The RNA-binding Proteins FMR1, Rasputin and Caprin Act Together with the UBA Protein Lingerer to Restrict Tissue Growth in *Drosophila melanogaster*

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**Abstract**

Appropriate expression of growth-regulatory genes is essential to ensure normal animal development and to prevent diseases like cancer. Gene regulation at the levels of transcription and translational initiation mediated by the Hippo and Insulin signaling pathways and by the TORC1 complex, respectively, has been well documented. Whether translational control mediated by RNA-binding proteins contributes to the regulation of cellular growth is less clear. Here, we identify Lingerer (Lig), an UBA domain-containing protein, as a growth suppressor that associates with the RNA-binding proteins Fragile X mental retardation protein 1 (FMR1) and Caprin (Capr) and directly interacts with and regulates the RNA-binding protein Rasputin (Rin) in *Drosophila melanogaster*. *lig* mutant organs overgrow due to increased proliferation, and a reporter for the JAK/STAT signaling pathway is upregulated in a *lig* mutant situation. *rin, Capr* or *FMR1* in combination as double mutants, but not the respective single mutants, display *lig*-like phenotypes, implicating a redundant function of Rin, Capr and FMR1 in growth control in epithelial tissues. Thus, Lig regulates cell proliferation during development in concert with Rin, Capr and FMR1.

**Introduction**

Understanding how cells and organs control their growth is a major endeavor in developmental biology. In *Drosophila melanogaster* and in mammalian systems, genetic studies have revealed a tight regulation mainly at two different layers. Whereas the Hippo and the Insulin receptor signal transduction pathways alter the transcription of growth-regulatory genes via the co-transcriptional factor Yorkie and the transcription factor FoxO, respectively, TORC1 controls translational initiation via 4EBP and S6K [1]. However, increasing evidence indicates that RNA-binding proteins like Fragile X mental retardation 1 protein (FMR1), mammalian cytoplasmic activation/proliferation associated protein (Caprin) and mammalian Ras-GTPase activating protein SH3 domain binding protein (G3BP) regulate growth and growth factors at the translational level [2–5].

In humans, loss of FMR1, a protein with one RGG RNA-binding and two KH domains, causes the most common form of inherited mental retardation, the fragile X syndrome (FXS). Analysis of FMR1 function in the model organisms mouse and *Drosophila* implicated FMR1 in cell proliferation, cell differentiation and apoptosis in reproductive organs and neuronal tissue via translational regulation of growth-regulatory proteins. For example, FMR1 knockout mice display increased proliferation of adult progenitor/stem cells in two-month-old mice, probably caused by increased protein levels of Cdk4, Cyclin D1, and GSK3β as a result of missing translational regulation [2]. In *Drosophila*, FMR1 maintains germline stem cells in oocytes using the miRNA bantam [6], and brains of *Fmr1* mutants display increased neuroblast proliferation rates with altered Cyclin E levels [7]. Recently, it was demonstrated that FMR1 associates with the RNA-binding protein Caprin in mice [8] and flies [9] to cooperate in binding to the same mRNA targets (at least in flies [9]).

In humans, Caprin-1 and Caprin-2 comprise the homologous region-1 (HR1) and the homologous region-2 (HR2), which contain RGG motifs. Caprin levels have been correlated with proliferation, e.g. in human T- or B-lymphocytes [10] and the chicken lymphocyte line DT40 [11]. In contrast, inhibition of cell proliferation has been observed e.g. by overexpression of GFP-Caprin-1 in NIH-3T3 cells [10]. Caprin interacts with another RNA-binding protein, G3BP, and binds to growth-associated mRNAs, such as *c-myc* and *cyclin D2* [4]. *Drosophila* Caprin (Capr), which shares the HR1 domain and three RGG motifs but lacks the HR2 domain, cooperates with FMR1 to regulate the cell cycle via the repression of the *CycB* and *Fruhstart* mRNAs at the mid-blastula transition in embryos [9].

G3BP consists of an NTF2-like domain and RNA-binding domains (RRM and RGG). It has been implicated in translational control and mRNA decay of growth factors in mammalian model systems. For example, in quiescent Chinese hamster fibroblasts, human G3BP has been reported to bind to the *c-myc* 3′ UTR and to mediate *myc* mRNA decay [12,13]. Furthermore, in a Filamin-C-RasGAP-dependent manner, G3BP regulates two RNA polymerase II kinases, Cdk7 and Cdk9, at the mRNA level.
Author Summary

Animal growth is orchestrated by controlled expression of growth-regulatory factors. This regulation is achieved at different molecular levels like transcription, translation initiation, and translational regulation. Whereas transcriptional control and translation initiation of growth components have been well studied, the role of translational control in this process is less well understood. Here, we describe Lingerer (Lig), an UBA domain-containing protein, as a new growth suppressor that associates with the three RNA-binding proteins Fragile X mental retardation protein 1 (FMR1), Rasputin (Rin) and Caprin (Capr). Drosophila FMR1, Rin and Capr orthologs are known translational regulators. In lig mutants and in FMR1, Capr and rin in combination as double mutants, organ size is increased due to excess proliferation. These data unveil a growth-regulatory function of Lig, and a redundant function of the RNA-binding proteins FMR1, Capr and Rin. Our findings demonstrate the involvement of mRNA-binding proteins in epithelial growth control and may also contribute to a better molecular understanding of the Fragile X mental retardation syndrome.

to control growth of cardiac myocytes [3]. However, in Drosophila, it is not known whether FMR1, Capr and Rasputin (Rin), the fly ortholog of G3BP, regulate cellular growth in epithelial tissues.

In this study, we identify the UBA domain-containing protein Lingerer (Lig) as a novel interaction partner of FMR1, Rin and Capr in flies and present genetic, biochemical and cell biological evidence that a complex of Lig with RNA-binding proteins restricts proliferation in growing tissues. Furthermore, we demonstrate that JAK/STAT signaling is activated in lig mutant cells.

Results

Lig suppresses tissue overgrowth by regulating cell number in a diet-dependent manner

In a tissue-specific genetic screen for suppressors of tissue growth [14], we recovered a complementation group consisting of three EMS-induced recessive lethal alleles based on increased eye and head size (Figure 1B and 1D). By subsequent mapping in combination with complementation tests, rescue experiments and sequencing, we identified lig as the gene responsible for the growth phenotype. lig encodes an conserved ubiquitin-associated (UBA) domain-containing protein. All three lig alleles, when placed over ligppi, a recessive lethal null allele [15], or over the deficiency Df(2R)Exel7094 uncovering the lig locus, resulted in lethality in an early pupal stage, forming long and slender pupae (Figure 1F and Figure S1A and S1B) as described for lig null mutants [15]. Both the lethality and the clonal overgrowth phenotype were rescued with one copy of a lig genomic rescue construct (Glig) (data not shown and Figure 1C, 1D and 1Z) but not with a genomic rescue construct containing a frameshift mutation in exon 10 (Gligg10) (data not shown and Figure 1Z). Sequence analysis of the lig protein-coding sequence of the EMS-induced alleles revealed small deletions (ligg1, ligg2) that result in premature stop codons and a point mutation (ligg3), respectively (Figure 1Z). We conclude that all three lig alleles represent null alleles.

To determine whether the lig overgrowth phenotype is due to increased cell number or enlarged cell size, we analyzed tangential sections of mosaic compound eyes composed of lig mutant clones and wild-type sister clones surrounded by heterozygous cells. In lig mutant ommatidia, all cell types were normally differentiated and structured and without cell size defects (Figure 1G), suggesting that the overgrowth phenotype is caused by more cells rather than larger cells. Analysis of adult lig mutant eyes revealed a variable ommatidia number. In most cases, the ommatidia number was increased as expected (Figure 1B and 1D), but in some cases, the ommatidia number was equal or even lower than the number in control eyes (Figure 1D). The ommatidia size was not altered in the lig mutant eyes (Figure S1C). The increased or reduced ommatidia number of lig mutant eyes was completely rescued to a control situation by the presence of the Glig transgene (Figure 1C and 1D), thus excluding a second-site mutation as the reason for the variability of the phenotype. Cellular growth is tightly linked to environmental factors like nutrient availability. The variability of the lig mutant eye phenotype might thus depend on food conditions. Indeed, animals raised on food with reduced yeast content (25% yeast and 40% yeast, respectively) were delayed and displayed eyes with a constant increase in ommatidia number (Figure 1I, 1N, S1E and S1H). In contrast, animals grown under normal food conditions (100% yeast) displayed a high variability (Figure 1K and 1N), and this effect was even more pronounced in flies from larvae that developed on food with increased yeast content (400%) (Figure 1M, 1M’ and 1N).

The diet-dependent phenotype of lig mutant eyes may be explained by varying amino acid levels or by altered developmental timing. To test the former possibility, larvae were grown on 40% yeast-containing food supplemented with the milk protein Casein to 100% protein content. However, this food condition did not increase the variability in lig mutant eyes (Figure S1G and S1H), excluding altered total amino acid levels as the reason for the variable lig phenotype. To investigate the latter possibility, we made use of a Minute mutation to reduce the developmental speed under normal food conditions and to generate eyes largely mutant for lig with the eyFLP/FRT system. In a second experiment, we induced the developing delay by raising the flies at 18°C. Interestingly, the ommatidia number of lig mutant eyes was stably increased only in the Minute experiment (Figure S1J and S1K) but variable at 18°C (Figure S1L). However, lig mutant eyes of flies raised on 25% yeast-containing food at 18°C produced a stable overgrowth phenotype (Figure S1M), excluding a temperature sensitivity of lig mutant cells. These results suggest that the diet-dependent phenotype of lig mutant eyes is not dependent on amino acid levels or developmental delay but is probably influenced indirectly by an unknown diet-sensitive process.

