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Drosophila Lin-7 is a component of the Crumbs complex in epithelia and photoreceptor cells and prevents light-induced retinal degeneration

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Abstract

The *Drosophila* Crumbs protein complex is required to maintain epithelial cell polarity in the embryo, to ensure proper morphogenesis of photoreceptor cells and to prevent light-dependent retinal degeneration. In *Drosophila*, the core components of the complex are the transmembrane protein Crumbs, the membrane-associated guanylate kinase (MAGUK) Stardust and the scaffolding protein *D*PATJ. The composition of the complex and some of its functions are conserved in mammalian epithelial and photoreceptor cells. Here, we report that *Drosophila* Lin-7, a scaffolding protein with one Lin-2/Lin-7 (L27) domain and one PSD-95/Dlg/ZO-1 (PDZ) domain, is associated with the Crumbs complex in the subapical region of embryonic and follicle epithelia and at the stalk membrane of adult photoreceptor cells. *D*Lin-7 loss-of-function mutants are viable and fertile. While *D*Lin-7 localization depends on Crumbs, neither Crumbs, Stardust nor *D*PATJ require *D*Lin-7 for proper accumulation in the subapical region. Unlike other components of the Crumbs complex, *D*Lin-7 is also enriched in the first optic ganglion, the lamina, where it co-localizes with Discs large, another member of the MAGUK family. In contrast to *crumbs* mutant photoreceptor cells, those mutant for *D*Lin-7 do not display any morphogenetic abnormalities. Similar to *crumbs* mutant eyes, however, *D*Lin-7 mutant photoreceptors undergo progressive, light-dependent degeneration. These results support the previous conclusions that the function of the Crumbs complex in cell survival is independent from its function in photoreceptor morphogenesis.

Keywords: Apical–basal polarity; Scaffolding protein; L27 domain; PDZ domain; Discs large; Ring canal; Light-dependent photoreceptor degeneration

Introduction

The Crumbs protein complex is highly conserved between *Drosophila* and vertebrates. In flies, the core components known so far include the transmembrane protein Crumbs (Crb), the cytoplasmic domain of which directly binds to the PDZ (PSD-95/Discs large/ZO-1) domain of the MAGUK protein Stardust (Sdt). Sdt, in turn, recruits the scaffolding protein *D*PATJ (protein associated with tight junction/Pals1-associated tight junction protein) via direct interaction between the L27 domain of *D*PATJ and the N-terminal L27 domain of Sdt (Fig. 1A) (see Richard et al., 2006b, for recent review). In epithelia, the complex is confined to the

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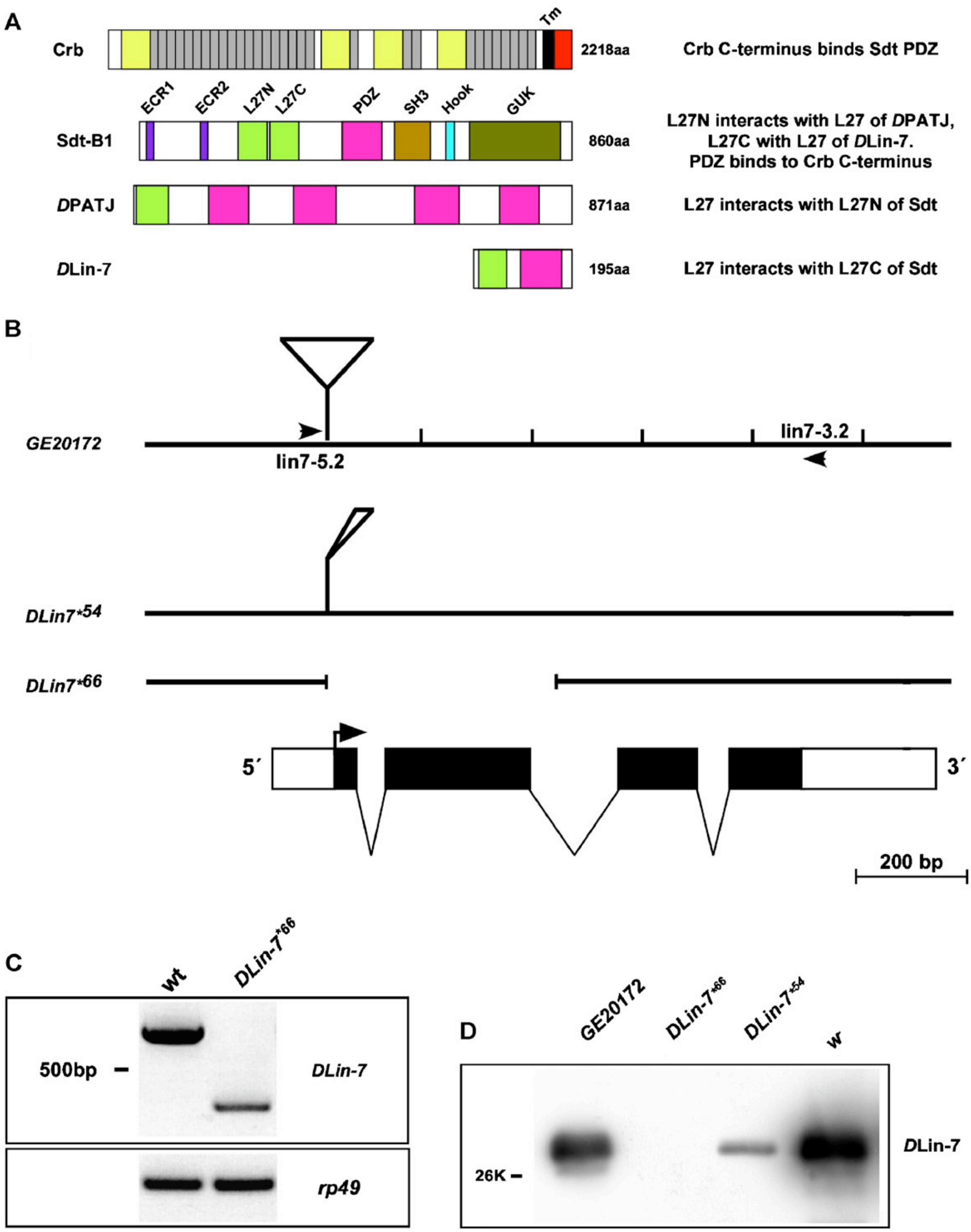
subapical region, a portion of the apical plasma membrane just apical to the zonula adherens. *Drosophila* embryos lacking *crb* or *sdt* fail to maintain polarity and integrity of ectodermally derived epithelia (Grawe et al., 1996; Tepass, 1996). In vertebrates, the core members of the complex, CRB1/2/3, MPP5/Pals1 and PATJ, are highly conserved. In epithelia CRB3 forms a complex with the two scaffolding proteins at the tight junction, hence at an equivalent position to that of the subapical region in *Drosophila* epithelia. Knockdown of any component of the complex in epithelial cells in culture impairs tight junction formation and adherens junction stability (reviewed in Shin et al., 2006).

