Artemis Regulates Cell Cycle Recovery from the S Phase Checkpoint by Promoting Degradation of Cyclin E*§

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Artemis, a member of the SNM1 gene family, is a known phosphorylation target of ATM, ATR, and DNA-PKcs. We have previously identified two serine residues in Artemis (Ser516 and Ser645) that are subject to phosphorylation by ATM and are involved in mediating recovery from the G2/M checkpoint in response to ionizing radiation. Here we show that these same sites are also phosphorylated by ATR in response to various types of replication stress including UV, aphidicolin, and hydroxyurea. We also show that mutation of the Ser516 and Ser645 residues causes a prolonged S phase checkpoint recovery after treatment with UV or aphidicolin, and that this delayed recovery process coincides with a prolonged stabilization of cyclin E and down-regulation of Cdk2 kinase activity. Furthermore, we show that Artemis interacts with the F-box protein Fbw7, and that this interaction regulates cyclin E degradation through the SCF/Fbw7 E3 ubiquitin ligase complex. The interaction between Artemis and Fbw7 is regulated by phosphorylation of Ser516 and Ser645 sites that occur in response to replication stress. Thus, our findings suggest a novel pathway of recovery from the S phase checkpoint in that in response to replication stress phosphorylation of Artemis by ATR enhances its interaction with Fbw7, which in turn promotes ubiquitylation and the ultimate degradation of cyclin E.

As an arm of the DNA damage response cell cycle checkpoints maintain genomic stability by allowing time for DNA repair processing to be completed before resumption of the cell cycle (1). Much progress has been made on the elucidation of the mechanisms that lead to the detection of structural alterations in DNA and the implementation of checkpoint pathways, however, the processes by which the cell cycle reinitiates are not as well understood. Resumption of cell cycle progress after genotoxic stress is usually referred to as the recovery process. Recent findings have shown that recovery is an active process and not simply an attenuation of the initial checkpoint response (2). Perhaps the best studied recovery process is the resumption of the cell cycle from the G2 checkpoint. In this mechanism the kinase Plk1 mediates degradation of Claspin, an activator of Chk1, and Wee1, a negative regulator of Cdk1 (3–5). Phosphorylation of these two substrates leads to ubiquitylation by the SCF⁷TrCP E3 ligase, and ultimate degradation by the proteosome (6, 7). Recently it has been shown that Plk1 is activated to promote recovery by phosphorylation of its Thr210 residue by the Aurora A kinase (8), although, how completion of DNA repair results in activation of Aurora A is not clear. Other mechanisms to induce resumption of the cell cycle include dephosphorylation of p53, Chk1, and γH2AX, and the induction of Cdc25B (9–12).

Cdk2 and its partners cyclin E and cyclin A are crucial regulators of G1/S transition and progression through S phase. Cyclin E accumulates in late G1 as a result of E2F-mediated transcriptional regulation, which has been previously activated by cyclin D-associated kinases. During S phase cyclin E is degraded by two independent pathways. Cyclin E unbound to Cdk2 is targeted by the Cul3-based E3 ubiquitin ligase (13), whereas Cdk2-bound cyclin E is targeted by the SCF/Fbw7 ubiquitin ligase in a process that requires phosphorylation of cyclin E (14–21). Interestingly, ectopic overexpression of cyclin E has been shown to accelerate entry into S phase, but somewhat paradoxically also slows progression through S phase (22–25). One possible mechanism by which overexpression of cyclin E prolongs S phase is an interference with pre-RC assembly during G1, which ultimately leads to lower levels of replication initiation (26).

Artemis is a member of the SNM1 gene family that is characterized by conserved metallo-β-lactamase and β-CASP domains (27). Artemis has roles in V(D)J recombination, non-homologous end-joining mediated repair of double strand breaks, and in cell cycle regulation after DNA damage (27–32). It is a known substrate both in vitro and in vivo of the phosphatidylinositol kinases ATM, ATR, and DNA-PK in response to many types of genotoxic agents (28, 30, 32–35). With regard to its cell cycle functions, we have shown previously that Artemis is involved in regulating the recovery from the G2 checkpoint in response to ionizing radiation (IR) through regulation of the activation of cyclin B-Cdk1 (28, 32). Furthermore, Artemis was shown to be phosphorylated on its Ser⁵¹⁶ and Ser⁶⁴⁵ residues by ATM, and mutation of these two sites to alanine led to a slower recovery from the G2/M checkpoint.

