TITLE: The *Drosophila* Dbf4 ortholog Chiffon forms a complex with Gcn5 that is necessary for histone acetylation and viability

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RUNNING TITLE: Drosophila Chiffon-Gcn5 complex

ABSTRACT:

Metazoans contain two homologs of the Gcn5-binding protein Ada2, Ada2a and Ada2b, which nucleate formation of the ATAC and SAGA complexes respectively. In *Drosophila melanogaster*, there are two splice isoforms of Ada2b: Ada2b-PA and Ada2b-PB. Here we show only the Ada2b-PB isoform is in SAGA; in contrast, Ada2b-PA associates with Gcn5, Ada3, Sgf29 and Chiffon forming the Chiffon Histone Acetyltransferase (CHAT) complex. Chiffon is the *Drosophila* ortholog of Dbf4, which binds and activates the cell cycle kinase Cdc7 to initiate DNA replication. In flies, Chiffon and Cdc7 are required in ovary follicle cells for gene amplification, a specialized form of DNA re-replication. Although *chiffon* was previously reported to be dispensable for viability, here we find that Chiffon is required for both histone acetylation and viability in flies. Surprisingly, we show that *chiffon* is a dicistronic gene that encodes distinct Cdc7- and CHAT-binding polypeptides. Although the Cdc7-binding domain of Chiffon is not required for viability in flies, Chiffon's CHAT-binding domain is essential for viability but is not required for gene amplification, arguing against a role in DNA replication.

SUMMARY STATEMENT:

The *Drosophila* ortholog of Dbf4, Chiffon, binds Gcn5 to form a novel histone acetyltransferase complex that is essential for viability in flies, but is not required for DNA replication.

INTRODUCTION:

Chromatin modifications impact both transcription and cell cycle events such as DNA replication (Li et al., 2007; Ma et al., 2015). In particular, histone acetylation contributes to transcription, and correlates with the timing of the initial step in DNA replication, origin firing. The histone acetyltransferase Gcn5 stimulates transcription by generating a permissive chromatin environment that facilitates chromatin remodeling by complexes such as SWI/SNF (Hassan et al., 2002; Weake and Workman, 2010). Gcn5 also stimulates origin firing when tethered to a late-firing origin in yeast (Vogelauer et al., 2002) and enhances the rate of DNA synthesis from a chromatin template *in vitro* (Kurat et al., 2017). In *Saccharomyces cerevisiae*, Gcn5's function in transcription is mediated predominantly through the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex (Grant et al., 1997). During evolution, there has been an expansion in the diversity of Gcn5-containing complexes (Spedale et al., 2012). In metazoans, including *Drosophila*, there are two homologs of the Gcn5-binding protein Ada2, Ada2a and Ada2b, which nucleate formation of the Ada2a-containing (ATAC) or SAGA transcription coactivator complexes respectively (Kusch et al., 2003; Wang et al., 2008). All Gcn5 complexes share Sgf29 and Ada3

subunits, which together with their respective Ada2 homolog, enable nucleosomal histone acetyltransferase activity (Balasubramanian et al., 2002; Grant et al., 1997; Spedale et al., 2012). The SAGA and ATAC complexes activate transcription during development and in response to signaling pathways or external stimuli (Spedale et al., 2012). In addition, ATAC has roles in cell cycle progression via acetylation of cyclin A, which promotes progression through mitosis (Orpinell et al., 2010).

In *Drosophila*, Ada2b has two splice isoforms that differ in their C-terminal regions but share a common N-terminal region (Pankotai et al., 2013; Qi et al., 2004). The short Ada2b-PA isoform binds Gcn5, and is necessary for histone H3 acetylation *in vivo* (Pankotai et al., 2013; Qi et al., 2004). Both Ada2b isoforms are required to fully complement *ada2b* mutations, although expression of the short Ada2b-PA isoform alone can support development and partially restore histone H3 acetylation (Pankotai et al., 2013). Although it has been assumed that the short Ada2b-PA isoform functions as part of the SAGA complex, our previous studies showed that the long Ada2b-PB splice isoform is associated with *Drosophila* SAGA (Weake et al., 2009). In this study, we describe a novel Gcn5-containing complex nucleated by the short Ada2b-PA isoform that contains the cell cycle regulatory protein, Chiffon: the Chiffon Histone Acetyltransferase, CHAT complex. Chiffon is the *Drosophila* ortholog of Dbf4 (Landis and Tower, 1999), and it binds and activates the cell cycle kinase Cdc7 (Stephenson et al., 2015). In yeast, Dbf4 and Cdc7 form the Dbf4-Dependent Kinase (DDK) complex that phosphorylates the Mcm helicase to initiate DNA replication (Lei et al., 1997; Weinreich and Stillman, 1999). While Dbf4 is essential for DNA replication in most organisms, previous studies found that *chiffon* null mutants were viable (Landis and Tower, 1999). Here, we show that indeed the Cdc7-binding activity of Chiffon is dispensable in flies. However, the C-terminal insect-specific domain of Chiffon that nucleates formation of the CHAT complex is required in flies for both histone H3 acetylation and viability, but not for DNA replication. Our data demonstrate that the DNA replication and histone acetylation activities of Chiffon can be genetically separated, and raises the question of why these two activities are encoded by the same gene. One possibility, although not tested in this study, is that the DDK and CHAT complexes are part of the same gene to coordinate their expression and/or levels during either the cell cycle or development. This could provide a mechanism to coordinate histone acetylation with DNA replication, potentially during particular developmental stages in flies.

RESULTS:

Identification of a novel Chiffon-Gcn5 complex in *Drosophila*

Drosophila Ada2b has two splice isoforms that differ in their C-terminal regions but share a common N-terminal region containing the zinc finger-like ZZ, SANT, and two of the three previously described ADA box domains (Pankotai et al., 2013; Qi et al., 2004) (Fig. 1A). Similar to published observations (Pankotai et al., 2013), we observed that expression of Ada2b-PA alone partially rescued adult viability in *ada2b* null mutants, although both Ada2b isoforms were required to fully complement lethality of the null $ada2b^{1}/ada2b^{842}$ allele combination (Table 1). Expression of single-copy transgenes for both Ada2b-PA and Ada2b-PB restored viability to the expected one third of $ada2b^{1}/ada2b^{842}$ progeny (30.9 ± 4.2%, $\chi^2 = 0.23$) compared to 20.7 ± 5.2% with expression of Ada2b-PA alone. In contrast, expression of Ada2b-PB alone did not restore viability to $ada2b^{1}/ada2b^{842}$ progeny. These data suggested that the Ada2b isoforms have non-redundant functions in flies, and that Ada2b-PA alone can partially support development. Thus, we sought to determine whether these Ada2b isoforms were both required in SAGA, or alternatively, if like Ada2a, each Ada2b isoform nucleated formation of a distinct Gcn5-containing complex. To distinguish between these alternatives, we purified the Ada2b-PA and Ada2b-PB isoforms from cultured S2 cells using tandem FLAG-HA affinity chromatography and examined the co-purifying proteins using Multidimensional Protein Identification Technology (MudPIT). Using this approach, the Ada2b isoforms could be distinguished by peptide spectra that mapped to their unique C-terminal regions. Ada2b-PB co-purified all other 19 SAGA subunits (Stegeman et al., 2016) but did not co-purify any peptide spectra specific to the short Ada2b-PA isoform (Fig. 1B). Similarly, SAGA-specific purifications using bait proteins such as Spt3 and Spt20 contained peptide spectra specific to Ada2b-PB, but not Ada2b-PA. Instead, Ada2b-PA co-purified Gcn5, Ada3 and Sgf29, but not Ada2b-PB or other SAGA subunits. Ada2b-PA also did not co-purify ATAC-specific subunits such as Atac1, Atac2 or D12 (Table S1). Epitope-tagging of Ada2b-PA did not disrupt its interaction with SAGA because similar results were observed with Ada2b isoforms tagged at either their shared N- or unique C-termini. These data suggest that Ada2b-PA associates with Gcn5, Sgf29 and Ada3 in a complex that is distinct from either ATAC or SAGA.

To identify other proteins in this Ada2b-PA complex, we examined the MudPIT data to find proteins that co-purified specifically with Ada2b-PA, but not with SAGA-specific subunits. A single protein, Chiffon (CG5813; FBgn0000307), co-purified with Ada2b-PA or Sgf29, but not with other SAGA subunits or with the negative controls (Fig. 1B, Table S1). Moreover, reciprocal purifications of C-terminally tagged Chiffon co-purified Gcn5, Ada3, Sgf29 and Ada2b-PA, but not Ada2b-PB. Chiffon is the *Drosophila* homolog of Dbf4, which binds and activates the cell cycle kinase Cdc7 to phosphorylate the Mcm helicase, initiating DNA replication (Landis and

Tower, 1999; Stephenson et al., 2015). The Chiffon-purified or Ada2b-PA purified complexes exhibited similar levels and specificity of *in vitro* histone acetyltransferase activity to *Drosophila* SAGA purified via Ada2b-PB, with predominant activity on histone H3 in core histones (Fig. 1C) and ability to acetylate histone H3 tail peptides (Fig. 1D). Thus, we conclude that Chiffon is a *bona fide* subunit of a novel histone acetyltransferase complex containing Ada2b-PA, Gcn5, Sgf29 and Ada3 that we named the Chiffon Histone Acetyltransferase (CHAT) complex.

