proliferation upon activation of Hh signalling. However, the cell cycle kinetics model cannot account for the reduced proliferation observed as a result of activation of Hh signalling in non-endodermal cells. Loss of Smo function resulted in distinct transcriptional profiles in endodermal and non-endodermal cells, suggesting that the proliferative response to Hh signalling is context dependent.

**Cxcr4a acts downstream of Smo to modulate Hh signalling outcome**

We identified the chemokine receptor Cxcr4a as a crucial determinant of the proliferative response to Hh signalling in the gastrula stage embryo, and show that Cxcr4a is required downstream of Smo to promote Hh-dependent endodermal cell proliferation. A recent study showed that activation of Hh signalling promotes the membrane localisation of, and signalling by, Cxcr4 in a human medulloblastoma cell line; thus, in these cells, Cxcr4C signalling depends on SMO function (Sengupta et al., 2012). We have found Smo-dependent and -independent roles for Cxcr4a in endodermal cell proliferation. Cxcr4a promotes the expression of gli1, and this effect depends on Smo function (Fig. 5B). However, overexpression of Cxcr4a restored proliferation in smo morphant endodermal cells (Fig. 4A). Although this indicates that in smo morphant cells Cxcr4a is a limiting factor, consistent with the findings of Sengupta et al. (Sengupta et al., 2012), it also argues that Cxcr4a has Smo-independent functions in the zebrafish endoderm. The reason for this discrepancy is unknown, but could reflect differences between zebrafish embryos and the human cancer cell lines used.

**Cxcr4a inhibits PKA activity**

Klein and co-workers showed that the proliferative effect of Cxcr4 in CGNPs is pertussis toxin sensitive, suggesting that Cxcr4 acts through Galpha to enhance Hh-dependent proliferation (Klein et al., 2001). Using Rluc PCA (Stefan et al., 2007), we show here for the first time that zebrafish Cxcr4a can inhibit PKA activity (Fig. 7B). PKA is a major negative regulator of Gli function, thus our results suggest that Cxcr4a promotes Hh-dependent cell proliferation through inhibition of PKA. One possibility is that Smo and Cxcr4a act in parallel to inactivate PKA, and that Cxcr4a enhances the effect of Smo in inhibiting PKA, leading to enhanced activity of Gli2 and/or Gli3 (Fig. 7D). Consistent with this model, we found that Cxcr4a promotes the expression of gli1, a direct transcriptional target of Hh signalling, and that this effect required Smo function. However, we detected no significant effects of Cxcr4a on ptc1 expression (Fig. 5C,D), arguing that Cxcr4a activity does not lead to a general increase in the transcription of Hh target genes.

An alternative, not mutually exclusive, possibility is that Cxcr4a inhibits PKA activity independently of Smo. Gli1 contains consensus PKA phosphorylation sites, and evidence from Drosophila indicates that PKA regulates Gli1 protein activity (Marks and Kalderon, 2011). Furthermore, PKA has been reported to inhibit the nuclear localisation and the transcriptional activity of Gli1 in cell culture (Sheng et al., 2006). This suggests that a
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second, and perhaps functionally more significant, role of Cxcr4a might be to inactivate PKA to promote Gli1 activity and subsequent cell proliferation (Fig. 7D). Such an effect would be independent of Smo, but require the presence of Gli1. Zebrafish embryos lacking Smo function retain some gll1 expression (Fig. 5) (Karlstrom et al., 2003), raising the possibility that Cxcr4a restores smo morphant endodermal cell proliferation by promoting the activity of residual Gli1 protein. Consistent with this model, Cxcr4a overactivation restored proliferation in smo morphant, but not gll1 morphant, endodermal cells (Fig. 4A). Furthermore, inactivation of PKA by dnPKA mRNA injections phenocopied cxcr4a overexpression in synergising with low levels of gll1 mRNA to cause a statistically significant increase in non-endodermal cell proliferation (Fig. 7C), suggesting that Cxcr4a inhibits PKA specifically to promote Gli1 activity.

