**Cdc45 Is a Critical Effector of Myc-Dependent DNA Replication Stress**

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**SUMMARY**

c-Myc oncogenic activity is thought to be mediated in part by its ability to generate DNA replication stress and subsequent genomic instability when deregulated. Previous studies have demonstrated a nontranscriptional role for c-Myc in regulating DNA replication. Here, we analyze the mechanisms by which c-Myc deregulation generates DNA replication stress. We find that overexpression of c-Myc alters the spatiotemporal program of replication initiation by increasing the density of early-replicating origins. We further show that c-Myc deregulation results in elevated replication-fork stalling or collapse and subsequent DNA damage. Notably, these phenotypes are independent of RNA transcription. Finally, we demonstrate that overexpression of Cdc45 recapitulates all c-Myc-induced replication and damage phenotypes and that Cdc45 and GINS function downstream of Myc.

**INTRODUCTION**

DNA replication involves the stepwise assembly of the prereplicative complex on chromatin (Machida et al., 2005; Méchali, 2010; Remus and Diffley, 2009). The origin recognition complex (ORC) is recruited first, followed by the Cdc6- and Cdt1-dependent loading of the mini chromosome maintenance (MCM)2–7 helicase. MCMs are present on DNA in excess, as inactive double hexamers (Blow et al., 2011; Evrin et al., 2009; Remus and Diffley, 2009). Replication initiation occurs upon the action of S phase CDKs and CDC7, which promote Cdc45 and GINS binding to and subsequent activation of the MCMs (Costa et al., 2011; Remus and Diffley, 2009). DNA is then unwound by the Cdc45-MCM2-7-GINS (CMG) complex (Fu et al., 2011; Remus and Diffley, 2009). DNA synthesis triggers signs of origin hyperactivation. Deregulated Myc also activates a DNA damage response (Dominguez-Sola et al., 2007; Sankar et al., 2009). Replication of DNA in the absence of Myc is impaired, whereas Myc overexpression triggers signs of origin hyperactivation. Deregulated Myc also activates a DNA damage response (Dominguez-Sola et al., 2007); however, the aberrant DNA structure that is responsible has not been identified.

Here, we used DNA combing to investigate the consequences of Myc overexpression on the spatiotemporal program of DNA replication, and on the stability of replication forks in Xenopus-cell-free extracts and mammalian cells. We show that Myc initiates premature origin firing, increases origin density, and leads to asymmetrical fork progression and DNA damage. We further demonstrate that Cdc45 overexpression phenocopies all Myc-dependent phenotypes and that the Myc-induced replication alterations are manifested only when Cdc45 or GINS are available, indicating that Myc functions upstream of the CMG complex in a pathway that leads to activation of replication origins. Finally, we provide insight into the mechanism by which Myc facilitates initiation of DNA replication.
protein on the timing of origin firing. To this end, we quantified the origin distance. Error bars represent SD of the mean. See also Figure S1.

Myc positively regulates DNA replication (Herold et al., 2009; Pellengris et al., 2002), and our previous studies helped identify the nontranscriptional role of Myc in replication. Myc directly binds to DNA and appears to influence the activity of replication origins (Dominguez-Sola et al., 2007). To get insights into the mechanism of Myc-induced replication, we used molecular DNA combing (Marheineke et al., 2009) and cell-free extracts derived from Xenopus eggs to monitor origin activity under normal or Myc-overexpressing conditions. The Xenopus system recapitulates semiconservative, chromosomal DNA replication but does not support RNA transcription (Srinivasan and Gautier, 2011).

The experimental strategy is depicted in Figure 1A. Replicating chromosomal DNA is sequentially labeled with two nucleotide analogs that can be differentially detected with specific antibodies. Addition of the nucleotide analogs at different times in the experiment allowed us to differentiate between stretches of early- (before 60 min) and late- (after 60 min) replicating DNA (Figure 1B), monitor fork movement, fork symmetry, and interorigin distance.

