Dual role of the *fringe connection* gene in both heparan sulphate and *fringe*-dependent signalling events

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The precise regulation of growth factor signalling is crucial to the molecular control of development in *Drosophila*. Post-translational modification of signalling molecules is one of the mechanisms that modulate developmental signalling specificity. We describe a new gene, *fringe connection* (*frc*), that encodes a nucleotide–sugar transporter that transfers UDP–glucuronic acid, UDP–*N*-acetylglucosamine and possibly UDP–xylose from the cytoplasm into the lumen of the endoplasmic reticulum/Golgi. Embryos with the *frc* mutation display defects in Wingless, Hedgehog and fibroblast growth factor signalling. Clonal analysis shows that *fringe*-dependent Notch signalling is disrupted in *frc* mutant tissue.

Secreted growth factors of the Wnt/Wingless (Wg), Hedgehog (Hh) and fibroblast growth factor (FGF) families play important roles in patterning the body plan in both *Drosophila* and vertebrates. Recently, it has become clear that the proper function of these molecules depends on the integrity of proteoglycan and glycosaminoglycan (GAG) biosynthesis. Proteoglycans are proteins modified on specific serine residues by the addition of GAGs synthesized in the Golgi. In *Drosophila*, mutations in enzymes involved in the biosynthesis of heparan sulphate proteoglycan (HSPG) side chains or in proteoglycan core proteins lead to defects very similar to those seen in mutations that affect the Wg, FGF or Hh signalling pathways[1–7]. HS-GAGs are repeating disaccharide units of *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) that are added to the growing HS chain by glycosyltransferases as UDP–sugar substrates. Several mutations that disrupt the biosynthesis of HSPGs have been identified. Mutations in *sugarless* (*sgl*) and *sulfateless* (*sfl*) disrupt Wg and FGF signalling. These genes encode a UDP–glucose dehydrogenase (the enzyme required for GlcA biosynthesis[1–3]) and an *N*-deacetylase/*N*-sulphotransferase (the enzyme that catalyses the addition of sulphate groups to HS (refs 4–6)), respectively. Hh signalling is blocked by mutations in *tout velu* (*ttv*), the *Drosophila* homologue of Ext1 (refs 7,8), which encodes an HS-specific glucuronosyltransferase that synthesizes the GAG chain[9].

Growth factor receptors themselves have also been shown to be modified by the addition of sugar chains. The variable addition of polysaccharide side chains could, in fact, work as a molecular switch to regulate the activity of growth factor receptors[10]. In *Drosophila*, the specificity of the Notch receptor for its ligands Serrate (Ser) and Delta (Dl) during determination of the dorsal–ventral boundary in the wing and eye imaginal discs is modified by the activity of the Fringe (Fng) protein[11]. Fng has recently been shown to encode an *N*-acetylglucosaminyltransferase[12,13]. The addition of GlcNAc by Fng to an *O*-linked fucose in Notch increases its affinity for Dl and inhibits its interaction with Ser (refs 14,15).

Here, we show that mutations in the *fringe connection* (*frc*) gene, which encodes a nucleotide–sugar transporter, affect not only Wg-, Hh- and FGF-dependent pathways but also the activity of Fng/Notch-dependent pathways. In an accompanying paper by Goto *et al.*[51] the same gene is called *ust74c*. We propose that Frc transports several crucial UDP–sugars into the Golgi apparatus, an essential process for the Fng-mediated modification of the Notch receptor, as well as for the synthesis of HS chains involved in Wg, Hh and FGF signalling. Consistent with this, we find that Frc transports UDP–GlcNAc and UDP–GlcA.

Results

We identified mutations in the *fringe connection* (*frc*) gene in a genetic screen for mutations associated with segment polarity phenotypes[16]. Embryos lacking both maternal and zygotic *frc* activity (*frc* mutant embryos) develop cuticles with severe segment polarity phenotypes (Fig 1a,b) reminiscent of mutations disrupting Wg and/or Hh signalling[17]. This result is supported by an analysis of the expression patterns of wg and engrailed (*en*) genes in frc mutant embryos. Initially the expression of *wg* and *en* is normal in *frc* mutant embryos, indicating that the pair-rule transcription-factor-dependent initiation of these genes is not affected (not shown). However, at stage 11, the segmentally repeated expression of *wg* (Fig. 1c,d) and *en* (Fig. 1e,f) in the epidermis fades prematurely. This phenotype is reminiscent of that observed in other segment polarity mutants and implies that *frc* plays a role in the maintenance of the *wg*/*hh*-dependent feedback loop in the embryonic epidermis. The *frc* embryonic homozygous maternal effect germ-line clone phenotype is fully paternally rescuable, and we therefore refer to them as rescued and non-rescued to distinguish the two classes of embryos.