In prior work (32) we have also shown that Artemis is phosphorylated in vivo in response to UV irradiation, however, the

* The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; IR, ionizing radiation; siRNA, small interfering RNA; IP, immunoprecipitation; HA, hemagglutinin; PBS, phosphate-buffered saline; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter.

† The on-line version of this article (available at http://www.jbc.org) contains supplementary Figs. S1–S3.

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plasms were gifts from B. Clurman (Fred Hutchinson Cancer Research Center, Seattle, WA). Artemis siRNAs were previously described (32). Cyclin E (1214563-H/904; 1214564) and ATR (1214725/747; 121426-H) siRNAs were purchased from Sigma.

**Immunoblotting, Immunoprecipitation, and IP Kinase Assays**—Immunoblotting, immunoprecipitation (IP), and IP kinase assays have been previously described (32). Briefly, cell lysates were prepared in RIPA buffer with protease inhibitors and 10 mM sodium fluoride, 0.2 mM sodium vanadate, 8 mM β-glycerophosphate, and 1 mM dithiothreitol. Cell lysates were then incubated with primary antibody for 2 h and protein A/G beads for an additional 45 min at 4°C. IP kinase assays were carried out in 10 mM MgCl₂, 1 mM dithiothreitol, 70 mM NaCl, 10 μM ATP, 0.1 μg/μl histone H1 (Millipore), and 5 μCi of [γ-32P]ATP (PerkinElmer Life Sciences) at room temperature for 30 min.

**In Vivo Ubiquitylation Assay**—pCMV-HA-ubiquitin was transfected into HEK293 cells to facilitate detection of ubiquitin conjugates. Transfection efficiency was monitored by cotransfection of 100 ng of pEGFP-N1 (Clontech) plasmid. Twenty-four h after transfection cells were treated with MG-132 for 4 h and cell lysates were prepared for immunoblotting.

**Flow Cytometry and BrdUrd Labeling**—Cell cycle analysis by flow cytometry and BrdUrd labeling were previously described (28). Briefly, cells were incubated with 10 μM BrdUrd for 15 or 30 min prior to harvesting, or released into regular medium and harvested at various time points. Cells were fixed in 70% ethanol and incubated in PBS containing 4% bovine serum albumin and 0.2% Triton X-100 for 1 h at room temperature. DNA was denatured in 2 N HCl for 20 min, washed with PBS, and resuspended in 0.1 M sodium tetraborate. Cells were exposed to monoclonal anti-BrdUrd (BD Transduction Laboratories) for 1 h, washed with PBS, and incubated with fluorescein isothiocyanate-conjugated goat antibodies to mouse IgG (Jackson Laboratories) for 30 min. After washing with PBS, cells were resuspended in PBS containing 40 μg/ml propidium iodide, and 50 μg/ml DNase-free RNase (Calbiochem). Fluorescence was measured on a FACS Calibur flow cytometer (BD Biosciences) using 488 nm laser excitation.

**RESULTS**

Artemis Is Phosphorylated by ATR on Ser^{516} and Ser^{645} in Response to Replication Stress—We have shown previously that in response to IR Artemis is rapidly phosphorylated on four serine residues, namely Ser^{516}, Ser^{534}, Ser^{538}, and Ser^{645} (28). Artemis phosphorylation on Ser^{516} and Ser^{645} is dependent on ATR. HEK293 cells stably expressing GST-Artemis were transfected with ATR or control siRNA, and 48 h later treated with UV (3 J/m²). Cells were harvested 2 h later for analysis.

![Artemis is phosphorylated by ATR on residues Ser^{516} and Ser^{645} in response to replication stress](image)

**EXPERIMENTAL PROCEDURES**

**Cells and Antibodies**—HEK293 cells stably expressing Artemis alleles, tissue culture protocols, and DNA transfections were previously described (28). Mouse monoclonal antibody against cyclin E (sc-247), and rabbit polyclonal antibodies to cyclin E (sc-481), Cdk2 (sc-163), and cyclin A (sc-751) were purchased from Santa Cruz Biotechnology, Inc. Antibodies to Chk1 and phosphorylated Chk1 (pS345) were obtained from Cell Signal Technology. ATR antibodies were described previously (32). FLAG (M2)–conjugated beads were purchased from Sigma. Artemis and Artemis phospho-specific antibodies were previously described (28).

