Chapter 4

Overexpression of Cyclin E1 or Cdc25A leads to replication stress, mitotic aberrancies and increased sensitivity to replication checkpoint inhibitors

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Chapter 4

Mitotic defects upon Cyclin E1/Cdc25A overexpression

ABSTRACT

Oncogene-induced replication stress, for instance as a result of Cyclin E1 overexpression, causes genomic instability and has been linked to tumorigenesis. To survive high levels of replication stress, tumors depend on pathways to deal with these DNA lesions, which represent a therapeutically actionable vulnerability. We aimed to uncover the consequences of Cyclin E1 or Cdc25A overexpression on replication kinetics, mitotic progression, and the sensitivity to inhibitors of the WEE1 and ATR replication checkpoint kinases.

We modeled oncogene-induced replication stress using inducible expression of Cyclin E1 or Cdc25A in non-transformed RPE-1 cells, either in a TP53 wild-type or TP53 mutant background. DNA fiber analysis showed Cyclin E1 or Cdc25A overexpression to slow replication speed. The resulting replication-derived DNA lesions were transmitted into mitosis causing chromosome segregation defects. Single cell sequencing revealed that replication stress and mitotic defects upon Cyclin E1 or Cdc25A overexpression resulted in genomic instability. ATR or WEE1 inhibition exacerbated the mitotic aberrancies induced by Cyclin E1 or Cdc25A overexpression, and caused cytotoxicity. Both these phenotypes were exacerbated upon p53 inactivation. Conversely, downregulation of Cyclin E1 rescued both replication kinetics, as well as sensitivity to ATR and WEE1 inhibitors.

Taken together, Cyclin E1 or Cdc25A-induced replication stress leads to mitotic segregation defects and genomic instability. These mitotic defects are exacerbated by inhibition of ATR or WEE1 and therefore point to mitotic catastrophe as an underlying mechanism. Importantly, our data suggest that Cyclin E1 overexpression can be used to select patients for treatment with replication checkpoint inhibitors.

INTRODUCTION

A common hallmark of cancer is the acquisition of genomic gains and losses as well as complex genomic re-arrangements, collectively termed genomic instability (1). Genomic instability drives intra-tumor heterogeneity, which is an important factor underlying therapy failure (2). Stalling or slowing of replication, commonly referred to as ‘replication stress’, is increasingly considered to be an important factor in fueling genomic instability in cancer (3,4). Although there are various factors that induce replication stress, a common cause in the context of cancer is the increased activity or elevated expression of oncogenes (5–7).

Amplification of CCNE1 (encoding for Cyclin E1) is frequently observed in genomically instable tumors, including high-grade serous ovarian cancer and triple negative breast cancer (TNBC) (8–13), and has been associated with a poor prognosis in these and various other tumor types (14–17). CCNE1 amplification has been linked to induction of replication stress, by causing collisions between the replication and transcription machineries (18), and by triggering aberrant firing of replication origins, which subsequently leads to depletion of the nucleotide pool (18,19). Combined, these effects can lead to stalling or collapse of replication forks (7). Oncogene-induced replication stress triggers a DNA damage response, with ensuing genetic pressure to inactivate TP53 (20). In good agreement with these observations, Cyclin E1 overexpression was demonstrated to exclusively induce genome instability in tumors lacking functional p53 (21–23).

Multiple oncogenic events were shown to exert their effects on DNA replication through direct or indirect elevation of Cyclin-dependent kinase-2 (CDK2) activity (24–27). CDK2 activity is important in regulating the ‘firing’ of replication origins (28–30), and is primarily controlled by the abundance of its Cyclin partner. Indeed, overexpression of Cyclin E1 elevates CDK2 activity (31). Importantly, CDK2 activity - determined by inhibitory phosphorylation of Tyr15 (32) - is catalyzed by the WEE1 kinase (33,34), and can be removed by the Cdc25A phosphatase (35). In line with this notion, overexpression of Cdc25A has been shown to result in CDK2 hyperactivation (36). Consequently, overexpression of either CCNE1 or Cdc25A leads to aberrant firing of replication origins and triggers a replication stress response (18).
Since replication stress hampers cell growth, cancers harboring oncogene-induced replication stress have apparently adapted to cope with replication stress. In order to find better treatments for tumors with oncogene-induced replication stress, it could be of great clinical interest to target pathways that allow tumors to deal with replication stress. Particularly interesting in this context are cell cycle checkpoint kinases. Previously, tumor cells with genome instability due to defective homologous recombination were shown to depend on the ATR and WEE1 replication checkpoint kinases for their survival (37,38). Furthermore, lymphomas driven by MYC amplification - which triggers profound replication stress - were shown to be highly sensitive to CHK1 inhibition (39). In order to optimally implement cell cycle checkpoint inhibitors in cancer treatment, and identify patients who benefit from such treatments, it is essential to understand how cancer cells deal with replication stress, and uncover the mechanisms underlying checkpoint kinase inhibitor-mediated cytotoxicity in cancer cells.

It is increasingly apparent that the resolution of replication stress is highly complex and not restricted to S-phase. Indeed, resolving late-stage replication intermediates was observed even when cells had already entered mitosis (40,41). In line with these observations, our recent data underscored the notion that PARP inhibitor-induced replication-mediated DNA lesions are transmitted into mitosis, and cause chromosome segregation defects and mitotic failure (42). Whether these findings hold true for other sources of replication stress is currently unknown. In this study, we assessed whether oncogene-induced replication stress as a result of Cyclin E1 or Cdc25A overexpression affects mitotic behavior of tumor cells and genome instability. Additionally, we studied whether replication stress can be targeted through inhibition of the cell cycle checkpoint kinase WEE1 and ATR.

RESULTS

Overexpression of Cyclin E1 or Cdc25A leads to slower replication kinetics and mitotic defects.

Cyclin E1 is often found to be overexpressed in cancers, specifically in TNBCs and high-grade ovarian cancers (43,44), which is accompanied by higher CCNE1 mRNA expression levels in these cancers (Suppl. Fig. 1A). To study the effects of Cyclin E1 overexpression on replication kinetics, we engineered hTERT-immortalized human retinal pigmented epithelial (RPE-1) cells to overexpress a truncated oncogenic version of Cyclin E1 in a doxycycline-dependent manner. Doxycycline treatment resulted in a ~70-fold increased expression of Cyclin E1 compared to endogenous levels (Fig. 1A and Suppl. Fig. 1B). In parallel, we evaluated the effects of Cdc25A overexpression, as this protein also leads to CDK2 hyperactivation, albeit through an alternative mechanism (Fig. 1A). To test whether overexpression of Cyclin E1 or Cdc25A affected replication dynamics, cells were treated with doxycycline for 48 hours, and cells were subsequently incubated with thymidine analogues CldU and IdU to label ongoing replication (Fig. 1B). Single DNA fibers were analyzed to measure replication kinetics. The IdU fiber tract length was reduced by 28% in Cyclin E1-overexpressing cells and 31% in Cdc25A-overexpressing cells, indicating a robust reduction of ongoing DNA synthesis speed compared to parental RPE-1-TP53wt cells (Fig. 1C).

