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TITLE:

Cyclin E1 as a Therapeutic Target in Women with High-Grade Serous Ovarian Cancer and Primary Treatment Failure

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14. ABSTRACT A significant number of women with high-grade serous ovarian cancer (HGSC) are intrinsically refractory to standard platinum-based treatment. We have previously shown that amplification of the cyclin E1 gene (<i>CCNE1</i>) in HGSC is associated with primary chemoresistance and poor clinical outcome. Therefore, we hypothesized that cyclin E1 is a key therapeutic target in HGSC, and that generation of a genetically engineered mouse (GEM) model of <i>CCNE1</i> -amplified HGSC will facilitate the development of novel therapeutic strategies. Here, we have generated two mouse strains with Cre-mediated expression of full-length or truncated <i>Ccne1</i> at the <i>Rosa26</i> locus. We plan to cross these mice with <i>Pax8-TetOCre-Tp53</i> mice in order to induce expression of <i>Ccne1</i> in the fallopian tube epithelium and drive the initiation and development of HGSC. Mouse models that closely resemble human disease have been powerful platforms for new therapies and understanding resistance mechanisms. Immune checkpoint inhibitors have shown substantial activity in melanoma and lung cancer, and it is now a priority to extend these findings to other solid cancers, including HGSC. The availability of an intact animal model of <i>CCNE1</i> is likely to be a substantial value in development of immune checkpoint inhibitors and other approaches to targeting <i>CCNE1</i> amplified tumours.					
15. SUBJECT TERMS Cyclin E1, <i>CCNE1</i> , amplification, genomic instability, high-grade serous ovarian cancer (HGSC), chemoresistance, mouse models, fallopian tube, homologous recombination (HR)					
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1. INTRODUCTION

A significant number of women with high-grade serous ovarian cancer (HGSC) are intrinsically refractory to standard platinum-based treatment. We have previously shown that amplification of the cyclin E1 gene (*CCNE1*) in HGSC is associated with primary chemoresistance and poor clinical outcome. Cyclin E1 complexes with CDK2 to regulate cell cycle G1/S transition. Deregulation of cell cycle control is thought to be a prerequisite for tumour development and several studies have shown accelerated entry into S phase due to constitutive expression of *CCNE1*. Cyclin E1 is also able to induce chromosome instability by inappropriate initiation of DNA replication and centrosome duplication. We have demonstrated that *CCNE1*-amplified tumour cells are highly sensitive to knockdown of *CCNE1* or *CDK2*. Our current goal is to further advance novel therapeutic approaches to targeting *CCNE1* amplification through the development and characterisation of genetically engineered mouse (GEM) models of *Ccne1*-amplified HGSC. We also aim to identify and characterise additional genetic events that enhance cyclin E1-mediated transformation in fallopian tube secretory epithelial cells (FTSECs), the likely cell of origin in HGSC, using a high-throughput CRISPR-mediated genome-wide loss of function (LOF) screen. Results generated from this study have significant potential for clinical translation in HGSC, as well as other solid tumours with high frequency of *CCNE1* amplification including gastric, breast, and esophageal cancer.

2. KEYWORDS

Cyclin E1, CCNE1, amplification, genomic instability, high-grade serous ovarian cancer (HGSC), chemoresistance, mouse models, fallopian tube, homologous recombination (HR)

3. ACCOMPLISHMENTS

(A) Major Goals of the Project

Site 1: Peter MacCallum Cancer Centre; Melbourne, VIC, Australia (Bowtell)

Site 2: University of Pennsylvania; Philadelphia, PA, USA (Drapkin)

Site 3: Dana-Farber Cancer Institute; Boston, MA, USA (Berhoukim)

SPECIFIC AIM 1: GENERATE A TRANSGENIC MOUSE MODEL	Timeline (Months)	Site 1 (PI)	Site 2 (Co-PI)	Site 3 (Co-PI)	% Completed
Major Task 1: Generate transgenic <i>CAG-LSL-Ccne1</i> mice					
Subtask 1: Generate transgenes using the CMV early enhancer/chicken beta-actin (<i>CAG</i>) promoter, followed by a <i>loxP-STOP-loxP</i> cassette to provide conditional expression of full-length and low molecular weight forms of mouse <i>Ccne1</i>	1-3	*			100%

Subtask 2: Rosa26 knock-in- CAG promoter-driven, full length or LMW Ccne1 cDNA will be knocked into the ROSA26 locus by homologous recombination	3-6	*			100%
Subtask 3: Southern Blot analysis to determine precise integration via homologous recombination in mouse ES cells. Blastocyst injection of targeted ES into sterile mouse embryos to generate a founder line. <i>*Outsourced to Ozgene</i>	6-12	*			67%
Major Task 2: Generate compound mutant mice by intercross and induce tumors	Months				
Subtask 1: Submit documents for local IRB and Animal Ethics review. <ul style="list-style-type: none"> Submission of institution approved animal protocols and related material for DoD's ACURO approval. Receive ACURO approval before initiating animal experiments. 	1-6	x	x		100%
Subtask 2: Interbreed breed CAG-LSL-Ccne1 founding mice to expand the colony and obtain homozygous stock. <ul style="list-style-type: none"> Evaluate observed genotype ratios of animals generated for evidence of intrauterine or perinatal mortality and survey tissues from neonatal and adult mice histologically for normal development 	12-18	x			35%
Subtask 3: Intercross to Pax8 Cre-deletor mice, also carrying <i>Tp53</i> (R270H) and <i>Pten</i> ^{-/-} alleles. Confirm genotype.	15-18	x			0%
Subtask 4: Ship compound <i>CAG-LSL-Ccne1, Ccne1; Tp53; Cre deletor</i> , and <i>Ccne1; Tp53; Pten; Cre deletor</i> mice to Drapkin lab and establish stock for investigation of early lesion studies	18-24	x	x		0%
Subtask 5: Administration of doxycycline (0.2mg/ml ad libitum) in the drinking water to induce expression of cyclinE1, mutant Tp53, and Pten deletion <ul style="list-style-type: none"> Monitor animals by ultrasound, body weight, and 	18-24	x			0%

physical examination on a weekly basis for tumor development. • Ship tumor blocks to Drapkin lab.					
Milestone #1: Established the Ccne1 model					
Major Task 3: Pathologically and genomically characterize tumours. Compare tumors in different transgenic and mutant backgrounds	Months				
Subtask 1: Evaluate Pax8, Stathmin-1, Ccne1, Ki-67, Pten and Tp53 expression compared to controls using immunohistochemistry	24-30		x		0%
Subtask 2: Evaluate DNA copy number by low coverage (~6X) whole genome sequencing (WGS). Compare copy number profiles to human HGSC with CCNE1 amplification to identify syntenic regions of gain or loss	24-36	x			0%
Subtask 3: Characterize the timing and penetrance of tumor development and investigate early lesions	24-36	x	x		0%
Milestone #2: Characterized tumours and co-authored manuscript.					
SPECIFIC AIM 2: INTERSECT FUNCTIONAL STUDIES IN FTSECS AND PAN-CANCER TUMOR GENOMIC DATA TO IDENTIFY GENES THAT COOPERATE WITH CCNE1 IN THE TRANSFORMATION OF FTSEC TO GUIDE THE DEVELOPMENT OF COMPOUND CYCLINE1 GEM MODELS.	Timeline	Site 1 (PI)	Site 2 (Co-PI)	Site 3 (Co-PI)	% Completed
Major Task 4: shRNA screen of FTSEC over expressing CCNE1 performed in Victorian Centre for Functional Genomics (VCFG)					
Subtask 1: Transduce cells at an MOI of ~ 0.3. Select cells by puromycin sensitivity and grow in soft agar. Isolate all the organoids, extract genomic DNA and perform next generation sequencing to identify shRNAs that are statistically abundant and potential drivers of the phenotype.	6-18	x			10%
Subtask 2: Utilise the VCFG bioinformatics pipeline for analysis of the sequencing data.	18-24	x			0%

Subtask 3: Orthogonal validation using CRISPR-mediated gene silencing	18-24	x			0%
<i>Milestone #3: Genes required in vitro for CCNE1-dependent FTSEC transformation and tumour cell survival identified</i>					
Major Task 5: Analyse shRNA and cDNA screen findings against pan-cancer data					
Subtask 1: Filter hits from the shRNA and cDNA screens using TCGA and ICGC pan-cancer data.	18-30			x	0%
<i>Milestone #4: In vitro screen observations supported by genomic data from human cancers</i>					

(B) Accomplishments Under These Goals

a. Major Activities

We primarily focused on the generation of transgenic *CAG-LSL-Ccne1* mice (Major Task 1) during the reporting period.

b. Specific Objectives

In order to complete the generation of transgenic *CAG-LSL-Ccne1* mice we had the following objectives:

- Identify a suitable promoter to drive *Ccne1* transgene expression in mice
- Design a vector for homology-mediated targeting of *Ccne1* to the *Rosa26* locus
- Perform successful ESC targeting and blastocyst injection to generate transgenic animals

c. Significant Results or Key Outcomes

Testing of the CAG & UBC promoters in FTSECs. As stated in the proposal, we planned to use the synthetic CAG promoter to drive expression of *Ccne1* at the *Rosa26* locus as it expressed across a wide range of mouse tissues and has been used extensively for transgene expression in mice. We also wanted to compare it to the human UBC promoter, which is also commonly used for transgene expression. As neither promoter had been targeted to FTSEC, we could not predict which would be more appropriate for our studies. Therefore as an initial guide, we generated GFP constructs with each promoter and transfected these into FTSEC lines to monitor expression. Under optimal transfection conditions, we found that the CAG promoter was able to drive strong GFP expression in 35.9% of transfected FTSECs versus the UBC promoter, which only stimulated GFP expression in 12.7% of cells (Figure 1). These results indicated that the CAG promoter was the most suitable for expression of *Ccne1* in mice. Subsequently, Ozgene approached us about pursuing the comparison of the CAG and UBC promoters *in vivo*, and agreed to generate an additional *UBC-LSL-Ccne1* mouse strain for direct comparison with the *CAG-LSL-Ccne1* at no cost.

Successful generation of UBC-LSL-CCNE1 and CAG-LSL-LMW_Ccne1 transgenic mice.

Generation of the *Ccne1* transgenic mice was outsourced to Ozgene. Targeting vectors (Figure 2) were designed and produced to our specifications and included: the CAG or UBC promoter, a LoxP-STOP-LoxP (LSL) cassette, a neomycin selectable marker, and the coding sequence (cDNA) for either full-length or a low molecular weight (LMW) isoform of *Ccne1* (Appendix A). Ozgene was able to successfully integrate *UBC-LSL-Ccne1* and *CAG-LSL-LMW_Ccne1* into the *Rosa26* locus in ES cells and generate homozygous stocks of mice (Figure 3 & Figure 4). These mice were subsequently shipped to the Peter MacCallum Animal Facility, where we have been able to successfully genotype, expand, and maintain colonies (Figure 5). Furthermore, to facilitate the generation of compound mutant mice, both the Bowtell and Drapkin laboratories have submitted animal ethics protocols and received approval from their respective Animal Ethics Committees (Appendix B & Appendix C).

Multi-copy CAG-LSL-Ccne1 transgenic mice. Ozgene also reported successful generation of *CAG-LSL-Ccne1* targeted ES cells and subsequent germline transmission in mice. However, in the course of further characterisation of the mice it became apparent that there are multiple integrants in the locus, perhaps up to 10 copies. We have obtained these mice and continue to characterise them while Ozgene has returned to generating single copy precise intergrants of the *CAG-LSL-Ccne1* transgene. It may be that the additional copies will provide enhanced expression that is serendipitously useful or be transcriptionally leaky and not appropriate for our studies. Will characterise the single and multiple copy animals until we have a clear outcome.

Optimization of soft agar growth conditions for FTSECs for the genome-wide screen (Aim 2). We have optimized conditions for soft agar growth of FTSECs in 6-well tissue culture dishes to facilitate positive selection in the genome-wide loss-of-function screen. We are currently working on establishing FTSECs with stably expression of Cas9.

FIGURES:

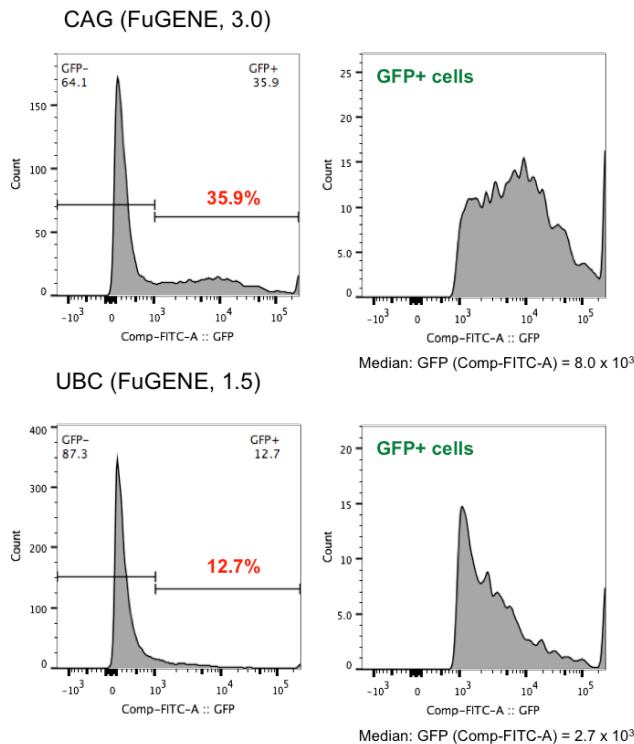


Figure 1. CAG and UBC driven expression of GFP in FTSECs. FACS analysis for GFP expression in FTSECs optimally transfected with vectors containing either the CAG or UBC promoter and a GFP reporter protein.

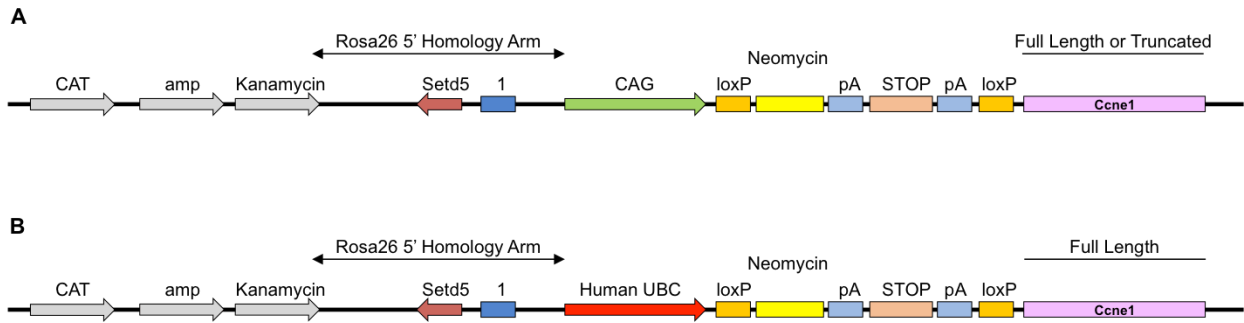


Figure 2. Targeting vectors used to facilitate insertion of *Ccne1* at the *Rosa26* locus. (A) The CAG promoter vector used to generate mice expressing full length or truncated *Ccne1*. (B) The UBC promoter used to generate mice expressing full length *Ccne1*. Note the presence of a loxP-STOP-loxP cassette facilitating conditional expression of *Ccne1* via Cre recombinase.

