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Identification of CDK10 as an Important Determinant of Resistance to Endocrine Therapy for Breast Cancer

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SUMMARY

Therapies that target estrogen signaling have transformed the treatment of breast cancer. However, the effectiveness of these agents is limited by the development of resistance. Here, an RNAi screen was used to identify modifiers of tamoxifen sensitivity. We demonstrate that CDK10 is an important determinant of resistance to endocrine therapies and show that CDK10 silencing increases ETS2-driven transcription of *c-RAF*, resulting in MAPK pathway activation and loss of tumor cell reliance upon estrogen signaling. Patients with ERα-positive tumors that express low levels of CDK10 relapse early on tamoxifen, demonstrating the clinical significance of these observations. The association of low levels of CDK10 with methylation of the *CDK10* promoter suggests a mechanism by which CDK10 expression is reduced in tumors.

INTRODUCTION

Approximately 70% of breast tumors express estrogen receptor α (ERα), and of these, the majority are dependent on estrogen signaling (EBCTCG, 1998). ERα acts as a hormone-dependent nuclear transcription factor; binding of estrogen to ERα causes a conformational change within the receptor that allows dimerization and binding to estrogen-responsive elements (EREs) located in the promoter region of ERα target genes. This ER/ERE interaction leads to the recruitment of cofactors that facilitate gene transcription (Mangelsdorf et al., 1995). Since most ERα-positive breast cancers are fully dependent on estrogen signaling, they can be effectively treated with antiestrogens such as tamoxifen or aromatase inhibitors that prevent estrogen synthesis (EBCTCG, 1998). Tamoxifen binds to ERα, blocking the interaction between estrogen and its receptor. This inhibition of ERα leads to cell cycle arrest at the G_1 cell cycle checkpoint, limiting cellular proliferation (Musgrove and Sutherland, 1994; Cariou et al., 2000). Despite its widespread use, the effectiveness of tamoxifen is limited by the development of drug resistance; all patients with metastatic disease and 40% of early-stage breast cancer patients treated with adjuvant tamoxifen eventually relapse with tamoxifen-resistant disease (Jordan, 1995). Aromatase inhibitors, which block the synthesis of estrogen, have recently been shown to have superior efficacy and improved tolerability for the treatment of postmenopausal, ERα-positive breast cancer patients compared to tamoxifen (Baum et al., 2003). Initially it was envisaged that resistance to these agents would be less frequent than for tamoxifen, but up to 50% of treated patients develop resistance to aromatase inhibitors (Anderson et al., 2007).

Two major mechanisms have been proposed by which resistance to endocrine therapy may occur. First, continued ERα signaling in the presence of an ERα antagonist or the absence of estrogen may occur, known as ligand-independent ERα activation (Shou et al., 2004). Second, the reliance of tumors upon ERα signaling may be circumvented by the activation of non-ERα growth-promoting pathways (El-Ashry et al., 1997;

SIGNIFICANCE

Understanding the molecular basis of the response to tamoxifen and other antiendocrine agents is of importance in delineating mechanisms of resistance to these clinically important therapies. We demonstrate that high-throughput loss-of-function screens have the ability to identify determinants of response to endocrine therapies that have clear clinical significance. The identification of CDK10 as a critical determinant of tamoxifen response and the molecular mechanisms by which CDK10 dysfunction modulates the response to tamoxifen may allow alternative therapeutic approaches. Our findings indicate that loss of CDK10 expression may be a major determinant of tamoxifen resistance.

Oh et al., 2001). The activity of signal-transducing kinases has been implicated in both of these mechanisms (Gee et al., 2001; Pérez-Tenorio et al., 2002). While the study of individual kinases has been informative, a complementary approach is to analyze all protein kinases simultaneously to uncover alternative mechanisms of resistance. Approaches such as high-throughput RNA interference (RNAi) screening now allow such systematic analysis to be performed (Iorns et al., 2007).

The development of RNAi libraries, which are composed of reagents that allow the selective silencing of specific transcripts, has made it possible to conduct high-throughput screens (HTSs) that interrogate phenotypes associated with the loss of function of many genes (Iorns et al., 2007). Furthermore, siRNA libraries have been used to identify key determinants of resistance to chemotherapeutic drugs such as paclitaxel (Whitehurst et al., 2007; Swanton et al., 2007). We used a library of synthetic siRNA oligonucleotide pools targeting the expression of all kinases in the human genome to identify those that, when silenced, cause tamoxifen resistance in vitro. We demonstrate that targeting CDK10 causes resistance to tamoxifen and other endocrine therapies in vitro and that low expression of CDK10 is associated with a poor clinical response to tamoxifen.

