

COP9 signalosome subunit 5 (CSN5/Jab1) regulates the development of the *Drosophila* immune system: effects on Cactus, Dorsal and hematopoiesis

Orit Harari-Steinberg¹, Rafael Cantera^{2,3}, Simona Denti⁴, Elisabetta Bianchi⁴, Efrat Oron¹, Daniel Segal⁵ and Daniel A Chamovitz^{1*}

Departments of ¹Plant Sciences and ⁵Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv, Israel

²Zoology Department, Stockholm University, Stockholm, Sweden

³Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay

⁴Laboratory of Immunoregulation, Institut Pasteur, Paris, France

The COP9 signalosome is a multifunctional regulator essential for *Drosophila* development. A loss-of-function mutant in *Drosophila* COP9 signalosome subunit 5 (CSN5) develops melanotic bodies, a phenotype common to mutants in immune signaling. *csn5^{null}* larvae accumulated high levels of Cactus that co-localizes with Dorsal to the nucleus. However, Dorsal-dependent transcriptional activity remained repressed in the absence of an inducing signal, despite its nuclear localization. Dorsal activity in mutant larvae and NFκB activity in CSN5 down-regulated mammalian cells can be induced following activation of the Toll/IL-1 pathway. *csn5^{null}* larvae contained more hemocytes than wild-type (wt) larvae. A large portion of these cells have differentiated to lamellocytes (LM), a hemocyte cell type rarely seen in normal larvae. The results presented here indicate that CSN5 is a negative regulator of Dorsal subcellular localization, and of hemocyte proliferation and differentiation. These results further indicate that nuclear localization of Dorsal can be uncoupled from its activation. Surprisingly, CSN5 is not necessary for immune-induced degradation of Cactus.

Introduction

Innate immunity in response to microbial infection is common to both plant and animal systems (reviewed in Nurnberger *et al.* 2004; Ausubel 2005). In *Drosophila*, septic infection induces cellular and humoral responses leading to local melanization in the hemolymph, phagocytosis and encapsulation by hemocytes, and synthesis and release to the hemolymph of anti-microbial peptides (AMPs) (reviewed in Hultmark 2003; Brennan & Anderson 2004). These responses are similar to those found in human innate immunity, and are controlled by similar signaling pathways, such as the Toll/TLR cascades (reviewed in Imler & Zheng 2004).

The humoral response to infection in *Drosophila*, characterized primarily by synthesis of AMPs in the fat body, the *Drosophila* equivalent to a liver, is mediated by

the Toll/IL-1 receptor (Wasserman 2000; Hultmark 2003; Brennan & Anderson 2004). The accepted model for Toll signaling in the fat body is as follows: Cactus, the homolog of mammalian IκB, forms a cytoplasmic complex with either Rel transcription factor Dorsal or Dif, the homologs of mammalian NF-κB. Following fungal or gram-positive bacterial infection, a proteolytic event activates a ligand of the Toll receptor, which in complex with Toll activates an intracellular signaling cascade resulting in the degradation of Cactus (Nicolas *et al.* 1998; Wu & Anderson 1998). The Rel protein relocates to the nucleus, where it can activate target AMP-encoding genes such as *Drosomycin (Drs)* (Wu & Anderson 1998; Manfrulli *et al.* 1999). During embryogenesis, signal-dependent degradation of Cactus requires its phosphorylation (Reach *et al.* 1996), which together with analogous IκB studies (Alkalay *et al.* 1995), suggest that once phosphorylated, Cactus is degraded in a proteasome-dependent fashion.

The cellular response to infection in *Drosophila* is mediated through different classes of hemocytes. The

*Correspondence: E-mail: dannyc@tauex.tau.ac.il

Communicated by: Xing-Wang Deng

Drosophila blood, also called the hemolymph, circulates in an open circulatory system. *Drosophila* hematopoiesis during larval development occurs in the lymph glands, which serve as a hemocyte reservoir (Rizki 1978; Shrestha & Gateff 1982; Lanot *et al.* 2001; Sorrentino *et al.* 2004). Hematopoiesis gives rise to three independent cell lineages: plasmatocytes (PL), phagocytes that resemble the monocyte/macrophage lineage, crystal cells, which play a critical role in defense-related melanization, and lamellocytes (LM) that encapsulate large invaders (Meister *et al.* 2000; Minakhina & Steward 2006). The regulation of hematopoietic cell proliferation and lineage specification in *Drosophila* involves several signaling pathways, including the Toll pathway.

Both dominant gain-of-function mutations in Toll, and loss-of-function mutations in Cactus, lead to constitutive activation of immune signaling with nuclear accumulation of Dorsal and induction of AMPs in the fat body, hemocyte proliferation and formation of melanotic bodies (Lemaitre *et al.* 1996; Qiu *et al.* 1998; Minakhina & Steward 2006). Down-regulation of the Toll-pathway caused by mutations in Toll, Tube or Pelle, on the contrary, lead to reduced hemocyte numbers (Qiu *et al.* 1998). However, mutations in Dorsal or Dif, two other key members of the Toll pathway, apparently have little or no effect on hemocyte numbers (Sorrentino *et al.* 2004).

The COP9 signalosome (CSN) is a conserved eight-subunit protein complex that functions in the ubiquitin-proteasome (Ub) system and thus has multiple roles in plants and animals. Within the Ub system, CSN regulates substrate phosphorylation and subcellular localization, and Cullin E3-ligase activity (Deng & Serino 2003). However, evidence from several systems indicates that there is an intricate equilibrium between the complex and specific individual subunits, which are also detected in forms independent of the CSN complex and may have independent activity. For example, CSN5, the subunit that contains deneddylase activity towards Cullin proteins, performs this enzymatic function only within the complex (Lyapina *et al.* 2001). However, CSN5 is also detected in smaller complexes and as a monomeric form, which may have independent signaling activities (Oron *et al.* 2002; Fukumoto *et al.* 2005). In *Drosophila*, null mutations in *Csn5* leave an intact CSN, indicating that CSN5 is not essential for complex formation (Oron *et al.* 2002).

There are several indications that the CSN may be involved in immune signaling: in *Arabidopsis*, *csn* mutants have defects in innate immunity (Liu *et al.* 2002); in human cells, the CSN co-purified with an I κ B α kinase activity (Seeger *et al.* 1998); and CSN3 interacts with IKK γ (Hong *et al.* 2001). To further explore the role of CSN5 in regulating immune signaling, we have analyzed *Drosophila csn5^{null}* mutants.

Results

csn5^{null} larvae are hypersensitive to bacterial immune challenge

Early developmental progression of *Drosophila csn5^{null}* mutants parallels that of wild-type (wt) strains and of their heterozygotic siblings, displaying no changes in body size, locomotor activity or in timing of the larval molts (Freilich *et al.* 1999). However, *csn5^{null}* larvae never pupariate and die as 10- to 13-day-old larvae (Freilich *et al.* 1999). These mutants developed massive melanotic capsules that first appear floating in the hemolymph during the third larval instar, (Fig. 1A) and progressively increase in size (Fig. 1B).

Since the melanotic capsules phenotype is reminiscent of phenotypes reported for immune response and hematopoiesis mutants, we hypothesized that CSN5 is involved in the regulation of immune signaling in *Drosophila*. To test this, we examined the resistance of *csn5^{null}* larvae to bacterial immune challenge (Table 1). The wt and *csn5^{null}* third instar larvae, 72 h after egg deposition (AED), a developmental stage when wt and mutants are still indistinguishable, were bacterially challenged by pricking them with a needle dipped in a bacterial cocktail and their viability was monitored 24 h later. The treatment had no effect on survival of wt larvae, but caused 40% mortality among mutant larvae. Pricking



Figure 1 *csn5^{null}* larvae display massive melanotic capsules. (A) four-day-old *csn5^{null}* larva with small melanotic capsules (arrow). (B) seven-day-old *csn5^{null}* larva with small and large melanotic capsules (arrows).

Table 1 *csn5^{null}* larvae are hypersensitive to bacterial immune challenge

Strain	Treatment	<i>n</i>	% Survival	Fisher's exact test
wt	UC	100	100	<i>P</i> = 1
	BC	100	99	
<i>csn5^{null}</i>	UC	346	73	<i>P</i> = 0.77
	P	70	71	
<i>csn5^{null}</i>	P	70	71	<i>P</i> < 0.001
	BC	257	44	
<i>csn5^{null}</i>	UC	346	73	<i>P</i> < 0.001
	BC	257	44	

Wild-type (wt) and *csn5^{null}* third instar larvae, 72 h AED and at the same developmental stage, were either unchallenged (UC) or bacterially challenged (BC) with a bacteria cocktail, and their viability was monitored 24 h later. As an additional negative control, *csn5^{null}* larvae were pricked with a sterile needle (P). Fisher exact test for similarity between two samples was carried out. *n* = number of larvae examined.

the larvae with a sterile needle, as a negative control, caused no mortality in either wt or mutant larvae. As an additional control, the experiment was also carried out on heterozygous *Csn5⁺/csn5^{null}* siblings of the mutants. These siblings also showed no sensitivity to the bacterial challenge (not shown). This indicates that *csn5^{null}* larvae are hypersensitive to bacterial immune-challenge, displaying a risk value of 1.678 (confidence interval 1.440–1.995) (see Experimental procedures). Given the compromised defense response of the *csn5^{null}* larvae, we further examined some important components of the humoral and cellular immune responses during the third larval instar.