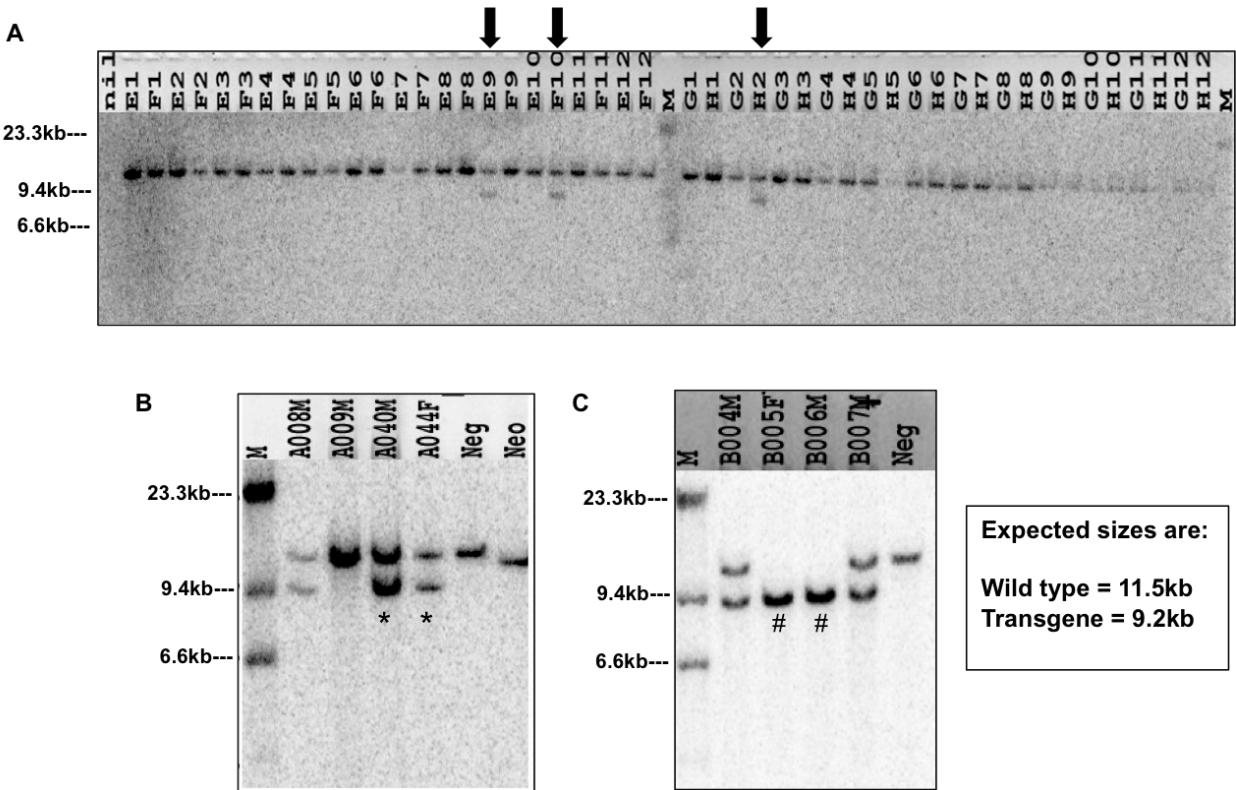


Figure 3. Successful ES cell targeting and germline transmission of *CAG-LSL-LMW_Ccne1*. South blot screening of: (A) targeted ES cells identified positive clones as indicated by the black arrows; (B) chimera mice identified successful germline transmission as indicated by *; (C) heterozygous founder mating identified homozygous offspring as indicated by #.

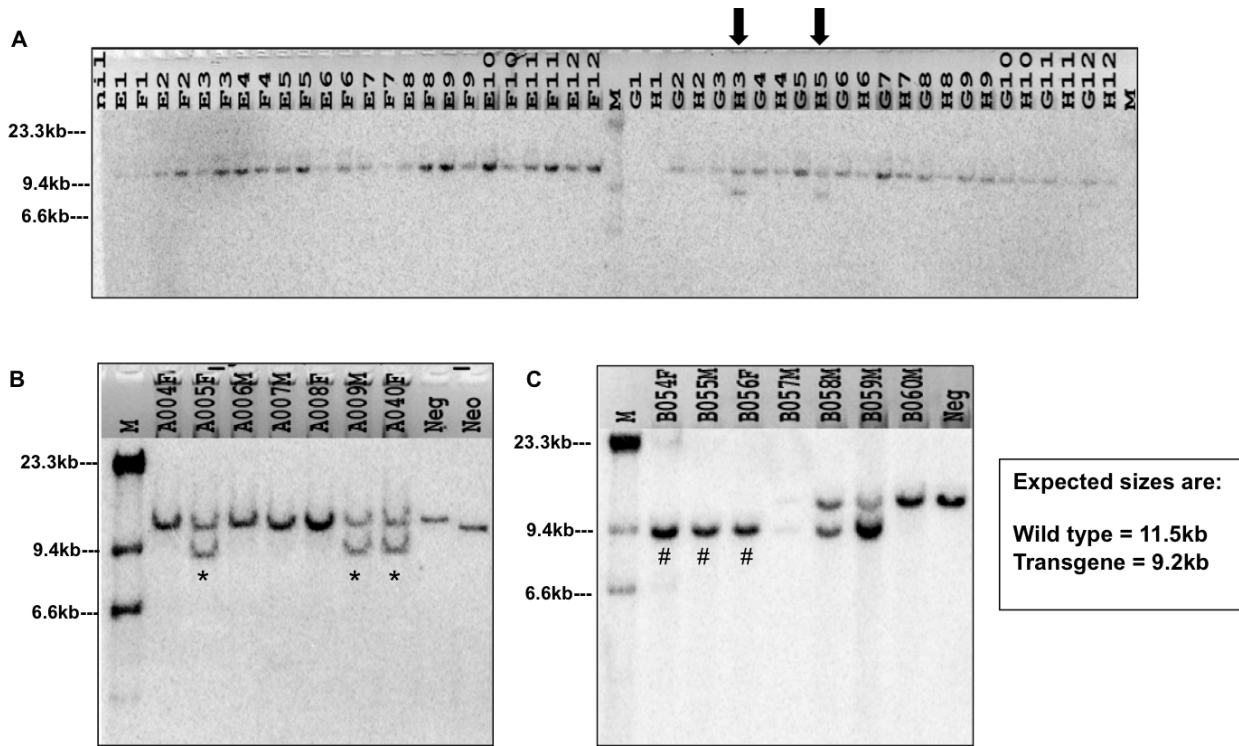


Figure 4. Successful ES cell targeting and germline transmission of *UBC-LSL-Ccne1*. South blot screening of: (A) targeted ES cells identified positive clones as indicated by the black arrows; (B) chimera mice identified successful germline transmission as indicated by *; (C) heterozygous founder mating identified homozygous offspring as indicated by #.

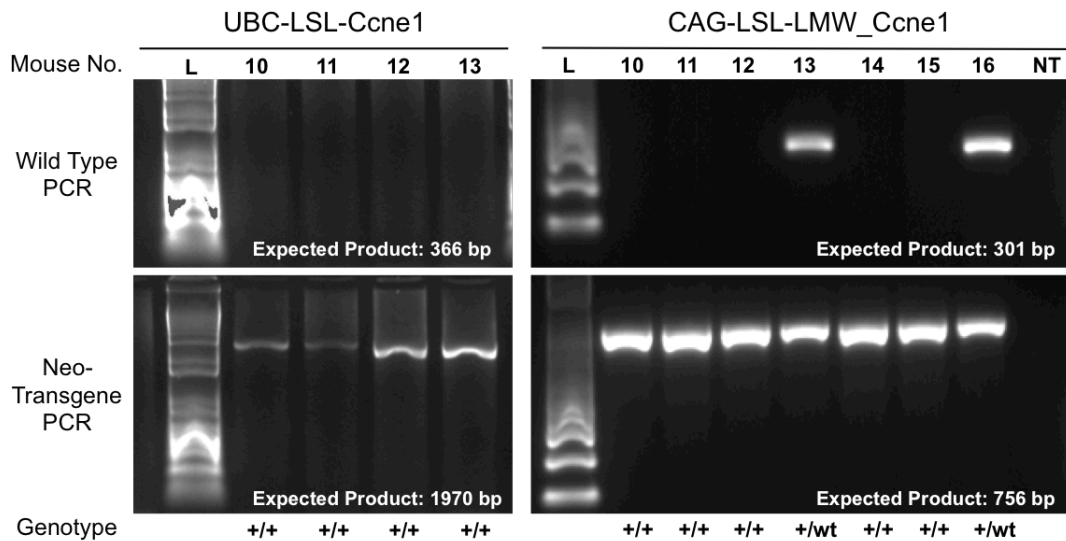


Figure 5. Genotyping PCR. Successful breeding of homozygous *UBC-LSL-Ccne1* and *CAG-LSL-LMW-Ccne1* mice at the Peter MacCallum Animal Facility.

d. Other Achievements

We identified AKT inhibition as a potential therapeutic combination with the CDK2 inhibitor dinaciclib in *CCNE1* amplified HGSC cell lines. The results of this study are shown in the attached manuscript (Appendix D: Au-Yeung et al, Clinical Cancer Research, 2016, in press) and are briefly described below.

We showed that CDK2 is a highly selective target for *CCNE1* amplified high-grade serous ovarian cancer (HGSC), using siRNA and conditional shRNA systems *in vitro* and *in vivo*. However, we found that dinaciclib, a small molecule CDK inhibitor, did not trigger amplicon dependent sensitivity in a panel of HGSC cell lines. This is likely due to the non-specific nature of small molecule CDK inhibitors such as dinaciclib, which although it is a potent inhibitor of CDK2, also inhibits CDK1, 5, 9 and 12. In order to improve on the efficacy of dinaciclib, we conducted a high throughput compound screen of over 4000 known drugs and targeted agents to identify selective synergistic combinations. We also aimed to identify combinations that may overcome drug resistance by including a CDK-inhibitor resistant cell line previously derived from a sensitive, *CCNE1*-amplified cell line. We obtained a number of hits, and focused particularly on a combination of dinaciclib and AKT inhibitors that were synergistic in *CCNE1*-amplified HGSC. We orthogonally validated the interaction between *CCNE1* and *AKT*, both in genomic data from TCGA and functionally in fallopian tube secretory cells, the target of HGSC transformation. In summary, our findings support CDK2 as a specific target for *CCNE1* amplified HGSC, and identify a combination of dinaciclib and AKT inhibitors that may inform the design of a rational clinical trial targeting an important subset of HGSC.

(C) Opportunities for Training and Professional Development

Nothing to Report

(D) Dissemination of Results to Communities of Interest

Nothing to Report

(E) Plans for Next Reporting Period to Accomplish the Goals

Our overall goal is to develop a GEM model of *Ccne1*-amplified HGSC to better understand disease initiation and to facilitate the development of novel therapeutic strategies. To this end, over the next year, we will finish generating the *CAG-LSL-Ccne1* mouse strain and begin generating compound mutant mice by intercrossing the *Ccne1* mouse strains with *Pax8-TetO-Cre-Tp53* mice (as outlined in the proposal). Once the compound mutant mice are established, we plan to induce *Ccne1* expression and *Tp53* deletion via doxycycline administration and systematically monitor mice for tumour development. The Drapkin and Bowtell laboratories will pathologically and genomically characterize any tumours that develop, respectively. Finally, we will finish optimization of conditions (transduction and soft agar growth) and carry out the high-throughput CRISPR-mediated gene knockout screen in FTSECs. Results from the screen will identify tumor suppressor genes that cooperate with *CCNE1* in the transformation of FTSECs, and guide the selection of alternative genes to develop additional compound GEM models using the *CAG-LSL-Ccne1-Tp53* strains.

4. IMPACT

(A) Impact on the Development of the Principal Discipline(s) of the Project

Nothing to Report

(B) Impact on Other Disciplines

Nothing to Report

(C) Impact on Technology Transfer

Nothing to Report

(D) Impact on Society Beyond Science and Technology

Nothing to Report

5. CHANGES/PROBLEMS

(A) Changes in Approach and Reason for Change

Selection of a CRISPR-Cas9 approach for the loss-of-function screen (Aim 2.1). At the time of the proposal submission, CRISPR-Cas9 was just emerging as a powerful tool for manipulating genomes, including genome-wide loss-of-function (LOF) screens. We had planned to perform an shRNA positive selection screen to identify tumour suppressors that cooperate with *CCNE1* in the transformation of FTSECs, however we indicated that we were open to adopting a CRISPR-Cas9 approach as the methodology evolved. Recent publications have compared CRISPR-Cas9 to shRNA-based genome-wide LOF screens in human cancer cell lines and found CRISPR-Cas9 to be superior in identifying essential/lethal genes, which suggests that complete gene inactivation may be necessary to identify cellular dependencies (Munoz et al *Cancer Discovery* 2016; Aguirre et al *Cancer Discovery* 2016). Therefore, we have elected to use a CRISPR-Cas9 approach in our genome-wide LOF screen. This screen will be performed in the Victorian Centre for Functional Genomics (VCFG), located at the Peter MacCallum Cancer Centre as previously described in the proposal. The overall workflow for the screen will remain as described for the shRNA screen including soft agar growth selection and next generation sequencing to identify guide RNAs (gRNAs) that are statistically abundant and potential drivers of the phenotype. The VCFG have obtained, expanded, and sequenced the human CRISPR Brunello lentiviral pooled library (two-vector system), which was designed using optimized metrics and consists of four gRNAs per gene for a total of 76,441 gRNAs (Doench et al *Nat Biotechnol.* 2016). The switch to a CRISPR-based approach results in a slight delay as the VCFG have obtained the libraries and developed virus. During this time we have optimized conditions for soft agar growth of FTSECs, and are currently generating the required FTSEC lines stably expressing Cas9. Since any intercross into mice of additional mutant alleles revealed by the CRISPR screen requires prior characterisation of the GEM mouse model, the delay in initiating the screen will not impact on the overall timeline for the study.

