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There was an error published in *Development* **138**, 755-765.

On p. 756, the *actin:GAL4* transgenic strain was erroneously attributed to Scheer and Campos-Ortega (1999). This line [which now appears on ZFIN with designation *Tg(actc1b:GAL4)*^{i269}] should have been attributed to Sudipto Roy and Franco di Giovine who generated it in the laboratory of Philip W. Ingham.

The authors apologise to readers for this mistake.

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Integration of Hedgehog and BMP signalling by the *engrailed2a* gene in the zebrafish myotome

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SUMMARY

Different levels and timing of Hedgehog (Hh) signalling activity have been proposed to specify three distinct cell types in the zebrafish myotome. Two of these, the medial fast-twitch fibres (MFFs) and the slow-twitch muscle pioneers (MPs) are characterised by expression of *eng1a*, *-1b* and *-2a* and require the highest levels of Hh for their specification. We have defined a minimal *eng2a* element sufficient to drive reporter expression specifically in MPs and MFFs. This element binds both Gli2a, a mediator of Hh signalling, and activated Smads (pSmads), mediators of bone morphogenic protein (BMP) signalling, in vivo. We found a strict negative correlation between nuclear accumulation of pSmad, and *eng2a* expression in myotomal cells and show that abrogation of pSmad accumulation results in activation of *eng2a*, even when Hh signalling is attenuated. Conversely, driving nuclear accumulation of pSmad suppresses the induction of *eng* expression even when Hh pathway activity is maximal. Nuclear accumulation of pSmads is depleted by maximal Hh pathway activation. We show that a synthetic form of the Gli2 repressor interacts with Smad1 specifically in the nuclei of myotomal cells in the developing embryo and that this interaction depends upon BMP signalling activity. Our results demonstrate that the *eng2a* promoter integrates repressive and activating signals from the BMP and Hh pathways, respectively, to limit its expression to MPs and MFFs. We suggest a novel basis for crosstalk between the Hh and BMP pathways, whereby BMP-mediated repression of Hh target genes is promoted by a direct interaction between Smads and truncated Glis, an interaction that is abrogated by Hh induced depletion of the latter.

KEY WORDS: BMP, Smad, BiFC, Engrailed, Patched, Gli, Sonic Hedgehog, Myotome, Muscle pioneers, Zebrafish

INTRODUCTION

Members of the Hedgehog (Hh) family of signalling molecules regulate a wide variety of processes during embryonic development, acting in some contexts as morphogens to specify cell identities (reviewed by Ingham and McMahon, 2001). In the zebrafish embryo, Hh signalling activity plays a crucial role in the specification of muscle cell types (reviewed by Ingham and Kim, 2005). Adaxial cells, a subpopulation of the paraxial mesoderm, are allocated to the slow-twitch muscle lineage early in development (Devoto et al., 1996; Hirsinger et al., 2004) by activating expression of the transcription factor Prdm1a in response to midline-derived Hh signals (Baxendale et al., 2004; Liew et al., 2008; Roy et al., 2001; von Hofsten et al., 2008), whereas the remaining myogenic progenitors are fated to differentiate into fast-twitch myofibres (Blagden et al., 1997; Du et al., 1997). In addition to regulating this early binary (slow versus fast fibre type) cell fate decision, Hh signalling also induces distinct cell types within each of these lineages: muscle pioneers (MPs), a sub-set of the slow-twitch fibres, and medial fast fibres (MFFs), a sub-set of fast-twitch fibres (Currie and Ingham, 1996; Roy et al., 2001; Wolff et al., 2003). Both of these Hh-dependent cell types are characterised by their expression of the *engrailed* (*eng*) *1a*, *eng1b* and *eng2a* genes (Ekker et al., 1992; Roy et al., 2001; Thisse et al., 2001) as well as by the expression of other genes, such as *Wnt11* (Thisse et al., 2001).

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Several lines of evidence suggest that MPs and MFFs are specified in response to prolonged and/or elevated levels of Hh signalling activity. In particular, exposure of embryos to increasing levels of the Hh pathway inhibitor, cyclopamine, causes a progressive elimination, first of MPs and then of MFFs, with loss of all slow-twitch fibres occurring only at the highest inhibitor concentrations (Wolff et al., 2003). At the same time, there is evidence that the induction of the MP fate by Hh activity can be suppressed by bone morphogenic protein (BMP)-mediated signalling (Du et al., 1997; Kawakami et al., 2005) an antagonism that parallels the opposing influences of BMP and Hh signalling on cell identity in the vertebrate neural tube (Liem et al., 2000; Patten and Placzek, 2002); how these two opposing signals might be integrated to elicit distinct cellular outcomes has remained unclear.

Activation of gene expression in response to Hh signalling is mediated by members of the Gli family of zinc finger transcription factors (Alexandre et al., 1996; Ericson et al., 1997; Ruiz i Altaba, 1998; Sasaki et al., 1997; Stamataki et al., 2005). Gli1 is a transcriptional activator, whereas both Gli2 and Gli3 are bifunctional, acting either to promote or repress transcription. Several lines of evidence suggest that varying levels of extracellular Hh activity are translated into different intracellular levels of the activator (Gli^A) and repressor (Gli^R) forms of Gli (Aza-Blanc et al., 1997; Pan et al., 2009; Wang et al., 2000; Wen et al., 2010). In this view, increasing concentrations of ligand leads to an increase in the intracellular Gli^A/Gli^R ratio, which in turn causes a progressive activation of target genes. Consistent with this, synthetic forms of Gli proteins with differing levels of transcriptional activating activities have been shown to recapitulate the induction of different neuronal cell types elicited by varying levels of Shh activity (Stamataki et al., 2005). Of the few bona fide transcriptional Hh targets that have been characterised in detail, all have been found to

have sequences similar or identical to the Gli consensus-binding site upstream of their promoters (Agren et al., 2004; Alexandre et al., 1996; Dai et al., 1999; Gustafsson et al., 2002; Laner-Plamberger et al., 2009; Sasaki et al., 1997; Winklmayr et al., 2010). Genome-wide chromatin immunoprecipitation (ChIP) screens, however, have identified hundreds of putative Gli/Hh targets, a significant proportion of which are not associated with Gli consensus-binding sites (Vokes et al., 2007; Vokes et al., 2008).

Here we describe the identification of a minimal element of the *eng2a* gene sufficient to drive reporter gene expression in the MP and MFF cells. Our findings indicate that the threshold response of the *eng2a* promoter is determined by its integration of the relative levels of BMP and Hh activity; they also suggest a novel mode of crosstalk between the Hh and BMP pathways, whereby nuclear accumulation of pSmad is modulated by Hh pathway activity via its interaction with the repressor forms of Gli proteins.

MATERIALS AND METHODS

Zebrafish strains and husbandry

Adult fish were maintained on a 14 hour light/10 hour dark cycle at 28°C in the AVA (Singapore) certificated IMCB Zebrafish Facility. Zebrafish strains used were: *disp1*^{tf18b} (Nakano et al., 2004; van Eeden et al., 1996), *ptc1*^{hu1602,} *ptc2*^{tj222} (Koudijs et al., 2008) and *smo*^{b641}(Varga et al., 2001). Transgenic strains used were *actin:GAL4* (Scheer and Campos-Ortega, 1999) and *Tg*(*PACprdm1:gfp*)^{i106} (Elworthy et al., 2008).

DNA constructs

Bacterial artificial chromosome and promoter constructs

Recombineering of bacterial artificial chromosomes (BACs) was as previously described (Lee et al., 2001). The 4, 8 and 12 kb promoter constructs were generated by gap repair (Lee et al., 2001). The 10 kb-egfp construct was made by *Sca*I digestion and blunt-ended ligation into the miniTol2 vector (Urasaki et al., 2006).

Upstream activation sequence (UAS) bi-cistronic constructs

UAS-turboFP635 (*tRFP*): tRFP isolated from pturboFP635-N1 by *Bam*HI/*Afl*II digest was blunt-end ligated into UAS-Tol2. *UAS-MCS* (multiple cloning site): multiple cloning and polyA of pCS2+ isolated by *Bam*HI/*Not*I digest and blunt end ligated into *Eco*RI digested UAS-Tol2. *UAS-MCS-UAS-tRFP*: *Hin*dIII/*Spe*I digested UAS-tRFP, was blunt-end ligated into *Spe*I digested UAS-MCS. *caALK3*: *Eco*RI/*Not*I digested mouse caAlk3 cDNA was blunt end ligated into *Eco*RI digested UAS-Tol2. *Hin*dIII/*Spe*I digested UAS-tRFP was blunt end ligated into *Spe*I digested UAS-caALK3-Tol2. Shh, Dorsalin-1 and dnPKA were cloned into the bi-cistronic UAS vector using a similar strategy.

Bimolecular fluorescence complementation constructs

Coding sequences for truncated Gli2a, Smad1, and Smad5 were PCR amplified from 22 hour embryonic cDNA. *Gli1* cDNA (Karlstrom et al., 2003) was obtained from S. Roy (IMCB, Singapore). PCR products were purified, digested and cloned into the pCS2+ Vn, pCS2+ Vc constructs (Harvey and Smith, 2009).

Morpholino injection

bmpr1ba Exon1-Intron1 Splice MO (5′-GTAAAATGTTGACA-CTCACCCAAGT-3′) was injected at a concentration of 1 nmol/μl in 2 nl drop size per embryo (2 pmol per embryo).

Trans-regulation screen

The 2 kb muscle enhancer and a 1.5 kb promoter fragment of *eng2a* were excised from the enhanced green fluorescent protein (eGFP) reporter constructs, blunted using T4 polymerase and ligated with *Eco*RV-digested pGL4.10 (Promega, USA). Transregulation screening was performed as previously described (Souren et al., 2009) using a set of 1152 full-length medaka cDNAs. Raw firefly luciferase luminescence values were normalised against an internal *Renilla* luciferase control and against the average value of each 96-well plate. Cut-off value for repressors was 0.33 and threefold activation for activators.

