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Gliolectin positively regulates Notch signalling during wing-vein specification in *Drosophila*

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ABSTRACT Notch signalling is essential for animal development. It integrates multiple pathways controlling cell fate and specification. Here we report the genetic characterization of Gliolectin, presumably a lectin, a cytoplasmic protein, significantly enriched in Golgi bodies. Its expression overlaps with regions where Notch is activated. Loss of *gliolectin* function results in ectopic veins, while gain of its function causes loss of wing veins. It positively regulates *Enhancer of split mβ,* a target of Notch signalling. These observations suggest that it is a positive regulator of Notch signalling during wing development in *Drosophila*.

KEY WORDS: *Glec, Notch, wing vein development, D/V boundary*

Introduction

Signalling pathways interact at different stages of development to give rise to a flattened wing in the adult *Drosophila*, starting from a monolayer sheet of cells called the wing imaginal disc. The five longitudinal veins found in *Drosophila* wings are positioned in the late third larval instar wing disc by the activity of numerous signalling pathways (reviewed by Blair, 2007). During wing disc patterning, Rhomboid (Rho), a membrane protein is expressed in stripes of cells that develop later into veins (Sturtevant *et al*., 1993). Rho interacts with Epidermal Growth Factor Receptor (EGFR) (Price *et al*., 1989; Sturtevant *et al*., 1993) leading to activation of the EGFR signalling pathway. Activation of Ras/EGFR signalling pathway in presumptive vein territories directs these cells into a vein differentiation pathway (Gabay *et al*., 1997; Diaz-Benjumea *et al*., 1990; De Celis, 1998; De Celis and Diaz-Benjumea, 2003). Vein differentiation continues during pupal development and involves the restriction of specific gene products and cell adhesion proteins to veins or intervein territories (Fristrom *et al*., 1993; Montagne *et al*., 1996).

Notch (N) signalling is involved in establishing the correct width of various wing veins (de Celis *et al*., 1994). *Delta (Dl),* a ligand that activates N, is found at higher levels in the provein regions resulting in the activation of N in the intervein regions (de Celis *et al.,* 1997b). Loss of *N* alleles display vein thickening in the wing and enhanced *rho* expression, while gain of function of *N* show thinner and incomplete veins with reduced or lost *rho* expression (Palka *et al*., 1990; de celis *et al*., 1994; Sturtevant *et al*., 1995; de Celis *et al*., 1997b).

Expression of *Dl* is under the transcriptional control of EGFR signalling pathway (Simcox *et al*., 1996; Schnepp *et al*., 1996; de Celis *et al*., 1997b) whereas the expression of *N* is independent of the formation of wing veins (de Celis *et al*., 1997b). In the absence of Dl, N is not activated and hence is unable to activate its downstream targets (de Celis *et al*., 1997b). The N target gene Enhancer of split mβ (E(Spl)mβ) represses *rho* expression when ectopically expressed in the wing imaginal disc and thus mimics gain of function of N. This is further confirmed by the fact that loss of vein phenotype caused by ectopic expression of E(Spl)mβ can be rescued by the simultaneous ectopic expression of Rho (de Celis *et al*.,1997b). Expression of E(Spl)mβ in the wing imaginal discs is complementary to that of Dl, being maximal in the presumptive intervein regions and low in the veins. As E(Spl)mβ is not expressed in the vein territory, the repression of *rho* (expressed in the presumptive veins) by N target gene E(Spl)mβ must be indirect (de Celis *et al*.,1997b). This mechanism restricts the regions (presumptive veins) where *rho* is expressed (Sturtuvent *et al*., 1995; de Celis *et al*., 1997b). Thus, N maintains the correct width of the veins by inhibiting vein development in the intervein cells.

An allele of *glec* (*glec*^{d04956}) has been shown to enhance the notched wing phenotype of *N* allele (N^{54L9}) (Hurlbut *et al*., 2009). In

Abbreviations used in this paper: D/V, dorso/ventral; Rho, rhomboid.

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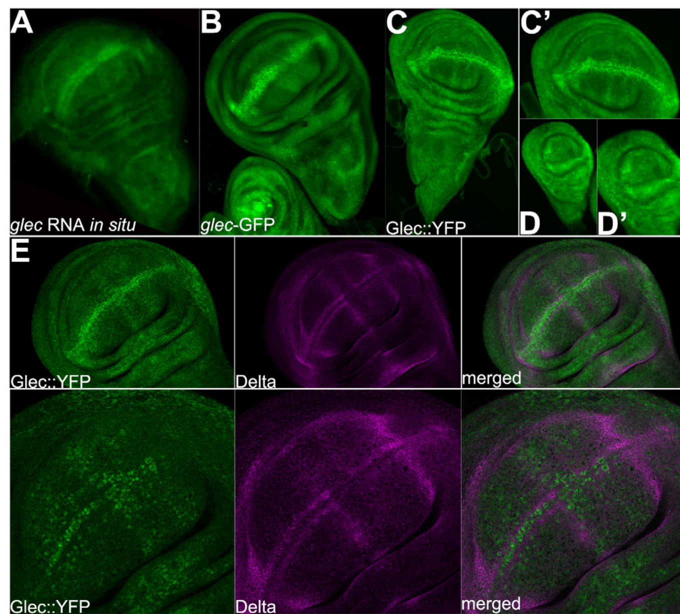