Deprioritization of *Pten* deletion from the breeding scheme. As stated in the proposal Aim 2.2, we plan to filter hits identified in the CRISPR-mediated LOF screen (and other previously performed cyclin E1 gain-of-function and LOF screens) to identify the most relevant genes to assess in mice. As an initial filter, we utilized pan-cancer data from TCGA and identified genes that are frequently altered by somatic genetic events (amplification or deletion), and for which these events are positively correlated with *CCNE1* amplification, suggesting cooperativity. Our preliminary results indicate that *PTEN* deletion does not significantly co-occur with *CCNE1*

amplification in human cancers (Table 1). Although loss of Pten significantly reduced tumour latency in PI Drapkin's *BRCA1/2* model we have deprioritized the crossing of *CAG-LSL-Ccne1-Tp53* mice with *Pten*^{-/-} mice, as it would not reflect the biology of human HGSC. It is quite possible that the combination of *Ccne1* over expression and *Tp53* deletion will be sufficient for generating HGSC in the mouse. However, in parallel, we will also consider other mutations/deletions identified in our loss-of-function screen, which may be introduced into the *CAG-LSL-Ccne1-Tp53* GEM model to enhance *in vivo* transformation.

Rank	Peak	Correlative p-value	Gene(s)
1	del_22q11.1	0.0051	<i>IL17RA, POTECH, CCT8L2, XKR3, CECR7</i>
2	amp_ERBB2	0.0212	<i>ERBB2</i>
3	del_KMT2C	0.0236	<i>KMT2C</i>
4	amp_(MCL1)	0.0254	<i>ENSA, GOLPH3L</i>
5	amp_17q11.2	0.0275	<i>FLOT2, ERAL2, FAM222B, PHF12, SEZ6</i>
106	del_PTEN	0.9966	<i>PTEN</i>

Table 1. Genetic Alterations That Correlate with CCNE1 Amplification. GISTIC peaks (from the "2015-06-01 stddata__2015_04_02 arm-level peel-off" analysis at <http://www.broadinstitute.org/tcga>) with the p-values for correlation of the peak event with amplification of *CCNE1* in the pan-cancer TCGA dataset.

(B) Actual or Anticipated Problems or Delays and Actions or Plans to Resolve Them

Relocation of Peter MacCallum Cancer Centre. In June 2016, Peter MacCallum Cancer Centre relocated to the new Victorian Comprehensive Cancer Centre building in the Parkville precinct of Melbourne. This relocation resulted in the Peter MacCallum Animal Facility being unable to accept external mouse importations from approximately April to August 2016, which impacted the shipment of the *Ccne1* mouse strains from Ozgene. To keep the project on track, we contracted Ozgene to interbreed the *UBC-LSL-Ccne1* and the *CAG-LSL-LMW_Ccne1* founding mice to expand the colonies and obtain homozygous stocks. We received homozygous breeding pairs for both strains in mid-August 2016, and have successfully continued to expand these colonies.

Relocation of the Drapkin Laboratory. During Year 1 of the grant, PI Drapkin accepted a new position at the University of Pennsylvania that required the relocation of his laboratory to Philadelphia, Pennsylvania. Due to the necessary shipment and reestablishment of the Drapkin laboratory mouse strains at the new animal facility, there has been a delay in receiving the *Pax8-TetOCre-Tp53* mice required for interbreeding with the *Ccne1* mice. We anticipate shipment of three breeding pairs of *Pax8-TetOCre-Tp53* mice to Peter MacCallum Cancer Centre in February 2017. Additionally, we have identified a laboratory in Australia that has an alternative *Pax8-TetOCre* mouse strain that can be imported to Peter MacCallum Cancer Centre if the delay in shipment from the Drapkin laboratory is longer than expected.

Multiple Integrations of Ccne1 in CAG-LSL-Ccne1 Mice. In May 2016, we were informed by Ozgene that in the process of reconfirming integration of *Ccne1* in the germline offspring, they identified that the *CAG-LSL-Ccne1* mice had multiple integrations of the vector at the *Rosa26* locus. Unfortunately, frozen stocks of targeted *CAG-LSL-Ccne1* mouse ES cells were not viable, and Ozgene had to begin *de novo* derivation of the strain. As of this report, Ozgene has achieved successful single integration and germline transmission of *CAG-LSL-Ccne1*, with heterozygous mice anticipated for shipment in late-January 2017. Although it will be important to have strain with single integration of *Ccne1*, we believe that *CAG-LSL-Ccne1* mice with multiple integrations (*CAG-LSL-Ccne1_multi*) may also prove useful given that patients with HGSC can

have more than eight copies of *CCNE1*. Therefore, we have elected to maintain the *CAG-LSL-Ccne1_multi* mouse strain (provided by Ozgene at no cost) and characterize it in parallel with the other established mouse strains as an interim measure.

(C) Changes That Had a Significant Impact on Expenditures

Nothing to Report

(D) Significant Changes in Use or Care of Human Subjects, Vertebrate Animals, Biohazards, and/or Select Agents

In regard to animal ethics protocol titled “Genetically Engineered Mouse Models of Ovarian Cancer”, IACUC protocol E561, we have submitted and received approval from the Peter MacCallum Animal Experimentation Ethics Committee to add an additional investigator (Dr. Jessica Beach). The amendment was approved 21 April 2016 and these changes were reported via email to ACURO on 1 September 2016.

6. PRODUCTS

(A) Journal Publications

Au-Yeung G, Lang F, Mitchell C, Jarman KE, Lackovic K, Aziz D, Cullinane C, Pearson RB, Mileskin L, Rischin D, Karst AM, Drapkin R, Etemadmoghadam D, Bowtell DD. Selective targeting of Cyclin E1 amplified high grade serous ovarian cancer by cyclin-dependent kinase 2 and AKT inhibition. *Clinical Cancer Research*, 2016. Advanced online publication. doi: 10.1158/1078-0432.CCR-16-0620.

Acknowledgement of federal support: Yes

(B) Other Products—Research Material

We have generated two of three planned GEM strains with mouse cyclin E1 (*Ccne1*; full length or truncated) expressed under the control of the *CAG* or human *UBC* promoter at the *Rosa26* locus. Furthermore, our targeting construct contained a *loxP-STOP-loxP* cassette allowing for tissue-specific, *Cre*-mediated excision of the cassette and conditional expression of *Ccne1*. These mouse strains may be beneficial in the generation of GEM models of other CCNE1-amplified cancers, such as gastric cancer.

Mouse Strain	Status
C57Bl6-Gt(ROSA)26Sor ^{tm1(LSL-CAG-Ccne1)Ozg}	Anticipated in February 2017
C57Bl6-Gt(ROSA)26Sor ^{tm1(LSL-UBC-Ccne1)Ozg}	Established
C57Bl6-Gt(ROSA)26Sor ^{tm1(LSL-CAG-LMWCcne1)Ozg}	Established

7. PARTICIPANTS

(A) Individuals who have worked on this project

The following individuals have contributed to the project:

Name:	David Bowtell
Project Role:	PI
Nearest Person Month Worked:	1
Contribution to Project:	No Change

Name:	Ronny Drapkin
Project Role:	Co-PI
Nearest Person Month Worked:	1
Contribution to Project:	No Change

Name:	Rameen Berhoukim
Project Role:	Co-PI
Nearest Person Month Worked:	1
Contribution to Project:	No Change

Dr. Dariush Etemadmoghdam was named under other personnel as the postdoctoral fellow on this project at Peter MacCallum Cancer Centre, however Dr. Etemadmoghdam has taken on a new role that means he no longer has the required time to commit to the project. In his place, we plan to deploy Dr. Jessica Beach, who also has the required and appropriate experience to be the postdoctoral fellow on this project (see below). As this role is not due to commence until year 2 of this project (1 October 2016) we anticipate that this personnel change will have no impact on the progress of the project or the budget.

Name:	Jessica A. Beach
Project Role:	Postdoctoral Fellow
Research Identifier:	orcid.org/0000-0001-9995-9892
Nearest Person Month Worked:	6 (anticipated each year)
Contribution to Project:	Dr. Beach will coordinate all aspects of the study being performed at Peter MacCallum Cancer Centre including animal breeding, animal model characterisation, and the high-throughput gene knockout screen.

(B) Changes in Active Other Support of the PI or Key Personnel

David Bowtell (PI)

Pending to Active Research Support:

APP1092856 (Bowtell) 2016 – 2020
 NHMRC Program Grant
 Improving Outcomes for Women with Epithelial Ovarian Cancer
 Role: Principal Investigator (40% effort)
 Awarded: \$374,632 AUD (annual direct costs)

OC150563 (Pike/Pearce) 2016 – 2019
 US Department of Defense Ovarian Cancer Research Program
 Multidisciplinary Ovarian Cancer Outcomes Group (MOCOG)
 Role: Key Personnel (5% effort Year 1, 2, 3; 4% effort Year 4)
 Awarded: \$216,418 (direct costs)

APP 14/TPG/1-15 (deFazio) 2015 – 2019
Cancer Institute NSW Translational Program Grant
INOVATe - Individualised Ovarian Cancer Treatment Through Integration of Genomic Pathology
into Multidisciplinary Care
Role: Chief Investigator E (5% effort)

Active to Completed Research Support:

APP1044447 (Bowtell) 2013 – 2015
NHMRC Project Grant
Novel Therapeutic Approaches to Ovarian Clear Cell Cancer
Role: Chief Investigator A

Ronny Drapkin (Co-PI)

Pending to Active Research Support:

No Number (Drapkin) 2013 – 2017
Tina Brozman Foundation
Ex vivo culture of fallopian tube epithelium for proteomic detection of biomarkers in ovarian
cancer
Role: Principal Investigator (5% effort)
Awarded: \$45,455 (annual direct costs)

No Number (Drapkin) 2016 – 2016
Tina Brozman Foundation
Tina Brozman Ovarian Cancer Consortium (TBOCC)
Role: Principal Investigator (5% effort)
Awarded: \$33,174 (annual direct costs)

Active to Completed Research Support:

R21 CA156021 (Drapkin) 2013 – 2015
NIH/NCI
Elafin as a biomarker in serous ovarian cancers and basal-like breast tumors
Role: Principal Investigator (15% effort)
Awarded: \$57,925 (annual direct costs)

Rameen Berhoukim (Co-PI)

Pending to Active Research Support:

5 R01 CA188228-02 (Beroukhim, Ligon) 2015 – 2020
NIH Project Grant
Genetic Evolution of Glioblastoma During Radiation and Temozolomide Therapy
Role: Principal Investigator (20% effort)
Awarded: \$448,351 (annual direct cost)

No Number (Beroukhim) 2015 – 2016
Ian's Friend's Foundation

Identifying Therapeutic Targets of MYB-QK1 Fusions in Pediatric Low-Grade Glioma Role: Key Personnel (1% effort) Awarded: \$100,000 (direct costs)	
No Number (Beroukhim) Cure Starts Now Characterizing resistance mechanisms to radiation and adjuvant therapy in DIPG Role: Key Personnel (1% effort) Awarded: \$90,909 (direct costs)	2016 – 2016
Footridge Award (Lindquist, Santagata, Beroukhim) KI-DF/HCC Bridge Program Modulating the heat-shock machinery to limit genetic heterogeneity and evolution of highly malignant cancers Role: Key Personnel (2% effort) Awarded: \$41,288 (Beroukhim portion, direct costs)	2016 – 2017
Innovation Grant (Beroukhim) Alex's Lemonade Stand Foundation Characterizing resistance mechanisms to BET-bromodomain inhibition of MYC-amplified medulloblastoma Role: Principal Investigator (12.5% effort) Awarded: \$125,000 (annual direct cost)	2015 – 2017
No Number (Beroukhim) Broad Institute SPARC (Scientific Projects to Accelerate Research & Collaboration) EvoSeq: A molecular time machine to study evolution Role: Key Personnel (2% effort) Awarded: \$185,000	2016 – 2016
No Number (Beroukhim) St. Baldrick's Foundation Intratumoral heterogeneity of resistance drivers in DIPG Role: Key Personnel (1% effort) Awarded: \$100,000 (direct costs)	2016 – 2017
No Number (Beroukhim) Pediatric Brain Tumor Foundation BRD4 as a therapeutic target in medulloblastoma Role: Principal Investigator (5% effort) Awarded \$100,836 (annual direct costs)	2016 – 2018
U24 CA210978-01 (Beroukhim, Cherniack) NIH Resource-Related Research Projects Center for the comprehensive analysis of somatic copy-number alterations in cancer Role: Principal Investigator (20% effort) Awarded: \$412,500 (annual direct costs)	2016 – 2021

(C) Other Partner Organizations

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDIX

Appendix A: *Ccne1* Sequences Used for Targeting

Appendix B: E561 Animal Ethics Approval, Peter MacCallum Cancer Centre (Bowtell)

Appendix C: 806138 Animal Ethics Approval, University of Pennsylvania (Drapkin)

Appendix D: Journal article in press, Au-Yeung et al. *Clinical Cancer Research* 2016

APPENDIX A: *Ccne1* Sequences Used for Targeting

CAG-LSL-*Ccne1* (1615_Truman)

