Development 129, 4399-4409 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV5043

COP9 signalosome subunits 4 and 5 regulate multiple pleiotropic pathways in *Drosophila melanogaster*

Efrat Oron[1,*], Mattias Mannervik[2], Sigal Rencus[3,*], Orit Harari-Steinberg[1], Shira Neuman-Silberberg[4], Daniel Segal[3,†] and Daniel A. Chamovitz[1,†]

[1]Department of Plant Sciences, Tel Aviv University, Tel Aviv 69978, Israel
[2]Department of Developmental Biology, Wenner-Gren Institute, Stockholm University, S-106 91 Stockholm, Sweden
[3]Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv 69978, Israel
[4]Molecular Genetics of Development, Faculty of Health Sciences, Ben-Gurian University of the Neger, Beer-Sheva 84105, Israel
*These authors contributed equally to this manuscript
†Authors for correspondence (e-mail: dannyc@tauex.tau.ac.il and dsegal@post.tau.ac.il)

Accepted 17 June 2002

SUMMARY

The COP9 signalosome (CSN) is an essential eight-subunit repressor of light-regulated development in *Arabidopsis*. This complex has also been identified in animals, though its developmental role remains obscure. CSN subunits have been implicated in various cellular processes, suggesting a possible role for the CSN as an integrator of multiple signaling pathways. In order to elucidate the function of the CSN in animals, a *Drosophila* model system has previously been established. Gel-filtration analysis with antibodies against CSN subunits 4, 5 and 7 revealed that these proteins act as a complex in *Drosophila* that is similar in size to the plant and mammalian complexes. Null mutations in either one of two subunits, CSN4 or CSN5, are larval lethal. Successful embryogenesis appears to be a consequence of maternal contribution of the complex. Biochemical analysis indicates that the different subunits are found in both CSN-dependent and CSN-independent forms, and that these forms are differentially affected by the mutations. Phenotypic characterization of these two mutants indicates that they show both shared and unique phenotypes, which suggest specific roles for each subunit. Both mutants have defective oocyte and embryo patterning, and defects in response to DNA damage, while *csn5* mutants develop melanotic tumors and *csn4* mutants have phenotypes reminiscent of defects in ecdysone signaling.

Key words: COP9 signalosome, Maternal effect, Embryo development, Oogenesis, *Drosophila*

INTRODUCTION

The COP9 signalosome (CSN) is an evolutionarily conserved multi-subunit protein complex that was originally identified in *Arabidopsis* as an essential master regulator of light-mediated development (Chamovitz et al., 1996). The CSN contains eight core subunits termed CSN1-CSN8 in order of descending molecular mass. In plants and animals, all of the subunits are found in a ~450 kDa protein complex (Deng et al., 2000). Some of the subunits are also found in smaller molecular mass forms that are independent of the COP9 signalosome (Freilich et al., 1999; Karniol et al., 1999; Kwok et al., 1998; Serino et al., 1999). For example, the complexed form of AtCSN5 is nuclear while the complex-independent form is cytoplasmic (Kwok et al., 1998). However the biological significance of these forms, and of those of other subunits, is still poorly understood. The CSN is related to two other multi-protein complexes, the regulatory lid of the 26S proteasome and eIF3, and evidence from several studies suggests a direct interaction between the complexes or their subunits (Glickman et al., 1998; Kwok et al., 1999; Wei et al., 1998; Yahalom et al., 2001).

The different CSN subunits interact with a variety of important signaling molecules, such as $p27^{kip}$, Jun, Vpr, MIF and the thyroid receptor, suggesting that the CSN functions at the juncture of kinase signaling and ubiquitin-dependent protein degradation (reviewed by Chamovitz and Segal, 2001; Kim et al., 2001; Schwechheimer and Deng, 2001). Consistent with this hypothesis, a CSN-associated kinase phosphorylates p53 to regulate its degradation by the proteasome (Bech-Otschir et al., 2001). It appears that substrates of the CSN kinase activity must bind to one of the CSN subunits in order to be phosphorylated. CSN5 for example, recruits Jun and p53 to the CSN, whereas phosphorylation of the interferon consensus sequence binding protein (ICSBP) requires binding to CSN2 (Bech-Otschir et al., 2001; Cohen et al., 2000; Naumann et al., 1999).

The CSN also has a direct link to ubiquitin-dependent protein degradation. The CSN regulates the SCF E3 ubiquitin-ligase complex by mediating the removal of the NEDD8 modification of the SCF cullin subunit (Lyapina et al., 2001). Cells of the fission yeast *Schizosaccharomyces pombe* deleted for SpCSN1 showed an accumulation of neddylated cullin, accompanied by a significant increase of SCF activity. Addition of purified mammalian CSN lead to a reduction in the

level of neddylated cullins. Furthermore, studies in *Arabidopsis* showed that the SCF mediated degradation of PSIAA6, a regulatory protein of the auxin response pathway, is compromised in CSN5-deficient plants (Schwechheimer et al., 2001). The exact role of the COP9 signalosome in these processes is still unclear.

While the CSN has been studied in vivo in *Arabidopsis*, with a few notable exceptions, most studies in animal systems have been limited to cell culture assays involving ectopic overexpression of CSN components. These studies have thus largely ignored the function of the COP9 signalosome as a whole, and have concentrated on individual subunits. To develop a system for studying the roles of the CSN in animal development, we have been using *Drosophila* as a model (Freilich et al., 1999). The *Drosophila* COP9 signalosome is highly conserved with its plant and mammalian counterparts. We have previously shown that CSN5 is essential for *Drosophila* development.

To further understand the role of the CSN and its subunits in *Drosophila* development, we have generated null mutations in two CSN subunits, CSN4 and CSN5, and examined the effect of these mutations on *Drosophila* development. Our results indicate that each subunit has common and unique functions that affect a variety of developmental processes, including oogenesis, embryo patterning, hematopoesis and molting.

MATERIALS AND METHODS

Antibody production

The entire coding region of CSN4 was cloned into pGEX 4t-1 (Pharmacia, Upsalla, Sweden) at the *Sal*I and *Not*I restriction sites. The resulting glutathione S-transferase-DCH4 fusion protein was over-produced in *Escherichia coli* strain BL-21 and found to be insoluble. The 12,000 ***g*** centrifugation pellet containing the fusion protein in inclusion bodies was separated on a 12.5% SDS-PAGE, and the fusion protein excised and injected either as a polyacrylamide suspension, or blotted and as pulverized nitrocellulose, to immunize rabbits (AniLab, Rehovot, Israel) for the production of polyclonal antibodies. For affinity purification of the resulting serum, the GST-CSN4 fusion protein was immobilized on a PVDF membrane and reacted with total sera. Antibodies were eluted from the membrane according to standard procedure.

Gel-filtration chromatography

Total soluble protein (200 μg) was applied to a Superose 6HR gel filtration column and eluted with gel filtration buffer at rate of 0.3 ml/minute (Freilich et al., 1999). The eluent was collected in 0.5 ml fractions starting from onset of the void volume. The fractions were concentrated with StrataClean resin beads (Stratagene). The concentrated samples were analyzed on SDS-PAGE followed by standard protein gel blot analyses.

D. melanogaster maintenance and hybrid dysgenesis

Canton-S (CS) served as the wild-type control. Strains were maintained and crosses were conducted on cornmeal-molasses medium at 25°C. Description of balancer chromosomes and markers can be found in FlyBase (The Fly Consortium, 1999) (http://flybase.bio.indiana.edu/). The CyO balancer that carries the *GFP* transgene is described elsewhere (Reichhart and Ferrandon, 1998). The TM3 *kr*-GFP balancer is described elsewhere (Casso et al., 1999).

The P element in the 10765 strain is inserted within the coding sequence of Csn4. Analysis of putative excision lines was performed with a primer derived from genomic sequences 5′ to the P element insertion (5′-TGCTTCCCATATGTAGCCACTT-3′) corresponding to nucleotides –2092 to –2070, where the P insertion site is at position 0, and a primer corresponding to CSN4 gene sequence +54 to +75 downstream of the P insertion site (5′-TGTTCG-TCAGCACAGTCTTCA-3′)

The P element in the 10765 strain carries the dominant *w+* eye color marker. Reciprocal crosses of the 10765 strain and a strain carrying the Δ2-3 stable source of transposase were conducted. Dysgenic *y w; l(2)K08018/CyO P[Δ 2-3*] male progeny, in whose germ line the *l(2)K08018*-associated P element was mobilized, were crossed to *y w; Gla/CyO* females. Offspring (F_2) that did not carry the transposase source were screened for males with white eyes indicative of loss of the *w+* marker caused by excision the P element from 10765. Individual putative excision males were crossed to *y w; Gla/CyO* females to establish balanced lines. 330 F2 flies were scored for white eyes, suggestive of excision of this P element, and lines were established from 268 independent putative excision flies, 68 of which were homozygous viable.

The hybrid dysgenesis protocol for *csn5* has been previously described (Freilich et al., 1999). $csn5^{1}$ and $csn5^{3}$ have been described (Suh et al., 2002). $csn5^{null}$, $csn5^{p}$, $csn5^{1}$ and $csn5^{3}$ were balanced over a *TM3* chromosome carrying the green fluorescent protein (GFP) marker gene under the actin-5 promoter. $csn4^{null}$ was balanced over a *CyO* chromosome carrying the same GFP marker. $csn5^{null}$ was also balanced over the *TM3* chromosome carrying the GFP marker gene under the *kr* promoter.

