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REVIEW

S-phase-coupled apoptosis in tumor suppression

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Abstract DNA replication is essential for accurate transmission of genomic information from parental to daughter cells. DNA replication is licensed once per cell division cycle. This process is highly regulated by both positive and negative regulators. Over-replication, under-replication, as well as DNA damage in a cell all induce the activation of checkpoint control pathways such as ATM/ATR, CHK kinases, and the tumor suppressor protein p53, which provide "damage controls" via either DNA repairs or apoptosis. This review focuses on accumulating evidence, with the emphasis on recently discovered Killin, that S-phase checkpoint control is crucial for a mammalian cell to make a life and death decision in order to safeguard genome integrity.

Keywords Killin · E2F · Geminin · S-phase control · DNA replication · p53 · Differential display

Introduction

DNA replication

DNA replication is a complex process that involves the coordinated activity of many different proteins. DNA replication occurs once per cell cycle and begins with the formation of the pre-replication complex (pre-RC) during the G1-phase of the cell cycle [1, 2]. This complex consists of the origin recognition complex (ORC), cell division cycle 6 (Cdc6), chromatin licensing and DNA replication factor 1 (Cdt1), and the mini-chromosome-maintenance 2–7 (Mcm2–7) complex. First, the ORC recognizes and binds to the origin of replication. The ORC then recruits Cdc6 and Cdt1, which in turn promote the bidirectional loading of the Mcm2–7 complex onto each origin of replication. During the S-phase, Mcm2–7 functions as a replicate helicase, which can unwind the double helix at the origin of replication. Mcm4, 6, and 7 constitute the core helicase, while Mcm2, 3 and 5 are regulatory subunits. The Mcm complex is loaded bidirectionally at each origin of replication and degraded when elongation is stalled. After the replication fork is formed, the Mcm2–7 complex dissociates from the origin so that it cannot be unwound.

The formation of the pre-RC, a critical step in replication licensing, has been shown to be dependent on cyclin-dependent kinase (CDK) levels. In the G1-phase, low levels of CDK allow the formation of the pre-RC [3]. However, increased CDK levels during the transition from G1 to S-phase lead to the disassembly of existing pre-RCs and inhibition of the formation of new pre-RC. In vertebrates, the pre-RC member Cdc6 is relocated from the nucleus by CDK phosphorylation. Moreover, CDK-mediated phosphorylation of Cdtl promotes its degradation during the S-phase. Prior to DNA synthesis, two single strands of DNA are immediately protected by binding of replication protein A (RPA) to form a bidirectional replication fork [3, 4]. Mcm10 plays a role in elongation by binding to the elongation factors such as DNA polymerase (pol) ε and pol δ. Mcm10 is also necessary for activation and disassembly of pre-RC [5].

First, DNA pol α and DNA primase are recruited to the origin and initiate the synthesis of short RNA primers. During DNA synthesis, this process occurs only once for the leading strand, but it occurs for each Okazaki fragment

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on the lagging strand. After synthesis of short RNA primers, DNA pol α is exchanged for DNA pol δ and ε, which have greater processivity. As the single-strand-binding protein replication factor C (RFC) binds to the primer template junction, proliferating cell nuclear antigen (PCNA) is immediately loaded at the primer end [6, 7]. Processivity is increased by binding of the ring-shaped PCNA (clamp structure) to DNA pol δ and ε, which results in activating the elongation of DNA synthesis. Another characteristic of these enzymes is proofreading (3′-to-5′-exonuclease activity). When pol ε reaches the 5′ end of a previously synthesized Okazaki fragment on the lagging strand, this fragment is displaced by the strand currently undergoing synthesis, leading to the generation of a flap structure. The flap structure is excised by the endonucleases Dna2 and flap endonuclease 2 (Fen2), and the resulting nick is sealed by DNA ligase I [8, 9].

Although much progress has been made in the understanding of eukaryotic DNA replication, events happen during DNA damage or replication errors that trigger cell death remain to be fully elucidated. Below, we summarize some of the major evidence that supports S-phase coupled cell death as a built-in fail-safe mechanism for preventing propagation of the damaged genome of a mammalian cell.

Replication checkpoint signaling

When replication is impeded by genotoxic stress caused by exogenous or endogenous events such as ionizing or ultraviolet radiation, smoke, chemical compounds such as hydroxyurea (HU) and aphidicolin (APH), or cellular metabolic by-products a signal transduction cascade is activated to prevent genomic instability [10]. The cascade consists of cell cycle checkpoints that are under the control of members of the phosphatidylinositol 3-kinase-related kinases (PIKK) family, such as ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR). When cell cycle checkpoint signals are induced, the cell cycle slows down to repair the damaged replicated chromosome; stalled DNA replication is restarted and DNA replication forks are stabilized. Failure of cell cycle checkpoints results in genomic instability caused by chromosomal abnormalities, which are hallmarks of cancer [11]. DNA damage can arise in any phase of the cell cycle. However, S-phase checkpoints are of particular importance because they prevent the bypass of lesion repair processes, thus enhancing the probability of error-free DNA replication. S-phase checkpoints are, in comparison with G1 and G2-phase checkpoints, of special importance [12].

There are three types of S-phase checkpoints: the DNA replication checkpoint, the intra-S-phase checkpoint, and the S-M checkpoint [13]. The DNA replication checkpoint inhibits the initiation of replication by the formation of complexes comprising single-stranded DNA (ssDNA) and RPA (ssDNA-RPA complexes); the complexes form in response to replication stress and they trigger cell cycle arrest. However, the intra-S-phase checkpoint is activated by DNA double-strand breaks (DSBs), which occur in response to exposure to ionizing radiation and leads to the suppression of DNA synthesis. The checkpoint can be divided into three steps, represented by sensor, mediator (or transducer), and effector molecules [14]. First, the DNA lesion is detected by sensor molecules such as ATR, ATM, MRN, and 9-1-1 complex. The signal is then amplified by mediator molecules and finally communicated to effector molecules, which phosphorylate target proteins. While cell cycle progression is arrested or retarded by the physiological response of the cell, other pathway triggers stabilize replication fork structure and activate repair.

