

The Fes/Fer non-receptor tyrosine kinase cooperates with Src42A to regulate dorsal closure in *Drosophila*

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Fes/Fer non-receptor tyrosine kinases regulate cell adhesion and cytoskeletal reorganisation through the modification of adherens junctions. Unregulated Fes/Fer kinase activity has been shown to lead to tumours in vivo. Here, we show that *Drosophila* Fer localises to adherens junctions in the dorsal epidermis and regulates a major morphological event, dorsal closure. Mutations in *Src42A* cause defects in dorsal closure similar to those seen in *dfer* mutant embryos. Furthermore, *Src42A* mutations enhance the *dfer* mutant phenotype, suggesting that *Src42A* and DFer act in the same cellular process. We show that DFer is required for the formation of the actin cable in leading edge cells and for normal rates of dorsal closure. We have isolated a gain-of-function mutation in *dfer* (*dfer^{gof}*) that expresses an N-terminally fused form of the protein, similar to oncogenic forms of vertebrate Fer. *dfer^{gof}* blocks dorsal closure and causes axon misrouting. We find that in *dfer* loss-of-function mutants β -catenin is hypo-phosphorylated, whereas in *dfer^{gof}* β -catenin is hyperphosphorylated. Phosphorylated β -catenin is removed from adherens junctions and degraded, thus implicating DFer in the regulation of adherens junctions.

KEY WORDS: *Drosophila*, Fes, Fer, Tyrosine kinase, Src, Adherens junction, Dorsal closure, β -catenin

INTRODUCTION

Fes/Fer non-receptor tyrosine kinases regulate numerous cellular processes, including cell adhesion, cytoskeletal reorganisation and intracellular signalling (Greer, 2002). Mammalian Fer has been implicated in the regulation of adherens junctions (AJs) in tissue culture (Kim and Wong, 1995; Piedra et al., 2003; Rosato et al., 1998; Xu et al., 2004). Adherens junctions (AJ) are responsible not only for adhesion between neighbouring cells, but also for providing a link between the plasma membrane and the actin cytoskeleton. AJs are rich in phosphotyrosine residues, and several tyrosine kinases and phosphatases are known to regulate their assembly, stability and function (Daniel and Reynolds, 1997; Muller et al., 1999; Taddei et al., 2002).

Cell adhesion and cytoskeletal reorganisation are the driving force behind many morphogenetic movements of embryonic development. For example, during dorsal closure in the *Drosophila* embryo, the epidermal sheets on each side of the embryo extend dorsally to meet and fuse at the dorsal midline, thereby enclosing the amnioserosa and yolk sac (for reviews, see Jacinto et al., 2002; Noselli and Agnes, 1999). Cell adhesion in the dorsal-most row of cells, the leading edge cells, is regulated in part through the modification of adherens junctions.

Intercellular adhesion is mediated by the transmembrane protein E-Cadherin, a homophilic adhesion molecule that binds to E-Cadherin on neighbouring cells. β -catenin binds to the cytoplasmic tail of E-Cadherin. β -catenin can also interact with the F-actin-binding protein α -catenin (reviewed by Perez-Moreno et al., 2003). α -catenin acts as a molecular switch regulating F-actin assembly (Drees et al., 2005), binding alternately to β -catenin and to F-actin (Yamada et al., 2005). p120-catenin (p120ctn) binds to the

juxtamembrane domain (JMD) of E-Cadherin (Daniel and Reynolds, 1995), and is thought to act as a regulator of adherens junction assembly and disassembly.

Both β -catenin and p120-catenin are regulated by phosphorylation. Fer binds constitutively to p120-catenin (Kim and Wong, 1995; Lilien et al., 1999) and can phosphorylate it in vitro (Kim and Wong, 1995). Phosphorylation of p120-catenin increases its affinity for Cadherin (Roura et al., 1999). When overexpressed or activated, Fer can also phosphorylate β -catenin and thereby disrupt adhesion (Piedra et al., 2003; Rosato et al., 1998): phosphorylation on Tyr654 disrupts its association with E-Cadherin (Roura et al., 1999); phosphorylation on Tyr142 blocks interaction with α -catenin (Piedra et al., 2003). In *C. elegans*, the Fer orthologue associates with β -catenin in vivo and depends upon β -catenin for localisation to cell-cell junctions (Putzke et al., 2005). Fer can also stabilise the cadherin complex by phosphorylating and activating the phosphatase PTP1B, which in turn keeps β -catenin (Tyr654) dephosphorylated (Xu et al., 2004). Thus Fer has the capacity to both positively and negatively regulate cadherin complex stability.

Several of the putative substrates of Fer are also substrates of the Src family of cytoplasmic tyrosine kinases (SFKs). Both p120ctn and Cortactin were initially identified as being substrates of Src (Kanner et al., 1991; Wu et al., 1991). Like Fer, the SFK Fyn binds constitutively to p120-catenin, and can phosphorylate β -catenin at Tyr142 (Piedra et al., 2003). Src is able to phosphorylate β -catenin at Tyr654 (Roura et al., 1999). Given the overlap in substrate specificity, Fer and SFKs may play overlapping or redundant roles in the regulation of cell adhesion and motility. Functional redundancy between different SFKs has been demonstrated in mammals and in insects. In mice, double mutations in SFKs lead to overt phenotypes, where single mutations do not (Stein et al., 1994). In *Drosophila*, members of the Src kinase family cooperate to regulate JUN kinase (JNK) activity (Takahashi et al., 2005; Tateno et al., 2000): double mutations in *Src42A;tec29*, and *Src42A;Src64* give a dorsal open phenotype, whereas single mutations do not.

Only a single member of the Fes/Fer family is found in *Drosophila*, DFer (Fps85D – FlyBase). The canonical form of DFer, p92dfer, was identified by similarity to other family

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members (Fig. 1B) (Katzen et al., 1991), and shares equal homology with Fes and Fer. Subsequently, a second cDNA encoding a short isoform, p45dfer, was discovered (Paulson et al., 1997) (Fig. 1B). Paulson et al. showed that DFer can transform vertebrate cells (Paulson et al., 1997), suggesting that the molecular pathways through which Fer signals are likely to be conserved.

Here, we show that DFer acts in conjunction with Src42A in the process of dorsal closure. *dfer* mRNA is specifically upregulated in the leading edge cells of the dorsal epidermis. DFer localises to adherens junctions and is required for the formation of the F-actin cable in leading edge cells, and for normal rates of dorsal closure. When mutations in *dfer* are combined with a mutation in *Src42A*, dorsal closure fails completely. We have isolated a gain-of-function *dfer* mutant (*dfer^{sof}*) that blocks dorsal closure and causes axon misrouting. The *dfer^{sof}* mutant expresses an N-terminally fused form of DFer, similar to oncogenic forms of Fer. β -catenin phosphorylation levels are reduced in *dfer* loss-of-function mutants and increased in *dfer^{sof}* mutants. This supports a role for DFer in the regulation of AJs and cell-cell adhesion, and may begin to explain its role in oncogenesis.

MATERIALS AND METHODS

Drosophila stocks

The *Drosophila* lines used in this study are: Wild type (Oregon-P; Bloomington Stock Centre), *hep¹* (Glise et al., 1995), UAS-puc (Martin-Blanco et al., 1998), UAS-GFP-Actin (Verkhusha et al., 1999), da-GAL4 (Wodarz et al., 1995), *Src42A^{myr1}* (Tateno et al., 2000), *puc^{E69}* lacZ (Martin-Blanco et al., 1998). The following stocks were generated in this study: UAS-DFerRB;*dfer^{Δ1}*/TM3,hb-lacZ, daGAL4,*dfer^{Δ1}*/TM3,GFP, *Src42A^{myr1}*/CyO,GFP;*dfer^{Δex1}*, *Src42A^{myr1}*/CyO,GFP;*dfer^{Δ1}*/TM3,GFP, UAS-puc,*dfer^{sof}*/TM3,GFP, daGAL4,*dfer^{sof}*/TM3,GFP, UAS-DFerRB;+;*dfer^{sof}*/TM3,GFP and UAS-GFP-Actin;*dfer^{sof}*/TM3,GFP. Transgenic flies containing UAS-DFerRB were generated as previously described (Brand and Perrimon, 1993), except that DNA was prepared using a Qiagen Midiprep Kit.

To generate DFer mutants by male recombination, w/Y;+;pGawB^{MZ465} homozygous males were mated en masse to yw hop6;+;ru st e ca/TM6B virgins at 27°C. Mosaic eyed yw hop6/Y;+;ru st e ca/pGawB F1 males were then crossed to w⁺;+;mwh th st ri roe p⁺ cu sr e⁺ virgins en masse and the progeny screened for recombinant scarlet males. In a typical crossing scheme, around 10,000 males were scored, to isolate 30 scarlet recombinants. Recombinants were then crossed to yw;+;D gl/TM3 p[Kr-GAL4, UAS-GFP] (Casso et al., 1999) (hereafter called TM3,GFP) virgins to generate a stock. *dfer^{sof}* was generated by imprecise excision. All fly crosses and egg collections were performed at 25°C unless otherwise stated.