We next tested whether the observed replication stress resulted in mitotic aberrations. To this end, we quantified the amount of chromatin bridges and lagging chromosomes during anaphase and telophase at 48 hours after induction of Cyclin E1 or Cdc25A overexpression in RPE-1-TP53wt cells (Fig. 1D). Doxycycline-induced Cyclin E1 or Cdc25A overexpression resulted in a 3-fold increase in mitotic aberrancies when compared to control cells (Fig. 1E). Both chromatin bridges and lagging chromosomes were increased in Cyclin E1 and Cdc25A-overexpressing cells (Fig. 1E). A third type of mitotic aberration, ultra-fine bridges (45), was only increased in Cdc25A-overexpressing cells (26% vs 14%) but not in Cyclin E1-overexpressing cells (11% vs 14%) (Suppl. Fig. 1C). To further investigate the mitotic aberrations induced by oncogene-induced replication stress, RPE-1-TP53wt cells overexpressing Cyclin E1 or Cdc25A were analyzed by live-cell microscopy. To this end, cells were transduced with EGFP-tagged Histone-H2B, treated with doxycycline to induce overexpression of Cyclin E1 or Cdc25A and were then followed for the duration of 48 hours, capturing images every 7 minutes using live cell microscopy (Fig. 1F). Overexpression of Cyclin E1 or Cdc25A did not significantly affect mitotic duration as measured by the time between nuclear envelope break-down (NEB) and anaphase entry (Fig. 1G), but did increase the frequency of mitotic aberrancies (23% in Cyclin E1-overexpressing cells and 19% in Cdc25A-overexpressing cells versus 12% and 3% in respective control-treated cells, Fig. 1H). Combined, these data show that both Cyclin E1 and Cdc25A-induced replication stress results in the formation of chromatin bridges and lagging chromosomes, whereas Cdc25A overexpression also increases ultra-fine bridge formation.
TP53 mutation exacerbates replication stress and mitotic defects

Since oncogene expression in genomically instable cancers is frequently associated with loss of TP53, we used CRISPR/Cas9 to mutate TP53 in RPE-1 cells (Fig. 2A). We selected two TP53-mutant clones and introduced the doxycycline-inducible Cyclin E1 and Cdc25A constructs or an empty vector to assess how p53-negative cells behave upon overexpression of these oncogenes (Fig. 2B, Suppl. Fig. 2A, B). Compared to endogenous Cyclin E1 levels, doxycycline treatment increased the expression by ~60-fold in clone #1 and ~38-fold in clone #2 (Suppl. Fig 2C). Like in TP53-wt cells, overexpression of Cyclin E1 or Cdc25A in RPE-1-TP53−/− cells reduced IdU tract length by 7-53% compared to untreated conditions (Fig. 2C, Suppl. Fig 2D).

We next analyzed the amounts of mitotic aberrancies. In line with previous reports, RPE-1-TP53−/− cells showed higher basal frequencies of mitotic aberrations when compared to RPE-1-TP53+ cells (17% vs 6%, Fig. 1D and 2D, Suppl. Fig 2E) (46). The percentage of mitotic aberrations increased from 17% to 41.1% in Cyclin E1-overexpressing cells and to 33.3% in
Figure 2: Mutation of TP53 exacerbates replication stress and mitotic defects.

a Schematic overview of CRISPR/Cas9 gene targeting in TP53 gene. The exon map and protein coding are based on Ensembl entry ENSG00000141510. Placement of the sgRNA sequence is indicated with a horizontal line under exon 4 and the wild type sequence. Sanger sequencing shows that the gRNA targeting exon 4 induced a -7 bp deletion and a +215 bp insertion in RPE-TP53 cl#1 and a +1 deletion and +2 insertion in RPE-TP53 cl#2, leading to frame-shifts in TP53. b RPE-1-TP53−/− cl#1 cells were engineered to overexpress empty, Cyclin E1 or Cdc25A constructs in a doxycycline-inducible manner. Immunoblot shows Cyclin E1, Cdc25A, p53 and Vinculin protein levels at 48 hours after addition of doxycycline (dox). RPE-1-TP53−/− cells were used as a positive control for p53. c Cells were treated with doxycycline for 48 hours, and were then labeled for 20 minutes with CldU (25 µM) and subsequently for 20 minutes with IdU (250 µM). Per condition at least 279 fibers were analyzed. Graphs show individual data points, median and interquartile range. p-values were calculated using the Mann-Whitney U test. d Quantification of anaphase or telophase cells containing chromatin bridges or lagging chromosomes. The bars represent mean and standard error or the mean (SEM) from 3 experiments, n≥25 per experimental condition; p-values were calculated using two-tailed Student’s t-test. e Representative examples of mitotic aberrancies observed in RPE-1-TP53−/− cells transduced with H2B-EGFP cells using live-cell microscopy. Scale bar represents 20 µm. f Duration of mitosis as measured by NEB breakdown to anaphase. Cells were pre-treated for 24 hours with doxycycline and subsequently followed with live-cell microscopy using 7 minute intervals for the duration of 48 hours. p-value was calculated using a Kruskal-Wallis test. g Quantification of aberrant mitoses in cells from panel f. p-values were calculated using absolute values, using Mann-Whitney U test.

Cdc25A-overexpressing cells (Fig. 2D). We did not observe an increase in the amount of ultra-fine bridges upon Cyclin E1 or Cdc25A overexpression in TP53 mutated cells (Suppl. Fig. 2F).

To confirm that the absence of p53 expression leads to elevated amounts of mitotic defects, we analyzed H2B-EGFP-expressing cells using live-cell imaging (Fig. 2E). Analogous to previous observations in TP53−/− cells, overexpression of Cyclin E1 or Cdc25A in RPE-1-TP53−/− H2B-EGFP cells did not result in a significant change in the duration of mitosis (Fig. 2F). We did observe more mitotic defects at baseline in RPE-1-TP53−/− cells than in RPE-1-TP53−/− cells (Fig. 1H and 2E). Although not statistically significant, Cyclin E1 and Cdc25A overexpression in TP53−/− cells did increase the percentage of mitotic defects (Fig. 2G). These data underscore that replication stress and mitotic errors are increased upon TP53 inactivation, and point towards exacerbation of this phenotype upon Cyclin E1 and Cdc25A overexpression.
**Cyclin E1 or Cdc25A overexpression induces genomic instability.**

Elevated levels of Cyclin E1 have previously been associated to structural chromosome defects (47,48). Moreover, overexpression of both Cyclin E1 and Cdc25A has been shown to result in loss of specific genomic regions (49–51). Furthermore, a mouse model of Cyclin E1 overexpression resulted in tumors with genomic instability (51). Indeed, we also observed correlations between mRNA expression of CCNE1 or CDC25A and copy number load in various tumor types (Suppl. Fig. 3A-C). However, since some of these observations could be explained by indirect effects, we employed single-cell whole genome sequencing to assess if and how the observed chromosome segregation defects upon short-term overexpression of Cyclin E1 or Cdc25A in RPE-1-TP53−/− cells translate into structural or numerical chromosome aberrations (52). Of note, we observed genomic deviations that arose in the process of engineering the TP53−/− cell lines, underscoring the importance of analyzing multiple clones (Suppl. Fig. 4A). Importantly, we observed increased numbers of focal copy number alterations (CNAs) upon induction of Cyclin E1 or Cdc25A overexpression for 5 days (Fig. 3A, B and Suppl. Fig. 4B-D). This increase was statistically significant in RPE-1-TP53−/− clone #1, but not in clone #2, possibly due to the limited number of cells that were analyzed, a relatively short treatment time, and lower levels of overexpression in clone #2 (Suppl. Fig. 2C). In RPE-1-TP53−/− clone #1, Cyclin E1 overexpression resulted in more copy number aberrations compared to empty vector control (Fig. 3B and Suppl. Fig. 4C, whereas Cdc25A overexpression resulted in more whole chromosome aberrations (Fig. 3C and Suppl. Fig. 4D). These data suggest that the increased mitotic errors upon Cyclin E1 or Cdc25A overexpression translate into genomic instability.