Beside the above-mentioned core components Crb/CRB, Sdt/MPP5/Pals1 and *D*PATJ/PATJ, which are associated whenever co-expressed in a given cell, additional proteins can be recruited into the complex. *Dm*Par-6/Par-6, a member of the conserved Par complex, can bind via its PDZ domain to the C-terminus of Crb/CRB3 or the N-terminus of Sdt (Hurd et al., 2003;



Kempkens et al., 2006; Lemmers et al., 2004). In the *Drosophila* embryo it is co-localized with the Crb complex in the subapical region of epithelia. The FERM (4.1-ezrin–radixin–moesin) domain-containing protein Yurt is a negative regulator of the complex, restricting the localization of the complex to the subapical region. Yurt can bind via its FERM domain to the cytoplasmic domain of Crb, which contains a conserved FERM-binding motif. Yurt is mainly confined to the baso-lateral membranes both in embryonic epithelia and in photoreceptor cells (PRCs). In both tissues, Crb is required to recruit Yurt transiently to the apical membrane in later stages of development. The zebrafish orthologue *mosaic eyes* encodes a structurally and functionally similar protein, which is required to confine the Crb complex to the apical site (Hsu et al., 2006). The mammalian Yurt orthologues EHM2 and EPB41L5 were shown to bind to the FERM-binding domain of CRB1 and CRB3, respectively. EPB41L5 can additionally interact with the Hook domain of MPP5 (Gosens et al., 2007; Laprise et al., 2006).

In PRCs of the adult fly, the Crb complex is localized at the stalk membrane, a specialized region of the apical membrane apical to the zonula adherens. This part of the membrane topologically corresponds to the sub-apical region of epithelial cells. Loss of function of *crb*, *sdt* or *DPATJ* in PRCs results in morphogenetic defects and in light-dependent degeneration (Berger et al., 2007; Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002; Richard et al., 2006a). Both in flies and zebrafish loss of Yurt/Mosaic eyes results in an expansion of the apical membrane of PRCs, a phenotype similar to that described for over-expression of Crumbs (Laprise et al., 2006). Strikingly, mutations in mammalian CRB1 result in retinal degeneration in the mouse, and Retinitis pigmentosa 12 (RP12)- and Leber congenital amaurosis (LCA)-related blindness, two severe forms of retinal dystrophies in humans (den Hollander et al., 1999; Mehalow et al., 2003; van de Pavert et al., 2004). In the zebrafish *Danio rerio*, PRCs mutant for *crb2b*, one of the zebrafish *crumbs* genes, exhibit a significant shortening of the inner segments, which constitute part of the apical membrane (Omori and Malicki, 2006). Furthermore, the zebrafish *sdt* orthologue *nagie oko* is essential for the patterning of the retina (Wei and Malicki, 2002). These results highlight the functional conservation of the Crumbs complex throughout evolution.

Both in *Drosophila* and vertebrates, Sdt/MPP5/Pals1 shows in vitro interaction with Lin-7, a small protein with an N-terminal L27 and a C-terminal PDZ domain. The proteins bind to each other via the single L27 domain of Lin-7 and the C-terminal L27 domain of Sdt/MPP5/Pals1 (Bachmann et al., 2004; Kamberov et al., 2000; Roh et al., 2002). In vertebrates, three different Lin-7 isoforms (also called MALS for mammalian LIN-7 or Veli, vertebrate homolog of LIN-7), encoded by separate genes, have been identified (Irie et al., 1999; Jo et al., 1999). They are expressed in a variety of epithelia and neurons, where they preferentially cluster at cell–cell junctions, such as tight junctions or synapses, respectively. In the mouse retina, Veli3 co-localizes with MPP5 (the Sdt orthologue) in the outer limiting membrane and with MPP4, another MAGUK protein, in the outer plexiform layer (Aartsen et al., 2006; Stöhr et al., 2005). In the mammalian kidney, the three MALS/Veli isoforms are differentially localized. MALS-3 is restricted to the baso-lateral membrane in cells of the collecting duct, but is localized both to the baso-lateral membrane and the tight junction in proximal tubule cells (Olsen et al., 2005b). This suggests that it may undergo interactions with different interacting partners. Mice lacking all three Lin-7 genes die perinatally and exhibit deficiencies in breathing and in synaptic transmission, but no major defect in external tissue morphogenesis (Olsen et al., 2005a). MALS-3 single knockout mice exhibit regional defects in epithelial polarization in the kidney, which is associated with disruption of tight junctions and results in the development of cystic and fibrotic tissue (Olsen et al., 2007). Similarly, knockdown

Fig. 1. Generation of *DLin-7* mutant alleles. (A) Protein structure and size of the core components of the Crumbs complex and their reciprocal interactions. The transmembrane protein Crb contains 30 EGF-like repeats (gray) and four laminin A globular domains (yellow). The 37-aa intracellular domain of Crb (red) contains a FERM-binding motif and terminates with the PDZ-binding motif -ERLI. The Sdt-B1 isoform is expressed in epithelia and photoreceptor cells and consists of two evolutionary conserved regions, ECR1 and ECR2 (lilac), two L27 domains (green), a PDZ (magenta), an SH3 (brown), a Hook (blue), and a GUK domain (olive). *D*PATJ possesses an N-terminal L27 domain and four PDZ domains. *D*Lin-7 includes an N-terminal L27 domain and a C-terminal PDZ domain. Their reciprocal interactions are described. All proteins, except Crb, are shown at the same scale. (B) Mobilization of the EP-element *GE20172* yielded two mutant *DLin-7* alleles. The hypomorphic allele $DLin-7^{*54}$ still contains remnants (447 bp) of the original P-element (P-element DNA not to scale), but leaves the genomic region intact, whereas the putative null allele $DLin-7^{*66}$ lacks a genomic region of 448 bp, including part of the first exon with the single translational start site and the complete second exon (black boxes, ORF; white boxes, UTR). (C) *DLin-7*-specific RT-PCR on total RNA from wild-type and $DLin-7^{*66}$ L3 larval body walls, respectively, using the primer pair lin7-5.2/3.2 (B; see Materials and methods). In $DLin-7^{*66}$ animals a truncated *DLin-7* transcript is produced, which lacks the first exon (including the translational start site) and the complete second exon. Transcripts from the *rp49* gene served as an internal loading control. (D) Western blot analysis of adult heads of flies from the following genotypes: *GE20172*, $DLin-7^{*66}$, $DLin-7^{*54}$ and w^-. The blot was probed with an anti-*D*Lin-7 antibody and overexposed to demonstrate lack of protein in $DLin-7^{*66}$ and still very low amounts of protein in $DLin-7^{*54}$. Protein amount per lane equals one adult head.

of Lin-7C/MALS-3 in epithelial cells in culture impairs tight junction formation and destabilizes Pals1 and PATJ (Straight et al., 2007).

Lin-7 was originally identified in *Caenorhabditis elegans*, together with Lin-2 and Lin-10, as part of a tripartite complex, which is essential for the baso-lateral localization of the receptor tyrosine kinase Let-23 in epithelial cells of the vulva (Kaech et al., 1998; Simske et al., 1996). *Drosophila Lin-7* encodes a single protein of approximately 27 kDa, which is expressed throughout development. It co-localizes apically with Crb/Sdt in epithelia of the imaginal discs, but forms a complex with the MAGUK Discs large in the larval neuromuscular junction (Bachmann et al., 2004). Given that the Crb/Sdt complex is required for polarity and adhesion of epithelial cells in the embryo, and photoreceptor morphogenesis and survival in the eye, we were interested to know whether *D*Lin-7 is of similar importance for these three processes. Therefore, we induced a null mutation in *D*Lin-7 by imprecise excision of a transposable P-element. From the three processes analyzed, *D*Lin-7 is only required for the survival of photoreceptors when exposed to light, but is dispensable for epithelial cell polarity in the embryo and photoreceptor morphogenesis. This result supports the notion already drawn from the analysis of different *sdt* alleles (Berger et al., 2007) that the function of the Crb complex in cell survival is independent from its function during morphogenesis.