In contrast to genes that act downstream of Wg, like *dishevelled* or *armadillo*, in which *en* expression fades at an early stage[18], *en* expression fades relatively late in *frc* mutant embryos (Fig. 1f, data not shown). This observation is consistent with a role for *frc* as an upstream component of Wg signalling or as a factor influencing the efficiency of signal transduction. In order to establish the epistatic relationships between *wg*, *hh* and *frc*, we misexpressed *wg* and *hh* in the ventral epidermis of *frc* mutant embryos. When *wg* (Fig. 1g) or *hh* (Fig. 1h) were expressed using a *prd–Gal4* driver[19], naked cuticle

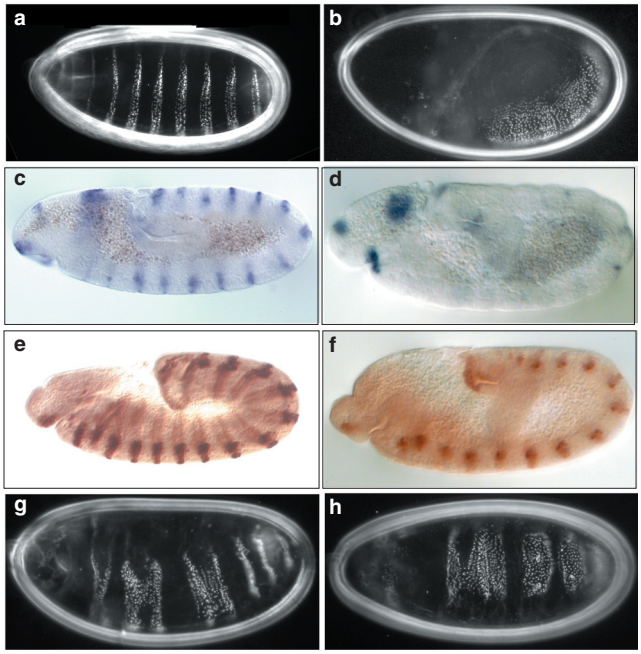


Figure 1 **Segment polarity phenotypes of *frc* mutant embryos.** Lateral (**a**–**f**) or ventro–lateral (**g**,**h**) views, anterior is to the left. Cuticle from (**a**) wild-type embryo and (**b**) cuticle of maternally and zygotically mutant $frc^{l(3)00073}$ embryo (termed *frc* mutant hereafter). *In situ* hybridization of *wg* mRNA from (**c**) wild-type and (**d**) *frc* mutant embryo at stage 11. Expression of *wg* mRNA in the epidermis fades prematurely compared with that in the wild type. Anti-En antibody staining of (**e**) wild-type and (**f**) *frc* mutant embryo at stage 11. Expression of *en* fades prematurely in epidermal cells compared with the wild type. Expression of *en* in the nervous system, which is not Wg dependent, is unaffected. **g**, Expression of a *UASwg* gene in *frc* mutant embryo using a *prd–Gal4* driver. Naked cuticle is induced in a pair-rule pattern. **h**, Expression of a *UAS–hh* gene in *frc* mutant embryo using a *prd–Gal4* driver. Naked cuticle is induced in a pair-rule pattern.

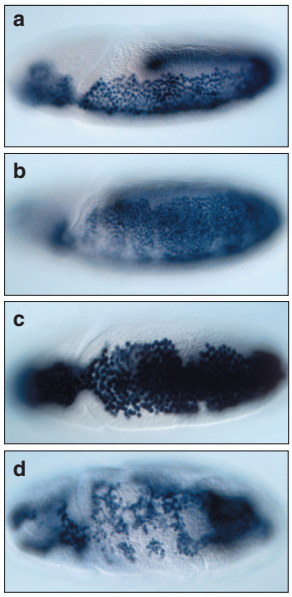


Figure 2 **Lateral migration of the mesoderm in *frc* mutant embryos.** All embryos are immunohistochemically stained with anti-Twi antiserum, which stains mesodermal cells. Ventral views, anterior is to the left. **a**,**b**, Lateral migration occurs evenly along the anterior–posterior axis in wild-type embryos (**a**, stage 9; **b**, stage 11). **c**, Lateral migration fails to occur in some regions along the anterior–posterior axis (arrow) in *frc* mutants at stage 9. **d**, Failure to migrate leads to holes in the layer of mesodermal cells in *frc* mutants at stage 11 (arrow).

was restored in a typical pair-rule pattern. This result indicates that, in the overexpression assays, *frc* is not an essential component of the Wg or Hh signalling cascades but rather potentiates the signalling efficiency of these factors.

The *frc* gene is required for FGF signalling in the embryo. Very similar observations have been made for mutations in the *sgl* (refs 1–3) and *sfl* (ref. 6) genes. Because these genes encode enzymes involved in HSPG biosynthesis, this suggests that *frc* might also be involved in this process. In support of this hypothesis, we find that FGF-dependent patterning events, which depend on HSPGs[6], are disrupted in *frc* mutant embryos. The lateral spreading of the mesoderm, which depends on FGF signalling[20,21], is disrupted in *frc* mutant embryos (Fig. 2a–d), as previously observed in *sgl* and *sfl* mutant embryos[6]. Taken together, our results suggest that loss of *frc* function interferes with the integrity of HSPGs.

