

Genomic analysis of COP9 signalosome function in *Drosophila melanogaster* reveals a role in temporal regulation of gene expression

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The COP9 signalosome (CSN), an eight-subunit protein complex, is conserved in all higher eukaryotes. CSN intersects the ubiquitin–proteasome pathway, modulating signaling pathways controlling various aspects of development. We are using *Drosophila* as a model system to elucidate the function of this important complex. Transcriptome data were generated for four *csn* mutants, sampled at three developmental time points. Our results are highly reproducible, being confirmed using two different experimental setups that entail different microarrays and different controls. Our results indicate that the CSN acts as a transcriptional repressor during development of *Drosophila*, resulting in achronic gene expression in the *csn* mutants. ‘Time shift’ analysis with the publicly available *Drosophila* transcriptome data indicates that genes repressed by the CSN are normally induced primarily during late embryogenesis or during metamorphosis. These temporal shifts are likely due to the roles of the CSN in regulating transcription factors. A null mutation in CSN subunit 4 and hypomorphic mutations in *csn5* lead to more severe defects than seen in the *csn5*-null mutants strain, suggesting that CSN5 carries only some of the CSN function.

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Introduction

The COP9 signalosome (CSN) is a highly conserved protein complex that in higher eukaryotes consists of eight subunits named CSN1 to CSN8 (Deng *et al*, 2000; Cope and Deshaies, 2003; Wei and Deng, 2003; Schwechheimer, 2004). With its discovery in *Arabidopsis*, the complex was originally described as a repressor of light-dependent growth in plants (Wei *et al*, 1994; Chamovitz *et al*, 1996). Subsequent identification and characterization of the CSN from mammalian cells, insects and yeast highlighted the complex as a general modulator of signal transduction (Seeger *et al*, 1998; Wei *et al*, 1998; Freilich *et al*, 1999; Mundt *et al*, 1999).

The CSN is involved in diverse cellular and developmental processes such as DNA repair, cell cycle regulation, MAPK signalling, hormone signalling, axonal guidance, and embryogenesis. (Doronkin *et al*, 2002; Maytal-Kivity *et al*, 2002, 2003; Mundt *et al*, 2002; Oron *et al*, 2002; Suh *et al*, 2002; Wee *et al*,

2002; Busch *et al*, 2003; Cope and Deshaies, 2003; Liu *et al*, 2003; Lykke-Andersen *et al*, 2003; Nielsen, 2003; Serino and Deng, 2003; Yan *et al*, 2003; Harari-Steinberg and Chamovitz, 2004). In regulating these processes, the CSN has been shown to impact transcription, protein translation and protein degradation.

The most studied CSN function is regulation of protein degradation. The CSN intersects the ubiquitin–proteasome pathway at a number of junctions: The CSN or CSN subunits directly interact with the proteasome (Kwok *et al*, 1999; Peng *et al*, 2003). The CSN also interacts with E3-ubiquitin ligases, removing Nedd8, a ubiquitin-like modifier, from cullin-based E3s (Lyapina *et al*, 2001; Cope *et al*, 2002; Wolf *et al*, 2003), thereby regulating ligase activity. This deneddylation activity resides in the JAMM/MPN+ domain of CSN5 (Cope *et al*, 2002). The CSN also mediates phosphorylation and deubiquitination of ubiquitin–proteasome pathway substrates, and as a consequence alters their stability and subcellular localization (reviewed in Harari-Steinberg and Chamovitz, 2004).

Certain CSN activities impinge on transcriptional regulation. Through its regulation of protein degradation, the CSN affects the stability (and activity) of transcription factors such as HY5 in plants and cJUN and p53 in animals (Naumann *et al*, 1999; Osterlund *et al*, 1999; Bech-Otschir *et al*, 2001). CSN subunits also directly affect transcriptional activity as corepressors or coactivators. For example, CSN2 was originally discovered as a corepressor of the thyroid hormone receptor, where as CSN5 acts as a coactivator of AP-1 target genes (Dressel *et al*, 1999; Akiyama *et al*, 2003; Groisman *et al*, 2003; Tenbaum *et al*, 2003). Moreover, CSN associates with chromatin through CSA and RNA polymerase II (Groisman *et al*, 2003), suggesting that it may mediate gene expression directly.

To better understand the function of CSN in a multicellular animal, we initiated a study in *Drosophila melanogaster* (Freilich *et al*, 1999). The *Drosophila* CSN is highly similar in subunit composition, subunit sequence, and biochemical properties to its human counterpart, and is essential for *Drosophila* development (Freilich *et al*, 1999; Oron *et al*, 2002). Loss-of-function mutants in *Drosophila* CSN4 and CSN5 display overlapping phenotypes in oogenesis and larval development. However, each mutant also has specific defects. For example, *csn5^{null}* mutants develop large melanotic masses in their hemolymph, whereas *csn4^{null}* mutants never show these masses, but instead display multiple abnormalities consistent with defects in steroid hormone signalling (Oron *et al*, 2002; Harari-Steinberg *et al*, 2007). Work in *Schizosaccharomyces pombe* also showed that not all *csn* mutants are equal (Mundt *et al*, 2002). This lack of complete phenotypic overlap is different from the case in *Arabidopsis* where loss-of-function mutants in different CSN subunits are phenotypically indistinguishable (Kwok *et al*, 1996; Ma *et al*, 2003; Dohmann *et al*, 2005). The lack of complete phenotypic overlap between the *csn* mutants can be explained by at least two non-exclusive hypotheses. First, each subunit can have different roles within the CSN complex, perhaps binding different interacting proteins. Second, each subunit can have distinct roles independent of the CSN.

Indeed, the exact forms of a 'functional' CSN are not clear. While some of the eight subunits are present only on a ~500 kDa 'core complex-dependent' form, some are present also in forms independent of the eight-subunit complex (which we refer to as 'complex-independent' forms). In *Drosophila*, while both CSN4 and CSN5 are detected in both complex-dependent and complex-independent forms, CSN4 is essential for CSN core complex stability, while CSN5 is not (Oron *et al*, 2002). We therefore refer to CSN4 as a 'core subunit' and to CSN5 as peripheral. Several studies have suggested various types of equilibria between these different forms of subunits (Tsuge *et al*, 2001; Oron *et al*, 2002; Fukumoto *et al*, 2005). This dynamic nature has consequences for experiment interpretation. It is still unclear in many cases which of the activities and phenotypes attributed to CSN reside in the complex as a whole and which reside in the individual subunits (or other complexed forms of the subunits).

One major caveat with many developmental studies employing mutants in CSN is the severe phenotypes of the mutants. The obvious severe morphological phenotypes observed, such as constitutive photomorphogenesis in *Arabidopsis* seedlings, embryo lethality in mice or larval lethality in *Drosophila*, may mask more subtle phenotypes arising from

more specific roles for the complex. Furthermore, such strong phenotypes may be downstream indirect consequences of earlier perturbations resulting from the lack of CSN function.

Transcriptome analysis could be useful in overcoming these limitations for several reasons. First, transcriptome analysis will identify global changes in gene transcription, and not only those that lead to obvious visible morphological changes. Second, transcriptome analysis at early developmental stages can identify changes in gene transcription that precede overt morphological changes. Third, such analysis could be useful for elucidating the underlying molecular basis for observed morphological phenotypes. The fact that in *Drosophila*, as opposed to *Arabidopsis*, mutations in different CSN subunits cause different morphological and molecular phenotypes, makes this model system ideal for studying questions regarding subunit-specific functions. The analysis reported here implies that the neddylation is only a part of CSN function. Developmental timing has a major effect on the expression profiles of the mutants, and our results suggest that mutations in CSN lead to a heterochronic shift in regulation of gene expression. We also show that genes involved in hormone signalling are misexpressed in CSN mutants in a development-dependant manner.

