

CCNE1-amplified ovarian cancer cells depend on CDK2 for both cell cycle progression
and homologous recombination

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Abstract

An intact cell cycle is important for normal cell growth but is often dysregulated in cancer. One factor that is often dysregulated is the cyclin-dependent kinase 2 (CDK2)/Cyclin E1 (encoded by the *CCNE1* gene) complex. Together, this complex drives the cell past the restriction point, committing it to DNA replication. Because DNA replication is an energy intensive process that must be completed with high fidelity, passage into S-phase and accurate duplication of the genome is normally tightly controlled. If either process becomes dysregulated, cells will exit the cell cycle permanently, or will re-wire to become dependent on particular proteins or pathways for survival.

Here, we investigate the role of CDK2 in *CCNE1*-amplified high grade serous ovarian cancer (HGSOC), a disease in which the core cell cycle machinery is dysregulated. High levels of Cyclin E1 drives cells prematurely into S-phase, resulting in increased DNA damage (Gorgoulis et al., 2005; Ohstubo & Roberts, 1993). HGSOC tumors harboring *CCNE1* amplification do not harbor defects in homologous recombination (HR) (Ciriello et al., 2012), suggesting they may rely on HR for repair. To investigate this directly, we utilize highly potent and specific inhibitors of CDK2, finding that CDK2 and Cyclin E1 are located directly at the site of stalled replication forks and that *CCNE1*-amplified HGSOC require HR to repair collapsed replication forks, suggesting that this complex has a direct role in genome maintenance. Furthermore, we show that CDK2 non-redundantly regulates HR in this cell type and that inhibition of CDK2 sensitizes cells to DNA damaging agents, suggesting that combination of agents targeting HR and CDK2 may provide clinical benefit.

CDK2 is dispensable in most cell types for proliferation and for repair, but *CCNE1*-amplified cells depend on CDK2 for both processes. Using a CRISPR based resistance screen, we identified p16 as a biomarker for CDK2 dependence in *CCNE1*-amplified cells. Through isogenic cell line engineering, we show that CDK4 is activated upon p16-loss and this activation can compensate for inhibition of CDK2. However, cells then become vulnerable to combination treatment with CDK2 and CDK4/6 inhibitors, highlighting that cell cycle plasticity has implications for patients who may progress on cell cycle targeted therapies. Taken together, these data support the benefit of CDK2 + CDK4/6 inhibitor combination therapy in HGSOC following the emergence of resistance.

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Abbreviations

AS	Analog sensitive
ATM	Ataxia-telangiectasia mutated
ATR	ATM and Rad3-related
ATRIP	ATR interacting protein
BIR	Break induced replication
CAK	Cyclin dependent kinase activating kinase
CDK	Cyclin dependent kinase
CtIP	CtBP- interacting protein
CO	Crossing over
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
D-loop	Displacement loop
DN	Dominant negative
DSB	Double-stranded break
ER	Estrogen receptor
FDA	Food and drug administration
FT-SEC	Fallopian tube secretory epithelial cell
G1	Gap phase 1
G2	Gap phase 2
GCR	Gross chromosomal rearrangement
GOF	Gain of function
HER2-	Human epidermal growth factor receptor 2 negative
HGSOC	High-grade serous ovarian cancer
HR	Homologous recombination
HR+	Hormone receptor positive
HRD	Homologous recombination deficiency
HU	Hydroxyurea
iPOND	Identification of proteins on nascent DNA
KO	Knockout
LOH	Loss of heterozygosity
M	Mitosis
MRN	Mre11-Rad50-Nbs1
NCO	Non-crossing over
NHEJ	Non-homologous end joining
PARP	Poly-ADP ribose polymerase
PDX	Patient derived xenograft
PLA	Proximity ligation assay
Plk	Polo-like kinase
Rb1	Retinoblastoma protein 1
RINGO	Rapid inducer of G2/M progression in oocytes
RPA	Replication protein A
RPE	Retinal pigment epithelial

S	Synthesis phase
sgRNA	Single-guide RNA
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
ssDNA	Single-stranded DNA
TF	Transcription factor
TLS	Translesion synthesis
WT	Wild-type

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Chapter 1 Introduction: The role of CDK2 in the cell cycle, oncogenesis, and DNA repair

Abstract

Cell cycle dependent kinases (CDKs), along with their regulatory cyclin partners, are critical components of the cell cycle machinery. Throughout metazoan evolution, higher order eukaryotic organisms have evolved distinct CDK/cyclin pairs to control different phases of the cell cycle. These CDKs and cyclins also have important and specialized roles during development, as particular CDKs are required for specific cell lineages. Moreover, several CDKs have been implicated in tumorigenesis, and as such are attractive therapeutic targets in oncology. In addition to their role in cell cycle progression, the CDKs have been implicated in high-fidelity repair, namely homologous recombination. As many cancer types have reliance on specific DNA repair pathways, understanding the role of CDKs in repair processes may aid in development of additional targeted therapies or rational drug combinations. Here, we provide a comprehensive review of CDK function in development, oncogenesis, and DNA repair.

Overview of the mammalian cell cycle

The cell cycle is fundamental to cellular growth, genome replication, and cellular division and is therefore tightly regulated. The main drivers of the cell cycle are cyclin dependent kinases (CDKs) which, along with their cyclin partners, act via substrate phosphorylation in a temporally controlled manner. While the CDK family is comprised of 20 serine/threonine kinases, four of them are mainly responsible for progressing the

cell cycle - the interphase CDKs: CDK4, CDK6, CDK2, and the mitotic CDK: CDK1 (Malumbres et al., 2009). The enzymatic activity of these kinases is regulated through cyclin binding, endogenous inhibitors, and phosphorylation, and these layers of control are fundamental to proper cell cycle progression (Figure 1.1). (Malumbres & Barbacid, 2009).

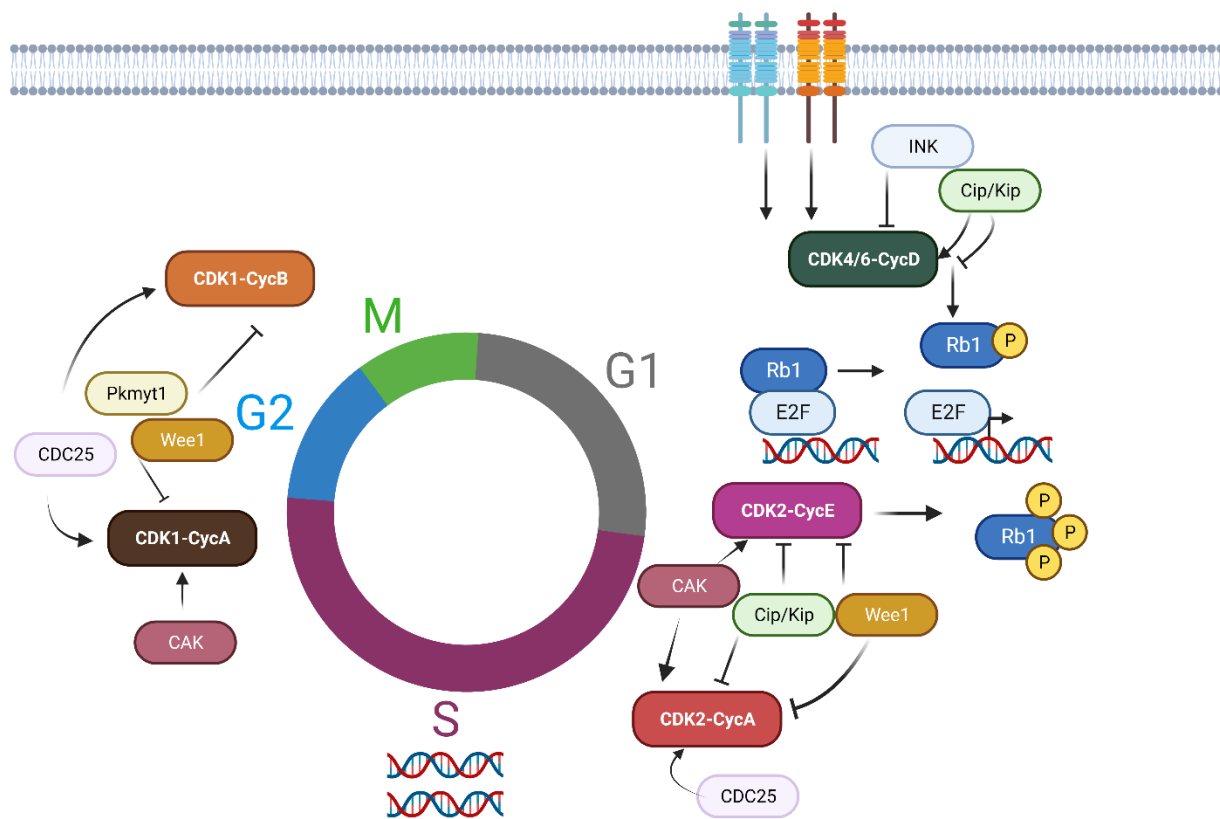


Figure 1.1: The mammalian cell cycle is tightly regulated. The mammalian cell cycle CDKs (CDK1/2/4/6) are regulated by cyclin binding, endogenous activator and inhibitors, and by phosphorylation. Image generated with BioRender.

A-, B-, D-, E-type cyclin function

Structure-function of cyclin-CDK binding

Cyclins are the major regulatory partners for CDKs and are named as such for their oscillation over the cell cycle (T. Evans et al., 1983). The cyclins that associate with the interphase and mitotic CDKs are A-, B-, D-, and E-type cyclins, each with specialized roles (Martínez-Alonso & Malumbres, 2020). The structural changes induced by Cyclin-CDK binding vary depending on the complex: in monomeric form, CDK2 structure has a N-terminal lobe rich in β -sheets, and a C-terminal lobe rich in α -helices that also houses the T-loop, a site of activation through post-translation modification. At the junction of these two lobes is an ATP-binding site (Jeffrey et al., 2000; Pavletich, 1999). Cyclins bind at the C-terminal end through hydrophobic interactions, and this binding event induces a conformational change in the CDK, exposing the catalytic cleft and T-loop concomitantly, resulting in active complex (Pavletich, 1999) (Figure 1.2). CDK4, on the other hand, appears to have additional layers of activation as T-loop phosphorylated CDK4-Cyclin D remains in an inactive conformation, and only becomes activated upon substrate recognition (Day et al., 2009; Takaki et al., 2009). Despite subtle differences in structure-function between CDK-cyclin pairs, cyclin binding is a baseline requirement for CDK activity and as such cyclins are the major regulators of CDKs (Malumbres et al., 2009; Martínez-Alonso & Malumbres, 2020).

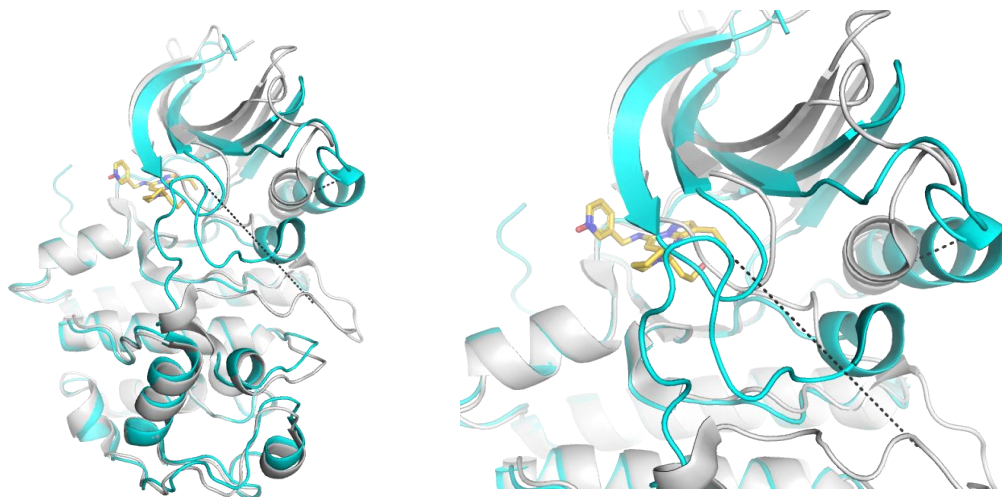


Figure 1.2: Structure of CDK2 apo versus complexed with Cyclin E. C-helix shifts ~6Å towards ATP site, and activation loop shifts ~20Å to interact with the P-loop in presence of Cyclin E1. Apo CDK2 is shown in blue. CDK2.in complex with Cyclin E1 shown in gray; Cyclin E1 hidden for clarity. Dinaciclib shown in gold. Right image is a zoomed in version of image on the left. Figures and measurements made using PyMol: The PyMOL molecular graphics system, Version 2.0 Schrödinger, LLC. Figure made by Deb Conrady.

D-type cyclins

The D-type cyclins were first discovered through their ability to rescue the cell cycle in cyclin-deficient yeast (Xiong et al., 1991) and were identified as mitogen sensors in human cells (Matsushime et al., 1991). In this way, the D-type cyclins act to integrate extracellular information with cell cycle activation. Cyclin D1, D2, and D3, encoded by the *CCND1*, *CCND2*, and *CCND3* genes respectively, become stabilized by major receptor tyrosine kinase (RTK) signaling, and bind to CDK4/6 to promote progression through the G1 phase of the cell cycle (Matsushime et al., 1992, 1994). The different isoforms of Cyclin D are tissue specific as revealed by knockout mouse studies (Table 1.1) (Y. J. Choi et al., 2012; Kozar et al., 2004). The major role of CDK4/6-cyclin D complexes are to phosphorylate the retinoblastoma protein 1 (RB1) to allow the transcription factor E2F to promote expression of target genes, many of which are involved in the cell cycle itself (Harbour, JW, Luo, RX, Dei Santi, A, Postigo, AA, and

Dean, 1999). The current understood mechanism of these phosphorylation events is to prime Rb1 for further phosphorylation by CDK2-Cyclin E (Rubin et al., 2020). When cells reach S-phase, Cyclin D1 is degraded by the proteasome, a process governed mainly by phosphorylation via GSK3 β and translocation from the nucleus to the cytoplasm (Diehl et al., 1998).

E-type cyclins

CCNE1 and *CCNE2*, the genes encoding the E-type cyclins are E2F target genes and are repressed by Rb1 until Rb1 is phosphorylated by CDK4/6-Cyclin D in G1 (Rubin et al., 2020). Cyclin E binds to CDK2 to transition the cells into S-phase (Koff et al., 1992) through complete phosphorylation of Rb1 along with DNA replication factors such as Cdc6 to enable Mcm2-7 mediated replication origin licensing (Coverley et al., 2002). Interestingly, Cyclin E interacts with the centrosome to promote centrosomal duplication in both kinase dependent and independent manners. CDK2-Cyclin E phosphorylates nucleophosmin on T199 to promote its dissociation from the centrosome and thus allow for duplication, while other studies demonstrated that Cyclin E1 mutants in which CDK2 binding was abolished but the centrosomal localization signal was retained were still able to promote entry into S-phase (Chan & Lim, 2015; Matsumoto & Maller, 2004). Non-catalytic associated functions of E-type cyclins may also have a role in oncogenesis and is an area of intense research (See CDK2 and Cyclin E1 in cancer).

Cyclin E1 and E2 have compensatory roles as E1^{-/-} and E2^{-/-} mice are viable with no obvious phenotype (Geng et al., 2003). Deletion of both E1 and E2, on the other hand, is embryonic lethal, with mice showing defects in cell types that require

endoreplication, such as trophoblasts and megakaryocytes (Geng et al., 2003).

Surprisingly, CDK2 knockout mice do not have overlapping phenotypes with Cyclin E knockout mice: while CDK2^{-/-} mice are defective in gonadal development (Berthet et al., 2003), stemming largely from incomplete meiosis (Chauhan et al., 2016; Palmer et al., 2020), Cyclin E knockout mice do not share this phenotype. Given that successful meiosis depends on recombination (Guillon et al., 2005; Szostak et al., 1983), these data suggest a non-redundant role for CDK2 in specific homologous recombination (HR) pathways in certain cell types that may be independent of E-type cyclins. Later research suggested that CDK2 binds with Speedy A, a Ringo protein that serves as an atypical CDK activator and whose loss leads to male and female infertility, phenocopying the CDK2^{-/-} mice (Mikolcevic et al., 2016; Tu et al., 2017). CDK2 regulation, then, may be cell type or tissue specific.

Cyclin E is degraded by two proteasome-dependent pathways depending on whether it is a monomer or in complex with CDK2. Cul3, an E3 ubiquitin ligase, rapidly targets monomeric Cyclin E for proteasomal degradation through recognition of a degron on the N-terminus, suggesting that Cyclin E is a labile and unstable protein (Davidge et al., 2019; Singer et al., 1999). When in complex with CDK2, Cyclin E is protected from Cul3-mediated ubiquitination, but this protection is reversed when CDK2 itself phosphorylates Cyclin E at specific residues (T62, T380), allowing Cyclin E recognition by second E3 ligase, Fbxw7 (Clurman et al., 1996; Koepp et al., 2001). CDK2 is therefore at least in part responsible for the degradation of Cyclin E.

A-type cyclins

The A-type cyclins, Cyclin A1 (*CCNA1*) and Cyclin A2 (*CCNA2*) were identified as crucial for the transition from S to G2 phase and from G2 to M phase, binding both CDK2 and CDK1 (Pagano et al., 1992). At the onset of S-phase, Cyclin A, an E2F target gene, levels increase as Rb1 is fully phosphorylated by CDK2-Cyclin E (Rubin et al., 2020). As Cyclin E levels decrease, Cyclin A levels increase, keeping CDK2 activity high, albeit with different target substrates. Unlike Cyclin E which promotes genome duplication, Cyclin A serves as guardian of the genome through interaction with Cdc6, Orc1, MCM5 to antagonize centrosomal duplication and DNA re-replication (Ferguson et al., 2010; X. Xu et al., 2017). Cyclin A2 deletion results in embryonic lethality, while Cyclin A1 knockout mice are viable, but males have defects in spermatogenesis (Liu et al., 1998; Murphy et al., 1997). In a negative feedback cycle at the end of S-phase, CDK2-Cyclin A phosphorylates E2F to halt its transcription (Schulze et al., 1995; M. Xu et al., 1994). As cells transition to G2 phase, Cyclin A begins to associate with CDK1 and the major role of this complex appears to be promoting CDK1-Cyclin B activity through the Bora-PLK1-CDC25 axis (Vigneron et al., 2018). After mitotic commitment, Cyclin A is degraded by the anaphase promoting complex (APC) (Elzen & Pines, 2001).

B-type cyclins

Cyclin B is regulated via multiple transcription factors and protein levels peak as cells transition from G2 to M (Hwang et al., 1995). Cyclin B levels accumulate in the cytoplasm and translocate to the nucleus only at the onset of mitotic entry (Pines & Hunter, 1991) in a PLK1 dependent manner (Jackman et al., 2003). Cyclin B activates CDK1 and acts as the mitotic kinase complex, phosphorylating substrates involved in mitosis, spindle assembly, and actin polarization (Dittrich & Dandekar, 2004). Cyclin B1,

but not Cyclin B2, deletion in mice is embryonic lethal after G2 arrest in early development (Brandeis et al., 1998). Of note, CDK1 deletion is also embryonic lethal, with embryos not progressing past the blastocyst stage (Santamaría et al., 2007). After successful completion of metaphase, Cyclin B is ubiquitinated by APC/C-Cdc20 to promote proteasomal degradation, allowing cells to initiate anaphase and complete mitosis (Peters, 2006). After successful division, a cell will only initiate another cycle if proper mitogenic signaling is present, thus activating Cyclin D1 and beginning the cycle again.

Gene	Phenotype	Reference(s)
CDK4	Insulin-insufficient diabetes from defects in pancreatic β -islet cells; infertile	(Rane et al., 1999)
CDK6	Impaired hematopoiesis; decreased erythrocytes, neutrophils, macrophages, granulocytes	(Malumbres et al., 2004)
CDK4/CDK6	Embryonic lethal (severe anemia)	(Malumbres et al., 2004)
CDK2	Gonadal defects, infertility	(Berthet et al., 2003)
CDK2/CDK4	Embryonic lethal in one report Mice survive but die shortly after birth from heart failure; CDK2 ^{-/-} conditional in a CDK4-null background survive	(Barrière et al., 2007; Berthet et al., 2006)
CDK2/CDK4/CDK6	Embryonic lethal	(Santamaría et al., 2007)
CDK1	Embryonic lethal	(Santamaría et al., 2007)

CCNA2	Embryonic lethal	(Murphy et al., 1997)
CCNA1	Male infertility	(Liu et al., 1998)
CCND1	Defect in mammary gland development, retinopathy, small in size	(Fantl et al., 1995; Sicinski et al., 1995)
CCND2	Hypoplastic testes, ovaries; granules and stellate interneurons decreased	(Huard et al., 1999; Sicinski et al., 1996)
CCND3	Thymocyte development defect	(Sicinska et al., 2003)
CCND1/CCND2	Small, neurological impairments from abnormal cerebella, and death within first 3 weeks	(Ciemerych et al., 2002)
CCND1/CCND3	Rapid death after birth thought to be from neurological impairment, asphyxiation, inability to suckle	(Ciemerych et al., 2002)
CCND2/CCND3	Embryonic lethality from profound anemia	(Ciemerych et al., 2002)
CCND1/CCND2/CCND2	Embryonic lethal	(Kozar et al., 2004)
CCNE1	Viable	(Geng et al., 2003; Parisi et al., 2003)
CCNE2	Viable	(Geng et al., 2003; Parisi et al., 2003)
CCNE1/CCNE2	Embryonic lethal, defects in trophoblast, megakaryocyte development	(Geng et al., 2003; Parisi et al., 2003)

Table 1.1: Phenotypes of CDK, cyclin homozygous knockout mice

Endogenous CDK inhibitors

While cyclins act as major activators of CDKs, endogenous inhibitors are also present in the cell to restrain CDK activity, and this balancing act allows for controlled cell cycle progression. The major inhibitory proteins are the INK4 family and the Cip/Kip family.

The INK4 family consists of 4 members: p15^{INK4b} (*CDKN2B*), p16^{INK4a} (*CDKN2A*), p18^{INK4c} (*CDKN2C*), and p19^{INK4d} (*CDKN2D*) (Parry et al., 1999). These polypeptides bind primarily to the CDK4 or CDK6 monomers and compete with Cyclin D binding, but do not bind CDK1 or CDK2 (Parry et al., 1995). While the Cyclin D half-life is roughly 30 minutes, the half-life of p16 is much longer, suggesting that, in cellular contexts where p16 is highly expressed, p16-CDK4 will be the dominant species. Indeed, this is the case in Rb-deficient cells with high p16 protein levels (Parry et al., 1995), which subsequently lose dependence on Cyclin D (Lukas et al., 1994). Structural analysis revealed that INK4 association with CDK4 or CDK6 misaligns critical residues for cyclin-CDK binding and distorts the ATP binding site, thus providing an explanation for the low kinase activity in INK4-expressing cells (Jeffrey et al., 2000). More recently, however, it was shown that p18^{INK4c} can interact with CDK6 in such a way to retain catalytic activity, but occlude the binding of ATP-competitive inhibitors, such as Palbociclib (Q. Li et al., 2022). These more recent insights suggest that INK-CDK association may induce graded levels of kinase activity in different cellular conditions.

The Cip/Kip protein family has three major members: p21^{Cip1} (*CDKN1A*), p27^{Kip1} (*CDKN1B*), and p57^{Kip2} (*CDKN1C*). This class of inhibitors can bind all interphase CDKs and can associate with the CDK-cyclin heterodimer (Pavletich, 1999). Interestingly, at

low levels, p21/p27/p57 can promote CDK-cyclin assembly, but at high concentrations will inhibit kinase activity (Labaer et al., 1997). In their inhibitory role, these proteins function by inserting a helix into the catalytic cleft, blocking ATP hydrolysis (Pavletich, 1999). On the other hand, loss of p21 and p27 in murine fibroblasts prevents CDK4-Cyclin D1 complex assembly (Cheng et al., 1999). A recent study by Guiley et al offered a structural basis for this dual role with p27: when phosphorylated at Y74, p27 can open the ATP-binding site on CDK4, promoting its activity, while the unphosphorylated form acts as an inhibitor (Guiley et al., 2019). Similarly, the importance of the stoichiometric balance of p21 with the different CDK-cyclin pairs was studied recently in the context of pharmacological CDK4/6 inhibitors, such as palbociclib, ribociclib and abemaciclib. Pack et al demonstrated that, upon CDK4/6 inhibitor binding, p21 immediately dissociates from CDK4/6 and binds to CDK2, inhibiting the cell cycle, suggesting that redistribution of major regulators contributes to phenotypes observed with CDK catalytic inhibitors (Pack et al., 2021). The delicate balance of endogenous CDK inhibitors is an important layer of CDK control.

CDK regulation through phosphorylation

In addition to activation via cyclin binding, and regulation through the INK4/Cip/Kip proteins, CDKs are also regulated through activating and inhibitory phosphorylation.

As previously mentioned, full CDK activation requires phosphorylation of the T-loop, a segment regulated by a threonine residue that is positioned across the catalytic site. When phosphorylated, the T-loop shifts, allowing the active site to be fully available (Morgan, 1995). Phosphorylation of the T-loop – T161 in CDK1 and T160 in CDK2 - in

human cells is regulated by the CDK activating kinase (CAK) CDK7 (Laroche et al., 2007). Mutation of this threonine residue to alanine completely abolishes CDK activity, highlighting that this layer of regulation is critical for cell cycle progression (Gu et al., 1992). Notably, CDK1 and CDK2 appear to have different requirements to reach a fully active state. While CDK2 can form a complex with cyclin in the absence of T-loop phosphorylation, CDK1 requires the phospho-threonine in order to bind cyclin (Laroche et al., 2007). These subtle differences in kinase activation may explain why CDK2 cannot substitute for CDK1 in CDK1 knockout animals, even when expressed from the CDK1 endogenous locus (Satyanarayana et al., 2008).

Inhibitory phosphorylation sites on the N-terminus of the CDKs (T14, Y15 for CDK1, CDK2) serve to suppress CDK activity. CDK2 is modulated at these inhibitory sites mainly by Wee1 kinase, while CDK1 is regulated by both Wee1 and Pkmyt1 (Boher et al., 1997; Gu et al., 1992). Wee1 and Pkmyt1 serve to constrain CDK activity until G2 phase, and these kinases are in turn also highly regulated by other kinases, such as Plk1, Ck2, and CDK1 in a feedback loop (van Vugt et al., 2004; Watanabe et al., 2004). The inhibitory phosphates are removed by the CDC25 phosphatase family, of which there are three isoforms: A, B, C. CDC25A was ascribed to S-phase regulation while CDC25B and CDC25C were traditionally thought to control mitotic entry, but more recent work has demonstrated that there is redundancy among the family members (Boutros et al., 2006). These phosphatases are regulated by sub-cellular localization, mainly through 14-3-3 binding and sequestration (Conklin et al., 1995). The balance between the Wee1 and Pkmyt1 kinases and the CDC25 phosphatases is essential for cell cycle control.

CDK2 and Cyclin E1 in tumorigenesis

Cell cycle dysregulation is a hallmark of cancer as alterations in this pathway decouple extracellular environmental clues and cell cycle progression. For example, mutations in Rb1 disable the restriction point, pushing cells into S-phase prematurely, thus increasing genomic instability (Hanahan, 2022). To safeguard against disruptions in the core cell cycle machinery, mammalian cells have evolved redundancy between CDKs and cyclins. However, during oncogenesis, some tumors evolve dependencies on specific CDK-cyclin pairs, driven by both the lineage and tumor genetic background.

Requirement of CDKs during development

While yeast have a single CDK to drive the cell cycle, mammalian cells have several CDKs to push cells through a round of division (Bloom & Cross, 2007). In a normal state, these CDKs, along with their activating cyclin partners, act in a sequential and tightly controlled manner to integrate extracellular signaling with the manufacturing of internal cellular resources, such as transcription of cell cycle related genes and nucleotide production, to ensure accurate DNA replication and proper division (see “A-, B-, D-, E-type cyclin function”) (Malumbres et al., 2009; Malumbres & Barbacid, 2005; Martínez-Alonso & Malumbres, 2020). Despite the CDKs having preferred roles during the cell cycle, they can often compensate for each other when another is lost, which is most evident during development.

Loss of individual interphase CDKs does not lead to embryonic lethality, but reveals tissue-specific requirements for each CDK. CDK4 deletion induces defects in β -islet cells in the pancreas, causing insulin-deficient diabetes (Rane et al., 1999), while

mice expressing an activating mutation in CDK4, R24C, which disrupts binding to INK4 proteins but not to Cyclin D, display pancreatic hyperplasia (Rane et al., 1999). The phenotypes of these mice suggest that CDK4 has a role in pancreatic development but is dispensable in other tissue types. Similarly, CDK6 KO mice are viable, but have defects in the heme compartment, with decreased erythrocytes, neutrophils, granulocytes, and macrophages, suggesting a role in blood cell maturation, but not other tissues (Malumbres et al., 2004). CDK2 knockout mice are viable but are infertile due to gonadal development defects, highlighting the requirement of CDK2 only in oogenesis and spermatogenesis (Berthet et al., 2003). Contrary to the interphase CDK KO phenotypes, mice lacking CDK1 fail to develop soon after the zygote stage (Santamaría et al., 2007). Indeed, even replacement of CDK1 with CDK2 at the CDK1 locus fails to rescue the CDK1 KO phenotype (Satyanarayana et al., 2008), highlighting important differences between the two related CDKs. CDK1, then, is the only essential and non-redundant CDK in the normal cell cycle.

While mammals can typically compensate for the loss of one CDK, they can also compensate for the loss of multiple. Mice lacking both CDK4 and CDK6 die during late-stage embryogenesis from severe anemia, but mouse embryonic fibroblasts (MEF) lacking both genes proliferate normally and respond to mitogen stimulation (Malumbres et al., 2004). On the other hand, studies on CDK2 and CDK4 double KO differ in findings. In one mouse study, CDK2^{-/-} CDK4^{-/-} was embryonic lethal as a result of heart defects (Berthet et al., 2006), while in another CDK2^{-/-} CDK4^{-/-} mice survived but died soon after birth from heart failure (Barrière et al., 2007). In the latter study, double KO mice had thin ventricular walls, low proliferating cardiomyocytes, and disorganized

capillaries (Barrière et al., 2007). The authors speculate that these mice are unable to tolerate hemodynamic changes that occur at birth. Although CDK2^{-/-} and CDK4^{-/-} mice died soon after birth, conditional CDK2 KO mice in a CDK4-null background showed no obvious phenotypes in adult mice, suggesting that, after development, cells can tolerate loss of both CDKs (Barrière et al., 2007). Perhaps most strikingly, Santamaria et al demonstrated that CDK1 can compensate for the loss of all three interphase CDKs until mid-gestation through non-canonical binding to D- and E- type cyclins to maintain Rb1 phosphorylation (Santamaría et al., 2007). These important developmental studies highlighted that, although individual CDKs have critical roles in specific tissue types, the loss of any one interphase CDK can often be compensated for by other family members.

CDK2 and Cyclin E1 in cancer

Despite compensatory activity of the interphase CDKs, certain cancers become dependent on one CDK. The genetic features of these tumors create an environment of unregulated cell cycle activity that is decoupled from mitogen sensing, driving transformation (Hanahan, 2022). Different oncogenic drivers influence CDK dependency.

The most well-known example of CDK dependency is the reliance on CDK4 in hormone-receptor positive (HR+), human epidermal growth factor receptor negative (HER2-) breast cancer. The estrogen receptor (ER), in its role as a transcription factor, drives expression of *CCND1* which contributes to the reliance on CDK4 in these tumor types (Cicatiello et al., 2004). Inhibition of CDK4/6 with small molecule inhibitors such as Palbociclib halt cell cycle proliferation in this specific tumor type, and the Food and

Drug Administration (FDA) approved Palbociclib for the treatment of HR+/HER2- breast cancer in 2015 (Fry et al., 2004). In this setting, CDK2 may be unable to immediately compensate for the loss of CDK4, presumably because enough *CCNE1* transcription has not occurred to fully activate CDK2 at the time of inhibition. However, studies after long-term treatment with CDK4/6 inhibitors demonstrate that CDK2 may be able to non-canonically bind to D-type cyclins to overcome CDK4/6 inhibition (Herrera-Abreu et al., 2016). It was also reported that patients that have high levels of *CCNE1* mRNA progress faster on Palbociclib than patients with lower levels of *CCNE1*, suggesting again that CDK2 may be able to compensate for the loss of CDK4/6 activity after some time (Turner et al., 2019). These studies imply that initial dependence on one CDK may be overcome by activation of another CDK following treatment with targeted therapy.

Like its role in development, CDK2 was traditionally viewed as dispensable in cancer. CDK2 knockdown or expression of the dominant negative (DN) CDK2 D145N in osteosarcoma, colon, and glioblastoma cell lines did not impact proliferation (Tetsu & McCormick, 2003). The authors of this study concluded that CDK4 was likely able to compensate for the loss of CDK2 as this kinase remained active and was able to phosphorylate Rb even at CDK2 preferred sites (Tetsu & McCormick, 2003; Zarkowska & Mitnacht, 1997). This pivotal study, along with the minimal phenotypes observed in CDK2 KO mice (Berthet et al., 2003), labeled CDK2 as a poor therapeutic target in cancer therapy.

The role of CDK2 in cancer emerged starting with the large-scale small-hairpin RNA (shRNA) studies completed by the Broad Institute and Novartis. These studies revealed that *CCNE1*-amplified cancers have an exquisite sensitivity to CDK2 depletion

(Cowley et al., 2014; McDonald et al., 2017). This relationship is most significant in ovarian cancer (Figure 1.3), where *CCNE1* amplification occurs in 20% of patients (Ciriello et al., 2012). As the previous study by Tetsu and McCormick tested CDK2 dependence in *CCNE1*-non-amplified cell lines only, this relationship was not identified. *In vitro* and *in vivo* ovarian cancer models that harbor *CCNE1*-amplification were later verified to rely on CDK2 for proliferation (Au-Yeung et al., 2017). Similar to CDK4-dependence in HR+/HER2- breast cancer, other CDKs cannot immediately compensate for the loss of CDK2 in *CCNE1*-amplified cancers.

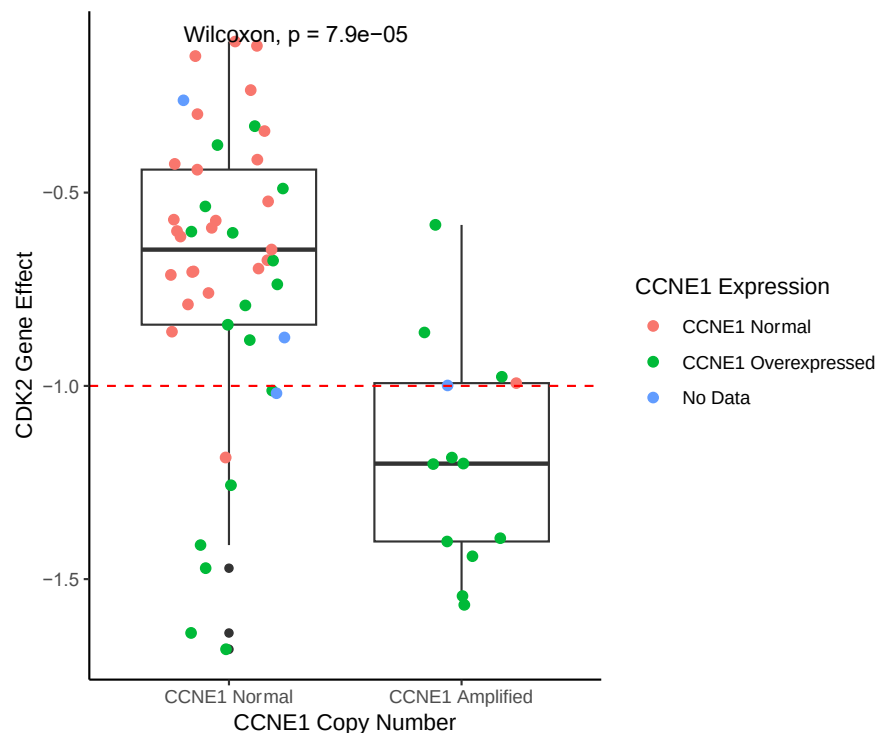


Figure 1.3: CCNE1-amplification is predictive of CDK2 dependency in ovarian cell lines. CCNE1-normal cell lines (copy number < 3) and CCNE1-amplified cell lines (copy number ≥ 3) were assessed for CDK2 dependency (“gene effect”). The red dotted line represents the threshold for dependency. Wilcoxon rank-sum test, $p = 7.9 \times 10^{-5}$. Data source = DepMap 1Q23; Figure created by Maxine Chen.

CCNE1 was identified as an oncogene as it was reported to transform rat and human fibroblast cells when overexpressed (Ohstubo & Roberts, 1993). These cells

were able to proliferate in the absence of mitogen stimulation, suggesting that high levels of Cyclin E1 can override the mitogen sensing requirement of Cyclin D to begin the cell cycle. The authors of this study also noted that cells with *CCNE1* overexpression had shortened G1-phase and prolonged S-phase compared to empty vector controls (Ohstubo & Roberts, 1993). Later studies in *CCNE1*-amplified breast cancer cell lines revealed that, rather than oscillating over the course of the cell cycle, Cyclin E1 protein levels remained relatively constant, and in active kinase complex, during the entire cell cycle (Gray-Bablin et al., 1996). These reports highlight that an abundance of Cyclin E1 can transform the cell and hijack the cell cycle into an altered state.

CCNE1-amplification is an early oncogenic event in high-grade serous ovarian cancer (HGSOC), and studies in the cell line of origin for HGSOC, fallopian tube secretory epithelial cells (FT-SECs), showed that *CCNE1*-amplification occurs following p53 gain-of-function (GOF) mutations (Karst et al., 2014). Importantly, p53 regulates p21 levels, a negative regulator of CDK2 (El-Deiry et al., 1993; C. Evans et al., 1996) (see “Endogenous CDK inhibitors”). *CCNE1*-amplification is mutually exclusive with homologous recombination deficiency (HRD) and is associated with poor prognosis as patients are typically resistant to standard of care (SOC) therapy such as platinum agents (Ciriello et al., 2012; Etemadmoghadam et al., 2010, 2013; P. Smith et al., 2023). Notably, *CCNE1* copy number alterations, once established, remain stable in the genome following clinical therapeutic intervention as recently reported in the BriTROC-1 study (P. Smith et al., 2023). *CCNE1*-amplification is likely then a driver event in HGSOC and influences response to clinical agents.

As high-fidelity replication relies on proper cell cycle progression and reliance on the restriction point, alterations in the Rb-E2F pathway, such as *CCNE1* amplification, can cause DNA damage and subsequent genomic instability (Halazonetis et al., 2008). *CCNE1* overexpressing fibroblast cells have decreased replication fork speed, increased intra-origin distance, and high levels of the DNA-damage marker γ H2AX which could all be rescued with an exogenous supply of nucleotides (Bester et al., 2013). In *CCNE1* overexpressing U2OS cells, origins that should remain dormant during G1 instead fire after the premature entry into S-phase, leading to replication-transcription collision, replication fork collapse, and genomic rearrangement (Ge et al., 2007; Jones et al., 2013; Macheret & Halazonetis, 2018). These increased levels of DNA breaks after *CCNE1* overexpression reveal a unique landscape of fragile sites, areas in the genome that are late-replicating and prone to breakage, that are distinct from aphidicolin-induced or *myc*-induced fragile sites (Miron et al., 2015). Indeed, a study in human mammary epithelial cells with exogenous *CCNE1* overexpression revealed breakage points in fragile sites that induced chromosomal arrangements that are frequently found in breast cancer tumors (Teixeira et al., 2015). These data suggest that cells entering S-phase prematurely do not have adequate resources for DNA replication, resulting in perturbations such as replication fork collapse that result in DNA damage.