**Plasmids and siRNAs**—Wild-type and mutant Artemis constructs were previously described (28). FLAG-Fbw7 expression constructs were previously described (32). FLAG-Fbw7 expression vectors and siRNAs were purchased from Sigma.

**In Vivo Ubiquitylation Assay**—pCMV-HA-ubiquitin was transfected into HEK293 cells to facilitate detection of ubiquitin conjugates. Transfection efficiency was monitored by cotransfection of 100 ng of pEGFP-N1 (Clontech) plasmid. Twenty-four h after transfection cells were treated with MG-132 for 4 h and cell lysates were prepared for immunoblotting.

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**RESULTS**

Artemis Is Phosphorylated by ATR on Ser^{516} and Ser^{645} in Response to Replication Stress—We have shown previously that in response to IR Artemis is rapidly phosphorylated on four serine residues, namely Ser^{516}, Ser^{534}, Ser^{538}, and Ser^{645} (28).
Two of these sites, Ser\textsuperscript{534} and Ser\textsuperscript{538}, undergo rapid phosphorylation and dephosphorylation within 1–2 h after exposure to DNA damage, whereas the other two sites, Ser\textsuperscript{516} and Ser\textsuperscript{645}, exhibit rapid and prolonged phosphorylation for up to approximately 24 h. We have also previously shown that Artemis is phosphorylated in response to UV irradiation (32), we therefore examined the kinetics of these phosphorylation events in response to UV. Phosphorylation at Ser\textsuperscript{516} and Ser\textsuperscript{645} was observable at 30 min, peaked from 2 to 12 h, and then slowly declined until 24 h (Fig. 1A). Rapid and prolonged phosphorylation of Artemis at Ser\textsuperscript{516} and Ser\textsuperscript{645} was also observed in vivo in response to aphidicolin and hydroxyurea (Fig. 1B and data not shown). Thus similar to IR, Artemis also exhibits rapid and prolonged phosphorylation at Ser\textsuperscript{516} and Ser\textsuperscript{645} in response to replication stress.

We have also shown previously that ATM was the principal kinase for phosphorylation of Ser\textsuperscript{516} and Ser\textsuperscript{645} in response to IR (28). To determine the kinase responsible for UV-induced phosphorylation at these two sites, we examined their phosphorylation after UV in the presence of caffeine, an inhibitor of ATM and ATR. Reduced phosphorylation was observed in the cells treated with caffeine (Fig. 1C). To further identify the kinase, we used siRNA to deplete ATR, and then examined the phosphorylation at each site. siRNA-mediated knockdown of ATR significantly reduced the phosphorylation at each site (Fig. 1D). These results indicate that ATR is the major kinase responsible for Artemis phosphorylation at the Ser\textsuperscript{516} and Ser\textsuperscript{645} sites in response to UV.

**Mutation of Ser\textsuperscript{516} and Ser\textsuperscript{645} Results in Prolonged S Phase Arrest**—We previously reported that HEK293 cells expressing a mutant of Artemis in which the serines at Ser\textsuperscript{516} and Ser\textsuperscript{645} had been mutated to alanine residues exhibited defective recovery from the G\textsubscript{2}/M cell cycle checkpoint after IR (28, 32). Because UV induces a significant S phase arrest, we examined this Artemis mutant (termed S516A/S645A) to determine whether these two phosphorylation sites also affect the recovery from the S phase checkpoint. As shown (Fig. 2A), cells expressing the S516A/S645A mutant showed an accumulation of cells in S phase 12–18 h after UV treatment compared with cells expressing wild-type Artemis. In addition, expression of a mutant Artemis (S516D/S645D) in which the serines at 516 and 645 were mutated to aspartic acid residues to mimic phosphorylation, resulted in an essentially wild-type phenotype. To further support these results, cells were incubated with BrdUrd for 15 min, treated with UV, and returned to regular medium. Twelve h later cells expressing the S516A/S645A mutant exhibited a higher S phase content compared with cells expressing wild-type Artemis or the S516D/S645D mutant (Fig. 2B).

