Cyclin F-Mediated Degradation of Ribonucleotide Reductase M2 Controls Genome Integrity and DNA Repair

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SUMMARY

F-box proteins are the substrate binding subunits of SCF (Skp1-Cul1-F-box protein) ubiquitin ligase complexes. Using affinity purifications and mass spectrometry, we identified RRM2 (the ribonucleotide reductase family member 2) as an interactor of the F-box protein cyclin F. Ribonucleotide reductase (RNR) catalyzes the conversion of ribonucleotides to deoxyribonucleotides (dNTPs), which are necessary for both replicative and repair DNA synthesis. We found that, during G2, following CDK-mediated phosphorylation of Thr33, RRM2 is degraded via SCFcyclin F to maintain balanced dNTP pools and genome stability. After DNA damage, cyclin F is downregulated in an ATR-dependent manner to allow accumulation of RRM2. Defective elimination of cyclin F delays DNA repair and sensitizes cells to DNA damage, a phenotype that is reverted by expressing a nondegradable RRM2 mutant. In summary, we have identified a biochemical pathway that controls the abundance of dNTPs and ensures efficient DNA repair in response to genotoxic stress.

INTRODUCTION

The ubiquitin-proteasome system allows the precise temporal and spatial regulation of a large variety of regulatory proteins. This tight control is accomplished through the specific, targeted degradation of proteins via ubiquitin ligases. The Skp1-Cul1-F-box protein (SCF) complexes are the canonical multisubunit E3 ubiquitin ligases and assemble by using Cul1 as a core scaffold (Cardozo and Pagano, 2004; Petroski and Deshaies, 2005). The small RING protein Rbx1 and an ubiquitin conjugating enzyme (UBC) are recruited via the C terminus of Cul1. The Cul1 N terminus, instead, binds the bridging factor Skp1 and a variable F-box protein, which determines substrate specificity. Each F-box protein can target multiple substrates, allowing the core SCF scaffold, by using different F-box proteins, to target hundreds of substrates for degradation (Jin et al., 2004). Of the 69 human F-box proteins, only a minority have established functions (Skaar et al., 2009).

Cyclin F (also known as Fbxo1) is the founding member of the F-box protein family and is essential for mouse development (Bai et al., 1996; Tetzlaff et al., 2004). In addition to an F-box domain, cyclin F contains a cyclin box domain, but, in contrast to typical cyclins, it does not bind or activate any cyclin-dependent kinases (CDKs) (Bai et al., 1996; D’Angiolella et al., 2010; Fung et al., 2002; Tetzlaff et al., 2004). However, like other cyclins, cyclin F protein levels oscillate during the cell division cycle, peaking in G2. Cyclin F localizes to both the centrosomes and the nucleus (D’Angiolella et al., 2010). During G2, centrosomal cyclin F targets CP110 for proteasome-mediated degradation to limit centrosome duplication to once per cell cycle (D’Angiolella et al., 2010). Additionally, cyclin F promotes the degradation of NuSAP1, a protein involved in mitotic spindle organization (Emanuele et al., 2011). The biological function of nuclear cyclin F remains unknown.

Ribonucleotide reductase (RNR) is a well-studied enzyme composed of two identical, large subunits (called RRM1, RR1, RR1, or R1) and two identical small subunits (called RRM2, RNR2, R2, or R2) (Nordlund and Reichard, 2006). A functional catalytic site is constituted when two RRM2 (ribonucleotide reductase family member 2) subunits are bound to two RRM1 (ribonucleotide reductase family member 1) subunits. RNR catalyzes the conversion of ribonucleotides to deoxyribonucleotides (dNTPs), which are used in the synthesis of DNA during replication and repair (Nordlund and Reichard, 2006). Because of this fundamental function, RNR is among
the most well-conserved (from prokaryotes to eukaryotes) and highly-regulated enzymes. Indeed, dNTP pool increases or imbalances produce a hypermutator phenotype (Hu and Chang, 2007; Kunz et al., 1998), and decreased dNTP levels interfere with proper DNA replication and repair (Nordlund and Reichard, 2006).

RNR activity needs to be coordinated with cell-cycle progression to preserve the fine balance between dNTP production and DNA replication. RRM1 levels are constant throughout the cell cycle and are always in excess of the level of RRM2, which fluctuates during the cell cycle (Chabes and Thelander, 2000 and Figure 1A). Therefore, the cell-cycle-dependent activity of RNR is regulated by RRM2 levels. The G1/S induction of RRM2 transcription is dependent on the transcription factor E2F1 (Chabes et al., 2004; DeGregori et al., 1995), and, to prevent RRM2 accumulation in G1, RRM2 levels are also kept in check by the APC/C^Cdh1 ubiquitin ligase (Chabes et al., 2003b). Notably, how RRM2 is degraded in the G2 phase of the cell cycle remains unknown.

Although RNR is a cytoplasmic enzyme, in response to genotoxic stress, it translocates from the cytoplasm to the nucleus to ensure the local availability of dNTPs at DNA damage sites for

Figure 1. Cyclin F and RRM2 Physically Interact and Colocalize to the Nucleus in G2
(A) RPE1-hTERT cells were synchronized in G0/G1 by 72 hr of serum starvation before release into fresh medium containing serum. Cells were collected at the indicated time points after serum readdition (SR), lysed, and immunoblotted as indicated.

(B) HEK-293T cells were transfected with an empty vector (EV) or the indicated FLAG-tagged F-box protein constructs (FBPs). Whole-cell extracts were immunoprecipitated (IP) with anti-FLAG resin, and immunoprecipitates were immunoblotted as indicated.

(C) HeLa cells were synchronized at G1/S by using a double-thymidine block before release into fresh medium. Cell lysates were generated at the indicated time points, immunoprecipitated with an antibody to cyclin F, and immunoblotted as indicated. Ten percent of the material used for immunoprecipitation (input) is shown on the right panels.

(D) U-2OS cells were synchronized at G1/S by using a double-thymidine block before release into fresh medium. Cells were fixed at 5 hr (S phase) and 9 hr (G2 phase) after release from the block and stained with an antibody to cyclin F (green) or RRM2 (red). Where indicated, cells where pretreated for 2 hr with Leptomycin B (LMB) before fixation. Confocal microscopy was used to visualize stained cells.

See also Table S1.
During G2, Cyclin F Interacts with RRM2 in the Nucleus

To identify substrates of the SCF\(^{\text{cyclin F}}\) ubiquitin ligase, FLAG-HA-tagged cyclin F was transiently expressed in either HeLa or HEK293T cells and immunopurified for analysis by Multidimensional Protein Identification Technology (MudPIT) (D’Angiolella et al., 2010; Flores and Washburn, 2006). MudPIT revealed the presence of peptides corresponding to Skp1 and Cul1, as well as peptides derived from RRM2 (Table S1). Combining both analyses, 23 total spectra, corresponding to five unique RRM2 peptides, were identified. In two additional experiments, we immunopurified cyclin F(1-270), a cyclin F mutant lacking the cyclin box, and although Skp1 and Cul1 still coimmunoprecipitated with cyclin F(1-270), binding to RRM2 was not detected (Table S1).

