

## Cyclin E2 Overexpression Is Associated with Endocrine Resistance but not Insensitivity to CDK2 Inhibition in Human Breast Cancer Cells

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### Abstract

Cyclin E2, but not cyclin E1, is included in several gene signatures that predict disease progression in either tamoxifen-resistant or metastatic breast cancer. We therefore examined the role of cyclin E2 in antiestrogen resistance *in vitro* and its potential for therapeutic targeting through cyclin-dependent kinase (CDK) inhibition. High expression of *CCNE2*, but not *CCNE1*, was characteristic of the luminal B and HER2 subtypes of breast cancer and was strongly predictive of shorter distant metastasis-free survival following endocrine therapy. After antiestrogen treatment of MCF-7 breast cancer cells, cyclin E2 mRNA and protein were downregulated and cyclin E2-CDK2 activity decreased. However, this regulation was lost in tamoxifen-resistant (MCF-7 TAMR) cells, which overexpressed cyclin E2. Expression of either cyclin E1 or E2 in T-47D breast cancer cells conferred acute antiestrogen resistance, suggesting that cyclin E overexpression contributes to the antiestrogen resistance of tamoxifen-resistant cells. Ectopic expression of cyclin E1 or E2 also reduced sensitivity to CDK4, but not CDK2, inhibition. Proliferation of tamoxifen-resistant cells was inhibited by RNAi-mediated knock-down of cyclin E1, cyclin E2, or CDK2. Furthermore, CDK2 inhibition of E-cyclin overexpressing cells and tamoxifen-resistant cells restored sensitivity to tamoxifen or CDK4 inhibition. Cyclin E2 overexpression is therefore a potential mechanism of resistance to both endocrine therapy and CDK4 inhibition. CDK2 inhibitors hold promise as a component of combination therapies in endocrine-resistant disease as they effectively inhibit cyclin E1 and E2 overexpressing cells and enhance the efficacy of other therapeutics. *Mol Cancer Ther*; 11(7); 1488–99. ©2012 AACR.

### Introduction

The introduction of tamoxifen as a treatment for hormone receptor-positive breast cancer led to significant decreases in breast cancer mortality over the last 30 years. Unfortunately, resistance to endocrine therapy occurs in more than 30% of patients and results in

disease progression (1). A pervasive feature of tamoxifen-resistant breast cancers is increased expression of genes related to proliferation, including cyclin D1, and gene sets regulated by MYC, E2F, and RB, suggesting a particularly important role for genes involved in the G<sub>1</sub>-S transition of the cell cycle (1–6). Consequently, endocrine-resistant breast cancer is one disease subtype in which cyclin-dependent kinase (CDK) inhibitors with specificity for the cyclin D1-associated kinases CDK4 and CDK6 may offer significant clinical benefit (7). A further understanding of the major drivers of proliferation in breast cancer cells and direct targeting of the pathways they control are likely to provide mechanisms for identifying and treating endocrine-refractory breast cancer.

Cyclins E1 and E2 (collectively referred to as cyclin E) drive proliferation by promoting the initiation of DNA replication and by activating CDK2. Cyclin E/CDK2, along with cyclin D/CDK4, phosphorylates RB to activate E2F-responsive genes and promote progression into S phase. Antiestrogens target these pathways through downregulation of cyclin D1 leading to loss of cyclin D1/CDK4 activity, downregulation of MYC leading to derepression of the MYC target gene p21<sup>Cip1/Waf1</sup>, and

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upregulation of p27<sup>Waf1</sup>. This leads to inhibition of both CDK2 and CDK4 activity and G<sub>1</sub>-S phase arrest (8).

Cyclin E2 expression has been associated with poor outcome in ER-positive breast cancer (9, 10), and cyclin E2 is included in gene signatures that predict disease progression in either tamoxifen-resistant breast cancer or metastatic breast cancer, whereas cyclin E1 is absent from these same signatures (3, 4, 11–14). Cyclin E1 overexpression can acutely reduce antiestrogen sensitivity *in vitro* (15–17), but cyclin E2 has not been studied in this context, although it is strongly estrogen regulated (18). In addition, CDK2 activation is a possible mechanism of resistance to a CDK4 inhibitor that preferentially inhibits ER-positive breast cancer cell lines and can overcome acquired resistance to tamoxifen (19–21). We therefore examined the roles of cyclin E1 and E2 in antiestrogen resistance in ER-positive breast cancer and as potential therapeutic targets in endocrine-resistant disease.

## Materials and Methods

### Patient cohorts and bioinformatics

Affymetrix HG-U133A and HG-U133PLUS2 microarray data from a total of 863 breast cancers (GSE3494 (22), GSE1456 (23), GSE7390 (24), GSE4922 (25), GSE6532 (26), and GSE36771) were normalized and combined into a single data set (27). Intrinsic subtypes were assigned using the Single Sample Predictor algorithm (28), on the basis of mean-centered data and Spearman correlation with subtype centroids. Survival analyses were restricted to patients with unambiguous treatment and survival information who had not received any chemotherapy. Additional details are provided in Supplementary Methods.

### Cell lines

MCF-7 cells (Michigan Cancer Foundation, Detroit, MI), T-47D cells (EG&G Mason Research Institute, Worcester, MA), MCF-7 cells originally obtained from AstraZeneca and their tamoxifen-resistant derivatives (29), and T-47D cells overexpressing cyclin E1 and cyclin E2 derived as described elsewhere (Caldon; submitted for publication), were all authenticated by STR profiling (CellBank Australia) and cultured for less than 6 months after authentication.

### Cell proliferation analysis

S-phase percentages were measured by flow cytometric analysis of propidium iodide-stained, ethanol-fixed cells. Bromodeoxyuridine (BrdUrd) incorporation was assayed using the Cell Proliferation ELISA, BrdUrd (colorimetric) Assay (Roche). Proliferation was also assessed by hemocytometer cell counts or AlamarBlue (Invitrogen).

### siRNA transfection

Gene-specific siRNAs [cyclin D1 smart pool (L-003210-00); cyclin E1 (L-003213-00); cyclin E2 (L-003214-00); CDK2 (L-003236-00)] and controls [On-Target Plus

siCONTROLS (D-001810-10, D-001810-1-4)]; siGENOME Nontargeting siRNA #2 (D-001210-02) were purchased from Dharmacon and transfections carried out as described previously (18).

