Schlafen-11 (SLFN11) inactivation in ~50% of cancer cells confers broad chemoresistance. To identify therapeutic targets and underlying molecular mechanisms for overcoming chemoresistance, we performed an unbiased genome-wide RNAi screen in SLFN11-WT and -knockout (KO) cells. We found that inactivation of Ataxia Telangiectasia- and Rad3-related (ATR), CHK1, BRCA2, and RPA1 overcomes chemoresistance to camptothecin (CPT) in SLFN11-KO cells. Accordingly, we validate that clinical inhibitors of ATR (M3444 and M6620) and CHK1 (SRA737) reseensitize SLFN11-KO cells to topotecan, indotecan, etoposide, cisplatin, and talazoparib. We uncover that ATR inhibition significantly increases mitotic defects along with increased CDT1 phosphorylation, which destabilizes kinetochore-microtubule attachments in SLFN11-KO cells. We also reveal a chemoresistance mechanism by which CDT1 degradation is retarded, eventually inducing replication reactivation under DNA damage in SLFN11-KO cells. In contrast, in SLFN11-expressing cells, SLFN11 promotes the degradation of CDT1 in response to CPT by binding to DDB1 of CUL4CDT2 E3 ubiquitin ligase associated with replication forks. We show that the C terminus and ATPase domain of SLFN11 are required for DDB1 binding and CDT1 degradation. Furthermore, we identify a therapy-relevant ATPase mutant (E669K) of the SLFN11 gene in human TCGA and show that the mutant contributes to chemoresistance and retarded CDT1 degradation. Taken together, our study reveals new chemotherapeutic insights on how targeting the ATR pathway overcomes chemoresistance of SLFN11-deficient cancers. It also demonstrates that SLFN11 irreversibly arrests replication by degrading CDT1 through the DDB1–CUL4CDT2 ubiquitin ligase.

Significance

Schlafen-11 (SLFN11) inactivation leads to chemoresistance of a broad range of DNA-damaging agents. We uncover an expanded Ataxia Telangiectasia- and Rad3-related (ATR)-mediated signaling network that overcomes chemoresistance by an unbiased genome-wide RNAi screen in SLFN11-knockout cells and is validated with clinically developing ATR/CHK1 inhibitors. ATR inhibition induces CDT1 phosphorylation, leading to mitotic catastrophe cell death in SLFN11-deficient cells. We identify a key role of SLFN11 for CDT1 degradation by binding to DDB1–CUL4CDT2 ubiquitin ligase during DNA damage, thereby blocking replication reactivation by which SLFN11-deficient cells cause chemoresistance. Our findings provide a rationale for combination therapy with ATR inhibitor and DNA-targeted drugs, and reveal how SLFN11 irreversibly arrests replication during DNA damage acting as a cofactor of CUL4CDT2 ubiquitin ligase.

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1To whom correspondence may be addressed. Email: ukhyun.jo@nih.gov or yves.pommier@nih.gov.

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It is important to note that the ATPas activity and replication stress to chemosensitivity to DNA-damaging agents (3–5) imply that the ATPas-helicase of SLFN11 interacts specifically in DNA damage response (DDR) to replication stress. Indeed, inactivation of the Walker B motif of SLFN11 by the mutation E669Q suppresses SLFN11-mediated replication block (5, 30). In addition, SLFN11 contains a binding site for the single-stranded DNA binding protein RPA1 (replication protein A1) at its C terminus (3, 31) and is recruited to replication damage sites by RPA1 (3, 3). The putative ATPas activity of SLFN11 is not required for this recruitment (5) but is required for blocking the replication helicase complex (CMG–CDC45) and inducing chromatin accessibility at replication origins and promoter sites (5, 30). Based on these studies, our current model is that SLFN11 is recruited to “stressed” replication forks by RPA filaments formed on single-stranded DNA (ssDNA), and that the ATPas/helicase activity of SLFN11 is required for blocking replication progression and remodeling chromatin (5, 30). However, underlying mechanisms of how SLFN11 irreversibly blocks replication in DNA damage are still unclear.

Increased RPA-coated ssDNA caused by DNA damage and replication fork stalling also triggers ATR kinase activation, promoting subsequent phosphorylation of CHK1, which transiently halts cell cycle progression and enables DNA repair (32). ATR inhibitors are currently in clinical development in combination with DNA replication damaging drugs (33, 34), such as topoisomerase I (TOP1) inhibitors, which are highly synergistic with ATR inhibitors in preclinical models (35). ATR inhibitors not only inhibit DNA repair, but also lead to unscheduled replication origin firing (36), which kills cancer cells (37, 38) by inducing genomic alterations due to faulty replication and mitotic catastrophe (33).

