

# Cell Cycle Proteins as Key Regulators of Postmitotic Cell Death

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Cell cycle progression in dividing cells, characterized by faithful replication of the genomic materials and duplication of the original cell, is fundamental for growth and reproduction of all mammalian organisms. Functional maturation of postmitotic cells, however, requires cell cycle exit and terminal differentiation. In mature postmitotic cells, many cell cycle proteins remain to be expressed, or can be induced and reactivated in pathological conditions such as traumatic injury and degenerative diseases. Interestingly, elevated levels of cell cycle proteins in postmitotic cells often do not induce proliferation, but result in aberrant cell cycle reentry and cell death. At present, the cell cycle machinery is known predominantly for regulating cell cycle progression and cell proliferation, albeit accumulating evidence indicates that cell cycle proteins may also control cell death, especially in postmitotic tissues. Herein, we provide a brief summary of these findings and hope to highlight the connection between cell cycle reentry and postmitotic cell death. In addition, we also outline the signaling pathways that have been identified in cell cycle-related cell death. Advanced understanding of the molecular mechanisms underlying cell cycle-related death is of paramount importance because this knowledge can be applied to develop protective strategies against pathologies in postmitotic tissues. Moreover, a full-scope understanding of the cell cycle machinery will allow fine tuning to favor cell proliferation over cell death, thereby potentially promoting tissue regeneration.

The mammalian cell cycle is an evolutionarily conserved process that results in DNA replication and cell division [1]. Cell cycle is typically divided into four phases (Figure 1): 1) G1 phase, the Gap 1 phase during which the cell acquires energy and prepares for DNA synthesis; 2) S phase, DNA Synthesis phase for replication of the genetic material; 3) G2 phase, the Gap 2 phase during which the cell checks the integrity of genetic material and prepares for mitosis; and 4) M phase, the Mitosis phase for division of the nucleus, cytoplasm, and plasma membrane.

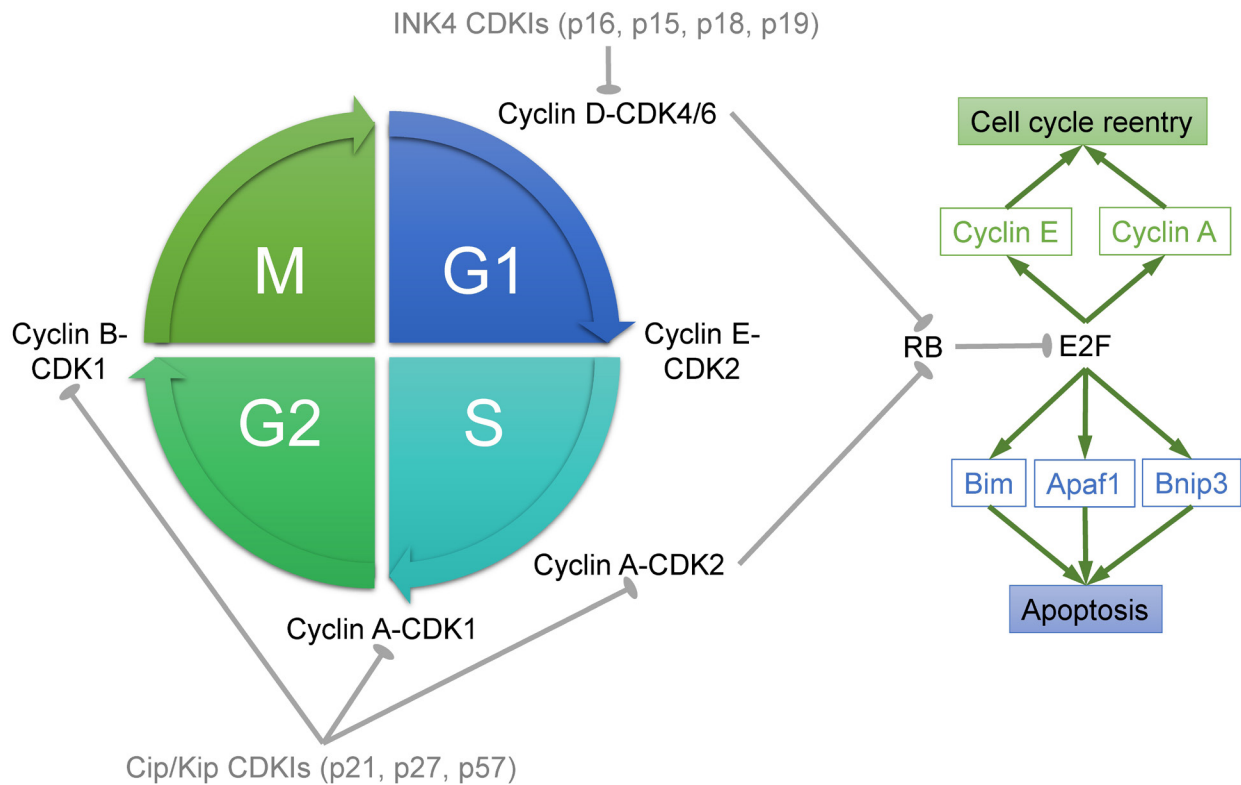
After completion of mitosis, a cell can temporarily or permanently exit the cell cycle and enter the Gap 0 (G0) phase. The majority of non-growing cells in the human body are in G0 phase.