To investigate whether the binding between RRM2 and cyclin F is specific, we screened a panel of human F-box proteins. Fifteen FLAG-tagged F-box proteins were expressed in HEK293T cells (with the proteasome inhibitor MG132 added for 6 hr prior to harvesting) and immunopurified to evaluate their interaction with RRM2. We found that the only F-box protein able to coimmunoprecipitate endogenous RRM2 was cyclin F (Figure 1B). Through the use of synchronized HeLa cells (monitored by immunoblotting for cell-cycle markers and flow cytometry), the interaction between endogenous cyclin F and RRM2 was observed exclusively in G2 and early M (Figure 1C), when the levels of cyclin F increase and RRM2 levels are drastically reduced (Figures 1A and 1C).

Because cyclin F localizes to both the nucleus and centrosomes (D’Angiolella et al., 2010) and RRM2 is largely cytoplasmic (Nordlund and Reichard, 2006), we asked where and when the two proteins could colocalize. Synchronized U-2OS cells were costained with antibodies to RRM2 and cyclin F. Although, RRM2 localized to the cytoplasm in S phase cells and virtually absent in G2 cells (Figure 1D). In contrast, our confocal images revealed that during G2, RRM2 enters the nucleus to interact with and localize to the nucleus (Figure 1D, bottom panels). These results suggest that during G2, RRM2 enters the nucleus to interact with and localize in the nucleus.

A CY Motif and Thr33 Are Required for RRM2 Binding to Cyclin F

Subsequently, we mapped the cyclin F binding motif in RRM2. A series of binding experiments, which used multiple RRM2 deletion mutants, narrowed the binding motif to a region of RRM2 located between amino acids 40–65 (Figures S2A–S2B). This region contains two putative CY motifs (RxL and RxI) (Figure 2B), which are established cyclin binding domains (Schulman et al., 1998). A mutant in the second motif (RRM2(RxA/A)) failed to coimmunoprecipitate endogenous cyclin F (Figure 2C), indicating that this CY motif, located at residues 49–51 of RRM2, mediates binding to cyclin F. In parallel, we also performed Ala scanning mutagenesis of the region encompassing amino acids 22–40 and found that, in addition to the CY motif, Thr33 is also necessary for an efficient binding to cyclin F (Figures 2B and 2C). Using this antibody, we found that cyclin F coimmunoprecipitated the phosphorylated form of endogenous RRM2 (Figures 1B and 1C). Significantly, RRM2 was phosphorylated on Thr33 predominantly in G2 and M (as indicated by the increase in cyclin B levels and Histone H3 phosphorylation on Ser10), when the levels of total RRM2 decrease and RRM2 interacts with cyclin F (Figures 1A and 1C).

Thr33 in RRM2 Is Phosphorylated by CDKs to Promote Binding to Cyclin F

The presence of a Pro at position 34 suggested that a Pro-directed kinase phosphorylates Thr33, and the phosphorylation of this residue in G2 and early M suggested that this kinase could be a CDK. In support of this hypothesis, we found that three different CDK inhibitors (NU6102, Roscovitine, and Alsterpaulone) reduced RRM2 phosphorylation on Thr33, whereas SB203580 (a p38 inhibitor), U0126 (a MEK inhibitor), and LY293646 (a DNA-PK inhibitor) had no effect (Figure 2D). Moreover, both Cdk1-cyclin B and Cdk2-cyclin A, but not Plk1 (another G2/M kinase), phosphorylated RRM2 on Thr33 in vitro, as shown by immunoblotting with the phospho-specific antibody (Figure 2E and data not shown). Importantly, CDK-dependent phosphorylation of RRM2 was necessary for its in vitro binding to cyclin F (Figure 2F).

Both Thr33 and the CY motif of RRM2 are highly conserved across species (Figure S2D), and both are necessary, but not sufficient, for a stable binding of RRM2 to cyclin F. RRM2(T33A), which contains an intact CY motif, did not efficiently bind cyclin F in vivo or in vitro (Figures S2C, 2F, and S2C), and wild-type RRM2 results obtained with CP110 (D’Angiolella et al., 2010). However, cyclin F(1-549), which retains the cyclin box domain but does not localize to the nucleus (Figure S1C), was unable to bind RRM2 (Figures S1A–S1B), although this mutant still interacted with CP110 (D’Angiolella et al., 2010), further confirming that the cyclin F and RRM2 interact in the nucleus.

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was unable to bind cyclin F in vitro without prior phosphorylation by a CDK (Figure 2F). However, RRM2(Rxl/Axl), in which Thr33 is intact, was unable to bind cyclin F, even though phosphorylation of Thr33 was unaffected (Figure 2C), and a peptide corresponding to amino acids 26–39 of RRM2 (i.e., a peptide that does not contain the CY motif) was unable to bind cyclin F irrespective of Thr33 phosphorylation.
of cyclin F are deleted (and the CY motif at 49–51 is presumably exposed), Thr33 becomes dispensable.

RRM2 Is Targeted for Degradation by SCF<sub>cyclin F</sub>

We noted that, compared to wild-type cyclin F, the cyclin F(LP/AA) mutant (in which the first two amino acids of the F-box domain were mutated to alanine) bound less Skp1 and Cul1 (as expected) and more RRM2 (Figure 2A). This result suggested that RRM2 is targeted for proteolysis by cyclin F because this mutant is unable to form an active SCF ubiquitin ligase and can sequester RRM2. To test whether cyclin F regulates the degradation of RRM2, we used three established siRNA oligos (D’Angiolella et al., 2010) to reduce the expression of cyclin F in synchronized HeLa cells. We also silenced Cyclin F expression in synchronized U-2OS and RPE1-hTERT cells by using the most effective of the three oligos. In all cases, depletion of cyclin F inhibited the G2-specific degradation of RRM2 (Figures 3A and S3A and data not shown). Significantly, compared to controls, the amount of RRM2 phosphorylated on Thr33 drastically increased, confirming that it is the phosphorylated form of RRM2 that is targeted by SCF<sub>cyclin F</sub>. Upon cyclin F depletion in G2 cells, RRM2 half-life increased (Figure S3B) and RRM2 accumulated mostly in the cytoplasm because of active nucleus-cytoplasm shuttling, as indicated by its nuclear accumulation following LMB treatment (Figure S3C). Moreover, in agreement with the siRNA results, we observed that Cyclin F<sup>−/−</sup> mouse embryonic fibroblasts (MEFs) (Tetzlaff et al., 2004) displayed RRM2 accumulation compared to parental Cyclin F<sup>+/−</sup> MEFs (Figure 3B). Finally, purified wild-type cyclin F, but not cyclin F(LP/AA), promoted the in vitro ubiquitylation of RRM2, but not RRM2(T33A) (Figures 3C and S3D and S3E), supporting the hypothesis that cyclin F directly controls the ubiquitin-mediated degradation of RRM2.

