Signal transduction by the Fat cytoplasmic domain

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SUMMARY
The large atypical cadherin Fat is a receptor for both Hippo and planar cell polarity (PCP) pathways. Here we investigate the molecular basis for signal transduction downstream of Fat by creating targeted alterations within a genomic construct that contains the entire fat locus, and by monitoring and manipulating the membrane localization of the Fat pathway component Dachs. We establish that the human Fat homolog FAT4 lacks the ability to transduce Hippo signaling in Drosophila, but can transduce Drosophila PCP signaling. Targeted deletion of conserved motifs identifies a four amino acid C-terminal motif that is essential for aspects of Fat-mediated PCP, and other internal motifs that contribute to Fat-Hippo signaling. Fat-Hippo signaling requires the Drosophila Casein kinase 1ε encoded by discs overgrown (Dco), and we characterize candidate Dco phosphorylation sites in the Fat intracellular domain (ICD), the mutation of which impairs Fat-Hippo signaling. Through characterization of Dachs localization and directed membrane targeting of Dachs, we show that localization of Dachs influences both the Hippo and PCP pathways. Our results identify a conservation of Fat-PCP signaling mechanisms, establish distinct functions for different regions of the Fat ICD, support the correlation of Fat ICD phosphorylation with Fat-Hippo signaling, and confirm the importance of Dachs membrane localization to downstream signaling pathways.

KEY WORDS: Dachsous, Drosophila, Fat, Hippo, PCP, Polarity

INTRODUCTION
Organ morphogenesis requires coordination of growth with patterning processes that orient cell behaviors. The Drosophila fat gene encodes an atypical cadherin that functions as a receptor for signal transduction pathways that regulate growth (Hippo signaling) and planar cell polarity (PCP) (reviewed by Thomas and Strutt, 2012; Staley and Irvine, 2012). Fat is regulated by two proteins expressed in gradients: Dachsous (D) and Four-jointed (Fj). Ds encodes an atypical cadherin that can function as a ligand for Fat (reviewed by Thomas and Strutt, 2012; Staley and Irvine, 2012). Fj is a Golgi-localized kinase that phosphorylates cadherin domains of Fat and Ds to modulate binding between them (Ishikawa et al., 2008; Brittle et al., 2010; Simon et al., 2010). Rather than responding solely to the level of Ds and Fj, Fat is also regulated by the slope and vector of their expression gradients, with the slope influencing Hippo signaling and the vector influencing PCP (Rogulja et al., 2008; Willecke et al., 2008; Thomas and Strutt, 2012).

Fat is one of several upstream pathways that impinge on Hippo signaling (reviewed by Pan, 2010; Halder and Johnson, 2011; Staley and Irvine, 2012). Most of these upstream inputs converge on the kinase Warts (Wts), which negatively regulates the transcriptional co-activator Yorkie (Yki). Hippo pathway activity promotes Wts activity, which promotes cytoplasmic localization of Yki. When fat, wts or other upstream tumor suppressors are downregulated, then Yki accumulates in the nucleus, increasing the transcription of genes that promote growth. Three genes have been identified as playing key roles in Fat-Hippo signal transduction: discs overgrown (dco), dachs and Zyxin (Zyx). Dco is Drosophila Casein kinase 1ε (Zilian et al., 1999). An antimorphic allele, dco3, specifically impairs Fat-Hippo signaling (Cho et al., 2006; Feng and Irvine, 2009). A portion of Fat is phosphorylated on its intracellular domain (ICD); this phosphorylation depends upon both Ds and Dco, suggesting that Fat ICD phosphorylation is a key step in Fat-Hippo signal transduction (Feng and Irvine, 2009; Sopko et al., 2009). Dachs is a myosin that downregulates Wts, and is required for the influence of fat or dco mutations on Hippo signaling (Cho and Irvine, 2004; Cho et al., 2006). Dachs localization is normally polarized in response to the Ds and Fj gradients (Mao et al., 2006; Rogulja et al., 2008; Ambegaonkar et al., 2012; Bosveld et al., 2012; Brittle et al., 2012). When Fat is overexpressed, Dachs membrane localization is reduced, whereas when fat is mutant, Dachs localizes to the membrane around the entire circumference of the cell (Mao et al., 2006). The correlation between Dachs localization and Fat activity suggests that regulation of Dachs localization is a key step in Fat signal transduction. Zyx affects Fat-Hippo signaling similarly to Dachs (Rauskolb et al., 2011). Zyx and Dachs can bind to each other, and binding of Dachs to Zyx stimulates Zyx-Wts binding (Rauskolb et al., 2011).

