A homologue of the *Drosophila* kinesin-like protein Costal2 regulates Hedgehog signal transduction in the vertebrate embryo

Shang Yew Tay[1,*], Philip W. Ingham[2] and Sudipto Roy[1,3,*,†]

[1]Institute of Molecular and Cell Biology, Proteos, 61 Biopolis Drive, Singapore 138673
[2]Centre for Biomedical Genetics, Department of Biomedical Science, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK
[3]Department of Biological Sciences, 14 Science Drive 4, National University of Singapore, Singapore 117543
*These authors contributed equally to this work
†Author for correspondence (e-mail: sudipto@imcb.a-star.edu.sg)

Accepted 2 December 2004

Development 132, 625-634
Published by The Company of Biologists 2005
doi:10.1242/dev.01606

Summary

Orthologues of nearly all of the core components of the Hedgehog signalling pathway, defined originally through genetic analysis in *Drosophila*, have now been discovered in vertebrates and shown to have highly conserved functions. The one striking exception to this rule is the kinesin-like protein Costal2, which plays a central role in controlling the activity of the zinc-finger-containing transcriptional regulator, Cubitus interruptus that modulates all Hedgehog-dependent target gene expression, but whose involvement in Hedgehog signalling has not been demonstrated in vertebrates. We report the cloning of a kinesin-related gene from the zebrafish that in structure as well as function, appears to represent the first vertebrate orthologue of *costal2*. Using a combination of genetic and biochemical analysis, we provide evidence that as in *Drosophila*, zebrafish Costal2 acts principally as an intracellular repressor of signal transduction, in conjunction with Suppressor of Fused, another protein that negatively regulates signalling in Hedgehog-responsive cells.

Key words: Hedgehog, Costal2, Suppressor of Fused, Zebrafish, Muscle

Introduction

During animal development, the Hedgehog (Hh) family of secreted lipid-modified glycoproteins plays essential instructive roles in guiding the specification of cell fates and regulating cell proliferation and survival in a context-dependent manner. In keeping with such manifold functions, inappropriate activity of the Hh pathway has been directly linked to a wide spectrum of congenital abnormalities and malignancies in humans (reviewed by McMahon et al., 2003). In *Drosophila*, where the intracellular mechanism of Hh signal transduction has been characterised extensively and most comprehensively, the kinesin-related protein Costal2 (Cos2), binds to microtubules and is thought to provide a platform for the assembly of a tetrameric cytoplasmic complex of proteins that includes the serine-threonine kinase Fused (Fu), the Gli-family transcriptional regulator Cubitus interruptus (Ci) as well as the PEST domain-containing protein, Suppressor of Fused [Su(fu)] (reviewed by Ingham and McMahon, 2001; Lum and Beachy, 2004; Robbins et al., 1997; Sisson et al., 1997). Cos2 interacts directly with Ci, and this Cos2-mediated sequestration of Ci on microtubules is believed to be essential for targeting the protein for sequential rounds of phosphorylation by three distinct kinases and subsequently, in a proteasome-mediated proteolytic cleavage, that generates a C-terminally truncated repressor form, Ci_{rep}, which antagonises Hh target gene expression (Ingham and McMahon, 2001; Lum and Beachy, 2004). Exposure to the Hh signal results in an alteration in the interaction among the components of the tetrameric complex at multiple levels. This includes the dissociation of the complex from the microtubule scaffold thereby alleviating the cytoplasmic sequestration, phosphorylation and processing of Ci, and culminating in the facilitation of the nuclear import of its full-length form and maturation into Ci_{act}, a labile transcriptional activator that induces the expression of Hh target genes.

There is evidence to indicate that among the primary events in this process of Ci activation by the Hh signal is the neutralisation of the inhibitory effects exerted by Cos2 on Ci, which results in the dissociation of the Cos2-Fu-Ci-Su(fu) complex from the microtubules (Ingham and McMahon, 2001; Lum and Beachy, 2004). Indeed, the cytoplasmic tail of Smoothened (Smo), a seven-pass serpentine transmembrane protein and component of the Hh receptor that is essential for the intracellular transmission of the Hh signal, interacts with Cos2 and has recently been implicated in the Hh-mediated abrogation of Cos2 activity during the process of intracellular signal transduction (Jia et al., 2003; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003).

In vertebrates, at least three distinct paralogous Gli proteins, the orthologues of *Drosophila* Ci, regulate the transcriptional responses to Hh, and their activities appear to be similarly modified by phosphorylation and proteolytic cleavage (Ingham and McMahon, 2001). Furthermore, the nuclear access of the activator and repressor forms of the Gli proteins and their

transcriptional regulatory functions have been shown to be controlled by analogous antagonistic effects of Su(fu) and Fu, raising the possibility that a similar or an identical cytoplasmic complex of proteins could perform an essential function in modulating the signalling pathway in Hh target cells of vertebrates as well as *Drosophila* (Ingham and McMahon, 2001). However, despite the remarkable overall evolutionary conservation of the signalling mechanism, in the absence of any evidence yet implicating the involvement a Cos2-like protein, the significance of such a cytoplasmic complex in vertebrate Hh signalling has not been fully appreciated.

We report the identification and functional characterisation of a kinesin-like protein in the zebrafish embryo that represents the first vertebrate orthologue of Cos2 and show that it plays a crucial and conserved role as an intracellular repressor of the Hh signalling pathway.

Materials and methods

Zebrafish strains

Wild-type and mutant strains of zebrafish were maintained under standard conditions of fish husbandry. The *syu*^{t4} (Schauerte et al., 1998; van Eeden et al., 1996) and *smu*^{b641} (Barresi et al., 2000; Varga et al., 2001) strains have been described previously.

Cloning of zebrafish *cos2*

We used the web-based CODEHOP (consensus-degenerate hybrid oligonucleotide primer) programme (http://blocks.fhcrc.org/codehop.html) to design appropriate primers for degenerate PCR based on sequence information derived from the *Drosophila*, mouse, Fugu and human homologues of *cos2*: Cos2FORWARD, 5′-AGATCGACAACCTGCGACARGARAARGA-3′; Cos2REVERSE, 5′-CAGCTGCATGTTCCGCTCRTGYTCYTT-3′.

RACE reactions were performed using reagents from Clontech according to the manufacturer's instructions. A full-length zebrafish *cos2* cDNA was constructed by piecing together 5′ and 3′ RACE products at their overlapping sequence. This cDNA was subsequently fused in frame with GFP at the 5′ end in the pCS2 mRNA expression vector. mRNA expression construct for *shh* was used as reported previously (Blagden et al., 1997). Approximately 2-3 nl of 0.5 mg/ml of synthetic mRNA of the different constructs was injected into each fertilised egg.

Antisense *cos2* MOs

The sequence of the antisense MOs are as follows: Cos2START, 5′-GCCGACTCCTTTTGGAGACATAGCT-3′; Cos2SPLICE, 5′-AAATACTCACAAATGCTGGCTTCCC-3′.

cos2 genomic sequences used for delineating the exon-intron boundaries were identified in the zebrafish BAC/PAC sequence database (BAC zK265M8; LG7) at the Wellcome Trust Sanger Centre. The MOs were used at a concentration of 1 mM each and 3-4 nl of the MO solution was injected into each fertilised egg. As a combination of the two MOs consistently gives a slightly stronger phenotype than when each is used singly, we have provided data that we obtained on using this mixture. The sequence and use of the *Su(fu)* MOs have been previously reported (Wolff et al., 2003). Consistent with published data from our laboratory as well as others, the effect of the MOs was most prominent in somites in the anterior two-thirds of the embryo, possibly owing to the degradation and dilution of the MOs over time. As a test for the specificity of the *cos2* morphant phenotype, the effect of the MOs was titrated by co-injection of synthetic *gfp-cos2* mRNA. This construct carries the *cos2* translational start sequence that is recognised by the Cos2START antisense MO, immediately downstream of the GFP coding sequence, with a single mismatch (CGCT**ATG** instead of AGCT**ATG**, *cos2* start codon in bold; see also Fig. 4) introduced to ensure in-frame fusion of the *cos2* ORF with that of *gfp*. However, the intronic sequence that is recognised by the Cos2SPLICE MO is completely absent from this chimaeric cDNA. These modifications possibly account for the ability of this construct to effectively titrate the inhibitory effects of the MOs. The MO injected *syu*^{t4} and *smu*^{b641} embryos were identified by their curled down tails and U-shaped posterior somites as described before (Wolff et al., 2003). The numbers of embryos of a particular genotype that exhibited a specific phenotype from among the total number of embryos of that genotype that were analysed for each MO experiment have been expressed as 'n' values. Quantification of MP cell numbers in embryos with different levels of Hh activity was carried out as described by Wolff et al. (Wolff et al., 2003).

