

MYC and the Control of DNA Replication

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The MYC oncogene is a multifunctional protein that is aberrantly expressed in a significant fraction of tumors from diverse tissue origins. Because of its multifunctional nature, it has been difficult to delineate the exact contributions of MYC's diverse roles to tumorigenesis. Here, we review the normal role of MYC in regulating DNA replication as well as its ability to generate DNA replication stress when overexpressed. Finally, we discuss the possible mechanisms by which replication stress induced by aberrant MYC expression could contribute to genomic instability and cancer.

NORMAL ROLE OF MYC IN CYCLING CELLS AND CONTROL OF THE G₁/S TRANSITION

C-MYC (MYC) and its cellular homologs are essential for the initiation and maintenance of normal cell cycles, both in vivo and in vitro (Shichiri et al. 1993; de Alboran et al. 2001; Prathapam et al. 2006), and only in rare cases is MYC dispensable for cell growth (Shichiri et al. 1993; Mateyak et al. 1997; Pierce et al. 2008; Steiger et al. 2008). MYC proteins are strongly induced during cell-cycle entry from quiescence, and are critical in the decision of cells to enter or exit cell cycle (Holzel et al. 2001). For example, resting lymphocytes require c-MYC expression to initiate and sustain the proliferative burst triggered by immune activation signals (de Alboran et al. 2001). During neurogenesis, the cerebellar primordium relies

on MYCN to sustain the rapid proliferation of neural progenitors (Knoepfler et al. 2002). This is also the case for MYC in other progenitor and transiently amplifying tissue compartments (Wilson et al. 2004; Muncan et al. 2006; Sansom et al. 2007; Laurenti et al. 2008). In addition, constitutive MYC expression is sufficient to promote cell-cycle entry (G₀ to S transition) and sustain replicative cycles in specific cellular settings, like mouse and rat fibroblasts, or postmitotic neurons (Kaczmarek et al. 1985; Eilers et al. 1991; Steiner et al. 1995; Mateyak et al. 1997). Although the mechanism by which MYC drives cell-cycle progression is not fully understood (Amati et al. 1998; Obaya et al. 1999), it is becoming increasingly clear that transcriptional and nontranscriptional mechanisms mediate the ability of MYC to initiate and sustain proliferative cycles.

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Transcriptional Control of Cell-Cycle Entry

As discussed by Dang (2013), Campbell and White (2014), and Morrish and Hockenbery (2014), a major biological end point of MYC activity is the increase in cell mass, achieved through its global transcriptional effects on cellular and mitochondrial metabolism and ribosomal biogenesis (Gomez-Roman et al. 2003; O'Connell et al. 2003; Grandori et al. 2005; Liu et al. 2008). Coupling of cell growth and cell-cycle progression could explain, at least in part, the ability of MYC to induce cell-cycle entry (Obaya et al. 1999; Schorl and Sedivy 2003). This is supported by the observations that inhibition of specific metabolic pathways activated in response to MYC activity (e.g., oxidative phosphorylation or nucleotide biosynthesis) can prevent MYC-dependent cell-cycle entry and progression in MYC^{-/-} cells (Liu et al. 2008; Morrish et al. 2008).

In cell culture, MYC activation also correlates with changes in expression levels of cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs) (Obaya et al. 1999; Hermeking et al. 2000). By favoring the relative abundance of activating (cyclin D1, Cdk-4, and Cdk-6) versus inhibitory (p15 and p21) complexes, MYC can promote cell-cycle entry and progression. Of note, some of these studies have been performed in engineered Rat1A MYC null cells, which have the unusual ability to slowly proliferate in absence of MYC (Shichiri et al. 1993; Holzel et al. 2001). Therefore, it is unclear whether these observations extend beyond this cell type.

D-type cyclins (D1, D2, and D3) associate with Cdk-4/Cdk-6 and drive exit from quiescence and commitment to cell cycle. In B lymphocytes, cyclin D2 is required for MYC-induced cell-cycle entry and proliferation in response to immune activating cues (de Alboran et al. 2001; Calado et al. 2012; Dominguez-Sola et al. 2012; Poe et al. 2012). Cyclin D2 transcriptional activation by MYC requires the PI3K pathway (Bouchard et al. 2001), and activation of the PI3K pathway is also required for cells to exit quiescence and commit to the initiation of DNA replication (Kumar et al. 2006; Marques

et al. 2008). The relationship between cyclin D2 and PI3K is an important example of the connection between external cues (e.g., growth factors) and the downstream effects of MYC activity.

Control of the G₁/S Transition by Cyclin E/Cdk-2 Activity

Although D-type cyclins and Cdk-4/6 are critical for promoting cell-cycle entry, they are dispensable during DNA replication, when cyclin E/Cdk-2 complexes are instead required (Sherr and Roberts 2004). Cyclin E/Cdk-2 complexes target several substrates directly involved in DNA replication, including proteins necessary for replication origin licensing (TopBP1, MCM) and origin firing in mammals and other organisms (Yu and Sicinski 2004). Once cyclin E/Cdk-2 reach critical levels, they are sufficient to drive cell-cycle completion even in the absence of active mitogenic signals (Sherr and Roberts 2004).

The relationship between MYC and cyclin E/Cdk-2 is complex and is only partially understood. For example, acute MYC depletion in cycling cells via genetic manipulation leads to cell-cycle arrest and subsequent quiescence, effects that can be rescued by constitutive expression of specific growth factor-like signals like those provided by cellular Src (c-Src). In this context, c-Src activates early G₁ regulators but cannot drive G₁/S transition and DNA synthesis unless MYC is reintroduced or cyclin E/Cdk-2 complex is expressed (Prathapam et al. 2006). These observations point to a rate-limiting role for MYC at the G₁/S transition and further suggest that MYC alters the activity of G₁/S-specific CDK complexes, consistent with several reports indicating that MYC regulates cyclin E/Cdk-2 activity.

The activity of cyclin E/Cdk-2 complexes is markedly decreased in myc^{-/-} cells (Obaya et al. 2002), and MYC appears to be required for cyclin E/Cdk-2 activation by either displacing or inactivating p27Kip1 via poorly defined mechanisms (Steiner et al. 1995; Vlach et al. 1996; Berns et al. 1997; Muller et al. 1997; Perez-Roger et al. 1997; Pusch et al. 1997; O'Hagan et al. 2000). p27Kip1 is a CDK inhibitor that is

loaded with cyclin E/Cdk-2 at origins of replication at each cell cycle and specifically blocks its activity. Degradation—or functional inactivation—of p27 at these sites is coupled to the initiation of DNA replication (Furstenenthal et al. 2001; You et al. 2002). Therefore, a potential mechanism by which MYC could induce origin firing and trigger DNA synthesis would involve the modulation of cyclin E/Cdk-2 complex activity at origins of replication (see below) (Srinivasan et al. 2013).