Most CHAT complexes do not contain Cdc7

Since Chiffon is the regulatory subunit of the cell cycle kinase Cdc7 (Stephenson et al., 2015), we next asked if Cdc7 was present in the CHAT complex. Only 7 peptide spectra were identified for Cdc7 using C-terminally tagged Chiffon as bait, which co-purified 89 – 158 peptide spectra for each of the other subunits of the CHAT complex: Ada2b-PA, Gcn5, Sgf29 and Ada3 (Fig. 1B). Thus, we next asked if Chiffon did in fact bind Cdc7 *in vivo*. We previously showed that the N-terminal domain of Chiffon (1 – 400aa) is sufficient to bind and stimulate Cdc7 kinase activity *in vitro* (Stephenson et al., 2015). Indeed, Cdc7 and Chiffon interact *in vivo* because N-terminally tagged Chiffon co-purified 93 peptide spectra for Cdc7, and Cdc7 reciprocally co-purified 176 peptide spectra for Chiffon. In contrast, N-terminally tagged Chiffon or Cdc7 co-purified fewer than 13 peptide spectra for other components of the CHAT complex such as Gcn5. There are two possibilities for the mutually exclusive binding of Cdc7 and CHAT subunits with Chiffon; first, Cdc7 blocks binding of CHAT subunits to Chiffon; or second, *chiffon* encodes two separate polypeptides that interact with Cdc7 or CHAT independently. Our data support the latter possibility because most peptide spectra for C-terminally tagged Chiffon map to its C-terminal region, whereas most peptide spectra for N-terminally tagged Chiffon map to its N-terminal region (Fig. 1E). These data suggest that very little full-length Chiffon exists in asynchronous cultured cells. However, a small fraction of Chiffon might interact with both Cdc7 and CHAT because we identified a few peptide spectra corresponding to Cdc7 in CHAT purifications (Ada2b-PA and Chiffon-C). Similarly, we also observed a few peptide spectra for CHAT subunits in Cdc7 or Chiffon-N purifications. Thus, we conclude that although a small fraction of Chiffon protein might interact with Cdc7 and CHAT simultaneously, most Chiffon interacts separately with either Cdc7 or CHAT, likely as two independent Chiffon polypeptides.

The insect-specific C-terminal domain of Chiffon directly binds Gcn5

To test if the N- and C-terminal domains of Chiffon could interact independently with Cdc7 and CHAT subunits, as suggested by the mass spectrometry data, we used a yeast two-hybrid approach to screen for interactions between different domains of Chiffon and each CHAT

subunit (Fig. S1). Using this approach, we identified a strong reciprocal interaction between the N-terminal Chiffon domain (1 – 400aa) and Cdc7 (Fig. 2A). This is consistent with both our MudPIT data and with the previous observation that the N-terminal 400aa of Chiffon is sufficient to bind Cdc7 *in vitro* (Stephenson et al., 2015). We also observed a weak unidirectional interaction between Gcn5 and the C-terminal Chiffon domain (1243 - 1695aa) (Fig. 2A). We were able to co-immunoprecipitate the recombinant C-terminal region (1400 - 1695aa) of Chiffon and Gcn5 under low salt (150 mM NaCl) conditions *in vitro* (Fig. 2B), further suggesting that Chiffon and Gcn5 interact directly, albeit weakly.

The C-terminal domain of Chiffon in *Drosophila* and other insects is much longer than other Dbf4 homologs and is not present in yeast or vertebrate Dbf4 (Landis and Tower, 1999; Tower, 2004) (Fig. 2C). Moreover, the C-terminal region of Chiffon that binds Gcn5 shares several highly conserved regions with other insects (Fig. S2). Since this insect-specific C-terminal domain interacted with Gcn5, we predicted that yeast or mammalian Dbf4 would be unlikely to interact with Gcn5. Indeed, we did not observe any peptides for Ada2, Gcn5, Sgf29 or Ada3 in TAP-purified Dbf4 from yeast cells (Fig. 3A). Moreover, human DBF4A and DBF4B co-immunoprecipitated CDC7, but not GCN5 or its paralog, PCAF, from HEK293T cells (Fig. 3B). Thus, the insect-specific C-terminal domain of Chiffon interacts directly with Gcn5, while the conserved N-terminal domain of Chiffon binds Cdc7.

Since Gcn5 is a component of all three of the SAGA, ATAC, and CHAT complexes in flies, and since Gcn5 binds the C-terminal domain of Chiffon, we wondered why Chiffon did not associate with either the SAGA or ATAC complexes (Fig. 1B, Table S1). To examine this question, we examined the interaction of each Ada2b isoform with all SAGA subunits except Nipped-A (Tra1) using yeast two-hybrid analysis (Fig. S3). Using this approach, we found that Ada2b-PB, but not Ada2b-PA, auto-activated when fused to the Gal4 DNA binding domain. This suggests that Ada2b-PB, but not Ada2b-PA, might interact with yeast transcriptional coactivators like SAGA to activate expression of the reporter genes in this assay. We also observed that Ada2b-PA interacted with the CHAT subunits Gcn5 and Ada3, and surprisingly also with the SAGA-specific subunit Spt7 (Fig. S3A). Further, Ada2b-PB fused to the Gal4 activating domain interacted with two additional SAGA-specific subunits that did not interact with Ada2b-PA: Spt3 and TAF12 (Fig. S3B). These data suggest a model in which the unique C-terminal region of the Ada2b-PB isoform binds SAGA through Spt3 and TAF12, enhancing binding of Spt7 to the Ada2b-PB N-terminal, which precludes Gcn5 binding to Chiffon (Fig. S3C). The Ada2b-PA isoform lacks the C-terminal region necessary for binding Spt3 and TAF12, preventing stable binding of Spt7 to

the N-terminal of Ada2b-PA, and instead enabling Gcn5 to bind Chiffon. This model is partially based on the observation that Ada2b-PA did not interact with Spt7 in our mass spectrometry data, even though it is capable of binding Spt7 by yeast two-hybrid. We further suggest that the Ada2b-PB C-terminal domain might also be capable of interacting with yeast SAGA, potentially via yeast Spt3 and TAF12. Although Ada2b-PA and Ada2b-PB interacted in one direction by yeast two-hybrid (Fig. S3B), our MudPIT data indicate that the Ada2b isoforms are not present in the same complex *in vivo* (Fig. 1B, Table S1). We conclude that the unique C-terminal regions of the Ada2b isoforms control protein-protein interactions that determine formation of the SAGA or CHAT complexes, and that the extended C-terminal domain in Ada2b-PB is necessary for SAGA formation (Fig. 3C).

Chiffon is necessary for histone H3 acetylation *in vivo*

Since the CHAT complex exhibits *in vitro* histone acetyltransferase activity against histone H3, we next asked if Chiffon was necessary for proper histone H3 acetylation *in vivo*. To do this, we used *Drosophila* ovary follicle cells in which specific regions of the genome undergo repeated bidirectional replication initiation to increase DNA copy number (Spradling and Mahowald, 1980). Chiffon is necessary for gene amplification in these cells (Landis and Tower, 1999; Stephenson et al., 2015; Zhang and Tower, 2004). We generated somatic mosaics for the *chiffon*^{ETBE3} null allele in ovaries using the FLP/FRT system and examined levels of different histone H3 acetyl marks by immunostaining. Notably, *chiffon*^{ETBE3} mutant cells showed decreased levels of histone H3 acetylated at lysine 14 (H3K14ac) relative to the adjacent GFP-positive cells (Fig. 4A). We also observed decreased levels of H3K9ac and H3K18ac, but not H3K23ac, in *chiffon*^{ETBE3} mutant cells (Fig. 4B). H3K18ac levels were only modestly reduced in *chiffon*^{ETBE3} clones, consistent with data showing that p300/CBP (*nejire*) is the major histone acetyltransferase for H3K18 in *Drosophila* and in mammalian cells (Jin et al., 2011; Tie et al., 2009). H3K14ac levels within *chiffon*^{ETBE3}, but not control *FRT40A*, clones were reduced to ~50% of the surrounding tissue (Fig. 4A, C). The nuclei in some *chiffon*^{ETBE3} clones appeared slightly more condensed using DAPI staining, suggesting that the reduced histone acetylation could be due to decreased DNA content in these cells. However, we and others have previously shown that *chiffon* is not essential for endoreplication, which determines the ploidy of follicle cells (Landis and Tower, 1999; Stephenson et al., 2015; Zhang and Tower, 2004). Moreover, some H3 acetyl marks such as H3K23ac were not reduced in *chiffon*^{ETBE3} clones (Fig. 4B). Together, these data demonstrate that Chiffon is required for full levels of histone H3 acetylation at lysines 9, 14 and 18 *in vivo*, suggesting that the CHAT complex contributes to bulk levels of

histone H3 acetylation in flies. Interestingly, *ada2b*^{1} clones showed only slightly lower levels of H3K14ac (not significant) when compared to *chiffon*^{ETBE3} clones, despite the fact that the *ada2b*^{1} allele removes both Ada2b isoforms (Fig. 4A, C). This suggests that the CHAT complex, rather than SAGA, might contribute to the majority of histone H3 acetylation in ovary follicle cells. To test if CHAT was also required in other cell types for histone H3 acetylation, we compared H3K14ac levels in *chiffon*^{ETBE3} and *ada2b*^{1} clones from imaginal discs. Similar to ovary follicle cells, most *chiffon*^{ETBE3} and *ada2b*^{1} clones from imaginal discs showed decreased levels of H3K14ac (Fig. S4). Although some large *chiffon*^{ETBE3} clones appeared to have little or no H3K14ac staining, other *chiffon*^{ETBE3} clones showed only moderate decreases in H3K14ac more similar to those observed in ovary follicle cells. Notably, some *chiffon*^{ETBE3} clones also appeared to contain fewer nuclei, suggesting that Chiffon might also contribute to cell number, potentially through DNA replication, in this cell type. While some *ada2b*^{1} clones showed only slight decreases in H3K14ac, other clones showed similar levels of H3K14ac to those observed in *ada2b*^{1} ovary follicle clones. Previous studies showed that mutations in the SAGA-specific subunit, *wda*, strongly reduced H3K9ac levels in *Drosophila* embryos (Guelman et al., 2006), suggesting that SAGA is necessary for full levels of histone H3 acetylation in embryos. It remains unclear whether SAGA and CHAT have overlapping or specialized functions with regard to histone H3 acetylation in *Drosophila*. However, our observation that the CHAT-specific Ada2b-PA isoform is sufficient to partially restore viability to *ada2b* null flies suggests that CHAT might compensate for some of SAGA's essential functions during development. Overall, these data indicate that *chiffon* is required for histone H3 acetylation *in vivo*, and that the CHAT complex contributes to histone H3 acetylation in several tissues in flies.