In situ hybridisation, antibody labelling and microscopy

In situ probes for zebrafish *ptc1* (Concordet et al., 1996) and *fkd4* (Odenthal et al., 2000), as well as antibodies that recognise Eng, β-tubulin (Developmental Studies Hybridoma Bank), Prox1 (gift of S. Tomarev), slow myosin heavy chain (MyHC; gift of F. Stockdale) and GFP proteins (Abcam) were used according to routine protocols. Histochemical staining was carried out using the Vectastain Elite kit or reagents from Roche. Confocal analysis was carried out with appropriate fluorophore-conjugated secondary antibodies using a Zeiss LSM confocal microscope. Images of muscle fibres and distribution of GFP-Cos2/β-tubulin in somitic cells represent projections of *z*-stacks accumulated using a 40× and a 63× oil immersion lens, respectively. For colocalisation studies of GFP-Cos2 and β-tubulin in 293T cells, a single scan was collected using a 100× oil immersion lens. Where necessary, preparations were counterstained with the DNA-binding dye ToPro-3 for highlighting the nuclei.

Cell culture, co-immunoprecipitation and western blotting

Mammalian 293T cells were transfected with pCS2-GFP, pCS2-GFP-Cos2, pCS2-Su(fu)-GFP and pcDNA-His-Gli1 either singly or in combination using the Qiagen Superfect transfection kit. Lysates from untransfected 293T cells or single transfections of pCS2-GFP-Cos2, pCS2-Su(fu)-GFP and pcDNA-His-Gli1, each containing ~40 μg of protein, were fractionated by SDS-PAGE. For co-immunoprecipitation reactions, lysates from untransfected 293T cells, pcDNA-His-Gli1 and pCS2-GFP single transfections, or co-transfections of pCS2-GFP, pCS2-GFP-Cos2 or pCS2-Su(fu)-GFP, together with pcDNA-His-Gli1, each containing ~500 μg of protein, were incubated with purified mouse anti-GFP antibodies (Sigma) overnight at 4°C. The immune complexes were collected by incubation with protein A-sepharose beads (Oncogene) for 1-4 hours at 4°C, followed by centrifugation. The immunoprecipitates were then washed twice with washing buffer and fractionated by SDS-PAGE. For single transfections, membranes were probed with mouse monoclonal anti-His (Santa Cruz Biotechnology) or anti-GFP antibodies, whereas for co-immunoprecipitations, only the anti-His antibodies were used.

Results

Molecular cloning of a vertebrate orthologue of *cos2*

To investigate the possible involvement of a Cos2-like protein in vertebrate Hh signalling, we initiated searches for homologous sequences in the available vertebrate genome databases. In the course of these searches, we were able to identify sequences in the mouse and human genome databases [annotated as kinesin family member 7 (Kif7)] that showed significant sequence similarity to the Cos2 protein of *Drosophila* (Fig. 1). Using this information as a starting point for our investigation, we designed a pair of degenerate PCR

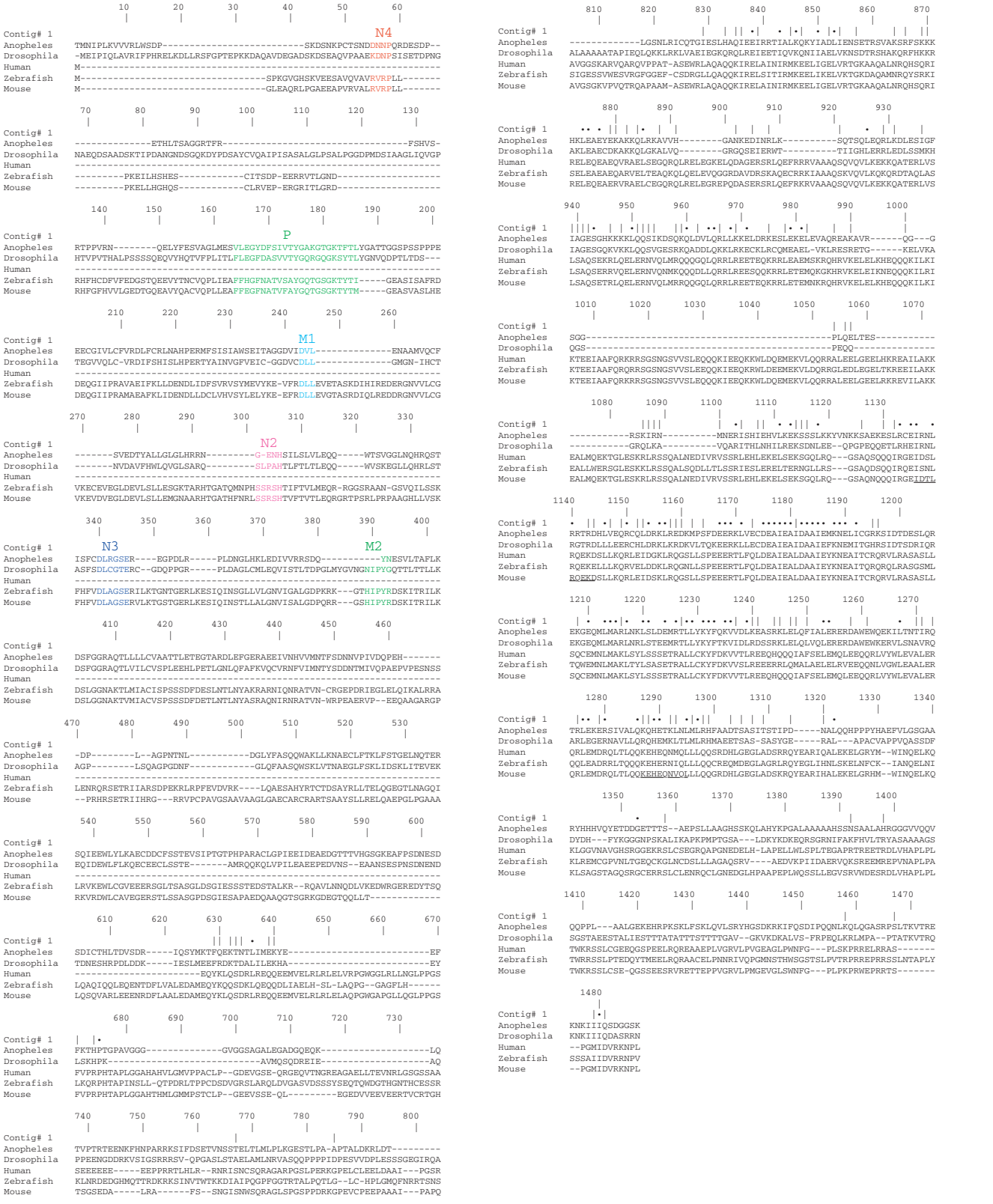


Fig. 1. Alignment of Cos2 sequences from insects and vertebrates. The nucleotide- (N4, P, N2 and N3) and microtubule-binding (M1, M2) motifs in the motor domain are different colours. The amino acid sequence corresponding to the degenerate primer pairs used to obtain a 480 bp nucleotide fragment of zebrafish *cos2* are underlined. Accession numbers: AAB66813 (*Drosophila*), XP_309818 (*Anopheles*), XP_133575 (mouse), XP_226596 (rat, not shown) and NP_940927 (human). Sequence of all Cos2 proteins other than that of *Drosophila* and zebrafish are predicted by automated computational analysis with supporting mRNA/EST evidence available for all, except *Anopheles*. Predictions for *Anopheles* and human Cos2 sequences are incomplete.

