

Wingless, Hedgehog and Heparan Sulfate Proteoglycans

Several recent papers published in *Development* challenge some of the previous views published by our group and others that: (1) the *Drosophila* glypican molecules Dally and Dally-like protein (Dlp) are involved in Wingless (Wg) signaling; and (2) *Tout-velu/Ext/Ttv* affects Hedgehog (Hh) but not Wg signaling. We discuss how to reconcile these new results (Desbordes and Sanson, 2003; Takei et al., 2004; Han et al., 2004b) with the previous studies, and highlight some of the difficulties inherent to the genetic studies of Heparan Sulfate Proteoglycans (HSPGs) in *Drosophila*.

Eight years ago, we identified mutations at five zygotic lethal loci: *sugarless (sgl)*, *sulfateless (sfl)*, *fringe connection (frc)*, *slalom (sll)* and *ttv* that were associated with a 'segment polarity' phenotype reminiscent of loss of Wg and/or Hedgehog (Hh) signaling (Perrimon et al., 1996). Characterization of these segment polarity genes showed that they encoded enzymes involved in glycosaminoglycan (GAG) biosynthesis (Häcker et al., 1997; Lin and Perrimon, 1999; Bellaïche et al., 1998; The et al., 1999; Selva et al., 2001; Lüders et al., 2003; Binari et al., 1997; Haerry et al., 1997; Goto et al., 2001). GAGs are unbranched polysaccharide chains that are synthesized on proteoglycan core proteins, such as glypicans and syndecans, in the Golgi apparatus and undergo complex modification reactions before the core protein to which they are attached is transported to the cell surface (reviewed by Perrimon and Bernfield, 2000; Selleck, 2001; Nybakken and Perrimon, 2002). Altogether, phenotypic characterization of mutations in the biosynthetic enzymes and substrate transporters involved in their production have now established that properly modified GAGs are crucial for the function of the Wg, Hh, fibroblast growth factor and Decapentaplegic (fibroblast growth factor and Dpp pathways will not be discussed here) pathways. With our current level of understanding, we can now clarify some of the puzzling results from earlier studies, which lead us to urge caution in interpreting some of the most recent findings.

The mutations in GAG biosynthetic enzymes were originally identified based on their 'segment polarity' phenotype. However, the fact that the Wg and Hh pathways are linked in a feedback loop during segment polarity determination makes it very difficult to decide which pathway is affected by a particular mutation (see Häcker et al., 1997; Desbordes and Sanson, 2003). Although some differences exist between loss of Hh or Wg signaling with respect to the timing of Engrailed (En) decay, i.e. in *wg* mutants En protein is completely absent by stage 11, whereas it only begins to disappear at that stage in *hh* mutants, this is not a reliable marker to use to determine whether a gene plays a role in either Wg or Hh signaling. In the case of hypomorphic mutations or mutations in accessory factors that are not essential for signal transduction, it cannot be determined whether delayed fading of En is due to complete loss of Hh signaling or to partial activity of the Wg pathway.

Studies using the UAS/Gal4 system have shown that ectopic expression of Wg or Hh in mutants in which GAG biosynthesis is abrogated still leads to activation of the respective pathways. These experiments have therefore not allowed us to pinpoint

the requirements of GAGs for either Wg or Hh signaling in the embryo.

The most conclusive results regarding the role of GAGs in different signaling pathways have been obtained in imaginal discs, where Wg and Hh act independently as morphogens to control patterning at the dorsoventral and the anteroposterior compartment boundaries, respectively. With regard to the role of GAGs in Hh signaling, all studies published to date consistently demonstrate a requirement for GAGs in Hh signaling and suggest that GAGs are required to facilitate spreading of the Hh morphogen through the field of imaginal disc cells (Bellaïche et al., 1998; Takei et al., 2004; Han et al., 2004b).

