The fat-like Gene of Drosophila Is the True Orthologue of Vertebrate Fat Cadherins and Is Involved in the Formation of Tubular Organs*

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Casimiro Castillejo-López‡§, Wilma Martinez Arias‡, and Stefan Baumgartner¶
From the Department of Cell and Molecular Biology, Lund University, BMC B13, S-22184 Lund, Sweden

Fat cadherins constitute a subclass of the large cadherin family characterized by the presence of 34 cadherin motifs. To date, three mammalian Fat cadherins have been described; however, only limited information is known about the function of these molecules. In this paper, we describe the second fat cadherin in Drosophila, fat-like (fl). We show that fl is the true orthologue of vertebrate fat-like genes, whereas the previously characterized tumor suppressor cadherin, fat, is more distantly related as compared with fl. Ftl is a large molecule of 4705 amino acids. It is expressed apically in luminal tissues such as trachea, salivary glands, proventriculus, and hindgut. Silencing of fl results in the collapse of tracheal epithelia giving rise to breaks, deletions, and sac-like structures. Other tubular organs such as proventriculus, salivary glands, and hindgut are also malformed or missing. These data suggest that Ftl is required for morphogenesis and maintenance of tubular structures of ectodermal origin and underlines its similarity in function to a reported lethal mouse knock-out of fat1 where glomerular epithelial processes collapse. Based on our results, we propose a model where Ftl acts as a spacer to keep tubular epithelia apart rather than the previously described adhesive properties of the cadherin superfamily.

The cadherins constitute important cell interaction molecules. Within this large family, the protocadherins represent the major subgroup (reviewed in Refs. 1 and 2). They are characterized by the presence of more than five cadherin repeats in their extracellular domains. Recent large scale phylogenetic analyses showed that some of the cadherin molecules exist forming their own subgroups, neither matching the classical cadherins nor the protocadherins (1). One such subgroup exist forming their own subgroups, neither matching the classical cadherins nor the protocadherins (1). One such subgroup comprises the large Fat cadherins that exhibit an unusually large size from 4000 to 5000 amino acids, mainly due the large amount of cadherin repeats. The first molecule of this subgroup was identified in Drosophila as the gene product of the fat (ft) locus (3). Subsequently, proteins of the Fat subgroup were also discovered in mammals, and so far the following three have been characterized: human and mouse Fat1, respectively (4–6), human Fat2 (7, 8), and rat Fat3 (9). The Fat cadherins are characterized by the presence of 34 cadherin repeats in the extracellular domain, followed by 2–6 EGF repeats and 1 laminin G domain (Fig. 1), with the exception of Drosophila Fat which has two G domains (3). All Fat molecules are transmembrane proteins. Intracellularly, the cytoplasmic domains show reduced homology between Fat cadherins of distant organisms, and the homologies to classical cadherins are weak. However, two blocks with limited homology to classical cadherins have been identified, and binding of the intracellular tail to β-catenin has been suggested (4).

The genomes of Drosophila and Caenorhabditis elegans each harbor two fat-like cadherins (10). Close inspection, however, shows that the C. elegans genes, cdh-3 and cdh-4, are more distantly related to vertebrate fat-like cadherins than the two Drosophila genes. Although both proteins contain a laminin G domain and an EGF-like repeat on the extracellular part, the number of repeats is irregular. They lack the constant number of 34 cadherin repeats as in the case in Drosophila (10, 11), making them less suitable as candidates for true Fat cadherins.

The first functional description of a Fat cadherin came from Drosophila fat shown to be involved in tumor suppression (3). fat1 mutants exhibit hyperplastic growth in imaginal disks, differentiation and morphogenetic defects, and premature pupal death. These results were interpreted as a failure to control cell proliferation upon lack of contact-dependent cell interactions. It was later reported that fat was involved in planar polarity. Two models have been proposed to explain how fat controls planar polarity in the eye (12, 13). In the first model, fat regulates the activity of Frizzled (13), and in the second, fat inhibits equator formation by an unknown mechanism (12). Mutations in the C. elegans cdh-3 gene affect morphogenesis of the single cell, hyp 10, which is involved in tail formation. However, no other major phenotype was observed in cdh-3 mutants, and the offspring is viable (11). Mutations in cdh-4 are also viable suggesting some redundancy between the two genes. Further clues to the function of Fat cadherins came from the report on the first mouse knock out of the fat1 gene (14). Embryonic lethality in fat1− mice is likely to be caused by defects in renal glomerular slit junctions and fusion of epithelial cell processes; however, no tissue overgrowth phenotype was observed. These results were contrasted to the tumor suppressor role of Drosophila fat mutants, concluding that significant functional differences might exist between Drosophila fat and vertebrate fat1. It was not considered, however, that yet another fat-like gene in Drosophila could be more similar in function to that of murine fat1.
In this paper we show that the second fat gene in *Drosophila*, termed *fat-like* (*ftl*), is the true orthologue of all vertebrate Fat cadherins characterized to date and not *Drosophila* fat. Moreover, we show that *ftl* is involved in formation of tubular ectodermal structures, specifically affecting the formation and maintenance of epithelia in tracheae, salivary gland, hindgut, and proventriculus. In this regard, the mutant phenotype of *ftl* has more resemblance to the mutant phenotype of murine *fat1* than *fat*, supporting our claim that *ftl* is the *Drosophila* orthologue of mammalian Fat cadherins.

