The Drosophila Cell Survival Gene discs lost Encodes a Cytoplasmic Codanin-1-like Protein, Not a Homolog of Tight Junction PDZ Protein Patj

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Summary

The Drosophila gene discs lost (dlt) has been reported to encode a homolog of the vertebrate tight junction PDZ protein Patj, and was thought to play a role in cell polarity. Using rescue experiments and sequence analyses, we show that dlt mutations disrupt the Drosophila Codanin-1 homolog, a cytoplasmic protein, and not the PDZ protein. Mutations in human Codanin-1 are associated with congenital dyserythropoietic anemia type I (CDA I). In Drosophila, the genomic organization of dlt is unusual. dlt shares its first untranslated exon with α-spectrin, and both genes are coexpressed throughout development. We show that dlt is not required for cell polarity but is needed for cell survival and cell cycle progression. Finally, we present evidence that the PDZ protein previously thought to be encoded by dlt is not required for viability. We propose to rename this PDZ protein after its vertebrate homolog, Patj (Pals-associated tight junction protein).

Introduction

In a large-scale mutagenesis experiment, we had previously identified a number of genes required for the formation of the embryonic central nervous system (CNS) axon pattern (Hummel et al., 1999a, 1999b). One of these mutations was mapped to a chromosomal region comprising some genes encoding well-characterized proteins, including α-Spectrin, Cdc37, and a cytosolic PDZ (PSD-95, Discs-large, ZO-1) domain protein reported to be mutated in discs lost (Bhat et al., 1999). Further genetic and phenotypic analyses suggested discs lost is critically important for epithelial polarity in Drosophila (Bhat et al., 1999). The chromosomal interval that uncovers this PDZ locus was subject to an intense and saturating genetic analysis (Sliter et al., 1989). Mutations in the dre1 complementation group lead to pupal lethality, and mutant third instar larvae are characterized by fewer or even no imaginal discs. Based on rescue experiments, Bhat et al. (1999) concluded that the dre1 complementation group encodes a cytosolic PDZ protein. In accordance with the mutant phenotype, the gene was renamed discs lost (dlt; Bhat et al., 1999).

It was reported that Dlt executes its function during the establishment of apico-basal polarity through a direct interaction with Crumbs (Bhat et al., 1999). Reduction of discs lost function by RNA interference resulted in a severe disruption of the columnar cell morphology of blastoderm cells (Bhat et al., 1999). Similarly, discs lost mutant cell clones showed some defects in cell polarity although localization of the β-catenin Armadillo was unchanged (Tanentzapf et al., 2000).

Here, we reinvestigated the function of discs lost and show that it does not encode the PDZ domain protein but a protein that is nested within the first intron of α-spectrin. In contrast to the previously reported findings, discs lost is not involved in the regulation of cell polarity but rather is required for cell survival and cell cycle progression. The protein encoded by discs lost is an evolutionarily conserved cytosolic protein. Mutations in a human homolog have been associated with congenital dyserythropoietic anemia type I (CDA I; Dgany et al., 2002). In Drosophila but not in vertebrates, discs lost is intimately linked to the α-spectrin locus. However, no genetic interaction with α-spectrin is evident. The vertebrate homolog of the PDZ protein previously thought to encode the Discs lost protein is called Patj (Pals-associated tight junction protein; Roh et al., 2002a, Lammers et al., 2002). We thus propose using the name Patj for its Drosophila homolog. Rescue experiments suggest that patj is not essential for development.

Results

Isolation of discs lost Mutations

In the course of generating P element-induced alleles of an unrelated gene, we identified three independent, lethal P element-induced discs lost mutations, which all failed to complement the original dltαsnt mutation (dltαsnt, dltαsnt, and dltαsnt; Figure 1; see Experimental Procedures for details). A previous analysis of the discs lost locus had suggested a complex genetic scenario (Bhat et al., 1999) and only a single EMS-induced discs lost mutation was available (dltαsnt). Thus, we conducted an F2 EMS-mutagenesis to isolate four EMS-induced discs lost alleles (dltαsnt, dltαsnt, dltαsnt, and dltαsnt). The original dltαsnt mutation (Sliter et al., 1989) as well as the dltαsnt allele lead to larval lethality. In contrast, homozygous dltαsnt, dltαsnt, or dltαsnt animals die as pupae. To discriminate whether the differences in the lethal phase are due to different allelic strengths or to background effects, we determined the lethal period of all discs lost alleles in trans to deficiencies. In this paradigm, all dlt alleles lead to pupal lethality (Bhat et al., 1999; Sliter et al., 1989; Table 1). This indicates that additional background mutations are responsible for the larval lethality that we observed for dltαsnt and dltαsnt mutant animals.