Molecular biology

The 3.3 kb full-length *dfer* cDNA (pBS-p92) (Katzen et al., 1991) was digested with *EcoRI* and *XbaI*, and cloned into pUAST (Brand and Perrimon, 1993) to create pUAS-DFerRB.

In MZ465, pGawB is inserted between bases 156733 and 156734 in GenBank genomic sequence AE003682 (TACTCGAAAC<pGawB>-ACTCGGGCCG); in *dfer^{Δex1}* mutants, bases 155504–156733 (ATTGCTAAT...TACTCGAAAC) are deleted; in *dfer^{Δ1}* mutants, bases 102998–156733 (ATTTCACAAT...TACTCGAAAC) are deleted.

Analysis of isoforms DFerRA and DFerRC is based on Gadfly Release 4.2 annotations (Celniker et al., 2002; Stapleton et al., 2002) and independent sequencing of cDNA clones RH14840 and AT17877. The existence of isoform DFerRD/p45dfer was originally proposed based on a cDNA, 9C13 (Paulson et al., 1997). However, comparison of 9C13 with the genomic sequence and the full-length cDNA for DFerRA (RH14840) reveals several frameshift mutations which, when removed, reveal an open reading frame upstream of the predicted translational start. This, combined with a lack of 5' ESTs aligned with the proposed transcriptional start site for

DFerRD, and the fact that DFerRD is not apparent on northern or western blots (Paulson et al., 1997), suggests that 9C13 is a partial cDNA of the long isoform DFerRA.

For RT-PCR, total RNA was prepared from *dfer^{sof}* homozygous mutant or wild-type embryos. First strand cDNA libraries were generated using the SMARTTM RACE Kit (Clontech), and analysed using 5' and 3' RACE with forward and reverse gene-specific primers. RT-PCR analysis of *dfer^{Δex1}* flies used the QIAshredder and RNeasy kits (QIAGEN) and SuperScript III RNase H-Reverse Transcriptase (Invitrogen). Mutants were mapped using direct PCR, inverse PCR and plasmid rescue on adult fly genomic DNA, and direct PCR on single embryo DNA using standard methods.

Immunohistochemistry

To generate DFer antibody, the N terminus of DFer was amplified using 5'-GCGCTCGAGATATGGGCTTCTCATCAGC-3' and 5'-GCGGAATTC-CTGGCGGCATAGGTCATCCTT-3' primers, and subcloned into the *XhoI* and *EcoRI* sites of the pRSETC vector (Invitrogen). DFer antibody (Eurogentec) was pre-adsorbed for several days at a dilution of 1:10 on *dfer^{Δex1}* homozygous mutant embryos and used at a final concentration of 1:100. Antibodies used in this study were: MAb phospho-Tyrosine (Cell Signalling; 1:100); MAb 1D4 anti-Fascin 2 (1:10), MAb 2D5 anti-Fascin 3 (Patel et al., 1987) (1:2), MAb 40-1a anti- β -galactosidase (Developmental Studies Hybridoma Bank, DSHB; 1:200), Rat anti-DECad, DCAD2 (Oda et al., 1994) (1:50), Mouse anti-arm (N2 7A1; DSHB; 1:10) and Rabbit anti-odd-skipped (Spana and Doe, 1996) (1:1000). F-actin in embryos was labelled with Alexa568-Phalloidin as described previously (Kaltschmidt et al., 2002).

Immunoprecipitation and western blotting

Five hundred stage 14 to 17 embryos were homogenised in 50 mM HEPES pH 7.6, 1 mM MgCl₂, 1 mM EGTA, 50 mM KCl, Roche complete EDTA-free protease inhibitor cocktail, Sigma phosphatase inhibitor cocktail I and Sigma phosphatase inhibitor cocktail II. Extracts were incubated for two hours at 4°C with Protein A beads coupled to anti-arm (N2 7A1). Immunoprecipitates were spun down and washed five times with the above buffer containing 0.01% Tween. The final immunoprecipitates were boiled in SDS-PAGE sample buffer and equal loadings were separated on a 4–15% SDS-PAGE BIO-RAD Ready gel, then transferred to PVDF membrane. Membranes were probed with mouse anti-arm (N2 7A1; 1:10) and mouse anti phospho-tyrosine (Cell Signalling; 1:10,000). For detection, we used horseradish peroxidase (HRP)-conjugated secondary antibodies and the ECL plus western blotting detection system (Amersham Biosciences), or, for quantification, Alexa680-conjugated secondary antibodies (Molecular Probes) and a LI-COR Odyssey Infrared Imager.

Whole embryo extracts for western blotting were prepared as for immunoprecipitation. Equal numbers of embryos were loaded onto 4–15% SDS-PAGE Ready gels, transferred as above and probed with anti-arm (N27A1; 1:10) or anti-DFer (1:2000). Loadings were confirmed by western blotting with anti-actin [Sigma (20-33); 1:1000].

Embryonic staging

To document the reduced rates of closure, we selected embryos at late stage 16. This was defined as the stage when the gut has undergone its two major constrictions, but prior to the successive lateral movements that disrupt the symmetry of the three gut sections. To ensure that *dfer^{Δ1}* mutants did not reach this morphology prematurely, we selected dechorionated embryos that were midway through germ band retraction (stage 12), and allowed them to develop to late stage 16 on apple juice agar plates. In all cases, *dfer^{Δ1}* mutant embryos (*n*=9) developed characteristic gut morphologies at similar rates to control embryos (*n*=28).

RESULTS

DFer is expressed in the leading edge cells of the dorsal epidermis

dfer is a complex locus encoding at least three protein isoforms: DFerRA, DFerRB/p92dfer and DFerRC (see Materials and methods for cDNA details). DFerRB/p92dfer was originally identified by Katzen et al. (Katzen et al., 1991), and has the canonical structure of

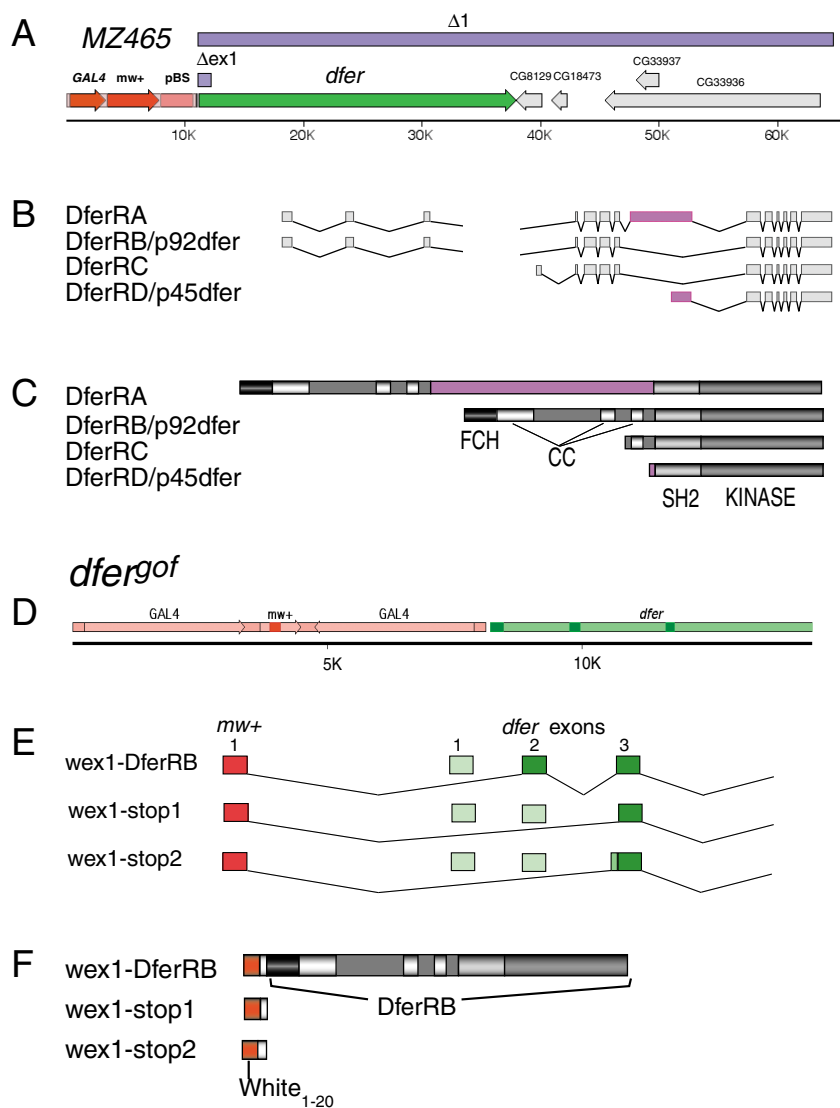


Fig. 1. Genomic organisation of the *dfer* locus.