>Mouse_CCNE1_FullLength_CDS

ATGCCAAGGGAGAGAGACTCGACGGACCACAGCAACATGAAAGAAGAAGGTGGCTCCGACCTTTCAGTCCGCTCCAG
AAAAAGGAAGGCAAATGTGGCCGTGTTTTTGCAGACCCAGATGAAGAAATTGCCAAGATTGACAAGACTGTGAAAA
GCGAGGATAGCAGTCAGCCCTGGGATGATAATTCAGCATGCGTGGACCCCTGCTCTTTCATCCCCACCCCTAACAAA
GAAGAGGACAATGAGCTTGAATACCCAGGACTGCATTTACAGCCTCGGAAAATCAGACCACCCAGAGCCTCCCCACT
TCCCGTCTTGAATTGGGGCAATAGAGAAGAGGTTTGGAGGATCATGTTAAACAAAAGAAAAGACTTACCTGAGAGATG
AGCACTTCTGCAGCGTCATCTCTCCTGCAGGCGAGGATGAGAGCAGTTCTTCTGGATTGGCTAATGGAGGTGTGC
GAAGTCTATAAGCTCCACAGAGAGACGTTCTACTTGGCACAGGACTTCTTTGATCGTTACATGGCATCACAAATAA
TATCATAAAAAACACTTTTTACAGCTTATTGGGATTTACAGCCTTATTTATTGCTTCAAAACTTGAGGAAATCTACCCTC
CAAAGTTGCACCAGTTTGCTTATGTTACAGATGGCGCTTGCTCCGGGGATGAAATTTCTTACCATGGAATTGATGATG
ATGAAGGCCCTTAAGTGGCGTCTAAGCCCTCTGACCATTGTGTCTGGCTGAATGTCTATGTCCAAGTGGCCTATGT
CAACGACACGGGTGAGGTGCTGATGCCTCAGTACCCACAGCAGGTCTTCGTGCAGATCGCAGAGCTTCTAGACCTGT
GCGTCTGGATGTTGGCTGCTTAGAATTTCTTATGGTGTCTCGCTGCTTCTGCTTTGTATCATTTCTCCTCACTG
GAGTTGATGCAGAAGGTCTCAGGTTATCAGTGGTGCACATAGAGAAGTGTGTCAAATGGATGGTTCCGTTCCGCAT
GGTTATCCGGGAGATGGGAAGTTCCAAGCTCAAGCACTTCCGGGGAGTCCCATGGAAGACTCCCACAACATCCAGA
CCCACACCAACAGCTTGGATTTGCTGGACAAAGCCCAAGCAAAGAAAGCCATATTGTGAGAACAGAATAGGATTTCT
CCTCCTCCGAGTGTGGTCTGACACCCCCACCCAGCAGTAAGAAGCAGAGCAGCGAGCAGGAGACAGAATGA

>Mouse_CCNE1_FullLength_Protein

MPRERDSTDHNSMKEEGGSDLSVRSRKRKANVAVFLQDPDEEIAKIDKTVKSEDSSQPWDDNSACVDPCSFIPTPNK
EEDNELEYPRTAFOQPRKIRPPRASPLPVLNWNREEVWRIMLNKEKTYLRDEHFLQRHPLLQARMRAVLLDWLMEVC
EVYKLRHRETFYLAQDFFDRYMASQHNIIKTLLQLIGISALFIASKLEEIYPPKLHQFAYVTDGACSGDEILTMELMM
MKALKWRLSPLTIVSWLNVYVQVAYVNDTGEVLMPOYPQOVFVQIAELLDLCVLDVGCLEFPYGVLAASALYHFSS
ELMQKVSQYQWCDIEKCVKWMVFPAMVIREMGSSKLRKHFVPMEDSHNIQTHTNSLDLLDKAQAKKAILSEQNRIS
PPPSVVLTPPPSSKKQSSSEQETE

CAG-LSL-*Ccne1*-Truncated (1616_Enoch)

>Mouse_CCNE1_T1_Truncated_CDS

ATGGACCCAGATGAAGAAATTGCCAAGATTGACAAGACTGTGAAAAGCGAGGATAGCAGTCAGCCCTGGGATGATAA
TTCAGCATGCGTGGACCCCTGCTCTTTCATCCCCACCCCTAACAAAAGAAGAGGACAATGAGCTTGAATACCCAGGA
CTGCATTTACAGCCTCGGAAAATCAGACCACCCAGAGCCTCCCCACTTCCCGTCTTGAATTGGGGCAATAGAGAAGAG
GTTTGGAGGATCATGTTAAACAAAAGAAAAGACTTACCTGAGAGATGAGCACTTTCTGCAGCGTCATCTCTCCTGCA
GGCAGGATGAGAGCAGTTCTTCTGGATTGGCTAATGGAGGTGTGCGAAGTCTATAAGCTCCACAGAGAGCAGTTCT
ACTTGGCACAGGACTTCTTTGATCGTTACATGGCATCACAAATAATATCATAAAAAACACTTTTTACAGCTTATTGGG
ATTTACAGCCTTATTTATTGCTTCAAAACTTGAGGAAATCTACCCTCCAAAGTTGCACCAGTTTGCTTATGTTACAGA
TGGCGCTTGCTCCGGGGATGAAATTTCTTACCATGGAATTGATGATGATGAAGGCCCTTAAGTGGCGTCTAAGCCCTC
TGACCATTGTGTCTGGCTGAATGTCTATGTCCAAGTGGCCTATGTCAACGACACGGGTGAGGTGCTGATGCCTCAG
TACCCACAGCAGGTCTTCGTGCAGATCGCAGAGCTTCTAGACCTGTGCGTCTGGATGTTGGCTGCTTAGAATTTCC
TTATGGTGTCTCGCTGCTTCTGCTTTGTATCATTTCTCCTCACTGGAGTTGATGCAGAAGGTCTCAGGTTATCAGT
GGTGCACATAGAGAAGTGTGTCAAATGGATGGTTCCGTTCCGCATGGTTATCCGGGAGATGGGAAGTTCCAAGCTC
AAGCACTTCCGGGGAGTCCCATGGAAGACTCCCACAACATCCAGACCCACACCAACAGCTTGGATTTGCTGGACAA
AGCCCAAGCAAAGAAAGCCATATTGTGAGAACAGAATAGGATTTCTCCTCCTCCGAGTGTGGTCTGACACCCCCAC
CCAGCAGTAAGAAGCAGAGCAGCGAGCAGGAGACAGAATGA

>Mouse_CCNE1_T1_Truncated_Protein

MDPDEEIAKIDKTVKSEDSSQPWDDNSACVDPCSFIPTPNKEEDNELEYPRTAFOQPRKIRPPRASPLPVLNWNREE
VWRIMLNKEKTYLRDEHFLQRHPLLQARMRAVLLDWLMEVCEVYKLRHRETFYLAQDFFDRYMASQHNIIKTLLQLIG
ISALFIASKLEEIYPPKLHQFAYVTDGACSGDEILTMELMMMKALKWRLSPLTIVSWLNVYVQVAYVNDTGEVLMPO
YPQOVFVQIAELLDLCVLDVGCLEFPYGVLAASALYHFSSLELMQKVSQYQWCDIEKCVKWMVFPAMVIREMGSSK
LRFVPMEDSHNIQTHTNSLDLLDKAQAKKAILSEQNRISPPPSVVLTPPPSSKKQSSSEQETE

APPENDIX B: E561 Animal Ethics Approval, Peter MacCallum Cancer Centre (Bowtell)



CANCER RESEARCH DIVISION

PETER MACCALLUM CANCER CENTRE ANIMAL EXPERIMENTATION ETHICS COMMITTEE (AEEC)	
Application For Approval To Use Animals In A Research Project	Date application received: 23/11/15 Resubmitted: 15/02/16

Office Use Only

Project Title AEEC Register Number

AEEC Permit Number


Genetically engineered mouse models of ovarian cancer

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DECLARATION BY AEEC CHAIRMAN

I certify that this project has been considered and approved by the Peter MacCallum Cancer Centre AEEC Chair on the 7th March 2016 and ratified by the full committee on the 26th May 2016.

The period of approval for this project is 07/03/16 to 06/03/19

AEEC Chairman Name:	A/Prof. Phillip Darcy
AEEC Chairman Signature:	
Date:	31/05/16

CONDITIONS OF APPROVAL

All matters pertaining to the conduct of the approved project are to be reported to the AEEC, which maintains oversight in accordance with licence conditions for the Licence SPPL078.

Any variation proposed to the project, and the reasons for that change, must be submitted to the AEEC for approval and must not be implemented until approval is granted.

A record of details of any animals used in the project must be retained.

The project should only be conducted in approved premises nominated on the Bureau of Animal Welfare Scientific Licence **SPPL078**.

The AEEC must also be notified in writing of;

- Any changes to approved investigators
- Any unexpected incidents or complications that result in deaths, euthanasia or pain and suffering for the animals used in the project. Details of the steps taken to deal with adverse incidents must be included in the notification.

OTHER CONDITIONS:

This approval is subject to the following special conditions:

APPENDIX C: 806138 Animal Ethics Approval, University of Pennsylvania (Drapkin)



IACUC Protocol Administration
<http://www.upenn.edu/animalwelfare>

Office of Animal Welfare
Science Center Building
3624 Market Street, Suite 301S
Philadelphia, PA 19104
Phone: 215-898-2615
Fax: 215-746-6308
Email: iacuc@pobox.upenn.edu

**UNIVERSITY OF PENNSYLVANIA
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)**
(Animal Welfare Assurance # A3079-01)

RONNY DRAPKIN
4333 - OB-Obstetrics and Gynecology
421 Curie Blvd
BRB II/III Suite 1215
Philadelphia, PA 19104-6160

10-Nov-2016

PRINCIPAL INVESTIGATOR : RONNY DRAPKIN
PROTOCOL TITLE : Using Genetically Engineered Mice for the Study High Grade Serous Ovarian Cancer
PROTOCOL # : 806138
3-YEAR APPROVAL PERIOD : 05-Nov-2016 – 05-Nov-2019

Dear DR. DRAPKIN:

The above referenced protocol was reviewed and approved by the Institutional Animal Care and Use Committee on **05-Nov-2016**. Protocols are valid for three years from the date of approval. However, please note that protocols with USDA-covered species will require annual continuing reviews. This study will be due for its next review on or before **05-Nov-2019**. Please log into the ARIES electronic protocol system (<https://aries.apps.upenn.edu/laProtocol/jsp/fast2.do>) on a routine basis to check the status of your protocol, and be cognizant of reminder notifications that will be sent out when new submissions are required.

Please note that the principal investigator should contact ULAR to verify animal housing availability and to coordinate activities for any special animal study needs (including special housing) or equipment requirements if this was not done during the planning phases of the protocol. IACUC protocol approval DOES NOT guarantee the availability of required resources for animal work.

Please take note of the following information:

Personnel Training: It is the responsibility of the Principal Investigator to ensure that all persons have completed all necessary training prior to participating in the research described in this protocol.

Submissions: Please note that all future submissions related to this protocol must be submitted within ARIES. No paper submissions will be accepted.

Amendments: If you wish to change any aspect of this study, such as personnel, sponsors, hazardous materials, drugs, or procedures, please submit an Amendment to the protocol within ARIES. The new changes cannot be initiated until IACUC approval has been given.

Completion of Study: Please notify the Office of Animal Welfare once the research has been completed so the protocol can be terminated. This will prevent you from receiving unnecessary reminder notifications for renewal submissions.

If you have any questions, please contact our office as indicated above. Thank you for your cooperation with the Committee.

Sincerely,

A handwritten signature in black ink that reads "Gregory R. Reinhard".

Gregory R. Reinhard, MBA, DVM, DACLAM
Director, Office of Animal Welfare

Digitally signed by Gregory R. Reinhard
Date: 2016.11.14 09:09:32 -05'00'

2 Q1 **Selective Targeting of Cyclin E1-Amplified**
 3 Q2 **High-Grade Serous Ovarian Cancer by Cyclin-**
 4 **Dependent Kinase 2 and AKT Inhibition** 

5 AU George Au-Yeung^{1,2}, Franziska Lang¹, Walid J. Azar¹, Chris Mitchell¹, Kate E. Jarman³,
 6 Kurt Lackovic^{3,4}, Diar Aziz⁵, Carleen Cullinane^{1,6}, Richard B. Pearson^{1,2,7}, Linda Mileschkin^{2,8},
 7 Danny Rischin^{2,8}, Alison M. Karst⁹, Ronny Drapkin¹⁰, Dariush Etemadmoghadam^{1,2,5}, and
 8 David D.L. Bowtell^{1,2,7,11}

9 **Abstract**

10 **Purpose:** Cyclin E1 (*CCNE1*) amplification is associated with
 11 primary treatment resistance and poor outcome in high-grade
 12 serous ovarian cancer (HGSC). Here, we explore approaches to
 13 target *CCNE1*-amplified cancers and potential strategies to over-
 14 come resistance to targeted agents.

15 **Experimental Design:** To examine dependency on *CDK2* in
 16 *CCNE1*-amplified HGSC, we utilized siRNA and conditional
 17 shRNA gene suppression, and chemical inhibition using dina-
 18 ciclib, a small-molecule *CDK2* inhibitor. High-throughput
 19 compound screening was used to identify selective synergistic
 20 drug combinations, as well as combinations that may over-
 21 come drug resistance. An observed relationship between
 22 *CCNE1* and the AKT pathway was further explored in genomic
 23 data from primary tumors, and functional studies in fallopian
 24 tube secretory cells.

25 **Results:** We validate *CDK2* as a therapeutic target by demon-
 26 strating selective sensitivity to gene suppression. However, we
 27 found that dinaciclib did not trigger amplicon-dependent sensi-
 28 tivity in a panel of HGSC cell lines. A high-throughput compound
 29 screen identified synergistic combinations in *CCNE1*-amplified
 30 HGSC, including dinaciclib and AKT inhibitors. Analysis of geno-
 31 mic data from TCGA demonstrated coamplification of *CCNE1* and
 32 *AKT2*. Overexpression of Cyclin E1 and AKT isoforms, in addition to
 33 mutant *TP53*, imparted malignant characteristics in untransformed
 34 fallopian tube secretory cells, the dominant site of origin of HGSC.