primers that allowed us to amplify an ~480 bp nucleotide fragment from embryonic zebrafish cDNA that exhibited striking homology to Cos2-like sequences from the mouse and human genomes (see Materials and methods and Fig. 1). We performed 5′ and 3′ sequence extension of this clone by RACE and assembled a full-length cDNA containing an ORF predicted to encode a kinesin-like protein of 1363 amino acids (Fig. 1). As with *Drosophila* Cos2 and other members of the kinesin heavy chain (KHC) superfamily of proteins (Goldstein, 1993; Robbins et al., 1997; Sisson et al., 1997), the N-terminal and C-terminal regions of zebrafish Cos2 have features of alternating α-helices and β-sheets that are thought to form globular structures – the motor and cargo binding domains, respectively. Moreover, the existence of a set of heptad repeats in the central stalk region of the molecule suggests potential homodimerisation through the formation of a parallel coiled coil, consistent with homodimers being the functionally active form of KHC molecules. Within the N-terminal motor domain, the motifs for nucleotide as well as microtubule binding are well conserved in the zebrafish as well as the mouse proteins; however, as has been reported previously (Lum et al., 2003; Sisson et al., 1997), these residues in the Cos2 proteins of insects are not strictly conserved, showing some variation from the consensus. In contrast to typical kinesins, whose homology with *Drosophila* Cos2 is mainly restricted to the highly conserved motor domain, the zebrafish Cos2 protein exhibits sufficient sequence homology with *Drosophila* Cos2 that extends throughout the length of the molecule (Fig. 1).

Expression pattern of the zebrafish *cos2* gene and subcellular distribution of its protein

We examined the expression pattern of *cos2* during embryogenesis using whole mount mRNA in situ hybridisation. Similar to the transcripts of the zebrafish *fu* and *Su(fu)* orthologues reported in our earlier study (Wolff et al., 2003), we observed a low level ubiquitous transcription of the gene at all developmental stages (Fig. 2A,C,D and data not shown). The levels of *cos2* expression remained unaltered in *slow-muscle-omitted* (*smu*) mutant embryos that are incapable of transducing Hh, owing to the complete lack of Smo activity (Fig. 2A) (Chen et al., 2001; Varga et al., 2001). Moreover, embryos with unrestrained Hh signalling effected through injection of synthetic mRNA encoding the zebrafish Sonic hedgehog (Shh) protein (Blagden et al., 1997; Du et al., 1997), exhibited no discernible variation in *cos2* expression pattern, indicating that its transcription is not regulated by Hh activity (Fig. 2B).

In *Drosophila*, *cos2* transcription also occurs rather ubiquitously in the developing embryo and limb imaginal disks and does not seem to be modulated by Hh activity (Sisson et al., 1997). Levels of the Cos2 protein, by contrast, are clearly modulated in cells in the embryo, as well as in the imaginal disks depending on their status of Hh signalling activity (Ogden et al., 2003; Ruel et al., 2003; Sisson et al., 1997). In the immediate non-availability of an antibody to detect the endogenous pattern of zebrafish Cos2 expression, we visualised its subcellular localisation by monitoring embryos injected with a synthetic mRNA that encodes an N-terminal GFP-tagged variant of the Cos2 protein. Although we observed expression of GFP-Cos2 in cells throughout the embryo, we chose to investigate its subcellular distribution in the paraxial mesodermal cells of the somites, which constitute a relatively uniform field of cells that do not respond to the Hh signal under normal circumstances (Wolff et al., 2004). In these cells, the localisation of GFP-Cos2 was restricted almost exclusively to the cytoplasm (Fig. 2E,F). Overexpression of the Shh protein is sufficient to instigate activation of the Hh pathway in these somitic cells (Blagden et al., 1997; Du et al., 1997). Such ectopic Hh signalling activity did not alter the subcellular distribution of GFP-Cos2, indicating that its localisation pattern does not change significantly in response to Hh pathway activity (data not shown).

In the epidermal cells of the *Drosophila* embryo, the distribution of Cos2 has been shown to mirror the distribution of microtubules in the cytoplasm, consistent with Cos2 being a microtubule binding protein (Sisson et al., 1997). We performed double-labelling studies with antibodies to β-tubulin to assess the relative distribution patterns of zebrafish Cos2 and microtubules within the cytoplasm of the somitic cells, and found a substantial overlap of the GFP signal with β-tubulin, reminiscent of the pattern in *Drosophila* embryos (Fig. 2G,H). In addition, high-resolution confocal microscopy of mammalian 293T cells transfected with a construct expressing the GFP-Cos2 fusion protein, showed significant

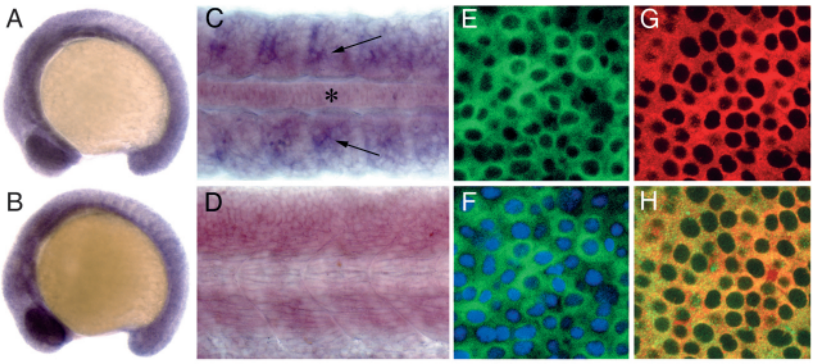


Fig. 2. Expression pattern of the zebrafish *cos2* gene and subcellular distribution of the Cos2 protein. (A) *cos2* expression in a 12-somite stage embryo derived from a cross of heterozygous *smu*^{b641} parent fish. The expression is indistinguishable in wild-type embryos and their homozygous mutant *smu* siblings. (B) *cos2* expression in a 12-somite stage wild-type embryo injected with Shh mRNA. (C) Flat mount of a 15 somite wild-type embryo with uniform expression of *cos2* in somitic cells (arrows) and the midline (asterisk). (D) Lateral view of the myotome of a wild-type embryo at 24 hours post fertilisation (hpf) showing *cos2* expression in muscle fibres. (E) Somitic cells of a two- to three-somite stage wild-type embryo injected with GFP-Cos2 RNA exhibiting cytoplasmic distribution of the fusion protein (*n*=5/5). (F) Superimposition of the GFP channel depicted in E with that of ToPro-3 fluorescence to highlight the nuclei. (G) Somitic cells of a two- to three-somite stage wild-type embryo injected with GFP-Cos2 RNA and stained with antibodies to β-tubulin showing the cytoplasmic distribution of microtubules. (H) Superimposition of the image in G with the pattern of GFP-Cos2 distribution reveals substantial overlap of the GFP signal and the microtubules. Panels illustrating embryos in this and subsequent figures are oriented anterior towards the left and dorsal towards the top, unless mentioned otherwise.

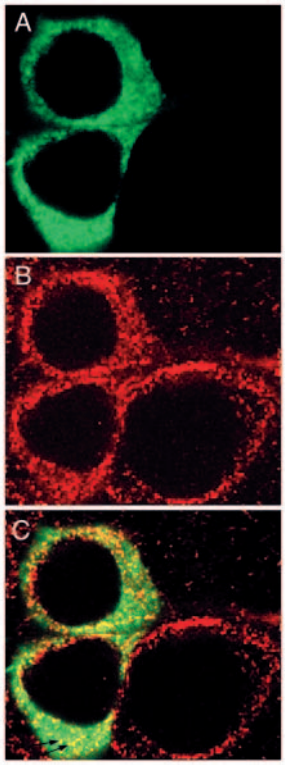


Fig. 3. Localisation of Cos2-GFP with microtubules in mammalian cells. (A) Distribution of GFP-Cos2 in transfected 293T cells. (B) Pattern of β-tubulin distribution in the same cells depicted in A. (C) Merged image of A,B. Arrows indicate distinct foci of GFP and β-tubulin co-localisation.

colocalisation of GFP with β-tubulin (Fig. 3A-C), further substantiating the view that zebrafish Cos2, like its *Drosophila* homologue, possibly functions by associating with microtubules.

