The N Terminus of the Retinoblastoma Protein Inhibits DNA Replication via a Bipartite Mechanism Disrupted in Partially Penetrant Retinoblastomas

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The N-terminal domain of the retinoblastoma (Rb) tumor suppressor protein (RbN) harbors in-frame exon deletions in partially penetrant hereditary retinoblastomas and is known to impair cell growth and tumorigenesis. However, how such RbN deletions contribute to Rb tumor- and growth-suppressive functions is unknown. Here we establish that RbN directly inhibits DNA replication initiation and elongation using a bipartite mechanism involving N-terminal exons lost in cancer. Specifically, Rb exon 7 is necessary and sufficient to target and inhibit the replicative CMG helicase, resulting in the accumulation of inactive CMGs on chromatin. An independent N-terminal loop domain, which forms a projection, specifically blocks DNA polymerase α (Pol- α) and Ctf4 recruitment without affecting DNA polymerases ϵ and δ or the CMG helicase. Individual disruption of exon 7 or the projection in RbN or Rb, as occurs in inherited cancers, partially impairs the ability of Rb/RbN to inhibit DNA replication and block G₁-to-S cell cycle transit. However, their combined loss abolishes these functions of Rb. Thus, Rb growth-suppressive functions include its ability to block replicative complexes via bipartite, independent, and additive N-terminal domains. The partial loss of replication, CMG, or Pol- α control provides a potential molecular explanation for how N-terminal Rb loss-of-function deletions contribute to the etiology of partially penetrant retinoblastomas.

Mutational inactivation or deletion of the retinoblastoma (Rb) tumor suppressor gene occurs in multiple cancer types, including retinoblastoma, osteosarcoma, and breast and small cell lung cancers, and deregulation or inactivation of regulatory components of the Rb pathway is a hallmark of human cancers (1). The Rb protein functions to harness a variety of cellular processes important in tumorigenesis, including regulation of the cell cycle, apoptosis, differentiation, stress responses, and DNA replication. The role of Rb in these processes derives to a large extent from interactions of proteins with the C terminus of Rb that contains a large pocket domain (1–5), and most Rb loss-of-function mutations compromise pocket structure and/or function and are highly penetrant alleles of inherited cancer in humans and mice (6).

Multiple observations indicate that the N-terminal domain of Rb (RbN) (residues 1 to 400) also plays an important role in growth suppression and tumorigenesis. Indeed, nearly 20% of cancer-associated in-frame mutations in Rb are located in the N-terminal region (6). These lesions leave an intact C-terminal pocket and generate stable forms of Rb that bind E2F transcription factors and localize to the nucleus in a fashion similar to that of wild-type Rb (wt-Rb) (6–10). Several in-frame RbN exon deletions in familial retinoblastomas have been reported, including individual losses of exon 4 (Ex4), Ex5, Ex7, or Ex9 (11–14). In-frame deletions and mutations have also been found in Rb exons 6 and 8 in prostate cancers and astrocytomas, respectively (15, 16). Furthermore, in contrast to pocket mutations, N-terminal in-frame deletions in Rb generally display partial penetrance for the development of retinoblastoma (6, 8, 11–14). For example, transgenic mice expressing Rb proteins with N-terminal in-frame deletions produce a partial-penetrance phenotype for tumor development (7). Finally, forced expression of such Rb alleles in mice can impair embryonic and postnatal development and cannot res-

cue the embryonic lethality of *Rb*^{−/−} mice (7). Thus, RbN must direct unique functions of Rb that harness tumorigenesis.

Rb regulates G₁ cell cycle transit through its interactions with E2F transcription factors (1). However, through unknown means, Rb also blocks the initiation and progression of S phase through inhibitory effects on DNA replication in an E2F-independent manner (17–19). For example, primary mouse embryo fibroblasts (MEFs) exhibit an intra-S checkpoint and inhibit DNA synthesis in response to DNA damage (20), yet *Rb*^{−/−} MEFs fail to elicit this checkpoint response and continue to replicate DNA (21–24). Indeed, following irradiation-induced DNA damage, Rb localizes to DNA replication initiation sites and suppresses hyperreplication (22). In *Drosophila melanogaster*, the dRb protein binds with dE2F to the *chorion ori* and interacts with the origin recognition complex (ORC) to suppress origin firing (25, 26). In both scenarios, it is unclear how Rb blocks DNA synthesis at replication sites.

The ability of Rb to control DNA replication has been suggested to be directed by its N-terminal domain. First, yeast two-hybrid and biochemical studies have shown that RbN directly binds to the C terminus of Mcm7, a subunit of the replicative CMG (Cdc45, MCM, and GINS) helicase, and Rb-Mcm7 com-

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plexes are observable *in vitro* and *in vivo* (27–29). Second, RbN can inhibit DNA replication *in vitro* when added to replicating extracts from *Xenopus laevis* oocytes (28, 30). Inhibition is manifest at both initiation and elongation steps and is associated with a reduction in replication protein A (RPA) loading, suggesting that the CMG helicase is one component of the replication apparatus inhibited by RbN (30). Third, incubation with the C-terminal domain of Mcm7 (Mcm7-CT) blocks the ability of RbN to suppress DNA replication (30). Fourth, in mammalian cells, a transforming growth factor β 1 (TGF- β 1)-to-Rb circuit acutely blocks S-phase entry by inhibiting the assembled CMG helicase at G₁/S, and perturbation of the Rb-Mcm7 interaction abrogates this arrest (27). Finally, Rb protein lacking RbN is compromised for blocking entry into S phase (31).

The mechanisms by which Rb suppresses DNA replication and helicase activity are unknown. Here we report a bipartite mechanism by which Rb inhibits DNA replication, where the exon 7 domain of RbN is required to inhibit CMG helicase activity at initiation and elongation steps, while a notable projection domain derived from part of exons 5 and 6 (10) suppresses DNA polymerase α (Pol- α) and Ctf4 recruitment to replisomes. The loss of either of these domains impairs the ability of RbN to suppress DNA replication, while the combined loss of both regions abolishes the ability of Rb to inhibit DNA replication and to block progression into S phase *in vivo*. Thus, the ability of Rb to directly harness DNA replication via specific N-terminal regions mutated in cancers contributes to its tumor suppressor functions.