Nontranscriptional Control of DNA Replication Initiation

An additional way by which MYC contributes to cell-cycle progression is by directly controlling DNA replication initiation and S-phase entry. The ability of MYC to directly promote DNA synthesis has been experimentally documented for a long time. Some early reports suggested that this biological function would involve direct interactions between MYC and the DNA replication machinery (Kaczmarek et al. 1985; Studzinski et al. 1986; Iguchi-Arigo et al. 1987, 1988; Hermeking et al. 1994; Lemaitre et al. 1995). However, the idea was later challenged (Gutierrez et al. 1987, 1988). Subsequently, the prevalent idea was that MYC effect on DNA synthesis was a consequence of its transcriptional activity on cell-cycle regulators, as described above. Nevertheless, different groups had reported the existence of protein–protein interactions between MYC and components of the prereplication protein complex (Table 1, and references therein). These findings, and the apparent overlap between specific origins of replication and MYC-binding sites (Iguchi-Arigo et al. 1988) pointed to a direct role for MYC in the initiation of DNA replication, which was characterized two decades later (Dominguez-Sola et al. 2007, and see below).

MYC CONTROL OF INITIATION OF DNA REPLICATION

DNA replication, the process by which the entire genome is accurately duplicated during S phase, requires the unwinding, copying, and re-

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assembling of three billions base pairs in human cells. Replication contributes significantly to genomic instability and chromosome alterations owing to the fragile replication intermediates generated at replication forks, the presence of obstacles on the DNA template, and potential replication errors.

Origin Assembly and Mechanism of Activation

Chromosomal replication in eukaryotes starts at multiple discrete loci, the origins of replication. Origins of replication are assembled and activated in a stepwise manner from proteins and protein complexes (Blow and Dutta 2005; DePamphilis et al. 2006). In addition to regulation of origin activity, some of these factors also participate in genome surveillance to activate checkpoints and DNA damage responses when the DNA replication machinery encounters problems. This concept is important to understand the consequences of tampering with normal DNA replication, and the significance of specific genetic interactions between MYC and some of these factors.

The mechanisms that control the timing, distribution, and efficiency of replication origins vary considerably among eukaryotes and are still not fully understood, especially in mammalian cells (Mechali 2010). In contrast, the mechanisms that regulate the initiation of a single origin of replication are conserved throughout evolution and well characterized. The functional unit that assembles on chromatin, the prereplicative complex (pre-RC) is a multiprotein complex conserved from yeast to human (Bell and Dutta 2002; Errico and Costanzo 2010; Costa et al. 2013). Pre-RC assembly starts with the binding of the origin recognition complex (ORC). In budding yeast, this six-subunit complex recognizes specific DNA sequences. In other eukaryotes, ORC proteins are thought to recognize specific DNA structures rather than specific sequences. ORC is loaded in late M phase or early G₁ phase of the cell cycle (Fig. 1). ORC, together with Cdc6 and Cdt1 recruit the minichromosome maintenance (MCM) hexameric helicase complex at origins. MCMs

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Table 1. Known functional interactions between MYC and the DNA replication and replication surveillance machinery

Factor	Functional interaction	Notes	References
Cdc6	Abrogation of E-box-dependent transcriptional activity Physical interaction and colocalization at assembled replication origins		Takayama et al. 2000a; Dominguez-Sola et al. 2007
Orc1	Abrogation of E-box-dependent transcriptional activity		Takayama et al. 2000b
Orc2	Colocalization at chromatin enhancers throughout cell cycle	<i>Drosophila</i>	Yang et al. 2013
ORC	Physical interaction and colocalization at assembled replication origins		Dominguez-Sola et al. 2007
Cdt1	Physical interaction and colocalization at assembled replication origins		Dominguez-Sola et al. 2007
Mcm2-7	Physical interaction and colocalization at assembled replication origins		Dominguez-Sola et al. 2007; Koch et al. 2007
Cdc45	Chromatin recruitment facilitated by MYC		Dominguez-Sola et al. 2007; Sankar et al. 2009; Srinivasan et al. 2013
Pol-α	Cosegregation in common functional protein complexes	Results challenged in a publication by Gutierrez et al. (1987)	Studzinski et al. 1986
BRCA1	Abrogation of MYC-dependent transcriptional activation, or active transcriptional repression of target genes	BRCA1 defects and MYC overexpression seem to coincide in some breast cancer subtypes (Ren et al. 2013)	Wang et al. 1998; Li et al. 2002; Kennedy et al. 2005
TopBP1	Competition for Miz-1 binding	Possible effects in DNA damage response (modulation of ATR checkpoint?)	Herold et al. 2008
WRN	Genetic interaction (see text)		Grandori et al. 2003
ATR	Genetic interaction (see text)		Murga et al. 2011

ORC, origin recognition complex.

are the catalytic components of the replicative DNA helicase responsible for unwinding genomic DNA bidirectionally. MCM assembly completes the formation of an inactive pre-RC.

Although the MCM complex harbors the catalytic activity that unwinds DNA, the formation of an active helicase requires the presence of Cdc45 and the GINS complex. These three components assemble into the CMG (Cdc45/MCM/GINS) complex, which is the active enzyme (Fig. 1). Productive activation of the heli-

case requires additional factors Sld2, Sld3, and Dbp11 in budding yeast, which are regulated by phosphorylation by Dbf4-dependent kinase (DDK) and by CDKs (Yabuuchi et al. 2006; Tanaka et al. 2007). In yeast, DDK phosphorylates MCM2-7 complex and promotes the loading of Sld3 and Cdc45. CDK promotes the loading of GINS, and phosphorylates Sld2 and Sld3 triggering the binding of these proteins to the BRCT tandem repeats of Dbp11. The combined actions of these kinases convert the pre-RC



