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HCLK2 Is Required for Activity of the DNA Damage Response Kinase ATR*[S]

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ATR is a protein kinase that orchestrates the cellular response to replication problems and DNA damage. HCLK2 has previously been reported to stabilize ATR and Chk1. Here we provide evidence that human HCLK2 acts at an early step in the ATR signaling pathway and contributes to full-scale activation of ATR kinase activity. We show that HCLK2 forms a complex with ATR-ATRIP and the ATR activator TopBP1. We demonstrate that HCLK2-induced ATR kinase activity toward substrates requires TopBP1 and vice versa and provides evidence that HCLK2 facilitates efficient ATR-TopBP1 association. Consistent with its role in ATR activation, HCLK2 depletion severely impaired phosphorylation of multiple ATR targets including Chk1, Nbs1, and Smc1 after DNA damage. We show that HCLK2 is required for and stimulates ATR autophosphorylation and activity toward different substrates *in vitro*. Furthermore, HCLK2 depletion abrogated the G_2 checkpoint and decreased survival of cells after exposure to DNA damaging agents and replicative stress. Overall, our data suggest that HCLK2 facilitates ATR activation and, therefore, contributes to ATR-mediated checkpoint signaling. Importantly, our results suggest that HCLK2 functions in the same pathway as TopBP1 but that the two proteins regulate different steps in ATR activation.

In response to DNA damage and replication stress, genomic integrity is protected by coordinated activation of the genome surveillance pathways, including cell cycle checkpoints, DNA repair, and chromatin remodeling (1). The DNA damage checkpoint cascade involves several unique and overlapping factors, classified as sensors, mediators, transducer, and effectors, that upon activation lead to the spatiotemporal assembly of multiprotein complexes at the sites of damage (2). Failure in checkpoint signaling can result in genome instability and predispose to cancer. DNA damage checkpoints operate at the G_1-S and G_2-M transitions and within the S phase of the cell cycle (3). The master regulators of the checkpoints are the two phosphoinositide 3-kinase-related protein kinases ataxia-telangiectasia mutated (ATM)[3] and ataxia-telangietascia and Rad3-related (ATR). Although ATM is mainly important for the response to DNA double-stranded breaks, ATR responds to a wide variety of DNA damage lesions and replication stress. ATR is activated by single-stranded DNA, which can be generated by replication intermediates or during processing of DNA lesions. The single-stranded DNA is recognized and coated by replication protein A, which subsequently recruits the ATR-ATRIP complex (4, 5).

Upon activation, ATR phosphorylates various downstream targets including Smc1, Nbs1, and Chk1, which in turn are responsible for coordinating cell cycle progression and DNA repair (4, 6). ATR activation and phosphorylation of targets has been shown to require DNA topoisomerase II-binding protein 1 (TopBP1). In addition, phosphorylation of the downstream effector kinase Chk1 requires another mediator, Claspin (6–8). Activation of Chk1 is crucial for efficient genome surveillance during S or G_2 phase of the cell cycle. Chk1 phosphorylates and inhibits the activity of the Cdc25 phosphatases. Cdc25s regulate cell cycle progression by activating cyclin-dependent kinases. Inhibition of Cdc25s has been implicated in the control of G_1, S, and G_2 DNA damage checkpoints (9).

Recently, much attention has been drawn to HCLK2 and its orthologs. It is an essential gene conserved throughout eukaryotes. Evidence from several organisms has indicated a role of HCLK2 and its orthologs in the DNA damage response. In *Caenorhabditis elegans*, two mutants (rad-5 and clk-2) were isolated with increased sensitivity to DNA-damaging agents and prolonged life span, respectively (10, 11). Later, it was shown that *rad-5* was allelic to *clk-2*, and cloning of the gene revealed that it encodes a protein homologous to *Saccharomyces cerevisiae* Tel2 and human HCLK2 (12, 13). Clk-2 is not required for recruitment of ATL-1 (*C. elegans* ATR) to sites of damage, which is consistent with what has later been shown in human cells (14, 15). In human cells a physical association between HCLK2 and ATR-ATRIP and Chk1 has been identi-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S8.

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[3] The abbreviations used are: ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangietascia and Rad3-related; TopBP1, topoisomerase II-binding protein 1; Gy, gray; sh-, short hairpin; siRNA, small interfering RNA; GST, glutathione *S*-transferase.

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fied, and HCLK2 was shown to be critical for the S-phase checkpoint possibly via stabilization of Chk1. In addition, depletion of HCLK2 impaired monoubiquitination of FANCD2 and homologous recombination (15). The *Schizosaccharomyces pombe* HCLK2 ortholog (Tel2p) is required for activation of the Mrc1 (a Claspin homologue)-mediated checkpoint in response to hydroxyurea, and in *S. cerevisiae* tel2 mutants have short telomeres, and Tel2 has been suggested to play a role in the activation and localization of Tel1 (ATM) to double-stranded breaks (16, 17). HCLK2/Tel2 has been shown to interact with the family of phosphatidylinositol 3-kinase-like kinases (18, 19), and in human and mouse this interaction has been suggested to stabilize the phosphoinositide 3-kinase-related protein kinases, thereby providing another layer of HCLK2-mediated regulation of the DDR (18).

Here, we report that in addition to previous findings, HCLK2 modulates the ATR-mediated checkpoint signaling very rapidly, already before an effect on ATR protein levels can be observed. This is accompanied by a decrease in ATR autophosphorylation and activity toward substrates in the absence of HCLK2. We demonstrate that HCLK2-induced ATR activity requires TopBP1 and vice versa. However, ATR autophosphorylation appears independent on TopBP1. Furthermore, we identify the region of HCLK2 required for association with ATR and TopBP1. Our data indicates that HCLK2 modulates ATR activity by promoting ATR-TopBP1 interaction and, thus, support the emerging evidence that HCLK2 is a *bona fide* component of the ATR activation machinery.

