Comparing and Contrasting the Effects of *Drosophila* Condensin II Subunit dCAP-D3 Overexpression and Depletion *in Vivo*

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**ABSTRACT** The Condensin II complex plays important, conserved roles in genome organization throughout the cell cycle and in the regulation of gene expression. Previous studies have linked decreased Condensin II subunit expression with a variety of diseases. Here, we show that elevated levels of Condensin II subunits are detected in somatic cancers. To evaluate potential biological effects of elevated Condensin II levels, we overexpressed the Condensin II subunit, dCAP-D3 in *Drosophila melanogaster* larval tissues and examined the effects on the mitotic- and interphase-specific functions of Condensin II. Interestingly, while ubiquitous overexpression resulted in pupal lethality, tissue specific overexpression of dCAP-D3 caused formation of nucleoplasmic protein aggregates which slowed mitotic prophase progression, mimicking results observed when dCAP-D3 levels are depleted. Surprisingly, dCAP-D3 aggregate formation resulted in faster transitions from metaphase to anaphase. Overexpressed dCAP-D3 protein failed to precipitate other Condensin II subunits in nondividing tissues, but did cause changes to gene expression which occurred in a manner opposite of what was observed when dCAP-D3 levels were depleted in both dividing and nondividing tissues. Our findings show that altering dCAP-D3 levels in either direction has detrimental effects on mitotic timing, the regulation of gene expression, and organism development. Taken together, these data suggest that the different roles for Condensin II throughout the cell cycle may be independent of each other and/or that dCAP-D3 may possess functions that are separate from those involving its association with the Condensin II complex. If conserved, these findings could have implications for tumors harboring elevated CAP-D3 levels.

**KEYWORDS** Condensin II; gene expression; mitosis; CAP-D3; development

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The three-dimensional organization of the genome is important for both efficient cellular division as well as for the regulation of gene transcription. In mitosis, proper packaging of DNA is essential for the symmetric segregation of genetic material. In interphase, the positioning of loci within the cell’s nucleus can largely affect both the spatial and temporal expression of genes (Holwerda and de Laat 2012; Van Borltle and Corces 2012; Smallwood and Ren 2013; Dowen and Young 2014; Gorkin *et al.* 2014; Dekker and Mirny 2016). DNA is organized in a hierarchical manner, with chromosomes occupying their own discrete territories (Croft *et al.* 1999; Bolzer *et al.* 2005; Cremer and Cremer 2010). It has also recently been determined that individual chromosomes are organized into topologically associated domains (TADs), which contain genomic regions of higher intrachromosomal interaction frequency (Nora *et al.* 2013). Disruption of TADs and TAD boundaries can negatively affect gene expression, leading to both acquired diseases as well as developmental defects (Lupiañez *et al.* 2015, 2016). In addition to intrachromosomal interactions, interactions between chromosomes...
through chromosome pairing or through contacts between interchromosomal loops can also influence gene expression (Apte and Meller 2012; Holwerda and de Laat 2012).

Condensin II is a multisubunit complex found in multicellular organisms that is necessary in both the establishment and maintenance of genome architecture throughout the cell cycle. In *Drosophila*, the Condensin II complex comprises two structural maintenance of chromosome (SMC) subunits, dSMC2 and dSMC4, and two chromosome-associated protein (CAP) subunits, the kleisin dCAP-H2 and the HEAT-repeat protein dCAP-D3 (Nasmyth and Haering 2005). Unlike mammalian Condensin II, the *Drosophila* CAP-G subunit has been shown to exclusively associate with the Condensin I complex (Herzog et al. 2013). Condensin II is most known for the role it plays in condensing DNA during mitosis (Steffensen et al. 2001; Ono et al. 2003, 2004; Hirota et al. 2004; Nasmyth and Haering 2005). Recent work has shown that Condensin II possesses important functions in interphase as well. In the fly, roles for Condensin II in chromosome territory formation and maintenance, TAD organization, and the repression of homologous chromosome pairing have been identified (Hartl et al. 2008; Bauer et al. 2012; Joyce et al. 2012; Li et al. 2015). Work from our laboratory in both *Drosophila* and human cells has shown that depletion of the CAP-D3 subunit of Condensin II also results in the deregulation of gene expression (Longworth et al. 2012; Schuster et al. 2013, 2015; Klebanow et al. 2016). Studies from other laboratories have also implicated CAP-H2 and CAP-G2 in gene regulation (Xu et al. 2006; Hartl et al. 2008; Rawlings et al. 2011). Chromatin-immunoprecipitation (ChIP) and ChIP-sequencing (ChIP-seq) experiments have identified Condensin II binding sites at regions flanking target gene loci (Rawlings et al. 2011; Longworth et al. 2012; Pan et al. 2013; Schuster et al. 2013, 2015; Klebanow et al. 2016), at enhancer elements (Dowen et al. 2013), and at TAD boundaries (Li et al. 2015; Yuen et al. 2017). Additionally, loss of Condensin II has been shown to affect the chromatin landscape proximal to the genes it regulates (Rawlings et al. 2011; Schuster et al. 2013), but the direct mechanisms by which Condensin II regulates gene expression and the extent to which the condensation function of Condensin II affects the gene regulatory functions are still not well understood.

Both genomic instability and the aberrant regulation of gene expression have been linked to cancer. Since Condensin II is a key regulator of genome architecture, plays a role in the regulation of gene expression, and has been implicated in tumor development in both mice (Woodward et al. 2016) and humans (Ham et al. 2007; Leiserson et al. 2015), we examined CAP-D3 expression patterns in human somatic cancers using the COSMIC database (http://cancer.sanger.ac.uk/cosmic). In finding that elevated CAP-D3 levels were present in many different somatic tumors, we sought to characterize the effects of higher dCAP-D3 expression levels in *vivo*. Here we show that overexpression of dCAP-D3 in *Drosophila* results in the formation of protein aggregates in both dividing and nondividing tissues. Our data also indicate that, similar to underexpression, overexpression of dCAP-D3 accompanied by aggregate formation slows mitotic progression in *vivo* as a result of lags identified in prophase. Interestingly, we also show that overexpression of dCAP-D3 results in changes to gene expression in a pattern opposite to what is observed in dCAP-D3-deficient tissues. Our findings juxtapose the effects of dCAP-D3 overexpression and depletion in dividing and nondividing tissues. If conserved, these results could shed light on possible mechanisms by which elevated CAP-D3 levels potentially contribute to both developmental defects and tumor development.