### Western blot analysis

Primary antibodies were CDC6 (180.2), CDK2 (M2), cyclin E1 (HE12), E2F1 (KH95), and MYC (9E10), Santa Cruz Biotechnology; cyclin D1 (DCS6, Novocastra); cyclin E2 (Cell Signaling); p21<sup>Cip1/Waf1</sup> (610233) and p27<sup>Kip1</sup> (610241), BD Transduction Laboratories;  $\beta$ -Actin (AC-15; Sigma) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, 4300; Ambion).

### Immunoprecipitation and kinase assays

Antibodies used for immunoprecipitation were p21<sup>Cip1/Waf1</sup> (M-19), p27<sup>Kip1</sup> (C-19), cyclin E1 (C-19), cyclin A (C-19), and CDK2 (C-19) from Santa Cruz Biotechnology and cyclin E2 from Epitomics. Kinase activity was measured in cyclin E1 and cyclin E2 immunoprecipitates using histone H1 substrate as previously described (30).

### Gene expression analysis

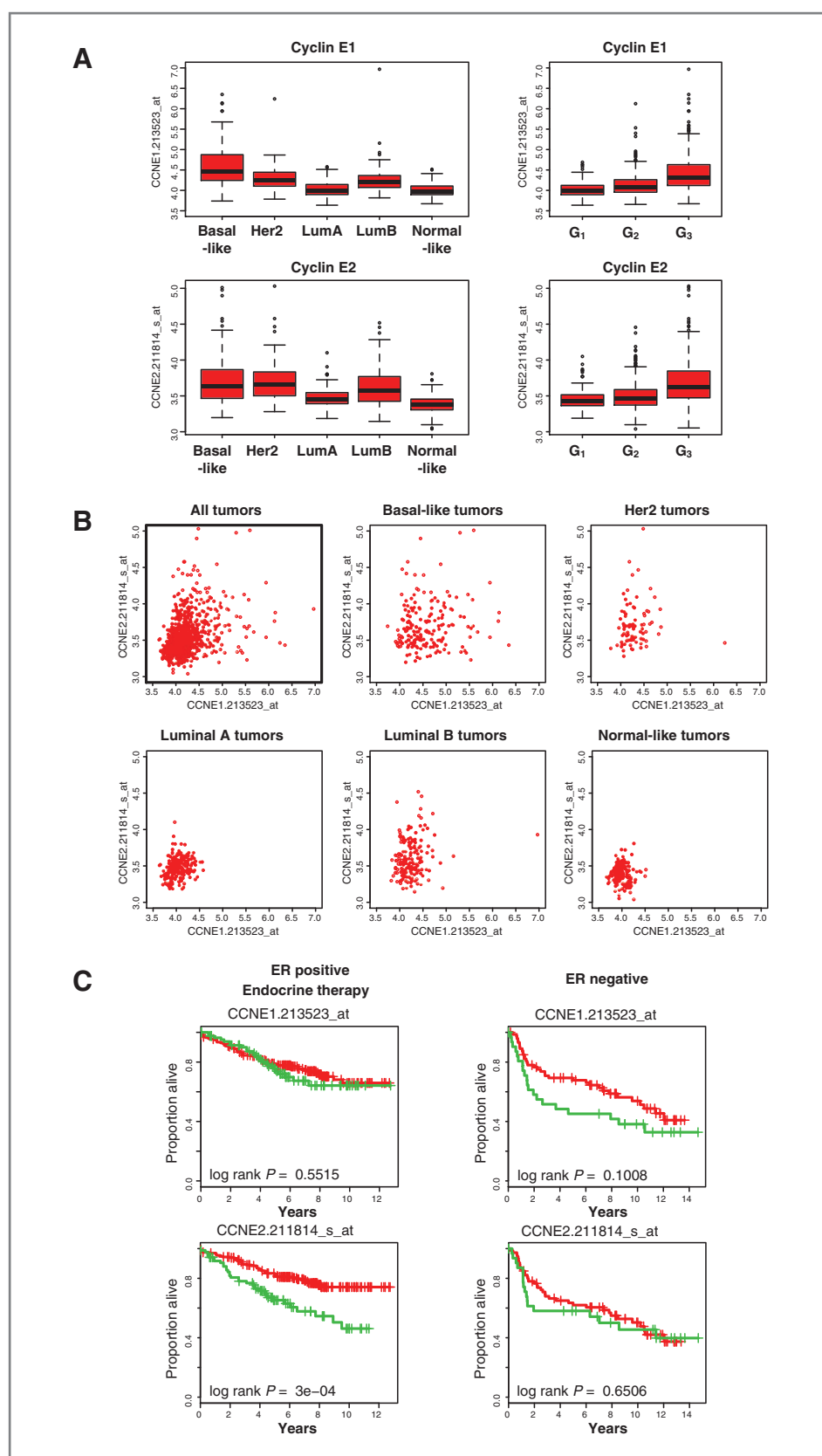
Quantitative reverse transcriptase PCR (qRT-PCR) used inventoried TaqMan probes cyclin E1 (Hs00180319\_m1), cyclin E2 (Hs00180319\_m1), and human RPLPO (4326314E; Applied Biosystems).

## Results

### Cyclin E1 and cyclin E2 have distinct associations with breast cancer subtypes and with distant metastasis-free survival

To explore the relationship between increased expression of cyclins E1 and E2, we used a database of transcript profiles from 863 breast cancer patients with carefully curated clinicopathologic data (detailed in Supplementary Methods). Both *CCNE1* and *CCNE2* increased in expression with increasing tumor grade, and there were significant differences in their expression between breast cancer subtypes (Fig. 1A, Supplementary Tables S1 and S2). The basal-like subtype expressed the highest levels of *CCNE1*, whereas levels of *CCNE2* were similar in Her2 and basal-like cancers (Fig. 1A, Supplementary Table S2). Scatter plots of *CCNE1* and *CCNE2* suggested that there was a tendency to overexpress *CCNE2* more strongly than *CCNE1* in the Her2 and luminal B breast cancers (Fig. 1B). In contrast, in the highly proliferative basal-like subtype, these cyclins were often co-ordinately overexpressed (Fig. 1B). A second *CCNE2* probe set gave similar results and, in addition, identified overexpression of *CCNE2* in some luminal A breast cancers (Supplementary Fig. S1, Supplementary Tables S1 and S2).

