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Report

DLin-7 Is Required in Postsynaptic Lamina Neurons to Prevent Light-Induced Photoreceptor Degeneration in *Drosophila*

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Summary

Inherited retinal degeneration in humans is caused by mutations in a wide spectrum of genes that regulate photoreceptor development and homeostasis. Many of these genes are structurally and functionally conserved in *Drosophila*, making the fly eye an ideal system in which to study the cellular and molecular basis of blindness [1, 2]. *D*Lin-7, the ortholog of vertebrate MALS/Veli, is a core component of the evolutionarily conserved Crumbs complex [3]. Mutations in any core member of the Crb complex lead to retinal degeneration in *Drosophila* [4]. Strikingly, mutations in the human ortholog, *CRB1*, result in retinitis pigmentosa 12 (RP12) and Leber congenital amaurosis, two severe retinal dystrophies [5, 6]. Unlike Crumbs, *D*Lin-7 is expressed not only in photoreceptor cells but also in postsynaptic lamina neurons. Here, we show that *D*Lin-7 is required in postsynaptic neurons, but not in photoreceptors such as Crumbs, to prevent light-dependent retinal degeneration. At the photoreceptor synapse, *D*Lin-7 acts as part of a conserved *D*Lin-7/CASK/DlgS97 complex required to control the number of capitate projections and active zones, important specializations in the photoreceptor synapse that are essential for proper neurotransmission [7]. These results are the first to demonstrate that a postsynaptically acting protein prevents light-dependent photoreceptor degeneration and describe a novel, Crumbs-independent mechanism for photoreceptor degeneration.

Results and Discussion

In the visual system, *D*Lin-7 (also known as Veli) is localized at the stalk membrane of the retina [8] (Figures 1A–1A″, arrow), colocalizing with other members of the evolutionary conserved Crb complex (Figures 1B–1B″ and data not shown) [9–12]. In addition, and in contrast to Crb, *D*Lin-7 is also detected in the optic neuropils (Figures 1A–1A″). *D*Lin-7 antibody specificity was confirmed by the lack of staining in D*lin-7* mutants (see Figures S1A–S1A″ available online). In the lamina, where photoreceptor cells (PRCs) synapse with monopolar neurons in a single cartridge [1], *D*Lin-7 was detected presynaptically and colocalized with Dlg [13] (Figures 1C–1C″). *D*Lin-7 is also expressed postsynaptically in the lamina monopolar cells (LMCs) L1 and L2 (Figures 1C–1C″, asterisk, and S1B–S1B″), which receive their major synaptic input from the PRCs R1–R6. No *D*Lin-7 was detected in glia cells (Figures S1C–S1C″).

White-eyed (*w*) homo- or hemizygous D*lin-7* mutant flies [*w;;*D*lin-7*$^{\#66}$/*Df(3R)*D*lin-7*^{20l}] showed PRC degeneration after 7 days of exposure to constant light [8] (compare Figures 2A and 2B; Figure S2A). Strikingly, removal of D*lin-7* only in PRCs by using the Flippase (FLP)-*F*LP *r*ecognition *t*arget (FLP-FRT) technique to induce mitotic clones did not induce any degeneration after 7 days in continuous light (Figure 2C). In contrast, reduction of D*lin-7* function by RNAi in both LMCs (Figure 2D) or in L1 or L2 neurons individually (Figures S2D and S2E) resulted in light-dependent retinal degeneration of PRCs. *D*Lin-7 expression in L1 and L2 neurons completely rescued PRC degeneration of D*lin-7* mutants (Figure 2E). In contrast, signs of degeneration were still observed upon PRC-specific expression of *D*Lin-7 in mutant flies (Figure 2F). Specificity of L1 and L2 driver lines was confirmed by analyzing *D*Lin-7 levels in the retina (Figure S2I). Taken together, these results indicate that *D*Lin-7 function is required in postsynaptic LMCs, but not in PRCs, to prevent light-induced PRC degeneration, in contrast to other members of the Crb complex, whose function is required in PRCs. This suggests that the mechanism by which *D*Lin-7 protects against light-dependent retinal degeneration is different from that mediated by Crb, which has recently been shown to involve correct rhodopsin trafficking via MyoV [14] and limitation of oxidative damage by regulating Rac1-NADPH oxidase complex activity [15].