Cyclin F-Mediated Degradation of RRM2 Prevents Genome Instability

Together, the results shown in Figures 1, 2, 3, S1, S2, and S3 demonstrate that cyclin F mediates the nuclear degradation of RRM2 in G2. To investigate the biological significance of this event, we analyzed synchronized HeLa, U-2OS, and RPE1-hTERT cells expressing either HA-tagged wild-type RRM2, HA-tagged RRM2(RxI/AxA), or HA-tagged RRM2(T33A). In G2 and M, wild-type RRM2 was degraded, whereas RRM2(RxI/AxA) and RRM2(T33A) were not (Figures 4A and 4BB and S4A–S4C), in agreement with their inability to efficiently bind cyclin F (Figure 2C). Importantly, the expression of stable RRM2 mutants induced an increase in the cellular concentration of dATP and dGTP, but not dCTP and dTTP (Figures 4C and S4D), creating an imbalance in dNTP pools. This result is consistent with a reduction of purine dNTPs, but not pyrimidine dNTPs, in cells treated with either hydroxyurea, an RNR inhibitor, or an siRNA to RRM2 (Håkansson et al., 2006; Lin et al., 2007; Lin et al., 2004), and it is likely due to a more efficient nucleotide salvage pathway for pyrimidine deoxyribonucleosides than purine deoxyribonucleosides (Reichard, 1988).

We also synchronized in prometaphase RPE1-hTert cells infected with either an empty retrovirus or a retrovirus expressing HA-tagged wild-type RRM2, HA-tagged RRM2(T33A), or
HA-tagged RRM2(RxI/AxA) (Figure 4D). Cells were released from the block, and, 3 hr after, the majority of cells were in G1, as indicated by the disappearance of Histone H3 phosphorylated on Ser10. In all cases, when cells reached the next G1, the levels of both endogenous and exogenous RRM2 decreased (Figure 4D), likely due to APC/CCdh1 (Chabes et al., 2003b), which does not require either T33 or the RxI motif to interact with RRM2 (Figures 2C and 2G). Yet, in G1, levels of RRM2(T33A) and RRM2(RxI/AxA) remained higher than endogenous RRM2 (Figure 4D). Consistent with this observation, the concentrations of purine dNTPs were higher in G1 cells expressing stable RRM2 mutants compared to wild-type cells (Figure 4E).

Failures in the control of dNTP levels have been shown to lead to genome instability (Chabes et al., 2003a; Hu and Chang, 2007; Ke et al., 2005; Kunz, 1988; Kunz et al., 1994; Meuth, 1989; Phear and Meuth, 1989; Xu et al., 2008). To test whether expression of the RRM2 mutants induced mutations, we measured the spontaneous mutation frequency of the gene encoding hypoxanthine phosphoribosyl transferase (HPRT). Because of the presence of HPRT, cells are sensitive to 6-thioguanine (6-TG), thus, the occurrence of resistant clones represents spontaneous mutations at the HPRT locus (Daré et al., 1995). Upon 6-TG selection, and after adjusting for plating efficiency, we observed that, compared to cells transfected with an empty vector or expressing wild-type RRM2, cells expressing stable RRM2 mutants developed approximately 20 and 40 times more resistant colonies, respectively (Figure 5A), corresponding to 20- and 40-fold increases in mutation frequency.

FIGURE 4. Expression of Stable RRM2 Mutants Induces an Increase in the Concentration of dATP and dGTP
(A) HeLa cells infected with either a retrovirus expressing HA-tagged RRM2 or HA-tagged RRM2(T33A) were synchronized by using a double-thymidine block. Samples were collected at the indicated times after release, lysed, and immunoblotted as indicated.
(B) HeLa cells infected with either a retrovirus expressing HA-tagged RRM2 or HA-tagged RRM2(RxI/AxA) were analyzed as in (A).
(C) RPE1-hTert cells infected with either a retrovirus expressing HA-tagged RRM2, HA-tagged RRM2(T33A), or HA-tagged RRM2(RxI/AxA) were enriched for the G2 and M populations and lysed to assess the concentration of dNTPs. Each data point represents the mean ± standard deviation (SD) of three separate experiments.
(D) RPE1-hTert cells infected with either an empty virus (EV) or a retrovirus expressing HA-tagged wild-type (WT) RRM2, HA-tagged RRM2(T33A), or HA-tagged RRM2(RxI/AxA) were incubated for 16 hr with nocodazole and subjected to a mitotic shake-off to isolate round, prometaphase cells, which were subsequently released into fresh medium. Samples were collected at the indicated times after release from the block, lysed, and immunoblotted as indicated.
(E) RPE1-hTert cells were treated as in (D). Three hours after release from the prometaphase arrest, cells were lysed to quantify dNTP concentrations. Each data point represents the mean ± SD of three separate experiments. See also Figure S4.
synchronized in G2 (Figures S5B and S5C). These events
in fact, similar protein oscillations were observed in cells
increased rate of mutation) (Figures 5B and S4E).

stable RRM2 mutants (i.e., unbalanced dNTP pools and
mimicked the phenotypes observed following expression of
and prevent genome instability. Accordingly, cyclin F silencing
dagation via cyclin F is required to maintain dNTP pool balance
inhibited both cyclin F degradation and RRM2 accumulation
(Figures S6A and S6B). When expressed at near physi-
ologic levels, exogenous cyclin F was downregulated similar
to the endogenous protein (Figure S6C). Analysis of the stability
of different truncation mutants suggested that cyclin F downre-
gulation upon DNA damage depends on a motif located between
amino acids 407 and 660 (data not shown) and a cyclin F
domain contains three SerGln (526, 534, 595) and four ThrGln
sites (427, 467, 472, 543), which are potential sites of phosphor-
lation upon DNA damage depends on a motif located between
amino acids 407 and 660 (data not shown) and a cyclin F

Figure 5. Cyclin F-Mediated Degradation of RRM2 Prevents
Genome Instability

(A) The frequency of mutations at the HPRT locus was determined in U-2OS
cells infected with a retrovirus expressing either RRM2 or the indicated
HA-tagged RRM2 mutants. Each data point represents the mean ± SD of three
separate experiments.

(B) The frequency of mutations at the HPRT locus was determined in U-2OS
cells transfected with siRNAs to either a nonrelevant mRNA (LacZ) or
cyclin F mRNA. Each data point represents the mean ± SD of three separate
experiments.

These experiments show that the regulation of RRM2 degra-
dation via cyclin F is required to maintain dNTP pool balance
and prevent genome instability. Accordingly, cyclin F silencing mimicked the phenotypes observed following expression of stable RRM2 mutants (i.e., unbalanced dNTP pools and increased rate of mutation) (Figures 5B and S4E).

Finally, we generated and sequenced HPRT cDNAs from 21
individual 6-TG resistant clones expressing stable RRM2
mutants. Mutations, including deletions, transversions, transi-
tions, and insertions were found in all 21 HPRT coding sequences in these clones. Interestingly, 15 of the 21 clones analyzed showed skipping of exon 8. This deletion has been reported to depend on mutations in the pyrimidine rich tract of intron 7 (Andersson et al., 1992), which is consistent with the increased levels of purines detected in cells expressing stable RRM2 or depleted of cyclin F (Figures 4C, 4E, and S4D).