MATERIALS AND METHODS

Cell culture, Rb mutant plasmids, and transfections. SaOS-2 cells were obtained from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; HyClone). Balb/MK cells were cultured and transfected as described previously (27). For SaOS-2 cells, transfections were performed as previously described (17, 31). Rb alleles were expressed in pCMV-based vectors containing hemagglutinin (HA) epitopes. A green fluorescent protein (GFP)-expressing plasmid was cotransfected in each sample. Forty-eight hours after transfection, SaOS-2 cells were analyzed by fluorescence-activated cell sorter (FACS) analysis for DNA content after separation for GFP-positive (transfected) and GFP-negative (nontransfected) populations. SaOS-2 cell transfections were performed in duplicate, and results were averaged, followed by normalization to values for empty-vector transfections. For mouse keratinocyte (MK) transfections, plasmid pCS2+XIRb (gift from Anna Philpott) (32) was expressed for 24 h.

Antibodies. All antibodies were rabbit polyclonal antibodies and raised against human homologues, unless otherwise specified. Mouse monoclonal anti-glutathione S-transferase (anti-GST), anti-His₆, and anti-HA antibodies were purchased from Covance. Monoclonal anti-Mcm7, anti-Rb (polyclonal antibody raised against mouse Rb), and anti-Psf2 antibodies were purchased from Santa Cruz; anti-beta-actin and anti-histone H3 were purchased from Abcam; and anti-Mcm3 was purchased from Cell Signaling. Anti-Mcm5 was generated and characterized as described previously (33). Anti-Orc2 was a gift from Rolf Knippers (Konstanz University, Germany). Anti-Ctf4 was a gift from Anindya Dutta (University of Virginia). Antibodies raised against *Xenopus laevis* proteins, anti-XMcm2, anti-XMcm4, anti-XMcm6, and anti-XMcm7, were gifts from Julian Blow (University of Dundee, United Kingdom). Anti-XRPA32, anti-XCdc45, and anti-XMcm10 were gifts from Johannes Walter (Harvard Medical School); anti-XRPA70 was a gift from William Dunphy (California Institute of Technology); anti-XPol-alpha, used for assessment of RbN experiments, was a gift from Karlene Cimprich (Stanford University); and anti-XPol-alpha, anti-XPol-delta, and anti-XPol-epsilon, used for assessment

of peptide experiments, were gifts from Shou Waga (Japan Women's University).

Preparation of extracts, *in vitro* DNA replication, and protein purification. *Xenopus laevis* egg extracts were prepared from Ca²⁺-ionophore-activated mature eggs as described previously (34). Cytosol and membrane fractions were snap-frozen and kept at -80°C until needed. Egg extracts reconstituted from frozen cytosol and membrane fractions were incubated with 500 sperm nuclei/ μl in triplicate. Radiolabeled nucleotides ($[\alpha\text{-}^{32}\text{P}]\text{dCTP}$) were added for 150 min at 22°C to measure DNA replication rates (35). Isolated DNA/sperm chromatin from the samples was precipitated on filters, and the levels of DNA replication were determined by scintillation counting, with results averaged and errors shown as standard errors of the means (SEM). Peptides for human RbN exons were synthesized at the Moffitt Cancer Center Proteomics Core facility. GST-RbN (residues 1 to 400), the GST-tagged Rb C-terminal domain (RbC) (residues 399 to 928), GST-Rb, and all mutant derivatives (all human species) were expressed in pGEX-4T. His₆-Mcm7-CT was expressed in pET28a. All proteins were purified from *Escherichia coli* as described previously (27). Protein amounts used for each replication reaction are indicated in each figure.

Chromatin pull-down assays, *in vitro* protein binding assays, and immunoblotting. Reconstituted *Xenopus* egg extracts were incubated with 2,500 sperm nuclei/ μl for studies with peptides (250 μM each) or with 1,250 sperm nuclei/ μl for studies with RbN mutant proteins (1.5 μM each). Following incubation for 80 min at 22°C , extracts were diluted in chromatin isolation buffer (36) containing 80 mM KCl (high salt) or 20 mM KCl (low salt) for assessment of CMG chromatin association. For analysis of DNA Pol- α and Ctf4 recruitment in the presence of RbN mutants or peptides, the same buffer was used but with 40 mM KCl or 80 mM KCl, respectively. Analysis of RPA and GST-RbN chromatin associations was performed by using buffer with 80 mM KCl. To isolate chromatin, diluted samples were layered over 1 M sucrose and subjected to centrifugation for 15 min at $16,000 \times g$. *In vitro* GST-RbN/His₆-Mcm7 protein binding assays were performed as described previously (27). Immunoblotting was performed by using enhanced chemiluminescence.

***In vitro* DNA elongation assays.** Elongation assays were performed by using a modification of a previously reported protocol (37), where a substitution of roscovitine for the p27^{Kip1} Cdk inhibitor protein was used to block the initiation of DNA replication (38). Briefly, fresh egg extracts containing sperm nuclei (4,000 nuclei/ μl) were incubated for 30 min at 22°C to allow the first round of DNA replication initiation and elongation to begin. The samples were diluted in buffer containing 250 mM KCl, 25 mM MgCl₂, 25 mM EGTA, 2 mM β -mercaptoethanol, 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, and 50 mM HEPES-KOH (pH 7.6) and spun through a 1 M sucrose cushion. The pellet containing chromatin preinitiated for DNA replication was resuspended at 500 nuclei/ μl in fresh egg extracts supplemented with 100 μM roscovitine to inhibit further initiation of DNA replication in the second nuclei that formed. DNA replication elongation in the second nuclei was assessed as described above, in the presence of 37.5 μM peptides or 375 nM RbN proteins.

RESULTS

The RbN domain inhibits DNA replication via interactions with Mcm7. Yeast two-hybrid analyses established that the RbN domain binds to the C terminus of Mcm7 (28), and full-length Rb or RbN protein inhibits DNA replication in *Xenopus* oocyte-derived *in vitro*-replicating extracts (28, 30). *Xenopus* oocytes and early-stage embryos do not contain endogenous Rb protein (32, 39), which we verified here using ectopically expressed *Xenopus laevis* Rb (Xl-Rb) in murine cells for comparison and antibody validation (Fig. 1A). Thus, the effects of added Rb in these extracts are not due to competition issues with endogenous Rb. Using this system to assess the effects of GST-RbN on DNA replication, we confirmed these findings and showed that RbN inhibition of DNA

