The COP9 signalosome is essential for development of *Drosophila melanogaster*

Shiri Freilich*, Efrat Oron*, Ya'ara Kapp†, Yael Nevo-Caspi†, Sara Orgad†, Daniel Segal† and Daniel A. Chamovitz*

The COP9 signalosome (originally described as the COP9 complex) is an essential multi-subunit repressor of light-regulated development in plants [1,2]. It has also been identified in mammals, though its role remains obscure [3–5]. This complex is similar to the regulatory lid of the proteasome and eIF3 [5,9–12] and several of its subunits are known to be involved in kinase signaling pathways [4,6–8]. No proteins homologous to COP9 signalosome components were identified in the *Saccharomyces cerevisiae* genome, suggesting that the COP9 signalosome is specific for multi-cellular differentiation [13]. In order to reveal the developmental function of the COP9 signalosome in animals, we have isolated *Drosophila melanogaster* genes encoding eight subunits of the COP9 signalosome, and have shown by co-immunoprecipitation and gel-filtration analysis that these proteins are components of the *Drosophila* COP9 signalosome. Yeast two-hybrid assays indicated that several of these proteins interact, some through the PCI domain. Disruption of one of the subunits by either a P-element insertion or deletion of the gene caused lethality at the late larval or pupal stages. This lethality is probably a result of numerous pleiotropic effects. Our results indicate that the COP9 signalosome is conserved in invertebrates and that it has an essential role in animal development.

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Received: 15 June 1999
Revised: 4 August 1999
Accepted: 13 September 1999

Published: 11 October 1999

Current Biology 1999, 9:1187–1190

0960-9822/99/$ – see front matter
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Results and discussion

We identified putative *Drosophila* orthologs of all eight published COP9 signalosome subunits (see Supplementary material). As the mammalian subunits have been named according to their size, with the largest subunit S1/Sgn1 and the smallest S8/Sgn8 [4,5], the *Drosophila* subunits will be referred to as Dch1 (*Drosophila* COP9 signalosome homolog 1) to Dch8. There are apparently two copies of the *dch1* gene, *dch1-1* and *dch1-2*; the former is probably a pseudogene. The Dch proteins have high levels of amino acid identity with their mammalian and plant counterparts; Dch1 to Dch6 are most highly conserved and Dch7 and Dch8 are less so (Table 1). Dch1-2, Dch2, Dch3, Dch4 and Dch7 each contain a PCI/PINT domain [9,11] and Dch5 and Dch6 contain an amino-terminal MPN domain [11]. These domains are common to subunits of the proteasome regulatory subcomplex, eIF3 and the COP9 signalosome.

Dch5 is the most highly conserved of the Dch proteins, having 72% amino acid identity with its putative orthologs mouse S5/Jab1 and *Arabidopsis* AJH1. Dch7 is highly similar to *Arabidopsis* FUS5, which encodes a phosphorylated component of the *Arabidopsis* COP9 signalosome [8]. No distinct cDNA was detected for Dch8, but a sequence putatively encoding an ortholog of *Arabidopsis* COP9 is found in the 5′ untranslated region of the fly gene that encodes the 65 kDa regulatory subunit of protein phosphatase [14]. Repeated analysis of this gene by Northern blotting [14] or PCR (our unpublished observations) failed to identify other forms of this transcript.

This high level of conservation implies that the COP9 signalosome also exists in *Drosophila*. We checked whether Dch7 can co-immunoprecipitate with Dch5. As shown in Figure 1, anti-Dch5 antibodies precipitate Dch7. The precipitation of Dch7 was specific to anti-Dch5 antibodies and was not seen with other antibodies. This result shows

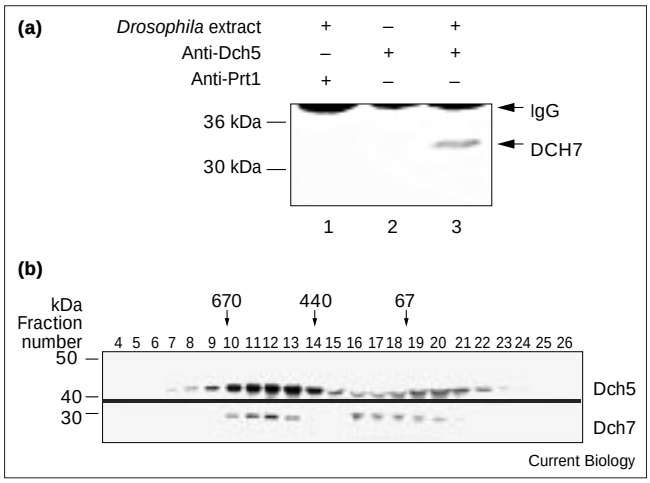
Table 1

Characterization of the *Drosophila* COP9 signalosome subunits.

Drosophila (MW)	Amino acid identity with COP9 signalosome subunits (%)			
	Mouse		*Arabidopsis*	
Dch1-1 (38.3)	S1 (Gps1)	36	FUS6	30
Dch1-2 (58.3)	S1 (Gps1)	53	FUS6	35
Dch2 (51.5)	S2 (Trip15)	83	–	–
Dch3 (50.6)	S3	50	–	–
Dch4 (46.5)	S4	68	–	–
Dch5 (37.1)	S5 (Jab1)	72	AJH1	59
Dch6 (37.2)	S6	58	–	–
Dch7 (30.1)	S7a/S7b	40	FUS5	26
Dch8 (18.3)	S8 (hCop9)	27	COP9	21

