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Inactivation of *dispatched* 1 by the *chameleon* mutation disrupts Hedgehog signalling in the zebrafish embryo☆

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Abstract

Searches of zebrafish EST and whole genome shotgun sequence databases for sequences encoding the sterol-sensing domain (SSD) protein motif identified two sets of DNA sequences with significant homology to the *Drosophila* dispatched gene required for release of secreted Hedgehog protein. Using morpholino antisense oligonucleotides, we found that inhibition of one of these genes, designated Disp1, results in a phenotype similar to that of the "you-type" mutants, previously implicated in signalling by Hedgehog proteins in the zebrafish embryo. Injection of *disp1* mRNA into embryos homozygous for one such mutation, *chameleon* (*con*) results in rescue of the mutant phenotype. Radiation hybrid mapping localised *disp1* to the same region of LG20 to which the *con* mutation was mapped by meiotic recombination analysis. Sequence analysis of *disp1* cDNA derived from homozygous *con* mutant embryos revealed that both mutant alleles are associated with premature termination codons in the *disp1* coding sequence. By analysing the expression of markers of specific cell types in the neural tube, pancreas and myotome of *con* mutant and Disp1 morphant embryos, we conclude that Disp1 activity is essential for the secretion of lipid-modified Hh proteins from midline structures.
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Keywords: Mutation; Zebrafish; Morpholino

Introduction

Hedgehog (Hh) signalling plays a fundamental role in the development of vertebrates and arthropods, underlying the induction and patterning of multiple tissues and organs in members of both phyla (McMahon et al., 2003). One of the unusual features of members of the Hh protein family is their covalent coupling to cholesterol, a lipid modification that constrains their release from cells (Porter et al., 1996a,b). Genetic analysis in *Drosophila* has identified a multipass transmembrane (TM) protein designated Dispatched (Disp) that is dedicated to the secretion of lipid-modified Hh from expressing cells (Burke et al., 1999). Strikingly, Disp shares significant sequence and topological similarity with the Hh receptor Patched (Ptc) (Hooper and Scott, 1989; Nakano et al., 1989) as well as other multi-pass membrane spanning proteins implicated in lipid transport or metabolism. All of these proteins are characterised by a sequence motif encompassing five TM domains known as the SSD. Although the function of the SSD remains enigmatic, most SSD containing proteins have been implicated in some form of intracellular trafficking (Kuwabara and Labouesse, 2002). In addition, a subset of SSD proteins shows significant sequence similarity with a large family of bacterial permeases, known as the RND family (Tseng et al., 1999). Recent studies have shown that conserved residues that are critical for permease activity are also essential for the activity of both Ptc and Disp in *Drosophila* (Ma et al., 2002; Taipale et al., 2002).

The zebrafish provides a highly tractable model for the analysis of Hh signalling in vertebrate development. In addition to the well documented properties of the zebrafish

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embryo, numerous mutations affecting Hh signalling have been isolated (Barresi et al., 2000; Chen et al., 2001; van Eeden et al., 1996; Karlstrom et al., 1999; Schauerte et al., 1998; Varga et al., 2001) and high resolution in vivo assays of pathway activity have been established. In particular, the specification of distinct cell types within the myotome has been shown to be regulated by Hh signalling in a manner that is sensitive both to the strength and timing of the signal (Wolff et al., 2003). Reflecting this, different mutants have differing effects on the specification of the Hh-dependent muscles (Lewis et al., 1999).

Here, we describe the isolation of two *disp* homologues from the zebrafish and show that one of these, *disp1*, is inactivated by the previously described *chameleon* (*con*) mutations (van Eeden et al., 1996). Using the *con* mutant alleles and morpholino oligonucleotides to eliminate *disp1* activity, we show that like its murine counterpart (Ma et al., 2002; Caspary et al., 2002; Kawakami et al., 2002), zebrafish *disp1* is essential for normal Hh signalling. By contrast, we show that *disp2* is dispensable for Hh activity. In addition, we present in vivo evidence that the dependence of Shh activity upon Disp1 is specific to its lipid-modified form.

Materials and methods

Zebrafish husbandry

All fish strains were maintained in a continuously recirculating system with 14 h day and 10 h light cycle at 28.5°C. All control and manipulated embryos were grown at the above temperature. Embryos were staged at according to Kimmel et al. (1995).

Mapping and linkage testing

Fish heterozygous for the both *con* alleles were outcrossed to the WIK strain and the pooled DNA from F2 homozygous mutants and siblings was analysed using SSLPs and SSCPs.

To map the *disp1* and *disp2* loci, we used the Zebrafish Radiation Hybrid Panel (Research Genetics) and the following PCR primers:

Disp1	stu1 TCAGAATTTCAAGTGTTTCGCTCCT
	stu2 TGGAGCAAGGCGGTACGCCACTCCATA
Disp2	zdisp5 ATCCTGCCTTGTGGAGTGGTGAAATTCCGA
	zdisp8 ATTCGACCCTCTGAATCAAACCGTAAGCCT

with an annealing temperature of 60°C for 35 cycles. Reactions were performed in triplicate.

Cloning disp1 and disp2 sequences

A short *disp1* sequence (cDNA; fw61e03.y1) was obtained from the EST database: http://www.ncbi.nlm.nih.gov/blast/Blast.cgi and full-length cDNA was obtained from RNAs of 30 hours post fertilisation (hpf) wild-type embryos by SMART RACE cDNA amplification Kit (Clontech). Disp2 sequences were obtained from whole zebrafish genome shotgun sequence databases http://www.sanger.ac.uk/Projects/D_rerio/). cDNAs were synthesised using several sets of primers. Fragments were subcloned into the pCR2.1-vector using the TOPO kit (Invitrogen) and sequenced in both directions.

Sequencing of the genomic disp1 gene from con mutants

Fragments of all *disp1* exons from homozygous *con* mutant DNAs were PCR amplified and mutations were mapped by genomic DNA direct sequencing. Mutations were also confirmed by *con* heterozygous DNA direct sequencing.

Embryo injections

Wild-type or mutant embryos were injected at the 1- to 2-cell stage with capped mRNA, or morpholino oligonucleotides using a micromanipulator and microinjector (Narishige). *disp1* cDNA encoding amino acids 1 to 1195 was cloned as a fragment into the *Xho*I and *Not*I sites of pSP64TXB (Tada and Smith, 2000). The resulting plasmid, pSP64T-Disp1 was linearised and transcribed using the "SP6 mMessage mMachine" kit (Ambion). 3 nl of in vitro synthesised mRNA was injected into embryos at the 1 cell stage.

Full-length Shh mRNA was generated as previously described (Krauss et al., 1993). A construct encoding an unmodified form of ShhN (Shhu) was generated by introduction of a stop codon immediately N-terminal to the autocleavage site into the full-length cDNA. This construct was sub-cloned into *Not*I and *Xho*I site of pSP64TXB vector.