Heat-shock treatment: flies of the desired cross were allowed to lay eggs for 24 hours in small tubes containing standard molasses medium. The larvae were heat-shocked at the second and third instar stages. Heat shock was administered by putting the vials with the larvae in a 37°C waterbath for 1.5 hours. Heat shock was performed twice a day on days 3 and 4 after egg laying. After the heat treatment the tubes were returned to 25°C.

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 on 470 nm. *Drosophila* larvae were immobilized for examination by placing them on ice for several minutes.

MMS treatment

First instar larvae were treated with 0.4% methyl methane sulfonate (MMS, Sigma) by feeding. After treatment, the larvae were examined for viability.

In situ analysis

Whole-mount in situ hybridization assays were performed on $csn5^{p}$ germline clone embryos with digoxigenin-labeled *snail*, *rhomboid*, *tolloid*, *knirps*, *Krüppel*, *giant*, *even-skipped* and *engrailed* antisense RNA probes as described previously (Jiang et al., 1991; Tautz and Pfeifle, 1989).

Ovaries were dissected in Ringer's solution on ice. Ovaries were fixed in 200 μl of 4% paraformaldehyde (PFA) in PBS, 20 μl DMSO and 600 μl heptane for 20 minutes. The ovaries were then briefly washed in PBS and mounted for observation. Staining was as previously described (Neuman-Silberberg and Schubach, 1993).

RESULTS

Generation of CSN4 and CSN5 null mutants

To elucidate the role(s) of the COP9 signalosome in *Drosophila* development, we took a genetic approach of

Table 1. Strains used

Strain	Molecular lesion	Protein present	Reference
csn4^{null}	Deletion of –375 to +55 relative to ATG of coding sequences	No	This work
csn5^{P}	P element insert 24 bp 5′ to the gene	Yes	Freilich et al., 1999
csn5^{null}	Deletion of 1st 1396 bp of coding sequence	No	Freilich et al., 1999, this work
csn5^{1}	E_{160}→V	Yes	Suh et al., 2002
csn5^{3}	T_{99}→I	Yes	Suh et al., 2002

analyzing mutants for the complex components. We have previously reported four alleles of *csn5*, *csn5^{P}*, *csn5^{1}*, *csn5^{3}* and *csn5^{null}* (Table 1) (Freilich et al., 1999; Suh et al., 2002). The *csn5^{null}* strain was isolated following imprecise P-element excision. *csn5^{null}* was further analyzed to ascertain the molecular basis of the null phenotype. Sequence analysis of the *Csn5* gene in this strain revealed a deletion from nucleotides –87 to 1396, where 1 is defined as the first nucleotide in the start codon, resulting in a deletion of the entire coding sequence, and not disrupting other known coding sequences. This *csn5^{null}* is distinct from those previously published [*CSN5^{N}* (Suh et al., 2002); *dch5-3* (Freilich et al., 1999)].

The 10765 strain contains a P element located in polytene bands 44A1-2, in dmCSN4 (The Fly Consortium, 1999). The chromosome carrying this insert is homozygous lethal and the lethality is not complemented by the chromosomal deletion Df(2R)CA53, which removes bands 43E6-44B6. Southern and PCR analysis confirmed that the single P element in the genome of the 10765 strain is inserted 55 bp downstream of the first ATG codon in the *Csn4* cDNA (not shown).

To verify that the lethality of the 10765-carrying chromosome is due to the P insertion in *Csn4*, the P element was mobilized through a hybrid dysgenesis protocol of crosses. Lines were established from 268 independent putative excision flies, and 68 of them were homozygous viable. PCR analysis on three of these homozygous viable lines indicated a normal size *Csn4* gene, and sequencing of one of the PCR products confirmed the excision of the P element and restoration of the *Csn4* gene sequence. These results demonstrate that the P insert in *Csn4* of the 10765 strain is the cause of the homozygous lethality of the chromosome carrying it. Thus, normal function of *Csn4* is essential for *Drosophila* development.

To further aid in our analysis, we have examined the homozygous lethal excision lines that were obtained in the hybrid dysgenesis. The mutant lines were balanced over a chromosome containing the gene for GFP. PCR analysis on individual homozygous mutant larvae (identified as non-GFP, see Materials and Methods) suggested a deletion of *Csn4*. Sequencing of one of these lines, called *csn4^{null}*, confirmed the deletion of base pairs –375 to +55 in the *Csn4* gene, where 1 is defined as the first nucleotide in the start codon, resulting in a true null allele. This was confirmed by immunoblot analysis on individual non-GFP larvae from this strain (not shown).

csn4^{null} and *csn5^{null}* mutants are larval lethal

To understand the roles of the CSN and its individual subunits in *Drosophila* development, the lethal phases of the mutants were analyzed. Homozygous mutant (i.e. non-GFP) embryos of *csn4^{null}* and *csn5^{null}* hatch normally and proceed through first and second larval instars without any apparent derailment

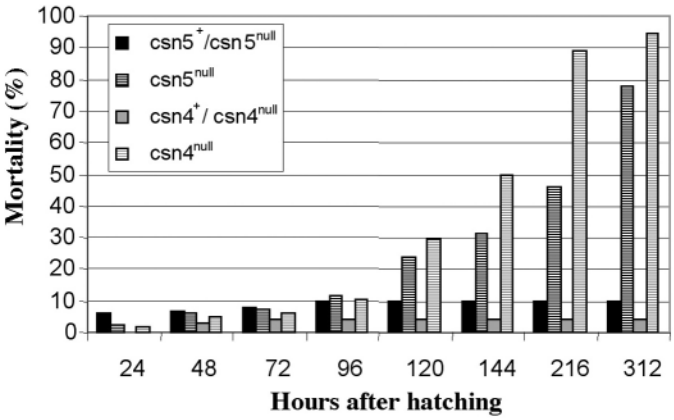

Fig. 1. Lethality of *csn4^{null}* and *csn5^{null}* mutants. Mutant homozygous individuals, identified as non-GFP, and non-mutant heterozygote siblings, identified as GFP positive, were scored for viability each day following laying. *n*=120 for each strain.

(Fig. 1). The development of *csn5^{null}* larvae appears to parallel that of the wild type, as the third instar is reached at the same time. However, during subsequent development, while 100% of the GFP-positive (i.e. heterozygous) siblings pupate, the *csn5^{null}* siblings remain in a prolonged third instar. Increased lethality in *csn5^{null}* is evident by 5 days after hatching, with most of the larvae dying by 13 days post-hatching. *csn4^{null}* larvae show increased lethality earlier than *csn5^{null}*. The third instar larvae remain much smaller than their GFP-positive siblings or csn5^{null} third instar larvae.

The CSN is essential for oogenesis

While the above analyses indicate that the CSN is necessary for larval development, given the wide range of proposed functions for the CSN, it is somewhat surprising that development is not derailed earlier than the larval stages in the *csn^{null}* strains. One explanation for this could be that the CSN and its subunits are maternally deposited. To check this possibility, non-fertilized wild-type eggs were analyzed by immunoblot analysis. CSN5 and CSN7 were detected in the unfertilized eggs, indicating that CSN components are maternally contributed. Gel filtration analysis demonstrated that CSN subunits exist in unfertilized eggs in a ~500 kDa complex, and also in smaller forms indicating that the CSN complex is maternally deposited (Fig. 2). To determine when the maternally deposited proteins are depleted, the *csn5^{null}* chromosome was balanced over a *kr*-GFP carrying chromosome, which allows the identification of non-GFP embryos in late embryogenesis (see Materials and Methods). Western blot analysis showed that *csn5^{null}* homozygous embryos in late stages of embryogenesis (identified as non-

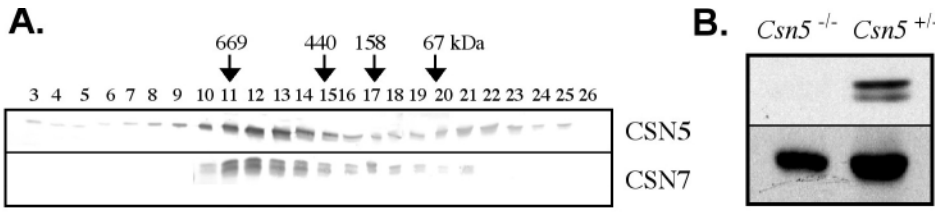

Fig. 2. The CSN is maternally deposited and is present until late embryogenesis. (A) Unfertilized eggs from virgin wild-type females were homogenized in gel-filtration buffer and total soluble protein was fractionated over a Superose 6 gel-filtration column (Pharmacia). Fractions (0.5 ml) were examined for the presence of CSN5 or CSN7 as indicated by immunoblot analysis with anti-CSN5 or anti-CSN7 affinity-purified antibodies. Positions of size markers are shown. (B) Proteins from *csn5*^{null} late-stage embryos (identified as non-kr-GFP) and heterozygote siblings (identified as kr-GFP positive) were analyzed for CSN5 and CSN7 as above.

GFP) contain CSN7, but no CSN5, while the GFP-positive siblings contain both proteins (Fig. 2B). This indicates that the maternal contribution of the CSN is depleted by late embryogenesis, and that the *csn5*^{null} larvae are thus truly null mutants.

