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# DSulfatase-1 fine-tunes Hedgehog patterning activity through a novel regulatory feedback loop

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ABSTRACT

Sulfs are secreted sulfatases that catalyse removal of sulfate from Heparan Sulfate Proteoglycans (HSPGs) in the extracellular space. These enzymes are well known to regulate a number of crucial signalling pathways during development. In this study, we report that DSulfatase-1 (DSulf1), the unique *Drosophila* Sulf protein, is a regulator of Hedgehog (Hh) signalling during wing development. DSulf1 activity is required in both Hh source and Hh receiving cells for proper positioning of Hh target gene expression boundaries. As assessed by loss- and gain-of-function experiments in specific compartments, DSulf1 displays dual functions with respect to Hh signalling, acting as a positive regulator in Hh producing cells and a negative regulator in Hh receiving cells. In either domain, DSulf1 modulates Hh distribution by locally lowering the concentration of the morphogen at the apical pole of wing disc cells. Thus, we propose that DSulf1, by its desulfation catalytic activity, lowers Hh/HSPG interaction in both Hh source and target fields, thereby enhancing Hh release from its source of production and reducing Hh signalling activity in responding cells. Finally, we show that *Dsulf1* pattern of expression is temporally regulated and depends on EGFR signalling, a Hh-dependent secondary signal in this tissue. Our data reveal a novel Hh regulatory feedback loop, involving DSulf1, which contributes to maintain and stabilise expression domains of Hh target genes during wing disc development.



## Introduction

Multicellular organisms develop through the specification of particular tissue types in well-defined spatial positions. Such patterning is largely mediated by secreted morphogens, such as FGFs, Wnts, Bone Morphogenetic Proteins (BMPs) and Hedgehog (Hh), which are produced locally and diffuse into adjacent tissues specifying distinct cellular fates in a dose dependant manner (Tabata and Takei, 2004). A remarkable feature of morphogen activities is the precision and robustness of the resulting cell fate patterns. Understanding how morphogen distribution is regulated and how their graded activities are established and maintained remains a major challenge. Extracellular matrix proteins belonging to the Heparan Sulfate Proteoglycans (HSPGs) family are known to play a major role in both stabilisation and transport of secreted molecules (Hacker et al., 2005; Yan and Lin, 2009). Based on mathematical modelling, it has also been proposed that HSPGs might be part of mechanisms that enhance robustness of morphogen patterning activities (Irons et al., 2010). HSPGs are formed by a core protein to which Heparan Sulfate (HS) glycosaminoglycan chains, composed of disaccharide units, are covalently attached. To date, most HSPGs studies have demonstrated the importance of HS chains in regulating their function (Bornemann et al., 2004; The et al., 1999). After polymerization, HS chains undergo modifications such as sulfate addition to the 2-O position of iduronic acid and N, 3-O and 6-O positions of the glucosamine HS units (Esko and Selleck, 2002; Ori et al., 2008). The importance of HS sulfation state for interactions with specific ligands was confirmed by genetic studies showing that mutations in genes encoding for either N-deacetylase/N-sulfotransferase or HS sulfotransferases that catalyse these critical sulfations, cause defects in various signalling pathways both in *Drosophila* and mice (Gorsi and Stringer, 2007; Ori et al., 2008). In this context, the characterization of Sulf proteins has attracted particular attention. Indeed, these secreted 6-O-endosulfatases, called Sulf proteins, are unique in their ability to catalyse removal of 6-O-sulfate within the HS chains in the extracellular space or in the Golgi, thus modulating the activity of different signalling pathways (Ai et al., 2003; Dhoot et al., 2001; Kleinschmit et al., 2010; Morimoto-Tomita et al., 2002). Sulfs have been involved in the regulation of Wnts, FGFs, BMPs and Shh morphogen activities in vertebrates (Danesin et al., 2006; Freeman et al., 2008; Lamanna et al., 2007; Otsuki et al., 2010). So far, Sulf enzyme activity has been reported to regulate positively or negatively the availability of ligands for binding to their receptors by modulating interactions of ligands or their antagonists with HS chains (Ai et al., 2003; Dhoot et al., 2001; Rosen and Lemjabbar-Alaoui, 2010; Viviano et al., 2004; Wang et al., 2004). Moreover, due to their ability to concomitantly modulate several signalling pathways within a given tissue, Sulfs have been proposed to