Materials and methods

Fly work and histology

The following fly stocks were used: wild-type (Oregon R); *DLin-7*^{*66} and *DLin-7*^{*54} (see below); *crb*^{11A22} (Jürgens et al., 1984); UAS-Myc-Crb$_{\mathrm{intra}}$ (Wodarz et al., 1995); UAS-Flag-*D*Lin-7 (Bachmann et al., 2004). UAS constructs were activated using *en*GAL4 (Han and Manley, 1993) for overexpression in an otherwise wild-type genetic background. Eyes mosaic for *crumbs* were generated by crossing *yw ey*FLP;;FRT82B w^+ *cl*3*R3*/*TM6B* females (Newsome et al., 2000) to *w*;;FRT82B *crb*^{11A22}/*TM6B* males. Preparation of semi-thin sections and analysis of light-induced retinal degeneration were performed as described (Johnson et al., 2002).

Generation of *DLin*-7 mutants

The EP line GE20172/G3768 is derived from the GenExel collection (GenExel Inc., South Korea). The P-element is inserted into the first exon 35 bp upstream of the translational start site at position 3R: 20891271 (release: r4.3). Imprecise excision of *GE20172* yielded two mutant alleles, $DLin{-}7^{*66}$ and $DLin{-}7^{*54}$.

RT-PCR

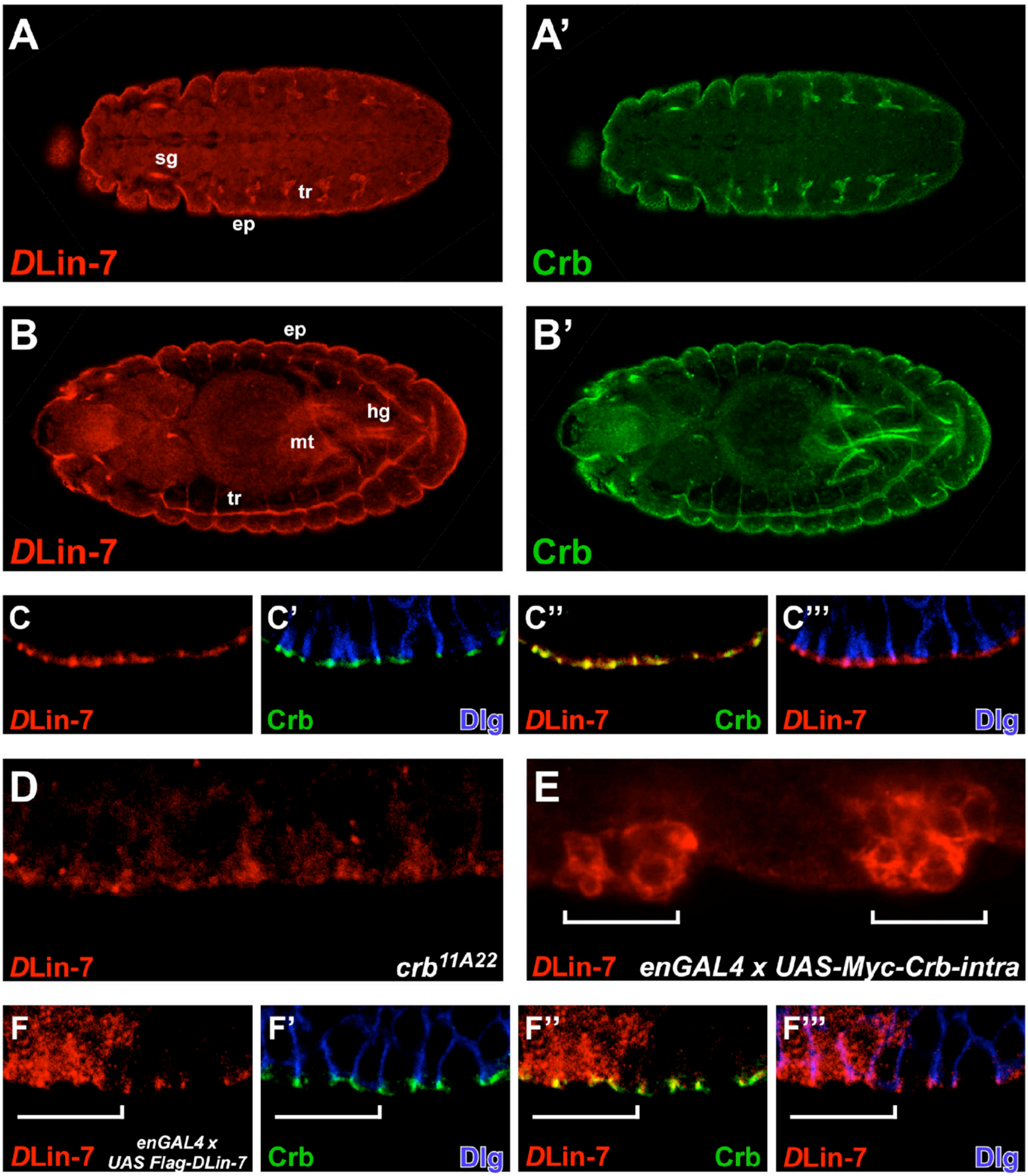
Total RNA from 20 body walls of third-instar larvae (wild-type and $DLin{-}7^{*66}$, respectively) was extracted using the NucleoSpin RNA II kit from Macherey & Nagel. Total RNA (500 ng) was used as template for RT-PCR (OneStep RT-PCR Kit, Qiagen). The primer pair for detection of the *DLin-7* transcript (lin7-5.2/3.2) has been described before (Bachmann et al., 2004). As an internal loading control transcripts from the *rp49* gene were detected using the primers rp49-5 (AGATC-GTGAAGAAGCGCACC) and rp49-3 (CGATCCG-TAACCGATGTTGG).

Antibodies and immunofluorescence analyses

For immunofluorescence analyses, embryos and ovaries were fixed and stained by standard protocols, respectively. For staining of adult retina and brain, cryosections were prepared and stained as described (Richard et al., 2006a). Rabbit anti-*D*Lin-7 was used at 1:500 (Bachmann et al., 2004). Other primary antibodies used were: mouse anti-Dlg^{4F3} (1:100; directed against