Upon Genotoxic Stress, Cyclin F Is Downregulated
and RRM2 Accumulates in an ATR-Dependent Manner

In response to DNA damage, RRM2 is recruited to the nucleus to
guarantee local availability of dNTPs for efficient DNA repair
synthesis (Chabes and Thelander, 2000; Lin et al., 2007; Niida
et al., 2010; Zhang et al., 2009), we found that in response to genotoxic stress, RRM2 accumulates in the nucleus, as detected by immunofluorescence staining of U-2OS cells (Figure S7A) or immunoblotting of the chromatin fractions from either HeLa or RPE1-hTERT cells (Figures 7A and S7B). Thus, we hypothesized that cyclin F downregulation is a prerequisite for the accumulation of nuclear RRM2. To test this hypothesis, we either infected HeLa cells with doxocy-
cline-repressible cyclin F constructs [wild-type cyclin F, cyclin
F(M309A)] (Figure 7A) or transiently expressed wild-type cyclin
F in RPE1-hTERT cells (Figure S7B), and subjected them to various forms of DNA damage. In contrast to control cells, RRM2 failed to accumulate in cells expressing wild-type cyclin F, whereas cells expressing the inactive cyclin F(M309A) mutant retained the ability to accumulate nuclear RRM2.

We then performed alkaline comet assays to monitor DNA
repair and found that cells expressing wild-type cyclin F, but
not cyclin F(M309A), displayed a reduced ability to repair
damaged DNA (Figure 7B). Accordingly, compared to control
cells, many more cells died (as judged by clonogenic survival)
after UV treatment when expression of wild-type cyclin F,
but not cyclin F(M309A) or cyclin F(LP/AA), was induced in
HeLa cells by removing doxycycline (Figures 7C and S7C).
Significantly, expression of a stable RRM2 mutant [either
RRM2[Rxl/AxA] or RRM2[T33A]), but not wild-type RRM2, reverted the UV sensitivity induced by the expression of cyclin F (Figure 7D and data not shown), indicating that the downregulation of cyclin F after genotoxic stress is required to allow RRM2 accumulation within the nucleus and, consequently, efficient DNA repair.

**DISCUSSION**

Here we report that RRM2 is targeted for degradation by SCFcyclin F during the G2 phase of the cell cycle (see model in Figure 7E). Failure to degrade RRM2 in G2 promotes imbalances in dNTP pools (both at G2/M and during the next G1) and an increased frequency of mutations (Figures 4, 5, and S4). Imbalances in dNTP pools result in increased base misincorporation during DNA synthesis and decreased proofreading due to enhanced polymerization rates (Mathews, 2006). Abnormal dNTP levels negatively affect the fidelity of DNA replication, producing an increase in gene mutation rate and genome instability. In agreement with these data, (1) elevated or imbalanced pools of dNTPs promote transformation and induce an increase in the rate of spontaneous mutations in cell systems (Chabes et al., 2003a; Hu and Chang, 2007; Ke et al., 2005; Kunz, 1988; Kunz et al., 1994; Meuth, 1989; Phear and Meuth, 1989), (2) overexpression of RRM2 induces the development of lung cancer in mice (Xu et al., 2008), and (3) elevated levels of RRM2 correlate with poor prognoses for cancer patients (Ferrandina et al., 2010; Grade et al., 2011; Jones et al., 2011; Kretschmer et al., 2011; Morikawa et al., 2010a; Morikawa et al., 2010b; Satow et al., 2010).

Oncogenic stress produces dNTP deficiencies and a consequent DNA replication stress typical of early oncogenic events (Bester et al., 2011). In contrast, expression of an RRM2 stable mutant increases the dNTP pool, but it does not induce DNA replication stress, as indicated by the lack of Chk1 phosphorylation or induction of 53BP1 bodies in G1 nuclei (Figure S4A and data not shown) (G1 nuclear 53BP1 bodies mark DNA lesions induced by replication stress [Lukas et al., 2011]). These findings suggest that mammalian cells may not have a checkpoint that senses dNTP pool increases, highlighting the risk of elevated RRM2 levels for the pathogenesis of cancer.

An interesting aspect of RRM2 degradation via SCFcyclin F is its regulation by CDKs. In fact, although cyclin F utilizes its
hydrophobic patch to recognize the CY motif in RRM2 (similar to other cyclin-substrate pairs), it does so only after RRM2 is phosphorylated by CDKs on Thr33, an event that appears to expose the CY motif (Figure 2 and S2B). Thus, the mode of RRM2 recognition by cyclin F is an exception to the rule that cyclin-substrate interactions do not require posttranslational modifications, highlighting the unique nature of RRM2 regulation. Interestingly, RRM2 is also phosphorylated by CDKs on Ser20 (Chan et al., 1993; Chan et al., 1999). However, in contrast to Thr33 phosphorylation, this modification occurs early in S phase, does not

Figure 7. Cyclin F Downregulation Is Required for Efficient DNA Repair
(A) HeLa cells infected with a pLVX Tet-Off lentivirus and either pLVX-Tight-puro Cherry-cyclin F or pLVX-Tight-puro Cherry-cyclin F(M309A) lentiviruses were treated with or without doxycyclin (DOXY) for 48 hr. Next, cells were treated with UV (4 J/m²) for the indicated times. After treatment, cell pellets were divided into chromatin and soluble fractions and immunoblotted as indicated.
(B) HeLa cells treated as in (A) were subjected to alkaline comet assays. Each data point represents the mean ± SD of three separate experiments in which at least 100 cells per sample were counted.
(C) HeLa cells were treated as in (A), except that the indicated doses of UV were used. A colony formation assay was performed 10 days after treatment. Each data point represents the mean ± SD of three separate experiments.
(D) HeLa cells preinfected with either pBabe HA-tagged RRM2 or pBabe HA-tagged RRM2(RxI/AxA) were infected as described in (A), and exposed to the indicated UV doses. A colony formation assay was performed 10 days after treatment. Each data point represents the mean ± SD of three separate experiments.
(E) A model of the regulation of DNA replication and repair by the cyclin F-RRM2 axis. During G2, after the last majority of DNA replication has occurred, cyclin F accumulates, thereby promoting RRM2 degradation in collaboration with a G2 CDK. DNA damage induces an ATR-dependent downregulation of cyclin F to allow accumulation of RRM2 for efficient DNA repair.
See also Figure S7.
affect RRM2 stability, and its function remains unknown (Chabes and Thelander, 2000).

