Roles for the Histone Modifying and Exchange Complex NuA4 in Cell Cycle Progression in *Drosophila melanogaster*

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ABSTRACT Robust and synchronous repression of E2F-dependent gene expression is critical to the proper timing of cell cycle exit when cells transition to a postmitotic state. Previously NuA4 was suggested to act as a barrier to proliferation in *Drosophila* by repressing E2F-dependent gene expression. Here we show that NuA4 activity is required for proper cell cycle exit and the repression of cell cycle genes during the transition to a postmitotic state *in vivo*. However, the delay of cell cycle exit caused by compromising NuA4 is not due to additional proliferation or effects on E2F activity. Instead NuA4 inhibition results in slowed cell cycle progression through late S and G2 phases due to aberrant activation of an intrinsic p53-independent DNA damage response. A reduction in NuA4 function ultimately produces a paradoxical cell cycle gene expression program, where certain cell cycle genes become derepressed in cells that are delayed during the G2 phase of the final cell cycle. Bypassing the G2 delay when NuA4 is inhibited leads to abnormal mitoses and results in severe tissue defects. NuA4 physically and genetically interacts with components of the E2F complex termed *Drosophila*, Rbf, E2F and Myb/Multi-vulva class B (DREAM/MMB), and modulates a DREAM/MMB-dependent ectopic neuron phenotype in the posterior wing margin. However, this effect is also likely due to the cell cycle delay, as simply reducing Cdk1 is sufficient to generate a similar phenotype. Our work reveals that the major requirement for NuA4 in the cell cycle *in vivo* is to suppress an endogenous DNA damage response, which is required to coordinate proper S and G2 cell cycle progression with differentiation and cell cycle gene expression.

KEYWORDS DNA damage; proliferation; H2Av; cell cycle checkpoint; cell cycle exit

A conserved eukaryotic transcriptional oscillator controls cell cycle gene expression in proliferating cells and underlies the well-studied Cyclin/Cdk cell cycle protein oscillator (Orlando *et al.* 2008; Oikonomou and Cross 2010). In metazoans, this oscillator depends upon transcriptional activation of several hundred cell cycle genes, initiated by E2F transcription factor complexes followed by E2F inhibition (or degradation) (Shibutani *et al.* 2008; Zielke *et al.* 2011; Sadasivam and Decaprio 2013). This generates an oscillation of chromatin opening and closing at cell cycle genes that is thought to properly coordinate G1/S and G2/M gene expression as well as the transition to a noncycling state (Litovchick *et al.* 2007, 2011; Forristal *et al.* 2014). Disruption of this

coordination can affect cell cycle progression by causing inappropriate gene expression (Reis and Edgar 2004; Wen *et al.* 2008; Forristal *et al.* 2014). While the oscillation of E2F activity is required for robust cell cycle gene expression (Dimova *et al.* 2003; Korenjak *et al.* 2012), E2F complexes are not absolutely essential for cell cycle progression or timely cell cycle exit in *Drosophila* (Frolov *et al.* 2001, 2003, 2005). In the absence of E2F activity there must be E2F-independent factors or mechanisms that allow sufficient cell cycle gene regulation for cell cycle progression and timely cell cycle exit.

Cells entering nonproliferative or quiescent states are thought to repress the transcriptional oscillator by association of the tumor suppressor retinoblastoma (RB) or RB family members with E2F. This association recruits a repressive complex termed the *Drosophila*, Rbf, E2F and Myb/Multi-vulva class B (DREAM/MMB) complex that is thought to promote the closing of chromatin and stable repression of cell cycle genes (Korenjak *et al.* 2004; Lewis *et al.* 2004; Litovchick *et al.* 2007; Sadasivam and Decaprio 2013). Although DREAM/MMB has no apparent histone exchange component itself, recent

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findings suggest that its role in transcriptional repression is linked with histone H2A variant (H2Av) localization to target gene bodies (Latorre *et al.* 2015). Perturbations in the DREAM/MMB complex shift cells from quiescence toward proliferation in mammalian tissue culture and in chondrocytes, but additional *in vivo* DREAM/MMB functions during terminal differentiation remain largely unknown (Litovchick *et al.* 2007, 2011; Forristal *et al.* 2014). Furthermore, *Drosophila* tissues still proliferate and exit the cell cycle normally in the complete absence of DREAM/MMB binding to chromatin, underscoring the importance of additional chromatin modulating factors in cell cycle progression and exit (Korenjak *et al.* 2012).

The Transition from Proliferation to a Postmitotic State in *Drosophila*

Pulses of the hormone ecdysone control the developmental stages of *Drosophila*, with terminal differentiation of most adult tissues occurring during metamorphosis at the pupal stage (Ashburner 1989). Since ecdysone pulses control the onset of metamorphosis, subsequent developmental events are relatively naturally synchronized *in vivo*. This includes the final cell cycles in the *Drosophila* eyes and wings. All cell types in the eye become postmitotic by 24 hr after pupa formation (APF) (Cagan and Ready 1989). In the wing, there is a temporary G2 arrest early in metamorphosis, such that most cells complete their final cell cycle between 12 and 24 hr APF (Schubiger and Palka 1987; Milan *et al.* 1996; O'Keefe *et al.* 2012). The synchronized cell cycle exit in the pupal fly wings and eyes provides a convenient *in vivo* context to identify genes that influence the proper timing of cell cycle exit.

The NuA4 Complex

We took advantage of the synchronized cell cycle exit in the pupal fly eyes and wings to perform an RNAi-based screen for genes involved in the proper timing of cell cycle exit. This screen identified multiple components of the Tip60/Nucleosome Acetyltransferase of Histone H4 (NuA4) complex as important regulators of proper cell cycle exit, which we subsequently also found to be important for proper cell cycle progression in proliferating tissues.

Tip60/NuA4 is a multisubunit complex conserved from yeast to humans, best characterized to open chromatin to promote gene expression (Doyon *et al.* 2004; Lu *et al.* 2009). Tip60/NuA4 has histone acetyltransferase (HAT), DNA helicase, histone reading and histone exchange activities, and plays an essential, conserved role in histone exchange for the H2Ax variant (H2Av in *Drosophila*) that becomes phosphorylated upon DNA damage (Kusch *et al.* 2004). NuA4 has been reported to engage many transcription factors, including Myc, p53, and E2F (McMahon *et al.* 2000; Frank *et al.* 2003; Legube *et al.* 2004; Taubert *et al.* 2004) to turn on target gene expression in proliferating cells. However, the NuA4 complex also acts as an essential repressor of gene expression in certain contexts, such as embryonic stem cells

(Fazio *et al.* 2008; Chen *et al.* 2013), and can promote closed chromatin formation in flies (Qi *et al.* 2006). Furthermore, NuA4 components paradoxically act as both tumor suppressors and oncogenes, leaving their positive vs. negative roles in cell cycle control unresolved (Sapountzi *et al.* 2006; Jude *et al.* 2015).

Several studies suggest roles for NuA4 and H2Av in regulation of E2F target genes and genetic interactions with DREAM/MMB components (Ceol and Horvitz 2004; Lu *et al.* 2007; DeBruhl *et al.* 2013; Latorre *et al.* 2015). Mammalian studies implicate Tip60/NuA4 as a transcriptional activator of E2F target genes, whereas a subset of NuA4 components in *Drosophila* cell culture were shown to repress E2F transcriptional targets (Lu *et al.* 2007). Here we reveal contradictory roles for NuA4 in cell cycle progression and cell cycle gene expression during development. We find the loss of NuA4 function: (1) compromises proliferation, (2) leads to accumulation of the DNA damage hallmark phosphoH2Av, (3) causes aberrant expression of a transcriptional program associated with a DNA damage response, and (4) disrupts the proper timing of cell cycle exit in pupal eyes and wings by delaying the final mitosis. We further show that NuA4's role in delaying cell cycle exit is independent of E2F/DP function as well as the DNA damage response pathway component p53 and that NuA4 promotes genetic stability in proliferating tissues.

Materials and Methods

Fly stocks and genetics

Details of the stocks and crosses are provided in Supplemental Material, File S1. To reduce lethality, some crosses used temperature-sensitive Gal80 (*tub-Gal80^{TS}*) (McGuire *et al.* 2004) to suppress transgene expression until later in development. In these cases, animals were raised at 18°, then shifted to 28° for Gal4 induction at the indicated times. For cell cycle gene reporter assays (PCNA-GFP, Mad-GFP, Stg-GFP), animals were raised at room temperature due to the lack of Gal80^{TS} in these stocks (23°). For inhibition of NuA4 in the pupal eye, GMR-GAL4 was used to overexpress the transgenes. For maximum GAL4 production with weak UAS-RNAi lines (*e.g.*, *domino^{RNAi}*), animals were raised at 28° and staged at 0 hr APF.

Clone size and lineage counting

Clone counts in pupal wings were performed as described (Buttitta *et al.* 2007). For *Tip60^{DN}* and *Tip60^{WT}* clone counts at L3, animals were reared in uncrowded vials and heat-shocked in a 37° water bath for 7.5 min. Mitotic Flp/FRT clones for the *dom^{-/-}* analysis were counted 48 hr after a 13-min (37°) heat shock. Clone areas and cell numbers were counted blind.

Immunostaining

Pupae were staged from white prepupae (0 hr after puparium formation). Development at 28° occurs 1.15 times faster than

at 25°, 1.25 times faster at 31°, and 2.2 times more slowly at 18° (Ashburner 1989; Buttitta *et al.* 2007). All incubation times were adjusted accordingly and the hours APF are presented as the 25° equivalent. Tissues were fixed with 4% paraformaldehyde/1× PBS for 30 min, washed twice in 1× PBS, 0.1% Triton X, 10 min each, and then used for immunostaining as described (Buttitta *et al.* 2007). Hoechst 33258 was used to label nuclei and tissues were mounted in Vectashield (Vector Laboratories). F-actin staining was performed in 1× PBS using 1:100 rhodamine-phalloidin (Invitrogen; R415) for 2–4 hr.

Antibodies

The antibodies used and their sources are provided in [File S1](#).

Microscopy

Images were obtained using a Leica DM1600 epifluorescence system with deconvolution (ImageQuant) or a Leica SP5 confocal. All images were cropped, rotated, and processed using Adobe Photoshop. All brightness/contrast adjustments were applied equally on the entire image.

Flow cytometry

Flow cytometry was performed on an Attune cytometer with tissue dissociation and gating/detection parameters as described (Flegel *et al.* 2013).