To ensure that the accumulation in S phase observed after UV treatment was not the result of effects in the G\textsubscript{1} or G\textsubscript{2}/M stages of the cell cycle, we synchronized cells at early S phase by treatment with aphidicolin, and then released them into regular medium without drug. At 12 h after release a greater accumulation was observed in the G\textsubscript{1}/S phase in cells expressing the S516A/S645A mutant compared with wild-type Artemis or the S516D/S645D mutant (Fig. 2C). In addition, we repeated this experiment, but introduced a 30-min pulse labeling of the cells with BrdUrd immediately before harvesting. Again this experiment showed that the S phase arrest was not due to effects in the G\textsubscript{1} or G\textsubscript{2}/M stages of the cell cycle. In contrast, cells expressing the S516A/S645A mutant exhibited a higher S phase content compared with cells expressing wild-type Artemis or the S516D/S645D mutant (Fig. 2D).

**FIGURE 2.** Mutation of Ser\textsuperscript{516} and Ser\textsuperscript{645} results in prolonged S phase arrest in response to replication stress. **A**, cell cycle analysis of HEK293 cells after UV treatment. Cells were treated with UV (3 J/m\textsuperscript{2}), incubated for the indicated times, and analyzed by FACS. **B**, same as in **A** except cells were pulse labeled with BrdUrd (15 min) immediately before UV irradiation. **C**, cell cycle analysis of HEK293 cells after aphidicolin treatment. HEK293 cells expressing wild-type (WT), S516A/S645A or S516D/S645D mutants Artemis were treated with aphidicolin for 14 h. Cells were then released into regular medium and harvested at the indicated times. **D**, same as in **C** except cells were pulse labeled with BrdUrd for 30 min prior to harvesting.
iment indicated that a greater fraction of cells expressing the S516A/S645A mutant were in S phase compared with the control cell lines (Fig. 2D). Finally, we compared cells expressing the S516A/S645A mutant with those expressing the S516D/S645D mutant and found that in the absence of DNA damage the former did not exhibit a prolonged S phase (Fig. S1). Taken together, these results demonstrate that phosphorylation of Ser516 and Ser645 of Artemis facilitates recovery from the replication checkpoint in response to DNA damage.

The target of the S phase checkpoint is the cell cycle kinase Cdk2. Using an IP kinase assay we found that the activity of this enzyme was reduced in cells expressing the S516A/S645A mutant particularly at 12 h after UV exposure when the greatest levels of S phase accumulation were observed (Fig. 3A). Thus, the Cdk2 kinase, which is principally responsible for driving cells through S phase, has reduced activity in cells expressing the S516A/S645A Artemis mutant.

Cyclin E and cyclin A are the principal cyclins that regulate Cdk2 activity during the S to G2 transition (38–40). Ectopic overexpression of cyclin E in specific cell lines is known to cause a delayed progression through S phase (22–25), and previous studies have shown that replication stress causes an increase in cyclin E levels and a slowing of DNA replication (41). We, therefore, examined cyclin E protein levels in cells expressing wild-type Artemis, and the S516A/S645A and S516D/S645D mutants after UV irradiation or release from aphidicolin. We observed significantly higher cyclin E levels in cells expressing the S516A/S645A mutant, whereas cyclin A levels were not significantly different (Fig. 3B). Overexpression of cyclin E could slow S phase progression by competing with cyclin A for binding to Cdk2. An examination of the interaction between cyclin A and Cdk2 by co-IP analysis showed that less cyclin A was bound to Cdk2 in cells expressing the S516A/S645A mutant (Fig. 3C). Thus a likely explanation for our findings is that the S516A/S645A mutant pre-
Depletion of Artemis stabilizes cyclin E and delays recovery from the S phase checkpoint.

