**Drosophila COP9 signalosome subunit 7 interacts with multiple genomic loci to regulate development**

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**ABSTRACT**

The COP9 signalosome protein complex has a central role in the regulation of development of multicellular organisms. While the function of this complex in ubiquitin-mediated protein degradation is well established, results over the past few years have hinted that the COP9 signalosome may function more broadly in the regulation of gene expression. Here, using DamID technology, we show that COP9 signalosome subunit 7 functionally associates with a large number of genomic loci in the *Drosophila* genome, and show that the expression of many genes within these loci is COP9 signalosome-dependent. This association is likely direct as we show CSN7 binds DNA in vitro. The genes targeted by CSN7 are preferentially enriched for transcriptionally active regions of the genome, and are involved in the regulation of distinct gene ontology groupings including imaginal disc development and cell-cycle control. In accord, loss of CSN7 function leads to cell-cycle delay and altered wing development. These results indicate that CSN7, and by extension the entire COP9 signalosome, functions directly in transcriptional control. While the COP9 signalosome protein complex has long been known to regulate protein degradation, here we expand the role of this complex by showing that subunit 7 binds DNA in vitro and functions directly in vivo in transcriptional control of developmentally important pathways that are relevant for human health.

**INTRODUCTION**

The COP9 signalosome (CSN) protein complex was originally described as a negative regulator of light-induced growth patterns in plants (1). Subsequently it was found to have a central role in the regulation of development of multicellular organisms (2–4). In higher eukaryotes, the complex contains eight subunits, termed CSN1, for the largest subunit, to CSN8 for the smallest. Six of the CSN subunits, CSN1, 2, 3, 4, 7 and 8, contain a motif termed PCI (Proteasome-CSN-Initiation factor 3). A number of studies have indicated that the PCI domains interact to stabilize and form the backbone of these multiprotein complexes (5–7). Some of the subunits, including CSN7 are detected also in forms independent of the CSN core-complex (8–10).

The most studied CSN function is regulation of protein degradation. The CSN regulates cullin-RING E3 ligase (CRL) activity by removal of the ubiquitin-like protein Nedd8, from the cullin subunit of CRLs. This deneddylation activity localizes to CSN5 and is dependent on the integrity of the complete CSN complex (11,12). However, CSN5-dependent deneddylation is apparently only one aspect of CSN function (5,9,13–16).

Many CSN activities impinge on transcriptional regulation. Through its regulation of protein degradation, the CSN affects the stability of transcription factors (17–19). Yet, several studies hint that the nuclear CSN regulates transcription directly. For example, CSN2 (Alien) interacts with the corepressor SIN3 (20,21), and several studies revealed an association of CSN subunits to specific genes (22–24). These results together lead to the hypothesis that the CSN functions more broadly in the regulation of gene expression (25).

This hypothesis was backed up by structural studies showing that CSN7 has characteristics of known nucleic acid-binding proteins (26,27). The PCI domain contains a putative nucleic acid-binding motif in its winged-helix subdomain, which is comprised of a canonical helix-turn-helix.

Here we show that *Drosophila* CSN7 indeed binds DNA and functionally associates with a large number of loci in the *Drosophila* genome. Furthermore, we show that the transcription of many of these genes in *vivo* is dependent on
the entire CSN complex. Disruption of CSN7 leads to cellular and developmental phenotypes, which correlate with the predicted functions of CSN7 targets. Our results open a new line of studies for understanding how the CSN functions as a transcriptional regulator of developmentally-regulated genes and has direct implications for CSN involvement in human disease.

MATERIALS AND METHODS

‘Fly growth conditions’ were as published (9) with the following additions: Canton-S (CS) strain was used as wild type. RNAi-csn7 (#40691), RNAi-csn8 (#50565) and RNAi-csn2 (#48044) were obtained from VDRC stock center. Csn7p (#18023), Actin-GAL4 (#3954) and Engrailed-GAL4 (#25752) were obtained from Bloomington stock center. Additional information including complementation analysis and transgenic flies is found in the Supplementary Methods.

Cell lines and cell culture

Drosophila Kc167, SR+ and S2 cell lines were cultured in SFX medium (Thermo Scientific HyClone) supplemented with 10% fetal bovine serum (FBS) (Biological Industries) and penicillin–streptomycin (Biological Industries), respectively, at 25°C. The RNAi procedures are detailed in the Supplementary Methods.

