Protein Phosphatase 2A promotes the transition to G0 during terminal differentiation in *Drosophila*

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Abstract

Protein phosphatase type 2A complex (PP2A) has been known as a tumor suppressor for over two decades, but it remains unclear exactly how it suppresses tumor growth. Here we provide data indicating a novel role for PP2A in promoting the transition to quiescence upon terminal differentiation in vivo. Using Drosophila eyes and wings as a model, we find that compromising PP2A activity during the final cell cycle prior to a developmentally controlled cell cycle exit leads to extra cell divisions and delayed entry into quiescence. By systematically testing the regulatory subunits of Drosophila PP2A, we find that the B56 family member widerborst (wdb) is required for the role of PP2A in promoting the transition to quiescence. Cells in differentiating tissues with compromised PP2A retain high Cdk2 activity when they should be quiescent, and genetic epistasis tests demonstrate that ectopic CyclinE/Cdk2 activity is responsible for the extra cell cycles caused by PP2A inhibition. The loss of wdb/PP2A function cooperates with aberrantly high Cyclin E protein, allowing cells to bypass a robust G0 late in development. This provides an example of how loss of PP2A can cooperate with oncogenic mutations in cancer. We propose that the wdb/PP2A complex plays a novel role in differentiating tissues to promote developmentally controlled quiescence through the regulation of CyclinE/Cdk2 activity.
Introduction

In adult metazoans, most terminally differentiated cells exit from the cell cycle and lie in a state of prolonged or permanent quiescence. The transition from active proliferation to quiescence in vivo is robust, often irreversible, and ensured by redundant cell cycle regulatory mechanisms (Buttitta et al., 2007; Firth and Baker, 2005; Nicolay et al., 2010; Pajcini et al., 2010; Wirt et al., 2010). By comparison, most studies of quiescence have been performed in cell culture where contact inhibition, drug treatments or withdrawal of mitogens induce a quiescent state which is most often readily reversible (Coller, 2011). While some of the key cell cycle regulators promoting quiescence in these contexts overlap (e.g. Retinoblastoma family members, Cyclin-dependent Kinase Inhibitors, CKIs), there must be critical differences between the reversible quiescence in cell culture and developmentally controlled robust cell cycle exit in vivo.

Recent work in mammalian cell culture has demonstrated that the level of Cdk2 activity after mitosis impacts the proliferation vs. quiescence decision for the next cell cycle, such that cells with low Cdk2 activity enter a quiescent “G0-like” state (Spencer et al., 2013). This suggests that mechanisms regulating Cyclin/Cdk2 activity during the final cell cycle in vivo could impact the timing and robustness of cell cycle exit in tissues. Consistent with this hypothesis, the loss of CKIs which inhibit CyclinE/Cdk2 complexes or loss of the F-box protein Fbw7, which regulates Cyclin E stability, can partially delay proper cell cycle exit in certain tissues (Chen and Segil, 1999; de Nooij et al., 1996; Fero et al., 1996; Kiyokawa et al., 1996; Lane et al., 1996; Minella et al., 2008; Moberg et al., 2001; Tane et al., 2014). But even in the presence of aberrantly high CyclinE/Cdk2, cell cycle exit is most often only delayed by one or two cell cycles in vivo, demonstrating the
robustness of developmentally controlled quiescence (Baumgardt et al., 2014; Buttitta et al., 2010; Loeb et al., 2005; Nakayama et al., 1996).

Determining which cell cycle regulators are required for developmentally controlled cell cycle exit \textit{in vivo} has posed some challenges. The redundant functions of multiple paralogs for each cell cycle regulator makes genetic analysis complicated, with studies often encompassing double or triple mutants (Gui et al., 2007; Wirt et al., 2010; Zindy et al., 1999). In addition the late stage of development where cell cycle exit occurs and the asynchronous nature of cell cycle exit in many tissues requires conditional genetic manipulations and timecourse analysis of samples. \textit{Drosophila} eyes and wings have been an advantageous system to study this process, as they undergo a relatively synchronized cell cycle exit during metamorphosis and have fewer paralogs with tools for precise conditional genetic manipulations. We used this system to examine cell cycle exit in terminally differentiating tissues and found that even in retinoblastoma family 1 deficient cells, CyclinE/Cdk2 overexpression delays but cannot bypass cell cycle exit (Buttitta et al., 2007), suggesting that additional downstream mechanisms ensure the transition from proliferation to quiescence \textit{in vivo} (Ehmer et al., 2014; Nicolay et al., 2010; Simon et al., 2009).

To identify additional mechanisms ensuring cell cycle exit, we examined \textit{Drosophila} homologs of several tumor suppressor proteins expected to play a role in promoting quiescence.

PP2A has been recognized as a tumor suppressor for over two decades (Janssens et al., 2005), but the molecular mechanism for PP2A in tumor suppression remains unknown. PP2A de-phosphorylates RB family members to inhibit cycling (Kolupaeva and Janssens,
and removes an essential activating phosphorylation on the Cdk2 T-loop in vitro (Poon and Hunter, 1995). We therefore examined whether PP2A may play multiple, redundant roles to promote the developmentally controlled robust cell cycle exit in vivo.

Here, we show that cells with reduced PP2A function fail to transition to a quiescent state at normal developmental time. Loss of PP2A function specifically during the final cell cycle leads approximately 10% of cells to perform an extra cycle before entry into permanent quiescence. Cells with compromised PP2A exhibit increased Cdk2 activity and aberrant E2F transcriptional activity. In the presence of high, oncogenic Cyclin E the loss of PP2A function allows cells to bypass a robust G0 mechanism during late stages in fly development. The PP2A enzyme is directed to distinct substrates via associations with different regulatory subunits, which can be highly dynamic during development. Here we show that the PP2A-B56 regulatory subunit widerborst (wdb) is specifically required for the PP2A-mediated transition between proliferation and quiescence. Furthermore, this new function for PP2A-B56 occurs even in the complete absence of RB/E2F/DP function, suggesting it acts through downstream targets directly on the cell cycle machinery to promote quiescence in vivo.

Results

Loss of PP2A delays the transition to quiescence in vivo.

We performed a small-scale RNAi-based screen of approximately 500 randomly chosen Harvard Transgenic RNAi Project (TRiP) lines to identify new potential tumor suppressor genes involved in the proper timing of the developmentally controlled
quiescence in the *Drosophila* eye. The primary screen was an adult eye-color based screen, an adaptation of the method described by Bandura and colleagues (Bandura et al., 2013). This was followed by a secondary, dissection-based screen to determine which hits from the initial screen effectively compromised cell cycle exit. Normally, the *Drosophila* eye becomes completely quiescent by 24h after pupa formation (24h APF) (Butitta et al., 2007). We therefore looked for RNAis that compromised quiescence, leading to ectopic cell cycles at 24h APF. We used the *Glass Multimer Reporter (GMR)*-*Gal4* (Ellis et al., 1993) driver to express UAS-controlled RNAi lines, and assayed for ectopic S-phases by 5-ethynyl-2'-deoxyuridine (EdU) incorporation (Buck et al., 2008) and ectopic expression of a cell cycle transcriptional reporter *PCNA-GFP* (Thacker et al., 2003) in eyes after normal quiescence from 24-30h APF (Fig. S1). Importantly, the *GMR-Gal4* driver activates the UAS-RNAi specifically during the final cell cycle in the eye, thereby avoiding earlier deleterious effects. We found that two independent RNAi lines to the *Drosophila* PP2A catalytic subunit *microtubule star (mts)* and one to the sole PP2A scaffold A subunit, *Pp2A-29B*, caused ectopic S-phases and cell cycle gene expression, at timepoints when the *Drosophila* eye should be fully quiescent (Fig. S1 A-C).