A, depletion of Artemis from HEK293 cells prolongs S phase arrest in response to replication stress. HEK293 cells were transfected with the indicated siRNAs. Forty h later they were treated with aphidicolin (2 μg/ml) for 14 h, released for the indicated times, and subsequently analyzed by FACS using MultiCycle software (Phoenix Flow Systems, San Diego, CA) to quantitate cell cycle distributions. Graphical analysis of the percentages of cell cycle phases is displayed in the lower panels. B, immunoblot analysis of Artemis depletion mediated by siRNA. C, depletion of Artemis up-regulates cyclin E expression. HEK293 cells were treated as described in A and subsequently examined by immunoblot analysis. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Artemis Depletion Stabilizes Cyclin E and Delays Recovery from the S Phase Checkpoint—As shown above, HEK293 cells that overexpress the S516A/S645A Artemis mutant exhibit a delayed progression through S phase after replication stress. To directly demonstrate that Artemis is required for normal recovery from the S phase checkpoint, we depleted endogenous Artemis by siRNA, exposed the cells to aphidicolin for 14 h, and then released them into regular medium. As shown (Fig. 4, A and B), depletion of Artemis resulted in a slower recovery from the aphidicolin treatment similar to that observed with overexpression of the S516A/S645A mutant. We next determined whether depletion of Artemis also resulted in stabilization of cyclin E as observed above with overexpression of the S516A/S645A mutant. Upon release from aphidicolin cyclin E is strongly stabilized in cells depleted of Artemis compared with cells treated with a control siRNA (Fig. 4C). These findings suggest that Artemis is required for degradation of cyclin E during recovery from the replication stress checkpoint, and that the S516A/S645A mutant acts as a dominant negative in this pathway.

Artemis Regulates Cyclin E Abundance through SCF\(^{Fbw7}\)-mediated Ubiquitylation—To elucidate the mechanism by which Artemis affects cyclin E abundance, we first examined the effect of the S516A/S645A mutation on cyclin E protein stability. Cycloheximide was added to HEK293 cells stably expressing wild-type Artemis or the S516A/S645A mutant 30 min before treatment with UV. Samples were collected at the indicated times and cyclin E levels were determined by immunoblotting (Fig. 5A). The Artemis S516A/S645A mutant exhibited a reduced cyclin E turnover compared with wild-type Artemis. Because cyclin E is degraded through ubiquitin-mediated proteolysis, we next assessed the ubiquitylation of cyclin E in vivo. In the presence of the proteasome inhibitor MG-132, levels of ubiquitinated cyclin E were far lower in cells expressing the S516A/S645A mutant compared with cells expressing wild-type Artemis (Fig. 5B). This result demonstrates that ubiquitylation of cyclin E is impaired by the expression of the S516A/S645A Artemis mutant.

Cyclin E is a substrate of the Skp1-Cul1-F-box (SCF) E3 ubiquitin ligase complex, and the F-box protein or substrate receptor for cyclin E is Fbw7/Cdc4 (16). Because Artemis affects cyclin E ubiquitylation in vivo we examined whether Artemis interacts with Fbw7. Fbw7 has three isoforms α, β, and γ, two of which, α and γ, have been implicated in the ubiquitylation of cyclin E, although the functions of these isoforms are distinctly different (21). To assay for an interaction between Artemis and Fbw7, we cotransfected HEK293 cells with GST-Artemis and FLAG-Fbw7 α, β, or γ isoforms, and performed reciprocal co-IPs. Interestingly, Artemis co-IPed with the Fbw7 α and γ isoforms, but not with the β isoform (Fig. 5C), which indicates that the interaction is specific for the former two isoforms. Because the phosphorylation state of Artemis affects the stabilization of cyclin E, we examined the interaction between Artemis and the Fbw7 α and γ isoforms after UV irradiation. As shown (Fig. 5D), UV irradiation increased the co-IP between these proteins. Furthermore, prior treatment of the cells with wortmannin decreased the interaction suggesting that phosphorylation of Artemis helped to promote the interaction with Fbw7 α and γ. To confirm this possibility, the co-IP wild-type and S516A/S645A or S516D/S645D isoforms, but not with the

Depletion of Artemis stabilizes cyclin E and delays recovery from the S phase checkpoint.
mutants of Artemis with Fbw7 α and γ were compared. As shown (Fig. 5E), after UV irradiation the wild-type protein interacted more efficiently than did the S516A/S645A mutant protein (upper panel). Interestingly, the S516D/S645D mutant appeared to exhibit an enhanced interaction particularly in the untreated samples (lower panel). Note, however, that phosphorylation of Artemis is not necessary for the interaction between Fbw7 isoforms and cyclin E (Fig. S3), suggesting that Artemis is required subsequent to cyclin E recruitment.