Anisotropy experiments

Expression and purification of CSN7 was as described (26). For binding assays, the DNA strands were synthesized and labeled using fluorescein attached through a six-carbon linker to the 5′ end of the oligonucleotide primer used for polymerase chain reaction (PCR) synthesis. CSN7 protein was serially diluted from 4000 to 25 nM in buffer solution consisted of 10 mM Tris–HCl, 1 mM EDTA, 0.1 mM dithiothreitol (DTT) and 75 mM KCl, to a final volume of 60 μL. The indicated DNA was added to a final concentration of 3 nM and in competition experiments to final concentration of 150 nM. The anisotropy was measured using a standard four-parameter binding curve using Sigmaplot (Systat). The equation used was:

\[
\Delta r = \min + \frac{\max - \min}{1 + (\frac{[CSN7]}{EC50})^{-\text{Hill slope}}}
\]

where \( r \) is anisotropy, max and min are the respective maximal and minimal anisotropy values and the EC50 we treat as the \( K_{D,\text{app}} \).

‘DamID’ was carried out in Kc167 cells as described (28). The pNDamMyc-CSN7 fusion construct is described in Supplementary Methods. Samples were hybridized to a custom NimbleGen Drosophila 385k array (29).

‘Immunohistochemistry’ techniques are described in the Supplementary Methods. Primary antibody used: αCSN7

![Figure 1. CSN7 interacts with DNA. Anisotropy binding profile for binding of full-length CSN7 to 3-nM fluorescein-labeled oligonucleotide derived from the Drosophila E2f promoter at 16°C (x). A 50-fold excess of unlabeled E2f oligonucleotide (-) at [150 nM] was added to 3 nM of fluorescein-labeled oligonucleotide as a competitor and titrated by CSN7. The \( K_{D,\text{app}} \) of CSN7 for this DNA fragment is 0.51 ± 0.03 μM with a Hill slope of 1.45, whereas the curve with unlabeled DNA had a \( K_{D,\text{app}} \) constant of 1.7 ± 0.16 μM with a Hill slope of 2.3. Due to the likelihood of multiple binding sites, caution should be exercised in overinterpreting these parameters as they represent an empirical characterization of the binding rather than providing a rigorous binding model.

Flow cytometry

Third instar larvae were dissected in phosphate buffered saline (PBS) and about 30–50 imaginal wing discs were dissociated in 500 μl Trypsin-EDTA x10 (Sigma), 1 μl Vybrant DyeCycle orange 5 mM (Invitrogen) in PBS for 2–4 h at room temperature. SR+ cells were grown for 4 days following RNAi treatment. Cells were harvested and precipitated on ice for 1 h followed by incubation with 1 μl Vybrant DyeCycle orange 5 mM (Invitrogen) for 30 min at room temperature. Fluorescence-activated cell sorting (FACS) analysis for cell-cycle profile was performed using the Becton Dickinson FACSort and CellQuest software.

‘Chromatin immunoprecipitation (ChIP)’ was modified from (30) and detailed in the Supplementary Methods. ChIP was performed using αCSN7 (2), αHistone-3 (Abcam, ab8580) or normal goat serum (NGS).

Computational analyses

For mapping to the genome and orthologs mapping, data were downloaded from Biomart (http://www.biomart.org/).

Data normalization. The Csn7 DamID data analyses were based on quintile normalization of the four samples and average of the results.

P-values and graphs. The genomic positions of Drosophila melanogaster open reading frames were downloaded from
Figure 2. Analyses of the genes bound by CSN7. (A) A global view on the DamID experiment. Black dots mark the positions of genes along the D. melanogaster genome; blue dots denote the positions of the most significant (top 10%) DamID signals; and red dots denote regions enriched in DamID signals, considering sliding windows of 1000 bp in length, and the top 49% of the scores. Dots are spaced minimally by 1000 bp. (B) CSN-dependent genes are preferentially enriched in the CSN7-DamID genes. Genes over- and underexpressed in csn mutants are significantly enriched in the CSN7-DamID results. Ten genes were both up- and downregulated, dependent on the mutant and stage; thus, the total number of genes in the graph (1770) is slightly larger than the actual total number of genes (1760). The $P$-values are based on hypergeometric distribution (which is based on the total number of genes, the number of gene in each set and the number of overlapping genes between the two sets). (C) Analyses of CSN7 enrichment in five chromatin types defined by Filion et al. (29) revealed a high overlap with ‘red’ and ‘yellow’ chromatin, while ‘black’ and ‘green’ chromatin were under-represented in the CSN7-DamID targets. ‘Blue’ chromatin was not affected by the CSN7-DamID. (D) Genes bound by CSN7 are significantly enriched in Rbf1 targets ($P$-value for overlap with CSN7-DamID: Embryo = 7.3255e$^{-11}$, Larvae = 4.2188e$^{-15}$).