Fig. 1. Expression pattern of Glec in the wing imaginal disc. (A) *Wild type wing disc showing Glec expression as determined by RNA* in situ *hybridization.* **(B)** *Wing disc of* glec-*GFP enhancer trap line showing GFP expression pattern.* **(C,D)** *Wing (C) and haltere (D) discs of Glec::YFP stained for YFP (C' and D' show the discs at higher magnification). Glec is expressed predominantly along the D-V axis of the wing imaginal discs. Please note the absence of Glec along the D-V boundary in the haltere imaginal disc (as reported by Prasad* et al., *2006).* **(E)** *Wing imaginal disc of Glec::YFP double stained for YFP (green) and Dl (magenta). Upper panel is at magnification 25X, while bottom panel is the same disc at 63X magnification. Note that expression patterns of Glec and Delta are mutually exclusive.*

this study we report that *gliolectin (glec)* is required for specification of vein/intervein regions in the *Drosophila* wing. We show that *glec* is expressed in the regions of wing imaginal disc where *N* is activated. Loss of function of *glec* shows ectopic veins, while gain of function of *glec* shows loss of wing vein. Glec positively regulates members of *N* signalling pathway and interacts with components of EGFR signalling pathway. Owing to its biochemical nature as a carbohydrate binding protein (Tiemeyer *et al.*, 1996) and its presence in Golgi bodies in the regions where N is activated, we propose that Glec might be modifying certain components of the *N* signalling cascade.

Results and Discussion

Glec is expressed in the D/V boundary in the wing imaginal disc

Differential expression of *glec* between wing and haltere has been shown earlier by RNA *in situ* (Prasad *et al.*, 2006; Fig. 1A). First we tested expression of protein trap lines of Glec. The Glec:YFP (DGRC Kyoto) and the *glec*-GFP (Enhancer Trap) show that although Glec is expressed at low levels ubiquitously in the entire wing imaginal disc, maximal expression of the protein is seen in the dorsal ventral (D-V) boundary (Fig. 1B).

Dl is expressed in the proveins in the third larval wing disc and also along two stripes adjoining the D-V boundary (Huppert *et al.,* 1997, de Celis *et al.,* 1997a,b). Double staining of Glec with Dl indicates a complementary expression pattern in the wing pouch (Fig. 1E). Along the D/V axis, N activates Ct and Wg in the D-V boundary (deCelis *et al.*, 1997a). Glec expression overlaps with that of Wg and Ct, although Glec is also expressed in cells abutting the D-V boundary (Fig. 2 A-C).

Presence of Glec in Golgi bodies

We then examined the sub cellular localization of Glec. Triple staining for Wg, Glec and DAPI suggested that Glec is present as punctate structures in the entire cell but is not present near or on the plasma membrane (Fig. 2C). Mouse homolog of *glec*, GM3 synthase localizes to the Golgi bodies of neurons (Stern *et al.*, 2000). We therefore examined the co-localisation of Glec with Galactosyl transferase (Galt), a Golgi body marker in the wing imaginal disc. The co-localization suggests that Glec is present in the Golgi bodies, although not exclusively (Fig. 2D).

Glec *functions to restrict wing vein development*

To enable spatio-temporal down regulation of *glec,* we generated two different transgenic RNAi lines that would generate double stranded RNA targeting non-overlapping regions of *glec* mRNA -