Dachs participates in both Fat-Hippo and Fat-PCP pathways, but it has been proposed that the influence of Dachs on Fat-Hippo signaling is related to the amount of Dachs localized to the membrane, whereas its influence on PCP is related to the direction in which Dachs membrane localization is polarized (Reddy and Irvine, 2008; Rogulja et al., 2008). One manifestation of Fat-PCP in the wing is the orientation of cell divisions, which contributes to wing elongation. In fat, ds or dachs mutants, cell division orientation is randomized, resulting in rounder wings (Baena-Lopez et al., 2008; Mao et al., 2011a). It has been proposed that Dachs myosin motor activity may contribute to the orientation of wing cell division by contracting cell apices, thereby altering cell geometry (Mao et al., 2011a). Modulation of tension along intercellular junctions also appears to contribute to influences of...
Dachs on PCP in the notum (Bosveld et al., 2012). A transcriptional co-repressor, Atrophin, has also been linked to some Fat-PCP phenotypes (Fanto et al., 2003; Li et al., 2009).

The central core of the Hippo pathway is conserved between Drosophila and mammals, but there is variation among upstream regulators (Pan, 2010; Halder and Johnson, 2011). Vertebrates have homologs of Fat (Fat4) and Ds (Dachs1), and deletion of Fat4 has been shown to affect Yap activity in a subset of CNS neurons in chick (Van Hateren et al., 2011). However, gene-targeted mutations in Dachs1 or Fat4 do not result in evident Hippo pathway phenotypes in mice, although they are consistent with influences of Dachs1 and Fat4 on PCP (Saburi et al., 2008; Mao et al., 2011b). Mammals do not, however, have an obvious Dachs homolog, and it remains unclear whether Fat signaling pathways in flies and mammals are related.

Here, we employ a structure-function approach to investigate signal transduction downstream of Fat. We show that Hippo and PCP pathways can be separated at the level of the Fat receptor. We identify point mutations in the Fat ICD that specifically impair Fat-Hippo signaling and reduce Fat phosphorylation, and identify a conserved four amino acid motif that is crucial for the effects of Fat on PCP. We also explore the relationship between Fat signaling and Dachs localization and provide direct evidence that Dachs localization influences both Hippo and PCP phenotypes.

**Materials and Methods**

**Drosophila genetics**

Rescuing activity of Fat transgenes was characterized by crossing fat(Gal4)[ft8]CyO,GFP; P[acman]-ft8/TM6b or ft8(CyO,GFPl; P[acman]-ft8/TM6b to ft8/Gal4,GFPl; CyO,GFPl. Constructs were amplified by copy induction to Table S2). Recombineering was similarly used to introduce deletion or inactivation of fat and categorized as <30°, 30°-90° or >90° if more than 10% of wing hairs showed a deviation. Only the regions anterior to L3 and proximal to the incomplete cross-vein were scored; costa and abdomens were scored normally. Points of crossing were estimated where possible for mutant wings with incomplete cross-veins, and categorized as <30°, 30°-90° or >90° if more than 10% of wing hairs showed a deviation.

To quantify cross-vein distance, the length of vein L4 between cross-veins was measured using ImageJ and divided by the length of vein L3 to obtain a relative length, and these were normalized to the wild-type ratio. For mutant wings with incomplete cross-veins, points of crossing were estimated where possible based on the direction of the incomplete cross-vein. Hair polarity phenotypes were evaluated by the angle of deviation from the normal axis, and categorized as <30°, 30°-90° or >90° if more than 10% of wing hairs showed a deviation. Only the regions anterior to L3 and proximal to the posterior cross-vein were scored; costa and abdomens were scored independently using similar criteria.

**Plasmids and constructs**

Following recombineering techniques, the Drosophila ft locus can be rescued by genomic Bac clones containing a 39 kb region encoding the ECD of Fat and Kugelei (also known as Fat-like or Fat2) (Tanoue and Takeichi, 2005). Within the ICD, only Fat4 exhibits significant similarity to Drosophila Fat and Kugelei (also known as Fat-like or Fat2) (Tanoue and Takeichi, 2005) (supplementary material Fig. S1). To assess the functional significance of this similarity, we investigated whether human Fat4 could rescue Drosophila fat mutants. As our goal was to investigate signal transduction by the ICD, we excluded potential differences between the ECDs by creating a hybrid transgene encoding the ECD of Drosophila Fat and the ICD of human Fat4 (Fat:Fat4) (supplementary material Fig. S2A,B). This was constructed within a fat genomic rescue construct (Feng and Irvine, 2009) by recombineering, and inserted using Flp-Cit-mediated site-specific recombination into the same location as we had previously inserted Drosophila fat (attP2 at 68A4) (Groth et al., 2004; Venken et al., 2006).

This Fat:Fat4 transgene could not rescue the lethality of Drosophila fat mutants. Moreover, examination of wing imaginal discs from fat mutant larvae expressing Fat:Fat4 revealed that they have the overgrown imaginal discs typical of fat mutants (supplementary material Fig. S2F), which indicates that they lack Drosophila Fat-Hippo signaling. In order to assess Fat-PCP signaling, we took advantage of the observation that the lethality and overgrowth phenotypes of fat mutants are suppressed by overexpressing Wts, whereas these flies still exhibit PCP phenotypes (Fig. 1A,E,H) (Feng and Irvine, 2007). Wing hairs normally point distally, but when fat activity is impaired wing hairs are misoriented and swirling patterns can be observed in the proximal wing. To quantify the effects on hair polarity, we...
classified wing hair PCP phenotypes as ‘normal’ when hair orientation was within 30° of its normal distal orientation, ‘weak’ when clusters of hairs (constituting >10% of hairs in the region examined) deviated in orientation by more than 30° but less than 90° from normal, and ‘strong’ when clusters of hairs deviated more than 90° from the normal orientation (Fig. 1M).