Mounting and scoring of adult wings

Mounting and microscopy of adult wings was performed as described (O'Keefe *et al.* 2012). For scoring phenotype severity, seven classes of defects (notched margin, ectopic vein, ectopic bristles, L4 truncation, loss of crossveins, L5 truncation, and smaller posterior compartment) were generated, and wings were scored according to the same rubric for the total number of defects.

Quantitative RT-PCR and RNA sequencing

For quantitative PCR (RT-qPCR), RNA was isolated from dissected tissues per the TRIzol manual, resuspended in water, and then treated with DNase I to remove contaminating DNA. Using 300 ng of RNA per sample, complementary DNA (cDNA) was synthesized using oligo(dT)₂₀ and the Superscript III First-Strand Synthesis System (Invitrogen; 18080051). qPCR using 0.5 µl of cDNA per reaction was then performed using the 7500 Fast Real-Time PCR and StepOnePlus Real-Time PCR Systems (Applied Biosystems).

For RNA sequencing (RNA-seq), heat-shocked animals were shifted to 28° either 48 or 78 hr prior to dissection at the wandering L3 stage. Twenty wing discs were dounce homogenized in TRIzol with 10 strokes of a tight pestle. Samples were vortexed at speed 10 for 1 min, incubated for 5 min at room temperature, and then frozen in liquid nitrogen. Samples were kept at –80° until they were pooled to make a total of 40 wing discs per replicate of each condition and genotype. The pooled RNA was phase separated with

chloroform and RNA was precipitated in isopropanol at –30° overnight prior to a 20-min 4° centrifugation at 12,000 × *g*. The RNA pellet was resuspended in 30 µl of RNase-Free water.

Using PolyA selection, the University of Michigan's Sequencing Core generated barcoded libraries for each sample and confirmed the quality via the Bioanalyzer and qPCR. Sequencing was performed with the Illumina HiSeq 2000 platform and high read quality was confirmed using FastQC. Reads were aligned to the BDGP6.82 *Drosophila melanogaster* genome using Rsubread (v1.21.5), with featureCounts resulting in >83% of the reads being successfully assigned to genes (Liao *et al.* 2014). Counts per million (cpm) were determined with edgeR (v3.13.4) and transcripts with low expression were identified and removed using the data-based Jaccard similarity index determined with HTSFilter (v1.11.0). The cpm were normalized using a trimmed mean of *M*-values (TMM) via calcNormFactors, voom transformed (Law *et al.* 2014), fit to a linear model (lmFit), and then differential gene expression calls were made with eBayes.

For significance of overlap with other datasets (Figure 4), hypergeometric probabilities were calculated using the hypergeometric distribution. In our case: (1) the population size equals the total number of genes that align to the genome and pass our filters for the RNA-seq; (2) the number of successes in the population is the total number of genes that are represented in both our dataset and the published dataset under comparison; (3) the sample size is the total number of genes upregulated by Tip60^{DN} and for which there are data in the published dataset under comparison; and (4) the number of successes in the sample equals the total number of genes that overlap in our Tip60^{DN} upregulated dataset and are significantly upregulated in the published dataset under comparison.

S2R⁺ RNA interference treatment

S2R⁺ cells were cultured as previously described (Zielke *et al.* 2014). (Note that in Figure 3 the graphs are color coded according to the indicated cell cycle phase, not according to the fluorescent ubiquitination-based cell cycle indicator (FUCCI) red/green readout.) RNAi was performed as described (Rogers and Rogers 2008) with the following modifications: cells were incubated in 1 ml of serum-free medium with 10 µg/ml of double-strand RNA (dsRNA) for 4–24 hr, followed by addition of 1 ml of 10% serum medium for 4 days. dsRNA was synthesized using the T7 Megascript Kit (Ambion) and primer sequences are provided in the supplement.

Reagent and data availability

Strains are available upon request. [File S1](#) contains seven supplemental figures and legends as well as detailed descriptions of all supplemental files and tables. Gene expression data are available at Gene Expression Omnibus under accession no. GSE81159.

Figure 1 NuA4 inhibition delays cell cycle exit. (A–I) Using *engrailed-Gal4* modified with a temperature sensitive Gal80 (*en^{TS}*), a UAS-driven HAT-inactive form of Tip60 (*Tip60^{E431Q}*, “dominant negative” Tip60, *Tip60^{DN}*) or the indicated UAS-RNAi lines were expressed in the posterior wing from L2. Pupa were collected at 0 hr APF and aged to 27 hr APF or the indicated stages while being kept at the permissive temperature for Gal4-induced transgene expression. The dotted line indicates the wing anterior/posterior boundary. NuA4 inhibition increased the number of mitoses (indicated by phospho-Ser10 histone H3, PH3) in the posterior wing, at time points when the wing is normally postmitotic. (J–N) Ectopic mitoses persist for <8 hr past the time of normal cell cycle exit. (N) Flow cytometry at the indicated time points (hours APF) showed an increased proportion of cells with a 4C DNA content (G2/M) in wings expressing *Tip60^{DN}*. (O) Schematic of the NuA4 complex. Multiple RNAi lines for the core subunits were tested (Table 1). Subunits required for proper cell cycle exit are shown in blue.

Results

Compromising NuA4 delays cell cycle exit

To identify genes involved in the proper timing of cell cycle exit in *Drosophila* eyes and wings, we screened ~2000 RNAi lines from the TRiP collection (Ni *et al.* 2011) for ectopic cell cycle activity in pupal eyes or wings at time points when these tissues are normally fully postmitotic (Buttitta *et al.* 2007). Our initial screen was performed in the eye and assayed a *PCNA-white* reporter transgene previously described as a readout for cell cycle activity (Bandura *et al.* 2013). This screen used the *Glass Multimer Repeats (GMR)* promoter to drive Gal4/UAS-induced RNAi expression in the eye during the transition to a postmitotic state. From 36 hits in the initial eye screen, we verified 12 RNAi lines that led to ectopic mitoses in both the eye and wing at time points after 24 hr APF, a developmental time point when these tissues should

be fully postmitotic. Four of these RNAi hits fell within the same complex, the NuA4 complex. We therefore systematically tested the core subunits of NuA4 with additional RNAi lines and mutant alleles. We found that multiple, independent RNAi lines to six NuA4 subunits expressed in the posterior wing from larval stages (using *engrailed-Gal4/UAS*, *en-Gal4*) resulted in ectopic mitoses (as detected by phospho-Ser10-histone H3, PH3) at time points after the normal transition to a postmitotic state (Figure 1, Table 1, Figure S1). The effect of *Tip60^{RNAi}* could be phenocopied by overexpression of a histone acetyltransferase (HAT)-inactive form of the catalytic subunit Tip60, (*Tip60^{E431Q}* also termed *Tip60^{DN}*) previously shown to act as a dominant negative and reduce H4 acetylation (Figure S1, A and B) (Lorbeck *et al.* 2011). By contrast, overexpression of a wild-type *Tip60* transgene (*Tip60^{WT}*) had no effect on the cell cycle or wing and eye development in any of our assays. Through

a time-course analysis (Figure 1, J–N), we confirmed that cells with inhibited NuA4 were not permanently arrested in an aberrant mitosis. Inhibition of NuA4 delayed cell cycle exit for 4–6 hr in both eyes and wings, after which all cells became postmitotic at 32 hr APF with a normal 2C DNA content as measured by flow cytometry (Figure 1N). The effects of NuA4 inhibition on cell cycle exit were compartment autonomous, as the anterior wing became postmitotic normally (Figure 1). We further verified the effects were cell autonomous by generating GFP-labeled clones of cells expressing *Tip60^{DN}* in the wing. Cells within the clone delayed cell cycle exit, while nonexpressing neighboring cells became postmitotic normally (Figure S1D). All six subunits with similar phenotypes are core NuA4 subunits, required for full function of the complex (Doyon and Cote 2004) (Figure 1O). Thus, NuA4 HAT activity is required for the proper timing of developmentally controlled cell cycle exit.

We next examined whether the delay of cell cycle exit associated with NuA4 inhibition was an indirect effect due to earlier developmental defects. Using temporal restriction of Gal4/UAS transgene activation via *engrailed*-Gal4 combined with a temperature-sensitive Gal80 (hereafter termed *en^{TS}*), we limited expression of *Tip60^{DN}* to the final cell cycle in the pupal wing. Inhibition of Tip60 HAT function during the final cell cycle recapitulated the cell cycle exit delay observed with RNAi to NuA4 subunits (Figure S1, C and E). Similar to the wing, inhibition of NuA4 HAT activity during the final cell cycle in the eye (using *GMR-Gal4*) also delayed the timing of cell cycle exit for several hours (Figure S1, F–H). To test whether the effects of *Tip60^{DN}* on cell cycle exit reflect a general requirement for H4 acetylation, we inhibited another major H4 HAT, *Drosophila* CREB binding protein (CBP) (Parker *et al.* 2008). Inhibition of CBP in the wing reduced overall levels of histone H4 acetylation, but did not delay cell cycle exit (Figure S1I), suggesting that NuA4 performs a specific function required for the proper timing of cell cycle exit.

Compromising NuA4 does not alter the timing of terminal differentiation

Since NuA4 has histone H4 acetyltransferase activity, it could be responsible for promoting gene expression globally. To determine if NuA4 inhibition simply slows developmental progression, we examined whether the 4- to 6-hr delay of cell cycle exit was also associated with a delay in the acquisition of characteristics associated with terminal differentiation. In the pupal wing, the formation of actin-rich hairs is a physical marker for terminal differentiation (Mitchell *et al.* 1983). At 25°, wing cells initiate hair formation at 32 hr APF (Mitchell *et al.* 1983; Fang and Adler 2010). Inhibition of Tip60 or other NuA4 components using *en^{TS}* did not alter wing hair initiation or growth (33 hr APF shown, Figure S2, A–C), when compared to the anterior nonexpressing compartment despite a 4- to 6-hr delay of cell cycle exit. We also examined whether Tip60/NuA4 inhibition altered the timing of differentiation in the eye (Figure S2, D–F). Terminal differentiation of many cell types in the late third

Table 1 RNAi lines for core NuA4 subunits

<i>en</i> > GAL4	EdU at 22 hr APF	PH3 at 27 hr APF	Stock nos.
<i>white</i> ^{RNAi}	N	N	BDSC: 35573
<i>Tip60</i> ^{RNAi}	Y	Y	BDSC: 28563
<i>E(Pc)</i> ^{RNAi}	Y	Y	BDSC: 28686
<i>Brd8</i> ^{RNAi}	Y	Y	v41530, v104879
<i>dom</i> ^{RNAi}	Y	Y	BDSC: 31054, v7789
<i>Nipped-A</i> ^{RNAi}	Y	Y	BDSC: 31255, 34849
<i>YL-1</i> ^{RNAi}	Y	Y	v21903, v107951
<i>Mrg15</i> ^{RNAi}	N	N	v43800, v43802
<i>Reptin</i> ⁰⁶⁹⁴⁵	N	N	BDSC: 11706
<i>Pontin</i> ^{5.1}	N	N	PMID: 16087886

RNAi lines for the core NuA4 subunits tested for a delay in cell cycle exit. N, no; Y, yes.

larval instar (L3) eye disc is spatially synchronized, following the morphogenetic furrow (MF) that sweeps from posterior to anterior across the tissue. Posterior to the MF, a new row of differentiated cells is established every 2 hr, which can be marked by the expression of postmitotic neural and cone cell markers (Treisman 2013). A 4- to 6-hr delay in development would be expected to cause a two- to three-row posterior shift in the onset of differentiation marker expression. We used heat-shock-induced recombination to create nonoverlapping GFP-labeled clones expressing *Tip60^{DN}* that span the MF and the first few rows of differentiated cells. We did not observe any delay in photoreceptor or cone cell marker expression upon NuA4 inhibition, compared to the surrounding nonclonal cells (Figure S2, D–F). This suggests that compromising NuA4 does not delay cell cycle exit simply by slowing overall developmental progression.