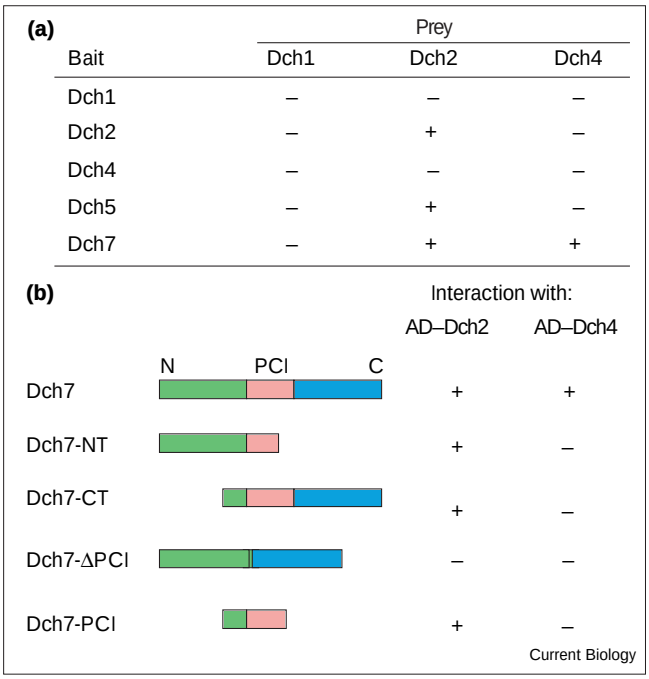
MW, molecular weight. If a protein has alternative designations, these are given in parentheses.

Figure 1



Dch5 and Dch7 are associated *in vivo*. (a) Dch7 co-immunoprecipitates with Dch5. Crude soluble protein extract from adult wild-type *Drosophila* flies (Canton-S strain) was precipitated in the presence of RIPA buffer with either affinity-purified anti-Dch5 antibodies (lane 3) or with affinity-purified antibodies against a protein that is not a part of the COP9 signalosome, Prt1 (lane 1). The precipitated proteins were separated on a 12.5% SDS–polyacrylamide gel, blotted and probed with affinity-purified anti-Dch7 antibodies diluted 1:10,000. IgG denotes immunoglobulin G. (b) Dch5 and Dch7 are present in the COP9 signalosome and in other forms. Wild-type adult flies were homogenized in gel-filtration buffer (PBS, 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, pH 7.5) and total soluble protein was separated over a Superose 6 gel-filtration column (Pharmacia). Fractions (0.5 ml each) were examined for the presence of Dch5 and Dch7 by immunoblot analysis with anti-Dch7 or anti-Dch5 affinity-purified antibodies diluted 1:10,000. Positions of size markers are shown.

Figure 2



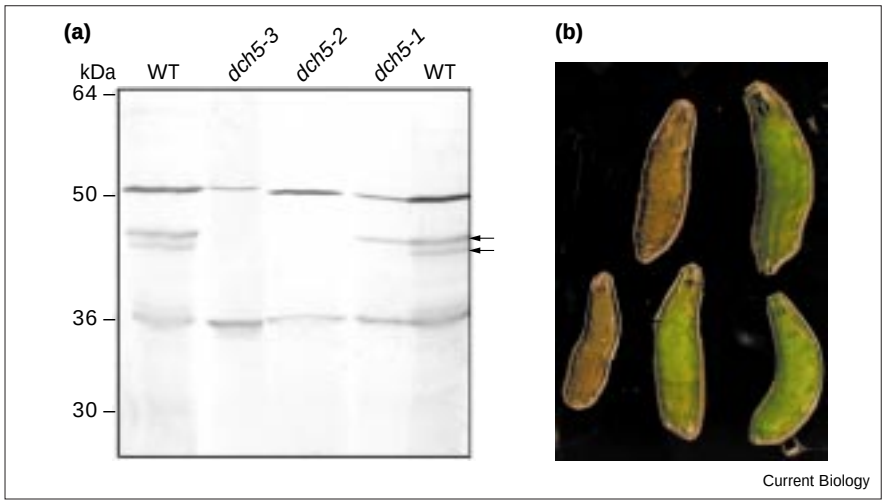
Protein–protein interactions in the *Drosophila* COP9 signalosome. (a) The indicated proteins were fused with LexA (bait) or the Gal4 activation domain (AD; prey) and interactions were tested in diploid yeast (see Supplementary material). None of the fusion proteins alone activated β-galactosidase, nor did they interact with either the activation domain or the LexA domain by themselves (not shown). (b) The PCI domain is essential for the interaction between Dch7 and Dch2. Dch7 was subdivided into four different fragments: an amino-terminal fragment (amino acids 1–157), Dch7-NT; a carboxy-terminal fragment (amino acids 79–278), Dch7-CT; a fragment containing most of the PCI domain (amino acids 79–157), Dch7-PCI; and a fragment with the deletion of amino acids 79–157, Dch7ΔPCI. N denotes the amino terminus and C the carboxyl terminus. The indicated portions of Dch7 were used as bait and specific interactions with AD–Dch2 and AD–Dch4 were tested as in (a).

that endogenous Dch5 and Dch7 associate, suggesting an association *in vivo*. We then checked whether this association is in the form of a larger protein complex. As shown in Figure 1b, Dch5 and Dch7 co-eluted from a gel-filtration column in fractions peaking around 500 kDa, similar to the elution profile of the plant and mammalian COP9 signalosomes [2,5]. Antibodies against both proteins also recognized proteins that were found in lower molecular weight fractions. Taken together, these results indicate that Dch5 and Dch7 are components of a complex in *Drosophila* similar to the COP9 signalosome in plants and mammals, and that they might be in equilibrium between complexed forms and monomeric or smaller complexed forms.