Identification of the discs lost Gene

The discs lost locus was previously shown to encode a protein with four PDZ domains (Figure 1; Bhat et al., 1999). To determine the molecular nature of the newly
Figure 1. The discs lost Locus Is Linked to α-spectrin

(A) Schematic view of the chromosomal region encompassing the genes α-spectrin, discs lost, cdc37, CG12020, and the PDZ encoding gene patj. The size of the two deficiencies and the insertion sites of the four P element-induced discs lost alleles are indicated. The boxes at the bottom of the panel represent the different rescue constructs used in this study. The extent of the 9.5 kb large P[cdc37] rescue fragment was judged from data by Cutforth and Rubin (1994).

(B) Alignment of Discs lost homologs from Drosophila melanogaster (D.m.), Drosophila pseudoobscura (D.p.), and human. Identical amino acids are shaded.
induced discs lost EMS alleles, we sequenced the genomic DNA encompassing the PDZ domain protein-encoding gene in two EMS-induced dlt mutations. Only third base changes were found, none of which resulted in an amino acid exchange (data not shown), indicating that discs lost may not correspond to the PDZ domain protein that is now called Patj.

In contrast to the EMS-induced mutations, all P element-induced discs lost alleles result in late embryonic, early larval lethality. Sequence analyses demonstrated that all three P element insertions occurred within the first intron of α-spectrin (Figure 1). The analysis of a large number of cDNA clones identified by the Berkeley Drosophila Genome Project (BDGP) demonstrated a rather unusual organization of the α-spectrin locus (Figure 1). Within its first intron, 3' to the P element insertion sites, resides a 1240 amino acid large open reading frame called CG32315 or vanaso (Fanara et al., 2002; but see below). Interestingly, this transcript shares the first noncoding exon with α-spectrin.

Given the genetic organization of the α-spectrin locus, it was not surprising that the genetic complementation analyses demonstrated that all P element-induced discs lost mutations do not complement mutations in α-spectrin. Thus, the P element-induced discs lost alleles are indeed double mutations, and the loss of α-spectrin function is likely to be responsible for the earlier lethal phase of the P element-induced dlt alleles compared to the EMS-induced dlt alleles. On reexamining the previously identified P element-induced discs lost allele (Bhat et al., 1999), we found that it too failed to complement α-spectrin mutations.

Because none of the EMS-induced discs lost alleles could be linked to sequence alterations in the genomic region encompassing the patj gene, we assumed that discs lost might correspond to the open reading frame CG32315 located within the first α-spectrin intron. To test this assumption, we performed rescue experiments using different CG32315 transgenes (Figure 1; Table 2).

To facilitate these experiments, we placed the CG32315 coding region under the control of the ubiquitin promoter (Brummel et al., 1994). As observed for GAL4-UAS-activated expression, ubiquitous expression of CG32315 in otherwise wild-type animals did not cause any dominant phenotype (data not shown). Using the ubi-CG32315 transgene, we were able to rescue EMS-induced discs lost mutations (dre1, N1, A1, and C1) in trans to the deficiencies Df(3L)Apt32 or Df(3L)My10. In addition, we were able to rescue all allelic combinations of discs lost alleles that we tested. We could even rescue homoygous dlt^{avt} mutants, which indicates that no other lethal mutations are present on the background of this chromosome. In all cases, lethality was completely rescued and fertile flies with normal appendages eclosed. Although we were not able to rescue homozygous dlt^{avt} animals, the rescue of dlt^{avt} in trans to the deficiencies clearly demonstrates that discs lost corresponds to CG32315 and not to the previously published PDZ domain protein.

### Sequence Analysis of discs lost Mutants

To further verify that discs lost is encoded by the transcription unit CG32315, we sequenced the corresponding genomic sequence in two mutant alleles. In dlt^{avt}, which is likely to represent a strong allele, we determined the CAA → TAA exchange at nucleotide 865 of the open reading frame, which leads to an early premature translational stop leaving only a small protein with 288 amino acids. In dlt^{avt} mutants, we detected a CAG → TAG exchange at position 1507 resulting in a truncated protein of 62 amino acids (Figure 1). In summary, these data clearly demonstrate that discs lost corresponds to CG32315 and not the previously published PDZ protein.

Recently, the CG32315 transcription unit has been identified as the quantitative trait locus vanaso that affects olfaction (Fanara et al., 2002). As the correlation to CG32315 is based upon a P element insertion affecting both α-spectrin and CG32315, we suggest keeping the phenotype-based name discs lost.