As aberrant Cyclin E1 expression can drive chromosomal instability through the mechanisms described above, cells must have secondary alterations to tolerate the replication stress imposed by this oncogene. Primary cells engineered to overexpress *CCNE1* increased WT p53 phosphorylation at serine 15 which was reversible in the

presence of Ataxia-telangiectasia mutated (ATM) inhibitors wortmannin and caffeine, suggesting a feedback mechanism to suppress cell cycle progression through the ATM pathway (Minella et al., 2002). The same study showed that when p53 or p21 were lost, cells were able to proliferate in the presence of high Cyclin E1 levels but did experience chromosomal instability and aneuploidy. Similarly, U2OS cells with exogenous *CCNE1* overexpression senesce 4-6 days following expression induction, again likely due to WT p53 and therefore intact p21. The senescence phenotype was rescued by inhibiting ATM, further strengthening the feedback loop between *CCNE1*-induced replication stress and DNA repair pathways (Bartkova et al., 2006). In the aforementioned FT-SEC model, only cells expressing GOF p53 R175H were able to tolerate *CCNE1* overexpression as cells with WT p53 underwent senescence after 2 passages (Karst et al., 2014). Karst *et al* reported that FT-SEC *CCNE1* overexpressing cells upregulate proteins involved in DSB repair as well as replication fork stability such as BRCA1, XRCC2, FANCD2, and BLM. Together these studies suggest that cells must disable the G1/S DNA damage checkpoint through p53 alterations to evade senescence and increase expression of genes involved in DSB repair/replication fork protection to tolerate *CCNE1* imposed replication stress.

Although cancer cells tolerate *CCNE1* induced senescence through additional genomic alterations, they may become reliant on these mechanisms and thus vulnerable to therapies that target them. In a human retinal pigment epithelial (RPE) cell isogenic model, *CCNE1* overexpression induced chromosomal instability as measured by replication fork speed decrease, appearance of anaphase bridges during mitosis, and copy number alteration increase. These cells became sensitive to Wee1 and ATR

inhibitors (Kok et al., 2020). A recent study in both cell lines and patient derived xenograft (PDX) models of *CCNE1* amplified ovarian and endometrial cancers suggested that the combination of Wee1 and ATR inhibitors might be a tractable strategy in this patient population (H. Xu et al., 2021). The efficacy of this combination may derive from the inability of a cell to stabilize the replication fork through ATR inhibition followed by premature mitotic entry through Wee1 inhibition. A second mechanism may stem from Wee1 restraining CDK2 activity to protect the replication fork as a recent report demonstrated that Wee1 inhibition promotes fork degradation via CDK2 mediated phosphorylation of the nuclease DNA2 (Elbæk et al., 2022). Another study showed that *CCNE1*-amplified ovarian cancer cell lines have basally higher levels of phosphorylated Chk1, a marker of replication stress, and were sensitive to a combination of PARP and ATR inhibition (H. Kim et al., 2020). Additionally, Pkmyt1 was identified as a potential drug target in a CRISPR screen in the RPE *CCNE1* overexpression isogenic model and was further validated in *CCNE1*-amplified cancer models through treatment with a catalytic inhibitor, RP-6306, that selectively targets this kinase (Gallo et al., 2022). The mechanism of action is through selective activation of CDK1 in a genomically unstable background (high levels of Cyclin E1), that pushes the cells through mitosis before DNA repair can occur. Lastly, an siRNA screen revealed that BRCA1 and BRCA2 depletion inhibited clonogenic growth in *CCNE1* amplified cells, suggesting that these cells are particularly reliant on HR (Etemadmoghadam et al., 2013). Notably, however, this relationship was not supported by the large-scale shRNA drop out screens (Cowley et al., 2014; McDonald et al., 2017), suggesting that synthetic lethality may only be revealed under particular conditions (e.g. clonogenic

survival versus logarithmic growth). Taken together, these results suggest that aberrant expression of *CCNE1* confers a reliance on proteins that interface between DNA repair and cell cycle progression.

Gaps in knowledge

While recent studies have suggested that *CCNE1*-amplified cancer cells are dependent on CDK2 for proliferation, it is not completely clear why the other CDK family members cannot compensate for the loss or inhibition of CDK2 in this specific genetic context. Although increased levels of Cyclin E1 protein enhance CDK2 activity, *CCNE1* overexpression alone does not account for dependency on CDK2 as roughly half of *CCNE1* overexpressed cell lines are not dependent on CDK2 (Figure 1.3). These data suggest that the presence of other, secondary biomarkers is required to reveal this dependence. Because nearly all *CCNE1*-amplified ovarian cell lines require CDK2 to proliferate, it is likely that *CCNE1*-amplification co-occurs with these putative, secondary oncogenic events and together drive dependence on CDK2.

As CDKs are regulated through a variety of mechanisms as detailed above, it is possible that alterations in or levels of endogenous regulators contribute to the dependency of *CCNE1*-amplified cell lines on CDK2. For example, expression of p16^{INK4a} has been shown to suppress CDK4/6 activity (Malumbres et al., 2004), and patient data reveals that *CDKN2A* (p16) loss is mutually exclusive with *CCNE1*-amplification (Figure 1.4). This mutual exclusivity may explain why CDK4/6 cannot compensate for the loss of CDK2 in the context of *CCNE1*-amplification.

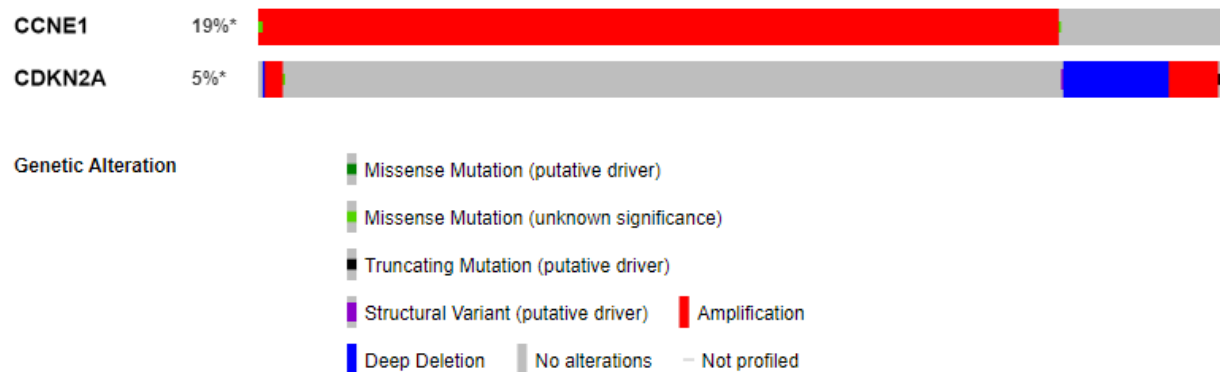


Figure 1.4: CCNE1 amplification is mutually exclusive with CDKN2A deletion in high grade serous ovarian cancer. Patient data from The Cancer Genome Atlas and Memorial Sloan Kettering Cancer Center. Each bar represents a patient. Log2Odds ratio: -1.016; p-value: 0.276; tendency: mutual exclusivity. Data generated on March 11, 2023 from cBioPortal.

CDK1 is sufficient to drive the mammalian cell cycle even in the absence of all interphase CDKs and can non-canonically pair with Cyclin E1 in CDK2 null mice (Aleem et al., 2005; Santamaría et al., 2007). Why, then, is CDK1 unable to compensate for CDK2 after genetic suppression in *CCNE1*-amplified cells? As recently reported by Gallo et al, *CCNE1*-amplified cell lines may depend on restraining CDK1 activity to prevent premature entry into mitosis (Gallo et al., 2022). As *CCNE1*-overexpression induces genomic instability and DNA damage via premature S-phase entry, the cell needs time to repair before entering mitosis (Bester et al., 2013; Halazonetis et al., 2008; Macheret & Halazonetis, 2018). The inhibitory kinases Pkmyt1 and Wee1 are responsible for maintaining inactive CDK1, and depletion of either kinase in *CCNE1*-amplified cells is lethal (Booher et al., 1997; Gallo et al., 2022; Heald et al., 1993; Mueller et al., 1995). It is thus possible that CDK1 is maintained in a generally inactive state to maintain genome integrity and cannot, at least acutely, compensate for CDK2 loss.

CDK and cyclin function in normal cells is well studied but remains an area of intense research. Recently, two studies suggest that even the basic tenants of cell cycle biology may need to be revisited. While CDK4 and CDK6 were previously thought to function only in G1-phase to initiate the cell cycle, new data points to roles also in S-phase (Arora et al., 2023; Cornwell et al., 2023). Arora et al (2023) demonstrated that CDK4/6 can compensate for CDK2 inhibition during S-phase by maintaining the phosphorylation of Rb, promoting transcription of Cyclin A2. Cornwell et al (2023) have challenged the restriction point model, showing that cells can exit the cell cycle at any time before mitosis if mitogen levels decrease. These studies were made possible by the development of CDK-specific sensors, single-cell imaging technologies, and well characterized CDK inhibitors (Freeman-cook et al., 2021; Spencer et al., 2013).

As newer technologies and chemical biology tools are developed, additional functions of CDKs, as well as the cellular “rules” that govern CDK activity, will be further elucidated. It is apparent that these same “rules” in normal cells are frequently broken or altered in cancer cells, highlighting the need for more research into specific disease indications. This information will be informative for our basic understanding of cellular proliferation and will drive the field toward developing novel therapies using specific biomarkers.

CDKs regulate homologous recombination

Double-stranded breaks (DSBs) are a serious type of lesion as open-ended DNA promotes gross chromosomal rearrangement (GCR) and thus are a threat to genomic integrity. Mammalian cells experience 10^5 DSBs per day and have thus evolved

mechanisms to repair these lesions (Collins, 1999). When cells experience double-stranded breaks (DSBs), whether through errors in replication or from exogenous forms of damage, two major pathways are responsible for repair – error-prone non-homologous end joining (NHEJ) and the more accurate homologous recombination (HR), the latter of which plays an essential role in replication-coupled repair. If DNA damage is persistent, a cell will either undergo apoptosis or senescence, unless they are able to proliferate with constant damage, sometimes leading to a cancerous state (Bartkova et al., 2006). These repair pathways are inherently linked to cell cycle stage, and research over the last decade has implicated CDK1 and CDK2 activity as essential for high-fidelity repair.

Repair pathway choice

Several factors contribute to the choice of repair pathway after a cell experiences a DSB, including the DNA state around the break and cell cycle stage.

First, the type of lesion around the break influences which repair factors are recruited to the site of DSBs. In a two-ended DSB with blunt ends, Ku70-Ku80 are typically recruited to the DNA to initiate NHEJ (Britton et al., 2013), and, if not displaced, Ku70-80 recruit other factors such as DNA-dependent protein kinase catalytic subunit (DNA-PKc), and scaffolding proteins like XRCC4 and XLF (Ahnesorg et al., 2006). These proteins serve to align the two broken DNA ends and bring them into close proximity. Nucleases such as Artemis perform short-range DNA processing to ensure compatibility between broken ends, and finally DNA ligase IV (LIG4) ligates the two ends (Nick McElhinny et al., 2000). NHEJ is a fast process and can function without a

sister chromatid or other homolog present. However, small insertions or deletions tend to occur during this process, and as such NHEJ is considered error prone.

Other factors work to promote NHEJ, such as p53 binding protein 1 (53BP1) and the Shieldin complex. 53BP1 is recruited to double stranded breaks to block DNA resection at the site of damage (Wong et al., 2010; Xie et al., 2007). RIF1 is also recruited to DSBs via N-terminal phosphorylation of 53BP1, and together RIF1 and 53BP1 cooperate to suppress HR in favor of NHEJ (Steger et al., 2013). The more recently discovered Shieldin complex acts in a similar manner to suppress HR by antagonizing BRCA2/RAD51 loading, and REV7, a member of Shieldin, cooperates directly with 53BP1 to promote NHEJ (Dev et al., 2018; Ghezraoui et al., 2018; Gupta et al., 2018). Interestingly, loss of 53BP1 restores HR in BRCA1 compromised cancer cells, reverting their sensitivity to PARP inhibitors or chemotherapy, highlighting how repair pathway choice can influence clinical responses (Bouwman et al., 2010; Wong et al., 2010).

While NHEJ is a fast-acting process, the initiation can be halted by Ku70-Ku80 displacement. Ku70-Ku80 has a low affinity for resected DNA with long 3' overhangs, and resection itself can dislodge the complex, pushing the cell toward HR (Mao et al., 2008; W. Wang et al., 2017). Early resection is catalyzed by the Mre11-Rad50-Nbs1 (MRN) complex, which requires the interaction between CtBP- interacting protein (CtIP) and Mre11 (Sartori et al., 2007). MRN induces short-range resection which can displace Ku70-Ku80, then allowing the long-range resection factor, Exo1, to create long 3' overhangs (Mimitou & Symington, 2008). Other factors, such as BRCA1/2, can interact with MRN-CtIP to tip the balance toward resection (Cruz-Garcia et al., 2014; Huertas &

Jackson, 2009). As single-stranded DNA (ssDNA) is inherently unstable, replication factor A (RPA) binds 3' overhangs, which is later displaced by Rad51 via other recombination intermediates such as BRCA2. Aided by BRCA1, Rad51 forms a synaptic complex of nucleofilaments around DNA and begins homology search to find an appropriate template for accurate repair. The synaptic complex will invade a homologous DNA strand and displace the complementary strand, forming a displacement loop (D-loop), catalyzed by Rad51-driven ATP hydrolysis and nucleofilament disassembly. DNA polymerases extend the invading strand, termed branch migration, which will now be complementary to the template strand (Z. Chen et al., 2008). After repair, the four-way junction formed during D-loop formation, termed a Holliday junction, is resolved through mechanisms that either result in crossing over (CO), termed “resolution”, or in non-crossover (NCO), termed “dissolution”. Holliday junction resolution occurs during meiosis to introduce genetic variation to progeny, while mitotic repair favors dissolution to maintain genome integrity in somatic cells (Bizard & Hickson, 2014). Although a longer process, HR, when properly regulated, typically results in high-fidelity repair and is crucial for genomic integrity.

Cell cycle phase inherently influences repair pathway choice as sister chromatids, which serve as homologous templates for damaged DNA, only exist during S and G2 phases. As such, a cell restricts HR to these phases to avoid repair from an allelic chromosome or from sequences with enough homology to initiate HR but that may result in chromosomal fusion events (Takata et al., 1998). During G1, 53BP1, along with the Shieldin complex, compete with HR factors such as BRCA1 to protect DNA ends from resection (Setiাপutra & Durocher, 2019). Recent studies have shown

that components of the Shieldin complex may even promote fill-in of resected ends to further push cells toward NHEJ (Mirman et al., 2022). Some HR protein levels are also kept at lower levels during G1 (Y. E. Choi et al., 2014; Orthwein et al., 2015), and their activation is in part controlled mainly by CDKs during S/G2, where CDK1 and CDK2 activity levels are at their highest (Ditano et al., 2021; Falck et al., 2012; Huertas & Jackson, 2009; Myers et al., 2007; Tomimatsu et al., 2014; H. Wang et al., 2013; Wohlbald et al., 2012).

Although cells prefer sister chromatids as partners for repair, it is possible for cells to repair from homologous (allelic) and non-homologous chromosomes. While repair between allelic chromosomes does not typically occur spontaneously during S-phase, a DSB on one allele may induce recombination with the other allele, often resulting in loss of heterozygosity (LOH). This was demonstrated in a study where DSBs were induced in the Rb1 locus on one allele via I-SceI cutting. The DSB resulted in allelic recombination rates at 100-fold over control, suggesting a mechanism to explain how tumor suppressors, like Rb1, can be lost in mammalian cells (Moynahan & Jasin, 1997). While genome integrity is paramount to avoid a malignant cell state, DNA repair pathway alterations or mismanagement can lead to tumorigenesis.

Replication-coupled repair

DNA replication occurs once per cell cycle after cells have committed to a round of division by passing through the restriction point (Pardee, 1989). Barriers to replication include unusual DNA structures, blocking lesions such as abasic sites, transcriptional collisions, and breakage at fragile sites (Cortez, 2019). Cells have evolved a variety of

mechanisms to deal with these obstacles. For this overview, HR-mediated mechanisms will be described.

While lesions can occur on both the leading and lagging strand during DNA synthesis, lesions on the leading strand tend to be more deleterious. Lagging strand lesions can be skipped over naturally by the primase activity of polymerase alpha ($\text{pol}\alpha$) primase and ssDNA will be filled in post-replication (Fumasoni et al., 2015; Mcinerney & Donnell, 2004). Leading strand damage, however, cannot be efficiently skipped over by priming activity, and must be resolved in an alternative manner (Taylor & Yeeles, 2018). If left unresolved, the CMG-helicase will become uncoupled from the polymerase, an event referred to as replication fork uncoupling, resulting in extended ssDNA which will be bound by RPA (Byun et al., 2005). This event triggers the ATR pathway, via ATR-interacting protein (ATRIP) associating with ATR, an interaction that requires phosphorylation by CDK2 (Myers et al., 2007; Zou & Elledge, 2003). ATR phosphorylates Chk1 to inhibit the Cdc25 phosphatase family, thus inhibiting CDK1/2 in a feedback loop and halting the cell cycle until DNA can be repaired (Zou & Elledge, 2003). If cells can repair the damage at the stalled fork, replication can resume; if left unresolved, the replication fork will collapse, requiring new origin firing to complete DNA synthesis (Petermann et al., 2010).

One method to overcome a stalled replication fork is to trigger fork reversal. Fork reversal is catalyzed by SMARCAL1, ZRAN3B, and HLTF to unwind the replication fork, annealing the new strands and re-annealing the parental strands (Betous et al., 2013; Ciccio et al., 2012; Unk et al., 2008). The resulting structure is termed a “chicken foot” and allows one of the new strands to act as an undamaged template to overcome the

fork-stalling lesion (Neelsen & Lopes, 2015). The reversed fork structure can be cleaved by the nuclease Mus81, and can result in mitotic aberrations under oncogene-induced stress contexts, such as *CCNE1* or *CDC25A* overexpression (Neelsen et al., 2013). Deletion of Mus81 rescued DSB formation and micronuclei in *CCNE1* and *CDC25A* overexpression systems, supporting the notion that high levels of reversed forks can lead to DSBs (Neelsen et al., 2013). As detailed earlier, DSBs can be repaired via HR (see Repair pathway choice).

A second, potentially more mutagenic method of overcoming a stalled or collapsed replication fork is to activate break-induced replication (BIR) (Davis & Symington, 2004; Malkova et al., 1996). Like classic HR, BIR begins with DNA end resection and the recruitment of RPA. In RAD51-dependent BIR, the D-loop, or “bubble”, migrates to displace newly synthesized DNA (Davis & Symington, 2004; Saini et al., 2013). However, synthesis during this process is asynchronous, causing the lagging strand to use the leading strand as a template, yielding a conservative DNA inheritance (Saini et al., 2013). Polymerase δ , thought to be more error prone, has been implicated in BIR (Lydeard et al., 2007). When used for replication, BIR is more mutagenic and induces more chromosomal rearrangements than regular, semi-conservative DNA replication as it can result in so-called “half cross-over” events that lead to chromosomal fusions (C. E. Smith et al., 2009). When cells experience replication stress, they can utilize BIR to overcome replication fork stalling. Constantino *et al* demonstrated that cyclin E1 overexpressing cells required POLD3 and POLD4, subunits of polymerase δ , to progress the cell cycle. Furthermore, segmental genomic duplications induced by cyclin E1 overexpression were dependent on POLD3

(Constantino et al., 2014). Another study in breast cancer found that tandem duplications were positively associated with *CCNE1* aberrations (Menghi et al., 2018). Together, these data suggest a role for BIR in tolerating *CCNE1*-induced replicative stress.

Although genomic integrity is paramount to survival, cells employ potentially mutagenic, HR-based repair pathways to complete replication when under stress. Both reversed fork resolution and BIR have been shown to yield high levels of DSBs, cross-over events resulting in LOH, as well as tandem duplications.

CDK1 and CDK2 regulate homologous recombination

CDK activity is inherently linked to DNA repair as cell cycle stage plays a major role in repair pathway determination. However, there is conflicting evidence to support that mammalian cells are dependent on CDK1, CDK2, or both for HR.

Even though cells and whole organisms can survive without CDK2 (Berthet et al., 2003; Tetsu & McCormick, 2003), CDK2 appears to have non-redundant functions in meiosis, a process largely analogous to homologous recombination. CDK2 knockout mice survive to adulthood, but both males and females are sterile (Berthet et al., 2003). CDK2 kinase activity is required for normal ovarian development as mice homozygous for either kinase dead mutant, $CDK2^{D145N/D154N}$ or $CDK2^{T160A/T160A}$, had normal mitotic cell progression, but failed to develop normal gametes (Chauhan et al., 2016). SpeedyA, a Rapid Inducer of G2/M progression in Oocytes (RINGO) protein, is an atypical activator of CDK2 that also appears to be required for proper meiosis, as the SpeedyA knockout mice phenocopy the CDK2 knockout mice (Mikolcevic et al., 2016).

SpeedyA/CDK2 function to tether telomeres to the nuclear envelope, a process that serves to align homologous chromosomes for CO events (Palmer et al., 2020; Tu et al., 2017). CDK2, then, has a specialized role during development in meiosis, a process inherently linked to homologous recombination.

The CDK1 homolog, Cdc28, is required in *S. cerevisiae* for initial end resection and subsequent HR, suggesting that at least CDK1 may play a role in this high-fidelity repair. In temperature sensitive mutant strains, Cdc28 was required for early-stage resection (Aylon et al., 2004), and in a parallel study, an analog sensitive Cdc28 mutant demonstrated that Cdc28 kinase activity is required for early-stage resection and recruitment of RPA and Rad51 downstream (Ira et al., 2004). These approaches suggest that CDK kinase activity, rather than a scaffolding function, are essential for proper HR.

As mammalian cells have multiple CDKs which have redundancy during development and in cancer cells (see Requirement of CDKs during development, CDK2 and Cyclin E1 in cancer), it was an open question which CDKs, if any, were essential for checkpoint control and HR. Using an inducible-knockdown system in non-small cell lung cancer cell lines, Johnson *et al* demonstrated that CDK1, but not CDK2, activity was required for S-phase checkpoint activation as measured by cell cycle analysis and Chk1/Chk2 phosphorylation (Johnson et al., 2009). Interestingly, only suppression of both CDK1 and CDK2 inhibited end resection in this cell system, suggesting compensatory activity for this particular step of repair. Lastly, this study suggested CDK1 is essential for BRCA S1497 and S1189/S1191 phosphorylation, which are required for BRCA1 localization to DSBs. Due to the requirement of CDK1 in later steps

of HR, inhibition or knockdown of CDK1 sensitized cells to cisplatin while CDK2 depletion had little effect on cell survival after cisplatin treatment (Johnson et al., 2009). In a follow-up study, the same group again implicated CDK1, but not CDK2, as essential for HR as measured by Rad51 foci. In concordance with that data, only CDK1 depletion or inhibition sensitized cancer cells to a PARP inhibitor (Johnson et al., 2011). While both studies show some promising evidence that CDK1 inhibition in combination with DNA damaging agents only inhibited cancer cell growth while sparing normal cells, CDK1 is thought to be an essential gene and is an unlikely candidate for cancer therapy (Santamaría et al., 2007). Nevertheless, these studies presented compelling evidence that CDK1 is the main regulator for HR while CDK2 was dispensable. Importantly, these studies were done in cancer cell lines that had intact core cell cycle machinery (e.g. NCI-H1299, A549, MDA-MB-436) (Johnson et al., 2009, 2011).

Other major studies have implicated CDK2 as a regulator for particular steps of HR. Initial end resection is initiated by the MRN complex after recruitment to broken DNA ends, and a requirement for that step is CtIP binding (see Repair pathway choice). CtIP has several residues that match the minimal CDK consensus sequence, S/T-P, including T847 and S327 (H. Wang et al., 2013). CDK2/Cyclin A2 were shown to have kinase activity against T847, and mutation of this site to alanine impaired DNA end resection and RPA recruitment. Additionally, treatment with Roscovitine, a CDK inhibitor that is slightly more selective for CDK2 over CDK1 (~15 fold), suppressed T847 phosphorylation, and phosphorylation of S4/8 on RPA, a marker of resection (Huertas & Jackson, 2009; Meijer et al., 1997). Similarly, both mutation of CtIP at S327 and treatment with Roscovitine abolished the CtIP-BRCA1 interaction, suppressing HR (L.

Chen et al., 2008). Yet another study demonstrated that the CDK activity was required for the CtIP-Nbs interaction, and subsequent ATM-mediated CtIP phosphorylation (H. Wang et al., 2013). Additionally, Buis *et al* suggested that the CDK2-Mre11 interaction was required for CtIP association and downstream phosphorylation that promotes CtIP-BRCA1 binding (Buis et al., 2012). CDK2-Cyclin E1 also directly affects BRCA1 levels, as well as BRCA1-BARD1 ubiquitin ligase activity (Hayami et al., 2005). Exo1, a long-range resection nuclease, also has several CDK-consensus sequences, and inhibition of CDK1/2 with Roscovitine suppressed recruitment of Exo1 to DSBs and prevented end resection. When Exo1 activity was inhibited, cells switched to NHEJ to complete repair, placing the CDKs as major regulators in pathway repair choice (Tomimatsu et al., 2014). Lastly, an unbiased *in situ* phosphorylation screen in HEK293 cells identified 117 candidate CDK2 substrates, including genes involved in DNA replication and repair such as ATRIP, BRCA1, and RAD54 (Chi et al., 2020). These data suggest that CDK2 has a role in DNA repair in mammalian cells.

Gaps in knowledge

While taken together these studies support a role for CDK1/2 in HR, comparison of these data is inappropriate, as different model cell lines were used in each of these studies, thus representing distinct genetics. These findings also suffer from the lack of selective CDK2 inhibitors or use of the analog sensitive (AS) mutant, which is reported to have lower kinase activity from deficient cyclin binding (Merrick et al., 2011) .

Moreover, the role of the CDKs in DNA repair has not been well studied in a context where the core cell cycle machinery is dysregulated, as is the case in *CCNE1*-amplified cells.

With the advancement of selective CDK inhibitors, studies to determine which CDKs are participating in the DNA damage response pathways are more easily possible. Phospho-proteomic studies will be especially illuminating in this context as specific substrates for particular CDKs can be identified following treatment with CDK-selective inhibitors. Additional approaches such as proximity ligation assays (PLA) or identification of Proteins On Nascent DNA (iPOND) can also be used in conjunction with the chemical biology tools to determine how CDKs regulate recruitment of repair complexes to stalled or collapsed replication forks (Dungrawala et al., 2015; Fredriksson et al., 2002). Given that the basic paradigms of cell cycle control are being challenged (Arora et al., 2023; Cornwell et al., 2023), it is likely that our understanding of how CDKs regulate other processes, such as DNA repair, will also continue to evolve.

Chapter 2 CDK2 regulates collapsed replication fork repair in *CCNE1*-amplified ovarian cancer cells via homologous recombination

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Abstract

CCNE1 amplification is a common alteration in high-grade serous ovarian cancer and occurs in 15–20% of these tumors. These amplifications are mutually exclusive with homologous recombination deficiency, and, as they have intact homologous recombination, are intrinsically resistant to poly (ADP-ribose) polymerase inhibitors or chemotherapy agents. Understanding the molecular mechanisms that lead to this mutual exclusivity may reveal therapeutic vulnerabilities that could be leveraged in the clinic in this still underserved patient population. Here, we demonstrate that *CCNE1*-amplified high-grade serous ovarian cancer cells rely on homologous recombination to repair collapsed replication forks. Cyclin-dependent kinase 2, the canonical partner of cyclin E1, uniquely regulates homologous recombination in this genetic context, and as

such cyclin-dependent kinase 2 inhibition synergizes with DNA damaging agents *in vitro* and *in vivo*. We demonstrate that combining a selective cyclin-dependent kinase 2 inhibitor with a DNA damaging agent could be a powerful tool in the clinic for high-grade serous ovarian cancer.

Keywords: CDK2, cyclin E1, homologous recombination, replication stress, DNA repair, cell cycle, ovarian cancer

Introduction

Ovarian cancer is the leading cause of death from gynecologic cancer, and the fifth leading cause of cancer-related deaths in women in the United States; in 2020, approximately 200,000 women worldwide died from ovarian cancer (IARC & WHO, 2020). This disease generally has a poor prognosis, with a 5-year survival rate of only one-third of patients (IARC & WHO, 2020). Patients with homologous recombination deficient (HRD) ovarian cancer, such as those with mutations in *BRCA1/2*, have showed clinical benefit from poly (ADP-ribose) polymerase (PARP) inhibitors, due to the synthetic lethal relationship between alternative non-homologous end joining (NHEJ), a pathway controlled in part by PARP, and homologous recombination (HR) (Bryant et al., 2005; Farmer et al., 2005). It has been previously reported that *CCNE1*-amplification, which occurs in 15–20% of high-grade serous ovarian cancer (HGSOC) patient tumors, generally does not co-occur with loss of function mutations of HR genes (Ciriello et al., 2012; Konstantinopoulos et al., 2015). It is believed that because *CCNE1*-amplified tumors have intact HR, they are resistant to PARP inhibitors and platinum agents, which is the standard of care for HGSOC (Etemadmoghadam et al., 2009). HGSOC patients

with *CCNE1*-amplified tumors, therefore, have limited therapeutic options and represent a high unmet medical need.

Cyclin-dependent kinase 2 (CDK2) is the canonical partner of cyclin E1, and this complex controls entry into the S-phase (Koff et al., 1992) via phosphorylation of the retinoblastoma protein 1 (RB1) to release E2F and allow for transcription of genes that will drive the cell cycle (Lundberg & Weinberg, 1998). Aberrant activation of the RB1-E2F pathway, such as through overexpression of *CCNE1*, increases double-stranded break (DSB) formation, leading to genomic instability (Frame et al., 2006; Gorgoulis et al., 2005). Specifically, *CCNE1* overexpression induces premature S-phase entry (Ohstubo & Roberts, 1993), increasing replication fork stalling and collapse via insufficient nucleotide pools (Bester et al., 2013), or replication-transcription collisions stemming from inappropriate origin firing (Macheret & Halazonetis, 2018). Although some studies have demonstrated a synthetic lethality between *CCNE1* amplification and HRD, such as through genetic suppression of *BRCA1/2* (Etemadmoghadam et al., 2013), other large-scale screens have not supported this relationship (Cowley et al., 2014; McDonald et al., 2017). The discrepancy in these results may stem from the experimental conditions (e.g. clonogenic assays vs logarithmic growth). More investigation is required to understand the molecular mechanism(s) underlying the mutual exclusivity of these two genomic events.

In addition to their role in cell cycle progression, the cyclin-dependent kinases (CDKs) are known regulators of HR in eukaryotic cells. Cyclin-dependent kinase 1 (CDK1) and CDK2 have been implicated in HR in human cells via substrate phosphorylation during each step of recombination, but with conflicting evidence to

support whether cells are dependent on CDK1, CDK2, or both for this pathway (Buis et al., 2012; Huertas & Jackson, 2009; Johnson et al., 2009, 2011; Tomimatsu et al., 2014; H. Wang et al., 2013; Wohlbald et al., 2012; X. Yu & Chen, 2004). These studies are in part confounded by the use of non-selective CDK inhibitors, making it difficult to ascribe specific functions to particular CDK family members. Moreover, the role of CDKs in DNA repair has not been well studied in a context where the core cell cycle machinery is dysregulated, as is the case in *CCNE1*-amplified cells.

Here, we provide evidence for a mechanism that could explain the mutual exclusivity of *CCNE1* amplification and HRD in HGSOC. We demonstrate that *CCNE1*-amplified HGSOC cells rely on HR to resume replication following induced fork collapse. We detected high levels of CDK2 and cyclin E1 at the site of stalled replication forks only in *CCNE1*-amplified cells, which places CDK2-cyclin E1 directly at barriers to replication. Lastly, we provide evidence that CDK2 is required for both signaling after replication fork stalling and HR in *CCNE1*-amplified cells, but is dispensable in *CCNE1* non-amplified cells. Inhibition of CDK2 at low doses sensitizes *CCNE1*-amplified cells to DNA-damaging agents both *in vitro* and *in vivo*. With the advancement of CDK2 inhibitors into the clinic, these studies suggest that the combination of CDK2 inhibition with DNA-damaging chemotherapy or PARP inhibitors may provide clinical benefit in HGSOC patients with *CCNE1*-amplified tumors.

Materials and methods

Cell lines and culture

OVCAR-3 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 20% fetal bovine serum. hTERT-RPE1 cells were maintained in Dulbecco's Modified Eagle Medium: F12 (DMEM: F12) supplemented with 10% fetal bovine serum and 0.01 mg/mL hygromycin B. A549 cells were maintained in F-12K Medium supplemented with 10% fetal bovine serum. COV644 and COV318 cells were maintained in DMEM supplemented with 10% fetal bovine serum. FUOV1 cells were maintained in Ham's F12 + DMEM at 1:1, and supplemented with 10% fetal bovine serum. MCF-7 cells were maintained in Eagle's Minimum Essential Medium (EMEM), and supplemented with 0.01 mg/mL human recombinant insulin and 10% fetal bovine serum. OVCAR-3, hTERT-RPE1, A549, and MCF-7 cells were purchased from the American Type Culture Collection. COV644 and COV318 were purchased from Sigma Aldrich. FUOV1 cells were purchased from DSMZ. Cell line identity was confirmed with short tandem repeat (STR) testing (Charles River). All cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Proliferation assay

OVCAR-3, COV644, or MCF-7 cells were seeded at 1000 cells/well in 384-well black, clear bottom plates and allowed to adhere overnight at 37°C/5% CO₂. The following day, cells were treated with compound in a 10-point dose response, 1:4 dilutions. After 5 days of incubation, CyQuant Direct Proliferation Assay was performed according to the manufacturer's instructions. Data were processed with GraphPad Prism using a four-parameter fit to calculate IC₅₀.

AlphaLISA assays

For the RB1 phosphorylation (T821/826) AlphaLISA, OVCAR-3 cells were seeded in serum-free DMEM in a 96-well plate (25,000 cells/well) the day before the assay. After overnight incubation, media were replaced with DMEM containing 10% FBS, and cells were treated with a dose response of compound (1:4 dilutions) for 18 hours. pRb AlphaLISA was performed according to the manufacturer's instructions. Plates were read on EnVision plate reader (Perkin Elmer). Data were processed with GraphPad Prism using a four-parameter fit to calculate IC₅₀.

For the Lamin phosphorylation (S22) AlphaLISA, OVCAR-3 or COV644 cells were seeded in DMEM containing 10% FBS in 384-well plates (3,000 cells/well for OVCAR-3; 12,500 cells/well for COV644). After overnight incubation, cells were treated with a dose response of compound (1:4 dilutions) for 2 hours. Media was aspirated from the plate, and cells were lysed in 10 µL of 1X AlphaLISA lysis buffer (Perkin Elmer Cat# AL003C) and placed on a plate shaker for 15 minutes. Anti-total lamin (SC-7292) was diluted to 0.2 nM, and anti-phospho Lamin (CST# 13448) was diluted to 0.3 nM in 1X immunoassay buffer (Perkin Elmer Cat# AL00F). 10 µL of each antibody was added to the plate and incubated for 1 hour at RT. Anti-rabbit acceptor beads (Perkin Elmer Cat# AL104R) were prepared at 5X and added to the plate (10 µL/well) and incubated for 1 hour at RT. Anti-mouse donor beads (Perkin Elmer Cat# AS104R) were prepared at 5X and added to the plate (10 µL/well) and incubated overnight in the dark. Plates were read on EnVision plate reader (Perkin Elmer). Data were processed with GraphPad Prism using a four-parameter fit to calculate IC₅₀.

Lentiviral transduction/stable cell line generation

Lentivirus encoding inducible non-targeting control shRNA, CDK2 shRNA, or CDK1 shRNA in the SMARTvector inducible lentiviral shRNA vector under the EF1 alpha promoter with puromycin resistance were purchased from Horizon Discovery. Stable OVCAR-3, FUOV1, COV318, hTERT-RPE1, A549, and COV644 cells were generated by exposing cells to lentivirus at a multiplicity of infection of 1:1 overnight in the presence of 5 µg/mL polybrene. After overnight incubation, media was replaced and cells were allowed to recover for 48 hours. Cells were passaged into selection media (Puromycin – OVCAR-3: 2 µg/mL, FUOV1: 0.5 µg/mL, COV318: 5 µg/mL, hTERT-RPE1: 10 µg/mL, A549 and COV644: 1 µg/mL) for 2 weeks before experiments were conducted.

Western blot

Cells were lysed in PhosphoSafe Lysis Buffer (EDM millipore, Cat#71296) and protein was quantified using the Pierce BCA Gold Kit (Thermo Fisher Scientific Cat#A53227). Lysate and lysis buffer were combined with 4X Loading Dye (Bio-Rad, Cat#1610747) and 2ME (Bio-Rad, Cat#1610710). Samples were loaded into Bio-Rad 4–20% Tris-Glycine, 18-well SDS-PAGE (Cat#5671094). Transfer was performed using Bio-Rad dry transfer system (Trans-Blot Turbo) (Nitrocellulose membrane, 0.2 µM). Blocking was performed using LI-COR Odyssey Blocking Buffer (Cat#927-70001). Primary antibodies (CDK2 1:500, p16 1:1000, CCNE1 1:500, pRbT821 1:500, Actin R 1:2000, p21 1:1000, CCNA2 1:500, pRb S807/811 1:1000, pRb S780 1:500, CtIP 1:1000, Actin M 1:5000, pH2A.X Ser139 1:1000, pCDK2 T160 1:1000, pRPA S4/8 1:1000, CDK1 1:1000, pCDK1 Y15 1:1000, pChk1 S317 1:500, Total Chk1 1:1000, Tubulin 1:10,000) were combined with Odyssey Blocking Buffer and 0.1% Tween-20, and incubated at 4°C ,

rotating. Secondary antibodies were combined with Odyssey Blocking Buffer and 0.1% Tween-20 and incubated at room temperature, rotating. All secondary antibodies were diluted to 1:10,000 (LI-COR Cat#926-33212, Cat#926-33213, Cat#926-68070, Cat#926-68071, Cat#926-68076). Signals were detected using LI-COR Odyssey CLx Imaging Studio.

CRISPR/Cas9 recombination assay

pBluescript SK II+ and LentiCRISPR v2 plasmids were purchased from Genscript. LentiCRISPR v2 plasmids expressing single guide (sgRNA) against ACTB or scrambled sgRNA were constructed according to the depositor's instructions. To construct HR donor vectors against ACTB, sequences from -1554 to +1527 relative to the stop codon of ACTB plus 3X FLAG directly 5' from the stop codon were synthesized and cloned into pBluescript SK II using restriction digest and ligation. Compounds were added to cells on the day of plasmid transfection. sgRNA/Cas9 and the donor plasmid were prepared in a 1:2 molar ratio; 1 µg of total DNA/well was transfected onto cells using Lipofectamine 2000 (Thermo Fisher Scientific Cat#11668019). Cells were harvested for Western Blot analysis 72 hours later.