EXPERIMENTAL PROCEDURES

Cell Culture and DNA-damaging Treatments—Human U-2-OS, HEK 293T, and HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen). Transfection of siRNAs was performed using Lipofectamine RNAi MAX (Invitrogen). For stable and transient DNA transfections, we used FuGENE 6 (Roche Applied Science) according to manufacturer's instructions. For generation of the stable shATR cell line with inducible small hairpin RNA expression, naive U-2-OS cells were transfected with shATR vector and a plasmid encoding the tetracycline repressor for 24 h and selected with puromycin (1 μg/ml; Sigma) and blasticidin (5 μg/ml; InvivoGen). Expression of the small hairpin RNA was achieved by incubating the cells with 2 μg/ml of doxycycline (BD Biosciences) for 72 h. Cells were exposed to ionizing radiation using an x-ray generator (Pantak HF160, 150hV, 16mA, dose rate of 2.18 Gy/min). Exposure to UV light was performed with a Stratalinker 1800 (Stratagene). To induce replication fork stalling, 2 mM hydroxyurea (Sigma) was added to the cells for 1 h. ATM inhibitor (KU55933, KuDOS pharmaceuticals) was used at a final concentration of 1 μM.

Plasmids and siRNA—The expression plasmids for HCLK2 were generated by inserting PCR-amplified HCLK2 cDNA (full-length and various fragments) in-frame with a FLAG tag into pFLAG-CMV-2 (Sigma). siRNA from Dharmacon were used to transiently down-regulate the expression of TopBP1 (5′-GUGGUUGUAACAGCGCAUC-3′), ATRIP (5′-AGAGAAACUGUUCCAAUUA-3′), and ATR (5′-TGAACTGTACGTGGAAAGG-3′). For down-regulation of HCLK2 we applied a previously published siRNA SmartPool from Dharmacon (15). As a control, a previously described siRNA against HSP70B (used in U-2-OS cells) and RISCFree siRNA (in HeLa cells), both from Dharmacon, were applied (20). An inducible small hairpin RNA expression vector targeting ATR was generated by annealing oligonucleotides (target sequence: 5′-TGAACTGTACGTGGAAAGG-3′) and ligating the fragment into BglII/HindIII-digested pSUPERIOR vector (OligoEngine).

Immunochemical Methods—Immunoprecipitation and immunoblotting were performed as described (6, 21). Antibodies used included phosphospecific rabbit polyclonal antibodies against Chk1-S317, Chk1-S345, Nbs1-S343, and Smc1-S966 (Cell Signaling); additional polyclonal antibodies included ATRIP, total Smc1 (Abcam), goat antibodies against ATR, γ-tubulin, and MCM6 (Santa Cruz). Rabbit polyclonal TopBP1 antibody was raised against recombinant His-tagged human TopBP1 peptide comprising amino acid residues 900–1200. In addition another rabbit polyclonal TopBP1 antibody was used (Abcam). The HCLK2 antibody used was a gift from S. Hekimi. In addition, mouse monoclonal antibodies against FLAG (clone 2, Sigma) and Chk1 (DSC 316) were applied.

Kinase Assay—Lysis, immunoprecipitation, and kinase reactions were performed as described earlier (22). The ATR, TopBP1, FLAG, and HCLK2 antibodies used for immunoprecipitations was described under "Immunochemical Methods." Immunoprecipitated proteins were mixed with kinase buffer containing 25 μM ATP, 15 μCi of [γ-^{32}P]ATP, and 8 μg of GST-p53$^{1-43}$ (residues 1–47) or GST-MCM2 (residues 1–285) and incubated for 30 min at 30 °C. Labeled proteins were separated by SDS-PAGE, and substrate phosphorylation was visualized and quantified by phosphorimaging. In experiments where immunoprecipitated HCLK2 was added to the kinase reactions (Figs. 1*D* and 5*A* and supplemental Figs. S5, *C* and *E*, and S8, *A* and *B*), HCLK2 was immunoprecipitated from 2.5 mg of cell lysate. In Fig. 5, *C* and *D*, and supplemental Fig. S8, *C* and *D*, 3 μg of GST-TopBP1 (ATR-activating domain) was added.

Flow Cytometry—To assay cell cycle distribution by DNA content, trypsinized cells were fixed in 70% ethanol, stained with 0.1 mg/ml propidium iodide, and analyzed by a FACSCalibur flow cytometer (BD Biosciences). Two parallel analyses to assay the number of mitotic cells were performed as previously described (23).

Clonogenic Survival Assays—U-2-OS cells were transfected with control, HCLK2, TopBP1, or HCLK2/TopBP1 siRNAs. 2 days after transfection, between 100 and 2000 cells (depending on radiation/drug dose to yield 30–200 colonies per dish) were seeded to 6-cm-diameter dishes, incubated for 20–24 h and treated with hydroxyurea (0, 0.25, 0.5, 1.0, or 1.5 mM, for 24 h), IR (0, 1, 2, or 4 Gy), or UV (0, 3 or 7 J/m^2). Subsequently, cells were incubated for additional 10 days and stained with crystal violet. Colonies containing more than 50 cells were scored as survivors. Survival fractions were calculated in each experiment as the average cloning efficiency (from at least two parallel dishes) after treatment and corrected for plating efficiency.

Computational Methods—Multiple sequence alignment of HCLK2 and many of its orthologs using ClustalW revealed a weak conservation between repetitive hydrophobic patterns dispersed along the central region of CLK2. By scanning the

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Swiss-Prot data base for human proteins with similar repetitive patterns using the Profile Pattern Search server, we found matches in HEAT-repeat regions of various HEAT repeat-containing proteins (*e.g.* Huntingtin, Importin β2, PP2A, FRAP, HEAT2, and HEAT5A). Hierarchical neural network (HNN) and PSIPRED predicted HCLK2 to contain 12 HEAT repeats (24).