The restriction of Crb expression to PRCs (Figures 1B–1B″) excludes its functional cooperation with *D*Lin-7 in LMCs. In order to understand the functional role of synaptic *D*Lin-7 in PRC survival, we investigated potential interaction partners. *Drosophila* calcium/calmodulin-dependent serine protein kinase CASK (also called CamGuk or Caki in *Drosophila*) and DlgS97 (Dlg/PSD95/SAP97 in vertebrates), two membrane-associated guanylate kinase (MAGUK) proteins, are localized to the lamina neuropil [8, 16, 17]. MAGUKs play key roles in synaptogenesis, synaptic function, and plasticity, and their misregulation results in a range of neurological disorders [18]. We therefore analyzed whether these synaptic proteins are also required for photoreceptor survival. *w;;cask*^{x313}/*cask*^{x307} (Figure 2G) and *w,dlgS97*^{flpV} or *w,dlgS97*^{flpV}/*yw dlgS97*^{138} mutant flies showed degenerative defects after 7 days of continuous light exposure (Figures 2H and S2F) comparable to those observed in D*lin-7* mutants (Figure 2B). Specific reduction of DlgS97 in laminar neurons also induced degeneration (Figures 2I and S2G). None of these flies showed retinal degeneration when kept in darkness for 7 days (data not shown).

To further analyze the relationship between *D*Lin-7 and CASK and DlgS97, we performed colocalization studies. Antibody staining showed partial colocalization of *D*Lin-7, DlgS97, and CASK in PRC terminals (Figures 3A–3A‴, arrow in inset, S3A, S3A′, and S3C–C″) and colocalization between *D*Lin-7 and CASK in laminar neurons (Figures S3B–S3B″). In lamina cartridges of *dlgS97* and *cask* mutants, *D*Lin-7 exhibited abnormal immunofluorescence localization (Figures 3F and 3G) as well as a significant reduction of total fluorescence

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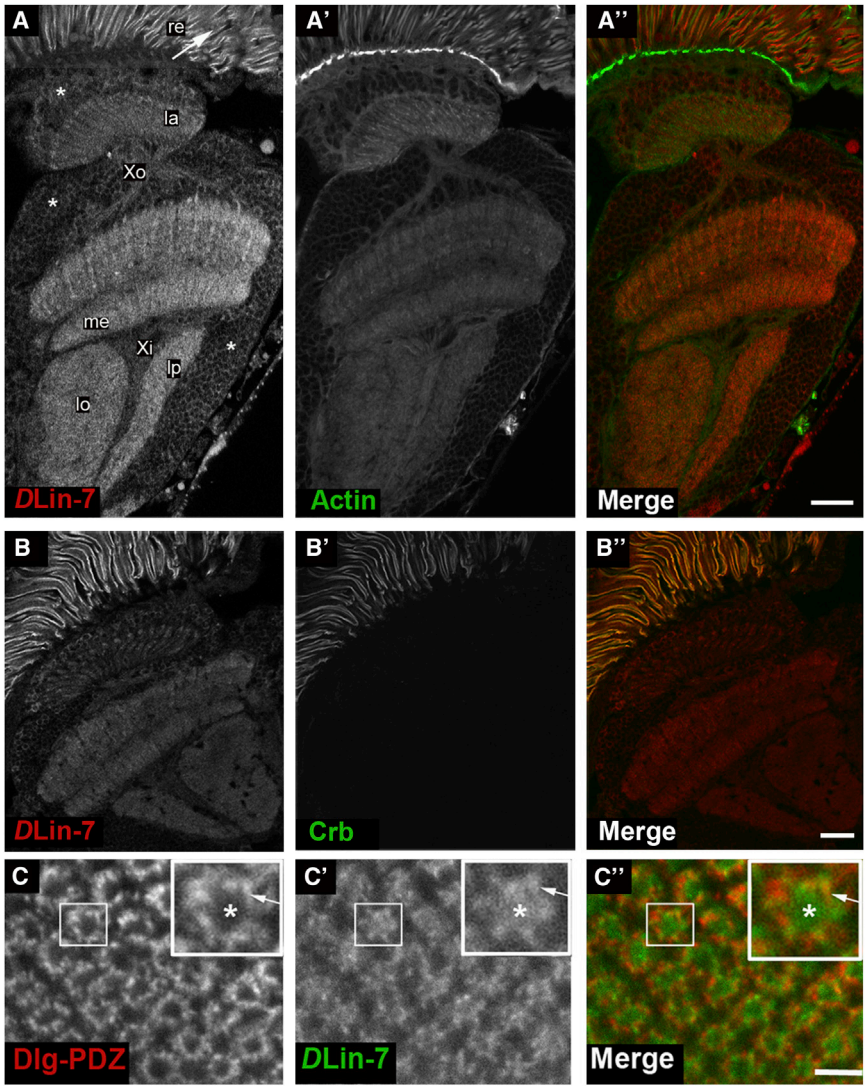

