**Drosophila liquid facets-Related** encodes Golgi epsin and is an essential gene required for cell proliferation, growth, and patterning

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**A B S T R A C T**

Epsin and epsin-Related (epsinR) are multi-modular proteins that stimulate clathrin-coated vesicle formation. Epsin promotes endocytosis at the plasma membrane, and epsinR functions at the Golgi and early endosomes for trans-Golgi network/endosome vesicle trafficking. In Drosophila, endocytic epsin is known as Liquid facets, and it is essential specifically for Notch signaling. Here, by generating and analyzing loss-of-function mutants in the liquid facets-Related (lqfR) gene of Drosophila, we investigated the function of Golgi epsin in a multicellular context. We found that LqfR is indeed a Golgi protein, and that like liquid facets, lqfR is essential for Drosophila viability. In addition, primarily by analyzing mutant eye discs, we found that LqfR is required for cell proliferation, insulin-independent cell growth, and cell patterning, consistent with a role in one or several signaling pathways. Epsins in all organisms share an ENTH (epsin N-terminal homology) domain, which binds phosphoinositides enriched at the plasma membrane or the Golgi membrane. The epsinR ENTH domain is also the recognition element for particular cargos. By generating wild-type and mutant lqfR transgenes, we found that all apparent LqfR functions are independent of its ENTH domain. These results suggest that LqfR transports specific cargo critical to one or more signaling pathways, and lays the foundation for identifying those proteins.

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**Introduction**

Epsins are multi-modular membrane-associated proteins that function in endosome trafficking in yeast and metazoans (reviewed in Wendland, 2002; Duncan and Payne, 2003; Legendre-Guillemin et al., 2004). The distinctive feature of all epsins is an ENTH (epsin N-terminal homology) domain that binds membrane phosphoinositides (Kay et al., 1998; Rosenthal et al., 1999; Itoh et al., 2001). There are two classes of epsins: endocytic epsins, known as vertebrate epsin-1 (Chen et al., 1998), yeast ent1p and ent2p (Wendland et al., 1999), and Liquid facets (Lqf) in Drosophila (Cadavid et al., 2000; Overstreet et al., 2003) and nematodes (Tian et al., 2004), and Golgi-associate epsins, known as yeast ent3p and ent5p (Duncan et al., 2003), and vertebrate epsin-Related (epsinR), also known as enthoprotin or Clint (Kalthoff et al., 2002; Wasiak et al., 2002; Hirst et al., 2003; Mills et al., 2003). Endocytic or Golgi epsin ENTH domains prefer to bind the phosphoinositides enriched in the plasma membrane and Golgi membranes, respectively (Duncan and Payne, 2003; Legendre-Guillemin et al., 2004). Both endocytic and Golgi epsins have a variety of motifs C-terminal to their ENTH domains. Endocytic epsins have motifs for interaction with ubiquitin, clathrin, the clathrin adapter complex AP-2, and EH-domain containing endocytic factors. Golgi epsins have clathrin-binding motifs, and also motifs for binding the Golgi-associated clathrin adapter proteins AP-1 and Gga (Duncan and Payne, 2003; Legendre-Guillemin et al., 2004).

Endocytic epsins have been studied more intensively than Golgi epsins. Most of the available data supports a model where endocytic epsin promotes clathrin-dependent endocytosis, acting either as a clathrin adapter, or as an accessory factor for the AP-2 adapter complex (reviewed in Wendland, 2002; Aguilar and Wendland, 2005). As a clathrin adapter, through its UIMs, epsin binds transmembrane proteins that use ubiquitin as an internalization signal, and recruits clathrin and other endocytic factors to the plasma membrane. As an accessory factor, in order to facilitate internalization of transmembrane proteins whose endocytic signals are amino acid motifs in their intracellular domains that bind the AP-2 adapter, epsin bound to AP-2 would bring clathrin and other proteins to the plasma membrane. Epsin’s ENTH domain may also promote vesicle formation by inducing membrane curvature (Ford et al., 2002). Yeast epsin ENTH domains also coordinate actin cytoskeleton rearrangement with endocytosis (Aguilar et al., 2006). In yeast and also in
vertebrate cell culture, endocytic epsin functions in internalization of a variety of different cargos (Chen et al., 1998; Wendland et al., 1999; Sigismund et al., 2005; Barriere et al., 2006; Wang et al., 2006). Although this is probably also the case in Drosophila, the only apparent requirement for Lqf is for endocytosis of Notch ligands, which is essential for Notch receptor activation (Overstreet et al., 2003, 2004; Wang and Struhl, 2004, 2005). Thus at least in Drosophila, endocytic epsin plays a pivotal role in Notch signaling, and epsin is therefore critical for virtually all aspects of cell determination and differentiation during development.

Golgi epsins promote vesicular trafficking mainly between endosomes and the trans-Golgi network (TGN) (reviewed in Duncan and Payne, 2003; Legendre-Guillemin et al., 2004). In higher organisms, epsinR promotes clathrin-coated vesicle formation and trafficking between the TGN and early endosomes, in both directions. One type of cargo known to be transported in an epsinR-dependent manner from the TGN to the early endosome is lysosomal proteins bound to mannose-6-phosphate receptors on their way to the lysosome. Also, epsinR-dependent retrograde trafficking from the early endosome to the TGN retrieves mannose-6-phosphate receptors, and also other resident Golgi membrane proteins (Mills et al., 2003; Saint-pol et al., 2004; see also Hirst et al., 2003). Yeast ent3p and ent5p are required for trafficking of carboxypeptidase S from the TGN to the vacuole, where it is processed into active form, and also for endosome-to-TGN transport of Kex2p, a protease required for α-factor mating pheromone maturation (Duncan et al., 2003). Another type of Golgi epsin cargo in both yeast and higher eukaryotes is SNARE proteins. In yeast, SNARES required for vesicle fusion at late endosomes are transported from the TGN to the late endosome in an ent3p-dependent manner (Chidambaram et al., 2008). In mammalian cells, although the biological rationale for this is unclear, SNAREs that function at late endosomes appear to depend on epsinR for early endosome-to-TGN transport (Chidambaram et al., 2008). Yeast ent3p and ent5p also function in sorting proteins within the multi-vesicular body, the late endosome whose internal vesicles eventually fuse with the vacuole (Friant et al., 2003; Eugster et al., 2004). Like yeast endocytic epsins, ent3p is also a factor in actin cytoskeletal organization (Friant et al., 2003).

The mechanism of Golgi epsin function is probably similar to that of endocytic epsin (Duncan and Payne, 2003; Legendre-Guillemin et al., 2004), with at least one notable difference. EpsinR is likely the clathrin adapter for SNARE cargo, but unlike endocytic epsin which recognizes ubiquitin internalization signals via its UIMs, EpsinR binds SNAREs directly with its ENTH domain (Hirst et al., 2004; Chidambaram et al., 2004; Miller et al., 2007). In the transport of other cargos, EpsinR and also ent3p/ent5p may function either as the key clathrin adapter, or as accessory proteins for the AP-1 clathrin adapter complex or Gga adapters (Duncan and Payne, 2003; Legendre-Guillemin et al., 2004; Costaguta et al., 2006).

No analysis of Golgi epsin function in a developmental context has been reported in a multicellular organism. Only through genetic characterization of the Drosophila endocytic epsin gene, lqf, was it revealed that endocytic epsin plays a critical and specific role in Notch signaling. We wondered whether Drosophila Golgi epsin is also essential, and if it functions in cell patterning. Here, we report an analysis of the mutant phenotype of flies with weak and strong loss-of-function alleles of the Golgi epsin gene, which we call liquid facets-related (lqfR). We found that lqfRΔ117 is indeed essential for Drosophila viability, and that more specifically, lqfRΔ117 is required for cell proliferation, insulin-independent cell growth, and cell fate determination in the developing eye. In addition, we used a transgene to generate flies that express only LqfR protein specifically lacking the ENTH domain. We found that all of the functions of lqfRΔ117 that we have detected are independent of the ENTH domain.

