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The role of ATR in G1 phase DNA damage response

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The role of ATR in G1 phase DNA damage response

Abstract:

Ataxia telangiectasia and Rad3-related (ATR) protein is an important regulator of DNA damage response. Previous studies have shown that ATR activation is restricted to S-G2 cell cycle phases. However, recent findings indicate that ATR can also be activated in G1 phase. Therefore our understanding of ATR signaling is incomplete. In present study we have shown that ATR contributes to cell cycle arrest in G1 phase by modulating DNA damage checkpoint. Additionally, we found out that hyperactivation of ATR signaling can delay cell cycle progression. Thus, cells need to carefully balance the level of ATR activation to allow diverse cell fate outcomes. As ATR inhibitors are currently investigated in several clinical trials for cancer treatment, understanding the interplay between ATR signaling and DNA damage response could potentially be translated into more effective use of ATR inhibitors.

Keywords: DNA damage response, ATR, ATM, cell cycle, checkpoints, cyclin-dependent kinase

CERCS: B230 Microbiology, bacteriology, virology, mycology; P310 Proteins, enzymology; B200 Cytology, oncology, cancerology

ATR valgu roll G1 faasi DNA kahjustuste vastuses

Lühikokkuvõte:

Ataksia-telangiektaasia ja Rad3-ga seotud (ATR) valk on DNA kahjustuste vastuse oluline regulaator. Varasemad uuringud on näidanud, et ATR valgu aktiivsus on piiratud rakutsükli S-G2 faasidesse. Seevastu hiljutised tööd näitavad, et ATR-i saab aktiveerida ka G1 faasis, kuid meil ei ole terviklikku arusaamist ATR valgu toimimisest. Käesolevas töös leiame, et ATR aitab kaasa rakutsükli peatamisele G1 faasis DNA kahjustuste kontrollpunkti kaudu ja et ATR valgu hüperaktiveerimine võib rakutsükli kulgemist takistada. Seega, rakud peavad hoolikalt tasakaalustama ATR aktiveerimise taset, et võimaldada rakkude mitmekesist saatust. ATR inhibiitorid on vähiravimitena mitmetes kliinilistes uuringutes, seega võib ATR aktiivsuse ja DNA kahjustuste vastuse vastastikkuse mõju mõistmine potentsiaalselt võimaldada ATR inhibiitorite tõhusamat kasutamist.

Võtmesõnad: DNA kahjustuste vastus, ATR, ATM, rakutsükkel, kontrollpunktid, tsükliinist sõltuv kinaas

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia; P310 Proteiinid, ensümoloogia; B200 Tsütoloogia, onkoloogia, kantseroloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

53BP1 - p53-binding protein 1

9-1-1 - Rad9–Rad1–Hus1, heterotrimeric clamp that promotes checkpoint signaling and repair at DNA damage sites

ATM - Ataxia-telangiectasia mutated

ATR - Ataxia telangiectasia and Rad3-related

ATRi - ATR inhibitor

ATRIP - ATR Interacting Protein

BRCA2 - BReast CAncer gene 2

BrdU - Bromodeoxyuridine

CDC25 - Cell division cycle 25 phosphatase

CDK - Cyclin-dependent kinase

Cdt1 - Chromatin licensing and DNA replication factor 1

Chk1 - Checkpoint kinase 1

Chk2 - Checkpoint kinase 2

CtIP - CtBP-interacting protein

DAPI - 4',6-diamidino-2-phenylindole

DDR - DNA damage response

DMEM/F-12 - Dulbecco's Modified Eagle Medium F12

DNA - Deoxyribonucleic acid

DNA-PKcs - DNA-dependent protein kinase

DSB - Double-strand break

EdU - 5-ethynyl-2-deoxyuridine

EXO1 - Exonuclease 1

FACS - Fluorescence-activated cell sorting

FBS - Fetal bovine serum

FITC - Fluorescein isothiocyanate

FUCCI - Fluorescent ubiquitination-based cell cycle indicator

GFP - green fluorescent protein

H2AX - H2A histone family member X

HR - Homologous recombination

MAPK - mitogen-activated protein kinase

MRE11 - meiotic recombination 11 homolog

MRN - Mre11, Rad50 and Nbs1

NCS - neocarzinostatin

NHEJ - Non-homologous end joining

OHT - 4-hydroxytamoxifen

p21 - p21WAF1/Cip1 is CDK inhibitor

p53 - tumor suppressor transcription factor

PBS Phosphate-buffered saline

PCNA - Proliferating cell nuclear antigen (PCNA)

RAD17 - Checkpoint Clamp Loader Component

RFC - replication factor C

RFC2-5 - complex of RFC2, RFC3, RFC4, and RFc5

RPA - Replication protein A

RPE - Retinal pigment epithelium

RT - Room temperature

siRNA - Small interfering RNA

ssDNA - Single-stranded DNA

TopBP1 - DNA Topoisomerase II Binding Protein 1

WT - Wild Type

XLF - XRCC4-like factor

XRCC4 - X-ray repair cross-complementing protein 4

γH2AX - phosphorylated H2AX

INTRODUCTION

Every day each of the $\sim 10^{13}$ cells in our body is exposed to various types of environmental DNA damage. In addition, DNA damage continually arises as a byproduct of normal cell metabolism. Accumulated DNA damage can drive cancer development and is a characteristic of most cancers. Paradoxically, currently used anticancer therapies, such as chemotherapy and irradiation therapy, eliminate cancer cells by inducing DNA damage. Thus, manipulating the DNA damage response of cells presents an opportunity to improve the outcomes of genotoxic therapies.

To cope with a constant threat to genome integrity, cells evolved a sophisticated protection mechanism known as DNA damage response (DDR). Upon detection of DNA breaks, the DDR activates DNA repair and cell cycle checkpoints that pause cell proliferation until the repair is complete. The main regulators of DDR signals are Ataxia-telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3-related (ATR) that can induce cell cycle arrest in G1 or G2 cell cycle phases. Previous studies have shown that ATR determines cell fate in response to double strand breaks (DSBs) in G2 phase, where it drives the induction of senescence. Recently we discovered that ATR is activated by DSBs in G1 phase, yet the role of ATR activation in G1 phase was unknown.

Here, we combine several single-cell techniques to definitively identify how ATR signalling is coupled to cell cycle checkpoints and how ATR affects DNA repair in G1 phase. Importantly, unravelling these questions will ultimately allow us to influence cell fate outcomes, thereby potentially leading to improved genotoxic therapies for the treatment of cancer.

1 Literature Overview

1.1 DNA damage

Cells in our body are constantly exposed to DNA damage caused by endogenous sources, such as replication stress and reactive oxygen species, or exogenous sources like UV light, ionizing radiation and environmental mutagens. There are many types of DNA damage, among which double-stranded break (DSB) is the most toxic type of lesion (Jackson and Bartek, 2009; Huang and Zhou, 2020).

To protect the genome integrity from such lesions, cells have evolved a complex signalling pathway known as DNA damage response (DDR). The function of DDR is twofold (Figure 1): it initiates cell cycle arrest to prevent propagation of a damaged genome and simultaneously activates DNA repair (Shaltiel et al., 2015). Defects in the DDR lead to genomic instability and promote carcinogenesis (Her and Bunting, 2018). Paradoxically, the induction of DNA damage is often used to kill cancer cells. Furthermore, deficiencies in the DDR may present therapeutic opportunities that allow elimination of cancer cells while causing little harm to normal cells. A successful example of using vulnerabilities in DDR against carcinogenesis would be the FDA-approved PARP inhibitor (Olaparib) for ovarian cancer patients Cerrato et al. (2016).

Given its importance in both carcinogenesis and cancer therapy it is essential to fully understand the DDR, which may allow us to improve efficacy of anti-cancer therapies.

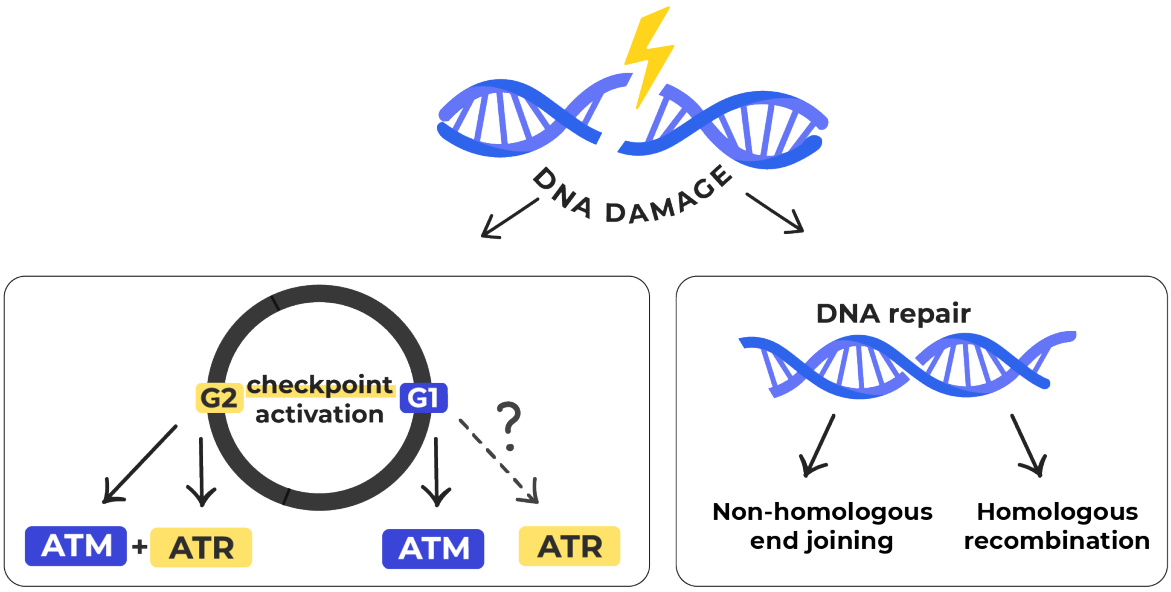

Figure 1. DNA damage response signalling. In the presence of DNA double-strand breaks, DNA damage response facilitate cell cycle arrest in G1 or G2 phases, by activating checkpoint responses, and simultaneously promotes DNA repair. Repair of the breaks mainly occurs through non-homologous end joining (NHEJ) or homologous recombination (HR). Checkpoints are regulated by ATM and ATR in G2 phase, and by ATM in G1 phase. Recent findings indicate that upon DSBs ATR is active in G1 phase and may play a role in G1 checkpoint.