(A) In *GAL4^{MZ465}*, pGawB (red) is inserted upstream of *dfer* (green). The deletions *dfer^{Δ1}* and *dfer^{Δex1}* are depicted (purple). (B) Exon structure of the four predicted *dfer* isoforms (a section of the third intron is deleted for clarity). Exon 1 is non-coding. Additional exons in *dferRA* and *dferRD* are in pink. (C) Predicted protein structure of Dfer isoforms. (D) Molecular map of the *dfer^{gof}* locus. pGawB (red) has undergone a rearrangement, duplicating and inverting GAL4, and deleting pBluescript (pBS) and most of mini-white (*mw+*). (E) *dfer^{gof}* produces three transcripts, splicing the first exon of mini white (red) to exons of *dfer* (green). Light green exons are spliced out. (F) One transcript (*wex1-DferRB*) is predicted to encode a White-Dfer fusion protein.

Fes/Fer family kinases, consisting of an FCH motif at the N terminus and three coiled-coil regions, an SH2 domain and a kinase domain (Fig. 1C). The longest isoform, DferRA, shares the promoter and exon structure of DferRB, but includes an additional exon, which encodes a novel protein domain that lies between the canonical N-terminal domain and the SH2 domain (Fig. 1C). A short isoform, DferRC, transcribed off a second promoter, encodes a truncated form of DferRB, lacking most of the N-terminal domain but including the third coiled-coil domain (Fig. 1C). A fourth isoform, DferRD/p45dfer, has been described (Paulson et al., 1997), which shares the SH2 and kinase domain, but has a distinct 10 residue N-terminal domain (Fig. 1C). Recent genome and EST sequence data, however, raise some doubt over the validity of this isoform (see Materials and methods for details).

As has been previously reported (Katzen et al., 1991), *dfer* expression is highly dynamic in a wide range of tissues, including the epidermis (Fig. 2A), central nervous system (Fig. 2B-E) and developing trachea (data not shown). In the epidermis, *dfer* is strongly expressed in the leading edge cells (Fig. 2A, arrowheads), beginning at late stage 13 and continuing throughout dorsal closure to the end of stage 15. In the CNS, *dfer* is expressed in the midline glia at stage 13 (Fig. 2B), and later in a segmentally repeated subset of cells (Fig.

2C) and in the dMP2 neurons (Fig. 2D,E). To investigate the distribution and localisation of Dfer protein, we raised an antibody against an N-terminal fragment of Dfer that excludes the SH2 and kinase domains. This fragment overlaps the predicted protein for the intermediate isoform DferRC by 30 residues. The antibody recognises ectopic expression of Dfer-RB using the GAL4/UAS system (Brand and Perrimon, 1993), and staining is lost in the *dfer^{Δex1}* mutant (Fig. 7C), which confirms the specificity of the antibody.

Dfer protein is ubiquitously expressed at relatively uniform levels throughout embryogenesis. Prior to dorsal closure, during stages 9-10, Dfer is predominantly localised to the cytoplasm, although some weak staining is seen around the perimeter of epidermal cells (data not shown). During stages 11-12, Dfer becomes localised to cell-cell junctions in the epidermis (Fig. 3A). Dfer becomes polarised in the leading edge cells as dorsal closure proceeds, as is seen for several other cell-cell junction proteins [e.g. Canoe (Kaltschmidt et al., 2002)]. Although initially present around the entire circumference of the cell, Dfer is lost from the border with the amnioserosa during stages 13-14 (Fig. 3B, arrow). Dfer protein is also apically enriched in other epithelial sheets such as the gut and trachea (data not shown), and is expressed in a subset of cells within the CNS (Fig. 7A, arrowheads).

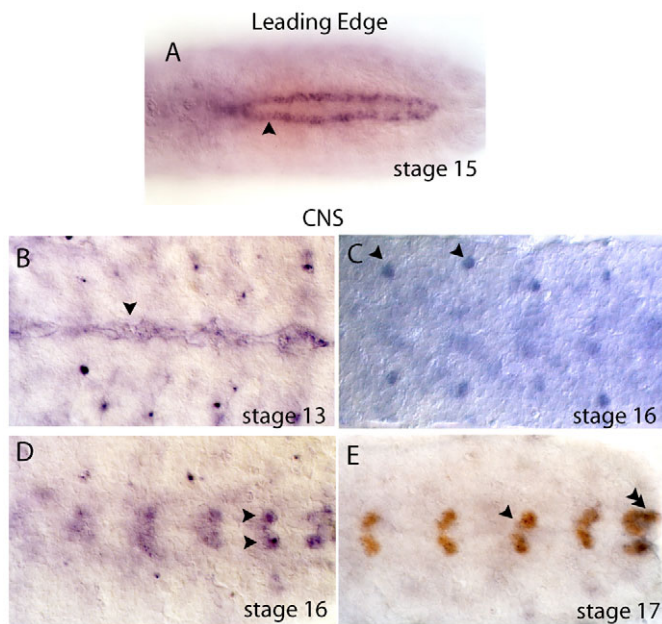


Fig. 2. *dfer* is expressed in leading edge cells and the CNS.

(A) *dfer* mRNA is expressed in the leading edge cells (arrowhead). (B) In the CNS, *dfer* is expressed in midline glial cells (arrowhead) and upregulated in a segmentally repeated, lateral, cell (C, arrowheads). (D) *dfer* is expressed in a pair of dorsal neurons, corresponding to the MP2 neurons (arrowheads). (E) *dfer* mRNA (black), Odd-skipped (brown), MP1s (single arrowhead), MP2s (double arrowhead).

DFer localises to the adherens junction in the dorsal epidermis

In vertebrates, Fer associates with the adherens junction components p120-catenin and β -catenin (Kim and Wong, 1995; Rosato et al., 1998). To test whether DFer also localises to adherens junctions, we co-stained embryos for DFer and DE-Cadherin (*Drosophila* E-Cadherin). In the dorsal epidermis (Fig. 3C-H), DFer extensively colocalises with DE-Cadherin, although the distribution is not identical. At the leading edge, DE-Cadherin can be seen around the apical circumference at stage 14, whereas at this stage DFer is lost from the leading edge itself (Fig. 3C-E). In the amnioserosa, DFer is only occasionally detected at cell-cell junctions, where again it colocalises with DE-Cadherin (data not shown). At the leading edge, DFer is apical to the more basal septate junction proteins, Fasciclin 3 (Fig. 3I,J) and Discs large.

Generation of loss-of-function *dfer* mutants

Expression of DFer in leading edge cells suggests that it might play a role in dorsal closure. In the GAL4 insertion line MZ465 (Hidalgo and Brand, 1997), a P-element has inserted upstream of the first, non-coding, exon of *dfer* (Fig. 1A). We generated mutations in *dfer* by imprecise excision of the P-element and by male recombination (see Materials and methods), and screened for loss of DFer protein expression (e.g. Fig. 7C). Nine recombinant lines were generated. In one line, *dfer* ^{Δ ex1}, the deletion removes only the promoter and first exon of the *dfer* gene (Fig. 1A). In the other eight lines, a region of over 50 kb is deleted, encompassing the entire *dfer* locus and the four genes proximal to *dfer* (CG8129, a threonine dehydratase; CG18473, a phosphotriesterase; CG33936, a zinc finger protein; and CG33937, GadFly release 4.2; Fig. 1A). One of these lines, Df(3R)*dfer* ^{Δ 1} (hereafter *dfer* ^{Δ 1}), was selected for further analysis.