**Materials and methods**

**Drosophila strains**

Flies were grown on standard media at 25 °C unless indicated otherwise. Strains from our laboratory’s collection, Bloomington Stock Center and individual stock collections:

Oregon R

w1118

yw

w; Sco/Cyo; MKRS/TM6B

MKRS/TM2, ry

TM3, ry Sb Δ2-3/Δ3[F Drake]C7, ry

lqfRΔ117/Δ117 ry/TM3 (FBal009480)

mirrorP69Df7/TM6B (FBal0083801; from D. Strutt)

l(3) SG621/TM6B (FBal0011236)

InR129/TM3 (FBal012280; from H. McNeill)

w; Act5C-gal4/Cyo (FBti0012293)

Df(3R) hh/TM3i (FBal0002795)

w; ro-gfp (on chr. 2) (Overstreet et al., 2004)

yw; hs-flp (FBti0015982)

w; FRT82B (FBti0002074)

w; FR7 82B Pw+ (FBti0001288)

yw; FRT82B GMR-hid (FBti0012710) I(3) CL-R1 (FBal0098712)/TM6B

w; FRT82B arm-lacZ (FBti0032911)/TM6B

w; FRT82B ubi-gfp (FBti0012695)/TM6B

FRT40A (FBti0002071) ubi-gfp/Cyo.

Strains generated for this work:

w; FRT82B lqfRΔ117/TM6B

w; FRT82B lqfRΔ117/TM6B

yw; ey-flp; Sco/Cyo; FRT82B GMR-hid I(3) CL-R1/TM6B

w; mirrorP69Df7/FRT82B lqfRΔ117/TM6B

yw; ey-flp; FRT82B arm-lacZ/TM6B

w; ro-gfp/Cyo; FRT82B lqfRΔ117/TM6B

yw; hs-flp; FRT82B arm-lacZ/TM6B

yw; ey-flp; Sco/Cyo; FRT82B/TM6B

w; FRT82B InR129/TM6B

w; FRT82B InR129 lqfRΔ117/TM6B

w; glfR+/CyO; FRT82B lqfRΔ117/TM6B

w; glfRΔENTH/Cyo; FRT82B lqfRΔ117/TM6B

w; Act5C-gal4/Cyo; FRT82B lqfRΔ117/TM6B

w; UAS-lqfRa-gfp/Cyo; FRT82B lqfRΔ117/TM6B

w; UAS-lqfRaΔENTH-gfp/Cyo; FRT82B lqfRΔ117/TM6B

yw; ey-flp; Sco/Cyo; gfp; FRT82B GMR-hid I(3) CL-R1/TM6B

w; Act5C-gal4, UAS-lqfRa-gfp/Cyo; FRT82B lqfRΔ117/TM6B

**Molecular biology**

Standard procedures were used for molecular biology. PCR of genomic DNA was performed on DNA from single flies as described (Overstreet et al., 2003). Restriction enzymes and phosphatases were from New England BioLabs, Boehringer Mannheim, and Promega Biotech. Automated fluorimetric DNA sequencing was performed in the DNA analysis facility of the Institute for Cell and Molecular Biology (ICMB), at UT Austin.

**Generation of lqfRΔ117 by P element mobilization**

lqfRΔ117 was generated by imprecise excision of the Pry+ element in lqfRΔ685 (Spradling et al., 1999). Approximately 200 crosses were set up where males of the genotype lqfRΔ685 ry/TM3, ry, Δ2–3 were
crossed with MKRS/TM2, ry females. Male progeny in which the Pry+ element had excised were identified as ry flies, whose genotype was Pry+Δ/ry TM2, ry or Pry+Δ/ry/MKRS. The Pry+Δ chromosome from a single male progeny from each cross was amplified and balanced by crossing the males individually to MKRS/TM2, ry females and stocks were generated from the Pry+Δ/ry TM2, ry progeny. Of the 200 Pry+Δ chromosomes, 9 were lethal in trans to Df(3R)hhl. Molecular lesions in 3 of the 9 chromosomes were identified using a variety of PCR primers flanking the Pry+ insertion site to amplify genomic DNA, and determining the DNA sequence of the PCR products whose sizes were smaller than that obtained from wild-type template DNA. lqfRΔ was chosen for further genetic analysis because its molecular lesion suggested that it might be a null allele of lqfR that did not affect surrounding genes.

Transgene construction and transformation

P element transformation of yw flies was performed in our laboratory using standard techniques, or by Genetic Services (Sudbury, MA) or Genetivision (Houston, TX).

pglqR+

This plasmid is Casper 4 (Thummel and Pirrotta, 1992) containing an ∼11.5 kb fragment of D. melanogaster genomic DNA that includes a wild-type lqfR gene: sequences from −663 to +10,905 relative to the lqfR translation start site. BAC clone 30J14 (BacPac Resources) was restricted with BssHII and the fragments ligated into containing an AscI site pBacI (Chen and Fischer, 2000) restricted with AscI. As the lqfR gene should be contained in an −12 kb BssHII fragment, a plasmid with a 12 kb insert (pGenBssHl) was identified and DNA sequence determination of one end of the insert confirmed its identity. The fragment was modified in order to eliminate most of the upstream gene, CG13850, as follows. pGenBssHl was used a PCR template to amplify an ∼1.1 kb fragment containing genomic DNA sequences from 663 bp 5′ to the insertion point of the Pry+ element in lqfRΔlqfR, to a downstream AatII site. The PCR primers used were 5′-GGGCGGCCATTACGGTCAAGAAGACC-3′ and 5′-AAATCTGGGGAGCCTGGG-3′. The amplified product was subcloned in pGEMT (Promega), its DNA sequence determined, and a plasmid with the correct sequence, pGEM-5′g2e2, was identified. An ∼1 kb Not I–AatII fragment of pGEM-5′g2e2 and an ∼9 kb AatII–HindIII fragment of pGenBssHl were ligated together into pBSKSI restricted with NotI and HindIII, to generate pBSKSI-g2e2. An ∼11.5 kb Not I–KpnI fragment from this plasmid was ligated into pCasper4 restricted with NotI and KpnI.

pglqR−ENTH

This plasmid is pglqR+ with a 461 bp deletion that includes exon 2, intron 2, and part of exon 3, and results in removal of 122 codons, corresponding to amino acids V32–R153 (Tweedie et al., 2009). The deletion was introduced by generating two PCR amplification products using pglqR+ as a template, and then by using both products simultaneously as templates for PCR. The first amplification product (∼420 bp) contains a restriction site unique to the gflqR+ DNA fragment (Avr II) and the deletion: it began at the Avr II site in intron 1 and included all the rest of intron 1 and also exon 3 sequences corresponding to the deletion breakpoint. The 3′ PCR primer included the deletion, meaning that in addition to intron 1 sequences, it contained 25 bp of exon 3 sequence, corresponding to the deletion breakpoint. The two primers used were 5′-GTACACCTTTTACGCTTTACGGAA-3′ (5′, contains Avr II site) and 5′-TTCGCGTGTGCAAGAAGCAGCGAC-3′ (3′ ENTH, contains deletion). The second amplification product (∼1000 bp) contains a restriction site, Sgr A1, unique to the lqfR+ genomic fragment: it began at the deletion breakpoint in exon 3 and included sequences up to the downstream Sgr Al site. The primers used were 5′-CCCACGTGGGAGAAGGCGAGAAGAACAGG-3′ (5′ENTH2) and 5′-AATCGTTCCGCGGCTTG-3′ (3′ENTH2, includes Sgr Al site). Both amplification products were used together as the template for PCR amplification with the primers 5′ENTH1 and 3′ ENTH2. As the two template DNAs have 23 bp of overlapping DNA, they will anneal and generate an ∼1.5 kb Avr II–Sgr Al template fragment that includes the deletion. The amplified product was restricted with Avr II and Sgr Al, and the resulting fragment was ligated into pglqRF+ restricted with the same enzymes. The DNA sequence of the insertion was confirmed.

