ATR and ATRIP: Partners in Checkpoint Signaling

David Cortez,[1,2] Saritha Guntuku,[1,2] Jun Qin,[1,3] Stephen J. Elledge[1,2,4]*

The checkpoint kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) transduce genomic stress signals to halt cell cycle progression and promote DNA repair. We report the identification of an ATR-interacting protein (ATRIP) that is phosphorylated by ATR, regulates ATR expression, and is an essential component of the DNA damage checkpoint pathway. ATR and ATRIP both localize to intranuclear foci after DNA damage or inhibition of replication. Deletion of ATR mediated by the Cre recombinase caused the loss of ATR and ATRIP expression, loss of DNA damage checkpoint responses, and cell death. Therefore, ATR is essential for the viability of human somatic cells. Small interfering RNA directed against ATRIP caused the loss of both ATRIP and ATR expression and the loss of checkpoint responses to DNA damage. Thus, ATRIP and ATR are mutually dependent partners in cell cycle checkpoint signaling pathways.

ATR is a member of the phosphatidylinositol kinase–related protein family that includes ATM. These kinases are essential for signaling the presence of DNA damage or replication blocks and activating cell cycle checkpoints (*1*, *2*). ATR is the sequence and functional homolog of the Rad3 and Mec1 checkpoint proteins from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively (*3*, *4*).

The function of ATM has been extensively studied in cell lines derived from ataxia telangiectasia patients that lack expression of the ATM protein. The lack of comparable cell lines for ATR has impaired analysis of its specific activities. Overexpression of catalytically inactive versions of ATR indicates that it is required for checkpoint responses after treatment of cells with agents that cause various forms of DNA damage or block replication (*5*–*8*). Furthermore, homozygous deletion of ATR in mice causes early embryonic lethality, suggesting that ATR has essential functions during development (*9*, *10*).

Rad3 and Mec1 function in cooperation with the Rad26 and DDC2 (also called LCD1 or PIE1) proteins, respectively (*11*–*14*). Rad26 binds to and is phosphorylated by Rad3, whereas DDC2 binds to and is phosphorylated by Mec1. Mutations in either Rad3 or Rad26 yield almost identical phenotypes, as do mutations in either Mec1 or DDC2. As yet, the functional roles of Rad26 and DDC2 are unclear. However, Rad3 and DDC2 are essential for transducing checkpoint signals to downstream proteins such as the Chk1 protein kinase (*11*–*14*).

In a search for substrates of ATM and ATR, we noticed that a protein with an apparent molecular size of 85 to 90 kD immunoprecipitated with ATR and incorporated ^{32}P when ATR immunoprecipitates were incubated with [γ-^{32}P]adenosine triphosphate ([γ-^{32}P]ATP) (Fig. 1A). To identify this phosphoprotein, we transiently overexpressed ATR tagged with a Flag epitope in 293T cells, immunoprecipitated the expressed ATR, and sequenced the coimmunoprecipitating proteins by mass spectrometry (Fig. 1B). In addition to peptides from heat shock protein 70, we identified two peptides, DSLHQTESVLEEQR and DTVLLLHGLSQK (*15*), that corresponded to two expressed sequence tags (ESTs) in the GenBank database. By comparison of overlapping cDNAs and genomic sequence, we assigned both EST sequences to the same gene and designed primers to amplify and clone a full-length cDNA. Sequencing of the cloned cDNA indicated that it encodes a 791–amino acid protein with a predicted molecular size of 86 kD containing a coiled-coil domain near its NH_2-terminus. We named this protein ATRIP for ATR-interacting protein.