We used the yeast two-hybrid assay to investigate the architecture of the COP9 signalosome by studying whether the Dch proteins can physically interact. The Dch7 fusion protein clearly interacted with both Dch2 and Dch4. The Dch5 fusion protein also interacted with Dch2, and Dch2 homodimerizes. Some of these interactions are different from those reported between COP9 signalosome components from *Arabidopsis* [7,12]. The *Arabidopsis* interactions were monitored in haploid cells, however, and not in diploids as in our assay. It is thus impossible to determine at this point whether this discrepancy results from a different architecture of the plant and *Drosophila* complexes, a different assaying system, or a general weakness of the two-hybrid assay to indicate authentic interactions.

As the PCI/PINT domain was identified in subunits of three similar complexes, it was proposed that this domain serves a scaffolding role, allowing for multiple protein interactions both within and between protein complexes [11]. We found that both the amino- and carboxy-terminal halves and the PCI domain of Dch7 interact in yeast with Dch2 but not with Dch4 (Figure 2b). Deletion of the PCI domain from Dch7 abolished its interaction with Dch2. The PCI domain of Dch7 is therefore necessary and sufficient for interaction with Dch2. The entire Dch7 protein is necessary for its interaction with Dch4.

Figure 3



(a) Dch5 levels in wild-type (WT) and mutant strains. Individual larvae were homogenized in 10 μl SDS sample buffer, separated on a 12.5% SDS–polyacrylamide gel and blotted with affinity-purified antibodies raised against Dch5. The arrows indicate the 44 kDa and 42 kDa proteins. The *dch5-1* strain is the original P-element insertion strain *l(3)L4032*; *dch5-2* and *dch5-3* are two independent P-element excision strains. (b) Typical *Drosophila* larvae from the *dch5-1*/TM3, *Ser*, GFP strain were photographed at the third instar stage. Green larvae have the *dch5-1*/TM3, *Ser*, GFP genotype, while the non-fluorescing larvae are *dch5-1*/*dch5-1* homozygotes. Magnification was 5.25 ×; enlargement of photograph 2.36 × for a total scale of 12.4 ×. Larvae were photographed under both fluorescent and bright-field conditions simultaneously.

To elucidate the developmental role of the COP9 signalosome in *Drosophila*, we analyzed mutants for the complex components. The *l(3)L4032* strain contains a P element located in polytene bands 89D1–89D2, in dch5 [15]. The chromosome carrying the l(3)L*4032* insert is homozygous lethal and the lethality is not complemented by the chromosomal deletion Df(3R)bxd100, which removes bands 89B6–89E2. Southern blotting and PCR confirmed that the single P element in the genome of the *l(3)L4032* strain is inserted 24 bp upstream of the cloned *dch5* cDNA. Flanking genomic sequences were obtained by inverse PCR, and no additional open reading frame was identified in this region. To verify that the lethality of the *l(3)L4032*-carrying chromosome is due to the P-element insertion in *dch5*, the P element was mobilized by hybrid dysgenesis (see Supplementary material). Lines were established from 73 independent putative excision flies, and 58 of them were homozygous viable. PCR analysis on four of these homozygous viable lines indicated a normal size *dch5* gene, and sequencing of one of the PCR products confirmed the excision of the P element and restoration of the *dch5* gene sequence. These results demonstrate that the P-element insert in the *dch5* gene of the *l(3)L4032* strain is the cause of the homozygous lethality of the chromosome carrying it. Thus, the normal function of the *dch5* gene, and presumably the COP9 signalosome, is essential for *Drosophila* development.

We then examined the homozygous lethal excision lines that were obtained in the hybrid dysgenesis. The mutant lines were maintained over a balanced chromosome containing the gene for green fluorescent protein (GFP). PCR analysis on individual homozygous mutant larvae (identified by their lack of GFP expression) suggested a deletion of *dch5*. To confirm that the non-GFP lines are truly null mutants of Dch5, individual non-GFP-expressing larvae from two of the homozygous lethal excision lines, *dch5-2* and *dch5-3*, and the original *l(3)L4032* strain (renamed *dch5-1*), were analyzed by immunoblotting for the presence of Dch5. As shown in Figure 3, anti-Dch5 antibodies recognize two specific proteins with apparent molecular masses of 44 and 42 kDa in the wild type. These bands were absent in individual non-GFP larvae from the excision lines. Interestingly, in homozygous *dch5-1* larvae, we detected the 44 kDa band, but not the 42 kDa one. This result is reminiscent of the behavior of the FUS5 subunit of the *Arabidopsis* COP9 signalosome. Whereas FUS5 is detected as a doublet in immunoblot analysis on the wild type, only one protein band is detected in mutants lacking the COP9 signalosome [8], possibly due to differential phosphorylation.

Homozygous mutant *dch5-1*, *dch5-2* and *dch5-3* embryos hatched normally and proceeded through the first and second larval instars without any apparent defect. Developmental retardation was apparent during the middle of the third larval instar: 10–30% of the larvae became increasingly sluggish and flaccid and did not reach pupation, while 100% of the GFP-positive siblings pupated. The majority of homozygous mutant larvae pupated but ceased to develop, and less than 10% reached a stage at which differentiated adult cuticular structures were apparent through the pupal case. None succeeded in eclosing. No cuticular abnormalities were apparent in these non-eclosing adults.

The COP9 signalosome appears to be ubiquitous in animal cells [5,13], suggesting a fundamental biochemical function. The lethality of the *dch5* mutants clearly indicates that Dch5, and presumably the COP9 signalosome, is essential for development to the adult stage. It is possible that the lethality results not from a lack of the

COP9 signalosome, but from the lack of a specific role fulfilled by the Dch5 monomer. We expect, however, that mutations in each component of the COP9 signalosome in *Drosophila* will have essentially identical phenotypes, regardless of whether the protein functions exclusively in the complex, or also as a monomer, just as in *Arabidopsis*.