Fig. 2. *D*Lin-7 expression in the embryo. (A, B) Ventral view of a stage-11 (A) and dorsal view of a stage-15 (B) wild-type embryo, respectively, each stained with anti-*D*Lin-7 (red) and anti-Crb (green). *D*Lin-7 and Crb co-localize in all epithelia of ectodermal origin like the salivary glands (sg), the tracheal system (tr), the epidermis (ep), the hindgut (hg), the foregut (out of focus) and the Malpighian tubules (mt). (C–C‴) Close-up view of the epidermis of a stage-13 wild-type embryo stained with anti-*D*Lin-7 (red, C, C″ and C‴), anti-Crb (green, C′, C″) and anti-Dlg (blue, C′, C‴). C′, C″ and C‴ show merged images. *D*Lin-7 co-localizes with Crb in the subapical region (C″), apical to the septate junction-specific form of Dlg (C‴). (D) Part of the epidermis of a stage-13 *crb*^{11A22} mutant embryo stained with anti-*D*Lin-7 antibody (red). Most of *D*Lin-7 is lost from the subapical region and distributed in the cytoplasm. (E) Part of the epidermis of a stage-13 wild-type embryo, overexpressing the intracellular domain of Crb in the posterior part of each segment (white brackets) by means of *en*Gal4, and stained with anti-*D*Lin-7 antibody (red). Overexpressed Crb$_{\mathrm{intra}}$ redistributes *D*Lin-7 to ectopic sites. (F–F‴) Gal4/UAS-mediated overexpression of Flag-*D*Lin-7 in the posterior part of each segment (white brackets) of a stage-14 wild-type embryo with close-up view on the epidermis. The embryo was stained with anti-*D*Lin-7 (red, F, F″ and F‴), anti-Crb (green, F′, F″) and anti-Dlg (blue, F′, F‴). F′, F″ and F‴ show merged images. Epithelial cell polarity is not affected as Crb is still localized apically (F′, F″) and the septate-junction component Dlg is still restricted to the lateral membrane domain (F′, F‴). In (A–B′), anterior is left. In (C–F), apical is down.

the second PDZ domain) (Developmental Studies Hybridoma Bank), rat anti-Crb$^{2.8}$ (1:100) (E. Theilenberg and E. Knust, unpublished), rabbit anti-*D*PATJ (1:500) (Richard et al., 2006a), rabbit-anti-Dlg-S97 (1:200; directed against the Dlg-S97-specific N-terminus) (Mendoza et al., 2003), and rabbit anti-Sdt^{MPDZ} (1:500) (Berger et al., 2007). Fluorescence-labeled secondary antibodies purchased from Jackson ImmunoResearch Laboratories, Inc. (fluorescein conjugates) or Molecular Probes, Inc. (Alexa-568 conjugates) were applied at a 1:200 dilution. Confocal imaging was performed on a Leica TCS NT confocal microscope. All images were



processed and mounted using Adobe Photoshop 7.0 and Deneba Canvas 9.0.

Western blot analysis and immunoprecipitations

Adult head protein lysates were prepared as follows: heads from 50 adult flies were homogenized on ice in 50 μl lysis buffer containing 20 mM Tris–HCl (pH 8), 150 mM NaCl, 10% glycerol, 2 mM EDTA, 10 mM CHAPS, and protease inhibitors (1 μM Pefabloc, 5 μM Leupeptin, 1 μM Pepstatin, 0.3 μM Aprotinin); the homogenate was cleared from cuticle debris by centrifugation for 2 min at 13,000 rpm. Until usage the supernatant was stored at −70 °C.

For immunoprecipitation 40 μl supernatant was loaded with 460 μl lysis buffer onto 30 μl of protein A Sepharose (Pharmacia Biotech) and preincubated overnight at 4 °C on a shaker. After brief centrifugation to precipitate the Sepharose beads, the supernatant was incubated for 2 h at 4 °C with 2 μl anti-*D*Lin-7 antibody or control antibody (2 μl rabbit anti-Serrate antibody (Thomas et al., 1991)), respectively. Fresh protein A Sepharose (30 μl) was added, followed by an overnight incubation at 4 °C on a shaker. The protein A Sepharose precipitate was washed four times in lysis buffer, supplied with 20 μl of 2× SDS sample buffer and boiled for 5 min. Equivalents of one adult head (input controls) or 10 adult heads (precipitates) were separated by SDS–PAGE and blotted onto nitrocellulose transfer membrane. Upon blockage in TBST/5% dry milk, the membrane was incubated overnight with the following antibodies, respectively: rabbit anti-*D*PATJ at 1:4000, rabbit anti-$\mathrm{Sdt}^{\mathrm{MPDZ}}$ at 1:2000 or mouse anti-Dlg^{4F3} at 1:5000. Peroxidase-conjugated secondary antibodies in combination with the ECL system (Amersham Pharmacia Biotech) were employed to detect immunoreactive bands.

Preparation of ovaries and subsequent immunoprecipitation were carried out according to the protocols described above with the following modifications: 25 ovaries were dissected for lysate preparation. Afterwards, 20 μl lysate was diluted in 480 μl lysis buffer and immunoprecipitation was performed with 2 μl anti-*D*PATJ antibody. Equivalents of one ovary (input control) or 5 ovaries (precipitates) were separated by SDS–PAGE and blotted onto nitrocellulose transfer membrane. *D*Lin-7 was detected using the rabbit anti-*D*Lin-7 antibody at 1:5000.

Results and discussion

In order to better understand the role of *D*Lin-7 in *Drosophila* and its possible relation to the Crumbs complex, we induced loss-of-function mutations in this gene. Therefore, we took advantage of an EP-element (*GE20172/G3768*, Genexel) located 35 bp upstream of the translational start site (Fig. 1B). Transposase-induced excision of the P-element was scored by the loss of the *white*$^{+}$ phenotype. The DNA of a total of 123 independent w^{-} flies was analyzed by PCR. Two lines were found that exhibited a partial and a complete excision of the P-element, respectively. Line *54 lacks most of the P-element insert except 447 bp, but leaves the genomic region of *DLin-7* intact. Line *66 removes the complete P-element plus 448 bp of genomic DNA (Fig. 1B, C), which includes part of the first exon and the complete second exon. Both lines as well as the original P-element line are homozygous viable and fertile. *D*Lin-7 protein is absent in flies of line *66 and strongly reduced in flies of line *54, but is only mildly affected in GE20172 (Fig. 1D). Therefore, we named the respective mutants $DLin\text{-}7^{*66}$ and $DLin\text{-}7^{*54}$.

As previously shown, *D*Lin-7 immunoprecipitates Sdt from extracts of embryos and interacts with Sdt in vitro (Bachmann et al., 2004). To further determine its tissue expression profile, wild-type embryos were stained with a *D*Lin-7-specific antibody. *D*Lin-7 is expressed in all embryonic epithelia derived from the ectoderm, i.e. the epidermis, the fore- and the hindgut, the tracheae, the salivary glands, and the Malpighian tubules (Fig. 2A, B). In these tissues, it co-localizes with Crb in the subapical region (Fig. 2C–C″). Unlike in the neuromuscular junction, it does not overlap with Discs large (Dlg), a component of the septate junction, in embryonic epithelia (Fig. 2C‴). Loss of Crb in the embryo prevents the apical accumulation of *D*Lin-7 and induces its diffuse distribution in the cytoplasm (Fig. 2D). Overexpression of the cytoplasmic domain of Crb in otherwise wild-type embryos recruits additional *D*Lin-7 to ectopic sites in the cell (Fig. 2E). In contrast, neither loss nor overexpression of *D*Lin-7 affects the apical localization of Crb or the lateral localization of Dlg (Fig. 2F–F‴ and data not shown). These results indicate that Crb is necessary and sufficient for the apical recruitment of *D*Lin-7 in epithelia and acts upstream of *D*Lin-7 in these tissues.