The replication licensing factor CDT1 orchestrates the initiation of replication by assembling prereplication complexes (pre-RC) in G1-phase before cells enter S-phase (39). Once replication is started by loading and activation of the MCM helicase, CDT1 is degraded by the ubiquitin proteasomal pathway to prevent additional replication initiation and ensure precise genome duplication and the firing of each origin only once per cell cycle (39, 40). At the end of G2 and during mitosis, CDT1 levels rise again to control kinetochore-microtubule attachment for accurate chromosome segregation (41). Deregulated overexpression of CTD1 results in rereplication, genome instability, and tumorigenesis (42). The cellular CDT1 levels are tightly regulated by the damage-specific DNA binding protein 1 (DBB1)–CUL4CDT2 E3 ubiquitin ligase complex in G1-phase (43) and in response to DNA damage (44, 45). How CDT1 is recognized by CUL4CDT2 in response to DNA damage remains incompletely known.

In the present study, starting with a human genome-wide RNAi screen, bioinformatic analyses, and mechanistic validations, we explored synthetic lethal interactions that overcome the chemoresistance of SLFN11-deficient cells to the TOP1 inhibitor camptothecin (CPT). The strongest synergistic interaction was between depletion of the ATR/CHK1-mediated DNA damage response pathways and DNA-damaging agents in SLFN11-deficient cells. We validated and expanded our molecular understanding of combinatorial strategies in SLFN11-deficient cells with the ATR (M4344 and M6620) and CHK1 (SRAT373) inhibitors in clinical development (33, 46, 47) and found that ATR inhibition leads to CDT1 stabilization and hyperphosphorylation with mitotic catastrophe. Our study also establishes that SLFN11 promotes the degradation of CDT1 by binding to DDB1, an adaptor molecule of the CUL4CDT2 E3 ubiquitin ligase complex, leading to an irreversible replication block in response to replicative DNA damage.

**Results**

**Genome-Wide RNAi Screen Identifies the ATR/CHK1 Pathway as Therapeutic Targets in SLFN11-Deficient Cells.** To identify clinically actionable therapeutic targets to overcome the chemoresistance of SLFN11-deficient cells, we carried out an unbiased genome-wide RNAi chemosensitization screen using transient transfection with a short-interfering RNA (siRNA) library and subsequent exposure to the TOP1 inhibitor and replication stress inducer CPT (Fig. 1A) (48). The screen was performed in isogenic DU145 SLFN11 WT and knockout (KO) prostate cancer cells, based on their prior characterization with respect to SLFN11 (4–6, 30) and with a drugable siRNA genome library targeting ~10,418 human genes (three siRNAs per gene) (SI Appendix, SI Materials and Methods). SLFN11 WT and KO cells were transfected in a 384-well plate format with the siRNA library and subsequently treated for 2 d with a range of CPT concentrations (0, 5, 10, 25, and 100 nM). After evaluating cell viability using a luminescence-based cell number assay, responses were converted to z-scores from three-individual siRNA-transfected wells (Dataset S1). The normalized z-score data in SLFN11 WT and -KO cells were plotted for each gene. Negative z-scores were considered synthetic-lethal interactions with CPT treatment. ATR, CHK1, BRCA2, and RPA1 were identified as the most potent hits in SLFN11 KO cells (Fig. 1B), whereas POLR2B, PSMD3, POLR2F, and POLR2D were found as significant hits in SLFN11 WT cells (Fig. 1C). Gene ontology (GO)-term enrichment analysis with the top 50 hit genes revealed that the hit genes in SLFN11 KO cells form a cluster associated with DNA repair (Fig. 1D and E), whereas hit genes in SLFN11 WT cells were categorized for RNA processing (SI Appendix, Fig. S1A and B).

To validate the relevance of the ATR/CHK1 pathway in SLFN11-deficient cells, we tested three independent siRNAs targeting ATR, CHK1, and BRCA2 in SLFN11 WT and -KO cells. Silencing of ATR, CHK1, or BRCA2 reversed CPT resistance of SLFN11 KO cells (Fig. 1D–F and SI Appendix, Fig. S1 C–E). These findings demonstrate that inhibition of the ATR/CHK1 and BRCA2 pathways could provide a strategy to overcome resistance to TOP1 inhibitors in SLFN11-deficient cancer cells.

**Clinical Inhibitors of the ATR/CHK1 Pathway Reverse the Resistance of SLFN11-Deficient Cells to a Broad Range of Clinically Used DNA-Damaging Agents.** Given the prevalence of replicative DNA stress in cancer cells and in response to treatment with chemotherapeutic agents, targeting ATR with small-molecule inhibitors is actively being pursued in clinical trials (33). To validate and extend our siRNA screening, we tested two ATR inhibitors in clinical development: M4344 (VX-803) and M6620 (VX-970; berzosertib) in SLFN11 KO cells and -WT cells. In the absence of CPT, both ATR inhibitors showed single-agent activity regardless of SLFN11 expression and M4344 showed greater potency than M6620 (SI Appendix, Fig. S2A).

For combination studies, we selected a nontoxic dose (25 nM) of M4344 to avoid secondary effects related to cell death. Consistent with the ATR targeting siRNA results, combining M4344 and CPT markedly sensitized the SLFN11 KO cells to avoid secondary effects related to cell death. Consistent with the ATR targeting siRNA results, combining M4344 and CPT markedly sensitized the SLFN11 KO cells to avoid secondary effects related to cell death.
resistance of the \textit{SLFN11}-KO to the clinically used TOP2 inhibitor etoposide, PARP inhibitor talazoparib and cisplatin in \textit{SLFN11}-KO cells, while the synergy was less in \textit{SLFN11}-WT cells.