### Discs lost Is Conserved in Vertebrates

The Discs lost protein comprises 1240 amino acids, and homologous, similar sized proteins are present in vertebrates but not in C. elegans, yeast, or plants. The closest discs lost homolog was found in Drosophila pseudoobscura, which is separated from Drosophila melanogaster by about 100 million years (Wang and Hey, 1996). The protein is 53% identical over the entire sequence length (Figure

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**Table 1. Lethal Phases of discs lost Mutant Animals**

<table>
<thead>
<tr>
<th>Allele</th>
<th>dlt^{avt}</th>
<th>dlt^{avt}</th>
<th>dlt^{avt}</th>
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<td>28%</td>
<td>32%</td>
<td>0%</td>
</tr>
<tr>
<td>Df(3L)Apt32</td>
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<td>24%</td>
<td>32%</td>
<td>29%</td>
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The phenotype of the different discs lost alleles was analyzed in homozygous condition as well as in trans to the deficiency Df(3L)Apt32. The percentage of mutant discs lost pupae is indicated. More than 100 pupae were counted in each case. A value less than 33% indicates partial larval lethality.

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**Table 2. Genetic Relationship between α-spectrin and discs lost**

<table>
<thead>
<tr>
<th>Allele</th>
<th>α-spectrin^{wor}</th>
<th>dlt^{avt}</th>
<th>dlt^{avt}</th>
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<tr>
<td>α-spectrin^{wor}</td>
<td>-</td>
<td>yes</td>
<td>-</td>
<td>P[ubi-α-spectrin]</td>
</tr>
<tr>
<td>discs lost^{avt}</td>
<td>yes</td>
<td>-</td>
<td>-</td>
<td>P[ubi-CG32315]</td>
</tr>
<tr>
<td>Df(3L)My10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>P[ubi-α-spectrin] + P[ubi-CG32315] + P[cdc37]</td>
</tr>
</tbody>
</table>

α-spectrin^{wor} is an α-spectrin null allele (Lee et al., 1993). discs lost^{avt} and discs lost^{avt} are EMS-induced discs lost alleles, and dlt^{avt} is a P element-induced mutation affecting α-spectrin and discs lost. P[ubi-α-spectrin] (Lee et al., 1993) and P[ubi-CG32315] are P element-based rescue constructs using the ubiquitin promoter to express the indicated transscripts. P[cdc37] is a genomic rescue (Cutforth and Rubin, 1994).
Figure 2. Discs lost Is a 140 kDa Large Cytoplasmic Protein
(A) Western blot of embryonic cell extracts. The UAS-GAL4 system was used to express the Discs lost protein. The following genotypes were used: rho
/H11022 dltmyc (expression of a Myc-tagged Discs lost protein in the rhomboid pattern; due to the addition of the Myc tag, the protein is slightly larger than the wild-type protein); da
/H11022 dlt (expression of the wild-type Discs lost protein in the daughterless pattern); wt (wild-type embryos). The Discs lost protein is about 140 kDa in size and can be seen as a faint band in wild-type embryos. Overexpression of the protein results in prominent staining.
(B) Whole-mount preparation of an embryo expressing Discs lost in the engrailed pattern.
(C) Higher magnification of one segment. Discs lost can be detected in the cytoplasm.
(D–I) Expression of a Myc-tagged Discs lost protein in the salivary glands (D–F) or the embryonic ectoderm (G–I). 1; calculations were performed using the BLAST 2 SEQUENCES program at NCBI; Tatusova and Madden, 1999). Homology spreads over the entire open reading frame but is less pronounced in the first quarter of the protein. The vertebrate homolog, Codanin-1, is a predicted cytosolic protein (Dgany et al., 2002), which has a similar size (1226 amino acids) and shows significant homology (25%; E \( \times 10^{-49} \)) over almost the entire length of the protein to both D. melanogaster and D. pseudoobscura Discs lost proteins (Figure 1). Again the first 300 amino acids are the least conserved. In D. melanogaster and D. pseudoobscura, the discs lost gene is linked to α-spectrin but lacks any α-Spectrin-related sequence motives. Human or mouse Codanin genes, however, are not linked to a spectrin locus. Mutations in the human Codanin-1 gene have been associated with CDA I (Dgany et al., 2002). CDA I is a rare recessive disorder affecting erythropoiesis. In CDA I patients, DNA synthesis is arrested in erythroid progenitor cells, and increased apoptosis has been observed (Tamary et al., 1996; Wickramasinghe, 1997).

discs lost Is Expressed Ubiquitously during Development
Due to the genomic organization, the transcription of discs lost and α-spectrin must be regulated by the same promoter elements. To determine whether splicing of the two transcripts is temporally and spatially regulated, we performed whole-mount in situ hybridization experiments using digoxigenin-labeled exon-specific RNA probes. During embryonic development both genes are ubiquitously expressed, and we could not detect specific differences between the expression patterns of α-spectrin and CG32315, suggesting that differential splicing is not involved in regulating the expression pattern of the two genes (Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/5/6/841/DC1). Similarly, we observed a ubiquitous expression in imaginal discs (Supplemental Figure S1), salivary glands, and fat body, and low levels of expression in the gut (data not shown).