1.2 DNA damage-induced checkpoint signalling

The two master kinases that regulate the DDR are ATM and ATR (Figure 2), which orchestrate a large network of proteins in order to maintain genomic integrity (Shaltiel et al., 2015). In the presence of DSBs, ATM and ATR activate cell cycle arrest in G1 or G2 phases to allow damaged cells time to either repair the DNA damage, or alternatively undergo apoptosis or senescence (Shaltiel et al., 2015). Once most of the damage is repaired the checkpoint is silenced, thus leading to cell cycle resumption (Shaltiel et al., 2015; Feringa et al., 2018). While ATM and ATR share one main goal, they are activated in response to different kinds of DSBs. ATM is activated by blunt DSB and does not require any pre-processing of the break. As such, ATM activation can occur throughout the cell cycle. In contrast, ATR activation requires resection of the DNA, which is restricted to S and G2 phases of the cell cycle. DNA end resection results in a 3' overhang of single stranded DNA and a free 5' end of the double stranded DNA flanking the break (Shaltiel et al., 2015).

1.2.1 G1 checkpoint

The G1 checkpoint is initiated upon DSBs recognition by Mre11, Rad50 and Nbs1 (MRN)-complex that facilitates ATM activation and subsequent ATM-dependent phosphorylation of H2AX (γH2AX) (Burma et al., 2001). This locally acts as a positive feedback loop amplifying ATM activity and promoting further γH2AX around the DSB site to recruit repair factors (Lou et al., 2006). ATM also activates its downstream effector Checkpoint kinase 2 (Chk2) (Matsuoka et al., 1998). ATM and Chk2 are both required to stabilize the tumor suppressor transcription factor p53 (Cheng and Chen, 2010). Stabilization of p53 initiates expression of p21WAF1/Cip1 (p21), which halts cell cycle progression by inhibiting Cyclin-CDK complexes (Harper, 1993). Loss of p53 or p21 leads to a complete abrogation of the G1 checkpoint (Deng et al., 1995; Hirao et al., 2002). In addition, ATM and Chk2 activate p38-dependent pathway to promote degradation of cell division cycle 25 (CDC25) phosphatase. Under normal conditions, CDC25 removes the inhibitory phosphorylation of CDK2 and positively regulates cell proliferation. CDC25 degradation in G1 thus inhibits the transition to S-phase (Shen and Huang, 2012).

1.2.2 G2 checkpoint

In contrast to the DSB-induced checkpoint in G1, a cell cycle arrest in G2 does not solely rely on ATM and Chk2 (Shaltiel et al., 2015). Although both of these kinases control the initiation of the G2 checkpoint (Painter and Young, 1980; Rainey et al., 2008), ATR and Chk1 are required for G2 checkpoint maintenance (Brown and Baltimore, 2003; Liu et al., 2010). With the onset of S-phase, activity of CDK rise and promote CDK2-dependent phosphorylation of ATR-interacting protein (ATRIP) and thus enhance ATR activity (Myers et al., 2007). Furthermore, progressive resection upon DSB in S-G2 attenuates ATM signalling and promotes ATR activation (Shiotani and Zou, 2009), therefore the checkpoint dependency shifts from ATM to ATR (Jaiswal et al., 2017).

While p53 and p21 are required for sustained cell cycle arrest, G2 checkpoint mostly relies on Wee1 kinase, which is expressed in S- phase (Chow et al., 2003; Watanabe et al., 1995). Activated Wee1 phosphorylates CDK1 on Tyr15 in the nucleus, inactivating kinase and causing a G2 cell cycle arrest (Heald et al., 1993; Watanable et al., 1995). CDC25C, on the other hand,

reflects Wee1 activity by dephosphorylating CDK1 on Tyr15 and reactivating it (Boutros et al., 2007; Bulavin et al., 2003). Chk1 and Chk2 phosphorylate Wee1 kinase and mark CDC25 phosphatase for degradation, thereby preventing CDK1 activation and mitotic entry (Beck et al., 2010; Hughes et al., 2013).

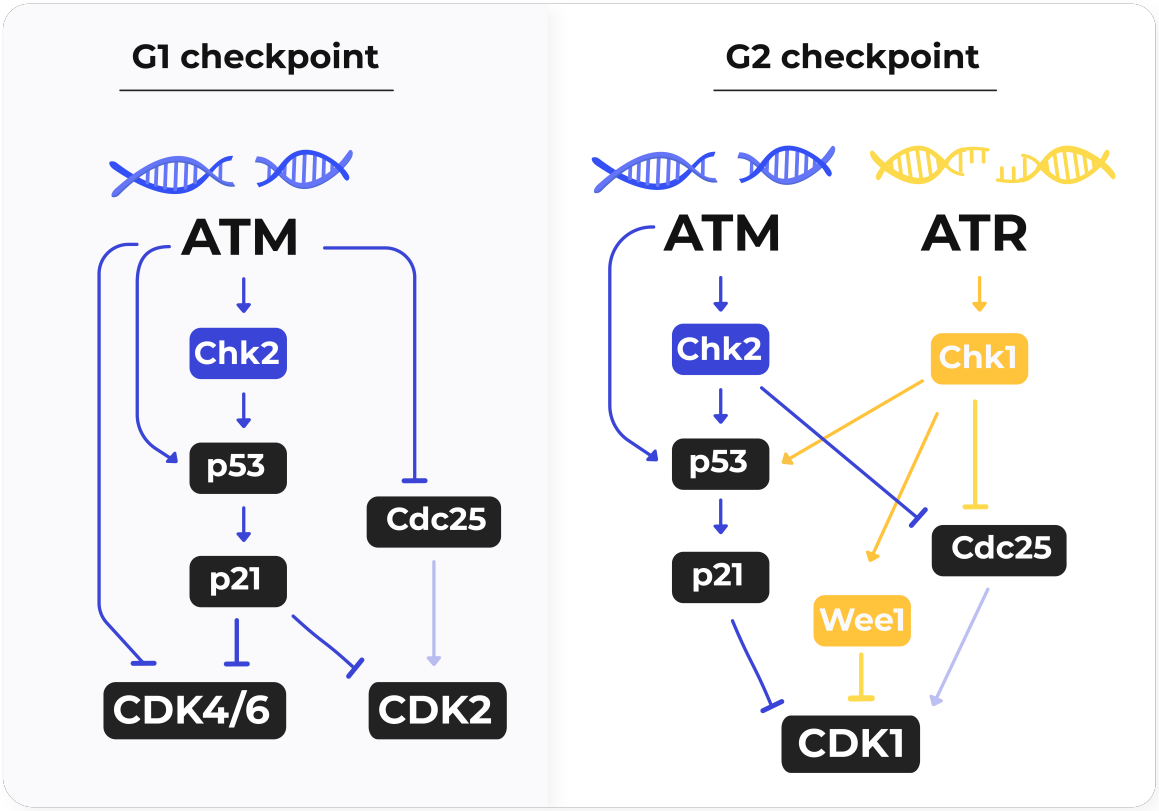

Figure 2. DNA-damage induced checkpoint signalling in G1 and G2 phases. ATM and ATR are activated in response to sensor proteins that identify DNA lesions, and trigger a cascade of events that facilitate cell cycle arrest. G1 checkpoint: G1 checkpoint: DSBs in G1 phase recruit ATM kinase, which leads to activation of Chk2 kinase, and both kinases are then required to stabilize p53. Once activated, p53 initiates different transcriptional programs, which includes targeting of a CDK inhibitor, p21. Expression of p21 supresses cyclin/CDK activity and induces a G1 cell cycle arrest. Additionally, Chk2 facilitates inhibition of Cdc25 phosphatase, that removes inhibitory phoshporylation from CDK complexes and is critical for CDK activation. G2 checkpoint: The rise of Cdk2 activity in S-G2 phases promotes DNA end processing by phosphorylating key exonucleases, such as CtIP and Exo1. Resected DNA ends activate ATR, and its activation is further promoted by direct CDK2-dependent phosphorylation. ATR phosphorylates downstream protein Chk1 and together with ATM/Chk2, they target p53, Wee1, and Cdc25 proteins, which halts cell cycle progression in G2.

1.3 DNA repair

The other function of the DDR is to initiate DNA repair. There are two main DSB repair pathways in mammalian cells: non-homologous end joining (NHEJ) and homologous recombination (HR). While NHEJ re-joins DSBs without a reference to the DNA sequence and functions throughout the cell cycle, HR requires extensive resection of DNA-ends, a template for repair (sister chromatid) and is restricted to S-G2 phases (Mao et al., 2008).

1.3.1 Non Homologous end-joining (NHEJ)

NHEJ is a dominant DSB repair mechanism. Upon detection of DSBs, the Ku70/Ku80 heterodimer binds to both ends of the break and recruits the DNA dependent protein kinase (DNA-PKcs) (Mahaney et al., 2009). Recruited DNA-PKcs forms a synaptic complex responsible for holding the two ends of the broken DNA molecule together (Jette and Lees-Miller, 2015). If necessary the DSB can be minimally processed by Artemis nuclease to create short microhomology repeats (Löbrich and Jeggo, 2017). Finally, the DNA ligase IV, X-ray cross-complementation group 4 (XRCC4), and XRCC4 like factor (XLF)/Cernunnos ligation complex carry out the ligation of the ends (Chang et al., 2017). Recent studies have shown that DNA-PKcs may switch between short-range and long-range states in synaptic reactions, implying that protein binding on DNA ends is a dynamic process until DNA ends are ligated (Scully et al., 2019).

1.3.2 Homologous recombination (HR)

To initiate HR DSB should be extensively resected. This includes two subsequent steps: short-range resection initiated by MRN-complex and CtIP followed by long-range resection with exonucleases that produce ssDNA tails (Zhao et al., 2020). The ssDNA is then rapidly coated with abundant Replication protein A (RPA) to remove secondary structure and prevent nucleolytic degradation (Chen and Wold, 2014). The RPA-bound 3' overhangs activate ATR which contributes to the activation of the cell cycle checkpoint (Choi et al., 2010). BRCA2 facilitates RPA displacement by RAD51, to form a nucleoprotein filament that searches for the homologous sequence on the undamaged sister strand and facilitates HR (Sung and Klein, 2006).