**Cyclin E1 and Cdc25A-induced mitotic aberrancies are exacerbated upon treatment with ATR and WEE1 inhibitors.**

The observation that Cyclin E1 or Cdc25A overexpression leads to replication stress, mitotic aberrations and ensuing focal copy number alterations likely indicated that replication-born DNA lesions remain unresolved when cells enter mitosis. Indeed, regardless of TP53−/− status, we observed that Cyclin E1-overexpressing cells, and to a lesser extent Cdc25A-overexpressing cells have increased amounts of the DNA damage marker pH2AX Ser139 (γH2AX) (Fig. 4A, B, Suppl. Fig. 5A, B). These cells also activated a replication stress response mediated by ATR, as measured by pATR Thr1989, pCHK1 Ser345 and pRPA32 Ser33) (Fig. 4A, B) and elevated WEE1 activity as measured by levels of pCDK Tyr15 (Fig. 4C, D, Suppl. Fig. 5A, B). Although the observed activation of the ATR and WEE1 kinases did not completely prevent mitotic errors from occurring, inhibiting this response could enforce premature mitotic entry (53), thereby exacerbate chromosome segregation errors in Cyclin E1- or Cdc25A-overexpressing cells. To test this, we induced overexpression of Cyclin E1 or Cdc25A in RPE-1-TP53−/− or RPE-1-TP53−/− cells for 48 hours, and subsequently treated the cells with ATR or WEE1 inhibitors for 8 hours. Upon overexpression of Cyclin E1 in RPE-1-TP53−/− cells, WEE1 inhibition, but not ATR inhibition, resulted in a significant increase of mitotic aberrancies (Fig. 4F-G). In Cdc25A-overexpressing RPE-1-TP53−/− cells, inhibition of ATR and WEE1 both enhanced the frequency of mitotic aberrancies (41.1% to 72.2% upon ATR inhibition and 25.6% to 77.8% upon WEE1 inhibition, Fig. 4E, F).

In the RPE-1 TP53−/− clones, both ATR and WEE1 inhibition increased the frequency of mitotic aberrancies in Cyclin E1-overexpressing cells (from 37.7% to 72.2% upon ATR inhibition and up to 87.8% upon WEE1 inhibition, Fig. 4G, Suppl. Fig. 5C), and in Cdc25A-overexpressing cells (from 37.7% to 81.1% upon ATR inhibition and to 82.7% upon WEE1 inhibition, Fig. 4G). We did not observe an increase in ultra-fine bridges upon inhibition of ATR or WEE1 in any of the tested conditions (Suppl. Fig. 5D, E).

We next used live cell microscopy to investigate whether chromosome segregation defects induced by ATR or WEE1 inhibition in Cyclin E1-overexpressing RPE-1-TP53−/− cells translated into altered mitotic fidelity and duration. Indeed, ATR inhibitor treatment in Cyclin E1-overexpressing RPE-1-TP53−/− cells increased the percentage of mitoses with chromatin bridges from 13% to 33%, and increased the percentage of lagging chromosomes from 7% to 22% (Fig. 4G). Similarly, WEE1 inhibition exacerbated the formation of chromatin bridges in Cyclin E1-overexpressing cells from 15% to 33% (Fig. 4G), and increased the percentage of lagging chromosomes from 9% to 14% (Fig. 4G). The induction of mitotic aberration by ATR and WEE1 inhibition was confirmed in RPE-1-TP53−/− H2B-EGFP using live cell microscopy (Fig. 4H). ATR nor WEE1 inhibition affected mitotic duration in Cyclin E overexpressing cells (Suppl. Fig. 5F). To measure premature mitotic entry upon ATR or WEE1 inhibition, cells were synchronized using a thymidine block. In line with previous reports, ATR inhibition accelerated entry into mitosis, leading to a burst in mitotic cells (53),
Chapter 4

Mitotic defects upon Cyclin E1/Cdc25A overexpression

Overexpression of Cyclin E1 or Cdc25A results in increased sensitivity to ATR and WEE1 inhibition.

Using MTT assays, we next examined whether the enhanced occurrence of mitotic aberrancies upon ATR or WEE1 inhibition in Cyclin E1 or Cdc25A-overexpressing cells is accompanied with increased sensitivity towards ATR or WEE1 inhibition. In line with the absence of increased mitotic aberrancies upon ATR inhibitor treatment in TP53<sup>wt</sup> Cyclin E1 overexpressing cells, we observed that Cyclin E1 overexpression only sensitized RPE-TP53<sup>-/-</sup> cells to ATR inhibition (Fig. 4I), indicating that loss of p53 function is required for ATR inhibitor sensitivity in Cyclin E1-overexpressing cells. In contrast, loss of p53 function was not required for WEE1 inhibitor sensitivity in Cyclin E1-overexpressing cells, although it did enhance sensitivity (Fig. 4I). Cdc25A overexpression sensitized both RPE-1-TP53<sup>wt</sup> and RPE-1-TP53<sup>-/-</sup> cells to ATR inhibition as well as to WEE1 inhibition (Fig. 4I). These data indicate that Cyclin E1 or Cdc25A overexpression sensitizes cells to inhibition of the ATR or WEE1 checkpoint kinases.

Reduction of Cyclin E1 levels diminishes replication stress and mitotic errors.

To test whether high expression levels of Cyclin E1 influenced DNA replication kinetics and sensitivity of cancer cells to ATR and WEE1 inhibitor, we aimed to downregulate Cyclin E1 expression in TNBC cancer cells. We first tested the sensitivity to ATR and WEE1 inhibition in three TNBC cell lines (MDA-MB-157, HCC1806 and HCC1569) that have a 19q12 amplification which encompasses the CCNE1 gene (55). HCC1806, and to a lesser extent HCC1569, were sensitive to both ATR and WEE1 inhibition (Suppl. Fig. 6A, Suppl. Fig. 6B). MDA-MB-157 cells did not display notable sensitivity to either drug (Suppl. Fig. 6A, B), and we therefore selected HCC1806 to test whether downregulation of CCNE1 could rescue the sensitivity to the cell cycle checkpoint inhibitors. Two doxycycline-inducible shRNAs targeting CCNE1 were transduced in these cells, and knockdown efficiency was assessed after 48 hours of doxycycline treatment (Fig 5A). Whereas shCCNE1#1 showed a near-complete depletion of Cyclin E1, shCCNE1#2 reproducibly resulted in a partial yet homogeneous knock-down throughout the cell population (Fig. 5A-C). In line with Cyclin E1 being a driver oncogene on the 19q12 amplicon, cell cycle analysis demonstrated that severe depletion of Cyclin E1 levels using shCCNE1#1 in whereas WEE1 inhibition did not (54) (Suppl. Fig. 5G). These data indicate that ATR inhibition may affect mitotic fidelity by premature mitotic entry, whereas the effects of WEE1 inhibition appear more complex.

Figure 3: Cyclin E1 or Cdc25A overexpression induces genomic instability.

a Genome-wide copy number deviation plots of RPE-TP53<sup>-/-</sup> cell#1 empty (n=47), RPE-TP53<sup>-/-</sup> cell#1 -Cyclin E1 (n=44) and RPE-TP53<sup>-/-</sup> cell#1 -Cdc25A cells (n=46). Cells were treated with doxycycline for 120 hours. After single cell sorting, genomic DNA was harvested for single-cell whole genome sequencing (sc-WGS). Each panel displays the individual cells in rows, and the chromosomes numbers from 1-X in columns. The modal copy number state is pictured in green, deviations of the modal copy number state, both focal and whole-chromosome, are colored red). b Copy-number alterations (CNAs) per cell were calculated according to the modal state. Medians with interquartile range are depicted and statistical analyses were performed using a One-sided Mann Whitney U test. c whole numerical chromosomes per cell were counter per single cell. Medians with interquartile range are depicted and statistical analyses were performed using a One-sided Mann Whitney U test.
HCC1806 cells reduced the percentage of cells in S-phase (Fig. 5D), which was accompanied by a near-complete loss of clonogenic potential (Fig. 5E-G).

