Structure-functional analysis of Stardust in the *Drosophila* eye

Inaugural Dissertation

zur

Erlangung des Doktorgrades der

Mathematisch-Naturwissenschaftlichen Fakultät der

Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Natalia Bulgakova

aus Novosibirsk, Russische Federation

Januar, 2007

Aus dem Institut für Genetik
der Heinrich-Heine Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Berichterstatter:	Prof. Dr. Elisabeth Knust
	Prof. Dr. Johannes H. Hegemann
Tag der mündlichen Prüfung:	20.04.2007

1. Introduction

1.1 Drosophila *Stardust: protein structure*

1.1.1 Stardust protein structure

Stardust (Sdt) belongs to the p55-like subfamily of membrane-associated guanylate kinase homologs protein family (MAGUKs). MAGUKs act as molecular scaffolding for signaling pathway components at the plasma membrane, and function by binding to the cytoplasmic termini of transmembrane proteins as well as to other signalling proteins through their multiple protein-protein interaction domains. Sdt protein contains two L27 domains, a PDZ, SH3, Hook and GUK domain (Figure 1.1, Bachmann *et al*., 2001).

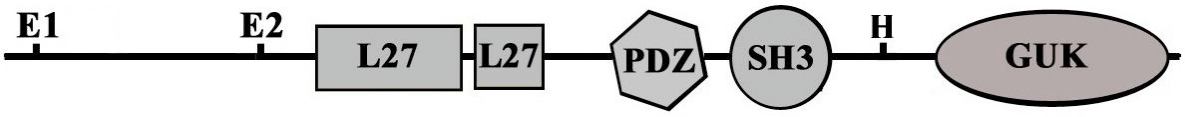


Figure 1.1. Domain structure of Sdt protein. E1 and E2 stand for ECR1 and ECR2. H – Hook domain.

1. L27 (for Lin-2/Lin-7 binding motif) domains were shown to interact with other L27 domains forming heterodimers (Li *et al*., 2004, Kempkens, 2005). In case of Sdt its N-terminal L27 domain interacts with *D*PATJ (Roh *et al*., 2002). The C-terminal L27 domain of Sdt was shown to interact with *D*Lin-7 protein (Bachmann *et al.*, 2004).
2. PDZ domains usually bind to the extreme C-terminal 4-aminoacid regions of different transmembrane and channel proteins. Rarely, PDZ domains can also interact with internal motifs of other proteins, or with other PDZ domains (Dimitratos *et al*., 1999). The PDZ domain of Sdt was shown to interact with the C-terminal ERLI motif of the Crb protein (Hong *et al*., 2001, Bachmann *et al*., 2001).
3. SH3 (from Src-homology 3) domains are also protein-protein interaction domains though their partners are not well characterised. It is known that SH3 domains interact with left-handed poly-proline helices (Cohen *et al*., 1995). For Sdt no interaction partners of its SH3 domain are known.

4. Hook domains interact with FERM domains of actin-associated proteins of the 4.1-like protein superfamily (Dimitratos *et al*., 1999). But for Sdt such interaction partners are also unknown.
5. GUK – for guanylate kinase – domains share a high homology with guanylate kinase that converts GMP to GDP using ATP as a donor of phosphate. Though MAGUK proteins highly diverge at GMP- and ATP-binding sites, in p55-like MAGUK subfamily, the subfamily that Sdt belongs to, these sites are intact. So they can bind nucleotides, but only for one of these proteins – for p55 – and only *in vitro* a low kinase activity was observed (Marfatia and Chishti, 1995). In addition to binding nucleotides, in several cases GUK-domains were shown to mediate protein-protein interaction (Satoh *et al*., 1997). For several MAGUK proteins, engagement of their PDZ domain by a peptide ligand influenced the binding properties of the GUK domain (Brenman *et al*., 1998). But for Sdt the function of its GUK domain is yet unknown. There is evidence that GUK domain may bind to the Sdt SH3 domain, and therefore form an interaction within the same Sdt protein or between two different Sdt proteins (Kempkens, 2005). Such intermolecular binding was previously demonstrated for human MAGUK protein hCASK (Nix *et al.*, 2000).

In the most N-terminal part of the Sdt protein there are two evolutionary conserved regions: ECR1 and ECR2. ECR1 is absolutely required for the binding of Sdt to *D*Par-6 protein, since when it is deleted the binding is blocked. Absence of ECR2 does not prevent Sdt protein from the binding to *D*Par-6 but strongly reduces the efficiency of the interaction between these two proteins (Wang *et al*., 2004).

1.1.2 Sdt protein isoforms

Alternative splicing is one of the important mechanisms to produce different gene products for a single gene comparing to other posttranscriptional modifications. MAGUK proteins are often presented by several isoforms, which are expressed tissue or stage specifically and probably provide tune regulation of the protein functions (see Sierralta and Mendoza, 2004, for review). For the Discs Large (Dlg) protein two different isoforms were described: Dlg-A and Dlg-S97. Dlg-A is the form specific for epithelial cells, while Dlg-S97 is expressed at neuro-muscular junctions. These two

forms of the protein differ in their N-terminal region (Mendoza *et al.*, 2003). Similar, the CG9326 gene encodes for two different forms of a MAGUK protein, which again differ in their N-terminal part and are differentially expressed during development (Bachmann A., personal communication).

At present three different isoforms of Sdt were described (Sdt-MAGUK1, Sdt-GUK and Sdt-B (Figure 1.2). The Sdt-MAGUK isoform is the longest one and encodes for a 142 kDa protein (Bachmann *et al.*, 2001). The Sdt-B in comparison to Sdt-MAGUK lacks the large portion of sequence in the N-terminal part of the protein and has a predicted size of 95 kDa. This is a result of alternative splicing, as the mRNA of this form lacks the large exon in the 5'-region and has a different transcription start site (Hong *et al.*, 2001). This form is thought to be the preliminary expressed one in the *Drosophila* embryo (Wang *et al.*, 2004). Sdt-GUK form lacks PDZ, SH3 and Hook domains, and has the same transcriptional start site as Sdt-B (Bachmann *et al.*, 2001).

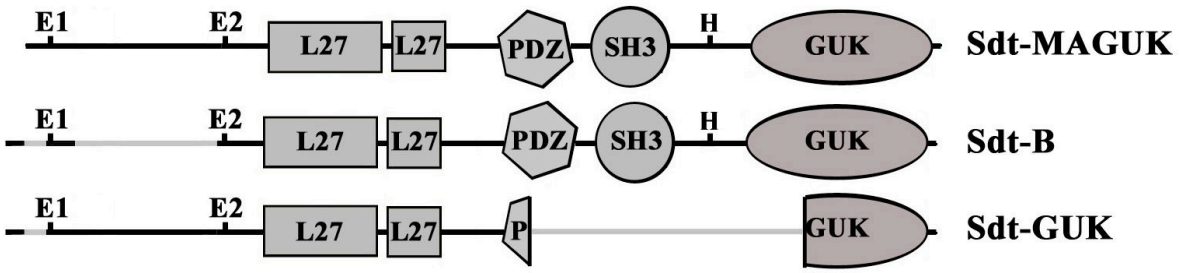


Figure 1.2. Scheme of the proteins encoded by three known Sdt isoforms. E1, E2 and H stand correspondingly for ECR1, ECR2 and Hook domain respectively. The parts that are absent in the proteins in comparison to other isoforms in each isoform are marked in grey.

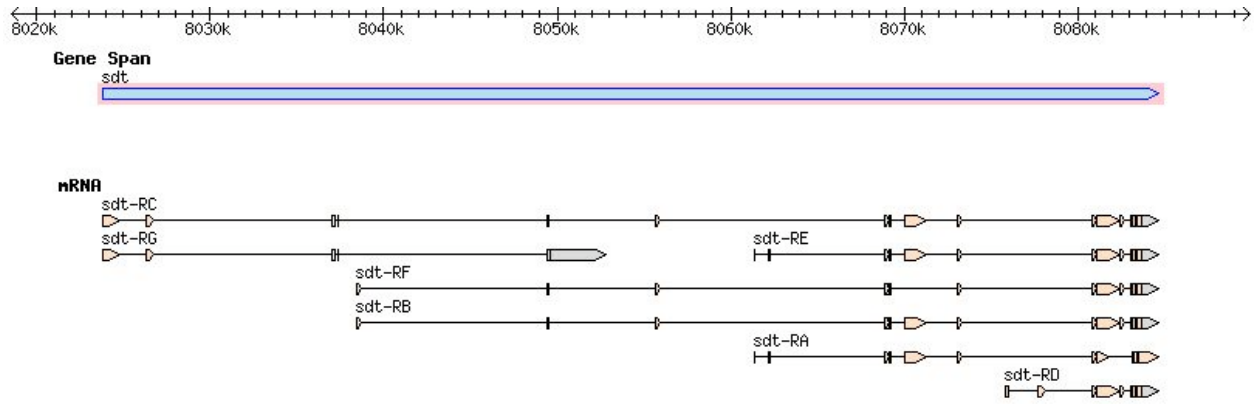


Figure 1.3. Prediction of Sdt isoforms based on ESTs. The numbers on the upper panel indicate the position in the genome according to the Release 3 of the *Drosophila* genome project.

In the FlyBase (http://flybase.bio.indiana.edu) several more isoforms of Sdt are predicted from expressed sequence tags (EST). They include the three already described forms – Sdt-MAGUK and Sdt-GUK (= sdt-RB and sdt-RD). Sdt-B as it was published in Hong *et al.*, 2001 is absent from the scheme. The only form that is similar to the published Sdt-B isoform, and has the same deletion in comparison to Sdt-MAGUK as Sdt-B, is Sdt-RF. The only difference between Sdt-B and Sdt-RF is that Sdt-RF has the same transcription start site as Sdt-MAGUK does. This isoform will be called Sdt-B2 in the following text.

All the other predicted isoforms, except sdt-RG, have the same 3'-part as Sdt-MAGUK, and represent the proteins with two L27 domains, a PDZ domain, a SH3 domain and a GUK domain. These isoforms differ mainly in the exons contained in the 5'-region. sdt-RE is very similar to Sdt-MAGUK in structure but has the same translational start site as Sdt-GUK. sdt-RC has an other translation start as the previously described two, but has the same domains as Sdt-MAGUK. sdt-RD, in addition to having an alternative translation start, also lacks the ECR motifs. All forms are schematically shown on Figure 1.3. The presence of different Sdt isoforms gives rise to the possibility that they may participate in the control of different functions, as well as in temporal and spatial control of these functions realization. At the same time there is the probability that not all forms of Sdt are described yet.

1.2 Crb-complex

The Crumbs (Crb) - complex is a highly conserved protein complex. It consists of four main members: Crb, *D*PATJ, Sdt and *D*Lin-7 proteins (Figure 1.4). Crb is the only transmembrane member of the complex, and has a large extracellular domain, a transmembrane domain and a short cytoplasmic domain. The extracellular domain of Crb is rather large and contains 30 EGF-like repeats (Tepass *et al*., 1990) and 3 Laminin A G domain-related repeats (Patthy, 1991). Though it is clear that the extracellular domain of Crb should provide an important function, there is nothing known so far about these functions or about its possible interaction partners. The short intracellular domain of Crb consists of only 37 amino-acids, and has on the most C-terminus a highly conserved PDZ-binding motif ERLI (Klebes and Knust, 2000). The ERLI-motif was shown to bind to the PDZ domains of Sdt (Hong *et al.*, 2001, Bachmann *et al*., 2001) and *D*Par-6 (Nam and Choi, 2003, Kempkens *et al*., 2006). In the middle part of the Crb intracellular domain there is a 4.1 protein/ezrin/radixin/moesin (FERM) domain binding consensus motif, which was shown to bind to *D*Moesin (Medina *et al*., 2002). Overexpression of the intracellular domain of Crb in the mutant embryos is sufficient to partially rescue the *crb* mutant phenotype (Wodarz *et al.*, 1995).

*D*PATJ, Sdt and *D*Lin-7 are membrane-associated cytoplasmic proteins. *D*PATJ contains a N-terminal L27 domain followed by four PDZ-domains (Bhat *et al.*, 1999, Pielage *et al*., 2003). The L27 domain, called also in literature MRE for MAGUK recruiting element, is required to provide binding between *D*PATJ and Sdt (Roh *et al.*, 2002, Kempkens, 2005). Function of the PDZ domains is nearly unclear; it was demonstrated that the third PDZ domain of *D*PATJ can bind to *D*Par-6 protein *in vitro* (Nam and Choi, 2003). *D*Lin-7 protein consists of two protein-protein interaction domains: an L27 and a PDZ domain. The L27 domain of *D*Lin-7 can bind to the C-terminal L27 domain of Sdt (*Bachmann et al.*, 2004). It is known that Lin-7-like proteins are involved in binding receptor tyrosine kinases, and are important for receptor localisation (Kaech *et al*., 1998). In addition to being a member of the Crb-complex, *D*Lin-7 co-localizes with Dlg at the postsynaptic side of neuromuscular junctions and interacts with it (Bachmann *et al.*, 2004).

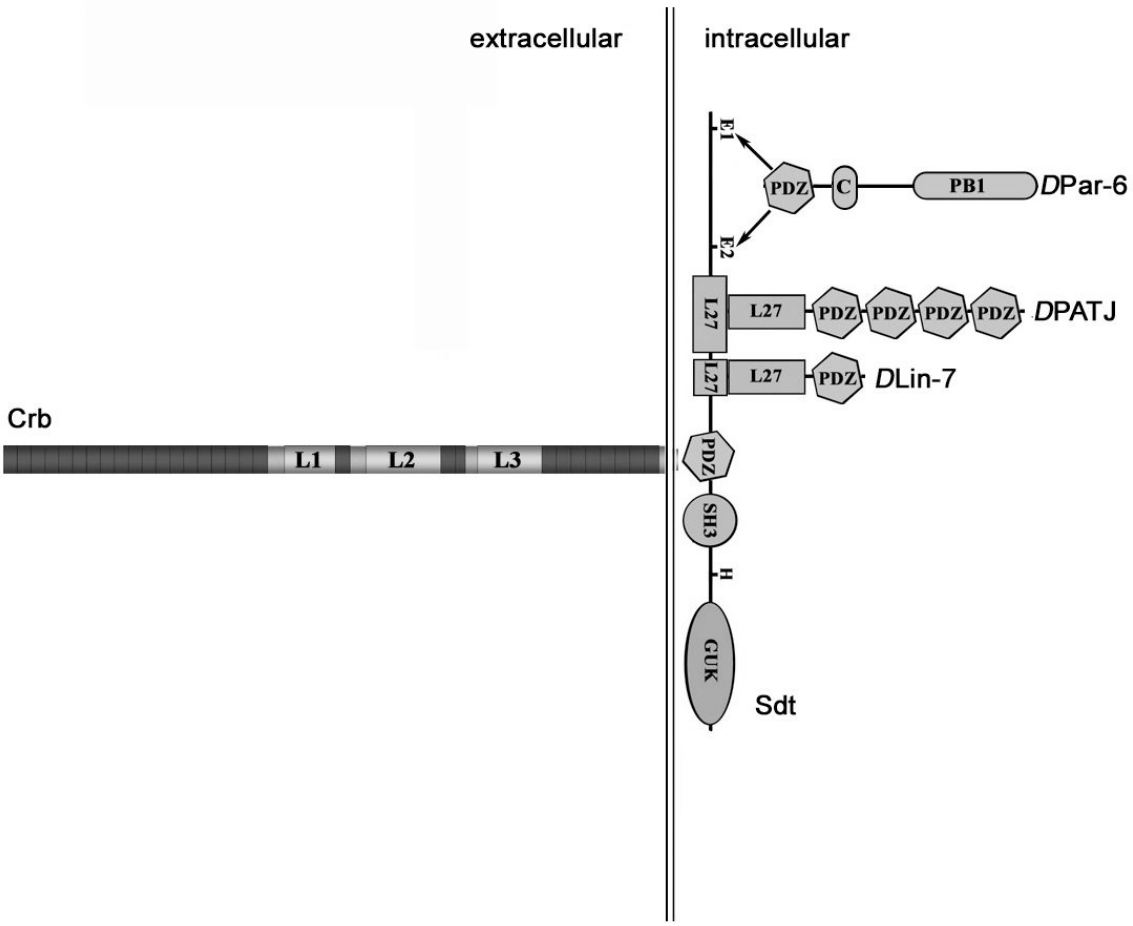


Figure 1.4. Schematic structure of the Crb-complex. Domains are indicated. L1-L3 - Laminin A G domain-related repeats, C – CRIB domain, H – Hook domain. Arrows indicate that the *D*Par-6 protein requires both ECR1 and ECR2 (E1 and E2 respectively) for binding to Sdt.

1.3 The Crb-complex and embryonic epithelial cell polarity

The function of the Crb-complex is the most extensively studied in *Drosophila* embryonic epithelial cells. One of the main characteristics of the epithelial cells is an apical-basal polarity. It provides a proper compartmentalization within the cell, localization of intracellular connections, and allows the formation of a continuous sheet of epithelial cells. The continuous epidermis does not only secret the cuticle that provides protection and stability for the larval body, but also fulfils specific tasks such as targeted uptake and secretion of molecules and the segregation of different tissue compartments.

Epithelial cells have two main features: they have a polarized phenotype and form highly elaborate cell-cell junctions. Polarization is reflected in the shape of the cell, the distribution of different organelles and molecules, and the alignment of cytoskeletal network. The plasma membrane of such cells is also polarized: it is subdivided into a basolateral and an apical domain (Figure 1.5). Adherens junctions (AJ's) are localized on the border between the apical and the basolateral membrane domains and encircle the cell forming the zonula adherens (ZA). Together with other junction types, AJ's provide close adhesion between epithelial cells. Core proteins that form the AJ's are the transmembrane *D*E-Cadherin and a number of associated cytoplasmic proteins like α-catenin or β-catenin/Armadillo (Oda *et al.*, 1994, Peifer, 1993, see also Rantsch, 1994, Garrod and Collins, 1992, for review). The extracellular domains of *D*E-Cadherin form dimers, thereby providing adhesion between neighbouring cells. The intracellular domain of *D*E-Cadherin binds to β-catenin. This, in turn, binds to α-catenin, and α-catenin binds to F-actin organizing actin network in the whole cell. The second type of junctions that can be found in epithelial cells is the septate junction (SJ's). SJ's are localized basal to the ZA, and have got their name because of their morphology on the ultrastructural level where they form a ladder-like structure connecting neighbouring cells. SJ' provide barrier function of the epithelium (Skaer et al., 1987). They are formed by a number of transmembrane proteins (e.g. Neurotactin) and of cytoplasmic proteins associated with them (e.g. Coracle, Discs Large, Scribble) (for review see Hortsch & Margolis, 2003).

The Crb-complex is required for the maintenance of apical-basal polarity in epithelial cells. It is localized immediately apical to the ZA in the so-called subapical region (SAR) in these cells (Tepass et al., 1990, Bachmann *et al.*, 2001). Loss-of-function of either *crb* or *sdt* leads to embryonic lethality. This is due to loss of apical-basal polarity in many ectodermally derived epithelia, which is followed by tissue disintegration and massive cell death (Tepass and Knust, 1990). This leads to a failure in the formation of a continuous shield of cuticle secreted by the epithelial cells of the epidermis. In mutant embryos only grains of cuticle can be seen, that was the reason for the names of the genes (Tepass & Knust, 1990). Different ectodermally derived epithelia are affected in the mutants to different extend. One of the reasons for such

phenotype is the failure to establish a proper ZA (Grawe *et al.*, 1996). Overexpression of the intracellular part of Crb protein in a wild-type background results in an enormous expansion of the apical membrane (Wodarz *et al.*, 1995).

The molecular basis of the function of the Crb-complex in the epithelial cells includes its interaction with two other protein complexes – the Discs Large (Dlg) and the Bazooka (Baz) -complexes. The Dlg-complex includes Dlg, Scribble (Scrib) and Lethal giant larvae (Lgl). All three components are cytoplasmic membrane associated proteins and localize at the basolateral membrane with an accumulation at the SJ's at later stages (Tepass *et al.*, 2001). In larvae homozygous for mutations in the corresponding genes cells of imaginal tissues lose their polarity and continue to proliferate, forming amorphous masses (Gateff, 1978, Wodarz, 2000). The Baz-complex includes Baz, *D*Par-6 and *D*aPKC. Baz and *D*Par-6 are scaffolding proteins, and *D*aPKC has a kinase activity (Muller H.-A. and Wieschaus E., 1996, Wodarz *et al.*, 2000). The Baz-complex localizes to the SAR in embryonic epithelial cells similar to the Crb-complex.

The polarity in epithelial cells is provided by the integrated activity of three polarity protein-complexes. Baz is the initial and critical regulator of apical-basal polarity. It is also the first complex of these three, which is localized during development (Harris & Peifer, 2004). The Dlg-complex allows basolateral membrane development by antagonizing the Baz-complex function and repressing Baz-complex's ability to promote apical membrane characteristics. The Crb-complex becomes localized the last and maintains Baz at the apical membrane by antagonizing repression of the Dlg-complex. (Bilder et al., 2003, Tanentzapf and Tepass, 2003). The sensitive balance between the activities of the Crb- and Dlg-complexes allows proper polarity to be achieved. When any of the members of the polarity complexes is mutated it leads to the shift in the tuned balance and loss of the polarity as a consequence. The interaction between Crb and Baz complexes can be direct. It was demonstrated that the ECR1 and the ECR2 regions in the Sdt N-terminus could bind to the *D*Par-6 protein *in vitro* (Wang *et al.*, 2004), and *D*aPKC phosphorylates the intracellular C-terminus of Crb *in vitro* (Sotillos et al., 2004).

Recently several new members of polarity cascade in epithelia were discovered. One of them is *D*Par-1, which was previously known to participate in establishment of anterior-posterior polarity in the fly oocyte (Cox *et al*., 2001). *D*Par-1 is localized laterally, and, in parallel with the Dlg-complex, inhibits the Baz-complex by phosphorylation of Baz (Benton, St. Johnston, 2003). At the same time *D*Par-1 is inhibited by *D*aPKC in mammalian systems (Hurov *et al*., 2004). The other protein that was recently shown to participate in apical-basal polarity is the CDC42 GTPase. The CRIB domain of *D*Par-6 binds to activated CDC42. This binding increases the affinity of the PDZ domain of *D*Par-6 for its ligands by about 13-fold (Peterson *et al.*, 2004). Through this binding *D*Par-6 is probably recruited to the apical membrane domain. After this, *D*Par-6 in turn recruits Baz and *D*aPKC to the complex (Hutterer *et al*., 2004). In vertebrate system CDC42 also regulates the binding between Par-6 and Pals1, the mouse homolog of Sdt (Hurd *et al*., 2003). Schematically the interactions between the protein complexes are presented on Figure 1.5.

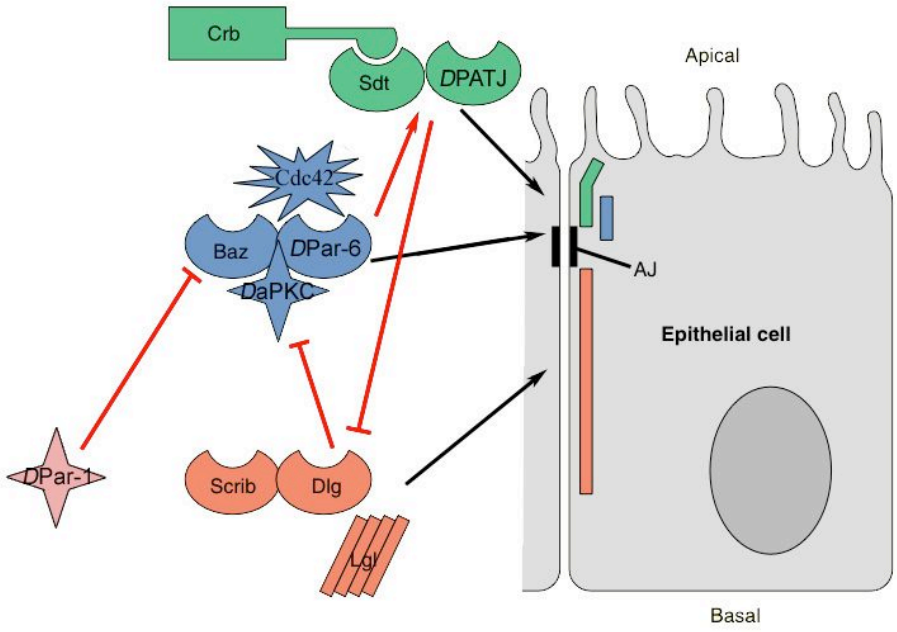


Figure 1.5. Model outlining the role of the Crb-complex in epithelial polarity. Black arrows show the localization of the protein complexes within the epithelial cell. Interactions between protein complexes are indicated in red. Modified from Bilder, 2003 and Bilder, 2004.

1.4 Role of the Crb-complex in the Drosophila eye

1.4.1 Stucture of *Drosophila* eye

Drosophila compound eyes consist of about 700-800 ommatidia. Each ommatidium represents an autonomous light-sensitive unit that is formed by 20 cells. These include pigment and cone cells and 8 photoreceptor cells (PRC) that are arranged in a stereotypic manner. PRCs are highly differentiated epithelial cells, and therefore are polarised along the apical-basal axis (Figure 1.6a-c). The basal membrane of the PRCs is differentiated into axon. The apical membrane of PRC is subdivided into the rhabdomere and the stalk membrane. The rhabdomere membrane is highly folded, forms the dense structure that is visible under the electron microscope and contains the photosensitive molecule – rhodopsin. In cross-sections rhabdomeres have a perfect round shape, and are organized within one ommatidium in a stereotypic trapezoid pattern (Figure 1.6d-e). The stalk membrane connects the rhabdomere with the ZA, and topologically corresponds to the SAR of embryonic epithelial cells. The members of the Crb-complex localize to the stalk membrane (Johnson *et al*., 2002, Richard *et al*., 2006, Figure 1.6f).

Figure 1.6.

Structure of the *Drosophila* adult eye.

A – Scanning electron micrograph of the complex eye of the fly consisting of multiple separated facets.

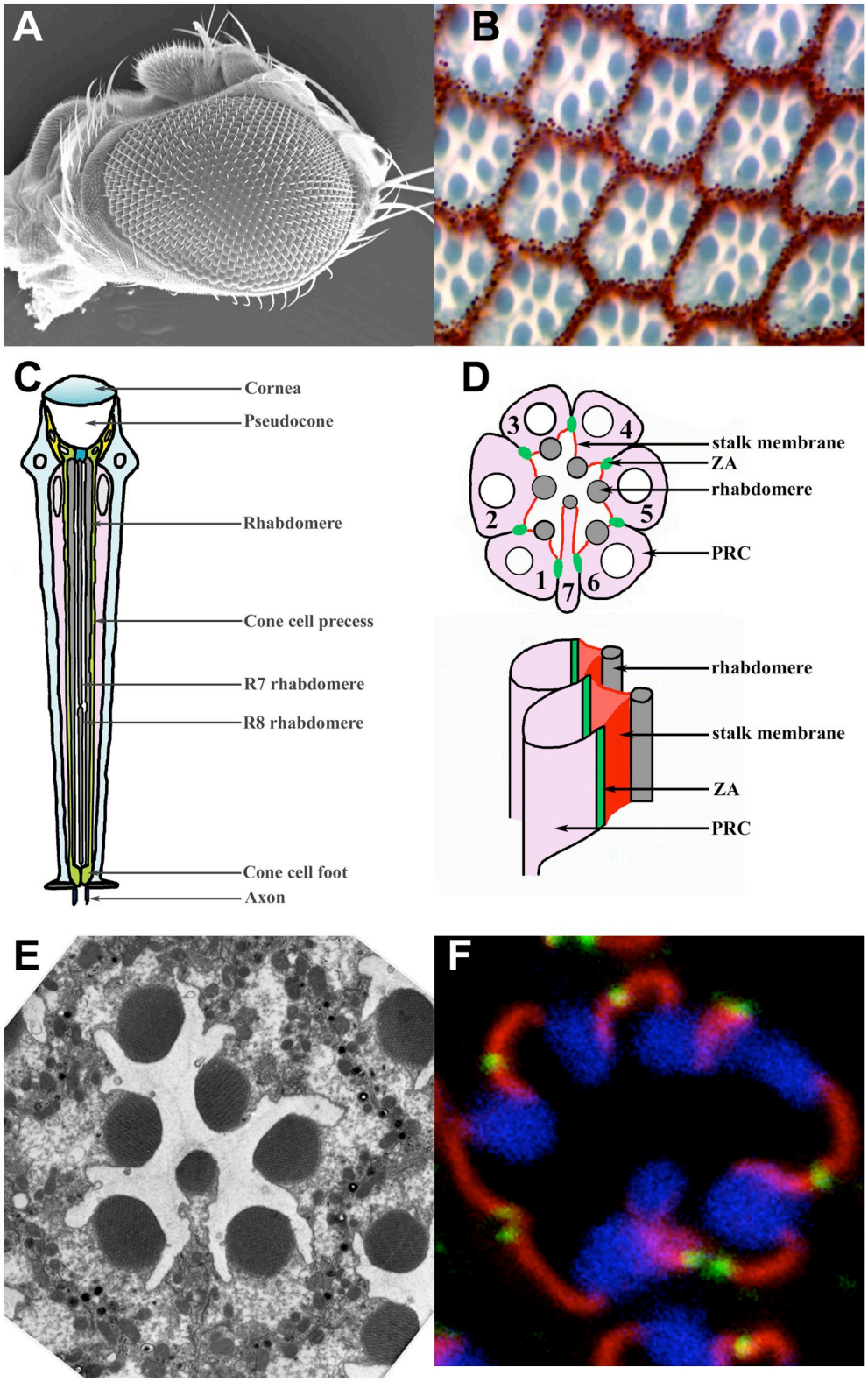
B – Crossection through an adult eye. Rhabdomeres are stained in blue. Individual ommatidia are separated by pigment cells containing large pigment vesicles.

C – Schematic longitudinal section through one ommatidium. Different cell types are marked by different colours. Blue – secondary pigment cells, yellow – primary pigment cells, green – cone cells, and rosy – PRCs. Rhabdomeres are marked in grey. Modified from Cagan & Ready, 1989.

D – Schematic cross-section through one ommatidium (accessory cells are excluded). Modified from Nam & Choi, 2003.

E – transmission electron micrograph of a cross-section through one ommatidium. Rhabdomeres form electron dense structures organised in a trapezoid pattern.

F – confocal micrograph of a cross-section through one ommatidium. Rhabdomeres are marked in blue with phalloidin, the ZA is stained in green with α-*D*E-Cad, and the stalk membrane is stained in red with α-Sdt-PDZ-C.


A
B
C
Cornea
Pseudocone
Rhabdomere
Cone cell precess
R7 rhabdomere
R8 rhabdomere
Cone cell foot
Axon
D
3
4
2
5
1
7
6
stalk membrane
ZA
rhabdomere
PRC
rhabdomere
stalk membrane
ZA
PRC
E
F

1.4.2 Development of the *Drosophila* eye.

1.4.2.1 Development of *Drosophila* photoreceptor cells.

Highly specialized PRCs differentiate from the typical epithelial cells of the eye imaginal discs. The differentiation process takes mainly place during *Drosophila* pupal development. In the third instar larvae the cells are organized in groups of eight cells under the control of different developmental signals. These cells will later differentiate into PRCs. Other cells will differentiate into accessory cells or die by apoptosis. After this the developing PRCs undergo a dramatical rearrangement (Figure 1.7). First, the apical membrane turns by 90° into the centre of the group of the cells belonging to one ommatidium. At this stage, the apical membrane is not yet subdivided into rhabdomere and stalk membrane precursors, and members of the Crb-complex co-localizes with F-actin as rhabdomere marker apically (Hong *et al.*, 2003). At about 50% of pupal development (50% p.d.) membrane domains separate, and the Crb-complex becomes localized only to the small region apical to the ZA, which will form a future stalk membrane. At about the same time, the apical membrane expands and reaches the contact with retinal floor (Cagan & Ready, 1989, Baumann, 2004). After this, at about 60% p.d. the cells themselves also elongate in the proximal direction. Elongation is accompanied by the decrease of the retinal floor surface. At this stage, the F-actin/myosin-II system is important for the cell elongation to ensure that the rhabdomeres are straight as the photoreceptors grow, and serves as a contractile system to keep the retinal floor flattened (Baumann, 2004).

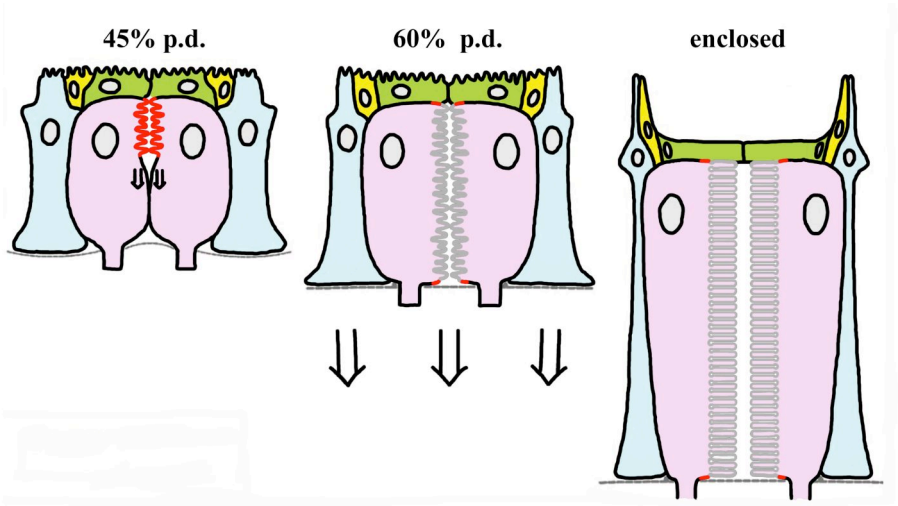


Figure 1.7. Scheme of ommatidial development. Age in per cent of p.d. is indicated. Different cell types are marked by different colours. Blue – secondary pigment cells, yellow – primary pigment cells, green – cone cells, and rosy – PRCs. Rhabdomeres are marked in grey, and stalk membrane – in red. Modified from Baumann, 2004.

1.4.2.2 Role of the Crb-complex in the development of photoreceptor cells.

Mutations of *crb, sdt* and *DPATJ* cause morphological defects in photoreceptor cells. In mutant eyes, the rhabdomeres are thicker as in wild type, and the shape of the cells is affected. In *crb* mutant eyes rhabdomeres do not elongate properly and reach only about a third of retina length. In mutants for *crb, sdt* and *DPATJ* the stalk membrane is reduced in length (Figure 1.8, Johnson *et al*., 2002, Pellika *et al*., 2002, Richard *et al*., 2006, Nam and Choi, 2006, Berger *et al*., 2006). In cases where the complex is still present, though in strongly reduced amounts (e.g in Sdt^{N5}), the morphology is unaffected (S. Berger, unpublished). The function of the Crb-complex in PRCs morphogenesis is yet unclear.

Absence of each of these proteins leads to the delocalization of the others in the adult eye, and to morphological defects. In *DPATJ* hypomorphic mutant the Crb-complex is detected at the forming stalk membrane till 70% p.d. Later it gradually delocalizes, and is undetectable in adult flies (Richard *et al*. 2006). This may indicate either that a remaining activity of *D*PATJ is sufficient at earlier stages of the development but not in later stages, or alternatively that not *D*PATJ but other

uncharacterized proteins are required for complex stabilization in early stages. In any case, these results give a hint to different mechanisms at <70% p.d. pupal eyes and in later stages that stabilize Crb-complex at its correct position. In contrast, the integrity of Crb-complex is lost at an early stage of pupal development in *crb* and *sdt* PRCs (Nam & Choi, 2003).

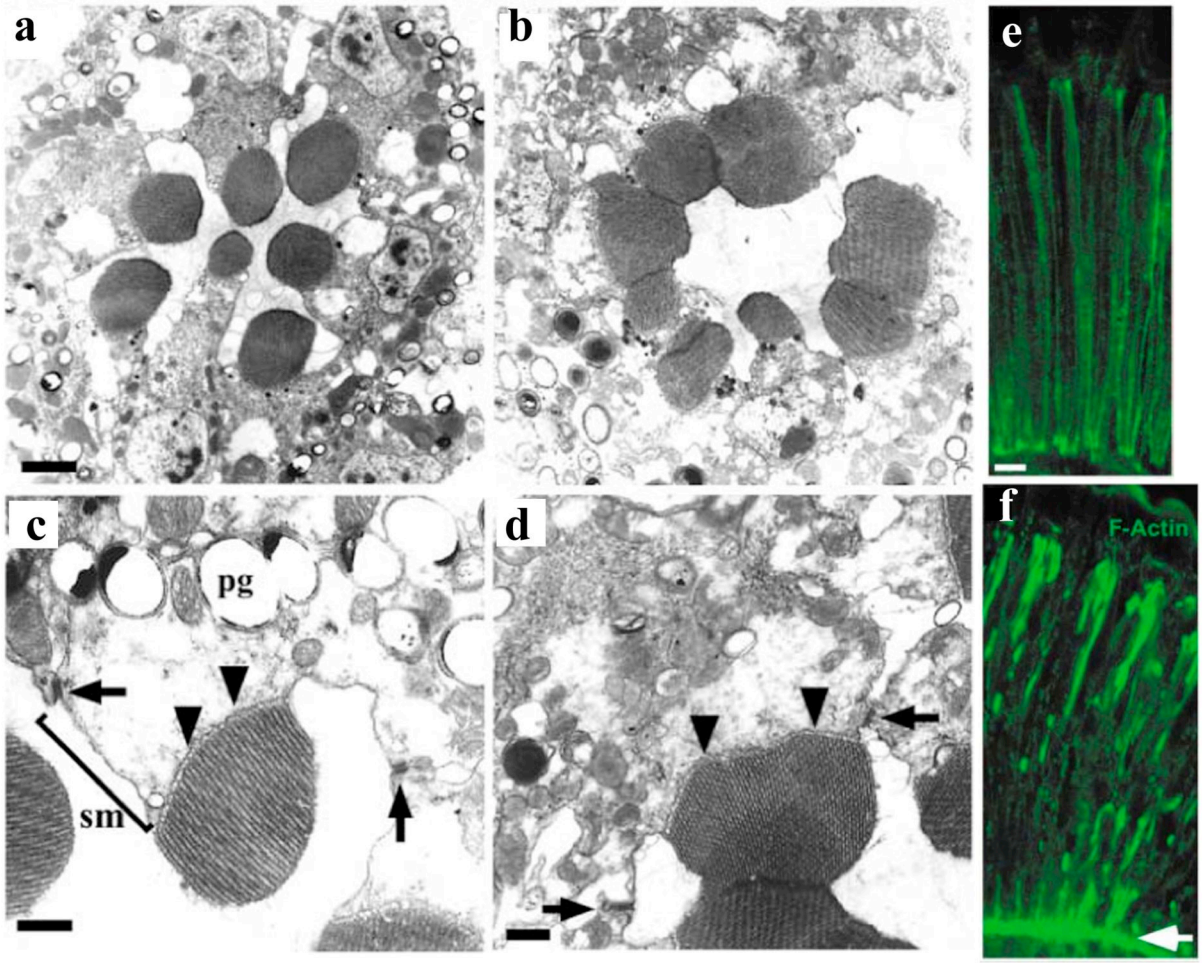


Figure 1.8. Morphological defects caused by mutations of Crb-complex members, on an example of *crb*^{11A22}. Electron micrographs of cross-sections through one ommatidium of wild-type (a, c) and mutant eye (b, d). Morphological defects in the rhabdomere shape and organization can be observed. On the higher resolution it can be seen that the stalk membrane (sm) in mutant PRC (d) is shorter then in wild-type (c). ZAs are marked with black arrows, rhabdomere base – with arrowheads. Confocal micrographs of longitudinal sections through a wild-type (e) and a mutant (f) eye. Rhabdomeres are marked in green by F-actin staining. Mutant rhabdomeres reach only about one third of the length of the retina in comparison to the wild type rhabdomeres reaching the basal lamina. The basal lamina is indicated by a white arrow in (f). Modified from Johnson *et al.*, 2002.

The Crb-complex organizes the pattern of several other proteins in development, and thus may regulate morphogenesis. Though AJs in the mutant PRCs are not affected, in *crb*$^-$ cells Arm accumulates in a distal part of the cells where the rhabdomeres are still present. Upon overexpression Crb recruits components of the AJs to ectopic sites, where it localizes. Overexpression of the Crb FERM-binding domain is sufficient for the delocalization of components of the AJs (Izaddoost *et al.*, 2002). At the same time the Crb-complex is required for the proper localization of members of the Baz-complex in the developing PRCs. *D*Par-6 and *D*aPKC colocalize with *D*PATJ at the apical membrane while about mid-pupal development. *D*aPKC not only colocalizes with *D*PATJ but also overlaps with the AJs. Baz localizes to the AJs at this stage. In *crb*$^-$ PRCs *D*Par-6 and *D*aPKC are strongly reduced or mislocalized. Baz is expanded basolaterally, similar as Arm (Nam and Choi, 2003). Later in development Baz becomes localized to the rhabdomere base (Pinal *et al.*, 2006). It was demonstrated that Baz binds to the phosphatase PTEN (Pinal *et al.*, 2006) and recruits it to AJs in developing PRCs (Pinal *et al.*, 2006). The main substrate for PTEN is phosphatidylinositol-3,4,5-tris-phosphate (PtdIns(3,4,5)P3). Action of PTEN in pupal PRCs leads to the accumulation of both PtdIns(3,4,5)P3 and PtdIns(4,5)P2 apical to the ZA where it activates S/T kinase Akt1. This can regulate directly or indirectly the assembly of F-actin and the formation of the rhabdomere. Mutations in *PTEN* result in a similar rhabdomere shape defect as in *crb* mutants (Pinal *et al.*, 2006). A schematic representation of the interactions is presented in Figure 1.9.

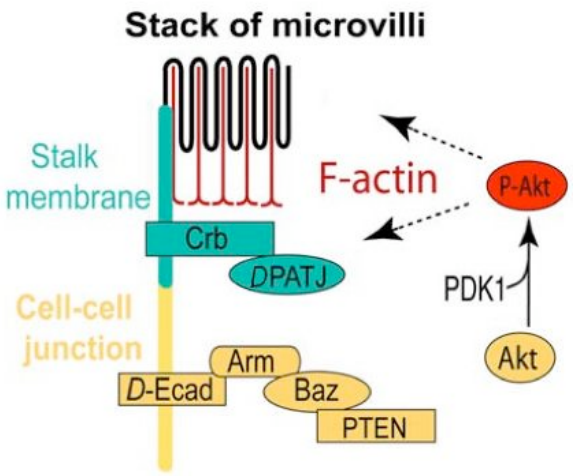


Figure 1.9. Schematic diagram of PTEN function in the *Drosophila* eye. PTEN is localized to the ZA by the Baz-complex where it regulates the PtdIns(3,4,5)P3 level and leads to the spatial activation of Akt. This precise regulation is critical for controlling apical membrane differentiation, including the positioning of Crb/*D*PATJ in the stalk membrane. Adopted from Pinal *et al.*, 2006.

The other protein that can be regulated by PtdIns(4,5)P2 accumulation is Moesin. It has an N-terminal FERM-domain and a C-terminal F-actin binding domain. Its binding sites are activated upon phosphorylation by PtdIns(4,5)P2. It is localized at the rhabdomere base in PRCs, and *moesin* mutations lead to morphological defects in the shape of the rhabdomere (Karagiosis and Ready, 2004). The last protein described to be connected with the Crb-complex in the regulation of PRCs morphology is β_{Heavy}-spectrin. It is localized to the stalk membrane in adult PRCs (e.g. Baumann and Lutz, 2006), and its localization is dependent on Crb-complex. In *crb*$^{-}$ PRCs β_{Heavy}-spectrin is delocalized (Pellikka *et al.,* 2002). Mutations of β_{Heavy}-spectrin result in defects in the shape of rhabdomere (Thomas *et al.*, 1998).

1.4.2.3 Development of the pigment cells.

Development of PRCs is accompanied by the development of various accessory cells (Figure 1.10). First, four cone cells surrounded by two primary pigment cells (1° p.c.) differentiate. They lie distal from the forming PRCs. Cone cells in the late pupal development secrete the cornea outside the eye. Excess interommatidial cells (IOC) are rearranged between 16% and 21% of pupal development, and as the result each cell has contact with at least two ommatidia. The sorting step is followed by the short cell death step when only twelve cells are left around PRCs in every ommatidium. These twelve cells form a typical hexagonal pattern. The six cells at the hexagon sides become secondary pigment cells (2° p.c.), three in the corners become tertiary pigment cells (3° p.c.), and other three differentiate to bristles (Reiter *et al.,* 1996).

It was shown that Crb-overexpression leads to defects in death of extra IOCs. It disrupts the continuous belt of *D*E-Cadherin on the border between 1° p.c. and IOC. As result the localization of the adhesion molecule irreC-rst is as well disrupted at AJs (Grzeschik and Knust, 2005). Mutations of *irreC-rst* lead to similar IOC death phenotype (Reiter *at al.*, 1996). It is not clear if the Crb-complex controls directly apoptosis or acts at the stage of the perception of the intracellular regulatory signals. The function of pigment cells is yet also elusive. It is usually thought that the only function of the pigment cells is to optically separate the individual ommatidia and to restrict the amount of light reaching each ommatidium.

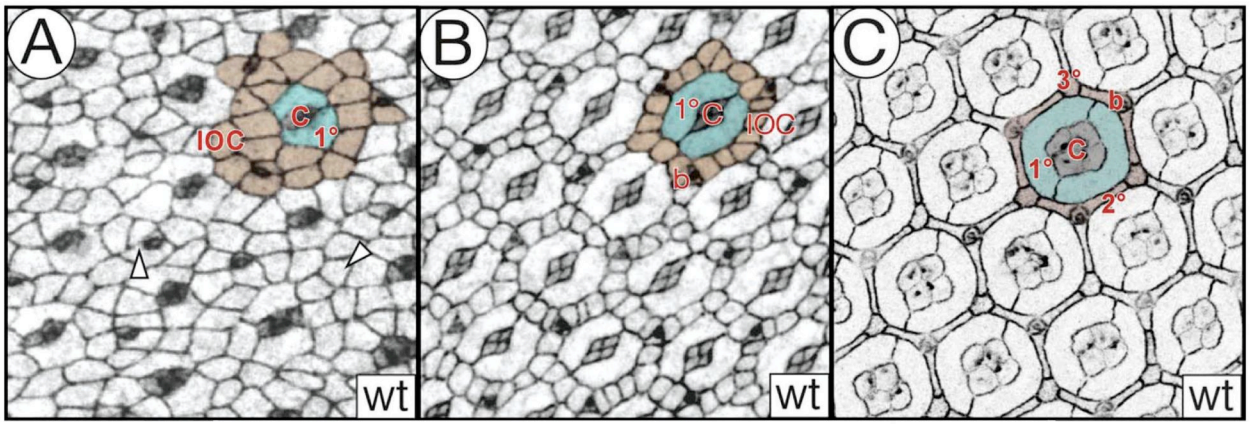


Figure 1.10. Development of the IOCs in wild-type eye imaginal discs. (A) At the onset of cell sorting (16% p.d.), formation of the primary pigment cells (1°; blue) around the cone cell quartet (c) is complete. Sorting of the interommatidial cells (IOC; brown) is in progress. Most ommatidial clusters are still separated by more than one row of IOCs (arrowheads). (B) At 21% p.d. cell sorting has been completed. The IOCs (brown) now form single rings of lattice cells, aligned head-to-tail, separating the ommatidial clusters from each other. b: bristle. (C) By 42% p.d. all surplus cells have been eliminated by apoptosis. The remaining cells are now arranged in a regular hexagonal pattern, in which secondary (2°) and tertiary (3°) pigment cells, as well as bristles (b), can be distinguished (from Grzeschik and Knust, 2005).

1.4.3 Phototransduction mechanisms

R1-R6 photoreceptor cells are sensitive to blue light and contain the membrane-bound photosensitive pigment Rhodopsin1 (Rh1) (Zuker *et al*., 1985). R7 cells contain UV-sensitive opsins, and R8 cells express blue-green rhodopsin (Chou *et al.*, 1996, Papatsenko *et al.*, 1997; Huber *et al.*, 1997). The light receptor molecule rhodopsin is composed of a protein, opsin, covalently linked to a chromophore, 3-hydroxy-11-cis-retinal. Upon absorption of a light photon the chromophore is isomerized from the 11-cis to the all-trans configuration. This change in the conformation of the chromophore leads to a change in the conformation of the protein and to the activation of its catalytic properties. Activated rhodopsin, or metarhodopsin, induces a signal transduction pathway and activates a heterotrimeric G protein of the G_q-family (for review see Zuker *et al.,* 1996). This leads to dissociation of the GTP bound $G_q\alpha$ subunit of this trimeric complex. In flies an effector enzyme activated by $G_q\alpha$ is phospholipase C (PLC)

encoded by the *norpA* gene. PLC catalyzes the breakdown of phospholipid phosphatidyl 4,5-bisphosphate (PIP2) into the two intracellular messengers inositol trisphosphate (IP3) and diacylglycerol (DAG). By an unknown mechanism this results in the activation of the cation-permeable channels Transient receptor potential (TRP) and Transient receptor potential-like (TRPL) and membrane depolarisation, both by releasing Ca^{2+} from IP3-sensitive stores and by activating Ca^{2+} influx through the channels in the plasma membrane (for review see Hardie, 2001). At the same time DAG is thought to modulate a photoreceptor cell-specific protein kinase C (encoded by *inaC*).

For efficient signal transduction, particularly to maximize the temporal resolution, it is important that the response is also rapidly inactivated. In *Drosophila* photoreceptor cells there are two means for the signal deactivation.

- First, activated metarhodopsin binds to Arrestin2 (Arr2) protein. For this binding metarhodopsin must be phosphorylated by rhodopsin kinase. Arr2 competes with $G_q\alpha$ for binding with metarhodopsin (Krupnick *et al*., 1997). Arr2 in turn is phosphorylated by the Calcium/calmodulin-dependent protein kinase II, which is activated upon the action of TRP and TRPL channels. Phosphorylation of Arr2 is necessary for its release from the membranes once rhodopsin has been photoconverted back to its inactive form (Alloway & Dolph, 1999) and dephosphorylated by Rh phosphatase (RdgC). Arr2 functions in the PRCs together with another visual arrestin – Arr1. It is present in 7-fold less amount in PRCs than Arr2, and loss of Arr1 by itself has no reported phenotype. Its only reported phenotype is to prolong the photoresponse by 10-folds in *arr1; arr2* double mutants (Dolph *et al*., 1993).
- Second, Ca^{2+} rapidly inactivates both classes of light-sensitive channels (Reuss *et al.,* 1997).

The phototransduction cascade is schematically presented in Figure 1.11.

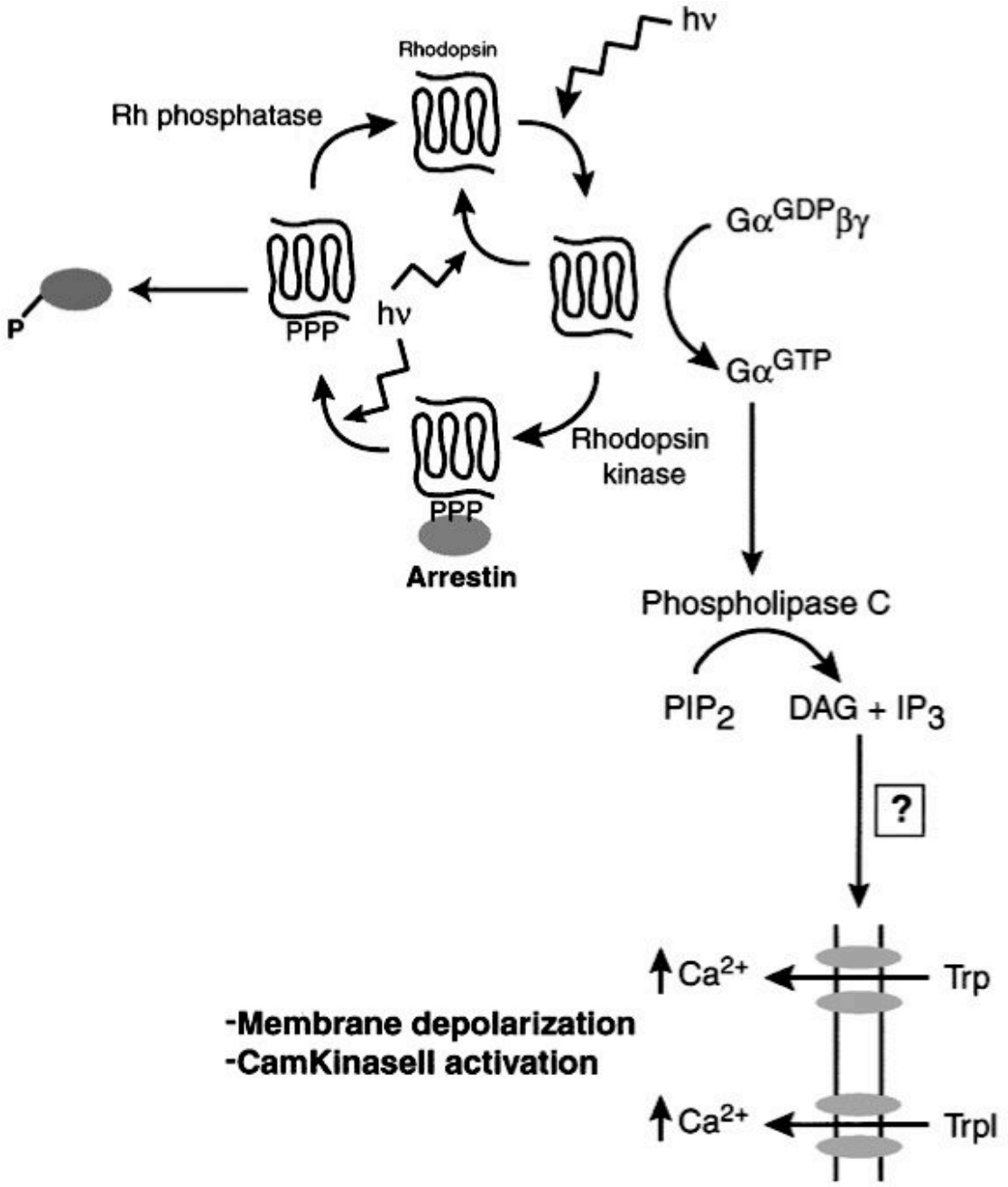


Figure 1.11. Scheme of the phototransduction cascade in *Drosophila*. Gαβγ refers to the heterotrimeric G protein. Abbreviations—PIP2: phosphatidylinositol 4,5 bisphosphate, DAG: diacylglycerol, IP3: inositol 3,4,5 trisphosphate, Rh: rhodopsin. Adopted from Orem and Dolph, 2002.

