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Sulf1 influences the Shh morphogen gradient during the dorsal ventral patterning of the neural tube in *Xenopus tropicalis*

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ABSTRACT

Genetic studies have established that heparan sulphate proteoglycans (HSPGs) are required for signalling by key developmental regulators, including Hedgehog, Wnt/Wg, FGF, and BMP/Dpp. Post-synthetic remodelling of heparan sulphate (HS) by Sulf1 has been shown to modulate these same signalling pathways. *Sulf1* codes for an N-acetylglucosamine 6-O-endosulfatase, an enzyme that specifically removes the 6-O sulphate group from glucosamine in highly sulfated regions of HS chains. One striking aspect of *Sulf1* expression in all vertebrates is its co-localisation with that of *Sonic hedgehog* in the floor plate of the neural tube. We show here that *Sulf1* is required for normal specification of neural progenitors in the ventral neural tube, a process known to require a gradient of Shh activity. We use single-cell injection of mRNA coding for GFP-tagged Shh in early *Xenopus* embryos and find that Sulf1 restricts ligand diffusion. Moreover, we find that the endogenous distribution of Shh protein in Sulf1 knockdown embryos is altered, where a less steep ventral to dorsal gradient forms in the absence of Sulf1, resulting in more a diffuse distribution of Shh. These data point to an important role for Sulf1 in the ventral neural tube, and suggests a mechanism whereby Sulf1 activity shapes the Shh morphogen gradient by promoting ventral accumulation of high levels of Shh protein.

Introduction

During embryogenesis, initially totipotent cells become progressively restricted in their developmental potential as they commit to develop along particular cell lineages. During this process, groups of progenitor cells are established and proliferate before differentiating as specific cell types. In the case of neural progenitors that form in the ventral spinal cord, several distinct pools of progenitor cells are induced by one signal, Sonic hedgehog (Shh), which acts as a morphogen to specify discrete precursor populations at specific positions along the dorsoventral axis of the developing neural tube (Briscoe and Ericson, 1999; Briscoe and Novitch, 2008). The class of progenitor pool specified depends on the local concentration of Shh perceived by the responding cells. The sources of Shh are localised ventrally, in the floor plate (FP) of the developing spinal cord and in the notochord (NC). High levels of Shh give rise to ventrally positioned motor neuron progenitors while lower concentrations give rise to the more dorsal interneurons; these progenitor populations arise in spatially distinct regions and express unique combinations of transcription factors that can serve as markers for specific cell types (Briscoe et al., 2000). Although further layers of complexity include the effects of the duration of Shh signalling (Dessaud et al., 2007) and the transcriptional output of responding cells (Ribes et al., 2010), the generation of positional identity in the ventral spinal cord by a Shh morphogen is supported by robust experimental evidence (Ingham and McMahon, 2001; Jessell, 2000).

Mature Shh protein is highly processed; after signal peptide cleavage and an internal cleavage removing the C-terminus of the pro-protein, the remaining 19kd N-terminal signalling domain is modified by the addition of a C-terminal cholesterol group and an N-terminal palmitate group (Mann and Beachy, 2004). The fatty nature of the Shh ligand, and the fact its cholesterol group has been shown to associate tightly with cell membranes (Peters et al., 2004), presents a question as to how this protein can diffuse to form a morphogen gradient. Some evidence suggests that this is accomplished by the formation of multimeric Shh complexes which relies on the lipid modifications (Zeng et al., 2001). High molecular weight, multimeric forms of Shh have been shown to be active (Callejo et al., 2006; Chen et al., 2004), while monomeric Shh lacking lipid modification can diffuse further but has lower activity. In addition, cell surface heparan sulphate proteoglycans (HSPGs) have been shown to be critical for the formation of multimeric Shh complexes and the establishment of a morphogen gradient (Guerrero and Chiang, 2007).

Heparan sulphate proteoglycans (HSPGs) are essential for hedgehog signalling (Lin, 2004). Shh binds to HSPGs via a Cardin–Weintraub protein–heparin interaction domain (Rubin et

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al., 2002; Farshi et al., 2011) deletion of this sequence results in a failure to generate high molecular weight, visible clusters of Shh (Vyas et al., 2008). HSPGs consist of a protein core to which glycosaminoglycan (GAG) chains are attached. These unbranched chains of disaccharide repeats can be differentially modified by sulfation; this results in a high degree of structural heterogeneity and allows HSPGs to bind many different proteins (Turnbull et al., 2001). The enzymes Sulf1 and Sulf2 can act at the cell surface to remodel HSPG structure by specifically removing a sulphate group from the 6-O position of glucosamine in heparan sulphate (HS) chains. This modification changes the affinity of HS for ligands and receptors and impacts cell signalling (Ai et al., 2006; Freeman et al., 2008; Lai et al., 2004).

Some recent work in *Drosophila* has pointed to a role for Sulf1 in influencing the activity of hedgehog (Hh) signalling in the wing imaginal disc (Wojcinski et al., 2011). The distribution of endogenous Hh was found to change in the absence of DSulf1, becoming more evenly distributed along the wing disc and not accumulating in the normal defined regions of high concentration (Wojcinski et al., 2011). Work in chick showed that Sulf1 overexpression enhanced cell surface accumulation of Shh and concluded that cell autonomous Sulf activity can promote the high levels of Shh signalling that is required for oligodendrocyte precursor cell (OPC) specification (Danesin et al., 2006). Consistent with this, analyses of Sulf1$^{-/-}$ mice revealed that in the absence of Sulf1 fewer OPCs form (Touahri et al., 2012). Shh is known to have higher affinity for highly sulphated heparin than it does to less sulphated HSPGs derived from tissues (Dierker et al., 2009) although any specific requirement for 6-O sulfated HS has not been determined.

Sulf1 is highly expressed in the floor plate of all vertebrate embryos investigated so far (Dhoot et al., 2001; Freeman et al., 2008; Gorsi et al., 2010; Ohto et al., 2002; Winterbottom and Pownall, 2009). Given that Shh signalling is known to require HSPGs, we have investigated whether Sulf1 has a role in the specification of neural progenitor cells in the developing ventral spinal cord that is known to require a gradient of Shh activity. Using morpholino knock-down of *Sulf1* in *Xenopus tropicalis* we have determined that Sulf1 is required for the normal dorsal–ventral patterning of the neural tube. In addition, we find that Sulf1 activity influences the distribution of GFP-tagged Shh in tissue explants. Our finding that endogenous *Sulf1* is required for the normal distribution of Shh protein suggests that one possible mechanism by which Sulf1 could be influencing the patterning of ventral neural tube is by shaping the gradient of the Shh morphogen.

Material and methods

in situ hybridisation and immunohistochemistry

Embryos for in situ hybridisation and immunohistochemistry were fixed in MEMFA for one hour at room temperature and then stored in methanol. Embryos were processed for whole mount in situ hybridisation as previously described (Harland, 1991). For synthesis of DIG labelled antisense RNA, templates were generated by linearising plasmid DNA and transcribing with the appropriate polymerase the plasmids used were Ptc2 (IMAGE:7615868), Shh (TNeu023n04), and Sulf1(7.7) Freeman et al., 2008.