CHAT-mediated histone acetylation is not required for gene amplification

Histone acetylation correlates with and contributes to localized replication at the amplified follicle-cell origins (Aggarwal and Calvi, 2004; Liu et al., 2012; McConnell et al., 2012). Moreover, mutations in *chiffon* eliminate gene amplification in follicle cells (Landis and Tower, 1999; Stephenson et al., 2015; Zhang and Tower, 2004). Therefore, we asked if CHAT-mediated histone acetylation was also necessary for gene amplification. As observed previously, *chiffon*^{ETBE3} clones lack the characteristic BrdU-foci indicative of chorion gene re-replication that are present in the wild-type cells adjacent to the clone or in the *FRT40A* control clone (Fig. 4A, D). To test if CHAT-mediated histone acetylation was required for gene amplification, we examined *ada2b*^{1} somatic ovary mosaics, which exhibit decreased levels of histone H3 acetylation similar to that observed in *chiffon*^{ETBE3} mutant cells (Fig. 4A, C). In

contrast to *chiffon*^{ETBE3} clones that lack detectable gene amplification, we observed multiple *ada2b*^{1} clones undergoing gene amplification (Fig. 4A, D). We note that there were more pyknotic nuclei in *ada2b*^{1} clones, suggesting that loss of both Ada2b isoforms increased cell death in follicle cells, potentially due to pleiotropic effects resulting from loss of both the SAGA and CHAT complexes. Supporting this, loss of *ada2b* in the germline cells of female flies results in arrested oogenesis and increased apoptosis, suggesting that proper histone acetylation is necessary for other aspects of egg development (Li et al., 2017). Despite this, these data suggest that Ada2b-PA, which is necessary for histone acetyltransferase activity of the CHAT complex, is not required for gene amplification.

Since our MudPIT data and binding studies suggested that the N- and C-terminal domains of Chiffon interacted independently with Cdc7 and the CHAT complex respectively, we wondered if expression of these domains would restore either gene amplification or histone acetylation in *chiffon* mutants. To test this, we generated flies expressing either full-length Chiffon (1 - 1695aa, Chiffon-FL), or its N-terminal (1 – 375aa, Chiffon-N) or C-terminal (401 - 1695aa, Chiffon-C) domains. Each Chiffon construct was expressed under control of *chiffon* genomic regulatory elements from transgenes inserted in the third chromosome *attP2* site (Fig. 5, *methods*). Both H3K14ac levels and gene amplification were restored in *chiffon*^{ETBE3} mutant clones by expression of a single copy of full-length Chiffon (Fig. 4A, C, and D). In contrast, the N-terminal Chiffon transgene rescued gene amplification, but not histone acetylation in *chiffon*^{ETBE3} clones. Further, the C-terminal Chiffon transgene partially rescued histone acetylation, but did not restore gene amplification in *chiffon*^{ETBE3} clones. To our surprise, a full-length Chiffon transgene that contained a stop codon at position 376 (Chiffon-FL*), separating the N-terminal Cdc7-binding domain from the C-terminal Gcn5-binding domain, fully restored both gene amplification and histone acetylation in *chiffon*^{ETBE3} clones. Since histone acetyltransferases function redundantly to stimulate follicle cell gene amplification (McConnell et al., 2012), we cannot exclude the possibility that CHAT functions redundantly with other histone acetyltransferases to stimulate origin activity. Indeed, although bulk H3K14ac was reduced in *chiffon*^{ETBE3} clones expressing the N-terminal Chiffon transgene, we observed residual H3K14ac foci that co-localized with the BrdU foci in half of the images (five of the ten images) analyzed for acetylation in this genotype. We also observed H3K14ac foci that co-localized with the BrdU foci in some of the *ada2b* clones (three of the nine images), but these were much fainter than those present in *chiffon*^{ETBE3} clones expressing the N-terminal Chiffon transgene. This suggests that other histone acetyltransferases target the amplified follicle-cell origins in the absence of CHAT, likely including SAGA. Thus, we conclude that the histone acetyltransferase activity of the CHAT

complex alone is not essential for the specialized gene amplification form of DNA replication that occurs in follicle cells.

CHAT-mediated histone acetylation is essential for viability in flies

To our surprise, a premature stop codon in one of the *chiffon* transgenes (Chiffon-FL*), that should have truncated the protein prior to the Gcn5-binding region, fully rescued histone acetylation in *chiffon* mutant cells. These data implied that there could be an internal translation start site within the single, large exon in the *chiffon* gene (Fig. 5). Indeed, we identified a potential consensus initiation codon sequence 393 aa from the end of the *chiffon* coding region, that would be expected to generate a ~43 kDa polypeptide. While *chiffon* has been reported to be dispensable for viability in flies, these conclusions were based largely on an allele containing a nonsense mutation at position 174, $chiffon^{WF24}$ (Landis and Tower, 1999). This mutation disrupts the Cdc7-binding domain and results in viable flies with phenotypes indicative of partially disrupted DNA replication such as rough eyes and female infertility. Although these data suggested that the Cdc7-binding activity of Chiffon was not essential for viability in flies, we wondered if this was also the case for the CHAT complex. Because Ada2b-PA was sufficient to partially restore viability to *ada2b* mutants (Table 1), we hypothesized that the CHAT complex is essential for development in flies.

To test this, we used CRISPR-Cas9 technology to generate a new null *chiffon* allele in which the entire *chiffon* coding region was replaced with a visible eye marker, 3xP3-DsRed (Fig. 5, $chiffon^{DsRed}$). We then crossed these $chiffon^{DsRed}$ flies with the $chiffon^{ETBE3}$ null allele generated by Landis *et al.* (Landis and Tower, 1999), or the *Df(2L)RA5* deficiency that spans the *chiffon* gene and removes several adjacent genes. Lethality in the $chiffon^{ETBE3}$ flies was previously attributed to a secondary mutation in the nearby *cactus* gene, which is also missing in the *Df(2L)RA5* deficiency. However, we found that combinations of any of these three *chiffon* alleles resulted in complete adult lethality (Table 2). We then expressed single-copy *chiffon* rescue transgenes expressing full-length Chiffon (Chiffon-FL) with or without the $chiffon^{WF24}$ mutation (174Q>X). If the *chiffon* rescue transgene fully restored Chiffon function, we would expect to observe one third of adult progeny lacking the balancer chromosome. Moreover, we would expect that female adult progeny with restored Chiffon function would be fertile due to restoration of Chiffon activity in ovary follicle cells. Indeed, expression of the wild-type full-length Chiffon transgene fully restored both viability and female fertility in all three allele combinations (Table 2). Moreover, similar to Landis *et al.*, the Chiffon-FL^{WF24} transgene fully restored viability, but not female fertility, in the $chiffon^{DsRed}$/$chiffon^{ETBE3}$ progeny; similar results were observed with

the other *chiffon* allele combinations. Thus, the *chiffon*^{WF24} mutation, which disrupts the Cdc7-binding domain of Chiffon, eliminates Chiffon function with respect to female fertility, but does not disrupt Chiffon's role in adult viability. Next, we asked if expression of the Chiffon-C domain, which partially rescued histone acetylation in *chiffon* clones but did not restore gene amplification, could restore adult viability. Indeed, consistent with the observations for the Chiffon-FL^{WF24} transgene, expression of the Chiffon-C domain restored viability, although not to the same extent as Chiffon-FL, but the resulting females were infertile. In contrast, expression of the Chiffon-N transgene did not restore viability, even though this transgene did rescue gene amplification in *chiffon* clones (Fig. 4A, D). Further supporting the possibility that *chiffon* contains an internal translation start site, a full-length Chiffon transgene that contained a stop codon at position 376 (Chiffon-FL*), separating the N-terminal Cdc7-binding domain from the C-terminal insect-specific region, fully complemented both viability and fertility in *chiffon* mutants. Thus, we asked if the Cdc7- and Gcn5-binding domains of Chiffon could function *in trans*. To test this, we expressed single copies of the Chiffon-N and Chiffon-C in combination, and found that this fully restored both viability and female fertility to *chiffon* mutants (Table 2). These data demonstrate that Chiffon, like Ada2b-PA, is essential for viability in flies. Moreover, the essential function of Chiffon relates to its histone acetyltransferase activity rather than Cdc7 activation.

Our genetic observations support the possibility that *chiffon* is a dicistronic gene that encodes two distinct polypeptides; although this type of gene structure is relatively rare in *Drosophila*, there are several examples of dicistronic genes in flies including *stoned* and *Adh* (Andrews et al., 1996; Brogna and Ashburner, 1997; Komonyi et al., 2009). The coding sequence for the 1695 aa Chiffon protein encoded by the RD or RB transcripts lies within a single exon (Fig. 5) and northern analysis previously identified a single 6.5kb *chiffon* transcript (Landis and Tower, 1999), suggesting that alternative splicing is unlikely to account for our observations. To test if a C-terminal product was generated from either of the Chiffon transgenes that contained premature stop codons, we immunoprecipitated the Chiffon-FL* and Chiffon-FL^{WF24} proteins via their C-terminal FLAG epitope tags, and performed western blotting with FLAG antibodies. We observed a ~ 48 kDa product that was recognized by FLAG antibodies in both the immunoprecipitations from Chiffon-FL* and Chiffon-FL^{WF24} lysates, but not from untagged embryo lysates (Fig. 6A). Further, both Chiffon-FL* and Chiffon-FL^{WF24} co-immunoprecipitated Gcn5, suggesting that the C-terminal product expressed by these transgenes interacted with the CHAT complex. Thus, the Chiffon C-terminal domain nucleates CHAT formation, can be expressed from an alternative translation start site in the *chiffon* gene, and is essential for viability in *Drosophila* (Fig. 6B).

DISCUSSION

Here we show that the *Drosophila* Dbf4 homolog *chiffon* is a dicistronic gene that encodes two distinct polypeptides from alternative translation start sites. Chiffon's two activities can be separated genetically; its N-terminal domain binds Cdc7 and its C-terminal domain binds the histone acetyltransferase Gcn5 (Fig. 6B). The interaction between Chiffon and Gcn5 forms the CHAT complex that is required for histone H3 acetylation and viability in flies. Thus, in addition to the Gcn5-containing SAGA and ATAC complexes, flies contain a third Gcn5-containing complex: CHAT. The CHAT complex is not present in yeast or human cells, and is likely to be specific to insects because it is nucleated by Chiffon's insect-specific C-terminal domain. Our mass spectrometry data suggest that most Chiffon interacts in a mutually exclusive manner with Cdc7 and CHAT, and transgenes that separate the N- and C-terminal domains of *chiffon* fully restore both functions. Thus, our data demonstrate that the DDK and CHAT complexes function independently in DNA replication and histone acetylation respectively.