To investigate whether the variable phenotype is induced by increased apoptosis in lig mutant eyes, we overexpressed the Drosophila inhibitor of apoptosis (DIAPI) or baculovirus caspase inhibitor p35 in lig mutant eyes to block apoptosis. Indeed, lig mutant eyes overexpressing DIAPI displayed an increased ommatidia number (Figure 1R and 1S) in comparison to the control (Figure 1O and 1S). Flies with lig mutant eyes overexpressing p35 were dying as pharate adult except for a few escapers that displayed massively overgrown eye structures (Figure 1U). These results are consistent with published data that DIAPI overexpression leads to reduced apoptosis rates without developmental consequences [16,17], whereas p35 overexpression abolishes virtually all apoptosis but causes an aberrant morphology probably due to “undead cells” that activate compensatory proliferation [reviewed in [17]). We conclude that lig mutant cells are sensitive to apoptosis.

To test whether Lig acts as a general growth regulator, we generated two independent RNAi lines against lig to downregulate lig specifically in different developing tissues (Figure 1Z). The functionality of both RNAi lines was established by ubiquitous expression (using da-Gal4 as driver) resulting in pupal lethality like
Figure 1. Lig regulates organ size during development. (A–C) Scanning electron micrographs of the control (A), lig mutant overgrown eyes (B), and lig mutant eyes rescued by one copy of Glig (C). The mutant eyes were generated by eyFLP/FRT-mediated mitotic recombination. Scale bar represents 100 μm. (D) Quantification of ommatidia number from two independent experiments. Statistical analyses were done with a Student’s t test (two-tailed, unpaired). Error bars indicate the standard deviations, (n) number of organs analyzed. Mean ± s.d. and p-values: Control (808 ± 27 and 780 ± 20), lig mutant eyes (856 ± 23; p = 0.0027 and 806 ± 28; p = 0.031) and lig mutant eyes with one copy of a genomic rescue transgene for lig (ND (not determined) and 768 ± 9; p = 0.0011). (E–F) lig1 in combination with ligPP1, a lig null mutant allele, causes long, slender pupae (F) in comparison to the control (E). Scale bar represents 500 μm. (G) Tangential eye sections of adult lig1 mosaic eyes reveal normal differentiation and cell size in lig1 mutant clones. The lig1 mutant cells are marked by the absence of pigmentation. (H–M) Scanning electron micrographs of adult control and lig1 mutant eyes generated by eyFLP/FRT-mediated mitotic recombination from flies grown on 25% (H–I), 100% (J–K) or 400% (L–M') yeast-containing food. Scale bar represents 100 μm. (N) Statistical analyses as described in Figure 1D: control (690 ± 39 and 726 ± 21) and lig1 mutant (729 ± 27; p = 0.022 and 789 ± 20; p = 2.35E-05) eyes at 25% yeast-containing food, control (763 ± 23 and 749 ± 23) and lig1 mutant (747 ± 47; p = 0.33 and 761 ± 43; p = 0.46) eyes at 100% yeast-containing food, and control (708 ± 38 and 719 ± 43) and lig1 mutant (688 ± 53; p = 0.35 and 700 ± 75; p = 0.48) eyes at 400% yeast-containing food. (O–R) Scanning electron micrographs of adult control (O), lig1 mutant (P), DIAP1 overexpressing (Q) and lig1 mutant DIAP1 overexpressing eye (R) generated by eyFLP, Actin-Flp out-Gal4/FRT-mediated mitotic recombination. Scale bar represents 100 μm.

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lig mutants (Figure S1O and S1P) and by compartment-specific reduction of Lig protein levels in the developing eye using the Drosophila CD2 driver line (Figure S1R and S1S). Expression of lig RNAi in developing eyes increased the ommatidia number (Figure S1U, S1V and S1W) without effecting cell size (Figure S1X) under normal food conditions, similar to the lig mutant situation. Consistently, Lig reduction in developing wings by means of RNAi induced overgrowth (Figure 1W, 1X and 1Y), identifying Lig as a general growth regulator.

Cells overexpressing lig undergo apoptosis

We next tested the effects of lig overexpression in the developing eye under different food conditions using the Gal4/UAS system. To this end, we generated two UAS-lig transgenic lines: UAS-ligR185C encodes a protein version with an amino acid exchange (published as wild type in [15]), including the 5′ and 3′ UTRs of lig. Overexpression of the transgenes in the proliferating cells of the developing eye led to smaller adult eyes with fewer ommatidia (Figure 2B, 2C, 2E, 2F, 2H, 2I and 2J), and similar effects were obtained for UAS-ligR185C(Figure S2B and S2C), suggesting that the amino acid exchange R185C represents a polymorphism. Whereas the overexpression induced by UAS-ligR185C mildly reduced the ommatidia number independently of the diet (Figure 2B, 2E, 2H and 2J), UAS-ligR185C(Figure S2Z and S2C), suggesting that the amino acid exchange R185C represents a polymorphism. Therefore, we tested whether Lig punctae localize to P-body. Therefore, we tested whether Lig punctae localize to P-body. Therefore, we tested whether Lig punctae localize to P-body. Therefore, we tested whether Lig punctae localize to P-body.

Lig interacts and co-localizes with the RNA-binding domain-containing proteins FMR1, Rin and Capr

To elucidate the function of Lig, we attempted to identify binding partners of Lig using affinity purification coupled with mass-spectrometry (AP-MS). In this experiment, HA epitope-tagged Lig interacted with Rin, FMR1 and DART1, a functional Arginine methyl transferase, in Drosophila cultured cells (Table S1). A complex including Lig, Rin, FMR1, Capr, and Orb (isoRNA binding), the Drosophila cytoplasmic polyadenylation element binding (CPEB) protein, has been identified by co-immunoprecipitation (CoIP) in ovarian extracts using Orb as bait [18]. To confirm the interactions observed in the AP-MS experiment, we performed co-localization experiments with overexpressed epitope-tagged proteins in cultured Drosophila cells. Lig, FMR1 and Rin localized in punctae in the cytoplasm and were not observed in the nucleus (Figure S3A, S3B and S3C). Co-overexpression of Lig, FMR1 and Rin (pairwise and all three together) (Figure S3A′, S3B′ and S3C′) or Lig and Capr revealed co-localization in cytoplasmic punctae (3C′′). In contrast, no co-localization was observed between Lig and DART1 (Figure S3F′′). To test whether the endogenous proteins of Lig, Capr, FMR1 and Rin co-localize in cultured Drosophila cells, we transfected the cells with a Cherry-tagged Rin genomic rescue transgene (GrafCherry) and performed antibody stainings to visualize Lig, FMR1 and Capr. Rin-Cherry was homogeneously distributed in the cytoplasm (Figure S3H). In some cases, we observed discrete punctae in the cytoplasm suitable for co-localization studies. Indeed, Lig, FMR1 and Capr co-localized with these punctae (Figure S3I′, S3J′ and S3K′). However, when we analyzed Lig and Capr localization in cultured Drosophila cells by antibody staining, Lig and Capr co-localized only within bigger dots in few cells (S3L′).
Figure 2. Eyes overexpressing lig are reduced in size and are partially rescued by CycE and DIAP1 co-overexpression. (A–I) Scanning electron micrographs of eyes overexpressing the indicated UAS transgenes under the control of ey-Gal4 during development from flies reared on 25% (A–C), 100% (D–F) and 400% (G–I) yeast-containing food, respectively. Scale bar represents 100 μm. (J) Statistical analyses as described in Figure 1D: ey-GFP (741 ± 30 and 753 ± 12), ey>lig (628 ± 31; p = 1.66E-10) and ey>ligR185C/UTR (448 ± 80; p = 4.35E-06) eyes at 25% yeast-containing food, ey-GFP (768 ± 14 and 768 ± 12), ey>lig (666 ± 30; p = 3.03E-05) and ey>ligR185C/UTR (336 ± 58; p = 6.71E-05) eyes at 100% yeast-containing food and ey-GFP (748 ± 34 and 785 ± 21), ey>lig (588 ± 33; p = 1.55E-06) and ey>ligR185C/UTR (193 ± 50; p = 1.18E-10) eyes at 400% yeast-containing food. (K–L) lig overexpressing clones (induced with the Actin-Flp out-Gal4 system and marked by GFP) in wing imaginal discs of third instar larvae undergo apoptosis as judged by Cleaved Caspase-3 staining (red) in comparison to the control (K–K’). Scale bar represents 50 μm. (M–R) The reduced size of eyes overexpressing ligR185C/UTR induced by ey-Gal4 (N) is partially rescued by co-overexpression of DIAP1, an inhibitor of apoptosis (P), or CycE (R). Overexpression of DIAP1 (O) or CycE (Q) has no effect on eye size in comparison to the control (M). Scale bar represents 100 μm. (S) Statistical analyses as described in Figure 1D: ey-GFP (770 ± 15 and 746 ± 14), ey>ligR185C/UTR (309 ± 34 and 135 ± 73), ey>GFP, DIAP1 (789 ± 17 and 790 ± 16), ey>ligR185C/UTR, DIAP1 (477 ± 47; p = 9.60E-06 and 410 ± 51; p = 7.97E-06), ey>GFP, CycE (753 ± 33 and 708 ± 23), ey>ligR185C/UTR, CycE...
Based on the localization experiments, we focused on the interaction between Lig, FMR1, Rin, and Capr. To test for direct interactions, we performed a yeast two-hybrid (Y2H) assay. Lig, FMR1, Rin, and Capr were N-terminally fused to the activation domain (AD) or to the DNA-binding domain (BD) of Gal4, respectively, and tested for autoactivity (Figure S4A). We used plates lacking adenine (ADE) to test for strong interactions and plates lacking histidine (His) for weak interactions. Lig interacted with Rin but not with FMR1 or Capr in the Y2H assay (Figure 4A, and data not shown), identifying Rin as a direct interaction partner of Lig. The interaction between Lig and Rin was only visible when Lig and Rin were tagged with the AD and the BD, respectively. To identify the interaction domain in Rin, we generated three Rin protein fragments: Rin1-175 consisting of the NTF2-like domain and the acid-rich region, Rin[129-495] containing the acid-rich region and six PspX motifs, and Rin[145-690] containing the RNA recognition motif (RRM) and Arginine-Glycine rich region (RGG) (Figure 4B and data not shown). In the Y2H assay, the fragment encompassing the NTF2-like domain interacted with Lig (Figure 4A). Proteins with NTF2-like domains like NTF2, TAP13/p15 and Importinβ have been shown to bind to FxFG, FG and GLFG repeats [22-24]. Recently, the structure of the Rin NTF2-like domain was resolved but binding sites for the FG motifs are not conserved [25]. However, analysis of Lig, which consists of a predicted UBA domain at the N-terminus and four conserved regions (CR2-4) [13] (Figure 4B), revealed two FGs in close proximity within the CR3 that could serve as a binding site for the NTF2-like domain of Rin. Indeed, when we mutated the FG repeat to a Leucine-Alanine (LA) repeat in Lig, the interaction between Lig and Rin was completely abolished (Figure 4A and 4B). Thus, Rin is a direct interaction partner of Lig and the interaction occurs via the NTF2-like domain of Rin and the FG repeat of Lig.