Loss of Cos2 activity induces ectopic Hh signalling in the myotome of the zebrafish embryo

Large-scale mutagenesis screens in the zebrafish have resulted in the isolation of a number of mutations that affect inductive signalling from the axial midline (Brand et al., 1996; van Eeden et al., 1996). Molecular characterisation of the affected loci has shown that most of them disrupt the activity of components required for the generation, release or transduction of the Hh signal (Chen et al., 2001; Karlstrom et al., 1999; Karlstrom et al., 2003; Nakano et al., 2004; Schauerte et al., 1998; Varga et al., 2001; Wolff et al., 2004). Using the T51 radiation hybrid (RH) panel, we mapped the zebrafish *cos2* gene on linkage group 7 (LG7), which shows a high degree of synteny to human chromosome 15 and mouse chromosome 7, with their respective putative homologues of *cos2* mapping within the syntenic interval (Fig. 4A; data not shown). As no zebrafish mutation has yet been mapped in the vicinity of the region of LG7 that contains *cos2*, we adopted the well established use of antisense morpholino oligonucleotides (MOs) (Nasevicius and Ekker, 2000) to 'knock down' the activity of the Cos2 protein and assess the effects of its loss-of-function on Hh signalling during embryonic development.

In a series of earlier studies, we showed that distinct muscle fibre-types – superficial slow-twitch fibres (SSFs), muscle pioneer (MP) slow fibres and medial fast-twitch muscle fibres (MFFs) – differentiate in the myotome of the zebrafish embryo in response to different levels and timing of Hh activity emanating from the midline (Lewis et al., 1999; Wolff et al., 2003; Wolff et al., 2004). These unique muscle identities are specified by the combinatorial effects of the Gli1 and Gli2 proteins, and serve as very sensitive cellular readouts of the status of the activities of individual components of the Hh pathway that modulate Gli function (Wolff et al., 2003). We used two different MOs designed against *cos2* mRNA – one targeted at the translational start and the other at the splice junction between the first coding exon and the succeeding intronic sequences (Fig. 4B; see also Materials and methods). Injection of either one or a combination of these MOs into wild-type embryos resulted in ectopic activation of the pathway, as evidenced by an upregulation and expansion in the domain of *patched1* (*ptc1*) expression in the muscle precursor cells (Concordet et al., 1996), which encodes the ligand-binding component of the Hh receptor complex and, in addition, is a direct and immediate transcriptional target of Hh activity in responding cells (Fig. 4C,D). Consistent with this expansion in the domain of *ptc1*, analysis of the specification of muscle cell identities in such morphant (i.e. MO injected) embryos revealed an increase in the population of all three Hh-dependent muscle fibre types as a consequence of Cos2 inactivation – the effects on the MPs and MFFs being more pronounced than the SSFs (Fig. 4E-H; Table 1). Such ectopic induction of Hh-dependent muscle fates was suppressed in embryos co-injected with the MOs, as well as synthetic mRNA encoding the GFP-Cos2 fusion protein (Fig. 4I, see Materials and methods for alterations in the MO recognition sequences in the *gfp-cos2* chimaeric cDNA), thereby confirming the specificity of the morphant phenotype and suggesting that it could result from improper translation and splicing of the endogenous *cos2* transcript.

Inactivation of Cos2 also triggers ectopic Hh signalling in the developing neural tube

Like the myotome, cell types within the ventral neural tube of all vertebrate embryos are specified in response to a graded activity of Hh proteins (McMahon et al., 2003). In the zebrafish, two distinct populations of cells, the medial and lateral floor-plate (MFP and LFP) cells, occupy the ventral most territories of the neural tube (Odenthal et al., 2000). Although Hh signalling is dispensable for the formation of the MFP, the requirement of Hh activity for the induction of the LFP fate is absolute as mutations in Hh signalling components that compromise signal transduction, concomitantly impair the development of LFP cells (Odenthal et al., 2000). In

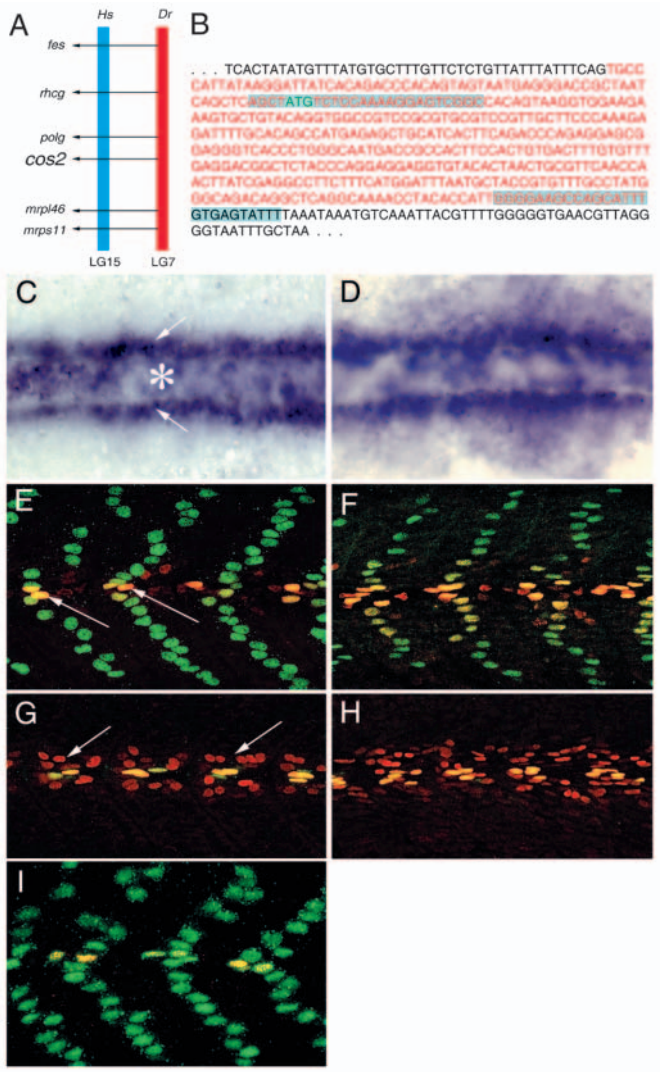
Table 1. Numbers of MP cells in each myotomal hemisegment of embryos with distinct levels of Hh activity

Wild type	4.1±0.6
shh mRNA into wild type	>25
smu	0.0
cos2 MOs into wild type	8.9±0.3
cos2 MOs and *Su(fu)* MOs into wild type	18.9±0.7
cos2 MOs and *Su(fu)* MOs into *smu*	2.9±0.3

The data represent the mean and s.d. of MPs quantified in hemisegments 5-8 of five embryos analysed for each genotype

contrast to these situations, Cos2 morphants exhibited an enlargement in the LFP, as exemplified by a discernible expansion in the domain of *fork head domain 4* (*fkd4*), a gene whose expression marks all cells of the LFP as well as the MFP (Odenthal et al., 2000) (Fig. 5A,B). In these embryos, we additionally observed a consistent upregulation of *fkd4* expression in cell populations of the ventral mid- and hindbrain territories that also require Hh signalling for their proper specification (Fig. 5C,D) (see also Karlstrom et al., 1999; Karlstrom et al., 2003). Taken together, all of these loss-of-function data provide evidence that Cos2 acts as a general intracellular repressor of Hh signalling in the zebrafish embryo, in a manner that reflects a conserved function akin to its homologue in *Drosophila*.

In humans, de-repression of HH pathway activity associated with loss of SU(FU), predisposes individuals to medulloblastomas and other neuroectodermal tumours (Taylor et al., 2002). In line with this, our demonstration that a vertebrate homologue of Cos2 has similar negative regulatory effect on Hh signal transduction during embryonic development, suggests that inactivation of its function in mammals could likewise be associated with such pathologies.