For immunohistochemistry on cryosections, embryos were rehydrated (100 mM Tris–Hcl + 100 mM NaCl (pH7.4)) for 30 min, mounted (25% cold water fish gelatin + 15% sucrose), cryosectioned and stored at −80 °C until required. Slides were dried for 1 h at room temperature, washed in acetone for 2 min, re-dried and washed in PBSTx. Samples were then blocked for 1 h at room temperature in PBSTx (2% BSA, 5% goat serum). For analysis of Shh, embryos were processed whole mount; fixed 20 min in MEMFA at 4 °C, washed in PBSTx then blocked as above. Both whole mount specimens and cryosections were incubated in primary antibody for 72 h at 4 °C. Primary antibodies were used at the following concentrations: Nkx2.2 (DSHB) 1/5, Nkx6.1 (DSHB)1/5, HB9 (DSHB) 1/10, Isl1 (DSHB) 1/5, phosphoH3 (Millipore) 1/500, Shh (DSHB) 1/5. Following washes in PBSTx, samples were incubated in 1/250 anti-mouse Alexa555 antibody (Life Technoligies) in block along with DAPI (1/50,000) for 90 min at room temperature (slides) or incubated overnight at 4 °C (whole mount). Slides were washed and mounted in Vectashield mounting medium. Whole mount samples were washed in PBSTx and refixed in 4% formaldehyde in PBS then processed for cryosectioning as described above.

Microinjection

Morpholino oligonucleotides (MOs) were designed by GeneTools and SulfMO was directed against the splice junction of exon 2/3 of *XtSulf1* (as described in Freeman et al., 2008; S1AMO 5′ ataagaaaactctcacctaactcc 3′) The SulfMO was heated at 55 °C for 5 min immediately before injection into the four animal blastomeres of *X. tropicalis* embryos at the eight cell stage in order to target the neural tube. *X. tropicalis* embryos were generated and cultured according to protocols on the Harland website (http://tropicalis.berkeley.edu/home/). The control MO was provided by GeneTools.

For the diffusion assays, *X. laevis* embryos were injected at the 2-cell stage with 10 nl (4 ng) of Sulf1 mRNA (or control mRNA) and then again at the 32-cell stage with a volume of 1.25 nl (1 ng) of Shh-GFP mRNA (and lineage tracer) into a single cell. In other experiments, one or two individual cells were injected with 1.25 nl (1 ng) of mRNAs at the 32-cell stage (described in Fig. 3).

Quantifying fluorescent images

Animal caps were cultured for three hours at 21 °C in NAM/2 before mounting on relief slides (Thermo Fisher Scientific) and imaged by confocal microscopy (LSM 710 (Carl Zeiss)) using Zen software (2008–2010) (Carl Zeiss). Fluorescence levels were quantified using the plot profile function in ImageJ. Images were manipulated using ImageJ and Adobe Photoshop CS5. For the quantification of animal cap data, a single 2 μm plane was taken from 8 control and 7 Sulf1 injected embryos and a 30 × 600 pixel rectangle was drawn starting from the source cells and oriented away from them. The mean pixel intensity across this box was then plotted as a function of distance. To quantify the level of Shh immunostaining in the neural tube, 12 μm Z-stacks were taken from 5 control and 7 Sulf1 knockdown embryos and the average grey level across the neural tube was plotted as a function of distance.

Results

Co-expression of Sulf1 with Shh in the floorplate is required for normal Shh signalling

Expression analysis of Sulf1(Freeman et al., 2008) and Shh (Khokha et al., 2005) during *X. tropicalis* development shows little overlap except in the floor plate of the neural tube (Fig. 1). At neural plate stages, Sulf1 is expressed in the paraxial mesoderm (Fig. 1A and C), while Shh is expressed in the midline of the open neural plate and in the underlying notochord (Fig. 1B and D). By neural tube closure (NF stage 21), Sulf1 and Shh are co-expressed

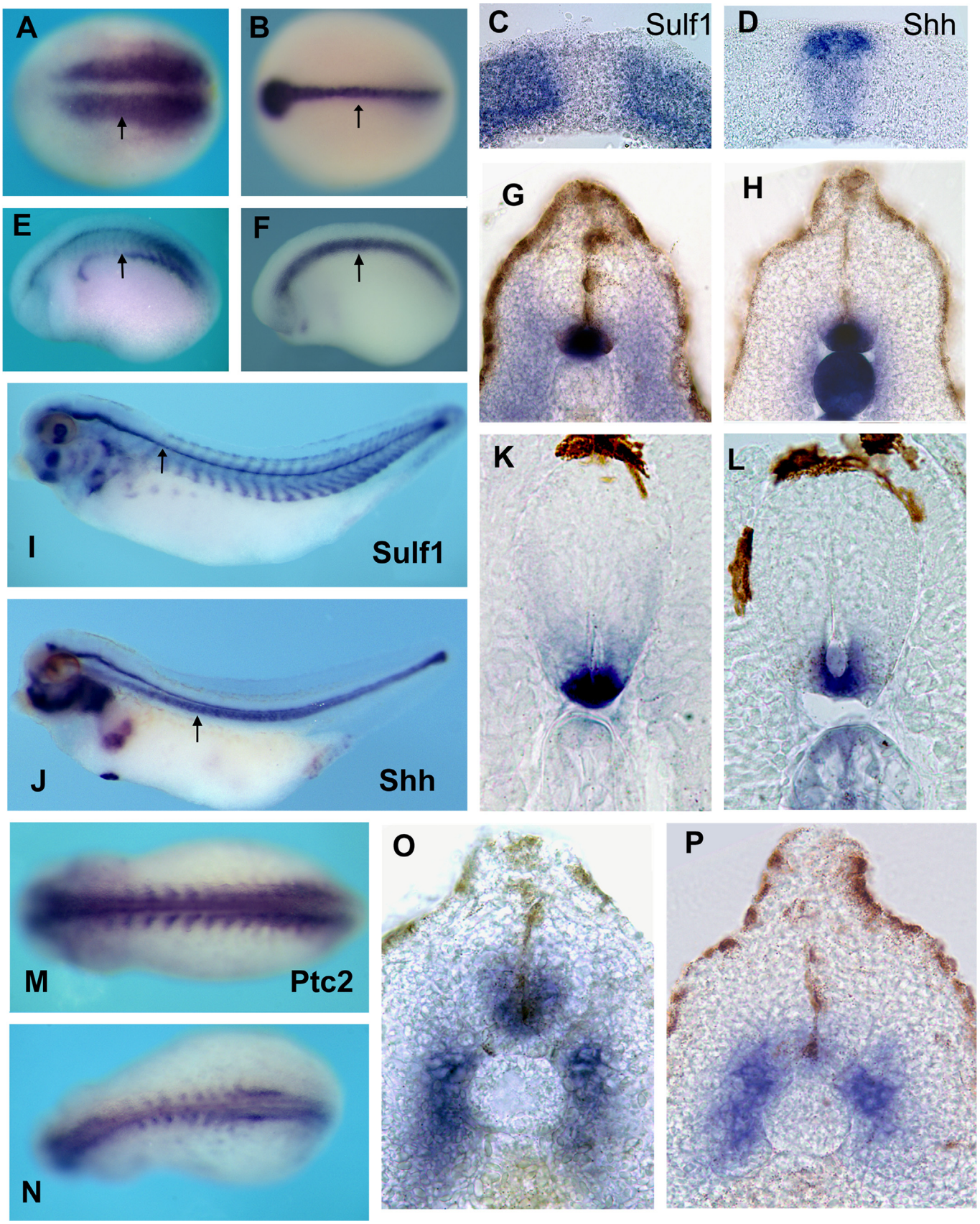

Fig. 1. Sulf1 is co-expressed with Shh in the floor plate of the neural tube in *Xenopus tropicalis* and is required for normal Shh signalling. in situ hybridisation shows the normal expression pattern of *Sulf1* (A,C,E, G, I, K) and *Shh* (B, D, F, H, J, L) at stages 15 (A–D) 23 (E–H), and 35 (I–L). Arrows in (A, B, E, F, I, J) indicate level where the vibratome sections were taken at these same stages and are shown in (C, D, G, H, K, L). *Sulf1* is expressed in the paraxial mesoderm at Stage 15 (A, C) while *Shh* is expressed in the floor plate and notochord (B, D). By stage 23, both *Sulf1* and *Shh* are expressed in the floor plate (E–H), and this co-expression is still apparent at stage 35 (I–L). Knockdown of Sulf1 using a splice blocking morpholino oligo shows a reduced level of ptc expression in the neural tube (N, P) as compared to those injected with an equal amount of a control morpholino (M, O).

in the floor plate (Fig. 1E–H), while maintaining distinct regions of expression in the somites and pronephros (Sulf1) and the notochord (Shh). At later tailbud stages, many different expression domains are apparent for Sulf1 and Shh, while their co-expression in the floor plate persists (Fig. 1I–L).

