E2F1 proteolysis via SCF-cyclin F underlies synthetic lethality between cyclin F loss and Chk1 inhibition

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Abstract

Cyclins are central engines of cell cycle progression in conjunction with cyclin-dependent kinases (CDKs). Among the different cyclins controlling cell cycle progression, cyclin F does not partner with a CDK, but instead forms via its F-box domain an SCF (Skp1-Cul1-F-box)-type E3 ubiquitin ligase module. Although various substrates of cyclin F have been identified, the vulnerabilities of cells lacking cyclin F are not known. Thus, we assessed viability of cells lacking cyclin F upon challenging them with more than 180 different kinase inhibitors. The screen revealed a striking synthetic lethality between Chk1 inhibition and cyclin F loss. Chk1 inhibition in cells lacking cyclin F leads to DNA replication catastrophe. Replication catastrophe depends on accumulation of the transcription factor E2F1. We find that SCF-cyclin F controls E2F1 activity during cell cycle and upon checkpoint inhibition to prevent DNA replication stress. Our findings pave the way for patient selection in the clinical use of checkpoint inhibitors.

Keywords cell cycle; checkpoints; Chk1; cyclin F; F-box proteins

Introduction

Cell cycle transitions are operated by the periodic oscillations of cyclins, which bind cyclin-dependent kinases (CDKs) to promote phosphorylation of target substrates and drive cell cycle progression. Entry into the S phase of the cell cycle is initiated by the activation of the transcription factor E2F1 that promotes accumulation of S phase cyclins and induces the transcription of genes required for DNA replication. The activity of E2F1 is restrained principally by the retinoblastoma (Rb) protein, which masks the E2F1 transactivation domain to keep it inactive. The control of Rb is crucial to prevent unscheduled cell cycle entry, a hallmark of cancer. Indeed, the loss of Rb is a common cancer event (Dyson, 2016).

Among the cyclins co-ordinating cell cycle progression, cyclin E/A and cyclin B, in partnership with CDK2 and CDK1, respectively, promote the progression of cells through S phase and G2 phase (Lim & Kaldis, 2013). Cyclin F is most similar to cyclin A but does not act as an activator of CDKs and is not able to bind to CDKs (Bai et al., 1994). Instead, cyclin F, also known as F-box only protein 1 (Fbxo1), is the founding member of the F-box family of proteins (Bai et al., 1996). Cyclin F, through the F-box domain, forms a functional Skp1-Cul1-F-Box (SCF) complex acting as an E3 ubiquitin ligase. The SCF<sup>cyclin F</sup> mediates the ubiquitylation and degradation of proteins important for cell cycle progression and genome stability (D’Angiolella et al., 2013; Klein et al., 2015).

In addition to the coordinated action of cyclins, cell cycle progression is monitored by the presence of checkpoints, which restrict CDK activity and prevent the execution of cell cycle phases if the previous one has not been completed.

Blocks in the progression of DNA replication fork promote the accumulation of single-stranded DNA (ssDNA) behind the fork, whose coating with the ssDNA-binding protein RPA represents a stimulus for the activation of the ATR kinase. Active ATR elicits a

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checkpoint response by phosphorylating Chk1 and initiating the signalling cascade that culminates with CDK inactivation. Chk1 exerts this function by controlling the ubiquitylation and subsequent degradation of Cdc25A phosphatase (Busino et al., 2003; Jin et al., 2003; Kotsantis et al., 2018). Cdc25A removes the inhibitory phosphorylation on Tyr15 of CDKs, mediated by the tyrosine kinase Wee1 to prompt CDK activation. Upon activation of Chk1, the degradation of Cdc25A is initiated and CDK activity is suppressed to restrain DNA replication and progression of the cell cycle (Bartek & Lukas, 2003). The checkpoint response allows DNA repair before committing to mitosis by restricting CDK activity until the S/G2 transition through Chk1 (Lemmens et al., 2018). If the blocks at the replication fork cannot be bypassed or repaired, the ssDNA gaps can be converted into double-stranded breaks (DSBs), which are more deleterious and could lead to gross chromosomal rearrangements resulting in cell death. Given their high proliferative capacity and compromised DNA repair, cancer cells have intrinsically higher replication stress and DNA damage. These observations have spurred the development of a number of inhibitors targeting the checkpoint response mediated by ATR and Chk1. Although ATR and Chk1 inhibitors are being evaluated in multiple clinical trials, the genetic predispositions that would sensitise or desensitise cancer cells to ATR and Chk1 inhibitors are not fully elucidated.

By screening the kinase chemogenomic set (KCGS) to identify vulnerabilities of cells lacking cyclin F, we discover a novel synthetic lethal interaction between cyclin F loss and Chk1 inhibition. We observe that the loss of cyclin F promotes DNA replication catastrophe after challenging cells with Chk1 inhibitors. The DNA replication catastrophe in cells lacking cyclin F after Chk1 inhibitors is promoted by the accumulation of E2F1. E2F1 is a novel substrate of cyclin F which is controlled at the G2/M transition and after checkpoint inhibition. By expressing a non-degradable version of E2F1, we show that E2F1 accumulation exacerbates Chk1 inhibitor sensitivity, promoting the accumulation of DSBs and cell death.

Results

KCGS screen identifies synthetic lethality between Chk1 inhibitors and cyclin F loss

Using CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9, we have previously generated cyclin F knock-out (CCNF K/O) cell lines (Mavrommati et al., 2018). Interestingly, these cells present a cell cycle profile comparable to the control parental cell line (Fig EV1A and B). To identify weaknesses of cells lacking cyclin F, we conducted a kinase inhibitor drug screen to assess differences in viability between CCNF K/O and parental cell lines (HeLa; Fig 1A). To this end, we used the KCGS, a set whose origins can be traced to the well-utilised kinase inhibitor collections PKIS and PKIS2 (Elkins et al., 2016; Drewry et al., 2017). The simple premise of KCGS is that screening a publicly available, well annotated set of potent and selective kinase inhibitors in disease-relevant phenotypic assays is an efficient way to elucidate biology and uncover dependencies (Jones & Bunnage, 2017). The current version of KCGS consists of 188 small molecule kinase inhibitors that cover 221 kinases. None of the members of KCGS is broadly kinome active, facilitating target deconvolution. The screen was run in duplicate with an average replicate correlation (Pearson’s) of 0.9467 and an average Z-Factor of 0.6040, demonstrating a good dynamic range between DMSO (vehicle-negative control) and controls (chemo-toxic-positive control) with robust reproducibility between replicates. To identify small molecule kinase inhibitors, which specifically target cyclin F-depleted cells, we normalised the cell viability results using a Z-score for each cell line and then expressed the difference between CCNF K/O cells and control cells as Z. The graph in Fig 1A and additional data in Table 1 highlight compounds to which CCNF K/O cells are sensitive or resistant (Dataset EV1). The most striking difference in viability between control cells and CCNF K/O cells was obtained with CCT244747, a selective Chk1 kinase inhibitor (Walton et al., 2012). Among the compounds which killed CCNF K/O cells, we also identified VE-822, an ATR inhibitor (Charrier et al., 2011). Similarly, in a parallel drug screen, a striking sensitivity of CCNF K/O cells to two other Chk1 inhibitors with unrelated structure, PF477736 and AZD7762, was observed (Fig 1B; Blasina et al., 2008; Zabludoff et al., 2008).

To confirm that the effect of CCT244747, PF477736 and AZD7762 was on target, we also tested the survival of the HeLa CCNF K/O with LY2603618 and UCN-01, two structurally unrelated Chk1 inhibitors. UCN-01 decreased cell proliferation in CCNF K/O cells compared to HeLa parental cells (Fig 1C). Upon treatment of cells with LY2603618, a very selective Chk1 inhibitor (King et al., 2014), we observed the most striking difference in cell viability. The IC50 of LY2603618 in normal HeLa cells was > 1 mM, whereas CCNF K/O cells had an IC50 of 400 nM, accounting for a significant fold difference in sensitivity across the two cell lines using LY2603618 (Fig 1D).

To establish that the difference in sensitivity between control and CCNF K/O cells was not due to proliferation defects of the CCNF K/O cells, we measured cell proliferation across 72 h comparing HeLa control to CCNF K/O. Overall, cell proliferation was not different in the 72-h time-frame between HeLa control and CCNF K/O (Fig 1E). However, upon addition of LY2603618, CCNF K/O cells stopped proliferating (Fig 1E), corresponding to an increase in cell death (Fig 1F).