Cell cycle progression is tightly controlled by a series of cyclins, cyclin-dependent kinases (CDKs†), and CDK inhibitors (CDKIs) [1]. In response to growth or mitogenic signals, the cell exits G0 and reenters G1 with increased expression of cyclin D (Figure 1). Cyclin D binds and activates CDK4 and CDK6, which phosphor-

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†Abbreviations: Apaf1, apoptotic protease-activating factor 1; BH3, Bcl 2 homology 3; CDKs, cyclin-dependent kinases; CDKIs, CDK inhibitors; Cip/Kip, CDK-interacting protein / kinase inhibitor protein; INK4, inhibitors of cyclin dependent kinase 4; NGF, nerve growth factor; NRCMs, neonatal rat cardiomyocytes; RB, retinoblastoma; RBL1, RB-like 1; RBL2, RB-like 2; Smac, second mitochondrial activator of caspases; UV, ultraviolet; XIAP, X linked inhibitor of apoptosis protein.

Keywords: cell cycle, apoptosis, neuron, cardiomyocyte, disease



**Figure 1.** Cell cycle proteins as key regulators of postmitotic cell death. Green arrows indicate activation; gray bar-headed lines indicate inhibition. CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; INK4, inhibitors of cyclin dependent kinase 4; Cip/Kip, CDK-interacting protein / kinase inhibitor protein; RB, retinoblastoma family of proteins.

ylate the retinoblastoma (RB) family of proteins, including RB1/p105, RB-like 1 (RBL1/p107), and RB-like 2 (RBL2/p130). CDK4/6-mediated RB phosphorylation derepresses the E2F family transcription factors, resulting in transcription of target genes such as cyclin E and cyclin A. The cyclin E-CDK2 complex then provokes G1/S transition, and cyclin A associates with CDK2 and CDK1 to regulate completion of S phase. The cyclin A-CDK1 complex also drives cell cycle progression through G2 phase, which is followed by cyclin B-CDK1-dependent onset and completion of mitosis.

CDK activity is restrained by CDKIs, which are further divided into two classes [2]. The INK4 (inhibitors of cyclin dependent kinase 4) family CDKIs, including p16<sup>INK4a</sup> (encoded by the *Cdkn2a* gene), p15<sup>INK4b</sup> (*Cdkn2b*), p18<sup>INK4c</sup> (*Cdkn2c*), and p19<sup>INK4d</sup> (*Cdkn2d*), primarily inhibit the kinase activities of CDK4 and CDK6 through direct interaction with CDK4/6 and prevention of cyclin D binding (Figure 1). In contrast, the Cip/Kip (CDK-interacting protein / kinase inhibitor protein) family CDKIs, including p21<sup>Cip1/Waf1/Sdi1</sup> (*Cdkn1a*), p27<sup>Kip1</sup> (*Cdkn1b*), and p57<sup>Kip2</sup> (*Cdkn1c*), can inhibit all cyclin-CDK complexes through binding to both cyclins and CDKs. Under certain conditions, however, the Cip/Kip CDKIs

may participate in the assembly of the cyclin D-CDK4/6 complexes and enhance cyclin D-dependent kinase activity, eventually resulting in activation of cyclin E-CDK2 [2].

The RB proteins are best known as inhibitors of G1/S transition, through interaction with the E2F family members (Figure 1). Specifically, RB1 binds to and sequesters the E2F1, E2F2, E2F3a transcriptional activators, thereby preventing them from initiating transcription of essential genes involved in cell cycle progression. Conversely, RBL1 or RBL2 associates with E2F4 and E2F5 transcriptional repressors, resulting in blockage of transcription [3].