Figure 1. *D*Lin-7 Colocalizes with Crb in the Retina, but Not in the Optic Lobe

(A–A″) Longitudinal section of *w* flies, showing *D*Lin-7 expression (red) in the stalk membrane of the retina (re; arrow), lamina (la), outer optic chiasm (Xo), medulla (me), lobula (lo), lobula plate (lp), inner optic chiasm (Xi), and cell cortex (*). Actin is shown in green.

(B–B″) *D*Lin-7 and Crb double staining shows colocalization in the stalk membrane of the retina and no staining of Crb (green) in the optic lobe (B′).

(C–C″) Lamina cross-sections show photoreceptor terminals marked by α-Dlg-PDZ (red) and α-*D*Lin-7 (green) in lamina cartridges. Partial colocalization (orange) of *D*Lin-7 and Dlg reveals presynaptic *D*Lin-7 in photoreceptor cell (PRC) terminals 1–6 (arrow) and postsynaptic *D*Lin-7 in lamina monopolar cells (LMCs) L1 and L2 (asterisk).

Scale bars represent 20 μm in (A)–(B″) and 5 μm in (C)–(C″). See also Figure S1.

(Figure 3H). CASK showed abnormal localization in D*lin-7* mutants (Figure 3J), while a reduction of total fluorescence occurred in *dlgS97* mutants (Figures 3L and 3M). DlgS97 fluorescence was also reduced in D*lin-7* and *cask* mutants (Figures 3O, 3P, and 3R).

Previous studies using yeast-two-hybrid assays indicated that *D*Lin-7 binds via its L27 domain to CASK and DlgS97 [3]. Another report showed that the stabilization of *D*Lin-7 at the neuromuscular junction (NMJ) depends on the MAGUK proteins Metro and DlgS97, while DlgS97 stabilization depends to a lesser extent on Metro and *D*Lin-7 [19]. To further characterize the molecular interactions in the visual system, we used *Drosophila* head extracts for immunoprecipitation studies. *D*Lin-7 was pulled down from extracts of *w* fly heads, but not from head extracts of *dlgS97* or *cask* mutants, when an anti-Dlg-PDZ antibody was used (Figure 3B). This indicates that *D*Lin-7 forms a complex with DlgS97 and CASK, and that *D*Lin-7 and DlgS97 require CASK for complex formation.

Our data suggest that all members of the tripartite complex require each other to different extents for their stabilization and/or localization in the lamina. Therefore, we propose a novel tripartite complex at the synaptic terminals of PRCs R1–R6 (Figure 3C), in which DlgS97 and *D*Lin-7 bind to CASK.

We further investigated structural defects at the PRC synapse that might have an impact on PRC survival. Transmission electron microscopy (TEM) analysis revealed that in D*lin-7*, *cask*, and *dlgS97* mutant laminas (Figures 4B–4D), terminals of photoreceptors R1–R6 (marked in violet) were arranged around LMCs L1 and L2 (marked in green) as in lamina cartridges of control *w* flies (Figure 4A). However, capitate projections (marked in blue, arrow in Figure 4A′) were defective. Capitate projections are invaginations from surrounding epithelial glia cells into photoreceptor terminals R1–R6 [20], which act as sites of clathrin-mediated endocytosis and have been linked to neurotransmitter recycling [21–24]. D*lin-7* and *cask* mutant flies had 32% fewer capitate projections compared with *w* control flies, whereas *dlgS97* mutant flies showed 61% fewer capitate projections compared with *w* and 43% fewer projections than *cask* and D*lin-7* mutant flies (Figure 4E). To determine whether the reduced number of capitate projections is causally linked with degeneration, we analyzed degeneration in eyes with reduced levels of *basigin* (*bsg*). Reduction of *bsg* also reduced the number of capitate projections [25] (Figures 4F and S4A), a phenotype that has been associated with decreased synaptic transmission [26]. Strikingly, knockdown of *bsg* also resulted in severe retinal degeneration (Figures 4G and S4B). Therefore, we conclude that the *D*Lin-7/Cask/DlgS97 tripartite complex ensures survival of PRCs by controlling the proper number of capitate projections and hence proper synaptic transmission.