What might be the function of this CHAT complex in flies? Gcn5 and another histone acetyltransferase, Esa1, stimulate DNA replication in yeast *in vitro* (Kurat et al., 2017). In addition, several histone acetyltransferases work together to stimulate follicle cell amplification in *Drosophila* (McConnell et al., 2012). However, our work argues against a role for the CHAT complex in DNA replication; although we cannot exclude the possibility that the CHAT complex functions redundantly with other histone acetyltransferases to stimulate DNA replication, CHAT is not essential for gene amplification in follicle cells. Since SAGA is required for proper gene expression in flies, and because the CHAT-specific Ada2b-PA isoform can restore viability to *ada2b* mutants, we propose that CHAT, like the SAGA and ATAC complexes, regulates gene expression in flies. In other organisms, Dbf4 levels fluctuate throughout the cell cycle to control activity of Cdc7 (Cheng et al., 1999; Oshiro et al., 1999): Dbf4 protein levels correlates with Cdc7 activity and increase at the G1-S transition, peak in S phase, and then become low during G1 phase when Dbf4 is degraded by the anaphase-promoting complex (Cheng et al., 1999; Oshiro et al., 1999). One possibility in flies is that Chiffon levels are also cell cycle regulated, and if so, CHAT complex expression could be controlled by Chiffon levels, potentially peaking in S phase. Thus, the DDK and CHAT functions of *chiffon* could have evolved as part of the same gene structure to coordinate DNA replication with expression of CHAT-target genes during the cell cycle in insects.

Although Dbf4 did not interact with Gcn5 in yeast or in human cells, some observations support a potential role for Dbf4 in gene expression in these organisms. For example, the C-terminal

domain of human Dbf4 (ASK) binds the chromatin-associated protein Lens epithelium-derived growth factor (LEDGF), which is associated with the MLL histone H3 methyltransferase complex (Hughes et al., 2010; Yokoyama and Cleary, 2008). Further, the C-terminus of yeast Dbf4 binds forkhead transcription factors (Fang et al., 2017). In addition, DDK phosphorylates Thr45 of histone H3 in budding yeast (Baker et al., 2010), demonstrating a direct role for DDK complexes in chromatin modification. Thus, while the CHAT complex might be specific to insects, Dbf4 orthologs could have a more general role in gene expression in addition to their essential DNA replication activity.

One unusual feature of Chiffon in flies is that its Cdc7-binding activity is dispensable for viability. Loss of either Dbf4 or Cdc7 disrupts DNA replication and mitosis in organisms from yeast to mammals, leading to growth defects and/or cell death (Labib, 2010). In flies, *Cdc7* is also an essential gene (Stephenson et al., 2015), and recent work showed that *Cdc7* is required for early embryonic nuclear cycles, consistent with its essential role in DNA replication (Seller and O'Farrell, 2018). However, our data show that Chiffon's Cdc7-binding activity is not essential for DNA replication or viability in flies, although it is required for follicle cell gene amplification in the ovary. These conclusions are consistent with the previous findings of Landis *et al.*, and are in stark contrast to the absolute requirement of Cdc7 and Dbf4 for DNA replication and cell viability in organisms from yeast to vertebrates (Labib, 2010; Landis and Tower, 1999). Despite this, *chiffon* is essential for development in flies but this is due to a requirement for the CHAT complex, likely due to its role in histone acetylation. Thus, our studies here raise the question of how *Drosophila* Cdc7 can function in the absence of its Dbf4 regulatory partner, since flies do not have any other detectable sequence homolog for Dbf4. While budding yeast possess only one homolog for Dbf4 and Cdc7, several organisms possess paralogs of DDK subunits with specialized functions in meiosis and development. In particular, the vertebrate Dbf4B paralog has specialized roles in early embryogenesis (Collart et al., 2017; Montagnoli et al., 2002; Silva et al., 2006; Yoshizawa-Sugata et al., 2005). If Chiffon, like Dbf4B, has a more specialized developmental role in DNA re-replication in ovary follicle cells, then our data suggest that there might be an alternative mechanism to regulate Cdc7 activity in flies.

MATERIALS AND METHODS:

Affinity purification, MudPIT analysis and histone acetyltransferase assays

Tandem FLAG-HA affinity purification and MudPIT analysis was conducted from stable *Drosophila* S2 cell lines as described previously (Stegeman et al., 2016). TAP purifications from *S. cerevisiae* was performed as described previously (Lee et al., 2004). To estimate relative

protein levels, distributed Normalized Abundance Factors (dNSAFs) were calculated for each non-redundant protein or protein group (Zhang et al., 2010). Briefly, shared spectral counts (sSpC) were distributed based on spectral counts unique to each protein (uSpC). Histone acetyltransferase assays were performed as previously described (Stegeman et al., 2016) using Flag-purified complexes and HeLa core histones or human histone H3 peptide (K5 - K23) as substrate.

Fly stocks and genetics

Genotypes for flies used in this study are described in Table S2. The *chiffon*^{ETBE3} (Landis and Tower, 1999) and *ada2b*^{1} (Qi et al., 2004) null alleles were used for somatic mosaic analysis. The null *ada2b*^{1} and *ada2b*^{842} (Pankotai et al., 2013) alleles that disrupt both Ada2b isoforms were used to assess adult survival. Ada2b rescue transgenes contain genomic *ada2b* enhancer sequences that begin -1878 bp from the transcription start site and extend +1782 bp to the end of the second exon. The alternative exon 3 and 4 sequences for each Ada2b isoform are fused directly to the 3′ end of exon 2. Constructs were generated in the pCa4B vector with the addition of the *Adh* 3′ UTR and polyadenylation signal sequences from the *pRmaHa3* vector. Transgenic flies were generated using the phiC31 site-specific integration system in the attP40 site on chromosome 2L. Chiffon rescue transgenes contain genomic *chiffon* enhancer sequences that span -3480 bp relative to the translation start site of the *chiffon-RD* transcript, and include the *chiffon* 3 ′UTR sequences that extend 1056 bp past the stop codon of the *chiffon-RD* transcript. Chiffon domain constructs encode the indicated number of amino acids relative to 1695aa full-length Chiffon based on the *chiffon-RD* transcript. Chiffon constructs were N and C-terminally epitope-tagged with 2xHA and FLAG respectively. Transgenic flies were generated in the attP2 site on chromosome 3L. The *chiffon*^{DsRed} allele was generated using CRISPR-Cas9 technology (Gratz et al., 2014). The following guide RNAs were used to target the *chiffon* 5088 bp exon for replacement: 5' GGAGGGAAACTTTATAGGAGTGG 3' and 5' GATGATGATTAGATGACACAGGG 3'. Flanking regions immediately upstream and downstream of the *chiffon* coding region (*chiffon-RD*) were cloned into the flyCRISPR vector pHD-DsRed-attP and used as a template for homologous recombination. Flies expressing DsRed were selected, and the insertion position of the 3xP3-DsRed-attP cassette was confirmed by PCR and sequencing. The *chiffon*^{ETBE3} allele was also confirmed by PCR and sequencing. The genomic positions of the regions deleted in each *chiffon* allele are: *chiffon*^{DsRed} chr2L, 16344356 – 16349852; and *chiffon*^{ETBE3} chr2L, 16344400 – 16351631.

Immunohistochemistry

Somatic clones were induced in egg chambers, and ovaries were dissected from adult females at 3 days post-eclosion, labeled with 5-bromo-2-deoxyuridine (BrdU), fixed, immunostained with anti-BrdU (BD Pharmingen, mouse) and either anti-H3K14ac (#07-353, Millipore, rabbit, 1:100), anti-H3K9ac (#Ab10812, Abcam, rabbit, 1:500), anti-H3K18ac (#Ab1191, Abcam, rabbit, 1:400) or anti-H3K23ac (#Ab47813, Abcam, rabbit, 1:700) antibodies, followed by Alexa^{568} and Alexa^{633} secondary antibodies (LifeTechnologies), and imaged as described previously (Stephenson et al., 2015). H3K14ac levels were quantified for 10 – 30 nuclei in each clone relative to a similar number of nuclei from the surrounding wild-type region of the tissue (GFP-positive). Acetylation levels were determined as average sum intensity values for nuclear-localized fluorescence using NIS-Elements Analysis software. Acetylation levels were quantified for four individual frames from a *z*-stack image of each egg chamber. These four frames were selected based on those images that contained the brightest H3K14ac signal in the wild-type region (GFP-positive) of the egg chamber. Somatic clones were induced in imaginal discs by heat shock for 30 min at 37°C 72h after egg laying. Imaginal discs were dissected from wandering third instar larvae and immunostained with anti-H3K14ac.

Phylogenetic Analysis

The following protein sequences were aligned using Clustal Omega (Sievers et al., 2011) and used to generate a neighbor-joining phylogenetic tree, which was plotted using *phytools* in R: *Bos taurus* XP_024836692.1 and XP_015324178.1, *Canis lupus familiaris* XP_532451.2 and XP_022278602.1, *Homo sapiens* NP_006707.1 and NP_663696.1, *Gallus gallus* XP_004939326.1 and XP_004948536.1, *Xenopus laevis* ABB16337.1 and BAC76421.1, *Mus musculus* NP_001177646.1, *Ceratitis capitata* XP_004521831.1, *Lucilia cuprina* XP_023301579.1, *Drosophila melanogaster* AAD48779.1, *Camponotus floridanus* EFN62957.1, *Pogonomyrmex barbatus* XP_011633258.1, *Linepithema humile* XP_012229084.1, *Apis mellifera* XP_016770645.1, *Apis florea* XP_003693265.1, *Tribolium castaneum* XP_008197891.1, *Schizosaccharomyces pombe* CAA19117.1 and CAB39799.1, *Aspergillus nidulans* AAD01519.1, *Saccharomyces cerevisiae* NP_010337.3, *Eremothecium gossypii* NP_986462.1, *Kluyveromyces lactis* XP_455609.1.