National Center for Biotechnology Information (NCBI). We employed two definitions related to the genes associated with the DamID peaks: these were defined as either (i) the 500 nt before and after the beginning of the ORF; or (ii) as genes mapped closest to the DamID peak (a definition that is used in all other analyses performed in the study).

For Figure 2A, genes were divided into groups according to their fold change in the $csn4^{null}$ mutant at time 60 h AED (4). For each group the mean signal defined based on the CSN7 DamID experiment over the genomic region defined above was computed.

In the case of definition 1, the $P$-value $k$-test when comparing the DamID experiment of the overexpressed genes to the ones that do not change significantly and when comparing the DamID experiment of the underexpressed genes to the ones that do not change significantly are ($P = 0.00977$; KS-test) for significant increase in fold change versus no significant change in fold change and ($P = 0.0113$; KS-test) for significant decrease in fold change versus no significant change in fold change and respectively. The difference in DamID signals between genes with significant increase in fold change and gene with significant decrease in fold change is not significant ($P = 0.067$ KS-test).

Enrichment $P$-value related to CSN7-DamID and differentially expressed genes (definition 2) in csn mutant experiments (Figure 2C) was computed based on hypergeometric $P$-value ($P = 5.5*10^{-5}$).
To compute the enrichment of genes regulated by CSN7 among orthologs of human disease genes, a list of 6548 human disease genes was downloaded from gene-card database 2013 (http://www.genecards.org/)(31). The enrichment P-value was computed based on hypergeometric distribution considering the following numbers: the number of human genes with Drosophila orthologs (5801 genes); the number of human disease genes with fly orthologs (2165 genes); the number of human genes (with fly orthologs) that are orthologous to DamID significant genes in fly (1014 genes); the number of human genes that (i) are disease genes, (ii) have Drosophila orthologs, (iii) their fly orthologs are DamID significant (410 genes).

To identify transcription factor (TF)-binding sites, we used the genome versions BDGP R5/dm3 and BDGP R4/dm2 of Drosophila (Apr. 2006 [BDGP R5/dm3] for the DamID experiment; Apr. 2004 [BDGP R4/dm2]—for the TFs), downloaded from the UCSC genome browser (http://genome.ucsc.edu/). Binding site positions of various TFs were downloaded from mod-ENCODE (www.modencode.org). We considered two TFs binding site datasets ‘Fly Transcription Factors by chIP-chip’ (http://intermine.modencode.org/release-32/template.do?name = TF_BindingSites&scope = all) and ‘REDfly transcription factor binding sites’ (http://www.flymine.org/release-37.1/template.do?name = Dataset_TF_BindingSites&scope = all). For each transcript start site, we considered the region of up to 2000 nt upstream and 2000 nt downstream, though the results are robust to small changes in these definitions. We computed for each TF a hypergeometric P-value considering the enrichment of the TF-binding sites in the same genes with CSN7-DamID-binding sites: to this end, we considered the total number of genes that include binding sites of the TF, the total number of genes that include binding sites of the CSN7-DamID, the total number of genes and the total number of genes that include binding sites of the CSN7-DamID and also binding sites of the TF. The P-values were filtered based to control for a false discovery rate of 5%.

‘To identify sequence motifs enriched in the CSN7-binding sites’, we employed the HOMER (Hyper-geometric Optimization of Motif Enrichment) tool (32). To this end, we compared the set CSN7-binding sites after adding 50 nt to both end of the sites to a background set composed in the following way: for each CSN7-binding site of length l, we added to the background set two sequences of length l, corresponding to the DNA regions 100 nt upstream and downstream to the binding site (as defined above); similar results were obtained for sites of distance 300 nt upstream and downstream to the binding site.