Because *CCNE2* was commonly overexpressed in luminal breast cancer, the relationship between *CCNE2* and distant metastasis-free survival in ER-positive breast cancer was examined. After endocrine therapy, patients with the highest *CCNE2* expression (top 30%) had shorter



**Figure 1.** Cyclin E1 and cyclin E2 association with breast cancer subtype, grade, and distant metastasis-free survival. Cyclin E1 and E2 probe sets from a total of 863 breast cancer patients analyzed using Affymetrix microarrays were analyzed to identify relationships with breast cancer subtype or histologic grade (A). B, relative expression of cyclin E1 and cyclin E2 probe sets in individual specimens from different breast cancer subtypes. C, Kaplan–Meier survival analysis of distant metastasis-free survival from ER-positive patients treated with endocrine therapy ( $n = 287$ ) and untreated ER-negative patients ( $n = 106$ ), using a 70% cut-off between low (green) and high (red) expression.

distant metastasis-free survival, but this was not seen in patients with high *CCNE1* expression (Fig. 1C). In contrast, despite the high expression of *CCNE1* and *CCNE2* in basal-like breast cancer, neither was associated with shorter distant metastasis-free survival in ER-negative breast cancer treated with surgery alone. The use of different cut-offs (stratification at 20th–80th percentiles) and a second *CCNE2* probe set gave similar results (Supplementary Fig. S1 and data not shown).

### Antiestrogens regulate cyclin E1/CDK2 and cyclin E2/CDK2 activity by independent pathways in estrogen-responsive cells

Because of the relationship between *CCNE2* expression and distant metastasis-free survival after endocrine therapy, we examined the role of cyclin E2 in the response of MCF-7 cells to antiestrogen treatment *in vitro*. The non-steroidal antiestrogen 4-hydroxytamoxifen (100 nmol/L) and the pure antiestrogen fulvestrant (1 nmol/L) caused significant decreases in cyclin E1/CDK2 and cyclin E2/CDK2 activities and an acute G<sub>1</sub> phase arrest (Fig. 2A and B). *CCNE1* levels were not affected by fulvestrant treatment, but cyclin E1 protein increased (Fig. 2C). By contrast, *CCNE2* and cyclin E2 protein expression were significantly downregulated by both 4-hydroxytamoxifen and fulvestrant (Fig. 2C). Thus, cyclins E1 and E2 are differentially regulated by both estrogen (18) and antiestrogens.

We next examined the effects of 48-hour antiestrogen treatment on cyclin E/CDK2 complexes (Fig. 2D). In lysates from control cells cultured in the presence of 100 nmol/L 17 $\beta$ -estradiol, cyclin E1 and cyclin E2 both coimmunoprecipitated CDK2 and p130, a pocket protein that is sequestered into active cyclin E/CDK2 complexes during estrogen-stimulated proliferation (31). Treatment with 10 nmol/L fulvestrant markedly decreased the levels of CDK2, p21<sup>Cip1/Waf1</sup>, p27<sup>Kip1</sup>, and p130 in cyclin E2 immunoprecipitates, as expected from the decreased overall level of cyclin E2. Fulvestrant treatment did not alter the amount of CDK2 in cyclin E1 immunoprecipitates, but it did increase the amount of p21<sup>Cip1/Waf1</sup> and p27<sup>Kip1</sup> and decreased the amount of p130 binding, indicating a shift to inactive cyclin E1/CDK2/p21<sup>Cip1/Waf1</sup> or cyclin E1/CDK2/p27<sup>Kip1</sup> complexes. Therefore, antiestrogen treatment predominantly affects cyclin E2/CDK2 via transcriptional downregulation of cyclin E2, in contrast with the previously described inhibition of cyclin E1/CDK2 activity by CDK inhibitor binding (8).

### Antiestrogen-resistant cells have high levels of cyclin E1 and cyclin E2 that contribute to proliferation

To investigate whether deregulated cyclin E expression might contribute mechanistically to endocrine resistance, we used TAMR cells, an *in vitro* model of 4-hydroxytamoxifen resistance (ref. 29; Supplementary Fig. S2A). Both cyclin E1 and E2 were overexpressed in the tamoxifen-resistant cells, compared with matched parental MCF-7 cells (designated MCF-7C, Fig. 3A), and

the associated kinase activities also increased (see Fig. 3C). This was not simply because of generalized overexpression of proliferative genes as TAMR cells did not overexpress cyclin D1 or MYC (Fig. 3A). Cyclin E2 mRNA and protein were no longer downregulated by either 4-hydroxytamoxifen or fulvestrant in TAMR cells (Fig. 3A and B, Supplementary Fig. S3B). These cells remained partially sensitive to fulvestrant (ref. 29; Supplementary Fig. S2A), and fulvestrant treatment reduced cyclin E-CDK2 activity, although to a lesser extent than in the parental MCF-7C cells (40%–50% vs. 80%–90%, Fig. 3C). The decrease in cyclin E-CDK2 activity in the absence of changes in cyclin E abundance is likely due to increased p21<sup>Cip1/Waf1</sup> and p27<sup>Kip1</sup> abundance after fulvestrant treatment (Fig. 3B).