The *frc* gene encodes an endoplasmic reticulum/Golgi (ER/Golgi) nucleotide–sugar transporter. Molecular characterization of *frc* identified a 1.6 kb cDNA (Fig. 3a) that has no detectable transcript in mutants as assessed by northern analysis (see Methods). The *frc* cDNA encodes a predominantly hydrophobic protein of 373 amino acids (Fig. 3b,c). Although *frc* is unique in *Drosophila*, it has significant sequence identity to the *Caenorhabditis elegans* gene *Sqv–7* (refs 22,23), the partial human cDNA *KIAA0260* and *LPG2* (ref. 24) from the parasite *Leishmania donovani* (Fig. 3b). All of these genes encode proteins with up to ten putative transmembrane domains (Fig. 3c) that belong to a family of nucleotide–sugar transporters, which are believed to transport nucleotide–sugars from the cytosol into the lumen of the ER and Golgi, where they serve as substrates for glycosyltransferases. Nucleotide–sugar transporters can have a great spectrum of substrate specificities and, among the Frc homologues, LPG2 and SQV7 have been functionally characterized and shown to be multispecific nucleotide–sugar transporters. LPG2 substrates have been shown to include GDP–mannose, GDP–arabinose and GDP–fucose[25], and SQV7 transports UDP–GlcA, UDP–GlcNAc and UDP–galactose[26].

In order to determine the substrate specificity of Frc, we tested different nucleotide–sugar substrates (Fig. 3d) in an *in vitro* vesicle transport assay (see Methods). Although Frc does not transport UDP–galactose, UDP–glucose, GDP–mannose or GDP–fucose, the uptake of UDP–xylose, UDP–GlcNAc and UDP–GlcA acid is increased two- to threefold in vesicles derived from *Leishmania* transfected with a *frc* expression construct (Fig. 3d). The validity of UDP–GlcA uptake is confirmed by the addition of DIDS, an anion transport inhibitor, which effectively suppressed transport (Fig. 3e). The uptake of UDP–GlcNAc is also inhibited by DIDS and its transport is competed by excess UDP–GlcA (Fig. 3f), suggesting that Frc specifically transports both nucleotide–sugars. UDP–GlcNAc uptake is saturable (Fig. 3g) with a K_m (7.8 μM) within the range reported for other nucleotide–sugar transporters[27]. These data suggest that *frc* encodes a multisubstrate nucleotide–sugar transporter with specificity for UDP–GlcA, UDP–GlcNAc and possibly UDP–xylose. The phenotypic similarities between *frc* mutants and *sgl* mutants, and the molecular identity of *frc* as a nucleotide–sugar transporter, suggest that both genes act in a common pathway. GlcA and GlcNAc are the two monosaccharide subunits that form the backbone of HS, and xylose is a substituent of the core region. Thus, we propose that *frc* activity is necessary to transport UDP–GlcA (produced by *sgl*), UDP–GlcNAc and UDP–xylose from their site of synthesis in the cytosol into the ER/Golgi, where they subsequently serve as substrates for the synthesis of HSPG side chains.