RESULTS

CSN7 binds DNA directly

As earlier biophysical and cell biology studies suggested that CSN7, or the CSN in general, binds nucleic acids (25), we carried out binding assays (33) to study the potential interaction of purified full-length CSN7 protein with a fluorescein-labeled DNA fragment, 70 bp in length. Steady-state fluorescence anisotropy of fluorescein-labeled DNA, derived from E2f, was monitored upon titration with the full-length CSN7 protein. Figure 1 (x) shows a representative binding isotherm. The anisotropy increases in a saturable manner, demonstrating binding of CSN7 to this DNA fragment. The anisotropy values suggest that multiple protein copies bind this fragment.

To examine binding specificity, the binding of CSN7 to the labeled DNA fragment was challenged with 50-fold excess of unlabeled DNA. As observed in Figure 1 (circles), this excess of unlabeled DNA fragment induced a marked shift in the titration curve indicating that the unlabeled DNA competitively binds CSN7 protein. Fitting of the binding data indicates that CSN7 interacts with this fragment with an apparent K_D of about 500 nM. This value is within the range of non-sequence-specific DNA-binding proteins (34). We have chosen to present a K_D(app constant since determination of bona fide affinity constants will require delimitation of a target DNA site and subsequent binding isotherms with these defined sites. Similar studies were carried out with a different labeled DNA fragment, derived from the CycE open reading frame, yielding similar anisotropy profiles (Supplementary Figure S1), while experiments using the maltose-binding protein yielded no increase in anisotropy providing a negative control.

While CSN7, like five other CSN subunits, contains a PCI domain, which harbors a putative nucleic acid-binding motif (21,22), this winged-helix subdomain on its own is not stable (21), so we could not further test if this domain alone is involved in nucleic acid binding.

Drosophila CSN7 associates with multiple genomic loci

The binding of CSN7 to DNA detected above, together with the earlier studies where other CSN subunits were detected in association with several specific genes (22–24), led us to hypothesize that CSN7 interacts with multiple regions in the Drosophila genome. Thus, as a starting point to identify the genomic regions bound by CSN7, we performed DamID chromatin profiling in Drosophila Kc167 cells (28,29). We determined that CSN7 binds to ~9400 regions genomewide and overlaps with gene-rich regions (Figure 2A), corresponding to ~1700 genes (Dataset S1). CSN7 binding was detected preferentially in promoter, exon and intron regions, while excluded from both the 5’ and 3’ UTR regions, and in regions defined as intergenic (Table 1, Supplementary Table S1). These data have been uploaded to the UCSC Genome Browser for visual integration with other datasets. Supplementary Figure S2A and B show screenshots from the browser at the resolution of chromosome 3, and the region of the e2f gene, which is further analyzed below.

CSN-dependent genes are enriched for CSN7 targets

To connect between the genomic loci bound by CSN7 and active gene regulation, we compared our DamID data to our previous transcriptome analyses that suggested a direct role for the CSN in transcriptional control (14), and with additional transcriptomic data generated in our lab. Specifically we determined whether CSN7 associates with the genes misregulated in mutants in CSN subunits [csn4null, csn5null, csn52; csn3null, csn130.1], and examined the CSN7.
The CSN7-DamID signal is enriched in genic regions

| Region          | Enrichment ratio | P-value |
|-----------------|------------------|---------|
| Promoter        | 1.4694           | <10^{-30} |
| Intron          | 1.1677           | <10^{-30} |
| Exon            | 1.0955           | <10^{-30} |
| Exon123         | 1.0747           | <10^{-30} |
| 5'-UTR          | 0.0038           | 1       |
| 3'-UTR          | 0.0003           | 1       |
| Non-gene        | 0.9009           | 1       |

Analysis of the intersection of the CSN7-DamID signal with various genic regions. Enrichment ratio is based on data presented in Supplementary Table S1. The enrichment ratio is the fraction of the genic region containing the CSN7-DamID signal relative to the amount of the CSN7-DamID signal predicted for that gene if normal distribution. A value > 1 corresponds to higher overlap between the genic region and the CSN7-DamID signal than expected, with the corresponding enrichment P-value.