1.4.4 Light-induced retinal degeneration

Retinal degeneration (RD) is an irreversible loss of photoreceptors over time. Light dependent retinal degeneration (LDRD) is a special type of RD when the loss of PRCs is due to exposure to light. So far, mutations in several members of the phototransduction cascade were shown to result in LDRD. The most widely characterized are *norpA*, *rdgC* and *arr2*^{p261S} mutants. RdgC is directly required for the release of the Rh from Rh-Arr2 complexes. NorpA is important for the activation of Ca^{2+}-channels and influx of Ca^{2+} into the cells. By this it leads to activation of Calcium/calmodulin-dependent protein kinase II and phosphorylation of Arr2. When Arr2 is not phosphorylated, Rh can't release from the Rh-Arr2 complex. The last mentioned mutant, *arr2*^{p261S}, results in an Arr2 protein that can't be phosphorylated, and the outcome is the same as in the previous case (Alloway *et al.,* 2000). So all these mutations result in formation of stable Rh-Arr2 complexes in the PRCs upon light exposure. These complexes are then endocytosed, and apoptosis follows (Alloway *et al.,* 2000, Kiselev *et al.,* 2000). In Kiselev *et al*., 2000 it was shown that Arr2 is required for the degeneration in *rdgC* mutants and that block of endocytosis by loss-of-function mutation in the Shibere Dynamin GTPase (Shi) partially suppresses light-dependent degeneration in *rdg*^{C306}. The same was shown for *norpA* mutants (Alloway *et al.,* 2000). This endocytosis by a presently unknown mechanism triggers apoptosis. Degeneration was significantly suppressed by overexpression of the p35 protein, which is a known inhibitor of apoptosis, in both *rdgC* and *norpA* mutants (Alloway *et al.,* 2000, Davidson and Steller, 1998).

PRCs also degenerate in response to constant light exposure in *crb, sdt* and *DPATJ* mutants (Johnson *et al.,* 2002, Richard *et al*., 2006*,* Nam and Choi, 2006, Berger, 2005). The degeneration in the case of mutations in Crb-complex members is caused probably by the same mechanism as for the mutations described before. The reduction of Rh1 content in these flies by vitamin A depletion prevents degeneration (Berger, 2005). It is yet unclear if in the case of Crb-complex mutations the formation of stable Arr2-Rh complexes is the main cause for the degeneration, or if other intermediate players are important to trigger apoptosis in this context.

The situation with *sdt* mutations is complex. All mutations were discovered as embryonic lethal, and behave as null alleles in embryos. Nevertheless, the mutations can be subdivided into four classes according their phenotypes in the adult eye.

- The first class of mutations results neither in morphological phenotype no in retinal degeneration. The example of mutations of this class is Sdt^{EH}, which is nonsense point mutation in exon 3 (here numbering of exons according to Hong *et al.,* 2001) and truncates Sdt-MAGUK and Sdt-GUK proteins, but should not affect Sdt-B.
- The second class of mutations (e.g. sdt^{K85}) results in both morphological defects and retinal degeneration. sdt^{K85} contains a point mutation leading to the stop-codon in the middle of L27-N (Berger S., 2005).
- The third class of mutations leads only to morphological defects but not to retinal degeneration. As example of the mutation of this class, sdt^{XP96} may serve. It has a point mutation between the Hook and the GUK domain in the exon-intron boundary. This mutation should block the splicing of the intron. The resulting mRNA contains a stop-codon in the very beginning of the unspliced intron (Berger S., 2005, Hong *et al.,* 2001).
- Mutations of the fourth class cause only retinal degeneration and do not affect eye morphology (except for the weak shortening of the stalk membrane). This class is represented by only one allele sdt^{N5}. It contains a point mutation in the C-terminal half of L27-N. This mutation results in a stop-codon (Berger S., 2005, Hong *et al.,* 2001).

Recently it was shown that not only mutant *Drosophila* eyes degenerate. The $white^{-}$ eyes that are otherwise wild type degenerate as well in the response to constant bright light illumination. In this case degeneration could not be prevented by mutations in *arr2*, which indicates that it can be caused by a different mechanisms than in the cases described before. It is not clear what triggers apoptosis in this case. The authors separate light-dependent retinal degeneration and blindness as independent parallel processes. In wild type, constant light leads to blindness through a multi-step process initiated by the formation of stable Rh1/Arr2 complexes and culminating with the loss of the light receptor, Rh1 (Lee and Montell, 2004b).

1.5 Crb-complex and human diseases.

Crb-complex proteins are highly conserved during evolution. The homologs of these proteins were found in several organisms starting with *C.elegans* up to humans. In humans three homologs of *Drosophila* Crb exist: CRB1, CRB2 and CRB3. Their structure is similar to that of *Drosophila* Crb, especially in the intracellular region. All have a short, 37 aa long intracellular domain. This domain carries in all cases the ERLI motif at its C-terminus and has a highly conservative FERM-binding domain (Richard *et al*., 2006b). The interactions within the Crb-complex are as well conserved. The Sdt orthologue Mpp5/Pals1 (membrane protein palmitoylated 5/Protein associated with Lin-seven-1) was found to bind to mouse Crb1 (Roh *et al.*, 2002) and mouse Crb3 (Makarova *et al*., 2003). Mpp5/Pals1 was found to interact with multiple proteins. Its C- and N-terminal L27 (Lin-2/Lin-7) domains bind to the L27 domain of Veli3/Lin-7 (Kamberov *et al.*, 2000) and to that of the multi-PDZ protein Patj (Pals1-associated tight junction protein, also called protein associated with tight junctions) respectively (Roh *et al.*, 2002). These are the homologs of *D*Lin-7 and *D*PATJ, correspondently. Additional members of the complex were identified in vertebrates in comparison to *Drosophila*. Using immunoprecipitation from murine retinal lysates, direct *in vivo* associations have been demonstrated between Mpp5/Pals1 and Mupp1 (a *D*PATJ homologue; multiple PDZ domain protein-1) and between Mpp5/Pals1 and the MAGUK protein Mpp3 but not between Mpp5/Pals1 and Mpp4 or Mpp3 and Mpp4, though *in vitro* data and coimmunoprecipitation experiments suggest that Mpp4 is part of the Crb-complex in murine retinas (Kantardzhieva *et al.*, 2005, Kantardzhieva *et al.*, 2006, Aartsen *et al.*, 2006).

In epithelial cells of mammals, the CRB-complex is localized at the tight junctions. These junctions have a function similar to that one of SJ, but are localized apically to ZA. Though in culture lines of epithelial cells of vertebrates knocking down of CRB-complex proteins delays the formation of TJs after the calcium switch, no general role of the CRB-complex in the maintenance of the apical-basal polarity in these cells was shown (Roh *et al*., 2002).

Similar to the *Drosophila* retina, the CRB-complex is expressed in vertebrate retinas. All three CRB proteins are expressed there. CRB1 expression is restricted to retina and brain. CRB2 and CRB3 are expressed in a wider range of tissue (Makarova *et al.*, 2003, van den Hurk *et al.,* 2005, Lemmers *et al*., 2002). In human mutations in the *Crumbs homologue 1* (*CRB1*) gene cause autosomal recessive retinitis pigmentosa (arRP) and Leber congenital amaurosis (arLCA) (den Hollander *et al*., 1999, den Hollander *et al.*, 2001). arRP is characterized by a progressive degeneration of photoreceptors. arLCA is the earliest and most severe form of all inherited retinal dystrophies, with blindness or severe visual impairment at birth. Up to 4% of arRP patients and 10-15% of arLCA patients carry mutations in the *CRB1* gene. Most mutations are located in the large extracellular domain of the CRB1 protein (for review see Richard *et al.,* 2006b).

No disease-causing variants were found in *CRB2* and *CRB3* in patients with arRP and arLCA so far. Probably, the wider expression pattern of these genes results in a more complex clinical phenotype or even early lethality. The similar wide function in development explains why no mutations of other members of the complex were found so far to be associated with retinal destrophies.

In the vertebrate retina, the photoreceptor cell layer is embedded in the retinal pigment epithelium (RPE) (Figure 1.12A). The apical side of each photoreceptor is characterized by a photosensitive outer segment supported by an inner segment, located above the AJs (Figure 1.12B). The CRB-complex localizes to the outer limiting membrane (OLM) at the subapical region (SAR), apically to the AJs that connect photoreceptor cells with each other and with Müller glia cells (Kantardzhieva *et al*., 2005, Figure 1.12B-C). Immuno-electron microscopy of murine retinas revealed strong Crb1 immunoreactivity at the SAR in Müller glia cells but hardly in photoreceptors, whereas Crb2, Crb3, Patj, Pals1 and Mupp1 are present in both cell types (van Rossum *et al.,* 2006). There are two *Crb1*-mutant mouse models (Mehalow *et al*., 2003, van de Pavert *et al*., 2004). In these mice focal regions of retinal disorganization consisting of photoreceptor loss and retinal folds can be detected. Adult *Crb1*$^{-/-}$ mice exposed to continuous white light (3000 lux) for 3 days showed significantly more foci of retinal disorganization (van de Pavert *et al*., 2004).

In zebrafish, the Mpp5/Pals1 orthologue *nagie oko* (*nok*) is essential for a proper adhesion of photoreceptors (Wei & Malicki, 2002). In addition, the zebrafish CRB2 orthologue Crb2a is involved in the *oko meduzy* (*ome*) phenotype that resembles the disrupted neuronal patterning of *nagie oko* (Omori & Malicki, 2006).

The similarity and high conservation of the whole complex between fly and human makes fly to be a powerful model to study the basic cell biological processes underlying Crb-complex function, and might lead to new therapeutic approaches for patients with retinal dystrophies caused by mutations in the Crb-complex.

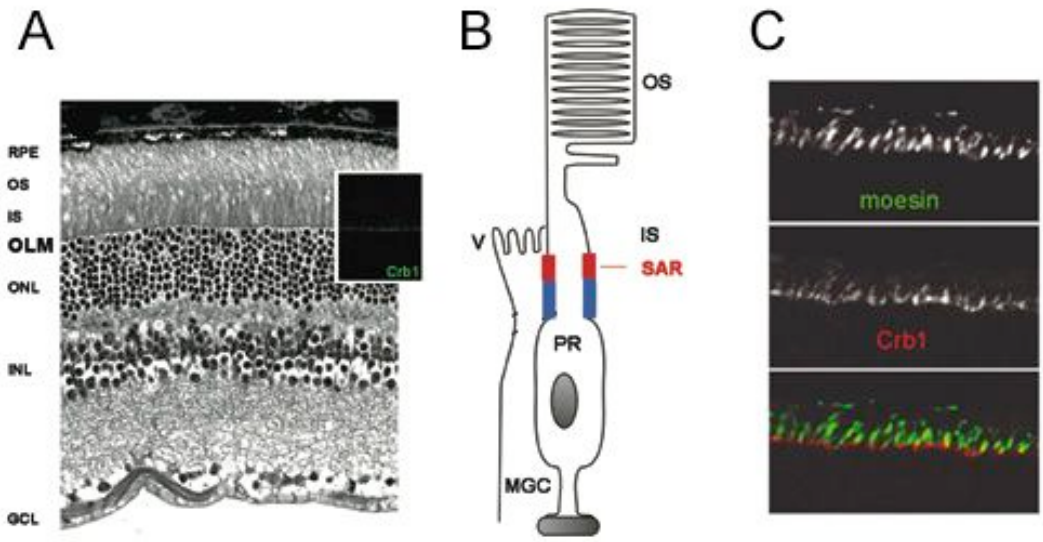


Figure 1.12. Structure of the vertebrate retina and CRB1 expression. Light microscopic picture of retinal layers from a wild-type mouse is shown in A. Photoreceptor outer and inner segments (OS-IS) localize below the retinal pigment epithelium (RPE). Basally to the outer limiting membrane (OLM), photoreceptor cell bodies make up the outer nuclear layer (ONL). Phototransduction is integrated through bipolar, amacrine and horizontal cells of the inner nuclear layer (INL) and further transmitted to the brain by the ganglion cell layer (GCL). The confocal image in the inserted frame displays Crb1 protein localization at the OLM. In B the schematic illustration of a photoreceptor cell (PR) and one Mueller glia cell (MLG) is demonstrated. Adherens junctions (blue) and the subapical region (SAR, red), where the CRB/Crb complex is localized, are shown. In C a confocal image of the retina is shown. CRB1 (red) is localized to the OLM. As a marker of the inner segments moesin was used. A and B modified from Richard *et al.*, 2006b, C is modified from van Rossum *et al.*, 2006.

1.6 Aim of the work.

In the previous works it was shown that Sdt is regulating the morphogenesis of the PRCs and prevents retinal degeneration. These two Sdt functions are independent of each other. The mechanisms of its functions are yet elusive. Two important features of Sdt may explain its multiple role in *Drosophila* eye – the existence of different splice variants and the wide potential to bind other proteins via its multiple protein-protein binding domains.

One aim of the present work is to identify the eye-specific splice-variants of *sdt*. The identification of the different splice-variants of *sdt* in the eye would give a strong hint that they may be required for different processes, especially if they will differ in their domain structure.

The second aim of this work is to characterize the relevance of different domains of the Sdt protein for functions and proper localization of the whole Crb-complex. For this, the functions of different domains will be analyzed *in vivo* by expression in flies of different forms of Sdt protein: full-length protein, deletion forms that lack one or several domains, and single domain forms.

For the better understanding of the phenotypes observed upon expression of Sdt-transgenes, the phenotypes of different *sdt* mutants will be further characterized. These mutants are well characterized at the adult stage (Berger, 2005), but not much is known about them at the pupal stage or on the level of protein expression.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals

All chemicals were obtained in pro analysis quality from the following companies: Acros,Geel, Belgium; Baker, Deventer, Netherland; Biomol, Hamburg; Bio-Rad, München; Difco, Detroit, USA; Fluka, Buchs, Switzerland; Gibco/BRL Life Technologies, Karlsruhe; Merck, Darmstadt; Roth, Karlsruhe; Serva, Heidelberg; Sigma-Aldrich, Steinheim. All solutions were made with destilled H_2O and autoclaved prior to use. Enzymes required for molecular work were purchased from: Roche Diagnostics, Mannheim; MBI Fermentas, St. Leon- Rot; Promega, Madison, USA

2.1.2 General laboratory equipment

Electroporation: Gene Pulser II and Puls Controller Plus (Bio Rad Munich); UV-spectrophotometer: Gene Quant II (Pharmacia Biotech, Cambridge, UK); Sonificator: Labsonic U (Braun Biotech, Melsungen); SDS PAGE & Western Blotting: Miniprotean 3 (Bio Rad, Munich); Centrifuge (Heraus biofuge fresco and pico); PCR machine (MS Research MiniCycler); Micropipets puller: Sutter P-97 (Sutter Instrument Co., USA); X ray film development: film: Fuji Super RX, Fuji, Tokyo, Japan, developer: Tenetal Roentogen, Tenetal, Norderstedt, fixation: Tenetal Roentogen Superfix, Tenetal, Norderstadt; Confocal microscope: Leica TCS NT, Leica, Heidelberg, and Zeiss 510Meta, Zeiss Jena; Light microscopy: Zeiss-Axiophot2, Zeiss Oberkochen; Microtome: REICHERT OM 2; Transmission electron microscope: ZEISS EM 109 (Zeiss); Apple Macintosh computers were used. Pictures were handled with Adobe Photoshop CS, Text and calculations were done with Microsoft Office 2003 Students Edition.

2.1.3 Buffers, solutions and media.

Ampicillin	10 mg/ml in H_2O
Blocking solution (Western)	5% dry milk in 1xTBST
Blocking solution (Northern)	2% Blocking reagent (Roche) in washing buffer
Coomassie staining solution	0.25% Coomassie Brilliant Blue, R250 50% methanol 10% ice acetic acid
DEPC-H_2O	Add 0.1% DEPC in H_2O, mix thoroughly, incubate for 12 hours and autoclave. This eliminates possible RNases
GST-binding buffer	500 ul 50% Glutathion Sepharose in 25 ml 15 mM DTT, 0.1% Tween-20
GST-elution buffer	10 mM Glutathion (reduced) 50 mM Tris, pH 8.0 250 mM KCl 2 mM DTT
GST-suspension buffer	500 ul 0.5 M PMSF 2 ug/ml leupeptin 2 ug/ml pepstatin A 2 ug/ml chymostatin 2 ug/ml aprotinin 1xPBS

GST-washing-buffer	100 mM NaCl 10 mM Tris, pH 8.0 1 mM EDTA500 500 ul 0.5 M PMSF 2 ug/ml leupeptin 2 ug/ml pepstatin A 2 ug/ml chymostatin 2 ug/ml aprotinin
Prehybridisation buffer (Northern)	5xSSC 50% formamid 0.1% sodiumlaurylsarcosin 0.02% SDS
Hybridisation buffer (Northern)	prehybridisation buffer with 2% blocking reagent (Roche)
Leadcytrate-solution	1.33 g leadnitrate 1.76 g sodiumcitrate (x 2 H_2O) Dissolve in 30 ml of H_2O with 7 ml 1M NaOH, pH 12.0 Adjust the total volume to 50 ml with H_2O
6xLoading buffer (DNA)	0.25% bromphenolblue 0.25% xylencyanol
2xLoading buffer (proteins)	4% SDS 200 mM DTT 100 mM Tris, pH 6.8 0.2% bromphenolblue 20% glycerol

LB-medium	1 g/l Bacto Trypton 5 g/l Bacto Yeast Extract 10 g/l NaCl (for plates: 15 g/l agar)
Lysis buffer (for protein extracts) (after U. Thomas)	50 mM Tris, pH 8.0 150 mM NaCl 0.5% Triton X-100 1 mM $MgCl_2$ (before use, add protein inhibitors, each in concentration of 2 ug/ml)
1xMOPS, pH 7.0	20 mM MOPS 5 mM sodium-acetate 1 mM EDTA
Mowiol	Mix 2.4 g Mowiol with 6 ml of glycerol and 6 ml of H_2O. Incubate for 2 hours at room temperature, add 12 ml 200 mM Tris (pH 8.5) and incubate at 50°C for 3 hours. Centrifuge 10 min at 4000 rpm and aliquote.
4% paraformaldehyde	Add 50 ml H_2O and 1 ml 1M NaOH to 4g paraformaldehyde. Incubate at 65°C till paraformaldehyde is fully dissolved. Add 10 ml PBS. Adjust pH to 7.4. Fill up to 100 ml volume with H_2O. Filter and aliquote. Store at –20°C.

PBS (pH 7.2)	3.97 g/l Na_2HPO_4 1.12 g/l KH_2PO_4 4 g/l NaCl
PBT	0.1% Tween-20 in PBS
PBX	0.1% Triton X-100 in PBS
0.1 M phosphate buffer, pH 7.2	36 ml 0.2 M Na_2HPO_4 14 ml 0.2 M NaH_2PO_4 50 ml H_2O
10x Ponceau S	2 g Ponceau S 30 g trichloraceticacid 30 g sulfosalicylacid
Running buffer for SDS-PAGE	25 mM Tris, pH 8.3 250 mM Glycin 0.1% SDS
20xSSC, pH 7.0	3 M NaCl 0.3 M Na-citrate
TBS	50 mM Tris, pH 7.5 150 mM NaCl
TBST	0.2% Tween-20 in TBS

Transfer buffer (Western blot)	5.82 g/l Tris
	2.93 g/l Glycin
	0.0375 g/l SDS
	20% methanol
SDS-polyacrylamid-minigel	2.5 ml 30% acrylamid/BIS (29:1)
(10% separating gel)	2.8 ml 1M Tris, pH 8.8
	38 ul 20% SDS
	2.1 ml H_2O
	30 ul 10% APS in H_2O
	8 ul TEMED
SDS-polyacrylamid-minigel	310 ul 30% acrylamid/BIS (29:1)
(stacking gel)	235 ul 1M Tris, pH 6.8
	10 ul 20% SDS
	1.3 ml H_2O
	10 ul 10% APS in H_2O
	5 ul TEMED
8% Stefaninis fixative	8% formaldehyde
	75 mM PIPES
	15% picric acid
TAE	40 mM Tris-acetate
	1 mM EDTA
Washing buffer (Northern)	100 mM maleic acid
PH 7.5	150 mM NaCl
	0.3% Tween-20

2.1.4 Antibodies

2.1.4.1 Primary antibodies

Antibodies	Description	Dilution	Reference
α-Sdt-PDZ	Polyclonal rabbit-antibodies	Eyes: 1:500-1:1000 Western: 1:15000	S. Berger
α-Sdt-N	Polyclonal rat-antibodies	Eyes: 1:200	This work (Eurogentec)
α-Sdt-PDZ-N	Polyclonal rat-antibodies	Eyes: 1:200-1:500	A. Bachmann, not published
α-*D*Patj	Polyclonal rabbit-antibodies	Eyes: 1:500	Richard *et al.*, 2006
α-Crb2.8	Polyclonal rat-antibodies	Eyes: 1:100 Embryos: 1:50	E. Knust, not published
α-*D*Lin-7	Polyclonal rabbit-antibodies	Eyes: 1:500	Bachmann *et al.*, 2004
α-Arm	Monoclonal mouse-antibodies	Eyes: 1:50	Riggleman *et al.*, 1990
α-*D*E-Cad	Polyclonal rat-antibodies	Eyes: 1:50	DSHB
α-FLAG	Monoclonal mouse-antibodies (M2 and M5)	Eyes: 1:200 Western: 1:1000	Sigma
α-myc	Polyclonal rabbit-antibodies	Eyes: 1:500	Upstate Biotechnology
α-GFP	Polyclonal rabbit-antibodies	Eyes: 1:500	Invitrogen
α-GFP	Monoclonal mouse-antibodies	Eyes: 1:25	Invitrogen
α-β_H-spectrin	Polyclonal rabbit-antibodies	Eyes: 1:2000	G. Thomas
α-Par-6	Polyclonal guinea pig	Eyes: 1:1000	Wodarz, not published

2.1.4.2 Secondary antibodies

Antibodies	Description	Dilution	Reference
α-mouse-Cy3	Polyclonal goat-antibodes	1:200	Dianova
α-rabbit-Cy3	Polyclonal donkey-antibodes	1:200	Dianova
α-rat-Cy3	Polyclonal donkey-antibodes	1:200	Dianova
α-mouse-Cy2	Polyclonal goat-antibodes	1:200	Dianova
α-rabbit-Cy2	Polyclonal donkey-antibodes	1:200	Dianova
α-rat-Cy2	Polyclonal donkey-antibodes	1:200	Dianova
α-mouse-Cy5	Polyclonal goat-antibodes	1:200	Dianova
α-rabbit-Cy5	Polyclonal goat-antibodes	1:200	Dianova
α-rat-Cy5	Polyclonal donkey-antibodes	1:200	Dianova
α-rabbit-HRP	Polyclonal goat-antibodies	1:1000	Jackson Immuno Research Laboratories
α-mouse-HRP	Polyclonal goat-antibodies	1:1000	Jackson Immuno Research Laboratories
α-DIG-HRP	Polyclonal rabbit-antibodies	1:10000	Jackson Immuno Research Laboratories

Alexa Fluor® 660 phalloidin or Alexa Fluor® 488 phalloidin (Molecular probes) were used for staining of the rhabdomeres.

2.1.5 Fly stocks

Oregon R and w^{1118} were used as wild type. For injections and generation of transgenic stocks w^{1118} was used.

2.1.5.1 Balancer chromosomes

Stock	Description	Reference
FM7	1st chromosome balancer	Lindsley and Zimm, 1992
CyO	2d chromosome balancer	Lindsley and Zimm, 1992
Tf	2d chromosome balancer	Lindsley and Zimm, 1992
TM3	3d chromosome balancer	Lindsley and Zimm, 1992
TM6	3d chromosome balancer	Lindsley and Zimm, 1992
SM6-TM6	Compound balancer between 2d and 3d chromosomes	Thomas Klein

2.1.5.2 *Sdt* mutant stocks

Three different *sdt* alleles were used in this work. They are all molecularly characterized and belong to different classes according to the phenotype in the eye. First, sdt^{K85} results in both morphological defects and retinal degeneration. It contains a point mutation leading to the stop-codon in the middle of L27-N (Berger S., 2005). Second, sdt^{XP96} leads only to morphological defects but not to retinal degeneration. It carries a point mutation between the Hook and GUK domain at the exon-intron boundary. This mutation should block the splicing of the intron. The resulting mRNA contains a stop-codon in the very beginning of the unspliced intron (Berger S., 2005, Hong *et al.,* 2001). And the third allele was sdt^{N5}. It cause only retinal degeneration and doesn't affect eye morphology (except for the weak shortening of the stalk membrane). It contains a point mutation in the 3'-part of L27-N. This mutation results in the stop-codon (Berger S., 2005, Hong *et al.,* 2001).

Stock	Description	Reference
sdt^{K85} / FM7	Initial *sdt*^{K85} stock	T. Hummel, Schneider, 1996
sdt^{N5} / FM7	Initial *sdt*^{N5} stock	C. Nüsslein-Vollhardt M. Seeger
sdt^{XP96} / FM7	Initial *sdt*^{XP96} stock	Wieschaus *et al.*, 1984
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7	Small eye clones production, crossing in the transgenes	S. Berger, unpublished
y w sdt^{N5} P{ry[+t7.2]=neoFRT}19A / FM7		
y w sdt^{XP96} P{ry[+t7.2]=neoFRT}19A / FM7		
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO	Large eye clones production, crossing in the transgenes	S. Berger, unpublished
y w sdt^{N5} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO		
y w sdt^{XP96} P{ry[+t7.2]=neoFRT}19A /FM7; Tp(1,2)sn / CyO		

2.1.5.3 Driver lines

GAL4-transgenes are introduced on the second chromosome in all driver-lines used.

Stock	Description	Reference
w; *GMR*GAL4	Drives expression in all cells of the eye. Expression starts in 3d instar larvae behind the morphogenetic furrow	Bloomington
*w; Rh1*GAL4	Drives expression in R1-R6 starting from late pupal development (about 70% p.d.)	C. Desplan
*w; 54*GAL4	Drives expression in all pigment cells starting from their specification	R. Cagan
*w; ey*GAL4	Drives expression from embryo stage till mid-pupal development	Bloomington

2.1.5.4 Sdt transgenic stocks

Stock	Description	Reference
w; CYO/Gla; UAS-MAGUK	Untagged Sdt-MAGUK isoform, 3^{d} chr.	Bachmann *et. al.,* 2001
w; UAS-MAGUK-15	FLAG-tagged Sdt-MAGUK, 3^{d} chr.	This work
w; UAS-Sdt-B	FLAG-tagged Sdt-B, 3^{d} chr.	This work
w; UAS-Sdt-ΔN	FLAG-tagged, 3^{d} chr.	This work
w; UAS-Sdt-ΔL27-C	FLAG-tagged, 3^{d} chr.	This work
w; UAS-Sdt-ΔPDZ-C	FLAG-tagged, 3^{d} chr.	This work
w; UAS-Sdt-ΔSH3-Hook	FLAG-tagged, 3^{d} chr.	This work
w; UAS-Sdt-ΔSH3-GUK	FLAG- and myc-tagged, 2^{d} chr.	This work
w; UAS-Sdt-ΔHook-GUK	FLAG- and myc-tagged, 3^{d} chr.	This work
w; UAS-Sdt-1-566	FLAG- and myc-tagged, 3^{d} chr	Ö. Kempkens
w; UAS-Sdt-1-L27-N	FLAG- and myc-tagged, 1st chr.	Ö. Kempkens
w; UAS-Sdt-1-L27-C	FLAG- and myc-tagged, 3^{d} chr.	Ö. Kempkens
w; UAS-Sdt-PDZ-C	HA-tagged, 3^{d} chr.	Ö. Kempkens
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7*;* UAS-MAGUK-15	Stock for the rescue experiments	This work
y w sdt^{XP96} P{ry[+t7.2]=neoFRT}19A / FM7*;* UAS-MAGUK-15	Stock for the rescue experiments	This work
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7*;* UAS-Sdt-B	Stock for the rescue experiments	This work

Stock	Description	Reference
y w sdt^{XP96} P{ry[+t7.2]=neoFRT}19A / FM7; UAS-Sdt-B	Stock for the rescue experiments	This work
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO; UAS-Sdt-ΔN	Stock for the rescue experiments	This work
y w sdt^{XP96} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO; UAS-Sdt-ΔN	Stock for the rescue experiments	This work
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO; UAS-Sdt-ΔL27-C	Stock for the rescue experiments	This work
y w sdt^{XP96} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO; UAS-Sdt-ΔL27-C	Stock for the rescue experiments	This work
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO; UAS-Sdt-ΔPDZ-C	Stock for the rescue experiments	This work
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO; UAS-Sdt-ΔSH3-Hook	Stock for the rescue experiments	This work
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO; UAS-Sdt-ΔHook-GUK	Stock for the rescue experiments	This work
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO; UAS-Sdt-1-L27-C	Stock for the rescue experiments	This work
y w sdt^{K85} P{ry[+t7.2]=neoFRT}19A / FM7; Tp(1,2)sn / CyO; UAS-Sdt-PDZ-C	Stock for the rescue experiments	This work

Stock	Description	Reference
w; *Rh*GAL4; UAS-MAGUK-15	Stocks for Sdt-overexpression using *Rh1*GAL4 in a wild type background	This work
w; *Rh*GAL4; UAS-Sdt-B		This work
w; *Rh*GAL4; UAS-Sdt-ΔN		This work
w; *Rh*GAL4; UAS-Sdt-ΔL27-C		This work
w; *Rh*GAL4; UAS-Sdt-ΔPDZ-C		This work
w; *Rh*GAL4; UAS-Sdt-ΔSH3-Hook		This work
w; *Rh*GAL4; UAS-Sdt-ΔHook-GUK		This work
w; *Rh*GAL4; UAS-Sdt-PDZ-C		This work
w; *Rh*GAL4; UAS-Sdt-1-L27-C		This work

2.1.5.5 Activator lines for making the clones

Stock	Description	Reference
GMRhid cl P{ry[+t7.2]=neoFRT}19A; UAS-FLP *ey*GAL4	Activator line for large eye clones	Newsome et. al., 1984
y w P{ry[+t7.2]=neoFRT}19A; P{ry[+t7.2]=eyFLP.N}5	Activator line for small eye clones	B. Dickson
*tub*GAL80 *hs*FLP FRT19A; *act*GAL4 UAS-GFP:CD8	MARCM	Bloomington Stock Center, stock 5138 Sweeney *et al.* 2002

2.1.5.6 Other stocks

w^+ *GMR*IR (13D), received from R.W Carthew (Lee & Carthew, 2003), was used for the retinal degeneration assay. This line carries a RNAi construct against the product of the *white* gene. The transgene is located on the first chromosome.

w; UAS-src-GFP was used as a control UAS-transgene. The transgene encodes Src fused to GFP. The transgene is integrated into the second chromosome.

2.2 Methods

2.2.1 Molecular methods

If not otherwise stated, standard methods were used for molecular cloning, transformation of bacteria, heterologous expression in bacteria and purification of these proteins like described in Molecular Cloning, A Laboratory manual, 2nd edition; Sambrook, Fritsch and Maniatis; Cold Spring Harbour Laboratory Press. Whenever kit-systems were used, protocols were followed according to the manufacturer instruction.

2.2.1.1 Cloning of different Sdt-constructs

Sdt-MAGUK cDNA cloned into pBluescript KS+/SK+ vector (Stratagene, Heidelberg, Amp^R) was used as a template for generation of all constructs except Sdt-B. For the generation of the constructs containing the deletions parts of the cDNA from 5'- and 3'-sides to the deletion were amplified, cloned separately into the pCRII-TOPO vector, and, afterwards, brought together into the pUAST-vectors using restriction/ligation technique. The schematic representation of the cloning procedure is demonstrated on Figure 2.1. Restriction sites were introduced into all of the used primers used for cloning. All these and further primers were checked for the formation of hairpins, dimers and crossdimers with other primers using NetPrimer program (http://www.premierbiosoft.com/netprimer).

RE05272 and RE14379 (FlyBase, http://flybase.bio.indiana.edu) were used for the cloning of Sdt-B construct. RE05272 contains nearly full sequence that encodes Sdt-B isoform. RE14379 contains the most 5'-region of this seuence. *Not*I and *Kpn*I enzymes were used to cut the 5'-part encoding Sdt-B isoform from RE14379 and to clone it into RE05272. The obtained whole Sdt-B sequence was amplified using the 5'-SdtNotI and 3'-M5Xho3 primer. Then it was cloned into pUAST-FLAG vector using *Not*I and *Xho*I enzymes.

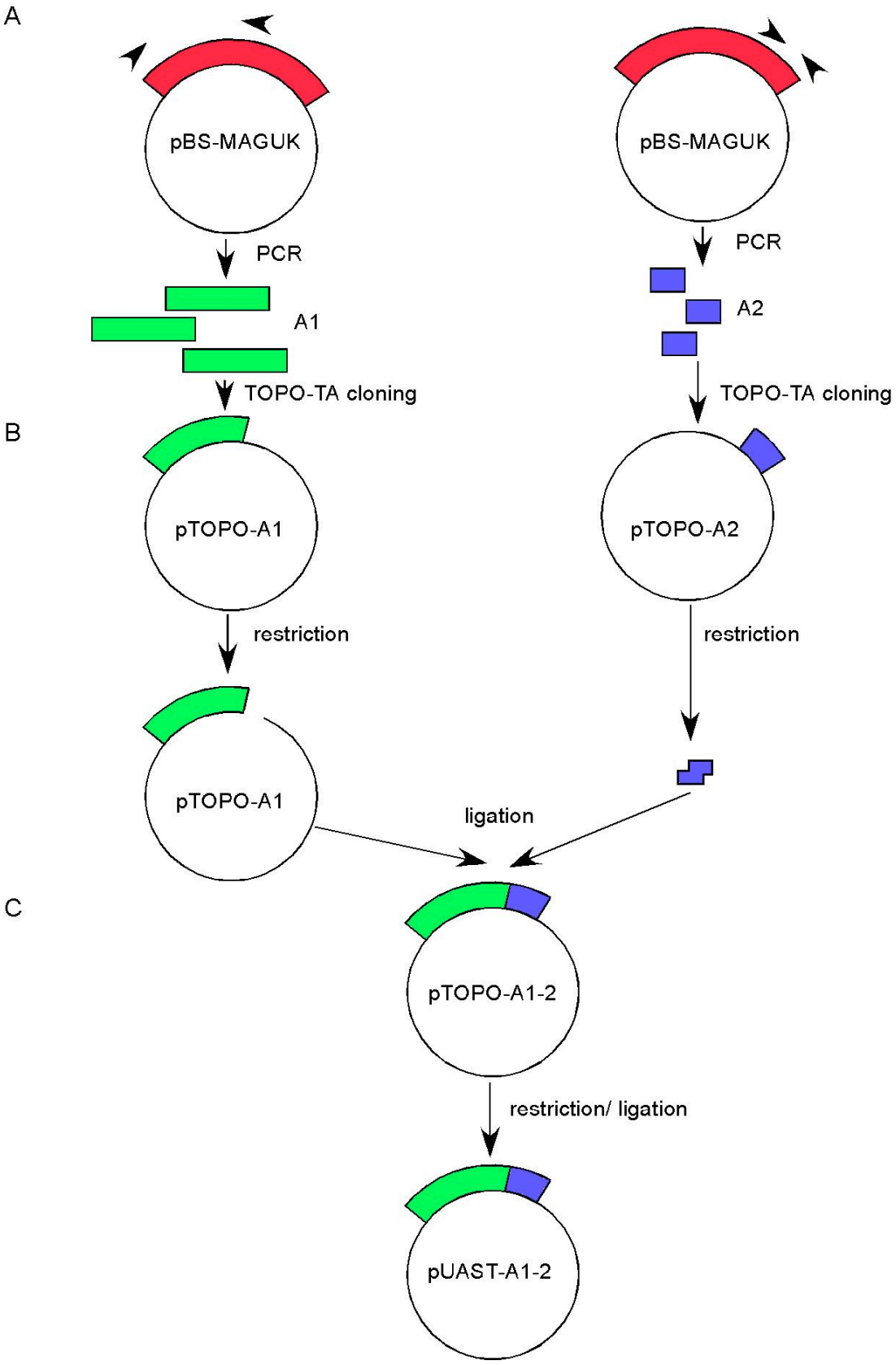

A
pBS-MAGUK
PCR
A1
TOPO-TA cloning
pBS-MAGUK
PCR
A2
TOPO-TA cloning
B
pTOPO-A1
pTOPO-A2
restriction
restriction
pTOPO-A1
ligation
C
pTOPO-A1-2
restriction/ ligation
pUAST-A1-2

Figure 2.1. The schematic representation of the cloning procedure that was used for cloning of the most constructs containing deletions.

A – Using specific primer pairs the parts that lay in 5'-direction (A1, green) and 3'-direction (A2, blue) of the deletion were amplified and cloned into pCRII-TOPO (pTOPO) vector using the TOPO-TA cloning system. Arrowheads mark the positions of different primers. Thus, two independent vectors were obtained: pCRII-TOPO containing 5'-part of the construct (pTOPO-A1) and pCRII-TOPO containing 3'-part of the construct (pTOPO-A2). Sdt-MAGUK cDNA (red) cloned into pBluescript KS+/SK+ vector (pBS-MAGUK) was used as a template for amplification.

B – 3'-part of the construct of interest (A2) was cut out of pTOPO-A2 using restrictases recognising the restriction sites introduced into the primers used for amplification. A2 was ligated into pTOPO-A1 linearized using the same restrictases. The insert in the resulting vector (pTOPO-A1-2) corresponds to the construct of interest.

C – The insert (A1-2) was cut out of pTOPO-A1-2 using restrictases recognising the restriction sites introduced into the primers used for amplification (*Not*I and *Xho*I in most cases) and was ligated into pUAST linearized using the same restrictases. The resulting construct was sequenced and used for trasgenesis.

2.2.1.2 Polymerase chain reaction (PCR)

For molecular cloning, DNA fragments were amplified by PCR according to Mullis and Faloona, 1987. Annealing temperature and amplification time of a standard protocol indicated below were adjusted according to the needs of the particular experiment. The reaction volume was set to 50ul in all reactions. A standard PCR protocol was used with 31 cycles of denaturation, annealing and elongation. Pfu-polymerase was used to minimise the number of mistakes by the polymerization.

Standard PCR mixure of the final volume 50 ul:

DNA (10-50 ng)	1 ul
dNTPs (10 mM)	2 ul
5'-primer (10 mM)	1 ul
3'-primer (10 mM)	1 ul
10xPCR buffer	5 ul
H_2O	39 ul
1 U Pfu-polymerase	1 ul

The standard program for PCR was the following:

Step	Duration	Temperature	Meaning
1	2 min	94°C	Initial denaturation of DNA
2	30 sec	94°C	Denaturation of DNA
3	1 min	Primer pair specific: 3-6°C under the melting temperature of the primer	Annealing of the primers to DNA
4	1 min for each 1 kb of the PCR product	72°C	Synthesis of DNA by Pfu-polymerase (elongation)
5	7 min	72°C	Final synthesis of the DNA
6	∞	4°C	End of reaction

Steps 2 to 4 were cycled and repeated for 30 times.

The following primers were used for the generation of the constructs:

Primer name	Primer sequence (5' -> 3')	Corresponding constructs and temperature (T_m)
5'-SdtNotI	GAGGCGGCCGCCGATTGTC	All constructs except Sdt-ΔN (66°C)
5'-SdtCL27XhoI	GACGTTCTCGAGTCTCTGGTCC	Sdt-ΔN (70°C)
5'-SdtCL27SmaI	TCCCCGGGTGAGTGCGTGCTT	Sdt-ΔL27-C (70°C)
5'-SdtPDZSalI	CAGTCGACTGGCCGGGTTGGGT	Sdt-ΔPDZ-C (74°C)
5'-SdtGUKSalI	GGATGAAACGTCGACGGAGGAG	Sdt-ΔSH3-Hook and Sdt-ΔHook (70°C)
3'-M5Xho3	GTTGATGGATCCTCGAGAAGATGG (A. Bachmann)	All constructs except Sdt-ΔSH3-GUK and Sdt-ΔHook-GUK (72°C)
3'-o.L27SmaI	AGACGCGGGAACGTCGTCGCTGCA (Ö. Kempkens)	Sdt-ΔL27-C (82°C)

Primer name	Primer sequence (5' -> 3')	Corresponding constructs and temperature (T_m)
3'-L27KT7	GCCACCGGACTGCTGAGTGTCGATCC (Ö. Kempkens)	Sdt-ΔPDZ-C (86°C)
3'-SH3SalINr2	GGGAGTCGACAGGAGCGTGGCTCCTTTGC	Sdt-ΔHook (96°C)
3'-SdtSH3XhoI2	CACTCGAGTGGCTCCTTTGCT	Sdt-ΔHook-GUK (66°C)
3'-SdtPDZXhoI2	CACCCACTCGAGCCAGTTGA	Sdt-ΔSH3-GUK and Sdt-ΔSH3-Hook (64°C)

2.2.1.3 mRNA extractions, RT-PCR and 5'-RACE.

polyA$^+$-RNA was isolated from heads of adult *white*$^-$ flies or from the differentially staged *white*$^-$ embryos using the MACS Kit (Miltenyi Biotec) following the protocol proposed by the providing company. RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen) with different Sdt-specific primer pairs. Annealing temperature and amplification time of a standard protocol indicated below were adjusted according to the needs of the particular experiment.

A typical mixture for the RT-PCR with the final volume of 50 ul was the following:

polyA$^+$-RNA (100 ng/ul)	7 ul
H_2O	23.5 ul
5xRT-Buffer	10 ul
dNTP Mix (10 mM)	2 ul
Gene-specific 5'-primer (10 mM)	3 ul
Gene-specific 3'-primer (10 mM)	3 ul
RNase inhibitor	1 ul
Omniscript reverse transcriptase (4 U/ul)	0.5 ul

RT-PCR was performed according to the following program.

Step	Duration	Temperature	Meaning
1	45 min	50°C	Reverse transcription
2	15 min	95°C	Inactivation of reverse transcriptase Activation of Hot Star Taq polymerase Initial denaturation of DNA
3	30 sec	94°C	Denaturation of DNA
4	1 min	Primer pair specific: 3-6°C under the melting temperature of the primer	Annealing of the primers to DNA
5	1 min for each 1 kb of the PCR product	72°C	Synthesis of DNA by Pfu-polymerase (elongation)
6	7 min	72°C	Final synthesis of the DNA
7	∞	4°C	End of reaction

Steps 3 to 5 were cycled and repeated for 30 times.

The following primers were used for the RT-PCRs:

Primer name	Primer sequence (5' ->3')	Temperature (T_m)
5'-Ex6-44654	GCGAACTGATAGCGGCCCTTACCC	68°C
5'-Sdt-Ex1-1	GCAACACTATCGCACGCACTC	66°C
5'-Sdt-A5-1	GAGATCGAACGGATTACAGATATG	68°C
5'-Sdt-i1	GCTCACCTGTATCATCCGCTG	66°C
5'-Sdt-i2	CCAAGTGCGTGACAAAGAACC	64°C
5'-Sdt-e1	GGCAGCGGCACAGACAACG	64°C
5'-Sdt-ExA2-1	GCCTTCGTCAATCGCCGTC	60°C
5'-Sdt-Ex4-1	GGAAATGCGAGCAGCGGC	60°C
5'-Sdt-k1	CCATCTCTGTCTCCACTTCCG	66°C
5'-Sdt-ExA1-1	CGGCGGACTGCGTTCTCG	62°C

Primer name	Primer sequence (5' ->3')	Temperature (T_m)
5'-Sdt-m1	GAAGTGGCAGCAGCAACG	58°C
5'-Sdt-Ex3-1	GCCTTCGCCTGTCACTGC	60°C
3'-Ex6-RA-44866	CAATGTCCTTCTGCAATCCGAG	66°C
3'-SdtPDZSH3	GCGTGATGAAGTGGTAGTCC	62°C
3'-SdtGUKdm	CCACCACCGTGCGTATGGCCTC	74°C
3'-Sdt-Ex4-1	CGACCATCCTTCTCCACCC	62°C
3'-Sdt-Ex1-1	GGTCAATCTCCCTGATGTCGC	66°C
3'-Sdt-e1	CCGTGGCACTGCTGGTCG	62°C
3'-Sdt-k1	GTGGATGTGGACTCGGATTC	62°C
3'-Sdt-Ex3-1	GCTGCCATTGCGGTTGTC	58°C

For 5'-RACE first the cDNA was generated using the BD Advantage2 PCR Kit (BD Biosciences Clontech, USA). Then 5'-RACE was performed using the SMART Race cDNA Amplification Kit (BD Biosciences Clontech, USA) on obtained cDNA. The procedure was performed according to the company recommendations. The following 3'-primers were used:

3'-Sdt-i1-RACE 5'-CGTGGCAGCGGATGATACAGGTG-3' T_m=74°C (Sdt-D isoform)

3'-Sdt-l1-RACE 5'-GAAGTGGCAGCAGCAACG-3' T_m=72°C (Sdt-C and Sdt-C2 isoform)

3'-Sdt-ExA5-1-RACE 5'-CGGCGATTCCATTGCTTTAGGATGC-3' T_m=76°C (Sdt-B isoform)

RT-PCR and 5'-RACE products were separated on agarose gels, eluted from the gels in elution buffer. The cleaning and the concentration of the products followed this. 1/10 V 3M Na-acetate (pH 5.2) and 5 V 96% ethanol were added to the PCR product. The mixture was thoroughly mixed and incubated at –20°C overnight. Then it was centrifuged at 13000 rpm for 30 min. Supernatant was removed, precipitate was washed in 70% ethanol, dried and diluted in 10 ul sterile H_2O. Afterwards DNA was sequenced directly without cloning by AGOWA (Berlin).

2.2.1.4 Isolation of genomic DNA and PCR on genomic DNA

Chelex-extraction was used for the isolation of genomic DNA (Walsh *et al.*, 1991). 5 embryos of the required phenotype were sorted out according to the phenotype and put into a clean eppendorf tube with 50 ul of 5% weight / volume Chelex in sterile H_2O. During the sorting process embryos were kept on ice. Then they were incubated for 5 min at 95°C, cooled on ice and centrifuged for 1 min at 13000 rpm. Supernatant was transferred into a new eppendorf tube, which was then stored at –20°C and used for the PCR.

PCR mixture for the DNA amplification using genomic DNA as a matrix was the following (for 50 ul volume):

Genomic DNA	5 ul
dNTPs (10 mM)	3 ul
3'-primer (10 mM)	2.5 ul
5'-primer (10 mM)	2.5 ul
10xPCR buffer	5 ul
Pfu polymerase (1U/ul)	1 ul
$MgCl_2$ (25 mM)	2 ul
H_2O	29 ul

The program for the PCR in this case was the following:

Step	Duration	Temperature	Meaning
1	2 min	94°C	Initial denaturation of DNA
2	30 sec	94°C	Denaturation of DNA
3	1 min	68°C for the first 10 cycles, then primer pair specific: 3-6°C under the melting temperature of the primer	Annealing of the primers to DNA
4	1 min for each 1 kb of PCR product	68°C	Synthesis of DNA by Pfu-polymerase (elongation)
5	7 min	68°C	Final synthesis of the DNA
6	∞	4°C	End of reaction

2.2.1.5 Electrophoresis in agarose gel

DNA fragments were separated according to standard protocols on 0.8-1% TAE-agarose gels in about 10 V/cm electronic fields. For the visualization of the DNA 5ul of ethidiumbromid (10mg/ml in H_2O) were added to each 100 ml of gel volume. DNA bands were visualized with the UV-transilluminator (λ=312 nm) and photographed with video camera and Gelprint 2000E system. The sizes of the bands were estimated by comparison with fragments of known sizes (1-kb Ladder, Gibco/BRL).

2.2.1.6 Elution of DNA from the agarose gels

DNA fragments were cut from the agarose gels using a clean razor blade. Time of exposure of DNA to UV-light was minimized and didn't exceed 30 sec. For the following elution of the DNA from the agarose gels NucleoSpin Extract Kit (Machery-Nagel) or E.Z.N.A. Gel Extraction Kit (Peqlab) were used.

2.2.1.7 Restriction of DNA

All restriction reactions were performed in the volume of 20 ul. Enzyme was added in the proportion of 1 U for 1 ug of DNA. For each enzyme enzyme-specific buffer was added in appropriate amount. Restriction reactions were incubated for 1 hour at 37°C.

2.2.1.8 Cloning of PCR products into vectors

All PCR products were first cloned into pCRII-TOPO vector using the TOPO-TA Cloning Kit (Invitrogen). PCR products used for the generation of the Sdt constructs were then re-cloned in frame to a modified pUAST vector (Brand and Perrimon 1993; Amp^R) carrying an N-terminal FLAG epitope using *No*tI and *Xb*aI introduced with the primer pair:

5`-AAGGAAAAAAGCGGCCGCCACCATGAGTTACATGCCAGCCCAGAAT-3` and 5`-GCTCTAGACTAACAATCGGTATCGTACCAGG-3` (vector was kindly provided by N. Fischer).

For the cloning of Sdt-ΔSH3-GUK and Sdt-ΔHook-GUK the other modified pUAST vector was used. In addition to the N-terminal FLAG, a C-terminal 8xmyc epitope was cloned into the vector. To create pUAST-FLAG-8xmyc eight myc-tag repeats were amplified with PCR from pBS vector (kindly provided by B. Dickson) with 5'pBS8myc 5'-CTACTCCCTCGAGATGATGCGG-3' and 3'pBS8myc 5'-CAATTAACCCTCACTAAACGGAAC-3' primers and cloned into pUAST-FLAG vector using *Xho*I and *Xba*I restriction sites. Products for the probes for the Northern analysis (see below) were cloned into pCR-TOPO vector and directly used for generation of the probes. The electrocompetent bacterial strain XL-1 Blue MRF was used for propagation of plasmids. Inserts in the obtained plasmids were sequenced using vector-specific primers by AGOWA (Berlin).

Sdt-MAGUK cloned into pGEX-4T-2 vector (GE Healthcare) in frame was used for the generation of the antibodies against the N-terminus of this isoform (kindly provided by Ö. Kempkens). The C-terminal part was cut out using *Eco*RI enzyme. The one *Eco*RI was located in the position 1698 bp of the MAGUK cDNA, the second one was in the polylinker region of the vector. This vector should result in the protein that has GST at its N-terminus and the total protein size about 90 kDa.

2.2.1.9 Generation of the probes for the Northern analysis

For the Northern analysis 7 different probes were used. The MAGUK-probe was the same as described before (Bachmann *et al.*, 2001). The probe directed against exon-3 was kindly provided by S. Berger. DNA for SH3- and exon-f probes was amplified by PCR using SdtMAGUK and SdtGUK cDNAs in pBluescript KS+/SK+ vector (Stratagene, Heidelberg, Amp^R) respectively. These full-length cDNA plasmids were generated and kindly provided by A. Bachmann. For generation of the SH3-probe the following primer pair was used:

5'-SdtPDZSH3	5'-GAAGGTGATGAGATACTGGAGG-3'	T_m=66°C
3'-SdtPDZSH3	5'-GCGTGATGAAGTGGTAGTCC-3'	T_m=62°C

For generation of the exon-A5-probe the following primer pair was used:

5'-SdtGUKNterm	5'-CCTAAAGCAATGGAATCGC-3'	T_m=56°C
3'-SdtGUKNterm	5'-CCTGGTCAATCTCCCTGATATC-3'	T_m=66°C

DNA for probes directed against exon-e and exon-i was amplified from genomic DNA using PCR. The following primers were used for the amplification of the exon-e probe:

5'-Sdt-e2	5'-CGACCAGCAGTGCCACGC-3'	T_m=62°C
3'-Sdt-e2	5'-CGTTGTCTGTGCCGCTGC-3'	T_m=60°C

For the amplification of exon-i the following primer pair was used:

5'-Sdt-i4	5'-GATTGTCGCCGCACCTCGTCG-3'	T_m=70°C
3'-Sdt-i1-RACE	5'-CGTGGCAGCGGATGATACAGGTG-3'	T_m=74°C

For the generation of the probe against exon-k the RT-PCR product was used as a template. This RT-PCR product was obtained using 5'-Sdt-k1 and 3'-Sdt-Ex1-1 (see above). It was re-amplified using 5'-Sdt-k1 (see above) together with:

3'-Sdt-k2	5'-GTGCCGAATGACCTCCGTG-3'	T_m=62°C

All this amplification products were cloned into pCRII-TOPO vector using the TOPO-TA Cloning Kit (Invitrogen) and sequenced. Digoxygenin-labelled RNA antisense probes were generated by *in vitro* transcription using the DIG RNA labelling Kit (Roche).

All the probes were tested using the spot-test and *in situ* hybridisation on fly embryos.

2.2.1.10 Northern analysis

- First, polyA$^+$-RNA from the heads or the embryos (see above) was separated on the agarose gel. 1.5 g agarose were mixed with 85 ml H_2O and 10 ml 10xMOPS for the preparation of the gel. It was then boiled in the microwave oven for 2 min at maximum intensity (800 W) and cooled to about 60-70°C. Then 5 ml of 37% formaldehyde was added to the mixture and the gel was poured.
- About 3 ug of RNA was used for each test. The RNA was prepared in the following way: x ul RNA were mixed with 10 ml formamid, 4 ul 37% formaldehyde, 3 ul 10xMOPS and 1ul ethidiumbromid (400 ug/ml). Then the mixture was incubated for 5 min at 70°C and cooled on ice. 3 ul RNA-probe buffer were added to the RNA before loading. 5 ul RNA marker (Promega) were loaded on the gel for the characterization of sizes of the RNA bands. The gel was running at 100V for 3 hours in 1xMOPS.
- The gel was photographed with a ruler on it for the further identification of the RNA band sizes.

- The gel was washed once for 15 min in distilled H_2O and twice for 15 min each time in 10xSSC. After this RNA was blotted to the membrane in the following way:
- A whatman-paper was put on the glass plate in the way that it was touching a reservoir with 10xSSC at both sides. The gel was put above and covered with nylon-membrane of the same size as the gel. All the bubbles between whatman-paper, gel and a nylon-membrane were removed. Three whatman-papers of the same size as the gel were put above. Then the structure was covered by with an approximately 6 cm thick stack of Apura-paper and a weight of about 0.5 kg was put above. The transfer was performed overnight.
- Transfer was checked under the UV-light. The membrane was left to dry and then RNA was fixed at the membrane by UV-irradiation (120 mJ).
- Membrane was incubated in at least 15 ml prehybridisation buffer for 1 hour at 68°C
- Membrane was incubated in hybridisation buffer with RNA-probes overnight at 68°C. The probes were prepared in the following way: first, 1.5 ug of antisense RNA-probe were added to 100 ul of hybridisation buffer, incubated for 5 min at 95°C and cooled on ice. Then they were mixed with the rest amount of hybridisation buffer after short centrifugation.
- Membrane was washed 2x5min at room temperature in 50 ml 2xSSC/ 0.1% SDS.
- Membrane was washed 2x15min at 68°C in 50 ml 0.1xSSC/ 0.1% SDS, equilibrated in washing buffer and blocked for 1 hour in blocking solution.
- Membrane was incubated for 1.5 hours with αDIG-HRP antibodies (1:10000) in blocking solution.
- Membrane was washed 2x15 min washing buffer.
- Membrane was washed 2x5 min in TBST and incubated for 90 sec with staining solution (5 ml solution A with 50ul solution B, BM Chemiluminescence Blotting Substrate, Roche).
- Staining was developed on the Roentgen film in the Roentgenfilmchamber (exposure time laid in the interval from several seconds to several minutes).

2.2.2 Biochemical methods

2.2.2.1 Isolation of the total protein from the *Drosophila* head/eye.

Heads were cut from the body with a sharp blade. Retinas were separated from the remaining head tissue with forceps in 1xPBS buffer. Then tissues were transferred in lysis buffer (for protein extracts) in the ratio of 1 head/ 2ul buffer or 1retina/ 1ul buffer in eppendorf tubes. They were thoroughly homogenized and stored on ice for 30 min. Then they were centrifuged at 10000 rpm for 1 min, supernatant was collected and used for the further analysis.

2.2.2.2 Induction of protein expression and isolation of the GST-fused protein from *E.coli*

In this work two proteins fused to GST were expressed in *E*.coli:

1. N-terminal part of the Sdt-MAGUK isoform (19-1698 bp, 7-566 aa) was cloned into expressing vector pGEX-4T-2 with *Bam*HI and *Eco*RI restriction sites. The resulting protein (GST-SdtN) had GST at its N-terminus and the total protein size about 90 kDa. This protein was used for generation of α-Sdt-N antibody.
2. The second protein contains 1-256 aa of Sdt-B isoform fused to GST. The pGSTag-Stardust(1-265) vector (kindly provided by B. Margolis) was used directly for induction of expression. This protein was used to check the specificity of α-Sdt-N antibody.