1.4 ATR activation

ATR is serine/threonine-protein kinase of about 300 kDa (Unsal-Kacmaz and Sancar, 2004) that is essential in proliferating cells (Saldivar et al., 2017). Although ATR has other functions, a general consensus is that ATR regulates specific events during S-phase in response to DNA damage and DNA replication stresses, such as cell cycle arrest, regulation of origin firing, maintaining replication fork stability etc.

As discussed earlier, ATR activation requires DNA end resection to generate a 3' single stranded DNA overhang. It is then recruited to 3' overhangs coated with RPA via a direct interaction between its binding partner ATRIP and RPA (Choi et al., 2010). However, ATR localization to RPA is not sufficient for its activation. Instead, full ATR activation requires several additional components such as topoisomerase II binding protein 1 (TOPBP1) (Kumagai et al., 2006) and RAD17, RAD9-RAD1-HUS1 (9-1-1 complex) (Zou et al., 2002) . TOPBP1, in particular, contains an ATR-activating domain (AAD) that functions as an allosteric activator of ATR (Thada and Cortez, 2019). TopBP1 is recruited to an ATR activating structure via MRN-mediated mechanisms, but its role in ATR activation requires interaction with the RAD9-HUS1-RAD1 (9-1-1) clamp complex, which is loaded onto ssDNA junctions by the RAD17/RFC2-5 clamp loader (Saldivar et al., 2017). Activated ATR phosphorylates a cascade of signalling molecules, including Chk1, to safeguard the integrity of the genome. (Saldivar et al., 2017).

DSB-induced ATR activation has been thought to be limited to S-G2 phases, when HR factors are available to perform long range resection (Saldivar et al., 2017). However, it has become evident that DSB resection is not limited to S-G2 phases, but can also occur in G1 phase. A previous study implicated that repair of complex DNA breaks in G1 phase requires extensive end processing by CtIP, MRE11, and EXO1, the core factors of HR (Averbeck et al., 2014). Moreover, recent data that obtained in our laboratory showed that ATR can be activated in response to resected DSBs in G1 phase (Supplementary 1). These findings indicate that our understanding of ATR signalling is incomplete, and that is what we address in this thesis.

2 THE AIMS OF THE THESIS

The aim of thesis is to understand the interplay between ATR signalling and the cell cycle machinery, in response to DNA damage. We know how ATR is activated, and we understand how ATR determines cell fate decisions in G2. Based on our discovery that ATR is activated in response to DSBs in G1 phase (Supplementary 1), we would also like to understand:

1. ATR contribution to the G1 cell cycle checkpoint
2. ATR involvement in DNA repair in G1 phase

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Cell culture

Cell lines used in the study are listed in Table 1. Cells were grown on culture dishes, or cover slips in Dulbecco's Modified Eagle Medium F12 (DMEM/F-12) (Thermo Fisher Scientific) supplemented with 12% fetal bovine serum (FBS) (Serana) and streptomycin/penicillin (Sigma Aldrich) at 37°C and 5% CO_2.

Table 1. Eukaryotic cell lines used in the study.

Description	Source
RPE WT	Lenno Krenning
RPE FUCCI	Lenno Krenning
RPE p21-GFP PCNA-mRuby	Lenno Krenning
RPE-TopBP1-AAD	Lenno Krenning
RPE HALO-53BP1 FUCCI	Anoek Friskes

3.1.2 Cell densites

One day prior to the live-cell imaging experiment, cells were plated in density of 25 000 per well on 8-well Nunc LabTek Chamber Slide (Thermo Fisher Scientific). For immunofluorescence experiments, 500 000 cells were seeded on a 6 cm dish (Corning) 24 hours before the experiment. For the FACS analysis $1\text{-}2 \cdot 10^6$ cells were plated on a 10 cm dish (Corning). For small interfering RNA (siRNA)-mediated gene silencing, 25 000 cells per well were seeded on a 8-well Nunc LabTek Chamber Slide (Thermo Fisher Scientific) one day before the experiment.

3.1.3 G1 cell cycle phase synchronization

For serum starvation experiments, cells were grown to 100% confluence, counted and plated in DMEM/F-12 with 12% FBS for 4 hours. When cells adhered, the media was replaced with serum-free DMEM/F-12 medium and cells were incubated at 37°C for an additional 36 h. After that, cells were incubated in DMEM/F-12 with 12% FBS for 4 hours and directly used in the experiment. Following this protocol, the percentage of G1 cells in the population was in the range of 90-98%.

Another approach to synchronise cells in G1 phase was to treat cells with dual CDK4/6 inhibitor Palbociclib. For that, Palbociclib was added to cells in the final concentration of 1 μM and incubated for at least 20 hours. This method resulted in 95-100% of G1 cells in a population.

3.1.4 Immunocytochemistry

Cells grown on coverslips received different treatments and were allowed to recover at 37°C. 30 minutes after, 10 μM of 5-ethynyl-2-deoxyuridine (EdU) was added to label G1-negative cells. During collection of timepoints, the cover slips were washed with phosphate-buffered saline (PBS) and fixed in 4% Formaldehyde for at least 10 min. The fixed cover slips were washed once in PBS and cells were permeabilized with 0.5% Triton-X for 5 minutes. This was followed by two additional washes in PBS and 1 h incubation in primary antibody (see Table 2). After that, the primary antibody was washed off twice with PBS and cells were incubated for 1 h in the dark with secondary FITC-conjugated antibody (see Table 3).

After antibody staining, cells were washed once in PBS and EdU staining buffer (100 mM Tris–HCl, pH 8.5, 1 mM $CuSO_4$), then the samples were stained for EdU. They were incubated for 30 min at RT in EdU staining buffer + 100 mM Ascorbic Acid + 1:1000 azide AF647. Lastly, samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:1000) to label the nucleus. After the stainings, cover slips were rinsed twice in PBS and mounted on glass slides using a drop of Vectashield (Vector Laboratories).

Table 2. Primary antibodies used in the study.

Name	Source	Product number	Manufacturer	Dilution
γH2AX (Ser 139)	Mouse	05-636	Millipore	1/600
Phospho-Chk2 (Thr 68)	Rabbit	2661	Cell Signaling Technology	1/600
Phospho-p53 (Ser 15)	Rabbit	9284	Cell Signaling Technology	1/600

Table 3. Secondary antibodies used in the study.

Name	Species	Product number	Manufacturer	Dilution
α-mouse-AF488	Goat	A11029	Invitrogen	1/1000
α-rabbit-AF568	Goat	A11011	Invitrogen	1/1000

3.1.5 Microscopy imaging

Fluorescent imaging was performed using a Zeiss Observer Z1 microscope equipped with AxioCam 506 mono-camera and 63x/1.4NA objective or DeltaVision Deconvolution microscope (applied precision) equipped with a 60X 1.45 NA oil objective and a 100X 1.40NA oil objective (Olympus) connected to a CoolSnap CCD camera. On average, each live-cell imaging experiment was 48 hours long, imaged every 15 minutes. Definite Focus was utilised to eliminate focus drift in images. During the whole experiment the temperature and CO_2 supply were kept at constant 37°C and 5%. Image analysis was performed using Fiji software (ImageJ 2.0.0-rc-49/1.51d).

3.1.6 Reagents

At the beginning DNA damage was induced with one of the two chemotherapeutic agents: Etoposide or Neocarzinostatin. Etoposide was added in the final concentration of 10 μM, for one hour and then washed away with fresh medium. Neocarzinostatin was used in the final concentration of 100 ng/μl. DNA-PKcs (M3814) and Ligase IV (SCR7) inhibitors were added at the beginning of the experiment as well, in the final concentrations of 1 μM.

Five hours after DSB induction, kinase inhibitors were added.

ATR kinase inhibitor (VE-822) was used at final concentrations of 200 nM.

ATR kinase inhibitor (VE-821) was used at final concentrations of 5 μM.

Chk1 inhibitor (CHIR 124) was added in the final concentration of 500 nM.

Chk2 inhibitor (Chk2 inhibitor II) was added in the final concentration of 10 μM.

To visualise p53-binding protein 1 (53BP1) foci in RPE HALO-53BP1 FUCCI cells, Halo ligand (Janelia Fluor® 646 HaloTag® Ligand) in the concentration of 200 nM was added to the cell for 15 minutes, then washed away, and after one hour of incubation cells could be imaged.

3.1.7 Small interfering RNA (siRNA) transfection

Transfection reactions were performed at a final concentration of 20 μM siRNA. First, OptiMEM (Gibco) was supplemented with 1:100 RNAiMAX (Invitrogen) and incubated at RT for 5 minutes. Next, siRNA was added to the same mix and incubated at RT for 20 minutes. This transfection mix was then added to the cells which were incubated for 24 hours. After incubation cells could be directly imaged. For TopBP1 and Chk1 depletion, cells were first synchronised in G1 phase

by contact inhibition (see G1 cell cycle phase synchronization by serum starvation). Cells were transfected with siRNA at the moment of serum withdrawal.

3.1.8 Fluorescence-activated cell sorting (FACS)

At the start of the experiment cells received different treatment as indicated in results and incubated for 6 hours. Before washing with PBS, BrdU (Sigma Aldrich) (1:1000) is added and the cells are incubated for 10 minutes. After incubation, cells are washed, trypsinized, and collected in media. After 3 rounds of spinning and washing, the cell pellet is resuspended in 70% Ethanol. 1 hour before proceeding to analysis, the pellet is resuspended in 1:40 RNase 1 (Sigma Aldrich) and 1:100 PI (Sigma Aldrich) to stain the DNA and incubated at 37C for 15 minutes in the dark. FACS analysis is performed using Attune NxT Flow Cytometer (Thermo Fischer Scientific). Data obtained is further analyzed using FlowJo software. To isolate G1 cells, FACS gates were set on small size populations and cells with low PCNA signal.

3.2 RESULTS

3.2.1 ATR contributes to maintenance of a G1 checkpoint

It is known that ATR is essential for regulating G2 checkpoint and drives the induction of senescence in G2 (Feringa et al., 2018). Given that ATR is active in G1 phase in response to DSBs (Supplementary 1 Figure 1), we aimed to investigate its role in the regulation of G1 cell cycle checkpoint.