First, we monitored the impact of adding recombinant Myc protein on the timing of origin firing. To this end, we quantified DNA fibers in which replication was initiated and completed within 1 hr (green only) and fibers in which replication was initiated after 1 hr and progressed until termination of the reaction at 2 hr (red only). The average length of DNA molecules was calculated using YO-YO-iodide staining, which stains both replicated and unreplicated DNA. We observed that DNA supplemented with either buffer (73.42 kb) or recombinant Myc (74.89 kb) did not significantly differ in length (p = 0.2407; Figure S1A). However, the distribution of replicated DNA (Figure S1B) showed that DNA replicated upon Myc overexpression was shorter than controls (83.18 kb for buffer versus 69.34 kb for Myc).

The addition of full-length Myc protein (75 nM) resulted in 25% of DNA fully replicating before 1 hr when compared to the 5% of early replicated fibers in the buffer control (Figure 1C). In contrast, addition of recombinant Myc protein with a C-terminal truncation removing the bHLH domain and thus abrogating DNA binding (amino acid residues 337–420; Mut Myc) had no significant impact on the timing of origin firing (Figure 1C). Myc therefore triggers early origin firing consistent with our previous data showing an early burst in radiolabeled nucleotide incorporation in Myc-supplemented extracts (Dominguez-Sola et al., 2007).

To evaluate the impact of Myc on the spatial distribution, i.e., density of origins, we next measured the distance between consecutive origins of replication. Origins that fire within the initial 60 min and continue to elongate past 60 min yield green tracks (digoxigenin-dUTP) flanked by orange tracks (digoxigenin-dUTP + biotin-dUTP). In DNA molecules where multiple origins fire, the fibers showed alternate stretches of green and red. The midpoint of each green track was scored as a replication origin. The distance from one origin to another is the “interorigin distance” (Figure 2A) measured in kilobases. We scored DNA fibers that contain two origins or more, which represented about 60% of the total DNA molecules. Comparison of DNA from buffer and Myc-supplemented extracts shows that origins are more closely spaced following addition of Myc and thereby caused a shift in the distribution of interorigin distances (Figure 2B). The mean distance between origins was 29.83 kb (SD = 4.2 kb) in control and 19.2 kb (SD = 2.3 kb) upon Myc overexpression (Figure 2B). The number of origins per DNA fiber was also greater upon Myc overexpression (Figure 2C) despite the fact that the average length of replicated DNA was slightly shorter in Myc-treated extracts than in control (Figure S1B).

Next, we investigated the effect of Myc overexpression on DNA replication in mammalian cells. We used B cells from mice expressing a Myc transgene (λ-Myc mice), a mouse model in which Myc deregulation promotes the development of immature B cell neoplasias (Kovalchuk et al., 2000). B cells were isolated from splenic mononuclear cell suspensions, stimulated to proliferate in vitro, and sequentially labeled with nucleotide analogs (5-chloro-2-deoxyuridine- green followed by 5-iodo-2-deoxyuridine- red). Interorigin distance was measured following the isolation and staining of combed DNA molecules. The DNA of B cells isolated from transgenic λ-Myc mice had shorter interorigin distances than their controls (15.04 kb versus 22.07 kb) (Figure 2D). Thus, constitutive overexpression of Myc in murine B cells resulted in a significant decrease in interorigin distance, as did the addition of recombinant Myc protein to Xenopus extracts.
Myc Causes Aberrant Fork Progression

Myc overexpression triggers a robust DNA damage response (Dominguez-Sola et al., 2007; Karlsson et al., 2003). The aberrant DNA structure that elicits this response is not known. We hypothesized that stalled or collapsed replication forks could be the structures responsible for Myc-dependent DNA damage and therefore sought to assess the impact of Myc on fork progression. Following origin firing, replication elongation typically proceeds symmetrically on either side of the origin. To assess fork symmetry, we scored DNA molecules that were at least 40 kb in length and contained a single origin that fired before 60 min (green), flanked by tracks indicating fork progression (red) on one or both sides (Figure 3A). The DNA molecules with a single origin represented about 10%–15% of the total population of DNA fibers analyzed. To quantify the symmetry of fork progression, the extent of biotin-dUTP incorporation to the right and left of the origin was measured (length of red tracks that flank the origin, l: left, r: right) and the ratio between them (fork progression ratio [FPR]) was computed (Figure 3A). Perfectly symmetric forks have an FPR of 1, whereas unidirectional forks (with a single red track) have an FPR of 0. In the absence of exogenous Myc, 50% of forks were largely symmetrical, with an FPR value between 0.75 and 1.0. In contrast, the addition of Myc resulted in 50% of forks being highly asymmetrical with FPR values between 0 and 0.25 (Figure 3B). Notably, Myc addition did not yield many forks with partial asymmetry (FPR values between 0.25 and 0.75). This observation suggests that Myc overexpression does not slow down fork progression but rather triggers fork-stalling events that occur in close proximity to the origin.