BL21 pLysS E.coli cells that lack both *ompT* and *lon* proteases were used for the induction. The induction was performed by adding of 0.1 M IPTG at 0.7 OD_{600} for 3 hours. It was followed by the isolation of the GST-fused protein according to the following protocol:

- Cells were precipitated by centrifugation at 5000 rpm for 10 min. The supernatant was discarded.
- Cells were resuspended in the 1/20 of the initial liquid volume in 1xPBS containing proteinase inhibitors in the concentration 2ug/ml each. To break the cell walls cells were sonified.
- Triton X-100 was added to the final concentration 1%.

- Solution was incubated with shaking for 30 min at 4°C, centrifuged for 15 min at 5000 rpm. Supernatant was collected.
- For the binding of the GST-fusion protein 100 ul Glutathion Sepharose 4B was added to each 10 ml of the protein extract. That was followed by the incubation for 1 hour at room temperature.
- Sepharose beads together with bound protein were precipitated by centrifugation for 5 min at 5000 rpm at 4°C, supernatant was discard and the precipitate was dissolved in 1.5 ml 1xPBS with proteinase inhibitors. This washing step was repeated 3-4 times.
- To separate the bound protein from the beads the equal amount of Glutathion elution buffer was added to the beads. This was followed by incubation for 1 hour at room temperature and centrifugation for 2 min at 13000 rpm. The supernatant was collected, protein concentration was measured, and the protein quality was analyzed by SDS-polyacrylamid gel electrophoresis followed by Coomassie staining.

The fusion protein was sent to the Eurogentec company (http://www.eurogentec.com/code/en/hp.asp) at a concentration of 250 ug/ml, where they performed injections into 2 rats and antibodies purification.

2.2.2.3 *In vitro* transcription/translation

Sdt prodeins encoded by different isoforms were used for the analysis of the specificity of the antibody-containing serum. They were generated using *in vitro* transcription/translation Kit (Roche, Mannheim) according to the protocol recommended by the company. Sdt-MAGUK and Sdt-GUK cloned in pBluescript KS+/SK+ vector (Stratagene, Heidelberg, Amp^R) were transcribed using T3-RNA-polymerase and translated. 2 ul of obtained protein solution were used for each Western analysis.

2.2.2.4 SDS-polyacrylamid gel electrophoresis and Western blot.

Proteins were mixed with 2xloading buffer and cooked for 5 min. They were separated in a 0,75 mm polyacrylamid gel in SDS-buffer system. The Mini-Trans-Blot system (BioRad) was used for the separation. The electrophoresis ran at the constant voltage 100V. The relative size of the proteins was determined using the protein standards (BioRad).

For the Western blot analysis separated proteins were transferred to the nitrocellulose membrane (Hybond ECL, Amersham). The transfer was performed at 5 mA/cm^2 for 1 hour at 4°C. Membrane was stained with 1X Ponceau S for as long as was required to detect the protein bands to check the transfer quality. Then the membrane was washed 3 times for 15 min in TBST, blocked for 30 min in 5% dry milk in TBST and incubated overnight with the primary antibodies in 5% dry milk in TBST. Next day the membrane was washed 3 times for 15 min in TBST, incubated for 2 hours with the secondary antibodies in 5% dry milk in TBST and washed 3 times for 15 min in TBST. After this the staining was developed using BM Chemiluminescence Blotting Substrate (POD) (Roche Diagnostics, for the details see above).

2.2.3 Histological methods

2.2.3.1 Immunocytochemistry on pupal eye discs

Pupae of the appropriate developmental stages were collected. The retina-brain complex was dissected in PBS and fixed for 25 min in 4% paraformaldehyde on ice. Then they were washed 3 times for 15 min in PBSap (PBS with 0.1% saponin) on ice. The retina-brain complexes were incubated overnight with the primary antibodies diluted in PBSap. Then they were washed 3 times for 15 min in PBSap, incubated for 2 hours at room temperature with secondary antibodies diluted in PBSap and again washed 3 times for 15 min in PBSap. Then they were embedded in glycerol/propylgallate and analysed with a confocal microscope.

2.2.3.2 Immunocytochemestry on the sections through adult eyes

Heads of adult flies were cut from the bodies and then cut in two halves between the eyes with a sharp blade. The eyes were then fixed for 30 min in 8% Stefaninis fixative on ice. Fixation was followed by thorough washing with 1xPBS (total time at least 30 min), cryopreservation for 30 min in 10% sucrose in 1xPBS on ice and overnight incubation in 25% sucrose in 1xPBS at 4°C.

Then the eyes were embedded in tissue-freezing medium (GSV1, Slee Technik, Germany) and rapidly frozen on dry ice. Cryosections (about 10um thick) were done with SLEE Cryotom (SLEE Technik, Germany) and collected on the Superfrost slides (Menzel Glaeser, Germany). Then sections were permeabilized 3 times for 15 min in PBT (PBS, 0,1% Triton X-100) and incubated overnight with the primary antibodies in PBT / 0,1% BSA. Then they were washed 3 times for 15 min in PBT, incubated with the secondary antibodies in PBT /0,1% BSA for 2 hours at room temperature, washed 3 more times for 15 min in PBT and mounted in Mowiol (Polysceinces, PA) containing DAPCO (Sigma). Then the slides were examined under the confocal microscope.

2.2.3.3 Fixation of the eyes for transmission electron microscopy

- Heads of the adult flies were cut from the bodies and then cut in two halves between the eyes with a sharp blade.

- Heads were fixed in 25% glutaraldehyde for 20 min at room temperature, and then washed 2 times for 20 min in 0,1M phosphate buffer, pH 7,2.
- Heads were simultaneously fixed in 1% osmium and 2% glutaraldehyde in 0,1M phosphate buffer pH 7,2, on ice in the dark, and washed 2 times for 20 min in 0,1M phosphate buffer, pH 7,2.
- Postfixation in 2% osmium in 0,1M phosphate buffer, pH 7,2 for 1 hour on ice in the dark followed by 3 times 10 min washing in H_2O.
- Heads were dehydrized by the washing in the following row of ethanol solutions: 30%, 50%, 70% and 96%, each for 5 min on ice.
- Washing 2 times for 10 min in 100% ethanol and 2 times for 10 min in 100% acetone.
- Heads were stored overnight in 1:1 araldite/ acetone.
- Then they were left under the hood for 2 hour to let acetone evaporate and put in the fresh araldite for 4 hours.
- Eyes were embedded in fresh araldite and left for 24 hours at 68°C for the polymerization.

2.2.3.4 Semi-thin sections of the adult eyes

The semi-thin sections (2,5 um) were done with a glass knife on the microtome REICHERT OM 2. Slides covered with gelatine were used. To prepare the slides, they were first washed with 96% ethanol, then put shortly in 0,5% gelatine in H_2O and dried. After cutting the sections were transferred into the drop of water on the slide. To stick the section to the slide the drop of water was evaporated by incubation of the slide at 98°C for 20 min.

2.2.3.4 Ultra-thin sections of the adult eyes

Ultra-thin sections (about 50 angstrom) were done with diamond knife on the microtome REICHERT OM 2. They were transferred from the water onto the nickel-nets and dried. Then they were stained with 2% uranylacetate for 5 min, washed in distilled water and contrasted in leadcitrate solution for 5 min. Then they were again washed thoroughly in distilled water and dried. Sections were analysed on the electron microscope EM109 (Zeiss).

2.2.4 Genetic methods

2.2.4.1 UAS-GAL4 system

The UAS-GAL4 system allows the ectopic expression of the transgene at the appropriate time-point in the appropriate tissue (Brand and Perrimon, 1993). The system consists of a driver-line and an effector-line. The driver-line contains the yeast transcription factor GAL4 gene under the control of specific *Drosophila* regulatory sequences. Expression of the GAL4 protein alone results in no phenotype, as GAL4 can't regulate the activity of *Drosophila* genes. To activate the gene expression GAL4 needs to bind to a special sequence, called upstream activating sequence (UAS). The effector line contains the gene of interest cloned behind 5 adjacent UAS sequences. Without GAL4 protein the expression of the gene of interest is minimal or absent. When flies from driver- and effector lines are crossed to each other, both components of the system come together. Then under the control of specific regulatory elements the expression of GAL4 starts, GAL4 binds to UAS, and activates the expression of the gene of interest (Figure 2.2). Several driver-lines resulting in GAL4 expression in different cell types of *Drosophila* eye were used.

- *GMR*GAL4 leads to GAL4 expression from third larval instar in all cells after the morphogenetic furrow.
- *Rh*GAL4 starts GAL4 expression at about 70% p.d. in R1-R6 rhabdomeres.
- *54*GAL4 starts expression in early pupal development in secondary and tertiary pigment cells.
- *ey*GAL4 starts GAL4 expression in late embryogenesis in eye imaginal disc precursors and in expressed in all the eye cells till about 50% p.d. (Beronja *et al.,* 2005).

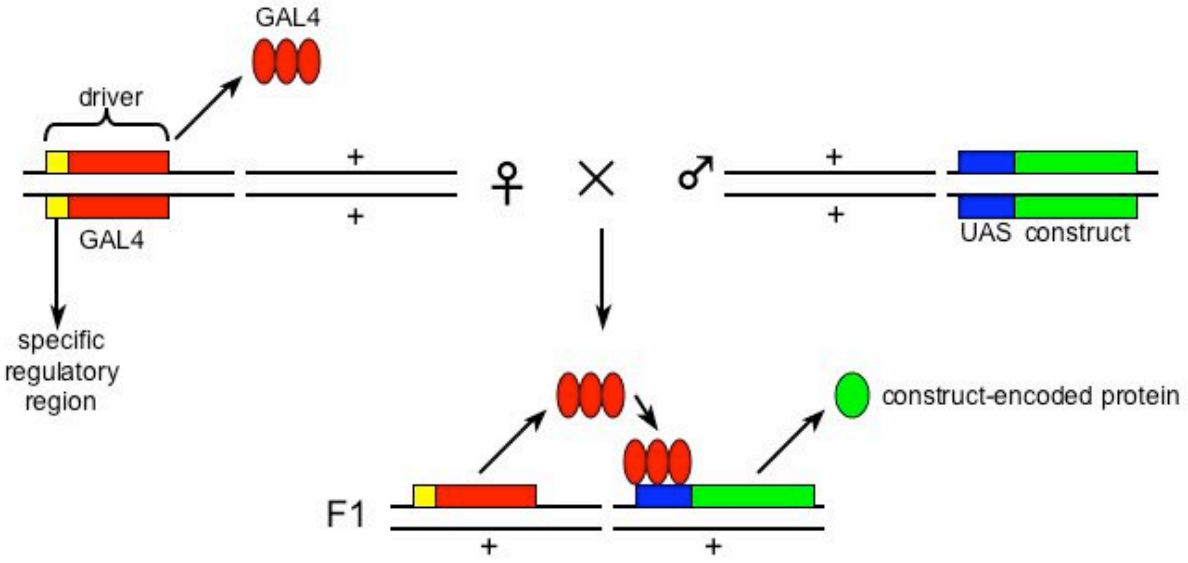


Figure 2.2. The UAS-GAL4 system allows tissue- and time-specific expression of the gene of interest. For its work two transgenes are required – the driver line and the one encoding the gene of interest with UAS promoter. When they are brought together in one animal GAL4 protein is produced under the control of specific regulatory elements, binds to UAS, and activates expression of the gene of interest.

2.2.4.2 Producing *sdt* clones in the eyes using FLP/FRT system

All *sdt* alleles are embryonic lethal, and therefore the *sdt* mutant clones have to be induced in the *Drosophila* eye to be able to analyze the phenotype causeed by absence of Sdt. For making the *sdt* mutant clones the FLP/FRT system (Xu und Rubin, 1993) was used. It is based on the mitotic recombination between FRT (FLP-recombinase-target) sequences induced by FLP-rekombinase (FLP). As Sdt is localized to the X chromosome two FRT-carrying X-chromosomes were used for the making of the clones. One carries the *sdt* mutation, *white*$^{-}$ and FRT19A. The other contains wild type versions of *sdt*, *white* and FRT19A. The *white* gene was required to be able to distinguish between mutant and wild type parts of the eyes, as the *sdt* homozygous cells were not able to produce pigment and resulted in the white clones. For the generation of the large eye clones a second X-chromosome was used that in addition to FRT19A carries *GMRhid cl*. As the result the sister clones of the *sdt* mutant

clones are homozygous for *GMRhid cl* and die. As the source of FLPase either *ey*FLP (under the direct control of *ey* regulatory elements) or *ey*GAL4 UAS-FLP (under the control of *ey* regulatory elements in the UAS-GAL4 system) were used. The system in work is shown on the Figure 2.3.

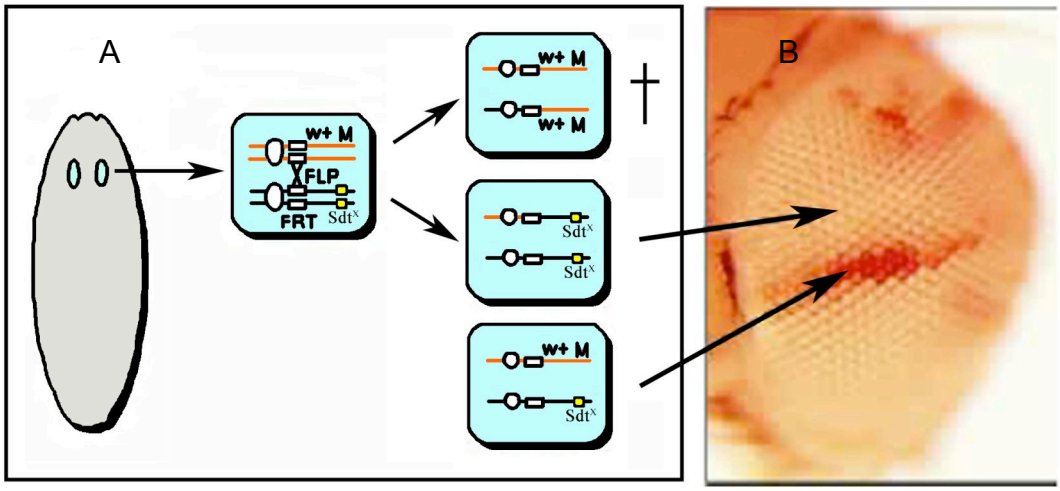
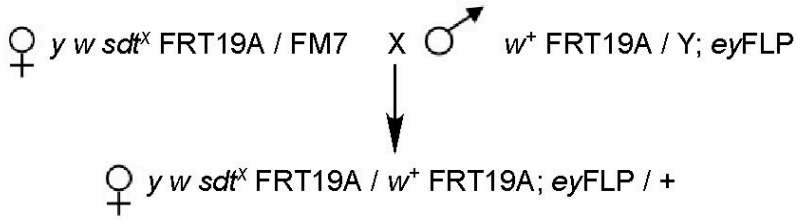


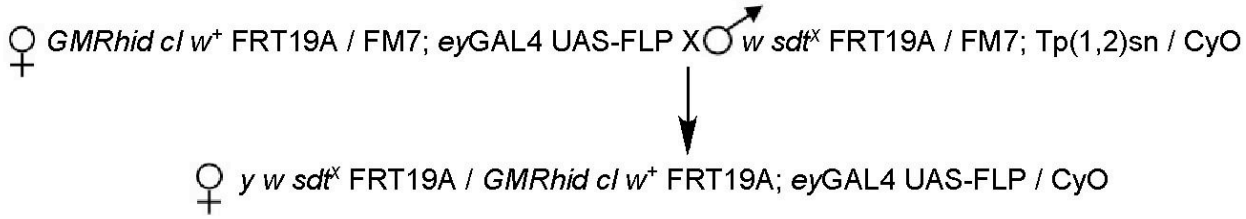
Figure 2.3. Induction of mitotic recombination using the FRT/FLP system with large *sdt* eye clones as an example (modified from Newsome *et al.,* 2000). A – schematic representation of the system. B – Photograph of the complex eye with the large eye clones. White parts of the eye correspond to the mutant tissue, red parts – to the *sdt* heterozygous tissue. By the increase of the temperature at which the flies are kept (to 25°C), it is possible to decrease the number of heterozygous cell to nearly no cells.

2.2.4.3 Crossings for making large or small eye clones

Induction of small eye clones:

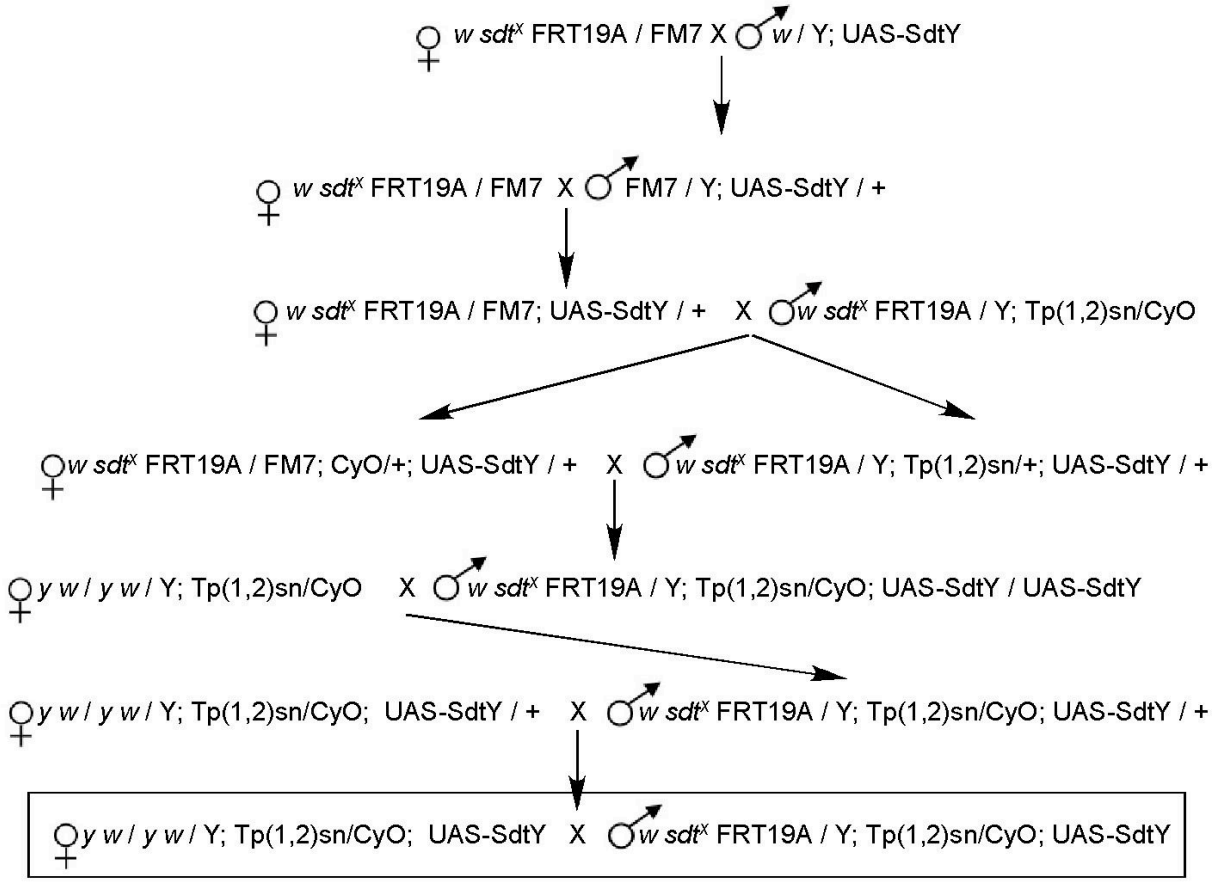


Crosses for the large eye clones:



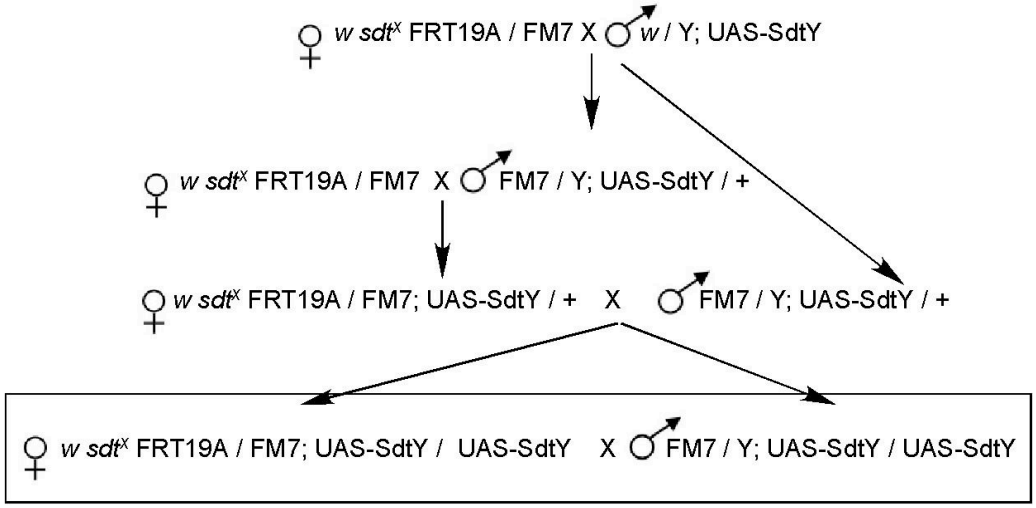
2.2.4.4 Crossings for bringing Sdt-transgenes in the *sdt* mutant background

The following scheme of crossings was used for the generation of the stocks carrying both *sdt* mutation and Sdt-transgene:



One the scheme *sdt*^{X} means any *sdt* mutation, and UAS-SdtY – any Sdt-encoding transgene.

For UAS-Sdt-MAGUK-15, UAS-Sdt-B and UAS-Sdt-1-L27-C the simplified scheme of crosses was used. The obtained flies can be used for inducing MARCM clones but not the large eye clones. It was the following:



The obtained stable stocks are marked in both cases by the rectangles. Flies homozygous for Sdt-encoding construct were collected by eye colour.

2.2.4.5 Mosaic analysis with a repressible cell marker (MARCM) technique

The MARCM system allows to simultaneously induce mutant clones, to express transgenes in these clones and to mark them with GFP expression (Sweeney *et al.*, 2002). The system is based on the FRT/FLP system. In addition to the standard FRT/FLP system, the counter chromosome with a wild-type version of the gene of interest contains *tub*GAL80 and *hs*FLP. On the other chromosome it carries *act*GAL4, UAS-GFP:CD8. To make the clones this stock is brought together with the stock that has a mutation of the gene of interest combined with FRT sequence. Then these animals are heat-shocked. In this work for making the clones the animals were heat-shocked at 3^{d} and 4^{th} days after egg laying each time for 1 hour 37°C. Heat-shock activates the expression of FLP, and it induces the exchanges between FRT sites. In the cells where such exchange occurred and that are homozygous mutant for the gene of interest, the *tub*GAL80 is excised. As GAL80 that is a repressor of GAL4 is now not

expressed, expression of GAL4 starts. In its turn it activates the expression of UAS-GFP, so the mutant clones are marked with GFP. At the same time it will activate the expression of any other UAS-transgene that is introduced in the system. The system is schematically presented on Figure 2.3.

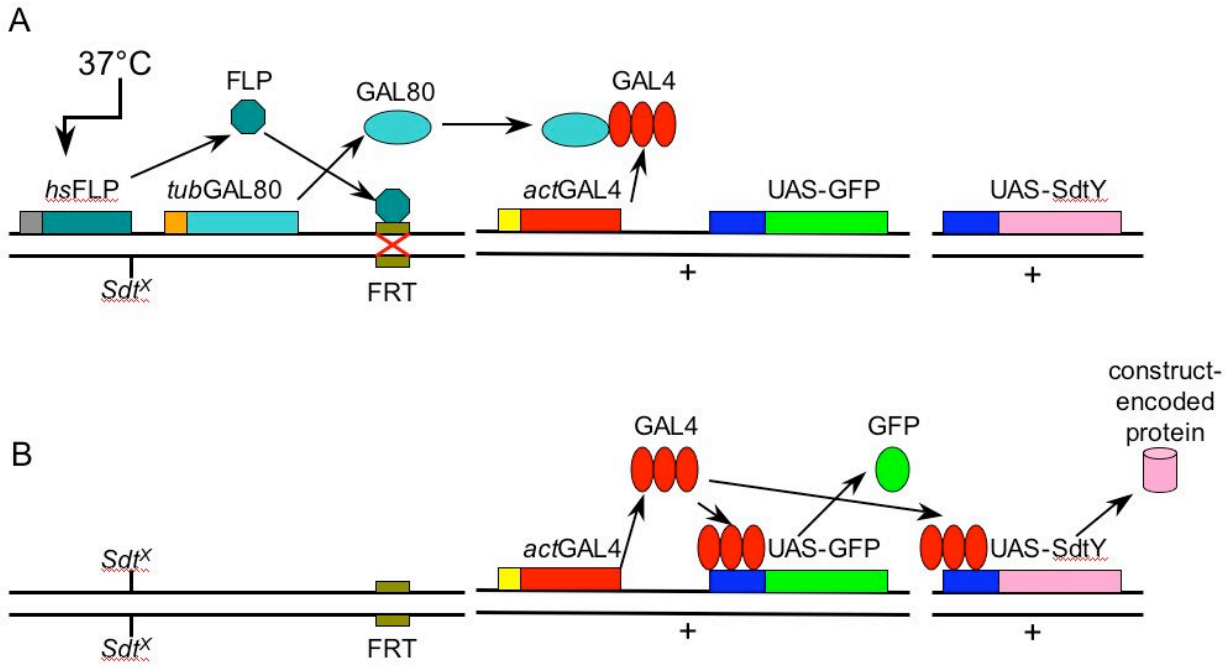


Figure 2.3. Scheme of the MARCM system. During heat-shock (A) in the cell FLP protein is produced to induce the exchange between FRT sequences (exchange is marked by **X**). At the same time GAL80 protein is still present to repress the GAL4 protein. After the recombination (B) in some cells *sdt* is homozygous (for the details see Figure 2.2). There is no GAL80, GAL4 is expressed and induces expression of both GFP and construct-encoded protein in these cells.

2.2.4.6 Assaying retinal degeneration in flies expressing the transgenes.

To analyze the induction of the retinal degeneration by constant light exposure, the flies were kept during a 21 days period in constant light. Its intensity was 17 umol/m^2s, the range of the wave-length was 380-710 nm. These parameters were reached by the combination of an incandescent lamp of 60 W and a neon lamp. The intensity was measured using a quantum sensor. The temperature was controlled and varied between 24°C and 26°C. The lamps were located on opposite sites of the vials, so that the flies were exposed to the light from all directions. The vials were changed once every two days to prevent the crawling of the larvae on the vials walls. The control flies were kept in the same temperature range in the light-isolated incubator.

3. Results

3.1 Sdt expression in adult Drosophila *heads*

The first step to understand the function of the Sdt protein in the *Drosophila* eye was to characterize its expression there. Berger, 2005 demonstrated for several *sdt* mutant alleles that although they express no protein and have a null phenotype in the embryo, in eyes mutant for these alleles Sdt protein is still produced. From these data one can conclude that the expression of Sdt differs between embryo and eye, and that probably there are isofoms of Sdt that are specific for the *Drosophila* eye, and are not expressed in the embryo.

3.1.1 Three different Sdt isoforms are predominantly expressed in adult *Drosophila* heads, only two of them are specific for the retina

To get an idea of Sdt expression in different tissues including the *Drosophila* retina, Western blot analysis was performed. Only the antibody raised against the PDZ-C domain of Sdt works in Western blots. Therefore this antibody was used throughout this work in Western blots. Lysates from embryos, bodies (without heads), pupal brain/retina complexes from staged pupae (40-60% p.d.), whole heads, and dissected retinas from adults were used (Figure 3.1A). In the embryo only a major band of 100 kDa was detected. This size corresponds to the predicted size of the Sdt-B isoform (Hong *et al*., 2001), and nicely agrees with the data from Wang *et al.* 2004, where they speculate that Sdt-B is the major isoform of Sdt in the *Drosophila* embryo. No protein could be detected in the bodies. There should be some protein present in the follicular cells in the ovaries of adult female flies, as it is known that Sdt is expressed there (Tanentzapf *et al.,* 2000), but probably the relative amount of Sdt protein was too low to detect it. In lysates from pupal brain/retina complexes 5 different Sdt proteins were detected. Two of them have sizes of about 110-120 kDa, two have sizes between 130 and 170 kDa, and the larger one has a size more than 170 kDa. In whole heads three different proteins were detected. Two of them have sizes between 110-120 kDa, and the third one is much bigger and has a size of more then 170 kDa.

In retinas, in comparison to whole heads, only the two smaller proteins but not the third large one were found.

The two bands of 110-120 kDa that can be detected in the retina were the most interesting for this work. To test, whether they are specific for *sdt*, lysates from retinas carrying sdt^{K85} large mutant eye clones were tested with α-Sdt-PDZ-C antibody on Western blots. sdt^{K85} was chosen because it seems to have a null phenotype in the eye: it results in both morphological defects and retinal degeneration, and no protein can be detected using immunocytochemistry (Berger, 2005). Indeed, the two proteins that can be detected in wild type retinas were completely absent from lysates of sdt^{K85} mutant retinas (Figure 3.1A), so it can be concluded that they are encoded by *sdt*.

The Sdt proteins in the retina have sizes between 110 and 120 kDa. Of the three published Sdt isoforms, Sdt-MAGUK has a size of 142 kDa, Sdt-B – 95 kDa, and only Sdt-GUK has a predicted size of 119 kDa (see 1.1.2). So the detected bands correspond in size to the Sdt-GUK isoform. The antibodies that were used are directed against the PDZ domain of Sdt. The Sdt-GUK isoform has a very short part of the PDZ domain (only 22 aa out of 72 aa). To see if these 22 aa are sufficient to be detected by the antibody in Western blots, Sdt-MAGUK and Sdt-GUK proteins that were translated *in vitro* were used. Both proteins were detected using the α-Sdt-PDZ-C antibody (Figure 3.1B). The lower band present in both Sdt-MAGUK and Sdt-GUK samples is unspecific, as it was also present in the negative control, where no vector was added in the *in vitro* transcription/translation reaction.

To prove or to rule out that at least one of the isoforms detected on the Western blot corresponds to Sdt-GUK, Northern analysis on head mRNA was performed. For the Northern blot two different probes were used. One of them is anti-sense to the part of the Sdt mRNA that encodes for the whole PDZ, SH3 and GUK domains. This probe is called MAGUK-probe and should recognize all three known Sdt isoforms (Bachmann *et al*., 2001). The second probe was directed against the part of the mRNA that is present in Sdt-MAGUK and Sdt-B isoforms, but absent in Sdt-GUK isoform. This probe is called SH3-probe (see 2.2.1.8). With both probes the same three RNAs were detected (Figure 3.1C). One has a size of 5,5 kb, the second one is of about 6 kb and the largest one is more then 6 kb. The detection of all three RNA bands with both

probes indicates that all head-specific Sdt isoforms contain an intact PDZ, SH3 and GUK domain, and makes it unlikely that the Sdt-GUK isoform is expressed in *Drosophila* heads.

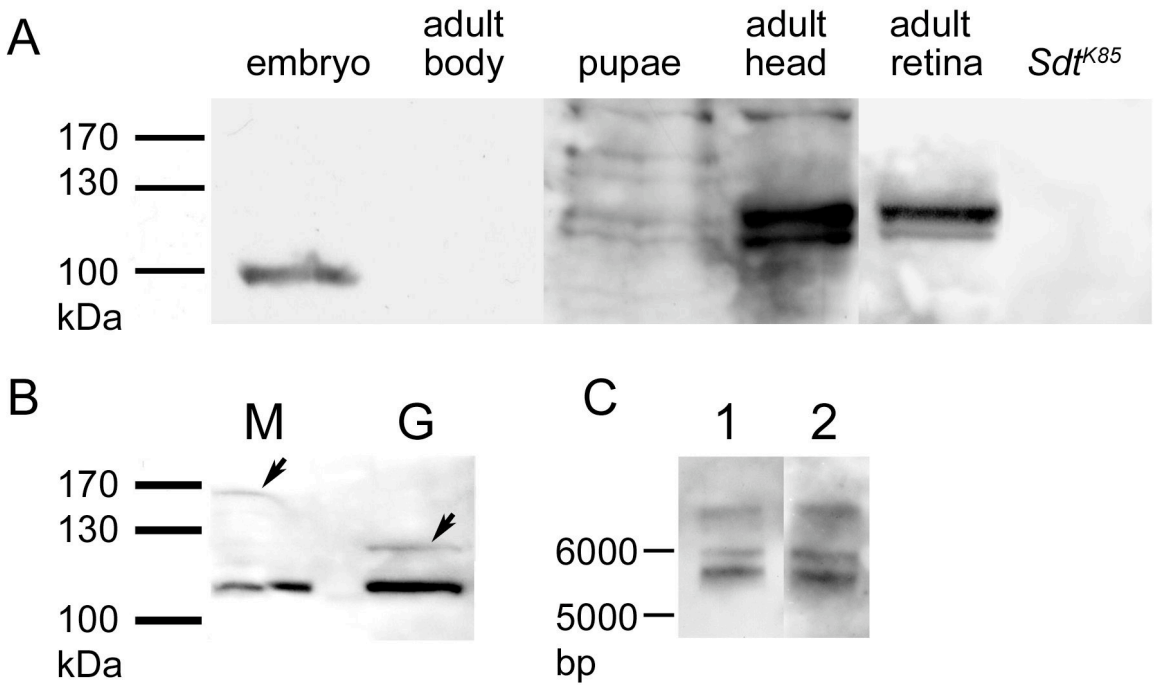


Figure 3.1. Expression of Sdt protein and mRNA.

A. – Sdt expression is dynamic and varies between different tissues and stages. Lysates from embryos, adult bodies, pupae, adult heads, adult retinas, and adult retinas mutant for Sdt^{K85} were used.

B. – Both Sdt-MAGUK (M) and Sdt-GUK (G) *in vitro* translated proteins are recognized by α-Sdt-PDZ-C antibody. Arrows indicate bands corresponding to *in vitro* translated proteins.

C – The same three mRNAs are detected on Northern blots with MAGUK-probe (1) and GUK-probe (2).

3.1.2 Identification of head-specific Sdt transcripts

The forms that are expressed in heads based on Western analysis seemed not to correspond to any of the three published ones. For a further characterisation of the Sdt isoforms that are expressed in *Drosophila* heads, an expanded RT-PCR analysis of the mRNA expressed in the heads was performed. Therefore, the prediction from Flybase was used as a basis for the RT-PCR analysis (see 1.1.2, Figure 1.3). At the same time one additional partial mRNA is included (identified and provided by Bachmann A.). It was called sdt-AB. Figure 3.2 depicts the modified scheme of the predicted isoforms. Where possible, the numbering of exons was kept as in Hong *et al.,* 2001. Otherwise additional exons are designated by letters (e, i, k, l, m).

To confirm that the GUK isoform is not expressed in heads, RT-PCR was done with the primers located around the part of the mRNA that is deleted in Sdt-GUK (5'-Ex6-44654 and 3'-SdtGUKdm). This primer pair should not amplify the GUK-like splice variants, only the MAGUK-like, as the 3'-primer is homologous to the sequence within the part that is deleted in Sdt-GUK. As a control 5'-Ex6-44654 primer was used together with 3'-SdtPDZSH3 (see Figure 3.2). The results of the RT-PCR are shown on Figure 3.3A. All amplified bands were sequenced (see Supplementary Data 6.2). In both cases the larger fragment corresponds to pre-mRNA, which contains rather small introns. The shorter fragments correspond to the Sdt-MAGUK version of this part of mRNA. Other fragments are unspecific. No Sdt-GUK-like variant of this part of mRNA was amplified.

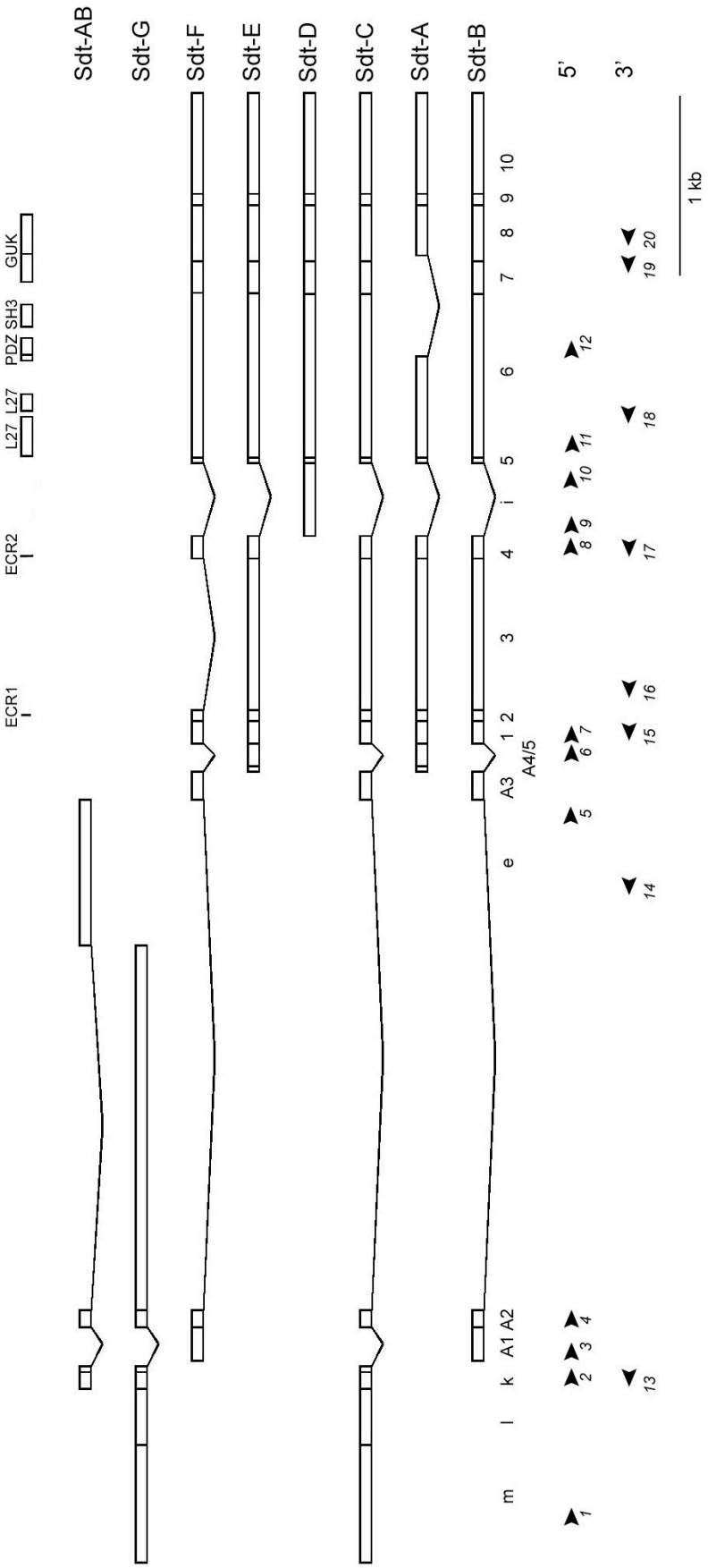

Sdt-AB
Sdt-G
Sdt-F
Sdt-E
Sdt-D
Sdt-C
Sdt-A
Sdt-B
GUK
SH3
PDZ
L27
L27
ECR2
ECR1
5'
3'
1 kb
m
l
k
A1
A2
e
A3
A4/5
i
1 2 3 4 5 6 7 8 9 10

Figure 3.2. Different transcripts of *sdt*.

Only the parts that are not spliced out at least in one of the forms are included, all the rest of the transcribed region is excluded from the scheme. Verticals indicate intron/exon boundaries, which are common to all RNA variants. Isoforms Sdt-A – Sdt-G are equal to the once predicted in FlyBase (see also 1.1.2, Figure 1.3). Letter R is excluded from the isoforms' names, as it indicates only that transcript but not the protein is concerned. Additionally the partial mRNA isolated by A. Bachmann is included (Sdt-AB). Regular numbers or letters designate exons.

The relative positions of different primers are depicted (5' – 5'primers, and 3' – 3'-primers, respectively). The primers are marked by the following italic numbers:

5'-primers:

1 – 5'-Sdt-m1

2 – 5'-Sdt-k1

3 – 5'-Sdt-ExA1-1

4 – 5'-Sdt-ExA2-1

5 – 5'-Sdt-e1

6 – 5'-Sdt-A5-1

7 – 5'-Sdt-Ex1-1

8 – 5'-Sdt-Ex4-1

9 – 5'-Sdt-i2

10 – 5'-Sdt-i1

11 – 5'-Ex6-44654

12 – 5'-SdtPDZSH3

3'-primers:

13 – 3'-Sdt-k1

14 – 3'-Sdt-e1

15 – 3'-Sdt-Ex1-1

16 – 3'-Sdt-Ex3-1

17 – 3'-Sdt-Ex4-1

18 – 3'-Ex6-RA-44866

19 – 3'-SdtPDZSH3

20 – 3'-SdtGUKdm

Above the transcripts, the projection of known domains is depicted. For more details see text.

Similar experiment was done to check the presence of exon 3 in the head-specific Sdt mRNAs. For the amplification the following primer pairs were used: 5'-Sdt-Ex1-1 and 3'-Ex6-RA-44866, and 5'-Sdt-Ex1-1 and 3'-Sdt-Ex4-1 (see Figure 3.2). As additional control the same 3'-primers were used together with 5'-Sdt-e1. The results of these RT-PCR are demonstrated on Figure 3.3B. All products were sequenced (see Supplementary Data 6.2). Specific products correspond to the Sdt splice-variants that lack exon 3. To control that our system allows amplification of rather large products that contain exon 3 mRNA from embryos was used. mRNA was prepared from the embryos of three different stages: 0-4 hours after egg laying (AEL), 4-11 hours AEL and 11-22 hours AEL. 5'-Sdt-Ex1-1 and 3'-Sdt-Ex4-1 were used for the amplification. RT-PCR products of both larger (about 1500 bp) and smaller (about 200 bp) size were detected when mRNA from 0-4 hour AEL embryos was used. These products correspond to both exon 3 containing and exon 3 lacking form. At later embryonic stages no larger band was detected (Figure 3.3C). This indicates that all the splice-forms of Sdt that are expressed in the head lack exon 3. Moreover, Sdt splice-forms containing exon 3 are present only in early embryogenesis.

To confirm that exon 3 is always spliced out in the head, additional primer pairs were used. The first primer pair was 5'-Sdt-Ex1-1 and 3'-Sdt-Ex3-1, and second primer pair was 5'-Sdt-Ex3-1 and 3'-Sdt-Ex4-1 (see Figure 3.2). In both cases one primer is complementary to the sequence of exon 3, and other is directed against the sequences of neighbouring exons. The first primer pair should amplify a 404 bp fragment, and the second one a fragment of 192 bp. In both cases no specific products were obtained from head mRNA (data not shown). To check that the absence of a product is not due to non-functional primers, 3'-Sdt-Ex3-1 was used for the amplification from mRNA of 0-4hours AEL embryos. It was used in pair with one of the following primers: 5'-Sdt-Ex1-1, 5'-Sdt-A5-1 or 5'-Sdt-k1. For all three combinations of primers specific products were obtained (Figure 3.3D). The conclusion from these data is that all Sdt splice-variants in the head lack exon 3.

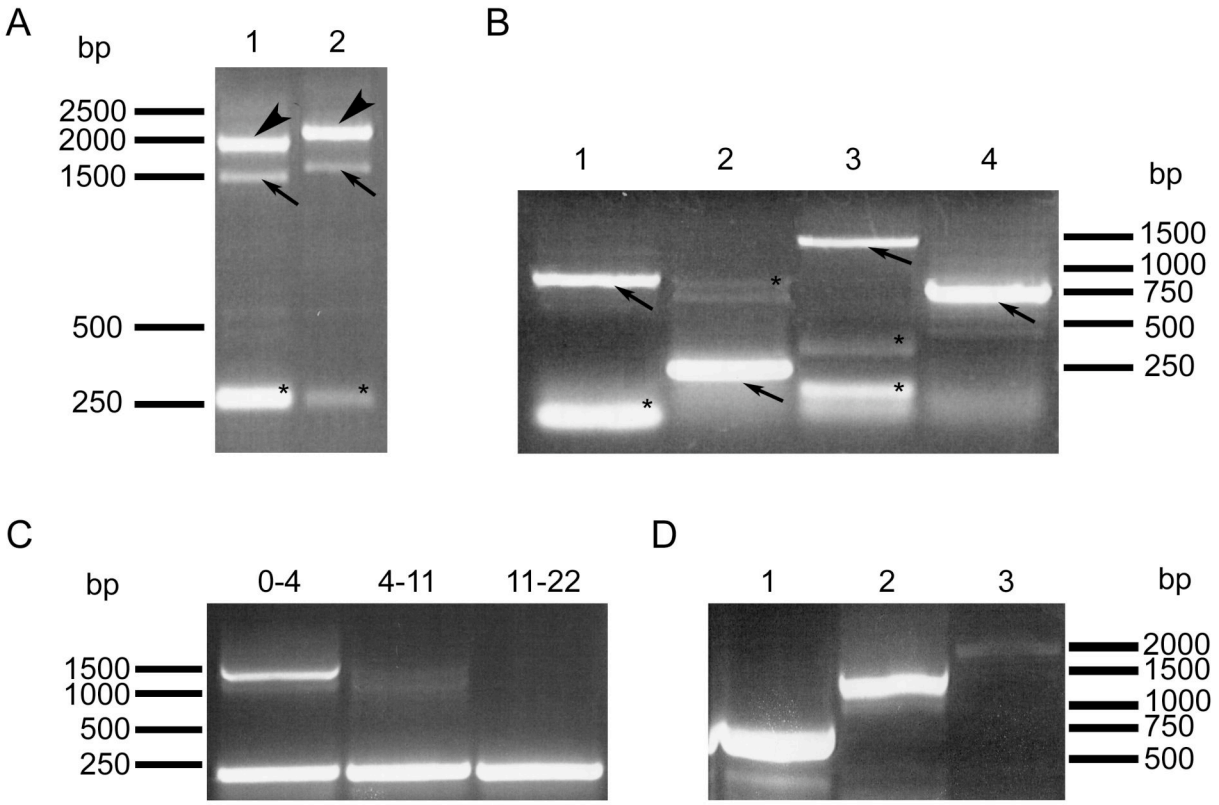


Figure 3.3. All Sdt splice-variants expressed in heads contain MAGUK-like 3'-part and lack exon 3.

A – The amplification with 5'-Ex6-44654 and 3'-SdtPDZSH3 (1), and 5'-Ex6-44654 and 3'-SdtGUKdm (2) resulted in both cases only in MAGUK-like variants of the 3'-part of the mRNAs. Arrows mark the products corresponding to this variant. Arrowheads mark the products amplified from pre-mRNA. Asterisks mark unspecific products.

B – Exon 3 cannot be amplified from head mRNA with any of the following primer pairs: 5'-Sdt-Ex1-1 and 3'-Ex6-RA-44866 (1), 5'-Sdt-Ex1-1 and 3'-Sdt-Ex4-1 (2), 5'-Sdt-e1 and 3'-Ex6-RA-44866 (3), and 5'-Sdt-e1 and 3'-Sdt-Ex4-1 (4). Arrows mark the products corresponding to this variant. Arrowheads mark the products amplified from pre-mRNA. Asterisks mark unspecific products.

C – RNA variants containing exon 3 are found in early embryos (0-4 hours AEL). 5'-Sdt-Ex1-1 and 3'-Sdt-Ex4-1 primer pair was used for amplification. Numbers indicate the age in hours AEL of the embryos that were used for the mRNA extraction.

D – Presence of exon 3 in the mRNA of embryos from 0-4 hours AEL embryos is confirmed with the use of the following primer pairs: 5'-Sdt-Ex1-1 and 3'-Sdt-Ex3-1 (1), 5'-Sdt-A5-1 and 3'-Sdt-Ex3-1 '(2), and 5'-Sdt-k1 and 3'-Sdt-Ex3-1 (3).

If the 3'-part of the mRNA is the same for all the splice-variants, then the difference between them should lay in the 5'-region. Several combinations of exons in the 5'-region are already found in ESTs (see 1.1.2), and more combinations are still possible. The different primers specific for the sequences of the different exons were used to characterize the 5'-parts of the mRNAs in the head. The results are presented on Figure 3.4A-C. The following primer pairs were tested: 5'-Sdt-ExA2-1 and 3'-Sdt-Ex1-1; 5'-Sdt-ExA1-1 and 3'-Sdt-Ex1-1; 5'-Sdt-k1 and 3'-Sdt-Ex1-1; 5'-Sdt-k1 and 3'-Sdt-e1; 5'-Sdt-ExA1-1 and 3'-Sdt-e1; 5'-Sdt-ExA2-1 and 3'-Sdt-Ex4-1; 5'-Sdt-A5-1 and 3'-Sdt-Ex4-1; 5'-Sdt-i2 and 3'- Ex6-RA-44866; 5'-Sdt-i2 and 3'-SdtPDZSH3; 5'-Sdt-m1 and 3'-Sdt-k1 (Figure 3.2). All the obtained products were sequenced. Sequences are presented in Supplementary Data (see 6.2). Schematically they are demonstrated on Figure 3.4D.

The most 5'-parts of some splice-variants were previously published. For the other forms (Sdt-D containing exon i, and Sdt-C with exon k and l) the most 5'-parts are predicted only on the base of ESTs. To get the full sequence of these splice-forms, 5'-RACE experiments were performed. As a control, exon A5 was used (Figure 3.5A). The obtained fragments were sequenced. For exon i and exon A5 the 5'-parts were equal to the published once.

To varify the 5'-part of Sdt-C-like mRNAs, 5'-RACE data were analyzed in the combination with the RT-PCR fragments obtained using 5'-Sdt-m1 and 3'-Sdt-k1 (3.4C). This RT-PCR product has a deletion in comparison to the predicted fragment. 5'-RACE primer lies within this deletion. Probably the amount of pre-mRNA was sufficient to get the product from it using 5'-RACE protocol. As result, 5'-RACE product was used only to get the most 5'-point of the mRNA. RT-PCR products were used for the analysis of the rest. The resulting cDNA starts at the 245th bp of predicted exon m. The last nucleotide in exon m corresponds to the 342th bp of the predicted one. Then it continues with the sequence from predicted exon l, starting with the 199th nucleotide of the predicted exon. 5'-RACE for these exons was repeated two times, and in both cases the same fragments were obtained. Schematically the modified region of exons l and m in the comparison to the prediction is shown on Figure 3.5B.

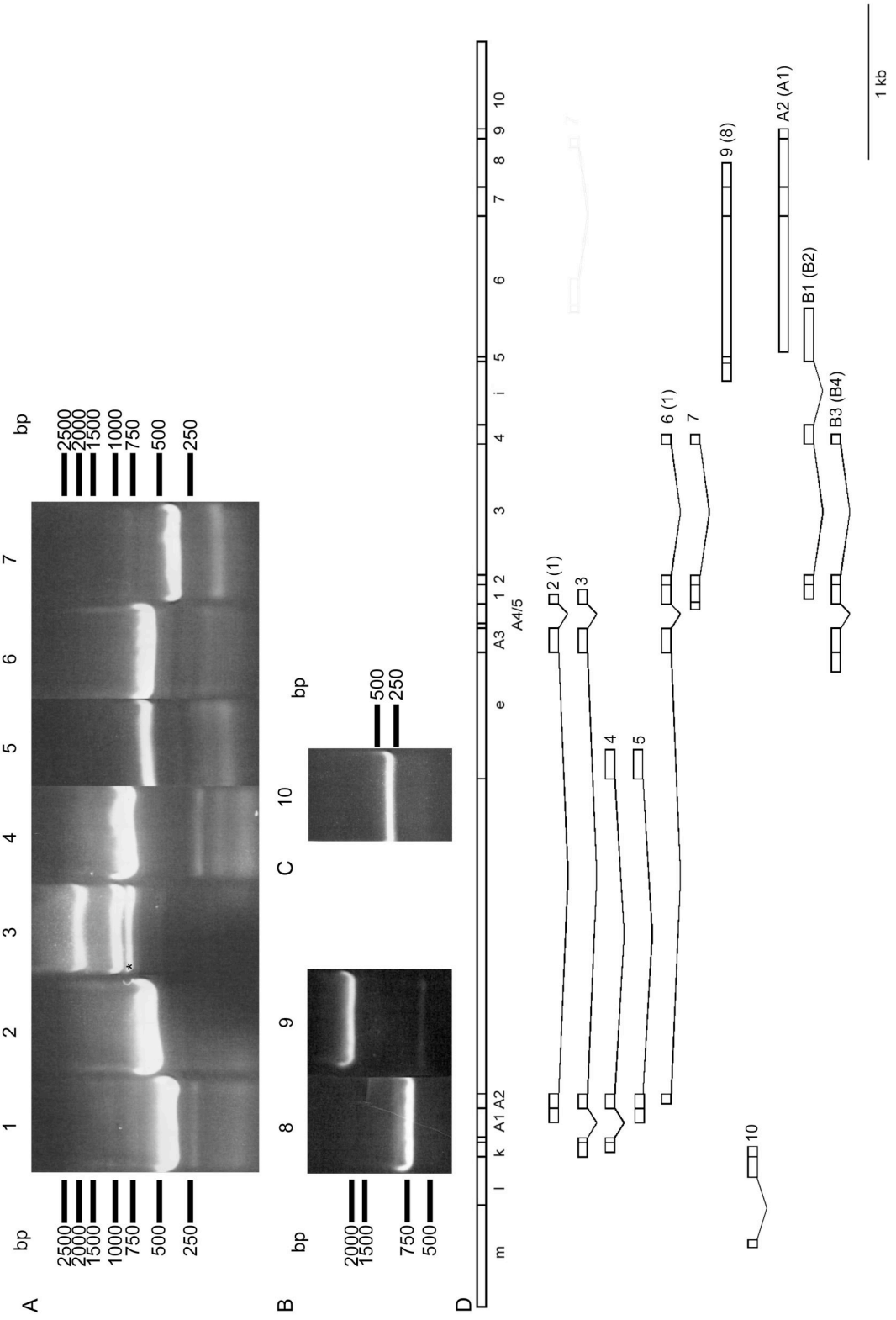

A
bp
2500
2000
1500
1000
750
500
250
1
2
3
4
5
6
7
*
B
bp
2000
1500
750
500
8
9
C
bp
500
250
10
D
m
l
k
A1
A2
e
A3
1
2
A4/5
3
4
i
5
6
7
8
9
10
2 (1)
3
4
5
6 (1)
7
9 (8)
10
A2 (A1)
B1 (B2)
B3 (B4)
1 kb

Figure 3.4. Different variants of 5'-parts of Sdt in the head.

A-C – results of different RT-PCRs using the following primer pairs: (1) – 5'-Sdt-ExA2-1 and 3'-Sdt-Ex1-1, (2) – 5'-Sdt-ExA1-1 and 3'-Sdt-Ex1-1, (3) – 5'-Sdt-k1 and 3'-Sdt-Ex1-1, (4) – 5'-Sdt-k1 and 3'-Sdt-e1, (5) – 5'-Sdt-ExA1-1 and 3'-Sdt-e1, (6) – 5'-Sdt-ExA2-1 and 3'-Sdt-Ex4-1, (7) – 5'-Sdt-A5-1 and 3'-Sdt-Ex4-1, (8) – 5'-Sdt-i2 and 3'- Ex6-RA-44866, (9) – 5'-Sdt-i2 and 3'-SdtPDZSH3, (10) – 5'-Sdt-m1 and 3'-Sdt-k1. Unspecific bands are marked with asterisk.