***In situ* proximity ligation assay for EdU (double stranded DNA)–protein interaction**

Cells were seeded onto microscope chamber slides the day before the experiment in DMEM supplemented with 10% FBS. On the day of the experiment, cells were incubated with 100 µM EdU for 10 minutes and treated with hydroxyurea (2 mM) for 2 hours. After treatment, cells were pre-extracted in CSK-100 buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM Pipes pH 6.8, 1 mM EGTA, 0.2% Triton X-100, 1×

antiproteases) for 5 minutes on ice under gentle agitation and fixed with 4% PFA/PBS for 20 minutes at room temperature (RT). Cells were permeabilized in 0.2% Triton X-100/PBS for 10 minutes at RT. Cells were incubated for 30 minutes at RT with Click-iT reaction cocktail (Thermo Fisher, Cat#C10269) to attach a biotin azide (ThermoFisher, Cat# B10184) to EdU. Next, cells were incubated with PLA blocking buffer (Sigma Aldrich) for 1 hour at 37 °C in a humidified chamber before addition of primary antibodies: rabbit anti-CCNE1 (Sigma-Aldrich, 1:250); mouse anti-CDK2 (Santa Cruz, 1:50); mouse anti-CCNA2 (Santa Cruz, 1:800); rabbit anti-biotin (CST, 1:200), and mouse anti-biotin (Sigma, 1:100). The negative control used only one primary antibody. Slides were incubated with secondary antibodies conjugated with PLA probes MINUS (anti-rabbit) and PLUS (anti-mouse) (Millipore Sigma). The incubation with all antibodies was done in a humidified chamber for 1 hour at 37°C. PLA probes MINUS and PLUS were ligated using two connecting oligonucleotides to form a template for rolling-cycle amplification. Following amplification, the products were hybridized with red fluorescence-labeled oligonucleotide. Samples were mounted in Duolink *In Situ* mounting medium with DAPI (blue) (Sigma-Aldrich, Cat# DUO82040). Images were acquired using the Nikon Ti inverted, spinning disk confocal microscope “Spinster” in the MicRoN (Microscopy Resources on the North Quad) core at Harvard Medical School. PLA spots per nuclei were quantified in each plane of the Z-stack using arivis Vision4D analysis software on the MicRoN Big Dipper Image Analysis work station. Images used in manuscript represent maximum projects of a Z-stack from ImageJ FIJI.

Enzyme assays

All CDK/cyclin assays were performed with 1 mM ATP. Untreated enzyme (0.65 nM for CDK2/cyclin E, 5 nM for CDK2/cyclin A2, 0.60 nM for CDK1/cyclin B, 1 nM for CDK4/cyclin D1, 1 nM for CDK6/cyclin D3, 15.0 nM for CDK7/cyclin H1/MNAT1, 3.0 nM for CDK9/cyclin T1) was incubated for 30 minutes in the presence or absence of compound. For CDK2/cyclin E and CDK1/cyclin B, the buffer used was 20 mM HEPES, pH 7.5, 0.0015% Brij 3510 mM MgCl₂, 0.01% BSA, and 2.0 mM DTT. For CDK2/cyclin A2, the buffer used was 50 mM HEPES, pH 7.5, 10 mM MgCl₂, 0.0015% Brij-35, and 2 mM DTT. For CDK7/cyclin H1/MNAT1 and CDK9/cyclin T1, the buffer used was 20 mM HEPES, pH 7.5, 0.01% Triton X-100, 10 mM MgCl₂, 0.01% BSA, and 2 mM DTT. For CDK4/cyclin D1 and CDK6/cyclin D3, the buffer used was 20 mM HEPES, pH 7.5, 0.0015% Brij 35, 10 mM MgCl₂, 0.01% BSA, and 2.0 mM DTT. After enzyme-inhibitor preincubation, the kinase reaction was initiated by the addition of fluorescent peptide substrate (for CDK2/cyclin E and CDK1/cyclin B, 3 μ M ProfilerPro Kinase Substrate 18, 5-FAM-QSPKKG-CONH₂). For CDK7/cyclin H1/MNAT1 and CDK9/cyclin T1, 5 μ M CTD3. For CDK4/cyclin D1 and CDK6/cyclin D3, 1 μ M ProfilerPro Kinase Substrate 34, 5-FAM-RRRFRPASPLRGPPK-COOH, and 1.0 mM ATP at 28°C for 30–60 minutes or until 10–20% total substrate peptide is phosphorylated in the absence of inhibitor. The kinase reactions were stopped by the addition of stop Buffer (75 mM HEPES, pH 7.5, 0.01125% Brij 35, 37.5 mM EDTA, and 0.15% of Coating Reagent 3 [Perkin Elmer]). The plates were read on a Caliper EZReader. Data were normalized to 0% inhibition (DMSO) and 100% inhibition (10 μ M staurosporine) controls, and the IC₅₀ calculated using XL fit.

NanoBRET target engagement assays

HEK293 cells were prepared to a final concentration of 100,000 cells/mL in 1% fetal bovine serum (FBS) Opti-MEM. Transfection mixtures were prepared with 1 µg of plasmid encoding NanoLuc-fused CDK and 9 µg of plasmid encoding cyclin, diluted in 1 mL Opti-MEM. FuGENE HD (Promega) was added to transfection mixture at a ratio of 30 µL/1 mL. After liposome formation, the lipid:DNA complex was added at a 1:20 ratio to HEK293 cells in suspension. Cells were plated in 384-well tissue culture plates and incubated overnight (20–30 hours) at 37°C/5% CO₂. Compounds were prepared from DMSO stock solutions and added to cell plates with a final top concentration of 10,000 nM. NanoBRET tracer solution was prepared from 100X tracer solution. For CDK1/cyclin B1, CDK9/cyclin T1, and CDK2/cyclin E1 assays, tracer K10 was used at a final concentration of 0.5 µM, 0.250 µM, and 0.125 µM, respectively. For CDK4/cyclin D1, and CDK6/cyclin D3, tracer K7 was used at a final concentration of 0.0625 µM. For CDK7/cyclin H1, tracer K7 was used at a final concentration of 0.5 µM. The plate was incubated at 37°C/5% CO₂ for 2 hours. NanoBRET substrate solution was prepared 15 minutes prior to the assay endpoint using serum-free OptiMEM. After the incubation was complete, NanoBRET substrate solution was added to the treated cell plate. The plate was then shaken and read using the Perkin Elmer Envision plate reader. The donor emission was measured at 450 nm, followed by acceptor emission at 610 nm. BRET ratio was generated by dividing the acceptor emission signal by the donor emission signal per sample, and converted to milliBRET by multiplying each raw value by 1000. Data were normalized to no drug (100% signal) and no tracer (0% signal), and fit using GraphPad Prism v8.0.2.

Cell cycle profiling

Reagents for EdU incorporation were included in the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Thermo Fisher Scientific Cat#C10420). Cells were treated with compound for 22 hours prior to EdU pulsing. EdU was added to cells in culture for a final concentration of 10 μ M, and incubated at 37°C in 5% CO₂ for 2 hours prior to collection and pelleting. Cells were incubated in the dark for 15 minutes with 100 μ L of Click-iT fixative, washed with 1% BSA PBS, and incubated for 15 minutes with 100 μ L 1x Click-iT saponin-based permeabilization agent, prepared by 1:10 dilution of stock solution in PBS supplemented with 1% BSA. The Click-iT reaction cocktail was prepared following manufacturer's instructions and added to cells for 30 minutes at room temperature in the dark. Cells were washed with 1% BSA. FxCycle Violet (Thermo Fisher Scientific Cat#F10347) was prepared in 1x saponin-based permeabilization reagent at a 1:2000 dilution, and added to cells for 30 minutes at room temperature in the dark. FACS analysis was performed using LSR Fortessa (BD Biosciences) and analyzed using FlowJo.

Synergy analysis

OVCAR-3 or COV644 cells were seeded in 96-well plates in complete media. The next day, BLU1851 was added in a 10-point dose response. After 24 hours of incubation, etoposide was added in a five-point dose response. The following day, compound containing media was aspirated, cells washed, and complete media added back. CyQuant was used 3 days later to determine cellular proliferation. HSA scores were determined using Synergy Finger (<https://synergyfinder.org/>).

***In vivo* tumor growth inhibition, pharmacokinetics, and pharmacodynamics**

Female NOD-SCID mice were purchased from Shanghai Lingchang BioTech Co., Ltd at 6–8 weeks old. Mice were housed in solid bottom polycarbonate ventilated cages at a temperature of 20–26°C in 40–70% humidity. Mice were kept on a 12-hour light, 12-hour dark cycle with regularly changed 100% filtered air. Municipal water and rodent diet were provided ad libitum. The general health of the animals was monitored through gross observation, regular body weight measurement, and monitoring of food and water intake. All procedures related to animal handling, care, and treatment were performed according to the protocol approved by the Institutional Animal Care and Use Committee of Shanghai Chempartner following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

OVCAR-3 cells were passaged through mice once, and a cell line was rederived from the tumor, named “T2A.” OVCAR-3 T2A cells were cultured in RPMI 1640 media supplemented with 20% FBS and 0.01 mg/mL insulin. To establish the OVCAR-3 xenograft model, 6×10^6 cells per mouse were implanted subcutaneously into the right hind flank of NOD-SCID female mice. Cells were suspended in 50% Matrigel and 50% RPMI 1640 media serum free media prior to implantation.

In OVCAR-3 tumor growth inhibition studies, animals were randomized and treatment began when tumors reached $\sim 200 \text{ mm}^3$ in size ($n =$ at least eight/group). BLU2256 was prepared in 20% (v/v) PEG 400, 80% (v/v) [20% (v/v) Solutol in 100 mM citrate buffer, pH 3, and dosed orally (PO) as a suspension once daily (QD) at 3 mg/kg, or BID at 5 mg/kg. Etoposide was prepared in 5% DMSO+40% PEG300+5%Tween 80+50%dd water and dosed intraperitoneally QD at 1.25 mg/kg for 3 days for each week of

the study. Olaparib was prepared in 10% HP- β -CD in water and dosed intraperitoneally QD at 50 mg/kg. Tumor sizes and body weight were measured twice weekly. Tumor sizes were measured in two dimensions using a caliper, and the volume (mm³) was calculated using the formula: $V = 0.5 a \times b^2$ where a and b are the long and short diameters of the tumor, respectively.

TCGA and DepMap data sources

We used publicly available data of patients with ovarian cancer from the TCGA to determine mutual exclusivity of *CCNE1* amplification and mutations in HR-related genes. To interrogate features with potential relationships to HR, we retrieved data from the DepMap 21Q4 public release (DepMap, Broad, 2021) of annotated cell lines from the CCLE. Specifically, fusion and copy number data were used to assess the landscape of gross chromosomal rearrangements across cancer types and tumor mutation burden estimated from mutation data and aneuploidy scores derived from ABSOLUTE copy number profiles were used to characterize differences in *CCNE1*-amplified versus non-amplified cell lines.

Statistical analysis of mutual exclusivity in cancer patients and fusion events in cancer cell lines

Mutual exclusivity of *CCNE1* amplification and HR-related genes was analyzed in patients with ovarian cancer with mutation and copy number data provided by TCGA. *CCNE1*-amplified tumors had GISTIC-called amplifications while HR-mutated tumors had either missense, inframe, or truncation mutations identified by whole exome sequencing (25). The Fishers' exact test was used to determine statistical significance

($P \leq 0.05$), while odds ratios were calculated to assess the magnitude and direction of effect.

Fusions identified in DepMap 21Q4 release were called from RNA-sequencing data using the STAR-Fusion pipeline version 1.6.0 and filtered as described in DepMap documentation (Haas et al., 2017). To create a more robust call set, we further filtered out fusions with fusion fragments per million <0.1 or a spanning fragment count of 0 with no long double anchor support. Copy number values greater than three were called as amplified, assuming diploidy. Differences in fusion distribution, tumor mutation burden, and aneuploidy were tested using the Wilcoxon rank-sum test ($P \leq 0.05$). Differences in the proportion of cell lines were tested using the Fishers' exact test ($P \leq 0.05$).

All statistical analyses were performed in R 3.6.3.

Results

CCNE1-amplified ovarian cancers have a high incidence of chromosomal fusion events

Co-occurrence of *CCNE1* amplification with *RB1* loss and somatic mutations in genes involved in HR, Fanconi anemia (FA), and DNA repair was assessed using patient data from The Cancer Genome Atlas (TCGA). In agreement with previous analyses, *CCNE1* amplification has a tendency toward mutual exclusivity with *RB1* loss and putative driver mutations in the HR and FA pathways (Ciriello et al., 2012) (Figure 2.1A, Figure S 2.1). We also analyzed *PIK3CA*, a frequently amplified oncogene in ovarian cancer, and observed that *PIK3CA* and *CCNE1* amplification are not mutually exclusive (Table S 2.1). Because *CCNE1* amplification is not generally mutually exclusive with other

oncogenic alterations, such as *PIK3CA* amplification, the mutual exclusivity between *CCNE1* amplification and HR may be governed by specific molecular mechanisms.

Because *CCNE1* amplification induces oncogenic stress and DSBs that can lead to allelic imbalances (Gorgoulis et al., 2005; Halazonetis et al., 2008), we assessed gross chromosomal rearrangements (GCRs) in *CCNE1*-amplified cell lines versus non-amplified cell lines using data from the Cancer Cell Line Encyclopedia (CCLE). Across all indications, we found that *CCNE1*-amplified lines are more prone to chromosomal fusions than non-amplified lines ($P = 9.1 \times 10^{-6}$) (Figure 1.1B), and ovarian *CCNE1*-amplified cell lines showed the same trend compared with non-amplified cell lines ($P = 0.032$) (Figure 2.1C). Furthermore, these fusions are more likely to occur at cyclin E1-induced fragile sites (Figure S 2.1A-B) (Miron et al., 2015). We also observed that *CCNE1*-amplified cells tend to have higher levels of aneuploidy compared with non-amplified cell lines across tumor types ($P = 2.2 \times 10^{-9}$) (Figure 2.1.D), and in ovarian cancer cell lines specifically ($P = 0.0044$) (Figure 2.1E). This observation is consistent with the role of cyclin E1 in endoreplication during development (Geng et al., 2003; Keck et al., 2007; Parisi et al., 2003). *CCNE1*-amplified cells, however, do not have higher mutational burden compared with non-amplified cells (Figure S 2.1C-D). Taken together, these data support that *CCNE1*-amplified cell lines may be encountering barriers to replication in *CCNE1*-induced fragile sites, leading to chromosomal fusion events.

***CCNE1*-amplified cells require CDK2 for CHK1 signaling, and HR for replication restart**

To choose model cell lines to interrogate the relationship between *CCNE1* amplification and HR, we measured protein levels of key cell cycle regulators in a panel of HR-proficient *CCNE1*-amplified and *CCNE1* non-amplified cells (Figure 2.2A, Table S 2.2). We chose OVCAR-3, FUOV1, and COV318 to represent ovarian cell lines with amplification of *CCNE1*, and COV644 to represent ovarian cell lines with two copies of *CCNE1*. We also included a diploid, immortalized cell line, hTERT-RPE1, to interrogate a non-cancerous cell line with an intact cell cycle (Bodnar et al., 1998), and A549, a cell line previously reported to be dependent on CDK1 for HR (Table S 2.2) (Johnson et al., 2011). As expected, *CCNE1*-amplified cell lines had increased levels of cyclin E1 protein (Figure 2.2A), consistent with patient data from the TCGA (Figure S 2.2A). *CCNE1*-amplified cell lines also had markers of active CDK2 (elevated phosphorylation

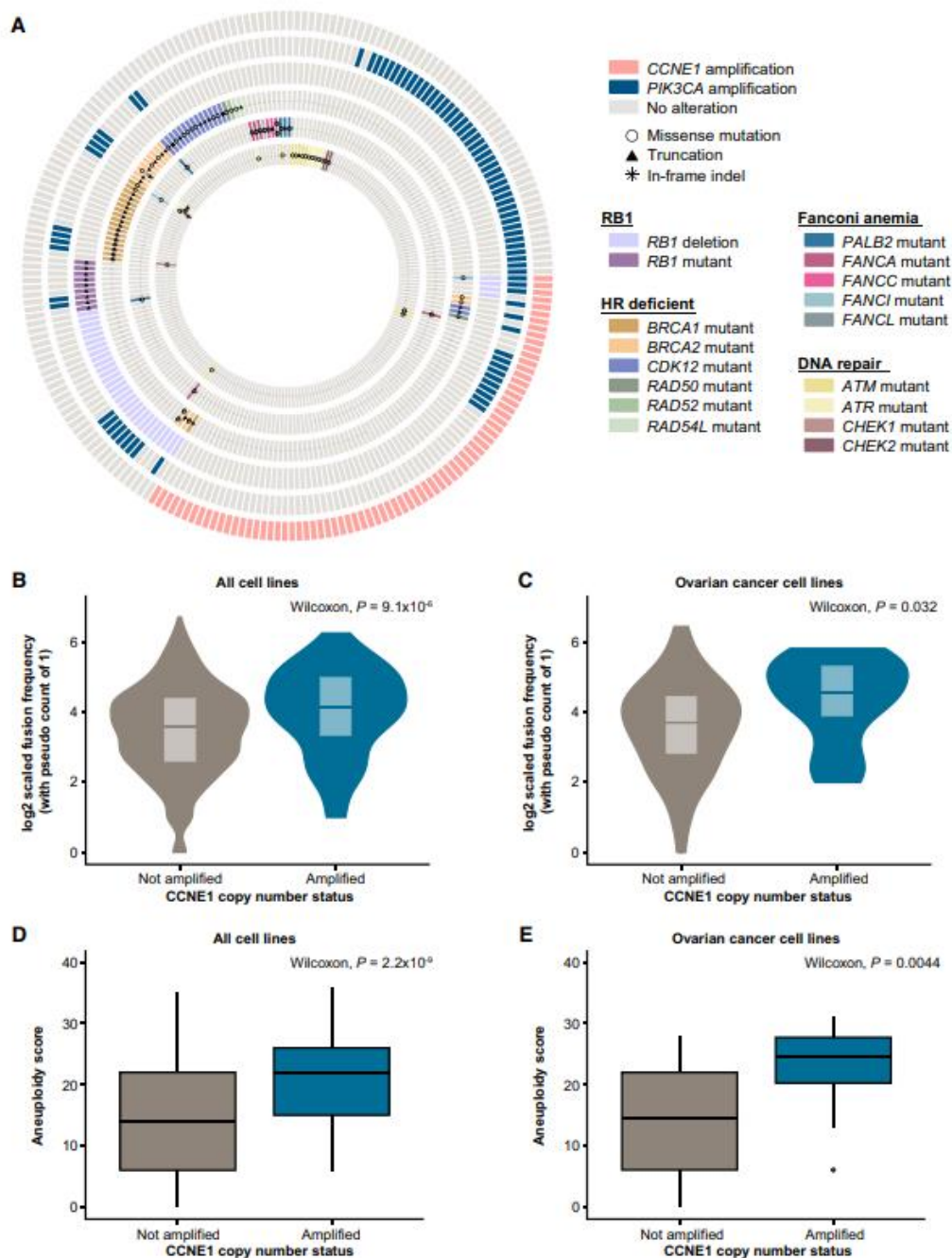


Figure 2.1: CCNE1-amplified cancer cell lines are prone to GCR

(A) Co-occurrence of CCNE1-amplification and somatic mutations in genes of interest in patients with ovarian cancer included in The Cancer Genome Atlas ($n=224$). Each ring represents a gene or gene category (outer to inner: CCNE1, PIK3CA, RB1, HR deficient, Fanconi anemia-related genes, DNA repair genes, Holliday junction genes,

replication fork genes). Each row of bars across the rings represents a patient with CCNE1-amplification and/or a somatic mutation in a gene of interest. The color of the bar specifies the gene being mutated; symbols within the bar represent the type of mutation. Patients with ovarian cancer with no amplifications or mutations in listed genes are not shown. **(B)** Distribution of fusion events in CCNE1-amplified and non-amplified cancer cell lines (n=1371) (Wilcoxon Rank Sum Test, $P = 9.1E-06$). **(C)** Distribution of fusion events in CCNE1-amplified and non-amplified ovarian cancer cell lines (n=64) (Wilcoxon Rank Sum Test, $P = 0.032$). **(D)** Difference in aneuploidy scores in CCNE1-amplified and non-amplified cancer cell lines (n=942) (Wilcoxon Rank Sum Test, $P = 2.2E-09$). For each box plot, the center line represents the median aneuploidy score of each cell line category. The upper and lower boundary of box plots represent the 75th percentile and 25th percentile of aneuploidy score, respectively. The extreme ends of the line are the minimum and maximum values, excluding outliers. Any outliers are represented by a solid black point. **(E)** Difference in aneuploidy scores in CCNE1-amplified and non-amplified ovarian cancer cell lines (n=48) (Wilcoxon Rank Sum Test, $P = 0.0044$). For each box plot, the center line represents the median aneuploidy score of each cell line category. The upper and lower boundary of box plots represent the 75th percentile and 25th percentile of aneuploidy score, respectively. The extreme ends of the line are the minimum and maximum values, excluding outliers. Any outliers are represented by a solid black point.

at T160, elevated levels of cyclin A2, low p21) (Figure 2.2A). Notably, CCNE1-amplified cell lines also had high levels of p16, a negative regulator of cyclin-dependent kinase 4/6 (CDK4/6) (Serrano et al., 1993), and elevated levels of pCDK1 Y15, an inhibitory phosphorylation site on CDK1 (Figure 2.2A) (Mueller et al., 1995). The protein levels of these markers indicate that CDK2 may be the predominantly active CDK in this setting.

Because the response to replication fork stalling is controlled mainly by the CDK2-ATRIP-ATR-CHK1 axis (Myers et al., 2007), we utilized a selective CDK2 inhibitor, BLU1851 (Figure S 2.2B), to interrogate this signaling cascade in CCNE1-amplified cells. BLU1851 is a potent and specific CDK2 inhibitor with limited off-target activity against the entire kinome, including other related CDK family members, such as CDK1 (Figure 2.2.B, Figure S 2.2C-H, Table S 2.3A-C) (Ramsden, P.D., Bifulco, N.J., Brooijmans, N., Perola, E., Vargas, R., Wenglowsky, S.M., Wilson, 2022). To keep

concentrations in a selective range over the key off-target CDK1 for our mechanistic experiments, we tested BLU1851 in cellular phosphorylation assays to measure CDK2 and CDK1 activity. We measured phosphorylation of Rb in OVCAR-3 as a CDK2 readout (Zarkowska & Mitnacht, 1997), and phosphorylation of lamin A/C as a CDK1 readout (Adhikari et al., 2012) and found that there is a large window between CDK2 and CDK1 activity (Figure S 2.2F-G). Lastly, because there was some activity on CDK4 in the enzyme assay (Figure S 2.2D, Table S 2.3A), we tested the anti-proliferative effect of BLU1851 on a CDK4-dependent cell line, MCF-7, and calculated an average $IC_{50} > 1 \mu M$, suggesting BLU1851 does not potently target CDK4 (Figure S 2.2H, Table S 2.3C). Based on these data, we chose CDK2-selective concentrations of BLU1851 in our studies.

Given the overall chromosomal instability and markers of hyperactive CDK2 observed in *CCNE1*-amplified cells, we hypothesized that these cells may be especially reliant on CDK2-CHK1 signaling in response to stalled or collapsed replication forks. We therefore measured CHK1 activation via S317 phosphorylation in both OVCAR-3 and COV644 cells after 2 hours and 24 hours of hydroxyurea (HU) treatment to stall or collapse replication forks, respectively, in the presence or absence of BLU1851, at a concentration below the proliferative half-maximal inhibitory concentration (IC_{50}) in OVCAR-3 (Table S 2.3C) to limit the effects of the compound on the cell cycle. While CHK1 signaling was expectedly activated in both cell lines following HU treatment, CDK2 inhibition suppressed pCHK1 S317 to a greater extent in OVCAR-3 cells compared with COV644 cells (Figure 2.2C, Figure S 2.2I). BLU1851 treatment also inhibited pCHK1 S317 in FUOV1 and COV318 to a greater extent than in hTERT-RPE1

or A549 cells (Figure S 2.2J). These data support a more prominent role for CDK2 in replication fork maintenance via CHK1 in *CCNE1*-amplified cells compared with non-amplified cells.

As an avenue for fork restart or repair, cells can use HR-mediated mechanisms such as DSB repair after nucleolytic cleavage of a regressed fork, template switching, and gap repair, or they can utilize alternative, more mutagenic pathways such as translesion synthesis (TLS) (Cortez, 2019). To first validate that we could prevent proliferative recovery from replication fork collapse via HU, we treated OVCAR-3 cells with the CHK1 inhibitor, rabusertib, at a concentration that would not inhibit proliferation on its own followed by a 24-hour pulse of HU (King et al., 2014) (Figure S 2.2K). Indeed, CHK1 inhibition suppressed the ability of OVCAR-3 cells to proliferate following HU treatment (Figure S 2.2K). We next utilized this assay to measure the ability of OVCAR-3 and COV644 cells to resume proliferation after HU-induced replication fork collapse by using small interfering RNA to knockdown the HR factor RAD51, or the TLS factor, REV1. COV644 cells were overall less efficient at recovering from HU, while OVCAR-3 cells were able to resume proliferation and reach the confluence of vehicle-treated controls (Figure 2.2E). However, when we depleted RAD51 in OVCAR-3 cells, we observed that cells were less efficient at proliferating following HU treatment compared with non-targeting control, while REV1 depletion had no significant effect (Figure 2.2 D-E, Figure S 2.2L). In contrast, neither RAD51 nor REV1 depletion in COV644 cells worsened the recovery from HU compared with non-targeting control, albeit with less efficient knockdown compared to OVCAR-3 (Figure S 2.2M-N). These data support the hypothesis that *CCNE1*-amplified cells rely, at least in part, on HR-mediated

mechanisms to repair collapsed replication forks, while non-amplified cells may utilize multiple repair pathways to compensate for the loss of one pathway.

Since *CCNE1*-overexpressing cells incur endogenously higher rates of DNA damage through accumulation of DSBs (Gorgoulis et al., 2005), and RAD51 knockdown impaired the ability of OVCAR-3 cells to recover from induced replication fork collapse, we reasoned that OVCAR-3 cells would be more sensitive to inhibitors that disrupt the HR pathway compared with COV644 cells if they do indeed utilize HR to combat replication stress. To test this, we treated cells with mirin, which inhibits the exonuclease activity of MRE11, streptonigrin, which inhibits RAD54 ATPase activity, and JH-RE-06, a REV1-REV7 protein-protein interaction inhibitor, to model inhibition of early-stage resection, later stage HR, and TLS, respectively (Deakyne et al., 2013; Dupré et al., 2008; Wojtaszek et al., 2019). OVCAR-3 cells were more sensitive to both mirin and streptonigrin compared with COV644 cells, while COV644 cells were more sensitive to JH-RE-06 (Figure 2.2F). These data suggest that OVCAR-3 cells require HR, but not TLS, for proliferation to a greater extent than COV644 cells.

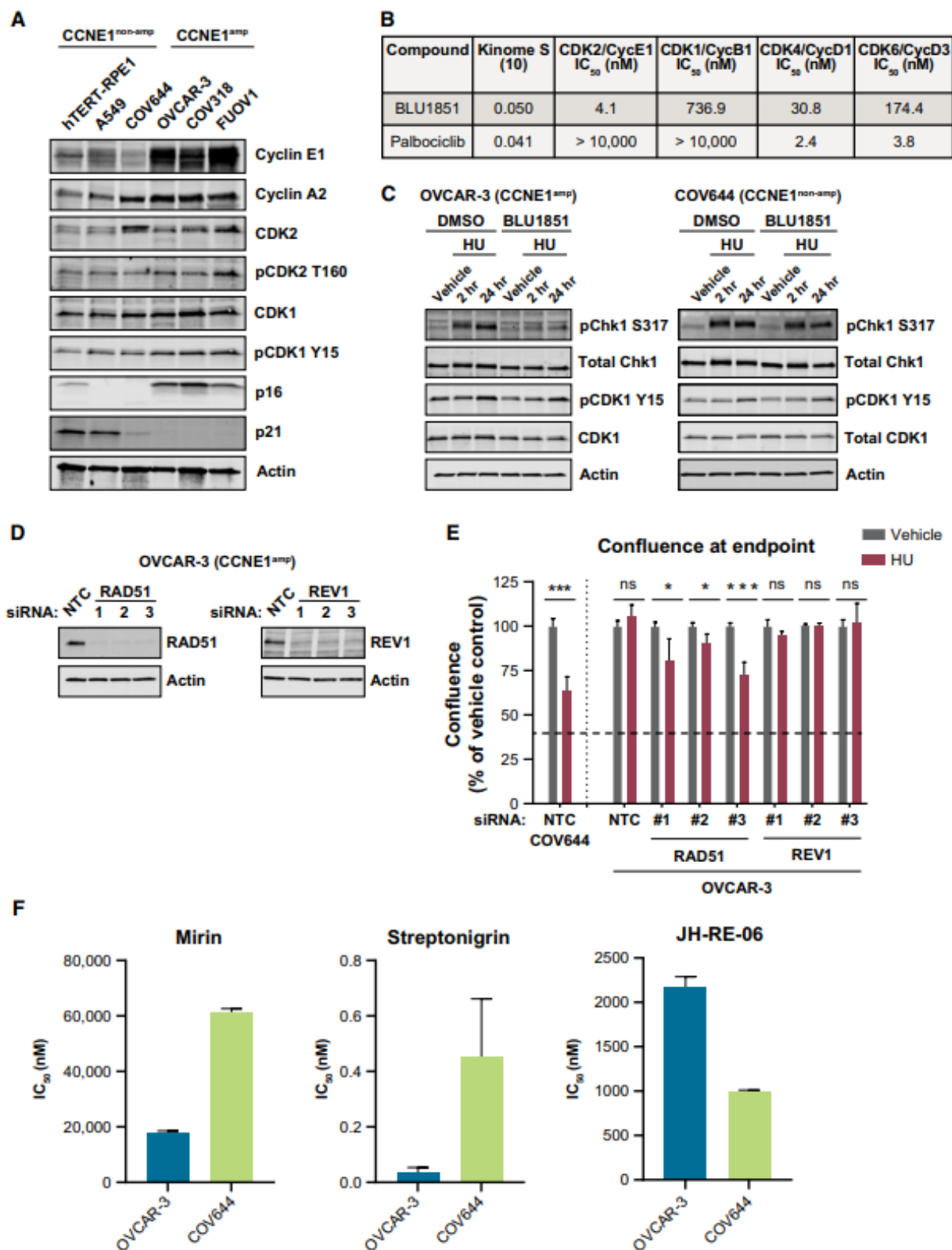


Figure 2.2: Replication fork stall signaling is dependent on CDK2, HR in CCNE1-amplified cells

(A) Western blot analysis of indicated antibodies in panel of CCNE1-amplified versus non-amplified cell lines. (B) Fraction of kinases with <10 % of control at 3 μ M among all the kinases tested (n = 468), measured by KINOMEScan platform, and IC₅₀ (nM) of indicated compounds in ATP-competition caliper enzyme assay run at 1 mM ATP. (C) Western blot analysis of indicated antibodies in OVCAR-3 and COV644 cells. Cells were treated with BLU1851 (100 nM) for 24 hours prior to addition of HU at indicated time points. (D) Western blot analysis of indicated antibodies. OVCAR-3 cells were transfected with non-targeting siRNA (NTC), RAD51 siRNA, or REV1 siRNA, and collected 48 hours later. (E) Cell confluency at experimental endpoint. Confluence was measured by IncuCyte live cell imaging and values were determined using an algorithm to detect cells from phase-contrast images. Cells were transfected with siRNA 24 hours before addition of HU (2 mM) for 24 hours. After the HU was washed out, cells were transfected again with indicated siRNAs and allowed to incubate for 5 additional days. HU-treated groups were normalized to vehicle-treated groups for each siRNA at the endpoint. The dotted line represents confluency of cells treated with constant HU exposure over the time course of the experiment. Error bars represent standard deviation (SD) in duplicate experiments. Statistical analysis: Student's t-test, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, ns = not significant. (F) Proliferative IC₅₀ (nM) of indicated compounds. Cells were treated with a 10-point dose response of indicated compounds and incubated for 5 days before CyQuant assay was performed. Error bars represent SD of at least duplicate independent experiments.

Cyclin E1 is present at the site of stalled replication forks in CCNE1-amplified cells

Since CCNE1-amplified cells are dependent on CDK2 for replication fork stall signaling, we measured the physical presence of CDK2 and its canonical cyclin partners, cyclin E1 and cyclin A2, at the site of stalled forks in two CCNE1-amplified lines, OVCAR-3 and FUOV1, and two non-amplified lines, COV318 and hTERT-RPE1. We used a proximity ligation assay (Fredriksson et al., 2002) to detect the presence of cyclin E1, cyclin A2, and CDK2 at 5'-ethylene-2'-deoxyuridine (EdU) labeled nascent DNA stalled by HU (Figure 2.3A) (Roy et al., 2018). Surprisingly, OVCAR-3 and FUOV1

cells not only had significantly more cyclin E1 at the site of stalled replication forks compared with COV644 cells, but also significantly more CDK2 and cyclin A2 (Figure 2.3B-C, Figure S 2.3A-B). The physical proximity of CDK2 along with its canonical cyclin partners suggests that catalytically active CDK2 has a role directly at the site of the stalled fork. Additionally, the detection of cyclin E1 at stalled forks in *CCNE1*-amplified cell lines suggests that cyclin E1 could be participating in non-canonical replication activities outside of its known functions in pre-replication complex assembly (Coverley et al., 2002). While COV644 cells had less CDK2 and cyclins present at stalled forks compared with OVCAR-3 and FUOV1 cells, hTERT-RPE1 did not display significant differences in cyclin A2 levels, but did have significantly fewer instances of CDK2 recruitment and trended toward less cyclin E1 levels (Figure 2.3B, Figure S 2.3A). Overall, these data suggest that CDK2 is acting physically at the site of stalled replication forks in the *CCNE1*-amplified setting.

CDK2 regulates homologous recombination in *CCNE1*-amplified ovarian cells

Because *CCNE1*-amplified cells require CDK2 for stalled fork signaling via CHK1 (Figure 2.4C, Figure S 2.2H), and may restart forks through HR (Figure 2.4E), we hypothesized that CDK2 may be the exclusive kinase regulating HR in tumors with this genetic background. To test this, we engineered stable cell lines expressing doxycycline-inducible CDK2 and CDK1 short hairpin RNA (shRNA) in a panel of *CCNE1*-amplified and non-amplified cell lines. After knockdown of CDK2 or CDK1 in each line, we treated cells acutely with the topoisomerase II inhibitor, etoposide, to induce DSBs (Long et al., 1985). We confirmed induction of DNA damage by p2AX

S139, and examined HR signaling via CtIP bandshift and pRPA S4/8 levels as a measure of DNA end resection (Fugger et al., 2015; Lossaint et al., 2013) (Figure 2.4A,

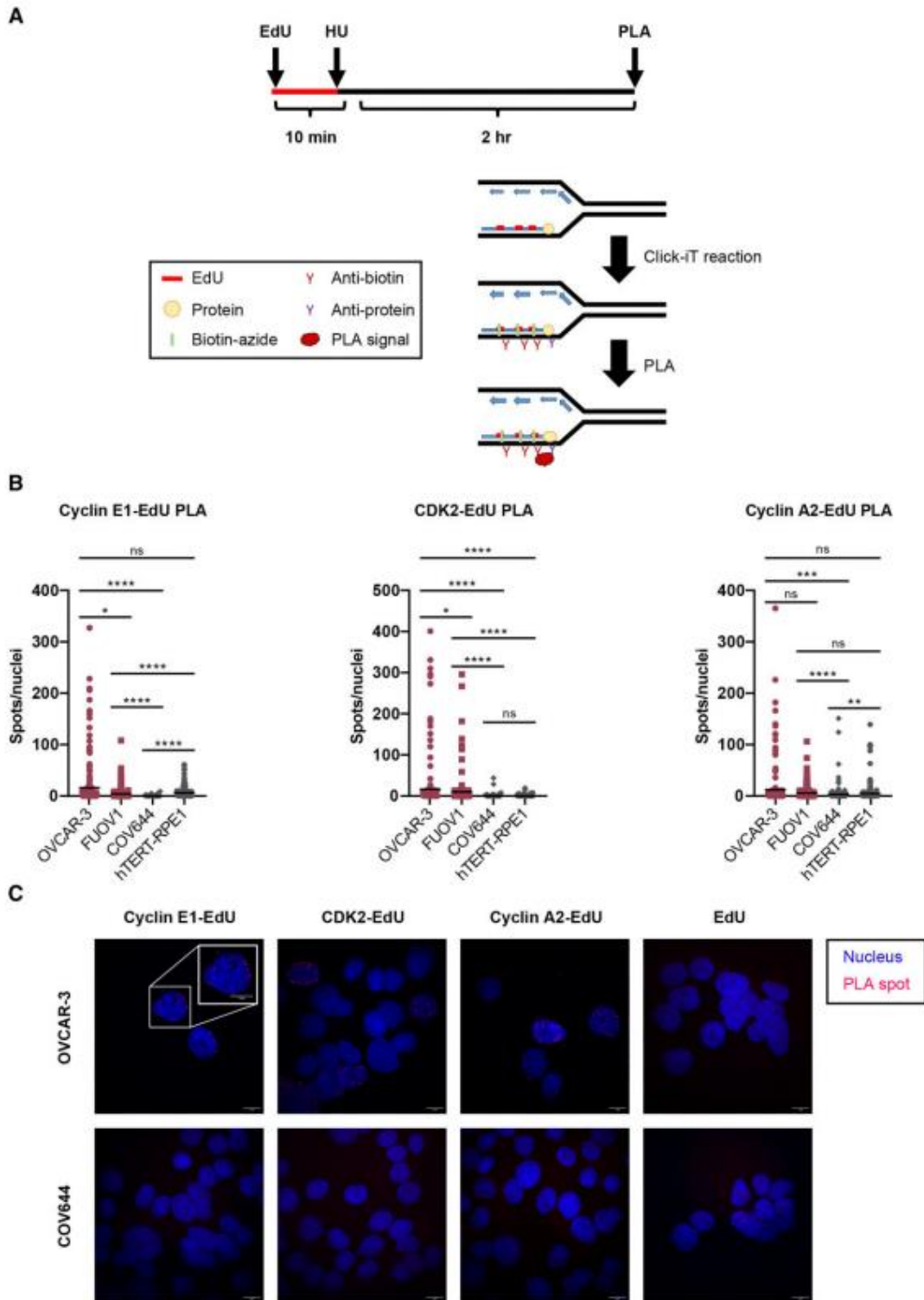


Figure 2.3: CDK2 and cyclin E1 are recruited to stalled replication forks in CCNE1-amplified cells

(A) Schematic of protein recruitment to stalled replication forks using EdU-proximity ligation assay (PLA). PLA was performed using anti-biotin with either anti-CDK2, anti-cyclin E1, or anti-cyclin A2 antibodies. The negative control excluded one primary antibody. (B) The graphs shows the mean number of PLA spots per nucleus in each indicated PLA assay. Bars represent the mean (NS = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** $P < 0.0001$, Mann-Whitney test; n = at least 100 cells/experiment). (C) Representative images of PLA for each noted interaction in OVCAR-3 and COV644 cells. PLA spots are shown in red and nuclei are shown in blue.