To confirm the RNAi results, we overexpressed a dominant negative form of *mts* (*mts^{DN}*) during the final cell cycle in fly eyes and found that it fully recapitulated the RNAi phenotypes. The *mts* dominant negative is a truncation which interacts non-productively with PP2A scaffolding (A) and regulatory (B) subunits, and serves as an effective competitive inhibitor when overexpressed (Evans et al., 1999). To test whether the role for PP2A in quiescence is eye-specific, we overexpressed *mts^{DN} in the posterior
wing during the final 1-2 cell cycles using *engrailed-Gal4*, modified with a temperature sensitive *Gal80^{TS} (en^{TS}, see methods for details). Similar to the eye, we observed ectopic S-phases by EdU incorporation and ectopic mitoses by staining for phosphorylation of Serine-10 on Histone H3 (PH3) at 24-28h APF, timepoints when few cell cycles are normally observed in the wing (Fig. 1A,B). Overexpression of a functional wild-type *mts* (*mts^{WT}) however had no observable effect on quiescence in these tissues (Fig. 1C-D), confirming that the observed phenotype is due to the loss of PP2A function. We performed a timecourse and quantification of the mitoses in wings expressing *mts^{DN} (Fig.1E, S1D) or PP2A RNAis (Fig. 1F), which revealed continued mitoses in eyes and wings until 37h APF, 13h after the normal cell cycle exit in these tissues (Buttitta et al., 2007; Milan et al., 1996; Schubiger and Palka, 1987). To confirm the staining results, we measured the DNA content of pupal eyes expressing *mts^{DN} by flow cytometry. As expected from the ectopic cell cycle markers, an increased proportion of cells containing greater than 2C DNA content was observed in *mts^{DN} expressing eyes compared to stage matched controls without transgene expression (Fig.1G). However after 37hAPF, eyes expressing *mts^{DN} exit the cell cycle with a normal G1 DNA content. Altogether our data suggests that inhibition of PP2A during the final cell cycle causes a temporary delay of the transition to quiescence in a compartment-autonomous manner.

**Inhibition of PP2A leads to an extra cell cycle during the delay of quiescence.**

We next investigated whether the delayed transition to quiescence caused by loss of PP2A leads to additional cell cycles or whether it is the result of a prolonged final cell cycle. To address this, we performed a clonal analysis to count the number of cells per
clone, reflecting the number of cell divisions before quiescence, using the heat shock (hs)-flp actin>stop>Gal4/UAS “flip-out” system. In brief, with this system a precisely timed heat-shock leads to random cis-recombination between FRT sites (indicated by >) flanking a stop codon. Cells where recombination occurs “flip-out” the stop codon to allow Gal4-mediated gene expression, which continues permanently in all daughter cells (Pignoni and Zipursky, 1997). In this manner, the number of daughter cells can be counted after the delayed quiescence at 37h APF. Non-overlapping clones were induced at 0h APF (just prior to the final cycle) by a low-level of heat shock at 37°C. Transgenes to manipulate PP2A activity, as well as GFP to mark clones, and an apoptosis inhibitor (to prevent loss of daughters which confounds clonal cell counts) were expressed and cells per clone were counted blind for at least 100 clones in the wing blade at 42-44h APF (Fig. 2A, S1G). Most (95%) control clones expressing GFP and P35 contain 2 cells or less per clone, as the induction of the recombination occurs during or just prior the final cycle. However, 15% of clones expressing mtsDN contain more than 2 cells per clone, which indicates that approximately 10% of mtsDN expressing cells undergo an extra cell cycle before entering G0. For comparison, when the G1 cyclin complex CyclinD/Cdk4 is directly overexpressed via Gal4/UAS, we observe 50% of cells performing an extra cell cycle, before becoming quiescent at 36h APF (Buttitta et al., 2007).

To determine whether PP2A inhibition also causes extra cell cycles in the eye, we examined the morphology of the fly eye at late pupal stages (40-42h APF). In a wild-type or control retinas (Fig. 2B), the apical ommatidial structure consists of four cone cells in the center surrounded by inter-ommatidial cells (IOCs) (Tomlinson and Ready, 1987).
The IOCs are shared by adjacent ommatidial cores and the number of IOCs can be quantified within an ommatidial group (OG) that covers a defined hexagonal area (Figure 2D) (Ou et al., 2007). When PP2A is inhibited during the final cell cycle in the eye, extra IOCs are observed (17.4±0.3 cells/OG) consistent with approximately 1 extra cell cycle per OG. We confirmed the extra cycles are not due to disruption of programmed cell death in the pupal eye, as the cell number is further increased when apoptosis is inhibited (Fig. 2E,F). The size of the adult eye is also increased when PP2A is inhibited (Fig. 2H). Our cell count data suggests that PP2A inhibition enlarges the eye partly by ectopic cell proliferation, but we also consistently observed an increase in cell size. An increase in cell size is consistent with known functions of PP2A in the TOR/S6Kinase (S6K) pathway (Bielinski and Mumby, 2007; Hahn et al., 2010).

PP2A counteracts the phosphorylation of S6K, which we used as an assay to confirm the activity of our mts transgenes (Fig. S2A). To test whether the increase in active phospho-S6K impacts the transition to quiescence, we overexpressed the GTPase, Rheb, which increases cellular growth, TOR signaling and phospho-S6K (Saucedo et al., 2003). We did not observe any delay in cell cycle exit in the pupal wing, nor extra IOCs in the retina when Rheb is overexpressed, despite increased phospho-S6K (Fig. S2). We therefore suggest the function of PP2A in the transition to quiescence is independent of its role in regulating phospho-S6K.

**Inhibition of PP2A leads to ectopic Cdk2 activity.**

Proper cell cycle exit in *Drosophila* eyes and wings is ensured by inhibition of E2F/DP-mediated transcription and suppression of Cyclin E/Cdk2 activity (Buttitta et al., 2007;
Firth and Baker, 2005). To examine whether cells with inhibited PP2A retain high Cdk2 activity, we used anti-MPM2 staining as an *in vivo* readout for ectopic Cdk2 activity at timepoints after normal cell cycle exit. MPM-2 antibodies detect nuclear Cdk2 phospho-epitopes on the histone locus body (HLB) which occur normally only during S-phase in proliferating cells, in addition to the well-described cytoplasmic epitopes present during mitosis (White et al., 2011; White et al., 2007). We generated GFP marked clones in eyes and wings expressing CyclinE/Cdk2 as a positive control, *mts*<sup>DN</sup> or wild-type *mts* (*mts*<sup>WT</sup>) during the final 1-2 cell cycles and examined MPM2 reactivity at 26h APF, two hours after normal cell cycle exit. We observed abundant nuclear HLB staining by MPM2 in cells expressing CyclinE/Cdk2 and *mts*<sup>DN</sup>, but no MPM2 staining in cells expressing *mts*<sup>WT</sup> (Fig. 3 A-C). This suggests that loss of PP2A leads to ectopic Cdk2 activity in normally postmitotic tissues.

We next tested whether loss of PP2A also leads to a failure to repress E2F/DP transcriptional activity during normal cell cycle exit. We used the E2F-responsive *proliferating cell nuclear antigen* (PCNA) promoter fused to GFP (Thacker et al., 2003) as a readout of ectopic E2F activity at timepoints after normal cell cycle exit. Compromising PP2A function in eyes during the final cell cycle led to ectopic E2F activity at 26h APF, a timepoint when little to no E2F activity should persist (Fig. 3D,E).

