The CAF-1 complex couples Hippo pathway target gene expression and DNA replication

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ABSTRACT The Hippo signaling pathway regulates tissue growth and organ development in many animals, including humans. Pathway activity leads to inactivation of Yorkie (Yki), a transcriptional coactivator that drives expression of growth-promoting genes. In addition, Yki has been shown to recruit chromatin modifiers that enhance chromatin accessibility and thereby enhance Yki function. Here, we asked whether changes in chromatin accessibility that occur during DNA replication could also affect Yki function. We found that depletion of the chromatin assembly complex-1 (CAF-1) complex, a histone chaperone that is required for nucleosome assembly after DNA replication, in the wing imaginal epithelium leads to increased Hippo pathway target gene expression but does not affect expression of other genes. Yki shows greater association with target sites when CAF-1 is depleted and misregulation of target gene expression is Yki-dependent, suggesting that nucleosome assembly competes with Yki for pathway targets post-DNA replication. Consistent with this idea, increased target gene expression is DNA replication dependent and newly replicated chromatin at target sites shows marked nucleosome depletion when CAF-1 function is reduced. These observations suggest a connection between cell cycle progression and Hippo pathway target expression, providing insights into functions of the Hippo pathway in normal and abnormal tissue growth.

INTRODUCTION

The Hippo signaling pathway regulates tissue growth and development by controlling proliferation and apoptosis (Pan, 2010). Central to the pathway is a multiple kinase cascade whose output is to phosphorylate Yorkie (Yki), a transcriptional coactivator (Huang et al., 2005). Phosphorylated Yki is retained in the cytoplasm and is therefore transcriptionally inactive; in contrast, when the kinase pathway is inactive, Yki translocates into the nucleus to promote transcription of pathway target genes (Dong et al., 2007; Oh and Irvine, 2008). Yki requires a DNA-binding partner such as Scalloped (Sd) to promote target gene transcription (Zhang et al., 2008). When Yki is absent, Sd serves as a Hippo pathway target transcription repressor (Koontz et al., 2013).

In addition to driving transcription by binding to DNA-binding partners, Yki is thought to recruit chromatin modifiers to regulate transcription. By mass spectrometry and communoprecipitation, Yki was shown to physically interact with chromatin modifying factors including the GAGA factor and the Brahma complex (Oh et al., 2013). More recently, Yki was shown to recruit the Trithorax-related (Trir) histone H3 lysine 4 (H3K4) methyltransferase complex through physically interacting with nuclear receptor coactivator 6 (Oh et al., 2014; Qing et al., 2014). These observations strongly suggest that Yki actively promotes changes in chromatin accessibility that in turn could...
affect Hippo pathway target expression in different developmental contexts. Conversely, it is likely that chromatin accessibility regulates Yki binding and thus Hippo pathway target expression, but this has not been examined.

The chromatin assembly complex-1 (CAF-1) complex, which is composed of Caf1-180, Caf1-105, and Caf1-55 in Drosophila, is required for replication-coupled nucleosome assembly (Smith and Stillman, 1989). Specifically, the CAF-1 complex has been shown to interact with PCNA and to assemble H3 and H4 histones into newly synthesized DNA (Shibahara and Stillman, 1999). Consistent with this notion, recent work has shown that Caf1-105 depletion in Drosophila S2 cells affects nucleosome deposition dynamics and chromatin accessibility of newly replicated chromatin (Ramachandran and Henikoff, 2016). In addition to its role as a histone chaperone, the CAF-1 complex also functions to maintain heterochromatin during DNA replication and restores nucleosomes on DNA after double-strand break repair (Muzina et al., 1999; Moggis et al., 2000). Together, these observations suggest that CAF-1 function can affect chromatin accessibility, particularly during DNA replication when genomic DNA is transiently free of nucleosomes or other binding factors. Interestingly, a recent study showed that loss of CAF-1 complex components results in increased expression of Notch signaling targets in actively replicating Drosophila follicle cells, raising the possibility that CAF-1 has functions that extend beyond post-DNA replication nucleosome assembly (Lo et al., 2019).

In this study, we asked whether changes in chromatin accessibility associated with CAF-1 function during DNA replication might also affect Hippo pathway target expression. We show that depletion of CAF-1 complex components affects expression of Hippo pathway targets in a Yki-dependent manner, but does not affect expression of other genes not associated with Hippo signaling. Furthermore, we demonstrate that alteration of the CAF-1 complex affects Yki’s accessibility to its target loci, and Hippo pathway target expression, in a DNA replication-dependent manner. Overall, our study uncovers a previously unknown link between DNA replication and Hippo pathway target expression through the CAF-1 complex.

RESULTS

Drosophila CAF-1 complex affects expression of Hippo pathway targets and growth

To ask whether changes in chromatin accessibility might affect Yki function, we investigated how depletion of the CAF-1 complex, which regulates post-DNA replication chromatin assembly (Shibahara and Stillman, 1999; Ramachandran and Henikoff, 2016), affects expression of known Yki target genes. Like other upstream components of Hippo signaling, Merlin (Mer) has been shown to be a target for Yki transcriptional activation as a part of a negative feedback loop that regulates pathway activity (Hamaratoglu et al., 2006). Mer has been extensively characterized in this context and therefore was used as a reporter for pathway activity in these studies.