Transcription regulators with binding sites enriched in the CSN7-DamID results

| Transcriptional regulators | Total # genes with binding sites | # CSN7-DamID genes with binding sites | P-value    |
|----------------------------|----------------------------------|--------------------------------------|------------|
| bric a brac 1              | 1358                             | 148                                  | 6.29E-12   |
| CTCF                       | 7812                             | 663                                  | 0          |
| C-terminal Binding Protein | 3183                             | 252                                  | 1.32E-05   |
| Dichaete                   | 3020                             | 297                                  | 0          |
| Disconnected               | 2018                             | 214                                  | 5.55E-16   |
| GATAe                      | 2361                             | 201                                  | 1.30E-06   |
| gooseberry-neuro           | 820                              | 96                                   | 1.29E-09   |
| Hairy                      | 2851                             | 257                                  | 7.19E-11   |
| Hucklebein                 | 3268                             | 267                                  | 4.53E-07   |
| Jumeau                     | 3069                             | 267                                  | 1.12E-09   |
| Nejire                     | 9635                             | 797                                  | 0          |
| Trithorax-like             | 7193                             | 640                                  | 0          |
| Ultrathorax                | 2863                             | 281                                  | 1.11E-16   |
| Zn finger homeodomain 1    | 755                              | 91                                   | 8.24E-10   |

The list of transcription regulators with the lowest P-values for enrichment in the CSN7-DamID results.

DamID-signal distribution for these genes relative to genes whose expression is not altered in these mutants.

We used two different statistical approaches in this analysis. In the first, we looked at the average DamID signal at the beginning of the open reading frame of all the CSN-dependent genes, relative to genes whose expression is not affected by the csn mutants. In the second, we compared DamID signals in different groups of genes, looking for significant enrichment in the CSN-dependent genes differentiated by up- or downregulation (see Supplementary Methods). Using both approaches, the CSN-dependent genes were preferentially enriched in the CSN7-DamID genes. For the first approach, the overlap of 389 genes was found to be significant (P = 5.5*10^{-3}). As seen in Figure 2B, the second approach also identified a significant overlap between the DamID and CSN-dependent genes. We found enrichment between the CSN-dependent and the CSN7-DamID genes only when considering all mutants together and not for a mutant in a particular subunit. This is likely due to the reduced statistical power of smaller groups comparisons to all genes. Nevertheless, groups of genes in all mutants were represented in the CSN7-DamID results (Supplementary Table S2).

Since CSN4 and CSN3 are essential for the integrity of the entire CSN complex (9), our finding that CSN7 preferentially binds to CSN-regulated genes strongly suggests an interaction of the CSN holo-complex with these loci.

CSN7 targets genes involved in central developmental processes

Filion et al. (29) identified five distinct chromatin types in Drosophila by DamID profiling of 53 different proteins. These chromatin types were arbitrarily given the colors red, yellow, black, blue and green where ‘red’ and ‘yellow’ chromatin are defined as transcriptionally active and are similar to the classic definition of euchromatin, and ‘green’ and ‘black’ chromatin are defined as heterochromatic or repressed chromatin. We analyzed these chromatin states for enrichment of CSN7 binding and found a high overlap with ‘red’ and ‘yellow’ chromatin, while ‘black’ and ‘green’ chromatin were under-represented in the CSN7-DamID targets. These over- and under-representations of CSN7-DamID targets were significant compared to other chromatin states, as well as a control cohort randomized version of the genome (Figure 2C). Thus, CSN7 in Kc167 cells preferentially targets transcriptionally active regions of the Drosophila genome.