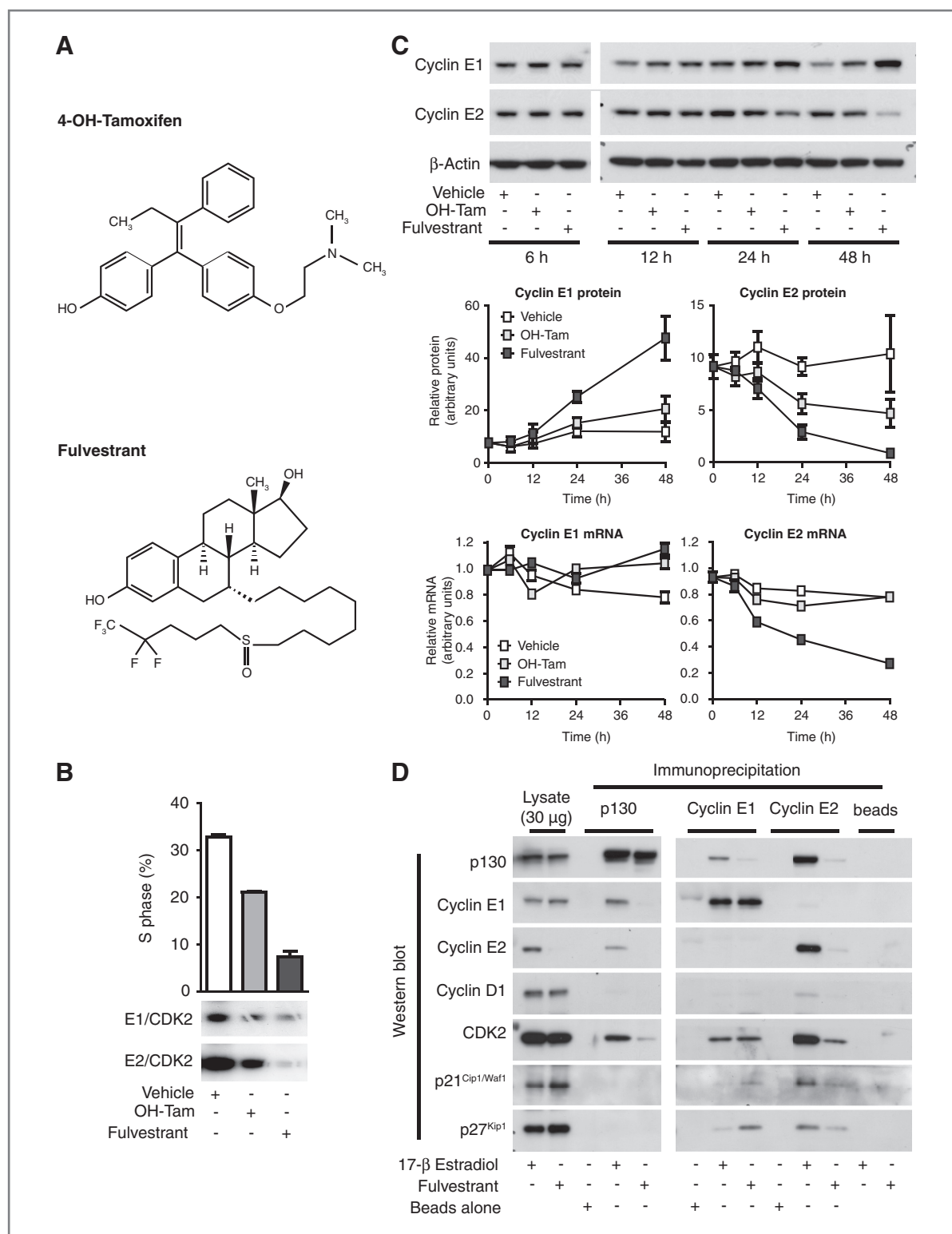
To investigate whether tamoxifen-resistant cells are dependent on overexpressed cyclin E, we used siRNAs to cyclins E1 and E2, alone and in combination (Fig. 3D). Cyclin E1 siRNA led to a small upregulation of cyclin E2 and vice versa; but in all cases, S phase was significantly reduced. Although the effects of the individual siRNAs were similar in MCF-7C and TAMR cells, the combination of cyclin E1 and E2 siRNA was significantly more effective in tamoxifen-resistant cells than in MCF-7C cells (Fig. 3D). Overall, these data suggested that TAMR cells may be more dependent on cyclin E than MCF-7C, pointing to CDK2 inhibition as a potential therapeutic avenue in endocrine-resistant breast cancer.

Because cyclin E2 lies downstream of cyclin D1 in estrogen-mediated proliferation in MCF-7 cells (18), we examined the effects of cyclin D1 siRNA on cyclin E2 expression. Cyclin D1 knockdown led to marked inhibition of proliferation and decreased expression of cyclin E2 in both MCF-7C and TAMR cells (Fig. 3D). However, cyclin E2 expression was maintained at higher levels in TAMR cells than in MCF-7C cells (Fig. 3D), suggesting partial decoupling between cyclin D1 and cyclin E2 as one mechanism for deregulation of cyclin E2 expression in this model of tamoxifen resistance.

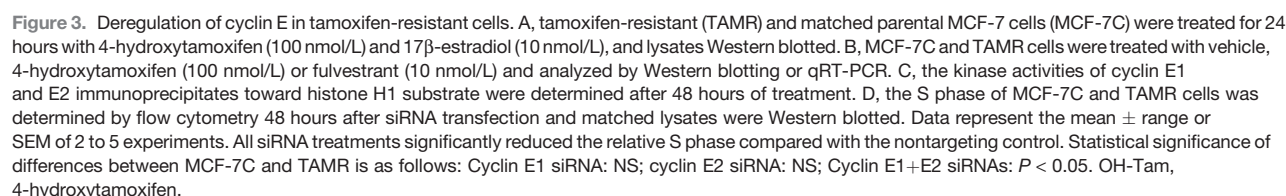
### Overexpression of either cyclin E1 or E2 causes antiestrogen resistance

Because the effect of cyclin E2 deregulation on antiestrogen sensitivity has not been investigated, we treated T-47D cells overexpressing matched levels of cyclins E1 and E2 with fulvestrant or 4-hydroxytamoxifen. In cells overexpressing cyclin E1 or cyclin E2, fulvestrant led to only a 25% to 30% reduction in S phase, compared with approximately 70% reduction in the vector control cell line (Fig. 4A). Vector, cyclin E1-, and cyclin E2-overexpressing cell lines had similar logarithmic growth rates under control conditions (Fig. 4A and B). After exposure to fulvestrant, the vector control cells were growth arrested but the cyclin E1- and cyclin E2-overexpressing cell lines continued to proliferate, although at a slower rate (Fig. 4B). The cyclin E1- and E2-overexpressing cells were also less sensitive to 4-hydroxytamoxifen (see Fig. 5C).

