The Molecular Chaperone Hsp90 Is Required for Cell Cycle Exit in Drosophila melanogaster

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Introduction

Proper development depends on the coordination of cell proliferation and differentiation to produce the correct number of cells in space and time. An important component of this is that cells generally exit the cell cycle in G1 and enter a permanently non-proliferative state when they terminally differentiate. In fact, most cells in adult metazoans have exited the cell cycle and lie in a quiescent state. Control of cell cycle exit is also relevant to cancer, as disruption of the postmitotic state can lead to tumorigenesis.

Cell divisions are primarily driven by oscillations in the activity of Cyclin/Cdk complexes [1]. S phase entry is promoted by the activity of Cyclin E/Cdk2 kinase. Cyclin A/Cdk1 and Cyclin B/Cdk1 complexes, once activated by Cdk5/Stg phosphatase, then induce the G2/M transition. These oscillations in Cyclin/Cdk activity are themselves controlled by oscillations in cell cycle gene expression and proteolysis. For example, the E2F/DP transcription factor stimulates the expression of many genes important for both S phase and mitosis, including Cyclins, Cdns and Cdk5/Stg phosphatase [2]. Additionally, the Anaphase-Promoting Complex/Cyclosome (APC/C), which is an E3 ubiquitin ligase, triggers the destruction of a plethora of proteins during mitosis, including Cyclins A and B, to initiate mitotic exit and re-entry into G1 [3].

The prevailing model for how cell divisions are halted upon terminal differentiation has invoked both inhibition of G1 Cyclin/Cdk activity by Cyclin-dependent kinase inhibitors (CKIs) and the repression of E2F activity. In several model systems, including humans, mice and flies, E2F/DP functions as either a transcriptional activator or repressor depending on which E2F family member is present in the dimer [4]. As a result, E2F activity can be inhibited by trading repressive E2F family members for activating ones in the E2F/DP dimers, or by association with Retinoblastoma (Rb) protein or Rb-related pocket proteins, which convert activating complexes into repressive ones.

Mammals possess eight E2F genes, three DP family members, three pocket proteins (Rb, p107 and p130), three Cip/Kip type CKIs (p21, p27 and p57) and four INK type CKIs (p15, p16, p18 and p19). The machinery controlling cell cycle progression in Drosophila melanogaster is highly conserved with that in vertebrates. However, convenient for studying the roles of these factors in cell cycle exit, flies possess a simpler system with fewer paralogs of each regulatory factor: one activator E2F (E2F1), one repressive E2F
Hsp90 Is Required for Cell Cycle Exit

Author Summary

Cells must permanently stop dividing when they terminally differentiate for development to occur normally. Maintenance of this postmitotic state is also important, as unscheduled proliferation of differentiated cells can result in cancer. To identify genes important for restraining cell proliferation during terminal differentiation, we performed a genetic screen in *Drosophila* and found that mutation of Hsp90 caused ectopic cell proliferation in differentiating tissues. Hsp90 is a molecular chaperone that is essential for viability in all eukaryotes and has been shown to facilitate the activity of hundreds of “client” proteins. Indeed, several inhibitors of Hsp90 are currently being tested in clinical trials for use as anti-cancer therapeutics due to their ability to silence multiple client oncoproteins simultaneously. Our data suggest that Hsp90 is necessary to halt cell proliferation during differentiation because the protein Cdh1, which is required for normal cell cycle exit, may be a client of Hsp90. As reduced Cdh1 function results in genomic instability and tumorogenesis, our work highlights the need to design more precisely targeted Hsp90 inhibitors for use as cancer treatments.

Studies in mammalian model systems indicate that restraining both Cyclin/Cdk and E2F activity constitute important elements of the cell cycle exit mechanism. For example, overexpression of E2F1 or E2F3, both of which are activating E2Fs, in quiescent mammalian cells can induce DNA replication [6]. Consistent with this, cells lacking all three mammalian pocket proteins fail to undergo senescence or arrest in response to serum starvation or contact inhibition [7,8]. Furthermore, mice mutant for pRB, the Rb-related pocket proteins p107 or p130, or CKIs experience ectopic proliferation in many tissues [9–11]. CKIs and Rb family members are also important for cell cycle exit in *Drosophila*. In the fly embryonic ectoderm, ectopic expression of E2F1 can induce an extra cell cycle [12]. Maintenance of cell cycle arrest in the embryonic epidermis requires the repressive activity of *Rbf*, because cells in *Rbf* mutants exit the cell cycle on time but some cells undergo an ectopic S phase later [13]. Importantly, restraining both Cyclin/Cdk and E2F activity was also found to be required for timely cell cycle exit in terminally differentiating neural and epithelial cells of the eye and wing [14,15]. In fact, ectopic expression of both G1 Cyclin/Cdk and E2F could completely prevent cell cycle exit in both tissues [15].

In addition, the APC/C has been shown to play an important role in cell cycle exit. In the *Drosophila* shattered mutant, which lacks a core APC/C component, cell cycle exit of differentiating photoreceptor cells of the larval eye is delayed [16]. Similarly, deletion of APC/C subunits in mice and fish results in ectopic cell proliferation in terminally differentiated tissues [17,18]. The APC/C complex is activated by either of two proteins: Cdc20/Fizzy (Fzy), which activates the complex during mitosis, and Cdh1/Fizzy-related (Fzr), which activates the APC/C from the end of mitosis through G1 phase [19]. It is specifically the G1 APC/C/Cdh1 that is required for normal exit to occur. Loss or inhibition of Fzr in *Drosophila* delays cell cycle exit in the embryonic ectoderm, larval eye, and terminally differentiating pupal eye and wing cells [20–24]. Consistent with this, knockout of Cdh1 causes cultured mammalian cells to have difficulty arresting in G1 and results in ectopic proliferation in mice *in vivo* [25,26].

Despite the proven role these cell cycle regulators play in establishing the postmitotic state, it is clear that additional unknown mechanisms exist that also enforce cell cycle exit upon terminal differentiation. The over-proliferation observed in mice lacking either pocket proteins or CKIs is tissue-specific, indicating that other factors must enforce cell cycle exit in the unaffected tissues [9–11]. In flies, mutation of *dap* alone has no effect cell cycle exit in differentiating pupal eye and wing cells [14,15] and repressive *Rbf/E2F1/DP* function is not required for permanent cell cycle exit *in vivo* [27–29]. Furthermore, although mouse fibroblasts lacking all three pocket proteins have difficulty arresting in G1, embryonic stem cells carrying the same mutations can exit the cell cycle when induced to differentiate [30]. In addition, it is unclear how cell cycle exit is coordinated with terminal cell differentiation, and how the cell signaling pathways that promote differentiation interface with the core cell cycle machinery.

