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Antagonistic Functions of Two Stardust Isoforms in *Drosophila* Photoreceptor Cells

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Membrane-associated guanylate kinases (MAGUKs) are scaffolding proteins that organize supramolecular protein complexes, thereby partitioning the plasma membrane into spatially and functionally distinct subdomains. Their modular organization is ideally suited to organize protein complexes with cell type- or stage-specific composition, or both. Often more than one MAGUK isoform is expressed by one gene in the same cell, yet very little is known about their individual in vivo functions. Here, we show that two isoforms of *Drosophila stardust*, Sdt-H (formerly called Sdt-B2) and Sdt-D, which differ in their N terminus, are expressed in adult photoreceptors. Both isoforms associate with Crumbs and PATJ, constituents of the conserved Crumbs–Stardust complex. However, they form distinct complexes, localized at the stalk, a restricted region of the apical plasma membrane. Strikingly, Sdt-H and Sdt-D have antagonistic functions. While Sdt-H overexpression increases stalk membrane length and prevents light-dependent retinal degeneration, Sdt-D overexpression reduces stalk length and enhances light-dependent retinal degeneration. These results suggest that a fine-tuned balance of different Crumbs complexes regulates photoreceptor homeostasis.

INTRODUCTION

The membrane-associated guanylate kinase (MAGUK) family comprises proteins that act as molecular scaffolds by bringing different proteins into proximity. The plasma membrane-associated protein complexes define spatially and functionally restricted subdomains that are often engaged in signal transduction or adhesion (reviewed in Dimitratos *et al*., 1999; Funke *et al*., 2005). MAGUK proteins have a characteristic domain organization that includes one to three postsynaptic density 95/disc-large/zona occludens (PDZ) domains, a Src homology SH3 and a guanylate kinase (GUK) domain. Some MAGUKs additionally contain one or two Lin-2/Lin-7 (L27) domains, a HOOK domain, or both (reviewed in Dimitratos *et al*., 1999; González-Mariscal *et al*., 2000). These protein–protein interaction domains allow the recruitment of a variety of proteins into membrane-associated protein scaffolds. Strikingly, many MAGUK-encoding genes generate several alternatively spliced transcripts, which give rise to proteins that differ in their domain organization. The different isoforms can be expressed in the same cell or in different cell types. *Drosophila polychaetoid* (*pyd*), for example, generates two alternatively spliced transcripts that differ by one exon (Wei and Ellis, 2001). Both transcripts encode MAGUK proteins that are expressed in embryonic and larval epithelial cells. The longer form localizes to adherens junctions, whereas the shorter form is broadly distributed along the plasma membrane. *Drosophila discs large* (*dlg*) encodes two major MAGUK forms, Dlg-A and Dlg-S97 (Mendoza *et al*., 2003). Dlg-A is expressed in epithelial cells where it is associated with septate junctions on the lateral plasma membrane. Dlg-S97 has an additional N-terminal L27 domain and is localized at larval neuromuscular junctions. Here, it forms a complex with Drosophila Lin-7 and the MAGUK *Metro*, and recruits other proteins, among them fasciclin II and shaker K^+ channels that are involved in synapse development and function (Tejedor *et al*., 1997; Thomas *et al*., 1997; Bachmann *et al*., 2004, 2010). *dlg* is one of the few cases in which different functions have been assigned to alternative transcripts. In most cases, however, the role of individual splice variants is not known and to our knowledge no data are available on the function of different MAGUK isoforms expressed in the same cell.

The *Drosophila* MAGUK Stardust (Sdt), a member of the MPP/p55 subfamily of MAGUKs, is a constituent of the highly conserved Crumbs (Crb) protein complex. In addition to a PDZ, an SH3, a GUK, a HOOK, and two L27 domains, it contains two evolutionary conserved regions (ECR1 and ECR2) in the N terminus (see Figure 1A). The PDZ domain of Sdt binds the C terminus of the transmembrane protein Crb. The two L27 domains interact with the L27 domains of *Drosophila* PATJ and Lin-7, respectively (Bachmann *et al*., 2001; Hong *et al*., 2001; Bulgakova *et al*., 2008). Beside the core components Crb, Sdt, PATJ, and Lin-7 that are always colocalized when expressed in the same cell, other proteins can transiently be recruited into the complex (reviewed in Bulgakova and Knust, 2009). For example, *Drosophila* Par-6 can bind to the N terminus of Sdt and the C terminus of Crb (Wang *et al*., 2004; Kempkens *et al*., 2006), and the 4.1/ezrin/radixin/moesin (FERM) family member Yurt can bind to the FERM-binding domain of the cytoplas-

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mic tail of Crb but only transiently colocalizes with Crb (Laprise *et al*., 2006). Furthermore, the FERM-binding domain of Crb has been implicated in the recruitment of the FERM domain protein Expanded in epithelial cells of imaginal discs and Schneider S2+ cells (Ling *et al*., 2010; Robinson *et al*., 2010).

In *Drosophila*, the Crb complex is required for the maintenance of apical–basal polarity in epithelial cells of the embryo (Tepass and Knust, 1993; Bachmann *et al*., 2001; Hong *et al*., 2001) and controls the morphogenesis and function of photoreceptor cells (PRCs) of *Drosophila* compound eyes. PRCs develop from epithelial cells of the eye imaginal disc. Cell fate determination occurs in the larval imaginal disc epithelium, during which groups of eight PRCs are formed, each of which will later form an ommatidium. At early pupal stages, the apical membranes of the developing PRCs are shifted from an apical to a lateral position. This is followed by a subdivision of the apical membrane into the apical-most rhabdomere and the stalk, a supporting region between the rhabdomere and the zonula adherens (ZA). The rhabdomere is the photosensitive organelle, which harbors rhodopsin and other components of the phototransduction signaling cascade. During the second half of pupal development, PRCs undergo a conspicuous elongation. Concomitantly, the rhabdomeral membrane is expanded and forms a highly pleated array of microvilli (Longley and Ready, 1995). During early stages of PRC development, members of the Crumbs complex localize on the entire apical membrane, where they transiently associate with Par-6 (Hong *et al*., 2003; Nam and Choi, 2003; Richard *et al*., 2006; Berger *et al*., 2007; Bulgakova *et al*., 2008). As the apical membrane becomes subdivided into rhabdomere and stalk, proteins of the Crumbs complex are restricted to the future stalk membrane (Pellikka *et al*., 2002; Nam and Choi, 2003). At late stages of pupal development they recruit the FERM domain protein Yurt to the stalk, which antagonizes the function of Crb (Laprise *et al*., 2006). Mutations in *crb*, *PATJ*, or *sdt* result in morphogenetic defects of PRCs, which are manifested by a failure to properly extend the rhabdomeres. In addition, the stalk membrane is reduced in length. Finally, loss of Crb, Sdt, PATJ, or Lin-7 in the *Drosophila* eye leads to progressive light-induced PRC degeneration (Izaddoost *et al*., 2002; Johnson *et al*., 2002; Pellikka *et al*., 2002; Hong *et al*., 2003; Nam and Choi, 2006; Richard *et al*., 2006; Berger *et al*., 2007; Bachmann *et al*., 2008).

Similar to many other MAGUK-encoding genes, *Drosophila sdt* gives rise to several alternatively spliced transcripts. To date, four different isoforms of Sdt have been described, Sdt-A (formerly described as Sdt-GUK1), Sdt-B (formerly called Sdt-A or Sdt-MAGUK1; Bachmann *et al*., 2001; Hong *et al*., 2001), Sdt-F (formerly called Sdt-B1; Wang *et al*., 2004), and Sdt-H (formerly called Sdt-B2; Berger *et al*., 2007; see Figure 1, A and B, and Supplemental Figure S1). Two isoforms, Sdt-B and Sdt-F, are expressed in epithelial cells of the embryo and the follicle epithelium (Tanentzapf *et al*., 2000; Bachmann *et al*., 2001; Hong *et al*., 2001; Wang *et al*., 2004; Berger *et al*., 2007; Horne-Badovinac and Bilder, 2008; Li *et al*., 2008). Sdt-F is expressed throughout oogenesis and embryogenesis, whereas Sdt-B is expressed only at early stages in the follicle and the embryo (Berger *et al*., 2007; Horne-Badovinac and Bilder, 2008). Sdt-B differs from Sdt-F with respect to the presence of one alternatively spliced exon, which encodes a region of 433 amino acids in the N terminus without any obvious domain structure (see Figure 1, A and B). This exon carries a localization signal responsible for apical targeting of its mRNA (Horne-Badovinac and Bilder, 2008). Sdt-H is predominantly expressed in heads of adult flies and differs from Sdt-F in its transcription–translation start site (Berger *et al*., 2007; see Figure 1, A and B). Finally, transcripts for Sdt-A, which lacks most of the PDZ domain, the SH3, Hook, and part of the GUK domain, were amplified from embryonic mRNA only (Bachmann *et al*., 2001). Until now, the functions of individual Sdt isoforms were not known. To gain more insight into the functions of different splice variants, we characterized two of them, which are expressed in adult PRCs, and analyzed their role with respect to stalk membrane length and light-dependent retinal degeneration.