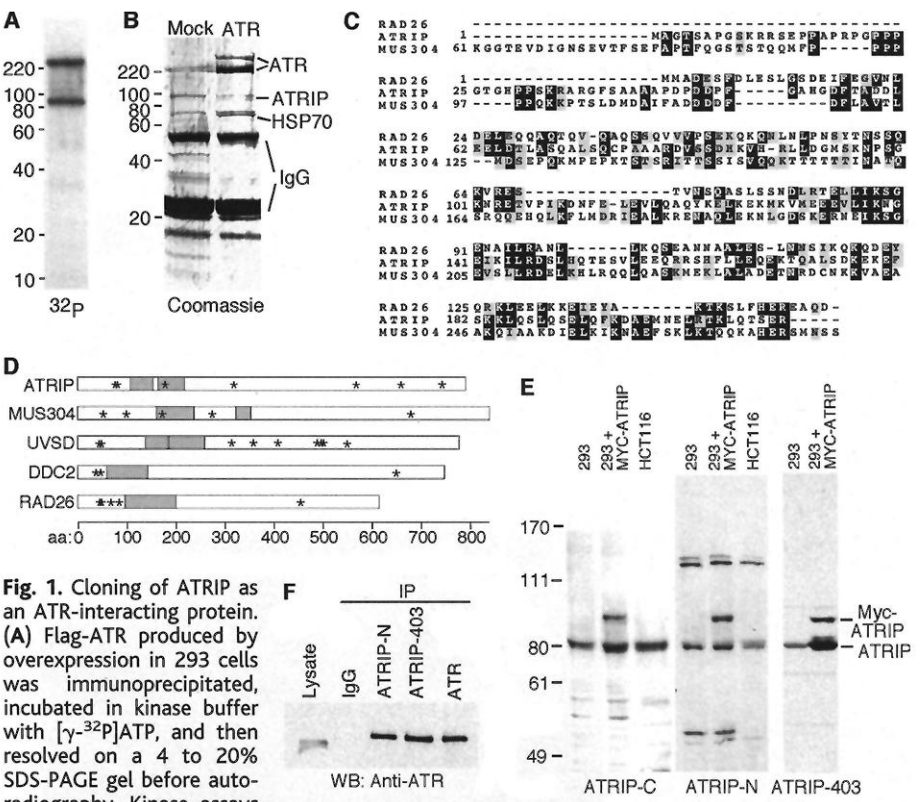

Fig. 1. Cloning of ATRIP as an ATR-interacting protein. **(A)** Flag-ATR produced by overexpression in 293 cells was immunoprecipitated, incubated in kinase buffer with [γ-^{32}P]ATP, and then resolved on a 4 to 20% SDS-PAGE gel before autoradiography. Kinase assays were performed as described (*21*). **(B)** Antibody to Flag immunoprecipitates from equal amounts of 293 cell lysate from either mock or Flag-ATR–transfected cells were resolved by SDS-PAGE and stained with Coomassie blue. Protein bands were trypsinized, and the recovered peptides were identified by mass spectrometry, as described (*22*). IgG, immunoglobulin G. **(C)** Alignment of ATRIP with *S. pombe* Rad26 protein and *D. melanogaster* Mus304 protein (*15*). **(D)** Schematic representation of Rad26 family members. Shaded boxes indicate predicted coiled-coil domains. Asterisks represent S/TQ locations that are potential phosphorylation sites for ATR or ATM. **(E)** Western blot analysis was done with three rabbit polyclonal antibodies to ATRIP on cell lysates from 293, HCT116, or 293 cells transfected with a CMV-Myc(3X)-ATRIP expression plasmid. **(F)** 293 cell lysates were immunoprecipitated with the indicated antibodies to either ATRIP or ATR and immunoblotted with antibodies to ATR.

[1]Verna and Mars McLean Department of Biochemistry and Molecular Biology, [2]Howard Hughes Medical Institute, [3]Department of Cell Biology, [4]Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.

*To whom correspondence should be addressed.

Blast searches revealed sequence similarity to the *Drosophila melanogaster* mus304 protein—a protein implicated in DNA damage checkpoint signal transduction (*16*). ATRIP also has weak sequence similarity to Rad26, DDC2, and *Aspergilllus nidulans* UVSD proteins (Fig. 1C). Each of these proteins contains a coiled-coil domain near the NH_2-terminus (Fig. 1D). RNA blotting indicated that ATRIP is expressed in all tissues tested (*17*). We also identified an alternatively spliced exon encoding amino acids 658 to 684 near the COOH-terminus. Reverse transcription polymerase chain reaction from two cell lines indicated that both forms were expressed. The significance of the alternative splicing is unknown.