To determine any requirement for Sulf1 in Shh signalling we injected an antisense morpholino oligo targeted against the exon2/intron2 boundary of Sulf1 (Freeman et al., 2008) and confirmed knock-down by rtPCR (Supplementary data Fig. S1). The expression of the hedgehog receptor *patched* is known to be a direct transcriptional response to Shh signalling (Alexandre et al., 1996) and in *Xenopus ptc2* is expressed in tissues known to be responsive to Shh (Takabatake et al., 2000) including the neural tube and somites (Fig. 1M and O). In embryos in which Sulf1 has been knocked down we find a reduced level of *ptc2* expression in the neural tube (Fig. 1N and P). This effect can be rescued by co-injecting mRNA coding for Sulf1 (Supplementary data, Fig. S1), indicating that the effects of knocking down *Sulf1* are specific and that Sulf1 is important for normal hedgehog signalling in the developing neural tube.

Sulf1 is required for the normal patterning of neural progenitors

Extensive literature describes the role of a Shh morphogen gradient in the specification of the distinct pools of neural progenitor cells that form along the dorsoventral axis of the vertebrate neural tube (Ericson et al., 1997; Dessaud et al., 2008). These progenitor pools can be identified by their expression of specific transcription factors (Briscoe et al., 2000). Here we use antibodies that recognise Nkx2.2 to mark cells that give rise to V3 interneurons (Fig. 2A–D and A′–D′), Nkx6.1 to mark cells that give rise to motor neurons, V3 and V2 interneurons (Fig. 2E–H and E′–H′), and HB9 to mark motor neurons (Fig. 2I–L and I′–L′). Islet-1 marks neurons in three distinct domains: ventrally, Islet-1 is expressed in a few motor neurons, while more dorsally it is expressed in some ventral interneurons and also in some dorsal interneuron cells (Fig. 2M–P and M′–P′). Sulf1 was knocked-down in *X. tropicalis* embryos and the expression of these neural markers was assayed by immunohistochemistry on cryosections taken at NF stages 23 (Fig. 2A–P) and 40 (Fig. 2A′–P′). In the absence of Sulf1, Nkx2.2$^+$ cells are found in a more ventral position, spanning the floor plate from which they are normally excluded. The ventral shift of Nkx2.2 at stage 23 is shown in Fig. 2C, D ($n=12$, 75% show this phenotype) and at stage 40 is shown in Fig. 2C′, D′ ($n=18$, 67% show this phenotype). A similar ventral shift in Nkx2.2 is observed in mice which lack either hedgehog co-receptors or other factors required for Shh signalling (Allen et al. 2007; Tenzen et al. 2006; Endoh-Yamagami et al., 2009).

At stage 23, Sulf1 knockdown does not seem to affect Nkx6.1 expression shown in Fig. 2G, H ($n=8$, 100% show this phenotype). However, at stage 40, in the absence of Sulf1, Nkx6.1 expression shows a ventral shift that is similar to Nkx2.2 and is shown in Fig. 2G′, H′ ($n=20$, 70% show this phenoptype). These data indicate that the P3 progenitors are forming in a more ventral position in the absence of Sulf1. A reduced number of HB9$^+$ cells is apparent in Sulf1 knockdown embryos at stage 23 shown in Fig. 2K, L ($n=13$, 77% show this phenoptype) as well as a loss of Islet-1$^+$ cells in this same region shown in Fig. 2O, P ($n=8$, 63% show this phenotype) is consistent with a reduced number of motor neuron progenitors at Stage 23. In contrast, at stage 40, the numbers of HB9$^+$ motor neurons in Sulf1 morphants are not reduced when compared with controls and have increased since stage 23, shown in Fig. 2K′, L′ ($n=19$, 68% show this phenotype). The expression of Isl1 in the motor neuron domain is no longer reliably detected at stage 40 and this is not changed in the Sulf1 knock-downs (Fig. 2M′–P′). The finding that embryos lacking Sulf1 display a decreased number of motor neuron progenitors early (stage 23), which increases later (stage 40), is consistent with a failure of the motor neuron to oligodendricyte precursor cell (OPC) switch described in Sulf1$^{-/-}$ mice (Touahri et al., 2012).

Overall these effects of Sulf1 knockdown are consistent with a reduction in Shh signalling perceived by the cells in the ventral NT. However, when Shh signalling is pharmacologically inhibited using the Smoothened inhibitor cyclopamine (Chen et al., 2002), a complete loss of Nkx2.2$^+$ cells and a dramatic reduction in the numbers of HB9$^+$ motor neurons is observed at stage 40 (Supplementary data Fig. S2). This demonstrates that while the depletion of Sulf1 is consistent with a reduction in Shh signalling, it does not represent a complete loss. A recent, similar analysis in zebrafish (Oustah et al., 2014) has used two different doses of cyclopamine and found that the higher dose completely blocks the expression of a motor neuron marker, while in a lower concentration of cyclopamine the expression this marker persists, but is ventrally shifted. This is similar to what we have found in *Xenopus* embryos lacking Sulf1. These data therefore point to a role for Sulf1 in modulating the level of Shh activity during the dorsoventral patterning of the neural tube.

Loss of Sulf1 results in fewer proliferative cells in the neural tube

In addition to the well characterised role for Shh in patterning neural progenitor cell type in the ventral neural tube, Shh also promotes progenitor cell proliferation (Ulloa and Briscoe, 2007). To analyse any requirement for Sulf1 in cell proliferation in the neural tube, immunohistochemistry for the marker phospho-Histone 3 was carried out on sections from *X. tropicalis* embryos injected with either control MO or our antisense MO targeted against Sulf1. Fig. 3 shows that in embryos lacking Sulf1, the number of mitotic cells in the neural tube at NF stage 22 is significantly decreased compared to controls. These data indicate that Sulf1 is required for the dual roles of Shh in neural patterning and progenitor cell proliferation.

Sulf1 affects the distribution of Shh

Genetic studies have shown that he diffusion of Hedgehog in the *Drosophila* wing disc requires the presence of heparan sulphate (Bellaiche et al., 1998; The et al., 1999). More recent studies suggest that modification of HSPGs by DSulf1 can change the distribution of Hedgehog in the wing disc (Wojcinski et al., 2011). To test whether this is the case in vertebrates, we carried out experiments where mRNA coding for Shh-GFP (Chamberlain et al., 2008), along with that of a lineage tracer, was injected into a single blastomere of a 32-cell stage *X. laevis* embryo to create a small clone of cells expressing Shh-GFP. After several hours of development animal cap explants were dissected and imaged by confocal microscopy to visualise the distribution of the Shh ligand in an intact field of embryonic cells. Control embryos analysed in these experiments were injected with mRNA coding for beta galactosidase or the catalytically inactive Sulf1CA; the results obtained using either control did not differ from uninjected embryos.