Crb, Sdt and *D*PATJ are also expressed in the subapical region of the follicle epithelium, a single-layered epithelium surrounding the 16-cell cysts in the egg chambers of the ovaries (Schneider et al., 2006; Tanentzapf et al., 2000; Tepass and Knust, 1990). The observation that *D*Lin-7 expression fully overlaps with Sdt (Fig. 3B–B″, C–C″) suggests that it is part of the Crb complex in these cells, comparable to that formed in embryonic ectodermal epithelia. This assumption is supported by the fact that *D*Lin-7 can be co-immunoprecipitated from extracts of ovaries with an anti-*D*PATJ antibody (Fig. 3A). It has been previously shown that *D*PATJ, a scaffolding protein containing a single L27 domain and four PDZ domains (Fig. 1A) or

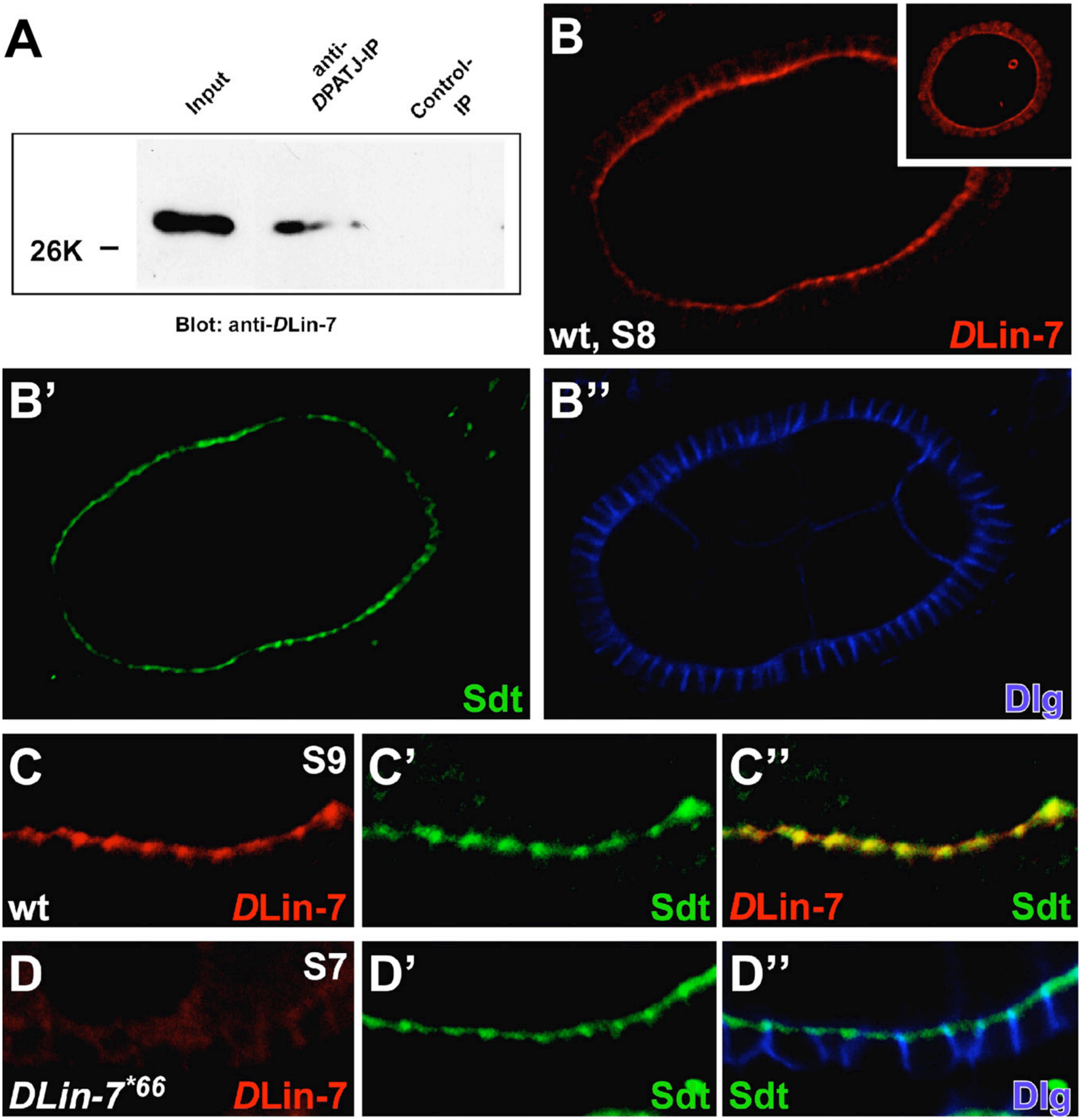
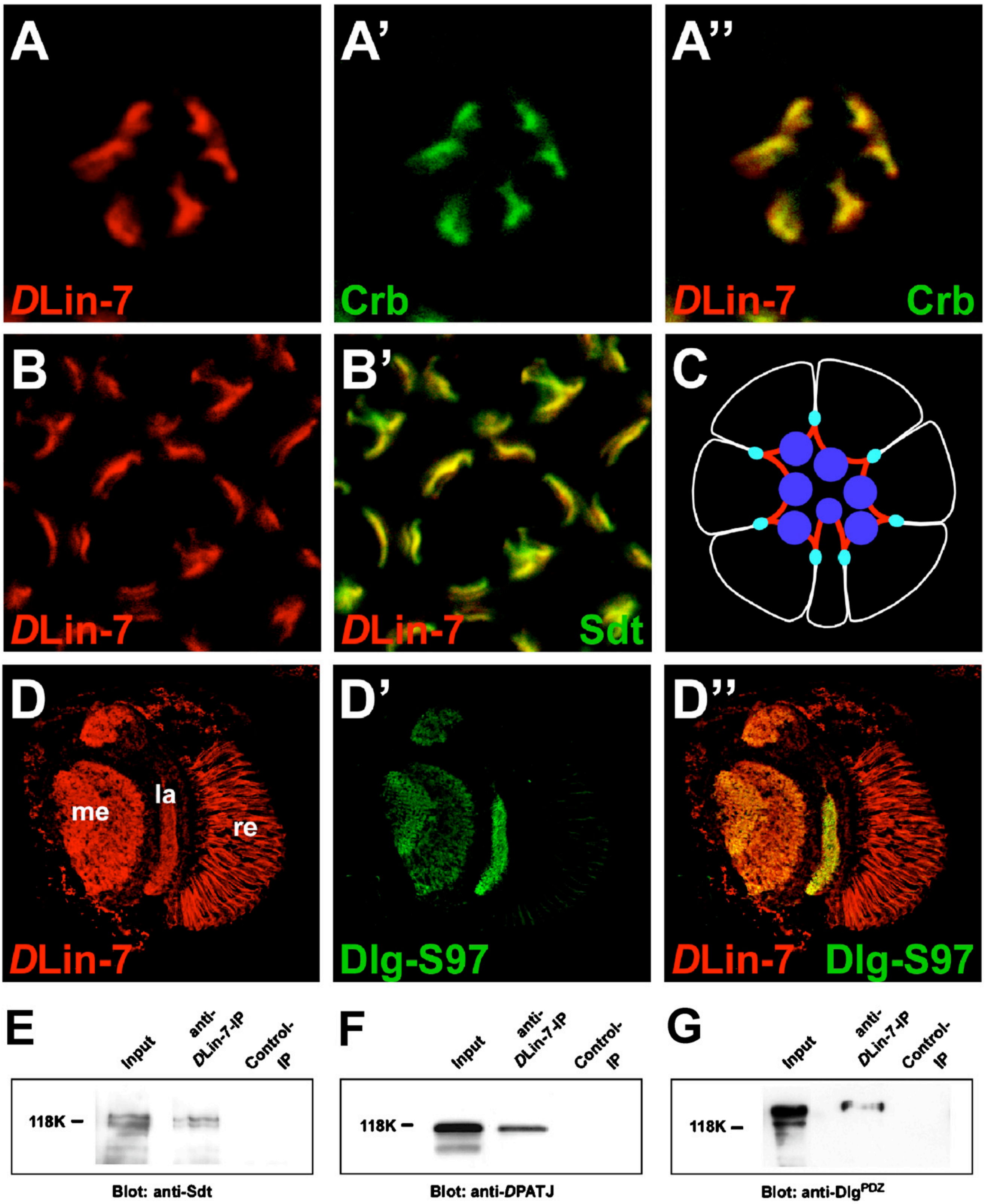