A G0 cell that permanently exits the cell cycle enters a postmitotic state, and is unable to undergo a new round of mitotic division. Cell cycle proteins also have important cell-cycle-independent functions in terminal differentiation, which is characterized by specific molecular marker expression, cellular morphological, and functional maturation [4]. Although permanent cell cycle exit and terminal differentiation are independent and separable events, they are usually coupled with each other because of the dual roles of cell cycle proteins in both processes [4]. During embryonic and postnatal development, major

cell types in postmitotic organs such as neurons in the brain and cardiomyocytes in the heart, withdraw from the cell cycle and undergo terminal differentiation. These terminally differentiated cells continue expressing many cell cycle proteins, but have extremely limited capacity for proliferation. In fact, aberrant cell cycle reentry in postmitotic cells frequently leads to apoptotic cell death, suggesting that cell cycle machinery may be repurposed as key regulators of cell death in postmitotic tissues.

Current evidence suggests that cell cycle proteins primarily regulate intrinsic apoptosis through the mitochondrial pathway, which is controlled by the Bcl-2 family members [5,6]. The Bcl-2 family proteins are divided into three groups: 1) anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1, etc.); 2) pro-apoptotic effectors (Bax, Bak, Bok); and 3) pro-apoptotic initiators, which are Bcl-2 homology 3 (BH3)-only proteins (Bad, Bid, Bik, Bim, Bmf, Noxa, Puma, Bnip3, etc.). In response to a variety of cytotoxic stimuli (oxidative, genotoxic stress, etc.), the BH3-only pro-apoptotic initiators bind and inhibit the anti-apoptotic proteins, resulting in activation of the pro-apoptotic effectors Bax and Bak. Bax and Bak then form oligomers and permeabilize the mitochondrial outer membrane, leading to release of apoptogenic factors including cytochrome *c* and second mitochondrial activator of caspases (Smac). Cytochrome *c* and apoptotic protease-activating factor 1 (Apaf1) form apoptosome and result in activation of caspase 9, whereas Smac represses the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP). These events eventually result in activation of caspase 3 and execution of apoptotic cell death. This review article will briefly summarize our current knowledge regarding the role of cell cycle machinery in the regulation of postmitotic cell death.

## NEURONS

During nervous system development, neuronal turnover is a physiological process and maintains homeostasis. As neuronal precursor cells finish the last round of cell division and exit the cell cycle, they terminally differentiate into neurons, which then undergo axonal elongation and form synapses with target tissues. During this process, approximately 50 percent of all neurons are removed via programmed cell death due to their high propensity to initiate apoptosis [7]. Fully differentiated mature neurons are less susceptible to cell death, and aberrant apoptosis most commonly occurs in pathological situations including acute trauma and ischemic stroke, as well as chronic neurodegenerative diseases such as Alzheimer, Huntington, and Parkinson diseases [8]. Therefore, identification of critical mediators of neuronal apoptosis may provide valuable drug target for intervention, allowing restriction of cell death and preservation of neurological function.

Signaling pathways involved in neuronal cell death have been reviewed in detail elsewhere [8]. In the past decades, a growing body of literature reveals that neuronal apoptosis is frequently accompanied by cell cycle reentry [9]. These findings have shed crucial light on the pro-apoptotic role of cell cycle reactivation in neurons. It is noteworthy that cell cycle reactivation does not always cause death in postmitotic neurons, but may lead to *de novo* tetraploid formation, which has been suggested as an early marker of neurodegeneration [10]. Particularly, when apoptosis is inhibited, neuronal cell cycle reactivation primarily results in mitosis and cell division [11]. Therefore, activation of the cell cycle machinery may result in different outcomes depending on the context.