As the maternally contributed CSN could allow for the prolonged development of the mutants, we eliminated this effect in order to reveal if the CSN has a role in early *Drosophila* development. To achieve this, we generated germline clones homozygous for *csn5*^{null} or *csn4*^{null} in otherwise heterozygous females via FLP-FRT mediated mitotic recombination. No embryos were derived from germline clones homozygous for either *csn5*^{null} or *csn4*^{null} mutations, indicating that both CSN4 and CSN5, and by association the COP9 signalosome, are necessary for oogenesis.

This was confirmed by dissection of ovaries from the females carrying the germline clones (Fig. 3). As expected, ovaries of 3-day-old positive control females (both CS and *w*/*w* hs-*FLP*; FRT *csn4*$^{+}$/FRT *OVO*^{D}) were fully developed (Fig. 3D,E), whereas in ovaries dissected from negative control females, carrying the *OVO*^{D} mutation, of the same age, oogenesis was found to be arrested at stage 4 (Fig. 3H,I). However, ovaries of females carrying germline clones of the *csn4*^{null} deletion arrest later, at stage 6 of oogenesis (Fig. 3B,F,J). Ovaries of females carrying germline clones of the *csn5* gene, either the *csn5*^{null} deletion (Fig. 3G,K) or the *csn5*^{1} point mutation (not shown), arrest at stage 5-6 of oogenesis. No mature egg chambers were seen in ovaries from these three germline clones. Homozygous germline clones were also generated for the *csn5*^{p} allele. This allele is generally weaker than the null allele, with ovaries containing more developed egg chambers, with few oocytes developing further into embryos (Fig. 3C,L). Thus, both CSN4 and CSN5 are essential for successful oogenesis.

csn5^{p} embryos are defective in embryo patterning

The *csn5*^{p} homozygous embryos derived from germline clones were further analyzed for changes in gene expression patterns to further examine the role of the CSN in embryo development. Genes involved in specifying cell fates along the dorsoventral and anteroposterior axes were examined. Dorsoventral patterning is dictated by the Dorsal transcription factor, which forms a concentration gradient along the dorsoventral axis. The *snail* gene is activated in ventral cells where the concentration of the Dorsal transcription factor is the highest. In mutant embryos derived from *csn5*$^{p/p}$ germline clones, the sharp on/off border of *snail* gene expression is maintained, but the pattern has changed so that a wavy border that expands dorsally is formed instead of the straight border observed in wild-type embryos (Fig. 4A,B). Therefore, CSN5 is probably involved in spatially localizing signals that pattern the oocyte, consistent with a requirement of *Csn5* for oogenesis (see above).

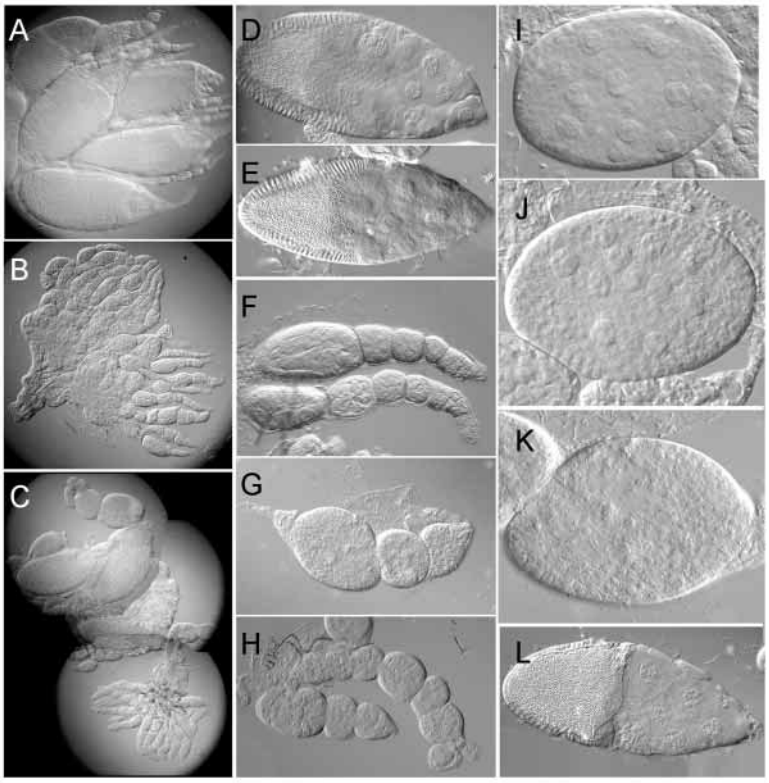

Fig. 3. CSN4 and CSN5 are essential for oogenesis. Ovaries (A-C), egg chambers (F-H), and the most developed stage oocytes (D,E,I-L) are shown for the following genotypes: wild type (A,D); *w*/*w* hs-*FLP*; FRT *csn4*$^{+}$/FRT *OVO*^{D}, heat-shocked (E); *w*/*w* hs-*FLP*; FRT *csn4*^{null}/FRT OVO^{D}, non heat-shocked (H,I); *w*/*w* hs-*FLP*; FRT *csn4*^{null}/FRT *OVO*^{D}, heat-shocked (B,F,J); *w*/*w* hs-*FLP*; FRT *csn5*^{null}/FRT *OVO*^{D}, heat-shocked (G,K); *w*/*w* hs-*FLP*; *csn5*^{p}/FRT *OVO*^{D}, heat-shocked (C,L). (A,D) Wild-type control; (E) negative control showing that heat shock does not effect oogenesis; (H,I) *OVO*^{D}-derived oocytes. (B,C,F,G,J-L) Germline clones of *csn4*^{null} (B,F,J) *csn5*^{null} (G,K) and *csn5*^{p} (C,L). *csn5*^{null} ovaries are essentially indistinguishable from *csn4*^{null} ovaries and are not shown. Staging of oocytes was according to position in ovariole.

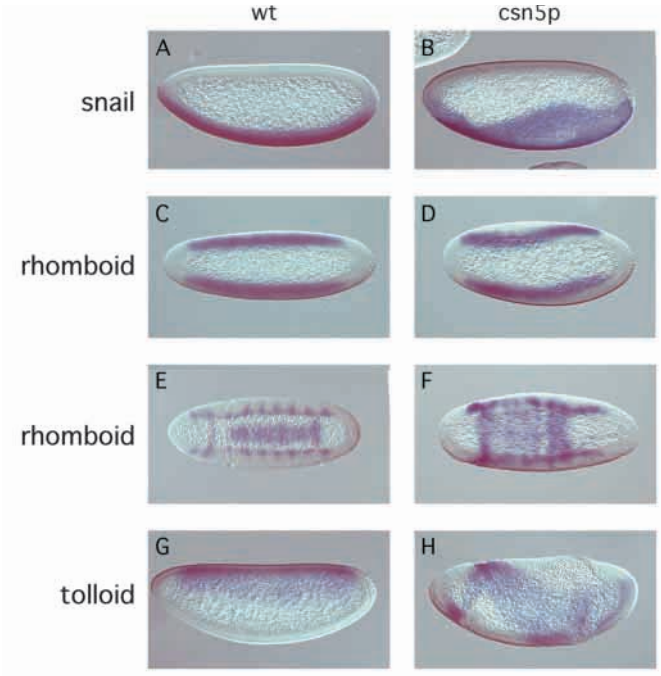

Fig. 4. Dorsoventral patterning defects in *csn5^p* mutant embryos. Embryos were hybridized with digoxigenin-labeled antisense RNA probes and are oriented with dorsal upwards and anterior towards the left (A,B,G,H) except C-F, which are dorsal views. Csn5p mutant embryos were derived from csn5p germline clones. (A,B) Wild-type (wt) and mutant (*csn5^p*) embryos undergoing cellularization after hybridization with a *snail* probe. In the mutant embryo, the *snail* expression domain forms on the ventral side as in wild type, but has a wavy border. (C,D) Cellularizing wild-type and mutant embryos after hybridization with a *rhomboid* probe. *rhomboid* is expressed in two lateral stripes in the presumptive neuroectoderm. In the mutant embryo, the two lateral stripes are wavy. (E,F) Wild-type and mutant embryo at the onset of gastrulation hybridized with the *rhomboid* probe. In addition to the two lateral stripes, *rhomboid* is expressed in the dorsal ectoderm in response to the *dpp* signaling molecule at this stage. In the *csn5^p* mutant embryo, the dorsal ectoderm staining is expanded, suggesting that CSN5 normally restricts *dpp* signaling. (G,H) Cellularizing wild-type and mutant embryos hybridized with a *tolloid* probe. *tolloid* expression is normally excluded from the ventral side of the embryo by the Dorsal protein. In the mutant embryo, the pattern is severely distorted, with *tolloid* expression observed in parts of both the ventral and dorsal half of the embryo.

The *rhomboid* gene is activated in the lateral parts of the embryo in response to a lower threshold of Dorsal concentration, and repressed in ventral parts by the Snail protein. As can be seen in Fig. 4C,D, *rhomboid* is activated by Dorsal and repressed by Snail in the *csn5^p* mutant. However, the pattern is again wavy instead of straight. At later stages of embryogenesis, *rhomboid* is expressed in the dorsal ectoderm in response to the signaling molecule Decapentaplegic (Dpp). In *csn5^p* mutants, the late *rhomboid* pattern is expanded compared with wild type (Fig. 4E,F).

The Dorsal protein is a repressor of *tolloid* expression in ventral parts of the embryo. In the *csn5^p* mutant embryo, a severe distortion in the *tolloid* expression pattern is observed that appears to represent a shift of the dorsoventral axis towards the anteroposterior (Fig. 4H). These data are consistent with a mislocalization of the Dorsal gradient in *csn5^p* mutant embryos, leading to misexpression of Dorsal target genes.