RESULTS

HCLK2 Contributes to ATR-mediated Signaling Early after DNA Damage—To study the role of HCLK2 in ATR-mediated phosphorylations, we down-regulated HCLK2 by siRNA, exposed the cells to various types of DNA damage or replicative stress, and determined the phosphorylation status of selected ATR targets 1 h post-treatment. Consistent with the previous reports (8), phosphorylation of Chk1 S317 was severely impaired after hydroxyurea and UV treatment. Surprisingly, however, phosphorylation of Nbs1 on S345 and Smc1 on S966 was also affected (Fig. 1*A*). We used a siRNA SmartPool for HCLK2 knockdown in this experiment but validated that each of the four HCLK2 siRNAs from the SmartPool resulted in similar defects in ATR phosphorylations (data not shown). This spectrum of phosphorylation defects is similar to what was earlier reported for either ATR or TopBP1 knockdown (6, 7), suggesting that HCLK2, unlike Claspin but similar to TopBP1, has a more general effect on ATR-mediated phosphorylation (6). To confirm that the phosphorylation defects observed after DNA damage in HCLK2-depleted cells were a general phenomenon and not restricted to U-2-OS cells, we also performed the experiment in HeLa cells and found a similar impairment in phosphorylation of ATR targets after UV exposure (supplemental Fig. S1). HCLK2 depletion also inhibited phosphorylations of Smc1 and Nbs1 after ionizing radiation (Fig. 1*A*), consistent with recent studies in mice and *S. cerevisiae* suggesting a role of Tel2 in ATM-mediated signaling after DNA damage (17, 18).

A recent study reported that the lack of HCLK2 destabilizes the family of phosphatidylinositol 3-kinase-like kinases including ATR (18). We could reproduce the decrease in ATR levels in our model cell line but only after extended periods (6 days) of HCLK2 depletion (supplemental Fig. S2*A*). In addition, we found a similar decrease in TopBP1 levels after 6 days HCLK2 depletion, suggesting that the reduction in protein levels might be a consequence of disrupting the ATR holo-complex. This was further supported by the fact that a long term depletion of any of the members of the TopBP1/ATR-ATRIP complex resulted in decreased protein levels of the other members (supplemental Fig. S2*B*). Interestingly, however, we found that the defects in ATR-mediated phosphorylation in HCLK2-depleted cells occurred already before a detectable drop in ATR levels (see the time points in supplemental Fig. S2*A*). Therefore, to exclude the influence of decreased ATR levels, all subsequent experiments we carried out did not exceed this time point. To further exclude that reduction in ATR protein level could account for the observed reduction in phosphorylation of ATR targets in HCLK2-depleted cells at early time points, we ectopically expressed FLAG-tagged ATR from a plasmid to increase ATR levels. Indeed, also under such circumstances ATR-mediated phosphorylation was still defective (supplemental Fig.

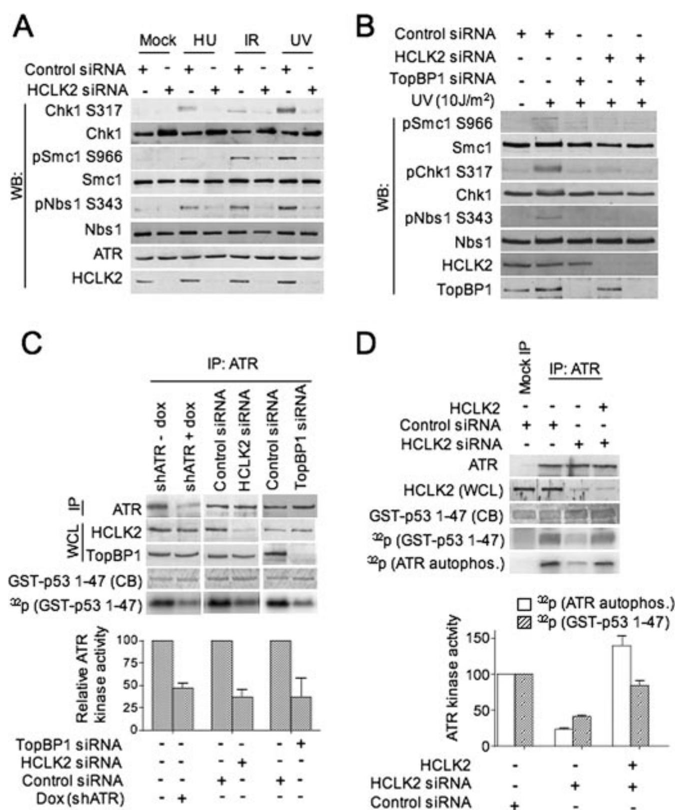

FIGURE 1. **HCLK2 is required for ATR kinase activity and for phosphorylation of its downstream targets.** *A*, U-2-OS cells were transfected with HCLK2 or control siRNA for 72 h, subjected to 2 mM hydroxyurea (*HU*), 3Gy, or 10J/m^2, and harvest 1 h later. Subsequently, total and phosphorylated proteins were analyzed by Western blotting (*WB*) with the indicated antibodies. *B*, U-2-OS cells were transfected with the indicated siRNAs for 72 h, subjected to 10 J/m^2, and harvested 1 h later. Subsequently, total and phosphorylated proteins were analyzed by Western blotting with the indicated antibodies. *C*, small hairpin RNA against ATR was induced with doxycycline (*dox*) for 72 h. In parallel U-2-OS cells were transfected with HCLK2, TopBP1, or control siRNA for 72 h. The cells were lysed and subjected to immunoprecipitation (*IP*) using an antibody against ATR. Immunoprecipitated ATR was incubated with substrate (GST-p53 1–47) and [γ-^{32}P]ATP. Kinase reactions were separated by SDS-PAGE, stained with Coomassie Blue (*CB*), and analyzed by phosphorimaging. Duplicate gels were blotted and probed with anti-ATR, anti-TopBP1, and anti-HCLK2 antibody. The diagram shows the relative ATR kinase activity toward the substrate. The experiment was performed twice; *the error* bars represent S.E. *WCL*, whole cell lysates. *D*, U-2-OS cells were transfected with HCLK2 or control siRNA for 72 h lysed and subjected to immunoprecipitation using an antibody against ATR. Immunoprecipitated ATR was incubated with immunoprecipitated HCLK2 or control immunoprecipitates, substrate (GST-p53 1–47), and [γ-^{32}P]ATP. HCLK2 was immunoprecipitated from cells depleted of ATR and TopBP1 (supplemental Fig. 5*A*) and incubated 10 min with 1 μM ATM inhibitor KU55933 before it was added to the kinase reaction. Kinase reactions were separated by SDS-PAGE, stained with Coomassie Blue, and analyzed by phosphorimaging. Duplicate gels were Western-blotted with the indicated antibodies. The diagram shows the relative ATR kinase activity toward the substrate. The experiment was performed three times; the *error bars* represent S.E.

