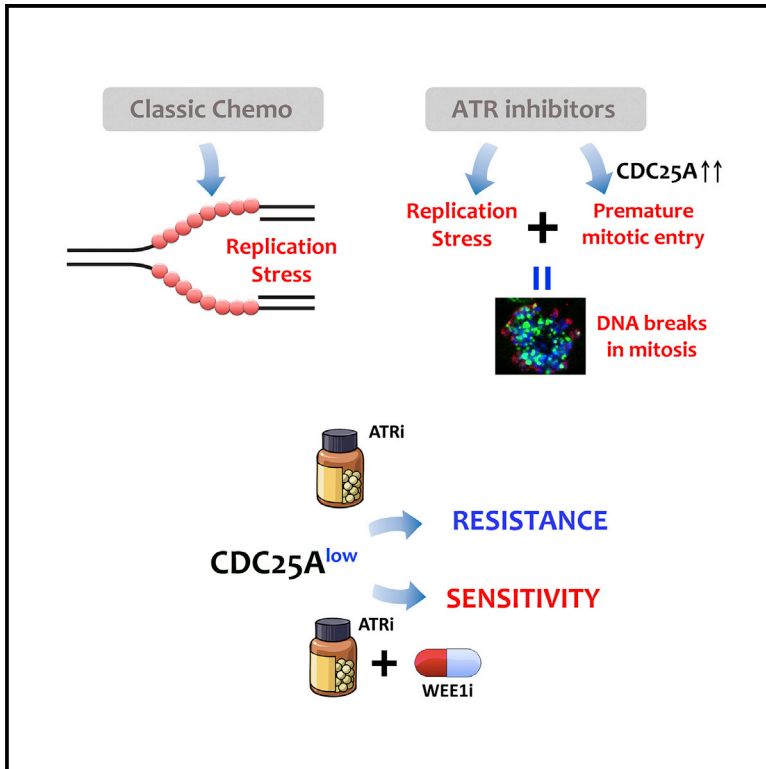


# A Genome-wide CRISPR Screen Identifies CDC25A as a Determinant of Sensitivity to ATR Inhibitors

## Graphical Abstract



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## In Brief

ATR inhibitors are being developed for anticancer therapy. By developing a platform for conducting CRISPR screenings in primary stem cells, Ruiz et al. reveal that low levels of CDC25A render mammalian cells resistant to ATR inhibitors.

## Highlights

- A stem cell line improves CRISPR-mediated deletions in primary mammalian cells
- CDC25A deficiency renders mammalian cells resistant to ATR inhibitors
- ATR inhibition generates replication stress and promotes premature mitotic entry
- The toxicity of ATR inhibitors is due to DSBs generated in mitosis



# A Genome-wide CRISPR Screen Identifies CDC25A as a Determinant of Sensitivity to ATR Inhibitors

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## SUMMARY

One recurring theme in drug development is to exploit synthetic lethal properties as means to preferentially damage the DNA of cancer cells. We and others have previously developed inhibitors of the ATR kinase, shown to be particularly genotoxic for cells expressing certain oncogenes. In contrast, the mechanisms of resistance to ATR inhibitors remain unexplored. We report here on a genome-wide CRISPR-Cas9 screen that identified CDC25A as a major determinant of sensitivity to ATR inhibition. CDC25A-deficient cells resist high doses of ATR inhibitors, which we show is due to their failure to prematurely enter mitosis in response to the drugs. Forcing mitotic entry with WEE1 inhibitors restores the toxicity of ATR inhibitors in CDC25A-deficient cells. With ATR inhibitors now entering the clinic, our work provides a better understanding of the mechanisms by which these compounds kill cells and reveals genetic interactions that could be used for their rational use.