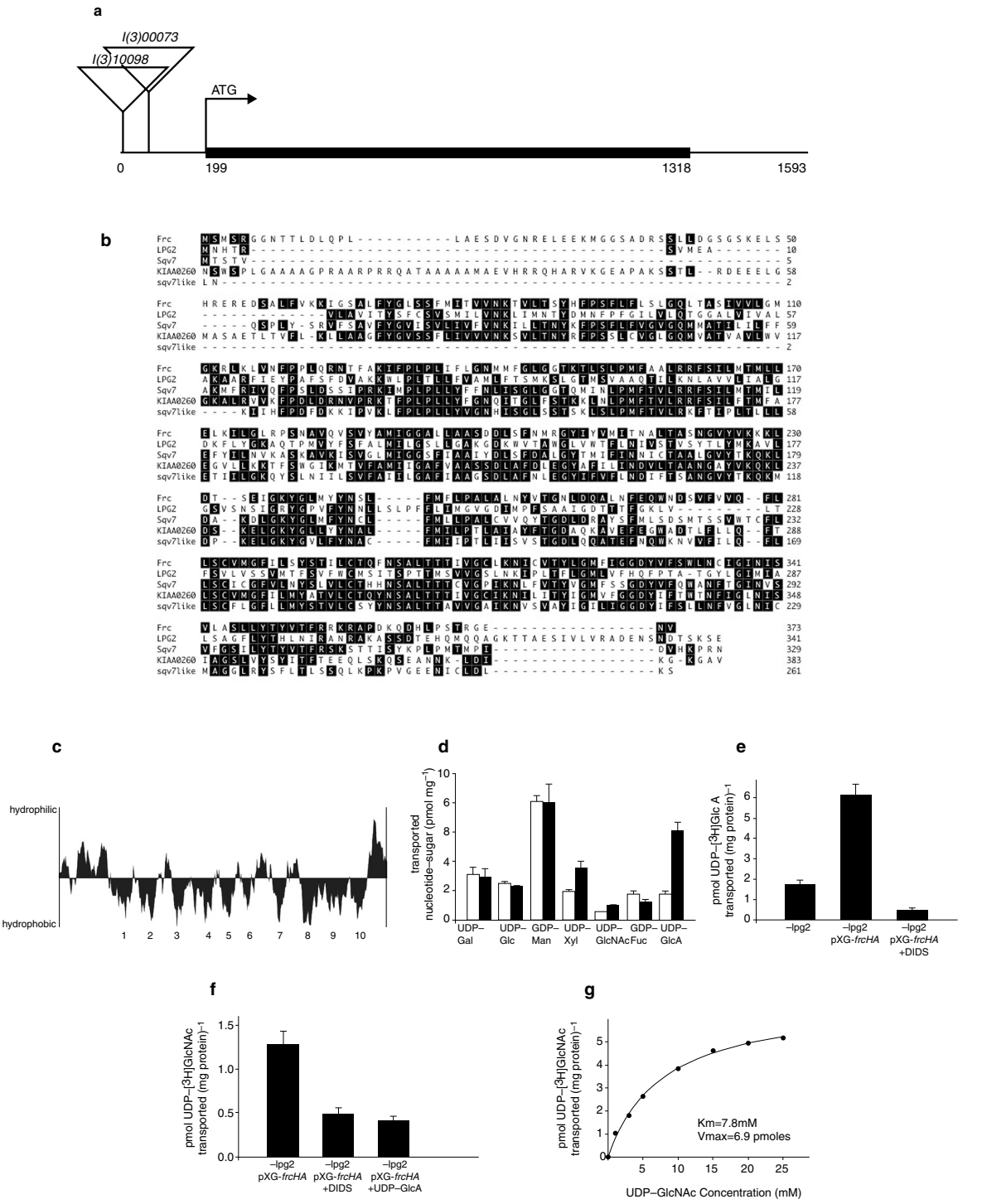


Figure 3 **Molecular and biochemical characterization of the *frc* gene. a**, The *frc* transcription unit. Frc is encoded by an intronless transcript of 1593 bp containing a 1119 bp open reading frame that starts at position 199. The *frc* gene has been named gene *CG3874* by the *Drosophila* genome project[50] and has been assigned protein ID AAF49343.1. P-element insertions have been mapped at nucleotide positions 10 ($frc^{l(3)10098}$) and 63 ($frc^{l(3)00073}$). **b**, Alignment of Frc with *Caenorhabditis elegans*, human and *Leishmania donovani* protein sequences. *Frc* shares 49% identity with the *Sqv7* gene of *C. elegans*, 47% identity with the partial human cDNA *KIAA0260*, 46% identity with the human partial cDNA coding for *Sqv7*-like and 18% identity with the *L. donovani* gene *LPG2*. **c**, Kyte–Doolittle hydrophobicity plot of Frc showing the ten putative transmembrane regions characteristic of nucleotide–sugar transporters. **d**, Transport activity of the UDP–galactose (UDP–Gal), UDP–glucose (UDP–Glc), GDP–mannose (GDP–Man), UDP–xylose (UDP–Xyl), UDP–*N*-acetylglucosamine (UDP–GlcNAc), GDP–fucose (GDP–Fuc) and UDP–glucuronic acid (UDP–GlcA) into *Leishmania* vesicles transfected with *frc* (filled bars) or mock transfected (open bars). A two- or threefold stimulation in transport was detected for UDP–Xyl, UDP–GlcNAc and UDP–GlcA. **e**, The import of UDP–[^{3}H]-GlcA into vesicles derived from *frc*-expressing *Leishmania* is increased threefold compared with *lpg2* vesicles. This activity can be blocked by the anion transport inhibitor DIDS. **f**, Transport of UDP–[^{3}H]-GlcNAc is increased greater than twofold in Frc vesicles. This activity is inhibited by DIDS and excess UDP–GlcA. **g**, The transport of UDP–[^{3}H]-GlcNAc into Frc vesicles is saturable with a K_m of 7.8 μM.