Dorsal is constitutively nuclear in *csn5^{null}* larval fat body

In larvae with mutations in other genes, the presence of melanotic bodies similar to those seen in *csn5^{null}* larvae has been correlated with nuclear localization and activity of Dorsal in fat body (Lemaitre *et al.* 1995) or in hemocytes (Huang *et al.* 2005). To first assess if CSN components are present in wt fat body, we examined the subcellular localization of CSN5 and CSN7. Both subunits were enriched in fat body nuclei, accumulating against the nuclear envelope, excluded from the nucleolus (Fig. 2A). A fraction of CSN5 was also present in the cytoplasm. This localization pattern was not affected by immune challenge (not shown).

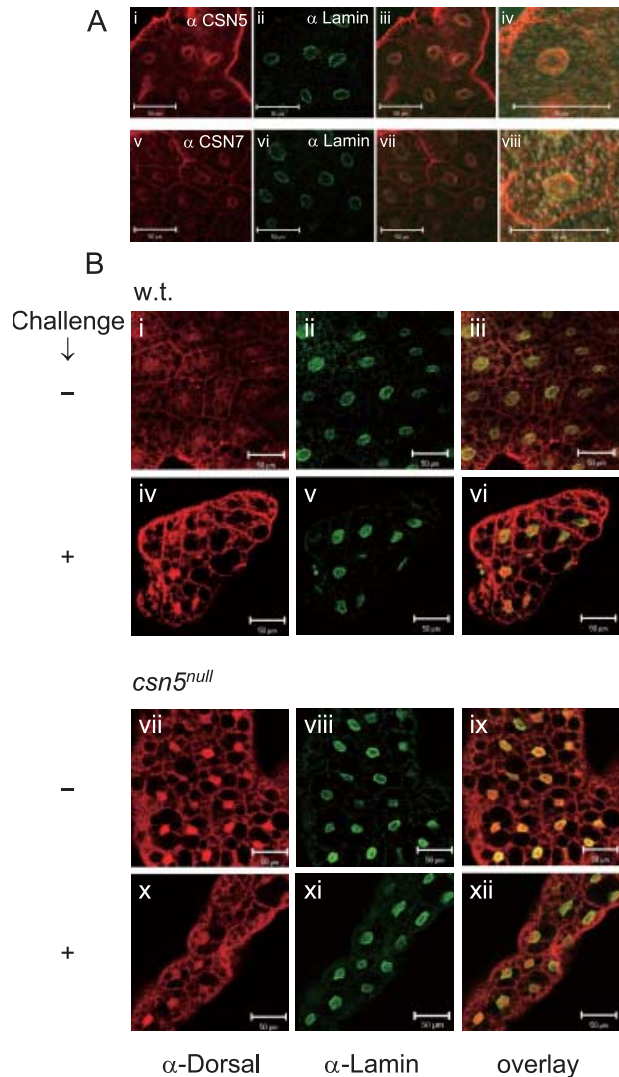


Figure 2 Dorsal is constitutively nuclear in *csn5^{null}* larvae fat body. (A) CSN5 (top row) and CSN7 (bottom row) are mainly nuclear in wt fat body. Anti-Lamin (ii, vi), overlay of anti-lamin and anti-CSN 5 or 7 (iii, vii); overlay with Nomarsky (iv, viii). (B) Subcellular localization of Dorsal in wt (i–iii, iv–vi) and *csn5^{null}* (vii–ix, x–xii) larvae with or without bacterial challenge. Anti-Lamin (ii, v, viii, xi), anti-Dorsal (i, iv, vii, x), Lamin and Dorsal signal overlay (iii, vi, ix, xii). Similar results were obtained for larvae 96 h AED (not shown). Bars = 50 μ m.

We then looked at the subcellular localization of Dorsal in the fat body of *csn5^{null}* larvae. In wt fat body of naive animals, as expected, Dorsal was mainly cytoplasmic (Fig. 2Bi–iii), but enriched in nuclei 1 h after a bacterial challenge (Fig. 2Biv–vi). In *csn5^{null}* larvae, Dorsal was constitutively nuclear, regardless of the immune challenge (Fig. 2Bvii–xii).

Dorsal activation is immune signal-dependent in *csn5^{null}* larvae

We next monitored Dorsal activity in *csn5^{null}* larvae by analyzing the transcript levels of one of its target gene, *Drosomyacin* (*Drs*). We also studied transcript levels of *Diptericin* (*Dpt*), another AMP gene, which is primarily under the control of the IMD signal pathway (Georgel *et al.* 2001). Any changes detected in transcription of both genes could be indicative of a general defect in immune signaling or in transcriptional repression. As expected, in wt larvae, *Drs* and *Dpt* transcript levels were undetectable in unchallenged conditions, and present in high amounts following bacterial challenge (Fig. 3). Surprisingly, despite the constitutive nuclear accumulation of Dorsal, transcript levels of *Drs* in *csn5^{null}* larvae mirrored those found in wt, as did *Dpt*. These results were confirmed by

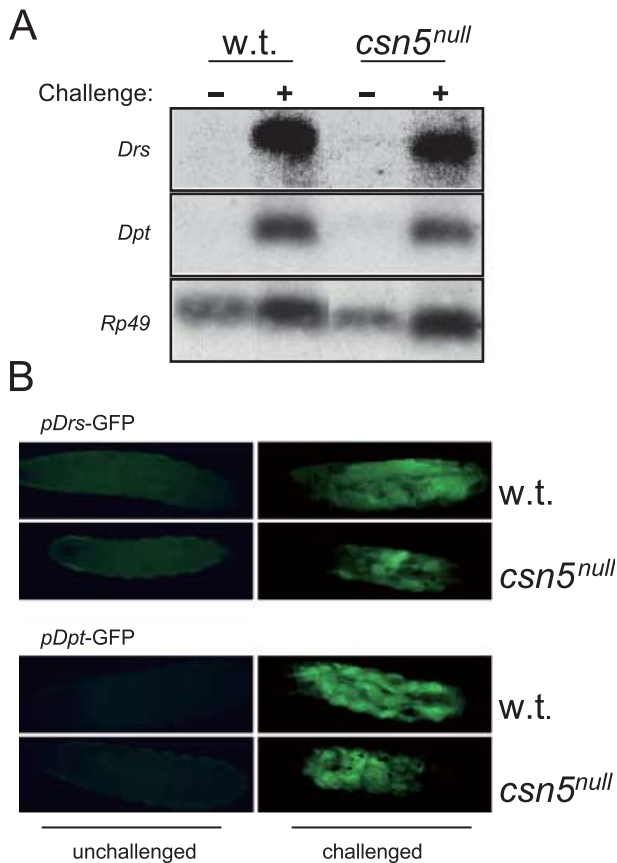


Figure 3 Induction of Drosomyacin is immune-signal dependent in *csn5^{null}* larvae. (A) Northern blots of total RNA samples from wt and homozygous *csn5^{null}* larvae before (-) and 4 h following bacterial infection (+) were probed with *Drs*, *Dpt* and *rp49* cDNA probes. (B) Induction of the GFP-tagged *Drs* or *Dpt* reporter genes in wt and *csn5^{null}* larvae.

employing *Drosophila* strains carrying a GFP-reporter gene under the *Drs* or *Dpt* promoters (Fig. 3B). An independent transcript profiling experiment on *csn5^{null}* larvae (Oron *et al.* 2005; Tuller *et al.* 2005) also did not identify any induction of other Dorsal targets such as *cactus* or *attacin* in *csn5^{null}* larvae 96 h AED (not shown). Furthermore, promoter analysis of the genes misregulated in *csn5^{null}* did not point to enrichment of Dorsal binding sites in the region extending from -400 to +200 bp relative to their annotated transcription start sites (not shown). These results indicate that Dorsal, although constitutively nuclear in *csn5^{null}* fat body, is inactive in the absence of immune signal and that CSN5 is not essential for Dorsal activation.