In the first set of experiments, Sulf1 was expressed throughout the animal hemisphere by injecting mRNA coding for Sulf1 into both blastomeres at the 2-cell stage; this was followed by the later injection of Shh-GFP into a single cell at the 32-cell stage (the cartoon in Fig. 4A and B shows only show the later injection). In controls, Shh-GFP is distributed as discrete punctae over several cell diameters (Fig. 4C and D). In explants where Sulf1 was expressed though out the field of cells, the distribution of Shh-GFP is greatly restricted (Fig. 4E and F) and the ligand forms elongated aggregates on cell membranes.

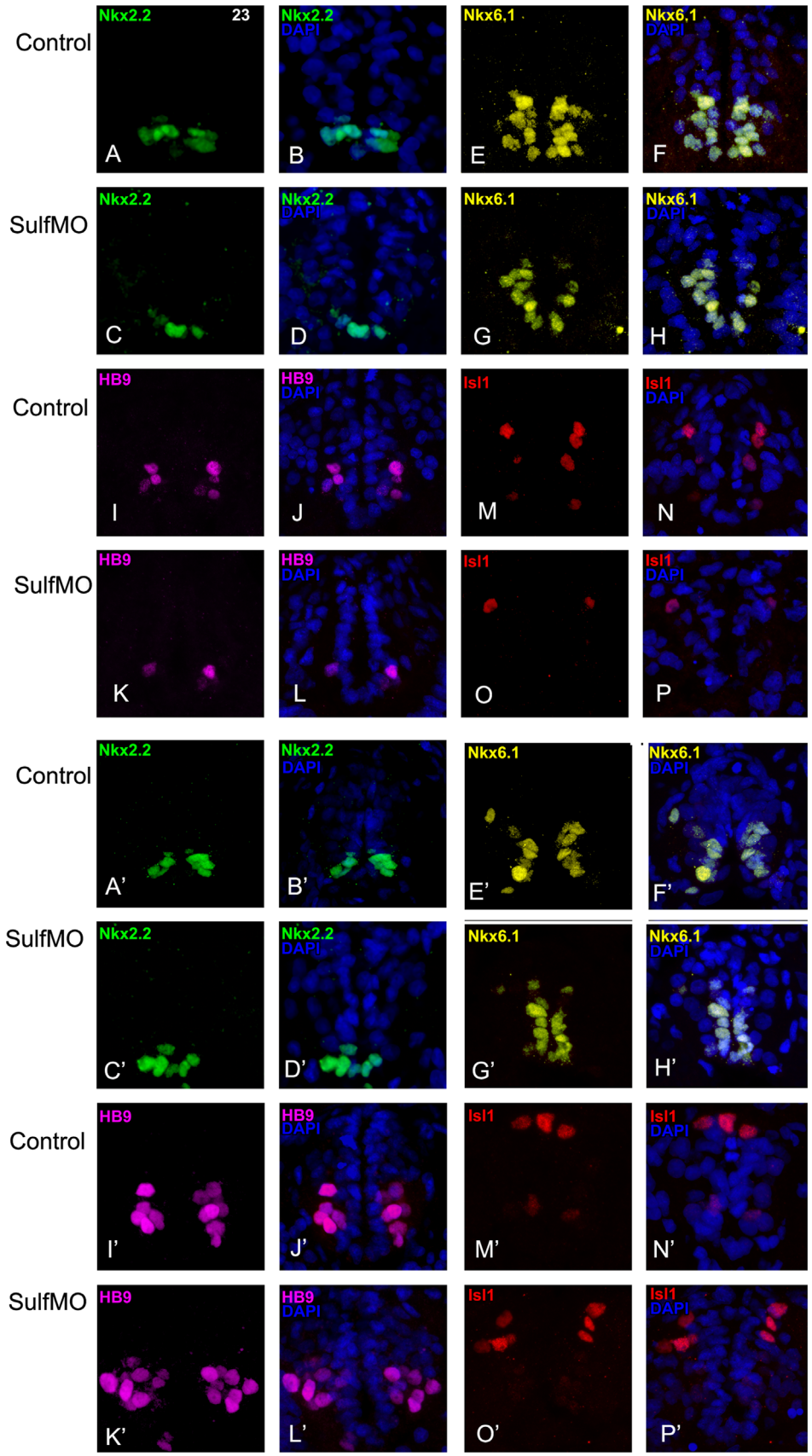

Fig. 2. Sulf1 is required for correct DV patterning in the vertebrate neural tube. Immunostaining for Nkx2.2 (A–D), Nkx6.1 (E–H), HB9 (I–L) and Isl1 (M–P) in control (A, B, E, F, I, J, M, N) and Sulf1 knockdown (C, D, G, H, K, L, O, P) *X. tropicalis* embryos at stages 23 (A–P) and stage 40 (A′–P′). At stage 23, the expression of Nkx2.2 is shifted ventrally in Sulf knockdown embryos (C, D; $n=12$, 75%) compared with controls (A, B; $n=8$, 100%). At stage 40, the expression of Nkx2.2 is also shifted ventrally in Sulf knockdown embryos (C′, D′; $n=18$, 67%) compared with controls (A′, B′; $n=11$, 100%). Sulf1 knockdown only leads to a small change in Nkx6.1 expression at stage 23 (G, H; $n=8$, 63%), compared to controls (E, F; $n=8$, 100%). At stage 40 the expression of Nkx6.1 is shifted ventrally (G′, H′; $n=20$, 70%), compared to controls (E,′ F′; $n=12$, 100%). HB9 staining similarly reveals differences between the stages, at stage 23, the expression of HB9 is reduced (K, L; $n=12$, 75%) compared with controls (I, J; $n=7$, 100%). Cells positive for HB9 in Sulf1 knockdown embryos increases at stage 40 (K′, L′; $n=19$, 69%). At stage 23, the expression of Isl1 reduced in the MN domain of Sulf knockdown embryos (O, P; $n=8$, 63%) compared with controls (M, N; $n=12$, 100%); whereas at stage 40 Isl1 is not reliably detectable in this domain in Sulf1 knockdowns (O′, P′; $n=2$, 100%) or in controls (M′, N′; $n=5$, 100%).