We also analyzed the number of “acutely” asymmetric forks (unidirectional fork progression) and observed that Myc-supplemented extracts had a significantly higher percentage of completely asymmetric forks (Figure 3C). These DNA molecules, in which replication did not progress bidirectionally following addition of biotin-dUTP, could result from DNA breakage or fork stalling or correspond to DNA molecules with an origin positioned close to the end of the DNA fiber. Since the YOYO-1 DNA dye is no longer detectable after the anti-biotin

Figure 3. Myc Overexpression Decreases Interorigin Distance in *Xenopus* Extracts and Mammalian Cells

(A) Representative images of DNA molecules scored in (B) and (C). White arrows indicate the putative position of replication origins. Scale bar is 10 kb. (B) The interorigin distance is plotted against the percentage of DNA molecules in buffer and Myc-supplemented extracts. Number of origins analyzed: N<sub>Buffer</sub> = 117, N<sub>Myc</sub> = 118. (C) Number of origins was plotted against DNA length (kb). The numbers on/beside the circle represent the number of DNA fibers with a given number of origins. (D) B cells isolated from wild-type or λ-Myc mice were sequentially labeled with CldU and IdU. The combed DNA from cells was stained for early- (green) and late-replicating tracts, and interorigin distances were plotted as described in (B). Error bars represent SD of the mean. See also Figure S1.
and anti-digoxygenin staining, we could not selectively count only those fibers that had unlabeled DNA at the end. However, since Myc expression results in increased origin density, the probability of an origin firing close to the end of a DNA fiber is higher in Myc-overexpressing extracts than in controls. Therefore, the contribution to fork asymmetry of origins positioned close to the end of a DNA strand is higher in Myc extracts than controls. Since the interorigin distance in Myc-supplemented extracts is one-third shorter than in controls (29.8 versus 19.2 kb), we applied a correction factor of 0.66 to the Myc value, which is shown as the dotted line in Figure 3C. Comparison of fork asymmetry in B cells isolated from wild-type or \( \lambda \)-Myc mice revealed that the percentage of asymmetric forks was higher in \( \lambda \)-Myc B cells (Figure 3D) than in controls (20.7% versus 7.39%). The presence of significantly more asymmetric forks upon Myc expression suggests that Myc increases the stalling or collapse of replication forks.

**Cdc45 Recapitulates Myc-Dependent DNA Replication Stress**

Since Myc overexpression increases the association of Cdc45 on chromatin (Dominguez-Sola et al., 2007), we decided to evaluate the role of Cdc45 in Myc-dependent replication stress. We initially assessed the consequences of Cdc45 overexpression on DNA replication. To do this, we used Cdc45-depleted extracts (Figure S2A) and first tested the activity of Cdc45 recombinant protein (Figure S2A); we found that recombinant Cdc45 was functional and rescued \( { }^{32}P \) incorporation in Cdc45-depleted extracts (Figure S2B). We then supplemented nondepleted extracts with Cdc45 (Figure 4A) and observed that overexpression of Cdc45 resulted in precocious origin firing (Figure 4B), increased origin density as reflected by decreased interorigin distance (Figure 4C), and increased asymmetry of replication forks (Figure 4D). Increased expression of Cdc45 thus recapitulated the phenotypes observed upon Myc overexpression. Next, we asked whether coexpression of Myc and Cdc45 has an additive or synergistic effect. We observed that cooverexpression of both proteins did not augment the overexpression phenotype, these data strongly suggest that Myc and Cdc45 might function in the same pathway.