To track G1-S phase transitions by live-cell fluorescent imaging, we employed RPE FUCCI cells, where the degradation motif of Cdt1 is fused to a red fluorescent protein, and the degradation motif of Geminin is fused to a green fluorescent protein. As such, the FUCCI system marks G1 and S/G2 phases with red and green fluorescence, respectively (Figure 3A). At the start of the experiment, we induced DNA damage with chemotherapeutic agents, neocarzinostatin (NCS) or etoposide (Figure 3B). Five hours post-DSBs induction we treated cells with small molecule kinase inhibitors of Chk2 (Chk2i) and ATR (ATRi) to study the contribution of kinases to a DNA damage-induced G1 arrest.

First, we assessed if the inhibition of Chk2 and ATR affects cell cycle progression from G1 into S-phase. We find that undamaged cells that received ATRi or Chk2i and the combination of two showed no delay in cell cycle progression compared to untreated conditions (Figure 3C). On the contrary, cell proliferation was significantly delayed in G1 cells damaged with NCS, with more than 40% of cells remaining in G1, 44 h post-DSB induction (Figure 3D). Consistent with our previous work (García-Santisteban et al., 2021), Chk2 inhibition 5 hours after DNA damage resulted in almost complete G1 checkpoint abrogation (Figure 3D). Interestingly, the ATR inhibition alone showed an elevated rate of S-phase entry compared to cells that received no checkpoint inhibitors and increased the number of cells that recovered from G1 arrest to 80% (Figure 3D). Furthermore, combined Chk2 and ATR inhibition facilitated even faster G1 exit and resulted in a complete recovery from cell cycle arrest, suggesting dual activation of ATR and Chk2 is needed to sustain G1 checkpoint. Similarly, inhibition of ATR five hours after etoposide-induced DSBs elevated the rate of S-phase entry compared to cells in which kinase was not inhibited (Figure 3E, 3F). These data suggest that ATR functionally contributes to the

G1 checkpoint.

As chemical inhibitors may have off-target effects, we assessed whether siRNA-mediated silencing of the ATR activating protein-TopBP1 and Chk1 exhibit similar effects on the G1 checkpoint. Since TopBP1 and Chk1 are essential during DNA replication, we first synchronized RPE FUCCI cells in G1 by serum starvation (see methods) and then depleted TopBP1 or Chk1 by siRNA transfection (see methods). We observed that TopBP1 depletion in G1 cells is non-lethal, S-phase entry of these cells was similar to untreated condition (Figure 3G). Surprisingly, depletion of Chk1 slowed cell cycle progression in the absence of DNA damage (Figure 3G), which possibly precludes analysis of the G1 checkpoint. We then induced DSBs by NCS and followed the progression of G1 cells into S-phase. Consistent with our data using chemical ATR inhibitor, depletion of TopBP1, thereby preventing DNA damage-induced activation of ATR, resulted in an increased checkpoint recovery from G1 phase (Figure 3H). On the other hand, upon induced DNA damage, Chk1-depleted cells display a delay in G1 checkpoint recovery and significantly reduced recovery (Figure 3H). An adverse effect of Chk1 depletion may reflect an as of yet uncharacterized contribution of Chk1 to normal cell cycle progression.

Collectively these data demonstrate that ATR contributes to the G1 checkpoint control, and dual inhibition of ATR and Chk2 is required to achieve complete checkpoint suppression.

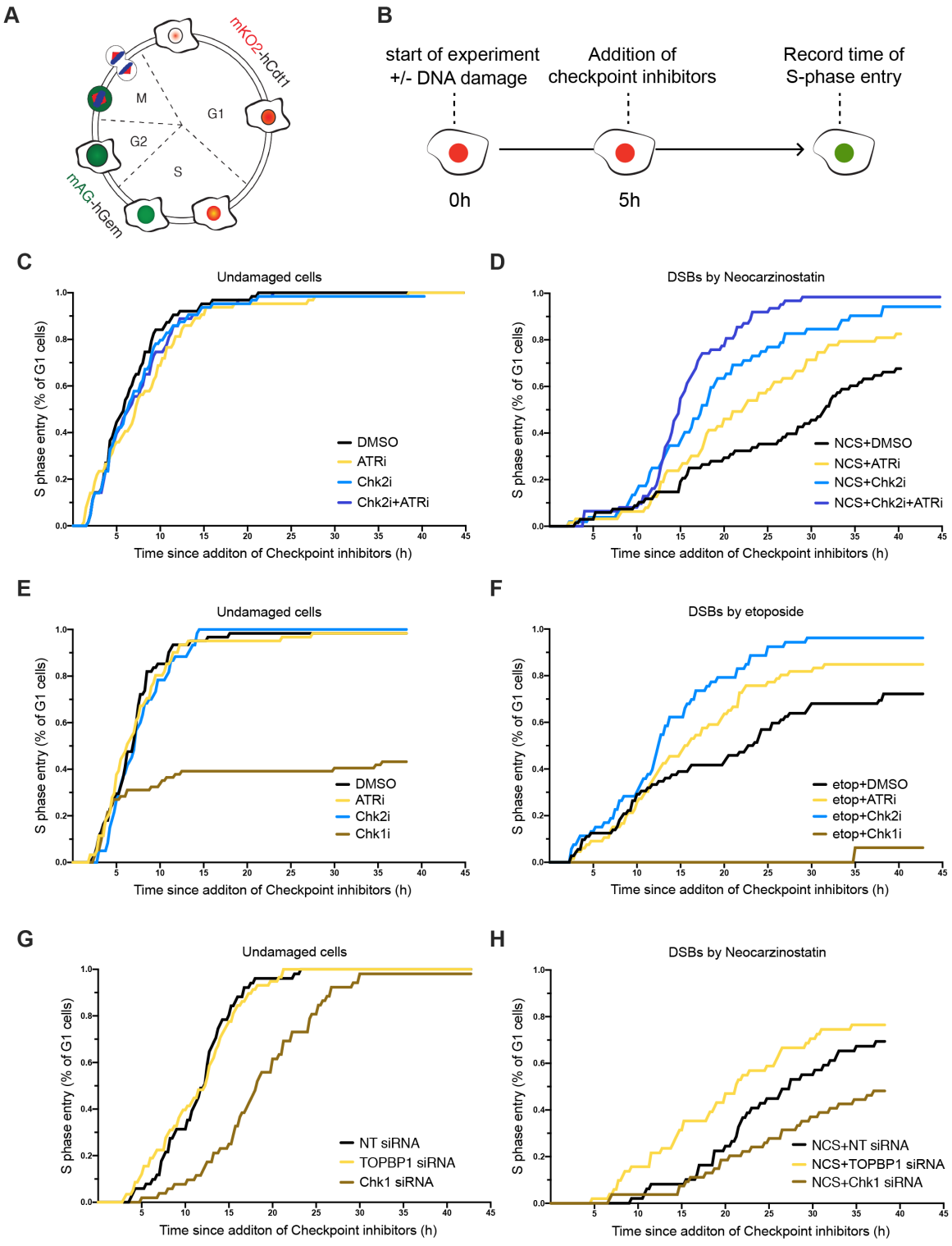

Figure 3. ATR contributes to maintenance of G1 checkpoint. [Figure caption is on the next page.]

Figure 3. ATR contributes to maintenance of G1 checkpoint. A) Schematic representation of the FUCCI system for the visualization of cell cycle progression (Sakaue-Sawano et al., 2008). The Cdt1 and Geminin degradation motifs are fused to mAG and mKO2 (a green and orange fluorescent protein, respectively) to mark G1 and S/G2 phases, respectively. B) Timeline of the experiment. RPE FUCCI cells, grown in chambered microscopy slides, were imaged every 15 minutes by real time microscopy. At the start of the experiment, we induced DNA damage, or left cells undamaged to assess unperturbed cell cycle progression. Five hours after, cells were treated with small molecule kinase inhibitors (Chk2i = Chk2 Inhibitor II, ATRi = VE822, Chk1i = CHIR124), and cell cycle progression was analysed for cells that were in G1 phase at the moment of inhibitor addition. Average 2 independent experiments. C) Same as (B). D) Same as (B), except immediately before the start of the experiment, we induced DNA double strand breaks with neocarzinostatin (NCS). E) Same as (B). F) Same as (B), except DNA double strand breaks were induced immediately before imaging, by the addition of etoposide. After 1 hour etoposide was washed out with a fresh medium. G) RPE FUCCI cells were grown in chambered microscopy slides, synchronised in G1 phase by serum starvation (see methods), transfected with the indicated siRNAs (see methods) and imaged every 15 minutes by real time microscopy. At the start of the experiment, we induced DNA damage, or left cells undamaged to assess unperturbed cell cycle progression. Cell cycle progression was analysed for cells that were in G1 phase at the start of the experiment. H) Same as (G), except DNA double strand breaks were induced by the addition of NCS immediately before imaging

3.2.2 Inhibition of NHEJ leads to ATR dependent G1 checkpoint regulation

Our data show that ATR contributes to the G1 DNA damage checkpoint. Since ssDNA is a key platform for ATR activation (Saldivar et al., 2017), we were interested if stimulation of resection events by inhibition of NHEJ repair factors would lead to a more ATR-dependent G1 checkpoint. As such, we assessed the ATR-dependency of the G1 checkpoint in cells in which we inhibited NHEJ by various means.

Our first candidate among factors engaged in NHEJ was 53BP1, because 53BP1 directly binds to DSBs and counteracts DNA resection to promote NHEJ in G1 (Mirman et al., 2018). We depleted 53BP1 using siRNAs in RPE Fucci cells and monitored the dynamics of S-phase entry. At first glance, the knockdown of 53BP1 alone nor with inhibited ATR had no effect on unperturbed cell proliferation (Figure 4A). However, when 53BP1-depleted cells were exposed to NCS, we observed a complete loss of the G1 checkpoint - cells continued cell cycle progression despite the presence of DNA damage (Figure 4B). Although additional inhibition of ATR led to a slightly faster S-phase entry (Figure 4B), the overall effect of 53BP1-depletion in abrogating the G1 checkpoint precluded a conclusion regarding ATR dependency of the G1 checkpoint.