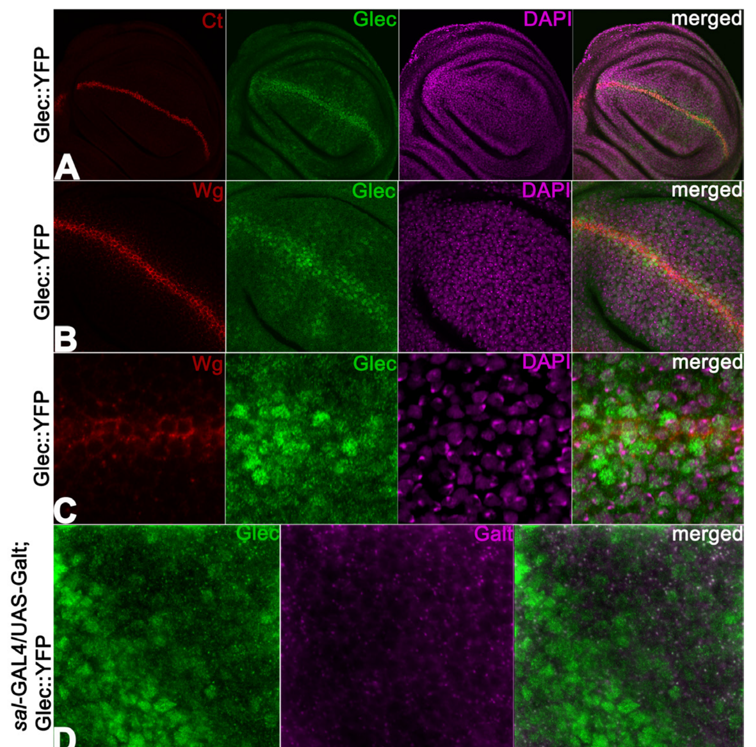

Fig. 2. Expression of Glec along the D-V boundary and its localisation in the Golgi bodies. (A) *Glec:: YFP wing disc triple stained for YFP (green), Ct (red) and DAPI (magenta). Glec expression overlaps with that of Ct in the D/V boundary.* **(B,C)** *Glec:: YFP Wing disc triple stained for YFP (green), Wg (red) and DAPI (magenta). Glec expression overlaps with that of Wg in the D/V boundary.* **(C)** *Magnified image of the D-V boundary. Note that Glec is neither present on the plasma membrane nor in the nucleus.* **(D)** *Glec::YFP wing imaginal disc double stained for YFP (green) and Galt (magenta). Please note co-localization of Glec and Galt (a marker for Golgi bodies) suggesting that Glec is a Golgi-localized protein.*

one targeting 5' end, while the other targeting the 3' end. To test the efficacy of these RNAi lines, we crossed them to *sal*–GAL4 driver in a background of Glec::YFP fusion lines. The expression of Glec was reduced considerably in the domain of *sal*-GAL4 and both the RNAi lines were equally efficient in down regulating *glec* expression (Fig. 3 A-C). All the experiments were independently carried out using both 5' and 3' targeting *glec*^{RNAi} lines and they both generated identical phenotypes. In the text we would be referring to these lines as *glec*^{RNAi} line.

Upon down regulation of *glec* using UAS-*glec*^{RNAi} (driven by *MS1096*-GAL4 driver), we observed ectopic veins in regions close to different longitudinal veins (Fig.3: D-E; penetrance 30%, n= 176 at 28 °C). This phenotype was also seen when *glec* was down regulated using *ap*-GAL4 driver (data not shown; penetrance 81 % at 28 °C, n= 20; down-regulation was also associated with considerable lethality). The penetrance of the phenotype was higher when down regulation was carried out using two copies of *glec*^{RNAi} driven by *MS1096*-GAL4 at 28 °C (penetrance 39 % at 25 °C, n= 73 and 74% at 28 °C, n= 135). We also used VDRC RNAi lines to down regulate *glec* using *MS1096*-GAL4 driver under similar conditions and observed identical phenotypes (data not shown; penetrance 64% at 28 °C, n= 72). However, the VDRC lines had two off targets and hence to avoid any discrepancies, we only used *glec*^{RNAi} lines that were generated by us, which didn't have any off targets, for further experiments.

We generated *UAS-Glec* transgenic lines to over-express Glec in a spatio-temporal manner under the control of various GAL4 drivers. We used *omb*-GAL4, *ptc*-GAL4 and *MS1096*-GAL4 drivers to ectopically express Glec in different regions of the wing. When *omb*-GAL4 driver was used to ectopically express Glec, second longitudinal veins (L2) and anterior cross veins (ACV) were lost (Fig. 3F). When *ptc*-GAL4 driver was used to ectopically express Glec, ACV was lost (Fig. 3G). Penetrance of these phenotypes was considerably higher at 28 °C. When we ectopically expressed Glec using *MS1096*-GAL4 at 25 °C, considerable loss of vein tissue was observed (Fig. 3H). When we increased the temperature to 28°C, vein tissue was completely lost in wings of most flies (Fig. 3I).

Considering varying degree of gain-of-function phenotype for Glec when expressed using mild to strong GAL4 drivers, we examined possible auto-regulation. We observed down regulation of endogenous levels of *glec* (as measured by the YFP expression in the Glec::YFP protein fusion line) in the regions where *sal*-GAL4 (Fig. 3J) is expressed. This suggested the presence of a auto-feedback loop due to which the endogenous levels of Glec may be lowered when the it is over-expressed.

We further tested specificity of the phenotype caused by the down- or up-regulation of Glec. Co-expression of UAS-*glec*^{RNAi} and UAS-Glec using *MS1096*-GAL4 driver resulted in complete suppression of each other's effect i.e. suppression of both the ectopic-vein and loss-of-vein phenotypes (penetrance 100% at 28 °C, n= 92) caused by UAS-*glec*^{RNAi} or UAS-Glec alone (data not shown).

Thus, both loss- and gain-of-function phenotypes associated with *glec* suggest that normal function of *glec* is to suppress the vein development pathway. This points to a possible role for Glec in positive regulation of N pathway and/or negative regulation of EGFR pathway. As Glec itself is expressed at maximal levels in the presumptive inter-vein region, its regulation of N would be cell-autonomous and that of EGFR pathway would be non-cell autonomous.