FIG 1 RbN inhibits DNA replication by impairing CMG helicase activity and DNA polymerase α recruitment. (A) Mouse keratinocytes (MK) were transfected (Tfx) with a plasmid expressing *Xenopus laevis* Rb (Xl-Rb). Total protein lysates from nontransfected and ~10,000 transfected cells were compared to total lysates from ~20,000 *X. laevis* (X.l.) embryonic nuclei for the presence of Xl-Rb protein by immunoblotting. Coomassie blue staining was used as a loading control. Note that Xl-Rb migrates slightly faster than murine Rb (*Mus musculus* Rb [Mm-Rb]) (39). Note also that the antibody used recognizes Xl-Rb but is not as efficient at recognizing Mm-Rb. (B to E) DNA replication was measured *in vitro* by using *Xenopus* oocyte extracts and sperm chromatin. (B) Bacterially purified human GST-RbN protein was titrated into reaction mixtures prior to nuclear assembly and the start of DNA replication. The control sample had no RbN protein added, and a boiled sample controlled for the lack of replication. Assays were performed in triplicate, and results were averaged; errors are SEM. Two asterisks represent a *P* value of <0.01 for significance calculations, with reference to the control. (C) Replication assays were performed with a fixed amount of GST-RbN protein preincubated with the indicated increasing amounts of bacterially purified His₆-Mcm7-CT protein. The control mixture had no proteins added. Assays were done in triplicate, and results were averaged; errors are SEM. One asterisk indicates a *P* value of <0.05, and two asterisks indicate a *P* value of <0.01, as determined by one-sided *t* test calculations, compared to data for assays where no Mcm7-CT was added. (D) Bacterially purified human His₆-Mcm7-CT protein was titrated into replication assay mixtures prior to nuclear assembly and the start of DNA replication. Control samples had no His₆-Mcm7-CT added. Assays were performed in triplicate, and results were averaged; errors are SEM. (E) Sperm chromatin was used to initiate DNA replication in oocyte extracts, with or without added GST-RbN. Chromatin was isolated and analyzed by immunoblotting for associations of RPA70, DNA Pol- α , DNA Pol- δ , and DNA Pol- ϵ .

replication is titratable (Fig. 1B). Furthermore, preincubation of GST-RbN with His₆-Mcm7-CT prevents RbN from inhibiting replication, and suppression of RbN inhibition by Mcm7-CT is also titratable (Fig. 1C). The effects of Mcm7 were due to interactions with RbN, as incubation of His₆-Mcm7-CT alone does not affect DNA replication in *Xenopus* extracts (Fig. 1D). As *Xenopus* extracts are transcriptionally inactive (40), RbN directly inhibits DNA replication independent of transcription. These results are consistent with a stoichiometric mechanism by which RbN inhibits DNA replication via interactions with the Mcm7 component of the CMG helicase.

RbN inhibits CMG helicase activity and recruitment of DNA polymerase α . RPA is a single-stranded DNA binding protein,

and loading of RPA onto chromatin is used as a surrogate measurement for CMG helicase functionality (38). As expected (30), incubation with RbN reduced the levels of RPA bound to *Xenopus* sperm chromatin (Fig. 1E). We reasoned that RbN might inhibit the binding of other components that are also needed for DNA replication. DNA polymerase α is a priming polymerase (41), and the inclusion of RbN significantly reduced the association of DNA polymerase α with chromatin in *Xenopus* extracts (Fig. 1E). RbN did not reduce the levels of DNA polymerase δ or ϵ bound to chromatin (Fig. 1E) but instead caused increases in the levels of both polymerases. Thus, the chromatin-associated reduction in the level of DNA polymerase α by RbN was specific. Collectively, these findings indicate that RbN suppresses DNA replication by

FIG 2 The F2 and F1 domains of RbN inhibit DNA replication by suppressing CMG helicase and DNA Pol- α , respectively. (A) Model of the crystal structure of the RbN domain. Exons lost in retinoblastoma are in color. The F1 and F2 finger domains are notated based on 3D modeling. See the text for discussion. (B) Peptides representing exons from RbN were analyzed for their inhibitory capacity relative to that of GST-RbN using *in vitro* *Xenopus* oocyte replication assays. Exon peptides are matched to the simplified line diagram shown in panel A. Assays were performed in triplicate with the molarities indicated for each peptide or GST-RbN, and results were averaged; errors are SEM. Significance for each assay was calculated with reference to the control. (C and D) Sperm chromatin was isolated after *in vitro* DNA replication assays were performed with or without RbN-derived peptides. Chromatin was analyzed by immunoblotting for RPA70 to measure CMG activity (C) or for DNA polymerases to assess chromatin recruitment (D).

blocking CMG helicase activity and by impairing the association of DNA polymerase α with replisomes.

Mechanism of RbN-directed inhibition of DNA replication.

In-frame exon deletions in the N terminus of Rb are associated with partially penetrant inherited retinoblastoma (6), and such *Rb* alleles are also partially penetrant in provoking tumor formation in transgenic mice (7). Given the effects of RbN, we hypothesized that Rb N-terminal mutants might be impaired in their ability to inhibit DNA replication.

Examination of the crystal structure and three-dimensional (3D) space-filling models of RbN (10) revealed two finger-like structures, F1 and F2 (Fig. 2A). The F1 finger has been called a “projection” domain (10) and is encoded by part of *Rb* exons 5 and 6, whereas the F2 “bulge” is encoded by *Rb* exon 7 (Fig. 2A).

The *Rb* exon 7-encoded F2 domain is lost in familial inherited retinoblastomas (14), while *Rb* exons 5 and 6 are lost or mutated in certain cancers (13, 15).

To test the roles of these domains in harnessing DNA replication, peptides representing Rb exon 4 (amino acids [aa] 128 to 167), exon 5 (aa 168 to 179), exon 7/F2 (aa 202 to 241), exon 9 (aa 287 to 313), and the projection/F1 domain (aa 173 to 187; from the end of the α -helix in exon 5 to the beginning of the α -helix in Ex6) were generated. These peptides were then tested to assess if they could directly inhibit *in vitro* DNA replication in the *Xenopus* cell-free system (Fig. 2B). Exon 4 and exon 9 peptides, and the exon 5 peptide, which contains only part of the projection/F1 domain, did not block DNA synthesis. In contrast, the peptide representing the entire projection/F1 domain inhibited DNA rep-

FIG 3 The F2 domain of RbN causes accumulation of inactive CMG helicases on chromatin. Sperm chromatin was isolated after *in vitro* DNA replication assays with or without RbN-derived peptides (A and B) or with or without GST-RbN (C) were performed. Chromatin was extracted with low-salt buffer (A) or high-salt buffer (B and C). Samples were analyzed by immunoblotting for the indicated components of the CMG helicase or actin, as a loading control. The data shown in panels A and B were derived from samples in the experiments shown in Fig. 2D and C, respectively. The samples used for panel C are the same as those used for Fig. 1E.

lication in a titratable manner, as did the exon 7/F2 peptide. Notably, when the F1 and F2 peptides were combined in the reaction mixtures, the inhibition of DNA synthesis that was achieved was additive and titratable (Fig. 2B, far right). We conclude that the projection/F1 and Ex7/F2 domains represent two functional regions of RbN that are capable of directly inhibiting DNA replication. Furthermore, the additive nature of these peptides in blocking DNA replication supports a model whereby F1 and F2 regulate DNA replication through independent mechanisms.