Another vital component of NHEJ that negatively regulates resection is DNA-dependent protein kinase (DNA-PKcs). DNA-PKcs is recruited to DSBs to form a synaptic complex responsible for holding the two ends of the broken DNA molecule together (Davis et al., 2014). Using RPE Fucci cells, we monitored how perturbations in NHEJ through inhibition of DNA-PKcs affect G1 checkpoint. In the absence of damage, inhibition of DNA-PKcs alone or in combination with ATRi showed no significant changes in cell cycle progression, demonstrating that the DNA-PKcs inhibitor is not toxic (Figure 4C). In contrast, inhibition of DNA-PKcs concurrently with the induction of DNA damage led to a complete cell cycle arrest (Figure 4D). Interestingly, consequent inhibition of ATR resulted in 40% cell recovery, confirming that ATR is important for G1 checkpoint (Figure 4D). Since we did not observe a complete G1 checkpoint rescue by ATRi, in cells impaired in DNA-PKcs signaling, we speculated that inactivated DNA-PKcs could act as a physical block on DNA ends and prevent end processing. Thus, in the following

experiments, we aimed to alter NHEJ through its downstream effectors.

Downstream of DNA-PKcs and central to the re-joining of DNA ends together is ligase IV (McElhinny et al., 2000). Therefore, we tested how inhibition of ligase IV affected the G1 checkpoint. Similar to earlier experiments, we induced DSBs using NCS, followed by single or dual inhibition of ligase IV and ATR 5 hours later. Treatment of undamaged cells with ligase IV or ATR inhibitors did not affect their normal proliferation (Figure 4E). However, while exposure to NCS delayed the progression of G1 cells into S-phase (Figure 4F), this delay was further amplified by simultaneous inhibition of ligase IV (which resulted in more than 50% of cells remaining arrested at 48 h (Figure 4F). Strikingly, dual inhibition of ligase IV and ATR completely reverted the effect of slow proliferation that was induced by inhibition of ligase IV (Figure 4F). Moreover, ATR-inhibition in ligase IV-inhibitor treated cells elevated the number of cells entering S-phase to 90%, which is similar to ATR-inhibition in cells that only received DNA damage (Figure 4F). These results demonstrate that impairment of NHEJ by inhibition of ligase IV shifts G1 checkpoint balance towards ATR dependency. This is likely caused by an increase in DNA end resection, which remains to be formally tested in our setting.

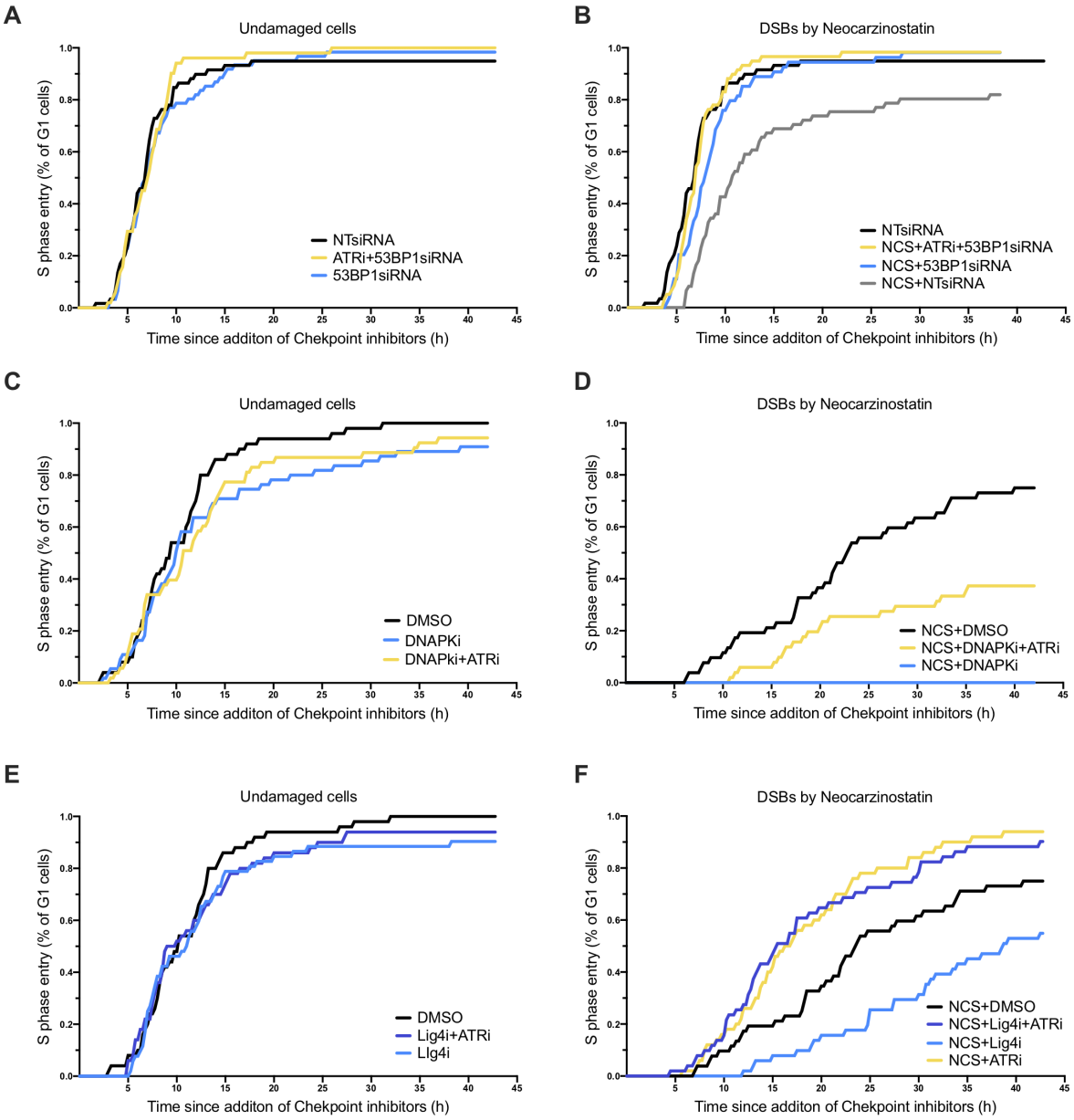

Figure 4. Inhibition of NHEJ leads to ATR dependent G1 checkpoint regulation. A) RPE FUCCI cells were transfected with the indicated siRNAs and imaged every 15 minutes by real time microscopy. B) Same as (A), except DNA double strand breaks were induced immediately before imaging, by the addition of NCS. C) RPE FUCCI cells were imaged every 15 minutes by real time microscopy. DNA-PKcs inhibitor (DNAPKi = M3814) was added at the start of the experiment. Five hours later the ATR inhibitor (ATRi = VE822) was added, and cell cycle progression was analysed for cells that were in G1 phase at the moment of ATR inhibitor addition. D) Same as (C), except DNA double strand breaks were induced immediately before imaging, by the addition of NCS. E) RPE FUCCI cells were imaged every 15 minutes by real time microscopy. Ligase IV inhibitor (LigIVi = SCR7) was added at the start of the experiment, five hours later, ATR inhibitor (ATRi = VE822) was added, and cell cycle progression was analysed for cells that were in G1 phase at the moment of ATR inhibitor addition. F) Same as (E), except DNA double strand breaks were induced immediately before imaging, by the addition of NCS.

3.2.3 ATR contributes to G1 arrest through activation of p53 and p21

The tumor suppressor p53 and its downstream effector p21 are critical components of G1 checkpoint (Harper, 1993; Harper et al., 1995). To better understand how ATR regulates G1 checkpoint we investigated a putative role for ATR in the induction of p53 and p21 in the G1 phase.

First, we measured the nuclear stabilization of p53 in G1 cells by immunofluorescence staining. To activate p53, we induced DSBs with NCS in the presence or absence of ATRi and fixed cells every hour up to 6 hours post-DSB-induction. Expectedly, p53 is rapidly stabilized in response to DNA damage (Figure 5A). Intriguingly, ATR inhibition prevented the increase of the p53 levels, suggesting that ATR affects the stabilization of p53.

Stabilized p53 initiates cell cycle arrest by transactivation of cyclin-dependent kinase inhibitor p21 (He et al., 2005). Since we observed that ATR inhibition reduces p53 activation, we wanted to assess if that also translates into altered levels of p21 levels. Therefore, we used RPE p21-GFP PCNA-mRuby cell line (Barr et al., 2017) to investigate the effect of ATR inhibition on p21 dynamics using live cell fluorescence experiments. We synchronized cells in G1 phase by CDK4/6 dual inhibitor Palbociclib during 20 h treatment (see methods) and assessed p21-levels in the presence or absence of DNA damage. In the absence of DNA damage, p21 retains relatively low expression (Figure 5B). In response to the induction of DNA damage by NCS cells rapidly activate p21 and maintain high p21 activity for over 44 h (Figure 5B). On the other hand, cells treated with ATRi at the moment of DSB induction show significantly decreased p21 induction. This shows that ATR contributes to the induction of p21 by p53, and suggests that ATR promotes a G1 cell cycle arrest through regulation of the p53/p21 pathway (Figure 5B).

Another approach to measuring the p21 levels specifically in G1 population without prior synchronization was to assess p21 levels in RPE p21-GFP PCNA-mRuby cells by FACS. Six hours prior to FACS-analysis, cells were treated with two different ATR-inhibitors, in the presence or absence of DNA damage. In the absence of DNA damage, p21-GFP levels were low and not affected by the addition of the ATR inhibitors VE821 and VE822 (Figure 5C). Expectedly, cells

treated with NCS showed a clear p21 induction (Figure 5C). Consistent with our live experiments, ATR inhibition decreased p21 induction, confirming that ATR contributes to the induction of p21 in G1 phase. Together these data support that ATR contributes to G1 cell cycle arrest through stabilization and activation of p53 resulting in expression of the CDK-inhibitor p21.