**EXPERIMENTAL PROCEDURES**

**Drosophila Stocks**—Inducible RNAi was achieved by P-element transformation of a transgene expressing a hairpin segment of *ftl*. Injections of the vector (UAS-ds-ftl) were performed by using w1118 as recipient stock. Twenty independent transgenic lines were established. The silencing effect of different transgenic lines was estimated by scoring the ratio (SB1/Sb) of the progeny from homozygous UAS-ds-ftl × tub-GAL4/TM3-Sb at 21 and 29 °C, respectively, expecting a reduction in viability because of *ftl* silencing. The progeny of UAS-act-GFP × tub-GAL4/TM3-Sb was used as a control cross. Lines 7749-50a and 7749-57a were chosen for knock-down analysis. Other drivers used were prd-GAL4/TM3-Sb, tub-GAL4/Sb, prd-GAL4/TM3-Sb, and act-Gal4/TM3-Sb. The last driver allowed us to unambiguously identify mutant embryos because of the lack of fluorescence. Fly stocks are described in FlyBase (flybase.bio.indiana.edu) and were obtained from the Bloomington Stock Center unless otherwise specified.

**RT-PCR**—The structure of the *fat-like* gene was determined by PCR using cDNA and genomic DNA as templates, followed by sequencing and computational analysis. The deposited genomic sequence (GenBank™ accession no. AC015229) and the predicted gene (CG7749; Ref. 19 and FlyBase) were used as a start point to verify the gene structure. Primers were designed to generate fragments suitable for direct sequencing of stretches of about 0.5 to 1 kb in length along the predicted sequence. A putative alternative splicing site at the C terminus of the sequence defines the intracellular region. The genomic organization of the gene was amplified with primers targeting exons 1 and 2 (5′–TTCTATCCGACGTAAACCAATGCCTC-3′ and 5′–ACTCCTCCAACTCAGAATCTTC-3′). The bands were cloned into pCR-2.1 (Invitrogen). Embryos of the crosses UAS-ds-ftl × da-GAL4 and UAS-act-GFP × da-GAL4 were compared. The RNA quality was assayed with primers specific to the blood cell transcript CG6639. 3′-End labeling of cDNA from embryos was performed by omitting the primary antiserum and by titration with an irrelevant antiserum at 1:20. The nucleotide sequences of the cDNA were determined with either the T7 RNA polymerase-binding site. The 3′-end fragment was amplified by using primers 5′–GGGCGACGAGTTCCGAAT-3′ and 5′–gggctaGGACGTAAACCAATGCCTC-3′, generating a 985-bp fragment. The 5′-end fragment was amplified using 5′–TTCTATCCGACGTAAACCAATGCCTC-3′ and 5′–AGGGCGACGAGTTCCGAAT-3′ and 5′–TAATACGACTCACTATAGGGAGACCACATTCCG-3′, generating a 343-bp fragment. The procedure for in situ hybridization was carried out according to Ref. 15. A sense probe was used as a negative control.

**Immunohistochemistry**—Embryos were fixed in 5% formaldehyde for detection with horseradish peroxidase or alkaline phosphatase and fixed for detection with fluorescein isothiocyanate. Primary antisera were used at the following concentrations: rabbit anti-Fit-ED at 1:20; rabbit anti-Fit-C at 1:50; the tracheal-lumen-specific mouse monoclonal antibody 2A12 at 1:5 (kindly provided by C. Samakovlis); and an antiserum against forhead raised in guinea pig at 1:2000 (kindly provided by P. Carreras). Detection was carried out by using biotinylated anti-rabbit at 1:300 and avidin-horseradish peroxidase (Vectastain Elite Kit, Vector Laboratories) and anti-mouse-AP at 1:500 (Promega) and anti-rabbit fluorescein isothiocyanate (Dako). For antibody staining, salivary glands were dissected in phosphate-buffered saline and fixed in 4% formaldehyde. After staining salivary glands were mounted in Vectashield mounting medium (Vector Laboratories). Control experiments for the specificity of the antisera were performed by omitting the primary antiserum and by titration with an excess of antigen when available.