The repression of E2F/DP-mediated transcription upon cell cycle exit is modulated by RB binding, which is inhibited by RB phosphorylation via active Cyclin/Cdk complexes or promoted by de-phosphorylation via phosphatases. In mammals, PP2A can modulate the phosphorylation state of the RB- related pocket protein, p107, to promote cell cycle exit in chondrocytes (Jayadeva et al., 2010; Kolupaeva et al., 2008; Kurimchak et al.,
2013). Thus, a plausible mechanism for PP2A regulation of the transition to quiescence could be through inhibition of *Drosophila* retinoblastoma family (Rbf)-mediated repression of E2F/DP transcriptional activity. To genetically test whether endogenous E2F/DP complexes are required for the delay of quiescence caused by PP2A loss, we used the MARCM system (Lee and Luo, 2001) to create *Dp* homozygous null mutant clones (Fig. S3), with and without PP2A inhibition. *Dp* null mutant cells exhibit defects in cell proliferation and *Dp* null mutant clones in larval wings are on average $3.27 \pm 0.21$ times smaller than wild-type clones generated in parallel (Nicolay and Frolov, 2008). We confirmed a similar phenotype for *Dp* mutant clones in pupal wings, which are $3.06$ times smaller than wild-type clones induced in parallel, Fig. 3G, Fig. S3) and *Dp* mutant clones in the pupal wing lack Dp protein (Fig. 3H,I). *Dp* null mutant clones expressing *mts*DN in pupal wings exhibit ectopic mitoses in wings at timepoints after normal cell cycle exit, while no mitoses were observed in any stage-matched *Dp* null mutant clones (Fig. 3G,H). This suggests that the delay of cell cycle exit upon inhibition of PP2A is epistatic to E2F/DP function, and reveals an additional role for PP2A in promoting quiescence independent of RB/E2F/DP complexes *in vivo*.

**Inhibition of PP2A function does not delay cell cycle exit by preventing APC/C activity**

The Anaphase Promoting Complex/Cyclosome (APC/C) promotes timely cell cycle exit in *Drosophila* eyes and wings by degrading residual Cyclin A and Cyclin B during the final G1 (Buttitta et al., 2010; Ruggiero et al., 2012; Tanaka-Matakatsu et al., 2007). Furthermore, the APC/C complex can cooperate with RB proteins to reinforce cell cycle
exit by promoting degradation of Skp2, which targets CKIs for destruction (Binne et al., 2007). PP2A can impact the APC/C indirectly by counteracting Cyclin B/Cdk1 phosphorylations (Hunt, 2013) as well as regulating the binding and stability of the APC/C inhibitor Emi (Wu et al., 2007), which functions similarly to *Drosophila Regulator of Cyclin A1 (Rca1)* (Grosskortenhaus and Sprenger, 2002). We therefore examined whether APC/C function may be inhibited when PP2A is compromised, leading to a delay in cell cycle exit. As a read-out for APC/C activity, we examined the levels of the known APC/C target, Cyclin B (CycB) by immunohistochemistry. GFP-marked clones with transgene expression were induced by the “flipout” Gal4/UAS/Gal80TS system and shifted to permissive temperature during late larval stages (Fig. 4A-C). As a positive control, we inhibited APC/C activity by expression of *Rca1* and observed clear CycB accumulation in GFP positive cells in the posterior of larval eye imaginal discs (Fig. 4A, A’). However, we observed no change in CycB levels in eyes with either PP2A loss-of-function via mtsDN expression or PP2A gain-of-function with mtsWT (Fig. 4B-4C’). We also extracted protein samples from late larval eye imaginal discs and performed western blots to measure total levels of Cyclins A and B. We found that neither gain-of-function PP2A nor loss-of-function PP2A significantly increased CycA or CycB levels (Fig. 4D,E).

We next examined whether CycB/Cdk1 complex activation may be altered by PP2A inhibition during cell cycle exit *in vivo*. The activation of the CycB/Cdk1 complex is triggered by the removal of inhibitory phosphates on Cdk1 (at Y14 and T15) by the phosphatase cdc25c, termed *string* in *Drosophila*. The activity of *string* is rate-limiting for entry into mitosis in the wings and eyes *in vivo* (Neufeld et al., 1998) and persistent
CycB/Cdk1 activity could delay proper cell cycle exit. However we did not observe significant effects on Cdk1 inhibitory phosphorylations under genetic manipulations of PP2A activity (Fig. 4F), in contrast to ectopic expression of *string*, which strongly reduces Cdk1 inhibitory phosphorylation as expected (Fig.4F).

**PP2A interacts with negative regulators of CyclinE/Cdk2 activity in vivo.**

Consistent with the evidence of ectopic Cdk2 activity when PP2A is compromised (Fig. 3 A-C), we also observed functional genetic interactions between known negative regulators of Cdk2 activity and PP2A in the fly eye (Fig. 5). The sole p21/p27 CKI in *Drosophila*, *dacapo (dap)*, is a major inhibitor of the CyclinE/Cdk2 complex upon cell cycle exit (de Nooij et al., 1996; Lane et al., 1996; Sukhanova and Du, 2008). We examined whether loss of *dap* cooperates with inhibition of PP2A to delay quiescence by quantifying IOCs in late pupal stages as described previously for Fig. 2. The loss of one copy of *dap* (using the *dap*4 null allele) enhanced the effect of *mts*DN expression (driven by *GMR*-Gal4) on the number of IOCs (18.7±0.3), compared to PP2A inhibition alone (17.3 ±0.2) (Fig. 5A-5D). We also observed a 15% increase in adult eye size in *dap* heterozygotes expressing *mts*DN compared to siblings with normal *dap*, while *mts*DN expression alone caused ~8% increase in adult eye size (Fig. 5E). In addition, we used MARCM system to create *dap* homozygous null mutant clones, with and without PP2A inhibition via expression of *mts*DN. In *dap* null mutant clones expressing *mts*DN, we also observed an increase in extra cells including an increase in lens-producing cone cells, which is rarely seen in wild-type clones expressing *mts*DN (Fig.5G-I, S4I). In a reciprocal experiment, we overexpressed *dap* together with *mts*DN during the final cell cycle using
the GMR-Gal4 driver, and observed a partial suppression of the enlarged eye phenotype caused by PP2A inhibition alone (Fig. 5F). This indicates that high Cdk2 activity is at least in part, required for the enlarged eye phenotype resulting from PP2A inhibition.

To confirm that the enhancement of the *dap* eye phenotypes by *mts*\(^{DN}\) were due to impacts on CyclinE/Cdk2 function, we next examined a different negative regulator of CyclinE for genetic interactions with PP2A. Cyclin E (CycE) protein level is controlled by the SCF complex with the ubiquitin ligase Fbw7, termed *archipelago* (*ago*) in *Drosophila*. Loss of *ago* in the fly leads to aberrant accumulation of CycE protein and temporarily delays cell cycle exit of the bristle precursors in the eye (Moberg et al., 2001). Consistent with our results from loss of one copy of *dap*, we found that loss of one copy of *ago* (using the *ago\(^{1}\)* allele) also enhanced the *mts*\(^{DN}\) large eye phenotype (Fig. S4).

We next examined whether modulation of PP2A activity itself could impact CycE protein levels or stability during the final cell cycle. We used *GMR-Gal4* to drive expression of *mts*\(^{DN}\) or *mts*\(^{WT}\) during the final cell cycle in the eye and extracted protein from larval eyes for western blot analysis. We observed no significant increase in CycE protein levels when PP2A was compromised (Fig S4). Altogether our genetic data indicates that PP2A acts through a pathway independent of RB/E2F/DP, and possibly in parallel to *dap* or *ago* to regulate CycE/Cdk2 activity.