FMR1, Rin and Capr synergize in growth control to inhibit proliferation in epithelial tissues

The physical interaction of Lig with the RNA-binding domain-containing proteins Rin, FMR1 and Capr suggested that Lig is involved in an RNA-regulatory network and regulates growth via Rin, FMR1 and Capr. To investigate this possibility, we first focused on Rin and FMR1 that we identified as binding partners in the AP-MS experiments. No growth phenotypes in FMR1, Rin and Capr synergize in growth control to inhibit growth in the AP-MS experiments. No growth phenotypes in FMR1, Rin and Capr suggested that Lig is involved in an RNA-regulatory network and regulates growth via Rin, FMR1 and Capr. To investigate this possibility, we first focused on Rin and FMR1 that we identified as binding partners in the AP-MS experiments. No growth phenotypes in FMR1, Rin and Capr, respectively, were observed in the lig mutant situation (Figure 5J and 5K), suggesting that the lig mutant phenotype, we never observed a variability of the ommatidia number in Figure 5S, S5A’ and S5A’’. Indeed, cells homozygous for either of the P-elements upregulate RinCherry, verifying both P-elements as alleles (Figure S5B, S5B’, and S5C’). Both P-elements placed over rin were viable without phenotypic alterations (data not shown). In the eyFLP/FRT experiment, rin but not the rin P-elements or FMR1 mutant eyes showed an increase in ommatidia number (Figure S5B-5D, S5E-5G) under normal food conditions (100% yeast). In contrast to the lig mutant phenotype, we never observed a variability of the ommatidia number in FMR1 or rin mutant eyes under normal food conditions. Thus, the single mutant phenotypes of FMR1 and rin did not display growth phenotypes similar to the effects caused by lig.

In a next step, we tested for functional redundancy using a double mutant situation of rin and FMR1 since FMR1 and Rin are dispensable for viability and are both RNA-binding proteins that co-localize in cultured Drosophila cells. Most rin, FMR1[P5113M] homozygous larvae died at an early stage but few escapers that reached the early pupal stage formed long, slender pupae (Figure 3F), reminiscent of the lig null mutant phenotype. Consistently, P[GacB]rin[P524A], FMR1[P534M] or P[GacB]rin[P5720] FMR1[P63M] over rin, FMR1[P63M] also resulted in long slender pupae (Figure S5K and S5Q). Note that pupae with the P-element FMR1D50M, rin2, and FMR1D113M mutants so far. To analyze a putative growth function of FMR1 and Rin, we used the FMR1 null mutant alleles FMR1[P5113M] and FMR1[P63M] and the rin null mutant allele rin+, respectively. Flies homozygous for the FMR1 alleles or the rin allele are viable and do not display obvious growth phenotypes. Note that rin contains a 13 kbp deletion removing the complete coding sequence of rin as well as the Rbp4 and Hhbp7F loci (Figure 5S). Hence, we attempted to identify additional rin alleles to exclude secondary effects of Rbp4 and Hhbp7F. We wondered whether the P-elements P[GacB]rin[P524A] and P[GacB]rin[P5720], inserted in the 5‘ UTR of rin, are rin alleles and tested them with a Cherry-tagged Rin genomic rescue transgene (rinCherry). In the course of the rin rescue experiments, we identified a Rin dosage-dependent regulation of rinCherry. Whereas Rin-Cherry was upregulated in rin mutant clones, Rin-Cherry was slightly downregulated in the sister clone, suggesting a tight regulation of rin to achieve wild-type levels of the gene product (Figure 5S, S5A’ and S5A’’). Indeed, cells homozygous for either of the P-elements upregulate RinCherry, verifying both P-elements as alleles (Figure S5B, S5B’, and S5C’). Both P-elements placed over rin were viable without phenotypic alterations (data not shown). In the eyFLP/FRT experiment, rin but not the rin P-elements or FMR1 mutant eyes showed an increase in ommatidia number (Figure S5B-5D, S5E-5G) under normal food conditions (100% yeast). In contrast to the lig mutant phenotype, we never observed a variability of the ommatidia number in FMR1 or rin mutant eyes under normal food conditions. Thus, the single mutant phenotypes of FMR1 and rin did not display growth phenotypes similar to the effects caused by lig.

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Figure 3. Lig co-localizes with Rin, FMR1, Capr and P-body components in cultured Drosophila S2 cells. (A–D’’) S2 cells co-transfected with GFP-lig (A’, A’’), GFP-FMR1 (A’, A’’), GFP-lig (B’, B’’), GFP-FMR1 (B’, B’’), GFP-lig (C’, C’’), and GFP-lig (D’, D’’), RFP-FMri1 (D’, D’’), and FLAG-rin (D’, D’’). Cells were stained with DAPI to visualize DNA (blue) and for the FLAG-tag to visualize FLAG-Rin (D’, D’’). Scale bars represent 25 μm. (E–F’’) Lig co-localizes with P-body markers in S2 cells transfected with GFP-DCP1 (E’, E’’), and GFP-lig (E’, E’’), and GFP-lig (F’, F’’). Cells were stained with DAPI (blue) to visualize DNA and with anti-Ago1 antibody (F’, F’’). Note that endogenous Ago1 is accumulating in GFP-Lig foci. Scale bars represent 25 μm.

doi:10.1371/journal.pgen.1003598.g003

Figure 4. Lig interacts with Rin in Y2H experiments. (A) Y2H interactions between Lig, Lig-FG-LA, Rin, Rin-1–175 and Rin-129–492. Lig fused to the AD does not interact with Rin fused to the BD. Lig binds to the fragment Rin-1–175 containing the NTF2-like domain but not to the fragment Rin-129–492 containing the PxxP motifs. Lig with mutated putative binding sites for Rin (FG repeat mutated to LA repeat; Lig-FG-LA) does not interact with Rin-1–175. (B) A linear protein model of Lig with the predicted UBA domain, the conserved regions (CR2-4) and the FG repeat is presented at the top. Linear protein models of Rin with the NTF2-like domain, the acid-rich domain, the PxxP motifs and the RNA-binding motifs (RNA recognition motif (RRM) and arginine-glycine rich region (RRG)) as well as the Rin fragments Rin-1–175 and Rin-129–492 are presented in the middle. A linear protein model of Lig with mutated FG (Lig-FG-LA) repeat is presented at the bottom.

doi:10.1371/journal.pgen.1003598.g004

yeast) (Figure S5O, 5O’ and 5P). Furthermore, overexpression of p35 in FMR1 rin double mutant eyes resulted in pharate adults except for some escapers displaying massively overgrown eyes (Figure 5R). Taken together, FMR1 rin double mutant eyes, but not the single mutants, displayed a lig like phenotype, suggesting a functional relationship between lig, FMR1 and rin.

Recently, Capr2 null mutants were described to be viable without morphological alterations, and Capr and FMR1 cooperatively regulate the cell cycle at the mid-blastula transition [9]. We wondered whether Capr acts redundantly with FMR1 and Rin in growth control in epithelial tissues. To characterize the Capr growth phenotype, we generated mutant eyes during development using the eyFLP/FRT technique or by downregulation of Capr via RNAi. Note that we used a Minute mutation instead of a cell lethal mutation on the FRT80 chromosome. CaprRNAi overexpression in clones resulted in a strong reduction of Capr protein (Figure S6A’), proving the functionality of the RNAi line. Both Capr2 null mutant eyes and eyes with downregulated Capr displayed slightly reduced eye size in comparison to the controls (Figure 6B, 6C, 6E and 6L). In contrast, downregulation of Capr in FMR1 rin mutant eyes resulted in overgrown eyes due to more ommatidia (Figure 6G, 6I and 6L). Furthermore, downregulation of Capr in FMR1 rin double mutant eyes resulted in late pupal lethality. Analysis of eyes and heads revealed strongly overgrown structures in pharate adults (Figure 6K), suggesting that FMR1, Rin and Capr act synergistically in growth regulation.