CSN5 is dispensable for NF- κ B activation in human cell lines

To determine if CSN5 has a role in mediating Toll-related signaling in mammals, we monitored the activity of the Dorsal homolog, NF- κ B, in human cell lines with reduced levels of CSN5. The levels of CSN5 were reduced at least 75% by transfection of a siRNA vector targeting CSN5 (iCSN5) in 293T and HeLa cells (Fig. 4A and not shown). NF- κ B activity in response to IL-1 or TNF α stimulation was monitored by measuring expression of a luciferase reporter gene under the control of a NF- κ B dependent promoter (IgGK promoter). IL-1 or TNF α treatment activated the NF- κ B promoter both in control and in CSN5 knockdown cells (Fig. 4B, C). This indicates that in human cells, as for the *Drosophila* fat body, a reduction in CSN5 levels does not affect NF- κ B activation.

Cactus over-accumulates and is mislocalized in *csn5^{null}* larvae

According to the accepted model, Dorsal activity is inhibited mainly by its cytoplasmic sequestering via its interaction with Cactus. The constitutive nuclear accumulation of Dorsal would thus suggest that Cactus is constitutively degraded in *csn5^{null}* larval fat body. On the other hand, the lack of Dorsal activity in unchallenged *csn5^{null}* larvae suggests that Dorsal is inhibited, possibly by still being complexed to Cactus. We therefore monitored the levels and subcellular localization of Cactus. Total protein extracts from *csn5^{null}* third instar larvae, as well as from fat body isolated from these larvae, displayed elevated levels of Cactus in comparison with wt larvae or their heterozygous siblings (Fig. 5A). In agreement with this finding, we observed higher immunofluorescence of anti-Cactus in the fat body of *csn5* mutants (see below, Fig. 5C) However, Cactus was apparently degraded in

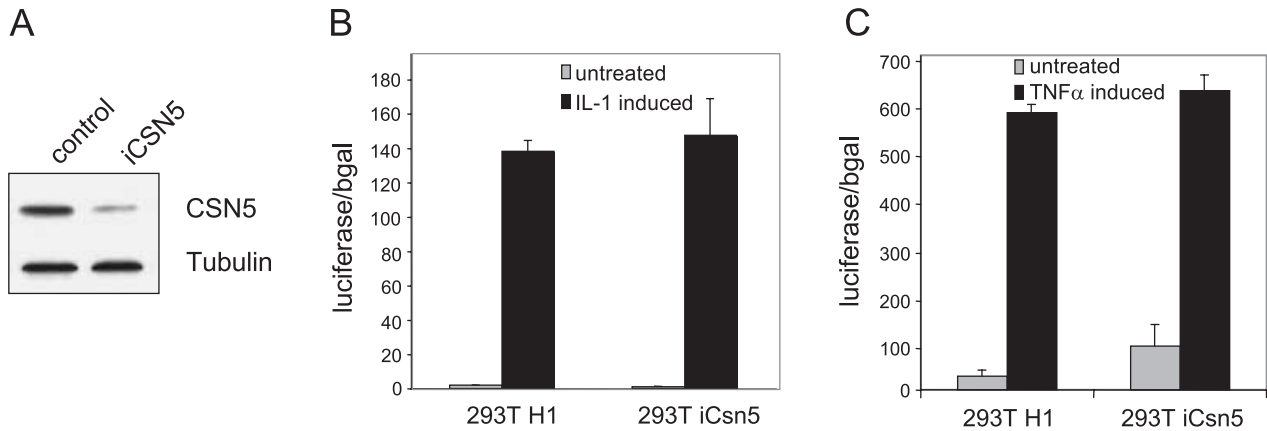


Figure 4 CSN5 is not required for IL-1 nor TNF α -dependent activation of NF κ B transcription. (A) Transfection of an shRNA construct against CSN5 (iCSN5, lane 2) down-regulates CSN5 protein levels in 293T cells, compared to control shRNA vector (H1, lane 1). Top: immunoblot with anti-CSN5. Bottom: immunoblot with anti-tubulin monoclonal Ab (Sigma). (B) Down-regulation of CSN5 does not impair NF- κ B activation by IL-1 treatment. 293T cells were transfected with the indicated shRNA constructs, with a synthetic NF- κ B luciferase reporter construct, and a β -galactosidase reporter construct as a control for transfection efficiency. Luciferase activity was measured in total lysates and normalized to galactosidase activity. Mean and standard deviations from three independent transfections are represented. (C) Down-regulation of CSN5 does not impair NF- κ B activation by TNF α treatment. Cells were transfected as in B and stimulated with TNF α .

csn5^{mut} following bacterial infection, as the Cactus band was not detected following infection (Fig. 5A), consistent with the immune inducibility of *Drs* in *csn5^{mut}* larvae. Additional high molecular weight species that cross-react with the anti-Cactus antibodies were also detected. These appear specific for Cactus as they over-accumulated in *csn5^{mut}*, and were not detected following immune challenge (Fig. 5A, right panel). The increase in Cactus in *csn5^{mut}* larvae was not due to increased *Cactus* transcript levels (Fig. 5B).

The subcellular localization of Cactus in fat body of *csn5^{mut}* larvae was then studied. As expected, Cactus was mainly cytoplasmic in unchallenged wt fat body (Fig. 5Ci–iv), but, surprisingly, accumulated in the nucleus of *csn5^{mut}* larvae, though an elevated cytoplasmic signal was also detected (Fig. 5Cv–viii). Thus, constitutive Cactus protein levels and localization are altered in *csn5^{mut}* larvae, while *Cactus* transcript levels and the immune-signal dependent degradation of Cactus protein are apparently unaffected.

csn5^{mut} larvae show hematopoietic phenotypes

Having ascertained that immune defenses are defective in *csn5^{mut}* mutants, though the humoral output is apparently normal, we then examined the cellular arm of the immune response. The formation of melanotic bodies similar to those seen in *csn5^{mut}* larvae has been correlated with hematopoietic defects, specifically arising from defects in Toll signaling (Lemaitre *et al.* 1995). To first assess if CSN components are present in wt hemocytes, we examined

the subcellular localization of CSN5 and CSN7 in these cells. Both subunits were enriched in PL nuclei. This localization pattern was not affected by immune challenge (not shown).

Observation of living mutant larvae under the microscope indicated that they contain abnormally large numbers of hemocytes, many of which are intimately associated with melanotic cells (Fig. 6A,B). To confirm this, circulating hemocytes were counted in blood samples extracted from mutant and wt larvae. As seen in Fig. 6, *csn5^{mut}* larvae contain more hemocytes than the wt (2.14-fold increase, $P < 0.05$). A similar ratio of 2.16 was obtained by a second counting method based on sampling representative microscope frames of fixed cells from different larvae. Hemocyte numbers from heterozygotic siblings mirrored those of the wt (not shown).

To determine which cell types are present in this population, the hemocytes were stained with specific monoclonal antibodies that allow identification of PLs, crystal cells or LMs. While the population of circulating hemocytes in unchallenged pre-wandering stage wt larvae was comprised mainly of PLs (Figs 6 and 7A), with a minor fraction of crystal cells, the hemocytes population of *csn5^{mut}* contained in addition an obvious population of LM (Figs 6 and 7B). While LM are rarely observed in normal wt larvae (Luo *et al.* 2002), they constituted ~10% of the hemocytes in *csn5^{mut}* larvae (899 LM out of 8307 cells counted from 11 larvae). Mutant larvae contained normal numbers of crystal cells, although these cells were larger compared with wt cells (Fig. 7C).