Cotransfection of Myc(3x)-tagged ATRIP cDNA with Flag-tagged ATR followed by reciprocal coimmunoprecipitation confirmed that the overexpressed proteins could associate in vivo (*17*). We produced antibodies to peptides from the NH_2-terminus (ATRIP-N) and COOH-terminus (ATRIP-C) of ATRIP and polyclonal antiserum to GST-ATRIP (amino acids 1 to 107) purified from bacteria (ATRIP-403). Each of these antibodies recognized a protein with an apparent size between 80 and 85 kD in lysates of human cells separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and a slightly larger protein in cells transfected with a Myc-ATRIP expression vector (Fig. 1E). The ATRIP-N and ATRIP-403 antibodies both coimmunoprecipitated ATR from 293T cell lysates (Fig. 1F). Under similar conditions, we have not detected an association of ATRIP with ATM (*17*). Mapping of the ATRIP domain that binds ATR indicated that there may be multiple interaction domains including the coiled-coil domain between amino acids 107 and 214 (*18*).

To confirm that ATRIP is the phosphoprotein that we originally immunoprecipitated with ATR, we analyzed whether ATRIP is an ATR substrate. Western blotting of cell lysates that were treated with phosphatase revealed a change in ATRIP migration on SDS-PAGE gels, suggesting that ATRIP is phosphorylated in vivo (Fig. 2A). The 85-kD protein phosphorylated during incubation with immunoprecipitated ATR comigrated with ATRIP detected by the ATRIP-N antibody (*17*). Furthermore, kinase assays with Flag-ATR immunoprecipitated from cells that also expressed Myc-ATRIP revealed ^{32}P-labeling of coimmunoprecipitated Myc-ATRIP (Fig. 2B). Finally, recombinant full-length ATRIP isolated from baculovirus-infected insect cells and an NH_2-terminal fragment of ATRIP isolated from bacteria could both be phosphorylated when placed in a kinase reaction with ATR isolated from human cells (Fig. 2C). These results suggest that the 85-kD protein that is phosphorylated in ATR kinase reactions is indeed ATRIP, although we cannot exclude the possibility that a second substrate or kinase with the same apparent size immunoprecipitated with ATR.

ATR localizes to intranuclear foci that may correspond to sites of DNA synthesis and repair after cells have been treated with agents that cause DNA damage or stalling of replication forks (*8*). Immunostaining with the ATRIP-403 antibody revealed that ATRIP is a diffuse nuclear protein that also redistributes to intranuclear foci after treatment of cells with ultraviolet (UV) radiation, hydroxyurea (HU), or ionizing radiation (Fig. 2D) (*17*). Costaining with an antibody to ATR showed strong colocalization of ATRIP and ATR after treatment of cells with UV or HU. Expression of a green fluorescent protein (GFP)–ATRIP fusion revealed similar intranuclear localization patterns (*17*), and small interfering RNA (siRNA) inhibition of ATRIP expression indicated that the ATRIP-403 antibody specifically recognizes ATRIP in stained cells.

To further elucidate the function of ATR and determine if ATRIP phosphorylation and localization in vivo are dependent on ATR, we created a conditional ATR-null cell line ($ATR^{flox/-}$) in which one allele of ATR is disrupted by the neomycin resistance gene and the second "flox" allele has lox sites flanking exon 2 (*18*). Before Cre expression, the amount of ATR protein expressed by the $ATR^{flox/-}$ cells was about 20% of that in wild-type cells. This may indicate that insertion of the lox sites partially disrupts proper transcription or splicing of this allele. After infection with adenovirus encoding Cre (Ad-Cre), exon 2 was deleted and the amount of ATR protein rapidly declined (Fig. 3A) (*18*).

We found that the amounts of ATRIP protein were proportional to those of ATR. ATRIP was expressed at about 20% of the