The role of GAGs in Wg signaling has been more difficult to assess as it is now apparent that their requirement in the Wg pathway is more subtle than in the case of Hh. Initial clues came from the observation that extracellular Wg protein does not accumulate at the surface of *sfl* mutant cells (Baeg et al., 2001). Mosaic clones mutant for *sll*, a PAPS transporter that is necessary for sulfation of GAGs, also show reduced levels of Wg protein (Lüders et al., 2003). Furthermore, studies on the α/β hydrolase enzyme encoded by *notum* (Gerlitz and Basler, 2002; Giraldez et al., 2002) suggest a role for GAGs in Wg signaling. Notum is expressed at the dorsoventral boundary of the wing in a similar manner to Wg and has been demonstrated to shape the Wg gradient most probably by modifying the glypicans Dally and/or Dlp (Giraldez et al., 2002).

The lesser requirement of GAGs for Wg signaling is apparent from the observation that expression of Wg target genes such as *distalless (dll)* is only slightly reduced in clones mutant for genes involved in GAG synthesis such as *botv* (Takei et al., 2004; Han et al., 2004b) or *sll* (U.H., unpublished). Using Dll expression as a marker with which to study the role of GAG enzymes in Wg signaling has led to conflicting results regarding the role of Ttv. The et al. (The et al., 1999), as well as Han et al. (Han et al., 2004b), reported that loss of *ttv* activity did not affect Wg signaling, while Takei et al. (Takei et al., 2004) detected a slight reduction. Both Takei et al. and Han et al., however, detected an effect on Wg extracellular accumulation (not analyzed by The et al.) at the surface for *ttv* mutant cells. The difficulties in pinpointing a role of Ttv in Wg signaling may be due to the fact that Ttv and Sotv collectively function as a co-polymerase.

In the context of patterning the embryonic epidermis, the different affinities of Hh and Wg for GAGs imply that the segment polarity phenotype of embryos deficient for GAG synthetic enzymes may reflect a loss of Hh signaling rather than of Wg signaling. This hypothesis is consistent with the decay of En in *sgl*, *sfl*, *ttv*, *frc* and *sll* mutant embryos, which occurs later than expected for *wg* loss-of-function mutations (e.g. Häcker et al., 1997). However, the late decay of En could also result from a partial loss of activity in both signaling pathways. Importantly, this possibility cannot be tested using overexpression experiments because, as shown in the studies of *sgl*, *sfl*, *ttv*, *frc* and *sll* mutants, the reduced signaling levels associated with the loss of GAGs are compensated for by overexpressing either the Hh or Wg ligands. The severe embryonic phenotype associated with loss of GAGs may result

from 'amplification' of a mild reduction in signaling activity in either one or both of the pathways by the regulatory loop between Hh and Wg signaling in the epidermis. These are important points to remember when considering the conclusions reached by Desbordes and Sanson on the role of glypicans in Wg signaling (Desbordes and Sanson, 2003).

Analysis of proteoglycan function in *Drosophila* has focused largely on the glypicans Dally and Dlp. Flies homozygous for a hypomorphic allele of *dally* show wing margin defects reminiscent of loss of Wg signaling (Nakato et al., 1995; Lin and Perrimon, 1999; Tsuda et al., 1999). The same allele also shows genetic interactions with components of the Wg signaling pathway (Lin and Perrimon, 1999; Tsuda et al., 1999) and with the PAPS transporter *sll* (Lüders et al., 2003) during wing imaginal disc development. In addition, overexpression of *dally* in wing imaginal discs leads to a slight accumulation of Wg adjacent to the dorsal ventral boundary (Strigini and Cohen, 2000). These observations suggest that the functions of GAGs in Wg signaling during wing disc development are at least in part mediated by Dally.