Figure S 2.4A-F). Strikingly, CDK2 depletion had little effect on HR signaling in the *CCNE1* non-amplified cell lines COV644, hTERT-RPE1, and A549, but suppressed HR signaling in the *CCNE1*-amplified cell lines OVCAR-3, COV318, FUOV1 (Figure 2.4A, Figure S 2.4A-F). CDK1 depletion had little impact on HR signaling in any cell line irrespective of *CCNE1* copy number (Figure S 2.4B-F). Consistent with the data from inducible CDK2 knockdown, the CDK2 inhibitor BLU1851 suppressed HR signaling as measured by the CtIP bandshift and pRPA S4/8 in *CCNE1*-amplified cell lines in a dose-dependent manner but had little effect in non-amplified cell lines (Figure 2.4B, Figure S 2.4G-H). While the selective CDK1 inhibitor, RO-3306 (Vassilev et al., 2006), had only a small effect in some cell lines, the CDK1/2 dual inhibitor, roscovitine (Meijer et al., 1997), suppressed HR signaling in most cell lines tested (Figure 2.4B, Figure S 2.4G-H). Taken together, these data suggest that CDK2 may be regulating HR non-redundantly in *CCNE1*-amplified cells.

To test if CDK2 inhibition can inhibit HR in *CCNE1*-amplified cells, we employed a CRISPR/Cas9-based recombination assay to induce a targeted DSB upstream of the beta-actin stop codon and provided a donor template with an inserted 3X-FLAG reporter (Yoshino et al., 2019) (Figure 2.4C). As expected from the signaling data (Figure 2.4B,

Figure S 2.4H), recombination was suppressed in OVCAR-3 and FUOV1 cells but not in COV644 or hTERT-RPE1 cells after treatment with BLU1851 (Figure 2.4D-E, Figure S 2.4I). Treatment with roscovitine, in contrast, inhibited recombination in all cell lines (Figure 2.4D-E, Figure S 2.4I). These data suggest that, in the *CCNE1*-amplified setting, CDK2 is uniquely required for recombination, while CDK1 and CDK2 may have redundant roles in non-amplified cells.

Because repair pathway choice is in part dictated by cell cycle phase (Aylon et al., 2004), and CDK2 inhibition arrests cells at the G1/S boundary in *CCNE1*-amplified cells (Figure S 2.4J), we tested if the suppression of HR signaling after CDK2 inhibition in *CCNE1*-amplified cells was a consequence of cell cycle arrest or if CDK2 was directly acting in the HR pathway. We compared cells arrested at G1/S with double thymidine block or with BLU1851 treatment. While both methods arrested cells in G1 as measured by flow cytometry (Figure S 2.4K), only treatment with the CDK2 inhibitor suppressed HR signaling after etoposide treatment (Figure S 2.4L). These data support that CDK2 is functioning in HR directly and the observed effects are not simply a consequence of cell cycle arrest.

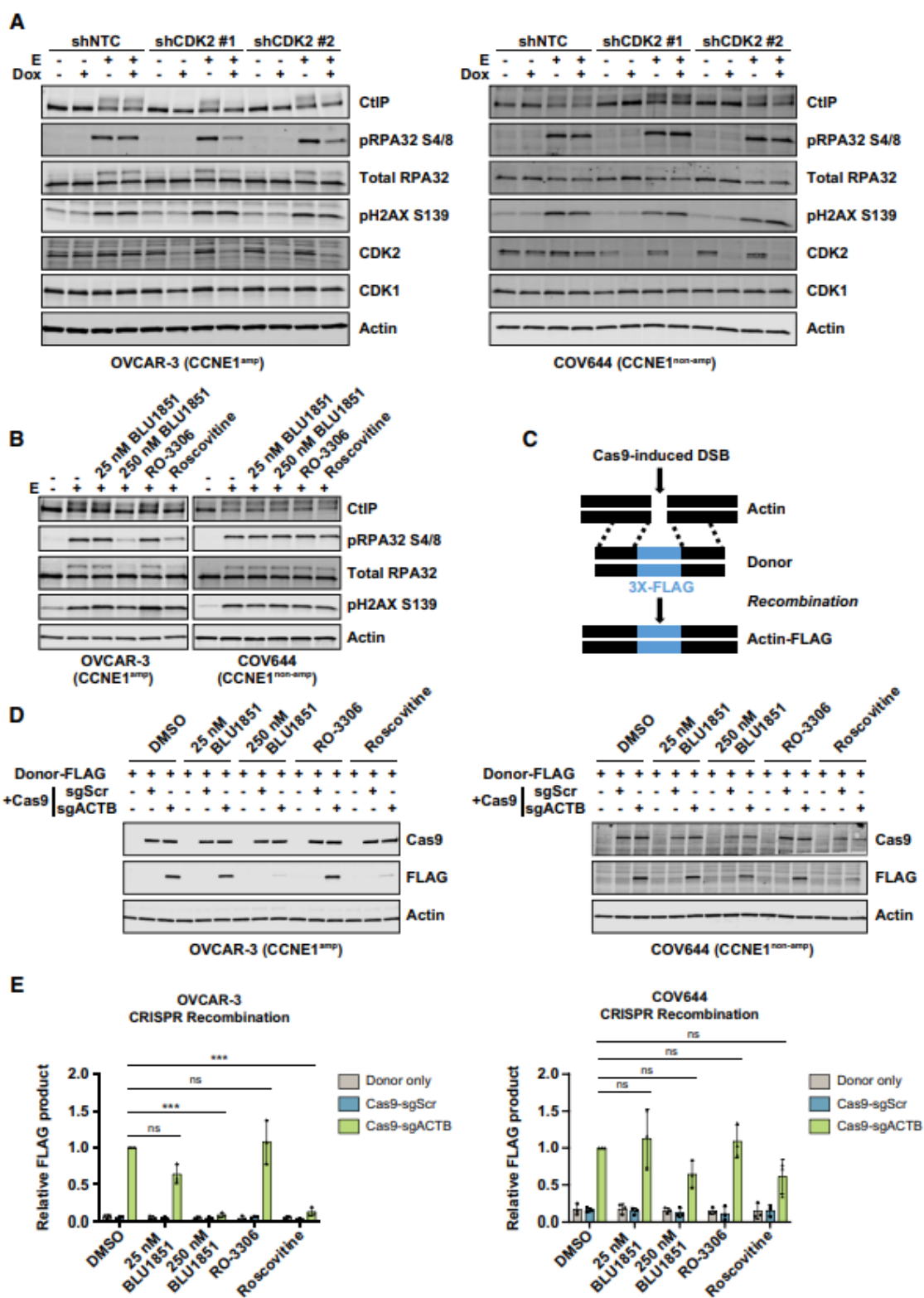


Figure 2.4: CDK2 regulates HR in *CCNE1*-amplified cell lines

(A) Western blot analysis with the indicated antibodies. Small hairpin RNA were induced by doxycycline (dox) for 96 hours prior to etoposide (E) treatment at 50 μ M for 2 hours. (B) Western blot analysis with the indicated antibodies. OVCAR-3 or COV644 cells were treated with indicated 25 nM or 250 nM BLU1851, 1 μ M RO-3306, or 10 μ M roscovitine for 24 hours before being treated with 50 μ M etoposide (E) for 2 hours. (C) Schematic of CRISPR recombination assay. Cas9 induces a double stranded break upstream of the actin stop codon. A donor plasmid with homology on either side of the cut site with an inserted 3X-FLAG tag is provided. (D) Western blot analysis with indicated antibodies. OVCAR-3 or COV644 cells were treated with 25 nM or 250 nM BLU1851, 1 μ M RO-3306, or 10 μ M roscovitine. Contemporaneously, cells were transfected with a 3X-FLAG donor plasmid plus a plasmid containing Cas9 and a single guide RNA (sgRNA), scramble control (sgScr), or a sgRNA targeting beta-actin (sgACTB). Cells were harvested 72 hours later. (E) Quantification of recombination product in (D) normalized to actin and to the DMSO sgACTB sample. Error bars represent SD in three independent experiments. Statistical analysis: One-way analysis of variance (ANOVA), * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, ns = not significant.

CDK2 inhibition sensitizes *CCNE1*-amplified cells to DNA damaging agents *in vitro* and *in vivo*

We have shown that CDK2 loss or inhibition suppresses HR signaling and recombination, and thus reasoned that combining CDK2 inhibition with a chemotherapy that induces DSBs, such as etoposide, should be synergistic. We tested a dose matrix of BLU1851 and etoposide in OVCAR-3 and COV644 cells *in vitro* and observed that the combination was synergistic in OVCAR-3 cells (highest single agent [HSA] = 13.97), but not in COV644 cells (HSA = 5.03) (Figure 2.5A). Notably, the strongest levels of synergy in OVCAR-3 cells were observed at low concentrations of both agents, below the proliferative IC_{50} . These same doses in COV644 cells trend toward antagonism. The combination effect at low doses in OVCAR-3 cells suggests that the underlying mechanism is based on the DNA damage function, rather than the proliferative function,

of CDK2. These data support the hypothesis that CDK2 regulates HR in *CCNE1*-amplified cells.

To test if a CDK2 inhibitor in combination with etoposide is anti-tumorigenic *in vivo*, we used the OVCAR-3 xenograft, a model previously used to validate CDK2 as an oncogenic driver in *CCNE1*-amplified ovarian cancer (Au-Yeung et al., 2017). In these studies, we used BLU2256, a second selective CDK2 inhibitor that suppresses HR signaling and recombination in *CCNE1*-amplified cells. BLU2256 is structurally related to BLU1851 and showed similar *in vitro* phenotypes, but with superior pharmacokinetics, making it more suitable than BLU1851 for *in vivo* studies (Figure S 2.5A-J, Table S 2.4A-C) (Ramsden, P.D., Bifulco, N.J., Brooijmans, N., Perola, E., Vargas, R., Wenglowsky, S.M., Wilson, 2022). BLU2256 demonstrated dose-dependent tumor growth inhibition in OVCAR-3 xenografts without a change in body weight (Figure 2.5B-C, Figure S 2.5K). We determined on-target activity of this agent by measuring the inhibition of RB1 phosphorylation, which was dose and time-dependent (Figure S 2.5L-M). Based on the dose-titration data, we determined that a 3 mg/kg QD dose was sub-efficacious with minor tumor growth inhibition, while 5 mg/kg twice daily (BID) was an efficacious dose, inducing tumor stasis. We tested both doses of BLU2256 in combination with a low-dose of etoposide (1.25 mg/kg) and observed there is only a benefit of combination treatment when CDK2 is inhibited sub-optimally at 3 mg/kg QD, consistent with the *in vitro* data (Figure 2.5D-E, Figure S 2.5N).

Because HR impairment is synthetically lethal with PARP inhibition (2,3) we reasoned that a CDK2 inhibitor, in combination with a PARP inhibitor should show greater anti-tumor activity compared to single agent. To test this, we treated mice

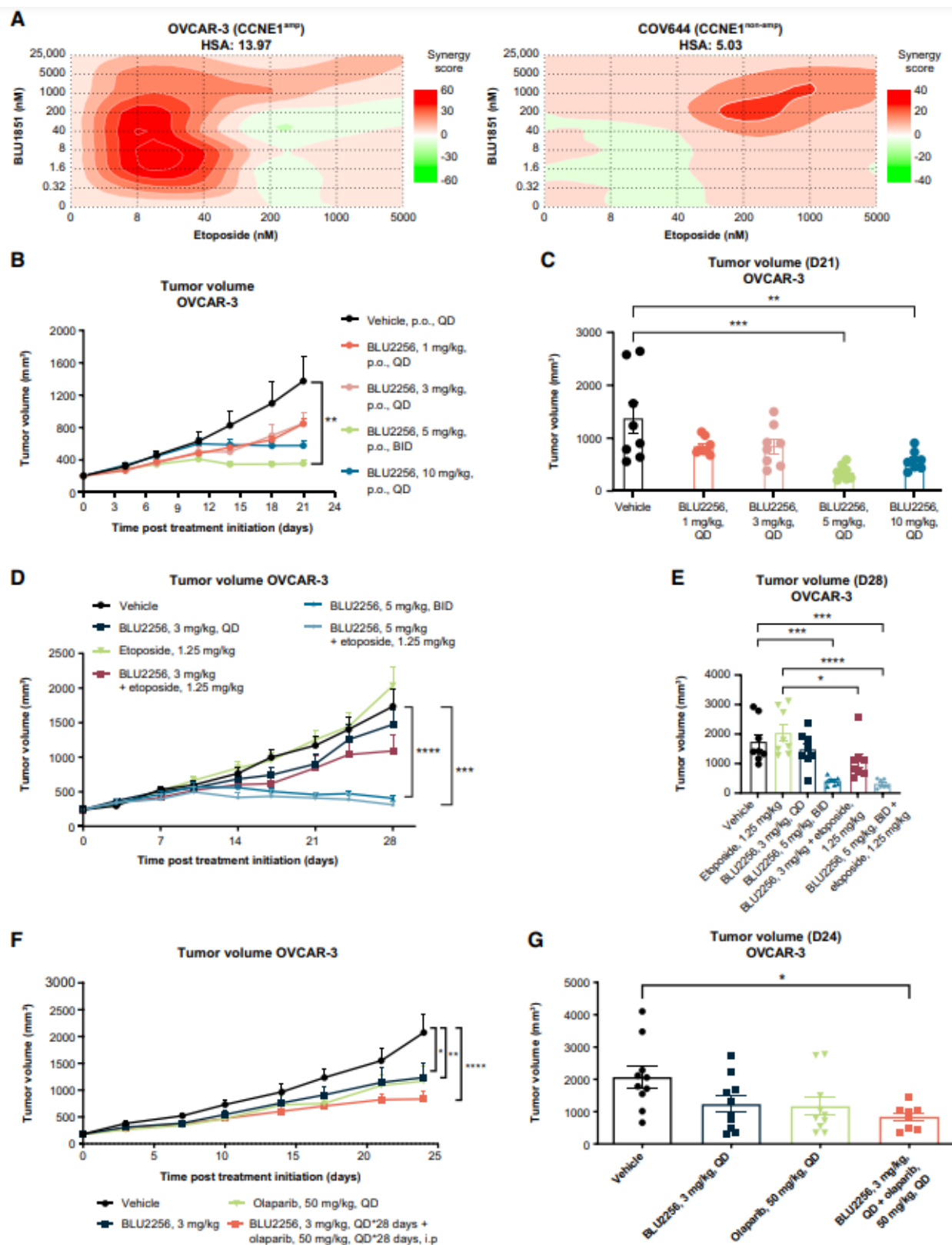


Figure 2.5: CDK2 inhibition sensitizes CCNE1-amplified cells to DNA damaging agents in vitro and in vivo

(A) Synergy score heat maps in OVCAR-3 or COV644 cells after combination treatment with BLU1851 and etoposide. Highest single agent (HSA) synergy model was used to calculate the synergy scores in Synergy Finder. A score <-10 is indicative of antagonism, a score between -10 and 10 is indicative of additivity, and a score >10 is indicative of synergy. Data are representative of at least four independent experiments. **(B)** Tumor volume over time in mice harboring OVCAR-3 xenografts treated with vehicle or BLU2256 at indicated doses QD for 3 weeks. $N = 8/\text{treatment group}$. A two-way ANOVA repeated measures (RM) was performed on day 21 to compare the tumor volume between treatment groups and vehicle groups. Comparisons between groups were carried out with Tukey multiple comparison test. $P < 0.05$ was considered to be statistically significant. $*$ = $P < 0.05$, $**$ = $P < 0.01$, $***$ = $P < 0.001$, $****$ = $P < 0.0001$. **(C)** Tumor volume at day 21 shown in (B). A one-way ANOVA RM was performed to compare the tumor volume between treatment groups and vehicle groups. Comparisons between groups were carried out with Tukey multiple comparison test. $P < 0.05$ was considered to be statistically significant. $*$ = $P < 0.05$, $**$ = $P < 0.01$, $***$ = $P < 0.001$, $****$ = $P < 0.0001$. **(D)** Tumor volume over time in mice harboring OVCAR-3 xenografts treated with vehicle, BLU2256 at indicated doses, Etoposide at 1.25 mg/kg (3 days on, 4 days off). $N = 8/\text{treatment group}$. A two-way ANOVA RM was performed on day 28 to compare the tumor volume between treatment groups and vehicle groups. Comparisons between groups were carried out with Tukey multiple comparison test. $P < 0.05$ was considered to be statistically significant. $*$ = $P < 0.05$, $**$ = $P < 0.01$, $***$ = $P < 0.001$, $****$ = $P < 0.0001$. **(E)** Tumor volume at day 28 shown in (D). A one-way ANOVA RM was performed to compare the tumor volume between treatment groups and vehicle groups. Comparisons between groups were carried out with Tukey multiple comparison test. $P < 0.05$ was considered to be statistically significant. $*$ = $P < 0.05$, $**$ = $P < 0.01$, $***$ = $P < 0.001$, $****$ = $P < 0.0001$. **(F)** Tumor volume over time in mice harboring OVCAR-3 xenografts treated with vehicle, BLU2256 at indicated doses, olaparib at 50 mg/kg QD . $N = 8-10/\text{treatment group}$. A two-way ANOVA RM was performed on day 24 to compare the tumor volume between treatment groups and vehicle groups. Comparisons between groups were carried out with Tukey multiple comparison test. $P < 0.05$ was considered to be statistically significant. $*$ = $P < 0.05$, $**$ = $P < 0.01$, $***$ = $P < 0.001$, $****$ = $P < 0.0001$. **(G)** Tumor volume at day 24 shown in (F). A one-way ANOVA RM was performed to compare the tumor volume between treatment groups and vehicle groups. Comparisons between groups were carried out with Tukey multiple comparison test. $P < 0.05$ was considered to be statistically significant. $*$ = $P < 0.05$, $**$ = $P < 0.01$, $***$ = $P < 0.001$, $****$ = $P < 0.0001$.

harboring the OVCAR-3 xenograft model with BLU2256 at 3 mg/kg QD, olaparib at 50 mg/kg QD, or the combination. We observed that the combination of BLU2256 and olaparib had better anti-tumor activity compared to the single agent treatments (Figure 2.5F-G, Figure S 2.5O). These data support a mechanism by which CDK2 inhibition suppresses HR in *CCNE1*-amplified cells and induces an anti-tumorigenic phenotype when in combination with DNA damaging agents.

Discussion

This study provides a mechanism to explain the mutual exclusivity between *CCNE1* amplification and HRD in HGSOC. We propose that *CCNE1*-amplified ovarian cancer cells rely on HR to resume replication after fork collapse. We also measured high levels of CDK2, cyclin E1, and cyclin A2 protein at the site of stalled replication forks in *CCNE1*-amplified cells which may indicate direct CDK2 catalytic activity at stalled forks. Lastly, we demonstrate that CDK2 is non-redundantly regulating stalled/collapsed replication fork signaling via CHK1 and HR following induced DNA damage in *CCNE1*-amplified cells. Inhibition of CDK2 in combination with etoposide showed better efficacy compared with a single agent at low doses *in vitro* and *in vivo*. This work suggests CDK2 inhibitors in combination with DNA damaging agents may have added clinical benefit.

While previous studies have noted the mutual exclusivity between *CCNE1*-amplification and HRD (Ciriello et al., 2012), the molecular process governing this relationship has not been adequately detailed. Our proposed mechanism that *CCNE1*-amplified cells utilize HR to combat replication stress is consistent with reports that HR genes are upregulated following *CCNE1* overexpression in fallopian tube secretory

epithelial cells, the presumed cell of origin for HGSOC (Karst et al., 2014). A similar mechanism may be apparent in cancer cell lines that grow despite the constant replication stress imposed by high cyclin E1 levels. The frequency of replication fork collapse in *CCNE1* overexpressing human fibroblast cells reveals unique fragile sites in the genome, many of which are instability hot spots in cancer and are prone to rearrangement (Miron et al., 2015). Consistent with this finding, our GCR analysis revealed that *CCNE1*-amplified cells are more likely to have chromosomal fusion events overall, and these fusion events are more likely to occur in *CCNE1*-induced fragile sites. The higher levels of aneuploidy observed in *CCNE1*-amplified cells are also consistent with the hypothesis that replication stress imposed by *CCNE1*-levels induces GCR. In contrast, we did not observe a higher mutational burden in *CCNE1*-amplified cells, which would be associated with more error-prone processes, such as NHEJ or replication restart through TLS. These data support a hypothesis that *CCNE1*-amplified cells have barriers to replication and may be using HR as a method to resume replication at the site of replication fork stall/collapse.

The role of CDK2 in this process has been less clear, as a recent report suggested that unrestrained CDK2 activity can promote excessive fork degradation (Elbæk et al., 2022), despite known roles in maintaining genome integrity (Ferretti et al., 2013; Sviderskiy et al., 2020). A major limitation and potential confounding factor in past studies is the use of non-selective kinase inhibitors to interrogate CDK functions, or the use of a CDK2 analog-sensitive mutant that has impaired cyclin binding and thus low kinase activity (Merrick et al., 2011). Here, we utilized specific and potent CDK2 inhibitors with selectivity not only over other CDK family members but also the entire

kinome, to more precisely assess how CDK2 is promoting repair signaling in *CCNE1*-amplified cells. Our study suggests CDK2 is required in *CCNE1*-amplified cells both for replication fork signaling, as well as HR but is dispensable in non-amplified cells. To our knowledge, this is the first report demonstrating exclusive dependence on CDK2 for DNA repair.

Prior to this study, neither cyclin A2 nor cyclin E1 had been detected at the site of stalled replication forks using methods such as isolation of proteins on nascent DNA (iPOND) (Dungrawala et al., 2015), potentially because this technique is not suited for transient or low abundance interactions. The data presented here indicate active CDK2 is directly associating with stalled replication forks, especially in the *CCNE1*-amplified setting. In contrast, CDK2 does not appear to be a prime actor in replication fork maintenance in *CCNE1* non-amplified cell lines. The physical presence of CDK2, cyclin A2, and cyclin E1 at stalled replication forks in *CCNE1*-amplified cells may indicate these complexes are functioning to promote repair and restoration of the fork. Determining the precise function of CDK2-cyclin E1 or CDK2-cyclin A2 at the site of stalled forks in *CCNE1*-amplified cells is an area of future research.

Loss of CDK2 function can typically be compensated for by other CDK family members, especially by CDK1 (Berthet et al., 2003; Margalef et al., 2018). Indeed, in previous reports, CDK1 was identified as essential for HR and synergized with DNA damaging agents, while CDK2 was dispensable (Johnson et al., 2009, 2011). The present study shows that CDK2 is essential for HR in the *CCNE1*-amplified context and selective inhibition of CDK2 at sub-efficacious doses can sensitize cells to DNA damaging agents. CDK2 is an attractive therapeutic target as a single agent in *CCNE1*-

amplified cancers as cell lines and xenograft models harboring the amplification fail to proliferate after knockdown or catalytic inhibition of CDK2 (Au-Yeung et al., 2017). When treated in combination with etoposide or olaparib, we demonstrated added anti-tumor activity when CDK2 inhibition was sub-efficacious. Because PARP inhibitors have had a major clinical impact in HR-deficient tumors, inducing an HRD phenotype to reveal synthetic lethality with a PARP inhibitor is attractive (Bryant et al., 2005; Farmer et al., 2005). These findings will also be important for clinical applications if patients receiving CDK2 inhibitors need to dose reduce, or to limit potential drug-drug interactions when treated in combination. These data support a CDK2-driven HR mechanism in *CCNE1*-amplified HGSOC.

Our data suggest a model in which *CCNE1*-amplified cell lines rely on HR to repair collapsed replication forks. In addition to its role as a cell cycle regulator, CDK2 non-redundantly coordinates HR in this genetic context. While *CCNE1*-amplified cells have engaged HR as a survival mechanism to combat replication stress, inhibiting this process leaves them vulnerable to synthetic lethality with chemotherapies. As CDK2 inhibitors enter the clinic for HGSOC (clinicaltrials.gov: NCT04553133, NCT05252416), rational drug combinations could open additional clinical avenues and improve patient outcome.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author contributions

V.B. conceived the study and designed the experiments. V.B. and S.M. performed and analyzed most cell-based experiments. M.C. performed all computational analyses. N.H. aided in data interpretation. P.R. designed and characterized the novel CDK2 inhibitors. H.J.W. supported the *in vivo* studies and performed statistical analysis. Y.J.C. supervised some aspects of the project. A.G. supervised most aspects of the project and provided editorial support in manuscript writing. V.B. wrote the manuscript with contributions from all the authors.

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data checking of information provided in the manuscript. However, ultimate responsibility for opinions, conclusions, and data interpretation lies with the authors.

Declaration of interests

V.B., M.C., N.H., P.R., and A.G. are current employees of Blueprint Medicines Corporation and receive stock options. H.J.W and Y.J.C were previous employees of Blueprint Medicines; S.M. has no disclosures.

Funding statement

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Supplementary Material

Materials

Cell lines

CELL LINE	SOURCE	CATALOG NUMBER
OVCAR-3	American Tissue Type Collection	HTB-161
hTERT-RPE1	American Tissue Type Collection	CRL-4000
A549	American Tissue Type Collection	CRL-185
COV644	Millipore Sigma	07071908
COV318	Millipore Sigma	07071903
FUOV1	DSMZ	ACC 444
MCF-7	American Tissue Type Collection	HTB-22

Western blot

ANTIBODY	SOURCE	CATALOG NUMBER
Cdk2	Santa Cruz Biotechnology	SC-6248, RRID:AB_627238

p16	Cell Signaling Technology	CST-80772, RRID:AB_2799960
Cyclin E1	Santa Cruz Biotechnology	SC-247, RRID:AB_627357
pRb S807/811	Cell Signaling Technology	CST-8516 , RRID:AB_11178658
Actin	Cell Signaling Technology	CST-8457, RRID:AB_10950489
p21	Cell Signaling Technology	CST-2947, RRID:AB_823586
Cyclin A2	Cell Signaling Technology	CST-4656, RRID:AB_2071958
CtIP	Cell Signaling Technology	CST-9201, RRID:AB_10828593
Actin	Cell Signaling Technology	CST-3700, RRID:AB_2242334
pH2A.X S139	Cell Signaling Technology	CST-9718, RRID:AB_2118009
pRPA S4/8	Bethyl	Bethyl #A300-245A, RRID:AB_210547
pCdk2 T160	Cell Signaling Technologies	CST-2561, RRID:AB_2078685
Cdk1	Santa Cruz Biotechnology	SC-54, RRID:AB_627224
pCdk1 Y15	Cell Signaling Technologies	CST-4539, RRID:AB_560953
pChk1 S317	Cell Signaling Technologies	CST-2344, RRID:AB_331488
Chk1	Cell Signaling Technologies	CST-2360, RRID:AB_2080320
RPA32	Cell Signaling Technologies	CST-2208 RRID:AB_2238543
Tubulin	Sigma-Aldrich	T9026, RRID:AB_477593
Rad51	Abcam	Ab1333524, RRID: AB_2722613
Rev1	Santa Cruz Biotechnology	SC-393022; RRID: Ab_2885169
Donkey anti-mouse	LI-COR	926-32212, RRID:AB_621847
Donkey anti-rabbit	LI-COR	926-32213, RRID:AB_621848
Goat anti-mouse	LI-COR	926-68070, RRID:AB_10956588
Goat anti-rabbit	LI-COR	926-68071, RRID:AB_10956166
Goat anti-rat	LI-COR	926-68076,

Phospho-Lamin S22 AlphaLISA

ANTIBODY	SOURCE	CATALOG NUMBER
pLamin S22	Cell Signaling Technologies	13448, RRID:AB_2798221
Total Lamin	Santa Cruz Biotechnology	SC-7292, RRID:AB_627875

Proximity ligation assay

ANTIBODY	SOURCE	CATALOG NUMBER
Biotin	Cell Signaling Technologies	5597, RRID:AB_10828011
Biotin	Sigma-Aldrich	SAB4200680
Cdk2	Santa Cruz Biotechnology	SC-6248; RRID: AB_627238
Cyclin E1	Sigma-Aldrich	SAB1400044, RRID:AB_184738
Cyclin A2	Santa Cruz Biotechnology	SC-271682, RRID:AB_10709300

Critical commercial assays

ASSAY	SOURCE	CATALOG NUMBER
Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit	Thermo Fisher Scientific	C140424
FxCycle Violet	Thermo Fisher Scientific	F10347
CyQuant Direct Cell Proliferation Assay	Thermo Fisher Scientific	C35011
Phospho-Rb (Thr821/826) AlphaLISA SureFire Ultra	Perkin Elmer	ALSU-PRB-B500

Compounds

ASSAY	SOURCE	CATALOG NUMBER
BLU1851	Blueprint Medicines	N/A
BLU2256	Blueprint Medicines	N/A
RO-3306	Selleck Chemicals	S7747
Roscovitine	Selleck Chemicals	S1153
Etoposide	Millipore Sigma	E1383
Hydroxyurea	Millipore Sigma	H8627
Rabusertib	Selleck Chemicals	S2626
Mirin	Selleck Chemicals	S8096

Streptonigrin	Sigma Aldrich	S1014
JH-RE-06	Selleck Chemicals	S8850
DMSO	Thermo Fisher Scientific	D12345
Doxycycline	Takara	631311

Plasmids

VECTOR	SOURCE
pBluescript II SK+	Genscript
pLentiCRISPRV2	Genscript
SMARTvector inducible shRNA	Horizon Discovery

siRNA

TARGET	SOURCE	CATALOG NUMBER
NTC	Horizon Discovery	D-001810-01-20
RAD51 #1	Horizon Discovery	J-003530-10
RAD51 #2	Horizon Discovery	J-003530-11
RAD51 #3	Horizon Discovery	J-003530-12
REV1 #1	Horizon Discovery	J-008234-07
REV1 #2	Horizon Discovery	J-008234-08
REV1 #3	Horizon Discovery	J-008234-05

sgRNA

TARGET	SOURCE	SEQUENCE
sgScramble	GenScript	5'-CCTGGGTTAGAGCTACCGCA-3'
sgACTB	GenScript	5'-CCGCCTAGAAGCATTGCGG-3'

shRNA

TARGET	SOURCE	CATALOG NUMBER
shNTC	Horizon Discovery	Cat# VSC6572 Lot# V20012406
shCdk2 #1	Horizon Discovery	Cat# V3SH7670-229933017; clone ID V3IHSHEG_9870667
shCdk2 #2	Horizon Discovery	Cat# V3SH7669-227445411; clone ID V3IHSHEG_7383061

shCdk1#1	Horizon Discovery	Cat# V3SH7669- 229925658; clone ID V3IHSHEG 9863308
shCdk1 #2	Horizon Discovery	Cat# V3SH7669- 229395843; clone ID V3IHSHEG 9333493

Figures

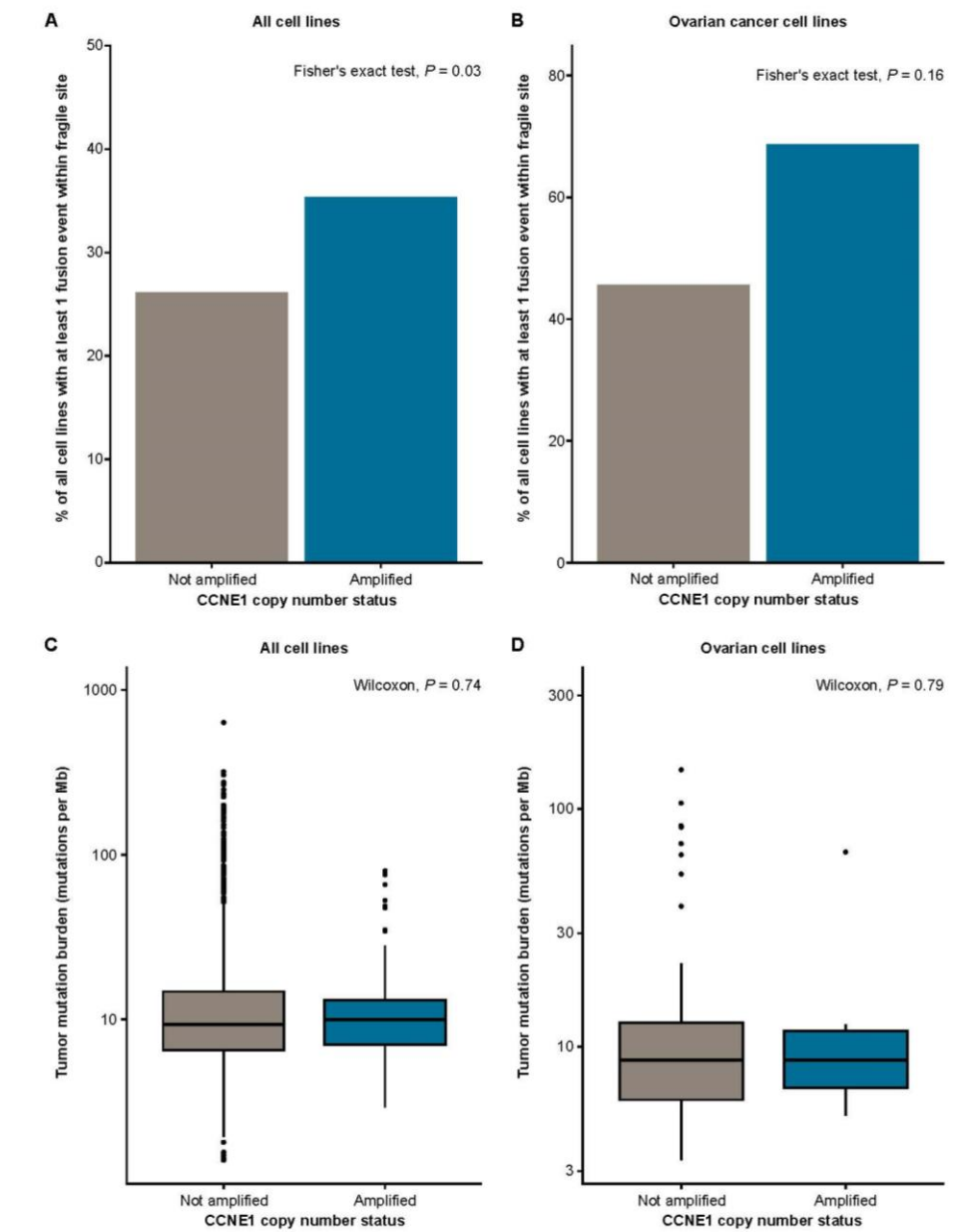
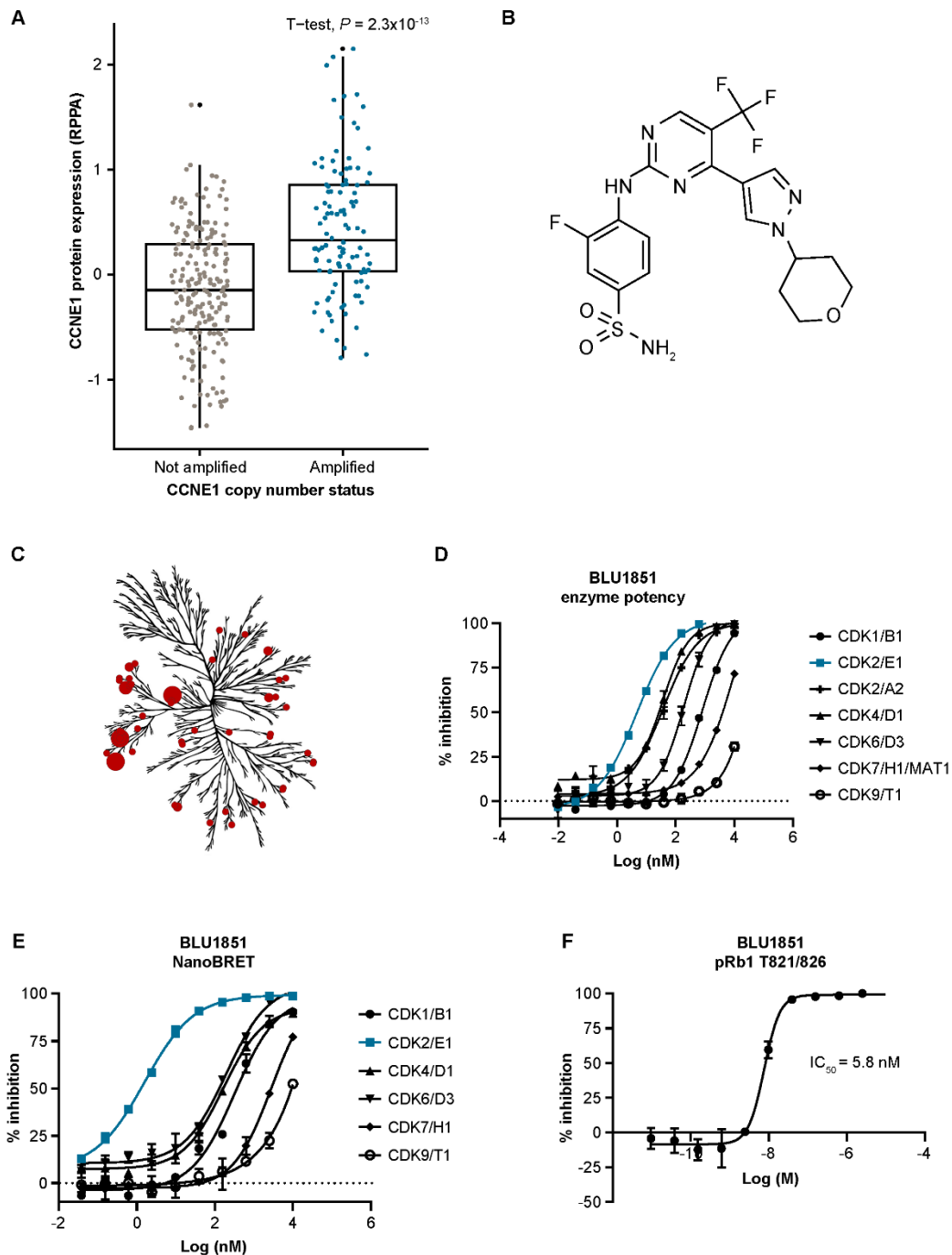
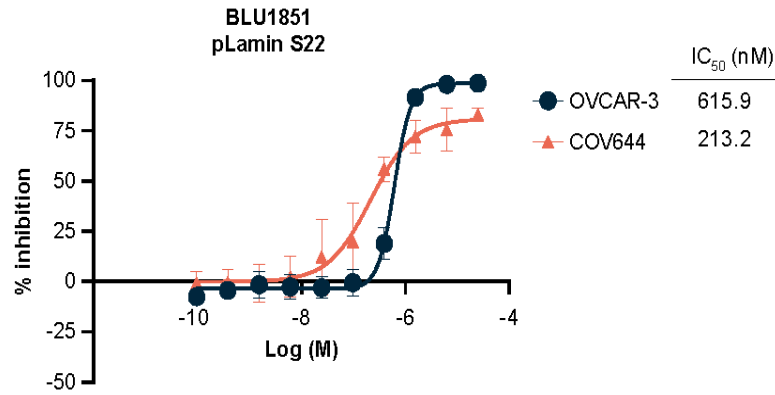
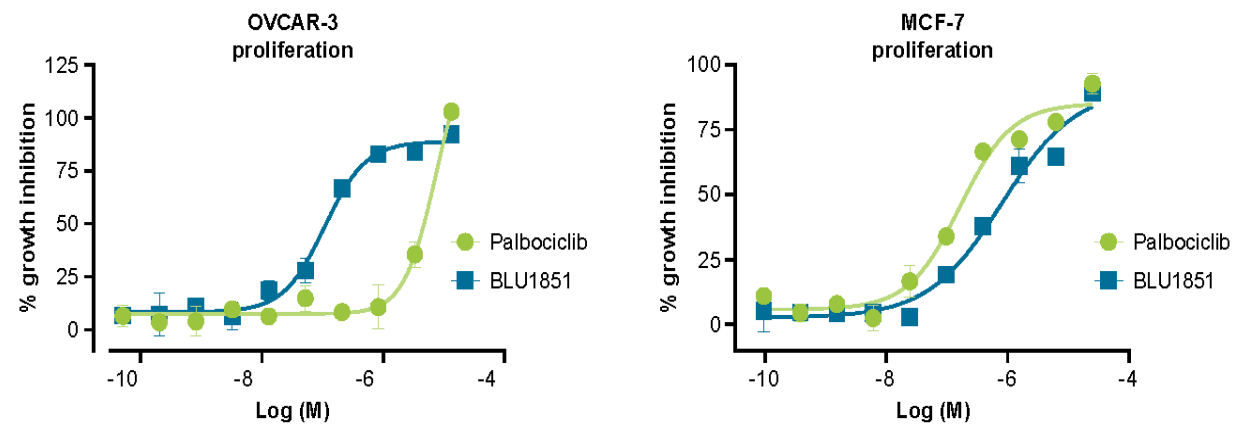
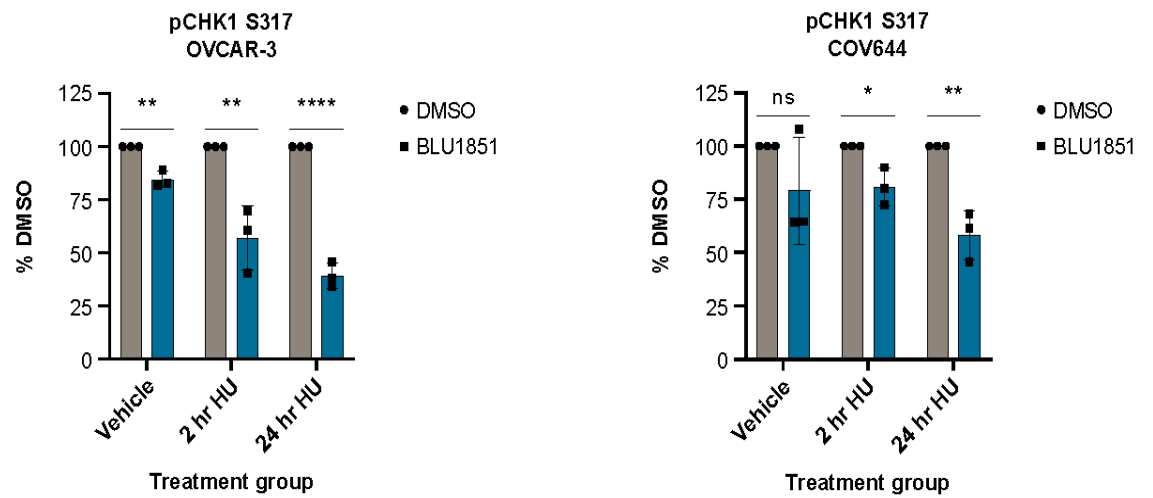


Figure S 2.1: *CCNE1*-amplified ovarian cancer cell lines are prone to GCR

(A) Percent of cell lines with at least one fusion event within a known fragile site in amplified and non-amplified cancer cell lines ($n=1371$) (Fisher's Exact Test, $P = 0.03$). (B) Percent of cell lines with at least one fusion event within a known fragile site in amplified and non-amplified ovarian cancer cell lines ($n=64$) (Fisher's Exact Test, $P =$

0.16). **(C)** Difference in estimated tumor mutation burden in CCNE1-amplified and non-amplified cancer cell lines (n=1371) (Wilcoxon Rank Sum Test, $P = 0.74$). **(D)** Difference in estimated tumor mutation burden in CCNE1-amplified and non-amplified ovarian cancer cell lines (n=64) (Wilcoxon Rank Sum Test, $P = 0.79$).