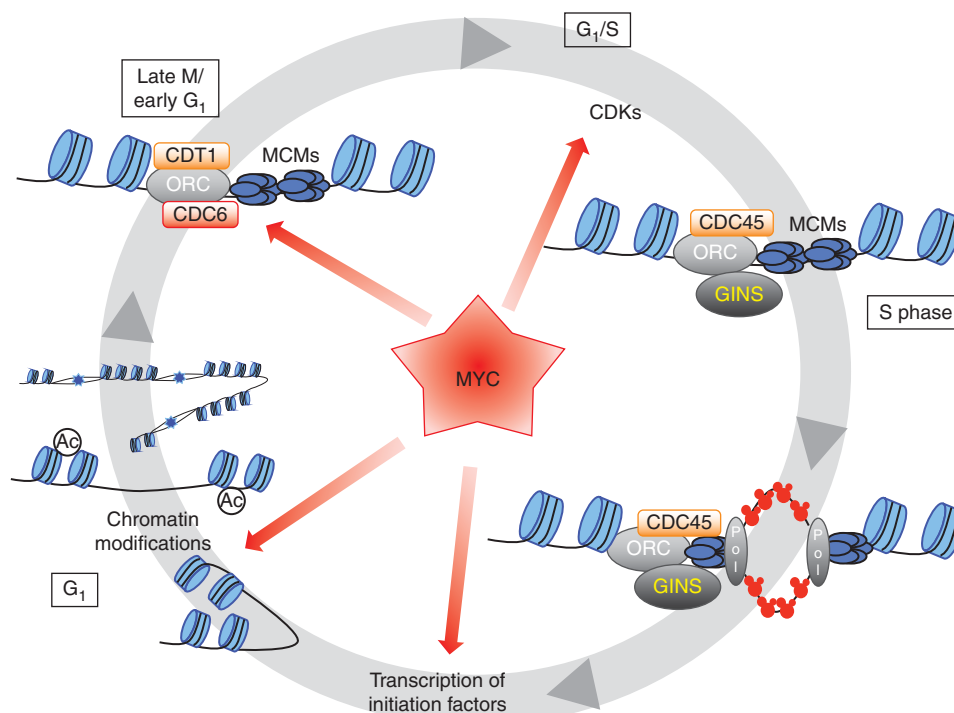


Figure 1. Regulation of origin assembly and origin firing by c-MYC. The stepwise assembly of the prereplicative complex takes place in late mitosis (M phase) or early G_1 with the binding of ORC (origin recognition complex), followed by the Cdt1- and Cdc6-dependent loading of the MCM (minichromosome maintenance) helicase. At the G_1/S transition, CDK (cyclin-dependent kinase) activity is required for the activation of the pre-RC into an initiation complex, coinciding with the assembly of the helicase cofactors CDC45 and GINS. Subsequently, DNA polymerases are recruited and DNA synthesis occurs on unwound DNA. Origins of replication are assembled within permissive regions of chromatin, which are nucleosome-free and open. MYC can influence several steps in these processes, as indicated by the red arrows. MYC physically interacts with the pre-RC proteins CDC6 and MCMs. MYC also regulates the activity of CDKs at origins and moreover, influences the generation of open chromatin, which in turn is thought to regulate the spatiotemporal pattern of origin positioning and firing. Finally, MYC also stimulates the transcription of many factors required for origin assembly and activation (see text for details).

into an “initiation” complex. RecQ4, Treslin/Ticrr, and TopBP1 are the vertebrate orthologs of Sld2, Sld3, and Dbp11, respectively (Mueller et al. 2011). They are thought to play similar roles as their yeast counterparts, yet the mechanistic details of their mode of action are not fully understood. On activation, the CMG complex undergoes significant structural changes that allow the hexameric MCM ring (the helicase engine) to switch from encircling dsDNA (MCM is loaded in G_1) to encircling ssDNA and unwinding DNA by strand exclusion (Pacek et al. 2006; Costa et al. 2011). As mentioned above, MYC physically interacts with several compo-

nents of the pre-RC providing a possible mechanism for MYC’s role in the initiation of DNA synthesis (Fig. 1; Table 1).

To ensure that DNA replication is taking place once and only once at each cell cycle (i.e., that no part of the genome is rereplicated), loading of the pre-RC is temporally uncoupled from its activation. Pre-RCs are assembled in late mitosis and G_1 when CDK activity is low and permissive for the stepwise assembly of its components. Activation of pre-RCs into functional initiation complexes requires CDK activity, which normally rises at the onset of S phase when DNA synthesis is initiated. Because elevat-

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ed CDK activity also inhibits further pre-RC assembly, CDKs play a dual role during DNA replication. They activate initiation of DNA replication and prevent pre-RC assembly once DNA synthesis has started. As discussed above, MYC is known to influence the activity of S-phase CDK, thus providing another possible mechanism by which MYC could regulate origin assembly or activation (Fig. 1).

Replication Timing and Origin Selection

In budding yeast, in which ORC complexes bind specific DNA sequences, the position and numbers of origins are primarily dictated by the genome's nucleotide sequence. In contrast, the positioning of origins of replication in other eukaryotes is largely independent of specific sequences. It is estimated that there are approximately 30,000 origins in human cells. The structural determinants of the positioning of mammalian cell origins are starting to be unraveled, thanks to complementary genome-wide origin mapping studies (Everitts and Collier 2012). Large numbers of origins have been predicted by computational approaches using strategies such as the genome-wide identification of discontinuities in nucleotide composition strand asymmetry (Huvet et al. 2007), or have been mapped experimentally by RNA-DNA hybrid analysis, nascent strand, or origin trapping methods (Mesner et al. 2006; Cadoret et al. 2008; Karnani et al. 2010). A consensus emerges from these diverse approaches as well as some differences. Origins are found in nucleosome-free regions, they are enriched at DNase-hypersensitive sites, and correlate with acetylated histone H4 and other chromatin marks (di- and trimethyl H3K4). Origins are also enriched near CpG islands. Notably, origins of replication are significantly enriched at transcription start sites including the c-MYC locus itself (Tao et al. 2000), although transcription per se is not required for origin activity (Cohen et al. 2003).