D – Schematic representation of all amplified fragments. The numbers 1-10 near the fragments corresponds to the products in lines 1-10 on pictures A-C. The product of the middle size is depicted for line 3. B1-B4 correspond to the RT-PCR products in lines 1-4 in Figure 3.3B. A1-A2 correspond to the RT-PCR products in lines 1-2 in Figure 3.3A. When the numbers is written in brackets then the sequence of the product marked by this number lays within the product marked with the number without brackets.

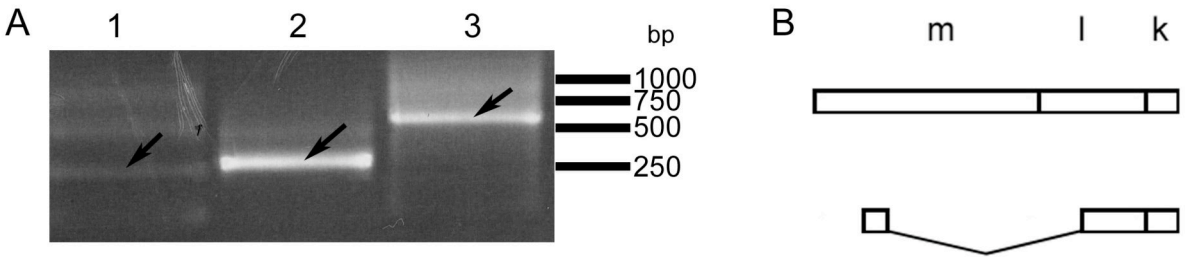


Figure 3.5. Analysis of most 5'-parts of different Sdt mRNAs.

A – 5'-RACE experiments using 3'-Sdt-ExA5-1-RACE (1), 3'-Sdt-l1-RACE (2) and 3'-Sdt-i1-RACE (3). For 1 and 3 the obtained products were equal to the published ones. For 5'-RACE with 3'-Sdt-ExA5-1-RACE (1) four times more amount of mRNA was required to obtain the product. Arrows indicate the specific 5'-RACE products. The other products were unspecific.

B – The schematic representation of 5'-region of Sdt-C-type isoforms. The upper panel shows exons m, l and k as they are predicted in databases. The lower panel shows this part of the mRNA as it was sequenced from 5'-RACE and RT-PCR products.

Combination of obtained sequences results in 6 different splice-variants of Sdt locus expressed in the head (summarized in Figure 3.6). Three of the obtained forms are similar to the Sdt-B isoform and are called Sdt-B1, Sdt-B2 and Sdt-B3. Sdt-B1 corresponds to the already characterised one (Hong *et al.*, 2001). Sdt-B2 has exons A1-A3 in its 5'-part instead of exons A4-A5. Sdt-B3 has exon e in its 5'-part in addition to exons A1-A3. The fourth isoform corresponds to sdt-RD in the FlyBase prediction and will be called Sdt-D. In comparison to Sdt-MAGUK or Sdt-B it lacks both ECR motifs. All other Sdt domains are present in this protein. The fifth and sixth splice-forms are similar to FlyBase sdt-RC and will be called Sdt-C1 and Sdt-C2. In comparison to Sdt-MAGUK they have exons k, l and m instead of exon A1 in their 5'-part. As well, they lack exon 3 as all the forms expressed in the head. Sdt-C2 in comparison to Sdt-C1 carries in addition exon e.

When Sdt-C1 and Sdt-C2 are analysed for the domain structure using the prediction program (Simple Modular Architecture Research Tool – SMART, http://smart.embl-heidelberg.de/), an additional PDZ domain at the N-terminus of the corresponding proteins shows up (see 3.7). It is encoded by amino-acids 8-105 of these proteins.

The sequences of the mRNAs of all these 6 splice-variants of *sdt* as well as of the proteins encoded by them are shown in the Supplementary Data (6.3 and 6.4 correspondently). For the proteins the domains are highlighted. The predicted features of different amplified isoforms are shown in Table 1.

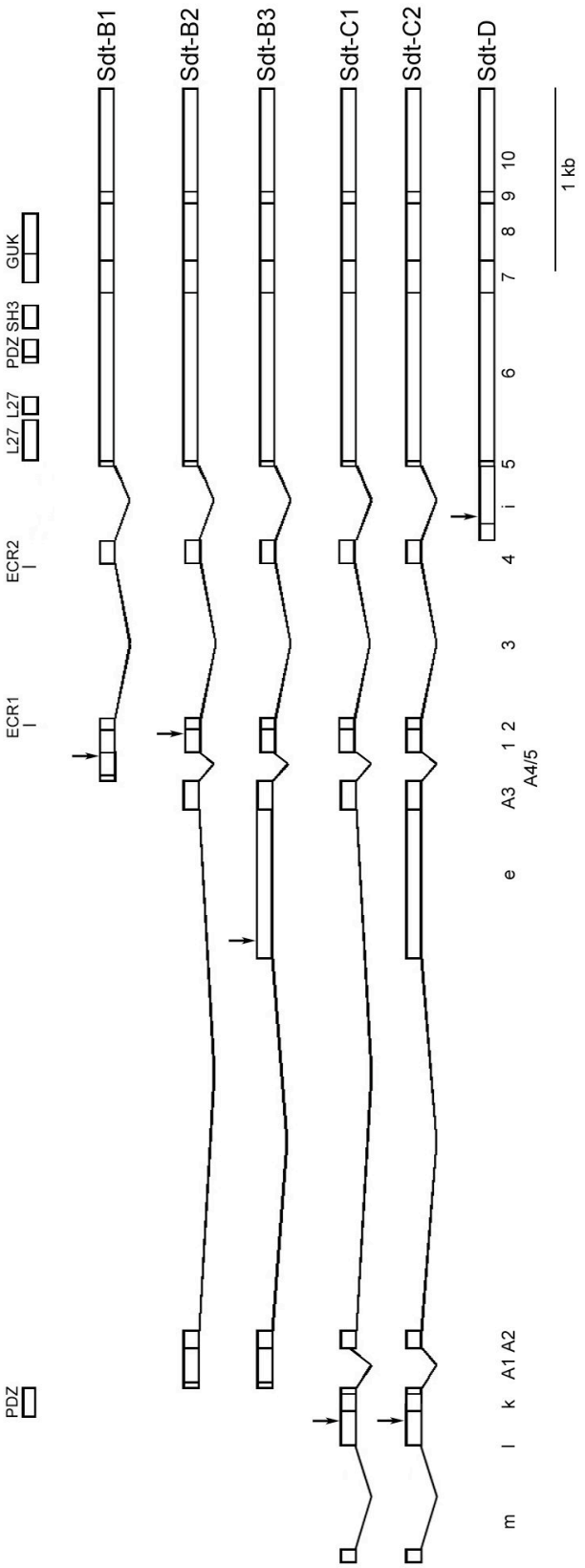


Figure 3.6. Sdt mRNA of six different splice-variants can be amplified from *Drosophila* heads. These forms include Sdt-B1, Sdt-B2, Sdt-B3, Sdt-C1, Sdt-C2 and Sdt-D. Verticals indicate intron/exon boundaries Above the transcripts, the projection of known domains is depicted.. Numbers or letters designate exons under the Sdt-D splice-variant. Arrows mark the predicted position of the translation start-site in each of the splice-variants. For more detailed description of these isoforms see text.

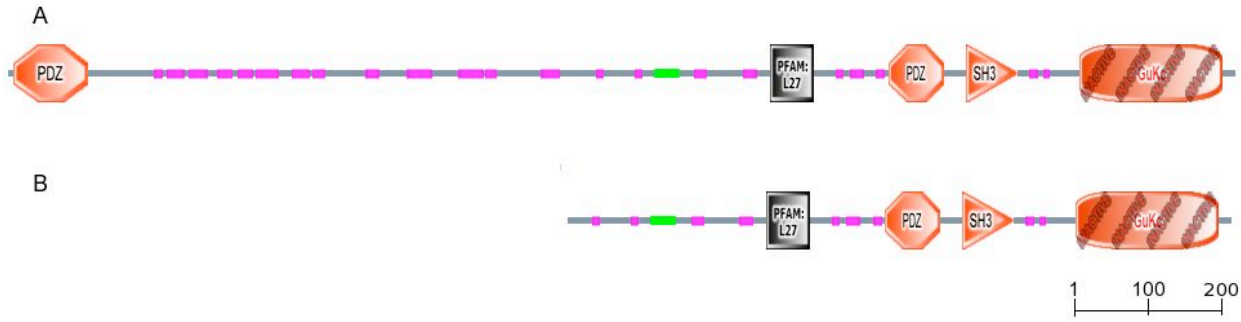


Figure 3.7. Prediction of the domain structure of Sdt-C1 (A) and Sdt-B2 (B) using SMART. The domain constitution of Sdt-C2 is similar to Sdt-C1. Sdt-B1, Sdt-B3 and Sdt-D have the same domains as Sdt-B2. In Sdt-C1 and Sdt-C2 an additional N-terminal PDZ domain is predicted. For all forms, only the N-terminal L27 domain is predicted, but not the C-terminal one. The protein scale in amino-acids is shown below the predictions.

Table1: Features of the six Sdt isoforms amplified from head mRNA:

Isoform:	Predicted transcript size, bp:	Predicted protein size, aa (kDa):	Predicted domains:
Sdt-B1	3984	934 (103)	ECR1, ECR2, L27-N, L27-C, PDZ-C, SH3, Hook, GUK
Sdt-B2	4507	895 (95)	ECR1, ECR2, L27-N, L27-C, PDZ-C, SH3, Hook, GUK
Sdt-B3	5749	1393 (153)	ECR1, ECR2, L27-N, L27-C, PDZ-C, SH3, Hook, GUK
Sdt-C1	5131	1173 (129)	PDZ-N, ECR1, ECR2, L27-N, L27-C, PDZ-C, SH3, Hook, GUK
Sdt-C2	6373	1587 (175)	PDZ-N, ECR1, ECR2, L27-N, L27-C, PDZ-C, SH3, Hook, GUK
Sdt-D	3865	879 (97)	L27-N, L27-C, PDZ, SH3, Hook, GUK

3.1.3 Identification of three Sdt splice variants predominantly expressed in adult head

Both on Northern and Western blots only three products of *sdt* were detected in *Drosophila* heads (see 3.1.1). This leads to the conclusion that out of 6 splice-forms of *sdt* expressed in the heads only three are expressed predominantly. Others are probably expressed in amounts below detection level. To distinguish the forms that are expressed predominantly, additional Northern analysis was performed. Several probes were generated so that they allow to distinguish between the different splice variants. They were complementary to individual exons in the 5'-parts of the different splice variants. These include exon i (for recognition of Sdt-D isoform), exon k (recognises Sdt-C1 and Sdt-C2), exon e (recognises Sdt-C2 and Sdt-B2) and exon A4 (recognises only Sdt-B1 form). As a probe that should recognise all the isoforms the MAGUK-probe was used (see 3.1.1) as this part of the mRNA is present in all splice-variants. The results of the Northern analysis are shown in Figure 3.8. Probes against exons k and e recognise mRNAs of the same size as the biggest mRNA recognized by the MAGUK-probe. That suggests that this mRNA encodes Sdt-C2 isoform, as this is the only splice-variant that contains both exons k and e.

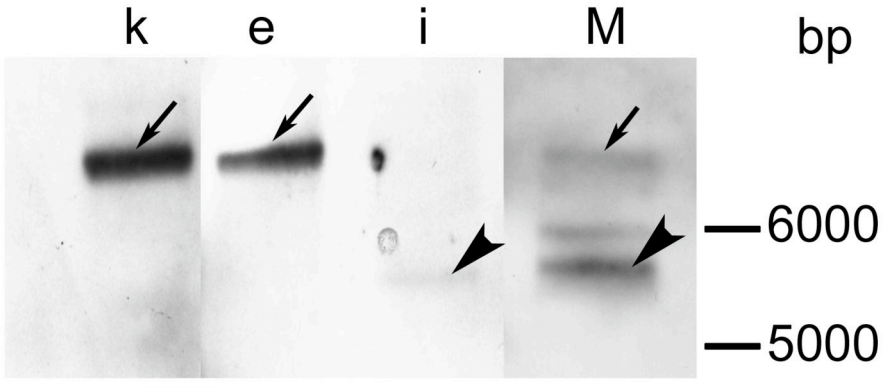


Figure 3.8. Identification of three Sdt splice variants that are predominantly expressed in heads. Northern blots were done using different antisense probes that are complementary to single exons: exon k, exon e, and exon i. As a control MAGUK-probe was used. The large mRNA of more than 6 kb (arrow) is recognized by probes against exons k and e, and MAGUK probe. The mRNA of about 5,5 kb is detected by the MAGUK-probe and the probe against exon i (arrowhead). Using other probes, no mRNA was detected.

The smallest mRNA that is detected by the MAGUK-probe is also detected by the probe representing exon i. The only splice-variant that should be recognised by this probe is Sdt-D. This means that the second predominantly expressed splice-form of *sdt* in *Drosophila* head is Sdt-D. The probe directed against exon A4 has not recognised any mRNA on the Northern blot. That indicates that the third predominantly expressed Sdt splice variant in the head is Sdt-B2.

3.1.4 Identification of two Sdt splice variants predominantly expressed in adult retina

On Western blots done from head lysates three different Sdt proteins were detected. However, in extracts from retinas only two bands out of these three were present, and the largest protein was absent. That indicates that the largest band is specific for head tissue, but is not present in the retina.

The previously generated antibody against Sdt (α-Sdt-PDZ-C, Berger, 2005) recognizes all Sdt isoforms expressed in the head, as all of them have a PDZ-C domain. This antibody can't distinguish between different isoforms of the protein. For this, additional antibodies against Sdt were used.

The first of these, α-Sdt-N antibody, was raised against a protein encoded by amino-acids 7-566 of Sdt-MAGUK isoform. This part includes mainly the region encoded by exon 3, which is absent in all isoforms expressed in the head, but it also includes regions encoded by exons 2 and 4 (the 32 C-terminal amino-acids from exon 2 and the 41 N-terminal amino-acids of exon 4). To check if this antibody can recognize Sdt-B isoforms, its specificity was checked on Western blots. The protein that lacks aminoacids encoded by exon 3, but contains aminoacids 1-256 of Sdt-B2 isoform fused to GST was used to check if the antibody could recognise it on Western blots. Antibody recognises this protein as well as the protein that contains exon 3 on Western blots (Figure 3.9A-B). That means that the few amino-acids provided by exon 2 and exon 4 are sufficient for the recognition of the Sdt-B protein by this serum. Two out of the three predominant isoforms expressed in the head contain exons 2 and 4, and should be visualized by α-Sdt-N.

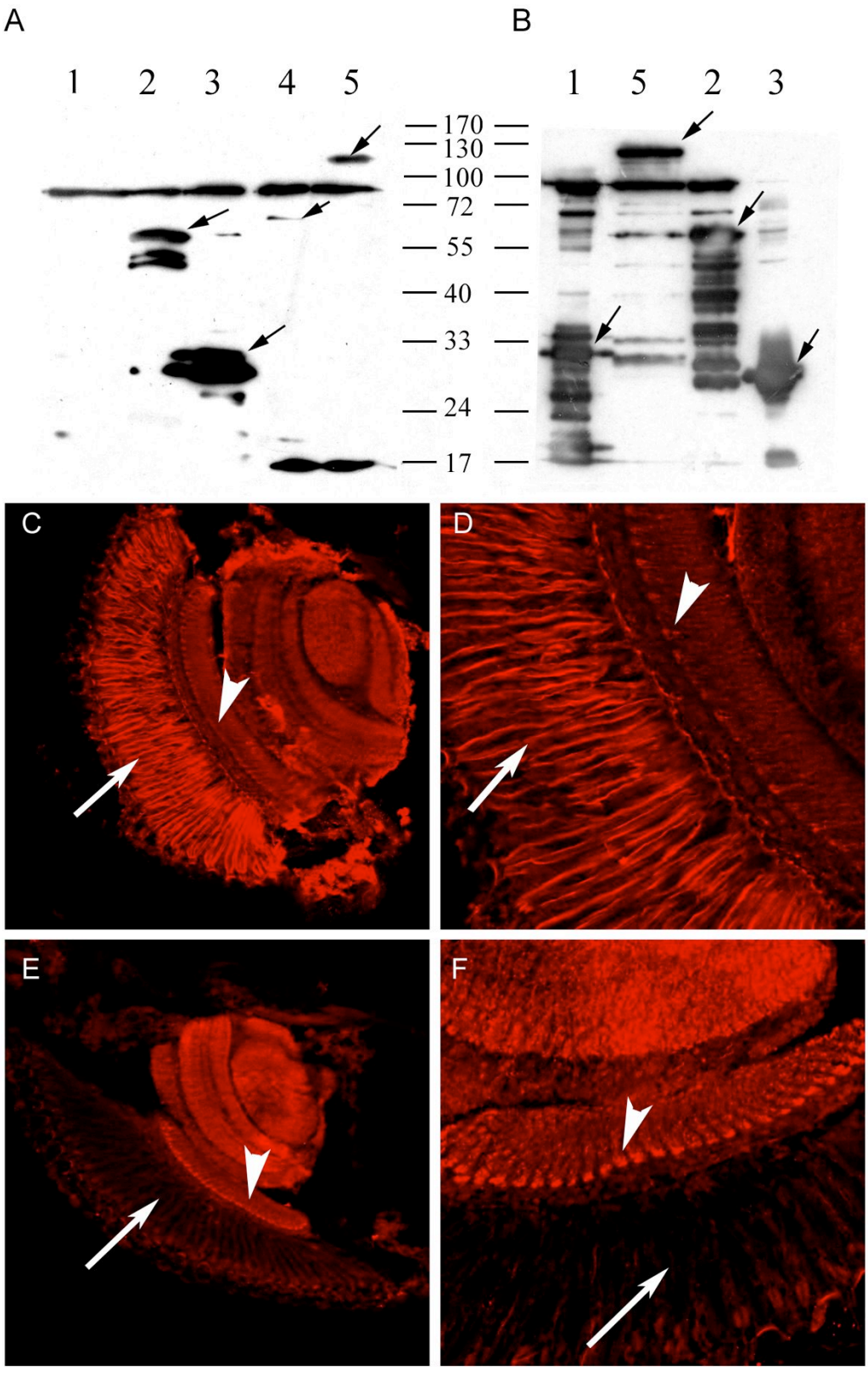

A
B
1 2 3 4 5
1 5 2 3
170
130
100
72
55
40
33
24
17
C
D
E
F

Figure 3.9. Expression of different Sdt isoforms in retina and brain.

Sdt-B isoform is recognized by α-Sdt-N antibody deluted 1:10000 (A) or 1:1000 (B). pGSTag-Stardust(1-256) with cleaved GST off (1), pGSTag-Stardust(1-256) with GST (2), GST (3), GST-SdtN containing GST (5) or with cleaved GST off (4) were used. pGSTag-Stardust(1-256) with cleaved GST off (1) is detected only when antibodies are used in high concentration. Other proteins are detected both in A and B. Black arrows indicate the proteins of interest in Western blots. In all cases the clear unspecific band of the size about 90 kDa is detected.

C-D – α-Sdt-N stains brightly the retina (corresponds to stalk membrane staining). Weak staining can also be observed in the lamina and other brain structures.

E-F –α-Sdt-PDZ-N stains the lamina, other brain regions, but not the retina.

In C-F white arrows mark retina regions. Arrowheads mark the stainings in the lamina.

In the head α-Sdt-N antibody stains stalk membrane in the *Drosophila* PRCs (Figure 3.9C-D, 3.11 A-A'). This staining is specific as it is absent in eyes mutant for sdt^{K85} (Figure 3.11 C-C'). At the same time α-Sdt-N stains also some parts of the brain including the lamina, where PRCs 1-6 project their neurons (Figure 3.9C-D). Additional controls are required to prove that the staining in the brain is specific (Figure 3.9C-D).

The third antibody against Sdt was raised against N-terminal PDZ domain (PDZ-N, Bachmann, 2000). This antibody should recognize only Sdt-C2 out of the Sdt isoforms predominantly expressed in the head. It stains the brain, including the lamina, but does not stain retina (Figure 3.9E-F). This gives a hint that Sdt-C2 can be the isoform that is specifically expressed in the brain, but not in the retina. To prove this, the additional experiments should be done including generation of clones null for Sdt in the brain and adjustment of the Western protocol for this antibody. Another hint that indicates that Sdt-C2 is brain specific is that in Western blots the largest Sdt protein with a size more than 170 kDa is not detected in extracts from retina. Only Sdt-C2 has predicted size about 175 kDa. All other isoforms should result in much smaller proteins.

To summarize the results, it can be concluded that Sdt-B2 and Sdt-D are expressed in the retina. The only important difference between these two isoforms that one of them (Sdt-B2) is able to bind to *D*Par-6 and other cannot (Wang *et al*., 2004). Whether this binding is of any importance in the adult eye is unknown at the present

time. There is a mild possibility left that Sdt-B1 is expressed instead of Sdt-B2, but that does not change the situation on the protein-structure level. In the optic lobes of the brain, Sdt-C2 is expressed. May be it is expressed alone or may be together with one/two other isoforms. It has an additional PDZ domain at its N-terminus – PDZ-N. The function and binding partners of this domain are currently unknown.

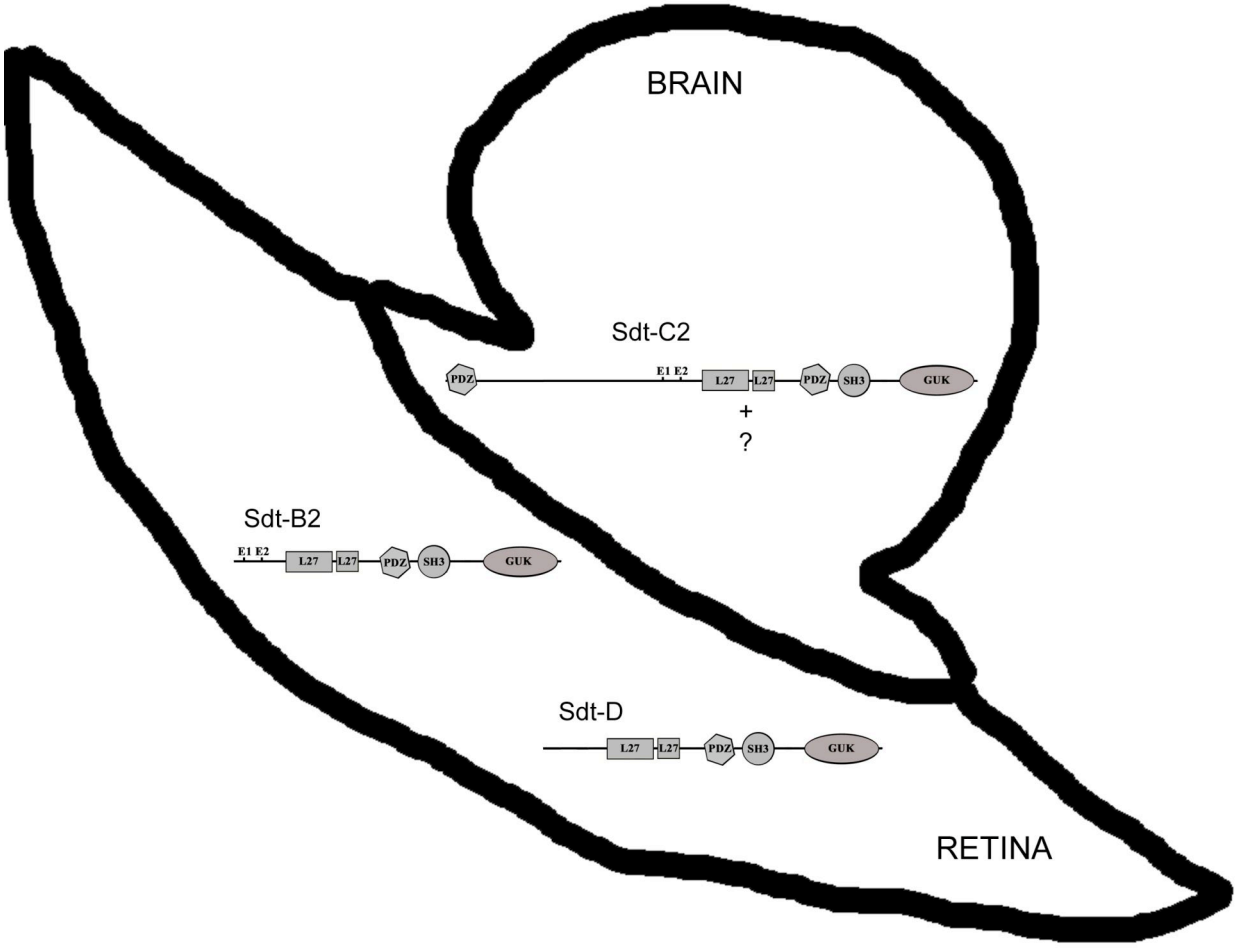


Figure 3.10. Summary of Sdt expression in the head. Sdt-B2 and Sdt-D are expressed in the retina, and are the predominant forms there. In the optic lobe Sdt-C2 is expressed, and is probably the predominant form there.

3.2 Sdt expression in different sdt alleles

Previously, the phenotypes of different *sdt* alleles in the eye were characterized by Berger (Berger, 2005). She analyzed their effects on PRCs morphology, their ability to induce light-dependent retinal degeneration, and their effects on expression and localization of Crb-complex members. According to her work, all *sdt* alleles were divided into four different classes depending on their behaviour in the eye (for more details see 1.4.5). Several important questions were still open. It is not known yet what is the expression of Sdt in these alleles on molecular level. Are there truncated proteins expressed? If no protein is detected on the immunochemistry level is it because there is no protein or because the protein is delocalized? Also, the analysis in the adult eyes should be supplemented by the analysis in the pupal eyes when eye morphology is established. To do this, three different *sdt* alleles were chosen that belong to three different classes of *sdt* mutations: sdt^{K85}, sdt^{XP96} and sdt^{N5} (for more information see 2.1.5.2).

3.2.1 Analysis of protein expression in *sdt* mutant eyes

sdt^{K85} carries a stop-codon in the middle of the L27-N domain and should result in a truncated protein that contains the N-terminal part, but lacks the PDZ, SH3 and GUK domains. Using α-Sdt-PDZ-C antibody no Sdt protein can be detected in PRCs mutant for this allele (Figure 3.11B-B'). Interestingly, no Sdt protein can be detected as well when α-Sdt-N antibody is used (Figure 3.11C-C'). That indicates that the truncated Sdt protein is instable in this mutant. In the second allele analyzed, sdt^{XP96}, no localized protein can be detected using both α-Sdt-PDZ-C and α-Sdt-N antibody (data not shown).

In the case of sdt^{N5}, Sdt is observed at the stalk membrane though in strongly reduced amounts. Furthermore, all three other components of the Crb-complex in the eye – Crb, *D*PATJ, *D*Lin-7 – are as well present at the stalk membrane, but also in reduced amount (Berger, 2005). This mutation should result in a stop-codon at the end of the L27-N domain, and should result in a truncated protein lacking the PDZ, SH3 and GUK domains. The previous analysis was done using α-Sdt-PDZ-C antibody. To

get more information on the nature of the Sdt, mutant eyes were stained using α-Sdt-N antibody. Similar to α-Sdt-PDZ-C antibody, α-Sdt-N antibody resulted in a faint staining at the stalk membrane (Fig. 3.11D-D' and 3.11E-E'). That indicates that both the N-terminus-containing Sdt and PDZ-domain-containing Sdt are expressed in the eyes mutant for this allele.

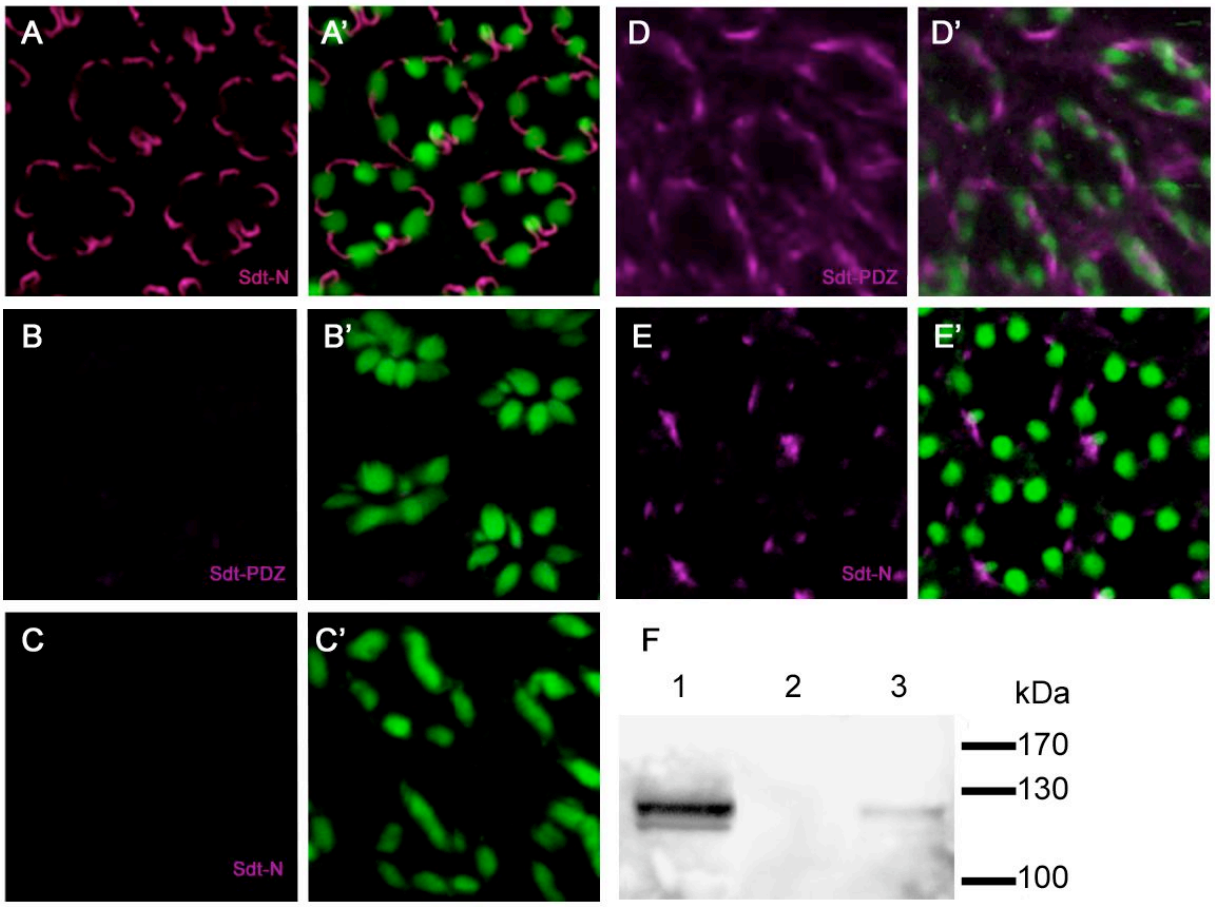


Figure 3.11. Expression of Sdt protein in *sdt*^{K85} and *sdt*^{N5}.

A-A' – α-Sdt-N antibody stains the stalk membranes in the wild type adult PRCs.

B, B', C, C' – In *sdt*^{K85} no Sdt protein can be detected with α-Sdt-PDZ-C (B-B', from Berger, 2005), or with α-Sdt-N (C-C').

D, D', E, E' – In *sdt*^{N5} low amount of protein is detected at the stalk membranes with both α-Sdt-PDZ-C (D-D', form Berger, 2005), and α-Sdt-N (E-E').

In A'-E' rhabdomeres are stained with phalloidin (green).

F – α-Sdt-PDZ-C antibody detects two bands in lysates from adult retinas of wild type flies (lane 1), no protein in lysates from retinas mutant for *sdt*^{K85} (lane 2), and low amount of two proteins in lysates from retinas mutant for *sdt*^{N5}, which have the same sizes as those in wild-type.

To further characterize the protein expressed in *sdt*^{N5}, additional Western blot analysis was performed. Induction of large eye clones (2.2.4.2) allows to obtain nearly 100% mutant retinas. Since it was shown that Sdt protein is expressed in brain (3.1.4), the retinas were carefully separated from the brain tissue, and used for Western analysis. As a control extracts from *sdt*^{K85} mutant retinas were used (Figure 3.11F, lane 2). Indeed, no protein can be detected on the Western blot in the lysates from *sdt*^{K85} mutant eyes. This proves that this mutation is null in the eyes. In lysates from *sdt*^{N5} eyes both retina-specific Sdt isoforms are detected though in strongly reduced amount (Figure 3.11F, lane 3). Both these protein seem to have the same size as the ones in the wild-type lysate (Figure 3.11, lane 1). The only possible explanation of this phenomenon is that the stop-codon introduced in *sdt*^{N5} does not result in 100% translation termination, so that in some cases read-through takes place. Examples of such mutations are already known in *Drosophila* (Chao *et al.,* 2003; Robinson and Cooley, 1997; for review see Namy *et al.,* 2004). To prove that this stop-codon is indeed leaky, additional experiments are required.

3.2.2 Expression of members of the Crb-complex in the pupal eye discs mutant for *sdt*

As the morphology of PRCs is established during pupal development, it was important to study the expression of Sdt and other Crb-complex members in mutant pupal eye discs. To assay the localization of these proteins in the pupal discs, pupal stages between 40% p.d. and 60% p.d. were chosen. These are the developmental stages when pigment cell sorting is already finished, but apical membrane of PRCs is not yet subdivided into rhabdomere and stalk membrane precursors, and the Crb-complex is localized throughout the apical membrane. At these stages, ZA markers like *D*E-Cad or Arm and the Crb-complex are already separated, though sometimes partial overlapping may be detected.

For the generation of *sdt* mutant clones in the pupal discs, the MARCM system was used. It allows the preliminary selection of the pupae that carry proper genotypes under the UV-stereomicroscope.

In *sdt*^{K85} mutant clones no Sdt can be detected apically already at early stages of pupal development (Figure 3.12A-A''). This is in line with very strong morphological defects caused by this mutation, and proves that in this case we deal with a real null mutation. In Figure 3.12A-A'' the staining obtained using α-Sdt-PDZ-C antibody is shown. α-Sdt-N antibody similarly results in no apical staining in mutant PRCs (data not shown). At the same time, both Crb and *D*PATJ are still present apically in the PRCs mutant for *sdt*^{K85} at these pupal stages (Figure 3.12B-B'' and 3.12C-C'' respectively). Crb staining in mutant PRCs looks a bit fainter than in neighbouring wild-type PRCs, but the difference is weak, and may be not put under consideration.

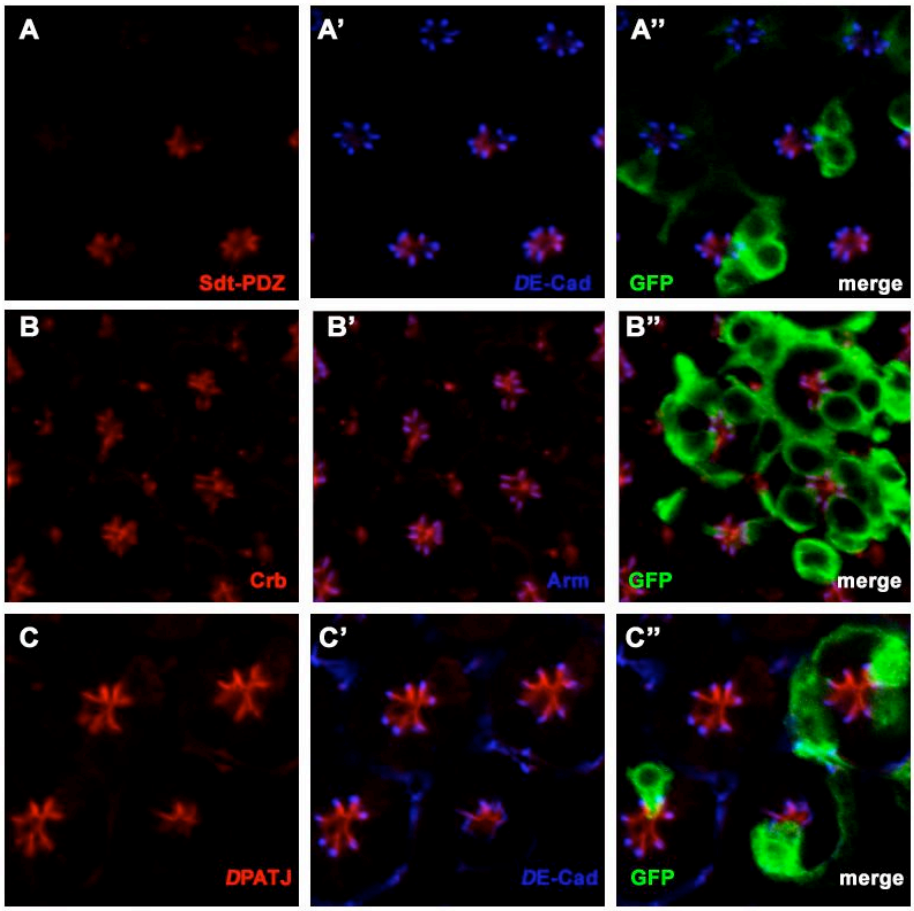


Figure 3.12. Expression of the Crb-complex in pupal *sdt*^{K85} PRCs at 40-60% p.d.
α-Sdt-PDZ-C (A-A''), α-Crb (B-B'') and α-*D*PATJ (C-C'') antibodies were used to stain pupal eye discs carrying the MARCM clones mutant for *sdt*^{K85}. In A', B' and C' staining for the Crb-complex members is shown together with ZA markers *D*E-Cad (A' and C') or Arm (B'). In A'', B'' and C'' GFP in green marks the mutant cells. Though no Sdt can be detected in mutant cells (A-A''), Crb and *D*PATJ are still localized apical (B-B'' and C-C'').

In the second mutant – *sdt*^{XP96} – Sdt protein is still detected apically in the developing PRCs at the stages of interest. But the amount of the detected protein is strongly reduced (Figure 3.13A-A''). Previous studies have already demonstrated that this allele is probably not a null, as no retinal degeneration under constant light exposure is caused by this mutation (Berger, 2005). This is in agreement with observation that some protein is produced. The low amount of detected protein in the pupal discs indicates that this protein is either unstable, or can't be properly transported to the apical membrane. Since no protein can be detected in adult PRCs at the stalk membrane the mutant protein is either fully delocalized from the apical membrane, or its amount stays under detection levels in mutant PRCs. Similar to *sdt*^{K85} Crb was localized apically at 40-60% p.d. in *sdt*^{XP96} (Figure 3.13B-B''). Crb staining was weaker in mutant PRCs in comparison to neighbouring wild-type PRCs at these stages. At earlier stages no difference in the amounts of Crb protein at apical membrane between mutant and wild-type PRCs were detected (around 30% p.d., Figure 3.13C-C'''). This indicates that the initial amount of Crb protein is the same in mutant and wild-type PRCs, but then, starting from mid-pupal development, this amount gradually decreases in mutant PRCs, and in adult eyes no protein can be detected at the stalk membrane of mutant PRCs. A similar situation was observed in the development of the PRCs mutant for a *D*PATJ hypomorphic allele (Richard *et al*., 2006). They show that Crb and Sdt are initially localized apically in proper amounts, but then their amounts decrease in mid-/late-pupal development, and no protein can be detected in adults.

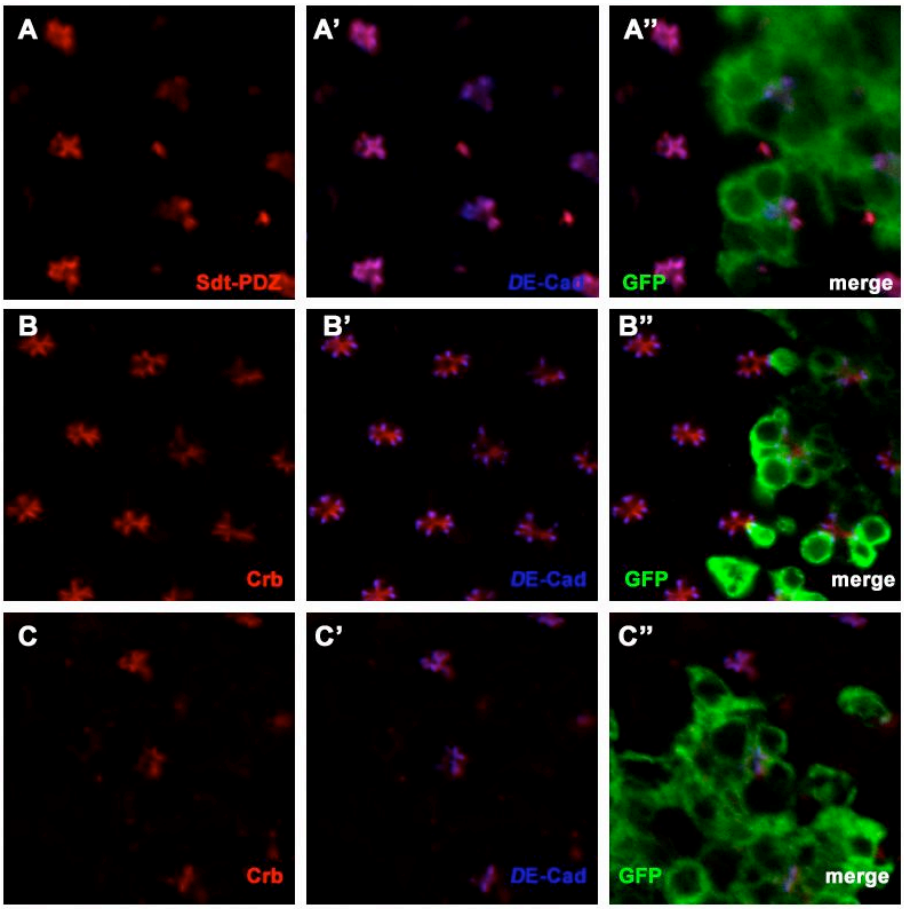


Figure 3.12. Expression of the Crb-complex in *sdt*^{XP96} PRCs in early pupae.

α-Sdt-PDZ-C (A-A'' – 40% p.d.), α-Crb (B-B'' – 50% p.d., C-C'' – 30% p.d.) antibodies were used to stain pupal eye discs carrying the MARCM clones mutant for *sdt*^{XP96}. The amount of Crb is reduced at 40-60% p.d. (B-B''), but is the same as in the wild-type at 30% p.d. (C-C''). In A', B' and C' staining for the Crb-complex members is shown together with ZA marker *D*E-Cad. In A'', B'' and C'' GFP marks the mutant cells in green.

The last allele where the expression of Crb-complex members was analyzed at pupal stage is *sdt*^{N5}. This mutation does not result in any morphological phenotype except for a slight reduction in the length of the stalk membrane (Berger S., personal communication). Moreover, in adult PRCs mutant for this allele, the members of the Crb-complex can still be detected, though in reduced amounts. So, it was logical to assume that the same would be true for the pupal PRCs. Indeed, all three proteins are still present apically in the mutant developing PRCs at 40-60% p.d. (Figure 13.14). The amount of all three proteins seems to be undistinguishable from neighbouring wild-type cells. The question arises if in later stages the amount of these proteins decreases, or if it stays the same. In the latter case the fainter staining would be caused be the dramatic increase in both dimensions of the surface that these proteins should cover.

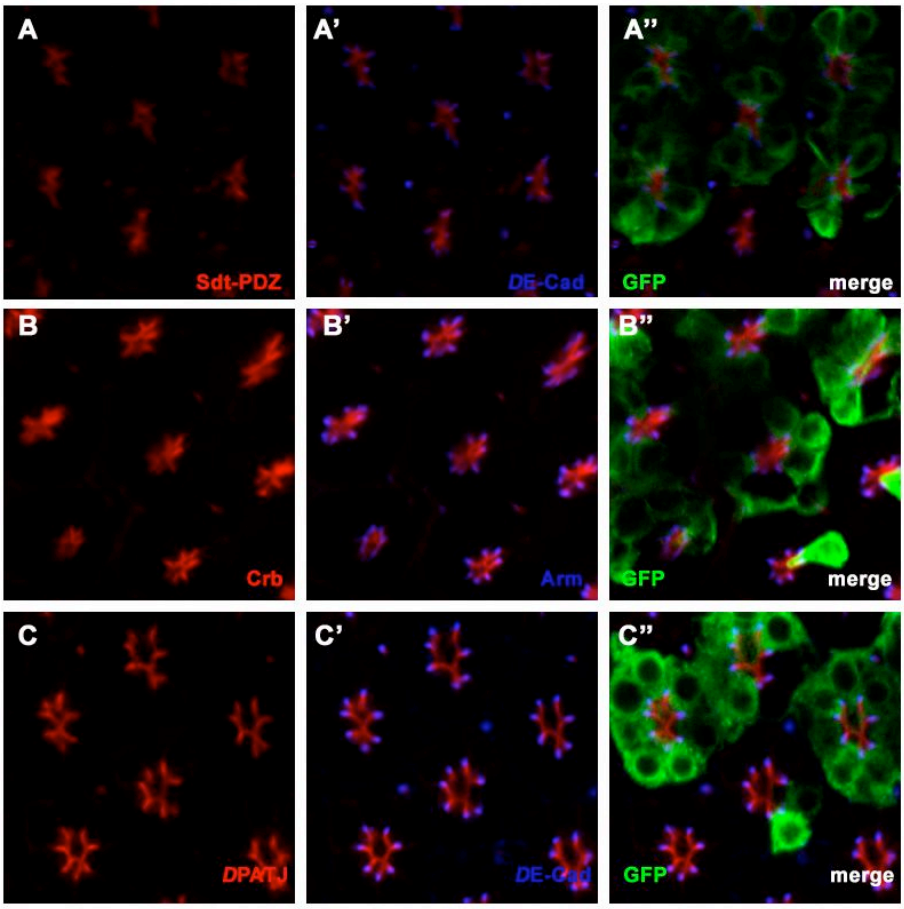


Figure 3.14. Expression of the Crb-complex in pupal *sdt*^{N5} PRCs at 40-60% p.d.
Sdt protein (A-A''), Crb protein (B-B'') and *D*PATJ (C-C'') are present in the amounts comparable to the neighbouring wild type cells. In A', B' and C' staining for the Crb complex is shown together with ZA markers *D*E-Cad (A' and C') or Arm (B'). In A'', B'' and C'' GFP marks the mutant cells (green).

To summarize the obtained data, it can be concluded that the differences in mutant phenotypes between the three analyzed *sdt* alleles are caused by the differences in their molecular nature. In sdt^{K85} no Sdt is present in PRCs throughout development. This results in a null phenotype – both morphological defects and retinal degeneration are observed in the PRCs mutant for this allele. In sdt^{XP96} though the protein is detected apically in the early pupal stages, no Sdt is localized apically later in development. This is sufficient to cause morphological defects observed in PRCs mutant for this allele. Some protein should be expressed but not localized in adult PRCs mutant for this allele, as they do not degenerate when exposed to constant light. In sdt^{N5} protein of proper size is produced and localized at the stalk membrane till the adult stage. This protein is sufficient to establish the proper morphology of the rhabdomeres, but can not prevent light-induced degeneration. The summary on these alleles is demonstrated in Table 2.

Table 2: The comparison of the three analyzed *sdt* alleles:

	sdt^{K85}	sdt^{XP96}	sdt^{N5}
Morphological defects of rhabdomeres	+	+	-
Retinal degeneration	+	-	+
Sdt localization in the adults	-	-	+*
Crb/*D*PATJ localization in the adults	-/-	-/-	+*/+*
Sdt expression in the adults	-	n.d.	+*
Sdt localization in the early pupae	-	+*	+
Crb/*D*PATJ localization in the early pupae	+/n.d.	+*/+	+/+

n.d. – not determined

* - protein is present in reduced amount

3.3 Structure-functional analysis of Sdt

To analyze the role of different domains of Sdt for its localization and function, a transgenic approach was used. Together with Ö.Kempkens several Sdt transgenes were made. They encode either different full-length isoforms of Sdt, or different Sdt variants that lack one or several domains (for example, Sdt-ΔPDZ), or single domains (for example, Sdt-PDZ). All constructs are schematically presented in the Figure 3.15. To be able to distinguish the proteins encoded by the constructs from endogenous Sdt, the transgene-encoded proteins were tagged. FLAG-, 8xMyc- or 3xHA-tags were used either alone or in combinations with each other. All constructs were injected into embryos, and for all of them transgenic flies were obtained. The list of all the transgenic stocks that were obtained including the specification of the tag and exact amino-acids encoded by the constructs in comparison to Sdt-MAGUK is presented in Supplementary Data (see 6.5). For each construct, one line with the strongest expression of the *white* marker gene and an insertion on the third chromosome was chosen. The third chromosome was preferred as all GAL4-drivers are located on the second chromosome, and *sdt* is located on the first. For some constructs a second independent insertion on the third chromosome was used as a control for some experiments. In all cases tested both independent insertions resulted in the same phenotype and the same localization of the construct-encoded proteins.

The first step in the analysis of the effects caused by the expression of the transgenes was to check if transgenes encode the proteins of the expected size. To check this, the constructs were expressed using the *GMR*GAL4 driver, since this driver results in the highest level of expression, and facilitates the detection of the transgene-encoded proteins. The lysates from these heads were probed either with α-Sdt-N or α-FLAG antibodies to detect the transgene-encoded proteins on Western blots. All tested lines express the proteins of about the predicted sizes (Figure 3.16). The transgenes were tested in embryo lysates by Ö.Kempkens, and also resulted in the expression of proteins of the expected sizes (data not shown).

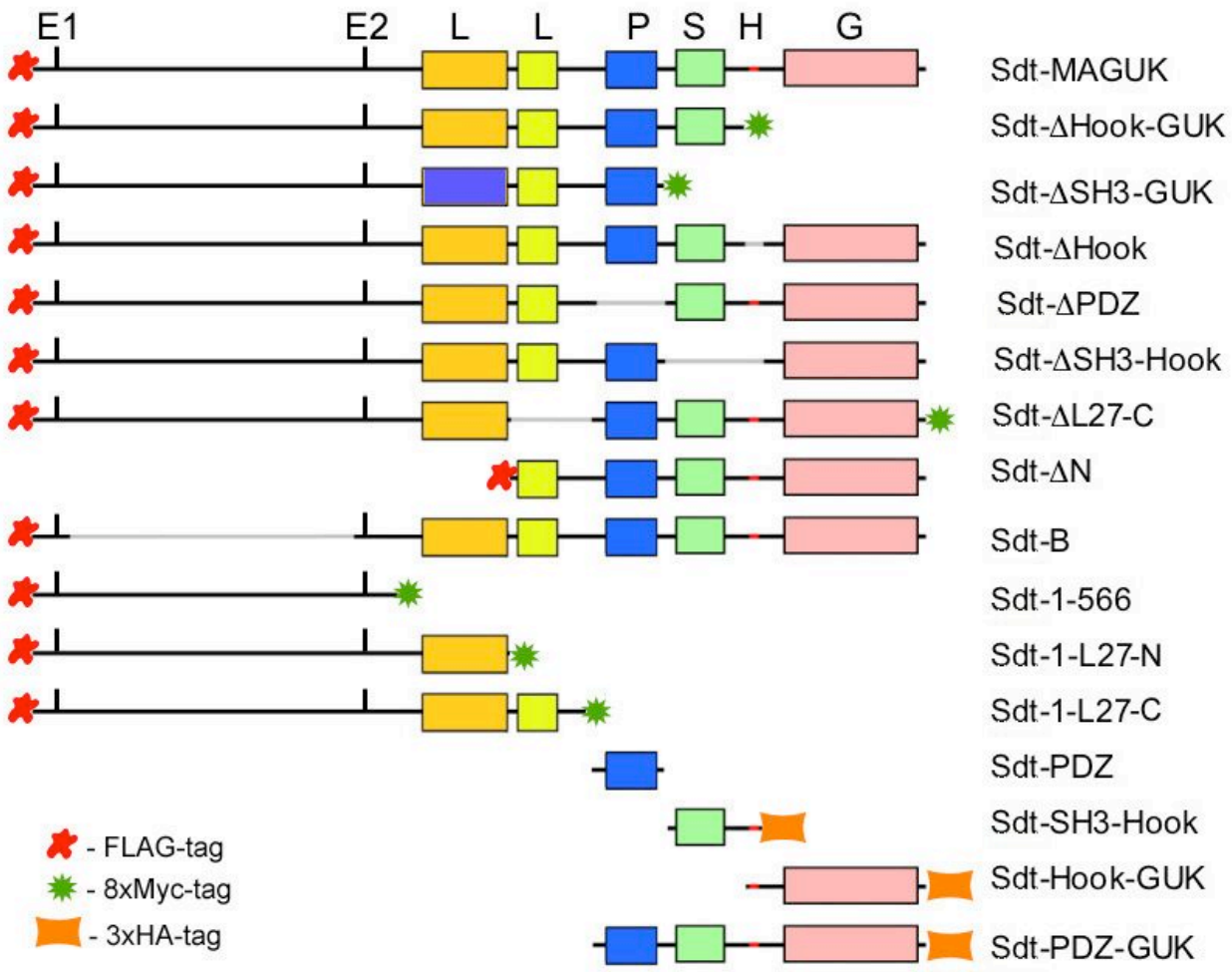


Figure 3.15. Sdt-constructs. The domains are marked. E1 – ECR1, E2 – ECR2, L – L27-domain, P – PDZ-domain, S-SH3-domain, H – putative Hook-domain, G – GUK-domain. The deleted sequences that lay within the constructs are marked with grey line. The tags are indicated.

The following steps were performed to study Sdt function in the eye:

1. Overexpression of the transgenes in a wild type background (in the presence of endogenous Sdt). This allows checking:
   - If the constructs are expressed and the size of the expressed protein is as expected;
   - The localization of the expressed protein in adult eyes or pupal eye discs;
   - If the construct-encoded protein can affect localization of its known interaction partners;
   - If the expression of the constructs results in dominant phenotypes.

2. Expression of the transgenes in a mutant background (in the absence of wild type protein). This allows checking:
   - The localization of the expressed protein in adult eyes or pupal eye discs;
   - If the construct-encoded protein can affect the localization of its known interaction partners;
   - It the expression can rescue the mutant phenotype.

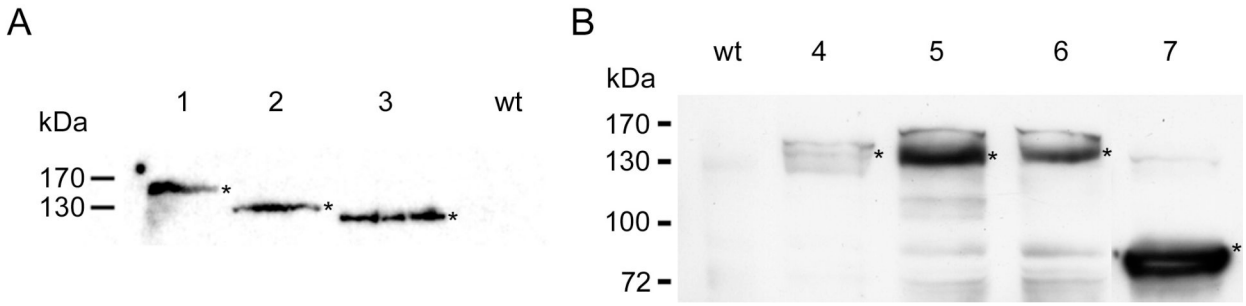


Figure 3.16. Expression of different transgenes using the *GMR*GAL4 driver results in expression of proteins of the expected sizes.