Materials and Methods

**Fly strains**

All stocks were maintained at 25° on standard dextrose medium. For RNA-sequencing (RNA-seq) experiments, the w1118 (Bloomington Stock Center 5905) stock served as a wild-type control. dCap-D3 mutant larvae for this experiment were homozygous for the hypomorphic c07081 allele which was generated in the w1118 line (Thibault et al. 2004) (w; dCap-D3c07081/c07081, Harvard Exelixis c07081). Quantitative real-time PCR (qRT-PCR) validation of RNA-seq-identified targets were also performed in these lines as well as in a line transheterozygous for the c07081 allele and Δ25 chromosome deletion. Progeny were generated by crossing heterozygous dCap-D3c07081/Cyo-GFP virgins with Δ25/Cyo-GFP males (Bloomington Stock Center 25). For live imaging experiments, w; P[(His2Av-mRFP1)] III.1 (Bloomington Stock Center 23650) larvae were used as wild-type controls. The His2Av-mRFP allele was crossed into dCap-D3c07081/Cyo-GFP flies to generate a stock with the genotype of dCap-D3c07081/c07081; P[(His2Av-mRFP1)] III.1 used for imaging analysis. Imaging was also performed in transheterozygous tissue generated by crossing dCap-D3c07081/cyo-GFP females with Δ25/cyo-GFP males. Ubiquitous overexpression and overexpression in the salivary glands and wing discs was accomplished by crossing β-tubulin-GAL4, SGS-GAL4, or Nubbin-GAL4 males with pUAST-GFP-dCAP-D3, pUAST-FLAG-HA-dCAP-D3, pUASg-dCAP-D3, pUASG-dCAP-D3, or pUAST-FLAG-HA virgins. GFP-dCAP-D3, FLAG-HA-dCAP-D3, FLAG-HA, and GFP overexpression stocks were generated using the gateway system as previously described in Longworth et al. (2012). pUASg-dCAP-D3 were acquired from FlyORF (M{UAS-Cap-D3.ORF} ZH-86Fb, F001285). For live imaging in the wing disc, Nubbin-GAL4 flies were crossed with w; P[(His2Av-mRFP1)] III.1 flies to generate a *Nubbin-GAL4; P[(His2Av-mRFP)] III.1 stock. Males from this stock were then crossed with pUAST-GFP-dCAP-D3 or pUAST-GFP (control) virgins to drive expression in the wing disc pouch.

**Immunofluorescence**

All tissues were fixed in 4% paraformaldehyde/0.5% Triton X-100 for 25 min with rocking at room temperature. Stained tissues were washed with 0.1% PBS-Triton, blocked in 0.1% PBS-Triton/1%BSA, and incubated with primary antibody in
block buffer [dCAP-D3, 1:50 (Longworth et al. 2008); lamin, 1:100 (Developmental Studies Hybridoma Bank ADL67-10); HA, 1:500 (Biolegend); CID, 1:500 (Blower and Karpen 2001); PH3, 1:500 (06570 Millipore, Bedford, MA, or abcam14955)] overnight at 4°. The following day, samples were washed and incubated with secondary antibody (1:500, Alexa Fluor; Thermo Fisher, San Jose, CA). Tissues were mounted in Vectashield with DAPI prior to imaging.

**Live imaging**

Protocols were adapted from what was previously described in Handke et al. (2014) and Poulton et al. (2014). Briefly, wing discs were dissected from third instar larva at room temperature and placed apical side up onto an oxygen-permeable LumoX dish 50 (Sarstedt) in a base media [Schneider’s media (GIBCO, Grand Island, NY), 5% fly extract (Drosophila Genomics Resource Center), 6.2 μg/ml bovine insulin, and 1% penicillin/streptomycin] supplemented with 200 μg/ml bovine insulin. Spacers of 1 μm in thickness were placed adjacent to the disc and a poly-L-lysine-coated coverslip was placed on top. Coverslips were sealed with Halocarbon oil 700 (Sigma Chemical, St. Louis, MO) around the edges. Discs were allowed to settle for 30 min, after which they were imaged with a 40× water objective for up to 3 hr.

**Imaging**

Unless otherwise noted, all imaging was performed using a Leica SP5 confocal/multi-photon microscope that was purchased with partial funding from National Institutes of Health Scientific Interest Groups grant 1S10RR026820-01.

**Immunoprecipitation and mass spectrometry**

Antibodies (4 μg GFP, 1 μl IgG preserum) were incubated with Protein A Dynabeads for 4 hr at room temperature with rocking. Six-hundred pairs of salivary glands per genotype were homogenized in either a low salt (150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 1% Triton, 1 mM DTT, 1× protease inhibitor cocktail) or high salt (300 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 0.1% Triton, 10% glycerol, 1 mM DTT, 1× protease inhibitor cocktail) lysis buffer. Samples were adjusted to 650 μl and 200 μl of lysate was used per IP, which was performed overnight at 4° with rocking. After washes, samples were boiled off of beads in 30 μl Laemmili buffer for 5 min. Equal volumes of sample were run on an SDS-polyacrylamide gel and stained with Gel CodeBlue (Thermo Fisher) overnight. Protein bands from control and IP samples were excised from the gel, reduced and alkylated with iodoacetamide prior to the in-gel digestion. All bands were digested using trypsin by adding 5 μl of 10 ng/μl trypsin in 50 mM ammonium bicarbonate and incubating overnight at room temperature. The peptides that were formed were extracted from the gel pieces. These extracts were evaporated in a SpeedVac and then resuspended in 1% acetic acid for liquid chromatography–mass spectrometry (LC-MS) analysis. The LC-MS system was a Finnigan LTQ-Orbitrap Elite hybrid mass spectrometer system (Thermo Fisher). The HPLC column was a Dionex 15 cm × 75 μm inner diameter Acclaim Pepmap C18, 2 μm capillary chromatography column. A 5-μl aliquot of the extract was injected and the peptides eluted from the column by an acetonitrile/0.1% formic acid gradient at a flow rate of 0.3 μl/min. The digest was analyzed using the data-dependent multitask capability of the instrument, acquiring full-scan mass spectra to determine peptide molecular weights and product-ion spectra to determine amino acid sequence in successive instrument scans. The data were analyzed by using centromere identifier (CID) spectra collected in the experiment to search the Drosophila reference sequence database (27,588 entries), using the programs Mascot (Matrix Science, London, United Kingdom) and Sequest (Thermo Fisher), and more specifically against the sequence of dCAP-D3 using the program Sequest. Protein and peptide identification was validated using the program Scaffold (Proteome Software, Portland, OR). Protein identifications were accepted if they could be established at >99.9% probability using the Protein Prophet algorithm and contained at least two identified peptides. The proteins identified in the control and IP samples were compared using the total number of spectra (spectral counts) identified in the LC-MS/mass-spectrometry analysis and putative dCAP-D3 binding partners are proteins preferentially identified in the IP sample compared to control. The Orbitrap Elite Instrument was purchased via a National Institutes of Health shared instrument grant, 1S10RR031537-01.

**Polytene squashes**

Salivary glands were dissected from larvae in fresh formaldehyde fixation buffer (0.15 M PIPES, 3 mM MgSO4, 1.5 mM EGTA, 1.5% NP-40, 2% formaldehyde). Dissection and fixation were both performed in 2–3 min. Glands were incubated in 0.2% Triton-PBS (PBT) for 2–3 min and then transferred to 50% glycerol for an additional 5 min. After transferring to a fresh drop of 50% glycerol on a coverslip, a poly-L-lysine-coated slide was placed over the top of the glands and squashing and spreading was carried out by pressing on the cover glass with a pencil. Slides were flash frozen in liquid nitrogen and the coverslip removed with a razor blade. Slides were rehydrated in PBT and blocked in PBT plus 0.2% BSA and 0.5% goat serum prior to the addition of primary antibody as described above. Slides were stained with DAPI (1:100) prior to imaging.