**PP2A inhibition cooperates with high Cyclin E to bypass robust cell cycle exit.**

High CyclinE/Cdk2 activity during the final cell cycle in fly tissues delays cell cycle exit, but after only a few extra cell cycles a robust cell cycle exit mechanism ensures
permanent quiescence (Baumgardt et al., 2014; Buttitta et al., 2010; Buttitta et al., 2007).

We asked whether PP2A inhibition could promote cells with aberrantly high CycE expression to override the robust transition to quiescence and maintain proliferation during later stages in development, as suggested by its known role as a tumor suppressor.

To test this, we used GMR-Gal4 to drive UAS-CycE expression together with the Baculoviral apoptosis inhibitor P35 (to minimize corrective apoptosis) with or without PP2A inhibition via mts\textsuperscript{DN}. We examined proliferation in the eye at late pupal stages, several hours after the normal robust exit that occurs even in the presence of de-regulated CycE (Fig. 6A-D). Pupal eyes expressing Cyclin E without any PP2A modulation exhibit few S-phases and mitoses at this late stage of development, while eyes expressing CycE together with mts\textsuperscript{DN} maintain high proliferation even after the stage normally associated with robust permanent cell cycle exit. To further confirm this, we isolated late pupal eyes and performed flow cytometry to examine the DNA content in eyes at 46h APF. When eyes overexpress CycE, only about 9% of cells from the entire retina exhibit an abnormal S/G2 DNA content. By contrast, when PP2A is compromised in stage-matched eyes over-expressing CycE, 27% of cells exhibit abnormal S/G2 DNA contents (Fig. 6F). This suggests that PP2A normally functions as a barrier to limit the bypass of cell cycle exit when CycE is de-regulated in vivo.

**The PP2A B56 subunit widerborst regulates the transition to quiescence in vivo.**

To identify the PP2A regulatory subunit responsible for promoting quiescence in differentiating tissues, we systematically tested each PP2A regulatory subunit in *Drosophila* for phenotypes in the eye (Supplemental Table 1). We used RNAi to
knockdown regulatory subunits during the final cell cycle, and compared the adult eye sizes of progeny (Fig. S5A). Inhibition of the *Drosophila* B56 epsilon homolog (also called PPP2R5E) *widerborst* (*wdb*) led to an enlarged eye phenotype, similar to what we observe with *mts*<sup>DN</sup> expression, whereas knockdown of the B55 homolog *twins* causes a decrease in eye size, perhaps due to defects in mitosis (Brownlee et al., 2011; Chen et al., 2007). To test directly whether *wdb* is required for cell cycle exit, we used a dominant negative form of *wdb*, *wdb*<sup>DN</sup> (Hannus et al., 2002). Expression of *wdb*<sup>DN</sup> driven by *en*<sup>TS</sup>-Gal4 during the final 1-2 cycles in the wing or *GMR-Gal4* driving *wdb*<sup>RNAi</sup> during the final cell cycle in the eye, leads to ectopic S-phases and mitoses in tissues at developmental timepoints that are normally quiescent (Fig. 7A,A’, Fig. S5C,D). We also observed ectopic E2F/DP transcriptional activity at normally postmitotic stages when *wdb* is knocked down (Fig. S5I). We quantified the mitotic index in pupal tissues expressing *wdb*<sup>DN</sup> at 26h APF (Fig. 7C), and found that the defect in cell cycle exit upon *wdb* inhibition is less dramatic than the defect caused by inhibition of *mts*. We suggest that either the dominant-negative *wdb* does not completely block *wdb* function, or *wdb* may not be the only PP2A regulatory subunit involved in the transition to quiescence and other subunits may provide some partially overlapping functions.

Consistent with our previous tests of genetic interactions between PP2A and negative regulators of CycE/Cdk2 activity, we observed that adult eye size is increased by 15% in *dap* heterozygotes expressing *wdb*<sup>DN</sup> compared to *dap*<sup>WT</sup> siblings (Fig. 7E). Inhibition of *wdb* alone causes an ~8% increase in adult eye size, suggesting that loss of one copy of *dap* synergizes with inhibition of *wdb*, similar to the genetic interaction we observed with *mts*.
We next tested whether *wdb* contributes to the role of PP2A as a barrier to limit the bypass of cell cycle exit when CycE is de-regulated *in vivo*. We examined the morphology of the ommatidial structure in pupal retinas expressing CycE or CycE + *wdb*<sup>DN</sup> as described previously for Fig. 6. The expression of *wdb*<sup>DN</sup> and CycE expression together dramatically enhances the number of IOCs (Fig. 7F,G), indicative of continued cycling in the late pupal retina. By contrast, when the B55 family regulatory subunit (*twins*) is knocked down by RNAi in the CycE expressing background, there is no obvious difference in IOC cell number compared to CycE expression alone (Fig. S5J-L). Our data thus indicate that *wdb* contributes to the role of PP2A as a barrier to limit proliferation when CycE is de-regulated in terminally differentiating tissues.