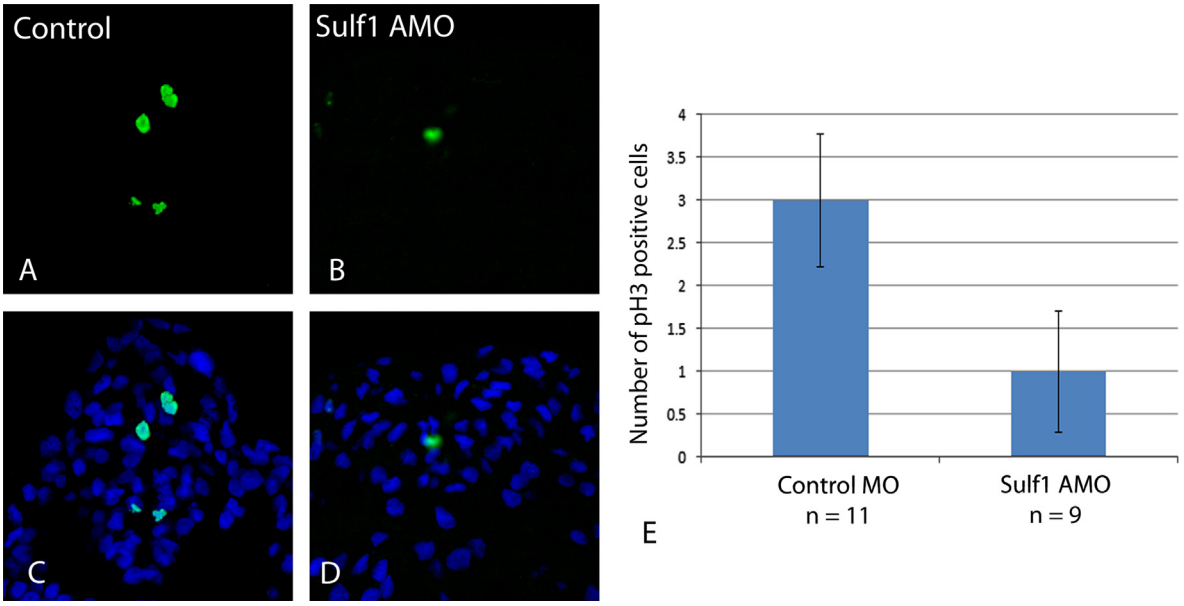

Fig. 3. Sulf1 is required for normal proliferation in the neural tube. (A, C) Antibody staining for the mitotic cell marker phosphor-Histone3 (pH3) reveals the normal number of cells in mitosis in the neural tube at NF stage 22 ($n=11$, range from 2 to 4 pH3 positive cells, average 3, median 3, standard deviation 0.77). (B, D) Antibody staining for pH3 when Sulf1 is knocked-down ($n=9$, range 0 to 2 cells, average 1, median 1, standard deviation 0.71). (E) Graph of the data where the reduction in pH3 positive cells in embryos lacking Sulf1 compared to controls is significant (Student's t-test $P<0.0001$).

To test the effects of Sulf1 on cells receiving, but not expressing Shh, mRNA coding for Sulf1 was injected into one blastomere, while an adjacent blastomere was injected with mRNA coding for Shh-GFP (Fig. 4G and H) and imaged as described above. Shh-GFP was present between cells in control embryos (Fig. 4I and J), while in regions of cells expressing Sulf1 no Shh-GFP is detected (Fig. 4K and L). The lack of observable Shh-GFP in regions where cells express high levels of Sulf1 suggests that Shh is not able to migrate through an environment deficient in 6-O sulphated HSPGs. This is consistent with the notion that Sulf1 activity lowers the affinity of HS for Shh, which is also supported by the results of a heparin competition assay demonstrating that Sulf1 treated heparin does not bind Shh with as high affinity as control heparin (Supplementary data Fig. S3).

In order to determine the effect of Sulf1 in cells that also produce Shh, mRNA coding for Shh-GFP was co-injected together with mRNA coding for Sulf1 (Fig. 4M and N). Again, when Shh-GFP was expressed alone (or with the control mRNAs described), it travelled freely as discrete punctae (Fig. 4O and P). However, when co-expressed with Sulf1, Shh-GFP diffusion was restricted and it tended to form aggregates (Fig. 4Q and R). To quantify the distribution of Shh observed in this experiment, ImageJ was used to measure the average pixel intensity over a set area in several experimental replicates. The distribution of pixels is shown graphically in Fig. 4S. Shh-GFP diffusion from control cells diffuses freely, however, when Sulf1 is co-expressed with Shh-GFP, it forms aggregates and does not travel as far; when represented graphically, a sharp drop off in Shh approximately 30 μm from the source cells can be seen. These data demonstrate that Sulf1 can modify the distribution of Shh ligand when co-expressed in signalling cells.

Sulf1 is required for the normal distribution of Shh protein in the ventral neural tube

The observation that Sulf1 activity can restrict the movement of a GFP tagged Shh ligand across a field of cells is consistent with findings that DSulf1 influences the distribution of Hh ligand in the Drosophila imaginal disc (Wojcinski et al., 2011). To determine whether the effects of Sulf1 knockdown on the patterning of neural progenitor cells can be explained by a change in the Shh morphogen gradient, we analysed the distribution of endogenous Shh ligand using the antibody 5E1. Immunohistochemical staining reveals the presence of the Shh protein in the notochord (NC), and ventrally within the neural tube (NT) and the floor plate (FP) (Fig. 4A, B). The level of Shh protein drops off sharply away from the Shh expressing region, and is not detected in the dorsal neural tube (Fig. 4C). We confirmed local effects on HS structure in response to Sulf1 knock-down using the antibody 10E4 that recognises highly sulphated HS and found that consistent with results in other systems (Ai et al., 2003; Hayano et al., 2012), there is more immunoreactivity in regions lacking Sulf1 (Supplemental data Fig. S4). Knockdown of Sulf1 leads to a change in the endogenous distribution of Shh protein, which can be detected further dorsally in a more diffuse pattern than in controls (compare Fig. 4C and G).

Quantification of the immunohistochemistry on several sets of knockdown and control embryos reveals a marked change in the distribution of Shh in the absence of Sulf1. The level of Shh in embryos injected with control morpholino oligo (blue) immediately adjacent to the FP is high, but drops to almost zero within ~20 μm. In the absence of Sulf1, the level of ligand adjacent to producing cells is lower than in controls, but this level remains higher much further from the source, only dropping to zero ~50 μm from the FP. These data indicate that the presence of Sulf1 in the floor plate is required for the sharp gradient of Shh that is essential for the normal positioning of neuronal subtypes in the developing neural tube.

Discussion

The level of Shh at distinct dorsoventral regions of the neural tube is a crucial factor contributing to the determination of populations of neural precursor cells and defines the position of specific neuronal subtypes. Interfering with Shh signalling affects the expression level and spatial distribution of transcription factors that specify the identity of these precursor populations (Briscoe and Ericson, 1999; Briscoe and Novitch, 2008). Sulf1