Here, we have performed a loss-of-function genetic screen in *Drosophila* to identify mutants defective in cell cycle exit. We demonstrate that *Hsp83* is required for timely cell cycle exit in terminally differentiating pupal eyes and wings. *Hsp83* is the sole *Drosophila* member of the Hsp90 family of molecular chaperones. Generally thought to be less promiscuous than other chaperones, Hsp90 acts to potentiate the functioning of more than 200 client proteins by promoting subtle changes in the structure of those clients [31,32]. Hsp90 is essential in eukaryotes, and known clientele of Hsp90 include several key signaling pathway proteins, transcription factors and cell cycle proteins. The activity of Hsp90 depends on ATP binding and hydrolysis, which is coupled to conformational changes in Hsp90 protein. In addition, association with co-chaperones and post-translational modifications of Hsp90 protein can modulate its activity.

Analysis of *Hsp83* mutant clones indicates that the cell cycle exit delay is primarily due to increased Cyclin/Cdk activity. In addition, the levels of several APC/C target proteins, including Cyclin A, are increased in cells lacking *Hsp83*. We found that removing one copy of an inhibitor of the APC/C/Cdh1 could suppress the *Hsp83* cell cycle exit phenotype. These data are consistent with a model in which Cdh1/Fzr is a client protein of Hsp83, and Hsp83 restrains Cyclin/Cdk activity after cell cycle exit by ensuring maximal activity of the APC/C/Cdh1. In this way, Hsp83 could provide yet another layer of control over the core cell cycle machinery to ensure that cells stop dividing on schedule during development.

Results

Identification of a mutant line, 6-55, that experiences ectopic E2F activity and delayed cell cycle exit

To identify unknown factors required for timely cell cycle exit, we performed a loss-of-function screen utilizing a *PCNA-miniwhite* reporter gene (Figure 1A). Briefly, this reporter consists of a portion of the *proliferating cell nuclear antigen (pcna)* promoter/enhancer sequence that is E2F-responsive, and is active in proliferating cells but inactive in non-dividing cells ([15,33] and data not shown). The *pcna* promoter/enhancer sequence induces expression of the *mini-white* gene, which is a truncated form of the *white* (+) gene necessary for red eye color. *White* must be expressed specifically between 1 and 2 days after puparium formation in order for the flies to exhibit red eye color [34]. Just prior to this phenocritical period for *white*, all cells in wild-type pupal eyes permanently exit the cell cycle and exhibit reduced levels of E2F activity by 24 hours after puparium formation (hr APF) [15,35].
Therefore, animals carrying the PCNA-miniwhite+ reporter only display dark red eye tissue if either ectopic E2F activity or ectopic cell proliferation continues after wild-type cells have become quiescent. We tested the use of PCNA-miniwhite+ to identify mutants that delay cell cycle exit by inducing mutant eye clones of archiplego (ago), which is the Drosophila homolog of mammalian Fbw7, encodes an F-box protein important for SCF-dependent degradation of Cyclin E and was previously shown to be required for timely cell cycle exit in the Drosophila pupal eye [36,37]. Compared to controls, flies with mutant ago eye clones displayed higher levels of PCNA-miniwhite+ expression (Figure 1B, C).

The screen was performed using ethyl methanesulfonate (EMS) as a mutagen, and eyeless (ey)-FLP was used to induce homozygous mutant tissue in the eyes only of flies heterozygous for mutations created on FRT chromosomes. After screening ~185,500 such animals, we identified and established 61 mutant lines (representing a maximum of 57 genetic complementation groups) that

![Figure 1. 6-55 mutant cells experience ectopic E2F activity.](image-url)
resulted in an increase in PCNA-miniwhite\textsuperscript{+} reporter gene expression in adult eyes. (For a detailed summary of this screen, refer to Table S1.) Three of the mutant lines (one complementation group) failed to genetically complement mutant alleles of ago, indicating that these lines were ago mutants. This confirmed the efficacy of the loss-of-function screen. In addition, a mutant line generated on chromosome arm 3L and designated as \textit{CCE\textsuperscript{6-55}} (6-55) also experienced increased expression of PCNA-miniwhite\textsuperscript{+} compared to controls when homozygosed using \textit{ey-FLP} (Figure 1D,E).

In the course of this study, we used multiple different types of mosaic analysis in larval and pupal tissues to analyze the 6-55 mutant line. FLP/FRT mitotic clones were induced to examine homozygous mutant cells within a heterozygous animal. These were induced in the eye using \textit{ey-FLP}, and were marked by the absence of either GFP or \textit{pi-Myc} labeling, in cases where a GFP+ transgene was to be visualized. To generate GFP-marked clones in which we could simultaneously overexpress transgenes, we used the MARCM system [38]. Finally, to create homozygous clones in which we could simultaneously overexpress a transgene was to be visualized. To generate GFP-marked pupal eyes and wings from animals containing a \textit{PCNA-miniwhite} line generated on chromosome arm 3L and designated as \textit{CCE\textsuperscript{6-55}} (6-55), indicating that these lines were ago mutants. This confirmed the efficacy of the loss-of-function screen. In addition, a mutant line generated on chromosome arm 3L and designated as \textit{CCE\textsuperscript{6-55}} (6-55) also experienced increased expression of PCNA-miniwhite\textsuperscript{+} compared to controls when homozygosed using \textit{ey-FLP} (Figure 1D,E).

To confirm that the observed increase in PCNA-miniwhite\textsuperscript{+} expression truly represented an increase in E2F-dependent transcription, we assayed 6-55 homozygous mutant clones in the pupal eye for increased expression of another E2F-responsive reporter, \textit{PCNA-GFP}, which contains the same \textit{pca} enhancer/promoter region as PCNA-miniwhite\textsuperscript{+} [33]. After 24 hr APF, increased \textit{PCNA-GFP} expression was apparent in some cells within 6-55 mutant clones, compared to the low level of \textit{PCNA-GFP} expression in either wild-type clones or the wild-type cells surrounding 6-55 mutant clones (Figure 1F–F\textsuperscript{0} and data not shown). This ectopic \textit{PCNA-GFP} expression was also not observed in 6-55 mutant clones that carried a \textit{PCNA-GFP} reporter lacking the E2F binding sites (Figure S1). These data confirm that E2F activity is increased in 6-55 mutant tissue.

We then wanted to determine whether these 6-55 mutant cells also underwent ectopic cell proliferation. To test this, we labeled pupal eyes and wings from animals containing 6-55 mutant MARCM clones with anti-phospho-Ser10 histone H3 (PH3) antibodies as a marker for mitosis [39]. At 28 hr APF, we never observed PH3 labeling in wild-type clones (Figure 2A–A\textsuperscript{0}). In contrast, a small number of 6-55 mutant cells at this time point labeled with PH3 antibodies (1% in pupal wings) (Figure 2B–B\textsuperscript{0} and D–D\textsuperscript{0}). In addition, homozygous FLP/FRT clones mutant for 6-55 exhibited BrdU incorporation, a marker for DNA replication, at 24 hr APF when the surrounding control cells were quiescent and did not incorporate BrdU (Figure 2E–E\textsuperscript{0}). We observed evidence of this ectopic cell proliferation until approximately 40 hr APF, but not at time points thereafter (Figure S2).