35 **Conclusions:** These findings suggest a specific dependency of
 36 *CCNE1*-amplified tumors for AKT activity, and point to a novel
 37 combination of dinaciclib and AKT inhibitors that may selectively
 38 target patients with *CCNE1*-amplified HGSC. *Clin Cancer Res*; 1–15.
 39 ©2016 AACR. 40

41

42 **Introduction**

43 Targeted therapies have changed the management of many
 44 cancers types, resulting in significant improvements in clinical
 45 response rates and survival (1). However, the antiangiogenic mAb
 46 bevacizumab (2, 3) and the PARP inhibitor olaparib (4, 5) have
 47 entered care in high-grade serous ovarian cancer (HGSC) recently,
 48 the development of targeted therapy to this disease has been
 49 relatively slow. 50

51 HGSCs are characterized by ubiquitous *TP53* mutations, geno-
 52 mic instability, and widespread copy number alterations, with
 53 relatively infrequent somatic point mutations of driver genes
 54 (6, 7). Structural aberration also contributes to loss of tumor
 55 suppressors such as *RBI* and *NF1* by gene breakage (8). Defects in
 56 the homologous recombination repair (HR) pathway are present
 57 in approximately 50% of HGSCs, primarily associated with germ-
 58 line and somatic mutations in *BRCA1*, *BRCA2*, and associated
 59 proteins (7). HR deficiency imparts platinum sensitivity in HGSC,
 60 and provides the basis for the use of PARP inhibitors that target
 61 compensatory DNA repair pathways (4, 9). Of HGSC with intact
 62 HR, amplification of *CCNE1*, which encodes the cell-cycle regu-
 63 lator cyclin E1, is the best characterized driver. *CCNE1* amplifi-
 64 cation or gain occurs in 20% of all HGSC tumors and is associated
 65 with primary treatment resistance and reduced overall survival
 66 in HGSC (10, 11). Patients whose tumors have *CCNE1*

Q3 ¹Division of Cancer Research, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia. ²Sir Peter MacCallum Department of Medical Oncology, University of Melbourne, Parkville, Victoria, Australia. ³Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia. ⁴Department of Medical Biology, University of Melbourne, Parkville, Victoria, Australia. ⁵Department of Pathology, University of Melbourne, Parkville, Victoria, Australia. ⁶Translational Research Program, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia. ⁷Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria, Australia. ⁸Department of Medical Oncology, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia. ⁹Department of Medical Oncology, Dana Farber Cancer Institute, Boston, Massachusetts. ¹⁰Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Penn Ovarian Cancer Research Center, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania. ¹¹Kinghorn Cancer Centre, Garvan Institute for Medical Research, Darlinghurst, New South Wales, Australia.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

D. Etemadmoghadam and D.D.L. Bowtell contributed equally to this article.

Q4 **Corresponding Author:** David D.L. Bowtell, Peter MacCallum Cancer Centre, Locked Bag 1, A's Beckett Street, Melbourne, Victoria 8006, Australia. Phone: 613-9656-1356; Fax: 613-9656-1414; E-mail: david.bowtell@petermac.org

doi: 10.1158/1078-0432.CCR-16-0620

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Translational Relevance

High-grade serous ovarian cancer (HGSC) patients with Cyclin E1 (*CCNE1*) amplification represent a group with high unmet clinical need. Novel therapies are needed to improve outcomes in these patients, given that *CCNE1*-amplified tumors are unlikely to respond to chemotherapy or PARP inhibitors, and are associated with poor overall survival. Here, we validate *CDK2* as a selective target for *CCNE1*-amplified cell lines. We performed a high-throughput compound screen and identified a number of potential therapeutic combinations. We focused on dinaciclib and AKT inhibitors, and demonstrate selective and potent activity in *CCNE1*-amplified HGSC. We further show cooperation between *CCNE1* and *AKT*, both in genomic data from TCGA and functionally in fallopian tube secretory cells. This study demonstrates approaches to target an important subset of solid cancers, and for the first time provides evidence to support the design of a rational clinical trial that targets *CCNE1*-amplified HGSC.

69 amplification represent a group with unmet clinical need, as they
70 are unlikely to benefit from PARP inhibitors by virtue of the
71 mutual exclusivity of *CCNE1* amplification and *BRCA1/2* muta-
72 tion (7, 12), and are less likely to respond to platinum agents.

73 In recent preclinical studies, we have shown a dependency on
74 *CDK2* (13) and HR activity (12) in *CCNE1*-amplified cell lines.
75 Although targeted agents have been effective in the clinical setting
76 across many cancers, the emergence of acquired resistance is
77 common (14). Indeed, we reported *in vitro* resistance to *CDK2*
78 inhibitors through selection of a polyploid population in the
79 *CCNE1*-amplified cell line OVCAR3 (13). Rational drug combina-
80 nations are a potential strategy to prevent resistance (15), and may
81 also facilitate improvements in the therapeutic window by reduc-
82 ing the doses of drugs required to achieve efficacy, resulting in
83 fewer side effects (16). We therefore used a high-throughput drug
84 screen to identify drug combinations that synergize with the
85 *CDK2* inhibitor dinaciclib (17) to selectively target *CCNE1*-
86 amplified HGSC, and to overcome resistance in a cell line that
87 has acquired resistance to *CDK* inhibitors *in vitro* (13). We
88 identified several synergistic combinations, including dinaciclib
89 and AKT inhibitors, and found that that this synergy extended
90 more generally to *CCNE1*-amplified HGSC cell lines. Our results
91 suggest targeting *CDK2* and the AKT pathway may be an impor-
92 tant approach to the clinical management of *CCNE1*-amplified
93 HGSC.

94 **Materials and Methods**

95 **Ethics statement**

96 All animal experiments were approved by the Peter MacCallum
97 Cancer Centre Animal Experimentation Ethics Committee and
98 conducted in accordance with the National Health and Medical
99 Research Council Australian Code of Practice for the Care and Use
100 of Animals for Scientific Purposes.

101 **Cell lines**

102 Ovarian cancer cell lines were obtained from the National
103 Cancer Institute Repository, actively passaged for less than 6
104 months, and authenticated using short-tandem repeat markers

106 to confirm their identity against the Cancer Genome Project
107 database (Wellcome Trust Sanger Institute, Cambridgeshire, Unit-
108 ed Kingdom) before use in experiments. Cells were maintained at
109 37°C and 5% CO₂ (v/v), and cultured in RPMI1640 media
110 containing 10% (v/v) FCS and 1% penicillin/streptomycin. Trans-
111 fection and drug sensitivity assays were performed in the absence
112 of antibiotics. Cell lines resistant to dinaciclib were generated
113 utilizing methods as described previously (13). Briefly, OVCAR3
114 cells were plated in 6-well plates and treated with dinaciclib at the
115 IC₅₀ dose for two 72-hour periods (media removed and fresh drug
116 added). Surviving cells were allowed to repopulate for 96 hours
117 and the process repeated once. Remaining cells were cultured in
118 media or in the presence of drug, and regularly monitored for
119 sensitivity to dinaciclib. Six independent cell lines were generated
120 in this fashion, and designated OVCAR3-RD1 to -RD6.

121 **Short hairpin-mediated *CDK2* knockdown**

122 Short hairpin-mediated knockdown of *CDK2* was performed
123 by cloning *CDK2*-specific shRNA into a lentiviral tetracycline-
124 inducible expression vector containing the optimized miR-E
125 backbone (18). The modified lentiviral vector pRRL-T3G-Tur-
126 boGFP-miRE-PGK-mCherry-IRES-rTA3 (also referred to as
127 LT3GECIR) system includes a red (mCherry) fluorescent marker
128 for transduction and a green (turboGFP) fluorescent marker for
129 induction. Five *CDK2*-specific shRNA constructs were cloned into
130 this system (see Supplementary Table S2 for sequences). For
131 lentiviral production, HEK293T cells were transfected with plas-
132 mid DNA combined with the Lenti-X packaging system (Clontech
133 Laboratories). Transfection, production of lentiviral particles, and
134 transduction of target cells was performed as described by the
135 manufacturer's protocol. Doxycycline was used to induce shRNA
136 expression, and transfection efficiency was validated by flow
137 cytometry (FACS), and knockdown of individual hairpins by
138 RT-PCR and Western blot analysis. The most efficient shRNA
139 construct was taken forward for *in vitro* and *in vivo* experiments.

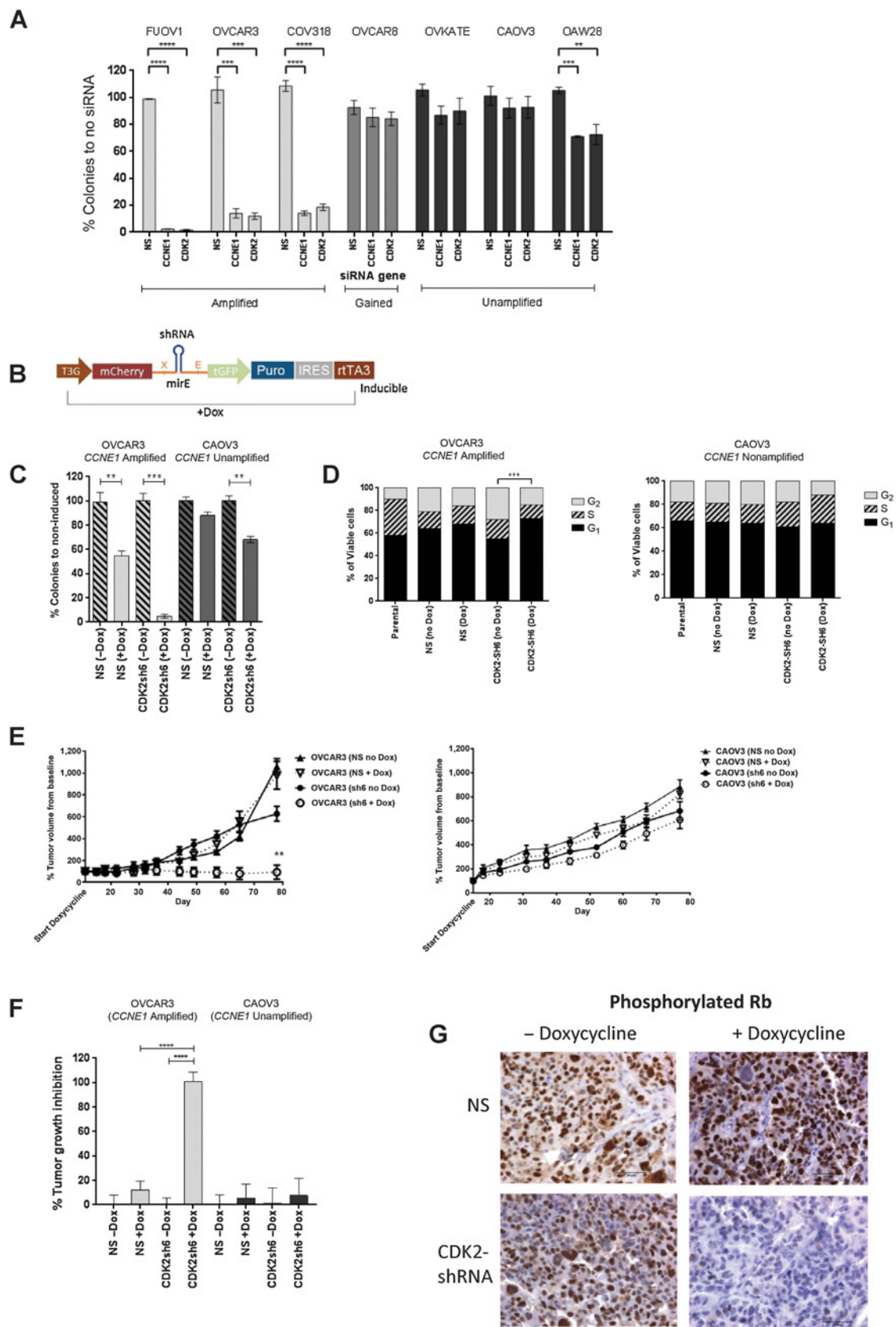
140 For *in vivo* experiments, xenograft tumors from transduced cells
141 were generated as described below. Once tumors reached 100
142 mm³, mice were randomized into two groups to receive either
143 normal food and water or doxycycline food and water (2 mg/mL
144 in 2% sucrose) as a means of reliable induction of shRNA
145 expression. Tumors were subsequently monitored as described
146 below.

147 **Cyclin E1 and AKT overexpression in Fallopian tube secretory
148 epithelial cells**

149 The immortalized fallopian tube secretory epithelial cell
150 (FTSEC) line FT282 was obtained from Ronny Drapkin (Univer-
151 sity of Pennsylvania, Philadelphia, PA; ref. 19). Derivative cell
152 lines were generated using pMSCV-mCherry-(empty) and
153 pMSCV-mCherry-*CCNE1*, encoding full-length *CCNE1*. Addi-
154 tional cell lines were generated with pMSCV-GFP-myr-AKT1,
155 pMSCV-GFP-myr-AKT2, and pMSCV-GFP-myr-AKT3, encoding
156 the three different isoforms of myr-AKT (20). Plasmids were
157 validated by sequencing, and expression of *CCNE1*, *AKT1*, *AKT2*,
158 and *AKT3* was validated by quantitative real-time PCR and
159 Western blotting. Primer sequences are listed in Supplementary
160 Table S1.

161 **High-throughput compound screen**

162 The compound library consisted of 73 targeted agents, 71
163 epigenetic agents, 208 kinase inhibitors, and 3,707 known drugs



287 Taken together, *CCNE1*-amplified HGSC appear selectively
 288 sensitive to siRNA- and shRNA-mediated knockdown of *CDK2*
 289 both *in vitro* and *in vivo*. These findings support our previous
 290 studies and point to *CDK2* as a potential therapeutic target in
 291 *CCNE1*-amplified HGSC.

292 **CDK2 inhibitor dinaciclib delayed tumor growth in *CCNE1*-** 293 **amplified HGSC xenografts**

294 Consistent with siRNA data, we previously showed in a limited
 295 number of cell lines selective sensitivity of *CCNE1*-amplified cell
 296 lines to dinaciclib, a potent *CDK2* inhibitor in advanced clinical
 297 development (13). However, in this study, when tested across a
 298 broader panel of HGSC cell lines, there did not appear to be a clear
 299 amplicon-dependent sensitivity (Fig. 2A), in contrast with the
 300 siRNA and shRNA data. Furthermore, activity *in vivo* was also seen
 301 in a xenograft model developed from a *CCNE1*-unamplified cell
 302 line, CAOV3 (Fig. 2A–D). The difference in amplicon-dependent
 303 sensitivity between gene suppression and pharmacologic inhibition
 304 may be due to the broad activity of dinaciclib, which, in
 305 addition to inhibiting *CDK2*, is also active against *CDK1*, 5, 9, and
 306 12 (17, 27).

307 In addition to *CDK2* inhibitors, we previously identified use of
 308 bortezomib, a proteasome inhibitor, as a potential therapeutic
 309 strategy for *CCNE1*-amplified HGSC (12). Although we did not
 310 observe amplicon-dependent sensitivity to dinaciclib, we investi-
 311 gated the interaction between dinaciclib and bortezomib to see
 312 whether the two drugs would be synergistic in combination.
 313 Using the Chou–Talalay methodology for drug combination
 314 studies (28), we did not observe a synergistic interaction with
 315 dinaciclib and bortezomib (Fig. 2E and F) in a panel of *CCNE1*-
 316 amplified and *CCNE1*-unamplified HGSC cell lines. Given this
 317 lack of synergism, we sought to identify selective synergistic drug
 318 combinations by adopting an unbiased high-throughput screen-
 319 ing approach.

320 **A high-throughput compound screen identifies synergistic drug** 321 **combinations**

322 We performed a high-throughput compound screen to identify
 323 combinations that would be synergistic in *CCNE1*-amplified cells,
 324 as well as combinations that would be selective in a *CDK* inhibi-
 325 tor-resistant cell line OVCAR3-R1-533533 (13). In the primary
 326 screen, 4,059 compounds (including duplicates) were combined
 327 with a fixed dose of dinaciclib as described in Materials and
 328 Methods. Dose–response curves were generated and manually

330 curated, and compounds where a curve could not be fitted were
 331 excluded from the analysis. A full list of EC_{50} values for each cell
 332 line and compound is given in Supplementary Tables S4 and S5.