S3*A*). In a kinase assay we tested and verified that the overexpressed FLAG-tagged ATR was active (supplemental Fig. S3*B*).

Because both TopPB1 and HCLK2 promote ATR-mediated phosphorylations in response to DNA damage, we wanted to compare their relative contributions to these processes. We used siRNA to deplete the two proteins either individually or in combination and assayed the phosphorylation status of Chk1, Nbs1, and Smc1 1 h after exposure of the cells to UV (Fig. 1*B*). We found that depleting cells for either TopBP1 or HCLK2 resulted in a severe and very similar impairment of Chk1/Nbs1/

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Smc1 phosphorylations. No synergistic effect of co-depleting TopBP1 and HCLK2 was observed, suggesting that HCLK2 and TopBP1 operate in a common pathway required for ATR-mediated phosphorylation events.

It has previously been shown that TopBP1 is required for ATR kinase activity (25). Because our data point to a similar role of HCLK2, we tested whether depletion of HCLK2 would directly affect the kinase activity of ATR in an i*n vitro* kinase reaction. To address this, we immunoprecipitated endogenous ATR from cells treated with control siRNA or siRNA against either HCLK2 or TopBP1 for 72 h and subjected these immunoprecipitates to an *in vitro* kinase assay (Fig. 1*C*). TopBP1 depletion resulted in decreased activity of ATR (Fig. 1*C*). Interestingly, we observed a similar decrease in ATR kinase activity upon HCLK2 depletion (Fig. 1*C*). ATR kinase assays are often performed without DNA damage, as there has been observed no apparent effect of checkpoint-inducing treatments on ATR activity after extraction and immunoprecipitation from human cells (Refs. 25–27 and Fig. S4, *A* and *B*). Indeed, we obtained similar results as reported above in cells exposed to replicative stress (data not shown).

We next wanted to address if HCLK2-like TopBP1 could stimulate the activity of ATR. To examine this possibility, we wanted to generate recombinant GST-tagged HCLK2, but despite many attempts we failed to obtained soluble recombinant HCLK2 protein. Instead, we used HCLK2 immunoprecipitated from cells depleted of ATR and TopBP1 (supplemental Fig S5*A*) and treated with ATM inhibitor before being added to the kinase reaction. Under these conditions the immunoprecipitated HCLK2 contained negligible kinase activity by itself (supplemental Fig. S5*B*). Importantly, and consistent with a role of HCLK2 in activation of ATR, we found that incubation with HCLK2 induced an increase in both ATR autophosphorylation and kinase activity toward the p53 substrate, almost completely rescuing the decrease caused by HCLK2 depletion (Fig. 1*D*). Activity of protein kinases is often associated with autophosphorylation. Consistent with our observations (supplemental Fig. S3*B*), it has previously been reported that ATR can phosphorylate itself and that this autophosphorylation is dependent upon the integrity of the catalytic domain (26–28). We observed that immunoprecipitated HCLK2 stimulated ATR kinase activity in a dose-dependent manner (supplemental Fig. S5*C*), and our method of measuring the stimulatory effects of HCLK2 on ATR kinase activity was further supported by experiments showing that FLAG-HCLK2 isolated by anti-FLAG antibodies and eluted with 3X-FLAG peptide also yielded a similar stimulation in ATR activity (supplemental Fig. S5*D*). In addition, we found that also *in vitro* translated green fluorescent protein-tagged HCLK2 stimulated ATR autophosphorylation and kinase activity toward the p53 substrate. (supplemental Fig. S5*E*). The specificity of the assay was verified by performing immunoprecipitations in cells depleted for ATR or by adding caffeine to the kinase reaction. In both cases a reduction in ATR kinase activity was observed (Fig. 1*C* and supplemental Fig. S5*F*). Similar amounts of ATR were immunoprecipitated from cells treated with control and HCLK2 siRNA (Fig. 1*D*), emphasizing that at early time point ATR levels did reduce in HCLK2-depleted cells in our cell model, supporting a role of HCLK2 in ATR activation *per se*.