Expression of genes that establish segmentation of the embryo along the anteroposterior axis was examined in *csn5^p* mutants. The gap genes *knirps* and *giant* are among the first genes to be expressed in the embryo and are dependent on the Bicoid and Hunchback protein gradients. In *csn5^p* mutant embryos, these gap genes are expressed in a pattern that resembles wild type, except for distortions that make cells on

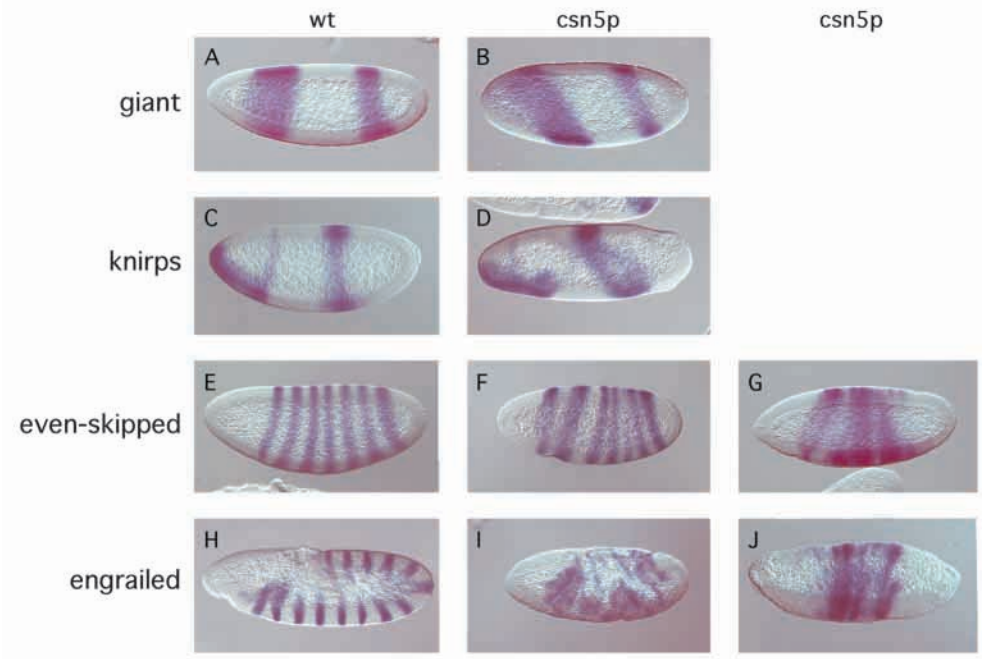

Fig. 5. Anteroposterior patterning in embryos derived from csn5p germline clones. Embryos, oriented with dorsal upwards and anterior towards the left, were hybridized with digoxigenin-labeled antisense RNA probes. (A,B) *giant* expression in wild-type (A) and mutant (B) embryo undergoing cellularization. In the mutant embryo, *giant* expression is tilted towards the anterior. (C,D) Cellularizing wild-type and mutant embryo after hybridization with a *knirps* probe. *knirps* expression is tilted towards the anterior in the mutant embryo. (E-G) *even-skipped* expression in wild-type and mutant embryos undergoing cellularization. The seven-stripe *even-skipped* pattern is altered to a variable extent in csn5p mutant embryos. The embryo in F has an expanded stripe 2 and diminished stripe 5, whereas the embryo in G is more severely affected. (H-J) Embryos undergoing germband elongation hybridized with an *engrailed* probe. The mutant embryos in I,J have failed to undergo the cell movements of gastrulation. The normal *engrailed* pattern includes 14 stripes that extend along the length of the germband. In mutant embryos, a striped *engrailed* pattern can no longer be observed.

the dorsal and ventral side of the embryo different with respect to anteroposterior patterning (Fig. 5B,D). This result indicates that maternal factors specifying anteroposterior fates have also been mislocalized.

Expression of genes acting later in the segmentation hierarchy, such as the pair-rule gene *even-skipped* and the segment polarity gene *engrailed* display more severe and variable defects in $csn5^{p}$ mutant embryos. Fig. 5F,G shows that the *even-skipped* pattern ranges from mild defects such as an expanded stripe 2 and diminished stripe 5 expression, to severe defects where individual stripes can hardly be discerned. Similarly, the *engrailed* pattern is variable in $csn5^{p}$ mutants, with two examples shown in Fig. 5I,J. It can also be seen that $csn5^{p}$ mutant embryos cannot undergo the cell movements required for gastrulation. For example, the embryo shown in Fig. 5J fails to undergo germband extension. The more severe defects in expression of genes downstream of the gap genes, than in expression of the gap genes themselves, suggests that CSN5 has an essential role in the embryo as well as in oogenesis.

Oocyte polarity is disrupted in *csn4* and *csn5* germ line clones

The defects in early embryo patterning described above suggest defects in patterning in the oocyte. To identify the specific processes in which CSN4 and CSN5 take part during oogenesis, the expression pattern of genes known to participate in determination of the embryonic axes was examined by in situ hybridization to the ovaries carrying germline clones of *csn4* or *csn5* mutations. Both CS ovaries and ovaries carrying germline clones of a wild type chromosome (*w*/*w* hs-*FLP*; FRT $csn4^{+}$/FRT OVO^{D}, following heat shock treatment) were used as wild-type positive controls in these experiments. The non-heat-shocked females (*w*/*w* hs-*FLP*; FRT $csn4^{null}$/FRT OVO^{D} and *w*/*w* hs-*FLP*; FRT $csn5^{null}$/FRT OVO^{D}) were used as negative controls, as the staining pattern in these ovaries reflects the effect of the OVO^{D} mutation on the expression pattern of the examined mRNA.

The expression patterns of three genes known to be crucial for axis formation and fate determination were examined, *oskar*, *bicoid* and *gurken*. *oskar* is a determinant of the posterior fate of the embryo; *bicoid* is a determinant of the anterior fate of the embryo; and *gurken* is essential for determination of both the anteroposterior and dorsoventral axis. The specific expression pattern of all three genes in the oocyte effect embryo patterning (Cooperstock, 1999; Johnstone, 2001).

In wild-type egg chambers, *oskar* is expressed at the posterior end of the oocyte throughout oogenesis and sometimes in the nurse cells of egg chambers from stage 7 and onwards, as previously reported (Fig. 6A) (Kim-Ha, 1991). This expression pattern is not affected in ovaries containing germline clones of $csn4^{null}$ (Fig. 6B), suggesting that *Csn4* is not required for proper expression pattern of *oskar* mRNA. However, ovaries containing germline clones of $csn5^{null}$ show no *oskar* staining at all (Fig. 6C). Ovaries containing germline clones of $csn5^{1}$ also show no *oskar* expression, with the exception of a few egg chambers that show either wild type or mislocalized, more anterior, *oskar* staining pattern (not shown). Germline clones of $csn5^{P}$ show *oskar* staining similar to wild type, with the nurse cells staining being stronger and more prevalent compared with that seen in wild-type ovaries (Fig. 6D).

bicoid is localized in the positive control ovaries at the anterior part of the oocyte, as previously reported (Fig. 6E) (St Johnston, 1989). Ovaries containing germline clones of either the $csn4^{null}$ deletion or the $csn5^{null}$ deletion show no or very diffuse *bicoid* staining (Fig. 6F,G), which may indicate a role for both CSN4 and CSN5, and by extension, the COP9 signalosome, in the regulation of *bicoid* expression. A similar result is observed in ovaries containing germline clones of $csn5^{1}$ gene, with the exception of a few egg chambers that were stained in a pattern similar to wild type (not shown). However, ovaries containing germline clones of $csn5^{P}$ show an anterior staining pattern of *bicoid* that is indistinguishable from the wild-type pattern (Fig. 6H).

The *gurken* staining pattern is the most complex, as it sets up the anteroposterior axis in early oogenesis, and then redistributes to set up the dorsoventral axis in later stages of oogenesis (van Eeden, 1999). In the positive control ovaries, *gurken* mRNA is expressed in the posterior end of young egg chambers and in the anterodorsal corner of older egg chambers, as previously reported (Fig. 6I,J) (Neuman-Silberberg and Schupbach, 1993). Ovaries containing germline clones of either $csn4^{null}$ or $csn5^{null}$ show no *gurken* staining (Fig. 6K,L). Ovaries containing germline clones of $csn5^{P}$ display a complex *gurken* staining pattern: while most of the egg chambers show a staining pattern similar to wild type, 10%-20% of the developed egg chambers (stage 8 onwards) display a staining pattern of an anterior ring (Fig. 8M). In the wild type, *gurken* mRNA has been shown to be normally expressed in a pattern of anterior ring at stage 7 of oogenesis, but to be localized to the anterodorsal corner from stage 8 onwards (Neuman-Silberberg and Schupbach, 1993). The aberrant *gurken* expression pattern in some egg chambers homozygous for $csn5^{P}$ may therefore indicate a problem in the transition of *gurken* mRNA from the anterior ring to the specific anterodorsal localization. The expression pattern of *gurken* mRNA in ovaries containing germline clones of $csn5^{1}$ was not examined.