By these criteria, 100% of fat mutant wings rescued by a wild-type Fat transgene have a normal PCP phenotype, whereas 100% of Wts-rescued fat mutant wings have a strong PCP phenotype in the proximal, anterior wing (Fig. 1E,F,L). Using the same criteria, all fat mutant abdomens rescued by a wild-type Fat transgene have normal hair polarity, whereas all Wts-rescued fat mutant abdomens have a strong PCP phenotype (Fig. 1H,I,K). In fat mutants expressing Fat:Fat4 and Wts, PCP hair phenotypes were substantially rescued in the wing compared with Wts-expressing fat mutants without Fat transgenes, and were completely rescued in the abdomen (Fig. 1G,J-L). Fat:Fat4 also partially rescued the reduced cross-vein spacing phenotypes of fat mutants (Fig. 1C,N). Reduction of the distance between the anterior and posterior cross-veins is a classic Fat pathway mutant phenotype (Fig. 1A), which we think reflects the influence of Fat-PCP signaling on wing elongation. Wing elongation is influenced by Fat-PCP signaling both during disc growth, when it polarizes cell divisions along the proximal-distal axis, and during pupal development, when it influences local cell rearrangements (Baena-López et al., 2005; Aigouy et al., 2010; Mao et al., 2011a). This partial rescue of fat PCP phenotypes by Fat:Fat4 implies that mechanisms involved in Fat-PCP signal transduction are conserved from Drosophila to humans, and that they rely on shared structural motifs.

**Identification of motifs required for distinct Fat signaling pathways**

The overall sequence identity between the Drosophila Fat and human FAT4 ICDs is less than 25%, and most of this similarity is contained within six clusters of sequence identity (annotated A through F, Fig. 2; supplementary material Fig. S1). The functional significance of these regions of similarity was evaluated by deleting them within the Drosophila Bac clone that encompasses the fat locus. We also constructed one ECD mutation (FatΔEGF), in which four EGF domains, the significance of which have not been examined, were removed (supplementary material Fig. S2B).
Transformed flies containing *fat* genomic rescue constructs with each of these seven mutations were obtained. None exhibited any dominant phenotypes. When they were crossed into *fat* mutant backgrounds, all six of the ICD deletions, but not FatΔEEGF, rescued lethality (Fig. 2). Animals rescued by three of the ICD deletions (FatΔA, FatΔB and FatΔC) appeared morphologically normal (Fig. 2C-E; supplementary material Fig. S3), which implies that, despite their evolutionary conservation, these motifs are not essential. Conversely, animals rescued by constructs containing the other three deletions exhibited phenotypes that indicate that they provide only partial Fat activity.

In assays of wing growth, FatΔD-rescued *fat* mutants had wings that were obviously larger (by 29%) than normal, indicating a deficit in Fat-Hippo pathway activity (Fig. 2F,V). FatΔE-rescued...
fat mutant wings, by contrast, were on average only 8% larger than controls, a subtle, but nonetheless statistically significant difference (Fig. 2G,V; supplementary material Table S1). FatΔF-rescued fat mutant wings were only 4% larger than controls, which implies that Fat-Hippo signaling was almost fully rescued (Fig. 2H,V).

Although the area of FatΔF-rescued fat mutant wings was similar to that of wild type, they were shorter and wider than normal wings (Fig. 2H; supplementary material Table S1), which is suggestive of a PCP defect as the shape of the wing is influenced by oriented cell divisions and rearrangements. A PCP defect in FatΔF-rescued fat mutant wings was also implied by the short distance between cross-veins, which was only 27% of the wild-type distance (Fig. 2H,W). FatΔD and FatΔE mutants, by contrast, were more effective at rescuing cross-vein spacing, at 73% and 87% of the wild-type distance, respectively (Fig. 2F,G,W). fat wings rescued by FatAΔ, FatAΔB, FatAΔC, FatAΔD or FatAΔE have negligible wing hair PCP phenotypes, indicating virtually complete rescue (Fig. 3; supplementary material Fig. S3). FatΔF-rescued wings, by contrast, exhibit hair PCP phenotypes. As the most proximal part of the anterior wing, the costa, was affected more severely than the wing blade, we scored these regions separately. Within the costa, most FatΔF-rescued wings exhibit a strong PCP phenotype, whereas in the rest of the wing the phenotype is mild (Fig. 3C,J,K). In the abdomen, a subtle phenotype was observed for FatΔE, whereas other transgenes fully rescued abdominal hair polarity (Fig. 3D-F,L; supplementary material Fig. S3).