Yeast two-hybrid assay

Yeast two-hybrid analysis was performed with the Matchmaker Gold Yeast two-hybrid system as per the manufacturers' instructions (Clontech). Three independent transformed colonies were replica plated on the different selective media for each interaction tested.

Co-immunoprecipitation and western blotting analysis

Recombinant proteins (500 ng) were incubated with glutathione-sepharose (#16100, Thermo Scientific) in the following buffer: 50 mM Tris pH 8.0, 150 mM NaCl, 0.1% NP-40. Embryo lysates (4 mg protein) were immunoprecipitated using FLAG-agarose in the following buffer: 50 mM Tris pH 8.0, 300 mM NaCl, 0.5% NP-40, 10% glycerol. C-terminally FLAG-tagged DBF4A, DBF4B or pENTER empty vector control (#CG801040, #CH874659, #P100001, Vigene Biosciences, Rockville MD) were transiently transfected into human embryonic kidney (HEK) 293T cells, and nuclear lysates (1 mg protein) immunoprecipitated using Flag M2 antibodies and Protein-G Dynabeads in the following buffer: 50 mM Tris pH 8.0, 150 mM NaCl and 0.2% IPEGAL with PMSF, aprotinin, leupeptin and pepstatin. The following antibodies were used for western blotting analysis: GST (#PC53, Millipore, rabbit, 1:1000), His-HRP (#MA1-21315, Invitrogen, mouse, 1:1000), FLAG-HRP (#A8592, Sigma, mouse, 1:5000), *Drosophila* Gcn5 (rabbit, 1:3000) (Kusch et al., 2003), Flag M2 (Sigma; 1:1000), human CDC7 (#Ab108382, Abcam, 1:1000), human PCAF (#Ab12188, Abcam, 1:500), human GCN5 (#Ab153903, Abcam, 1:1000). HEK293T cells were obtained from the American Type Culture Collection (ATCC) and were tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza).

ACKNOWLEDGEMENTS: We thank John Tower, Scott Hawley, Mattias Mannervik, Imre Boros, and the Bloomington Drosophila Stock Center (NIH P40OD018537) for flies. The following undergraduate students contributed to the yeast two-hybrid experiments: Jose Adorno-Cancel, Evan Baker, William Delacruz, Shohei Fujikawa, Brianna Kennedy, Macey Lee, Emily Olson, Delayna Pagan, Josie Rhodes, Karissa Rulon, Ana Stenstrom and Samantha Villagomez.

FUNDING: The authors thank the Purdue University Center for Cancer Research (American Cancer Society Institutional Research Grant, IRG #58-006-53; NIH P30 CA023168) for funding to support this work. This work was initiated with support from the National Institutes of Health GM99945-01 to Susan M. Abmayr and Jerry L. Workman. Microscopy studies were performed in the Bindley Bioscience Imaging Facility funded by the NIH-funded Indiana Clinical and Translational Sciences Institute.

AUTHOR CONTRIBUTIONS: ET performed immunostaining experiments, yeast two-hybrid analysis and recombinant protein experiments. BA assisted with yeast two-hybrid analysis. RS performed immunostaining experiments and embryo immunoprecipitations. AA performed co-immunoprecipitations in human cell lines, with supervision from ED. SS, LF and MW performed

514 the MudPIT analysis. VW conceived the project, designed the experiments, performed the
515 complex purifications and *in vitro* assays, and wrote the manuscript in collaboration with ET.

516 **CONFLICT OF INTEREST:** The authors declare no competing financial interests.

517 **DATA AVAILABILITY:** The complete MudPIT dataset (raw files, peak files, search files, as well
518 as DTASelect result files) can be obtained from the MassIVE database via
519 ftp://massive.ucsd.edu/MSV000081791 using ProteomeXChange accession: PXD008391. All
520 raw and supporting data including detailed protocols have been deposited at the Purdue
521 University Research Repository (PURR) as a publically available, archived data set and can be
522 accessed using https://doi.org/10.4231/R72V2DD0 . Any additional data or material required for
523 analysis are available from the corresponding author on reasonable request.

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FIGURE LEGENDS:

Figure 1. Identification of a novel Chiffon-Gcn5 complex in *Drosophila.* (A) The *ada2b* gene encodes two splice isoforms, Ada2b-PA and Ada2b-PB, resulting from alternative use of 3′ splice acceptor sites in exon 3 (splice site, SS) that generate a frame-shift following amino acid 330 (*, stop codon). Ada2b isoforms differ only in their highlighted C-terminal regions (red/blue). (B) Heat map showing the relative spectral abundance of SAGA subunits, Chiffon, and Cdc7 expressed as distributive normalized spectral abundance factor (dNSAF) in tandem FLAG-HA purifications from S2 cells using the indicated bait proteins (N/C epitope tag shown in brackets). Control 1, untagged; Control 2, CG6459 (non-specific bait). Bait proteins new to this study are highlighted in red. The dNSAF scale represents abundance of subunits on a scale from yellow (high) to blue (low) with subunits that were not identified shown in white. dNSAF values used to generate the heat map are provided in Table S1. The number of spectra specific to each protein isoform (distributed spectra, dS) are shown in each box. Data for each bait protein represent sum of two technical MudPIT experiments. (C) The histone acetyltransferase activity of FLAG-purified CHAT (via Ada2b-PA or Chiffon) or SAGA (Ada2b-PB) complexes containing equivalent amounts of Gcn5 as determined by western blot with anti-Gcn5 antibody (left panel) were assayed using core histones as substrate. Incorporation of ^{3}H-acetyl CoA was assayed by fluorography (right upper panel) and the migration of histone H3 was determined by Coomassie staining (right lower panel). The negative control lane consists of histones and ^{3}H-acetyl CoA with no complex added. (D) Histone acetyltransferase activity of the indicated complexes was quantified by scintillation counting of ^{3}H-acetyl CoA incorporated into core histones or H3 tail peptides as in panel C. Mean ± s.d. is shown for 3 independent histone acetyltransferase assays relative to no complex control. (E) Heat map showing the percentage of total spectra mapping to each region of full-length Chiffon (1695aa) purifications using the indicated bait proteins as in panel B. The conserved Dbf4 N and C motifs in Chiffon are indicated by the grey shaded boxes.

Figure 2. The insect-specific C-terminal domain of Chiffon directly binds Gcn5. (A) Yeast two-hybrid assay was performed to test the pair-wise interaction of each CHAT subunit with Chiffon. The Gal4 activating domain (AD) was fused to Cdc7, Gcn5, Ada3, Sgf29 or Ada2b-PA, and the Gal4 DNA binding domain (DBD) was fused to either the N-terminal (1 – 400aa) or C-terminal (1243 – 1695aa) domains of Chiffon. Empty plasmids expressing only the AD or DBD were used to test for auto-activation of each protein. Three independent transformed yeast colonies were patched on media lacking leucine and tryptophan to test for presence of the AD

and DBD plasmids, and on media lacking leucine, tryptophan, adenine and histidine to test for interaction. (B) Glutathione-sepharose pull-down of recombinant GST-Gcn5 and the C-terminal domain of Chiffon (1400 – 1695aa) tagged with His followed by western blotting with antibodies against GST and His. Representative data from 3 experiments are shown. (C) Phylogenetic tree constructed using Neighbor-Joining method showing Dbf4 homologs from fungi, insects and vertebrates based on Clustal-Omega multiple sequence alignment of full-length proteins. Shading represents protein length (amino acid, aa).

Figure 3. Dbf4 does not bind Gcn5 in yeast or humans. (A) Table showing proteins identified in Chiffon and Dbf4 purifications from *Drosophila melanogaster* (tandem FLAG-HA) or *Saccharomyces cerevisiae* (TAP-tagged). Sequence coverage (percentage) and number of spectra are shown for each protein. (B) FLAG-tagged human DBF4A or DBF4B were immunoprecipitated from HEK293T cell extracts using FLAG antibodies, and analyzed by western blotting using the indicated antibodies. Control, empty vector. Representative data from 3 experiments are shown. (C) Schematic showing subunit composition of the SAGA, CHAT and DDK complexes. Interactions between subunits are based on the yeast two-hybrid analysis from Fig. S1 and Fig. S3, which suggest that Ada2b-PB binds Spt3 and TAF12 via its unique C-terminal domain to nucleate SAGA formation. In contrast, CHAT formation is nucleated by the binding of Chiffon's C-terminal to Gcn5, which precludes association of other SAGA subunits. Chiffon interacts with Cdc7 via its N-terminal domain to form the DDK complex, and the DDK and CHAT complexes appear to be largely separate *in vivo*.

Figure 4. Chiffon is necessary for histone H3 acetylation *in vivo.* (A) Mosaic egg chambers were generated using the FLP/FRT system for *chiffon*^{ETBE3} and *ada2b*^{1}, their respective controls, *FRT40A* and *FRT82B*, and for *chiffon*^{ETBE3} clones expressing single copies of the indicated Chiffon rescue transgenes. Maximum intensity projection images showing BrdU incorporation, α-H3K14ac and DAPI staining from amplification-stage egg chamber follicle cells containing representative clones, marked by the absence of GFP and outlined in white. Scale bars, 20 μm. (B) Mosaic egg chambers for *chiffon*^{ETBE3} were examined for H3K9ac (n = 5), H3K18ac (n = 6) or H3K23ac (n = 7) as in panel A. Representative images are shown for each histone modification. (C) Boxplots showing relative H3K14ac levels in mutant clones versus GFP-positive control regions. 10 - 30 nuclei were quantified per region for 9 - 10 independent animals (red dots indicate clone analyzed from individual animal; X, mean). *p*-values for the indicated comparisons were determined by ANOVA + Tukey-HSD; ns, not significant. (D) The percentage of clones undergoing gene amplification (BrdU-positive foci) in amplification-stage egg

chambers from the indicated genotypes was determined. Several genotypes showed clones that were composed entirely or partially of pyknotic nuclei, which did not undergo gene amplification. The number of independent animals and clones examined for each genotype is shown above the plot (animal/clones).