To further establish that the synergistic effect of M4344 was related to ATR, we tested a structurally different ATR inhibitor (M6620, VX-970, berzosertib), which is undergoing clinical trials in combination with TPT (34). M6620 also strongly reversed the chemoresistance of \textit{SLFN11}-KO cells to all tested DNA-damaging agents, albeit at slightly higher concentration (100 nM) compared with 25 nM of M4344 (\textit{SI Appendix}, Fig. S2 B–G).

\textbf{CHK1}, a key downstream protein kinase for the ATR-mediated DNA repair pathways, can also be targeted by...
Fig. 2. Clinical inhibitor of the ATR pathway consistently resensitizes SLFN11-deficient cells to clinical DNA damaging agents. (A–C, Upper) DU145 SLFN11-WT and -KO cells were treated with M4344 (25 nM) and the indicated concentrations of the TOP1 inhibitors CPT, TPT, and indotecan (LMP400) for 72 h. Cell viability was analyzed by CellTiter-Glo. Error bars represent SD (n = 3). (Lower) Combination Index (CI) versus Fa (fraction affected) calculated from cell viability data. (D) Combination treatment of M4344 (1 nM) and the indicated concentrations of CPT in the isogenic WT and SLFN11-KO leukemic lymphoblasts CCRF-CEM cells. (E) Combination treatment of M4344 (25 nM) and the indicated concentrations of CPT in small-cell lung cancer DMS114 cells. (F–H) DU145 cells were treated with M4344 (25 nM) and the indicated concentrations of etoposide, talazoparib, and cisplatin for 72 h. Cell viability was analyzed by CellTiter-Glo. Error bars represent SD (n = 3).
selective small molecule inhibitors for anticancer treatment (47). Given that ATR activates CHK1, we hypothesized that the CHK1 inhibitor in clinical trials, SRA737, could also overcome chemoresistance in SLFN11-KO cells. Accordingly, SRA737 reversed the resistance of SLFN11-KO cells (SI Appendix, Fig. S2 H–O). Together, our findings show that combining clinical ATR/CHK1 inhibitors with widely using clinical DNA-damaging agents is highly effective for overcoming the chemoresistance of cancer cells that do not express SLFN11.

**ATR Inhibition Leads to Mitotic and Genomic Damage in Cells Lacking SLFN11.** To assess how the combination of ATR inhibitors sensitizes SLFN11-deficient cells, we assessed the phosphorylation status of signaling molecules of the ATR-mediated DDR pathway in SLFN11-WT and -KO cells after treatment with M4344 and CPT. As expected, M4344 preferentially decreased the CPT-induced autophosphorylation of ATR, and phosphorylation of CHK1, CHK2, and RPA32 in both SLFN11-WT and -KO cells, demonstrating that M4344 efficiently blocks the CPT-induced DDR (SI Appendix, Fig. S3A). In addition, combination with M4344 and CPT enhanced γH2AX, a marker of DNA damage and apoptosis (49) in SLFN11-KO cells to levels comparable to SLFN11-WT cells, consistent with increased DNA damage by M4344 under conditions that resensitized the SLFN11-KO cells to CPT (Fig. 3 A and SI Appendix, Fig. S3A). In addition, single-cell analyses by immuno-fluorescence staining of cells treated with M4344 and CPT showed that the strong γH2AX signals originated from multifragmented small nuclei in SLFN11-KO cells (Fig. 3A). SLFN11-KO cells exhibited over 50% multinucleated cells, whereas SLFN11-WT cells showed less than 10% (Fig. 3B). These results indicate that ATR inhibition by M4344 might lead to mitotic catastrophe with nuclear fragmentation in SLFN11-KO cells treated with CPT.

Hypothesizing that M4344 triggered abnormal replication and the premature entry of SLFN11-KO cells in S-phase, leading to improper chromosome segregation, we measured ssDNA, which is known to arise in response to replication stress (36) in cells treated with M4344 and CPT. To do so, we stained the cells with bromodeoxyuridine (BrdU) antibodies without denaturation following treatment with M4344 and CPT. To further investigate the molecular process leading to CDT1 degradation, we examined the molecular process leading to CDT1 degradation in response to replication stress to allow cells to compensate for stalled replication forks and finish genome duplication (36, 37, 50), we investigated whether ATR inhibition was associated with molecular alterations that could explain the catastrophic effects on both DNA replication and mitosis in SLFN11-KO cells. As shown in Fig. 3F, the levels of CDT1 (Cdc10-dependent transcript 1 protein), a licensing factor for the pre-RC (39, 51) were differentially affected by M4344 in SLFN11-KO and SLFN11-WT cells in response to CPT. While CDT1 levels were profoundly reduced in the SLFN11-WT cells treated with CPT alone or in combination with 4344, in the SLFN11-KO cells treated with CPT, CDT1 levels remained high and were even higher in the presence of M4344 (Fig. 3F). Additionally, CDT1 immunoblotting showed a slower migrating band in the SLFN11-KO cells treated with M4344 in the presence of CPT. We confirmed these results by fluorescence-conjugated quantification analysis (SI Appendix, Fig. S3F) and in a different isogenic CCRF-CEM SLFN11-WT and -KO cells (SI Appendix, Fig. S3G). Together, these experiments show that CDT1 degradation by CPT-induced damage is reduced in the absence of SLFN11 and that ATR inhibition further enhances CDT1 persistence in cells lacking SLFN11.