The RbN exon 7/F2 domain inhibits CMG helicase activity, and the projection/F1 domain inhibits recruitment of DNA polymerase α . To test if the F2 or F1 peptides blocked CMG helicase activity or Pol- α recruitment, *Xenopus in vitro* replication assays were performed with or without each of these peptides, and sperm chromatin was isolated after an 80-min incubation. The loading of RPA and DNA Pol- α onto sperm chromatin was then assessed by immunoblotting. Incubation with the F2 peptide selectively reduced RPA association, indicating that this domain inhibits CMG helicase activity (Fig. 2C), whereas the F1 peptide impaired the recruitment of DNA Pol- α (Fig. 2D). Neither peptide affected the association of the leading-strand DNA polymerases δ and ϵ with chromatin (Fig. 2D); thus, the inhibitory effect of the F1 peptide on DNA Pol- α recruitment is specific. Notably, the addition of both the F2 and F1 peptides reduced both RPA and DNA Pol- α recruitment (Fig. 2C and D, far right). Thus, the F2 and F1 domains of RbN elicit specific, independent, and additive inhibitory effects on the DNA replication machinery.

The RbN F2 domain triggers accumulation of CMG helicases without affecting assembly. To test if Rb-directed inhibition of CMG helicase activity was due to a failure of the CMG helicase complex to assemble on chromatin, sperm chromatin was incubated with *Xenopus* oocyte extracts in the presence and absence of

F2 or other peptides (Fig. 3A and B) or RbN protein (Fig. 3C). CMG complexes were then allowed to assemble prior to the start of DNA replication, and chromatin was isolated by using low-stringency and high-stringency buffers. Immunoblot analyses demonstrated that none of the peptides blocked the association of CMG components (Mcm2-7 and Mcm10) with chromatin under low-stringency conditions. Rather, incubation with the F2 peptide specifically caused an enrichment of these CMG components on chromatin (Fig. 3A). This effect was even more apparent under high-stringency conditions, where CMG components (Mcm2-7, Mcm10, Cdc45, and Psf2) were tightly retained on chromatin when incubated with F2 peptide (Fig. 3B). Similarly, the addition of the RbN protein to the reaction mixtures caused an enrichment of CMG components on chromatin (Fig. 3C). Note also that RbN caused an enrichment of DNA polymerases δ and ϵ on chromatin (Fig. 1E), although individual peptides from RbN were not able to recapitulate this effect (Fig. 2D). CMG components normally become progressively lost from chromatin during active DNA replication (42), resulting in easier extraction. Given that F2 and RbN block helicase function, the accumulation of CMGs results from an inability to become activated and extracted rather than a gain of additional CMG interactions. Thus, RbN and F2 promote the accumulation of tightly bound and inactive CMG helicase complexes on chromatin.

Loss of the F1 and F2 domains blocks the ability of RbN to inhibit DNA replication. As the F2 and F1 peptides recapitulated the inhibition of DNA replication by RbN, we reasoned that these domains would be necessary for suppressing DNA synthesis. To test this, RbN proteins lacking exon 4, exon 5, exon 7 (F2), exon 9, or the projection/F1 domain (Fig. 4A) were generated and tested for activity in *in vitro* DNA replication assays. The loss of exon 4, exon 5, or exon 9 did not appreciably affect the ability of RbN to

FIG 4 Deletion of the F1 and F2 domains abolishes RbN-mediated suppression of DNA replication, CMG helicases, and DNA Pol- α . (A) Schematic showing structures of in-frame deletion mutants of RbN that were tested. (B) RbN deletion mutants were analyzed for their inhibitory capacity relative to that of wild-type RbN using *in vitro* *Xenopus* oocyte replication assays. All proteins contained GST tags and were purified from bacteria. Equal addition of RbN proteins (100 nM each) was verified by immunoblotting with anti-GST antibody (bottom). Results are averages of data from triplicate samples. Errors are SEM. Significance for mutant RbN data was calculated with reference to wt-RbN results. (C) Sperm chromatin was isolated after *in vitro* DNA replication assays were performed with or without wild-type GST-RbN or the indicated GST-RbN mutant derivatives. Samples were analyzed by immunoblotting for chromatin association of RPA70, RPA32, and DNA Pol- α . Anti-Orc2 immunoblotting was used as a loading control. (D) Replication assays in which RbN proteins were tested alone or added in pairs to rescue partial replication inhibition of single RbN mutants were performed. Equal molar amounts of each protein (100 nM) were added to reaction mixtures. Results are averages of data from triplicate samples. Errors are SEM. Significance was calculated against control results and between results for single RbN mutants and results from combined assays using the same mutant RbN protein.

suppress DNA replication, although the loss of exon 4 or exon 5 somewhat diminished the inhibitory capacity (Fig. 4B). Deletion of F2 or F1 significantly impaired RbN-directed inhibition of DNA replication, which was totally abolished in a deletion mutant lacking both the F2 and F1 domains (Fig. 4B, far right). Thus, the F1 and F2 domains of RbN contribute independent and additive functions in suppressing DNA replication.

To assess if the F1 or F2 domain affected CMG activity or DNA Pol- α recruitment, *in vitro* DNA replication assays were performed in the presence or absence of RbN proteins containing in-frame deletions, chromatin was isolated, and the RPA and DNA Pol- α association was determined by immunoblotting (Fig. 4C). Analysis of Orc2 levels served as a loading control. Wild-type RbN blocked DNA Pol- α recruitment and the association of RPA,

as measured via RPA32 and RPA70 association (Fig. 4C, left), indicating that the CMG helicase was inhibited. RbN proteins lacking exon 4, exon 5, or exon 9 also blocked DNA Pol- α recruitment and CMG helicase activity. Notably, RbN protein lacking the F2 domain blocked DNA Pol- α recruitment but not the association of RPA, whereas RbN protein lacking the F1 domain impaired CMG activity but not DNA Pol- α recruitment (Fig. 4C). Finally, RbN missing both the F1 and F2 domains was completely deficient in blocking RPA and DNA Pol- α recruitment (Fig. 4C, far right), consistent with the dual-deletion mutant being severely deficient for blocking DNA replication. Thus, the F2 and F1 domains of RbN block CMG helicase activity and DNA Pol- α recruitment, respectively.