We further tested if the CSN7-DamID targets are enriched for binding sites of known transcription regulators (TR). Binding site positions of various TRs were based on the ChiP-chip data from modENCODE (35). We considered the region of up to 2000 nt upstream and 2000 nt downstream to the start site of each transcript, and mined for an overlap between TR-binding sites and the CSN7-DamID signal. We computed for each TR a hypergeometric P-value considering the enrichment of the TR-binding sites in the
Table 3. Signaling pathways enriched in CSN7-DamID genes

| Ontology grouping      | Genes present in CSN7-DamID | P-value     |
|------------------------|----------------------------|-------------|
| Functional GO clusters |                            |             |
| Imaginal disc development | 104                        | 3.50E-10    |
| Cytoskeleton organization | 109                        | 1.10E-08    |
| Cell morphogenesis      | 102                        | 8.40E-08    |
| Regulation of transcription | 148                        | 8.20E-05    |
| Ion binding             | 304                        | 8.40E-04    |
| Chromatin organization  | 47                         | 3.20E-06    |
| Gamete generation       | 138                        | 2.00E-08    |
| Nucleotide binding      | 227                        | 1.70E-05    |
| Cell cycle              | 111                        | 2.00E-03    |
| Cell motion             | 72                         | 3.80E-06    |
| Regulation of cell development | 46                      | 1.70E-09    |
| Tissue morphogenesis    | 60                         | 1.10E-05    |
| ATP binding             | 113                        | 1.30E-04    |
| KEGG pathways           |                            |             |
| Dorso-ventral axis formation | 29                      | 2.21E-07    |
| PDGF signaling pathway  | 27                         | 3.06E-06    |
| Jak-STAT signaling pathway | 22                      | 4.09E-06    |
| Gonadotropin releasing hormone receptor pathway | 28 | 5.42E-05 |
| Integrin signaling pathway | 28                      | 9.43E-05    |
| Interleukin signaling pathway | 18                      | 2.86E-04    |
| PI3 kinase pathway      | 19                         | 4.65E-04    |
| EGF receptor signaling pathway | 24                      | 1.04E-03    |
| Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade | 11 | 1.23E-03 |
| Progesterone-mediated oocyte maturation | 25 | 1.27E-03 |
| Notch signaling pathway | 16                         | 2.50E-03    |
| FGF signaling pathway   | 22                         | 3.49E-03    |
| Wnt signaling pathway   | 36                         | 3.60E-03    |
| Ras pathway             | 14                         | 1.55E-02    |
| MAPK signaling          | 13                         | 1.80E-02    |
| Ubiquitin-mediated proteolysis | 39                      | 1.80E-02    |
| Endothelin signaling pathway | 21                      | 1.97E-02    |
| FAS signaling pathway   | 9                          | 2.22E-02    |
| Circadian rhythm        | 9                          | 3.03E-02    |
| Hedgehog signaling pathway | 11                      | 3.66E-02    |

Ontology groupings were found using the David Bioinformatics Database, Panther or Babelomics servers.

same genes with CSN7-DamID-binding sites. The P-values were filtered to a false discovery rate of 5% (36).

The CSN7-DamID targets are significantly enriched for binding of at least one of 23 distinct TRs with central roles in Drosophila development (Table 2). These include both sequence-specific transcription factors such as CTCF (37), and transcriptional corepressors/activators like CtBP (38).

Ullah et al. (2007) showed that CSN4 associates with the Retinoblastoma family protein Rbf1 on specific promoters in both Drosophila S2 cells and in embryos. Recently it has been shown using chromatin IP technology that Rbf1 associates with multiple target genes involved in diverse signaling pathways (39,40). This led us to ask if there is an overlap between the CSN7 and the Rbf1-binding profiles. As seen in Figure 2D, there is a significant overlap between the CSN7 DamID targets in Kc167 cells and the Rbf1 ChiP targets in both embryo and larvae (Dataset 3). This overlap was identified for targets that were detected with complementary approaches (DamId for CSN7 versus ChiP for Rbf1) and using different starting material (Kc167 cells for CSN7 versus embryos and larva for Rbf1), emphasizing the robustness of the overlap.

In an attempt to discover if CSN7 associates with specific nucleic acid sequences, we searched for enriched sequence motifs using a computational tool for finding consensus sequence motifs (HOMER (32)). To this end, we compared the set of CSN7-DamID sites to background sequences at a distance of 100 (x) nt from the CSN7-DamID sites and with identical size (more details in the Supplementary Methods). Following a few iterations of this approach with different x (100, 300) we did not succeed in finding a sequence motif that appears in the CSN7-DamID sites but not in the background sequences; all the motifs enriched in the CSN7-DamID sites also appeared in at least 42% of the background sequences. While a negative result, from this we conclude that the overall binding of CSN7 to the chromatin does not appear to be sequence specific.