Lig synergizes with FMR1, Rin and Capr and controls rin expression at the transcriptional level

The similarity of the lig and the FMR1, rin or Capr phenotypes in combination of double mutants prompted us to genetically test whether Lig regulates growth via FMR1, Rin and Capr. We downregulated lig via RNAi in FMR1, rin or Capr mutant eyes induced by the eyFLP/FRT system. Note that lig RNAi eyes did not consist of more ommatidia under reduced food conditions (Figure 7B and 7H) in comparison to flies raised under normal conditions (Figure S1W). Reduced Lig levels in FMR1 or rin mutant eyes increased the eye size due to more ommatidia (Figure 7D, 7F and 7H). Flies with Capr mutant eyes and reduced lig were dying as pharate adults with increased and disturbed eye structures (Figure 7J). We conclude that Lig cooperates with FMR1, Rin and Capr in growth control. The fact that the single mutants of FMR1, rin or Capr have no or minor effects on growth regulation, whereas the double mutants have similar effects like lig mutants, suggests that Lig modulates FMR1, Rin and Capr function in concert.

Next we checked the localization and protein levels of FMR1, Capr and Rin in lig mutant clones induced by the hsFLP/FRT system in developing eyes. Whereas FMR1 showed no localization or abundance alterations (Figure 7K”) and Capr only a slight upregulation (Figure 7L”) in lig mutant cells, Rin-Cherry levels were reduced in lig mutant clones (Figure 7M’”), indicating that Lig mainly regulates Rin levels.

Vice versa, Rin-Cherry levels were upregulated in lig overexpressing clones in eye imaginal discs (Figure S7A’ and S7A”). Recently, Rin has been identified as substrate for ubiquitination in the central nervous system [26]. To test whether Lig regulates Rin at the protein level, we induced lig null mutant clones in eye imaginal discs expressing a HA-tagged Rin under the control of an UAS promoter. In this situation, Lig was not able to regulate Rin (Figure S7B” and S7B”), excluding Lig as stabilizer of the Rin protein. We then investigated whether Lig regulates rin at the transcriptional and/or translational level. Lig overexpression in S2 cells was able to increase Rin-Cherry expression by GrinCherry
Figure 5. Rin cooperates with FMR1 to suppress growth. (A–C) Scanning electron micrographs of adult control (A), FMR1<sup>D113M</sup> (B) and rin<sup>2</sup> (C) mutant eyes generated by eyFLP/FRT-mediated mitotic recombination. Scale bar represents 100 μm. (D) Statistical analyses as described in Figure 1D: control (724±13 and 766±18), FMR1<sup>D113M</sup> (728±19; p = 0.68 and 772±17; p = 0.56) and rin<sup>2</sup> (761±13; p = 1.85E-04 and 801±17; p = 2.16E-03). (E–F) FMR1<sup>D113M</sup> rin<sup>2</sup> homozygous animals die in the early pupal stage forming long, slender pupae (F) in comparison to the control (E). Scale bar represents 300 μm. (G–J) Scanning electron micrographs of adult eyes from control (G), FMR1<sup>D113M</sup> rin<sup>2</sup> (H), P(fmr1.14); FMR1<sup>D113M</sup> rin<sup>2</sup> (I) and Grin<sup>Cherry</sup>; FMR1<sup>D113M</sup> rin<sup>2</sup> (J) mutant eyes generated by eyFLP/FRT-mediated mitotic recombination. Scale bar represents 100 μm. (K) Statistical analyses as described in Figure 1D: control (744±37 and 772±40; p = 1.02E-04) mutant eyes at 25% yeast-containing food. (L–O) Scanning electron micrographs of adult control (D), Capr2<sup>FRT80</sup> (E), CaprRNAi (F) and Capr2<sup>FRT80</sup> CaprRNAi (G) mutant eyes generated by eyFLP/FRT-mediated mitotic recombination from flies grown on 25% yeast-containing food. Scale bar represents 100 μm.

Figure 6. Capr cooperates with FMR1 and Rin to suppress growth. (A–B) Scanning electron micrographs of adult control (A) and Capr<sup>+</sup> (B) eyes generated by eyFLP/FRT Minute mediated mitotic recombination from flies reared on 25% yeast food (A–B). Scale bar represents 100 μm. (C) Statistical analyses as described in Figure 1D: control (772±22 and 765±15) and Capr<sup>+</sup> (754±18; p = 0.11 and 738±26; p = 0.04) mutant eyes from flies raised on 25% yeast-containing food. (D–K) Scanning electron micrographs of adult control (D), Capr<sup>RNAi</sup> (E), FMR1<sup>D113M</sup> (F), Capr<sup>RNAi</sup> FMR1<sup>D113M</sup> (G), rin<sup>2</sup> (H), Capr<sup>RNAi</sup> rin<sup>2</sup> (I), FMR1<sup>D113M</sup> rin<sup>2</sup> (J) and Capr<sup>RNAi</sup> FMR1<sup>D113M</sup> rin<sup>2</sup> (K) eyes generated by eyFLP, Actin-Flp out-Gal4/FRT-mediated mitotic recombination. (Q–R) Scanning electron micrographs of adult control (760±20; p = 1.17E-03 and 787±16; p = 4.45E-02) and Grin<sup>Cherry</sup>, FMR1<sup>D113M</sup> rin<sup>2</sup> (760±35; p = 9.4E-03 and 785±33; p = 7.87E-02) identical control in the second experiment of (D) and (K). (L–O) Scanning electron micrographs of adult control and FMR1<sup>D113M</sup> rin<sup>2</sup> eyes generated by eyFLP/FRT-mediated mitotic recombination from flies grown on 25% (L–M) or 400% (N–O') yeast-containing food. Scale bar represents 100 μm.

Lig regulates a reporter of the JAK/STAT pathway

We demonstrated that Lig regulates cell proliferation in concert with the mRNA binding proteins FMR1, Rin and Capr. To investigate which growth signaling pathway is altered, we tested readouts for various signaling pathways in lig mutant clones in wing and eye imaginal discs. FMR1 binds to the miRNA bantam to control the fate of germline stem cells [6]. bantam miRNA is a known target of the Hippo signaling pathway [27,28] and inhibits the pro-apoptotic gene hid [29]. If Lig regulates the Hippo pathway and/or bantam miRNA, we would expect an upregulation of a minimal Hippo response element (DIA4.3-GFP) and downregulation of the bantam sensor. In both experiments we did not observe any alteration of the reporter signal (Figure S8A, S8B). Consistently, overexpression of lig did not upregulate the bantam sensor (S8C). Furthermore, FMR1 was reported to
Figure 7. Lig cooperates with FMR1, Rin and Capr in growth control and regulates rin at the transcriptional level. (A–G) Scanning electron micrographs of adult control (A), ligRNAi II (B), FMR1D113M (C), ligRNAi II FMR1D113M (D), rin2 (E), ligRNAi II rin2 (F) eyes generated by eyFLP Actin-Flp out-Gal4/FRT-mediated mitotic recombination from flies grown at 25% yeast content (A–F). Scale bar represents 100 μm. (G) Statistical analyses as described in Figure 1D: control (714 ± 630 and 720 ± 630), ligRNAi II expressing (738 ± 632 and 706 ± 23), FMR1D113M mutant (737 ± 10 and 729 ± 34), ligRNAi II expressing FMR1D113M mutant (775 ± 636; p = 0.0051 and 806 ± 23; p = 0.00046), rin2 mutant (767 ± 634 and 760 ± 40), ligRNAi II expressing rin2 mutant (821 ± 645; p = 0.03 and 817 ± 69; p = 0.082) eyes from flies raised on 25% yeast-containing food. (H–J) Scanning electron micrographs of adult control (H), ligRNAi II (I) and ligRNAi II Capr2 (J) eyes generated by eyFLP Actin-Flp out-Gal4/FRT Minute-mediated mitotic recombination from flies grown on 25% yeast-containing food. (K–M') Negatively marked 72 h old lig' mutant clones (induced with the hsFLP/FRT system) in eye imaginal discs of third instar larvae (K', L' and M'). FMR1 levels (visualized by immunostaining) remain unchanged (K''). Rin-Cherry levels expressed from the GrinCherry transgene are autonomously decreased in the lig1 mutant clones (M''). Imaginal discs are stained with DAPI (blue) to visualize the DNA. Scale bar represents 25 μm. (N) S2 cells transfected with GFP-Lig and GrinCherry have increased levels of Rin-Cherry in comparison to S2 cells overexpressing GFP and GrinCherry. S2 cells overexpressing GFP-Lig upregulate a transcriptional reporter consisting of the rin promoter followed by a Cherry coding sequence and the 3' 9UTR of rin. Conversely, a translational reporter consisting of an ubi promoter followed by the 5' UTR of rin, a Cherry protein coding sequence and the 3' UTR of rin is not affected. Genotypes: (A) y eyFLP, Act>CD2>Gal4/y w; FRT82 cl w'/FRT82 (B) y w eyFLP, Act>CD2>Gal4/y w; UAS-ligRNAi [51D]++; FRT82 cl w'/FRT82 (C) y w eyFLP, Act>CD2>Gal4/y w; FRT82 cl w'/FRT82 FMR1D113M (D) y w eyFLP, Act>CD2>Gal4/y w; UAS-ligRNAi [51D]++; FRT82 cl w'/FRT82 (E) y w eyFLP, Act>CD2>Gal4/y w; FRT82 cl w'/FRT82 rin' (F) y w eyFLP, Act>CD2>Gal4/y w; UAS-ligRNAi [51D]++; FRT82 cl w'/FRT82 rin' (H) y w eyFLP, Act>CD2>Gal4/y w; UAS-CG1315RNAi (control)++; M(3)Rp517 FRT80+ (I) y w eyFLP, Act>CD2>Gal4/y w; UAS-ligRNAi [51D]++; M(3)Rp517 FRT80+ (J) y w eyFLP, Act>CD2>Gal4/y w; UAS-ligRNAi [51D]++; M(3)Rp517 FRT80+ (K, L) y w hsFLP/y w; FRT42 ubiGFP/FRT42 lig' (M) y w hsFLP/y w; FRT42 ubiGFP/FRT42 lig'; GrinCherry [86Fb]/+. doi:10.1371/journal.pgen.1003598.g007
regulate cbl mRNA, a negative regulator of the EGFR, to control germline cell proliferation in ovaries [30]. However, a transcriptional reporter for pointed expression, a target of the EGFR pathway, was not changed in lig mutant clones in eye imaginal discs (Figure S8D’’). Recently, increased Insulin signaling has been observed in FMR1 mutant brains using pAkt as readout [31]. In lig mutant clones in eye imaginal discs, we observed neither an increase of pAkt nor a recruitment of pAkt to the membrane, a sign for active Insulin signaling (Figure S8E’’). The Rin ortholog G3BP is involved in human c-myc mRNA decay by an intrinsic endonuclease activity [12,13]. However, we did not detect any alterations of Myc levels in lig mutant clones (Figure S8F’’).