Results

Transcription profiling of CSN mutants

Ma *et al* (2003) showed that in *Arabidopsis*, mutations in CSN1 and CSN8 lead to essentially identical transcriptomes, which correlates well with their identical morphological phenotypes. However, the non-overlapping phenotypes of the *Drosophila* mutants in *csn4* and *csn5*, and the different structural roles of these proteins in the CSN complex (Table I), suggest that the transcriptomes of *Drosophila csn4* and *csn5* mutants should be different. Furthermore, as these mutations are obviously pleiotropic, we reasoned that there could be numerous misregulated pathways that are not morphologically noticeable, and that any transcriptional misregulation could be developmentally specific. To clarify these issues, we initiated a global analysis of transcription profiles on our available CSN mutants.

Two rounds of microarray analyses were carried out. In the first round, the spotted microarrays used contain roughly half of the *Drosophila* estimated coding sequences (GEO GPL4285). Four mutants (*csn4^{null}*, *csn5^{null}*, *csn5¹*, *csn5³*) in two CSN subunits (CSN4 and CSN5) were examined. The two null mutants are true genomic null alleles arising from gene deletion generated by imprecise excisions of P-elements contained in the genes (Freilich *et al*, 1999; Oron *et al*, 2002). Both *csn5¹* and *csn5³* contain missense mutations in the CSN5 gene, maintain a CSN5 protein and were defined molecularly and phenotypically as hypomorphic mutants (Suh *et al*, 2002). mRNA populations were isolated from mutant larvae at three time points, 60, 72, and 96 h after egg deposition (AED), and compared with mRNA populations from age-matched wild-type (wt) larvae. The rationale for choosing these time points is outlined in Table II. Four biological repeats that included dye reversals were conducted for each experiment. The resulting data was standardized and analyzed using the FDR procedure (Reiner *et al*, 2003). The expression levels of 1362 genes, representing ~20% of the genes on the

Table I Summary of the genetic and biochemical analysis of the *Drosophila* CSN

Mutant	Visible phenotypes							Biochemical phenotypes		
	Lethal stage (hours AED)	DNA damage sensitivity	Oocyte defects	Axonal differentiation	Axonal guidance	Melanotic tumors	Molting defects	CSN complex	CSN5 monomer	CSN7 monomer
WT	—	No	No	Normal	Normal	No	No	Present	Present	Present
<i>csn4^{null}</i>	96	Yes	Yes	Defective	ND	No	Yes	Absent	Present	Present
<i>csn5^{null}</i>	> 120 ^a	Yes	Yes	Defective	ND	Yes	No	Present	Absent	Present
<i>csn5¹</i>	96–120	Yes	Yes	Normal	Defective	No	Yes ¹	Present	Absent	Absent
<i>csn5³</i>	96–120	Yes	Yes	Normal	Defective	No	Yes ¹	Present	Absent	Absent

Based on (Freilich *et al*, 1999; Oron *et al*, 2002; Suh *et al*, 2002). AED=after egg deposition. 1—see Figure 7.
^a*csn5^{null}* continue to live during a prolonged third larval instar but never pupariate.

Table II Rationale for time points sampled

Time point	Rationale
60 h AED (mid L2)	Mutant larvae molt from L1 to L2 concurrently with wt (at 48 h AED). There are no apparent differences in body size, phenotype, or behavior between the mutants and wt.
72 h AED (L2/L3 transition)	Differences between <i>csn4^{null}</i> and <i>csn5^{null}</i> mutant larvae are first observed during the second molt at the L2/L3 transition (~72 h AED), where <i>csn4^{null}</i> larvae are unable to molt properly and display a double mouth hook phenotype. <i>csn5¹</i> and <i>csn5³</i> larvae are smaller than <i>csn5^{null}</i> larvae.
96 h AED (mid L3)	Growth arrest of <i>csn4^{null}</i> , <i>csn5¹</i> , and <i>csn5³</i> larvae, with obvious differences in body size observed relative to <i>csn5^{null}</i> and WT. <i>csn5^{null}</i> larvae develop melanotic capsules.

AED=after egg deposition.

Table III Summary of genes identified in the different experimental conditions using the 6k or 12k chip (shown in parentheses)

Mutant	Hours AED	No. of regulated genes > 1.85-fold, FDR < 0.05		
		Up	Down	Total
<i>csn4^{null}</i>	60	176 (493)	61 (210)	273 (703)
	72	191	179	370
	96	187	190	377
<i>csn5¹</i>	60	281	100	381
	72	139	198	337
	96	292	263	555
<i>csn5³</i>	60	293 (739)	95 (297)	388 (1036)
	72	122	153	275
	96	209	193	402
<i>csn5^{null}</i>	60	37 (131)	41 (187)	78 (318)
	72	108	52	160
	96	94	85	179

microarray, were found to change significantly (FDR < 0.05- and > 1.85-fold change) in at least one experiment (Table III). This first round of experimentation is referred to below as '6k' reflecting the number of genes on the chip.

The second round of microarray experiments differed from the first both in the microarray used and in experimental design. The spotted microarrays used in this second round of experiments contain 12144 cDNAs representing most of the *Drosophila* estimated coding sequences (GEO GPL1908). Three mutants (*csn4^{null}*, *csn5^{null}*, *csn5³*) and the wt were examined at 60 h AED. Each sample was hybridized together with a common reference (CR) comprised of a pooled wt RNA sample from multiple larval stages. Four biological repeats including dye reversals were conducted for each experiment. The resulting data were standardized and analyzed using the FDR procedure

(Reiner *et al*, 2003). Differentially expressed genes were determined by comparing mutant versus CR to wt versus CR. The expression levels of 2816 genes, representing ~20% of the genes on the microarray, were found to change significantly (FDR < 0.05- and > 1.85-fold change) in at least one experiment (Table III). This second round of experimentation is referred to below as '12k', reflecting the number of genes on the microarray.

We first determined the overlap between differentially expressed genes detected in the 6k and 12k experiments. The genes on the 6k microarray used in the first round of experimentation are also present on the 12k microarray used in the second round of experimentation. Using a strict cutoff of FDR < 0.05 for both sets of experiments, ~60% of the genes detected in the first round of experimentation were also detected in the second round. This is the minimal overlap based on genes with > 1.85-fold change in gene expression in both experimental sets. When these limitations were relaxed such that we consider genes with > 1.5-fold change if the same gene has a 1.85-fold change in the second experiment, the overlap is much greater (~90%). As seen in Figure 1, not only are the same genes identified but also their direction of differential expression remains the same, that is genes found to be upregulated on the 6k microarray were also detected as upregulated in the 12k microarray. This overlap, based on experiments carried out at different times with *Drosophila* grown in different laboratories and at different times, and with different experimental designs shows a high robustness of the results.