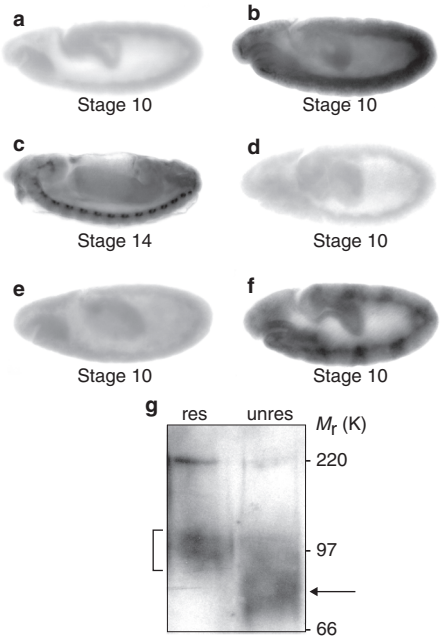


Figure 4 **The *frc* gene is required for HSPG biosynthesis.** Wild-type embryos (**a**–**c**), $frc^{l(3)00073}$ mutant embryos (**d**,**e**) and $frc^{l(3)00073}$ mutant embryos (**f**) expressing *frc* in the *prd* domain stained with an antibody against heparan sulphate (HS; antibody 3G10) before (**a**,**d**) or after (**b**,**c**,**e**,**f**) heparinase-III treatment. The stage of embryonic development is indicated. Wild-type expression of HS is mostly in the central nervous system by embryonic stage 14, providing an internal measure for 3G10 staining (**c**). **g**, Western blot from a 7% SDS–polyacrylamide gel for the HS proteoglycan Dally-like from paternally rescued (res) and unrescued (unres) germ-line clone embryos. Females carrying homozygous *frc* germ-line clones were crossed with *frc/TM3, Ser, actinGFP* males and paternally rescued embryos were unambiguously identified by the expression of the green fluorescent protein (GFP). The predicted molecular weight of core (unmodified) Dally-like protein is 80 kDa, which is consistent with what we observed when Dally-like western blots are prepared from wild-type embryos treated with HNO_2 to remove the glycosaminoglycans from the core protein (data not shown). The bracket shows that the molecular weight of HS-modified Dally-like in rescued embryos has a mobility range from 85 kDa to 110 kDa, which is similar to the wild type[29]. In *frc* mutant embryos derived from unrescued *frc* homozygous germ-line clones, the mobility of Dally-like is shifted to lower sizes, indicating that it is not modified as it is in the wild type.

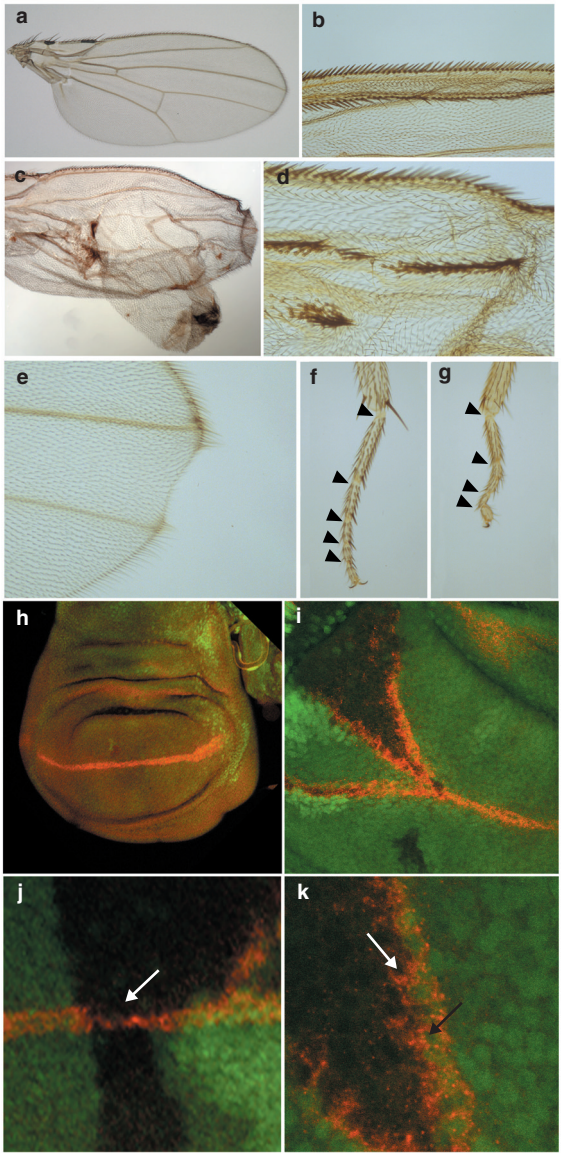
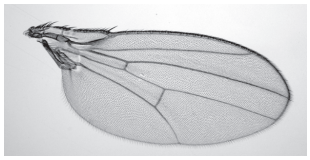