**Figure 8. JAK/STAT signaling is activated in lig mutant cells.** (A–C) lig mutant clones (induced with the FLP/FRT system, 72 h old, marked by the lack of lacZ staining) in eye (A and A’), antenna (B and B’) and wing (C and C’) imaginal discs of early third instar larvae. The JAK/STAT signaling reporter 10xSTAT92E-GFP is upregulated in lig mutant clones in the posterior side of the eye imaginal disc (A’ and A’’), antenna imaginal disc (B’ and B’’) and in the hinge region of the wing disc (C’ and C’’). Note that the reporter signal is autonomously increased in the mutant clones. Scale bars represent 50 µm. (D) Schematic representation of the interactions shown in this study (left) and a working model of a Lig/Rin/FMR1/Caprin complex (right). Genotypes: (A–C) y w hsFLP/y w; FRT42 arm-lacZ/FRT42 lig1; 10xSTAT92E-GFP/+.

doi:10.1371/journal.pgen.1003598.g008
Seneless (Sens), two target genes of the Wnt signaling pathway in *Drosophila*. We did not observe any alterations of the Dll expression pattern in wing imaginal discs (Figure S6G') or of the Sens expression patterns in wing (Figure S6H') and eye imaginal discs (Figure S8I'), arguing against an involvement of Lig in Wnt signaling. We also tested Hedgehog, Notch and JAK/STAT signaling. Whereas Ptc and Gut patterns, targets of the Hedgehog and Notch signaling pathway, respectively, were not altered in *lig* mutant clones (Figure S8J'' and S8K''), a JAK/STAT reporter (10xSTAT92E-GFP) was upregulated in *lig* mutant clones. GFP expression from the 10xSTAT92E-GFP reporter was autonomously increased in *lig* mutant clones in the posterior region of eye discs (Figure 8A''), in antenna discs (Figure 8B'') and in the pleura and hinge regions of wing discs (Figure 8C'') of early third instar larvae. Consistent with our findings, Lig was identified as a negative regulator of JAK/STAT signaling in an RNAi based screen in cultured *Drosophila* Kc cells [32]. To determine whether Lig has an effect on STAT92E protein levels, we analyzed STAT92E expression in *lig* mutant clones in eye imaginal discs. We did not observe any alteration of STAT92E levels in the posterior region but an upregulation of STAT92E in the anterior region of the eye imaginal disc (Figure S8L'). Thus, based on the autonomous effects on the 10xSTAT92E-GFP reporter and on STAT92E levels, Lig regulates intracellular components of the JAK/STAT signaling pathway rather than the ligands.

**Discussion**

We have identified Lig as a new growth suppressor in eye and wing epithelial tissues. Whereas eyes mutant for lig consist of more ommatidia without cell size defects, eyes overexpressing lig have a reduced cell number due to increased apoptosis and reduced cell cycle progression. *lig* mutant eyes are sensitive to apoptosis (resulting in a variable phenotype under normal food conditions) but are able to cope with the overgrowth situation when the flies develop under suboptimal growth conditions. Similarly, the reduced eye phenotype of *lig* overexpressing eyes was partially rescued under suboptimal growth conditions or by expression of DIAP1, suggesting that the starvation response impacts on the apoptosis rates in imaginal discs. However, we cannot exclude other indirect effects that might be triggered by starvation.

In addition to our findings, *lig* mutants have previously been characterized for their behavioral phenotype in the copulation process [15] and their putative role in neuronal tissues [33]. Lig is conserved from flies to humans, the human orthologs being ubiquitin associated protein 2 (UBAP2) and ubiquitin associated protein 2 like (UBAP2L). UBAP2 has been identified in a Y2H screen as a direct interaction partner of the zona pellucida 3 (ZP3) protein that is involved in sperm binding and acrosomal exocytosis [34]. UBAP2L has been reported to accumulate at ubiquitin-rich aggregates upon proteasome inhibition in human neuroblastoma tissue culture cells, suggesting that the UBA domain is functional [35]. It is currently unknown whether the Lig orthologs are involved in growth regulation, and no interaction partners have been identified except for ZP3.

Several lines of evidence indicate that Lig interacts with FMR1, Capr and Rin, and via these interactions functions to regulate growth: (i) Lig associated with FMR1 and Rin in an AP-MS experiment, (ii) Lig co-localized with FMR1, Capr and Rin, (iii) Lig directly interacted with Rin in a Y2H experiment, (iv) Lig transcriptionally regulated Rin levels, and (v) FMR1, Capr or rin mutant eyes synergistically increased the eye size (Figure 8D).

The interaction between Lig and Rin, Capr and FMR1, three RNA-binding proteins, and the co-localization with P-body components suggests that Lig regulates the translation and/or stability of specific mRNAs of growth-regulatory genes via FMR1, Capr and Rin function. Indeed, the *Drosophila* FMR1 and orthologs of Rin are involved in translational regulation of growth-regulatory genes in certain tissues. For example, FMR1 binds *bantam* miRNA, an inhibitor of the pro-apoptotic gene *hid* [29], and regulates *cdk*, which encodes a component of the EGFR signaling pathway, in germline stem cells [30]. However, *bantam* miRNA is not regulated by FMR1 in epithelial cells [36], and Lig was unable to regulate a *bantam* miRNA reporter. Furthermore, the expression of a *pointed* transcriptional reporter was unchanged in *lig* mutant clones, suggesting that *cdk* regulation by FMR1 is specific to the germline or has only subtle effects in the developing eye. The Rin ortholog G3BP controls *myc* [12,13], *CyclinD2* [4], *edk* and *edk9* mRNA [3]. However, it is not known whether this function is conserved for Rin, and we did not observe any alterations of *Myc* protein levels in *lig* mutant clones. It will be important to identify mRNAs that are regulated by FMR1, Capr and Rin in epithelial tissues during development, and to determine whether Lig mediates specificity for certain mRNAs.

To identify the signaling pathway that is regulated by Lig, we used readouts for the Hippo, EGFR, Insulin, Hedgehog, Wnt and JAK/STAT signaling pathways. We observed no alterations of all analyzed pathways except for the highly conserved JAK/STAT signaling pathway. The pathway is composed of four modules: the ligands, Upd, Upd2 and Upd3, the receptor Domeless (*Dome*), the receptor-associated Janus kinase (JAK) Hopscotch (*Hop*), and the signal transducer and activator of transcription (STAT) STAT92E (reviewed in [37]). The involvement of Lig in the JAK/STAT signaling pathway leads to a number of assumptions and questions in the context of our findings. First, the autonomous effect of Lig on the 10xSTAT92E-GFP reporter suggests that Lig regulates the intracellular components (*Dome*, *Hop* or STAT92E) or modifiers thereof rather than expression of the ligands, which would result in non-autonomous effects. Second, the physical and genetic interactions of Lig with the mRNA binding proteins FMR1, Capr and Rin raises the question whether Lig directly impacts on the JAK/STAT pathway or whether it modulates the JAK/STAT signaling via FMR1, Capr and Rin. So far, we cannot exclude either option. However, it was recently demonstrated that *upd* and *STAT92E* mRNAs are targets for posttranscriptional regulation via the miRNA pathway [38,39]. It will be interesting to determine whether FMR1, Capr or Rin are involved in this process in the case of STAT92E.

Our data provide evidence that FMR1, Capr and Rin function in a redundant manner in epithelial tissues in growth control, suggesting that they regulate either overlapping sets of mRNAs or different mRNAs encoding proteins with redundant functions. Examples for the former have been described for FMR1, Capr and G3BP, the human ortholog of Rin. In *Drosophila*, FMR1 cooperates with Capr, and both proteins bind to the same mRNAs *frs* and *CycB* [9]. Similarly, G3BP forms a complex with human Caprin and both interact with *myc* and *CycD* mRNAs [4]. Both examples suggest a redundant regulation of these targets. There is no direct evidence for the latter possibility. However, G3BP associates with and translationally regulates *tau* mRNA in neuronal cells [40,41]. In *Drosophila*, FMR1 negatively regulates *futsch* mRNA [42], and the *futsch* mutant phenotype is suppressed by overexpression of *Tau* [43], suggesting a redundant function of Tau and Futsch.

