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Mechanisms controlling cell cycle exit upon terminal differentiation

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Summary

Coordinating terminal differentiation with permanent exit from the cell cycle is critical for proper organogenesis, yet how the cell cycle is blocked in differentiated tissues remains unclear. Important roles for Retinoblastoma family proteins and Cyclin-dependent kinase inhibitors have been delineated, but in many cases it remains unclear what triggers cell cycle exit. This review focuses on describing recent advances in deciphering how terminal differentiation and exit from the cell cycle are coordinated.

Introduction

Terminal differentiation is usually coupled with permanent exit from the cell cycle and represents the most common cellular state in adult animals. Yet it remains unclear how cells exit the cell cycle during normal development, or maintain the non-proliferative state in adults. Upon terminal differentiation cells become refractory to proliferative signals, including those that promoted proliferation prior to differentiation. Current models for cell cycle exit invoke repression of Cyclin/Cdk activity by Cyclin dependent kinase inhibitors (CKIs), or repression of E2F-mediated transcription by retinoblastoma (Rb) family members, as the proximal mechanisms by which cell cycle progression is arrested (Fig. 1). Mutant studies in a number of organisms support this by demonstrating that the loss of various Rbs or CKIs leads to unscheduled cell proliferation in many tissues [1–4]. But several unresolved issues persist. For example, how is Rb-family and CKI activity coordinated with the process of terminal differentiation? How is cell cycle exit so robustly maintained in differentiated tissues, and do Rbs and CKIs really constitute the only essential cell cycle blockades?

Senescence vs. quiescence vs. cell cycle exit upon terminal differentiation

Exit from the cell cycle upon terminal differentiation shares many characteristics with other quiescent states, but also appears to be distinct. Senescence, quiescence and terminal differentiation are all characterized by prolonged cell cycle arrest with a G1 DNA content, the presence of hypophosphorylated Rb proteins that mediate inhibitory E2F activity, and often, high CKI activity [5,6]. However, quiescence, a state mostly characterized in cell culture, is a more easily reversible than senescence or differentiation induced exit *in vivo*. In quiescent tissue culture cells, differentiation also seems to be inhibited [7] and thus may more closely resemble the quiescence of stem cells *in vivo*.

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Senescence is a largely non-reversible state characterized by distinct cell morphology, formation of senescence-associated heterochromatin foci and expression of senescence-associated genes [8,9]. Senescence been characterized primarily in cell culture, but recent work has demonstrated that cellular senescence can and does exist *in vivo*, and is suggested to be induced either as a barrier against cancer [10,11], or as a result of normal aging [12]. Newer characterizations of senescence demonstrate that it involves an activated DNA damage checkpoint [13,14], and therefore seems to be a state distinct from terminal differentiation.

Terminal differentiation by contrast, has been characterized both in cell culture and *in vivo*, and appears to be more reversible in some cell types than others. Regeneration accompanied by de-differentiation, as observed in amphibian limbs, is a classic example of cell cycle exit reversal *in vivo* [15]. Yet reversal of exit in other cell types can be quite difficult [16]. It is not clear why cell cycle exit and differentiation are more or less reversible in different cell types, but continuing work examining the mechanisms of exit in different tissues will hopefully clarify this issue.

Coordinating cell cycle exit with terminal differentiation

Perhaps the most pressing question in the field of terminal differentiation is: how do the developmental signals triggering differentiation impinge on cell cycle regulators? One answer that seems to come up again and again is that differentiation signals can transcriptionally regulate CKIs to trigger cell cycle exit. One of the best-characterized examples of this involves regulation of the Cip/Kip type CKIs (p21, p27 and p57) by basic Helix Loop Helix (bHLH) transcription factors. Cip/Kip CKIs are frequently expressed in a manner spatially and temporally coordinated with terminal differentiation and initiation of exit. Examples of bHLH proteins such as MyoD or Hes1 regulating Cip/Kip expression both *in vitro* and *in vivo* exist [17–22]. In some of these cases however, the same bHLH proteins are used in earlier developmental events before terminal differentiation. So how is bHLH activity upon terminal differentiation distinguished from earlier activity in cells that continue to proliferate? A recent study in *Drosophila* [23] demonstrates that combinatorial regulation by bHLH activity together with EGF signaling might distinguish the proper time to induce cell cycle exit (Fig. 1). In this case both EGF signaling through the ETS transcription factor Pointed and specific bHLHs are required together at the promoter of the p21/p27 homolog, Dacapo, for proper activation in certain *Drosophila* neurons. Combinatorial mechanisms are not limited to bHLH-dependent regulation however. A similar mechanism has also been described for regulation of mammalian p21 by a combination of Tgfβ signaling though Smad transcription factors and developmental regulation of FoxO activity [24]. Such combinatorial control provides a satisfying mechanism to coordinate differentiation with exit, but it is important to note that this regulation may not actually be required for cell cycle exit *in vivo*. For example in the *Drosophila* eye, Dacapo acts redundantly with Rb like proteins in *Drosophila* (Rbfs) to promote cell cycle exit in neurons and loss of one or the other pathway can be tolerated [25,26]. How the Rb proteins are regulated upon differentiation is not known, but such redundancy has been described in a number of organisms and highlights the robustness of mechanisms that ensure cell cycle exit, a topic we will return to later in this review.