We first observed that RNA interference (RNAi)-mediated depletion of Caf1-180 using the hh-Gal4 driver led to increased Mer accumulation as detected by antibody staining (Figure 1, A and A’). To determine whether the increased Mer staining we observed is due to increased Mer transcription, we performed in situ hybridization with a Mer-specific RNA probe and found that Caf1-180 depletion led to increased Mer mRNA levels (Figure 1, B and B’; Supplemental Figure S1A).

To extend these initial observations, we asked whether Caf1-180 depletion also affects the expression of other known Hippo pathway target genes (Figure 1, C–F’). In the wing disk, RNAi depletion of Caf1-180 led to up-regulation of death-associated inhibitor of apoptosis 1 (Diap1)-lacZ (Diap1-lacZ), a well-characterized Yki target (Zhang et al., 2008). Caf1-180 depletion also led to increased expression of f-j5.1-lacZ (fj-lacZ), ban3-GFP, and CycE, three additional Hippo pathway targets (Huang et al., 2005; Cho et al., 2006; Sopko et al., 2009; Matakatsu and Blair, 2012). Similar results were observed using nonoverlapping Caf1-180 RNAi lines (Supplemental Figure S1, B–C’). Additionally, we made mitotic clones using the null Caf1-180(1;2) allele and found that Mer staining and fj-lacZ reporter expression increased in the mutant clones, consistent with RNAi results (Figure 1, G–H’). Interestingly, Caf1-180 depletion did not consistently cause increased expanded expression (Supplemental Figure S1, D–E’).

If Caf1-180 depletion allows greater expression of yki target genes, then it also seems likely that Caf1-180 depletion should result in tissue overgrowth. However, this prediction is nuanced by the fact that Caf1-180 has an important role in postreplication chromatin assembly and therefore its loss should have pleiotropic and likely deleterious effects. Consistent with this notion, we found that strong RNAi-mediated Caf1-180 depletion led to severe tissue loss (unpublished data), and Caf1-180 mitotic mutant clones are noticeably smaller than their sister clones (Figure 1, G and H). For this reason, we used a weak Gal4 driver, rubbin-Gal4 (rub-Gal4), to deplete Caf1-180 moderately throughout the entire wing blade to assess its effect on growth. Under this driver wing imaginal disks expressing Caf1-180 RNAi displayed significant overgrowth (Figure 1, I, J, and L). While these animals survived to the adult stage, their wings were severely misshapen (unpublished data).

Next, we asked whether Caf1-180 affects Hippo pathway targets as part of the CAF-1 complex by asking whether depletion of the other subunits of the CAF-1 complex, Caf1-105 and Caf1-55, also affect Hippo pathway target expression. RNAi-mediated depletion of Caf1-105 or Caf1-55 led to increased Mer, Diap1-lacZ, and ban3-GFP expression (Figure 1, M–O”). Consistent with experiments done with hh-Gal4, we conducted parallel experiments with apterous-Gal4 (ap-Gal4) and obtained similar results (Supplemental Figure S1, F–H”). Taken together, our results showing that depletion of each of the three components of the CAF-1 complex led to up-regulation of Hippo pathway targets in a similar manner indicate that Caf1-180 functions as a part of the CAF-1 complex to affect Hippo pathway target expression.

The CAF-1 complex preferentially affects Hippo pathway targets

Our observations that several characterized Hippo pathway targets are affected similarly by CAF-1 complex depletion raise the question of whether the CAF-1 complex preferentially affects Hippo pathway target expression or instead has a more general effect on transcription in the developing wing. To explore this possibility, we examined the effect of Caf1-180 depletion on expression of genes that are not known to be regulated by Yki. Using available transcriptional reporters and antibodies, we found that Caf1-180 depletion did not affect expression of Moeisin (a protein that is structurally similar to Mer), Dac-lacZ (a target of BMP signaling; Figure 2, A–B”), Lamin, Macroglobulin complement-related (Mer), Coracle (Cora), Diaphanos, beta-tubulin, or spaghetti squash (Supplemental Figure S2, A–F”). In contrast, as a control we consistently observed that Caf1-180 depletion caused increased Mer staining in the same tissues.

To expand the number of testable targets and as a complementary approach to immunofluorescence staining, we used real time quantitative PCR (RT-qPCR) to examine the effect of Caf1-180 depletion on Hippo pathway-related and -unrelated genes. Starting
with known pathway targets, we first confirmed that Mer, Diap1, ban, and CycE transcript level increased in Caf1-180-depleted wing imaginal disks using a twofold increase as a threshold (Figure 2C). To examine CAF-1’s effect beyond these well-characterized Hippo pathway targets, we examined additional Hippo pathway targets that were implicated from previous genome-wide studies (Oh et al., 2013; Zhang et al., 2017). We defined a set of high-confidence Yki targets as genes that were 1) up-regulated in wts depleted tissue, 2) up-regulated when yki is overexpressed, and 3) identified as Yki-binding targets in both ChiP and DamSeq data sets. We randomly selected 10 genes from this list and examined the effect of Caf1-180 depletion on their expression (Figure 2D). Interestingly, Caf1-180 depletion led to greater than twofold up-regulation of all but one of these genes. We then examined 15 genes that have not been
implicated as Hippo pathway targets (Figure 2E). Using the twofold cutoff, Caf1-180 depletion did not lead to up-regulation of any these genes. Collectively, our data strongly suggest that the CAF-1 complex preferentially affects Hippo pathway targets. An explanation for increased yki target gene expression on Caf1-180 depletion is that Caf1-180 normally promotes Hippo pathway function through an unknown mechanism and thereby reduces Yki nuclear localization and transcriptional function. To test this idea, we asked whether depleting Caf1-180 affects the subcellular localization of endogenously expressed Yki-YFP, which we previous have shown acts as a biosensor for Hippo pathway kinase activity (Xu et al., 2018). We found no change in Yki-YFP’s localization or abundance (Supplemental Figure S2, G and G’) on Caf1-180 depletion, suggesting that depletion of the Caf1-180 does not affect activity the Hippo pathway kinase cascade and instead acts at the level of Yki itself.