The primary S-phase checkpoint kinase, ATR, senses a wide variety of DNA lesions induced by UV radiation and reagents such as HU and methyl methanesulfonate (MMS); ATR effectively interrupts DNA replication [15]. In contrast to ATR, ATM kinase is activated in response to DSBs that result from ionizing radiation [16, 17]. MRN, a core protein kinase complex, is composed of Mre 11, Rad50, and Nbs1; MRN is localized to DSBs independently of ATM; however, MRN complexes are substrates for ATM and can induce ATM activation [13]. When active ATM is loaded to the site of DSBs by MRN complexes, Rad50 and Nbs1 induce the exonuclease activity of Mre11. Another sensor molecule, the 9-1-1 complex, consists of three proteins (Rad19, Rad1, and Hus1) that form a ring-shaped structure, similar in structure and function to the sliding clamps of PCNA [18, 19]. In response to DNA lesions, Rad17 forms a complex with 9-1-1, which opens the PCNA clamp complex and loads the complex onto the DNA. The Rad17/9-1-1 complex binds not only to RPA but also to the stalled replication fork; the recruitment of RAD17/9-1-1 occurs later than the recruitment of the ATR/ATR-interacting protein (ATRIP) complex [20].

The initial response to DSBs is the activation of ATM, followed by the activation of ATR, which results from ssDNA (a consequence of a DSB) that retains the intra-S-phase checkpoint. This in turn, blocks late origin firing. It is suggested, therefore, that ATR and ATM co-operate, even if they are activated by different types of DNA lesions [21]. The co-operation is represented by two parallel pathways that function during the intra-S-phase checkpoint: the ATM-Chk2 and ATM/MRN pathways. The signal detected by sensor molecules is amplified by a mediator or transducer molecule that plays a critical role in transmitting the signal to its targets. Claspin mediates Chks at a stalled replication fork and activates ATR, leading to a slower proliferation of cells and the up-regulation of CDC25A, which in turn, is dependent on Rad17 and

FANCD2 [22, 23]. The second mediator group forms foci at sites of damage resulting from DNA lesions, and has motifs such as BRCT and FHA that transmit the signal to Chk1 and Chk2. In response to DNA damage, H2AX phosphorylated by ATR/ATM forms foci at sites of damaged DNA; the H2AX holds the broken chromatin end and leads to the accumulation of MDC1, which is an important regulator of the damaged chromatin [24, 25]. Another mediator molecule, MDC1, is required for the recruitment of Nbs1 and binds to phosphorylated H2AX via its BRCT domains; this molecule is required for the recruitment of Nbs1, which functions as an anchor for the MRN complexes at the sites of DNA breaks [26, 27]. A component of the cohesion complex, SMC1, required for sister chromatid cohesion during the S-phase, is activated by phosphorylation by ATM and ATR [28]. Phosphorylation of Chk1 plays a critical role in checkpoint signaling, whereas activation of SMC1 is important for cell survival; therefore, the activation of SMC1 and Chk1 regulates two branches of the checkpoint response in the event of DNA damage [29]. The mediator molecules amplify and transmit signals to effector molecules. Chk1 and Chk2 are two major effectors, activated by the phosphorylation of their substrates [30, 31]. Although these kinases share similar biochemical functions related to phosphorylation of their target proteins, they differ in their activations of checkpoint pathways. While Chk1 is activated by ATR and ATM in response to cellular stress such as UV light, stalled replication, and some other compounds, the active form of Chk2 (the dimerized form) can phosphorylate CDC25. Chk1 is strongly expressed only in the S and G2-phases, whereas Chk2 is present through the entire cell cycle.

Apoptosis

Cells are removed by apoptosis if DNA damage is not repairable and the cell cannot tolerate cellular stresses. Consequently, the inactivation of apoptotic pathways induces genomic instability and chromosomal abnormality, which may manifest as cancer. Apoptosis is initiated either through an extrinsic pathway, which is mediated by death receptors on the cell surface, or through an intrinsic pathway, known as the mitochondrial apoptotic pathway [32]. DNA-damaged cells are eliminated by the intrinsic pathway, in which the Bcl-2 family of proteins plays a critical role in the regulation of apoptosis.

Apoptosis is regulated by two Bcl-2 subfamilies, depending on the type of influence: pro-apoptotic or anti-apoptotic [33]. Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl1/A-1, and Bcl-B proteins, all of which possess four Bcl-2 homology domains, have anti-apoptotic characteristics. Among pro-apoptotic proteins, Bax, Bak, and Bok share the Bcl-2 "multidomain protein" homology, whereas Bim, Bad, and Bid are members of the "BH3-only protein" domain. In normal cells, anti-apoptotic Bcl-2 family proteins suppress the activation of pro-apoptotic sub-families. The pro-apoptotic BH3-only proteins, on the other hand, are activated by apoptotic stimuli and lead to the activation of Bax and Bak. Two models have been proposed for the activation of Bax and Bak, direct and indirect; these models describe how Bax and Bak render the mitochondrial membrane permeable. In the direct model, sensitizer BH3-only proteins (such as Bad, Bik, Puma, and Noxa) are activated by apoptotic stimuli, followed by the release of activator BH3-only proteins such as Bim and Bid; the BH3-only proteins bind to effector proteins such as Bax and Bak, which activate processes leading to oligomerization [34]. According to this model, the sensitizer proteins are unable to bind to effector proteins. In the indirect model, BH3-only proteins bind to anti-apoptotic proteins, which suppress Bax and Bak. This leads to the displacement and release of Bax and Bak, which trigger their oligomerization [35]. The activation process of Bax occurs in two steps: initially Bax translocates to the mitochondria and then triggers a conformational change in the N-terminus. This results in the oligomerization and permeabilization of the mitochondrial outer membrane, followed by the release of apoptotic proteins from the mitochondrial intermembrane space into the cytosol, such as cytochrome c, AIF, Hsp60, endonuclease G, and Smac/Diablo; these molecules are involved in cysteine aspartyl-specific protease (caspase) activation cascades. This results in the amplification of apoptotic signaling [36]. Cytochrome c binds and activates APAF1, which triggers conformational changes and forms an apoptosome. The apoptosome, in turn, activates the initiator caspase (caspase-9). Smac/Diablo and HfrA2/Omaz also activate caspase cascades by the suppression of caspase inhibitors such as Xiap. In both apoptotic pathways (intrinsic and extrinsic), caspases are activated by the release of apoptogenic factors from the mitochondrial intermembrane into the cytosol [37]. The apoptotic caspases are divided into initiators and effectors, depending on the mode of activation. First, the initiator caspases, such as capase-8 and caspase-9 are activated at the apoptosome (intrinsic pathway) or the death-inducing signaling complex (DISC) (extrinsic pathway), followed by a caspase-9-mediated cleavage in the intrinsic pathway, which leads to the activation of effector caspases (caspase-3 and caspase-7). This results in the destruction of cells [32, 33].