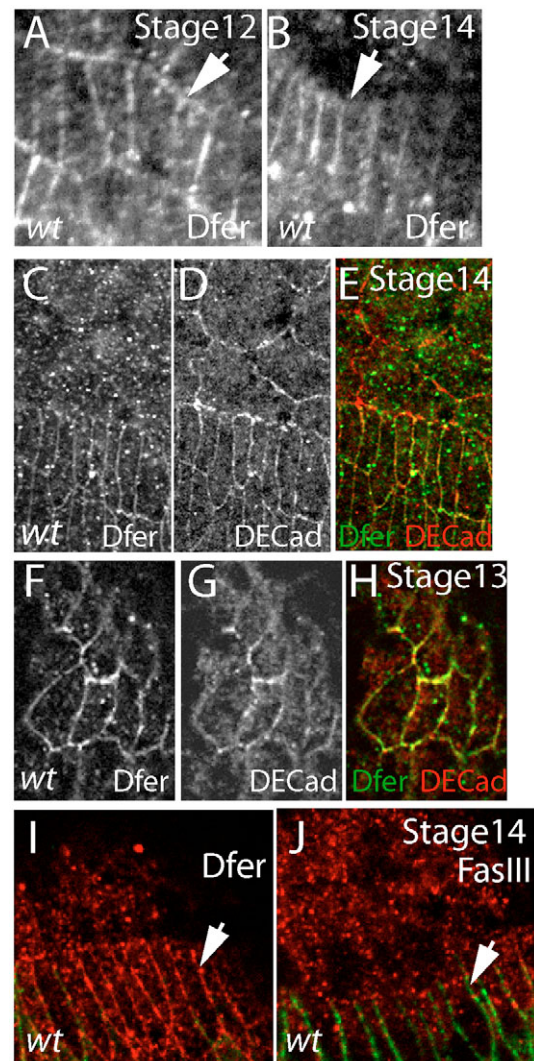


Fig. 3. DFer colocalises with DE-Cadherin and is apical to Fasciclin 3.

(A) At stage 12, DFer protein localises to cell-cell junctions (arrow) in leading edge cells and the dorsal epidermis. (B) DFer is lost from the border with the amnioserosa by stage 14 (arrow). (C-E) DFer colocalises with DE-Cadherin in the dorsal epidermis at stage 14, but is largely absent from the adherens junctions in the amnioserosa. (F-H) At stage 13, DFer overlaps extensively with DE-Cadherin. (I, J) At stage 14, the cell-cell junction staining of (I) DFer (red, arrow) is apical to (J) Fas3 (green, arrow). Confocal sections in I and J are 1 μ m apart.

Both *dfer* ^{Δ 1} and *dfer* ^{Δ ex1} still express functional GAL4 in patterns similar to that of MZ465. *dfer* ^{Δ 1} homozygotes are embryonic lethal in 53% of cases ($n=180$) with the remainder dying during larval and pupal development. *dfer* ^{Δ ex1} homozygotes are both viable and fertile. All results in this study using *dfer* ^{Δ ex1} are from embryos derived from *dfer* ^{Δ ex1} homozygous parents.

dfer ^{Δ 1} is an RNA and protein null for all DFer isoforms. In *dfer* ^{Δ ex1} mutants, *dfer* mRNA is lost from the CNS and leading edge, but tracheal expression is still evident, as is a low level of ubiquitous staining in the epidermis. DFer protein is not detected in the CNS (Fig. 7C), but some cell-cell junction staining is faintly visible in the epidermis. The first, non-coding, exon and promoter are deleted in *dfer* ^{Δ ex1} mutants; however, mRNA transcripts starting at the second exon, which encodes the translational start, are present. Western

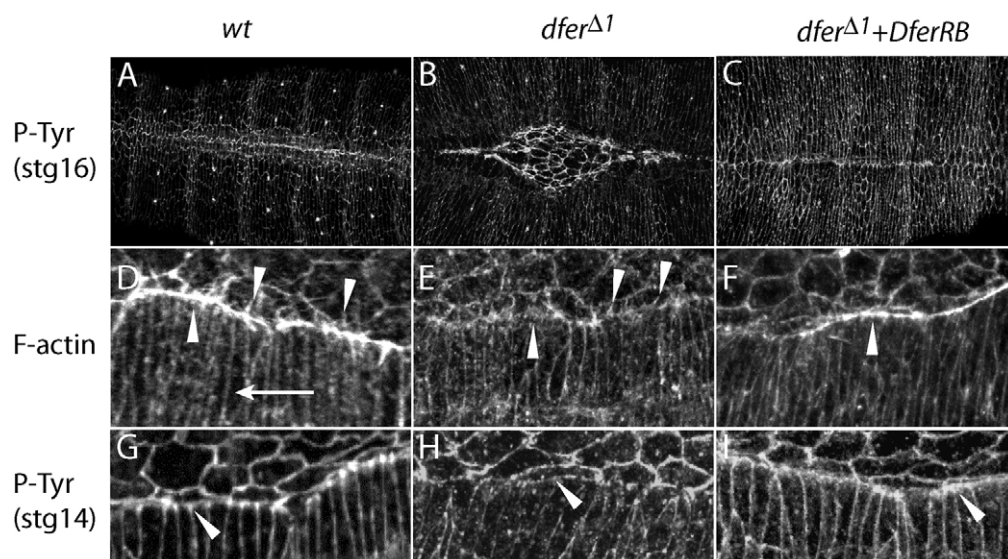


Fig. 4. DFer is required for normal leading edge morphology and normal rates of closure. (A,B) Late stage 16 wild-type embryos (A) have been dorsally closed for approximately three hours, whereas late stage 16 *dfer*^{Δ1} embryos (B) are dorsally open (white, anti-phospho-tyrosine). (C) DFerRB rescues dorsal closure (late stage 16 UAS-DFerRB/+;daGAL4,*dfer*^{Δ1}/*dfer*^{Δ1}). (D) At stage 14, leading edge cells elongate dorsoventrally (arrow), develop a thick F-actin cable (up arrowhead), and extend filopodial and lamellipodial protrusions (down arrowheads). (E) In *dfer*^{Δ1} embryos, the F-actin cable is greatly reduced (up arrowhead), but filopodia are still present (down arrowheads). (F) DFerRB expression largely restores the F-actin cable (arrowhead; white, Alexa568-Phalloidin; UAS-DFerRB/+;daGAL4,*dfer*^{Δ1}/*dfer*^{Δ1} embryos). (G) At stage 14, the leading edge is enriched for P-Tyr, particularly at the contact points between adjacent leading edge cells (arrowhead). (H) In *dfer*^{Δ1} embryos, P-Tyr staining is reduced (arrowhead). (I) In rescued embryos, P-Tyr staining is restored (arrowhead).

blots show that full-length DFer protein is produced in *dfer*^{Δ^{ex1}} mutants (see Fig. S1A in the supplementary material). Thus, *dfer*^{Δ^{ex1}} is a hypomorphic mutation in which *dfer* mRNA expression is lost in a subset of tissues and DFer protein levels in the dorsal epidermis are reduced.

DFer is required for normal leading edge cell morphology and for dorsal closure

During dorsal closure in wild-type embryos, the leading edge and neighbouring cells elongate along the dorsoventral axis (Fig. 4D, arrow). The profile of the leading edge changes from an irregular scalloped to a straightened edge as the actomyosin contractile cable forms (Fig. 4D, up arrowhead). Phosphotyrosine (P-Tyr) levels increase along the leading edge, particularly at the contact points between neighbouring cells or actin nucleating centres (ANC) (Kaltschmidt et al., 2002) (Fig. 4G, arrowhead). As closure continues, leading edge cells extend filopodial and lamellipodial processes (Fig. 4D, down arrowheads) that zip up the epidermal sheets that meet at the dorsal midline (reviewed by Jacinto et al., 2002).

In *dfer*^{Δ1} mutants, the actomyosin cable is reduced and the leading edge maintains an irregular profile during closure (Fig. 4E, up arrowhead). P-Tyr levels at the leading edge are also decreased, consistent with the loss of a cytoplasmic tyrosine kinase (Fig. 4H). These morphological differences are accompanied by a slower rate of closure. In wild-type embryos, dorsal closure is complete by the end of stage 15. *dfer*^{Δ1} mutant embryos are still open dorsally three hours later, at the end of stage 16 (90% *n*=10, Fig. 4B; see Materials and methods for staging details). Closure eventually completes, although 2% (*n*=180) of cuticles from *dfer*^{Δ1} mutants exhibit an anterior hole (Table 1). By contrast, *dfer*^{Δ^{ex1}} mutants appear to close normally and exhibit normal leading edge morphology, F-actin and P-Tyr staining.

To confirm that the morphological differences in *dfer*^{Δ1} mutants are due to loss of DFer, and not to the four other genes within the deletion, we used the GAL4/UAS system to express the canonical form of DFer, DFerRB, ubiquitously throughout the embryo in the *dfer*^{Δ1} background. In these embryos, the morphology of leading edge cells is largely restored to normal (Fig. 4F,I) and closure is completed at rates similar to wild type. All late stage 16 embryos are

Table 1. Embryonic lethality and cuticle defects in *dfer* and *src42A* mutants

Genotype	<i>n</i>	Embryonic lethality (%)	Morphology of unhatched embryos				No cuticle (%)
			Normal (%)	Small anterior hole* (%)	Anterior hole + scab [†] (%)	Large anterior hole [‡] (%)	
<i>dfer</i> ^{Δ1}	180	53	50.6	1.7	0	0.6	0
<i>src42A</i> ^{myri}	191	63	24.6	37.7	0	0.5	0
<i>src42A</i> ^{myri} ; <i>dfer</i> ^{Δ^{ex1}}	152	100	0	94.7	0	5.3	0
<i>src42A</i> ^{myri} ; <i>dfer</i> ^{Δ1}	102	100	0	30.4	28.4	30.4	10.8

*Small anterior hole or malformed mouth parts.