MATERIALS AND METHODS

Fly Strains, Generation of Sdt Transgenes, and Clonal Analysis

Flies were kept at 25°C. sdt^{K85}, classified as null allele in PRCs (Berger *et al*., 2007), was used. Because red pigments strongly inhibit light-induced retinal degeneration of *crb* and *sdt* mutant eyes (M. Richard, S. Berger, personal communication), the previously published transgenes (cloned in pUAST-w^+ vector) could not be analyzed. Therefore, corresponding cDNAs were cloned into the pUAST-y^+ vector (Perrin *et al*., 2003), producing transgenic flies with white eyes. The Sdt-H transgene was generated using the pUAST-w^+-FLAG-Sdt-H vector (Bulgakova *et al*., 2008) digested with XbaI and AatII, and cloned into pUAST-y^+. The N terminus of Sdt-D was amplified from mRNA by using 5′-Sdt-D-N primer: CGAAAAGGTACCTGTCCACTTTGCAC with an introduced KpnI restriction site (underlined) and 3′-Sdt-PDZSH3: GCGTGATGAAGTGGTAGTCC. The resulting product was digested with KpnI and cloned in pUAST-w^+-FLAG vector (Bulgakova *et al*., 2008). Part of the construct encoding the FLAG-tag and the N terminus of Sdt-D was cloned into pUAST-w^+-FLAG-Sdt-H vector (Bulgakova *et al*., 2008) by using EcoRI, thereby replacing the N terminus of Sdt-H by that of Sdt-D (pUAST-w^+-FLAG-Sdt-D). Finally, Sdt-D was recloned into pUAST-y^+ vector in the same way as Sdt-H. Injections of embryos and establishment of transgenic flies were performed by BestGene (http://www.thebestgene.com/HomePage.do). *Rh1*GAL4 (marked with ry^+) or *GMR*GAL4 (w^-) (Bloomington *Drosophila* Stock Center, Indiana University, Bloomington, IN) was used to drive expression of Sdt-transgenes. The *Rhodopsin-1* (*Rh1*) promoter activates moderate levels of GAL4 in the six outer PRCs, starting at ~70% pupal development (Kumar and Ready, 1995; Sheng *et al*., 1997). *GMR*GAL4 is expressed in all cells of the eye (Hay *et al*., 1994). For overexpression and rescue experiments, stocks with X-chromosomal integration sites of Sdt-H and Sdt-D (either *y* or sdt^{K85} FRT19A) were used. Large sdt^{K85} clones expressing Sdt-encoding transgenes were generated by crossing w^+ *GMR-hid cl FRT19A*/FM7; *Rh1*GAL4; *ey*FLP females (modified from Newsome *et al*., 2000) to *w UAS*Sdt-H sdt^{K85} FRT19A/Y; *Tp(1;2) sn*$^+$*72d/CyO* or *w UAS*Sdt-D sdt^{K85} FRT19A/Y; *Tp(1;2) sn*$^+$*72d/CyO* males. Mosaic analysis with a repressible cell marker clones, in which mutant cells are marked with green fluorescent protein ([GFP]; Lee and Luo, 2001), were induced by a 2 h heat shock (37°C) at 48–72 and 72–96 h of development in offspring of *hsFLP, tubG80 FRT19A/Y; Act-Gal4 UAS-CD8::GFP/CyO* females crossed to *w UAS*Sdt-H sdt^{K85} FRT19A/Y; *Tp(1;2) sn*$^+$*72d/CyO w UAS*Sdt-D sdt^{K85} FRT19A/Y; *Tp(1;2) sn*$^+$*72d/CyO* or males (where Sdt-X is any Sdt transgene). For the measurements of the stalk membrane length, two stocks with integration sites of Sdt-D on first and second chromosomes were used [named Sdt-D and Sdt-D(2), respectively].

Generation of Sdt Monoclonal Antibody (mAb), Immunoprecipitation, and Western Blot Analysis

mAb B8-1 was raised against glutathione transferase-Sdt-PDZ fusion protein (Berger *et al*., 2007) by the Antibody Facility of the Max-Planck Institute for Molecular Cell Biology and Genetics. Immunoprecipitation from adult heads were performed at 4°C, essentially as described previously (Bachmann *et al*., 2004). For each genotype, 20 heads were homogenized in 150 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Triton, 1 mM $MgCl_2$, 1 mM Pefabloc, 5 mM leupeptin, 1 mM pepstatin, and 0.3 mM aprotinin). After a 1-h extraction, the homogenate was cleared from cuticle debris by centrifugation for 3 min at 3000 × *g*. Then, 130 μl of the supernatant was loaded onto 30 μl of protein G-Sepharose (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), which was preincubated for 2 h with 3 μl of α-FLAG antibody (~12 μg; Sigma Chemie, Deisenhofen, Germany), mixed, and rotated overnight. The protein G-Sepharose precipitate was washed three times for 15 min in lysis buffer (with intervening centrifugation steps, 3 min at 700 × *g*) and then supplied with 15 μl of SDS sample buffer.

For protein extraction from retinas, heads were first cut in halves with a scalpel, and the retinas were dissected using forceps. The optic lobes, including the lamina, which slightly differs from w^- retina tissue in color and structure, were removed using fine forceps. Only the eyes with intact morphology and properly removed lamina were used for protein lysates. Western

blots were performed as described previously (Berger *et al*., 2007). Membranes were stained using mouse α-Sdt-PDZ (B8-1; 1:1000), mouse α-Crb-Cq4 (1:100; Tepass and Knust, 1993), rabbit α-PATJ (1:5000); Richard *et al*., 2006), rabbit α-Lin-7 (1:5000; Bachmann *et al*., 2004), mouse α-FLAG-M2 (1:1000; Sigma Chemie), and mouse anti-α-tubulin (1:2000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Peroxidase-conjugated secondary antibodies (Dianova, Hamburg, Germany) were used 1:1000 in combination with the enhanced chemiluminescence system (GE Healthcare). Alternatively, IRDye 800CW goat α-mouse immunoglobulin (Ig)G (1:5000) or IRDye 680 donkey α-rabbit IgG (1:5000), in combination with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE), was used. For quantification of protein amounts, the blots were scanned using the LI-COR system, background was subtracted, and the intensity of each band was measured using ImageJ (http://rsb.info.nih.gov/ij/). Intensities of the Crb bands were normalized to those of α-tubulin bands. The ratio of the amounts of Crb protein in flies, overexpressing Sdt-transgenes or carrying an *sdt* mutant allele, and in wild-type flies, was used for statistical analysis. For each Western blot, protein lysates were made new.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and 5′-Rapid Amplification of cDNA Ends (RACE)

Poly(A)$^+$ RNA was isolated from heads of adult *white* flies as described previously (Berger *et al*., 2007). Total RNA was obtained from individual isolated eyes using the Mini RNA Isolation I kit (Zymo Research, Orange, CA). RT-PCR and Northern blot analysis were performed as described previously (Berger *et al*., 2007). 5′-RACE was performed on poly(A)$^+$ RNA isolated from heads of adult *white* flies. cDNA was generated using the BD Advantage2 PCR kit (Clontech, Mountain View, CA), followed by 5′-RACE using the SMART Race cDNA amplification kit (Clontech). All products of RT-PCR were sequenced. Primer sequences for all exon-specific primers are available upon request.

Confocal and Transmission Electron Microscopy

Immunohistochemistry on pupal eye discs and adult eyes (frozen sections) was done as described previously (Richard *et al*., 2006). The following antibodies were used: mouse α-Sdt-PDZ (B8-1, 1:500; this work); rabbit α-Sdt-PDZ (1:500; Berger *et al*., 2007); mouse α-FLAG-M2 (1:100; Sigma Chemie); rat α-Crb (1:100); rabbit α-PATJ (1:500; Richard *et al*., 2006); guinea pig α-*D*Par-6 (1:1000; kindly provided by A. Wodarz; University of Göttingen, Germany) rat α-*D*E-Cad (1:50; (Developmental Studies Hybridoma Bank); rabbit α-GFP (1:500; Invitrogen, Carlsbad, CA); and Cy2-, Cy3- (Dianova), or Alexa-647 (Invitrogen)–conjugated secondary antibody. Rhabdomeres were visualized by labeling F-actin with Alexa-660-phalloidin (1:40; Invitrogen). Sections for transmission electron microscopy were prepared as described previously (Richard *et al*., 2006) and analyzed with a Tecnai 12 Biotwin (FEI, Hillsboro, OR [formerly Philips]) and photographed with a TemCam F214A-CS-HS-2 digital camera (Tietz, Germany) at 4800× magnification.