E2F transcription factors in cell cycle regulation and apoptosis

E2F1 was originally identified as a transcription factor essential for E1A-dependent activation of the adenovirus

E2 promoter [38, 39]. E2F proteins are primarily maintained in an inactive state through sequestration into protein complexes. E1A binding results in the dissociation of E2F from this complex. Free E2F can then mediate E1A actions through transactivation of both cellular and viral E2F-dependent genes [40–42].

The E2F family comprises eight members, which can be further sub-divided into two groups on the basis of whether their DNA binding activity leads to the activation or repression of transcription [43–45]. E2F1, E2F2, and E2F3 have been reported to activate transcription in over-expression assays. Conversely, E2F4 and E2F5 have been shown to prevent transcriptional activation [45–47]. One DNA binding domain and one DP dimerization domain (DIM) are present on E2F1–E2F6. The C-terminal domain of E2F1–E2F5 contains a transactivation domain and sequences that are essential for binding to pocket proteins [48]. The pocket protein family consists of pRB, p107, and p130. Each pocket protein binds to different members of the E2F family in vivo and has been shown to modulate E2F transcriptional activity [45]. pRb primarily binds with the transcriptional activators E2F1, E2F2, and E2F3, while p130 mainly associates with the transcriptional repressor E2F5. In contrast to other E2F family members, E2F4 can bind to all pocket proteins but is regulated mainly by p107 and p130 [47, 48]. While nuclear localization signals (NLS) are present in the transcriptional activators E2F1, E2F2, and E2F3, the transcriptional repressors, E2F4 and E2F5, contain two leucine/isoleucine-rich hydrophobic nuclear export signals (NES) [46]. Thus, unbound E2F4 and E2F5 are localized in the cytoplasm and can be imported into the nucleus after binding with pocket proteins [47]. E2F4 and E2F5 are detected in G0 cells, while E2F1, E2F2, and E2F3 are primarily detected in actively dividing cells [48].

The major role of transcription factor E2F1 is to transit cells from the G0/G1-phase to the S-phase of the cell cycle [49, 50]. Several genes are needed to transactivate the cell through the S-phase, such as DHFR, thymidine kinase, and DNA polymerase α [49, 51, 52]. Beyond its principal role of transactivating cells into the S-phase, E2F1 is additionally involved in the regulation of DNA replication through the activation of S-phase checkpoints [43, 49, 53]. E2F1 is stabilized by exposure to ionizing radiation or treatment with a DNA-damaging agent, due to the phosphorylation of E2F1 by ATM and ATR, leading to S-phase delay and apoptosis.

The N-terminus of E2F1 has several binding domains, such as the cyclin A/cdk2 binding domain, DNA binding domain, and transactivation domain, which play important roles in the inhibition of DNA replication to suppress genomic instability [54, 55]. The over-expression of the E2F1 N-terminus results in an arrested S-phase, and thus, the checkpoint function of E2F1 appears to be regulated by its N-terminus [56]. By serial deletion assay, it was found that the first 59 amino acids of E2F1 play a role in the inhibitory effect. Therefore, it was suggested that the cyclin A/cdk2 binding, DNA binding domain, and transactivation domains have no effect on the inhibition of DNA replication. The full-length E2F1 and its N-terminus construct suppressed DNA replication, whereas N-terminal deletion of the E2F1 construct did not; thus, E2F1 appears to inhibit the elongation phase and not the initiation of DNA replication. Additionally, E2F1 cDNA has a *cis*-acting inhibitory element, which was confirmed by in vitro SV40 DNA replication assays using two deletion constructs of E2F1 [56]. One was a C-terminal deletion construct, which expressed the first 139 amino acids of E2F1. The other was the same construct, but containing the entire E2F1 cDNA sequence, including the stop codon introduced between bases 411 and 412 of the E2F1 cDNA. Analysis of the replication level using the deleted construct, which expressed only the first 139 amino acids of the entire cDNA sequence, showed dramatically decreased replication, similar to what is observed using the wild-type E2F1 plasmid [56]. However, the replication level using the deleted construct without the entire cDNA sequence showed increased replication, similar to what is observed using the control plasmid pCDNA3. The ability of the C-terminal of E2F1 cDNA to bind to the N-terminus of the E2F1 protein, which was confirmed by gel shift assay, indicates that the N-terminus of E2F1 belongs to a DNA binding domain and functions as a *cis*-acting inhibitory element involved in the inhibition of DNA replication. The C-terminal of E2F1 may also inhibit the activity of proteins involved in replication elongation, such as topoisomerase I, or the activity of processivity factors such as PCNA, RF-C, and DNA polymerase sigma, resulting in the inhibition of DNA replication. This sequence could contain the sites where the S-phase checkpoint is activated [56].

Phosphorylation of Nbs1 by ATM is required for the activation of the S-phase checkpoint. The Mre11 complex (composed of Mre11, Rad50, and Nbs1) plays a role in the activation of the S-phase checkpoint [57]. It is co-localized with PCNA at the replication fork throughout the S-phase, suggesting that the Mre11 complex suppresses genomic instability as a negative regulator of the initiation of DNA replication in response to DNA damage. The interaction between Nbs1 and E2F1 was observed by yeast two-hybrid screening [58]. By serial deletion analysis, it was found that the C-terminus of E2F1 between amino acids 284 and 416 binds to Nbs1, which contains the transactivation and Rb-binding domains. Neither of these domains in E2F1 alone could bind to Nbs1. The first 221 amino acids of the N-terminal of Nbs1 contain an FHA and BRCT domain, which are

involved in S-phase regulatory functions of the Mre11 complex. When this region is deleted, Nbs1 cannot interact with either E2F1 or Mre11, indicating that the N-terminus of Nbs1 binds to E2F1 [58].

Nbs lymphoblastoid cells, which contain mutant Nbs1 (657del5) and Nbs1p70, in which the first 221 amino acids are deleted, showed radio-resistant DNA synthesis (RDS), even when Nbs1 was phosphorylated at serine 343 in response to ionizing radiation, indicating that Nbs1 phosphorylation is necessary, but not sufficient, for the activation of the S-phase checkpoint [59–62]. Immunoprecipitation showed that the Mre11 complex-E2F1 binding affinity was reduced in both A-TLD2 (R663X Mre11 mutant) and A-TLD3 (N117S Mre11 mutant), but remained intact in A-T cells, whereas RB immunoprecipitated with E2F1 in both types of A-TLD cells, suggesting that defective Nbs1–E2F1 interactions are intrinsic to the mutant Mre11 complex [58]. Reduced levels of Nbs1–E2F1 complex in A-TLD cells may result in lower levels of Nbs1 and localization of these mutant cells in the cytoplasm, leading to an inhibition of the activation of the S-phase checkpoint. Abrogation of the interaction between Nbs1 and E2F1 compromises the activation of the S-phase checkpoint in response to DNA damage, and compromises the capacity to suppress the initiation of replication. Neither phosphorylation of Nbs1 nor Nbs1–E2F1 interaction alone was able to activate S-phase checkpoint activation [58].