After genotoxic stress, the levels of cyclin F in both p53-positive and p53-negative cells rapidly drop, allowing the recruitment of RRM2 to chromatin for efficient DNA repair synthesis (Figures 6, 7E, SS, and S6). This function is consistent with reports indicating a role for RNR in guaranteeing availability of dNTPs at the sites of DNA damage (Lin et al., 2007; Niida et al., 2010; Zhang et al., 2009). The timing of RRM2 accumulation following DNA damage parallels the timing of DNA repair (Figures 6, 7A, and 7B). The rapid accumulation of RRM2 protein requires cyclin F downregulation, which occurs in an ATR-dependent, but Chk1- and transcription-independent, manner (Figures 6 and SS and SSD). However, if DNA damage persists, RRM2 upregulation also relies on Chk1- and E2F1-dependent transcription (Zhang et al., 2009). Moreover, upon persistent genotoxic stress, a different RNR subunit, called RRM2B or p53R2 (which normally substitutes for RRM2 to form an active RNR complex necessary for the synthesis of mitochondrial DNA [Bourdon et al., 2007; Wang et al., 2011]), accumulates much later (48–72 hr later) after DNA damage in a p53-dependent manner (Håkansson et al., 2006; Tanaka et al., 2000).

We have previously shown that cyclin F controls centrosome duplication and prevents chromosome instability by promoting the degradation of CP110 (D’Angiolella et al., 2010). Our current study reveals that cyclin F controls the cellular dNTP pools and prevents genome instability by preventing RRM2 degradation. Thus, cyclin F is a hub that coordinates and synchronizes centrosome duplication with DNA replication to ensure proper cell division. Interestingly, hydroxyurea (HU) inhibits RNR and induces centrosome duplication in certain cell types (Balczon et al., 1995; Meraldi et al., 1999). The fact that cyclin F, but not CP110, is degraded after HU treatment (D’Angiolella et al., 2010) may explain why HU (by blocking RNR) induces dissociation of centrosome duplication from DNA replication.

In addition to the fundamental implications for our understanding of cell physiology, our studies have clinical relevance because they provide insight into the response to genotoxic stress caused by HU, gemcitabine, fludarabine phosphate, and cladribine, RNR-inhibiting drugs that are used in the treatment of various cancers, including leukemia, melanoma, metastatic ovarian cancer, nonsmall cell lung cancer, and pancreatic cancer (Shao et al., 2006). Moreover, because the failure to downregulate cyclin F in response to DNA damage blocks nuclear accumulation of RRM2 and induces cell death, we propose that the inhibition of cyclin F degradation may be useful for enhancing the chemosensitivity of cancer cells to DNA damage-based therapies.

**EXPERIMENTAL PROCEDURES**

**Biochemical Methods**

Extract preparation, immunoprecipitation, and immunoblotting have been previously described (Bassermann et al., 2008; Guardavaccaro et al., 2008).

Subcellular fractionation was performed as described (Ballabeni et al., 2004). Briefly, soluble fraction was extracts by using CSK buffer (0.5% Triton X-100, 10 mM Pipes [pH 6.8], 100 mM NaCl, 1.5 mM MgCl2, 300 mM sucrose, 1 mM aprotinin, 1 mM leupeptin, and 1 mM PMSF). Cells lysates were centrifuged at 1,700 rcf for 4 min. After centrifugation, cell pellets were washed and insoluble proteins were extracted by using CSK buffer containing 250 mM NaCl and Turbo Nuclease (Ambion).

**Purification and MudPIT Analysis**

HEK293T cells were transfected with constructs encoding either FLAG-HA-tagged cyclin F or FLAG-HA-tagged cyclin (1–270). Cell lysis, immunopurification, and MS/MS analysis have been previously described (D’Angiolella et al., 2010).

**Immunofluorescence Microscopy**

Cells were fixed with PFA 4% for 10 min, permeabilized with PBS containing 1% Triton X-100 for 10 min and blocked for 1 hr in PBS containing 0.1% Triton X-100 3% BSA prior to incubation with primary antibodies. (Please note that PFA does not allow detection of centrosomal cyclin F.) Secondary antibodies were donkey and conjugated with Alexa Fluor fluorochromes (Invitrogen). DAPI was used to stain DNA. Slides were mounted with Prolong-Gold (Invitrogen). Confocal microscopy was performed by using a Zeiss LSM 510, equipped with Zeiss LSM 510 software.

**Establishment of Tet-Off HeLa Cells Expressing Cherry Cyclin F and pBabe HA-Tagged RRM2**

pLVX-Tight-puro Cherry-cyclin F, pLVX-Tight-puro Cherry-cyclin (FM309A), pLVX-Tet-Off lentiviruses were produced according to the manufacturer instruction (Clontech). HeLa cells (preinjected or not with pBabe vectors expressing wild-type or mutant RRM2) were infected with lentiviruses; positive clones were selected by using puromycin (1 μg/ml) and G418 (800 μg/ml), and grown in the presence of Doxycycline (2 μg/ml). Doxycycline removal induced Cherry-cyclin F expression.

**Gene Silencing by Small Interfering RNA**

The sequences of the oligonucleotides numbers 1, 2, and 3, corresponding to the cyclin F mRNA, were CCAGUUGUGUGCGUGAUU, UAGCCUACCU CUACAGAUU, and GCACCGGCGUUAUCAGUGAA, respectively. A dsRNA oligo to LacZ mRNA (CGUACGCGGAAUACUUCGA) served as a negative control (Duan et al., 2012).

**Determination of dNTP Pool in Whole-Cell Extracts**

U-2OS and RPE1-hTERT cells (5 × 10⁶) were washed twice with cold PBS and extracted by using 1 ml of ice-cold 60% methanol for 1 hr at −20°C, followed by centrifugation for 15 min at 14,000 g. The supernatant was dried under vacuum; the pellet was dissolved in 200 μl of sterile water and stored at −20°C. Determination of the dNTP pool size was based on DNA polymerase-catalyzed incorporation of radioactive dNTP into the synthetic oligonucleotide template as described (Sherman and Fyfe, 1989).

**Determination of Mutation Rates**

For stable transfection U-2OS cells were infected with pBabe retroviruses expressing HA-tagged RRM2, HA-tagged RRM2 (T33A), and HA-tagged RRM2 (Rx/AxA). After infection, cells were selected by using puromycin for 2 days. Mutation frequencies were determined by HPRT mutation as described (Xu et al., 2008). 6-TG resistant clones were subcultured in 24-well format. When individual clones reached confluence, RNA was extracted and cDNA was synthesized with Fast Lane cDNA synthesis kit (Qiagen), according to manufacturer’s instruction. The HPRT open reading frame was PCR amplified by using the following primers: 5'-CTGGACGGT CAGCCCGCGC-3' and 5'-TAAATAATTTTTCTTTTCAAGATTTAAACACATCC GCC-3'. HPRT mutations were identified via direct sequencing of PCR products by using the following primers: 5'-CGGCCCGGCGGT-3' and 5'-GGCTCATAGTGCACATATAAAGA-3'.