pUAS-lqfRa

Because we initially had difficulty obtaining a full-length lqfRa cDNA using RT-PCR, this plasmid was generated using cDNA for exons 1–5, and genomic DNA for exon 6. A DNA fragment containing exons 1–5 was generated using reverse transcribed (Superscript II and oligo-dT, Invitrogen) total embryo RNA (prepared with TriReagent, Ambion) as the template for PCR with primers 5′-GCAAGACGTGCAAATGTCGCCACAC and 5′-AGCCCTTGGAAGGCGAGAAAG. The amplified product was cloned into pGEMT and its DNA sequence confirmed to generate pGEM-epsinR-l. Next, a 450 bp fragment containing 5′ sequences of lqfR cDNA with an AscI site just upstream of the ATG and an in-frame Pml I site just downstream, was generated by PCR using pGEM-epsinR-l as template, the mutagenic primer 5′-GCGCGCCATGCAACGCGTGGTGGATAAATTCATC, and a primer that includes the downstream Bso BI site. The amplified product was ligated into pGEM and its sequence confirmed, to generate pGEM-E2Epml. A 450 bp Asc I–Bso BI fragment of pGEM-E2Epml and a 900 bp Bso BI–Eco RI fragment were ligated together into pBSKSI restricted with Asc I and Eco RI to generate pBSKSI-D-epsinR-l. Next, an in-frame Sap I site was introduced at the 3′ end of exon 5 (there is a Sap I site that spans the exon 5/6 splice junction) by using pBSKSI-D-epsinR-l as template for PCR with the primers 5′-TTGCTGAGACTGCCAGGC and 5′-CAAGATGTGGCAATGAAATGTATC. The 355 bp amplification product was ligated into pGEM and its sequence confirmed to generate pGEM-e2 Sap. Using genomic DNA as template, 5′ sequences of exon 6 (1.85 kb) were amplified with the primers 5′-TGTGCTAAGACCTGGCCACCC and 5′-CGAAGATGTCGCCAAATGGAATATC, and 3′ sequences of exon 6 (1.9 kb) were amplified with the primers 5′-CCCTGTTGGAAATTTGATG and 5′-ATACCTGGGATTAGC. Each amplified fragment was ligated into pGEMT and its sequence was confirmed, to generate pGEM-exon6A and pGEM-exon6B, respectively. A 350 bp Eco RI–Sap I fragment of pGEM-e2 Sap, and a 900 bp Sap I–Bam HI fragment of pGEM-exon6A, were ligated together into pBSKSI restricted with Eco RI and Bam HI to generate pBS-exon6RIBam. A 400 bp Bam HI–Bts I fragment of pGEM-exon6A and a 1.35 kb Bts I fragment of pGEM-exon6B were ligated together into pBSKSI-D-epsinR-l at the 3′ end of exon 6. As the two template DNAs have 23 bp of overlapping DNA, they will anneal and generate an ∼1.5 kb Avr II–Sgr Al template fragment that includes the deletion. The amplified product was restricted with Avr II and Sgr Al, and the resulting fragment was ligated into pglqRF+ restricted with the same enzymes. The DNA sequence of the insertion was confirmed.

pUAS-lqfRa-gfp

A 3′ fragment of lqfRa cDNA extending from the Sna BI site to the final codon was amplified by PCR using pUAS-lqfRa as a template, and the primers 5′-TTTACGTTAATAACGGAAAAATT–3′ and 5′-ACGGTCGGACCGCTTCTAC-3′. An in-frame Mlu I site was inserted in place of the stop codon using a mutagenic primer. After ligation into pGEMT, the fragment sequence was verified, and then the subclone was restricted with Sna BI and Not I to generate a 450 bp fragment. A 3.7 kb Asc I–Sna BI fragment of pUAS-lqfRa was isolated, and ligated with the 450 bp Sna BI–Not I fragment into pBSKSI restricted with Asc I and Not I, to generate pBS-lqfRAlm. gfp sequences were amplified as an Mlu I–Asc I fragment using p8036
(Harris and Macdonald, 2001) as a template, and the primers 5'-ACGCTGATGAGGAGGAG-3' and 5'-GGCGCCCTTGGATTGATGTC-3', which generated a stop codon prior to the Asc I site. The amplified product was ligated into pGEM and its sequence verified, to generate pGEM-3′-GFP. A 750 bp Mlu I–Not I fragment containing gfp was isolated from pGEM-3′-GFP and ligated into pBS-lqfRaMlu restricted with Mlu I and Not I, to generate pBS-lqfRa-gfp. A 5.0 kb Bss HII fragment of pBS-lqfRa-gfp was isolated and ligated into pUAS-XA restricted with Asc I, to generate pUAS-lqfRa-gfp.

pUAS-lqfRaΔENTH-gfp

A central fragment of lqfRa, extending from just 3′ of the ENTH domain to the downstream Bgl II site, was generated by PCR using pUAS-lqfRa as the template and primers 5′-CATATGAGTACATCGGCTGAGCAG-3′ and 5′-AGATCTTGACGCTGGTTCGGCTG-3′. An in-frame Nde I site was inserted at the 5′-end of the fragment by the 5′-primer. The amplification product was ligated into pGEM, and its sequence verified to generate pGEM-4. A 900 bp Eco RI–Nhe I fragment of pGEM-4 and a 4.2 kb Nhe I–Asc I fragment of pUAS-lqfRa-gfp were ligated together into pBSKII restricted with Eco RI and Asc I to generate pBS-E2aΔENTH-gfp. A 4.7 kb Asc I fragment of pBS-E2aΔENTH-gfp was ligated into pUAS, restricted with Asc I to generate pUAS-lqfRaΔENTH-gfp.

Generation of anti-LqfR

The antigen used to generate anti-LqfR is present in both the a and b isoforms, and contains amino acids M1–P396 (Tweedie et al., 2009), which includes the ENTH domain and some downstream amino acids (exons 1–4 and part of exon 5). The plasmid used to express the antigen in bacteria, pET28a-comm, was generated as follows. A 5′ lqfRa fragment extending from the start codon to the downstream Eco RI site was generated by PCR using pBS-D-epsinRa (see above) as the template and the primers 5′-CATATGAGTACATCGGCTGAGCAG-3′ and the 17′ promoter primer (Promega). The 5′ primer inserted an Nde I site just upstream of the start codon. The amplification product was ligated into pGEM, and its sequence verified to generate pGEM-comm. A 1.3 kb Nde I–Eco RI fragment of pGEM-comm was ligated into pET28a restricted with Eco RI and Nde I to generate pET28a-comm. The LqfR protein was expressed in E. coli Codon-Plus RIL (Strategene), and the purified using Chelating Sepharose Fast Flow (Pharmacia Biotech). The purified antigen was sent to Pocono Farms (Canadensis, Pa) where it was used to generate antisera in two guinea pigs.