**RNA isolation**

RNA was isolated from Drosophila larval tissue using standard Trizol methods. Each salivary gland and wing disc sample consisted of 50 and 150 tissues, respectively. Samples were cleaned and concentrated using the QIAGEN (Valencia, CA) Mini RNeasy Kit.

**RNA-seq**

RNA was isolated as described above. Directional (wing disc) or nondirectional (salivary gland) complementary-DNA (cDNA) libraries (50 bp, paired end) were made at the University of Chicago Genomics Core and sequenced on an Illumina
HiSeq2500, according to standard protocols. Raw data files (48–158 million paired reads per sample) are available under the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus accession number GSE116419. Adapters and low quality bases were trimmed with Trim Galore version 0.3.3 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and controlled for quality with FastQC version 0.11.3 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before alignment to the Drosophila genome assembly BDGP6 [Flybase PB2015_01 released February 24, 2015, with Ensembl 6.04 annotations (dos Santos et al. 2015)] using BWA aln/sample (version 0.7.12) (Li and Durbin 2009) with default settings. Unmapped reads were aligned using TopHat version 2.0.8b (Kim et al. 2013) (-r 150 –mate-std-dev 100 –b2-sensitive –I 5000) to retrieve splice junctions. HTSeq version 0.6.1 (Anders et al. 2015) was used to count transcripts. Picard tools (version 1.119; http://broadinstitute.github.io/picard) were used to convert formats and to merge alignment files. Prior to differential expression (DE) analysis, gene-level counts were filtered such that at least one sample had >0.5 read per million mapped (11,021/16034, 68.7% retained). Thereafter, expression differences were assessed separately comparing dCap-D3 mutants to wild type in each case. After a first-pass DE analysis with edgeR version 3.16.5 (McCarthy et al. 2012), a control set consisting of genes with false discovery rate (FDR) >0.1 and absolute log2 fold change (logFC) <1.1 was used with RUVg (estimating the factors of unwanted variation using control genes) (k = 1) to identify unwanted variation [RUVSeq version 1.8.0 (Risso et al. 2014)]. The second-pass edgeR analysis included the RUVg correction factor in the model matrix. Genes with FDR <0.05 and absolute logFC >1 were retained for further analysis. DAVID version 6.8 (Huang et al. 2009) was used for gene ontology (GO) enrichment analysis of the DE gene lists using all Drosophila melanogaster genes as background.

qRT-PCR

cDNA was made with 1 μg of RNA per 50 μl reaction using the TaqMan Reverse Transcription Kit (Life Technologies). qRT-PCR was performed as described in Longworth et al. (2012) using a Roche LightCycler480. Primer sequences are as follows: CG32073-F: GCCATTGGCCTGTAATTCCGTT, CG32073-R: GTAGTCGAGTGCCAGTGC AGTT; CG32071-F: CTGGTTG TCTGTCTCTGCA, CG32071-R: CAGCCGTTAGTTAGT GG TG; CG13560-F: AAGTACGGCCACTTGCAAGCA, CG13560-R: GAAAGACTCTAGTCGAG; CG17147-F: CTGGCTACGG GGACATCG, CG17147-R: CCAGCAAGGTTAGTTCCGTT; CecC-F: CAATCGGAAGCGCGTGGCTG, CecC-R: GGCGAT TCCAGTCCCT GAATTG; HP1D3csd-F: GGGAGTTCAAA AGTCGCTCAG, HP1D3csd-R: GCCGGACGT; Rp49-F: TACAGG CCAAGATCGTGAAAG, Rp49-R: GACGCACTCTGTGTTGG CATACC.

Data availability

Supplemental Material, File S1 contains supplemental methods and supplemental file legends. Figures S1–S10 are supplemental figures. Table S1 is a list of DE genes identified by RNA-seq. Table S2 is a list of commonly deregulated target genes. Files S2–S7 are movies captured from live imaging experiments. Gene expression data are available under the NCBI Gene Expression Omnibus accession number GSE116419. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.6884690.

Results

CAP-D3 expression levels are elevated in human somatic cancers

Condensin II plays an important role in organizing the genome throughout the cell cycle. As loss of CAP-D3 is associated with chromosome missegregation (Hartl et al. 2008; Longworth et al. 2008), we examined CAP-D3 levels in a panel of 9144 somatic cancers (COSMIC database) and were surprised to find that a greater percentage of tumors exhibited elevated rather than reduced levels of CAP-D3, based on Z-scores calculated from a population norm (Figure 1A) (Forbes et al. 2017). In fact, elevated levels of CAP-D3 were observed in 507 of the 9144 year (5.5%) somatic cancers tested, whereas only 91 of the 9144 tumors (1%) exhibited decreased levels of CAP-D3. Of the 507 tumors with increased CAP-D3 expression, the highest number were found in the lung (19%), breast (11%), and central nervous system (9%) (Figure 1B). Increased levels of other Condensin II subunits were also observed in tumors (Figure 1A), whereas lower levels of the tumor suppressor protein p53 was observed more often in somatic tumors (Figure 1A).

Overexpression of dCAP-D3, in vivo, results in the formation of nucleoplasmic aggregates

It is possible that elevated levels of Condensin II subunits are more frequently observed in tumors because higher Condensin II levels are required to accommodate the increase in cellular division that occurs in tumors. Interestingly, through The Cancer Genome Atlas (TCGA) analysis, we observed that individuals possessing low-grade gliomas (a type of central nervous system tumor with a low mitotic index) expressing higher levels of Condensin II subunits, on average, exhibited lower survival rates compared to individuals with low-grade gliomas expressing lower levels of Condensin II subunits (Figure S1, A–C). In contrast, TCGA analysis of survival rates for patients possessing glioblastomas (a type of high-grade glioma with a high mitotic index) expressing higher levels of Condensin II subunits did not reveal any significant differences when compared to patients possessing glioblastomas expressing lower levels of Condensin II subunits (Figure S1, D–F). These analyses demonstrate that elevated levels of Condensin II subunits are observed in tumors with lower numbers of mitotic events and may negatively affect patient survival.