Mutations in one CSN subunit do not affect expression of other CSN subunits

As an initial validation of the arrays and experimental conditions, we examined the expression levels of genes

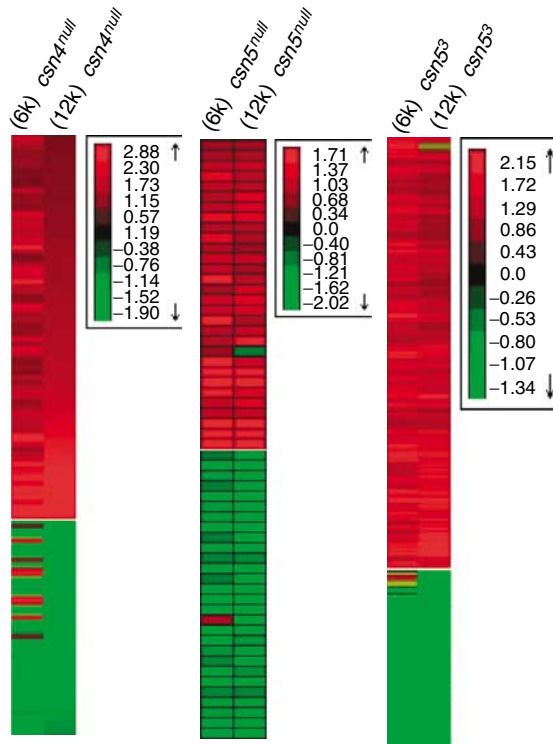


Figure 1 Comparison of the '6k' and '12k' microarray analyses. Heat map analysis of genes present on both chips. The results show genes with > 1.85-fold differential expression and FDR < 0.05 at 60 h AED in at least one of the experimental setups.

encoding CSN subunits (Figure 2A). As expected, *csn4* and *csn5* are not expressed in all experiments using *csn4^{null}* or *csn5^{null}*, respectively. No other genes encoding CSN subunits are misregulated in these strains, nor are any of the *csn* genes significantly misregulated in the two hypomorphic *csn5* alleles examined. In addition to showing that mutations in one CSN subunit do not significantly affect the expression of the mRNAs encoding the other subunits, these results validate the experimental setup by showing that expected down-regulated genes defined by the null mutation are detected.

Mutations in *csn4* and *csn5* lead to unique but overlapping expression profiles

We first examined the overall gene expression overlap between the mutants at different time points using the first round of experiments with the 6k microarray, to determine if the phenotypic differences are manifested in transcriptome differences. The entire data set of 1362 genes was subjected to hierarchical clustering analysis. Several trends are obvious from this analysis. First, Figure 2B shows a dendrogram based on the gene expression profile clustering. The clades center on developmental time points rather than individual mutants; for example, the expression profile of *csn4^{null}* at time 96 is more closely related to *csn5^{null}* at 96 than it is to the profile for *csn4^{null}* at time 72 or time 60. This indicates that different changes occur throughout development, but these changes are similar among the different mutants.

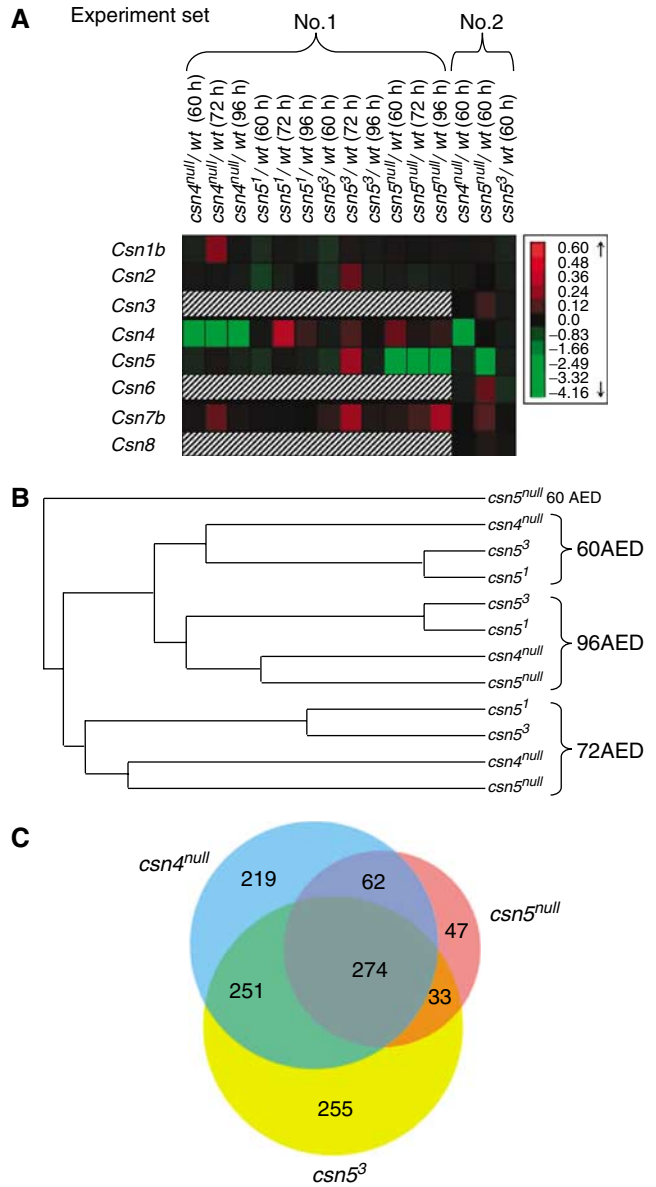


Figure 2 General expression profile analyses of *csn* mutants. Expression profiles of four *csn* mutants were analyzed. For (B) and (C), only genes in experiments using the 6k chip, with > 1.85-fold differential expression and FDR < 0.05 were considered. (A) Heat map of the genes encoding CSN subunits. The genes encoding CSN3, 6 and 8 are not present on the 6k chip and are marked with the hatched box. (B) Dendrogram showing expression profile relatedness following hierarchical clustering of the expression profiles of the four mutants at three time points. (C) Venn diagram showing numbers of common differentially expressed genes between *csn4^{null}*, *csn5^{null}*, and *csn5³*.

Second, the expression profiles of *csn5¹* and *csn5³* are very closely related (> 80% overlap). *csn5¹* and *csn5³* are both hypomorphic alleles: *csn5¹* is mutated at the carboxyl border of the MPN domain and *csn5³* contains a mutation in the middle of the MPN domain (Suh *et al*, 2002). However, both point mutations lead to essentially identical morphological phenotypes and similar transcriptome profiles. Because of this overlap, in some of the subsequent analyses, including the second round of microarray analysis ('12k'), we used only one of these mutants for comparison.

Third, the expression profiles of *csn4^{null}*, *csn5^{null}*, and *csn5³* partially overlap, consistent with each mutation affecting CSN activity leading to mutual underlying molecular effects, while subunits also have additional functions that are not shared with the entire CSN (Figure 2C). While fewer genes are misregulated in *csn5^{null}* relative to the other mutants, most of the expression profile of *csn5^{null}* is shared with both *csn4^{null}* and *csn5³*. The difference between *csn5^{null}* and the other mutants is especially obvious at 60 h AED. At this time point, where the mutants are developmentally indistinguishable from the wt (Figure 3A), *csn4^{null}*, *csn5¹*, and *csn5³* have >280 misregulated genes each, while *csn5^{null}* shows only 75 misregulated genes (Table III).

The CSN represses temporal regulation of gene expression

We examined the distribution of up- and downregulated genes in the mutants at the different time points. As seen in Table III and Figure 3B, for mutants *csn4^{null}*, *csn5¹*, and *csn5³*, the number of up- and downregulated genes was more or less equal at 72 and 96 h AED, while at 60 h AED, in both experimental sets, there were more up- than downregulated genes. Very few genes are misregulated at all in *csn5^{null}* at 60 h AED (Table III), although a similar skew toward upregulated genes is also noticed in *csn5^{null}* at 72 h as opposed to 96 h AED.

At 60 h AED, all four mutants are morphologically indistinguishable from each other and from wt in terms of body size and behavior (Figure 3A). We therefore reasoned that at this time point the underlying molecular differences between the mutant and wt larvae represent more primary effects of the mutations. We further hypothesized that similar to the role of the *Arabidopsis* CSN as a general repressor of environmentally induced gene expression, the prevalence of up- rather than downregulated genes in the mutants at 60 h AED indicates that the *Drosophila* CSN is a general repressor of various developmental/temporal cues that induce gene expression. In absence of the CSN repressor activity, as in the mutants, these genes would be expressed achronically. If this hypothesis is true, then groups of genes that are upregulated (derepressed) in the mutants at 60 h AED should be induced at different developmental stages in the wt. In other words, these genes would normally be repressed at 60 AED relative to other developmental stages (before or after 60 AED) where the transcription is induced. As the experimental setup of the 12k experiment included a common control, we could check the behavior in the wt of the genes that are misregulated at T_{60} in the mutants. This could not be determined in the first round of experimentation (6k) owing to experimental design. As seen in Figure 4, most of the genes that are upregulated in the mutants at T_{60} are normally downregulated in the wt at T_{60} relative to the common control.