Protein blots

Eye disc protein extracts were generated and analyzed on Western blots as described (Chen et al., 2002). The blots were probed with anti-LqfR diluted 1:1000 and mouse mAbE7 (anti-β-tubulin from DSHB) at 1:100. Secondary antibodies were HRP-anti-guinea pig (Jackson) at 1:20,000, and HRP-anti-mouse (Santa Cruz Biochemicals) used as a secondary antibody. Total RNA was purified from adult flies or third instar larval eye disc using TriReagent (Ambion), and reverse transcribed using Superscript II RT (Invitrogen), 5 μg RNA, 2 pmole primer (5′-GTCTTCTACCATGCG-3′; from exon 6). The RT reaction (2 μl) was used as a template for PCR using Platinum PCR Supermix (Invitrogen), and the following primers: 5′-GGAGAGGCGCGGGTGTAACGGCAG-3′ (exon 1) and 5′-TGATTACGCAATGCGCATATCC-3′ (exon 6). After agarose gel electrophoresis, a band of the expected size for a lqfRa template was obtained (1146 bp) and its structure confirmed by DNA sequencing. A no-RT control reaction yielded a light band of the size expected for a genomic DNA template (1623 bp). The structure of lqfRb (that exon 5 is connected to exon 7) was demonstrated by a 3′-RACE experiment (Ambion 3′-RACE kit) and the primer 5′-TCCGCCATGGGCGCTGACTCC-3′ (exon 5). The DNA sequence of the resulting 1.5 kb was determined and it contained lqfRb sequences from 1.3 kb downstream of the start codon through the 3′-UTR.

Analysis of eyes, nota, and salivary glands

Plastic sectioning of adult eyes was as described (Tomlinson and Ready, 1987). Scanning electron microscopy of adult eyes was performed on flies dehydrated in 70%, 100% ethanol, critical point dried in CO2, and coated with Pt/Pd, using a Zeiss Supra 40VP FE-SEM. For immunostaining, eye discs were fixed in PEMs and antibody incubations and washes were in PBST (see Fischer-Vize et al., 1992). Primary antibodies used were: guinea pig anti-LqfR (1:100), mouse monoclonal anti-p120 (1:200; Calbiochem), rabbit anti-Lava (1:2000; from John Sisson, UT Austin), rat monoclonal anti-Elav (1:9; Developmental Studies Hybridoma Center [DSHB]), mouse monoclonal anti-β-gal (1:50, DSHB), Secondary antibodies (1:500; Molecular Probes) were: Alexa488-anti-mouse, Alexa568-anti-mouse, Alexa633-anti-rat, Alexa633-anti-rabbit, Alexa488-anti-mouse, Alexa568-anti-guinea pig. TOPRO-3 (Molecular Probes) was used 1:1000. Salivary glands were dissected in PBS, fixed in 4% paraformaldehyde (45 min), incubated with Alexa488-phalloidin (used 1:100 in PBST, Molecular Probes) and mounted in Vectashield (Vector). Wings were dehydrated in 70% ethanol and mounted in DPX (Fluka). Wing and eye sections were photographed with a Zeiss Axioskop equipped with an Axiocam HRC. Note and eye sections were photographed in whole flies using an Olympus SZX12 microscope.

Fig. 1. Structure of the lqfR locus. (A) A diagram of the two alternate splice forms of lqfR mRNA (a and b) is shown. Grey bars are exons 1–7, and the lines connecting them indicate introns. Triangles indicate start and stop codons. (B) Diagram of the two different proteins encoded by the a and b transcripts in (A). Each protein has an epsilon N-terminal homology (ENTH) domain, two motifs for interaction with AP-1, and two clathrin-binding motifs (CBM). (C) A blot of eye disc protein extracts from w1118 (wild-type) and lqfR/b (third instar larvae probed with antibodies to LqfR and β-tubulin is shown. Different amounts (1×–4×) of the same extracts were loaded into each of three lanes. The lines and numbers at the right indicate the positions and approximate sizes (kb) of markers. A similar blot probed with preimmune serum from the guinea pig that generated anti-LqfR had no signal.
equipped with a SPOT idea (Diagnostic Instruments) digital camera. Immunofluorescent tissues were photographed with a Leica TCSSP2 or SP2AOBS confocal microscope. Images were processed with Adobe Photoshop.

Calculation of rhabdomere size and box plots

In order to control for differential effects of sample preparation on rhabdomere size between samples, the relative sizes of wild-type and mutant rhabdomeres were assessed in individual mosaic facets. Using images of eye sections in Adobe Photoshop, the number of pixels in each mutant rhabdomere and each wild-type rhabdomere of R1–R6 was counted, and the average of each was calculated, and used to compute the ratio of mutant/wild-type size. This ratio represents each data point (n). Box plots were calculated using GraphPad Prism software (version 3.0).

Quantitation of LqfΔENTH protein produced by genomic transgene

The data for these experiments is in Fig. S6. In order to determine if the LqfΔENTH protein produced by the glqfRΔENTH transgene accumulates to similar levels as the endogenous LqfR protein, we first used anti-LqfR to visualize the proteins in blots of extracts from animals with one copy of the transgene and one copy of the endogenous gene. The signals from the endogenous LqfR proteins and the LqfRΔENTH proteins were of similar strength. However, the polyclonal antibody was generated to amino acids 1–396 of LqfR, and the ENTH-less protein is missing 122 of those amino acids. The polyclonal antibody signal could rely heavily on antibodies that recognize epitopes missing in the ENTH-less protein. If so, the similar signal strength obtained for the LqfR and LqfRΔENTH bands could indicate that LqfRΔENTH is present at much higher levels than LqfR. In order to determine whether anti-LqfR gives similar signals for similar amounts of LqfR or LqfRΔENTH proteins, we used anti-GFP to detect LqfRΔENTH-GFP and LqfRΔENTH-GFP relative to tubulin, and then used anti-LqfR to detect the same proteins. We found that the signal strength for the full-length and ENTH-less proteins, relative to tubulin, was similar for both anti-GFP and anti-LqfR. Thus, we conclude that in flies containing the glqfRΔENTH transgene, LqfRΔENTH is not grossly overexpressed relative to endogenous LqfR. Protein blots were performed as described above, with rabbit anti-GFP at 1:1000 (Cell Signaling Technologies).

Fig. 2. Expression pattern and subcellular localization of LqfR in eye discs. Confocal images of immunolabeled w1118 (wild-type) third instar larval eye discs are shown. (A–A″) Expression of LqfR and the Golgi protein p120 are shown in Z-sections (A–A″) and apical XY-sections (B–B″). In (C–C″), enlargements of the area in the box in (B) are shown. (D–F″) Expression of LqfR and the Golgi protein Lava lamp (Lava) are shown in Z-sections (D–D″) and apical XY-sections (E–E″). In (F–F″), enlargements of the area in the box in (E) are shown. The apical XY-sections show the peripodial epithelium; the dark circles are nuclei. In both A and D, LqfR is detected mainly in the peripodial epithelium. Differences in the appearance of these two panels represent disc-to-disc variation. Scale bar in A: 40 μm in (A–A″), 20 μm in (B–B″, E–E″).
The Drosophila Golgi epsin gene, liquid facets-Related, encodes two similar proteins by alternate splicing

The Drosophila gene encoding Golgi epsin (CG42250), located on chromosome 3R at polytene position 94A12, was identified by sequence similarity to the vertebrate gene and referred to as epsin-2 (Lloyd et al., 2000) or epsin-like (Tweedie et al., 2009). Because the endocytic epsin gene is named liquid facets, and the vertebrate Golgi epsin gene is called epsin-Related, we call the gene liquid facets-Related (lqfR). The lqfR gene contains seven exons. As diagrammed in Fig. 1A, we found that lqfR mRNA is alternately spliced to generate one transcript (lqfRa) that contains all seven exons, and another transcript (lqfRb) that lacks exon 6. This model is supported by analysis of mRNA and protein produced by the lqfR locus. By RT-PCR of eye disc mRNA or whole adult fly mRNA, we amplified transcripts in which exons 1 through 6 were joined (lqfRb), and also transcripts in which exons 1 through 5 were joined with exon 7 (lqfRc) (data not shown; see Materials and methods). In addition, we generated an antibody in guinea pigs to bacterially produced protein encoded by the open-reading frame in exons 1 to 5, which is expected to recognize the proteins produced by both transcripts. On protein blots of third instar larval eye disc protein extracts, the antibody recognizes two proteins of approximate sizes 70 kD and 150 kD (Fig. 1C) predicted by the open-reading frames in lqfRa and lqfRb transcripts (Fig. 1B). The antibody is likely to be specific for LqfR protein in this assay, as the levels of both the 70 kD and the 150 kD protein are reduced by a factor of ~1/3 in eye discs homozygous for a hypomorphic allele of lqfR called lqfRΔ117 (Fig. 1C and Materials and methods; see below).