The role of PKA in vertebrate Hh signalling

Loss of PKA activity causes strong activation of Hh signalling in vertebrates and mimics maximal Hh pathway activation in mouse neural tube patterning (Tuson et al., 2011). Consistent with this, injection of dnPKA into non-endodermal cells resulted in reduced cell proliferation, thus phenocopying ptc MO injections (Fig. 6B). Surprisingly, dnPKA expression did not significantly increase endodermal cell proliferation compared with controls (Fig. 6A); thus, dnPKA cannot phenocopy ptc MO injections in the endoderm. However, dnPKA was able to induce maximal proliferation in the endoderm in the absence of Smo (Fig. 6A), similar to what was found upon ptc MO-mediated activation of Hh signalling in these cells (Fig. 2E). This suggests that in the absence of PKA activity, Smo exerts some antagonistic effect on cell cycle progression. As maximal levels of endodermal proliferation were also obtained with ptc MO injections in the presence of Smo, this suggests that loss of PKA activity itself negatively affects the activity of Smo.

In Drosophila, PKA plays both positive and negative roles in Hh signalling. Drosophila Smo protein contains several PKA phosphorylation sites, and phosphorylation of Smo by PKA is required for maximal Smo activation (Jia et al., 2004; Zhang et al., 2004; Apionshev et al., 2005; Zhou et al., 2006). The PKA phosphorylation sites are not conserved in vertebrate Smo (Zhang et al., 2004). However, recent studies have shown that activation of PKA by cholera toxin or forskolin treatment can induce the accumulation of Smo in the proximal part of the cilium (Wilson et al., 2009; Milenkovic et al., 2009; Wen et al., 2010). Moreover, inhibition of PKA activity using small molecule inhibitors can block Shh-induced activation of ptc1 and gll1 (Milenkovic et al., 2009). These results suggest that PKA might play both positive and negative roles also in vertebrate Hh signalling.

Tuson et al. did not detect any defects in the trafficking or activity of Smo in Pkac null embryos, suggesting that complete inactivation of PKA is sufficient to mimic all Hh signalling (Tuson et al., 2011), in contrast to our results. This discrepancy could reflect incomplete inactivation of PKA by the injected dnPKA protein in our experiments, as compared with the genetic model used by Tuson and colleagues. Alternatively, cell proliferation could require higher levels of Hh pathway activation, or there could be subtle differences in the mechanism of pathway activation required for these processes (Chan et al., 2009).

Our results suggest that Cxcr4a is required for higher proliferation rates in endodermal cells, and that Cxcr4a promotes Hh-dependent cell proliferation by inactivating PKA. Barzi and colleagues reported that inactivation of PKA at the primary cilium is required for Shh-dependent cell proliferation, and that Hh signalling may specifically inactivate PKA at the primary cilium (Barzi et al., 2010). Cxcr4 has been shown to accumulate in the plasma membrane (Sengupta et al., 2012). It is tempting to speculate that Hh-dependent cell proliferation might require PKA activation both at the cilium, to regulate Gli2, and outside the peri-ciliary space, to promote Gli1 activity (Marks and Kalderon, 2011; Sheng et al., 2006).

Overexpression of Cxcr4a in non-endodermal cells did not increase proliferation in our assay. As we did not detect any significant differences in gll1 mRNA levels between endodermal and non-endodermal cells, this appears to be inconsistent with our model. However, the non-endodermal sample contains a relatively small population of presomitic mesodermal cells with very high levels of gll1 expression (Karlstrom et al., 2003), raising the possibility that high levels of gll1 transcripts from these cells mask a much lower expression of gll1 in other mesodermal and ectodermal cells. Supporting this possibility, near-maximal proliferation in non-endodermal cells was observed with low levels of gll1 mRNA in combination with either Cxcr4a or dnPKA overexpression. Alternatively, the proliferative outcome might depend on the state of Hh pathway or Smo activation, as suggested by the different endodermal responses to inactivation of PKA in the presence and absence of Smo.

Conclusions

Our results suggest that Cxcr4a specifically promotes the activation by Hh signalling of a transcriptional programme required for cell cycle control, but not other Hh target genes involved in patterning. We propose that this occurs through coordinated regulation of PKA activity by Hh signalling and Cxcr4a. The parallel activation of Cxcr4a might thus be one mechanism by which Hh signalling can direct distinct transcriptional outcomes to control both patterning and proliferation during embryonic development.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References

Aanstad, P., Santos, N., Corbit, K. C., Trinh, L. A., Scherz, P. J., Salvenmoser, W., Huiskens, J., Reiter, J. F. and Stainer, D. Y. R. (2009). The extracellular domain of Smo has been required for ciliary localisation and maximal Hh pathway activation. Curr. Biol. 19, 1034-1039.