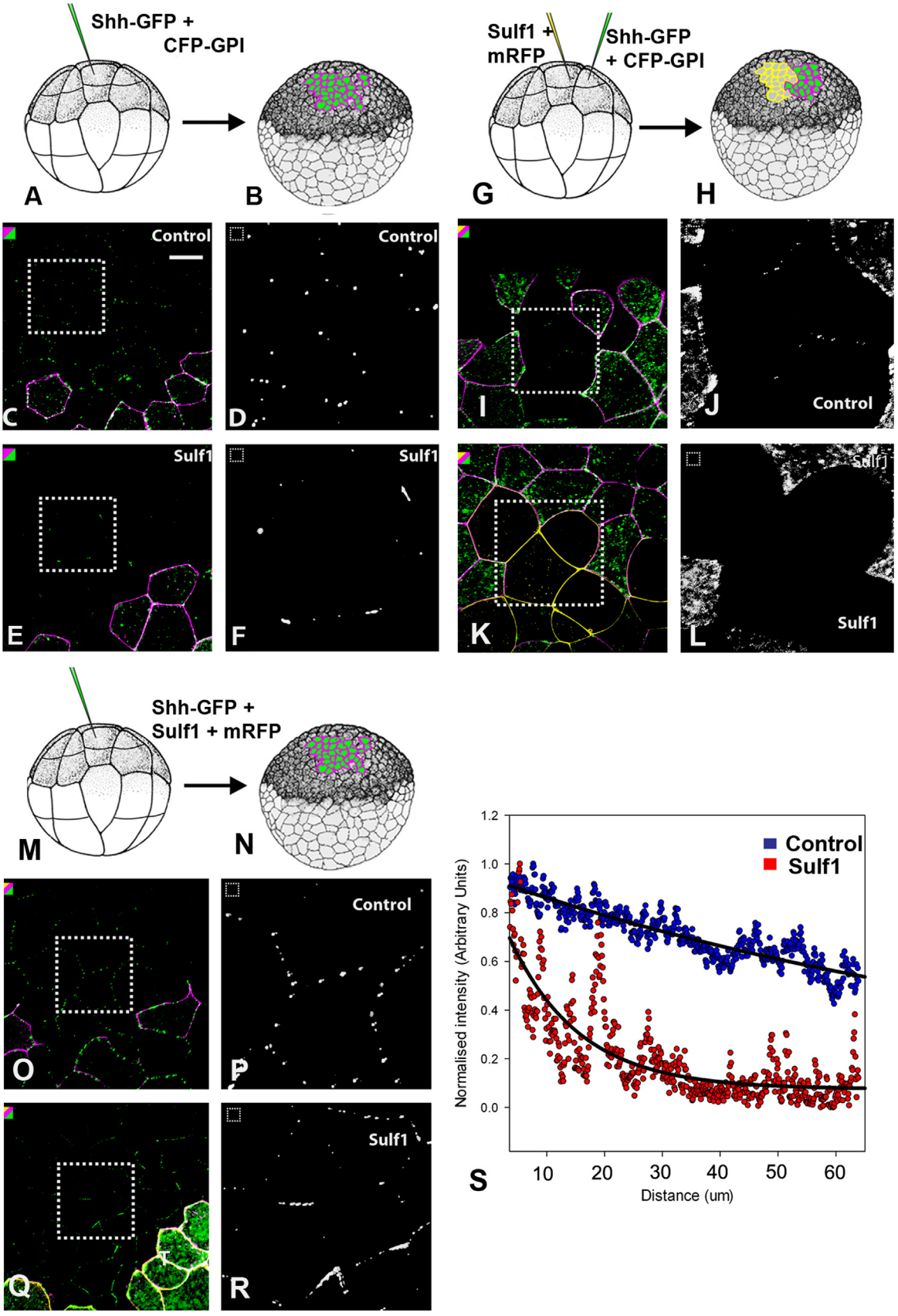

Fig. 4. Sulf1 affects the diffusion of Shh-GFP in embryo explants. Shh-GFP is shown as green, and is co-expressed with the membrane tethered lineage marker, CFP-GPI which is shown as magenta. Sulf1 mRNA was co-injected with membrane RFP, which is shown in yellow. The cartoons in Fig. 4 illustrate the experiments done in the panels below. (A–D) Shh-GFP expressed in a subset of cells labelled with CFP-GPI (magenta) is able to diffuse away from its site of synthesis forming discrete puncta around cells at a distance from its source. (E–F) When Sulf1 is expressed globally, Shh-GFP is less able to diffuse away from its source. When Sulf1 (yellow) is expressed in cells adjacent to a source of Shh-GFP (magenta) (G–J), diffusion of Shh-GFP is completely abolished within the Sulf1 expressing region (K, L). Shh-GFP displays a reduction in its diffusion when it is co-expressed with Sulf1 (M–R). 50□M squares are shown at a 10 fold magnification in adjacent panels (D, F, J, L, P, R) revealing that while Shh-GFP forms discrete puncta in controls (D, P), large aggregates of Shh-GFP form when Sulf1 is expressed either globally (F) or co-expressed with Shh-GFP (R). Magnified images show only Shh-GFP which is depicted in white to improve contrast. (S) Fluorescence levels were quantified using the plot profile function in ImageJ and fluorescent intensity is shown as a function of the distance from the source cells in embryos co-injected with Shh-GFP and control mRNA (blue) versus embryos co-expressing both Shh-GFP and Sulf1 (red). Scale bar is 20 μM.

knockdown in *Xenopus* results in the disruption of the regional expression of these key homeobox transcription factors, demonstrating that loss of Sulf1 affects the dorsoventral patterning of the neural tube.

Early establishment of the floorplate

Classic embryology using chick and mouse embryos established a model where Shh signalling from the notochord induces the formation of the floor plate (for example Yamada et al., 1991; Placzek et al., 1993; Roelink et al., 1994). Other data indicate that there are two cell lineages under distinct development control that give rise to floor plate. The medial floor plate (MFP) lineage derives from midline precursor cells in the organiser while the lateral floor plate (LFP) lineage arises later and depends on Shh signalling from the medial floor plate and notochord (Odenthal et al., 2000). In *Xenopus*, the MFP was shown to derive from two separate populations of progenitors, one of which depends on Notch signalling (Peyrot et al., 2011). This study also showed that the inhibition of Shh signalling results in the loss of Nkx 2.2 expression in the LFP of the neural tube, consistent with findings presented here. *Sulf1* is not expressed in the floor plate until after neural tube closure in *Xenopus*, so it plays no role in these very early inductive events, however, like *Shh* itself, *Sulf1* expression is likely to be part of the response to floor plate induction and we have found that it is important for the subsequent patterning of the ventral neural tube.

Later events: the MN to OPC switch

A recent paper describes a later function for Sulf1 in promoting the switch from motor neuron to oligodendrocyte fate (Touahri et al., 2012) that is driven by high levels of Shh (Danesin et al., 2006). The pMN (motor neuron progenitor) domain gives rise to motor neurons (MNs) first and, later in development, to most of the oligodendrocyte precursor cells (OPCs). Sulf1 has been shown to trigger this switch by locally increasing levels of Shh activity at this time (Touahri et al., 2012). In this study, Sulf1$^{-/-}$ mice were found to have dramatically fewer Olig2+ cells in the mantle zone compared to wild type, indicating a failure of the MN to OPC switch in the absence of Sulf1. Our work found an increase in cells positive for the MN marker HB9 at NF stage 40, as compared to stage 23, in *Xenopus* lacking Sulf1 which is consistent with a failure of the MN to OPC switch described in Sulf1$^{-/-}$ mice (Touahri et al., 2012). To corroborate this, we attempted to detect OPCs in our Sulf1 knockdown embryos, unfortunately not one the four antibodies available against Olig2 was effective in *Xenopus*.

A role for Sulf1 in the early neural patterning: frogs vs mice

Another conclusion from Touahri et al. (2012) was that Sulf1 is dispensable for the early patterning of the neural tube in mice, which clearly disagrees with our data (Fig. 2). This work also shows that in the progenitor domain, located adjacent to the lumen of the neural tube, there is no difference in the number of Olig2+ cells in Sulf1$^{-/-}$ compared to controls. The lack of any effect on progenitor cells in Sulf1$^{-/-}$ mouse embryos is in contrast to the changes we see in the expression of key transcriptional regulators in the very early neural tube in *Xenopus*. It is possible that this difference is simply due to the timing of the two analyses: our experiments use embryos at NF stage 23 which is only a few hours after neural tube closure, Touahri et al. (2012) present data from embryonic day 12.5; in the mouse the neural tube begins to close at embryonic day 8. Alternatively, the difference could reflect the distinct timing of development of the two organisms. The temporal activities of other important developmental regulators are known to be different in amniotes and *Xenopus;* for instance, the myogenic regulatory genes are expressed from gastrula stages in frogs and fish but are not activated until somitogenesis in amniotes (Pownall et al., 2002). Indeed, the earliest expression of *Sulf1* in mice that has been described is at day 9.5 (Lum et al., 2007), while *Xenopus Sulf1* displays both maternal and early zygotic expression (Freeman et al., 2008). It is possible that the early activity of Sulf1 that is essential for neural patterning in *Xenopus* is not important until later in the mouse. However, this conclusion would require more extensive expression analysis of transcription factors important for progenitor specification in early mouse embryos lacking *Sulf1*. While our work was under review, the Soula group has reported that zebrafish embryos lacking Sulf1 show disrupted neural patterning (Oustah et al., 2014). Sulf1 knockdown zebrafish initially show a complete loss of Nkx2.2a in the ventral neural tube, but later (at 36hpf) Nkx2.2a is expressed but is shifted ventrally compared to controls, consistent with what we see in *Xenopus*. In another part of this same study, chick explants were used to visualise Shh ligand using the antibody 5E1, and similar to our findings (Fig. 5), their work also reports the reduced accumulation of Shh ligand when Sulf1 is inactivated. The data together provide further evidence that Sulf1 plays an important role in mediating neural patterning in response to Shh signalling.