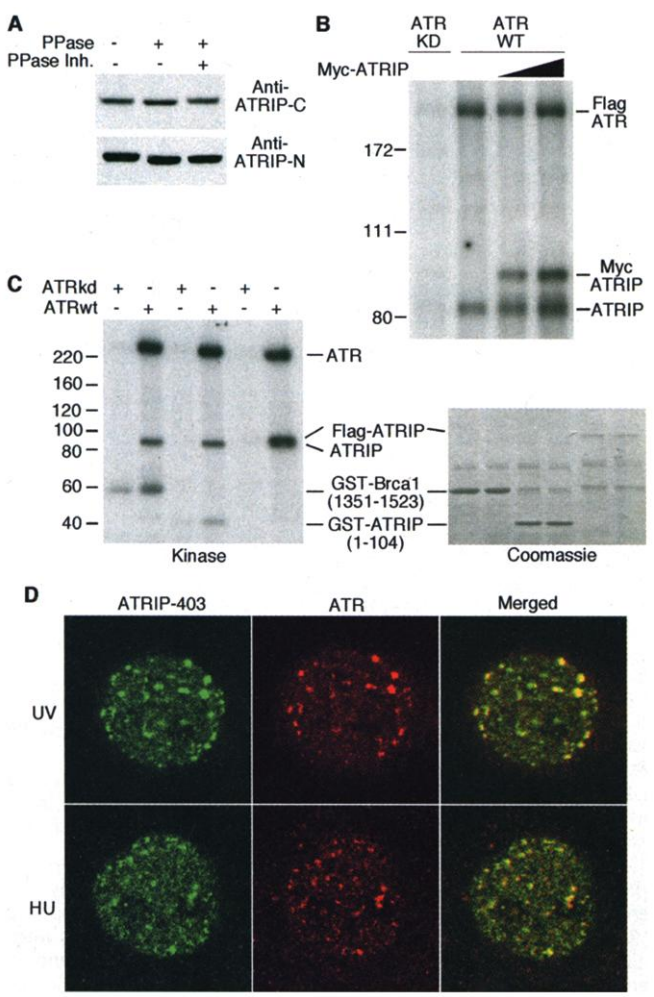

Fig. 2. ATRIP is an ATR substrate and colocalizes with ATR to intranuclear foci after DNA damage or replication blocks. (**A**) Mock-treated, phosphatase-treated, or phosphatase + phosphatase inhibitor–treated 293 cell lysates were immunoblotted with ATRIP-N or ATRIP-C antibodies. (**B**) 293 cells transfected with either Flag-tagged catalytically inactive ATR or wild-type ATR expression vectors as well as various amounts of Myc-ATRIP expression vector where indicated. Flag immunoprecipitates were incubated in kinase buffer with [γ-^{32}P]ATP and then resolved on a 4 to 20% SDS-PAGE gel before autoradiography. (**C**) Recombinant Brca1 or ATRIP fragments were purified from *Escherichia coli* or full-length Flag-ATRIP protein purified from insect cells after baculovirus infection was incubated in kinase buffer containing [γ-^{32}P]ATP and either wild-type or catalytically inactive Flag-ATR immunoprecipitates. The kinase reaction was resolved by SDS-PAGE before staining with Coomassie blue and autoradiography. (**D**) HeLa cells exposed to UV light (40 J/m^2) or 2 mM HU for 5 hours were fixed with paraformaldehyde, permeabalized with Triton X-100, and then immunostained with goat polyclonal antibody to ATR antibody and rabbit polyclonal ATRIP-403 antibody. After incubation with appropriate Cy3- and fluorescein isothiocyanate (FITC)–conjugated secondary antibodies, fluorescent images were captured on a confocal microscope. Each image represents a single section of the cell nucleus.

wild-type amount in $ATR^{flox/-}$ cells. Less ATR and ATRIP were expressed after Ad-Cre infection to delete the flox allele (Fig. 3A). Stable expression of Flag-ATR in the $ATR^{flox/-}$ cell line restored ATR and ATRIP expression to about wild-type levels (Fig. 3B). The loss of ATRIP expression after deletion of ATR precluded further analysis of phosphorylation and localization dependencies.

The loss of ATR protein from the $ATR^{flox/-}$ cells was maximal at day 4 after Ad-Cre infection. By day 6 after infection, ATR levels actually increased, and by day 10, they had returned to the starting levels (*17*). We also noticed that the $ATR^{flox/-}$ cells lost viability starting at day 5 after Cre infection, whereas $ATR^{+/+}$ cells infected with the same amount of virus showed no signs of toxicity. Therefore, we suspected that deletion of ATR caused cell death and that cells that had not been infected by the Ad-Cre virus eventually overtook the culture. On day 6 after infection, 28% of the cells underwent apoptosis, as indicated by a less than 2n DNA content (Fig. 3C). Plating efficiency of the Ad-Cre–infected $ATR^{flox/-}$ cells was reduced by about 85 to 90% compared with Ad-GFP–infected $ATR^{flox/-}$ cells. Finally, when we analyzed the genotype of the surviving colonies derived from the Ad-Cre–infected cells, we found that 60 out of 60 clones analyzed retained ATR exon 2, indicating that they had not undergone Cre-mediated recombination (*18*). In contrast, 90% of the surviving colonies derived from limiting dilution plating of Ad-Cre–infected $ATR^{flox/-}$ cells that stably expressed the Flag-ATR cDNA had deleted exon 2. These results indicate that ATR is an essential gene in this human somatic cell line.