As an additional assay for inappropriate cell proliferation in 6-55 mutant cells, we used flow cytometry. 24–28 hr APF pupal eyes and wings containing either wild-type or 6-55 GFP-expressing MARCM clones were isolated and the cells were dissociated and stained with the DNA dye Hoechst 33342. Flow cytometric analysis revealed that both the GFP-expressing wild-type control MARCM clone cells and the non-GFP-expressing non-clone cells from all samples possessed a G1 DNA content, indicative of proper cell cycle exit (Figure 2F–G and data not shown). However, the 6-55 MARCM clones contained a small proportion of cells (~10%) with a G2 DNA content (Figure 2F–G). Combined, these data indicated that at least some 6-55 mutant cells experience a delay in cell cycle exit upon terminal cell differentiation.

A mutation in \textit{Hsp83} is responsible for the cell cycle exit phenotype in 6-55

Although clones of 6-55 homozygous mutant cells could be produced, animals composed entirely of 6-55 homozygous mutant cells died as pupae (data not shown). We exploited this lethal phenotype for the initial genetic mapping of the molecular lesion responsible for the 6-55 mutant phenotypes. A combination of deficiency and recombination mapping restricted the region of interest in which the gene corresponding to 6-55 resided to an approximately 50 kb region (see Materials and Methods for details). Approximately 40 kb of those 50 kb were sequenced in both the 6-55 mutant strain and the isogenic parental strain mutagenized to create 6-55. This analysis revealed only one base pair change in 6-55 within those 40 kb: a C to T change at 3L coordinate 3195636, within the \textit{Hsp83} protein coding region, which would be predicted to result in a proline (P) to serine (S) change at residue 380 in the \textit{Hsp83} protein (Figure 3A).

\textit{Hsp83} is the \textit{Drosophila} homolog of mammalian \textit{Hsp90}, a molecular chaperone with an established role in optimizing the function of over 200 known client proteins [32,40]. Although its expression is induced after cellular stress, \textit{Hsp83} also plays an important role in facilitating the activation and/or maturation of its client proteins under non-stress conditions. The proline residue mutated in 6-55 is conserved in the \textit{Hsp90} family proteins in other organisms, from yeast to humans [40]. Further, this proline lies within the catalytic loop, a short stretch of 21 amino acids shown to be essential for viability and ATP hydrolysis in yeast \textit{Hsp90} [31,41]. Therefore, it is likely that the 6-55 mutation is a loss-of-function mutation for \textit{Hsp83} and that a similar mutation in the corresponding residue in other organisms would also result in loss-of-function phenotypes.

To confirm that \textit{Hsp83} is indeed non-functional in 6-55, we performed genetic complementation tests against known mutant alleles of \textit{Hsp83}. 6-55 failed to genetically complement nine alleles of \textit{Hsp83} (Table 1). The 6-55 mutation in trans to two of these alleles (\textit{Hsp83}\textsuperscript{565g} and \textit{Hsp83}\textsuperscript{ps35}) was lethal, and was semi-lethal in trans to two other alleles that carry identical molecular lesions (\textit{Hsp83}\textsuperscript{19} and \textit{Hsp83}\textsuperscript{13E}). In addition, five other \textit{Hsp83} mutant alleles (\textit{Hsp83}\textsuperscript{gs3}, \textit{Hsp83}\textsuperscript{ed3}, \textit{Hsp83}\textsuperscript{1D}, \textit{Hsp83}\textsuperscript{gs4} and \textit{Hsp83}\textsuperscript{3A}) produced viable but male-sterile flies when combined with 6-55. These phenotypes are all known \textit{Hsp83} mutant phenotypes, and are consistent with the genetic complementation that has observed previously with multiple \textit{Hsp83} alleles [42]. Further, two pieces of evidence led us to believe that the mutation in 6-55 is a hypomorphic mutation. First, the stronger alleles of \textit{Hsp83} are lethal in trans to other \textit{Hsp83} alleles, whereas weaker alleles are often male-sterile in trans to each other [42]. In addition, null alleles of \textit{Hsp83} have been reported to be embryonic lethal; however, 6-55 is pupal lethal [42,43]. Therefore, the 6-55 allele behaves genetically like a partial loss-of-function mutation in \textit{Hsp83}.

Although these data indicated that 6-55 contains a mutation in \textit{Hsp83} that is responsible for the lethal phenotype, it was still formally possible that the lesion in \textit{Hsp83} was not responsible for the cell cycle exit phenotype we observed in 6-55 mutant clones. To determine if \textit{Hsp83} is required for timely cell cycle exit, we generated flies that carried both the 6-55 mutation and a genomic rescue construct that contains the entire coding sequence of \textit{Hsp83} [44]. The presence of this genomic rescue construct rescued both the lethality of the 6-55 homozygotes (data not shown) and the cell cycle exit phenotype, as we never observed any \textit{PH3} positive cells after 24 hr APF in flies with 6-55 MARCM clones that also carried the rescue transgene (compare Figure 3F–F\textsuperscript{0} with G–G\textsuperscript{0}). It should be noted that the \textit{Hsp83} genomic rescue construct also
contains the entire coding sequence for the neighboring genes CG14966 and CG32276 [44]. Although we have not sequenced these genes in the mutant strain to confirm that they are unaltered, both reside in a region that remains intact in the deficiency strain Df(3L)BSC23 that failed to complement 6-55. As a consequence, it is unlikely that either of these genes could correspond to 6-55.

To confirm that Hsp83 is required for cell cycle exit, we expressed RNAi directed against Hsp83 either in postmitotic cells

Figure 2. 6-55 mutant cells undergo ectopic cell proliferation. (A–D’) Clones homozygous for a wild-type FRT80B chromosome (A–A’, C–C’) or an FRT80B chromosome carrying the 6-55 mutation (B–B’, D–D’) and also overexpressing GFP and P35, an inhibitor of apoptosis, were induced using the MARCM system. Pupal tissues were then isolated at 28 hr APF and labeled with anti-PH3 antibodies (red) to visualize mitosis. The clones were marked with GFP (green) and DNA was stained with either Hoechst or DAPI (blue). White arrows indicate mitotic 6-55 mutant cells. (E–E’) 24 hr APF FLP/FRT homozygous 6-55 pupal eye clones marked by the absence of GFP (green) were labeled with BrdU (red) to assay for DNA replication. The white arrow in E and E’ indicates a 6-55 mutant cell that has incorporated BrdU. (F, G) Flow cytometry was also used to assess the cell cycle profile of 24–28 hour APF eyes (F) and wings (G) containing 6-55 homozygous MARCM clones marked with GFP and overexpressing P35. GFP-marked clone cells are represented by the green traces, while control non-clone cells are represented by the black traces. At this time point, almost 100% of wild-type cells in both eyes and wings have a G1 (2N) DNA content, whereas approximately 10% of 6-55 clone cells exhibit a G2 (4N) DNA content. All scale bars are 25 μm.