The activity of zebrafish Cos2 is epistatic to Shh and Smo

To explore this interpretation further and obtain additional evidence that the effects of Hh-dependent cell fate determination on inhibition of Cos2 function arise specifically from a de-repression of the Hh pathway, we injected the MOs into embryos compromised to varying degrees in their ability to transduce Hh, either because of deletion of the *shh* gene (Schauerte et al., 1998; van Eeden et al., 1996) or as a consequence of mutations in the Smo protein that render it completely non-functional (Chen et al., 2001; Varga et al., 2001). Although embryos that lack *shh* activity lack all MP cells (Lewis et al., 1999; Schauerte et al., 1998), a muscle fibre that forms in response to the highest levels of Hh activity (Wolff et al., 2003), this cell type was effectively restored in the mutant embryos injected with the *cos2* MOs (Fig. 6A-C). Moreover, in *smu* mutant embryos, where no Hh-dependent muscle cell-types are specified (Barresi et al., 2000; Wolff et al., 2003), loss of Cos2 activity resulted in the restoration of some SSFs [cells that require the lowest levels of Hh (Wolff et al., 2003)], indicating that inhibition of Cos2 is sufficient for triggering the activity of Gli_{act}, even under conditions where Smo-dependent signal transduction is completely abolished (Fig. 6D-F).

Cos2 and Su(fu) collaborate to negatively regulate intracellular Hh signal transduction in the zebrafish embryo

All of these effects of the *cos2* MOs on de-repression of the Hh pathway are relatively limited when compared with the effects on myotomal cell fate specification that occur in response to ectopic expression of Shh itself (Blagden et al.,

Fig. 4. Loss of Cos2 function results in de-repression of the Hh pathway in the myotome of the zebrafish embryo. (A) Diagrammatic representation of the synteny between regions of human (Hs; LG15) and zebrafish (Dr; LG7) chromosomes bearing the *cos2* genes. *fes*, proto-oncogene tyrosine protein kinase; *rhcg*, Rh type-C glycoprotein; *polg*, DNA polymerase γ subunit 1; *mrpl46*, mitochondrial ribosomal protein L46; *mrps11*, mitochondrial ribosomal protein S11. (B) Nucleotide sequence of the first protein coding exon of *cos2* and the flanking introns. The start codon is indicated in green. The sequences targeted by the start and splice MOs are highlighted. (C) Flat mount of a two-somite stage wild-type embryo, showing the pattern of *ptc1* transcription. The precursors of the SSFs and MPs (arrows) and the neural plate (asterisk) are indicated. (D) Similar flat mount of a *cos2* morphant with expanded domain of *ptc1* expression in the somitic mesoderm (*n*=9/16). (E) Lateral view of the myotome of a 24 hpf wild-type embryo showing the SSFs (stained for expression of the homeodomain protein Prox1, green) and the MPs [stained for expression of the homeodomain proteins of the Engrailed (Eng) family, red]. Within the slow muscle lineage, Prox1 is expressed in SSFs as well as MPs, while Eng proteins are expressed exclusively in the MPs. Prox1- and Eng-positive MP nuclei (yellow) are indicated (arrows). (F) A similar stage *cos2* morphant with supernumerary MP cells in the myotome (*n*=6/7). (G) Medial view of the myotome of a 24 hpf wild-type embryo. MFFs that surround the MPs and express low levels of Eng are indicated (arrows). (H) MFFs are increased in numbers in *cos2* morphants (*n*=5/7). (I) Myotome of an embryo co-injected with *cos2* MOs and *cos2* sense mRNA, showing effective suppression of supernumerary MP induction that is observed in *cos2* morphants (*n*=12/12).

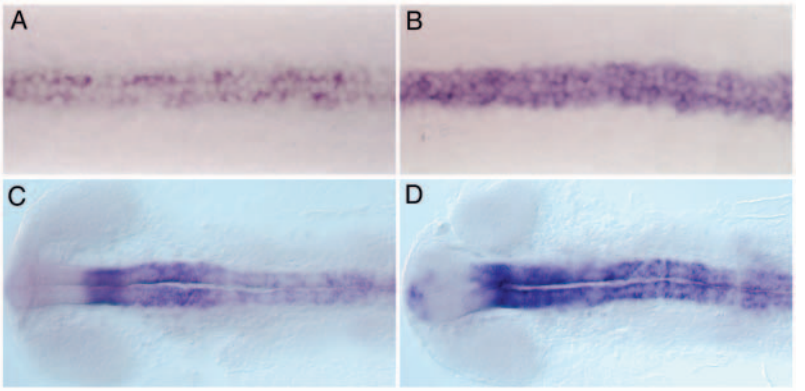


Fig. 5. Inactivation of Cos2 function induces ectopic Hh signalling in the ventral neural tube. (A) *fkd4* expression in the ventral neural tube (MFP and LFP cells) of a 22-somite stage wild-type embryo. (B) Upregulation of the levels and expansion in the domain of *fkd4* expression in the ventral neural tube of a 22-somite stage *cos2* morphant (*n*=16/22). (C) Expression pattern of *fkd4* in ventral cell populations of the developing mid- and hindbrain of a 22-somite stage wild-type embryo. (D) Increased levels and enlargement of the domain of *fkd4* in ventral cell populations of the developing mid- and hindbrain of a 22-somite stage *cos2* morphant embryo (*n*=13/22).

1997; Du et al., 1997) (Table 1). In these circumstances, almost the entire myotome is converted to the SSF and MP fate in response to high levels of Gli_{act} activity that is induced by high levels of Shh. As Cos2 is an intracellular component of the Hh pathway, loss of its activity is expected to result in a cell-autonomous upregulation of signalling, independently of the availability of the Hh ligand. Indeed, in *Drosophila*, *cos2* mutant cells far away from the source of Hh exhibit cell-autonomous upregulation of signalling activity (Sisson et al., 1997). However, although signalling levels are clearly elevated in *cos2* mutant cell clones in *Drosophila*, it does not attain the maximal intensity that ensues when Hh itself is misexpressed or when the activity of Ptc, which suppresses Smo signalling in the absence of Hh, is lost (Ingham and McMahon, 2001; Wang et al., 2000; Wang and Holmgren, 1999). In this context, there is some evidence to indicate that the activity of Su(fu) plays a redundant role in inhibiting Ci: in the absence of Cos2, Ci, which is now untethered from the microtubules, nevertheless continues to remain complexed with Su(fu), which interferes with its nuclear access as well as transcriptional activity within the nucleus, thereby preventing full activation of the pathway (Ingham and McMahon, 2001; Wang et al., 2000; Wang and Holmgren, 1999). An alternative view, gleaned from more recent findings, suggests that loss of Cos2 activity prevents high threshold levels of Hh signalling because apart from its negative influence on the tethering and processing of Ci, it also acts positively within the transduction cascade by somehow counteracting the restraining influence of Su(fu) on the nuclear access and transcriptional activity of Ci_{act} (Jia et al., 2003; Lum et al., 2003; Wang et al., 2000).

Given this scenario, we analysed whether the phenotypic effects resulting from the de-repression of Hh signalling as a consequence of the loss of Cos2 activity could be further potentiated by the simultaneous inhibition of the activity of the zebrafish Su(fu) homologue. Indeed, embryos co-injected with MOs directed against *Su(fu)* as well as *cos2* exhibit high levels of ectopic Hh signalling that represents a marked enhancement of the effects observed when either the Cos2 or Su(fu) proteins are individually inactivated with a striking increase in the numbers of all muscle cell fates that depend upon Hh activity (Fig. 7A,B; Table 1) (see also Wolff et al., 2003). We also examined the effects of the concurrent loss of Cos2, as well as Su(fu), in *smu* mutant embryos that lack all Hh signalling activity. Like Cos2, inhibition of Su(fu) alone in the absence of Smo-mediated signalling, results in a weak de-repression of the pathway as manifest by the differentiation of a few SSFs in the myotome (Fig. 7C). By contrast, simultaneous loss of Cos2 and Su(fu) from *smu* embryos leads to the differentiation of substantial numbers of SSFs and MFFs, as well as more sporadic MPs – cell types that are otherwise completely eliminated in the absence of Smo activity (Fig. 7D-F; Table 1).