Ectopic expression studies using a *UAS-dlp* transgene have clearly shown that Dlp can trap extracellular Wg and prevent activation of the Wg signaling pathway (Baeg et al., 2001, Giraldez et al. 2002), suggesting that Dlp plays a role in Wg signaling during wing development. Experiments addressing the role of Dlp and Dally in Hh signaling during imaginal disc development suggest a partially redundant role for both genes in patterning at the anteroposterior boundary of the wing (Han et al., 2004a). In the embryo, *dally* and *dlp* are expressed in a segmentally repeated pattern in partially overlapping groups of cells in the epidermis, suggesting a role for both genes in mediating GAG function. Accordingly, removing *dlp* activity by RNAi leads to a segment polarity phenotype (Baeg et al., 2001; Desbordes and Sanson, 2003).

The situation with *dally* is less clear. Hypomorphic *dally* alleles, as well as RNAi against *dally*, have been reported to show weak segment polarity phenotypes (Lin and Perrimon, 1999; Tsuda et al. 1999). However, as pointed out recently by Desbordes and Sanson, and by Han et al. (Han et al., 2004a), this observation is likely to be incorrect, as the dsRNA against *dally* most probably interfered with *dlp* as well, which was not identified at the time of the *dally* experiment. Consequently, evidence supporting a non-redundant embryonic function of *dally* is currently not available.

Importantly, Desbordes and Sanson have reported that RNAi against *dlp* is able to block Hh, but not Wg, signaling in the embryonic epidermis. With regard to Hh signaling, overexpression of Hh in embryos that lack Dlp cannot activate the Hh pathway, implying that the Dlp core protein is essential for Hh signaling. This result is surprising in view of the non-essential role of *sgl*, *sfl*, *ttv*, *sll* or *frc* in Hh signaling (see above), and suggests that the Dlp core protein itself may be able to interact directly with Hh. Consistent with a role for Dlp in Hh signaling, Lum et al. (Lum et al., 2003) found that RNAi against Dlp blocked the ability of cells in culture to respond to Hh if Hh was provided from the media, but not if it was expressed in responding cells. Because mature Hh is anchored to the plasma membrane by lipid modification, the latter experiment suggests that Dlp functions to concentrate Hh and thus facilitates its interaction with the Hh receptor Patched. These results contrast with the *in vivo* experiments of

Desbordes and Sanson who concluded that Dlp was essential for Hh signaling.

Another conclusion put forward by Desbordes and Sanson, based on overexpression of Wg in *dlp* or *dally* mutant backgrounds, is that neither Dally nor Dlp is required for Wg signaling in the embryonic epidermis. As discussed earlier, GAGs are required to a lesser extent for Wg signaling than for Hh signaling in imaginal discs, and the overexpression of Wg may compensate for the lack of GAGs that are normally attached to the proteoglycan. Thus, Desbordes and Sanson's data show that glypicans are not essential for Wg signal transduction but, taking into account the data from imaginal discs, that Dally and Dlp are very probably necessary as accessory factors for Wg signaling during embryonic patterning *in vivo*.

Altogether, Desbordes and Sanson's results indicate that Hh is more sensitive to proteoglycans than is Wg signaling and that Dlp is probably playing a more important role than Dally. Further studies, however, are needed in order to understand fully the role of the Dlp core protein and its associated GAG chains. In particular, the unexpected observation that Dlp is absolutely required for Hh signaling *in vivo* will need to be further substantiated and understood at the molecular level in the context of the roles of Patched and Smoothed in Hh signaling.

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Response

Do glypicans play a role in Wingless signalling in *Drosophila*?

While Perrimon and Häcker argue in favour of a role of glypicans in Wingless (Wg) signalling in *Drosophila* (Perrimon and Häcker, 2004), I present reasons why I believe this hypothesis, although still tenable, is not demonstrated by the current evidence.