ChIP assays

Whole-embryo chromatin from 14- to 16-somite stage (ss) AB embryos was extracted as previously described (von Hofsten et al., 2008). Anti-phospho-Smad1/5/8 antibody (Cell Signalling Technology, USA), rabbit polyclonal anti-Gli2a antibody (raised against the zebrafish Gli2a N-terminal fragment from 323-406 amino acids) and purified polyclonal IgG antibody (as negative control) were used for the ChIP assays. Immunoprecipitations were carried out as previously described (von Hofsten et al., 2008) with minor modifications: for pSmad and corresponding IgG ChIPs, 30 μl of Protein G Dynabeads (Invitrogen, USA) were pre-bound to 10 μl and 2 μl of antibody, respectively, at 4°C, followed by incubation with chromatin for 2.5 hours at 4°C. For Gli2a and corresponding IgG ChIPs, the protocol remained the same except that 20 μl of Protein A PureProteome beads (Millipore, USA) were pre-bound to 4 μg and 2 μl of antibody, respectively. Quantitative PCR (qPCR) analysis of ChIPed material was carried out using SYBR Fast reagents (KAPA Biosystems, USA) on a Bio-Rad MyiQ2 platform (Bio-Rad, USA), according to the manufacturer's instructions. ChIP experiments were carried out in biological triplicate and each qPCR reaction was performed in duplicate.

Antibody staining and image analysis

Antibody staining was performed as previously described (Elworthy et al., 2008) at the following dilutions: mAb F59 (anti myosin heavy chain 1; Hybridoma Bank) 1:50; mAb 4D9 (anti-engrailed; DHSB) at 1:50-1:200; mAb F310 (anti-fast myosin light chain; DSHB) at 1:50; rabbit polyclonal anti-turboFP635 (Evrogen) at 1:750, rabbit polyclonal anti-N-Smad1/5/8 (Santa Cruz Biotechnology) at 1:500; mAb mCherry (Living Colors, Clonetech, USA) at 1:500; rabbit polyclonal anti-pSmad1/5/8 (Cell Signalling) at 1:100 dilution. Rhodamine coupled anti-mouse secondary antibody (Invitrogen, USA) at 1:500 and Cy5 and FITC anti-rabbit-coupled secondary antibodies (Invitrogen, USA) at 1:1000 dilution. Specimens were imaged with a 60× oil immersion objective on an Olympus Fluoview confocal microscope. Images were acquired using Olympus FV10-ASW software and analysed using ImageJ software (http://rsbweb.nih.gov/ij/). Unless otherwise stated, images shown represent merged stacks of between eight and twelve parasagittal optical sections derived from the trunk region closest to the embryonic yolk extension.

RESULTS

Identification of cis-regulatory elements controlling *eng2a* expression

To define the sequence elements controlling *eng2a* gene expression, we identified three BAC clones containing the *eng2a* gene locus (CH211-150E22, DKEY-251D18 and DKEY-182G13). These BACs were modified by homologous recombination in bacteria (Lee et al., 2001) to insert reporter genes 3′ to the start codon of the *eng2a* coding region (Fig. 1A). Embryos injected with these modified BACs recapitulated the endogenous pattern of *eng2a* expression, in the mid-hindbrain boundary (MHB), mandibular arch, MPs and MFFs. To delineate the cis-regulatory elements more precisely, we made several reporter constructs containing differing lengths of the *eng2a* promoter region (1.5, 4, 8, 10 and 12 kb upstream of *eng2a* ATG, all derived from the CH211-150E22 BAC) and tested their activities in transgenic zebrafish embryos (Fig. 1). The 1.5 and 4 kb fragments displayed no activity in embryos, whereas the 8, 10 and 12 kb fragments drove robust expression within all three early expression domains (MHB, MPs and MFFs). We generated several stable transgenic lines with all but one of these constructs (Table 1), the patterns of expression of which confirmed that cis elements driving expression in the MHB, MP and MFFs lie within the 8 kb of *eng2a* start codon (Fig. 1).

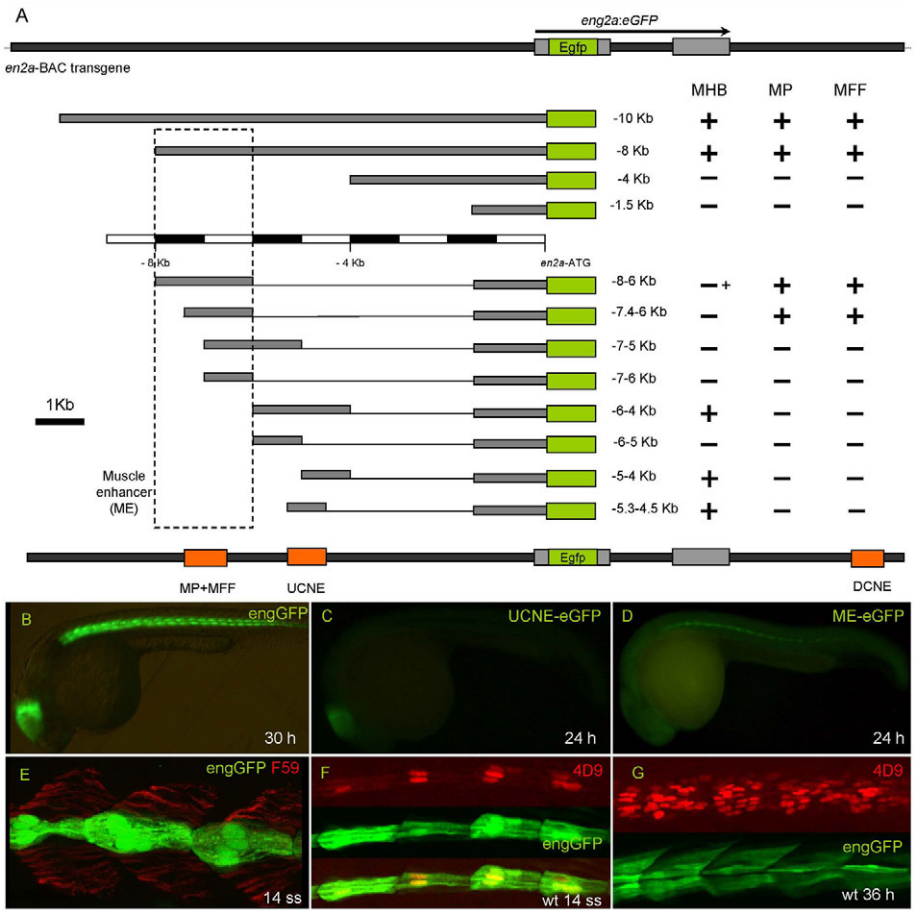

Fig. 1. Identification of cis-regulatory elements in the zebrafish *eng2a* gene. (**A**) Schematic representation of the *eng2a:eGFP* BAC transgene, its deletion derivatives and the enhancers that they identify; the activity of each construct in the MHB, MP cells and MFF is indicated by a + or – sign (+ indicates low-level expression). The dotted box indicates the limits of the 2 kb fragment referred to throughout as the ME. (**B-D**) Whole-mount lateral views (anterior to left, dorsal up) of: a 30 hour *Tg*(*eng2a:eGFP*)^{i233} embryo showing eGFP expression in the MHB and MP/MFF population (B); a 30 hour *Tg*(*eng2a:eGFP*)^{i243} embryo showing eGFP expression restricted to the MHB (C); a 30 hour *Tg*(*eng2a:eGFP*)^{i236} embryo showing eGFP expression restricted to the MP/MFF population (D). Note that in D the levels of eGFP expression are relatively low, such that only the MP expression is clearly visible at low magnification. (**E**) Parasagittal optical section of anterior trunk region of a 14 ss *Tg*(*eng2a:eGFP*)^{i233} embryo showing myotomal expression restricted to a subset of the slow fibres revealed by staining with the mAb F59 antibody. (**F**,**G**) Similar preparations of F, a 14 ss *Tg*(*eng2a:eGFP*)^{i233} embryo stained with mAb 4D9 showing coincidence of expression (lower merged image) of the transgene (middle image) and the endogenous Eng proteins (upper image), and G, a 36 hour *Tg*(*eng2a:eGFP*)^{i233} stained with mAb 4D9; the transgene is now expressed in both MPs and multinucleate MFFs. UCNE/DCNE: upstream/downstream conserved non-coding element (conserved from fish to mammals).

Minimal enhancer elements with activity in MPs and MFFs

To identify the minimal enhancer element(s) that mediate the response to Shh activity, we made constructs carrying fragments of varying lengths from the –8 to –4 kb region cloned upstream of a 1.5 kb *eng2a* 'minimal' promoter driving eGFP that alone displays no activity in transgenic embryos (Fig. 1A). We identified several distinct enhancer elements using this approach (Fig. 1A); an approximately 800 bp highly conserved non-coding element (CNE) between –5.3 and –4.5 kb region upstream of *eng2a* drove expression specifically in the MHB region (Fig. 1A,C), consistent with previous reports of the equivalent CNE in mouse acting as an MHB enhancer (Logan et al., 1993). A 2 kb fragment (from –8 to –6 kb) was sufficient to drive eGFP expression principally in MPs and MFFs (Fig. 1A,D), some lines retaining low level residual expression in the MHB region (data not shown); a deletion of 600 bp from the 5′ end of this fragment retained activity in both the MPs and MFFs, whereas deletion of a further 400 bp abolished all activity (Fig. 1A). The 2 kb fragment (from –8 to –6 kb upstream of *eng2a* translation start site) therefore harbours sequences that we refer to henceforth as the *eng2a* muscle enhancer (ME). We note that the location of this element is distinct from that of the previously identified mandibular muscle enhancer adjacent to the MHB enhancer of the mouse *En2* gene (Degenhardt et al., 2002; Logan et al., 1993) and the analogous 'muscle enhancer' identified upstream of the *Amphioxus engrailed* gene (Beaster-Jones et al., 2007).