Chromatin modifications, and more specifically histone acetylation, can influence origin assembly and activity (Aggarwal and Calvi 2004; Danis et al. 2004; Miotto and Struhl

2010) and could potentially regulate DNA replication dynamics. Notably, MYC influences histone and chromatin acetylation and associates with several chromatin modifying enzymes (Frank et al. 2001; Knoepfler et al. 2006; Martino et al. 2008) and could therefore influence the position and the activity of origins through this mechanism (Fig. 1). For example, MYC binds to an E box within the Lamin B origin to recruit the MLL1 (mixed lineage leukemia 1) methyltransferase, which modifies surrounding nucleosomes by methylating H3K4. These marks subsequently recruit the HBO1 histone acetylase, resulting in the generation of a nucleosome-free open chromatin region and the loading of MCM at the origin (Swarnalatha et al. 2012).

Studies, mostly performed in yeast, are thus consistent with the idea that modification of the chromatin landscape can directly alter the spatiotemporal program of DNA replication. In agreement with a role for histone acetylation in origin activity, deletion of the Rdp3 histone deacetylase in yeast advances the firing of late origins (Knott et al. 2009). Conversely, deletion of the forkhead box transcription factor Fkh1 and Fkh2 delays the firing of early origins (Knott et al. 2012). Finally, Rif1 deletion results in delayed firing of early origins and advanced firing of late origins, thus compressing S phase (Hayano et al. 2012). Notably deletion of Rif1 suppresses the lethality of DDK deletion. Rif1 also controls replication timing in human and mouse cells (Cornacchia et al. 2012; Yamazaki et al. 2012) in a way that is similar to yeast. Rif1-depleted cells show increased chromatin-bound Cdc45 in G₁ suggesting that Rif1, a chromatin remodeling enzyme, normally prevents Cdc45 loading.

Loading onto chromatin of Cdc45, along with Treslin and the GINS protein complex, is required for the activation of assembled origins of replication. Cdc45, GINS, and Treslin are low abundance proteins and are loaded only at selected origins. Thus, the regulation of their association to replication origins is essential in determining the pattern and timing of origin firing in different organisms, from yeast to humans (Mantiero et al. 2011; Tanaka et al. 2011).

As described above, chromatin remodeling enzymes, such as Rif1, regulate Cdc45 binding to chromatin, which is a rate-limiting step of origin activation (Pryde et al. 2009; Wong et al. 2011; Cornacchia et al. 2012; Knott et al. 2012; Yamazaki et al. 2012).

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Work in *Xenopus* cell-free extracts and mammalian cells recently showed that MYC directly influences DNA replication initiation by promoting recruitment of Cdc45 to chromatin, in a manner independent of MYC transcriptional activities (Dominguez-Sola et al. 2007; Sankar et al. 2009; Srinivasan et al. 2013). Through this mechanism, MYC regulates the number of replication origins activated early in S phase, which is proportional to the number of MYC molecules available in the cell. MYC cellular protein concentration is low suggesting that MYC is limiting under physiologic conditions (Waters et al. 1991; Rosales et al. 2013). The precise molecular events by which MYC controls Cdc45 recruitment to chromatin are not yet fully understood, although they could involve the modulation of cyclin E/Cdk-2 activity at origins (Srinivasan et al. 2013). Notably, Treslin mutants insensitive to cyclin E/Cdk-2-dependent phosphorylation are severely deficient in DNA replication (Kumagai et al. 2010, 2011). Because GINS/Treslin is critical for proper recruitment of Cdc45 to origins (Kumagai et al. 2010), GINS/Treslin could regulate the ability of MYC to recruit Cdc45 to replication origins.

The interaction between Treslin and TopBP1 is essential for the recruitment of Cdc45 and GINS to replication origins and the initiation of DNA replication (Boos et al. 2011; Kumagai et al. 2011; Mueller et al. 2011). TopBP1 is a BRCT domain-containing protein with essential functions in DNA replication and for the activation of ATR during checkpoint responses (Kumagai et al. 2006). In addition, TopBP1 physically interacts with Miz-1 (ZBTB17), a POZ-ZF protein required for MYC-dependent transcriptional repression of specific gene targets like p21 (CDKN1A) (Herold et al. 2002; Seoane et al. 2002; van de Wetering et al. 2002;

Wu et al. 2003; further detailed by Eilers and colleagues in Wiese et al. 2013). The interaction between Miz-1 and TopBP1 is disrupted following UV irradiation, or by MYC (Herold et al. 2002, 2008). These observations could reflect the physiological engagement of TopBP1 at origins and its essential role in regulating initiation of DNA replication.

MYC, REPLICATION STRESS, AND TUMORIGENESIS

Challenges to DNA Replication: Stress

DNA replication stress is used widely to refer to conditions that challenge the normal process of DNA replication. For example, stress can be generated experimentally by decreasing the nucleotide pool or by inhibiting/slowing down the replicative polymerases. However, DNA replication stress is also intrinsic to this physiological process and can be exacerbated by the deregulation of oncogenes. The resulting “oncogene-induced” stress often has a replication component (see below).

When faced with replicative stress, cells stall replication forks to avoid their collapse or damage. Broken forks are indeed a source of double-strand breaks and are prone to illegitimate recombination. For example, hydroxyurea (an inhibitor of ribonucleotide reductase) and aphidicolin (an inhibitor of replicative DNA polymerases) induce gene rearrangements with microhomologies (Arlt et al. 2009, 2011). Furthermore, deep sequencing studies reveal complex chromosome rearrangements thought to arise from replication fork failure associated with genetic disorders (Lee et al. 2007; Carr et al. 2011). Therefore, checkpoint pathways that ensure replication fork stability are most critical to cell viability (Segurado and Tercero 2009).

Major challenges to DNA replication progression are physical barriers and obstacles along the DNA molecule. These include problematic genomic sequences (palindromes, G-quartet, telomeric repeats, tRNA genes), proteins bound to DNA and DNA adducts, including RNA polymerases, which can cause replication fork stalling. One of the most frequent problems is

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thought to be collision between a replication fork and transcribing RNA polymerases. MYC deregulation has the unique potential to trigger such collisions because it acutely stimulates replication and is a general activator of transcription (reviewed in Bermejo et al. 2012). Fork stalling is more pronounced following head-on collisions with an RNA polymerase than following co-directional encounters (Pomerantz and O'Donnell 2008). As mentioned above, open, nucleosome-free zones within acetylated chromatin favor the assembly of origins, but these chromatin features are also characteristic of transcription start sites. Initiating transcription and replication from the same open chromatin regions has the potential advantage of allowing codirectionality in these processes. Nonetheless, the most recent genome-wide analysis did not find evidence for transcription and replication codirectionality, suggesting that head-on collisions might not be the major problem for replication forks (Necsulea et al. 2009). Instead, the presence of a transcription bubble could be the most problematic encounter (Azvolinsky et al. 2009). It has been proposed that replicating through nuclear membrane tethered genes might also be a significant obstacle to faithful replication (Everitts and Collier 2012).