MAGUKs have been reported to play key roles in active zones, electron-dense structures at the presynaptic site that are specialized to attract synaptic vesicles for fusion with the plasma membrane [7, 26]. Bruchpilot (Brp) is a structural component of the T-bar ribbon (Figure 4A′, arrowhead), and its localization correlates with active zones (Figure 4H) [7, 27]. Active zones were affected in all three mutants

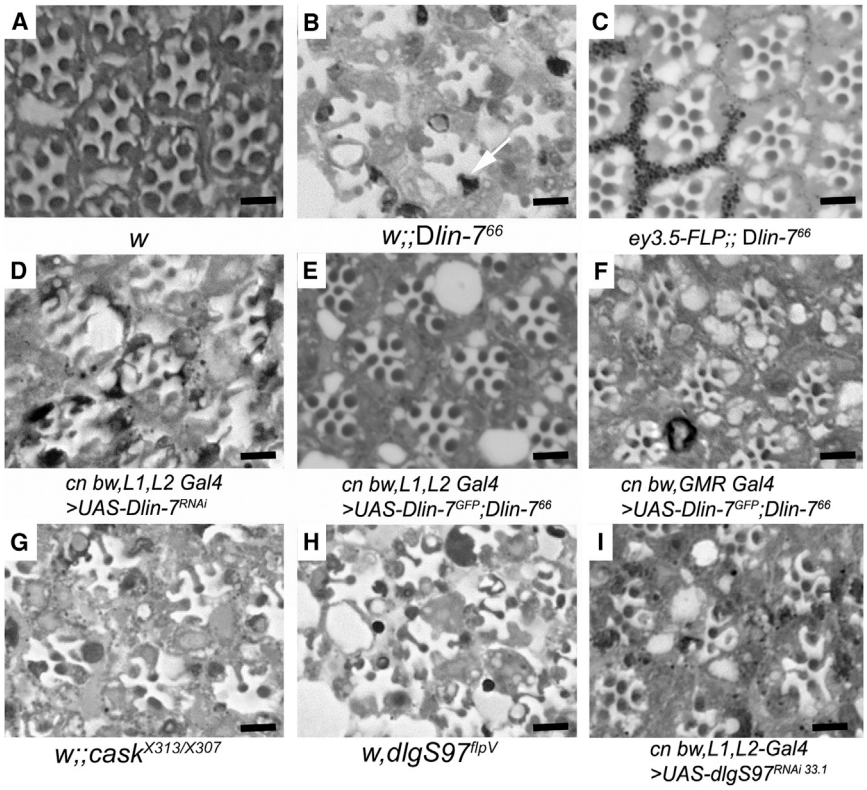

Figure 2. *D*Lin-7 in LMCs L1 and L2 Prevents Photoreceptor Degeneration

Cross-sections of retinas after 7 days of continuous light exposure.

(A and B) D*lin-7* mutant flies (B) exposed to constant light for 7 days undergo PRC degeneration (indicated by the loss of rhabdomeres [dark structures leaking into the PRC cytoplasm]; arrow), in contrast to *w* control eyes (A), which remain unaffected.

(C and D) Flies with D*lin-7* mutant PRCs but wild-type optic lobes (ey3.5-FLP;;D*lin-7*^{66}) do not show retinal degeneration under continuous light (C). In contrast, PRCs of flies expressing *RNAi*-D*lin-7* in LMCs L1 and L2 (D) undergo degeneration similar to that observed in D*lin-7* mutant flies (B), but less severe.