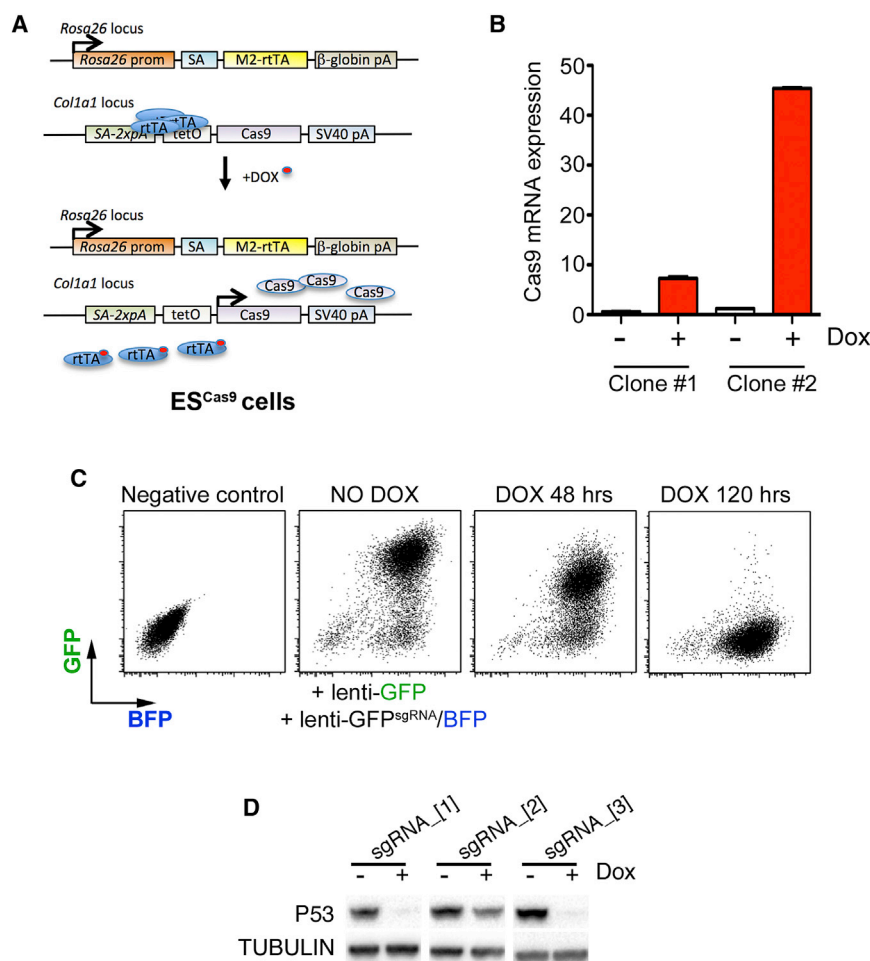
## INTRODUCTION

Targeting DNA repair enzymes is an active area of drug development in cancer therapy. The interest was fueled by the discovery of synthetic lethal interactions, such as the selective toxicity of poly(ADP-ribose) transferase inhibitors for cells lacking BRCA tumor suppressors (Bryant et al., 2005; Farmer et al., 2005). One widespread feature of cancer cells is the presence of replication stress (RS), which is driven by the underlying oncogenes and is responsible for a large fraction of the genomic rearrangements found in cancer cells (Halazonetis et al., 2008; Lecona and Fernández-Capetillo, 2014). RS stands for the accumulation of single-stranded DNA (ssDNA) at stalled replication forks, which can promote the nucleolytic breakage of the fork and subsequent recombination events, as well as overall replication catastrophe through the exhaustion of ssDNA-binding proteins

(Toledo et al., 2013). In mammals, RS is sensed and suppressed by a signaling cascade initiated by the ATR kinase (Cimprich and Cortez, 2008; López-Contreras and Fernandez-Capetillo, 2010). Recent evidence has also revealed the existence of a backup pathway, controlled by DNAPK and CHK1 kinases, that limits ssDNA in conditions of limited ATR activity (Buisson et al., 2015). We previously hypothesized that targeting ATR should be particularly deleterious for cancer cells experiencing high levels of oncogene-induced RS. Accordingly, mice with reduced ATR levels are refractory to the development of various tumors (Murga et al., 2011; Schoppy et al., 2012), and ATR inhibitors are preferentially toxic for cells expressing MYC or CYCE oncogenes, or lacking tumor suppressors such as ATM or P53 (Kwok et al., 2015; Reaper et al., 2011; Toledo et al., 2011). In addition, other cancer-associated conditions, such as the use of the alternative lengthening of telomeres pathway for telomere maintenance, also increase sensitivity to ATR inhibitors (Flynn et al., 2015). In contrast to mutations that sensitize to these compounds, whether resistance to ATR inhibitors can occur remains unknown.

## RESULTS

In order to develop genome-wide clustered regularly interspaced short palindromic repeats (CRISPR) screens, we first developed murine embryonic stem cells (ESCs) carrying a doxycycline (Dox)-inducible Cas9 cDNA (ES<sup>Cas9</sup>). We used a previously developed system whereby the Cas9 cDNA under the control of a tetracycline responsive operator (tetO) was placed at the 3' UTR of the ubiquitously expressed *Col1a1* locus, and the expression of reverse tetracycline-controlled transactivator (rtTA) was driven by the ROSA26 promoter (Beard et al., 2006) (Figure 1A). This two-tier system provides a stringent expression of Cas9, thereby preventing nuclease activity until Dox addition. Two clones showing clear Dox-inducible Cas9 expression were selected for further experiments (Figures 1B, S1A, and S1B). To determine the efficiency of CRISPR-Cas9 editing in these cells, we co-infected a clone of ES<sup>Cas9</sup> cells with lentiviruses expressing GFP and with lentiviruses expressing both a *Gfp*-targeting single guide RNA (sgRNA) and blue fluorescent protein (BFP). After sorting and before the addition of Dox, cells expressing both GFP and BFP dominated the culture. Forty-eight hours after Dox,



**Figure 1. Efficient and Dox-Inducible Gene Knockouts in ES<sup>Cas9</sup> Cells**

(A) Scheme illustrating the two-allele system used for the generation of ES<sup>Cas9</sup> cells. In this previously described system (Beard et al., 2006), the Cas9 cDNA is placed under the control of a tet-responsive sequence (tetO) at the *Col1a1* locus. At the same time, rtTA is expressed from the *Rosa26* locus, providing Dox-inducible activation of Cas9 expression.