Figure 4: ATR and WEE1 inhibition cause mitotic aberrancies.

a, b RPE-TP53wt (panel a) and RPE-TP53-/- c1#1 (panel b) cells were treated with doxycycline for 72 hours to induce overexpression of Cyclin E or Cdc25A. Control cells (RPE-TP53wt) were then left untreated or were treated with ATR inhibitor (ATRI, VE-822, 1 µM) for 2 hours, followed by a 6 hour treatment with hydroxyurea (HU, 1 mM) and immunoblotted for ATR-responses proteins pATR, pCHK1, pPRR and pH2AX, and for WEE1-response marker pCDK (Tyr15). Vinculin serves as a loading control.

c, d RPE-TP53wt (panel c) and RPE-TP53-/- c1#1 (panel d) were treated with doxycycline for 72 hours to induce overexpression of Cyclin E or Cdc25A. Control cells (RPE-TP53wt) were then left untreated or were treated with ATR inhibitor (ATRI, VE-822, 1 µM) for 2 hours, followed by a 6 hour treatment with hydroxyurea (HU, 1 mM) and immunoblotted for WEE1 response protein pCDK (Tyr15). f, g RPE-1-TP53-/- c1#1 cells induced to express Cyclin E1 or Cdc25A were treated with WEE1 inhibitor (WEE1i, MK-1775, 0.1 µM) for 8 hours if indicated. The percentages of anaphase or telophase cells containing chromatin bridges or lagging chromosomes were quantified. The bars represent mean and SEM from 3 experiments, n>25 per experimental condition; p-values were calculated using two-tailed Student's t-test.

g RPE-1-TP53-/- c1#1 cells induced to express Cyclin E1 or Cdc25A were treated as in panels e and f. The percentages of anaphase or telophase cells containing chromatin bridges or lagging chromosomes were quantified. The bars represent mean and SEM from 3 experiments, n>25 per experimental condition; The p-values were calculated by one-way ANOVA (P<0.0001) and followed by Sidak's Multiple Comparison Test.

h RPE-1-TP53-/- c1#1 -Cyclin E1-H2B-EGFP cells that showed aberrant mitoses. Cells were pre-treated for 24 hours with doxycycline. Cells were then treated with ATR inhibitor (VE-822, 0.25 µM) or WEE1 inhibitor (MK-1775, 0.1 µM), and subsequently followed with live-cell microscopy using 7 minute intervals for 48 hours. p-values were calculated using a Mann-Whitney U test.

i RPE-1TP53wt and RPE-1TP53-/- c1#1 cell lines were induced to express Cyclin E1 or Cdc25A, and were treated for 3 days with ATR inhibitor (VE-822) in a range from 0 µM to 3.2 µM, or WEE1 inhibitor (MK-1775) in a range from 0 µM to 1.28 µM. Subsequently, relative cell survival was assessed using MTT conversion as a proxy. Plots include mean and standard error of the means (SEM) of 3 biological replicates. Reported p-values were calculated by a Student's t-test comparing the area under the curve of doxycycline untreated samples to the curve of the doxycycline treated samples.
Figure 5: Reducing Cyclin E1 overexpression diminishes replication stress and mitotic errors.

\(a\) HCC1806 cells transduced with inducible Cyclin E1 construct (shCCNE1#1 or shCCNE1#2) or control shRNA (shLuc) were treated with doxycycline for 2 days, and immunoblotted for Cyclin E1 and β-Actin. Cyclin E1 protein levels were measured and normalized to shLuc-Dox controls (f) and relative average diameter of colonies counted (g) in panel i, relative to Luc-Dox control. Bars represent the mean and standard error of the mean (SEM) mitotic fraction of 2 independent experiments. \(p\)-values were calculated using two-tailed Student’s \(t\)-test. \(b\) Cells were treated with doxycycline for 48 hours and sequentially labeled for 20 minutes with CldU (25 µM) and 20 minutes with IdU (250 µM). Representative DNA fibers of doxycycline-treated samples are shown. \(c\) Quantification of IdU-DNA fiber lengths as described in panel h. Per condition, at least 466 fibers were analyzed and corresponding medians with interquartile range are shown. \(p\)-value was calculated using Mann-Whitney \(U\) test. \(d\) γH2AX intensity as measured by flow cytometry in cells treated with and without doxycycline for 48 hours. Means and SEM normalized to the untreated luciferase condition are shown from 3 biological replicates. \(e\) Cyclin E1 knock-down was induced by doxycycline treatment for 48 hours. Cells were then fixed and the percentage of mitotic aberrancies was quantified. Data represents mean and SEM of three independent experiments; at least 30 mitoses were analyzed for each experimental condition. The \(p\)-values were calculated by one-way ANOVA (\(P<0.0001\)) and followed by Sidak’s Multiple Comparison Test. \(i\) Duration of mitosis as measured by NEB breakdown to anaphase. HCC1806 H2B:EGFP cells were pre-treated for 48 hours with doxycycline and subsequently followed with live-cell microscopy in 7 minute intervals for the duration of 48 hours. \(p\)-value was calculated using a Kruskal-Wallis test and subsequently followed with live-cell microscopy using 7 minute intervals for 48 hours. Duration of mitosis is shown. \(j\) Quantification of aberrant mitoses in cells from panel i. \(p\)-values were calculated using absolute errors, using Mann-Whitney \(U\) test.

To evaluate the effects of Cyclin E1 downregulation on replication kinetics, we analyzed DNA fibers of HCC1806 cells (Fig. 5H). Interestingly, knockdown of Cyclin E1 cells resulted in increased DNA synthesis speed in HCC1806 cells, as judged by IdU tract length (Fig. 5i). In addition, flow cytometry analyses demonstrated a reduction of intensity of the DNA damage and replication stress marker γH2AX upon Cyclin E1 knock-down (Fig. 5j). We next tested whether the observed reduction of replication stress levels in the Cyclin
E1 knock-down cells also resulted in a reduction of mitotic aberrancies. Of note, the base-line frequency of mitotic errors in untreated HCC1806-shLuc cells was ~50% (Fig. 5K), which is 10-fold higher than in non-transformed RPE-1-TP53 WT cells (Fig. 1E). Partial depletion of Cyclin E1 resulted in a dramatic reduction of mitotic errors to ~20% (Fig. 5K). Live-cell microscopy demonstrated that while mitotic duration was similar in all conditions (Fig. 5L), the percentage of mitotic errors is reduced ~1.5-fold (Fig. 5M). Combined, our data show that reducing Cyclin E1 expression levels in a Cyclin E1 overexpressing TNBC model, reduces replication stress levels and mitotic errors.

**ATR and WEE1 inhibitor sensitivity in Cyclin E1 overexpressing cells**

We next investigated how downregulation of Cyclin E1 impacts on replication stress and ATR and WEE1 inhibitor sensitivity. ATR or WEE1 inhibition increased γH2AX intensity levels (Suppl. Fig. 6C, D), although levels of mitotic errors were not further increased, likely because of the high base-line levels of mitotic errors in the HCC1806 cell (Suppl. Fig. 6E). Importantly, partial Cyclin E1-depletion consistently lowered γH2AX intensities and mitotic aberrances observed in ATR or WEE1 inhibitor-treated cells (Suppl. Fig. 6E). Moreover, treatment with ATR and WEE1 inhibitor increased the mitotic fraction of HCC1806 cells approximately 2-fold, which was completely rescued by depletion of Cyclin E1 (Fig. 6A). Moreover, we observed Cyclin E1 depletion to confer resistance to ATR or WEE1 inhibition (Fig. 6B, C). Similarly, partial Cyclin E1 knockdown using shCCNE1#2 resulted in increased clonogenic survival of WEE1 inhibitor-treated HCC1806 cells (Fig. 6D–F). Combined, our data indicate that Cyclin E1 overexpression is not only sufficient to drive sensitivity to ATR and WEE1 inhibition, but is also required for these effects.

**DISCUSSION**

In this report, we investigated the effects of oncogene-induced replication stress on mitotic fidelity and on the sensitivity to cell cycle checkpoint kinase inhibitors. We demonstrated that overexpression of Cdc25A or Cyclin E1 resulted in severe replication stress, which was associated with the induction of chromatin bridges and lagging chromosomes during mitosis. Furthermore, we observed that oncogene-induced replication stress sensitized cells to ATR and WEE1 checkpoint kinase inhibitors. ATR and WEE1 inhibition exacerbated the mitotic aberrancies induced by Cyclin E1 or Cdc25A overexpression and increased cell death. Finally, we show downregulation of Cyclin E1 in TNBC cells to result in rescue of replication kinetics and reduced cytotoxicity of ATR and WEE1 inhibitors.

Our findings are in line with earlier reports in which ATR inhibitor sensitivity was associated with Cdc25A expression, and WEE1 inhibitor sensitivity was associated with Cyclin E expression (56, 57). Importantly, our data point towards a critical role for mitotic segregation defects in cell death following oncogene-induced replication stress. Furthermore, our data indicate that exacerbation of chromosome segregation defects during mitosis upon ATR and WEE1 inhibit is associated with cytotoxicity of these drugs in cells harboring oncogene-induced replication stress, which was previously reported for PARP inhibitors (42).