Similar to the *dch5* mutants, *Arabidopsis* mutants lacking the COP9 signalosome are also lethal, though only after successful embryogenesis and several days of growth. The successful early development in both *Drosophila* and plants that lack components of the COP9 signalosome therefore suggests that the complex does not fulfill an essential housekeeping function, the removal of which would probably lead to lethality at the embryonic stages, but rather is necessary for later developmental stages. Mutations in the *Arabidopsis* COP9 signalosome lead to numerous pleiotropic phenotypes and general transcriptional misregulation [16–18]. We thus expect that detailed analysis of the *Drosophila* mutants will show a number of phenotypes, which may mask the earliest lesion resulting from lack of COP9 signalosome function.

We speculate that the CO9 signalosome fulfills an essential function needed for cellular differentiation, perhaps through regulating mitogen-activated protein (MAP) kinase signaling or cell-cycle control. Supporting data for this hypothesis comes from the analysis of individual subunits in various systems. The COP9 signalosome was hypothesized to serve as an additional regulator of proteasome activity [5]. The transcription factor c-Jun and the cyclin-dependent kinase $p27^{Kip1}$, proteins regulated by ubiquitin-dependent protein degradation [19], interact with the mammalian Dch5 ortholog Jab1 [20,21]. The mammalian COP9 signalosome may phosphorylate c-Jun [4], implying that the complex is involved in regulating the proteasome-directed degradation of c-Jun [4]. Several other COP9 signalosome subunits were previously identified by their involvement in the MAP kinase signaling pathway [6,8,22]. Taken together, this body of data suggests an involvement of the COP9 signalosome in both the stress-activated MAP kinase signaling pathway mediated by the c-Jun/AP-1 transcription complex, and in cell-cycle control through $p27^{Kip1}$.

Supplementary material

Supplementary material including deduced Dch protein sequences and additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank Michael Glickman for critical reading of the manuscript, and Xing-Wang Deng and Ning Wei for communicating results prior to publication. We are indebted to Nir Ohad for assistance with microscopy, and acknowledge the technical assistance of Ruth Werczberger in maintaining *Drosophila* strains, and Etan Winter in constructing the yeast two-hybrid cassettes. This research was funded by a grant from The Israel Science Foundation founded by The Israel Academy of Sciences and Humanities (42/97-1). D.A.C. is the recipient of a Yigal Alon Young Scientist Award.

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Supplementary material

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Current Biology 11 October 1999, 9:1187–1190

Supplementary material and methods

Cloning and characterization of Drosophila *COP9 signalosome subunits*

In order to identify potential *Drosophila* orthologs of the known COP9 signalosome subunits, we used the available amino acid sequences for the subunits of the *Arabidopsis* [S1] or mammalian COP9 signalosome [S2] for searches against the *Drosophila* database (FlyBase) [S3] by TBLASTN search [S4]. Expressed sequence tag (EST) clones that encode putative homologs for all eight published COP9 signalosome subunits were identified and the longest clones were fully sequenced using a fluorescence-based automatic sequencer. Conceptual COP9 signalosome proteins from *Drosophila*, *Arabidopsis*, mouse and/or human were aligned with the CLUSTALV alignment program in order to determine the *Drosophila* coding region and to identify the conserved amino acid domains. Amino acid similarity and identity between amino acid sequences was determined using the MACHSHADE program following alignment by CLUSTALV. PCI and MPN domains were detected with PRODOM [S5] (Figure S1).

A genomic sequence was identified in FlyBase, the predicted gene product of which, Dch1-1, is highly similar to Dch1-2. But Dch1-1 lacks the carboxy-terminal third, no expressed sequence tag has been identified for *dch1-1* and expression analysis failed to reveal a *dch1-1* transcript (data not shown), suggesting that *dch1-1* is either a pseudogene or is expressed at very low levels.

Dch1-2 is highly similar to *Arabidopsis* FUS6 and human GPS1/S1/SGN1. *Arabidopsis fus6* mutants develop photomorphogenically in the dark [S6] and GPS1 represses G-protein- and MAP kinase-mediated signal transduction [S7]. Dch2 was previously identified as *Drosophila* Alien [S8], and is highly similar to human TRIP15. The *alien* gene was identified from its proximity to the *rolling stone* gene, and no *alien* mutant has been reported. Expression of *alien* is predominantly found in the muscle attachment sites (apodemes) during *Drosophila* embryogenesis. TRIP15 was isolated by its ability to interact with the thyroid receptor in the absence of hormone [S9]. A functional correlation between Alien and TRIP15 has not been established. In addition, Dch2 displays moderate similarity in its carboxy-terminal region to the mammary tumor-associated protein Int6 [S10], which was later found to be the p48 subunit of the mammalian eIF3 complex [S11]. The *Arabidopsis* ortholog of Int6 was identified in purified COP9 signalosome preparations [S12]. Dch3 and Dch4 are similar to the 26S proteasome regulatory subunits p58 and p55, respectively. The carboxy-terminal 40 amino acids of Dch4 are also similar to the 26S proteasome regulatory subunit 9/p44.5.

Dch5 is the most highly conserved of the Dch proteins, sharing approximately 75% amino acid identity with its putative vertebrate and plant orthologs S5/Jab1 and Ajh1. Jab1 was originally identified by its ability to interact with c-Jun [S13], and has recently been shown to interact with and regulate subcellular localization and amounts of $p27^{Kip1}$ [S14]. In addition, Dch5 is similar to the yeast proteins Pad1 and Rpn11/Mpr1 over its entire amino terminus. This region has been defined as the MPN domain (Mpr1/Pad1 N terminus [S15]). The MPN domain spans approximately 140 residues, occurs typically at the amino terminus of proteins, and has a structure predicted to be α/β type [S16]. The MPN domain is a signature of the Mov34 family of proteins [S17], and the amino terminus of Dch5 is similar to the *D. melanogaster* Mov34 homolog, the role of which in fly development is unknown. The Mov34 locus was initially described in mice as a retroviral integration mutation in the murine germ line causing embryonic lethality [S18]. Subsequently, it was shown to encode the 26S proteasome subunit p40 [S19]. Moreover, Dch5 is also similar to the p47 subunit of mammalian eIF3, which also contains an MPN domain [S17].