We also analyzed the organization of intercellular ridges within the wing, the formation of which reflects polarized cellular organization (Doyle et al., 2008). Like wing hairs, ridges are influenced by both Frizzled and Fat-PCP pathways; however, ridge orientation is regulated separately from hair polarity (Hogan et al., 2011). In wild type, wing ridges run along the proximal-distal axis in the posterior wing, but along the anterior-posterior axis in the anterior wing (Fig. 3G; supplementary material Fig. S4) (Doyle et
al., 2008). When Fat signaling is disrupted by viable mutations, or RNAi of ds or fat, or in Wts-rescued fat mutants, then ridge orientation in the posterior wing is altered such that they now run in an anterior-posterior direction (Hogan et al., 2011) (supplementary material Fig. S4). Expression of fat genomic constructs with ΔA, ΔB, ΔC or ΔE mutations rescued posterior ridge orientation back to its wild-type, proximal-distal orientation (supplementary material Fig. S4), whereas neither FatΔD nor FatΔF was able to rescue ridge orientation (Fig. 3H,I).

The observation that some altered Fat transgen proteins are preferentially deficient in a subset of Fat signaling activities while retaining relatively normal activity in others implies that Fat signals through distinct downstream pathways that diverge at the level of Fat itself. As a genetic test of this proposal, we performed complementation tests. The results of these experiments were consistent with the hypothesis of separable Fat activities. For example, FatΔF largely rescued the overgrowth phenotype of FatΔD (adult wings were 7% larger than wings rescued by wild-type transgenes; Fig. 2I,V), and FatΔD provided substantial rescue of the PCP phenotypes of FatΔC (cross-vein spacing was 62% of wild type, and hair polarity was rescued; Fig. 2I,W; Fig. 3J-L; supplementary material Fig. S3). Conversely, mutations that impair the same Fat activity are not expected to complement. Thus, Fat:Fat4 failed to rescue the overgrowth of FatΔD-expressing wings (33% larger than wild type; Fig. 2I,V), but did partially rescue the cross-vein spacing defect (66% of wild type; Fig. 2K,W) and hair polarity phenotypes (Fig. 3; supplementary material Fig. S3) of FatΔF.

Since FatΔEGF was unable to rescue the lethality of fat mutations, we overexpressed Wts to assess PCP phenotypes. However, no PCP activity of FatΔEGF was detected, as by all of the criteria described above Wts-rescued fat mutants expressing ΔEGF were indistinguishable from Wts-rescued fat mutants without any Fat transgene (Fig. 1D,K-N; supplementary material Fig. S2G, Fig. S3A,K, Fig. S4G).

It has been reported that some Fat-PCP activity could be provided by overexpression of a Fat construct lacking the ICD in fat mutants, but not in ds fat double mutants (Casal et al., 2006; Matakatsu and Blair, 2012), presumably owing to an ability of the ECD of Fat to interact with Ds, and an ability of Ds to influence PCP independently of the Fat ICD. To evaluate whether the Fat ECD could provide Fat activity under endogenous expression conditions, we created and characterized a Fat isoform missing the ECD could provide Fat activity under endogenous expression conditions, we created and characterized a Fat isoform missing the ECD largely rescued the overgrowth phenotype of FatΔD-expressing animals (7% larger than wings rescued by wild-type transgenes; Fig. 2I,K-N; supplementary material Fig. S2G, Fig. S3A,K, Fig. S4G). FatΔICD largely rescued the lethality of fat mutants, and these animals had overgrown imaginal discs, consistent with the expected lack of Fat-Hippo pathway activity. However, when this lethality was rescued by Wts overexpression, partial PCP activity was detected. Cross-vein spacing can only be roughly estimated when Fat-Hippo activity is severely compromised because the cross-veins are often incomplete. Nonetheless, FatΔICD appeared to increase cross-vein spacing (Fig. 1N; supplementary material Fig. S2B). FatICD partially rescued abdominal and wing hair polarity (Fig. 1K,L; supplementary material Fig. S3L), providing more Fat-PCP activity than FatΔEGF but less than Fat:Fat4 or FatΔF.

**The influence of deletion mutations on Fat protein levels, localization and phosphorylation**

To investigate the molecular basis for effects on Fat activity, we examined the localization, levels and potential modifications of the Fat proteins encoded by these transgenes. FatΔEGF, which had no detectable Fat activity in any assay, was expressed, but immunostaining revealed that it was mislocalized, as it was detected within the cytoplasm and failed to accumulate at the normal subapical membrane location of Fat proteins (Fig. 4B,E).
Thus, the complete lack of Fat activity associated with this transgene is likely to reflect misfolding and mislocalization. By contrast, all of the Fat transgenes that provided some Fat activity appeared to be expressed at normal levels and localized normally to the subapical membrane (Fig. 4; data not shown).