To exclude the possibility that the phenotype of cell death induced by Chk1 inhibitors was limited to CCNF K/O cell lines, we also tested sensitivity of U-2-OS cells to LY2603618 after knockdown of cyclin F by siRNA. Although treatment of U-2-OS cells with LY2603618 at 500 nM caused more cell death in U-2-OS cells compared to HeLa cells, U-2-OS cells where cyclin F levels were diminished by siRNA had significantly reduced cell survival and increased cell death (Fig 1G and H). In addition, non-transformed cell line RPE-1 showed increased sensitivity to Chk1 inhibitors upon siRNA of cyclin F (Fig EV1C).

Since ATR is upstream of Chk1, it was not surprising that the drug screen above also identified ATR inhibitors. Indeed, inhibition of ATR using VE-821 also promoted cell killing in the CCNF K/O, although the difference between HeLa control and CCNF K/O cells was only observed at high doses and was less pronounced compared to Chk1 inhibitors (Fig EV1D). Similar results were obtained after inhibition of ATR in U-2-OS cells and cyclin F depletion by siRNA (Fig EV1E). The data presented above highlight a novel synthetic lethal interaction between the loss of cyclin F and checkpoint deple- tion. The sensitivity of cyclin F loss to Chk1 inhibition was striking and investigated further.
Loss of cyclin F causes DNA replication catastrophe after Chk1 inhibition

The inhibition of ATR and Chk1 promotes accumulation of ssDNA, which is exacerbated over time due to active DNA replication. The ssDNA saturates the capacity of RPA to bind to it and is exposed to the action of nucleases. Nucleases convert ssDNA to DSBs, which promote the activation of DNA-PK and ATM kinases required for DSB repair. The accumulation of DSBs can be detected by markers of DNA-PK and ATM kinase activation and is an indication of DNA replication catastrophe (Toledo et al., 2013).

To gain insights into the mechanism of synthetic lethality between loss of cyclin F and Chk1 inhibition, we compared the response of HeLa parental cells and CCNF K/O cells after treatment with Chk1 inhibitor. In contrast to parental cells that showed accumulation of RPA phosphorylation on Serine (S) 33, a surrogate marker of ATR activation, at 6 h, CCNF K/O cells already showed increased RPA S33 phosphorylation after 2 h of treatment (Fig 2A).
While ATR activation can be attributed to accumulation of RPA-coated ssDNA, further activation of DNA-PK and ATM can be associated with formation of DSBs leading to DNA replication catastrophe detected by phosphorylation of γ-H2AX, phosphorylation of RPA on S4 and S8 and phosphorylation of KAP1 on S824 at the 16-h time point as reported previously (Toledo et al., 2013). In the CCNF K/O cell line, phosphorylation of γ-H2AX, phosphorylation of RPA on S4 and S8 and phosphorylation of KAP1 on S824 were already present at 6 h after treatment with the Chk1 inhibitor LY2603618. Activation of the DSB markers continued thereafter, accumulating profoundly at 16 and 24 h of treatment compared to control cells (Fig 2A). It is worth mentioning that the overall levels of Chk1 and RPA were unchanged in CCNF K/O cells compared to control cells, indicating that the differences observed were due to upstream phosphorylation events rather than a change in the total levels of these proteins (Fig 2A). The phenotype of significant accumulation of single-stranded DNA (ssDNA) and DSB markers was recorded in the U-2-OS cell line by knockdown of cyclin F using siRNA in the presence of Chk1 inhibitors (Fig EV2A).

To confirm that CCNF K/O cells indeed encountered DNA replication catastrophe, we analysed chromatin bound RPA and γ-H2AX formation in response to Chk1 inhibition in different cell cycle phases by Fluorescence Activated Cell Sorting (FACS). In accordance with the induction of profound DNA replication stress in the CCNF K/O, the γ-H2AX signal initiated in replicating cells (Fig 2B–D) at early time points, and γ-H2AX presence and levels increased as cells progressed from early S to mid-late S phase (Figs 2E and F, and EV2B and C). At 24 h after Chk1 inhibitor treatment, about 15% of cells were found to be highly γ-H2AX positive but Edu negative, having S phase DNA content of 2-4n (Fig 2G). These cells are arrested in S phase, likely due to the extensive accumulation of DNA damage detected by γ-H2AX staining. In addition, we observed excessive RPA loading on chromatin starting from 6 h of treatment of Chk1 inhibitors in CCNF K/O cells (Fig EV2D). Consistent with the previous observation of DNA replication catastrophe induced by checkpoint inhibition, the accumulation of RPA on chromatin reached its peak before DSβ generation (Fig EV2D). The conversion of ssDNA (high RPA loading) to DSBs (γ-H2AX) was confined to cells with the highest degree of chromatin-loaded RPA (Fig 2H). The shape of the plot of the nuclear ratio of RPA to γ-H2AX following Chk1 inhibition in CCNF K/O treatment was consistent with DNA replication catastrophe induced by checkpoint inhibition (Fig 2H).

It is important to mention that γ-H2AX formation in CCNF K/O cells upon Chk1 inhibitor treatment was significantly reduced after expression of GFP-cyclin F (Fig 3A and B), demonstrating that the formation of DSBs is mediated specifically by cyclin F loss.

Although the evidence provided so far is indicative of DNA replication catastrophe in CCNF K/O cells upon Chk1 inhibition, we sought direct evidence of single-stranded breaks (SSBs) and DSB accumulation. Therefore, we conducted alkaline and neutral comet assays in CCNF K/O cells treated with Chk1 inhibitors. Treatment of CCNF K/O cells with Chk1 inhibitors induced a drastic increase in SSBs and DSBs readily visible in the tail images (Fig 3C) and quantified in the olive tail moment (Fig 3D and E). Our data support a model where loss of cyclin F predisposes cells to DNA replication catastrophe upon challenging cells with Chk1 inhibitors.

### Table 1. Top hits of the viability screen comparing HeLa to CCNF K/O using the KCGS.

| Compound name | Δ z-score | Target |
|---------------|-----------|--------|
| **100 nM**    |           |        |
| C10 VE-822    | −0.61484  | ATR    |
| B17 CCT251545 | −0.56548  | CDK8, CDK19 |
| I03 GSK236394 | −0.4252   | PIK3CB |
| **Less sensitive** |         |        |
| A20 GW784752X | 1.016439  | GSK3b  |
| A21 GWS80509X | 1.052113  | VEGFR2 |
| E22 GW296115X | 1.443446  | PDGFR  |
| **1 μM**      |           |        |
| B08 CCT244747 | −2.88143  | CHK1   |
| E16 B00614644 | −0.56505  | MAPKAPK2, MAPKAPKS, PIM3, PHKG2 |
| I05 SB-725317 | −0.55738  | CDK2/cyclinA, LTK |
| **Less sensitive** |         |        |
| C11 SB-590885 | 1.036447  | B-raf  |
| B03 JNK-IN-7  | 1.164645  | JNK    |
| E22 GW296115X | 1.180556  | PDGFR8 |
| D15 GW814408X | 1.364238  | GSK3b  |
| H16 GSK1070916| 1.572064  | Aurora kinase |
| D12 CA93.0    | 2.072085  | GAK1   |
| **10 μM**     |           |        |
| H19 GW807982X | −1.06323  | GSK3b  |
| I20 TPKI-39   | −0.86181  | CIT, KIT, PDGFRB, CSF1R, PDGFR, DDR1, FLI1, TIE2 |
| C13 GSK1838705| −0.73301  | IGF1R, IR |
| **Less sensitive** |         |        |
| G14 PFE-PKIS 3| 0.838916  | TNK2, TYRO3, YES, FGFR1,2,3 |
| H11 CCT241533 | 0.859239  | CHK2   |
| G09 GW440135  | 0.866643  | KIT, NEK7, MEK5, PDGFRB, VEGFR2, NEK9, PDGFR, FLT4 |

To identify novel substrates of cyclin F that mediate DNA replication stress after checkpoint inhibition, we performed immunoprecipitation of cyclin F and identified interacting partners by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LC-MS/MS was conducted in the absence and presence of MLN4924, an inhibitor of Nedd8 activating enzyme, which prevents the activity of SCF complexes (Soucy et al., 2009). Results are summarised in Table 2 and include components of G1/S phase transition regulators and

E2F1 promotes DNA replication catastrophe in CCNF K/O cells treated with Chk1 inhibitors