$csn4^{null}$ and $csn5^{null}$ larvae are sensitive to MMS

Mutations in *SpCSN1* lead to cell cycle check point defects in *S. pombe* (Mundt et al., 1999), while mutations in SpCSN4 or SpCSN5 do not (Mundt et al., 2002). Furthermore, various in situ studies have implicated CSN in regulating the cell cycle (Yang et al., 2002; Chamovitz and Segal, 2001). To examine if CSN4 or CSN5 have a role in cell cycle regulation in vivo, we checked the mutants for sensitivity to the DNA-damaging agent methylmethane sulfonate (MMS). First and second instar larvae were supplemented with medium containing 0.4% MMS, and survival was monitored in relation to untreated larvae. As seen in Fig. 7, while the MMS treatment had no obvious effect on either wild type or heterozygous GFP-positive siblings, both the $csn5^{null}$ and $csn4^{null}$ larvae were sensitive to the MMS, with only ~20% of the larvae surviving 4 days following the treatment. This suggests that CSN4 and CSN5, and by association, the COP9 signalosome, are necessary for responses to DNA damage in *Drosophila*.

$csn4^{null}$ and $csn5^{null}$ larvae have unique phenotypes

While null mutations in both *csn4* and *csn5* lead to larval lethality, both strains also have unique phenotypes that suggest the involvement of distinct developmental pathways. The

developmental retardation in *csn4*^{null} larvae at the second to third instar transition is associated with obvious molting defects. This is best illustrated in the mouth hooks of *csn4*^{null} third instar larvae. The second instar mouth parts fail to detach from the newly formed third instar mouth hooks giving a double mouth hook phenotype (Fig. 8B). Some of the mutant larvae also have defective ecdysis, as the shed second instar cuticle also remains attached at the head (Fig. 8C). This phenotype was seen in ~20% of the *csn4*^{null} second to third instar larvae. By contrast, none of the *csn4*$^{null/+}$ or *csn5*^{null} larvae showed obvious molting defects.

The *csn5*^{null} third instar larvae develop conspicuous melanotic capsules in their hemolymph (Fig. 8D,E). The capsules begin to appear floating freely in the hemoceol 4 days after the second molt, and increase in frequency until over 80% of the non-GFP (*csn5*^{null}) larvae contain these capsules by the late stages of the third instar. None of the GFP-positive (heterozygous) siblings shows melanization. The number, size and localization of the capsules are variable. This phenotype was not seen in the P-element insertion line *csn5*^{p}, and rarely seen in point mutations *csn5*^{1} and *csn5*^{3}.

Biochemical analysis of the CSN

The overlapping yet unique phenotypes of the *csn4*^{null} and *csn5*^{null} mutants led us to question the basis of these differences. In *Arabidopsis*, all reported mutations in CSN subunits lead to essentially identical phenotypes (Karniol et al., 1999; Misera et al., 1994; Serino et al., 1999; Wei and Deng, 1992; Wei et al., 1994). If the phenotypes reported above were solely due to a loss of the CSN complex, then we would have expected that the two mutants would have identical phenotypes. Alternatively, it is possible that each CSN subunit also has specific roles.

To further analyze the CSN in *Drosophila* development, antibodies were generated against CSN4 and affinity purified. Gel filtration analysis on total protein extracts from different developmental stages of wild-type *Drosophila* indicates that CSN4, like CSN5 and CSN7, is present in both CSN-dependent and CSN-independent forms (Fig. 9). The CSN-independent form of CSN4 in larvae is approximately the size of the CSN4 monomer, or slightly larger. A CSN4 doublet is detected in the CSN-dependent fractions (~500 kDa). Both bands are CSN4 specific as they are absent in the *csn4*^{null} mutant (not shown). For CSN7, the CSN-corresponding peak is followed by a gradual decrease in CSN7 protein levels. We refer to this gradual decrease profile as a ‘low molecular weight shoulder’. CSN5 from larvae is found in two distinct peaks, one corresponding to the intact CSN complex, with the second peak around the monomer sizes in fraction 21-23.

To correlate these results with the unique phenotypes described above, we analyzed the effects of the mutations in *Csn4* and *Csn5* on the complex. Protein extracts from *csn5*^{null}, *csn5*^{1}, csn5^{3} and *csn4*^{null} mutant larvae were separated over a gel filtration column, and the resulting fractions analyzed by immunoblot. The null mutation in *csn4* results in a complete loss of the CSN-dependent forms of CSN5 and CSN7, leaving CSN-independent forms of the two proteins. For CSN5, this form appears to be the monomeric size that is also seen in wild-type larvae. Although CSN7 also is present in CSN-independent forms, CSN7 is affected by the *csn4*^{null} mutation. In wild-type larvae, CSN7 is found in a continuum of sizes ranging from 500 kDa to 67 kDa; in *csn4*^{null}, CSN7 is only slightly larger than the predicted monomer size (27 kDa), peaking around 60 kDa. Therefore, the phenotypes seen in the *csn4*^{null} mutant could be a result of the loss of the entire CSN, the CSN-independent forms of CSN4, and/or the changes in CSN7.

The analysis of the *csn5* mutants was more surprising. Although we had hypothesized that a null mutation in *Csn5* would lead to a loss of the entire COP9 signalosome, both CSN4 and CSN7 are found as a large molecular weight complexed forms in *csn5*^{null} larvae. This indicates that CSN5 is a peripheral component of the COP9 signalosome in *Drosophila* and is not necessary for the integrity of the complex. However CSN4 is affected by the lack of CSN5 as CSN-independent form of CSN4 is absent in *csn5*^{null}. CSN7 appears unaffected by the lack of CSN5.

In the *csn5*^{1} point mutation, an apparently normal sized CSN is detected, indicating that the mutated protein can incorporate into the complex. However, the point mutation appears to affect the CSN-independent forms of CSN5 and CSN7. The CSN-independent form of CSN5 is larger than in the wild type, eluting two fractions earlier than the wild type. The large CSN7 ‘shoulder’ seen in fractions 16-20 in the wild type, is absent in the *csn5*^{1} mutant. The gel filtration results for the *csn5*^{3} mutant are similar to *csn5*^{1} as the CSN7 shoulder is not detected. In addition, no CSN-independent forms of CSN5 are detected.

DISCUSSION

We have shown, through the analysis of mutants in two subunits of the *Drosophila* COP9 signalosome, that the CSN is involved in the regulation of diverse developmental processes. Furthermore, we have shown that individual subunits may have specific functions that are dependent on the in vivo form of the protein.

In wild-type *Drosophila* larvae, CSN4, CSN5 and CSN7 are present in both complex-dependent and complex-independent forms. This is similar to the situation for these three subunits in *Arabidopsis*, further highlighting the remarkable conservation of this complex between the plant and animal kingdoms (Karniol et al., 1999; Kwok et al., 1998; Serino et al., 1999). While the elution profiles for CSN4 and CSN5 show two distinct separate peaks, the elution profile of CSN7 reveals one major peak corresponding to the intact COP9 signalosome, followed by a gradual decrease in CSN7 protein levels in following fractions giving rise to a ‘low molecular weight shoulder’. We postulate that this ‘shoulder’ comprises multiple CSN7-containing complexes of variable molecular weights. These smaller complexes appear to be dependent on other CSN subunits as they are absent in the *csn5* point mutation strains. Further analysis is needed to determine the identity of these complexes.

Analysis of the mutant larvae reveals that CSN4 is essential for the assembly and/or stability of the COP9 signalosome as *csn4*^{null} mutants lack the CSN-dependent forms of CSN5 and CSN7. AtCSN4 has a similar central position in the complex (Serino et al., 1999). Furthermore, CSN4 appears to affect the CSN-independent forms of CSN7, suggesting that there is an equilibrium between the various forms of the CSN subunits.

Surprisingly the situation in the *csn5*^{null} mutant is strikingly different from any CSN mutant described so far in

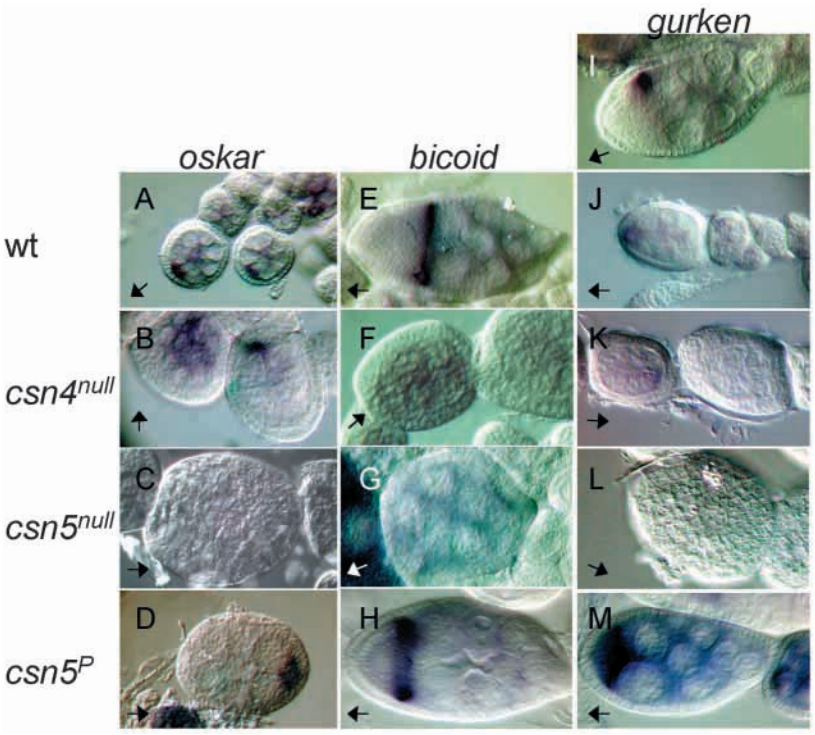

Fig. 6. Axis patterning in *csn4* and *csn5* mutant oocytes. Oocytes were hybridized with digoxigenin-labeled antisense RNA probes. (A-D) *oskar* expression in wild-type (A), $csn4^{null}$ (B), $csn5^{null}$ (C) and $csn5^{P}$ (D) mutant oocytes. (E-H) *bicoid* expression in wild-type (E), $csn4^{null}$ (F), $csn5^{null}$ (G), and $csn5^{P}$ (H) mutant oocytes. (I-M) *gurken* expression in wild-type (I,J), $csn4^{null}$ (K), $csn5^{null}$ (L) and $csn5^{P}$ (M) mutant oocytes. Late (I) and early (J) *gurken* patterns are shown for the wild type. The arrow indicates the posterior.