More specifically, we further hypothesized that these genes would normally be upregulated early in development and repressed at 60 AED, but not repressed in the mutants; alternatively, they would normally be upregulated later in development, but derepressed early in the mutants. To test this hypothesis, we used the available data sets from the developmental time-course expression profiling of wt

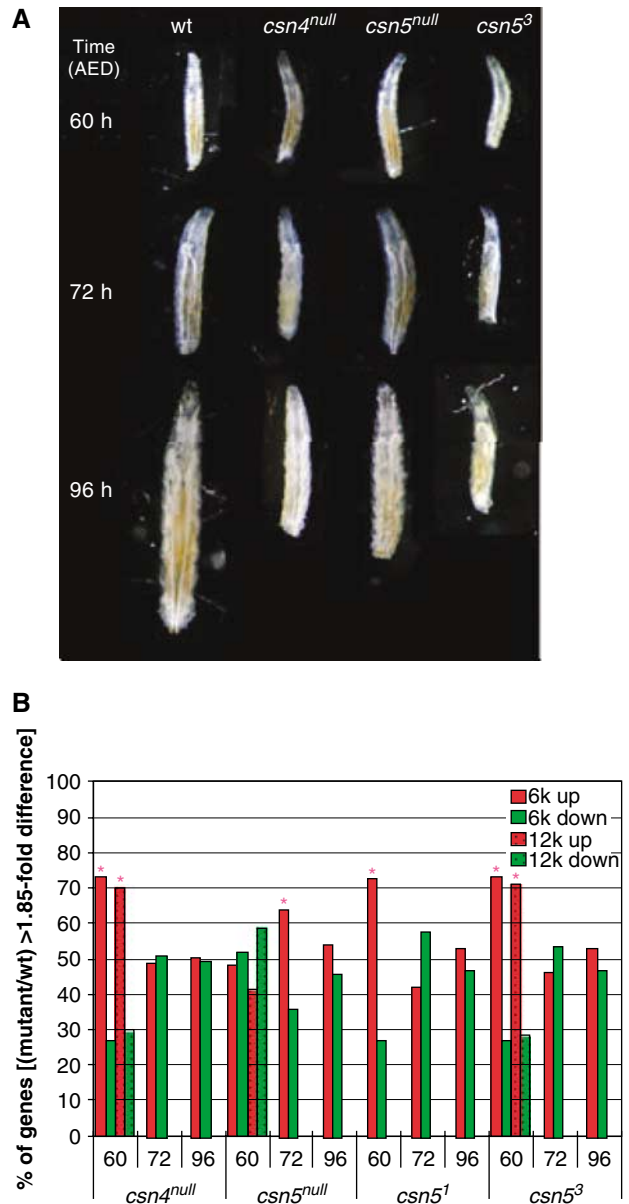


Figure 3 (A) Morphology of *csn* mutants and wt at 60, 72, and 96 h AED. (B) Distribution of up- and downregulated genes in the mutants at the time points checked. The stars designate time points with more up- than downregulated genes. Only genes with > 1.85-fold differential expression and FDR < 0.05 were considered.

Drosophila (Arbeitman et al, 2002) (Figure 5). A total of 54 and 33% of the genes in the 6k and 12k microarrays respectively that are upregulated in the *csn* mutants at 60 h AED are present in the Arbeitman et al (2002) data set. Briefly, we searched for the normal time of induction of genes that were derepressed in the *csn* mutants at 60 AED. We mined for time points (T) at which the misregulated genes (g) at 60 h AED (T_{60}) in the *csn* mutants fit the following criterion: $g_n^{wt}(T_j) > 2g_n^{wt}(T_{60})$, where T_j is the normal time of induction of a gene n that is upregulated at T_{60} in the mutant. Of the genes upregulated in the mutants at T_{60} , >85% fit the requirements above, that is, are upregulated in the wt at a different developmental time relative to T_{60} (Figure 6A). Similarly, of the genes upregulated in

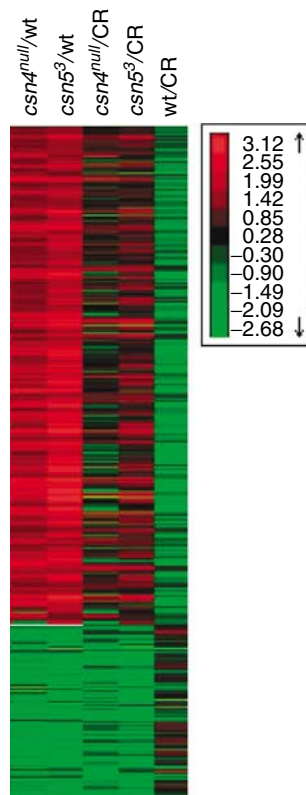


Figure 4 Genes upregulated in the mutants at T_{60} are normally downregulated in the wt. The first two columns on the left show the expression levels of genes in *csn4^{null}* and *csn5³* relative to wt, such that the red represents genes upregulated and the green downregulated genes. The next two columns show the expression levels of genes in *csn4^{null}* and *csn5³* relative to the common reference. The first column on the right shows the expression levels of the same genes in wt relative to the common reference. Note that most of the genes identified as upregulated in the mutants in the first two columns are green (downregulated) in the wt relative to the common reference.

csn5^{null} at T_{72} , >85% fit the same requirements. A total of 81% of the genes identified as time shifted in the 6k experiment were also identified in the 12k experiment (Supplementary Table I).

Figure 6B shows the normal expression pattern in the wt of selected genes that are upregulated at T_{60} in the mutants. Three classes of genes are identified. The first class comprises genes normally upregulated before T_{60} . A clear example here is *apontic* (*apt*), which encodes a Myb-homeodomain transcription factor involved in a number of early developmental processes including heart and central nervous system development. *apt* is strongly induced during embryogenesis and repressed in later development. *apt* levels are highly induced in *csn4^{null}*, *csn5¹*, and *csn5³* at T_{60} . Interestingly, although *apt* is not induced in *csn5^{null}* at T_{60} , it is induced in this mutant at T_{72} , providing further evidence for a delay in phenotypic development in *csn5^{null}* relative to the other mutants. The second class contains genes that are normally upregulated later in development, as seen in CG8505. CG8505 encodes a structural component of the cuticle and is normally induced only during late metamorphosis. CG8505 levels are increased in *csn4^{null}*, *csn5¹*, and *csn5³* at T_{60} . The third class of genes is induced in both early and late development, relative to T_{60} . An example of this class, *epithelial membrane protein* (*emp*) is

involved in apoptosis connected to autophagy and defense responses. *emp* is normally induced in late embryogenesis and during metamorphosis, but is induced in *csn4^{null}*, *csn5¹*, and *csn5³* at T_{60} , and in *csn5^{null}* at T_{72} .

To determine if CSN-dependent repression of gene expression is time specific, we plotted the number of genes identified in each time point as a function of T_j (Figure 7). As a control, we sampled random groups of genes from the data set. In agreement with our hypothesis, we find that transcript levels of genes upregulated in the mutants normally increase in the wt predominantly at one of two time windows—either during late embryogenesis (16–24 h) or late larval/early metamorphosis (96–126 h). The transcript levels of genes upregulated in *csn5^{null}* at T_{72} normally increase in the wt also in these same two time windows. Random sampling indicated that these windows are enriched above a 95% confidence interval, and shows that in the wt, the largest group of genes is normally induced, relative to T_{60} , very early in development (<12 h AED).