Fig. 3. *D*Lin-7 expression during oogenesis. (A) Co-immunoprecipitation assays from wild-type ovary protein extracts demonstrate that *D*Lin-7 forms a complex with the Crb complex member *D*PATJ. In the input lane, 20% of the protein amount used in the co-immunoprecipitation assays was loaded. (B–B″) A stage-8 wild-type egg chamber stained with anti-*D*Lin-7 (red, B), anti-Sdt (green, B′) and anti-Dlg (blue, B″). *D*Lin-7 is expressed apically from stage 2 onwards in the follicle epithelium. It can also be detected in the ring canals of the nurse cells and the oocyte (inset in B). (C–C″) Close-up view of the follicle epithelium of a wild-type stage-9 egg chamber, double-labeled with anti-*D*Lin-7 (red, C) and anti-Sdt (green, C′). *D*Lin-7 and Sdt co-localize in the subapical region of the follicle cells (C″ = merged image). (D–D″) The follicle epithelium of a stage-7 *DLin-7*^{*66} mutant egg chamber, stained with anti-*D*Lin-7 (red, D), anti-Sdt (green, D′) and anti-Dlg (blue, D″). Neither the localization of the Crb complex component Sdt (green), nor cell polarity in general are affected, as revealed by correct localization of Dlg (blue), a marker for the lateral membrane, (D″ = merged image). In (C–D), apical is up.

its mammalian orthologue PATJ is indirectly connected to *D*Lin-7/Lin-7 via interaction with Sdt/Pals1/MPP5. The latter contains two *L*27 domains (Fig. 1A), which directly bind to *D*PATJ/PATJ and *D*Lin-7/Lin-7, respectively, as demonstrated by GST-pulldown assays and/or yeast two-hybrid interactions (Feng et al., 2005;



Roh et al., 2002) (Ö. Kempkens and E. Knust, unpublished). Surprisingly, *D*Lin-7 can also be detected on the ring canals (Fig. 3B, inset), actin-rich intercellular bridges that connect the nurse cells with each other and with the oocyte. Loss of *D*Lin-7 in follicle cells does not influence the apical localization of other members of the Crb complex in the follicle epithelium, and has no effect on the localization of Discs large at the lateral membranes (Fig. 3D–D″). This demonstrates that, similarly as in embryonic epithelia (data not shown), *D*Lin-7 is not essential for the maintenance of tissue integrity and polarity of the follicle epithelium.

Components of the Crb complex are also expressed in adult PRCs, where they are restricted to the stalk membrane, a specialized portion of the apical membrane between the zonula adherens and the rhabdomere. *D*Lin-7 distribution perfectly overlaps with that of Crb, Sdt and *D*PATJ at the stalk membrane (Fig. 4A, B and data not shown) (see Fig. 4C for a schematic drawing to explain the staining patterns). The co-localization of *D*Lin-7 with Crb, Sdt and *D*PATJ at the stalk membrane suggests that *D*Lin-7 is a component of the Crb complex in PRCs of adult flies. In fact, the *D*Lin-7-specific antibody co-immunoprecipitates Sdt and *D*PATJ from extracts of fly heads (Fig. 4E, F). Similarly as in embryos, absence of *D*Lin-7 has no effect on the correct localization of Crb, Sdt or *D*PATJ (Fig. 5A–C). In contrast, localization of *D*Lin-7, Sdt and *D*PATJ at the stalk membrane strictly depends on Crb (Fig. 5D–F). Unlike Crb and *D*PATJ, *D*Lin-7 is also expressed in the optic ganglia, in particular in the lamina and the medulla. Here, it co-localizes with Dlg-S97 (Fig. 4D–D″), an isoform of Dlg also expressed in the neuromuscular junction, but not in epithelial cells (Bachmann et al., 2004; Mendoza et al., 2003). Although both the Dlg-A and the Dlg-S97 isoforms are expressed in the head (see input lane in Fig. 4G), *D*Lin-7 specifically pulls down the larger isoform, Dlg-S97.

The localization of a Lin-7 member at both the apical and the baso-lateral plasma membrane of the same cell has already been observed in another tissue. Expression studies in the kidney showed that, similar as in *Drosophila* PRCs, MALS-3 is localized both at the baso-lateral membrane and at the apical tight junction. In these cells, it forms a complex with the baso-lateral MAGUK Dlg and the tight junction-associated members of the CRB3/Pals1/PATJ complex, respectively. As a result of this, loss of MALS-3 disrupts both the DLG- and the CRB–protein complexes and leads to loss of apical–basal polarity (Olsen et al., 2007).

Crb, *D*PATJ and some forms of Sdt ensure proper morphogenesis of PRCs and prevent light-induced retinal degeneration. Exposure of flies with eyes mutant for any of these genes exhibits a gradual devolution of their rhabdomeres, and most PRCs undergo apoptosis after 5–7 days of constant illumination (Berger et al., 2007; Johnson et al., 2002; Richard et al., 2006a). To find out whether *DLin-7* is involved in similar processes, we analyzed sections of eyes of adult flies, which were kept either in the dark or were exposed to constant light for 7 days. Strikingly, unlike *crb* mutant PRCs, eyes lacking *D*Lin-7 exhibit a perfect wild-type morphology of their PRCs and rhabdomeres (compare Fig. 6B and C). After 7 days of light exposure, however, *DLin-7* mutant PRCs undergoes extensive degeneration, which is less severe than taking place in *crb* mutant PRCs (compare Fig. 6E and F). Degeneration of both *crb* and *D*Lin-7 PRCs is strictly dependent on the absence of pigment, since w^+; $DLin\text{-}7^{*66}$ or w^+; crb^{11A22} PRCs do not degenerate (data not shown).