Inhibition of NuA4 leads to ectopic cell cycle gene expression

NuA4 is a chromatin remodeler and components of NuA4 have been shown to repress E2F-dependent gene expression in cultured *Drosophila* S2 cells (Lu *et al.* 2007). We therefore examined whether NuA4 inhibition altered cell cycle gene repression in pupal wings and eyes during cell cycle exit. We examined three reporters previously shown to be accurate readouts of endogenous cell cycle gene regulation including transcriptional repression at cell cycle exit; a PCNA-GFP transgene, which reports E2F-dependent G1–S gene expression, and the Mad2-GFP and Stg-GFP protein traps that report endogenous levels of G2–M phase cell cycle regulators (Thacker *et al.* 2003; Wen *et al.* 2008; Inaba *et al.* 2011). Inhibition of Tip60/NuA4 in the posterior pupal wing by RNAi or *Tip60^{DN}* expression led to ectopic PCNA-GFP and Mad2-GFP expression at stages that are normally postmitotic (Figure 2, A–G). NuA4 inhibition during the final cell cycle in the eye also caused a subset of interommatidial cells to exhibit ectopic PCNA-GFP expression at postmitotic pupal stages (Figure 2H). In clones with Tip60/NuA4 inhibited in the late L3 eye, we observed higher and prolonged Stg-GFP expression in the G1-arrested cells of the morphogenetic furrow as well as the postmitotic region posterior to the MF

Figure 2 NuA4 is essential for proper cell cycle gene repression at cell cycle exit. (A–G) Using *en-Gal4*, the indicated transgenes were expressed in the posterior wing. RNAi to *white* (*w^{RNAi}*) and *Tip60^{WT}* serve as negative controls that do not affect the cell cycle. Pupa were collected at 0 hr APF and aged to 27 hr APF. The dashed line indicates the wing anterior/posterior boundary. (B, C, and F) NuA4 inhibition resulted in high levels of PCNA-GFP expression. (D, E, and G) A G2/M reporter Mad2-GFP, also accumulated when Tip60 HAT activity was inhibited. (H) Tip60^{DN} was expressed during the final cell cycle in the eye using *GMR-Gal4/UAS*, resulting in PCNA-GFP expression in the interommatidial bristle precursors at 27 hr APF. (I–K) Using heat-shock induced *flipout-Gal4* we generated clones expressing *Tip60^{DN}* in the larval eye in a background carrying a String-GFP protein trap insertion (Stg-GFP). Cells expressing *Tip60^{DN}* posterior to the MF increased Stg-GFP expression (MF indicated by arrowhead, posterior is at top.) (L) Using *flipout-Gal4* in combination with *Gal80^{TS}* (*flipout^{TS}*) we generated large, overlapping clones of cells expressing Gal4 24 hr after embryo hatching, but restricted the UAS-induced expression of *Tip60^{DN}* or *Tip60^{WT}* to pupal stages only (from 0 hr APF) using temperature shifts. In pupa expressing *Tip60^{DN}* staged to 26 hr APF, quantitative RT-PCR revealed aberrant *pcna* and *mad2* in the wing, compared to the *Tip60^{WT}*-expressing controls. By contrast, *e2f1* transcript is reduced by *Tip60^{DN}*. Levels of *stg* in the pupa wing are unchanged by *Tip60^{DN}*. (M) E2F1 protein levels are decreased in 27 hr APF pupal wings expressing *Tip60^{DN}* in the posterior. *P*-values were determined by an unpaired *t*-test; **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001.

(Figure 2, I–K). To confirm that the reporters reflect the endogenous transcripts, we performed quantitative RT-PCR for cell cycle genes in pupal wings at 26 hr APF expressing *Tip60^{WT}* and *Tip60^{DN}*. Transcripts for *pcna* and *mad2* were increased 1.5–1.8 fold in wings expressing *Tip60^{DN}*, consistent with our reporter assays (Figure 2L). However, we did not observe an increase in *stg* expression in pupal wings. Unlike the eye, endogenous *stg* levels normally peak at the time of cell cycle exit in the pupal wing (Buttitta *et al.* 2007), and NuA4 inhibition may not increase *stg* beyond the already high levels of expression at this stage. The ectopic expression of the known E2F targets *pcna* and *mad2* raised the possibility that NuA4 impacts E2F1 expression or activity. Surprisingly, expression of *Tip60^{DN}* reduced both *e2f1* transcript and E2F1 protein levels in the pupa wing (Figure 2, L and M). Altogether our data suggest that NuA4 function is needed for the proper shut-off of specific cell cycle genes at cell cycle exit, but seemingly

paradoxically is also required for the proper maintenance of E2F1 levels.

Compromising NuA4 disrupts cell cycle progression

Our finding that NuA4 is required both for the proper shut-off of cell cycle gene expression as well as the proper maintenance of E2F1 levels, raised the question of whether the delayed cell cycle exit observed upon NuA4 inhibition is due to extra cell cycles or the result of a slowed final cell cycle. To address this, we performed a clonal lineage tracing experiment in the wing blade to quantify the number of cell divisions occurring before the delayed cell cycle exit. Nonoverlapping, GFP-labeled clones expressing *Tip60^{DN}* were induced at 0 hr APF during the final cell cycle in the wing. Pupa wings were examined at 36 hr APF, (12 hr after normal exit and 4–6 hr after the delayed cell cycle exit) and the number of cells per clone were counted blind for at least 100 clones per genotype

(Figure 3A). We also coexpressed the apoptosis inhibitor P35, to avoid the effects of apoptosis on counting cell divisions. Control clones expressing *GFP* + *P35* averaged 2.22 ± 0.08 cells/clone, while clones expressing *Tip60^{DN}* + *P35* averaged 2.11 ± 0.05 cells/clone (Figure 3A). We confirmed that this method is sensitive enough to detect even one extra cell cycle in the wing, as clones overexpressing *CycD/Cdk4* + *P35* caused one extra cell cycle in ~50% of clones resulting in 2.59 ± 0.11 cells/clone. Thus, inhibition of Tip60 HAT activity delays cell cycle exit by slowing the final cell cycle, rather than by causing additional cycles.

We next examined whether loss of Tip60/NuA4 function prolongs the cell cycle during asynchronous proliferation in the larval wing. We created nonoverlapping GFP-marked clones and compared cells/clone 30 hr and 46 hr after induction for *Tip60^{DN}* + *P35* vs. *Tip60^{WT}* + *P35* (Figure 3B). Clonal cell counts for *Tip60^{WT}* averaged 4.26 ± 0.12 cells/clone at 30 hr, corresponding to a normal cell doubling time (DT) of approximately 15 hr in the wandering L3 larval wing (Reis and Edgar 2004). By contrast, clones expressing *Tip60^{DN}* averaged 3.39 ± 0.11 cells/clone (Figure 3B), corresponding to an average DT almost 4 hr longer than the control (18.93 hr). This indicates that Tip60 HAT activity is required for proper cell cycle progression.

Slowed cell cycle progression was also observed upon inhibition of other NuA4 subunits. RNAi to E(Pc) or Nipped-A in the presence of P35 to block apoptosis, resulted in smaller clones in the larval wing (Figure 3C) despite little change to cell size (Figure S3A). *domino* (*dom*) null clones generated by mitotic recombination were also significantly smaller than their *dom^{+/+}* sibling twinstot clones, even when one copy of the H99 locus was removed to minimize cell death (Figure 3D and Figure S3, B–D). Using flow cytometry to measure DNA content, we determined that *dom^{-/-}* cells accumulated in the late S/G2 phase of the cell cycle (with >2C DNA content) in proliferating L3 wings (Figure 3D'). We observed similar results with RNAi to the other NuA4 subunits and *Tip60^{DN}*, when expressed in the posterior L3 wing (Figure 3E). To ensure the changes in cell cycle phasing were not due to nonautonomous effects on the non-Gal4-expressing anterior compartment of the wing, we also performed posterior-to-posterior wing comparisons of flow cytometry profiles to control genotypes, including *w¹¹¹⁸*, *white^{RNAi}*, and *Tip60^{WT}* (Figure 3F). We consistently observed an increase in cells with >2C DNA content when NuA4 is inhibited, although we also found a mild compensatory nonautonomous effect on the cell cycle distribution in the anterior wing as well (Figure 3G). This, together with the slowed cell cycle progression, suggests that full Tip60/NuA4 function is required for proper S/G2/M progression *in vivo*, consistent with data from other organisms (Clarke *et al.* 1999; Choy *et al.* 2001; Li *et al.* 2004; Hu *et al.* 2009; Tapias *et al.* 2014).

A previous study of the NuA4 subunits *Brd8*, *YL-1*, and *dom* in cultured *Drosophila* S2 cells described an effect of NuA4 on E2F activity, but did not describe a cell cycle phenotype (Lu *et al.* 2007). To examine whether NuA4 impacts cell cycle

progression in S2 cells, we used an S2 cell line containing the Fucci reporters and performed RNAi to NuA4 subunits for 5 days, followed by E2F reporter assays, flow cytometry, and cell counts (Zielke *et al.* 2014). NuA4 inhibition in S2 cells had the previously described effects on an E2F-Luciferase reporter (not shown), but also reduced EdU incorporation during S phase and lengthened the S/G2 transition (Figure S3, E–G). This resulted in altered cell cycle phasing, mainly by increasing the proportion of cells in late S and early G2 (Figure 3H). Altogether our data suggest inhibition of NuA4 leads to cell cycle alterations that slow S/G2 cell cycle progression.