In response to DNA damage, DNA replication could be slowed, stopped for repair, or induced to undergo apoptosis by activation of the S-phase checkpoint by E2F1, which inhibits the elongation of DNA replication at the DNA target sequence, suggesting that E2F1 may directly sense S-phase-related DNA damage.

Deregulation of E2F by inactivation of Rb or enforced E2F1 expression results in the accumulation of caspases-2, 3, 7, 8, and 9 through a direct transcriptional mechanism [63]. By up-regulation of the initiator caspase, caspase-8, E2F1 might sensitize cells to death-inducing ligands such as tumor necrosis factor (TNF)-α [64]. E2F1 can induce apoptosis by inhibiting activation of anti-apoptotic signal factors, including Bcl-2 and its family member myeloid leukemia cell differentiation protein 1 (Mcl-1), in a p53 family-independent mechanism [65]. Both factors are inhibited by binding to the DNA-binding domain of E2F1. Moreover, E2F1 can inhibit the activation of another anti-apoptotic signal, necrosis factor (NF)-κB, in a p53-independent manner. This inhibition occurs through the down-regulation of TNF receptor-associated factor 2 (TRAF2) protein levels, which is involved in the survival signal. Thus, E2F1 induction is important as a fail-safe mechanism to eliminate defective cells [66, 67].

Geminin as a sensor for accurate DNA replication

Geminin was initially discovered in an expression cloning screen to identify degraded proteins from mitotic *Xenopus laevis* egg extracts [68, 69]. At about the same time, a second geminin was identified in a screening for early patterning proteins from blastomeres of L-cell *Xenopus* embryos. The proteins encoded by the two cDNAs are 89% identical at the amino acid level and seem to have similar properties. Because of their apparent similarities, the protein was named geminin, in honor of the astrological sign for twins.

Geminin has two NLSs in its C-terminus and a destruction box sequence at its N-terminus [68]. The coiled-coil domain lies in the central region, which is responsible for protein dimerization [70]. Geminin acts as an inhibitor of DNA replication by direct binding to Cdtl, which is essential for pre-RC formation [71]. The resulting dimerization inhibits the recruitment of Mcm2–7 by Cdtl on the chromatin [72, 73]. Mutations in this domain inhibit binding to Cdt1.

The destruction box in geminin may play a critical role in cell cycle control [74]. Levels of geminin are low in the G1-phase but begin to accumulate during the S and G2-phase. Geminin expression levels subsequently decline at the metaphase–anaphase transition due to APC-mediated ubiquitination [68]. The conserved N-terminal destruction box of geminin is required for APC-mediated ubiquitination and proteolysis in mammalian cells and *Xenopus* eggs [68]. Mutation of the D-box inhibits APC-mediated ubiquitination and leads to stabilization of geminin [74]. In contrast to higher eukaryotes, geminin is expressed throughout the cell cycle in early embryonic *Xenopus* and *Drosophila* cells. In these species, if geminin binds to Cdtl during interphase, it may be degraded by APC-mediated ubiquitination, which in turn is dependent on CDK activity. Therefore, CDK acts not only as an inhibitor of pre-RC formation but also promotes the inactivation of geminin upon exit from metaphase. Interestingly, in contrast to other APC ubiquitination targets, degradation of geminin does not require the proteasome. Therefore, a second modification of geminin may occur after ubiquitination.

The mutation in the D-box of geminin inhibits its degradation and leads to its stabilization and accumulation in human cells, which causes a decrease in cell proliferation and a cell-cycle block in the G1-phase. In accordance with its role in proliferation, geminin has also been reported to be involved in the development of neural tissue in *Drosophila* and *Xenopus*. The homeobox transcription factor Six3 was found to be a Cdtl competitor in eye development. Competition between Six3 and Cdtl for geminin binding determines the extent of proliferation and differentiation [75], and this observation has led to the

suggestion that geminin is necessary for cell proliferation in vivo. The regulation of geminin is dependent on cell type. Although a homolog of geminin was identified in *Drosophila*, no homolog has been found in yeast. The DNA replication mechanism may differ in higher eukaryotes, but the possibility that yeast express a geminin homolog of very low homology has not been excluded [76].

Knockdown of geminin has been attempted using methods including antisense technology, mutagenesis, RNA interference, and small interference RNA [77–79]. Because of the complexity of different regulatory mechanisms in embryonic and cultured cells, studies to date have not yet established whether geminin is necessary to maintain a normal cell cycle [80]. Depletion of geminin induces DNA re-replication in normal human cells and in some tumor cells, and geminin depletion is sufficient to induce genomic instability. This is because over-replication within the same cell cycle induces the activation of ATR/ATM and CHK1/CHK2 kinase checkpoint pathways, accompanied by formation of γH2AX and RAD51 nuclear foci; this prevents cells with re-replicated DNA from entering into mitosis [80, 81]. Disruption of the G2/M checkpoint suppresses the accumulation of over-replicated cells and causes apoptotic cell death.

In contrast, geminin depletion in HeLa [3, 82], MCF10 [83], and 293T cells [84] does not induce DNA re-replication. For instance, HeLa cells do not dramatically change phenotype after geminin knockdown. This is because very efficient ubiquitination and proteolysis control Cdtl levels in the S-phase, preventing DNA re-replication [85].

Geminin is essential for maintaining genomic integrity by preventing re-replication. Depletion of geminin is sufficient to induce genomic stability. Because genomic stability seems to be one of the major features of human cancer, geminin may act as a putative tumor suppressor gene [3, 82].

Over-expression of geminin induces apoptosis by inhibiting replication licensing via a cell type-dependent mechanism [86]. For example, over-expression of geminin in U2OS (p53+/Rb+) cells results in localization of PCNA in replication foci and in low levels of BrdU incorporation. This suggests that the cells enter the S-phase, but that replication is blocked due to activation of an intra-S-phase checkpoint. In cells experiencing this early S-phase arrest, cyclin E is up-regulated, whereas cyclin A is down-regulated. In Saos2 (p53−/Rb−) cells, geminin over-expression leads to DNA synthesis and accumulation throughout the late S and G2/M-phases, with approximately normal levels of cyclin A and low levels of cyclin E. These patterns are consistent with loss of an intra-S-phase checkpoint, as though cells are unable to complete DNA replication due to an insufficiency of the licensed origins [86].