In wild-type eyes, the signal transduction cascade induced by the activation of rhodopsin is turned off by the formation of a complex between the activated form of rhodopsin, metarhodopsin, and arrestin2. Following further modifications of both proteins, the metarhodopsin–arrestin2 complex dissociates, which allows rhodopsin to return to its inactive state. In a subset of retinal degeneration mutants, such as *arr*2, *norpA*, *rdgB* or *rdgC*, the dissociation of the metarhodopsin–arrestin2 complex does not occur, and the complex is endocytosed, resulting in apoptosis of the cells by a still unknown mechanism (Alloway et al., 2000; Kiselev et al., 2000). Light-induced retinal degeneration in these mutants can be rescued by feeding larvae with a vitamin A-depleted medium (Alloway et al., 2000; Kiselev et al., 2000), which reduces rhodopsin levels by over 95% (Nichols and Pak, 1985). Light-induced retinal degeneration of *w*; $DLin-7^{*66}$ flies can be prevented when the larvae were raised and adult animals kept on food that lacks vitamin A, a precursor of rhodopsin in flies

Fig. 4. *D*Lin-7 expression in the adult eye and brain. (A, B) Cross-sections of adult wild-type *Drosophila* eyes double-stained with anti-*D*Lin-7 (red, A and B), anti-Crb (green, A′) and anti-Sdt (green: B′), respectively. *D*Lin-7 co-localizes with Crb complex components at the stalk membrane. A″ and B′ are merged images. (C) Schematic diagram of a cross-section through an adult photoreceptor cell: red: stalk membrane, blue: rhabdomere, turquoise: zonula adherens. (D–D″) Longitudinal sections of an adult wild-type *Drosophila* brain double-labeled with anti-*D*Lin-7 (red, D) and anti-Dlg-S97 (green, D′). *D*Lin-7 is strongly expressed in the retina (re), the lamina (la) and the medulla (me). In the lamina and medulla it co-localizes with Dlg-S97 (D″ = merged image). (E–G) Co-immunoprecipitation assays from head protein extracts reveal that in the adult eye *D*Lin-7 forms a complex with Sdt (E) and *D*PATJ (F). Note that there are two major Sdt proteins of about 120 kDa present (Berger et al., 2007). In the adult brain *D*Lin-7 forms a complex specifically with Dlg-S97 (G, upper band), but not Dlg-A (lower band). In the input lanes, 10% of the protein amount used in the co-immunoprecipitation assays was loaded.

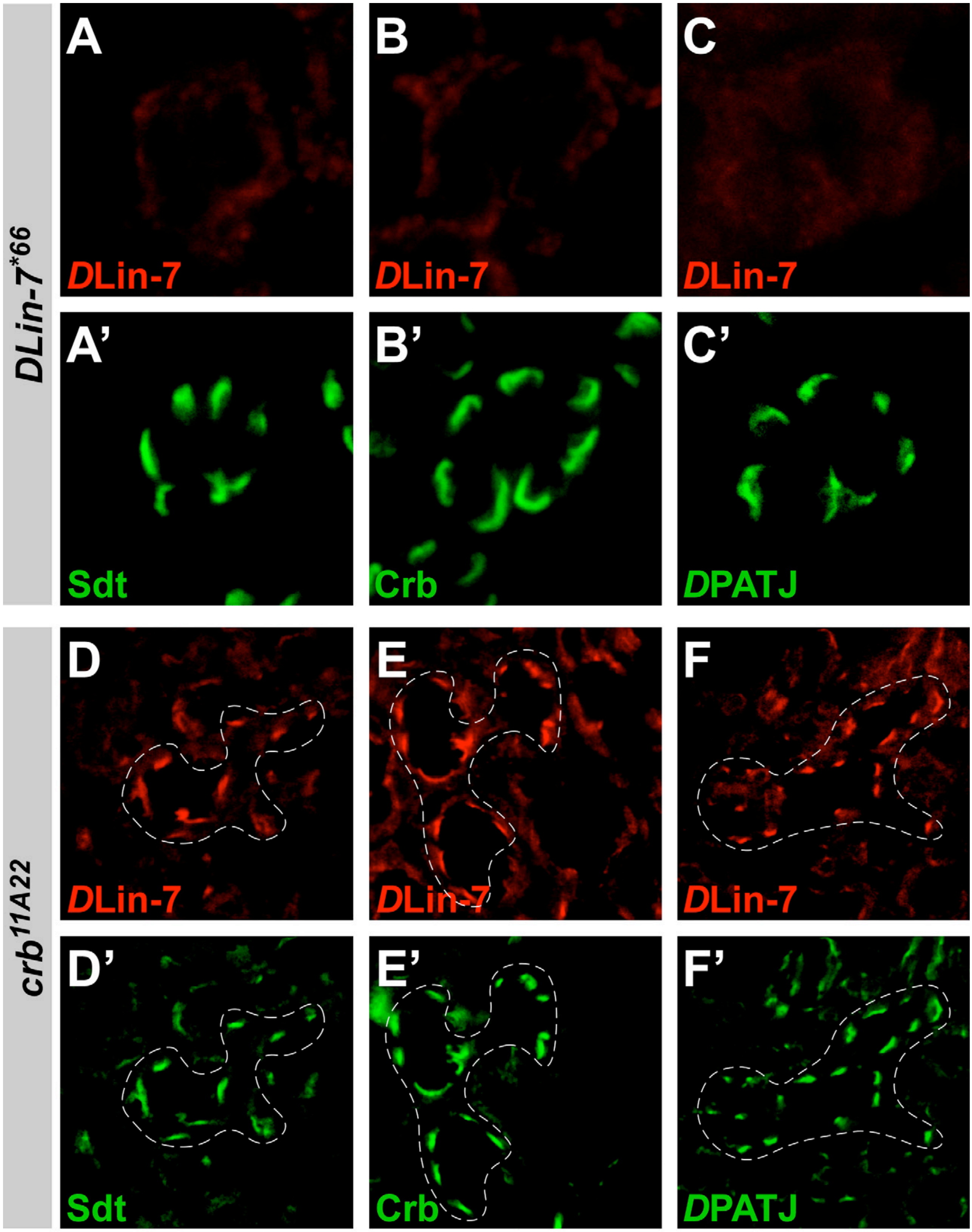


Fig. 5. Relationship between *D*Lin-7 and *crumbs*. (A–C) Cross-sections of adult *DLin-7*^{*66} mutant *Drosophila* eyes double-stained with anti-*D*Lin-7 (red, A–C), anti-Sdt (green, A′), anti-Crb (green: B′), and anti-*D*PATJ (green: C′), respectively. Loss of *D*Lin-7 protein in *DLin-7*^{*66} mutants does not affect other core components of the Crb complex. (D–F) Adult *Drosophila* eyes with *crb*^{11A22} mutant clonal areas, stained with anti-*D*Lin-7 (D–F), anti-Sdt (D′), anti-Crb (E′) and anti-*D*PATJ (F′). *D*Lin-7 (D–F), Sdt (D′) and *D*PATJ (F′) are lost from the stalk membrane. *crb*^{11A22} mutant areas are marked by the absence/delocalization of Crb or Crb complex members, and display morphological aberrations (compare with Fig. 6). White dashed lines encircle wild-type areas.

