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News and Commentary

Origin licensing and programmed cell death: a hypothesis

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Initiation of DNA replication in eukaryotic cells is regulated by an 'origin licensing' mechanism that requires the sequential assembly of proteins into pre-replicative complexes (pre-RCs) at origins of replication, the sites on chromosomes where DNA synthesis begins (reviewed in reference[1]). Activation of origins in S-phase by cyclin-dependent kinases (CDKs) coincides with the inactivation and partial disassembly of pre-RCs, and CDKs and other proteins block their re-assembly until the end of mitosis, when CDK activity is destroyed (Figure 1, top panel). The reciprocal relationship between pre-RCs and CDK activity provides a stringent, fail-safe mechanism for ensuring that DNA is replicated just once per cell cycle. Although the details are sometimes different, this basic mechanism for regulating chromosome duplication appears to be conserved in all eukaryotes.

Recent experiments[2] suggest the hypothesis that, in addition to regulating DNA replication, origin licensing plays an important role in regulating cell death. They show that multiple p53-independent, proteasome and/or caspase-dependent pathways destroy the replication initiation protein Cdc6, which is required for the assembly and/or maintenance of pre-RCs, at an early stage of apoptosis. The proteasome-dependent destruction of Cdc6, which was induced by the DNA-damaging drug adozelesin, occurs upstream of, or parallel to, the action of caspases. This pathway is conserved in budding yeast, where mutations in pre-RC proteins that interact with Cdc6 had previously been shown to alter the sensitivity of budding yeast cells to this drug.[3] In mammalian cells, the caspase-dependent destruction of Cdc6 was mediated by an extrinsic apoptotic pathway induced by TNF-α.

How might the destruction of Cdc6 contribute to cell death? In yeast and mammals, Cdc6 and some other proteins in pre-RCs are absent from quiescent cells, and licensing complexes are assembled upon entry into the cell cycle. In mammals, this occurs when proliferative signals activate the expression of licensing proteins regulated by the E2F family of transcription factors, downstream of cyclin D1 synthesis and the subsequent phosphorylation of members of the retinoblastoma protein (Rb) family. Thus, pre-RC assembly occurs at or just beyond the point where many of the pathways that regulate proliferation converge. In fact, these pathways may physically converge on origins of replication. In yeast,[4–7] *Xenopus*[8,9] and humans,[10] for instance, pre-RC proteins, including Cdc6, have been shown to interact with, and in some cases inhibit, CDKs required for progression through mitosis. Recent studies in mammals[11] and *Drosophila*[12] have also detected physical associations between components of pre-RCs and Rb and/or E2F, both of which have been implicated in DNA damage responses and apoptosis in addition to their roles in regulating proliferation. In *Drosophila*, a component of pre-RCs also regulates chromosome condensation and cohesion in preparation for mitosis.[13] All of these findings suggest that in eukaryotic cells, Cdc6 and other components of pre-RCs form a nexus for cell cycle regulation where DNA replication is coordinated with mitosis downstream of signaling pathways that regulate cell proliferation, and perhaps DNA damage responses and cell death.

One might predict, therefore, that loss of integrity of pre-RCs during apoptosis in proliferating cells would result in aberrant regulation of the cell cycle. In fact, accumulating evidence suggests that disregulation of the cell cycle is an important component of mammalian cell death pathways. At the simplest level, there is the longstanding observation that cycling cells are more sensitive to a variety of apoptotic triggers compared to quiescent cells. A clear *in vivo* example of this relationship is provided by studies of epithelial cells in the small intestine of mice exposed to ionizing radiation and other cytotoxic agents.[14] While cells in the proliferative compartment of this tissue are sensitive to these apoptosis inducers, they become refractile once they undergo a defined transition to quiescence.

The sensitization of proliferating cells to apoptosis induced by some apoptotic triggers can be duplicated by the forced overexpression of a number of oncoproteins in quiescent cells. Some studies have established direct connections between elevated levels of oncoproteins and the activity of CDKs during apoptosis. In fact, a compelling argument can now be made that the activation of CDKs is, in many cases, a requisite feature of programmed cell death in mammals (reference[15] and references therein). Perhaps the strongest argument may be the observation that many aspects of apoptosis are inhibited when CDK activity is blocked by dominant-negative CDK mutants.[16] This has now been observed in a variety of systems and in association with a number of different apoptotic triggers, including those required for normal differentiation processes.[17]

Interestingly, in yeast (reference[18] and references therein) and *Xenopus*,[19,20] the elimination of Cdc6 or the failure to form pre-RCs in G1 cells results in a CDK-dependent reductional anaphase when cells pass through mitosis in the absence of DNA replication. Recent experiments in budding yeast suggest this is due to the loss of a mitotic checkpoint that at least partly depends on inhibitory