**Inhibition of PP2A increases the T-loop phosphorylation of Cdk2.**

Our data suggest that PP2A may promote quiescence by limiting Cdk2 activity during the final cell cycle *in vivo* to restrict proliferation in terminally differentiating tissues. To test this hypothesis, we compromised PP2A function *in vivo* by expressing *mts*<sup>DN</sup> and CycE in the posterior larval eye under the control of the *GMR-Gal4* promoter, followed by immunoprecipitation of CycE to measure effects on CycE/Cdk2 kinase activity. When *mts*<sup>DN</sup> is expressed in the posterior larval eye, we observe a 20-40% increase in CycE/Cdk2 kinase activity after normalization to the amount of CycE pulled down (Fig. 8A). One interpretation of this result is that PP2A knockdown leads to an increase in CycE/Cdk2 activity, however it is also possible that the observed increase in CycE/Cdk2 kinase activity is a result of the increased proliferation we observe when CycE is expressed under conditions where PP2A is compromised (e.g. Fig 6B,D) and not a direct
effect of PP2A on CycE/Cdk2 activity. To distinguish whether the increased Cdk2 activity is due to an immediate effect of PP2A inhibition on CycE/Cdk2, we performed a kinase assay in *Drosophila* S2R+ cultured cells, where we can use short-term treatments with the pan-PP2A inhibitor Okadaic Acid (OA) to discern immediate versus indirect effects of PP2A inhibition on CycE/Cdk2 activity. We performed a timecourse and dosage test of OA treatment in S2R+ cells and confirmed that with 30 minutes of OA treatment, PP2A activity is inhibited as assessed by increased phosphorylation of S6Kinase. We therefore performed a timecourse of OA treatment on S2R+ cells transiently transfected with a CycE expression vector and performed CycE/Cdk2 kinase assays as described above. We found that with 30min of OA treatment, S2R+ cells exhibit a mild increase in CycE/Cdk2 activity (Fig. 8C), consistent with a direct effect of PP2A on CycE/Cdk2 activity. However upon longer OA treatments (2h shown), cells exhibit a reduction in CycE/Cdk2 kinase activity and a slower migrating form of CycE protein is immunoprecipitated (Fig.8D). In mammalian cells, PP2A/B55 can dephosphorylate the N- and C-terminal phosphodegrons of CycE1 (Tan et al., 2014). Thus the slower migrating form of CycE we observe may be due to inhibition of PP2A/Twins (B55) by OA in *Drosophila* which impacts the measured CycE/Cdk2 activity. Altogether, our data suggests that short-term inhibition of PP2A can increase CycE/Cdk2 activity, while a prolonged loss of PP2A function impacts CycE/Cdk2 in a complex manner, due to differing functions of multiple PP2A complexes. We suggest there may be smaller contribution of PP2A/B55 complexes to the overall PP2A activity during the final cell cycle *in vivo*, compared to actively proliferating S2R+ cells *in vitro.*
PP2A can bind and remove an activating phosphate on the T-loop of human Cdk2 in vitro (Poon and Hunter, 1995) and the T-loop and critical activating phosphorylation sites are conserved between mammals and Drosophila. To test whether PP2A complexes limit CyclinE/Cdk2 activity after mitosis by removing the Cdk2 T-loop phosphorylation, we turned to murine cell lines where cells can be synchronized in M-phase and Cdk2 phospho-T-loop specific antibodies are available. We synchronized NIH 3T3 mouse embryonic fibroblasts (MEFs) in M-phase with a nocodazole treatment to de-polymerize microtubules. We then released cells from the mitotic arrest and performed a time-course analysis of Cdk2 T-loop phosphorylation in cells treated with the pan-PP2A inhibitor Okadaic Acid (OA) versus vehicle only. We observed that 8h after release from a mitotic arrest, treatment with OA for 30 minutes increases T-Loop phosphorylation 2-fold over a vehicle treated control (Fig. 8F). We next tested whether a similar OA treatment in asynchronously proliferating mouse fibroblasts could lead to an increase in Cdk2 T-loop phosphorylation. We observed a mild increase (20%) on Cdk2 T-loop phosphorylation in 3T3 MEFs, while we detected no effect on Cdk2 T-loop phosphorylation in primary asynchronous MEFs. This suggests that redundant mechanisms may limit the effect of PP2A on the T-loop in primary cells. However, we observed a 50% increase in Cdk2 T-loop phosphorylation in p27-knockout (p27KO) primary MEFs treated with OA (Fig.8G), suggesting that PP2A may preferentially act on Cdk2 complexes that are not bound to Cdk inhibitors. We also observed increased levels of Cdk2 in p27KO MEFs suggesting the role of PP2A may be fully revealed under conditions where Cyclin E/Cdk2 levels are high, but need to be rapidly inhibited. This is consistent with the genetic interactions we observed in Drosophila between PP2A and the p27 homolog dacapo.
An interaction between *Drosophila* Wdb and Cdk2 in a yeast two hybrid assay has been reported (Stanyon et al., 2004). To confirm whether PP2A/Wdb complexes interact with CycE/Cdk2 complexes, we performed an immunoprecipitation of endogenous Cyclin E with a V5-tagged Wdb in proliferating S2R\(^+\) cells (Fig. S6A). We observe a mild enrichment of Cyclin E in samples with Wdb-V5 pulled down, compared to controls and mock precipitations. The enrichment of CycE may be mild because PP2A/Wdb interacts with many substrates in a transient manner throughout the cell cycle. We propose that only a fraction of the precipitated Wdb-V5 complexes at a given time from asynchronously proliferating cells will therefore contain endogenous CycE. To examine this in more detail, we next transfected CycE and Wdb-V5 expression vectors in S2R\(^+\) cells, and examined cells for co-localization of the proteins during the cell cycle. We found that Cyclin E and Wdb-V5 co-localize in the cytoplasm during mitosis (Fig.S6B). Cyclin E is predominantly nuclear, but becomes dispersed in the cytoplasm during nuclear envelope breakdown in mitosis, while Wdb is predominantly in the cytoplasm. This suggests that PP2A/Wdb complexes most likely interact with Cyclin E/Cdk2 complexes transiently during or just after mitosis, before nuclear envelope re-formation. This is consistent with our results in 3T3 cells, which suggest that the maximal effect of PP2A on the Cdk2 T-Loop occurs about 8 h after a mitotic release. Altogether, our studies suggest that B56/PP2A can act to restrict CycE/Cdk2 activity after mitosis to promote quiescence *in vivo*. 
Discussion

We identify a new role for PP2A in promoting quiescence during the transition to a permanently postmitotic state in *Drosophila* wings and eyes. In our studies we observe that approximately 10% of cells undergo an extra cell cycle when PP2A functions are compromised. While this effect may appear small, the cell cycle exit mechanism *in vivo* is so robust that cells completely lacking major cell cycle regulators such as the RB family member *rbf1* or the sole p21/p27-type CKI *dacapo* only exhibit a mitotic or S-phase index of 9% or less in eyes and wings (Buttitta et al., 2007; Sukhanova and Du, 2008). As with other cell cycle regulators that act redundantly to promote cell cycle exit, we see synergism when PP2A is compromised under conditions de-regulating the G1-S Cyclin, Cyclin E (Firth and Baker, 2005).

Cells with reduced PP2A function exhibit ectopic cell cycle markers until 13h after normal exit timing, which is roughly consistent with the one extra cell division we measure by clonal lineage analysis. Importantly, the ectopic proliferation phenotypes we observe are the result of manipulating PP2A functions specifically during the final 1-2 cell cycles, without disturbance of prior PP2A mitotic functions during active proliferation.

**PP2A impacts the proliferation-quiescence decision *in vivo***

Recent studies on PP2A in the proliferation-quiescence decision have revealed that PP2A activates the retinoblastoma protein related family member p107 by dephosphorylation to promote growth arrest in chondrocytes (Kolupaeva et al., 2008; Kurimchak et al., 2013). Another group recently found a second mechanism for PP2A to promote quiescence,
whereby PP2A/B56 inhibits Ras signaling during G2 phase which limits subsequent Myc expression and reduces Cyclin E expression in the following G1 (Naetar et al., 2014). This promotes quiescence by limiting Cyclin E, which would otherwise disrupt the association of RB family members with E2F/DP complexes by phosphorylation. Our data however suggest there is yet another mechanism during the final cell cycle in vivo, independent of Ras/ERK signaling (Fig. S7), dMyc (Fig. S7), Cyclin E levels (Fig. S4), and RB/E2F/DP function (Fig. 3), which promotes timely entry into quiescence. This additional mechanism acts directly on the cell cycle machinery, downstream or in parallel to RB/E2F/DP function, which appears to be critical for the extremely robust type of developmentally controlled G0 observed in vivo.

**B56 regulatory subunits promote quiescence in vivo.**

The emergence of multiple pathways for PP2A to promote quiescence may be due to PP2A’s broad functions, with impacts on various substrates in different cell cycle phases (Janssens et al., 2005; Mumby, 2007; Westermarck and Hahn, 2008; Yang and Phiel, 2010). In normal development, cells enter into quiescent state in response to developmental signals, while in cell culture serum deprivation is most often used for the synchronization in G0, via disrupted metabolic signals (Naetar et al., 2014). It may be that in these different biological contexts, PP2A acts upon different targets to influence the proliferation-quiescent decision. PP2A is directed to distinct targets via the regulatory subunit, which has dynamic associations and localizations during the cell cycle. It is therefore important to note that consistent with the recent work of Naetar et. al., we also independently identified a B56 regulatory subunit (wdb) as the main PP2A regulatory
subunit promoting quiescence in vivo. However our data demonstrates that \textit{wdb} acts via a different mechanism to promote permanent cell cycle exit in vivo.