LqfR is present at the Golgi

We used the LqfR antibody to detect the protein in developing eyes by immunofluorescence. The Drosophila eye develops in third instar larvae from the eye imaginal disc, which consists of a monolayer of columnar epithelial cells (the eye disc proper) that will form the eye, and an overlying layer of squamous epithelial cells, called the peripodial epithelium (Wolff and Ready, 1993). The peripodial epithelium does not contribute structurally to the adult eye, but functions in disc development by signaling to the columnar cells beneath, and is also required for disc eversion during pupation, a process where the disc unfolds to become the adult eye (Gibson and Schubiger, 2001). The LqfR antibody produces no fluorescence above background in eye disc cells homozygous for a null allele of lqfR called lqfRΔ117 (Fig. S1). By contrast, we detect a signal in wild-type eye discs, and we find that the anti-LqfR signal overlaps significantly with that of two Golgi markers, p120 (Stanley et al., 1997) and Lava lamp (Sisson et al., 2000) (Fig. 2). LqfR and p120 accumulate mainly in the peripodial epithelium (Figs. 2A–C′) and also basally in the eye disc proper (Figs. 2A–A′). The LqfR and p120 signals are both diffuse and punctate in the cytoplasm, and remarkably overlapping (Figs. 2B–C′). Lava lamp accumulates in puncta throughout the apical/basal plane of

Fig. 3. Molecular structure and external morphological phenotype of mutant lqfR alleles. (A) A diagram of the lqfR genomic DNA region, and the molecular structures of the two mutant alleles used in this work (lqfRΔ136 and lqfRΔ117), and two genomic DNA transgenes (glqfR and glqfRΔ136) is shown. The scale bar at the top is in increments of 1000 bp. The black bars represent exons, and the arrows indicate the start site and direction of transcription of lqfR and flanking genes (Tweedie et al., 2009). The open red triangle indicates the P element insertion site in lqfRΔ136, which is 94 bp upstream of the start codon (Tweedie et al., 2009). The extent of the deletion in lqfRΔ117 (~469 to +4081 bp relative to the start codon) is indicated by a break in the black line. Red bars indicate the extents of the genomic DNA in each of the transgenes, with a break indicating a deletion. (B–D) Scanning electron micrographs of adult eyes are shown. (E, F) Adult wings are shown. (G, H) The adult notum (dorsal thorax) is shown. Arrows indicate anterior scutellar bristles. (wt = w1118, lqfRΔ = FRT82B lqfRΔ136; lqfRΔ117 = ey-FLP; FRT82B lqfRΔ117/FRT82B GMR-hid (J3)Cl-8).
the eye disc proper (Fig. 2D). Some of the Lava lamp and LqfR puncta are coincident, and others are adjacent to each other (Figs. 2E–F). We conclude that LqfR is a Golgi protein.

**lqfR is an essential gene**

The Berkeley *Drosophila* Genome Project generated a mutant allele of *lqfR* (called l(3)03685) that has a P element insertion in the 5′-untranslated region (Spradling et al., 1999). We refer to this allele as *lqfR*P3685 (Fig. 3A). At 25 °C, *lqfR*P3685 is semi-lethal; most *lqfR*P3685 homozygotes die fully formed in their pupal cases. Rare escapers eclose and many have obvious morphological defects. The eyes are kidney-shaped and rough, especially at the dorsal/ventral axis (Figs. 3B, C). The wings are notched (Figs. 3E, F), and the wing hair patterns may suggest planar cell polarity defects (Fig. S2). Duplications of the anterior scutellar bristles on the notum are observed also (Figs. 3G, H). At 18 °C, *lqfR*P3685 homozygous flies eclose and appear normal morphologically. As described above, *lqfR*P3685 is hypomorphic; *lqfR*P3685 homozygotes produce LqfRa and LqfRb proteins at reduced levels. The results of several experiments indicate that the temperature-sensitive semi-lethality and morphological defects observed in these flies is due to the *lqfR*P3685 mutation. First, one copy of a transgene containing genomic DNA corresponding to a wild-type *lqfR* gene (PglqfR+) complements all defects in the homozygotes (data not shown). Second, a UAS-lqfRa transgene (contains a lqfRa cDNA) expressed using the ubiquitous ActSC-gal4 driver (ActSC-lqfRa) also complements the *lqfR*P3685 mutant phenotype observed (data not shown). Finally, precise excision of the P element in *lqfR*P3685 results in viable flies with apparently normal morphology (Materials and methods).

We mobilized the P element in *lqfR*P3685 in order to generate a null allele by imprecise excision. In this way we obtained *lqfR*Δ117, in which the transcription start site and most of the *lqfR* gene is deleted (Fig. 1A). The mutant phenotype of *lqfR*Δ117 is more severe than that of *lqfR*P3685. At 25 °C, *lqfR*Δ117 homozygotes die as third instar larvae, and *lqfR*Δ117/lqfRΔ117 animals die as pupae and no escapers are observed. (Maternal LqfR is provided to oocytes [data not shown] and it is possible that the maternal protein enables the animals to survive past embryogenesis.) Also, homozygous *lqfR*Δ117 adult eyes generated

![Fig. 4. Eye development defects in lqfR mutants. (A, B) Confocal images of third instar larval eye discs immunolabeled with anti-Elav which labels R-cell nuclei. The arrow in (A) indicates the morphogenetic furrow. Discs are oriented with anterior at top and posterior at bottom. (C, D) Adult eyes that express w+ under the control of mirror are shown. The arrows indicate the D/V axis. The genotype in (C) is ey-flp; mirrP69Df7 FRT82B lqfRΔ117/TM6B, and in (D) is ey-flp; mirrP69Df7 FRT82B lqfRΔ117/FRT82B GMR-hid l(3)CL-R1. (E, F) Apical sections of adult eyes through the D/V axis, or equator, marked by the red line. Numbers in (E) are R-cells, and in (F), asterisks indicate ommatidia with additional R-cells, and dots indicate ommatidia missing an R-cell. (G–G″) Confocal images of a third instar larval eye disc (ey-flp; ro-gfp/+; FRT82B lqfRΔ117/FRT82B arm-lacZ) immunolabeled with anti-β-galactosidase. GFP expression is shown in (G), and *lqfR*Δ117 clones are outlined. The clones are marked by the absence of β-galactosidase expression, shown in (G′) and (G″). (wt = w1118; lqfRΔ = FRT82B lqfRΔ117) Scale bar in A: 30 μm in (A, B), 100 μm in (C, D), 10 μm in (E–G″).]
using FLP/FRT-induced mitotic recombination and GMR-hid (Stowers and Schwarz, 1999) are much more severely malformed and smaller than lqfR\textsuperscript{P3685} homozygous eyes generated the same way (Fig. 3D and data not shown). Finally, unlike lqfR\textsuperscript{P3685} homozygotes, lqfR\textsuperscript{A117} third instar larvae have small salivary glands that have small cells (Fig. S3), and no imaginal discs (data not shown). All of the aspects of the mutant phenotype described above are due to loss of lqfR function, as they are complemented by one copy of PglqfR+ or Act5C>lqfRa (data not shown). 

lqfR+ is required for morphogenetic furrow movement and cell patterning

The kidney shape of lqfR\textsuperscript{P3685} eyes suggests that lqfR+ might play a role in controlling movement of the wave of morphogenesis, called the morphogenetic furrow.