To identify novel substrates of cyclin F that mediate DNA replication stress after checkpoint inhibition, we performed immunoprecipitation of cyclin F and identified interacting partners by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LC-MS/MS was conducted in the absence and presence of MLN4924, an inhibitor of Nedd8 activating enzyme, which prevents the activity of SCF complexes (Soucy et al., 2009). Results are summarised in Table 2 and include components of G1/S phase transition regulators and
Figure 2.
many components of DNA replication. We speculated that these interacting partners accumulate in CCNF K/O cell lines and exacerbate DNA replication stress upon Chk1 inhibition. Since components of pre-RC (Cdc6, Cdt1 and Geminin), components of the DNA replication elongation machinery (TICRR and MTBP), G1/S cell cycle transcriptional regulators (E2F1 and E2F3) and exonucleases (Exo1) have all been previously linked to the induction of DNA replication stress, we focused our study on these factors (Hills & Diffley, 2014; Kotsantis et al., 2018). It is important to mention that although E2F1 was not identified in the LC-MS/MS, E2F1 deregulation by Rb loss was previously shown to promote synthetic lethality in combination with Chk1 inhibition (Witkiewicz et al., 2018). To seek a potential substrate of cyclin F which could mediate sensitivity to Chk1 inhibitors, we performed siRNA of Exo1, Cdc6, Cdt1, Geminin, MTBP, TICRR, E2F1 and E2F3 in HeLa control and CCNF K/O cells (Fig EV3A–D) after treatment with Chk1 inhibitors and determined the extent of DNA replication stress by measuring γ-H2AX levels and RPA accumulation on chromatin, respectively, by FACS. We obtained a complete rescue of γ-H2AX and RPA accumulation only after siRNA of E2F1 but not after siRNA of any factor indicated above, suggesting that E2F1 was the main determinant of Chk1 inhibitor sensitivity upon cyclin F deletion (Fig 4A and B). Upon close observation of the DNA damage present exclusively in S phase cells, we noted that siRNA of E2F1 specifically resulted in a reduction of DNA damage in CCNF K/O cells (Fig 4C). In addition, E2F1 depletion did not rescue DNA damage in HeLa parental cells (Fig 4C). This experiment points out that the rescue effect of E2F1 depletion on DNA damage is not simply due to cell cycle arrest and is a specific feature of CCNF K/O cells. A different siRNA oligo targeting E2F1 but not an siRNA targeting Exo1 rescued sensitivity of CCNF K/O cells to Chk1 inhibitors (Fig EV3E). Furthermore, high expression of exogenous E2F1 in HeLa cells was sufficient to mediate DNA replication stress after Chk1 inhibition, as visualised by increased levels of γ-H2AX intensity detected by FACS analysis (Figs 4D and EV3F). All the surrogate markers of ssDNA and DSB formation (phosphorylation of RPA on S33, phosphorylation of RPA on S4 and S8, γ-H2AX and phosphorylation of KAP1 at S824) were increased in CCNF K/O cells upon treatment with Chk1 inhibitors as observed above. However, siRNA of E2F1 was sufficient to restore the levels of these markers to basal levels present in HeLa controls (Fig 4E).

A similar set of experiments to the ones described above was conducted upon siRNA of cyclin F and treatment with ATR inhibitors. We observed DNA replication stress upon cyclin F depletion and ATR inhibition, as detected by the appearance of phosphorylation of RPA on S4, S8 and γ-H2AX. The DNA replication stress observed in cyclin F-depleted cells treated with ATR inhibitor was rescued by concomitant depletion of E2F1 (Fig EV3G). Furthermore, we conducted a comet assay to detect directly DNA damage in cells treated as above. The comet assay highlighted that ATR inhibition induced increased DNA damage after siRNA of cyclin F. The DNA damage present in cells with reduced cyclin F levels was rescued fully by concomitant depletion of E2F1 (Fig EV3H). CCNF K/O cells died more upon ATR inhibition (Fig EV3I); however, the viability of cells lacking cyclin F upon ATR inhibition was fully rescued by concomitant depletion of E2F1 (Fig EV3I). We conclude that DNA replication catastrophe induced by cyclin F depletion and checkpoint inhibition is promoted by E2F1.

E2F1 is a ubiquitylation substrate of SCFcyclin F degraded at the G2/M transition and after checkpoint inhibitors

The experiments presented above suggest that E2F1 could be accumulating in CCNF K/O cells. Therefore, we asked whether E2F1 was regulated by cyclin F and measured the levels of E2F1 in CCNF K/O cells or after knockdown of cyclin F by siRNA. CCNF K/O cell lines had a prominent increase in the levels of E2F1 compared to control (Fig 5A). Furthermore, siRNA of cyclin F induced a significant increase of E2F1 in U-2-OS cells (Fig 5B).

We used normalised gene expression data to compare HeLa cells with the CCNF K/O in order to identify differentially expressed genes. Genes with adjusted P < 0.05 were considered significantly differentially expressed (Dataset EV2). Gene set variance analysis (GSVA) of three different data sets related to E2F1 and E2Fs target genes (Ishida et al., 2001; Schaefer et al., 2009; Liberezon et al., 2015) was significantly increased in CCNF K/O cells compared to HeLa (Fig 5C). Gene set enrichment analysis (GSEA) confirmed a statistically significant enrichment of these gene sets (Fig EV4A). Our data point out that E2F1 protein levels and target genes are increased in CCNF K/O cells.

E2F1-dependent transcription is required to promote G1/S phase transition, and the activity of E2F1 is reduced in G2/M.
Figure 3.
Figure 3. Loss of cyclin F causes SSBs and DSBs after Chk1 inhibition.

A Representative plots of γ-H2AX intensity versus DAPI signal of CCNF K/O cells transfected with empty GFP and GFP-CCNF untreated (top panel) and after treatment with 1 μM Chk1i (LY263036) for 20 h (bottom panels).
B Normalised γ-H2AX intensity in CCNF K/O cells transfected with either GFP empty vector or GFP-Cyclin F and treated with 1 μM Chk1i for 20 h. GFP-positive cells were gated. Data from three independent experiments were plotted with mean % ± SD.
C Representative images of neutral and alkaline comet assays from HeLa and CCNF K/O cells treated with Chk1i. Cells were treated with 1 μM Chk1i for 24 h before being harvested for neutral and alkaline comet assays as indicated.
D Quantification of comets in alkaline gels. At least 100 cells, across two slides, were analysed in each condition in two biological replicates. Data are shown as medians, with 25/75/percentile range (box) and 10–90/percentile range (whiskers). Fold changes of median values are shown in bold. P-values were calculated using the Mann–Whitney test (two-tailed). Olive tail moment = (Tail mean – Head mean)*Tail/DNA/100.
E Quantification of comets in neutral gels. At least 100 cells, across two slides, were analysed in each condition in two biological replicates. Data are shown as medians, with 25/75/percentile range (box) and 10–90/percentile range (whiskers). Fold changes of median values are shown in bold. P-values were calculated using the Mann–Whitney test (two-tailed). Olive tail moment = (Tail mean – Head mean)*Tail/DNA/100.

Table 2. High confidence interacting partners/substrates of cyclin F. Data shown correspond to MASCOT protein outputs including: protein score, N. Significant sequences and emPAI value.