Figure 5. Map of *chiffon* gene structure. Schematic of *chiffon* locus showing nearby genes including *cactus*. The gene structure for *chiffon* is shown in the inset shaded box as coding sequences (black boxes), untranslated regions (grey boxes), and introns (lines). There are three annotated splice isoforms for *chiffon*: RA encodes a 1711aa protein, RB and RD encode 1695aa proteins from a single ~5kb exon. An overlapping gene, *CG42231*, shares a promoter with *chiffon* but differs in its reading frame and encodes a separate polypeptide. The genomic regions deleted/mutated in each of the indicated *chiffon* alleles ($chiffon^{DsRed}$, $chiffon^{ETBE3}$, and $chiffon^{WF24}$) are shown by the dotted arrows. The *chiffon* rescue transgenes are shown by the black boxes at the base of the panel. Rescue constructs contain the indicated *chiffon* 5' and 3' regulatory regions (black boxes) and the *chiffon* coding sequences. The conserved Dbf4 N-terminal domain and C-terminal insect-specific Gcn5-binding domain are indicated by the shaded boxes overlaying the rescue constructs, and the position of each nonsense mutation in the rescue constructs is indicated by an asterisk.

Figure 6. An internal translation start site in *chiffon* expresses a C-terminal product that binds Gcn5. (A) C-terminally FLAG-tagged Chiffon-FL* or Chiffon-FL^{WF24} transgenes, that contain premature stop codons at amino acids 376 or 174 respectively, were immunoprecipitated from embryo lysates using FLAG antibodies. Co-immunoprecipitated proteins were analyzed by SDS-PAGE and western blotting with antibodies against FLAG (Chiffon) and Gcn5. *, non-specific bands present in w^{1118} control. Representative data from 3 experiments are shown. (B) Schematic illustrating the two polypeptides encoded by *chiffon*. The first start codon encodes full-length Chiffon (1695aa) with the conserved Dbf4 Cdc7-binding domain in its N-terminal region. The N-terminal Chiffon product binds Cdc7, nucleates DDK formation, and is necessary for gene amplification. An alternative internal ribosome entry site generates a C-terminal product containing the insect-specific Gcn5-binding domain that nucleates CHAT formation, and is essential for histone acetylation, and development. Our data suggest that two mechanisms might control production of the alternative Chiffon products that nucleate DDK versus CHAT complex formation: (1) translational switching between cap-dependent and IRES-dependent start sites; and/or (2) proteolytic cleavage of full-length Chiffon.

SUPPLEMENTAL FIGURE LEGENDS:

Figure S1. The N-terminal domain of Chiffon interacts with Cdc7, and the C-terminal domain of Chiffon interacts with Gcn5. (A) Schematic showing the Chiffon domains tested by yeast two-hybrid analysis. The conserved Dbf4 N, M, and C motifs are indicated by shaded grey boxes. (B) Yeast two-hybrid assay was performed to test the interaction of the CHAT subunits Gcn5, Ada2b-PA, Sgf29, Ada3, and Cdc7 with different Chiffon domains. The Gal4 activating domain (AD) or the Gal4 DNA binding domain (DBD) were fused to the indicated proteins. Empty plasmids expressing only the AD or DBD were used to test for auto-activation of each protein (*, auto-activation). Cells were patched on media lacking leucine and tryptophan to test for presence of the AD and DBD plasmids, and on media lacking leucine, tryptophan, adenine and histidine to test for interaction. Three independent transformed yeast colonies were patched for each interaction tested.

Figure S2. The C-terminal, Gcn5-binding domain of Chiffon shares regions of conservation within insect species. Insect Dbf4 homologs were aligned using Clustal Omega. The aligned region contains 1211 – 1695aa of *Drosophila* Chiffon, which includes the region that interacts with Gcn5 by yeast two-hybrid assay (1243 – 1695aa) and by co-immunoprecipitation using recombinant proteins (1400 – 1695aa). Regions of potential conservation within insects are underlined in red. Dbf4 homologs from the following insect species were used to generate this alignment: Diptera: *Drosophila melanogaster*, *Lucilia cuprina* (Australian sheep blowfly) and *Ceratitis capitata* (Mediterranean fruit fly). Coleoptera: *Tribolium castaneum* (Red flour beetle). Hymenoptera: *Apis mellifera* (Western honey bee), *Apis florea* (Dwarf honey bee), *Linepithema humile* (Argentine ant), *Pogonomyrmex barbatus* (Red harvester ant), *Camponotus floridanus* (Florida carpenter ant).

Figure S3. Ada2b isoforms interact with overlapping and distinct SAGA subunits. (A, B) Yeast two-hybrid assay was performed to test the interaction of Ada2b-PA or Ada2b-PB with the indicated SAGA subunits, Chiffon domains, and Cdc7. The Gal4 activating domain (AD) or the Gal4 DNA binding domain (DBD) were fused to the indicated proteins. Empty plasmids expressing only the AD or DBD were used to test for auto-activation of each protein (*, auto-activation). We did not test Ada2b-PB/DBD in combination with other SAGA subunits by yeast two-hybrid because Ada2b-PB auto-activated when fused to the DBD; in contrast, Ada2b-PA did not auto-activate. Cells were patched on media lacking leucine and tryptophan to test for presence of the AD and DBD plasmids, and on media lacking leucine, tryptophan, adenine and histidine to test for interaction. Three independent transformed yeast colonies were patched for

each interaction tested. ND, not determined. (C) Model for differential binding of Ada2b isoforms with SAGA or CHAT. Both Ada2b isoforms bind Gcn5 and Ada3 and can interact with Spt7 by yeast two-hybrid; however, only Ada2b-PB associates with Spt7 *in vivo*. By yeast two-hybrid analysis, Ada2b-PB, but not Ada2b-PA, interacts with Spt3 and TAF12. We propose that Ada2b-PB binds Spt3 and TAF12 via its unique C-terminal domain (highlighted in blue), stabilizing association of Spt7 with its common N-terminal domain. Binding of Spt3, TAF12 and Spt7 to Ada2b-PB promotes formation of SAGA and prevents Gcn5 from binding Chiffon, potentially via steric clashes. In contrast, Ada2b-PA does not interact with Spt3 or TAF12 because it contains an alternative C-terminal domain that lacks the necessary binding regions (highlighted in red). Although Ada2b-PA is capable of binding Spt7 via its N-terminal region, this association is destabilized in the absence of Spt3 or TAF12. Instead, in the absence of Spt7 binding, Gcn5 interacts with the C-terminal of Chiffon and promotes CHAT complex formation. It is possible that Chiffon binding to Gcn5 might also prevent Ada2b-PA from binding Spt7. Notably, Ada2b-PB, but not Ada2b-PA, auto-activates expression of the reporter genes when fused to the DBD, suggesting that the unique C-terminal domain of Ada2b-PB may also interact with yeast SAGA subunits. Although by yeast two-hybrid Ada2b-PA and Ada2b-PB showed an interaction in one direction (panel B, AD-Ada2b-PB + DBD-Ada2b-PA), we never observed peptide spectra for Ada2b-PA isoforms in Ada2b-PB purifications (Table S1), suggesting that this interaction does not occur *in vivo*.

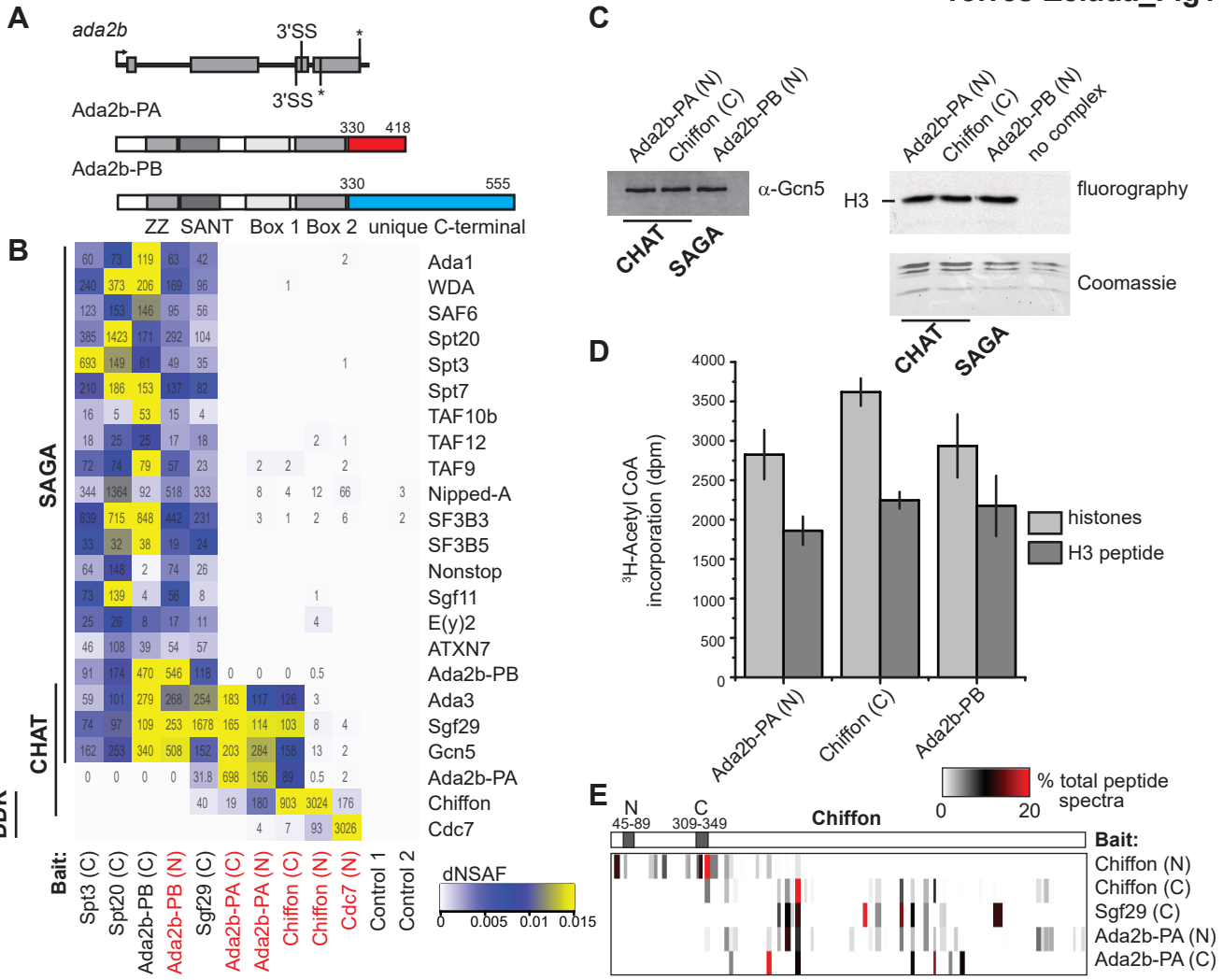
Figure S4. Chiffon is necessary for histone H3 acetylation in imaginal discs. Mosaic imaginal discs were generated using the FLP/FRT system for $chiffon^{ETBE3}$ (n = 10) and $ada2b^{1}$ (n = 3). Representative maximum intensity projection images for each allele showing α-H3K14ac and DAPI staining from imaginal discs containing multiple clones, marked by the absence of GFP and outlined in white. Scale bars, 20 μm.