**RNA Interference**—For dsRNA production, two cDNAs fragments from *ftl* were amplified by using specific primers, flanked at the 5′-end with the T7 RNA polymerase-binding site. The 3′-end fragment was amplified using 5′–TTCTATCCGACGTAAACCAATGCCTC-3′ and 5′–TAATACGACTCACTATAGGGAGACCACATTCCG-3′, generating a 985-bp fragment. PCR products were purified and used as template to produce dsRNA with the T7 Transcript kit (Roche Applied Science). The quality and concentration of dsRNA were checked by agarose gel electrophoresis. Pre-cellular embryos were injected as described previously (16) by using 1.5–5 μl dsRNA dissolved in injection buffer. Embryos were incubated under oil for 2 days before immunostaining. Surviving larvae were chilled at 4 °C before visualization of the trachea.

**Transgenic RNAi Interference Construct**—A pUAST-variant containing genomic and cDNA sequences in the opposite direction was constructed to produce a dsRNA against *fitl*. The genomic fragment including introns 15–17 was amplified by using primers 5′–ggggegcGATGGACATAAACAATCGCT-3′ and 5′–gggctgAGTCGAC-CCATCGGTTCCTCA-3′, and the corresponding cDNA by using primers 5′–gggctacAAAGTGCGGCAAATG-3′ and 5′–gggctgaGTGAAATACCGATCAGTCTC-3′. The bands were cloned into pCR-2.1 and sequenced. The genomic insert was released with NotI and the cDNA with KpnI/Xhol and then ligated together into pUAST NptI. The identity of the construct was confirmed by restriction analysis and sequencing. Upon injection, 20 transformed lines were obtained and isolated to homozygosity. The effect on viability upon induction of the transgene was monitored at 29 °C (18) in a cross using driver tub-GAL4/1Sb. In the progeny, the proportion between Sb1/UBAS- ds-ftl (not expressing the transgene) and tub-GAL4/UAS-ds-ftl was taken as indicator for the strength of the allele. Two independent lines (7749-50a and 7749-57a) of the genotype tub-GAL4/UAS-ds-ftl showing reduced viability were subsequently used for mutational analysis.

**RESULTS**

**Structure of the Drosophila fat-like (ftl) Gene**—In a search for large cadherin molecules in *Drosophila*, we noticed the presence of a large protein sequence containing a series of cadherin repeats within the genome, later annotated as CG7749 by the *Drosophila* Genome Consortium (19, 20). Because it contains high similarity to an already existing large transmembrane cadherin, Fat (3), we decided to call CG7749 *fat-like* (*ftl*). The *ftl* gene encodes a large transmembrane protein containing 34 cadherin ectodomains along with six EGF repeats and one laminin G domain (Fig. 1A). A predicted secretory signal is found at the N terminus, and a single transmembrane sequence defines the intracellular region. The genomic organization is shown in Fig. 1A. Similar to other cadherin-like genes, multiple ectodomains are encoded in a single large exon (7). Most interesting, 17 of the 18 introns are of similar length ranging from 55 to 65 bp. The gene produces a single transcript coding for a 4705-aminio acid protein. The annotated CG7749 protein sequence differs from our experimental results in the C-terminal part, where a short exon was found generating a
frameshift and an extension of the protein by 63 amino acids (Fig. 1, B and C). To confirm the presence of this particular exon within the fli mRNA, we conducted a series of RT-PCR experiments using RNA isolated from various stages during the Drosophila life cycle (Fig. 1D). This analysis confirmed the presence of this exon and excluded alternative splicing. Moreover, during the course of this work, a poly(A) tail-containing cDNA, GM08777, was made available to us, which also confirmed our result. Finally, an antiserum raised against the C-terminal-most part of the protein detected the same structures as the ectodomain antiserum, suggesting that the newly established exon/intron structure is indeed correct.