333 EC_{50} values from the primary screen were used to make two
 334 pair-wise comparisons (Fig. 2G and H): (i) dinaciclib plus library
 335 compound comparing OVCAR3 (*CCNE1*-amplified) versus
 336 SKOV3 (*CCNE1*-unamplified) and (ii) dinaciclib plus library
 337 compound comparing OVCAR3 (parental) and OVCAR3-R1
 338 (*CDK* inhibitor resistant). At the time of undertaking the screen,
 339 SKOV3 was a commonly used ovarian cancer cell line; however,
 340 recent studies have demonstrated that SKOV3 is unlikely to
 341 resemble HGSC (26). Therefore, any potential hits identified in
 342 the screen were subsequently validated using only HGSC cell
 343 lines.

344 Library compounds where the ratio of EC_{50} was less than 0.5
 345 were selected as hits for a secondary screen involving a total of 64
 346 compounds (Supplementary Table S6 and S7). Compounds that
 347 appeared to have an additive effect with dinaciclib were selected as
 348 hits from the secondary screen and carried forward for further
 349 testing.

350 The final part of the screen involved assessing the level of
 351 synergy between the library compound hits and dinaciclib involv-
 352 ing an 11-point titration of each compound. Using the Chou–
 353 Talalay methodology of constant-ratio drug combinations, a
 354 series of combination indexes were generated to identify syner-
 355 gistic interactions.

356 In the OVCAR3 parental cell line, there were no synergistic
 357 combinations identified between dinaciclib and the library com-
 358 pounds (Supplementary Table S8). In the OVCAR3-R1 cell line,
 359 there were a number of synergistic interactions identified (Sup-
 360 plementary Table S8). Nonselective BH3-mimetic agents ABT-263
 361 and ABT-737 were synergistic in combination with dinaciclib,
 362 suggestive of a class effect. This was validated further in an
 363 independently derived dinaciclib-resistant cell line, OVCAR3-
 364 RD6 (Fig. 3A–B and Supplementary Fig. S4A–S4C). There was
 365 no synergistic interaction noted in the combination between
 366 dinaciclib and ABT-199 (Fig. 3C), a selective Bcl-2 antagonist.
 367 The combination of dinaciclib and ABT-737 resulted in a dose-
 368 dependent increase in apoptosis, observed only in *CDK* inhibi-
 369 tor-resistant cell lines as demonstrated by increase in PARP
 370 cleavage products on Western blot analysis (Fig. 3D). Mcl-1
 371 protein expression was not observed in the OVCAR3-RD6 cell
 372 line resistant to dinaciclib (Fig. 3D). Real-time PCR demonstrated
 373 upregulation of antiapoptotic genes in the dinaciclib and

Figure 1.

CDK2 knockdown via siRNA and shRNA *in vitro* and *in vivo* results in selective reduction in clonogenic survival and tumor growth arrest in *CCNE1*-amplified HGSC. A, Clonogenic survival after transfection with *CCNE1* and *CDK2* siRNAs in panel of HGSC cell lines. Average percentage of discrete colonies formed after 7 to 10 days relative to no siRNA controls shown ($n = 3$). Error bars, SEM. Statistical significance (t test) calculated by comparison with nonsilencing (NS) siRNA in the same cell line. **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$. B, Schematic of conditional LT3GECIR lentiviral vector showing inducible transcripts produced by vector. C, Clonogenic survival after induction of a nonspecific or *CDK2*-targeting shRNA in OVCAR3 (*CCNE1*-amplified) and CAOV3 (*CCNE1*-unamplified). The average percentage of discrete colonies formed after 7 to 10 days relative to no induction shown ($n = 3$). Statistical significance (t test) calculated by comparison with noninduced (–Dox) in the same cell line; **, $P < 0.01$, ***, $P < 0.001$. D, Cell-cycle analysis following *CDK2* knockdown with inducible shRNA. Proportion of cells in G_1 , S , or G_2 phase for propidium iodide (PI)-stained cells analyzed by flow cytometry 72 hours after induction with doxycycline. Mean of three independently performed experiments shown. Statistical significance (t test) calculated by comparison with noninduced (–Dox) in the same cell line. ***, $P < 0.001$. E, Mean percentage change in tumor volume \pm SEM following induction of a nonspecific (NS) or *CDK2* (sh6) shRNA in subcutaneous xenograft tumors grown in immunocompromised mice, generated from OVCAR3 and CAOV3. Induced and noninduced groups as marked, $n = 5$ per group. **, $P < 0.001$, unpaired t test comparison of mean percentage tumor volume change. F, Percentage tumor growth inhibition following induction of nonspecific (NS) or *CDK2* (sh6) shRNA with doxycycline. Bars, mean \pm SEM, $n = 5$ mice per group. Statistical analysis performed with ANOVA followed by Dunnett *post hoc* test to compare the percentage tumor growth inhibition between the treatment groups. ****, $P < 0.0001$. G, IHC assessment of phospho-Rb with or without doxycycline treatment in OVCAR3 xenograft tumor.

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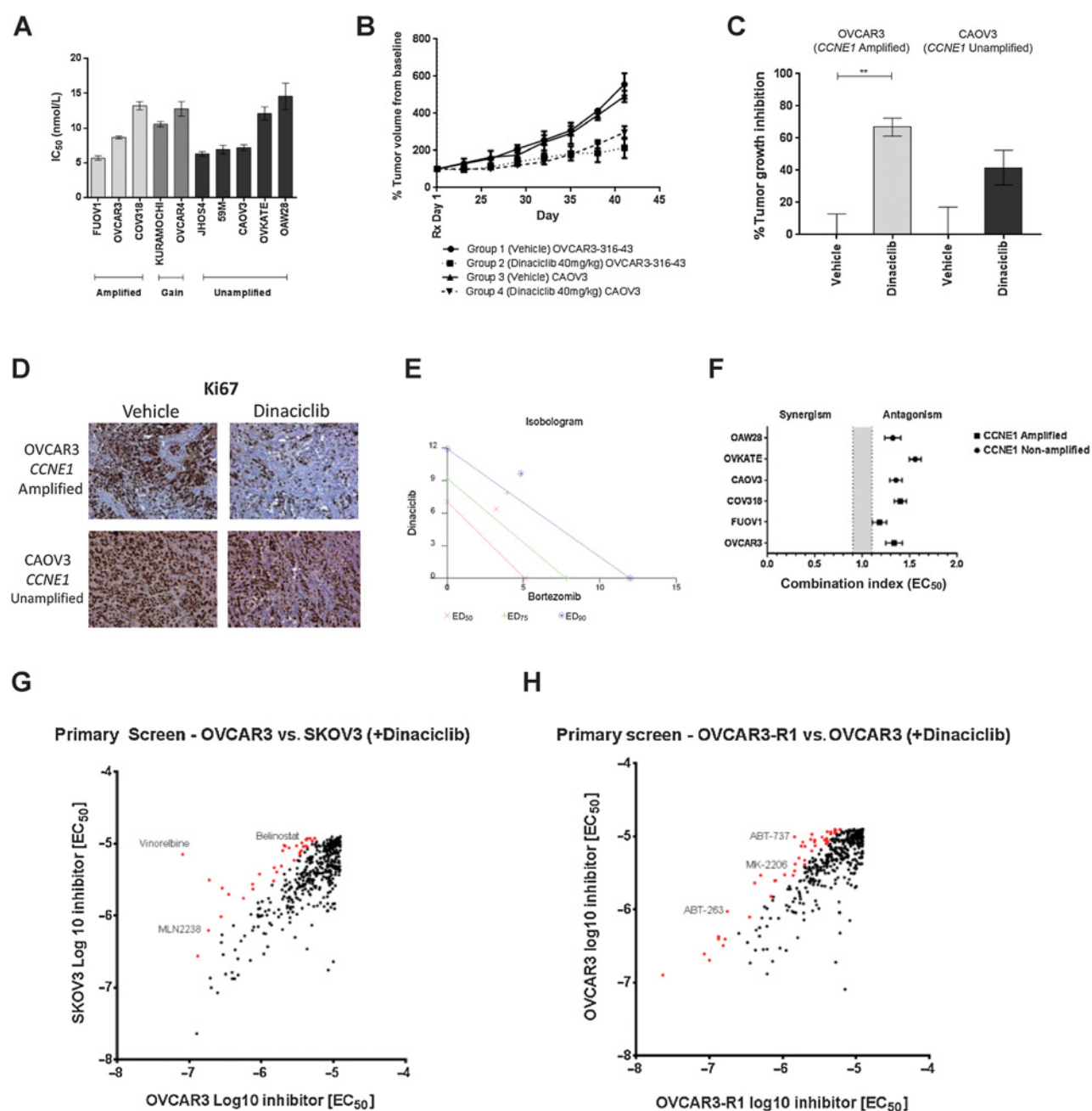


Figure 2.

CDK inhibitor dinaciclib results in modest tumor growth inhibition *in vivo* but is not synergistic in combination with bortezomib *in vitro*. **A**, Mean IC₅₀ values for a panel of HGSC cell lines treated with dinaciclib generated from dose-response curves following standard MTS cell proliferation assays. Error bars, SEM, *n* = 3 experiments. **B**, *In vivo* effects of dinaciclib. Immunocompromised mice bearing OVCAR3 (*CCNE1*-amplified) or CAOV3 (*CCNE1*-unamplified) tumor xenografts were treated with vehicle or drug as described in Materials and Methods. Plots represent mean tumor volume change from baseline ± SEM, *n* = 5 mice per group. **C**, the percentage tumor growth inhibition following 21 days of treatment with vehicle or dinaciclib. Bars represent mean ± SEM, *n* = 5 mice per group. Statistical analysis performed with ANOVA followed by Dunnett *post hoc* test to compare the percentage tumor growth inhibition between the treatment groups. **, *P* < 0.01. **D**, Immunohistochemical analysis of Ki67 expression in OVCAR3 and CAOV3 tumor xenograft harvested 24 hours after dose of vehicle or dinaciclib. **E**, Formal assessment of synergy between dinaciclib and bortezomib using Chou-Talalay Isobologram analysis. Figures are generated with CalcuSyn 2.0. Data are normalized, with connecting line at X and Y corresponding to combination index = 1, representing line of additivity. Data points above the line are antagonistic, along or near the line are additive and points below the line are synergistic. **F**, Combination indexes for a panel of HGSC cell lines tested against dinaciclib in combination with bortezomib. Values represent mean ± SEM, *n* = 3. **G–H**, Scatter plots showing EC₅₀ values for library compounds in combination with dinaciclib from primary screen for the comparison between *CCNE1*-amplified and unamplified (**G**) and resistant versus parental (**H**). Data points in red represent compounds taken forward for secondary screen.

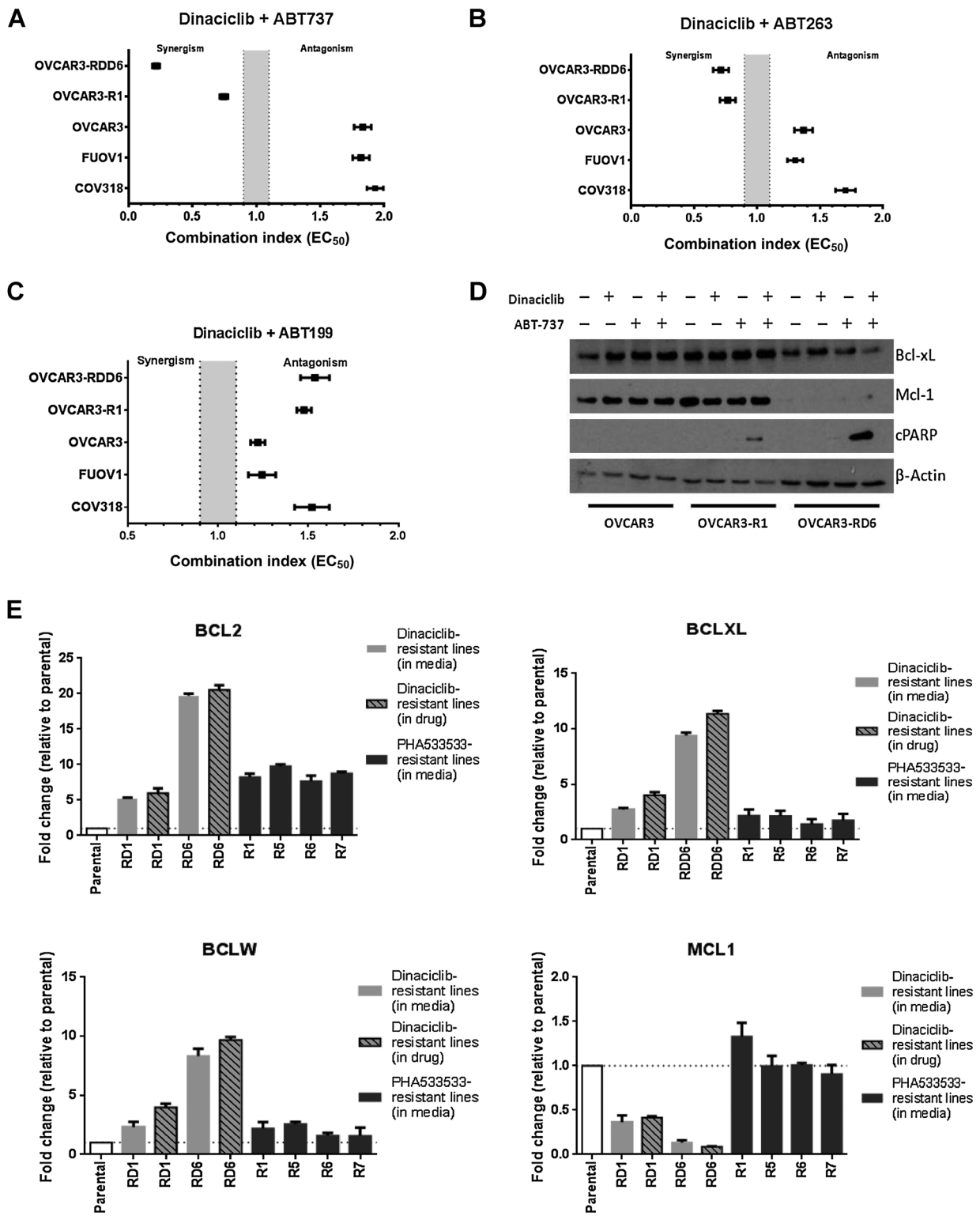


Figure 3. Dinaciclib in combination with nonselective BH3 mimetics are synergistic in CDK inhibitor-resistant cell lines. Combination indexes for parental and CDK inhibitor-resistant cell lines tested against dinaciclib in combination with ABT-737 (A), ABT-263 (B), ABT-199 (C). Values represent mean \pm SEM, $n = 3$. D, Western blot analysis demonstrating protein expression of Bcl-XL, Mcl-1, and PARP cleavage products in OVCAR3 parental and CDK inhibitor-resistant cell lines after treatment with dinaciclib and ABT-737. E, Expression of antiapoptotic proteins as assessed by quantitative real-time PCR. R-lines signify cell lines resistant to PHA533533. RD lines signify cell lines resistant to dinaciclib. Bars represent mean \pm SEM, $n = 3$.