Eye disc cells in third instar larvae proliferate until ommatidial assembly begins. The morphogenetic furrow forms at the posterior of the disc and moves anteriorly into the undifferentiated cells, which cease dividing as the furrow approaches. Posterior to the furrow, rows of cells assemble stepwise into ommatidia, beginning with the eight photoreceptor cells (Wolff and Ready, 1993). Kidney-shaped eye have been observed in mutants where furrow movement is hampered (Chanut et al., 2000). To monitor furrow movement in lqfR\textsuperscript{P3685} eye discs, we examined expression of Elav, a nuclear protein expressed in all photoreceptors, beginning at the fourth row of assembling ommatidia posterior to the furrow (Robinow and White, 1991). The idea is that if furrow movement slows in lqfR mutant discs, ommatidia closest to the furrow will be at more advanced stages of development than they normally would be. In lqfR\textsuperscript{P3685} eye discs, not only are ommatidia near the furrow abnormally mature, but the rows of assembling ommatidia are strikingly V-shaped, rather than straight as in wild-type discs (Figs. 4A, B). We conclude that the kidney shape of lqfR\textsuperscript{D} eyes is due to the furrow stopping, first at the dorsal/ventral axis (the equator), and then later more laterally in the disc.

Externally, lqfR\textsuperscript{P3685} eyes are rough along the equator, suggesting that there might be patterning defects in the ommatidia there (Fig. 3C). Tangential sections through the equator of lqfR\textsuperscript{P3685} eye reveal ommatidia with a wide variety of defects, including ommatidia with too many or too few photoreceptors, irregular polarity, and ommatidial fusions (Figs. 4E, F). Away from the equator, lqfR\textsuperscript{P3685} ommatidia are normal (data not shown). Dorsal/ventral polarity itself, however, is still present, even in lqfR\textsuperscript{A117} eyes. An enhancer trap insertion in the mirror gene, where the white+ gene is expressed only in cells in the dorsal half of the eye (Brodsky and Steller, 1996), was used as a dorsal marker (Fig. 4C). In lqfR\textsuperscript{A117} eyes generated using mitotic recombination and GMR-hid, the small eye that remains has dorsal and ventral halves in their normal positions (Fig. 4D).

One example of a mutation that results in morphogenetic furrow arrest and kidney-shaped eyes is a dominant gain-of-function allele of the rough gene, called ro\textsuperscript{DGO}, in which rough is overexpressed (Chanut et al., 2000). We wondered whether the lqfR\textsuperscript{P3685} mutant eye phenotype could be caused by rough overexpression. To determine if rough is overexpressed in lqfR− cells, we generated lqfR\textsuperscript{A117} homozygous clones in lqfR\textsuperscript{A117}/lqfR+ eye discs. The eye discs also contain a reporter for rough gene expression: a rough-gfp (ro-gfp) transgene that expresses GFP under the control of the rough promoter (Overstreet et al., 2004). We observed that GFP levels were higher within the lqfR\textsuperscript{A117} clones than in the surrounding lqfR+ cells (Figs. 4G–H). In order to test if the elevated GFP level within the clones is due to overexpression from the rough promoter, or to GFP stabilization, we generated lqfR\textsuperscript{A117} clones in eye discs that express GFP from the ubiquttin promoter. We found that GFP levels are not elevated in these clones (Fig. S4), and thus we conclude that the absence of lqfR+ activity results in rough overexpression.

lqfR+ is required for cell proliferation in the eye imaginal disc

The absence of imaginal discs observed in lqfR\textsuperscript{A117} larvae is typical of mutants in genes required for cell proliferation (Shearn et al., 2004). To test whether lqfR is required for cell proliferation, we used FLP/FRT-induced mitotic recombination and GMR-hid (Stowers and Schwarz, 1999) to generate homozygous eyes. In lqfR\textsuperscript{P3685} homozygous eyes, cell proliferation was reduced compared to wild-type eye discs. A twin spot (2XlacZ; solid outline) is shown. The twin spot is 1.3× larger in area, and has 1.3× as many nuclei as the clone. (C, C') Apical confocal images of third instar larval eye discs of the genotype lqfR−/lqfR−; FRT82B/FRT82B arm-lacZ, immunothesized with anti-β-gal and incubated with DNA stain TOPRO-3. A lqfR− clone (lacZ−; dotted outline) and lqfR− twin spot (2XlacZ; solid outline) are shown. The twin spot is 3.3× larger in area, and has 3.3× as many nuclei as the clone. Raw data for these and other control and experimental clones, at these and other focal planes, is in Table S1.
To test if \( lqfR^- \) cells divide more slowly than wild-type cells in the eye disc, we used mitotic recombination in a \( lqfR^{A117}/lqfR^+ \) disc to generate a \( lqfR^{A117}/lqfR^{A117} \) cell and a \( lqfR^+ / lqfR^+ \) cell, born at the same time (Fig. 5A). The descendants of the \( lqfR^{A117} \) homozygous cell and those of its \( lqfR^+ \) homozygous sister are marked so as to distinguish them from each other and also from the \( lqfR^{A117}/lqfR^+ \) background cells that did not undergo mitotic recombination (Fig. 5A). The \( lqfR^{A117} \) cell clone is marked by the absence of \( \beta\)-galactosidase (\( \beta\)-gal) expression, and its \( lqfR^+ \) twin spot is marked by a \( \beta\)-gal expression level two-fold higher than that in the background \( lqfR^{A117}/lqfR^+ \) cells (Fig. 5A). After several rounds of cell division, the cell number in the mutant clone and twin spot should be similar, unless the mutation has an effect on cell proliferation.

As described above, undifferentiated eye disc cells proliferate as a monolayer until the third instar larval stage when the morphogenetic furrow moves anteriorly across the eye disc. Cell division stops as cells enter the furrow, and then about 4 rows posterior to the furrow, cells not yet recruited into ommatidia undergo one additional division (Wolff and Ready, 1993). Therefore, in the mutant clones and twin spots, mitotic recombination and most subsequent cell division occurred anterior to the morphogenetic furrow. We examined four mutant clones and twin spots posterior to the furrow in third instar eye discs. First, we measured the sizes of the clones and twin spots, and found that the \( lqfR^{A117} \) clones had proportionally fewer (Fig. 5C and Table S1). Thus, the number of cells in the mutant clones and twin spots because the clones are smaller than the wild-type twin spots because they have fewer cells, not because the mutant cells are smaller. One potential caveat to this experiment is that if the \( \beta\)-gal initially present in the \( lqfR^{A117}/lqfR^+ \) cell perdures in the \( lqfR^{A117}/lqfR^{A117} \) daughter cell after mitotic recombination, and remains at detectable levels through several subsequent cell divisions, the clone could appear to have fewer cells than it actually does because the cells in which \( \beta\)-gal persists are indistinguishable from the \( lqfR^{A117}/lqfR^+ \) background cells. To determine whether perdurance of \( \beta\)-gal could explain the difference between clone and twin spot size, we generated clones and twin spots in exactly the same manner, except both were \( lqfR^+ / lqfR^+ \). We examined two wild-type clones and wild-type twin spots, and they were the same size and contained the same number of cells (Figs. 5B, B’ and Table S1). Thus, the number of cells in \( lqfR^{A117} \) clones is smaller than in their twin spots because the clones are \( lqfR^- \). We conclude that cells lacking \( lqfR^+ \) either divide more slowly than wild-type cells, or tend to die.