A possible explanation for these observations is that acceleration of mitotic entry upon ATR and WEE1 inhibition, leaves cells with oncogene-induced replication stress with insufficient time to resolve replicative lesions. Subsequently, mitotic entry commences in the presence of severe DNA lesions, which precludes proper chromosome segregation and leads to cell death. Indeed, cells in which ATR or WEE1 inhibition induced mitotic chromosome segregation defects showed a proportional increase in inhibitor-induced cytotoxicity. Specifically, RPE-1 cells with Cdc25A overexpression showed more chromosomal segregation defects and sensitivity to ATR and WEE1 inhibition in both TP53 WT and TP53 null settings. Conversely, Cyclin E1 overexpressing cells were only sensitive to both agents when TP53 was mutated. These observations are in good agreement with a role for p53 signaling in preventing genomic instability following Cyclin E1 amplification (20, 48, 58, 59).

An explanation for why Cdc25A overexpressing cells are sensitive to ATR and WEE1 inhibitors in a TP53 wild-type setting could lie in checkpoint abrogation resulting from Cdc25A overexpression (60). Furthermore, whereas Cyclin E1 overexpression only leads to CKD2 activation, Cdc25A affects multiple CDKs, including CDK1 (61). As a consequence, Cdc25A amplification de-regulates both S-phase and G2/M progression (62). Interestingly, our study demonstrates that WEE1 inhibition sensitizes tumor cells regardless of TP53 mutations status. WEE1 inhibition was reported earlier to be primarily effective in TP53 mutant cells (63), which was attributed to a defective G1/S checkpoint in TP53 mutant cells, leading to increased reliance on their G2/M checkpoint.
**Chapter 4 Mitotic defects upon Cyclin E1/Cdc25A overexpression**

**Figure 6: Cyclin E1 overexpression is required for ATR and WEE1 inhibitor sensitivity.**

- **a** HCC1806 cell lines were induced to express Cyclin E1 shRNA for 2 days and were then treated with 0.25 µM of ATR inhibitor (ATRi, VE-822) or 0.1 µM of WEE1 inhibitor (WEE1i, MK-1775) for 8 hours. Cells were then fixed and stained for DNA content (propidium iodine) and for mitotic population (MPM2) and analysed using flow cytometry. Bars represent the mean and SEM of 4 independent experiments, normalized to untreated Luc – dox; p-values were calculated using two-tailed Student’s t-test comparing the area under the curve of doxycycline untreated samples to the doxycycline treated samples.
- **b** Doxycycline-inducible HCC1806 cells were plated in 6-well plates and allowed to attach for 24 hours. Subsequently, cells were treated with doxycycline and 0.05 µM of ATR inhibitor (ATRi, VE-822) or 0.08 µM of WEE1 inhibitor (WEE1i, MK-1775). After 11 days, surviving colonies were stained. Bars represent the mean and SEM of clonogenic survival from panel d. Bars represent the mean and SEM of clonogenic survival, relative to the non-doxycycline treated controls of 2 independent experiments; p-values were calculated using two-tailed Student’s t-test.
- **c** Quantification of colony diameter, relative to non-treated shLuc cells of 2 independent experiments. Bars represent the mean and SEM; p-values were calculated using two-tailed Student’s t-test.

However, recent reports have shown that TP53 mutation status alone does not explain responses of tumors to WEE1 inhibition, which underscore that WEE1 inhibitor sensitivity is more complex and multifactorial (64–66).

As overexpression of Cyclin E1 leads to replication stress, increased mitotic aberrations, and sensitivity to inhibition of ATR or WEE1, we wondered whether normalization of Cyclin E1 levels in TNBC cells harboring CCNE1 amplification reduced these effects (67). We observed that downregulation of Cyclin E1 resulted in elevated DNA replication speed, and diminished cytotoxic effects of ATR or WEE1 inhibition. These findings are in line with previous observations that Cyclin E1 overexpression contributes to the increased origin initiation and collisions between the replication and transcription machineries, which negatively impact replication speed and lead to replication fork collapse (18,68). Such lesions create a dependence on replication checkpoint signaling, and explain the sensitivity of Cyclin E1-overexpressing cells to ATR and WEE1 inhibitors (66,69), as well as the reversal of ATR and WEE1 inhibitor sensitivity upon Cyclin E1 downregulation.

Our data supports the notion that expression of replication stress-inducing oncogenes could be used as criteria to select patients for treatment with replication checkpoint kinase inhibitors, including ATR and WEE1. To test their value as biomarkers, it would be insightful to test ATR and WEE1 sensitivity in tumors harboring amplifications of different replication stress-inducing oncogenes, including CCNE1 (70), which is being used in a clinical trial to select patients for WEE1 inhibitor treatment (clinicaltrials.gov identifier: NCT03253679). In this context, cancers that currently lack drug targets are of particular interest, as these are difficult to treat, including triple-negative breast cancer.

Taken together, this study reports that replication stress induced by overexpression of Cyclin E1 and Cdc25A results in the formation of lagging chromosomes and chromatin bridges, which is further exacerbated by inhibition of ATR or WEE1 kinases, and results in exacerbated tumor cell killing. Conversely, normalization of Cyclin E1 levels restores replication kinetics and reduces the cytotoxicity from inhibition of ATR or WEE1 kinases. These insights could therefore help to guide novel treatment strategies for targeting genomically unstable tumors harboring oncogene amplifications.
## MATERIALS AND METHODS

### Cell lines

hTERT-immortalized human retinal pigmented epithelial (RPE-1), human embryonic kidney 293 (HEK293T), HCC1806, HCC1569 and MDA-MB-157 cell lines were obtained from the American Type Culture Collection (#CRL4000, #CRL3216, #CRL2335, #CRL2330 and #HTB24) and regularly checked for mycoplasma and authenticated using STR profiling. RPE-1, HEK293T and MDA-MB-157 cells were cultured in Dulbecco’s Minimum Essential Media (DMEM, Thermofisher), complemented with 10% (v/v) fetal calf serum (FCS), 1% penicillin and 1% streptomycin (Gibco). HCC1806 and HCC1569 cells were maintained in Roswell Park Memorial Institute medium (RPMI, Thermofisher) complemented with 10% FCS and 1% penicillin/streptomycin. All cells were grown at 37°C in 20% O₂ and 5% CO₂ in a humidified incubator.

### Mutagenesis

CRISPR/Cas9 was used to mutate TP53 in RPE-1 cells. To this end, a single guide RNA (sgRNA) (5’-CTGTCATCTTCTGTCCCTTC-3’) targeting exon 4 was cloned into pSpCas9(BB)-2A-GFP, which was provided by Feng Zhang (PX458, plasmid #48138, Addgene) (71). Next, RPE-1 cells were transfected with PX458 and selected with Nutlin-3a (Axon Medchem, 10 μM) for 3 weeks. The viable cells were sorted into monoclonal lines using a MoFLO XDP or Sony cell sorter. TP53 mutations in exon 4 were confirmed by Sanger sequencing and lack of p53 expression was confirmed by Western blot analysis. The reading frame of TP53 was shifted by a 7 basepair deletion and a +217 bp insertion in Clone#1 and a -1 deletion and a +2 insertion in Clone#2 (Fig. 2A).