Next, we examined the molecular process leading to CDT1 hyperphosphorylation by cyclin-Dependent Kinases and Stabilization of Hyperphosphorylated CDT1 in Cells Lacking SLFN11. Because ATR can regulate replication origin firing in response to replication stress to allow cells to compensate for stalled replication forks and finish genome duplication (36, 37, 50), we investigated whether ATR inhibition was associated with molecular alterations that could explain the catastrophic effects on both DNA replication and mitosis in SLFN11-deficient cells, while ATR inhibition is ineffective in the SLFN11-proficient cells, which irreversibly arrest replication independently of ATR.

Delayed CDT1 Degradation Leads to Chemoresistance through Replication Recovery in SLFN11-Deficient Cells. Given that CDT1 is rapidly degraded upon completion of its licensing role during normal cell cycle and in response to radiation-mediated DNA
Inhibition of ATR leads to increased DNA damage and chromosomal defects with CDT1 hyperphosphorylation in SLFN11-deficient cells treated with CPT. (A) Confocal immunofluorescence staining of γH2AX in DU145 SLFN11-WT and -KO cells treated with CPT (100 nM) and M4344 (25 nM) for 24 h. (Upper) Representative images (γH2AX green). (Magnification, 63×). (Lower) γH2AX signal quantified by ImageJ. Error bars represent SD (n ≥ 50); ****P < 0.0001 Student’s t test; ns, not significant. (B) Confocal immunofluorescence staining of nuclei in cells treated with CPT and M4344 for 24 h. (Upper) Representative images (magnification, 63×) of nuclei/single cell, labeled with DAPI (blue). (Lower) Percentages of multinucleated cells after drug treatment (n = 100). (C) Confocal immunofluorescence staining of metaphase alignment in SLFN11-KO cells after treatment with CPT and M4344 for 16 h. Mitotic spindles were stained with α-tubulin (green) and chromosomes with DAPI (blue). (Magnification, 63×). (D) Cell cycle distribution analyzed by flow cytometry after treatment with CPT and M4344 for 24 h. Data are presented as mean values. Error bars represent SD (n = 3). (E) Relative EdU incorporation analyzed by flow cytometry. Cells were treated with CPT and M4344 for 24 h and pulsed with Edu (10 μM) for 30 min prior to harvesting. Error bars represent SD (n = 3). **P < 0.009, ****P < 0.0001 Student’s t test; (**P < 0.009, ****P < 0.0001 Student’s t test). (F) Protein expression levels of DNA replication initiation factors after treatment with CPT and M4344 for 24 h. (G) Retardation of CDT1 electrophoretic migration in response to combined treatment with CPT and M4344 in SLFN11-KO cells is due to hyperphosphorylation. SLFN11-KO cells were treated with CPT and M4344 for 24 h and then incubated with SAP (1 U/μL). (H) CDT1 hyperphosphorylation in response to combined treatment with CPT and M4344 is mediated by CDK. SLFN11-KO cells were treated with CPT, M4344 and roscovitine (20 μM) for 24 h and then analyzed by Western blotting. (I) CDT1 is hyperphosphorylated in time-dependent manner in response to combined treatment with CPT and M4344.
damage to keep genomic integrity (40, 45, 54), we examined whether alternations of CDT1 stability could be involved in the response to CPT and be modulated by SLFN11 and ATR. As shown in Fig. 3F, we observed the disappearance of CDT1 in response to CPT, particularly in the SLFN11-expressing cells. We confirmed this differential response after short treatment with CPT (Fig. 4A). While CDT1 protein levels remained stable in the SLFN11-KO cells, CDT1 disappeared in a CPT concentration-dependent manner in the SLFN11-WT cells. Time-course experiments also confirmed the limited CDT1 degradation in SLFN11-KO cells (Fig. 4B and C) and the disappearance of CDT1 at 2 to 4 h at the higher concentrations of CPT in SLFN11-WT DU145 cells (SI Appendix, Fig. S4 A and B). SLFN11-dependent CDT1 suppression was also observed in human small-cell lung cancer DMS114 SLFN11-WT and -KO cells and in human embryonic kidney HEK293 SLFN11-proficient and the HEK293T cells, which are SLFN11-deficient and chemoresistant (SI Appendix, Fig. S4 C–E). These results show that SLFN11 promotes CDT1 degradation. Yet, no difference in half-life of the CDT1 protein was observed in SLFN11-WT and -KO cells treated with the protein synthesis inhibitor cycloheximide (CHX) (SI Appendix, Fig. S4F), indicating that SLFN11-dependent degradation of CDT1 is a specific response to DNA damage.