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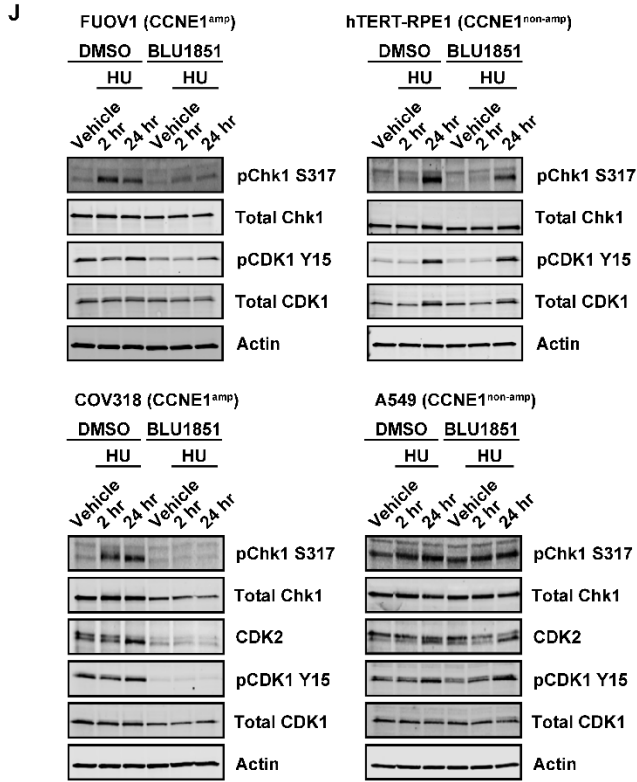
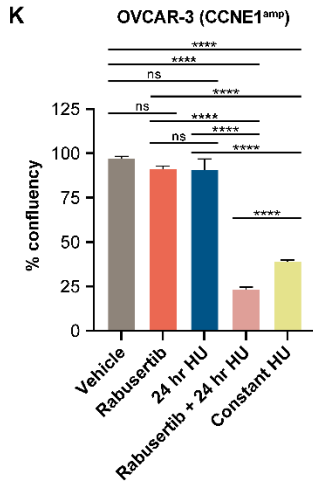
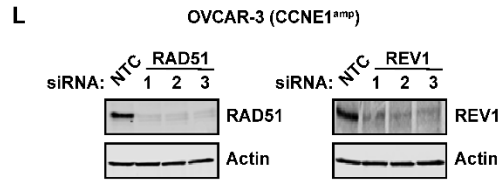
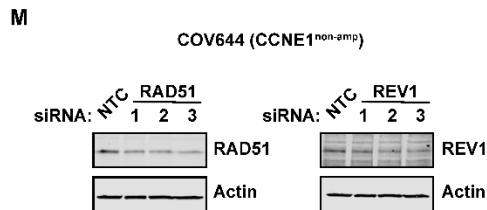
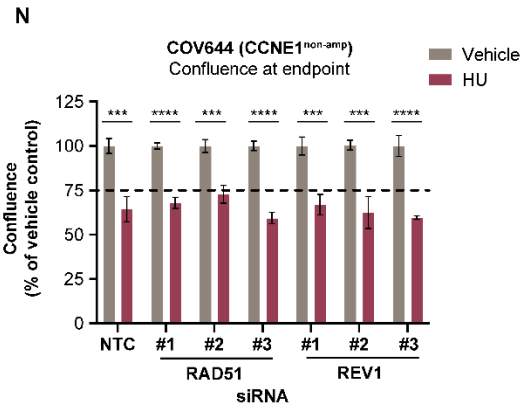


Figure S 2.2: Replication fork stall signaling is dependent on CDK2, HR in *CCNE1*-amplified cells

(A) Cyclin E1 protein expression is strongly associated with *CCNE1* copy number amplification among TCGA patients with ovarian cancer (n=304) (Welch Two Sample t-test, $P = 2.3E-13$). **(B)** Chemical structures of BLU1851. **(C)** Kinome tree for BLU1851 at 3 μ M (S-Score = 0.050). **(D)** Dose-response curves for BLU1851 enzyme inhibition for indicated CDK family members. Error bars represent SD of at least duplicate independent experiments. **(E)** NanoBRET assessment of BLU1851 target engagement in indicated CDK complexes expressed in HEK-293 cells. Error bars represent SD of at least duplicate independent experiments. **(F)** Dose-response curve for pRb T821/826 inhibition by BLU1851 in OVCAR-3 cells. Error bars represent SD of at least duplicate independent experiments. **(G)** Dose-response curve for pLamin S22 inhibition by BLU1851 in OVCAR-3 and COV644 cells. Error bars represent SD of duplicate independent experiments. **(H)** Dose-response curves in MCF-7, OVCAR-3 cells. Cells were treated with a 10-point dose response of indicated compounds and incubated for 5 days before CyQuant assay was performed. Error bars represent SD of at least duplicate independent experiments. **(I)** Quantification of pCHK1 S317 normalized to actin from Figure 2C. Error bars represent SD in three independent experiments. Statistical analysis: Student's T-test, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, ns = not significant. **(J)** Western blot analysis of indicated antibodies in FUOV1, COV318, hTERT-RPE1, and A549 cells. Cells were treated with BLU1851 (100 nM) for 24 hours prior to addition of hydroxyurea (HU) (2 mM) at indicated time points. **(K)** Confluency of OVCAR-3 at experimental endpoint. OVCAR-3 cells were treated for 2 hours with rabusertib (500 nM) before the addition of HU (2 mM) for 24 hours. After 24 hours, HU was washed out and cells were allowed to recover in the presence of rabusertib. Confluence was calculated using IncuCyte. Error bars represent SD in duplicate experiments. Statistical analysis: Student's t-test, *** = $P < 0.001$, **** = $P < 0.0001$, ns = not significant. **(L)** Western blot analysis of indicated antibodies. OVCAR-3 cells were treated as in Figure 4E in an independent experiment, and collected at day 7 after initial transfection. **(M)** Western blot analysis of indicated antibodies. COV644 cells were transfected with non-targeting siRNA (NTC), RAD51 siRNA, or REV1 siRNA, and collected 48 hours later. **(N)** Cell confluency at experimental endpoint. Confluence was measured by IncuCyte live cell imaging and values were determined using an algorithm to detect cells from phase-contrast images. Cells were transfected with siRNA 24 hours before addition of HU (2 mM) for 24 hours. After the HU was washed out, cells were transfected again with indicated siRNAs and allowed to incubate for 5 additional days. HU-treated groups were normalized to vehicle-treated groups for each siRNA at the endpoint. The dotted line represents confluency of cells treated with constant HU exposure over the time course of the experiment. Error bars represent SD in duplicate experiments. Statistical analysis: Student's t-test, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, ns = not significant.

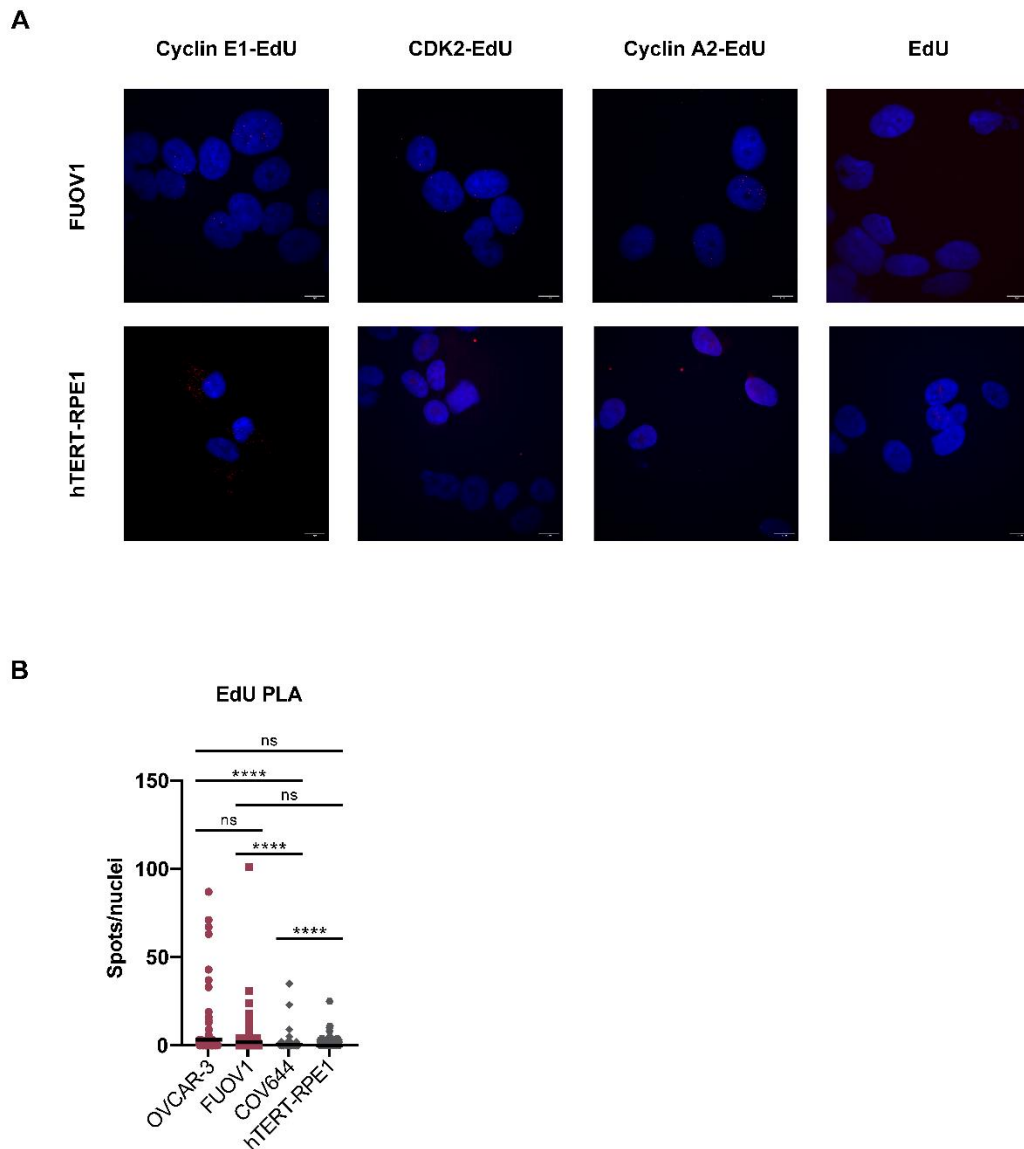
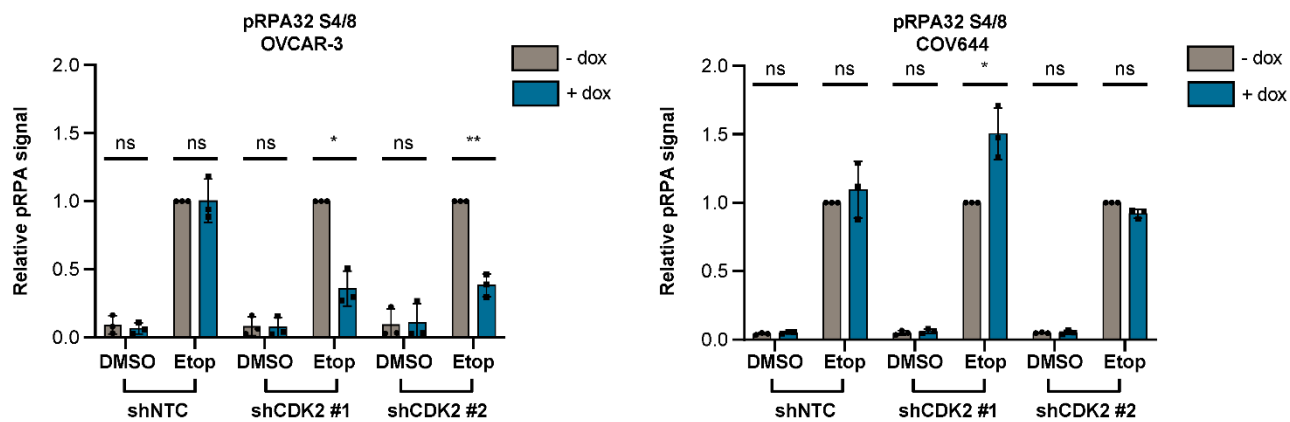


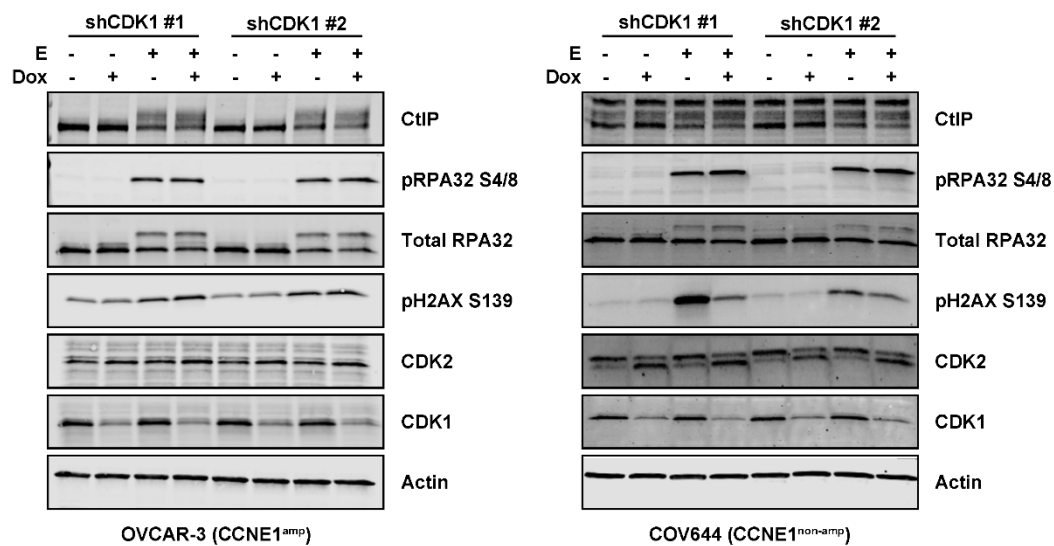
Figure S 2.3: CDK2 and cyclin E1 is recruited to stalled replication forks in *CCNE1*-amplified cells

(A) Representative images of PLA for each noted interaction in FUOV1 and hTERT-RPE1 cells. (B) The negative control excluded 1 primary antibody. The graph shows the mean number of PLA spots per cell in each PLA assay. Bars represent the mean (ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, ***** = $P < 0.00001$, Mann-Whitney test; n = at least 75 cells/experiment)

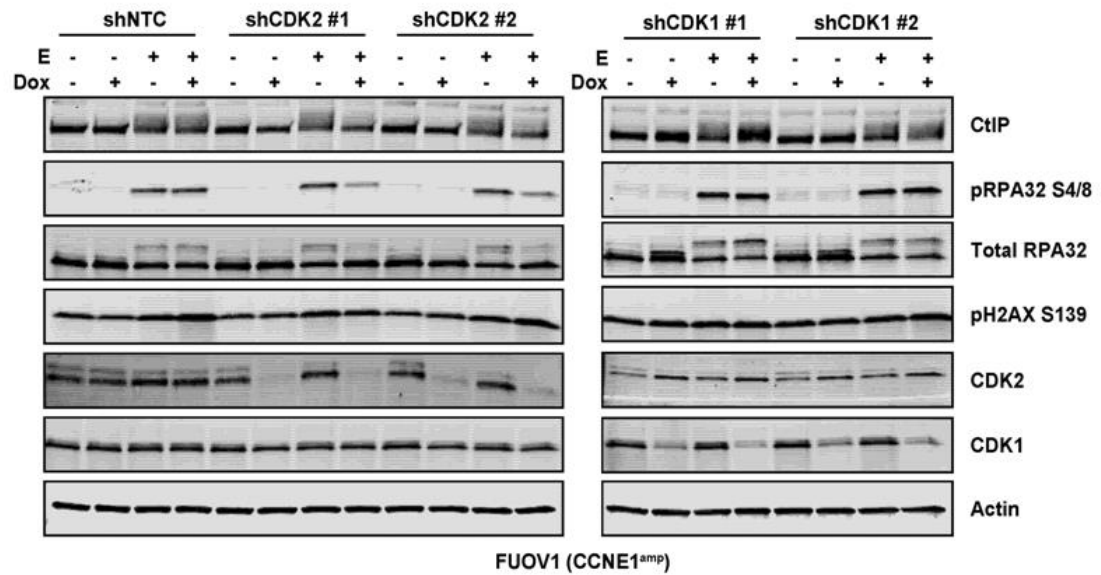
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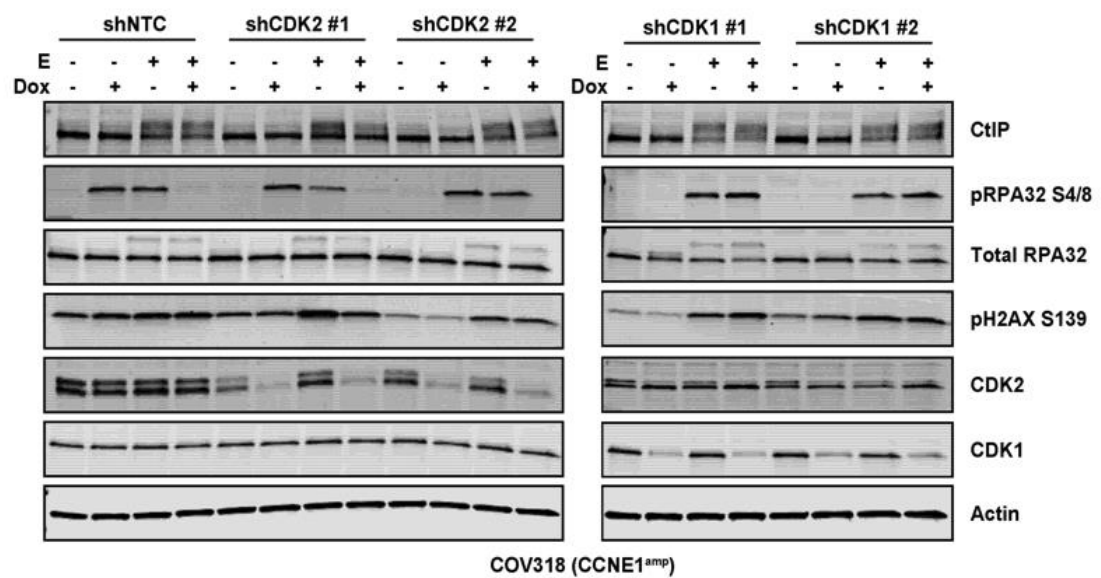
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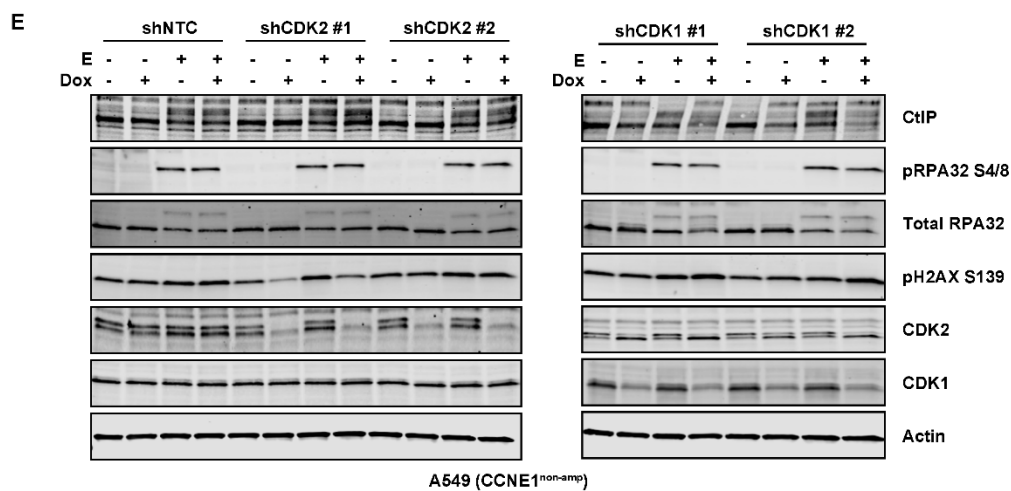
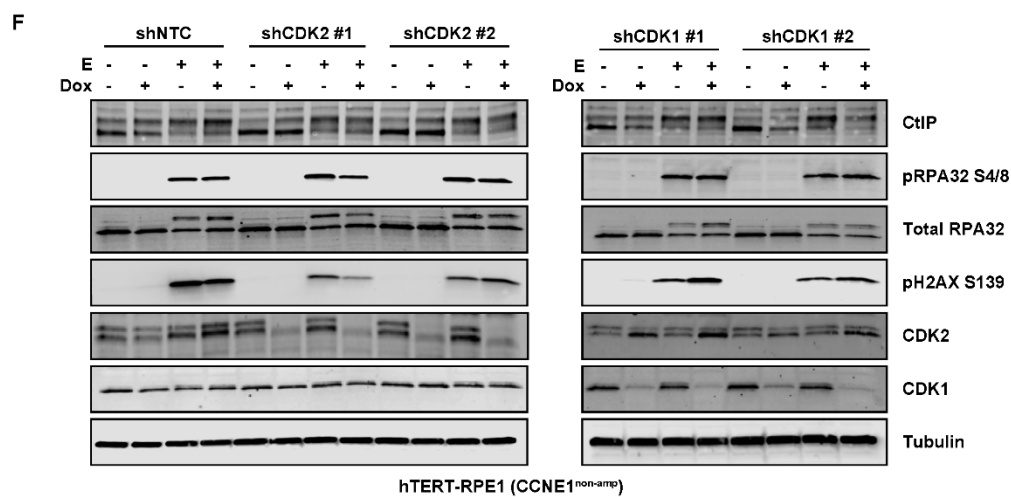
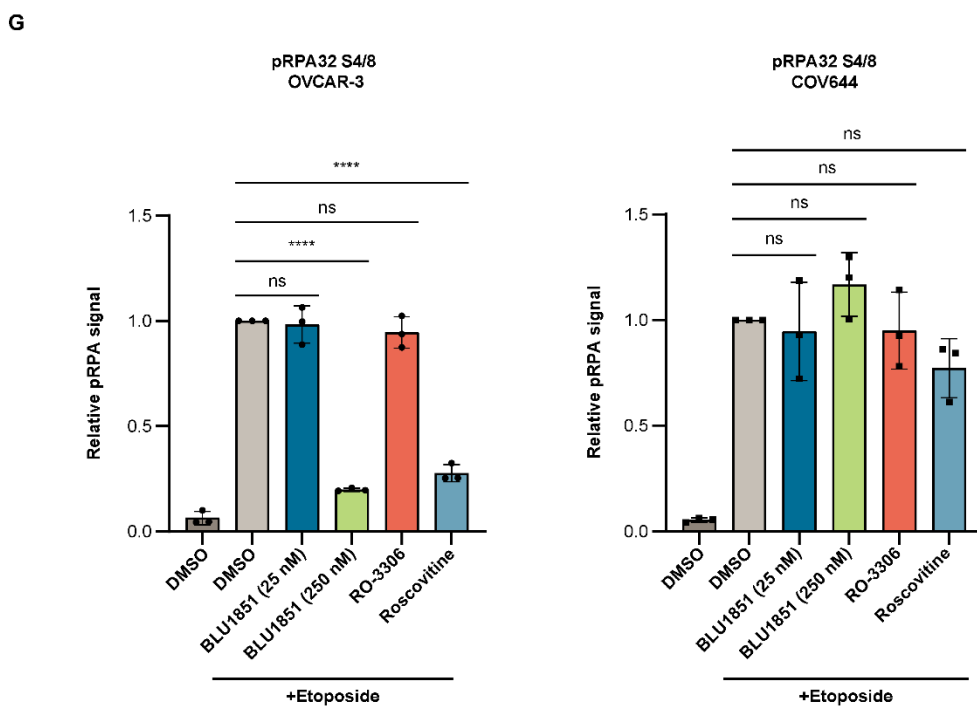


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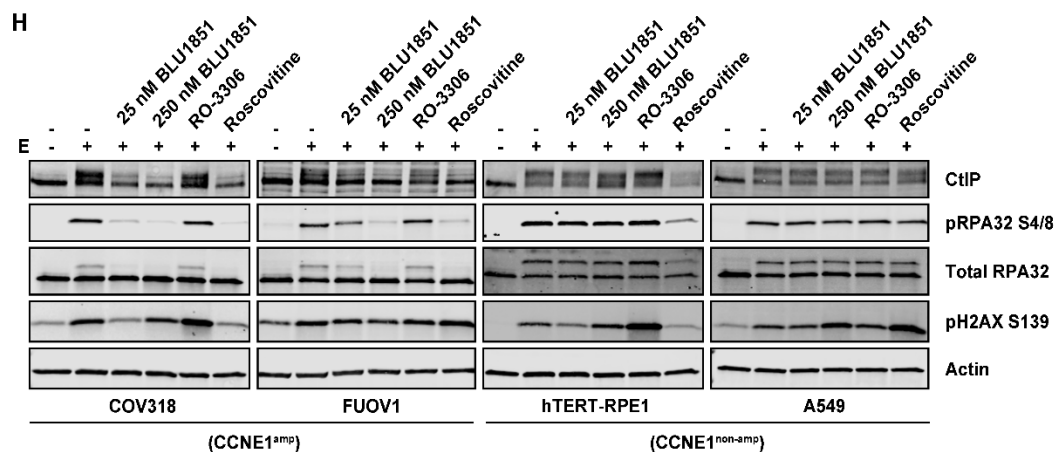
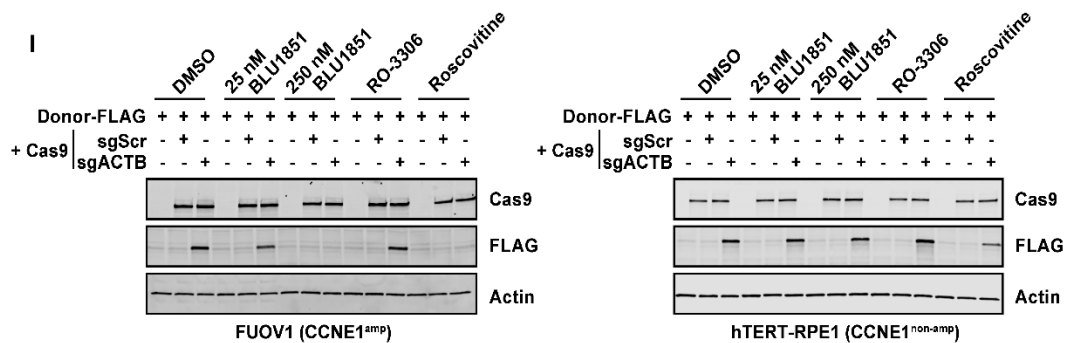
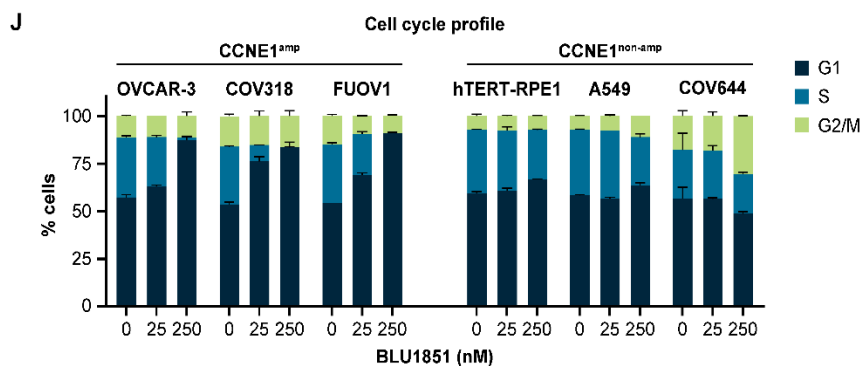
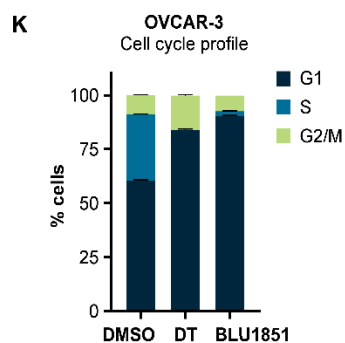
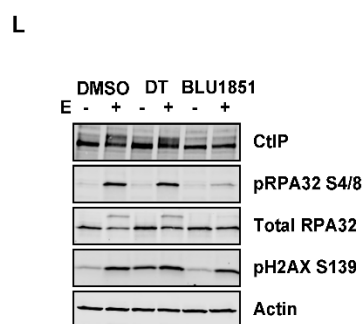
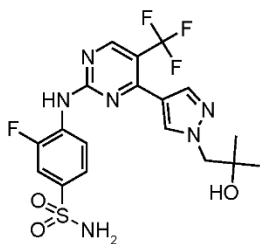
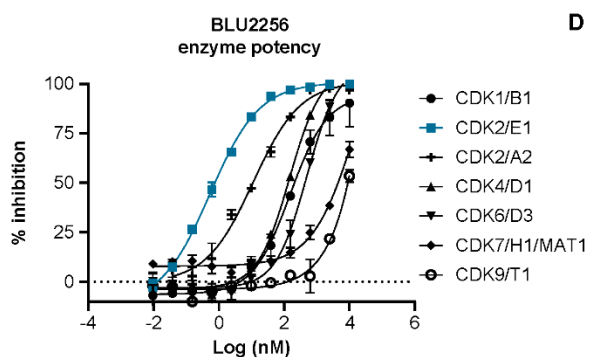
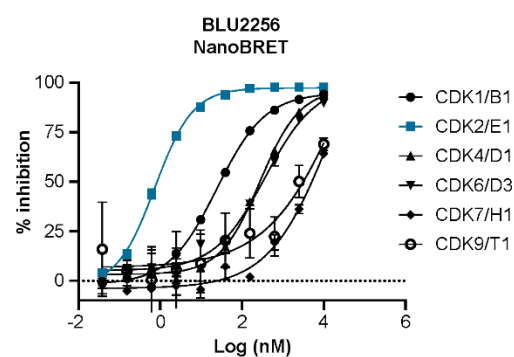
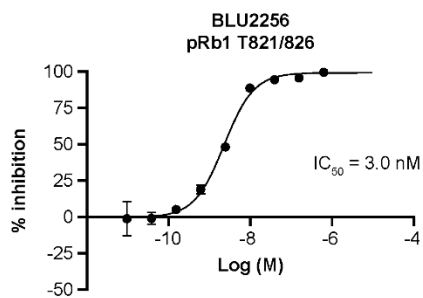
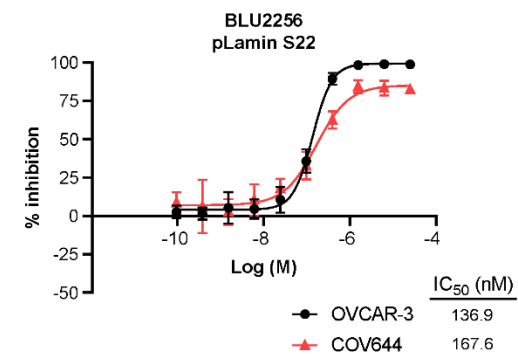
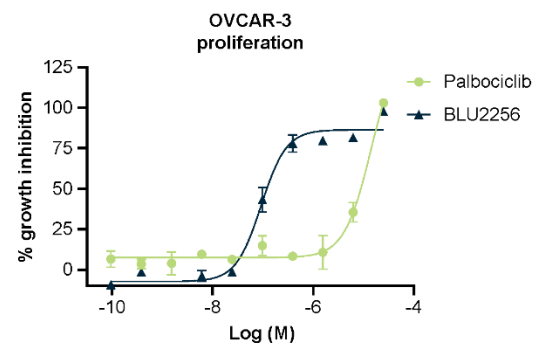
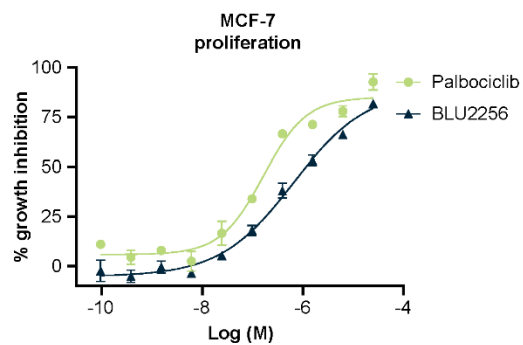


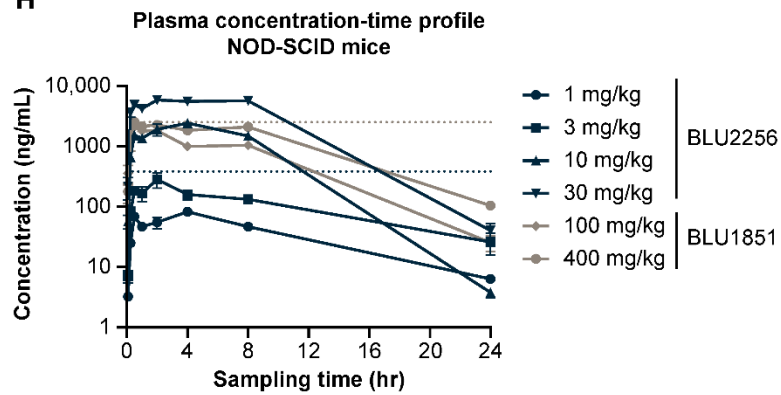
Figure S 2.4: CDK2 regulates HR in *CCNE1*-amplified cells

(A) Quantification of pRPA normalized to actin from Figure 4A. Error bars represent SD in three independent experiments. Statistical analysis: Student's T-test, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, ns = not significant.