| Name                                                                 | Cyclin F | Cyclin F + MLN4924 |
|----------------------------------------------------------------------|----------|--------------------|
|                                                                      | Score    | No.sign peptides  | Empai  | Score    | No.sign peptides  | Empai  |
| Cyclin F (F-box only protein)                                        | 1,973    | 18                 | 2.04   | 1,993    | 28                 | 5.39   |
| Cell division control protein 6 homolog (CDC6-related protein)       |          |                    |        |          |                    |        |
| (Cdc18-related protein) (HsCDC18) [p62(cdc6)] (HsCDC6)               | 107      | 3                  | 0.25   | 193      | 9                  | 0.95   |
| M-phase phosphoprotein 9                                             | 56       | 2                  | 0.07   | 162      | 8                  | 0.33   |
| Geminin                                                              | 52       | 1                  | 0.22   | 247      | 8                  | 7.88   |
| Ankyrin repeat domain-containing protein 17 (gene trap ankyrin repeat protein) (serologically defined breast cancer antigen NY-BR-16) | 51       | 1                  | 0.02   | 100      | 5                  | 0.09   |
| Zinc finger protein 31B (endocrine regulatory protein)               | 50       | 3                  | 0.06   | 126      | 8                  | 0.16   |
| Exonuclease 1 (hExo1) (EC 3.1.16) (exo nuclease I) (hExoI)           | 50       | 2                  | 0.1    | 113      | 6                  | 0.35   |
| Protein red (cytokine IK) (IK factor) (protein RER)                  | 45       | 1                  | 0.07   | 53       | 2                  | 0.15   |
| DNA replication factor Cdt1 (double-parked homolog) (DUP)            | 71       | 2                  | 0.17   | 140      | 10                 | 1.17   |
| Opioid growth factor receptor (OGFr) (protein 7-60) (zeta-type opioid receptor) | 60       | 1                  | 0.07   | 37       | 1                  | 0.07   |
| Heterogeneous nuclear ribonucleoprotein A3 (hnRNP A3)               | 59       | 1                  | 0.13   | 41       | 1                  | 0.13   |
| Cullin-1 (CUL-1)                                                     | 53       | 2                  | 0.11   | 91       | 3                  | 0.17   |
| Ribonucleoside-diphosphate reductase subunit M2 (EC 1.17.4.1) (ribonucleotide reductase small chain) (ribonucleotide reductase small subunit) | 1        | 0                  | 0       | 61       | 1                  | 0.11   |
| Treslin (TopBP1-interacting checkpoint and replication regulator)    | 31       | 1                  | 0.02   | 68       | 2                  | 0.05   |
| TopBP1-interacting, replication-stimulating protein                  |          |                    |        |          |                    |        |
| Casein kinase II subunit alpha 3 (CK II alpha 3) (EC 2.7.11.1) (casein kinase II alpha 1 polypeptide pseudogene) | 1        | 0                  | 0       | 50       | 1                  | 0.12   |
| BRISC and BRCA1-A complex member 1 (mediator of RAP80 interactions and targeting subunit of 40 kDa) (new component of the BRCA1-A complex) | 45       | 1                  | 0.14   | 1        | 0                  | 0      |
| Zinc finger protein 8 (zinc finger protein HF.18)                    | 1        | 0                  | 0       | 39       | 3                  | 0.24   |
| Protein aurora borealis (HsBora)                                    | 1        | 0                  | 0       | 38       | 2                  | 0.17   |
| Mdm2-binding protein (hMTBP)                                        | 1        | 0                  | 0       | 46       | 1                  | 0.05   |
| Transcription factor E2F3 (E2F-3)                                   | 1        | 0                  | 0       | 42       | 1                  | 0.1    |
| Transcription factor ETV6 (ETS translocation variant 6) (ETS-related protein Tel1) | 1        | 0                  | 0       | 37       | 1                  | 0.09   |

(Budhavarapu et al., 2012). We speculated that E2F1, like other cyclin F substrates, was degraded at the G2/M transition so we measured levels of E2F1 in synchronised cells. While in HeLa cells the levels of E2F1 were high in S phase and reduced in G2/M, in CCNF K/O cells E2F1 levels remained high across the cell cycle (Fig EV4B).
The experiments presented above show that E2F1 levels and transcriptional targets are increased in CCNF K/O cells; therefore, we tested whether E2F1 is directly regulated by cyclin F through ubiquitylation. To prove that the regulation of E2F1 by cyclin F was not due to different levels of expression of cyclin F and E2F1 which were comparable in the input (Fig EV4C). Furthermore, the interaction data, cyclin F WT promotes the ubiquitylation of E2F1, while cyclin F AF and M309A mutants were not promoting E2F1 ubiquitylation (Fig 5D). In accordance with the interaction data, cyclin F WT mediates DNA replication catastrophe in cyclin F K/O cells treated with Chk1 inhibitors.

- Figure 4. E2F1 mediates DNA replication catastrophe in cyclin F K/O cells treated with Chk1 inhibitors.

**A** Percentage [%] of γ-H2AX-positive cells in HeLa and CCNF K/O cell lines after transfection of the indicated siRNAs and treatment with Chk1i for 20 h. Percent of γ-H2AX-positive cells was plotted as mean ± SD.

**B** Percentage [%] of hyper-RPA-positive cells in HeLa and CCNF K/O cell lines after transfection of the indicated siRNAs and treatment with Chk1i for 20 h. Percent of hyper-RPA-positive cells was plotted as mean ± SD.

**C** Percentage [%] of γ-H2AX-positive cells in S phase cells by DAPI content after Chk1i (LY2603618). Related to the experiments presented in (A).

**D** Relative γ-H2AX intensity in cells transfected with either empty vector (EV) or HA-E2F1, treated with Chk1i for 20 h. γ-H2AX fluorescence in HA-positive cells was normalised to the empty vector (EV) transfected cells and compared to cells not expressing HA. Related to Fig EV5F.

**E** HeLa and CCNF K/O cells transfected with the indicated siRNA and treated with 1 μM Chk1i (LY2603618) for 20 h were harvested and lysed using SDS. Indicated proteins were resolved by SDS-PAGE and detected by WB. TFIIH is a loading control.

Data information: Data are presented as mean ± SD, with at least three independent experiments. P-values (*P < 0.05, **P < 0.005, ***P < 0.0005) were calculated by paired and two-tailed t-test.

Promote E2F1 ubiquitylation. We have previously shown that cyclin F interacts with its substrates using the cyclin domain and a mutant of cyclin F (M309A) in critical residues for the cyclin domain folding is unable to do so (D’Angiolella et al, 2010, 2012). The interaction between cyclin F and E2F1 was abolished by a single point mutation in the cyclin domain of cyclin F (Fig 5D). In accordance with the interaction data, cyclin F WT promotes the ubiquitylation of E2F1, while cyclin F AF and M309A mutants were not promoting E2F1 ubiquitylation (Fig 5E). The difference in ubiquitylation of E2F1 was not due to different levels of expression of cyclin F and E2F1 which were comparable in the input (Fig EV4C). Furthermore, the half-life of E2F1 was increased in CCNF K/O cells (Fig EV4D), providing strong evidence that E2F1 is a novel ubiquitylation substrate of cyclin F.
Figure 5.
It was previously shown that E2F1 is degraded upon challenging cells with ATR inhibitors (Buisson et al., 2015); therefore, we tested whether E2F1 was degraded upon treatment with Chk1 inhibitors as well, and if the degradation was dependent on cyclin F. E2F1 is down-regulated upon challenging cells with Chk1 (Fig EV4E). E2F1 down-regulation was prevented by treating cells with MG-132 or MLN4924 (Fig EV4E). In CCNF K/O cells, E2F1 degradation was not triggered by Chk1i (Fig EV4E). While the degradation of E2F1 was quickly elicited in HeLa parental cells upon Chk1i, CCNF K/O cells started with high levels of E2F1, which remained unaffected over the course of the experiment (Fig 5F). The results above indicated that cyclin F is controlling degradation of E2F1 upon Chk1 inhibition. To establish that the effect of cyclin F on E2F1 upon Chk1 inhibition was direct, we measured interaction between cyclin F and E2F1 upon treating cells with Chk1 inhibitors. The interaction between cyclin F and E2F1 was increased upon Chk1 inhibition (Fig S5 and quantified in H). Furthermore, ubiquitylation of E2F1 was triggered by treatment with Chk1 inhibitors (Fig S5). The increased ubiquitylation of E2F1 observed upon Chk1 inhibition was reduced by concomitant depletion of cyclin F using siRNA (Fig S5). Our findings reveal that E2F1 is a novel ubiquitylation substrate of cyclin F degraded at the G2/M transition and upon Chk1 inhibition.

A mutant of E2F1 lacking a CY motif is not ubiquitylated by cyclin F and promotes DNA replication stress and cell death after Chk1 inhibitors

Similarly to RRM2 and CP110, previously identified substrates of cyclin F (D’Angiolella et al., 2012), E2F1 is recruited by cyclin F using the cyclin domain, indicating the existence of a CY motif (RxL—cyclin binding domain) (Schulman et al., 1998) in E2F1 mediating the interaction with cyclin F. We identified an RxL at position 89–91 in E2F1 which promotes interaction with cyclin F; indeed, an E2F1 lacking this region failed to interact with cyclin F (Fig 6A). In accordance with the lack of interaction with cyclin F, E2F1 Δ RxL was also not ubiquitylated by cyclin F (Figs 6B and EV5A).

To test the stability of E2F1 and its effect on DNA replication stress, E2F1 WT and Δ RxL were exogenously expressed in cells under the control of a weak retroviral promoter (pBabe), which maintain expression of E2F1 to near-endogenous levels. In these cells, when comparing the half-life of E2F1 WT and ΔRxL, we observed a significant increase of E2F1 ΔRxL half-life (Fig 6C and quantified in D). The increased stability of E2F1 ΔRxL corresponded to high levels of E2F1 ΔRxL during cell cycle progression. Indeed, while E2F1 WT was degraded in G2/M, E2F1 ΔRxL was not (Fig EV5B), in agreement with E2F1 ΔRxL not being targeted for degradation by cyclin F.