Figure 5 **Frc function during imaginal disc development. a**, Wild-type adult wing. **b**–**e**, Clones of *frc* mutant cells in wing imaginal discs have the ability to organize duplications of the wing margin (**b**), outgrowths of additional wings from the wing blade (**c**), an additional dorsal–dorsal margin inside the wing (**d**) and wing nicks (**e**). All phenotypes arise on the dorsal surface of the wing. **f**, Wild-type adult leg. Black arrowheads show the joints between tarsal segments. **g**, With *frc* mutant clones in leg discs, the result is the loss of joints (here, between tarsi 2 and 3) and leg shortening. Clones of *frc* mutant cells (unstained in a field of green GFP-marked heterozygous and homozygous wild-type cells) in wing imaginal discs stained with antiserum against Wg (red). Clones in the dorsal compartment (up) lead to the activation of Wg along the clone borders in over 90% of the wing discs examined with clones in the dorsal compartment. **h**, Merged image of a wild-type wing disc. (**i**) A *frc* mutant clone with Wg outlining the clone borders. **j**, Clones that cross the dorsal–ventral compartment boundary showed a reduction in the level of *wg* expression (white arrow), as reported for *fng* mutant clones[33], even though clones in the ventral compartment (down) showed no phenotype. **k**, A close up of (**i**) shows that homozygous mutant (frc^-, white arrow) and heterozygous (frc^+, black arrows) cells on either side of the clone border express *wg*.

Loss of Frc activity interferes with normal HSPG biosynthesis. The requirement for the transport activity of Frc in HSPG biosynthesis was confirmed by examining the deposition of HS and the post-translational modification of the HSPG Dally-like, which is a member of a class of GPI-linked proteoglycans known as glypicans (refs 28, 29) in *frc* mutant embryos. In wild-type embryos, treatment with heparinase III to expose the 3G10 HS epitope yields ubiquitous staining at stage 10 (Fig. 4a,b)[7]. In *frc* mutant embryos, 3G10 staining is significantly reduced relative to the wild type after heparinase III treatment (Fig. 4b,e) and only slightly greater than untreated (Fig. 4d), suggesting that HS biosynthesis is impaired in the absence of *frc*. Staining by 3G10 is recovered in a typical pair-rule pattern when *frc* is expressed under the control of *prdGAL4* in *frc* mutant embryos (Fig. 4f). This result shows that the transport activity of Frc specifically restores HS biosynthesis in *frc* embryos.

Consistent with these results, we find that the mobility of Dally-like in an SDS gel is significantly altered in non-paternally rescued *frc* embryos derived from mothers that carry *frc* germ-line clones (Fig. 4g). Dally-like from rescued embryos shows a mobility ranging from 85 kDa to 110 kDa, as previously reported for modified Dally-like, whereas, in non-rescued embryos, it shows a broader mobility that concentrates at 80 kDa, which corresponds to the

a



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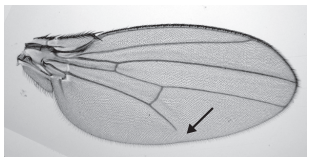

c

	+/+	$frc^{l(3)10098}$/+	$frc^{l(3)00073}$/+	fng^{13}/+
Notch Ax^{E2}/+	8.7% (n=76)	95% (n=19)	30% (n=108)	62% (n=34)

Figure 6 **The *frc* gene enhances the *Notch Abruptex* phenotype**. Wings obtained from females heterozygous for Ax^{E2} (ref. 36) on the X chromosome and wild type or heterozygous for $frc^{l(3)10098}$ on chromosome 3 were scored for the frequency of a failure of vein 5 to reach the wing margin (compare wild type in **a** to arrow in **b**). The vein phenotype in Ax^{E2} heterozygous mutant flies is variable and is strongly enhanced in a *frc* heterozygous background (**c**). The number of wings examined for each genotype (*n*) is shown in brackets. Similar results were obtained with heterozygous Ax^{71d} (ref. 37), another *Ax* allele of the same class (data not shown).

Dally-like unmodified core size[29]. The mobility shift of Dally-like from *frc* unrescued embryos is similar to that observed when the HSPG is treated with HNO_2 to remove the GAGs (data not shown). Taken together, these results show that the transport activity of Frc is required for the biosynthesis of HSPGs.

Clonal analysis in imaginal discs reveals that Frc is important for Fng-dependent Notch signalling. Surprisingly, when we investigated the function of *frc* during imaginal development, we found a phenotype not previously seen in either *sgl* or *sfl* mutant clones. Clones of *frc* homozygous mutant cells in wing discs were often associated with duplications of the anterior wing margin (Fig. 5b), wing outgrowths (Fig. 5c), ectopic margin bristles inside the wing blade (Fig. 5d) and occasional wing nicks (Fig. 5e). In contrast to the wild-type margin, ectopic margins were always mirror-image dorsal–dorsal margins (Fig. 5d) and the wing outgrowths always originated from the dorsal surface (Fig. 5c). An *frc* clone in the leg results in a shortened leg that lacks joints between tarsal segments (Fig. 5g)[30,31]. These phenotypes are strikingly similar to those associated with mutations in the *Drosophila* gene *fringe* (*fng*).