Replication Stress and Preneoplasia

Replication stress, as seen by robust staining for activated (phosphorylated) forms of the DNA damage response (DDR) proteins ATM, Chk1, Chk2, γ H2AX, and p53, as well as 53BP1 foci, is a common phenomenon in early, preneoplastic lesions, which are the earliest detectable morphological evidence of tumor initiation and are associated with increased risk of cancer (Bartkova et al. 2005; Gorgoulis et al. 2005). Replication stress has been primarily documented in epithelial and melanocytic preneoplasias but not in other tissues (Bartkova et al. 2005; Gorgoulis et al. 2005; Di Micco et al. 2006; Kuilman et al. 2010). This could either reflect tissue specificity or the fact that routine histopathological analysis of superficial, epithelial lesions is generally more thorough. The constitutive DDR elicited by replication stress is thought to be

responsible for the elevated level of genomic instability observed in tumors, especially solid tumors (Negrini et al. 2010). In turn, this potentially leads to the accumulation of point mutations, deletions, and chromosome rearrangements (Hartwell and Kastan 1994).

These observations strongly support the concept that several (but not all) oncogenes can trigger DNA replication stress followed by activation of persistent DDR and subsequent genomic instability in tumors (what has been conceptualized as “oncogenic stress”). Furthermore, forced expression of certain oncogenes recapitulates the DDR activation seen in preneoplastic lesions, in both cell culture and xenograft tumor models (see Table 2). Importantly, replication stress and subsequent DDR, both in experimental settings and in preneoplastic lesions, are intimately associated with the activation of cell-intrinsic tumor-suppressor mechanisms, checkpoints, senescence, and apoptosis, which have been clearly shown in different preneoplastic lesions (Bartkova et al. 2005, 2006; Gorgoulis et al. 2005; Di Micco et al. 2006). These tumor suppressor mechanisms are believed to be responsible for the delayed progression of preneoplastic lesions to overt cancers, and not surprisingly, are frequently and selectively lost during tumor progression (Bartek et al. 2007; Halazonetis et al. 2008). However, genetic inactivation of tumor suppressor pathways such as the ATM-p53 axis occurs subsequent to the activation of the DDR in preneoplastic lesions (Bartek et al. 2007; Halazonetis et al. 2008).

The mechanistic connection between replication stress and senescence or apoptosis is, however, poorly understood. Senescence and apoptosis appear to be context dependent and are mutually exclusive responses to persistent replication stress and DDR triggered by oncogenes (d'Adda di Fagagna 2008). One possibility is that factors involved in the control of DNA replication or DDR might also be involved in the control of senescence or apoptosis responses. For example, Cdk-2 differentially modulates the ability of MYC to trigger cellular senescence and replication stress. Specifically, loss of Cdk-2 in cells and tissues with deregulated MYC expression triggers a senescence response that is

Table 2. Factors known to induce replication stress in experimental settings

Factor	Function	Associated features	Original references
Ras	Membrane-associated small GTPase	Cellular senescence Coincides with Cdc6 up-regulation	Di Micco et al. 2006
Cyclin E	S-phase cyclin		Bartkova et al. 2005
Cdc25A	Dual specificity phosphatase, required for G ₁ to S transition		Bartkova et al. 2005
E2F1	Transcriptional activator of DNA replication and cell-cycle genes		Bartkova et al. 2005
MYC	Transcriptional amplifier DNA replication initiation factor	Cellular senescence in some cell types	Grandori et al. 2003; Dominguez-Sola et al. 2007
Mos	Serine/threonin kinase, activated MAP kinase cascade	Cellular senescence	Bartkova et al. 2005
Cdc6	Prereplication complex component	Cellular senescence	Bartkova et al. 2005; Di Micco et al. 2006
Cdc45	Preinitiation complex, required for GINS loading and replication origin firing	Epistatic to MYC	Srinivasan et al. 2013

not associated with increased replication stress (Campaner et al. 2010), perhaps owing to the requirement of Cdk-2 activity in MYC-dependent replication (Srinivasan et al. 2013). Similarly, MYC and Cdk-2 synergize to suppress senescence downstream from Ras hyperactivation (Hydbring et al. 2010), and MYC is also required to suppress BRAF-induced senescence in melanoma (Zhuang et al. 2008). Synergy between MYC and Cdk-2 relies in part on Cdk-2-dependent MYC phosphorylation at Ser62 by Cdk-2, a critical MYC residue normally phosphorylated in response to Ras activation (Sears et al. 2000; Hydbring et al. 2010). The ability of Cdk-2 to dictate the cellular responses to MYC deregulation seems to also be supported by the observation that this kinase is also essential for MYC-dependent apoptosis (Deb-Basu et al. 2006).

Limiting MYC-Dependent Replication Stress

As outlined above, replication stress triggers diverse cellular responses, including DDR and senescence, which normally determine the fate of cells with deregulated oncogenes in a cell context-dependent manner. Indeed, the outcomes of oncogene- and MYC-induced replication stress are determined by the status and

the activity of specific factors and signaling pathways (Fig. 2; see also Table 2).

WRN

WRN is a member of the RecQ family of DNA helicases, commonly mutated in Werner syndrome, a genetic disease characterized by premature aging and cancer predisposition. WRN can resolve aberrant DNA intermediates arising at DNA replication forks and thus plays a crucial role in the response to replication stress (Fig. 2) (Sidorova 2008). Notably, loss of WRN activity synergizes with MYC deregulation to induce replication stress and cellular senescence (Grandori et al. 2003; Robinson et al. 2009; Moser et al. 2012). WRN is required to allow MYC overexpressing cells to grow or to form tumors (Grandori et al. 2003; Moser et al. 2012). MYC deregulation in absence of WRN leads to activation of cellular senescence similar to that found in preneoplastic lesions on oncogene deregulation (Grandori et al. 2003, 2004). Furthermore, Emu-MYC mice with deficient WRN helicase activity show a significant delay in lymphoma onset, coinciding with signs of increased DNA damage and senescence in the tumor cells (Moser et al. 2012). The role of WRN is not