Agathocleous, M., Locker, M., Harris, W. A. and Perron, M. (2007). A general role of hedgehog in the regulation of proliferation. Cell Cycle 6, 156-159.

Apionshev, S., Karanayeva, N. M., Marks, S. A., Kalderon, D. and Tomlinson, A. (2005). Drosophila Smoothened phosphorylation sites essential for Hedgehog signal transduction. Nat. Cell Biol. 7, 86-92.
Ayers, K. L. and Therond, P. P. (2010). Evaluating Smoothened as a G-protein-coupled receptor for Hedgehog signalling. Trends Cell Biol. 20, 287-298.

Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, D. (2005). The hedgehog-PKA pathway regulates two distinct steps of the differentiation of retinal ganglion cells: the cell-cycle exit of retinoblasts and their neuronal maturation. Development 132, 1539-1553.

Milenkov, L., Scott, M. P. and Rohatgi, R. (2009). Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium. J. Cell Biol. 187, 365-374.

Mizoguchi, T., Verkade, H., Heath, J. K., Kuroiwa, A. and Kikuchi, Y. (2008). Sdf1/Cxcr4 signaling controls the dorsal migration of endodermal cells during Drosophila gastrulation. Development 135, 252-261.

Nair, S. and Schilling, T. F. (2008). Chemokine signaling controls endodermal migration during zebrafish gastrulation. Science 322, 89-92.

Ogden, S. K., Fei, D. L., Schilling, N. S., Ahmed, Y. F., Hwa, J. and Robbins, D. J. (2008). G protein Galpha functions immediately downstream of Smoothened in Hedgehog signalling. Nature 456, 967-970.

Oliver, T. G., Grasfeder, L. L., Carroll, A. L., Kaiser, C., Gillingham, C. L., Lin, S. M., Wickramasinghe, R., Scott, M. P. and Wechsler-Reya, R. J. (2003). Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. Proc. Natl. Acad. Sci. USA 100, 7231-7236.

Pan, Y., Bai, C. B., Joyner, A. L. and Wang, B. (2006). Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Mol. Cell. Biol. 26, 3365-3377.

Pan, Y., Wang, C. and Wang, B. (2009). Phosphorylation of Gli2 by protein kinase A is required for Gli2 processing and degradation and the Sonic Hedgehog-regulated mouse development. Dev. Biol. 326, 177-189.

Pasca di Magliano, M. and Hebrok, M. (2003). Hedgehog signalling in cancer formation and maintenance. Nat. Rev. Cancer 3, 903-911.

Price, M. A. and Kalderon, D. (1999). Protein kinase A required for Gli2 processing in Drosophila embryos. Nature 401, 43-50.

Robu, M. E., Larson, J. D., Nasevicius, A., Beiraghi, S., Bremner, C., Farber, S. A. and Eckner, C. S. (2007). p53 activation by knockdown technologies. PLoS Genet. 3, e79.

Roy, S., Qiao, T., Woff, C. and Ingham, P. W. (2001). Hedgehog signalling pathway is essential for pancreas specification in the zebrafish embryo. Curr. Biol. 11, 1358-1363.

Sengupta, R., Dubuc, A., Ward, S., Yang, L., Northcott, P. and Somerfield, I., Kroll, K., Luo, J., Taylor, M. D. and Wechsler-Reya, R. J. (2012). Cxcr4 activation defines a new subgroup of sonic hedgehog-driven medulloblastoma. Cancer Res. 72, 122-132.

Sheng, T., Chi, S., Zhang, X. and Xie, J. (2006). Regulation of Gli1 localization by the cAMP/protein kinase A signaling axis through a site near the nuclear localization signal. Dev. Biol. 294, 153-162.

Shukumatova, A. and Neumann, C. J. (2005). Shh directs cell-cycle exit by activating p57Kip2 in the zebrafish retina. EMBO Rep. 6, 563-569.

Stefan, E., Malleshaiah, M. K., Breton, B., Ear, P. H., Bachmann, V., Beyermann, M., Bouvier, M. and Michnick, S. W. (2007). Quantification of dynamic protein complexes using Remilla luciferase fragment complementation applied to protein kinase A activities in vivo. Proc. Natl. Acad. Sci. USA 104, 16191-16192.

Stefan, E., Malleshaiah, M. K., Breton, B., Ear, P. H., Bachmann, V., Beyermann, M., Bouvier, M. and Michnick, S. W. (2011). PKA regulatory subunits mediate synergy among conserved G-protein-coupled receptor cascades. Nat. Commun. 2, 598-607.