Although both SLFN11-WT and KO cells were arrested in S-phase by CPT (Fig. 3D), SLFN11-WT cells were mostly arrested in early S-phase with accumulation of cyclins E and A, whereas SLFN11-KO cells were arrested in late S-phase with accumulation of cyclins A and B (SI Appendix, Fig. S5 D and E), implying that SLFN11-KO cells treated with CPT maintain some replication. To assess whether the differential CDT1 stability between the SLFN11-WT and -KO cells was coupled with DNA replication, we performed time-course experiments with EdU pulse-labeling. As shown in Fig. 4D, at 4 h DNA synthesis was suppressed by CPT regardless of SLFN11 expression. However, at later time points, DNA synthesis (EdU+ cells) recovered and even rebounded at 24 h in the SLFN11-KO cells, while in the SLFN11-WT cells DNA replication was continuously suppressed (Fig. 4D and SI Appendix, Fig. S4G).

We also examined whether the replication recovery in SLFN11-KO cells was accompanied by the firing of new replication origin using the DNA fiber-combing assays at the time point of the replication recovery phase (i.e., at 16 h of CPT treatment). As shown in Fig. 4E, CPT treatment significantly blocked origin firing in SLFN11-WT cells, whereas new origins in the SLFN11-KO cells were comparable to untreated cells, indicating that the new origin firing activity contributes to the replication recovery of SLFN11-KO cells.

To determine whether the replication recovery of SLFN11-KO cells was dependent on CDT1, we depleted endogenous CDT1 by siRNA before CPT treatment (SI Appendix, Fig. S4H). Silencing of CDT1 significantly reduced the replication recovery of SLFN11-KO cells (Fig. 4F and G). Because the origins licensed by CDT1 are subsequentially fired by phosphorylation and activation of the Mcm2 helicase by Cdc7 kinase (39, 55), we tested whether replication recovery in SLFN11-KO cells was impaired by inhibition of Cdc7 kinase. We first treated cells with CPT and subsequently added a Cdc7 inhibitor before the replication recovery phase. Replication recovery in SLFN11-KO cells was blocked by the Cdc7 inhibitor (Fig. 4H and I and SI Appendix, Fig. S4I). We also confirmed that Cdc7 inhibitor could overcome chemoresistance in SLFN11-KO cells (SI Appendix, Fig. S4 J and K). Similarly, chemoresistance to CPT was reversed by the WEE1 inhibitor AZD1775 (SI Appendix, Fig. S4L). Collectively, these data show that SLFN11-mediated replication arrest in response to DNA damage is linked with the degradation of CDT1, and that the absence of CDT1 degradation in SLFN11-deficient cells allows those cells to recover replication and gain resistance against CPT.
The C Terminus ATPase/Helicase Domain of SLFN11 Binds and Activates DDB1–CUL4 in Response to Replicative DNA Damage. To understand how SLFN11 interacts with DDB1, we performed IP with antibodies specifically targeting the N- and C-terminus regions of SLFN11 in 293T cells, which are SLFN11-deficient (2). We transiently cotransfected them with DDB1-Flag and two different SLFN11 expression constructs corresponding to deletions of the N terminus (1 to 452 aa) or the C terminus (450 to 901 aa) (Fig. 5D). The SLFN11 C-terminal deletion-mutant failed to interact with DDB1 whereas the N-terminal deletion-mutant retained the ability to interact with DDB1 (Fig. 5D). Furthermore, transfection of the C-terminal deletion-mutant failed to suppress EdU incorporation in CPT-treated cells (SI Appendix, Fig. S5F). These data indicate that the C-terminal region of SLFN11 is required for the interaction of SLFN11 with DDB1 and suppression of DNA replication.

Given that the C-terminal region of SLFN11 contains a Walker B/ATPase motif critical for the nuclear DDR functions of SLFN11 (3, 5), we tested whether mutation/inactivation of this ATPase motif (E669Q) (5) would affect SLFN11 binding to DDB1 whereas the N-terminal deletion-mutant.

Fig. 4. Defective CDT1 degradation causes replication recovery in SLFN11-deficient cells. (A) DU145 SLFN11-WT and -KO cells were treated with the indicated concentrations of CPT for 4 h. Protein levels were analyzed by Western blotting. (B) DU145 SLFN11-WT and -KO cells were treated with CPT (100 nM) for the indicated times. Levels of protein expression were analyzed by Western blotting. (C) Quantitation of CDT1 as shown in B. Bars show the mean band intensity from triplicate experiments, normalized to GAPDH. *P < 0.0274, **P < 0.0077. (D) Percentage of EdU+ S-phase cells in the time course treatment of CPT was determined by flow cytometry. Error bars represent SD (n = 3). *P < 0.0335, **P < 0.0025, ***P < 0.0005 Student’s t test. (E, Upper) Labeling protocols for the DNA combing assay. Cells were treated with CPT (100 nM) for 16 h and then pulse-labeled sequentially with IdU (100 μM) and CldU (100 μM) for 1 h. (Lower) Frequencies of new origins only labeled by the CldU pulse. Error bars represent SD (n = 3). ***P < 0.0009 Student’s t test. (F) CDT1-dependent replication recovery. SLFN11-KO cells were transfected with siControl (Ctrl) or siCDT1 for 48 h, and then treated with CPT for 24 h. Cells were incubated with EdU (10 μM) for 30 min prior to harvest. The percentage of EdU+ and DAPI+ cells was determined by flow cytometry. (G) Quantification of the EdU+ cells in S-phase. Error bars represent SD (n = 3). **P < 0.008 Student’s t test.