Another transcriptional mechanism coordinating terminal differentiation and cell cycle exit through the *Drosophila* homeobox transcription factor Prospero (Pros) has been elaborated recently. In neuroblasts, Pros is asymmetrically localized at the cytoplasmic membrane. Upon division, Pros is inherited by one daughter cell, the Ganglion Mother Cell (GMC), where it enters the nucleus and directs terminal differentiation. Previous studies of Pros in the *Drosophila* embryo had shown that Pros inhibits transcription of Cyclin E and the cdc25 homolog String, and also induces the CKI Dacapo in differentiating neurons. But it remained unclear whether this was a an indirect effect of Pros on differentiation, or a more direct effect

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on these cell cycle genes [27]. New work [28] investigating the genome-wide binding of Pros on chromatin confirms that Pros has a direct role in transcriptionally inhibiting several key cell cycle genes including *e2f1*, *cyclin E* and *string*, making it an effective blockade (Fig. 1). Importantly, Pros also has a direct role in activating a number of terminal differentiation genes in neurons, making it a dual-function signal [28].

Pros expression is highly restricted to certain cell types, and is therefore not likely to have a universal role in cell cycle exit. Pros could be a common mechanism for cell cycle exit when there is direct differentiation from asymmetric division of a multipotent progenitor without a transiently amplifying progenitor pool. Recent work in the *Drosophila* gut has delineated a stem cell differentiation pathway where a similar pattern of an asymmetric division followed by differentiation without intervening mitoses occurs, and Pros is specifically expressed in one of the resulting post-mitotic cell types [29,30], where it might coordinate terminal differentiation and cell cycle exit. Importantly, the mammalian Pros homolog, Prox1, regulates exit in certain cell types in the mammalian retina [31]. Other Homeobox proteins could play similar roles in other cell types. The search for such dual-function transcription factors will continue.

Destroying the destroyer: protein degradation and timely cell cycle exit

Recent work suggests new roles for the protein degradation machinery in promoting cell cycle exit. The Anaphase promoting complex/Cyclosome (APC/C) and Skp-Cullin-F-box (SCF) ubiquitin ligase complexes have been recognized as cell cycle blockades that promote quiescence. APC/C has long been known to have function in mediating exit from mitosis and entry into G1 or a quiescent state. The large APC/C complex associates with activators Cdc20 or Cdh1, which activate the ubiquitin ligase activity and specify targets such as mitotic Cyclin/Cdks for degradation [32]. In addition to limiting mitotic Cyclin/Cdk activity in proliferating cells, it had previously been shown that the APC/C also targeted Skp2, for degradation [33, 34]. Binne *et al*. have now connected this degradation of the degradation machinery to the cell cycle exit pathway by showing that hypophosphorylated Rb associates with the APC/C specifically when activated by Cdh1 [35]. This promotes degradation of Skp2, a component of the SCF^{skp2} complex responsible for degradation of p27 and p21. The Rb/APC/C interaction thus results in accumulation of p27 (and possibly p21), allowing Rb to act in a second, E2F-independent manner, to inhibit the cell cycle (Fig. 1). Importantly, using a mutated Rb that cannot bind Cdh1, Binne *et al*. demonstrate that the Rb/APC/C interaction is required for Rb to inhibit the cell cycle in proliferating cells.