Most known cell cycle functions for PP2A in \textit{Drosophila} involve the B55 regulatory subunit \textit{twins} and its roles in regulating mitotic entry and exit (Brownlee et al., 2011; Chabu and Doe, 2009; Chen et al., 2007; Wang et al., 2013; Wang et al., 2011). Consistent with this, when we manipulate PP2A activity in early tissues such as the actively proliferating larval wing or eye, we also observe disruptions of mitosis. An inhibitory role for PP2A in the Hippo signaling pathway which regulates tissue growth, survival and proliferation has also been described (Ribeiro et al., 2010). However the role for PP2A inhibiting Hippo signaling acts via B''' regulatory subunits and exactly opposite to the growth and cell cycle phenotype we observe here. The requirement for \textit{wdb} during the final cell cycle to promote quiescence implies that the PP2A enzyme complexes may switch from predominantly B55 (\textit{twins}) to B56 (\textit{wdb}) during the final cell cycle, mitotic exit and the subsequent G0 arrest. Understanding how the switches in PP2A regulatory subunits are regulated during the cell cycle and in response to developmental signals will be an important area for future study.

**PP2A inhibits CyclinE/Cdk2 activity during the final cell cycle to promote quiescence.**

A recent study monitoring single-cell cycle dynamics in cell culture demonstrated that thresholds of Cdk2 activity after the completion of mitosis regulate the subsequent proliferation-quiescence decision (Spencer et al., 2013). Our data suggest a role for PP2A in limiting Cdk2 activity during the final cell cycle in vivo to restrict proliferation in
terminally differentiating tissues. Inhibition of PP2A during the final cell cycle leads to ectopic Cdk2 activity as detected by the anti-MPM2 epitopes at the HLB (Fig. 3) and genetically cooperates with Cyclin E inhibitors, ago and dacapo. In mammalian cells, PP2A inhibition after mitosis leads to an increase in the activating Cdk2 T-loop phosphorylation. It is possible that PP2A and Cyclin E/Cdk2 also share downstream targets in cell cycle regulation, similar to the role of PP2A/B55 complexes in reversing Cdk1 phosphorylation of mitotic targets. However, we could not confirm any effect of PP2A genetic manipulations on the phosphorylation of Drosophila Rbf, an important target of Cyclin E/Cdk2 activity for cell cycle exit in flies (Meyer et al., 2000) (Fig.S6C). We suggest that PP2A/Wdb acts to modulate Cyclin E/Cdk2 activity during the final cell cycle to help promote rapid and timely induction of G0 during development.
Fig. 1. PP2A promotes the timely transition to quiescence in vivo. (A-D) Expression of a dominant negative mts (mts$^{DN}$ in A,B) or wildtype mts (mts$^{WT}$ in C,D) was restricted to the posterior wing during late larval stages using the engrailed-Gal4/temperature-sensitive Gal80,UAS system (en$^{TS}$). Pupal tissues were dissected at 24h APF and labeled with EdU for 1h to visualize S-phases (A,C) or labeled with anti-Phospho-histone H3 Ser10 (PH3) to visualize mitoses at 28h APF (B,D). In regions where PP2A function is compromised by mts$^{DN}$, cells continue cycling when they should be postmitotic. (E,F) Quantification of ectopic mitoses in pupal tissues at different time points during normally postmitotic stages reveal a delay of cell cycle exit by about 10h. (G) Flow cytometry was used to assess the DNA content of 25h APF eyes with mts$^{DN}$ expression (green trace) or controls (black trace). The arrow indicates an increase in cells with non-G1 DNA content. Bar = 50um.
Fig. 2. PP2A inhibition during the final cell cycle leads to extra cell divisions during tissue development. (A) Clonal-lineage analysis in the wing was used to measure the number of cell cycles before entry into quiescence. GFP marked clones were induced at the start of metamorphosis, 0h APF during the final cell cycle using the hs-Flp
actin>Gal/UAS system. Wings were examined 42-44h later and cells/clone were quantified for at least 100 clones/genotype. Clones also express the apoptosis inhibitor P35 to prevent apoptosis. Approximately 10% of cells undergo an extra cell cycle when PP2A is inhibited during the final cell cycle to generate an increase in clones with >2 cells. (B-F) GMR-Gal4/UAS was used to drive expression of the indicated transgenes in the eye, specifically during the final cell cycle. Quantification of interommatidial cell (IOC) number was performed at 40-42h APF in retinas stained for Dlg to reveal cell morphology. Cell types of IOC were labeled as: B, bristles; 2\textsuperscript{o}, secondary pigment cell; 3\textsuperscript{o}, tertiary pigment cell. IOCs are shared by adjacent ommatidia and the number of IOCs was quantified within an ommatidial group (OG) that covers a defined hexagonal area (bordered by yellow dots in C,D). The secondary pigment cells crossed by the hexagonal boundary were counted as half. At least 15 OG’s were scored from independent samples for each genotype (B). Representative examples are shown for w\textsuperscript{1118} (C), GMR>mts\textsuperscript{DN} (D), GMR>P35 (E) and GMR>P35 + mts\textsuperscript{DN} (F). (G-I) The lateral surface area of adult fly eyes were measured and compared between w\textsuperscript{1118}, GMR (G) and GMR>mts\textsuperscript{DN} (H). N=8 for I. P-values were determined by Student’s t test (**P< 0.01).
Fig. 3. Inhibition of PP2A leads to ectopic Cdk2 and E2F activity during the final cell cycle. (A–C’) Pupal wings containing clones expressing the indicated transgenes at 24h APF via the hs-flp actin>Gal4/UAS system, were stained with MPM2 antibody. MPM2 recognizes subnuclear foci (arrows) corresponding to Cdk2 phosphorylated epitope(s) at the Histone Locus Body. This is in contrast to the cytoplasmic staining
(arrowhead) that indicates mitotic MPM2 phospho-epitopes. (A,A’) CyclinE/Cdk2 overexpression results in MPM2 foci within clones. (B,B’) Inhibition of PP2A function via expression of mts<sup>DN</sup> leads to MPM2 subnuclear foci. (C,C’) No ectopic MPM2 foci are observed in clones expressing mts<sup>WT</sup>. (D,E) Pupal eyes were assessed at 26h APF, a stage normally postmitotic, for E2F transcriptional activity using the PCNA-GFP reporter transgene. (E) Expression of mts<sup>DN</sup> during the final cell cycle via GMR-Gal4/UAS leads to ectopic E2F activity, when tissues should be postmitotic. (F) Quantification of the PCNA-GFP reporter intensity was normalized to DNA content and compared between control (w<sup>1118</sup>) and mts<sup>DN</sup>. P-values were determined by Student’s t test (**P< 0.01). Wild type or Dp<sup>a3</sup> null mutant clones were induced using the MARCM system by a 20 min heat shock at 37°C at early L3 stage. Clones were examined and measured at 24-26h APF. A scatter plot (G) of clone sizes reveals that the average area of dDp<sup>a3</sup> null mutant clones compared to wild-type control MARCM clones generated in parallel. (H-I’) Wild type or Dp<sup>a3</sup> null mutant clones were stained with Dp antibody. Dp<sup>a3</sup> mutant clones lack Dp protein. (J-K’) Loss of PP2A delays cell cycle exit independent of E2F activity. Dp null mutant clones (J,J’) or Dp null mutant clones expressing mts<sup>DN</sup> (K,K’) were induced as above, and assayed for ectopic mitoses via anti-PH3 at a time normally postmitotic, 26h APF. Clones were marked by GFP. White lines outline the clones and yellow arrows indicate ectopic mitoses within the clones.
Fig. 4. APC/C activity is not compromised by reduced PP2A function during the final cell cycle. (A-C’) Late L3 instar larval eye imaginal discs were isolated and stained with anti-Cyclin B in red, DNA in blue. Clones were induced by the hs-Flp actin-Gal4/UAS system and marked by GFP. As a positive control, overexpression of Rca1 (A,A’) resulted in accumulation of Cyclin B protein via inhibition of the APC/C. By contrast, no obvious change in Cyclin B level was observed for either mts<sup>DN</sup> overexpression (B,B’) or wildtype mts overexpression (C,C’). Note that Cyclin B staining is observed in R8 photoreceptors as previously described (Ruggeiro et al., 2012). (D-F) Western blots of Cyclin B, Cyclin A, or phospho-Cdk1 (p-Cdk1) levels with mts<sup>DN</sup> or wildtype mts overexpression. Protein samples were collected from either late L3 instar
larval eye imaginal discs with transgene expression under control of *GMR-Gal4* (D,E) or late L3 instar larval heads with transgene expression induced by the *hs-Flp actin>*Gal4/UAS system (F). Altering PP2A activity did not increase Cyclin B or Cyclin A, nor strongly alter the ratio of p-Cdk1/total Cdk1. Note that expression of the Cdk1 phosphatase Stg significantly reduces pCdk1, and serves as a positive control. Bar graphs show the quantification of signal intensities from two independent experiments.
Fig. 5. PP2A genetically interacts with negative regulators of CyclinE/Cdk2 activity in vivo. (A-D) The number of IOCs is modulated by PP2A and dacapo in pupal eyes. 