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Fig. 4. Defective CDT1 degradation causes replication recovery in SLFN11-deficient cells. (A) DU145 SLFN11-WT and -KO cells were treated with the indicated concentrations of CPT for 4 h. Protein levels were analyzed by Western blotting. (B) DU145 SLFN11-WT and -KO cells were treated with CPT (100 nM) for the indicated times. Levels of protein expression were analyzed by Western blotting. (C) Quantitation of CDT1 as shown in B. Bars show the mean band intensity from triplicate experiments, normalized to GAPDH. *P < 0.0274, **P < 0.0077. (D) Percentage of EdU+ S-phase cells in the time course treatment of CPT was determined by flow cytometry. Error bars represent SD (n = 3). *P < 0.0335, **P < 0.0025, ***P < 0.0005 Student’s t test. (E, Upper) Labeling protocols for the DNA combing assay. Cells were treated with CPT (100 nM) for 16 h and then pulse-labeled sequentially with IdU (100 μM) and CldU (100 μM) for 1 h. (Lower) Frequencies of new origins only labeled by the CldU pulse. Error bars represent SD (n = 3). ***P < 0.0009 Student’s t test. (F) CDT1-dependent replication recovery. SLFN11-KO cells were transfected with siControl (Ctrl) or siCDT1 for 48 h, and then treated with CPT for 24 h. Cells were incubated with EdU (10 μM) for 30 min prior to harvest. The percentage of EdU+ and DAPI+ cells was determined by flow cytometry. (G) Quantification of the EdU+ cells in S-phase. Error bars represent SD (n = 3). **P < 0.008 Student’s t test.
Fig. 5. SLFN11 promotes CDT1 degradation by binding to the CUL4–DDB1 complex in response to replicative DNA damage. (A) Representative interactors of SLFN11. DU145 cells were treated with CPT for 16 h. Nuclear cell lysates were immunoprecipitated with anti-SLFN11 antibody and probed with anti-Flag antibody. (Bottom) Same as above but using anti-SLFN11 antibody (E4) that detects the C terminus region of SLFN11. Interacting proteins were identified by Western blotting. (Middle) 293T cells transfected with DDB1-Flag and indicated SLFN11 constructs were treated with CPT (10 nM) and MG134 for 72 h. Cell viability was analyzed by CellTiter-Glo. (B) HEK293 cells transfected with a DDB1-Flag construct for 2 d were treated with CPT (10 nM) and MG134 for 4 h. Cells were immunoprecipitated with anti-SLFN11 antibody (D2) that detects the N terminus region of SLFN11. Interacting proteins were profiled by Western blotting. (C) Interaction between endogenous SLFN11 and DDB1 was assessed by immunoprecipitation with anti-SLFN11 antibody (D2) and analyzed by mass spectrometry. (D) HEK293 cells transfected with a DDB1-Flag construct for 2 d were treated with CPT (10 nM) and MG134 for 4 h were lysed and immunoprecipitated with anti-Flag antibody. Interacting proteins were profiled by Western blotting. (E) Interaction between endogenous SLFN11 and DDB1 was assessed by immunoprecipitation with anti-SLFN11 antibody (D2) and analyzed by mass spectrometry. (Middle) HEK293 cells transfected with a DDB1-Flag construct for 2 d were treated with CPT (10 nM) and MG134 for 4 h were lysed and immunoprecipitated with anti-Flag antibody. Interacting proteins were profiled by Western blotting. (F) Interaction between endogenous SLFN11 and DDB1 was assessed by immunoprecipitation with anti-SLFN11 antibody (D2) and analyzed by mass spectrometry. (Middle) HEK293 cells transfected with a DDB1-Flag construct for 2 d were treated with CPT (10 nM) and MG134 for 4 h were lysed and immunoprecipitated with anti-Flag antibody. Interacting proteins were profiled by Western blotting. (G) Interaction between endogenous SLFN11 and DDB1 was assessed by immunoprecipitation with anti-SLFN11 antibody (D2) and analyzed by mass spectrometry. (Middle) HEK293 cells transfected with a DDB1-Flag construct for 2 d were treated with CPT (10 nM) and MG134 for 4 h were lysed and immunoprecipitated with anti-Flag antibody. Interacting proteins were profiled by Western blotting. (H) Interaction between endogenous SLFN11 and DDB1 was assessed by immunoprecipitation with anti-SLFN11 antibody (D2) and analyzed by mass spectrometry. (Middle) HEK293 cells transfected with a DDB1-Flag construct for 2 d were treated with CPT (10 nM) and MG134 for 4 h were lysed and immunoprecipitated with anti-Flag antibody. Interacting proteins were profiled by Western blotting. (I) Interaction between endogenous SLFN11 and DDB1 was assessed by immunoprecipitation with anti-SLFN11 antibody (D2) and analyzed by mass spectrometry. (Middle) HEK293 cells transfected with a DDB1-Flag construct for 2 d were treated with CPT (10 nM) and MG134 for 4 h were lysed and immunoprecipitated with anti-Flag antibody. Interacting proteins were profiled by Western blotting.
DDB1. Similar binding was observed after transfection of SLFN11-WT and SLFN11-E669Q (Fig. 5G). To test whether the Walker B/ATPase motif was required for CDT1 degradation, we used human leukemia K562 cells, which are deficient for SLFN11 (25), and which were previously engineered to stably express SLFN11-WT or SLFN11-E669Q (5). SLFN11-WT complementation resulted in CDT1 degradation, whereas complementation with the SLFN11-E669Q mutant failed to induce CDT1 degradation after CPT treatment (Fig. 5E). As previously shown for PARP inhibitors (4), the K562 cells expressing the SLFN11-E669Q mutant failed to reverse the CPT resistance of K562 cells compared with those expressing SLFN11-WT (Fig. 5F). Furthermore, treatment with the ATR inhibitor M4344 significantly abolished the chemoresistance of the SLFN11-E669Q-complemented cells like SLFN11-deficient parental cells (Fig. 5F). Taken together, these results show that the Walker B/ATPase motif of SLFN11 in the C-terminal domain of SLFN11 is critical for the SLFN11-induced degradation of CDT1 and growth arrest in response to CPT.