Identification of Smad5 as a negative regulator of the *eng2a* muscle enhancer in BHK21 cells

In silico analysis of the *eng2a* ME revealed an absence of consensus Gli-binding sites. To identify trans-acting factors controlling the activity of the enhancer, we employed a transregulation screening methodology that exploits the availability of a medaka unigene cDNA library (Souren et al., 2009). The 2 kb

Table 1. Stable transgenic lines carrying the reporter constructs used to delineate the *eng2a* regulatory elements

Reporter constructs	Allele designation
–10 kb-eGFP	*Tg(eng2a:eGFP)^{i233}*
–8 kb-eGFP	*Tg(eng2a:eGFP)^{i234}*
–8-6 kb-eGFP (ME)	*Tg(eng2a:eGFP)$^{i235, i236}$*
–7.4-6 kb-eGFP	*Tg(eng2a:eGFP)^{i237}*
–7-5 kb-eGFP	*Tg(eng2a:eGFP)^{i238}*
–7-6 kb-eGFP	*Tg(eng2a:eGFP)^{i239}*
–6-4 kb-eGFP	*Tg(eng2a:eGFP)^{i240}*
–6-5 kb-eGFP	*Tg(eng2a:eGFP)^{i241}*
–5-4 kb-eGFP	*Tg(eng2a:eGFP)^{i242}*
–5.3-4.5 kb-eGFP	*Tg(eng2a:eGFP)^{i243}*
–7.4-7.2 kb-eGFP	*Tg(eng2a:eGFP)^{i244}*
–8-6.8 kb-eGFP	*Tg(eng2a:eGFP)^{i245}*
–8-6.6 kb-eGFP	*Tg(eng2a:eGFP)^{i246}*
–7.8-6.8 kb-eGFP	*Tg(eng2a:eGFP)^{i247}*
–7.8-6.6 kb-eGFP	*Tg(eng2a:eGFP)^{i248}*
–7.6-6.8 kb-eGFP	*Tg(eng2a:eGFP)^{i249}*
–7.6-6.6 kb-eGFP	*Tg(eng2a:eGFP)^{i250}*
–7.4-6 kb-eGFP	*Tg(eng2a:eGFP)^{i251}*

eng2a ME was cloned upstream of the firefly luciferase reporter (here referred to simply as *eng2a-ME:luciferase*); BHK21 cells were co-transfected with this construct along with a cytomegalovirus (CMV):*Renilla* luciferase reporter and medaka cDNAs cloned downstream of the CMV promoter and assayed for dual luciferase activity 2 days later. The CMV:*Renilla* luciferase served as a control for cell viability and transfection efficiency. Potential regulators were ranked by their ability to modulate the expression of *eng2a-ME:luciferase* normalised against the *Renilla* luciferase activity. Around 1200 full-length medaka cDNA clones selected on the basis of GO (Gene Ontology) terms implicating them in embryonic development were screened for their ability either to enhance or suppress the activity of the ME in BHK21 cells. Among the strongest negative regulators was Smad5, a transcription factor that mediates BMP signalling (Graff et al., 1996; Liu, 1996; Suzuki et al., 1997; Thomsen, 1996). Given the previously reported ability of BMP signalling to suppress *eng* expression and MP specification (Du et al., 1997; Kawakami et al., 2005), we focused our analysis on this candidate trans-regulator.

Activated Smads accumulate within nuclei of *engrailed* negative muscle progenitors

Phosphorylation of Smad proteins by activated BMP receptors leads to their accumulation in the nucleus, where they can either activate or repress target gene transcription (Hoodless, 1996; Kretzschmar et al., 1997). We used an antibody that recognises the C-terminally phosphorylated forms of Smads 1, 5 and 8 (hereafter collectively referred to as 'pSmad'), to analyse their distribution in the zebrafish myotome. Before the onset of *eng* expression, pSmad is detectable in the dorsal- and ventralmost adaxial cells (see Fig. S1A-A″ in the supplementary material) and by the time Eng proteins are detectable (12-14 ss), accumulates in the nuclei of all pre-migratory slow muscles, identified either by expression of slow myosin heavy chain (marked by mAb F59) or *prdm1a:GFP* reporter (Elworthy et al., 2008), with the notable exception of the MP cells (Fig. 2A-C). By 20 ss, when the adaxial cells have completed their lateral migration and formed a superficial layer of slow fibres on the surface of the myotome, they no longer accumulate pSmad (Fig. 2D-F). Instead, high levels of pSmad are detectable in somitic cells adjacent to the neural tube and along the hypochord (Fig. 2E,F). This later pSmad accumulation is specific to differentiating fast-twitch muscles, which after the lateral migration of adaxial cells are displaced next to the neural tube and hypochord. High levels of nuclear pSmad are also detected in broader domains at the most dorsal and ventral extremes of the somite (Patterson et al., 2010).

Attenuation of Smad activity lowers the threshold response of cells to Hh

The BMP receptor-encoding gene *bmpr1ba* is expressed specifically in the developing somites of zebrafish embryos (Nikaido et al., 1999); embryos injected with morpholino oligonucleotides (MO) that target the exon1-intron1 splice junction of the *bmpr1ba* transcript showed a significant reduction in pSmad accumulation in muscle cells (Fig. 3A,B), consistent with its mediating the response of myoblasts to BMP. Strikingly, such embryos also showed a concomitant expansion of *eng2a* expression in slow-twitch muscle fibres (Fig. 3C,D,G) and medially located fast-twitch fibres towards the dorsal and ventral edges of the myotome (Fig. 3A,B; data not shown). These findings suggest that reduction of BMP activity, and hence of Smad activation, allows cells to activate *eng2a* expression in response to levels of Hh signalling lower than those normally required in the wild-type context. To confirm this inference, we investigated the effect of attenuating BMP signalling in embryos with compromised Hh activity. The Dispatched1 (Disp1) protein is required for efficient secretion of Hh proteins (Burke et al., 1999; Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002); accordingly, zebrafish embryos homozygous for the *disp1^{tf18b}* mutant allele have reduced numbers of slow-twitch muscle fibres and essentially lack MPs and MFFs (Nakano et al., 2004). In homozygous *disp1^{tf18b}* mutant embryos injected with the *bmpr1ba* MO, expression of the *eng2a* transgene was restored in both slow and fast-twitch muscle fibres (Fig. 3E,F). Thus reduced levels of Hh activity are sufficient to induce *eng2a* expression when Smad activity is attenuated. Depletion of BMPR1ba in *smo* mutant embryos, by contrast, failed to activate *eng* expression, consistent with the absolute dependence of *eng* activation on Hh pathway activity (Fig. 3H).

Activated Smads bind to a repressive element within the *eng2a* muscle enhancer

We next asked if the repression of *eng2a* expression by pSmad is mediated by its direct interaction with the ME. Quantitative PCR amplification of fragments around the *eng2a* locus of ChIPed DNA from embryonic nuclear extracts using the pSmad antibody, revealed a robust enrichment for the *eng2a* ME sequences relative to the adjacent exonic regions of the *eng2a* gene (Fig. 4A). The enrichment levels peaked close to a central position within the 2 kb fragment (–8094 to –6020 bp upstream of the *eng2a* ATG) and were similar to that of the upstream region of *np63*, a known BMP target gene (Bakkers et al., 2002). We derived a Smad consensus-binding site from a collection of consensus sites previously collated (Henningfeld et al., 2000) and searched for instances of this position weight matrix (PWM) in the 2 kb enhancer fragment. The instance with the highest score was found at position –7231 bp (Fig. 4C,E), contained within one of the fragments most enriched for pSmad. The motif, GCCACACA, differs in two positions from the published consensus: at the second position it contains a C instead of a G, and the central G at position 5 is a C. Analysis of the ME sequence using the Matinspector software revealed the presence of a second motif, CTAGTCACGCGCCACAC, that matches the consensus for the E2F-Smad-binding site (Chen et al., 2002) at position –6883 bp, located within a fragment that showed

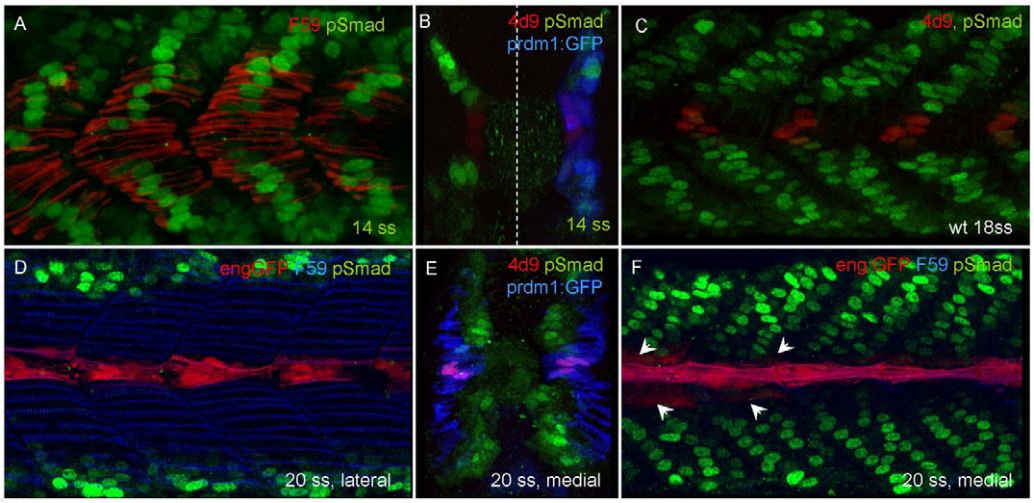

Fig. 2. Activated Smads display a dynamic pattern of nuclear accumulation during zebrafish myotomal development. (**A**,**B**) Medial parasagittal optical and transverse sections of 14 ss embryos showing nuclear accumulation of pSmad (green) in slow myoblasts, marked by F59 (red) staining in A and *prdm1a:GFP* expression (blue) in B; the blue channel has been suppressed on the left-hand side of this panel to aid visualisation of the red and green signals. Note absence of accumulation in cells closest to the midline in both cases. These latter cells are the presumptive MPs. (**C**) 18 ss wild-type embryo showing the mutually exclusive pattern of Eng (4D9) and pSmad accumulation. (**D-F**) Lateral and medial parasagittal optical sections and transverse section of 20 ss *Tg*(*eng2a:eGFP*)^{i233} embryos. After the outward migration of slow fibres labelled with F59 (blue) in D, F, and *prdm1:GFP* (blue) in E, pSmad accumulates in a subset of medially located fast fibres, except for those at the midline, closest to the notochord that express the transgene (red in D,F) and endogenous Eng, revealed by 4D9 (red in E). Note that at this stage, most *eng* expression is still restricted to MPs, but is starting to initiate in MFFs in more anterior somites (arrows in F).

the highest (~4×) level of enrichment (Fig. 4A,C,E); transgenic lines carrying deletions of this region of the ME showed ectopic expression of the eGFP reporter in the myotome, consistent with its acting as a repressive element (Fig. 4C,D).