There is clear evidence that glycosaminoglycans (GAGs) affect the distribution of the Wg and Hedgehog (Hh) ligands in *Drosophila* epithelia. By doing so, they influence Hh signalling, and to a lesser extent Wg signalling. This is illustrated by the segment polarity phenotypes exhibited by seven genes required for GAG synthesis, *sugarless* (*sgl*), *sulfateless* (*sfl*), *tout-velu* (*ttv*), *brother of tout-velu* (*botv*), *sister of tout-velu* (*sotv*), *fringe connection* (*frc*) and *slalom* (*sll*) (Binari et al., 1997; Goto et al., 2001; Häcker et al., 1997; Haerry et al., 1997; Han et al., 2004b; Lin and Perrimon, 1999; Luders et al., 2003; Selva et al., 2001; The et al., 1999). The segment polarity phenotypes of *sgl* and *sfl* were attributed to a failure in Wg signalling (Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997), whereas the *ttv* embryonic phenotype was attributed to a loss of Hh signalling (The et al., 1999). However, because it is difficult to separate Wg and Hh signalling in the embryonic epidermis, these conclusions were based on the signalling requirement of these genes in other tissues, which could well be different. Thus, in my view, the current experimental data fail to distinguish unambiguously between a role for *sgl*, *sfl*, *ttv*, *sotv*, *botv*, *frc* and *sll* in Hh signalling or Wg signalling, or in both, in the embryonic epidermis.

A key question is what is the identity of the proteins that carry the GAG and that are required for Wg and Hh signalling? The prime candidates are the glypicans, and the function of the enzymes involved in GAG synthesis has often been equated in the literature with the function of the two fly glypicans, Dally and Dally-like (Dlp). However, the aforementioned enzymes could modify other proteins involved in Wg and Hh signalling, and it remains to be proven that the segment polarity

phenotypes exhibited by *sgl*, *sfl*, *ttv*, *sotv*, *botv*, *frc* and *sll* are the result of a failure in the signalling activity of Dally and Dlp.

A first step towards answering this question is to determine whether the glypicans have a function in Wg and Hh signalling. If Dally and Dlp are required non-redundantly for at least one of these two pathways, *dally* and *dlp* mutants should exhibit segment polarity phenotypes in embryos.

We have shown using RNAi that loss of Dlp produces a clear segment polarity phenotype (Desbordes and Sanson, 2003). Moreover, Han and colleagues (Han et al., 2004a) have published the first mutation in *dlp*, and have shown that removing both zygotic and maternal contributions of the gene causes a full segment polarity phenotype, thus confirming our RNAi results. These results demonstrate without ambiguity that *dlp* is a bona fide segment polarity gene.

By contrast, there is no evidence as yet that *dally* mutants have a segment polarity phenotype. Early work from Baeg et al. (Baeg et al., 2001), Lin and Perrimon (Lin and Perrimon, 1999) and Tsuda et al. (Tsuda et al., 1999) used RNAi in an attempt to ablate *dally* function, and found mild segment polarity phenotypes that they interpreted as being the consequence of a loss of Wg signalling. In our study, we used RNAi to silence *dally* (using shorter dsRNA fragments), and found the same proportion of weak segmentation defects, because of the injection process, in the control and in the experimental embryos (Desbordes and Sanson, 2003). We showed using RT-PCR that *dally* mRNA levels were strongly reduced, thus confirming that our RNAi procedure was effective. This result suggests that, as acknowledged by Perrimon and Häcker (Perrimon and Häcker, 2004), the original RNAi results for *dally* are likely to have been an artefact.

Although RNAi affects both maternal and zygotic mRNA pools, it will not affect the protein pool contributed maternally, so we cannot exclude the possibility that some maternal Dally protein is sufficient to rescue a segment polarity phenotype in our experiments. This issue will only be resolved by making

germline clones with a null mutation in *dally*, in order to remove both maternal and zygotic contributions of the gene. A putative null mutation in *dally* has recently been isolated by Han et al. (Han et al., 2004a), but no germline clone analysis has yet been reported.