Glec *positively regulates the Notch signalling pathway and represses EGFR signalling pathway*

N mediates the repression of vein development by activation of its target gene *E(Spl)m*β (de Celis *et al.*, 1997b) in the intervein region. We examined *E(Spl)m*β expression in wing imaginal discs where *glec* was down- or up-regulated using a its lacZ reporter transgene *E(Spl)m*β-lac Z. We observed

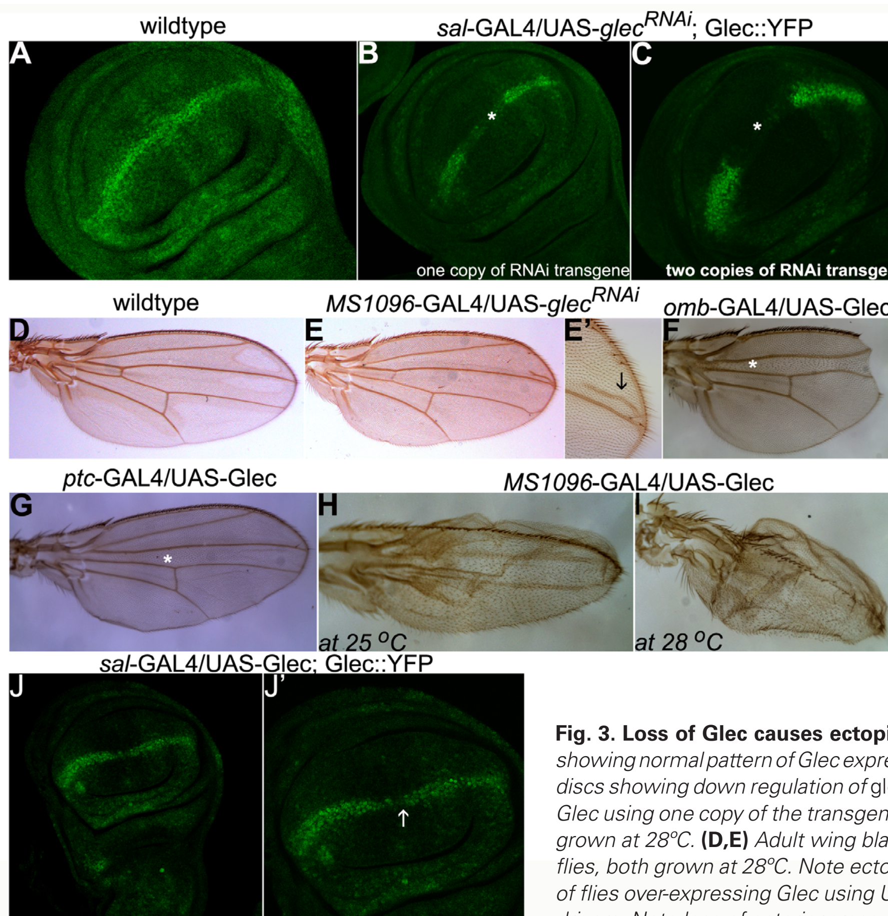

Fig. 3. Loss of Glec causes ectopic wing vein development. (A) *Glec::YFP wing disc - showing normal pattern of Glec expression.* **(B,C)** sal-*GAL4/UAS-B-C,* glec^{RNAi}; *Glec::YFP wing discs showing down regulation of* glec *along A-P axis (asterix). (B) shows down regulation of Glec using one copy of the transgene and (C) shows with two copies of the transgene, both grown at 28ºC.* **(D,E)** *Adult wing blades of wildtype (D) and* MS1096-*GAL4/UAS*-glec^{RNAi} *(E) flies, both grown at 28ºC. Note ectopic vein close to L3 in E (arrow).* **(F-I)** *Adult wing blades of flies over-expressing Glec using UAS*-glec *and* omb- *(F),* ptc- *(G) and* MS1096- *(H, I) GAL4 drivers. Note loss of anterior-cross vein (asterix) in (F,G) and complete loss of wing veins in (H,I). The experiment was carried out at 28ºC in (F, G and I) and at 25ºC in (H).* MS1096-*GAL4 expresses in the dorsal compartment.* **(J)** *Wing disc of* sal-*GAL4/UAS-Glec; Glec::YFP grown at 28ºC. A reduction in Glec expression in the centre of the wing pouch (arrow), suggests negative feedback loop.*

down regulation of its expression in dorsal compartment of the wing imaginal disc in response to down regulation of *glec* using *MS1096*-GAL4 driver (Fig. 4B).

When over-expressed using *MS1096*-GAL4, Glec caused increased levels of *E(Spl)m*β in the ventral compartment (Fig. 4C). We observed higher levels of *E(Spl)m*β in the anterior side of the ventral compartment and in the intervein between L3 and L4 (Fig. 4C). Intriguingly, we found that the levels of *E(Spl)m*β was significantly reduced in the dorsal compartment. We attribute this discrepancy to the variation in spatio-temporal expression pattern of GAL4 line used for this study. *MS1096*-GAL4 is strongly expressed in the dorsal compartment of the wing imaginal disc, which begins from early third instar larval stage, while its expression in the ventral compartment starts from late third larval instar stage and levels are much lower than in the dorsal compartment (Wang *et al*., 1999). This could be the reason for the effect of RNAi-mediated down regulation of *glec* on *E(Spl)m*β was detectable only in the dorsal compartment.

If Glec positively regulate N signalling, its ectopic expression in the dorsal compartment in the early third instar larval stage would activate N, which in turn would increase the levels of *E(Spl)m*β. However, prolonged strong activation of N may have negative impact on its own signalling. Activation of N would result in the down regulation of EGFR/Ras signalling in the early third instar larva. As *Dl* is under the transcriptional control of the EGFR/Ras signalling pathway, loss of EGFR/Ras signalling would reduce the expression of *Dl*, which in turn would lead to decreased activation of N, resulting in reduced expression of *E(Spl)m*β expression.