## Neuronal cyclins-CDKs

In a variety of experimental systems and neurodegenerative disease patient samples, neuronal apoptosis is associated with up-regulation of cyclins-CDKs, and most strikingly, activation of the cyclin D-CDK4/6 complex [9]. Specifically, cyclin D1 expression and associated CDK activity in neurons are dramatically increased upon challenge with the pro-apoptotic treatments [12-16]. Transfection of cyclin D1 is sufficient to induce apoptosis in many cell types [15]. Overexpression of dominant negative forms of CDK4 or CDK6, but not those of CDK2 or CDK3, protects neurons from apoptosis induced by nerve growth factor (NGF) deprivation [17], the DNA damaging agents (ultraviolet (UV) irradiation, arabinoside, and camptothecin) [13], or  $\beta$ -amyloid protein [18]. However, expression of dominant negative form of CDK2 is able to suppress apoptosis in cortical neurons after proteasomal inhibition [19]. Moreover, silencing of either CDK4/6 or CDK2 inhibits apoptosis in postmitotic cortical neurons in response to oxidative stress induced by H<sub>2</sub>O<sub>2</sub> [20]. Most recently, overexpression of Cyclin E and CDK2 has been shown to induce p53-dependent apoptosis in differentiated neurons [11]. Although cyclin D1 most commonly partners with CDK4, it has been shown that cyclin D1 mediates cell cycle-related neuronal apoptosis by repressing CDK5 [21]. In addition, CDK1 expression and activation are augmented by traumatic spinal cord injury [22] or cerebral ischemia/reperfusion injury [23]. Genetic deletion of CDK1 or pharmacologic inhibition of CDK1 confers marked neuroprotection both *in vivo* and *in vitro* [22,23].

Neuronal cyclins-CDKs regulate apoptosis frequently through the Bcl-2 family members. There is evidence that activation of CDK4 exacerbates neuronal cell death through augmented transcription of Bim (Figure 1) [24], a pro-apoptotic BH3-only protein essential for triggering Bax/Bak-mediated mitochondrial damage [5]. Mechanistically, Bim transcription is mediated by the E2F and myb family transcription factors [24], which are necessary

for neuronal apoptosis upon NGF withdrawal or DNA damage [25], and are sufficient to induce apoptosis while overexpressed in cortical neurons [26]. Additional transcription factors involved in Bim regulation in neurons include c-Jun and FoxO family members [27]. Moreover, the cyclin B1-CDK1 complex may cause apoptotic neuronal death through phosphorylation of mitochondrial Bcl-xL and inhibition of ATP synthase activity [28] and upregulation of the BH3-only proteins Puma and Noxa [29].

CDK activation during neuronal apoptosis is accompanied by S-phase cell cycle reentry in response to oxidative stress [20], or genotoxic stress [30]. Under these stress conditions, postmitotic cell cycle reentry appears to depend on the DNA damage response pathway, because genetic or pharmacologic inhibition of the DNA damage sensing kinase ataxia telangiectasia mutated (ATM) prevents cell cycle activation and eventually suppresses neuronal apoptosis [30]. Along similar lines, DNA damage-induced CDK2 activation is dependent on checkpoint kinase 1 (Chk1), an effector kinase downstream of ATM and Rad3-related (ATR) [31]. For pro-apoptotic stimuli that do not target DNA, such as the broad spectrum protein kinase inhibitor staurosporine or the microtubule-depolymerizing agent colchicine, induction of neuronal apoptosis is not associated with cell cycle reentry [30]. Despite the detrimental function, activation of CDK4/6 has been shown to be necessary for DNA repair [20].

The small molecule CDK inhibitors flavopiridol and olomoucine also attenuate neuronal apoptosis evoked by NGF withdrawal, DNA-damaging agents [32,33],  $\beta$ -amyloid protein [18], or proteasomal inhibitors [19]. CDK activity, but not DNA synthesis, is essential for neuronal apoptosis, because apoptotic death of neurons is suppressed by G1/S blockers deferaxamine and mimosine, but not by DNA synthesis inhibitors such as aphidicolin and N-acetylcysteine [32]. Additional CDK inhibitors, such as roscovitine or CR8, also confer neuroprotection after traumatic injury [22,34]. Despite the anti-apoptotic effect of CDK inhibitors in neurons following NGF deprivation and DNA damage, no protection is detected in apoptosis evoked by oxidative stress through SOD1 depletion [33]. It is noteworthy that the anti-apoptotic action of CDK inhibitors is only restricted in differentiated postmitotic neurons, because treatment with CDK inhibitors paradoxically exacerbates apoptosis in proliferation-competent cells [35].