**Figure 3. Myc Causes Replication Fork Stalling in Xenopus Extracts and Mammalian Cells**

(A–D) The DNA fibers that had only one origin and contained forks that progressed either uni- or bidirectionally were analyzed.

(A) Representative images of DNA molecules that incorporated digoxigenin (green) or biotin (red) in control (buffer) or upon Myc overexpression (Myc). Scale bar is 10 kb.

(B) Fork progression ratio (FPR) of DNA fibers in control and Myc-supplemented extracts. FPR is calculated by measuring the lengths of red tracts and dividing the smaller value by the larger one.

(C) Fraction of DNA fibers that had unidirectional fork progression in control and Myc-supplemented extracts. The dashed line for Myc represents the corrected value. \( n \geq 100 \); the \( p \) value reflects the adjusted value.

(D) B cells isolated from wild-type or \( \lambda \)-Myc mice were isolated and processed as in Figure 2D. Symmetric forks were graphed as in Figure 3C. Error bars represent SD of the mean.
Cdc45 protein associates with MCM2-7 and GINS to form the CMG complex, which controls origin firing and DNA unwinding. Thus, we tested whether GINS overexpression triggers DNA replication stress. We supplemented extracts with purified, recombinant GINS complex (Boskovic et al., 2007) and monitored the impact on DNA replication. We found that GINS overexpression increased origin density as reflected by decreased interorigin distance (Figure 4E). We also observed more asymmetry of replication forks in presence of added GINS, although the difference was not statistically significant (p = 0.1694) possibly due to sequence divergence between *Xenopus* and human GINS used in these experiments (Figure 4F).

**Myc-Induced DNA Replication Stress Requires Cdc45 and GINS**

To further test the idea that Myc and the CMG complex work in the same pathway, we next investigated whether Myc-induced replication stress depended on Cdc45. We hypothesized that Myc increases origin firing through increased recruitment of Cdc45 to chromatin and that manifestation of the Myc phenotype requires Cdc45. To test this, we experimentally created conditions where Cdc45 becomes rate limiting. We first established partial Cdc45-depletion conditions (one round of depletion for 15 min) in which Cdc45 levels are reduced to approximately 20% of control levels (Figure S2A) but are sufficient to support chromosomal DNA replication to levels comparable to that observed in control extracts (Figure S2C). Reducing Cdc45 levels to about 20% of controls did not hinder normal replication (Figure S2C), but reduced the overall pool of available Cdc45 (Figure S2A). We then overexpressed Myc in both mock-depleted extracts and in extracts partially depleted of Cdc45. As anticipated, overexpressing Myc in mock-depleted extracts resulted in the characteristic Myc phenotypes of early origin firing (Figure 5A), decreased interorigin distance (Figure 5B), and increased fork stalling (Figure 5C). In contrast, all these phenotypes were abrogated when Cdc45 was rate limiting (Figures 5A–5C and S3).

Next, we evaluated the potential requirement of GINS for Myc-dependent DNA replication stress. Since GINS are essential for DNA replication (MacNeill, 2010), we established...
conditions for partial depletion of GINS using specific antibodies against Psf2 (Boskovic et al., 2007). This strategy, although reducing the amount of available GINS (Figure S2D), successfully preserved genomic DNA replication. Under these conditions, we found that overexpression of Myc in GINS-depleted extracts did not significantly alter interorigin distance (Figure 5D) and did not significantly affect the degree of fork asymmetry (Figure 5E). We conclude that Myc-dependent DNA replication stress requires the downstream activity of the CMG complex.