It is also possible that elevated Condensin II levels might actually result in phenotypes that could lead to tumor development and/or progression. To investigate this second
possibility, we sought to assess the consequences of higher CAP-D3 levels on cellular division and in transcriptional regulation in vivo in *D. melanogaster*. Toward this end, we overexpressed dCAP-D3 using a GFP-tagged *Cap-D3* allele, which we have previously published to be able to rescue a loss-of-function dCAP-D3 phenotype caused by the loss of dCAP-D3-mediated ability to repress enhancer activity and gene transcription (Klebanow et al. 2016). dCAP-D3 overexpression was confirmed by Western blotting using the ubiquitous β-tubulin-GAL4 driver to promote expression of a GFP-dCAP-D3 or GFP control transgene (Figure S2). Using the SGS-GAL4 and Nubbin-GAL4 drivers, we expressed either GFP-dCAP-D3 or GFP in the salivary glands and in the wing disc pouch to characterize the effects of dCAP-D3 overexpression in a nondividing and dividing tissue type, respectively. Immunofluorescence experiments showed that overexpression of dCAP-D3 in both tissues resulted in the formation of GFP-dCAP-D3 aggregates that were not present when only GFP was expressed (Figure 2, A and B). The aggregates were found solely in the nucleus and appeared to predominantly inhabit the interchromatin space in cells from both tissues, however faint GFP-dCAP-D3 signal was observed colocalizing with DAPI. The formation of GFP-dCAP-D3 aggregates was most likely not an artifact of GFP-tag fusion to dCAP-D3 protein, as overexpression of FLAG-HA tagged and untagged dCAP-D3 also resulted in aggregate formation (Figure S3 and Figure S4). Upon examination of aggregate numbers, we observed a significant variation ranging between 1 and 10 GFP-dCAP-D3 aggregates present in each salivary gland nucleus (Figure S5). Interestingly, only one GFP-dCAP-D3 aggregate was ever observed in a given wing disc nucleus, with some cells exhibiting a complete absence of GFP-dCAP-D3 aggregates. To determine if this discrepancy in aggregate number between salivary glands and wing discs was a result of using GAL4 drivers of different strengths, we used β-tubulin-GAL4 to ubiquitously overexpress GFP-dCAP-D3 at higher levels in both tissues. In both tissues, the aggregates appeared to be more interconnected, suggesting that higher expression driven by the stronger β-tubulin-GAL4 driver may increase the size of and/or interactions between aggregates. Salivary gland nuclei from these larvae, however, still exhibited a higher number of distinct GFP-dCAP-D3 aggregates and each wing disc nucleus still contained one GFP-dCAP-D3 aggregate, with some nuclei exhibiting a complete absence of aggregates (Figure 6, A and B). Together, the observed difference in aggregate number combined with the complete absence of aggregates in some wing disc cells suggests that there may be a tissue- or cell-specific component acting to regulate the formation of these GFP-dCAP-D3 structures.

**Figure 1** CAP-D3 levels are altered in somatic cancers. (A) Percentage of somatic cancers that exhibit elevated and lower levels of Condensin II proteins using the COSMIC database (http://cancer.sanger.ac.uk/cosmic). (B) Pie chart of different types of cancers that exhibit elevated CAP-D3 levels. Data were obtained using the COSMIC database.
Both underexpression and overexpression of dCAP-D3 significantly affect mitotic timing

Condensin II functions to ensure the uniform condensation of chromosomes prior to cellular division (Ono et al. 2003, 2004; Hirota et al. 2004; Longworth et al. 2008). This function is essential for facilitating equal chromosome segregation (Hartl et al. 2008; Longworth et al. 2008). Work in HeLa and DT-40 chicken cells has shown that depleting CAP-D3 can alter mitotic progression (Hirota et al. 2004; Abe et al. 2011; Bakhrebah et al. 2015). To determine whether this occurs in vivo, we performed live cell imaging in wing discs expressing an RFP-tagged histone variant (His2aV-mRFP) explanted from third instar larvae that expressed the previously characterized (Longworth et al. 2008, 2012) mutant dCap-D3c07081/c07081 allele. From imaging analyses, we observed an increase in the average time that it took dCAP-D3-deficient cells to progress from the initial onset of observed condensation to the formation of the metaphase plate (Figure 3, A and B; File S2; and File S3). Similar to what has been shown in other organisms, decreasing dCAP-D3 expression also appeared to affect chromosome segregation, as a small but significant lag from metaphase-plate formation to the onset of anaphase was also observed (Figure 3, A and B). While omitted from the averaging, we witnessed extreme events in which chromosomes took up to 50 min to resolve when dCAP-D3 levels were depleted (File S4). Imaging analyses of wing discs explanted form dCap-D3c07081/Δ25; His2aV-mRFP larvae recapitulated the metaphase to anaphase lags observed in dCap-D3c07081/c07081 discs, but not the prophase to metaphase lags (Figure S7 and File S5). This is surprising as we have previously reported that dCap-D3c07081/Δ25 larval brains exhibit condensation defects (Longworth et al. 2008). As the Δ25 allele is a second chromosome deletion that results in the loss of 63 other genes and regulatory RNAs in addition to dCap-D3, including the B-type lamin lamDm0, it is possible that decreased expression of these other factors could influence mitotic progression, thus skewing the prophase timing results. Indeed, the nuclear lamina has been shown to be important for regulating chromosome movement during meiotic prophase in Caenorhabditis elegans (Link et al. 2018), but whether it plays similar roles in mitotic cells is unknown.

Since we identified lags in mitosis in these developing discs, we asked whether dCap-D3c07081/c07081 flies would also exhibit gross changes in development. To test this, we analyzed eclosion rates in both wild-type and dCap-D3 mutant flies. We found that, on average, dCap-D3 mutants took significantly longer to emerge from their pupal casing compared to their wild-type counterparts (Figure S8A), suggesting that lags in mitosis at the cellular level may be contributing to a more global developmental delay. Additionally, we also observed that adult dCap-D3 mutant males and females (Figure S8B) exhibited a decrease in overall body weight compared to wild-type adults, which may imply that a decrease in the rate of mitosis could translate to a decrease in cell number.

Next, to determine the effects of increased levels of dCAP-D3 on mitotic progression, we used Nubbin-GAL4 to drive...
expression of GFP-tagged dCAP-D3 in the wing disc pouch in third instar larvae of the w; His2Av-mRFP background. Using only cells harboring GFP-dCAP-D3 aggregates for analysis, we unexpectedly again observed a lag in the time it took from the onset of DNA condensation to metaphase plate formation when dCAP-D3 was overexpressed as compared to GFP expression alone (Figure 3, C and D; File S6; and File S7). This, however, was not the case for cells that overexpressed GFP-dCAP-D3 but lacked aggregates, as a significant change in mitotic timing was not observed in these cells (Figure 3, C and D). Notably, while dCAP-D3-deficient wing disc cells exhibited lags in both early and late mitosis (Figure 3A), cells that overexpressed dCAP-D3 actually exhibited a significant acceleration in timing from metaphase to sister chromatid resolution when aggregates were present, but no change in timing when they were absent. (Figure 3, C and D; File S6; and File S7). Interestingly, GFP-dCAP-D3 aggregates disappeared after the appearance of the metaphase plate, which may be due to degradation or dissipation into the cytoplasm as a result of partial nuclear envelope breakdown. Like dCAP-D3-deficient cells, however, delays in total mitotic time were again only observed in dCAP-D3-overexpressing wing disc cells that possessed aggregates (Figure 3, C and D). The fact that timing defects are observed only in cells where aggregates are present suggests that it is the aggregation of dCAP-D3 that drives the phenotype.