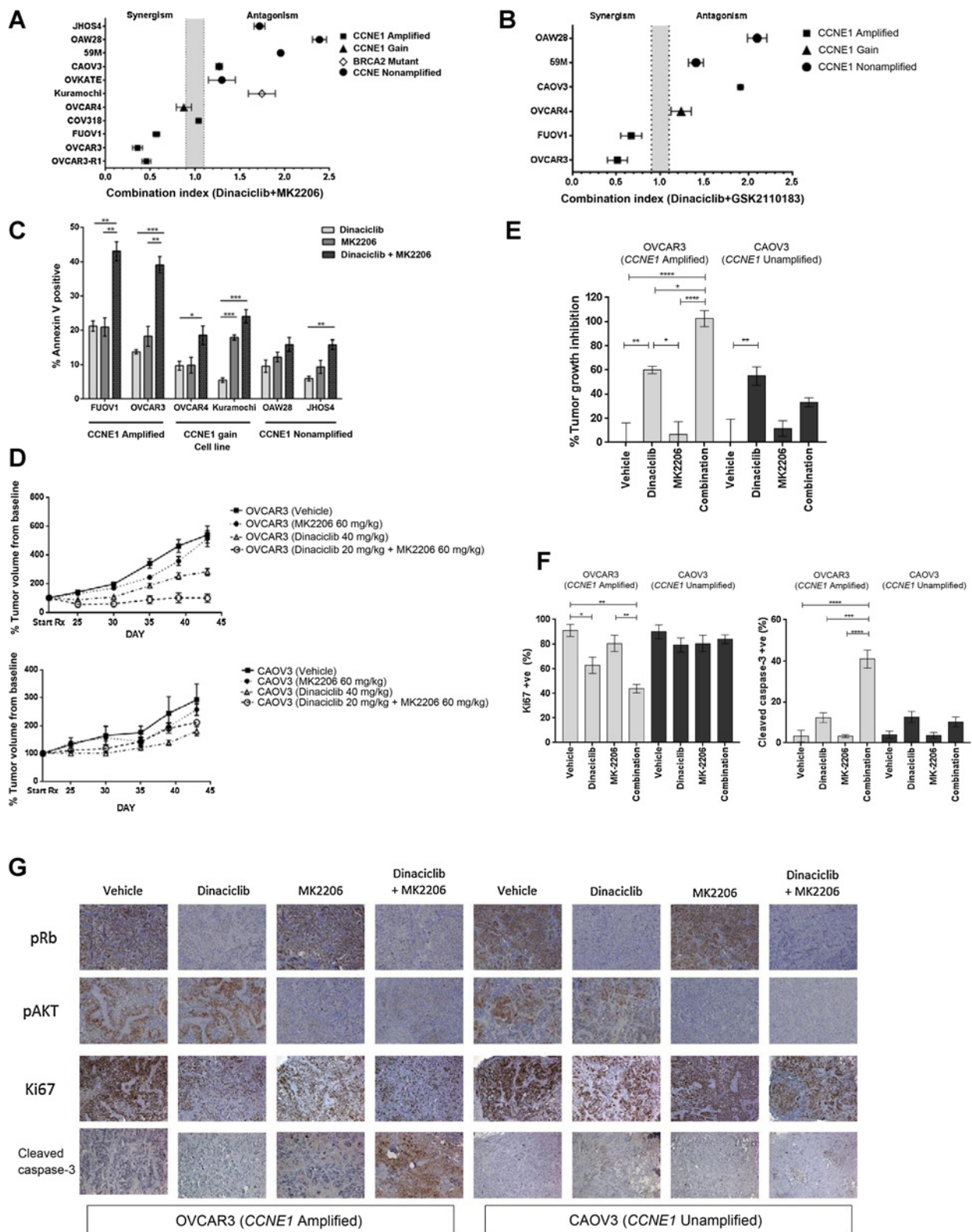


Figure 4. Dinaciclib in combination with two AKT inhibitors are synergistic *in vitro* and *in vivo* models of *CCNE1*-amplified HGSC. Combination indexes for a panel of HGSC cell lines tested against dinaciclib in combination with MK-2206 (A) and GSK2110183 (B). Values represent mean ± SEM, (Continued on the following page.)

376 PHA533533-resistant cell lines (Fig. 3E), but downregulation of
 377 *MCL1* in the dinaciclib-resistant OVCAR3-RD cell lines. Dinaci-
 378 cliclib is reported to have a greater effect on CDK9 compared with
 379 PHA533533 (13). Given that *MCL1* is regulated by CDK9 activity
 380 (29), this may explain the reduction of *MCL1* levels in the
 381 presence of dinaciclib. However, it is unclear why reduced *MCL1*
 382 expression is also apparent in OVCAR3-RD cell lines even when
 383 grown in the absence of dinaciclib.

384 MK-2206, a pan-AKT inhibitor, was identified as a synergistic
 385 drug combination in the CDK inhibitor-resistant cell line,
 386 OVCAR3-R1. In validating this interaction between dinaciclib
 387 and MK-2206, we observed that this combination was also
 388 synergistic in *CCNE1*-amplified cell lines FUOV1 and parental
 389 OVCAR3 (Fig. 4A). This effect was similarly observed with another
 390 AKT inhibitor, GSK-2110183 (Fig. 4B), that was not included in
 391 the original high-throughput screen library. Exposure to dinaci-
 392 cliclib and MK2206 resulted in significantly higher number of
 393 apoptotic cells in *CCNE1*-amplified cell lines, indicated by per-
 394 centage of Annexin V-positive cells measured by FACS (Fig. 4C).
 395 This result was similarly observed on Western blot analysis, with
 396 appearance of PARP cleavage products following treatment of
 397 OVCAR3 cells with the combination of dinaciclib and MK-2206
 398 (Supplementary Fig. S4D). As dinaciclib targets several CDKs in
 399 addition to CDK2 (17), we used siRNA knockdown of CDK2,
 400 CDK1, or CDK9 to determine the specificity of the synergistic
 401 effect of dinaciclib and MK-2206. We found that the synergy
 402 observed was predominantly mediated through CDK2 (Supple-
 403 mentary Fig. S4E).

404 **Dinaciclib and MK-2206 are selectively synergistic in *CCNE1*-**
 405 **amplified cell lines *in vivo***

406 The *in vivo* effect of dinaciclib and MK-2206 was assessed using
 407 xenograft models from *CCNE1*-amplified and unamplified cell
 408 lines, OVCAR3 and CAOV3, respectively. The combination was
 409 significantly more effective than each single agent alone in the
 410 *CCNE1*-amplified model (Fig. 4D and E), whereas there was no
 411 statistically significant effect of the combination compared to
 412 single-agent treatment in the *CCNE1*-unamplified model. After a
 413 treatment period of three weeks with dinaciclib and MK-2206,
 414 xenograft tumors began regrowing within 10 days of treatment
 415 cessation. Rechallenge with the same drug combination resulted
 416 in significant tumor regression (Supplementary Fig. S4F), indi-
 417 cating continued sensitivity to the combination. Consistent with
 418 this effect on tumor growth, treatment with dinaciclib and MK-
 419 2206 resulted in inhibition of cell proliferation and induction of
 420 apoptosis, as assessed by Ki67 and cleaved caspase-3 IHC on
 421 tumors harvested at 24 hours (Fig. 4F and G). Taken together, the
 422 high-throughput screen identified a novel combination of dina-
 cliclib and MK-2206 that appeared to be selectively synergistic in
CCNE1-amplified HGSC cell lines both *in vitro* and *in vivo*.

***CCNE1* and *AKT2* are frequently coamplified in primary HGSC samples**

We sought to investigate whether there was evidence for an interaction between *CCNE1* amplification and the AKT pathway in primary tumor samples. Analysis of TCGA dataset indicated that *CCNE1* and *AKT2* amplification events cooccur ($P < 0.001$; Supplementary Fig. S5). This observation was not seen with other isoforms of AKT or genes in the AKT pathway. To examine the relationship between *CCNE1* amplification and the AKT pathway further, we made use of data from Project Achilles, a genome-wide shRNA screen of synthetic lethality in 216 cancer cell lines (24). The abundance of shRNA sequence relative to a reference pool was measured by microarray to identify genes essential for survival. We analyzed the effect of shRNA-targeting genes within the AKT pathway, restricting the analysis to HGSC cell lines, classified according to *CCNE1* copy number or expression. A statistically significant dependence on genes in the AKT pathway, including *AKT2*, was observed, indicated by a depletion of shRNAs targeting these genes in cell lines with *CCNE1* amplification or overexpression (Fig. 5). *CDK2* was included in the analysis as a control, and consistent with our previous analysis, was shown to be required in *CCNE1*-amplified cells (13).

Cyclin E1 and AKT overexpression cooperates to promote uncontrolled growth in FTSECs

Previously, Karst and colleagues demonstrated that cyclin E1 overexpression combined with *TP53* mutation in FTSECs resulted in increased proliferation, colony-forming ability, and colony formation in soft agar (19). However, cyclin E1 overexpression alone did not result in complete transformation, suggesting that additional events are required.

We examined the interaction between cyclin E1 and AKT overexpression in FTSECs by overexpressing the myristoylated, active forms of AKT1, AKT2, and AKT3 (20). Expression of each AKT isoform and cyclin E1 was validated with Western blot analysis (Fig. 6A) and RT-PCR (Supplementary Fig. S6A). Overexpression of AKT isoforms led to increased expression of AKT downstream targets (Supplementary Fig. S6B). AKT2 and cyclin E1 overexpression alone or in combination showed a trend toward increased proliferation compared with empty vector alone (Fig. 6B), and AKT2 or AKT3 overexpression in combination with cyclin E1 showed a trend toward enhanced clonogenic colony formation in comparison with overexpression of cyclin E1 alone (Fig. 6C). There was a significant increase in soft agar colony formation with the overexpression of AKT2 or AKT3 in combination with

(Continued.) $n = 3$. C, HGSC cell lines were cultured *in vitro* with dinaciclib, MK-2206, or the combination for 24 hours and then analyzed using flow cytometry for Annexin V/propidium iodide positivity. Bars, mean \pm SEM, $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired *t* test. D, *In vivo* effects of vehicle, dinaciclib, MK-2206, or combination. Immunocompromised mice bearing OVCAR3 (*CCNE1*-amplified) or CAOV3 (*CCNE1*-unamplified) tumor xenografts were treated with vehicle or drug as described in Materials and Methods. Plots represent mean tumor volume change from baseline \pm SEM, $n = 5$ mice per group. E, Percentage tumor growth inhibition following 21 days of treatment with vehicle, dinaciclib, MK-2206, or the combination. Bars, mean \pm SEM, $n = 5$ mice per group. Statistical analysis performed with ANOVA followed by Dunnett *post hoc* test to compare the percentage tumor growth inhibition between the treatment groups. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. F, Quantitation of immunohistochemical staining for Ki67 and cleaved caspase-3. Bars, mean percentage of Ki67 or cleaved caspase-3-positive cells relative to background number of cells measured \pm SEM, $n = 3$ in each group. Statistical analysis performed by ANOVA with Tukey multiple comparison test to compare between treatment groups. G, Subcutaneous tumors were obtained after 24 hours of treatment and were examined by IHC for biomarker analysis. Rb phosphorylation was inhibited by dinaciclib, but not MK-2206 treatment. AKT phosphorylation was inhibited by MK-2206, but not dinaciclib treatment. Proliferation (Ki67) was inhibited and apoptosis (cleaved caspase-3) was induced by the combination of dinaciclib and MK-2206 in *CCNE1*-amplified xenograft model (OVCAR3).

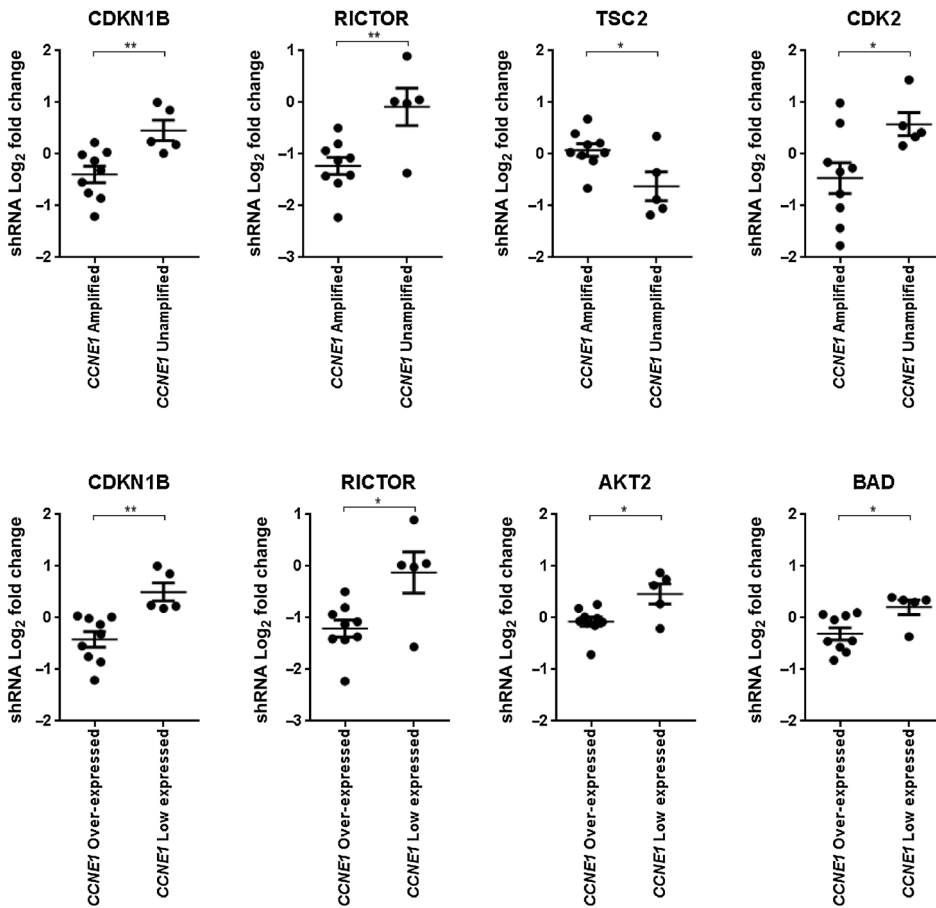


Figure 5. *CCNE1* and *AKT2* are coamplified in primary HGSC samples. Dot plots of median shRNA abundance for each gene targeted by shRNA in HGSC cell lines, stratified by *CCNE1* copy number or expression. Depletion of shRNA abundance within a group suggests requirement for maintained expression of its target gene. Only genes with a statistically significant difference are shown; see Supplementary Table S3 for the list of genes and cell lines analyzed. Statistical significance (*t* test) calculated by comparison between *CCNE1*-amplified and unamplified or *CCNE1* overexpressing and low expressing cell lines; *, $P < 0.05$; **, $P < 0.01$.