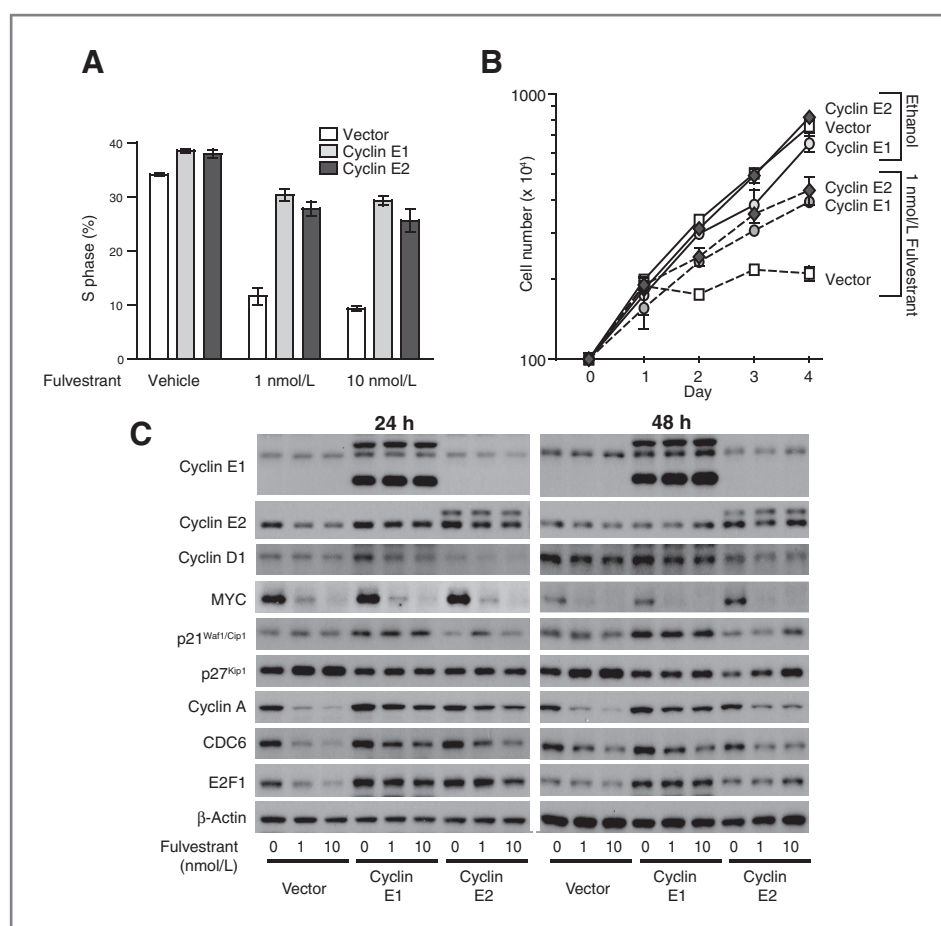




**Figure 2.** Antiestrogen regulation of cyclins E1 and E2. **A**, structures of the antiestrogens 4-hydroxytamoxifen and fulvestrant. **B–D**, MCF-7 cells were treated with antiestrogens 4-hydroxytamoxifen (100 nmol/L) and fulvestrant (1 nmol/L). **B**, S-phase fraction after 24-hour antiestrogen treatment (mean  $\pm$  range of duplicate experiments). The kinase activities of cyclin E1 and E2 immunoprecipitates toward histone H1 substrate were determined at the same time point. **C**, representative Western blots and densitometry of triplicate experiments, with loading corrected using  $\beta$ -actin levels. Matched lysates were analyzed in triplicate by qRT-PCR for cyclin E1 and E2 mRNA expression. Triplicate experiments were pooled and error bars represent SEM. **D**, lysates of cells treated with 17 $\beta$ -estradiol (100 nmol/L) or fulvestrant (10 nmol/L) for 48 hours were immunoprecipitated and then Western blotted using the indicated antibodies. OH-Tam, 4-hydroxytamoxifen.



sing cells, nor was it affected by fulvestrant treatment of these cells (Fig. 4C, Supplementary Fig. S3), although p21<sup>Cip1/Waf1</sup> expression was increased by approximately 1.5-fold in cyclin E1-overexpressing cells, consistent with previous data (16). Similarly, there were no major changes in the level or fulvestrant regulation of p27<sup>Kip1</sup> in cyclin E1- and cyclin E2-overexpressing cells (Fig. 4C,



**Figure 4.** Effect of overexpression of cyclins E1 and E2 on antiestrogen-induced growth arrest. T-47D cells overexpressing cyclin E1, cyclin E2, or vector control were treated with fulvestrant or vehicle. A, S-phase fraction after 24 hours of treatment (mean and range of duplicate experiments). B, cell number was determined by hemocytometer cell counting (mean  $\pm$  SEM of duplicate experiments, in which duplicate counts were carried out on triplicate treatments). C, cell lysates collected at 24 and 48 hours of fulvestrant treatment were Western blotted. Data are representative of quadruplicate experiments.