Given that the F1 and F2 domains provided independent and

FIG 5 The F1 and F2 domains of RbN suppress elongation of DNA replication. (A) RbN deletion mutants and wild-type RbN were analyzed for their ability to inhibit elongation steps during DNA replication. Elongation was measured in the presence of 100 μ M roscovitine in a second nucleus that formed around preinitiated sperm chromatin isolated after DNA replication in the first set of nuclei. RbN proteins were not present before or during DNA replication in the first set of nuclei and were added just prior to nuclear envelope formation in the second set of nuclei. Roscovitine inhibited >80% of DNA replication in the second set of nuclei that formed (data not shown), with the remainder being derived from elongation at established replisomes and normalized to 100% (control, no protein added). Experiments were performed in triplicate, and results were averaged; errors are SEM. Percentages shown are percentages of the control. One-sided *t* test calculations for statistics are shown, comparing mutant RbN results to those for wt-RbN. (B) Elongation assays were performed by using RbN alone; a combination of RbN mutants, each missing F1 or F2; or no protein (control). Equal molar amounts of each protein were used. Results are averages of data from three experiments; errors are SEM. Significance was considered a *P* value of <0.0007 for both comparisons to the control. (C) Elongation assays were performed as described above for panel A but using RbN-derived peptides. Experiments were performed in triplicate, and results were averaged; errors are SEM. Percentages shown are percentages of the no-peptide control. One-sided *t* test calculations for statistics are shown, comparing peptide results to those for the control lacking a peptide.

partial inhibitory functions toward DNA replication, we asked if RbN proteins lacking F1 or F2 could produce full inhibitory function over DNA replication when added together or with RbN. Whereas RbN lacking F1 or F2 again caused only partial suppression of DNA replication, the addition of wild-type RbN alongside RbN- Δ F1 or RbN- Δ F2 resulted in an inhibition of replication similar to that with RbN alone (Fig. 4D). Likewise, the coaddition of RbN- Δ F1 and RbN- Δ F2 resulted in an effective suppression of replication. These results demonstrate that each RbN mutant protein can provide in *trans* an independent and additive inhibitory function, suggesting that multiple RbN molecules can access the replisome to elicit additive inhibitory effects.

The F1 and F2 domains of RbN suppress elongation of DNA replication. RbN has also been reported to inhibit the elongation steps of DNA replication (30). To assess if Rb uses similar mechanisms to inhibit elongation steps, a *Xenopus in vitro* replication

assay was used that specifically measures elongation, which, in contrast to initiation, is Cdk independent (38, 43, 44). Briefly, sperm chromatin was mixed with oocyte extracts and allowed to form nuclei and to begin replicating. Chromatin was then retrieved, which stops ongoing replication activity, and this preinitiated chromatin was transferred to a second oocyte extract containing the Cdk small-molecule inhibitor roscovitine. RbN deletion mutant proteins or peptides were then added to these second extract/chromatin samples. The extracts then formed a new nuclear envelope, and DNA replication resumed from established replisomes.

Consistent with elongation measurements using other approaches (30), RbN efficiently suppresses the elongation of DNA replication (Fig. 5A). The loss of exon 4, exon 5, or exon 9 did not disrupt the ability of RbN to inhibit elongation. In contrast, the loss of the F2 or F1 domain partially impaired this function of

FIG 6 F2 mediates chromatin and Mcm7 binding by RbN, and F1 blocks Ctf4 recruitment. (A) Sperm chromatin was isolated after *in vitro* DNA replication assays were performed in the presence of wild-type GST-RbN or the indicated GST-RbN mutant derivatives. Samples were analyzed by anti-GST immunoblotting for equal inputs of RbN proteins and for chromatin association of the GST-RbN proteins after isolation. Histone H3 probing was used as a chromatin recovery control. (B) *In vitro* protein binding assays were performed with purified GST-RbN proteins bound to glutathione beads in the presence of bacterially expressed His₆-Mcm7. After incubation, beads were recovered, and samples were analyzed by immunoblotting for bound Mcm7 protein with anti-His₆ antibodies. (C) Sperm chromatin was isolated after *in vitro* DNA replication assays were performed with or without RbN-derived peptides. Chromatin was analyzed by immunoblotting for the indicated proteins to assess chromatin recruitment. Actin was used as a loading control. (D) Sperm chromatin was isolated after *in vitro* DNA replication assays were performed with or without wild-type GST-RbN or the indicated GST-RbN mutant derivatives. Samples were analyzed by immunoblotting for chromatin association of Ctf4. Immunoblotting with anti-GST verified equal inputs of GST-RbN proteins in each sample. Histone H3 probing was used as a chromatin recovery control.

RbN, and the deletion of both F1 and F2 abolished the ability of RbN to inhibit elongation (Fig. 5A). As was the case for the initiation reactions (Fig. 4D), the coaddition of RbN-ΔF1 and RbN-ΔF2 resulted in a suppression of elongation similar to that achieved with wt-RbN alone (Fig. 5B), indicating that both mutant proteins provide additive inhibitory functions in *trans*. Furthermore, while exon 4, exon 5, and exon 9 peptides have essentially no inhibitory effect on elongation, the F1 and F2 peptides impaired elongation to at least a modest extent (Fig. 5C) and, when added together, were effective at blocking elongation (Fig. 5C, far right). Thus, the F1 and F2 domains of RbN are necessary and sufficient for inhibiting both the initiation and elongation steps of DNA replication.

F1 and F2 mediate Ctf4 abrogation and chromatin/Mcm7 interactions, respectively, by RbN. To gain further insight into the mechanisms by which F1 and F2 mediate RbN regulation of DNA replication, we next determined if the loss of F1 or F2 disrupted the ability of RbN to interact with chromatin. Wild-type RbN or RbN lacking F1, F2, or both F1 and F2 was incubated with extracts and sperm chromatin templates as described above for replication assays, and after assembly steps, the chromatin was recovered by centrifugation and extracted with detergent buffer to assess RbN chromatin affinity by immunoblotting. The loss of F2 but not F1 significantly reduces the ability of RbN to bind tightly to chromatin (Fig. 6A), and the loss of F2 renders RbN deficient for direct interactions with Mcm7 (Fig. 6B). These results suggest that the inability of RbN-ΔF2 to inhibit the helicases is derived, at least in

part, from a reduced ability of RbN to interact efficiently with helicases on chromatin.