### DNA cloning and retroviral infections

RPE-1-TP53wt and RPE-1-TP53mut cell lines were engineered to express Cdc25A or Cyclin E1 in a doxycycline-dependent manner. To this end, human CDC25A was PCR amplified from FLAG-CDC25A-WT, which was a gift from Peter Stambrook (72), using the following oligos: forward: 5’-CGCGGCCGCCATGGAACTGGGCCCGGAGCCC-3’, reverse: 5’-GATGAATTCTCACAGCTTCTTCAGACG-3’. Human CCNE1 was PCR amplified from Rc-CycE, which was a gift from Bob Weinberg (Plasmid #8963, Addgene) (73), using the following oligos: forward: 5’-CGCGGCCGCCATGAAGGAGGACGGCGGCGCG-3’, reverse: 5’-GATGAATTCTCACAGCTTCTTCAGACG-3’. The resulting fragments were cloned into pJET1.2/blunt, GeneJET, (ThermoFisher). CDC25A and CCNE1 were subcloned into pRetroX-Tight-Pur using NotI and EcoRI restriction sites. Subsequently, cell lines harboring pRetroX-Tet-On Advanced were transduced with pRetroX-Tight-Pur containing CDC25A, CCNE1 or empty plasmid. For transduction, HEK293T cells were transfected with 10 μg of pRetroX-Tet-On Advanced, 2.5 μg of pMDg and 7.5 μg of pMDg/p as described previously (74). After transduction, RPE-1 cell lines were selected for 7 days using geneticin (G418 Sulfate, 800 μg/mL, Thermofisher). Next, cell lines harboring pRetroX-Tet-On Advanced were transduced with pRetroX-Tight-Pur vectors containing CDC25A or CCNE1, and selected for 2 days with puromycin dihydrochloride (5 μg/mL, Sigma). To obtain cells stably expressing Histone H2B-EGFP, indicated RPE-1 cell lines were transduced as previously described (42).

### RNA interference

For identifying endogenous Cyclin E1 on immunoblots, a SMARTpool siRNA mix (Dharmacon, Horizon Inspired Cell Solutions) for CCNE1 was transfected at a final concentration of 80 nM with Oligofectamine (Invitrogen) according to the manufacturer’s instructions. To down-regulate CCNE1 in HCC1806 cells, lentiviral shRNA interference sequences were clones into the Tet-pLKO-puro plasmid (a gift from Dimitri Wiederschain, #21915, Addgene, (75)) following the depositor’s protocol. shCCNE1#1 was designed to target exon 8 (5’-GCTTGTTCAGGAGATGAAATT-3’) and shCCNE1#2 (sh#2) was designed to target exon 7 (5’-CGGTATATGGCGACACAAGAA-3’). A control shRNA targeting luciferase (5’-AGAGCTGTTTCTGGAGGACC-3’) was included in the experiments.

### Western blotting

After pretreatment with doxycycline, ATR inhibitor VE-822 (Axon), WEE1 inhibitor MK1775 (Axon MedChem) or Hydroxyurea (Sigma) at the indicated doses, cells were washed in PBS and lysed in MPER lysis buffer (Pierce), complemented with protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein concentration was quantified using the Pierce BCA Protein Quantification Kit (Thermo Scientific). Lysates were resolved by SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon). Membranes were incubated overnight at 4°C with primary antibodies in Tris-buffered saline (Tris) containing 0.05% Tween-20 (Sigma) with 5% skimmed milk (Sigma). The following primary antibodies were used for Western blot analysis: mouse anti-Cdc25A (Santa Cruz Biotechnology, Sc-7389, 1:500), mouse anti-Cyclin E1 (1:1200, Bachem, ab3927, 1:1000), mouse anti-p53 ([DO-1], Santa Cruz Biotechnology, Sc-
Chapter 4

Mitotic defects upon Cyclin E1/Cdc25A overexpression

126, 1:1000), rabbit-anti-vinculin ([EPR8185], Abcam, ab129002, 1:2500), rabbit-anti-phospho ATM/ATR (Thr1989) Merck Millipore ABE462, 1:500), rabbit-anti-Phospho-Chk1 (Ser345) ([I33D3], Cell Signaling, #2348, 1:500), Rabbit anti-phospho RPA32 (S33) (Bethyl Laboratories 1:1000), rabbit-anti-Phospho-Histone H2AX (Ser139) ([20E3], Cell Signaling, #9718, 1:1000), rabbit-anti- Recombinant Anti-CDK1 + CDK2 + CDK3 + CDK5 (phospho Y15) ([EPR7875], Abcam, ab133463, 1:1000) and mouse anti- beta-actin (MpBiomedicals, 69100, 1:10000). Subsequently, membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (1:2000, DAKO), and visualized with Lumi-Light (Roche Diagnostics). Images were captured with the ChemiDoc MP imaging system (Bio-Rad), and analyzed with the analyze gel module of the FIJI software.

Flow cytometry
Flow cytometry was performed as described in (53). Cells were stained with MPM2 antibody (Merck Millipore, 05-368, 1:000) and anti-γH2AX (Cell Signaling, #9718, 1:200), in combination with Alexa-488-conjugated and Alexa-647-conjugated secondary antibodies (1:200).

Single-cell whole-genome analysis
RPE-1-TP53<sup>wt</sup> and RPE-1-TP53<sup>-/-</sup> cell lines harboring doxycycline-inducible Cdc25A or Cyclin E1 were treated with doxycycline (1 µg/ml) for 120 hours. Single-cell sequencing was performed as described in (53,76).

MTT assays
RPE-1-TP53<sup>wt</sup> or RPE-1-TP53<sup>-/-</sup> cell lines harboring doxycycline-inducible Cdc25A or Cyclin E1, transduced with H2B-EGFP were seeded in eight-chambered cover glass plates (Lab-Tek-II, Nunc). Cells were left untreated or treated with doxycycline (1 µg/ml) for 48 hours, and were subsequently imaged for 48 hours under the same treatment on a Delta Vision Elite microscope (20x objective with 0.75 NA). Every 7 minutes, 10 to 15 images in the Z-plane were acquired with an interval of 0.5 µm. Mitotic entry was defined by nuclear envelope break-down, and mitotic duration was defined as time between nuclear envelope break-down and anaphase entry. Image analysis was done with SoftWorX software (Applied Precision/GE Healthcare).

Detailed descriptions of the following techniques are available in the Supplemental methods.

- DNA fiber analysis
- Immunofluorescence microscopy
- Flow cytometry
- Single-cell whole-genome analysis
- Clonogenic survival assays
- TCGA data set and CNA burden

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Data availability: All sequencing data have been deposited at the European nucleotide archive under accession no. PRJEB32207.

Conflict of Interest Statement: M.v.V. has acted on the scientific advisory board of Repare Therapeutics, which is unrelated to this work. The other authors declare no conflict of interest.
REFERENCES


SUPPLEMENTAL METHODS

DNA fiber analysis
RPE-1-TP53<sup>−/−</sup> or RPE-1-TP53<sup>−/−</sup> cell lines harboring doxycycline-inducible Cdc25A and Cyclin E1 were pre-treated with doxycycline (1 µg/ml) for 48 hours, and subsequently pulse-labeled with CdU (25 µM) for 20 minutes at 37 °C. Subsequently, cells were washed three times with pre-warmed medium and then pulse-labeled with IdU (250 µM) for 20 minutes at 37 °C. After labeling, cells were harvested by trypsinization and re-suspended in cold PBS. Next, 2 µl of cell suspension was lysed on a microscopy slide by addition of 8 µl lysis solution (0.5% sodium dodecyl sulfate, 200 mM Tris [pH 7.4], 50 mM EDTA). After 5 minutes of incubation at room temperature, DNA fibers were spread by tilting the microscope slide, and were subsequently air-dried and fixed in methanol/acetic acid (3:1) for 10 minutes. Slides were washed twice in PBS, and DNA was denatured in 2.5M HCl for 75 minutes. DNA fibers were incubated in blocking solution (5% BSA in PBS) for 30 minutes, prior to incubation in primary antibodies (rat anti-BrdU, 1:1000, Abcam, ab6326; mouse anti-BrdU, 1:250, BD Biosciences, Clone B44) for 60 minutes at room temperature. After three washing steps in blocking solution, slides were incubated with secondary antibodies (Alexa488-conjugated anti-rat and Alexa594 or 647-conjugated anti-mouse, 1:500) for 1 hour at room temperature. Images were acquired on a Leica DM-6000B (63x immersion objective with 1.30 NA) fluorescence microscope, equipped with Leica Application Suite software. Per condition, the lengths of at least 250 IdU tracts were measured using ImageJ software. Statistical analysis was performed using the non-parametric Mann-Whitney U test with GraphPad Prism version 8.