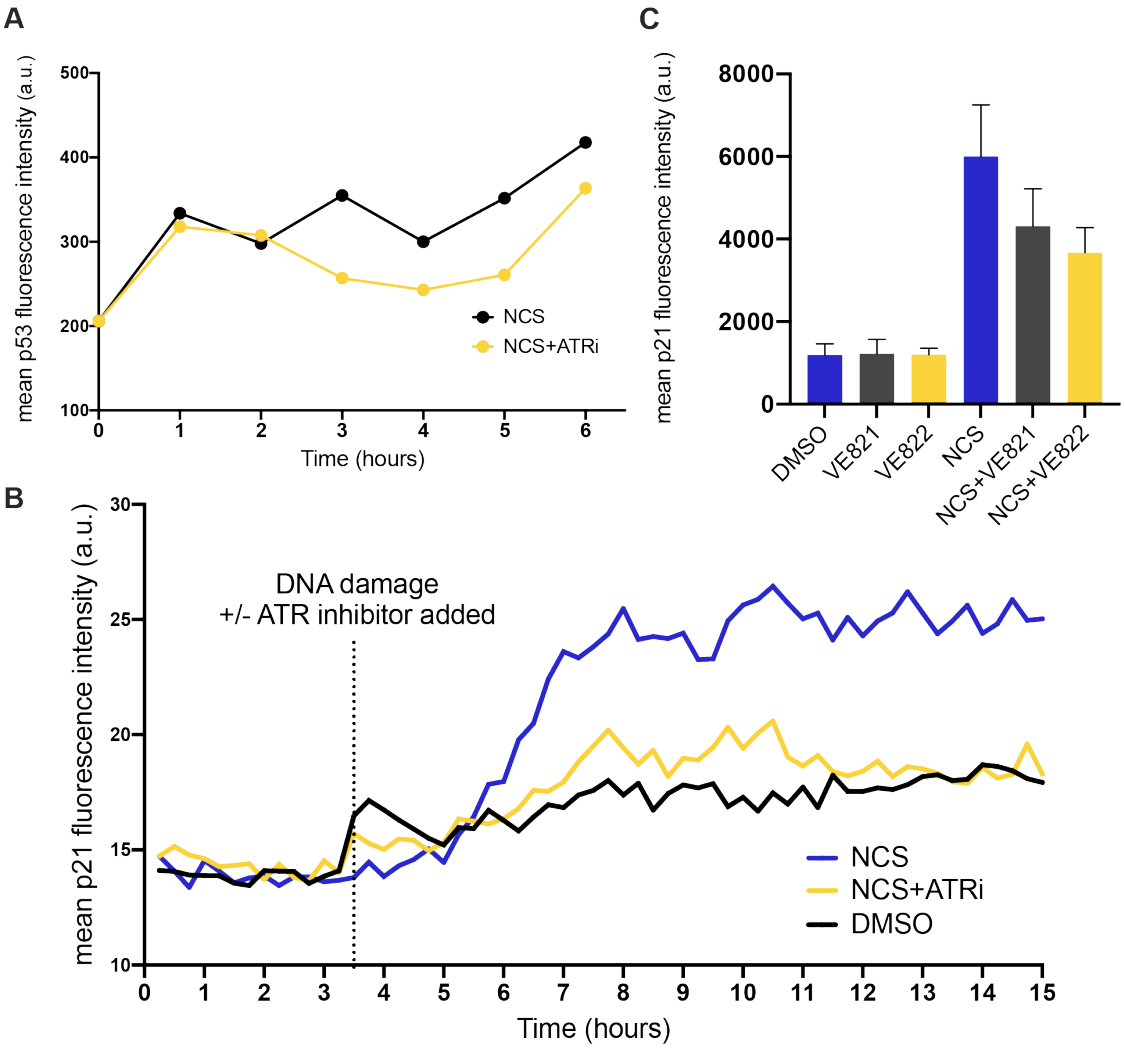

Figure 5. ATR contributes to activation of p53 and p21. A) Quantification of p53 immunofluorescence signal in RPE WT cells. DNA double strand breaks were induced at the start of the experiment, by the addition of NCS. Where indicated, the ATR inhibitor VE822 was added simultaneously. p53-levels were visualized by immunofluorescent staining of p53. Each time point represents mean p53 fluorescence intensity of more than 50 G1 cells. B) Quantification of mean p21 fluorescence intensity in RPE p21-GFP cells. RPE p21-GFP PCNA-mRuby cells were synchronised in G1 by 20 h Palbociclib treatment and then imaged every 15 minutes by real time microscopy. DNA double strand breaks were induced at the start of the experiment, by the addition of NCS. ATR inhibitor (ATRi = VE822) was added simultaneously. C) FACS analysis of p21-GFP fluorescent intensity. RPE p21-GFP cells were grown in culture dishes, then subjected to indicated treatments for six hours, and sorted. Bar charts represent the mean p21 fluorescent intensity of the G1 population.

3.2.4 ATR signaling can delay G1 progression by itself

Having established that ATR contributes to the G1 checkpoint (Figure 3), we aimed to investigate whether ATR signaling is sufficient to drive G1 cell cycle arrest.

To assess that, we used previously generated in our lab RPE1 cells expressing an ATR activating domain TopBP1 under 4-hydroxytamoxifen (OHT)-inducible promoter (Toledo et al., 2008). Activated by OHT treatment, TopBP1 translocates to the nucleus and activates ATR. This system (RPE-TopBP1-AAD) represents a tool to specifically activate ATR-signaling in absence of ATM- or DNA-PKcs-dependent signaling that occurs in response to double strand breaks.

In order to verify that translocation of TopBP1 promotes ATR activation, we examined the pan-nuclear distribution of the phosphorylated γH2AX a well-known target of DDR kinases (Ward and Chen, 2001). As expected, no H2AX phosphorylation was identified in G1 phase RPE-TopBP1 cells without tamoxifen (Figure 6A). On the other hand, the addition of OHT stimulated increased phosphorylation of H2AX in TopBP1-infected cells (Figure 6A). As a control, we assessed γH2AX-levels in cells damaged with Etoposide. Notably, the levels of γH2AX accumulation in activated RPE-TopBP1 cells corresponded to the levels of γH2AX induced by etoposide, demonstrating that TopBP1-mediated ATR signaling was proportional to DDR signaling (Figure 6A).

To ascertain that the induction of ATR signaling does not alter ATM signaling, we measured nuclear levels of Chk2, a downstream target of ATM (Chaturvedi et al., 1999). The induction of ATR signaling by OHT does not elevate Chk2 (Figure 6B), as opposed to etoposide treatment. Furthermore, the ATR-dependent H2AX phosphorylation was not affected by inactivation of ATM but completely abolished upon ATR inhibition (Figure 6C). These results demonstrate the system's strong ATR specificity. In conclusion, the generated RPE-TopBP1 cell line allowed us to precisely manipulate ATR activity without ATM signalling.

Having a reliable mechanism for inducible ATR activation, we next asked if ATR-activation by itself could slow down proliferation. We assessed the growth dynamics of RPE-TopBP1-AAD

and RPE WT cells in the presence or absence of OHT over seven days. While the treatment with tamoxifen did not alter the proliferation of RPE WT, it significantly limited the growth of RPE-TopBP1 cells (Figure 6D). These results indicate that increased ATR signaling can drive a temporary cell cycle arrest. As ATR could arrest cells in both G1 or G2 phases, we next assessed whether ATR would slow down G1-progression, by measuring the fraction of cells that fail to enter into S-phase within 20 hours (Figure 6E). We observed a decreased amount of S-phase cells (BrdU positive cells) in RPE-TopBP1 population treated with tamoxifen as opposed to untreated cells, thus confirming that artificial ATR activation induces cell cycle arrest specifically in G1.

Together, this data suggest that we can inducibly activate ATR and its hyperactivation can drive a temporary G1 arrest.