Dch6 shares 58% amino acid identity with the mouse COP9 signalosome subunit 6. Like Dch5, Dch6 is also a member of the Mov34 family of proteins and contains an MPN domain in the first 88 amino acids. Dch7 is highly conserved with *Arabidopsis* FUS5, which encodes a phosphorylated component of the plant COP9 signalosome [S20], and also is similar to Acob, a preinduction gene product required for asexual sporulation in *Aspergillus nidulans* [S21].

Antibody production

The entire coding regions of Dch5 and Dch7 subunits were cloned into pGEX 4t-1 (Pharmacia) at the *Sal*I and *Not*I restriction sites. A primer was synthesized to create a *Sal*I restriction site at the 5′ UTR of Dch7 (5′-AAACAAATTAGTCGACTTGAAGTTA-3′). The plasmid constructs were verified by sequencing. The resulting glutathione-S-transferase (GST)–Dch5 and GST–Dch7 fusion proteins were over-produced in *Escherichia coli* strain XL1-blue and found to be insoluble. The $12,000 \times g$ centrifugation pellet containing the fusion protein in inclusion bodies was separated on a 12.5% SDS–polyacrylamide gel, the fusion protein was recovered by electroelution and used to immunize rabbits (AniLab) for the production of polyclonal antibodies. For affinity purification of the resulting serum, Dch5 and Dch7 were expressed in pET28a (Novagen). The expressed proteins were solubilized from inclusion bodies with 6 M Guanidine HCl, 0.1 M sodium phosphate, 0.01 M Tris–HCl pH 8.0 and immobilized on an N-hydroxysuccinimide Hi-Trap column (Pharmacia). Anti-Dch5 and anti-Dch7 antibodies bound to Dch5 and Dch7, respectively, were eluted with low pH buffer (2 M glycine, 1 mM EGTA pH 2.5) for 30 min. The resulting affinity-purified anti-Dch5 antibodies were neutralized by addition of 1 M Tris–HCl pH 8.8.

Co-immunoprecipitation analysis

Wild-type adult flies (Canton-S strain) were homogenized with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Na-deoxychelate, 0.1% SDS, 50 mM Tris–HCl pH 8.0) containing 100 μM PMSF, with 400 μl buffer per 100 mg flies. After two centrifugations at $20,800 \times g$ in a table-top centrifuge for 20 min at 4°C, the supernatant was incubated with affinity-purified anti-Dch5 antibodies for 1 h at room temperature. After a short spin, 25 μl of protein-A beads (Sigma) were added to the supernatant for 1 h incubation at 4°C. The beads were then washed six times with 0.2% Tween-20 in PBS. Precipitated proteins were eluted in SDS sample buffer, and further analyzed by standard 12.5% SDS–PAGE followed by transfer to polyinylidene difluoride membranes, and probed with affinity-purified anti-Dch7 antibodies. Bound antibodies were detected with alkaline phosphatase-coupled secondary antibodies, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

Gel-filtration chromatography

The column was washed with gel-filtration buffer at a rate of 0.3 ml/min. The eluant was collected in 0.5 ml fractions starting from the onset of the void volume. The fractions were concentrated with resin beads (Stratagene). The concentrated samples were analyzed by SDS–PAGE followed by standard western blotting.

Figure S1

The deduced amino acid sequences of the putative *Drosophila* COP9 signalosome subunits. The coding regions were determined according to comparison of the putative *Drosophila* open reading frame with the amino acid sequences of known *Arabidopsis* and human COP9 signalosome subunits. The PCI domains (in Dch1-1, Dch1-2, Dch2, Dch3, Dch4, and Dch7) and MPN domain (in Dch5 and Dch6) are underlined. Domains were determined according to [S5].

Dch1-2
MPVLPMPPLMQNAVEPMQVDIAPPNEDNENNEEQQIVVENPSIDLEVFANQYAGIVRLHRLIYVADVCPVLAVEALKMAIT
YVQTTYNVNLYQVLHKRLSDLNAGNAPAPPANAGGDQAGAAAPGPLAAAPLPDIAAQPVAQAQGQAQPAVEKDAFAYDAAW
VDTKMKKAALKLEKLDSDLKNYKSNSIKESIRRGHDDLADHYLSCGDLTNALKCYSRARNYCTSGKHVVNMCLNVIKVSIY
LQNWAHVMSYISKAESTPDFAEGSKEANAQVHTRLECAAGLAELQQKKYKVAAKHFLNANFDHCDFPEMISTSNVAVYGGF
CALATFDRQELKRLVIASTSFKLFLELEPQLRDIIFKFYESKYASCLTLLDEIRDNLLVDMYIAPHVTTLYTKKRNRALIQ
YFSPYMSADMHKMAMAFNSSVGDLENEVMQLILDGQIQARIDSHNKILFAKEADQRNSTFERALIMGKQYQRHTRMLVLRA
AMLKSHIHVKSISREGGSNHGAELCVSAGSSTTAQFARI