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serve as integrators of morphogen activities (Freeman et al., 2008; Otsuki et al., 2010). In *Drosophila*, wing development is a paradigm for studying integration of signalling pathways during development. The adult wing blade originating from the wing pouch of the wing imaginal disc is characterised by the stereotyped alternation of vein (named L1 to L5) and intervein tissues. The positioning and elaboration of ectodermal veins in the wing pouch of the disc rely on at least four different signalling pathways: Hh, Decapentaplegic (Dpp), Epidermal Growth Factor Receptor (EGFR) and Wingless (Wg) (Blair, 2007). Hh signalling is required to pattern the imaginal disc epithelium along the antero-posterior (AP) axis (Crozatier et al., 2004). Subsequent activation of Hh-dependant secondary signals such as Dpp and EGFR are further responsible for positioning the four provein domains (L2 to L5) corresponding to the prospective adult longitudinal veins (Blair, 2007). Hh is produced by cells of the posterior (P) compartment and diffuses in the anterior (A) compartment where it activates target genes in a dose dependant manner: *engrailed* (*en*), *patched* (*ptc*), *collier* (*col*/*knot*) and *decapentaplegic* (*dpp*), recognised as high-, mid- and low-level target genes, respectively (Crozatier et al., 2004; Strigini and Cohen, 1997). Col specifies the presumptive L3-L4 intervein domain in a cell autonomous way but also contributes to induce L3 and L4 provein cells in adjacent domains by modulating EGFR signalling (Crozatier et al., 2002; Vervoort et al., 1999). Likewise, Dpp, recognised as a long distance signalling molecule, is involved in positioning L2 and L5 provein domains (Blair, 2007). In the wing disc, the dorsal–ventral (DV) patterning depends on Wg signalling (Neumann and Cohen, 1997). Interestingly, the 6-O-endosulfatase DSulfatase-1 (DSulf1) has recently been reported to regulate Wg signalling in this process (Kleinschmit et al., 2010; You et al., 2011). Moreover, Kleinschmit and collaborators proposed that DSulf1 also contributes to regulate Dpp signalling in this tissue (Kleinschmit et al., 2010).

Here, we show that DSulf1 is a novel modulator of Hh signalling required for correct antero-posterior (AP) patterning of the wing. By analysing *Dsulf1* null mutants, we first evidenced a mild Hh gain-of-function wing phenotype. Unexpectedly, depleting DSulf1 in either Hh producing or receiving cells of the posterior (P) and anterior (A) compartments, respectively, led to more severe and opposite Hh phenotypes. Indeed, DSulf1 behaves as a positive regulator of Hh in its source but down-regulates Hh signalling activity in its responding field. We provided evidence that DSulf1 regulates Hh distribution by locally lowering its concentration threshold at the apical pole of *Dsulf1*-expressing cells in both compartments, indicating that DSulf1 promotes the release of the morphogen from the cell surface. Our functional data further involved the glypicans, Dally and Dally-like (Dlp), as potential substrates of DSulf1 activity in the wing disc. Together, our findings lead to the proposal that DSulf1 by reducing Hh/HSPG interaction prevents local Hh retention in producing cells, then promoting Hh release that results in a higher Hh activity in the receiving field. Concomitantly, it controls the morphogen activity in Hh receiving cells, again by reducing its concentration at their apical pole that results in lowering Hh signalling. Finally, we found that *Dsulf1* expression is controlled by the EGFR signalling, itself positioned by Hh in the central region of the wing imaginal disc. Therefore, DSulf1 is part of a novel Hh regulatory feedback loop that contributes to define accurate Hh target gene expression domains during wing development. Our results highlight the importance of modulating 6-O-sulfation state of HSPGs to fine-tune Hh patterning activity and bring novel experimental support to the emerging morphogen concept viewing positional specification as a dynamic process driven by feedback adaptation mechanisms.