The CSN7-DamID targets were further analyzed for enrichment of Gene Ontology (GO) terms. One hundred sixty GO terms were found to be significantly enriched in the CSN7-DamID targets (Dataset S2). To distill this data, we employed the ‘Functional Annotation’ tool of the DAVID Bioinformatics Database (41), which revealed that CSN7 associates preferentially with genes involved in central developmental and related pathways, including those involved...
CSN7 is essential for wing disc development and G1-S cell-cycle progression

To experimentally test these bioinformatic predictions, we examined whether loss of CSN7 function impacts these biological processes and affects expression of the target genes through analyses of Drosophila carrying a null mutation in Csn7. Bloomington stock center strain (#18203, termed here csn7<sup>0</sup>) has a P-element insertion in the second intron of the Csn7 gene and when homozygous results in a complete loss of Csn7 transcript and protein (Supplementary Figure S2); thus, csn7<sup>0</sup> should be considered a null allele. Similar to mutations in other CSN subunits (14, 42), csn7<sup>0</sup> is recessive lethal, with double mouth-hooks appearing after the second ecysis concurrent with the primary lethal phase, and melanotic tumors developing during the third instar stage in surviving larvae, which then arrest 120 h after egg deposition (Supplementary Figure S3). These phenotypes are linked solely to the Csn7 locus, as all phenotypes were complemented by the expression of a UAS-Csn7-GFP transgene driven by an Act-GAL4 driver (Supplementary Figure S4).

Furthermore, targeted reduction of Csn7 expression via a Csn7-RNAi transgene under the control of the Act-Gal4 driver resulted in pupal lethality, which was preceded by the appearance of melanotic tumors (Supplementary Figure S3). As expected, expression of this transgene was effective in reducing both transcript and protein levels of Csn7.

Next, we examined the role of CSN7 in imaginal disc formation and cell-cycle progression, two of the GO groupings identified as enriched in Csn7-DamID targets. Imaginal wing discs in third instar csn7<sup>0</sup> larvae are severely reduced in size, which correlates with the enrichment of genes associated with imaginal discs identified by DamID. As the size of the imaginal wing discs from the csn7<sup>0</sup> mutant discs precluded further analyses, we specifically targeted CSN7 using UAS-Csn7-RNAi transgene under either Act-Gal4 or En-Gal4. While these wing discs were of normal size, they were developmentally defective as the folds in the wing pouch and hinge were not readily evident (Figure 3A and B).

To garner additional information about the nature of this wing phenotype, we carried out flow cytometry (FACS) analysis on dissociated cells from wing discs expressing the Csn7-RNAi transgene under either Act-Gal4 or En-Gal4 control. Expression of the Csn7-RNAi transgene under either driver resulted in a cell-cycle profile consistent with a proliferation defect: the G1 fraction was increased at the expense of G2 relative to the wild type (Figure 3C–D). A similar G1 enrichment was obtained for Drosophila SR<sup>+</sup> cells transfected with a Csn7-RNAi construct (Figure 3E), further indicating that CSN7 preferentially promotes G1/S progression. This cell-cycle phenotype is likely a result of loss-of-function of the entire CSN complex as RNAi-mediated downregulation of CSN2 or CSN8 resulted in similar G1 delays (Figure 3F).

Endogenous CSN7 associates with the regions identified by DamID

The bioinformatic analysis above clearly illustrated that CSN-dependent genes are enriched in the Csn7-DamID dataset. To further connect between the genes bound by CSN7 and the phenotypes described above, we determined
if the expression of the genes within the CSN7-DamID loci is also misregulated upon RNAi-mediated reduction of CSN7. We examined by qRT-PCR the mRNA levels of three key developmental genes involved in cell-cycle regulation and wing disc development, CyclinE, E2f and Scalloped (Sd). mRNA levels were checked in Kc167 and S2 cells where we reduced CSN7 levels via RNAi. As shown in Figure 4A and Supplementary Figure S6, the levels of CyclinE and E2f were reduced when Csm7 was silenced in both cell types, while Sd was reduced only in Kc167 cells.