**Comet Assay**

Alkaline comet assays were performed by using a Trevigen’s Comet Assay kit (4250-050-k) according to the manufacturer’s instructions. DNA was stained with SYBR Green, and slides were photographed by using a Zeiss Axiovert 200 M microscope, equipped with a cooled Retiga 2000R CCD (Qimaging). Tail moments were analyzed as reported previously (Park et al., 2008) by using the Tritek Comet Score Freeware.
Supplemental Information

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at doi:10.1016/j.cell.2012.03.043.

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Clonogenic Assay

Tet-off HeLa cells expressing Cherry-cyclin F or Cherry-cyclin F(M309A) were irradiated with varying doses of UV-C in the presence or absence of doxycycline (2 μg/ml) and then washed with PBS. Ten days after a third incubation, surviving colonies were counted and their relative numbers were expressed as percentages of the untreated cells as described (Franken et al., 2006).


SUPPLEMENTAL INFORMATION

EXTENDED EXPERIMENTAL PROCEDURES

Cell Culture and Cell-Cycle Synchronization
HeLa, U-2OS, RPE1-hTERT, HEK293T, and HCT116, cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). NHF1-hTert (ATM+/+) and AT cells (ATM−/−; GM0252A-hTert) were grown as described by (Heffernan et al., 2002). HCT116 ATR−/−/Flox cells (Martin et al., 2011) cyclin F Flox/− and cyclin F−/− MEFs (Tetzlaff et al., 2004) have been previously described. For synchronization at G1/S, HeLa and U-2OS cells were cultured in the presence of 2 μM thymidine (Sigma) for 16 hr, washed twice with PBS, and cultured in fresh medium without thymidine for 8 hr. After another 16 hr in thymidine, cells were washed twice with PBS and cultured in fresh medium. siRNA oligos were transfected between the first and second thymidine block. To trap cells in prometaphase, nocodazole (100 ng/ml) was added five hours after the release from the thymidine block. To enrich the G2 and M populations, asynchronously growing cells were treated with nocodazole for 16 hr. Where indicated, 10 μM MG132 was added for 3–6 hr prior to harvesting the cells.

Plasmids
Cyclin F plasmids and mutants have been previously described (D’Angiolella et al., 2010). RRM2 was amplified by PCR using a cDNA library generated from HEK293T cells and cloned in pcDNA3.1 either with a FLAG or HA tag. For retrovirus production, HA-tagged RRM2, HA-tagged RRM2(T33A), and HA-tagged RRM2(RKx/AXx) were subcloned in two retroviral pBABE-based vectors (one bearing a resistance to puromycin and one to hygromycin). For lentiviral production, Cherry-cyclin F WT and Cherry-cyclin F(M309A) were cloned in pLVX-Tight Puro. RRM2 and cyclin F mutants were generated either by using the QuikChange Site-directed Mutagenesis kit (Stratagene) or by standard PCR procedures. All cDNAs were fully sequenced.

Transient Transfections
U-2OS, HEK293T, and HCT116 cells were transfected by using Exgene 500 (Fermentas) and RPE1-hTERT cells by using X-tremeGene-HP (Roche) according to the manufacturer’s instructions. siRNA duplexes were transfected into subconfluent U-2OS or HeLa cells by using HiPerfect reagent (QIAGEN) according to the manufacturer’s instructions.

Chemical Inhibitors and Genotoxic Agents
The following kinase inhibitors were used: NU6102 (200 nM), Roscovitine (10 μM), DMAT (200 nM), LY293646 (1 μM), Alsterpaullone (400 nM), UO126 (30 μM), SB203580 (10 μM), Caffeine (2 mM), 4-hydroxytamoxifen (+4-OHT; 1 μM). The following genotoxic agents were used: Doxorubicine (0.5 μM), Camptothecin (2 μM), Methyl methanesulfonate (0.01%), and Neocarzinostatin (0.1 μg/ml).

In Vitro Kinase and Binding Assays
FLAG-tagged wild-type RRM2 and RRM2(T33A) proteins were in vitro transcribed/translated in a wheat germ extract (Promega). Purified recombinant Cdk1-cyclin B and Cdk2-cyclin A were obtained from Millipore. Proteins were incubated for 60 min in a kinase reaction buffer (50 mM Tris-HCl pH 7.5, 10 μM MgCl2, 0.6 mM DTT, 0.01% Triton X-100, and 2 mM ATP) at 30°C. Reactions were stopped with Laemmli buffer and samples were incubated for 5 min at 95°C. After the reaction mixtures were resolved by SDS-PAGE, phosphorylated proteins were detected by immunoblotting. For binding assays, immediately after the kinase reaction, in vitro translated/translated HA-tagged cyclin F was added to the samples and incubated for 30 min at 4°C. Next, anti-HA-coupled beads were added together with a binding assay buffer (50 mM Tris-HCl pH. 7.5, 250 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 50 mM NaF, 2 M β-glycerophosphate, 200 μM okadaic acid, and protease inhibitors). Samples were incubated for 60 min at 4°C, washed, resolved by SDS-PAGE, and analyzed by immunoblotting.

In Vitro Ubiquitylation Assay
In vitro ubiquitylation assays were performed in a volume of 30 μl containing 0.1 μM E1 (Boston Biochem), 10 ng/μl Ubch3, 10 ng/μl Ubch5c, 1 μM ubiquitin aldehyde, 2.5 μg/μl ubiquitin (Sigma), and either SCFCyclin F or SCFCyclin F(LP/AA) complex in a ubiquitylation buffer (50 mM Tris [pH 7.6], 2 mM ATP, 5 mM MgCl2, 0.6 mM DTT, okadaic acid 0.1 μM). In vitro translated 35S labeled wild-type RRM2, RRM2(T33A) or RRM2(1-200), a mutant encompassing the first 200 amino acids of RRM2 (in which Thr33 was or was not mutated to Ala), were used as substrates. The reactions were incubated at 30°C for the indicated times and analyzed by autoradiography. SCFCyclin F used in the experiments shown in Figures 3C and S1 was obtained mixing purified, recombinant cyclin F-Skp1 complex with a purified, recombinant Cul1(NTD)-Cul1(CTD)-Rbx1 complex [in which Cul1(CTD) was neddylated]. The cyclin F-Skp1 complex was produced by coexpressing insect cells (S9) with baculoviruses encoding His-tagged cyclin F and HA-tagged Skp1. Cells were then lysed and proteins subjected to Ni-NTA affinity purification. The Cul1(NTD)-Cul1(CTD)-Rbx1 complex was a gift from Ning Zheng (University of Washington) and was produced, purified, and neddylated as described in (Li et al., 2005). Briefly, proteins were produced by cotransforming E. Coli with constructs encoding His-tagged Cul1(NTD), His-tagged Cul1(CTD), and GST-tagged Rbx1. Bacteria were then lysed and proteins subjected to glutathione-Sepharose 4B affinity purification. After glutathione elution, GST was removed from Rbx1 and His tags were cleaved off from the two Cul1 domains by thrombin digestion. For the ubiquitylation reaction shown in Figure S5D, FLAG-tagged cyclin F or FLAG-tagged cyclin F(LP/AA) were transfected into HEK293T cells. Twenty-four hours after transfection, cells were incubated with MG132 for three hours, prior to lysis. Anti-FLAG M2 agarose beads were used to
immunoprecipitate the SCF cyclin F complex. The beads were washed four times in lysis buffer and two times in ubiquitylation reaction buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl₂, and 1 mM DTT). Finally, beads were then used for in vitro ubiquitylation assays.