Analysis of Retinal Degeneration and Length of Stalk Membranes

Length of stalk membranes and survival of rhabdomeres after exposure to constant light showed some variability in different control strains (Supplemental Tables S1 and S2). To detect changes caused by expression of any transgene, the genotype of control flies was as close as possible to the genotype of the flies expressing the transgene. To obtain the control flies, we crossed Sdt-H or Sdt-D homozygous transgenic flies to *Rh1*GAL4/CyO or *GMR*GAL4/CyO animals. Progeny of these crosses carrying a transgene and the CyO balancer chromosome differ only with respect to one chromosome from flies having both the transgene and the driver. The former were used as control, and the latter were used to detect the effect of the respective transgene.

For measurements of the stalk membrane, the stalk membranes of 10 ommatidia, obtained from three eyes of three different individuals of each genotype were hand-traced using ImageJ (http://rsb.info.nih.gov/ij/). Because the length of the stalk membrane differs with respect to the proximal–distal extension, all sections used for measurements were taken at ~50-μm depth of the retina, i.e., in the distal portion that contains R7. Only stalk membranes from R1 to R6 with clearly identifiable ZAs were measured.

To assay retinal degeneration, flies were kept for 7 d in the dark or in constant light (1830 lux). The number of outer PRCs with clearly detectable rhabdomeres in each ommatidium was counted in semithin sections (see Figure 5D). In each section, 30–40 ommatidia were counted. Six eyes from different individuals were analyzed for each genotype. For each genotype, the mean number of PRCs with intact rhabdomeres and ommatidium was quantified for flies kept for 7 d in the light or in the dark. To determine survival rate of PRCs, we used the ratio of these mean numbers. Nonparametric Mann–Whitney–Wilcoxon test was used to compare different distributions of stalk membrane length and PRCs survival. Statistical analyses were performed using Prism software (GraphPad Software, San Diego, CA; http://www.graphpad.com/prism/prism.htm).

RESULTS

Sdt-H and Sdt-D Are Expressed in the Adult Retina

Drosophila sdt spans a genomic region of ~61 kb and encodes several protein isoforms as a result of alternative splicing and transcription initiation. Different names were used for the same isoforms in different publications. To be more consistent with the nomenclature of Sdt isoforms, we now use the names assigned by Flybase throughout the text. From the multiple transcripts predicted in Flybase (http://flybase.bio.indiana.edu; Supplemental Figure S1), expression of four isoforms has been confirmed previously (Bachmann *et al*., 2001; Hong *et al*., 2001; Wang *et al*., 2004; Berger *et al*., 2007; Horne-Badovinac and Bilder, 2008) (Figure 1, A and B; data not shown). Of these isoforms, Sdt-H is expressed in heads of adult flies (Berger *et al*., 2007; Figure 1C). To determine which of the other isoforms are expressed in the retina of adult flies, we performed multiple RT-PCR experiments using mRNA from whole fly heads or adult retinas. Using exon-specific primers (Supplemental Figure S1), we confirmed that several transcripts were expressed in adult heads, among them three isoforms (Sdt-K, Sdt-I, and Sdt-J) not described so far (summarized in Supplemental Figure S2). Beside Sdt-H, only one additional mRNA species was amplified from mRNA of isolated adult retinas (Figure 1, C and D), suggesting that only two Sdt isoforms are expressed in the retina of adult flies. Sequence analysis of RT-PCR and 5′-RACE products confirmed that this second mRNA encodes Sdt-D, one of the isoforms predicted in Flybase (Figure 1, A and B, and Supplemental Figure S1). A major part of Sdt-D, including the two L27 domains, the PDZ, SH3, HOOK, and GUK domain, was identical to that of Sdt-H. The N terminus of Sdt-D differs from that of Sdt-H in that it maintains only the ECR2 motif but lacks the ECR1 motif due to an alternative transcription/translation start site (Figure 1, A and B). The Sdt-D–specific 137-amino acid N-terminal region, which lacks ECR1 present in all other isoforms described so far, is conserved in several *Drosophila* species, among them *D. sechelly*, *D. yakuba*, *D. ananassae*, and *D. persimilis*, with amino acid identities of 99, 70, 52, and 43%, respectively. In particular, the last 24 C-terminal amino acids of this region are 100% identical in all five species (with the exception of one T to S exchange in *D. persimilis*). No particular domains are predicted in the Sdt-D–specific N terminus when using the SMART program (http://smart.embl-heidelberg.de/).

Using wild-type retinas ("wild type" means w^{1118} sdt^+ throughout the text), two distinct protein bands of ~115 and 120 kDa were detected on Western blots (Figure 1E). To verify that these bands correspond to the two different Sdt isoforms as suggested from RT-PCR experiments, we expressed UAS-transgenes encoding FLAG-tagged Sdt-H or Sdt-D by using *GMR*GAL4, which activates GAL4 in all cells of the eye. The expressed gene products were analyzed by Western blots, by using either α-Sdt-PDZ, which detects both Sdt isoforms, or α-FLAG antibodies (Figure 1E). Transgene-encoded Sdt-H and Sdt-D correspond to the lower and upper band detected in retinal extracts from wild-type flies, respectively (Figure 1E). From these data, we conclude that Sdt-H and Sdt-D are the predominant Sdt isoforms in adult retinas. The predicted sizes of the proteins encoded by *UAS*Sdt-D and *UAS*Sdt-H transgenes are 95.3 and 102.9 kDa, respectively. Strikingly, when expressed from transgenes in flies, both proteins run at higher molecular weight in Western blots, and Sdt-D has even a slightly higher molecular weight than Sdt-H (Figure 1E). This might be a result of stable posttranslational modifications of the proteins in vivo,