Two partially overlapping antisense morpholino oligonucleotides for each of *disp1* and *disp2* were purchased from Gene Tools, LLC. The sequences of the morpholinos, which were designed by the manufacturer are:

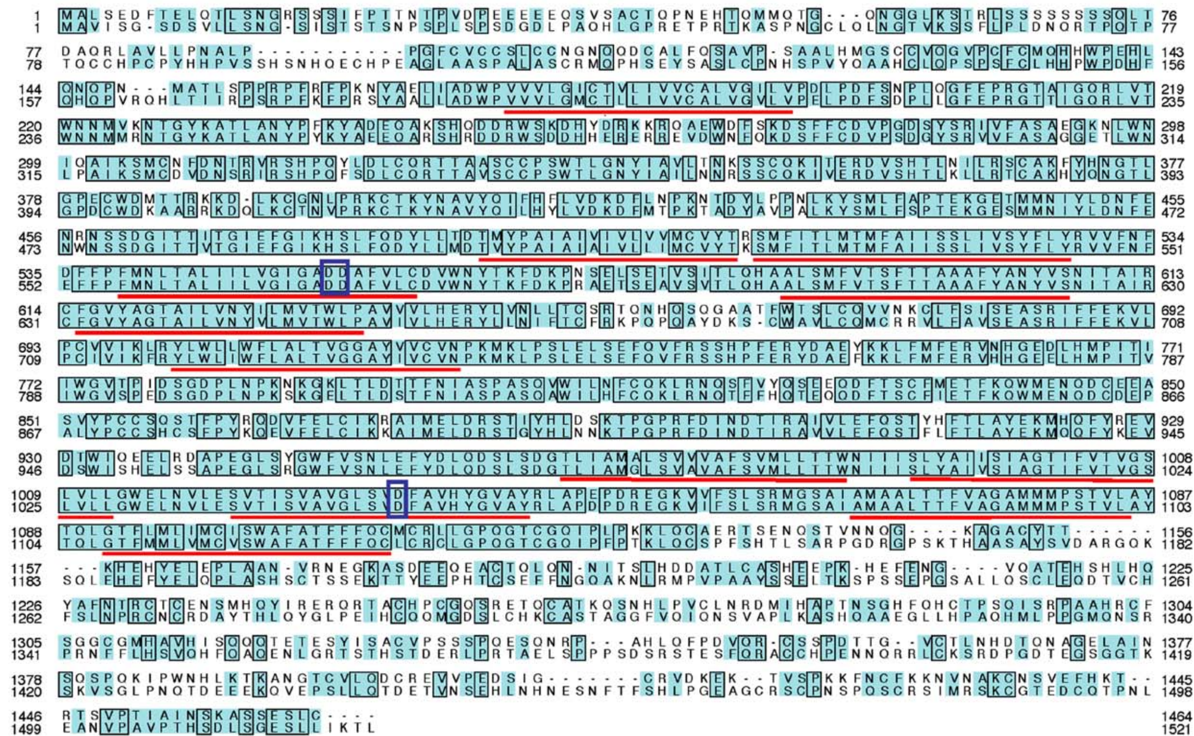
Disp1	AGTGAAGTCCTCGCTCAGAGCCATG
	ATCATTGTATGTACTGACCTGGTAC
	ATACTACATACCCGCGTATTGTCGA
Disp2	TGGACCCGCTTTCCATGCTGGAGTA

The oligonucleotides were dissolved in water at a stock concentration of 2 mM (16.7 mg/ml).

About 4–5 nl of 1:2, 1:4 and 1:10 dilutions in water, respectively were injected into 1-cell stage embryos.

Immunohistochemistry and microscopy

Antibody labellings were done essentially as previously described (Roy et al., 2001a) Eng proteins were



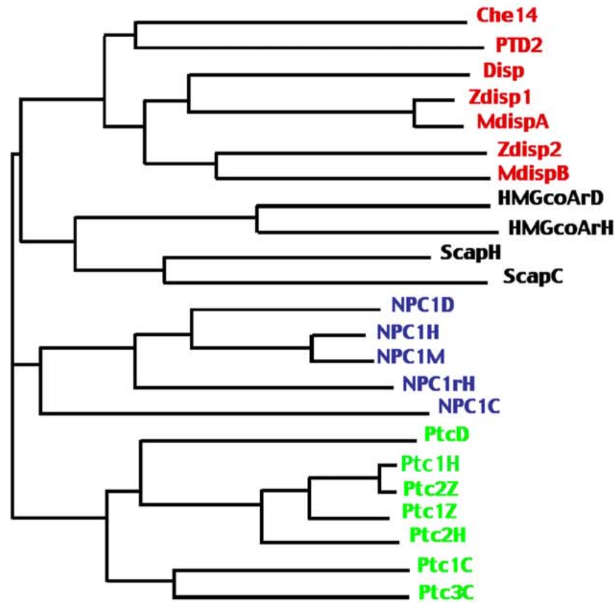


Fig. 1. (a) Alignment of the zebrafish Disp1 (upper) and mouse DispA (lower) amino acid sequences. Transmembrane domains are underlined in red and amino acid residues critical for mouse DispA function are indicated by blue boxes. Phylogeny showing the relationships between members of the five major SSD family members, Patched (Ptc), Dispatched (Disp), SREBP cleavage activating protein (SCAP) and HMG CoA reductase (HMGCoAr) as well as the *Caenorhabditis elegans* Che 14, PTD, Ptc-related Disp-like and human NPC1rH, NPC-related protein. C, *C. elegans*; H, *Homo sapiens*; D, *Drosophila melanogaster*; M, *Mus musculus*; Z, zebrafish; (b) radiation hybrid mapping of the zebrafish *disp1* and *disp2* genes. *disp1* maps to LG20, close to z1534 and to fa28a07.s1, corresponding to the *Hlx*1 gene. The human *Hlx*1 gene similarly maps close to *DISPA* on chromosome 1q. *disp2* maps to LG17, close to the z11341 marker and to wz5785, corresponding to the *IVD* gene that in humans maps close to *DISPB* to Chromosome 15 q.