\( w^{1118}, GMR \) (A) \( GMR>mts^{DN} \) (B) \( dap^{+/+}, GMR>mts^{DN} \) (C) pupal retinas were isolated at 42h APF and stained for Discs Large protein (Dlg), to determine the numbers of IOCs.
IOC quantification is shown in D. Loss of one dap allele exacerbates the ectopic cell proliferation in pupal retinas caused by PP2A inhibition alone. (E) The lateral surface area of each adult eye was measured, and compared to the area of dap+/dap+; GMR-Gal4/+ control siblings. The change in eye size is presented as the percentage change from the control siblings. All animals were raised under identical conditions within the same vials. Positive values represent increases in eye size. (F) The area of each adult eye for the indicated genotypes was measured at the lateral view, and normalized to total head size by measurements of the distance between fronto-orbital to postvertical bristles, as animals were raised in parallel but in separate vial crosses. (G-I) GFP labeled mutant clones were induced using the MARCM system at the early third instar larval stage. In dap mutant clones and dap mutant clones expressing mtsDN, extra cone cells were quantified at 41h APF (I). Yellow arrows indicate examples of ommatidia with extra cone cells. P-values were determined by Student’s t test (*p<0.05; **p<0.01) N=10.
**Fig. 6. Loss of PP2A function cooperates with high CyclinE to bypass cell cycle exit.**

(A-D) Pupal eyes expressing Cyclin E and the apoptosis inhibitor P35 under control of GMR-Gal4 were stained for mitoses (with anti-PH3) or S-phases (via EdU incorporation) at 40-44h APF. Eyes with mtsDN expression in the presence of high Cyclin E exhibit an increase in both EdU and PH3 staining (B,D), compared to the high Cyclin E control (A,C). (E-F) pupal eyes expressing mtsDN alone under control of GMR-Gal4 were stained for mitoses or S-phases at 40-44h apf. (G) EdU was quantified within a central area of the pupal eye and compared between genotypes (N=6 **P< 0.01 by Student’s t test). (H) FACS analysis of DNA content was performed on 46h APF pupal eyes with high Cyclin E and mtsDN expression and compared to controls with high Cyclin E. Cells with an S/G2 DNA content are increased when PP2A function is compromised.
Fig. 7. The B56/wdb regulatory subunit promotes the transition to quiescence in terminally differentiating tissues. (A-B) Dominant negative wdb (wdb\textsuperscript{DN}) was expressed in the posterior wing from mid-L3 using en-Gal4/Gal80\textsuperscript{TS}. Pupal tissues were labeled with EdU for 1h at 23h APF to visualize S-phases (A) or labeled with anti-PH3 at 26h APF to visualize mitoses (B). Bar = 50um. (C,D) Quantification of ectopic mitoses in pupal wings from 24-26h APF (C) and eyes at 27h APF (D). (E) Quantification of adult eye sizes show an increase of >8% when wdb\textsuperscript{DN} is expressed during the final cell cycle using GMR/Gal4. Loss of one allele of dap enhances this phenotype, while loss of one allele of dap alone increases eye size <8%. (F,G) Pupal retinas were isolated at 42h APF and stained for Dlg to visualize IOCs in the sensitized GMR>CyclinE+P35 background (F) IOC numbers increase, forming multiple layers between cone cell clusters in this background when wdb\textsuperscript{DN} is expressed (G).
Fig. 8. PP2A affects Cdk2 T-loop phosphorylation. (A,B) CycE/Cdk2 activity was measured via an in vitro kinase assay using Histone H1 as a substrate. Protein samples were collected from larval eyes co-expressing Cyclin E, the apoptosis inhibitor P35, with...
or without mts\textsuperscript{DN} under control of GMR-Gal\textsuperscript{4}. CycE was immunoprecipitated, while mock precipitations (without CycE antibody) were preformed with the same lysate. (C,D) S2R+ cells transfected with a CycE expression vector were treated with 50nM OA for either 30min or 2h versus vehicle (DMSO) only. Kinase assays were performed on immunoprecipitated CycE. (E) The T-loop phosphosite of Cdk2 is conserved in human, mouse and \textit{Drosophila}. (F) Murine 3T3 cells were arrested in G2/M using nocodazole and subsequently released from arrest. A timecourse was performed, to examine endogenous Cdk2-T-loop phosphorylation in samples treated with vehicle only or the PP2A inhibitor Okadaic Acid (OA) for 30 minutes at the indicated timepoint after nocodazole release. An outline of the experimental procedure is shown at left. A representative blot of total Cdk2 and phospho-Cdk2 after nocodazole release is shown at right. The line graph shows quantification of the OA treated/vehicle treated phospho-Cdk2 ratio with 2-3 independent biological replicates at each timepoint. Error bars indicate s.e.m. (G) In asynchronously proliferating murine 3T3s treatment with OA causes a 20% increase in Cdk2 T-loop phosphorylation, while primary p27\textsuperscript{WT} MEFs show no increase. In contrast, asynchronously proliferating p27\textsuperscript{KO} MEFs exhibit a 50% increase in Cdk2 T-loop phosphorylation upon OA treatment.
Materials and Methods

Fly stocks

w^{1118}

y w hsflp^{122};+;UAS-CycE,UAS-Cdk2 (Buttitta et al., 2007)
y w hsflp^{122};UAS-CycD,UAS-Cdk4;+ (Datar et al., 2000)
y w hsflp^{122};UAS-CycA;+ (Jacobs et al., 2001)
y w hsflp^{122}; UAS-Stg/CyO-GFP; + (Neufeld et al., 1998)
y w hsflp^{122};+;UAS-Dacapo (Neufeld et al., 1998)
y w hsflp^{122}; +; UAS-HA-Rca1/TM6B
y w hsflp^{122};+;UAS-Rbf (Neufeld et al., 1998)
y w hsflp^{122};+;UAS-Rbf^{RNAi}

w;tub>CD2>gal4,UAS-GFP;tub-gal80^{TS},UAS-Diap (UAS-Diap from (Lohmann et al., 2002))
w;UAS-P35;act>CD2>gal4,UAS-GFP_{NLS} (Neufeld et al., 1998)
FRT42D,Dp^{n3}/CyO-GFP;+ (Frolov et al., 2005)
w;FRT42D,dap^{4}/CyO-GFP (Lane et al., 1996)
w;FRT82B ago^{1}/TM6B (Moberg et al., 2001)
y w hsflp^{122},tub-gal4,UAS-GFP;FRT42D tub-gal80
y w hsflp^{122},GMR-gal4;PCNA-GFP (Bandura et al., 2013)
w; GMR-gal4, UAS-CycE; GMR-P35 (kindly provided by H. Richardson)
w; GMR-gal4; Dr/TM6B
w; en-gal4,UAS-GFP; tub-gal80^{TS}
UAS-P35;+;sb/TM6B
UAS-mts\textsuperscript{DN} (Chabu and Doe, 2009)
UAS-mts\textsuperscript{WT} (Wang et al., 2009)
UAS-wdb\textsuperscript{DN} (Hannus et al., 2002)