[†]Anterior hole and dorsal puckering/scab.

[‡]Large anterior hole/dorsal open.

closed ($n=10$, Fig. 4C). Our results show that Dfer is required for the normal morphology of leading edge cells and for normal rates of closure.

Dfer acts with Src42A to regulate dorsal closure

Vertebrate Fes/Fer kinases and members of the Src family kinases share some substrates, such as p120ctn, β -catenin (Piedra et al., 2003) and the Arp2/3 activator Cortactin (Kim and Wong, 1998; Wu and Parsons, 1993), and play similar roles in regulating cell-cell adhesion (Frame et al., 2002; Matsuyoshi et al., 1992; Rosato et al., 1998). Src family kinases are functionally redundant in several developmental processes, including dorsal closure (Takahashi et al., 2005; Tateno et al., 2000): single mutations in *Src42A*, *tec29A* and *Src64C* do not exhibit dorsal holes, whereas double mutants, such as *tec29A*, *Src42A*, do (Tateno et al., 2000). Therefore, we tested whether *Src42A* and Dfer act together in dorsal closure.

Src42A single mutants do not exhibit dorsal holes, but show defects in mouthpart formation (Tateno et al., 2000) and epithelial organisation following closure (Takahashi et al., 2005). We find that *Src42A* embryos also exhibit defects in leading edge cells that are similar to, but less severe than, *dfer* ^{Δ 1} mutants: the actomyosin cable

is disrupted (compare Fig. 5D, arrowhead, with Fig. 4D), P-Tyr staining is weaker than in wild type (compare Fig. 5G, arrowhead, with Fig. 4G), and dorsal closure is slightly defective. Eight percent ($n=25$) of embryos show a very small dorsal hole at late stage 16 when analysed by confocal microscopy, and the remainder show an irregular arrangement of epidermal cells that is reflected later in the arrangement of dorsal hairs (Fig. 5A). Embryonic lethality is 63% ($n=191$), with 60% of the unhatched embryos showing malformed mouthparts and a small anterior hole (Fig. 5M).

When the *Src42A* and *dfer* ^{Δ 1} mutants are combined, leading edge cells have a more irregular profile (Fig. 5E, arrowhead) and P-Tyr staining is weaker (Fig. 5H, arrowhead). When analysed by confocal microscopy, most embryos are still undergoing dorsal closure by late stage 16 (85%, $n=34$; Fig. 5B). Embryonic lethality is 100% ($n=152$), and embryos have breaks and irregularities in the dorsal hair pattern, and a small anterior hole near the mouthparts (95%, $n=152$; Fig. 5K, arrowhead). In the remaining embryos, dorsal closure fails completely, leaving a large anterior hole (5%, $n=152$; Fig. 5N, arrowhead). When the *dfer* deficiency, *dfer* ^{Δ 1}, and *Src42A* are combined, these defects are further enhanced. The leading edge becomes highly irregular with a complete loss of the F-actin cable (Fig. 5F, arrowhead) and a substantial reduction in P-Tyr staining

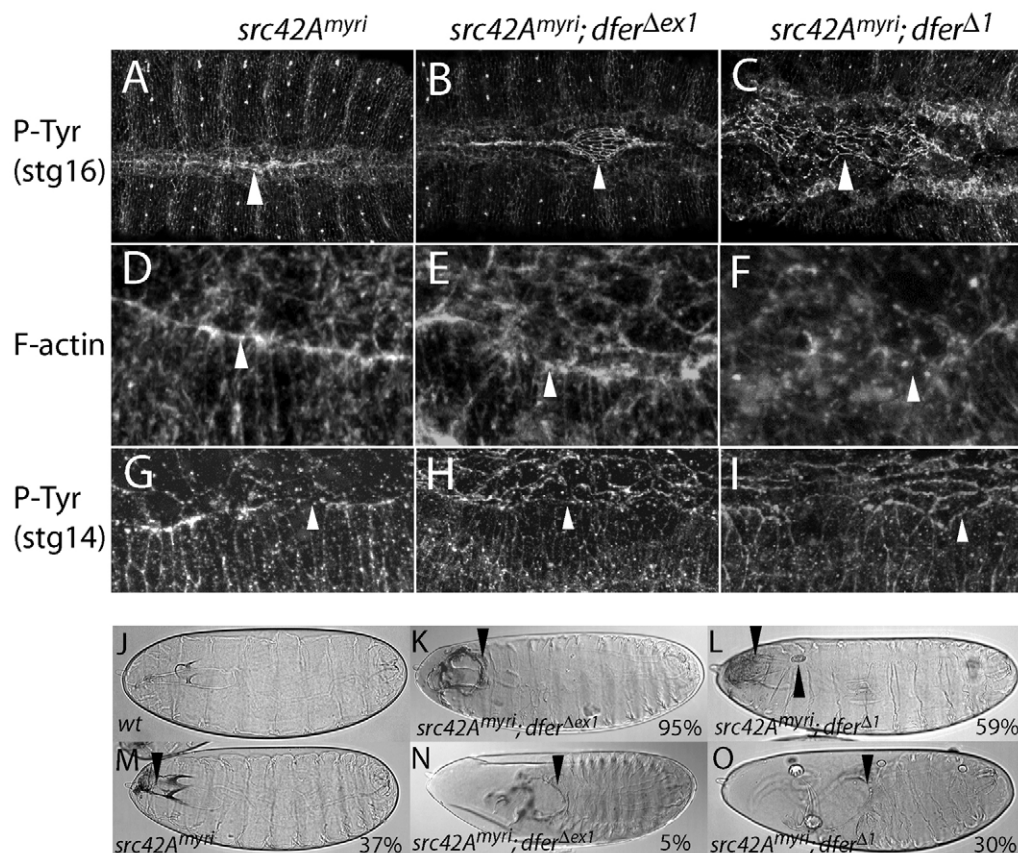


Fig. 5. Dfer and Src42A cooperate during dorsal closure. (A–C) Late stage 16 *Src42A*^{*myri*} embryos (A) are closed but have an irregular arrangement of epidermal cells, whereas *Src42A*^{*myri*}; *dfer* ^{Δ ex1} mutants (B) are still closing and *Src42A*^{*myri*}; *dfer* ^{Δ 1} mutants (C) are dorsally open (white, anti-phospho-tyrosine). (D–F) The F-actin cable (arrowheads) is slightly disrupted in *Src42A*^{*myri*} mutants (D, compare with Fig. 4D), further disrupted in *Src42A*^{*myri*}; *dfer* ^{Δ ex1} mutants (E) and completely lost in *Src42A*^{*myri*}; *dfer* ^{Δ 1} embryos (F; white, Alexa568-Phalloidin). (G–I) P-Tyr staining (arrowheads) is progressively reduced in *Src42A*^{*myri*} (G), *Src42A*^{*myri*}; *dfer* ^{Δ ex1} (H) and *Src42A*^{*myri*}; *dfer* ^{Δ 1} (I) mutants. (J) Wild-type cuticle preparation. (K, N) *Src42A*^{*myri*}; *dfer* ^{Δ ex1} mutants typically show a small anterior hole (K; 95%, $n=152$; arrowhead), but are occasionally anterior open (N; 5%, arrowhead). (L, O) *Src42A*^{*myri*}; *dfer* ^{Δ 1} mutants have a small to medium anterior hole (L; 59%, $n=102$, down arrowhead), often with scabs along the dorsal midline (up arrowhead), or dorsal closure fails (O), yielding an anterior open phenotype (30%, down arrowhead). (M) *Src42A*^{*myri*} mutants fail to hatch (63%, $n=191$), with 37% showing a small anterior hole near the mouthparts (arrowhead).

(Fig. 5I, arrowhead). When analysed by confocal microscopy, embryos exhibit a large dorsal hole at late stage 16 ($n=5$; Fig. 5C). Cuticle preparations from these embryos show a large anterior hole (30%, $n=102$; Fig. 5O) or a small anterior hole (59%, $n=102$; Fig. 5L, down arrowhead), often with small scabs along the dorsal midline (Fig. 5L, up arrowhead). The remaining embryos do not secrete a cuticle (11%, $n=102$). Therefore, when either DFer or Src42A expression is reduced, leading edge cell morphology is compromised and closure is delayed, but when both are removed dorsal closure fails completely.