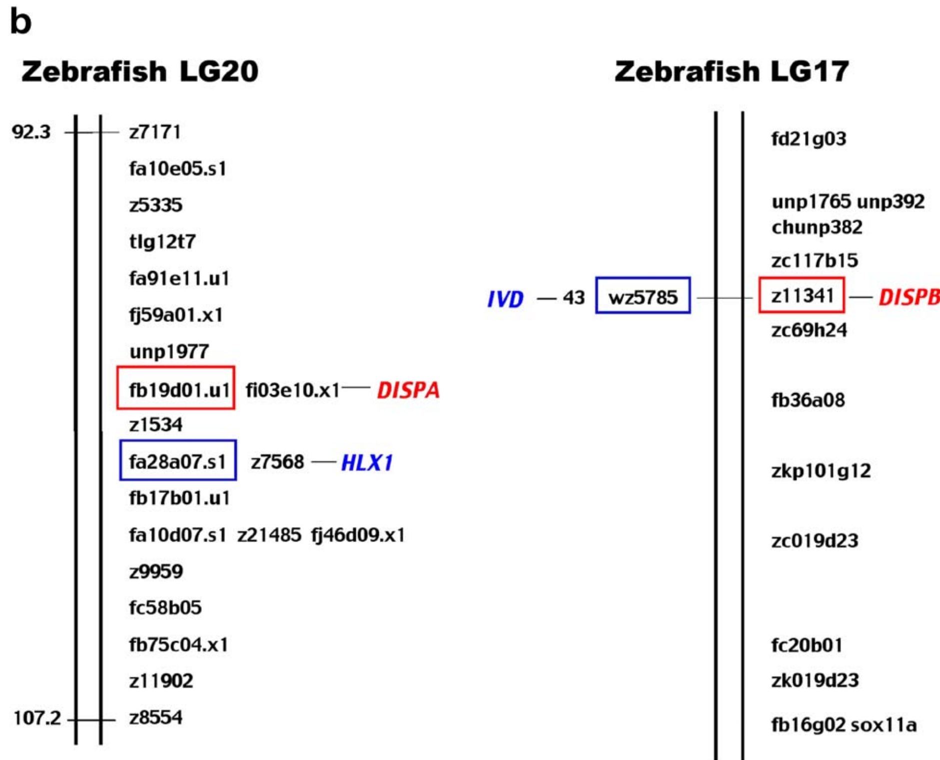


Fig. 1 (*continued*).

detected with mAb 4D9 and Islet 1 with 40.2D6 (or 39.4D5) (Developmental Studies Hybridoma Bank, University of Iowa); Prox1 was detected using human anti-Prox1 antiserum (Glasgow and Tomarev, 1998). Stainings were either developed colorimetrically using the Vectastain Elite Kit (Vector labs) or using appropriate fluorophore coupled secondary antibodies or tertiary conjugates (Jackson Immuoresearch). Embryos were cleared in 70% glycerol, mounted on bridged coverslips and analysed using a Zeiss Axioplan microscope or a Leica SP confocal microscope. Captured images were assembled using Adobe Photoshop.

Results

Identification, isolation and mapping of zebrafish dispatched homologues

The *Drosophila* Disp protein is characterised by possession of a sterol-sensing domain (SSD), a motif found in several multipass membrane-spanning proteins including the Hedgehog receptor Patched (Ptc), the nematode trafficking protein Che14 and NPC1, a protein implicated in cholesterol transport identified through its association with the human Niemann Pick syndrome (Kuwabara and Labouesse, 2002). In the course of a screen for SSD containing proteins in the zebrafish, we identified two sets of DNA sequences encoding SSDs with significant sequence identity to that of *disp*. Full-length cDNAs for each gene were assembled by PCR amplification of embryonic cDNA and the nucleotide sequences of each determined (GenBank accession numbers: AY512779 and AY512780). The longest open reading frames of each cDNA encode predicted proteins of 1465 aa and 1476 aa, respectively, both of which have 12 putative transmembrane domains. Alignment of the sequences of both cDNAs with that of *Drosophila* Disp (see Supplementary material) revealed 46% and 33% identity, respectively, between the transmembrane regions of the *Drosophila* and fish proteins and 43% between the two fish proteins. The overall identity between the entire sequence of the fish and fly proteins, however, is less than 10%.

Alignment with the recently characterised murine Disp proteins (Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002) indicates that they most likely represent the zebrafish orthologues of DispA and DispB (Fig. 1a and Supplementary material). The genomic location of the *disp1* and *disp2* genes was determined by radiation hybrid (RH) mapping using the TN51 RH panel. This revealed that *disp1* is located on LG20 close to the marker fb19d01.u1, whereas *disp2* maps to LG17close to the marker z11341 (Fig. 1b). The regions to which both genes map show significant levels of synteny to the chromosomal regions around the

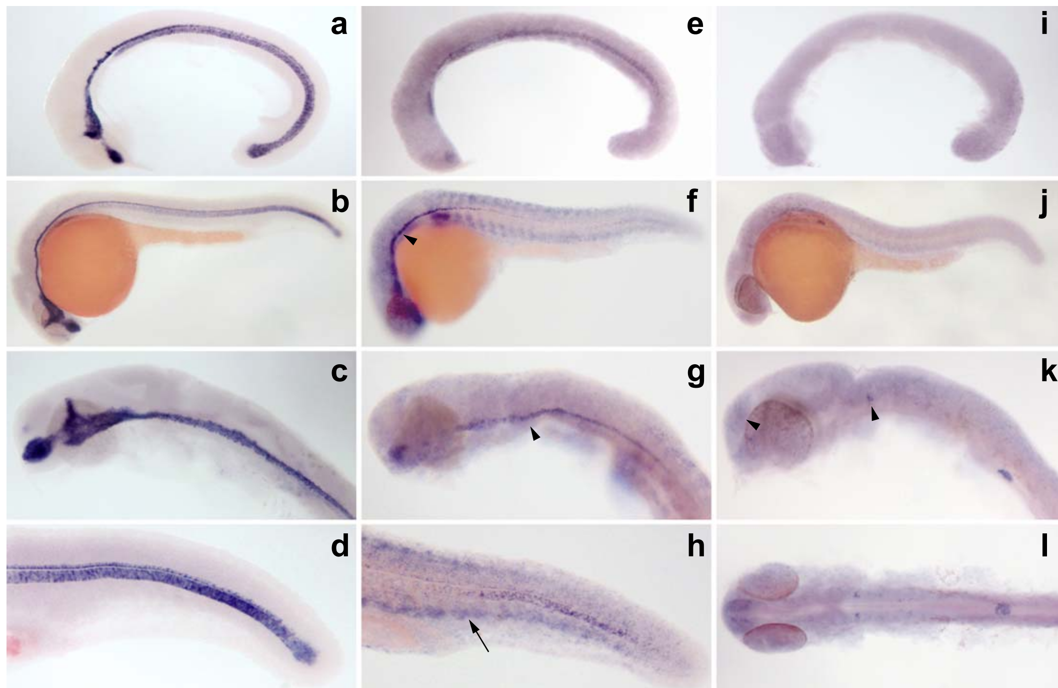


Fig. 2. Expression of *disp1* and *disp2* during embryogenesis. The expression of both genes at 18 and 24 hpf is compared to that of *shh* (panels a–d) at the same stages. Transcripts of both genes can be detected throughout the embryo at both stages shown: *disp1* (panels e–h) accumulates at elevated levels in the notochord at 18 h and subsequently in the ventral floor of the brain (arrowheads in f, g) and edges of the myotome (h). *disp2* (panels i–l) accumulates at elevated levels in a discrete region of the gut and in cells in the telecephalon and ventral hindbrain (arrowheads in k) at 24 h.