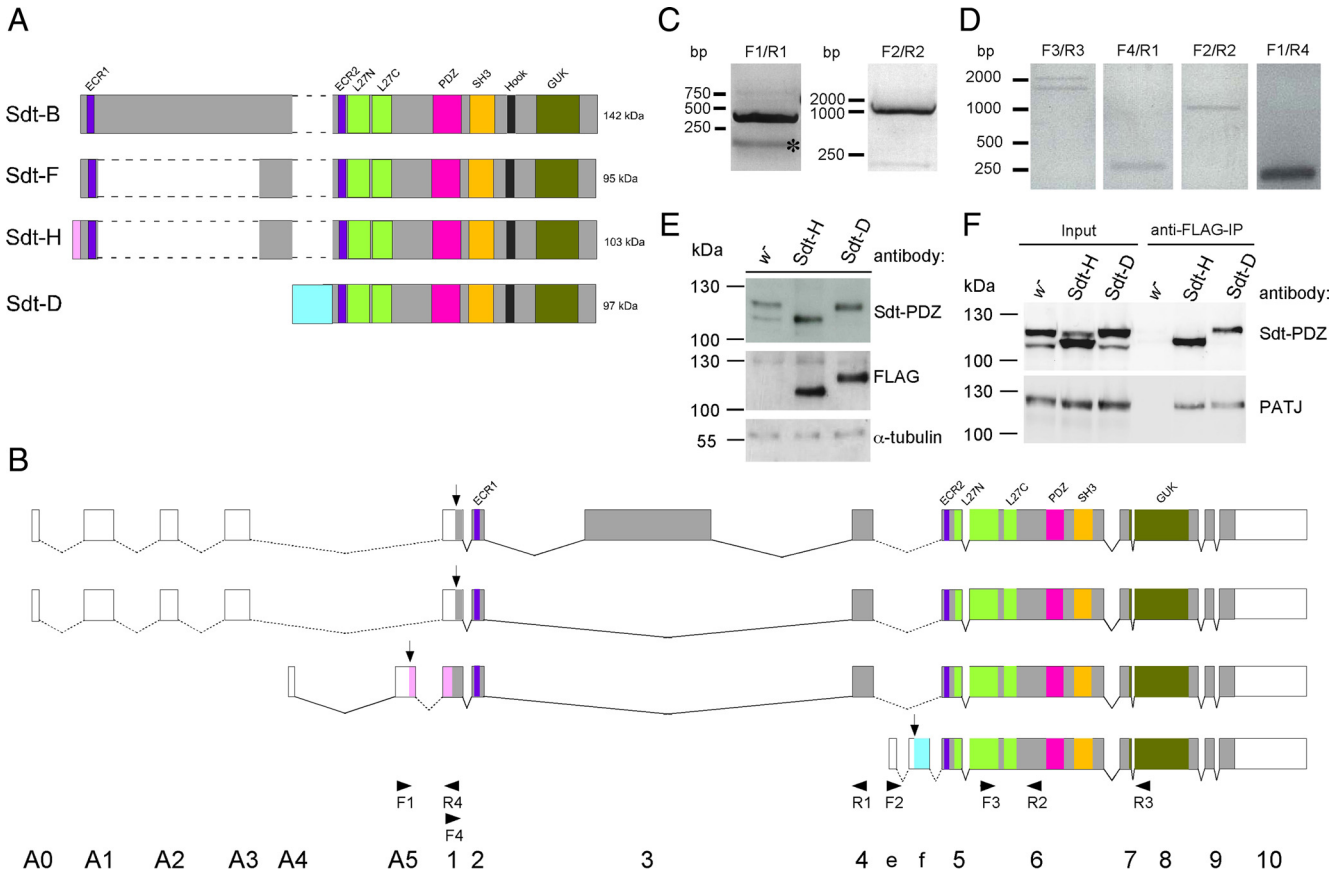

Figure 1. Structure of Stardust isoforms and their expression. (A) Protein structure of the three previously described different Sdt isoforms (previously used names in parentheses): Sdt-B (previously called Sdt-MAGUK1 or Sdt-A), Sdt-F and Sdt-H (previously called Sdt-B1 and Sdt-B2, respectively; Bachmann *et al.*, 2001; Hong *et al.*, 2001; Wang *et al.*, 2004; Berger *et al.*, 2007), and the novel Sdt-D isoform. (B) Schematic representation of *sdt* splice variants and locations of primers used for RT-PCR (arrowheads). Colors correspond to those in A, the light blue rectangle depicts the alternative N terminus of Sdt-D. Exons with numbers are according to (Hong *et al.*, 2001; Berger *et al.*, 2007), novel exons e and f are indicated, untranslated 5′ and 3′ exons are in white, and arrows indicate translation start sites. Introns represented by hatched lines are not to scale. (C and D) Results from RT-PCR experiments from adult heads (C) or isolated retinas (D). Primers used for amplification are indicated on top of each lane (for genomic positions, see B). (C) The 5′ termini corresponding to Sdt-H and Sdt-D are amplified from adult heads. Band marked by asterisks indicates nonspecific products. (D) Sdt-H and Sdt-D are expressed in the adult retina. Only variants encoding the PDZ, SH3, and GUK domain and lacking exon 3 are expressed in the retina (first and second lane, respectively). The larger bands in the first lane still contain the intron. The 5′ termini of the RNA contain the same exons as Sdt-D or Sdt-H splice variants (third and forth lane, respectively). (E) Western blot of eye extracts of wild-type flies, *GMR*GAL4 > FLAG-tagged Sdt-H and *GMR*GAL4 > FLAG-tagged Sdt-D. FLAG-tagged proteins have the same size as endogenously expressed Sdt protein, which differ from the predicted size (see A). (F) Sdt-H and Sdt-D coimmunoprecipitate PATJ but not the respective other Sdt isoform. Wild-type eyes or eyes expressing FLAG-tagged Sdt-H or Sdt-D using *Rh1*GAL4 were used.

although we currently do not know the nature of these modifications. One target of modification could be the specific N terminus of Sdt-D, which could result in a higher molecular weight of Sdt-D relative to Sdt-H.

Both Sdt-H and Sdt-D Are Members of Distinct Crb Complexes at the Stalk Membrane

It was demonstrated previously that Sdt-H, when expressed in sdt^{K85} mutant photoreceptor cells (we used the null allele sdt^{K85} throughout this work), localizes at the stalk membrane in PRCs of adult flies and that multiple domains of Sdt are required for its proper localization (Bulgakova *et al.*, 2008). The N terminus, including both ECR motifs and the N-terminal L27 domain, is needed to bring the protein apically to the rhabdomere base, whereas the PDZ, SH3, and GUK domains are needed to target the protein to the stalk membrane. Sdt-D protein contains the L27, PDZ, SH3, and GUK domains and the ECR2 motif but lacks the ECR1 motif (Figure 1). Similar to Sdt-H, Sdt-D localizes at the stalk membrane when expressed with *Rh1*GAL4 in a sdt^{K85} mutant background (Figure 2, A′ and A″). Hence, the ECR1 motif is not required for targeting Sdt to the stalk membrane in adult PRCs. Similar to Sdt-H (Bulgakova *et al.*, 2008), Sdt-D also rescued the localization of the core-members of the Crb complex at the stalk membrane in sdt^{K85} PRCs (Figure 2, A and B, data not shown). This behavior is consistent with the presence of the binding sites for the core members of the Crb complex in Sdt-D.

*Rh1*GAL4 induces a mosaic expression pattern of the Sdt-D and Sdt-H–encoding transgenes in sdt^{K85} mutant PRCs (Figure 2, A and B). Transgene-encoded Sdt could only be detected in some PRCs but was undetectable in others. In addition, protein levels varied. In cells with high levels of Sdt-H protein, it was associated with the stalk membrane, but it also distributed in the cytoplasm (Figure 2B). In contrast, mosaic expression was not observed when the same

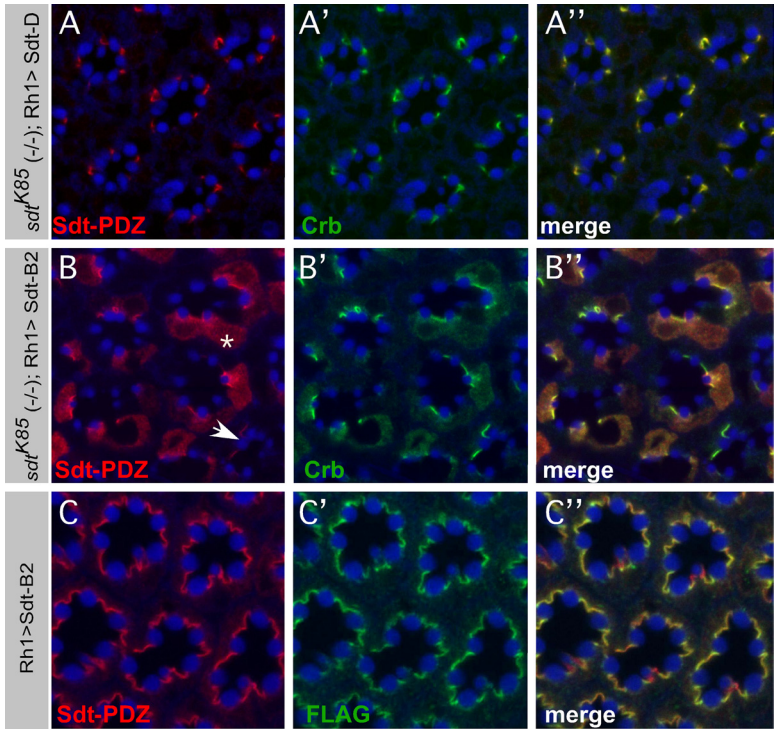

Figure 2. Localization of transgene-encoded Sdt proteins and proteins of the Crb complex in adult photoreceptor cells. Optical cross sections of adult *Drosophila* eyes stained with anti-Sdt-PDZ (red) and with anti-Crb (A), anti-PATJ (B), or anti-FLAG (C) (green), and phalloidin to highlight the F-actin–rich rhabdomeres (blue). (A) Sdt-D localizes at the stalk membrane when it is expressed in sdt^{K85} mutant PRCs. (B) Sdt-H localizes at the stalk membrane when expressed in sdt^{K85} mutant PRCs. The expression is mosaic in A and B, and the areas shown here contain a lot of PRCs expressing the transgenes. Other regions of the retina contain even less transgene-expressing cells. In some cells, Sdt-H protein is detected in large amounts so that some protein stays in the cytoplasm (asterisk in B). In cells with moderate amounts of Sdt-H, the protein localizes at the stalk membrane (arrow in B). (C) Sdt-H localizes at the stalk membrane when it is expressed in wild type using *Rh1*GAL4. Similar amounts of transgene-encoded Sdt protein are detected in all expressing cells in C. Note that the stalk of R7 is not labeled by anti-FLAG antibody in C, because *Rh1*Gal4 is only expressed in the six outer PRCs.

transgenes were expressed using *Rh1*GAL4 in wild-type or in a sdt^{K85}/+ heterozygous background: transgene-encoded protein was detected in all outer PRCs at constant levels, as judged from immunohistochemical staining (Figure 2C; data not shown). In all cases analyzed, the transgene-encoded protein as well as the core members of the Crb complex localized at the stalk membrane in adult PRCs (Figure 2; data not shown). Both Sdt-H and Sdt-D coimmunoprecipitated PATJ, but one Sdt isoform did not coimmunoprecipitate the respective other isoform (Figure 1F). This suggests that both isoforms are constituents of two separate Crb complexes.