(B) Levels of Cas9 mRNA evaluated by RT-PCR (normalized to levels of GAPDH mRNA) in the two clones of ES<sup>Cas9</sup> cells used in this study. The high stringency of the system prevents cleavage in the absence of Dox. Data are represented as mean  $\pm$  SD ( $n = 3$ ). See also Figures S1A and S1B.

(C) FACS analysis illustrating the loss of GFP signal in ES<sup>Cas9</sup> cells that were made GFP positive by infection with a lentiviral construct expressing GFP and simultaneously infected with a lentivirus expressing a GFP-targeting sgRNA together with BFP. Doubly infected cells are thus BFP and GFP positive and gradually lose GFP expression after the addition of Dox. See also Figure S1C.

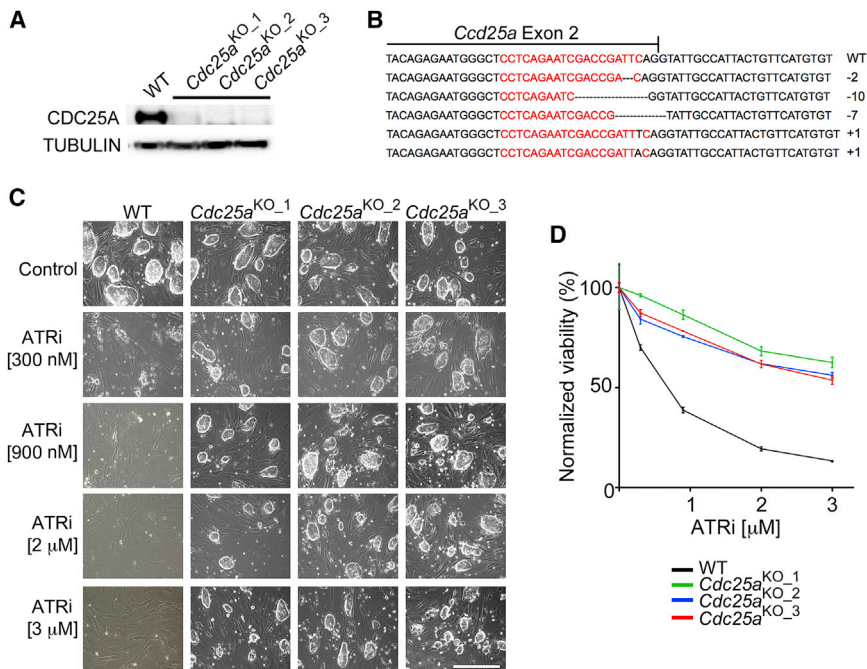
(D) Western blot illustrating the Dox-dependent loss of P53 expression observed in ES<sup>Cas9</sup> cells that were infected with lentiviruses expressing three independent P53-targeting sgRNAs. TUBULIN levels are shown as a loading control. Dox was used at 2  $\mu$ g/ml.

the GFP (but not BFP) signal started to decrease and was virtually absent by 5 days of treatment (Figure 1C). To further characterize the efficiency of the system at endogenous loci, three different lentiviruses carrying sgRNAs against P53 were used to independently infect ES<sup>Cas9</sup> cells. After infection, a Dox-inducible reduction in P53 levels was detectable with all sgRNAs (Figure 1D). Together, these results revealed that ES<sup>Cas9</sup> cells provide a very efficient platform for obtaining nullizygous mutations in primary mammalian cells by CRISPR-Cas9 editing and prompted us to conduct forward genetic screenings using this system.

To perform our screenings, we used a recently described library targeting 19,150 mouse genes with 87,897 sgRNAs (Koike-Yusa et al., 2014). ES<sup>Cas9</sup> cells were infected with the library at a low MOI to enrich in cells carrying a single lentiviral copy and sorted for BFP expression (Figure S1C). Infected cells were subsequently treated with Dox for 10 days to induce CRISPR-mediated mutations. To confirm the quality of the library and as proof of principle, we first conducted a screening for resistance against 6-thioguanine (6TG), an inert compound that is converted to toxic inside cells by the action of hypoxanthine-guanine phosphoribosyltransferase (HPRT). Five million cells from the mutagenized library were exposed to 20  $\mu$ M 6TG

for 7 days, a time and dose at which no wild-type (WT) ES<sup>Cas9</sup> cells survive. Four of 5 of the resistant clones that were isolated carried sgRNAs targeting *Hprt* (Table S1). Using this library, a second screening was performed exposing the cells for 9 days to 0.9  $\mu$ M of an ATR inhibitor (ATRI) developed by our group in a previous chemical screen (Toledo et al., 2011), again a dose and time at which no WT cells survive. A 3-day treatment with 300 nM ATRI suffices to kill all WT ES<sup>Cas9</sup> cells, so that our screening was performed under highly stringent conditions. Five of 7 of the resistant clones that could be isolated in this screen carried sgRNAs that targeted the phosphatase CDC25A (Table S1). To strengthen our observations, we generated a new library using an independent clone of ES<sup>Cas9</sup> cells and repeated the screen. In this case, 6 of 6 of the 6TG-resistant clones carried sgRNAs targeting *Hprt* (Table S1). As for ATRI, we obtained 16 resistant clones, 6 of which targeted *Cdc25a*. This second library also identified sgRNAs targeting *Cnot8*, a member of the CCR4-NOT deadenylase complex (Collart and Panasenko, 2012), in a large fraction of the resistant clones (6 of 16) (Table S1). Given the recurrence of *Cdc25a*-targeting sgRNAs in both libraries, we focused this study in understanding the mechanism by which CDC25A deficiency limits the toxicity of ATR inhibitors.