A *dfer* gain-of-function mutant disrupts dorsal closure

Fes/Fer family non-receptor tyrosine kinases were first identified as the retroviral oncogenes, *v-fps* and *v-fes*, from avian and feline sarcomas, respectively (Shibuya et al., 1980; Snyder and Theilen, 1969). In *v-fes* and *v-fps*, a fragment of the viral GAG protein is

fused to the N terminus of the endogenous protein. The N terminus of Fes/Fer is implicated in the regulation of autophosphorylation (Orlovsky et al., 2000), and N-terminal fusions result in a constitutively active kinase. Activated kinases have also been created by the introduction of an N-terminal myristoylation sequence (Greer et al., 1994), and by point mutations in the first coiled-coil domain (Cheng et al., 2001). These are thought to disrupt intramolecular autoregulatory interactions.

We isolated a third *dfer* mutant, *dfer^{gof}*, which behaves as a gain-of-function mutant. Homozygous *dfer^{gof}* mutants are embryonic lethal and exhibit a number of embryonic defects, including a large dorsal hole (Fig. 6B) and an aberrant midline crossing of axons in the CNS (Fig. 7E, arrowhead). DFer protein is expressed at higher levels than in wild-type embryos (Fig. 7A,B), and when the levels of DFerRB are further increased in *dfer^{gof}* mutants, the midline-crossing defect is enhanced (Fig. 7F). By contrast, expression of DFerRB in a wild-type background has no effect on dorsal closure or CNS development. *dfer^{gof}* mutants express an N-terminally modified form of DFerRB, similar to the activated forms of Fes/Fer kinases, such as *v-fps* (see below). This appears as an extra band, slightly larger than the canonical DFer isoform (see Fig. S1B in the supplementary material).

In *dfer^{gof}* mutants, dorsal closure starts to arrest at stage 13, with only the most anterior and posterior segments meeting at the dorsal midline at stage 16 (Fig. 6B). The leading edge and dorsal epidermal cells fail to elongate (Fig. 6D). The actomyosin cable still forms and creates a straightened leading edge, albeit one reduced in thickness

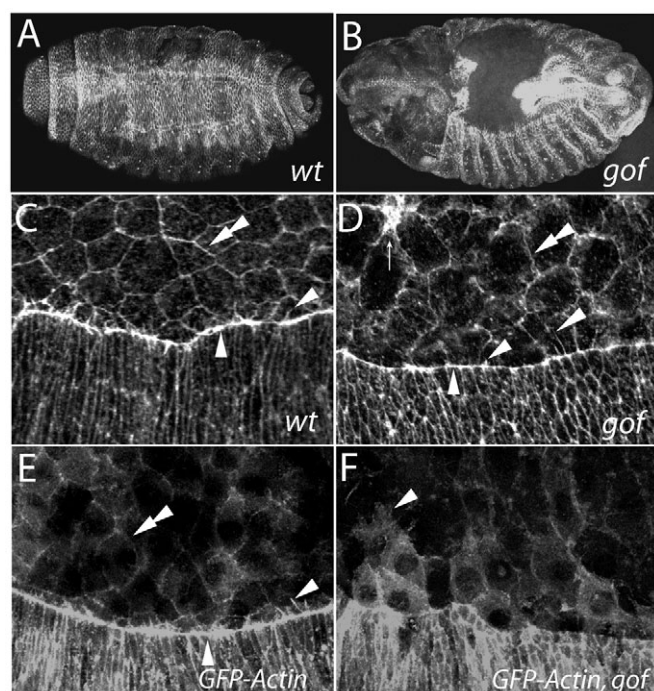


Fig. 6. A *dfer* gain-of-function mutant affects dorsal closure, the morphology of leading edge and amnioserosal cells. (A,B) Late stage 16 wild-type embryo (A), stained for the cell adhesion molecule Fasciclin 3, is dorsally closed, whereas an equivalently staged *dfer^{gof}* embryo (B) is still open. (C) At stage 14, wild-type leading edge cells develop an actomyosin cable (up arrowhead; white, Alexa568-Phalloidin), and numerous filopodia (down arrowhead). F-actin is enriched at amnioserosal cell borders (double arrowhead; same embryo as is shown in Fig. 4D). (D) In *dfer^{gof}* mutants, the actomyosin cable is thinner (up arrowhead), and the filopodia less abundant (down arrowheads). Amnioserosal cells show weaker F-actin staining at cell-cell junctions (double arrowhead). Contraction of individual amnioserosal cells still occurs (up arrow). (E) In wild-type embryos, GFP-Actin highlights the actomyosin cable (up arrowhead), filopodia (down arrowhead) and cortical enrichment in amnioserosal cells (double arrowhead; UAS-GFP-Actin/+; daGAL4/+; stage 14). (F) In *dfer^{gof}* embryos, the actomyosin cable and filopodia are reduced, and amnioserosal cells show less cortical actin and a more motile morphology (arrowhead; UAS-GFP-Actin/+; daGAL4, *dfer^{gof}/dfer^{gof}*; stage 14).

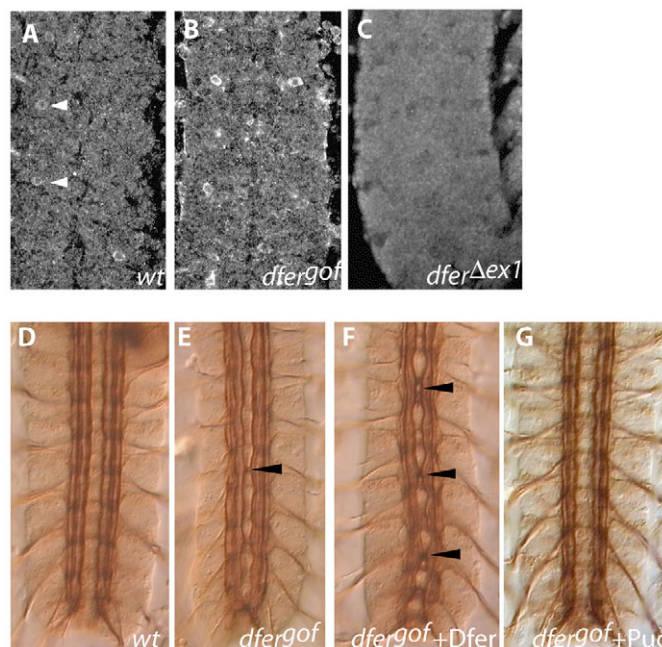


Fig. 7. The *dfer* gain-of-function mutant disrupts axon guidance at the ventral midline of the CNS. (A,B) Fillets of stage 16 embryos immunostained on the same slide show significantly higher levels of DFer protein in the CNS of *dfer^{gof}* mutants (B) than in wild type (A). (C) DFer protein is not expressed in the CNS of *dfer^{Δex1}* mutant embryos. (D) In wild-type embryos, Fasciclin 2 (brown) is expressed on longitudinal axons. (E,F) In *dfer^{gof}* mutants, axons cross the midline aberrantly (E, arrowhead), a phenotype that is enhanced when the levels of DFerRB are increased (F, arrowheads; UAS-DFerRB/+; *dfer^{gof}*). (G) Expression of the JNK inhibitor Puc rescues the midline phenotype of *dfer^{gof}* mutants (UAS-Puc, *dfer^{gof}/dfer^{gof}*).

(Fig. 6D, up arrowhead). The F-actin-rich filopodia that extend from the leading edge are also much less extensive (Fig. 6D, down arrowheads).

The amnioserosa is also affected in *dfer^{gof}* mutant embryos. In wild-type embryos, F-actin staining becomes increasingly strong at the perimeter of amnioserosal cells as they progressively contract (Fig. 6C, double-down arrowhead). In *dfer^{gof}* mutants, F-actin staining is much less concentrated at cell-cell junctions, and the cell cortices are irregular (Fig. 6D, double arrowhead). Accelerated contraction of isolated amnioserosal cells still occurs (Fig. 6D, arrow).

To characterise further *dfer^{gof}* mutants, we expressed GFP-actin (Verkhusa et al., 1999) ubiquitously in wild-type and *dfer^{gof}* backgrounds. The leading edge actomyosin cable and filopodia are reduced in *dfer^{gof}* mutants, and less GFP-Actin is concentrated at the cell-cell junctions of amnioserosal cells. In addition, the amnioserosal cells exhibit more lamellipodia (Fig. 6F).