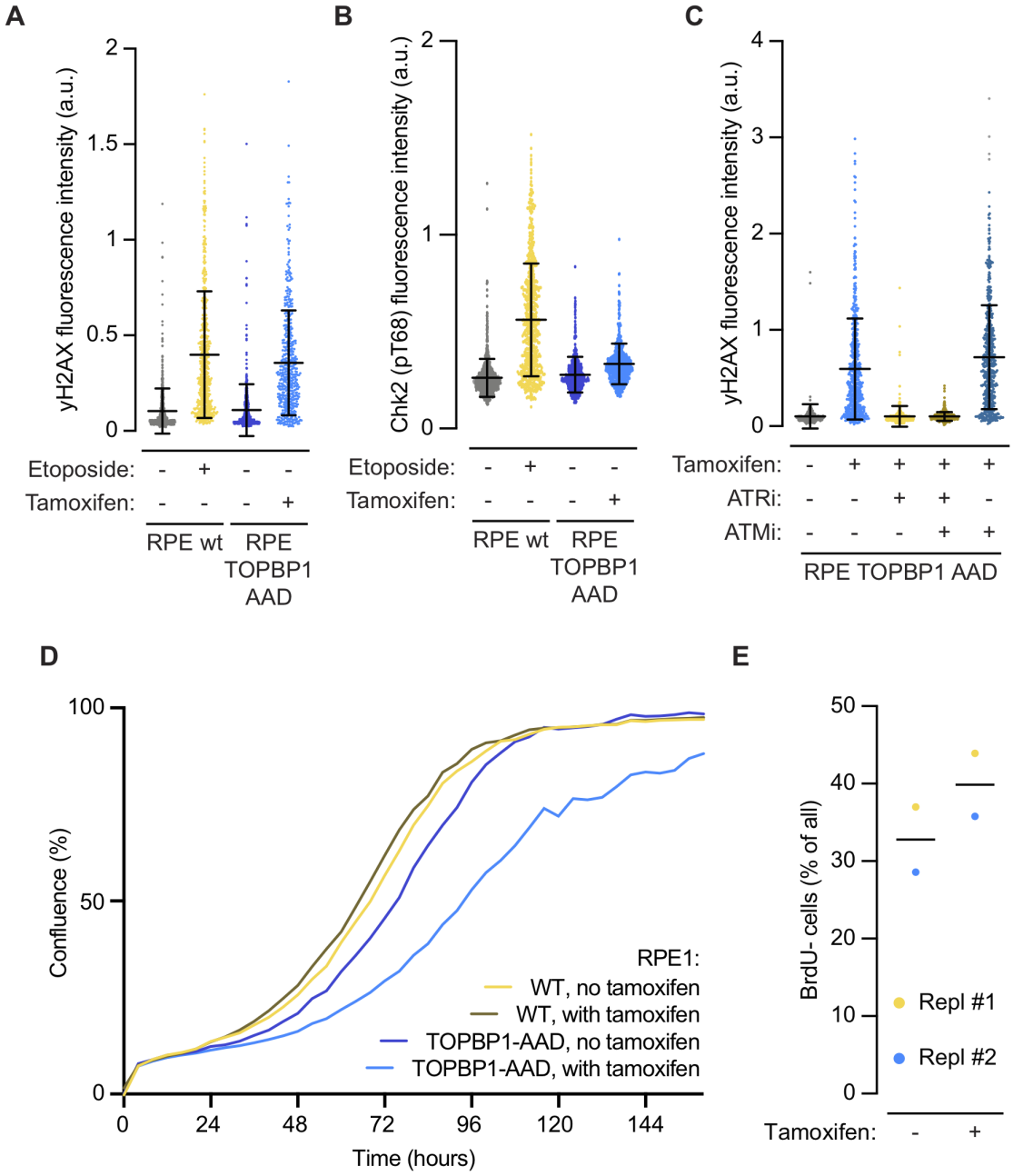

Figure 6. Artificial activation of ATR can delay cell cycle progression to S-phase. A) γH2AX levels in RPE1 wild type cells following etoposide-induced DSBs and RPE-TopBP1-AAD cells following 5 μM OHT (tamoxifen) treatment, resulting in artificial ATR activation. WT RPE cells were damaged by etoposide and cells were fixed 3 hours later. RPE1 TopBP1-AAD cells, which express a TopBP1-ATR-activating domain were treated with 5 μM OHT (tamoxifen) for 3 hours and then fixed. Fixed cells were stained with γH2AX antibody, and γH2AX-intensity was assessed using immunofluorescence. B) Chk2 pT68 levels in RPE1 wild type cells damaged with etoposide and fixed 3 hours later, compared to RPE-TopBP1-AAD cells treated with 5 μM OHT (tamoxifen) for 3 hours. C) Comparison of γH2AX-levels in RPE-TopBP1-AAD cells that were treated for 3 hours with 5 μM OHT (tamoxifen), in the presence of an ATR inhibitor, an ATM inhibitor, or both. D) 250 RPE-TopBP1-AAD cells were plated per well in a 96-well plate. The cells were imaged every 4 hours over the course of a week, and the confluency of the cells was analyzed using an IncuCyte Zoom. E) RPE-TopBP1-AAD cells were treated with BrdU, in the presence or absence of 5 μM OHT (tamoxifen), for 20 hours. Then, cells were fixed and stained for BrdU analysis using FACS.

3.2.5 ATR does not contribute to repair in G1

So far, we have shown that ATR activation in G1 phase contributes to the checkpoint regulation. Also, our data indicate that ATR is activated in response to DNA end resection in G1 phase (Supplementary 1 Figure 1). It was previously suggested that ATR is involved in DNA repair in G1 phase (Gamper et al., 2013). Therefore, we assessed if ATR-signalling contributes to the repair of DNA damage in G1-phase in RPE cells.

To study whether ATR is involved in DSB-repair in G1 phase, we monitored the dynamics of 53BP1, an important regulator of the DSB repair, in RPE HALO-53BP1 cells expressing the FUCCI system. The RPE HALO-53BP1 FUCCI cell line was kindly provided by Anoek Friskes, and allowed us to simultaneously visualize the DNA repair kinetics and cell cycle progression by live-cell imaging (Figure 7A).

Having an elegant system for accessing DSB repair kinetics, we induced DNA damage by NCS in G1 synchronized RPE HALO-53BP1 FUCCI cells and analysed the amount of 53BP1-foci over 8 hours, both in unperturbed cells and upon ATR inhibition. ATR inhibition did not affect the recruitment dynamics of 53BP1 (Figure 7B), indicating that ATR is not directly involved in DSB repair in G1.

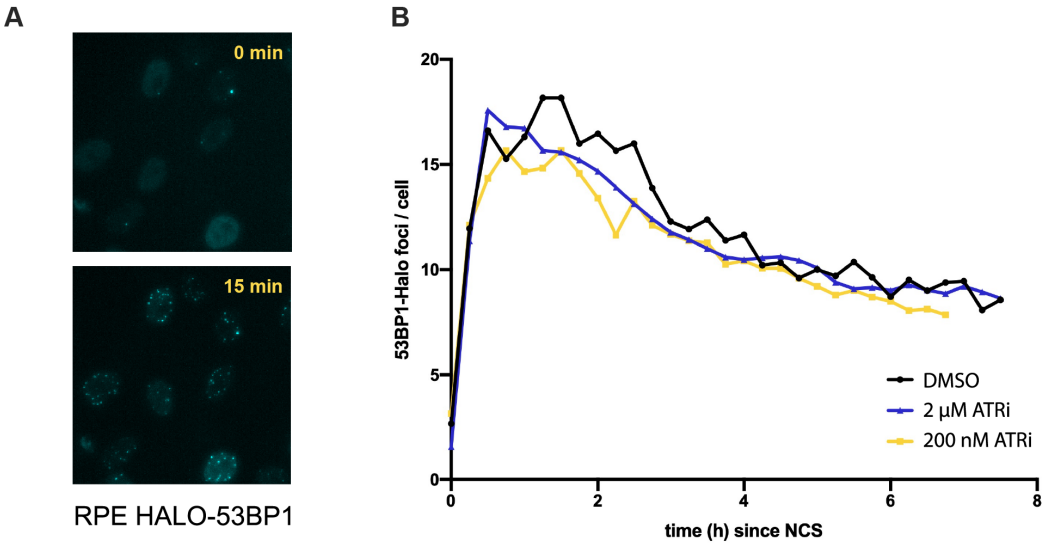

Figure 7. ATR does not contribute to repair in G1. A) Representative images of RPE HALO-53BP1 cells expressing the FUCCI system. Top picture depicts the amount of 53BP1 foci (cyan dots) before DNA damage (0 min) and bottom - 15 minutes after NCS treatment. B) Quantification of 53BP1 foci per cell. RPE HALO-53BP1 FUCCI cells were synchronised in G1 by 20 h Palbociclib treatment. DNA double strand breaks were induced immediately before imaging, by the addition of NCS. ATR (ATRi = VE822) inhibitors were added at the start of experiment as well.

3.3 DISCUSSION

Classically, DSB-induced ATR activation has been thought to be limited to S-G2 phases, when HR factors are available to perform long range resection (Saldivar et al., 2017). However, it has become evident that DSB resection is not limited to S-G2 phases, but can also occur in G1 phase. A previous study showed that resection signals can be detected in around 20–40% of human G1 cells (Yajima et al., 2013). Another study implicated that repair of complex DNA breaks in G1 phase requires extensive end processing by CtIP, MRE11, and EXO1, the core factors of HR (Averbeck et al., 2014). Since extensive DNA end resection has been shown in G1 for complex breaks, it is expected to occur at some rate also in non-complex breaks. In line with that, Peterson et.al (2013) showed that CtIP is phosphorylated at a conserved site (T859 in human) by ATR to promote DSB processing. This raised a question whether DNA resection in G1 may lead to ATR activation. Indeed, recent data obtained in our laboratory showed that ATR can be activated in response to resected DSBs in G1 phase (Supplementary 1). Collectively, these observations indicate that some breaks in G1 phase are resected and lead to ATR activation.

Upon DNA damage in G2 phase ATR regulates cell cycle arrest, therefore we asked ourselves whether resection-dependent ATR signaling in G1 can influence cell cycle outcome. We subjected cells to different genotoxic treatments and observed that ATR reinforces the G1 checkpoint (Figure 3). This result demonstrates that the cell fate decisions in G1 are not solely regulated by ATM and Chk2, as conventionally believed, but rather dual cooperation of Chk2 and ATR allows to achieve a complete control over G1 checkpoint. Thus, ATR is involved in cell fate determination throughout the cell cycle. Moreover, we noticed that upon inhibition of NHEJ by DNA-PKcs all cells were arrested in G1 phase, but when we additionally inhibited ATR, 40% of those cells recovered from the arrest (Figure 4). These findings indicate that some cells arrest in an ATM-dependent manner while in others the arrest is ATR-dependent.

Next, we showed that we could tune the G1 checkpoint regulation towards ATR by inhibiting NHEJ factor ligase IV (Figure 4). Interestingly, upon inhibition of ligase IV 50% of the cells remained arrested and this arrest was terminated by ATR inhibition. This data suggests that there could be a potential therapeutic window for ligase IV inhibitors, since most cancer cells

do not have a functional G1 checkpoint and will proliferate despite unrepaired DNA breaks, but normal cells, on the other hand, will remain arrested due to present ATR activation. In line with that, we show that ATR hyperactivation can independently lead to cell cycle arrest (Figure 6). These results highlight the importance of DNA damage-induced ATR activation and the possibility of modulating the G1 checkpoint by ATR signaling only. For future research, it would be interesting to evaluate if continuous ATR-signaling by itself, or in combination with DNA damaging agents, can cause the induction of senescence.

Another interesting finding emerged from studying the contribution of ATR to the regulation of p53 and p21 DNA damage response. The results showed that ATR inhibition restricts full activation of both p53 and p21, providing mechanistic insight into G1 checkpoint regulation by ATR. However, whether this event is carried out by direct phosphorylation of p53 by ATR or through the regulation of counteracting proteins, such as KAP1, remains unknown. Furthermore, it is of great interest to investigate whether ATR mimics ATM activation in G1 and contributes to the p53-independent activation of p21 through p38 MAPK or targets CDC25 phosphatase for degradation, preventing early S-phase entry.To identify proteins involved in ATR-mediated signaling in the G1 phase, we could synchronise RPE WT cells in G1 by CDK4/6 inhibitor, induce DNA damage and inhibit ATR simultaneously, and with all proper controls analyse ATR specific events by phosphoproteomics.