Protein Structure and Phylogenetic Analysis of the Ftl Protein—The Drosophila genome contains two 34-cadherin repeat genes, the tumor suppressor gene fat (ft) (3), and fli (this study). In mammals, related molecules with 34-cadherin repeats have been described (4, 6, 8, 9, 21, 22). In order to clarify their evolutionary relationship, we compared their predicted amino acid sequences. We observed, as have others (9), that the overall similarity between Ftl and mammalian Fat cadherins is considerably higher than to Fat itself. Further detailed analysis of the organization and sequence of the cadherins repeats strengthens this postulate. The alignment of the 34-cadherin repeats of Ftl is shown in Fig. 2.

We perceived also that the 6th cadherin repeat (EC6, Fig. 2) differs from the consensus cadherin repeat, and it is substantially longer. A similar feature was seen in mammalian Fat cadherins (22) as well as in the 6th cadherin repeat of protocadherins (7). The EC6 repeat contains the motif YX6E, also known to be conserved in protocadherin and Fat cadherins, but this motif is clearly missing in Fat. The signatures RGD or HGD, localized in the middle of the first repeat for protocadherins, show that the cadherin repeats of Fat-like cadherins are more similar to protocadherins repeats than the fat cadherin modules.

Moreover, Fat contains two laminin G domains, a feature never observed in any vertebrate Fat cadherin, which further indicates that Fat is a more distantly related molecule to mammalian Fat cadherins than is Ftl.

In contrast to the well conserved classical cadherins containing the catenin-binding domain reviewed in Ref. 1, the cytoplasmic part of the Fat cadherins shows very low sequence
similarities. However, the conservation is significant when orthologue genes of closely related species are compared (Fig. 2B). The predicted open reading frame contained within *Anopheles gambiae* sequence EAA03222 not only shows structural similarities to Ftl but also at least two of the predicted introns are in the same position as compared with *Drosophila* (introns 16 and 17). The cytoplasmic introns 15 and 18 are still not annotated in the *Anopheles* sequence, but in both cases the downstream amino acid region is of low complexity indicating the presence of a yet unidentified intron or acquisition of a repetitive sequence. The amino acid domains with at least 75% identity in all the *fat-like* genes are underlined in Fig. 2B. The putative PDZ domain binding sequence theonine-glutamic-valine at the C terminus (23) is present in the predicted *A. gambiae* Ftl protein, but its functional significance is questioned here because it is absent in both mammalian Fat cadherins and Ftl.

A phylogenetic tree of the amino acid sequences of fat cadherin genes is presented in Fig. 3. In order to include the available partial sequence of *Anopheles*, a comparison was performed only from the middle of the 21st cadherin repeat through the C terminus. Similar clustering was obtained by excluding the *Anopheles* sequence and using the complete polypeptides. As an important result, we can conclude that the *Drosophila ft* tumor suppressor gene is more distantly related to mammalian Fat cadherins than *ftl*. As such, Fat may represent an evolutionary divergent molecule and probably is functionally different among the Fat cadherins so far described in eukaryotes.

**Expression Analysis**—By using digoxigenin-labeled RNA
probes, the spatial expression of the ftl gene was examined. ftl transcripts were first detected at stage 11 as a weak band at the location of the salivary gland placode (Fig. 4A). Shortly after this stage, transcription at this site becomes stronger, and a new pattern in the tracheal dorsal trunk becomes obvious (Fig. 4, B and C). The evolution of this pattern is dynamic, being first detected at the posterior end as a continuous expression in the dorsal trunk cells, whereas in more anterior segments, a patchy expression in individual dorsal trunk cells is observed (Fig. 4, E, J, and K). This expression pattern fits the observation that tracheal morphogenesis occurs in posterior tracheal structures ahead of more anterior ones (24). Most interestingly, the ventral side of the dorsal trunk shows stronger levels of transcription (Fig. 4, J and M, arrowheads). At stage 14, after fusion of all dorsal trunk cells, strong transcription is observed in the lateral trunk proceeding in a dorsal to ventral direction (Fig. 4, G and M). At stages 15 and 16, transcription of ftl is strong in the lateral trunks (Fig. 4, H, I, and N). Before hatching, at stage 17, the ganglionic branches are labeled (Fig. 4O). No transcripts were detected in the imaginal discs (data not shown).