How do these observations relate to differentiation induced cell cycle exit *in vivo*? Although the studies of Binne *et al*. were performed in cells either undergoing senescence or quiescence *in vitro*, hypophosphorylated Rb does accumulate when cyclin/cdk activity is low *in vivo*, a hallmark of cell cycle exit. Furthermore, genetic studies of APC/C components *in vivo* suggest an important role in promoting properly timed cell cycle exit [36–39]. We must point out though, that different SCF components have also been shown to play roles in promoting timely exit in a number of organisms, through distinct SCF complexes that promote degradation of Cyclin E [40,41], thus protein degradation is important for timely cell cycle exit through at least two distinct pathways. Moreover, in all of these APC/C and SCF mutant studies *in vivo*, cell cycle exit is only temporarily delayed, demonstrating that while the protein degradation machinery may play a role in triggering cell cycle exit, additional mechanisms eventually compensate to ensure cell cycle exit.

Maintaining cell cycle exit: Switching things up at the chromatin

Studies characterizing Rb-family/E2F complexes on the DNA have identified a number of interactions with chromatin remodeling factors that can contribute to E2F-dependent gene

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repression. These include Rb-family dependent recruitment of a Histone Deacetylase (HDAC) complex, association with a SWI/SNF ATP-dependent nucleosome remodeling complex, and Rb-family association with a histone methyltransferase (HMT) complex (reviewed in [42]). These associations suggest an obvious mechanism by which E2F-dependent cell cycle genes can be stably repressed by the Rb family members (Rb, p107 and p130) upon cell cycle exit. It has remained unclear, however, which associations are functionally relevant for repression of cell cycle genes upon terminal differentiation.

One hint was provided by the characterization of a repressive complex from *Drosophila* termed the dREAM complex, which contains Rb/E2F complexes, Myb, Myb-interacting proteins, and at least in one study, contained the HDAC Rpd3 [43,44]. A new characterization of the homologous DREAM complex in human cells shows its specific recruitment to E2F-regulated cell cycle genes in quiescent cells in culture [45]. Knockdown of DREAM components prevented repression of cell cycle genes upon serum starvation, demonstrating functional relevance in a quiescent state, although the effect was mild, ranging from a 1.5–3-fold increase in expression, suggesting other factors may compensate. Interestingly, the *Drosophila* dREAM complex specifically represses developmentally regulated E2F targets with no known cell cycle functions [43]. Since the studies on the human complex were performed on cultured cells in a reversible quiescent state, further work will also be needed to determine whether the human DREAM complex plays a role in maintaining cell cycle gene repression upon terminal differentiation.

Rb also associates with the HMT Suv39H, which methylates Histone H3, thereby creating a binding site for Heterochromatin Protein 1 (HP1) family members. HP1s play important roles in establishing and maintaining repressive heterochromatin (reviewed in [42]) and Rb can recruit HP1 to the Cyclin E promoter and repress Cyclin E expression in proliferating cells in culture [46]. New work extends these observations to differentiating cells by suggesting a role for Rb-family association with an HMT complex in a model of terminal differentiation [47]. Panteleeva *et al*. demonstrate an increase in Hp1α expression and association with the Rb-family member p130/E2F complexes at E2F responsive cell cycle gene promoters upon differentiation of neurons in the cerebellum. This association may be important for repression of cell cycle genes upon differentiation, as knockdown of Hp1α in a cell culture model of neural differentiation partially impairs cell cycle arrest and terminal differentiation.

One interesting aspect of the study by Panteleeva *et al*., is that they observe a developmental switch in HP1 isoform expression, from HP1γ which does not associate with p130/E2F complexes, to HP1α upon neural differentiation. This is reminiscent of another study [48] which identified a developmental switch in the composition of a neural specific SWI/SNF complex upon terminal differentiation *in vivo*. While this study does not demonstrate a direct effect of this subunit switch on the chromatin of cell cycle genes, they do find that the switch in subunits is essential for cell cycle exit and differentiation of neurons *in vivo*. This study along with the others described above suggests that developmentally regulated changes in chromatin modifying complexes are likely to play an important role in maintaining cell cycle exit upon terminal differentiation.