Both Sdt-H and Sdt-D Interact with Par-6 in Pupal Photoreceptor Cells

Par-6 localizes apically in pupal PRCs, its localization depends on Sdt and it can transiently be recruited into the Crb complex (Nam and Choi, 2003; Bulgakova *et al*., 2008). In sdt^{K85} mutant pupal PRCs, which do not express Sdt protein (Figure 3A), Par-6 is strongly reduced apically, and in some cases it is detected basal to the ZA (Figure 3, B–B″; Berger *et al*., 2007). When Sdt-H is expressed in sdt^{K85} mutant pupal PRCs, Sdt protein was apically enriched but could also be detected basolaterally as well as in cytoplasm (Figure 3, C–C″). The ectopic localization may be caused by high expression levels (compare the amount of Sdt protein in Sdt-H–expressing cells with that in wild-type neighboring cells in Figure 3, C–C″). In contrast, apical localization of Par-6 was completely rescued by expression of Sdt-H in sdt^{K85} pupal PRCs, and no Par-6 protein was detected anywhere else in the cells (Figure 3, D–D″; Bulgakova *et al*., 2008). It was proposed previously that the ECR1 motif of the mammalian Sdt orthologue Pals1 is necessary, although not sufficient, for binding Par-6. The minimum binding region was mapped to amino acid 21-140 of Pals1 (Wang *et al*., 2004). This region corresponds to amino acid 9-213 of Sdt-H. Although Sdt-D lacks ECR1, it contains amino acid 118-s213 of the minimum Par-6–binding region. We tested the ability of Sdt-D protein to rescue Par-6 localization when expressed in pupal sdt^{K85} PRCs. Similar to Sdt-H, Sdt-D protein was enriched apically when expressed in pupal sdt^{K85} PRCs, but some protein also was detected basal to the ZA and in the cytoplasm (Figure 3, E–E″). Surprisingly, Par-6 localization was rescued by Sdt-D expression, and no Par-6 was detected basal to the ZA (Figure 3, F–F″). Although most Par-6 is detected at the ZA, where it might be additionally stabilized by binding to Bazooka, some Par-6 is detected at the apical membrane (Figure 3F, arrow). Together with previous results, which showed that the N terminus of Sdt, including ECR1, ECR2, and the N-terminal L27 domain, is necessary for rescuing Par-6 localization in pupal sdt^{K85} PRCs (Bulgakova *et al*., 2008), these results allow the conclusion that the N terminus of Sdt-D containing the ECR2 domain is sufficient for binding Par-6 and rescuing its localization. Strikingly, Par-6 is not colocalized with Sdt at the stalk membrane in adult PRCs but is rather localized basally (Figure 3, G–G″). This localization is not affected in PRCs lacking Sdt (Figure 3, H–H″) or in those overexpressing Sdt-H or Sdt-D (data not shown).

Overexpression of One Sdt Isoform Leads to Down-Regulation of the Other Isoform

Because all *sdt* mutations mapped so far affect either Sdt-B, which is an embryo-specific isoform, or all isoforms (Hong *et al*., 2001; Berger *et al*., 2007), we performed overexpression studies and rescue experiments to identify any individual function of Sdt-H and Sdt-D in PRCs. For this, we used either *Rh1*GAL4 that activates moderate levels of GAL4 in the six outer PRCs under the control of the *Rhodopsin 1* promoter, starting at ~75% pupal development; or *GMR*-GAL4 that is expressed throughout PRC development. When Sdt-H was overexpressed in a wild-type background with *Rh1*GAL4 or *GMR*GAL4, the amount of Sdt-D was strongly down-regulated. Similarly, Sdt-D overexpression

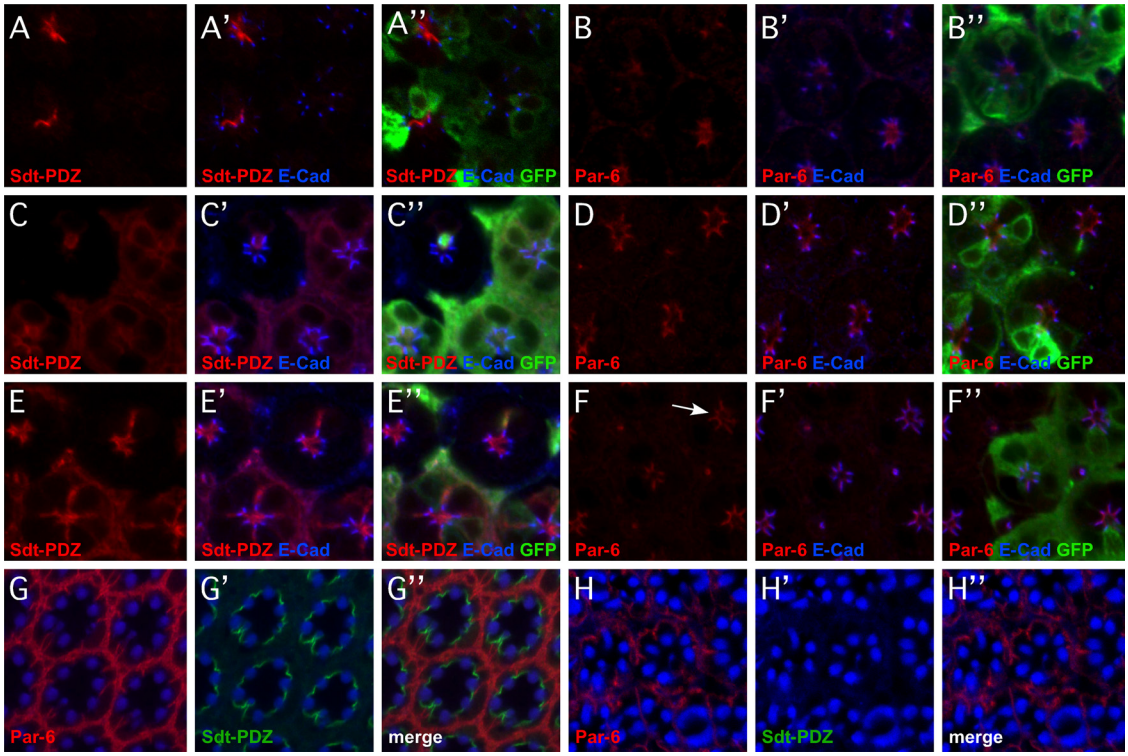

Figure 3. Localization of Sdt proteins encoded by transgenes and Par-6 in pupal photoreceptor cells. Optical cross-sections of pupal *Drosophila* eye discs (A–F) or adult PRCs (G and H). Sdt (A) is not detectable, and Par-6 (B) is delocalized and/or down-regulated in sdt^{K85} mutant pupal PRCs. Sdt-H (C) and Sdt-D (E) are enriched apically but are also detected basolaterally and in cytoplasm when expressed in sdt^{K85} mutant pupal PRCs. Apical localization of Par-6 is rescued when Sdt-H (D) or Sdt-D (F) is expressed in sdt^{K85} mutant pupal PRCs. Par-6 localizes at basolateral membrane in adult PRCs in wild-type eyes (G) as well as in sdt^{K85} mutant eyes (H). Sections are stained with anti-Sdt-PDZ (red in A, C, and E; green in G and H), with anti-Par-6 (red in B, D, F, G, and H), with anti-E-Cad (blue in A–F) to mark ZA, with anti-GFP (green in A–F) to mark the mutant clones and phalloidin to highlight the F-actin–rich rhabdomeres (blue in G and H).

reduced Sdt-H levels (Figures 1, E and F, and 4A). The degree of down-regulation is less pronounced with *Rh1*GAL4 than with *GMR*GAL4, due to either lower expression level or to later onset of expression of *Rh1*GAL4 (compare Figure 1, E and F). Reduction of Sdt-D levels upon overexpression of Sdt-H in outer PRCs (by using *Rh1*GAL4) is only possible when Sdt-D, or at least a major portion of it, is also expressed in outer PRCs. Together with the fact that Sdt-H is expressed in outer PRCs (as revealed by detection with an antibody that recognizes the Sdt-H but not the Sdt-D isoform; Bulgakova *et al*., 2008), we conclude that both Sdt-H and Sdt-D are coexpressed in outer PRCs.

When Sdt proteins were expressed with *Rh1*GAL4 in a $sdt^{K85}/+$ heterozygous background, the respective other isoform was not detectable by Western blot (Figure 4A). These data suggest that the amount of Sdt protein is reduced in *sdt* heterozygous eyes in comparison with wild-type eyes. This assumption was confirmed by Western blots (Figure 4A). At the same time, the level of Crb was also reduced in $sdt^{K85}/+$ heterozygous eyes (Figure 4B). Quantification of the data showed that the amount of Crb in $sdt^{K85}/+$ eyes is only 51.9 ± 2.8% of the amount present in wild-type eyes (Figure 4B′). This result is consistent with previous observations showing that *sdt* regulates the amount of Crb in photoreceptor cells (Bulgakova *et al*., 2008). The level of Crb is partially restored upon overexpression of Sdt-H or Sdt-D in $sdt^{K85}/+$ eyes (67.1 ± 2.8 and 65.4 ± 2.5%, respectively). However, when Sdt-H or Sdt-D is expressed in wild-type eyes, the amount of Crb protein is not changed (103.9 ± 6.9 and 97.7 ± 2.7%, respectively; Figure 4B′). We next tested to what extent expression of Sdt-H or Sdt-D affects the amount of other core components of the Crb complex. When either Sdt-H or Sdt-D was overexpressed in a wild-type or a $sdt^{K85}/+$ heterozygous background, the amount of PATJ and Lin-7 was not changed compared with the control (Figure 4C; data not shown).