The CAF-1 complex affects Hippo pathway target expression through Yki
The CAF-1 complex is thought to limit chromatin accessibility by promoting nucleosome deposition behind replication forks in S-phase nuclei (Kaufman et al., 1995). Given this, we wondered whether the effect of CAF-1 complex component depletion on Hippo pathway target gene expression might reflect changes in accessibility of Yki to its target loci on chromatin. As an initial test of this idea, we examined how simultaneously depleting CAF-1 complex components and yki would affect Hippo pathway targets. Since yki is an essential gene for growth in the wing imaginal disk, we transiently depleted yki using hh-Gal4 in combination with Tub-Gal80TS, which allows Gal4-driven expression only at restrictive temperature, for 48 h before tissue collection. Using Mer staining as a readout of Hippo pathway target expression, depletion of yki alone had no discernable effect while, as expected, depletion of Caf1-180 resulted in a dramatic increase in Mer staining (Figure 3, A–C). Interestingly, codepletion of yki strongly attenuated the effect of Caf1-180 depletion on Mer expression (Figure 3D). Similarly, codepletion of yki and Caf1-180 or Caf1-105 suppressed Diap1-lacZ up-regulation caused by depletion of Caf1-180 or Caf1-105 alone (Figure 3, G–M).

The observation that codepletion of CAF-1 components and Yki suppressed expression of Yki target genes raises the question of whether overgrowth induced by Caf1-180 RNAi (Figure 1, I and J) also requires Yki. To test this, we depleted Caf1-180 in animals heterozygous for a yki null allele (ykiB5) (Huang et al., 2005). Reduction in yki dosage substantially suppressed the Caf1-180 RNAi phenotype in the wing imaginal disk (Figure 1, K and L). Collectively, the above data suggest that the CAF-1 complex affects Hippo pathway target expression in a yki-dependent manner.

The CAF-1 complex restricts Yki’s interaction with Hippo pathway target loci
One possible explanation for the increased expression of yki regulated genes we observed is that Yki has greater access to its target loci when the CAF-1 complex is depleted. To test this idea, we conducted chromatin immunoprecipitation (ChIP) experiments to...
examine Yki occupancy on target loci comparing normal cells to those depleted for CAF-1 components. For these experiments, we used a YFP-tagged Yki transgene expressed under its endogenous promoter (yki-YFP) in the background of yki^{B5} (Su et al., 2017; Xu et al., 2018). Using anti-GFP antibodies, we could then efficiently immunoprecipitate Yki from different genotypes. Homozygous yki^{B5}, 2018). Using anti-GFP antibodies, we could then efficiently immunoprecipitate Yki from different genotypes. Homozygous yki^{B5} flies show no growth defect and were compared with (yki-YFP) yki^{B5} animals in which the CAF-1 complex component Caf1-180 was depleted using nub-Gal4. As negative controls, we used Glyceraldehyde 3 phosphate dehydrogenase 1 (Gapdh1) and Pyruvate dehydrogenase E1 alpha subunit (Pdha), which should be expressed independently of Yki activity.

We first focused on two well-characterized yki target regulatory regions: ban, whose Yki regulatory region has been mapped and used for a similar ChIP assay (Parker and Struhl, 2015), and Diap1, which has a defined enhancer element, 2B2C that is regulated by yki and sd (Huang et al., 2005; Wu et al., 2008; Zhang et al., 2008; Koontz et al., 2013). In (yki-YFP) yki^{B5} imaginal disks, sequences including the CATTCA motif of ban, to which Yki binds, were strongly enriched relative to the Gapdh1 and Pdha negative controls (Figure 3N). Strikingly, in Caf1-180-depleted imaginal disks, Yki pulled down an average of 2.2% of the input chromatin at ban, an approximately fourfold enrichment over the (yki-YFP) yki^{B5} controls ($p < 0.01$, $n = 3$ independent experimental replicates). In a similar manner, Yki occupancy at the Diap1-2B2C target site increased approximately threefold, from an average of 0.57% of the input chromatin in control tissue to 1.80% in Caf1-180-depleted tissue (Figure 3N).

To extend these observations, we conducted parallel experiments on Myc and lz, two additional Hippo pathway targets with previously characterized Yki-binding sites (Neto-Silva et al., 2010; Milton et al., 2014). Both studies also identified adjacent control chromosomal regions that do not bind Yki. Consistent with the observations for ban and Diap1, we observed significantly increased Yki binding in response to Caf1-180 depletion at the Yki-binding sites within the Myc and lz loci (Figure 3N). In contrast, in our experiments the control regions displayed ~10-fold less ChIP signal in Yki-YFP IPs compared with Yki-binding sites. Additionally, Yki binding at control regions was not significantly altered by Caf1-180 depletion alone, Student’s t test, $n = 7$ disks quantified). (N) Caf1-180 depletion enhanced Yki binding to its target loci. ChIP data are represented as the percentage of input DNA. Lz and Myc controls are regions near their respective gene’s regulatory regions and acts as negative controls. Gapdh2 and Pdha provide additional negative controls. Three biological replicates are shown.