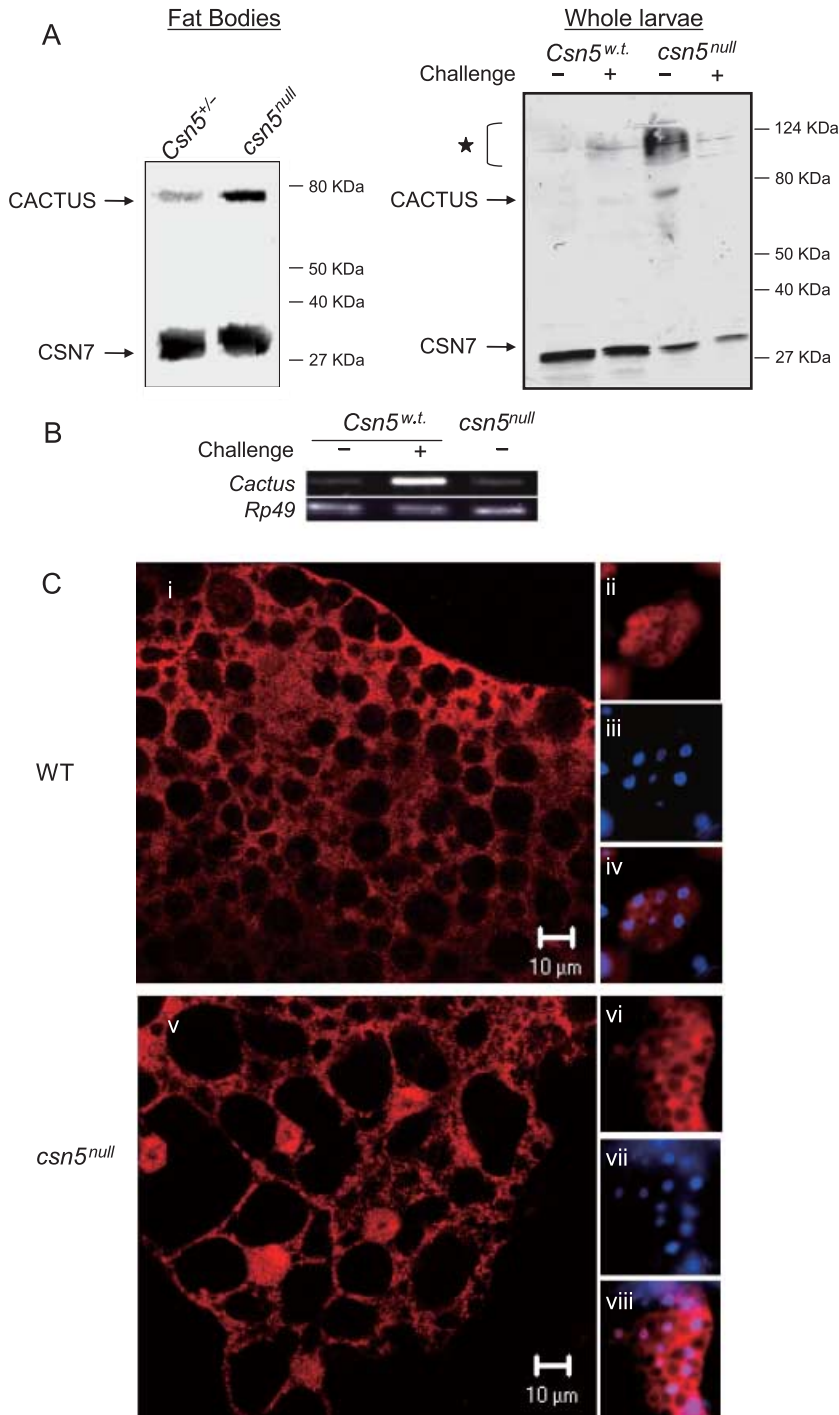


Figure 5 The absence of Csn5 affects Cactus levels and localization, but not its immune-signal dependent degradation. (A) Western blot of total protein extracts from homozygous *Csn5^{null}* and their heterozygous siblings, taken from isolated fat bodies (left) and whole larvae (right), either before (-) or after bacterial infection (+), probed with anti-Cactus. The antibody recognizes high molecular weight forms (*). Anti-Csn7 was probed as a loading control. (B) RT-PCR products of *Cact* and *Rp49* in naive (-) and bacterially challenged (+) larvae. (C) Cactus subcellular localization in fat body of wild-type (i-iv) and *Csn5^{null}* (v-viii) larvae. Enriched nuclear staining of Cactus is clearly seen in the confocal image from the *Csn5* mutant (v) but not from the wt (i). Epifluorescence images (ii-iv, vi-viii) of independent samples were taken to clearly visualize with anti-Cactus (ii and vi) and Hoechst stained nuclei (iii and vii). The overlap of the Cactus and Hoechst staining in the *Csn5* mutant (viii) is visualized as fuscina. In wt fat body nuclei, there is little overlap between Cactus and Hoechst staining (iv). Bars = 10 μ m.

Discussion

We have shown here that subunit 5 of the COP9 signalosome (CSN5) is involved in both the humoral and cellular immune responses in *Drosophila* (Fig. 8). Melanotic capsule formation in *Csn5^{null}* larvae provided the first

important clue regarding the biological function of CSN5 in hematopoiesis (Oron *et al.* 2002). This "leukemia-like" phenotype is further characterized by increased cell numbers and abnormal shapes among circulating hemocytes. As humoral activation of Toll signaling is preserved in *Csn5^{null}* larvae despite abnormal subcellular localization of

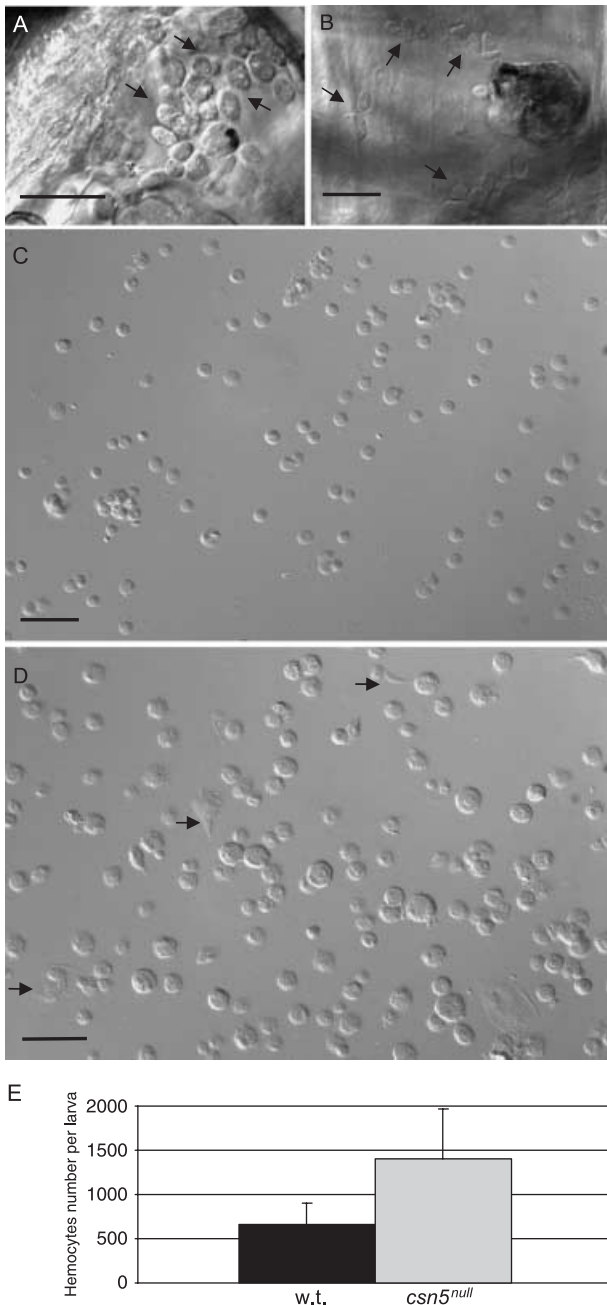


Figure 6 *csn5^{null}* larvae display an excess of circulating hemocytes. (A, B) Micrographs through the body wall of living *csn5^{null}* larvae showing high numbers of circulating hemocytes (arrows), some of which are associated with melanotic cells. (C, D) Hemocytes in fixed blood samples from wt (C) and *csn5^{null}* (D) larvae. The small round cells are plasmatocytes and the large, flat cells are lamellocytes (black arrows). Nomarski optic images. (E) Graph showing the average number of circulating hemocytes per individual in both strains. Hemocyte numbers were determined as described in the Experimental procedures. Bars represent 50 μm .

Cactus and Dorsal, we suggest that abnormal hematopoiesis is the basis for the immune-challenge hypersensitivity of *csn5^{null}* larvae. A reasonable hypothesis for further experimentation would be that *csn5^{null}* larvae display abnormal phagocytosis leading to immune hypersensitivity.

Hemocyte numbers change in the course of normal larval development (Lanot *et al.* 2001), and as a result of immune stimulation (Rizki & Rizki 1992). Unchallenged *csn5^{null}* homozygous larvae have approximately twice as many hemocytes as the wt or heterozygous larvae of the same stage, suggesting that CSN5 functions as a repressor of hemocyte proliferation. Two different cell counting methods were used on independent samples to minimize intrinsic variability in hemocyte numbers sometimes noticed even for the same strain. As the strains analyzed were not isogenic, this caveat must be kept in mind. However, our conclusion is further supported by the finding that a proportion of *csn5^{null}* hemocytes remain mitotically active following their release into the hemolymph (Harari-Steinberg 2006). We thus propose that one function of CSN5 is to modulate hemocyte cell division preventing their over-proliferation during larval development (Fig. 8A).