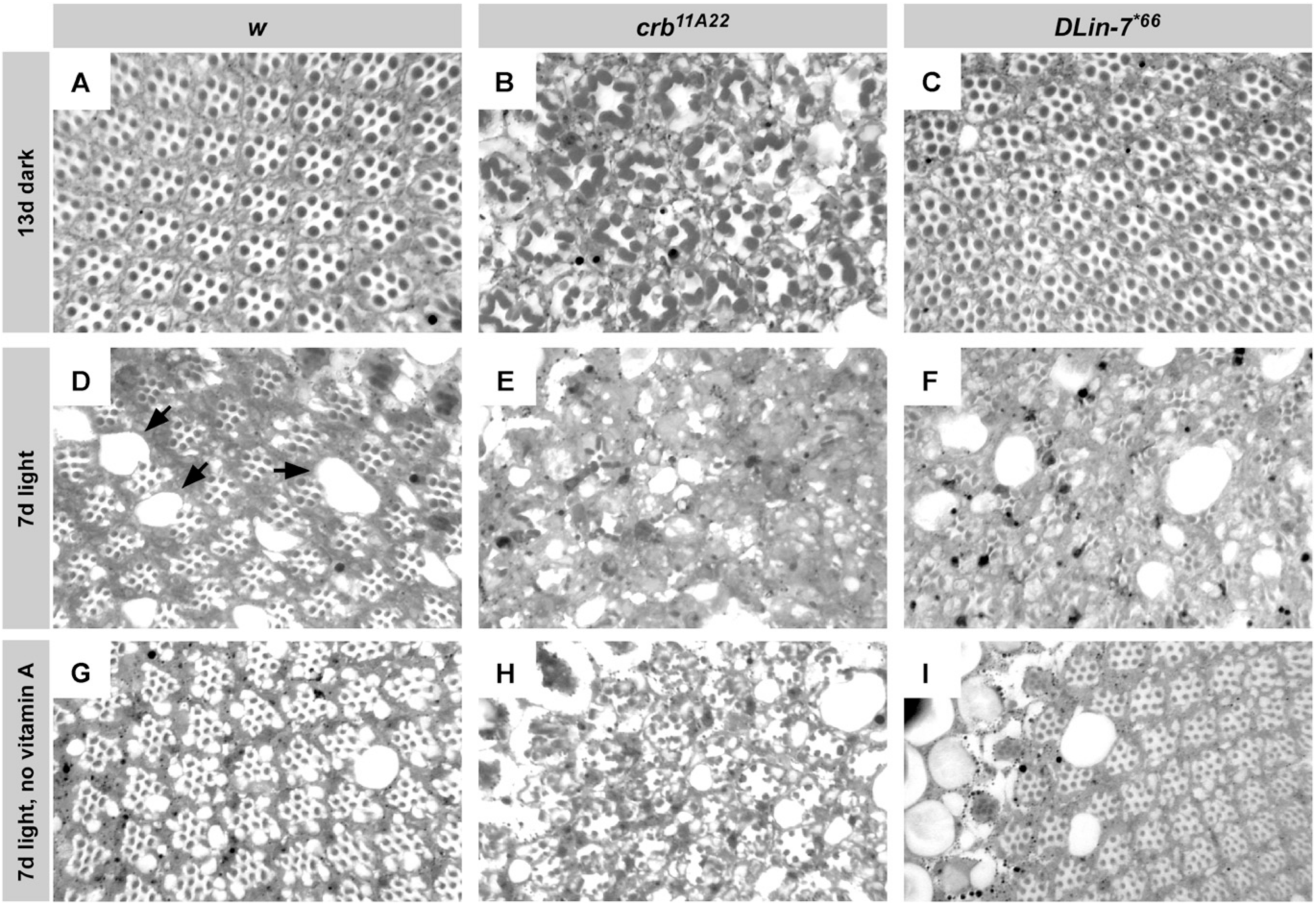


Fig. 6. *DLin-7* is required to prevent light-induced retinal degeneration. Semi-thin sections of eyes of *w* (A, D, G), crb^{11A22} (B, E, H) and $DLin\text{-}7^{*66}$ (C, F, I) mutant flies kept for 13 days in the dark (A–C), exposed to constant light for 7 days (D–F) or exposed to constant light for 7 days, but raised in a medium that lacks vitamin A (G–I). Unlike crb^{11A22} mutant photoreceptor cells, $DLin\text{-}7^{*66}$-mutant PRCs show a wild-type morphology when kept in the dark for 13 days (compare A with B and C). When exposed to constant light illumination for 7 days, $DLin\text{-}7^{*66}$ mutant eyes (F) undergo retinal degeneration similar to, but less severe, than *crb* mutant eyes (E). *w* control eyes (D) remain unaffected. When exposed to constant light, loss of the *white* gene always leads to "holes" in the adult eyes (black arrows in D), regardless of the genetic background (compare D with E–I). This phenotype does not result from retinal degeneration, its origin is unclear. Lack of vitamin A in the medium prevents light-dependent retinal degeneration in crb^{11A22} (H) and $DLin-7^{*66}$ (I) mutant eyes. Note that in all cases, including the wild type (*w*), the size of the rhabdomeres is significantly reduced when animals are raised in a vitamin *A*-depleted medium (compare, for example, A and G).

(Fig. 6I). The degree of rescue is similar to, or even better than that observed in *crb* mutant PRCs under the same condition (compare Fig. 6H and I) (Johnson et al., 2002) or that of *sdt* mutant PRCs (Berger et al., 2007). More experiments are required to explain the relationship between the function of the Crb-mediated membrane-associated protein scaffold and retinal degeneration and its rescue.

Data presented here show that, although *D*Lin-7 is part of the Crumbs complex in all tissues tested, loss of its function has no obvious effect on the localization of Crb, Sdt and *D*PATJ, nor on the maintenance of epithelial cell polarity or the morphogenesis of PRCs. Similarly as the other members of the complex, however, *D*Lin-7 prevents light-dependent retinal degeneration. This result supports the conclusion already drawn from the analysis of different *sdt* alleles (Berger et al., 2007) that the function of the Crb complex in PRC survival is independent from its function during morphogenesis. Unlike Crb, Sdt and *D*PATJ, however, which are restricted to the stalk membrane in PRCs, *D*Lin-7 can also be found in the first and second optic ganglion. Currently, we cannot decide whether the light-dependent retinal degeneration in *DLin*-7 mutants is due to its removal from the stalk membrane or from the lamina (or both). Crb, Sdt and *D*PATJ localization are unaffected in *DLin*-7 mutant PRCs, while *D*Lin-7 is lost in all the three mutants. Therefore, a likely explanation would be that it is the loss of *D*Lin-7 from the stalk membrane that causes retinal degeneration. In this case, however, one would expect that the *DLin-7* mutant phenotype is as strong as, for example, the *crb* mutant phenotype, which was not observed. Alternatively, light-dependent retinal degeneration could be the consequence of the removal of *D*Lin-7 from the lamina. Loss of *D*Lin-7 eliminates Metro, a MAGUK related to MPP4, which is mainly localized in the lamina of PRCs and not at the stalk membrane. Loss of *metro* also results in light-dependent retinal degeneration (A. Bachmann, E. Knust and U. Thomas, manuscript in preparation). Hence, we cannot rule out that retinal degeneration observed in *D*Lin-7 mutant eyes is due to the absence of *D*Lin-7 and Metro at the synapses of the first optic ganglion, which may impair synaptic function.

The possible importance of the localization of *D*Lin-7 at the synapse is emphasized by the observation that mouse Veli3, one of the three mammalian Lin-7 orthologues, is localized in the outer plexiform layer of the retina (in addition to the localization in the outer limiting membrane), a region rich in synapses (Stöhr et al., 2003, 2005). Here, it is associated with MPP4 at the presynaptic plasma membrane and presynaptic vesicles. Loss of MPP4 prevents proper localization of PSD-95 and Veli3 at the presynaptic membrane (Aartsen et al., 2006), and has been associated with an impairment of Ca^{2+} homeostasis and of synaptic transmission in PRCs (Yang et al., 2007). Similarly, the knockout of all the three Lin-7 genes exhibits defects in presynaptic neurotransmitter release (Olsen et al., 2005a). It would be interesting to analyze whether these animals display any defects in their retina, which are similar to those described for mice lacking CRB1.

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