Expression of Sdt-H or Sdt-D Has Opposite Effects on Stalk Membrane Length

In $sdt^{K85}/+$ retinas, in which the level of Crb and Sdt is reduced (Figure 4, A and B), the stalk membrane is shorter (Figure 5A; data are summarized in Supplemental Table S1). A similar effect was observed in flies with only half the dose of *crb* (Pellikka *et al*., 2002), suggesting that the amount of Crb complex influences the length of the stalk. It has been shown previously that expression of Sdt-H in sdt^{K85} PRCs by using *Rh1*GAL4 partially rescues the length of the stalk membrane (Bulgakova *et al*., 2008). Because one aim of the current study was to test the effect of Sdt proteins on light-dependent degeneration, which is only observed in a *w* background, we generated new Sdt-encoding transgenes carrying y^+ as selection marker. No significant increase of stalk membrane length in sdt^{K85} PRCs was detected with the new Sdt-H transgene (data not shown). The most likely explanation of this result is the mosaic expression of the new transgene in *sdt* homozygous mutant PRCs, resulting in the expression of transgene-encoded Sdt protein in only some mutant PRCs (Figure 2, A and B). In contrast, the transgene used previously showed uniform expression in all outer PRCs with the same GAL4 line (Bulgakova *et al*., 2008).

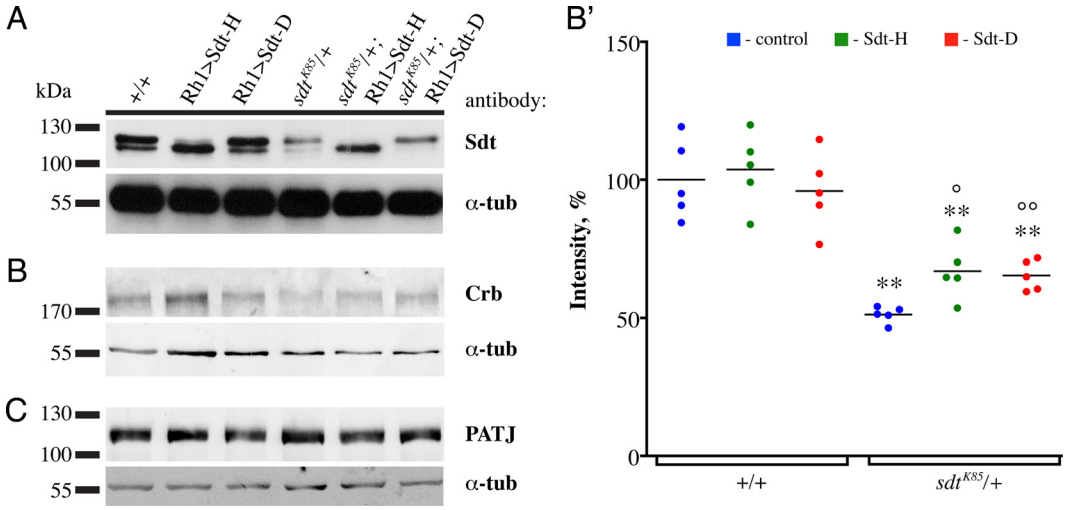

Figure 4. Expression of Sdt-H or Sdt-D from transgenes and their effects on constituents of the Crb complex. Western blots of extracts from wild-type (w^-) or sdt^{K85} heterozygous eyes ($sdt^{K85}/+$), and eyes expressing Sdt-H– or Sdt-D–encoding transgenes in a wild-type or a sdt^{K85} heterozygous background, probed with anti-Sdt-PDZ antibody (A), anti-Crb antibody (B), or anti-PATJ antibody (C). Anti-α-tubulin staining was used as a loading control. (B′) Effects of Sdt-H or Sdt-D expression and sdt^{K85} mutation on total amount of Crb protein in the eye. Amount of Crb protein for the following genotypes is depicted (left to right): +/+, *UAS*Sdt-H/+; *Rh1*GAL4/+, *UAS*Sdt-D/+; *Rh1*GAL4/+, $sdt^{K85}/+$; *Rh1*GAL4/+, sdt^{K85} *UAS*Sdt-H/+; *Rh1*GAL4/+, and sdt^{K85} *UAS*Sdt-D/+; *Rh1*GAL4/+. Statistically significant differences (two-tailed p-value, $p < 0.01$) in comparison with wild-type flies are marked **. Statistically significant differences in comparison to $sdt^{K85}/+$ flies are marked °° for two-tailed p-value, $p < 0.01$ and ° for two-tailed p-value, $p < 0.05$.

Therefore, we investigated the influence of Sdt-H and Sdt-D overexpression on stalk membrane length in a w^- sdt^+ background, where these proteins are uniformly expressed (Figure 2, C–C″).

The average lengths of the stalk membrane in different control strains, which are wild type for *sdt* (i.e., sdt^+), as measured from electron microscopic pictures, is close to 2 μm and ranges from 2.04 ± 0.06 to 2.13 ± 0.04 μm (Supple-

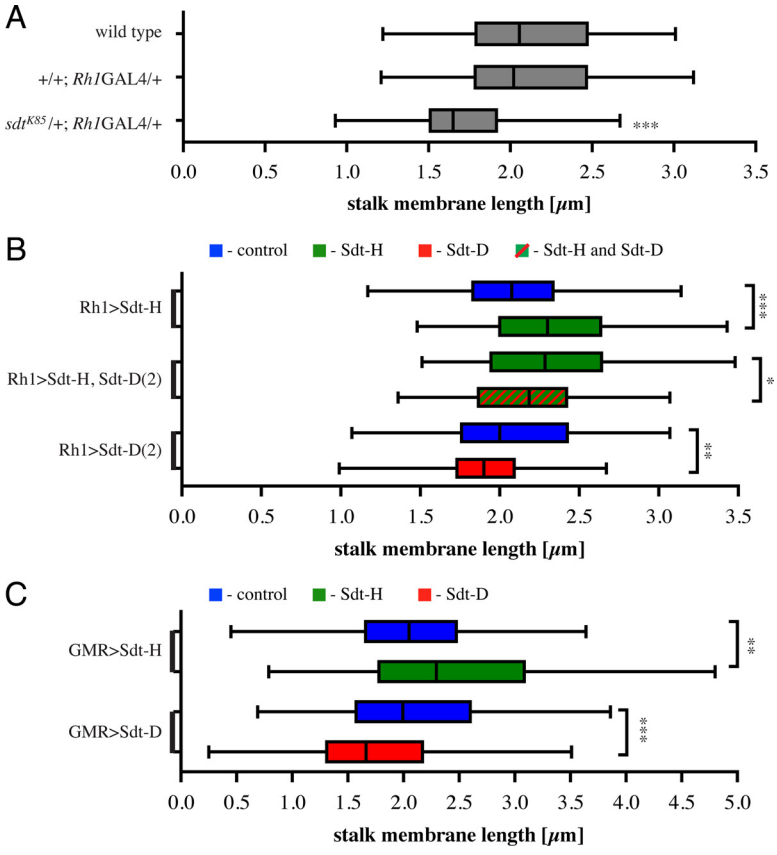

Figure 5. Stalk membrane length of different genotypes. (A) Effect of removing one copy of sdt^+ on the length of the stalk membrane. (B) Changes of stalk membrane length upon expression of Sdt-H or Sdt-D or both with *Rh1*GAL4. The following genotypes are depicted (top to bottom): *UAS*Sdt-H/+; CyO/+ (blue) and *UAS*Sdt-H/+; *Rh1*GAL4/+ (green) (F1 from *UAS*Sdt-H × *Rh1*GAL4/CyO); *UAS*Sdt-H/+; *Rh1*GAL4/CyO (green) and *UAS*Sdt-H/+; *Rh1*GAL4/*UAS*Sdt-D(2) (green/red) (F1 from *UAS*Sdt-H/FM7; *UAS*Sdt-D(2)/CyO × *Rh1*GAL4); and CyO/Sdt-D(2) (blue) and *Rh1*GAL4/*UAS*Sdt-D(2) (red) (F1 from *UAS*Sdt-D(2)/CyO × *Rh1*GAL4). For each genotype, the plot depicts the median (line), lower and upper quartiles (box), and maximum and minimum values of the sample (whiskers). (C) Changes of stalk membrane length upon expression of Sdt-H or Sdt-D with *GMR*GAL4. Distributions of stalk membrane lengths in *UAS*Sdt-H/+; *GMR*GAL4/+ (green) and *UAS*Sdt-D/+; *GMR*GAL4/± (red) are compared with the corresponding controls *UAS*Sdt-H/+; CyO/+ (F1 from *UAS*Sdt-H × *GMR*GAL4/CyO) and *UAS*Sdt-D/+; CyO/+ (F1 from *UAS*Sdt-D × *GMR*GAL4/CyO) (blue). Exact average values and numbers of the stalk membranes measured are summarized in Supplemental Table S1. The choice of the proper control for each case is described in *Materials and Methods*. Statistically significant differences in comparison with control are marked *** for two-tailed p-value, $p < 0.0001$; ** for two-tailed p-value, $p < 0.01$; and * for two-tailed p-value, $p < 0.05$.