Finally, as shown above (Fig. 1), ATR is required for the phosphorylation of Artemis at Ser516 and Ser645 in response to replication stress. We, therefore, examined whether ATR affected the interaction between Artemis and Fbw7 isoforms, and whether it affected the ubiquitylation of cyclin E. As shown (Fig. 6, A and B), in both cases depletion of ATR by siRNA reduced Artemis-Fbw7 interactions and reduced cyclin E ubiquitylation to levels observed with the expression of the S516A/S645A mutant after exposure to UV irradiation. Taken together, these findings indicate that Artemis is involved in the ubiquitylation of cyclin E by the SCFFbw7 E3 ligase through an interaction with the α and γ isoforms of Fbw7, and that phosphorylation of Artemis on residues Ser516 and Ser645 by ATR positively regulates this association.

DISCUSSION

As we and others have shown previously Artemis is a substrate of both ATM and ATR, and is phosphorylated by at least one of these kinases in response to various types of genotoxic stress (28, 30, 32–35, 42–45). In this report we demonstrate that Artemis is phosphorylated at residues Ser516 and Ser645 in response to UV irradiation or aphidicolin exposure by the ATR kinase. Cells that express a mutant of Artemis with these residues converted to alanine exhibit a pro-

FIGURE 5. Artemis regulates cyclin E stability via SCFFbw7-mediated ubiquitylation. A, Artemis phosphorylation affects cyclin E stabilization at the post-translational level. HEK293 cells expressing wild-type or the S516A/S645A mutant were irradiated with UV (3 J/m²) in the presence of 25 μg/ml cycloheximide (CHX), and harvested at the indicated times for immunoblot analysis. C indicates no treatment. B, Artemis phosphorylation regulates ubiquitylation of cyclin E. HEK293 cells expressing wild-type Artemis or the S516A/S645A mutant were transfected with HA-ubiquitin and 24 h later irradiated with UV. Cells were incubated for an additional 4 h with or without MG-132 (10 μM), and then harvested for immunoblot analysis. Enhanced green fluorescent protein (EGFP) was co-transfected with HA-ubiquitin as an internal control for transfection efficiency. C, Artemis interacts with Fbw7 isoforms in vivo. HEK293 cells were transfected with GST-Artemis and FLAG-Fbw7 isoforms and reciprocal co-IPs were performed using GST (upper panel) or FLAG (lower panel) antibodies as indicated. GST-β-glucuronidase (GUS) was used as negative control. D, the interaction between Artemis and Fbw7 isoforms is enhanced after UV irradiation. HEK293 cells expressing GST-Artemis were transfected with FLAG-Fbw7 isoforms. Twenty-four h later cells were mock treated or irradiated with UV at the indicated doses, and harvested for co-IP experiments after an additional 1.5 h of incubation. Wortmannin (2.5 μM) was added 20 min before UV irradiation. E, Artemis phosphorylation is required for the interaction between Artemis and Fbw7 isoforms. HEK293 cells were transfected with GST-tagged Artemis wild-type, S516A/S645A or S516D/S645D mutants, and FLAG-Fbw7 isoforms. Cells were treated with UV and harvested at the indicated time points for co-IP experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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A

GST-Artemis + + + +
Flag-Fbw7α + + + +
Flag-Fbw7γ + + + +
ATR siRNA + + + +
Control siRNA + + + +

IP: Flag

GST
Flag
ARTEMIS
GAPDH

B

Artemis-wt + + + +
Artemis-S516/645A
ATR siRNA + + + +
Control siRNA + + + +

Ub-Cyclin E
Cyclin E
GAPDH
EGFP
ATR

Figure 6. ATR is required for the Artemis-mediated degradation of cyclin E. A, depletion of ATR by siRNA reduces the interaction between Artemis and Fbw7 isoforms α and γ. HEK293 cells were transfected with GST-Artemis and with either control or ATR siRNA. Forty-eight h later cells were transfected with UV (30 J/m²), incubated for 1.5 h, and harvested for co-IP assays. B, depletion of ATR reduces ubiquitylation of cyclin E by wild-type Artemis. HEK293 cells expressing wild-type Artemis or the S516A/S645A mutant were transfected with HA-ubiquitin and the indicated siRNA, and 24 h later irradiated with UV. Cells were incubated for an additional 4 h with MG-132 (10 µM), and then harvested for immunoblot analysis. Enhanced green fluorescent protein (EGFP) was co-transfected with HA-ubiquitin as an internal control for transfection efficiency. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