other organisms. While the mutant is a true null, and no CSN5 protein is detected, CSN4 and CSN7 are detected in high molecular weight fractions, similar to the wild type. As CSN5 is a single copy gene in *Drosophila*, we would expect that the complex detected in $csn5^{null}$ would be slightly smaller (by 38 kDa). However, our gel filtration system cannot detect such small changes in complex size. Thus, we conclude that the *Drosophila* CSN5 is not essential for CSN assembly and/or stability, leading us further to postulate that in *Drosophila*, CSN5 is a peripheral component of the CSN complex.

However, the $csn5^{null}$ mutation does affect other CSN subunits. In $csn5^{null}$ larvae, the CSN-independent form of CSN4 is abolished. This suggests that the CSN-independent form of CSN4 is somehow stabilized by CSN5, possibly by CSN-independent interactions between the two proteins. This resembles the effect described for mutations in the *COP1* and *DET1* loci on *Arabidopsis* CSN5 (Kwok et al., 1998). In these mutants the CSN5 proteins were not detected as monomers whereas the complex form was present. However, as opposed to CSN4, COP1 and DET1 are not components of the CSN and it was postulated that COP1 and DET1 somehow regulate the stability of the CSN5 monomer.

A similar situation can be seen in both *Drosophila csn5* point mutation lines with respect to CSN7. In larvae homozygous for either $csn5^{1}$ or $csn5^{3}$ point mutations, the low molecular weight shoulder of CSN7 is not detected, leaving CSN7 in the complex-dependent form only. These results are not due to changing protein concentrations, as the experiments were repeated three times, each time with equal protein concentration. This is interesting in light of the fact that CSN7 profile appears normal in $csn5^{null}$ mutants, suggesting that the point mutations in CSN5 somehow disrupt the interaction of CSN7 with other proteins.

The COP9 signalosome in *Drosophila* development

Our phenotypic analysis of the *csn4* and *csn5* mutants indicates that the CSN and its subunits are involved in the regulation of diverse developmental signaling cascades. Evidence for involvement in hematopoiesis and/or cell cycle regulation comes from the melanotic capsule phenotype of the $csn5^{null}$ mutants. There are at least four categories of biological defects that can lead to the formation of melanotic capsules in *Drosophila* (termed 'melanotic tumors') (Dearolf, 1998). As the melanotic capsules in $csn5^{null}$ appear floating in multiple positions in different larvae, and as the capsules are large and composed of multiple cells (not shown), they probably result from derailment of the immune response and/or cell cycle. Indeed, *Drosophila* mutants for cell cycle proliferation exhibit both melanotic capsules and an abnormally extended larval stage followed by lethality, similar to the lethal stage in the csn^{null} mutants.

Additional evidence for a defect in cell cycle regulation in the *csn* mutants was seen in the MMS study. Both the $csn4^{null}$ and $csn5^{null}$ mutants are hypersensitive to MMS treatment, suggesting a role of the CSN in cell cycle regulation through the regulation of the DNA-integrity checkpoint. One potential

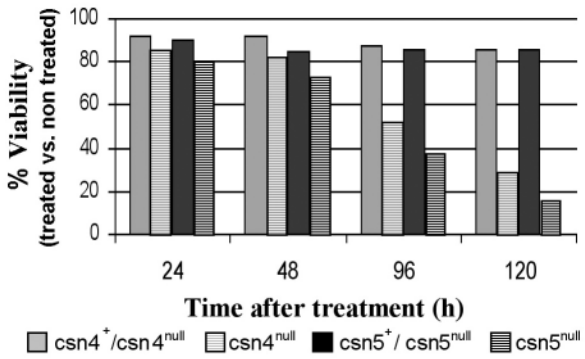

Fig. 7. $csn4^{null}$ and $csn5^{null}$ mutants are hypersensitive to MMS. First instar larvae of $csn4^{null}$, $csn5^{null}$, and their respective heterozygotic siblings were fed with yeast extract supplemented with methyl methane sulfonate (MMS). After treatment, the larvae were observed for viability. Viability=$(x_M/t_M)/(x_c/t_c)\times100$, where x_M is the number of living MMS-treated individuals, t_M is the total number of MMS-treated individuals (including dead individuals), x_c is the number of living non-treated individuals and t_c is the total number of non-treated individuals for each strain. Starting numbers were 180 larvae for each strain.

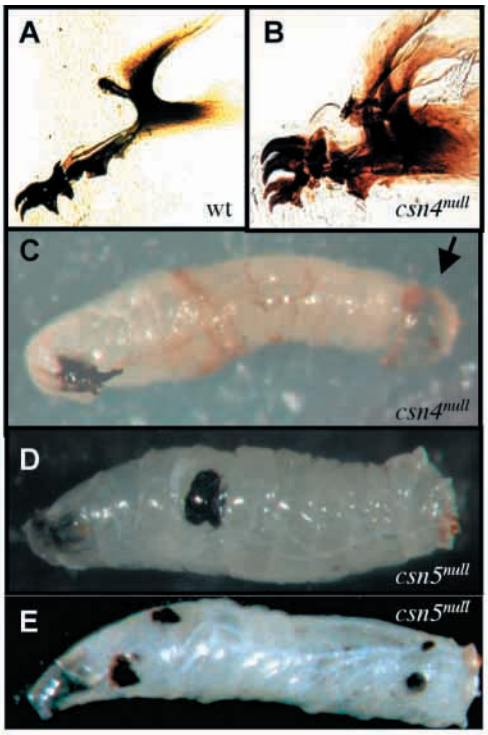

Fig. 8. Unique phenotypes of *csn* mutants. Dissected mouth hooks from a (A) third instar wild-type larva, similar to $csn4^{+/null}$ heterozygote larva, and from a (B) third instar $csn4^{null}$ homozygote larva. (C) Third instar $csn4^{null}$ homozygote larva attached to the second instar molt (arrow). (D,E) $csn5^{null}$ homozygote larvae showing melanotic capsules.

caveat with this interpretation is that the MMS sensitivity can also arise from defects in DNA damage repair machinery, and not specifically connected to the cell cycle checkpoint. However, this hypothesis does fit with other studies that showed a role for CSN components in regulating proteins such as p53, Jun, MIF and $p27^{kip}$ (Bech-Otschir et al., 2001; Chamovitz and Segal, 2001; Mundt et al., 1999; Mundt et al., 2002; Yang et al., 2002).

However, in *Drosophila*, the CSN is probably not directly involved in the checkpoint machinery or the DNA repair signaling pathways, as mutations in this machinery, such as *chk1* and *mei41*, are more sensitive to MMS than are our mutants (Sibon et al., 1999) (N. Egoz, N. Madar and D. S., unpublished). We therefore suggest that the CSN modulates the activity of the checkpoint regulators, and that in the absence of the complex, these regulators work at a lower efficiency. As the *Drosophila* cell cycle machinery is highly similar to that of mammals, we suggest that the *Drosophila* CSN is involved in cell cycle regulation via regulation of Dacapo and p53. Further work must be undertaken before a direct relation between these components in *Drosophila* is established.