(E and F) D*lin-7* mutant flies expressing *D*Lin-7 in L1 and L2 neurons do not show degeneration (E), whereas D*lin-7* mutant flies expressing *D*Lin-7 in PRCs do show degeneration (F).

(G and H) *cask*^{x313}/*cask*^{x307} (G) and *dlgS97*^{flpV} (H) mutants for synaptic proteins show PRC degeneration comparable to that observed in D*lin-7* (B).

(I) Silencing *dlgS97* in L1 and L2 neurons leads to light-dependent degeneration.

Scale bars represent 5 μm. See also Figure S2.

analyzed here. Specifically, D*lin-7*, *cask*, and *dlgS97* mutants exhibited ~3-fold higher Brp fluorescence levels (Figures 4I–4K and 4M) than *w* flies. Quantification of Brp staining by particle analysis revealed more and larger Brp-positive particles in D*lin-7*, *cask*, and *dlgS97* mutants (Figures 4I–4K, 4N, and 4O). In contrast, *crb* mutant PRCs exhibited no significant difference from wild-type PRCs with respect to the intensity, number, and size of Brp-positive spots (Figures 4L–4O). Therefore, the phenotype observed in D*lin-7*, *cask*, and *dlgS97* uncovers a novel mechanism to prevent light-dependent photoreceptor degeneration, which acts in postsynaptic neurons.

In conclusion, we have identified a novel tripartite complex, formed by *D*Lin-7, CASK, and DlgS97, that controls the number and size of active zones in the terminals of PRCs R1–R6, linking synaptic transmission and photoreceptor survival. To the best of our knowledge, the data presented here are the first to define a mechanism acting at terminals of PRCs R1–R6 rather than in PRCs themselves to prevent light-dependent retinal degeneration. Although *D*Lin-7 is part of the Crb complex at the stalk membrane of PRCs, its function in these cells is not required to prevent light-dependent retinal degeneration. Instead, *D*Lin-7 acts in postsynaptic L1 and L2 LMC neurons to ensure PRC survival under light stress. It is tempting to speculate that the *D*Lin-7/CASK/DlgS97 complex provides a postsynaptic scaffold that is required to support *trans*-synaptic signaling or adhesion, which in turn influences presynaptic differentiation and homeostasis. A precedent for such retrograde effects has been described for the NMJ, where postsynaptic defects in the glutamate receptor (GluR) subunit DGluR-IIA or the transmembrane protein teneurin induce presynaptic defects [28].

The reduction of capitate projections in laminas mutant for D*lin-7*, *cask*, and *dlgs97* suggests that the *D*Lin-7/CASK/DlgS97 complex controls proper formation and/or stabilization of glial invaginations into PRC terminals, and that the supply of neurotransmitter might be critical for synaptic function, particularly under light stress. Previous results have shown the importance of capitate projections and MAGUK proteins such as CASK in synaptic transmission [24, 25, 29]. Additionally, we show that reduction of capitate projections caused by depletion of Bsg also causes light-induced degeneration, supporting a pivotal role for *D*Lin-7, CASK, and DlgS97 in capitate projection maintenance and photoreceptor survival during light stress. Our data may be extrapolated to vertebrates, since in the vertebrate retina the *D*Lin-7 ortholog Veli3 colocalizes with the MAGUKs MPP5 and MPP4 [30, 31], and knockout of all three Veli genes in mice results in defective presynaptic neurotransmitter release [31]. Moreover, CASK-deficient neurons also exhibit impaired synaptic function in mice [32]. Interestingly, *bsg* knockout mice also show retinal degeneration [33, 34]. These reports, together with our data, suggest the existence of a mechanism linking neurotransmitter supply and PRC survival that is conserved between mammals and flies.

D*lin-7*, *cask*, and *dlgS97* mutant lamina cartridges showed more and larger active zones. This phenotype is similar to that described in *Drosophila* NMJs of *dlgS97* mutants [19, 35] and supports the general role of submembranous protein scaffolds in architecting synaptic junctions. In the *Drosophila* NMJs, a CASK/Neurexin complex has been linked to the regulation of synaptic bouton formation [36], and *cask* mutants exhibit fewer synapses per bouton and reduced synaptic density [37].