### Neuronal CDKIs

Treatment of neurons with multiple apoptosis inducers reduce levels of the CDKIs including the INK4 family CDKI p16 [16], the Cip/Kip family members p21 [36], and p27 [14]. Overexpression of p16, p21, or p27, suppresses apoptotic death of cultured postmitotic neu-

rons in response to a variety of stimuli including NGF withdrawal [17], serum starvation [15], proteasomal inhibition [19], UV irradiation, or the DNA-damaging drugs arabinoside and camptothecin [13], neurotoxin ethylcholine aziridinium [36], or oxidative stress [37]. These data further support important roles of CDK4/6 and CDK1/2 in neuronal apoptosis, because the INK4 and Cip/Kip CDKIs primarily inhibit activities of CDK4/6 and CDK1/2, respectively [2]. Mice lacking both p19 and p27 die at around postnatal day 18, due to uncontrolled neuronal cell divisions and apoptosis within all parts of the brain [38].

### Neuronal RB/E2F

Homozygous deficiency of RB1 results in embryonic lethality partly due to defects in neurogenesis, which is characterized by massive neuronal cell death [39-41]. Initial research suggests that loss of RB1 provokes apoptosis through p53 and E2F1-mediated transcription of Apaf1 (Figure 1) [42,43]. However, follow-up studies revealed that apoptosis in the central nervous system of RB1-deficient mice was caused by placental defects rather than neuronal cell autonomous functions of RB1 [44,45]. Indeed, conditional knockout of RB1 in the central nervous system does not induce apoptosis in mitotically active neuronal precursor cells [46,47]. Intriguingly, acute deletion of RB1 in both embryonic and adult postmitotic neurons triggers apoptosis without inducing expression of classical E2F1 target genes, such as Apaf1 or Puma, suggesting RB1 likely maintains survival of postmitotic neurons through other mechanisms [48]. It is noteworthy that peripheral nervous system apoptosis in RB1 null mice appears to be cell autonomous, and is not due to placental defects [47]. Upon induction of neuronal apoptosis, RB1 is transiently phosphorylated and then gradually degraded in a CDK activity-dependent manner [14,18,19]. The decreased level of RB1 may also be explained by caspase-dependent cleavage [49].

Similar as RB1-deficient animals, adult RBL1/p107-null mice also have elevated numbers of apoptotic cells in the brain [50]. The function of RB1 and RBL1 appears to be non-redundant, because apoptosis is most pronounced in RB1 and RBL1 double knockout mice than in animals deficient in either gene [51]. The predominant RB family member associated with E2F complexes, however, turns out to be RBL2/p130 [52]. The RBL2-E2F4 complex promotes neuron survival by inducing gene silencing via recruitment of the chromatin modifiers histone deacetylase 1 (HDAC1) and Suv39H1 [52]. Interestingly, knockdown of RBL2 is sufficient to induce apoptosis of cortical neurons [52].

In the postmortem human tissue from patients with Parkinson disease, the dopaminergic neurons express high levels of E2F1 and undergo aberrant cell-cycle ac-



tivation [53]. Neuronal E2F1 activation and cell cycle S phase reentry following dopaminergic neurotoxin injection results in cell death rather than mitotic division [53]. As a result, disruption of E2F1 attenuates neurotoxin-induced dopaminergic cell death [53]. Overexpression of E2F1 induces transcription of b-/c-myb [26], which further mediate Bim transcription and result in neuronal apoptosis [24,25]. Alternatively, E2F1 may also mediate DNA damage-induced apoptosis through direct transcription of Apaf1 [54]. In sharp contrast to the pro-apoptotic role of transcriptional activator E2F1, the transcriptional repressor E2F4 is anti-apoptotic as silencing of E2F4 leads to spontaneous apoptosis in cortical neurons [52], whereas overexpression of E2F4 promotes neuronal survival following hypoxia/reoxygenation [55].

## CARDIOMYOCYTES

Heart disease is currently the number one cause of death in the United States [56]. Many cardiac pathologies, including myocardial infarction, ischemia/reperfusion injury, heart failure, and cancer treatment-related cardiotoxicity, are characterized by cardiac cell death [6]. Accounting for only ~32 percent of the total cell number, cardiomyocytes occupy as much as 85 percent of the myocardial volume [57]. As the major contracting cells in the heart, cardiomyocytes directly contribute to the cardiac contractile function, which is essential for maintaining adequate perfusion through pumping blood into circulation. Therefore, loss of cardiomyocytes through apoptotic cell death, either acute or chronic, impairs cardiac function and eventually results in heart failure.

Mammalian cardiomyocytes exit the cell cycle and become terminally differentiated soon after birth [58]. Yet expression of the cell cycle regulators, including CDKs and CDKIs, persists into adulthood [59,60]. To date, the scientific knowledge regarding cardiac cell cycle molecules are mostly focused on cell cycle activity-dependent events, such as cardiomyocyte proliferation during embryonic cardiac development, physiological hypertrophy during postnatal development, and pathological hypertrophy in disease conditions [58]. By contrast, the role of cell cycle machinery in cardiomyocyte apoptosis has been much less appreciated.