Peptide Cross-Linking
The following peptides: RRM2 (26-39) (SLVDKENTPPALSG), phospho-RRM2 (26-39) (SLVDKEntp-PPALSG), RRM2 (30-60) (KENTPPALSGTRVLASKTARRIFQEPTEPK), phospho-RRM2 (30-60) (KEntp-PPALSGTRVLASKTARRIFQEPTEPK), RRM2 (40-60) (TRVLASKTARRIFQEPTEPK), p27 (180-198) (NAGSVEQTPKPGLRRTQ), or phospho-p27 (180-198) (NAGSVEQTPKPGLRRTQ) were conjugated to CNBr-activated Sepharose-4B according to the manufacturer’s instruction.

mRNA Quantification
Total RNA was extracted from cells by using RNase Mini Kit (QIAGEN), following the manufacturer instructions. 500 ng of total RNA was retro-transcribed by using the Superscript III kit (Invitrogen), following the manufacturer instructions. The cDNAs obtained after the retro-transcription was used as templates for qPCR. The reaction was performed with a Roche480 thermal cycler, by using the Absolute Blue QPCR SYBR Green Mix from Thermo Scientific. Primers for cyclin F were 5'-CCCCGAAGATGTGCTCTTTCA-3' (forward) and 5'-GCCTTCATTGTAGAGGTAGGCT-3' (reverse). Primers for RRM2 were 5'-GGGAGCGATTTAGCCAAGAA-3' (forward) and 5'-CACAAGGCATCGTTTCAATGG-3' (reverse). In each reaction, amplification of GAPDH (primer forward: 5'-GCTTGCCCTGTCCAGTTAAT-3' and reverse: 5'-TAGCTCAGCTGCACCCTTTA-3') or 18S RNA (primer forward: 5'-GACGCGAGGATTGACAGG-3' and reverse: 5'-ACCCACGGAATCGAGAAAGA-3') was used for normalization. To measure mRNA half-lives, cells were incubated in the presence of 2 μM actinomycin D diluted in DMEM.

SUPPLEMENTAL REFERENCES
Figure S1. Mapping of the RRM2 Binding Domain on Cyclin F, Related to Figure 2

(A) Schematic representation of cyclin F mutants. Binding to endogenous RRM2, binding to endogenous CP110, centrosomal localization, and nuclear localization are indicated for cyclin F constructs with a (+).

(B) HEK-293T cells were transfected with an empty vector (EV), FLAG-tagged wild-type (WT) cyclin F, or the indicated FLAG-tagged cyclin F mutants. Whole-cell extracts were immunoprecipitated (IP) with anti-FLAG resin, and immunocomplexes were probed with antibodies to the indicated proteins.

(C) U-2OS cells were transfected with FLAG-tagged cyclin F or FLAG-tagged cyclin F(1-549), collected 48 hr after transfection, and stained with anti-FLAG antibody to visualize cyclin F (red). DNA was stained with DAPI (blue). This result, together with the results listed in (A), shows that cyclin F(1-549) does not localize to the nucleus, and indicates that cyclin F contains an NLS (nuclear localization signal) between amino acids 550 and 600.
Figure S2. Mapping of the Cyclin F Binding Domain on RRM2, Related to Figure 2

(A) Schematic representation of RRM2 mutants. Binding of RRM2 to endogenous cyclin F is indicated with symbol (+).

(B) HEK-293T cells were transfected with an empty vector (EV), FLAG-tagged wild-type (WT) RRM2, or the indicated FLAG-tagged RRM2 mutants. Whole-cell extracts (WCE) were immunoprecipitated (IP) with anti-FLAG resin, and immunocomplexes were probed with antibodies to the indicated proteins.

(C) HCT116 and HCT116 p53−/− cells were transfected with either an empty vector (EV), FLAG-tagged wild-type (WT) RRM2, or the indicated FLAG-tagged RRM2 mutants. Whole-cell extracts (WCE) were immunoprecipitated (IP) with anti-FLAG resin, and immunocomplexes were probed with antibodies to the indicated proteins.

(D) Alignment of the amino acid regions containing Thr33 and the RxI motif in RRM2 homologs.
Figure S3. Cyclin F Promotes the Ubiquitylation and Degradation of RRM2, Related to Figure 3

(A) U-2OS cells were transfected with siRNAs to either a nonrelevant mRNA (LacZ) or cyclin F mRNA. Cells were synchronized at G1/S by a double-thymidine block and collected at the indicated times after release from the block. Cell lysates were immunoblotted with antibodies to the indicated proteins.

(B) U-2OS cells were treated as in (A) and 9 hr after the release from the double-thymidine block, cycloheximide (CHX) was added for the indicated times. Cell lysates were immunoblotted with antibodies to the indicated proteins.

(C) U-2OS cells were synchronized at G1/S by using a double-thymidine block before release into fresh medium. Cells were fixed 9 hr after release (when they were in G2) and stained with an antibody to cyclin F (green) or RRM2 (red). Where indicated, cells were pretreated for two hours with Leptomycin B (LMB) before fixation. DAPI was used to visualize nuclei. Confocal microscopy was used to visualize stained cells. The experiment shows that in cyclin F-depleted cells, RRM2 accumulates mostly in the cytoplasm, due to its active nucleus-cytoplasm shuttling. Notably, centrosomal cyclin F (D’Angiolella et al., 2010) is not detectable when using PFA fixation for detection of nuclear RRM2.

(D) HEK293T cells were transfected with either an empty vector (EV), FLAG-tagged cyclin F, or FLAG-tagged cyclin F(LP/AA). Twenty-four hours after transfection, cells were collected and lysed. Extracts were immunoprecipitated with an anti-FLAG resin and used in ubiquitylation assays with 35S-labeled, in vitro-transcribed/translated RRM2 (prephosphorylated with Cdk1-cyclin B) as substrate. The bracket indicates a ladder of bands corresponding to polyubiquitylated RRM2, detected by autoradiography. Cyclin F and Skp1 present in the immunoprecipitates were also detected by immunoblotting (bottom panels).

(E) Insect cells (Sf9) were coinfected with baculoviruses encoding His-tagged cyclin F and HA-tagged Skp1, lysed, and subjected to Ni-NTA affinity purification. E. coli expressing His-tagged Cul1-NTD, His-tagged Cul1-CTD, and GST-tagged Rbx1 were lysed and subjected to glutathione-Sepharose 4B affinity purification. After glutathione elution, the GST tag was removed from Rbx1 and His tags were cleaved off from the two Cul1 domains by thrombin digestion. An aliquot of the two purified complexes was separated by SDS-PAGE, and the gel was stained with Coomassie Blue.
Figure S4. Defects in RRM2 Degradation Induce an Increase in the Cellular Concentration of dATP and dGTP, Related to Figure 4

(A) RPE1-hTert cells infected with either a retrovirus expressing either HA-tagged wild-type (WT) RRM2 or HA-tagged RRM2(T33) were serum starved for 72 hr before release into fresh medium containing serum. Samples were collected at the indicated times after serum re-addition (SR), lysed, and immunoblotted with antibodies to the indicated proteins.