doi:10.1371/journal.pgen.1003835.g002
Figure 3. 6-55 is a mutant allele of Hsp83. (A) A portion of the ABI sequencing traces from the isogenized parental FRT80B strain and 6-55 homozygous mutant animals. Red arrows indicate the C to T base pair change at 3L coordinate 3195636, which is within the Hsp83 protein coding region. (B–E') RNAi directed against w (B–C') or Hsp83 (D–E') was expressed in either 28 APF pupal eyes (B, D) or the dorsal half of 26 APF pupal wings (C–C', E–E'). Pupal tissues were then stained with PH3 (red) to assay for ectopic mitoses and Hoechst to visualize DNA (blue). GFP (green) was also expressed in the dorsal half of the wing (C, E). (F–G) Homozygous 6-55 MARCM clones overexpressing GFP (green) and P35 were induced in 24–30 APF pupal wings. The animals in G–G' also carry one copy of the genomic rescue construct for Hsp83. These wings were labeled with Hoechst (blue) (F, G), anti-PH3 (red) (F–F', G–G'), and antibodies against Geminin protein (gray) (F', G'). White arrows in F and F' indicate mitotic cells.

doi:10.1371/journal.pgen.1003835.g003
in the eye using GMR-GAL4 or in the dorsal half of the wing using apertus (ap)-GAL4 and assayed for ectopic cell proliferation with anti-PH3 antibodies in 28 hr APF pupal tissues. At this time point, we observed multiple mitotic cells in both eyes and wings, which were not seen in controls in which an RNAi against the white gene was expressed, or in the ventral half of the wing (Figure 3B–E’ and data not shown). Together, these multiple pieces of data indicated that a loss-of-function hypomorphic mutation in Hsp83 is responsible for the delay in cell cycle exit seen in the 6-55 mutant cells.

The cell cycle defect caused by mutation of Hsp83 is more severe after cell cycle exit than in proliferating tissues

We then wondered whether this cell cycle effect of Hsp83 loss-of-function was specific to cell cycle exit or whether a reduction in Hsp83 function could also cause ectopic cell divisions in proliferating tissues. To test this, we induced 6-55 mutant MARCM clones in larvae and assayed these clones for cell cycle defects in proliferating 4th larval instar imaginal discs. Compared to controls, there was no obvious difference in PH3 labeling in 6-55 mutant clones in the proliferating regions of eye and wing imaginal discs (Figure 4A–A’). We did occasionally observe ectopic PH3 labeling within 6-55 mutant clones in the non-proliferating regions of the eye disc posterior to the morphogenetic furrow (MF) (Figure 4C–C’). In addition, it is important to note that when mutant clones spanned the MF, there was no effect on the timing of expression of Elav, a marker of neuronal differentiation (data not shown). This indicated that differentiation occurs in the 6-55 mutant cells with proper timing, and therefore that the ectopic cell proliferation we saw in these tissues was not due to either delayed development or faulty differentiation.

We also used flow cytometry to assess the cell cycle profiles of 6-55 mutant MARCM clones in larval eye and wing discs. In eye discs, 6-55 mutant clones had a cell cycle profile that was indistinguishable from clones made using a wild-type FRT chromosome and the wild-type non-clone cells within the mutant mosaic discs (Figure 4I and data not shown). In wing discs, however, there was a mild change in cell cycle phasing in 6-55 mutant clones compared to controls, such that a larger proportion of the mutant cells had a G2 DNA content and fewer had a G1 DNA content (Figure 4J). This is consistent with either a shortening of G1 phase in these mutant cells or a lengthening of G2. Overall, these data demonstrated that a reduction of Hsp83 function sufficient to delay cell cycle exit has only a very mild effect in actively proliferating cells.

The cell cycle exit defect in Hsp83 mutant cells is caused primarily by an increase in Cyclin/Cdk activity

It had previously been shown that the suppression of both Cyclin/Cdk activity and E2F transcriptional activity are necessary for timely cell cycle exit to occur in terminally differentiating tissues [14,15,45]. Clearly, at least some Hsp836-55 mutant cells possess inappropriate E2F activity after wild-type cells have exited the cell cycle (Figure 1). However, we also wanted to determine if Hsp836-55 mutants experience ectopic Cyclin/Cdk activity after cell cycle exit. To test this, we utilized two reporters for Cyclin/Cdk activity: anti-phospho-histone H1 antibodies, which detect phospho-epitopes of multiple Cyclin/Cdk complexes in several systems, but which have been shown to specifically respond to Cyclin E/Cdk2 activity in Drosophila endoreplicating follicle cells [46,47], and the MPM2 antibody, which detects a small focus of nuclear staining at the histone locus body (HLB) in response to Cyclin E/Cdk2 activity [48,49] and Figure 5A–A’). At both early and late time points after wild-type cells had exited the cell cycle (26–28 hr APF) and a later time point (44 hr APF), Hsp836-55 mutant cells exhibited labeling with both of these markers of Cyclin/Cdk activity, whereas the control cells surrounding the Hsp836-55 mutant clone did not (Figure 5B–F’). This indicated that cells with reduced Hsp83 function retain Cyclin/Cdk activity after wild-type cells have exited the cell cycle.

Given that Hsp836-55 mutant cells experienced increases in both E2F activity and Cyclin/Cdk activity compared to controls in differentiating tissues, we wondered which of these two effects represented the primary effect of loss of Hsp83. At early time points after cell cycle exit in this system (24–36 hr APF), ectopic Cyclin/Cdk activity can still activate E2F activity, and ectopic E2F activity is still capable of stimulating Cyclin/Cdk activity [15]. However, after 36–40 hr APF, crosstalk between Cyclin/Cdk activity and E2F activity is eliminated and neither one can activate the other [15]. Therefore, we determined whether the ectopic E2F activity in Hsp836-55 mutant clone cells, which had been observed at early times after cell cycle exit, continued after prolonged exit. At 44 hr APF, activation of the PCNA-GFP reporter was no longer visible in Hsp836-55 mutant clones, indicating that ectopic E2F activity was no longer present in Hsp836-55 cells at this time (Figure 5G–G’). This suggested that increased Cyclin/Cdk activity is the primary defect responsible for the cell cycle exit delay in Hsp836-55 mutant cells, and that the observed increase in E2F activity was merely a secondary effect caused at early time points after cell cycle exit due to ectopic Cyclin/Cdk activity. Consistent with this interpretation, there were still markers of ectopic Cyclin/Cdk activity at 44 hr APF, a time when Hsp836-55 mutant cells no longer divided. However, the timing of cell proliferation in Hsp836-55 mutants (until approximately 40 hr APF) was similar to that seen when either Cyclin E/Cdk2 or Cyclin D/Cdk4 were overexpressed in pupal tissues [15].