Although SLFN11 has not yet been the focus of extensive and systematic high-resolution genomic sequencing studies, analysis of the TCGA database revealed that the same residue (E669) used for inactivating the Walker B/ATPase motif of SLFN11 is mutated in five patient samples as E669K mutation (Fig. 5H). To determine whether this patient mutation exhibits the same phenotype as the E669Q mutation, a SLFN11-E669K construct was generated by site-directed mutagenesis and transfected HEK293T cells (SI Appendix, Fig. S5A) to analyze the effects of the E669K mutation on CPT-induced CDT1 degradation and cytotoxicity. The SLFN11-E669K mutant construct was defective in inducing CDT1 degradation compared with SLFN11-WT (SI Appendix, Fig. S5H). It also failed to sensitize cells to CPT (Fig. 5F). From these findings, we conclude that the Walker B/ATPase motif of SLFN11 is required both for chemosensitivity and CDT1 degradation.

**Discussion**

The present study elucidates the coordination of SLFN11 and ATR in the cellular responses to replication stress. Although both are recruited to stressed replication forks by binding to RPA filaments (3, 5, 31, 32, 36), their impact on replication is strikingly different (Fig. 6). While ATR produces a transient replication arrest allowing the cell to repair and resume replication (32), SLFN11 irreversibly blocks replication and cellular proliferation (6). In this study, we relate this divergence to the differential molecular effects of SLFN11 and ATR on the pre-RC and mitotic licensing factor CDT1 and show a previously unsuspected activating role of SLFN11 on the replication-associated DDB1–CUL4CDT2 E3 ligase complex.

**Overcoming the Chemoresistance of SLFN11-Deficient Cancer Cells by Targeting the ATR/CHK1 Pathway.** SLFN11 is genetically inactivated in ~50% of all cancer lines and cancer tissues (6, 25) and studies are ongoing in various research institutions to establish clinically whether SLFN11 inactivation determines resistance to anticancer treatments as it does in preclinical models (20). In this study, we demonstrate that inhibition of the ATR pathway in SLFN11-deficient cancer cells overcomes chemoresistance to a broad range of clinical DNA-damaging agents targeting TOP1 (TPT, irinotecan [LMP400]), TOP2 (etoposide), PARP (talazoparib), DNA (cisplatin), WEE1 (AZD1775), and Cdc7 (PHA-767491). We first used a genome-wide RNAi screening approach to identify pathways that are synthetic lethal in cells lacking SLFN11 and treated with the TOP1 inhibitor CPT, and found that the top genetics hits were inactivation of ATR as well as depletion of other key components in the ATR pathway. We next validated the clinical applicability of our finding with clinical inhibitors of the ATR/CHK1 pathway combined with various classes of chemotherapeutic DNA-targeted drugs. These results provide a rationale for the clinical testing and potential clinical use of ATR/CHK1 inhibitors combined with DNA-targeted drugs, as well as SLFN11 as a potential predictive biomarker of sensitivity to ATR/CHK1 inhibitors.