However, western blotting revealed an intriguing effect of the ∆D mutation on Fat protein mobility. In wild type, a fraction of Fat is phosphorylated on its ICD. This phosphorylation is visible as a mobility shift that results in the 95 kDa cleavage product appearing as a smeared doublet, with the reduced mobility of the upper band dependent upon phosphorylation (Feng and Irvine, 2009; Sopko et al., 2009). A similar smeared doublet was observed for all of the Fat deletion constructs that provide significant Fat-Hippo activity (∆A, ∆B, ∆C, ∆E and ∆F) (Fig. 4M,N). For ∆D, by contrast, the relative fraction of the 95 kDa product that appeared in the upper band was reduced and the fraction in the lower band was increased, mimicking the influence of dco3 or ds mutations on Fat mobility (Feng and Irvine, 2009; Sopko et al., 2009) (Fig. 4M,N).

Identification of Dco phosphorylation sites

Independently of the analysis of conserved sequence motifs, the correlation between Fat phosphorylation and Fat-Hippo signaling led us to pursue the identification of Dco phosphorylation sites on Fat. As a similar Dco-dependent mobility shift of Fat is detected both in vivo and in cultured cells (Feng and Irvine, 2009; Sopko et al., 2009), we first used a cultured cell assay. The mobility of Fat constructs containing deletions of parts of the ICD was examined by western blotting lysates from cells co-expressing Dco or a mutant isoform that fails to phosphorylate Fat (Dco3). A Fat polypeptide containing amino acids 172-415 of the ICD (ft-STI-11:FVH) was shifted by co-expression with Dco, whereas constructs encompassing further deletions of this region were not affected (Fig. 5A).

Since this suggested that either recognition by Dco, or the ability to detect a mobility shift, was lost when this region was further truncated, we turned to site-specific mutagenesis. Among a series of 20 constructs (P1 to P20) in which clusters of potential phosphorylation sites within this region were mutated (supplementary material Fig. S5), the mobility shift of 18 constructs appeared normal, whereas that of P14 and P15 was impaired (Fig. 5B; supplementary material Fig. S6). The ten Ser residues affected in P14 or P15 were then changed to Ala, alone and in combinations within 13 additional constructs (P21 to P33). This identified three Ser residues as contributing to the Dco-dependent mobility shift (Fig. 5B; supplementary material Fig. S6). When any of these Ser residues was individually mutated to Ala (P25, P26, P27), the mobility shift was reduced, and when all three were changed to Ala (P32) the mobility shift was eliminated (Fig. 5B). The introduction of a phosphomimetic residue, aspartic acid, into these sites (P15D, P32D) was sufficient to introduce a modest, Dco-independent, mobility shift (supplementary material Fig. S6B).

However, when the P15, P15D, P32 or P32D amino acid substitutions were engineered into fat genomic clones, the resulting transgenic flies all fully rescued fat mutants (Fig. 2Q,V; data not shown). Thus, these phosphorylation sites are not essential for Fat activity, even though the P32 mutation impaired the mobility shift of Ft-95 [the predominant C-terminal polypeptide produced by Fat processing (Feng and Irvine, 2009)] in vivo (Fig. 4N). To reconcile this with the evidence that Dco-dependent phosphorylation is linked to Fat activity, we hypothesized that there are multiple Dco sites on Fat, some of which visibly influence its mobility but are
Development 140 (4)

not required for signal transduction, whereas others are required for signal transduction but do not affect mobility.

Thus, we made additional fat genomic constructs in which clusters of Ser and Thr residues were changed to Ala by mutagenesis and recombining. Three constructs, each containing six to ten point mutations, were successfully created and transformed: Fat-mI, Fat-mIV and Fat-mV (supplementary material Figs S1, S5). Fat-mIV has virtually normal Fat activity, as rescued fat mutants did not appear significantly different from wild type (Figs 2, 3). By contrast, both Fat-mI and Fat-mV have only partial Fat activity, as rescued animals have overgrown wings (Fig. 2S, T, V). Fat-mV-rescued wings were similar in all respects to FatΔD-rescued wings (Figs 2, 3; supplementary material Figs S3, S4). Moreover, when the mobility of Fat-mV was examined, the slower migrating hyperphosphorylated form of Ft-95 was reduced compared with the faster migrating unphosphorylated form (Fig. 4N).

The region spanned by the point mutations in Fat-mV overlaps the region removed in Fat-ΔD (Fig. 2; supplementary material Fig. S1), and together they implicate this as a crucial region for the influence of Fat on Hippo signaling. They also both alter Fat mobility in a manner consistent with reduced phosphorylation of the Fat ICD (Fig. 4M, N), suggesting this as a region required for Fat phosphorylation in vivo. As this region overlaps a Dco binding site, we considered the possibility that they might reduce binding to Dco, thereby indirectly affecting other sites. However, introduction of the ΔD or mV mutations into a Fat ICD construct had no effect on its ability to bind Dco (Fig. 5C).