### Cardiac cyclins-CDKs

Myocardial infarction, commonly known as heart attack, is a form of myocardial damage caused by lack of oxygen supply to the heart muscle due to obstruction of coronary blood flow. Insufficient blood flow, or ischemia, results in myocardial tissue hypoxia (reduced oxygen level) and eventually leads to cardiac cell death. In response to hypoxia, primary neonatal rat cardiomyocytes (NRCMs) undergo apoptosis that appears independent

of critical Bcl-2 family members including Bcl-2, Bax, Bcl-xL, and Bad [61]. Cardiomyocyte apoptosis following *in vitro* hypoxia or *in vivo* ischemia/reperfusion is accompanied by upregulation of cyclin A and activation of cyclin A/CDK2, without altering protein levels of cyclin B, cyclin E, c-myc, and CDK2 [61-63]. Interestingly, hypoxia fails to upregulate cyclin A in non-myocytes, and hence does not induce apoptosis in these cells [61,63]. Activation of cyclin A-CDK2 mediates hypoxia or ischemia/reperfusion-induced apoptosis, because: 1) overexpression of cyclin A [61], but not CDK2 [62,64], is sufficient to induce apoptosis in NRCMs; and 2) hypoxia or ischemia/reperfusion-induced apoptosis is suppressed by dominant-negative CDK2 or CDK3 adenovirus infection [62,63], or the CDK1/2 inhibitors butyrolactone I (olomoucine) or roscovitine [61,63].

*In vivo* studies further demonstrate that myocardial CDK2 is activated in multiple pathological conditions, including myocardial ischemia [65,66], reperfusion injury [63,65], and anthracycline-related cardiomyopathy [64]. All these pathological challenges are associated with increased levels of CDK2 protein and phospho-CDK2 (T160) [64,65]. Phosphorylation of CDK2 at T160 is mediated by CDK-activating kinase (CAK) [67], and has been widely used as a marker of CDK2 activation [31,68]. Germline knockout of CDK2, or pharmacologic inhibition of CDK2 with the CDK inhibitor roscovitine, significantly protected against myocardial ischemia/reperfusion injury and anthracycline cardiotoxicity [64,65]. Mechanistically, CDK2 activation exacerbates cardiomyocyte apoptosis through augmenting the expression of Bim (Figure 1) [64]. Based on a significant change in Bim mRNA level, CDK2 likely regulates Bim transcription [64]. Future studies are warranted to identify the transcription factors responsible for CDK2-dependent Bim expression in cardiomyocytes.

In contrast to cyclinA/CDK2, current evidence argues against a major role of cyclin D-CDK4/6 in hypoxia-induced cardiomyocyte apoptosis [62]. Firstly, hypoxia does not induce activation of CDK4 and CDK6, and if anything, it appears to reduce protein levels of CDK4 and CDK6. Secondly, overexpression of CDK4 or CDK6 fails to induce apoptosis in NRCMs. Lastly, hypoxia-induced apoptosis is not modulated by overexpression of wild-type or dominant-negative CDK4/6 [62]. In response to doxorubicin challenge, cardiac-specific transgenic expression of cyclin D2 does not significantly alter cardiomyocyte apoptosis and cardiac function in the acute phase, albeit it facilitates recovery in the late stage partly due to enhanced cardiomyocyte cell cycle reentry [69]. In primary mouse cardiomyocytes isolated at postnatal day 7 and postmitotic 60-day-old human-induced pluripotent stem-cell-derived cardiomyocytes (hiPSC-CMs), overexpression of CDK1/cyclin B1/aurora kinase B results in

cell division followed by rapid cell death [70]. However, expression of CDK1/cyclin B1/CDK4/cyclin D induces stable cardiomyocyte proliferation without triggering apoptosis [70]. These interesting findings raise the possibility that the cell cycle machinery could be harnessed to enhance myocardial regeneration (cardiomyocyte proliferation) and protect against myocardial degeneration (cardiomyocyte apoptosis). To achieve this goal, it is crucial to tease apart the cell cycle molecules that are specifically involved in propelling cardiac cell division and death.