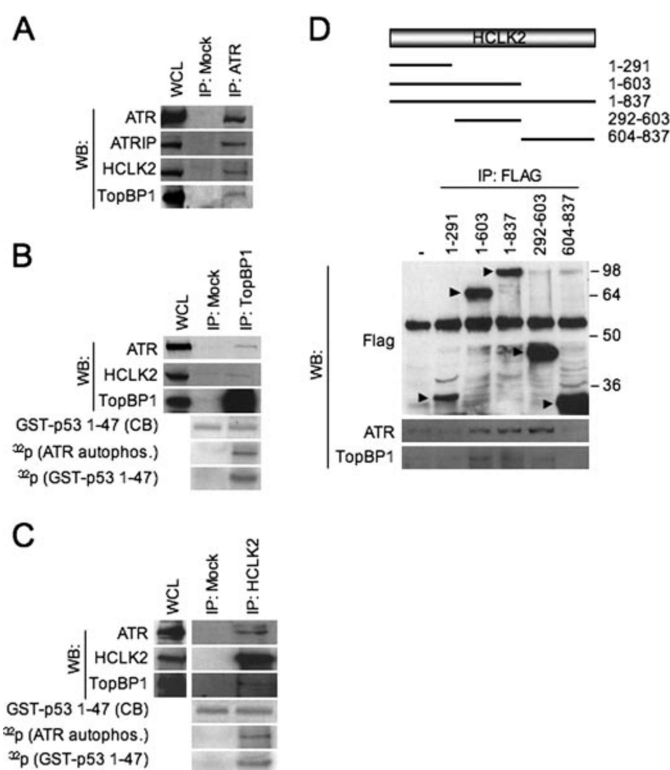

FIGURE 2. **The domain required for ATR association resides within the 291–603-amino acid region of HCLK2.** *A*, HEK293T cells were lysed and immunoprecipitated (*IP*) with ATR antibody. The levels of ATR, TopBP1, ATRIP, and HCLK2 in the immunoprecipitates were assayed by Western blotting (*WB*). *WCL*, whole cell lysates. *B*, HEK293T cells were lysed, immunoprecipitated with TopBP1 antibody, and Western-blotted with the indicated antibodies. *C*, HEK293T cells were lysed, immunoprecipitated with HCLK2 antibody, and Western blotted with the indicated antibodies. *D*, a schematic map shows the FLAG-tagged HCLK2 fragments used. HEK293T cells were transfected with empty vector or vectors encoding the indicated FLAG-tagged HCLK2 proteins. Protein levels were analyzed by Western blotting with the indicated antibodies.

ATR Association of HCLK2 Resides within Residues 292–603 of HCLK2—Because depletion of either TopBP1 or HCLK2 resulted in similar defects in ATR kinase activity and codepletion of these factors did not show any apparent synergistic effects, we wanted to test if the three proteins associated *in vivo*. Indeed, immunoaffinity purifications identified HCLK2 in a complex containing fractions of ATR-ATRIP and TopBP1. The associations of HCLK2, TopBP1, and ATR were shown both for overexpressed FLAG-tagged and endogenous HCLK2 and verified by reciprocal immunoprecipitations using antibodies against ATR and TopBP1 (Fig. 2, *A*–*D*). Importantly, even though it is a minor fraction of the total amount of ATR that associates with HCLK2 and TopBP1, our results show that this fraction of ATR is active (Fig. 2, *B* and *C*). The associations between ATR, TopBP1, and HCLK2 were unaffected by DNA damage (supplemental Fig. S6*A*; data not shown). This is similar to previous studies observing no effect on the amount of ATR-ATRIP associating with TopBP1 after ionizing radiation (29). By overexpressing various fragments of HCLK2 fused to a FLAG tag, we found that the 292–603 amino acid region of HCLK2 is required for association with ATR. Within this frag-

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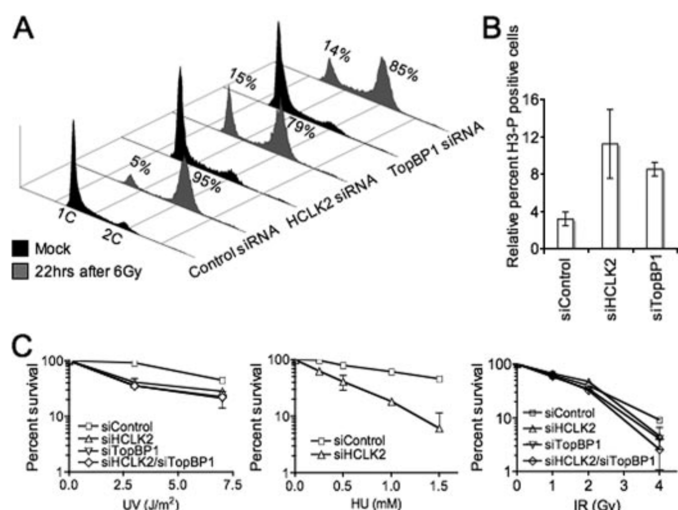

FIGURE 3. **Depletion of HCLK2 impairs the G_2 checkpoint and sensitizes the cells to UV, hydroxyurea, and high doses of ionizing radiation.** *A*, U-2-OS cells were treated with the indicated siRNAs for 50 h and then irradiated with 6 Gy and harvested 22 h later. Cell cycle profile was visualized by propidium iodide staining and analyzed using Modfit software. *B*, the diagram indicates nocodazole and irradiated cells in M-phase relative to the number of cells in M-phase in the nocodazole but non-irradiated sample. U-2-OS cells were treated with siRNA for 57 h, then nocodazole was added, and cells were irradiated with 6 Gy as indicated. Cells were harvested and analyzed by fluorescence-activated cell sorter 15 h later. M-phase cells were detected by staining for the mitotic marker phosphorylated histone 3. The experiment was repeated twice; the *error bars* represent S.E. *C*, clonogenic survival of U-2-OS cells transfected with control siRNA or siRNA against HCLK2, TopBP1, or both and then treated with the indicated doses of UV, hydroxyurea (*HU*), or IR. The experiments were carried out in duplicate. The *error bars* indicate the S.E.