The ‘time-shifted’ genes were analyzed for two GO criteria, ‘biological process’ and ‘molecular function’ (Table IV). Several GO definitions are statistically more prevalent among the time-shifted genes. While a more detailed GO analysis of the entire data set will be presented elsewhere, several trends are obvious from this initial analysis. First, >70% of the ‘time-shifted’ genes with a known GO definition are clearly involved in some aspect of development or signaling (Supplementary Table I). Second, several of the GO groups identified as significantly enriched are closely associated with cellular stress responses including oxidoreductase activity, electron transport and the antibacterial humeral response. Indeed, this last group is especially interesting in light of the melanotic tumor phenotype of *csn5^{null}* (Oron *et al*, 2002) and the implication of *Arabidopsis* CSN in plant defense responses (Azevedo *et al*, 2002; Liu *et al*, 2002). Consistent with problems in molting (see Figure 8), genes involved in cuticle formation (chitin metabolism and binding) are also enriched in the time-shifted genes. One other group worth mentioning are genes involved in amine metabolism. These genes could potentially affect a wide range of responses, including timing of gene expression, through their essential function in dopamine biosynthesis (De Luca *et al*, 2003; Jordan *et al*, 2006).

Ecdysone-regulated genes are misregulated in *csn* mutants

In light of the abnormal molting phenotype of *csn4^{null}* (Oron *et al*, 2002) and that CSN2 was shown to interact with *Drosophila* Ecdysone receptor (EcR) (Dressel *et al*, 1999), we previously suggested that the CSN is involved in steroid hormone signaling in *Drosophila* development (Oron *et al*, 2002). To further explore this hypothesis, we examined groups of genes present on the 6k microarray that had been implicated in ecdysone action (Andres *et al*, 1993). Indeed, a group of genes implicated in ecdysone signaling are misexpressed in *csn* mutants (Figure 8B).

All mutants analyzed showed misregulation of this group of genes. However, although these genes are misexpressed in

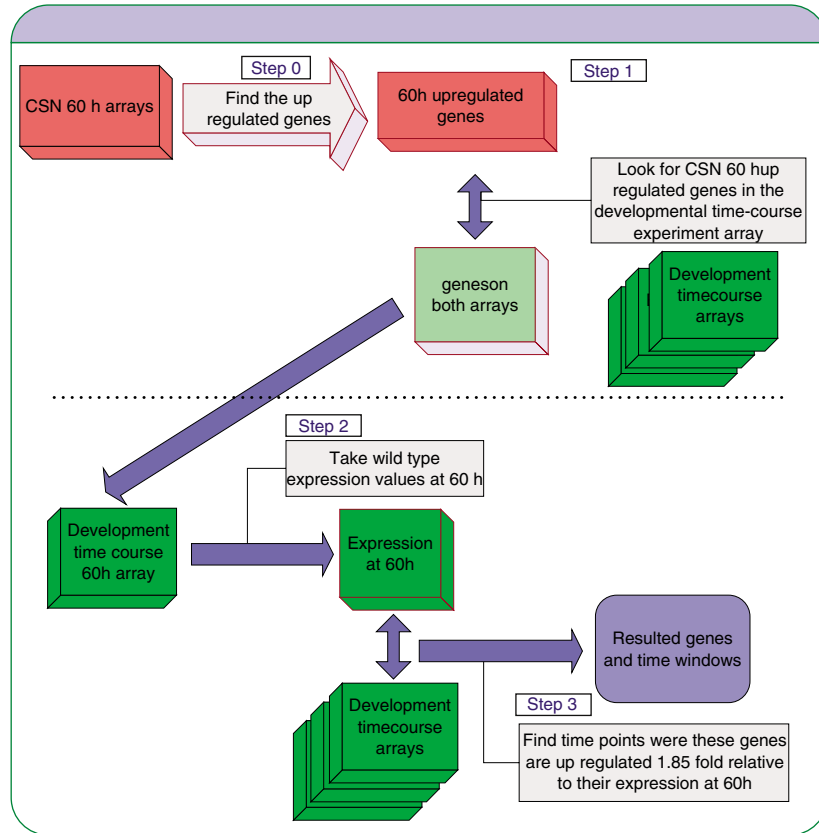


Figure 5 Overview of time-shift analysis, comparing the data presented here with that shown in Arbeitman *et al* (2002).

both *csn4* and *csn5* mutants, we find a difference in the onset of their misexpression. While in *csn4^{null}* and the *csn5* hypomorphs, a majority of these genes show abnormal expression already at 60 h AED, in *csn5^{null}*, the misexpression is evident only at 72 h AED, as seen for *Ftz-f1*, *ImpL1*, and *ImpE2* (Figure 8B). This raises the possibility that the reason that *csn5^{null}* does not display the molting phenotypes clearly observed in *csn4^{null}* is not because CSN5 and CSN4 regulate different pathways, as had been proposed, but rather because of different temporal disruption of the same pathways. That is, the loss of both CSN4 and CSN5 results in deregulation of ecdysone signaling, but this deregulation manifests earlier in *csn4^{null}* than in *csn5^{null}*.

To validate these results, RNA was isolated from larvae at 60 h AED from newly established cultures of each mutant and the wt and was used as a template for quantitative reverse transcription-PCR (RT-PCR) (Figure 8C). The transcript levels of three representative genes, *Ecr* and *ftz transcription factor 1* (*ftz-f1*), whose gene products physically interact with the CSN2 (Dressel *et al*, 1999) and *Preintermolt Gene 1* (*pig1*) were found to be upregulated relative to the wt in *csn4^{null}* at 60 AED and downregulated in *csn5^{null}*, confirming the microarray results.

As the transcriptome of the *csn5* hypomorphic mutants was similar to that of *csn4^{null}* in general (Figure 2) and specifically with regard to the timing of ecdysone-related genes, we further examined these mutants for morphological changes. As seen in Figure 8D, *csn5³* larvae at 72 h AED display double mouth

hook phenotypes similar to those seen in *csn4^{null}* (Oron *et al*, 2002). This phenotype was not observed in *csn5^{null}*. Thus, from the transcriptome results, we succeeded in predicting a morphological change that was confirmed experimentally.

Discussion

We present here the first genomic analysis of animal mutants in the COP9 signalosome. Using two different experimental setups, entailing two different cDNA arrays, different controls, and carried out with strains grown in different labs and at different times, we have obtained similar results indicating that (1) the CSN appears to act as a transcriptional repressor at 60 h AED, (2) the genes repressed by the CSN are normally induced at earlier or later developmental time points, (3) CSN5 carries only part of CSN functions, and (4) the hypomorphic mutations in *csn5* are more severe than the null mutation.