Immunofluorescence microscopy
Indicated cells were seeded on glass coverslips in 6-well plates for 24 hours. Subsequently, cells were treated with doxycycline (1 µg/ml) for 48 hours. Then, cells were treated with MK-1775 (100 nM) or VE-822 (250 nM) for 8 hours if indicated, and were subsequently fixed in 4% formaldehyde in PBS. Following permeabilizing for 5 minutes (0.1% Triton X-100 in PBS), cells were incubated with blocking buffer (3% BSA and 0.05% tween in PBS), cells were incubated overnight with mouse anti-PICH (1:1000, Novus Biologicals, NBP2-13969), mouse anti-γH2AX (1:400, Millipore, 05-636) or mouse anti-Cyclin E1 ([HE12], Abcam, ab3927, 1:1000), and were then treated with Alexa-488 or Alexa-647-conjugated secondary antibodies and counterstained with DAPI. Images were acquired on a Leica DM6000B microscope using a 63x immersion objective (PL S-APo, numerical aperture: 1.30) with LAS-AF software (Leica). Using ImageJ software, Cyclin E1 staining intensity was measured in the nuclei which were selected based on the DAPI channel by the ‘Analyze Particle’ tool.

Flow cytometry
Cells where either analyzed as asynchronous cultures or were synchronized at the G1/S cell cycle transition using a double-thymidine block. Specifically, cells were treated with thymidine (2mM, Sigma) for 17 hours, washed twice with pre-warmed PBS, and were incubated in pre-warmed warm medium for 9 hours. Subsequently, cells were again incubated in thymidine for 17 hours, after which cells were washed with PBS and released in pre-warmed medium containing VE-822 (0.250 µM) or MK-1775 (0.1 µM), and harvested at indicated time points. When indicated, cells were trapped in mitosis using an 8-hour incubation with nocodazole (250 ng/ml, Sigma). Cells were then fixed in ice-cold ethanol (70%) for at least 16 hours and stained with MPM2 antibody (Mercck Millipore, 05-368, 1:000) and anti-γH2AX (Cell Signaling, #9718, 1:200), in combination with Alexa-488-conjugated and Alexa-647-conjugated secondary antibodies (1:200). DNA staining was performed using propidium iodide in the presence of RNase. For S-phase analysis, prior to fixation asynchronous cells were incubated with 10 µM of EdU (Invitrogen) for 45 minutes. Cells were permeabilized with 0.5% Triton-X-100 for 30 minutes and washed with 3% BSA-PBS. EdU click reaction was performed at room temperature by incubation for 30 minutes with staining cocktail final dilution of 43mM Tris-HCl pH 7.5, 1.6 mM CuSO<sub>4</sub>-5H<sub>2</sub>O, 25 µM ATTO 488 Azide (ATTO-TEC GmbH) and 1 mM Ascorbic Acid. At least 10,000 events per sample were analyzed on a FACScalibur or LSR-II (Becton Dickinson). Data was analyzed using FlowJo software.

Single-cell whole-genome analysis
RPE-1-TP53<sup>−/−</sup> cells and RPE-1-TP53<sup>−/−</sup> cell lines harboring doxycycline-inducible Cdc25A or Cyclin E1 were treated with doxycycline (1 µg/ml) for 120 hours. Subsequently, cells were lysed and the G1 population was single-cell sorted into 96-well plates (48 cells per sample) using a Hoechst/Propidium iodide double staining. To perform sample preparation and generate Illumina-based libraries, a Bravo automated liquid handling platform (Agilent Technologies) was employed, as described previously (1) a state in which cells have an abnormal number of chromosomes, has been proposed to play a role in neurodegeneration in AD patients. Several studies using fluorescence in situ hybridization have shown that the brains of AD
patients contain an increased number of aneuploid cells. However, because the reported rate of aneuploidy in neurons ranges widely, a more sensitive method is needed to establish a possible role of aneuploidy in AD pathology. RESULTS In the current study, we used a novel single-cell whole genome sequencing (scWGS). The libraries were sequenced on a NextSeq 500 sequencer (Illumina) and analyzed using AneuFinder software as previously described (2). A state in which cells have abnormal numbers of chromosomes, and is found in two out of three cancers. In a chromosomal instable p53 deficient mouse model with accelerated lymphomagenesis, we previously observed whole chromosome copy number changes affecting all lymphoma cells. This suggests that chromosome instability is somehow suppressed in the aneuploid lymphomas or that selection for frequently lost/gained chromosomes out-competes the CIN-imposed mis-segregation. RESULTS To distinguish between these explanations and to examine karyotype dynamics in chromosome instable lymphoma, we use a newly developed single-cell whole genome sequencing (scWGS). RPE-1-TP53 WT -Empty or RPE-1-TP53 WT-/- Empty (negative control) cells were employed as a reference to determine the deviation from the modal copy number state per sample and per bin. The focal copy number alterations (CNAs) scores were obtained from the bins that deviated from the modal copy number of the negative control.

Clonogenic survival assays
HCC1806 cells were seeded in 6-well plates (at approximately 232 or 500 cells per well) and allowed to adhere for 24 hours. Subsequently, cells were treated with doxycycline (1 µg per ml) in the presence or absence of VE-822 (0.05 µM, Axon MedChem) or MK-1775 (0.08 µM, Axon MedChem). After 11 or 14 days, cells were fixed in methanol and stained in staining buffer (50% methanol, 29.95% water, 20% Acetic acid, and 0.05% of Coomassie Brilliant Blue). Images of colonies were obtained using an EliSpot reader (Alpha Diagnostics International) with vSpot Spectrum software. The number and size of colonies were measured using ImageJ software. Statistical analysis was performed using the non-parametric Mann-Whitney U test with GraphPad Prism 6.

TCGA data set and CNA burden
From TCGA, we obtained the pre-processed and normalized level 3 RNA-seq (version 2) data for 34 cancer datasets available at the Broad GDAC Firehose portal (downloaded January 2017 https://gdac.broadinstitute.org/). For each sample, we downloaded RNA-Seq with Expectation Maximization (RSEM) gene normalized data (identifier: illuminahiseq_rnaseqv2-RSEM_genes_normalized) (3). RNA-Seq expression level read counts were normalized using FPKM-UQ (Fragments per Kilo-base of transcript per Million mapped reads upper quartile normalization, NCI Genomic Data Commons (GDC), n.d.). The RNA-Seq expression level read counts for each of the samples were log2-transformed. Publicly available inferred CNA burden data for TCGA were obtained from http://www.genomicinstability.org/ (4), however, CNAs do not always translate proportionally into altered expression levels. By reanalyzing >34,000 gene expression profiles, we reveal the degree of transcriptional adaptation to CNAs in a genome-wide fashion, which strongly associate with distinct biological processes. We then develop a platform-independent method-transcriptional adaptation to CNA profiling (TACNA profiling). The association between CNA burden and mRNA expression of CCNE1, CDC25A for the TCGA-dataset were quantified using Spearman correlation coefficient. This analysis was conducted separately for different cancer types.

Supplementary Figure 1: Related to figure 1

a TGCA analysis of Cyclin E1 and Cdc25A mRNA expression in pan cancer, breast cancer (ER+/HER2-), and TNBC breast cancer. b Longer exposure of Cyclin E1 immunoblot presented in Fig 1a in RPE-1-TP53wt cell lines induced to express Cyclin E1 or Cdc25A. Black arrow indicates an aspecific band, red arrow indicates endogenous Cyclin E1. c RPE-1-TP53wt were treated for 48 hours with doxycycline to induce expression of Cyclin E1 or CDC25A. The percentages of anaphase or telophase cells containing ultra-fine bridges (n=3, n>25 per experiment) were quantified. p-values were calculated using two-tailed Student's t-test.