Chromosome condensation has previously been shown to begin at centromeres and progress toward telomeres as mitosis continues (Hendzel et al. 1997). To determine whether altering dCAP-D3 levels perturbs this order, we immunostained dCAP-D3-deficient and -overexpressing wing discs with antibodies against the phosphorylated serine 10 residue of histone 3 (PH3) to detect condensed chromatin and centromere protein A (CENP-A/CID) to label centromeres (Figure 4, A and B). Our results show that regardless of dCAP-D3 levels, wing disc cells exhibit higher PH3 levels at centromeres, suggesting that dCAP-D3 is not involved in facilitating the directionality of condensation. Interestingly, we also observed that GFP-dCAP-D3 aggregates in prophase wing discs were consistently located next to PH3-dense (centromeric) chromosomal regions (Figure 4, A and B). In Vivo Overexpression of dCAP-D3

Figure 3 Altering levels of dCAP-D3 influences mitotic progression. (A) Plot showing mitotic progression times in control (His2aV-mRFP). Blue ●: three discs, n = 32 cells, and dCap-D3 mutant (dCap-D3c07081/c07081; His2aV-mRFP). Green ●: three discs, n = 25 cells wing discs. (B) Stills of a representative mitotic event in a control (top row) and in a dCap-D3 mutant (bottom row) wing disc. (C) Plot showing mitotic progression times in discs expressing control GFP (Nubbin-GAL4 > pUASt-GFP). Orange ●: three discs, n = 30 cells, discs expressing GFP-dCAP-D3 protein where aggregates are observed (Nubbin-GAL4 > pUASt-GFP-dCAP-D3). Purple ●: four discs, n = 20, and discs expressing GFP-dCAP-D3 protein where aggregates are not observed. Lilac ●: three discs, n = 20 cells. (D) Stills of a representative mitotic event in a wing disc expressing GFP (top row), a wing disc expressing GFP-dCAP-D3 where aggregates are present (middle row), and a wing disc expressing GFP-dCAP-D3 where aggregates are not observed (bottom row). * P < 0.05.
4C), separating the more condensed from the less condensed DNA. This localization pattern was similar to that of endogenous dCAP-D3 in wild-type cells, which also appeared to aggregate, although in much smaller aggregations, and localize more strongly to less condensed DNA (Figure 4D).

As with the dCap-D3 mutants, we also attempted to assess the effects of overexpression of dCAP-D3 on eclosion rates, as well as total body weight using the ubiquitous β-tubulin-GAL4 driver. However, the use of this driver to overexpress dCAP-D3 resulted in lethality at the pupal stage. Together, these data show that overexpression of dCAP-D3 can phenocopy dCAP-D3 deficiency in the regulation of mitotic timing, specifically in prophase, which could potentially contribute to developmental delays.

**Overexpression of dCAP-D3 does not grossly affect genome organization in interphase**

Interphase genome organization is also modulated by Condensin II. In fact, Drosophila dCAP-H2 overexpression has been linked to polytene chromosome disassembly, chromatin reorganization, and both centromeric and nuclear envelope defects (Hartl et al. 2008; Bauer et al. 2012; Buster et al. 2013). Furthermore, in Drosophila S2 cells, overexpression of dCAP-H2 has been shown to cause the compaction of individual chromosomes into globules during interphase (Buster et al. 2013). To test whether overexpression of dCAP-D3 would result in a similar phenotype, we transfected GFP-dCAP-D3 protein (green, Nubbin-GAL4 > pUAST-GFP-dCAP-D3). DAPI staining of nuclei is shown in blue. Yellow dotted lines outline nuclear perimeter. (D) Immunostaining for PH3 (red) and dCAP-D3 (green) in wild-type (w1118) wing discs. Yellow dotted line outlines less condensed DNA; white dotted line outlines more condensed DNA. Blue + denote PH3-positive and CID-positive DNA.

**Figure 4** dCAP-D3 does not influence the directionality of condensation and localizes to less condensed mitotic DNA. (A) PH3 (gray) and CID (red) immunostaining in wing disc cells expressing GFP (Nubbin-GAL4 > pUAST-GFP) or GFP-dCAP-D3 (Nubbin-GAL4 > pUAST-GFP-dCAP-D3). DAPI staining of nuclei is shown in blue. Yellow dotted lines outline nuclear perimeter. (B) PH3 (gray) and CID (green) immunostaining in wild-type (w1118) and dCap-D3 mutant (dCap-D3c07081/c07081) wing discs. DAPI staining of nuclei is shown in blue. Yellow dotted lines outline nuclear perimeter. (C) PH3 (gray) immunostaining in wing discs expressing control GFP (green, Nubbin-GAL4 > pUAST-GFP) or GFP-dCAP-D3 protein (green, Nubbin-GAL4 > pUAST-GFP-dCAP-D3). DAPI staining of nuclei is shown in blue. Yellow dotted lines outline nuclear perimeter. (D) Immunostaining for PH3 (red) and dCAP-D3 (green) in wild-type (w1118) wing discs. Yellow dotted line outlines less condensed DNA; white dotted line outlines more condensed DNA. Blue + denote PH3-positive and CID-positive DNA.
changes to the nuclear envelope between GFP- and GFP- 
dCAP-D3-expressing cells (Figure S9B). Additionally, mea- 
urements revealed that dCAP-D3 overexpression did not 
not have a significant effect on nuclear size (Figure S9C).

Since overexpression of dCAP-D3 did not result in gross 
changes to DNA morphology in S2 cells or in vivo, we won- 
dered whether GFP-dCAP-D3 was localizing to DNA. To test 
this, polytene squashes were prepared from GFP and GFP- 
dCAP-D3 salivary glands and immunostained for dCAP-D3. 
Results revealed that GFP-dCAP-D3 does, in fact, localize to 
polytene chromatin in a pattern that does not overlap with 
DAPI dense regions, similar to what we have published pre-
viously for endogenous dCAP-D3 (Longworth et al. 2008) 
(Figure 5). Furthermore, IP of GFP-dCAP-D3 in salivary 
glands coupled with mass spectrometry successfully preci-
pitated GFP-dCAP-D3 and identified a number of putative 
binding partners previously shown to associate with DNA, 
including Dodeca-satellite-binding protein 1 and histone 
H2B (Table 1). Surprisingly, we failed to coprecipitate GFP- 
dCAP-D3 and the other Condensin II subunits, however, this 
result is similar to experiments in which IP of dCAP-H2 in 
embryos and ovaries did not coprecipitate dCAP-D3, but did 
pull down dSMC4 and dSMC2 (Herzog et al. 2013). Taken 
together, these data suggest that dCAP-D3 aggregates formed 
upon overexpression in nongrowing tissues did not seem to 
associate with the other Condensin II subunits and did not 
impair significant global changes to DNA organization in 
interphase.