To study the function of ATRIP, we used siRNA to inhibit its expression. Reduction of ATRIP expression also resulted in less ATR expression. Both Western blotting and immunolocalization analysis revealed that ATRIP is required for continued expression of the ATR protein (Fig. 4, A and B). ATR RNA levels were unaffected by transfection of siRNA targeting ATRIP (Fig. 4C). Three independent, 21–base pair RNA duplexes targeting ATRIP yielded similar results, whereas two control duplexes had no effect on either ATRIP or ATR expression (*17*). Therefore, ATRIP and ATR are mutually dependent on each other for expression.

We were unable to reduce the amount of ATRIP in HeLa cells below 15 to 20% of that in wild-type cells. This amount of reduction yielded no detectable checkpoint defects, which was not unexpected because about the same amount of protein is observed in the $ATR^{flox/-}$ cells, which also have no detectable checkpoint defects before excision of exon 2. Therefore, we used the siRNA method to interfere with ATRIP expression in the

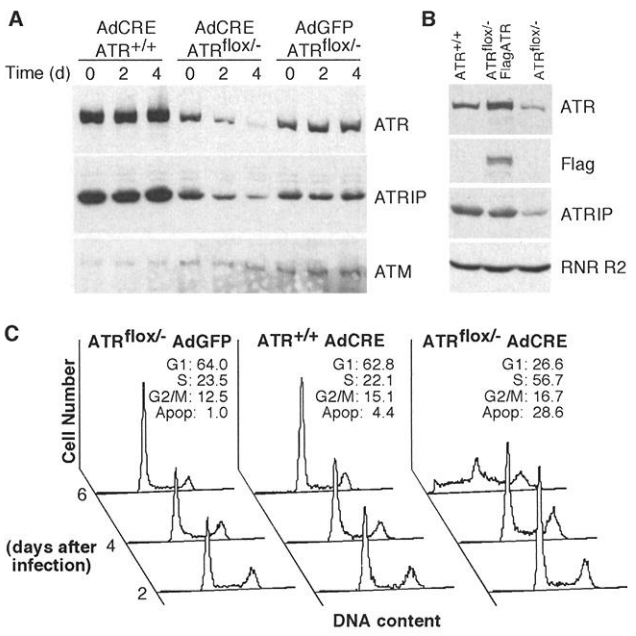

Fig. 3. Requirement of ATR for cell viability and ATRIP expression. (**A**) Lysates prepared from the indicated cells after infection with adenovirus encoding either the Cre recombinase or GFP were separated by SDS-PAGE, blotted, and probed with the indicated antibodies. (**B**) $ATR^{flox/-}$ cells were complemented with Flag-ATR by transfection of CMV-Flag-ATR (in pCDNA3.1Zeo; Invitrogen) and selection of a stable clonal cell line. Lysates from the indicated cell lines were blotted with antibodies for ATR, Flag, ATRIP, and the R2 subunit of ribonucleotide reductase. (**C**) $ATR^{+/+}$ or $ATR^{flox/-}$ cells were infected with Ad-Cre or Ad-GFP virus. DNA content was measured by flow cytometric analysis of propidium iodine–stained cells at the indicated times after infection.