We further investigated if the genomic regions identified as bound by the CSN7-Dam fusion protein for these three genes (see Supplementary Figure S2 for specific example of e2f) are indeed occupied by endogenous CSN7 by performing ChIP with anti-CSN7 antibodies. Anti-histone H3 served as a positive control and NGS as a negative control. As seen in Figure 4B, the regions identified by DamID of all three genes mentioned above were bound with CSN7 and histone H3, but not control IP. While the IP signal above background for the three genes varied, possibly due to affinity issues, they were significant, ranging from 4.6 to 35.7 standard deviations (see legend of Figure 4B for P-values). The validity of the IP results vis a vis the DamID results is further supported by the negative control where anti-histone H3 precipitated a second region of CyclinE, 2.5 kb downstream of the DamID-predicted CSN7-occupied region, while anti-CSN7 did not.

**DISCUSSION**

We present here the first global genomic description of a CSN subunit binding to chromatin. While a number of earlier studies had indicated that certain CSN subunits associate with specific genes, it was unclear from these studies whether these results were indicative of a broader function of the CSN in regulating gene expression on the chromatin, or rather were isolated incidents (22–24). Using DamID technology, we showed that Drosophila CSN7 associates with ~10% of Drosophila protein-encoding genes. Thus, the data presented here expand the arsenal of functions attributed to the CSN. Not only does CSN7 associate with chromatin, but it is associated with genomic regions known to be misregulated in the absence of the CSN. Thus, CSN7, and by extension the entire CSN, could now be considered a transcriptional regulator.

Defining the CSN as a transcriptional regulator echoes back 20 years to the original description of the cop9 mutant in Arabidopsis. Wei and Deng (43) reported high-level expression of light-inducible genes in the absence of light in these mutants, and hypothesized that COP9 acts by altering the promoter activities of these genes. Later the CSN was found not only to be a signal-dependent repressor of transcription, but also a signal-dependent inducer of some of the same genes (44). While work in many labs established the paradigm of the CSN regulating gene expression through modulating the stability of transcription factors (17,18), the results presented here reopen the door for the CSN having a direct role in global transcriptional regulation on the chromatin. This role likely involves a direct interaction between the CSN and DNA, as clearly shown in the anisoptry studies with CSN7.

Importantly though, we do not propose that CSN7 is a sequence-specific DNA-binding protein, but rather functions as a co-regulator of transcription that is recruited to targets by specific factors. Indeed, CSN7 interacts in vitro with oligonucleotides derived from both regions predicted to bind CSN7 and regions not identified as binding CSN7, suggesting that the affinity is for DNA in general. This is also supported by the in vitro Kd, which is higher than that of sequence-specific DNA-binding proteins, but in line with other general binding proteins, and by our inability to identify enriched sequences in the CSN7-DamID sites. We propose that the specificity in CSN7 targeting is mediated through interacting proteins. Indeed, CSN subunits have
been conspicuous for their ability to fish-out key transcriptional regulators in protein–protein interaction studies (45–48). Thus while each interacting protein may have a specific target site, the presence of multiple target sites in our data precluded the possibility of detecting these through bioinformatic analysis.

Of course, these two roles proposed for the CSN, chromatin binding and regulation of TR stability, are not mutually exclusive. It is conceivable that the CSN regulates TR stability directly on the chromatin, similar to the activity of the proteasome on chromatin (49). Indeed many of the transcriptional regulators whose binding sites are enriched in the CSN7-DamID targets (Table 1) are either substrates of the proteasome [e.g. Hairy (50)] or components of the degradation machinery [e.g. bric—a-brac (51)], such that the function of the CSN on the chromatin may be intimately connected with its role in protein degradation.

Yet, other evidence does point to a proteasome-independent role for the CSN. Indeed an N-terminal fragment of human CSN1 translocates to the nucleus and inhibits gene expression from a number of specific promoters (10). Although the mechanism of this inhibition is still unclear, it might be related to the fact that this part of CSN1 interacts with SAP130—a member of the DDB1 protein family and an established component of the transcription machinery (20). This fits very well with other observations for ubiquitin-like modifications that regulate chromatin structure and gene activity independent of degradation (52).

Regardless of the mechanism, the results presented here have implications for the role of the CSN in human biology. Considering the number of key signaling pathways bound by CSN7, it is not surprising that so many human disease genes have homologs with significant binding signal of CSN7. Thus further elucidating the mechanism by which the CSN is recruited to different loci could have major implications for biomedical research.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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