RESULTS

Tamoxifen Resistance High-Throughput siRNA Screen

To identify nonredundant determinants of tamoxifen resistance, we designed a robust, high-throughput RNA interference screen targeting 779 known and putative kinases (Figure 1A). The screen involved transfecting an ERα-positive, tamoxifen-sensitive breast cancer cell line (MCF7) with a 96-well plate arrayed library of siRNA duplexes. Twenty-four hours after transfection, cells were divided into replica plates, and half were treated with 4OH tamoxifen (the active tamoxifen metabolite) and half with vehicle (Figure 1A). Seven days later, cell viability in each plate was measured to assess the effect of each siRNA on cell growth (as assessed in the vehicle-treated plates) and also the effect of exposure to 4OH tamoxifen. A medium-term time course of drug treatment was chosen as opposed to short-term treatments (1–2 days) commonly used in chemoresistance screens to increase the sensitivity of the screen. We used a library of siRNA arrayed as SMARTpools; each SMARTpool (contained within one well of a 96-well plate) was composed of four distinct siRNA species targeting different sequences of the same target transcript.

We validated the performance of MCF7 cells in the high-throughput format as follows: (1) transfection of MCF7 cells with siRNA targeting genes essential for cellular viability (such as *PLK1*) was observed to cause a reduction in viability of more than 90%, compared to transfection with a nontargeting siRNA, siControl, indicating that high-efficiency transfection could be achieved; and (2) transfection of MCF7 with siControl did not reduce cellular viability more than 20%, compared to mock-transfected cells, indicating that these cells could be transfected without excessive nonspecific toxicity, which would reduce the sensitivity of the screen. The cell numbers plated for transfection and at the division of cells into replica plates after transfection and before 4OH tamoxifen/vehicle treatment were also titrated. This ensured efficient transfection and prevented cells reaching confluence after vehicle treatment, as this would have the potential to mask 4OH tamoxifen sensitivity. We used the Celltiter Glo (Promega) method of cell viability measurement, as opposed to other methods (MTT, MTS assay), as this reagent generated the most reproducible and sensitive measurements of difference in viability. Following optimization, the screen was repeated twice with internal duplication, and comparison of data from each screen revealed high reproducibility (Figure 1B) with the results approximating a normal distribution (Figure S1). Data from both screens were combined in the final analysis (Figure 1C). The 20 siRNAs causing the most significant resistance to 4OH tamoxifen are listed in Table S1.

Validation and Exclusion of Off-Target Effects

In addition to silencing specific transcripts, off-target effects of siRNAs can also occur (Iorns et al., 2007). To validate the specificity of the effects observed, the most potent tamoxifen resistance causing hits were reassayed using each of the four different siRNA species that comprise the SMARTpools. It is generally considered that observation of a phenotype caused by two distinct siRNA species indicates that it is unlikely to be the result of an off-target effect (Echeverri et al., 2006).

Of the four most potent resistance causing hits, three were judged likely to be on-target: *Cyclin-Dependent Kinase 10* (*CDK10*, Entrez GeneID 8558), *CDC2-related protein kinase 7* (*CRK7*, Entrez GeneID 51755), and *Mitogen-Activated Protein Kinase Kinase 7* (*MAP2K7*, Entrez GeneID 5609) (Figure 1D). *TTK1* was not re-examined because of the excessive toxicity associated with silencing of this gene in the absence of 4OH tamoxifen. We further investigated the mechanism of resistance to tamoxifen induced by CDK10 silencing.

CDK10 Silencing Causes Resistance to Tamoxifen and Estrogen Deprivation

CDK10 gene silencing by siRNA was confirmed by quantitative PCR and western blotting (Figures 2A and 2B and Figure S2A). The two CDK10-targeting siRNAs that caused the most significant effects on tamoxifen sensitivity were also shown to cause the most significant CDK10 silencing (Figure 2C). To confirm the validity of the results from the HTS, dose-response curves were performed (Figure 2D). CDK10 silencing significantly decreased sensitivity to tamoxifen (siControl SF_{60} = 28 nM, CDK10 SMARTpool SF_{60} = 488 nM; a 17-fold reduction in sensitivity). Further confirmation that CDK10 silencing causes tamoxifen resistance was provided by replicating the dose-response effect with an ONTARGETplus SMARTpool (Dharmacon) targeting CDK10, which also significantly decreased tamoxifen sensitivity (Figure 2E and Figure S2B). The siRNAs that comprise ONTARGETplus SMARTpools have been chemically modified to minimize off-target effects (Jackson et al., 2006). We also investigated whether the CDK10 effect on tamoxifen sensitivity could be trivially explained by an increase in cellular proliferation or by resistance to apoptosis. In fact, CDK10 silencing resulted in a small decrease in cell viability and did not cause resistance to apoptosis (Figures S3 and S4).