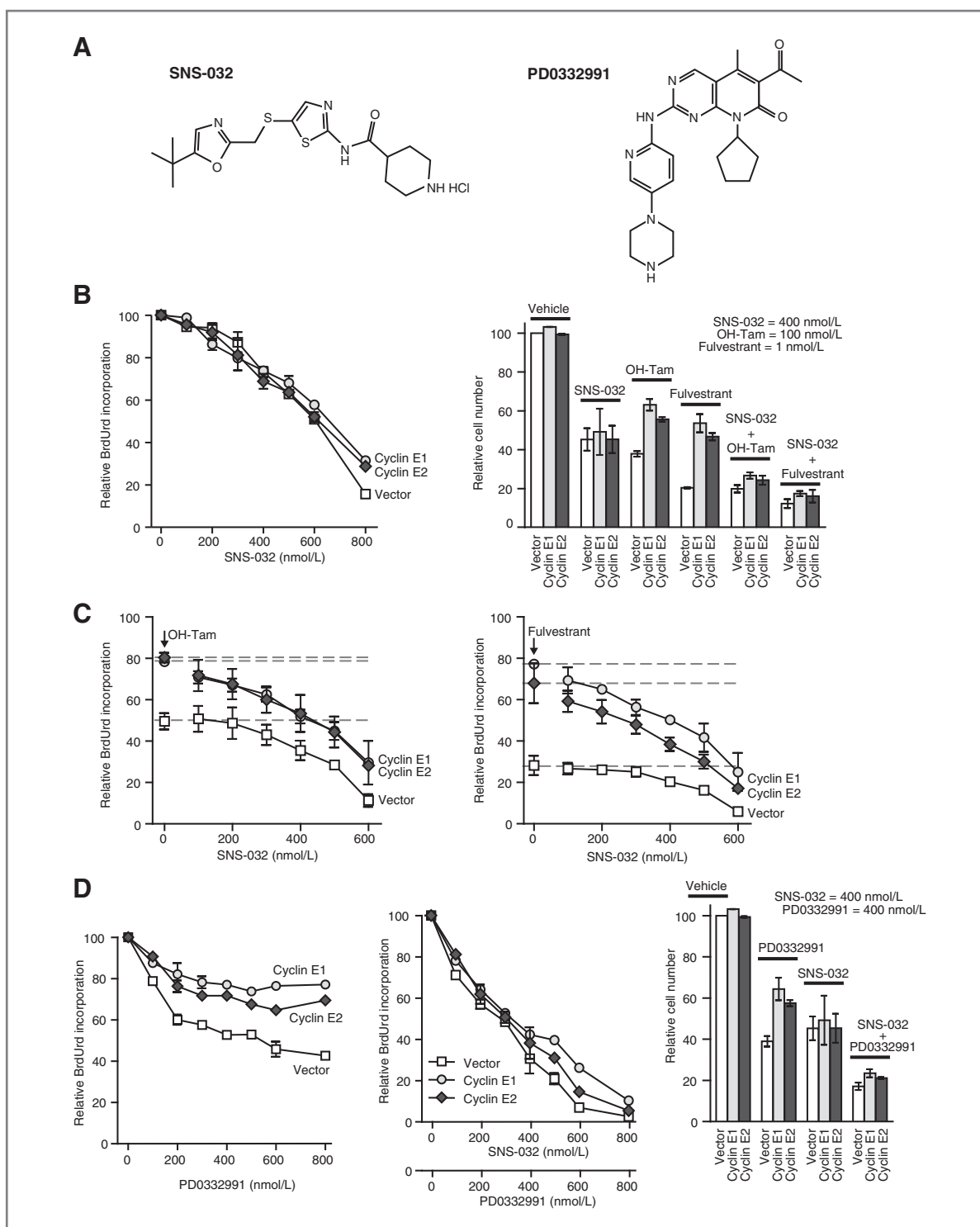
Supplementary Fig. S3). Thus, deregulation of cyclin D1, MYC, p21<sup>Cip1/Waf1</sup>, or p27<sup>Kip1</sup> does not seem to contribute to fulvestrant resistance following cyclin E2 overexpression. However, overexpression of cyclin E1 or cyclin E2 led to a 1.5- to 2-fold increase in the expression of E2F1 and dampened the fulvestrant-mediated downregulation of the E2F targets E2F1, cyclin A, cyclin E2, and CDC6, so that their expression was maintained at levels similar to those in untreated vector control cells (Fig. 4C, Supplementary Fig. S4). This indicated that cyclin E overexpression is accompanied by deregulation of E2F signaling that may contribute to antiestrogen resistance *in vitro*.

#### CDK2 inhibitors decrease the proliferation of cyclin E1- or cyclin E2-overexpressing cells

To examine CDK2 inhibition as a potential therapeutic modality in cyclin E-overexpressing cells, we used SNS-032, a compound that potently inhibits CDK2, CDK7, and CDK9 (ref. 32; Fig. 5A). SNS-032 treatment reduced BrdUrd incorporation and relative cell number in vector, cyclin E1- and cyclin E2-overexpressing T-47D cell lines to a similar degree and with similar concentration dependence ( $IC_{50}$  values of 600–700 nmol/L; Fig. 5B). Treatment with SNS-032 in combination with either 4-hydroxytamoxifen or fulvestrant reduced both

BrdUrd incorporation and relative cell number more effectively than either SNS-032 or antiestrogen treatment alone (Fig. 5B and C). In the presence of 4-hydroxytamoxifen or fulvestrant, SNS-032 concentrations of 300 nmol/L or more, or 400 nmol/L or more, respectively, were required to inhibit BrdUrd incorporation in vector control cells (Fig. 5C). However, in cyclin E-overexpressing cells, the lowest concentration of SNS-032 tested, 100 nmol/L, reduced BrdUrd incorporation in combination with either 4-hydroxytamoxifen or fulvestrant (Fig. 5C), suggesting that the effects of SNS-032 may be potentiated by the presence of an antiestrogen.

CDK2 activation has been implicated in resistance to CDK4 inhibition in leukemia cells (20), suggesting that cyclin E overexpression may reduce sensitivity to CDK4 inhibition. Consistent with this idea, the selective CDK4/6 inhibitor PD0332991 (Fig. 5A) was less effective at reducing BrdUrd incorporation and relative cell number of cyclin E1- or cyclin E2-overexpressing cells than vector control cells (Fig. 5D). The combination of SNS-032 and PD0332991 resulted in greater decreases in BrdUrd incorporation and relative cell number than either compound alone (Fig. 5D). Furthermore, the CDK4 inhibitor resistance of cells overexpressing cyclin E was no longer evident when



**Figure 5.** Effect of CDK inhibitors on cells overexpressing cyclin E1 or E2. **A**, structures of the CDK inhibitors SNS-032 and PD0332991. **B**, cyclin E1, cyclin E2, and vector cell lines were either (i) treated with SNS-032 in multiwell plates for 48 hours at the indicated concentrations, followed by measurement of BrdUrd incorporation per well (left), or (ii) treated with SNS-032 and/or 4-hydroxytamoxifen or fulvestrant and analyzed after 4 days by hemocytometer cell counts (right). **C**, cells were treated with 100 nmol/L 4-hydroxytamoxifen or 1 nmol/L fulvestrant in combination with SNS-032 over a concentration range and BrdUrd incorporation measured after 48 hours. Data are mean  $\pm$  SEM of cells analyzed in triplicate in duplicate experiments. **D**, cells were treated with PD0332991 alone or in combination with SNS-032. Either (i) BrdUrd incorporation was measured after treatment for 48 hours at the indicated concentrations (mean  $\pm$  SEM of triplicate wells in duplicate experiments), or (ii) cells were analyzed after 4 days by hemocytometer cell counts. Bar charts in **B** and **D** are from the same experiments, and data from vehicle and SNS-032 are presented in both panels for clarity. Data represent the mean  $\pm$  SEM of duplicate cell counts in duplicate experiments. OH-Tam, 4-hydroxytamoxifen.



these cells were treated with a combination of SNS-032 and PD0332991 (Fig. 5D).