To verify that CDC25A deficiency confers resistance to ATR inhibition, we first analyzed CDC25A expression in three independent ATRI-resistant clones isolated from the screen. Western blotting revealed the absence of CDC25A protein expression in



**Figure 2. Resistance to ATR Inhibition in CDC25A-Deficient ES<sup>Cas9</sup> Cells**

(A) Western blot illustrating the loss of CDC25A expression in three independent ATRi-resistant clones.

(B) Examples of the mutations in *Cdc25a* identified in ATRi-resistant ES<sup>Cas9</sup> cells shown in (A). Note that all mutations include small insertions or deletions that change the reading frame in exon 2, precisely at the 3' of the sgRNA sequence (in red).

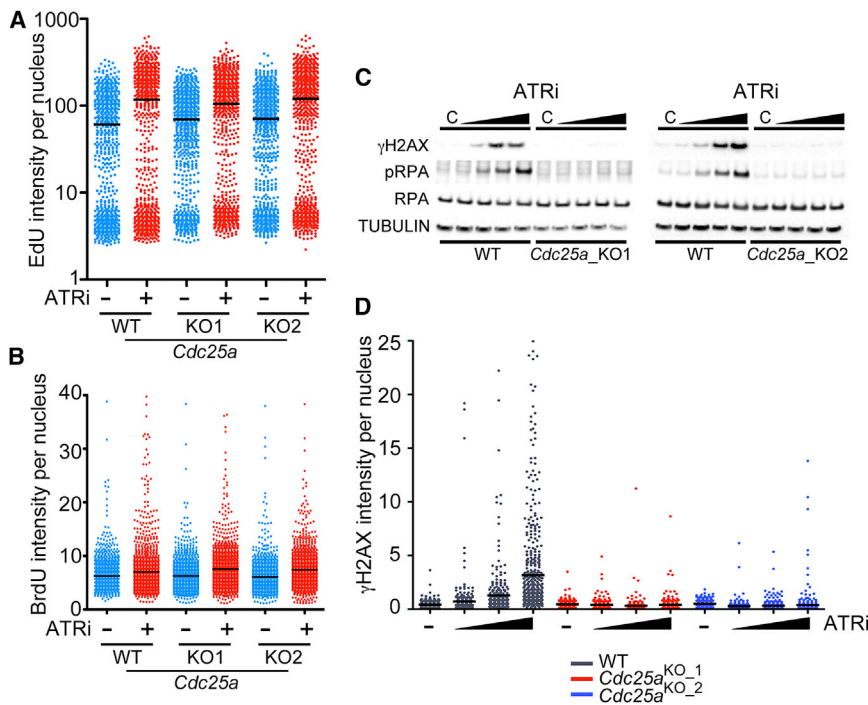
(C) Representative pictures of the resistance to ATRi observed in CDC25A-deficient ES<sup>Cas9</sup> cells exposed to the compound for 72 hr. ES cells are grown on top of a feeder layer of growth-arrested murine embryonic fibroblasts, which are unaffected by the treatment. The scale bar (white) represents 25  $\mu$ m.

(D) XTT viability assay in WT and CDC25A-deficient ES<sup>Cas9</sup> cells exposed to ATRi for 24 hr at the indicated doses. Data are representative of three independent experiments. Error bars indicate SD. See also Figure S2.

these cells (Figure 2A), all of which carried small deletions or mutations at the sequence of *Cdc25a* targeted by the sgRNA (Figure 2B). Multiple lines of evidence confirmed a high degree of resistance against ATR inhibitors in CDC25A-deficient cells. First, whereas chronic exposure to ATRi kills all WT ES<sup>Cas9</sup> cells at doses above 300 nM, CDC25A-deficient cells were able to expand at doses up to 3  $\mu$ M (Figure 2C). XTT viability assays confirmed the resistance of the mutant clones to ATRi (Figure 2D). Moreover, in all clones, CDC25A deficiency also conferred resistance to an independent ATR inhibitor (AZ-20) (Foote et al., 2013) and to the CHK1 inhibitor UCN-01 (Figure S2A). Finally, and to discard any potential off-target effects of the sgRNA identified in the screen, we generated additional CDC25A-deficient cells by infecting ES<sup>Cas9</sup> cells with lentiviruses expressing four independent sgRNA sequences targeting *Cdc25a*. After selection, all pools of infected cells acquired resistance to ATRi in response to Dox (Figure S2B). Consistent with our findings in mouse cells, CDC25A heterozygosity in HAP1 cells (Figures S2C–S2E) or its depletion by small interfering RNA (siRNA) in five additional human cancer cell lines (Figures S2F and S2G) invariably led to resistance to ATRi. Finally, we also tested whether increased CDC25A expression enhanced sensitivity to ATR inhibition. Indeed, CDC25A overexpression sensitized NIH 3T3 cells to ATRi (Figures S2H and S2I). Moreover, CDC25A expression is highest in tumors that are already known to be particularly dependent on the ATR/CHK1 response, such as Burkitt lymphomas, acute lymphoblastic or myeloid leukemias, and diffuse large B cell lymphomas (Figure S2J) (Derenzini et al., 2015; Kwok et al., 2015; Murga et al., 2011; Santos et al., 2014; Sarmento et al., 2015; Schoppy et al., 2012). Together, these results reveal that CDC25A levels determine the sensitivity of mammalian cells to ATR inhibitors.

