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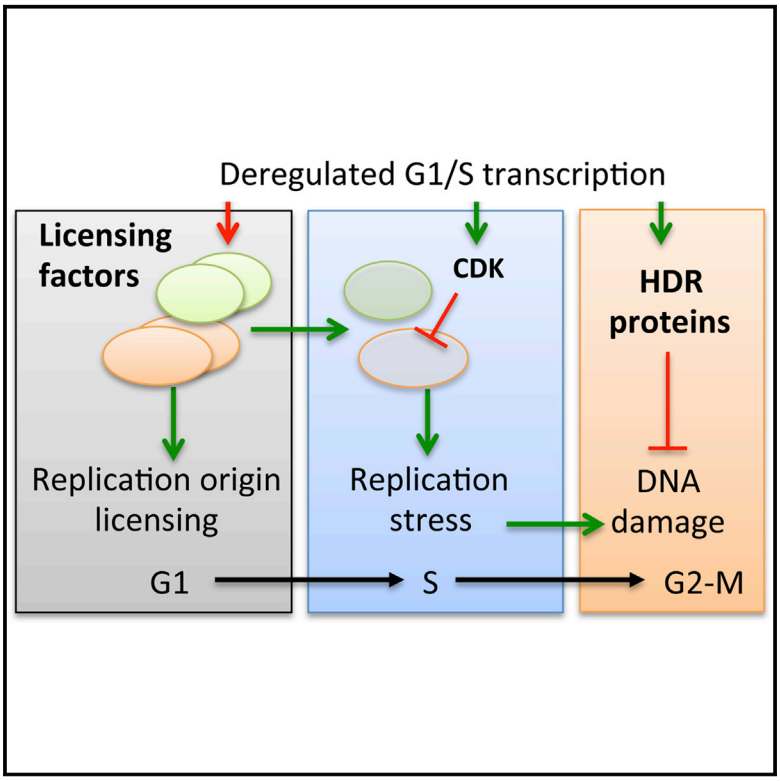
Tolerance of Deregulated G1/S Transcription Depends on Critical G1/S Regulon Genes to Prevent Catastrophic Genome Instability

Graphical Abstract



Highlights

- Deregulated G1/S transcription causes replication stress that is well tolerated
- Deregulation creates a critical requirement for homology-directed repair
- The G1/S target Cig2 becomes essential to inhibit G1/S target Cdc18 licensing factor
- Nonessential genes acquire essential functions during deregulated G1/S transcription

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In Brief

Here, Caetano et al. show that cells with deregulated G1/S transcription become differentially dependent on G1/S targets involved in the replication control and DNA damage repair pathways, uncovering specific vulnerabilities of these cells. Given that G1/S transcription is one of the most commonly deregulated networks in cancer, their findings provide a better understanding of how deregulation might expose specific vulnerabilities to direct therapeutic approaches.

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Tolerance of Deregulated G1/S Transcription Depends on Critical G1/S Regulon Genes to Prevent Catastrophic Genome Instability

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SUMMARY

Expression of a G1/S regulon of genes that are required for DNA replication is a ubiquitous mechanism for controlling cell proliferation; moreover, the pathological deregulated expression of E2F-regulated G1/S genes is found in every type of cancer. Cellular tolerance of deregulated G1/S transcription is surprising because this regulon includes many dosage-sensitive proteins. Here, we used the fission yeast *Schizosaccharomyces pombe* to investigate this issue. We report that deregulating the MBF G1/S regulon by eliminating the Nrm1 corepressor increases replication errors. Homology-directed repair proteins, including MBF-regulated Ctp1^{CtIP}, are essential to prevent catastrophic genome instability. Surprisingly, the normally inconsequential MBF-regulated S-phase cyclin Cig2 also becomes essential in the absence of Nrm1. This requirement was traced to cyclin-dependent kinase inhibition of the MBF-regulated Cdc18^{Cdc6} replication origin-licensing factor. Collectively, these results establish that, although deregulation of G1/S transcription is well tolerated by cells, nonessential G1/S target genes become crucial for preventing catastrophic genome instability.

INTRODUCTION

Uncontrolled cell proliferation is an invariable characteristic of human cancer. The primary regulation of cell proliferation in most eukaryotic cells is imposed during the G1-to-S transition of the cell cycle. Activation of G1/S transcription is required to drive entry into S phase, which commits a cell to a new division cycle. Sequential activation (during G1) and inhibition (during S phase) of G1/S transcription factors is tightly controlled in most eukaryotic cells. In mammalian cells, G1/S cell-cycle-regulated transcription is under the control of the E2F family of transcription factors (E2F1–E2F8), which are regulated by the pocket proteins p107 and p130 and the tumor suppressor pRB (Attwooll et al., 2004; Chen et al., 2009; Dimova and Dyson, 2005; Trimarchi et al., 1998). Elevated levels of E2F-dependent transcription, which are found in every type of cancer, allow cancer cells to sustain proliferation in the absence of growth factors and render them insensitive to growth-inhibitory signals (Chen et al., 2009).

The G1/S transcriptional network comprises a large number of dosage-sensitive coregulated genes that encode proteins involved in many essential cellular functions, such as cell division control, DNA replication, cell growth, and maintenance of genome stability. Deregulated G1/S transcription is thought to be a prominent driving force for early stages of cancer development (Hills and Diffley, 2014). Surprisingly, however, these cells can tolerate deregulated G1/S transcription without suffering catastrophic genome instability, and how they do so is somewhat of a mystery.

As G1/S transcriptional regulation is largely conserved in eukaryotic organisms, we turned to the fission yeast *Schizosaccharomyces pombe* to more fully explore the consequences of constitutive G1/S transcription and establish how cells tolerate deregulated G1/S transcription. The G1/S transcriptional program in fission yeast involves <40 genes that are regulated by a single transcription factor complex, *M*lul cell-cycle *B*ox binding *F*actor (MBF). The core components of MBF include two homologous DNA-binding, zinc-finger proteins (Res1 and Res2) and the protein encoded by the START gene *cdc10*$^{+}$ (Aves et al., 1985; Caligiuri and Beach, 1993; Miyamoto et al., 1994; Tanaka et al., 1992; Zhu et al., 1997). Although there is no sequence or obvious structural homology between the yeast and mammalian G1/S transcription factor, recent data suggest that the mechanisms of regulation are conserved (Bertoli et al., 2013b; Cooper, 2006; Cross et al., 2011). As in mammalian cells, G1/S targets in fission yeast are enriched for genes that encode for proteins involved in DNA replication, DNA repair, and cell-cycle progression. The mechanism by which MBF-dependent transcription is confined to G1 largely depends on an autoregulating negative-feedback loop involving the products of the MBF targets *nrm1*$^{+}$

