Integration of Hedgehog and BMP signalling by the *engrailed*2a gene in the zebrafish myotome

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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*)^269^] 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.
Integration of Hedgehog and BMP signalling by the engrailed2a gene in the zebrafish myotome

Ashish K. Maurya1,2, Haihan Tan1, Marcel Souren1,3, Xingang Wang1, Joachim Wittbrodt3 and Philip W. Ingham1,2,*

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 morphogenetic 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 Pdm1a 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; Thissle et al., 2001) as well as by the expression of other genes, such as Wnt11 (Thissle et al., 2001).

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 (Li 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 (Gli1A) and repressor (Gli1R) forms of Gli (Aza-Blanc et al., 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 (Gli1A) and repressor (Gli1R) forms of Gli (Aza-Blanc et al., 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 (Gli1A) and repressor (Gli1R) forms of Gli (Aza-Blanc et al., 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.

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have sequences similar or identical to the Gli consensus-binding site upstream of their promoters (Agren et al., 2004; Alexandre et al., 1996; Dui 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 in the AVA (Singapore) were described previously (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 reporter construct was made by ScaI digestion and blunt-ended ligation into the miniTol2 vector (Urasaki et al., 2006).

Upstream activation sequence (UAS) bi-cistronic constructs

Recombining 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 Scal digestion and blunt-ended ligation into the miniTol2 vector (Urasaki et al., 2006).

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).
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 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 Eng2 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

<table>
<thead>
<tr>
<th>Reporter constructs</th>
<th>Allele designation</th>
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<tbody>
<tr>
<td>–10 kb-eGFP</td>
<td>Tg[eng2a:eGFP]223</td>
</tr>
<tr>
<td>–8 kb-eGFP</td>
<td>Tg[eng2a:eGFP]224</td>
</tr>
<tr>
<td>–6.6 kb-eGFP (ME)</td>
<td>Tg[eng2a:eGFP]225</td>
</tr>
<tr>
<td>–7.4 kb-eGFP</td>
<td>Tg[eng2a:eGFP]226</td>
</tr>
<tr>
<td>–7.5 kb-eGFP</td>
<td>Tg[eng2a:eGFP]227</td>
</tr>
<tr>
<td>–7.6 kb-eGFP</td>
<td>Tg[eng2a:eGFP]228</td>
</tr>
<tr>
<td>–6.4 kb-eGFP</td>
<td>Tg[eng2a:eGFP]229</td>
</tr>
<tr>
<td>–6.5 kb-eGFP</td>
<td>Tg[eng2a:eGFP]230</td>
</tr>
<tr>
<td>–5.4 kb-eGFP</td>
<td>Tg[eng2a:eGFP]231</td>
</tr>
<tr>
<td>–5.3−4.5 kb-eGFP</td>
<td>Tg[eng2a:eGFP]232</td>
</tr>
<tr>
<td>–7.4−7.2 kb-eGFP</td>
<td>Tg[eng2a:eGFP]233</td>
</tr>
<tr>
<td>–8−6.8 kb-eGFP</td>
<td>Tg[eng2a:eGFP]234</td>
</tr>
<tr>
<td>–8.6−6 kb-eGFP</td>
<td>Tg[eng2a:eGFP]235</td>
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<tr>
<td>–7.8−6.8 kb-eGFP</td>
<td>Tg[eng2a:eGFP]236</td>
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<td>Tg[eng2a:eGFP]238</td>
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<tr>
<td>–7−6 kb-eGFP</td>
<td>Tg[eng2a:eGFP]239</td>
</tr>
<tr>
<td>–7.4 kb-eGFP</td>
<td>Tg[eng2a:eGFP]240</td>
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<td>–758</td>
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**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., 2002), we focused our analysis on this candidate trans-regulator.

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

Phosphorylation of Smads 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 eng2a expression when Smad activity is attenuated. eng expression and MP specification (Du et al., 1997; Kawakami et al., 2005), we focused our analysis on this candidate trans-regulator.

**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 disp1MO mutant allele have reduced numbers of slow-twitch muscle fibres and especially lack MPs and MFFs (Nakano et al., 2004). In homozygous disp1MO 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 BMP1Rba 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 ChpPed 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 (Bakker 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, CTAGTCACCACCCACAC, 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)$_{233}$ 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 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$^{ii}$ 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
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 disp1tf18b heterozygotes that also carry the actin:GAL4 driver and the Tg(eng2a:eGFP)i233 transgenes. The transient transgenic resultant disp1tf18b 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 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 bmp1ba MO was simultaneously injected with the constructs (Fig. 6E,F), implying that the

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 chromatim and also against IgG-precipitated chromatim. 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.
762 RESEARCH ARTICLE

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...
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 Gli8 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 GliR 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 GliR 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 GliR levels in response to Shh acts principally to deplete nuclear activity was abrogated reinforces the notion that the reduction of GliR levels in response to Shh acts principally to deplete nuclear activity but rather the relative levels of GliA and pSmad present to activate 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.

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Integration of Hedgehog and BMP signals