CSN5 appears also to be necessary for proper hemocyte differentiation. In wt larvae, LM differentiation is normally repressed but rapidly induced as an immune response to parasites (Lanot *et al.* 2001; Sorrentino *et al.* 2004). LM differentiation is also induced during metamorphosis, when LM have been proposed to have a role in remodeling larval tissues (Rizki & Rizki 1978). Consequently, very few LM are observed in normal larvae prior to metamorphosis (Luo *et al.* 2002; Evans *et al.* 2003). The abnormally large numbers of LM in mutant larvae indicates that CSN5 normally represses their differentiation during larval life. Although it is generally unclear what seeds formation of melanotic capsules, it is clear that in our larval cultures the melanotic capsules generated within *csn5* mutant larvae are not a response to parasitism or penetration of other foreign bodies into their body cavity; hence, the encapsulation reaction in *csn5* mutant larvae may represent an immune response to self tissues. Furthermore, the physical association of LM with melanotic bodies from the earliest stage of melanization suggests a direct relationship between the abnormally high number of LM and the melanotic phenotype. At this stage, however, we cannot discern whether the increased numbers of LM are induced by the melanotic bodies or if melanization itself is initiated by the misregulation of LM differentiation.

Our microarray analysis indicates that hemocyte over-proliferation and differentiation phenotypes of *csn5^{null}* mutants are also reflected at the molecular level (Oron *et al.* 2005; Tuller *et al.* 2005). For example, *Pendulin*, a

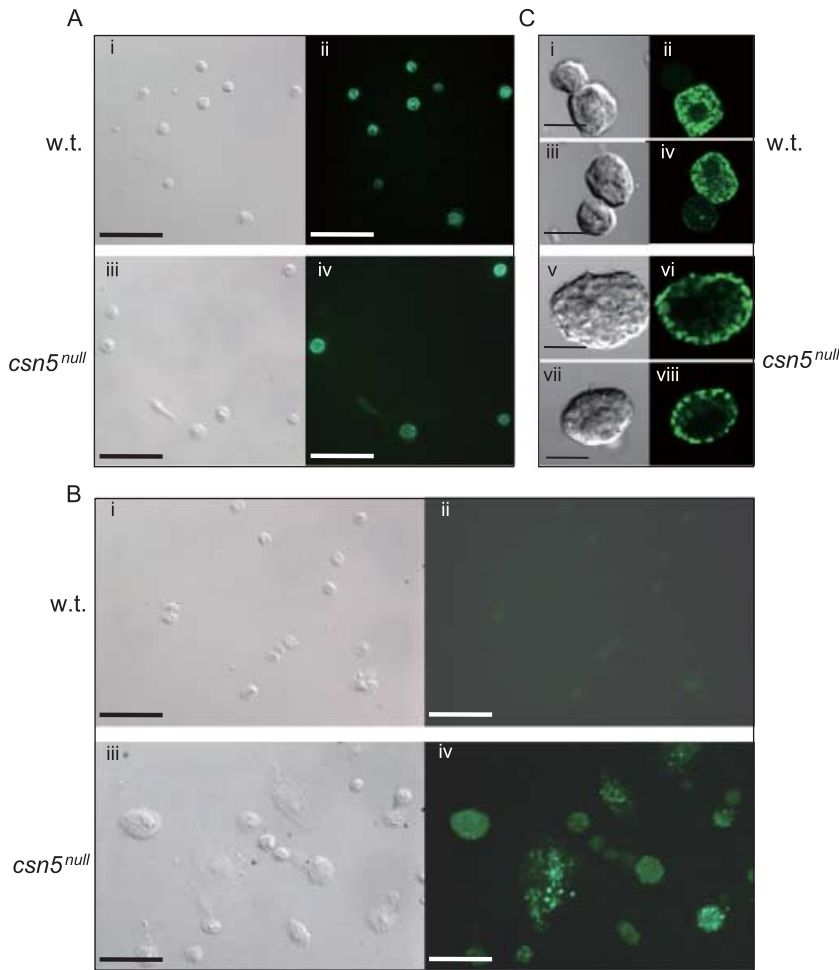


Figure 7 Hemocyte classes in *csn5^{null}* and wt larvae. Fixed hemocytes of wt and *csn5^{null}* larvae were stained with antibodies specific for plasmatocytes (A), lamellocytes (B) or crystal cells (C). Paired Nomarski (i, iii, v, vii) and fluorescence (ii, iv, vi, viii) images of individual crystal cells are shown wt (i–iv) and *csn5^{null}* (v–viii). Crystal cells from *csn5^{null}* are larger in the mutant (v: 17.27 × 22.78 μm; vii: 11.83 × 14.88 μm) than in wt larvae (i: 10.69 × 11.07 μm; iii: 10.69 × 12.02 μm). Bar represents 50 (A, B) or 10 (C) micron.

blood cell tumor suppressor (Kussel & Frasch 1995), is strongly down-regulated in *csn5^{null}*. A loss-of-function mutation of *pendulin* causes lethality, melanotic bodies formation, abnormal growth of larval tissues, enlarged lymph glands and over-proliferation of abnormal hemocytes (Kussel & Frasch 1995). These phenotypes resemble the phenotypes of *csn5^{null}* mutants suggesting that some of the hematopoietic phenotypes of *csn5^{null}* larvae are the result of the *pendulin* suppression.

On the other hand, the up-regulation of several genes involved in hematopoiesis may simply represent higher mRNA levels due to increased hemocyte numbers. For example, *Cg25C*, expressed in all hemocytes, is 3.5-fold increased in *csn5^{null}*. Similarly, transcript levels of *Dox-A3*, which is expressed specifically in LMs (Irving *et al.* 2005) are increased -fold in *csn5^{null}* mutants, reflecting the increased numbers of LMs in the mutant.

In humoral signaling, CSN5 appears to be essential for the steady-state regulation of Cactus and Dorsal in *Drosophila* larval fat body, but dispensable for Toll/IL-1

activation in fat body. Our data indicate that the same is probably true for the homologous pathway in mammalian cells. We propose a model in which CSN5 is involved in Cactus and Dorsal regulation at two levels (Fig. 8B).

First, CSN5 is a negative regulator of Cactus and Dorsal nuclear accumulation in the fat body in unchallenged conditions. The Cactus–Dorsal complex is mainly cytoplasmic in wt fat body cells under naive conditions but, in the absence of Csn5, both Cactus and Dorsal are found mainly in the nucleus. Nuclear localization of Cactus in wt larvae has been previously reported in larval brain cells in response to dark–light cycle (Cantera *et al.* 1999b) and also in somatic muscles of *dorsal* mutant larvae (Cantera *et al.* 1999a). However, while the subcellular distribution of Cactus has been extensively studied during embryogenesis and in relation to the immune response during larval and adult stages, nuclear accumulation of Cactus in fat body cells has not been reported so far. For lack of immunoprecipitating antibodies we could not directly confirm that Cactus and Dorsal remain bound to

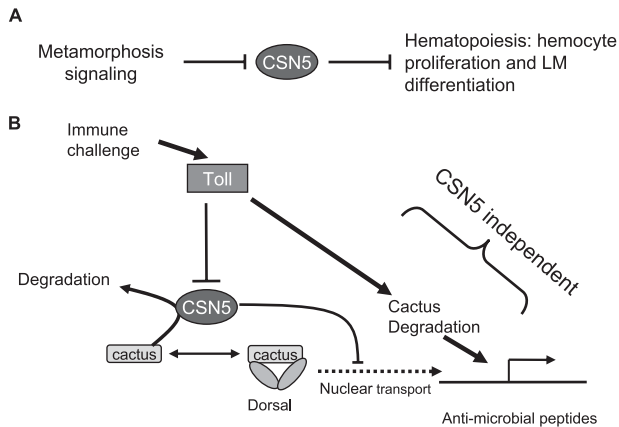


Figure 8 Model for the role of CSN5 in immune regulation. (A) CSN5 is a negative regulator of hematopoiesis. In the absence of a developmental or environmental signal, CSN5 represses hematopoiesis, maintaining low levels of circulating plasmatocytes and crystal cells, and repressing lamellocyte (LM) differentiation. Following the proper signal, hemocytes proliferate and some differentiate to LM. (B) CSN5 has two roles in humoral immune regulation: the over-accumulation of Cactus in the *csn5* mutants indicates that CSN5 has a positive role in promoting the degradation of Cactus, which we propose to be the “unbound” form, and a negative role on the nuclear accumulation of Cactus and Dorsal, as indicated by their nuclear localization in unchallenged *csn5* mutants. We propose that in wt larvae, immune-challenge releases the CSN5-mediated repression of Dorsal nuclear localization, and promotes CSN5-independent degradation of Cactus.

each other in the *csn5^{null}* fat body nuclei. However, our data support this hypothesis as Dorsal transcriptional activity on the *drosomyacin* gene in unchallenged larvae remains repressed, and Dorsal targets are not preferentially induced in *csn5^{null}* larvae, as shown by transcript profiling experiments (Oron *et al.* 2005; Tuller *et al.* 2005), despite nuclear localization of Dorsal. In mammals, I κ B α is also found in the nucleus, where it not only serves to transport NF κ B back to the cytoplasm, but also inhibits DNA binding of NF κ B (Arenzana-Seisdedos *et al.* 1995; Turpin *et al.* 1999). Furthermore, a recent study shows that nuclear I κ B α can also directly repress transcription (Aguilera *et al.* 2004). Our results add to the accumulating evidence which points to a repressive role for Cactus in the nucleus of larval fat body.