longed S phase and a failure to recover normally from the S phase checkpoint. There are a number of possible mechanisms that could lead to this phenotype. One is that a failure to repair UV-induced lesion would lead to a prolonged checkpoint response. However, whereas Artemis has been reported to have a role in the repair of double strand breaks, no such function has been ascribed for Artemis in the repair of photolyses. Furthermore, Artemis-deficient cells are not sensitive to fork blocking adducts such as those induced by mitomycin C or etoposide (31, 35). In addition, our results indicate that there is not a prolonged activation of Chk1 in cells expressing the S516A/S645A mutant. Rather, our results indicate that the abnormal recovery from the S phase checkpoint is due to stabilization of cyclin E that preferentially occurred in cells expressing the mutant form of Artemis in comparison to the wild-type protein. Previous studies have shown that ectopic overexpression of cyclin E results in a more rapid transition from the G1 to S phase, but a slower progression through S phase (22–25). Thus, the abnormal stabilization of cyclin E observed due to expression of the S516A/S645A mutant likely accounts for the failure of cells to properly recover from the S phase checkpoint, and indicates that phosphorylation of Artemis at Ser516 and Ser645 is necessary for degradation of cyclin E after genotoxic stress. Depletion of Artemis by siRNA also resulted in the same phenotypic consequences, thus adding support for this model. Our results further show that the reduced degradation of cyclin E during S phase checkpoint recovery in cells expressing the S516A/S645A mutant results in impaired Cdk2 activity concomitant with a reduced interaction between Cdk2 and cyclin A. Thus, high levels of cyclin E appear to interfere with the association between Cdk2 and cyclin A, which is required for reinitiation of DNA replication during checkpoint recovery.

Cyclin E is ubiquitinated and ultimately degraded during S phase by the SCF<sup>Fbw7</sup> E3 ligase complex and the 26 S proteosome (16). Of the three alternative splice isoforms of Fbw7 only two, α and γ, participate in the ubiquitylation of cyclin E, isoforms with this mutant protein were reduced, whereas the interactions appeared to be enhanced in cells expressing the S516D/S645D mutant. Nevertheless, the degradation of cyclin E was not enhanced by the S516D/S645D mutant, thus suggesting that Artemis is necessary, but not sufficient, for triggering checkpoint recovery. This is a reasonable scenario because phosphorylation of Artemis occurs rapidly after UV irradiation, whereas the degradation of cyclin E during checkpoint recovery occurs hours later. Thus, our findings indicate that in addition to its well recognized role in the initiation of the replication checkpoint, ATR, via Artemis, also regulates recovery from the checkpoint. This mechanism is highly similar to our published findings showing that ATM regulates recovery from the G<sub>2</sub>/M checkpoint after IR via phosphorylation of Artemis (28, 32).

A trivial explanation for these findings, namely that Artemis is a substrate of the SCF<sup>Fbw7</sup> complex and that its overexpression competes with cyclin E is not supported by our results. Overexpression of wild-type Artemis causes increased ubiquitylation of cyclin E and increased interaction with Fbw7α and Fbw7γ, results, which are contrary to the predictions of this model. Rather, Artemis appears to interact with the SCF<sup>Fbw7</sup> complex to promote ubiquitylation of cyclin E, perhaps acting as a substrate specificity factor to enhance rapid degradation of cyclin E during checkpoint recovery. This model is further supported by our finding that DNA damage-induced phosphorylation of Artemis by ATR enhances its interaction with Fbw7 and the mult ubiquitylation of cyclin E. Interestingly, it has recently been shown that cyclin E is a direct phosphorylation substrate of ATR in response to DNA damage indicating that ATR may regulate cyclin E through multiple pathways (46).

Artemis has, in cooperation with DNA-PKcs, a defined role in the cleavage of DNA hairpins at coding joints during V(D)J recombination (29, 44, 47). We have previously shown that Artemis also functions in the recovery from the G<sub>2</sub>/M checkpoint after IR treatment via regulation of the activation of cyclin B-Cdk1 (28), which showed initially that Artemis has a role in...
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cell cycle regulation. Here we have demonstrated an additional function for Artemis in the recovery from the S phase checkpoint via regulation of cyclin E. Furthermore, we show that the mechanism of this regulation of cyclin E by Artemis is as an interacting partner of the SCFbw7 E3 ligase complex. Taken together, these findings define a dramatically novel role for Artemis as a component of the ubiquitin-proteosome system.

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