Over-expression of geminin in IMR90 primary fibroblasts leads to very different results than those observed in the cancer cell lines. Although geminin blocks cell proliferation and reduces the levels of Mcm2 on chromatin, cells are arrested in the G1-phase before entry into the S-phase, with reduced levels of cyclins A and E and no detectable apoptosis. This appears to happen because primary cells can respond directly to the absence of sufficient licensed replication origins by blocking entry into S-phase with or without a 'licensing checkpoint' [86].

Geminin is also known to interact with Plk1 (polo-like kinase 1), an enzyme that is essential in mitosis [87]. Plk1 increases during the S-phase and peaks during the M-phase [88]. It phosphorylates Emi, which is then degraded, leading to activation of the APC/C. This degrades mitotic proteins, such as cyclin B1 and geminin, causing cells to exit the M-phase when mitosis is complete [89–91]. The depletion of Plk1 and geminin leads to up-regulation of Emi1, which inhibits the APC/C and DNA pre-RC. This disrupts the formation of DNA pre-RC and activation of DNA damage-sensing kinases, leading to apoptosis [87].

Work in primary cells and cancer cell lines has shown that during apoptosis, two sites at the carboxyl terminus of geminin (C1 and C2) are cleaved by caspase-3, resulting in the creation of two truncated forms of geminin [92]. The cleavage is likely mediated by CKII phosphorylation. Truncated geminin caused by C1 cleavage is involved in induction of apoptosis. Truncated geminin caused by C2 cleavage is unable to interact with the chromatin remodeling factor Brahma (Brm), a catalytic subunit of the SWI/SNF chromatin remodeling complex; however, it maintains the ability to interact with the cell cycle regulator Cdt1 [92].

An important first step is understanding why the checkpoint signal is activated during over-expression of geminin in cancer cell lines. One possibility is a reduced number of active replication origins, followed by a stalling of replication forks during the course of undisturbed S-phases. This would account for the chromatin-bound PCNA observed in geminin-expressing U2OS cells [86].

Another possibility is that replication may proceed at near-normal rates until certain replication origins are inactivated due to reductions in Mcm2–7 levels. This would prevent the cell from completing DNA replication, arresting mitosis late in the S-phase and leading to apoptosis [86].

The knockdown of geminin in *Drosophila* and mammalian cells induced re-replication and activated checkpoint pathways, which inhibit entry into mitosis [93, 94]. During mitosis, high levels of CDK activity are sufficient to inhibit the relicensing of DNA replication independent of geminin levels. However, geminin plays a critical and specific role in S-phase suppression of

relicensing, when CDK levels alone are insufficient. Geminin has been suggested to positively regulate licensing during mitosis by binding and stabilizing Cdtl and to act as a negative regulator by inhibiting relicensing [93]. The expression levels of geminin and Cdtl remain complementary throughout the cell cycle. This balance may play a critical role in the regulation of proliferation in the cell cycle, with geminin acting as both a negative regulator by inhibiting pre-RC complex formation in the S-phase and as a positive regulator by maintaining the basal expression level of Cdtl through ubiquitin-mediated degradation [77]. Surplus Cdt1 causes re-replication, DNA damage, and genomic instability. This results in the activation of checkpoint proteins such as the ATM/ATR kinases and the tumor suppressor p53, whereas surplus geminin triggers cell cycle arrest. Thus, a balance between geminin and Cdt1 expression is important for genomic stability [94, 95].

Killin as a missing link in p53-mediated S-phase control and apoptosis

Usually, cells only replicate once per cell cycle and replication cannot occur again before mitosis. When cells are damaged, at least two factors affect whether cells undergo growth arrest or apoptosis. First, cells are arrested in the G1 and G2-phases as a result of activation of p21, which binds and suppresses CDK and blocks cell cycle progression [96–98]. During the cell cycle arrest, cells can repair the damage before continuing the cell cycle. In this case, p21 plays an anti-apoptotic role. Since p53 stimulates p21 expression, this may be a p53-mediated mechanism. Consequently, deletion of p21 from colon cancer cells or mouse embryonic fibroblasts allows p53 to induce apoptosis [99–102]. Second, cells that undergo apoptosis in response to p53 can overcome p21-mediated protection because pro-apoptotic factors require cells to enter the S-phase [103–105]. The supporting evidence for S-phase-coupled apoptosis includes findings that forced entry into S-phase by unrestricted E2F activity can activate caspases, and as a result, induce apoptosis [106]. Conceivably, DNA damage, and consequently cell cycle arrest, can occur at any phase of the cell cycle. For example, p53 can induce growth arrest via either p21 during G1 or p21, GADD45, and 14-3-3 at the G2/M transition.

Killin was discovered in an attempt to systematically identify p53 target genes through high-throughput fluorescent differential display (FDD) screening using two tetracycline inducible cell lines [107–111], the DLD-1 colon cancer cell line [112, 113] and the p53-null human lung carcinoma cell line H1299 [114]. Both cell lines contained tetracycline-regulated expression of the wild-type p53 tumor suppressor gene and underwent apoptosis within 24–48 h after removal of tetracycline. A 4.1-kb full-length Killin cDNA was isolated from a human kidney cDNA library [115]. After complete sequencing the cDNA (GenBank accession no. EU552090) was shown to encode a small 20-kDa basic protein of 178 amino acids residues with an apparent pI of 11.3. Given its alkaline pI and the existence of two putative nuclear localization domains, Killin may be a nuclear protein. In fact, its localization was confirmed by Western blot and immunofluorescent microscopy. Additionally, Western-blot analysis showed that Killin was induced not only by ectopic p53 expression but also by activation of endogenous p53 in response to genotoxic stress, such as doxorubicin or 5-FU treatment.