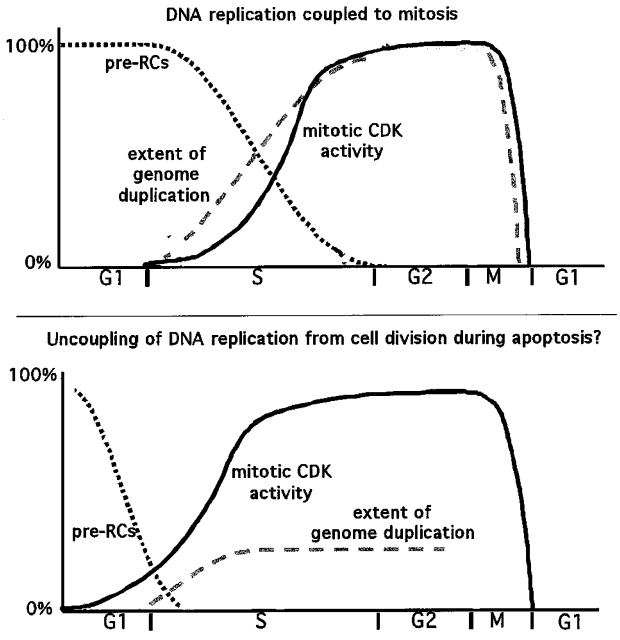

Figure 1 Top panel: Coupling of DNA replication to the cell division cycle by the reciprocal regulation of pre-RCs and mitotic CDKs. Experiments in yeast and *Xenopus* suggest the possibility that before they are activated for initiation, pre-RCs inhibit mitotic CDKs. As pre-RCs are activated in S-phase to establish replication forks, they are partly disassembled, and mitotic CDK activity increases. Activated mitotic CDKs block the re-assembly of pre-RCs until this activity is eliminated at the end of mitosis, ensuring a single round of genome duplication per cell cycle. Bottom panel: The disassembly of pre-RCs during apoptosis in G1 or early S-phase blocks the establishment of sufficient numbers of replication forks to completely duplicate the genome at the same time it allows mitotic CDKs to be activated. Increasing mitotic CDK activity inhibits the re-assembly of pre-RCs, even if the apoptotic stimulus is withdrawn, thus committing cells to an irreversible cell cycle arrest

interactions between Cdc6 and mitotic CDKs to restrain mitosis in cells with unreplicated DNA.[5] This checkpoint may also require other initiation proteins.[21] Although a similar role in mitosis for Cdc6 and other initiation proteins has not been described in mammals, conservation in humans of Cdc6 sequences required for inhibiting mitotic CDKs in *S. cerevisiae*[5] and of the interaction between Cdc6 and a CDK[10] required for progression to mitosis[22] suggest this role is likely to exist. Thus, one could also predict that the proteasome and/or caspase destruction of Cdc6 before pre-RCs have been activated for initiation of DNA replication might contribute to the unscheduled activation of CDKs frequently observed during apoptosis. This would provide an irreversible cell cycle arrest: once these CDKs are activated by the loss of Cdc6, pre-RCs cannot re-assemble until after mitosis, when mitotic CDK activity is destroyed – but in the absence of functional pre-RCs, initiation of DNA replication cannot occur, and chromosomal duplication cannot be completed (Figure 1, bottom panel). In essence, the loss of integrity of licensing complexes during apoptosis might serve to 'hijack' the uniquely irreversible origin licensing mechanism for blocking re-initiation of DNA replication until the next cell cycle, in order to ensure a commitment to cell death.

In fact, in addition to the inappropriate activation of CDKs, numerous other features of apoptosis also suggest it coincides with loss of the coordinated regulation of DNA replication and cell division. These observations led to earlier theories that during apoptosis, DNA replication becomes uncoupled from the cell division cycle, leading to a catastrophic, lethal mitosis (reviewed in[23]). However, the irreversible events associated with the disassembly of pre-RCs in G1 or early S-phase cells would precede mitosis. Although some cells may eventually proceed completely through the cell cycle without completing DNA replication, particularly cells with defective checkpoints, the molecular events of the ensuing catastrophic mitosis which was the focus of earlier models may be irrelevant to an earlier CDK-dependent commitment to cell death.

Our model may help to explain some aspects of the seemingly paradoxical relationship between proliferative pathways and programmed cell death. It predicts that at least in some cases, apoptosis in cycling cells and cells overexpressing oncogenic proteins may be related to the expression of licensing proteins in these cells, and not in quiescent cells that are generally more refractile to apoptosis. Although correlative in nature, consistent with this possibility is the fact that all the cells in the apoptosis-sensitive proliferative compartment of intestinal epithelium described above express Cdc6, but this expression becomes undetectable precisely at the transition from the proliferative to the quiescent state, where cells become refractile to apoptosis.[24]