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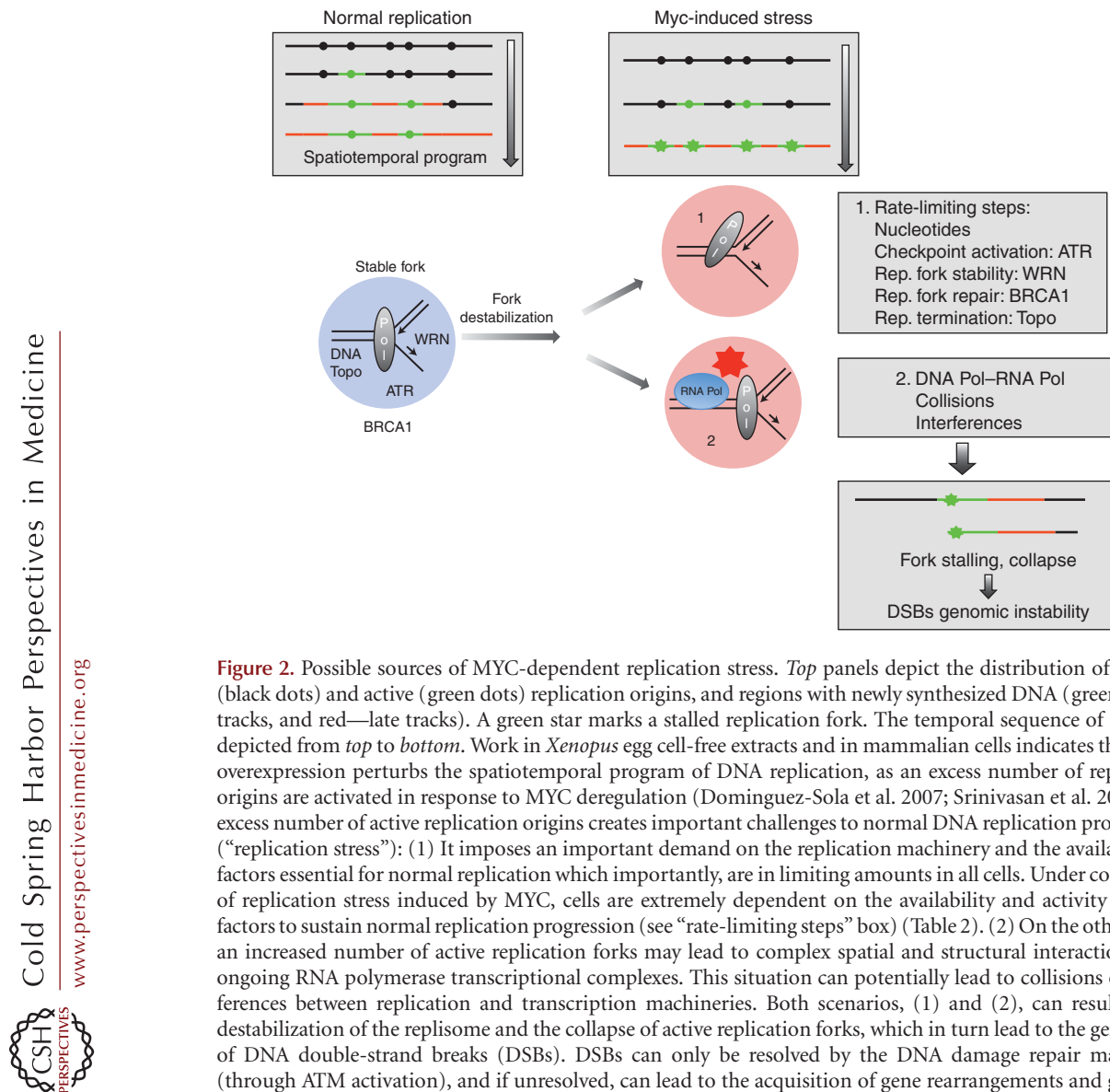


Figure 2. Possible sources of MYC-dependent replication stress. *Top* panels depict the distribution of inactive (black dots) and active (green dots) replication origins, and regions with newly synthesized DNA (green—early tracks, and red—late tracks). A green star marks a stalled replication fork. The temporal sequence of events is depicted from *top* to *bottom*. Work in *Xenopus* egg cell-free extracts and in mammalian cells indicates that MYC overexpression perturbs the spatiotemporal program of DNA replication, as an excess number of replication origins are activated in response to MYC deregulation (Dominguez-Sola et al. 2007; Srinivasan et al. 2013). An excess number of active replication origins creates important challenges to normal DNA replication progression (“replication stress”): (1) It imposes an important demand on the replication machinery and the availability of factors essential for normal replication which importantly, are in limiting amounts in all cells. Under conditions of replication stress induced by MYC, cells are extremely dependent on the availability and activity of these factors to sustain normal replication progression (see “rate-limiting steps” box) (Table 2). (2) On the other hand, an increased number of active replication forks may lead to complex spatial and structural interactions with ongoing RNA polymerase transcriptional complexes. This situation can potentially lead to collisions or interferences between replication and transcription machineries. Both scenarios, (1) and (2), can result in the destabilization of the replisome and the collapse of active replication forks, which in turn lead to the generation of DNA double-strand breaks (DSBs). DSBs can only be resolved by the DNA damage repair machinery (through ATM activation), and if unresolved, can lead to the acquisition of gene rearrangements and genomic instability. Rep., replication; RNA pol, RNA polymerase II; Pol, DNA polymerase.

restricted to limiting MYC-induced replication stress, as deregulation of other oncogenes capable of inducing replication stress (i.e., cyclin E and E2F1) leads to cell death in absence of WRN, or in absence of MUS81, an endonuclease required to process stalled forks and certain replication intermediates in response to replication stress (Franchitto et al. 2008; Murfun et al. 2013; Neelsen et al. 2013).

ATM and ATR

The timing of origin firing during DNA replication is regulated by the activity of ATM and ATR, which control the density of active replication origins allowing replication to proceed normally and be completed at the end of S phase (Shechter et al. 2004; Shechter and Gautier 2005). Interestingly, ATM and ATR show strong

genetic interactions with MYC deregulation during tumorigenesis.