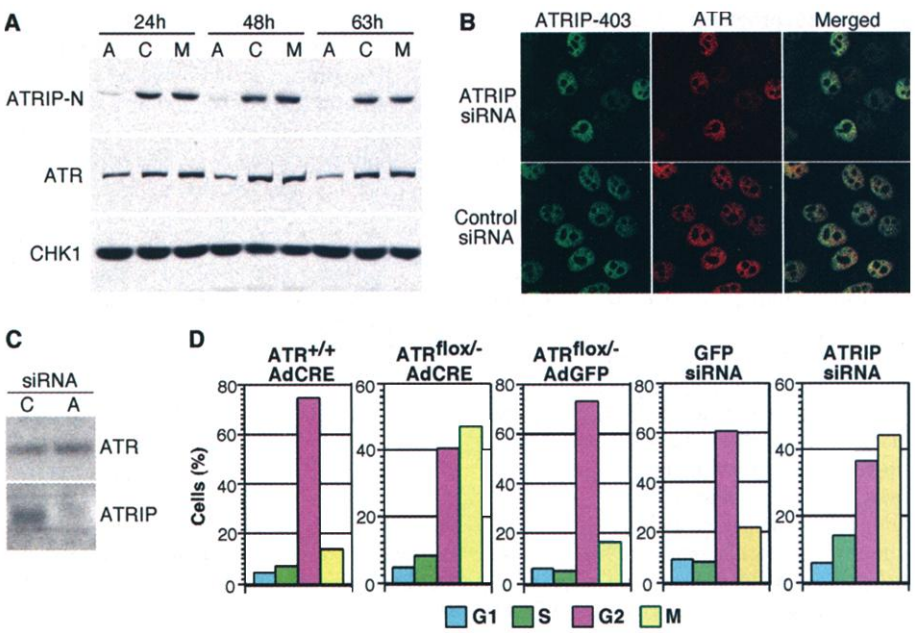

Fig. 4. Requirement of ATRIP for ATR expression and the G_2-M DNA damage checkpoint. HeLa cells were transfected with 21-nucleotide siRNAs targeting ATRIP (A) or GFP (C), or they were mock transfected (M) (*23*, *24*). (**A**) Immunoblots of cell lysates prepared at the indicated times after transfection were performed with antibodies directed against ATRIP, ATR, or CHK1. (**B**) The transfected cells were fixed and stained with antibodies against ATRIP and ATR and the appropriate FITC- and Cy3-conjugated secondary antibodies. (**C**) Total RNA from transfected cells was separated by electrophoresis, blotted onto nitrocellulose, and probed with portions of either the ATR or ATRIP cDNA. (**D**) $ATR^{flox/-}$ or $ATR^{+/+}$ cells were infected with Ad-GFP or Ad-Cre viruses, or $ATR^{flox/-}$ cells were transfected with siRNAs targeting ATRIP or GFP three times over a 3-day period. Three days after infection or the initial transfection, the cells were exposed to 8 Gy of γ-irradiation, and nocodazole (1 μg/ml) was added to the medium. Sixteen hours after irradiation, the cells were harvested, stained with propidium iodide for DNA content analysis with flow cytometry, or fixed with Camoy's fixative. The percentage of mitotic cells was determined by counting 600 DAPI-stained cells. The percentage of G_2 cells was determined by subtracting the percentage of cells in mitosis from the percentage of cells in G_2-M as determined by flow cytometry. Alternatively, the percentage of cells that were in M phase was determined by staining with propidium iodide and antibody to phospho-histone H3 (Cell Signaling) followed by FITC-conjugated secondary antibody, and the percentage of G_1, S, G_2, and M phase cells was determined by flow cytometry (*25*). Phospho-histone H3 staining and DAPI staining of mitotic figures yielded similar percentages of mitotic cells in multiple experiments.

$ATR^{flox/-}$ cells, which already had reduced ATRIP expression. Transfection of siRNA in HCT116 cells effectively reduced ATRIP expression (*17*). Transfection of control siRNAs in $ATR^{flox/-}$ cells, Ad-Cre infection of $ATR^{+/+}$ cells, or Ad-GFP infection of $ATR^{flox/-}$ cells had no effect on the ability of these cells to delay entry into mitosis after ionizing radiation. However, transfection of siRNAs against ATRIP yielded a profound γ-irradiation–induced G_2-M checkpoint defect that was similar to that seen in the $ATR^{flox/-}$ cells treated with Ad-Cre (Fig. 4D). About 40% of Cre-infected or ATRIP siRNA-transfected $ATR^{flox/-}$ cells enter mitosis 16 hours after irradiation compared with 20% of control cells. These results are consistent with checkpoint defects of cells overexpressing catalytically inactive ATR protein (*6*). Thus, ATR and ATRIP are essential for a normal DNA-damage–induced delay of mitosis initiated by ionizing radiation.