Dch1-1
MDNGEETMLHLPSYADRYTDIPRLIRLKFIAQVCPELSVLALELALNHVKTTYNVKLYDELYKTLCVEVDRKYPNQSKGNE
ELHTTGGSEPSTSSGRGRVVVPYDSYWVEDNIMEATLMLQELDAELNFKKSNSGSSYVRRILEEIGDHHEKSGNLQMAVKF
YARARPYCTSSENVINMFRNLIRVSIYMENWWHVLTYIDEAKQYAYGFENLAQEVPARLSCVAGLAHLGLKIYKSAAQYFL
STPYGRYDYDKIVAPEDVTLYAGLCALATFDRETLQLNAINSEAFKPFFQLSPKMWTILAKFYAGEFDACMTLLREIENHV
RLDVYLSPH

Dch2
MSDNDDDFMCDDDEDYGLEYSEDSNSEPDVDLENQYYNSKALKEEEPKAALASFQKVLDLENGEKGEWGFKALKQMIKINF
RLCNYDEMMVRYKQLLTYIKSAVTRNHSEKSINSILDYISTSKNMALLQNFYETTLDALRDAKNDRLWFKTNTKLGKLYFD
RSDFTKLQKILKQLHQSCQTDDGEDDLKKGTQLLEIYALEIQMYTVQKNNKKLKALYEQSLHIKSAIPHPLIMGVIRECGG
KMHLREGEFEKAHTDFFEAFKNYDESGSPRRTTCLKYLVLANMLMKSGINPFDSQEAKPYKNDPEILAMTNLVNSYQNNDI
NEFETILRQHRSNIMADQFIREHIEDLLRNIRTQVLIKLIRPYKNIAIPFIANALNIEPAEVESLLVSCILDDTIKGRIDQ
VNQVLQLDKINSSASRYNALEKWSNQIQSLQFAVVQKMA

Dch3
MGSALENYVNQVRTLSASGSYRELAEELPESLSLLARNWSILDNVLETLDMQQHSLGVLYVLLAKLHSASTANPEPVQLIQ
LMRDFVQRNNNEQLRYAVCAFYKTCHLFTEFVVQKNLSILGIRIISRAIDQIRQLETQLTPIHADLCLLSLKAKNFSVALP
YLDADITDISTVAAECKTQQQQQSQHADANNDAKYFLLYFYYGGMIYTAVKNYERALYFFEVCITTPAMAMSHIMLEAYKK
FLMVSLIVEGKIAYIPKNTQVIGRFMKPMANYYHDLVNVYANSSSEELRIIILKYSEAFTRDNNMGLAKQVATSLYKRNIQ
RLTKTFLTLSLSDVASRVQLASAVEAERYILNMIKSGEIYASINQKDGMVLFKDDPEKYNSPEMFLNVQNNITHVLDQVRQ
INKMEEEIILNPMYVKKALGSQEDDLTSQHPKTFSGDPTD

Dch4
MAANYGISTAALRSQQMGLINFTGTHKDQADKYRQLLKTVLTNTGQELIDGLRLFVEAIVNEHVSLVISRQILNDVGSELS
KLPDDLSKMLSHFTLEKVNPRVISFEEQVAGIRFHLANIYERNQQWRDAATVLVGIPLETGQKQYSVECKLGTYLKIARLY
LEDNDSVQAELFINRASLLQAETNSEELQVLYKVCYARVLDYRRKFIEAAQRYNELSYRKIVDQGERMTALKKALICTVLA
SAGQQRSRMLATLFKDERCQHLPAYGILEKMYLERIIRRSELQEFEALLQDHQKAATSDGSSILDRAVFEHNLLSASKLYN
NITFEELGALLDIPAVKAEKIASQMITEGRMNGHIDQISAIVHFENRELLPQWDRQIQSLCYQVNSIIEKISVAEPDWMDN
LN

Dch5
MDSDAAQKTWELENNIQTLPSCDEIFRYDAEQQRQIIDAKPWEKDPHFFKDIKISALALLKMVMHARSGGTLEVMGLMLGK
VEDNTMIVMDAFALPVEGTETRVNAQAQAYKYMTAYMEAAKKVGRMEHAVGWYHSHPGYGCWLSGINVSTQMLNQTYQEPF
VAIVVDPVRTVSAGKVCLGAFRTYPKGYKPPNEEPSEYQTIPLNKIEDFGVHCKQYYPLEISYFKSALDRRLLDSLWNKYW
VNTLGSSGLLTNTEYTTGQIMDLSEKLEQSENFLGRGTDVNEKRSEDKLSKATRDCSRSTIELIHGLMAQIVKDKLFNKVG
LGK

Dch6
MSAKPSTSSSAAAGSSMAVDKTADQNPQPQGNIMAAAGTSGSVTISLHPLVIMNISEHWTRFRAQHGEPRQVYGALIGKQK
GRNIEIMNSFELKTDVIGDETVINKDYYNKKEQQYKQVFSDLDFIGWYTTGDNPTADDIKIQRQIAAINECPIMLQLNPLS
RSVDHLPLKLFESLIDLVDGEATMLFVPLTYTLATEEAERIGVDHVARMTSNESGEKSVVAEHLVAQDSAIKMLNTRIKIV
LQYIRDVEAGKLRANQEILREAYALCHRLPVMQVPAFQEEFYTQCNDVGLISYLGTLTKGCNDMHHFVNKFNMLYDRQGSA
RRMRGLYY

Dch7
MTQDMLLGNEEPSKSKETFLEKFCVLAKSSTGAALLDVIRQALEAPNVFVFGELLAEPSVLQLKDGPDSKHFETLNLFAYG
TYKEYRAQPEKFIELTPAMXKKLQHLTIVSLAIKAKSIPYALLLSELEIDNVRHLEDIIIEAIYADIIHGKLFQNTRILEV
DYAQGRDIPPGYTGQIVETLQAWVNSCDXVSNXXEMQIKXANAEKSKRLINKERVEQDLINLKKVLKSQTSDSDESMQXDT
HGPGTSGGXGQSEXRKKPSKLRNPRSAAVGLKFSK