Hsp836-55 mutant cells accumulate proteins targeted for degradation by the APC/C

As Hsp836-55 mutant cells experienced ectopic Cyclin/Cdk activity after they should have exited the cell cycle, we wanted to determine if these cells contained higher Cyclin protein levels than quiescent control pupal eye and wing cells. To test this, we labeled pupal eyes and wings containing either Hsp836-55 or wild-type MARCM clones with antibodies against Cyclin A, Cyclin D, and Cyclin E. In addition, we looked at expression of a Cyclin B-

**Table 1. The 6-55 mutation fails to genetically complement mutant alleles of Hsp83.**

| Hsp83 allele | Nature of the mutation | Phenotype in trans to 6-55 |
|--------------|------------------------|--------------------------|
| JS22         | P element insertion    | Lethal                   |
| PS82         | P element insertion    | Lethal                   |
| 19F2         | Missense (R48C)        | Semilethal               |
| 13F3         | Missense (R48C)        | Semilethal               |
| e6A          | Missense (S592F)       | Male sterile             |
| e6D          | Missense (E317K)       | Male sterile             |
| e1D          | Missense (S386L)       | Male sterile             |
| e3A          | Missense (S5574C)      | Male sterile             |
| e4A          | Missense (S655F)       | Male sterile             |

doi:10.1371/journal.pgen.1003835.t001
Figure 4. Mutation of Hsp83 only causes mild cell cycle effects in proliferating cells. (A–H') Clones homozygous for a wild-type FRT80B chromosome (A–A', B–B', E–E', F–F') or an FRT80B chromosome carrying the 6-55 mutation (C–C', D–D', G–G', H–H') and also overexpressing GFP and P35 were induced using the MARCM system. Imaginal discs were isolated from wandering third instar larvae and labeled with anti-PH3 antibodies (red) to label mitosis (A, C, E, G) or anti-Geminin (white) (B', D', F', H'). In all cases, the clones were marked with GFP (green) and DNA was stained with Hoechst (blue). White arrows in D' and H' indicate increased Geminin protein levels within 6-55 clones in regions of the disc containing non-proliferating cells. (I, J) Flow cytometry was also used to assess the cell cycle profile of 6-55 homozygous MARCM clones marked with GFP and overexpressing P35 in larval imaginal eye discs (I) and wing discs (J). GFP-marked clone cells are represented by the green traces, while
GFP fusion protein driven by the endogenous Cyclin B promoter (CycB-GFP) in FLP/FRT Hsp836-55 and control clones. We observed increased levels of both Cyclin A and CycB-GFP in Hsp836-55 mutant clones compared to wild-type clones and surrounding control cells (Figure 6A–E). No increase in Cyclin D protein was observed, and increased Cyclin E protein was evident in only some cells of the eye and not at all in the wing blade (data not shown). These data suggested that increases in Cyclin A and/or Cyclin B may be responsible for the ectopic Cyclin/Cdk activity present in Hsp836-55 mutant tissue.

Cyclin A and Cyclin B are both well-established targets of the Anaphase Promoting Complex/Cyclosome (APC/C) [50–52]. The increased protein levels we had observed of both of these led us to hypothesize that APC/C function might be reduced in Hsp83 mutant cells. Indeed, reducing the function of either a core component of the APC/C or one of its activators, Cdh1/Fzr, has been shown to delay cell cycle exit [16,20,21,23,24]. To test for a potential APC/C defect, we assayed control and Hsp836-55 mutant clones for Geminin protein, another known target of the APC/C. As can be seen in Figure 6F–G, Geminin protein levels were also

control non-clone cells are represented by the black traces. The cell cycle profile of 6-55 mutant cells in eye discs was indistinguishable from that of controls, but fewer 6-55 mutant wing disc cells had a G1 (2N) DNA content than control cells. Disc images in A–A’, C–C’, E–E’, G–G’ are single confocal sections. Anterior is to the right. Yellow arrowheads mark the position of the morphogenetic furrow (MF) in A–D’. Dashed yellow lines outline a single clone spanning the MF in A’, B’, C’, D’ and outline all clones in E’, F’, G’, H’.

doi:10.1371/journal.pgen.1003835.g004

Figure 5. Hsp83 mutant cells experience ectopic Cyclin/Cdk activity. (A–B’) Labeling of 26 hr APF pupal wings containing MARCM clones (GFP, green) with MPM2 antibody (red in A, B; gray in A’, B’), which is a reporter for Cyclin/Cdk activity. (A, A’) As a control, cyclin E overexpression in clones resulted in small foci of MPM2 staining only within clone cells. (B, B’) MPM2 staining of a pupal wing containing a clone of cells mutant for Hsp83. Note the presence of foci within the clone but not in the surrounding control cells. (C–F’) Pupal eyes containing Hsp83 mutant clones were labeled with two markers of Cyclin/Cdk activity: MPM2 (C–D’) and P-H1 (E–F’). (C, D) MARCM mutant clones also overexpressing P35 are marked by the presence of GFP (green). Staining for MPM2 is red in C, D and gray in (C’, D’). (E, F) FL-P35/FRT mutant clones are marked by the absence of GFP (green). Staining for P-H1 is red in (E, F) and gray in (E’, F’). DNA stain is blue (E’, F’). Positive feedback between Cyclin/Cdk activity and E2F activity is still intact in the 26–28 APF eyes in (C–C’, E–E’), but is gone in the 44 APF eyes in (D–D’, F–F’). (G–G’) 44 APF pupal eyes were assayed for a reporter of E2F activity, P-Myb/pcna-GFP (green in G, gray in G’). Hsp83 mutant FLP/FRT clones are marked by the absence of pi-Myc labeling (red in G). Dashed yellow lines outline the clones. All scale bars are 25 μm.

doi:10.1371/journal.pgen.1003835.g005
dramatically increased in Hsp83 mutant clones compared to control clones or neighboring wild-type cells. Importantly, the observed increase in Geminin levels in Hsp83-55 mutant clones could be rescued by the Hsp83 genomic rescue construct (Figure 3F and G). We also assayed Geminin levels in third instar larval discs (Figure 4B–B9, D–D9, F–F9, H–H9). Consistent with our previous experiments in larval discs, there was no apparent difference in Geminin levels between mutant and control clones in proliferating cells. However, Hsp83-55 clones in the differentiating cells posterior to the MF in the eye and in the G1-arrested cells in the posterior half of the zone of non-proliferating cells (ZNC) exhibited increased Geminin [53,54]. Altogether, these data indicate that proteins targeted by the APC/C for degradation accumulated in Hsp83 mutant cells.