By DNA sequencing and genomic database search it was revealed that Killin is localized in close proximity to the PTEN tumor-suppressor gene on human chromosome 10 [116]. The intergenic region separating the two genes (based on transcriptional start sites) is only 194 bp in length and contains a divergent promoter with a high-consensus p53-binding site. Interestingly, PTEN was previously shown to be modulated by p53 as well, although, unlike Killin, the basal level of PTEN expression appears to be constitutive [116–120]. It was confirmed that Killin is a direct p53 target gene by both ChIP and dual luciferase reporter assays using a 140-bp intergenic region containing the conserved p53-binding site [115]. The Killin promoter not only was shown to bind to p53 but also conferred $\approx$70-fold increase in wild-type p53-dependent luciferase activity, whereas an expression vector encoding a DNA-binding mutant p53 (R248W) failed to activate the promoter. Moreover, mutations within the conserved p53-binding site in the Killin promoter greatly decreased the p53-dependent promoter strength. Taken together, these results confirmed that Killin was a direct transcriptional target of p53 [115].

On closer inspection of the minimal 41-aa Killin peptide sequence essential for DNA binding in vitro and killing of bacteria in vivo, it was noted that multiple WXXR and KXXW motifs were present [121]. Although a theoretical protein-folding prediction could not provide the definitive secondary structure of the Killin/N8–50 peptide, conceivably these regular motifs would bring R, K, and W residues along the same surface for DNA binding should the peptide fold into binary α helices that are connected by the single proline residue within the peptide sequence [115]. The binary DNA-binding fingers could allow Killin to bind to more than one DNA template, causing it to tangle up, which may explain why the DNA-Killin/N8–50 peptide complex had dramatically retarded mobility on the gel. Conceivably, tryptophan (W) may interact with purine or pyrimidine bases, whereas basic amino acid residues arginine (R) and lysine (K) may interact with phosphates in the DNA. The tight binding of Killin to DNA may prevent DNA synthesis machinery from accessing or moving along

the template, thus leading to inhibition of DNA synthesis and S-phase arrest.

The Killin protein is highly conserved (95–100%) in large mammals (cow, pigs, dogs, and chimps), while rodents exhibited some local sequence homology at the DNA level without the corresponding Killin ORF [115]. No sequence homology of Killin was found in fly, worm, zebra fish, and yeast. Thus, Killin may be unique to large mammals that have longer life spans than rodents, rendering a mouse KO model for Killin irrelevant without proving the existence of the gene.

Killin is sufficient and necessary for triggering cell growth arrest and subsequently apoptosis

To determine whether Killin is sufficient for triggering cell growth arrest and apoptosis, several experiments were performed including cell proliferation, fluorescent microscopy, and FACS analysis using DLD-1 colon cancer cells with inducible tetracycline-regulated expression of GFP-Killin [115].

GFP-Killin was shown to cause rapid cell growth arrest within 24 h after tetracycline removal, whereas GFP alone had little effect. Interestingly, unlike p53-mediated growth arrest, which occurs primarily at G1 via p21, FACS analysis indicated there was little decrease in S-phase DNA content or increase in either G1 or G2 DNA content during the first 48 h of cell growth arrest after the induction of Killin. This rather surprising finding suggested that Killin may function as an inhibitor of DNA replication and causes S-phase arrest. However, massive apoptosis was observed by FACS analysis and fluorescence microscopy 2–3 days after tetracycline removal and induction of GFP-Killin. This finding suggested that Killin-induced growth arrest is coupled to cell death, in contrast to G1 arrest mediated by p21, which prevents cells from undergoing apoptosis.

To determine whether Killin is necessary for p53-mediated apoptosis, RNAi technology was used to selectively knock down Killin mRNA expression in H1299 cells, which contain an inducible wild-type p53 gene, that were used for the initial FDD screening [122]. Cells were stably transfected with either the pSUPER control RNAi vector or pSUPER-Killin. Cells receiving the latter treatment had diminished Killin protein expression and a marked blockade of p53-mediated apoptosis, manifested by dramatic inhibition of both caspase 3 activation and caspase-dependent PARP cleavage, and as observed altered by FACS cell cycle profiles. The reduction in Killin had little effect on p53-induced p21 expression, and the cells were arrested in the G1-phase. Additionally, FACS analysis revealed a concomitant decrease in both S and G2/M content. This is consistent with the prediction that Killin is a p53-dependent checkpoint control during the S-phase. Thus, Killin appears to be an integral part of p53-mediated apoptosis, functioning in both S and G2/M cell cycle control [115].

In addition to being necessary for exogenous p53-induced apoptosis, Killin also appears to be a vital part of endogenous p53-mediated apoptosis. RNAi knockdown of Killin expression completely blocked apoptosis (mediated by caspase 8 and PARP cleavages) after cells were given a 5-fluorouracil (5-FU) treatment [115]. PUMA and Bax are two proteins that have previously been shown to be necessary for cell apoptosis mediated by exogenous p53 overexpression [123]. However, it was not clear whether they were also downstream effectors of endogenous Killin-mediated p53 apoptosis. To investigate this possibility, a 5-FU treatment was given to wild-type HCT116 cells and derivatives containing homologous deletions of p21, p21 and PUMA, and p21 and Bax. The results suggested that, unlike Killin knockdown, deletion of PUMA or Bax alone was insufficient to completely abolish p53-dependent apoptosis. However, both genes (especially PUMA), seemed capable of partially blocking p53-mediated caspase activation. These results suggested that PUMA works with an as-yet-unidentified gene [or gene(s)] as a downstream effector of Killin in p53-mediated apoptosis [115].

Killin is a general DNA synthesis inhibitor with high-affinity to DNA

In bacteria cells induction of Killin by IPTG caused immediate growth arrest of the bacterial hosts within 30 min when the expressed protein was barely detectable by Western-blot analysis using a His-tag monoclonal antibody [109]. Based on its extremely toxic effect of low-level Killin expression in bacteria it is predicted that Killin is a general DNA synthesis inhibitor, given that bacteria have naked DNA. To overcome the difficulty in the expression and purifying of Killin protein, full-length Killin with predicted 20-kDa molecular mass was produced by in vitro transcription and translation and was shown to be able to bind to both single- and double-stranded DNA templates.

To better define the functional domain of Killin for DNA binding, deletional mutagenesis was then conducted by PCR from both the N and C termini of Killin [115]. It was revealed that the minimal sequence from 8- to 49-aa residues were essential for Killin's toxicity in bacteria. The same Killin deletion mutants fused to GFP were also tested for their ability to cause apoptosis (nuclear condensation) in H1299 cells. The results were consistent with those seen with toxicity assays in bacteria. It is interesting to note that the minimum essential region of Killin contained multiple

WXXR or KXXW motifs and was rich in basic amino acids.