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**Fig. 6.** Proposed model for SLFN11-mediated replication reactivation block and synthetic lethality of ATR/CHK1 inhibitors in SLFN11-negative cancer cells. In SLFN11-proficient cells, SLFN11 is recruited to chromatin by DNA damage. It subsequently binds to DDB1 and promotes the degradation of CDT1 and other substrates as a cofactor of activated DDB1–CUL4CDT2. Consequently, CDT1-mediated replication recovery is irreversibly blocked, leading to sensitivity to DNA-damaging agents. In SLFN11-deficient cells, conversely, DDB1–CUL4CDT2-mediated degradation of CDT1 is reduced. CDT1 then promotes replication recovery by activating dormant origins, resulting in chemoresistance. Treatment of ATR/CHK1 inhibitor reverses chemoresistance of SLFN11-deficient cells by inducing premature replication and mitosis through CDT1 phosphorylation, which induces mitotic catastrophe and cell death.

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SLFN11 promotes CDT1 degradation by CUL4 in response to replicative DNA damage, while its absence leads to synthetic lethality with ATR/CHK1 inhibitors.
development of these agents with ATR and CHK1 inhibitors in chemoresistant SLFN11-negative tumors.

Indeed, based on the fact that epigenetic gene silencing is a common cause of SLFN11 inactivation in tumorigenesis, reactivation of SLFN11 by the DNA demethylating drug 5-aza-2'-deoxycytidine, the EZH2 inhibitor EPZ011989 and the HDAC inhibitors entinostat or romidepsin reactivates SLFN11-deficient cells to clinical DNA-damaging agents (11, 21, 25, 26). Although epigenetic therapies can efficiently reexpress SLFN11, they also reactivate normally silenced genes related to genome instability, carcinogenesis and antiapoptotic pathways (59, 60).

Targeting selectively SLFN11-negative cancer cells with ATR or CHK1 inhibitors is an alternative approach and potential synthetic-lethal strategy to overcome resistance in SLFN11-deficient cancers. We recently reported that the preclinical ATR inhibitor VE-821 can overcome resistance of SLFN11-deficient cancer cell lines to the clinical PARP inhibitors olaparib and talazoparib (4). Here, we demonstrate the importance of the ATR-SLFN11 connection by showing that genetic inactivation of ATR, CHK1, or RPA1 selectively sensitizes SLFN11-KO cells to CPT and that clinical inhibitors of ATR and CHK1 selectively sensitize SLFN11-KO cells not only to CPT but also to other Food and Drug Administration-approved clinical anticancer agents (TPT, etoposide, cisplatin, and talazoparib).

Potent and selective ATR inhibitors are in clinical development (33, 61). Given that ATR responds and protects cells against replication stress as a primary sensor, combinations of ATR inhibitors are actively studied with chemotherapeutic drugs that induce replication stress. Here, we tested two clinical ATR inhibitors, M6620 (VE-822, VX-970, berzosertib) and M4344 (also known as VX-803). M4344 is a potent and tight-binding inhibitor of ATR, orally bioavailable and currently undergoing phase 1 trial (NCT02278250). Both M6620 and M4344 showed high synergy at nontoxic nanomolar concentrations with clinical DNA-damaging agents in SLFN11-deficient cancer cells. Consistent with the ATR inhibitor and genetic screen results, we found that the CHK1 inhibitor SRA737, a new orally bioavailable small molecule in clinical trials (NCT02797964, NCT02797977) (46), also overcame the chemoresistance of SLFN11-KO cells. Considering that several ATR/CHK1 inhibitors are currently in clinical trials (33), our results provide rationale for their combinations with clinical DNA-damaging agents in multidrug-resistant SLFN11-deficient tumors.

At the molecular level, inhibition of the ATR/CHK1 pathway enhances replication stress induced by DNA-targeted anticancer agents by preventing the transient inactivation of CDKs (SI Appendix, Fig. S6), thereby abrogating the replication and G2 checkpoints, eventually leading to unscheduled replication origin firing (62), defective DNA repair, mitotic catastrophe, and cell death (33, 61). Our study provides a previously unnoted insight regarding the molecular mechanism by which inhibiting ATR impinges on the replication licensing factor CDT1 in SLFN11-KO cells. Indeed, we found that, instead of being degraded in ATR, CHK1, or RPA1 selectively sensitize cancer cell lines to the clinical PARP inhibitors olaparib and deficiency may contribute to alteration of the ATR pathway (27). In conclusion, the differential activity of ATR inhibitors depending on SLFN11 expression offers opportunities to gain new molecular insights on the molecular cross-talks between the cell survival (ATR) and death (SLFN11) pathways (SI Appendix, Fig. S6) and provides a rationale for the clinical development of ATR inhibitors in combination with chemotherapeutic agents targeting DNA replication.

Materials and Methods
All materials including cell lines, reagents such as inhibitors, antibiotics, DNA oligos for CRISPR/CAS9 editing, RNA interference, and gene cloning can be found in SI Appendix. A genome-wide RNAi screen analysis was performed using the Silencer Select Human Druggable siRNA library (Thermo Fisher Scientific, Grand Island, NY). All detailed methods including RNAi screen, cell viability assay, siRNA transfection, apoptosis assay, immunofluorescence microscopy, flow cytometry, Western blot analysis, DNA combing assay, and mass spectrometry are described in the SI Appendix.

Data Availability. All study data are included in the article and supporting information.

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