#### **CDK2 inhibitors decrease proliferation of tamoxifen-resistant cell lines and enhance the effects of CDK4 inhibition or tamoxifen treatment**

Because CDK2 inhibition significantly decreased the proliferation of cells overexpressing cyclin E, its potential in combination with either CDK4 inhibition or antiestrogen treatment of tamoxifen-resistant MCF-7 cells was investigated. We first used CDK2 siRNA to determine whether MCF-7C and tamoxifen-resistant cells depend on CDK2 for proliferation. Knockdown of CDK2 increased the expression of cyclin E1, but not cyclin E2, and reduced the S phase fraction of both MCF-7C and TAMR cells (Fig. 6A). As expected from their greater dependence on cyclin E, tamoxifen-resistant cells were more dependent on CDK2 ( $P < 0.03$ ). Treatment with SNS-032 had similar effects on BrdUrd incorporation in the 2 cell lines [ $IC_{50}$  130–170 nmol/L (MCF-7C) vs. 170–190 nmol/L (TAMR), Fig. 6B], but PD0332991 was more potent in TAMR cells ( $IC_{50}$  160 nmol/L) than in the parental MCF-7C cells ( $IC_{50}$  >200 nmol/L, Fig. 6B). The addition of SNS-032 to PD0332991 was more effective than PD0332991 alone in both MCF7 and TAMR cells (Fig. 6B). Measurement of cell number after 4 to 5 days exposure yielded similar results (data not shown).

As observed in T-47D cells (Fig. 5), treatment with SNS-032 in the presence of a fixed concentration of 4-hydroxytamoxifen reduced BrdUrd incorporation in MCF-7C cells compared with SNS-032 alone (Fig. 6C). In TAMR cells, SNS-032 was more potent in the presence of 4-hydroxytamoxifen than alone, so that the  $IC_{50}$  was significantly reduced ( $P < 0.0217$  for 100 nmol/L 4-hydroxytamoxifen;  $P < 0.0001$  for 1  $\mu$ mol/L 4-hydroxytamoxifen; Fig. 6C), again suggesting that the effects of SNS-032 may be potentiated by the presence of an antiestrogen.

## **Discussion**

### **Cyclin E2 overexpression in breast cancer: relationship with endocrine resistance**

Cyclin E1 and cyclin E2 are coexpressed and coregulated in many contexts (33), but accumulating evidence indicates that this is not the case in breast cancer cells. First, in contrast with the well-studied estrogen/anti-estrogen regulation of cyclin E1-CDK2 activity by p21<sup>Cip1/Waf1</sup> and p27<sup>Kip1</sup> (8), cyclin E2-CDK2 regulation following estrogen or antiestrogen treatment occurs predominantly via transcriptional regulation of cyclin E2 expression (ref. 18 and this study). Second, *CCNE2*, but not *CCNE1*, is included in gene signatures associated with poor outcome in breast cancer (3, 4, 11–14). Finally, we have identified overexpression of *CCNE2*, but not *CCNE1*, in the luminal B and Her2 subtypes. Because subtypes expressing similar levels of *CCNE2*

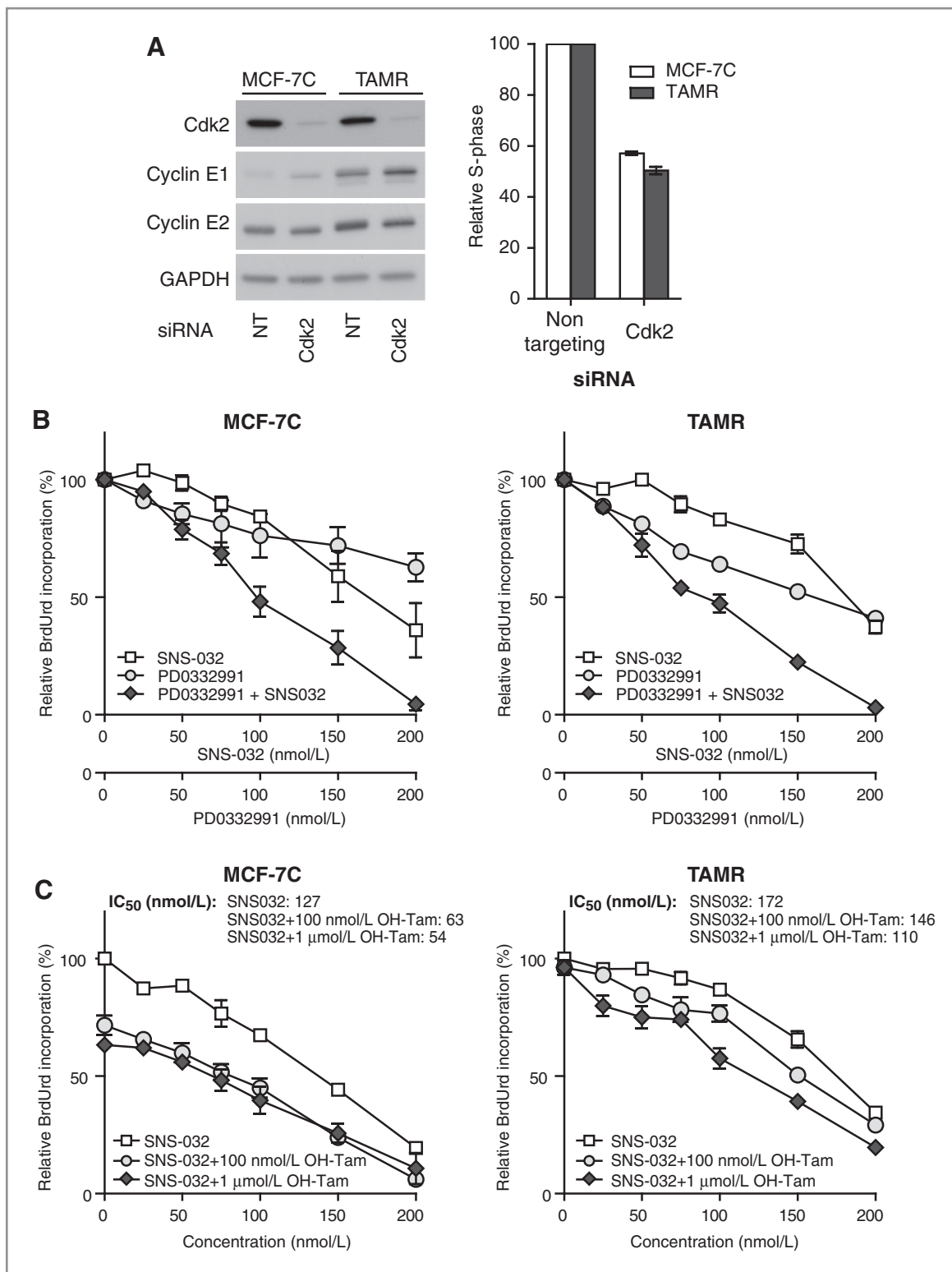
differ in indices of proliferation (11, 34), Fig. 1) and *CCNE2* and Ki67 expression are not correlated (9), high *CCNE2* expression in breast cancer is not simply a surrogate for increased proliferation. Instead, it may, in part, reflect amplification of the region encompassing *CCNE2* at 8q22.1, which occurs in at least 20% of breast cancer and is associated with elevated *CCNE2* expression (35, 36).