Dch8
VYQQLLAIYLYQNKLADAKLLWMRVPANLRDDKELIQLNLLNIALQNNNYADFFKHIKYEWSERVKSPVEDLLNKQREELF
KLMGSAYMSIYQHNLLELSLMSEDELKHACAALNWTEELDGDRVILKPKVQEAPPARGNDDQLLKLTEFVTFLEN

Current Biology

Construction of cassettes for the yeast two-hybrid assay

The coding regions of *dch1-2*, *dch2*, *dch4*, *dch5* and *dch7* were cloned into pEG202 to make in-frame fusion proteins with LexA. The plasmids generated were designated pEG-Dch1-2, pEG-Dch2, pEG-Dch4 and pEG-Dch7, respectively. The coding regions of *dch1-2*, *dch2*, *dch4*, *dch5* and *dch7* were also cloned into pJG4-5 to make in-frame fusions with the transcription-activation domain. The plasmids generated were designated pJG-Dch1-2, pJG-Dch2, pJG-Dch4 and pJG-Dch7, respectively. For cloning, we used restriction sites in the pBluescript (for *dch1-2*, *dch2*, *dch4*, *dch5 and dch7* in EST clones Id17230, Id14719, Id11968, Id19430 and Id09490, respectively) or pGEXDch7 polylinkers. When necessary, PCR with specific oligonucleotides was used to generate in-frame restriction sites (Table S1).

For the construction of Dch7-NT, two primers were used, a primer specific for the pEG202 vector found upstream of the cloning site (P6) and a primer generating a *Nco*I restriction site at amino acid 157 of Dch7 (P7). A pEG-Dch7 template was used for a PCR amplification

Table S1

Primers used for cloning the *dch* genes.

Plasmid	Insert source	Strategy for PCR oligonucleotides	Sites
pEG-Dch1-2	ld17230	T7	*Bam*HI
		P1 5′-GAAGCAGGATCCCCGTGC-3′	*Bam*HI
pJG-Dch1-2	ld17230	T7	*Xho*I
		P2 5′- TTGAATTCGTTGCAAATAGAA-3′	*Eco*RI
pEG-Dch2			
pJG-Dch2	ld14719	T7	*Xho*I
		P3 5′-TTGAATTCATGTCCGACAACGATGAT-3′	*Eco*RI
pEG-Dch4			
pJG-Dch4	ld11968	T7	*Xho*I
		P4 5′-GGTATAGAATTCAATATGGC-3′	*Eco*RI
pEG-Dch5	ld19430	Polylinker sites from pBLUESCRIPT	*Eco*RI–*Xho*I
pJG-Dch5	ld19430	Polylinker sites from pBLUESCRIPT	*Eco*RI–*Xho*I
pEG-Dch7	pGEXDch7	N.N.	*Eco*RI–*Xho*I
pJG-Dch7	pGEXDch7	N.N.	*Eco*RI–*Xho*I
pEG-Dch7-NT	pEGDch7	P6 5′-CGTCAGCAGTTCACCATTG-3′	*Eco*RI
		P7 5′-TCCAAAATCCATGGGTTCTGGA-3′	*Nco*I
pEG-DH7-CT	ld09490	T7	*Xho*I
		P8 5′-ACCTATTCCCATGGGGCACATA-3′	*Nco*I

The ld designation is for cDNA clones obtained through FlyBase. The underlined sequences designate engineered restriction sites. N.N., not necessary.

reaction and the PCR product was restricted and cloned into pEG202 at the *Eco*RI–*Nco*I restriction sites. For the construction of Dch7-CT, the T7 primer and P8, which generates a *Nco*I site at amino acid 79 of Dch7, were used. The Dch7 EST clone (ld09490) was used as a template for PCR amplification and the product restricted and cloned into pEG202 at the *Nco*I–*Xho*I restriction sites. For the construction of Dch7-PCI, Dch7-NT and Dch7-CT were used as templates for a single PCR reaction with primers T7 and P6. The PCR product was cloned into pEG202 at the *Eco*RI–*Xho*I restriction sites, thus generating pEG-Dch7$_{79,157}$ containing two *Nco*I restriction sites. The PCI fragment was deleted by a *Nco*I restriction to generate Dch7-PCI. For the construction of Dch7-PCI, pEG-Dch7$_{79,157}$, was used as template for a PCR reaction with primers P7 and P8.

All PCR reactions were performed under the following conditions: 2 min at 94°C; 1 min at 94°C, 1 min at 55°C, 1 min at 72°C for 29 cycles; 10 min at 72°C, with Expand High Fidelity polymerase (Boehringer Mannheim) in a PTC-100 thermal cycler (MJ Research).

Yeast two-hybrid assay

The pEG-Dch plasmids were transformed into yeast strain L-40 (*Mat***a**, *his*$^{-}$, *leu*$^{-}$, *trp*$^{-}$, *ade*$^{-}$, *ura*$^{+}$, *8-lexA-op*) that contains a *lacZ* reporter gene under the control of *lexA* operator. The pJG-Dch plasmids were transformed into the yeast strain EGY48 (*Mat*α, *his*$^{-}$, *leu*$^{-}$, *trp*$^{-}$, *ade*$^{+}$, *ura*$^{-}$). Yeast cells were transformed with LiCl. The different strains were mated on YPD media and transferred for selection to synthetic complete media without histidine and tryptophan. As the medium contains X-gal, the interactions between the proteins were determined by the appearance of blue colonies.