The ability of the Caf1-1 complex to affect Hippo pathway target expression depends on DNA replication
In Drosophila S2 cells, histone occupancy is reestablished quickly after replication, in part due to the Caf1-1 complex’s chaperone function. Indeed, reducing the abundance of Caf1-1 complex components impairs post-DNA replication chromatin reestablishment
Given our results suggesting that Yki has greater access to its target sites when CAF-1 function is depleted, we wondered whether impairment of postreplication chromatin assembly might underlie Yki’s increased accessibility to target loci. If so, then the increased Hippo pathway target expression we have observed in response to CAF-1 complex depletion should be DNA replication dependent.

To test whether the CAF-1 complex’s ability to affect expression depends on DNA replication, we asked whether simultaneously depleting Caf1-180 and inhibiting DNA replication would suppress up-regulation of Mer or Diap1-lacZ staining characteristic of Caf1-180 depletion alone. To inhibit DNA replication, we depleted Origin Replication Complex (ORC) components by RNAi. Although most available transgenic RNAi lines for other ORC components produce no observable phenotype when expressed in the wing using hh-Gal4, we found that depletion of Orc4 using two nonoverlapping RNAi transgenes led to decreased DNA staining in the posterior compartment of the wing (Figure 4C” and Supplemental Figure S3, A–B’). Orc4 depletion alone had no effect on Mer or Diap1-lacZ, simultaneously depleting Caf1-180 and Orc4 attenuated Mer and Diap1-lacZ up-regulation compared with Caf1-180 depletion alone (Figure 4, A–D” and G; Supplemental Figure S3, C–F).

As a second means of inhibiting DNA replication, we expressed dacapo (dap), a cell cycle regulator whose ectopic expression blocks cell cycle progression (De Nooij et al., 1996). The dap expression using hh-Gal4 also resulted in decreased DAPI staining, suggesting that DNA replication was slowed and had little or no effect on Mer or Diap1-lacZ up-regulation compared with Caf1-180 depletion alone (Figure 4, E–G).

We also noticed that simultaneous depletion Caf1-180 and Orc4 (or coexpression of dap) resulted in synergistic undergrowth that was more substantial than Caf1-180 depletion alone. This decreased cell

**FIGURE 4**: The relationship between DNA replication and Yki target gene expression. (A–F”) Orc4 depletion or dap expression attenuates Yki target up-regulation caused by Caf1-180 depletion. Mer staining (A–F), diap1-lacZ expression (A”–F”), and DAPI staining (A”–F”) in the indicated genotypes. DAPI staining shows reduced DNA in Orc4 depleted (C”, D”) or dap expressing (E”, F”) wing disks. Larvae were incubated in restrictive temperature for 48 h before dissection. Approximate location of the anterior–posterior expression boundary is marked by dashed yellow lines. (G) Quantification of Yki target staining intensity in the indicated genotypes. Bars display ratios of posterior/anterior fluorescence intensity, represented as mean ± SEM (**p < 0.01 for comparison of the indicated genotype to Caf1-180 depletion alone, Student’s t test, n = number of disks quantified).
number was accompanied by a decrease in DAPI staining (Figure 4, D" and F"), potentially complicating quantification of Yki target expression levels. To examine the possibility that codepletion of Caf1-180 and Orc4 nonspecifically decreases gene expression, we conducted parallel experiments on Mcr and Cora. We found that neither Orc4 depletion alone or Orc4 and Caf1-180 codepletion affected Mcr (Supplemental Figure S3, G–I') or Cora (Supplemental Figure S3, J–L') abundance, suggesting that Caf1-180 and Orc4 specifically affected Hippo pathway targets. Collectively, our observation that inhibiting DNA replication suppressed the effect of Caf1-180 depletion on Hippo pathway targets suggests that CAF-1 antagonizes Yki function in a DNA replication-dependent manner and is consistent with the idea that CAF-1–mediated nucleosome assembly after DNA replication inhibits the access of Yki to its target sites.

Differential timing of postreplication histone deposition may contribute to CAF-1’s preferential effect at Yki targets

The CAF-1 complex is believed to function as a generic histone chaperone that deposits histone H3/H4 onto DNA behind the replication fork. However, the data presented here suggest that CAF-1 preferentially affects the expression of Hippo pathway targets in a Yki- and DNA replication–dependent manner. To explore how CAF-1 does this, we analyzed published MINCE-seq data from Drosophila S2 cells (Ramachandran and Henikoff, 2016) to see how nucleosome landscape changes postreplication at Yki targets. MINCE-seq is a metabolic labeling method that labels <10 kb of newly replicated chromatin for each active replication fork (with 10 min of labeling), followed by micrococcal nuclease treatment and DNA sequencing. Additionally, MINCE-seq can be used to profile nucleosomal footprints from both newly replicated and steady state chromatin from the same sample. In this study, MINCE-seq was also performed after knocking down CAF-1 to analyze changes in the nucleosome landscape behind the replication fork when replication-coupled nucleosome assembly was impaired (Ramachandran and Henikoff, 2016). We reanalyzed these data to compare nucleosome gains behind the replication fork for 22 genes (Figure 2E and Supplemental Table S1) that are not affected by CAF-1 knockdown to four genes (Figure 2C) that are up-regulated on Caf1-180 knockdown in our experiments. We observed no significant change in nucleosome occupancy 10 min postreplication at the nontarget genes (Figure 5A) in response to CAF-1 depletion. However, we observed a strong impairment of nucleosome gain at transcription start sites (TSS) of the four verified Yki targets.