Mechanisms of Myc-Dependent DNA Damage

Our lab and others have previously described how Myc-dependent DNA replication stress results in DNA damage, a known consequence of Myc deregulation in different cell types (Dominguez-Sola et al., 2007; Karlsson et al., 2003). Given that Cdc45 is required for Myc-dependent replication stress, we next compared the ability of Myc and Cdc45 to trigger DNA damage. First, we monitored phosphorylation of the H2AX variant, which is a marker of double-stranded breaks (DSBs) and aberrant DNA structures following phosphorylation by ATM, DNA-PKcs, or ATR. As shown in Figure 6A, we confirmed that Myc expression resulted in elevated γ-H2AX. Notably, addition of recombinant Cdc45 protein to extracts also resulted in increased levels of γ-H2AX on chromatin (Figure 6A). The increase in γ-H2AX correlated with increased levels of the auto-phosphorylated form of ATM (p-ATM) in extracts overexpressing Myc or Cdc45 (Figure 6A), thus indicating the concomitant activation of ATM (Bakkenist and Kastan, 2003) upon Myc or Cdc45 overexpression. We conclude that Cdc45 recapitulates all aspects of Myc-dependent replication phenotypes: altered DNA replication patterns and DNA damage. To assess whether Myc was required for Cdc45-dependent DNA damage, we first established Myc-depletion conditions that allowed DNA replication to proceed with similar kinetics as control, undepleted extracts (data not shown). We then monitored DNA replication (interorigin distance) in these Myc-depleted extracts that were supplemented with either buffer or recombinant Cdc45. We found that addition of Cdc45 protein to Myc-depleted extracts shortened the interorigin distance when compared with buffer-supplemented extracts (Figure S4). This strongly suggests that Cdc45 exerts its function downstream of Myc. Next, we assessed whether Myc-dependent DNA damage, i.e., γ-H2AX and p-ATM, required Cdc45. We monitored chromatin-bound γ-H2AX and p-ATM following addition of Myc to control extracts or extracts partially depleted of Cdc45. Myc-dependent DNA damage was abrogated in extracts that were deficient in Cdc45 (Figure 6B). Therefore, both Myc-dependent replication
stress and subsequent DNA damage require the activity of Cdc45 downstream of Myc.

Next, we attempted to gain insights into the mechanism by which Myc recruits Cdc45 to chromatin. Myc is known to antagonize p27, the CDK2/cyclin inhibitor (Vlach et al., 1996), and CDK2/cyclin E complexes, in turn, modulate p27 (Xic1) activity at Xenopus replication origins (Furstenthal et al., 2001). Moreover, origin activation and firing require the local activity of CDK2/cyclin E complexes, which results in the recruitment of Cdc45 and GINS to primed origins (Mimura and Takisawa, 1998). Therefore, we hypothesized that Myc overexpression could lower the threshold of CDK activity required for origin activation and Cdc45 recruitment. To test our hypothesis experimentally, we added recombinant p27 protein at a concentration that significantly inhibited both DNA replication (Figure 6C), and Cdc45 loading onto chromatin (Figure 6D, lane 3). Under these inhibitory conditions, we overexpressed Myc and found that Cdc45 loading was restored (Figure 6D, compare lanes 3 and 4). This strongly suggests that Myc facilitates Cdc45 recruitment to chromatin and to replication origins by antagonizing the inhibitory activity of p27.

DISCUSSION

Aberrant DNA replication initiation is a critical source of genomic instability leading to chromosomal alterations (Davidson et al., 2006; Kawabata et al., 2011; Lebofsky and Walter, 2007; Shima et al., 2007). Our previous study demonstrating a nontranscriptional role for Myc in regulating the initiation of DNA replication prompted us to explore the mechanisms by which this commonly deregulated oncogene influences DNA replication. Notably, while several studies have documented the transcriptional functions of Myc in regulating S phase entry (Alexandrow and Moses, 1998; Classon et al., 1987; Heikkila et al., 1987; Pelengaris et al., 2002), our approach using Xenopus cell-free extracts allows us to specifically address the nontranscriptional functions of Myc in regulating replication and triggering oncogenic DNA damage (Dominguez-Sola et al., 2007; Lebofsky and Walter, 2007).