These data strongly suggest that ATRIP is the functional human homolog of the Rad26 family of genes. ATRIP associates with ATR, is a substrate of ATR in vitro and a phosphoprotein in vivo, and colocalizes with ATR to sites of DNA synthesis and repair after treatment of cells with DNA-damaging agents or replication inhibitors. Furthermore, interference with ATRIP function generates the same G_2-M checkpoint defect as observed after deletion of ATR. ATRIP expression is dependent on ATR, and ATR expression is dependent on ATRIP. This mutual dependency for expression suggests that the amount of ATR and ATRIP in cells is tightly coordinated and may indicate that these proteins form a stable complex with each other at a fixed stoichiometry.

ATR function is required for the viability of undamaged, proliferating cells and in cells exposed to DNA-damaging agents. In this respect, ATR is similar to MEC1, which is essential for viability because of difficulties in the proper coordination of DNA replication (*19*, *20*). We did observe an increase in the percentage of S phase cells after Ad-Cre infection of the $ATR^{flox/-}$ cells (see Fig. 3C), perhaps reflecting a requirement for ATR and ATRIP signaling to ensure successful DNA replication.

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15. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Two Essential DNA Polymerases at the Bacterial Replication Fork

Etienne Dervyn,[1] Catherine Suski,[1] Richard Daniel,[2] Claude Bruand,[1] Jérome Chapuis,[1] Jeff Errington,[2] Laurent Jannière,[1] S. Dusko Ehrlich[1]*

DNA replication in bacteria is carried out by a multiprotein complex, which is thought to contain only one essential DNA polymerase, specified by the *dnaE* gene in *Escherichia coli* and the *polC* gene in *Bacillus subtilis*. *Bacillus subtilis* genome analysis has revealed another DNA polymerase gene, $dnaE_{BS}$, which is homologous to *dnaE*. We show that, in *B. subtilis*, $dnaE_{BS}$ is essential for cell viability and for the elongation step of DNA replication, as is *polC*, and we conclude that there are two different essential DNA polymerases at the replication fork of *B. subtilis*, as was previously observed in eukaryotes. $dnaE_{BS}$ appears to be involved in the synthesis of the lagging DNA strand and to be associated with the replication factory, which suggests that two different polymerases carry out synthesis of the two DNA strands in *B. subtilis* and in many other bacteria that contain both *polC* and *dnaE* genes.

The paradigm of the bacterial replication fork is that of *Escherichia coli* (*1*). The fork includes the DNA polymerase III holoenzyme, which contains 10 different subunits. One of the subunits, α, is the catalytic DNA polymerase. There are two α molecules in the holoenzyme, each of which copies a different DNA strand. The holoenzyme from *B. subtilis* is not as well characterized, but was reported to contain a similar number of polypeptides, including a catalytic DNA polymerase subunit (*2*). The *E. coli* and *B. subtilis* catalytic subunits of the holoenzyme, encoded by *dnaE* and *polC* genes, respectively, are prototypes of two different DNA polymerase classes within the so-called family C, which groups replicate DNA polymerases from eubacteria (*3*). The *B. subtilis* genome sequence (*4*) indicated the existence of two other genes encoding family C DNA polymerases, in addition to *polC*, both of the *E. coli* class. One of these genes, *yorI*, is carried on a prophage that can be eliminated from *B. subtilis* (*5*) and thus is not essential for cell growth. The other gene, referred to here as $dnaE_{BS}$, is encoded chromosomally. Genes homologous to *polC* and $dnaE_{BS}$ are present in all fully sequenced genomes of bacteria belonging to the *Bacillus/Clostridium* group and in the genome of a thermophilic microorganism species called *Thermotoga maritima*. $dnaE_{BS}$ encodes a protein with DNA polymerase activity (*6*), as does its homolog from *Streptococcus pyogenes* (*7*).

The *polC* gene is essential for *B. subtilis* cell growth (*8*). We show here that $dnaE_{BS}$ is also essential. First, inactivation of the gene by recombination with an insertional plasmid vector, pMUTIN (*9*), carrying an internal segment of the gene, was not successful. The failure was not due to a polar effect of insertion, which we know because a downstream

[1]Génétique Microbienne, Institut National de la Recherche Agronomique, Jouy-en-Josas, 78352 Cedex, France. [2]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, UK.

*To whom correspondence should be addressed. E-mail: ehrlich@jouy.mra.fr