In *dfer^{gof}* mutants the P-element, pGawB, has undergone a rearrangement, duplicating and inverting the GAL4 gene, deleting pBluescript, and all but the promoter and first exon of the mini-white gene (Fig. 1D). As predicted from this map, *dfer^{gof}* still expresses GAL4. In fact, GAL4 is expressed at higher levels and in more tissues, such as the epidermis and the amnioserosa, than in the original starting line, GAL4^{MZ465} (data not shown). We detect three new fusion transcripts in which the first exon of the mini-white gene is spliced to the beginning of the second exon of *dfer*, (*wex1-DferRB*), to the beginning of the third exon (*wex1_stop1*), or to an alternate splice acceptor in intron2 (*wex1_stop2*) (Fig. 1E). The second and third of these transcripts encode short proteins comprising the first 24 residues of White, followed shortly thereafter by stop codons. The first transcript encodes a predicted fusion protein in which the first 24 residues of White (MGQEDQELLIRGGSKHPSAEHLNN) are followed by 12 novel amino acids (RAATQIGSNESI) and the entire DferRB protein. This chimaeric protein is strikingly similar to the oncogenic forms of Fes, such as the Fujinami sarcoma virus protein GAG-Fps, in

which part of the retroviral GAG sequence is fused to the N terminus of the entire Fps gene (Shibuya et al., 1980) (see also Discussion).

Dfer cooperates with Src42A to activate dpp expression in leading edge cells

In wild-type embryos, dorsal closure is initiated by activation of the JNK pathway in leading edge cells, resulting in the transcription of two JNK pathway targets, *decapentaplegic* (*dpp*), a TGF- β homologue, and *puckered* (*puc*), a dual specificity phosphatase (Glise and Noselli, 1997; Hou et al., 1997; Martin-Blanco et al., 1998; Riesgo-Escovar and Hafen, 1997). Dpp signals to the neighbouring epidermal cells, causing them to elongate dorsoventrally. Puc inhibits Jun kinase, initiating a negative-feedback loop. Given that both Src and Dfer function in dorsal closure, and that Src is an upstream regulator of the JNK pathway, we tested whether Dfer also regulates the JNK pathway.

We first assayed whether the activity of the JNK pathway in leading edge cells is altered in *dfer* loss-of-function mutants. In *dfer^{Δ1}* and *dfer^{Δex1}* mutants, *dpp* expression levels appear normal. In *Src42A* mutants, *dpp* expression is slightly reduced, becoming patchy from stage 13 onwards (Fig. 8B). In *Src42A;dfer^{Δ1}* double mutants, *dpp* expression in the leading edge is reduced further and is almost abolished by stage 13 (Fig. 8C). This suggests that Dfer facilitates Src42A-mediated JNK signalling. However, neither DferRB nor *wex1-DferRB* is able to induce ectopic expression of *dpp*, suggesting that Dfer is not itself sufficient to activate the pathway. Furthermore, JNK activation is normal in leading edge cells of *dfer^{gof}* mutants (Fig. 8E,G).

dfer transcription is upregulated in LE cells, as are *dpp* and *puc*. Although *dfer* transcription occurs at a later stage than that of *dpp* and *puc*, we tested whether *dfer* might nonetheless be a transcriptional target of the JNK pathway. In loss-of-function mutants for the Jun kinase kinase *hemipterous* (*hep*) (Glise et al., 1995), *dpp* is lost in leading edge cells (see Fig. S2B in the supplementary material). *dfer*, however, is still expressed (Fig. S2E

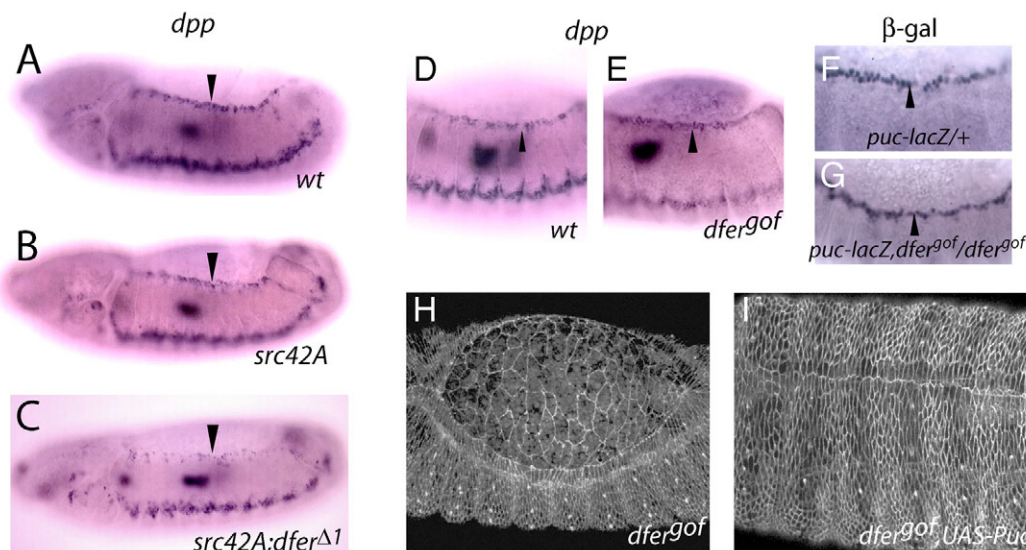


Fig. 8. Dfer interactions with the JNK pathway. (A) Stage 13 wild-type embryos express *dpp* in leading edge cells (arrowhead) and in the midgut. (B,C) In *Src42A^{myr1}* embryos (B), *dpp* expression in leading edge cells (arrowheads) is slightly reduced and expression is almost lost in *Src42A^{myr1};dfer^{Δ1}* double mutants (C). (D-G) *dpp* (E) and *puc-lacZ* (G) expression in leading edge cells of *dfer^{gof}* mutants (arrowheads) is comparable to wild type (D,F). (H,I) Phosphotyrosine staining in *dfer^{gof}/dfer^{gof}* (H) and *UAS-puc, dfer^{gof}/dfer^{gof}* (I) embryos. (I) Expression of the JNK inhibitor Puc with *dfer^{gof}* serving as the GAL4 driver, rescues the dorsal open phenotype of *dfer^{gof}* mutants.

in the supplementary material). When a constitutively active form of Hep, UAS-hep^{CA} (Adachi-Yamada et al., 1999), is expressed in the engrailed pattern, *dpp* is upregulated in the posterior half of each segment, but *dfer* is not (see Fig. S2C,F in the supplementary material). Therefore, *dfer* is not a JNK target.

Surprisingly, although *dfer* is not regulated by the JNK pathway, we find that the *dfer^{gof}* phenotype is rescued by expression of the JNK inhibitor Puckered (Puc). *UASpuc, dfer^{gof}* embryos close at normal rates (100% embryos closed at stage 16, *n*=6), resulting in a regular arrangement of epidermal cells (*n*=5; Fig. 8I). Axon misrouting at the ventral midline is also rescued (Fig. 7G). As DFer is unlikely to act through the JNK pathway, Puc may suppress the *dfer^{gof}* phenotype by dephosphorylating either DFer^{gof} itself or its downstream targets.

Tyrosine phosphorylation of β -catenin is altered in *dfer* mutants

DFer localises to AJs where it may regulate the phosphorylation state, and hence stability, of AJ proteins. To test whether DFer regulates the phosphorylation of β -catenin, we determined the extent of β -catenin tyrosine phosphorylation in *dfer* mutants. β -catenin was immunoprecipitated from *yw*, *dfer Δ 1* and *dfer^{gof}* embryos, and probed with anti-armadillo (β -catenin) and anti-phospho-tyrosine (Fig. 9A,B). In *dfer Δ 1* embryos, β -catenin tyrosine phosphorylation is reduced nearly fivefold with respect to control embryos (Fig. 9A). Conversely, tyrosine phosphorylation is significantly increased in *dfer^{gof}* embryos (Fig. 9B). Much less β -catenin is recovered from *dfer^{gof}* embryos, suggesting that hyperphosphorylated β -catenin is removed from AJs and degraded. This is confirmed by whole-mount staining of *dfer^{gof}* embryos, where the levels of β -catenin are

decreased in the epidermis of stage 13 embryos (Fig. 9C). DE-Cadherin levels may also be slightly reduced in stage 13 *dfer^{gof}* embryos, but the result is more variable. Western blots on total extracts from late stage *dfer^{gof}* embryos also show a reduction in the levels of β -catenin (Fig. 9D).