A major difficulty in analysis of mutants in the COP9 signalosome is determining which of the many phenotypes arise directly from the lesions in the CSN, and which are pleiotropic downstream effects. Indeed, the severe phenotypes of mutants in the CSN in *Drosophila*, mouse, and *Arabidopsis* all converge on death. But mutations in CSN itself are not likely the direct cause of death, as null mutations in the CSN in several organisms including *S. pombe*, *Caenorhabditis elegans*, and *Aspergillus nidulans* are not lethal (Mundt *et al*, 1999; Maytal-Kivity *et al*, 2002; Busch *et al*, 2003). That mutation in

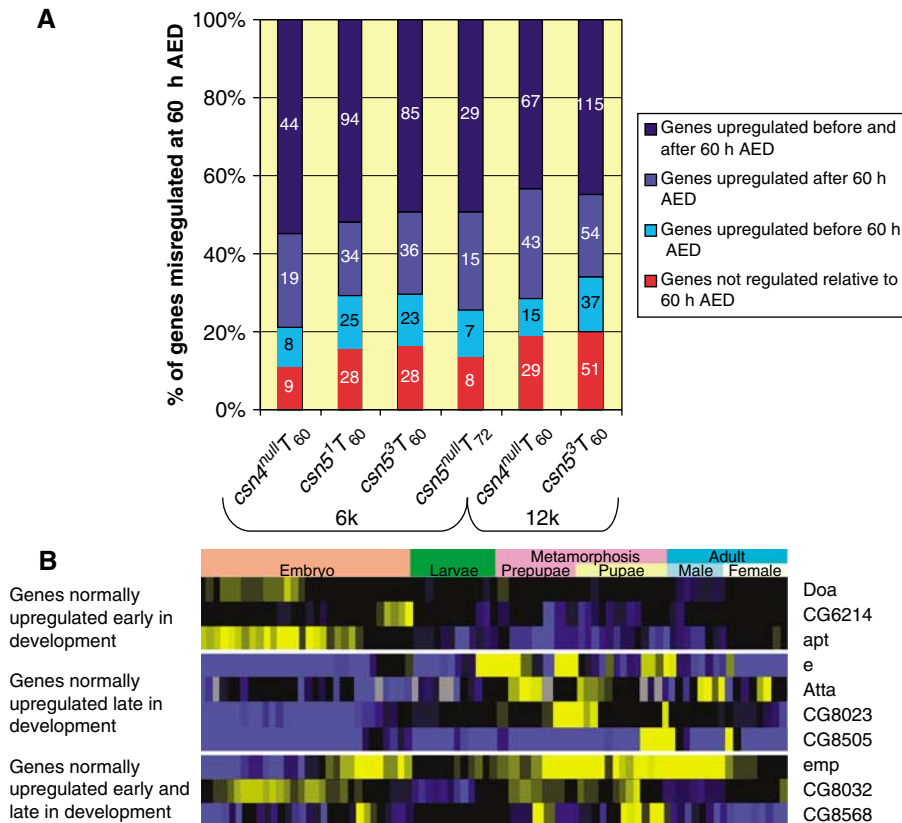


Figure 6 Genes upregulated at 60 h AED in the mutants are putatively time shifted. **(A)** Percentage of upregulated genes that are putatively time shifted in the first (6k) and second (12k) rounds of microarray analysis, and their predicted normal time of induction. The number in each bar is absolute number of genes. **(B)** Snapshot of wt developmental time-course data (based on data in Arbeitman *et al*, 2002) for genes putatively time shifted in the *csn* mutants. Representative time-shifted genes that were identified in both the first and second sets of experimentation are shown for each category (see Supplementary information for complete lists). Yellow shows upregulation while blue shows downregulation.

CSN leads to accumulating downstream effects can be seen in the transcription profiles of later developmental stages. The number of misregulated genes increases with time in all mutants and the overlap between the mutants also increases. This is likely due to each mutant approaching developmental arrest and subsequent death.

Can such analysis help us in elucidating the contribution of individual subunits to CSN function? Comparison of the profiles of the null mutants in *csn4* and *csn5* with the point mutations in *csn5* can help answer this question. While fewer genes are misregulated in *csn5^{null}* relative to the other mutants, about two-thirds of the expression profile of *csn5^{null}* is shared with *csn4^{null}*, *csn5¹*, and *csn5³*. This suggests that part of the molecular phenotype of *csn4^{null}*, *csn5¹*, and *csn5³* is due to the absence of CSN5 activity, presumably its deneddylase activity. However, as CSN4 is essential for CSN complex integrity, while CSN5 is not (Oron *et al*, 2002; Dohmann *et al*, 2005), the additional genes misregulated in *csn4^{null}* could represent functions dependent on the entire CSN complex (which remains intact in *csn5^{null}*) but not connected to CSN5-mediated deneddylation. As the overlap between *csn4^{null}*, and *csn5¹*, and *csn5³* extends beyond those genes shared with *csn5^{null}*, that is, that these mutants affect the expression of genes not dependent on CSN5 itself, this suggests that the

point mutations in *csn5* affect the entire CSN complex, leading to a molecular and physical phenotype that mimics loss of the entire complex.

As the *Arabidopsis* CSN is a negative regulator of transcription factor stability, we hypothesized that a lack of CSN activity in *Drosophila* would lead to transcription factor stabilization and increase in target gene activation. The first evidence supporting this was the larger number of up- than down-regulated genes early in development before any obvious morphological difference between the mutants and wt. This enrichment of upregulated genes was not detected at later time points, further indicating that the transcription profiles at these times reflect mainly secondary effects of loss of CSN function. As a number of transcription factors are induced at T₆₀ in the mutants (e.g. *dei1*, *ken*, *Mef2*, *ftz-f1*, *bun*, *Rel*, and *apt*), it is likely that some of the expression profile of the mutants in later development is due to induction of target genes for these transcription factors.

Comparison of the upregulated genes with the developmental transcriptional data provided further evidence for the CSN as a repressor of developmentally regulated transcription. The time-shift analysis developed here revealed that the vast majority of the genes upregulated in the *csn* mutants early in development are normally induced at other specific stages.

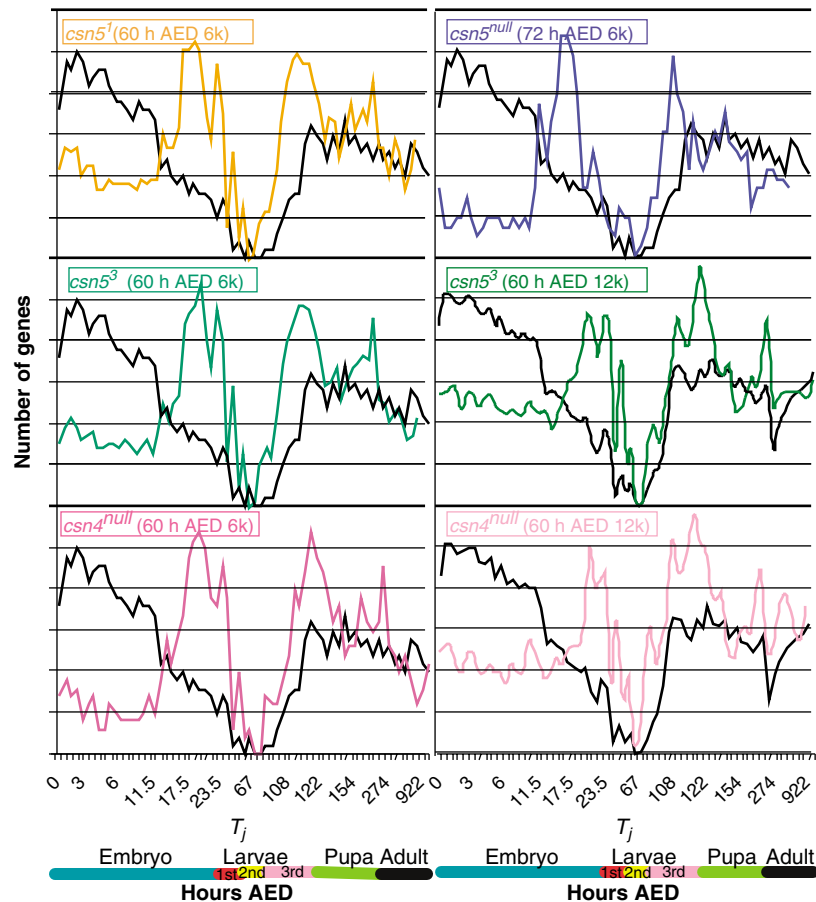


Figure 7 Time-shift analysis. For each mutant, the wt expression levels of putatively time-shifted genes identified in Figure 6A were compared relative to their expression levels at T_{60} such that at T_{60} , the relative value=1 (base line). The y-axis shows the number of genes upregulated (e.g. relative value > 1) at a particular time point. The black lines represent the average of > 100 random gene groups that were sampled. Note that for $csn5^{null}$, the analysis was carried out using T_{72} . The total number of genes in each analysis is different as detailed in Figure 6A and is presented here as normalized.