Sulf1 is required for Shh promotion of proliferation

Some data suggest that HS is not required for Shh patterning of the neural tube. In the Shh protein, an N-terminal Cardin–Weintraub (CW) motif is important for its interactions with HSPGs. Genetically altered mice have been generated with a mutation in this domain (Shh^{Ala}) which reduces Shh–HSPG interaction (Chan et al., 2009). Unlike Shh$^{-/-}$ mice, mice homozygous for Shh^{Ala} do not display the cyclopia or limb defects typical of mice lacking Shh, suggesting that this mutation does not affect the ability of Shh to pattern the embryo. The Shh^{Ala} mice do show growth defects, with an overall reduction in body weight and a decrease in brain size resulting from reduced cell division in the neural tube. While this mutation does affect signal transduction downstream of Shh, the expression of the immediate early targets *Ptch1* and *Gli1* as well as the neural patterning genes *Isl1*, *Nkx2*.2 and *Nkx6*.1 are not altered in Shh^{Ala} mice. These data may suggest that the interaction of the CW domain of Shh with HSPGs is not important for its ability to pattern the neural tube, only for Shh regulation of cell proliferation. Our findings in *Xenopus* show that Sulf1 knockdown results in embryos with an altered distribution of Shh protein (Fig. 4), disrupted expression of key regulators of dorsal ventral neural tube patterning (Fig. 2), and reduced cell proliferation in the neural tube (Fig. 3). Recently another domain important for HS binding has been identified in the Shh protein. Whalen et al. (2013) determined the crystal structure of Shh complexed with heparin and found that a Shh dimer forms a continuous stretch of positive amino acids that interact with the heparin chain and this interaction appears to hold the dimer together. They conclude that the CW motif and the newly described “core GAG-binding site” both contribute to Shh interactions with HSPGs. It is yet to be determined whether engineering mutations in this domain will cause neural patterning defects in mice.

A mechanism for Sulf1 activity in the neural tube

Our findings are consistent with the notion that Sulf1 promotes high levels of Shh signalling by increasing the local accumulation of Shh ligand. One possible model is that Sulf1 activity in the floorplate allows for the retention of Shh on cell surface HS, limiting Shh diffusion away from its source. This creates the steep

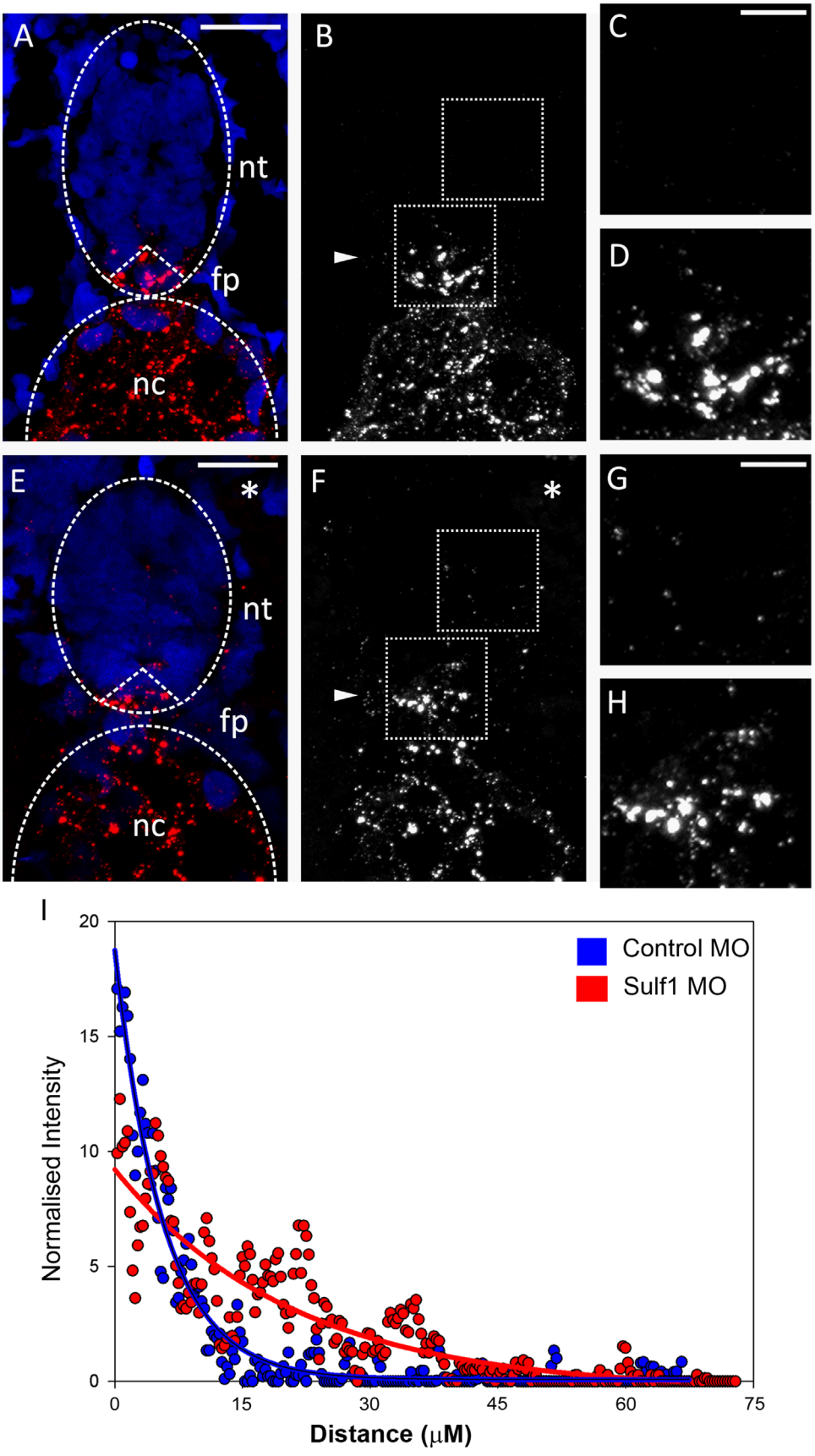

Fig. 5. Sulf1 is required for the normal distribution of Shh protein *in vivo*. (A–D) Immunostaining embryos unilaterally injected with a control morpholino with 5E1reveals Shh protein in the notochord (NC) and the neural tube (NT), with highest levels in the floor plate (FP). In these control embryos the level of Shh protein in the neural tube drops off sharply away from the FP and is not detected within the dorsal neural tube (C). (E–H) Unilateral knockdown of Sulf1 *in vivo* leads to a change in the distribution of Shh protein on the injected side (*) where there is reduced Shh protein detected which is detected much further dorsally in a more diffuse pattern than in controls (compare G with C). (I) Quantification of the immunohistochemistry reveals a marked change in the diffusion of Shh away from its source in the absence of Sulf1 expression. The level of Shh in controls embryos (blue) immediately adjacent to Shh producing cells is high; this drops to almost zero within ~20 μm. In the absence of Sulf1(red), the level of ligand adjacent to producing cells is lower than in controls, but this level remains higher much further from the source, only dropping to zero ~50 μm from the source cells. Graph represents average grey level across the width of the neural tube on the injected side. Area measured for quantification shown. Mean values from a number of samples are shown (CMO $n=5$, AMO $n=7$).

gradient of Shh signalling necessary for proper patterning of ventral cell types in the neural tube. Fig. 6 is a model depicting our findings *in vivo*, illustrating graphically that Sulf1 activity is required in the floorplate to promote a high level of Shh protein in the ventral neural tube and that there is a ventral shift of neural precursor populations in the embryos lacking Sulf1. Our model suggests that this ventral shift is due to the flattening of the Shh gradient.