Gli2a protein interacts with the *eng2a* muscle enhancer in vivo

Gli1 and Gli2a function redundantly to activate *eng* expression (Wolff et al., 2003), and the finding that Hh activity is required for *eng2a* transcription even in the absence of pSmad implies that this effect may be direct. Accordingly, we used ChIP analysis to ask whether the *eng2a*-ME also interacts with Gli proteins. Using a polyclonal antibody specific for the zebrafish Gli2a protein (see Materials and Methods for details), we found a consistent enrichment across the central region of the ME, including both putative Smad-binding sites. The levels of enrichment were significantly lower than that of the upstream region of *ptc1*, a well-established Hh target gene (Agren et al., 2004; Alexandre et al., 1996; Hallikas et al., 2006; Vokes et al., 2007), which served as positive control. This may reflect the absence of close matches to the Gli consensus-binding site from the ME, in contrast to the *Ptc1* upstream regulatory region, but is also probably influenced by the relatively small number of cells that express the *eng2a* in response to Gli2a activity.

Nuclear accumulation of pSmad is antagonised by Hh pathway activity

Our findings indicate that Hh signalling activates *eng2a* transcription in cells with low levels of nuclear pSmad. These levels could be set uniquely in response to BMP activity; alternatively, they might themselves be modulated by Hh signalling. To explore this issue further, we investigated the effect of de-regulated Hh pathway activity on pSmad accumulation. Full activation of the intracellular Hh pathway in zebrafish embryos can be achieved by overexpression of the Shh ligand (Krauss et al., 1993) or by mutational inactivation of the Shh receptor Ptc-1 and its paralogue Ptc-2 (Koudijs et al., 2008; Koudijs et al., 2005). Shh mis-expressing (data not shown) and *ptc1*; *ptc2* double mutant embryos both showed a transformation of the entire myotome to the slow-twitch fibre fate with a substantial increase in Eng^{+ve} muscle pioneer cells in the central region of the myotome. In both cases, all Eng^{+ve} cells were devoid of nuclear pSmad staining. Strikingly, however, the accumulation of pSmad persisted dorsally and ventrally and was no longer restricted to cells closest to the midline, as in wild type, but now extended more laterally (Fig. 5A-A″,B,B′, compared with Fig. 2). This is consistent with the previously reported ability of Hh to activate *smad1* transcription (Dick et al., 1999). Inhibiting the activity of protein kinase A (PKA), which promotes the conversion of full-length Gli to the truncated Gli^{R} forms, also results in activation of Hh target genes in the zebrafish embryo (Concordet et al., 1996; Hammerschmidt et al., 1996). Injection of embryos with mRNA encoding a dominant-negative form of the PKA regulatory subunit (dnPKA) essentially replicated the effects of *ptc1*; *ptc2* double mutant or Shh mRNA injection on pSmad accumulation (see Fig. S1F in the supplementary material).

Cell-autonomous activation of pSmad suppresses induction of Eng transcription by Hh activity

The incomplete induction of *Eng* expression within the myotome even under conditions of maximal Hh pathway activation, suggests a dominant role of pSmad-mediated repression in the regulation of *eng2a* transcription. To explore this further, we asked whether nuclear accumulation of pSmad proteins is sufficient to block the activation of *eng2a* expression (and MP/MFF differentiation) in all cells in which the Hh pathway is maximally activated. We used a

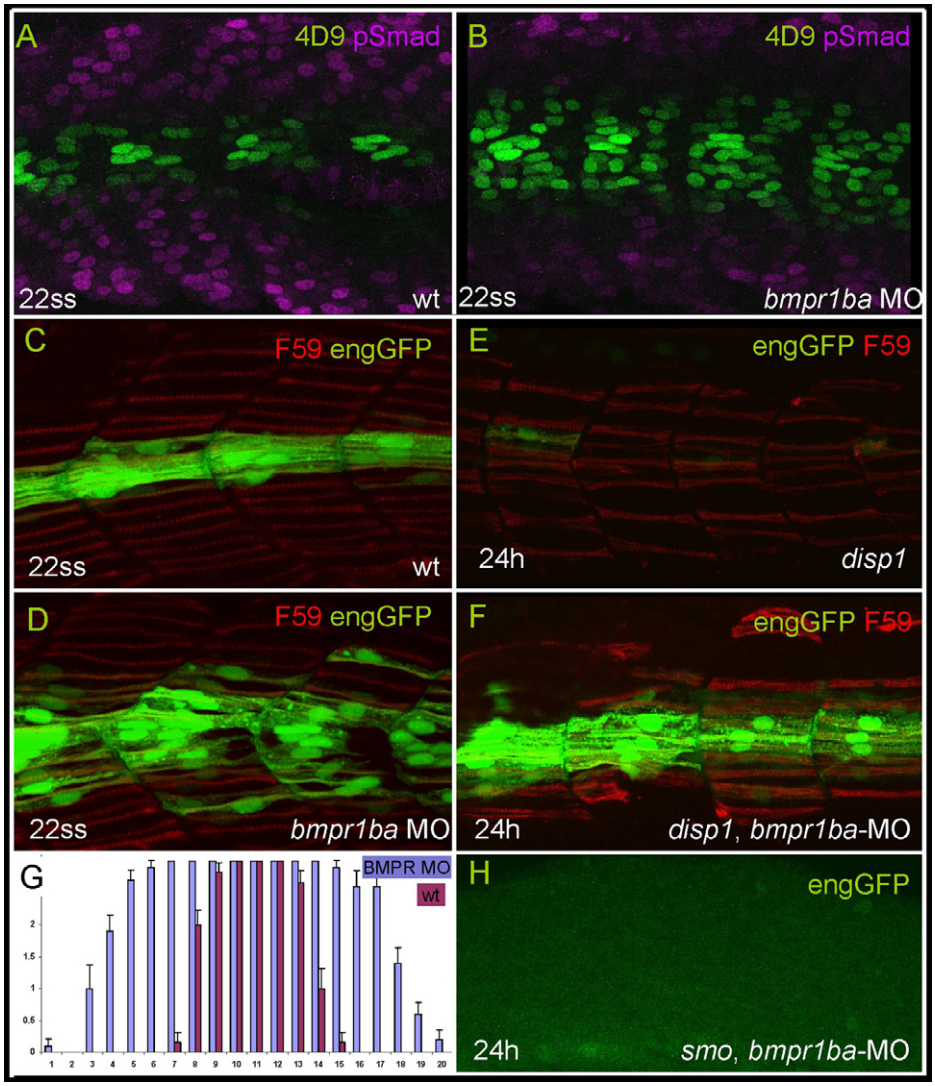

Fig. 3. Inhibition of Smad activity de-represses *engrailed* expression. (**A**,**B**) Parasagittal optical sections of 22 ss embryos showing distribution of Eng (4D9) and pSmad in wild type (A) and *bmpr1ba* morphants (B). Somites shown are at the level of the yolk sac. (**C**,**D**,**G**) Attenuation of BMPR1ba causes ectopic expression of *eng2a*. The histogram in G compares eGFP expression in *Tg*(*eng2a:eGFP*)^{i233} embryos injected with *bmpr1ba* morpholino, with that in non-injected controls; bars represent the frequency of eGFP expressing slow fibres at different dorsoventral positions. Positions 8-13 represents the horizontal myoseptum. Morphants display a wide expansion of the eGFP domain. Data based on three consecutive somites at the end of yolk extension in 22 ss embryos. *x* axis, dorsoventral position; *y* axis, number of eGFP^{+} fibres. Error bars indicate s.d. Representative examples are shown in C,D. (**E**) Parasagittal optical sections of a 24 hour *Tg*(*eng2a:eGFP*)^{i233};*disp1*^{tf18b} mutant embryo displaying reduced slow fibres (F59; red) and near absence of *eng2a:eGFP*. (**F**) Similarly staged *Tg*(*eng2a:eGFP*)^{i233}; *disp1*^{tf18b} mutant embryo that was injected with *bmpr1ba* morpholino at the one- to two-cell stage, showing robust rescue of *eGFP* expression. (**H**) Parasagittal optical sections of a 24 hour *Tg*(*eng2a:eGFP*)^{i233}; *smo*^{b641} mutant embryo injected with *bmpr1ba* morpholino at one- to two-cell stage, showing complete absence of *eng2a:eGFP* expression.

constitutively active form of the mouse BMP receptor (caALK3) (Wieser et al., 1995) to drive high-level accumulation of pSmads. Misexpression of caALK3 was targeted to myotomal progenitor cells using the *actin:GAL4* driver and visualised by tRFP expressed from the same transgene (see Materials and methods for details). As predicted, mosaic expression of the caALK3 in embryos injected with dnPKA mRNA resulted in the cell-autonomous accumulation of pSmad and repression of *eng2a* expression, irrespective of position within the myotome (Fig. 5E and see Fig. S1C,C′ in the supplementary material). The caALK3 transgene also potently and autonomously suppressed the induction of *eng2a* by Shh in the fast fibres (Fig. 5F).