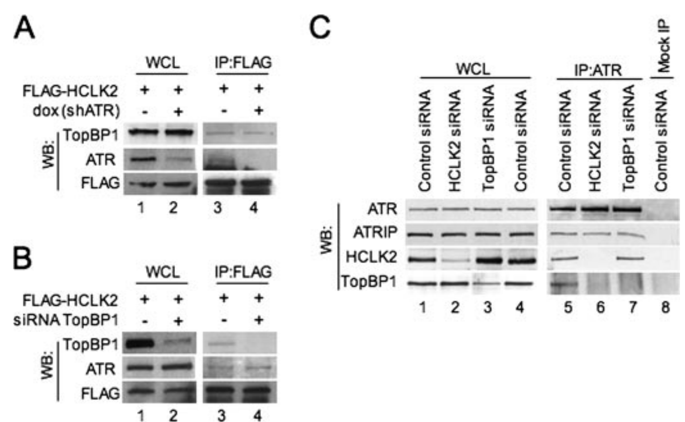

FIGURE 4. **HCLK2 is required for efficient ATR-TopBP1 association.** *A*, down-regulation of ATR was induced or not by incubating shATR U-2-OS cells with doxycycline (*dox*) for 48 h, and then cells were transfected with wild-type FLAG-HCLK2 constructs. Twenty-four hours later cells were lysed, immunoprecipitated (*IP*) with FLAG antibody, and Western blotted (*WB*) with the indicated antibodies. *WCL*, whole cell lysates. *B*, U-2-OS cells were transfected with TopBP1 or control siRNA for 48 h, then cells were transfected with wild-type FLAG-HCLK2 constructs and after 24 h cells were lysed and immunoprecipitated with FLAG antibody. Immunoprecipitates and whole cell lysates were Western-blotted with the indicated antibodies. *C*, U-2-OS cells were treated with control siRNA or siRNA against HCLK2 or TopBP1 for 72 h, then cells were lysed and immunoprecipitated with ATR antibody, and Western blotted with the indicated antibodies.

ment, we were not able to narrow down the region responsible for the interaction any further (data not shown). The binding to TopBP1 resides within residues 1–603 (Fig. 2*D*). Like Tel2, HCLK2 mainly consist of α-helices and is structurally similar to proteins from the ARM repeat superfamily. HCLK2 is predicted to contain 12 HEAT repeats, five of which are found within the HCLK2 fragment required for the interaction with ATR (supplemental Fig. S6, *B* and *C*).

HCLK2 Is Required for the G_2 DNA Damage Checkpoint—The G_2 DNA damage checkpoint prevents the entry of cells into mitosis in the presence of DNA damage. Chk1 regulates the late G_2 checkpoint by controlling the activity of the M-phase promoting cyclin B/cyclin-dependent kinase 1 complex (30). We wanted to address whether the failure of ATR to phosphorylate and activate Chk1 in response to DNA damage in HCLK2-depleted cells would also result in a defect in the G_2 checkpoint. First, we used siRNA to knockdown HCLK2 or TopBP1, irradiated the cells with 6 Gy, and analyzed the DNA profile by propidium iodide staining 22 h later (Fig. 3*A*). Cells treated with control siRNA arrested in G_2, whereas both HCLK2 and TopBP1-deficient cells escaped the G_2 arrest prematurely as evident by a decreased number of cells in G_2 and an increased number of cells in G_1 compared with the control. We also quantified the G_2 leakage by using nocodazole to trap the cells that escaped the G_2 checkpoint in M-phase and measured the percentage of cells positive for the mitotic marker, phosphorylated histone 3 (pS10). When comparing the amount of phosphorylated histone 3-positive cells in the irradiated and nocodazole-treated sample with those treated only with nocodazole, the relative percentages were 3.2, 11.2, and 8.5% for cells treated with control, HCLK2, and TopBP1 siRNA, respectively (Fig. 3*B*). This is consistent with a role of HCLK2 in the G_2 checkpoint.

Cells deficient for ATR are sensitive to genotoxic stress (31). Similarly, cells depleted for HCLK2 or TopBP1 were found to be hypersensitive to the cytotoxic effects of UV when compared with control cells in a clonogenic survival assay (Fig. 3*C*). HCLK2-TopBP1 double knockdown cells did not exhibit increased sensitivity to UV, supporting the previous results indicating that HCLK2 and TopBP1 function in a common pathway. HCLK2-depleted cells also showed increased sensitivity to hydroxyurea (effects of depleting TopBP1 was not tested). Ionizing radiation had a modest effect on survival after HCLK2 and TopBP1 depletion but only at the highest dose tested (4 Gy; Fig. 3*C*).

HCLK2 Facilitates the Association between ATR and TopBP1—To examine the cause of the checkpoint defect and reduced activation of ATR in HCLK2-depleted cells, we wanted to dissect the nature of the ATR-ATRIP/TopBP1/HCLK2 complex in more detail. To test if TopBP1 could associate with HCLK2 independently of ATR, the ability of HCLK2 to copurify with TopBP1 was analyzed in U-2-OS cells conditionally depleted for ATR and transiently transfected with FLAG-tagged HCLK2 (Fig. 4*A*). HCLK2 and TopBP1 associated to a similar degree in samples with or without ATR (Fig. 4*A*, *lane 3* and *4*). Similarly, we could not detect any significant impact of TopBP1 depletion on the ATR-HCLK2 association (Fig. 4*B*, *lane 3* and *4*). In contrast, we did reproducibly observe a reduction in the association between ATR and TopBP1 in the absence of HCLK2 (Fig. 4*C*, *lane 6*), suggesting that HCLK2 facilitates efficient ATR-TopBP1 association.