We next tried to address the mechanism by which loss of CDC25A promoted resistance to ATR inhibitors. First, we evaluated whether ATR inhibition generated RS and/or chromosomal breakage in CDC25A-deficient cells. To determine whether this was the case, we first analyzed replication dynamics in response to ATRi. In vertebrates, depletion of ATR leads to increased origin firing and, as a consequence, slower progression at each individual fork (Eykelboom et al., 2013). Consistent with an increase in origin firing, chemical inhibition of ATR in ES<sup>Cas9</sup> cells led to a rapid overall increase in EdU incorporation rates as measured by high-throughput microscopy (HTM), which was, however, not affected by CDC25A deficiency (Figure 3A). Moreover, ATR inhibitors induced equivalent amounts of ssDNA in WT and *Cdc25a*<sup>−/−</sup> ES<sup>Cas9</sup> cells (Figure 3B). Next, we monitored the impact of ATR inhibition at individual replication forks by stretched DNA fiber analyses. Despite CDC25A-deficient cells presented a slightly lower density of replication forks in unchallenged conditions, the addition of ATRi increased origin firing and decreased fork rates in both WT and *Cdc25a*<sup>−/−</sup> ES<sup>Cas9</sup> cells (Figures S3A and S3B). Surprisingly, and in contrast to the modest reduction observed in CDC25-deficient ES<sup>Cas9</sup> cells on the amount of ATRi-induced RS, chromosomal breakage induced by the drug was fully abrogated in *Cdc25a*<sup>−/−</sup> cells, measured by western blotting against  $\gamma$ H2AX and RPA32-S4/8P (Figure 3C), as well as by quantifying  $\gamma$ H2AX and 53BP1 nuclear foci by HTM (Figures 3D and S3C). Hence, despite the fact that ATR inhibitors generate RS in both WT and mutant cells, this is only translated into cytotoxic DNA double-strand breaks (DSBs) in the presence of CDC25A. Whereas we cannot discount that some cell death might be due to RS instead of DSB, we do not think that an altered RS response or intrinsic resistance to RS can account for the resistance to ATRi in CDC25A-deficient cells. Accordingly, *Cdc25a*<sup>−/−</sup> ES<sup>Cas9</sup> cells show normal levels





**Figure 3. ATR Inhibition Generates RS, but Not DSB, in CDC25A-Deficient Cells**

(A) HTM analysis of the EdU signal per individual nucleus in wild-type (WT) and CDC25A-deficient ES<sup>Cas9</sup> cells exposed to ATRi for 1 hr (900 nM).

(B) HTM analysis of the BrdU signal accumulated in ssDNA per individual nucleus in WT and CDC25A-deficient ES<sup>Cas9</sup> cells exposed to ATRi for 4 hr (900 nM).

(C) Western blot illustrating the levels of H2AX (γH2AX) and RPA (S4/8) phosphorylation in WT and CDC25A-deficient ES<sup>Cas9</sup> cells exposed to ATRi for 4 hr (0, 0.3, 0.9, 2, and 3 μM). TUBULIN and total RPA levels are shown as loading controls.

(D) HTM analysis of the γH2AX signal per individual nucleus in WT and CDC25A-deficient ES<sup>Cas9</sup> cells treated (or not) with ATRi (0.3, 0.9, and 2 μM; 4 hr).

See also Figure S3.