### Cardiac CDKs

Hypoxia-induced cardiomyocyte apoptosis is associated with a progressive downregulation of the Cip/Kip family CDKs p21, p27, and the INK4 family CDK p16, possibly by caspase 3-dependent cleavage [61,62]. However, hypoxia-induced apoptosis in neonatal cardiomyocytes is hindered only by overexpression of p21 or p27, but not by elevated level of p16 [62]. In adult cardiomyocytes, elevated p21 level is also associated with resistance to hydrogen peroxide-induced apoptosis [71]. These data further support a critical role of CDK2 over CDK4/6 in cardiomyocyte apoptosis induced by hypoxia or oxidative stress, because CDK2 is inhibited by p21 or p27, and CDK4/6 is inhibited by p16.

In response to the anthracycline anticancer drug doxorubicin, p21 protein level is transiently elevated, followed by a gradual decline below basal level that accompanies cardiomyocyte apoptosis [72]. Doxorubicin-induced mitochondrial outer membrane permeabilization and myocyte apoptosis are suppressed by overexpression of p21, but exacerbated by silencing of p21 [72]. While the CDKI p21 represses Bim [72], CDK2 appears to augment Bim expression, which is necessary for doxorubicin-induced apoptosis [64]. These findings underscore the importance of Bim in the execution of apoptosis downstream of altered cardiac CDK2 activity.

Similar as p21, p27 also protects against cardiomyocyte apoptosis, as shown in an animal model of myocardial infarction [73], and in cultured NRCMs after glucose deprivation [74]. The protective effect of p27 is due, at least in part, to enhanced autophagy [74]. An independent study has confirmed p27-mediated apoptosis inhibition and autophagy activation in cardiomyocytes [75]. At present, it remains unclear how p27 promotes autophagy, and whether its CDK inhibitory function is necessary for activation of autophagy [76].

### Cardiac RB/E2F

In agreement with the findings that hypoxia induces activation of cyclin A/CDK2, the well-established CDK substrate RB1 is hyper-phosphorylated upon hy-

poxia stimulation in a CDK2-dependent manner [62]. CDK2-mediated RB1 hyper-phosphorylation results in inhibition of RB1 [77]. RB1 plays an anti-apoptotic role in cardiomyocytes because overexpression of constitutively active RB1 represses the key apoptotic proteins caspases 3 and 7 and protects against hypoxia-induced cardiomyocyte apoptosis [62]. Moreover, cardiac-specific deletion of RB1 significantly increases apoptosis and infarct size following myocardial ischemia/reperfusion [65], further supporting an anti-apoptotic function of RB1 in cardiomyocytes. It is noteworthy that cardiac-specific deletion of RB1 does not cause cardiomyocyte apoptosis during normal development [78].

RB1 directly binds and inhibits the E2F transcription factor E2F1 [3]. Contrary to RB1, overexpression of E2F1 results in massive, spontaneous apoptotic death of cardiomyocytes, as evaluated by TUNEL assay and annexin V staining [79]. Interestingly, E2F1 overexpression increases levels of cyclin A/E, CDK1/2, and hyper-phosphorylated RB1, without upregulation of CDK4/6 protein expression and kinase activity, suggesting that CDK4/6 are likely not involved in E2F1-mediated cell death [79]. Mechanistically, overexpression of E2F1 induces cardiomyocyte apoptosis through resulting in a 6.2-fold increase in transcription of Bnip3 (Figure 1), an E2F1 target gene encoding a BH3-only pro-apoptotic protein [80]. E2F1 is essential in hypoxia-induced apoptosis because hypoxia stimulation markedly increases luciferase activity of the E2F-1 promoter, which has E2F response element [61,62]. The pro-apoptotic role of E2F1 in cardiomyocytes is further supported by the findings that overexpression of transcriptionally inactive E2F1 suppresses hypoxia-induced apoptosis [62].

Although mice deficient in E2F1 are resistant to cardiomyocyte apoptosis following myocardial ischemia/reperfusion injury, expression of many downstream E2F1 target genes including caspase 3, Apaf1, and c-myc are not significantly altered, except a modest downregulation of Bnip3 [81]. These results suggest that endogenous E2F1 may not directly control transcription of its classic pro-apoptotic target genes in cardiomyocytes to regulate ischemia/reperfusion-induced apoptosis [81]. Rather, the pro-apoptotic function of E2F1 is attributed, at least in part, to FOXO1-dependent transcription of FasL, Bcl-6 [81]. These studies are in agreement with the observation that apoptotic gene expression in the heart is independent of E2F1 or E2F2 [82].