Pan-cancer
Breast cancer (ER+/HER2-), (n = 554)
Breast cancer (TNBC), (n = 146)
Ovarian carcinoma, (n = 307)

RPE-1-TP53wt
siCCNE

Cyclin E1
Cdc25A

Cyclin E1
(long exposure)

Cyclin E1
endogenous

Cdc25A

empty

Cyclin E1
Cdc25A
dox

n=0.0196

Pan-cancer
Breast cancer (ER+/HER2-), (n = 554)
Breast cancer (TNBC), (n = 146)
Ovarian carcinoma, (n = 307)
to overexpress empty, Cyclin E1 or Cdc25A constructs in a doxycycline-inducible manner. Immunoblot shows Cyclin E1, Cdc25A, p53 and Vinculin protein levels at 48 hours after addition of doxycycline (dox). RPE-1-TP53<sup>wt</sup> cells were used as a positive control for p53. c RPE-1-TP53<sup>−/−</sup> cells were treated as in b. Immunoblot shows Cyclin E1, Cdc25A, p53 and Vinculin protein levels at 48 hours after addition of doxycycline (dox). d Cells were treated as in b and subsequently labeled for 20 minutes with CldU (25 µM) and for 20 minutes with IdU (250 µM). At least 473 were analyzed. Graphs show individual data points, median and interquartile range. p-values were calculated using the Mann-Whitney U test. e RPE-1-TP53<sup>−/−</sup> cl#2 were treated for 48 hours with doxycycline to induce expression of Cyclin E1 or Cdc25A. Cells were stained with α-Tubulin (red) and counterstained with DAPI (blue). Quantification of anaphase and telophase cells containing chromatin bridges and/or lagging chromosomes. The bars represent the mean and standard error or the mean (SEM) from 3 experiments, n>25 per experimental condition; p-values were calculated using two-tailed Student’s t-test. f RPE-1-TP53<sup>−/−</sup> cells were engineered
Supplementary Figure 3: mRNA expression of Cyclin E1 and Cdc25A are correlated with copy number alterations in various tumor types

a, b CCNE1 or Cdc25A read count were correlated to copy number load using Spearman’s correlation in panel a triple negative breast cancer and in panel b ovarian carcinomas. c Spearman’s correlation coefficient was calculated between copy number load and read counts of CCNE1 or Cdc25A for patient samples from various tumor types in TCGA data.

Supplementary Figure 4: Cyclin E1 or Cdc25A overexpression induces genomic instability, related to figure 3.

a RPE-1-TP53+/− (n=44), RPE1-TP53−/− #c1 (n=47), RPE1-TP53−/−#cl2 (n=42) were treated with doxycycline for 120 hours. After single cell sorting, genomic DNA was harvested for single-cell whole Genome Sequencing (sc-WGS). Genome-wide copy number plots were generated using AneuFinder software. Each panel displays the individual cells in rows, and the chromosomes numbers from 1-X in columns. Absolute copy number states for each cell are depicted in different colors. b Genome-wide copy number deviation plots of RPE-TP53−/− cl#2 empty (n=42), RPE-TP53−/− cl#1 Cdc25A (n=48) and RPE-TP53−/− cl#1- Cdc25A cells (n=43). Cells were treated as in a. The modal copy number state is pictured in green, deviations of the modal copy number state, both focal and whole-chromosome, are colored red. c Copy-number alterations (CNAs) per cell were calculated according to the modal state. Medians with interquartile range are depicted and statistical analyses were performed using a One-sided Mann Whitney U test. d Whole numerical chromosomes per cell were counter per single cell. Medians with interquartile range are depicted and statistical analyses were performed using a One-sided Mann Whitney U test.
**Supplementary Figure 5:** ATR or WEE1 inhibition do not affect ultra-fine bridge formation or mitotic timing, related to figure 4

* a, b RPE-TP53<sup>wt</sup> (a) and RPE-TP53<sup>-/-</sup> cl#1 (b) cells were treated as described in Fig. 4a, and immunoblotted for Cyclin E and Cdc25A overexpression. Vinculin serves as a loading control. RPE-1-TP53<sup>wt</sup> cells induced to express Cdc25A or Cyclin E1 were treated with ATR inhibitor (VE-822, 0.25 µM) or WEE1 inhibitor (MK-1775, 0.1 µM) for 8 hours if indicated. The percentages of anaphase or telophase cells containing chromatin bridges or lagging chromosomes were quantified. The bars represent mean and SEM from 3 experiments, n>30 per experimental condition; The p-values were calculated by one-way ANOVA (P<0.0001) and followed by Sidak’s Multiple Comparison Test. d RPE-1-TP53<sup>wt</sup> were treated as described in c. cells were stained for PICH and α-Tubulin and counterstained with DAPI. The percentages of anaphase or telophase cells containing ultra-fine bridges were quantified. The bars represent the mean and standard error or the mean (SEM) from 3 experiments n>25 per experimental condition; p-values were calculated using two-tailed Student’s t-test. e RPE-TP53<sup>-/-</sup> cl#2 cells were treated as described in panel c. The percentages of anaphase or telophase cells containing ultra-fine bridges were quantified. The bars represent the mean and standard error or the mean (SEM) from 3 experiments n>25 per experimental condition; p-values were calculated using two-tailed Student’s t-test.

**f** Duration of mitosis in RPE-1-TP53<sup>wt</sup> cells harboring doxycycline-inducible Cdc25A or Cyclin E1, transduced with H2B-EGFP. Cells were pre-treated for 24 hours with doxycycline, after which cells were treated with 0.25 µM of ATR inhibitor (ATRI, VE-822) or 0.1µM of WEE1 inhibitor (WEE1i, MK-1775), and subsequently followed with live-cell microscopy for 48 hours using 7-minute intervals. Duration of mitosis was measured as the time between nuclear envelope breakdown (NEB) and anaphase entry. A Gaussian curve was fitted to the data, and a p-value was calculated using a Kruskal-Wallis multiple-comparison test. g RPE-TP53<sup>wt</sup> were treated for 48 hours with doxycycline to induce expression of Cyclin E1 or CDC25A and subsequently incubated with thymidine (2mM) for 17 hours. Cells were then released for 9 hours in pre-warmed growth media and again treated for 17 hours with thymidine prior to release in growth media supplemented with DMSO, 0.25 µM of ATR inhibitor (ATRI, VE-822) or 0.1µM of WEE1 inhibitor (WEE1i, MK-1775). Cells were then fixed and indicated time points and stained for DNA content (propidium iodine) and for MPM2 and analysed using flow cytometry a minimum of 20,000 events was analyzed per sample.
Chapter 4

Mitotic defects upon Cyclin E1/Cdc25A overexpression

Supplementary Figure 6: ATR and WEE1 inhibitor sensitivity in triple-negative breast cancer cells, related to figure 5

a, b Triple-negative breast cancer cell lines MDA-MB157, HCC1569 and HCC1806 were treated for 3 days with ATR inhibitor VE-822 in a range from 0 µM to 3.2 µM (a), or WEE1 inhibitor (MK-1775) in a range from 0 µM to 1.28 µM (b). Subsequently, MTT conversion was analyzed. Averages and standard error of the means (SEM) of 3 biological replicates are plotted.

c Example of flow cytometry plot measuring γH2AX intensity and DNA content in HCC1806 cells. d HCC1806 cell lines were induced to express Cyclin E1 shRNA for 2 days and were then treated with 0.25 µM of ATR inhibitor (ATRi, VE-822) or 0.1 µM of WEE1 inhibitor (WEE1i, MK-1775) for 8 hours. Cells were then fixed and stained for DNA content (propidium iodine) and for γH2AX and analyzed using flow cytometry a minimum of 20,000 events was analyzed per sample. Bars represent the mean and standard error of the mean (SEM) mitotic fraction of 3 independent experiments, normalized to untreated Luc – dox. DMSO-control cells are the same as in figure 5j. p-values were calculated using two-tailed Student’s t-test.

e Cyclin E knock-down was induced by doxycycline for 48 hours. Cells were then fixed and the percentage of mitotic aberrancies was quantified. DMSO-control cells are the same as in figure 5k. At least 30 mitoses were analyzed for each experimental condition. Data represents mean and SEM of three independent experiments; The p-values were calculated by one-way ANOVA (P<0.0001) and followed by Sidak’s Multiple Comparison Test.