NuA4 inhibition deregulates cell cycle gene expression during active proliferation

A readout of E2F transcriptional activity, PCNA-GFP is increased upon NuA4 inhibition in pupal wings (Figure 2). We observed similar increases in PCNA-GFP and Stg-GFP reporters when NuA4 was inhibited in the proliferating late L3 wing, despite the slowed proliferation and prolonged S and G2 phases under these conditions (Figure 4, A–H). Similar to the pupa wing, expression of *Tip60^{DN}* in the posterior L3 wing caused a reduction in E2F1 protein levels (Figure 4, I and J). Since E2F1 is degraded in S phase but accumulates during G1 and late G2 phases (Shibutani *et al.* 2008; Zielke *et al.* 2014) cells in a prolonged late S/early G2 would be expected to have lower E2F1 protein levels. However we were puzzled by the apparently contradictory results of a delay in S/G2 progression, accompanied by higher levels of Stg. This suggested that compromising NuA4 may activate a checkpoint to offset the increased Stg. Stg acts on the CycB/Cdk1 complex to promote its activity by removing the inhibitory Cdk1 phosphorylation catalyzed by the Wee and Myt kinases. However, we observed no obvious changes in total Cdk1 or Wee levels by immunofluorescence. Instead, we found that expression of *Tip60^{DN}* reduced CycB protein within 24 hr of expression in the larval wing, while *Tip60^{WT}* expression had no effect on CycB (Figure 4, K–M). RNAi to other NuA4 subunits also recapitulated this effect, leading to a ~15–20% reduction in CycB protein levels, which we also confirmed by RT-qPCR (Figure S4, A and B). By contrast, we did not observe consistent changes in another G2/M regulator CycA (Figure S4, C–E). We suggest that the reduced levels of CycB may contribute to the slowed S/G2 progression when NuA4 is compromised. Importantly, a previous study of Tip60 localization on the genome by DNA adenine methyltransferase identification (DamID) resolved one of the strongest peaks of Tip60 binding on the entire chromosome to the 5' region of the *cycB* gene, suggesting the regulation of *cycB* by Tip60 could be direct (Figure 4N) (Filion *et al.* 2010).

The transcriptional response to Tip60 inhibition

To get a global view of the gene expression changes caused by inhibition of Tip60, we performed RNA-seq on late L3 wings expressing *Tip60^{WT}* or *Tip60^{DN}*. We used *flipout^{TS}* to restrict expression of *Tip60^{WT}* or *Tip60^{DN}* to 48 hr or 78 hr prior to

Figure 3 Compromising NuA4 disrupts cell cycle progression. (A) Using heat-shock inducible *flipout-Gal4*, we generated nonoverlapping clones of cells coexpressing *Tip60^{DN}*, an inhibitor of apoptosis *P35*, and *GFP* at 0 hr APF. Pupa were aged to 36 hr APF and the average number of cells per clone was counted blind from a minimum of 100 clones per genotype. (B) Clones expressing *Tip60^{WT}* or *Tip60^{DN}* and *P35* were generated in the proliferating L3 wing for 30 hr, to measure cell DT. *Tip60^{DN}* expression lengthened cell doubling time by ~4 hr (*Tip60^{WT}*: 15 hr, *Tip60^{DN}*: 18.93 hr). (C) Clones expressing the indicated UAS-RNAi line and *P35* were induced for 72 hr and measured in the late L3 wing. (D) FRT-mediated mitotic recombination was used to generate *domino* null (*dom^{LO5537}*) mosaic wings (Tea and Luo, 2011). Sibling clones generated from the same recombination event (+/+ and -/-) were measured after 48 hr in the late L3 wing. A heterozygous deficiency for the proapoptotic *H99* locus (*H99^{+/-}*) could not rescue the small size of *dom^{-/-}* clones. (D') Flow cytometry was performed on late L3 wings to examine the DNA content of GFP-labeled *dom^{-/-}* cells (gray). Cells lacking *dom* (gray trace) exhibit an increased proportion of >2C DNA content (cells in S/G2/M phases) compared to *dom^{+/-}* and *dom^{+/+}* cells (black trace). (E–G) Flow cytometry was performed on late L3 wings with *en^{TS}*, UAS-*Tip60^{DN}*, or the indicated UAS-RNAi with GFP. (E) NuA4 inhibition increased the proportion of cells with >2C DNA content (green trace) compared to the non-Gal4 expressing cells (black trace). (F) Quantification of flow cytometry replicates are shown. NuA4 inhibition in the posterior wing (green bars, F) increases the >2C (S/G2/M) population compared to the posterior wing of control genotypes (gray bars, F). (G) NuA4 inhibition in the posterior wing also non-autonomously increased the proportion of anterior cells with 2C DNA content (blue bars G) compared to the anterior wing of control genotypes (gray bars, G). (H) RNAi to NuA4 subunits in S2R⁺ FUCCI cells (Zielke *et al.* 2014). Inhibition of NuA4 increased cell DT and the proportion of cells in S and G2, with the exception of *Brd8*. *P*-values were determined with an unpaired *t*-test; **P*-value < 0.05, ****P*-value < 0.001.

RNA extraction. *Tip60^{WT}* expression caused minimal changes in the transcriptome compared to control wings expressing Gal4/GFP alone (112–133 transcripts of the entire genome),

while *Tip60^{DN}* expression for 48 hr and 78 hr led to a largely overlapping program of 860 genes that significantly change expression >1.4-fold compared to *Tip60^{WT}*.

of IR response genes that are also upregulated upon *Tip60^{DN}* expression, encompassing both p53-dependent and p53-independent targets with Tip60 binding. (R–T) NuA4 inhibition leads to an accumulation of pH2Av foci in the absence of exogenous DNA damage. (1) Filion *et al.* (2010) (2) Xu *et al.* (2014).

Tip60 acts as a transcriptional coactivator with several factors, including nuclear hormone receptors, Myc and E2F (Brady *et al.* 1999; Frank *et al.* 2003; Taubert *et al.* 2004; Hattori *et al.* 2008). We were therefore surprised to find that the majority of genes with changed expression (72.5%) are upregulated when Tip60 HAT function is inhibited (Figure 4O). We observed few significant changes to core G2 cell cycle regulators, with the exception of a mild (1.4-fold) upregulation of *tribbles*, a mitotic inhibitor involved in String degradation (Mata *et al.* 2000; Seher and Leptin 2000). Also consistent with our RT-qPCR results, *cycB* (and *cycB3*) were mildly reduced (32–41%) at both time points. We examined the genes altered by *Tip60^{DN}* for overlap with Tip60 binding based on published Tip60 chromatin immunoprecipitation (ChIP)-seq and Tip60-DamID data (Filion *et al.* 2010; Xu *et al.* 2014). We found that 35% of upregulated and 33% of downregulated genes are also bound by Tip60 in *Drosophila* cells, suggesting our dataset includes targets of both direct and indirect Tip60 regulation.

We next compared the genes upregulated by *Tip60^{DN}* to transcriptional targets identified for the DREAM/MMB

complex (Georlette *et al.* 2007). Due to the known roles for Tip60/NuA4 in DNA damage signaling (reviewed by Squatrito *et al.* 2006; Sun *et al.* 2010; Gursay-Yuzugullu *et al.* 2015) we also included a comparison to a transcriptional response (both p53 dependent and p53 independent) to DNA damage by ionizing radiation (IR) in wings (van Bergeijk *et al.* 2012) (Figure 4P). We found a significant overlap with the genes upregulated by *Tip60^{DN}* expression and the DNA damage response (DDR) profile (104 of 634 genes, File S2), and a lesser overlap with the DREAM/MMB regulated genes (68 of 634 genes). The DNA damage response also partially overlaps with DREAM/MMB regulated genes, as several DNA repair and synthesis enzymes are regulated by DREAM, but only 24% (14 of 48) of the shared DREAM/DNA damage response genes are also significantly upregulated by *Tip60^{DN}* (File S3). Thus, the inhibition of Tip60/NuA4 results in the upregulation of >100 genes associated with a DNA damage response. These include the known direct targets of p53 such as *reaper*, *eiger*, and *Xrp1* (Brodsky *et al.* 2000; Akdemir *et al.* 2007; Link *et al.* 2013), as well as several

p53-independent targets such as *Gadd45*, *Snap25*, and *Traf4* (van Bergeijk *et al.* 2012). Figure 4Q shows a subset of targets, their regulation by p53 and DREAM, as well as whether they overlap with known locations of Tip60 binding. Importantly orthologs of several of these targets (e.g., *Snap25*, *Gadd45*, *Traf4*, *trbl*, and *eiger*) are also upregulated in mammalian cells in response to replication stress induced by aphidicolin treatment (Mazouzi *et al.* 2016).

Tip60/NuA4 may act to quench DNA damage signaling in *Drosophila* via its modification and replacement of phosphorylated H2A variant (pH2Av) with an unmodified variant (Kusch *et al.* 2004). Consistent with this, Tip60/NuA4 inhibition in the proliferating wing (using *en^{TS}*) caused a significant increase in pH2Av foci in the absence of any exogenous DNA damaging agents (Figure 4, R–T, Figure S4F). This suggests that Tip60/NuA4 is required to limit or prevent a DNA damage response due to some form of endogenous stress. To confirm a role for NuA4 in resolving pH2Av after DNA damage, we treated S2R⁺ cells with RNAi to Tip60 or E(Pc) and examined the resolution of pH2Av foci after 16 hr of recovery from a pulse of the DNA damaging agent camptothecin (CPT). Cells with reduced NuA4 exhibited a significant inability to resolve pH2Av foci compared to cells treated with a GFP control RNAi (Figure S4, G–I). Altogether, our data suggest that unresolved pH2Av, and/or possibly unrepaired DNA damage, contributes to the transcriptional response and cell cycle defects we observe when NuA4 is inhibited.