DISCUSSION

Fps/Fer has been implicated in oncogenesis from its first isolation in tumour causing avian and feline retroviruses. Here, we show that DFer localises to adherens junctions during *Drosophila* embryogenesis, where it regulates leading edge morphology and dorsal closure. In *dfer* null mutants, P-Tyr staining is reduced at the leading edge, in particular at the actin-nucleating centers. Two potential substrates of DFer, p120ctn and β -catenin, are localised to adherens junctions (Kim and Wong, 1995; Myster et al., 2003). We find that in *dfer Δ 1* mutants β -catenin phosphorylation is reduced. Conversely, β -catenin is more highly phosphorylated in *dfer^{gof}* mutants, demonstrating that the role of Fer in the phosphorylation of β -catenin is conserved in *Drosophila*. Interestingly, the overall level of β -catenin at cell-cell junctions is lower in *dfer^{gof}* mutants, suggesting that phosphorylated β -catenin is lost from AJs and subsequently degraded.

dfer mutants also exhibit a disorganised and reduced F-actin cable at the leading edge. Formation of the F-actin cable appears to depend on adherens junctions, as F-actin nucleation begins at the level of the AJs and the F-actin cable is disrupted in DE-Cadherin mutants (Takahashi et al., 2005). Recently it has been suggested that elevated levels of cytoplasmic α -catenin near stable AJs could favour the formation of F-actin bundles (Drees et al., 2005). DFer may contribute to the formation of the F-actin cable by phosphorylating

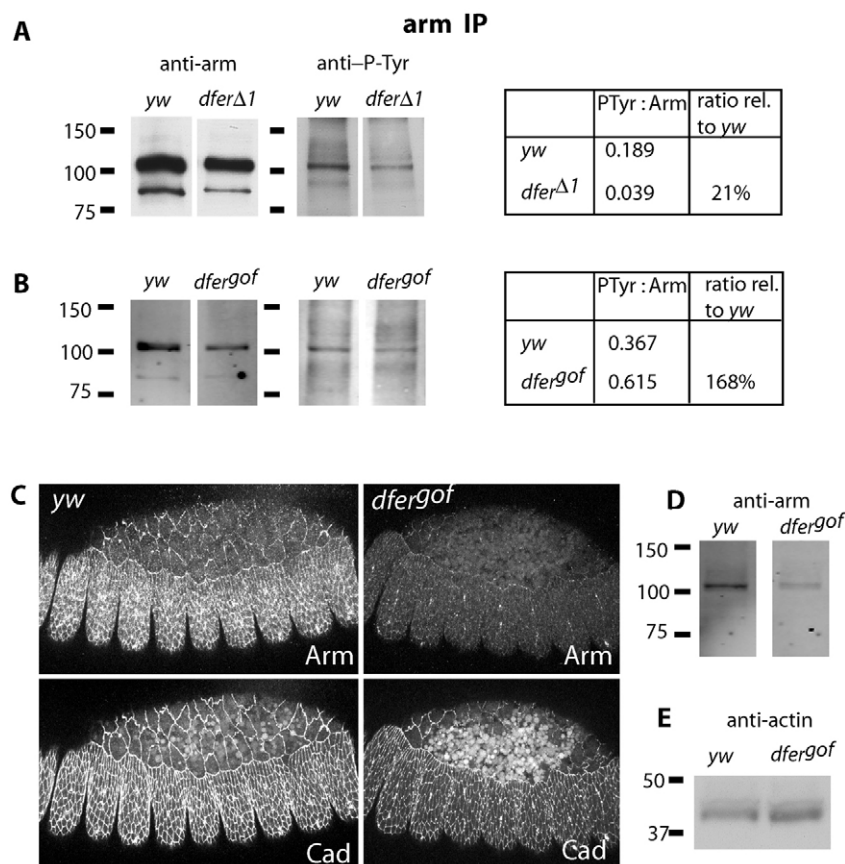


Fig. 9. β -catenin levels and phosphorylation state are altered in *dfer* mutants. (A,B) β -catenin immunoprecipitated from (A) *dfer Δ 1* and (B) *dfer^{gof}* late stage embryo extracts, probed with anti- β -catenin (Arm) and anti-phosphotyrosine. β -catenin is hypophosphorylated in *dfer Δ 1* embryos (21% relative to yw control embryos) and hyperphosphorylated in *dfer^{gof}* embryos (168% relative to yw). (C) Levels of β -catenin are reduced in the epidermis of *dfer^{gof}* embryos. DE-Cadherin may also be reduced to a lesser degree. (D) Western blots on total embryo extracts confirm that β -catenin is depleted in *dfer^{gof}* mutants. (E) Loading control (anti-actin).

β -catenin, reducing its affinity for α -catenin, and thereby increasing the local levels of cytoplasmic α -catenin (see Fig. S3 in the supplementary material). If DFER promotes stable F-actin bundles then the regulated loss of DFER from the leading edge at stage 14 may enable the more motile Arp2/3 regulated F-actin filopodia to form and complete dorsal closure by 'zipping up'.

We have shown that DFER and Src42A cooperate during dorsal closure. DFER localises to AJs and regulates β -catenin phosphorylation. In *Drosophila*, Src42A binds and phosphorylates β -catenin, although this may not be direct (Takahashi et al., 2005). Consequently, the more severe phenotypes seen in the *dfer*; *Src42A* loss-of-function mutants are most likely due to a combined loss of phosphorylation on at least two different tyrosine residues of β -catenin.

dfer mRNA is upregulated in leading edge cells. This, together with reports that vertebrate v-Fps and Fes mediate JNK pathway activation (Li and Smithgall, 1998), suggested that *dfer* might activate the JNK pathway during dorsal closure. Although DFER itself cannot induce *dpp* expression, it does play a supporting role in the maintenance of *dpp* levels, as revealed in the *Src42A* mutant background. A similar failure in the maintenance of *dpp*, as opposed to its induction, is seen in mutants of the Wnt pathway (Morel and Arias, 2004). Given the comparable phenotypes, and the fact that phosphorylated β -catenin is reduced in *dfer* mutants, it is possible that DFER contributes to the maintenance of *dpp* via the Wnt pathway.

We isolated a novel, gain-of-function mutation, *dfer^{gof}*, in which a fragment of the White protein is fused to the N terminus of DFER. This protein, Wex1-DFERB, is analogous to oncogenic forms of Fps in which part of the viral GAG protein is fused to the N terminus of the endogenous proto-oncogene, generating an activated kinase. Although *dfer^{gof}* mutants express DFER at higher levels, this alone seems unlikely to account for the observed defects, as overexpression of DFERB gives no obvious embryonic phenotype (M.J.M. and A.H.B., unpublished).

In *dfer^{gof}* mutants, the leading edge cells fail to elongate and the F-actin-rich filopodia are greatly reduced. The overall levels of the AJ junction components DE-Cadherin and β -catenin are reduced, and β -catenin is hyperphosphorylated. This suggests that AJs are disrupted in *dfer^{gof}* mutants. By contrast, it is interesting that the morphology of amnioserosal cells is shifted to a more motile appearance: F-actin is reduced at the cortex and there is an increase in the number of filopodia, perhaps because of a disruption of cell-cell junctions. In vertebrates, Fer has the capacity to both positively and negatively regulate cadherin-complex stability. This dual function may reflect a difference in binding partners present at AJs in different tissues.

Although loss of *dfer* does not appear to affect axon guidance, *dfer^{gof}* mutants have a clear CNS phenotype in which axons aberrantly cross the midline. A similar phenotype is seen with overexpression of the abelson tyrosine kinase, which antagonises the receptor Robo (Bashaw et al., 2000). *dfer^{gof}* mutants also disrupt axon guidance in the PNS, with some general misrouting of motor nerves and some overly large synapses (data not shown). In vertebrates, Fer associates with N-Cadherin in elongating neurites, where it can coordinately regulate N-Cadherin and integrin adhesion (Arregui et al., 2000). Fer has been shown to be concentrated in growth cones of stage 2 hippocampal neurons and is required for neuronal polarisation and neurite development (Lee, 2005). Similar to the leading edge, DFER may be required at growth cones to regulate filopodial extensions. In chick retinal cells, the phosphatase PTP1B when phosphorylated by Fer, localises to the catenin-binding domain of N-Cadherin (Xu et al., 2004). Interestingly, the

Drosophila homologue of PTP1B, DPTP61F, is expressed in the CNS and binds to the axon guidance molecule Dock (Clemens et al., 1996; Walchli et al., 2000).

Strikingly, all of the phenotypes associated with *dfer^{gof}* mutants are rescued by expression of the Puckered tyrosine phosphatase. Given that JNK pathway activity appears normal in *dfer^{gof}* mutants, Puckered may target DFER itself, or its substrates, at least one of which we have shown to be hyperphosphorylated in *dfer^{gof}* mutants. We have demonstrated a role for DFER during embryonic development in the regulation of AJ stability, in the formation of the contractile leading edge during dorsal closure, and in axon guidance. It cooperates with Src42A to regulate β -catenin phosphorylation at AJs. We isolated a gain-of-function mutant with structural similarity to oncogenic forms of vertebrate Fer. Unregulated Fer activity leads to oncogenesis, possibly through unregulated epidermal to mesenchymal transition. We have shown that activated DFER, or loss of DFER together with Src42A, disrupts AJs. This may provide a model for studying oncogenesis in the whole organism.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/16/3063/DC1>

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