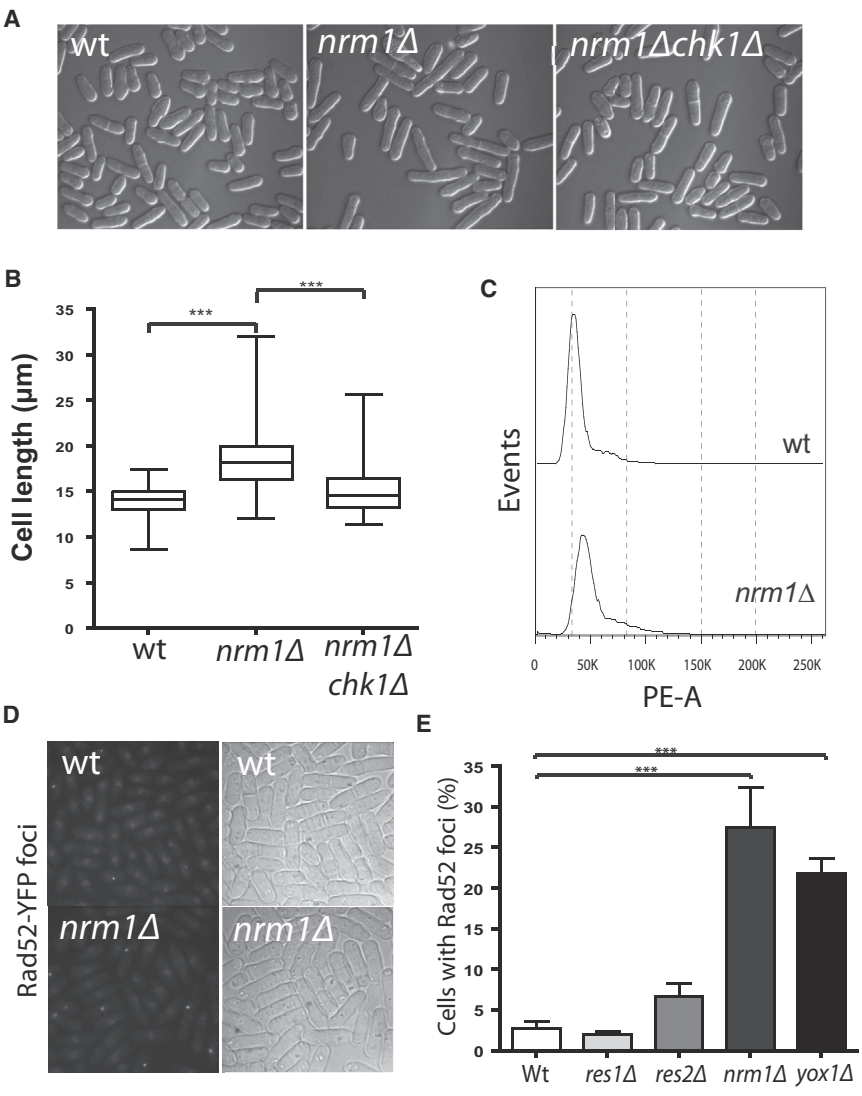

Figure 1. Constitutive Expression of G1/S Transcription Activates the DNA Damage Checkpoint

(A) Microphotographs showing wild-type, *nrm1Δ*, and *nrm1Δchk1Δ* cells.

(B) Whisker plot displaying the maximum, minimum, and quartile lengths of 100 cells immediately before division for the strains described in (A). Statistical treatment: unpaired, two-tailed Student's t test. *p < 0.05, **p < 0.005, ***p < 0.001.

(C) Flow cytometry of the wild-type and *nrm1Δ* cells shown in (A).

(D) Fluorescent microphotographs of Rad52-YFP foci in wild-type and *nrm1Δ* cells.

(E) Bar graph displaying the quantification of Rad52 foci for the strains described in (D). Error bars correspond to the SD of three independent biological experiments. Statistical treatment: unpaired, two-tailed Student's t test. *p < 0.05, **p < 0.005, ***p < 0.001.

See also Figures S1–S3.

and *yox1*$^{+}$ (Aligianni et al., 2009; de Bruin et al., 2006). Activation of MBF-dependent transcription results in accumulation of the G1/S transcriptional repressors Nrm1 and Yox1, which bind and repress MBF-dependent transcription as cells progress into S phase. Consequently, disruption of the negative-feedback loop via inactivation of Nrm1 and/or Yox1 results in constitutive expression of MBF targets throughout the cell cycle (Aligianni et al., 2009; de Bruin et al., 2006).

The constitutive expression of MBF targets in *nrm1Δ* cells was shown to have modest effects on growth rate and cell viability (Aligianni et al., 2009; de Bruin et al., 2006). This outwardly mild phenotype was contrary to expectations, as previous studies (Nishitani and Nurse, 1995; Yanow et al., 2001) had established that ectopic promoter-driven constitutive overexpression of just one MBF target, the replication origin-licensing factor Cdc18^{Cdc6}, causes cell death through repeated rounds of DNA replication in the absence of intervening mitoses. Here, we address this puzzle in experiments that reveal significant genome instability in *nrm1Δ* cells, with both genome protection factors and MBF-regulated expression of S-phase cyclin Cig2 playing crucial roles in maintaining viability in *nrm1Δ* cells. These studies show how cells cope with the deregulated activities of G1/S transcription factors, and in doing so uncover specific vulnerabilities of these cells that reveal the importance of coregulating proteins with opposing functions.

RESULTS

Checkpoint Activation in *nrm1Δ* Cells

Yox1 binds to MBF promoters through Nrm1 to repress G1/S transcripts outside of G1/S (Aligianni et al., 2009). Deletion of *nrm1*$^{+}$ or *yox1*$^{+}$ deregulates the MBF transcriptional program (Aligianni et al., 2009; de Bruin et al., 2006). We used the *nrm1Δ* mutant throughout our studies, although we obtained similar results for *yox1Δ* and *nrm1Δyox1Δ* cells when we tested them. Whereas *nrm1Δ*, *yox1Δ*, and *nrm1Δyox1Δ* cells are viable, they display an elongated phenotype indicating a cell-cycle delay (Nurse et al., 1976). Measurements of septated cells using time-lapse imaging confirmed that *nrm1Δ*, *yox1Δ*, and *nrm1Δyox1Δ* cells were significantly elongated compared with the wild-type (Figures 1A, 1B, S1A, and S1B). Interestingly, the range of cell-division lengths was much larger in the mutants. These observations suggest that stochastic events caused by a deregulated MBF transcriptional program delay cell-cycle progression in the mutants. Importantly, flow cytometry showed that *nrm1Δ*, *yox1Δ*, and *nrm1Δyox1Δ* cells had a single 2C DNA peak typical of the wild-type, indicating that the cell-cycle delay was occurring after the onset of S phase (Figures 1C and S1C). The slightly rightward displacement of the peak in the mutants is likely due to cell elongation causing increased background. To