Apart from segment polarity phenotypes, what is known about the putative requirement of the two glypicans in Hh signalling and Wg signalling? I believe it is now clear that Dlp has a non-redundant role in Hh signalling in the embryo and in cultured cells (Lum et al., 2003; Desbordes and Sanson, 2003; Han et al., 2004a). For reasons that are yet unclear, Dally can take over the role of Dlp in Hh signalling in wing imaginal discs, i.e. in this tissue the two glypicans are redundant for Hh signalling (Han et al., 2004a).

There are hints that glypicans could contribute to Wg signalling, but in my view the evidence is circumstantial and firmer experimental results are needed. Mutant alleles of *dally* were available for a while (Nakato et al., 1995), but only very weak segmentation defects were observed in zygotic mutants (Tsuda et al., 1999), or in mutants deficient in both maternal and zygotic contribution, suggesting that these *dally* alleles could be hypomorphic (Lin and Perrimon, 1999). In the wing disc, the same *dally* mutations produce a very low percentage of wing notches (3%) (Lin and Perrimon, 1999) (see also Nakato et al., 1995), a phenotype associated not only with defects in Wg signalling, but also in Notch signalling. Thus, in the absence of null alleles of *dally*, its role in Wg signalling remains uncertain.

Likewise, the evidence that Dlp is involved in Wg signalling is not compelling. In 2001, Baeg and colleagues used RNAi to ablate *dlp* function and found a mild segment polarity phenotype (Baeg et al., 2001). Whereas a segment polarity phenotype can be due to either a loss in Hh or Wg signalling (or both), the researchers interpreted this phenotype as resulting from a failure in Wg signalling. One reason for this interpretation was that Dally, and also Sgl and Sfl, two enzymes involved in GAG synthesis, had already been implicated in Wg signalling (Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999). However, the current evidence for *dally* is weak, and the segment polarity phenotype of *sgl* and *sfl* mutants cannot be attributed unambiguously to one signalling pathway. Another reason to link Dlp function with Wg signalling is that overexpression of Dlp alters the distribution of Wg at the surface of the wing cells and causes notching phenotypes in the adult wing (Baeg et al., 2001). However, the notching phenotype suggests a negative role in the Wg pathway and the segment polarity phenotype suggests a positive role, which was, and still is, puzzling.

Two publications have recently re-examined a putative role of the glypicans in Wg signalling, and neither detected a role for Dally and Dlp in this pathway (Desbordes and Sanson, 2003; Lum et al., 2003). Lum and colleagues (Lum et al., 2003) show that in *Drosophila* cultured cells, RNAi silencing of *dally* or *dlp* does not affect Wg signalling. We obtained the same result, also using RNAi silencing, but this time in vivo, in the embryonic epidermis (Desbordes and Sanson, 2003). We used heterologous expression to uncouple Wg and Hh pathways, and tested the requirement of Dlp and Dally in Wg signalling. We found that the secretion of smooth cuticle and the expression of Engrailed, two targets of Wg signalling in the embryonic epidermis, are not abolished after silencing Dlp or Dally or both by RNAi (Desbordes and Sanson, 2003).

It is important to stress that both studies, ours and the one by Lum and colleagues (Lum et al., 2003), do not prove that the glypicans are not involved in Wg signalling, they simply do not provide supportive evidence for a role in this pathway. There are two ways in which a requirement for Dally or Dlp in Wg signalling could be missed in our experiments with the embryonic epidermis. First, as mentioned above, RNAi does not affect the maternal protein stores, and thus some maternal Dlp and/or Dally protein could be rescuing Wg signalling. Embryos that lack maternal and zygotic contribution of both glypicans will need to be generated to settle this issue definitively. In these experiments, Hh signalling will have to be rescued by transgene expression, in order to test specifically a requirement in Wg signalling (otherwise the defect in Hh signalling caused by lack of Dlp could mask an additional effect on Wg signalling).