discs lost Is a Cytoplasmic Protein
The predicted molecular weight corresponds well to the size determined by Western blot analyses (140 kDa) using antisera generated against a bacterial expressed Discs lost GST-fusion protein (Figure 2). High levels of Discs lost protein were only detected following ectopic expression of an UAS-dlt construct in the daughterless or rhomboid pattern. Due to the addition of an 8 x Myc tag, the Discs lost-Myc protein is slightly larger than the wild-type protein on the Western blot (rho
/H11022 dltmyc; Figure 2). The identity of this protein band was verified by using an anti-Myc antibody (data not shown). The Myc-tagged version of the protein is still able to rescue the discs lost phenotype to full viability when expressed ubiquitously during development. The specificity of the antisera was further confirmed by RNA interference (RNAi) experiments (see below).

Unfortunately, antisera generated against different domains of the Discs lost protein did not allow the detection of the protein in wild-type embryos. However, when we ectopically expressed discs lost using the engrailed GAL4 driver, we could detect the protein in the engrailed expression domain. In these cells, the Discs lost protein is localized in the cytoplasm (Figures 2B and 2C). Similar results were obtained when we analyzed the expression of the Myc-tagged Discs lost protein in salivary gland cells or ectodermal cells. Discs lost protein never colocalized with α-Spectrin, which demarcates the cell membrane (Figures 2D–2I).

discs lost Mutant Phenotype
Mutant discs lost animals are characterized by a variable loss of imaginal discs. In addition, it was reported that discs lost mutations lead to a disruption of cell polarity (Bhat et al., 1999). We confirmed the variable loss of
imaginal discs and noted that eye imaginal discs preferentially lacked their antennal part (Figures 3A–3C). Because cell polarity markers such as Armadillo or the Patj protein previously assigned to the discs lost locus are still expressed in their normal apical domain in mutant epithelial cells, polarity does not appear to be disrupted (Figures 3A and 3B, and data not shown). As the discs lost alleles may not be null alleles, we determined the phenotype of the My10 deficiency, which fails to complement discs lost mutations. Because Df(3L)My10 removes cdc37 (Bhat et al., 1999) as well as α-Spectrin (Tanentzapf et al., 2000; see below), we complemented these genes by crossing the corresponding rescue constructs (P[ubi-α-spectrin] and P[cdc37]) into the background of the Df(3L)My10. Again, larvae with small eye discs were recovered that preferentially lacked the antenna part, suggesting that the EMS alleles are strong hypomorphic or null mutations in discs lost. These eye discs lacked expression of the Patj protein, and the severity of the imaginal disc phenotype was comparable to the phenotype of homozygous mutant discs lost larvae (Figures 3B and 3C). α-Spectrin expression outlines regular-shaped cell clusters, suggesting that some patterning occurred in these rudimentary discs (Figure 3C, arrowhead in insert). These analyses did not indicate any function of discs lost in cell polarity, and further support our finding that discs lost does not encode the Patj protein.

Clonal Analysis of discs lost
To further address whether discs lost affects cell polarity, we analyzed mutant clones in the follicular epithelium (Figures 3D and 3E). Mutant cell clones were detected by the lack of GFP expression. α-Spectrin expression was used to assess the cellular phenotype. As observed in the homozygous mutant condition, the loss of discs lost in epithelial cells does not alter PDZ protein expression or localization, indicating that cell polarity was not affected (Figures 3D and 3E). We confirmed this result by assaying several other markers. In discs lost mutant cell clones, the apical localization of Crumbs is unchanged (Figure 3E). Similarly, the distribution of the cell polarity markers SAS and Armadillo was not affected (Supplemental Figure S2).

To assess the consequences of the loss of discs lost in the eye discs, we used the Minute FRT approach to generate large mutant cell clones (Newsome et al., 2000; Simpson, 1979). When homozygous mutant discs lost clones were generated, the mutant eyes were significantly smaller than wild-type eyes (Figures 4A–4C). However, this phenotype was extremely variable. We found some flies with almost normal sized mutant eyes and in the most extreme situation a small and a large eye developed in the same fly (Figure 4C). These phenotypes indicated that discs lost function is required to a variable extent for cell proliferation or survival.

The reduction of eye size suggested that discs lost mutant cell clones had a growth disadvantage during larval development. To directly examine the growth behavior, we generated discs lost mutant cell clones in the absence of a Minute mutation. We used heat shock-induced expression of the Flp recombinase and tested cell proliferation by BrdU labeling. discs lost mutant cells were able to incorporate BrdU normally, and the size of the mutant cell clone was comparable to the wild-type twin spot. Thus, discs lost mutant cells are at least initially able to divide at rates similar to wild-type cells (Figure 4E). However, during pupal development, these cells must have a growth disadvantage, as mutant cell clones never appear in the adult eye.