mental Table S1). This length is consistent with previously published results for wild-type PRCs (Pellikka *et al.*, 2002; Laprise *et al.*, 2006; Richard *et al.*, 2006; Berger *et al.*, 2007). However, to exclude the influence of genetic background, we used genotypes that were as closely related to the experimental genotype as possible as controls (see *Materials and Methods*). Overexpression of Sdt-H using either *Rh1*GAL4 or *GMR*GAL4 induced an increase of stalk membrane length (Figure 5, B and C, green). In contrast, *GMR*GAL4-induced expression of Sdt-D resulted in a shortening of the stalk membrane (Figure 5C, red). The Sdt-D transgene with an integration site on the first chromosome shows only low expression levels in a w^- background using *Rh1*GAL4 (Figure 4A), and its expression has no effect on the length of the stalk membrane (data not shown). To confirm the effect of Sdt-D expression on the stalk membrane length we tested another transgenic line with an insertion site on the second chromosome, called Sdt-D(2). This line exhibits higher expression levels as judged by immunohistochemistry (data not shown). Overexpression of Sdt-D(2) with *Rh1*GAL4 leads to a significant reduction in the length of the stalk membrane (Figure 5B, red). As expected, overexpression of Sdt-D(2) together with Sdt-H with *Rh1*GAL4 restored the length of the stalk membrane, which was not significantly different from wild type (Figure 5B, green/red). All data are summarized in Supplemental Table S1. No differences in the amount of Crb or other core components of the Crb complex were detected in eyes overexpressing Sdt-H or Sdt-D compared with wild type (Figure 4B′; data not shown). This suggests that changing the ratio between the two Sdt isoforms is sufficient to affect the length of the stalk membrane, even when the total amount of the Crb complex at the stalk membrane is not changed.

Expression of Sdt-H or Sdt-D Has Opposite Effects on Light-induced Retinal Degeneration

Another phenotype associated with *sdt* mutations in adult PRCs is light-induced retinal degeneration (Berger *et al.*, 2007). However, due to mosaic expression of the transgenes used here when expressed in *sdt* mutant PRCs (Figure 2, A and B), the rescuing abilities of the transgenes could not be analyzed in adult eyes mutant for *sdt*. Therefore, to study the function of the *sdt* transgenes in preventing light-dependent degeneration, we took advantage of the observation that eyes mutant for *white* (w^{1118}) degenerate under constant light conditions (Lee and Montell, 2004). We adjusted the light conditions so that ~60% of w^{1118} PRCs survived after 7 d of exposure to constant light. These conditions allowed detecting any enhancement or suppression of degeneration as a result of transgene activation. To quantify the effects of the *sdt* transgenes on light-dependent degeneration of w^{1118} eyes, the control genotypes were as close to the experimental genotype as possible to avoid effects caused by genetic background. Unlike the length of the stalk membrane, light-dependent degeneration in w^{1118} flies did not depend on the dose of *sdt*. This conclusion is drawn from the observation that the rate of PRC survival seen in flies with two or one copy of sdt^+ is similar (compare the distributions for wild type or $sdt^{K85}/+$ heterozygous controls (blue in Figure 6A; values are summarized in Supplemental Table S2).

Expression of Sdt-H in outer PRCs by using *Rh1*GAL4 in a sdt^+ homozygous (w^-) or a $sdt^{K85}/+$ heterozygous back-

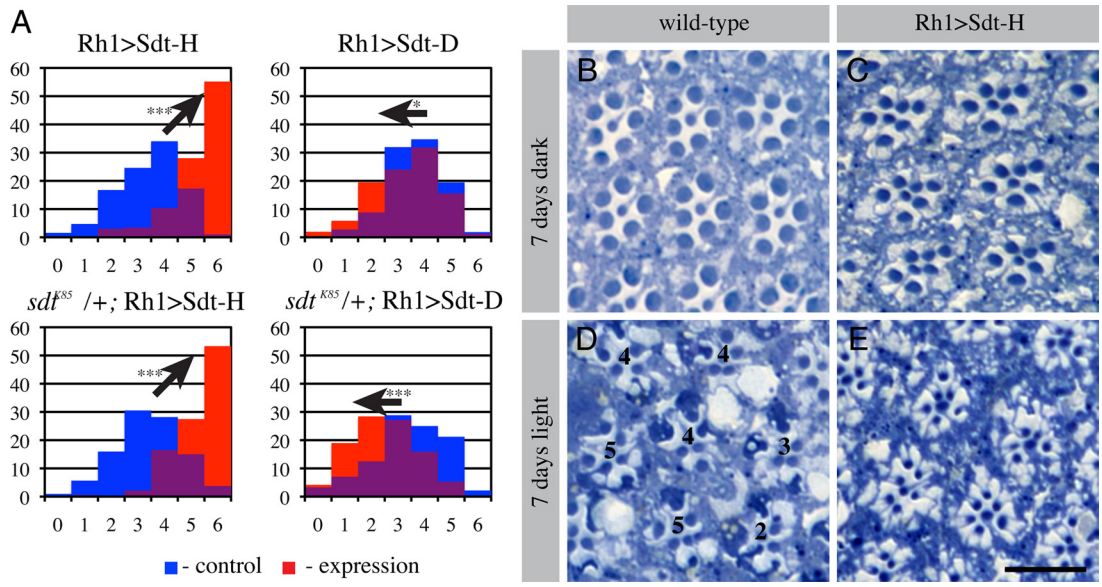

Figure 6. Effects of Sdt-H and Sdt-D expression on retinal degeneration. (A) Diagrams demonstrate the effects of Sdt-H and Sdt-D expression on the number of intact rhabdomeres of outer PRCs per ommatidium after 7 d of exposure to the light, in eyes with two (top diagrams) and one (bottom diagrams) copy of sdt^+. Distributions in experimental conditions are depicted in red, in corresponding controls in blue, and an overlay between distributions in violet. Genotypes are as follows (experimental conditions and control): Rh1>Sdt-H: *UAS*Sdt-H/+; *Rh1*GAL4/+ (red) and *UAS*Sdt-H/+; CyO/+ (blue). $sdt^{K85}/+$; Rh1>Sdt-B2: *UAS*Sdt-H sdt^{K85} FRT19A/+; *Rh1*GAL4/+ (red) and *UAS*Sdt-H sdt^{K85} FRT19A/+; CyO/+ (blue). Rh1>Sdt-D: *UAS*Sdt-D/+; *Rh1*GAL4/+ (red) and *UAS*Sdt-D/+; CyO/+ (blue). $sdt^{K85}/+$; Rh1>Sdt-D: *UAS*Sdt-D sdt^{K85} FRT19A/+; *Rh1*GAL4/+ (red) and *UAS*Sdt-D sdt^{K85} FRT19A/+; CyO/+ (blue). The arrows indicate the direction of the shift in distribution. Statistically significant differences in comparison to the respective control are marked *** for two-tailed p-value, $p < 0.0001$ and * for two-tailed p-value, $p < 0.05$. (B–E) Cross sections through w^{1118} eyes (B and D) and eyes expressing Sdt-H using *Rh1*GAL4 in w^{1118} background (C and E). Flies were kept for 7 d in the dark (B and C) or in constant light (D and E). The degeneration occurring in w^{1118} is completely blocked by expression of Sdt-H (compare D and E). To quantify degeneration, the mean number of PRCs with clearly detectable rhabdomeres was measured. Example of quantification is represented in D (numbers indicate survived PRCs). In all cases, *Rh1*GAL4 was used. Bar, 10 μm.

ground nearly completely blocked light induced degeneration (Figure 6A; compare Figure 6, D and E). In addition, the rhabdomeres of the outer, *Rh1*-expressing PRCs were reduced in size in comparison to control flies and to flies kept in the dark (Figure 6, C and E). When Sdt-D was expressed in sdt^{+} homozygous eyes using *Rh1*GAL4, the survival of PRCs was slightly but significantly reduced in comparison with the control. However, when Sdt-D was expressed in a $sdt^{K85}/+$ background, the decrease in survival of PRCs was stronger (Figure 6A; values are summarized in Supplemental Table S2). This difference correlates with the observation that Sdt-D expression completely removed the Sdt-H isoform only in $sdt^{K85}/+$ flies but not in wild-type background (Figure 4A). Together, these results support the conclusion that Sdt-H and Sdt-D have opposite effects: more Sdt-H inhibits light-induced retinal degeneration, whereas more Sdt-D enhances it.