**dCAP-D3 overexpression results in deregulation of 
gene expression**

Since our results showed that GFP-dCAP-D3 could localize to 
chromatin (Figure 5), we next tested the effects of overex-
pressing dCAP-D3 on gene expression in both the wing disc 
and salivary gland. While we have previously published that 
dCAP-D3 deletion results in transcriptional changes at the 
level of the whole organism (Longworth et al. 2012), gene 
targets of dCAP-D3 in wing disc and salivary glands have not 
been identified. Therefore, we performed RNA-seq in dCap-
D3<sup>-/-</sup> third instar larval wing discs and salivary 
glands to identify targets specific to each tissue. From these 
experiments, in which dCAP-D3 levels were depleted, we 
identified 1781 and 1224 deregulated target genes in the 
salivary gland and wing disc, respectively, based on a logFC 
>1 (fold change >2) and FDR <0.05. (Figure 6A and Table 
S1). Wing disc and salivary gland samples could be separated 
by principal component analysis while dCAP-D3 expression 
status further partitioned samples within tissues (Figure 
S10). A total of 465 genes were found to be deregulated in 
both tissue types, with 119 concordantly upregulated and 
274 concordantly downregulated. The remaining 72 shared 
target genes were deregulated in opposite directions in the 
two tissues (Figure 6B and Table S2).

GO analysis was performed to determine the types of gene 
programs and pathways affected by dCAP-D3 depletion (Fig-
ure 6C). The deregulation of genes involved in proteolysis, 
serine type endopeptidase activity, defense response to Gram-
positive bacteria, oxidation reduction, iron and heme bind-
ing, oxidoreductase and monoxygenase activity, and chitin 
metabolism was observed in both tissues. Similar to what 
was previously published for the whole larvae, we observed 
that many of the deregulated immune response genes were 
actually upregulated when dCAP-D3 levels were depleted in 
the larval tissues, contrary to the downregulation we had 
previously reported in the adult fly (Longworth et al. 
2012). This might be expected, as dCAP-D3 deficiency 
has been shown to result in chromosome instability in developing 
larval tissues and others have linked chromosome instability
Table 1 Putative binding partners of GFP-dCAP-D3

| Gene name                          | Gene symbol | Accession number | Low salt |   |   |
|------------------------------------|-------------|------------------|----------|---|---|
| Chromosome-associated protein D3, isoform A | CAP-D3      | 24581942         | 16       | 391 | 0 | 34 |
| Larval serum protein 1 gamma        | Lsp1γ       | 17647601         | 10       | 112 | 21 | 126 |
| Zipper, isoform B                   | zip         | 24762818         | 9        | 96  | 6  | 109 |
| Heat shock protein cognate 3, isoform B | Hsc70-3     | 24641404         | 18       | 72  | 22 | 53  |
| Heat shock protein cognate 4, isoform E | Hsc70-4     | 28571719         | 20       | 56  | 17 | 36  |
| β-Coatomer protein                  | βCOP        | 17647193         | 0        | 54  | 0  | 5   |
| Rudimentary, isoform A              | r           | 24642586         | 0        | 43  | 0  | 0   |
| Gamma-coatomer protein, isoform A   | γCOP        | 17864148         | 0        | 39  | 0  | 3   |
| Larval serum protein 1 β            | Lsp1β       | 17136306         | 0        | 33  | 14 | 66  |
| Oligosaccharide transferase Δ subunit | OstΔ        | 19922246         | 0        | 31  | 4  | 0   |
| β'-Coatomer protein                 | β' COP      | 24584107         | 0        | 30  | 0  | 11  |
| Ribosomal protein S27A              | RpS27A      | 17136574         | 0        | 26  | 0  | 2   |
| CG12715                            | CG12715     | 24641658         | 0        | 28  | 0  | 13  |
| Calcium ATPase at 60A, isoform A    | Ca-P60A     | 17136664         | 0        | 28  | 0  | 0   |
| Glutamine–fructose-6-phosphate aminotransferase 2 | Gfat2      | 21357745         | 0        | 26  | 0  | 2   |
| Hemomucin                          | Hmu         | 17137194         | 0        | 21  | 0  | 15  |
| Actin 87E, isoform A                | Act87E      | 17137090         | 19       | 21  | 13 | 13  |
| sec63                              | sec63       | 24660036         | 0        | 19  | 0  | 0   |
| Regulatory particle non-ATPase 1    | Rpn1        | 21356859         | 0        | 15  | 0  | 8   |
| AP-1gamama, isoform A               | AP-1γ       | 45549353         | 0        | 0   | 0  | 0   |
| Eukaryotic translation initiation factor 4G, isoform A | elf4G       | 161076325        | 0        | 14  | 0  | 0   |
| UDP-N-acetyl-α-α-galactosamine:polypeptide | GalNAc-T2  | 24643052         | 0        | 11  | 0  | 6   |
| N-acetylglactosaminyltransferase 2, isoform A | Veil        | 19922444         | 0        | 10  | 1  | 11  |
| CG30069                            | CG30069     | 116008309        | 3        | 10  | 0  | 0   |
| Glycoprotein 93                     | Gp93        | 21357739         | 0        | 9   | 5  | 55  |
| CG6453, isoform A                   | GCS2β       | 19921464         | 0        | 8   | 1  | 4   |
| Karyopherin β 3, isoform A          | Karyβ3      | 17737759         | 0        | 7   | 1  | 17  |
| Polypeptide GalNAc transferase 6, isoform A | pgant6     | 24656262         | 0        | 7   | 0  | 3   |
| Calnexin 99A, isoform A             | Cnx99A      | 24651030         | 0        | 7   | 0  | 2   |
| GalNAc-T1, isoform A                | GalNAc-T1   | 19922324         | 0        | 7   | 0  | 2   |
| Terribly reduced optic lobes, isoform L | trol        | 386763700        | 0        | 7   | 0  | 0   |
| CG9318, isoform A                   | CG9318      | 19921598         | 0        | 6   | 0  | 0   |
| Glutactin, isoform A                | Glit        | 17137794         | 0        | 6   | 0  | 0   |
| V-ATPase 69 subunit 2, isoform A    | Vha68-2     | 24583984         | 0        | 5   | 3  | 53  |
| Salivary gland secretion 3          | Sps3        | 17737563         | 1        | 5   | 1  | 24  |
| CG30463, isoform A                  | CG30463     | 24654219         | 0        | 5   | 0  | 0   |
| α-Coatomer protein, isoform B       | αCOP        | 24655452         | 0        | 4   | 0  | 12  |
| Asparyl-tRNA synthetase, isoform C  | Aats-asp    | 442623508        | 6        | 4   | 1  | 11  |
| β-Adaptin                          | AP-1-2β     | 17647183         | 0        | 4   | 0  | 0   |
| β-Tubulin at 56D, isoform B         | βTub56D     | 24655737         | 0        | 4   | 0  | 0   |
| α-Tubulin at 84B                   | αTub84B     | 17136564         | 0        | 3   | 0  | 0   |
| GTP-binding protein                | Gtp-bp      | 24641198         | 0        | 3   | 0  | 0   |
| White                              | w           | 17136592         | 0        | 3   | 0  | 0   |
| Δ-Coatomer protein, isoform A       | δCOP        | 21355081         | 0        | 2   | 0  | 8   |
| Regulatory particle non-ATPase 2, isoform A | Rpn2      | 24650984         | 0        | 2   | 0  | 8   |
| SLY-1 homologous, isoform C         | Slh         | 45552183         | 0        | 2   | 0  | 0   |
| Coro, isoform A                     | coro        | 24586098         | 2        | 1   | 9  | 12  |
| Mitochondrial trifunctional protein α-subunit, isoform A | Mtpα        | 19921000         | 0        | 1   | 0  | 7   |
| PolyA-binding protein, isoform C    | pAbp        | 24654797         | 0        | 1   | 0  | 4   |
| Glutamyl-prolyl-tRNA synthetase, isoform A | Aats-glupro | 24649466         | 0        | 1   | 0  | 3   |
| Heat shock protein 83, isoform A    | Hsp83       | 17647529         | 0        | 2   | 0  | 0   |
| Elongation factor 2b, isoform A     | EF2         | 24585709         | 0        | 0   | 0  | 0   |
| Tudor-SN, isoform A                 | Tudor-SN    | 20130403         | 0        | 0   | 0  | 0   |
| CG3523, isoform A                   | FASN1       | 19920632         | 0        | 0   | 0  | 0   |
| TER94, isoform C                    | TER94       | 161076486        | 0        | 2   | 3  | 36  |
| CG8036, isoform D                   | CG8036      | 24645119         | 0        | 3   | 0  | 34  |
| CG1516, isoform G                   | PCB         | 24652216         | 0        | 0   | 0  | 17  |
| Ghost, isoform B                    | Sec24CD     | 442625387        | 0        | 0   | 0  | 17  |
| Protein disulfide isomerase, isoform A | Pdi        | 17647799         | 0        | 0   | 0  | 17  |