Another possibility is, Glec is negatively auto-regulated when it is over-expressed, leading to loss of N signalling and increased EGFR signalling. This could be the reason for the observed low levels of *E(Spl)m*β expression in the dorsal compartment. As over-expression of Glec in the ventral compartment is only in the later stages of the third larval instar stages, we may have been able to observe increased levels of *E(Spl)m*β, reflecting the role of Glec as a positive regulator of N signalling. Consistent with this we also observed activation of *E(Spl)m*β expression on the ventral side of the D-V boundary, in the regions where *Dl* is normally expressed (and where *E(Spl)m*β is normally absent).

Earlier studies have shown that N suppresses the expression of

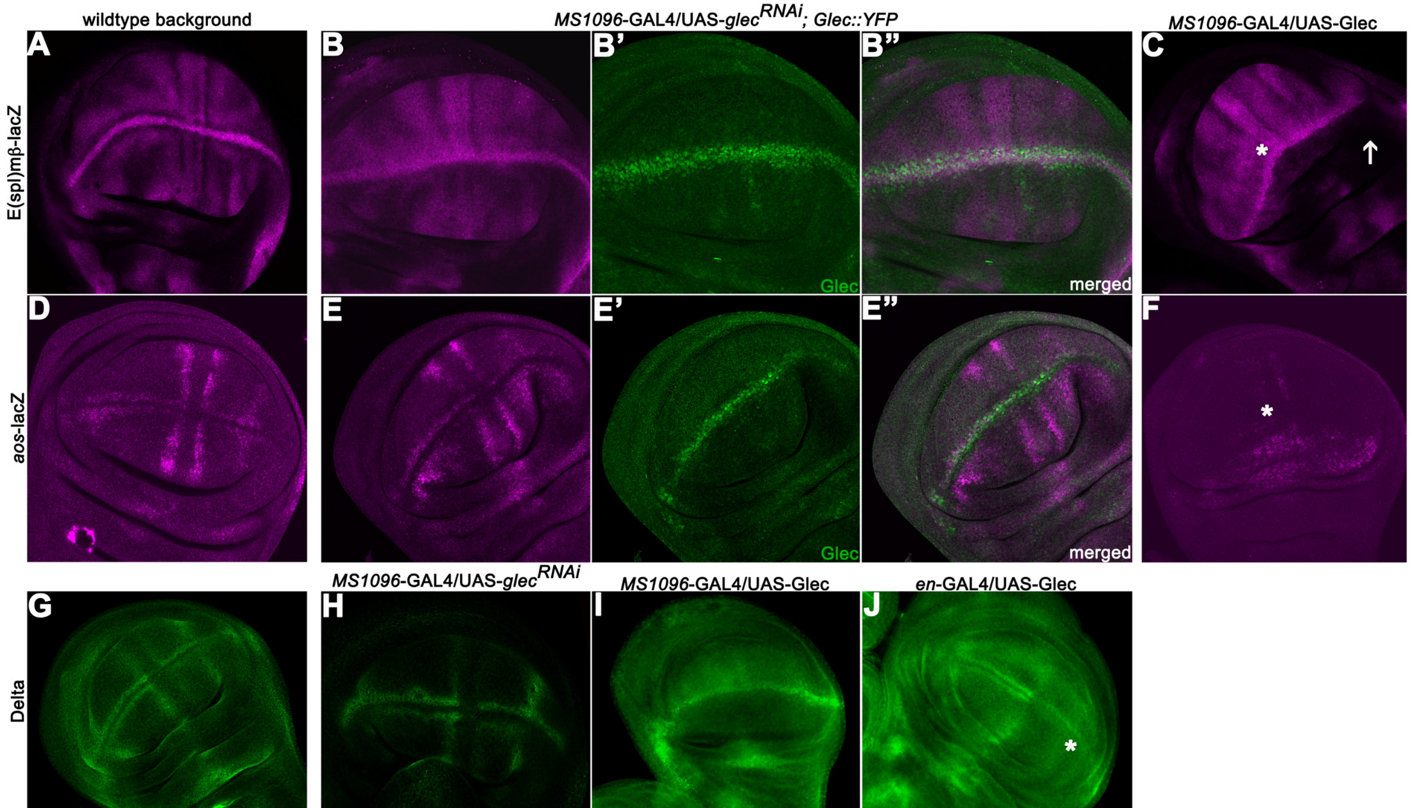

Fig. 4. Glec regulates the expression of *E(spl)mβ*. (A) E(spl)mβ-*lacZ wing disc stained for β-galactosidase. In the wild type background,* E(spl)mβ *is expressed in the presumptive intervein regions and in the D/V boundary.* **(B)** MS1096-*GAL4/UAS-*glec*RNAi*; E(spl)mβ*-lacZ/Glec::YFP (raised at 28°C) wing disc stained for YFP (green) and β-galactosidase (magenta). Note reduced levels of E(spl)mβ-lacZ in the dorsal compartment.* **(C)** MS1096-*GAL4/UAS-Glec;* E(spl)mβ*-lacZ (raised at 25°C) wing disc stained for β-galactosidase. Note increased levels of* E(spl)mβ*-lacZ in the ventral compartment (asterix) and its reduced levels in the dorsal compartment (arrow).* **(D)** aos*-lacZ wing disc stained for β-galactosidase showing Aos expression in the wild type background.* **(E)** MS1096-*GAL4/UAS-*glec*RNAi*; aos*-lacZ/Glec::YFP (raised at 28°C) wing disc stained for YFP (green) and β-galactosidase (magenta). Note ectopic activation of Aos, but, only in the interviein region.* **(F)** MS1096-*GAL4/UAS- Glec;* aos*-lacZ (raised at 25°C) wing disc. Note ectopic activation of Aos in the dorsal compartment, and its repression in the ventral compartment (asterix).