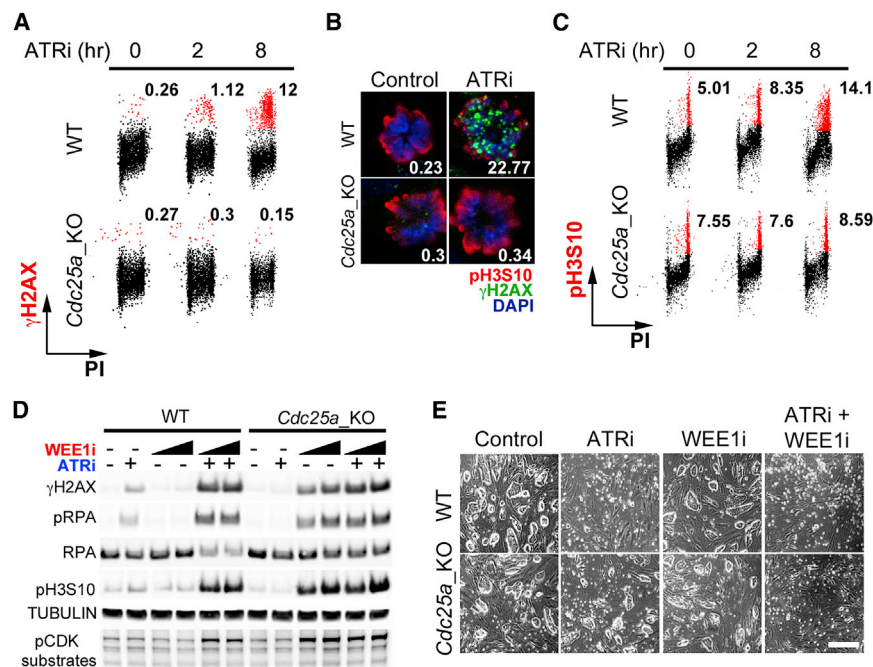
of CHK1 phosphorylation upon exposure to the RS-inducing agent hydroxyurea and are not resistant to this drug (Figures S3D and S3E).

CDC25A was originally discovered as a mitosis-promoting factor (Russell and Nurse, 1986), which promotes M phase entry by eliminating inhibitory phosphorylations in CDK1 that are placed by the WEE1 kinase (Galaktionov and Beach, 1991). Later studies revealed that in response to DNA damage, CHK1-dependent phosphorylation of CDC25A targets it for degradation, thereby activating the checkpoints that limit the expansion of damaged cells (Falck et al., 2001; Mailand et al., 2000; Peng et al., 1997; Sanchez et al., 1997). Hence, we first asked whether ATR inhibition would increase CDC25A levels. In fact, exposure to ATRi led to a dose-dependent increase in CDC25A levels in ES<sup>Cas9</sup> cells (Figure S4A). Of note, whereas we could validate that CNOT8 deficiency renders ES<sup>Cas9</sup> cells resistant to ATRi, this phenotype was not linked to CDC25A expression (Figures S4B and S4C).

Recent studies have indicated that the generation of DSBs by RS is due to unreplicated regions of the genome reaching mitosis (reviewed in Mankouri et al., 2013). Consistently, fluorescence-activated cell sorting (FACS) analyses revealed that ATRi-induced γH2AX occurred in cells with a late G2/M DNA content, which was again absent in *Cdc25a*<sup>-/-</sup> ES<sup>Cas9</sup> cells (Figure 4A). Moreover, immunofluorescence analyses confirmed the presence of ATRi-induced γH2AX foci in mitotic cells, identified by the presence of H3-S10 phosphorylation, in WT but not in CDC25A-deficient cells (Figure 4B). We thus hypothesized that chromosomal breaks induced by ATR inhibitors could be due to a premature entry into M phase of cells undergoing RS provoked by an increase of CDC25A levels. Supporting this view, FACS analyses revealed that ATR inhibitors promoted mitotic

entry in ES<sup>Cas9</sup> cells, an effect that was largely abolished in CDC25A-deficient cells (Figure 4C). Of note, prolonged exposure to ATR inhibitors led to H3-S10 phosphorylation in WT cells with an S phase DNA content, consistent with the premature activation of the mitotic kinases in cells still undergoing DNA replication. ATR inhibitors also induce some mitotic entry in *Cdc25a*<sup>-/-</sup> ES<sup>Cas9</sup> cells, although at later times and to a much lesser extent. Thus, whereas CDC25A upregulation is the main mechanism responsible for the premature mitotic entry induced by ATRi, ATR also limits mitotic entry through CDC25A-independent mechanisms. In this regard, and given the known roles of the CDC25A homolog CDC25B in the G2/M transition, we tested the impact of CDC25B deficiency in the response to ATR inhibitors. However, ATR inhibition did not increase the levels of CDC25B (Figure S4A), and CDC25B deletion in ES<sup>Cas9</sup> cells or siRNA-mediated depletion in human cancer cell lines did not increase the resistance to ATR inhibitors (Figures S4D–S4G). Finally, and to further document how ATR inhibitors induce cell death, we recorded videos of H2B-enhanced GFP expressing WT and *Cdc25a*<sup>-/-</sup> ES<sup>Cas9</sup> cells exposed to ATR inhibitors (Figure S4H). These analyses revealed that ATRi-induced cell death occurred frequently at mitosis or shortly during the following interphase. Interestingly, the low levels of cell death that are observed in CDC25A-deficient cells exposed to ATRi also tend to occur after passage through mitosis, suggesting that the same mechanism of ATRi-induced cell killing applies to these cells.