Cell cycle regulators with roles in terminal differentiation

Cases of dual roles in cell cycle and terminal differentiation are not limited to developmental transcription factors like Pros, but can also be found among cell cycle regulators themselves. A classic example of a cell cycle regulator with cell cycle independent functions in differentiation is Rb. Rb associates with a number of tissue-specific transcription factors either directly or indirectly, in an E2F independent manner, to potentiate their differentiation inducing activity. Some examples include Rb association with MyoD and Mef2 in muscle [49,50], association with CBFA1 and Runx2 in bone [51,52], and association with C/EBP family

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transcription factors in cell culture models of adipocyte and macrophage differentiation [53, 54]. Roles for Rb in terminal differentiation aren't always E2F-independent however. Recent work has demonstrated a function for Rb in directing neural migration in the mammalian brain through inhibition of E2F3, as well as induction of terminal differentiation in specific interneurons of the retina by inhibition of the E2F3a isoform, roles that appear distinct from Rb and E2F3 effects on the cell cycle [55,56]. The finding that Rb/E2F complexes can regulate differentiation targets is not surprising in light of previous studies of Rb/E2F transcriptional targets in *Drosophila*, where a large class of non-cell cycle developmental targets were identified, as well as a chromatin remodeling complex likely to control their expression [43, 44,57].

Cell cycle genes moonlighting as terminal differentiation inducers go beyond Rb and E2F. Studies in the last year have also described additional roles for the $p27^{Kip}$ CKI and the APC/C in differentiation. Xenopus p27 has a domain separate from the Cyclin/Cdk inhibitory domain that promotes differentiation of neurons and muscle [58,59], and mammalian p27 can promote neural differentiation through stabilization of Ngn2, even when its binding to Cyclin/Cdks is inhibited. This binding deficient mutant (p27ck-) also plays a role in neural migration, through regulation of Rho [60]. The APC/C^{Cdh1} complex, the same shown to interact with Rb, has also been suggested to play a role in terminal differentiation of neurons by regulating the stability of Inhibitor of Differentiation or ID proteins, which can regulate axon growth in culture [61].

Differentiation and cell cycle exit, independent events or not?

The dual roles of cell cycle regulators in terminal differentiation have complicated genetic studies and led to confusion about whether cell cycle exit and terminal differentiation are separable events. Although many studies have demonstrated that cells can exit the cell cycle without differentiating, it has remained controversial whether cells can terminally differentiate without exiting the cell cycle. The answer to this question is likely to be tissue specific, since the roles for cell cycle regulators in terminal differentiation are cell-type specific. Some apparent examples of separability exist [25,26,62] (Fig. 2), but difficulty arises in how to determine whether cells are fully terminally differentiated or not. Current definitions of terminal differentiation often rely on molecular markers, and the discovery of new, more specific markers can change the criteria for assaying terminal differentiation. In general, specific cellular morphologies (such as the formation of multinucleated myotubes in the case of muscle), or functions (such as firing in the case of neurons) is held to be the gold standard, and in this case the hair cells of the mammalian inner ear provide an excellent *in vivo* example in which exit and terminal differentiation can indeed be separated [62]. Loss of Rb in hair cells leads to continued entry into S-phase and proliferation, even after hair cells exhibit functional mechanotransduction (Fig. 2) [62]. Thus cell cycle exit and terminal differentiation are indeed separable, but may not appear so by genetic studies, in cases where cell cycle regulators also play essential functions in terminal differentiation.

Excessive redundancy

Do Rbs and CKIs constitute the only essential downstream cell cycle blockades for cell cycle exit upon terminal differentiation? Mammals have 3 Rb family members and 7 Cip/Kip or Ink type CKIs. The sheer number of paralogs has made answering this question difficult in mammals. Studies knocking out all three Rb family members have been able to delineate their roles in mouse embryonic fibroblasts [63,64]. Although these triple knockout or "TKO" fibroblasts fail to senesce or undergo quiescence upon contact inhibition, they can undergo cell cycle arrest in response to serum starvation [65].

Drosophila have a simpler system with only two Rb family members, a single Cip/Kip type CKI and no INK homologs. Investigations into this question in *Drosophila* suggest that Rb/

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E2F repressive activity is not required for cell cycle exit *in vivo* [66–68] and that Rb/E2F repression and CKI activity act redundantly to ensure exit in neurons and non-neural cells in the eye [25,26]. Other cell types however, such as the epithelial cells of the wing, still exit from the cell cycle upon differentiation even when E2F activity is high and the CKI Dacapo is absent [25], indicating that additional cell cycle blocking mechanisms must act in these cells. Currently we do not know what these additional mechanisms are, but since Cyclin/Cdk activity seems to be inhibited even when Cyclin and Cdk expression levels are quite high, a novel mechanism repressing CDK activity may be turned on at the onset of terminal differentiation in certain epithelial cells.