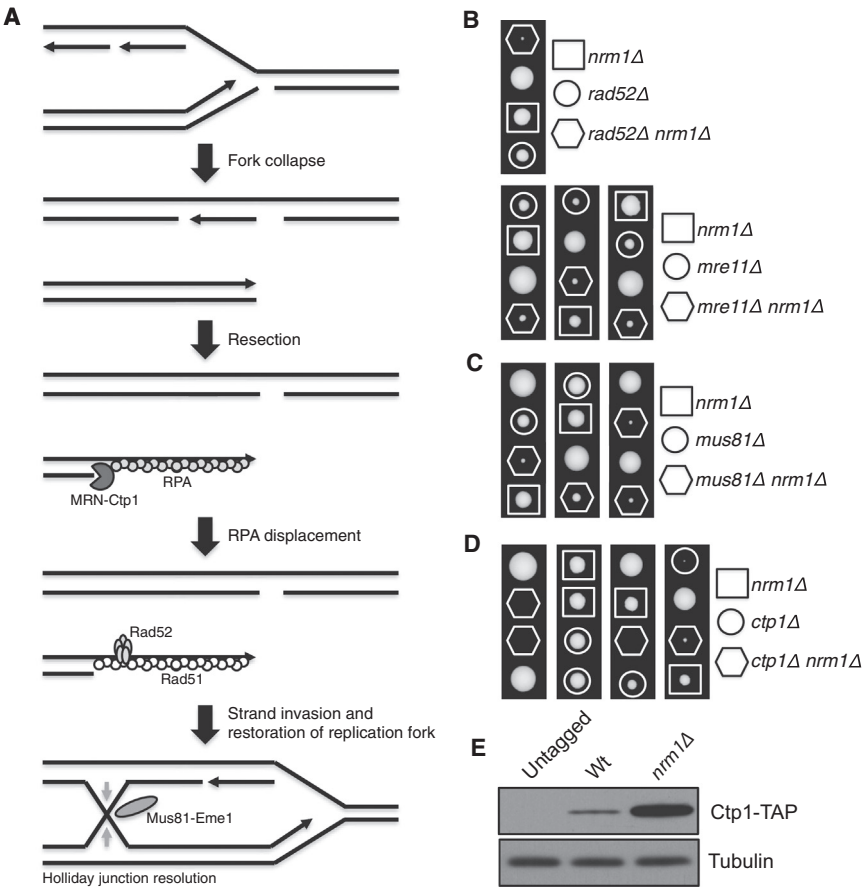

Figure 2. HDR Is Crucial for Viability of *nrm1Δ* Cells

(A) Diagram of HDR pathways.

(B–D) Tetrad dissection of the indicated mutants.

(E) Western blot analysis of the protein levels of Ctp1 in untagged, wild-type, and *nrm1Δ* cells. Ctp1 was tagged with TAP and detected with anti-TAP antibodies. Tubulin was used as the loading control.

determine the nuclear DNA content more accurately, we carried out all subsequent fluorescence-activated cell sorting (FACS) analyses using a method that removes most of the cytoplasmic material, leaving so-called ghosts (Carlson et al., 1997).

Cell elongation often indicates activation of a DNA damage checkpoint, which typically occurs in a stochastic manner. To investigate this possibility, we examined the phosphorylation state of the DNA damage checkpoint effector kinase Chk1, as detected by the reduction of Chk1 electrophoretic mobility, which correlates with Chk1 activation (Walworth and Bernards, 1996). A weak phospho-Chk1 signal was detected in *nrm1Δ* cells that appeared to be above that of the wild-type but below that of an *mre11Δ* mutant that is unable to repair spontaneous double-strand breaks (DSBs) (Figure S2A). To more definitively address the role of Chk1, we created a *nrm1Δ chk1Δ* strain. This experiment revealed that inactivation of Chk1 in *nrm1Δ* cells reduced the average cell length upon division (Figures 1A and 1B), indicating that Chk1 is significantly responsible for the mitotic delay in *nrm1Δ* cells.

Chk1 delays the onset of mitosis by controlling the inhibitory tyrosine-15 phosphorylation of Cdc2 (Rhind et al., 1997). Chk1 directly inhibits Cdc25 tyrosine phosphatase activity and promotes expression of the *mik1*$^{+}$ gene, which encodes a tyrosine-15 protein kinase (Baber-Furnari et al., 2000; Christensen et al., 2000; Furnari et al., 1999). As the *mik1*$^{+}$ gene is also an MBF target (Dutta and Rhind, 2009; Ng et al., 2001; Rustici et al., 2004) that is overexpressed in *nrm1Δ* cells (see below), we investigated whether Mik1 was required for the delayed onset of mitosis in *nrm1Δ* cells. Indeed, analysis of *nrm1Δ mik1Δ* cells revealed that the cell elongation observed in *nrm1Δ* cells required Mik1 (Figures S2B and S2C). Collectively, these experiments establish that Chk1 and Mik1 are required for the mitotic delay of *nrm1Δ* cells.

Increased Rad52 DNA Repair Foci in *nrm1Δ* Cells

The above data suggested that constitutive G1/S transcription in *nrm1Δ* cells increases spontaneous DNA damage. To investigate this possibility, we monitored foci formed by Rad52 (previously named Rad22), which is required for homology-directed repair (HDR) of damaged DNA (Mortensen et al., 2009). Stalled and collapsed replication forks are thought to be the primary source of Rad52 foci in cycling cells (Noguchi et al., 2003). Inactivation of Nrm1 or Yox1 results in a significant accumulation of cells with Rad52 foci (Figures 1D, 1E, and S1D). To assess whether the level of deregulated G1/S transcription correlates with the formation of DNA lesions, we monitored Rad52 foci in *res2Δ* and *res1Δ* MBF mutants. Whereas inactivation of Nrm1 or Yox1 results in persistent high expression of MBF transcripts throughout the cell cycle, abrogation of the MBF repressor Res2 or activator Res1 results in constitutive intermediate or low expression of the majority of MBF targets, respectively (Dutta et al., 2008). Elimination of the MBF repressor Res2, but not the activator Res1, results in an increase in cells with Rad52 foci (Figures 1D, 1E, and S1D). However, whereas *res2Δ* cells are elongated compared with the wild-type, the increase in Rad52 foci is significantly lower in *res2Δ* cells than in *nrm1Δ* or *yox1Δ* cells. These data indicate that the degree of deregulated G1/S transcription correlates with the amount of consequent DNA damage.

Increased Requirement for Repair of Damaged Replication Forks in *nrm1Δ* Cells

To investigate whether the increased Rad52 foci in *nrm1Δ* cells correlates with a greater requirement for DSB repair (Figure 2A), we used tetrad analysis to assess genetic interactions involving Nrm1 and central HDR proteins. A *rad52Δ nrm1Δ* double mutant

grew very poorly compared with either single mutant, indicating that the increased incidence of Rad52 foci reflects a critical requirement for Rad52-depedent HDR in *nrm1Δ* cells (Figure 2B). A comparable negative genetic interaction was also detected with a mutation that eliminates Mre11 (previously named Rad32), a subunit of the evolutionary conserved Mre11-Rad50-Nbs1 (MRN) protein complex that binds DSBs and initiates resection (Williams et al., 2008; Figure 2B).

Rad52 and Mre11 are essential for all HDR pathways in mitotic cells (Stracker and Petrini, 2011), whereas Mus81-Eme1 Holliday junction resolvase is specifically required for HDR of damaged replication forks (Boddy et al., 2000, 2001). This difference is likely explained by the formation of Holliday junctions through HDR of one-ended DSBs at broken replication forks (Figure 2A). Our tetrad analysis revealed that Mus81 was crucial for viability in the *nrm1Δ* background (Figure 2C). These data suggest that *nrm1Δ* cells suffer substantially increased rates of replication fork collapse, which makes HDR proteins essential for survival.