Lig impacts on Rin and slightly on Capr but not on FMR1 levels. However, only FMR1, Capr or Rin mutants in combina-
tion as double mutants resulted in a lig like phenotype, suggesting that the activity of FMR1 and Capr is altered (probably at the posttranslational level) in a lig mutant situation. Our AP-MS experiments also revealed DART1 as a physical binding partner of Lig. Arginine methyl transferases are able to methylate RGG motifs and thereby modulate the binding capability to mRNAs [44,45]. Interestingly, FMR1 contains a conserved RGG domain that can be methylated in Drosophila and humans. In humans, protein methyltransferase 1 (PRMT1), the ortholog of DART1, mediates the arginine methyltransferase activity of FMR1 to alter its binding affinity to mRNAs [46] (Figure 8D). Furthermore, G3BP1, the mouse ortholog of Rin, contains a RGG domain that is methylated by PRMT1 after stimulation of the Wnt signaling pathway to modulate the binding to β-Catenin mRNA [5]. The RGG domain of Rin is weakly conserved and lacks the RGG motifs. It is thus unclear whether Rin can be methylated in the truncated arginine-glycine-rich region. Like FMR1 and G3BP, Caprin contains RGG domains, and it was identified as binding partner of PRMT8, which is closely related to PRMT1 at the sequence level [47]. Further experiments are required to resolve whether Lig is involved in a DART1-mediated methylation of FMR1 and Rin under certain conditions, or whether Lig alters the activity of FMR1 and Capr by another mechanism.

Lig, FMR1, Rin and Capr have been identified as interactors of Orb in Co-IP experiments [18], suggesting a complex formation of these proteins. Complex formation has been reported for G3BP and Caprin in human cell lines [4] and for Capr and FMR1 in Drosophila [9] and mouse neurons [8] so far (Figure 8D). We were able to demonstrate that Rin, Capr and FMR1 have a redundant function in the eye, and that they localize in the same subcellular structure in cultured Drosophila cells. This raises the question whether the three RNA-binding proteins Capr, Rin and FMR1 are functionally related only in the eye. Systematic analyses of the phenotypes of double mutant combinations will reveal the tissues in which these RNA-binding proteins exert redundant and non-redundant functions. Furthermore, it will be interesting to determine whether Rin and Capr contribute to phenotypes associated with the FXS.

Materials and Methods

Fly stocks and culture conditions

EMS-induced lig mutant alleles were recovered in an unbiased eyFLP/FRT cell lethal screen [14]. lig harbors a small deletion of 5 bp (nucleotides 3959163–3959167) and an insertion of an adenine at position 3959174. lig includes a small deletion of 17 bp (nucleotides 3958424–3958440). The nucleotide positions are indicated with EcoRI and then self-ligated. The resulting inverted repeats lig3959163, lig3959167 (gift from Yamamoto lab) into the pUAST attb 

Additional fly strains used in this study were: nubbin-Gal4 [51], da-Gal4 (BSDC), DE-Gal4 [52], ey-Gal4 (insertion on 2nd chromosome) [53], UAS-CycE [54], EP-Diap1 (BSDC), P(Fm1.14) [55], UAS-p35 (BDSC), DIAP1-GFP4.3 [56], 1oxSTAT92E-GFP [57], MIR33 bantam sensor (gift from Stephen Cohen), pnt-lacZ (P[lacW]pntS0998, former stock collection of Szeged, No. 121625).

Genetic experiments were conducted at 25°C. Food with 100% yeast consists of 7.5 g sugar, 5.5 g corn, 1 g flour, 0.8 g Agar, 1.5 ml Nipagin/Nipasol and 10 g fresh yeast filled up to 100 ml with tap water. For fly food with 25% or 40% yeast, the yeast amount was reduced to 2.5 g and 4 g yeast, respectively. 3.3 g Casein was used to substitute 40% yeast-containing food to 100% amino acid-containing food. For fly food with 400% yeast, the yeast amount was 40 g fresh yeast. 10 ml of food was filled into vials with a diameter of 29 mm. For experiments with different food conditions, 100–150 embryos of each cross were collected from apple agar plates and distributed to individual vials.

Analysis of adult flies

To assess the ommatidia number, flies were exposed to dimethyl ether for 7–10 min before taking scanning electron micrographs with a JEOL 6360 VP microscope. The ommatidia number was counted using a semi-automated ommatidia counter software (Ommatidia counter, version 0.3, programmed by Vasco Medic, SciTrackS GmbH). Pictures from pupae and adult wings were taken with a Keyence VHX-1000 microscope. Tangential eye sections of adult eyes were done as previously described [58].

Cloning and generation of transgenic fly lines

The Gig was subcloned from pCasSpr-R-Gig [15] into the gattv vector using the restriction sites XhoI and XbaI. The framesshift in the Gig construct was obtained as a spontaneous mutation during the subcloning.

For the lig RNAi lines, the regions I (308 bp) and II (252 bp) were amplified with the primer pairs Lig_RNAI_FB, Lig_RNAI_NB and Lig_RNAI_FC, Lig_RNAI_RC, respectively, using pENTR-lig as template. The fragments were first digested with EcoRI and then self-ligated. The resulting inverted repeats were cloned into a modified gattv vector, attB-genXP3. attB-genXP3 was generated by cloning a fragment of the pMF3 vector containing the promoter, restriction sites for subcloning of the hairpin and the polA signal, into the gattv vector using the restriction sites NotI and BamHI.

The lig sequence was subcloned from pUAST-lig [gift from Yamamoto lab] into the pUAST attb vector using the restriction site EcoRI. The lig coding region was cloned into pUAST-lig [56] and cloned into the pENTR vector. Site-directed mutagenesis was used to obtain the lig coding region without the C53T substitution that causes the amino acid exchange R185C. Analysis of UAS-lig [57] revealed similar phenotypes as observed for UAS-lig (Figure S2B and S2C),
suggesting that the amino acid exchange R185C represents a polymorphism. pENTR-lig$_{F4+L4}$ was generated by site-directed mutagenesis with the primers LigF$_{LA}$ and LigR$_{LA}$ using pENTR lig as template.

The coding sequence of rin was cloned into pENTR. LR reaction was used to subclone the coding sequences from pENTR-lig and pENTR-rin into the Gateway vectors pUAST-W-attb and pUAST-HW-attb. The gattB-Grin and gattB-GrinCherry vectors were cloned in two and three steps, respectively. A fragment of 7.2 kbp from the P[acman] BAC 13D12 [59] was subcloned into a modified gatB vector using BamHI and AgeI restriction sites. In the second step, a PCR-amplified fragment of 4.6 kbp (using the primer pair Rin$_{FA}$, gRin$_{R}$) was subcloned into the gatB vector containing the 7.2 kbp Grin fragment using the restriction sites AgeI and NotI, resulting in the construct gattB-Grin. A cherry coding sequence including a stop codon was fused to the third exon of rin without stop codon and to the 3' UTR of rin by fusion PCR.

Transgenic flies were generated with the site-specific phiC31 integration system using vas-phiC31-zh2A; ZH-attP-44F, vas-phiC31-zh2A; ZH-attP-51D and vas-phiC31-zh2A; ZH-attP-86Fb embryos [60].

**Cell culture, transfection, Western blot and AP-MS**

S2 cells were cultured and transfected according to standard protocols.

The coding sequences of FMR1, Capr and DART1 were cloned into pENTR. LR reactions were performed to subclone the coding sequences from pENTR-GFP, pENTR-FMR1, pENTR-Capr, pENTR-DART1, pENTR-rin, pENTR-lig, pENTR-lig$_{R^{HDC}}$ into the Gateway vectors pMHW, pAGW, pARW and pAFW. GFP-DCP1 was used as a P-body marker [61].

For the rin translational reporter construct, the two parts of the 5' UTR of *rin* were amplified with the primer pairs EcoRI$_{R}$ Rin$_{F}$, Rin$_{RA}$ and Rin$_{FB}$, NotI$_{R}$ Rin$_{R}$, respectively, from genomic DNA of *y* white flies, fused by fusion PCR and subcloned into the gatB vector containing an *ubi* promoter using the restriction sites EcoRI and NotI. The coding sequence of cherry fused to the 3' UTR of *rin* was amplified with the primer pair NotI$_{R}$ Cherry$_{F}$, XbaI$_{R}$ Rin$_{R}$ from the template gattB-RinCherry and subcloned into the gatB-ubi-5' UTR *rin* vector using the restriction sites NotI and XbaI.

For the *rin* transcriptional reporter, the ubi-5' UTR of *rin* from the translational reporter was replaced with the *rin* promoter that was amplified with the primer pair gattB$_{R}$, Rin$_{RG}$ from the template gattB-GrinCherry.

Western blots were performed according to standard protocols. AP-MS analysis was done as described in [62].