We next assessed whether the F1 domain interfered with other aspects of replisome assembly involved in DNA polymerase α recruitment. Recruitment of DNA polymerase α involves its tethering to replisomes and CMG helicases by Ctf4/And1 (45–48). Sperm chromatin was incubated with extracts in the presence or absence of F1, F2, or exon 5 peptides, and replisomes were allowed to assemble, followed by the isolation of chromatin and extraction with detergent buffer. The inclusion of the F1 peptide again specifically blocked the chromatin association of DNA polymerase α but not DNA polymerase ϵ and also blocked the binding of Ctf4 (Fig. 6C). The inclusion of RbN also blocked the association of Ctf4 with chromatin, and the specific loss of F1 rendered RbN unable to block Ctf4 association (Fig. 6D). We conclude that the F1 domain of RbN interferes with the assembly of replisomes by abrogating the recruitment of DNA polymerase α and the tethering factor Ctf4.

The F1 and F2 domains contribute to antiproliferative functions of Rb, and RbC regulates RbN in a phosphorylation-dependent manner. We next determined whether full-length Rb was dependent on the F1 and F2 domains for suppressing DNA replication. *In vitro* DNA replication assays were performed in the presence or absence of wild-type Rb or Rb lacking F1, F2, or both F1 and F2. Whereas full-length Rb was efficient at inhibiting DNA replication, Rb lacking F1 or F2 was only partially capable of inhibiting DNA replication, and the loss of both F1 and F2 rendered

Rb completely deficient in its inhibitory capacity (Fig. 7A). Thus, in the context of full-length Rb, the F1 and F2 domains contribute functions necessary for inhibiting DNA replication *in vitro*.

It was interesting that the inclusion of the C-terminal domain of Rb (RbC) did not override the diminished inhibitory effects of the loss of F1 and/or F2 in the context of the full-length Rb protein, since it has been shown that RbC alone (containing the large pocket) is capable of inhibiting mammalian cells during S phase (18), and RbC in isolation is tumor suppressive in mouse and culture studies (1, 6). To address this, we determined the effects of RbC (residues 399 to 928) on *in vitro* DNA replication using oocyte extracts. Intriguingly, RbC in isolation is indeed capable of suppressing DNA replication although not as effectively as RbN (compare Fig. 7B and 1B). The mechanism by which RbC achieves replication suppression remains unclear (18) but may contribute to previously reported observations for RbC-induced growth suppression. Nonetheless, the results described above for the more physiologically relevant full-length Rb proteins indicate that even if RbC contains an inhibitory function in isolation, in the context of Rb, RbC does not provide an inhibitory function toward DNA replication. However, as discussed below, RbC provides a regulatory function toward RbN.

The expression of wild-type Rb triggers G₁ arrest in Rb-null SaOS-2 osteosarcoma cells (17, 31). Although hyperphosphorylation impairs the tumor suppressor function of Rb and prevents it from interacting with E2F complexes (49), the forced expression of an Rb protein containing C-terminal phosphomimetic glutamate substitutions at 10 serine or threonine sites (called Rb-M10E) continues, intriguingly, to provoke at least some level of G₁ arrest (31) despite this form of Rb being deficient for E2F binding (49). Notably, the loss of the N terminus abrogates the ability of Rb-M10E to elicit this block to G₁/S transit (31). These results indicate that Rb-M10E remains at least partially capable of inhibiting an unknown target(s) in late G₁, and the N terminus is required. Based on our findings here, we reasoned that N-terminal domains involved in CMG helicase and DNA Pol- α regulation might be required for this proliferative arrest. SaOS-2 cells were transfected with wild-type Rb, Rb-M10E, or Rb-M10E lacking the F1, F2, or F1 and F2 domains. Exogenous protein expression levels for each Rb derivative were similar (Fig. 7C, bottom). As expected for an Rb protein mimicking hyperphosphorylated Rb, Rb-M10E activity was diminished relative to that of wild-type Rb in inducing G₁ arrest (Fig. 7C), and the loss of F1 or F2 individually reduced the ability of Rb-M10E to elicit G₁ arrest. Notably, the combined loss of F1 and F2 abolished the ability of Rb-M10E to induce any level of G₁ arrest (Fig. 7C, far right). These results indicate that the ability of Rb to effectively suppress G₁/S transit requires the presence of an intact N-terminal domain and specifically requires the F1 and F2 domains.

An important reason for using Rb-M10E in the above-described experiment was that the inclusion of the 10 glutamates removed E2F targeting and suppression from the interpretations (49), allowing a focus on the remaining inhibitory effects of Rb derived from its N terminus. Interestingly, although such Rb phosphorylation is generally thought to result in an inactive form of Rb with respect to regulating cell cycle progression, an interpretation of our results was that phosphorylated Rb was active at suppressing cell cycle transit via negative effects on DNA replication. However, we suspected that this was not the case and that the ability of Rb to inhibit DNA replication was likely compromised

by phosphorylation. We reasoned that our ability to observe a G₁ arrest phenotype in SaOS-2 cells with Rb-M10E was potentially a derivative of the overexpression of high levels of Rb-M10E, allowing any remaining G₁ arrest effect derived from F1/F2 to be seen under such experimental conditions with elevated protein levels despite a suppressive set of phosphomimics. In addition, it has been shown that phosphorylated Rb is not effective at suppressing DNA replication in mammalian cells and must be dephosphorylated in order to efficiently elicit S-phase inhibition (22).

Based on the above-described concepts, we predicted that nonphosphorylated Rb was likely the more effective suppressive form of Rb toward DNA replication and that phosphorylation (via glutamate mimics) would diminish this effect. However, we also predicted that higher levels of Rb-M10E might overcome such suppressive modifications of Rb and become inhibitory in direct biochemical analyses. To test this, we used *Xenopus* replication assays to compare the abilities of bacterially purified Rb-M10E and nonphosphorylated Rb to inhibit DNA replication. We show in Fig. 7A that wild-type Rb (nonphosphorylated) was highly effective at inhibiting DNA replication when tested at a concentration of 5 nM. At this concentration, wild-type Rb was again highly effective at suppressing DNA replication, whereas Rb-M10E was not capable of such inhibition (Fig. 7D, left). At the same 5 nM concentrations, Rb-M10E proteins lacking F1, F2, or F1 and F2 were equally ineffective at suppressing DNA replication. However, similar testing of Rb-M10E derivatives at twice the molarity (10 nM) demonstrated that under such biochemical conditions, Rb-M10E began to display inhibitory effects toward DNA replication albeit not as effectively as nonphosphorylated wild-type Rb (Fig. 7D, right). Intriguingly, at a concentration of 10 nM, Rb-M10E lacking F1/F2 remained ineffective at suppressing replication, while mutants lacking F1 or F2 displayed partial effects. The latter results obtained at higher molarities are remarkably similar to those found in our SaOS-2 experiments using Rb-M10E derivatives, indicating that the results obtained by mammalian cell analysis and frog extract testing are consistent when the expression/protein levels of Rb-M10E derivatives are taken into consideration. We conclude that the phosphorylation of Rb on its C terminus diminishes the ability of Rb to function as an effective DNA replication inhibitor and that even if such phosphorylation-derived suppression of Rb is overcome at higher molarities, the ability of Rb to negatively regulate DNA replication clearly remains dependent on the F1 and F2 domains. A corollary of these results is that Rb protein added to extracts is unlikely to be appreciably phosphorylated by embryonic kinases that are present during analysis of DNA replication because such Rb molecules would become ineffective.