In addition to cell cycle regulation, Chk1 inhibitors triggered the degradation of the endogenous and exogenous E2F1, while a mutant of E2F1 lacking the CY motif was not degraded, demonstrating that the degradation of E2F1 is operated by cyclin F through the CY motif (Fig 6E). Most importantly, upon addition of Chk1 inhibitors there was a significant accumulation of DNA damage markers of ssDNA and DSBs, which was more prominent in cells expressing a non-degradable version of E2F1 (Fig 6E). Finally, the expression of E2F1 promoted more cell death upon Chk1 inhibition (Fig 6F). The cell death observed upon expression of E2F1 was significantly exacerbated by expressing a non-degradable version of E2F1, demonstrating that the stabilisation of E2F1 was promoting DNA replication stress and cell death upon treatment with Chk1 inhibitors (Fig 6F). Overall, the use of a non-degradable version of E2F1 (ΔRxL) solidly establishes that the DNA replication stress and cell death observed in CCNF K/O cells are mediated by accumulation of E2F1.

Discussion

Here, starting from a drug screen, we identify a novel synthetic lethal interaction between cyclin F loss and Chk1 inhibition. Our study highlights that CCNF K/O cells, upon Chk1 inhibition, undergo DNA replication catastrophe, which is followed by cell death. We observe that synthetic lethality is promoted by the accumulation of E2F1, present in cyclin F-depleted cells. The concomitant depletion of E2F1 prevents DNA replication catastrophe in CCNF K/O cells. We further show that E2F1 is ubiquitylated by cyclin F during cell cycle progression and upon Chk1 inhibition. Using a non-degradable E2F1 (ΔRxL), we recapitulate increased DNA damage and cell death upon Chk1 inhibition. We propose a model where cyclin F can restrict E2F1 activity by controlling its levels when cells are treated with Chk1 inhibitors. The degradation of E2F1 operated by cyclin F
Figure 6. A mutant of E2F1 lacking a CY motif is not ubiquitylated by cyclin F and promotes DNA replication stress and cell death after Chk1 inhibitors.

A HEK293T cells transfected with HA-E2F1 wild type (WT) and HA-E2F1 lacking a CY motif (ΔRXL) were harvested for immunoprecipitation using HA antibodies. Immunoprecipitates were immunoblotted as indicated.

B HEK293T cells were cotransfected with MYC-tagged ubiquitin and HA-E2F1 or HA-E2F1 ΔRXL lacking the CY motif as indicated (+). HA-E2F1 was immunoprecipitated with an anti-HA resin under denaturing conditions and immunoblotted.

C HeLa cells stably expressing HA-E2F1 WT and HA-E2F1 ΔRXL under the control of a retroviral promoter were treated with cycloheximide (CHX) at 50 μg/ml for the indicated minutes (min).

D Quantification of E2F1 half-life in (C).

E HeLa cells stably expressing an empty vector (EV), HA-E2F1 WT and HA-E2F1 ΔRXL under the control of a retroviral promoter were treated with Chk1i for the indicated hours (h). Indicated proteins were resolved by SDS–PAGE and detected by WB. H3 was used as a loading control.

F Relative survival (%) of HeLa cells stably expressing an empty vector (EV), HA-E2F1 WT and HA-E2F1 ΔRXL under the control of a retroviral promoter measured using resazurin and compared to EV treated with Chk1i at the indicated concentrations.

Data information: Data are presented as mean ± SD, with at least three independent experiments. P-values (*P < 0.05, **P < 0.005) were calculated by paired and two-tailed t-test.
suppresses DNA replication progression. The concomitant loss of cyclin F and Chk1 prompts uncontrolled E2F1 activity in the absence of checkpoint control inducing DNA replication catastrophe.

The expression of a non-degradable version of E2F1 recapitulated the phenotype of cyclin F depletion giving strong proof that the DNA replication stress promoted by loss of cyclin F upon Chk1 inhibitors is mostly mediated by E2F1 accumulation. However, the phenotype of cyclin F loss was more striking than the single deregulation of E2F1. While this could be also ascribed to technical issues related to the generation of CCNF K/O cell lines and the expression of E2F1 near to physiological levels, it is also possible to speculate that other cyclin F substrates could play an additive role in inducing DNA replication catastrophe. Interestingly, it has very recently been shown that cyclin F targets E2F1 and its paralogs E2F2 and E2F3 for degradation (Clijsters et al., 2019). While we have excluded E2F3 as a mediator of DNA replication catastrophe in CCNF K/O (Fig 4A and B), it is plausible that E2F2 accumulation could also play a role.

Our data are in accordance with the observation that Rb loss was shown to promote increase sensitivity to Chk1 inhibition due to deregulated DNA replication (Witkiewicz et al., 2018). The observed phenotype of increased DNA replication stress and accumulation of DNA damage upon Chk1 inhibition in Rb-deficient breast cancer cell lines (Witkiewicz et al., 2018) closely resembles the DNA replication catastrophe mediated by cyclin F loss and E2F1 deregulation.

The mechanism of DNA replication catastrophe induced by E2F1 accumulation in cyclin F-depleted cells could be seen as oncogene-induced DNA replication stress, mostly studied in models of cyclin E overexpression. It has been shown that cyclin E accumulation leads to reduced DNA replication origin assembly (Ekholm-Reed et al., 2004). Furthermore, cyclin E overexpression alters the normal DNA replication programme forcing the formation of intragenic origins of replication. This process predisposes cells to the deleterious consequences of transcription replication collisions and results in the formation of DSBs (Macheret & Halazonetis, 2018). It is possible that the accumulation of E2F1 in CCNF K/O cells also predisposes the cells to the deleterious consequences of cyclin E deregulation and contributes to the induction of DNA replication catastrophe observed upon Chk1 inhibition. However, there are also substantial differences, as cyclin E overexpression has not been connected to Chk1 inhibitor sensitivity. Recent evidence suggests that transcription and DNA replication initiation sites are closely linked (Chen et al., 2019). We found that cyclin F regulates E2F1 only in the G2/M phase of the cell cycle. Given the central role of E2F1 in the transcription of genes essential for DNA replication initiation, it is tantalising to speculate that cyclin F regulates a late DNA replication programme, which entails E2F1 depletion. It is worth pointing out that the DNA replication stress induced by cyclin F depletion does not rely on overexpression systems. Therefore, the CCNF K/O system of DNA replication catastrophe promoted by Chk1 inhibition could be a more physiological system compared to cyclin E overexpression to study deregulated DNA replication and warrants further investigation.

Chk1 inhibitors are being evaluated in clinical trials in a variety of solid tumours. Inactivating mutations in cyclin F detected as diploid truncating mutations have been identified in stomach adenocarcinoma, lung adenocarcinoma, medulloblastoma, squamous cell carcinoma of the head and neck, glioblastoma, mucinous adenocarcinoma of the colon and rectum, colon carcinoma and cutaneous melanoma (Cerami et al., 2012; Gao et al., 2013). In light of our observations, we believe that patients should be selected based on cyclin F status. Given the striking synthetic lethality we report, patients with cancers bearing truncating mutations in cyclin F could benefit significantly from treatment with Chk1 inhibitors.

Interestingly, our drug screen reveals a number of positive and negative drug interactions, which could be further investigated. Cyclin F is amplified in 10-15% of breast invasive ductal carcinoma (Cerami et al., 2012; Gao et al., 2013). It is likely that amplification of cyclin F hampers the effectiveness of Chk1 inhibitor treatment. In the future, it would be crucial to establish cancer models that mimic cyclin F amplification both in vivo and in vitro.

**Materials and Methods**

**Cell lines**

HEK293, U-2-OS, MCF7, HeLa and HeLa CCNF K/O cells were cultured in DMEM (Sigma) containing 10% FBS (Gibco) and 100 U/ml penicillin, 100 mg/ml streptomycin (Millipore). HEK293, U-2-OS and HeLa cell lines were purchased from the ATCC. CCNF K/O cells were generated previously from HeLa cells (Mavrommatis et al., 2018). MCF7 was obtained from the Chapman laboratory.