In summary, we have identified a novel tripartite complex of *D*Lin-7, CASK, and DlgS97 that is required for proper active-zone formation and structural organization of the photoreceptor synapse. It was recently shown that changes in active-zone formation occur in *Drosophila* disease models of neurodegeneration and neuropsychiatric disorders [38, 39].

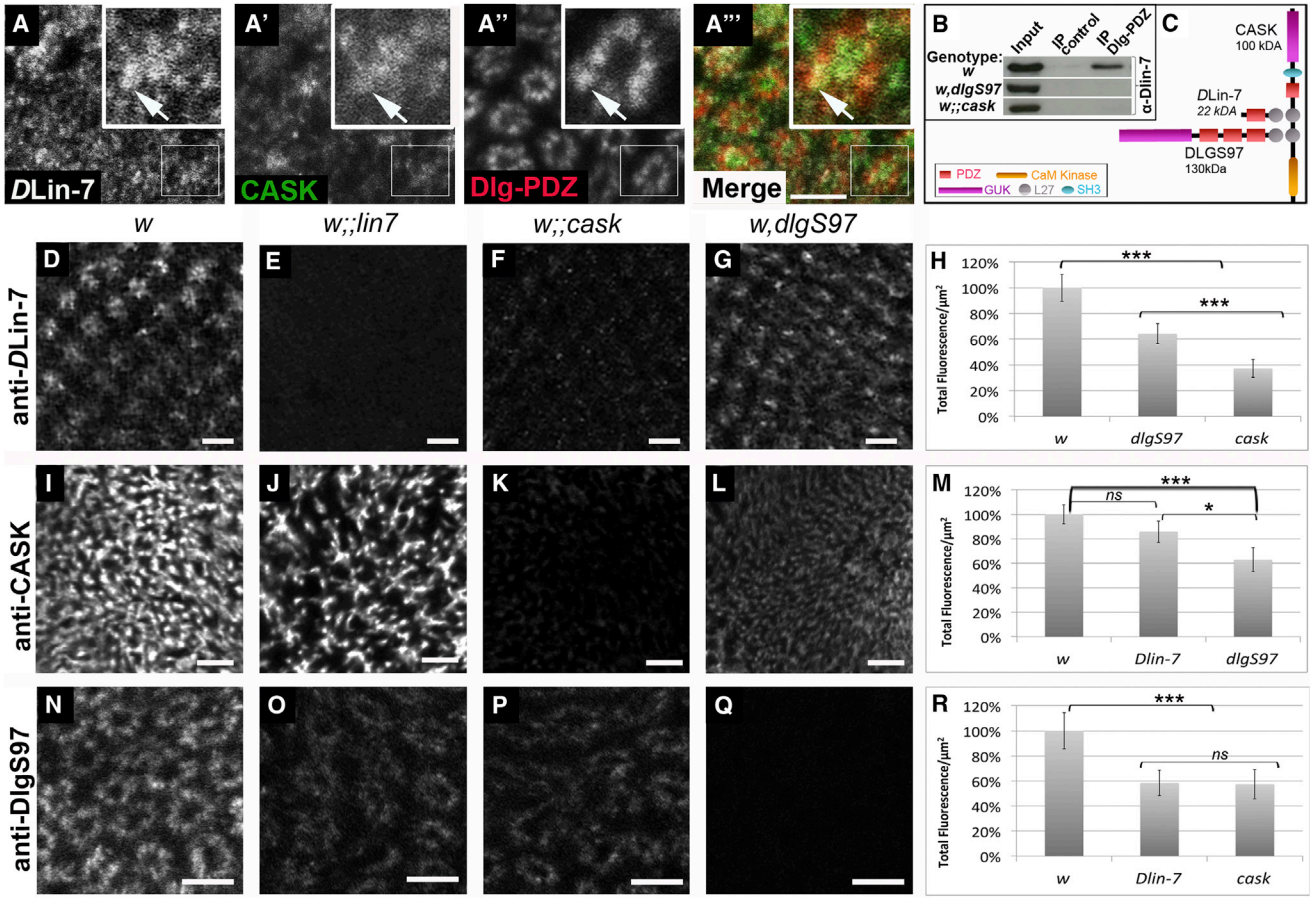

Figure 3. *D*Lin-7, CASK, and DlgS97 Form a Complex in the Lamina and Require Each Other for Stabilization

(A–A‴) *D*Lin-7 (gray) staining in *w* lamina cartridges. CASK (A′, green) localizes to L1 and L2 LMC neurons and their spines, and Dlg (A″, red) in photoreceptor terminals. Merge (A‴) shows partial colocalization of *D*Lin-7, CASK, and Dlg (arrow, light yellow structure) at the margin of photoreceptor terminals toward the LMCs. Scale bar represents 5 μm.