As observed in the follicular epithelium, discs lost mutant imaginal disc cells did not show any cell polarity...
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cell clones of considerable size in the eye imaginal disc, we were not able to find any discs lost mutant cell clones in the adult compound eye, suggesting that discs lost affects survival of differentiated cells. Only when neighboring cells carried a Minute mutation, which affects ribosomal function and thus leads to a growth disadvantage of these cells, were mutant discs lost cells able to survive to adulthood.

Disruption of discs lost Function by RNAi
The above-mentioned phenotypes tended to be relatively variable. This may be due to a perdurance of maternal Discs lost protein or RNA. To overcome the problems of RNA perdurance, we inhibited discs lost function by RNA interference (RNAi). Here, in vivo expression of double-stranded RNA (UAS-dlt RNAi) is able to interfere efficiently with endogenous gene expression (Lee and Carthew, 2003). To control the efficiency of the RNAi treatment, we expressed discs lost ubiquitously using (D) Expression of discs lost dsRNA during eye development (eyGAL4-UASdltRNAi) results in a discs lost phenocopy. (E) Mutant discs lost cells are able to incorporate BrdU, suggesting that DNA synthesis during proliferation is not affected.

defects. In Figure 5A, a confocal section shows the folds of a wing imaginal disc. Mutant discs lost cells can be recognized by the absence of β-galactosidase expression. No alterations in the apical localization of the Patj protein were detected (Figure 5A, arrowheads). Using different cellular markers, we could not find any alterations in cell polarity or shape (data not shown). The DNA content of mutant cells was similar to wild-type cells (Figure 5).

In the eye imaginal discs, neuronal cells differentiate following a series of inductive signaling events in the wake of the morphogenetic furrow (Freeman, 1996). The induction and later the differentiation of these neuronal cells require the formation and maintenance of polarized cells. Both processes proceed normally in mutant discs lost cells (Figure 5C). Although we could detect mutant

discs lost Is Required for Survival and Proliferation
Two possible scenarios may lead to the reduction in cell number observed in all discs lost mutant animals. The proliferation of cells may be disturbed due to defects in cell cycle progression, or the loss of discs lost could

<table>
<thead>
<tr>
<th>DNA</th>
<th>β-gal</th>
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<tbody>
<tr>
<td>dlN1</td>
<td>Patj</td>
<td>A</td>
</tr>
<tr>
<td>dlN1</td>
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</tr>
<tr>
<td>dlN1</td>
<td>α-Spectrin</td>
<td>C</td>
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</table>

Figure 5. Clonal Analysis of discs lost
Mutant discs lost clones were induced using heat shock-dependent Flipase expression. (A and B) Wing discs. (C) Eye disc. (A) The similar size of the discs lost mutant cell clones compared to the wild-type twin spots indicates normal proliferation rates. The DNA content (assayed using a YOYO-1 stain) is not changed and the expression pattern of the Patj protein is not affected (arrowheads). (B and C) No differences in the size of the mutant cells were detected and neuronal differentiation in the eye disc was not disturbed (C).
lead to an increase in apoptosis. To discriminate between these possibilities, we inhibited discs lost function by RNAi in a stripe across the wing disc epithelium using a patched-GAL4 driver (Wilder and Perrimon, 1995; Wodarz et al., 1995). Reduction of discs lost function led to a reduction in cell number in the corresponding wing domain (Figures 6A and 6B). Whereas in wild-type wings about 18 cells separated the longitudinal veins L3 and L4, only 16 cells were found following discs lost RNAi expression (Figure 6B). Concurrently, we noted a slight increase in cell size. This phenotype was subsequently used to quantify the effects of different genetic backgrounds (Figure 6I).

When we removed one copy of discs lost in this genetic background, the number of cells surviving dlt RNAi was further reduced (Figure 6C). This result again demonstrates that the gene located in the first intron of α-spectrin corresponds to the discs lost gene. As expected, the dlt RNAi phenotype is dose dependent. When we expressed higher levels of discs lost RNAi, the number of cells was further reduced to ten and the crossvein 1 was missing (Figure 6D). In extreme cases, we observed necrotic cells in the patched expression domain (data not shown). Acridine orange staining, which labels dying cells, showed that reduction of discs lost function in the patched domain leads to cell death (see Supplemental Figure S3).