Strong evidence for the involvement of the *Drosophila* CSN in steroid-hormonal signaling in vivo comes from analysis of the $csn4^{null}$ mutant that displays molting defects including a double mouth hook phenotype. Mutations in signal transduction molecules that disrupt the ecdysone-signaling pathway lead to similar defects (Bender et al., 1997; Freeman, 1999; Li and Bender, 2000). Ecdysone signaling is mediated by the ecdysone receptor (EcR). Conditional mutations in the EcR lead to developmental arrest during the larval molts with two pairs of larval cuticular derivatives, including mouth hooks and spiracles (reviewed by Kozlova, 2000). Our $csn4^{null}$ larvae display a similar phenotype regarding the molt to the third larval instar. This suggests that the involvement of the CSN in the hormonal signaling pathway is stage specific. A role for the CSN in ecdysone signaling was earlier suggested when interaction-trap studies showed that another COP9 signalosome subunit, CSN2, interacts with the EcR (Dressel et al., 1999), implying that CSN2 functions as a co-repressor of the EcR. We propose that in

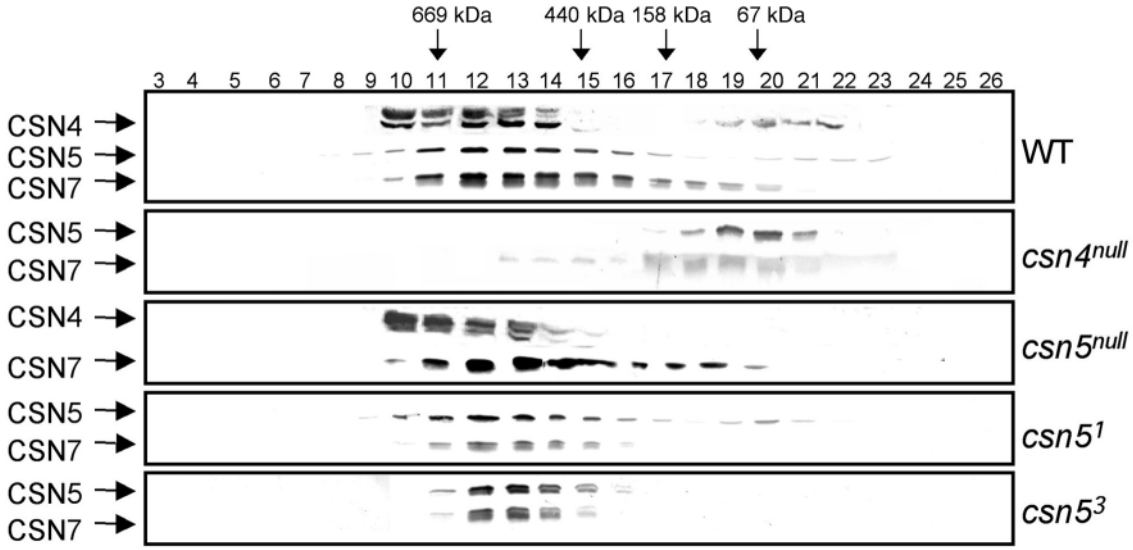

Fig. 9. Gel filtration analysis of the COP9 signalosome and its subunits in the wild-type and mutant strains. Third instar larvae from wild-type and mutant strains were homogenized in gel-filtration buffer and total soluble protein was fractionated over a Superose 6 gel-filtration column (Pharmacia). Fractions (0.5 ml) were examined for the presence of CSN4, CSN5 or CSN7, as indicated, by immunoblot analysis with anti-CSN4, anti-CSN5 or anti-CSN7 affinity-purified antibodies. Positions of size markers are shown.

csn4^{null}, the EcR-mediated signaling is damaged at least in part by the absence of CSN4. In this scenario, in the *csn4*^{null} mutant, in the absence of an intact CSN complex, excess CSN-independent CSN2 is free to interact with the EcR, thereby causing repression of the EcR pathway, while in *csn5*^{null}, where an intact CSN complex remains, no derailment of ecdysone signaling is apparent. This predicts that mutations in additional subunits that disrupt CSN structure would lead to a similar molting defect. As HsCSN2 was also identified through interaction-trap screens as a protein that binds the thyroid hormone receptor (Lee et al., 1995), the CSN may be a general regulator of steroid hormone signaling in diverse species.

Evidence for a role of the COP9 signalosome in axis formation comes from our analysis of *csn5*^{p} mutant embryos and oocytes derived from germline *csn4* and *csn5* mutant clones. Establishment of both the anteroposterior and the dorsoventral embryonic axes can be traced back to oocyte polarization during mid-stages of oogenesis (Riechmann and Ephrussi, 2001). The early gene expression defects observed in *csn5*^{p} mutant embryos are consistent with an involvement of CSN5 in the localization of a patterning molecule such as the TGFα-like Gurken within the oocyte. For example, the *rhomboid* expression pattern in *csn5*^{p} mutants is reminiscent of weakly dorsalized embryos derived from *fs(1)K10* mothers, where *gurken* mRNA is mislocalized to the ventral side of the oocyte (Roth and Schupbach, 1994). Consistent with this, in some *csn5*^{p} stage 8 oocytes, *gurken* mRNA is not restricted to the anterior-dorsal cortex, but expands ventrally. Interestingly, in contrast to embryos derived from *fs(1)K10* mutant oocytes, where segmentation genes are expressed normally along the anteroposterior axis, *csn5*^{p} mutant embryos also exhibit alterations in the expression patterns of early acting segmentation genes, suggesting a misexpression of *bicoid* and *oskar* mRNA. However, although neither *bicoid* nor *oskar* is expressed in the *csn5*^{null} oocytes, in the *csn5*^{p} oocytes, *bicoid* expression is indistinguishable from wild type, and *oskar* is highly similar to the wild type, being slightly overexpressed. One possibility is that while *bicoid* and *oskar* mRNA expression appears normal, the defect in *csn5*^{p} embryos results from abnormal translation and/or protein localization of the *bicoid* and *oskar* gene products. Alternatively, the expression patterns we observe in *csn5*^{p} mutant embryos are not caused by mislocalization of patterning molecules in the oocyte, but are caused by embryonic factors. Indeed, the s*nail* and early *rhomboid* expression patterns are suggestive of a defect in the Dorsal gradient. However, it is difficult to envisage how a signal-independent regulation of Dorsal nuclear translocation would result in a wavy expression boundary of Dorsal target genes. In addition, the width of the ventrolateral *rhomboid* expression domain reflects the slope of the Dorsal protein gradient. In *csn5*^{p} mutants, the wavy ventrolateral *rhomboid* stripes are of normal width, indicating that the Dorsal gradient is formed with a normal slope but at a shifted position. This contrasts with the expression patterns caused by mutations in *cactus* and *dorsal* group genes, which change the slope of the Dorsal gradient.

Genes acting later in the segmentation hierarchy, such as the *even-skipped* and *engrailed* genes, show much more severe disruptions in their expression patterns in csn5^{p} mutants than do the early acting gap genes. The CSN5 protein thus appears to function in controlling gene expression in the embryo as well as patterning the oocyte. The variability in the altered expression patterns makes it difficult to assign a specific function to CSN5 in anterior-posterior patterning. A role for CSN5 in regulating genes induced by the TGFβ molecule Dpp, is revealed by our results on *rhomboid* expression. The width of *rhomboid* expression in the dorsal ectoderm is dependent on the strength of the Dpp signal (Ashe et al., 2000). The expanded *rhomboid* pattern observed in *csn5*^{p} mutant embryos indicates that CSN5 is a negative regulator of Dpp signaling, consistent with a recent report showing that CSN5 antagonizes TGFβ signaling by inducing degradation of the common intracellular transducer of TGFβ factors, Smad4 (Wan et al., 2002).

As was shown in later development, the phenotypes of *csn4*^{null} and *csn5*^{null} strains are not completely equal. While oogenesis is arrested in both mutants, *oskar* patterning is unaffected in *csn4*^{null}, while it is completely abrogated in *csn5*^{null}. With regard to the *csn4*^{null} mutant ovaries, it should be pointed out that ecdysone also has a role in oogenesis (Carney and Bender, 2000; Kozlova, 2000), and therefore the defects observed may also result from pleiotropic effects of the *csn4* mutation on ecdysone signaling. Thus, throughout *Drosophila* development, it appears that CSN-independent forms of CSN5 have additional functions including the regulation of *oskar* expression, hematopoesis and axonal guidance (Suh et al., 2002), while the entire COP9 signalosome, or closely related subcomplexes, are necessary for other oocyte and embryo patterning processes, ecdysone regulation, responses to DNA damage and eye disc differentiation.

The results presented here clearly show that the COP9 signalosome and its individual subunits are involved in the regulation of diverse developmental pathways. The mechanism for this control is still unclear, though we can hypothesize that it involves the regulation of degradation of other central regulators. One known target of the CSN is Cul1, and recently the deneddylase activity associated with the CSN was shown to be dependent or closely associated with CSN5 (G. Cope and R. Deshaies, personal communication). However the diverse phenotypes described here cannot be explained solely by defects in Cul1 regulation. Elucidating the targets of CSN regulation, and further determining the relationship between the CSN and its individual subunits are critical for understanding CSN function in *Drosophila*.

We thank Prof. S. L. Zipursky from UCLA for providing *csn5*^{1}*and csn5*^{3}, Prof. Raymond Deshaies from Caltech for communicating results prior to publication, Ruth Werczberger for maintaining *Drosophila* strains, Dr Yael Nevo-Caspi for assistance throughout, Nirit Egoz for assistance in the MMS study, and Dr Nir Ohad for assistance in microscopy and photography. This research was partially funded by grants from the Swedish Research Council and the Magn. Bervall Foundation (to M. M.), and from The Israel Science Foundation founded by The Israel Academy of Sciences and Humanities (519/002) (to D. S. and D. A. C.).

REFERENCES

Ashe, H. L. Mannervik, M. and Levine, M. (2000). Dpp signaling thresholds in the dorsal ectoderm of the Drosophila embryo. *Development* **127**, 3305-3312.

Bech-Otschir, D., Kraft, R., Huang, X., Henklein, P., Kapelari, B., Pollmann, C. and Dubiel, W. (2001). COP9 signalosome-specific

phosphorylation targets p53 to degradation by the ubiquitin system. *EMBO J.* **20**, 1630-1639.

Bender, M., Imam, F. B., Talbot, W. S., Ganetzky, B. and Hogness, D. S. (1997). Drosophila ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* **91**, 777-788.

Carney, G. and Bender, M. (2000). The Drosophila ecdysone receptor (EcR) gene is required maternally for normal oogenesis. *Genetics* **154**, 1203-1211.