α-Sdt-N (A) or α-FLAG (B) were used to visualize the expressed proteins encoded by the transgenes.

In wild type (wt) both of these antibodies recognize no proteins on Western blots.

When Sdt-MAGUK (1, predicted size 142 kDa), Sdt-ΔHook-GUK (2, predicted size 115 kDa), Sdt-ΔSH3-GUK (3, predicted size 103 kDa), Sdt-ΔPDZ (4, predicted size 125 kDa), Sdt-ΔSH3-Hook (5, predicted size 127 kDa), Sdt-ΔL27-C (6, predicted size 127 kDa), Sdt-ΔN (8, predicted size 67 kDa) are expressed, protein of expected size are detected on Western blots The appropriate bands are indicated with asterisks.

3.3.1 Protein localization in pupal eye discs

The same stages of pupal development as were used for analysis of *sdt* alleles– 40-60% p.d. – were used to analyse the localization and function of the transgene-encoded proteins.

3.3.1.1 Proteins encoded by all constructs localize properly in pupal eye discs in the presence of endogenous Sdt.

First, the localization of different transgene-encoded proteins was checked in an otherwise wild type background. For this purpose, the *GMR*GAL4 driver was used, as it is the only one that drives the expression early enough and is strong enough. Since the Sdt proteins encoded by the transgenes were tagged with different tags, antibodies against these tags were used for the analysis of the protein localization. For the following constructs the localization of the protein encoded by them was apical and indistinguishable from the localization of the endogenous Sdt: both full-length constructs, Sdt-ΔN, Sdt-Δ1-L27-C, Sdt-ΔPDZ, Sdt-ΔSH3-Hook, Sdt-ΔHook-GUK, Sdt-ΔSH3-GUK, Sdt-PDZ, Sdt-1-566, Sdt-1-L27-N and Sdt-1-L27-C. One example is shown in Figure 3.17. From these data it can be concluded that no single domain is absolutely necessary for targeting the Sdt protein to the apical membrane at 40-60% of pupal development in the presence of endogenous Sdt. At the same time the data show that the PDZ domain alone is sufficient for apical localization of Sdt. But 1-566 aa of the Sdt are sufficient as well.

The expression of all the constructs mentioned above had no influence on the localization of other members of the Crb-complex (data not shown). At the same time no expansion or delocalization of DE-Cad or β-catenin was detected, as it was published for *sdt* mutants.

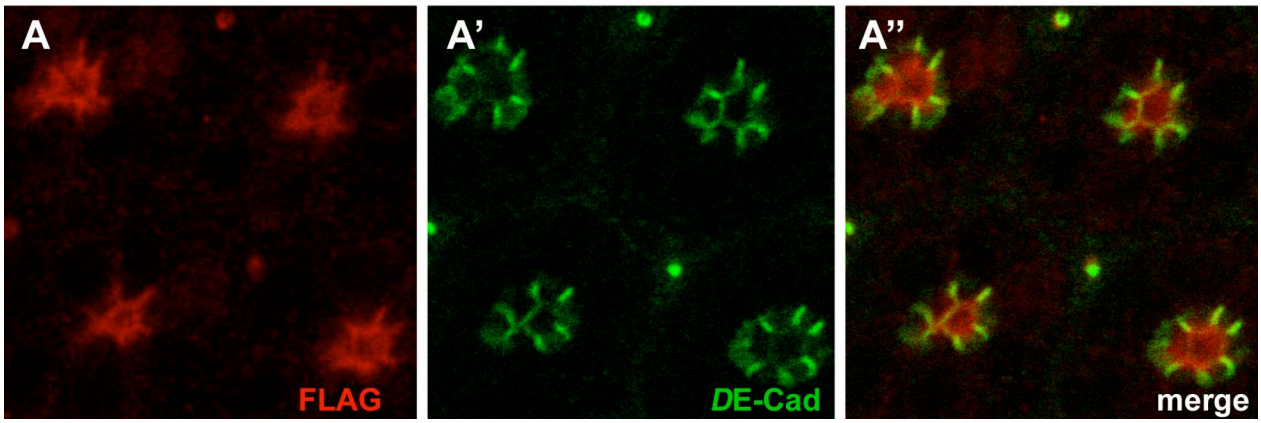


Figure 3.17. All Sdt proteins encoded by different transgenes localize apically during 40-60% p.d. when expressed in a wild-type background. Example for Sdt-ΔPDZ expressed using *GMR*GAL4 is shown in A, in A' the *D*E-Cad staining is shown. No expansion or delocalization of *D*E-Cad were detected when any of the constructs was expressed. Merge is demonstrated in A''.

3.3.1.2 The PDZ domain of Sdt is sufficient and necessary for apical localization of Sdt in pupal eye discs.

Next, the localization of the protein encoded by the different transgenes in the absence of endogenous Sdt was analysed. The MARCM system was used (see 2.2.5.5). sdt^{K85} was used as a *sdt* mutation because it behaves as a null in the eye. It results in both morphological defects and retinal degeneration, and no Sdt protein can be detected in adult PRCs or in pupal eye discs at the stages of interest (see 3.2.2 and Berger, 2005).

Localization of the protein encoded by the following transgenes was analysed in mutant clones: both full-length constructs, Sdt-ΔHook-GUK, Sdt-ΔPDZ, Sdt-ΔSH3-Hook, Sdt-ΔL27-C, Sdt-ΔN, Sdt-1-L27-C, and Sdt-PDZ. Only proteins encoded by Sdt-ΔPDZ and Sdt-1-L27-C were abnormally localized. Proteins encoded by these two constructs were localized not only apically, but also basolaterally. No apical enrichment in protein localization was detected (Figure 3.18A-A''). At the same time defects in Arm localization were observed, similar as they were observed when Crb was overexpressed (Izaddoost *et al.*, 2002). These defects are caused by the

overexpression in the mutant background, but not by *sdt* mutation itself, since in PRCs mutant for this allele no significant defects in *D*E-Cad or Arm localization were detected (see Figure 3.12). Interestingly, no defects in Arm localization were detected when these transgenes were expressed in a wild type background (data not shown). For all other constructs tested, the localization of the proteins encoded by them was indistinguishable from wild type localization. In some cases some protein was detected at the basolateral membrane, but its amount was much less as that of apically localized protein, and can be caused by increased total amount of the Sdt protein in the cells (Figure 3.18B-B''). These data lead to the conclusion that in the absence of endogenous Sdt protein, the PDZ domain of the transgene-encoded Sdt protein is necessary and sufficient for apical Sdt localization.

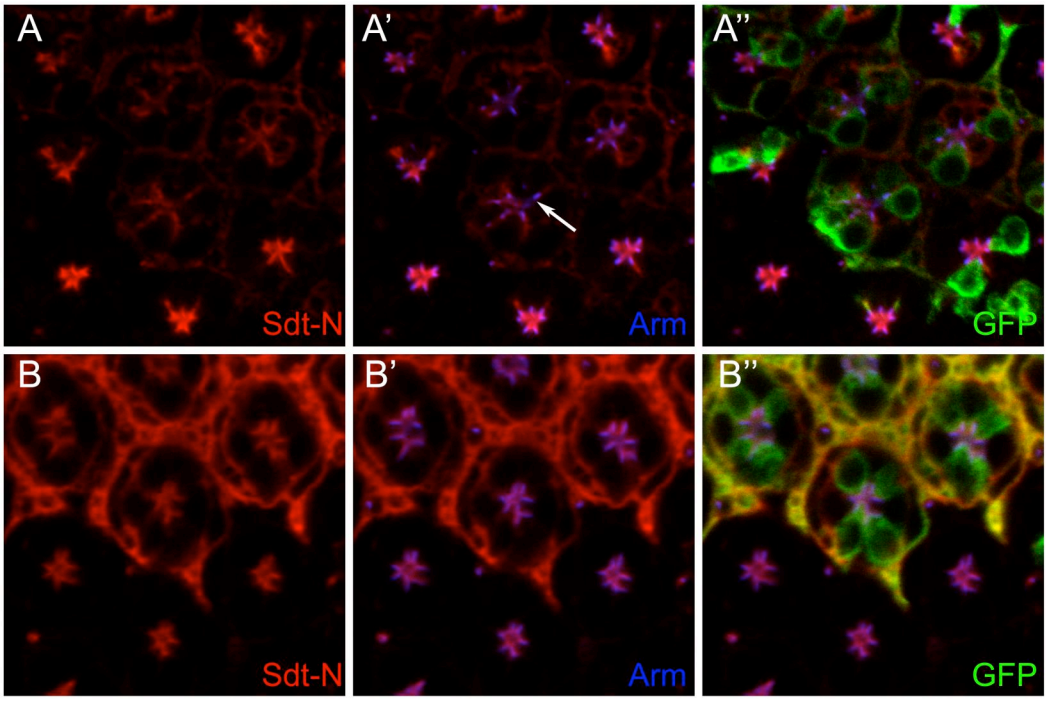


Figure 3.18. The PDZ-domain of Sdt is necessary and sufficient the targeting the protein apically in 40-60% p.d. eye discs in the absence of endogenous Sdt.

A-A'' – α-Sdt-N (red), α-Arm (blue) and α-GFP (green) staining of the pupal eye disc at 45% p.d. carrying MARCM clone that is mutant for sdt^{K85} and expresses Sdt-ΔPDZ. The Sdt is localized ubiquitously around all plasma membrane. In A' the expansion of Arm localization is indicated by an arrow. GFP marks mutant clones

B-B'' - α-Sdt-N (red), α-Arm (blue) and α-GFP (green) staining of the pupal eye disc at 45% p.d. carrying MARCM clone that is mutant for sdt^{K85} and expresses Sdt-ΔN. When PDZ-domain is present, transgene-encoded Sdt is localized apically.

Whenever the transgene encodes Sdt localized apically, except for Sdt-PDZ, the amount of apically localized Sdt was equal or slightly reduced in comparison to neighbouring wild type cells. At the same time the expression level in pigment cells was higher than the level of the endogenous Sdt in the wild-type pigment cells in the same disc (Figure 3.18B-B''). In the case of Sdt-PDZ the overall level of expression was much lower than in other cases. This may indicate that in the developing PRCs, but not in pigment cells at 40-60% p.d., a mechanism exists that controls the level of Sdt protein in the cells.

3.3.1.3 Apical localization of Crb and *D*PATJ is independent of Sdt

Next, the influence of Sdt on the localization of other members of the Crb-complex was checked. When Sdt-ΔPDZ was expressed, Crb protein was still localized apically as in the wild-type PRCs, though no Sdt protein can bind to Crb in this case (Figure 3.19A-A''). These data, together with the observed apical localization of Crb in pupal PRCs mutant for sdt^{K85}, demonstrate that binding of Crb to Sdt is not required for Crb apical localization in developing PRCs at 40-60% p.d. Similarly, when this transgene was expressed, *D*PATJ was localized properly (3.19B-B''), though Sdt encoded by the transgene was delocalized (see 3.3.1.2) and able to bind to *D*PATJ since it has the L27-N domain. Protein encoded by Sdt-ΔPDZ is able to bind to *D*PATJ in adult PRCs (see 3.3.3.2), since both proteins co-localize and are delocalized from the apical membrane. This indicates that in pupal eye discs before 60% of p.d. Sdt protein has no influence on localization of *D*PATJ. It was also shown that *D*PATJ localization is independent of Sdt at this stage of pupal development by expressing Sdt-ΔN transgene. Though the protein encoded by this transgene is localized apically, it cannot bind to *D*PATJ since it lacks the L27-N domain (see also 3.3.3.2). This means that in this case there is no Sdt protein that can bind to *D*PATJ. Despite this, *D*PATJ is localized apically as in the neighbouring wild-type PRCs (Figure 3.19C-C''). This confirms that apical localization of *D*PATJ at these stages is independent of Sdt.

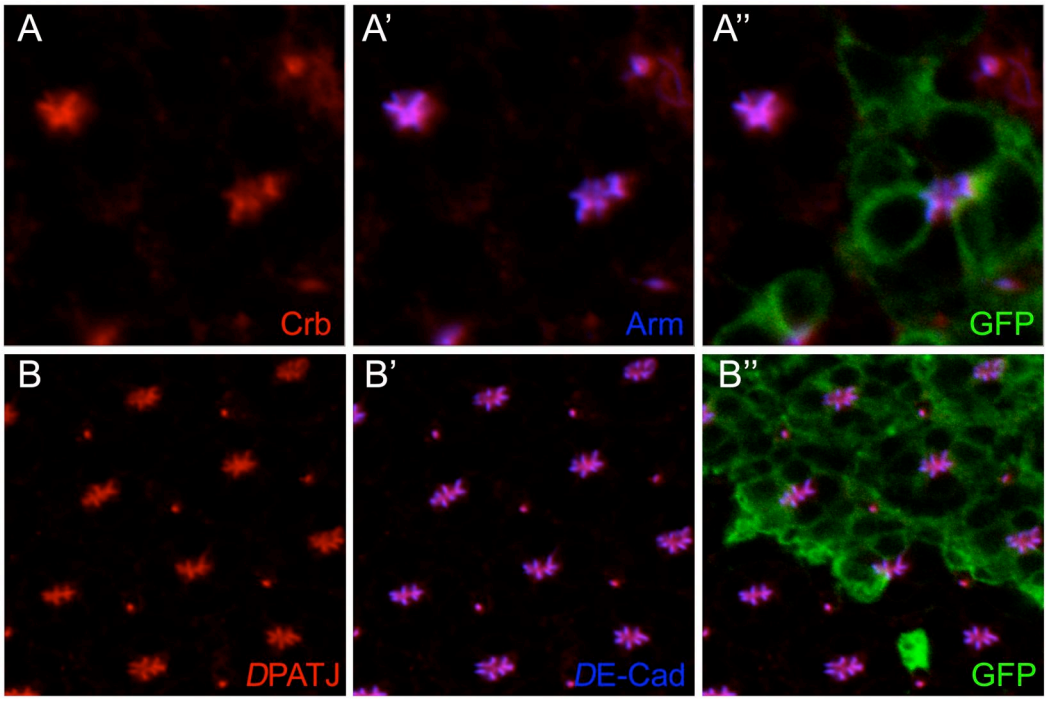


Figure 3.19. Crb and *D*PATJ apical localization does not depend on Sdt in 40-60% pupal eye discs. Crb (A-A'') and *D*PATJ (B-B'') localize apically in clones mutant for sdt^{k85} and expressing Sdt-ΔPDZ. In A' localization of Arm is demonstrated. In B' *D*E-Cad marks ZA. GFP in green marks *sdt* mutant clones that express the construct in A'' and B''.

3.3.2 Protein localization in adult eyes

3.3.2.1 The intact L27-C-GUK part is required for the efficient localization of the protein to the stalk membrane in the adult eyes

The constructs were expressed in wild type background using either *GMR*GAL4 or *Rh1*GAL4 drivers. The *GMR*GAL4 drives expression starting from the third instar larvae stage in all cells behind the morphogenetic furrow. The *Rh1*GAL4 drives expression in R1-R6 PRCs starting from the late pupal development (about 70% p.d.). As the *Rh1*GAL4 shows rather weak expression, two copies of the driver and effector lines were used to increase the signal and to be able to visualize the expressed proteins. To assay the localization of the transgene-encoded proteins the antibody recognizing the tag, fused to the proteins, was used.

Expression of Sdt-MAGUK, Sdt-B and Sdt-ΔN resulted in proteins localized at the stalk membrane. Upon Sdt-B and Sdt-ΔN expression some protein was also present in other parts of the cells. This may be caused by higher expression levels of these transgenes and excess of protein in the cells. Expression level may be connected with the site at which the P-element is integrated, and can be scored by the expression of the marker gene *white*. In the case of Sdt-B and Sdt-ΔN, the eye colour of heterozygous animals was darker in comparison to Sdt-MAGUK. Anyway, most of the protein encoded by these three constructs was localized at the stalk membrane. This results shows that full-length Sdt, when overexpressed in a wild-type background, goes to the stalk membrane in adult eyes, and the technique allows to investigate the mechanisms of Sdt localization. On other side, these data indicate that in the presence of wild type protein, the N-terminus of Sdt-MAGUK isoform is not important for the localization of protein, and hence targeting of Sdt to the stalk membrane of adult eyes is independent of binding to *D*PATJ and *D*Par-6. At the same time it gives an indication that both isoforms expressed in the retina are localized to the stalk membrane as they both have the L27-C till GUK part of Sdt-MAGUK, which is also contained in Sdt-ΔN. An example of the localization of the proteins encoded by these three construct is shown in Figure 3.20A-A'.

The remaining constructs can be grouped into three classes according to the severity of delocalization of the protein encoded by each of them. Localization was affected least in Sdt-ΔSH3-Hook, Sdt-ΔHook-GUK, and Sdt-ΔSH3-GUK. Large portion of the protein was localized to the stalk membrane. The rest of the protein accumulated at the rhabdomere base. Example of such localization is presented in Figure 3.20B-B'. The proteins encoded by Sdt-ΔPDZ or Sdt-1-L27-C constructs represent the next type of localization. The protein was localized ubiquitously at all plasma membranes including the basolateral membrane compartment (Figure 3.20C-C'). This is very similar to what was detected for these constructs at pupal stage when they were expressed in a mutant background. And last, in case of Sdt-ΔL27-C the protein mainly stayed in the cytoplasm (Figure 3.20D-D'). The same cytoplasmic localization was observed for all remaining short constructs (Sdt-PDZ and Sdt-1-L27-N). Upon Sdt-ΔPDZ and Sdt-ΔL27-C expression nearly no protein was detected at the stalk membrane, when they were expressed using *GMR*GAL4. In contrast, when *Rh1*GAL4 was used, some protein enrichment at the apical membrane was detected. As example, protein localization for Sdt-ΔL27-C expressed using *Rh1*GAL4 is shown on Figure 3.20E-E'. These data indicates that these proteins can reach the stalk membrane, but its delivery is inefficient. The difference between localization for different driver-lines can be explained by the difference in their strength of expression. One copy of *GMR*GAL4 leads to stronger expression then two copies of *Rh1*GAL4 (this speculation is based on the relative strength of staining).

In several cases it was possible to visualize the endogenous Sdt separately from the one encoded by the transgene (for example, using α-Sdt-PDZ-C antibody for Sdt-ΔPDZ, or using α-Sdt-N antibody for Sdt-ΔN construct). In all these cases the endogenous protein was localized correctly at the stalk membrane (data not shown). These data demonstrate that at least in these cases there is no intra-molecular interaction between different Sdt molecules, and between proteins encoded by the transgenes and endogenous ones.

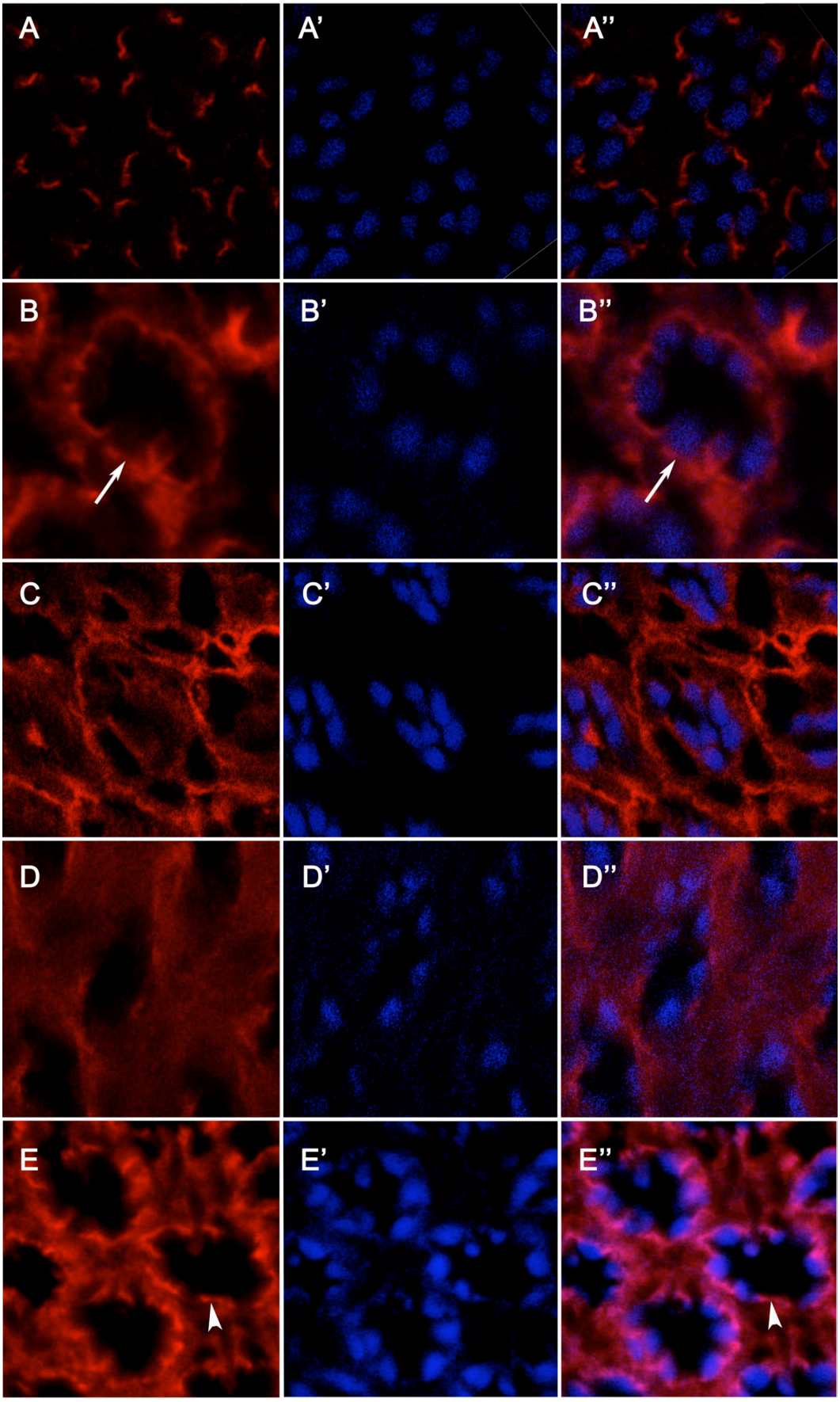

A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''

Figure 3.20. Localization of the proteins encoded by Sdt constructs in adult eyes in the presence of endogenous protein. The examples for the following transgenes are shown:
A-A'' – Sdt-MAGUK expressed using *GMR*GAL4.
B-B'' – Sdt-ΔHook-GUK expressed using *GMR*GAL4. Localization at the rhabdomere base is marked by arrow.
C-C'' – Sdt-ΔPDZ expressed using *GMR*GAL4.
D-D'' – Sdt-ΔL27C expressed using *GMR*GAL4.
E-E'' – Sdt-ΔL27-C expressed using *Rh1*GAL4. Some enrichment at the stalk membrane is detected (arrowhead), although a lot protein is within the cytoplasm.
In A-E staining with α-FLAG antibody is shown. These antibodies recognize specifically the proteins encoded by the constructs. In A'-E' rhabdomeres are stained with phalloidin. In A''-E'' the merged pictures are depicted.

To conclude, all parts of the Sdt protein including the L27-C to the GUK domain are required for efficient delivery of the protein to the stalk membrane. When any domain in this region is absent, the protein can no longer efficiently localize to the stalk membrane. The L27-C domain is important for the transition from the cytoplasm to the membrane, as when this domain is absent, most of the protein stays in the cytoplasm of the PRCs. The PDZ domain is required for localization of Sdt protein to the apical membrane compartment. When the PDZ domain is missing, most of the transgene-encoded protein is localized throughout all the plasma membrane. The GUK and the SH3 domains together are important for the restriction of the localization to the stalk membrane. When one of them or both are absent, the protein is localized at the stalk membrane and at the rhabdomere base. At the same time this requirement is not obligatory, since some protein can reach the stalk membrane in all these cases.

3.3.2.2 *D*PATJ and *D*Lin-7, but not Crb, delocalize together with Sdt

The next question was how the ectopic localization of construct-encoded Sdt-proteins influences the localization of its known direct interaction partners. To address this question, stainings with α-Crb, α-*D*Lin-7 and α-*D*PATJ antibodies were done.

In all cases, Crb was localized at the stalk membrane in the adult eyes, independent of the localization of transgene-encoded Sdt protein. As an example, Crb

localization in the eye overexpressing Sdt-ΔL27-C is shown on Figure 3.21A-A''. This indicates that the mechanism to localize Crb to the apical membrane is independent on Sdt.

For *D*PATJ and *D*Lin-7 the situation was different. These two proteins fully co-localized with Sdt encoded by the transgenes with several exceptions (example in Figure 3.21B-B''). *D*Lin-7 was properly localized when any of the transgenes lacking L27-C were expressed. Example for Sdt-ΔL27-C is shown in Figure 3.21C-C''. *D*PATJ was not delocalized when Sdt lacking the L27-N domain was expressed. As an example, Sdt-ΔN expressed by *GMR*GAL4 is shown. For this construct when the flies were kept at 25°C the expression level was high enough so that some of transgene-encoded Sdt protein was localized not at the stalk membrane but in ectopic sites. However, *D*PATJ was never observed in these ectopic sites together with Sdt (Figure 3.21D-D''), although *D*Lin-7 was (3.21E-E''). Previously the bindings of the Sdt L27-N domain to *D*PATJ and of the L27-C domain to *D*Lin-7 were shown only in *in vitro* studies like yeast two-hybrid binding assay and GST-pull down. These data now give the first *in vivo* confirmation of these bindings. The localization of transgenes-encoded Sdt proteins and its interaction partners is summarized in Table 3.

Figure 3.21. *D*PATJ and *D*Lin-7 but not Crb delocalizes together with Sdt.

A-A'' – Sdt-ΔL27-C expressed using *Rh1*GAL4. Crb (green) localizes to the stalk membrane though Sdt (red) encoded by the transgene is mostly delocalized and able to bind Crb.

B-B'' – Sdt-ΔPDZ expressed using *GMR*GAL4 *D*PATJ (green) is delocalized around all plasma membrane together with transgene-encoded Sdt (red).

C-C'' – Sdt-ΔL27-C expressed using *Rh1*GAL4. *D*Lin-7 (red) is localized at the stalk membrane, although Sdt (green) encoded by the transgene is mostly delocalized.

D-D'' – Sdt-ΔN expressed using *GMR*GAL4. *D*PATJ (green) is localized at the stalk membrane, although Sdt (red) encoded by the transgene is partially delocalized.

E-E'' – Sdt-ΔN expressed using *GMR*GAL4. *D*Lin-7 (green) is partially delocalized together with Sdt (red), encoded by the transgene.

α-FLAG stains the proteins encoded by the constructs in A, B, D and E. In C' α-Sdt-N was used to stain both endogenous protein and protein encoded by the construct. Stalk membrane staining in this case is due mainly to the localization of the endogenous protein there. Rhabdomeres are stained with phalloidin (blue). In A''-E'' the merged pictures are depicted.

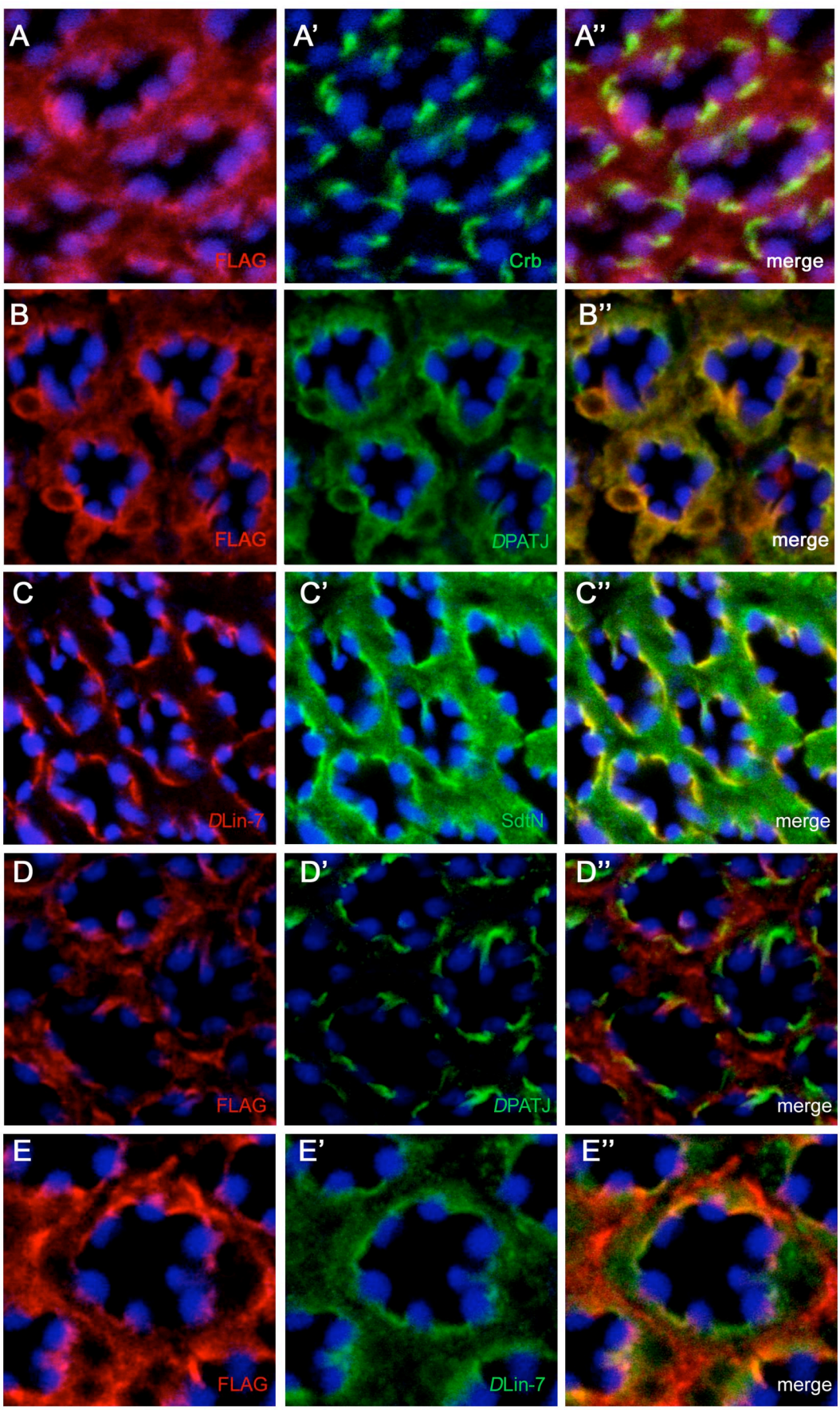

A
FLAG
A'
Crb
A''
merge
B
FLAG
DPATJ
B''
merge
C
DLin-7
C'
SdtN
C''
merge
D
FLAG
D'
DPATJ
D''
merge
E
FLAG
E'
DLin-7
E''
merge

Table 3. Localization of the transgene-encoded Sdt proteins and their interaction partners in adult eyes upon overexpression.

Construct: Tag[1]	Protein localisation in adult eyes				
	Construct-encoded Sdt	Endogenous Sdt	*D*PATJ	Crb	*D*Lin-7
Sdt-MAGUK FLAG	wt	n.d.	wt	wt	wt
Sdt-B FLAG	wt	n.d.	wt	wt	Wt
Sdt-ΔGUK-Hook FLAG	sm+rbase	n.d.	sm+rbase	wt	
Sdt-ΔSH3-GUK FLAG	sm+rbase	n.d.	sm+rbase	wt	sm+rbase
Sdt-ΔPDZ FLAG	membrane	wt*	membrane	wt	membranes
Sdt-ΔSH3-Hook FLAG	sm+rbase	n.d.	sm+rbase	wt	sm+rbase
Sdt-ΔL27-C FLAG and 8xmyc	cytoplasmic	n.d.	cytoplasmic	wt	wt
Sdt-ΔN FLAG	wt	wt**	wt	wt	wt
Sdt-1-L27-N FLAG and 8xmyc	cytoplasm	n.d.	cytoplasm	wt	n.d.
Sdt-1-L27C FLAG and 8xmyc	cytoplasmic	n.d.	cytoplasmic	wt	n.d.
Sdt-PDZ 3xHA	cytoplasmic	wt**	wt	wt	cytoplasmic

[1] – the tags that were used for the visualization of the construct-encoded proteins.

sm – stalk membrane, rbase – rhabdomere base, wt – wild type, n.d. – not determined

* - determined using α-Sdt-PDZ antibody.

** - determined using α-Sdt-N antibody.

3.3.2.3 Multiple domains of the Sdt protein are required for the stabilization of the Crb-complex at the stalk membrane

The next question to answer was what happens in adult eyes with the construct-encoded Sdt protein expressed in a mutant background, and what is the influence of the expression of different constructs on the assembly of the Crb-complex at the stalk membrane in adult PRCs. For this the MARCM system was used. In the PRCs mutant for the chosen *sdt* allele – sdt^{K85} – none of the members of the Crb-complex is properly localized.

The first important question was if full-length Sdt-MAGUK or Sdt-B can, in absence of other Sdt isoforms, localize to the stalk membrane in adult eyes. Indeed, both proteins were localized correctly to the stalk membrane (Figure 3.22A-A' and 3.22B-B'). Moreover, they both were able to fully restore the Crb-complex in the mutant PRCs. The members of the Crb-complex analyzed include Crb, *D*PATJ, *D*Lin-7 and β-spectrin. All of these proteins are localized correctly at the stalk membrane in the PRCs that are mutant for sdt^{K85} and express either Sdt-MAGUK or Sdt-B (Figure 3.22C-F and 3.22C'-F'). It gives hope, that these transgenes will also rescue the mutant phenotype, as all proteins seem to be present where they should be. Additional experiments are required to prove this.

The next construct analysed is Sdt-ΔL27-C. The only difference of the protein encoded by this transgene from Sdt-MAGUK is that it can't bind to *D*Lin-7. The protein encoded by this construct was also localized at the stalk membrane. For further analysis, two different antibodies against Sdt were used – α-Sdt-PDZ-C and α-Sdt-N (Figure 3.23A-A' and 3.23B-B'). α-Sdt-N antibody resulted in staining that has a similar or even brighter level as in the neighbouring wild-type PRCs. At the same time, α-Sdt-PDZ-C resulted in a weak staining that was much fainter than in neighbouring wild-type cells. This may be explained by using the data about the Sdt isoforms expressed in wild-type eyes. Only one of them – Sdt-B2 – but not Sdt-D should be recognized by α-Sdt-N antibody. At the same time, Sdt-B2 lacks sequence encoded by exon 3, but Sdt-ΔL27-C has this sequence. This sequence was included in the protein that was used for raising α-Sdt-N antibody and should be recognized by them. So α-Sdt-N antibody

should bind to Sdt-ΔL27-C much stronger then to Sdt-B2 that represents only part of the Sdt proteins in PRCs.

Other Crb-complex members were localized to the stalk membrane, when Sdt-ΔL27-C was expressed in sdt^{K85} mutant PRCs, except for *D*Lin-7 (3.23C-F and 3.23C'-F'). These data confirm the binding of L27-C to *D*Lin-7, and indicate that *D*Lin-7 is not required for complex assembly/maintenance at the stalk membrane. It was shown that in *D*Lin-7 mutant eyes other Crb-complex proteins are localized as in wild type. Also mutations of the *D*Lin-7 have no effect on eye morphology (A. Bachmann, personal communication). We speculate that when Sdt-ΔL27-C is expressed in sdt^{K85} mutant PRCs, at least the morphological defects induced by this mutation should be rescued.

When any of the other constructs was expressed, neither Sdt protein nor any other protein of the Crb-complex was detected at the stalk membrane or in other cell compartments (example is demonstrated at Figure 3.24). The obtained stainings are the same as those of sdt^{K85} mutant PRCs. It indicates that the proteins encoded by these constructs as well as all Crb-complex components are unstable at the stalk membrane in adult PRCs.

β_H-spectrin localization was analyzed for all constructs when they were expressed. In the wild type it localizes to the stalk membrane (GFP-negative part in Figure 3.22F-F', 3.23F-F' and 3.24F-F'). In the sdt^{K85} mutant ommatidia it is delocalized with enrichment at rhabdomere base (data not shown, but the localization is the same as in 3.24F-F' in the GFP-positive part). When the Sdt constructs were expressed in this background, the behaviour of β_H-spectrin was the same as of the other Crb-complex members. When Sdt was detectable at the stalk membrane in adult PRCs, β_H-spectrin was localized as well (Figure 3.22F-F' and 3.23F-F'). When no Sdt was detected, β_H-spectrin was delocalized in the same manner as in mutant PRCs (Figure 3.24F-F'). From this it can be concluded that the stalk membrane localization of β_H-spectrin is fully dependent on the Crb-complex. Probably, it binds to it via the FERM-domain of Crb (Medina *et al*., 2002). When Sdt is absent at the stalk membrane, Crb is delocalized, and results in β_H-spectrin delocalization.

To conclude from this part, the part of Sdt including ECR1, ECR2, L27-N as well as the PDZ, the SH3 and the GUK domains are all required for the

stabilization/maintenance of the Crb-complex in the adult photoreceptor cells. When the PDZ domain is absent, Sdt protein is already delocalized at pupal stage. When any other of these domains is absent, the localization is the same as in wild type at pupal stage, but the Crb-complex is delocalized from the stalk membrane or degraded at later stages. The mechanism of this process is currently unclear. The summarized data on the localization of transgene-encoded proteins and the members of the Crb-complex upon expression of the transgenes in sdt^{K85} mutant background are demonstrated in Table 4.

Table 4: Localization of the members of the Crb-complex and proteins encoded by the transgenes upon expression in sdt^{K85} mutant background:

Construct	Localization in pupal eye discs:		Localization in adult eyes:	
	Constructs-encoded protein	Crb/ *D*PATJ	Constructs-encoded protein	Other members of the Crb-complex
Sdt-MAGUK	+	+/+	+	+
Sdt-B	+	+/+	+	+
Sdt-ΔL27-C	+	+/+	+	+*
Sdt-ΔN	+	+/+	-	-
Sdt-ΔSH3-Hook	+	+/+	-	-
Sdt-Δ Hook-GUK	+	+/+	-	-
Sdt-ΔPDZ	-*	+/+	-	-
Sdt-1-L27-C	-*	+/+	-	-

+ - protein(s) is (are) localized properly.

+* - proteins are localized properly except for *D*Lin-7.

- - protein(s) is (are) delocalized and cannot be detected.

-* - protein is delocalized but can be detected in other parts of the cells.

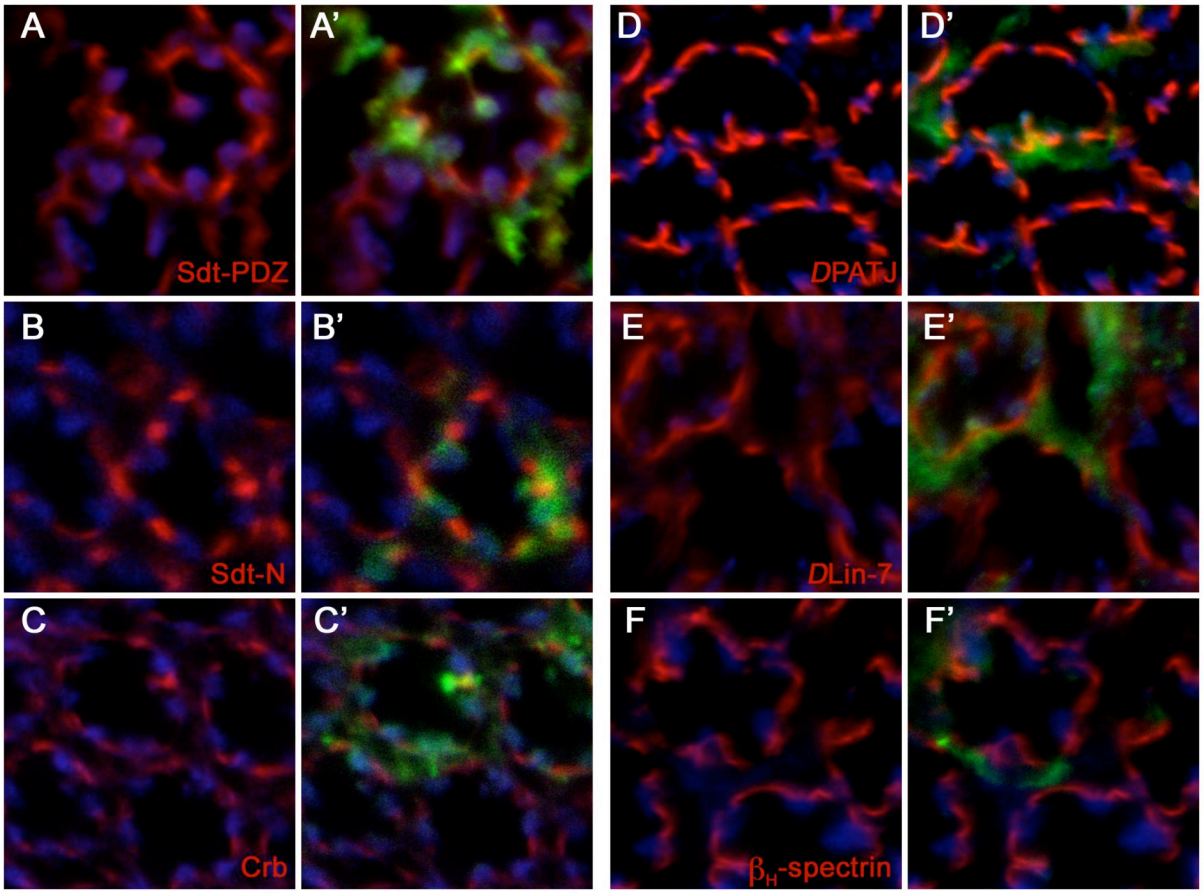


Figure 3.22. Sdt-MAGUK and Sdt-B expression in *sdt*^{K85} mutant background result in a fully assembled Crb-complex. All pictures represent Sdt-B expression. Sdt (A and B), Crb (C), *D*PATJ (D), *D*Lin-7 (E) and β_H-spectrin (F) localize at the stalk membrane. In A'-F' the GFP staining is shown. The GFP-positive cells are *sdt*^{K85} mutant and express Sdt-B. Rhabdomeres are stained by phalloidin (blue).

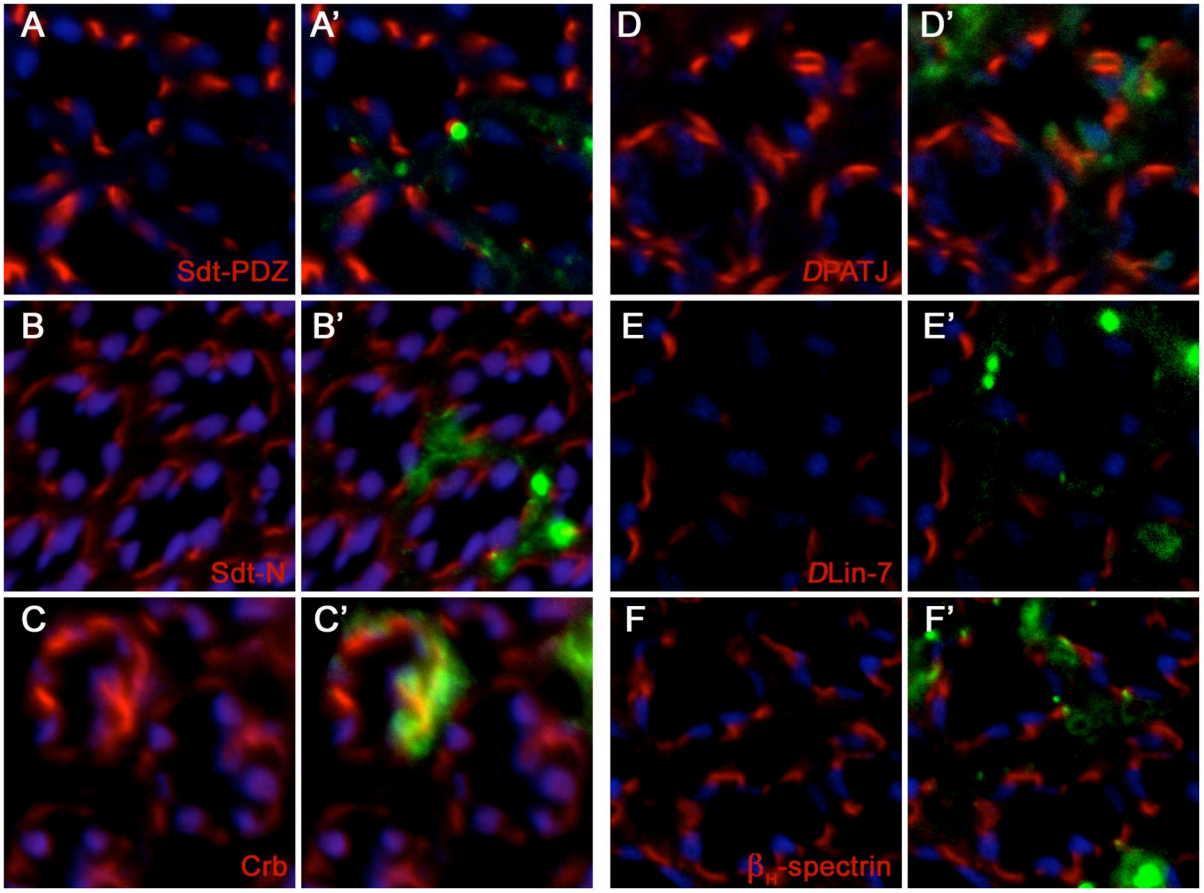


Figure 3.23. Sdt-ΔL27-C results in correct Crb-complex at the stalk membrane when it is expressed in *sdt*^{K85} mutant background. Sdt (A and B), Crb (C), *D*PATJ (D), and β_H-spectrin (F) localize at the stalk membrane. The only protein that is absent from the stalk membrane is *D*Lin-7 (E). In A'-F', the GFP staining is shown. The GFP-positive cells are *sdt*^{K85} mutant and express the transgene. Rhabdomeres are stained by phalloidin (blue).

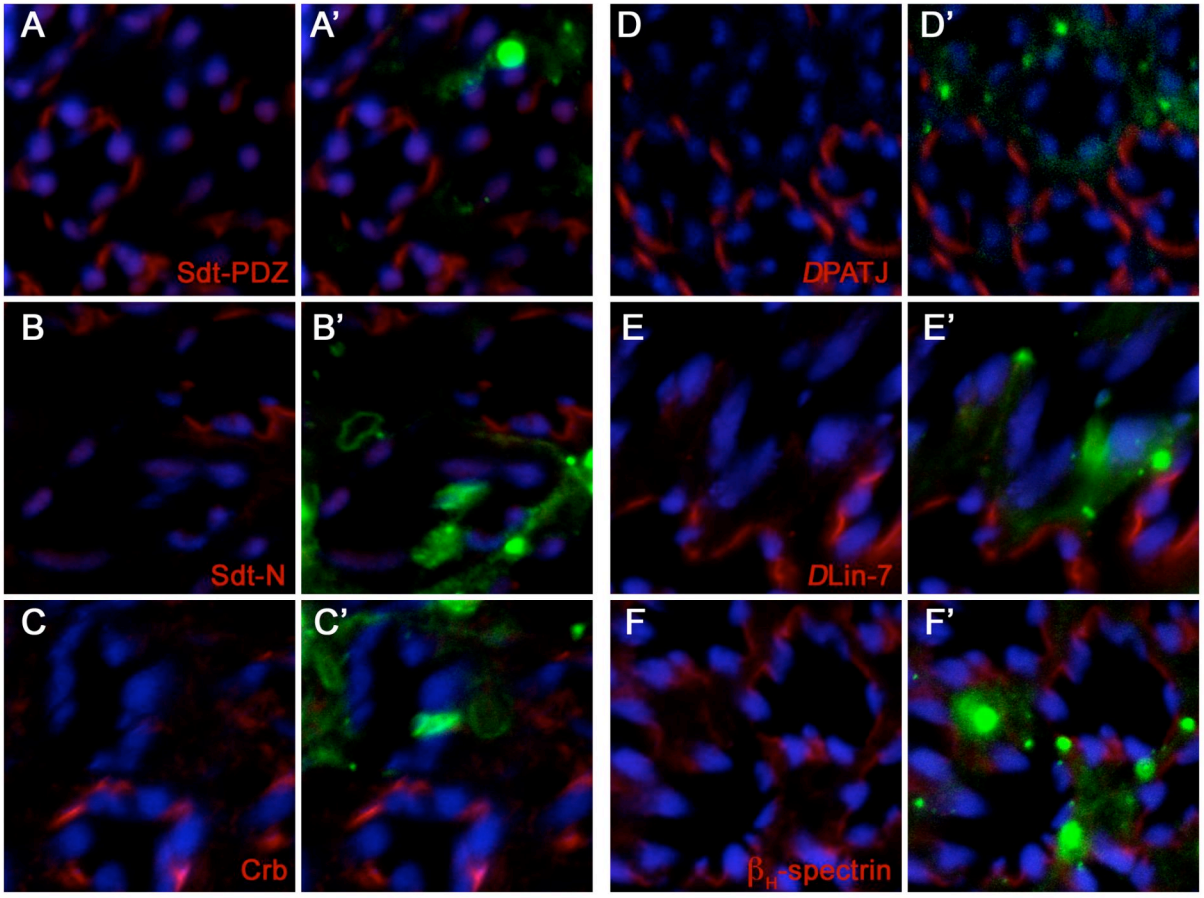


Figure 3.24. None of the Crb-complex members is localized at the stalk membrane when any other Sdt-transgene, except Sdt-MAGUK, Sdt-B and Sdt-ΔL27-C, is expressed in *sdt*^{K85} mutant background. No Sdt (A and B), no Crb (C), no *D*PATJ (D), no *D*Lin-7 (E) can be detected at the stalk membrane. In A the example of the expression of Sdt-ΔN was used, in B - Sdt-ΔSH3-Hook, in C – Sdt-PDZ, in D - Sdt-ΔN, in E - Sdt-ΔSH3-Hook, and in F - Sdt-ΔHook-GUK. In A'-F' the GFP staining is shown. The GFP-positive cells are *sdt*^{K85} mutant and express the respective constructs. Rhabdomeres are stained by phalloidin (blue).

3.3.3 Various, but weak dominant morphological defects are induced by overexpression of Sdt-constructs

3.3.3.1 Dominant effects of constructs expression in pupal eye discs

Previously it was observed that the overexpression of full-length Crb or of Crb lacking the extracellular domain (Crb-intra) in pupal eye discs causes defects in pigment cell sorting. In these discs additional secondary and tertiary pigment cells are observed. Another typical defect was an increased number of bristle cells. These cells were often surrounded by four but not three interommatidial cells (IOCs). Crb performs this via its action on the adherent junctions (AJs), since the disruption of the continuous belt of *D*E-Cad between 1° p.c. and IOC was observed (Grzeschik and Knust, 2005, Grzeschik, 2003). The defects were detected when *GMR*GAL4 or *sev*GAL4 were used. Overexpression of Crb-intra transgene lacking four C-terminal amino-acids (Crb-intra-ΔERLI) also induces similar defects though to a lower extent (Grzeschik, 2003), although Crb-intra-ΔERLI cannot bind to Sdt.

To understand the function of the Crb-complex in this process, it was important to check if Sdt overexpression can cause similar defects. Therefore, different Sdt constructs were expressed in a wild type background using the *GMR*GAL4 driver, and the pattern of pigment cells was analyzed in each case. The membrane of pigment cells was stained with antibody against the *D*E-Cad protein. *GMR*GAL4 in combination with UAS-src-GFP transgene was used as a control. UAS-src-GFP encodes Src protein fused to GFP under the control of UAS promoter. In these control flies no defects in the organization of pigment cells were ever detected (Figure 3.25A-A').

When the full-length Sdt-MAGUK construct was expressed, defects similar to the ones observed upon Crb-intra overexpression were found (Figure 3.25B-B'). These defects included increased number of 2°p.c., 3°p.c. and bristle cells. Bristle cells were often surrounded by additional IOCs, which is never observed in the control. In comparison to Crb overexpression, no change in number or orientation of 1°p.c. or cone cells was observed. The lack of defects in 1°p.c or cone cells number may be caused either by low expression level of Sdt-transgenes, or by lower input of Sdt

generally in pigment cell sorting (resulting in generally weaker phenotype) than that of Crb, or by absence of requirement for Sdt function in these particular cells.

To determine what domain of Sdt is important for causing the observed defects, different constructs were tested: Sdt-ΔN, Sdt-Δ1-L27-C, Sdt-ΔPDZ, Sdt-ΔSH3-Hook, Sdt-ΔHook-GUK, Sdt-ΔSH3-GUK, Sdt-PDZ, Sdt-1-566, Sdt-1-L27-N and Sdt-1-L27-C. Neither Sdt-PDZ nor Sdt-1-566 induced any defects (Figure 3.25C-C'), while expression of all other transgenes induced variable defects. For some of the constructs, like Sdt-Δ1-L27-C, the defects were stronger than those induced by full-length construct (Figure 3.25D-D'). For others, like Sdt-ΔPDZ, they were weaker. Sdt-ΔPDZ results in lower expression level as Sdt-Δ1-L27-C (compare 3.16B4 and 3.16B6). So, probably, the strength of observed phenotype depends mainly on the amount of protein expressed by the respective construct. In some discs a disruption of the continuous belt of *D*E-Cad between primary pigment cells and IOCs was detected (for example, see 3.25D-D'). The same phenotype was observed upon Crb overexpression (Grzeschik & Knust, 2005). In the case of Sdt overexpression this disruption seems to be weaker. No correlation between pigment cell sorting phenotype and disruption of *D*E-Cad belt was observed, thus, the relation between these two phenotypes remains elusive.

As Sdt-1-L27-N but not Sdt-1-566 is able to interact with the pigment cell sorting process, it can be concluded that the L27-N domain and, thus, binding of Sdt to *D*PATJ, is important for the observed phenotype. But since Sdt-ΔN is also able to induce these defects, the L27-N domain is not the only one required for this function. The second domain is probably not the PDZ-domain, as expression of PDZ-domain alone does not interfere with pigment cell sorting.