If loss of discs lost function would lead to apoptosis, the mutant discs lost phenotype may be rescued by expression of the caspase inhibitor baculovirus p35, which counteracts apoptosis (Hay et al., 1994). However, coexpression of discs lost RNAi and p35 did not alter the number of surviving cells (Figure 6E). Instead, we frequently observed that the dorsal and ventral wing
blades detached and that the L3 vein appeared broader (Figure 6E, arrow). Similar, although slightly weaker phenotypes were obtained following coexpression of discs lost RNAi and Drosophila myc (dmyc), which affects cell growth (Johnston et al., 1999; Prober and Edgar, 2000, 2002). Thus, Discs lost might be part of a pathway required for cell survival because mutant cells cannot be rescued by blocking caspase activity or increasing cellular growth.

Alternatively, the discs lost phenotype could be explained by a failure in cell cycle progression. Thus, we tested whether expression of cyclin E, which prompts additional mitoses by forcing cells into the S phase (Knoblich et al., 1994; Neufeld et al., 1998), might rescue the discs lost mutant phenotype. Expression of cyclin E in the patched domain led to a slightly enlarged wing compartment (Figures 6H and 6I). However, coexpression of both cyclin E and discs lost RNAi resulted in a dramatic loss of wing cells (Figure 6G), which was more than twice as severe as compared to expression of discs lost RNAi alone. When discs lost RNAi was expressed in a heterozygous cycE mutant background, the number of wing cells was decreased only very moderately (Figure 6I). Because Cyclin E prompts transition from G1 to S phase, these data may indicate that a reduction of discs lost function results in an arrest in G1 phase. Reduction of the level of Cyclin E does not overcome this arrest. However, when cells are forced into S phase by ectopic Cyclin E expression they die. Coexpression of discs lost RNAi and M phase promoting genes (cyclin A or string) had little or no effect. This suggests that discs lost is required for both G1 and S phase and that in the absence of discs lost, S phase cannot be completed and thus mitosis cannot be initiated (Figures 6F and 6I).

Discussion

Based on several arguments, we propose that discs lost does not correspond to the PDZ protein as previously reported but to a conserved protein related to the human Codanin-1 protein encoded by CG32315. First, we were able to rescue the discs lost mutant phenotype to full viability by expression of the CG32315 transcript. Second, we failed to detect sequence alterations in the patj gene encoding the PDZ domain protein but detected nucleotide changes leading to a premature translational stop in the transcription unit CG32315 of discs lost mutant animals. Third, RNAi-mediated inhibition of CG32315 function is sensitive to the discs lost gene dose. Fourth, we were able to phenocopy the discs lost mutant phenotype by RNA interference of CG32315. Finally, by using a rescue approach, we show that the Patj protein is not essential for viability (see below).

discs lost Regulates Proliferation and Survival

The decision between proliferation and survival is an important aspect of cellular regulation during the formation of a well-sized animal organ (Conlon and Raff, 1999). The mutant phenotype of discs lost is characterized by a dramatic reduction in the number of imaginal disc cells and consequently in the size of the respective adult tissues. The imaginal discs of Drosophila larvae are simple epithelia that within 4 days grow from 50 to 50,000 cells. During imaginal disc development, cell growth can be uncoupled from cell division. When cell division is increased, cell size is reduced, whereas when cell division is blocked, cells continue to grow to a larger size and exhibit multiple trichomes (Figure 6; Katzen et al., 1998; Neufeld et al., 1998; Szuplewski et al., 2003; Weigmann et al., 1997). These phenotypes can be detected following discs lost inactivation, suggesting that discs lost function is required for the control of cell cycle progression.

In addition, we have noted prominent cell death in epithelial cells expressing a discs lost RNAi construct. A similar phenotype can be induced by the deregulation of the cell cycle in imaginal discs. In these experiments, programmed cell death can be efficiently blocked by the caspase inhibitor baculovirus p35 (Asano et al., 1996; Hay et al., 1994; Milan et al., 1997; Neufeld et al., 1998). However, coexpression of p35 did not rescue the defects caused by the inhibition of discs lost function. The coexpression of p35 even slightly enhanced the severity of the discs lost phenotype. Crossvein 1 was generally missing and the L3 vein increased in size (compare Figures 6B and 6E, arrow). Thus, discs lost regulates cell survival independent of the caspase-induced apoptosis pathway.

