New *Myc*-anisms for DNA Replication and Tumorigenesis?

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The *c-Myc* proto-oncogene is an essential activator of cell proliferation and one of the genes most commonly deregulated in cancer. Although these activities of c-Myc are thought to result from its function as a transcription factor, the scientific literature contains hints that this is not the whole story. A new paper in *Nature* by Dominguez-Sola et al. reports the surprising observation that c-Myc promotes DNA replication via a nontranscriptional mechanism, and that c-Myc deregulation causes DNA damage predominately during S phase. These results identify c-Myc as a new DNA replication factor and suggest an alternative model for its role in cell growth and tumorigenesis.

c-Myc is a DNA-binding protein of the helix-loop-helix class that must interact with Max to bind DNA and regulate transcription (Cowling and Cole, 2006). Importantly, there is some discordance between c-Myc domains that are required for its transcriptional activity versus those involved in promoting cell proliferation and tumorigenesis. For example, a deletion of the N-terminal Myc Box I within its transcriptional activation domain is reportedly defective for cellular transformation in vitro but retains transcriptional activity (Herbst et al., 2005). These observations have provoked suspicions that c-Myc may contribute to oncogenic transformation via nontranscriptional activities.

To achieve efficient DNA replication, human cells activate tens of thousands of origins in each cell cycle. By separating replication initiation into two steps, cells insure that each origin undergoes only one initiation event in each S phase and that the genome is duplicated precisely once in each cell cycle (Arias and Walter, 2007). In the first step ("licensing"), which occurs in early G1, prereplicative complexes (pre-RCs) assemble at origins via the sequential binding of the origin recognition complex (ORC), Cdc6, Cdt1, and the MCM2-7 helicase (Figure 1). In the second step, which occurs at the G1/S transition, S phase-specific kinases cooperate with numerous factors including Cdc45 to activate the MCM2-7 helicase, leading to origin unwinding and replisome assembly. Importantly, MCM2-7 vacates the origin during initiation, and reinitiation is blocked because de novo MCM2-7 loading in S phase is strictly prohibited due to inhibitors of licensing such as Geminin.

The first clue that c-Myc might regulate DNA replication came from Dominguez-Sola et al.'s observation that ORC, Cdc6, Cdt1, and MCM2-7 coprecipitate with c-Myc from mammalian cell extracts. Because these pre-RC components normally do not associate with each other in the absence of DNA, this result raises the interesting possibility that c-Myc might stabilize interactions among these factors. Dominguez-Sola et al. (2007) further show that c-Myc and its dimerization partner, Max, bind within close proximity of two highly efficient origins in vivo and that it colocalizes with DNA replication foci early, but not late, in S phase.

In an experimental tour de force involving cell fusion assays, Dominguez-Sola et al. build a case that c-Myc's regulation of DNA replication is independent of transcription. Thus, when a mouse cell arrested in G1 is fused with a HeLa cell rich in S phase-promoting activities, the mouse nucleus within the heterokaryon enters S phase, but *only* if it contains c-Myc. To neutralize c-Myc-dependent gene expression, cells are fused in the presence of cycloheximide, an inhibitor of translation. Although powerful, this experiment cannot exclude the possibility that DNA replication in the mouse nuclei is due to c-Myc-dependent gene expression that occurred *prior* to cell fusion.

To rule out transcriptional effects, Dominguez-Sola et al. turned to a cell-free system derived from *Xenopus* egg extracts (Walter et al., 1998). In this approach, DNA is incubated in a cytosolic egg extract that supports pre-RC formation and therefore mimics G1, followed by addition of a nucleoplasmic S phase extract that promotes replication initiation while preventing reinitiation. Strikingly, immunodepletion of c-Myc from the G1 extract alone is sufficient to significantly inhibit DNA replication, and reconstitution with recombinant c-Myc rescues the defect. Because there is no transcription or protein synthesis in these extracts, this result appears to provide definitive evidence that c-Myc promotes DNA replication by a nontranscriptional mechanism. Notably, truncation of the C-terminal helix-loop-helix domain prevents rescue, suggesting that DNA binding by c-Myc is important for its role in DNA replication. In the future, it will be inter-

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esting to examine whether known *c-Myc* alleles that affect transformation but not transactivation are compromised for DNA replication.