Casso, D., Ramirez-Weber, F. A. and Kornberg, T. B. (1999). GFP-tagged balancer chromosomes for Drosophila melanogaster. *Mech. Dev.* **88**, 229-232.

Chamovitz, D. A. and Segal, D. (2001). JAB1/CSN5 and the COP9 signalosome. A complex situation. *EMBO Rep.* **2**, 96-101.

Chamovitz, D. A., Wei, N., Osterlund, M. T., von Arnim, A. G., Staub, J. M., Matsui, M. and 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.

Cohen, H., Azriel, A., Cohen, T., Meraro, D., Hashmueli, S., Bech-Otschir, D., Kraft, R., Dubiel, W. and Levi, B. Z. (2000). Interaction between ICSBP and CSN2 (TRIP15) – a possible link between IRF signaling and the COP9/signalosome. *J. Biol. Chem.* **275**, 39081-39089.

Cooperstock, R. L. (1999). RNA localization and translational regulation during axis specification in the Drosophila oocyte. *J. Cell Sci.* **112**, 4389-4396.

Dearolf, C. R. (1998). Fruit fly 'leukemia'. *Biochim. Biophys. Acta* **1377**, M13-M23.

Deng, X. W., Dubiel, W., Wei, N., Hofmann, K., Mundt, K., Colicelli, J., Kato, J., Naumann, M., Segal, D., Seeger, M. et al. (2000). Unified nomenclature for the COP9 signalosome and its subunits: an essential regulator of development. *Trends Genet.* **16**, 202-203.

Dressel, U., Thormeyer, D., Altincicek, B., Paululat, A., Eggert, M., Schneider, S., Tenbaum, S. P., Renkawitz, R. and Baniahmad, A. (1999). Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. *Mol. Cell. Biol.* **19**, 3383-3394.

Freeman, M. R. (1999). The dare gene: steroid hormone production, olfactory behavior, and neural degeneration in Drosophila. *Development Suppl.* **126**, 4591-4602.

Freilich, S., Oron, E., Kapp, Y., Nevo-Caspi, Y., Orgad, S., Segal, D. and Chamovitz, D. A. (1999). The COP9 signalosome is essential for development of *Drosophila melanogaster*. *Curr. Biol.* **9**, 1187-1190.

Glickman, M. H., Rubin, D. M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V. A. and Finley, D. (1998). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* **94**, 615-623.

Jiang, J., Kosman, D., Ip, Y. T. and Levine, M. (1991). The dorsal morphogen gradient regulates the mesoderm determinant twist in early Drosophila embryos. *Genes Dev.* **5**, 1881-1891.

Johnstone, O. (2001). Translational regulation and RNA localization in Drosophila oocytes and embryos. *Annu. Rev. Genet.* **35**, 365-406.

Karniol, B., Malec, P. and Chamovitz, D. A. (1999). Arabidopsis *FUSCA5* encodes a novel phosphoprotein that is a component of the COP9 complex. *Plant Cell* **11**, 839-848.

Kim, T.-H., Hofmann, K., von Arnim, A. G. and Chamovitz, D. A. (2001). The PCI complexes: pretty complex interactions in diverse signaling pathways. *Trend Plant Sci.* **6**, 379-386.

Kim-Ha, J. (1991). oskar mRNA is localized to the posterior pole of the Drosophila oocyte. *Cell* **66**, 23-35.

Kozlova, T. (2000). Steroid regulation of postembryonic development and reproduction in Drosophila. *Trends Endocrinol. Metab.* **11**, 276-280.

Kwok, S. F., Solano, R., Tsuge, T., Chamovitz, D. A., Ecker, J. R., Matsui, M. and Deng, X. W. (1998). Arabidopsis homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic *cop/det/fus* mutations. *Plant Cell* **10**, 1779-1790.

Kwok, S. F., Staub, J. M. and Deng, X. W. (1999). Characterization of two subunits of Arabidopsis 19S proteasome regulatory complex and its possible interaction with the COP9 complex. *J. Mol. Biol.* **285**, 85-95.

Lee, J. W., Choi, H.-S., Gyuris, J., Brent, R. and Moore, D. D. (1995). Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid receptor. *Mol. Endocrinol.* **9**, 243-253.

Li, T. and Bender, M. (2000). A conditional rescue system reveals essential functions for the ecdysone receptor (EcR) gene during molting and metamorphosis in Drosophila. *Development* **127**, 2897-2905.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D. A., Wei, N. and Deshaies, R. J. (2001). Promotion of NEDD8-CUL1 Conjugate Cleavage by COP9 Signalosome. *Science* **3**, 3.

Misera, S., Muller, A. J., Weiland-Heidecker, U. and Jurgens, G. (1994). The FUSCA genes of Arabidopsis: negative regulators of light responses. *Mol. Gen. Genet.* **244**, 242-252.

Mundt, K. E., Liu, C. and Carr, A. M. (2002). Deletion mutants in COP9/signalosome subunits in fission yeast *Schizosaccharomyces pombe* display distinct phenotypes. *Mol. Biol. Cell* **13**, 493-502.

Mundt, K. E., Porte, J., Murray, J. M., Brikos, C., Christensen, P. U., Caspari, T., Hagan, I. M., Millar, J. B., Simanis, V., Hofmann, K. et al. (1999). The COP9/signalosome complex is conserved in fission yeast and has a role in S phase. *Curr. Biol.* **9**, 1427-30.

Naumann, M., Bech-Otschir, D., Huang, X., Ferrell, K. and Dubiel, W. (1999). COP9 signalosome-directed c-Jun activation/stabilization is independent of JNK. *J. Biol. Chem.* **274**, 35297-35300.

Neuman-Silberberg, F. S. and Schupbach, T. (1993). The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. *Cell* **75**, 165-174.

Reichhart, J. M. and Ferrandon, D. (1998). Green balancers. *D. I. S.* **81**, 201-202.

Riechmann, V. and Ephrussi, A. (2001). Axis formation during Drosophila oogenesis. *Curr. Opin. Genet. Dev.* **11**, 374-383.

Roth, S. and Schupbach, T. (1994). The relationship between ovarian and embryonic dorsoventral patterning in Drosophila. *Development* **120**, 2245-2257.

Schwechheimer, C. and Deng, X. (2001). COP9 signalosome revisited: a novel mediator of protein degradation. *Trends Cell Biol.* **11**, 420-426.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W. L., Lyapina, S., Deshaies, R. J., Gray, W. M., Estelle, M. and Deng, X. W. (2001). Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIR1 in mediating auxin response. *Science* **292**, 1379-1382.

Serino, G., Tsuge, T., Kwok, S., Matsui, M., Wei, N. and Deng, X. W. (1999). Arabidopsis *cop8* and *fus4* mutations define the same gene that encodes subunit 4 of the COP9 signalosome. *Plant Cell* **11**, 1967-1980.

Sibon, O. C., Laurencon, A., Hawley, R. S. and Theurkauf, W. E. (1999). The Drosophila ATM homologue mei-41 has an essential checkpoint function at the midblastula transition. *Curr. Biol.* **9**, 302-312.

St Johnston, D., Driever, W., Berleth, T., Richstein, S., Nusslein-Volhard, C. (1989). Multiple steps in the localization of bicoid RNA to the anterior pole of the Drosophila oocyte. *Development Suppl.* **107**, 13-19.

Suh, G. S. B., Poeck, B., Chouard, T., Oron, E., Segal, D., Chamovitz, D. A. and Zipursky, S. L. (2002). Drosophila JAB1/CSN5 acts in photoreceptor cells to induce glial cells. *Neuron* **33**, 1-20.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81-85.

The Fly Consortium (1999). The FlyBase database of the Drosophila genome projects and community literature. *Nucleic Acids Res.* **27**, 85-88.

van Eeden, F. (1999). The polarisation of the anterior-posterior and dorsal-ventral axes during Drosophila oogenesis. *Curr. Opin. Genet. Dev.* **9**, 396-404.

Wan, M., Cao, X., Wu, Y., Bai, S., Wu, L., Shi, X., Wang, N. and Cao, X. (2002). Jab1 antagonizes TGF-{beta} signaling by inducing Smad4 degradation. *EMBO Rep.* **3**, 171-176.

Wei, N. and Deng, X. W. (1992). COP9: a new genetic locus involved in light-regulated development and gene expression in arabidopsis. *Plant Cell* **4**, 1507-1518.

Wei, N., Kwok, S. F., von Arnim, A. G., Lee, A., McNellis, T. W., Piekos, B. and Deng, X. W. (1994). Arabidopsis COP8, COP10, and COP11 genes are involved in repression of photomorphogenic development in darkness. *Plant Cell* **6**, 629-643.

Wei, N., Tsuge, T., Serino, G., Dohmae, N., Takio, K., Matsui, M. and Deng, X. W. (1998). The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. *Curr. Biol.* **8**, 919-922.

Yahalom, A., Kim, T. H., Winter, E., Karniol, B., von Arnim, A. G. and Chamovitz, D. A. (2001). Arabidopsis eIF3e (INT-6) associates with both eIF3c and the COP9 signalosome subunit CSN7. *J. Biol. Chem.* **276**, 334-340.

Yang, X., Menon, S., Lykke-Andersen, K., Tsuge, T., Di, X., Wang, X., Rodriguez-Suarez, R. J., Zhang, H. and Wei, N. (2002). The COP9 signalosome inhibits p27(kip1) degradation and impedes G1-S phase progression via deneddylation of SCF Cul1. *Curr. Biol.* **12**, 667-672.