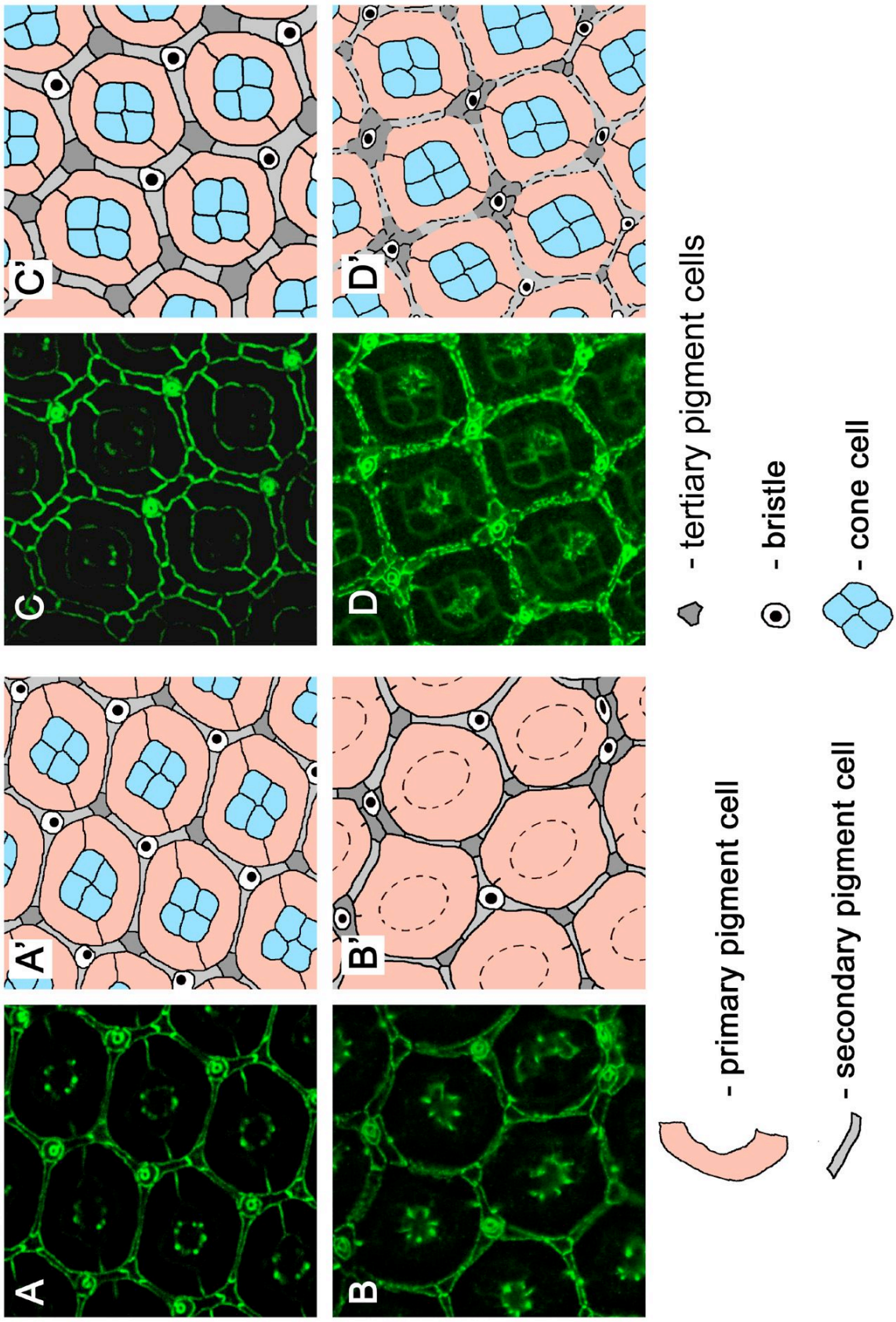

A
A'
B
B'
C
C'
D
D'
- primary pigment cell
- secondary pigment cell
- tertiary pigment cells
- bristle
- cone cell

Figure 3.25. Overexpression of Sdt induces defects in pigment cell sorting.
A-A' – Wild-type. The cells of the ommatidia are organized in a stereotypic pattern, composed of PRCs. primary, secondary and tertiary pigment cells, cone cells and bristles
B-B' – Overexpression of Sdt-MAGUK by the *GMR*GAL4 driver. Disorganization in the pattern of IOCs is observed.
C-C' – Overexpression of Sdt-PDZ by the *GMR*GAL4 driver. IOCs are organized properly. The same is observed, when Sdt-1-566 constructs is overexpressed.
D-D' – Overexpression of Sdt-Δ1-L27-C by the *GMR*GAL4 driver. Defects in pigment cell sorting are stronger in comparison to Sdt-MAGUK, and the disruption of the continuous *D*E-Cad belt is observed.
In A, B, C and D the stainings of the pupal discs with α-*D*E-Cad are shown. In A', B', C' and D' the same discs are presented schematically. Different cell types are marked with different colours. The cells of unknown nature were marked as tertiary pigment cells. In black lines the *D*E-Cad localization is depicted (punctuate in D'). In D' in some cases cell boundaries were indicated based on speculation.

3.3.3.2 Dominant effects on the morphology of adult eyes upon overexpression of Sdt-transgenes

It is known that the absence of Sdt protein results in morphological defects in photoreceptor cells (see 1.4.2.2). To understand the function of Sdt better and to distinguish which domains of Sdt are required for the establishment of the morphology of PRCs, different Sdt constructs were expressed on the wild type background.

When the full-length proteins or variants lacking one or several domains were expressed using the *GMR*GAL4 driver, very strong defects in the morphology of ommatidia were detected (example for Sdt-MAGUK is presented in Figure 3.26B in comparison to the control flies in Figure 3.26A). This activator line provides very high level of expression, especially when the flies are kept at 25°C. So the effects that were observed may be explained not only by direct effects of Sdt on PRCs development, but also by indirect effect caused by extreme loading of the cells with Sdt protein. The second negative feature of the *GMR*GAL4 driver-line is that it interferes with eye morphology on its own in heterozygous state without additional UAS effector line (Kramer and Staveley, 2003, personal observations). The eyes of these flies display a slightly rough phenotype, which is much stronger when flies are kept at 25°C in comparison to 18°C. For these two reasons, we decided to use the much weaker *Rh1*GAL4 driver to study effects of Sdt expression on PRCs morphology. This driver

line starts to be expressed relatively late in pupal development, at about 70% p.d., when the rhabdomere precursors have already separated from the stalk membrane precursors. It drives expression only in R1-R6 PRCs. This driver line was checked for its ability to induce morphological defects in PRCs. In control *Rh1*GAL4 / UAS-src-GFP flies, no morphological defects were detected (Figure 3.26A). Different constructs were checked whether they are capable to induce the defects in PRCs morphology alone, without GAL4 activation. This could be either due to leakage of expression in the absence of GAL4, or due to the site of the P-element insertion, causing additional mutations. Fortunately, none of the chosen lines displays any morphological defect when heterozygous and in the absence of GAL4.

When different Sdt constructs were expressed with *Rh1*GAL4, mild morphological defects in the eye were observed. These defects can be divided into two classes: changes of rhabdomere shape and absence of rhabdomeres (Figure 3.26C and 3.26D). Rhabdomeres were often partially or fully split into two parts in the middle. Missing rhabdomeres were rarely observed, but as all control lines never display such phenotype, it seems to be specific and dependent on transgene-encoded Sdt expression. The number of mis-shaped rhabdomeres was counted, and the percent of mis-shaped rhabdomeres in comparison to the total number of counted R1-R6 rhabdomeres was measured for different constructs. The percent of ommatidia missing one rhabdomere was counted. No ommatidium that lacks more than one rhabdomere was detected. The obtained numbers are summarized in Table 5.

In the case of Sdt-MAGUK, *ey*GAL4 was also used to check the effects of this transgene on morphology. *ey*GAL4 starts expression very early in the development. Its activity can be detected already in the precursors of the eye imaginal discs in the embryo. This driver stops its expression in the first half of pupal development (Beronja *et al*., 2005). When this driver was used, the percent of mis-shaped rhabdomeres was lower than in the case of *Rh1*GAL4 driver. At the same time the amount of missing rhabdomeres was strongly increased (see Table 5). This phenomenon may be explained by the earlier action of the *ey*GAL4 driver. It drives expression at the stages when rhabdomeres are not yet specified, whereas *Rh1*GAL4 acts when they are

already established. The data indicate that proper morphogenesis of rhabdomeres is influenced by overexpression at early and late stages of pupal development.

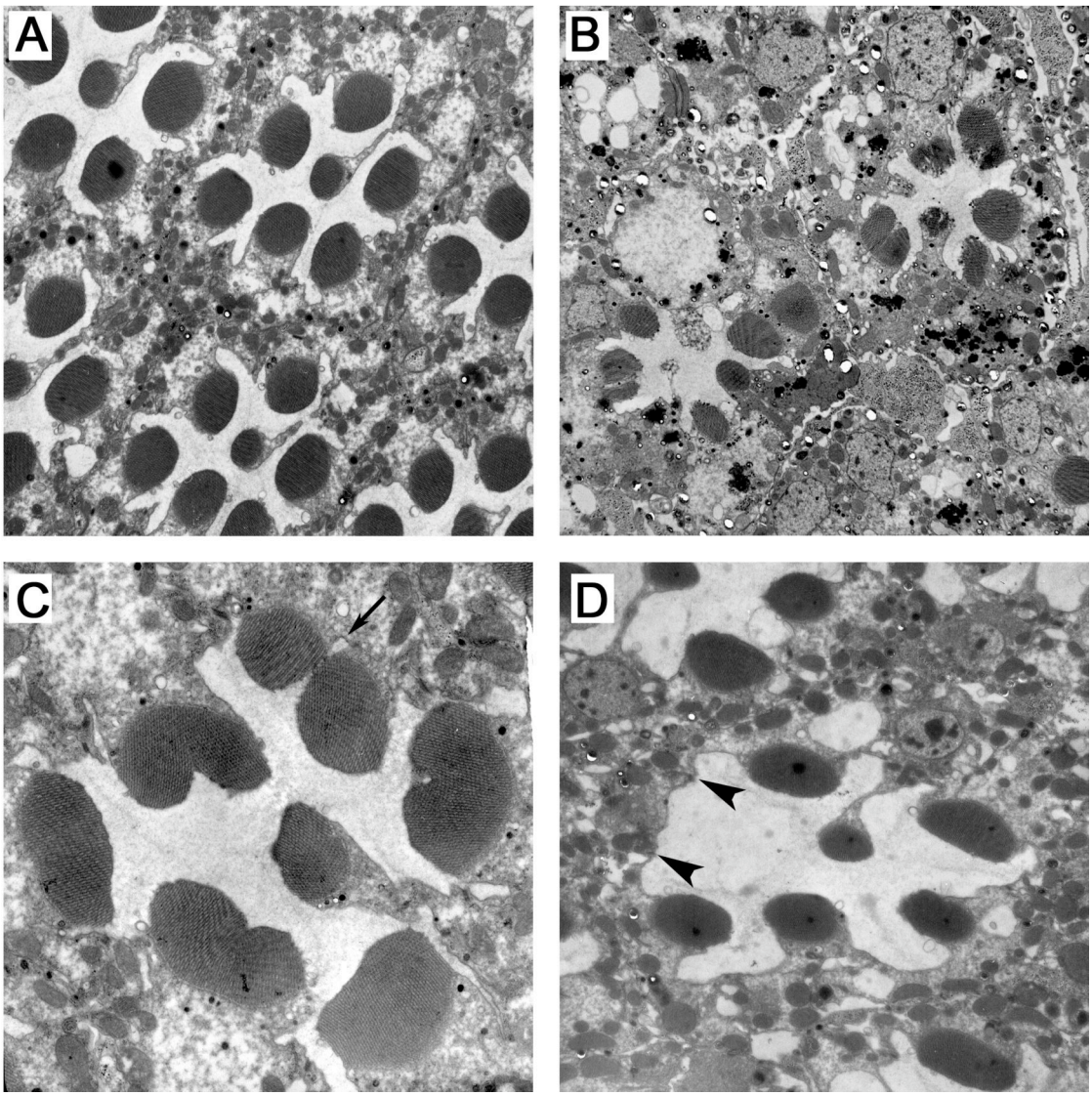


Figure 3.26. Expression of Sdt on the wild type background results in morphological defects.

A – In the control flies (*Rh1*GAL4 / UAS-src-GFP) no morphological defects are observed.

B – *GMR*GAL4/ +; +/ Sdt-MAGUK. Most of the Sdt constructs result in strong PRCs disorganization when are expressed using *GMR*GAL4.

C – *Rh1*GAL4; Sdt-ΔL27-C. Some rhabdomeres are partially or fully (arrow) split into two parts.

D – *Rh1*GAL4; Sdt-MAGUK. A rhabdomeres of R2 PRC is absent, although the cell is present. It can be detected by two ZA contacts with R1 and R3 PRCs (arrowheads).

All transgenes encoding proteins that lack one or few domains were able to induce morphological defects. The PDZ domain alone causes them as well. This means that the PDZ domain and binding of Sdt to Crb are required for the Crb-complex to induce this dominant phenotype. No defects were observed when Sdt-1-566 was expressed. But when Sdt-1-L27-N was expressed, defects were detected. From this it can be concluded that the L27-N domain and its binding to *D*PATJ are required for Sdt function to induce this dominant phenotype.

Table 5. Morphological defects induced by expression of Sdt constructs on wild-type background

Construct	Percent of split rhabdomeres R1-R6		Percent of ommatidia with one missing rhabdomere	
	*Rh1*GAL4	*ey*GAL4	*Rh1*GAL4	*ey*GAL4
Sdt-MAGUK	9	0.3	1	7,5
Sdt-ΔN	11	n.d.	0	n.d.
Sdt-ΔL27-C	15,6	n.d.	2,5	n.d.
Sdt-ΔPDZ	2,4	n.d.	0	n.d.
Sdt-ΔHook-GUK	2,1	n.d.	0	n.d.
Sdt-ΔSH3-GUK	2,4	n.d.	0	n.d.
Sdt-B	6,4	n.d.	0	n.d.
Sdt-1-L27-N	8,0	n.d.	0,5	n.d.
Sdt-1-L27-C	5,0	n.d.	0	n.d.
Sdt-PDZ	8,0	n.d.	0	n.d.
Sdt-1-566	0	n.d.	0	n.d.
Control	0	n.d.	0	n.d.

n.d – not determined.

3.3.4 Effects of Sdt expression in the wild type background on retinal degeneration in the constant light

The second independent function of Sdt in the eye is prevention of light-dependent retinal degeneration. To determine the domains of Sdt required for performing the function in retinal degeneration, different transgenes were expressed in a wild type background. It was previously shown that the presence of red pigment prevents degeneration. The eyes mutant for *crb* or *sdt* do not degenerate in constant light when they are in a *white*$^{+}$ background (Berger, Richard, personal communication). All Sdt constructs are marked with *mini-white*. As result all flies carrying these constructs have eyes that contain red pigment. Moreover, the colour of the eye varies from light orange till nearly red. This makes the comparison of the effects caused by the expression of different constructs nearly impossible. To avoid this problem, the constructs and the GAL4-driver lines were brought together with the *GMR*wIR (Lee & Carthew, 2003) in one animal. This is a transgene that encodes RNAi against the product of the *white* gene under the control of the *GMR* promoter. This results in expression of double-stranded RNA in all cells of the eye, and in efficient silencing of the *white* gene. To further unify the conditions, only females were taken for the analysis, as both the *white* gene and the *GMR*wIR are located on the first chromosome, and the efficiency of the silencing may be different between different sexes.

Sdt transgenes were expressed by using either *Rh1*GAL4 or *54*GAL4 driver. The latter induces the expression in pigment cells starting from early stages of pupal development (Figure 3.27A and 3.27B). Although low expression of this driver in the PRCs cannot be excluded, it is lower than the detection level. This driver was chosen to check if there is a role of Sdt in pigment cells for the prevention of retinal degeneration. Both Sdt and Crb are expressed in the pigment cells at least at the pupal stage (Figure 3.27C-C' and 3.27D-D'). In adult eyes it was not possible to detect the Crb-complex in pigment cells, but since they are very thin it cannot be excluded that the proteins are there in low concentration.

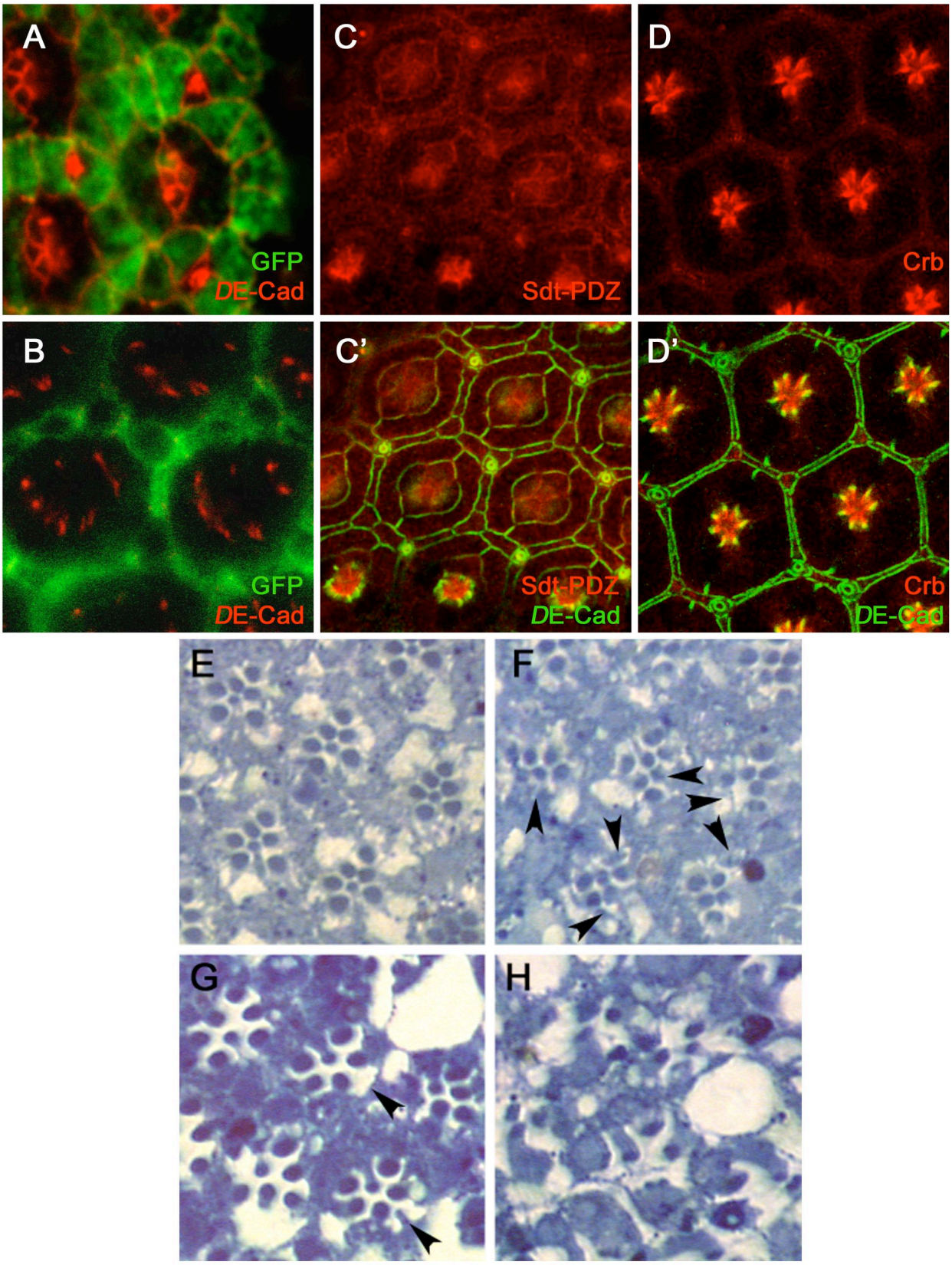

A
GFP
DE-Cad
C
Sdt-PDZ
D
Crb
B
GFP
DE-Cad
C'
Sdt-PDZ
DE-Cad
D'
Crb
DE-Cad
E
F
G
H

Figure 3.27. Expression of Sdt-constructs on the wild type background modifies the rate of the light-induced retinal degeneration.

A – Expression of GFP in eye discs from the *54*GAL4/ UAS-src-GFP pupae at about 25% p.d.

B – Expression of GFP in eye disvs from the *54*GAL4/ UAS-src-GFP pupae at about 90% p.d.

In A and B the boundaries of pigment cells and ZA in PRCs are stained with α-*D*E-Cad antibody (red).

C-C' – Sdt (red) is expressed in the pigment cells of the eye discs of the wild-type pupae.

D-D' – Crb (red) is expressed in the pigment cells of the eye discs of the wild-type pupae.

In C-D *D*E-Cad (green) marks the boundaries of pigment cells and ZA in PRCs.

F – Eyes from *GMR*wIR/ +; *54*GAL4/ + flies exhibit the wild-type morphology after 21 days in the dark conditions.

F – Eyes from *GMR*wIR/ +; *54*GAL4/ + flies exhibit the first signs of the degeneration after 21 days of constant light exposure. Rhabdomeres have cloudy appearance. Some PRCs lack their rhabdomere. Examples of these disorganizations are marked with arrowheads.

G – Eyes from *GMR*wIR/ +; *54*GAL4/ +; Sdt-MAGUK/ + flies after 21 days of constant light exposure. The overall appearance of the retina is better preserved (compare to F), though in some PRCs defects are detected.

H – Eyes from *GMR*wIR/ +; *54*GAL4/ +; Sdt-ΔL27-C/ + flies after 21 days of constant light exposure. The retina is much stronger affected as in the control (compare to F).

It was shown that the retinas of w^- flies degenerate after long exposure to constant light (Lee and Montell, 2004b). In comparison to *crb* or *sdt* mutant retinas, w^- retinas require longer exposure to constant light for the onset of degeneration. Additional expression of different Sdt constructs in a wild type background may not only induce the early onset of degeneration but for some constructs it is possible that they inhibit the degeneration occurring in wild type. To be able to distinguish between both cases, the flies expressing the construct and the control flies were kept in constant light for three weeks. That is a time point when wild type (w^-) eye just starts to degenerate, and the first signs of degeneration can be detected (Figure 3.27F in comparison to the dark raised flies in 3.27E). Females of w^+ *GMR*wIR / +; *Rh1*GAL4 / +; UAS-src-GFP / + or w^+ *GMR*wIR / w^-; *54*GAL4 / +; UAS-src-GFP / + genotype were used as control. Most PRCs in the control look still normal after 21 days of exposure to constant light, but in some the rhabdomeres have either cloudy appearance or are already absent.

When the Sdt constructs were expressed, differences in comparison to the control could be detected. For example, eyes expressing Sdt-MAGUK with the *54*GAL4 driver showed a rather wild-type morphology in comparison to control eyes (Figure 3.27G). In contrast, in other cases, for example, for Sdt-ΔPDZ expressed using the *Rh1*GAL4 driver, it seemed that the degeneration level is higher then in control eyes (Figure 3.27H).

Table 6: Influence of the expression of the different Sdt constructs on the rate of the light-induced retinal degeneration.

Construct	Mean number of rhabdomeres pro ommatidium	
	*54*GAL4	*Rh1*GAL4[1]
Sdt-MAGUK	6,0**	4,1
Sdt-ΔPDZ	4,8***	3,2***
Sdt-ΔL27-C	4,7***	4,1
Sdt-ΔN	6,3***	3,3***
Control	5,5	4,3

[1] – for these driver only the rhabdomeres in R1-R6 were counted.

** - P>0,99

*** - P>0,999

It was important to quantify the observed effects. The number of the rhabdomeres pro ommatidium that have an intact morphology was chosen as criterium. This number was counted in 120 ommatidia for each genotype (20 ommatidia in 6 eyes from 6 different flies). Only rhabdomeres of R1-R6 were counted when using *Rh1*GAL4 as driver. The mean was counted for each genotype, and the means of different genotypes were compared with a control using Student-test. The data are presented in Table 6. There was a significant difference in the degeneration rate between eyes expressing Sdt constructs and control eyes in several cases. The expression of Sdt-MAGUK or Sdt-ΔN significantly decreased the degeneration rate in

comparison to the control, when these constructs were expressed in pigment cells. At the same time expression of Sdt-MAGUK in PRCs had no significant rescuing effect on the degeneration, and expression of Sdt-ΔN even enforced the degeneration. Expression of Sdt-ΔPDZ significantly increased the degeneration level both when the construct was expressed in pigment cells or in PRCs. Sdt-ΔL27-C behaved oppositely to Sdt-ΔN and caused significant increase in degeneration level when it was expressed in pigment cells. When it was expressed in the PRCs it had no effect on the degeneration rate and behaved like Sdt-MAGUK.

To summarize the data, increased level of Sdt seems to be important in pigment cells but not in PRCs. The absence of the L27-N domain in overexpressed protein has effect on degeneration rate in comparison to full-length Sdt overexpression, when the corresponding transgene is expressed in PRCs. In contrast, the absence of the L27-C has an influence on degeneration rate only upon overexpression in pigment cells. The PDZ domain is important in both cell types. This may indicate that in the pigment cells the formation of the Crb-Sdt-*D*Lin-7 complex results in prevention of degeneration, whereas in PRCs this function is provided by the other triple complex Crb-Sdt-*D*PATJ. Although all four proteins were shown to be important for the prevention of retinal degeneration (Johnson *et al*., 2002, Berger, 2005, Richard *et al*., 2006, Bachmann, personal communication), the obtained data give the first indication that there are different requirements and functions of the Crb complex in pigment cells and PRCs, and that pigment cells may have other functions in the prevention of light-dependent retinal degeneration than the simple absorption of the excess light by the pigments.

4. Discussion

4.1 Sdt encodes multiple isoforms that are dynamically expressed throughout development

Sdt is a scaffolding protein, and its main function is to organize other proteins in functional complexes. For several scaffolding proteins the existence of several isoforms was already shown. For example, for *Drosophila* Dlg two different forms were described: Dlg-A and Dlg-S97. Dlg-A is the form specific for epithelial cells, while Dlg-S97 is expressed at neuro-muscular junctions. These two forms of the protein differ in their N-terminal region (Mendoza *et al.*, 2003). Similarly, the CG9326 gene encodes for two different forms of a MAGUK protein, which differ in their N-terminal part and are expressed differentially during development (Bachmann A., personal communication).

When different splice-variants of one scaffolding protein are expressed in different tissues, it may result in slightly different protein-complexes organized by them in these tissues. Different complexes may have different functions, and provide the processes that are required in this exact tissue at this exact time point. As result, the alternative splicing of transcript encoding scaffolding protein may be an important mechanism to regulate the development, differentiation and function of different cell types.

In the present work it was shown that Sdt expression is dynamic during development. Different isoforms are expressed at different developmental stages. For example, in pupal brain/retinas complexes at 40-60% p.d. five different Sdt proteins are detected on Western blots. This tissue undergoes multiple morphological events at this stage, and the Crb-complex participates in several of them – establishment of proper PRCs morphology, regulation of the sorting of pigment cells and, probably, additional functions presently unknown, provided by the expression of at least some of the members of the Crb-complex in the brain. Multiple splice-variants of *sdt* may be required for the fine-tuning of each of these functions.

The same situation can be observed in the embryo. Though on Western blots only one major band of Sdt can be detected (see Figure 3.1), on Northern blots

multiple bands are recognized by the Sdt-MAGUK probe (Peters K., personal communication). The functions of different splice variants are yet elusive, but they may be expressed in different epithelia, providing differences in their features and development. This question requires further investigations, but may help to understand better the development of the fly.

The main focus of this work was the fly retina. The results allow suggesting the existence of two predominant Sdt isoforms in the adult fly retina – Sdt-B2 and Sdt-D (see Figure 3.15). These two forms have an identical C-terminus that includes two L27 domains, PDZ, SH3, GUK domains and a putative Hook-domain. The only difference between these two forms lies in the N-terminus. Sdt-B2 contains ECR1 and ECR2 in its N-terminus, and, thus, can potentially bind to *D*Par-6 protein. Sdt-D lacks ECR1 and ECR2 and hence cannot bind to *D*Par-6, but may have additional binding sites in its specific N-terminus that never was characterized before.

Sdt-B2 is the only predominant form that can be recognized by Sdt-N antibody. Using this antibody results in stalk membrane staining. So, it can be concluded that Sdt-B2 is expressed in PRCs, though the expression of this isoform in the pigment cells cannot be excluded. For Sdt-D there is no direct indication that it is expressed in PRCs at all. All three possibilities remain: it is expressed only in PRCs, it is expressed only in pigment cells, and it is expressed in both PRCs and pigment cells. The generation of the antibodies that are specific to this splice-variant will allow to discriminate between these possibilities. The expression of full-length Sdt-B2 and Sdt-D transgenes cell-type specifically in the mutant background, or knocking down specifically one of the splice-variants in one cell type, will permit to identify the function of each of these splice-variants in each cell type.

These data lead to the question if the binding to *D*Par-6 of one of the Sdt variants in the retina is of functional significance. Preliminary data indicate that the *D*Par-6 is localized in vesicles in the adult PRCs (Figure 4.1A). This localization is similar of that of Rh1-immunopositive large vesicles (RLV) (Satoh & Ready, 2005, Figure 4.1B). Rh1 accumulates in rhabdomeres in dark-raised flies. Already after 8 min of light-exposure some Rh1 is presented in the cytoplasm in RLV that increase in size

till 30 min of exposure to light. At the same time the amount of Rh1 detected in rhabdomeres diminishes.

In addition to Rh1, several other proteins are transported into and out of signalling membrane in a light-dependent manner. This transport ensures long-term adaptation of photoreceptor cells to light. Three main proteins/protein complexes important for light signal transduction are known to exhibit such translocations (reviewed in Frechter & Minke, 2006). These are Arr2/Arr1, $G_q\alpha$ and TRPL/*D*moesin (Satoh & Ready, 2005, Lee *et al.,* 2003, Kosloff *et al*., 2003, Cronin *et al.,* 2004, Chorna-Ornan *et al*., 2005, for function of these proteins see also 1.4.3).

The stalk membrane is important for the light-induced transport of several proteins (Cronin *et al.*, 2006). One of them is TRPL. It localizes to the rhabdomere base in dark-raised flies. After 2 hours of light exposure it is present at the rhabdomere base and at the stalk membrane. After 6 hours of light-exposure it is found throughout the basolateral membrane of all photoreceptor cells. Mutations in *Rh1* and *PLC* disrupt initial transport of TRPL to the stalk membrane. Mutations in *TRP* and *inaC* do not interfere with transport of TRPL to the stalk membrane but block the translocation to the basolateral membrane (Cronin *et al.*, 2006). In dark-raised flies, TRPL is in association with *D*moesin, which also localizes in such flies to the rhabdomere base. Upon light-exposure, the TRPL/*D*moesin complex dissociates, *D*moesin is dephosphorylated and translocates from the membrane to the cytoplasm (Chorna-Ornan *et al*., 2005).

All together, it is possible that Sdt-B2 may transiently bind to *D*Par-6, and, thus, regulates light-induced transport of proteins (schematically presented in Figure 4.1C). To confirm this hypothesis co-staining of *D*Par-6 positive vesicles with Rh1 antibodies, and analysis of these vesicles in different dark/light conditions should be performed. Also it would be interesting to analyze the effects of *sdt* mutations on the formation of RLV.

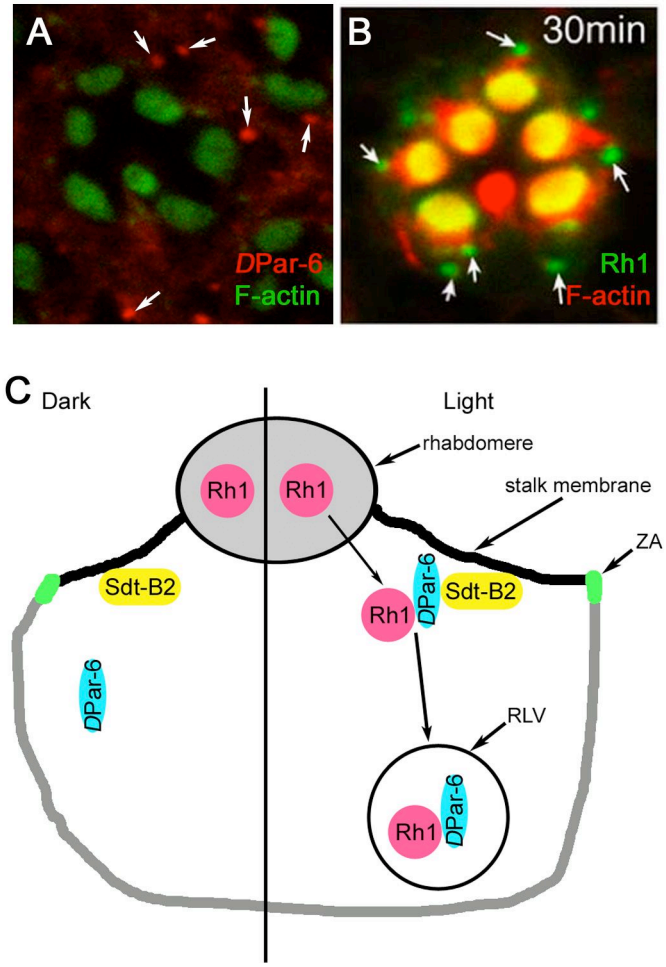


Figure 4.1. Possible model of the Sdt/*D*Par-6 interaction in the adult eye. *D*Par-6 is localized in vesicles in the PRCs of the adult eyes after about 20 min exposure to the light (A). These vesicles are similar to RLV observed when the eyes are exposed to the light (B, from Satoh & Ready, 2005). In C the model proposes, that the Sdt-B2 variant might interact with *D*Par-6 transiently via ECR1+ECR2 in the light, but not in the darkness. This interaction may be important for the transport of Rh1 to RLV, and *D*Par-6 may be associated with this transport. The interaction between Rh1 and *D*Par-6 not necessarily should be direct.

4.2 The mechanisms to localize Sdt are different in pupal eye discs and adult eyes

The proper localization of a protein within the cell is important for its proper function. For example, when a transgene encoding a full-length Crb protein is expressed in a *crb* mutant background, it localizes throughout all the plasma membrane. These data may explain only partial rescue that is obtained with this transgene (Wodarz *et al.*, 1995).

In this work I aimed to understand the mechanisms that provide proper Sdt localization in the eye. For this different Sdt transgenes were expressed either in a wild-type or in a *sdt* mutant background. In a wild-type background, both the PDZ domain of Sdt and ECR1+ECR2 are sufficient for the targeting the protein to the apical membrane at 40-60% p.d. (see 3.3.1.1). When the transgenes were expressed in a sdt^{k85} mutant background, only the PDZ domain was sufficient and required for the apical localization of Sdt at this stage (see 3.3.2.2). The difference between these two cases may be explained by the existence of a positive feedback that exists between *D*Par-6 and Sdt at this developmental stage. It is known that *D*Par-6 at 40-60% p.d. co-localizes with the Crb-complex apically to the ZA in developing PRCs, and that this localization is dependent on the Crb-complex (Nam & Choi, 2003). Then the following hypothesis can be proposed. When Sdt-1-566 is expressed in the wild-type background, endogenous Sdt is recruited apically by the Crb via its PDZ domain. The presence of endogenous Sdt at the apical membrane allows apical localization of *D*Par-6 via ECR1 and ECR2. Then *D*Par-6, in turn, binds to Sdt-1-566 and localizes this protein to the apical membrane. In the absence of endogenous Sdt, it is possible that *D*Par-6 is delocalized. Then the Sdt protein lacking the PDZ domain, though it can bind to *D*Par-6, is delocalized. This hypothesis is presented schematically in Figure 4.2A.

To sum up, the existing data on the localization of different construct-encoded Sdt proteins at pupal stage allow to propose the following localization mechanism for Sdt proteins. Crb serves as the initial cue to localize Sdt apically in the pupal PRCs. Then apical localization of Sdt provides apical localization of *D*Par-6. *D*Par-6, in turn,

provides a positive feedback, and either recruits more Sdt protein to the apical position or additionally stabilizes Sdt that is already apically localized.

In the adult PRCs, the situation differs from what is detected in pupal discs. When the Sdt-transgenes are expressed on the wild type background, none of the single domains is sufficient for the proper efficient targeting of the Sdt protein to the stalk membrane (3.3.2.1). The L27-C, PDZ, SH3 and GUK domain are all important for this. Though in the cases in which one domain is absent some Sdt protein is still able to reach the stalk membrane, most of the protein end up at ectopic sites. From the obtained data it can be concluded that different domains and interactions are important at different stages of Sdt transport to the stalk membrane, but this requirement is not absolute, and in all cases some Sdt protein can proceed to the stalk membrane. The L-27-C domain is important for Sdt to become associated with the plasma membrane, since when this domain is absent a large portion of the protein stays in the cytoplasm. The PDZ domain, similar to its function in pupal discs, is required for apical Sdt localization in adult eyes. When this domain is absent, Sdt is localized to a large extent at all plasma membranes. The SH3 and GUK domains act together to restrict the apical localization of Sdt to the stalk membrane. When any or both of these domains are absent, Sdt protein is localized not only to the stalk membrane, but also to the rhabdomere base. When the transgenes are expressed in a sdt^{K85} mutant background, only in the cases of two full-length constructs and Sdt-ΔL27-C it is possible to detect Sdt. In all three cases the detected proteins were localized apically. When Sdt-PDZ antibody is used to detect the protein encoded by Sdt-ΔL27-C, only a weak staining is detected (see Figure 3.23A-A'). This antibody should recognize all Sdt proteins expressed in the head and transgene-encoded protein with the same efficiency. The low amount of the detected protein encoded by Sdt-ΔL27-C in comparison to the neighbouring wild-type ommatidia can be explained by the low efficiency of the transport of the transgene-encoded protein in this case, similar to what is detected when this transgene is expressed in a wild-type background. Then only the protein that succeeds to reach the stalk membrane, where it binds to other members of the Crb-complex, and, probably, to some other proteins, would be stabilized, all remaining protein would be degraded.

To conclude from this, in adult PRCs the control of quality of Sdt is much stronger than in pupal discs. In pupal discs only the PDZ domain is necessary and required, in adult eyes the complete part from L27-C to GUK is important (summarized in Figures 4.2B and 4.2C). Truncated or mutated protein often properly localizes in pupal stages, in adult stages it is not localized. Probably, that is what is happening in sdt^{XP96} mutant PRCs. The truncated protein generated in this mutant lacks the GUK domain that is important for proper protein localization in adult but not pupal PRCs. As result, the protein is detected apically in PRCs carrying this mutation at the pupal stage (see Figure 3.13), but is not detectable in adult PRCs (Berger, 2005).

The mechanism to localize Sdt is different in embryonic epithelium, where the N-terminus, including ECR1, ECR2 and the L27-N domain, is crucial for Sdt localization to the subapical region (Kempkens, 2005). These data show that the localization of Sdt to its correct position is strongly regulated during fly development, and may serve as an additional mechanism to control its functions in development. One of the possibilities remains, that Sdt may perform some of its functions not directly at the place of its final localization, but during its transport to the final position, and that these functions differ from stage to stage and from tissue to tissue.

The main remaining question is at what stage in pupal development the change in the localization mechanism occurs. It is tempting to propose that this happens at about 70% p.d., when the apical membrane of the PRCs becomes subdivided in rhabdomere and stalk membrane precursors. For *DPATJ* hypomorphic mutant it was shown that the remaining truncated protein could be localized apically in pupal discs. Moreover, in pupal discs of this mutant, similar to *sdt* mutants, other members of the Crb-complex localize properly before 70% p.d. After this stage all proteins of the Crb-complex become gradually delocalized. This stage corresponds to the time point when the apical membrane becomes subdivided, and localization of the Crb-complex is restricted to the stalk membrane precursor (Richard *et al*., 2006). Probably, in the case of Sdt the same happens, and the change in the localization mechanism occurs at this stage. Currently, it is unclear what underlies this change, and what biological meaning does it have.

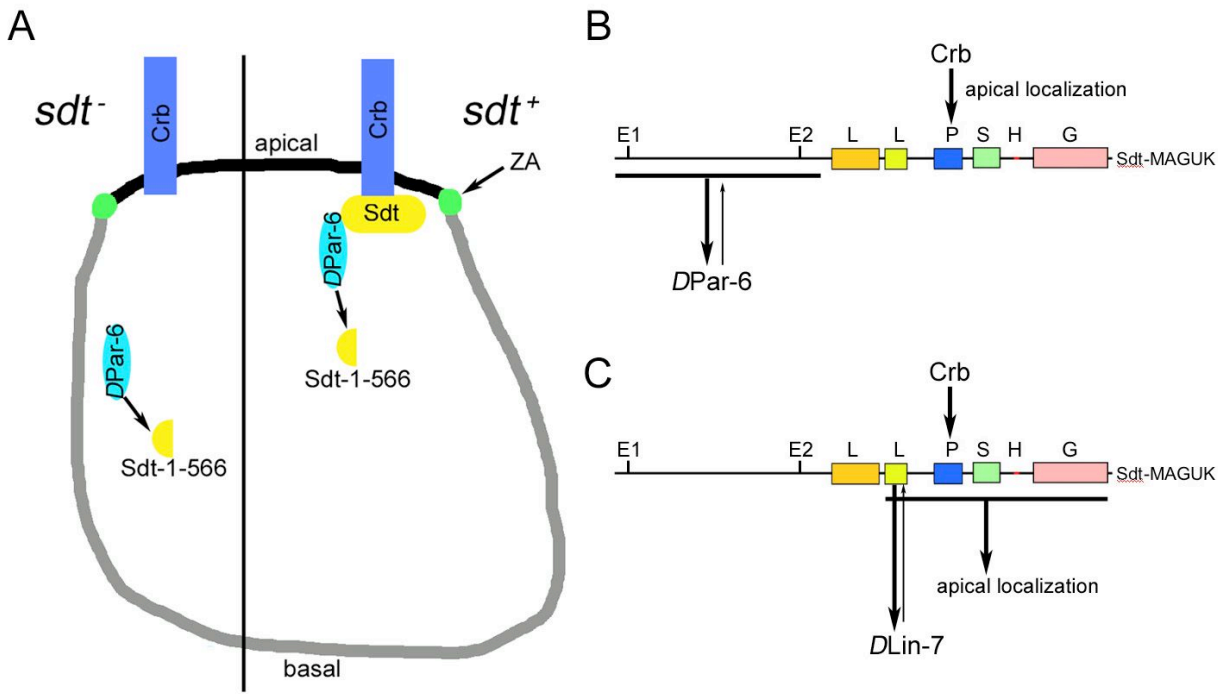


Figure 4.2. The mechanisms to localize Sdt in pupal discs and in adult eyes are different. In pupal eyes the PDZ domain of Sdt is sufficient and required.

A – The model shows how the Sdt protein in the presence of endogenous Sdt can be localized. Binding to Crb via its PDZ domain localizes endogenous Sdt apically. Then Sdt brings *D*Par-6 protein apically, and *D*Par-6, in turn, localizes Sdt-1-566. In the absence of Sdt, *D*Par-6 cannot be localized apically, and cannot bring Sdt-1-566 to the apical membrane.

B – The scheme shows the mechanism to localize Sdt in PRCs in pupal eye discs. In pupae, binding of the PDZ domain of Sdt to Crb is necessary and sufficient for its apical localization. Sdt brings *D*Par-6 apically, *D*Par-6 probably sends a positive feedback signal to further stabilize Sdt at the apical membrane, but this is not absolutely necessary for proper Sdt localization.

C – The scheme shows the mechanism to localize Sdt in PRCs in adult eyes. The L27-C to GUK part of Sdt is required for its proper and efficient localization. From interaction studies it is known that binding to Crb is necessary to bring Sdt to the apical membrane, but Sdt has no effects on Crb localization. Binding to *D*Lin-7 is important as well, but Sdt is also influencing the localization of *D*Lin-7 (see more in 4.3).

E1 – ECR1, E2 – ECR2, L – L27 domains, P – PDZ domain, S – SH3 domain, H – putative Hook-domain, and G – GUK domain.

4.3 Localization hierarchy within members of the Crb-complex

Studies of transgene-encoded proteins are a powerful tool to investigate events and interactions that are required for the proper protein localization, and for its function for the localization of other proteins. In previous works, the interdependence of members of the Crb-complex in their localization was already demonstrated. Thus, in *crb* mutant eyes or embryos the other members of the complex are lost from their positions (Klebes & Knust, 2000, Nam & Choi, 2003). The same is true for *DPATJ* mutation (Richard *et al.,* 2006), and for sdt^{XP96} mutations (Nam & Choi, 2003). The *in vitro* studies demonstrated possible interactions within the complex. The L27-C domain of Sdt was shown to bind to *D*Lin7, the L27-N – to *D*PATJ, and the PDZ domain – to the C-terminal ERLI motif of Crb (see 1.1.1).

In this work the interactions of Sdt with other members of the Crb-complex were confirmed *in vivo*. Indeed, the L27-C domain of Sdt binds to *D*Lin-7. Sdt proteins encoded by the transgenes are able to delocalize *D*Lin-7 as long as they contain the L27-C domain. The same was shown for the binding of Sdt to *D*PATJ via the L27-N domain (see 3.3.2.2). Unfortunately, there is no direct evidence that Sdt binds to Crb via its PDZ domain, as in none of the cases of Sdt overexpression Crb delocalization from the stalk membrane in adult eyes or from the apical membrane of pupal discs was observed. The information obtained by the expression of Crb-encoding constructs may serve as a confirmation. Constructs encoding the intracellular domain of Crb are able to delocalize Sdt, but the ones that lack the ERLI motif are not (S. Klose, personal communication). But since at the same time *D*PATJ is delocalized in the same manner (Klebes & Knust, 2000), additional experiments are required to confirm that a direct binding between Crb and Sdt occurs *in vivo*.

In eyes mutant for any of members of the Crb-complex, except for *D*Lin-7, the Crb-complex is delocalized from the stalk membrane. None of the proteins can be detected in the PRCs anymore. So, it was impossible to investigate the hierarchy between the proteins responsible for proper localization of the complex. It was however possible to do so, when the additional effects of complex stabilization were absent. When the constructs were expressed in a wild-type background, all proteins

were still detectable, though delocalized. Localization of *D*Lin-7 and *D*PATJ was fully dependent on the localization of Sdt encoded by the transgene, and on its L27-C and L27-N domains, respectively. At the same time, Crb was always properly localized to the stalk membrane, independent of Sdt localization (see 3.3.2.2).

These observations together with the fact that localization of the Crb-complex to the stalk membrane is highly dependent on Crb, a model of the events that assemble the complex at the stalk membrane of adult eyes is proposed (see Figure 4.3A). Crb protein is brought to the stalk membrane by a currently unidentified protein/protein complex Y. Then it brings other complex-members to this position by recruiting Sdt via the PDZ domain. In adult eyes, localization of Sdt to the stalk membrane does not fully depend on its binding to Crb. Other factors, probably binding to the SH3/GUK domain of the Sdt, are as well important for Sdt localization (protein Z on the Figure 4.3A). Sdt, in turn, localizes other proteins like *D*Lin-7 and *D*PATJ. These proteins provide a feedback to localize Sdt efficiently to the stalk membrane. When Sdt cannot bind to any of them, only low amount of Sdt can reach the stalk membrane, most of the protein ends up in other cell compartments (see 3.3.2.2).

A similar analysis was performed in the developing pupal eye. The situation at 40-60% p.d. differs from the one in adult PRCs. A common feature is that Crb is always localized apically, independent of Sdt. But there were several differences. Sdt localization was fully dependent on binding to Crb and the presence of the PDZ domain of Sdt. At the same time localization of *D*PATJ does not depend on Sdt localization. When Sdt lacks the PDZ domain, but still has the L27-N domain, Sdt is delocalized, but *D*PATJ is still localized properly. It was shown that in *crb* mutant PRCs at this stage of development both Sdt and *D*PATJ are delocalized (Nam & Choi, 2003, Richard, personal communication). From these data and the data on *DPATJ* mutant (Richard, 2006) a model of localization events in the pupal eye discs at 40-60% p.d. is suggested (Figure 4.3B). Crb protein is localized independently of all other proteins of the Crb-complex, may be by the same factor Y as in adult eyes. Then it serves as a master protein that localizes other complex members like *D*PATJ and Sdt. Probably, Sdt also localizes *D*Par-6 at this developmental stage. *D*Par-6 is not required for the localization of Sdt, but probably provides a positive feedback to stabilize Sdt apically

(see 4.2). To complete the model, the localization of the other members of the Crb-complex, like *D*Lin-7 or β_H-spectrtin, and their dependence on transgene-encoded Sdt, should be assayed.

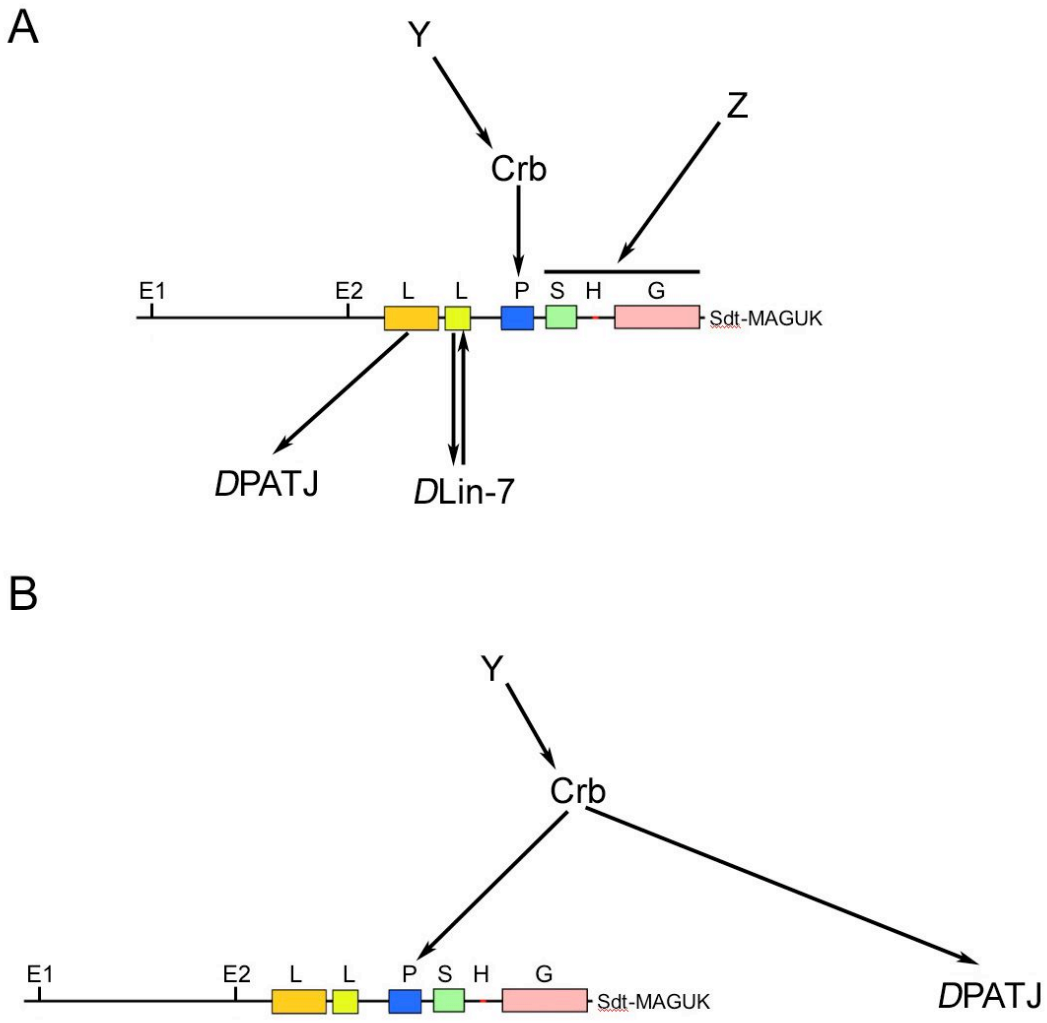


Figure 4.3 Localization hierarchies within the members of the Crb-complex in adult PRCs (A) and pupal eye discs (B).

A – In adult eyes Crb is localized independently of other proteins by unknown factor Y. Crb localizes Sdt together with unknown protein/ proteins Z binding to SH3-GUK part of Sdt. Sdt localizes *D*PATJ and *D*Lin-7. *D*Lin-7 provides a positive feedback to ensure the efficient Sdt localization.

B – In pupal eye discs Crb is similarly targeted apically independent of other members of the Crb-complex, probably, by the same factor Y. There it recruits Sdt and *D*PATJ. They are not important for each other localization at this stage.

E1 – ECR1, E2 – ECR2, L – L27 domains, P – PDZ domain, S – SH3 domain, H – putative Hook-domain, and G – GUK domain.

4.4 Sdt is a key protein to stabilize the Crb-complex at the stalk membrane in adult flies

In addition to its role in proper localization of the Crb-complex in adult PRCs, Sdt is also important for the maintenance of the Crb-complex in its position. In sdt^{K85} mutant eyes, none of the members of the Crb-complex is detected at the stalk membrane (Berger, 2005). The same is true for *crb* and *DPATJ* mutants. In *DLin-7* mutant adult PRCs all the other proteins of the Crb-complex are still properly localized to the stalk membrane (Bachmann, personal communication). In pupal eyes in the absence of Sdt, both Crb and *D*PATJ are detectable apically at pupal stage (see 3.2.2). These data indicate that, though Sdt is required for maintenance of the complex in adult eyes, it is not required in pupal eyes before formation of the stalk membrane. To understand what domains of Sdt protein are required for stabilization of the Crb-complex at the stalk membrane, different Sdt-transgenes were expressed in a *sdt* mutant background.

The obtained data demonstrate a requirement of several domains of Sdt for the Crb-complex stabilization. First, Sdt should be able to bind to Crb, since when the Sdt-encoding transgenes that lack the PDZ domain are expressed, no Crb-complex was detected at the stalk membrane. L27-N domain is of similar importance as well as the binding of Sdt to *D*PATJ. The L27-C domain was shown to be not important for the stabilization of the Crb-complex at the stalk membrane. This fully corresponds to the data on the *DLin-7* phenotype, where the Crb-complex is still localized to the stalk membrane (A. Bachmann, personal communication).

Interestingly, a strong requirement of the SH3 and GUK domains for the stabilization of the Crb-complex was shown. When any or both of these domains are absent, none of the members of the Crb-complex is detected at the stalk membrane (see 3.3.2.3). It was demonstrated that the binding of a PDZ domain to its ligand might influence the binding capacity of the GUK domain of the MAGUK proteins (Brenman *et al*., 1998). In case of Sdt this effect was not demonstrated, but it may be the same, and, similarly, binding of the L27-N domain to *D*PATJ may have the same influence on the GUK domain. For Sdt, an interaction between its SH3 and GUK domains was shown *in vitro* (Kempkens, 2005). These data lead to a model of the function of Sdt in

the maintenance of the Crb-complex at the stalk membrane in PRCs of adult *Drosophila* eyes (Figure 4.4). The GUK and/or SH3 domain of Sdt bind to an unknown protein P. This protein may be the same as protein Z (see Figure 4.3A), or may be a different one. This binding is mediated by inter- or intramolecular interactions between the SH3 and GUK domains of Sdt. At the same time, the binding of the PDZ domain of Sdt to Crb and the L27-N of Sdt to *D*PATJ are necessary for the interaction between protein P and Sdt. When Sdt is bound to Crb and *D*PATJ and has an intact SH3/GUK part, it binds to protein P. Then protein P stabilizes Sdt, and, as a consequence, the whole Crb-complex at the stalk membrane.