The increased wing size (18%) of Fat-mI-rescued animals was less than that for Fat-mV, and overall it exhibited only modest defects in PCP (Figs 2, 3; supplementary material Fig. S3). Nonetheless, the increased wing size implies that these point mutations impair Fat-Hippo signaling, and this region overlaps with a part of the Fat ICD identified by Matakatsu and Blair as important for Hippo signaling (Matakatsu and Blair, 2012). One curious feature of Fat-mI is that it actually appeared to increase Fat phosphorylation in vivo, based on the mobility of Fat on SDS-PAGE gels (Fig. 4N).

**Influence of fat mutations on Dachs localization**

To investigate whether defects in Fat activity associated with ICD mutations correlate with effects on Dachs, we made clones of cells expressing a tagged Dachs isoform (Dachs:Cit) (Ambegaonkar et al., 2012) in fat mutants expressing fat genomic constructs representative of different phenotypic classes (FatΔD, FatΔF and Fat:Fat4) and compared them with fat mutants lacking a Fat transgene or expressing a wild-type Fat transgene.

Dachs:Cit localizes to the membrane around the entire circumference of the cell in fat mutant animals, whereas expression of wild-type V5:Fat rescues normal Dachs polarization (Fig. 6A, E). In both Fat:Fat4-rescued and FatΔD-rescued animals, an intermediate Dachs membrane localization phenotype was observed. Some clones of cells appeared to have increased Dachs membrane localization, and for some clones Dachs was detectable on the membrane completely surrounding the circumference of cells, as in fat mutants (Fig. 6B-D). By contrast, in other cases a polarized Dachs localization profile was detected. In FatΔF-rescued animals, a novel Dachs localization profile was observed, in which Dachs localization was usually polarized but the direction of polarization was variable (Fig. 6C).

To quantify these effects, Dachs localization images collected from fat mutants and from fat mutants rescued by Fat+, Fat:Fat4, FatΔD or FatΔF transgenes were assigned random numbers and then scored together without knowledge of the genotypes. In this blind scoring, Dachs:Cit clones were first categorized as either non-polarized (Dachs localizes to the membrane around the circumference of the clone), multi-directional (Dachs localizes to the membrane around only part of a clone, but without a consistent direction of polarization), or unidirectional (Dachs is polarized in one direction). Then, among the unidirectional clones, the direction in which Dachs was polarized was scored. To simply this analysis, only clones in the medial two-thirds of the wing pouch were scored because in this region the distal polarization of Dachs points towards the dorsal-ventral compartment boundary, which can be identified by expression of Wg. In animals with Fat transgenes, 94% of clones were scored as unidirectional, and the vast majority of these were scored as having distally localized Dachs (Fig. 6F, G). Conversely, in fat mutant animals, only 13% of clones were scored as unidirectional, and of these few unidirectional clones only half were scored as having distally localized Dachs (Fig. 6F, K). Fat:Fat4-rescued and FatΔD-rescued animals were both intermediate in terms of the fraction of clones scored as non-polarized or unidirectional (Fig. 6F, H, J). FatΔF-rescued animals had a smaller fraction of non-polarized clones than Fat:Fat4 or FatΔD, and the largest fraction of multi-directional of all the genotypes (Fig. 6F). Moreover, among the unidirectional clones, the direction of polarization was partially randomized (Fig. 6I). These observations identify a correlation between the influence of Fat ICD mutations on Hippo or PCP signaling and their influence on Dachs localization.

Recent studies have revealed that Fat and Ds are themselves partially polarized in wing cells (Ambegaonkar et al., 2012; Brittle et al., 2012). To investigate whether the partial randomization of polarity in FatΔF occurs at the level of Fat localization, we took advantage of the observation that the polarized localization of Fat results in an anisotropy of Fat staining along proximal-distal interfaces as compared with anterior-posterior interfaces (Brittle et al., 2012). No difference in this anisotropy of localization was detected for a wild-type Fat construct versus FatΔF (Fig. 6L), which suggests that this mutation affects downstream signal transduction rather than Fat localization.

**Influence of directed Dachs membrane localization on Hippo and PCP signaling**

To confirm the importance of Dachs membrane localization and to distinguish it from other potential influences of Fat, we sought to localize Dachs to the membrane independently of fat mutation. One approach for membrane targeting is to attach a peptide sequence for lipidation. However, when a myristylation signal was attached to Dachs (Myr:Dachs), rather than activating Dachs it appeared to create a dominant-negative protein, as expression of Myr:Dachs pheno-copied dachs mutations (Fig. 7E, P). Dachs normally exhibits very discrete localization at the subapical membrane, near E-cadherin, whereas Myr:Dachs is broadly localized on membranes throughout the cell (Fig. 7G, H). Thus, we sought an alternative approach that would target Dachs to the correct location.