To analyze the requirement of Discs lost for cell proliferation, we tried to modulate the discs lost mutant phenotype by coexpressing CycE or Dmyc. Expression of CycE (and to a weaker extent Dmyc) is sufficient to activate cell proliferation (Jiao et al., 2001). Dmyc, unlike its vertebrate homolog, functions primarily as a regulator of cell growth, and overexpression of dmyc in the wing disc leads to an increase in cell size (Johnston and Gallant, 2002; Johnston et al., 1999; Trumpp et al., 2001). When discs lost RNAi and dmyc are coexpressed no alterations in the phenotypic strength are observed. In contrast, coexpression of cyclin E, which promotes G1/S phase transition, leads to an enhancement of the phenotype that now resembles the discs lost loss-of-function phenotype (Figure 6). A possible explanation for these results is that a reduction of Discs lost protein levels leads to a prolonged G1 phase and to a lower survival rate once the cells enter S phase. If these cells are now prematurely forced to enter S phase by ectopic expression of Cyclin E, they eventually die. To test whether discs lost mutant cells can complete S phase, we coexpressed cyclin A and string, which are both regulators of M phase initiation (Neufeld et al., 1998). Because coexpression of the M phase promoting factors had little or no effect, discs lost seems to be required for the progression through S phase during the cell cycle.

The analysis of discs lost mutant cell clones suggested that discs lost is also required for cell survival of differentiated cells independent of cell division. Although discs lost mutant cell clones could be detected during larval development, they were absent in the resulting adult compound eyes.

Discs lost Function Is Conserved throughout Evolution

Mutations in the human Discs lost homolog Codanin-1 cause an inherited disorder associated with morphological and functional abnormalities of erythropoiesis called
congenital dyserythrozipoietic anemia type I (CDA I; Dgany et al., 2002). Patients with CDA I present with moderate to severe macrocytic anemia. Cell division of the erythroblasts appears to be arrested in late S phase (Wickramasinghe, 1997, 2000). Interestingly, these phenotypes are only detectable in about 60% of the erythroblasts, whereas the remaining cells have a rather normal appearance (Wickramasinghe, 2000). This resembles the discs lost phenotype, which is primarily manifested in the fast dividing imaginal cells.

To test whether discs lost affects Drosophila hematoipoiesis, we analyzed the number of blood cells (hemocytes) in discs lost mutant larvae. Drosophila hematopoiesis occurs in two waves. At first, presumptive hemocytes are specified in the anterior mesoderm and colonize the entire embryo during midembryogenesis (Tepass et al., 1994). Later, the lymph gland, which is the larval hematopoietic organ, generates hemocytes that are found in the larval hemolymph (Lanot et al., 2001). In mutant discs lost third instar larvae, we noted a 30% decrease in the number of blood cells (data not shown), opening the possibility to set up a Drosophila model system to study CDA I.

Function of the PDZ Protein

Previously, the discs lost mutation was assigned to a PDZ protein (Figure 1), which localizes to the subapical domain of the cell membrane in epithelial cells (Bhat et al., 1999; Figure 3). Interaction of this PDZ domain protein with cell polarity proteins was shown in flies and vertebrates (Bhat et al., 1999; Lemmers et al., 2002; Roh et al., 2002a, 2002b). Because discs lost does not encode the PDZ protein, the function of this protein remained unclear. To generate animals lacking the patj gene, we utilized the deficiency Df(3L)My10 (Bhat et al., 1999) and restored the different gene functions using a set of transgenes.

Homozygous Df(3L)My10 animals carrying both the P[ubi-spec] and the P[cdc37] transgenes are pupal lethal and develop a discs lost phenocopy indicating that no functional Discs lost protein is expressed (Figures 1 and 3C). When the functions of α-spectrin, discs lost, and cdc37 were complemented in the background of Df(3L)My10, we were able to generate viable and fertile females (Figure 1; see Experimental Procedures for crosses). In these females, the patj coding region is absent, as determined by PCR and antibody staining of ovaries (Supplemental Figure S4, and data not shown). Similarly, Tanentzapf et al. (2000) failed to detect Patj expression in Df(3L)My10 mutant cell clones. Flies were then mated to Df(3L)My10/TM6 males and normal homozygous Df(3L)My10 flies emerged, indicating that recombination had occurred between the P[ubi-discs lost] and the P[cdc37] rescue constructs (see Experimental Procedures). These flies very likely develop without any maternal Patj protein, which may have contributed to the rescue in the first generation. All rescued males were sterile. Whether this phenotype is linked to the loss of Patj expression or due to the loss of additional genes that are affected by the deficiency remains to be determined. These results argue against the notion that the Patj protein exerts important functions during the establishment or maintenance of apico-basal polarity.

Experimental Procedures

Genetics

The mutation dre1 was isolated by Sliter et al. (1989); Df(3L)My10 was described by Bhat et al. (1999). All crosses were performed on standard fly food, and unless indicated, at 25°C. Three P element-induced discs lost alleles were generated in a local hopping experiment starting from EP(3)3081, which is located –10 kb 3′ of the α-spectrin gene. discs lost alleles were generated using 25 mM EMS according to standard procedures (Ashburner, 1989). About 8,000 chromosomes were tested for lethality in trans to one of the newly induced P element-induced discs lost alleles (P1, P2, or P4).