Hh pathway activation induces Eng expression cell-autonomously

We next asked whether the activation of *eng* transcription is a cell-autonomous response to Hh pathway activity. Plasmid DNA encoding the *UAS:dnPKA* transgene was injected into newly fertilised eggs derived from *disp1*^{tf18b} heterozygotes that also carry the *actin:GAL4* driver and the *Tg*(*eng2a:eGFP*)^{i233} transgenes. The transient transgenic resultant *disp1*^{tf18b} homozygous embryos exhibited a highly mosaic expression of the dnPKA transgene (visualised by tRFP expression); significantly, expression of the *eng2a:eGFP* transgene was activated in a majority of the isolated dnPKA-expressing muscle fibres (Fig. 5C,D). Clones of dnPKA in the myotome of wild-type embryos also cell autonomously induced *eng2a:eGFP* (see Fig. S1D,D′ in the supplementary material). From this we conclude that the activation of *eng2a* transcription is a cell-autonomous response to Hh pathway activity.

Truncated Gli2a can interact with Smad1 in the nuclei of embryonic myotomal cells

Our data imply that Hh pathway activity modulates the nuclear accumulation of pSmad and that this effect is mediated at the level of the Gli proteins. Previous studies have reported that truncated, but not full-length, forms of Gli can be co-precipitated with Smads from tissue culture cells transfected with constructs encoding tagged forms of both proteins (Liu et al., 1998). This suggests a possible mechanistic basis for the observed modulation of pSmad accumulation by Hh signalling, whereby the truncated repressor form but not the full-length forms of Gli might promote the nuclear accumulation of pSmads. To explore this possibility we employed the bimolecular fluorescence complementation (BiFC) assay (Hu et al., 2002; Saka et al., 2007) to investigate the potential of the two proteins to interact directly in an in vivo

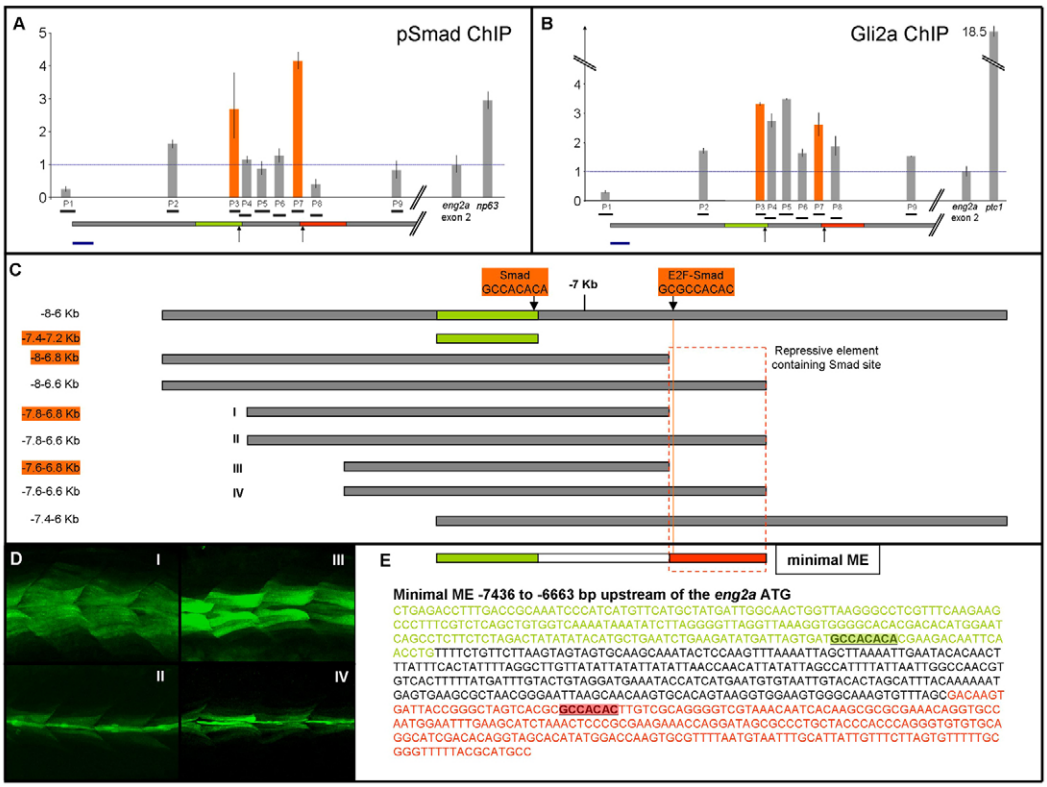

Fig. 4. Phospho-Smads and Gli2a bind the ME in the zebrafish embryo. (**A**) qPCR detection of DNA fragments (P1-P9) within the *eng2a* ME (horizontal grey bar: the regions shaded green and red correspond to the correspondingly coloured sequences shown in E) in chromatin precipitated from 14 ss embryo nuclear extracts using the pSmad antibody. Fragment sizes (bp): P1,146; P2, 119; P3, 102; P4, 120; P5, 145; P6, 124; P7, 117; P8, 117; P9, 114. The *y*-axis indicates fold changes normalised to a fragment within the second exon of the *eng2a* gene. Enrichment of a fragment upstream of the *np63* transcription unit provides a positive control. Orange bars denote enrichment of fragments containing putative Smad-binding sites, indicated in C and E. Enrichments are normalised against input chromatin and also against IgG-precipitated chromatin. Error bars represent s.d. derived from qPCR repeats from the same ChIP preparation; the enrichments shown are from a single ChIP. Similar enrichments were obtained in biological repeats of ChIP preparations. (**B**) qPCR detection of DNA fragments (P1-P9; as described above) within the *eng2a* ME in chromatin precipitated from 14 ss embryo nuclear extracts using the αGli2a antibody. Enrichment of a fragment upstream of the *ptc1* transcription unit serves as a positive control. (**C**) ME deletion constructs assayed for activity in stable transgenic lines (listed in Table 1). Fragment lengths boxed in orange indicate those that show ectopic expression. The dotted orange box indicates a putative repressor element revealed by this analysis. (**D**) Examples of ectopic eGFP expression in embryos from transgenic lines *Tg*(*eng2a:eGFP*)^{i247} (I) and *Tg*(*eng2a:eGFP*)^{i249} (III) and normal expression in lines *Tg*(*eng2a:eGFP*)^{i248} (II) and *Tg*(*eng2a:eGFP*)^{i250} (IV). (**E**) Nucleotide sequence of the *eng2a* minimal ME showing the two putative Smad-binding sites: the more distal of these (highlighted in green) lies in a region (shown in green) absolutely required for ME activity; the more proximal site (highlighted in red) lies in a region required for ME repression. Scale bar: 200 bp in A and B.

context. BiFC exploits the ability to reconstitute a fluorescent protein (modified non-aggregating forms of Venus) from its two halves (Vn-N terminal fragment and Vc-C terminal fragment), neither of which fluoresces in isolation (Hu et al., 2002; Saka et al., 2007). We first established the interaction assay in myotomal cells using our Vn-Smad1 along with previously described Vc-Smad4 constructs (Harvey and Smith, 2009). We cloned both constructs into a bi-cistronic UAS vector and targeted their expression to myoblasts using the *actin::GAL4* line; this resulted in a robust and characteristically punctate fluorescence signal, both in the cytoplasm and around the nuclei of muscle cells (Fig. 6A). We next generated fusions of Vn and Vc to full-length Gli1 and Gli2 as well as to a truncated form of Gli2 (which lacks the activation domain but retains the repressor and zinc finger domains) and inserted these into the bi-cistronic UAS vectors along with the complementary tagged Smad1 (Vn-Smad1, Vc-Smad1). As a control for the specificity of intracellular location, we also generated bi-cistronic vectors carrying the same Gli constructs and appropriately tagged forms of SuFu, a protein known to interact with Gli proteins in both *Drosophila* and mammalian cells. As anticipated, co-expression of Glis with SuFu resulted in the generation of fluorescent signal in muscle fibres; notably the signal was largely excluded from the nuclei (Fig. 6B and see Fig. S2A,C in the supplementary material), consistent with the proposed role of SuFu in sequestering Gli proteins in the cytoplasm (Dunaeva et al., 2003; Humke et al., 2010; Pearse et al., 1999; Stone et al., 1999). Co-expression of the full-length forms of Gli1 or Gli2a with Smad1 also resulted in significant levels of fluorescence in some fibres; in both cases the signal was localised predominantly in the cytoplasm although some nuclei were labelled with the Gli2a-Smad1 pair (Fig. 6C,E and see Fig. S2D in the supplementary material). By contrast, co-expression of the truncated Gli2a and Smad1 tagged pairs resulted in robust levels of fluorescence at high frequency (Fig. 6D,E); notably, this signal was largely abolished when the *bmpr1ba* MO was simultaneously injected with the constructs (Fig. 6E,F), implying that the