Loss of ATM accelerates the development and progression of MYC-driven tumors, coinciding with impaired p53 activation and reduced apoptosis (Pusapati et al. 2006; Shreeram et al. 2006; Maclean et al. 2007; Reimann et al. 2007). Similarly, addition of caffeine—a known ATM inhibitor—to *Xenopus* cell-free extracts with MYC overexpression results in bypass of MYC-dependent checkpoints, and replication proceeding despite active replication stress (Dominguez-Sola et al. 2007). ATM activation in response to DNA breaks requires its acetylation by Tip60 (KAT5) (Sun et al. 2005), a histone acetyltransferase recruited to chromatin by different transcription factors including MYC. Tip60 is also involved in chromatin transactions critical to the DDR (Ikura et al. 2000; Frank et al. 2003; Squatrito et al. 2006), and is required to mount an effective DDR on MYC deregulation. Tip60 haploinsufficiency synergizes with MYC deregulation in the development of lymphomas in Emu-MYC mice, coinciding with the abrogation of the DDR in tumor cells, despite the integrity of p53 function (Gorrini et al. 2007). The genetic interaction between MYC, ATM, and Tip60 suggests that the inability to elicit a functional DDR on MYC deregulation facilitates MYC-dependent tumorigenesis.

The role of ATR has been studied in patients of Seckel syndrome, a rare human autosomal recessive disorder caused by homozygous or heterozygous compound mutations in the *ATR* gene (O'Driscoll et al. 2003). ATR is a crucial factor in the cellular response to replication stress, and its acute inhibition is deleterious to proliferating cells (Cimprich and Cortez 2008; Branzei and Foiani 2010; Couch et al. 2013). Notably, MYC-induced lymphomagenesis driven by the Emu-MYC transgene is inhibited in a faithful mouse model of Seckel syndrome, coinciding with an exacerbation of MYC-induced replicative stress, massive DNA damage, robust checkpoint activation, and cell-cycle arrest in tumor cells (Murga et al. 2011).

Overall, the distinct contributions of ATM and Tip60, or WRN and ATR during MYC-dependent tumorigenesis reflect the specific roles

of these factors in the response to MYC deregulation. ATR is essential for the viability of cycling cells, and plays a crucial role during DNA replication by acting locally at active replication forks to monitor fork progression, maintain replisome stability and engage the checkpoint signaling machinery (Cimprich and Cortez 2008). Comparably, WRN is thought to resolve specific replication intermediates during normal DNA replication (Enomoto 2001; Pichierri et al. 2011). In contrast, ATM is not required for cell-cycle progression, and mostly responds to DNA double-strand breaks, which can be a consequence of collapsed replication forks or defects in ATR activity (Cimprich and Cortez 2008; Branzei and Foiani 2010). Thus, both ATR and WRN seem to sit upstream of ATM, which in turn would promote checkpoint activation and DNA damage repair in response to DNA DSBs arising from unresolved replication intermediates.

BRCA1

MYC has been shown to interact with BRCA1 tumor suppressor (see Table 1) (Kennedy et al. 2005). The functional outcome of this interaction is thought to be transcriptional, but it is conceivable that BRCA1 limits MYC-induced DNA damage, especially double-strand breaks (DSBs). BRCA1 tumor suppressor activity is associated with its ability to facilitate homology-dependent repair of DSBs. Of note, MYC deregulation correlates best with the basal-like breast cancer type (Alles et al. 2009), the breast tumor class that is associated with BRCA1 loss.

Nucleotides

Alterations of the cellular nucleotide pools can promote mutagenesis, genome instability, and tumorigenesis, mainly because physiologic nucleotide levels are limiting for normal DNA replication progression (Poli et al. 2012). Increased DNA replication can severely reduce cellular nucleotide pools, which can limit normal replication progression and can trigger replication stress. This phenomenon is caused by an imbalance of factors required for replication progres-

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sion. In agreement with this idea, experiments in cell culture show that addition of nucleosides to cell culture reduces oncogene-induced replication stress and subsequent DNA damage (Bester et al. 2011). This is consistent with other studies showing how cells may adapt to chronic replication stress by up-regulating their nucleotide pools, thus avoiding activation of checkpoints (Poli et al. 2012). Such adaptive response might be of special significance in cells under replication stress induced by MYC deregulation, as MYC is known to regulate nucleotide pools and stimulate nucleotide biosynthesis (Liu et al. 2008; Mannava et al. 2008). This particular trait could explain why MYC-dependent replication stress fails to activate robust checkpoints in mammalian cells (Dominguez-Sola et al. 2007).

Replication Stress as a Source of Cancer-Related Gene Rearrangements and MYC-Dependent Genomic Instability

As detailed by Kuzyk and Mai (2014) and Gabay et al. (2014), most malignancies triggered by MYC deregulation show clear signs of genomic instability. In vitro experiments, and in vivo experiments in MYC transgenic mice originally showed that transient MYC expression results in activation of the DDR, genomic instability, and chromosomal aberrations typically seen in MYC-dependent human tumors (Felsner and Bishop 1999; Fest et al. 2002; Kuttler and Mai 2006; Prochownik 2008). Induction of karyotypic abnormalities in cells with MYC deregulation occurs via pathways that are in part independent of its transcriptional activity (Menssen et al. 2007).

Genetic rearrangements associated with oncogenic stress/activation can occur as a result of telomere shortening and deprotection when telomerase becomes rate limiting during replication. This phenomenon yields DSBs, which favor aberrant telomere fusions and breakage-fusion cycles, resulting in additional damage and subsequent chromosome rearrangements (Artandi and DePinho 2010). In addition, DSBs may arise following alteration of the normal process of DNA replication and the generation of DNA replication stress by oncogenes

such as MYC. Replication stress has been recently linked experimentally to the appearance of genomic instability in cancer (Barlow et al. 2013; Burrell et al. 2013). Abnormal DNA replication events could promote the acquisition of tandem duplications in basal-like breast cancers and analogous rearrangements in different tumor types (Stephens et al. 2009; Dereli-Oz et al. 2011). In this scenario, replication stress would leave behind a DNA “footprint,” in a similar fashion to what has been proposed for UV radiation, alkylating agents, or tobacco exposure (Pleasant et al. 2010; Alexandrov et al. 2013a,b).