Zebrafish Cos2 physically associates and forms a complex with Gli1

In light of these multiple lines of genetic evidence linking zebrafish Cos2 with Hh signalling, we wished to investigate its capacity, if any, to interact physically with one or other of the intracellular components of the pathway that participate in

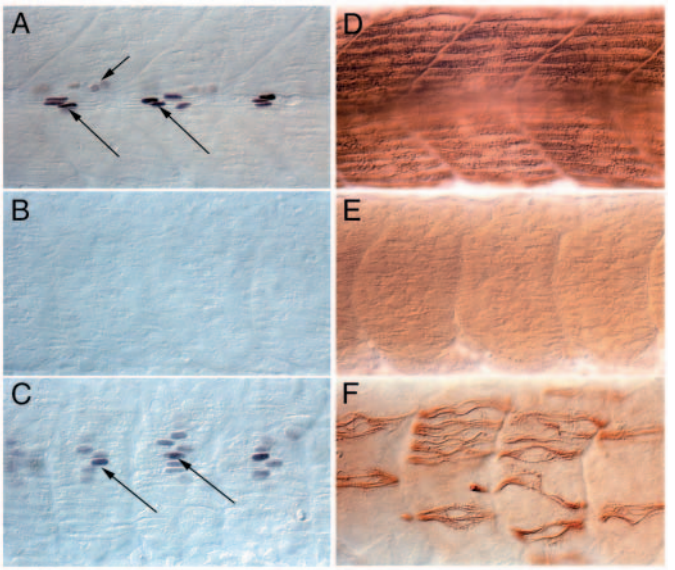


Fig. 6. Cos2 is epistatic to *shh* and *smo*. (A) Eng expression in the MPs (long arrows) and MFFs (short arrow) revealed by histochemistry in a 24 hpf wild-type embryo. (B) Eng expression is absent from the myotome of a 24 hpf *shh* mutant embryo. (C) Restoration of Eng-expressing MP cells (arrows) in the myotome of a *shh* mutant embryo injected with *cos2* MOs (*n*=9/12). (D) Myotome of a 24 hpf wild-type embryo stained with antibodies to slow MyHC showing the pattern of the SSFs. (E) Slow MyHC immunoreactivity is completely absent from the myotome of a 24 hpf *smu* mutant embryo, consistent with the lack all SSFs as well as MPs. Like *syu* embryos depicted in B, *smu* mutants also lack all Eng expression from the myotome (data not shown) (see also Barresi et al., 2000; Wolff et al., 2003). (F) Slow fibres are restored in significant numbers in *smu* mutants injected with *cos2* MOs (*n*=15/21).

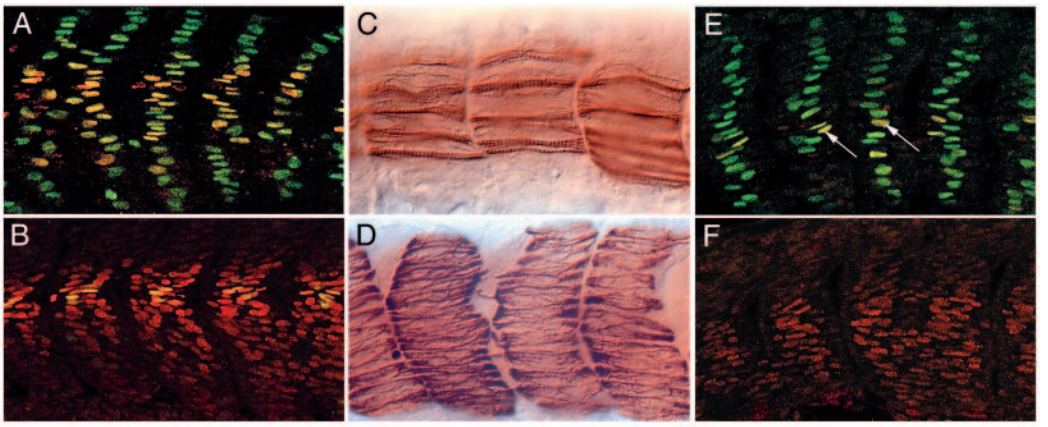
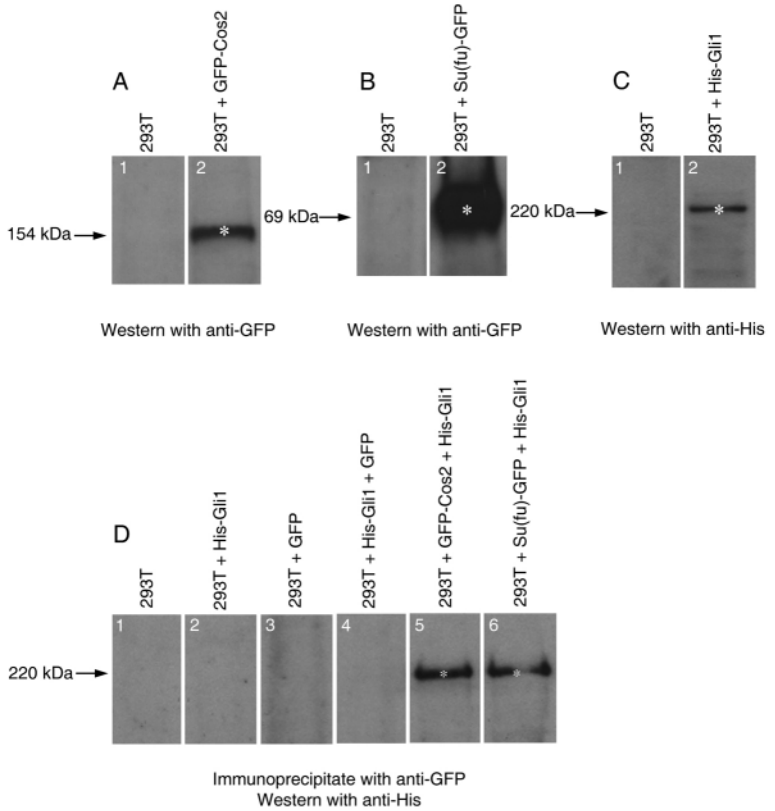


Fig. 7. Loss of Su(fu) activity enhances de-repression of Hh signalling in Cos2 morphants. (A) A 24 hpf wild-type embryo injected with MOs against *Su(fu)* and *cos2* and stained with antibodies against Prox1 and Eng, showing very large numbers of supernumerary MP cells in the myotome (*n*=18/23). Compare with Fig. 4E,F. (B) The embryo depicted in A in medial view, showing extensive expansion in the numbers of Eng expressing MFFs (*n*=20/23). Compare with Fig. 4G,H. (C) Injection of *Su(fu)* MO into *smu* mutants restores some slow fibres (*n*=16/22). Compare with Fig. 6D,E. (D) Injection of *Su(fu)* as well as of *cos2* MOs into *smu* embryos results in the recovery of substantial numbers of slow fibres (*n*=16/19). Compare with Fig. 6D,E. (E) A *Su(fu)* and *cos2* MO co-injected *smu* embryo, stained with anti-Eng and anti-Prox1 antibodies, showing restoration of SSFs as well as MPs (arrows; *n*=10/18). (F) A *Su(fu)* and *cos2* MO co-injected *smu* embryo, showing recovery of Eng expressing MFFs (*n*=16/18).

transducing the signal to the nucleus. Such interactions of *Drosophila* Cos2 with members of the tetrameric cytoplasmic complex, principally Ci, are a central determinant of signalling activity. In the zebrafish, the Ci homologue Gli1 plays a crucial role in activating the transcription of Hh-dependent target genes (Karlstrom et al., 2003; Wolff et al., 2003) and the activity of mammalian Gli1 has been shown to be modulated, in tissue culture cells, through its physical association with the Su(fu) protein (Ding et al., 1999; Kogerman et al., 1999; Murone et al., 2000; Pearse et al., 1999; Stone et al., 1999). We therefore used the cell culture system to establish an association between the zebrafish Cos2 and Gli1 proteins. To this end, we first analysed the ability of zebrafish Su(fu) and Gli1 to physically interact by expressing GFP-Su(fu) and a His-tagged version of Gli1 in mammalian 293T cells followed by co-immunoprecipitation and western blotting. In such assays, we could readily immunoprecipitate Gli1 together with Su(fu), as had been documented previously for the mammalian counterparts of these two proteins (Fig. 8A-D). Using the same assay, we could demonstrate the efficient immunoprecipitation of Gli1 with GFP-tagged Cos2 (Fig. 8A-D). Identical results were obtained with a MYC-tagged variant of Cos2 and His-Gli1 (data not shown). Based on these observations, we conclude that formation of a Gli-Cos2 complex could represent an evolutionarily conserved and a fundamental step in the transduction of the Hh signal in insects as well as vertebrates.