(B) *D*Lin-7 is immunoprecipitated with α-Dlg-PDZ antibody from extracts of *w*, but not from *w,dlgS97*^{flpV} and *w;;cask*$^{x313/x307}$ mutant, adult fly heads.

(C) Schematic drawing of the suggested CASK, DlgS97, and *D*Lin-7 tripartite complex based on immunoprecipitation data and their binding affinities to corresponding L27 domains (see text for details).

(D–R) Lamina cross-sections of *w*, *w;;Dlin-7*^{66}, *w;;cask*^{x313}/*cask*^{x307}, and *w,dlgS97*^{flpV} mutants stained with α-*D*Lin-7 (D–G), α-CASK (I–L), and α-DlgS97 (N–Q) antibodies, and quantification of the respective fluorescence intensities (H, M, and R). *D*Lin-7 staining is reduced in *cask* and *dlgS97* mutants (F–H). CASK appears mislocalized in D*lin-7* mutants (J) and is reduced in *dlgS97* mutants (L and M). DlgS97 is reduced in D*lin-7* and *cask* mutants (O–R).

Scale bars represent 5 μm. Error bars were calculated by SD. See also Figure S3.

Therefore, we believe that our data on retinal degeneration caused by impaired synaptic stability contribute to a better understanding of the molecular basis of human retinal dystrophies.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.05.060.

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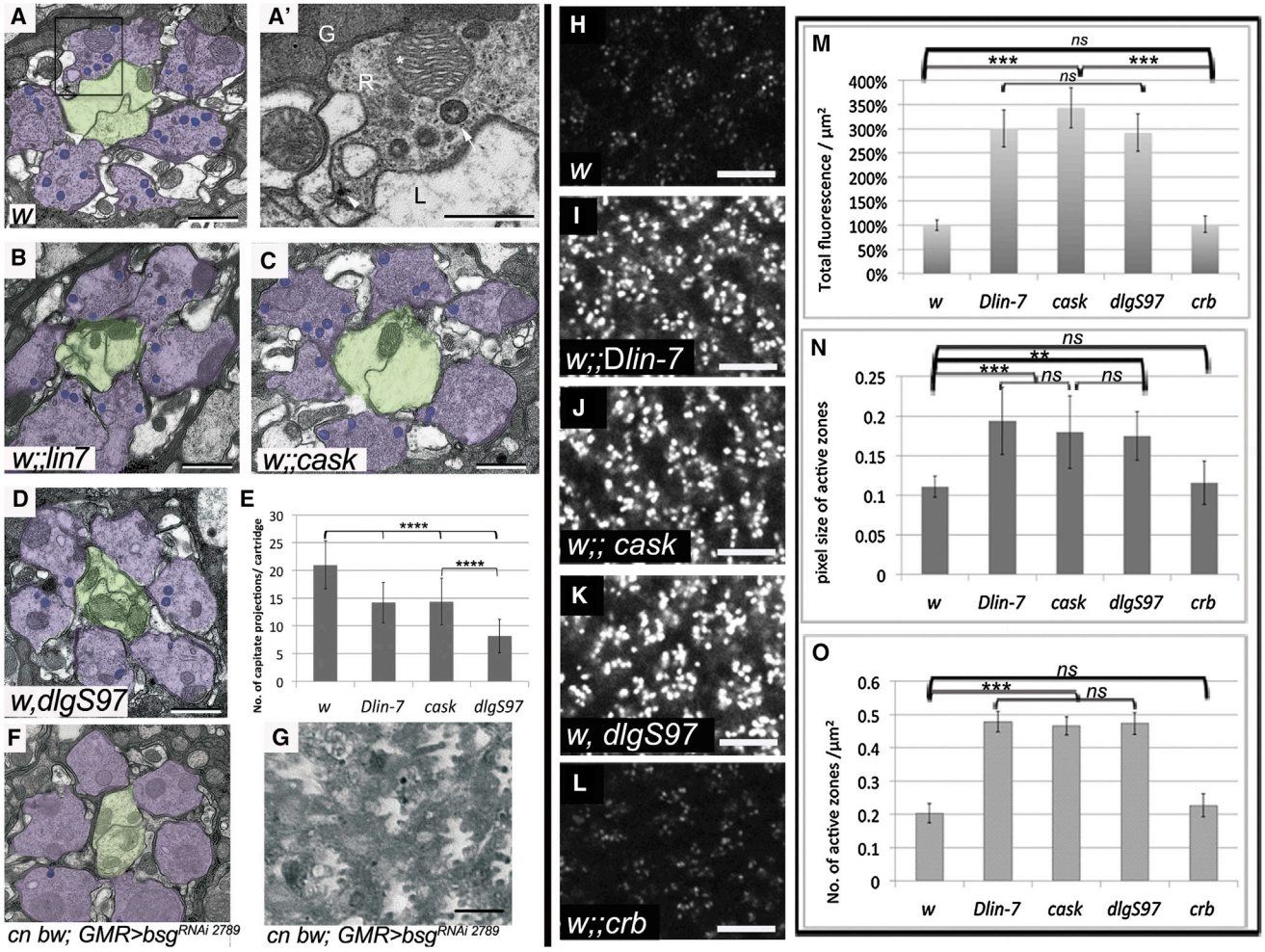