To extend these observations, we sought to compare the behavior of different Yki-binding sites in response to CAF-1 depletion. Yki has been shown to bind to several DNA-binding proteins including Gaga factor (Trl), Mad, and Sd to activate specific genes (Wu et al., 2008; Zhang et al., 2008; Oh and Irvine, 2011). The direct effects of
the CAF-1 complex would manifest as nucleosomes replacing these DNA-binding proteins behind the replication fork, preventing Yki access to DNA postreplication. We asked which of the DNA-binding protein sites are most affected by CAF-1 knockdown. To do this, we identified the subset of all Yki ChIP sites from Oh et al. (2013) that had strong motifs for Trl, Mad, and Sd (Supplemental Table S2). When we plotted the average nucleosome landscape at steady state over sites of each transcription factor (TF), we observed nucleosome depletion (Figure 5, B–D, input data sets), pointing to TF binding. We then asked how this nucleosome landscape changes postreplication by plotting the average nucleosome landscape postreplication over sites of each (Figure 5, B–D, pull-down data sets). In controls (dsGFP), we observed nucleosome gain at Trl, Mad, and Sd sites postreplication (Figure 5, B–D). CAF-1 knockdown impaired postreplication nucleosome gains at Sd sites to a significant extent, while Trl and Mad sites instead appeared to show slight gains (Figure 5, B–D). To confirm these observations, we calculated net change in postreplication nucleosome occupancy for each binding site ±50 base pairs and plotted the distribution of the nucleosome occupancy (Figure 5E). We observed no difference or slight increase in nucleosome occupancy replication for Trl and Mad in CAF-1 knockdown cells compared with control, indicating that postreplication nucleosome dynamics at these sites are not affected by the absence of CAF-1. However, we observed a significant decrease in nucleosome gain postreplication at Sd sites on knockdown of CAF-1, indicating that Sd sites are highly sensitive to CAF-1 function postreplication.

A possible explanation for differential sensitivity to CAF-1 depletion at different binding sites is that CAF-1-independent nucleosome assembly also occurs postreplication at Trl and Mad sites (Ray-Gallet et al., 2011), while Sd-binding sites are more dependent on CAF-1 for nucleosome deposition postreplication. Alternatively, if Trl and Mad sites have higher rates of nucleosome turnover throughout the cell cycle, nucleosome loss postreplication might be obscured. To explore this possibility, we calculated DNA replication-independent nucleosome turnover at Mad, Trl, and Sd sites using CATCH-IT (Teves and Henikoff, 2011) and found that Mad and Trl sites display significantly higher rates of replication-independent nucleosome turnover than observed at Sd sites (Figure 5F). Regardless of the precise mechanism, taken together these results suggest that Sd targets are protected by nucleosomes postreplication, and impairment of this process could allow Sd and its partner Yki to bind these genes postreplication.

The CAF-1 complex affects repressive histone modifications in a yki-dependent manner

Thus far, our data suggest that the CAF-1 complex influences Yki’s accessibility to its target loci and as a result affects Yki target gene expression. We next explored how these seemingly transient changes in chromatin accessibility might lead to sustained changes in gene expression. In addition to driving transcription by interacting with DNA-binding partners such as Sd, recent studies showed that Yki can recruit activating chromatin modifiers such as Trl to increase chromatin accessibility at target sites (Oh et al., 2013, 2014; Qing et al., 2014). The observation that Caf1-180 depletion causes greater Yki chromatin accessibility led us to wonder whether Caf1-180 depletion might also affect chromatin activation through Yki.

Although Yki has a relatively small number of validated transcriptional targets, previous genome-wide studies have reported thousands of chromatin binding peaks, some of which colocalize with binding peaks of chromatin modifiers, including Trl (Oh et al., 2013). This observation led us to wonder whether the effects of CAF-1 complex component depletion might have widespread effects on chromatin state. To assess this, we used antibodies against specific chromatin modifications to stain whole imaginal disks in which different CAF-1 components were depleted in a defined region of the tissue. A similar approach was used previously to assess the effects of Brt1 loss on H3K4 methylation (Xuan et al., 2013). This approach allows side-by-side, internally controlled comparisons of staining in wild type and RNAi-depleted tissues. All of the antibodies used have been validated for immunofluorescence and to be specific for particular histone modifications (Subbanna et al., 2013; Fei et al., 2015; Rothbart et al., 2015; Chiacchiera et al., 2016; Le et al., 2016).