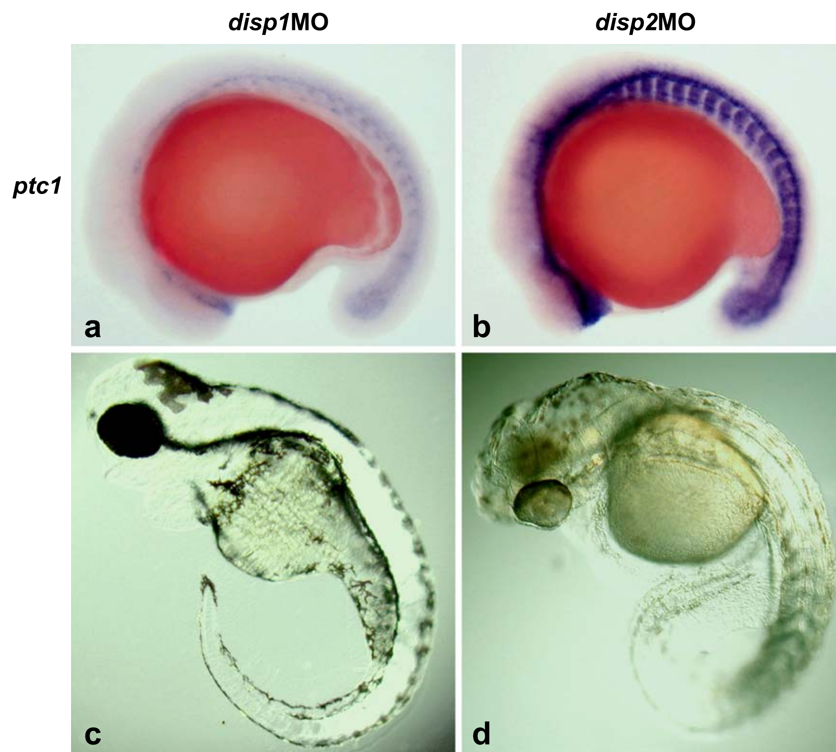


Fig. 3. *disp1* and *disp2* are essential for normal development but *disp2* is dispensable for *ptc1* expression. Expression of the Hh target gene *ptc1* is significantly diminished in *disp1* morphants at 18 hpf (a) but is unaffected in *disp2* morphants (b). Embryo injected with morpholino oligonucleotides specific for *disp1* or *disp2* shows similar ‘curly down’ phenotypes at 48 hpf (c, d). *disp2* morphants are completely immotile at this stage, *disp1* morphants exhibit blocky somites typical of ‘you-type’ mutants.

loci of the human *DISPA* and *DISPB* genes (not shown; see also Ma et al., 2002).

Expression of disp genes during zebrafish embryogenesis

The spatiotemporal expression patterns of *disp1* and *disp2* were investigated by in situ hybridisation of DIG labelled probes to embryos fixed at different developmental stages (Figs. 2e–l). Both transcripts accumulate at relatively low levels compared to those of other genes encoding Hh pathway components, for example *sonic hedgehog* (shh) itself (Figs. 2a–d). Transcripts are detectable from fertilisation onwards (data not shown) and are ubiquitously distributed throughout the embryo at all stages examined (up to 36 hpf). Some modulation of transcripts is, however, apparent in both cases; levels of *disp1* are elevated specifically in the notochord at 18 hpf and subsequently in the ventral floor of the brain as well as at the dorsal and ventral margins of the myotome (Figs. 2e–h). By contrast, *disp2* mRNA accumulates at higher levels specifically in cells of the telecephalon and ventral hindbrain as well as in a discrete patch of cells in the gut at 24 hpf (Figs. 2i–l).

Inhibition of disp gene functions by antisense morpholino oligonucleotides

To investigate the possible functions of the Disp proteins in the zebrafish embryo, morpholino oligonucleotides (MOs) directed against sequences around the predicted translational start site of each mRNA were injected into newly fertilised embryos (Nasevicius and Ekker, 2000). In both cases, dramatic effects on gross morphology were observed, embryos injected with either morpholino exhibiting a characteristic downward curl of their tails at 48 hpf (Figs. 3c,d), an effect characteristic of embryos in which Hh signalling is compromised (van Eeden et al., 1996). To determine whether either or both morphant phenotypes reflect a disruption of Hh signalling, injected embryos were fixed at 24 hpf and hybridised with probes for the Hh target gene *ptc1* (Concordet et al., 1996). Embryos injected with the *disp1*MO showed a significant reduction in the levels of *ptc1* mRNA accumulation (Fig. 3a), whereas expression of *ptc1* appeared unaffected in *disp2* MO injected embryos (Fig. 3b).