A recent study of structural changes in Rb due to C-terminal phosphorylation demonstrated that single phosphorylation of T373 via glutamate substitution caused a conformational change in Rb such that the N-terminal domain folded back onto RbC, resulting in the disruption of E2F interactions (49). Since T373E is one of the 10 sites altered in Rb-M10E, we asked whether the T373E substitution itself contributed to the loss of Rb suppression of DNA replication. Rb-M10E was compared to Rb-M9E (with wild-type T373) and to Rb containing only the single T373E change [Rb(T373E)]. Rb-M9E suppressed Rb inhibitory effects similarly to Rb-M10E (Fig. 7E), indicating that the T373E change is not dominant in abrogating Rb function and that one or more of the remaining C-terminal phosphomimicking changes in Rb-

FIG 7 The F1 and F2 domains of Rb contribute to G₁ cell cycle arrest. (A) *In vitro* replication assays were performed by using bacterially purified full-length Rb that lacked F1, F2, or both, and results were compared to those for wild-type Rb (5 nM in each reaction mixture). Experiments were performed in triplicate, and results were averaged; errors are SEM. (B) Replication assays were performed with bacterially purified RbC titrated into reaction mixtures. Assays were done in triplicate; errors are SEM. Immunoblotting with anti-GST verified the loading of the GST-RbC protein. (C) The ability of Rb proteins containing deletions of F1, F2, or both domains to block G₁/S progression was measured by using a G₁ arrest assay with SaOS-2 cells. Plasmids expressing Rb proteins with 10 phosphomimicking glutamates at C-terminal residues (M10E) or wild-type Rb were cotransfected into asynchronous SaOS-2 cells with a GFP expression vector. After 48 h, cells were collected and analyzed for cell cycle phases by FACS analysis. DAPI (4',6-diamidino-2-phenylindole) staining was visualized after sorting of populations into GFP-positive (transfected) and GFP-negative (nontransfected) cells. The increase in the number of G₁ (2N) cells for each Rb protein expressed is plotted after averaging of results from three independent experiments performed in duplicate; errors are SEM. Data for samples were normalized to values for empty-vector transfections and are graphed as a percentage of the wild-type Rb control, which produced an ~30% increase in G₁ arrest (called 100%). Similar expression levels of HA-tagged Rb proteins were measured by immunoblotting with anti-HA, shown below the graph. (D) *In vitro* replication assays were

M9E cause the loss of Rb inhibitory effects. Interestingly, relative to wild-type Rb, the single change of T373 for glutamate causes a small but statistically significant loss of the inhibitory capacity of Rb (Fig. 7E). Altogether, these results indicate that in the context of full-length Rb, phosphorylation of RbC contributes a negative regulatory role toward the function of RbN in inhibiting DNA replication. Importantly, the change in the Rb structure elicited by T373E (49) can diminish some but not all of the inhibitory capacity of Rb toward DNA replication. The latter result identifies a single phosphorylation site change in Rb that abolishes E2F interactions (49) but leaves intact some of the inhibitory effects of Rb toward DNA replication.

DISCUSSION

An intact N-terminal domain of Rb is required for tumor suppression in transgenic mice (7), and N-terminal loss-of-function in-frame exon deletions are associated with partially penetrant retinoblastoma (8, 10–14). Since C-terminal loss-of-function mutations in Rb produce nearly full penetrant disease, growth-regulatory defects arising from N-terminal in-frame exon deletions of Rb are arguably distinct in function from C-terminal deficiencies. Here our studies establish that the N terminus of Rb is required for blocking the initiation and elongation of DNA replication, specifically by blocking CMG helicase activity and DNA Pol- α recruitment.

Our findings demonstrate that the inhibition of DNA replication by Rb involves functional contributions from two domains of RbN, the F2 and the projection/F1 domains (Fig. 7F). Both domains are necessary and sufficient for the suppression of DNA replication, and both domains act independently of one another and are additive in blocking DNA initiation and elongation. The F2 domain is required to inhibit CMG helicase activity during initiation and elongation steps, while the projection/F1 domain prevents the proper association of DNA Pol- α and Ctf4 with replisomes. These functions are manifest in cell-free biochemical systems, and the F1/F2 domains are required for the *in vivo* regulation of G₁/S transit by Rb. The precise biochemical mechanisms by which F2 and F1 elicit their effects are unknown. The projection/F1 domain adopts a protrusion structure (10), which we speculate may sterically block or disrupt DNA Pol- α and Ctf4 interactions with replisomes. The CMG helicase has several activities, including ATP hydrolysis and helicase strand separation functions, which may be disabled by interactions with the F2 domain. Consistent with this, the loss of F2 renders RbN less efficient at binding to replicating chromatin and the Mcm7 subunit. Alternatively, the F2 domain may act indirectly, for example, by preventing the association of a CMG-activating factor during initiation steps. This would seem unlikely, however, since the F2 domain is capable of inhibiting the CMG helicase even during elongation.

The partial loss of the inhibitory capacity of RbN in blocking DNA replication in the absence of the F2 domain mirrors the

biological effects of the in-frame deletion in Rb exon 7 on the partial penetrance of this allele in provoking cancer (14). Similarly, studies with transgenic mice have shown that an in-frame N-terminal loss of a portion of Rb (Rb Δ N1 mutant), which overlaps almost all of Ex7/F2, also generates partially penetrant phenotypes in tumor incidence and in defects in embryonic development (7). Our findings provide explanations for the partial-penetrance effect of Rb alleles with Ex7/F2 loss. For example, the F2 domain encoded by exon 7 is required only for CMG control, and the F1 domain retained in this allele would partially harness DNA replication via polymerase control. Furthermore, the control of CMG activity may not be critical for all biological processes involving Rb, where CMG control by Rb at G₁/S may be less necessary to harness tumorigenesis, while CMG regulation may be required of Rb for the control of replication following DNA damage (21, 22). Finally, there could be compensation by Rb family members, where p107 and p130 share significant sequence similarity with Rb in their N-terminal regions (1, 6) and where p107 has been shown to bind to the replicative helicase (28).