When both E cyclins are measured in parallel, *CCNE1* is generally more strongly associated with poor patient outcome in ER-negative breast cancer, whereas *CCNE2* is more strongly associated with poor outcome in ER-positive breast cancer (9, 10). Here we have identified a strong relationship between high *CCNE2* levels and shorter distant metastasis-free survival following endocrine therapy. This relationship was significant across a range of cut-offs but was most apparent when the data were dichotomized at the 70th or 80th percentile, likely the result of the skewed *CCNE2* distribution seen in Fig. 1B. In a previous study, *CCNE2* was not significantly associated with shorter distant metastasis-free survival following tamoxifen therapy (10), perhaps because of the smaller cohort (110 patients compared with 287 here) but more likely because stratification at the median less accurately defines *CCNE2* overexpression.

Although cyclin E1 can reduce sensitivity to antiestrogens when overexpressed in experimental models and, in one study, *CCNE1* was predictive of response to endocrine therapy (37), in the large cohort analyzed here *CCNE1* was less commonly overexpressed in luminal breast cancers than *CCNE2*. However, truncated forms of the cyclin E1 protein are expressed in ER-positive breast cancer and can also cause resistance to endocrine therapies (17, 38). Both cyclin E1 and cyclin E2 overexpression impaired some antiestrogen responses, that is, downregulation of E2F target genes and inhibition of proliferation. There are, however, mechanistic differences between the overexpression of cyclin E1 and cyclin E2, as only cyclin E1 overexpression leads to upregulation of both cyclin D1 and p21<sup>Waf1/Cip1</sup> (Fig. 4), and although the activity of cyclin E2/CDK2 complexes is inhibited by p27<sup>Kip1</sup> (39), the truncated forms of cyclin E1 that confer insensitivity to antiestrogens are resistant to p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> (17). Our investigations revealed that cyclin E2 overexpression dampens antiestrogen-mediated downregulation of E2F target genes and reduces antiestrogen sensitivity *in vitro*. Cyclin E2 is also deregulated in a well-characterized model of antiestrogen resistance, TAMR cells. The more frequent high expression of *CCNE2* in the luminal B subtype may then be one reason for the relative endocrine resistance of luminal B breast cancer compared with luminal A breast cancer (40).

### **CDK inhibition as a therapeutic approach in endocrine-resistant breast cancer**

Promising preclinical studies showing that luminal breast cancer cell lines, including tamoxifen-resistant variants, are sensitive to CDK4 inhibition (5, 7, 19, 41)



**Figure 6.** Effect of CDK inhibition on tamoxifen-resistant cells. **A**, forty-eight hours after siRNA transfection, the S phase of MCF-7C and TAMR cells was determined by flow cytometry and matched lysates were Western blotted. Data represent mean  $\pm$  range of duplicate experiments. Other data from the same experiments is included in Fig. 3D. **B**, MCF-7C and TAMR cells were treated with PD0332991 and SNS-032 for 48 hours at the indicated concentrations. Data presented are mean  $\pm$  SEM of triplicate wells from duplicate experiments. **C**, MCF-7C and TAMR cells were treated for 48 hours with either 100 nmol/L or 1  $\mu$ mol/L 4-hydroxytamoxifen, in combination with SNS-032. Data presented are mean  $\pm$  SEM of triplicate experiments. OH-Tam, 4-hydroxytamoxifen.

have led to a phase II clinical trial of CDK4 inhibition in combination with letrozole in advanced breast cancer (NCT00721409). CDK2 inhibition has also been proposed as a potential therapeutic approach in endocrine-resistant disease as it impairs the proliferation of both ER-positive and ER-negative breast cancer cell lines, including cultured and xenografted endocrine therapy-resistant cell lines (refs. 38, 42–45, this study). A phase II clinical trial of dinaciclib, which targets CDK1, CDK2, CDK5, and CDK9, indicated a partial response in 2 of 19 breast cancer patients (46), both of whom had ER-positive disease.

Activation of CDK2 has been implicated in acquired resistance to CDK4 inhibition (20), and we show here that overexpression of cyclin E1 or E2 reduces sensitivity to CDK4 inhibition in breast cancer cells. In at least some endocrine-resistant breast cancers, cyclin E overexpression and activation of CDK2 may impair response to treatment with CDK4 inhibitors, raising the question of whether combined inhibition of multiple CDKs may be more effective than inhibition of CDK4 alone in endocrine-resistant breast cancer (7). Here we show that combining two CDK inhibitors with complementary specificity is more effective than either one alone and essentially prevents the proliferation of both antiestrogen-sensitive and antiestrogen-resistant cells in culture. Thus it may be therapeutically advantageous to target multiple CDKs, either by the use of a pan-CDK inhibitor or combination therapy.

CDK inhibitors are most likely to enter clinical practice in combination with existing therapies, rather than as single agents. Although antiestrogens inhibit CDK2 activity through several well-documented mechanisms (8), the addition of SNS-032 enhanced the potency of both 4-hydroxytamoxifen and fulvestrant. Decreases in ER expression or transcriptional activity provide a potential mechanism for this effect. Phosphorylation by CDK2 activates ER $\alpha$ , and this effect is enhanced by CDK7 (47), so that inhibition of these CDKs inhibits

ER $\alpha$  transcriptional activity (44, 48). In addition, the CDK inhibitor roscovitine decreases ER $\alpha$  expression (44, 48).

## Conclusions

We have shown here that cyclin E2 downregulation is necessary for antiestrogen inhibition of cell proliferation, and that cyclin E2 overexpression is associated with endocrine resistance in breast cancer, adding further weight to evidence for deregulation of the cell cycle as a common cause of endocrine resistance (1). Both antiestrogen-sensitive and antiestrogen-resistant cells were sensitive to inhibition of CDK2, and this was enhanced in the presence of either tamoxifen or fulvestrant. Collectively these data suggest that further investigation of compounds that inhibit CDK2 may be warranted in breast cancer, particularly in combination with endocrine therapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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