Misexpression Studies

To determine the gain-of-function phenotype of discs lost, we employed the UAS/GAL4 system (Brand and Perrimon, 1993). Transgenes were generated, as follows. UAS-CG32315: the cDNA clone L1D18420 was subcloned into pUAST and used for germline transformation. Overexpression of a full-length CG32315 protein did not cause any obvious developmental defects. When two UAS-CG32315 transgenes were expressed in the breathless, Krüppel, or dpp pattern, viability was reduced; however, flies that eclosed showed no obvious mutant phenotype. The same cDNA was inserted into Casper-ubi (Brummel et al., 1994) to generate a P[ubi-discs lost] transgene. A UAS construct directing the expression of a C-terminal Myc-tagged Discs lost protein was generated by fusion of an 8′-Myc tag sequence at position 1192 of the open reading frame (48 C-terminal amino acids are missing in this construct). A discs lost RNAi construct was generated by inserting bases 168–584 of the open reading frame into the pWIZ vector (Lee and Carthew, 2003).

Further Fly Strains Used in This Study

dtt10, dtt11, dtt12, dtt14, this work; P[ubi-α-spectrin], α-spectrin″ (Lee et al., 1993); P[cdc37 minigene], cdc37 E(sev)3B (Cutforth and Rubin, 1994); dtt10 (Sliter et al., 1989); dtt12, Df(3L)My10 (Bhat et al., 1999); Df(3L)Apr32 (Wang et al., 1994); dgal4 (Wodarz et al., 1993); en-GAL4, ptc-GAL4, arm-GAL4, ey-GAL4, ey-Fip; M FRT80B, hs-Fip; ubi-GFP, FRT80B, hs-Fip; arm-lacZ FRT80B (Bloomington Stock Collection); UAS-dmyc (Zaffran et al., 1998; kindly provided by M. Noll); UAS-cyCE (Lane et al., 1996); UAS-p35 (Hay et al., 1994); UAS-string (Neufeld et al., 1998); UAS-cycH-AHA (Grosskortenhaus and Sprenger, 2002).

Rescue Experiments

The following crosses were done: virgin females P[ubi-spec]/P[ubi-spec]; CyO/Sp;TM2/TM6 were crossed to w/Y; P[cdc37]/P[cdc37]; Df(3L)My10/10 TM6. Males. From the offspring, the following males and 3C). When the functions of ptc, P[ubi-spec]; arm-GAL4, Df(3L)My10/10 TM6. Only females were rescued, which had the genotype P[ubi-spec]/w; P[cdc37]/P[ubi-dtt]; Df(3L)My10/3L My10. These rescued females were fertile. When crossed to Df(3L)My10, we obtained viable flies indicating that recombination took place between the P[cdc37] and P[ubi-dtt] transgenes in the female germline. All males were sterile.

DNA Work

DNA sequences flanking the P element insertion sites were cloned by inverse PCR. To identify the molecular nature of the discs lost mutations, heterozygous mutant DNA was sequenced. Details are available on request. Sequencing was performed using an ABI310 sequencer according to the manufacturer’s instructions; data analyses were done using Lasergene software. cDNA clones were identified by the BDGP.
Histology

Immunohistochemistry and whole-mount in situ hybridizations were performed according to standard procedures (Hummel et al., 2000; Tautz and Pfeifle, 1989). Anti-Discs lost antisera were induced in rabbits against pGEX fusion proteins carrying amino acids 12–557 or 622–1124. Both antisera gave comparable results. Mutant follicle cell clones were induced in 2- to 3-day-old females heat shocked at 37°C for 1 hr. The tissue was dissected 6–8 days following heat shock and fixed in 8% (Crumb, SAS, Armadillo) or 4% (Patj, α-Spectrin) PFA/PBS for 20 min at room temperature.

The following antibodies were used: anti-Patj antisera (Tantzap et al., 2000); anti-SAS antisera (Schonbaum et al., 1992; Wodarz et al., 1999); Mab 9E10 anti-Myc; Mab 3A9 anti-α-Spectrin, anti-Crumb (Coq), anti-armadillo (Developmental Hybridoma Bank, Iowa); anti-β-galactosidase (Promega, Cappel); rabbit anti-Myc (Santa Cruz), anti-GFP Alexa488 (Molecular Probes); and anti-BrDu (Becton-Dickinson). Images were taken on a Zeiss Axioptohor or a Leica confocal microscope.

Acknowledgments

We thank R. Carthew for sending the pWIZ plasmid prior to publication; M. Bhat, L. Goldenstein, E. Hafen, C. Lehner, M. Noll, F. Sprenger, and U. Tepass for help in the generation of flies. This work was supported by the fellowship of the Boehringer Ingelheim Fonds (to J.P.) and a grant of the DFG (to C.K.).

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