Tuson, M., He, C. and Anderson, K. V. (2011). Protein kinase A acts at the basal body of the primary cilium to prevent GlI2 activation and ventralization of the mouse neural tube. Development 138, 4921-4930.

Ungar, A. R. and Moon, R. T. (1996). Inhibition of protein kinase A phosphorylates of hedgehog in the CNS of wild-type and cyclops mutant embryos. Dev. Biol. 178, 186-191.

Karlstrom, R. O., Tyurina, O. V., Kawakami, A., Shiokawa, N., Talbot, W. S., Sasaki, H. and Schier, A. F. (2003). Genetic analysis of zebrafish gli1 and gli2 reveals divergent requirements for gli genes in vertebrate development. Development 130, 1549-1564.

Kenney, A. M. and Rowitch, D. H. (2000). Sonic hedgehog promotes G1 cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol. Cell. Biol. 20, 9055-9067.

Kenney, A. M., Cole, M. D. and Rowitch, D. H. (2003). Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors. Development 130, 15-28.

Kenney, A. M., Wildlund, H. R. and Rowitch, D. H. (2004). Hedgehog and PI-3 kinase signaling converge on Nmyc1 to promote cell cycle progression in cerebellar neuronal precursors. Development 131, 217-228.

Klein, R. S., Rubin, J. B., Gibson, H. D., DeHaan, E. N., Alvarez-Hernandez, X., Segal, R. A. and Luster, A. D. (2001). SDF-1 alpha induces chemotaxis and enhances Sonic hedgehog-mediated proliferation of cerebellar granule cells. Development 128, 1971-1981.

Krauss, S., Concordet, J. P. and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. Cell 75, 1431-1444.

Liu, W., Ohmeyer, J. T., Lane, M. E. and Kalderon, D. (1995). Function of protein kinase A in hedgehog signal transduction and Drosophila imaginal disc development. Cell 80, 553-562.

Locke, M., Agathocleous, M., Amato, M. A., Parain, K., Harris, W. A. and Perron, M. (2006). Hedgehog signaling and the retina: insights into the mechanisms controlling the proliferative properties of neural precursors. Genes Dev. 20, 3036-3048.

Marks, S. A. and Kalderon, D. (2011). Regulation of mammalian Gli proteins by Costal 2 and PKA in Drosophila reveals Hedgehog pathway conservation. Development 138, 2533-2542.

Makki, I., Yamaguchi, M., Tonou-Fujimori, N., Komori, A. and Okamoto, H. (2005). The hedgehog-PKA pathway regulates two distinct steps of the differentiation of retinal ganglion cells: the cell-cycle exit of retinoblasts and their neuronal maturation. Development 132, 1539-1553.
Wang, B., Fallon, J. F. and Beachy, P. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell 100, 423-434.

Wang, G., Wang, B. and Jiang, J. (1999). Protein kinase A antagonizes Hedgehog signaling by regulating both the activator and repressor forms of Cubitus interruptus. Genes Dev. 13, 2828-2837.

Wen, X., Lai, C. K., Evangelista, M., Hongo, J. A., de Sauvage, F. J. and Scales, S. J. (2010). Kinetics of hedgehog-dependent full-length Gli3 accumulation in primary cilia and subsequent degradation. Mol. Cell. Biol. 30, 1910-1922.

Westerfield, M. (2005). The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio). Eugene, OR: University of Oregon Press.

Wilson, C. W., Chen, M. H. and Chuang, P. T. (2009). Smoothened adopts multiple active and inactive conformations capable of trafficking to the primary cilium. PLoS ONE 4, e5182.

Wolff, C., Roy, S. and Ingham, P. W. (2003). Multiple muscle cell identities induced by distinct levels and timing of hedgehog activity in the zebrafish embryo. Curr. Biol. 13, 1169-1181.

Zhang, C., Williams, E. H., Guo, Y., Lum, L. and Beachy, P. A. (2004). Extensive phosphorylation of Smoothened in Hedgehog pathway activation. Proc. Natl. Acad. Sci. USA 101, 17900-17907.

Zhou, Q., Apionishev, S. and Kalderon, D. (2006). The contributions of protein kinase A and smoothened phosphorylation to hedgehog signal transduction in Drosophila melanogaster. Genetics 173, 2049-2062.