MBF-Regulated Ctp1 Is Critical in *nrm1Δ* Cells

The onset of S phase marks a point in the cell cycle when the favored mechanism of DSB repair switches from nonhomologous end joining (NHEJ) to HDR. Indeed, collapsed replication forks cannot be repaired by NHEJ. In fission yeast, this switch is largely controlled through the Nrm1-regulated expression of *ctp1*$^+$, which is orthologous to human CtIP and budding yeast Sae2 (Limbo et al., 2007). Ctp1 interacts with the MRN protein complex to initiate DNA end resection, which is required for HDR of DSBs (Langerak et al., 2011; Lloyd et al., 2009; Williams et al., 2009). Having found that elimination of Nrm1 creates a critical requirement for several constitutively expressed HDR proteins (e.g., Rad52 and Mre11), we tested whether Ctp1 was required in *nrm1Δ* cells. In this case, tetrad analysis revealed an especially strong negative genetic interaction between *nrm1Δ* and *ctp1Δ* mutations (Figure 2D). The enhanced negative genetic interaction of *nrm1Δ* with *ctp1Δ* as compared with *mre11Δ* might be explained by the sustained and unproductive interaction of the MRN complex with DSBs in *ctp1Δ* cells (Limbo et al., 2011), which might interfere with alternative repair pathways. As an MBF target, *ctp1*$^+$ is strongly expressed during meiosis (Mata et al., 2002). Moreover, Ctp1 hyperaccumulates in *nrm1Δ* cells (Figure 2E). These effects might explain the exceptional requirement for Ctp1 in *nrm1Δ* cells.

Increased Mitotic Defects in *nrm1Δ* Cells

To further investigate the consequences of deregulated G1/S transcription, we assessed whether there were increased mitotic or cell division abnormalities in *nrm1Δ* cells. These abnormalities might be expected to occur if abnormal replication intermediates or collapsed replication forks persist in these cells. Indeed, we observed an increase in several morphological defects related to genomic instability: cells with more than two nuclei and one septum, trinucleated cells presenting more than one septum, cells presenting chromosome missegregation (scattered DNA), cells with a cut phenotype, and cells with accumulated chitin, among others (Figure S3A). In addition, in *nrm1Δ* cells, there was a statistically significant increase in cells presenting an aberrant cell morphology compared with wild-type cells (Figure S3B). The appearance of cells with mitotic abnormalities suggests that while persistent G1/S transcription increases replication-associated DNA damage that is repaired by HDR, it also causes the formation of other DNA structures (perhaps replication intermediates) that cannot segregate efficiently during mitosis, leading to chromosomal instability.

Cig2 Regulates Cdc18 Abundance in *nrm1Δ* Cells

Having found that constitutive MBF activity in *nrm1Δ* cells increases replication fork collapse, we next investigated the source of replication stress in *nrm1Δ* cells (Figure 3A). We focused on the MBF-regulated gene *cdc18*$^+$, which encodes the replication origin-licensing factor Cdc18^{Cdc6}, because constitutive overexpression of *cdc18*$^+$ from an ectopic promoter causes continuous DNA replication in the absence of intervening mitoses, leading to cell death (Nishitani and Nurse, 1995; Yanow et al., 2001). It is surprising that cells can tolerate the high constitutive expression of *cdc18+* that occurs in *nrm1Δ* cells. However, Cdc18 activity in wild-type cells is constrained to late G1 and early S phase by two mechanisms: (1) MBF-regulated transcription of *cdc18*$^+$ and (2) cyclin-dependent kinase (CDK)-dependent targeted destruction of Cdc18 activated after the onset of S phase. MBF-regulated expression of *cig2*$^+$, which encodes a B-type cyclin analogous to Cyclin A in mammalian cells (Fisher and Nurse, 1996; Mondesert et al., 1996), contributes to CDK-mediated destruction of Cdc18 (Jallepalli et al., 1997; Lopez-Girona et al., 1998). We hypothesized that in *nrm1Δ* cells, Cdc18 protein levels might be controlled by concurrent overexpression of Cig2, thereby averting a loss of replication control. With this model in mind, we investigated whether *nrm1Δ* cells depend on high levels of Cig2 to prevent the accumulation of DNA damage by targeting Cdc18 for destruction.

To control Cig2 expression, the endogenous copy of the *cig2*$^+$ gene was put under the control of the *nmt41* promoter, which is a modified version of the *nmt1* (for *no message in thiamine 1*) promoter (Maundrell, 1990). As expected, the addition of thiamine repressed *cig2*$^+$ mRNA expression in wild-type and *nrm1Δ* cells containing the *nmt41-cig2* construct (Figure 3B). Cig2 protein levels in these *nmt41-cig2* cells were well below those in the wild-type (compare lanes 6 and 8 with lane 2 in Figure 3C). Interestingly, when thiamine was depleted from *nmt41-cig2* cells, the *nrm1Δ* allele increased the total abundance of Cig2 protein but reduced the level of *cig2*$^+$ expression relative to *nrm1*$^+$ *nmt41-cig2* cells (compare lanes 5 and 7 in Figure 3C), suggesting that overexpressed MBF-regulated genes promote Cig2 protein degradation in *nrm1Δ* cells.

Importantly, we observed that thiamine-mediated repression of *cig2*$^+$ expression in *nmt41-cig2 nrm1Δ* cells led to a substantial increase in Cdc18 protein (compare lanes 7 and 8 in Figure 3C) without affecting *cdc18*$^+$ mRNA abundance (Figure S4A). Since expression of both *cdc18*$^+$ and *cig2*$^+$ is tightly regulated by MBF, as confirmed by increased Cdc18 and Cig2 proteins in *nrm1Δ* cells (lanes 1–4 in Figure 3C), these results suggest that the deregulated overexpression of *cig2*$^+$ in *nrm1Δ* cells plays an important role in limiting the increase in Cdc18 protein. Other MBF targets might also promote proteasome-dependent destruction of Cdc18, but elimination of Cig2 is sufficient to increase Cdc18.

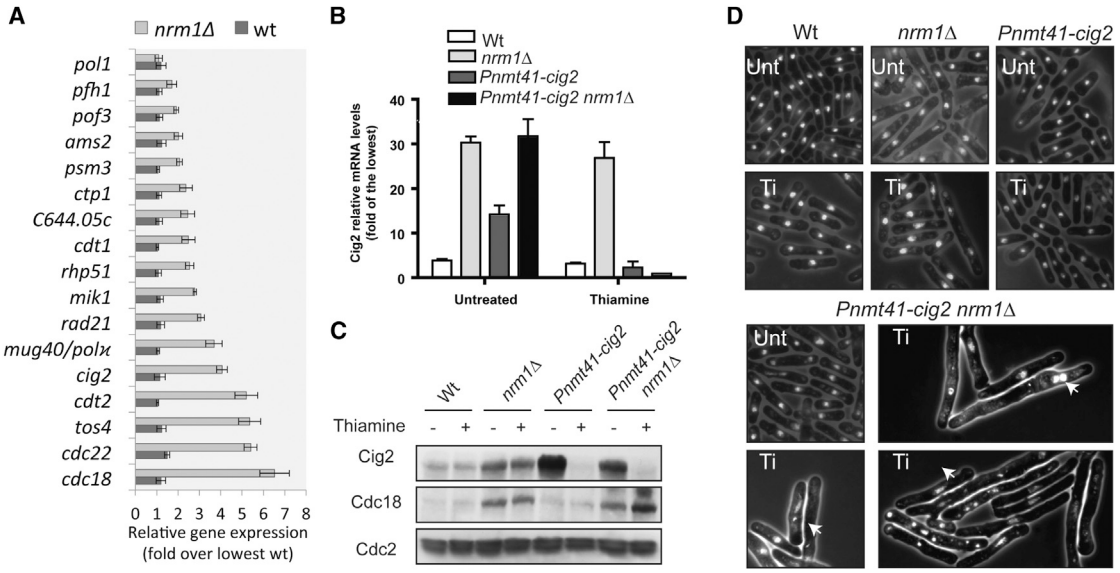

Figure 3. *nrm1*Δ Cells Depend on High Levels of Cig2 to Prevent Cdc18 Hyperaccumulation

(A) Bar graph representing relative mRNA levels of selected MBF-dependent transcripts in wild-type and *nrm1Δ* cells.