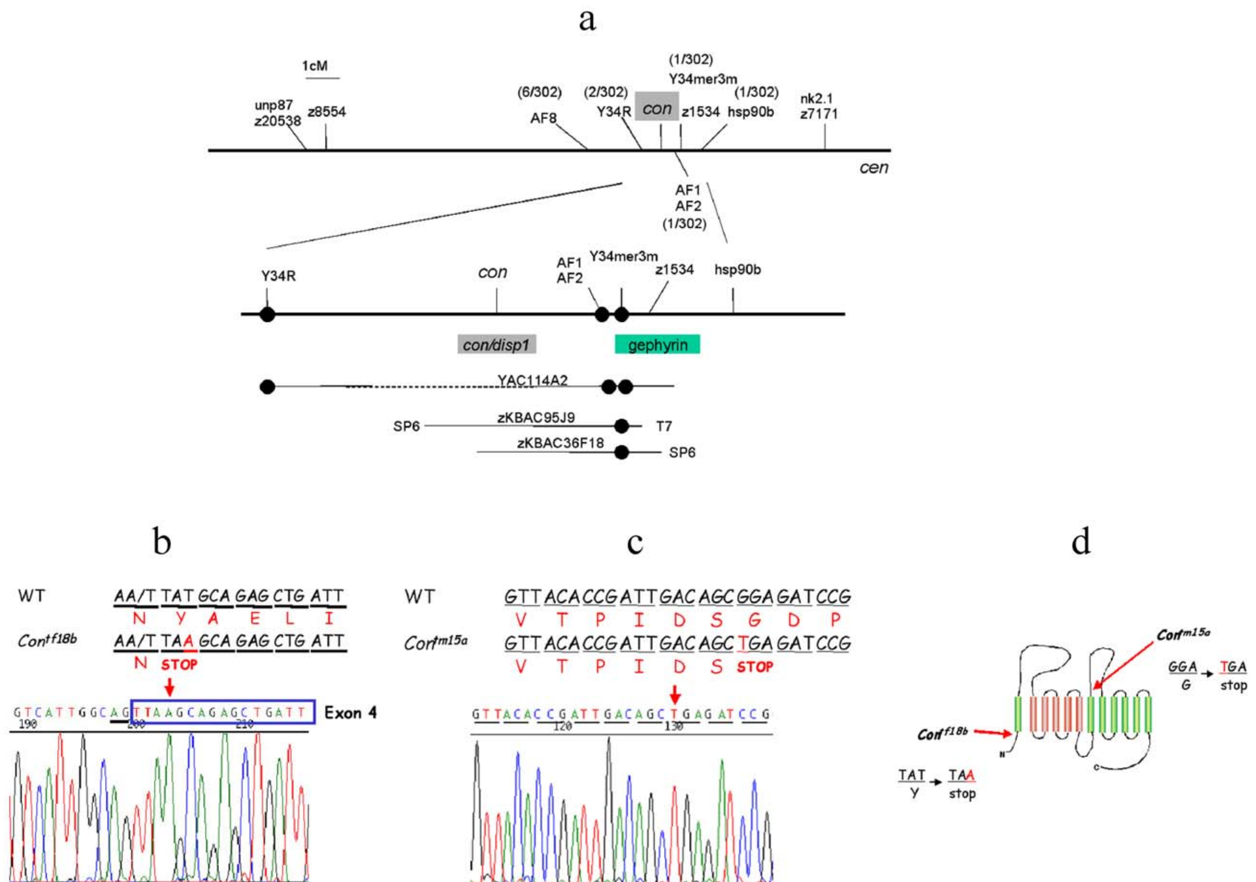


Fig. 4. Genetic mapping and sequences analysis of *chameleon* mutations. (a) The results of 302 meioses in *con* heterozygous fish were analysed by SSCP and SSLP. The locus was located between the markers Y34R and Y34mer3m, close to the z1534 marker (see Fig. 1b). (b, c) Sequence traces of *disp1* from the con^{tf18b} and con^{tm15a} alleles. These reveal that both are associated with single base changes that introduce stop codons predicted to truncate the protein as indicated in the schematic representation in panel (d).

disp1 mRNA rescues the chameleon mutant phenotype

The phenotypic similarity between the *disp1* MO injected embryos and embryos homozygous for "you-type" mutations prompted us to investigate whether injection of *disp1* mRNA could rescue any of the latter. Specifically, we focused on the *you* and *con* mutants, since neither of these have been characterised at the molecular level. Injection of *disp1* mRNA into *you* homozygous embryos had no effect on their phenotype; by contrast, *con* homozygotes injected with the same mRNA showed a complete suppression of the mutant phenotype (n = 40). This implies that *disp1* acts either downstream of the *con* gene or alternatively is itself mutated by the *con* mutations. Injection of *disp1* mRNA into embryos lacking Shh activity, derived from the progeny of *sonic-you* (*syu*) heterozygotes (Schauerte et al., 1998), had no effect on the expression of their mutant phenotype (data not shown).

Chameleon mutant alleles map to the disp1 locus and are associated with stop codons in the disp1 coding region

To investigate whether the *con* gene does in fact correspond to *disp1*, we used meiotic recombination analysis to map the *con* locus. A total of 302 meioses were analysed; two recombinants were detected between the *con* allele and the marker Y34R and 1 between the marker Y34mer3m, locating *con* to the same region of LG20 as *disp1* (Fig. 4a). Consistent with this, the finished genome sequence has subsequently revealed Y34mer3m and *disp1* to be approximately 50 kb apart http://www.sanger.ac.uk/Projects/D_rerio/).

The identity of *con* and *disp1* was confirmed by sequencing the *disp1* coding region from embryos homozygous for either mutant *con* allele. The con^{tf18b} allele is associated with a T to A transversion that introduces a stop codon at position 164 (Fig. 4b). This is predicted to result in a truncation of the protein before the first transmembrane domain (Fig. 4d) and should therefore result in a complete loss of Disp1 function. The con^{tm15a} allele is associated with a G to T transversion that introduces a stop codon at position 781, at the beginning of the second large extracellular loop (Figs. 4c, d).

Inactivation of Disp1 function disrupts Hedgehog signalling activity

To assess the effect of loss of Disp1 function on processes that require Hh signalling, we first analysed con^{tf18b} mutant embryos for the expression of a variety of genes marking distinct domains within the ventral neural tube. These spatially restricted domains are known to be established in response to Hh signals emanating from the axial midline (Briscoe and Ericson, 2001). Expression of *nkx2.2*, which is normally restricted to cells flanking the midline floor plate (FP) (Barth and