Cardiac E2F1 promoter is repressed by the E2F4/RBL2 complex, which recruits HDAC1 and inhibits transcription of pro-apoptotic genes including E2F1, Apaf1, and p73 $\alpha$  [83]. Indeed, E2F4-deficient heart exhibit higher levels of apoptosis, increased expression of E2F1, Apaf1, and p73 $\alpha$ , and impaired ventricular function [83].

## OTHER POSTMITOTIC CELLS

Adult skeletal muscle generates force through contraction of the postmitotic, multinucleated muscle fibers (myofibers), which are differentiated from myoblasts at embryonic day 14. RB1 insufficiency impairs myoblast cell cycle withdrawal and subsequent terminal differentiation during mouse development, resulting in massive apoptosis [84]. Consistently, inactivation of RB1 plus RBL1 or RBL2, or triple knockout of all RB proteins, increases myoblast cell death [85]. Expression of the adenovirus E1A oncogene in terminally differentiated, *in vitro* cultured myotubes, increases levels of cyclin E, cyclin A, CDK2, and b-myb [86], reactivates the cell cycle, and eventually results in cell death beginning at 4 days after infection [87]. Moreover, forced expression of E1A, cyclin D1-CDK4, or double depletion of p21 and p27, induces cell cycle reentry, DNA damage, and apoptosis in terminally differentiated myotubes, but not in non-differentiated fibroblasts and myoblasts, indicating a critical role of the differentiation state in cell cycle-related death. Interestingly, apoptosis response is an integral part of myofiber dedifferentiation following muscle injury in newts, which have remarkable regenerative capability throughout their entire lifespan [88]. A brief induction followed by inhibition of apoptosis in terminally differentiated myotubes has been shown to generate dedifferentiated regenerative progenitors [88]. These studies suggest that cell cycle reactivation followed by inhibition of apoptosis at the right time could be an exciting approach to induce regeneration in postmitotic tissues.

The lens fiber cells are terminally differentiated, postmitotic cells filled with high concentrations of crystallin proteins to ensure lens transparency and prevent visual impairment. Homozygous deletion of RB1 in the mouse leads to aberrant lens fiber cell apoptosis [89], which is not caused by reduced placental transport function [45]. Moreover, conditional knockout of RB1 in ocular lens also triggers apoptosis [47]. Lens fiber cell apoptosis resulted from RB1 disruption is abolished by deletion of p53 [89], E2F1 [90], or Apaf1 [43]. Transgenic expression of E2F1 or E2F2 in mice is sufficient to induce transcription of cyclins A2, B1, and E in lens fiber cells, and cause cell cycle reentry and subsequent apoptosis (Figure 1) [91].

Cochlear hair cells are terminally differentiated cells located in inner ear and transduce sound into electrical signals. Acute inactivation of RB1 in cochlear hair cells provokes rapid cell cycle reentry and apoptosis [92]. Disruption of the CDKs p19 and p21, synergistically triggers hair cell S-phase reentry, resulting in activation of the DNA damage response pathway, p53-mediated apoptosis, and eventually hearing loss [93,94]. Recent studies have identified CDK2 as an important mediator

of noise and cisplatin-induced cochlear hair cell apoptosis and hearing loss, both of which are prevented in CDK2-knockout mice, or in mice treated with CDK2 inhibitors [95,96]. Similarly, forced S-phase reentry of the inner ear supporting cells, by ectopic expression of cyclin D1 or c-myc, also results in DNA damage, cell cycle arrest, or apoptosis [97,98].

## CONCLUSIONS AND PERSPECTIVES

To date, the majority of studies suggest that maintenance of the postmitotic state of terminally differentiated cells is critical for survival. Abnormal cell cycle activation frequently results in DNA damage and apoptotic death in postmitotic cells. Since many forms of injuries and toxicities in postmitotic tissues are associated with inappropriate cell cycle reentry, cell cycle inhibitors may represent a promising class of protective agents in mature, postmitotic organs.

Although it is becoming increasingly clear that cell cycle reactivation in terminally differentiated cells often triggers apoptosis, the underlying mechanisms may not be exactly the same among different tissues or organs. Depending on cell types, cell cycle reentry has been shown to be driven by distinct cyclin-CDK complexes, and apoptosis may also be initiated by various pro-apoptotic molecules. In order to achieve tissue-specific protection, future studies are warranted to identify the signaling pathways involved in cell cycle-related death in each postmitotic cell type.

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