* **(G-J)** *Wing discs stained for Dl Wildtype (G),* MS1096-*GAL4/UAS-*glec*RNAi* *(two copies of RNAi transgene, raised at 28°C; H),* MS1096-*GAL4/UAS-Glec (raised at 25°C; I) and* en-*GAL4/ UAS-Glec (raised at 25°C; J). Note down regulation of delta along the D-V boundary (but, not in proveins) in (H) and its complete loss in the dorsal compartment in (I). The down regulation of delta is seen only in posterior compartment when Glec is ectopically expressed using* en-*GAL4 (asterix in J).*

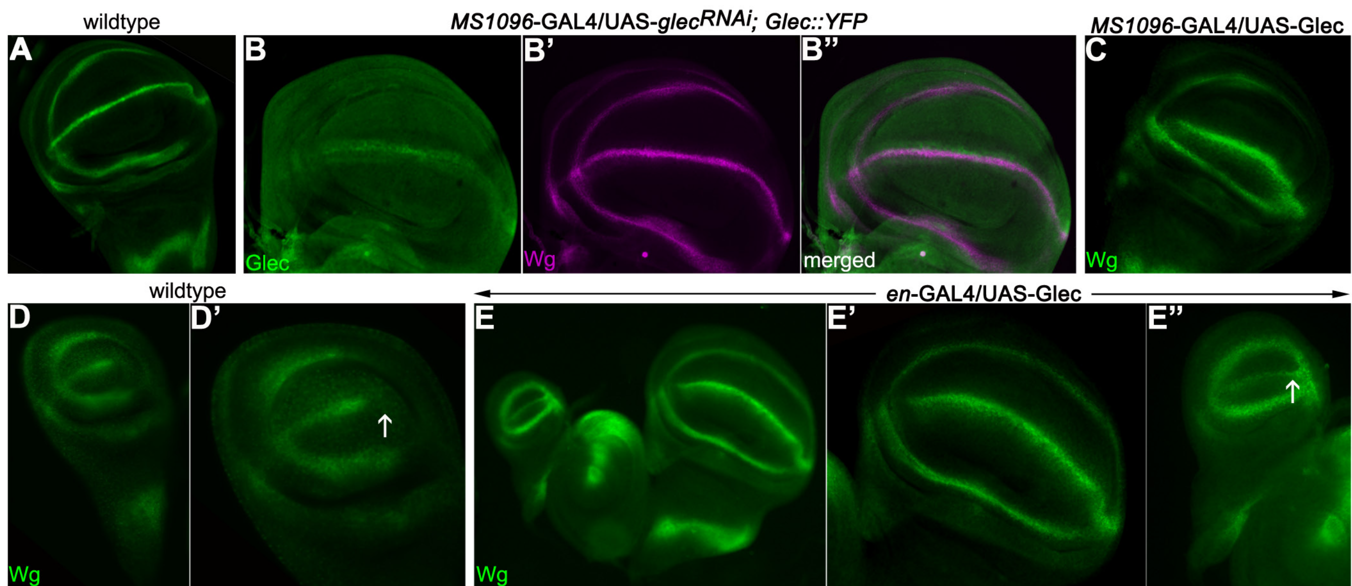

Fig. 5. Glec positively regulates Wg, a target of Notch. *Wildtype wing* **(A)** *and haltere* **(D)** *discs stained for Wg. Note absence of Wg expression in the D/V boundary of the posterior compartment of haltere disc (Arrow).* **(B)** MS1096-*GAL4/UAS*-glec^{RNAi}*; Glec::YFP (raised at 28ºC) wing disc stained for Wg (magenta). No change in Wg expression levels/patterns was observed on reduction of* glec *transcript.* **(C)** MS1096-*GAL4/UAS-Glec (raised at 25ºC) and* en-*GAL4/UAS-Glec (raised at 28ºC). Wing* **(C, E)** *and haltere discs* **(E)** *stained for Wg.* **(E' , E")** *The same wing and haltere discs as in (E), but at higher magnification. Note increased levels of Wg in the dorsal compartment of wing disc and in the posterior compartment of haltere disc (Arrow).*

EGFR in the presumptive intervein cells. This is by activating the expression of *E(Spl)m*β, which in turn represses the expression of components of EGFR in the intervein cells. This helps to keep EGFR expression confined to the presumptive vein cells (de Celis *et al.,* 1997b). We examined the effect of down-regulation of *glec* on the EGFR pathway by using *aos*-lacZ, a reporter transgene that directly reflects EGFR activity. We observed upregulation of *aos*-lacZ in the presumptive intervein regions (Fig. 4E) in response to the down-regulation of *glec* in wing imaginal discs using *MS1096*-GAL4 at 28 °C. Conversely, over-expression of Glec caused down regulation of Aos in presumptive vein regions (Fig. 4F). However, consistent with the effect of over-expression of Glec on the expression pattern of *E(Spl)m*β, we also observed increased levels of *aos*-lacZ in the dorsal compartment.