We have described some examples of how CKIs may be regulated by differentiation signals and their downstream transcription factors, but how are Rb proteins temporally regulated? In general Rb regulation is thought to be at the level of phosphorylation rather than transcription. The current model is that CKIs induced upon terminal differentiation trigger cell cycle exit by inhibiting CDK phosphorylation of Rbs. This leads to accumulation of hypophosphorylated Rbs which associate with E2F and recruit repressive chromatin modifying complexes to E2F bound promoters, thereby inhibiting cell cycle gene expression. So how does hypophosphorylated Rb accumulate when CKIs are absent, or in aberrant situations when Cyclin/CDK activity remains high? Previous investigations have searched for phosphatases that act on Rbs, and identified Protein Phosphatase 1 (PP1) as a potential Rb phosphatase [69–71]. However recent studies in *Drosophila* have demonstrated that PP1 is not required for regulation of Rbs [72]. How Rb phosphatases could be regulated by differentiation signals is also not clear. Thus, an essential connection between differentiation signals and Rb-family members still needs to be made.

Conclusions

The extent of redundancy in mechanisms ensuring cell cycle exit upon terminal differentiation is beginning to be fully appreciated. Given the double and even triple redundancy in exit mechanisms; will we ever be able to delineate a common cell cycle exit mechanism in all tissues? While no particular pathway appears to be universally dedicated to specifically direct cell cycle exit, the types of mechanisms used may be categorized by cell type or lineage. Hopefully, this will allow us to make some sense, or even predictions, of which exit mechanisms are used in different developmental contexts.

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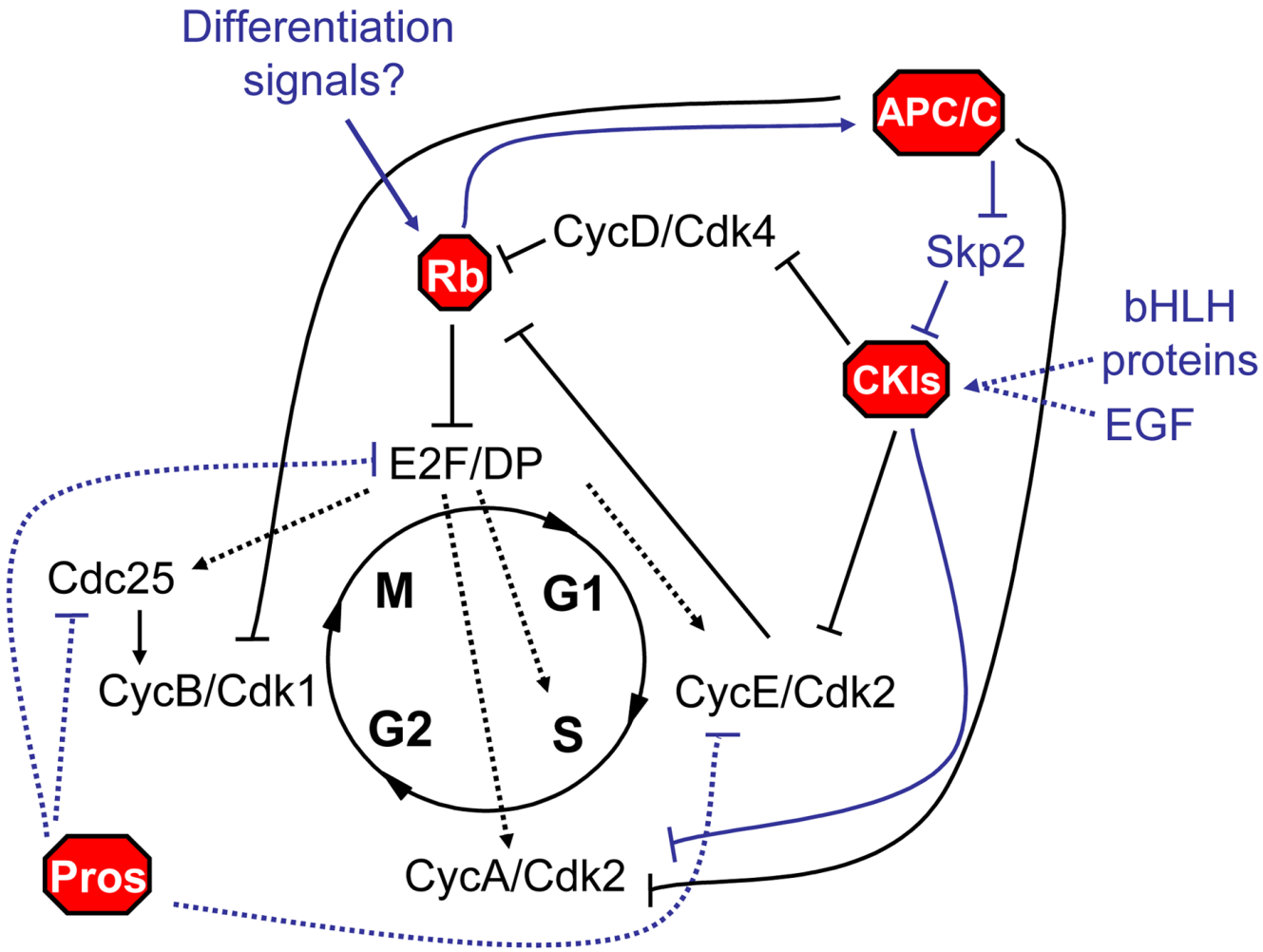