**Antibodies and chemicals**

| Antibody | Company         | Cat. No.  |
|----------|-----------------|-----------|
| CCNA     | a kind gift of Michele Pagano |          |
| CCNB     | Invitrogen      | AHF-0052  |
| CCNE1    | Merck Millipore | 05-363    |
| CCNF (C-20) | Santa Cruz     | sc-952    |
| Cdc6 (180.2) | Santa Cruz     | sc-9964   |
| Chk1 (2G1D5) | Cell Signalling Technologies | 23605     |
| E2F1     | Santa Cruz      | sc-251    |
| E2F3 (PC37) | Santa Cruz     | sc-69684  |
| Exo1    | GeneTex         | GTX109891 |
| Geminin (FL-249) | Santa Cruz | sc-13015  |
| GFP      | Roche           | 11814-460-001 |
| γ-H2AX(Ser139) | Novus Biologica | NB 100-2280 |
| H3       | Cell Signalling Technologies | 9715     |
| HA-HRP   | Roche           | 12013819001 |
| KAP1     | Abcam           | ab10484   |
| MTBP (K20) | Santa Cruz     | sc-47173  |
| p53 (DO-1) | Santa Cruz     | sc-126    |
| p53R2 (C-18) | Santa Cruz     | sc-10843  |
| pCDK1 (Thr14/15yr) | Santa Cruz | sc-12340  |
| pCHK1 S296 | Abcam         | ab79758   |
| pCHK1 S317 | GeneTex       | GTX22834  |
| PCNA (PC10) | Santa Cruz     | sc-56     |
| PH histone H3 (Ser10) | Millipore | 06-570    |
| pKAP1 S824 | Abcam         | ab70369   |
for 1 min at a flow rate of 20 μl/min before being chromatographic separated on a 50 cm-long Easy-Spray column (ES803, Thermo Fischer) with a gradient of 2–35% acetonitrile in 0.1% formic acid and 5% DMSO with a 250 nl/min flow rate for 60 min. The Q-Exactive HF was operated in a data-dependent acquisition (DDA) mode to automatically switch between full MS-scan and MS/MS acquisition. Survey-full MS scans were acquired in the Orbitrap mass analyser over an m/z window of 375–1,500 and at a resolution of 60k (AGC target at 36 ions). Prior to MSMS acquisition, the top twelve most intense precursor ions (charge state ≥ 2) were sequentially isolated in the Quad (m/z 1.2 window) and fragmented on the HCD cell (normalised collision energy of 28). MS/MS data were obtained in the orbitrap at a resolution of 30,000 with a maximum acquisition time of 45 ms, an AGC target of 5e5 and a dynamic exclusion of 27 s.

The raw data were searched against the Human UniProt-SwissProt database (June 2018; containing 20,361 human sequences) using Mascot data search engine. The search was carried out by enabling the Decoy function, while selecting trypsin as enzyme (allowing 1 missed cleavage), peptide charge of +2, +3, +4 ions, peptide tolerance of 10 ppm and MS/MS of 0.05 Da; #13C at 1; carbamidomethyl (C) as fixed modification; and deamidated (NQ), oxidation (M), phospho (ST), phospho (Y) as a variable modification. MASCOT outputs were filtered using an ion score cut off of 20 and a false discovery rate (FDR) of 1%. A qualitative analysis was carried on with proteins identified with two or more peptides. LC-MS/MS raw data could be made available by the authors upon request.

Kinase and drug screen

HeLa and CCNF K/O cells were seeded at 1,000 cells per well in 384-well plates 1 day before treatment with the KCGS library at 10, 1 and 0.1 μM concentrations. Relative cell survival was determined using resazurin 3 days after treatment. Z-scores were calculated for all samples, and the difference between HeLa versus CCNF K/O cells was plotted.

Relative cell proliferation assay

U-2-OS cells were seeded at 2,000 cells per well to 96-well plate in hexaplicate 1 day before siRNA transfection. Cells were treated 1 day after siRNA transfection with indicated concentrations of ATRi or Chk1i. HeLa and CCNF K/O cells were seeded at 2,000 cells per well to 96-well plate in hexaplicate 1 day before treatment with indicated concentrations of ATRi or Chk1i. Relative cell proliferation was determined 3 days after treatment using resazurin 30 μg/ml in complete media after 1- to 2-h incubation.

Cell counting and survival assay

U-2-OS cells were seeded at 20,000 cells per well to 12-well plate 1 day before transfection with siRNA. Cells were treated 1 day after siRNA transfection with indicated concentrations of Chk1i and analysed 3 days later. HeLa and CCNF K/O cells were seeded at 20,000 cells per well to 12-well plate 1 day before treatment with 1 μM Chk1i. Cells were counted, viability was determined using PI staining at 0.2 g/ml 24, 48 and 72 h by FACS Attune (Life Technologies), and data were analysed using FlowJo software (TreeStar).
Flow cytometry of chromatin bound proteins, cell cycle analysis and EdU staining

To analyse cell cycle profile, cells were pulse-labelled with 10 μM EdU for 30 min, harvested by trypsinisation and fixed in 70% ethanol. For analysis of chromatin bound proteins, cells were pulse-labelled with 10 μM EdU for 30 min where indicated, harvested by trypsinisation, washed in PBS, pre-extracted in pre-extraction buffer (25 mM HEPESE pH 7.4, 50 mM NaCl, 1 mM EDTA, 3 mM MgCl2, 0.3 M sucrose, 0.5% Triton X-100) 5 min on ice, centrifuged and fixed with 4% PFA 15 min at room temperature (RT). Fixed cell samples were permeabilised with 0.5% Triton X-100 for 15 min and blocked in 1% BSA. Click reaction was performed in buffer containing 0.1 M Tris–HCl pH 8.5, 0.1 M sodium ascorbate, 2 mM CuSO4, 10 μM Alexa 647-azide 30 min at room temperature. Cells were washed in PBS and incubated with primary antibodies for 2 h at RT before incubation with secondary antibodies 1 h at RT. Cells were resuspended in DAPI 5 μg/ml in PBS, and data were acquired using FACSc FlowJo (TreeStar) and analysed using FlowJo (TreeStar).

Immunoprecipitation and immunoblotting

HEK293 cells were seeded to 10-cm plates 1 day before plasmid transfection (5 μg/plate using PEI-MAX 40 kDA at ratio 6:1). After 20 h, cells were treated with 1 μM MLN4924 or 1 μM Chk1i for 4–5 h. Cells were washed twice with PBS, harvested to lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM β-glycerophosphate, 10% glycerol, 1% Tween-20, 0.1% NP-40) containing protease inhibitors (Sigma) and okadaic acid, incubated on ice for 30 min and centrifuged for 30 min at 20,000 g 4°C. Supernatant was incubated at 4°C with either Flag M2 beads or HA beads for 1 and 2 h, respectively, before washing four times with lysis buffer. Immunoprecipitates were eluted using 2× LDS buffer (Life Technologies) supplemented with β-mercaptoethanol and boiled for 5 min at 95°C. Whole cell lysates were harvested using 2×SB (62.5 mM Bis-Tris pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS), boiled and sonicated, and protein concentration was evaluated using BCA protein kit (Thermo) in most cases. Cell lysate or immunoprecipitate was resolved in 10% Bis-Tris gels, transferred to nitrocellulose membrane (Millipore) and immunoblotted.

DNA constructs, siRNA and transfections

CCNF gene was subcloned from Flag-cyclin F (D’Angiolella et al, 2012) into pYFP-C1. The product YFP-cyclin F was fully sequenced verified. HA-E2F1 plasmid was a kind gift of Nick La Thangue. Plasmid transfections were carried using PEI (PEI-MAX 40kDA, Polyscience) at ratio 6:1.

All siRNAs were purchased from Ambion: NC (4390843), CCNF (s2527, s2528, s2529), cdc6 (s2744), Cdt1 (s37724), Geminin (s27307), MTBP (s25788), TICRR (s40361), E2F3 (s4411), E2F1 (s4405, s4406), Exo1 (s17502) and transfected 10 nM. siRNA transfections were performed using HiPerFect (Qiagen) following manufacturer instructions.