D. melanogaster *maintenance and hybrid dysgenesis*

Canton-S (CS) served as the wild-type control. Mutant strains were obtained through the appropriate stock centers. Strains were maintained and crosses were conducted on cornmeal–molasses medium at 25°C. A description of balancer chromosomes and markers can be found in FlyBase [S1,S22]. The TM3, *Ser* balancer that carries the *GFP* transgene is described by Reichhart and Ferrandon [S23].

The P element in the *l(3)L4032* strain is oriented with its 3′ end towards the 5′ end of the *dch5* and flanking DNA sequence data was available only for that side of the P-element insert [S22]. To obtain genomic sequences flanking the other side (upstream) of this insert, DNA of *l(3)L4032/TM3, Ser* flies was digested with *Hin*P1I, self-ligated and subjected to inverse PCR using primers from the long terminal repeat of the P element and from the *dch5* gene. Sequencing of the resultant 865 bp amplified fragment yielded a genomic sequence 429 nucleotides from the 5′ end of *dch5* (upstream of the P-element insertion site; Figure S2). Inverse PCR was primed with the *dch5*-specific primers Pry4 5′-CAATCATATCGCTGTCTCACTCA-3′ and Plw3-1 5′-TGTCGGCGTCATCAACTCC-3′. Analysis of putative excision lines was performed with a primer derived from this sequence (PYaara2: 5′-GGGTGCAGGAACTGACAACTTAA-3′) corresponding to nucleotides 1–429, where the P-element insertion site is at position 0, and a primer corresponding to the *dch5* gene sequence +644 to +666 downstream of the P-element insertion site (PYaara1: 5′-ATACGTGCGGAATGCACCCAGA-3′).

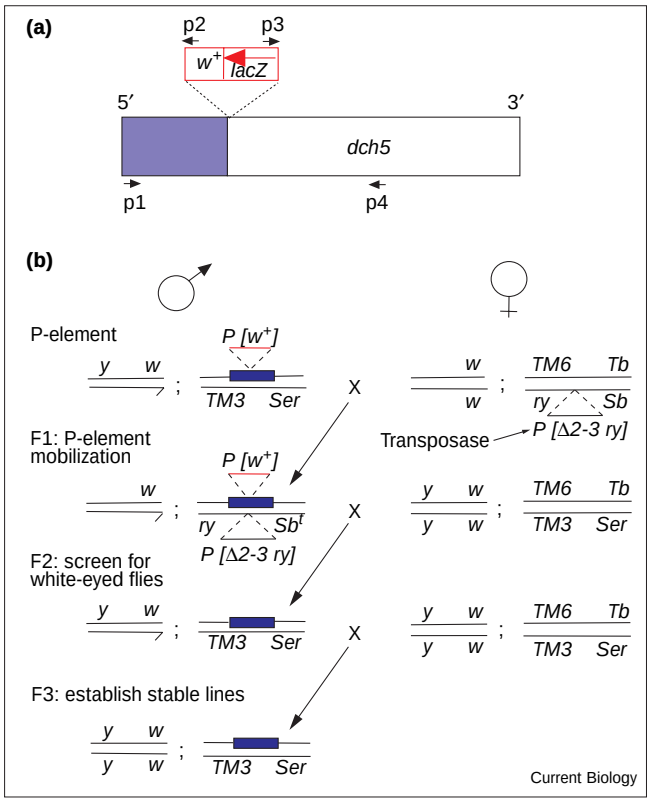
The P element in the *l(3)L4032* strain carries the dominant *w*$^{+}$ eye color marker. Males from the *l(3)L4032* strain were crossed to females carrying the Δ2-3 stable source of transposase. Dysgenic *y w; l(3)L4032/ry Sb P*[*Δ2-3*] *ry*$^{+}$ male progeny, in the germ line of which the *l(3)L4032*-associated P element was mobilized, were crossed to *y w; TM6, Tb/TM3, Ser* females. Their offspring (F2) that did not carry the transposase source were screened for males with white eyes, indicative of loss of the *w*$^{+}$ marker due to excision of the P element from *l(3)L4032*. Individual putative excision males were crossed to *y w; TM6, Tb/TM3, Ser* females to establish balanced lines. About 2,000 F2 flies were scored for white eyes and lines were established from 73 independent putative excision flies, 58 of which were homozygous viable.

We also examined the homozygous lethal excision lines that were obtained in the hybrid dysgenesis. Wild-type, *dch5-1*, *dch5-2*, and *dch5-3* were balanced over a *TM3* chromosome carrying the green fluorescent protein (GFP) marker gene under the actin-5 promoter.

Microscopy

Drosophila from different developmental stages were examined using a Zeiss Axioplan 2 fluorescent microscope. GFP was visualized using filter set 13 for GFP (#487913) at an excitation of 470 nm. *Drosophila* larvae were immobilized for examination by placing them for several minutes on ice.

Figure S2



The P element in *Dch5-1* causes lethality. (a) Structure of the P-element insertion in the *Dch5* mutant strain *l(3)L4032*. The P element (red box, containing *lacZ* transcribed in the direction shown by the arrow) is inserted 24 bp upstream of the putative Dch5 initiation codon. Upstream flanking sequences (purple box) were obtained by inverse PCR. Small arrows indicate P-element-specific and genomic primers used for PCR: p1, PYaara2; p2, P-element 3′; p3, P-element 5′; p4, PYaara1 (see Supplementary materials and methods). (b) Hybrid dysgenesis protocol. The series of crosses aimed at mobilization of the P element in the *l(3)L4032* strain are shown. Mobilization occurred in the germ line of the dysgenic flies (F1 males) and excision events were detected in the F2 generation as white-eyed (*w*) flies. Red line, P element; blue box, *Dch5*. Not drawn to scale.

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