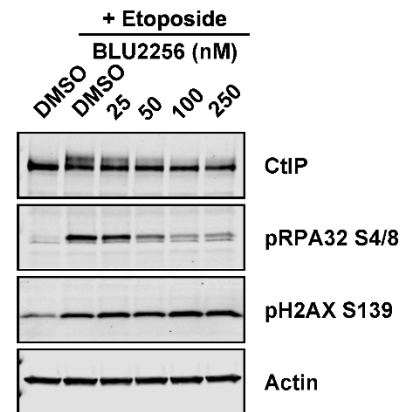
(B) Western blot analysis with the indicated antibodies. Small hairpin RNAs (shRNAs) targeting CDK1 were induced by doxycycline (dox) for 96 hours prior to etoposide (E) treatment at 50 μ M for 2 hours in OVCAR-3 or COV644 cells. (C) Western blot analysis with the indicated antibodies. shRNAs targeting CDK2, CDK1, or a non-targeting control (NTC) were induced by doxycycline (dox) for 96 hours prior to etoposide (E) treatment at 50 μ M for 2 hours in FUOV1 cells. (D) Western blot analysis with the indicated antibodies. shRNAs targeting CDK2, CDK1, or a NTC were induced by dox for 96 hours prior to etoposide (E) treatment at 50 μ M for 2 hours in COV318 cells. (E) Western blot analysis with the indicated antibodies. shRNAs targeting CDK2, CDK1, or a NTC were induced by doxycycline (dox) for 96 hours prior to etoposide (E) treatment at 50 μ M for 2 hours in A549 cells. (F) Western blot analysis with the indicated antibodies. shRNAs targeting CDK2, CDK1, or a NTC were induced by doxycycline (dox) for 96 hours prior to etoposide (E) treatment at 50 μ M for 2 hours in hTERT-RPE1 cells. (G) Quantification of pRPA normalized to actin from Figure 4B. Error bars represent SD in three independent experiments. Statistical analysis: Student's T-test, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, ns = not significant. (H) Western blot analysis with the indicated antibodies. Cells were treated with indicated 25 nM or 250 nM BLU1851, 1 μ M RO-3306, or 10 μ M roscovitine for 24 hours before being treated with 50 μ M etoposide (E) for 2 hours. (I) Western blot analysis with indicated antibodies. Cells were treated with 25 nM or 250 nM BLU1851, 1 μ M RO-3306, or 10 μ M roscovitine. Contemporaneously, cells were transfected with a 3X-FLAG donor plasmid plus a plasmid containing Cas9 and a single guide RNA (sgRNA) scramble control (sgScr) or a sgRNA targeting beta-actin (sgACTB). Cells were harvested 72 hours later. (J) Cell cycle profiles after 24 hours of treatment with BLU1851 at indicated concentrations in asynchronously growing cells. Error bars represent SD in duplicate independent experiments. (K) Cell cycle profile of OVCAR-3 cells after double-thymidine block or 24 hour treatment with 250 nM BLU1851. Error bars represent SEM of duplicate independent experiments. (L) Western blot analysis with indicated antibodies after double-thymidine block or 24-hour treatment with 250 nM BLU1851.

A**B****C****D****E****F****G**

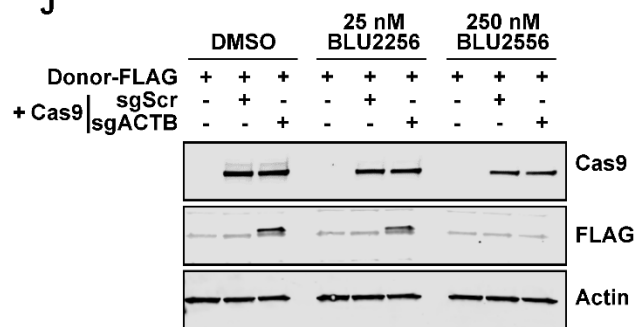
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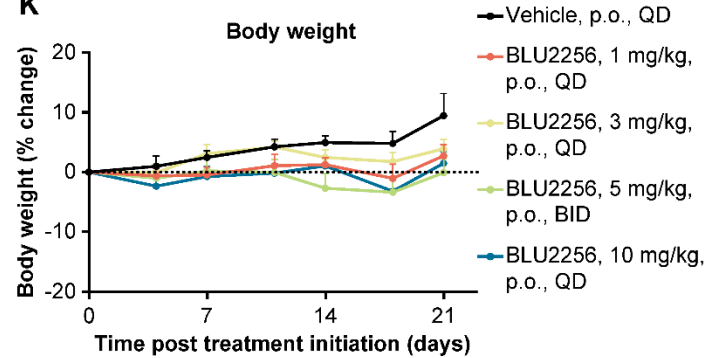
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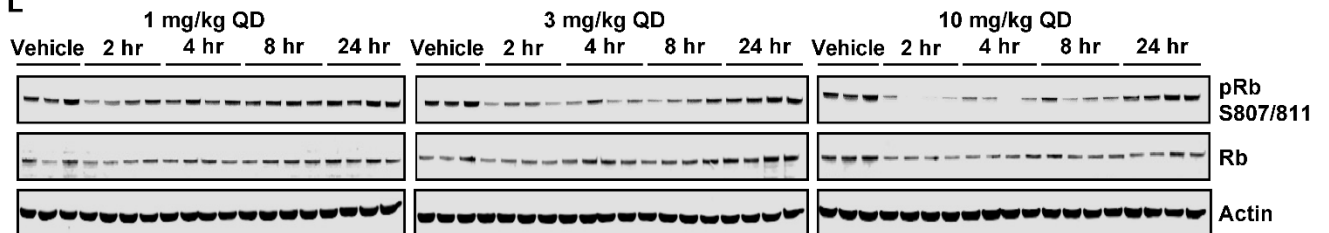
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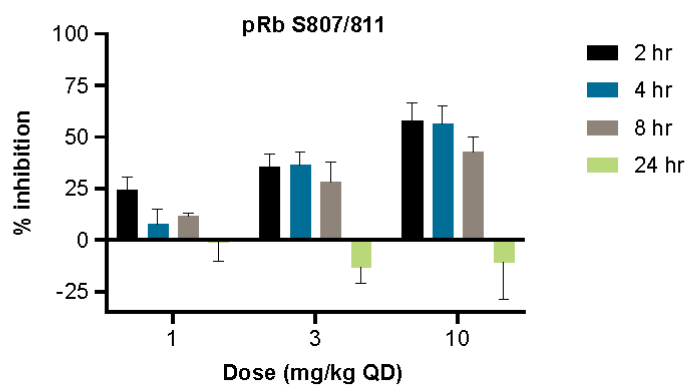
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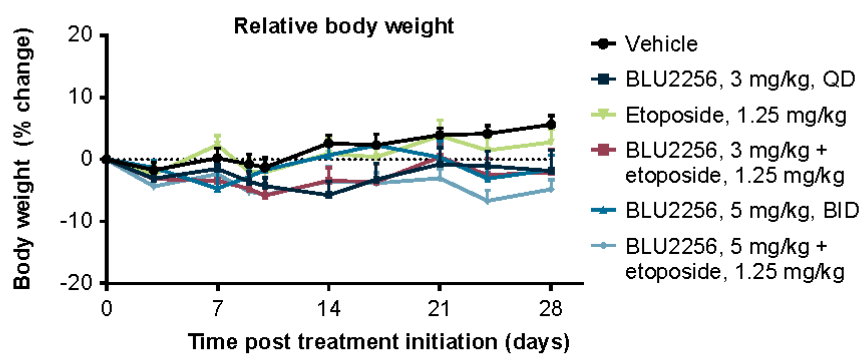
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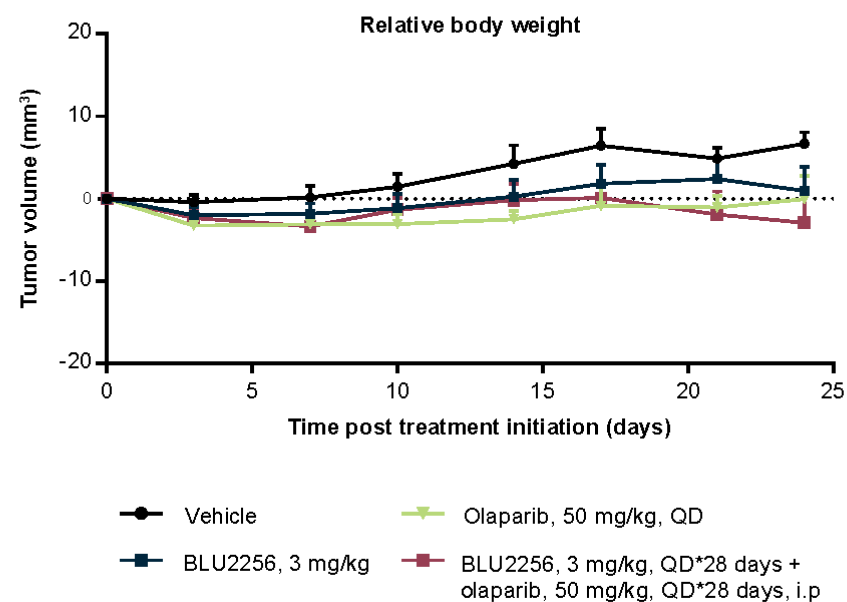


Figure S 2.5: BLU2256 is a selective CDK2 inhibitor

(A) Chemical structures of BLU2256. **(B)** Kinome tree for BLU2256 at 3 μ M (S-Score = 0.040). **(C)** Dose-response curves for BLU2256 enzyme inhibition for indicated CDK family members. Error bars represent SD of at least duplicate independent experiments. **(D)** NanoBret assessment of BLU2256 target engagement in indicated CDK complexes expressed in HEK-293 cells. Error bars represent SD of at least duplicate independent experiments. **(E)** Dose-response curve for pRb T821/826 inhibition by BLU2256 in OVCAR-3 cells. Error bars represent SD of at least duplicate independent experiments. **(F)** Dose-response curve for pLamin S22 inhibition by BLU1851 in OVCAR-3 and COV644 cells. Error bars represent SD of duplicate independent experiments. **(G)** Dose-response curves in MCF-7, OVCAR-3 cells. Cells were treated with a 10-point dose response of indicated compounds and incubated for 5 days before CyQuant assay was performed. Error bars represent SD of at least duplicate independent experiments. **(H)** Plasma concentrations of BLU2256 or BLU1851 at indicated doses. NOD-SCID mice were dosed once p.o. with indicated compound and plasma was collected at 0.5, 2, 4, 8, and 24 hours. The dashed lines indicate the in vitro proliferative IC₅₀ in OVCAR-3 cells corrected for plasma protein binding. Error bars represent SEM. N = 3 mice/timepoint. **(I)** Western blot analysis with the indicated antibodies. OVCAR-3 cells were treated with indicated concentrations of BLU2256 for 24 hours before being treated with 50 μ M etoposide (E) for 2 hours. **(J)** Western blot analysis with indicated antibodies. OVCAR-3 cells were treated with 25 nM or 250 nM BLU2256. Contemporaneously, cells were transfected with a 3X-FLAG donor plasmid plus a plasmid containing Cas9 and a single guide RNA (sgRNA), scramble control (sgScr), or an sgRNA targeting beta-actin (sgACTB). Cells were harvested 72 hours later. **(K)** Relative body weight over treatment time. The percent body weight change is calculated by normalizing to day 0 per treatment group. Error bars represent SEM. N = 8 mice/group. **(L)** Western blot analysis of indicated antibodies from tumors treated with BLU2256 at indicated doses. **(M)** Quantification of Western blot for pRb S807/811 normalized individually to actin and then to vehicle group to calculate % inhibition. Error bars represent SEM. N = at least 3/group. BW, body weight. **(N)** Relative body weight over treatment time. The percent body weight change is calculated by normalizing to day 0 per treatment group. Error bars represent SEM. N = 8 mice/group. BW, body weight. **(O)** Relative body weight over treatment time. The percent body weight change is calculated by normalizing to day 0 per treatment group. Error bars represent SEM. N = 8-10 mice/group. BW, body weight.

Tables

Gene	Category	Genes in category	Log ₂ odds ratio	P value	Tendency
CCNE1	HR deficient	BRCA1, BRCA2, CDK12, RAD50, RAD52, RAD54L	-1.03	0.1148	Mutual exclusivity
CCNE1	Fanconi anemia	PALB2, FANCA, FANCC, FANCI, FANCL	-1.77	0.2397	Mutual exclusivity
CCNE1	DNA repair	ATM, ATR, CHEK1, CHEK2	-1.21	0.2647	Mutual exclusivity
CCNE1	RB1	RB1	-1.311	0.057	Mutual exclusivity
CCNE1	PIK3CA	PIK3CA	0.10	0.4425	Co-occurrence

ATM, ataxia-telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related; BRCA1, breast cancer type 1; BRCA2, breast cancer type 2; CCNE1, cyclin E1; CDK12, cyclin-dependent kinase 12; CHEK 1, checkpoint kinase 1; CHEK 2, checkpoint kinase 2; FA, Fanconi anemia; FANCA, Fanconi anemia, complementation group A; FANCC, Fanconi anemia, complementation group C; FANCI, Fanconi anemia, complementation group I; FANCL, Fanconi anemia, complementation group L; HR, homologous recombination; HRD, homologous recombination deficiency; PALB2, partner and localizer of BRCA2; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; RAD50, radiation sensitive 50; RAD52, radiation sensitive 50; RAD54L, radiation sensitive 54-like; RB1, RB transcriptional corepressor 1.

Table S 2.1: CCNE1-amplification trends toward mutually exclusivity with HRD, FA, and DNA repair pathway mutations

Co-occurrence of CCNE1 amplification and alterations in genes of interest in patients with ovarian cancer included in the TCGA. Odds ratio, p-value, and tendency were calculated using cBioPortal.

Cell line	CCNE1 copy number	p53 status	BRCA1/BRCA2/PIK3CA status
hTERT-RPE1	2.00	WT	WT
A549	3.10	WT	WT
COV644	2.38	WT	WT
OVCAR-3	12.3	R248Q	WT
COV318	6.70	I195F	WT
FUOV1	23.50	H179D	WT

Table S 2.2: CCNE1, p53, BRCA status for model cell lines

CCNE1 copy number and p53, BRCA1, BRCA2, and PIK3CA mutations sourced from CCLE. CCNE1 copy numbers and p53 status for A549, COV644, OVCAR-3, COV318, and FUOV1 cells retrieved from CCLE DepMap release 21Q2 were reported as $\log_2(\text{copy number}/\text{ploidy}+1)$. Retransformed copy number estimates are reported in the CCNE1 copy number column assuming diploid status. CCNE1 copy number for hTERT-RPE1 is assumed to be copy number normal based on its diploid status.

A

Compound	CDK2/ CycE1	CDK2/ CycA2	CDK1/ CycB1	CDK4/ CycD1	CDK6/ CycD3	CDK7/CycH1/MNAT1	CDK9/ CycT1
BLU1851	4.1	36.4	736.9	30.8	174.4	4,087.5	>10,000

B

Compound	CDK2/ CycE1	CDK1/ CycB1	CDK4/ CycD1	CDK6/ CycD3	CDK7/ CycH1	CDK9/ CycT1
BLU1851	2.3	337.0	214.7	167.5	2,600.3	9,398.3

C

Compound	MCF-7 IC₅₀ (nM)	OVCAR-3 IC₅₀ (nM)
Palbociclib	149.8	>25,000
BLU1851	1,357.8	282.8

CDK, cyclin-dependent kinase; CDK1, cyclin-dependent kinase 1; CDK2, cyclin-dependent kinase 2; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; CDK7, cyclin-dependent kinase 7; CDK9, cyclin-dependent kinase 9; Cyc, cyclin; CycA2, cyclin A2; CycB1, cyclin B1; CycD1, cyclin D1; CycD3, cyclin D3; CycE1, cyclin E1; CycH1, cyclin H1; CycT1, cyclin T1; IC₅₀, half-maximal drug inhibitory concentration; MNAT1, MNAT CDK-activating kinase assembly factor 1

Table S 2.3: BLU1851 is a selective CDK2 inhibitor

(A) IC₅₀ (nM) of BLU1851 in the ATP-competition caliper enzyme assay in indicated CDK complexes. (B) IC₅₀ (nM) of BLU1851 in the NanoBRET assay performed in HEK-293T cells transfected with indicated CDK complexes. (C) IC₅₀ (nM) of indicated compound in MCF-7 and OVCAR-3 proliferation assays. IC₅₀'s represent the mean of two independent replicates.

A

Compound	CDK2/ CycE1	CDK2/ CycA2	CDK1/ CycB1	CDK4/ CycD1	CDK6/ CycD3	CDK7/Cyc H1/MNAT1	CDK9/ CycT1
BLU2256	0.7	11.3	153.6	117.0	394.6	>6,666.7	9,107.0

B

Compound	CDK2/ CycE1	CDK1/ CycB1	CDK4/ CycD1	CDK6/ CycD3	CDK7/ CycH1	CDK9/ CycT1
BLU2256	5.3	84.9	579.1	735.5	4,422.9	3,702.6

C

Compound	MCF-7 IC₅₀ (nM)	OVCAR-3 IC₅₀ (nM)
Palbociclib	149.8	>25,000
BLU2256	1,282.4	82.5

CDK, cyclin-dependent kinase; CDK1, cyclin-dependent kinase 1; CDK2, cyclin-dependent kinase 2; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; CDK7, cyclin-dependent kinase 7; CDK9, cyclin-dependent kinase 9; Cyc, cyclin; CycA2, cyclin A2; CycB1, cyclin B1; CycD1, cyclin D1; CycD3, cyclin D3; CycE1, cyclin E1; CycH1, cyclin H1; CycT1, cyclin T1; IC₅₀, half-maximal drug inhibitory concentration; MNAT1, MNAT CDK-activating kinase assembly factor 1.

Table S 2.4: BLU2256 is a selective CDK2 inhibitor

(A) IC₅₀ (nM) of indicated compound in the ATP-competition caliper enzyme assay. (B) IC₅₀ (nM) of indicated compound in the NanoBRET assay performed in HEK-293T cells. (C) IC₅₀ (nM) of indicated compound in MCF-7 and OVCAR-3 proliferation assays. IC₅₀'s represent the mean of two independent replicates.

Chapter 3 CRISPR resistance screen identifies p16 as a requirement for CDK2 dependence in *CCNE1*-amplified ovarian cancer cell lines

This data included in this chapter was compiled by Victoria Brown, Maxine Chen, and Sydney Moore, with supervision from Alexandra Grassian.

Abstract

CCNE1-amplification occurs across many different aggressive tumor types but is especially prevalent in high grade serous ovarian cancer (HGSOC). Although these tumor types tend to be intrinsically resistant to standard of care therapies such as platinum agents and Poly-ADP ribose polymerase (PARP) inhibitors, they are especially vulnerable to inhibition of cyclin dependent kinase 2 (CDK2), and several CDK2 inhibitors are in phase I/II clinical trials in this disease indication. To reveal potential mechanisms of resistance to CDK2 inhibitors, we performed a CRISPR resistance screen in a *CCNE1*-amplified cell line and identified *CDKN2A* (p16^{INK4A}) loss as the top hit. We demonstrate that loss of p16 activates CDK4 and allows this kinase to compensate for inhibition of CDK2 in the *CCNE1*-amplified setting. Importantly, this resistance can be overcome with combination treatment of a CDK2 inhibitor with a CDK4/6 inhibitor, suggesting a potential path forward if this resistance mechanism emerges in the clinic.

Introduction

The cell cycle is tightly regulated to promote faithful DNA replication and prevent DNA damage that can be passed onto daughter cells. Cyclin dependent kinases (CDKs)

along with their regulatory binding partners, the cyclins, act as main regulators of the cell cycle. Mammals have three interphase CDKs, CDK2, CDK4, and CDK6, that act mainly via the phosphorylation of the retinoblastoma protein, Rb1, to facilitate E2F-mediated transcription of genes to drive the cell cycle (Lundberg & Weinberg, 1998; Zarkowska & Mitnacht, 1997). CDK1 serves as the mitotic kinase, regulating the G2/M transition (Malumbres & Barbacid, 2005).

Studies in individual CDK knockout mice have demonstrated that other CDKs can typically compensate for the loss of one CDK, with exceptions for specialized roles in development (Berthet et al., 2003; Malumbres et al., 2004; Rane et al., 1999). In fact, CDK1 can compensate for the loss of all three interphase CDKs as CDK2^{-/-}/4^{-/-}/6^{-/-} knockout (KO) mice survive until mid-gestation through non-canonical pairing with D- and E-type cyclins, maintaining the phosphorylation of Rb1 (Santamaría et al., 2007). On the other hand, no CDK can compensate for the loss of CDK1 as CDK1 deletion is embryonic lethal, halting development at the blastocyst stage (Santamaría et al., 2007). These compensatory activities protect cells against disruption in the core cell cycle machinery. However, cancer cells often have a highly dysregulated cell cycle, driving dependence on particular CDKs (Malumbres & Barbacid, 2009).

Increased CDK4 activity can drive dependence on CDK4 for proliferation, especially in breast cancer. Cyclin D1, the canonical partner of CDK4, is itself an oncogene as overexpression of Cyclin D1 in the mammary glands of mice induces hyperplasia and carcinoma (T. C. Wang et al., 1994). Cyclin D1 and associated kinase activity is also required for tumor maintenance as acute deletion of Cyclin D1 induces cellular senescence in *ErbB2* driven tumors (Y. J. Choi et al., 2012). Like Cyclin D1,

CDK4 is required for oncogenic transformation and tumor maintenance of breast cancer cells, and mice overexpressing a CDK4 mutant that cannot bind INK family proteins, R24C, develop hyperplasia in a variety of tissues, including the mammary gland (Reddy et al., 2005; Sotillo et al., 2001; Q. Yu et al., 2006). CDK4, then, became an attractive drug target, with the development of three CDK4 inhibitors approved for the treatment of estrogen receptor positive (ER+)/human epidermal growth factor receptor 2 negative (HER2-) breast cancer in combination with hormone therapy – Palbociclib, Ribociclib, and Abemaciclib (Finn et al., 2015; Slamon et al., 2018; Sledge et al., 2017). Notably, these inhibitors also target the highly homologous kinase, CDK6 to varying degrees.

Despite clinical benefit versus hormone therapy alone, patients often develop resistance to CDK4/6 inhibitors. While these resistance mechanisms continue to emerge, one avenue toward resistance is activation of CDK2. CDK2 is dispensable in most cells as demonstrated by knockdown studies as well as the viable CDK2 KO mouse (Berthet et al., 2003; Tetsu & McCormick, 2003). Retrospective analysis of patients in the Palbociclib clinical trial PALOMA-3 revealed that those patients with high *CCNE1* mRNA expression levels progressed faster on Palbociclib than patients with lower levels of *CCNE1* expression (Turner et al., 2019). As CDK2-Cyclin E1 are canonical partners, these data suggest that CDK2, in the context of *CCNE1* high expression, may compensate for the pharmacological inhibition of CDK4. Preclinical evidence appears to support this as Palbociclib resistant cell lines tend to have increased Cyclin E1 protein levels and become sensitive to the combination of Palbociclib and genetic depletion of either CDK2 or Cyclin E1 (Herrera-Abreu et al.,

2016). CDK2 inhibitors, then, may be an attractive therapy to address, or to prevent, CDK4/6 inhibitor resistance.

Although CDK2 is redundant in most contexts, *CCNE1*-amplified cells, especially high grade serous ovarian cancer (HGSOC), are especially sensitive to genetic suppression of CDK2 (Au-Yeung et al., 2017). HGSOC is a high unmet medical need as these patients are typically resistant to chemotherapy and poly-ADP ribose polymerase (PARP) inhibitors given the mutually exclusivity of *CCNE1*-amplification and homologous recombination deficiency (HRD) (Ciriello et al., 2012; Etemadmoghadam et al., 2009, 2010; Konstantinopoulos et al., 2015). We have previously reported the development of a CDK2-selective inhibitor, BLU-222, for the treatment of *CCNE1*-amplified HGSOC, and clinical trials are underway to evaluate its efficacy in patients (Brown et al, 2022).

Given that patients typically develop resistance to kinase inhibitors, we performed a pooled whole genome Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) screen to identify resistance mechanisms to CDK2 inhibitors in HGSOC. Here, we report that deletion of *CDKN2A*, the gene that encodes the INK family protein p16, was the top hit in a screen of two chemically distinct CDK2 inhibitors, BLU1851 and PF-4091. The loss of p16 leads to activation of CDK4, allowing cells to bypass CDK2 inhibition. Treatment with CDK4/6 inhibitors re-sensitizes cells to CDK2 inhibition, suggesting that, similar to CDK4/6 inhibitor resistance, suppression of all interphase CDKs may be able to address cancer progression in the CDK2-inhibitor resistant setting.

Results

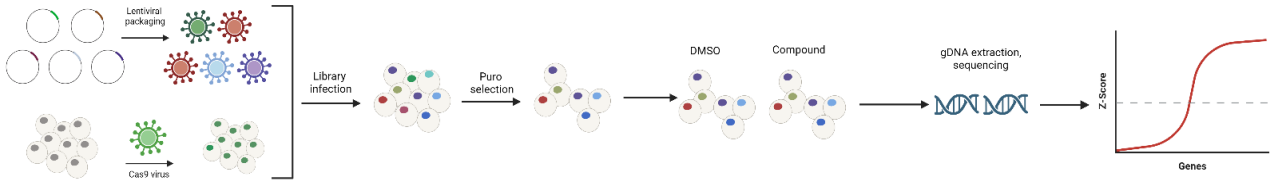
CRISPR screening identifies *CDKN2A* loss as a resistance mechanism to CDK2 inhibitors

We performed a genome-wide CRISPR/Cas9 pooled knockout screen to identify genes regulating sensitivity to CDK2 inhibition in *CCNE1*-amplified HGSOC cells. We chose OVCAR-3 cells as our model as this *CCNE1*-amplified cell line was previously shown to be sensitive to CDK2 depletion and inhibition (Au-Yeung et al., 2017; Brown et al., 2023). To reduce the number of false positives that may derive from a particular chemotype, we utilized two chemically distinct CDK2 inhibitors that both induced an anti-proliferative effect in OVCAR-3 cells (Figure S 3.1A-B). A whole genome single-guide RNA (sgRNA) library, containing ~80,000 constructs (4 sgRNAs/gene), was transduced into OVCAR-3 Cas-9 cells. After three days of Puromycin selection, cells were treated with either DMSO, BLU1851 (200nM), or PF-4091 (200nM) for 7 days. At the experimental end point, genomic DNA was extracted and analyzed for sgRNA abundance (Figure 3.1A). Quality control checks such as total read count, read counts per guide, concordance between replicates, and positive/negative control evaluation were performed (Figure S 3.1C-E).

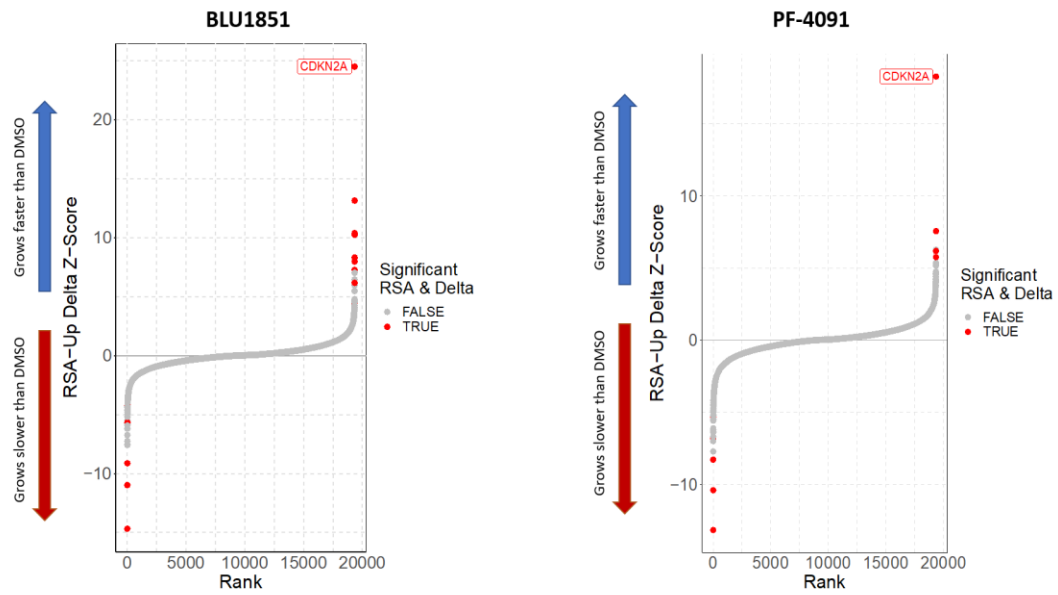
From the library screening, we identified 15 genes whose loss conferred a statistically significant growth advantage under BLU1851 treatment, and 10 genes whose loss conferred a growth advantage under PF-4091 treatment. The top hit that conferred resistance to both CDK2 inhibitors was *CDKN2A*, the gene encoding p16 (Figure 3.1B). To build additional confidence in *CDKN2A* as an important regulator that would drive CDK2 sensitivity, we measured CDK2 essentiality in ovarian cancer cell

lines based on both *CDKN2A* copy number and expression from Dependency Map (<https://depmap.org/portal/>). In concordance with the CRISPR screen data, both *CDKN2A* copy number and expression predicted CDK2 dependence as cell lines that either have copy number loss or low expression of *CDKN2A* were statistically more likely to be resistant to CDK2 knockout ($p = 0.0079$, $p = 0.0017$, respectively) (Figure 3.1C). Taken together, these data support that *CDKN2A* may be required for CDK2 dependence in *CCNE1*-amplified HGSOC.

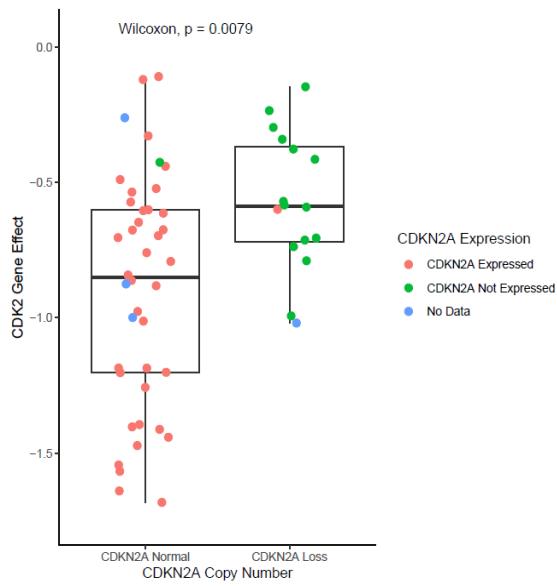
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B



C



D

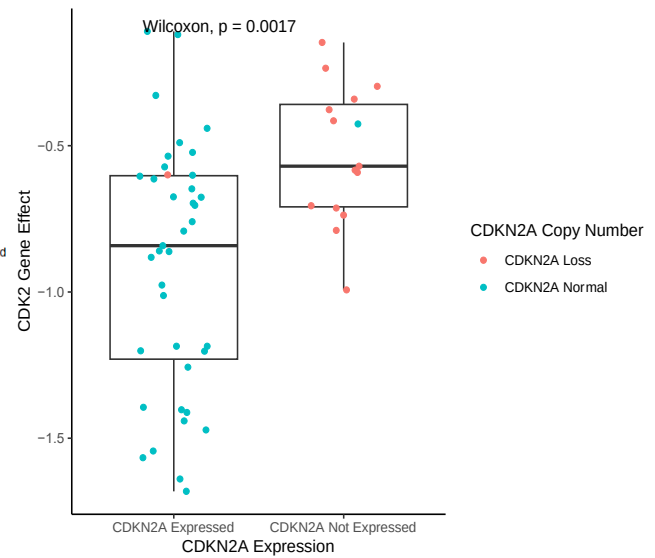


Figure 3.1: CDKN2A is the top hit in CDK2i CRISPR resistance screen

(A) Schematic of CRISPR resistance screen. Stable expressing Cas9 OVCAR-3 cells were transduced with an sgRNA library and subjected to puromycin selection for 3 days before treatment with DMSO, BLU1851 (200nM), or PF-4091 (200nM). After 7 days, genomic DNA was extracted and sequenced for the presence or absence of sgRNAs. Figure created with BioRender.

(B) Identified hits in CRISPR screen for BLU1851 (left) and PF-4091 (right). Significant hits are those >1 standard deviation away from the mean (represented by dotted lines).

(C) Ovarian cancer cell lines with $n=2$ copies of CDKN2A ("CDKN2A normal") or those with $n<2$ copies of CDKN2A ("CDKN2A loss") were assessed for CDK2 dependency ("gene effect"). Wilcoxon rank-sum test, $p = 0.0079$. Data source = DepMap 1Q23

(D) Ovarian cancer cell lines with CDKN2A expression in the top three quartiles ("CDKN2A expressed") or those with CDKN2A expression in the bottom quartile ("CDKN2A not expressed") were assessed for CDK2 dependency ("gene effect"). Wilcoxon rank-sum test, $p = 0.0017$. Data source = DepMap 1Q23

CDKN2A loss increases CDK4 activity

To investigate the mechanism by which *CDKN2A* loss yields resistance to CDK2 inhibition, we engineered OVCAR-3-Cas9+ stable cells to stably express sgRNAs targeting the *CDKN2A* locus. All sgRNAs tested showed marked decrease of p16 protein levels, but sgCDKN2A #1 and sgCDKN2A #2 appeared most robust (Figure 3.2A). Because *CDKN2A* encodes also for p14 via alternative splicing and a distinct reading frame (W. Y. Kim & Sharpless, 2006), we measured the effect on p14 expression after p16 knockout and noted differential effects between the sgRNAs (Figure 3.2A). Since sgCDKN2A #1 and sgCDKN2A #2 have different effects on p14 expression, we reasoned that similar phenotypes between the two sgRNAs, then, could be attributed to p16 loss. We therefore moved forward with subsequent biochemical and phenotypic analysis utilizing sgCDKN2A #1 and sgCDKN2A #2.

CDK4 is activated upon binding to its canonical regulatory partner, Cyclin D1, but its activity can also be suppressed by p16 (Matsushime et al., 1992, 1994; Parry et al., 1999). In cellular contexts where p16 protein levels are high, CDK4-p16, rather than CDK4-Cyclin D1, are reported to be the dominant complex (Parry et al., 1995). We therefore hypothesized that, in the absence of p16, we should observe more Cyclin D1 associating with CDK4. Indeed, after immunoprecipitation of CDK4, we measure higher levels of Cyclin D1 co-immunoprecipitating with CDK4 in sgCDKN2A cells compared to sgNTC cells (Figure 3.2B). Notably, we also tested the possibility that CDK4 may be non-canonically associating with Cyclin E1 given the high levels of Cyclin E1 protein in OVCAR-3 cells, but did not co-immunoprecipitate Cyclin E1 with CDK4 (Figure 3.2B). In the absence of p16, more Cyclin D1 associates with CDK4, suggesting CDK4 may be more catalytically active.

We noticed that, although we pulled down more Cyclin D1 with CDK4 in the sgCDKN2A cells, basal protein expression of CDK4 was lower compared to sgNTC (Figure 3.2B). Since CDK protein stability was recently reported to be affected by distinct complexes (Wu et al., 2021), we measured CDK4 stability using cellular engagement thermal shift assay (CETSA) (Jafari et al., 2014) with an AlphaLISA readout in both OVCAR-3 sgNTC and sgCDKN2A cells. We observed that CDK4 was less stable in the absence of p16 as the T_{m50} in sgNTC cells was 52.8°C while it was 43.2°C on average in sgCDKN2A cells (Figure 3.2C) Using an orthogonal Western blot based method, we observed the same trend that CDK4 becomes more unstable in the absence of p16 (

Figure S 3.2A-B). To test whether Palbociclib stabilizes CDK4, we pretreated sgNTC and sgCDKN2A cells with Palbociclib before CETSA-AlphaLISA analysis and observed that Palbociclib induces a right-shift in sgCDKN2A cells but not in sgNTC cells, suggesting that Palbociclib can stabilize CDK4 only in the absence of p16(Figure 3.2C). Together these results suggest that, in the absence of p16, the CDK4 ATP-binding pocket becomes more accessible, allowing Palbociclib to bind.

As Wu and colleagues recently reported, thermo-unstable CDK6 is able to be accessed by ATP-competitive small molecule CDK4/6 inhibitors, presumably by opening of the catalytic pocket. To assess ATP-pocket accessibility in CDK4, we treated OVCAR-3 sgNTC or sgCDKN2A cells cells with XO44, a covalent occupancy probe (Zhao et al., 2017). XO44 binds to a conserved lysine residue in the ATP-pocket, and structurally harbors an alkyne group, allowing for a click-chemistry reaction with a labeled azide. If XO44 is able to bind and access the ATP-pocket, a shift in molecular weight will be observed on a Western blot (Figure S 3.2C). XO44 bound CDK2 in both the presence and absence of p16, but only bound CDK4 when p16 was deleted (Figure 3.2D). These data suggest that, in the context of p16 loss, CDK4 binds more Cyclin D1, becomes thermally unstable, and has an accessible ATP-binding pocket.

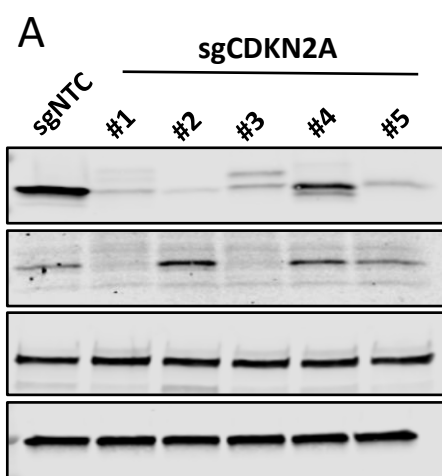
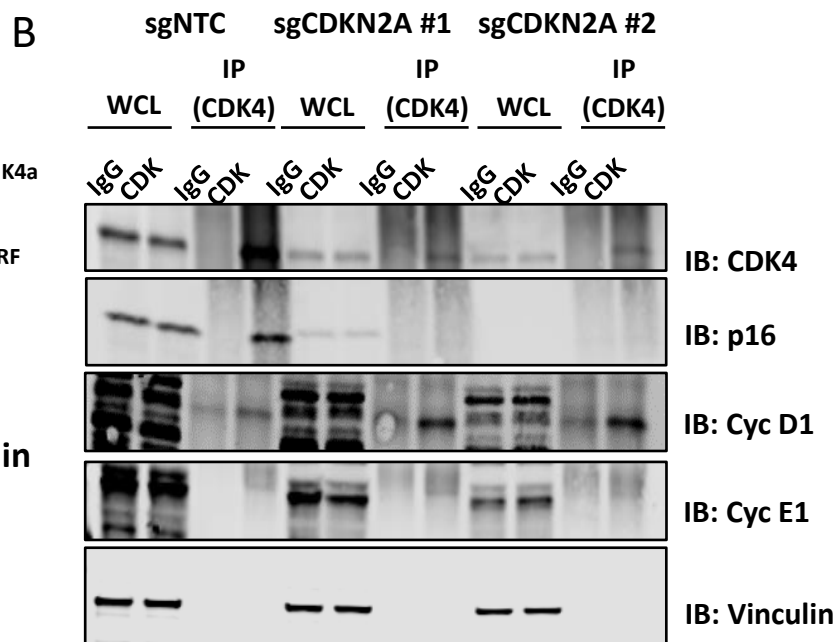
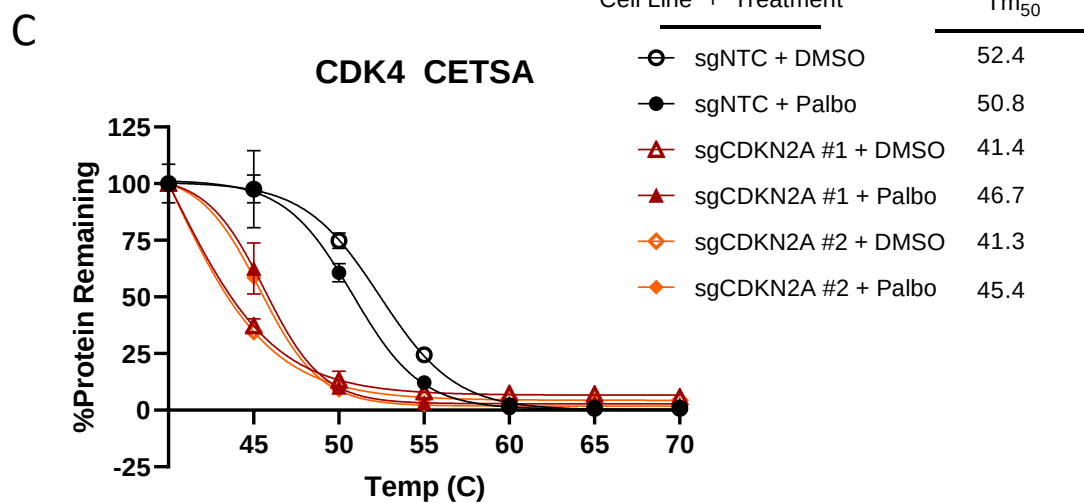
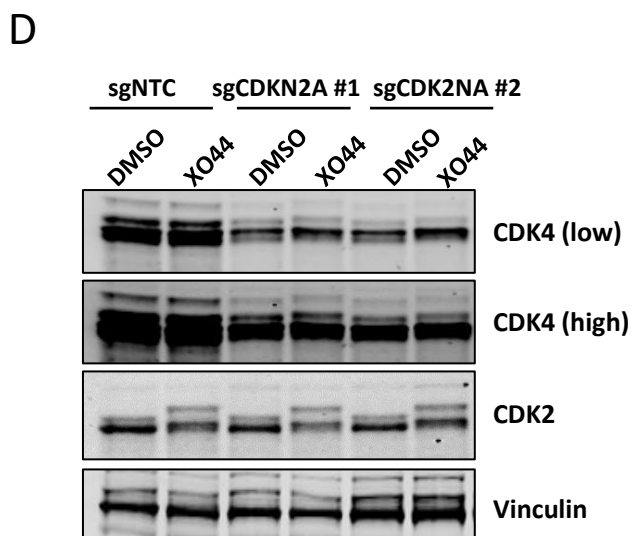


Figure 3.2: Loss of p16 destabilizes CDK4, allows Palbociclib to bind

(A) Western blot of indicated markers in OVCAR-3-Cas9 cells transduced with sgRNAs targeting CDKN2A.