Protein Location during Embryogenesis—In general, large proteins and in particular cadherins undergo proteolytic cleavage in vivo (25). In order to address the question of a possible cleavage of Ftl, we raised two polyclonal antisera, one against the N-terminal (anti-Ftl-ED) and the other against the C-terminal part of the predicted polypeptide (anti-Ftl-CD). Both antisera revealed identical staining patterns, confirming the location of the transcripts in the trachea and salivary glands (Fig. 4). The protein was detected first at stage 14 during tracheal lumen formation (Fig. 5A). However, the earlier patchy appearance seen with the transcript was not observed (Fig. 4, E, J, and K). The relatively large delay between the detection of the transcript and the protein might be explained in part by the enormous length of the protein and the time required for its transport to the apical membrane. Later in

Fig. 4. Expression of the ftl gene. Embryos are oriented with anterior to the left and dorsal side up unless otherwise noted. A, stage 11 embryo (stages are those of Ref. 44). The first staining is observed in the salivary gland placodes. B, stage 12 embryo. Staining in the salivary glands becomes more prominent, and at the same time, the first signs of tracheal expression become apparent, starting from posterior to anterior on the dorsal trunk. C, late stage 12 embryo. the dorsal trunk expression expands toward the anterior. D, late stage 12 embryo. In the horizontal section expression is strong in the salivary glands. E, stage 13 embryo. Anteriorly, the expression is still in patches, and posteriorly, the dorsal trunk cells show merged expression. F, stage 14 embryo. The dorsal trunk cells show merged expression, and the lateral trunk cells start to show ftl expression. G, stage 15 embryo. All lateral trunk cells shows staining. H and I, stage 16 embryo. For the lateral and dorsal view, respectively, staining is observed in most branches. J, high magnification of a stage 12 embryo, similar to B. Anteriorly, staining is patchy (arrows), and posteriorly, staining is observed continuously. Note that the staining is stronger on the ventral side of the forming tube (arrowhead). K, high magnification of a late stage 12 embryo. The focus on middle part of the embryo (embryo similar to C), the patchy staining becomes stronger. L, high magnification of stage 14 embryo, similar to F. The patchy staining shows almost complete fusion. M, high magnification of a stage 15 embryo, similar to embryo in G. The lateral trunk cells show staining. Note that staining is still stronger on the ventral side of the tube (arrowhead). N, high magnification of a stage 16 embryo, similar to H and I. O, high magnification of a stage 17 embryo. Most branches show ftl staining, including ganglionic branches (gb). Bars in I and O correspond to 100 and 20 μm, respectively.
development, the protein was localized in all tracheal branches, including dorsal tracheae (Fig. 5C) and ventral ganglionic tracheae (Fig. 5C). Both antisera also detected the protein in two other organs of ectodermal origin, the hindgut and the proventriculus (Fig. 5D and E). The staining was more intensive at the edges of both these structures, in between where the midgut will form. In salivary glands dissected from 3rd instar larvae, Ftl showed punctuated staining at the plasma membrane, a characteristic of apically located proteins (Fig. 5F).

Although at the tissue level both sera show colocalization, there are clear differences at the subcellular level as follows: Ftl-ED against the ectodomain binds intensively to a structure within the tracheal lumen (Fig. 5G), whereas the Ftl-CD antiserum produces a signal at the apical membrane (Fig. 5H). The tracheal lumen staining of Ftl-ED is interpreted as staining of the luminal ECM. To our knowledge this is the first experimental demonstration of the topological orientation of such a molecule.

Silencing of Fat-like Transcript Impairs Tubular Tissue Formation—In an attempt to analyze the function of ftl during Drosophila embryogenesis, we decided to use the double-strand RNA interference technique (16). To this end, both N- and C-terminal RNAi probes (Fig. 1A) were synthesized and injected into preblastoderm staged embryos, and the development of the embryos was monitored. Injected embryos did not show any dramatic effect on the shape of the larva nor was viability significantly affected. However, for a consistent percentage of the embryos, the tracheal system was affected (Fig. 6). Specifically, silencing of ftl created deletions in the dorsal trunk of the embryo (Fig. 6A) or of the larva (Fig. 6B). In some cases, the formation of doughnut-like structures was observed in larvae (Fig. 6, C and D, white arrowheads) or in embryonic tracheae after staining with the luminal antibody 2A12 (Fig. 6E).