Dl plays an important role in the cross talk between EGFR and N pathways. In the vein-intervein specification, Dl is expressed in the presumptive vein regions, which is directly regulated by the EGFR singling pathway at the transcriptional level, while it acts as a ligand to activate N in the adjacent intervein regions. However, along the DV axis, N signalling positively regulates Dl expression (de Celis *et al*., 1997a). Consistent with this mode of interactions, we observed loss of Dl in dorsal cells adjacent to the D/V boundary when *glec* is down regulated using *MS1096*-GAL4 driver. However, loss of *glec* did not affect Dl expression in the proveins. Even when *glec* was down regulated with two copies of *glec*^{RNAi} transgenes using *MS1096*-GAL4 at 28 °C, we did not see any effect on Dl expression in the proveins, while its expression was further down regulated along the D/V boundary compared to when single copy of *glec*^{RNAi} transgene was used. This suggests that Glec is primarily a regulator of N pathway, and the observed effect on EGFR/Ras pathway is due to down regulation of N pathway (Fig. 4H).

Over-expression of Glec using *MS1096*- or *en*-GAL4 driver at 25 °C down regulated Dl expression (Fig. 4 I,J). This was expected since, as described above, over-expression of Glec in the dorsal compartment during early 3rd instar larval stages would lead to loss of N, either due to negative auto-regulation of Glec itself and/or negative feedback through EGFR pathway.

It is intriguing, while loss of function of *glec* results in the activation of *N* signalling pathway, ectopic expression of Glec using MS1096-GAL4 seems to exhibit, in the dorsal compartment of the wing imaginal disc, phenotypes that would resemble down regulation of *N* leading to ambiguity in conclusions. We have tried to explain these discrepancies on the basis of spatio-temporal differences in expression of MS1096 GAL4 driver between the dorsal and ventral compartment of the wing imaginal disc. This explanation may be considered as an extrapolation of the limited amount of data that we have.

However, a clearer picture emerges if we focus on the ventral compartment of the wing imaginal disc in the cases where Glec is ectopically expressed using MS1096-GAL4 driver. As mentioned earlier, MS1096-GAL4 expression in the ventral compartment begins in the late third instar larval stage and is mild. The effects of Glec over expression using MS1096-GAL4 in the ventral compartment complement the effects induced by its down regulation in the dorsal compartment by the same GAL4 driver.

Glec *positively regulates* wingless, *a target of the Notch pathway*

N activates the expression of *E(Spl)m*β via Su(H) (Bailey *et al*., 1995; Lecourtois *et al*.,1995). E(Spl)mβ serves as a repressor of various transcription factors by interaction with Groucho *(*Gro). E(spl) proteins, in general and E(Spl)mβ, in particular, are required only for a subset of N functions. For example, these genes do not mediate the effect of N in various regions such as the D-V boundary per se. Asymmetric distribution of ligands of N, Dl and Serrate (Ser) results in the activation of N only at the D-V boundary. Activated N

in turn induces the expression of genes like *ct* and *wg* along the boundary, which are essential for the growth and differentiation of wing (Rulifson *et al*., 1995; Couso *et al*., 1995; Diaz- Benjumea *et al*., 1995; de Celis *et al*., 1996; Micchelli, *et al*., 1997).

We did not observe any change in Wg expression when *glec* was down regulated using *MS1096*-, *sal*- or *ptc*-GAL4 driver either at 25 °C or 28 °C (Fig 5 B), although down regulation of *glec* negatively affects E(Spl)mβ expression (Fig. 4B). Ectopic expression of Glec using *MS1096*-GAL4 driver resulted in increased levels of Wg in the dorsal compartment (Fig. 5C). Levels of Wg in the dorsal compartment were maximal near the D-V boundary and decreased towards the hinge region (unlike the expression of *wg* in the complete dorsal pouch when an activated form of N is expressed using *ap*-GAL4 (Juan *et al*., 2012). It is likely that under normal circumstances, Glec regulates N signalling only in the context of intervein specification, while increased levels of Glec may affect the expression of E(Spl)mβ-independent targets of N, such as Wg in the D/V boundary.

Ectopic expression of Glec using *ptc*-GAL4 and *sal*-GAL4 did not activate Wg or Ct (data not shown). This appeared contradictory to the results we obtained using *MS1096*-GAL4 driver. As discussed earlier, Glec is auto-regulated. We, therefore, infer that while using mild GAL4 drivers such as *sal*-GA4, the feedback loop manages to over ride the effect of exogenous Glec by reducing the amount of endogenous Glec. However, when strong GAL4 drivers are used, exogenous Glec itself might be sufficient to break this feedback loop and starts activating N targets.