**Histology and antibodies**

Pupae, staged from white pre-pupae (0 hr after pupa formation, hr APF) at 25°C, were dissected and fixed as described (Buttitta et al., 2007). Pupal cuticle was removed from wings post fixation. Note that the wing hinge and notum were excluded from our quantifications. Hoechst 33258 (Molecular Probes, 1 μg/ml) labels nuclei. Antibodies Used: Rabbit α-phospho-Ser10-histoneH3 (PH3, Upstate, 1:4,000), mouse α-MPM2 (Upstate, 1:200), rabbit α-GFP (Molecular Probes, 1:1,000), mouse α -CycB (DSHB,F2F4, 1:100), mouse α-Discs Large (DSHB,4F3, 1:100), anti-\textit{Drosophila} Dp (gift from Dr. M. Frolov). Appropriate secondary antibodies were Alexa 488, 568 or 633 conjugated (Molecular Probes) or HRP conjugated (Jackson ImmunoResearch) and used at 1:4,000. EdU incorporation was performed using Click-iT EdU Alexa Fluor 555 Imaging Kit from Life Technologies.

**IOC counting**

The IOCs are shared by adjacent ommatidial cores, and the number of IOCs is quantified within an ommatidial group (OG) that covers a defined hexagonal area (bordered by yellow dashed dots in Fig.2) with its apices being the adjacent ommatidial centers. Those secondary pigment cells crossed by the dashed lines were counted as half. At least 15
OG’s were scored from independent samples for each genotype. (Method from (Ou, Wang, Jiang, & Chien, 2007))

**Clonal analysis**

Clones were induced by heat shock for 7 min. at 37°C between 48-70hr AED in a *hsflp; tub>CD2>Gal4, UAS-GFP; tub-Gal80\textsuperscript{TS}, UAS-Diap* background. Animals were aged at 18°C (permissive for Gal80\textsuperscript{TS}, Gal4 OFF), and shifted to 28°C (non-permissive for Gal80\textsuperscript{TS}, Gal4 ON) at late L3 instar larval stage, collected at 0h APF and aged to different stages in metamorphosis. Experiments using *engrailed*-Gal4 with Gal80\textsuperscript{TS} were carried out in the same way, except that experiments restricting transgene expression to the final cell cycle were shifted to 28°C at 0h APF. By phenotypic analyses and GFP visualization, we confirmed complete inhibition of Gal4 in the lines used here with Gal80\textsuperscript{TS} at 18°C, and we detected activation of target genes within 6 hr of shifting to 29°C. Development at 28°C proceeds 1.15 times faster than at 25°C, and 2.2 times more slowly at 18°C (Ashburner, 1989). All incubation times were adjusted accordingly. Hours APF are presented as the equivalent time at 25°C for simplicity. Loss-of-function clones were generated using MARCM (Lee and Luo, 2001). Larvae were heat shocked for 20 min at 37°C at early third larval instar, collected for staging at 0 hr APF, aged at 25°C and dissected at the indicated times.

**Clonal cell counts to quantify cell divisions**

Non-overlapping clones labeled with GFP, expressing the indicated transgenes, were induced at 0 h APF (white prepupae) with 2 min heat-shock at 37°C. Wings were
dissected and fixed 40-42 h later, nuclei were labeled with 1 µg/ml Hoechst 33258, and GFP-positive cells per clone were scored blind on a Leica DMI6000 microscope. Cells per clone were counted blind for at least 100 clones in the wing blade and the average cell number per clone reflects the number of cell divisions that undergoes before exit. We excluded clones in the wing margin, hinge, notum area, and hemocytes in the veins. Transgenic expression of an apoptosis inhibitor (UAS-P35) was used in the clonal cell count experiments, including all controls.

**Flow Cytometry**

Dissociation of cells from staged, dissected pupae and FACS were carried out as described (Flegel et al., 2013). All experiments were carried out at least three times; representative examples are shown.

**Western Blotting and Kinase Assays**

Antibodies used: rabbit anti-CycE (Santa Cruz, sc-33748), goat anti-CycE (Santa Cruz, sc-15905), mouse α-CycA (DSHB,A12, 1:1000), mouse anti-CycB (DSHB, 1:1000), rabbit anti-phospho Cdc2(Cell Signaling, 1:1000), rabbit anti-Cdc2 (Upstate, 1:1000), anti-phospho S6K(Thr398)(Cell Signaling, 1:333), anti-dmyc (Santa Cruz, sc-28207), anti-dpERK (Sigma, M8159,1:500), anti-pERK (Cell Signaling, 1:1000), anti-HA probe (Santa Cruz, sc-805), anti-mouse phospho-Cdk2T^{160} (Cell Signaling, 1:500), anti- mouse Cdk2(Santa Cruz, M2, 1:1000), anti-*Drosophila* Rbf (DX3), anti alpha-tubulin (DSHB,12G10, 1:1000), beta-tubulin (Sigma, 1:1000) or anti-mouse GAPDH (Cell Signaling, 14C10, 1:2000) were used as loading controls with the appropriate HRP-
conjugated secondary antibody. Enhanced Chemiluminescence-detection (Amersham) followed by digital imaging (to prevent signal saturation, Bio-Rad) was performed and band signal intensity was quantified using NIH Image J. For kinase assays, cell lysates were collected either from late L3 imaginal discs or S2R^+ cells transfected with pMT-Cyclin E. For the S2r cells, 30min or 2h OA (50nM) treatment was performed before cell harvest. See details in (Guest et al., 2011).

**Microscopy**

Images were obtained using a Zeiss LSM 510 confocal or Leica DMI6000 epifluorescence system with deconvolution (ImageQuant). All images were cropped, rotated and processed using Adobe Photoshop. For brightness/contrast the Auto Contrast function was used. All brightness/contrast adjustments were applied equally on the entire image. Adult eye images were obtained using Leica MZ10F microscope and a Nikon Ds-Vi1 digital camera. All adult eye images were measured using Nikon NIS Elements D software and processed with Adobe Photoshop.

**Cell culture**

*Drosophila melanogaster* S2R+ cells were cultured at 25°C in Schneider’s insect medium supplemented with 10% fetal bovine serum (FBS). NIH3T3, p27WT and p27KO mouse embryonic fibroblast cells were cultured at 37°C, 5%CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For cell cycle synchronization, NIH 3T3 cells were treated with 200ng/uL Nocodazole for 18-20h. The
constructs pMT-Wdb-V5 and pMT-Cyclin E were transiently transfected using Fugene (Roche) and expressed by Copper induction (0.5mM) in S2R+ cells.

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References


Grosskortenhaus, R. and Sprenger, F. (2002). Rca1 inhibits APC-Cdh1(Fzr) and is required to prevent cyclin degradation in G2. Dev Cell 2, 29-40.