## Materials and methods

### *Mutant and transgenic Drosophila strains*

The following strains were used: wild-type (OregonR); lacZ expressing enhancer trap allele of *dpp* (*dpp-lacZ*$^{P10638}$) (Blackman et al., 1991); *dally*$^{gem}$; *dally*$^{MH32}$; *dlp*$^{1}$; *dlp*$^{2}$; *dlp*$^{MH20}$; *ci-Gal4* (a gift from G. Struhl); *UAS-EGFR*$^{DN}$ (Freeman, 1996); *UAS-EGFR*$^{CA}$ (Queenan et al., 1997); *UAS-rho* (de Celis et al., 1997); *UAS-Ci*$^{act}$ also named *UAS-Ci*$^{PKA}$ (Méthot and Bassler, 2000); *UAS-GFP:Dally* (Eugster et al., 2007); *UAS-GFP:Dlp* (Han et al., 2004b). We used either *UAS-sulf1* (Kamimura et al., 2006) or new *UAS-sulf1* transgenic lines inserted on ATTP platforms on 2nd or 3rd chromosome (Sulf1 open-reading-frame amplified by genomic PCR from *UAS-sulf1* flies and inserted into the *pUASattIns* vector). *sulf1*$^{\Delta P1}$ line was generated by local hopping using the strain carrying P-element inserted in the *Dsulf1* locus (*PSulf1*$^{GT\text{-}000656}$) and selected for imprecise excision (Kleinschmit et al., 2010). *UAS-HhNp:HRP* line was constructed similarly to *UAS-HhNp:GFP* line with addition of the HRP tag before the auto-proteolytic cleavage site of Hh, allowing further additions of palmitic acid and cholesterol moieties (Torroja et al., 2004).

### *Clonal analysis*

Mutant clones were induced by FLP-mediated mitotic recombination (Xu and Rubin, 1993). *sulf1*$^{\Delta P1}$ was recombined onto *FRT82B* chromosome and crossed to *w,hsflp; FRT82B, UbGFP, RpS3/TM6B, Tb*$^{1}$, *RpS3* being a homozygous cell lethal Minute mutation. The larvae were heat shocked at first instar and dissected at late L3 stage. To localise clones of cells lacking *Dsulf1* expression or over-expressing DSulf1 in adult wings, we visualised GFP positive cells in wings dissected from very young adults (less than 1 h after emerging from pupa). EGFR$^{DN}$ clonal cells were induced by the GAL4/UAS system (Brand and Perrimon, 1993; Ito et al., 1997) in *y,w,hsFLP1; Act>y*$^{+}$*>Gal4,UAS-GFP* strain.

### *In situ hybridization (ISH)*

*Dsulf1* expression was monitored using digoxigenin-labelled (DIG, ROCHE) antisense RNA probe, synthesised from SD04414 cDNA. Experiments combining ISH and immunohistochemistry (IHC) were performed as previously described (Kozopas et al., 1998), except that a Proteinase K treatment followed by a post-fixation step was added. Probes were detected using anti-DIG antibody conjugated to alkaline phosphatase (1:1000, ROCHE) and revealed using Fast Red (ROCHE).

### *Immunohistochemistry and image capture*

Wing discs were fixed either in 4% PAF in PBS or in an Absolute Ethanol/1% Acetic Acid solution (EtOH/AA) (Tuckett and Morriss-Kay, 1988). Antibodies were used at the following dilutions: mouse anti-Col, 1:50 (Dubois et al., 2007); rabbit anti-β-gal, 1:1000 (Cappell); mouse anti-Wingless 4D4, 1:200 (Hybridoma Bank); rabbit and mouse anti-GFP, 1:500 (Torrey, ROCHE); mouse anti-Invected 4D9, allowing detection of Engrailed, 1:50 (Hybridoma Bank); rabbit anti-Hh, 1:200 (Taylor et al., 1993); mouse anti-Dally, 1:200 (Abcam); mouse anti-Dallylike I3Q8, 1:50 (Hybridoma Bank) and mouse anti-Ptc Apa1, 1:100 (Hybridoma Bank). Secondary antibodies conjugated to Alexa Fluor fluorescent dyes (Molecular Probes) were used. Detection of HhHRP was done on living tissues by incubation in tyramide alexa fluor 488, 1:100 (Molecular Probes) for 20 min and further fixed in 4% PAF in PBS. Discs were mounted in polyvinyl alcohol 4–88 (Fluka). Fluorescence imaging was obtained from a Leica Sp5 confocal microscope. Captured images were assembled using Adobe Photoshop.

### *Quantifications*

In Figs. 1 to 4, expression of Hh target genes in wing discs was quantified by counting cell rows of positive cells in the A compartment. Cell countings were performed every 20 μm from dorsal to ventral borders of the wing pouch and results were expressed as mean