Comet assay

Alkaline and neutral comet assays were performed in parallel on the same cell set. Per condition, 50,000 trypsinised cells were mixed with 1% low melting point agarose (37°C) and added to microscope slides pre-coated with 1% normal melting point agarose. For the alkaline assay, cells were lysed for 1 h at 4°C in a buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 1% (v/v) DMSO and 1% (v/v) Triton X-100, pH 10.5. Slides were placed in an electrophoresis tank and covered with 4°C electrophoresis buffer (300 mM NaOH, 1 mM EDTA, 1% (v/v) DMSO, pH >13) for 30 min, to allow DNA to unwind. Electrophoresis was performed at 25 V for 25 min at 300 mA. Slides were neutralised in 0.5 M Tris–HCl pH 8.1 and allowed to dry overnight. For the neutral assay, cells were lysed for 2 h at 4°C in a buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 1% (v/v) sodium laurely sarcosinate, 10% (v/v) DMSO and 0.5% (v/v) Triton X-100, pH 9.5. Slides were washed in TBE buffer (4°C), and electrophoresis was performed in TBE buffer (4°C) for 25 min at 25 V. Slides were allowed to dry overnight. For both assays, cells were rehydrated in H2O, stained with SYBR Gold for 30 min and allowed to dry. Analysis was performed using the Nikon NIE microscope and Andor Komet 7.1 software. Two microscope slides were analysed per condition, with at least 100 cells per condition per biological replicate. Data were expressed as Olive tail moment: (Tail.mean – Head.mean)*Tail% DNA/100. P-values were calculated using the two-tailed Mann–Whitney test.

RNA sequencing

800,000 cells were seeded into 10-cm dish in triplicate for each cell line. The next day, cells were around 80% confluence and had no uneven distribution. Dishes were then taken out from incubator and proceeded to cell lysing one at a time. For lysing cells, cells were quickly washed with 5 ml PBS twice and transferred immediately onto dry ice to flash-freeze. After transferring the frozen dish onto ice, 600 μl lysing solution (buffer RLT) from the RNA extraction kit was added and quickly spread all over the dish. Cell lysates were then collected into a 1.5-ml Eppendorf tube and stored on ice. Once collected and lysed all samples, all lysates were equilibrated to room temperature and proceeded to subsequent RNA extraction according to the kit manual (RNeasy Mini Kit from Qiagen, 74106). Sample concentrations, 260/280 and 260/230 ratios, were measured via NanoDrop as a quality control before being diluted to 200 ng/µl and stored in –80°C. ERCC ExFold RNA spike-in mixes (Ambion) were added prior to library preparation using the QuantiSeq Forward Kit (Lexogen, 015.96) using 500 ng of starting material to minimise the PCR amplification step. Samples prepared as biological triplicates were sequenced on HiSeq 2500 (Illumina) carried out using Wellcome Trust Genomic Service, Oxford.

RNA-seq data analysis

The quality control of raw sequencing reads was performed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc). Raw fastq files were trimmed of poly-A using Cutadapt (Martin, 2011). All trimmed reads were aligned to the hg19 genomic reference using STAR software (Dobin et al, 2013). Counts per gene from STAR were used as input for differential gene expression analysis using Limma (Ritchie et al, 2015).
Gene set enrichment analysis

Gene set enrichment analysis (GSEA) analyses were carried out using GSEA software (javaGSEA 3.0) (Subramanian et al., 2005) with 1,000 permutations and default parameters using PID_E2F_PATHWAY, ISHIDA_E2F_TARGETS and HALLMARK_E2F_TARGETS gene signatures. Gene set variation analysis (GSVA) analyses were performed using the GSVA package using same E2F gene signatures (Hanzelmann et al., 2013). The gene sets were obtained from the Molecular Signatures Database (MSigDB; http://software.broadinstitute.org/gsea/msigdb/index.jsp).

In vivo ubiquitylation assay

HEK293T cells were seeded into 10-cm dishes. When reaching 60% confluency, cells were transfected with plasmids as indicated in each experiment (for a 10-cm dish, 1 μg HA-E2F1, 2 μg Flag-cyclin F or 5 μg myc-ubiquitin was transfected or cotransfected). Thirty hours after transfection, cells were harvested via scraping and washed with PBS once. Cell pellet from each dish was then thoroughly lysed and boiled in 300 μl ubiquitin lysis buffer (2% SDS, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, supplemented with protease inhibitors). After cooling down to room temperature, cell lysates were then subjected to sonication until losing viscosity. Lysates were then boiled again and centrifuged at 20,000 × g myc-ubiquitin was transfected or cotransfected).

Cell cycle synchronisation via double thymidine release (DTR)

300,000 cells were seeded into each 10-cm dishes (seven dishes per cell line). For HeLa and CCNF K/O cell lines, 24 h later seeding, cells were subjected to the first thymidine block (2 mM final concentration) for 16 h. Cells were then washed three times with PBS and once with full media before being released into fresh complete media for 8 h. A second thymidine block was then performed via the same condition. Sixteen hours after, 0 time points were collected before the rest of the cells were washed and released into fresh complete media. After releasing cells from the second thymidine block, cells were collected at different time points as indicated. At 7-h time point, 200 nM Nocodazole was added to the uncollected cells to prevent cells from entering the next cell cycle.

Cycloheximide (CHX) chase

250,000 cells were seeded into each well of a 12-well plate (5 wells per cell line). Sixteen hours after, cycloheximide was added to cell culture as indicated to a final concentration of 50 μg/ml to block translation. Cells were collected via scraping and subjected to straight WB for protein half-life estimation.

Statistical analysis

To evaluate statistical significance, $P$-value was determined using GraphPad Prism. Paired two-tailed $t$-test was used for relative survival assays. Two-way ANOVA test was used to evaluate statistical significance of cell proliferation in cell counting assay. Unpaired two-tailed $t$-test was used to evaluate statistical significance of percentage of γ-H2AX and hyper-RPA-positive cells. $P$-values are marked by asterisks in figures as follows: *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

Image plotting in Fig 2H

The RPA2 against γ-H2AX dot plots in Fig 2H was originally generated using FlowJo V10 software, in which γ-H2AX-positive events were colour in blue, whereas the rest are in grey. The original X-axis (RPA2 intensity) and Y-axis (γ-H2AX) of each plot were removed and replaced with common RPA2 and γ-H2AX axes. Plots from different time points were then assembled against time axes in a staggered offset manner using Adobe Illustrator. Please refer to Fig EV2D for original data from the same experiment.

Data availability

All raw data generated in this study are collected in Datasets EV1 and EV2. Data from the drug screen of CCNF K/O lines are uploaded as Dataset EV1 and summarised in Table 1. RNA-seq data are collected in Dataset EV2 and have also been deposited in the European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena/sub mit/sra/#studies) with primary accession number PRJEB33738 and secondary accession number ERP116555.

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Author contributions

KB and HY designed the experiments, acquired, analysed and interpret data. RF contributed to the investigation and analysis of data with ATR inhibitors. SH and GD contributed to the investigation and analysis of comet assays. DE, DHD, CIW and SBH helped with the execution and analysis of KCGS screening data. BMK and IV ran and analyzed LC-MS/MS. JC and FMB conducted the RNA-seq and EV2. Data from the drug screen of CCNF K/O lines are uploaded as Dataset EV1 and summarised in Table 1. RNA-seq data are collected in Dataset EV2 and have also been deposited in the European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena/sub mit/sra/#studies) with primary accession number PRJEB33738 and secondary accession number ERP116555.