NuA4 inhibition does not delay cell cycle exit through the p53-dependent DNA damage response

To test whether DNA damage signaling could cause a delay in cell cycle exit, we overexpressed p53 in the posterior wing with *en^{TS}* starting at L3 and examined pupal wings for mitoses at 26–30 hr APF. Overexpression of p53 is sufficient to induce a compartment autonomous delay of cell cycle exit, with similar timing to NuA4 inhibition (Figure 5A). We next tested whether p53 is necessary for the cell cycle exit delay caused by loss of NuA4. We coexpressed a DNA-binding defective p53 (*p53^{DN}*) with *Tip60^{DN}* in the posterior wing (Brodsky *et al.* 2000) or expressed *Tip60^{DN}* or *Brd8^{RNAi}* in a p53 null background (*p53^{5A-1-4/11-1B-1}*) (Rong *et al.* 2002; Xie and Golic 2004). We verified that these p53 alleles were functional and blocked p53-dependent apoptosis in the wing (Figure S5, A–C), and loss of p53 does not affect the normal timing of cell cycle exit (Figure S5, D and E). However, despite the inhibition or loss of p53, the cell cycle exit delay caused by NuA4 inhibition persisted (Figure 5, B–D). Instead we observed a severe enhancement of wing defects and a reduction in wing size and animal survival, suggesting Tip60/NuA4 and p53 may be required in parallel for proliferation and survival in the absence of exogenous DNA damage (Figure 5E, Figure S5F). Loss of p53 did not prevent the reduction in CycB levels in the wing caused by NuA4 inhibition (Figure S5, G–I), and inhibition of p53 by *p53^{DN}* could not rescue the poor growth or survival of *dom^{-/-}* cells in the proliferating larval wing (Figure S5J). Knockdown of the

DDR kinases upstream of p53, *ATM* (*mei-41*), *chk1* (*grp*), and *ATR* (*tefu*), also did not suppress the delayed cell cycle exit caused by *Tip60^{DN}* expression. However similar to our results with p53, the posterior wing size was significantly reduced by coexpression of *ATM* and *ATR^{RNAi}* with *Tip60^{DN}*, creating notches in the wing blade (Figure S5, K–M). Because *ATM* and *ATR* can act redundantly in *Drosophila* (Bi *et al.* 2005; Joyce *et al.* 2011; Kondo and Perrimon 2011), we also attempted to simultaneously knock down *ATM* with expression of *Tip60^{DN}* in an *ATR* mutant background. However we were unable to recover any animals of the correct genotype, suggesting possible synthetic lethality. Finally, blocking apoptosis, the most downstream component of DDR signaling, also could not suppress the delayed cell cycle exit caused by NuA4 inhibition (Figure S5, N and O). This suggests that the effects of NuA4 on cell cycle progression act independent of, but in parallel to, components of the canonical DDR pathway.

NuA4 inhibition does not delay cell cycle exit through altered E2F/DP function

Tip60 and NuA4 subunits genetically interact with components of a repressive RB/E2F/DP DREAM-like complex in *Caenorhabditis elegans*, while in *Drosophila* cell culture, specific NuA4 components have been shown to repress E2F target gene expression (Ceol and Horvitz 2004; Lu *et al.* 2007). Our results *in vivo* suggest that the effects of the NuA4 complex on cell cycle genes are complicated, as we see context-dependent evidence of both activating (e.g., *cycB* and *e2f1*) and repressive (e.g., *pcna*, *stg*, *mad2*, and *tribbles*) effects on cell cycle gene expression. Tip60 physically interacts with the E2F dimerization partner DP as assayed by co-immunoprecipitation (Figure S6A), consistent with the reported interactions between Dom and E2F in both *Drosophila* and mammalian cells (Lu *et al.* 2007). We therefore examined whether E2F/DP function could be required for the delayed cell cycle exit caused by Tip60/NuA4 inhibition.

We used a severe *Dp* hypomorphic mutation over a null allele (*Dp^{a1/a4}*) to obtain pupal wings with normal developmental timing (Royzman *et al.* 1997). The strong loss of *Dp* function neither suppressed nor enhanced the delay of cell cycle exit caused by *Tip60^{DN}* in the pupa wing (Figure 6, A–C). The loss of *Dp* also did not suppress the S/G2 cell cycle phase elongation in the larval wing or alter the reduction in CycB caused by Tip60 inhibition (Figure S6, B–D). In contrast, the loss of *Dp* enhanced a phenotype of ectopic Elav⁺ neurons at the posterior wing margin (PWM) caused by *Tip60^{DN}* or inhibition of another NuA4 subunit Brd8 (Figure 6, D–H and Figure S6E). This is a phenotype previously associated with the loss of repressive components of the DREAM/MMB complex, which is thought to be independent of the cell cycle functions of DREAM/MMB (Rovani *et al.* 2012). The loss of *Dp* also enhanced the severity of defects caused by *Tip60^{DN}* in adult wings (Figure S6F), and the loss of another DREAM/MMB component, *Rbf*, is synthetically cell lethal with *Brd8^{RNAi}* in pupal eyes and wings (Figure S6G). Altogether our data suggest that while NuA4 and

Figure 5 NuA4 inhibition does not delay cell cycle exit through p53. Pupa expressing the indicated transgenes in the posterior wing (*en-GAL4/UAS*) were collected at 0 hr APF, aged to 25–28 hr APF, and stained for mitoses using PH3. The dashed line indicates the anterior/posterior boundary. (A) Overexpression of *p53^{WT}* was sufficient to delay cell cycle exit similar to NuA4 inhibition in the posterior wing (compare to Figure 1). (B–D) Overexpression of a p53 allele that cannot bind DNA (*p53^{DN}*) or p53 null alleles (*p53^{5A-1-4/11-1B-1}*) did not prevent the delay of cell cycle exit caused by NuA4 inhibition in the posterior wing. Bar = 100um (E) Loss of p53 strongly enhanced the number of adult wing defects, suggesting loss of NuA4 function and p53 cooperate to increase genetic instability (The full details of the adult wing defect scoring are described in *Materials and Methods*).

DREAM/MMB genetically interact in some contexts, the impacts of NuA4 loss on cell cycle progression and cell cycle exit appear to be independent of E2F/DP function.

Delaying the final G2/M transition impacts the proper repression of cell cycle genes at cell cycle exit

NuA4 inhibition elongates late S/early G2 phases (Figure 3) and reduces the expression of a major G2/M regulator CycB in proliferating tissues (Figure 4). We therefore directly examined whether slowing the final cell cycle in the pupal wing could recapitulate the cell cycle exit defects we observe when NuA4 is inhibited. We used *en^{TS}* to prolong the final G2/M transition, through the expression of the Cdk1 inhibitory kinase Wee, or an RNAi to Cdk1. Prolonging the final G2/M effectively delayed cell cycle exit with timing similar to that observed when we compromise NuA4 function (up to 30 hr APF, Figure 7, A and B), while prolonging the final cell cycle with an RNAi to *e2f1* or the expression of a dominantly active Rbf, *Rbf²⁸⁰*, did not perturb the overall timing of cell cycle exit (Figure S7, A–D). Other genetic conditions known to prolong

the cell cycle such as generating Minute^{+/-} clones or slowly dividing *Dp* null mutant clones also did not detectably delay cell cycle exit in the pupal wing (Martin and Morata 2006; Sun and Buttitta 2015). This demonstrates that simply slowing cell cycles during the proliferative phases or slowing the final G1/S does not delay cell cycle exit in the wing.

What, then is the biological consequence of slowing the final G2/M? We find that delaying the final mitosis results in a failure to properly shut down the expression of cell cycle genes in the pupal wing. Elongation of the final G2 phase via expression of Wee kinase or Cdk1 inhibition led to ectopic PCNA-GFP and Mad2-GFP expression for several hours after they are normally repressed (PCNA-GFP shown, Figure 7, C–D). This was striking in the case of *cdk1^{RNAi}*, where expression led to an early arrest of cell proliferation in the posterior wing, but with ubiquitously high PCNA-GFP expression that persisted for several hours beyond the transition to a post-mitotic state (Figure 7D). Why does an elongation of the final G2/M transition lead to aberrant expression of cell cycle genes? The lengthening of either gap phase in the proliferating larval wing results in an accumulation of E2F1 activity that drives a compensatory shortening of the next gap phase to maintain the overall cell doubling time. This is termed “cell cycle compensation” and is dependent upon the negative regulation of E2F1 activity by Cyclin/Cdks (Reis and Edgar 2004). This creates a situation where G2 elongation, for example by Wee expression, results in increased E2F transcriptional activity that can be detected by the PCNA-GFP reporter (Figure S7E). Our results suggest that cell cycle compensation also occurs during the final cell cycle in the pupal wing, but due to the absence of a subsequent cell cycle, this results in aberrant E2F activity and cell cycle gene expression in tissues entering into a postmitotic state. Based on these results, we suggest that the aberrant cell cycle gene expression and derepression of E2F targets observed when NuA4 is inhibited may be due in part to the S/G2 delay and subsequent activation of the cell cycle compensation mechanism.

We also observed additional consequences of altering the final G2 phase on cell fate at the posterior wing margin. A developmentally regulated G2 arrest is essential for proper cell fate decisions in sensory organ precursors (SOPs), as disruption of proper Cdk1 activity in SOPs can alter cell division timing and asymmetric cell fate decisions (Fichelson and Gho 2004; Ayeni *et al.* 2016). Consistent with this, we observed that inhibition of Cdk1 activity was sufficient to fully recapitulate the ectopic neuron phenotype in the posterior wing margin, similar to that observed when components of the DREAM or the Myb component of MMB are inhibited (Rovani *et al.* 2012) (Figures 6F and Figure 7, E and F). We suggest this phenotype is similar to that shown previously in SOPs of the notum where a delay in G2 caused a cell fate change in the pI SOP to the pIIb fate, which produces a neuron and sheath cell (Fichelson and Gho 2004). Importantly, activation of the DDR pathway via p53 expression in the posterior wing is also sufficient to recapitulate this phenotype (Figure 7G), suggesting that multiple pathways

Figure 6 NuA4 affects cell cycle exit independent of DP function. (A) *Dp^{a1/a4}* wings with *en-Gal4/UAS-GFP* exhibit normal cell cycle exit. (B and C) Expression of *Tip60^{DN}* in the posterior wing delays cell cycle exit (as shown by PH3) in *Dp^{a1/+}* and *Dp^{a1/a4}* wings. All wings shown are at 27 hr APF. (D) *Tip60^{DN}* expression in the posterior pupal wing leads to ectopic neurons (as indicated by Elav staining, arrowheads) at the posterior wing margin (indicated by the dashed line). (E) *Brd8^{-/-}* (*Brd8^{G19099}*) clones in the pupal wing often contain ectopic neurons at the posterior wing margin (clone boundaries are indicated by dotted lines). (F and G) *Dp* mutant wings (*Dp^{a1/a4}*) occasionally exhibit ectopic neurons at the posterior wing margin, which becomes strongly enhanced when *Tip60^{DN}* is expressed in the posterior wing using *en-Gal4/UAS*. (H) Quantification of wings with ectopic posterior neurons in the indicated genetic backgrounds.

impacting timely S/G2 progression converge on this phenotype. Altogether our results suggest that the Tip60/NuA4 complex is required for proper S/G2 progression, likely through its roles in modulating CycB levels and p21^{Av} removal. This impacts proper cell cycle exit, cell identity in the posterior wing, and as described further below, is essential for genetic stability (Figure 7H).