The experiments in *Xenopus* egg extracts suggest the interesting possibility that c-Myc defines a novel G1-specific step in replication initiation (Figure 1). Thus, c-Myc must be supplied by the G1 extract, since the presence of c-Myc in the S phase extract is not sufficient to promote DNA replication. Paradoxically, however, the major replication event that occurs in G1, pre-RC formation, is not affected in the absence of c-Myc. These observations suggest that c-Myc controls a replication step in G1 that is independent of pre-RC formation and that precedes origin activation in S phase. An interesting question is why c-Myc cannot exert its function in S phase. Perhaps there exist S phase-specific factors that inhibit the replication activity of c-Myc, analogous to the S phase inhibition of pre-RC formation by Geminin. Based on these considerations, c-Myc could represent a novel licensing factor. It will be important to address whether the execution point for c-Myc in mammalian cells similarly occurs in the G1 phase of the cell cycle.

To explore potential mechanisms of c-Myc-induced tumorigenesis, Dominguez-Sola et al. examined the effects of c-Myc overexpression on DNA replication. In *Xenopus* egg extracts and in tissue culture cells, excess c-Myc stimulates DNA replication above normal levels. In cells, this is manifested as an increase in replication foci, and in extracts, as an enhanced initial rate of DNA replication. Based on elevated chromatin binding by Cdc45 (Figure 1), excess c-Myc appears to promote the initiation step of replication. Interestingly, although excess c-Myc promotes an elevated rate of DNA replication in early S phase, DNA replication does not go to completion in egg extracts. This result suggested that the enhanced firing of early origins might cause DNA damage and a checkpoint-dependent blockade of late S phase initiation events. This model is supported by the fact that excess c-Myc promotes γ-H2A.X phosphorylation, a marker of DNA double-strand break formation, and that complete DNA replication is restored by inhibition of checkpoint kinases. Based on these results, it is tempting to speculate that the genomic instability observed in c-Myc-overexpressing cancer cells is related to deregulation of replication.

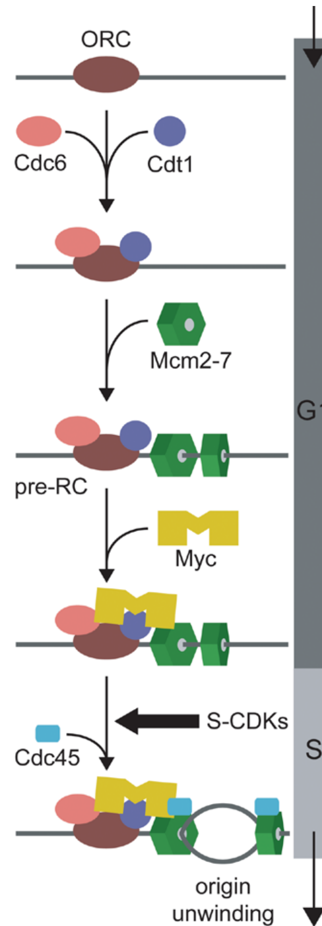

Figure 1. Mechanism of Replication Initiation and Putative Role of Myc in This Process
Origins are licensed in early G1 phase via the ORC-dependent assembly of prereplication complexes on DNA. At the G1/S transition, protein kinases promote replication initiation by stimulating Cdc45- and MCM2-7-dependent origin unwinding. Myc appears to act after pre-RC formation and before initiation.

It now appears there are several different mechanisms by which inappropriate origin usage can give rise to genomic instability and cancer. First, when too few origins of replication are engaged in S phase, DNA damage results, likely because cells enter mitosis with incompletely replicated chromosomes (Lengronne and Schwob, 2002; Shima et al., 2007; Tanaka and Diffley, 2002). Second, when the same origins are used repeatedly in the same S phase due to unrestrained licensing, overreplication results, and this is a possible cause of DNA damage and tumorigenesis (Davidson et al., 2006; Seo et al., 2005). Dominguez-Sola et al. add a third variation on this theme. Their results demonstrate that enhanced origin usage early in S phase causes DNA damage even in the absence of rereplication. Perhaps factors required for DNA synthesis are exhausted when the number of active replication forks exceeds a certain threshold, leading to replication fork collapse. If correct, this model would explain why, in most organisms, only a fraction of replication origins are activated at any time in S phase.

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