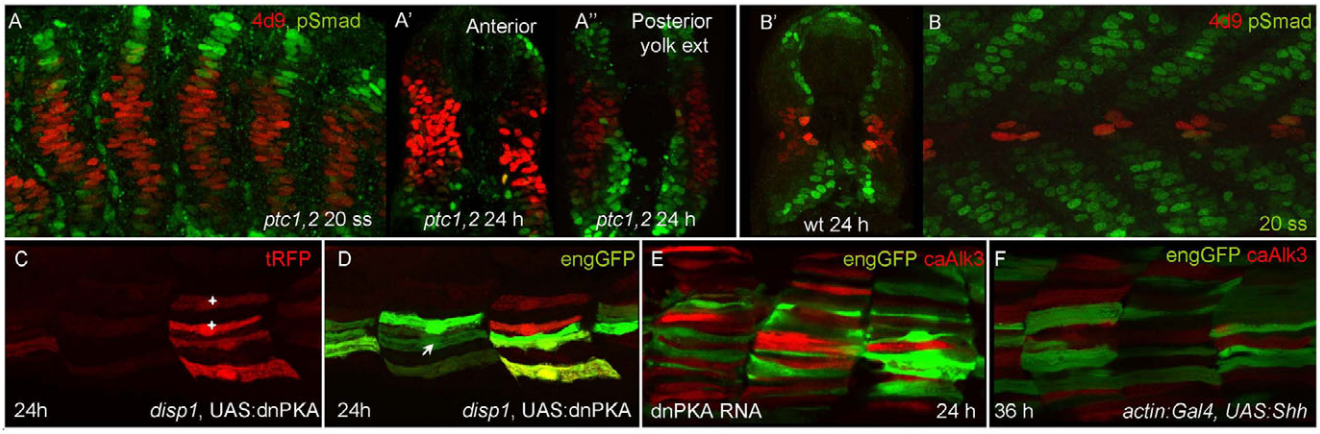

Fig. 5. Modulation of Smad accumulation and Eng expression by Hh and BMP pathway activity. (**A-A″**) Parasagittal optical section and transverse sections at different levels along the rostrocaudal axis of *ptc1*^{hu1602};*ptc2*^{tj222} double homozygous mutant embryo at 20 ss and 24 hours, respectively, showing vastly expanded MPs (revealed by 4D9, red) and reciprocal loss of pSmad accumulation (green) compared with wild-type embryo at a similar stage (**B**,**B'**). Note expansion in domain of pSmad accumulation dorsally and ventrally in *ptc* mutants, especially at more caudal levels; compare with 24 hour wild-type embryo (B'). (**C**,**D**) Parasagittal optical sections of *Tg*(*actin:GAL4*); *Tg*(*eng2a:eGFP*)^{i233}; *disp1*^{tf18b}mutant embryo injected with *UAS:dnPKA;tRFP* at one- to two-cell stage. Most tRFP expressing fibres are also eGFP^{+ve}, consistent with an autonomous effect of Hh pathway activity on *eng2a* induction. Note that not all fibres expressing tRFP (C) ectopically activated *eng2a:eGFP* (*); conversely, one eGFP^{+ve} fibre (marked by an arrow in D) was tRFP^{-ve}; this possibly represents a fibre induced by residual Hh signalling activity, as is sometimes observed in *disp1*^{tf18b}mutant embryos. (**E**,**F**) Merged images of 24 hours *Tg*(*eng2a:eGFP*)^{i233} embryos expressing caALK3 under *actin:GAL4* control in which the Hh pathway has been activated by injection of dnPKA mRNA or of *UAS:Shh* DNA. (E) dnPKA injection results in the ectopic activation of the *eng2a:eGFP* transgene (green) but this is suppressed in cells expressing caALK3 (red). Similarly, (F) late expression of Shh activates the *eng2a:eGFP* transgene in fast twitch fibres but this is suppressed by caAlk3 (red). On average, only 1.4 out of 13 tRFP^{+ve} fibres also expressed eGFP (*n*=22 hemisegments from seven embryos).

interaction was dependent upon phosphorylation of Smad1. Co-expression of truncated Gli2a and Smad4 resulted in very weak complementation both in the nuclei and cytoplasm that was detectable only by 40 hours, in contrast to 24 hours for all the other tagged pairs (see Fig. S2B in the supplementary material).

DISCUSSION

Several lines of evidence have shown that the specification of distinct muscle cell types is mediated by variation in the levels and timing of Hh exposure (Ingham and Kim, 2005; Wolff et al., 2003), whereas gain- and loss-of-function experiments have implicated

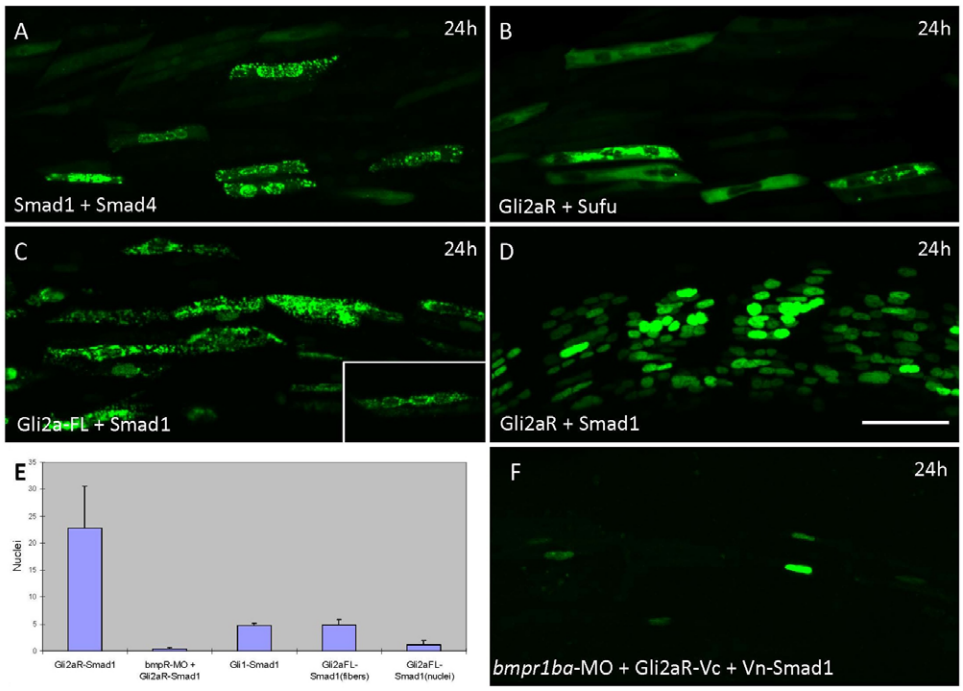

Fig. 6. Bi-fluorescence complementation assay for in vivo protein-protein interactions. Parasagittal sections of live embryos injected with constructs expressing Vn- and Vc-tagged protein pairs from the same UAS promoter and imaged at 24 hours. (**A**) Smad1/Smad4: note speckled appearance of signal, which shows a cytoplasmic and perinuclear localisation. (**B**) Gli2aR/SuFu; signal is localised to the cytoplasm and excluded from the nucleus. (**C**) Gli2a-FL/Smad1; note speckled appearance of signal, which shows a cytoplasmic and perinuclear localisation (see inset). (**D**) Gli2aR/Smad1; most fibres in each somite are positive for the Venus signal, localised exclusively to the nucleus. (**E**) Histogram showing frequency of Venus^{+ve} fibres in embryos injected with different Gli/Smad combinations. Error bars indicate s.d. based on ≥5° hemisegments from five different embryos. (**F**) Gli2aR/Smad1 co-injected with *bmpr1ba* morpholino: the majority of fibres in each somite are negative for Venus.

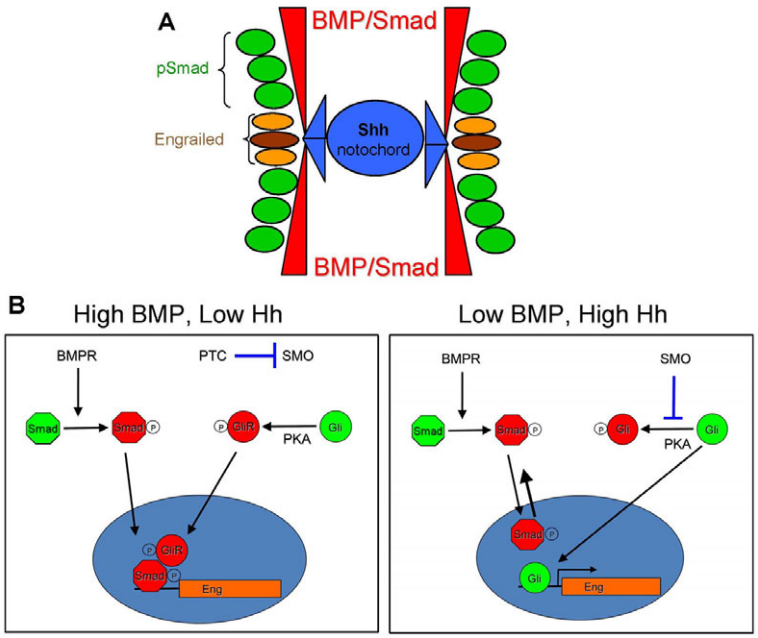

Fig. 7. Patterning of the zebrafish myotome by antagonistic gradients of Shh and BMP and model for crosstalk between the two pathways. (**A**) Schematic representation of a transverse section of the developing embryo showing the inferred distributions of Shh and BMP activities and their effects on myoblast identity. The most medially located myoblasts are postulated to respond to BMP activity emanating from dorsal and ventral regions of the somite by accumulating pSmad in their nuclei, with the exception of those closest to the notochord, the source of Shh. These cells activate *eng* expression in response to Gli^A activity and depletion of nuclear pSmad, both of which are promoted by high levels of Shh. (**B**) A model for the modulation of pSmad accumulation and regulation of the *eng2a* ME by Shh activity. In cells receiving relatively low levels of Shh and relatively high levels of BMP, accumulation of pSmad in the nucleus is potentiated by repressor forms of the Gli proteins (red) generated in response to phosphorylation of full-length Glis. Accumulated pSmad binds to the *eng2a* ME, repressing its transcription. In cells exposed to high levels of Shh and relatively low levels of BMP, Gli processing is abrogated: this results in depletion of pSmad from the nucleus and hence from the ME, favouring Gli^A (green) binding and activation of transcription.