DISCUSSION

The results presented here are the first to show in vivo functions of two Sdt isoforms, Sdt-H and Sdt-D, in the *Drosophila* retina. Strikingly, the two isoforms, which only differ in their N terminus, exhibit opposite effects on stalk membrane length and light-dependent retinal degeneration upon overexpression. It was reported previously that the N terminus of *Drosophila* Sdt-B, Sdt-F, and of the vertebrate orthologue protein associated with Lin-7/membrane palmitoylated protein-5/ (Pals1/MPP5) binds Par-6 in vitro (Wang *et al*., 2004; Bulgakova *et al*., 2008). In particular ECR1 of Pals1 turned out to be particularly important for Par-6 binding. Here we show, however, that the loss of Par-6 localization in *sdt* mutant pupal PRCs could be rescued by Sdt-D, which lacks ECR1. This suggests that in vivo, ECR2 may be sufficient for binding Par-6 in PRCs or that recruitment of Par-6 in pupal PRCs by Sdt occurs indirectly. The latter is supported by the observation that Par-6 is recruited only apically in *sdt* mutant PRCs expressing Sdt-H or Sdt-D, although the transgene-encoded proteins are also localized basolaterally and in the cytoplasm. This indicates that Sdt is necessary for Par-6 localization, but not sufficient. A similar role has been described for Sdt with respect to stabilization of Crb in adult PRCs. Here, Sdt is necessary for Crb localization at the stalk membrane, but it cannot recruit Crb to ectopic places. In contrast, Sdt can recruit PATJ to any site when overexpressed (Bulgakova *et al*., 2008). Interestingly, the zebrafish Sdt orthologue Nagie oko lacking ECR1 could rescue defects of *nagie oko* mutant embryos in the retinal pigment epithelium, but it failed to do so in the myocardium and the neural retina (Bit-Avragim *et al*., 2008).

Data presented here are consistent with the conclusion that besides Sdt-H, Sdt-D is also localized at the stalk membrane in wild-type PRCs, because the antibody used, which is directed against the PDZ domain common to both of them, detects Sdt proteins only at the stalk membrane. Yet, our data suggest that Sdt-H and Sdt-D define separate Crb complexes, each of which only contains one Sdt isoform. Homo- and heterodimerization has been shown to occur between MAGUK family members, such as hDlg1 and MPP7 (Stucke *et al*., 2007) or MPP4 and MPP5 (Kantardzhieva *et al*., 2005). Our data demonstrate that at least in PRCs the two Sdt isoforms do not interact with each other. Although we have shown previously that Sdt-H is expressed in all PRCs (Bulgakova *et al*., 2008), we cannot exclude the possibility, due to a lack of a Sdt-D-specific antibody, that Sdt-D is expressed in only a subset of PRCs. However, mutual down-regulation of one isoform upon overexpression of the other exclusively in outer PRCs strongly suggests that the two isoforms are coexpressed in outer PRCs of the adult retina. The opposite effects of Sdt-H and Sdt-D on stalk membrane length and prevention of retinal degeneration, and the influence of one isoform on the level of the respective other isoform suggests that a fine-tuned ratio between Sdt-H and Sdt-D, and hence between two Crb complexes, is required for the two processes. Thus, changing the ratio between the two isoforms has no affect on the total amount of the Crb complex but influences the length of the stalk membrane and cell survival.

Until now, two conditions are known that result in an elongation of the stalk membrane, namely, Crb overexpression or loss of *yurt* (Pellikka *et al*., 2002; Laprise *et al*., 2006; Richard *et al*., 2009). Because the amount of Crb is not changed upon Sdt overexpression as revealed by Western blot, we can rule out that the elongation observed upon Sdt-H overexpression is mediated by an increase in Crb. Loss of function of the FERM protein Yurt, which binds to the FERM-binding domain of Crb, leads to an increase in stalk membrane length (Laprise *et al*., 2006). Similarly, vertebrate orthologues Mosaic eyes (Moe)/EPB4.1L5 bind to vertebrate Crb, and in zebrafish it negatively regulates the apical membrane of PRCs (Hsu *et al*., 2006; Laprise *et al*., 2006; Gosens *et al*., 2007a). The FERM domain of mammalian EPB41L5 also can bind the HOOK domain of the Sdt orthologue Pals1/MPP5 (Gosens *et al*., 2007a). Because both Sdt-H and Sdt-D contain the HOOK domain, any regulation of stalk membrane length by Yurt should include more complex regulatory interactions. Alternatively, the ratio between the Crb/Sdt-H and the Crb/Sdt-D complexes at the stalk could determine stalk membrane length, e.g., by recruiting different interaction partners.

It is well established that genes encoding members of the MAGUK family are often controlled by alternative splicing, both in vertebrates and invertebrates, and give rise to various protein isoforms, which can have different functions (Sierralta and Mendoza, 2004). This has been demonstrated for the vertebrate MAGUKs synapse-associated protein-97 (SAP97), postsynaptic density protein-95 (PSD-95; also known as SAP90), and PSD-93/Chapsyn110. Two isoforms with different N termini have been reported for each of them. One isoform contains an L27 domain, whereas the other contains a cysteine doublet that can be palmitoylated. Although the α-isoforms of PSD-95 and SAP97 regulate the strength of the synapse in an activity-independent manner, the function of the respective β-isoforms depends on the activity of the synapse (Cho *et al*., 1992; Müller *et al*., 1995; Schlüter *et al*., 2006). So far, alternative splice variants have not been described for the vertebrate Sdt orthologue MPP5/Pals1. Instead, the vertebrate genome encodes several members of the MPP/p55 subfamily, which can be coexpressed in a cell and then often colocalize. For example, vertebrate MPP5/Pals1 colocalizes with members of the Crb complex in the cortical neuroepithelium (Kim *et al*., 2010) and in PRCs apical to the outer limiting membrane (OLM). MPP5, in turn, interacts with its family members MPP4 and MPP1, which also localize apical to the OLM (Kantardzhieva *et al*., 2005; Stöhr *et al*., 2005; van Rossum *et al*., 2006; Gosens *et al*., 2007b).

Our data not only show that two Sdt isoforms colocalize in the same cell but also that they have antagonistic function upon overexpression. Several cases have been reported, including transcription factors, signaling molecules and receptors, in which different isoforms, synthesized as a result of alternative splicing, have opposite functions. During mouse chondrogenesis, for example, two isoforms of the homeodo-

main transcription factor Prx1 are expressed. Although Prx1a overexpression decreases apoptosis when expressed in developing cells in culture, Prx1b overexpression increases apoptosis (Peterson *et al*., 2005). To our knowledge, Sdt is the first MAGUK family member for which antagonistic functions of two isoforms are shown in vivo. Antagonistic function also has been shown for the MAGUKs PSD-95 and MPP3, members of the DLG and the MPP/p55 subfamily, respectively, which are, however, encoded by two separate genes. Although PSD-95 increased the Ca^{2+}-induced desensitization of the 5-hydroxytryptamine (5-$HT)_{2C}$ receptor in mice cortical neurons in primary cultures and in heterologous cells, MPP3 prevented desensitization. These effects correlated with the observation that interaction of the 5-HT_{2C} receptor with the PDZ domain of PSD-95 facilitated receptor internalization, whereas its interaction with MPP3 stabilized the receptor at the membrane (Gavarini *et al*., 2006).

In summary, our results are the first to suggest that a tight stoichiometry between two protein isoforms can be essential to regulate cell shape, such as the length of the stalk membrane, and homeostasis. Future studies are required to determine whether this regulation occurs at the transcriptional or posttranscriptional level. Given the observation that MAGUKs play key roles in assembling multimolecular signaling complexes (Dimitratos *et al*., 1999), it is tempting to speculate that the two Crb–Sdt complexes may be engaged in different signaling events. This has been recently shown for the T cell receptor, which can recruit either of two MAGUKs, Dlgh1 or Carma1, into a signaling complex, thereby activating two different mitogen-activated protein kinases, p38 or c-Jun NH_2-terminal kinase, respectively (Blonska *et al*., 2007; Round *et al*., 2007). PRCs of *Drosophila* are ideally suited to further dissect the signaling events downstream of the Crb–Sdt complex.

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