Discussion

Ever since the discovery of the Hh genes in different groups of vertebrates almost a decade ago, a large number of studies have resulted in the generation of an explosive amount of information on the roles of Hh signalling in a fascinating diversity of biological processes (McMahon et al., 2003). However, a

Fig. 8. Zebrafish Cos2 physically associates with Gli1. (A-C) Western blots of lysates from 293T cells transfected with GFP-Cos2 (A), Su(fu)-GFP (B) and His-Gli1 (C) constructs, showing the expected bands (marked with asterisk) of the fusion proteins (lane 2). Untransfected 293T cell lysates served as controls (lane 1). In contrast to Cos2 and Gli1, we consistently observed higher levels of Su(fu) protein expression in these transfection experiments. (D) Lanes 1-6 show lysates from untransfected 293T cells (1) or those transfected with various constructs (2-6), immunoprecipitated with anti-GFP and probed with anti-His antibodies, respectively. The expected 220 kDa band of His-Gli1 (marked with asterisk) is observed only in lanes 5 and 6.

substantial proportion of our understanding of the basic mechanistic elements of the signal transduction process itself in these organisms has relied and progressed through the isolation and functional characterisation of the homologues of components originally identified through systematic genetic screens in *Drosophila* (Ingham and McMahon, 2001). The orthologue of the only core member that has not yet been distinguished and linked to the signalling pathway in vertebrates is the kinesin-related protein Cos2, perhaps largely owing to the ambiguity about the specific kinesin family gene that could be representative of a true Cos2 orthologue. In our current analysis, we have provided several lines of evidence for a crucial role of a Cos2-like protein in regulating Hh signal transduction in the zebrafish embryo. First, we have shown that this kinesin-related protein shares a significant degree of sequence similarity to *Drosophila* Cos2 to warrant recognition as a Cos2 homologue. Second, like loss-of-function mutations in *Drosophila cos2* that de-repress the Hh pathway, inactivation of this protein also elicits ectopic Hh signalling in the somites and the ventral neural tube – two regions in vertebrate embryos where Hh plays an essential role in instructing the specification of cell fates. In addition, using biochemical studies, we have demonstrated that this kinesin is indeed able to form a complex with Gli1, the activity of the fly homologue of which (Ci) is modulated through a similar association with *Drosophila* Cos2. All of these data are consistent with and supportive of the view that this kinesin protein does represent the first vertebrate orthologue of *Drosophila* Cos2.

We have noted that in contrast to situations where the Hh ligand is mis-expressed, the effects of the loss of activity of zebrafish Cos2 in instigating ectopic signalling is relatively mild. MO-mediated gene inactivation dramatically reduces, but does not completely eliminate, the translation of gene products (Nasevicius and Ekker, 2000), and this could explain the restricted de-repression of the Hh pathway that is observed in the *cos2* morphants. In addition, the endogenous Cos2 protein could be highly stable – its sustained activity counteracting the effects of the MOs. Moreover, we cannot rule out the existence of an additional paralogue(s) of Cos2 in the zebrafish genome in which case, like the multiple Hh, Ptc and Gli proteins of vertebrates, they could have overlapping functions in the regulation of the Hh pathway. Although we are presently unable to distinguish between these possibilities, we have nevertheless shown that the negative regulatory effect of zebrafish Cos2 on Hh signalling is subject to dramatic enhancement when the activity of the Su(fu) protein is also eliminated. Su(fu) is a known antagonist of Gli function in flies as well as vertebrates and it plays a dedicated role in restraining the nuclear access, as well as the transcriptional activities of the Gli proteins. Although it is conceivable that the augmentation of signalling levels in *cos2*; *Su(fu)* double morphants again reflects the additive effects of inefficient MO activity [because maximal activation of the Hh pathway in *Drosophila* also requires the simultaneous loss of Cos2 and Su(fu) function], it seems parsimonious to conclude that our results more likely point to a collaborative effect of Cos2 and Su(fu) in regulating Gli activity that represents a conserved event in the Hh signalling cascade.

There is now overwhelming support from investigations in *Drosophila* for the activity of the C-terminal intracellular tail of Smo in mediating the communication of Hh with the intracellular components of the pathway through an association with the Cos2 protein (Jia et al., 2003; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003). Thus, Cos2 not only serves as a scaffold for the assembly and interaction of molecules like Fu, Ci and Su(fu) with each other, but could also act to route the signal from the Smo receptor at the membrane to these proteins in the cytoplasm. In this connection we note that there are conflicting reports on the requirement of the cytoplasmic tail of mammalian Smo for normal Hh signalling (Jia et al., 2003; Murone et al., 1999). In addition, the stability as well as activity of Smo seems to be regulated by Hh very differently in flies and mammals (Kalderon, 2000; Taipale et al., 2002). Moreover, in contrast to *Drosophila*, our understanding of the precise function of Fu, Su(fu) and the Gli proteins and their inter-molecular interactions during the signalling process in vertebrates is rather incomplete. Even more intriguingly, recent genetic as well as biochemical screens for Hh pathway components in mammals and the zebrafish have led to the isolation of a number of new constituents (Bulgakov et al., 2004; Wolff et al., 2004), the roles of which have either not been elucidated or whose activities are not required for regulating Hh signalling in flies, pointing to the possibility that a substantial degree of diversification of the signalling mechanism has occurred in different groups of animals.

Of particular interest in this regard is the observation that mutations in intraflagellar transport (IFT) proteins, which includes a kinesin family member unrelated to *Drosophila* Cos2, Kif3a, affect Hh signal transduction in the mouse embryo (Huangfu et al., 2003). However, in contrast to zebrafish Cos2, the activities of these proteins appear to be required positively within the signalling cascade. Currently, it is unclear how these IFT proteins interface with the other more conserved intracellular players of the Hh pathway, especially Su(fu), Fu and Gli. It is also possible that the IFT proteins have assumed a committed role in Hh signalling exclusively in mammals because mutations in their homologues seem not to result in any obvious defects in Hh-dependent developmental processes in the zebrafish embryo (Sun et al., 2004; Tsujikawa and Malicki, 2004). In light of all of these new but disparate findings, our discovery of an obligate role of a Cos2 orthologue in regulating Hh signal transduction in the zebrafish is noteworthy, as it closes a major gap in the mechanistic parallels that exist between the signalling cascades that operate in flies and vertebrates. In addition, it provides an essential framework for further investigations directed at understanding the details of the interactions between the Gli proteins, Cos2 and other members of the cytoplasmic complex, and if and how these associations are modulated by the activity of Hh proteins during development of the vertebrate embryo.

We thank D. Sutton for assistance with sequencing; C. Wolff for RH mapping and for his encouragement and interest; S. Elworthy for advice on the use of the CODEHOP programme for degenerate PCR reactions; W. Y. Leong for sharing with us her expertise on cell culture and protein biochemistry; H. P. Liew for help with sequence alignments; and R. Karlstrom for the zebrafish Gli1 expression plasmid. This work was supported by funds from the Institute of Molecular and Cell Biology and the Agency for Science, Technology and Research (A*STAR) in Singapore (S.R.) and a Wellcome Trust programme grant (P.W.I.).

References

Barresi, M. J., Stickney, H. L. and Devoto, S. H. (2000). The zebrafish slow-muscle-omitted gene product is required for Hedgehog signal transduction and the development of slow muscle identity. *Development* **127**, 2189-2199.

Blagden, C. S., Currie, P. D., Ingham, P. W. and Hughes, S. M. (1997). Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. *Genes Dev*. **11**, 2163-2175.

Brand, M., Heisenberg, C. P., Warga, R. M., Pelegri, F., Karlstrom, R. O., Beuchle, D., Picker, A., Jiang, Y. J., Furutani-Seiki, M., van Eeden, F. J. et al. (1996). Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. *Development* **123**, 129-142.

Bulgakov, O. V., Eggenschwiler, J. T., Hong, D-H., Anderson, K. V. and Li, T. (2004). FKBP8 is a negative regulator of mouse sonic hedgehog signalling in neural tissues. *Development* **131**, 2149-2159.

Chen, W., Burgess, S. and Hopkins, N. (2001). Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity. *Development* **128**, 2385-2396.