The mechanistic basis of the CSN5-dependent repression on Cactus–Dorsal nuclear localization is unclear, and indeed the nuclear localization of CSN5 might suggest that this regulation is indirect. However, a direct role involving either a cytoplasmic fraction of CSN5, or regulation of nuclear import or export, must also be considered. Consistently, CSN5 is essential for proper

subcellular localization of signaling molecules such as p27^{kip1} and COP1 (Chamovitz *et al.* 1996; Bianchi *et al.* 2000; Tomoda *et al.* 2002).

Second, CSN5 is a negative regulator of Cactus steady state levels. Neither RT-PCR analysis, nor microarray analysis, detected significant changes in *Cactus* transcript levels, indicating that the increase in Cactus protein observed in *csn5^{null}* larvae is due to post-transcriptional mechanisms. Previous studies on *Drosophila* embryos (Belvin *et al.* 1995) and human cells (Van Antwerp & Verma 1996) demonstrated an equilibrium between Dorsal/NF κ B-bound and unbound Cactus/I κ B α . Unbound Cactus/I κ B α is unstable and marked for protease-dependent degradation. This steady-state degradation is promoted by CK2 (Schwarz *et al.* 1996; Liu *et al.* 1997; Packman *et al.* 1997). As CK2 activity co-purifies with CSN (Uhle *et al.* 2003), we propose that CSN5 negatively regulates steady-state Cactus levels by promoting the immune signal-independent degradation of unbound Cactus (Fig. 8B).

It is surprising that CSN5 is not essential for immune-dependent degradation of Cactus as the most central role postulated for CSN5 is deneddylation of the Cullin subunit of the SCF ubiquitin ligase complex. This activity is necessary for controlled degradation of specific regulatory proteins (Cope & Deshaies 2003). Neddylation and the coordinated activity of SCF^{B-TrCP} towards I κ B α (Read *et al.* 2000) and p105 (Amir *et al.* 2002) control NF κ B transcriptional activity. The *Drosophila* homolog, SCF^{slimb}, was suggested to be required for Cactus degradation in embryos, but may be dispensable in Toll signaling in larval fat body (Khush *et al.* 2002; Leulier *et al.* 2003).

Given the importance of SCF complexes in promoting signal-dependent degradation of I κ B α (Read *et al.* 2000), and the regulation by CSN of SCF activity (Cope & Deshaies 2003), we had hypothesized that CSN5 would be required for signal-dependent degradation of Cactus. Unexpectedly, our findings show that while Cactus over-accumulates in the absence of CSN5, degradation of Cactus and activation of Dorsal/NF κ B in response to immune stimulation can still occur in the absence of CSN5. This suggests that SCF dependent degradation of Cactus does not require CSN5 under physiological conditions.

Signal-dependent activation of Dorsal/NF κ B classically requires both Cactus/I κ B α degradation and Dorsal/NF κ B nuclear localization. These two steps however can be uncoupled (Uv *et al.* 2000; Bhaskar *et al.* 2002). In *TPP* naive larva, Dorsal is constitutively nuclear and active even though Cactus remains at high levels in the cytoplasm (Nicolas *et al.* 1998). In *csn5^{null}* larvae, Cactus also accumulates to high levels, but co-localizes

with Dorsal in the nucleus, where Dorsal activity remains repressed in the absence of an immune signal. Consistently, microarray analysis did not reveal preferential induction of Dorsal targets in *csn5^{null}* mutants despite constitutive Dorsal nuclear localization.

Our results clearly indicate a role for CSN5 in mediating immune responses in *Drosophila* larvae. The phenotypes described could be the result of a loss of CSN5 as a component of the CSN complex, or the result of a loss of the CSN-independent forms of CSN5 (Oron *et al.* 2002). As additional available *csn* mutants (e.g. *csn4^{null}*, *csn5¹*) die at earlier stages before immune competence, this question could not be further pursued.

Experimental procedures

Flies

Growth conditions and strains are as in Oron *et al.* (2002). Egg depositions lasted for 4 h. All larvae were taken for analysis at 110 h after egg deposition (AED). *Drs-GFP* (Ferrandon *et al.* 1998) and *Dpt-GFP* (Tzou *et al.* 2000) flies were crossed to *csn5^{null}* background to create *Drs-GFP; csn5^{null}/TM3*, *Ser act-GFP* and *csn5^{null} Dpt-GFP/TM3*, *Ser act-GFP*, respectively.

Immune response and statistical analysis

Third instar larvae, 72 h AED, were challenged by pricking with a needle dipped in *E. coli* and *Micrococcus luteus* cultures, and collected 30 min, 60 min, 4 h, and 24 h post-infection for analysis of Cactus degradation, Dorsal localization, RNA expression, and viability, respectively. For the survival experiments, two controls were used, Canton-S, and heterozygote *Csn5⁺/csn5^{null}* siblings of the mutants. Relative risk value shows the ratio of the risk of death relative to exposure in the mutant versus the control group.

Immunohistochemistry

Larval fat bodies were dissected and immunostained as described (Cantera *et al.* 1999b). Primary antibodies: α -Dorsal (Gillespie & Wasserman 1994) at 1 : 1500; α -Cactus (Reach *et al.* 1996) at 1 : 1000; α -Lamin (Harel *et al.* 1989) at 1 : 2; α -CSN5 and α -CSN7 at 1 : 1500 (Freilich *et al.* 1999). Secondary antibodies conjugated to Cy2 or Cy3 (Jackson Laboratories) were used at 1 : 1000.

Hemocytes immunostaining

Pre-wandering stage larvae were washed in water, dried and the integument was disrupted in the latero-posterior region into a cold drop of PBS on a microscope glass slide. The cells were allowed to settle and adhere for 10 min and fixed for 10 min by addition of an equal volume of cold 4% paraformaldehyde, followed by four washes in PBS. Two hours blocking and over-night primary antibodies incubations were performed at 4 °C in buffer containing PBS, Triton-X, and 5% normal goat serum. The

following primary antibodies were used: α -CSN7 and α -CSN5 diluted 1 : 1500 (Freilich *et al.* 1999), monoclonal α -crystal cell, HC12F6 (Johansson *et al.* 2005) diluted 1 : 1, monoclonal α -plasmatocytes, P1 diluted 1 : 10, and monoclonal anti-lamellocytes, L1 (Kurucz *et al.* 2003) diluted 1 : 5. Secondary antibodies as above or FITC (Molecular Probe) at 1 : 250 in PBS for 2 h. The cells were then washed in PBS and mounted in 50% glycerol in PBS for microscopy.

Hemocytes counting

Circulating hemocytes were extracted from eight mutant larvae and eight wt larvae, of same chronological age, with wt at early wandering, in phosphate-buffered saline, fixed and total hemocyte numbers counted using a Zeiss Axioplan-2 microscope. Alternatively, circulating hemocytes were fixed as above, and random microscope frames of fixed cells from different larvae were counted.

Immunoblotting

A 100 μ g total protein was analyzed on 7.5% SDS-PAGE, transferred to PVDF membrane (Immobilon-P) and incubated with α -Cactus at 1 : 1000 dilution and α -CSN7 at 1 : 10 000.

Fluorescence microscopy and laser confocal microscopy

Epi-fluorescence imaging was performed using a Zeiss Axioplan-2 imaging fluorescence microscope equipped with an AxioCam cooled charge-coupled device camera, or with an Olympus SZX 12 fluorescent stereoscope equipped with an eGFP filter, equipped with a DVC-1310 color digital camera (DVC, Austin, TX, USA). Cy2/Cy3 double staining was viewed with a Zeiss LSM510 confocal microscope, imaging in multitracking mode using 488 and 543 nm laser lines with 545 nm dichroic filter, a 505–530 nm band pass and 560 nm long-pass filters. Image analysis was performed with Zeiss AxioVision, Zeiss LSM-5 image browser, and Adobe Photoshop 6.0.