Studies of Zyx have identified it as a component of the Fat-Hippo pathway and suggested a model in which Dachs acts at the membrane in association with Zyx (Rauskolb et al., 2011). Thus, we constructed a Zyx:Dachs fusion protein, expressed under UAS control. This fusion protein exhibited a Zyx-like
localization profile, as it localized to the subapical membrane around the entire circumference of the cell, rather than exhibiting the polarized localization characteristic of Dachs (Fig. 7J). When expressed in the developing wing under nub-Gal4 control, it resulted in a strong wing overgrowth phenotype (Fig. 7F), and, like reductions of fat, it decreased Wts levels (Fig. 7K). These overgrown wings did not flatten properly, and hence it was difficult to compare their size with wings co-expressing wild-
type forms of Zyx and Dachs, which also overgrow (Fig. 7D), but Zyx:Dachs-expressing wings nonetheless appeared to be slightly larger. A stronger activation of Yki was also evident when comparing wing discs expressing the Zyx:Dachs fusion protein with wing discs co-expressing Zyx and Dachs – the discs became more highly folded, which can be a consequence of overgrowth, and a Yki target gene, ex-lacZ, was highly expressed (Fig. 7Q). The consequences of fusing Zyx and Dachs was even more dramatic when PCP was examined, as co-overexpression of Zyx and Dachs does not have significant effects on hair polarity (Fig. 7S), whereas expression of Zyx:Dachs resulted in a strong disturbance of wing hair polarity (Fig. 7T). Thus, targeting Dachs to the membrane by fusing it with Zyx phenocopies both the Hippo and PCP phenotypes of fat mutants.

DISCUSSION
Distinct regions of Fat required for Hippo and PCP signaling
Our results indicate that the effects of Fat on wing growth versus PCP can be separated at the level of Fat itself: a four amino acid deletion at the C-terminus of Fat (FatΔF) impairs PCP but does not affect wing growth, whereas deletion or point mutations within the D motif (FatΔD, Fat-mV) result in wing overgrowth but have weaker effects than FatΔF on PCP. Matakatsu and Blair (Matakatsu and Blair, 2012) also recently reported that they could separate regions of Fat required for Hippo and PCP activities, but identified completely different regions.

Matakatsu and Blair used UAS-driven expression, whereas we used genomic constructs. Because these large constructs are more difficult to manipulate, we did not undertake a detailed analysis of the entire ICD, but focused on candidate regions. However, our approach had the advantage that expression under endogenous conditions could identify activities that are missed when proteins are overexpressed. Thus, we observed wing overgrowth when region D was mutated, but this region was not identified by Matakatsu and Blair. Nonetheless, the ΔD and mV mutations only partially impair Fat-Hippo activity, as they rescue the lethality of fat mutants, and additional Hippo activity is presumably provided by regions identified by Matakatsu and Blair (Matakatsu and Blair, 2012), which are not conserved in Fat4. Hence, our combined studies imply that multiple regions of the Fat ICD contribute to Fat-Hippo signaling.

PCP was first recognized for its effects on the orientation of hairs on the body of the fly, but is now understood to encompass a wider range of cellular polarization. Matakatsu and Blair only examined hair polarity in their assessments of PCP, whereas we also considered Dachs polarization, ridge orientation and cross-vein spacing (because cross-vein spacing is also reduced by Fat overexpression, it could not be assessed by their approach). Outside of the costa, deletion of the F region had only minor effects on hair polarity. Instead, the PCP phenotypes of FatΔF were most noticeable when cross-vein spacing, Dachs localization or ridge orientation was examined. Thus, the assignment of PCP activity to distinct regions of the Fat ICD by our studies does not represent a disagreement, but rather emphasizes that there are different types of PCP that can be genetically separated.

The ability to analyze PCP phenotypes for Fat constructs that do not rescue lethality depends upon Wts overexpression. The wing hair PCP phenotype of these animals is restricted to the proximal wing. Moreover, they have strong disruptions of abdominal PCP, but typically only part of each abdominal segment is affected. Indeed, the hair phenotype of Wts-rescued fat mutants appears to be similar to that described for fat mutants rescued by γFatICD, a construct that contains just the Fat ICD, which has been interpreted as a partial rescue of hair polarity (Matakatsu and Blair, 2012). We suggest, therefore, that some of the hair PCP phenotype ascribed to fat could reflect effects on transcription of downstream target genes, mediated via downregulation of Wts. Our results also support the conclusion that some of the influence of Fat on PCP reflects an activity of the ECD as a ligand for Ds, indicating that Ds-Fat-PCP signaling is bidirectional, with Fat and Ds acting as both receptor and ligand for each other. Previous experiments showed that, when overexpressed, the Fat ECD could influence PCP in a Ds-dependent fashion (Casal et al., 2006; Matakatsu and Blair, 2012); we have now confirmed that FatICD partially rescues PCP phenotypes even at endogenous expression levels.