(B) Bar graphs represent the relative mRNA levels for transcripts *cig2*$^+$ of wild-type, *nrm1Δ*, *nmt41-cig2*, and *nmt41-cig2 nrm1Δ* cells 0 hr and 24 hr after addition of thiamine. Transcript levels are shown as the fold induction of the lowest signal measured. Bars and error bars represent the average value and SD, respectively, obtained by qRT-PCR of triplicate biological samples.

(C) Whole-cell lysates of wild-type, *nrm1Δ*, *nmt41-cig2*, and *nmt41-cig2 nrm1Δ* cells grown without thiamine and in the presence of thiamine for 24 hr were resolved. Cig2p and Cdc18-HA were detected by anti-Cig2 and anti-HA antibodies, respectively. Cdc2 is shown as the loading control.

(D) Fluorescent microphotographs of wild-type, *nrm1Δ*, *nmt41-cig2*, and *nmt41-cig2 nrm1Δ* cells stained for their nucleus (Hoechst 33242) and septum (calcofluor) before (untreated [Unt]) and after (Ti) treatment with thiamine. White arrows indicate examples of cells with aberrant phenotypes. Bars and error bars represent the average value and SD, respectively, of three independent biological experiments.

High Cig2 Levels Are Critical for Preventing Accumulation of DNA Damage in *nrm1*Δ Cells

To test whether high levels of Cig2, as observed in *nrm1Δ* cells, are required to maintain genomic stability, we analyzed the effects of repressing *cig2*$^+$ expression in the *nmt41-cig2 nrm1Δ* background. We found that repression of *cig2*$^+$ expression caused gross genomic aberrations, including an elongated phenotype, filamentous growth, and missegregated chromosomes, all of which are symptomatic of genome instability (Figure 3D). These findings agree with the significant increase in Rad52 foci formation observed in *nrm1*Δ cells when Cig2 was downregulated and the small decrease in Rad52 foci in *nrm1Δ* cells when Cig2 was induced (Figures 4A and 4B). These data strongly indicate that high levels of Cig2 are critical for preventing genomic instability in *nrm1*Δ cells.

Low Levels of Cig2 Cause DNA Rereplication in *nrm1Δ* Cells

High levels of Cig2 in *nrm1Δ* cells are required to maintain genome stability and prevent the hyperaccumulation of Cdc18. Since overexpression of Cdc18 alone induces rereplication, these data suggest that high levels of Cig2 in *nrm1Δ* cells avert the loss of replication control. To test this, we monitored DNA content after repression of Cig2 expression in *nrm1Δ P41nmt1cig2* cells. The DNA profile of *nrm1Δ* cells after growth in the presence of thiamine reveals an increase in DNA content (>2C DNA content), which is indicative of DNA rereplication (Figure 4B). In addition, a cohort of cells (6.11% of cells) showed a reduction in DNA content corresponding to cells that underwent chromosomal missegregation and displayed a so-called cut phenotype. These data show that high levels of Cig2 in *nrm1Δ* cells are required to reduce Cdc18 levels and prevent DNA rereplication. Overall, the data indicate that the concurrent increase of Cig2 with Cdc18 in *nrm1Δ* cells is required to maintain genome integrity and cell viability.

Cig2 Is Crucial in *nrm1Δ* Cells

In *cig2Δ* cells, there is only a modest G1-to-S delay because the major B-type cyclin Cdc13 readily substitutes for loss of Cig2. Remarkably, tetrad dissection revealed that whereas the *nrm1Δ* and *cig2Δ* single mutants formed colonies that were comparable to wild-type cells, the *nrm1Δ cig2Δ* double mutants were either unviable or formed very small colonies (Figure 5A). In the double-mutant spores that formed small colonies, microscopic observation revealed highly elongated cells and associated morphological defects (Figure 5B). Examples include, but are not limited to, missegregated and broken chromosomes, multiseptated cells, and filamentous growth. Furthermore, *nrm1Δcig2Δ* cells showed a significant increase in the formation of one or more Rad52 foci compared with *nrm1Δ* cells (Figures 4C and 5C). It is important to note that the viable *nrm1Δcig2Δ* strains recovered by tetrad analysis grow very poorly.

Cig2/Cdc2 has been reported to phosphorylate MBF on its DNA-binding subunit Res1 at residue S130, and loss of S130

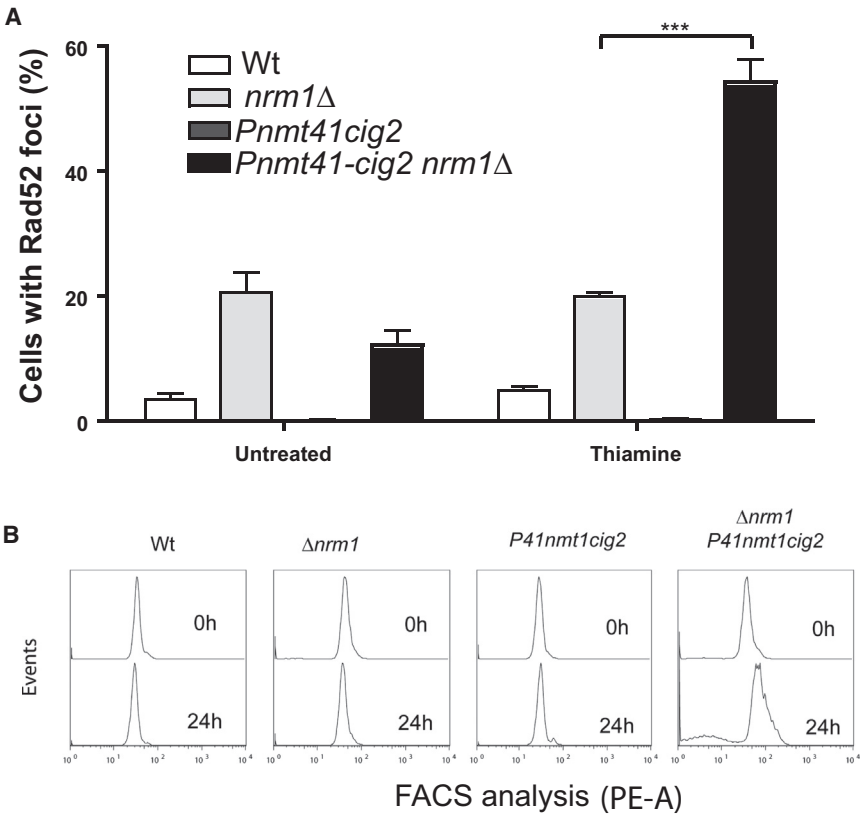

Figure 4. High Levels of Cig2 in *nrm1Δ* Cells Prevent Genomic Instability and Rereplication

(A) Quantification of YFP foci of wild-type, *nrm1Δ*, *nmt41-cig2*, and *nmt41-cig2 nrm1Δ* cells expressing Rad52-YFP at 0 hr (Unt) and 24 hr (Ti) after treatment with thiamine. Fluorescent microphotographs are shown in Figure S4B.