RNA analysis

Total RNA was isolated using TRI REAGENT (MRC, Cincinnati, OH, USA). For Northern analysis, 10 μ g RNA were blotted on Hybond nylon filters (Amersham Pharmacia Biotech). Random-primed *Drs* and *rp49* probes were amplified with specific primers. For RT-PCR, SuperScript II Reverse transcriptase (Invitrogen) was used for first strand cDNA synthesis using oligo(dT) on 5 μ g RNA. A 1 : 200 dilution of cDNA was amplified for 25 (for *Cact*) or 20 (for *rp49*) cycles. These conditions were within the linear PCR phase. PCR primers surrounded introns.

Small interference RNA

The human CSN5 target sequence was cloned in the mammalian expression vector pSUPER. A scrambled CSN5 oligo was cloned in the same vector as a control.

Luciferase reporter assay

The 293T cells were transfected in triplicate samples with I κ B- κ -ConA-luciferase reporter gene CMV- β -galactosidase, and either pSUPER-scrambled iCSN5 or pSUPER-iCSN5 constructs using FuGENE6 reagent (Roche Molecular Biochemicals). TNF α stimulation (10 ng/mL) was performed on day 4. Luciferase activity was analyzed after 20 h in total cell lysates and normalized to β -galactosidase activity.

Acknowledgements

We thank R. Steward, S. Wasserman, Y. Gruenbaum, R. Weil, D. Agami, D. Ferrandon and J.-L. Imler for providing reagents and strains; R. Peri, Y. Amir and A. Bronstein for technical assistance; R. Elkon for promoter analysis; M. Mannervik, Y. Galanty and A. Orian for scientific input; A. Sharon, S. Yalovsky and N. Ohad for use of microscopes. This work was supported by grants from the Israel Science Foundation (519/00) to D.S. and D.A.C., from the Swedish Science Council to R.C. and from French ARC (3402) to E.B.

References

- Aguilera, C., Hoya-Arias, R., Haegeman, G., Espinosa, L. & Bigas, A. (2004) Recruitment of I κ B α to the hes1 promoter is associated with transcriptional repression. *Proc. Natl. Acad. Sci. USA* **101**, 16537–16542.
- Alkalay, I., Yaron, A., Hatzubai, A., Orian, A., Ciechanover, A. & Ben-Neriah, Y. (1995) Stimulation-dependent I κ B α phosphorylation marks the NF- κ B inhibitor for degradation via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA* **92**, 10599–10603.
- Amir, R.E., Iwai, K. & Ciechanover, A. (2002) The NEDD8 pathway is essential for SCF(β -TrCP)-mediated ubiquitination and processing of the NF- κ B precursor p105. *J. Biol. Chem.* **277**, 23253–23259.
- Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M.S., Bachelier, F., Thomas, D. & Hay, R.T. (1995) Inducible nuclear expression of newly synthesized I κ B α negatively regulates DNA-binding and transcriptional activities of NF- κ B. *Mol. Cell. Biol.* **15**, 2689–2696.
- Ausubel, F.M. (2005) Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol.* **6**, 973–979.
- Belvin, M.P., Jin, Y. & Anderson, K.V. (1995) Cactus protein degradation mediates *Drosophila* dorsal-ventral signaling. *Genes Dev.* **9**, 783–793.
- Bhaskar, V., Smith, M. & Courey, A.J. (2002) Conjugation of Smt3 to dorsal may potentiate the *Drosophila* immune response. *Mol. Cell. Biol.* **22**, 492–504.
- Bianchi, E., Denti, S., Granata, A., Bossi, G., Geginat, J., Villa, A., Rogge, L. & Pardi, R. (2000) Integrin LFA-1 interacts with the transcriptional co-activator JAB1 to modulate AP-1 activity. *Nature* **404**, 617–621.
- Brennan, C.A. & Anderson, K.V. (2004) *Drosophila*: the genetics of innate immune recognition and response. *Annu. Rev. Immunol.* **22**, 457–483.
- Cantera, R., Kozlova, T., Barillas-Mury, C. & Kafatos, F.C. (1999a) Muscle structure and innervation are affected by loss of Dorsal in the fruit fly, *Drosophila melanogaster*. *Mol. Cell. Neurosci.* **13**, 131–141.
- Cantera, R., Roos, E. & Engstrom, Y. (1999b) Dif and cactus are colocalized in the larval nervous system of *Drosophila melanogaster*. *J. Neurobiol.* **38**, 16–26.
- Chamovitz, D.A., Wei, N., Osterlund, M.T., von Arnim, A.G., Staub, J.M., Matsui, M. & Deng, X.W. (1996) The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch. *Cell* **86**, 115–121.
- Cope, G. & Deshaies, R.J. (2003) COP9 signalosome: a multifunctional regulator of SCF and other cullin-based ubiquitin ligases. *Cell* **114**, 663–671.
- Deng, X.W. & Serino, G. (2003) The COP9 signalosome: regulating plant development through the control of proteolysis. *Annu. Rev. Cell Dev. Biol.* **19**, 261–286.
- Evans, C.J., Hartenstein, V. & Banerjee, U. (2003) Thicker than blood: conserved mechanisms in *Drosophila* and vertebrate hematopoiesis. *Dev. Cell* **5**, 673–690.
- Ferrandon, D., Jung, A.C., Criqui, M., Lemaitre, B., Uttenweiler-Joseph, S., Michaut, L., Reichhart, J. & Hoffmann, J.A. (1998) A drosomycin-GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependent on the Toll pathway. *EMBO J.* **17**, 1217–1227.
- Freilich, S., Oron, E., Kapp, Y., Nevo-Caspi, Y., Orgad, S., Segal, D. & Chamovitz, D.A. (1999) The COP9 signalosome is essential for development of *Drosophila melanogaster*. *Curr. Biol.* **9**, 1187–1190.
- Fukumoto, A., Tomoda, K., Kubota, M., Kato, J.Y. & Yoneda-Kato, N. (2005) Small Jab1-containing subcomplex is regulated in an anchorage- and cell cycle-dependent manner, which is abrogated by ras transformation. *FEBS Lett.* **579**, 1047–1054.
- Georgel, P., Naitza, S., Kappler, C., Ferrandon, D., Zachary, D., Swimmer, C., Kopczynski, C., Duyk, G., Reichhart, J.M. & Hoffmann, J.A. (2001) *Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis. *Dev. Cell* **1**, 503–514.
- Gillespie, S.K. & Wasserman, S.A. (1994) Dorsal, a *Drosophila* Rel-like protein, is phosphorylated upon activation of the transmembrane protein Toll. *Mol. Cell. Biol.* **14**, 3559–3568.
- Harari-Steinberg, O. (2006) *The role of COP9 signalosome subunit 5 in the regulation of immune responses in the fruit fly Drosophila melanogaster*. Ph.D. Thesis, Tel Aviv University.
- Harel, A., Zlotkin, E., Nainudel-Epszteyn, S., Feinstein, N., Fisher, P.A. & Gruenbaum, Y. (1989) Persistence of major nuclear envelope antigens in an envelope-like structure during mitosis in *Drosophila melanogaster* embryos. *J. Cell Sci.* **94**, 463–470.
- Hong, X., Xu, L., Li, X., Zhai, Z. & Shu, H. (2001) CSN3 interacts with IKK γ and inhibits TNF- but not IL-1-induced NF- κ B activation. *FEBS Lett.* **499**, 133–136.
- Huang, L., Ohsako, S. & Tanda, S. (2005) The lesswright mutation activates Rel-related proteins, leading to overproduction of larval hemocytes in *Drosophila melanogaster*. *Dev. Biol.* **280**, 407–420.
- Hultmark, D. (2003) *Drosophila* immunity: paths and patterns. *Curr. Opin. Immunol.* **15**, 12–19.