As final proof of this model, we forced mitotic entry in CDC25A-deficient cells by activating CDK1 through WEE1 inhibition. In agreement with our hypothesis, WEE1 inhibitors generated DSB and forced mitotic entry in CDC25A-deficient cells (as measured by H2AX and H3S10 phosphorylation by western blotting) (Figure 4D). Moreover, WEE1 inhibitors sensitized CDC25A-deficient cells to ATR inhibitors (Figure 4E). Both WEE1 and ATR inhibitors reduced the inhibitory phosphorylation of CDK1 at



**Figure 4. ATRi Induces Premature Mitotic Entry and DNA Breakage in a CDC25A-Dependent Manner**

(A) Representative FACS profiles showing the distribution of  $\gamma$ H2AX-positive cells (x, DNA content; y,  $\gamma$ H2AX) of WT and CDC25A-deficient ES<sup>Cas9</sup> cells exposed to ATRi (900 nM) for the indicated times. Numbers indicate the percentage of  $\gamma$ H2AX-positive cells (red) in each case.

(B) Representative image of the presence of DSBs in mitotic ES<sup>Cas9</sup> cells exposed to ATRi (900 nM). Mitotic cells were identified by antibodies against pH3S10 (red). DSBs were identified by  $\gamma$ H2AX (green) foci. DAPI was used to stain DNA. Numbers in white indicate the percentage of pH3S10-positive cells presenting  $\gamma$ H2AX foci. The scale bar (white) represents 2.5  $\mu$ m.

(C) Representative FACS profiles showing the distribution of pH3S10-positive cells (x, DNA content; y, pH3S10) of WT and CDC25A-deficient ES<sup>Cas9</sup> cells exposed to ATRi (900 nM) for the indicated times. Numbers indicate the percentage of pH3S10-positive cells (red) in each case.

(D) Western blot illustrating the levels of H2AX ( $\gamma$ H2AX), RPA (S4/8), and H3 (S10) phosphorylation in WT and CDC25A-deficient ES<sup>Cas9</sup> cells exposed to ATRi (300 nM) and/or WEE1i (100 or 300 nM) for 4 hr. An antibody detecting phospho-

phorylated CDK substrates was also used as measure of overall CDK activity. TUBULIN and total RPA levels are shown as loading controls.

(E) Representative pictures of cultures of WT and CDC25A-deficient ES<sup>Cas9</sup> cells exposed to ATRi (300 nM) and/or WEE1i (100 nM) for 72 hr. The scale bar (white) represents 25  $\mu$ m.

See also Figure S4.

Tyr15, particularly when combined together (Figure S4I). Of note, prolonged exposure to ATRi reduced CDK1-Y15P levels even in CDC25A-deficient cells, consistent with our earlier observations suggesting that whereas CDC25A plays a central role, ATR can limit mitotic entry through CDC25A-dependent and CDC25A-independent mechanisms. Altogether, the results presented here suggest that the toxicity of ATR inhibitors is due to a combination of (1) the generation of RS and (2) promotion of the premature mitotic entry of cells bearing RS, where DNA breaks arise. Forced mitotic entry is a consequence of the premature activation of CDK1, which is largely mediated by an increase in CDC25A phosphatase levels.

## DISCUSSION

We report here on a cellular platform for the development of forward genetic screenings in primary mammalian cells using CRISPR-Cas9, which we have used to identify mechanisms of resistance to ATR inhibitors. Two independent screenings identified sgRNAs targeting *Cdc25a* and *Cnot8* that conferred resistance to these compounds. Whereas we focused in this study on CDC25A given that it is a well-known oncogene (Ray and Kiyokawa, 2008), we also confirmed the resistance to ATRi in the *Cnot8* mutant clones isolated from the screening, although to a lesser extent than that observed in CDC25A-deficient cells. Given the pleiotropic roles of the CCR4-NOT complex on RNA metabolism, its link to ATR biology remains to be determined, although it seems independent from CDC25A, as ATRi-induced CDC25A upregulation occurs normally in CNOT8-deficient cells

(Figure S4C). With regard to CDC25A, this oncogene is frequently overexpressed in various cancers, which we suggest could confer sensitivity to ATR inhibitors. Whether reduced levels of CDC25A will emerge as a mechanism of resistance to ATR inhibitors in cancer therapy remains to be seen, but our findings suggest that CDC25A levels could be used to identify patients more likely to respond to this therapy. Supporting this view, CDC25A levels correlate with sensitivity to ATRi in the human cancer cell lines used in this study (Figures S2E and S2G), and as mentioned before, CDC25A expression is highest in tumors known to be sensitive to ATRi (Figure S2J).

With regard to the use of ATR inhibitors for cancer therapy, our work indicates that a combination with WEE1 inhibitors could overcome such resistance and suggests the potential of ATRi/WEE1i drug combinations. Along these lines, recent work has identified that WEE1 inhibitors are preferentially toxic for cancer cells with low levels of the ribonucleotide reductase subunit RRM2 (Pfister et al., 2015), which has been recently shown to be key for the essential roles of ATR in mammalian cells (Buisson et al., 2015; Lopez-Contreras et al., 2015). In addition to WEE1 inhibitors, our work suggests that ATR inhibitors should synergize not only with other RS-inducing drugs, such as cisplatin or gemcitabine (Prevo et al., 2012; Vendetti et al., 2015), but also with other strategies that promote mitotic entry.

