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; Gonzalez-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 cytoplasm-
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 zona 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., 2001; 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 (Tantenzapf 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. sdtK85, 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 (Ferrari et al., 2003), producing transgenic flies with which the Sdt-H transgene was generated using the pUAST-w–FLAG–Sdt-H vector (Bulgakova et al., 2008) digested with Xbal and Aattl and cloned into pUAST-y. The N terminus of Sdt-D was amplified from mRNA by using 5'-Sdt-D-N primer: CGAAGACTACCGCTCCACTTGCAC with an introduced restriction site (underlined) and 3'-Sdt-D-PDZSH3:GGATGGAAGTGTTGACC. 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). 

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 MgCl2, 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 Chemical, 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 optical lobes, including the lamina, which slightly differs from the 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 (BR8-1; 1:1000), mouse α-Crb-Cq4 (1:100; Tepass and Knust, 1993), rabbit α-PATJ (1:5000; Richard et al., 2006), rabbit α-Lamin B (kindly provided by T. Saito; University of Göttingen), 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 (IgG) (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 in 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 isolated eyes using the Mini RNA Isolation kit (Zymo Research, Orange, CA). The PCR and RACE primer set used was 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 (BR8-1; 1:5000; this work); rabbit α-Crb–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-DPar-6 (1:1000; kindly provided by A. Wodarz; University of Göttingen, Germany); rat α-Crb and rabbit α-FLAG (1:1000; Sdt-K–FLAG antibodies); 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 F211A-CHS-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 Sdt isoform, the genotype of control flies was as close as possible to the genotype of flies carrying the respective transgene, the mutant allele, and in wild-type flies, was used for statistical analysis. For each group, the mean number of PRCs with intact rhabdomeres and ommatidium was quantified. 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.html).

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 contains an 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. sechellia, 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 α1118 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 GMIRGal4, 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 UASSdt-D and UASSdt-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,
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 sdtK85 mutant photoreceptor cells (we used the null allele sdtK85 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 Rh1GAL4 in a sdtK85 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 sdtK85 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.

Rh1GAL4 induces a mosaic expression pattern of the Sdt-D and Sdt-H–encoding transgenes in sdtK85 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
Transgenes were expressed using *Rh1GAL4* in wild-type or in a *sdt*<sup>K55</sup>/+ 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 communoprecipitated PATJ, but one Sdt isoform did not communoprecipitate 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*<sup>K55</sup> mutant pupal PRCs, which do not express Sdt protein (Figure 3A), Par-6 is strongly enriched 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*<sup>K55</sup> 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*<sup>K55</sup> 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 118-213 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*<sup>K55</sup> PRCs. Similar to Sdt-H, Sdt-D protein was enriched apically when expressed in pupal *sdt*<sup>K55</sup> 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*<sup>K55</sup> 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 *Rh1GAL4* 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 *Rh1GAL4* or *GMR-GAL4*, the amount of Sdt-D was strongly down-regulated. Similarly, Sdt-D overexpression
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 sdtK85/D/H11001 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; data not shown). Expression of Sdt-H or Sdt-D Has Opposite Effects on Stalk Membrane Length

In sdtK85/D/H11001 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 sdtK85/D/H11001 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 sdtK85/D/H11001 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).

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Therefore, we investigated the influence of Sdt-H and Sdt-D overexpression on stalk membrane length in a w+/H11002 sdt/H11001 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+/H11001), as measured from electron microscopic pictures, is close to 2.04 ± 0.06 μm and ranges from 2.04 to 2.13 μm (Supplemental Figure 4).

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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 Rh1GAL4. The following genotypes are depicted (top to bottom): UASsdt-H/+; CyO/+ (blue) and UASSdt-H+; Rh1GAL4/+ (green) (F1 from UASSdt-H × Rh1GAL4/CyO); UASSdt-H+; Rh1GAL4/CyO (green) and UASSdt-H+; Rh1GAL4/UASSdt-D2 (green/red) (F1 from UASSdt-H/FM7; UASSdt-D2/CyO × Rh1GAL4); and CyO/Sdt-D2 (blue) and Rh1GAL4/UASSdt-D2 (red) (F1 from UASSdt-D2/CyO × Rh1GAL4). 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 GMRGAL4. Distributions of stalk membrane lengths in UASSdt-H+; GMRGAL4/+ (green) and UASSdt-D+/+; GMRGAL4/+ (red) are compared with the corresponding controls UASSdt-H+; CyO/+ (F1 from UASSdt-H × GMRGAL4/CyO) and UASSdt-D+/+; CyO/+ (F1 from UASSdt-D × GMRGAL4/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 Rh1GAL4 or GMRGAL4 induced an increase of stalk membrane length (Figure 5, B and C, green). In contrast, GMRGAL4-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 Rh1GAL4 (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 Rh1GAL4 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 Rh1GAL4 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 (w1118) degenerate under constant light conditions (Lee and Montell, 2004). We adjusted the light conditions so that ~60% of w1118 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 w1118; Rh1GAL4 or sdtK85; Rh1GAL4; CyO; w1118 mutant PRCs (Figure 2, A and D), the rescuing abilities of the transgenes could not be 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.
ground nearly completely blocked light induced degeneration (Figure 6A; compare Figure 6, D and E). In addition, the rhodobin 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+/+ background, the decrease in survival of PRCs was slightly but significantly reduced in comparison with the control. However, when Sdt-D was expressed in a sdt+/+ background, the survival of PRCs was strongly (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+/+ 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 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)/EPB41L5 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 stalk membrane length by Yurt should include more complex regulatory interactions. Alternatively, the ratio between stalk membrane length by Yurt should include more complex regulatory interactions. Alternatively, the ratio between stalk membrane length by Yurt should include more complex regulatory interactions. Alternatively, the ratio between stalk membrane length by Yurt should include more complex regulatory interactions. Alternatively, the ratio between stalk membrane length by Yurt should include more complex regulatory interactions.

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 Prx1α overexpression decreases apoptosis when expressed in developing cells in culture, Prx1β 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 Ca2+-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-HT2C 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, Dlg1 or Carma1, into a signaling complex, thereby activating two different mitogen-activated protein kinases, p38 or c-Jun NH2-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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References


Rotational Function of Sdt Isoforms