Concordet, J. P., Lewis, K. E., Moore, J. W., Goodrich, L. V., Johnson, R. L., Scott, M. P. and Ingham, P. W. (1996). Spatial regulation of a zebrafish patched homologue reflects the roles of sonic hedgehog and protein kinase A in neural tube and somite patterning. *Development* **122**, 2835-2846.

Ding, Q., Fukami, S-i., Meng, X., Nishizaki, Y., Zhang, X., Sasaki, H., Dlugosz., A., Nakafuku, M. and Hui, C-c. (1999). Mouse Suppressor of fused is a negative regulator of Sonic hedgehog signalling and alters the subcellular distribution of Gli1. *Curr. Biol.* **9**, 1119-1122.

Du, S. J., Devoto, S. H., Westerfield, M. and Moon, R. T. (1997). Positive and negative regulation of muscle cell identity by members of the hedgehog and TGF-beta gene families. *J. Cell Biol.* **139**, 145-156.

Goldstein, L. S. (1993). With apologies to scheherazade: tails of 1001 kinesin motors. *Annu. Rev. Genet*. **27**, 319-351.

Huangfu, D., Liu, A., Rakeman, A. S., Murcia, N. S., Niswander, L. and Anderson, K. V. (2003). Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature* **426**, 83-87.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signalling in animal development: paradigms and principles. *Genes Dev*. **15**, 3059-3087.

Jia, J., Tong, C. and Jiang, J. (2003). Smoothened transduces Hedgehog signal by physically interacting with Costal2/Fused complex through its C-terminal tail. *Genes Dev.* **17**, 2709-2720.

Kalderon, D. (2000). Transducing the hedgehog signal. *Cell* **103**, 371-374.

Karlstrom, R. O., Talbot, W. S. and Schier, A. F. (1999). Comparative synteny cloning of zebrafish you-too: mutations in the Hedgehog target gli2 affect ventral forebrain patterning. *Genes Dev.* **13**, 388-393.

Karlstrom, R. O., Tyurina, O. V., Kawakami, A., Nishioka, 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.

Kogerman, P., Grimm, T., Kogerman, L., Krause, D., Undén, A. B., Sandstedt, B., Toftgård, R. and Zaphiropoulous, P. G. (1999). Mammalian Suppressor-of-Fused modulates nuclear-cytoplasmic shuttling of GLI-1. *Nat. Cell Biol.* **1**, 312-319.

Lewis, K. E., Currie, P. D., Roy, S., Schauerte, H., Haffter, P. and Ingham, P. W. (1999). Control of muscle cell-type specification in the zebrafish embryo by Hedgehog signalling. *Dev. Biol*. **216**, 469-480.

Lum, L. and Beachy, P. A. (2004). The Hedgehog Response Network: Sensors, Switches, and Routers. *Science* **304**, 1755-1759.

Lum, L., Zhang, C., Oh, S., Mann, R. K., von Kessler, D. P., Taipale, J., Weis-Garcia, F., Gong, R., Wang, B. and Beachy, P. A. (2003). Hedgehog signal transduction via Smoothened association with a cytoplasmic complex scaffolded by the atypical kinesin, Costal-2. *Mol. Cell*. **12**, 1261-1274.

McMahon, A. P., Ingham, P. W. and Tabin, C. J. (2003). Developmental roles and clinical significance of hedgehog signalling. *Curr. Top. Dev. Biol*. **53**, 1-114.

Murone, M., Rosenthal, A. and de Sauvage, F. J. (1999). Sonic hedgehog signalling by the patched-smoothened receptor complex. *Curr. Biol*. **9**, 76-84.

Murone, M., Luoh, S-M., Stone, D., Li, W., Gurney, A., Armanini, M., Grey, C., Rosenthal, A., de Sauvage, F. J. (2000). Gli regulation by the opposing activities of Fused and Suppressor of Fused. *Nat. Cell Biol.* **2**, 310-312.

Nakano, Y., Kim, H. R., Kawakami, A., Roy, S., Schier, A. F. and Ingham, P. W. (2004). Inactivation of dispatched 1 by the chameleon mutation disrupts Hedgehog signalling in the zebrafish embryo. *Dev. Biol.* **269**, 381-392.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet*. **26**, 216-220.

Odenthal, J., van Eeden, F. J. M., Haffter, P., Ingham, P. W. and Nusslein-Volhard, C. (2000). Two distinct cell populations in the floor plate of the zebrafish are induced by different pathways. *Dev. Biol*. **219**, 350-363.

Ogden, S. K., Ascano, M., Jr, Stegman, M. A., Suber, L. M., Hooper, J. E. and Robbins, D. J. (2003). Identification of a functional interaction between the transmembrane protein Smoothened and the kinesin-related protein Costal2. *Curr. Biol.* **13**, 1998-2003.

Pearse, R. V. 2nd, Collier, L. S., Scott, M. P. and Tabin, C. J. (1999). Vertebrate homologues of *Drosophila* suppressor of fused interact with the gli family of transcriptional regulators. *Dev. Biol*. **212**, 323-336.

Robbins, D. J., Nybakken, K. E., Kobayashi, R., Sisson, J. C., Bishop, J. M. and Therond, P. P. (1997). Hedgehog elicits signal transduction by means of a large complex containing the kinesin-related protein costal2. *Cell* **90**, 225-234.

Ruel, L., Rodriguez, R., Gallet, A., Lavenant-Staccini, L. and Therond, P. P. (2003). Stability and association of Smoothened, Costal2 and Fused with Cubitus interruptus are regulated by Hedgehog. *Nat. Cell Biol.* **5**, 907-913.

Schauerte, H. E., van Eeden, F. J., Fricke, C., Odenthal, J., Strahle, U. and Haffter, P. (1998). Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish. *Development* **125**, 2983-2993.

Sisson, J. C., Ho, K. S., Suyama, K. and Scott, M. P. (1997). Costal2, a novel kinesin-related protein in the Hedgehog signalling pathway. *Cell* **90**, 235-245.

Stone, D. M., Murone, M., Luoh, S-M., Ye, W., Armanini, M. P., Gurney, A., Phillips, H., Brush, J., Goddard, A., de Sauvage, F. J. and Rosenthal, A. (1999). Characterisation of the human Suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli. *J. Cell Sci.* **112**, 4437-4448.

Sun, Z., Amsterdam, A., Pazour, G. J., Cole, D. G., Miller, M. S. and Hopkins, N. (2004). A genetic screen in zebrafish identifies cilia genes as a principal cause of cystic kidney. *Development* **131**, 4085-4093.

Taipale, J., Cooper, M. K., Maiti, T. and Beachy, P. A. (2002). Patched acts catalytically to suppress the activity of Smoothened. *Nature* **418**, 892-897.

Taylor, M. D., Liu, L., Raffel, C., Hui, C. C., Mainprize, T. G., Zhang, X., Agatep, R., Chiappa, S., Gao, L., Lowrance, A. et al. (2002). Mutations in SUFU predispose to medulloblastoma. *Nat. Genet*. **31**, 306-310.

Tsujikawa, M. and Malicki, J. (2004). Intraflagellar transport genes are essential for differentiation and survival of vertebrate sensory neurons. *Neuron* **42**, 703-716.

van Eeden, F. J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A. et al. (1996). Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. *Development* **123**, 153-164.

Varga, Z. M., Amores, A., Lewis, K. E., Yan, Y. L., Postlethwait, J. H., Eisen, J. S. and Westerfield, M. (2001). Zebrafish smoothened functions in ventral neural tube specification and axon tract formation. *Development* **128**, 3497-3509.

Wang, G., Amanai, K., Wang, B. and Jiang, J. (2000). Interactions with Costal2 and suppressor of fused regulate nuclear translocation and activity of cubitus interruptus. *Genes Dev*. **14**, 2893-2905.

Wang, Q. T. and Holmgren, R. A. (1999). The subcellular localisation and activity of Drosophila cubitus interruptus are regulated at multiple levels. *Development* **126**, 5097-5106.

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.

Wolff, C., Roy, S., Lewis, K. E., Schauerte, H., Joerg-Rauch, G., Kirn, A., Weiler, C., Geisler, R., Haffter, P. and Ingham, P. W. (2004). iguana encodes a novel zinc-finger protein with coiled-coil domains essential for Hedgehog signal transduction in the zebrafish embryo. *Genes Dev*. **18**, 1565-1576.