The largest group of genes is normally induced at late embryogenesis. That lack of CSN function should predominantly affect genes regulated at late embryogenesis is especially interesting considering that CSN subunits are maternally contributed. Both CSN4 and CSN5 are maternally contributed to the egg and this contribution abates by late embryogenesis (Oron *et al*, 2002). The overlap between loss of maternally contributed CSN and normal induction pattern of misregulated genes suggests that the increase in gene derepression reflects a primary response caused by the mutations.

Maternally contributed CSN is a potential stumbling block in the analysis of our results. The use of germline clones to remove the maternal contribution was not useful as this resulted in similar oogenic arrest at stages 5–6 for $csn4^{null}$, $csn5^{null}$, and $csn5^1$ (Oron *et al*, 2002). While maternally contributed CSN is depleted by late embryogenesis, we do not know the exact timing of this depletion, and indeed, a slight difference in persistence of the maternally contributed subunit could have drastic developmental effects. Certainly, exact timing of changes in transcriptional regulation has major phenotypic consequences. For example, while both $csn4^{null}$ and $csn5^{null}$ show misregulation of ecdysone-associated genes,

only $csn4^{null}$ shows an ecdysone-related phenotype, probably due to the earlier appearance of misregulation of these genes.

However, if differences in maternal contribution were the major cause of the phenotypic differences, we would expect that maternally contributed CSN5 would persist in the null background as in the hypomorphic backgrounds, leading to similar timing of phenotypic changes, which is not the case. Alternatively, the $csn5^{null}$ mutant larvae display mutant phenotypes later than the hypomorphic $csn5$ and $csn4^{null}$ mutants not because of the difference in maternal contribution but rather because of the persistence of a CSN5-less CSN complex, which we hypothesize maintains partial function. $csn4^{null}$ larvae lack the entire CSN complex and thus this partial function would be missing. Apparently, the mutated CSN5 protein in the hypomorphic alleles also disrupts CSN function.

This possibility is supported by the morphological and transcriptome phenotypes of $csn5^1$ and $csn5^3$, which are much more similar to $csn4^{null}$ than $csn5^{null}$. These mutants maintain a CSN that contains the mutated CSN5 (Oron *et al*, 2002), but apparently the point mutations in CSN5 have a negative effect on the entire CSN, such that a mutated CSN5 in the complex is worse than a lack of CSN5. However, this is not a classic

Table IV Gene ontology classifications for 'time-shifted' genes

GO category	No. of genes (array)	% of array	No. of genes (time shift)	% of time-shifted genes	P-value	
<i>Biological process</i>						
6k	Chitin metabolism	23	0.44	6	4.26	6.75E-06
	N-acetylglucosamine metabolism	25	0.48	6	4.26	3.66E-05
	Glucosamine metabolism	25	0.48	6	4.26	4.60E-05
	Amino sugar metabolism	25	0.48	6	4.26	4.60E-05
	Amine metabolism	111	2.13	12	8.51	1.21E-04
	Cellular polysaccharide metabolism	31	0.60	6	4.26	1.31E-04
	Electron transport	113	2.17	11	7.80	1.63E-04
	Nitrogen compound metabolism	115	2.21	12	8.51	1.63E-04
	Polysaccharide metabolism	31	0.60	6	4.26	2.30E-04
	Antibacterial humoral response	3	0.06	2	1.42	3.32E-03
	Cellular carbohydrate metabolism	103	1.98	8	5.67	5.28E-03
12k	Electron transport	203	5.86	18	20.22	5.58E-06
	Antibacterial humoral response	13	0.59	5	10.64	2.25E-05
	Amine metabolism	178	4.54	15	14.56	9.17E-05
	Catecholamine metabolism	7	0.32	3	6.38	9.29E-04
	Response to starvation	2	0.06	2	2.25	3.60E-03
	Amino-acid metabolism	105	3.5	8	11.27	4.04E-03
	Chitin metabolism	52	2.36	5	10.64	5.98E-03
	Nitrogen compound biosynthesis	27	0.78	4	4.49	6.89E-03
	N-acetylglucosamine metabolism	57	2.59	5	10.64	8.54E-03
	Glucosamine metabolism	57	1.9	5	7.04	1.34E-02
	Cellular polysaccharide metabolism	62	2.07	5	7.04	1.83E-02
	Polysaccharide metabolism	62	1.79	5	5.62	2.51E-02
<i>Molecular function</i>						
6k	Pattern binding	21	0.40	7	4.96	7.71E-06
	Oxidoreductase activity	207	3.97	19	13.47	1.00E-05
	Carbohydrate binding	33	0.63	8	5.67	1.22E-05
	Iron ion binding	64	1.22	10	7.09	2.64E-05
	Polysaccharide binding	18	0.34	6	4.25	4.57E-05
	Monooxygenase activity	39	0.74	8	5.67	4.65E-05
	Chitin binding	16	0.30	5	3.54	3.97E-04
	Tetrapyrrole binding	34	0.65	6	4.25	7.13E-04
	Heme binding	34	0.65	6	4.25	8.97E-04
	Oxidoreductase activity, acting on CH-OH group of donors	27	0.53	5	3.55	2.04E-03
12k	Oxidoreductase activity	340	5.83	32	19.75	4.21E-06
	Monooxygenase activity	63	1.69	13	11.5	1.48E-04
	Iron ion binding	108	4.27	17	19.54	1.76E-04
	Tetrapyrrole binding	58	1	11	6.79	7.17E-04
	Heme binding	58	1.55	11	9.73	1.14E-03
	Structural constituent of cuticle	40	0.69	6	3.7	3.44E-01
	Oxidoreductase activity, acting on CH-OH group of donors	45	1.21	6	5.31	7.45E-01
	Pattern binding	51	0.88	5	3.09	1.71E-02

The entire group of 'time-shifted' genes identified in Figure 6 were subjected to GO analysis using *Babilomics FatiGO* (Al-Shahrour *et al*, 2006). Only significantly enriched GO groups are shown.

dominant-negative effect, as heterozygotes for the point mutation (i.e. *csn5^l/+*) are completely wt in phenotype, consistent with a hypomorph, and any effect is only seen once the normal CSN is not present. Biochemically, while these point mutations do not affect CSN complex stability, they do lack CSN-mediated Cul1 deneddylation activity, similar to the null mutants (not shown), suggesting some type of neo-morphic action, and influence the stability of the complex-independent forms of other CSN subunits. For example, these mutants lack the complex-independent forms of both CSN5 and CSN7 (Oron *et al*, 2002). We thus conclude that the phenotypes detected are likely consequences of primary lesions and not simply mistiming of maternal genes.

This has major implications for our understanding of CSN function. The most celebrated function of the CSN is as a deneddylase or deubiquitinase. Both functions are dependent on CSN5, which carries the catalytic activity (Cope *et al*, 2002). However, if these were the only, or even most central, roles for the CSN, we would expect that the transcriptome phenotype of the *csn5^{null}* mutant would be very similar to that of the *csn4^{null}* mutant. As *csn4^{null}* and the hypomorphic *csn5* mutants affect more genes, we conclude that the entire CSN has additional functions that are not dependent on CSN5.