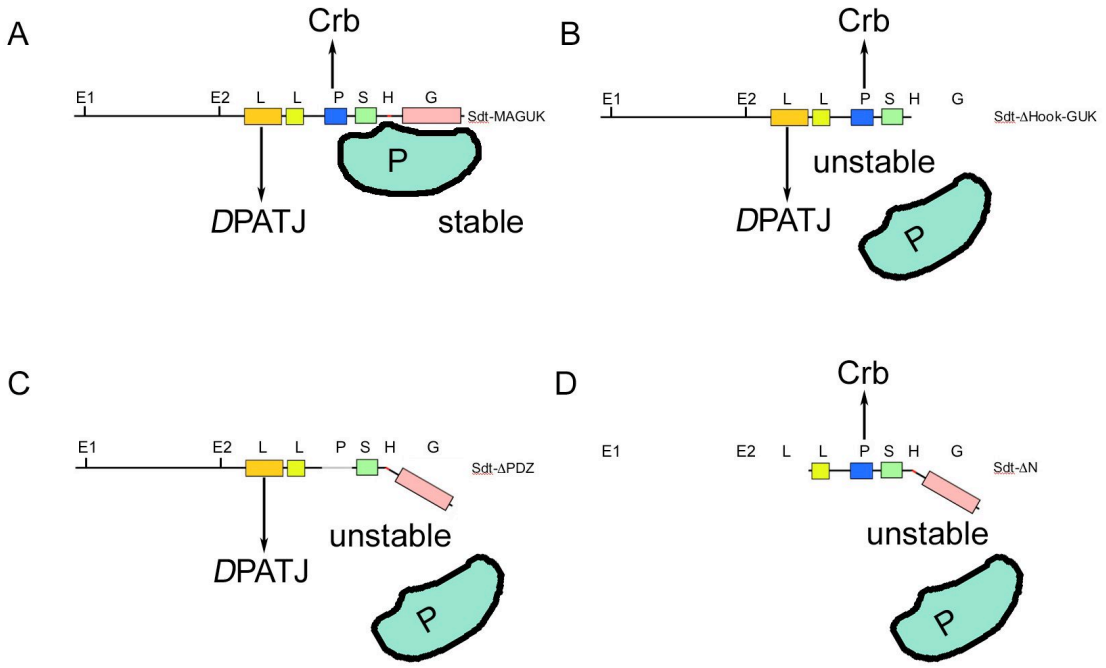


Figure 4.4. Sdt is important for the stabilization of the Crb-complex at the stalk membrane in the adult eyes. In the wild type (A) both SH3 and GUK domains of Sdt bind to unknown protein P, that may be the same as protein Z in Figure 4.3A. This binding occurs only when both PDZ domain and L27-N domain of Sdt are bound to their ligands. Then protein P stabilizes the whole complex in its position. Different examples are shown (B-D), when deleterious Sdt-transgenes are expressed on the *sdt* mutant background. When Sdt-ΔGUK (B) or Sdt-ΔSH3 is expressed protein P cannot bind to Sdt anymore (B), and the Crb-complex is unstable. When Sdt-ΔPDZ (C) or Sdt-ΔN (D) is expressed, PDZ or L27-N domains of Sdt are absent and cannot bind to their ligands. As the result the conformation of SH3-GUK part of Sdt is affected and does not allow binding to protein P. The result is the same as in B – the Crb-complex is unstable and delocalized from the stalk membrane.

For a better understanding of this process at the molecular level, the identification of protein P is required. Unfortunately, the previously performed yeast-two-hybrid screens did not result in any promising candidates (Ö. Kempkens, S. Berger, personal communication).

4.5 Crb-complex function in pigment cells sorting

The sorting of pigment cells is a complex process that includes elimination of excess of pigment cells, their specialization, and multiple morphological events that result in the specific cell shape of each of pigment cell type. In previous studies it was shown that overexpression of Crb interferes with this process at the step of pigment cells elimination. In pupal eyes that overexpress Crb, additional pigment cells are detected. These cells include mainly secondary, and tertiary pigment cells and bristle cells (Grzeschik, 2003).

It was shown that the disturbance of pigment cell sorting caused by Crb overexpression depends on its FERM-binding domain (Grzeschik, 2003). When this domain is modified these defects are stronger. At the same time, neither the ERLI motif of Crb (Grzeschik, 2003) nor the PDZ domain of Sdt (this work) is important for this phenotype. However, it was shown that expression of Sdt in a wild type background induces defects in pigment cell sorting, similar to that produced by overexpression of Crb (see 3.3.3.1). One possibility to explain the data is that the phenotype caused by Sdt overexpression is independent of the phenotype caused by Crb overexpression. The other possibility is that Sdt can modulate the Crb function in this process indirectly via its interaction with *D*PATJ and other unknown proteins. Such indirect action will as well explain lower penetrance of pigment cell sorting defects observed upon Sdt overexpression in comparison to Crb overexpression.

The question remains how Crb and Sdt can influence pigment cell sorting on the molecular level. It was shown that Crb overexpression disrupts the continuous belt of *D*E-Cad between primary pigment cells and interommatidial cells. When Sdt was expressed in a wild type background, the same disruption was observed in some discs though not as strong as in case of Crb overexpression (see Figure 3.25D-D'). It may be that the Crb-complex is influencing pigment cell sorting by stabilization AJs in

pigment cells, and AJ proteins, in turn, control this process. It was shown that different signalling molecules are localized to AJs, including β-catenin, or Arm - member of Wingless-signalling pathway. Alternatively, stabilization of AJs in pigment cells might have nothing to do with the function of the Crb-complex in pigment cell sorting. It was recently shown that the Crb-complex may directly interact with Notch-signalling in wing imaginal discs (Herranz *et al*., 2006), and Notch-signalling is known to control multiple processes in eye development. To answer what is the exact function of the Crb-complex here, additional studies are required.

4.6 Role of Sdt in the establishment of photoreceptor cells morphology

When a construct encoding only the PDZ domain of Sdt is expressed in a wild type background, defects in PRCs morphology are detected. They can be explained in the following way. The transgene-encoded PDZ domain binds to the ERLI motif of Crb. It is assumed that the number of Crb molecules is not increased in comparison to the wild type (as least no increase can be detected on the level of confocal microscopy). Therefore, only less Crb molecules are available to bind to endogenous wild type Sdt. The proper complex assembly happens to a lower extent than in wild type, and as result, it cannot perform its function as efficient as in wild type.

The other domain that is necessary for Sdt function in rhabdomere morphogenesis is the L27-N. When Sdt-1-566 is expressed, no morphological phenotype is observed. When Sdt-1-L27N is expressed, morphological defects are detected. This domain binds to *D*PATJ. That means that binding of Sdt to *D*PATJ in appropriate amounts is important for proper PRC morphogenesis, and in this case the same explanation as in the case of the PDZ domain can be applied, namely that overexpression of a Sdt protein that contains the L27-N domain acts in a dominant-negative way.

The obtained data indicate that the amount of the assembled Crb-complex is important for its proper function. Since the morphology is established during pupal development, all data obtained from *sdt* mutations confirm this observation. In sdt^{N5} the amount of the Crb-complex in adult eyes is strongly reduced. But in pupal eye

discs up to 60% p.d. no significant reduction in the amount of Crb-complex members can be detected. Even the level of Sdt seems to be nearly as in the wild type. In the case of this mutation, no morphological defects are observed in the adult eyes (with respect to rhabdomere morphology). In comparison, in *sdt*^{XP96} mutant pupal eye discs there is still Sdt protein, but its amount is strongly reduced. Less Sdt can bind Crb and *D*PATJ and only reduced amount of the Crb-complex is assembled. As result, morphological defects are observed in adult eyes. This interpretation is consistent with previous data, since mutations in both *crb* and *DPATJ* cause morphological defects and also disrupt the triple Crb-Sdt-*D*PATJ complex.

In light of these results, other observations seem to be important. When different constructs were expressed on a mutant background, the overall amount of Sdt protein in the developing PRCs seems to be nearly the same and equals that of Sdt in the neighbouring wild type cells. At the same time, the amount of Sdt detected in the surrounding pigment cells varies strongly – from a very faint staining to an extremely bright one. This suggests that a mechanism to regulate the amount of apically localized Sdt in the developing photoreceptor cells exists. If the amount of complex bound Sdt is crucial for establishment of PRCs morphology, as proposed before, it is important to have a mechanism that controls this amount. Unfortunately, the structure-functional analysis performed did not allow the determination of the exact domain of Sdt required for the control of its amount.

Morphological defects were detected when both *Rh1*GAL4 and *ey*GAL4 were used to overexpress Sdt. *ey*GAL4 stops expression relatively early in the first half of pupal development (Beronja *et al.,* 2005). *Rh1*GAL4 starts to be expressed at about 70% p.d. That means that expression of Sdt during a wide period of development influences rhabdomere morphology. The minimum range would be from about 30% to 75% p.d.

To sum up the obtained data, the following model of Sdt action during morphogenesis of PRCs is proposed (see Figure 4.5A). During pupal stages Sdt is expressed, localized apically, and by some unknown mechanism the amount of Sdt protein in the developing PRCs is controlled. Sdt at this stage provides a link between Crb and *D*PATJ. Though Crb and *D*PATJ are brought apically independently of Sdt

(see 3.3.1.3), Sdt may be required to build the direct link between them and/or to regulate the amount of assembled Crb-complex. These proteins, in turn, provide the further binding to unknown proteins, or perform the morphogenetic functions themselves. *D*Lin-7 does not play any role, as it was demonstrated that in *DLin-7* mutant eyes there are no morphological defects (A. Bachmann, personal communication). May be that one of the downstream targets of the Crb-complex in this case is Baz-complex. If so, the further regulatory events may be controlled by PTEN protein, an interaction partner of Baz (Pinal *et al.*, 2006). PTEN is regulating the PIP2/PIP3 ratio, and, thus, regulates actin cytoskeleton (Pinal *et al.*, 2006). On the other side, it is possible that the function of the complex is provided by *D*PATJ. No interaction partners of its four PDZ domains are known at present, and it cannot be excluded that one of them regulates morphogenesis. The last possibility is that Crb itself, when stabilized at the stalk membrane by other members of the Crb-complex, may perform the morphogenetic function via its extracellular domain. Recently, it was demonstrated that the expression of the extracellular domain of Crb in a *crb* mutant background is sufficient to rescue the defects in rhabdomere morphology, but not the stalk membrane length (M. Richard, personal commubication). The most likely scenario, however, is that all these three different pathways are playing a role in the establishment of proper morphology of PRCs. Thus, it can be concluded, that Sdt acts as a scaffold and organizes multiple morphogenic pathways at the apical membrane of the developing PRCs.

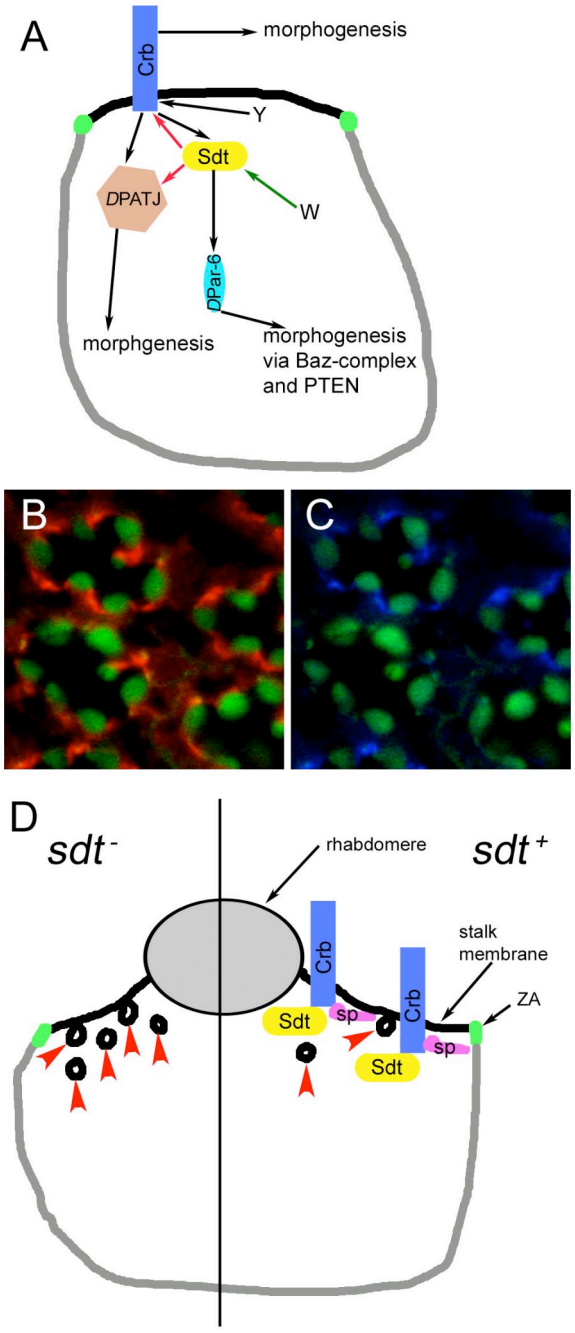


Figure 4.5. Sdt acts as a scaffold to organize multiple morphological pathways in developing eye.

A – Crb brings Sdt and *D*PATJ apically in PRCs of pupal eye. The amount of Sdt is control by unknown protein W. Crb is localized apically by unknown protein Y. Sdt also localizes apically *D*Par-6 and provides link between Crb and *D*PATJ. Then Crb via its extracellular domain, *D*PATJ via its PDZ domains, and *D*Par-6 via interaction with Baz-complex regulate the morphogenesis of rhabdomeres.

B-D – Crb-complex regulates stalk membrane length, probably, by regulation of β_H-spectrin.

B-C – In sdt^{N5} amount of β_H-spectrin at the stalk membrane is strongly reduced (red staining in B). Small eye clones mutant for sdt^{N5} are marked by the absence of Sdt protein (C). The remaining amount of Sdt in the mutant cells is too low to be detected in comparison to the neighbouring wild-type cells.

D – The model of regulation of the length of the stalk membrane by β_H-spectrin mediated by the Crb-complex. Sdt stabilizes Crb at the stalk membrane. Crb localizes β_H-spectrin. β_H-spectrin regulates the rate of endocytosis. When no Sdt is localized apically, then no Crb protein is at the stalk membrane. This leads to the absence of β_H-spectrin, and formation of more endocytic vesicles (marked by red arrowheads). As result, the length of the stalk membrane is decreased.

To prove this hypothesis, several additional experiments should be done. First, the existence of a mechanism that controls the level of Sdt in the developing PRCs should be proved. To do this, the constructs may be expressed using the *elav*GAL4 driver. This driver expresses GAL4 in all photoreceptor cells, but not in pigment or cone cells, starting from the determination of their fate. Then the amount of expressed protein in the pupal eye discs of staged pupae can be analyzed semi-quantitative on Western blot. It was shown that in adult eyes the constructs result in different expression level when they are expressed in all cells (Figure 3.16). If a mechanism to control the amount of Sdt in PRCs in pupal eyes exists, then all constructs expressed using the *elav*GAL4 will result in similar amount of the transgene-encoded protein at pupal stage, independent on the general efficiency of expression of each transgene.

Second, to confirm that the main function of Sdt in establishment of rhabdomere morphology is providing a link between Crb, *D*PATJ and *D*Par-6, constructs should be analyzed for their capability to rescue the *sdt* mutant phenotype. The full-length transgenes and Sdt-ΔL27-C will be probably able to rescue it, as they restore the complete Crb-complex. It is more interesting to see if the constructs that express apically localized protein in pupal eyes, but not in adult eyes, also can rescue the morphological defects caused by sdt^{K85}, at least to some extent. If the hypothesis is correct, they will do so, except for Sdt-ΔN, as they can provide a connection between Crb, *D*PATJ and *D*Par-6 at least until 60% of pupal development. The remaining transgenes should not rescue morphological defects, if the hypothesis is true. To check the rescue large eye clones mutant for sdt^{K85} and expressing Sdt-transgenes have to be analysed.

Separately from regulation of rhabdomere morphology, the question remains concerning the role of Sdt and the Crb-complex in regulation of the stalk membrane length. It was demonstrated that the morphology of the rhabdomeres and the stalk membrane length are not necessarily connected. In sdt^{N5} mutant eyes the rhabdomere morphology remains normal, but the stalk membrane is reduced in length (Berger, personal communication). Although the Crb-complex is still localized at the stalk membrane in PRCs mutant for sdt^{N5}, its amount is strongly reduced (see 3.2.1, Figure 3.11). One of the possible candidates to perform the function of the Crb-complex in the

regulation of the stalk membrane length is β_H-spectrin. Its localization to the stalk membrane is absolutely dependent on the presence of the Crb-complex there. It is delocalized in the *sdt* mutants. Preliminary data indicate that also in sdt^{N5} the amount of β_H-spectrin at the stalk membrane in the adult PRCs is strongly reduced in comparison to wild type (Figure 4.5B and 4.5C). When Sdt-encoding constructs were expressed in a sdt^{K85} mutant background, β_H-spectrin was detected at the stalk membrane exclusively in cases when both Sdt and Crb were also detected at the stalk membrane. In all other cases it was delocalized (see 3.3.2.3, Figure 3.25). In a recent paper of G. Thomas (Phillips & Thomas, 2006) it was demonstrated that β_H-spectrin regulates the endocytosis rate at the stalk membrane. The decrease in β_H-spectrin amount enhances endocytosis. From these data, the role of the Crb-complex in the control of the stalk membrane length can be suggested. Crb binds to β_H-spectrin, and localizes it to the stalk membrane. This localization as well as the one of Crb is stabilized in the adult eyes by Sdt. β_H-spectrin, in turn, controls the level of endocytosis at the stalk membrane, thereby regulating its size. When it is absent, more endocytic vesicles bud from the stalk membrane, more membrane is internalized, and the length of the stalk membrane decreases (schematically shown in Figure 4.5D).

This model gives rise to several interesting question. One is, if the expression of those transgenes that result in an assembled Crb-complex in the adult eyes can rescue the length of the stalk membrane and other morphological defects. If they do so, but others do not (especially the ones that result in proteins localized correctly at the pupal but not at the adult stage), this will serve as a powerful confirmation of the model. The second question is, if knocking down Sdt in adult stage (for example, using RNAi technique) results in the gradual decrease in the stalk membrane length that has initially the correct size. When the endocytic model is true, the knocking down of Sdt will result in the delocalization of the whole complex, including β_H-spectrin. Then the endocytosis rate at the stalk membrane will increase, and the stalk membrane length, oppositely, will decrease.

These experiments together with some others will give an answer on the role of this controlled endocytosis at the stalk membrane, and the role of stalk membrane length in PRCs morphology and light-induced retinal degeneration.

4.7 Crb-complex and the retinal degeneration

The role of the Crb-complex in prevention of light-induced retinal degeneration is independent of its role in PRCs morphogenesis. Thus, eyes mutant for *sdt*^{XP96} have defects in PRCs morphology, but do not degenerate in constant light. Oppositely, *sdt*^{N5} mutation results in retinal degeneration, but the eyes mutant for this mutation have nearly wild type morphology, except for a reduced length of the stalk membrane (Berger, 2005, Berger, personal communication).

In this work different Sdt-encoding transgenes were expressed in a wild type background in either PRCs or pigment cells. Concomitantly, a construct that encodes double-stranded RNA of *white* gene was expressed. The latter inhibits the pigment production in pigment cells. Resulting eyes are white coloured. The expression of Sdt-MAGUK or Sdt-ΔN in pigment cells significantly decreased the degeneration rate in comparison to the control. At the same time expression of Sdt-MAGUK in PRCs had no significant effect on degeneration, and expression of Sdt-ΔN even enhanced degeneration. That indicates that the increased amount of Sdt in pigment cells protects against degeneration in the PRCs. In PRCs, the amount of the Sdt protein does not matter, or, alternatively, in PRCs, similar to pupal stages, mechanisms may exist that control the amount of Sdt.

Expression of the L27-N domain in pigment cells and, therefore, binding of Sdt to *D*PATJ does not prevent retinal degeneration, but plays an important role when expressed in PRCs. Oppositely, expression of the L27-C domain of Sdt in pigment cells and, therefore, its binding to *D*Lin-7 is important for the prevention of light-induced retinal degeneration, but has no function in PRCs (see Figure 4.6).

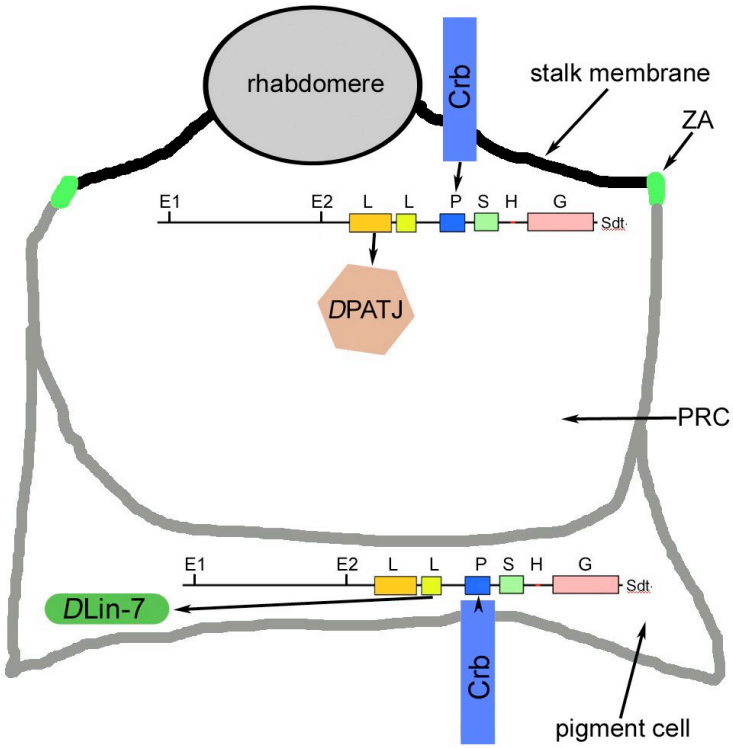


Figure 4.6. Sdt has a role in pigment cells as well as in PRCs of *w* ˉ eyes in prevention of light-induced retinal degeneration. In PRCs, the formation of triple Crb-Sdt-*D*PATJ complex is required to prevent the degeneration. In pigment cells ,Crb-Sdt-*D*Lin-7 is important for this function.

These data give the first indication that both pigment cells and PRCs may have an input in the control of light-dependent degeneration, and that these functions may be provided by different mechanisms. The role of the pigment cells remains elusive, and in this work it is shown for the first time, that the pigment cells may have important function, and not only restrict the light obtained by the single ommatidium. Obtained data indicate that the *Drosophila* eye is even closer to the mammalian eye in the mechanisms responsible for the degeneration than it was thought before. Recently, it was shown that CRB1, one of the three *Drosophila* homologs of Crb in mammals, is expressed exclusively in Mueller glia cells. CRB1 localization in Mueller glia cells depends on Pals1 – a mammalian homolog of Sdt (van Rossum *et al.*, 2006). Mueller glia cells surround mammalian photoreceptor cells, similar as the pigment cells in the *Drosophila* eye do. Mutations of *CRB1* result in retinal degeneration (den Hollander *et al.*, 1999). To conclude, in present work it was shown that pigment cells are important

for prevention of light-induced retinal degeneration, and may have a function that is similar to the function of Mueller glia cells in mammals.

Unfortunately, it is extremely difficult to perform an experiment to rescue the retinal degeneration by the expression of the Sdt-transgenes in either pigment cells or PRCs in a mutant background. Both the UAS-constructs and all GAL4-drivers are marked with *mini-white* gene, and the resulting eyes are red. Recently it was demonstrated, that the red pigment protects the eyes from degeneration, sice *crb* or *sdt* mutant eyes in a *white*$^{+}$ background do not degenerate. These data, together with observations that overexpression of Sdt in pigment cells delays the onset of degeneration, give a hint, that the Crb-complex acts in the same pathway that causes the degeneration in *white*$^{-}$ but otherwise wild-type eyes. To investigate this mechanism further, the generation of additional transgenes is required. An alternative marker, such as *yellow*$^{+}$ should be used. One of the possible approaches is to use the RNAi technique. Constructs encoding double-stranded RNA against Sdt products, or against specific exons of Sdt alternative splice-variants, can be used. The possibility to knock-down specific transcripts of a gene was shown by Celotto and Graveley in 2002 on an example of *Dscam* gene that, similarly to *sdt*, results in multiple alternative transcripts. Then these RNAi-constructs will be expressed in a cell-type specific manner, and the importance of specific splice-variants of Sdt, and the Crb-complex in general, for the prevention of light-induced retinal degeneration may be investigated.

An important question remains. Nothing is known so far about the mechanisms, how the Crb-complex prevents light-induced retinal degeneration. Degeneration is independent of rhabdomere morphology or the length of the stalk membrane. In *sdt*^{XP96}, rhabdomere morphology is affected, and the stalk membrane length is decreased (Hong *et al*., 2003), but no retinal degeneration is induced by the exposure to light. That means that the Crb-complex should have an independent function in this process. Presently, the most likely mechanism involves a light-induced transport of proteins participating in light-perception or light-adaptation. Recently, it was shown that the transport of several of these proteins, for example TRPL, occurs through the stalk membrane (Cronin *et al.*, 2006). Moreover, TRPL is bound to *D*moesin in the darkness (Chorna-Ornan *et al*., 2005). *D*moesin is able to bind to Crb via the FERM-binding

domain of Crb (Medina *et al*., 2002). This provides a direct link between the Crb-complex and light-dependent transport of TRPL, and indicates that the Crb-complex may interfere with the protection against constant light by regulating the transport of important proteins. To investigate this possibility, changes in light dependent transport of TRPL and, probably, other proteins should be analyzed in eyes mutant for *sdt* alleles that belong to the different classes of *sdt* mutations. The same should be done in eyes expressing different Sdt-transgenes in a wild-type background, including both the transgenes that have no effect on the degeneration when expressed in PRCs, and the transgenes that have.

4.8 Summary

The Crumbs- (Crb-) complex is required for two independent processes in the *Drosophila* eye. It assures the proper morphogenesis of the apical membrane of PRCs, and it prevents light-induced retinal degeneration in adult flies. Stardust (Sdt) plays a key role in the regulation of the different functions of the Crb-complex in the *Drosophila* eye. Three major functions of Sdt have been demonstrated in this work:

1. Sdt provides a link between the transmembrane Crb protein and other members of the complex as *D*PATJ, *D*Lin-7 and, probably, *D*Par-6. It recruits them to the stalk membrane in adult eyes. In pupal stage, it is not required for localization of *D*PATJ, but may still be important for its proper connection to Crb. The connection between different members of the Crb-complex is required for proper morphogenesis of photoreceptor cells and for prevention of light-induced degeneration.
2. Sdt is crucial for the stabilization of the complex at the stalk membrane in the adult flies. For this function, its L27-N, PDZ, SH3 and GUK domains are necessary. At pupal stages, Sdt is not required for the stabilization of the Crb-complex at the apical membrane. The change in the requirement for Sdt protein is likely to occur when the apical membrane becomes subdivided into rhabdomere and stalk.
3. Sdt is expressed in several splice-variants, both at pupal and adult stages. The alternative splice-variants might provide the fine-tuning of the Crb-complex composition, and thus provide its proper function at different stages in the retina.

Two additional interesting aspects came out of this work. First, the requirement of Sdt for proper localization of the Crb-complex is different before the stalk membrane formation and after. Before 60% p.d., Sdt has only minor function. Both Crb and *D*PATJ are localized properly. In adult photoreceptor cells, Sdt is crucial for localization of the cytoplasmic members and for the stability of the Crb-complex. Second, the Crb-complex might have a function not only in photoreceptor cells, but also in pigment cells, and the mechanisms controlled by the Crb-complex in different cell types may be different. In pigment cells, a link between Crb and *D*Lin-7 provided by Sdt is necessary for prevention of light-induced retinal degeneration. In photoreceptor cells, a link between Crb and *D*PATJ provided by Sdt is required for this function.

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6. Supplementary data

6.1 Abbreviations

α-	anti-
AJ(s)	Adherent junction(s)
ECR	evolutionary conserved region
GUK	Guanylate-kinase homolog
IOC	Interommatidia cells
L27	Lin-2/Lin-7 binding motif
MAGUK	membrane-associated guanylate kinase homolog
min	minutes
p.d.	pupal development
p.c.	pigment cells
PDZ	postsynaptic density 95/ discs large/ zonula occludens1
PRCs	photoreceptor cells
Rh1	Rhodopsin1
RLV	Rh1-immunopositive large vesicles
SAR	subapical region
SH3	*Src* homology 3
SJ	septate junctions
ZA	zonula adherence

6.2 Sequences of different fragments obtained using RT-PCR and 5'-RACE

* marks the primer that was used for sequencing.

6.2.1 5'-Ex6-44654* / 3'-SdtPDZSH3 (middle size band, about 1500 bp)

CGGGTCGCGTTTCGGCCGCCCACAGTGTCCTGGCCAGCGCCAGTGTGGCCCAT
GTCCTGGCCGCTCGCACCGCAGTACTGCAGCGACGACGTTCCCGCGTCTCTGGT
CCACTGCATCACAGTTCCCTCGGATTGCAGAAGGACATTGTGGAGCTGCTAACGC
AATCGAATACGGCGGCGGCCATCGAGCTGGGCAACCTGCTGACCAGCCATGAAA
TGGAGGGTCTGCTACTGGCCCACGATCGCATTGCCAATCACACGGATGGCACGC
CCTCGCCAACGCCCACGCCCACTCCGGCAATTGGAGCGGCGACGGGATCGACA
CTCAGCAGTCCGGTGGCTGGACCCAAGAGAAATCTAGGTATGGTGGTGCCACCG
CCCGTGGTACCACCGCCACTGGCGCAACGCGGAGCAATGCCACTGCCTCGCGG
GGAGTCGCCACCGCCGGTTCCCATGCCACCACTGGCTACAATGCCCATGTCCAT
GCCCGTTAATCTGCCGATGAGTGCGTGCTTTGGCACGCTAAACGATCAGAACGA
CAACATTCGTATCATCCAGATCGAGAAGTCAACGGAGCCGCTGGGCGCCACAGT
GCGCAACGAGGGTGAGGCGGTGGTCATCGGTAGGATTGTGCGTGGT

6.2.2 5'-Ex6-44654 / 3'-SdtPDZSH3* (middle size band, about 1500 bp)

GCGGCGGAGAACGCTCCGAGTCGGCCATCAGACGTTGGCGCAACTCATGTCTTC
CAATGTTGGGCGGGCCGATGAGGACGATGGGCCGCTTGTGGGTAGCGCGGGGA
TAGTACAAGGCCACCTCCTCGTAGGTGAGTATCTCCTCCGGATCCGTTTCATCCG
GCGCCGTGGTGGCGTATAAAGGATATCCCGCCTCGGAGCTGGCCTTCTTCTTCTT
TTTGCGACCCTTGCGCGCACACAGGAGCGTGGCTCCTTTGCTGCCCGATCCGTC
CTTACCGCGGGATCGCGCCAGACCCGCCTCCTCCGCAATGGCCAGCTTCATGGT
CTCGCGCTGATGCTGGAACGACTGACTAGGAATAAGACCGGCCAGCGTCTGGTC
CTCCTCGCCCTCGCGATACGCCTGCCACCAGTTGGGATCCTCGCGGCTAATCAC
GTGCAGTACATCGCCCTTCTGGAAGCTGATGCCCAGCTCCCGGCAGGGTATATA
CAGATCGTCCTCTGGATCGTAGTCGAAATGCGCCCGTACGTGCAACACGGCGGT
GTCACGATGTGCTCCACCCAACCCGGCCAGTTGACTGCCCGTTGTTCCGCCCAT

CACGCCGACGGACGGTGGACTACCCGCTGGGACGATCAGGAAGGTCAGGGTGC
CTTGCATGGCGCCCAACAGGGCGCACACTTCGTTCACCGTC

6.2.3 5’-Ex6-44654* / 3’-SdtPDZSH3 (largest band, about 2000 bp)

ACGCTGGCGGGTCGCGTTTCGGCCGCCCACAGTGTCCTGGCCAGCGCCAGTGT
GGCCCATGTCCTGGCCGCTCGCACCGCAGTACTGCAGCGACGACGTTCCCGCG
TCTCTGGTCCACTGCATCACAGTTCCCTCGGATTGCAGAAGGACATTGTGGAGCT
GCTAACGCAATCGAATACGGCGGCGGCCATCGAGCTGGGCAACCTGCTGACCAG
CCATGAAATGGAGGGTCTGCTACTGGCCCACGATCGCATTGCCAATCACACGGA
TGGCACGCCCTCGCCAACGCCCACGCCCACTCCGGCAATTGGAGCGGCGACGG
GATCGACACTCAGCAGTCCGGTGGCTGGACCCAAGAGAAATCTAGGTATGGTGG
TGCCACCGCCCGTGGTACCACCGCCACTGGCGCAACGCGGAGCAATGCCACTG
CCTCGCGGGGAGTCGCCACCGCCGGTTCCCATGCCACCACTGGCTACAATGCCC
ATGTCCATGCCCGTTAATCTGCCGATGAGTGCGTGCTTTGGCACGCTAAACGATC
AGAACGACAACATTCGTATCATCCAGATCGAGAAGTCAACGGAGCCGCTGGGCG
CCACAGTGCGCAACGAGGGTGAGGCGGTGGTCATCGGTAGGATTGTGCGTGGT
GGAGCGGCGGAAAAGTCGGGACTGTTGCACGAAGGTGATGAGATACTGGAGGT
CAACGGTCAGGAGTTGC

6.2.4 5’-Ex6-44654 / 3’-SdtGUKdm* (middle size band, about 1600 bp)

AAAGCGGCGCGCCAAAATATCCGCCTCGAAGGCCTGTCGCGTGATGAAGTGGTA
GTCCACTCCGGGCACTTCGCCCTCCCTGCGGGCTCGTGATGTGTGTGGCACTGC
GGCGGAGAAACGCTCCGAGTCGGCCATCAGACGTTGGCGCAACTCATGTCTTCC
AATGTTGGGCGGGCCGATGAGGACGATGGGCCGCTTGTGGGTAGCGCGGGGAT
AGTACAAGGCCACCTCCTCGTAGGTGAGTATCTCCTCCGGATCCGTTTCATCCGG
CGCCGTGGTGGCGTATAAAGGATATCCCGCCTCGGAGCTGGCCTTCTTCTTCTTT
TTGCGACCCTTGCGCGCACACAGGAGCGTGGCTCCTTTGCTGCCCGATCCGTCC
TTACCGCGGGATCGCGCCAGACCCGCCTCCTCCGCAATGGCCAGCTTCATGGTC
TCGCGCTGATGCTGGAACGACTGACTAGGAATAAGACCGGCCAGCGTCTGGTCC
TCCTCGCCCTCGCGATACGCCTGCCACCAGTTGGGATCCTCGCGGCTAATCACG

TGCAGTACATCGCCCTTCTGGAAGCTGATGCCCAGCTCCCGGCAGGGTATATAC
AGATCGTCCTCTGGAT

6.2.5 5’-Ex6-44654* / 3’-SdtGUKdm (largest band, about 2100 bp)

GCTGGCGGGTCGCGTTTCGGCCGCCCACAGTGTCCTGGCCAGCGCCAGTGTGG
CCCATGTCCTGGCCGCTCGCACCGCAGTACTGCAGCGACGACGTTCCCGCGTCT
CTGGTCCACTGCATCACAGTTCCCTCGGATTGCAGAAGGACATTGTGGAGCTGCT
AACGCAATCGAATACGGCGGCGGCCATCGAGCTGGGCAACCTGCTGACCAGCCA
TGAAATGGAGGGTCTGCTACTGGCCCACGATCGCATTGCCAATCACACGGATGG
CACGCCCTCGCCAACGCCCACGCCCACTCCGGCAATTGGAGCGGCGACGGGAT
CGACACTCAGCAGTCCGGTGGCTGGACCCAAGAGAAATCTAGGTATGGTGGTGC
CACCGCCCGTGGTACCACCGCCACTGGCGCAACGCGGAGCAATGCCACTGCCT
CGCGGGGAGTCGCCACCGCCGGTTCCCATGCCACCACTGGCTACAATGCCCATG
TCCATGCCCGTTAATCTGCCGATGAGTGCGTGCTTTGGCACGCTAAACGATCAGA
ACGACAACATTCGTATCATCCAGATCGAGAAGTCAACGGAGCCGCTGGGCGCCA
CAGTGCGCAACGAGGGTGAGGCGGTGGTCATCGGTAGGATTGTGCGTG

6.2.6 5’-Ex6-44654 / 3’-SdtGUKdm* (largest band, about 2100 bp)

AACAGTCCATCTTCTCCATTTGGAAATTCAACTCACCGTAGTAGGCCTTCTCATAT
TCACCGTGCTCCACAAAGCGGCGCGCCAAAATATCCGCCTCGAAGGCCTGTCGC
GTGATGAAGTGGTAGTCCACTCCGGGCACTTCGCCCTCCCTGCGGGCTCGTGAT
GTGTCTACGGAAAATGGCAAAGCGCAAGGATTACTAAAACGTATTTAAGGAGAAT
ATTCTAAGGGGATGGATTTGGTGTA

6.2.7 5’-Sdt-e1 / 3’-SdtPDZSH3*

GCGATGCTGCCAAAGGAGTCCAGGGTATGTTTGCTCATCACCTCATCGAAGGCG
CTCGATGAGGGCGAGGCCGTGTCCTTGCGTCCCTGATACAGGCCATCCACAACG
CACAGGAAATCATCGGTGATGCTGCTGTTGTTGCTGCTGCTGCTGCTGCTGATGC
CAACTATGTGGCTATTGTTGTTGCTATTGCTGTTGCCATTCGGATCGCTGCTCAAT

GTTGCCTGCAACTTGTGGCTGGGCGTCTGCTGTTGTGCCACCTGTGTTGCTGCC
ATTGCGGTTGTCGCTTGATGTTGCTTCTGTGGTGGACGGGGTGGCGGATATCGG
GGTGGCGTCTTGTTGCGTGCGATGAACGTGTCCGGACAATCGACGGCCATCTCA
CGGTGTGGACCGTTATCTTGGTTCACGGACAACATGGTCAACATTTCCTGGTCAA
TCTCCCTGATGTCGCCCGAGTGCGTGCGATAGTGTTGCTGCTGGAGCGGATGTT
GCTGCTGCAGCTGCTGCTGCAGTTGCAGCTGCTGCTGGTGGTTCAAATGATGCT
GACCGCTTATGTAGTCATCCGGCAGACATGGCTTCAAATCGTCGCCATCCAGCAC
AACAATGCTCGATCCGGAGCGCCGGCGATTCCCTCTGGGATCCTGCCGCAAACT
CTGATCGCCCAGTAAAAGTTCAGTTCTTCCAATTGATATGCTACTTGTGGGACTGT
TCTGTTGCTGCTGCTGCTGCTGAAGTTGCAACTGCTGCTGTTGCTGCAACTC

6.2.8 5’-Sdt-e1* / 3’-SdtPDZSH3

ACAGCACGAATGCATCAGCAGCAGCGATCGCCAGCAGCAGCAACAGATCACAGC
AACAGCAGCAGCAGCAGCATCAGTTGCTGAGTGCCGCCTACGAGTTGCAGCAAC
AGCAGCAGTTGCAACTTCAGCAGCAGCAGCAGCAACAGAACAGTCCCACAAGTA
GCATATCAATTGGAAGAACTGAACTTTTACTGGGCGATCAGAGTTTGCGGCAGGA
TCCCAGAGGGAATCGCCGGCGCTCCGGATCGAGCATTGTTGTGCTGGATGGCGA
CGATTTGAAGCCATGTCTGCCGGATGACTACATAAGCGGTCAGCATCATTTGAAC
CACCAGCAGCAGCTGCAACTGCAGCAGCAGCTGCAGCAGCAACATCCGCTCCAG
CAGCAACACTATCGCACGCACTCGGGCGACATCAGGGAGATTGACCAGGAAATG
TTGACCATGTTGTCCGTGAACCAAGATAACGGTCCACACCGTGAGATGGCCGTC
GATTTGTCCGGACACGTTTCATCGCACGCAACAAAGACGCCCCCCCCGATATCCC
GCCCACCCCCGTCCCCCACAGAAAGCAACATCAAAGCGACAACCGCAATGGCAG
CAACACAGGTGGCACAACAGCAGACGCCCAGCCACAAGTTGCAGGCAACATTGA
GCAGCGATCCGAATGCAACAGCAATAGCAACAACAATAGCCACATAGTTGGCATC
AGCAGCAGCAGCAGCAGCAACAACAGCAGCATCACCGATGATTT

6.2.9 5’-Sdt-Ex1-1 / 3’-Ex6-RA-44866*

GCGGGACGTCGTCGCTGCAGTACTGCGGTGCGAGCGGCCAGGACATGGGCCAC
ACTGGCGCTGGCCAGGACACTGTGGGCGGCCGAAACGCGACCCGCCAGCGTGC

TCAGTCCGCTCTTGCTCAGCTGGTTTTGCAGGCGGGTAAGGGCCGCTATCAGTT
CGCCATAGCCATCGATCTGTTCCGCCTGCGGCTGGTCTTCGTCGGGCAGATATG
CCTCATTCTCCACGCCAACCACCTGGGTGGCCAGCGTTGCCTGCTGCTGCTGCT
GGGCAACCGCTTTACCGGGCGTAGCCGTATCCTGAAGCGCCTTCAGCTTCCTGG
AGCCACAAAGACTGTTCCGCAGGAATTCATTTTGCTGCGCTATGCGCTCCTCCTC
CTCGCGTCGTCGGCGCAGTTGCTCCTGGTATTTCTTGATGCTGTCCAGCTGCTCC
AATGTGGGCTCAACAATCTGGGCGATCTGCTGATGCTGCAGCGGATGCGTTGTG
GCGCCGCTGCTGCCGCTGCTCGCATTTCCCGGCGCCCTGCGATCCGGCAGCTG
TGGTGCGGGTGAGCAATTGACCAGCCGACCATCCTTCTCCACCCGCAGATGATC
ACGTGGCGGCGGTACGGGCTTCGCATTCCCATTGAGCTGTGGTGGACGGGGTG
GCGGATATCGGGGTGGCGTCTTGTTGCGTGCGATGAACGTGTCCGGACAATCGA
CGGCCATCTCACGGTGTGGACCGTTATCTTGGTTCA

6.2.10 5’-Sdt-i1* / 3’-Ex6-RA-44866

TGCGTCGCGGCATTCAAATTCAACGATCCTCGACGCGTTTGCGACAACAACAGGA
GCAACTGCGCCGACGACGCGAGGAGGAGGAGCGCATAGCGCAGCAAAATGAAT
TCCTGCGGAACAGTCTTCGTGGCTCCAGGAAGCTGAAGGCGCTTCAGGATACGG
CTACGCCCGGTAAAGCGGTTGCCCAGCAGCAGCAGCAGGCAACGCTGGCCACC
CAGGTGGTTGGCGTGGAGAATGAGGCATATCTGCCCGACGAAGACCAGCCGCA
GGCGGAACAGATCGATGGCTATGGCGAACTGATAGCGGCCCTTACCCGCCTGCA
AAACCAGCTGAGCAAGAGCGGACTGAGCACGCTGGCGGGTCGCGTTTCGGCCG
CCCACAGTGTCCTGGCCAGCGCCAGTGTGGCCCATGTCCTGGCCGCTCGCACC
GCAGTACTGCAGCGACGACGTTCCCGCGTCTCTGGTCCACTGCATCACAGTTCC
CTCGG

6.2.11 5’-Sdt-Ex1-1* / 3’-Sdt-Ex4-1

CGCCGGCGCTCCGGATCGAGCATTGTTGTGCTGGATGGCGACGATTTGAAGCCA
TGTCTGCCGGATGACTACATAAGCGGTCAGCATCATTTGAACCACCAGCAGCAGC
TGCAACTGCAGCAGCAGCTGCAGCAGCAACATCCGCTCCAGCAGCAACACTATC
GCACGCACTCGGGCGACATCAGGGAGATTGACCAGGAAATGTTGACCATGTTGT

CCGTGAACCAAGATAACGGTCCACACCGTGAGATGGCCGTCGATTGTCCGGACA
CGTTCATCGCACGCAACAAGACGCCACCCCGATATCCGCCACCCCGTCCACCAC
AGCTCAATGGGAATGCGAAGCCCGTACCGCCGCCACGTGATCATCTGCGGGT

6.2.12 5’-Sdt-ExA2-1* / 3’-Sdt-Ex4-1

TGGCGGCCTTGAACCGGGTCACGCCCGTCGACATCACCCAGCTGTCCAAGAAGT
TACTAGAAGACGTCATAGTCCATCAAGCCAGTGATGTGCATTCATACAGCACGAA
TGCATCAGCAGCAGCGATCGCCAGCAGCAGCAACAGATCACAGCAACAGCAGCA
GCAGCAGCATCAGTTGCTGAGTGCCGCCTACGAGTTGCAGCAACAGCAGCAGTT
GCAACTTCAGCAGCAGCAGCAGCAACAGAACAGTCCCACAAGTAGCATATCAATT
GGAAGAACTGAACTTTTACTGGGCGATCAGAGTTTGCGGCAGGATCCCAGAGGG
AATCGCCGGCGCTCCGGATCGAGCATTGTTGTGCTGGATGGCGACGATTTGAAG
CCATGTCTGCCGGATGACTACATAAGCGGTCAGCATCATTTGAACCACCAGCAGC
AGCTGCAACTGCAGCAGCAGCTGCAGCAGCAACATCCGCTCCAGCAGCAACACT
ATCGCACGCACTCGGGCGACATCAGGGAGATTGACCAGGAAATGTTGACCATGT
TGTCCGTGAACCAAGATAACGGTCCACACCGTGAGATGGCCGTCGATTGTCCGG
ACACGTTCATCGCACGCAACAAGACGCCACCCCGATATCCGCCACCCCGTCCAC
CACAGCTCAATGGGAATGCGAAGCCCGTACCGCCGCCACGTGATCATCTGCGG

6.2.13 5’-Sdt-ExA1-1* / 3’-Sdt-Ex1-1

TTTGTTTTGAGTGCGCGCACGCGTTGGCGTCTCGTTTTTGGTACGTGGTTTGCAA
TCGTTTCGTTCTTTTTTTTTCGGTGTAACAAATTTGGTTTGTTGTTGTTTTATTGGG
GTGCAGCAGCAGCGGCAGCAGCAGAAAAAGCAGCAGCAGCGCAGCAGGCCTGG
GACATTGGCAACTCGGTGCAGGACGCCTTCGTCATCGCCGTCGAGGAGCACGCC
CGCGAACGTCTGCAGAGATTGGCGGCCTTGAACCGGGTCACGCCCGTCGACATC
ACCCAGCTGTCCAAGAAGTTACTAGAAGACGTCATAGTCCATCAAGCCAGTGATG
TGCATTCATACAGCACGAATGCATCAGCAGCAGCGATCGCCAGCAGCAGCAACA
GATCACAGCAACAGCAGCAGCAGCAGCATCAGTTGCTGAGTGCCGCCTACGAGT
TGCAGCAACAGCAGCAGTTGCAACTTCAGCAGCAGCAGCAGCAACAGAACAGTC
CCACAAGTAGCATATCAATTGGAAGAACTGAACTTTTACTGGGCGATCAGAGTTT

GCGGCAGGATCCCAGAGGGAATCGCCGGCGCTCCGGATCGAGCATTGTTGTGC
TGGATGGCGACGATTTGAAGCCATGTCTGCCGGATGACTACATAAGCGGTCAGC
ATCATTTGAACCACCAGCAGCAGCTGCAACTGCAGCAGCAGCTGCAGCAGCAAC
ATCCGCTCCAGCAGCAACACTATCGCACGCACTCGGGCG

6.2.14 5'-Sdt-k1 / 3'-Sdt-Ex1-1* (middle size fragment).

GCTGCTGGAGCGGATGTTGCTGCTGCAGCTGCTGCTGCAGTTGCAGCTGCTGCT
GGTGGTTCAAATGATGCTGACCGCTTATGTAGTCATCCGGCAGACATGGCTTCAA
ATCGTCGCCATCCAGCACAACAATGCTCGATCCGGAGCGCCGGCGATTCCCTCT
GGGATCCTGCCGCAAACTCTGATCGCCCAGTAAAAGTTCAGTTCTTCCAATTGAT
ATGCTACTTGTGGGACTGTTCTGTTGCTGCTGCTGCTGCTGAAGTTGCAACTGCT
GCTGTTGCTGCAACTCGTAGGCGGCACTCAGCAACTGATGCTGCTGCTGCTGCT
GTTGCTGTGATCTGTTGCTGCTGCTGGCGATCGCTGCTGCTGATGCATTCGTGCT
GTATGAATGCACATCACTGGCTTGATGGACTATGACGTCTTCTAGTAACTTCTTGG
ACAGCTGGGTGATGTCGACGGGCGTGACCCGGTTCAAGGCCGCCAATCTCTGCA
GACGTTCGCGGGCGTGCTCCTCGACGGCGATGACGAAGGCGTCCTGCACCGAG
TTGCCAATGTCCCAGGCCAGTCGTGAATCATTCTTCTTTCGCACACGCAGACAAA
TTGTGCAGGATTTGATGCAGTCGTGTATGTGCCGAATGACCTCCGTGCGCGATAT
ATTCTCCAGGGTCAGGCCATTGACCTCGAGTATCTCATCGCCCACTTCCAGATTA
TCGCGATCGGGCGGTGAGCCGTATAGTTTAATTTTACCCTCCACATTTTCGACGA
GTATGATGACATAGCCCGATAGTTCGACAACTTGTTGCTTGCCCTGGGACGAGGA
GATCGTTGAGGCCGACGGCGTGCCCCGAGGATTGTGCGCTTAAGTTGGAGCGG
CTGCTGTTCGCTTGCATCGCGCAAGGCAGAGGCGCCACTTCAGCGGGGGCTCCT
GTTCCCTCTTATCCGGTTGCTCTTCCTGTTGCTCACGAGTTCAGCTG

6.2.15 5'-Sdt-k1* / 3'-Sdt-Ex1-1 (middle size fragment).