Figure 4. The Number of Capitate Projections and Active Zones Is Decreased in D*lin-7*, *cask*, and *dlgS97* Mutant Laminas

(A–D) Transmission electron microscopic images of cross-sectioned cartridges taken in the proximal third of the lamina. The morphology of lamina cartridges of *w;;*D*lin-7*^{66} (B), *w;;cask*^{x313}/*cask*^{x3077} (C), and *w,dlgS97*^{flpV} (D) mutants is similar to that of control *w* flies (A; boxed area shown at higher magnification in A′; asterisk indicates a mitochondrion, arrowhead indicates a T-bar ribbon). PRCs are marked in violet, LMCs L1 and L2 in green, and capitate projections in blue and by arrow in (A′). Cartridges are surrounded by glial cells (white "G" in A′). D*lin-7*, *cask*, and *dlgS97* mutant photoreceptor terminals show significantly decreased numbers of capitate projections compared with *w* control laminas.

(E) Statistical analysis shows that D*lin-7* and *cask* mutant cartridges have ~30% and *dlgS97* mutant cartridges ~60% fewer capitate projections.

(F and G) Silencing of *basigin* (*bsg*) in PRC leads to reduced number of capitate projections (F) and light-dependent degeneration (G).

(H–L) Brp staining is significantly increased in *w;;*D*lin-7*^{66} (I), *w;;cask*^{x313}/*cask*^{x307} (J), and *w,dlgS97*^{flpV} (K) laminas compared with *w* controls (H). In contrast, *crb*^{11A22} eye clones show Brp staining comparable to that in control flies (L).

(M) Quantification of total fluorescence shows 3.0-fold, 3.4-fold, and 2.9-fold increases in *w;;*D*lin-7*^{66}, *w;;cask*^{x313}/*cask*^{x307}, and *w,dlgS97*^{flpV}, respectively.

(N) Analysis of Brp-positive spot numbers shows that *w;;*D*lin-7*^{66}, *w;;cask*^{x313}/*cask*^{x307}, and *w,dlgS97*^{flpV} laminas contain ~2.3 times more active zones than *w* laminas, while the number is not changed in the lamina of flies with large *crb*^{11A22} eye clones.

(O) Brp-positive spots are 1.8 times larger in size in *w;;*D*lin-7*^{66} lamina compared with *w* controls, and ~1.6 times larger in *w;;cask*^{x313}/*cask*^{x307} and *w,dlgS97*^{flpV} mutant laminas. The *w* controls and flies with large *crb*^{11A22} eye clones have Brp-positive spots of similar size.

Scale bars represent 900 nm in (A)–(D) and 5 μm in (G) and (H)–(L). Error bars were calculated by SD. See also Figure S4.

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