Second, as pointed out by Perrimon and Häcker (Perrimon and Häcker, 2004), rescue of *dally* and *dlp* RNAi by transgene expression of Wg could be a consequence of overexpression. This suggestion is based on the observation that overexpressing Wg in mutants for *sgl* restores some smooth cuticle and some Engrailed expression in mutant embryos (Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997; Pfeiffer et al., 2002). However, it is important to note that this rescue is dose sensitive, i.e. lower expression of the ligand does not result in rescue (Häcker et al., 1997), and two publications show that the rescue is only partial (Binari et al., 1997; Haerry et al., 1997). In addition, these experiments examined only the cell-autonomous response to Wg signalling. Indeed, Wg is overexpressed either in every cell (*hs-wg*) or in large domains of the epidermis (*prd-Gal4*), and the rescuing effect is examined in the cells that overexpress Wg. In our experiments, we used two different Gal4 drivers (Desbordes and Sanson, 2003). Using *armadillo-Gal4*, we looked at cell-autonomous signalling by driving expression of Wg in every embryonic cell. Here, a requirement for Dlp and/or Dally in Wg signalling could conceivably be masked by a high dose of ligand, as found for *sgl* mutants. However, the second driver we used, *single-minded-Gal4*, drives Wg expression in a thin stripe of neural cells, in the ventral midline. This experiment has two advantages: (1) the ligand is expressed at physiological levels (we could not detect a difference in levels of *wg* transcription at the midline compared with *wg* endogenous transcription); and (2) we are looking at non-autonomous cell signalling because Wg is secreted by the neural cells, whereas expression of target genes is monitored in adjacent epidermal cells. If Dally or Dlp were required for the secretion or transport of Wg from the neural cells (the source of ligand) to the epidermal cells, RNAi-mediated silencing of *dally* and *dlp* should prevent expression of target genes, which is not observed. This suggests that neither Dally nor Dlp, alone or together, are required for Wg ligand secretion, movement or reception in the embryonic cells, unless, again, there are enough maternal glypican molecules to rescue these functions.

It is possible that the glypicans affect Wg distribution, but without having a major impact on the efficiency of Wg signalling in embryonic or cultured cells. Indeed, in some instances, an alteration in the distribution of Wg at the cell surface has been associated with changes in Dally and Dlp levels. As mentioned above, overexpression of Dlp alters the distribution of Wg at the surface of the wing cells (Baeg et al.,

2001) [however, Strigini and Cohen (Strigini and Cohen, 2000) report that overexpression of Dally does not have a significant effect] and *dally* RNAi affects Wg distribution in *Drosophila* cultured cells (Lum et al., 2003).

In conclusion, I think that the evidence so far for a role of the fly glypicans in Wg signalling is not compelling. The recent isolation of null mutations in *dally* and *dlp* (Han et al., 2004a) (Jean-Paul Vincent, personal communication) should, in the near future, allow a thorough examination of this issue in embryos and in wing discs.

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Response

The role of Ttv in Wg signaling

Perrimon and Häcker's correspondence (Perrimon and Häcker, 2004) provides a concise review on the genetic studies of Heparan Sulfate Proteoglycans (HSPGs) and raises useful hints for future research. Among the topics discussed in this letter, they have summarized the recent reports on the role of GAGs for Wg signaling as follows:

Using Dll expression as a marker to study the role of GAG enzymes in Wg signaling has led to conflicting results regarding the role of Ttv. The et al. (The et al., 1999), as well as Han et al. (Han et al., 2004b), reported that loss of *ttv* activity did not affect Wg signaling, while Takei et al. (Takei et al., 2004) detected a slight reduction.

I do not think that the results reported by Han et al. conflict with those by Takei et al. because both groups observed and reported the same phenotype: levels of Dll (a marker for the long-range signaling activity of Wg) were slightly reduced in *ttv* mutant cells. Based on the same observations, Takei et al. simply concluded that Wg signaling was affected in *ttv* mutant cells, but Han et al. interpreted that Ttv was required only for Wg distribution but not for Wg signaling because levels of Senseless (a marker for the short-range

signaling activity of Wg) were not reduced in *ttv* mutant cells.

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