- Imler, J.L. & Zheng, L. (2004) Biology of Toll receptors: lessons from insects and mammals. *J. Leukoc. Biol.* **75**, 18–26.
- Irving, P., Ubeda, J.M., Doucet, D., Troxler, L., Lagueux, M., Zachary, D., Hoffmann, J.A., Hetru, C. & Meister, M. (2005) New insights into *Drosophila* larval haemocyte functions through genome-wide analysis. *Cell. Microbiol.* **7**, 335–350.
- Johansson, K.C., Metzendorf, C. & Soderhall, K. (2005) Microarray analysis of immune challenged *Drosophila* hemocytes. *Exp. Cell Res.* **305**, 145–155.
- Khush, R.S., Cornwell, W.D., Uram, J.N. & Lemaitre, B. (2002) A ubiquitin-proteasome pathway represses the *Drosophila* immune deficiency signaling cascade. *Curr. Biol.* **12**, 1728–1737.
- Kurucz, E., Zettervall, C.J., Sinka, R., Vilmos, P., Pivarsci, A., Ekengren, S., Hegedus, Z., Ando, I. & Hultmark, D. (2003) Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **100**, 2622–2627.
- Kussel, P. & Frasch, M. (1995) Pendulin, a *Drosophila* protein with cell cycle-dependent nuclear localization, is required for normal cell proliferation. *J. Cell Biol.* **129**, 1491–1507.
- Lanot, R., Zachary, D., Holder, F. & Meister, M. (2001) Postembryonic hematopoiesis in *Drosophila*. *Dev. Biol.* **230**, 243–257.
- Lemaitre, B., Meister, M., Govind, S., Georgel, P., Steward, R., Reichhart, J.M. & Hoffmann, J.A. (1995) Functional analysis and regulation of nuclear import of dorsal during the immune response in *Drosophila*. *EMBO J.* **14**, 536–545.
- Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M. & Hoffmann, J.A. (1996) The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* **86**, 973–983.
- Leulier, F., Marchal, C., Miletich, I., Limbourg-Bouchon, B., Benarous, R. & Lemaitre, B. (2003) Directed expression of the HIV-1 accessory protein *Vpu* in *Drosophila* fat-body cells inhibits Toll-dependent immune responses. *EMBO Rep.* **4**, 976–981.
- Liu, Y., Schiff, M., Serino, G., Deng, X.-W. & Dinesh-Kumar, S.P. (2002) Role of SCF ubiquitin-ligase and the COP9 signalosome in the N gene-mediated resistance response to tobacco mosaic virus. *Plant Cell* **14**, 1483–1496.
- Liu, Z.P., Galindo, R.L. & Wasserman, S.A. (1997) A role for CKII phosphorylation of the cactus PEST domain in dorsoventral patterning of the *Drosophila* embryo. *Genes Dev.* **11**, 3413–3422.
- Luo, H., Rose, P.E., Roberts, T.M. & Dearolf, C.R. (2002) The Hopscotch Jak kinase requires the Raf pathway to promote blood cell activation and differentiation in *Drosophila*. *Mol. Genet. Genomics* **267**, 57–63.
- Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N. & Deshaies, R.J. (2001) Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* **292**, 1382–1385.
- Manfrulli, P., Reichhart, J.M., Steward, R., Hoffmann, J.A. & Lemaitre, B. (1999) A mosaic analysis in *Drosophila* fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. *EMBO J.* **18**, 3380–3391.
- Meister, M., Hetru, C. & Hoffmann, J.A. (2000) The antimicrobial host defense of *Drosophila*. *Curr. Top. Microbiol. Immunol.* **248**, 17–36.
- Minakhina, S. & Steward, R. (2006) Melanotic mutants in *Drosophila*: pathways and phenotypes. *Genetics* **174**, 253–263.
- Nicolas, E., Reichhart, J.M., Hoffmann, J.A. & Lemaitre, B. (1998) *In vivo* regulation of the I κ B homologue cactus during the immune response of *Drosophila*. *J. Biol. Chem.* **273**, 10463–10469.
- Nurnberger, T., Brunner, F., Kemmerling, B. & Piater, L. (2004) Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol. Rev.* **198**, 249–266.
- Oron, E., Mannervik, M., Rencus, S., Harari-Steinberg, O., Neuman-Silberberg, S., Segal, D. & Chamovitz, D.A. (2002) COP9 signalosome subunits 4 and 5 regulate multiple pleiotropic pathways in *Drosophila melanogaster*. *Development* **129**, 4399–4409.
- Oron, E., Tuller, T., Lee, L., Rencus, S., Edgar, B.A., Segal, D., Chor, B. & Chamovitz, D.A. (2005) Elucidating the developmental roles of the COP9 signalosome in *Drosophila melanogaster*. In: *46th Annu. Dros. Res. Conf.* (San Diego), pp. 462C.
- Packman, L.C., Kubota, K., Parker, J. & Gay, N.J. (1997) Casein kinase II phosphorylates Ser468 in the PEST domain of the *Drosophila* I κ B homologue cactus. *FEBS Lett.* **400**, 45–50.
- Qiu, P., Pan, P.C. & Govind, S. (1998) A role for the *Drosophila* Toll/Cactus pathway in larval hematopoiesis. *Development* **125**, 1909–1920.
- Reach, M., Galindo, R.L., Towb, P., Allen, J.L., Karin, M. & Wasserman, S.A. (1996) A gradient of cactus protein degradation establishes dorsoventral polarity in the *Drosophila* embryo. *Dev. Biol.* **180**, 353–364.
- Read, M.A., Brownell, J.E., Gladysheva, T.B., Hottelet, M., Parent, L.A., Coggins, M.B., Pierce, J.W., Podust, V.N., Luo, R.S., Chau, V. & Palombella, V.J. (2000) Ned8 modification of cul-1 activates SCF β TrCP-dependent ubiquitination of I κ B α . *Mol. Cell. Biol.* **20**, 2326–2333.
- Rizki, T.M. (1978) The circulatory system and associated cells and tissues. In: *The Genetics and Biology of Drosophila* (eds M. Ashburner & T. R. F. Wright), pp. 397–452. New York: Academic Press.
- Rizki, T.M. & Rizki, R.M. (1978) Larval adipose tissue of homoeotic bithorax mutants of *Drosophila*. *Dev. Biol.* **65**, 476–482.
- Rizki, T.M. & Rizki, R.M. (1992) Lamellocyte differentiation in *Drosophila* larvae parasitized by *Leptopilina*. *Dev. Comp. Immunol.* **16**, 103–110.
- Schwarz, E.M., Van Antwerp, D. & Verma, I.M. (1996) Constitutive phosphorylation of I κ B α by casein kinase II occurs preferentially at serine 293: requirement for degradation of free I κ B α . *Mol. Cell. Biol.* **16**, 3554–3559.
- Seeger, M., Kraft, R., Ferrell, K., Bech-Otschir, D., Dumdey, R., Schade, R., Gordon, C., Naumann, M. & Dubiel, W. (1998) A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. *FASEB J.* **12**, 469–478.
- Shrestha, R. & Gateff, E. (1982) Ultrastructure and cytochemistry of the cell types in the larval hematopoietic organs and hemolymph of *Drosophila melanogaster*. *Dev. Growth Differ.* **24**, 65–82.
- Sorrentino, R.P., Melk, J.P. & Govind, S. (2004) Genetic analysis of contributions of dorsal group and JAK-Stat92E pathway

- genes to larval hemocyte concentration and the egg encapsulation response in *Drosophila*. *Genetics* **166**, 1343–1356.
- Tomoda, K., Kubota, Y., Arata, Y., Mori, S., Maeda, M., Tanaka, T., Yoshida, M., Yoneda-Kato, N. & Kato, J.Y. (2002) The cytoplasmic shuttling and subsequent degradation of p27Kip1 mediated by Jab1/CSN5 and the COP9 signalosome complex. *J. Biol. Chem.* **277**, 2302–2310.
- Tuller, T., Oron, E., Makavy, E., Chamovitz, D.A. & Chor, B. (2005) Time-window analysis of developmental gene expression data with multiple genetic backgrounds. In: *Algorithms in Bioinformatics* (eds R. Casadio & G. Myers), pp. 53–64. Berlin: SpringerVerlag.
- Turpin, P., Hay, R.T. & Dargemont, C. (1999) Characterization of I κ B α nuclear import pathway. *J. Biol. Chem.* **274**, 6804–6812.
- Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J.M., Lemaitre, B., Hoffmann, J.A. & Imler, J.L. (2000) Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. *Immunity* **13**, 737–748.
- Uhle, S., Medalia, O., Waldron, R., Dumdey, R., Henklein, P., Bech-Otschir, D., Huang, X., Berse, M., Sperling, J., Schade, R. & Dubiel, W. (2003) Protein kinase CK2 and protein kinase D are associated with the COP9 signalosome. *EMBO J.* **22**, 1302–1312.
- Uv, A.E., Roth, P., Xylourgidis, N., Wickberg, A., Cantera, R. & Samakovlis, C. (2000) Members only encodes a *Drosophila* nucleoporin required for rel protein import and immune response activation. *Genes Dev.* **14**, 1945–1957.
- Van Antwerp, D.J. & Verma, I.M. (1996) Signal-induced degradation of I κ B α : association with NF- κ B and the PEST sequence in I κ B α are not required. *Mol. Cell. Biol.* **16**, 6037–6045.
- Wasserman, S.A. (2000) Toll signaling: the enigma variations. *Curr. Opin. Genet. Dev.* **10**, 497–502.
- Wu, L.P. & Anderson, K.V. (1998) Regulated nuclear import of Rel proteins in the *Drosophila* immune response. *Nature* **392**, 93–97.

Received: 21 September 2006

Accepted: 1 November 2006

Intentionally Left Blank