Finally, by assessing the kinetics of 53BP1 foci disappearance as a measure of DNA repair, we show that ATR is not involved in DNA repair in G1 phase (Figure 7). Although it has been suggested that ATR may promote DNA repair (Gamper et al., 2013), that study was conducted in U2OS cells that have a defective p53 and an unstable G1 checkpoint. Furthermore, the researchers draw their conclusions based on colocalization of 53BP1 and RPA, with no evidence that colocalization is impaired upon ATR inhibition. On the contrary, the HALO-53BP1 FUCCI system used in this work allowed us to study the difference in 53BP1 localization upon ATR inhibition directly, and we observed no difference. Nevertheless, it is interesting to investigate whether activation of ATR is important to prevent premature recovery from the G1 checkpoint to limit the toxicity of the breaks. To test that, we could plate RPE p21-GFP PCNA-mRuby cells

for clonogenic outgrowth and access if ATR inhibition in G1 phase leads to a decreased cell survival. For that, we would need to induce the DNA damage and inhibit ATR for different durations, then isolate cells in G1 phase using FACS and let cells to grow for 1-2 weeks before fixation and visualizing growing colonies using crystal violet staining. Additionally, we could trace cell fates of RPE-FUCCI cells that received DNA damage and ATR inhibitor in G1 phase, and cell fates of their potential daughter cells for several days. Importantly, for this setting, ATR inhibitors should be washed away after a few hours, because ATR is essential in suppressing replication stress in S-phase and its inhibition in S-G2 leads to premature mitotic entry. Finally, we could assess if RPE HALO-53BP1 cells that received DNA damage and an ATR inhibitor in G1 phase exhibit an increased amount of unresolved breaks (53BP1 foci) upon S-phase entry. These experiments would allow us to elucidate if ATR promotes cellular fitness when facing DSBs in G1 phase.

In addition to investigating the role of ATR in G1 phase, we observed several interesting discoveries that require further investigation.

Namely, we found that the downstream effector of ATR, Chk1, is important in the unperturbed G1 phase. As a result, the toxic effect of Chk1 depletion did not allow us to implicate CHK1 in the G1 checkpoint. Although the exact role of Chk1 in the G1 phase remains unknown, it regulates DNA replication in the S-phase (Pabla et al., 2011). We know that preparation for DNA replication begins as soon as cells complete mitosis, in the G1 phase. That includes activation of a transcriptional program that induces expression of many S-phase related genes, as well as the assembly of replication complexes (Limas and Cook, 2019). Therefore, it is tempting to speculate that during G1 phase, Chk1 holds a function affecting the initiation of DNA replication. However, further investigation is needed to validate this hypothesis.

Another interesting observation was that the 53BP1 protein not only regulates DSB repair pathway choice by limiting nucleolytic processing of DNA ends, but also plays a role in the G1 checkpoint. Since 53BP1 was discovered as a p53 binding protein, the p53-53BP1 relationship remained unknown for a long time. Recently, Cuella- Martin et al. (2016) demonstrated

that 53BP1 mediates interactions between p53 and the deubiquitinating enzyme USP28, thus enhancing p53-dependent transcriptional programs. Moreover, 53BP1-/- mice cells show an impaired Chk2 activation (Ward et al., 2003). These findings support the notion that loss of 53BP1 results in an ineffective p53-dependent cell-cycle checkpoint response.

We also observed that damaged cells failed to recover from G1 arrest upon inhibition of DNA-PKcs. This raised a question if the persistence of DSBs due to the loss of DNA-PKcs activity leads to a sustained cell cycle arrest, or if there is crosstalk between DNA-PKcs and ATM that enhances checkpoint signalling in the absence of DNA-PKcs. Indeed, it has been shown that loss of DNA-PKcs leads to hyperactivation of ATM and p53 responses to DNA damage (Finzel et al., 2016). Other studies also suggest that DNA-PKcs phosphorylates p53 (Dip and Naegeli, 2005; Jack et al., 2004), and loss of DNA-PKcs leads to repressed activity of p53 downstream targets, such as p21, in response to DNA damage (Kachnic et al., 1999). Evidently, the role of DNA-PKcs is incompletely characterized and more work is required to further elucidate its contribution to cellular functions other than NHEJ.

4 SUMMARY

ATR is an essential gene and is also involved in suppressing replication stress caused by DNA damage and oncogene activation. Due to the higher degree of DNA damage, cancer cells are more reliant on ATR-dependent stress responses than normal cells. Currently, multiple ATR inhibitors are entering clinical trials. Therefore, a complete understanding of ATR signaling throughout the cell cycle is required to improve the efficacy of such anti-cancer therapies.

The results presented in this study cast a new light on ATR activation in DNA damage response. By tracing cell fate outcomes of individual cells, we were able to examine the interplay between ATR signaling and the cell cycle machinery in response to DNA damage.

Our experimental data showed that DSB-induced ATR activation in G1 phase influences cell fate decisions by modulating the G1 checkpoint. Moreover, we demonstrated that ATR inhibition in the presence of DNA damage significantly decreases the activation of p53 and p21 proteins, responsible for the induction of cell-cycle arrest. These findings provide mechanistic insight into the regulation of G1 checkpoint by ATR. Furthermore, we could increase the G1 checkpoint dependency on ATR and we show that ATR hyperactivation in G1 can slow down cell cycle progression. These results collectively demonstrate that ATR is involved in G1 checkpoint control. Thus it is essential to accurately balance DSB-induced ATR signaling to allow diverse outcomes such as complete recovery or cell cycle arrest.

Lastly, we observed that ATR activation does not play an essential role in the DNA repair in G1 phase. However, the G1 checkpoint regulation by ATR could be vital in reducing the toxicity of DNA breaks by providing cells extra time for DNA repair.

In conclusion, this study challenges the conventional idea that ATR activation is limited to S-G2 phases and broadens our understanding of ATR signaling throughout the cell cycle. We observed that DSB-induced ATR activation is crucial to allow diverse outcomes such as cell cycle arrest or proliferation. We suggest that this newly discovered function of ATR in G1 checkpoint is important to consider when designing ATR inhibitors and improving cancer therapy.

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SUPPLEMENTARY 1

ATR is activated by DNA double strand breaks in G1 phase

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The repair of DSBs through Homologous Recombination (HR) is restricted to S/G2 phases of the cell cycle (*8*), due to the inhibition of end-resection in G1 (*1–3*, *4*). However, end-resection can be involved in the repair of complex DSBs in G1 phase (*5*, *6*), and persistent DSBs are liable to end-resection in G1 phase (*7*). As ATR is activated in response to ssDNA resulting from end-resection (in S/G2 phases), this raises the possibility that ATR is activated in response to resected DSBs in G1 phase. To investigate this, I treated RPE cells with the radiomimetic drug Neocarzinostatin (NCS), which rapidly causes DSBs that are subsequently repaired (*9*). Three hours after the induction of DSBs I fixed cells and stained them for γH2AX, a general marker for DNA damage, RPA32 phosphorylated at serine 33, which is ATR-dependent (*10*), and EdU, to allow cell cycle stratification. Interestingly, NCS induced RPA pS33-foci in G1 phase cells, albeit to a lesser extent than G2 phase cells (Figure 1A and 1B). Next, I concomitantly treated RPE cells with NCS and the ATR inhibitor (ATRi) VE822. Inhibition of ATR in damaged cells decreased RPA pS33 fluorescence, confirming that NCS causes ATR activation in G1 phase (Figure 1C, left panel). Concomitant induction of DSBs and inhibition of MRE11, which is required for the initiation of end-resection (*8*), also decreases ATR activation in G1 phase. Finally, I tested whether ATR is activated in response to DSBs induced by the Topoisomerase II inhibitor Etoposide, and observed similar ATR- and MRE11-dependent phosphorylation of RPA (Figure 1C, right panel). Collectively, these results show that ATR can be activated in response to resected DSBs that occur in G1 phase.

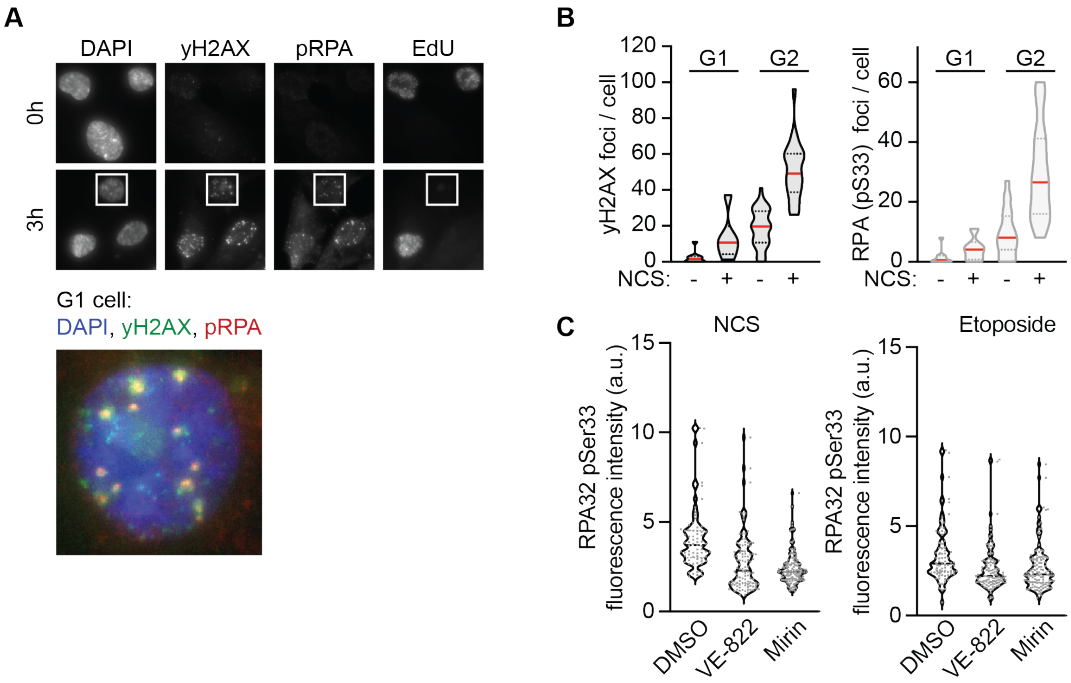

Figure 1. ATR is activated by DNA double strand breaks in G1 phase.
A) RPE cells were treated with 100ng/ul of neocarzinostatin (NCS). At the same time, EdU was added to allow visualization of DNA replication. Cells were fixed 3 hours later, and stained for γH2AX, as general marker for DNA double strand breaks, RPA32 pS33, as a marker for DNA end-resection and ATR activation, and EdU, to visualize replicating cells.
B) Quantification of the amount of γH2AX- and RPA32 pS33-foci of the cells in (A), stratified for G1 or G2 phases based on total DNA content and EdU-negativity.
C) RPE cells were treated with NCS or etoside, in the presence of the indicated inhibitors, as well as EdU. Cells were fixed 3 hours later and stained for RPA32 pS33 and EdU. The total nuclear intensity of RPA32 pS33 was measured in G1 phase cells treated with NCS (left panel) or etoposide (right panel).

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