We also ectopically expressed Glec in the posterior compartment of wing imaginal disc using *en*-GAL4, which is a weaker driver. We did not see any change in the levels of Wg in the posterior compartment of the wing disc (Fig. 5E). However, we noticed the expression of Wg at the D-V boundary of haltere disc in the posterior compartment, where Wg is normally repressed (Fig. 5 D,E). We reason that in the wing disc, the total levels of Glec is maintained by the feed back loop operating and hence ectopic expression of Glec in the wing imaginal discs may not show a over expression phenotype at the levels of Wg. However, Glec is not expressed in the haltere discs. When Glec is ectopically expressed in the posterior compartment of the haltere disc, the exogenous Glec must be sufficient to activate the expression of Wg. Once again we noticed only a context specific activation of Wg and we found ectopic Wg only in the posterior D/V boundary. This means Glec can activate Wg expression only in regions where N is normally activated. This suggests that Glec may function upstream of E(Spl) and Su(H), effectors of N and downstream of Dl and Ser, ligands of N.

The 'gain of function' analysis i.e. over expression of Glec clearly suggests that Glec potentiates N signalling. By contrast, the 'loss of function' experiments involving RNAi lines did not yield precisely complementary results. This could be due to low penetrance and poor efficiency of the RNAi lines. Another interesting possibility might involve an auto-regulatory feedback mechanism that compensates for loss of Glec. A detailed clonal analysis using *glec* mutants will enable to further substantiate the claim that Glec modulates N signalling pathway both in the D-V and non D-V cells of the wing disc.

Glec is predicted to be a carbohydrate binding protein or a lectin (Tiemeyer *et al*., 1996). A number of proteins are post-translationally modified in the Golgi bodies. N is one of the most studied amongst the various proteins that are post-translationally modified in Golgi bodies. At the D-V boundary, the ligands of N, namely Dl and Ser are responsible for activating N. Once N is produced in the endoplasmic reticulum, it interacts with O-fucosyl transferase and is subsequently transferred to the Golgi body. In the Golgi, it is processed by Furin like convertase and glycosylated by various glycosyl transferases. The extracellular domain of N protein has multiple glycosylation sites, which are modified in the Golgi bodies (reviewed by Bray, 2006; Fortini, 2009). Thus, the presence of N ligands at specific locations and modulation of N by post-translational modification enables specific activity of N even when its expression pattern is not spatially/temporally modulated. It is possible that Glec is involved in the post-translational modification of N in the Golgi bodies and thereby making it more sensitive to bind to one of its ligands. Further biochemical characterization is required to identify possible mechanism by which Glec activates N in the Golgi.

In summary, our observations are consistent with the possibility that N receptor is a direct target of Glec. Our data, however, do not rule out that Glec could also be involved in modulating activity/localization of other critical components of N pathway including its ligands such as Delta. 'In vitro' biochemical analysis will help distinguish between these different likely (and not necessarily mutually exclusive) scenarios.

Materials and Methods

Genetics

Combinations of various GAL4 drivers and UAS lines was carried out employing standard genetic methods. cDNA of *glec* was obtained by reverse transcription from total RNA of *Drosophila* embryos at stage 10; which was used to generate UAS-*glec* and UAS *glec* RNAi lines. Cloning strategies, sequences of primers that were used are described in the supplementary text.

Fly stocks were obtained from various sources: GAL4 drivers used in this study are MS1096-GAL4 (Capdevila *et al.,* 1994); *omb*-GAL4 (personal communication to Flybase, Calleja, 1996.10.16); *ptc*-GAL4 (Brand and Perrimon, 1993); *sal*-GAL4 (Thomas *et al*., 1995) and *ap*-GAL4 (Calleja *et al*., 1996). Other fly strains that were used are *argos*-lacZ (*aos*-lacZ, Freeman *et al*., 1992); *E(Spl)m*β-lacZ (Cooper *et al*., 2000); UAS-Galactosyl transferase::RFP (Galt-RFP, Rikhy and Lippincott-Schwartz, personal communication to Flybase); Glec::YFP protein fusion line (obtained from Kyoto Stock Center) and enhancer trap line of *glec*-GFP (Buszczak *et al*., 2007).

Histology

Immunochemical staining on imaginal discs were performed as described earlier (Patel *et al.,* 1989). Antibodies used were rabbit anti-GFP (1:3000; Invitrogen); chicken anti-GFP (1:500; Invitrogen); mouse anti-β-galactosidase (1:500; 40-1a, DSHB); mouse anti-Delta (1:500; Qi *et al.,* 1999); mouse anti-Wingless (1:200; Brook and Cohen, 1996); mouse anti-Cut (1:10 Blochlinger *et al*., 1993) and Rabbit anti-RFP (1:1000; Invitrogen). Antibodies against Wingless (Wg), Delta (Dl), Cut (Ct) and β-galactosidase (βgal) were obtained from DSHB, Iowa, USA. The secondary antibodies conjugated with different fluorophores were obtained from Invitrogen. Fluorescent images were taken using Zeis LSM 710 or Zeiss LSM 780 confocal microscope. Adult wings were processed for imaging as described in Shashidhara *et al.,* (1999).

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