In trying to understand the specific roles of individual subunits, we looked at transcriptome overlap. The lack of complete overlap between mutants could be due to several

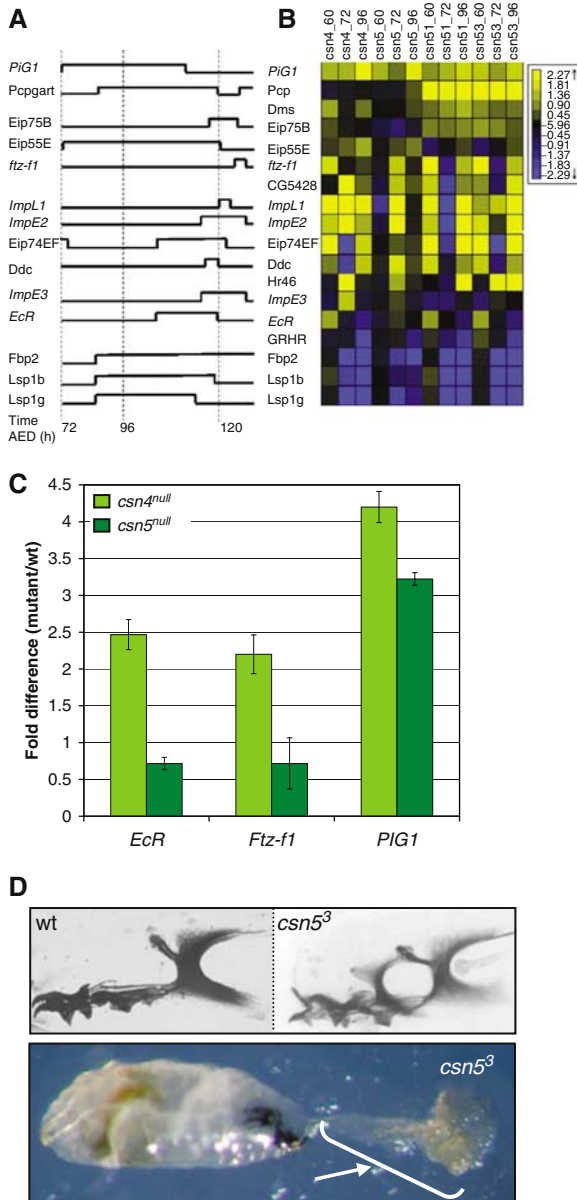


Figure 8 Ecdysone-regulated genes are misexpressed in *csn* mutants. **(A)** wt expression pattern of ecdysone-related genes during larval development (adapted from Andres *et al* (1993)). **(B)** Heat map of expression levels of the same genes in (A), plus additional genes. **(C)** Expression at 60 h AED of three ecdysone-regulated genes (*EcR*, β *Ftz-1*, and *PIG1*) in two CSN mutants (*csn4^{null}* and *csn5^{null}*) was validated using QRT-PCR. Each bar shows the relative mRNA expression of mutant versus wt with associated standard errors. Data shown are averages of quadruplicate QRT-PCR measurements. *Rp49* was used for normalization. **(D)** *csn5³* mutant L3 larvae contain a double set of mouth hooks. The top two panels show mouth hooks from wt (left) and *csn5³* (right) larvae dissected following the second molt (~80 h AED). The bottom panel illustrates that *csn5³* larva is unable to undergo the second/third instar molt, resulting in an L2 cuticle (designated by the arrow) attached through the L2 mouth parts to the L3 mouth parts.

factors: (1) it could represent true differences between mutants; (2) it could represent differences in the strains not connected to the CSN lesion; and (3) it could be a result of inherent error in the transcriptome analysis. While our results discussed above clearly point to phenotypic differences arising

from differences in effects on timing of gene expression, finding clear and specific differences in gene regulation aside from timing between the mutants is not trivial. When the experiments were repeated with different controls and with strains grown at different times and places, we found a very large overlap between profiles for the same mutants, indicating a robustness of our results. However, the drift in results is large enough to explain a major part of the transcriptome differences between any two mutants. Determining subunit-specific effects will necessitate an expanded analysis that includes more mutants, to find out specific differences between CSN subunits.

Materials and methods

Fly stocks and growth conditions

wt and mutant strains were maintained on standard medium and all experiments were performed at 25°C. Fly stocks: *w¹¹¹⁸* was used as wt. Three mutant alleles of *csn5* and one of *csn4* were utilized. *csn5^{null}* and *csn4^{null}* were generated by imprecise P-element excision, resulting in protein-null mutations (Freilich *et al*, 1999; Oron *et al*, 2002). *csn5¹* and *csn5³* alleles were generated by EMS mutagenesis and encode the missense mutations E160V and T100I respectively (Suh *et al*, 2002). *csn5* and *csn4* mutations were balanced over *TM3*, *Ser GFP* and *CyO*, *GFP* respectively, to permit sorting of homozygous mutant larvae.

Transcriptional profiling

For all microarray experiments, larvae were staged from egg deposition, collected in 2-h time windows following hatching and raised at a density of 50 per vial at 25°C. Age-matched mutants and wt larvae were collected at 60, 72, and 96 h time points AED. Total RNA was extracted with TRIzol reagent (Invitrogen) followed by RNeasy (Qiagen) clear up. cRNA targets were generated and coupled to either Cy3 or Cy5 fluorophores according to 'FHCRC Genomics Resource DNA Array Laboratory' protocols. Hybridization and scanning were performed by the FHCRC Genomics Resource DNA Array Laboratory. Expression profiles were performed using spotted microarrays (GEO GPL4285='6k' and GEO GPL1908='12k'). Microarray images were quantified using GenePix Pro software (Axon Instruments). Statistical analysis was conducted in R (R 2.2.1—The R Development Core Team). Raw expression readings were log-transformed using the RSVN package (Huber *et al*, 2002); within-array expression measurement biases were removed by applying LOWESS normalization to expression log ratios; and the FDR was controlled by computing FDR adjusted *P*-values (Reiner *et al*, 2003) to test for differential expression of each gene in each CSN mutant and comparing them to 0.05. Data were generated from four or five independent replicates (two with one dye orientation and two/three with the reversed dye orientation).

Heat maps and clustering analysis were performed with EXPANDER (EXpression Analyzer and DisplayER) gene expression analysis and visualization tool Version 2 (Shamir *et al*, 2005). GO analysis employed Babelomics (Al-Shahrour *et al*, 2006).

Time-shift analysis

The definition and mathematical properties of a 'time shift' as well as the algorithms and heuristics employed are described in detail in Tuller *et al* (2005).

The expression data have been deposited in the GEO database under accession number GSE7303.

Quantitative RT-PCR

Quantitative real-time reverse transcription-PCR (qPCR) reactions were carried out to validate changes in expression of three ecdysone-related genes (*EcR*, *Ftz-1*, and *PIG1*). RNA was extracted from *w¹¹¹⁸*,

csn4^{null}, and *csn5^{null}* larvae as described above. cDNA synthesis was carried out using Superscript II (Invitrogen).

Four qPCR primer pairs were designed to generate intron-spanning products using Primer Express Version 1.5 software:

EcR: (F) 5'-GCCACTATTCCGCTACTACCTGA-3' (R) 5'-ATATAACGGCCAACCTGATTGTACG-3'; *Ftz-f1*: (F) 5'-GATCTGAAGGTCGACGACCAA-3' (R) 5'-CGTTATGGATTTCGATGATGCAG-3'; *PIG1*: (F) 5'-GAGTCCAGTGCACGAACAGT-3' (R) 5'-CGGGAACCATCGTCCACATC-3'; and *RP49*: (F) 5'-TAAGCTGTCCACAAATGGC-3' (R) 5'-ACCGATGTTGGGCATCAGATA-3'.

qPCR analysis was performed on an ABI Prism[®] 7000 Sequence Detection System (Applied Biosystems) using SYBR Green[®] I chemistry. Samples were in triplicate and normalized for RNA levels based on *rp49* expression. Analysis was performed with the ABI Prism 7000 SDS software RQ study Application v1.1 using the $\Delta\text{-}\Delta C_t$ method, which determines fold changes in gene expression relative to a comparative sample (age matched *w¹¹¹⁸*).

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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