472 cyclin E1 compared with overexpression of cyclin E1 alone
 473 (Fig. 6D). These findings support an interaction between cyclin
 474 E1 and AKT pathway to promote uncontrolled growth in FTSECs,
 475 and may explain synergism observed between dinaciclib and MK-
 476 2206 in *CCNE1*-amplified HGSC.

477 **Discussion**

478 HGSC patients with *CCNE1* amplification have a clear unmet
 479 need in terms of effective therapies. In this study, we validate
 480 *CDK2* as a selective target in *CCNE1*-amplified HGSC using
 481 shRNA-mediated gene suppression *in vitro* and *in vivo*. However,
 482 we did not observe similar amplicon-dependent specificity to
 483 dinaciclib, a small-molecule inhibitor targeting CDKs. This may
 484 be due to the nonspecificity of inhibitors such as dinaciclib or a
 485 role for kinase-independent activities of *CCNE1* in amplified
 486 HGSC (30). Our findings highlight the potential differences
 487 between inhibition of kinase activity and complete suppression
 488 of *CCNE1* or *CDK2* gene expression.

489 In addition to *CDK2*, dinaciclib targets *CDK1*, 5, 9, and 12
 490 (17, 27). *CDK9* phosphorylates the carboxyl-terminal repeat
 491 domains of RNA polymerase II, and inhibition of *CDK9* by
 492 dinaciclib results in rapid downregulation of mRNA transcripts
 493 and proteins with short half-lives such as the antiapoptotic *BCL2*
 494 family member, *Mcl1* (17). Preclinical studies have indicated
 495 dinaciclib-mediated targeting of *Mcl-1* may be an effective ther-
 496 apeutic approach in a number of different cancers (17). Inhibition

of *CDK2* kinase activity may also differ significantly from com-
 plete suppression of gene expression, resulting in varying down-
 stream and compensatory effects (31, 32). Studies with knockout
 experiments indicate that *CDK2* functions appear redundant with
CDK1, although in our studies, we did not observe upregulation
 of *CDK1* expression following *CDK2* knockdown *in vitro* or *in vivo*
 (data not shown).

Although we observed a difference in the amplicon-dependent
 sensitivity of *CDK2* gene suppression compared with pharmaco-
 logic inhibition, dinaciclib remains a potent *CDK2* inhibitor with
 single-agent activity in *CCNE1*-amplified HGSC cell lines and is
 one of the most clinically advanced *CDK2* inhibitors (33). There-
 fore, to more effectively target *CCNE1*-amplified HGSCs, we
 performed a combinatorial drug screen to identify compounds
 that would synergize with dinaciclib. We also sought to identify
 compounds that may potentially overcome resistance to dinaci-
 clib, a common occurrence in the clinical use of targeted small-
 molecule inhibitors, by testing a cell line that was resistant to *CDK*
 inhibitors. Dinaciclib in combination with MK-2206, an *AKT*
 inhibitor, was identified as a synergistic combination in targeting
CDK inhibitor-resistant cell lines. This supported our previous
 work that identified increased *AKT1* copy number and upregula-
 tion of genes in the *AKT* pathway as a potential mechanism
 of resistance to *CDK2* inhibitors (13). In validating this finding,
 we observed selective, potent synergism between dinaciclib and
 MK-2206 *in vitro* and *in vivo* models of *CCNE1*-amplified HGSCs,
 including parental OVCAR3 cells. This interaction was not

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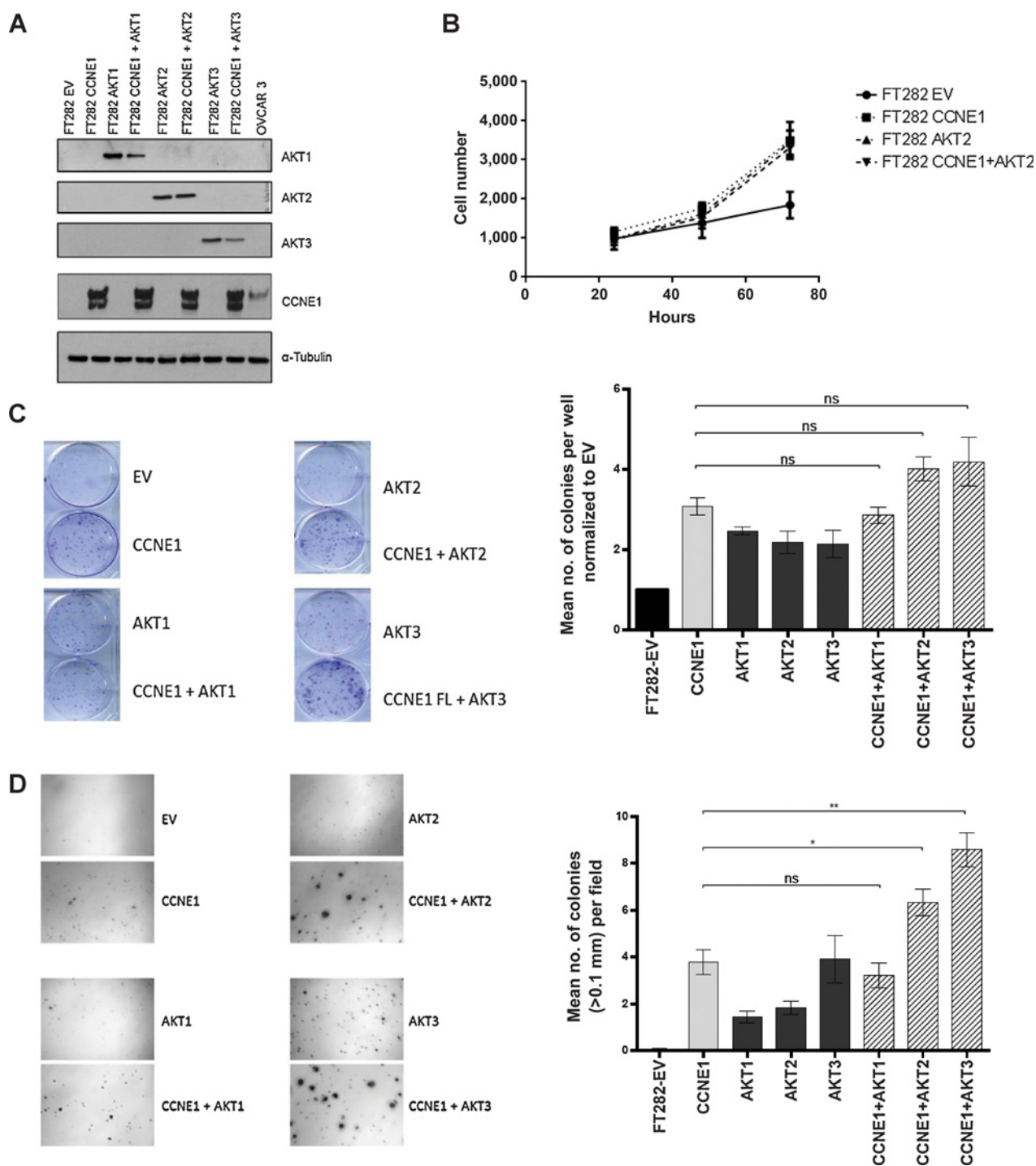


Figure 6.

Cyclin E1 and AKT overexpression cooperates to promote uncontrolled growth in FTSECs. A, Western blot analysis of fallopian tube secretory cells transduced with cyclin E1, empty vector, and AKT1, AKT2, and AKT3 overexpression constructs. Blots are representative of three independently performed experiments. B, Proliferation assay of fallopian tube secretory cells (FT282) transduced with empty vector (EV), cyclin E1 (CCNE1), AKT2, and both cyclin E1 and AKT2 (CCNE1+AKT2). Plots represent mean of three independently performed experiments, error bars represent SEM. C, Clonogenic survival assay of FT282 cells transduced as labeled. Images (left) show cells fixed and stained with crystal violet. Bar chart represents mean of three independently performed experiments, error bars represent SEM. Statistical significance (*t* test) calculated by comparison with FT282 cells transduced with cyclin E1 (FT282-CCNE1). D, Anchorage-independent assay of FT282 cells transduced as labeled. Images (left) represent cells fixed with 2% paraformaldehyde and captured using an Olympus IX81 live cell imager. Bar chart represents mean of three independently performed experiments, error bars represent SEM. Statistical significance (*t* test) calculated by comparison with FT282 cells transduced with cyclin E1 (FT282-CCNE1); *, *P* < 0.05, **, *P* < 0.01.

527	initially observed in the primary high-throughput screen. How-	589
528	ever, the use of SKOV3 cell line as a comparator in the screen may	590
529	be a potential confounder, as the selection of compounds as hits	591
530	from the primary screen was based on a difference in the EC ₅₀	592
531	values between the two cell lines tested, OVCAR3 and SKOV3.	593
532	Recently, multiple studies characterizing the genomic profile of	594
533	commercially available ovarian cancer cell lines have shown that	595
534	many of these cell lines, including SKOV3, may not accurately	596
535	resemble HGSC (26, 34–36).	597
536	Synergism between dinaciclib and MK-2206, as well as another	
537	AKT-specific inhibitor GSK2110183, but an absence of a syner-	
538	gistic combination with other inhibitors of the PI3K–AKT–mTOR	
539	pathway suggests that the interaction with <i>CCNE1</i> may be specific	
540	to AKT. Analysis of genomic data from patients demonstrated a	
541	significant cooccurrence of <i>CCNE1</i> and <i>AKT2</i> amplification,	
542	which may in part be explained by colocalization on chromosome	
543	19q. However, FUOV1, which has <i>CCNE1</i> -amplification without	
544	<i>AKT2</i> -amplification (25), was equally sensitive to the combina-	
545	tion of dinaciclib and AKT inhibitors. Coexpression of <i>AKT2</i> or	
546	<i>AKT3</i> with cyclin E1 in a <i>TP53</i> -mutant FTSEC cell line resulted in	
547	increased proliferation and anchorage-independent growth.	
548	Analysis of data from Project Achilles indicates that HGSC cell	
549	lines that have <i>CCNE1</i> amplification or overexpression are depen-	
550	dent on multiple genes within the AKT pathway. We previously	
551	performed a pathway analysis of genes coexpressed with <i>CCNE1</i>	
552	amplification and observed an enrichment of genes involved in	
553	AKT signaling (12). Collectively, these data suggest a specific	
554	dependency of <i>CCNE1</i> -amplified tumors for AKT activity.	
555	Dinaciclib and MK-2206 have previously been shown to be	
556	active against pancreatic adenocarcinoma (37). In <i>KRAS</i> -mutant	
557	pancreatic cancer patient–derived xenografts, Hu and colleagues	
558	(37) demonstrated efficacy of dinaciclib combined with MK-	
559	2206. They proposed that sensitivity was due to the effect of	
560	dinaciclib on CDK5, and in turn, inhibition of RAL pathway. On	
561	the basis of these results, a phase I clinical trial (NIH Trial	
562	NCT01783171) of dinaciclib and MK-2206 has been initiated in	
563	patients with advanced pancreatic cancer. While this trial will	
564	provide safety and recommended dosing of the combination,	
565	patients are not preselected on the basis of tumoral <i>CCNE1</i>	
566	amplification, and the mechanism of interaction and biomarkers	
567	that predict response are likely to be different in pancreatic cancer	
568	compared with HGSC.	
569	Other combinations were also identified from the high-through-	
570	put screen. In particular, nonselective BH3-mimetic compounds	
571	ABT-737 and ABT-263 were synergistic in combination with dina-	
572	ciclib in CDK inhibitor–resistant cell lines. There was no synergistic	
573	interaction between dinaciclib and the Bcl-2–specific antagonist,	
574	ABT-199, indicating that the targeting of multiple antiapoptotic	
575	proteins is potentially required to overcome resistance to CDK2	
576	inhibitors. This observation is supported by upregulation of mul-	
577	tiiple genes in this pathway including BCL-2, BCL-XL, and BCL-W in	
578	resistant cell lines. However, the use of ABT-737 or ABT-263 in	
579	combination with dinaciclib <i>in vivo</i> is hindered by significant	
580	toxicities, particularly hematologic (Joel Levenson, personal com-	
581	munication), and are therefore unlikely to have clinical utility.	
582	Biomarker-driven trials in HGSC are needed to improve clinical	
583	outcomes. HGSC patients with <i>CCNE1</i> amplification are a subset	
584	that requires different treatment approaches, given that they have	
585	HR-proficient tumors, and as such, are likely to have poor	
586	responses to platinum-based chemotherapy and PARP inhibitors.	
587	However, targeted therapies when used alone may not be sufficient	
	to induce selective, cytotoxic effects, and often result in the	
	development of resistance. Combination therapies may poten-	
	tially be a strategy to overcome these limitations. High-throughput	
	drug screening is an unbiased approach to identify novel thera-	
	peutic strategies, and we have identified dinaciclib and MK-2206	
	as a combination that may prove to selectively target patients with	
	<i>CCNE1</i> -amplified HGSC. Further work incorporating additional	
	clinically relevant models and novel combinations will inform the	
	design of rational clinical trials targeting <i>CCNE1</i> -amplified HGSC.	
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	No potential conflicts of interest were disclosed.	Q6 599
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	R.B. Pearson, R. Drapkin	604
	Acquisition of data (provided animals, acquired and managed patients,	605
	provided facilities, etc.): G. Au-Yeung, F. Lang, W.J. Azar, K.E. Jarman,	606
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	Analysis and interpretation of data (e.g., statistical analysis, biostatistics,	608
	computational analysis): G. Au-Yeung, F. Lang, W.J. Azar, K. Lackovic,	609
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	Bowtell	617
	Other [created material integral to the study (engineered primary human cell	618
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