Fng activity is needed to determine the dorsal–ventral axis of the wing[32]. Fng modifies the specificity of the Notch receptor for its ligands, Ser and Dl, rendering Notch-expressing cells less responsive to Ser and more responsive to Dl (refs 14,15). Binding of Ser to Notch activates the receptor and results in the upregulation of its target gene *wg*, which then specifies the wing margin[11]. The *fng* gene is expressed in the dorsal compartment of the wing disc and activates *wg* expression at the border of *fng*-expressing and non-*fng*-expressing cells. Although *fng* mutant clones in the ventral compartment have no phenotypes, clones of *fng* mutant cells generated in the dorsal compartment activate *wg* at the new border of fng^+ and fng^- cells, and induce ectopic wing margin[33].

The *fng* gene encodes a protein with weak homology to a family of glycosyltransferases[34] and has recently been shown to be involved in the glycosylation of Notch[12,13,35]. Our results support the model that *frc* activity is required to supply *fng* with a sugar substrate to allow activation of the Notch receptor. In the absence of *frc* activity, the substrate is missing, thereby mimicking the generation of a border of *fng* activity. According to this model, *wg* expression should be induced at the border between wild-type and *frc* mutant cells. When such clones were examined, Wg expression is detected at the border of the clone (Fig. 5i) in both frc^+ and frc^- cells (Fig. 5k), a finding consistent with the creation of a *fng* activity border by the *frc* clone. Ventral *frc* clones have no phenotype but those crossing the compartment border reduce *wg* expression (Fig. 5j).

Thus, both the molecular nature of *frc* and the mutant phenotypes suggest that Frc regulates the activity of Fng at the substrate level. This conclusion is further supported by the genetic interactions between *Abruptex* (*Ax*) and *frc* mutations (Fig. 6). *Ax* is an enigmatic gain-of-function class of *Notch* mutations that cause wing vein loss or gapping[36,37]. In a wild-type background, a single copy of Ax^{E2} yields the *Ax* phenotype (loss of wing vein (Fig. 6b, arrow)) with a low penetrance of 8.7%. However, a single copy of either *frc* allele in the Ax^{E2}/+ background enhances the penetrance of vein loss to 95% and 30%. The *fng* gene[13], a strong loss-of-function allele[33], enhances Ax^{E2}/+ vein loss to 60%, suggesting that both *frc* and *fng* interact with *Notch* in a similar fashion. In support of this hypothesis, several potential *O*-linked fucosylation sites in Notch (the substrate for Fng glycosyltransferase activity) map to the region of Notch to which the *Ax* mutations map[12,35].

Discussion

We have identified and characterized *fringe connection*, a segment polarity gene that encodes a nucleotide–sugar transporter. This protein catalyses the transfer of UDP–GlcA, UDP–GlcNAc and possibly UDP–xylose from the cytoplasm into the ER/Golgi. We find that *frc* mutant embryos have defects in the biosynthesis of HS and HS-proteoglycans that account for the observed block in embryonic Wg/Hh and FGF signalling. Clonal analysis in imaginal discs revealed that *frc* is also important for *fng*-mediated Notch signalling.

Our analysis indicates that Frc transports UDP–GlcA and UDP–GlcNAc from the cytosol into the Golgi. This activity of Frc explains the *fgf*-, *wg*- and *hh*-like mutant phenotypes of embryos that lack both maternal and zygotic *frc* activity, because both UDP–GlcA and UDP–GlcNAc are substrates for HSPGs, which have been shown to be required for the signalling activity of these pathways. With regard to the Fng-like phenotypes associated with *frc* imaginal disc mutant clones, several possibilities can be envisioned. Recently, Fng has been shown to function as an *N*-acetylglucosaminyltransferase that acts at the first step in elongation of a Notch *O*-linked fucose sugar chain on Notch[12,13,35,38]. Thus, Frc could affect the direct glycosylation of the Notch receptor by the Fng glycosyltransferase. However, our data do not distinguish whether the UDP–GcNAc or UDP–GlcA transported by Frc serves directly as a substrate for Fng, or whether Frc supplies a substrate for a more complex modification that requires Fng. These modifications might involve the glycosylation of Fng itself to alter its glycosyltransferase activity, because Fng has several potential *N*-linked glycosylation sites[32], or an as-yet-unidentified protein or lipid that influences the interaction of Notch with its ligands. In order to distinguish between these possibilities, a comparison of the glycosylation states of Notch, Fng and perhaps other candidate proteins or lipids needs to be performed with *frc* mutant and wild-type tissue.

Homologues of *fng* have been identified in higher vertebrates and mammals. In the chick, *Radical Fringe* (*R-Fng*) is expressed specifically in dorsal cells of the limb bud, where it appears to play a role analogous to *Drosophila fng* in the wing[39,40]. In the mouse, *Lunatic Fringe* (*L-Fng*) is expressed in a striped pattern in the presomitic mesoderm, and *L-Fng* mutants exhibit defects in the formation of somite boundaries[41,42]. All these developmental processes require the activation of the Notch receptor in addition to *fng*. Although homologues of *frc* have not yet been identified in the

chick or mouse, the presence of human homologues makes their existence very likely. Together, these findings suggest that Frc, Fng and Notch are essential components of a universal mechanism for developmental boundary formation. These observations underscore the importance of polysaccharide modifications as a general mechanism for the modulation of ligand–receptor interactions. □

Methods

Genetics.