Myc Alters Overall DNA Replication Kinetics

Using molecular DNA combing, we observe that Myc overexpression triggers premature and more synchronous firing of replication origins than in control, untreated conditions. This is supported by the significant increase in DNA fibers that are fully replicated early. Consistent with the analysis of replication kinetics by radionucleotide incorporation, Myc accelerates S phase entry. Since Myc does not alter fork progression rate (Dominguez-Sola et al., 2007), our results indicate that Myc alters the intrinsic temporal program of DNA replication (Czajkowsky et al., 2008; Gilbert et al., 2010). Notably, Myc also influences the spatial regulation of DNA replication initiation, as seen by the decrease in interorigin distance. In addition, we note that B cells stimulated to proliferate in vitro have a rapid doubling time.
Origin Firing and Myc
The spatiotemporal program of origin firing is thought to be regulated by several mechanisms including origin interference, activation of dormant origins, and localized activity of protein kinases. Interference is a mechanism by which newly fired origins inhibit firing of adjacent potential origins. Interference decreases with increased distances from the fired origins (Lebofske et al., 2006). It is thought that origin interference helps in achieving complete replication of the genome by regulating origin spacing. The activation of dormant origins within unreplicated DNA then allows completion of S phase and is critical to achieve full replication during normal S phase (Lucas et al., 2000) or under stress conditions (Anglana et al., 2003; Courbet et al., 2008; Ge et al., 2007; Woodward et al., 2006). Myc could alter origin interference early by allowing clusters of origins to fire, thus disrupting the normal spacing and timing program (Berezney et al., 2000). Alternatively, since Myc triggers DNA damage (see below), it is conceivable that Myc-dependent increase in origin density could be due to firing of dormant origins as a consequence of replication stress. However, our findings do not favor this theory. In replication assays using radionucleotide incorporation, Myc-induced replication burst occurs very early. In fact, as replication proceeds, overall replication actually slows down due to checkpoint activation (Domínguez-Sola et al., 2007). Also, in DNA-combing experiments, Myc expression is accompanied by an increase of early firing origins (“green-only” fibers). The data suggest that the “green-only” tracks result from excessive early firing and not from the increased firing of dormant origins activated later in response to fork stalling. By triggering premature firing of normally silent origins, Myc expression could accelerate the precocious depletion of origins such that no dormant origins would be left to fire in case of replication problems. It is also possible that Myc can render origins more sensitive to origin-triggering factors whose concentration may otherwise limit firing early in S phase. Based on our results, we propose that one mechanism by which Myc stimulates origin firing is by lowering the threshold of CDK2/cyclin E activity required to antagonize the CDK inhibitor p27. Some studies suggest that Myc could affect CDK2 activity through multiple mechanisms. It has been proposed that Myc directly activates cyclins, which are loaded in excess (Hyrien et al., 2003), Cdc45 and GINS are essential for DNA replication: they are part of the complex that unwinds DNA at each replication fork and is rate limiting in Xenopus (Hashimoto et al., 2012). However, our present study and others (Edwards et al., 2002) indicate that Cdc45 may not be involved in resolution of aberrant replication intermediates could become limiting when too many origins are activated at once. Indeed, there is evidence that Myc overexpression challenges the normal physiology of DNA replication forks because loss of the WRN RecQ helicase, a protein that is critical for replication fork stability and restart (Amazzalorso et al., 2010; Sarkies et al., 2012; Sidorova et al., 2008), sensitizes cells to Myc expression and triggers cellular senescence (Grandori et al., 2003; Moser et al., 2012; Robinson et al., 2009).

CMG and Myc-Dependent DNA Replication Stress
Cdc45 and GINS are essential for DNA replication: they are part of the complex that unwinds DNA at each replication fork and tethers DNA polymerase to the initiation complex (Gambus et al., 2006; Hashimoto et al., 2012; Jares and Blow, 2000; Mimura and Takisawa, 1998; Pacek et al., 2006). In contrast to MCMs, which are loaded in excess (Hyrien et al., 2003), Cdc45 is rate limiting in yeast (Edwards et al., 2002; Tanaka et al., 2011) and is thought to be a better marker for active replication forks (Hashimoto et al., 2012). However, our present study and others (Edwards et al., 2002) indicate that Cdc45 may not be rate limiting in Xenopus. Furthermore, excess Cdc45 is known to activate dormant origins (Wong et al., 2011).