Both HCLK2 and TopBP1 Are Required for ATR Activity—Previously we showed (Fig. 1*D*) that reconstitution with

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HCLK2 significantly increases ATR activity in cells depleted for HCLK2 but proficient for TopBP1. However, if HCLK2 promotes association between ATR-ATRIP and TopBP1, one would expect that incubation with HCLK2 would not be sufficient to rescue ATR activity in cells co-depleted for both HCLK2 and TopBP1. Indeed, under such conditions we observed only a very modest rescuing effect on ATR kinase activity toward the p53 substrate when reconstituting with immunopurified HCLK2 (Fig. 5*A*). Surprisingly, the addition of HCLK2 did rescue the autophosphorylation of ATR even in the absence of TopBP1 (Fig. 5*A*). Interestingly, like HCLK2, recombinant TopBP1 (ATR-activating domain, residues 972–1192) or endogenous immunoprecipitated TopBP1 could only stimulate the activity of ATR toward the substrate in cells proficient for HCLK2 (Fig. 5, *B* and *C*, and supplemental Fig. S7). If cells were codepleted for HCLK2 and TopBP1, incubation with recombinant TopBP1 had no stimulatory effect on either ATR substrate phosphorylation or ATR autophosphorylation (Fig. 5*C*). In HCLK2-proficient cells TopBP1 stimulated ATR activity toward the substrate but had no apparent effect on the autophosphorylation status of ATR (Fig. 5*B* and Fig. S7). The requirement for TopBP1 and HCLK2 for ATR activity toward its substrate appears to be a general phenomenon as TopBP1 and HCLK2 are also required for the phosphorylation of another established ATR target, MCM2, *in vitro* (supplemental Fig. S8, *A–D*). In all experiments TopBP1 and/or HCLK2 were effectively depleted (beyond detection by Western blotting), and similar amounts of ATR were immunoprecipitated from cells treated with control siRNA or TopBP1 and/or HCLK2 siRNA. Together these data suggest that both TopBP1 and HCLK2 are required for ATR activity and for efficient targeting of its substrates.

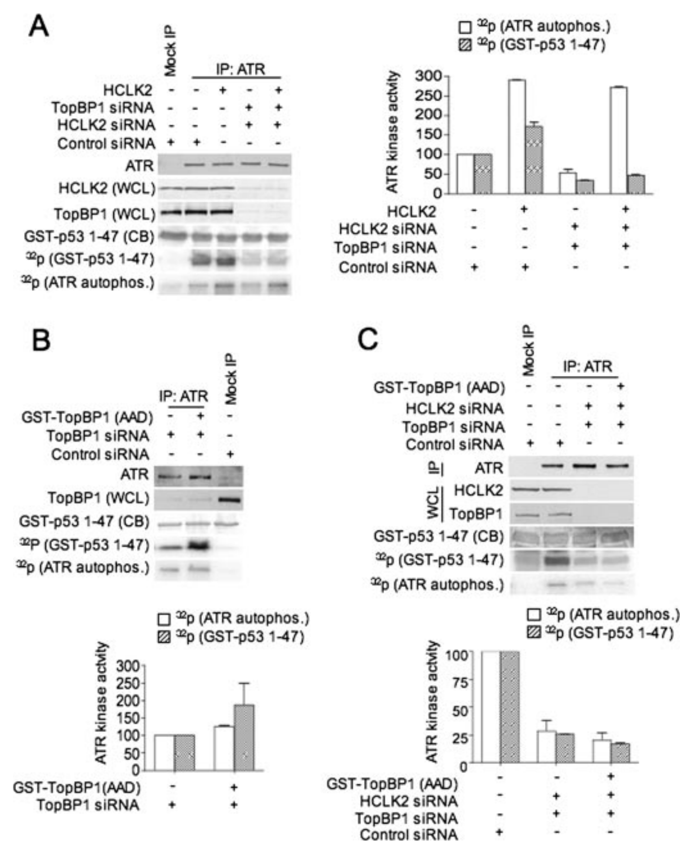

FIGURE 5. **HCLK2 is required for TopBP1-mediated activation of ATR and vice versa.** *A*, U-2-OS cells were cotransfected with HCLK2 and TopBP1 or control siRNA for 72 h. Lysates were subjected to immunoprecipitation (*IP*) using an antibody against ATR. Immunoprecipitated ATR was incubated with immunoprecipitated HCLK2 or control immunoprecipitates, substrate (GST-p53 1–47), and [γ-^{32}P]ATP. HCLK2 was immunoprecipitated from cells depleted of ATR and TopBP1 (supplemental Fig. S4*A*) and incubated 10 min with 1 μM ATM inhibitor (KU55933) before it was added to the kinase reaction. Kinase reactions were separated by SDS-PAGE, stained with Coomassie Blue (*CB*), and analyzed by phosphorimaging. *WCL*, whole cell lysates. Duplicate gels were Western-blotted with the indicated antibodies. The graph shows the relative ATR autophosphorylation and activity toward the substrate. The experiments were carried out in duplicates. The *error bars* indicate the S.E. *B*, U-2-OS cells were transfected with TopBP1 or control siRNA for 72 h. Lysates were subjected to immunoprecipitation using an antibody against ATR. Immunoprecipitated ATR was incubated with recombinant GST-tagged TopBP1 (ATR-activating domain, residues 972–1192) or GST alone (both 3 μg), substrate (GST-p53 1–47), and [γ-^{32}P]ATP. Kinase reactions were separated by SDS-PAGE, stained with Coomassie Blue, and analyzed by phosphorimaging. Duplicate gels were Western-blotted for the indicated antibodies. The diagram shows the relative ATR autophosphorylation and activity toward the substrate. The experiment was performed twice; the *error bars* represent S.E. *C*, U-2-OS cells were transfected with the indicated siRNAs for 72 h. Lysates were immunoprecipitated using an antibody against ATR. Immunoprecipitated ATR was incubated with recombinant GST-tagged TopBP1 (ATR-activating domain) or GST alone, substrate (GST-p53 1–47), and [γ-^{32}P]ATP. Kinase reactions were separated by SDS-PAGE, stained with Coomassie Blue, and analyzed by phosphorimaging. Duplicate gels were Western-blotted with the indicated antibodies. The diagram shows the relative ATR autophosphorylation and activity toward the substrate. The experiment was performed twice; the *error bars* represent S.E.