In order to confirm our RNAi injection results and to avoid such variation concomitant with single injections, we decided to construct transgenic lines employing the GAL4/UAS-inducible RNA interference system as described (17). The effect upon viability after induction of the transgene was monitored at 29 °C (18) in a cross using driver tub-GAL4/Sb. In the progeny, the proportion between ds-ftl/Sb (not expressing the transgene) and ds-ftl/tub-GAL4 was taken as indicator for the

**Fig. 5. The expression pattern of the Ftl protein.** Embryos are oriented with anterior to the left and dorsal side up unless otherwise noted. Staining patterns from A–F were done using the Ftl-CD antiserum. A, stage 14 embryo. Stages are those of Ref. 44; the staining pattern follows the one observed in the in situ hybridization analysis (Fig. 4), and the protein is detected continuously on the dorsal trunk and some lateral trunk cells. B, stage 17 embryo, slightly dorsal view. Staining is on most branches, also on the dorsal branch (arrow). C, stage 17 embryo, lateral view. Staining is also seen on the ganglionic branches (arrow). D and E, stage 16 embryo. The focus is on interior organs; Ftl is also observed in the esophagus and proventriculus (arrowhead) and in the hindgut (arrow). F, salivary gland from a 3rd instar larva. Staining is on the surface of salivary gland cells. G, high magnification of the dorsal trunk of a stage 17 embryo using the ectodomain antiserum (Ftl-ED). Staining is observed in the lumen. H, stage 17 embryo, similar to G, staining using the cytoplasmic antiserum (Ftl-CD). Staining is on the apical side of the lumen. Bars in F and H correspond to 100 and 5 μm, respectively.
At 21 °C, any of the lines showed reduced viability. Temperature-dependent transgenic RNAi has been shown to be a common feature in Drosophila (18). Two independent lines, showing bias against the genotype tub-Gal4/pUAST-inverted repeat (12 and 19% emergence, versus 52% of the control tub-Gal4/UAS-GFP), were subsequently used for mutational analysis. To determine the efficiency of the system and to quantify the reduction in expression strength of the allele. Fig. 6. Silencing of flt by RNA interference causes defects in the tracheal system and tubular structures of ectodermal origin. Injection of dsRNA against flt (A–E) produced deletions in the dorsal trunk of the embryo (A, arrow) and in larvae (B, arrow). Formation of doughnut-like structures (white or black arrowheads) were observed in larvae (C and D) or in embryonic trachea after staining with the luminal antibody 2A12 (E). Inducible expression of dsRNA against flt (G–M and P). The driver line prd-GAL4 was used for partially silencing of flt (G and H and J and K), and tracheae were visualized with anti-Flt antisera (P–H) or luminal monoclonal antibody 2A12 (I–M). The ubiquitous driver tub-GAL4 generated more severe phenotypes (L and M). Wild-type embryos (F and I) show normal tracheal branches, whereas a variety of defects are observed in the silenced embryos; deletions in the dorsal trunk (G and H and J–L), formation of doughnut-like structures (G, arrowhead and insert), or complete lack of tracheae (M). N, model to explain the formation of doughnut-like structures. I, due to cell-autonomous silencing, flt is knocked down only in a subset of tracheal cell (indicated by gray color); the trachea collapses at these sites (II) and eventually fuse and degenerate (III). The remaining intact cells form two unicellular tubes (IV). I, silencing in all cells leads to complete collapse, followed by disintegration of tracheae. O and P, immunostaining of stage 13 embryos with an anti-forkhead antiserum. In wild-type embryo (O), the serum recognized the proventriculus (pv), salivary glands (sg), hindgut (hg), Malpighian tubes, and dispersed hemocytes. P, in flt knock-down embryos, the salivary glands are missing, and the proventriculus and hindgut are highly disorganized. Q, analysis of the degree of silencing of flt using the da-GAL4 driver line by semi-quantitative RT-PCR. RT-PCR was performed on RNA isolated from embryos from the cross da-GAL4 × UAS-ds-flt and analyzed after 24, 26, 28, and 33 cycles (left lanes) and compared with wild-type embryos (right lanes). Only at higher cycle numbers, a band representing an at least 20-fold decrease in intensity was detected suggesting that silencing was notable but not complete.
of ftl, we collected RNA from embryos of a da-GAL4/UAS-ds-ftl cross and analyzed the transcriptional activity using RT-PCR. As is evident in Fig. 6Q, there is considerable reduction of the RNA in the mutant; however, the reduction is not complete. Another more ubiquitous and putatively stronger driver line, tubulin-GAL4 (26), generated more severe phenotypes (Fig. 6, L–N). In 20% of these knock-down embryos, the formation of most tracheal structures was abolished and most tracheae appeared collapsed (Fig. 6L), suggesting that fat-like is important for the structure and maintenance of the trachea. In extreme cases, the trachea formation was completely inhibited (Fig. 6M). In the remaining embryos, tracheae did develop, but single branches or fusion cells were missing, and numerous breaks were found (data not shown). The phenotype was attenuated when the silencing was induced with the pair-rule driver prd. Staining with the ftl antibody showed that the dorsal trunk was disrupted in cells devoid of Ftl (Fig. 6, F–H). By using the luminal antibody 2A12 truncations and/or defects on invagination also became evident (Fig. 6, I–K). In rare occasions, hole formation was observed (Fig. 6G, insert). We interpreted these formations as cell-autonomous silencing in the two opposite cells out of the four cells that can make up the trunk. Based on these results, we propose a model that implies a collapse of the tube and degeneration through contact of the silencing cells. The remaining cells eventually form independent single cell tubes (Fig. 6N, I–IV). Following the model, complete ablation is observed when all four cells are knocked down (Fig. 6N, I').