A mutagenesis screen for genes required for imaginal disc development identified a recessive lethal mutation, \( l(3)5G62^b \) (a.k.a. \( A9, \) and \( l(3)XII-10 \)) that was localized to a map position close to \( lqfR \), and like \( lqfR \) mutants, results in disc-less third instar larvae (Shearn et al., 1971; Shearn and Garen, 1974). We found that \( l(3)5G62^b \) fails to

![Fig. 6. Comparison of rhabdomere size in \( lqfR^- \) and \( InR^- \) single and double mutants. (A) A box plot comparing rhabdomere sizes in \( lqfR^{A117}, \) \( InR^{339}, \) and \( lqfR^{A117} \) \( InR^{339} \) cells. \( lqfR \) is \( lqfR^{A117}, \) and \( InR \) is \( InR^{339}. \) Each ommatidium mosaic for wild-type and mutant R-cells provided a single data point, which was calculated as the average size of the mutant rhabdomeres divided by the average size of the wild-type rhabdomeres (R1–R6 only). The boxes represent the values corresponding to 50% of the data points, and the line within each box is the median. The lines above and below each box extend to the highest and lowest data points. (n = the number of mosaic facets scored, ** = 0.001 by 1-way ANOVA) (B–D) Sections of adult eyes containing facets mosaic for wild-type R-cells and R-cells of the indicated genotypes, that serve as examples of the raw data used to generate the graph in (A). Genetically wild-type R-cells are marked by the presence of pigment granules that appear as black puncta (arrow in panel D). Dots indicate genetically mutant rhabdomeres in mosaic facets, and facets composed of all mutant R-cells are circled. Genotypes are: (B) ey-flp; \( FRT82B \) \( lqfR^{A117}/FRT82B \) \( Pw^+; \) (C) ey-flp; \( FRT82B \) \( InR^{339} \) \( lqfR^{A117}/FRT82B \) \( Pw^+; \) (D) ey-flp; \( FRT82B \) \( InR^{339} \) \( lqfR^{A117}/FRT82B \) \( Pw^+. \)
complement \(lgfR^{117}\). Analysis of cell division cycles in \(\{f(3)SG62\}\) mutants led to the conclusion that the cells did not die, that the integrity of their chromosomes was unaffected, and that the cell cycle was generally slower in the mutant cells (Gatti and Baker, 1989).

\(lgfR^{+}\) is required cell autonomously for insulin-independent cell growth in the eye

We were curious to determine how the complete absence of \(lgfR^{+}\) activity would affect cells in the adult eye. We generated marked \(lgfR^{0/117}\) homzygous clones in a \(lgfR^{+/}\)/\(lgfR^{0/117}\) background in adult eyes. Because \(lgfR^{+}\) is required for cell proliferation, we expected the clones to be small and they were. We found that ommatidia within the clones composed of all \(lgfR^{0/117}\) cells \((n=18)\) were sometimes normally constructed, with eight photoreceptors arranged in a trapezoid, but the mutant ommatidia as a whole and all the R-cell rhombodemes within them appeared smaller than the wild-type cells surrounding them (Fig. 6B). At the clone borders there were ommatidia mosaic for \(lgfR^{-}\) and \(lgfR^{+}\) photoreceptors. In 354 mosaic ommatidia examined, the \(lgfR^{-}\) cells are usually smaller than wild-type (a photoreceptor of normal size was observed in only 8/354 facets) and the \(lgfR^{+}\) cells were of normal size. The small cell size was due to the \(lgfR^{-}\) mutation, because a \(glfR^{+}\) + transgene complements the clone mutant phenotype (data not shown). We conclude that \(lgfR^{+}\) is required cell autonomously for cells in the adult eye to attain their normal size.

The appearance of the \(lgfR^{-}\) retinal clones is similar to that of mutants in the insulin pathway (Figs. 6B, C), a major regulator of cell size (reviewed in Oldham and Hafen, 2003). As \(lgfR^{+}\) function in cell growth is autonomous, its role in the insulin pathway could be in the signal receiving cells, in which the insulin receptor is activated. In the \(lgfR^{-}\) clones, rhombodeme size appears to be a reflection of cell size. Individual R-cells are not outlined, but their collective cytoplasm, which ring the rhombodemes, appear reduced in size relative to wild-type in the \(lgfR^{-}\) ommatidia (Fig. 6B). In previous studies of \(InR\) mutants (Brogiolo et al., 2001), and mutants in other genes in the insulin pathway, similar observations were made of R-cells in mutant clones (see Fig. 6C). In these studies, the sizes of mutant cells in other tissues where the cells were outlined were obviously smaller. Similarly, as described above, we find that \(lgfR^{0/117}\) salivary glands have small cells (Fig. S3). Thus, in the experiments described below, we measured rhombodeme size and used it as indicator of cell size.

We performed a genetic experiment to determine if the small size of \(lgfR^{0/117}\) cells was due to a defective insulin pathway. Retinal cell clones doubly mutant for an \(InR\) null and a \(lgfR^{+}\) null allele (\(InR^{230}/\)\(lgfR^{0/117}\) cells) were generated and compared with \(InR^{230}\) clones and \(lgfR^{0/117}\) clones. We measured the decrease in rhombodeme size relative to wild-type in each single mutant and in the double mutant. If the
lqfR — cell size defect is due solely to a malfunction in insulin signaling, then lqfR−→lnr− double mutant cells should not be smaller than cells with either mutation alone. Conversely, if lqfR+ regulates cell size through a different pathway, then lqfR−→lnr− cells could be smaller than lqfR− or lnr− single mutant cells, if the effects of failure of each pathway are additive. We found that lqfR<sup>ΔENTH</sup> R-cell rhomboodies are on average 45% the size of wild-type (Figs. 6A, B), that lnr<sup>Δ339</sup> rhomboodies are on average 30% the size of wild-type (Figs. 6A, C). Double mutant lnr<sup>Δ339</sup> lqfR<sup>ΔENTH</sup> rhomboodies are on average 23% of wild-type size (Figs. 6A, D), and are significantly smaller than those of either single mutant (Fig. 6A). We conclude that the cell growth defect in lnr− retinas cannot be due solely to a role of LqfR in insulin signaling.

A mutant LqfR protein lacking the ENTH domain complements all apparent morphological aspects of the lqfR mutant phenotype

We found that an ENTH-less endocytic epsin (Lqf) protein, when overexpressed, complements all obvious aspects of the lqfR mutant phenotype (Overstreet et al., 2003). We were curious to know if, like the ENTH domain of Lqf, the LqfR ENTH domain is dispensable in Drosophila. We constructed two transgenes, one that expresses full-length LqfRa fused at the C-terminus to GFP (UAS-lqfRa-gfp), and one that expresses an ENTH-less LqfRa-GFP (UAS-lqfRa<sup>ΔENTH</sup>-gfp). We generated GFP fusions because we expected that in this case, the ENTH-less protein would not function, and we wanted to be able to determine if this was due to its failure to localize to the Golgi. Contrary to our expectation, when overexpressed ubiquitously with an Act5C-gal4 driver, one copy of either transgene rescues to wild-type all apparent aspects of the mutant phenotype of lqfR<sup>ΔENTH</sup> homozygotes (Figs. 7A, B, E and F and data not shown) including lethality, and rescues the nearly absent lqfR<sup>ΔENTH</sup> homozygous eyes generated using CMR-hid (Figs. 7H, K, L). The full-length GFP-tagged LqfRa protein localizes similarly to the endogenous protein; it is diffuse in the cytoplasm and also in p120-positive puncta (Fig. S5). The ENTH-less GFP-tagged LqfRa is more diffuse in the cytoplasm than the full-length protein, but is also present in p120-containing puncta (Fig. S5).