Allele combination	Transgene (hemizygous)	Balancer siblings	Rescue flies	Total flies	χ^2	Mean ± s.d. (n ≥ 4 crosses)	Rescued female fertility
ada2b^{1} / *ada2b*^{842}	No transgene	987	0	987	2.47E-109	0 ± 0%	ND
	Ada2b-PA	726	202	928	7.77E-14	20.7 ± 5.2%	Fertile
	Ada2b-PB	670	0	670	7.83E-75	0 ± 0%	ND
	Ada2b-PA+Ada2b-PB	498	226	724	**0.23**	30.9 ± 4.2%	Fertile

Table 1. Flies carrying the indicated *ada2b* null alleles were crossed and the surviving adult progeny were scored for presence of the balancer chromosome (TM3). Adult progeny carried one copy of each indicated transgene. We would expect one third of adult progeny to lack the balancer chromosome if *ada2b*$^{-/-}$ flies expressing any of the hemizygous transgenes were viable. The mean percentage of rescued flies that lack the balancer chromosome +/- s.d. is presented for ≥ 4 independent crosses each with ≥ 100 scored flies; *p*-value, Chi-squared test. Chi-squared values that were not significantly different from the expected ratio of viable flies are highlighted in bold. Fertility was examined for rescued females; ND, not determined.

Allele combination	Transgene (hemizygous)	Balancer siblings	Rescue flies	Total flies	χ^2	Mean ± s.d. (n ≥ 4 crosses)	Rescued female fertility
chif^{DsRed} / *chif*^{ETBE3}	No transgene	1060	0	1060	2.82E-117	0 ± 0%	ND
	Chiffon-FL	618	300	918	**0.67**	33.0 ± 3.5%	Fertile
	Chiffon-N	871	0	871	1.03E-96	0 ± 0%	ND
	Chiffon-C	1027	279	1306	4.45E-20	21.6 ± 5.1%	Infertile
	Chiffon-FL^{WF24}	1010	462	1472	**0.11**	31.5 ± 1.6%	Infertile
	Chiffon-FL*	1093	563	1656	**0.57**	34.0 ± 1.4%	Fertile
	Chiffon-N + Chiffon-C	556	251	807	**0.18**	31.4 ± 2.4%	Fertile
chif^{DsRed} / *Df(2L)RA5*	No transgene	1095	0	1095	4.40E-121	0 ± 0%	ND
	Chiffon-FL	1026	546	1572	**0.24**	34.7 ± 1.4%	Fertile
	Chiffon-N	1103	0	1103	5.94E-122	0 ± 0%	ND
	Chiffon-C	1420	443	1863	2.17E-18	23.7 ± 4.4%	Infertile
	Chiffon-FL^{WF24}	575	262	837	**0.21**	31.9 ± 3.6%	Infertile
	Chiffon-FL*	852	402	1254	**0.34**	31.7 ± 3.5%	Fertile
chif^{ETBE3} / *Df(2L)RA5*	No transgene	1319	0	1319	1.92E-145	0 ± 0%	ND
	Chiffon-FL	455	262	717	**0.07**	36.7 ± 3.9%	Fertile
	Chiffon-N	820	0	820	3.67E-91	0 ± 0%	ND
	Chiffon-C	1310	260	1570	3.90E-45	16.2 ± 5.4%	Infertile
	Chiffon-FL^{WF24}	544	236	780	**0.07**	30.2 ± 2.6%	Infertile
	Chiffon-FL*	1172	357	1529	1.21E-16	23.5 ± 2.3%	Fertile

Table 2. Flies carrying the indicated *chiffon* null alleles were crossed and the surviving adult progeny were scored for presence of the balancer chromosome (CyO). Adult progeny carried one copy of each indicated transgene. We would expect one third of adult progeny to lack the balancer chromosome if *chiffon*$^{-/-}$ flies expressing any of the hemizygous transgenes were viable. The mean percentage of rescued flies that lack the balancer chromosome +/- s.d. is presented for ≥ 4 independent crosses each with ≥ 100 scored flies; *p*-value, Chi-squared test. Chi-squared values that were not significantly different from the expected ratio of viable flies are highlighted in bold. Fertility was examined for rescued females; ND, not determined.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3'SS *
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Ada2b-PB
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CHAT
DDK
Ada1
WDA
SAF6
Spt20
Spt3
Spt7
TAF10b
TAF12
TAF9
Nipped-A
SF3B3
SF3B5
Nonstop
Sgf11
E(y)2
ATXN7
Ada2b-PB
Ada3
Sgf29
Gcn5
Ada2b-PA
Chiffon
Cdc7
Bait:
Spt3 (C)
Spt20 (C)
Ada2b-PB (C)
Ada2b-PB (N)
Sgf29 (C)
Ada2b-PA (C)
Ada2b-PA (N)
Chiffon (C)
Chiffon (N)
Cdc7 (N)
Control 1
Control 2
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C
Ada2b-PA (N)
Chiffon (C)
Ada2b-PB (N)
α-Gcn5
CHAT
SAGA
Ada2b-PA (N)
Chiffon (C)
Ada2b-PB (N)
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H3
fluorography
Coomassie
CHAT
SAGA
D
3H-Acetyl CoA incorporation (dpm)
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3500
3000
2500
2000
1500
1000
500
0
histones
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Ada2b-PA (N)
Chiffon (C)
Ada2b-PB
E
N
C
45-89
309-349
Chiffon
% total peptide spectra
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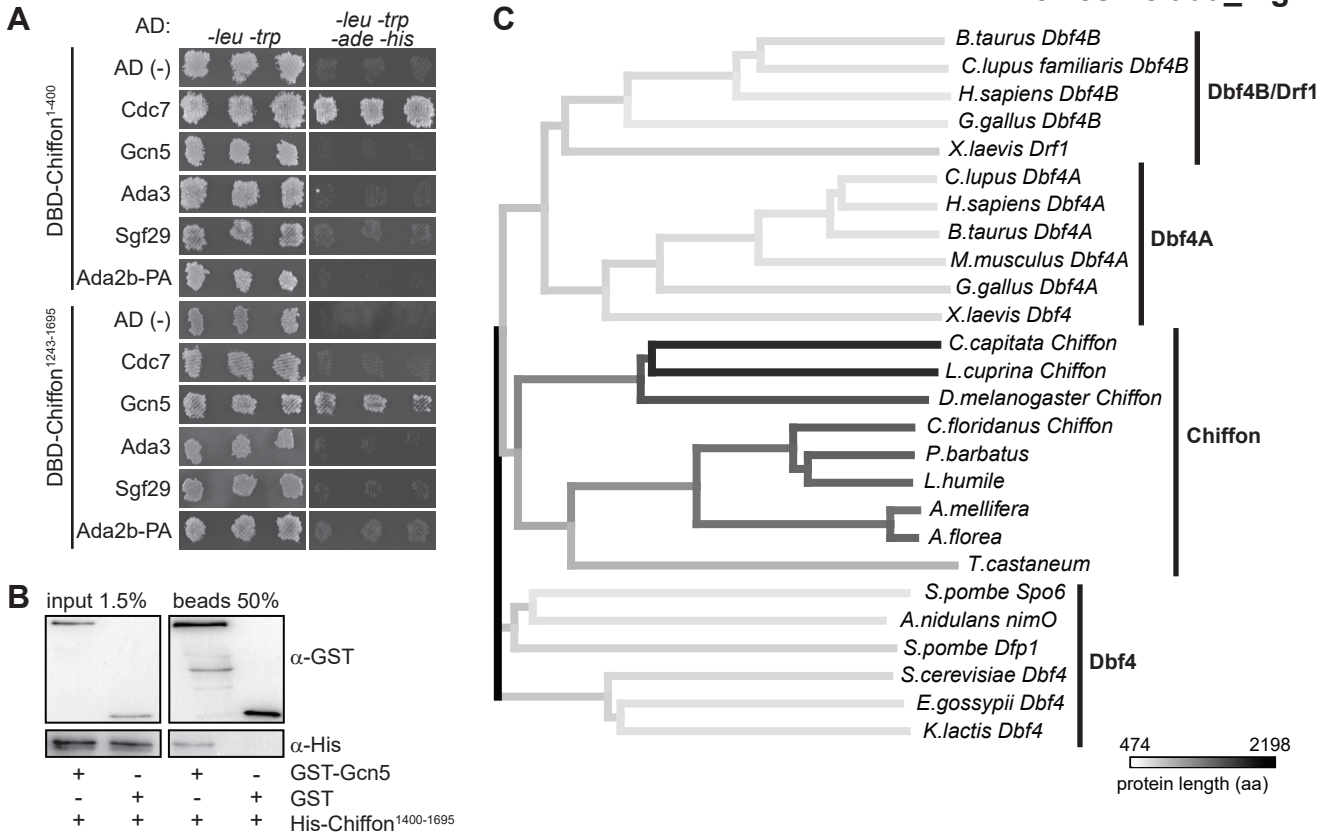
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AD (-)
Cdc7
Gcn5
Ada3
Sgf29
Ada2b-PA
B
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beads 50%
α-GST
α-His
+ - + - GST-Gcn5
- + - + GST
+ + + + His-Chiffon1400-1695
C
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C.lupus familiaris Dbf4B
H.sapiens Dbf4B
G.gallus Dbf4B
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L.humile
A.mellifera
A.florea
T.castaneum
Chiffon
S.pombe Spo6
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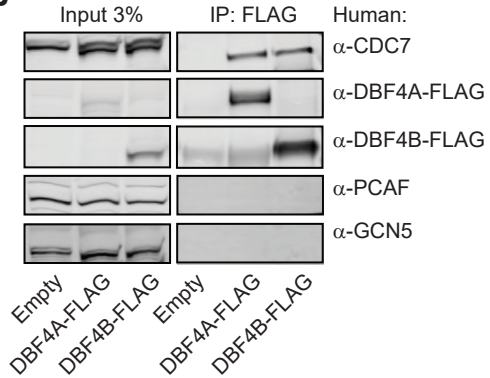
S.pombe Dfp1
S.cerevisiae Dbf4
E.gossypii Dbf4
K.lactis Dbf4
Dbf4
474
2198
protein length (aa)