(B) DNA FACS profile of wild-type, *nrm1Δ*, *nmt41-cig2*, and *nmt41-cig2 nrm1Δ* cells before and after addition of thiamine. Cells were treated with thiamine for 24 hr.

phosphorylation causes prolonged activation of G1/S transcription (Ayté et al., 2001). Given the role of Cig2 in negatively regulating MBF activity, deletion of *cig2*$^+$ may lead to enhanced levels of MBF transcription in a *nrm1Δ* background and hence more genomic instability. However, the levels of the MBF targets *cdc18*$^+$ and *cdt1*$^+$ in *nrm1Δcig2Δ* double mutants are similar to those observed for *nrm1Δ* cells, which argues against this option (Figure 5D).

Both Cig2 and Cdc13 CDKs prevent rereplication by targeting the DNA replication licensing factor Cdc18 for degradation via direct phosphorylation through the ubiquitin ligase SCF (Kominami et al., 1998; Lopez-Girona et al., 1998). The crucial role for Cig2 in *nrm1Δ* cells could be explained if Cdc13 B-type cyclin levels were reduced in the *nrm1Δ* background. However, western blot analysis established that Cdc13 levels were not reduced in *nrm1Δ* cells (Figure 4D), indicating that Cdc13 is unable to efficiently substitute for Cig2 when G1/S transcription is deregulated in *nrm1Δ* cells. This result agrees with the observation that a *cig2Δ* deletion suppresses a *cdc18* temperature-sensitive mutation, showing that Cig2 plays a significant role in promoting the timely degradation of Cdc18 in a *cdc13*$^+$ background (Kominami et al., 1998; Lopez-Girona et al., 1998).

High Levels of Cig2 in *nrm1Δ* Cells Prevent Cdc18-Dependent Induction of Rereplication

Based on the above considerations, we expected inactivation of Cig2 in a *nrm1Δ* background to promote inappropriate origin refiring and thereby cause genome instability. The DNA profile of a viable *nrm1Δ cig2Δ* double mutant recovered from a tetrad dissection, as indicated by the flow-cytometry profile shown in Figure 6A, reveals that the majority of cells had a >2C DNA content, with another substantial fraction appearing to have a <1C DNA content. These defects are consistent with both replication abnormalities being caused by hyperaccumulation of Cdc18 activity (e.g., replication origin relicensing causing rereplication of all or part of the genome) and chromosome segregation abnormalities. In support of this interpretation, we found that the *res2Δ* mutation, which impairs MBF activity, resulting in significantly lower levels of *cdc18* expression in both wild-type and *nrm1Δ* cells (Figure 6B), suppresses the lethality of the *nrm1Δcig2Δ* double mutant (Figure 6C). These results suggest that the reduced viability and morphological defects observed for *nrm1Δ cig2Δ* mutants are the result of rereplication due to an increase in *cdc18*$^+$ expression and concurrent Cdc18 hyperaccumulation.

To further investigate whether the synergistic negative interactions of *nrm1Δ* and *cig2Δ* mutations are connected to CDK-dependent phosphorylation of Cdc18, we tested whether mutations of the CDK sites in Cdc18 impair growth in *nrm1Δ* cells. Since mutations of the CDK sites in an otherwise normal *cdc18*$^+$ locus causes a checkpoint-dependent cell-cycle block, for this experiment we used the *cdc18-T6A chk1Δ* strain (Fersht et al., 2007). This *cdc18-T6A* mutant has six CDK sites mutated to alanine (T10A, T46A, T60A, T104A, T134A, and T374A) and is expressed at the endogenous locus under the native promoter. Tetrad dissection of the *cdc18-T6A chk1Δ* × *nrm1Δchk1Δ* cross showed that *cdc18-T6A* is lethal with *nrm1Δ* (Figure 6D), revealing that phosphorylation-dependent inhibition of Cdc18 is required for the viability of *nrm1Δ* cells.

DISCUSSION

The synchronized expression of genes in a regulon facilitates the coordinated activities of proteins charged with a specific task, be it a relatively simple process such as the uptake and

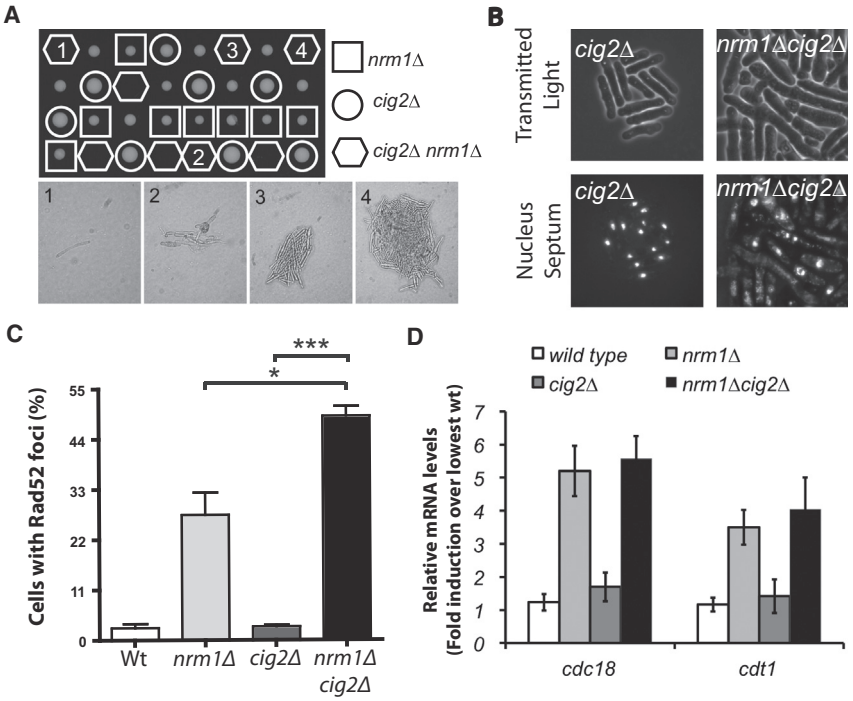

Figure 5. Genome Instability Is Enhanced in *nrm1Δcig2Δ* Mutants Compared with *nrm1Δ* and *cig2Δ* Single Mutants

(A) Tetrad dissection of *nrm1Δ* mutants crossed with a *cig2Δ* mutant. Microphotographs of the indicated double *nrm1Δcig2Δ* mutants are shown.

(B) Microphotographs displaying the aberrant morphology of the *nrm1Δcig2Δ* mutant. Cells were stained for nucleus and septum using Hoechst 33242 and calcofluor, respectively.

(C) Quantification of Rad52 foci in wild-type, *nrm1Δ*, *cig2Δ*, and *nrm1Δcig2Δ* cells. Statistical treatment: unpaired, two-tailed Student's t test. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$.

(D) Bar graph representing relative mRNA levels, as fold over the lowest value measured in wild-type cells, of the MBF-dependent transcripts *cdc18* and *cdt1* in wild-type, *nrm1Δ*, *cig2Δ*, and *nrm1Δcig2Δ* cells. Error bars correspond to the SD of three independent biological experiments.

metabolism of a specific nutrient or a much more complicated cellular event such as genome duplication. Some of the genes will be essential for the task, while others may have nonessential functions in quality control, efficiency improvement, or process termination. Here, we examined the G1/S regulon in fission yeast and found that normally dispensable genes can acquire essential functions during pathological constitutive expression of the G1/S regulon.