DISCUSSION

HCLK2 is conserved throughout eukaryotes, and studies in various species have suggested a role of HCLK2 and its orthologs in the DNA damage response. In mammalian cells, prevention of unscheduled Chk1 degradation and stabilization of ATR and ATM by interaction with HCLK2 has been suggested to explain why HCLK2 is essential for many aspects of ATR and ATM-mediated checkpoint signaling (15, 18). Here, we demonstrate that HCLK2 is required for ATR-mediated signaling, not only by stabilization of ATR and/or Chk1, but also by promoting ATR kinase activity *per se*. We provide evidence that HCLK2 induces and is required for ATR autophosphorylation and activity toward different substrates. Furthermore, phosphorylation of ATR target proteins after DNA damage and replicative stress depends on HCLK2, and cells depleted for HCLK2 show defects in the G_2 checkpoint and decreased viability after genotoxic stress. Overall, our data suggest that HCLK2 activates ATR and that this activation is necessary for ATR-mediated checkpoint signaling. Importantly, our results indicate that HCLK2 functions in the same pathway as the well known ATR-mediator TopBP1 but that the two proteins regulate different steps in ATR activation.

TopBP1 and HCLK2 depletion results in similar phenotypes regarding ATR substrate phosphorylation, *in vitro* kinase activity, cell cycle arrest, and survival after exposure to DNA-damaging agents, suggesting that the two proteins work in the same pathway. Moreover, we show that HCLK2 depletion results in a deficient G_2 checkpoint (Fig. 3, *A* and *B*) and, in addition, HCLK2 knockdown has been shown to impair the S-phase checkpoint, monoubiquitination of FANCD2 and homologous recombination, all of which is consistent with a role of HCLK2 in ATR activation (15).

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Studies have indicated that activation of human ATM involves autophosphorylation followed by dissociation of inactive dimers into active monomers and interaction with the Mre11-Rad50-Nbs1 complex (32–34). Autophosphorylation of ATR has also been reported (28). We observed that depletion of HCLK2 in addition to decreasing ATR activity toward target substrates also decreased autophosphorylation (Fig. 1*D* and supplemental Fig. S8*A*). Consistent with existing data, we did not detect any obvious changes in autophosphorylation of ATR upon incubation with TopBP1 (Fig. 5*B*). Importantly, even though neither HCLK2 nor TopBP1 incubation could rescue ATR activity toward two different substrates in cells codepleted for HCLK2 and TopBP1, HCLK2 incubation did rescue the autophosphorylation of ATR (Fig. 5*A*). In addition, TopBP1 association with the ATR-ATRIP complex appears to require HCLK2, as we observed a decreased association in cells depleted for HCLK2 (Fig. 4*C*). Overall, these data provide evidence that both HCLK2 and TopBP1 are essential for ATR activation and substrate phosphorylation but implies that the two proteins work at different steps in ATR activation. One possibility could be that HCLK2 induces, potentially by promoting ATR autophosphorylation, a conformational change in the ATR-ATRIP complex, allowing TopBP1 to bind more efficiently and mediate phosphorylation of ATR target proteins. Binding of TopBP1 to ATR-ATRIP was reported by Mordes *et al.* (29) to decrease the K_m of ATR for its substrates, potentially by altering the conformation of the ATR kinase domain such that substrates can assess ATR more easily.

We did not observe any increase in ATR activity after DNA damage or replicative stress (Fig. S4, *A* and *B*). This is consistent with previous studies and probably reflects the relative transient nature of the interaction between ATR-ATRIP, TopBP1, and HCLK2 observed here and which has also been reported and discussed by others (25, 35, 36). It is possible that the experimental conditions applied during extraction and immunoprecipitation of protein complexes from cell extracts dislodge portions of TopBP1 and HCLK2 from ATR-ATRIP. ATR-ATRIP and TopBP1 are recruited to sites of DNA damage, which can be readily detected as foci by immunofluorescence. Because HCLK2 associates with both proteins, one would expect to find HCLK2 in these foci as well, but we (and others) have not been able to detect any HCLK2 focus formation in response to genotoxic stress. However, by using salt fractionation we found an increase in HCLK2 bound to chromatin in response to DNA damage (data not shown), suggesting that HCLK2-like ATR and TopBP1 indeed gets recruited to sites of DNA damage. It is a minor fraction of total HCLK2 that accumulate at chromatin, which might explain why it is difficult to detect any foci formation using immunofluorescence.

Consistent with a recent report (Takai *et al.* (18)), we also found a decrease in the ATR level at late time points (6 days) after HCLK2 knockdown (Fig. S2*A*). However, the defects in ATR-mediated phosphorylations and ATR kinase activity identified in the present study were observed when ATR levels were still normal. In addition, overexpression of kinase active ATR did not rescue the ATR signaling in HCLK2-depleted cells (Fig. S3*A*), suggesting that decreased ATR levels are not the cause of the early checkpoint defects but, rather, the consequence of removing one of the interacting partners from the ATR-ATRIP-TopBP1-HCLK2 complex for extended periods of time. Loss of protein stability is commonly seen when a protein cannot interact with one of its usual partners. In support of this, we found that removing ATR or TopBP1 for 6 days resulted in a reciprocal decrease in protein levels (Fig. S2). How this complex destabilization is regulated is not yet clear, as both we and others noted that the decreased protein levels could not be rescued by proteasome inhibitor and no apparent change was observed on the mRNA level (data not shown and Takai *et al.* (18)). A role of HCLK2 in checkpoint signaling besides stabilization of phosphoinositide 3-kinase-related protein kinases is further supported by findings in *S. cerevisiae.* Here, depletion of Tel2 (*S. cerevisiae* HCLK2) was found to result in defects in Tel1 (*S. cerevisiae* ATM) signaling even when Tel1 was overexpressed (16, 17).

In conclusion, our data support a role of HCLK2 as a *bona fide* component in the regulation of ATR activity. Thus, both TopBP1 and HCLK2 are required for ATR activity and substrate phosphorylation; however, the regulation appears to occur at different levels. We propose that HCLK2 induces, potentially by promoting ATR autophosphorylation, a conformational change in the ATR-ATRIP complex enabling binding of TopBP1 and, hence, full activity of ATR toward its substrates.

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