Torres-Zelada_Fig3

A

Peptides identified in Dbf4 purifications % coverage (spectral count)	*Drosophila*		*S. cerevisiae*
	C-tag	N-tag	
Dbf4/Chiffon	42%(903)	45%(3024)	41%(888)
Cdc7	8%(7)	43%(93)	24%(290)
Ada2(b)	27%(89)	10%(1)	-
Ada3	39%(126)	6%(3)	-
Gcn5	36%(158)	12%(13)	-
Sgf29	56%(103)	20%(8)	-

B



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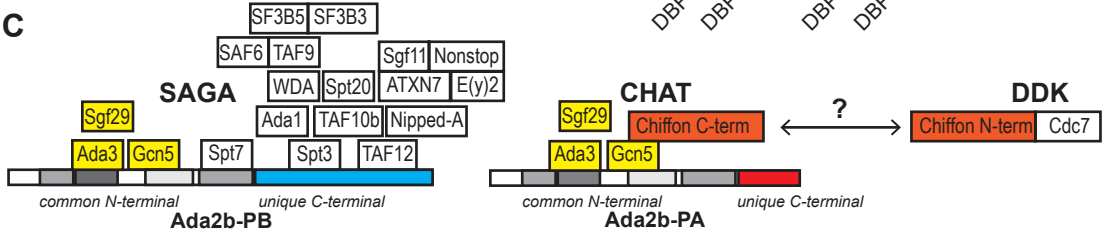

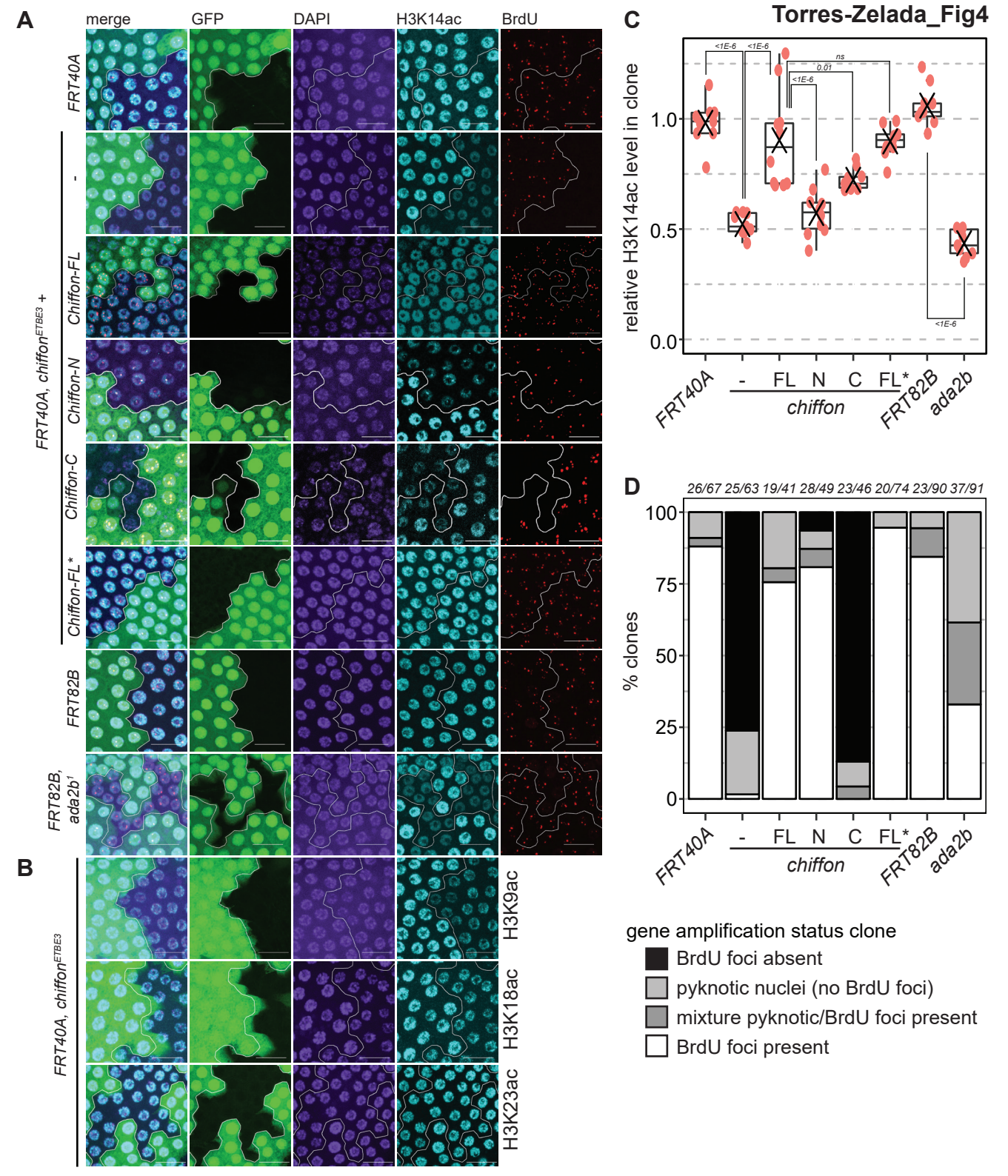
Torres-Zelada_Fig4
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Chiffon-FL
Chiffon-N
Chiffon-C
Chiffon-FL*
FRT40A, chiffonETBE3 +
FRT82B
FRT82B, ada2b1
B
FRT40A, chiffonETBE3
H3K9ac
H3K18ac
H3K23ac
C
relative H3K14ac level in clone
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0.5
0.0
<1E-6
<1E-6
ns
0.01
<1E-6
<1E-6
FRT40A
-
FL
N
C
FL*
chiffon
FRT82B
ada2b
D
26/67 25/63 19/41 28/49 23/46 20/74 23/90 37/91
% clones
100
75
50
25
0
FRT40A
-
FL
N
C
FL*
chiffon
FRT82B
ada2b
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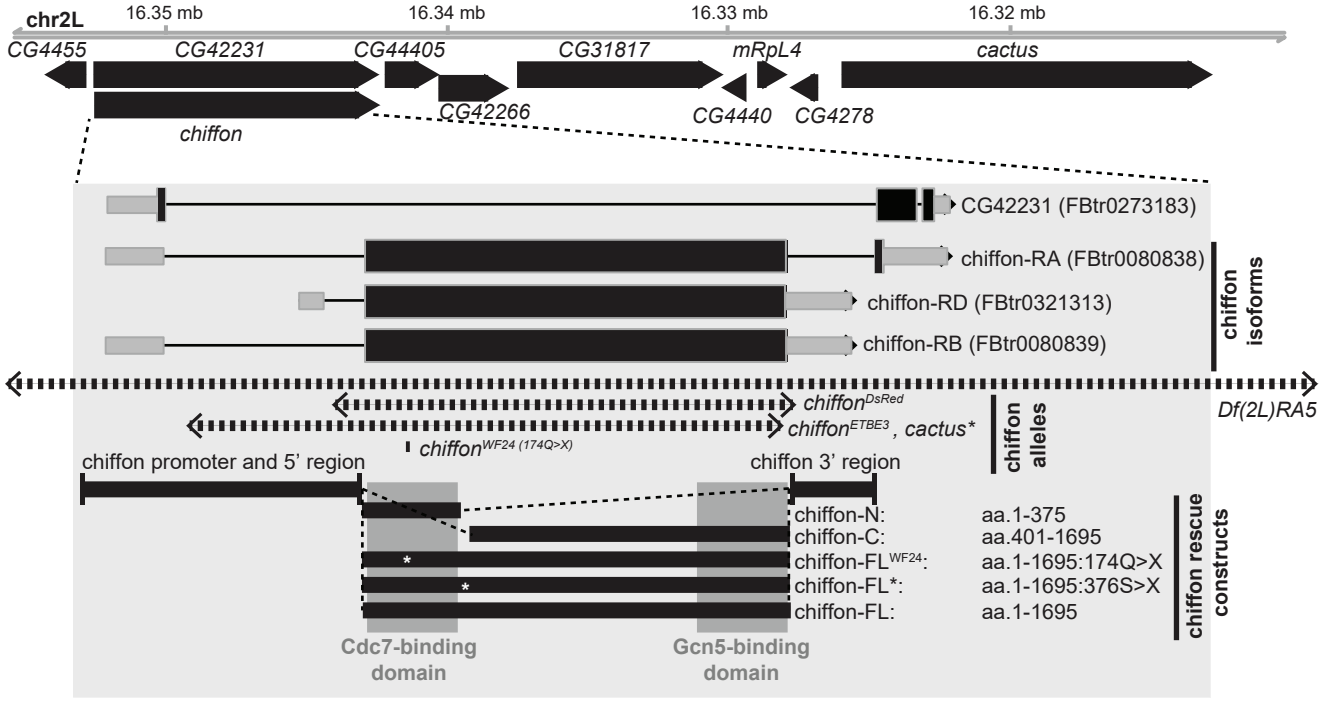
mixture pyknotic/BrdU foci present
BrdU foci present

Torres-Zelada_Fig5



Torres-Zelada_Fig6