The presence of increased amounts of Cyclin A, Cyclin B and Geminin in Hsp83 mutant cells could be due to decreased degradation of these proteins because of hindered APC/C function, but also could be due to increased expression of these proteins. All three are targets of E2F, which was ectopically active in Hsp83 mutant cells at time points shortly after wild-type cells have stopped dividing (Figure 1D–F). To test whether Cyclins A and B and Geminin accumulated in Hsp83 mutant cells due to ectopic E2F activity, we assayed Hsp83 mutant clones for CycB-GFP and Geminin at 44 hours APF, a time when E2F was no longer active in these clones (Figure 5G–G9). We found that Geminin and, to a lesser extent, CycB-GFP were still present in Hsp83 clones at this time point (Figure 6H–I9). Based on this, we feel it is unlikely that Cyclin A, Cyclin B and Geminin accumulated in Hsp83 mutant cells due to ectopic E2F-dependent transcription.

An additional situation that could lead to accumulation of APC/C target proteins for reasons other than reduced APC/C function is if the Hsp83 mutant cells were arrested in the G2 phase of the cell cycle. Cyclins A and B and Geminin are all abundant in G2 because the APC/C is not active during this cell cycle stage. To determine whether the Hsp83 mutant cells containing elevated levels of these proteins were simply arrested in G2, we quantified the number of mutant cells ectopically expressing Geminin and found that in 29–30 hr APF wings, 45% of Hsp83 mutant cells labeled with antibodies against Geminin (data not shown). Conversely, based on our FACS analysis, only ~10% of cells within Hsp83 mutant clones in 24–28 APF wings were in G2 (Figure 2G). These data indicate that the majority of Hsp83 cells containing elevated levels of Geminin were in G1 phase, and...
Hsp90 Is Required for Cell Cycle Exit

Several mechanisms have been implicated in controlling permanent cell cycle exit upon terminal differentiation. E2F and Rh family members influence cell proliferation by transcriptional regulation, Cyclin/Cdkks and CKIs regulate cell proliferation via control of cell cycle protein phosphorylation, and the APC/C targets pro-proliferative proteins for proteasome-dependent degradation. However, it has been clear that additional factors must exist that modulate the activity of known cell cycle regulators to ensure timely cell cycle exit. In this study, we provide evidence of an additional layer of control imposed on the cell cycle machinery by the molecular chaperone Hsp90.

A role for Hsp90 in cell cycle exit

Cells mutant for the Drosophila Hsp90 homologue, Hsp83, or expressing an RNAi against Hsp83 experienced a delay in cell cycle exit. Hsp83 mutant cells also experienced ectopic increases in both E2F and Cyclin/Cdk activity. Our data indicate that the increased Cyclin/Cdk activity was the primary defect, which secondarily caused an increase in E2F activity only at time points shortly after wild-type cells have exited the cell cycle. We have further found that the Hsp83 mutant cells contained increased levels of Cyclin A, Cyclin B and Geminin proteins, consistent with reduced APC/C function. This finding has led us to hypothesize that Cdh1/Fzr may be a client protein of Hsp83. The cell cycle exit effect resulting from Hsp83 knock-down was suppressed by reducing the dosage of Rea1, which provided support to our hypothesis.

Although the number of cells undergoing mitosis in the mutant and RNAi clones was significantly increased compared to control clones (1–1.24% versus 0%), Hsp83 loss-of-function resulted in a rather small number of cells delaying cell cycle exit. Why was this? One reason is that the cell cycle exit mechanism is quite robust. To provide a comparison, rhf1 mutant clones in pupal tissues exhibit a mitotic index of ~9% only from 24–28 APF and dnp mutant cells do not experience any cell cycle exit delay [15]. Although the Hsp83 mutant cells have a mitotic index of only ~1%, ectopic cell divisions continue until approximately 40 APF, timing consistent with one extra cell division. In addition, direct overexpression of Rea1 to inhibit Fzr delays cell cycle exit with similar timing as the Hsp83 mutation, and also results in a modest increase in mitotic index in pupal tissues [23]. It is not surprising that Hsp83[53]–[55], a hypomorphic mutation in something that we believe optimizes the function of Cdh1/Fzr, would produce a much more subtle effect.

To our knowledge, this is the first report providing evidence that Cdh1/Fzr could be an Hsp90 client. In budding yeast, it has been demonstrated that another chaperone, the CCT chaperonin, is required for the folding of both Cdc20 and Cdh1 and therefore is necessary for all APC/C activity [57]. While the CCT chaperonin functions in the bulk folding of nascent proteins, the Hsp90 family of chaperones generally promotes more subtle structural changes to potentiate the function of its clientele. We propose that Hsp90 is specifically required to optimize the function of APC/C[Cdh1] and not APC/C[Cdc20]. Indeed, the fact that we are able to observe Hsp90 mutant cells undergoing mitosis indicates that the function of the APC/C[Cdc20] is intact, as cells lacking functional Cdc20/Vfy arrest in mitosis [57–59].

Several studies have demonstrated that mutation or inhibition of fzr can delay cell cycle exit [20–24], but it is not clear which target protein(s) of the APC/C[Cdh1] are responsible for this delay. One likely candidate for the crucial target protein that causes the delay in cell cycle exit is Cyclin A. In Drosophila, Cyclin A normally functions during mitosis, but when ectopically expressed it can also induce entry into S phase [55,60]. Cyclin A overexpression can drive the G1/S transition even in the absence of Cyclin E, suggesting that Cyclin A/Cdk complexes can directly phosphorylate Cyclin E/Cdk2 targets important for S phase. Further, in mammals it has been demonstrated that Cdk1 is the only essential Cdk and that it is sufficient to drive the entire cell cycle in the absence of interphase Cdkks [61]. Indeed, direct overexpression of Cyclin A can cause one complete ectopic cell cycle in differentiating pupal eyes and wings [15]. However, the extra cell cycle induced in the Drosophila embryonic epidermis by mutation of fzr cannot be rescued by also mutating cyclin A [62], suggesting there may be other crucial target proteins of the APC/C[Cdh1] in addition to Cyclin A that must be restrained in order to initiate cell cycle exit. APC/C targets that could potentially play a role in cell cycle exit include Cyclins B and B3, Cdc25/Syg phosphatase and the DNA replication factor Orc1. Further studies are needed to address whether restraining these target proteins comprises an important part of the cell cycle exit requirement for APC/C[Cdh1].
Hsp90: A post-translational mechanism to control the activity of proteins in a cell cycle-dependent manner

Our model is that Hsp90 facilitates the function of the Cdhl/Fzr. What could be the purpose for the regulation of the APC/CCdhl? Unlike other ubiquitin ligases that recognize their targets only when they have been post-translationally modified, for example by phosphorylation or hydroxylation, the APC/C recognizes different unmodified substrates at distinct points in the cell cycle [3]. Although it is not entirely understood how this change in substrate specificity occurs, it is clear that it is partially dependent on which co-activator, Cdc20/Fzy or Cdhl/Fzr, is associated with the complex [3]. While Cdc20/Fzy is expressed periodically and this may partially control activation of the APC/C [63], Cdhl/Fzr is not periodically transcribed [19,63]. Cdhl/Fzr is known to be negatively regulated by both Cyclin/Cdk phosphorylation and binding of inhibitors [3]. In addition, activation of Cdhl/Fzr by interaction with Hsp90 in a cell cycle-dependent manner could potentially also be responsible for the timely activation of the APC/C.