Myc overexpression results in increased binding of Cdc45 on chromatin (Domínguez-Sola et al., 2007), and the increased dosage of Cdc45 allows late origins to fire earlier in the S phase (Wong et al., 2011). In this study, we have demonstrated that Cdc45 is a critical regulator of Myc-driven DNA replication stress. Cdc45 overexpression, and to a lesser extent overexpression of GINS, recapitulates all the phenotypes of Myc overexpression: increase in early origin firing, decreased interorigin distance, increase in asymmetrical forks, and subsequent DNA damage. Our data are in agreement with studies demonstrating that increasing Cdc45 levels leads to increased origin usage (Wong et al., 2011; Wu and Nurse, 2009). Importantly, when Cdc45 or GINS are depleted such that normal replication is
allowed but these factors become rate limiting. Myc overexpression no longer alters DNA replication timing and fork stability, nor does it trigger DNA damage. The abrogation of Myc-dependent replication stress phenotypes in a Cdc45- or GINS-deficient setting indicates that the CMG complex acts downstream of Myc and is required for the DNA replication stress generated by Myc. Furthermore, and consistent with Myc and Cdc45 operating in a single pathway, we show that coexpression of Myc and Cdc45 does not exacerbate the phenotypes of Myc or Cdc45 expression alone. Therefore, our work suggests that Myc overexpression enhances Cdc45 recruitment to replication origins in a manner that does not require transcription; excess Cdc45 on chromatin then hyperactivates origin firing, which, in turn, results in fork stalling that could lead to genomic instability. Our studies are consistent with the idea that CDK2/cyclin E is a limiting factor for origin activation, and that the threshold of CDK2/cyclin E activity required for origin firing is, at least in part, controlled by Myc. Our studies further raise the possibility that Cdc45 and possibly the CMG complex could collaborate with Myc overexpression in tumor development or, when overexpressed or amplified, could act as oncogenes.

**EXPERIMENTAL PROCEDURES**

All animal experiments were approved by the Institutional Animal Care and Use Committee at Columbia University Health Sciences Center under the supervision of the Institute of Comparative Medicine.

**Preparation of Xenopus Interphase Extracts**

Xenopus extracts were prepared as described earlier (Srinivasan and Gautier, 2011). Briefly, eggs were extracted by centrifugation to obtain the cytosol/membrane fractions and supplemented with cycloheximide (20 µg/ml), creatine phosphate, creatine phosphokinase, and protease inhibitors cocktail before use.

**Preparation of Sperm Chromatin**

Sperm chromatin was prepared as described previously (Srinivasan and Gautier, 2011). For radioactive replication assays, 10,000 nuclei were used for a 10 µl reaction. For replication assays in DNA-combing experiments, 40,000 nuclei were used per 20 µl of extract.

**Isolation, Induction, and Labeling of B Cells**

B cells were isolated from spleens of nonimmunized, 3-month-old, wild-type or λ-Myc mice, using the B cell isolation kit (Miltenyi Biotec). B-cell-enriched fractions were cultured (0.5 × 10⁶ cells/ml) in RPMI (Life Technologies) with 10% fetal bovine serum (Hyclone), 53 nM B-mercaptoethanol, 1% sodium pyruvate, 1% glutamine, 10 mM HEPES, and antibiotics. Cells were induced by 20 µg/ml lipopolysaccharide (Sigma-Aldrich) and 5 ng/ml interleukin-4 (RBD Systems), grown for 2 days, and then labeled with 5-chloro-2-deoxyuridine (green) (2 hr) followed by 5-iodo-2-deoxyuridine (green) (2 hr). Labeled cells were processed for DNA combing and immunofluorescence staining.

**Radioactive Replication Assay I**

The reaction was set up with 10 µl of extract, 0.1 µl of P³² dCTP, and 10,000 sperm nuclei. Where indicated, recombinant protein (Myc, Cdc45, p27, or GINS) was added. The samples were processed, electrophoresed, and analyzed as described previously (Srinivasan and Gautier, 2011).