A conserved motif required for Fat-PCP signaling
Fat4 and Dachs1 mutant mice have phenotypes that are consistent with effects on PCP (Saburi et al., 2008; Mao et al., 2011b), but the molecular mechanisms involved are unknown. The ability of Fat:Fat4 to rescue fat PCP phenotypes indicates that there are conserved mechanisms of Fat-PCP signaling involving the Fat ICD. Among the conserved sequence motifs, the four amino acid F motif is clearly required for PCP, which implicates it as being involved in a conserved PCP mechanism. These four amino acids resemble a PDZ domain-binding motif, suggesting that it might interact with a PDZ domain-containing protein.

A striking feature of FatΔF-rescued fat mutants is the partial randomization of Dachs polarization. The observation that Dachs can be polarized, but in a variable direction, suggests that there are multiple steps involved in establishing Dachs polarization, i.e. control over whether Dachs localization is polarized can be mechanistically uncoupled from control over the direction in which Dachs is polarized.

Role of Dachs localization in Fat signaling
The observation of both randomized Dachs localization and rounder wings with more closely spaced cross-veins in FatΔF-rescued animals extends the correlation between polarized Dachs localization and PCP phenotypes, and is consistent with the hypothesis that Dachs polarization directs polarized cell behaviors. We also found a rough correlation between the decreased Fat-Hippo pathway activity of Fat:Fat4 or FatΔD and increased detection of Dachs at the subapical membrane. These results are at least generally consistent with the hypothesis that the direction in which Dachs localization is polarized influences PCP, whereas the amount of Dachs on the membrane influences Hippo signaling (Mao et al., 2006; Reddy and Irvine, 2008; Rogulja et al., 2008).

These and other studies have identified a correlation between Dachs localization and Fat signaling, but could not prove that altered Dachs localization is a cause rather than a consequence of Fat signal transduction, nor separate the role of Dachs localization from other potential effects of Fat. We have now directly confirmed the importance of Dachs localization by creating a Zyx:Dachs fusion protein, the expression of which in otherwise wild-type animals phenocopies fat mutants both for wing growth and PCP phenotypes.

Role of Fat phosphorylation in Fat-Hippo signaling
Despite our extensive investigation of potential phosphorylation sites, the role of Fat receptor phosphorylation in signal transduction remains elusive. Fat is directly phosphorylated by Dco, which is
Fig. 7. Influence of membrane-tethered Dachs on wing growth and PCP. (A-F) Adult wings from animals expressing nub-Gal4 and (B) UAS-Zyx, (C) UAS-dachs, (D) UAS-Zyx UAS-dachs, (E) UAS-myr:dachs or (F) UAS-Zyx:dachs. (G-J) Localization of membrane-tethered Dachs constructs, showing clones of cells expressing (G,H) Myr:Dachs:V5 (green) or (I,J) Zyx:Dachs:V5 (green) in (G,I) horizontal or (H,J) vertical section. (K) Western blot on lysates of wing discs expressing tub-Gal4 and UAS-dachs, UAS-Zyx, UAS-Zyx:dachs, or UAS-RNAi-fat, or mutant for fat, as indicated. Loss of Fat activity reduces Wts protein levels (Cho et al., 2006). GAPDH serves as a loading control. Mean Wts levels from four independent experiments, normalized to Wts levels in fat mutants, were: UAS-dachs 3.3, UAS-Zyx 3.6, UAS-Zyx:dachs 3.3, UAS-RNAi-fat 1.8, wild type 5.0, and fat mutant 1.0. (L-Q′) Wing discs from animals expressing (L) en-Gal4 (marked by UAS-GFP, green) and (M) UAS-Zyx, (N) UAS-dachs, (O) UAS-Zyx UAS-dachs, (P) UAS-myr:dachs or (Q) UAS-Zyx:dachs, stained for ex-lacZ (red). (L′-Q′) ex-lacZ channel only. (R-T) Anterior proximal wing from animals expressing (R) nub-Gal4 and (S) UAS-Zyx UAS-dachs and (T) UAS-Zyx:dachs.
required for Fat-Hippo signaling, and acts genetically upstream of dachs and Zyx (Cho et al., 2006; Feng and Irvine, 2009; Sopko et al., 2009; Rauskolb et al., 2011). Moreover, Fat ICD phosphorylation correlates with Fat activity, as it is reduced in dco3 or ds mutants, or when there are mutations in Fat that impair Fat-Hippo signaling (FatΔD, Fat-mV). However, other Fat ICD mutations impair Fat phosphorylation without affecting Fat signaling (Fat-P32, Fat-P15). Moreover, dco3 does not affect Dachs localization, but FatΔD, which impairs Fat-Hippo signaling and Fat phosphorylation, does affect Dachs localization. To reconcile these observations, we propose that Dco normally blocks the ability of Zyx and Dachs to inactivate Wts through a mechanism that is independent of the influence of Fat on Dachs localization. Fat might be the key substrate of Dco in this process, but our results are equally consistent with the possibility that phosphorylation of the Fat ICD by Dco is a consequence, not a cause, of Fat receptor activation. Thus, although it can serve as a marker of Fat activity, the biologically important substrate of Dco might be some other protein.

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