In order to determine whether overexpression of the ENTH-less protein was necessary for it to provide all LqfR function, we also constructed a genomic DNA transgene similar to UAS-lqfRa, in which the ENTH domain coding sequences are deleted above, in which the ENTH domain coding sequences are deleted (glqfR<sup>ΔENTH</sup>, Fig. 3A). One copy of a glqfR<sup>ΔENTH</sup> transgene that is not grossly overexpressed (Materials and methods, Fig. S6) also rescues to wild-type the mutant phenotype of lqfR<sup>ΔENTH</sup> homozygotes, including lethality (Fig. 7D and data not shown). We conclude that the ENTH domain of LqfR is dispensable for all of its obvious non-redundant functions in Drosophila.

Discussion

We have generated flies with null mutations in the Golgi epsin gene, lqfR, and analyzed several aspects of their mutant phenotype and also those of hypomorphs. We found that lqfR is essential for viability of the organism. Further experiments in the eye imaginal disc revealed that lqfR is needed for cell proliferation, insulin-independent cell growth, patterning at the D/V axis, advancement of the morphogenetic furrow, and regulation of expression of the rough transcription factor. Finally, we find that all of these functions of lqfR are independent of the ENTH domain.

What does the lack of a requirement for the ENTH domain mean?

Endocytic epsin (Lqf) functions without its ENTH domain, but overexpression of the ENTH-less protein may be required (Overstreet et al., 2003). Here, we found that even when expressed by its own promoter, an ENTH-less lqfR gene can substitute for all apparent functions of endogenous lqfR. Like Lqf, LqfR is able to localize to the appropriate (Golgi) membrane without the ENTH domain. Also, if the LqfR ENTH domain induces curvature of clathrin-coated vesicles as proposed for the ENTH domain of endocytic epsin (Ford et al., 2002), this function is not essential to the major role of LqfR in Drosophila. The Golgi epsin ENTH domain also serves as the recognition element for SNARE cargos (Miller et al., 2007), and thus we can infer that SNARES are not the cargo relevant to the critical function of LqfR.

Is lqfR required in a specific signaling pathway?

We think that lqfR is likely required for cell signaling because signaling pathways control both cell proliferation/growth and patterning (Baker, 2007), and in the absence of lqfR, there are defects in both of these aspects of cell behavior. Also, the observation that expression levels of a transcription factor are altered in lqfR mutants is consistent with a signaling pathway defect. Virtually all signaling pathways have an endosomal component (Piddini and Vincent, 2003; Le Roy and Wrana, 2005; Fischer et al., 2006), and moreover, Wnt signaling is known to require endosome-to-TGN trafficking specifically (Port et al., 2008; Belenkaya et al., 2008; Franc-Marro et al., 2008). As a single signaling pathway may control cell proliferation and growth and also patterning, it seems possible, although by no means necessary, that all aspects of the lqfR mutant phenotype could be due to misregulation of a single pathway.

Wnt signaling (Wingless [Wg] in Drosophila) requires the Golgi transmembrane protein Wntless (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Wntless is a Golgi protein that promotes secretion of the ligand Wg, which accompanies Wg to the plasma membrane. Wntless is subsequently endocytosed, and sorted for recycling back to the TGN through the action of the retromer complex (Port et al., 2008; Belenkaya et al., 2008; Franc-Marro et al., 2008). Protein sorting by retromer in the early endosome may be followed by epsinR-dependent clathrin-coated vesicle formation (Popoff et al., 2007). Thus, it seems possible that along with Wntless and the retromer proteins, LqfR might promote Wg signaling.

Can the lqfR mutant eye phenotype be explained by wg loss-of-function? (The role of Wg in eye disc morphogenesis is reviewed in Legent and Treisman, 2008.) Early in eye disc development, Wg and Hh signaling collaborate in the dorsal half of the eye disc to set up the D/V axis. Later, by diffusing from the dorsal and ventral disc margins, Wg organizes the gradients of Dachsous and Four-jointed proteins, which participate in defining planar cell polarity in the eye. Damping of Wg signaling could lead to patterning disruptions at the equator in weak lqfR mutants. Wg emanating from the dorsal and ventral margins also positions the morphogenetic furrow centrally by repressing expression of decapentaplegic (dpp) (see below), and promotes proliferation of cells anterior to the morphogenetic furrow that will become head capsule. Reduction of Wg activity in the eye disc results in initiation of ectopic furrows at the lateral margins of the eye disc, and a larger eye at the expense of head tissue. Essentially the opposite is observed in lqfR mutant eyes: halting of the furrow and a tiny eye. Also, the cell size defect observed in adult eyes is cell autonomous. Thus, some, but not all aspects of the lqfR eye phenotype can be explained easily by weak Wg signaling alone.

One hypothesis that may explain most features of the lqfR mutant eye phenotype is that rough is overexpressed in lqfR eye discs. Excessive Hh signaling results in arrest of the morphogenetic furrow through overexpression of rough (Chanut et al., 2000). Morphogenetic furrow progression is controlled mainly by Hh and Dpp (reviewed in Baker, 2007). Posterior to the furrow, differentiating R-cells express Hh, which activates Dpp expression, and both ligands diffuse anteriorly. In cells anterior to the furrow, Dpp arrests the cell cycle in G1, and Hh subsequently initiates R-cell differentiation. Hh signaling is able to arrest the cell cycle also, but as Dpp diffuses faster than Hh, Dpp...
normaly plays this role. As the R-cells differentiate, they start to express Hh, and this cycle moves the furrow forward. Anterior to the furrow, through Notch, Hh activates expression of the proneural transcription factor Atonal, which is required for subsequent Hh expression by R-cells posterior to the furrow. Rough expression is also activated by Hh, and it blocks Atonal expression in some cells. The roPom allele appears to be hypersensitive to Hh activity, and thus Rough is overexpressed and blocks Atonal in too many cells, leading to loss of Hh expression posterior to the furrow, and the furrow stops (Chanut et al., 2000). Thus, overactivity of Hh in lqfR discs could result in rough overexpression and halting of the furrow. Third, the D/V axis in the eye disc is defined by a stripe of cells in which Notch is activated. Setting up of the Notch stripe requires Hh, which is expressed only dorsally and defines the dorsal half of the eye disc (Legent and Treisman, 2008). Hh overactivity sometimes results in enlargement of the dorsal area of the eye, but not always, and as in lqf, the D/V boundary is still present (Thomas and Ingham, 2003). As Hh can negatively regulate the cell cycle anterior to the furrow (Baker, 2007), it seems that Hh overactivity could account for the cell proliferation defect.

By what mechanism could LqfR negatively regulate Hh signaling? (The mechanism of Hh signaling is reviewed in Kalderon, 2005, and Rohatgi and Scott, 2007.) Hh signaling is transduced by the transmembrane protein Smoothened (Smo), which is negatively regulated (through mechanisms that are not entirely clear) by the Hh receptor Patched (Ptc) when it is not bound to Hh. Binding of Hh to Ptc relieves negative regulation of Smo. Hh/Ptc induces Smo phosphorylation, thereby inducing Smo to switch into active conformation (Zha et al., 2007). Hh/Ptc also binds the glypican Dally-like (Dlp), which serves as an endocytosis signal. Hh/Ptc endocytosis results in Smo translocation to the plasma membrane and signaling (Gallet et al., 2008; Beckett et al., 2008). LqfR could regulate Smo negatively through recycling of Ptc to the plasma membrane, or by promoting the transport of Smo or Dlp away from the plasma membrane, possibly to the lysosome instead. This role for LqfR would be similar to that of yeast ent3p/ent5p in the multi-vesicular body.

Further experiments are required to determine if LqfR plays any role at all in Wntless endosome-to-TGN recycling, in Hh signaling, or in one or several other pathways. The mutants and phenotypic characterization described here suggest strongly that like Lqf, LqfR is involved in signaling, and provide the tools for testing these and other hypotheses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.03.029.

References