Promoting G2 progression when NuA4 is inhibited leads to aberrant mitoses and genetic instability

A reduction in CycB occurs within 24 hr of *Tip60^{DN}* expression in proliferating wings. To determine whether increasing CycB/Cdk1 activity could suppress the G2 elongation and proliferation defects caused by Tip60/NuA4 inhibition, we co-overexpressed String to increase the activity of the remaining CycB/Cdk1. In L3 wings, coexpression of *stg* with *Tip60^{DN}* effectively suppressed the effect of *Tip60^{DN}* on increased S and G2 populations (Figure 8A). However, when we examined the pupal wing, we found that the delay in cell cycle exit persisted even in the presence of high levels of Stg (Figure 8, B–D). Closer examination of the PH3 staining in wings coexpressing *Tip60^{DN}* and *stg* revealed many mitotic defects, including defects in chromosome alignment, condensation, anaphase bridges, and missegregated chromosomes,

defects we do not observe in wings expressing *Tip60^{DN}* alone (Figure 8, E and F). Inhibition of Tip60/NuA4 function leads to defects in cell cycle progression that elongate the late S/early G2 phases of the cycle. This elongation may occur in part through a reduction in CycB expression and activation of a DNA damage response, which effectively increases the chances for repair prior to mitosis. When cells with compromised NuA4 are forced into mitosis without delay by high CycB/Cdk1 activity, severe mitotic defects occur ultimately resulting in defects in the adult wing (Figure 8G). Thus NuA4 plays an essential role in S/G2 progression to promote genetic stability.

Discussion

Here we show that compromising the Tip60/NuA4 complex *in vivo* leads to a delay of cell cycle exit and ectopic cell cycle gene expression in terminally differentiating cells. NuA4 components physically and genetically interact with DREAM components and can impact specific targets of RB/E2F repression (Lu *et al.* 2007). However, we find that other essential roles for NuA4 in promoting S- and G2-phase progression lead to slower cycling and confounding indirect effects on E2F cell cycle targets *in vivo* when NuA4 is compromised.

Figure 7 Prolonging the final G2 delays cell cycle exit, derepresses cell cycle genes, and causes ectopic neurons at the posterior wing margin. (A and B) Using *en^{TS}* the indicated transgenes were expressed in the posterior wing. Inhibition of Cdk1 activity by expression of *wee* or *cdk1* RNAi in the posterior wing delays the timing of cell cycle exit similar to loss of NuA4. (C and D) Using *en-GAL4/UAS* inhibition of Cdk1 activity in the posterior wing leads to ectopic E2F activity similar to inhibition of NuA4. (E and F) Inhibition of Cdk1 activity leads to Elav⁺ ectopic neurons in the posterior wing margin. (G) Overexpression of p53 (*p53^{WT}*) also led to Elav⁺ ectopic neurons in the posterior wing margin. Bar = 100μm. (H) Schematic of the Tip60/NuA4 functions described in our study.

When NuA4 is inhibited, cells accumulate pH2Av and exhibit transcriptional changes associated with a DNA damage response. In addition, E2F1 and CycB levels are reduced when NuA4 is inhibited. This leads to an elongation of the cell cycle, coupled with derepression of specific RB/E2F targets, resulting in a paradoxical cell cycle gene expression program where certain cell cycle genes become highly expressed in cells that are exiting the cell cycle. We suggest that NuA4 acts as an essential component to coordinate proper pH2Av removal with S/G2 phase completion prior to mitotic entry, in addition to its other roles in modulating chromatin for gene expression.

The Tip60/NuA4 complex and cell cycle gene expression

NuA4 components directly localize to the promoters of many cell cycle genes in several organisms, and most often Tip60/NuA4 is thought to promote cell cycle gene expression via HAT activity (Li *et al.* 2004; Taubert *et al.* 2004; Tapias *et al.* 2014). We show that the Tip60/NuA4 complex in *Drosophila* impacts cell cycle gene expression in complex ways. Tip60/NuA4 is required to prevent the expression of a cluster of DNA damage response and specific cell cycle genes, but is also required for the normal expression of *cycB* (Figure 4, Figure S4). Tip60/NuA4 binds to the *cycB* promoter and 5' intron, suggesting that *cycB* could be directly regulated by NuA4 (Filion *et al.* 2010). Interestingly, *cycB* is located in the genome as a divergently paired gene (DPG) (Yang and Yu 2009) with *stall*, which is upregulated 6.5-fold upon

Tip60 inhibition. Tip60/NuA4 may therefore act to direct the proper transcription of certain DPGs with differential expression patterns. Consistent with this idea, we noted that 18 of the 20 strongest Tip60 binding peaks in the genome (Filion *et al.* 2010) are associated with DPGs.

While specific components of NuA4 have been shown to repress certain E2F targets in cell culture, a repressive function for the Tip60 subunit on expression of many other genes was unexpected, as HAT activity is usually associated with increased gene expression. Some of the effects we observe on gene expression are indirect, likely due to an inability to remove pH2Av from chromatin and resolve DNA damage signaling (Figure 4, Figure S4). However, Tip60 HAT activity may also act on chromatin to directly repress target genes, as 35% of genes upregulated by Tip60^{DN} in the *Drosophila* wing have Tip60 binding sites (Filion *et al.* 2010; Xu *et al.* 2014). Tip60/NuA4 has been shown to catalyze histone exchange to incorporate the H2A variant H2AZ in yeast (Auger *et al.* 2008; Altaf *et al.* 2010), and the H2A variant in *Drosophila* H2Av, has been associated with gene repression (Swaminathan *et al.* 2005; Qi *et al.* 2006; Hanai *et al.* 2008). Recent work in *C. elegans* has revealed that target genes of RB/E2F repression, including cell cycle genes bound by the DREAM complex, exhibit deposition of the H2Av homolog H2A.Z, along the gene body (Latorre *et al.* 2015). H2A.Z localization on the gene body is associated with repression as opposed to H2A variant localization at promoters, which is associated with transcriptional activation (Barski *et al.* 2007; Hardy *et al.* 2009), and loss of H2A.Z at the majority of DREAM-bound genes in *C. elegans* derepresses their expression. Thus the function of NuA4 in H2Av incorporation could act to directly repress gene expression. While we did not observe large changes in expression of most *Drosophila* DREAM targets in our RNA-seq data (File S4), compromising Tip60/NuA4 function may prevent proper H2Av deposition at the 17% of genes affected by Tip60^{DN} that overlap with *Drosophila* DREAM-regulated genes. Consistent with this idea, we found that 60% of the genes upregulated by Tip60 inhibition that are shared with DREAM targets exhibit significant H2Av localization on the gene body, including *pcna* and *mad2* (modencode.org, dataset no. 4953).

Elongation of G2 phase can lead to an upregulation of E2F activity and activation of the E2F transcriptional reporter PCNA-GFP (Figure 7, Figure S7). This is due to cell cycle compensation, where E2F1 protein accumulates during the G2 delay, resulting in an increased E2F transcriptional output (Reis and Edgar 2004). When NuA4 is inhibited, we observe hallmarks of increased E2F1 activity and cell cycle delay, but without an accumulation of E2F1. Instead we observe a reduction in E2F1 protein and transcript levels consistent with a delay in late S/early G2 phase when E2F1 is degraded (Shibutani *et al.* 2008) (Figure 2, Figure 4). This suggests that either NuA4 inhibition acts on the cell cycle reporters in an E2F-independent manner, possibly by the H2Av mechanism described above, or that the reduced levels of E2F1 may somehow have increased activity when NuA4 is

Figure 8 Accelerating G2/M when NuA4 is inhibited leads to severe mitotic defects. (A–D) Using *en*^{TS} the indicated transgenes were expressed in wings starting at L2. (A) Cell cycle phasing was examined by flow cytometry at the late L3 stage. *Tip60*^{DN} expression increases the proportion of cells in S/G2 compared to *Tip60*^{WT} but coexpression of *stg* with *Tip60*^{DN} forces cells through G2/M, similar to *stg* expression alone. (B–D) Pupae were collected at 0 hr APF and aged to 26–28 hr APF at the permissive temperature for transgene expression. (B) *stg* expression does not alter normal cell cycle exit, (C) while *Tip60*^{DN} expression delayed cell cycle exit as observed by ectopic PH3 staining. (D) Coexpression of *stg* with *Tip60*^{DN} did not rescue the delayed cell cycle exit, but defects in mitosis occurred when cells with inhibited NuA4 were forced through G2. (E) Delayed mitoses in *Tip60*^{DN}-expressing wings

appeared relatively normal in prometaphase, metaphase, and telophase. (F) By contrast, many defective mitoses were observed in wings coexpressing *stg* and *Tip60*^{DN}, including anaphase bridges (arrowhead) and improperly segregated DNA. (G) Coexpression of *stg* with *Tip60*^{DN} increased the number of defects observed in the adult wing. *P*-values were determined with an unpaired *t*-test; ***P*-value <0.01, ****P*-value <0.001.

inhibited. E2F1 activity can be increased through reduced levels of the repressor Rbf or increased phosphorylation of Rbf by Cyclin/Cdks. We examined whether there were higher levels of G1 and S Cyclins CycE and CycA when NuA4 was inhibited (Figure S4, CycE data not shown) but did not observe any significant differences from controls. We also did not see significant changes in expression of other direct or indirect negative E2F1 regulators including *ago*, *dacapo*, *e2f2*, *Rbf*, *Rbf2*, *cycD*, or *cdk4* in our RNA-seq data. Because we could not ultimately rule out the possibility of a change in Rbf phosphorylation or protein levels, we genetically eliminated *Rbf* in cells expressing *Brd8*^{RNAi}. We found that this combination was strongly synthetically cell lethal, suggesting the two pathways may act independently but in parallel (Figure S6G).