(continued)
with activation of innate immune signaling in *Drosophila* wing discs (Longworth et al. 2008; Liu et al. 2015). While not tested, changes in chromatin landscape at different stages of development (i.e., adult vs. larval stage) could also account for the change in the direction of deregulation. While most GO terms unique to the wing disc included immune response genes, structural proteins, and extracellular matrix genes, uniquely enriched GO terms in the salivary glands included flavonoid processes and response to insecticide/DTT.

Randomly chosen upregulated targets were validated by qRT-PCR in tissue from both *dCap-D3c07081/c07081* and *dCap-D3c07081/D25* larvae (Figure 6, D and E). The D25 deletion is a genomic deletion on the second chromosome that encompasses *dCap-D3* and was expressed to confirm the observed changes in transcript levels were not due to bystander allele effect. Results from qRT-PCR experiments confirmed that the transcript levels of dCAP-D3 target genes identified by RNA-seq were changed in the expected direction.

Finally, to test whether dCAP-D3 overexpression affected target gene expression, qRT-PCR was performed on cDNA synthesized from RNA isolated from salivary glands and wing discs expressing GFP or GFP-dCAP-D3. In salivary glands, we found that overexpression of dCAP-D3 resulted in the downregulation of three target genes (*CG32071*, *CG32073*, and *CG13560*) whose expression was upregulated in dCAP-D3-deficient tissue (Figure 6F). We observed a similar phenomenon in the wing disc, with dCAP-D3 overexpression resulting in the downregulation of three target genes (*CecC*, *CG13560*, and *HP1D3csd*) whose expression was upregulated in dCAP-D3-deficient tissue (Figure 6G). These data demonstrate that overexpression of dCAP-D3 results in the deregulation of gene expression in both dividing and nondividing tissues and can occur in a manner that is opposite to what is observed when dCAP-D3 levels are depleted.

### Discussion

Prior to this work, the effects of elevated dCAP-D3 expression on the major functions of Condensin II, including mitosis, DNA organization, and transcriptional regulation, were unknown. However, our findings that elevated levels of CAP-D3 are found in many somatic cancers clearly demonstrates that elevated levels of CAP-D3, regardless of the reason for these higher levels, could have very important biological consequences. Our data now show that in *Drosophila*, overexpression of dCAP-D3 in developing larval tissues results in the formation of nucleoplasmic aggregates that act in a dominant-negative manner to slow mitotic prophase, similar to what is observed in dCAP-D3-deficient tissue. Interestingly, and in contrast to events observed in dCAP-D3-deficient tissues, overexpression of dCAP-D3 accelerates the metaphase to anaphase transition, and it is also during this period that the nucleoplasmic aggregates disappear. Notably, alterations to mitotic timing are only observed in cells harboring GFP-dCAP-D3 aggregates, which suggests it is the physical aggregation of the protein that is required for this phenotype. Future studies examining human tumor samples for the presence of CAP-D3 aggregates will reveal more about how and why these aggregates are formed. Our findings also demonstrate that while dCAP-D3 overexpression does not seem to affect global chromosome organization during interphase, it does result in changes to transcript levels of dCAP-D3 target genes.
genes in an opposite manner to changes observed in dCAP-D3-deficient tissues. Furthermore, should CAP-D3 overexpression have a conserved effect in human somatic tumors, these phenomena could lead to inefficient partitioning of daughter chromosomes and/or deregulation of important homeostatic transcriptional programs which could further promote tumorigenesis.

A major question that arises from this work is whether or not the dCAP-D3 aggregates possess significant functions that directly affect cellular homeostasis. Our studies are not the first to describe aggregation of Condensin II proteins upon overexpression. CAP-H2 overexpression in human cells was previously shown to result in the formation of aggregates that colocalize with markers of senescence-associated heterochromatin foci, and knockdown of CAP-H2 was subsequently found to inhibit oncogene-induced senescence (Yokoyama et al. 2015). The fact that, in our studies, dCAP-D3 aggregates are present in dividing cells as they enter into mitosis, however, argues against the fact that they play a role in senescence. Additionally, immunochemistry experiments showed that dCAP-D3 aggregates did not appear to colocalize with heterochromatin markers in salivary glands (data not shown).

Our data correlating the disappearance of the dCAP-D3 aggregates at the metaphase to anaphase transition with a change in the observed effects of dCAP-D3 overexpression on mitotic timing suggests that the aggregates may interfere with the condensation functions of the Condensin II complex, but this dominant-negative effect is lost once the aggregates disappear. This effect is also not observed when aggregates