Human cancer-associated missense mutations and in-frame deletions are also manifest in the Rb projection/F1 domain and neighboring exons. For example, exon 6 is mutated in prostate cancers (15) and retinoblastomas (10), and exons 4 and 5 are lost in inherited retinoblastomas (8, 11, 13). Such alterations might produce subtle effects on structural features of the F1 domain that partially promote tumor development, as proposed previously (10). Furthermore, previous studies using transgenic mice have shown that a larger deletion encompassing exons 4 and 5 and part of the projection/F1 domain (Rb Δ N4 mutant) has partially penetrant activity in promoting tumor development (7). Thus, as underscored by our studies, both the F1 and F2 domains play important roles in tumor suppression by Rb.

Much of the results obtained here are derived from testing Rb functions in embryonic egg extracts, and as such, a degree of caution must be reserved for our interpretations. Although this frog extract system is a powerful tool for analyzing metazoan DNA replication mechanisms and is currently the only practical means of assessing *in vitro* cell-free biochemical functions of Rb toward higher eukaryotic DNA replication activities, important caveats must be considered. By nature, the embryonic system is biologically different from somatic mammalian cells, having fewer limitations on DNA replication control than somatic cells. For example, transcription does not occur in early embryonic cell cycles (50), Rb is not present (32, 39), and cell cycles occur without intervening G₁/G₂ phases (50). The duration of early embryonic S phase is $\sim 1/10$ the duration of somatic S phase (51), and DNA replication is highly efficient, being able to replicate many DNA substrates, even those in the absence of chromatin (52). However, the basic enzymatic machinery involved in replicating DNA in these extracts is highly conserved with both mammalian and yeast

performed to compare the inhibitory effects of bacterially purified wt-Rb (full length and nonphosphorylated) to purified Rb proteins with 10 glutamates on the C terminus (M10E derivatives). Rb-M10E variants also lacked F1, F2, or both, as indicated. The Rb protein levels tested were 5 nM (left graph) or 10 nM (right graph) final concentrations. Equal Rb protein levels were verified, as shown below each graph (anti-GST immunoblotting). Assays were done with 3 to 6 replicates, and results were averaged; errors are SEM. *P* values (all <0.01) were obtained by using one-sided statistical testing. (E) Replication assays were performed to compare the inhibitory effects of wt-Rb to those of Rb-M10E, Rb-M9E (same glutamate substitutions as M10E except that T373 is normal), and Rb(T373E) (single glutamate at residue 373). Protein loading (5 nM each) using anti-GST immunoblotting is shown. Assays were done in triplicate, and results were averaged; errors are SEM. (F) Model proposed based on the findings of this study.

counterparts and is subject to similar kinase and other regulatory activities (42, 44, 53–55). Intriguingly, it is arguably a physiological benefit for these early embryonic S phases not to have endogenous Rb protein present, which might limit the replicative capacity of embryonic cells during a time of necessary rapid cell cycle division and embryonic growth.

Despite the above-mentioned limitations, there is a remarkable number of similarities between the findings obtained for Rb control of DNA replication in mammalian somatic cells and results obtained here by using biochemical testing with metazoan frog extracts. Rb has long been known to provide a negative function in regulating DNA replication in mammalian cells (17, 21, 22, 27, 28). Although the molecular nature of this Rb inhibitory function toward DNA replication has remained elusive, it has been shown to be independent of E2F regulation and occurs during S phase and late G₁ phase (17–19, 21, 22, 27). Rb (by way of RbN) makes direct physical contacts with the replicative CMG helicase *in vitro* and *in vivo* (27, 28). An interaction between Rb and the CMG helicase occurs in late G₁ phase (27), and evidence suggests that Rb can target the mammalian helicase under certain conditions during S phase (22). Intriguingly, this Rb-CMG interaction is required for the inhibition of the mammalian helicase *in vivo* in response to growth-inhibitory signals from TGF- β 1 (27). Such Rb-inhibited CMG helicases that are intact but nonfunctional accumulate on chromatin in mammalian cells (27), much the same as results obtained here by using frog extracts, where RbN-inhibited CMG helicases accumulate in an inactive but intact state. In addition, Rb mediation of TGF- β 1 arrest in late G₁ is also associated with a failure of the replicative polymerases to become associated with chromatin (27), similar to what was found in the extract system. RbN in particular has been shown to mediate a G₁/S transit block in mammalian cells (6, 31), consistent with RbN having a primary inhibitory function toward DNA replication in extract assays in the context of full-length Rb. Finally, the ability of Rb phosphorylation to suppress the inhibitory function of Rb toward DNA replication *in vitro* is consistent with findings in mammalian cells, where Rb inhibits S-phase progression following dephosphorylation by PP2A (22). In the latter situation, dephosphorylated Rb becomes associated with regions of chromosomes where MCMs/origins reside and DNA replication is cosuppressed (22). These consistencies strongly suggest that data from biochemical analyses of Rb/RbN using the frog extract system are likely of physiological relevance to the mechanisms used by Rb in mammalian cells to control DNA replication.

It is important to keep in mind that the temporal interaction of Rb with the helicase or other aspects of the replication machinery in mammalian cells is likely more complex than that illustrated here using biochemical assays with embryonic extracts. For example, it is possible that Rb interacts with somatic helicases in G₁ phase prior to its heightened late-G₁-phase phosphorylation and that some phosphorylation on Rb may occur without disrupting preformed Rb-CMG interactions until further sites are modified. Indeed, progressive phosphorylation on specific Rb residues is known to regulate E2F interactions with Rb, some of which prevent E2F binding, while others disrupt prebound E2F (49). Our approach here necessarily involved testing of prephosphorylated (mimicked) Rb derivatives, overexpressed transiently or added directly to replicative machinery as it formed, and thus, our methods cannot distinguish these possibilities. Caveats appreciated, our work provides an initial framework for understanding the

complexities by which Rb negatively regulates metazoan CMG helicases and has elucidated two functional domains (F1 and F2) within the Rb amino-terminal region that mediate Rb control over DNA replication.

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