Figure 1. Molecular stop signs: Multiple mechanisms prohibit cell cycle progression upon terminal differentiation

Negative regulators of the cell cycle act as blockades, preventing proliferation upon terminal differentiation. Recent research has identified new signals impinging on known blockades such as the retinoblastoma proteins (Rbs) and Cyclin dependent kinase inhibitors (CKIs), as well as new negative regulators such as Prospero-like homeobox transcrption factors (Pros), and a new post-mitotic role for the Anaphase Promoting Complex/Cyclosome (APC/C). Regulation can be at the level of transcription (dotted lines) or post-transcriptionally (solid lines). Recently identified pathways highlighted in blue are discussed in this review.

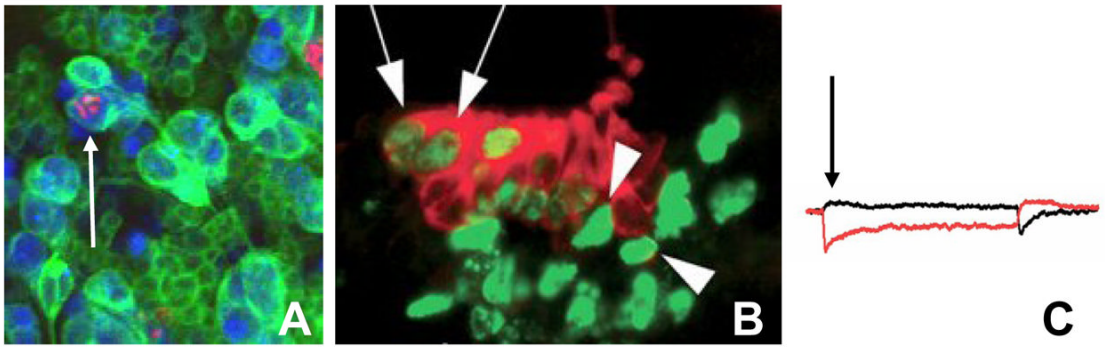

Figure 2. Terminal differentiation and cell cycle exit are separable

Although terminal differentiation and cell cycle exit are coordinated, in some contexts they are separable. A. For example a neuron in the late *Drosophila* retina can continue cycling and undergo mitosis (indicated by phosphohistone H3 in red) while maintaining characteristics of terminal differentiation, such as expression of Elav (in blue) and projection of an axon (cell membrane in green, indicated by an arrow) if both E2F and Cyclin/Cdk activities are kept high [25]. B. Hair cells of the mouse inner ear lacking Rb expression also continue cycling upon terminal differentiation, providing another example where differentiation and cell cycle exit are separable. Myo7A expression in red labels hair cells of the cochlea, while BrdU in green labels cells in S-phase. Co-labeled cells differentiate but continue into S-phase (arrow) C. Recorded transduction currents in $Rb^{-/-}$ hair cells demonstrate mechanosensitivity [B and C adapted from 62].

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