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**Figure 6** Overexpression of dCAP-D3 regulates gene expression in larval salivary glands and wing discs. (A) Table summarizing the number of deregulated genes identified from RNA-seq experiment performed in dCAP-D3-deficient (dCAP-D3<sup>10791x10791</sup>) salivary glands and wing discs. Significant targets were identified based on a logFC >1 (fold change >2), FDR <0.05. For salivary glands, three biological replicates were used for both wild-type (w<sup>1118</sup>) and dCAP-D3 mutant (dCAP-D3<sup>10791x10791</sup>) samples. For wing discs, three biological replicates were used for wild-type (w<sup>1118</sup>) and two for dCAP-D3 mutant (dCAP-D3<sup>10791x10791</sup>) samples. (B) Venn diagrams showing the number of unique and shared upregulated (green), downregulated (red), and discordantly (gray) altered dCAP-D3 target genes. (C) Summary of DAVID GO analysis of genes presented in (A) using FDR <0.05. (D and E) qRT-PCR validation of three upregulated dCAP-D3 target genes identified from RNA-seq in the (D) salivary glands and (E) wing discs (blue is w<sup>1118</sup>, green is dCAP-D3<sup>10791x10791</sup>, pink is dCAP-D3<sup>10791x10791</sup>). (F and G) qRT-PCR in GFP- (orange) and GFP-dCAP-D3- (purple) expressing (F) salivary glands and (G) wing discs to detect transcript levels of dCAP-D3 target genes. SG = salivary glands, WD = wing discs.
were not present from the onset of prophase. One possibility is that the dCAP-D3 aggregates may be amplifying our ability to visualize a structure that is actually present in cells expressing normal levels of CAP-D3. Figure 4 shows that dCAP-D3 aggregates present in mitotic wing disc cells are localized proximal to, but not overlapping with, DNA that is heavily stained with PH3 (i.e., more condensed DNA). In fact, the aggregates seem to separate the more condensed DNA from the less condensed DNA. Interestingly, immunostaining performed with primary antibody to dCAP-D3 in mitotic wing disc cells shows the appearance of structures that look like much smaller aggregates of dCAP-D3 protein, suggesting that these aggregates can form from endogenously expressed dCAP-D3 protein, although at a drastically reduced level (Figure 4). Recently, using double-stranded DNA tethered to a surface, yeast Condensins were shown to use the energy provided from ATP hydrolysis to extrude DNA loops in real time (Ganji et al. 2018). These studies provided in vitro evidence for a model in which the kleisin and HEAT-repeat subunits of the Condensin complex could anchor a DNA strand at one region and then extrude the rest of the strand through the Condensin ring. Another recent article combined Hi-C data from synchronized chicken DT-40 cells with polymer simulations to examine the formation of mitotic chromosomes (Gibcus et al. 2018). These studies showed that immediately following entry into mitosis, chromosomes lose their interphase organization and are folded into arrays of loops positioned around a central axis (Gibcus et al. 2018). Excitingly, they also demonstrated that Condensin II is required for both the formation of the loop arrays and for reorganization of the arrays in prometaphase into shapes that resemble spiral staircases. If these phenomena are conserved and applicable to the configuration of chromosomes in Drosophila wing discs in prophase/prometaphase, then GFP-dCAP-D3 overexpression may allow us to visualize Condensin II complexes organizing the mitotic DNA into condensed, helical loop arrays on the centromere-proximal side of the Condensin II aggregate network, and the less condensed, potentially less looped DNA on the telomere-proximal side. In this scenario, the abnormally high levels of dCAP-D3 could result in a tighter association with DNA, making it harder for Condensin II to move along the DNA as it is being progressively looped and condensed, thus increasing the duration of prophase. Further studies involving overexpression of CAP-D3 proteins unable to bind to Condensin II subunits, combined with Hi-C analyses in these cells, will help to confirm or refute these hypotheses.

The cell-specific mechanisms that regulate formation of dCAP-D3 aggregates and the mechanisms that facilitate their disappearance remain unknown. dCAP-H2 is degraded by the Slimb ubiquitin ligase in Drosophila, but Slimb does not affect levels of dCAP-D3 (Buster et al. 2013). To date, a similar mechanism involved in dCAP-D3 degradation has not been identified. Alternative to active degradation of dCAP-D3 protein occurring at the onset of metaphase, it is also possible that the GFP-dCAP-D3 aggregates are breaking down due to diffusion of the protein away from the nucleus, since Drosophila cells have been shown to undergo a partial nuclear envelope breakdown at the end of prophase (Paddy et al. 1996).

Recently, Drosophila Condensin II was shown to promote the separation of sister chromatids during interphase in Cohesin-depleted cells, in line with Condensin II’s known role as an antipairer (Senaratne et al. 2016). Our data also show that when dCAP-D3 is overexpressed and aggregates are present, the protein behaves differently after the onset of metaphase after the aggregates have disappeared, resulting in phenotypes that are opposite to those seen in cells deficient for dCAP-D3 expression. Since we observe an acceleration in the transition from metaphase to anaphase when dCAP-D3 is overexpressed (Figure 3), it is possible that dCAP-D3 may act to speed up sister chromatid separation, thus significantly decreasing the timing of this mitotic window.

Whether Condensin II’s condensation and transcriptional regulatory functions are linked currently remains an unanswered question in the field. Evidence exists suggesting that defects in condensation can directly affect gene expression (Rawlings et al. 2011); however, whether Condensin II subunits can influence transcription through an alternative mechanism has never been directly tested. In our studies, we observe that overexpression of dCAP-D3 results in changes to target gene transcript levels that are opposite to those observed in dCAP-D3-deficient tissues (Figure 6). It is possible that all observed changes in transcriptional regulation could be a result of disruption to local nuclear architecture. For example, depletion of dCAP-D3 could cause local decondensation, while overexpression could cause local hyper-condensation, thus resulting in transcriptional changes in opposing directions. However, IP-mass spectrometry experiments performed by both our laboratory (Table 1) and others fail to show that overexpressed dCAP-D3 protein associates with other Condensin II members. Therefore, during interphase, it is possible that dCAP-D3 could be regulating gene expression through a secondary mechanism, independently of Condensin II. Further support for this idea is the fact that overexpression of dCAP-D3 in interphase cells does not result in similar phenotypes caused by dCAP-H2 overexpression. Additional studies aimed at identifying key residues needed to cause the phenotypes associated with dCAP-D3 overexpression and for association with Condensin II would provide insight as to how intertwined its condensation/Condensin II-specific and gene regulatory functions truly are.

Finally, aberrant expression and activity of Condensin II has been linked to a number of diseases, including cancer. Both loss of Condensin II subunits and disruption of their loading onto chromatin in mice has been reported to drive lymphogenesis (Atchison 2014; Woodward et al. 2016; Ishak et al. 2017). Here, we report data derived from the COSMIC database, which show that expression levels of Condensin II subunits are more commonly elevated than decreased in many somatic tumors (Figure 1), and TCGA data showing elevated Condensin II levels correlate with lower survival rates in patients with low-grade glioma (Figure S1, A–C). Additionally,
increased levels of CAP-G2 correlate with poor prognosis in cases of nonsmall cell lung cancer (Zhan et al. 2017). Condensin II subunit depletion in several organisms has been shown to result in lagging chromosomes and increases in DNA damage (Hartl et al. 2008; Longworth et al. 2008; Lee et al. 2011; Green et al. 2012; Schuster et al. 2013; Bakhrehbah et al. 2015). Our in vivo studies demonstrate that dCAP-D3 overexpression causes an acceleration of the metaphase to anaphase transition. In other studies, this has been shown to result in the bypassing of the spindle checkpoint, which can lead to increases in chromosome missegregation and aneuploidy (Meraldi et al. 2004). Future analyses of lagging chromosome generation and karyotypes in human cells over-expressing CAP-D3 would determine whether the increased metaphase to anaphase transition leads to chromosomal instability. It is also possible that higher CAP-D3 levels may promote tumorigenesis through the aberrant regulation of genes necessary for the development and the maintenance of homeostasis. Our data provide insight into how overexpression of the genome-organizing protein dCAP-D3 affects cellular homeostasis in both mitosis and in interphase and, if conserved, these findings could shed light on the possible mechanisms by which elevated CAP-D3 levels could influence tumor development and/or tumor progression.

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