The Tip60/NuA4 complex and DNA damage signaling

Tip60/NuA4 plays multiple roles, both positive and negative, at several levels within the DNA damage response and repair pathway. Tip60 can acetylate the ATM kinase to promote its activation and phosphorylation of targets in response to DNA damage (Sun *et al.* 2005; Sun *et al.* 2007). Tip60 also acetylates p53 protein to modulate its activity (Tang *et al.* 2006), as well as acts with p53 at the chromatin to promote expression of target genes (Legube *et al.* 2004). We find that inhibition of Tip60/NuA4 leads to high levels of pH2Av, a hallmark of high ATM/ATR kinase activity, as well as activation of several direct transcriptional targets of p53 (Figure 4). This demonstrates that activating ATM and p53 target expression are not the key roles for Tip60/NuA4 *in vivo* in *Drosophila*. Instead, our results are consistent with an essential role for the Tip60/NuA4 complex in acetylating and

exchanging phospho-H2Av for H2Av to resolve DNA damage signaling (Kusch *et al.* 2004) (Figure 4, Figure S4). We show that Tip60/NuA4 inhibition or loss leads to an accumulation of pH2Av, possibly in response to endogenous DNA damage. We believe this is why loss of Tip60/NuA4 leads to ectopic activation of DNA damage response genes encompassing both a p53-dependent and p53-independent response (Figure 4), as well as enhances the loss-of-function phenotypes for multiple components of the DNA damage response pathway (Figure 5). We suggest a delayed progression through S/G2 becomes essential when NuA4 is inhibited, to allow for pH2Av removal or DNA/chromatin repair. This is demonstrated by the severe mitotic defects and genetic instability that occur when NuA4 is compromised but the cell cycle delay is bypassed (Figure 8).

NuA4 and cancer

Previous studies have paradoxically characterized NuA4 as both a tumor suppressor and an oncogene (Gorini *et al.* 2007; Judes *et al.* 2015). Maintenance of proper cell cycle gene expression may in part underlie some roles for NuA4 in cancer, while others may be due to its roles in DNA damage signaling (Squattrito *et al.* 2006). Our data suggest Tip60/NuA4 may behave as a tumor suppressor by ensuring genetic stability (Figure 4, Figure 8). However, we also observe cell cycle gene upregulation when NuA4 is compromised (Figure 3). While the increased cell cycle gene expression we observe upon NuA4 inhibition was insufficient to cause extra or accelerated cell cycling in our experiments, additional hits (which may be caused by increased genetic instability) could cooperate with compromised NuA4 to further deregulate E2F targets or bypass the slowing of the cell cycle due to the late

S/G2 phase elongation. This is of concern for the development and use of Tip60 HAT and bromodomain inhibitors in cancer therapy (Gao *et al.* 2014; Sanchez *et al.* 2014), which could cause further genetic instability and impair NuA4-dependent cell cycle gene repression and cell cycle exit.

NuA4 inhibitors may be highly effective in some contexts, however. Inhibition of Brd8 by RNAi in *Rbf* null cells strongly enhances their elimination in pupal eyes and wings, even more strongly than a combination previously shown to completely bypass cell cycle exit (Figure S6G) (Buttitta *et al.* 2010). This is reminiscent of other *Rbf* synthetic lethal genes such as TSC1 and -2 that lead to a lethal accumulation of DNA damage when *Rbf* is lost, which can effectively eliminate RB mutant cancer cells (Li *et al.* 2010; Gordon *et al.* 2013). This suggests NuA4 inhibitors could be highly effective for elimination of cancer cells that have lost or inactivated RB.

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Supporting Information

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Roles for the Histone Modifying and Exchange Complex NuA4 in Cell Cycle Progression in *Drosophila melanogaster*

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Fig S1. Compromising NuA4 delays the timing of the final cell cycle

(A-C, E, I) Using *en^{TS}*, *Tip60^{DN}* or the indicated RNAis were expressed in the posterior wing from the second larval instar. Pupa were collected at 0hr after puparium formation (APF) and aged to 27h APF at the permissive temperature for Gal4-induced transgene expression. The dashed line indicates the anterior/posterior boundary. (A) *Tip60^{RNAi}* in the posterior wing recapitulates the delayed cell cycle exit phenotype of *Tip60^{DN}* (compare to Fig. 1D, mitoses indicated by PH3). (B) *Tip60^{DN}* expression compromises endogenous H4 HAT activity as indicated by an antibody for H4K5/8/12/16 acetylation. (C, D) Using heat-shock induced flipase (*hs-flp*) with a *tub>stop>Gal4* transgene combined with *Gal80^{TS}*, (*flipout^{TS}*) we generated clones of cells expressing Gal4 beginning at 24 h after embryo hatching, but restricted the UAS-induced expression of *Tip60^{DN}* to stages after 0h APF using temperature shifts to limit cell death (the clonal border and wing anterior-posterior boundary are respectively indicated by the dotted and dashed lines). In pupa staged to 26h APF, clonal *Tip60^{DN}* expression delays cell cycle exit (as indicated by PH3) in a manner similar to that observed with *en^{TS}*. (C, E) Using *en^{TS}* we restricted *Tip60^{DN}* expression in the posterior wing to the final cell cycle in the wing (from 0h APF). Inhibition of Tip60 during the final cell cycle delayed cell cycle exit. (F-H) Inhibition of Tip60/NuA4 during the final cell cycle in the eye was performed using *GMR-GAL4/UAS*. Inhibition of Tip60/NuA4 delayed cell cycle exit in the pupal eye, as indicated by persistent mitoses and S phases (indicated by EdU labeling) in interommatidial cells 3h after normal cell cycle exit. (I) RNAi of CBP does not delay cell cycle exit, despite reducing H4 acetylation. p-values were determined with an unpaired t-test, * p-value<0.05, ** p-value<0.01.

Fig S3. NuA4 is needed for proper proliferation

(A) Cell size (as measured by Forward Scatter in flow cytometry) is not significantly altered by RNAi to NuA4 components (compare to cell cycle alterations in Fig. 3E). *Brd8*^{RNAi} mildly increases cell size, likely due to the increased proportion of cells in G2 in the wing. (B-D) FRT-mediated mitotic recombination was used to generate *domino* null (*dom*^{LL05537}, *dom*^{-/-}) clones in the proliferating L3 wing. (C) Heterozygosity for the pro-apoptotic *H99* locus (*H99*^{+/-}) increased the recovery of small lacZ-negative *dom*^{-/-} clones, but did not rescue their poor growth. (D) LacZ-negative *dom*^{-/-} cells do cycle, as indicated by EdU labeling (arrowheads). (E) RNAi to *Tip60* and *domino* in S2R+ cells decreased EdU incorporation (provided in a 30 min. pulse). However, consistent with the results shown in Fig. 3H, RNAi to *Brd8* did not compromise proliferation in S2R+ cells, as it does *in vivo*. (F) Cell proliferation is reduced for S2R+ cells exposed to RNAi for *Tip60* and *E(Pc)* (presented as % increase from day 4). RNAi to GFP did not affect cell proliferation. (G) An EdU pulse-chase experiment was performed in dsRNA treated S2R+ cells to measure the duration of G2. Cells were labeled with EdU for 1hr and DNA content of EdU+ cells was quantified by flow cytometry for the indicated timepoints following EdU removal. Cells exposed to RNAi to *Tip60*, *E(Pc)* or *dom* and labeled with EdU during the pulse, accumulated in G2 for 6-9 hours after the pulse. Vehicle indicates a mock RNAi treatment without any dsRNA added.

Fig S4. Loss of NuA4 function alters expression of specific cell cycle genes

(A,C-F) Using *en-Gal4/UAS* the indicated transgenes were expressed in the posterior larval wing. The dashed line indicates the anterior/posterior boundary (left side is posterior). (A) CycB is reduced 15-20% in the posterior wing when NuA4 is inhibited (n=5 discs). (B) Using *flipout-Gal4* in combination with *Gal80^{TS}* (*flipout^{TS}*) we generated large, overlapping clones of cells expressing Gal4 24h after embryo hatching, but restricted the UAS-induced expression of *Tip60^{DN}* or *Tip60^{WT}* to 24h or 48h prior dissection at wandering L3 larval stages using temperature shifts. We confirmed by quantitative RT-PCR that *Tip60^{DN}* expression in L3 wings caused aberrant mRNA expression of *pcna* and reduction of *cycB*, compared to the *Tip60^{WT}* expressing controls. (C-E) Levels of CycA were not significantly affected by NuA4 inhibition compared to controls expressing *white^{RNAi}*, which exhibited no cell cycle phenotypes in any of our FACS assays. (F) Inhibition of Tip60 HAT activity or expression of *Brd8^{RNAi}* led to formation of phospho-H2Av foci in L3 wings in the absence of exogenous DNA damaging agents. (G-I) Inhibition of NuA4 in S2R+ cell culture by dsRNA to *Tip60* and *E(Pc)* decreased the cells' ability to recover from the DNA damage inducing agent Camptothecin (CPT). NuA4 inhibition resulted in more pH2Av+ cells compared to the *GFP^{RNAi}* control. p-values were determined by an unpaired t-test, *p-value<0.05 *** p-value<0.001.

Fig S5. NuA4 inhibition delays cell cycle exit independent of several DDR components

Using *en-GAL4/UAS* the indicated transgenes were expressed in the posterior wing. The dashed line indicates the anterior/posterior boundary (left is posterior). (A-C) *p53*-dependent apoptosis (as indicated by staining for the cleaved caspase, Dcp1) induced by 40 Grays of irradiation is substantially reduced by *en-Gal4* expression of a *p53* allele that cannot bind DNA (*p53^{DN}*) or in a *p53* null background (*p53^{5A-1-4/11-1B-1}*) compared to the *w¹¹¹⁸* control. (D,E, K-O) Pupa expressing the indicated transgenes in the posterior wing were collected at 0hr APF, aged to 25-28h APF, and stained for mitoses using PH3. (D, E) Loss of *p53* or expression of *p53^{DN}* in the posterior wing, does not alter cell cycle exit in the pupal stages nor does it reduce the size of the posterior wing (27h APF shown). (F) Loss of *p53* severely enhances larval and pupal lethality when combined with NuA4 loss of function transgenes driven by *en-Gal4*. Numbers indicate eclosed adults for each genotype. (G-I) *p53^{-/-}* mutant larvae expressing RFP only, or RFP with *Tip60^{DN}* or *Brd8^{RNAi}* in the posterior L3 larval wing were stained for CycB protein. Loss of *p53* does not rescue the reduction in CycB levels. (J) *en-Gal4/UAS* driven expression of *p53^{DN}* in the posterior larval wing cannot rescue the small size of LacZ negative *dom^{-/-}* clones. (K-O) Pupa expressing the indicated transgenes under control of *en^{TS}* in the posterior wing were collected at 0hr APF, aged to 25-28h APF, and stained for mitoses using PH3. (K-M) RNAi knockdown of the DDR kinases *tefu/ATM*, *grp/chk1*, and *mei-41/ATR* in combination with *Tip60^{DN}* expression could not prevent the cell cycle exit delay in the posterior wing caused by inhibition of NuA4. (N, O) Blocking apoptosis by co-expression of P35 did not prevent the cell cycle exit delay when NuA4 is inhibited in the posterior wing.