**Primers used in this study**

| Primer | Sequence |
|--------|----------|
| Rin$_{F}$ | 5'-CACCATGCTATGGATGATGCAGCC-3' |
| Rin$_{R}$ | 5'-GGGACGTCCGTATATGGGCTC-3' |
| FMR1$_{FB}$ | 5'-CACCATGGAAAGATCCTCCTGCTGTG-3' |
| FMR1$_{RB}$ | 5'-GGCATGCAGGTTCTAG-3' |
| Rin$_{FA}$ | 5'-ATTCTAGATGTTGCTTGATGGATGATAGG-3' |
| Rin$_{RA}$ | 5'-GGTGGCCACACCATCCTGAT-3' |
| Rin$_{F}$ | 5'-TAAATATGTCATTGGTTTACTGG-3' |
| Rin$_{R}$ | 5'-TACACAGTCGTCTAGGACTTTTGC-3' |
| Rin$_{FA}$ | 5'-GGAAGATCTCCTCGTG-3' |
| Rin$_{RB}$ | 5'-ATTCTAGATGTTGCTTGATGGATGATAGG-3' |
| Rin$_{F}$ | 5'-TACACAGTCGTCTAGGACTTTTGC-3' |
| Rin$_{R}$ | 5'-TAAATATGTCATTGGTTTACTGG-3' |

**Antibody stainings**

S2 cells or eye imaginal discs were fixed in 4% PFA at RT for 20 min and blocked with 2% NDS in 0.3% PBT or 1% BSA in 0.3% PBT (only for rabbit a-Cleaved Caspase-3 antibody). The following primary and secondary antibodies were used: mouse a-Ago1 (1:300) [63], rabbit a-Cleaved Caspase-3 (1:1000, Cell Signaling, Catalog no. 9661). mouse a-Lig-N (1:300) [15], mouse a-a-FLAG (Sigma, F1804), mouse a-HA (Convance, MMS-101R), mouse a-gfp (Roche, 1181466001), mouse a-GrinCherry (Abcam, ab125096), rabbit a-Akt (Ser 473) (1:300, Cell signaling, 9275s), rabbit a-Myc (1:5000) [65], mouse a-Dil (Ian Duncan; gift from K. Basler), guinea pig a-Sens (PGP5, 1:800, H. Bellen, Baylor College of Medicine, Houston; gift from K. Basler), rabbit a-Ptc (1:100, DSHB), mouse a-Cut 2B10 (1:100, DSHB), rabbit a-STAT92E (1:1000) [66], goat a-rabbit Cy3 (GE Healthcare, PA43004), goat a-mouse Cy3 (GE Healthcare, PA45002), mouse a-Cy5 (GE Healthcare, PA45002), mouse a-HRP (Jackson ImmunoResearch, 115-035-003).

Pictures were taken using a Leica SPE or SP2 confocal laser scanning microscope.

**Yeast two-hybrid assay**

Yeast two-hybrid analysis was carried out using Invitrogen’s ProQuest Two-Hybrid System with Gateway Technology according to the manufacturer’s instructions. Full-length cDNAs and the cDNA fragments of lig, FMR1, Capr, and rin, and lig$_{26}^{1333}$ lig$_{L4}$, lig$_{101R}$, rin$_{129–492}$, respectively, were cloned into the Ga4 DNA-binding domain vector pDEST 32 as well as into the Ga4 activation domain vector pDEST 22. Plasmids were transformed into yeast strain AH109 and plated on SD-Leu-Trp-Ade and SD-Leu-Trp-His (supplemented with 2 mM 3-AT), respectively.

**Supporting Information**

Figure S1 Effective downregulation of lig during development. (A–B) Animals mutant for lig$_{L4}$ (A) or lig$_{L4}$ (B) in combination with lig$_{26}$ die as long, slender pupae. Scale bar represents 500 μm. (C) Statistical analysis of the size of seven ommatidia as described in Figure 1D: control (0.095±0.0022 and 0.09±0.0055) and lig$_{L4}$ mutant (0.097±0.005; p = 0.3 and 0.09±0.0017; p = 0.5) eyes of flies raised on 25% yeast-containing food. (D–G) Scanning electron micrographs of adult control and lig$_{L4}$ mutant eyes generated by eyFLP/FRT-mediated mitotic recombination from flies grown on 40% yeast food (D–E) or 40% yeast and 60% Casein-containing food (F–G). Scale bar represents 100 μm. (H) Statistical analysis as described in Figure 1D: control (749±29 and 721±24) and lig$_{L4}$ mutant (721±38; p = 0.025 and 765±35; p = 0.02) eyes at 40% yeast-consuming food. (I–J) Scanning electron micrographs of eyFLP/FRT Minute-induced adult control or lig$_{L4}$ mutant eyes (I–J) from flies grown on 100% yeast-consuming food. Scale bar represents 100 μm. (K) Statistical analysis as described in Figure 1D: control (806±23 and 806±21).
781±14) and lig^I (837±30; p = 0.028 and 844±45; p = 0.0091).

Statistical analysis as described in Figure 1D: control (717±19 and 753±23) and lig^I mutant (713±60; p = 0.042 and 755±38; p = 0.91) eyes from flies raised on 100% yeast-fermenting food at 18°C. M Statistical analysis as described in Figure 1D: control (653±23) and lig^I mutant (750±32; p = 0.003) eyes from flies raised on 25% yeast-fermenting food at 18°C. N Statistical analysis of the transgenes UAS-lig^RNAi (O) or UAS-lig^RNAi (P) under the control of da-Gal4 causes lethality. Scale bars are shown in (N). Scale bar represents 500 μm. Q–S Comparison-specific expression of the transgenes UAS-lig^RNAi (U) or UAS-lig^RNAi (V) driven by DE-Gal4 in the developing eye results in reduction of Lig (green) in the dorsal compartment (marked with RFP (red)) in comparison to the control (Q). Scale bar represents 50 μm. T–V Eyes expressing the transgenes UAS-lig^RNAi (U) or UAS-lig^RNAi (V) are larger than the control (T). Scale bar represents 100 μm. The expression of the transgenes was induced with the Actin-Flip out technique in combination with eYFP, W. Statistical analysis as described in Figure 1D: control (761±14 and 780±20), UAS-lig^RNAi (784±25; p = 0.035 and 799±21; p = 0.101) and UAS-lig^RNAi (809±7; p = 3.95±0.8 and 800±16; p = 0.061). X Statistical analysis of the size of seven ommatidia as described in Figure 1D: control (0.99±0.0021 and 0.992±0.0049), UAS-lig^RNAi (0.094±0.0044; p = 0.58 and 0.993±0.008; p = 0.78) and UAS-lig^RNAi (0.095±0.0035; p = 0.17 and 0.096±0.0069; p = 0.25) expressing eyes. Genotypes: (A) y w; lig^PP/FRT42 lig^b (B) y w; lig^PP/FRT42 lig^b (D, F, L) control and M (control) y w eyFLP/y; FRT42 P{SupPr-P}VhaAC43^R20272 (ch) FRT42 (E, G, L) (lig^I) and M (lig^I) y w eyFLP/y; FRT42 P{SupPr-P}VhaAC43^R20272 (ch) (I) y w eyFLP/y; FRT42 M(2)53/FRT42 (J) y w eyFLP/y; FRT42 M(2)53/FRT42 lig^b (N) y w; da-Gal4/UAS-CG1315;Rin^RNAi (control) (O) y w; da-Gal4/UAS-CG1315;Rin^RNAi (control) (P) y w; da-Gal4/UAS-CG1315;Rin^RNAi (control) (Q) y w; da-Gal4 UAS-CG1315;Rin^RNAi (control) (R) y w; da-Gal4 UAS-RFP/UAS-CG1315;Rin^RNAi (control) (S) y w; da-Gal4 UAS-RFP/UAS-CG1315;Rin^RNAi (control) (T) y w; eyFLP, Act>CD2>Gal4/ y w; UAS-CG1315;Rin^RNAi (control)/+ U y w; eyFLP, Act>CD2>Gal4+/ y w; UAS-CG1315;Rin^RNAi (control)/+ / y w; UAS-CG1315;Rin^RNAi (control)/+. (TIF)

**Figure S2** Overexpression of lig^R135C causes a similar eye phenotype to overexpression of lig^I. (A–B) Scanning electron micrographs of eyes overexpressing the indicated UAS transgenes (A–B). Scale bar represents 100 μm. (C) Statistical analyses as described in Figure 1D: ey-GFP (790±17 and 770±15), ey-GFP;lig^R135C (708±20; p = 1.43E-04 and 707±15; p = 6.00E-06). The phenotype caused by lig^R135C is very similar to the phenotype caused by lig^I (Figure 2E). (D–F) lig^I overexpressing clones (induced with the Actin-Flip out-Gal4 system) in comparison to the control (D–F). Scale bar represents 50 μm. (F–G) Pictures of wings expressing the indicated UAS transgenes under the control of nubbin-Gal4 (F and G). Scale bar represents 100 μm. (H) Statistical analysis as described in Figure 1D: nubbin-Gal4 (276838±12458 and 274887±13574), nubbin-Gal4 (98346±8035; p = 3.1E-13 and 97511±5953; p = 7.33E-12). Genotypes: (A) w/y w; ey-Gal4/UAS-GFP (B) w/y w; ey-Gal4/UAS-GFP; UAS-lig^R135C/D (C) y w hslFP/y; w; UAS-GFP/+; Act>CD2>Gal4/ UAS-GFP/+ (E) y w hslFP/y; Act>CD2>Gal4/ UAS-GFP/+; Act>CD2>Gal4/ UAS-GFP/+ (F) y w hslFP/y; Act>CD2>Gal4/ UAS-GFP/+; Act>CD2>Gal4/ UAS-GFP/+; Act>CD2>Gal4/ UAS-GFP/+ (G) y w/y; nubbin-Gal4/UAS-GFP (H) y w/y; nubbin-Gal4/UAS-GFP (I) y w/y; nubbin-Gal4/UAS-GFP (J) y w/y; nubbin-Gal4/UAS-GFP (K) y w/y; nubbin-Gal4/UAS-GFP (L) y w/y; nubbin-Gal4/UAS-GFP (M) y w/y; nubbin-Gal4/UAS-GFP (N) y w/y; nubbin-Gal4/UAS-GFP (TIF)