(B) U-2OS cells infected with a retrovirus expressing either HA-tagged wild-type (WT) RRM2, HA-tagged RRM2(T33A), or HA-tagged RRM2(Rxi/AxA) were either grown asynchronously (AS) or enriched for the G2 and M populations (G2/M) as described in Experimental Procedures. Cell lysates were then collected and immunoblotted with antibodies to the indicated proteins.

(C) RPE1-hTert cells infected with a retrovirus expressing either HA-tagged wild-type (WT) RRM2, HA-tagged RRM2(T33A), or HA-tagged RRM2(Rxi/AxA) were either grown asynchronously (AS) or enriched for the G2 and M populations (G2/M) as described in Experimental Procedures. Cell lysates were then collected and immunoblotted with antibodies to the indicated proteins.

(D) U-2OS cells infected with a retrovirus expressing either HA-tagged wild-type (WT) RRM2, HA-tagged RRM2(T33A), or HA-tagged RRM2(Rxi/AxA) were either grown asynchronously (AS) or enriched for the G2 and M populations (G2/M) as described in Experimental Procedures. Cell lysates were then collected and immunoblotted with antibodies to the indicated proteins.

(E) U-2OS cells infected with a retrovirus expressing either HA-tagged wild-type (WT) RRM2, HA-tagged RRM2(T33A), or HA-tagged RRM2(Rxi/AxA) were enriched for the G2 and M populations as described in Experimental Procedures and lysed to quantify dNTP concentrations. Each data point represents the mean ± SD of three separate experiments.

(F) U-2OS cells transfected with siRNAs to either a nonrelevant mRNA (LacZ) or cyclin F mRNA. Cells were enriched for the G2 and M populations as described in Experimental Procedures and lysed to quantify dNTP concentrations. Each data point represents the mean ± SD of three separate experiments. Similar results were obtained in HeLa cells (data not shown).
Figure S5. Upon Genotoxic Stress, Cyclin F Is Downregulated and RRM2Accumulates in Both Asynchronous and G2-Synchronized Cells, Related to Figure 6

(A) Cell-cycle analyses of HeLa and RPE1-hTert cells subjected to different forms of genotoxic stress. DNA content was monitored by flow cytometry.

(B) HeLa cells were synchronized at G1/S by using a double-thymidine block. Cells were then released from the block to allow progression toward G2. Six hours postrelease, cells were pulsed for one hour with either solvent (−) or doxorubicin (DRB) (+). Cells were collected at the indicated time after DRB treatment, lysed, and analyzed by immunoblotting with antibodies to the indicated proteins.

(C) The RRM2 mRNA levels of cells treated as in (B) were analyzed by qPCR with triplicate measurements (± SD). The amount of RRM2 mRNA present in nontreated cells (0 hr) was set as 100.

(D) HeLa and RPE1-hTert cells were transfected with the indicated siRNA oligos and treated with doxorubicin (DRB). Cells were collected at the indicated time after DRB treatment, lysed, and analyzed by immunoblotting with antibodies to the indicated proteins.
Figure S6. In Response to Genotoxic Stress, Whereas the Levels and Stability of Cyclin F mRNA Are Unchanged; Cyclin F Protein Is Degraded by the Proteasome, Related to Figure 6

(A) The levels of cyclin F mRNA in HeLa cells incubated for the indicated times with or without doxorubicin (DRB) or camptothecin (CPT) were analyzed by using qPCR in triplicate measurements (± SD). The amount of cyclin F mRNA present in nontreated cells (0 hr) was set as 100. The bottom graph shows the cyclin F mRNA levels of cells incubated for the indicated times in the presence or absence of doxorubicin (DRB), plus or minus actinomycin D.

(B) RPE1-hTERT cells were treated with the indicated DNA damaging agents: NCZ (neocarzinostatin), CPT (camptothecin), doxorubicin (DRB), or UV (8 J/m²). Cells were collected four hours after treatment and lysed for immunoblotting as indicated. Where indicated, the proteasome inhibitor MG132 was added for three hours prior to harvesting the cells.

(C) RPE1-hTert cells were infected with either pBabe HA-tagged wild-type cyclin F or the following pBabe HA-tagged cyclin F mutants: Cyclin F(4 > A) (TTSS467/472/526/595AAAA), cyclin F(5 > A) (TTSTS467/472/526/543/595AAAAA), cyclin F(6 > A) (TTSSTS467/472/526/534/543/595AAAAAA), cyclin F(7 > A) (TTTSSTS427/467/472/526/534/543/595AAAAAAA). Cells were either left untreated (UT) or treated with 10 J/m² UV. Three hours after treatment, cells were lysed and immunoblotted as indicated.

(D) RPE1-hTert cells were infected with either pBabe GFP or pBabe GFP fused to amino acids 407–660 of cyclin F. Cells were then either left untreated (UT) or treated with the indicated DNA damaging agents: camptothecin (CPT), DRB (doxorubicin), or UV (8 J/m²). Cells were collected four hours after treatment and lysed for immunoblotting as indicated. Where indicated, the proteasome inhibitor MG132 was added for three hours prior to harvesting the cells.
Figure S7. Forced Expression of Cyclin F Inhibits the Genotoxic Stress-Mediated Accumulation of RRM2 on the Chromatin, and Sensitizes Cells to DNA Damage, Related to Figure 7

(A) U-2OS cells were treated with camptothecin (CPT) for four hours and, where indicated, pretreated for two hours with Leptomycin B (LMB) before fixation. Cells were then stained with an antibody to cyclin F (green) or γH2AX (red). DAPI was used to visualize nuclei. Confocal microscopy was used to visualize stained cells.

(B) RPE1-hTert cells transiently transfected with an empty vector (EV) or FLAG-tagged cyclin F were treated with neocarzinostatin (NCS), camptothecin (CPT), or Doxorubicin (DRB) for the indicated times. After harvesting, cells were fractionated into soluble and chromatin fractions, and lysates were immunoblotted for the indicated proteins.

(C) Left panel: HeLa cells infected with a pLVX Tet-Off lentivirus and either a pLVX-Tight-puro Cherry-cyclin F or a pLVX-Tight-puro Cherry-cyclin F(LP/AA) lentivirus were treated with or without doxycycline (DOXY) for 48 hr. Next, cells were treated with the indicated doses of UV. A colony formation assay was performed 10 days after treatment. Each data point represents the mean ± SD of three separate experiments. Right panel: HeLa cells were transfected with the indicated siRNA oligos and treated with the indicated doses of UV. A colony formation assay was performed 10 days after treatment. Each data point represents the mean ± SD of three separate experiments.