One of the mechanisms by which replication stress can impact on genome stability is by promoting the appearance of DSBs during S phase (Branzei and Foiani 2010). S-phase DSBs are then carried over into mitosis and can cause cytokinesis failure and abnormal chromosome segregation (Ichijima et al. 2010; Burrell et al. 2013; Neelsen et al. 2013). Defects in chromosome segregation result in chromosomal instability (CIN), which is a common feature in solid cancers of epithelial origin (Schvartzman et al. 2010). It has been proposed that CIN could have a main impact during preneoplastic stages, by facilitating the loss of tumor suppressor genes (Nowak et al. 2002; Michor et al. 2005; Negrini et al. 2010). In agreement with this notion, the contribution of replication stress to chromosomal instability in colon cancer, a solid cancer commonly studied as a model of CIN, appears to be most significant at the progression point between adenoma (the preneoplastic stage) and carcinoma (Burrell et al. 2013).

DNA replication stress has also been proposed to arise from overreplication. Loss of the once-per-cell-cycle regulation of DNA synthesis results in rereplication of DNA segments owing to persistent origin activation. This phenomenon, which can result in the generation of tandem duplications and gene amplifications (Green et al. 2010; Black et al. 2013), can be detected on activation of the K-Ras oncogene (Di Micco et al. 2006). However, rereplication events are not observed on MYC deregulation in *Xenopus* cell-free extracts or in mammalian cells (Dominguez-Sola et al. 2007), suggesting the existence of specific differences in how on-

cogene deregulation disturbs the normal control of DNA replication.

Specific features at the chromosomal region under stress seem to also be important in determining the outcome of the cellular responses to replication stress (Branzei and Foiani 2010). In most mammalian cell types, induction of replication stress alone by oncogene deregulation fails to activate effective checkpoints. In contrast, oncogene-induced replication stress is able to trigger checkpoint responses in ATR- or WRN-deficient cells (see above). Hence, it is possible that the type of lesions induced by oncogene-induced replication stress remain “cryptic” and undetected as the cell cycle proceeds in cells with functional responses (Ichijima et al. 2010). But importantly, not all regions in the genome are equally sensitive to the challenges imposed by replication stress. Replication stress preferentially targets DNA regions located in so-called fragile sites (Bartkova et al. 2005; Gorgoulis et al. 2005; Bartek et al. 2007; Halazonetis et al. 2008), which are defined as genomic regions that selectively accumulate structural abnormalities in response to replication defects (Casper et al. 2002; Durkin and Glover 2007). A fraction of these fragile sites, known as “common fragile sites,” correspond to DNA segments enriched in A-Trich sequences and located in late replicating regions of the genome (Glover 2006; Durkin and Glover 2007), which are characterized by a paucity of replication origins. This architecture makes them extremely vulnerable to defects in replication progression, as they tend to be left unreplicated (Letessier et al. 2011) and can serve as sites of genetic rearrangements at mitosis. A second group of fragile sites is associated instead to early replicating regions, which accumulate damage under replication stress and on MYC deregulation (Barlow et al. 2013). “Early replicating fragile sites” constitute a challenge in situations in which DNA replication initiation is exaggerated, as it occurs on MYC deregulation (Dominguez-Sola et al. 2007; Srinivasan et al. 2013). Notably, a significant fraction of fragile sites overlaps with regions recurrently involved in gene rearrangements in cancer mouse models and in human tumor samples, suggesting that genomic structural abnormalities in cancer can also

be the result of replication stress downstream from oncogene activation (Barlow et al. 2013).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

As detailed in this article, the ability of MYC to control DNA replication initiation can provide an alternative explanation to some critical biological effects of MYC in normal and in cancer cells with deregulated MYC alleles. Nonetheless, the relative contribution of this activity to the spectrum of MYC biological effects remains to be explored.

A critical outcome of MYC activity at the onset of DNA replication is to enforce the recruitment of Cdc45 to chromatin. It will be important to assess whether MYC modulates the activity of protein complexes (e.g., Treslin/TopBP1/RecQL4) that influence Cdc45 loading and/or dictates the chromatin context (acetylation or other histone modifications). In turn, these studies could shed light on the significance of known interactions between MYC and some of these factors (e.g., TopBP1), and help understand the functional significance of certain cancer-associated mutations, like those found in RecQL4 (Fang et al. 2013) (refer to the COSMIC database, cancer.sanger.ac.uk/cancergenome/projects/cosmic). Similarly, understanding the molecular and functional relationship between MYC and Cdk-2 should provide important insights into how MYC regulates replication initiation, but most notably, on the possible connection between replication stress and senescence, which as of now, remains a mystery.

A second important question is to understand the genome-wide consequences of MYC on DNA replication initiation, as it would condition our understanding on the global control of DNA replication and the extent of the genomic abnormalities caused by MYC-dependent replication stress. MYC localizes at validated origins of replication in mammalian cells (Dominguez-Sola et al. 2007; Sankar et al. 2009; Swarnalatha et al. 2012), and in *Drosophila*, MYC colocalizes with Orc2, a key element of the pre-RC, throughout the genome (Yang et al. 2013). However, no genome-wide analysis correlating

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the location of active origins of replication and MYC-bound sites has been reported to date. Given the unparalleled extent of MYC binding across the genome (Fernandez et al. 2003; Orian et al. 2003; Lin et al. 2012; Nie et al. 2012) and our limited knowledge on how origins of replication are selected in every cell cycle to initiate productive DNA synthesis, the implications for such studies would be far-reaching. The close relationship between MYC chromatin binding and global transcription could provide important clues to understand how the long appreciated connection between transcription and replication origin selection is established (MacAlpine et al. 2004; Kohzaki and Murakami 2005; Sasaki et al. 2006; Sequeira-Mendes et al. 2009; Karnani et al. 2010; Dellino et al. 2013). Moreover, these studies would also help us understand how MYC influences cell differentiation and facilitates cellular reprogramming (Wilson et al. 2004; Cartwright et al. 2005; Leon et al. 2009; Smith et al. 2010), because it is plausible that changes in the distribution, choice, and timing of active replication origins throughout the genome would underlie cell fate transitions (Gilbert 2010; Ryba et al. 2011).

Given the prevalence of oncogene-dependent replication stress in early neoplasms, and the causal relationship between replication stress and genomic instability in cancer cells, it will be critical to understand the contribution of MYC-dependent replication stress to tumorigenesis. Indeed, and as detailed by Gabay et al. (2014), the molecular mechanisms that underlie MYC tumorigenic potential, and those that explain its addictive nature in tumor cells remain poorly understood. Altogether, we anticipate that these studies will bring us closer to defining a unifying theory on the role of MYC in normal and cancer cells.

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