**Figure S3** Lig does not co-localize with DART1, and endogenous Lig, FMR1 and Capr co-localize with Rin-Cherry. (A–F) S2 cells co-transfected with GFP-lig (A, N), REP-FMR1 (B, B), REP-FMR1 (C, C), REP-DART1 (D, D), and GFP-FMR1 (E, F, F) and HA-lig^R135C (E, E, E) and HA-lig^R135C (F, F, F), S2 cells stained with DAPI (blue) to visualize DNA and with phosHA to visualize HA-Lig (E, E, E) (F, F, F) S2 cells co-transfected with GFP-lig^R135C (F, F, F) and REP-DART1 (F, F, F) do not reveal any co-localization. S2 cells were stained with DAPI (blue) to visualize DNA. Scale bar represents 25 μm. (G–H) Untransfected S2 cells stained for endogenous Ago1. S2 cells were stained with DAPI (blue) to visualize DNA. Scale bar represents 25 μm. (H–K) S2 cells transiently transfected with GinCherry to express Rin-Cherry at endogenous levels. In most of the cells Rin-Cherry is homogeneously in the cytoplasm of transfected cells (H and I). In few cells Rin-Cherry forms punctae (I′, ′) and Kn′ and localizes with Lig (l and l′). FMR1 (j and j′) and Capr (K′ and K′′). S2 cells were stained with DAPI (blue) to visualize DNA. Scale bar represents 25 μm. (L–M) Untransfected S2 cells stained for endogenous Lig (L and L′) and Capr (L′ and L′′). Lig and Capr localize in bigger punctae but not in cells with small punctae. S2 cells were stained with DAPI (blue) to visualize DNA. Scale bar represents 25 μm. (TIF)

**Figure S4** Lig and Rin fragments display no autotoxicity in Y2H experiments. (A) Negative controls for Y2H interactions between Lig, Lig^R1-4, Lig^1-3, Lig^1-2, and the empty vector. Lig, Rin, Rin^1-75, Rin^1-29 and lig^1-4 used to the AD and to the DBD, respectively, do not show autotoxicity. (TIF)

**Figure S5** Analysis of rin homoplastic alleles and the genomic rescue transgene GinCherry. (A–C) Negatively marked 72 h old rin^I (A, A′), P{GawB}rin^NP1410 (B, B′) and P{GawB}rin^NP1420 (C, C′) mutant clones (induced with the FLP/FRT system) in eye imaginal discs of third instar larvae. Rin-Cherry levels expressed from the GinCherry are autonomously increased in the rin mutant clones (A, B, and C′). The scale bar represents 50 μm. (D–F) Scanning electron micrographs of adult P{GawB}rin^NP1410 (E) and P{GawB}rin^NP1420 (F) eyes generated by eyFLP/FRT-mediated mitotic recombination. The scale bar represents 100 μm. (G) Statistical analyses as described in Figure 1D: control (762±35 and 767±18), P{GawB}rin^NP1410 (732±9 and 731±17) and P{GawB}rin^NP1420 (713±20 and 711±29). (H–S) The long slender pupae formed by FMR1^D1035/D1035 rin^NP1410^K and FMR1^D1035 rin^NP1420 rin^NP1420 (K) and FMR1^D1035 rin^NP1420 (F) eyes generated by eyFLP/FRT-mediated mitotic recombination. The scale bar represents 100 μm. (TIF)
FMRI D113M P(GamB)rin NPS420 (M) y w eyFlp/w; Grin Cherry [44F]/+; FRT22 FMRI D113M rin / FRT22 FMRI D113M P(GamB)rin NPS420 (N) y w; FRT2/2 FRT2 FMR1 D113M P(GamB)rin NPS420 (O) y w/y w; FRT2/2 P(GamB)rin NPS420/ FRT2 P(GamB)rin NPS420 P(GamB)rin NPS420 Q y w eyFlp/w; FRT2/2 FMRI D113M rin / FRT2/2 FMRI D113M P(GamB)rin NPS420 (R) y w; Grin Cherry [44F]/+; FRT2/2 FMRI D113M rin / FRT2/2 FMRI D113M P(GamB)rin NPS420 (S) y w eyFlp/w; Grin Cherry [44F]/+; FRT2/2 FMRI D113M rin / FRT2/2 FMRI D113M rin / FRT2/2 FMRI D113M rin / FRT2/2 FMRI D113M rin / FRT2/2 FMRI D113M rin / FRT2/2 FMRI D113M rin (T–control) y w eyFlp/w; FRT2 cl w/ FRT2 (T - FMR1 D113M rin ) y w eyFlp/w; FRT2 cl w/ FRT2 FMR1 D113M rin (T - Grin; FMR1 D113M rin ) y w eyFlp/w; Grin [44F]/+; FRT2 cl w/ FRT2 FMRI D113M rin / FRT2 FMRI D113M rin / FRT2 FMRI D113M rin (TIF)

Figure S6 CaprRNAi strongly reduces Capr levels. (A–A’) CaprRNAi overexpressing cells (induced with the Actin-Flp out-Gal4 system and marked by GFP) in eye imaginal discs of third instar larvae reduce Capr levels as judged by Capr staining (red) (A–A’). Scale bar represents 50 μm. Genotypes: (A) y w eyFlp/w; UAS-CaprRNAi/+; Act>CD2>Gal4, UAS-GFP/+ (TIF).

Figure S7 Lig regulates Rin levels but not at the protein level. (A–A’) lig overexpressing cells (induced with the Actin-Flp out system and marked by GFP) (A and A’) display increased levels of Rin-Cherry expressed from the Grin Cherry transgene (red) (A’ and A”). Scale bar represents 50 μm. (B–B’) Negatively marked 72 h old lig mutant clones (induced with the FLP/FRT system) in eye imaginal discs of third instar larvae reduce Capr levels as judged by Capr staining (red) (A–A’). Scale bar represents 50 μm. Genotypes: (A) y w hsFlp/y w; UAS-CaprRNAi/+; Act>CD2>Gal4, UAS-GFP/+ (TIF).

Figure S8 Lig does not regulate bantam miRNA, EGFR signaling, Myc, Hippo signaling, Insulin signaling, Wnt signaling, and Hedgehog signaling. (A–A’) lig mutant clones (induced with the FLP/FRT system) 72 h old, marked by the lack of lacZ staining in red) in eye imaginal discs of third instar larvae (A and A”) do not display an upregulation of a minimal Hippo response element (DIAP1-GFP4.3; green; A’ and A”). Scale bar represents 50 μm. (B–C”) Negatively marked 72 h old lig mutant clones (induced with the FLP/FRT system; no lacZ (red); B and B”) and lig overexpressing cells (induced with the Gal4/UAS system using DE-Gal4) marked with RFP (C and C”) in eye imaginal discs of third instar larvae do not impact on a bantam miRNA reporter (B’, B”, C’ and C”). Scale bar represents 50 μm. (D–K”) Negatively marked 72 h old lig mutant clones (induced with the FLP/FRT system; no GFP (green) in wing (G, G”, H, H”, J, J”, K and K”) or eye (D, D”, E, E”, F, F”, I and I”) imaginal discs do not change expression or localization of pnt-lacZ (D’ and D”), pAkt (E and E”), Myc (F and F”), Dil (G and G”), Sens (H’, H”, I’ and I”), Fc (J’ and J”) and Cut (K’ and K”). Scale bars represent 50 μm. (L–L”) Negatively marked 72 h old lig mutant clones (induced with the FLP/FRT system; no GFP (green) in eye imaginal discs stained for STAT92E (L and L”). Scale bars represent 50 μm. Genotypes: (A) y w hsFlp/y w; FRT2 arm-lacZ/FRT2 lig2; DIAP1-GFP4.3/+ (B) y w hsFlp/y w; FRT2 arm-lacZ/FRT2 lig2; MIR33 bantam reporter/+ (C) y w/y w; UAS-lig/+; MIR33 bantam reporter/DE-Gal4, UAS-RFP (D) y w hsFlp/y w; FRT2 ubiGFP/FRT2 lig2; pnt-lacZ/+ (E, F, G, H, I, J, K, L) y w hsFlp/y w; FRT2 ubiGFP/FRT2 lig2 (TIF).

Table S1 Lig interaction partners identified in AP-MS experiments. HA-GFP and HA-Lig expressed under the control of a metallothionein-inducible promoter in cultured Drosophila S2 cells were used as bait for AP-MS analyses. The unique and total peptide numbers identified in two biological replicates are indicated for HA-GFP (control) and HA-Lig. FlyBase ID and gene symbols of the corresponding genes are listed. (XLSX)

Acknowledgments

We thank C. Rottig for isolating the lig alleles; A. Bär, A. Strässle, J. Demmer and I. Vuilleux for technical assistance; V. Medic for programming the Ommatidia counter 0.3 software; the Bloomington stock center, D. Yamamoto, O. Papoulas, G. Dryfuss, T. Jongens, M. C. Sionmi, E. Izzurralde, K. Nairz, S. Cohen, D. Stein, E. Bach, S. Hou, J. Bischof and K. Baden for flies and reagents; and A. Wepf and M. Gstaiger for help with the AP-MS analysis.

Author Contributions

Conceived and designed the experiments: RB HS EH. Performed the experiments: RB. Analyzed the data: RB HS. Contributed reagents/materials/analysis tools: RB HS. Wrote the paper: RB HS.

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