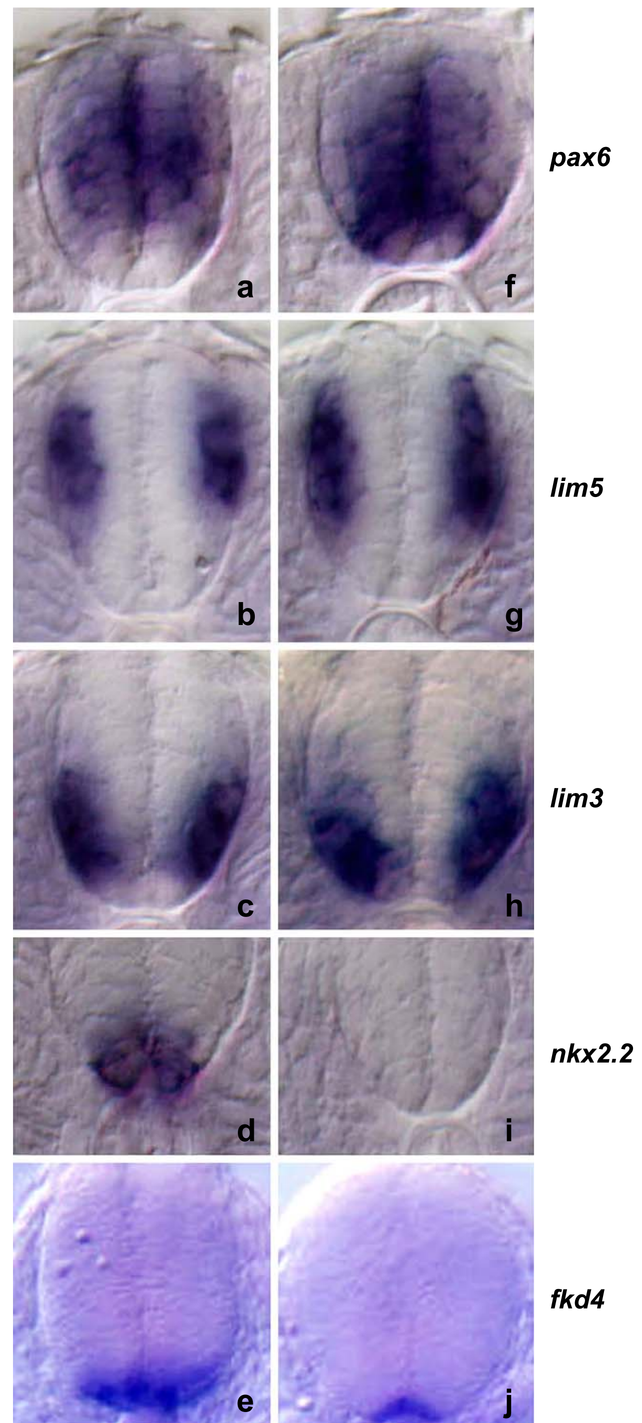


Fig. 5. Altered expression of Hh target genes in the neural tube of *con* mutants Thick sections through the trunk region of 24 hpf wild type (a–e) and *con* homozygous mutant (f–j) embryo hybridised with probes for genes expressed at different dorsoventral levels of the neural tube. Note the ventral shift of *pax6*, *lim5* and *lim3* expression domains in mutant embryos. Expression of *nkx2.2* which is confined to the lateral floor plate (LFP) in wild type (d) is completely eliminated in *con* mutants (i). *fkd4* is expressed in the LFP and medial floor plate (MFP) in wild type (e): in *con* mutants, only the MFP expression persists (j).

Wilson, 1995) is completely absent from the ventral neural tube of con^{tf18b} homozygotes (Figs. 5d, i). Expression of *fkd4* which is normally expressed in these so-called lateral FP cells as well as in the medial FP (Odenthal et al., 2000) is still detectable in con^{tf18b} homozygotes, but is restricted to the medial FP (Figs. 5e, j). The *pax6*, *lim3* and *lim5* genes are all normally expressed in distinct domains dorsal to the lateral FP. In each case, these domains are shifted ventrally in con^{tf18b} homozygous embryos (Figs. 5a–c, f–h).

We also noted that in *con* mutant embryos, expression of *nkx2.2* is absent from the region of the gut corresponding to the pancreatic primordium (Biemar et al., 2001) (Figs. 6b, e), consistent with our previous demonstration that Hh signalling is required for pancreas induction in the zebrafish embryo (Roy et al., 2001b). Expression of Islet 1, by contrast, is reduced but not absent (Figs. 6c, f) as is the region of elevated *pdx1* expression that distinguishes the pancreas from the duodenum in wild type embryos (Roy et al., 2001b) (Figs. 6a, d). However, injection of the *disp1*morpholinos into *con* mutant embryos resulted in the complete elimination of Islet 1 and elevated *pdx1* expression (Figs. 6g, h), a phenotype resembling that of embryos lacking smoothened activity (Roy et al., 2001b) (Figs. 6j, k). This latter finding implies that maternally derived *disp1* mRNA can partially compensate for the loss of zygotic *disp1* function in *con* mutant embryos (see Discussion).

We next analysed the differentiation of the myotome in con^{tf18b} mutant embryos. Three distinct muscle cell fates have been shown to arise in response to different levels of Hh activity, thus providing a sensitive in vivo assay for the strength of Hh signalling (Wolff et al., 2003). The first cells to become committed to a myogenic fate are the adaxial cells, so-called because they flank the notochord in the presomitic mesoderm. These cells express *myoD* in response to Hh signalling from the notochord. In the complete absence of Hh signalling, *myoD* fails to be expressed in adaxial cells (Barresi et al., 2000; Chen et al., 2001). In con^{tf18b} mutants, by contrast, expression of *myoD* is initiated but subsequently diminishes in more rostrally located cells. The same effect is seen in wild type embryos injected with *disp1* MO (data not shown); however, injection of the same concentration of MO into *con* mutant embryos results in complete suppression of *myoD* expression (Fig. 7g).

Adaxial cells give rise to the slow muscle lineage (Devoto et al., 1996), as reflected by their Hh-dependent expression of the slow Myosin Heavy Chain isoform encoded by the *slowMyHC* gene (Blagden et al., 1997; Lewis et al., 1999; Fig. 7b). In con^{tf18b} mutant embryos, expression of *slowMyHC* is suppressed in the adaxial cells in the presomitic mesoderm, but is detectable, albeit at much reduced levels as somites form and mature (Fig. 7e). The slow muscle lineage gives rise to two distinct populations of fibres—the superficial slow fibres (SSFs) and the muscle pioneers (MPs); the latter are induced by high levels of Hh activity. All slow muscle fibres express the homeodomain protein Prox1 (Glasgow and Tomarev, 1998; Roy et al., 2001a) but only the MPs express the Eng homeodomain proteins (Ekker et al., 1992; Roy et al., 2001a) (Fig. 7c). Homozygous con^{tf18b}mutants and *disp1* morphants have reduced numbers of SSFs, as judged by the expression of Prox1 and *slowMyHC*, and lack nearly all MPs. Injection of *disp1* MO into *con* mutants results in a further suppression of *slowMyHC* expression (Fig. 7h) and a complete loss of differentiated slow fibres, as evidenced by the absence of Prox1 expression (Fig. 7i). Eng proteins are also expressed in medially located fast muscle fibres in response to submaximal levels of Hh signalling (Wolff et al., 2003). These so-called medial fast fibres (MFFs) are also absent from con^{tf18b} homozygotes (Figs. 7f, i).