Fig S6. NuA4 and DREAM genetically and physically interact

(A) FLAG-tagged Tip60 co-immunoprecipitates endogenous Dp (arrowhead) in S2 cells. Controls were transfected with empty vector. (B) Using *en*-Gal4/UAS, either GFP alone, or GFP+Tip60^{DN} was expressed in *Dp^{a1/a4}* mutant larval wings and cell cycle phasing was examined by flow cytometry. *Dp^{a1/a4}* mutant wings show an increase in the G2/M population with GFP expression alone, but Tip60^{DN} expression in *Dp^{a1/a4}* mutants further increases the proportion of cells in late S/early G2. (C,D) *Dp^{a1/a4}* mutant larvae expressing GFP only, or GFP with Tip60^{DN} in the posterior L3 larval wing were stained for CycB protein. The dashed line indicates the anterior/posterior boundary (left side is posterior). (E) Overexpression of Tip60 in the posterior wing (*en*-Gal4/UAS-*Tip60^{WT}*) does not cause ectopic posterior wing margin neurons in the pupal wing at 27hAPF as indicated by Elav staining (this is a control for Fig. 6D). (F) Loss of *Dp* enhances the adult wing defects caused by expression of *en*-Gal4/UAS-*Tip60^{DN}*. The adult wing defect scoring system is described in the Methods. (G) Loss of NuA4 function enhances the lethality of *Rbf* loss in pupal clones, to a degree even more severe than that caused by a genetic combination causing a complete bypass of cell cycle exit, *Rbf^{-/-}; CycE/Stg* (BUTTITTA *et al.* 2010).

Fig. S7 Slowing the final cell cycle does not delay cell cycle exit

Using *en^{TS}* the indicated transgenes were expressed in the posterior wing. The dashed line indicates the anterior/posterior boundary. (A, B) Slowing the cell cycle with RNAi to *e2f1* or expression of a constitutively repressive Rbf, *Rbf²⁸⁰*, in the posterior wing did not alter the timing of cell cycle exit. (C) By contrast, co-expression of *Rbf²⁸⁰* with *wee* delayed the timing of cell cycle exit in a manner similar to *wee* alone. (D) RNAi to *e2f1* in the L3 posterior wing (left) effectively reduced E2F activity as shown by PCNA-GFP. (E) By contrast, expression of *wee* in the L3 posterior wing increased PCNA-GFP, similar to inhibition of NuA4 (compare to Fig. 4A-E).

File S1. Supplemental Materials and Methods:

Drosophila stocks and genetics:

Inhibition of NuA4 in the posterior wing:

w/y, w, hs-FLP; en-GAL4, UAS-GFP; UAS-Tip60^{WT or DN} or UAS-NuA4^{RNAi}/ tub-gal80^{TS}

w/y, w, hs-FLP; en-GAL4, UAS-GFP/+; UAS-P35/ UAS-Tip60^{DN} UAS-NuA4^{RNAi}

Inhibition of NuA4 during the final cell cycle in the eye:

w/y, w, hs-FLP; GMR-GAL4/+; UAS-Tip60^{WT or DN} or UAS-NuA4^{RNAi}/ PCNA-GFP

Clonal lineage analysis:

w/ y, w, hs-FLP; UAS-P35/+; UAS-Tip60^{WT or DN} or UAS-NuA4^{RNAi} /act>CD2>GAL4, UAS-GFP^{NLS}

Clones to assess terminal differentiation in the eye and qRT-PCR

w/ y, w, hs-FLP; tub>CD2>GAL4, UAS-GFP/+; tub-gal80^{TS}, UAS-dIAP/ UAS-Tip60^{DN} or UAS-NuA4^{RNAi}

Fluorescent reporters and inhibition of NuA4

w/ y, w, hs-FLP; en-GAL4, UAS-His2Av::RFP/+; mad2-GFP, mRFP-Nup107/ UAS-NuA4^{transgene}

y, w/ y, w, hs-FLP/+; PCNA-GFP, act>CD2>GAL4/ UAS-NuA4^{transgene}

y, w, hs-FLP/+; stg-GFP^{YD0246}, act>CD2>GAL4, UAS-His2Av::RFP/ UAS-NuA4^{transgene}

RNAi and over-expression lines (also refer to Table 1)

y, w, hs-FLP/+; UAS-Tip60^{E431Q}B/Tm6B

y, w, hs-FLP; UAS-Tip60^{WT}/CyO-GFP; +

y, w, hs-FLP; UAS-CycD, UAS-cdk4/CyO-GFP; +

UAS-Wee (NEUFELD et al. 1998), BDSC 28368 *UAS-Cdk1^{RNAi} UAS-Stg* (on II) (NEUFELD et al. 1998), BDSC 6583: *w¹¹¹⁸;+; PGUS-p53.Ct3.1/TM6B, Tb¹*, BDSC 6584: *w¹¹¹⁸; PGUS-p53.2.1*, BDSC 27277: *y, v;+; UAS-grp^{RNAi}*, BDSC 31635: *y, v;+; UAS-atm^{RNAi}*

Mutant alleles:

dom^{LL05537} (TEA AND LUO 2011) *Dp^{a1}* (ROYZMAN et al. 1997) *Dp^{a4}* (FROLOV et al. 2005)

p53^{5A-1-4/11-1B-1} (RONG et al. 2002; XIE AND GOLIC 2004) *Brd8^{G19099}* (BDSC#31838)

Antibodies and reagents:

Anti-phospho-Ser10 histone H3: 1:4000 rabbit (Millipore #06-570) or 1:1000 mouse (Cell Signaling #9706), Anti-GFP: 1:1000 chicken (Life Technologies A10262) or 1:1000 rabbit (Life Technologies A11122), Anti-Elav: 1:100 rat (DSHB 7E8A10), Anti-Cut: 1:100 mouse (DSHB 2B10). Anti- CycB 1:100 (DSHB, F2F5) Anti-CycA 1:100 (DSHB, A12), Anti-pH2Av 1:100 (DSHB, UNC93-5.2.1) Anti-DP rabbit (1:1,000, kindly provided by Dr. M. Frolov) mouse anti-FLAG (1:1,000, Sigma). Secondary antibodies to the appropriate animals were labeled with Alexa fluor 488, 568 or 633 (Invitrogen) and used at 1:2,000. Co-immunoprecipitation was performed as described (SUN AND BUTTITTA 2015) using 3X10⁶ S2R+ cells transiently transfected with pMT-Tip60^{FLAG}. Mouse anti-FLAG-agarose (Sigma) was used to immunoprecipitate endogenous DP which was detected by western blot with Rabbit anti-DP.

Flow cytometry, EdU labeling and Camptothecin treatment: For flow cytometry cells were stained with DyeCycleViolet (for samples run live in Fig.3) or FX Cycle Violet for fixed cells (Supp.Fig.3) and were run on the Attune flow cytometer (Life Technologies). EdU incorporation was performed on S2R+ cells (without FUCCI reporters) and detected using reagents from the Click-IT EdU 488 flow cytometry kit (Life Technologies). For Camptothecin (CPT) treatment, S2R+ cells were treated with the indicated dsRNA for 3 days. On the 4th day 10 μ M CPT was added for 6 hours, cells were washed and allowed to recover in fresh media without CPT for 16h. Cells were subsequently fixed with 4% paraformaldehyde/PBS and stained for pH2Av.

Supplemental Legends: Descriptions for additional Supplemental Files 2-5.

File S2. Supplemental Table 1. Genes induced by *Tip60^{DN}* and the DNA damage response

Transcriptional program induced by *Tip60^{DN}* expression that also overlaps with the DNA damage response (DDR) from (VAN BERGEIJK *et al.* 2012).

File S3. Supplemental Table 2. Genes induced by *Tip60^{DN}*, DREAM, and the DNA damage response

Transcriptional program induced by *Tip60^{DN}* expression that overlaps with both the DNA damage response and targets of DREAM (GEORLETTE *et al.* 2007; VAN BERGEIJK *et al.* 2012).

File S4. Supplemental Table 3. Genes induced by *Tip60^{DN}* that are also targets of DREAM

Transcriptional program induced by *Tip60^{DN}* expression that overlaps with targets of the DREAM complex (GEORLETTE *et al.* 2007).

File S5. Supplemental Table 4. Primer sequences for NuA4 dsRNA synthesis

Primers used for *in vitro* T7 transcription of dsRNA used for inhibition of NuA4 subunits in S2R+ cells.

File S2. Supplemental Table 1. Genes induced by *Tip60*^{DN} and the DNA damage response.
(.xlsx, 14KB)

Available for download as a .xlsx file at
www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.188581/-/DC1/FileS2.xlsx

File S3. Genes induced by *Tip60*^{DN}, DREAM, and the DNA damage response. (.xlsx, 10KB)

Available for download as a .xlsx file at
www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.188581/-/DC1/FileS3.xlsx

File S4. Genes induced by *Tip60*^{DN} that are also targets of DREAM. (.xls, 314KB)

www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.188581/-/DC1/FileS4.xls

File S5. Supplemental Table 4. Primer sequences for NuA4 dsRNA synthesis

Primers used for *in vitro* T7 transcription of dsRNA used for inhibition of NuA4 subunits in S2R+ cells.

NuA4 dsRNA	5' to 3' primer sequence
T7-Brd8-fwd1	TAATACGACTCACTATAGG GAT AAA CCA AAA TCT GTG GAG
T7-Brd8-rev1	TAATACGACTCACTATAGG GTC TTT TCT TGC ATT GTT ACT G
T7-E(Pc)-fwd1	TAATACGACTCACTATAGG CAA CAA CAA CAA CAA TAC CG
T7-E(Pc)-rev1	TAATACGACTCACTATAGG CAG CTC ATT GCA GAT GTC TA
T7-Dom-fwd1	TAATACGACTCACTATAGG CTT TGT GGA AGA AAC TGG AG
T7-Dom-rev1	TAATACGACTCACTATAGG CGG ACT CTT CAG GTA CTC AG
T7-Tip60-fwd1	TAATACGACTCACTATAGG TTA AGC CCT GGT ATT TCT CA
T7-Tip60-rev1	TAATACGACTCACTATAGG TGA GAT CAC GTC CTC TTT TT