We next examined whether silencing of Ftl affects other embryonic structures where the protein is expressed. By using the tubulin GAL4 driver and antibodies against the transcription factor forkhead (fkh) (27), we observed a variety of phenotypes showing alterations in the proventriculus, hindgut, and salivary glands. In most cases these organs were disorganized or absent (Fig. 6, O and P).

**DISCUSSION**

We have characterized the second Fat cadherin gene in Drosophila, fat-like. Analysis of the structure and amino acid composition outlines a subfamily of 34-cadherin repeat genes characterized by the presence of two conserved cysteines at the beginning of repeat 6 and at the end of repeat 28. During embryogenesis, the transcript is expressed in salivary glands and tracheal cells, and the protein is localized on the apical side of the epithelia. Silencing by RNAi reveals abnormalities in the development of tubular structures. Knocked down embryos, using ubiquitous driver lines, show a wide range of morphological defects ranging from small deletions of the trachea (Fig. 6, J and K) to complete lack of tubular structures (Fig. 6, M and P). In principle, this variation could be explained by incomplete penetration of the silencing effect and/or variation in the maternal contribution. An alternative explanation might reside in the function of the gene that could be compensated by other molecules. We favor the latter alternative, first because classical mutants affecting tube formation also show variable phenotypes, thereby reflecting the view that different processes and various environments interplay with each other in tube development (28). Second, in the mftl1 knockout mouse, only some mutants showed distinctive morphological defects as holo-prosencephaly (14). In particular, the variation in the eye phenotype reported in the same study was remarkable, often showing severe eye phenotypes on one side and normal eye development on the other side.

**Fat-like as the Prototype of Vertebrate Fat Cadherins—**Our comparative analysis clearly shows that Ftl is more similar to vertebrate fat cadherins than Fat itself. This is best documented in the dendrogram where Drosophila Fat-like clusters, together with Anopheles Fat-like, are in a group closer to vertebrates Fat cadherins and distinct from Fat (Fig. 3). The C. elegans fat-like-related molecules, Cdh-3 and Cdh-4, are poorly clustered suggesting that the onset of development of modern Fat cadherins may have occurred about 430 million years ago when insects emerged (29).

The conserved feature of a 34-cadherin repeat array associated with EGF and laminin G domains in the membrane-proximal region make fat and fat-like cadherins unique. Cadherins of similar size exist in Drosophila, but they lack EGF and laminin G domains such as the Dachsous protein (30), or the number of cadherin domains is lower as was shown for DN-cadherin which harbors only 18 cadherin repeats (31). The expression of ftl in tubular tissues resembles the expression of vertebrate Fat cadherins in cells adjacent to ventricular zones. Mouse fat1 is expressed, among other sites, in the respiratory epithelium, kidney glomeruli, and in cells next to the ventricles of the central nervous system (4, 6, 22, 32). Rat fat2 shows a more restricted expression in the postnatal cerebellum (8), and fat3 also shows expression in fetal central nervous system, in particular to the spinal cord, suggesting that this cadherin modulates the extracellular space of the axons (9). These neural expression patterns are clearly not observed in Drosophila ftl or fat.

**The Function of Fat-like—**In light of the tumor suppressor role for Fat, it was surprising to note that the structurally similar Ftl was found in tissues that give rise to tubular structures where morphogenesis is mainly due to changes in cellular shape rather than to cellular proliferation. In our functional analyses with ftl, we did not observe any tumor formation. The previously proposed tumor suppressor role of vertebrate Fat cadherins was only based on sequence similarity with Fat. Just recently, this issue has been addressed by functional studies to show that mice lacking mFAT1 did not reveal any changes in cellular overgrowth (14).