**Replication Assay II and DNA Combing**

Twenty microliters of undepleted, mock-depleted, or partially depleted extracts was supplemented with 2,000 sperm nuclei/µl and 0.4 µl digoxigenin dUTP (1 mM stock Roche) and incubated at 21°C for 1 hr. After the first hour, biotin dUTP (0.4 µl of 1 mM stock) was added, and the reaction was incubated at 21°C for 1 hr; reaction was stopped with ice-cold 1× PBS. After terminating the reaction, DNA was processed and combed onto silanized coverslips. The incorporated digoxigenin (green) and biotin (red) were detected by immunofluorescence staining. Technical details have been carefully described earlier (Marheineke et al., 2009). For DNA combing, we used a custom-built DNA-combing device that immerse silanized coverslips on the MES/DNA solution for 5 min and lifted them at the rate of 300 µM per second. For the analysis of replication, we used DNA molecules that were 40 kb or longer and had incorporated either digoxigenin (green) or both digoxigenin and biotin (reddish-orange). Only DNA fibers that exhibited continuous staining or had gaps <2 kb were analyzed. Experiments were repeated at least three times.

**Immunofluorescence Staining**

Detection of digoxigenin-dUTP and biotin-dUTP (extracts) and of the IdU and CldU incorporation (B cells) is described earlier (Marheineke et al., 2009).

**Protein Preparation and Purification**

Preparation of Myc:Flag-tagged recombinant Xenopus Myc protein (both wild-type and mutant) was prepared as previously described (Domínguez-Sola et al., 2007). Recombinant Myc protein was used at a final concentration of 75 nM in overexpression experiments.

**Preparation of Cdc45 Protein**

Recombinant Cdc45 was prepared by amplifying the Cdc45-encoding baculovirus in Sf9 cells and infecting High Five cells to produce recombinant protein. Cells were harvested, and protein was purified using standard nickel column purification techniques (Life Technologies).

**Preparation of Recombinant p27 Protein**

The pGEX-p27 plasmid (a kind gift from Dr. R. Yew) was used to transform BL21 cells; p27 is expressed as a GST-fusion protein. The BL21 cells were cultured at 27°C and induced with isopropyl-[1-D-thiogalactopyranoside for 6 hr before harvesting. Standard GST protein purification methods were used to isolate p27 protein.

**Depletion of Xenopus Extracts**

Cdc45 serum (a generous gift from Dr. J. Walker) was used to deplete the extract of Cdc45. Protein A beads were incubated with Cdc45 serum overnight. Xenopus extract was added to the washed beads, incubated for 15 min at 4°C, and recovered by low-speed centrifugation through a cellulose column. One round of depletion was sufficient to render the extract Cdc45 deficient; two rounds of depletion for 15 min each was performed to obtain extracts devoid of Cdc45. Depletion of Myc and GINS was performed using Myc and GINS (Psi2, a kind gift from Dr. Mendež) antibodies.

**Western Blotting and Detection of Chromatin-Bound Proteins**

Depletion of Cdc45/GINS or Myc in the extracts was confirmed by western blotting. To detect chromatin-bound proteins, reactions were assembled with 50 µl of extract and 10,000 sperm nuclei/µl and recombinant protein wherever indicated. Chromatin was isolated through a sucrose cushion; bound proteins were resolved by SDS-PAGE and detected with indicated antibodies (Srinivasan and Gautier 2011).

**Microscopy**

The Zeiss Palm Microbeam Fluorescent Microscope with a 100× objective and 10× eye piece was used to capture images. Images were taken in a series using an automated stage with 10% overlap and then stitched to generate a comprehensive image.

**Data analysis**

Data analysis was carried out using ImageJ 1.46d software (http://imagej.nih.gov/ij/index). The pixel to micrometer ratio used was 0.09 for multiple-tiled images and 0.06 for individual images and was in accordance with the calibration of the microscope. A conversion factor of 1 µm = 2 kb was applied to obtain DNA length. All error bars represent SD of the mean. The two-tailed t test was used to determine significance.
SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.04.002.

LICENSING INFORMATION

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