One observation that does not easily fit this model is that Sulf1 reduces the affinity of heparin for Shh (Supplementary data Fig. S3). However, HS is much more heterogeneous than heparin, and the specific sulfation pattern of HS is known to be an important factor contributing to the ability of HS to bind many proteins. Sulf1 has been shown to reduce the binding of heparin to both Wnt8 (Ai et al., 2003) and to FGF2 (Wang et al., 2004). However, this biochemical effect does not indicate how Sulf1 will impact cell processes. In these cases, the effects of Sulf1 on signalling are opposite: Wnt signalling is enhanced and FGF signalling is inhibited by Sulf1. Our competition assay suggests that, as with other signalling molecules, Sulf1 can reduce the binding affinity of heparin for Shh, while our experiments with Shh-GFP and our *in vivo* studies indicate that Sulf1 increases the local accumulation of ligand and is required for high level Shh signalling in the neural tube. It is therefore likely that the effects of Sulf1 are not binary such that the presence of Sulf1 does not completely abolish Shh: HS binding, but instead influences HS dependent incorporation of Shh into higher ordered multimers or into lipoprotein particles (Palm et al., 2013; Goetz et al., 2006).

The core GAG binding domain in the Shh protein (Whalen et al., 2013) is positioned opposite to the fatty modifications such that oligomers of Shh can assemble by associating with HSPGs after it is secreted from cells (Vyas et al., 2008). Shh proteins bind with high affinity to highly sulfated HS (such as heparin) and structural studies showed that a Shh dimer can assemble on an HS chain every 15 sugar residues (Whalen et al., 2013). As HS chains can consist of up to a hundred disaccharide repeats, this model predicts the formation of very large Shh multimers. Distinct GAG sulfation patterns affect the interaction of Shh with HS and would therefore influence the formation and or release of higher ordered protein assemblies. In the presence of Sulf1, Shh may not be released as efficiently via this HS dependent route, due a reduced affinity for Sulf-modified HS. The cell surface Shh that accumulates may be recycled and subsequently exported via an alternative route more akin to the basolateral release of Hh described in Drosophila. In the Drosophila wing disc, basolaterally and apically released Hh ligands are significantly different in their appearance; apical Hh is present as discrete puncta, while basolateral Hh is contiguous and is tightly localised to the membrane (Ayers et al., 2010).

In chick, Sulf1 overexpression leads to Shh accumulation at the cell surface and the induction of Nkx2.2 expression in a cell-autonomous manner (Danesin et al., 2006). This indicates that Sulf1 can also influence the level of Shh reception. A mechanism whereby Sulf1 modifies the association of Shh with glypicans could explain how Sulf1 promotes hedgehog signalling in receiving cells. In both mouse and Drosophila, endocytosis of hedgehog proteins complexed with glypicans has been found to influence the level of hedgehog signalling (Capurro et al., 2008; Ayers et al., 2012). When internalisation of a glypican is associated with a ligand/receptor complex (Shh/Ptc), this increases hedgehog signalling. Sulf1 may influence the association of specific glypicans with the Shh ligand to facilitate Ptc binding, thus promoting Shh endocytosis in a complex with Ptc and increasing hedgehog signalling. Ptc is also known to sequester hedgehog ligand and restrict its movement; if Sulf1 promotes Shh/Ptc association, this would explain why in the absence of Sulf1 Shh diffuses far from its source, whereas in the presence of Sulf1, the movement of Shh is restricted. Sulf1 promoting the association of Shh with Ptc is similar to the "catch and present" model used to explain the enhancement of canonical Wnt signalling by Sulf1 (Ai et al., 2003). This model takes into account that Sulf1 treated heparin has lower affinity for Wnt ligands, while it enhances Wnt activity; we report here similar findings for Shh. We show that Shh diffuses further in the ventral neural tube when Sulf1is depleted which could reflect a reduction in Shh/Ptc association and sequestration.

It is unlikely that a universal mechanism for the establishment of a hedgehog gradient exists, as the diverse cellular environments differ between tissue types and species. Additionally, the requirement for long versus short range signalling also differs between developmental settings (Ayers et al., 2010; Gallet et al., 2003).

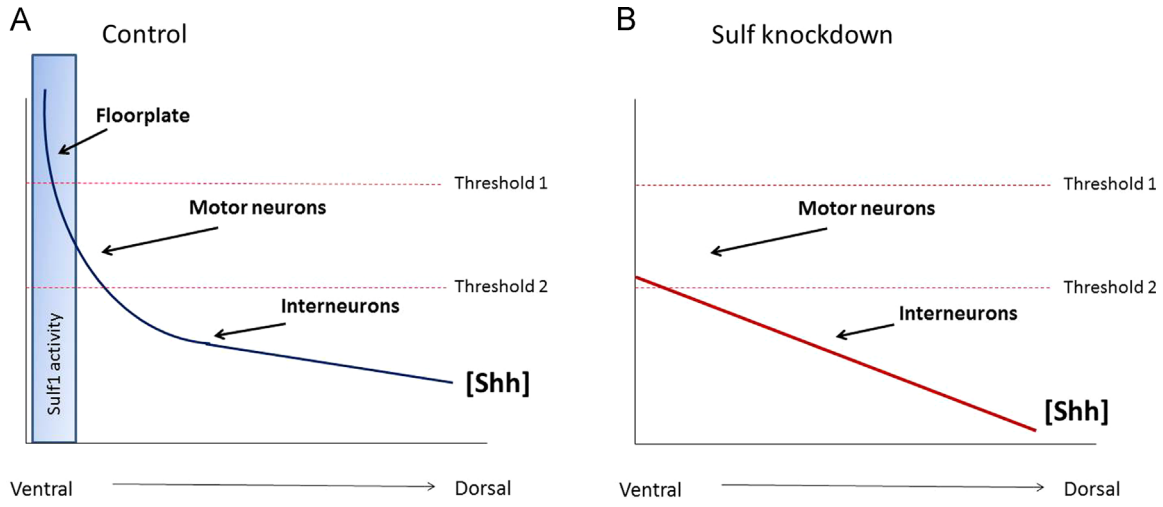

Fig. 6. A model for Sulf1 activity in patterning the ventral neural tube. (A) During normal development, *Sulf1* (blue box) is co-expressed with *Shh* in the floorplate of the neural tube. The activity of Sulf1 promotes the local accumulation of Shh ligand in the ventral neural tube and creates a steep gradient of Shh that falls off quickly in more dorsal regions (blue line). Very high levels of Shh, above Threshold 1, induce the formation of floorplate, while levels of Shh above Threshold 2 are required for the establishment of V3 interneuron and motor neuron precursor populations (pV3s and pMNs). Below the second threshold other interneuron progenitors form in response to a lower level of Shh signalling that occurs more dorsally. (B) When Sulf1 is knocked-down (no blue box), high levels of Shh ligand fail to accumulate in the ventral neural tube so that the gradient of Shh morphogen flattens (red line). Ventral levels of Shh fall below the threshold necessary for floorplate induction and the level needed for pV3 and pMN specification is shifted ventrally.

However, Sulf1 is co-expressed with hedgehog ligand in both the floorplate of the vertebrate neural tube and in the Drosophila wing disc, two regions known to be patterned in response to a hedgehog morphogen gradient. The establishment of the hedgehog morphogen gradient in the Drosophila wing disc requires HSPGs and Sulf1 (Callejo et al., 2006; Wojcinski et al., 2011). Our work provides the first *in vivo* evidence of a similar role for Sulf1 in shaping the Shh morphogen in the vertebrate neural tube and reveals a conserved requirement for Sulf1 in modulating the distribution of Shh.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.04.010.

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