How could a protein such as Hsp90 that is expressed ubiquitously and at high levels regulate another protein so that it only acts at a specific place and time? Hsp90 function is modulated both by association with co-chaperones and via post-translational modifications, including phosphorylation, acetylation and S-nitrosylation, all of which can direct Hsp90 to particular client proteins [32]. A recent study has even indicated that a specific phosphorylation event plays a role during exit from the cell cycle [64]. Although it has not been demonstrated that this specific phosphorylation event plays a role during exit from the cell cycle, these data combined with data indicating that Hsp90 is subject to many post-translational modifications [32,65] raise the possibility that a particular combination of modifications on Hsp90 could create a G1-specific Hsp90 activity that optimizes the function of clients important for cell cycle exit in G1.

Hsp90 inhibition and cancer

Hsp90 is needed to protect a number of mutated and overexpressed oncoproteins, such as ErbB2/HER2, v-Src, Akt and Bcr-Abl, from misfolding and degradation [32,66]. Therefore, inhibition of Hsp90 is an efficient way to silence multiple oncogenic signaling pathways simultaneously. As a result, several Hsp90 inhibitors are currently being developed and tested as anti-cancer therapeutics. However, the pleiotropic effects of Hsp90 inhibitors may complicate their clinical efficacy. For example, Hsp90 inhibition results in the release and activation of the heat shock transcription factor 1 (HSF1), and HSF1 has a clear role in supporting the proliferation and survival of transformed cells [67–69]. In addition, in Drosophila, it had been demonstrated that mutation or inhibition of Hsp90 relieves the suppression of transposon activity, resulting in de novo mutations and revealing that Hsp90 inhibition can be mutagenic [70]. Our findings, which suggest that APC/C-Cdh1 function is reduced in the absence of Hsp90, identify an additional mechanism by which Hsp90 inhibition could promote genomic instability and carcinogenesis. Mouse and human cells lacking Cdh1/Fzr exhibit multiple markers of genomic instability, including chromosome breaks, anaphase bridges and aneuploidy [26,71]. Further, Cdhl/Fzr heterozygous mice displayed an increased propensity to develop epithelial tumors, suggesting a tumor suppression function for Cdhl/Fzr [26]. Overall, these data highlight a need to target Hsp90 inhibitors not only to tumor cells, but specifically to Hsp90-oncoprotein client interactions within tumor cells. Current efforts by researchers to design Hsp90 inhibitors that disrupt specific Hsp90-co-chaperone interactions, or to develop Hsp90 isoform- and cell location-specific inhibitors are underway [32]. This approach of designing more precisely targeted Hsp90 inhibitors will be important to reduce undesirable pro-proliferative and mutagenic effects of Hsp90 inhibition.

Materials and Methods

Fly stocks and genetics

All crosses were performed at 25°C. For the screen, w; FRT40A, w; FRT42D, w; FRT80B, and w; FRT82B strains were first isogenized for the corresponding chromosome. Males were then mutagenized with 15–25 mM ethyl methanesulfonate (EMS) and crossed to y w; ey-FLP; FRT40A (or 42D or 80B or 82B) tester virgin females which also carried the PCNA-minicatché reporter. Males were removed from the crosses after four days and discarded. F1 progeny were screened for patches of darker red eye tissue. Such flies were first re-tested by crossing again to the tester stock. If the ectopic PCNA-minicatché phenotype was observed in the progeny of a mutant, a stock was then established by crossing to either y w; ey-FLP GMR-lacZ FRT42D (2)el-R111/CyO, y; + (for 2nd chromosome stocks) or y w; ey-FLP GMR-lacZ FRT80B RpS177/TM6B, y; + (for 3rd chromosome stocks).

For complementation tests with known Hsp83 alleles, y w; ey-FLP GMR-lacZ FRT80B (3)CCE525/TM6B, y; + animals were crossed to balanced stocks of known lethal alleles of Hsp83. If non-balancer progeny were not observed, the allelic combination was scored as lethal. Allelic combinations that produced very few non-balancer progeny (1–2 per 50 offspring) were scored as semilethal. For crosses that produced viable non-balancer progeny, male sterility was tested by individually crossing 10 non-balancer male progeny from each cross to virgin females. If none of these crosses produced progeny the allelic combinations were scored as male sterile.

Fly stocks used:

w; FRT40A
w; FRT42D
w; FRT80B
w; FRT82B
y w; ey-FLP GMR-lacZ FRT40A; PCNA-minicatché
y w; ey-FLP GMR-lacZ FRT42D; PCNA-minicatché
y w; ey-FLP GMR-lacZ FRT80B
y w; ey-FLP GMR-lacZ FRT82B
y w; ey-FLP GMR-lacZ FRT42D (2)el-R111/CyO, y; +
y w; ey-FLP GMR-lacZ FRT80B RpS177/TM6B, y; +
y w; ey-FLP GMR-lacZ FRT80B (3)CCE525/TM6B, y; +
w; FRT80B ago1/TM6B
w; FRT80B ago1/Y TM6B
cdc2E1-23/CyO
cdc2E1-24/CyO
cdc2E1-25/CyO
y; cdc2E287/CyO
y; cdc2E287/+/CyO
y; cdc2E287/+/CyO
y; cdc2E205/+/CyO
y; cdc2E205/+/CyO
w; cdc2E205/+/CyO
w; cdc2E205/+/CyO
CG12002GMR-lacZ FRT800/TM2
y w; ey-FLP GMR-lacZ FRT80B
w; FRT80B hs-piM75C
PCNA-GFP, FRT80B hs-piM75C/TM6B
PCNA-ASN2ZII-GFP, FRT80B hs-piM75C/TM6B
y w; hs-FLP tub-GAL4 UAS-GFP; FRT80B tub-GAL200/TM6B
y; UAS-P35/CyO, act-GFP; FRT80B (3)CCE525/TM6B
y; UAS-P35/CyO, act-GFP; FRT80B
dh, hs-piM75C/TM2
w; Hsp8319F2/TM3, Ref
dh
w; Hsp8319F2/TM3, Ref
dh
Generation of PCNA-miniwhite transgenic flies

The pcna promoter was amplified from genomic DNA using primers CCCAAGCTTTCCAAACCAGTGCGACCGCCGC and CATGGAATTCTGTTGTTTATTATTTAAATACTGATGACG. This PCR product was digested with HindIII and EcoRI and cloned into pBlueScript II HindIII/EcoRI sites, creating the vector pBS-miniwhite. The mini-white gene was amplified from pUAST using primers CGCGAATTCATGGGCCAACGACGTACCCCTTCTG and ACTGGAATTCATGGGCCAACGACGTACCCCTTCTG. This PCR product was digested with EcoRI and PstI and cloned into pBlueScript II EcoRI/PstI sites, creating the piggyBac vector pBSII-ITR1.1k-ECFP to generate pBa-