In order to investigate DNA binding kinetics in vitro, a peptide was chemically synthesized that was 42 amino acid residues in length corresponding to Killin N8–50 and was introduced to 32P-end-labeled oligonucleotide probes used as DNA templates. The Killin/N8–50 peptide was able to bind to the double-stranded DNA and to an artificial replication fork with an apparent Kd of 1–2 μM. The peptide had slightly higher affinity to the single-stranded DNA template with an apparent Kd of 0.5 μM. However, since each single-stranded DNA probe may contain more than one binding site for the Killin peptide, the actual Kd could be lower. Regardless, this quantitative biochemical binding study provided strong evidence that Killin was indeed a high-affinity DNA-binding protein [115].

Killin inhibits DNA synthesis in Vitro and in Vivo

To determine whether Killin/N8–50 peptide binding to DNA has any consequences in DNA replication, in vitro eukaryotic DNA replication assays were performed. This assay uses a soluble cell-free system derived from a mammalian cell nuclear extract that is capable of replicating exogenous plasmid DNA molecules containing the simian virus 40 (SV40) origin of replication [124]. Replication in the system depends completely on the addition of the SV40 large T antigen. Using this assay, we showed that the Killin/N8–50 peptide could greatly inhibit DNA replication [115]. The requirement of a higher concentration of Killin/N8–50 peptide for the inhibition of DNA replication than that seen in the in vitro DNA-binding assays was most likely because of the high concentration of chromosomal DNA present in the nuclear extracts used as a source of the SV40 large T antigen. Such chromosomal DNA would conceivably compete against the plasmid template for Killin peptide binding, thus competitively inhibiting plasmid DNA replication. This prediction was consistent with results obtained by decreasing the amount of nuclear extract used for the assay.

Killin inhibits DNA replication in vivo

To test whether Killin directly blocks DNA replication in vivo, a BrdU incorporation assay was employed [125]. This was done by transiently transfecting an RFP-Killin expression vector into Cos-E5 cells, which were then pulse-labeled with BrdU for 30 min to mark S-phase cells. After immunostaining for BrdU (in green), it showed that few of the RFP-Killin cells had BrdU signals, as predicted, in contrast to truncated RFP-Killin and RFP-transfected control cells [115] (Fig. 1).

However, this finding suggested only that a majority of RFP-Killin-expressing cells could not enter the S-phase, rather, they were arrested at the S-phase. Interestingly, we were also able to find a few rare cells that had both BrdU incorporation and RFP-Killin expression in the same nuclei. When BrdU-labeled DNA replication foci (in green) were overlaid with that of RFP-Killin (in red) in the same cell, an amazing picture emerged: BrdU and RFP-Killin showed an essentially mutually exclusive nuclear pattern, with a majority of replication foci appearing blocked by RFP-Killin when compared to truncated RFP-Killin and RFP control. The beads-on-string nuclear appearance of RFP-Killin is consistent with Killin being a high-affinity DNA-binding protein with a preference for ssDNA. We believed these were rare S-phase cells expressing a rate-limiting amount of Killin when BrdU was added, so there was not enough RFP-Killin to block all replication foci. However, in reality, even if one replication becomes blocked (e.g., by endogenous Killin induced by p53 activation), which could be much harder to visualize, the cell may still not be able to complete S-phase. Similar results were obtained in H1299 cells. This crucial piece of evidence strongly supports that Killin directly blocks DNA replication in vivo.

Interestingly, p21 was markedly induced by GFP-Killin, which could explain the cell growth arrest observed prior to apoptosis. To shed light on the mechanism of p21 induction, another major p53 target gene as well as p53 itself were tested and found both were induced by GFP-Killin but not GFP control. Although, the DLD-1 colon cancer cell line is known to have one wild-type and one mutant p53 allele (Ser241 → Phe), little is known about whether the mutation resulted in loss of function of the wild-type p53 allele [115]. If Killin functions as a component of a positive feedback loop regulating p53, it would be predicted that Killin expression in the parental HCT116 cells will lead to the induction of wild-type p53, which proved to be the case.

To understand the molecular mechanism of Killin activated apoptosis and whether it is coupled to S-phase arrest, the status of Chk1 and Chk2 have been evaluated, which are key signal transducers or sensors within the complex network of genome integrity checkpoints [115]. It is conceivable that the S-phase arrest and stalled replication forks caused by Killin could activate these kinases. Upon induction of GFP-Killin, both Chk1 (total and phospho-) and Chk2 levels were indeed shown to be markedly increased. At this moment, it was revealed that the activation of Chk1 was most likely through Ser317 phosphorylation. p53 is known to be activated by Chk1 and 2 through phosphorylation. In fact the increase in total p53 level was likely due to phosphorylation of p53 at multiple sites at Ser9, Ser46, and Ser392.

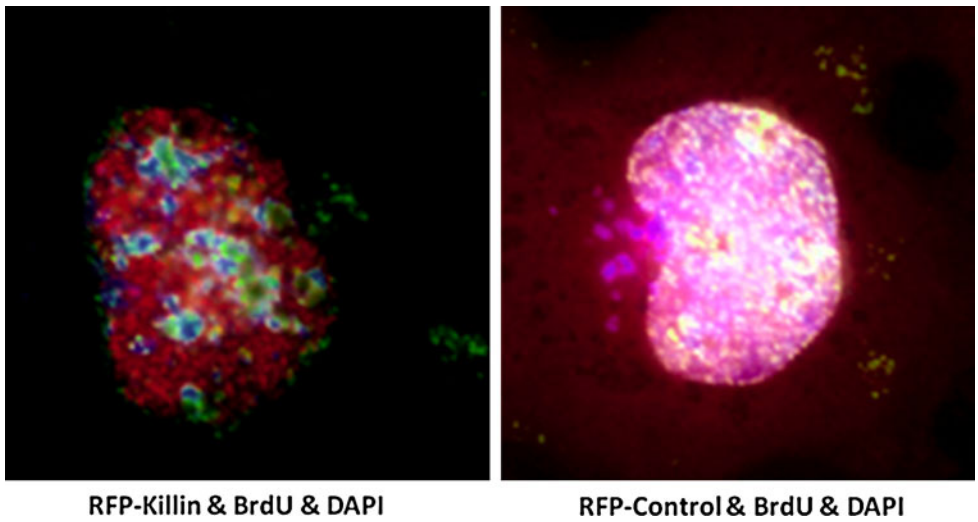

Fig. 1 RFP-Killin inhibits DNA replication in vivo. The RFP-Killin in-frame fusion protein (*upper*), RFP control (*middle*) or truncated RFP-Killin (*low*) expression vectors were transiently transfected into Cos-E5 cells by using FUGEN-6. Twenty-four hours after transfection, S-phase cells undergoing DNA replication were visualized after 30-min pulse label with BrdU followed by FITC-labeled anti-BrdU antibody staining (in *green*), under a Zeiss fluorescent microscope (×40). For cells in which the RFP-Killin (*red*) and BrdU signals (*green*) colocalized, the bulk of DNA replication foci (origins of replication) were missing in the area where the RFP-Killin foci reside. The overlay of fluorescent signals from BrdU labeling with RFP-Killin (merge) always exhibit a mutually exclusive pattern, in contrast to control cells transfected with RFP alone. DAPI was used to stain DNA (nuclei). Results shown were representative of multiple cells from at least two independent experiments