BMP signalling in attenuating the response of muscle progenitors to Hh activity (Du et al., 1997; Kawakami et al., 2005). The mechanistic basis of this interplay between the two pathways, however, has remained obscure, although Kawakami et al. (Kawakami et al., 2005) proposed that BMP signalling might attenuate Hh signalling at the level of Gli-Smad interactions. Our analysis of the regulation of the *eng2a* gene, a key marker of MP and MFF identity, has now revealed a direct link between Smad activity and a putative Hh target gene. Crucially, we found that phosphorylated forms of Smads bind to and repress specific Hh-responsive enhancer elements upstream of the *eng2a* promoter: moreover, we showed that nuclear accumulation of pSmads was abrogated in Eng^{+ve} cells inferred to be exposed to the highest levels of Hh signalling activity.

Given that loss of pSmad activity is not itself sufficient to activate *eng* expression in the absence of Hh activity, it follows that Hh function is required both to activate as well as de-repress the *eng2a* ME. The finding that chromatin around the ME was enriched for Gli2a as well as pSmad implies that both proteins act directly on the enhancer. Strikingly, the fragments containing the putative pSmad-binding sites are among those also enriched by anti-Gli2a. Whether or not Gli2a and pSmad compete with one another in this interaction remains to be determined: it is, however, notable that point mutation of the more distal pSmad-binding site eliminated ME activity (data not shown), consistent with an overlap of repressive and activating elements.

Notably, high-level nuclear accumulation of pSmad was restricted in the developing myotome of wild-type embryos, being limited initially to the adaxial cells and, subsequently, largely to fast-twitch muscle progenitors that lie closest to the midline. Significantly, these cell populations are known to express elevated levels of *smad1* transcript in response to Hh signalling activity (Dick et al., 1999). By contrast, in embryos in which the Hh pathway had been activated ubiquitously, either through mutation of the *ptc1* and *ptc2* genes or by injection of mRNA encoding Shh or the dnPKA regulatory subunit, there was an expansion in the domain of pSmad accumulation, which now extended to the lateral edges of the somite both dorsally and ventrally. However, accumulation in a broad central sector of the somite was completely abolished, concomitant with the ectopic activation of the *eng2a* and other *eng* genes. We take this to imply that the levels of pSmad are modulated at several distinct levels: first, through the upregulation of the gene encoding Smad1 (and possibly Smad5 and 8) by Hh pathway activity; second, through the spatially restricted activity of the BMP signalling pathway; and third, through the relative levels of Gli^R present in the nucleus. In this view, the highest levels of Smad protein expression should be found in cells responding to Hh signalling: if such cells are close to a source of BMP, significant levels of pSmad will be generated and accumulate in the nucleus. A potential candidate for such a source of BMP is the *radar* gene product, which is expressed at the right time and place to promote the phosphorylation of Smads in the dorsal and ventral regions of the somite (Rissi et al., 1995). If, however, cells are close to a source of Hh activity, such nuclear accumulation will be abrogated (Fig. 7A). A similarly negative effect of Hh signalling on Smad nuclear accumulation has previously been reported in *Drosophila*; in this case, high levels of Hh activity were shown to cause the repression of the BMP type I receptor encoded by the *thick veins* gene (Tanimoto et al., 2000). We consider such a mechanism to be unlikely in the zebrafish myotome, as the effects of high levels of Hh activity can be overcome by overexpression of a BMP ligand itself (Du et al., 1997) (our data). Moreover, there is no evidence for a corresponding spatial modulation of expression of the BMP receptors thus far described in zebrafish (Monteiro et al., 2008; Nikaido et al., 1999). In any event, if Hh signalling did act by repressing BMP receptor transcription, then high levels of ectopic Shh would be expected to convert the entire myotome into Eng^{+ve} fibres, which is not the case. Instead, we propose that pSmad accumulation could be dependent upon a direct and specific interaction with the truncated, repressive forms of the Gli proteins, the levels of which are reduced in response to Hh signalling activity or by inhibiting PKA (Humke et al., 2010; Pan et al., 2009; Wang et al., 2000; Wen et al., 2010) and which have previously been reported to interact directly with Smads when overexpressed in tissue culture cells (Liu et al., 1998). In this view, pSmad would accumulate in cells that receive relatively low levels of Hh signal

(viz. the adaxial cells and medially located fast-twitch progenitors) in which significant levels of Gli^R are predicted to persist (Fig. 7B), but not in those cells closest to the notochord that receive high levels of Hh signal (the MPs and MFFs), where the levels of Gli^R should be severely reduced if not abolished (Fig. 7C). Our finding that interaction of tagged forms of truncated but not full-length Gli2a with Smad1 is restricted exclusively to the nuclei of myotomal cells is entirely consistent with this model. We note that an analogous molecular interaction has previously been proposed to underlie the observed crosstalk between Shh and Wnt signalling in the amniote neural tube. In this case, potentiation of Wnt signalling by Shh is postulated to be mediated by abrogation of a direct interaction between β-catenin and the truncated repressor form of the Gli3 protein (Ulloa et al., 2007). A strong prediction of our model is that Hh pathway activity should act cell autonomously to activate *eng* transcription; in line with this, we found that isolated cells expressing dnPKA do indeed express the *eng2a* transgene.

Our finding that the reduced levels of Shh signalling due to inactivation of *disp1* were sufficient to activate *eng2a* when pSmad activity was abrogated reinforces the notion that the reduction of Gli^R levels in response to Shh acts principally to deplete nuclear pSmad. In *disp1* mutants, the levels of Gli^A should be reduced, whereas levels of Gli^R are predicted to be increased. Thus, the restoration of *eng* expression by abrogation of Smad phosphorylation supports the argument against a direct role of Gli^R in *eng* repression and supports our view that it acts via pSmad. Taken together, it follows that it is not the absolute level of Gli^A activity but rather the relative levels of Gli^A and pSmad present within the nucleus that is crucial for the correct activation of the *eng* gene.

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Competing interests statement
The authors declare no competing financial interests.