Immunohistochemistry and microscopy

Pupae, staged from white prepupae (0 hr APF) at 25°C, or imaginal discs from wandering third instar larvae were dissected and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. They were then washed in PBS+0.1% Triton X-100 at least two times 10 min each. The pupal cuticle was removed from wings after fixation. Larval tissues or 24–36 hr APF pupal tissues were blocked in PBS+0.1% Triton X-100+1% BSA (PAT) for 1 hr. Pupal tissues 36–44 hr APF were blocked in PBS+0.3% Triton X-100+1% BSA overnight. All samples were incubated with primary antibody overnight at 4°C, washed with PBS+0.3% Triton X-100+0.1% BSA+2% NGS three times 20 min each, and incubated with secondary antibody conjugated to Alexa Fluor 405, 568 or 633 (Invitrogen) 1:1000–2500 for 4 hr at room temperature. 1 µg/ml Hoechst 33258 (Invitrogen) or 0.5 µg/ml 4′,6-Diamidino-2-phenylindole (DAPI) (Sigma) were used to label nuclei and Vectashield (Vector Laboratories) was used as mounting medium.

For BrdU labeling, pupae were dissected at room temperature in Ringier’s solution (Tübingen and Düsseldorf). Tissues were incubated in 100 µg/ml BrdU in Ringier’s for 1 hr and then washed briefly in PBS. Fixation and labeling with α-GFP antibody was performed as usual. Following this, samples were fixed again with 100% methanol for 6 min, hydrolyzed in 2N HCl+0.1% Triton X-100 for 30 min, neutralized two times 5 min in 100 mM Borax, and washed at least three times with PBS+0.1% Triton X-100. The samples were then blocked and labeled with α-BrdU antibody per usual protocol. EdU labeling was performed similarly to the procedure for BrdU labeling, incubating for 1 hr with 10 µM EdU and using the Click-it EdU Alexa Fluor 555 imaging kit (Invitrogen) to visualize incorporation.

Wing hinge and notum, as well as head capsule and antenna, were excluded from our analyses. Confocal images were collected either on a Zeiss LSM 510 microscope (Figures 1F–F, 2E–E, 3D–D, 4A–D, 5C–D, 6C–E, 7A–D) or Leica TCS SP5 microscope (Figures 2A–D, 4A–D, 5C–D, 6C–E, 7A–D). Antibodies used were: rabbit α-Myc 9E10 (1:100; Santa Cruz Biotechnology), mouse α-Myc 1:100 (1:100; Santa Cruz Biotechnology), mouse α-phospho-Ser10-histone H3 (PH3; 1:1000; Millipore), rabbit α-Geminin (1:100; provided by B. Calvi), mouse α-MPM2 (1:200; Millipore), rabbit α-phospho-histone H1 (1:100; Millipore), rabbit α-CycA (1:500; provided by D. Glover), rat α-EVL-A (1:200; Developmental Studies Hybridoma Bank).

Flow cytometry

All clones were induced at 24–48 hr after egg deposition (AED) by a 45 min heat shock in a 37°C water bath. Discs were dissected from wandering third instar larvae or from staged pupae. Dissociation of cells and FACS were performed as previously described [72]. All experiments were carried out at least three times and representative examples are shown.

Clonal analysis

Loss-of-function clones in Figure 1, Figure 2E–E, Figure 3E–E and Figure S1 were generated using mitotic recombination [73] and flipase expressed from the eyeless promoter (ey-FLP). Clones in Figure 6C–E were induced by mitotic recombination using a heat shock-inducible flipase (hs-FLP). All other clones were generated using MARCM [38]. MARCM mutant clones in Figure 2A–D, Figure 4, Figure 5C–D, Figure 6A–B, F–G, I–I′ and Figure S2 also overexpress P35 to inhibit apoptosis. For MARCM or hs-FLP experiments, larvae were heat shocked at 24–48 hr AED for 45 min. in a 37°C water bath. Larvae were collected as wandering third instars for dissection; pupae were collected for staging a 0 hr APF, aged at 25°C, and dissected at the indicated times. For experiments where mutant clones were marked by the absence of pi-Myc, pupae were heat shocked to induce expression of pi-Myc in a 37°C water bath for 30 min., beginning 90 min. prior to dissection.

For the genetic suppression experiment (Figure T), w1118 UAS-Dcr-2; UAS-P35/Cyo act-GFP; act>CD2>GAL4/TM6B virgins were crossed to the following males:
Figure 7. Removing one copy of Rac1 suppresses the Hsp83 RNAi cell cycle exit phenotype. hs-FLP was used to induce flip-out clones expressing Hsp83 RNAi, Dcr-2, P35 and GFP in 28 hr APF pupal wings. These wings were then labeled with anti-PH3 and the mitotic cell cycle exit phenotype. Figure S1 PCNA-GFP reporter activation in 6-55 mutant clones is dependent on the E2F binding sites. (A) 6-55 mutant mitotic clones marked by the absence of pi-Myc marker (red) in a pupal eye after 24 hr APF. (B) A mutant PCNA-GFP reporter lacking the E2F binding sites in the pea enhancer/promoter sequence is not expressed in 6-55 mutant clones. (C) Merged image. Yellow arrows indicate the positions of two mutant clones. (TIF)
H–H”; red). Clones are marked by the presence of GFP (green) and DNA was stained with DAPI [blue]. White arrows indicate the presence of proliferation markers in 6–55 mutant clones. All scale bars are 25 μm.

**Table S1** Results of the *PCNA-miniwhite* EMS screen. Summary of the loss-of-function screen results per chromosome arm. The number of F1 progeny screened, number of stocks established that exhibited *PCNA-miniwhite*+ expression, and the number and identity of mutant lines with a confirmed cell cycle exit delay are indicated.

(DOC)

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**Acknowledgments**

We are grateful to B. Duronio, M. Fraser, B. Calvi, S. Rutherford, D. Glover, I. Hariharan, the Bloomington Stock Center, the Vienna Drosophila RNAi Center, the National Institute of Genetics, the Yale FlyTrap collection and the Developmental Studies Hybridoma Bank for fly stocks and antibodies. We would also like to thank the members of the Edgar lab, in particular L. Buttitta and N. Zielke for helpful discussions and A. de la Cruz for assistance with flow cytometry.

**Author Contributions**

Conceived and designed the experiments: JLB HJ BAE. Performed the experiments: JLB HJ DWN. Analyzed the data: JLB BAE. Contributed reagents/materials/analysis tools: HJ DWN. Wrote the paper: JLB BAE.
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