Deregulated G1/S Transcription

Activation of G1/S transcription factors by CDKs is a universal mechanism for controlling cell-cycle progression and cellular proliferation. Constitutive G1/S transcription causes unscheduled entry into S phase and replication stress in mammalian cells, which is thought to result from insufficient stockpiling of replication factors, but it is unclear how these cells avoid catastrophic genome instability. Here, we use fission yeast to investigate the effect of eliminating Nrm1, which is a repressor of the MBF G1/S transcription factor. Constitutive G1/S transcription causes replication stress that is well tolerated in *nrm1Δ* cells, but it creates a critical requirement for HDR proteins, notably the MBF-regulated factor Ctp1 (CtIP/Sae2), which is required for resection of DSBs, and Mus81-Eme1 endonuclease, which resolves Holliday junctions that form during repair of damaged replication forks. Strikingly, the cyclin A analog Cig2, which is an MBF target that is completely dispensable in *nrm1*$^{+}$ cells, is essential in *nrm1Δ* cells. Our studies indicate that the requirement for Cig2-CDK is explained by its ability to inhibit the replication origin-licensing factor Cdc18^{Cdc6}, which is also an MBF target. This study shows that cells with deregulated G1/S transcription become differentially dependent on G1/S targets involved in the replication control and DNA damage repair pathways, uncovering specific vulnerabilities of these cells. Given that G1/S transcription is one of the most commonly deregulated networks in cancer, the work presented here provides a better understanding of how deregulation might expose specific vulnerabilities to direct novel therapeutic approaches.

Deregulation of G1/S Transcription Results in an Increase in Replication Factors, Causing Replication Stress

The work presented here provides insights into how deregulated G1/S transcription leads to replication stress and genome instability. In fission yeast cells, the G1 interval is exceedingly short when the cells are grown in nutrient-rich media. Therefore, the consequences of deregulated G1/S transcription can be studied in this model organism independently of the accelerated S-phase entry observed in most other eukaryotic cells. Our work shows that irrespective of its role in driving S-phase entry, deregulation of G1/S transcription outside of G1 directly causes replication stress by increasing replication factors. Replication control mechanisms in eukaryotic cells are centered on regulating the activity of the DNA replication licensing factors. While there may be some significant differences between yeast and higher eukaryotes with regard to the precise mechanism by which deregulation of G1/S transcription leads to replication stress, the basic principles are likely the same. Given the critical role of replication stress in oncogene transformation and chromosomal instability, a detailed understanding of how deregulation of G1/S transcription in human cells can lead to replication stress should provide insights into cancer development.

Exploiting One of the Most Commonly Deregulated Networks in Cancer

Identifying specific vulnerabilities of cancer cells caused by deregulated network control creates a potentially large therapeutic window for damaging cancer cells without affecting normal cells.

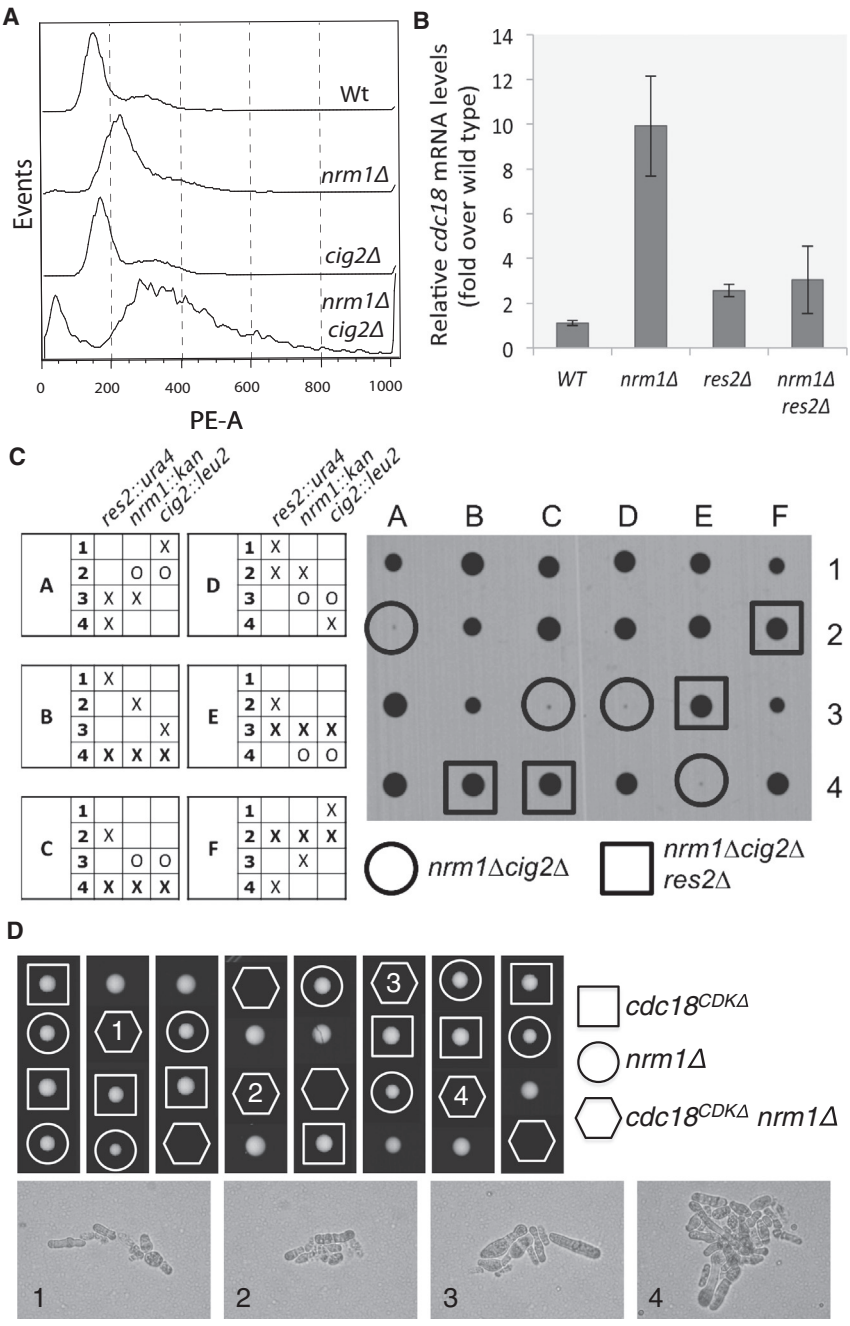

Figure 6. Cig2 Is Essential in *nrm1Δ* Cells to Prevent Cdc18-Induced Rereplication

(A) DNA FACS profile for wild-type, *nrm1Δ*, *cig2Δ*, and *nrm1Δ cig2Δ* cells. DNA was stained with propidium iodine.

(B) Bar graph representing the relative *cdc18* mRNA levels in wild-type, *nrm1Δ*, *res2Δ*, and *nrm1Δres2Δ* cells.

(C) Tetrad dissection of *nrm1Δres2Δ* mutants crossed with a *cig2Δ* mutant.

(D) Tetrad dissection of *nrm1Δ chk1Δ* mutant (*nrm1Δ*) crossed with a *cdc18-T6A chk1Δ* mutant (*cdc18*$^{CDK\Delta}$).

we aimed to exploit one of the most commonly deregulated networks in cancer, the G1/S transcription network. Our work reveals how cells cope with deregulated G1/S transcription, and shows that nonessential replication control (Cig2) and genome protection proteins (Ctp1) whose transcription is also deregulated become crucial for cell survival. In cancer research parlance, *nrm1Δ* cells become addicted to the "nononcogene" activities of Cig2 and Ctp1 (Luo et al., 2009).