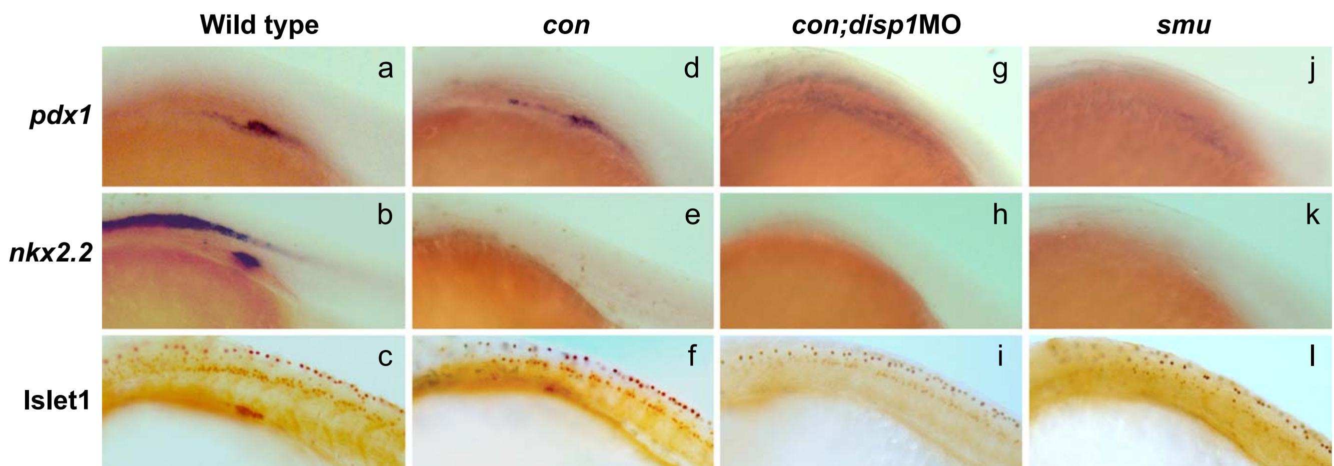


Fig. 6. Aberrant pancreas development in the absence of Disp1 function. In wild type embryos at 24 hpf, the pancreatic primodium is marked by elevated levels of *pdx1* expression (a) and by expression of *nkx2.2* (b): at this stage, endocrine β cells can also be distinguished by their expression of Islet 1 protein (c). In *con* homozygotes, the elevated levels of *pdx1* persist (d) but *nkx2.2* expression is lost (e), while the number of Islet1-positive cells is reduced (f). Injection of *disp1* MOs into *con* homozygous embryos results in a marked reduction of *pdx1* expression levels (g) similar to that seen in *smu* mutants (j), as well as the loss of *nkx2.2* (h) and complete elimination of Islet-1-positive cells (i).

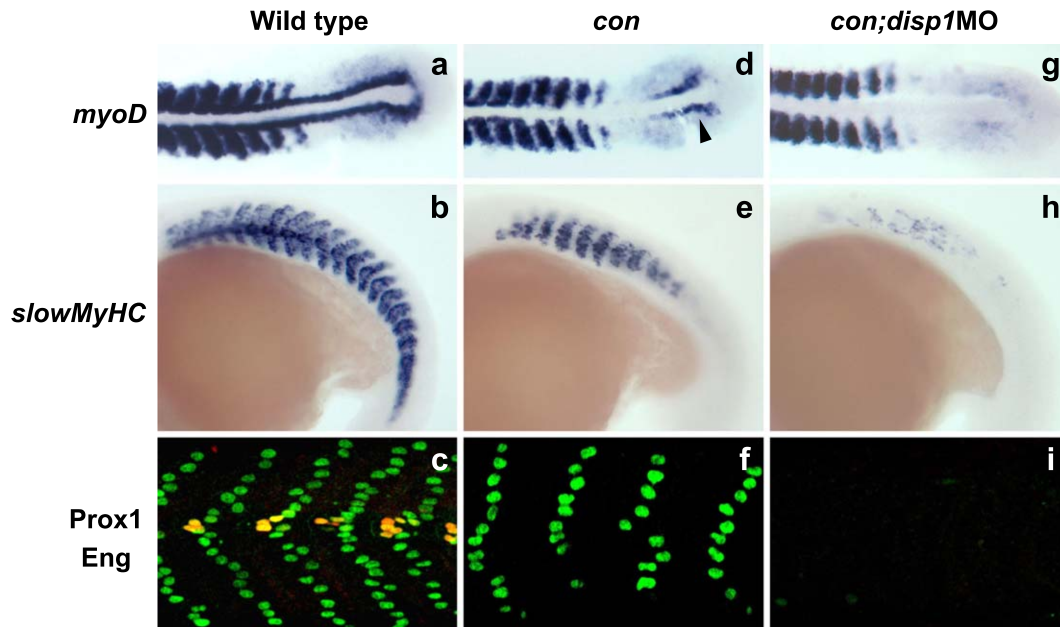


Fig. 7. Hh-dependent myogenesis is disrupted by loss of Disp1 activity. Adaxial cells express *myoD* in response to midline-derived Hh activity (a) and give rise to the cells of slow muscle lineage, characterised by their expression of *slow MyHC* (b) and the transcription factor Prox1 (c) (green). A subset of slow muscle cells, the muscle pioneers (MP) also express the Eng homeodomain proteins (c: red signal-appearing yellow in the merged image). In *con* mutant embryos, the adaxial expression of *myoD* is initiated close to the tail bud (arrowhead in panel d) but is then lost. There is a significant reduction in the levels of *slow MyHC* expression (e) and a concomitant reduction in the numbers of differentiated slow muscle fibres at 24 hpf (f). Note also the complete absence of Eng-positive MP cells. *con* mutants injected with *disp1* MOs exhibit a complete suppression of adaxial *myoD* expression (g) and a severe reduction in *slow MyHC* gene expression (h). Such embryos are devoid of all Prox1 and Eng expression at 24 hpf (i) indicative of a complete absence of slow muscle fibres.

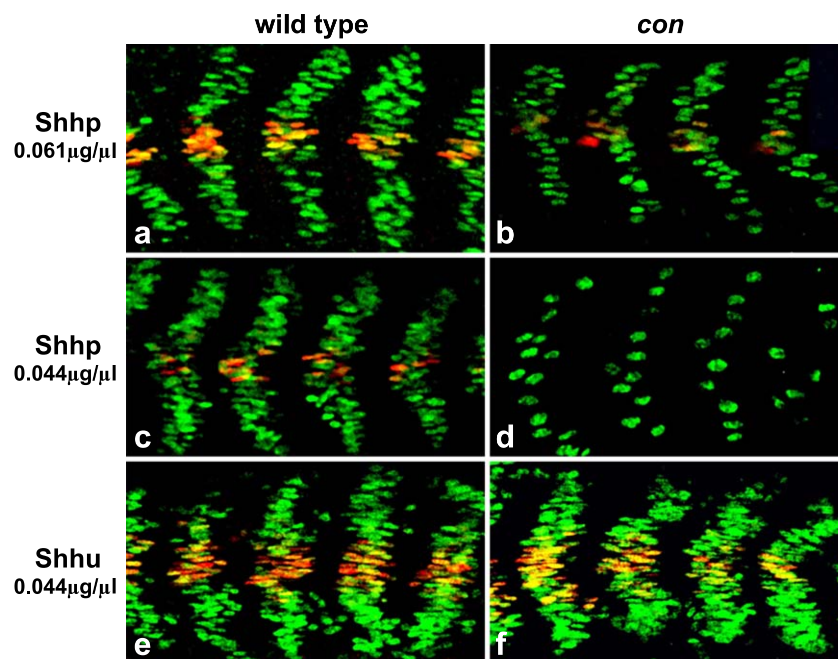


Fig. 8. Disp1 is specifically required for the activity of cholesterol-modified Shh. (a, b) Prox1 (green) and Eng (red) expression in the myotome of wild-type (a) and *con* mutant (b) embryos injected with 0.061 μg/μl full-length *shh* mRNA. Note the increased numbers of Prox1-positive cells in both cases (cf. Fig. 7 panels c and f) and the restoration of Eng expression in the *con* mutants. (c) Wild type embryo injected with 0.044 μg/μl full-length *shh* mRNA showing similar increase in Prox1- and Eng-positive cells. By contrast, *con* mutant embryo (d) shows no response to this lower concentration of message. Wild type (e) and *con* mutants (f) embryos injected with 0.044 μg/μl truncated Shh showing identical increases in Prox1- and Eng-positive cells.