Mutations in *frc* are represented by two non-complementing P-element insertions, $frc^{l(3)00073}$ and $frc^{l(3)10098}$, located in 74C1-2 on the third chromosome[43]. Homozygotes for either insertion die at the late pupal stages and northern-blot analysis of RNA derived from $frc^{l(3)00073}$ and $frc^{l(3)10098}$ pupae have less than 5% of the wild-type *frc* transcript levels (not shown). Embryos from germ-line clones die during embryogenesis with strong segment polarity and mesodermal patterning defects that can be paternally rescued to viability. The germ-line clone phenotypes are equally severe over *Df(3)81k19*, which uncovers this region, indicating that $frc^{l(3)00073}$ and $frc^{l(3)10098}$ behave as genetic nulls. Excision of the P elements using a Δ2-3 transgene as a transposase source leads to a full reversal of the germ-line clone phenotype and restoration of viability in $frc^{l(3)00073}/frc^{l(3)10098}$ transheterozygotes. The lethality of $frc^{l(3)00073}/frc^{l(3)10098}$ transheterozygotes is also rescued in the presence of *actin–Gal4* or *UAS–frc* transgenes. These results show that the P-element insertions disrupt the *frc* transcription unit and that the observed phenotypes are associated with the insertions. *In situ* hybridization to whole-mount embryos using a *frc* cDNA as a probe showed ubiquitous expression of *frc* transcripts throughout embryogenesis. Overexpression experiments using several Gal4 driver lines to express *frc* in various embryonic tissues as well as imaginal discs did not lead to detectable phenotypes. The genetic interaction between *frc* and *fng* alleles with *Ax* was determined by crossing *Ax* females to males and analysing the wings of female progeny. Wings were mounted in Euparal (ASCO Laboratory).

Molecular biology.

Genomic DNA from the *frc* locus was recovered by P-element plasmid rescue of $frc^{l(3)00073}$ and was used to isolate genomic and cDNA clones. Mapping of the P-element insertions revealed that both P elements are inserted within 60 bp of each other in the 5′ untranslated region of the 1.6 kb cDNA (Fig. 3a). The size of the cDNA corresponds well with the transcript size observed in northern blot analyses (not shown).

Misexpression, immunohistochemistry, mosaic and western analyses.

The *UAS–frc* transgene was constructed by cloning the *frc* cDNA into pUAST. The *UAS–wg*, *UAS–hh*, *prd–Gal4* and *act–Gal4* lines have been previously described[19,44–46]. Antibodies against En and Wg were obtained from the Developmental Studies Hybridoma Bank. The anti-HS staining using 3G10 (Seikagaku) was performed as previously described[7]. Homozygous mutant imaginal disc clones were generated by crossing $frc^{l(3)00073}$ FRT^{2A} and $frc^{l(3)10098}$ FRT^{2A} to *e22c–Gal4*, *UAS–Flp*; *ubn–GFP*, FRT^{2A}/*SM5-TM6B*[47]. Dally-like western analysis of SDS embryo extracts[29] and HNO_2 treatment of embryo extracts[48] were performed as described.

Expression of pXG-frcHA in *Leishmania*.

A *frcHA* transgene was cloned into the pXG vector. pXG-*frcHA* was transfected into the *Leishmania donovani* $lpg2^-$ knockout strain[25] using an electroporator (0.45 kV, 500 μF) and selected with G418 (50 μg ml^{-1}). Expression of *frcHA* was confirmed by anti-haemagglutinin western blot analysis (not shown).

Transport assay.

Cells were washed twice with PBS, harvested and resuspended in homogenization buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.1 mM TLCK, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonylfluoride, 0.5 mM 2,3-dimercaptopropanol). *Leishmania* microsomal vesicles were prepared as previously described[25,49]. Transport assays were started by mixing 100 μl vesicle protein with 100 μl of reaction buffer (homogenization buffer containing 10 mM $MnCl_2$, 4 mM $MgCl_2$ and 3.3 mM (0.8 μCi) [^{3}H]-nucleotide–sugar). After incubation at 28°C for 6 min, the samples were placed on ice, diluted with 1.5 ml washing buffer (10 mM Tris HCl, pH 7.4, 0.25 M sucrose) and applied to a filtration apparatus (Millipore 1225 Sampling Manifold) containing HAWP filters (24 mm diameter; 0.45 μm pore size). The filters were washed with 25–30 ml of washing buffer and radioactivity on the filters was measured by scintillation counting. The amount of [^{3}H]-nucleotide–sugar that was nonspecifically bound to the outside of the vesicles was determined by measuring the radioactivity associated with the vesicles at the start of incubation of vesicles with solute.

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