Previous studies have shown that Yki can modulate H3K4 methylation, an activation mark, at the diap1 and ex loci through its ability to recruit histone methyltransferases through interactions with NcoA6 (Oh et al., 2014; Qing et al., 2014). Therefore, we wondered whether the CAF-1 complex affects Hippo pathway target expression in part by preventing activating histone modifications such as H3K4 methylation. Using hh-Gal4, we depleted Caf1-180 in the posterior compartment of the wing imaginal epithelium, leaving the anterior compartment as an internal control. As expected, antibody staining of neuronal disks using anti-H3K4me1 and anti-H3K4me3 resulted in distinctly nuclear staining. However, we found that Caf1-180 depletion in the posterior of the wing did not noticeably affect staining for these activating marks when compared with anterior control cells (Supplemental Figure S4, A and B’).

The CAF-1 complex also has been implicated in heterochromatin formation, which is thought to repress transcription by promoting the accumulation of repressive modifications (Huang et al., 2010). For this reason, we next asked whether depletion of the CAF-1 complex might reduce repressive histone marks in the wing imaginal epithelium. We focused on H3K27 methylation, which is associated with repressed chromatin states and regulated by HP1 (Jamieson et al., 2016), because Caf1-180 was previously shown to affect HP1-dependent heterochromatin (Huang et al., 2010).

To examine whether the CAF-1 complex affects H3K27 methylation, we depleted Caf1-180, again using the hh-Gal4 driver and stained using H3K27me2- and H3K27me3-specific antibodies. Interestingly, Caf1-180 depletion led to decreased staining of H3K27me2 and H3K27me3 (Figure 6, A–B’ and G–H’), but in marked contrast did not reduce global histone H3 levels and instead led to moderately increased H3 staining (Supplemental Figure S4, C and C’). Previous work in mammalian cells has shown that under conditions of CAF-1 depletion, the HIRA complex functions in postreplication nucleosome assembly, replacing H3.1 with H3.3 (Ray-Gallet et al., 2011). We speculate that the pan-H3 antibody we used has a preference for H3.3, resulting in greater staining under conditions of CAF-1 depletion.

The observation that Caf1-180 depletion leads to decreased levels of the H3K27me2 and H3K27me3 chromatin modifications suggests two (not mutually exclusive) models: 1) that the CAF-1 complex promotes deposition of these repressive marks, or 2) that the increased binding of Yki to chromatin in response to CAF-1 depletion leads to decreased repressive marks. To address these possibilities, we reduced yki dosage in half (ykiΔy/∆y) and again stained Caf1-180-depleted imaginal disks with modification-specific antibodies. We found that decreased yki dosage suppressed the effect of Caf1-180 depletion on H3K27me2 and H3K27me3 staining (Figure 6, C–C”, F, I–I”, and L). This result suggests that Yki regulates transcription not only by promoting activating histone modifications but also by preventing repressive modifications. Consistent with published work (Huang et al., 2010), we found that Caf1-180 depletion decreased staining for H3K9 methylation.
Interestingly, the effect of Caf1-180 depletion on H3K9 methylation was not yki dependent (Supplemental Figure S4, E and E') suggesting that the CAF-1 complex might regulate different histone modifications by different mechanisms.

To further examine the possibility that Yki can affect repressive chromatin modifications, we next asked whether activating Yki by attenuating Hippo pathway activity also affects repressive chromatin marks. In wing imaginal disks where wts was depleted by RNAi using hh-Gal4, we observed decreased H3K27me2 and H3K27me3 staining. Quantification of H3K27me2 staining intensity (F) is shown as posterior/anterior ratios represented as mean ± SEM (***p < 0.001, Student’s t test, n = 10 for each genotype). (G–L) Mosaic wing imaginal disks of the indicated genotypes stained for H3K27me3. As seen for H3K27me2, Caf1-180 (H–H') or wts (J–J') depletion results in decreased H3K27me3 staining, while heterozygosity for yki (I–I'', K–K'') suppresses this effect. Quantification of H3K27me3 staining intensity (L) is shown as posterior/anterior ratios represented as mean ± SEM (***p < 0.001, Student’s t test, n = 10 for each genotype).

To further examine the possibility that Yki can affect repressive chromatin modifications, we next asked whether activating Yki by attenuating Hippo pathway activity also affects repressive chromatin marks. In wing imaginal disks where wts was depleted by RNAi using hh-Gal4, we observed decreased H3K27me2 and H3K27me3 staining.
staining in the posterior compartment in comparison to the anterior compartment (Figure 6, D–D”, F, J–J”, and L). Similar results were observed with hpo depletion (Supplemental Figure S4, H and H’ and K–K’) and in hpo and wts mitotic clones (Supplemental Figure S4, L–O’”). To ask whether this decrease was Yki dependent, we reduced yki activity in the background of hpo or wts depletion and stained for H3K27me2 and H3K27me3. Interestingly, wts or hpo depletion in yki heterozygotes displayed a significantly weaker effect on H3K27 methylation staining when compared with controls (Figure 6, E, F, K, and L; Supplemental Figure S4, H–K’). Collectively, these data strongly suggest that Yki can affect repressive histone marks, and that this activity can be regulated by Hippo signaling.

To address the possibility that Hippo pathway activity affects functioning of the CAF-1 complex, we used CRISPR-Cas9-based gene editing to insert GFP in-frame at the 3’ end of the Caf1-180’s coding sequence (Caf1-180-GFP). Homozygous Caf1-180-GFP animals showed normal growth and development, and Caf1-180-GFP was effectively depleted by Caf1-180-specific RNAi expression (Supplemental Figure S4, P–P”). However, depletion of hpo or Mer did not affect Caf1-180’s localization or abundance (Figure 6, M–O). Likewise, Mer overexpression did not affect Caf1-180’s abundance (Supplemental Figure S4, Q–Q”). Collectively, these data suggest that the CAF-1 complex function is not regulated by the Hippo pathway.