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Conflict of interest

The authors declare that they have no conflict of interest.
References

Bai C, Richman R, Elledge SJ (1994) Human cyclin F. EMBO J 13: 6087–6098
Bai C, Sen P, Hofmann K, Ma L, Goebi M, Harper JW, Elledge SJ (1996) SKP1
connects cell cycle regulators to the ubiquitin proteolysis machinery
through a novel motif, the F-box. Cell 86: 263 – 274
Bartek J, Lukas J (2003) Chk1 and Chk2 kinases in checkpoint control and
cancer. Cancer Cell 3: 421 – 429
Blasina A, Hallin J, Chen E, Arango ME, Kraynov E, Register J, Grant S,
Ninkovic S, Chen P, Nichols T et al (2008) Breaching the DNA damage
checkpoint by PF-00477736, a novel small-molecule inhibitor of
checkpoint kinase 1. Mol Cancer Ther 7: 2394 – 2404
Budhavarapu VN, White ED, Mahanic CS, Chen L, Lin FT, Lin WC (2012)
Regulation of E2F1 by APC/C Cdh1 via K11 linkage-specific ubiquitin chain
formation. Cell Cycle 11: 2030 – 2038
Buisson R, Boisvert JL, Benes CH, Zou L (2015) Distinct but concerted roles
of ATR, DNA-PK, and Chk1 in counting replication stress during S Phase.
Mol Cell 59: 1011 – 1024
Busino L, Donzelli M, Chiesa M, Guardavaccaro D, Ganoth D, Dorrello NV,
Hershko A, Pagano M, Draetta GF (2003) Degradation of Cdc25A by
beta-TrCP during S phase and in response to DNA damage. Nature 426:
87 – 91
Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A,
Byrne CJ, Heuer ML, Larsson E et al (2012) The cBio cancer genomics
portal: an open platform for exploring multidimensional cancer genomics
data. Cancer Discov 2: 401 – 404
Charrier JD, Durrant SJ, Golec JM, Kay DP, Knecht RM, MacCormick S,
Mortimore M, O’Donnell ME, Pinder JL, Reaper PM et al (2011) Discovery
of potent and selective inhibitors of ataxia telangectasia mutated and
Rad3 related (ATR) protein kinase as potential anticancer agents. J Med
Chem 54: 2320 – 2330
Chen YH, Keegan S, Kahli M, Tonzi P, Fenyo D, Huang TT, Smith DJ (2019)
Transcription shapes DNA replication initiation and termination in human
cells. Nat Struct Mol Biol 26: 67 – 77
Cljsters L, Hoencamp C, Calis JA, Marzio A, Handgraaf SM, Cuitino MC,
Rosenberg BR, Leone G, Pagano M (2019) Cyclin F controls cell-cycle
transcriptional outputs by directing the degradation of the three activator
E2Fs. Mol Cell 74: 1264 – 1277 e1267
D’Angiolla V, Donato V, Vijayakumar S, Saraf A, Florens L, Washburn MP,
Dynlacht B, Pagano M (2010) SCF(Cyclin F) controls centrosome
homeostasis and mitotic fidelity through CP110 degradation. Nature 466:
138 – 142
D’Angiolla V, Donato V, Forrester FM, Jeong YT, Pellacani C, Kudo Y, Saraf A,
Florens L, Washburn MP, Pagano M (2012) Cyclin F-mediated degradation
of ribonucleotide reductase M2 controls genome integrity and DNA repair.
Cell 149: 1023 – 1034
D’Angiolla V, Esencay M, Pagano M (2013) A cyclin without cyclin-
dependent kinases: cyclin F controls genome stability through ubiquitin-
mediated proteolysis. Trends Cell Biol 23: 135 – 140
Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleksi C, Jha S, Batut P,
Chaisson M, Gingeras TR (2013) STAR: ultrafast universal RNA-seq aligner.
Bioinformatics 29: 15 – 21
Drewry DH, Wells CI, Andrews DM, Angell R, Al-Ali H, Axtman AD, Capuzzi SJ,
Elkins JM, Ettmayer P, Frederiksen M et al (2017) Progress towards a
public chemogenomic set for protein kinases and a call for contributions.
PLoS ONE 12: e0181585
Dyson Nj (2016) RB1: a prototype tumor suppressor and an enigma. Genes
Deu 30: 1492 – 1502
Ekholm-Reed S, Mendez J, Tedesco D, Zetterberg A, Stillman B, Reed SI (2004)
Deregulation of cyclin E in human cells interferes with prereplication
complex assembly. J Cell Biol 165: 789 – 800
Elkins JM, Fedele V, Szklarz M, Abdul Azeez KR, Salah E, Mikolajczyk J,
Romanov S, Sepetov N, Huang XP, Roth BL et al (2016) Comprehensive
characterization of the published kinase inhibitor set. Nat Biotechnol 34:
95 – 103
Gao J, Aksoy BA, Dogrusoz U, Dresnner G, Gross B, Sumer SO, Sun Y,
Jacobsen A, Sinha R, Larsson E et al (2013) Integrative analysis of
complex cancer genomics and clinical profiles using the cBioPortal. Sci
Signal 6: p11
Hanzelmann S, Castelo R, Guinney J (2013) GSEA: gene set variation analysis
for microarray and RNA-seq data. BMC Bioinformatics 14: 7
Hills SA, Diffley JF (2014) DNA replication and oncogene-induced replicative
stress. Curr Biol 24: R435 – R444
Ishida S, Huang E, Zuzan H, Spang R, Levine G, West M, Nevins JR (2001) Role
for E2F in control of both DNA replication and mitotic functions as
revealed from DNA microarray analysis. Mol Cell 21: 4684 – 4699
Jin J, Shirogane T, Xu L, Nalepa G, Qin J, Elledge SJ, Harper JW (2003) SCF-beta-TRCP links Chk1 signaling to degradation of the Cdc25A protein
phosphatase. Genes Dev 17: 3062 – 3074
Jones LH, Bunnage ME (2017) Applications of chemogenomic library
screening in drug discovery. Nat Rev Drug Discov 16: 285 – 296
King C, Diaz H, Barnard D, Bardo D, Clawson D, Blosser W, Cox K, Guo S, Marshall
M (2014) Characterization and preclinical development of LY2603618: a
selective and potent Chk1 inhibitor. Invest New Drugs 32: 213 – 226
Klein DK, Hoffmann S, Ahlskog JK, O’Hanlon K, Quaas M, Larsen BD, Rolland
B, Rosner H, Walter D, Kousholt AN et al (2015) Cyclin F suppresses
B-Myb activity to promote cell cycle checkpoint control. Nat Commun:
5800
Kotsantis P, Petermann E, Boullon SJ (2018) Mechanisms of oncogene-
induced replication stress: jigsaw falling into place. Cancer Discov 8:
537 – 555
Lemmens B, Hegarat N, Akopyan K, Sala-Gaston J, Bartek J, Hochegger H,
Lindquist A (2018) DNA replication determines timing of mitosis by
restricting CDK1 and PLK1 activation. Mol Cell 71: 117 – 128 e113
Liberson A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P
(2015) The Molecular Signatures Database (MSigDB) hallmark gene set
collection. Cell Syst 1: 417 – 425
Lim S, Kaldis P (2013) Cdk5, cyclins and CKIs: roles beyond cell cycle
regulation. Development 140: 3079 – 3093
Macharet M, Halazonetis TD (2018) Intragenic origins due to short G1 phases
underlie oncogene-induced DNA replication stress. Nature 555: 112 – 116
Martin M (2011) Cutadapt removes adapter sequences from high-throughput
sequencing reads. EMBnetjournal 17: 10 – 12
Mavrommatis I, Faedda R, Galasso G, Li J, Burdova K, Fischer R, Kessler BM,
Carrero ZI, Guardavaccaro D, Pagano M et al (2018) beta-TrCP- and casein
kinase II-mediated degradation of cyclin F controls timely mitotic
progression. Cell Rep 24: 3404 – 3412
Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) limma
powers differential expression analyses for RNA-sequencing and
microarray studies. Nucleic Acids Res 43: e47
Schaefer CF, Anthony K, Krupa S, Buchoff J, Day M, Hannay T, Buetow KH
(2009) PID: the pathway interaction database. Nucleic Acids Res 37:
D674 – D679
Schulman BA, Lindstrom DL, Harlow E (1998) Substrate recruitment to cyclin-
dependent kinase 2 by a multipurpose docking site on cyclin A. Proc Natl
Acad Sci USA 95: 10453 – 10458
Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, Brownell JE, Burke KE, Cardin DP, Critchley S et al (2009) An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature* 458: 732–736
Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES et al (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102: 15545–15550
Toledo LI, Altmeyer M, Rask MB, Lukas C, Larsen DH, Povlsen LK, Bekker-Jensen S, Mailand N, Bartek J, Lukas J (2013) ATR prohibits replication catastrophe by preventing global exhaustion of RPA. *Cell* 155: 1088–1103
Walton MI, Eve PD, Hayes A, Valenti MR, De Haven Brandon AK, Box G, Hallsworth A, Smith EL, Boxall KJ, Lainchbury M et al (2012) CCT244747 is a novel potent and selective CHK1 inhibitor with oral efficacy alone and in combination with genotoxic anticancer drugs. *Clin Cancer Res* 18: 5650–5661
Witkiewicz AK, Chung S, Brough R, Vail P, Franco J, Lord CJ, Knudsen ES (2018) Targeting the vulnerability of RB tumor suppressor loss in triple-negative breast cancer. *Cell Rep* 22: 1185–1199
Zabludoff SD, Deng C, Grondine MR, Sheehy AM, Ashwell S, Caleb BL, Green S, Haye HR, Horn CL, Janetka JW et al (2008) AZD7762, a novel checkpoint kinase inhibitor, drives checkpoint abrogation and potentiates DNA-targeted therapies. *Mol Cancer Ther* 7: 2955–2966

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