Our model might also help to explain reports of an increased sensitivity to apoptosis of mammalian cells in G1,[25] as well as the G1-specific and proliferation-dependent sensitivity of budding yeast cells to the DNA-damaging drug adozelesin.[3] Once sufficient replication forks have been established in S-phase to completely replicate the genome, the destruction of pre-RCs may occur without consequence. The increased sensitivity of G1 cells is also reflected in studies of proliferating intestinal epithelial cells to cytotoxic agents described above. Although all the cells in the proliferative compartment express Cdc6 and are sensitive to apoptotic triggers, the more slowly dividing stem cells that less frequently enter S-phase from G1 are exquisitely sensitive compared to daughter progeny cells, which are more frequently in S-phase until they undergo the transition to quiescence[14] marked by the loss of Cdc6.[24]

This model has implications for designing effective therapeutic strategies for treating cancer. Like stem cell populations in the intestinal epithelium, tumor cells often proliferate slowly and thus reside in a prolonged G1 phase from which they infrequently enter S-phase. This is particularly the case at early stages of tumor progression. The vast majority of these cells stain positively for pre-RC proteins like Cdc6 and Mcm2. In fact, the presence of these proteins is now considered to be a defining characteristic of the proliferative state, and is the basis for the superior efficacy of these proteins as markers for neoplastic and even preneoplastic cells (reference[26] and references therein). Clearly, cell death pathways that target licensing proteins in G1 cells might be expected to eliminate a much

larger fraction of tumor cells at earlier stages of cancer compared to pathways that depend on S-phase-specific events. Unfortunately, this model also predicts the frequent profound sensitivity to apoptosis of stem cell populations, which, as is the case in intestinal epithelium, have licensed origins, but rarely enter S-phase.[26]

Conservation in budding yeast of a proteasome-dependent pathway that targets specific proteins for destruction during cell death suggests some form of apoptosis may occur in this organism. Whether programmed cell death occurs in yeast has been controversial, although accumulating evidence suggests this is the case.[27] In fact, recent studies suggest that apoptosis in budding yeast is regulated by the proteasome,[28] as well as by caspase-related proteases.[29] Programmed cell death also occurs in prokaryotes, including *Baccilus subtilis*, where it plays an important role in sporulation.[30] Interestingly, sporulation in *Baccilus subtilis* is regulated by the replication initiation protein DnaA. This regulatory pathway serves as a primitive origin licensing mechanism that may require conversion of DnaA molecules from the active to inactive state to signal that initiation of DNA replication has occurred before sporulation and subsequent death and disassembly of the mother cell can begin.[31]

This brings us to the question of how the proteasome-dependent destruction of Cdc6 and/or CDK activation might be related to classic caspase-dependent apoptosis in mammals, which may be absent from some simpler organisms. Several lines of evidence clearly point to a role for CDKs downstream of caspases.[15] These include, for instance, the discovery that apoptosis can require the caspase cleavage of CDK inhibitors, including $p27^{kip1}$.[32] The inhibitory role of $p27^{kip1}$ may be spatially constrained to pre-RCs through interactions between its target CDK and Cdc6.[9] However, other studies provide equally compelling evidence that CDK activation occurs upstream of caspases and/or the mitochondrial events that often trigger caspase activation.[17,33] The fact that Cdc6 is destroyed by either proteasome or caspase-dependent pathways depending on the apoptotic trigger may shed some light here. The proteasome-dependent pathway that responds to a DNA damaging drug clearly occurs either upstream of, or parallel to, caspase activation.[2] Perhaps this is a more primitive pathway that exists in parallel with pathways that require caspases and other effectors of apoptosis, such as p53. In the case of apoptotic triggers that directly activate caspases, such as the death domain-containing cytokine signal pathways, the caspase-dependent pathways may supercede the requirement for the proteasome in a parallel pathway. This would place the requirement for CDKs downstream of caspase activation in these cases.

Whatever their role in apoptosis, the existence of multiple mechanisms that target Cdc6 for destruction early in this process, at least one of which is highly conserved, suggests that Cdc6 and origin licensing provide a critical link between cell cycle regulation and cell death. Our model attempts to rationalize the destruction of Cdc6 in the context of aspects of the origin licensing mechanism that may be uniquely suitable for this role – its regulation of DNA replication downstream of multiple redundant, but converging, proliferative (and oncogenic) pathways and its ratchet-like mechanism for coordinating DNA replication with mitosis through the reciprocal regulation of pre-RCs and mitotic CDKs. In apoptotic cells, the irreversible nature of the licensing mechanism's block to re-initiation before mitosis may help to ensure an irreversible cell cycle arrest when invoked before DNA replication is complete. It is important to emphasize, however, that a participatory role for Cdc6 destruction in apoptosis has not been proved. Clearly, there are other possible models, and the complexity of cell death suggests that, even if correct, this model may not apply in all cases. However, we hope it provides a springboard for investigating some of the interesting possibilities.

Note added in proof

Pelizon *et al.* have determined that inhibition of Cdc6 destruction by caspases attenuates apoptosis, indicating this destruction plays a causal role in cell death (Pelizon *et al.*, EMBO Reports in press).

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