**DISCUSSION**

In this study, we have explored the functional relationship between CAF-1, a histone chaperone complex that assembles nucleosomes after DNA replication, and expression of Hippo pathway targets. We found that depletion of CAF-1 components leads to increased expression of Yki target genes in a Yki-dependent manner, that this effect depends on DNA replication, and that decreased CAF-1 function results in increased accessibility of Yki to its target loci. Our results further indicate that binding sites for Sd, the major DNA-binding cofactor for Yki-mediated transcription, display greater dependence on CAF-1 for nucleosome assembly after replication when compared with other TF-binding sites. We believe that as a consequence, depletion of CAF-1 complex components leads to increased Hippo pathway target expression, while not affecting expression of many nonpathway-related genes (Figure 7). Together, these observations reveal a previously uncharacterized role of the CAF-1 complex and a poorly understood mode of Yki target gene regulation.

Although initially it seems surprising that a ubiquitous nucleosome chaperone has relatively specific transcriptional effects, this is not the first example of CAF-1 mutations with specific developmental phenotypes. In Caenorhabditis elegans, CAF-1 mutations have been shown to disrupt the MI-e3D asymmetric cell fate decision in the nervous system resulting in extra e3D-like cells (Nakano et al., 2011). This defect was attributed to altered postreplication nucleosome assembly, possibly relating to asymmetric nucleosome assembly on leading and lagging strands behind the replication fork. While we have no evidence from our studies suggesting replicative asymmetry, the notion that delayed nucleosome reassembly post-DNA replication results in altered transcription of specific genes is common to both cases. Additionally, recent work in mammalian cells has shown that the CAF-1 complex regulates pluripotency in adult cells by preferentially affecting Sox2 binding to its target loci (Cheloufi et al., 2015), and studies in Drosophila have shown that CAF-1 modulates expression of target genes downstream of Notch signaling (Yu et al., 2013; Lo et al., 2019). Taken together, these results highlight the interplay between assembly of nucleosomes and the assembly of TF complexes on newly replicated DNA in mitotically active cells.

What remains unclear is why certain transcriptional complexes, such as those associated with Yki and Notch signaling, seem to be particularly affected by competition with assembling nucleosomes. By examining the post-DNA replication nucleosome dynamics at the DNA-biding sites of three different Yki partners, Sd, Trl, and Mad, we found that nucleosome deposition is more severely affected by CAF-1 depletion at Sd-binding sites than at the binding sites of the other Yki partners. This observation suggests that Sd-binding sites are more dependent on CAF-1 for postreplication nucleosome assembly. Additionally, we found that replication-independent nucleosome turnover is higher at Trl- and Mad-binding sites when compared with Sd sites, which could contribute to the relatively greater sensitivity of Sd sites to CAF-1 depletion. Further studies are required, but these findings again suggest that interactions between TFs and chromatin assembly have a complex role in the functioning of signaling pathways in development.

It is important to note that in addition to its role in promoting transcription of Hippo pathway targets, Sd interacts with Tendu-domain-containing Growth Inhibitor to repress pathway targets in the absence of Yki (Koontz et al., 2013). This raises the question of whether nucleosome depletion affects both Sd’s default repression function and its transcriptional activation function. While our work does not directly answer this question, it seems likely that Sd interaction with DNA is affected by its binding partner and as a result CAF-1 depletion might not affect Sd’s functions in promoting and repressing target site expression in the same way.

Previous studies of Yki function have shown that Yki has the ability to recruit chromatin modifiers, including Trl, to its target genes, thereby promoting increased chromatin accessibility and target gene expression. While these studies provided evidence only for local chromatin alterations at known Yki-binding sites, we took advantage validated antibodies and the internally controlled tissue mosaicism afforded by the Gal4-UAS system to look for subtle but more global effects on histone methylation by tissue staining. Although we
did not observe effects from CAF-1 depletion in the wing imaginal disk on the abundance of activating histone marks, we did observe a marked decrease in repressive methylation marks at H3K27. These results were particularly surprising for the following reasons. First, the effects of CAF-1 depletion on H3K27 methylation were strongly suppressed by heterozygosity for a yki null allele, suggesting that these effects are mediated by Yki. This observation suggests that Yki has a more widespread role in regulating chromatin accessibility than previously has been recognized. Second, given published estimates of ~3000 Yki-binding targets in the fly genome (Oh et al., 2013; Zhang et al., 2017), our results suggest that Yki could have an important role in suppressing the spread of repressive marks. Previous work has shown that Yki can promote local recruitment of activating histone marks (Oh et al., 2014; Qing et al., 2014) but has not examined repressive marks. Third, while CAF-1 depletion has widespread effects on repressive histone marks, reduction in these marks does not appear to have widespread transcriptional effects and instead seems to have specific effects on Hippo pathway targets at least in the wing imaginal disk. We do not currently fully understand the role of Yki in regulating chromatin accessibility or the impact of this role in expression of its targets, but these results clearly indicate the importance of further studies to address these questions.