In order to investigate whether fat-like has a function in imaginal disc patterning or cell polarity similar to fat, several larval GAL4 driver lines were tested. By crossing these lines to the UAS-ds-ftl silencing lines, we were unable so far to detect any phenotype in any larval or adult tissue (data not shown). Therefore, it seems unlikely that ftl plays a function during growth of imaginal disks or during the establishment of planar polarity. It should also be noted that although no ftl mRNA was detected in disks, ftl expression may have been low and may have escaped detection.

The adhesion role of classical cadherins is well established (32). Overexpression in cell cultures of the full-length rat MEGF1/fat2 indicated a homophilic binding activity (8). However, overproduction of protein made it difficult to estimate the relevance of the binding strength. In homophilic interactions, the presence of a cleavable pro-sequence might be crucial for transport of the protein to the surface (25). It has been suggested that the absence of this pro-sequence in Fat cadherins makes strong homophilic adhesions unlikely (6). In any case, they would extend a very large distance from the plasma membrane and presumably be incompatible with strong intercellular adhesion. An estimation of the length of the Ftl ectodomain, based on crystallization data from five consecutive cadherin repeats (34), shows that the tip of Ftl could well extend 160 nm away from the cell membrane. The same report also showed that the array of cadherin repeats is quite linear and rigid which would reduce the calculated length of the Ftl ectodomain only very little. Although Ftl is in fact a huge molecule, the tips would hardly reach the other side of the trachea lumen taking into account that an average lumen measures about 2000 nm in diameter. These topological restrictions make homophilic interactions through the lumen unlikely, at least when the tra-
Drosophila Fat-like Cadherin Required for Tubular Epithelia

The basic organization of trachea consists of a simple monolayer epithelium with an apical cuticle and a lamina on the basal side (24). The cuticle contains regular folds known as taenidia. They are arranged in a helical pattern and are thought to maintain a certain flexibility within tracheal epithelia and at the same time keep the lumen open (35). A number of genes are responsible for integrity and maintenance of this epithelium such as crumbs, a large EGF protein (36), shotgun, a DE-cadherin (37), and the transcription factor hind-sight (38). hind-sight has been shown to be involved mostly in cell death of the amnioserosa and tracheal epithelia formation (38). The hind-sight mutants show irregular taenidia folds leading to collapse of the trachea. The trachea displays sac-like structures, a feature also observed in ftl silencing (Fig. 6G). This suggests that hind-sight might be involved in the same signaling pathway as ftl.

According to our expression studies it is obvious that Ftl is not a molecule involved in the primary establishment of tracheal structures, rather it appears active at a time point when the tracheal anlagen is already established. The RNA appears specifically polarized within cells at the side where new branches are to be established (Fig. 4, J–M). It is tempting to speculate that the luminal content of the trunk is somehow projected toward the secondary branches, and this process might be mediated by ftl.

Recently, two interesting molecules residing in the luminal ECM of tracheae have been characterized, dampy (39) and piopio (40). They are involved in maintenance and formation of the tracheal tube. In addition, they also showed adhesive properties in other tissues such as the wing (41, 42). Both proteins are secreted apically, and it has been proposed that Dumpy, through its enormous size of 800 nm, acts as a ruler setting the diameter of secondary branches (39). It is possible that these molecules interact with Ftl directly or through the ECM. Our silencing analysis suggests, however, that Ftl is necessary for keeping epithelia apart as a spacer, rather than determining the tube diameter. Consistent with this proposal is the finding that the Ftl-ED antiserum stains the lumen (Fig. 5G).

The expression studies and functional analysis of mFat1 in mice reveal valuable insights as to the function of its true orthologue in Drosophila. In the mammalian kidney, slit junctions provide the gaps between the process components in the glomeruli. The expression of mouse fat1 at the slit junction suggests a role as both an adhesion molecule and as a spacer of the junction (32). In the mouse knock-out the slit junctions disappear, generating a flattened sheet overlying the basement membrane (14). We thereby infer a similar behavior in Drosophila ftl mutants whereby tracheal epithelia fuse because of degeneration of the luminal space. Further subcellular analysis using transmission electron microscopy and fourth-dimensional confocal microscopy might help to shed some light on this issue.

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The fat-like Gene of Drosophila Is the True Orthologue of Vertebrate Fat Cadherins and Is Involved in the Formation of Tubular Organs
Casimiro Castillejo-López, Wilma Martinez Arias and Stefan Baumgartner

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