(B) Immunoprecipitation of CDK4 complexes. Cell lysates from OVCAR-3 sgNTC or sgCDKN2A cells were either immunoprecipitated with an antibody specific for CDK4 or with immunoglobulin G (IgG). The immunoprecipitated complexes were analyzed via Western blot for the presence of CDK4, p16, Cyclin D1, Cyclin E1.

(C) Cellular thermal shift assay for CDK4 in OVCAR-3 sgNTC and sgCDKN2A cells treated with DMSO or Palbociclib (10uM). Cells were subjected to increasing temperatures (40-70C, 5-degree increments) before being processed by AlphaLISA for CDK4 levels. Error bars represent SD of two independent experiments.

(D) Western blot analysis of indicated markers after treatment with DMSO or XO44 (10uM) for 30 minutes.

CDK4/6 inhibition can overcome CDK2 inhibitor resistance in the context of p16 loss

To measure how CDKN2A deletion influences cell cycle regulation after CDK2 inhibition, in CCNE1-amplified cells, we measured Rb1 phosphorylation at CDK-targeted residues serine 807/811 (Zarkowska & Mitnacht, 1997) after treatment with BLU1851. Expectedly, BLU1851 treatment suppresses pRb1 S807/811 in sgNTC cells in a dose-dependent manner (Figure 3.3.A, Figure S 3.3A). In contrast, BLU1851 does affect pRb1 S807/811 levels in sgCDKN2A cells (Figure 3.3.A, Figure S 3.3A). Given that CDK4-Cyclin D1 association is enhanced in sgCDKN2A cells (Figure 3.3B), and the CDK4 ATP-binding pocket may be more accessible in this genetic background (Figure 3.3C-D), we reasoned that Palbociclib may be able to target CDK4 and re-sensitize sgCDKN2A cells to CDK2 inhibition. Indeed, Palbociclib treatment in combination with BLU1851 yields the greatest pRb1 S807/811 inhibition in sgCDKN2A cells (Figure 3.3A,

Figure S 3.3A). We observed the same phenotypes in another set of sgCDKN2A isogenic lines in the COV318 background, a *CCNE1*-amplified HGSOC cell line (Figure S 3.3B-D).

Because passage through the restriction point and continued cell cycle progression is mediated by the CDK-E2F-Rb axis (Nevins, 1992; Sherr, 1994), we measured the cell cycle profile of sgNTC and sgCDKN2A cells after treatment with BLU1851, Palbociclib, or the combination. In agreement with the Rb1 phosphorylation data, sgNTC cells arrested at the G1/S boundary after treatment with BLU1851 in a dose-dependent manner while the addition of Palbociclib did not further increase the percentage of cells in G1 (Figure 3.3B). On the other hand, sgCDKN2A cells were resistant to BLU1851, but re-sensitized to BLU1851 after Palbociclib treatment (Figure 3.3B). Notably, sgCDK2NA cells had a higher percentage of cells in S-phase compared to sgNTC cells, consistent with a dysregulated restriction point (Figure 3.3B).

Lastly, we measured cellular proliferation in a long-term colony formation assay to determine if the combination of BLU1851 and Palbociclib has a durable phenotype. Consistent with the Rb1 phosphorylation and cell cycle data, we observed that sgCDKN2A cells were resistant to BLU1851, but sensitive to the combination of BLU1851 + Palbociclib compared to sgNTC cells (Figure 3.3C). These results suggest that Palbociclib can re-sensitize cells to CDK2 inhibition following p16 loss.

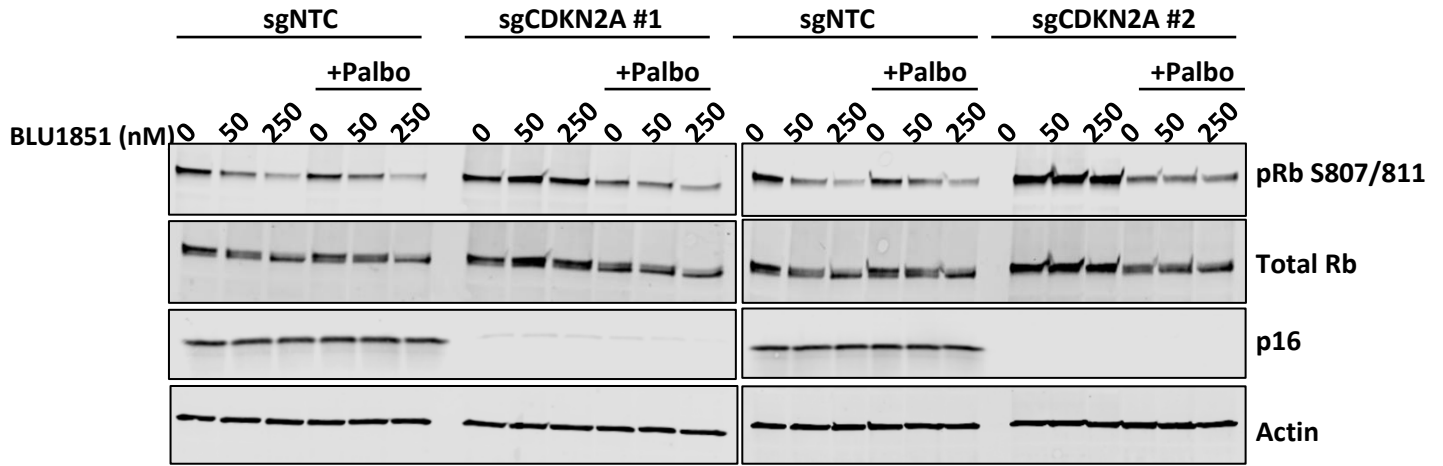
Given the data suggesting that CDK4 activity drives the resistance to CDK2 inhibition in OVCAR-3 sgCDKN2A cells, we engineered cells to overexpress the CDK4 R24C mutant that cannot bind p16 to determine if we could phenocopy p16 loss (Reddy et al., 2005; Sotillo et al., 2001; Q. Yu et al., 2006). Stable overexpression of either wild-

type (WT) or R24C CDK4 did not affect major cell cycle regulator levels, such as Cyclin D1, p16, total or phosphorylated Rb1 (Figure S 3.3E). Consistent with literature reports, the R24C mutant did not co-immunoprecipitate with p16, while the WT CDK4 did (Figure S 3.3F). Although we observed very minor resistance to BLU1851 as measured by phospho-Rb1 inhibition (Figure S 3.3G-H), this was not enough to induce a phenotypic effect in short-term cell cycle profile nor long term colony formation assays (Figure S 3.3I-J). These data may suggest that simple overexpression of mutant CDK4 without depletion of endogenous WT CDK4 disrupts stoichiometric ratios in the cell, or that both CDK4 and CDK6 need to be liberated from p16 to induce resistance.

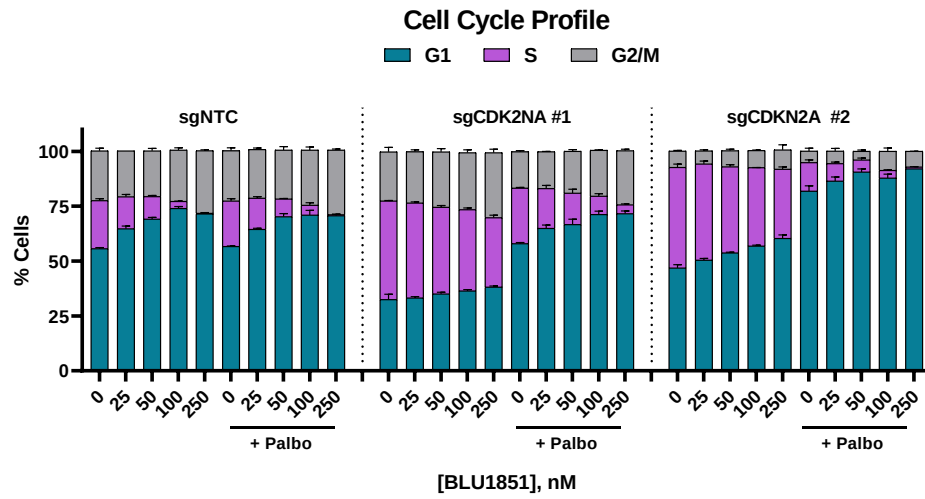
Expression of Cyclin E1, p16 sensitizes HR+ breast cancer cells to CDK2 inhibition

Since loss of p16 induced resistance to CDK2 inhibition in *CCNE1*-amplified cells, likely through the activation of CDK4, we tested if p16 overexpression could induce resistance to CDK4/6 inhibition and alter sensitivity to CDK2 inhibition in HR+ breast cancer cells. HR+ breast cancer cells are among the most sensitive to CDK4/6 inhibitors in preclinical studies, and CDK4/6 inhibitors have transformed the HR+ breast cancer treatment landscape (Finn et al., 2009, 2015; Slamon et al., 2018; Sledge et al., 2017). T47D cells, a common model for HR+ breast cancer, is one such cell line that is sensitive to CDK4/6 inhibition (Finn et al., 2009). We overexpressed p16, Cyclin E1, or both in T47D cells and evaluated protein expression (Figure 3.4A). While we observed increased expression of p16 or Cyclin E1 when expressed alone, the expression of both proteins was increased when they were overexpressed contemporaneously (Figure 3.4A).

A



B



C

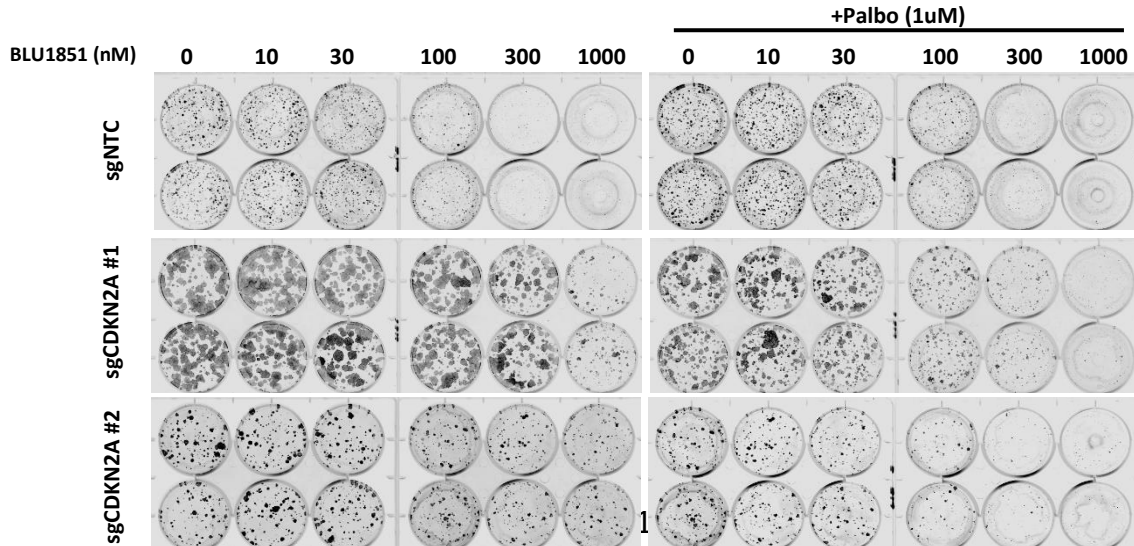


Figure 3.3: Palbociclib re-sensitizes OVCAR-3 CDKN2A knock-out cells to CDK2 inhibition.

(A) Western blot analysis of indicated markers in sgNTC, sgCDK2NA OVCAR-3 cells after 24-hour treatment with BLU1851, Palbociclib (1 μ M), or both. Blots are representative of duplicate experiments.

(B) Cell cycle profile of sgNTC and sgCDK2NA cells after 24-hour treatment with BLU1851, Palbociclib (1 μ M), or both. Error bars represent SD of two replicates.

(C) Colony formation assays in sgNTC and sgCDKN2A cells after 14 days of treatment with BLU1851, Palbociclib (1 μ M), or both. Cells were stained with crystal violet at experimental endpoint. Images are representative of duplicate experiments.

We next measured the CDK4-CyclinD1-p16 complex by CDK4 immunoprecipitation in the T47D engineered system to determine if overexpression of p16 and/or Cyclin E1 affected complex formation. We tested three antibodies for CDK4 IP to choose one that did not disrupt the interaction of CDK4 with Cyclin D1 or with p16. Given that the interactions with p16 occur at the N-terminal region of CDK4 (Jeffrey et al., 2000), we reasoned that the Bethyl antibody, which targets the C-terminal region, would not disrupt the interaction of CDK4 with p16. Indeed, we observed that the Bethyl antibody successfully immunoprecipitated CDK4 and co-immunoprecipitated Cyclin D1 and p16 (Figure 3.4B.). The other two antibodies, sourced from Abcam and SantaCruz Biotechnology, target the N-terminal region of CDK4, and as such did not co-immunoprecipitate p16 (Figure 3.4B). Consistent with our data in sgCDKN2A OVCAR-3 cells, overexpression of p16 in conjunction with Cyclin E1 diminished the interaction of CDK4 with Cyclin D1 (Figure 3.4B). We also explored the possibility that CDK2 may interact with Cyclin D1 when CDK4 is bound by p16 by measuring Cyclin D1 co-immunoprecipitation with CDK2. While we observed an increase in the CDK2-Cyclin E1 interaction when Cyclin E1 was overexpressed, CDK2 did not bind Cyclin D1 in any of

the engineered cell lines (Figure S 3.4A). Taken together, these results suggest that p16 and Cyclin E1 combined overexpression inactivate CDK4 and increase CDK2 activity.

We reasoned that changes in CDK-cyclin complexes would alter the sensitivity to CDK inhibitors in the T47D engineered cell lines as observed in the isogenic OVCAR-3 system (Figure 3.2A-C). Because palbociclib is an approved therapy for HR+ breast cancer, and there are ongoing clinical trials with the CDK2 inhibitor PF-4091 in patients who have progressed on Palbociclib (NCT04553133), we measured the anti-proliferative effect of these clinically relevant agents in the T47D engineered cell lines. Relative to empty vector controls, Cyclin E1 overexpression alone induced a notable right-shift in sensitivity, but the co-overexpression of p16 and Cyclin E1 demonstrated the greatest shift (Figure 3.4C). In contrast, the overexpression of p16 or Cyclin E1 did not alter sensitivity to PF-4091, while the combined overexpression sensitized cells relative to empty vector control (Figure 3.4C). The lack of change when p16 or Cyclin E1 were overexpressed alone may be due to insufficient protein levels when not expressed contemporaneously. We further validated this finding by measuring the cell cycle profile in empty vector control cells and cells overexpressing both Cyclin E1 and p16 following treatment with Palbociclib or PF-4091. Consistent with the proliferation assay, empty vector control cells arrested at G1/S following Palbociclib treatment, but had no change following PF-4091 treatment (Figure 3.4D). T47D cells overexpressing both p16 and Cyclin E1 were insensitive to Palbociclib but showed dose-dependent G1/S arrest following PF-4091 treatment (Figure 3.4D). These results suggest that, if

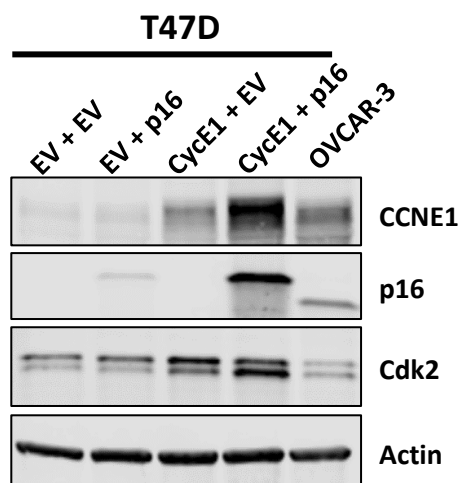
CDK2 is activated when Cyclin E1 is overexpressed, CDK2 can bypass CDK4 p16-mediated inhibition.

Discussion

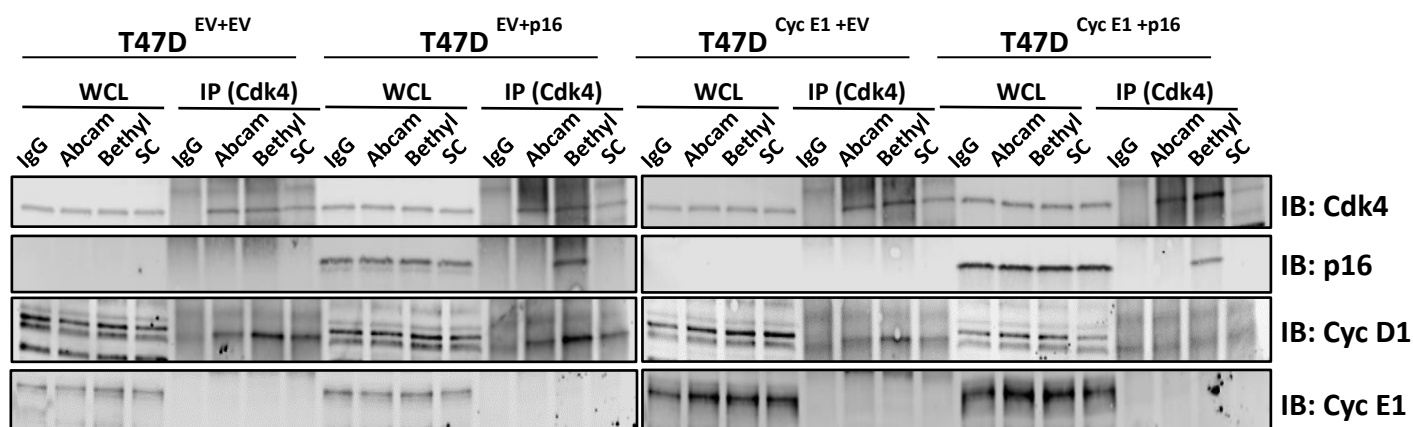
In this study, we uncovered the molecular features that govern CDK2-dependence in *CCNE1*-amplified HGSOC. Our CRISPR screen revealed major cell cycle regulators, such as *CDKN2A*, as requirements for CDK2 sensitivity in this disease. Loss of this gene allows the cell to maintain a proliferative state when CDK2 activity is suppressed by CDK2-specific small molecule inhibitors. As such, these genes can act as additional biomarkers to predict lack of CDK2 inhibitor sensitivity in HGSOC.

CDKs are known to compensate for each other during development and in some cancer types. *CCNE1*-amplification in HGSOC, however, renders the cells dependent on CDK2 for proliferation (Au-Yeung et al., 2017). *CCNE1*-amplification and *CDKN2A* loss is mutually exclusive in primary tumors in this disease type, suggesting it may play a role in disease maintenance or genetic dependence (Cerami et al., 2012; J. Gao et al., 2013). *CDKN2A* codes for p16, an endogenous negative regulator of CDK4/6, that acts to outcompete cyclin D binding, and high levels of p16 occlude the CDK4 catalytic pocket thus blocking the binding of CDK4/6 inhibitors, (Green et al., 2019; Parry et al., 1999; Pavletich, 1999)

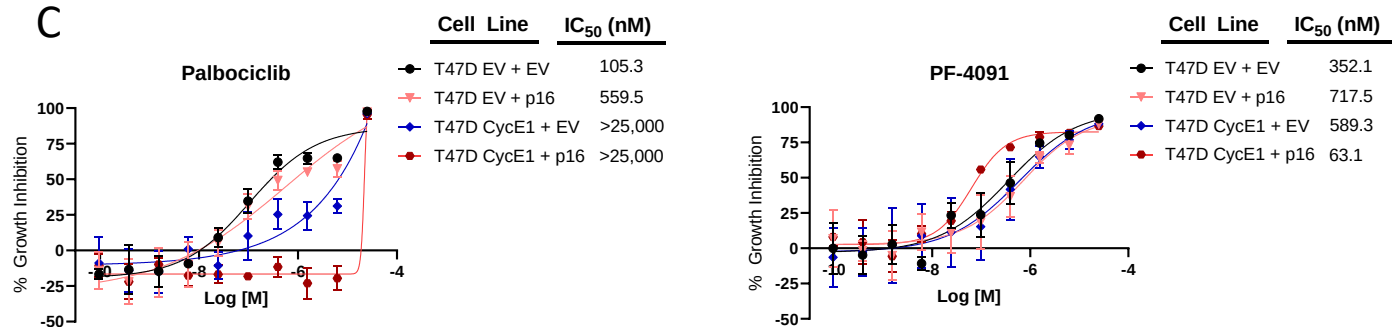
A



B

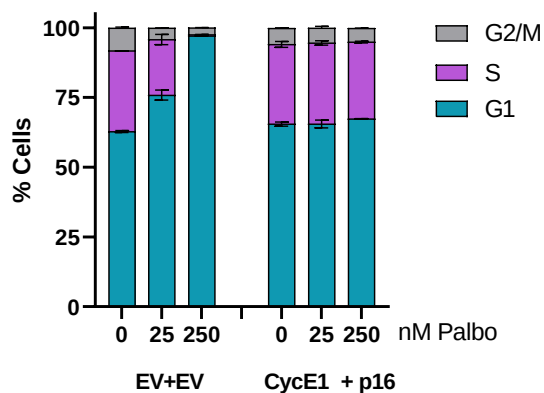


C



D

T47D engineered cells
Cell Cycle Profile (Palbociclib treatment)



T47D engineered cells
Cell Cycle Profile (PF-4091 treatment)

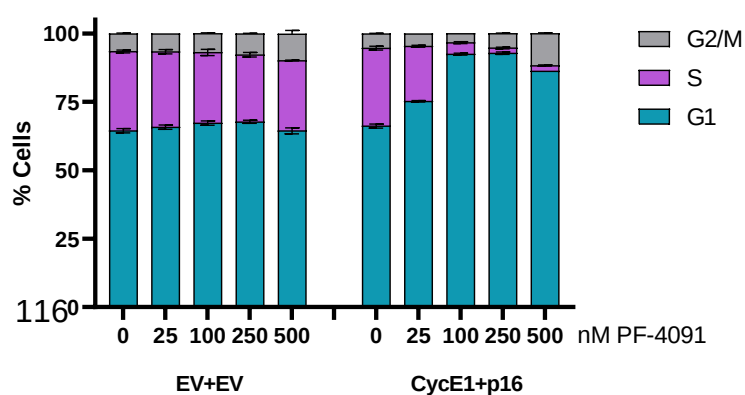


Figure 3.4: T47D cells overexpressing Cyclin E1 and p16 are sensitized to CDK2 inhibition, resistant to CDK4/6 inhibition

- (A)** Western blot analysis of indicated markers in T47D cells engineered to overexpress p16 and/or Cyclin E1. OVCAR-3 cells were used as a high cyclin E1 control.
- (B)** CDK4 immunoprecipitation in T47D engineered cells and Western blot of indicated markers.
- (C)** Dose-response curves of cells treated with Palbociclib (top) or PF-4091 (bottom). Cells were treated with a 10-point dose response of indicated compounds and incubated for 5 days before CyQuant assay was performed. IC50s are listed for each cell line in nM. Error represents SD of two independent experiments.
- (D)** Cell cycle profile of T47D EV+EV and CyclinE1+p16 cells after 24-hour treatment with Palbociclib (left) or PF-4091 (right). Error bars represent SD of two independent experiments.

Conversely, mechanisms by which p16 expression is reduced, such as low transcriptomic levels of *CDKN2A* and/or increased methylation at the *CDKN2A* promoter, increase the sensitivity to CDK4/6 inhibitors (Finn et al., 2009; P. Li et al., 2019). Non-tumor cells can rely on CDK4/6-Cyclin D when CDK2 is inhibited, but high p16 levels in HGSOC led to the near complete endogenous inhibition of CDK4/6, thus generating a CDK2-dependent state (Arora et al., 2023). Our study suggests that, in the presence of p16, cells become dependent on CDK2, which is likely the reason CDK2 was not a hit in a recent CRISPR screen using a *CCNE1*-overexpression isogenic system in hTERT-RPE1 cells, as RPE cells have low levels of p16 relative to *CCNE1*-amplified HGSOC lines (Brown et al., 2023; Gallo et al., 2022). Our study highlights the need to perform disease relevant experiments in models that accurately recapitulate the genetic and proteomic environment.

Mechanistically, our study demonstrates that p16 loss allows a cell cycle bypass mechanism through CDK4-Cyclin D1 destabilization and subsequent activation. Notably, cells lacking p16 have an increased percentage of cells in S-phase, which we hypothesize to be from uncontrolled G1/S transition via maintained hyperphosphorylation of Rb. Acceleration into S-phase can increase DNA damage load from low nucleotide pools and increased replication-transcription collisions (Bester et al., 2013; Halazonetis et al., 2008; Macheret & Halazonetis, 2018). An important area of future research is to measure DNA damage load in p16 loss cells, and test combination treatments of CDK2 inhibitors and DNA damaging agents to better understand secondary effects that follow *CDKN2A* loss.

The CDK4 R24C mutant, which abolishes the CDK4-p16 interaction, has been shown to immortalize murine fibroblasts, and increase incidence of tumor formation in several tissue types (Sotillo et al., 2001). The overexpression of the R24C mutant did not confer full resistance to CDK2 inhibition in our studies; one possibility is that exogenous overexpression of this mutant without depleting endogenous expression of CDK4 does not capture biologically relevant stoichiometric ratios. A future experiment is to engineer endogenous CDK4 to carry the R24C mutation. An alternate explanation for the lack of phenotype is that both CDK4 and CDK6 need to be liberated from p16 in order to bypass CDK2 inhibition; one way to begin to test this is to use the protein-protein interaction inhibitor, P18IN003, that disrupts the interaction of INK family protein members with both CDK4 and CDK6 (Y. Gao et al., 2015; Palafox et al., 2022). If it is indeed the case that both CDK4 and CDK6 must be catalytically active, treatment with P18IN003 should confer resistance to CDK2 inhibition.

Importantly, sensitivity to CDK2 inhibition in the absence of p16 can be restored with CDK4/6 inhibitor treatment. This has clinical implications as patients whose tumors have lost p16 expression after CDK2 inhibitor therapy may derive benefit from combination treatment with approved CDK4/6 inhibitors. The reverse is also true: patients who progress on CDK4/6 inhibitors with high p16 levels may have improved outcome with a combination treatment of CDK2 inhibitor and CDK4/6 inhibitor. In previous studies, p16 levels alone were a poor predictor of CDK4/6 efficacy in HR+ breast cancer patients (Finn et al., 2015). An expression analysis in a follow-up study with Palbociclib suggested that high CCNE1 levels may be part of an intrinsic mechanism of resistance to CDK4/6 inhibitors (Turner et al., 2019). Our study suggests that a multi-variate biomarker signature that includes both p16 and Cyclin E1 levels could predict response to CDK4/6 inhibitor therapy in breast cancer, and CDK2 inhibitor therapy in HGSOC, and stratify patients who may respond better to combination.

Methods

Cell lines and culture

OVCAR-3 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 20% fetal bovine serum. HEK293T cells were maintained in DMEM supplemented with 10% fetal bovine serum. T47D cells were maintained in RPMI 1640 media supplemented with insulin and 10% fetal bovine serum. All cells were maintained in a humidified incubator at 37°C with 5% carbon dioxide (CO₂).

Stable cell line generation

Cas9 stable cell line generation

OVCAR-3 or COV318 cells were plated at 2×10^5 cells/well in 6-well plates and incubated overnight at 37°C 5%CO₂ for 24 hours. Transduction with Cas9 pR-EF1-Cas9-2A-Blast (Cellecta, Lot# SVC9EB-VS) was performed at a 1:1 MOI with 5ug/mL polybrene. Cells were incubated overnight at 37°C 5%CO₂. Media was/ refreshed with polybrene-free replete media. After 48 hours, selection was started using 10 µg/mL Blasticidin. Cas9 expression was validated after two weeks by Western blot.

sgRNA stable cell line generation

5.0×10^6 HEK-293T cells were plated in 10% DMEM and incubated at 37°C, 5% CO₂ for 24 hours. Transfection was performed by combining 1µg sgRNA construct, 5µg Cellecta Packaging mix (Cat#CPCP-K2A), and Opti-MEM mixture 1:1 with Lipofectamine 2000 (ThermoFisher Scientific, Cat# 11668-019) in Opti-MEM. Following 20 minutes of incubation, transfection mixture was added dropwise to cells, and incubated for 24 hours. OVCAR-3 Cas9 or COV318 Cas9 cells were plated at 2×10^5 cells per well in replete media + Blasticidin 24 hours prior to transduction. Virus was harvested by media filtration using 0.45µM vacuum filters (Corning, Cat#430514), and virus production was verified using Takara Lenti-X GoStix™ Plus (Cat# 631281). Transduction was performed using 5 µg/mL polybrene in replete media +10ug/mL Blasticidin. After 24 hours, media was refreshed polybrene-free media. After 48 hours, selection was started using 10 µg/mL Blasticidin + 2 µg/mL Puromycin (OVCAR-3) or + 5ug/mL Puromycin (COV318).

CyclinE1 + p16 stable overexpression

5.0x10⁶ HEK-293T cells were plated in 10% DMEM and incubated at 37°C, 5% CO₂ for 24 hours. Transfection was performed by adding 7µg expression construct (empty vector, p16, or Cyclin E1) to lyophilized packaging mix/lipid according to manufacturer's instructions (Takara, Cat# 631275). Cells were transfected and allowed to incubate overnight 37°C, 5% CO₂. The next day, additional media was added to the plates. 24 hours later, virus was harvested by media filtration using 0.45µm vacuum filters (Corning, Cat#430514), and virus production was verified using Takara Lenti-X GoStix™ Plus (Cat# 631281). Transduction was performed using 5 µg/mL polybrene in replene media. After 24 hours, media was refreshed polybrene-free media. After 48 hours, selection was started using 1 µg/mL Puromycin or 800 µg/mL G418 (T47D).

CRISPR/Cas9 resistance screen

~100M OVCAR3-Cas9 cells were transduced at MOI=0.5 with Collecta's 80K shRNA CRISPR-KO lentiviral library (Cat# KOHW-80K-P). Three days post lentiviral infections, cells were subjected to puromycin selection to enrich for transduced cells, and expanded for 14 days. After day 14, cells were split into 6 samples (~50M cells each) and treated with DMSO, 250nM BLU1851, or 250nM PF-4091 in duplicate for 7 days. After treatment was completed, cells were harvested and processed for NGS for detection of sgRNA enrichment. Starting from the raw sgRNA counts in each sample, sgRNA fold-changes were calculated in two steps. First, the raw sgRNA counts in each sample were divided by the total sgRNA counts in that sample. The same normalization was applied to sgRNA counts in plasmid. Then, the sgRNA normalized counts in each sample were divided by the normalized sgRNA counts in plasmid to yield the individual sgRNA fold-changes. To create gene-level knock-out scores, first, the median sgRNA

fold-change values were calculated for each gene. Then in each sample, the median sgRNA fold-changes were used to calculate gene z-scores. Gene z-scores were used to evaluate the impact of each gene's knock-out on the proliferation. A negative score indicates stronger dependency on the gene whereas a positive score indicates a positive proliferation effect. Replicates from the same treatment condition were merged by taking the average gene z-score. To identify treatment-specific hits, z-score deltas were calculated for each gene by subtracting the gene's treatment z-score from its DMSO z-score. The z-score deltas were then sorted to rank the top hits. Positive delta z-scores signified potential resistance mechanisms whereas negative delta z-scores indicated potential synergistic effects.

Immunoprecipitation

CDK4, CDK2 IP

Cells were washed with PBS after trypsinization (1×10^6 /IP) and lysed on ice in Pierce IP lysis buffer (ThermoFisher Cat# 87787) supplemented with HALT protease and phosphatase inhibitors (ThermoFisher Cat# 78430). Lysates were centrifuged at 14,000 rpm for 10 min and the protein concentration was quantified using BCA (Thermo Fisher Cat# A53225). Lysates were incubated overnight at 4C with 10ug anti-CDK4 antibody (Bethyl #A304-225A), anti-CDK2 antibody (SC-6248) or isotype control (Cell Signaling #3900) with gentle rotation. Lysates were incubated with 50uL of twice washed Protein A/G magnetic beads (Thermo Fisher Cat# 88802) for 2 hours at room temperature with gentle rotation. After incubation with beads lysates were placed on a magnet and washed three times with lysis buffer. 1X laemmli buffer with 2-ME (BioRad) was used

for elution. Samples were heated at 95C for 5-10 minutes and run using Western blot protocol.

FLAG IP

Cells were washed with PBS after trypsinization (1×10^6 /IP) and lysed on ice in Pierce IP lysis buffer (ThermoFisher Cat# 87787) supplemented with HALT protease and phosphatase inhibitors (ThermoFisher Cat# 78430). Lysates were centrifuged at 14,000 rpm for 10 min and the protein concentration was quantified using BCA (Thermo Fisher Cat# A53225). Lysates were incubated overnight at 4C with 20uL of twice washed anti-FLAG M2 magnetic beads (Sigma#M8823) with gentle rotation. After incubation with beads lysates were placed on a magnet and washed three times with lysis buffer. 3X-FLAG peptide was added after final wash at a concentration of 100ng/uL and samples were shaken for 30 minutes. Elution was combined with 4X laemmli buffer with 2-ME (BioRad). Samples were heated at 95C for 5-10 minutes and run using Western blot protocol.

Cellular thermal shift assay

Western blot method

For basal CDK4 thermal stability assay, cells resuspended in 1 ml PBS containing HALT protease + phosphatase inhibitor cocktail (ThermoFisher Cat# 78430) were aliquoted with 100 μ l cell suspension each into seven PCR tubes (0.8×10^6 /cells/tube). PCR tubes were heated on a temperature gradient (40-70 °C) for 3 min in a thermal cycler (Applied Biosystems). Tubes were removed and incubated at room temperature for 3 minutes after which they were snap-frozen in liquid nitrogen. Cells were frozen and

thawed twice using liquid nitrogen and a thermal cycler at 25 °C. Cell lysates were centrifuged at 14,000 x g for 10 min at 4 °C. Supernatants were collected for Western blots (20% of supernatant run/gel).

For Palbociclib induced thermal shift assay, cells were treated with 10 µM Palbociclib or DMSO for 1 hour before harvesting as above.

AlphaLISA method

OVCAR-3 cells were plated at 40,000 cell/mL in 15uL/well in Hank's Balanced Salt Solution with calcium chloride and magnesium chloride (Gibco Cat# 14025-092) into PCR-plates (Applied Biosystems Cat#A32811). Palbociclib was diluted to 2X final concentration (20uM) in HBSS. Either DMSO or Palbociclib in HBSS was added to the plate (15uL/well). Cells were incubated for 1 hour at 37°C/5%CO₂. Using a 96-well thermal cycler (Applied Biosystems), cells were incubated on a temperature gradient ranging from 40-70 °C for 3 minutes and then placed on ice. CETSA lysis buffer #2 (Perkin Elmer Cat# CETSA-BUF2) was prepared was 2X with MilliQ water. 30uL of diluted lysis buffer was added to plate. Plate was placed on a plate shaker for 30 minutes at room temperature. 10uL of lysate from each well was moved to a 384-OptiPlate (Perkin Elmer). The CDK4 AlphaLISA kit (Perkin Elmer Cat# ALSU-TCDK4) was then performed according to manufacturer's instructions.

Western blot

Cells were lysed in PhosphoSafe Lysis Buffer (EDM millipore, Cat#71296) and protein was quantified using Pierce BCA Gold Kit (Thermo Fisher Scientific Cat#A53227).

Lysate and lysis buffer were combined with 4X Loading Dye (Bio-Rad, Cat##1610747)

and 2ME (Bio-Rad, Cat#1610710). Samples were loaded into Bio-Rad 4-20% Tris-Glycine, 18-well SDS-PAGE (Cat#5671094). Transfer was performed using BioRad dry transfer system (Trans-Blot Turbo) (Nitrocellulose membrane, 0.2 μ M). Blocking was performed using LI-COR Odyssey Blocking Buffer (Cat#927-70001). Primary antibodies (Cas9 1:1000, CDK2 1:500, CDK4 1:1000, p14: 1:1000, p16 1:1000, Cyclin E1 1:500, Cyclin D1 1:500, FLAG 1:500, Actin R 1:2000, Actin M 1:5000, Vinculin 1:5000, pRb S807/811 1:1000, total Rb 1:1000, Tubulin 1:10,000) were combined with Odyssey Blocking Buffer and 0.1% Tween-20, and incubated at 4°C, rotating. Secondary antibodies were combined with Odyssey Blocking Buffer and 0.1% Tween-20 and incubated at room temperature, rotating. All secondary antibodies were diluted to 1:10,000 (LI-COR Cat#926-33212, Cat#926-33213, Cat#926-68070, Cat#926-68071, Cat#926-68076). Signals were detected using LI-COR Odyssey CLx Imaging Studio.

Cell cycle profiling

Reagents for EdU incorporation were included in the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Thermo Fisher Scientific Cat#C10420). Cells were treated with compound for 22 hours prior to EdU pulsing. EdU was added to cells in culture for a final concentration of 10 μ M, and incubated at 37°C in 5% CO₂ for 2 hours prior to collection and pelleting. Cells were incubated in the dark for 15 minutes with 100 μ L of Click-iT fixative, washed with 1% BSA PBS, and incubated for 15 minutes with 100 μ L 1x Click-iT saponin-based permeabilization agent, prepared by 1:10 dilution of stock solution in PBS supplemented with 1% BSA. The Click-iT reaction cocktail was prepared following manufacturer's instructions and added to cells for 30 minutes at

room temperature in the dark. Cells were washed with 1% BSA. FxCycle Violet (Thermo Fisher Scientific Cat#F10347) was prepared in 1x saponin-based permeabilization reagent at a 1:2000 dilution, and added to cells for 30 minutes at room temperature in the dark. FACS analysis was performed using LSR Fortessa (BD Biosciences) and analyzed using FlowJo.