Disp1 function is specifically required for full activity of cholesterol-modified Shh

Studies in *Drosophila* have implicated Disp specifically in the release of lipid-modified Hh protein from secreting cells (Burke et al., 1999). To investigate whether Disp1 is similarly required for release of lipid-modified Hh proteins in the fish embryo, we compared the response of *con*^{tf18b} mutant embryos to ectopically expressed processed (Shhp) and un-modified (Shhu) protein. mRNA encoding full-length or C-terminally truncated Shh was transcribed in vitro and injected at varying concentrations into 1-cell stage embryos derived from *con*^{tf18b} parents. Injected embryos were fixed at 24 hpf and the differentiation of the different Hh-dependent muscle cell types analysed by staining with antibodies against Prox1 and Eng. Wild type sibling embryos showed a transformation of their entire myotome to the slow twitch muscle identity following the injection of mRNA encoding either Shhu or Shhp at 0.061 and 0.044 μg/μl, with increased numbers of MPs being induced by the higher concentration of Shhp (Figs. 8a, c, e). The response of *con*^{tf18b} homozygotes to Shhp, however, was significantly attenuated compared to wild type, with no detectable response at the lower concentration (Figs. 8b, d). Simultaneous injection of the *disp1* MO with the Shhp-encoding message, reduced, though did not eliminate, the response of the *con* homozygotes to the higher concentration of full-length *shh* mRNA (data not shown). By contrast, the response of *con*^{tf18b} embryos to Shhu was indistinguishable from wild type following injection of the truncated *shh* mRNA at both concentrations (Fig. 8f and data not shown).

Discussion

Large scale mutant screens in the zebrafish have yielded several groups of mutations with similar phenotypes (Driever et al., 1996; Haffter et al., 1996). Members of one such group, the you-type mutants, were isolated from their distinctive somite morphology and hypothesised to disrupt the signalling between the axial and paraxial mesoderm mediated by Sonic Hedgehog (van Eeden et al., 1996). Molecular analyses have revealed that two of these mutations, *sonic you* (*syu*) and *you-too* (*yot*), disrupt the genes encoding the Shh signal and the transcription factor Gli2, respectively (Karlstrom et al., 1999; Schauerte et al., 1998). Subsequently, an additional mutation with a you-type phenotype has been identified and shown to disrupt a third component of the Hh pathway, the serpentine protein smoothened (Chen et al., 2001; Varga et al., 2001). Here, we have shown that one of two zebrafish homologues of Dispatched—which in *Drosophila* is involved in the secretion of Hh from secreting cells—is inactivated by mutant alleles of *con*, another member of the you-type group.

Recent studies in *Drosophila* have identified Disp-dependent and -independent modes of signalling (Gallet et al., 2003). Thus, in the embryo, transcription of the target genes *wingless* (*wg*) and *patched* (*ptc*) in cells anterior to the Hh-expressive domain is abolished in the absence of *disp*, whereas transcription of *rhomboid* posterior to Hh-expressing cells is unaffected. This differential requirement for Disp appears to correlate with the route by which the Hh protein exits the cell, with Disp function apparently exclusively required for apical secretion of the protein. At first sight, the *con* phenotype might suggest that a similar dual mechanism of Hh protein release operates in the zebrafish: for instance, while MP induction is abolished in *con* mutants, the differentiation of the superficial slow muscles is only partially suppressed. This is unlikely to be due to the hypomorphism of the mutant alleles since both are associated with stop codons that cause premature termination of translation. Injection of a *disp1* MO into *con* homozygotes, however, results in a significant enhancement of the mutant phenotype, similar to that typical of *smu* or *yot* homozygotes in which all Hh signalling is effectively abolished. This presumably represents the effects of eliminating all zygotic and maternally derived Disp1 activity from the embryo; in line with this, *disp1* transcript is detectable from fertilisation onwards, indicating that it is indeed expressed both maternally and zygotically.

It follows from these findings that endogenous Hh signalling in the zebrafish embryo is critically dependent upon Disp1 activity. In this respect, the secretion of Hh proteins in the zebrafish and mouse appears to be under similar regulation since in the latter, targetted inactivation of *mDisp1* also results in a phenotype resembling that caused by complete loss of Hh signalling activity (Kawakami et al., 2002; Ma et al., 2002). In *Drosophila*, apical targetting of Hh correlates with the incorporation of the protein into distinct lipid microdomains, consistent with the specific requirement for Disp function for secretion of lipid-modified Hh (Gallet et al., 2003). Indeed, it had previously been shown that unmodified Hh can be effectively released from cells deficient in Disp function in the wing imaginal disc (Burke et al., 1999). Using the induction of different muscle cell types as an assay, we investigated whether Disp1 is similarly specifically required for the secretion of lipid-modified Hh in the zebrafish. Our results are consistent with such a mechanism: thus, although high levels of exogenous Shh are able to elicit a response in *con* mutant embryo, at lower levels, the response becomes significantly attenuated relative to wild type. By contrast, similar levels of unmodified Shh appear equally efficient in inducing slow muscle specification in either wild type or *con* mutants. These findings provide in vivo confirmation of the conclusions of Ma et al. (2002) who showed that murine DispA (but not DispB) can increase the efficiency of secretion of lipid-modified Shh by transfected tissue culture cells. The fact that Shhp can elicit a response when overexpressed, even when both zygotic and maternal Disp1 activity is abrogated, may reflect the ability of some protein to exit the cell when it is present at very high levels. It may also be

relevant that in the mouse, activation of Hh target genes in tissues that express high levels of Hh proteins—such as the notochord—still occurs in the absence of DispA activity (Caspary et al., 2002; Ma et al., 2002). Thus, in the fish, high-level ectopic Shh expression might act in an autocrine manner that is similarly independent of Disp1 activity.

By contrast to Disp1/*con*, our morpholino knock down experiments reveal that the second Disp homologue, Disp2, though necessary for normal embryonic development, is dispensable for Hh signalling in the zebrafish. This is in line with the finding that the murine Disp2 orthologue is unable to rescue the *Drosophila disp* mutation or potentiate secretion of Shh by tissue culture cells (Ma et al., 2002). Further studies will be required to elucidate the cellular role of Disp2.

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