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References
Agren, M., Kogerman, P., Kleman, M. I., Wessling, M. and Toftgard, R. (2004). Expression of the PTCH1 tumor suppressor gene is regulated by alternative promoters and a single functional Gli-binding site. *Gene* **330**, 101-114.
Alexandre, C., Jacinto, A. and Ingham, P. W. (1996). Transcriptional activation of *hedgehog* target genes in *Drosophila* is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. *Genes Dev.* **10**, 2003-2013.
Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, T. B. (1997). Proteolysis that is inhibited by *hedgehog* targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053.
Bakkers, J., Hild, M., Kramer, C., Furutani-Seiki, M. and Hammerschmidt, M. (2002). Zebrafish Np63 Is a direct target of Bmp signalling and encodes a transcriptional repressor blocking neural specification in the ventral ectoderm. *Dev. Cell* **2**, 617-627.
Baxendale, S., Davison, C., Muxworthy, C., Wolff, C., Ingham, P. W. and Roy, S. (2004). The B-cell maturation factor Blimp-1 specifies vertebrate slow-twitch muscle fiber identity in response to Hedgehog signalling. *Nat. Genet.* **36**, 88-93.
Beaster-Jones, L., Schubert, M. and Holland, L. Z. (2007). Cis-regulation of the amphioxus *engrailed* gene: insights into evolution of a muscle-specific enhancer. *Mech. Dev.* **124**, 532-542.
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.
Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K. A., Dickson, B. J. and Basler, K. (1999). Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signalling cells. *Cell* **99**, 803-815.
Caspary, T., Garcia-Garcia, M. J., Huangfu, D., Eggenschwiler, J. T., Wyler, M. R., Rakeman, A. S., Alcorn, H. L. and Anderson, K. V. (2002). Mouse Dispatched homolog1 is required for long-range, but not juxtacrine, Hh signalling. *Curr. Biol.* **12**, 1628-1632.
Chen, C. R., Kang, Y., Siegel, P. M. and Massague, J. (2002). E2F4/5 and p107 as Smad cofactors linking the TGFβ receptor to c-myc repression. *Cell* **110**, 19-32.
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.
Currie, P. D. and Ingham, P. W. (1996). Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. *Nature* **382**, 452-455.
Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M. and Ishii, S. (1999). Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *J. Biol. Chem.* **274**, 8143-8152.
Degenhardt, K., Rentschler, S., Fishman, G. and Sassoon, D. A. (2002). Cellular and cis-regulation of En-2 expression in the mandibular arch. *Mech. Dev.* **111**, 125-136.
Devoto, S. H., Melancon, E., Eisen, J. S. and Westerfield, M. (1996). Identification of separate slow and fast muscle precursor cells *in-vivo*, prior to somite formation. *Development* **122**, 3371-3380.
Dick, A., Andrea, M. and Matthias, H. (1999). Smad1 and Smad5 have distinct roles during dorsoventral patterning of the zebrafish embryo. *Dev. Dyn.* **216**, 285-298.
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-β gene families. *J. Cell Biol.* **139**, 145-156.
Dunaeva, M., Michelson, P., Kogerman, P. and Toftgard, R. (2003). Characterization of the physical interaction of Gli proteins with SUFU proteins. *J. Biol. Chem.* **278**, 5116-5122.
Ekker, M., Wegner, J., Akimenko, M. A. and Westerfield, M. (1992). Coordinate embryonic expression of three zebrafish engrailed genes. *Development* **116**, 1001-1010.
Elworthy, S., Hargrave, M., Knight, R., Mebus, K. and Ingham, P. W. (2008). Expression of multiple slow myosin heavy chain genes reveals a diversity of zebrafish slow twitch muscle fibres with differing requirements for Hedgehog and Prdm1 activity. *Development* **135**, 2115-2126.
Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V. and Jessell, T. M. (1997). Graded sonic hedgehog signalling and the specification of cell fate in the ventral neural tube. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 451-466.
Graff, J. M., Bansal, A. and Melton, D. A. (1996). Xenopus Mad proteins transduce distinct subsets of signals for the TGF-β superfamily. *Cell* **85**, 479-487.
Gustafsson, M. K., Pan, H., Pinney, D. F., Liu, Y., Lewandowski, A., Epstein, D. J. and Emerson, C. P., Jr (2002). *Myf5* is a direct target of long-range Shh signalling and Gli regulation for muscle specification. *Genes Dev.* **16**, 114-126.
Hallikas, O., Palin, K., Sinjushina, N., Rautiainen, R., Partanen, J., Ukkonen, E. and Taipale, J. (2006). Genome-wide prediction of mammalian enhancers based on analysis of transcription-factor binding affinity. *Cell* **124**, 47-59.
Hammerschmidt, M., Bitgood, M. J. and McMahon, A. P. (1996). Protein kinase A is a common negative regulator of Hedgehog signalling in the vertebrate embryo. *Genes Dev.* **10**, 647-658.
Harvey, S. A. and Smith, J. C. (2009). Visualisation and quantification of morphogen gradient formation in the zebrafish. *PLoS Biol.* **7**, e1000101.
Henningfeld, K. A., Rastegar, S., Adler, G. and Knochel, W. (2000). Smad1 and Smad4 are components of the bone morphogenetic protein-4 (BMP-4)-induced transcription complex of the Xvent-2B promoter. *J. Biol. Chem.* **275**, 21827-21835.
Hirsinger, E., Stellabotte, F., Devoto, S. H. and Westerfield, M. (2004). Hedgehog signalling is required for commitment but not initial induction of slow muscle precursors. *Dev. Biol.* **275**, 143-157.
Hoodless, P. A. (1996). MADR1, a MAD-related protein that functions in BMP2 signalling pathways. *Cell* **85**, 489-500.
Hu, C. D., Chinenov, Y. and Kerppola, T. K. (2002). Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* **9**, 789-798.
Humke, E. W., Dorn, K. V., Milenkovic, L., Scott, M. P. and Rohatgi, R. (2010). The output of Hedgehog signalling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. *Genes Dev.* **24**, 670-682.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signalling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.
Ingham, P. W. and Kim, H. R. (2005). Hedgehog signalling and the specification of muscle cell identity in the zebrafish embryo. *Exp. Cell Res.* **306**, 336-342.
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.
Kawakami, A., Nojima, Y., Toyoda, A., Takahoko, M., Satoh, M., Tanaka, H., Wada, H., Masai, I., Terasaki, H., Sakaki, Y. et al. (2005). The zebrafish-secreted matrix protein you/scube2 is implicated in long-range regulation of hedgehog signalling. *Curr. Biol.* **15**, 480-488.
Kawakami, T., Kawcak, T., Li, Y. J., Zhang, W., Hu, Y. and Chuang, P. T. (2002). Mouse *dispatched* mutants fail to distribute hedgehog proteins and are defective in hedgehog signalling. *Development* **129**, 5753-5765.
Koudijs, M. J., den Broeder, M. J., Keijser, A., Wienholds, E., Houwing, S., van Rooijen, E. M., Geisler, R. and van Eeden, F. J. (2005). The zebrafish mutants *dre*, *uki*, and *lep* encode negative regulators of the hedgehog signalling pathway. *PLoS Genet.* **1**, e19.
Koudijs, M. J., den Broeder, M. J., Groot, E. and van Eeden, F. J. (2008). Genetic analysis of the two zebrafish *patched* homologues identifies novel roles for the hedgehog signalling pathway. *BMC Dev. Biol.* **8**, 15.
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.
Kretzschmar, M., Liu, F., Hata, A., Doody, J. and Massague, J. (1997). The TGF-β family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. *Genes Dev.* **11**, 984-995.
Laner-Plamberger, S., Kaser, A., Paulischta, M., Hauser-Kronberger, C., Eichberger, T. and Frischauf, A. M. (2009). Cooperation between GLI and JUN enhances transcription of JUN and selected GLI target genes. *Oncogene* **28**, 1639-1651.
Lee, E. C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D. A., Court, D. L., Jenkins, N. A. and Copeland, N. G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* **73**, 56-65.
Liem, K. F., Jr, Jessell, T. M. and Briscoe, J. (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. *Development* **127**, 4855-4866.
Liew, H. P., Choksi, S. P., Wong, K. N. and Roy, S. (2008). Specification of vertebrate slow-twitch muscle fiber fate by the transcriptional regulator Blimp1. *Dev. Biol.* **324**, 226-235.
Liu, F. (1996). A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature* **381**, 620-623.
Liu, F., Massague, J. and Ruiz i Altaba, A. (1998). Carboxy-terminally truncated Gli3 proteins associate with Smads. *Nat. Genet.* **20**, 325-326.
Logan, C., Khoo, W. K., Cado, D. and Joyner, A. L. (1993). Two enhancer regions in the mouse En-2 locus direct expression to the mid/hindbrain region and mandibular myoblasts. *Development* **117**, 905-916.
Ma, Y., Erkner, A., Gong, R., Yao, S., Taipale, J., Basler, K. and Beachy, P. A. (2002). Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched. *Cell* **111**, 63-75.
Monteiro, R., van Dinther, M., Bakkers, J., Wilkinson, R., Patient, R., ten Dijke, P. and Mummery, C. (2008). Two novel type II receptors mediate BMP signalling and are required to establish left-right asymmetry in zebrafish. *Dev. Biol.* **315**, 55-71.
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.
Nikaido, M., Tada, M. and Ueno, N. (1999). Restricted expression of the receptor serine/threonine kinase BMPR-IB in zebrafish. *Mech. Dev.* **82**, 219-222.
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.
Patten, I. and Placzek, M. (2002). Opponent activities of Shh and BMP signalling during floor plate induction in vivo. *Curr. Biol.* **12**, 47-52.
Patterson, S. E., Bird, N. C. and Devoto, S. H. (2010). BMP regulation of myogenesis in zebrafish. *Dev. Dyn.* **239**, 806-817.
Pearse, R. V., 2nd, Collier, L. S., Scott, M. P. and Tabin, C. J. (1999). Vertebrate homologs of *Drosophila suppressor of fused* interact with the gli family of transcriptional regulators. *Dev. Biol.* **212**, 323-336.
Rissi, M., Wittbrodt, J., Délot, E., Naegeli, M. and Rosa, F. M. (1995). Zebrafish Radar: a new member of the TGF-β superfamily defines dorsal regions of the neural plate and the embryonic retina. *Mech. Dev.* **49**, 223-234.
Roy, S., Wolff, C. and Ingham, P. W. (2001). The *u-boot* mutation identifies a Hedgehog-regulated myogenic switch for fiber-type diversification in the zebrafish embryo. *Genes Dev.* **15**, 1563-1576.
Ruiz i Altaba, A. (1998). Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. *Development* **125**, 2203-2212.
Saka, Y., Hagemann, A. I., Piepenburg, O. and Smith, J. C. (2007). Nuclear accumulation of Smad complexes occurs only after the midblastula transition in Xenopus. *Development* **134**, 4209-4218.
Sasaki, H., Hui, C., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for *HNF-3beta* floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-1322.
Scheer, N. and Campos-Ortega, J. A. (1999). Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. *Mech. Dev.* **80**, 153-158.
Souren, M., Martinez-Morales, J., Makri, P., Wittbrodt, B. and Wittbrodt, J. (2009). A global survey identifies novel upstream components of the Ath5 neurogenic network. *Genome Biol.* **10**, R92.
Stamataki, D., Ulloa, F., Tsoni, S. V., Mynett, A. and Briscoe, J. (2005). A gradient of Gli activity mediates graded Sonic Hedgehog signalling in the neural tube. *Genes Dev.* **19**, 626-641.
Stone, D. M., Murone, M., Luoh, S., Ye, W., Armanini, M. P., Gurney, A., Phillips, H., Brush, J., Goddard, A., de Sauvage, F. J. et al. (1999). Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli. *J. Cell Sci.* **112**, 4437-4448.
Suzuki, A., Chang, C., Yingling, J. M., Wang, X. F. and Hemmati-Brivvanlous, A. (1997). Smad5 induces ventral fates in *Xenopus* embryo. *Dev. Biol.* **184**, 402-405.
Tanimoto, H., Itoh, S., ten Dijke, P. and Tabata, T. (2000). Hedgehog creates a gradient of DPP activity in *Drosophila* wing imaginal discs. *Mol. Cell* **5**, 59-71.
Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degrave, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X. Q. et al. (2001). Expression of the zebrafish genome during embryogenesis (NIH R01 RR15402). ZFIN Direct Data Submission (http://zfin.org).
Thomsen, G. H. (1996). Xenopus mothers against decapentaplegic is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor. *Development* **122**, 2359-2366.
Ulloa, F., Itasaki, N. and Briscoe, J. (2007). Inhibitory Gli3 activity negatively regulates Wnt/beta-catenin signalling. *Curr. Biol.* **17**, 545-550.
Urasaki, A., Morvan, G. and Kawakami, K. (2006). Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. *Genetics* **174**, 639-649.
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.
Vokes, S. A., Ji, H., McCuine, S., Tenzen, T., Giles, S., Zhong, S., Longabaugh, W. J., Davidson, E. H., Wong, W. H. and McMahon, A. P. (2007). Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning. *Development* **134**, 1977-1989.
Vokes, S. A., Ji, H., Wong, W. H. and McMahon, A. P. (2008). A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. *Genes Dev.* **22**, 2651-2663.
von Hofsten, J., Elworthy, S., Gilchrist, M. J., Smith, J. C., Wardle, F. C. and Ingham, P. W. (2008). Prdm1- and Sox6-mediated transcriptional repression specifies muscle fibre type in the zebrafish embryo. *EMBO Rep.* **9**, 683-689.
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.
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.
Wieser, R., Wrana, J. L. and Massague, J. (1995). GS domain mutations that constitutively activate TβR-I, the downstream signalling component in the TGF-β receptor complex. *EMBO J.* **14**, 2199-2208.
Winklmayr, M., Schmid, C., Laner-Plamberger, S., Kaser, A., Aberger, F., Eichberger, T. and Frischauf, A. M. (2010). Non-consensus GLI binding sites in Hedgehog target gene regulation. *BMC Mol. Biol.* **11**, 2.
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.