Finally, our work sheds light on the mechanisms of cell killing by ATR inhibitors. The results presented illustrate that the toxicity of these compounds is a combination of two activities: their capacity to (1) induce RS and (2) force premature mitotic entry. This second activity is not present in other RS-inducing

chemotherapeutic drugs, which might offer an advantage to ATR inhibitors in certain conditions. Bringing cells with RS into mitosis would promote the cleavage of stalled forks and the subsequent generation of toxic DSBs. This model is consistent with recent data showing that RS is converted into DSB during mitosis (Min-ocherhomji et al., 2015). Interestingly, a prolonged exposure to ATR inhibitors leads to the phosphorylation of mitotic markers such as H3S10 in cells with DNA content lower than G2 (Figure 4C). Thus, premature mitosis might occur as early as in S phase, which would support earlier observations of replication catastrophe induced by ATR inhibitors (Toledo et al., 2013).

In summary, our work provides proof of principle of the value of ES<sup>Cas9</sup> cells as an efficient platform for CRISPR-Cas9 based genome-wide screenings, clarifies the mechanism by which ATR inhibitors kill cells, and has led to the identification of CDC25A as a potential biomarker to optimize the rational use of ATR inhibitors in cancer therapy.

## EXPERIMENTAL PROCEDURES

### Generation of ES<sup>Cas9</sup> Cells

The previously described KH2 ESC line (Beard et al., 2006) contains an flanked neomycin resistance gene and an ATGless hygromycin resistance gene without promoter at the *Col1a1* locus. In addition, this cell line also contains an M2rtTA transactivator targeted at the endogenous *Rosa26* locus. A Flp-dependent recombination event using the pBS31 plasmid provides a PGK promoter and an ATG initiation codon to the hygromycin gene as well as the gene of interest under a tetO minimal promoter into the *Col1a1* locus. This recombination eliminates the neomycin resistance gene and confers a new resistance to the successfully targeted cell. Thus, to generate the ES<sup>Cas9</sup> cells, KH2 cells were electroporated with pBS31-Cas9 and a plasmid encoding for Flp. Twenty-four to 48 hr after electroporation, cells were incubated with 140  $\mu$ g/ml of hygromycin to select cells in which recombination took place. Resistant clones were picked, amplified, and tested for Dox-inducible Cas9 expression.

### Generation of Genome-wide Mutant Libraries and Screening

Two independent genome-wide mutant libraries were generated with two different ES<sup>Cas9</sup> clones, as previously described (Koike-Yusa et al., 2014), with minor modifications. Briefly,  $5 \times 10^6$  ES<sup>Cas9</sup> cells were infected in suspension for 1 hr at 37°C with the genome-wide lentiviral library at a MOI of 0.3 and plated on a fresh layer of feeder cells. Three days after the infection, a total of  $2 \times 10^6$  BFP-positive cells (estimated library coverage of 20x) were sorted and cultured to establish each of the cell libraries. Next, we maintained a minimum of  $5 \times 10^6$  cells (estimated library coverage of 50x) with 2  $\mu$ g/ml of Dox for a total of 10 days to induce Cas9 expression allowing gene editing. To perform the 6TG screening, a total of  $5 \times 10^6$  untreated or Dox-treated cells were incubated with 6TG at 20  $\mu$ M for 7 days. Resistant clones were picked and amplified on culture media without 6TG. To perform the ATRi screening, a total of  $5 \times 10^6$  untreated or Dox-treated murine ESCs (mESCs) were incubated with ATRi (0.9  $\mu$ M) for 9 days. Resistant clones were picked and amplified on culture media with ATRi (0.3  $\mu$ M) for 5 additional days before using culture media without ATRi. The ATRi used in this study was found in a screening reported previously (Toledo et al., 2011). To identify the sgRNA sequences inserted in the resistant mESC clones, we amplified by PCR the fragment flanking the U6-sgRNA cassette from the lentiviral vector, subcloned it in a TOPO vector, and sequenced by Sanger sequencing (see Table S1 for sgRNA sequences). Finally, to generate newly deficient mESCs for CDC25A or CDC25B, we independently infected ES<sup>Cas9</sup> cells with at least three different lentiviral supernatants encoding specifically designed sgRNAs targeting either *cdc25a* or *cdc25b*. After infection, the pool of ES<sup>Cas9</sup> cells, usually infected with a percentage above 90% of efficiency, was divided in two and either untreated or treated with 2  $\mu$ g/ml of Dox for 1 week to allow gene editing before performing experiments. See the Supplemental Information

for a full list of primers and antibodies, as well as the other methods used in this study.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2016.03.006>.

## AUTHOR CONTRIBUTIONS

C.M.-R. and S.R. designed and participated in most of the experiments in this study. V.L. performed DNA replication analyses. M.M. performed CDC25A overexpression experiments. M.V.-S. provided technical help. S.O. helped in the generation of ES<sup>Cas9</sup> cells. O.F.-C. and S.R. coordinated the study and wrote the manuscript.

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