Comparisons with Mammalian G1/S Transcription

In mammalian cells, G1/S transcription depends on the E2F family of transcription factors (E2F1–E2F8) and their coregulators, the pocket proteins (pRb, p107, and p130), whereas fission yeast requires the MBF transcription factor complex and the coregulators Nrm1 and Yox1 (Bertoli et al., 2013b). Although the yeast and mammalian proteins are not sequence orthologs, our work and that of others has shown that the basic molecular mechanisms are conserved from yeast to human. Activation of G1/S transcription initiates negative-feedback loops that subsequently inactivate transcription, creating a wave of expression that peaks at the G1-to-S transition. The autoregulatory negative-feedback loop where transcriptional repressors (G1/S targets themselves) accumulate and bind to G1/S promoters to turn off transcription during the progression to S phase was only recently discovered in yeast (Aligianni et al., 2009; de Bruin et al., 2006). In mammalian cells, a similar mechanism has been proposed to turn off transcription during S phase. E2F6, E2F7, and E2F8 are repressors of the E2F transcriptional program and, much like Nrm1 and Yox1 in fission yeast, they are G1/S targets themselves. E2F6 was recently shown to be involved in a negative-feedback loop to turn off transcription during the G1-to-S transition (Bertoli et al., 2013a), and E2F7 and E2F8 have also been implicated (Di Stefano et al.,

This mechanism-based approach has already been successful. For example, studies have shown that cells harboring BRCA1-2 mutations, which increase the risk of developing cancer, are specifically dependent on intact checkpoint functions involving DNA repair by Poly (ADP-ribose) polymerase (PARP) (Bryant et al., 2005; Farmer et al., 2005). Therefore, drugs that target PARP function are particular damaging to BRCA mutant cancer cells without affecting BRCA wild-type cells. However, the power of this approach depends entirely on our gaining detailed knowledge about the complex regulatory networks that are deregulated in any particular disease. In the work presented here,

2003; Lyons et al., 2006). Future research will reveal whether deregulation of G1/S transcription by inactivation of these negative-feedback loops in mammalian cells also creates a specific requirement for genome protection and S-phase cyclin activities.

Deregulation of G1/S transcription, which is found in most cancer types, generally involves increased levels of E2F-dependent transcription and likely a subsequent coordinated increase in replication factors. It will be important to establish how this increase of replication factors, resulting from the many different ways G1/S transcription can be deregulated in mammalian cells, contributes to replication stress and subsequent genomic instability. Such information could then be used to direct novel therapeutic approaches.

EXPERIMENTAL PROCEDURES

General Methods

The basic methods we used for working with fission yeast have been described elsewhere (Forsburg and Rhind, 2006).

Promoter Switch of Cig2 to the *nmt1-P41*-Inducible Promoter

Cig2 was placed under the control of the *nmt41* promoter at the endogenous locus using the PCR-based homologous recombination integration method (Bähler et al., 1998). In brief, the plasmid pFA6a-KANMx6-*P41nmt1* was amplified using TTTTGGTTACAAACAACTAGATATATTTCTATACGTTGATAAAA GGGTAATTTATCAATCCATATTTCAT GAATTCGAGCTCGTTTAAAC and TGT TTTCATCTTGATAACTATGCTTATTGATTTTAGAACCAACAGGCTTTGAAATT GAATAGAGAGCCATGATTTAACAAAGCGACTATA primers. The generated PCR product was then transformed into wild-type cells using the lithium acetate transformation protocol, and positive clones were detected by their ability to grow in kanamycin-YES plates. P41nmt1 tagging of *cig2*$^+$ was confirmed by PCR for insertion of the *P41nmt1::kan* cassette and by RT-PCR for detecting reduced expression levels of *cig2*$^+$ following addition of thiamine.

Quantitative RT-PCR

Total RNA was prepared using the RNeasy Plus Kit (QIAGEN) as indicated in the manufacturer's manual. Transcript levels were determined by quantitative RT-PCR (qRT-PCR) using the iScript One-Step RT-PCR kit with SYBR Green Supermix (Bio-Rad). RT-PCR reactions were run on a Chromo-4 Real-Time PCR Detector (Bio-Rad) and the experimental values obtained were analyzed using MJ Opticon analysis software v.3.0. Furthermore, data were normalized against actin and investigated using the C(t) method.

Flow Cytometry

The samples shown in Figures 1C and S1C were prepared as previously described (Sabatinos and Forsburg, 2009). Briefly, exponentially growing cells were fixed in 1 ml 70% ethanol, and 300 μl of this mixture was washed in 3 ml 50 mM Na citrate. Pellets resuspended in 0.5 ml 50 mM Na citrate containing 0.1 mg/ml RNase A (Sigma) and left at room temperature for 2 hr. A volume of 0.5 ml 50 mM Na citrate containing 8 μg/ml propidium iodide (Sigma) was subsequently added to 0.5 ml samples. Samples were sonicated for 30 s (Brason Sonifier 450 Sonicator, output control 4) prior to flow-cytometry analysis. Samples for all other FACS analyses were prepared as described previously (Carlson et al., 1997). In short, most of the cytoplasmic material was removed by exposing the cells to Triton X-100 and hypotonic conditions after cell wall digestion. For all experiments, 20,000 single events were analyzed for FSC, SSC, and DNA content using a BD LSR II flow cytometer and Flow_Jo v.9.2 software.

Rad52 Foci Quantification

For each strain, 25 ml cultures were grown for a total time of 4 hr in Edinburgh minimal medium at 25°C and fixed in ice-cold 70% ethanol. Fixed cells were washed in sterile deionized water, mounted on glass slides using Vectashield mounting medium, and captured using a Zeiss Axioskop fluorescence microscope set with a 100× objective, phase contrast 3, attached to a Hamamatsu Orca-ER digital camera connected to OpenLab software. Cells containing Rad52 foci were quantified using ImageJ software (v.1.46j).

Cell Size Quantification

Using images obtained from time-lapse experiments, the length of 100 cells was measured from cell tip to cell tip immediately before fission with the use of Volocity v.5.5.1 software.

Cell Staining with Hoechst 334 and Calcofluor

Exponentially growing cells were fixed with 1 ml 70% ice-cold ethanol and stored at −20°C until use. Cells were rehydrated in 1 ml of PBS and centrifuged for 1 min at 3,000 rpm, and the supernatant was discarded. The pellet was resuspended in 100 μl 0.02125 mg/ml calcofluor and incubated at room temperature for 5 min. Cells were then washed with 1 ml of PBS three times. The resulting pellet was resuspended in 5 μl 2 μg/ml Hoechst (prepared in ddH_2O) and mounted onto a microscope slide using Vectashield as the mounting medium. Images were captured using a Zeiss Axioskop fluorescence microscope set with a 100× objective, phase contrast 3, attached to a Hamamatsu Orca-ER digital camera connected to OpenLab software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.11.039.

AUTHOR CONTRIBUTIONS

C.C., O.L., R.A.M.d.B., and P.R. designed research. C.C., S.F., S.C., C.D., and O.L. performed research. C.C., O.L., S.F., S.K., P.R., and R.A.M.d.B. analyzed data. C.C., P.R., and R.A.M.d.B. wrote the paper.

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