Materials

Cell Lines

Cell line	Source	Catalog Number
OVCAR-3	American Tissue Type Collection	HTB-161
COV318	Millipore Sigma	07071903
T47D	American Tissue Type Collection	HTB-133
HEK293T	American Tissue Type Collection	CRL-3216

Antibodies

Western Blot

Antibody	Source	Catalog Number
Cas9	Cell Signaling Technology	CST-14697, RRID: AB_2750916
CDK2	Santa Cruz Biotechnology	SC-6248, RRID:AB_627238
CDK4	Cell Signaling Technology	CST-12790, RRID: AB_2631166
Cyclin D1	Cell Signaling Technology	SC-8396, RRID: AB_627344
p14	Cell Signaling Technology	CST-2407, RRID: AB_490785
p16	Cell Signaling Technology	CST-80772, RRID:AB_2799960
Cyclin E1	Santa Cruz Biotechnology	SC-247, RRID:AB_627357
pRb S807/811	Cell Signaling Technology	CST-8516, RRID:AB_11178658

Total Rb	Cell Signaling Technology	CST-9309; RRID:AB_823629
Actin	Cell Signaling Technology	CST-3700 RRID: 2242334
Actin	Cell Signaling Technology	CST-8457, RRID:AB_10950489
Actin	Cell Signaling Technology	CST-3700, RRID:AB_2242334
FLAG	Sigma-Aldrich	F1804, RRID:AB_262044
Tubulin	Sigma-Aldrich	T9026, RRID:AB_477593
Vinculin	Sigma-Aldrich	V9131 RRID: AB_477629
Donkey anti-mouse	LI-COR	926-32212, RRID:AB_621847
Donkey anti-rabbit	LI-COR	926-32213, RRID:AB_621848
Goat anti-mouse	LI-COR	926-68070, RRID:AB_10956588
Goat anti-rabbit	LI-COR	926-68071, RRID:AB_10956166

Critical Commercial Assays

Assay	Source	Catalog Number
Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit	ThermoFisher Scientific	C10419
FxCycle Violet	ThermoFisher Scientific	F10347
CyQuant Direct Cell Proliferation Assay	ThermoFisher Scientific	C35011

Compounds

Compound	Source	Catalog Number
BLU1851	Blueprint Medicines	N/A
PF-4091	Synthesized at ChemPartner	N/A
Palbociclib	MedChem Express	HY-50767
DMSO	Thermo Fisher Scientific	D12345

Plasmids

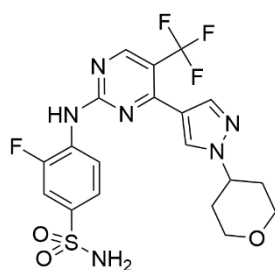
Vector	Source
pLVX-IRES-EF1 α -Puro	Genscript
pRSG-U6-(sgRNA)-EF1-GFP-puro	Cellecta
pRBBC-CMV-Cas9-2A-Blast	Cellecta

sgRNA

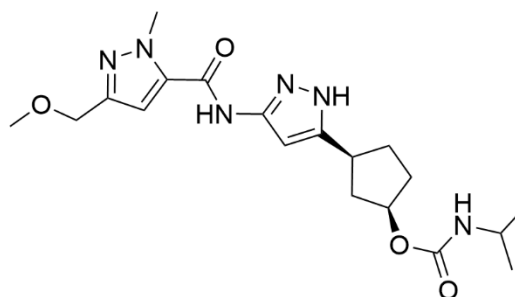
Target	Source	Sequence
sgNTC	Cellecta	5'- GGCAGTCGTTCTGGTTGATAT-3'
sgCDKN2A #1	Cellecta	5'-CGGGTCGGGTGAGAGTGGCG -3'
sgCDKN2A #2	Cellecta	5'-CACCGAATAGTTACGGTCGG-3'
sgCDKN2A #3	Cellecta	5'-GATGATGGGCAGCGCCCGAG -3'
sgCDKN2A #4	Cellecta	5'-GTGGCCAGCCAGTCAGCCGA-3'
sgCDKN2A #5	Cellecta	5'-TGTCGCACGGTACCTGCGCG-3'

Supplementary Figures

A

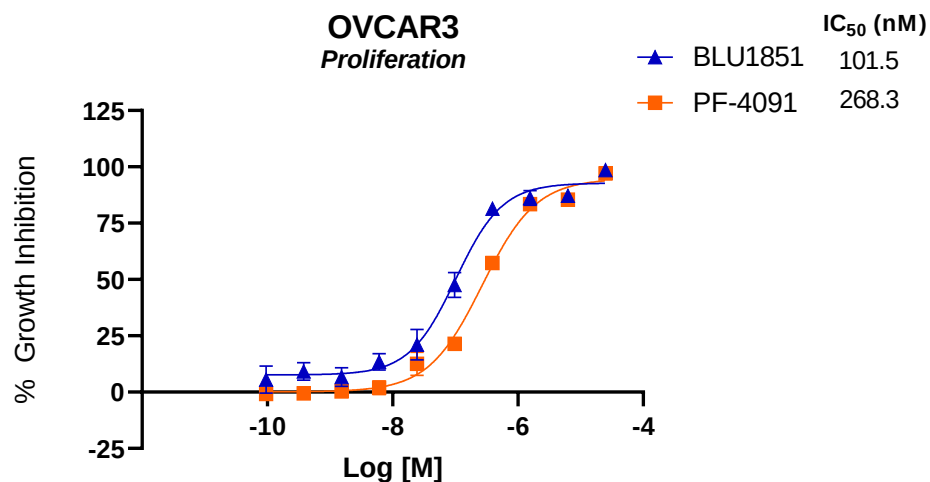


BLU1851



PF-4091

B



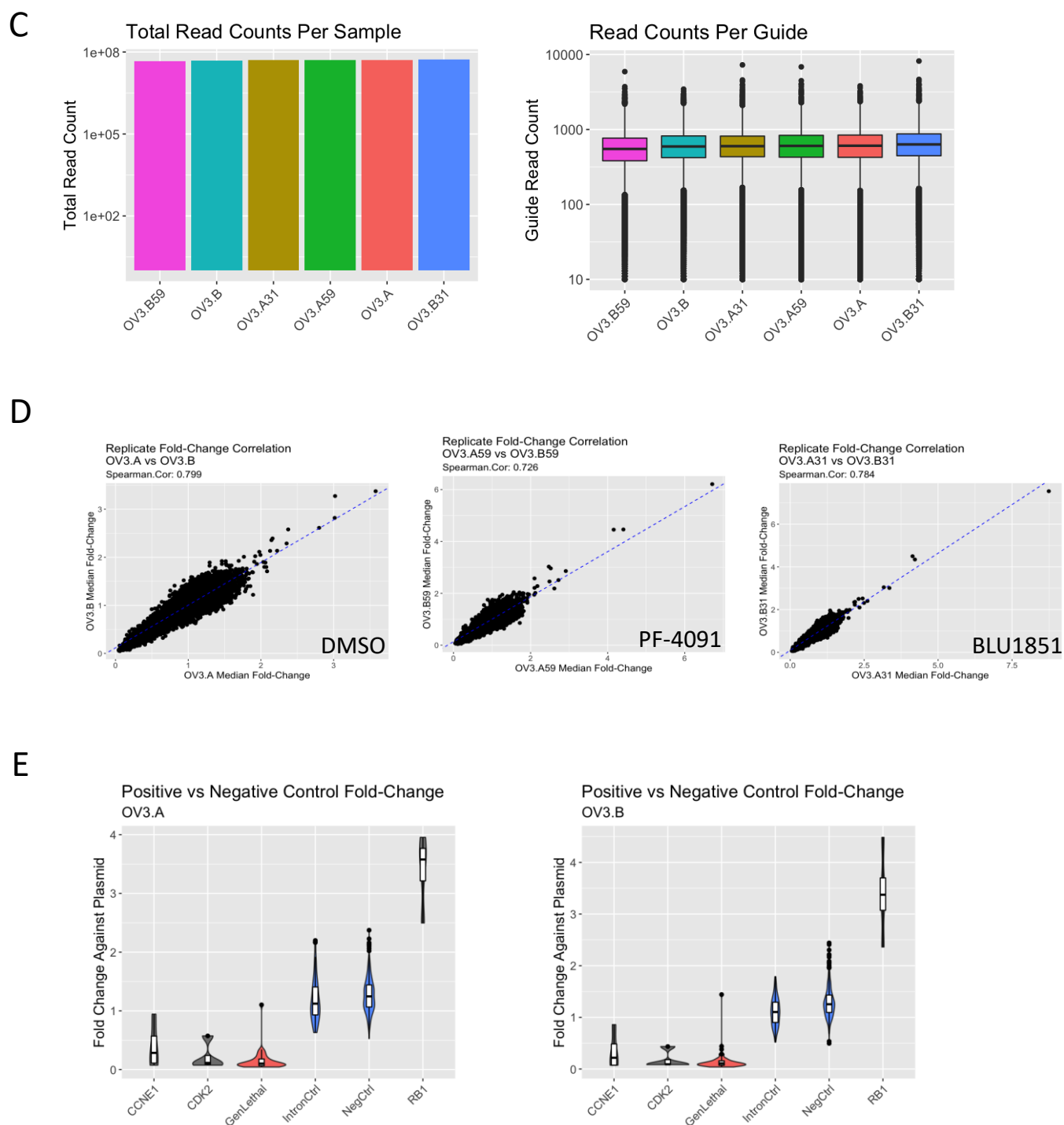


Figure S 3.1: BLU1851 and PF-4091 have a similar anti-proliferative effect in OVCAR-3 cells.

(A) Chemical structure of BLU1851 and PF-4091.

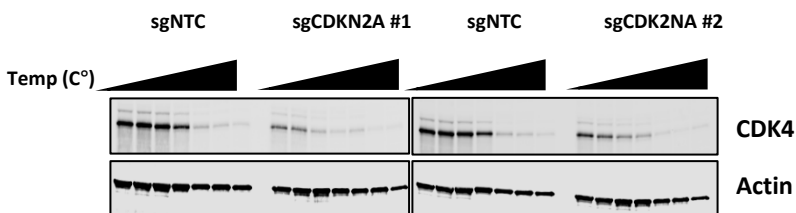
(B) Dose response curves OVCAR-3 cells. Cells were treated with a 10-point dose response of indicated compounds and incubated for 7 days before CyQuant assay was performed. Error bars represent SD of duplicate independent experiments.

(C) Total read counts per sample and read counts per guide.

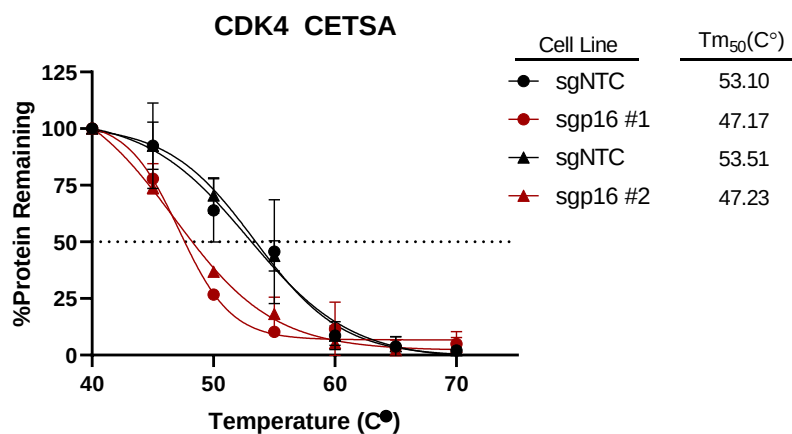
(D) Spearman correlation between replicates.

(E) Fold change of positive and negative controls in DMSO samples. CCNE1 and CDK2 are essential genes in OVCAR-3 cells and rank near lethal control, while the negative and intronic controls are neither enriched nor lost. Rb1 sgRNAs are enriched.

A



B



C

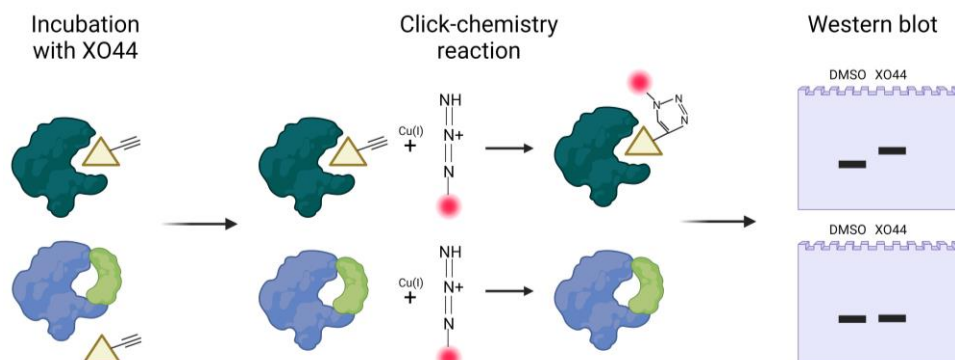
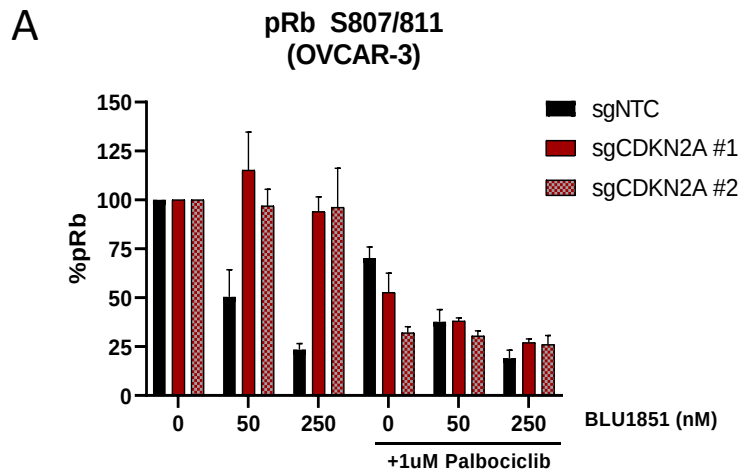


Figure S 3.2: p16 loss destabilizes CDK4

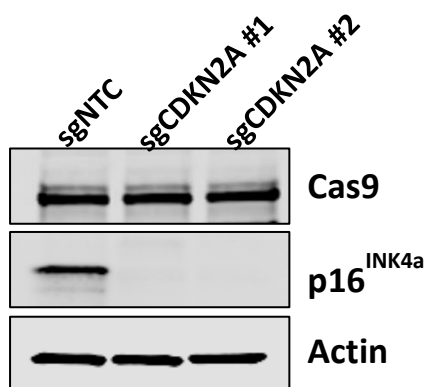
(A) Cellular thermal shift assay for CDK4 in OVCAR-3 sgNTC and sgCDKN2A cells. Cells were subjected to increasing temperatures (40-70C, 5-degree increments) before being processed by Western blot analysis for indicated markers.

(B) Quantification of Western blots as represented in (A). Error represents SD of two independent replicates. The melting temperature at which 50% of the protein is lost (T_{m50}) is listed.

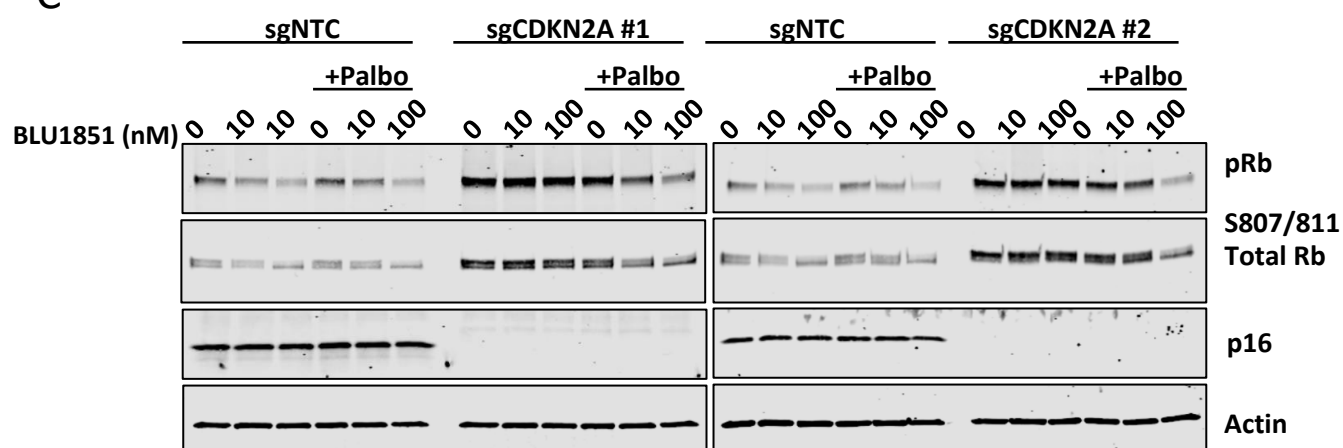
(C) Schematic of click chemistry reaction with XO44. If XO44 is able to bind, a labeled azide can be added via a click-chemistry reaction. Proteins that are bound by XO44 will show a shift to a higher molecular weight on Western blot.



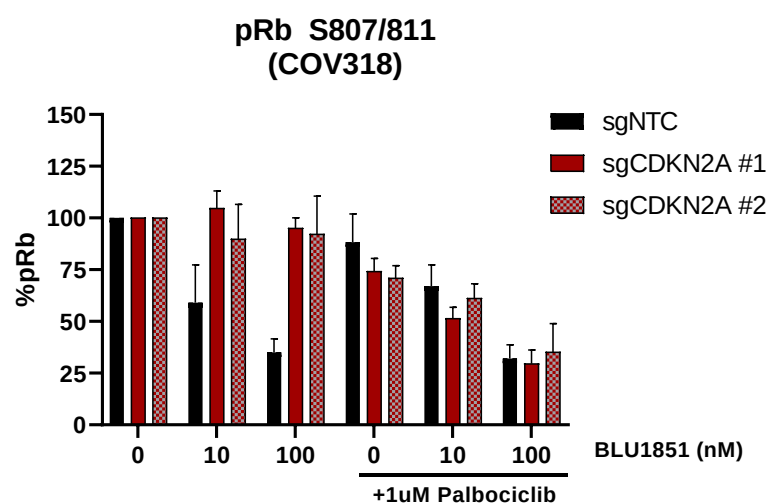
B

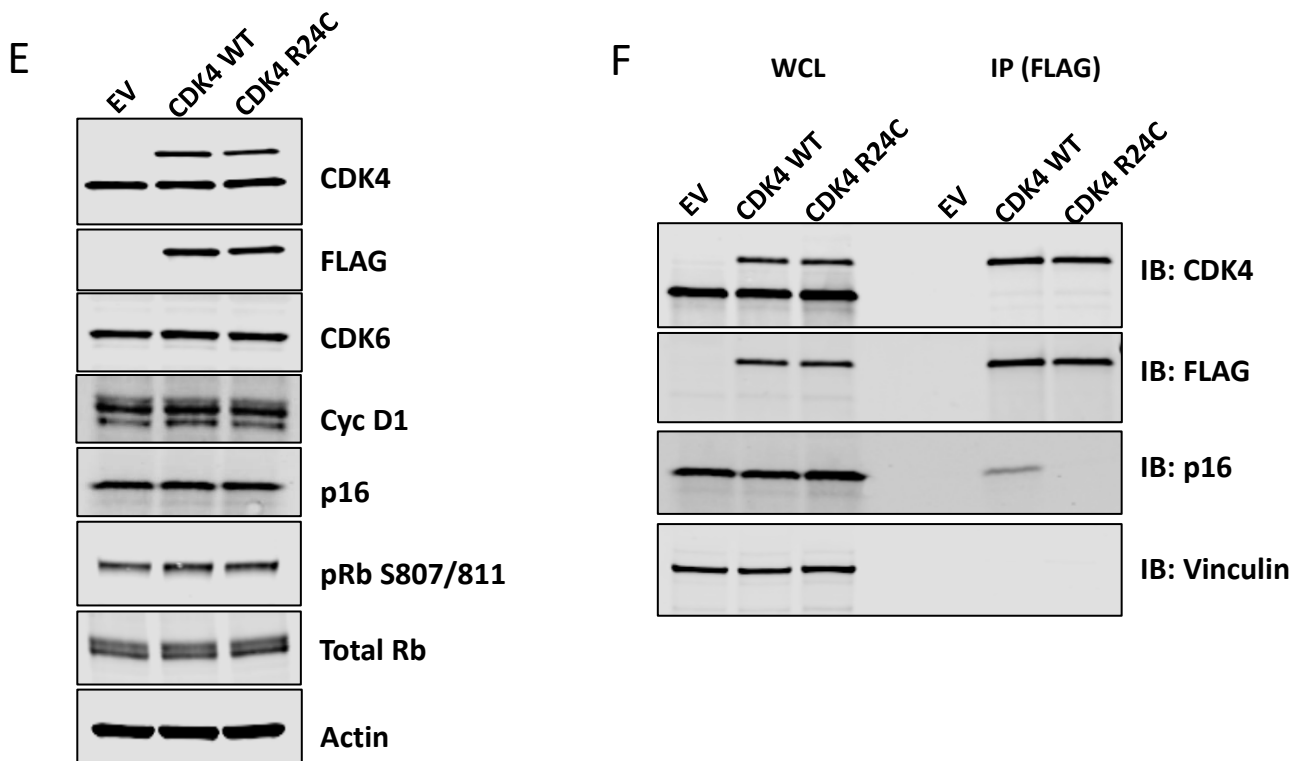
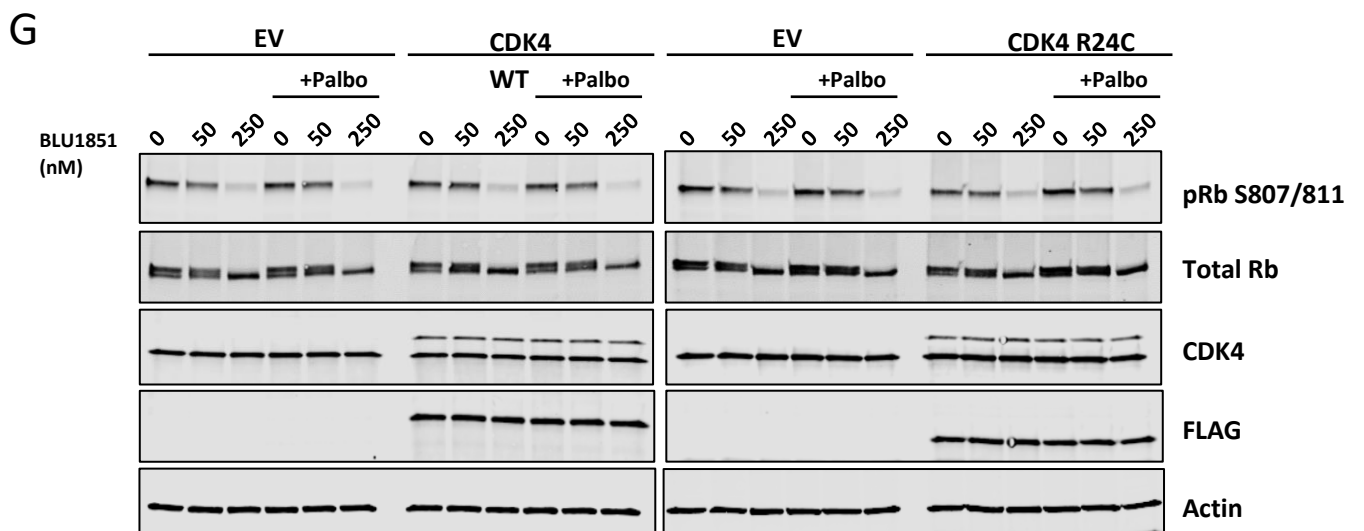


C

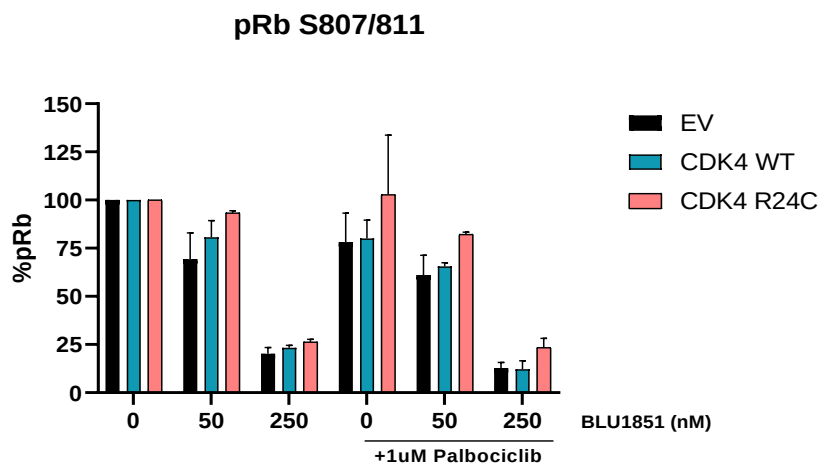


D

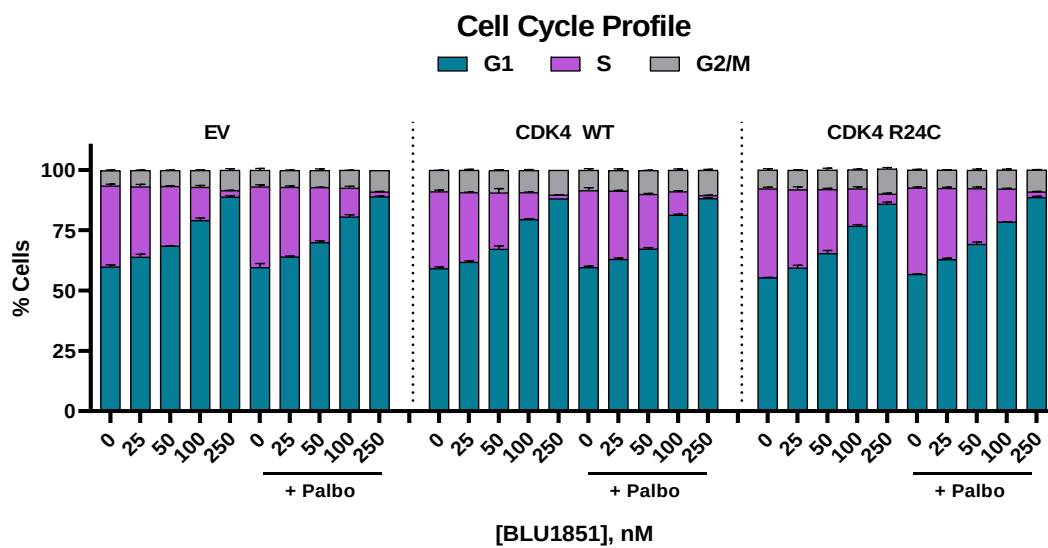




H



I



J

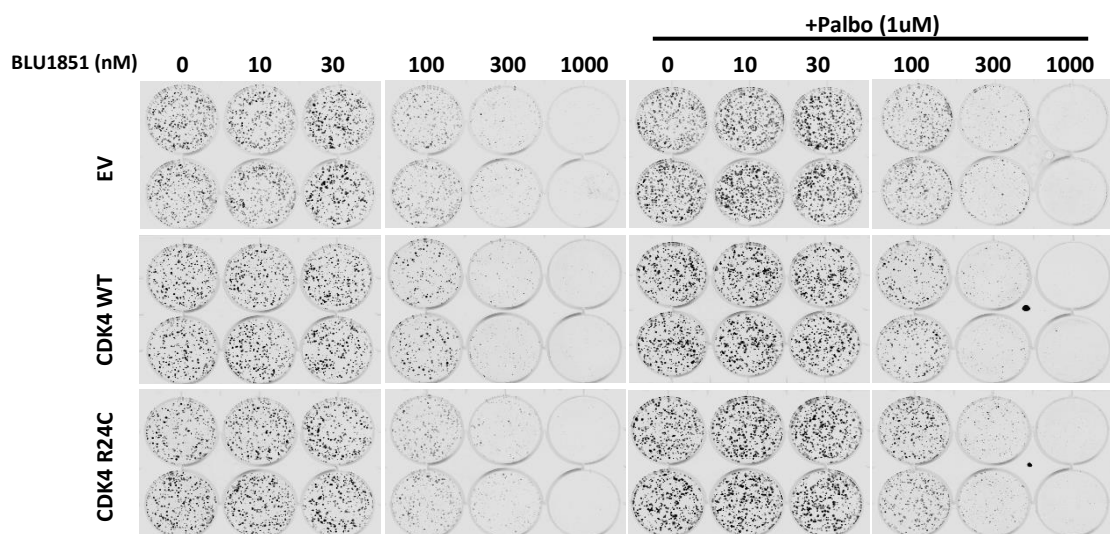


Figure S 3.3: CDK4 R24C expressing cells are not resistant to CDK2 inhibition.

(A) Quantification of Fig 3A. pRb1 levels were normalized per cell line to the vehicle control group. Error bars represent SD of two independent experiments.

(B) Western blot analysis of indicated markers. COV318 Cas9 stable cells were transduced with sgRNAs targeting CDKN2A.

(C) Western blot analysis of indicated markers in sgNTC, sgCDK2NA COV318 cells after 24-hour treatment with BLU1851, Palbociclib (1uM), or both.

(D) Quantification of (C). pRb1 levels were normalized per cell line to the vehicle control group. Error bars represent SD of two independent experiments.

(E) Western blot analysis of indicated markers in empty vector (EV), CDK4 wild-type (WT), and CDK4 R24C OVCAR-3 cells.

(F) Western blot analysis of indicated markers after FLAG immunoprecipitation (IP) in EV, CDK4 WT, and CDK4 R24C cells. Whole cell lysate (WCL) was included as a control.

(G) Western blot analysis of indicated markers in EV, CDK4 WT, CDK4 R24C OVCAR-3 cells after 24-hour treatment with BLU1851, Palbociclib (1uM), or both.

(H) Quantification of Fig S3G. pRb1 levels were normalized per cell line to the vehicle control group. Error bars represent SD of two independent experiments.

(I) Cell cycle profile of EV, CDK4 WT, CDK4 R24C cells after 24-hour treatment with BLU1851, Palbociclib (1uM), or both.

(J) Colony formation assays in EV, CDK4 WT, CDK4 R24C cells after 14 days of treatment with BLU1851, Palbociclib (1uM), or both. Cells were stained with crystal violet at experimental endpoint.

A

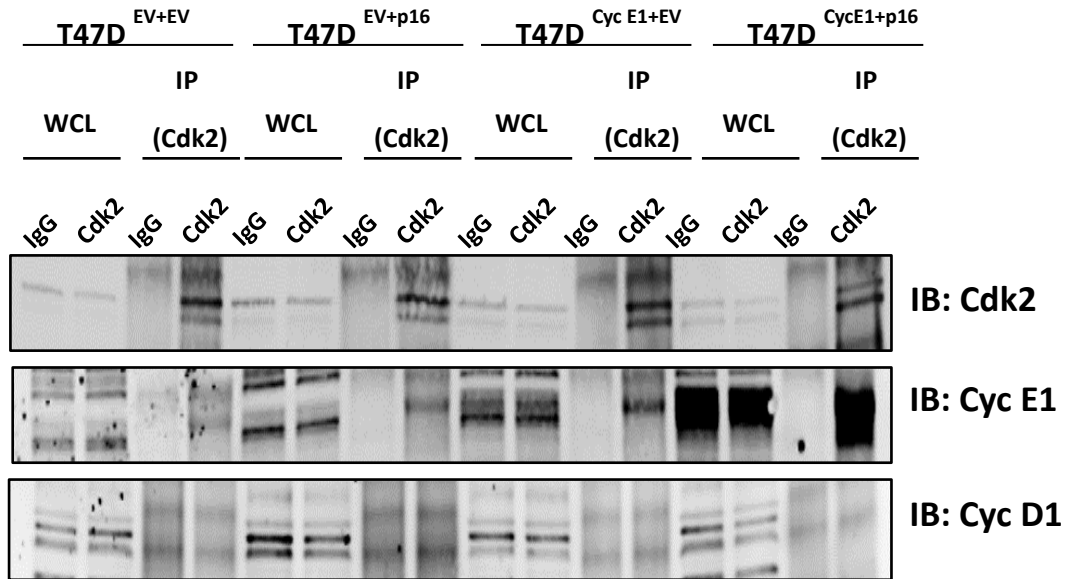


Figure S 3.4: CDK2 associates with Cyclin E1, but not Cyclin D1, in T47D cells

(A) Western blot analysis of indicated markers after CDK2 immunoprecipitation (IP) in T47D cells overexpressing p16, Cyclin E1, or both. Whole cell lysate (WCL) was included as a control.

Chapter 4 Perspectives

This dissertation details the role and requirements of CDK2 in both homologous recombination and cell cycle progression in *CCNE1*-amplified high-grade serous ovarian cancer (HGSOC). This chapter outlines the main findings and remaining questions for each.

CCNE1-amplified ovarian cancer cells rely on homologous recombination for repair of collapsed forks

Previous to the study outlined in chapter 2 (Brown et al., 2023), there has been no report, to our knowledge, to explain the mutual exclusivity between homologous recombination (HR) deficiency and *CCNE1*-amplification. Our results suggest that *CCNE1*-amplified HGSOC cells rely on HR to repair collapsed replication forks, likely due to the increased replication stress, and therefore high DNA damage load, imposed by Cyclin E1 levels. To follow up on these findings, it would be useful to know if *CCNE1*-amplified cells are able to tolerate more mismatches during recombination compared to wild-type (WT) cells. Our computational analysis showed that there is high incidence of gross-chromosomal rearrangement (GCR) in *CCNE1*-induced fragile sites specifically, but, while we hypothesize that these rearrangements could be mediated through recombination, efforts to show this computationally using traditional “rules” of recombination were not convincing (Rubnitz & Subramani, 1984). An experimental way to address this would be to utilize the Assay for Site Specific Homologous Recombination Assay (ASHRA) reporter system (Yoshino et al., 2019), modified so that the donor construct has decreasing levels of homology. The formation of a

recombination product could then be compared between WT and *CCNE1*-amplified cells. Learnings from these experimental models could then be applied computationally to strengthen the mechanistic hypothesis behind GCR in these cells. Once validated in cells, the next step would be to computationally analyze patient data and test if so-called “risky” HR may be occurring in patient tumors. If so, this might suggest novel players in HR in this disease area or could suggest HR occurring outside of S-phase. Both scenarios would inform on potential novel disease targets in this disease area.

CDK2 and Cyclin E1 localize to the sites of stalled replication

A second point of novelty in our study was the identification of CDK2 and Cyclin E1 at stalled replication forks. While previous methods had failed to detect levels of these complexes at the site of stalled forks (Dungrawala et al., 2015), utilization of the PLA method allowed us to measure these low abundant and/or transient interactions. To build on this finding, the precise role of CDK2 and cyclin E1 at stalled forks should be elucidated. A leading hypothesis is that CDK2/E1 activates ATR via ATRIP directly at the site of stalled forks, but the order of recruitment, and definitive experiments have not yet been done. A second hypothesis concerns potential kinase independent functions of Cyclin E1. One study suggests that Cyclin E1 imposes replication stress and accelerates entry into S-phase in a CDK2-independent manner (Matsumoto & Maller, 2004), and so it is plausible that Cyclin E1 has kinase independent roles at the stalled replication forks. To address this, cyclin E1 mutants that can or cannot bind CDK2 could be used in a FLAG-EdU PLA to determine if CDK2 binding is a requirement of Cyclin E1 localization to stalled forks. If results indicate that Cyclin E1 can localize to the site of stalled forks independently of CDK2, it would suggest a unique function of this cyclin. In

cases where tumor cells are dependent on Cyclin E1, but not CDK2 (e.g. liver cancer) (Geng et al., 2018), targeting Cyclin E1 alone through small molecule or degrader approaches could be a tractable therapeutic option.

CDK2 regulates HR non-redundantly in CCNE1-amplified HGSOC and combines with DNA damaging agents

The final novel discovery from the study in chapter 2 was that CDK2 non-redundantly regulates HR in *CCNE1*-amplified HGSOC. This was significant as CDK2 was previously shown to be dispensable in other cancer types in HR (Johnson et al., 2009, 2011). Because of this, CDK2 inhibition, at low doses, combines with DNA damaging agents or poly-(ADP-ribose) polymerase PARP inhibitors, which has implications for treatment of *CCNE1*-amplified HGSOC patients as combination treatment could offer clinical benefit. Although our study showed CDK2 is responsible for HR in this genetic context, it is not clear why CDK1 cannot compensate for CDK2 loss or inhibition. We hypothesize that the higher levels of CDK1 inhibitory phosphorylation may prevent CDK1 from participating. Indeed, a recent study suggests that *CCNE1*-amplified HGSOC cells may rely on restraining CDK1 activity to prevent entry into mitosis before DNA repair has taken place (Gallo et al., 2022). Activation of CDK1 with a PKMYT1 inhibitor may allow CDK1 to compensate for CDK2 in HR and is a strong experimental approach to begin to address this remaining question.

p16 expression is required for CDK2-dependence in HGSOC; CDK4/6 inhibition re-sensitizes p16 loss cells to CDK2 inhibition

The investigation presented in chapter 3 explored biomarkers of response/resistance to CDK2 inhibition in *CCNE1*-amplified HGSOC. Employing a whole genome Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based resistance screen, we identified *CDKN2A*, which encodes p16, as the top hit conferring resistance to two pharmacologically distinct CDK2 inhibitors. Mechanistically, when p16 is lost, CDK4 is activated and can bypass CDK2 inhibition. The reverse is also true – when p16 is co-overexpressed with Cyclin E1, cells that were previously dependent on CDK4, become resistant to CDK4 inhibition, and sensitized to CDK2 inhibition. Although *CDKN2A* loss and *CCNE1* amplification are mutually exclusive events in treatment naïve HGSOC patients, it may be an avenue of resistance to CDK2 inhibitors. Moreover, high levels of *CCNE1* mRNA or Cyclin E1 protein occur in the absence of amplification. In these instances, *CDKN2A* loss may be more common, and could act as an important secondary biomarker to predict response to CDK2 inhibitors in the clinic. There are cell lines that represent this scenario that could be used in follow up experiments to better understand how p16 levels may influence CDK2 inhibitor efficacy. Importantly, CDK4/6 inhibition re-sensitizes p16-null cells to CDK2 inhibition. Thus, although resistance to CDK2 inhibitors may emerge via p16 loss, there is a possible therapeutic avenue to address it. Further, combination treatment in patients that have high cyclin E1 levels in the absence of amplification and p16 loss may show the best efficacy. Given recent evidence that CDK4/6 act later in the cell cycle than previously thought and can compensate for CDK2 inhibition, at least in the short term, it is conceivable that several resistance mechanisms to CDK2 inhibition may converge on CDK4/6 (Cornwell et al., 2023; Spencer et al., 2013). Understanding how CDK4/6 can

become activated in addition to p16 loss will be important for refining patient selection strategies for CDK2 inhibitors, as single agent or in combination with CDK4/6 inhibitors.

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