Killin is directly involved in p53-mediated cell growth arrest coupled with cell apoptosis [115]. Compelling evidence from cell biological, genetic, and biochemical analysis of the gene suggests the following possible mechanism of action for Killin in mediating p53 tumor-suppressor functions. Upon induction by p53 during S-phase, Killin functions in the cell nucleus as a DNA synthesis inhibitor via its high affinity to both double- and single-stranded DNA (e.g., at the replication forks) and thereby causes S-phase arrest, which in turn triggers subsequent cell apoptosis. Thus, Killin-mediated checkpoint control at S-phase would complement that at G1 mediated by p21 and G2-M-phase by p21, GADD45, and 14-3-3 and provides a foolproof mechanism for p53 in preventing precancerous cells from replicating their DNA content [96, 115, 126]. Therefore, Killin represents a p53 target gene that is directly and functionally involved in S-phase checkpoint control, which is coupled to apoptosis, in contrast to p21-mediated G1 arrest, which is anti-apoptotic. The unique function of Killin in coupling S-phase arrest with apoptosis may also help explain why p21-mediated G1 arrest can be anti-apoptotic. Conceivably, prevention of cells from S-phase entry by p21 would spare cells from Killin-mediated inhibition of DNA synthesis. It is predicted that p21-deficient cells will be very sensitive to Killin-induced apoptosis and to p53 activation, which may now be experimentally tested. Without stalled replication forks caused by Killin, apoptosis may be avoided. The high affinity of Killin to both double- and single-stranded DNA could also be reconciled with the beads-on-string distribution pattern of RFP-Killin in S-phase nuclei. Future efforts are needed to determine how Killin-mediated DNA replication arrest triggers the activation of caspases and apoptosis.

The extremely close proximity of *killin* and *pten* is also of great interest, because it would make *killin* a candidate tumor-suppressor gene. *pten* was originally identified as a candidate tumor suppressor by positional cloning from the chromosome 10q23 region, which is frequently deleted in a variety of human tumors. Although *pten* is encoded by multiple exons spanning >100 kb, Killin resides in a single exon of only 4.1 kb with a <200-bp intergenic region. In fact, 50% of the human glioma cell lines with which *pten* deletions were initially mapped had deletions beyond the Killin locus [116–119]. The genomic DNA probe commercially available for FISH analysis of *pten* deletion or loss of heterozygosity spans the Killin locus, suggesting that many previously reported pten deletions in human cancers may also have *killin* deleted. The extremely short 194-bp intergenic region connecting the two genes contains a divergent promoter that appears to be p53-responsive for both *pten* and *killin*, with the latter shown here to be completely p53-dependent. One logical prediction for a major p53 target gene, such as Killin, would be that such a gene could be a tumor suppressor on its own. This is supported by the non-overlapping mutation spectra in human tumors for p53 and the *pten* region.

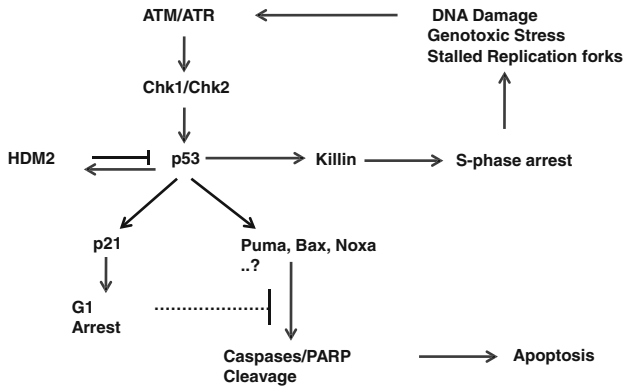

Fig. 2 A positive-feedback model showing the role of Killin in p53-mediated apoptosis. Genotoxic stress (e.g., radiation or the 5-FU treatment) is used to activate p53 via the ATR/ATM and Chk kinase checkpoint pathways, which function to sense and control DNA damage/repair. Subsequent induction of the p53 target gene, p21, will lead to growth arrest of cells that are in the G1-phase, whereas cells that have already entered the S-phase can only be stopped by Killin. The stalled replication forks caused by Killin further activates ATR/ATM and Chk1/Chk2 kinase checkpoint pathways, leading to persistent activation of p53 and induction of its apoptotic target genes (including known genes, such as *PUMA*, *Bax*, *Noxa*, etc., as well as those that may have yet to be identified). In contrast, this positive feedback loop cannot be activated in p21-arrested G1

Thus, there is growing evidence indicating that Killin plays an integral role as a nuclear inhibitor of DNA replication. Such activity is both a necessary and essential part of p53-mediated apoptosis, leading us to hypothesize that Killin is the long sought-after missing link between p53 activation and the S-phase check point control designed to eliminate replicating precancerous cells, should they escape G1 blockage mediated by p21 (Fig. 2). This would help explain the apparent paradox of p21 acting as both a growth and death inhibitor. The G1 arrest triggered by p21 can prevent cells from entering the S-phase, thereby escaping the apoptotic process through S-phase checkpoint control mediated by Killin.

Summary

While the mammalian cell growth is stringently regulated at multiple checkpoints at different phases of the cell cycle, the life and death decision of a cell is made primary during in the S-phase when genetic codes need to be faithfully copied. Any potential jeopardy at this most critical stage of cell cycle, such as forced S-phase entry by E2F activation, induces p53 dependent apoptosis, so does the transcriptional knockdown of geminin, an inhibitor of DNA replication. Yet why S-phase cells are prone to apoptosis is still not clearly understood. Killin, a p53-specific target gene essential for p53-mediated apoptosis, has all the attributes to be one of the missing links that directly trigger S-phase specific apoptosis. Future work along this pathway should shed more light on the intricate regulatory network that acts as a stringent control to ensure no detrimental mutations are passed onto any daughter cells that could endanger the well being of an organism.

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