TCCACATCAGAATCTGATCTCCTTCTCCGCCACCCATTGTCAGGCGCAGCCAACA
GGTGAGACTTTGATGCCAATTGGAGTTCGTCTTGGAGCTTAGACAGCTGAACTCG
TGGAGCAACAGGAAGAGCAACCGGATAAGGGGGAACAGGAGCCCCCGCTGAAG
TGGCGCCTCTGCCTTGCGCGATGCAAGCGAACAGCAGCCGCTCCAACTTAAGCG

CACAATCCTCGGGCACGCCGTCGGCCTCAACGATCTCCTCGTCCCAGGGCAAGC
AACAAGTTGTCGAACTATCGGGCTATGTCATCATACTCGTCGAAAATGTGGAGGG
TAAAATTAAACTATACGGCTCACCGCCCGATCGCGATAATCTGGAAGTGGGCGAT
GAGATACTCGAGGTCAATGGCCTGACCCTGGAGAATATATCGCGCACGGAGGTC
ATTCGGCACATACACGACTGCATCAAATCCTGCACAATTTGTCTGCGTGTGCGAA
AGAAGAATGATTCACGACTGGCCTGGGACATTGGCAACTCGGTGCAGGACGCCT
TCGTCATCGCCGTCGAGGAGCACGCCCGCGAACGTCTGCAGAGATTGGCGGCC
TTGAACCGGGTCACGCCCGTCGACATCACCCAGCTGTCCAAGAAGTTACTAGAA
GACGTCATAGTCCATCAAGCCAGTGATGTGCATTCATACAGCACGAATGCATCAG
CAGCAGCGATCGCCAGCAGCAGCAACAGATCACAGCAACAGCAGCAGCAGCAG
CATCAGTTGCTGAGTGCCGCCTACGAGTTGCAGCAACAGCAGCAGTTGCAACTTC
AGCAGCAGCAGCAGCAACAGAACAGTCCCACAAGTAGCATATCAATTGGAAGACT
GAACTTTTACTGGGCGATCAGAGTTTGCGG

6.2.16 5’-Sdt-e1* / 3’-Ex6-RA-44866

TACAGCACGAATGCATCAGCAGCAGCGATCGCCAGCAGCAGCAACAGATCACAG
CAACAGCAGCAGCAGCAGCATCAGTTGCTGAGTGCCGCCTACGAGTTGCAGCAA
CAGCAGCAGTTGCAACTTCAGCAGCAGCAGCAGCAACAGAACAGTCCCACAAGT
AGCATATCAGTTGGAAGAACTGAACTTTTACTGGGCGATCAGAGTTTGCGGCAGG
ATCCCAGAGGGAATCGCCGGCGCTCCGGATCGAGCATTGTTGTGCTGGATGGCG
ACGATTTGAAGCCATGTCTGCCGGATGACTACATAAGCGGTCAGCATCATTTGAA
CCACCAGCAGCAGCTGCAACTGCAGCAGCAGCTGCAGCAGCAACATCCGCTCCA
GCAGCAACACTATCGCACGCACTCGGGCGACATCAGGGAGATTGACCAGGAAAT
GTTGACCATGTTGTCCGTGAACCAAGATAACGGTCCACACCGTGAGATGGCCGT
CGATTGTCCGGACACGTTCATCGCACGCAACAAGACGCCACCCCGATATCCGCC
ACCCCGTCCACCACAGCTCAATGGGAATGCGAAGCCCGTACCGCCGCCACGTGA
TCATCTGCGGGTGGAGAAGGATGGGTCGGCTGGTCAATTGCTCACCCGCACCAC
AGCTGCCGGATCGCAGGGCGCCGGGAAATGCGAGCAGCGGCAGCAGCGGCGC
CACAACGCATCCGCTGCAGCATCAGCAGATCGCCCAGGTTGTTGAGCCCACATT
GGAGCAGCTGGACAGCATCAAGAAATACCAGGAGCAACTGCGCC

6.2.17 5’-Sdt-k1* / 3’-Sdt-e1

TCCACATCAGAATCTGATCTCCTTCTCCGCCACCCATTGTCAGGCGCAGCCAACA
GGTGAGACTTTGATGCCAATTGGAGTTCGTCTTGGAGCTTAGACAGCTGAACTCG
TGGAGCAACAGGAAGAGCAACCGGATAAGGGGGAACAGGAGCCCCCGCTGAAG
TGGCGCCTCTGCCTTGCGCGATGCAAGCGAACAGCAGCCGCTCCAACTTAAGCG
CACAATCCTCGGGCACGCCGTCGGCCTCAACGATCTCCTCGTCCCAGGGCAAGC
AACAAGTTGTCGAACTATCGGGCTATGTCATCATACTCGTCGAAAATGTGGAGGG
TAAAATTAAACTATACGGCTCACCGCCCGATCGCGATAATCTGGAAGTGGGCGAT
GAGATACTCGAGGTCAATGGCCTGACCCTGGAGAATATATCGCGCACGGAGGTC
ATTCGGCACATACACGACTGCATCAAATCCTGCACAATTTGTCTGCGTGTGCGAA
AGAAGAATGATTCACGACTGGCCTGGGACATTGGCAACTCGGTGCAGGACGCCT
TCGTCATCGCCGTCGAGGAGCACGCCCGCGAACGTCTGCAGAGATTGGCGGCC
TTGAACCGGGTCACGCCCGTCGACATCACCCAGCTGTCCAAGAAGCTGCAGCAA
ACGAAGAGCGGCACTGCGACCAGTCAGCGCCAGGATCTCAGCTTCCTGAACGAA
TCGACGCCGATCTATGTGACATCCTTCACGAGCAACCAGATCACCTGCAGCAGTT
CCACAATGACGACGGCCACCGCCGGCGGTCCGATTAGTGCACCATCGCTGGCG
ACGGCCACTACCACCGTGCCGACTGCATCCTCCCACACCACCACGGTGGTGGCC
CAGATCGAGCACGGTGCATCGGCGCTGGTCAGTGCCGCTGTGCAGCAGCCACT
GCAGCGGATCGCAATGCGAATAGCACAACGTCGGCGGCCCTAAAACAGACGGCC
AATTGCATTGGGA

6.2.18 5’-Sdt-i2* / 3’-Ex6-RA-44866

TATGCATACGAAAGATTGTCGCCGCACCTCGTCGCGCCACGCAGACAAAGATCG
ATGAAAGTGCAGCTCGAAAATGGTCGTGTCCACTTTGCACATAACGCTGGAGAAC
AACGGCAATGTTGCTGCGGGGCAGCCTGCTGCCGTTGCTGCTGCCAGTGTCGTT
GGGGCAGCAACATCGCCGACCAAGTTTGCCGTGCTGTCGGCGCAGCAGCTGCA
ACTGCAACTGCAGCAGCAGCAGCAGCAGCAGAACGAAAAGGAGAACCATATGAA
CAATAATAACAATAACAGCGACTATTGTGATATGAATGGCAATGGAGTTGGAATGG
GAAACGGTGGTAGTGGTGGACCGGGATCTCTGACCCCCCAATCCCCCGACCAAT

ATGTCGGCTCACCTGTATCATCCGCTGCCACGCCCCAGCGCACCGCCTCCGCCC
TGCGTCGCGGCATTCAAATTCAACGATCCTCGACGCGTTTGCGACAACAACAGGA
GCAACTGCGCCGACGACGCGAGGAGGAGGAGCGCATAGCGCAGCAAAATGAAT
TCCTGCGGAACAGTCTTCGTGGCTCCAGGAAGCTGAAGGCGCTTCAGGATACGG
CTACGCCCGGTAAAGCGGTTGCCCAGCAGCAGCAGCAGGCAACGCTGGCCACC
CAGGTGGTTGGCGTGGAGAATGAGGCATATCTGCCCGACGAAGACCAGCCGCA
GGCGGAACAGATCGATGGCTATGGCGAACTGATAGCGGCCCTTACCCGCCTGCA
AAACCAGCTGAGCAAGAGCGGACTGAGCACGCTGGCGGGTCGCGTTTCGGCCG
CCCACAGTG

6.2.19 5’-Sdt-i2 / 3’-SdtPDZSH3*

GGGCGGGCCGATGAGGACGATGGGCCGCTTGTGGGTAGCGCGGGGATAGTACA
AGGCCACCTCCTCGTAGGTGAGTATCTCCTCCGGATCCGTTTCATCCGGCGCCG
TGGTGGCGTATAAAGGATATCCCGCCTCGGAGCTGGCCTTCTTCTTCTTTTTGCG
ACCCTTGCGCGCACACAGGAGCGTGGCTCCTTTGCTGCCCGATCCGTCCTTACC
GCGGGATCGCGCCAGACCCGCCTCCTCCGCAATGGCCAGCTTCATGGTCTCGC
GCTGATGCTGGAACGACTGACTAGGAATAAGACCGGCCAGCGTCTGGTCCTCCT
CGCCCTCGCGATACGCCTGCCACCAGTTGGGATCCTCGCGGCTAATCACGTGCA
GTACATCGCCCTTCTGGAAGCTGATGCCCAGCTCCCGGCAGGGTATATACAGAT
CGTCCTCTGGATCGTAGTCGAAATGCGCGCGTACGTGCAACACGGCGGTGTCAC
GATGTGCTCCACCCAACCCGGCCAGTTGACTGCCCGTTGTTCCGCCCATCACGC
CGACGGACGGTGGACTACCCGCTGGGACGATCAGGAAGGTCAGGGTGCCTTGC
ATGGCGCCCAACAGGGCGCACACTTCGTTCACCGTCTTGCCACGCAACTCCTGA
CCGTTGACCTCCAGTATCTCATCACCTTCGTGCAACAGTCCCGACTTCTCCGCCG
CTCCACCACGCACAATCCTACCGATGACCACCGCCTCACCCTCGTTGCGACTGT
GGCGCCCAGCGGCTCCGTTGACTTCTCGATCTGGATGATAC

6.2.20 5’-Sdt-i2* / 3’-SdtPDZSH3

CGTGTCCACTTCTGCACATAACGCTGGAGAACAACGGCAATGTTGCTGCGGGGC
AGCCTGCTGCCGTTGCTGCTGCCAGTGTCGTTGGGGCAGCAACATCGCCGACCA
AGTTTGCCGTGCTGTCGGCGCAGCAGCTGCAACTGCAACTGCAGCAGCAGCAGC
AGCAGCAGAACGAAAAGGAGAACCATATGAACAATAATAACAATAACAGCGACTA
TTGTGATATGAATGGCAATGGAGTTGGAATGGGAAACGGTGGTAGTGGTGGACC
GGGATCTCTGACCCCCCAATCCCCCGACCAATATGTCGGCTCACCTGTATCATCC
GCTGCCACGCCCCAGCGCACCGCCTCCGCCCTGCGTCGCGGCATTCAAATTCAA
CGATCCTCGACGCGTTTGCGACAACAACAGGAGCAACTGCGCCGACGACGCGAG
GAGGAGGAGCGCATAGCGCAGCAAAATGAATTCCTGCGGAACAGTCTTCGTGGC
TCCAGGAAGCTGAAGGCGCTTCAGGATACGGCTACGCCCGGTAAAGCGGTTGCC
CAGCAGCAGCAGCAGGCAACGCTGGCCACCCAGGTGGTTGGCGTGGAGAATGA
GGCATATCTGCCCGACGAAGACCAGCCGCAGGCGGAACAGATCGATGGCTATGG
CGAACTGATAGCGGCCCTTACCCGCCTGCAAAACCAGCTGAGCAAGAGCGGACT
GAGCACGCTGGCGGGTCGCGTTTCGGCCGCCCACAGTGTCCTGGCCAGCGCCA
GTGTGGCCCATGTCCTGGCCGCTCGCACCGCATACTGCAGCGACGACGTTCCCG
CGTCTCTGGTCCACTGCATCACAGTTCC

6.2.21 5’-Sdt-l1* / 3’-Sdt-k1

TTTCAACGCAACGTCGTGGCAATTCCAAATCGGCGGCAGACGCGGGCGGGCGCA
GCGCACGGGCGGATTGCACAAATTATCAAGTTAATTTTAAGGAAAACGAAACGCA
CAACAAAAAAGCCAAAATAGATCCAGTAATAAAACTAATTTAAAATAATCACCTAAG
TCAACAAACTGCAGTTTCAGCAGCGCAAAACCGAGAACACCAAGTCTTACTAAGG
AACTATTCGACTAAGAGCGTTAACCAAATCAAGCTGTTGTATTTAACTGCAATCCA
AAGACGTAATGCTTTCGATCTGATGACATTCGACTACGGGCGCCATGAGCATCAG
TGCCACCTGGTTTTGGGTGCCATCTCTGTCTCCACTTCCGAATACGAATCCGAAT
CCGAATCCGAGCCCACATCCACACATGCGCACCCACCACAGCACGGTCAACCCC
ATCCCCACCAGCAACCCCAGCCACACTCGCATCCCCACGCGAATCCGAGTC

6.2.22 3’-Sdt-ExA5-1-RACE

CGATCTCTGTCGCCCTTTCTCACTTTTTCTGCGTCTTTCCCTCTCTGTGTCTGACT
TTCTATTACAACTTTTTT

6.2.23 3’-Sdt-I1-RACE

GTGATTAGTTGCGTTTGCCGTATTTTTAGCCCGTTCCGGAGGATTTATTACGTGCA
ATCCGCCCGTGCGCTGCGCACGCCCGCGTCTGCCGCCGATTTGGAATTGCCACG
ACGTTGCGTTGAAATGTTGCGCGTAACTCCCCGCGTACTCTGCGTTGATACCACT
GCTTGCCCTATA

6.2.24 3’-Sdt-i1-RACE

TTCTGCTGCTGCTGCTGCTGCAGTTGCAGTTGCAGCTGCTGCGCCGACAGC
ACGGCAAACTTGGTCGGCGATGTTGCTGCCCCAACGACACTGGCAGCAGCAACG
GCAGCAGGCTGCCCCGCAGCAACATTGCCGTTGTTCTCCAGCGTTATGTGCAAA
GTGGACACGACCATTTTCGAGCTGCACTTTCATCGATCTTTGTCTGCGTGGCGCG
ACGAGGTGCGGCGACAATCTTTCGTATGCATATGGCCTTATCTAATCGCTTATCG
GTTCTTTGTCACGCACTTGGCCCGCACAACAGCGAAGAGAATAAAACAAACGGTT
AAAATTGGTCGAAAAAATCAACAAAAAAAAATTGCGGCCGATCGCAAAACGCGTA
AAAAAACACCTCCCCGCGTACTCTGGGTTGAAACCCCTGCTTGCCCTAAAGGGA

6.3 mRNA of six Sdt splice-variants expressed in the head

For all splice variants bold letters mark the coding sequences.

6.3.1 Sdt-B1

3986 bp

ATTCTATGTTCGTACCGCGAGGCAGCAGAAATGTGTAAACCAAAAGGATAAACCA
ACAAAAAAAAACAATATTTAGAAACCAAAAAAAAAAGTTGTAATAGAAAGTCAGAC
ACAGAGAGGGAAAGACGCAGAAAAAGTGAGAAAGGGCGACAGAGATCGAACGG
ATTACAGAT**ATGCGCATCCTAAAGCAATGGAATCGCCGGCGCTCCGGATCGAGC**
ATTGTTGTGCTGGATGGCGACGATTTGAAGCCATGTCTGCCGGATGACTACATA
AGCGGTCAGCATCATTTGAACCACCAGCAGCAGCTGCAACTGCAGCAGCAGCT
GCAGCAGCAACATCCGCTCCAGCAGCAACACTATCGCACGCACTCGGGCGACA
TCAGGGAGATTGACCAGGAAATGTTGACCATGTTGTCCGTGAACCAAGATAAC
GGTCCACACCGTGAGATGGCCGTCGATTGTCCGGACACGTTCATCGCACGCAA
CAAGACGCCACCCCGATATCCGCCACCCCGTCCACCACAGCTCAATGGGAATG
CGAAGCCCGTACCGCCGCCACGTGATCATCTGCGGGTGGAGAAGGATGGTCG
GCTGGTCAATTGCTCACCCGCACCACAGCTGCCGGATCGCAGGGCGCCGGGAA
ATGCGAGCAGCGGCAGCAGCGGCGCCACAACGCATCCGCTGCAGCATCAGCA
GATCGCCCAGATTGTTGAGCCCACATTGGAGCAGCTGGACAGCATCAAGAAAT
ACCAGGAGCAACTGCGCCGACGACGCGAGGAGGAGGAGCGCATAGCGCAGCA
AAATGAATTCCTGCGGAACAGTCTTCGTGGCTCCAGGAAGCTGAAGGCGCTTC
AGGATACGGCTACGCCCGGTAAAGCGGTTGCCCAGCAGCAGCAGCAGGCAAC
GCTGGCCACCCAGGTGGTTGGCGTGGAGAATGAGGCATATCTGCCCGACGAAG
ACCAGCCGCAGGCGGAACAGATCGATGGCTATGGCGAACTGATAGCGGCCCTT
ACCCGCCTGCAAAACCAGCTGAGCAAGAGCGGACTGAGCACGCTGGCGGGTC
GCGTTTCGGCCGCCCACAGTGTCCTGGCCAGCGCCAGTGTGGCCCATGTCCTG
GCCGCTCGCACCGCAGTACTGCAGCGACGACGTTCCCGCGTCTCTGGTCCACT
GCATCACAGTTCCCTCGGATTGCAGAAGGACATTGTGGAGCTGCTAACGCAATC
GAATACGGCGGCGGCCATCGAGCTGGGCAACCTGCTGACCAGCCATGAAATG
GAGGGTCTGCTACTGGCCCACGATCGCATTGCCAATCACACGGATGGCACGCC
CTCGCCAACGCCCACGCCCACTCCGGCAATTGGAGCGGCGACGGGATCGACA

CTCAGCAGTCCGGTGGCTGGACCCAAGAGAAATCTAGGTATGGTGGTGCCACC
GCCCGTGGTACCACCGCCACTGGCGCAACGCGGAGCAATGCCACTGCCTCGC
GGGGAGTCGCCACCGCCGGTTCCCATGCCACCACTGGCTACAATGCCCATGTC
CATGCCCGTTAATCTGCCGATGAGTGCGTGCTTTGGCACGCTAAACGATCAGAA
CGACAACATTCGTATCATCCAGATCGAGAAGTCAACGGAGCCGCTGGGCGCCA
CAGTGCGCAACGAGGGTGAGGCGGTGGTCATCGGTAGGATTGTGCGTGGTGGA
GCGGCGGAGAAGTCGGGACTGTTGCACGAAGGTGATGAGATACTGGAGGTCA
ACGGTCAGGAGTTGCGTGGCAAGACGGTGAACGAAGTGTGCGCCCTGTTGGGC
GCCATGCAAGGCACCCTGACCTTCCTGATCGTCCCAGCGGGTAGTCCACCGTC
CGTCGGCGTGATGGGCGGAACAACGGGCAGTCAACTGGCCGGGTTGGGTGGA
GCACATCGTGACACCGCCGTGTTGCACGTACGGGCGCATTTCGACTACGATCC
AGAGGACGATCTGTATATACCCTGCCGGGAGCTGGGCATCAGCTTCCAGAAGG
GCGATGTACTGCACGTGATTAGCCGCGAGGATCCCAACTGGTGGCAGGCGTAT
CGCGAGGGCGAGGAGGACCAGACGCTGGCCGGTCTTATTCCTAGTCAGTCGTT
CCAGCATCAGCGCGAGACCATGAAGCTGGCCATTGCGGAGGAGGCGGGTCTG
GCGCGATCCCGCGGTAAGGACGGATCGGGCAGCAAAGGAGCCACGCTCCTGT
GTGCGCGCAAGGGTCGCAAAAAGAAGAAGAAGGCCAGCTCCGAGGCGGGATA
TCCTTTATACGCCACCACGGCGCCGGATGAAACGGATCCGGAGGAGATACTCA
CCTACGAGGAGGTGGCCTTGTACTATCCCCGCGCTACCCACAAGCGGCCCATC
GTCCTCATCGGCCCGCCCAACATTGGAAGACATGAGTTGCGCCAACGTCTGAT
GGCCGACTCGGAGCGTTTCTCCGCCGCAGTGCCACACACATCACGAGCCCGCA
GGGAGGGCGAAGTGCCCGGAGTGGACTACCACTTCATCACGCGACAGGCCTTT
GAGGCGGATATTTTGGCGCGCCGCTTTGTGGAGCACGGTGAATATGAGAAGGC
CTACTACGGCACATCACTGGAGGCCATACGCACGGTGGTGGCCAGCGGCAAGA
TCTGTGTGCTCAACCTGCATCCGCAGAGCCTTAAGCTGCTGCGCGCCTCTGACC
TCAAGCCGTACGTGGTGCTGGTGGCGCCGCCCAGCTTGGACAAGCTGCGCCAA
AAGAAGCTGCGCAACGGCGAACCCTTCAAGGAGGAAGAGCTCAAAGACATCAT
TGCCACGGCCAGGGATATGGAGGCCCGTTGGGGTCACCTATTCGACATGATCA
TAATCAACAACGACACGGAGCGCGCCTACCACCAACTGCTGGCCGAGATCAAC
TCGCTGGAACGCGAGCCCCAATGGGTGCCCGCCCAGTGGGTGCACAACAATCG
CGACGAGTCATAATGGGTTCTAAAGCACCCGCAACCACCACACTACCATCTTCCC

TAGGATCCATCAACACCCAAGAAATGAATAGCAAACCCAAACTCTTTGTAAATACA
ATTTTCAATTGAGCCATGAAGCGGAGAGCAGGATCGATTCCAGTCCAGTAGTTCG
GTTTGCCTGCCGATGCGATTAATCCCAACCCCTCCAATATTATATATAAATATATAT
ATATTTATACTATTCGTTGCATATGTGCATTTTAGTCTTAGAGAGAGATCGAGTTTT
AATTGTGTGTACTCCTGTTTTAAAAGCATTCCAACCTCTTTATGTATGTTCACAAAA
TTTGTTGTTCGATTAATCGATTTCGGGAATGCCACAGAGGAAATGGTGATGCTGA
AATCAGTGATTTCAAAAACCATATTGCTTCTTGTGGCACTTCCGTTTTCCTCTTTTT
TTTTACCACTACCACTAACTTATATACTGTAAAAGAATGTTTTTTTTTTTTTTGTATT
TAAATTAAGCTTGGACACTTCCTTAATTTCAAAGGGCAAATTGAAATTGTAAAATAA
CGAATCAGCAAAGGAAGGCCAGAATCGATATGATAGTACCCCTAGTACCCAATAA
AATGTGTAATAATACCCATAAGCGACTCCGAATGTTAACAGAGCCAGAACAATCC
GTAGGCAGCTGTGCGAGGGGAGATCGATCCGTCGGATATAGATTCATATTCATCT
TCAGATTCAAATTCAAAAGCAGAATCAGAAACATACAGAGAAACCGAATCAGAAG
CGCAGATACAGATGCAGATGCAAGTGTTGCAATTACGTAATCTATTTACTTTATAA
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6.3.2 Sdt-B2

4507 bp

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6.3.3 Sdt-B3

5749 bp.

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6.3.4 Sdt-C1

5131 kb.

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6.3.5 Sdt-C2

6373 bp

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GGCTATGTCATCATACTCGTCGAAAATGTGGAGGGTAAAATTAAACTATACGGC
TCACCGCCCGATCGCGATAATCTGGAAGTGGGCGATGAGATACTCGAGGTCAA
TGGCCTGACCCTGGAGAATATATCGCGCACGGAGGTCATTCGGCACATACACG
ACTGCATCAAATCCTGCACCATTTGTCTGCGTGTGCGAAAGAAGAATGATTCAC
GACTGGCCTGGGACATTGGCAACTCGGTGCAGGACGCCTTCGTCATCGCCGTC
GAGGAGCACGCCCGCGAACGTCTGCAGAGATTGGCGGCCTTGAACCGGGTCA
CGCCCGTCGACATCACCCAGCTGTCCAAGAAGCTGCAGCAAACGAAGAGCGG
CACTGCGACCAGTCAGCGCCAGGATCTCAGCTTCCTGAACGAATCGACGCCGA
TCTATGTGACATCCTTCACGAGCAACCAGATCACCTGCAGCAGTTCCACAATGA
CGACGGCCACCGCCGGCGGTCCGATCAGTGCACCATCGCTGGCGACGGCCAC
TACCACCGTGCCGACGGCATCCTCCCACACCACCACGGTGGTGGCCCAGATCG
AGCACGGTGCATCGGCGCTGGTCAGTGCCGCTGTGGCAGCAGCCACTGCAGC
GGATCGCAATGCGAATAGCACAACGTCGGCGGCCCTAAAACAGACGGCCAATT
GCATTGGGAATAGCACAAGCAGTCTTGGCACGACCAGCACCACCAGCAGCCAA
TCGACCAGCAGTGCCACGGGCCACATCTACCAGACCAGTCAGGCGCAGCAGC

AGCAGTTGCAACAGTTGCAGCAGCAGCTTGCGGCAGCAGCGGCGGCTGGAAA
GCCATTGCAGGCCAAATCCCTGCTGGCCAGCAGCTTGCAACATCTGGCCGAGG
AGGTGGACAACGAGGATCTGGACGATGATGACGATGTGGATGGGGCAAACTAT
TGTGGCATAACCTATATAAGCTACAACAACAAACATGCCCAGTTGCCGACGACA
ACATTGCCGGCGACGACCGCATTGCCAGCGGCAGCTGCATCATTGGCCACAAC
GGCGGCGATTTACCAACAGAGGCAGCAGCAACAACATCAGCAGCAGCAGCAA
CAGCAGCAGCAGCCGGTGCACCACCACAACCACCCACCAACGGCGAGCCAAC
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AGTTTCGTGGATGCCCAGACATCCACATCACCGCTGATGGCACAGCAGCTTCAT
TCGCAGCATGCGGATGTGGATGCAGCGCCACCATCGTCGTCATCATCATCAGC
GGTGGTGGTGGTGGAGCGGCATGTGCATGGCACCACCACGCCCAAAACGGAG
TACTCCACGGCCATATCCAGTGGCCAACTGCAACAGGCCTTTGCCGAATTGCAG
CTCCACTCGAGCAACAATAATGCAACACAGCAGCAGCAGCAACATTTACTTTTA
AGCAACAACAATAATAGCAATAATTCAATGGCAGCGGCACAGACAACGGCATC
TCTGATGAAGAATTGTGATCTACTGATATCGAACAATCTGTATCCACCGAGAAG
AGAGTTACTAGAAGACGTCATAGTCCATCAAGCCAGTGATGTGCATTCATACAG
CACGAGTGCATCAGCAGCAGCGATCGCCAGCAGCAGCAACAGATCACAGCAA
CAGCAACAGCAGCAGCAGCAGCATCAGTTGCTGAGTGCCGCCTACGAGTTGCA
GCAACAGCAGCAATTGCAACTTCAGCAGCAGCAGCAGCAACAGAACAGTCCCA
CAAGTAGCATATCAATTGGAAGAACTGAACTTTTACTGGGCGATCAGAGTTTGC
GGCAGGATCCCAGAGGGAATCGCCGGCGCTCCGGATCGAGCATTGTTGTGCTG
GATGGCGACGATTTGAAGCCATGTCTGCCGGATGACTACATAAGCGGTCAGCA
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ATCCGCTCCAGCAGCAACACTATCGCACGCACTCGGGCGACATCAGGGAGATT
GACCAGGAAATGTTGACCATGTTGTCCGTGAACCAAGATAACGGTCCACACCG
TGAGATGGCCGTCGATTGTCCGGACACGTTCATCGCACGCAACAAGACGCCAC
CCCGATATCCGCCACCCCGTCCACCACAGCTCAATGGGAATGCGAAGCCCGTA
CCGCCGCCACGTGATCATCTGCGGGTGGAGAAGGATGGTCGGCTGGTCAATTG
CTCACCCGCACCACAGCTGCCGGATCGCAGGGCGCCGGGAAATGCGAGCAGC
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TTGTTGAGCCCACATTGGAGCAGCTGGACAGCATCAAGAAATACCAGGAGCAA

CTGCGCCGACGACGCGAGGAGGAGGAGCGCATAGCGCAGCAAAATGAATTCC
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ACGCCCGGTAAAGCGGTTGCCCAGCAGCAGCAGCAGGCAACGCTGGCCACCC
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CCACGCCCACTCCGGCAATTGGAGCGGCGACGGGATCGACACTCAGCAGTCC
GGTGGCTGGACCCAAGAGAAATCTAGGTATGGTGGTGCCACCGCCCGTGGTAC
CACCGCCACTGGCGCAACGCGGAGCAATGCCACTGCCTCGCGGGGAGTCGCC
ACCGCCGGTTCCCATGCCACCACTGGCTACAATGCCCATGTCCATGCCCGTTAA
TCTGCCGATGAGTGCGTGCTTTGGCACGCTAAACGATCAGAACGACAACATTCG
TATCATCCAGATCGAGAAGTCAACGGAGCCGCTGGGCGCCACAGTGCGCAACG
AGGGTGAGGCGGTGGTCATCGGTAGGATTGTGCGTGGTGGAGCGGCGGAGAA
GTCGGGACTGTTGCACGAAGGTGATGAGATACTGGAGGTCAACGGTCAGGAGT
TGCGTGGCAAGACGGTGAACGAAGTGTGCGCCCTGTTGGGCGCCATGCAAGGC
ACCCTGACCTTCCTGATCGTCCCAGCGGGTAGTCCACCGTCCGTCGGCGTGAT
GGGCGGAACAACGGGCAGTCAACTGGCCGGGTTGGGTGGAGCACATCGTGAC
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ACGTGATTAGCCGCGAGGATCCCAACTGGTGGCAGGCGTATCGCGAGGGCGA
GGAGGACCAGACGCTGGCCGGTCTTATTCCTAGTCAGTCGTTCCAGCATCAGC
GCGAGACCATGAAGCTGGCCATTGCGGAGGAGGCGGGTCTGGCGCGATCCCG
CGGTAAGGACGGATCGGGCAGCAAAGGAGCCACGCTCCTGTGTGCGCGCAAG
GGTCGCAAAAAGAAGAAGAAGGCCAGCTCCGAGGCGGGATATCCTTTATACGC
CACCACGGCGCCGGATGAAACGGATCCGGAGGAGATACTCACCTACGAGGAG
GTGGCCTTGTACTATCCCCGCGCTACCCACAAGCGGCCCATCGTCCTCATCGGC

CCGCCCAACATTGGAAGACATGAGTTGCGCCAACGTCTGATGGCCGACTCGGA
GCGTTTCTCCGCCGCAGTGCCACACACATCACGAGCCCGCAGGGAGGGCGAA
GTGCCCGGAGTGGACTACCACTTCATCACGCGACAGGCCTTTGAGGCGGATAT
TTTGGCGCGCCGCTTTGTGGAGCACGGTGAATATGAGAAGGCCTACTACGGCA
CATCACTGGAGGCCATACGCACGGTGGTGGCCAGCGGCAAGATCTGTGTGCTC
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CGTGGTGCTGGTGGCGCCGCCCAGCTTGGACAAGCTGCGCCAAAAGAAGCTGC
GCAACGGCGAACCCTTCAAGGAGGAAGAGCTCAAAGACATCATTGCCACGGCC
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GACACGGAGCGCGCCTACCACCAACTGCTGGCCGAGATCAACTCGCTGGAACG
CGAGCCCCAATGGGTGCCCGCCCAGTGGGTGCACAACAATCGCGACGAGTCAT
AATGGGTTCTAAAGCACCCGCAACCACCACACTACCATCTTCCCTAGGATCCATC
AACACCCAAGAAATGAATAGCAAACCCAAACTCTTTGTAAATACAATTTTCAATTGA
GCCATGAAGCGGAGAGCAGGATCGATTCCAGTCCAGTAGTTCGGTTTGCCTGCC
GATGCGATTAATCCCAACCCCTCCAATATTATATATAAATATATATATATTTATACTA
TTCGTTGCATATGTGCATTTTAGTCTTAGAGAGAGATCGAGTTTTAATTGTGTGTA
CTCCTGTTTTAAAAGCATTCCAACCTCTTTATGTATGTTCACAAAATTTGTTGTTCG
ATTAATCGATTTCGGGAATGCCACAGAGGAAATGGTGATGCTGAAATCAGTGATT
TCAAAAACCATATTGCTTCTTGTGGCACTTCCGTTTTCCTCTTTTTTTTTACCACTA
CCACTAACTTATATACTGTAAAAGAATGTTTTTTTTTTTTTTGTATTTAAATTAAGCT
TGGACACTTCCTTAATTTCAAAGGGCAAATTGAAATTGTAAAATAACGAATCAGCA
AAGGAAGGCCAGAATCGATATGATAGTACCCCTAGTACCCAATAAAATGTGTAATA
ATACCCATAAGCGACTCCGAATGTTAACAGAGCCAGAACAATCCGTAGGCAGCTG
TGCGAGGGGAGATCGATCCGTCGGATATAGATTCATATTCATCTTCAGATTCAAAT
TCAAAAGCAGAATCAGAAACATACAGAGAAACCGAATCAGAAGCGCAGATACAGA
TGCAGATGCAAGTGTTGCAATTACGTAATCTATTTACTTTATAAAATATATACTATA
CGGATATACATACACATAAATTATAAATCGAAGCGTACTTAATACAATTAATTTTTA
ACAAAACGAATGCAAGAGCAAAGTGAAAACTAAGAAATAAAACCGTTCATAAGAG
AGAAACAACAAAATCAGTAAGTCATTGCCAAAAAAAAAAAAAACAAGAAAAACGAG

6.3.6 Sdt-D

3865 bp.

AGTTGTTTTTCTACGCGTTCTGCGATCGGCCGCAATTTTTTTTTGTTGATTTTTTCG
ACCAATTTTAACCGTTTGTTTTATTCTCTTCGCTGTTGTGCGGGCCAAGTGCGTGA
CAAAGAACCGATAAGCGATTAGATAAGGCCATATGCATACGAAAGATTGTCGCCG
CACCTCGTCGCGCCACGCAGACAAAGATCGATGAAAGTGCAGCTCGAAA**ATGGT**
CGTGTCCACTTTGCACATAACGCTGGAGAACAACGGCAATGTTGCTGCGGGGC
AGCCTGCTGCCGTTGCTGCTGCCAGTGTCGTTGGGGCAGCAACATCGCCGACC
AAGTTTGCCGTGCTGTCGGCGCAGCAGCTGCAACTGCAACTGCAGCAGCAGCA
GCAGCAGCAGAACGAAAAGGAGAACCATATGAACAATAATAACAATAACAGCG
ACTATTGTGATATGAATGGCAATGGAGTTGGAATGGGAAACGGTGGTAGTGGT
GGACCGGGATCTCTGACCCCCCAATCCCCCGACCAATATGTCGGCTCACCTGT
ATCATCCGCTGCCACGCCCCAGCGCACCGCCTCCGCCCTGCGTCGCGGCATTC
AAATTCAACGATCCTCGACGCGTTTGCGACAACAACAGGAGCAACTGCGCCGA
CGACGCGAGGAGGAGGAGCGCATAGCGCAGCAAAATGAATTCCTGCGGAACA
GTCTTCGTGGCTCCAGGAAGCTGAAGGCGCTTCAGGATACGGCTACGCCCGGT
AAAGCGGTTGCCCAGCAGCAGCAGCAGGCAACGCTGGCCACCCAGGTGGTTG
GCGTGGAGAATGAGGCATATCTGCCCGACGAAGACCAGCCGCAGGCGGAACA
GATCGATGGCTATGGCGAACTGATAGCGGCCCTTACCCGCCTGCAAAACCAGC
TGAGCAAGAGCGGACTGAGCACGCTGGCGGGTCGCGTTTCGGCCGCCCACAG
TGTCCTGGCCAGCGCCAGTGTGGCCCATGTCCTGGCCGCTCGCACCGCAGTAC
TGCAGCGACGACGTTCCCGCGTCTCTGGTCCACTGCATCACAGTTCCCTCGGAT
TGCAGAAGGACATTGTGGAGCTGCTAACGCAATCGAATACGGCGGCGGCCATC
GAGCTGGGCAACCTGCTGACCAGCCATGAAATGGAGGGTCTGCTACTGGCCCA
CGATCGCATTGCCAATCACACGGATGGCACGCCCTCGCCAACGCCCACGCCCA
CTCCGGCAATTGGAGCGGCGACGGGATCGACACTCAGCAGTCCGGTGGCTGG
ACCCAAGAGAAATCTAGGTATGGTGGTGCCACCGCCCGTGGTACCACCGCCAC
TGGCGCAACGCGGAGCAATGCCACTGCCTCGCGGGGAGTCGCCACCGCCGGT
TCCCATGCCACCACTGGCTACAATGCCCATGTCCATGCCCGTTAATCTGCCGAT
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GATCGAGAAGTCAACGGAGCCGCTGGGCGCCACAGTGCGCAACGAGGGTGAG
GCGGTGGTCATCGGTAGGATTGTGCGTGGTGGAGCGGCGGAGAAGTCGGGAC
TGTTGCACGAAGGTGATGAGATACTGGAGGTCAACGGTCAGGAGTTGCGTGGC
AAGACGGTGAACGAAGTGTGCGCCCTGTTGGGCGCCATGCAAGGCACCCTGAC
CTTCCTGATCGTCCCAGCGGGTAGTCCACCGTCCGTCGGCGTGATGGGCGGAA
CAACGGGCAGTCAACTGGCCGGGTTGGGTGGAGCACATCGTGACACCGCCGT
GTTGCACGTACGGGCGCATTTCGACTACGATCCAGAGGACGATCTGTATATACC
CTGCCGGGAGCTGGGCATCAGCTTCCAGAAGGGCGATGTACTGCACGTGATTA
GCCGCGAGGATCCCAACTGGTGGCAGGCGTATCGCGAGGGCGAGGAGGACCA
GACGCTGGCCGGTCTTATTCCTAGTCAGTCGTTCCAGCATCAGCGCGAGACCAT
GAAGCTGGCCATTGCGGAGGAGGCGGGTCTGGCGCGATCCCGCGGTAAGGAC
GGATCGGGCAGCAAAGGAGCCACGCTCCTGTGTGCGCGCAAGGGTCGCAAAA
AGAAGAAGAAGGCCAGCTCCGAGGCGGGATATCCTTTATACGCCACCACGGCG
CCGGATGAAACGGATCCGGAGGAGATACTCACCTACGAGGAGGTGGCCTTGTA
CTATCCCCGCGCTACCCACAAGCGGCCCATCGTCCTCATCGGCCCGCCCAACA
TTGGAAGACATGAGTTGCGCCAACGTCTGATGGCCGACTCGGAGCGTTTCTCC
GCCGCAGTGCCACACACATCACGAGCCCGCAGGGAGGGCGAAGTGCCCGGAG
TGGACTACCACTTCATCACGCGACAGGCCTTTGAGGCGGATATTTTGGCGCGCC
GCTTTGTGGAGCACGGTGAATATGAGAAGGCCTACTACGGCACATCACTGGAG
GCCATACGCACGGTGGTGGCCAGCGGCAAGATCTGTGTGCTCAACCTGCATCC
GCAGAGCCTTAAGCTGCTGCGCGCCTCTGACCTCAAGCCGTACGTGGTGCTGG
TGGCGCCGCCCAGCTTGGACAAGCTGCGCCAAAAGAAGCTGCGCAACGGCGA
ACCCTTCAAGGAGGAAGAGCTCAAAGACATCATTGCCACGGCCAGGGATATGG
AGGCCCGTTGGGGTCACCTATTCGACATGATCATAATCAACAACGACACGGAG
CGCGCCTACCACCAACTGCTGGCCGAGATCAACTCGCTGGAACGCGAGCCCCA
ATGGGTGCCCGCCCAGTGGGTGCACAACAATCGCGACGAGTCATAATGGGTTC
TAAAGCACCCGCAACCACCACACTACCATCTTCCCTAGGATCCATCAACACCCAA
GAAATGAATAGCAAACCCAAACTCTTTGTAAATACAATTTTCAATTGAGCCATGAA
GCGGAGAGCAGGATCGATTCCAGTCCAGTAGTTCGGTTTGCCTGCCGATGCGAT
TAATCCCAACCCCTCCAATATTATATATAAATATATATATATTTATACTATTCGTTGC
ATATGTGCATTTTAGTCTTAGAGAGAGATCGAGTTTTAATTGTGTGTACTCCTGTTT

TAAAAGCATTCCAACCTCTTTATGTATGTTCACAAAATTTGTTGTTCGATTAATCGA
TTTCGGGAATGCCACAGAGGAAATGGTGATGCTGAAATCAGTGATTTCAAAAACC
ATATTGCTTCTTGTGGCACTTCCGTTTTCCTCTTTTTTTTTTACCACTACCACTAACT
TATATACTGTAAAAGAATGTTTTTTTTTTTTTTGTATTTAAATTAAGCTTGGACACTT
CCTTAATTTCAAAGGGCAAATTGAAATTGTAAAATAACGAATCAGCAAAGGAAGGC
CAGAATCGATATGATAGTACCCCTAGTACCCAATAAAATGTGTAATAATACCCATA
AGCGACTCCGAATGTTAACAGAGCCAGAACAATCCGTAGGCAGCTGTGCGAGGG
GAGATCGATCCGTCGGATATAGATTCATATTCATCTTCAGATTCAAATTCAAAAGC
AGAATCAGAAACATACAGAGAAACCGAATCAGAAGCGCAGATACAGATGCAGATG
CAAGTGTTGCAATTACGTAATCTATTTACTTTATAAAATATATACTATACGGATATA
CATACACATAAATTATAAATCGAAGCGTACTTAATACAATTAATTTTTAACAAAACG
AATGCAAGAGCAAAGTGAAAACTAAGAAATAAAACCGTTCATAAGAGAGAAACAA
CAAAATCAGTAAGTCATTGCCAAAAAAAAAAAAAACAAGAAAAACGAG

6.4 Proteins encoded by six Sdt splice-variants in the head

The following colors are used for the marking of the following domains: ECR1, **ECR2**, **L27-N**, **L27-C**, **PDZ-C**, **SH3**, putative **Hook**, **GUK**, **PDZ-N**.

6.3.1 Sdt-B1

934 aa, 103 kDa

MRILKQWNRRRSGSSIVVLDGDDLKPCLPDDYISGQHHLNHQQQLQLQQQLQQQHP
LQQQHYRTHSGDIREIDQEMLTMLSVNQDNGPHREMAVDCPDTFIARNKTPPRYPP
PRPPQLNGNAKPVPPPRDHLRVEKDGRLVNCSPAPQLPDRRAPGNASSGSSGATTH
PLQHQQIAQIVEPTLEQLDSIKKYQEQL**RRRREEEE**RIAQQNEFLRNSLRGSRKLKAL
QDTATPGKAVAQQQQQATLATQVVGVENEAYLPDEDQPQAEQID**GYGELIAALTRL**
QNQLSKSGLSTLAGRVSAAHSVLASASVAHVLAARTAVLQRRRSRVSGP**LHHSSL**
GLQKDIVELLTQSNTAAAIELGNLLTSHEMEGLLLAHDRIANHTDGTPSPTPTPTPAI
GAATGSTLSSPVAGPKRNLGMVVPPPVVPPPLAQRGAMPLPRGESPPPVPMPPLAT
MPMSMPVNLPMSACFGTLNDQNDNIRIIQIEKST**EPLGATVRNEGEAVVIGRIVRGGA**
AEKSGLLHEGDEILEVNGQELRGKTVNEVCALLGAMQGTLTFLIVPAGSPPSVGVM
GGTTGSQLAGLGGAHRDTAV**LHVRAHFDYDPEDDLYIPCRELGISFQKGDVLHVISR**
EDPNWWQAYREGEEDQTLAGLIPSQSFQHQRETMKLAIAEEAGLARSRGKDGSGS
KGATLLCARKG**RKKKKKA**SSEAGYPLYATTAPDETDPEEILTYEEVALYYPRAT**HKR**
PIVLIGPPNIGRHELRQRLMADSERFSAAVPHTSRARREGEVPGVDYHFITRQAFEA
DILARRFVEHGEYEKAYYGTSLEAIRTVVASGKICVLNLHPQSLKLLRASDLKPYVVL
VAPPSLDKLRQKKLRNGEPFKEEELKDIIATARDMEARWGHLFDMIIINNDTERAYH
QLLAEINSLEREPQWVPAQWVHNNRDES

6.3.2 Sdt-B2

859 aa, 95 kDa

MLTMLSVNQDNGPHREMAVDCPDTFIARNKTPPRYPPPRPPQLNGNAKPVPPPRDH
LRVEKDGRLVNCSPAPQLPDRRAPGNASSGSSGATTHPLQHQQIAQIVEPTLEQLDSI
KKYQEQLRRRREEEERIAQQNEFLRNSLRGSRKLKALQDTATPGKAVAQQQQQATL
ATQVVGVENEAYLPDEDQPQAEQIDGYGELIAALTRLQNQLSKSGLSTLAGRVSAA
HSVLASASVAHVLAARTAVLQRRRSRVSGPLHHSSLGLQKDIVELLTQSNTAAAIEL
GNLLTSHEMEGLLLAHDRIANHTDGTPSPTPTPTPAIGAATGSTLSSPVAGPKRNLG
MVVPPPVVPPPLAQRGAMPLPRGESPPPVPMPPLATMPMSMPVNLPMSACFGTLN
DQNDNIRIIQIEKSTEPLGATVRNEGEAVVIGRIVRGGAAEKSGLLHEGDEILEVNGQE
LRGKTVNEVCALLGAMQGTLTFLIVPAGSPPSVGVMGGTTGSQLAGLGGAHRDTAV
LHVRAHFDYDPEDDLYIPCRELGISFQKGDVLHVISREDPNWWQAYREGEEDQTLA
GLIPSQSFQHQRETMKLAIAEEAGLARSRGKDGSGSKGATLLCARKGRKKKKKASS
EAGYPLYATTAPDETDPEEILTYEEVALYYPRATHKRPIVLIGPPNIGRHELRQRLMAD
SERFSAAVPHTSRARREGEVPGVDYHFITRQAFEADILARRFVEHGEYEKAYYGTSL
EAIRTVVASGKICVLNLHPQSLKLLRASDLKPYVVLVAPPSLDKLRQKKLRNGEPFK
EEELKDIIATARDMEARWGHLFDMIIINNDTERAYHQLLAEINSLEREPQWVPAQWV
HNNRDES

6.3.3 Sdt-B3

1393 aa, 153 kDa

MTTATAGGPISAPSLATATTTVPTASSHTTTVVAQIEHGASALVSAAVAAATAADRNA
NSTTSAALKQTANCIGNSTSSLGTTSTTSSQSTSSATGHIYQTSQAQQQQLQQLQQQ
LAAAAAAGKPLQAKSLLASSLQHLAEEVDNEDLDDDDDVDGANYCGITYISYNNKHA
QLPTTTLPATTALPAAAASLATTAAIYQQRQQQQHQQQQQQQQQPVHHHNHPPTAS
QLNRATAPAPLQLGGPVNPSFVDAQTSTSPLMAQQLHSQHADVDAAPPSSSSSSAV
VVVERHVHGTTTPKTEYSTAISSGQLQQAFAELQLHSSNNNATQQQQQHLLLSNNNN
SNNSMAAAQTTASLMKNCDLLISNNLYPPRRELLEDVIVHQASDVHSYSTSASAAAIA
SSSNRSQQQQQQQQQHQLLSAAYELQQQQQLQLQQQQQQQNSPTSSISIGRTELL
LGDQSLRQDPRGNRRRSGSSIVVLDGDDLKPCLPDDYISGQHHLNHQQQLQLQQQL
QQQHPLQQQHYRTHSGDIREIDQEMLTMLSVNQDNGPHREMAVDCPDTFIARNKTP
PRYPPPRPPQLNGNAKPVPPPRDHLRVEKDGRLVNCSPAPQLPDRRAPGNASSGSS
GATTHPLQHQQIAQIVEPTLEQLDSIKKYQEQLRRRREEEERIAQQNEFLRNSLRGSR
KLKALQDTATPGKAVAQQQQQATLATQVVGVENEAYLPDEDQPQAEQIDGYGELIAA
LTRLQNQLSKSGLSTLAGRVSAAHSVLASASVAHVLAARTAVLQRRRSRVSGPLH
HSSLGLQKDIVELLTQSNTAAAIELGNLLTSHEMEGLLLAHDRIANHTDGTPSPTPTP
TPAIGAATGSTLSSPVAGPKRNLGMVVPPPVVPPPLAQRGAMPLPRGESPPPVPMP
PLATMPMSMPVNLPMSACFGTLNDQNDNIRIIQIEKSTEPLGATVRNEGEAVVIGRIVR
GGAAEKSGLLHEGDEILEVNGQELRGKTVNEVCALLGAMQGTLTFLIVPAGSPPSV
GVMGGTTGSQLAGLGGAHRDTAVLHVRAHFDYDPEDDLYIPCRELGISFQKGDVLH
VISREDPNWWQAYREGEEDQTLAGLIPSQSFQHQRETMKLAIAEEAGLARSRGKDG
SGSKGATLLCARKGRKKKKKASSEAGYPLYATTAPDETDPEEILTYEEVALYYPRAT
HKRPIVLIGPPNIGRHELRQRLMADSERFSAAVPHTSRARREGEVPGVDYHFITRQA
FEADILARRFVEHGEYEKAYYGTSLEAIRTVVASGKICVLNLHPQSLKLLRASDLKPY
VVLVAPPSLDKLRQKKLRNGEPFKEEELKDIIATARDMEARWGHLFDMIIINNDTERA
YHQLLAEINSLEREPQWVPAQWVHNNRDES

6.3.4 Sdt-C1

1173 aa, 129 kDa

MQANSSR**SNLSAQSSGTPSASTISSSQGKQQVVELSGYVIILVENVEGKIKLYGSPP**
DRDNLEVGDEILEVNGLTLENISRTEVIRHIHDCIKSCTICLRVRKKNDSRLAWDIGNS
VQDAFVIAVEEHARERLQRLAALNRVTPVDITQLSKKLLEDVIVHQASDVHSYSTSASA
AAIASSSNRSQQQQQQQQQHQLLSAAYELQQQQQLQLQQQQQQQNSPTSSISIGRT
ELLLGDQSLRQDPRGNRRRSGSSIVVLDGDDLKPCLPDDYISGQHHLNHQQQLQLQ
QQLQQQHPLQQQHYRTHSGDIREIDQEMLTMLSVNQDNGP**HREMAVDCP**DTFIARN
KTPPRYPPPRPPQLNGNAKPVPPPRDHLRVEKDGRLVNCSPAPQLPDRRAPGNASS
GSSGATTHPLQHQQIAQIVEPTLEQLDSIKKYQEQL**RRRREEEE**RIAQQNEFLRNSLR
GSRKLKALQDTATPGKAVAQQQQQATLATQVVGVENEAYLPDEDQPQAEQID**GYGE**
LIAALTRLQNQLSKSGLSTLAGRVSAAHSVLASASVAHVLAARTAVLQRRRSRVSG
P**LHHSSLGLQKDIVELLTQSNTAAAIELGNLLTSHEMEGLLLAHDRIANHTDGTPSPT**
PTPTPAIGAATGSTLSSPVAGPKRNLGMVVPPPVVPPPLAQRGAMPLPRGESPPPVP
MPPLATMPMSMPVNLPMSACFGTLNDQNDNIRIIQIEKST**EPLGATVRNEGEAVVIGR**
IVRGGAAEKSGLLHEGDEILEVNGQELRGKTVNEVCALLGAMQGTLTFLIVPAGSPP
SVGVMGGTTGSQLAGLGGAHRDTAV**LHVRAHFDYDPEDDLYIPCRELGISFQKGDV**
LHVISREDPNWWQAYREGEEDQTLAGLIPSQSFQHQRETMKLAIAEEAGLARSRGK
DGSGSKGATLLCARKG**RKKKKKA**SSEAGYPLYATTAPDETDPEEILTYEEVALYYPR
AT**HKRPIVLIGPPNIGRHELRQRLMADSERFSAAVPHTSRARREGEVPGVDYHFITR**
QAFEADILARRFVEHGEYEKAYYGTSLEAIRTVVASGKICVLNLHPQSLKLLRASDL
KPYVVLVAPPSLDKLRQKKLRNGEPFKEEELKDIIATARDMEARWGHLFDMIIINNDT
ERAYHQLLAEINSLEREPQWVPAQWVHNNRDES

6.3.5 Sdt-C2

1587 aa, 175 kDa.

MQANSSRSNLSAQSSGTPSASTISSSQGKQQVVELSGYVIILVENVEGKIKLYGSPP
DRDNLEVGDEILEVNGLTLENISRTEVIRHIHDCIKSCTICLRVRKKNDSRLAWDIGNS
VQDAFVIAVEEHARERLQRLAALNRVTPVDITQLSKKLQQTKSGTATSQRQDLSFLNE
STPIYVTSFTSNQITCSSSTMTTATAGGPISAPSLATATTTVPTASSHTTTVVAQIEHGA
SALVSAAVAAATAADRNANSTTSAALKQTANCIGNSTSSLGTTSTTSSQSTSSATGHI
YQTSQAQQQQLQQLQQQLAAAAAAGKPLQAKSLLASSLQHLAEEVDNEDLDDDDDV
DGANYCGITYISYNNKHAQLPTTTLPATTALPAAAASLATTAAIYQQRQQQQHQQQQ
QQQQQPVHHHNHPPTASQLNRATAPAPLQLGGPVNPSFVDAQTSTSPLMAQQLHS
QHADVDAAPPSSSSSSAVVVVERHVHGTTTPKTEYSTAISSGQLQQAFAELQLHSSN
NNATQQQQQHLLLSNNNNSNNSMAAAQTTASLMKNCDLLISNNLYPPRRELLEDVIV
HQASDVHSYSTSASAAAIASSSNRSQQQQQQQQQHQLLSAAYELQQQQQLQLQQQ
QQQQNSPTSSISIGRTELLLGDQSLRQDPRGNRRRSGSSIVVLDGDDLKPCLPDDYIS
GQHHLNHQQQLQLQQQLQQQHPLQQQHYRTHSGDIREIDQEMLTMLSVNQDNGPH
REMAVDCPDTFIARNKTPPRYPPPRPPQLNGNAKPVPPPRDHLRVEKDGRLVNCSP
APQLPDRRAPGNASSGSSGATTHPLQHQQIAQIVEPTLEQLDSIKKYQEQLRRRREE
EERIAQQNEFLRNSLRGSRKLKALQDTATPGKAVAQQQQQATLATQVVGVENEAYLP
DEDQPQAEQIDGYGELIAALTRLQNQLSKSGLSTLAGRVSAAHSVLASASVAHVLA
ARTAVLQRRRSRVSGPLHHSSLGLQKDIVELLTQSNTAAAIELGNLLTSHEMEGLLL
AHDRIANHTDGTPSPTPTPTPAIGAATGSTLSSPVAGPKRNLGMVVPPPVVPPPLAQ
RGAMPLPRGESPPPVPMPPLATMPMSMPVNLPMSACFGTLNDQNDNIRIIQIEKSTE
PLGATVRNEGEAVVIGRIVRGGAAEKSGLLHEGDEILEVNGQELRGKTVNEVCALL
GAMQGTLTFLIVPAGSPPSVGVMGGTTGSQLAGLGGAHRDTAVLHVRAHFDYDPE
DDLYIPCRELGISFQKGDVLHVISREDPNWWQAYREGEEDQTLAGLIPSQSFQHQR
ETMKLAIAEEAGLARSRGKDGSGSKGATLLCARKGRKKKKKASSEAGYPLYATTAP
DETDPEEILTYEEVALYYPRATHKRPIVLIGPPNIGRHELRQRLMADSERFSAAVPHT
SRARREGEVPGVDYHFITRQAFEADILARRFVEHGEYEKAYYGTSLEAIRTVVASGKI
CVLNLHPQSLKLLRASDLKPYVVLVAPPSLDKLRQKKLRNGEPFKEEELKDIIATAR
DMEARWGHLFDMIIINNDTERAYHQLLAEINSLEREPQWVPAQWVHNNRDES

6.3.6 Sdt-D

879 aa, 97 kDa.

MVVSTLHITLENNGNVAAGQPAAVAAASVVGAATSPTKFAVLSAQQLQLQLQQQQQ
QQNEKENHMNNNNNNSDYCDMNGNGVGMGNGGSGGPGSLTPQSPDQYVGSPVS
SAATPQRTASALRRGIQIQRSSTRLRQQQEQLRRRREEEERIAQQNEFLRNSLRGSR
KLKALQDTATPGKAVAQQQQQATLATQVVGVENEAYLPDEDQPQAEQID**GYGELIAA**
LTRLQNQLSKSGLSTLAGRVSAAHSVLASASVAHVLAARTAVLQRRRSRVSGP**LH**
HSSLGLQKDIVELLTQSNTAAAIELGNLLTSHEMEGLLLAHDRIANHTDGTPSPTPTP
TPAIGAATGSTLSSPVAGPKRNLGMVVPPPVVPPPLAQRGAMPLPRGESPPPVPMP
PLATMPMSMPVNLPMSACFGTLNDQNDNIRIIQIEKST**EPLGATVRNEGEAVVIGRIVR**
GGAAEKSGLLHEGDEILEVNGQELRGKTVNEVCALLGAMQGTLTFLIVPAGSPPSV
GVMGGTTGSQLAGLGGAHRDTAV**LHVRAHFDYDPEDDLYIPCRELGISFQKGDVLH**
VISREDPNWWQAYREGEEDQTLAGLIPSQSFQHQRETMKLAIAEEAGLARSRGKDG
SGSKGATLLCARKG**RKKKKKA**SSEAGYPLYATTAPDETDPEEILTYEEVALYYPRAT
HKRPIVLIGPPNIGRHELRQRLMADSERFSAAVPHTSRARREGEVPGVDYHFITRQA
FEADILARRFVEHGEYEKAYYGTSLEAIRTVVASGKICVLNLHPQSLKLLRASDLKPY
VVLVAPPSLDKLRQKKLRNGEPFKEEELKDIIATARDMEARWGHLFDMIIINNDTERA
YHQLLAEINSLEREPQWVPAQWVHNNRDES

6.5 Established UAS-Sdt effector-stocks

Stock	Chromosome	Crorresponding construct	Encoded region (aa)* Tags used
15893	X	UAS-Sdt-MAGUK	1-1289, FLAG
15812	X	UAS-Sdt-MAGUK	1-1289, FLAG
15615	II	UAS-Sdt-MAGUK	1-1289, FLAG
15913	II	UAS-Sdt-MAGUK	1-1289, FLAG
1595	II	UAS-Sdt-MAGUK	1-1289, FLAG
15912	III	UAS-Sdt-MAGUK	1-1289, FLAG
15431	III	UAS-Sdt-MAGUK	1-1289, FLAG
15892**	III	UAS-Sdt-MAGUK	1-1289, FLAG
23777	X	UAS-Sdt-ΔSH3-GUK	1-928, FLAG, 8xMyc
23712	II	UAS-Sdt-ΔSH3-GUK	1-928, FLAG, 8xMyc
23964	II	UAS-Sdt-ΔSH3-GUK	1-928, FLAG, 8xMyc
231111**	II	UAS-Sdt-ΔSH3- GUK	1-928, FLAG, 8xMyc
3394	X	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
3351	X	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
33651	II	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
33361	II	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
33121	II	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
33141	II	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
33551	II	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
3391	II	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
33241	III	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
33212	III	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
33181	III	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
33284	III	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
33143	III	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc

Stock	Chromosome	Crorresponding construct	Encoded region (aa)* Tags used
33221**	III	UAS-Sdt-ΔHook-GUK	1-1035, FLAG, 8xMyc
4a142	X	UAS-Sdt-ΔHook	1-1035 and 1096-1289, FLAG
4a121	X	UAS-Sdt-ΔHook	1-1035 and 1096-1289, FLAG
4a141	II	UAS-Sdt-ΔHook	1-1035 and 1096-1289, FLAG
4a482	II	UAS-Sdt-ΔHook	1-1035 and 1096-1289, FLAG
4a233	III	UAS-Sdt-ΔHook	1-1035 and 1096-1289, FLAG
4a481	III	UAS-Sdt-ΔHook	1-1035 and 1096-1289, FLAG
4a13	III	UAS-Sdt-ΔHook	1-1035 and 1096-1289, FLAG
4a92**	III	UAS-Sdt-ΔHook	1-1035 and 1096-1289, FLAG
57373	X	UAS-Sdt-ΔPDZ	1-760 and 928-1289, FLAG
57204	II	UAS-Sdt-ΔPDZ	1-760 and 928-1289, FLAG
57642	III	UAS-Sdt-ΔPDZ	1-760 and 928-1289, FLAG
57371**	III	UAS-Sdt-ΔPDZ	1-760 and 928-1289, FLAG
63154	X	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
63371	X	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
63672	X	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
63591	X	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
6323	II	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
63311	II	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
63592	II	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
63221	II	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
63152	III	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
6321	III	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
63431	III	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
63133**	III	UAS-Sdt-ΔSH3-Hook	1-924 and 1069-1289, FLAG
7f452	X	UAS-Sdt-ΔL27-C	1-681 and 818-1289, FLAG
7f4522	II	UAS-Sdt-ΔL27-C	1-681 and 818-1289, FLAG

Stock	Chromosome	Crorresponding construct	Encoded region (aa)* Tags used
7761	III	UAS-Sdt-ΔL27-C	1-681 and 818-1279, FLAG, 8xMyc
77372**	III	UAS-Sdt-ΔL27-C	1-681 and 818-1279, FLAG, 8xMyc
81122	III	UAS-Sdt-ΔN	683-1289, FLAG
B314	II	UAS-Sdt-B	1-42 and 476-1289, FLAG
B171	II	UAS-Sdt-B	1-42 and 476-1289, FLAG
B312**	III	UAS-Sdt-B	1-42 and 476-1289, FLAG
B172	III	UAS-Sdt-B	1-42 and 476-1289, FLAG

* - aminoacids counting is based on Sdt-MAGUK isoform.

** - these stocks were used for detailed analysis.

Acknowledgements:

This work was done in the group of Prof. Dr. Elisabeth Knust. I would like to thank her for the supervision and good advices.

I thank Melisande Richard and Sandra Berger for fruitful discussion of my results, Andre Bachmann and Özlem Kempkens, who helped me a lot to get familiar with different molecular and biochemical techniques, and Ferdy Grawe for his help with everything concerning electron mictroscopy

I thank all members of the lab for nice, friendly atmosphere in the lab.

I also thank my parents (Alexander Bulgakov and Nadezhda Bulgakova) and my husband (Boris Klyuch) for encouragement and a lot of help.

Finally, I thank all members of the Genetic Institute.

Erklärung:

Die hier vorgelegte Dissertation habe ich eigennständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 15.01.07

Natalia Bulgakova