An additional intriguing aspect of our findings is the observation that inhibiting DNA replication suppresses the effects of CAF-1 depletion on Yki target gene expression. This result is consistent with the idea that CAF-1 competes with transcriptional activators, including Yki, as the chromatin reassembles after the replication fork (Ramachandran and Henikoff, 2016). It also raises the question of whether expression of Hippo pathway targets, or specifically changes in expression of those targets, is coupled to DNA replication during normal development. Such coupling could have important implications for Hippo pathway regulation of tissue growth by stabilizing pathway output for the duration of the cell cycle. Alternatively, our observations also raise the possibility that Yki-mediated transcription could be regulated by the cell cycle, perhaps being greatest immediately after S-phase in G2 and relatively quiescent in G1. Elucidation of the precise effects of the competition we have described between the transcriptional co-activator Yki and the nucleosome assembly factor CAF-1 for access to Hippo pathway target genes will require more precise analysis of the transcriptional profiles of these targets.

**MATERIALS AND METHODS**

*Drosophila* culture and genetics

All *Drosophila* culture was performed at 25°C unless otherwise noted. For experiments using conditional knockdown or expression, larvae carrying tubGal80TS together with a Gal4 driver (i.e., hh-Gal4) were reared at 18°C for 48–72 h and then shifted to 29°C. Late third-instar larvae were then dissected as usual. To induce somatic mosaic clones, larvae were heat shocked 60–84 h after egg laying in a 37°C culture and genetics.

**Wing imaginal disk immunostaining and in situ hybridization**

Wandering third instar larvae were dissected in Schneider's Drosophila Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum to collect wing imaginal disks, which were fixed in 2% paraformaldehyde in PBS for 20 min. Primary antibodies were incubated overnight at 4°C. The following primary antibodies were used: guinea pig anti-mer (1:10,000) (Lajeunesse et al., 1998), mouse anti-β-galactosidase (1:500, DSHB), mouse anti-CycE (1:10) (Richardson et al., 1995), rabbit anti-H3K27me2 (1:1000, Cell...
Expression analysis by qPCR
RNA was extracted from 200 wandering third larvae wing imaginal disks using Direct-zol RNA Miniprep (Zymo Research) with in-column DNase treatment. RNA (1 μg) was used with iScript cDNA Synthesis Kit (Bio-Rad Laboratories) to carry out reverse transcription according to manufacturer's protocol. qPCR was performed using the iQaq Universal SYBR Green Supermix (Bio-Rad Laboratories) on a CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories) in triple technical replicates and subsequently analyzed with manufacturer's CFX Manager software. Primers for qPCR are listed in Supplemental Table S3.

Chromatin immunoprecipitation and downstream analysis
Wing imaginal disk ChIP was done as previously described (Webber et al., 2013) with the following modifications: 200–250 wandering third instar larvae wing imaginal disks were used for each ChIP experiment; a guinea pig anti-GFP (1:2000, this study) was used to immunoprecipitate Yki-YFP; protein A magnetic beads (Thermo Fisher Scientific) were used to capture the guinea pig anti-GFP antibody; DNA was purified using the NucleoSpin Gel and PCR Cleanup Kit (Macherey-Nagel).

ChIP-qPCR was performed using the same reagents and equipment as above. Primers to amplify Diap1, ban, Myc, and Iz regulatory regions, as well as Myc and Iz control regions, were published previously (Neto-Silva et al., 2010; Milton et al., 2014; Parker and Struhl, 2015). ChIP signals were calculated as percentage of input for both experiment and no antibody mock controls. Mock control ChIP signal was below or close to the detection limit in the qPCR experiments, and therefore was not presented in the figures.

Post-DNA replication nucleosome change analysis
To evaluate nucleosome changes postreplication, we utilized published MINCE-seq data sets (Ramachandran and Henikoff, 2016) generated from Drosophila S2 cells that were either treated with GFP dsRNA (mock, denoted as dsGFP) or Caf1-105 dsRNA (denoted as dsCaf1). MINCE-seq input measures steady state nucleosome profile, and MINCE-seq pull down measures nucleosome profile within 10 min of passage of the replication fork. The GEO accessions for the four data sets used in the analysis presented in this study are shown in Table 1.

For analysis of nucleosome profiles from these data sets, fragments between 134 and 160 base pairs were used. For Figure 5 (B–D), Yki ChIP-binding sites were obtained from GSE38594 (GSE38594_Yki_E8-16.bed.gz). Motifs within ChIP peaks were identified using FIMO (Grant et al., 2011). Motifs were obtained from Fly Factor Survey (Zhu et al., 2011). The center of the nucleosome-depleted region (NDR) around the identified motif was used as the reference position for calculating average nucleosome profiles.

Replication-independent nucleosome turnover analysis
To evaluate replication-independent nucleosome turnover at Yki-binding sites, we used published CATCH-IT data sets (Teves and Henikoff, 2011). Fragments between 120 and 174 base pairs from CATCH-IT input and pull-down data sets were used (data sets listed below). We calculated log 2 ratio of normalized CATCH-IT pull-down read density to normalized CATCH-IT input read density at each 10-base pair interval in the genome. CATCH-IT ratio was calculated ± 50 base pairs around the center of the NDR around the identified motifs of Sd, Mad, and Trl at Yki-binding sites. These sites were same as those used for analysis of MINCE-seq data sets. The GEO accessions of CATCH-IT data sets used in this study are shown in Table 2.

Live Imaging. Live imaging of Caf1-180-GFP was performed as previously described (Xu et al., 2018).

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