To confirm whether the CDK10 silencing effect was specific to tamoxifen or common to other forms of ERα antagonism, we analyzed the effect of CDK10 siRNA on sensitivity to estrogen deprivation, a model for aromatase inhibition (Santen et al., 2005). CDK10 silencing significantly decreased sensitivity to

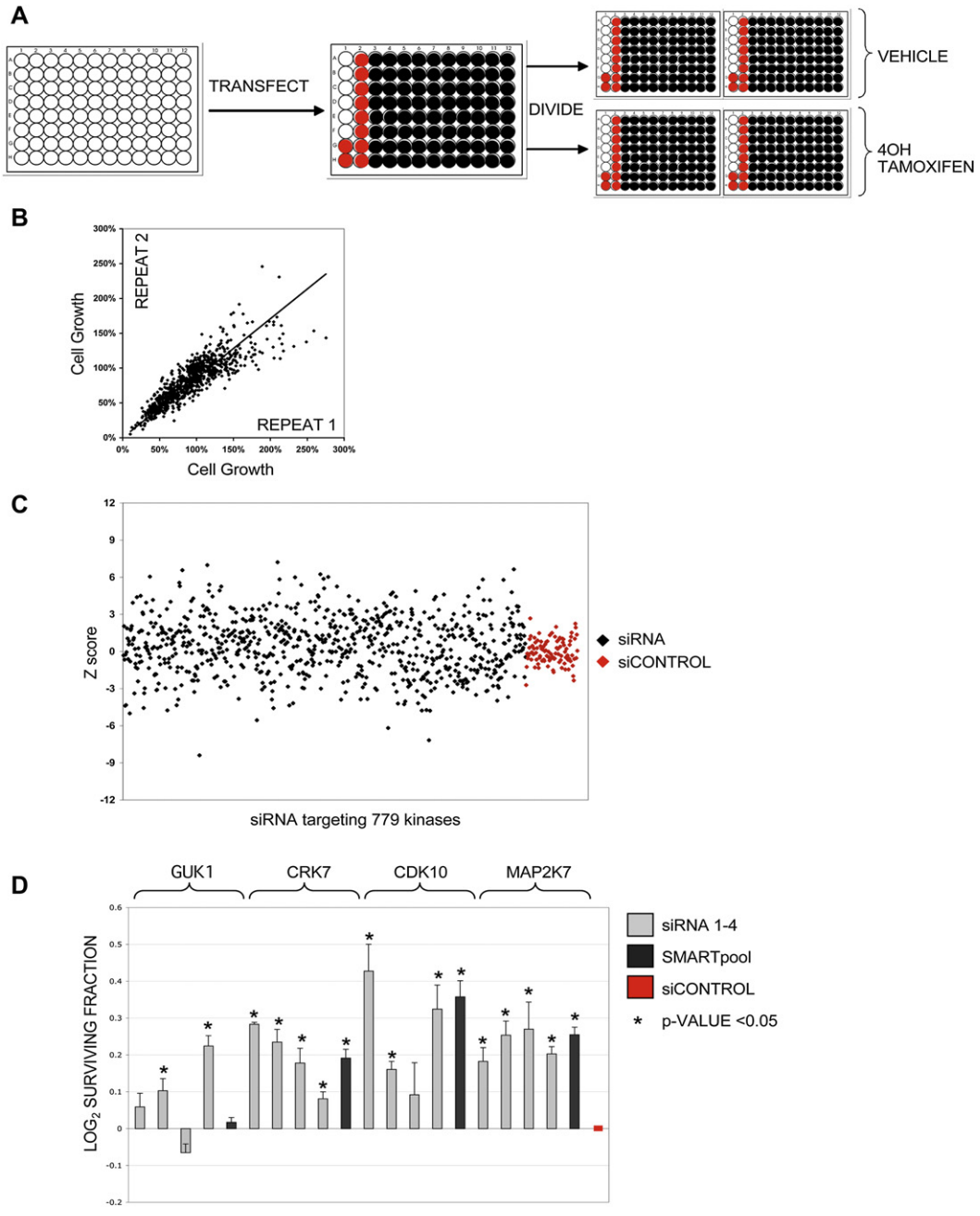

Figure 1. A Tamoxifen Resistance Screen with a Protein Kinase siRNA Library

(A) High-throughput screen (HTS) method. MCF7 cells plated in 96-well plates were transfected with siRNA. Each transfection plate contained 80 experimental siRNAs (SMARTpools of four different siRNA targeting the same gene), indicated in black, supplemented with ten wells of nontargeting siControl, indicated in red. Transfected cells were divided into four replica plates, half treated with ethanol vehicle alone and half with 4OH tamoxifen at 50 nM, the SF_{80} of MCF7. Cell viability was assessed after 7 days of 4OH tamoxifen exposure using CellTiter-Glo Luminescent Cell Viability Assay (Promega).

(B) Reproducibility of HTS method. Correlation of the effect of siRNA on cell growth in vehicle-treated plates from two replicates of the entire screen. Spearman correlation coefficient, $r^2 = 0.71$.

(C) Scatter plot of averaged Z scores from tamoxifen resistance screen carried out in duplicate. Black diamonds, siRNA SMARTpools targeting 779 protein kinase genes, Red diamonds, siControl.

(D) Validation of resistance hits from the tamoxifen HTS. Tamoxifen sensitivity assay repeated in triplicate with the four different siRNAs originally in each SMARTpool and the SMARTpool, all targeting the same kinase. Surviving fractions following tamoxifen treatment are shown, including those after transfection with siControl (Red). CRK7 and MAP2K7 revalidate with all four siRNAs; CDK10 revalidates with three siRNAs. *$p < 0.05$ compared to siControl (Student's t test). Error bars represent the standard error of the mean (SEM).

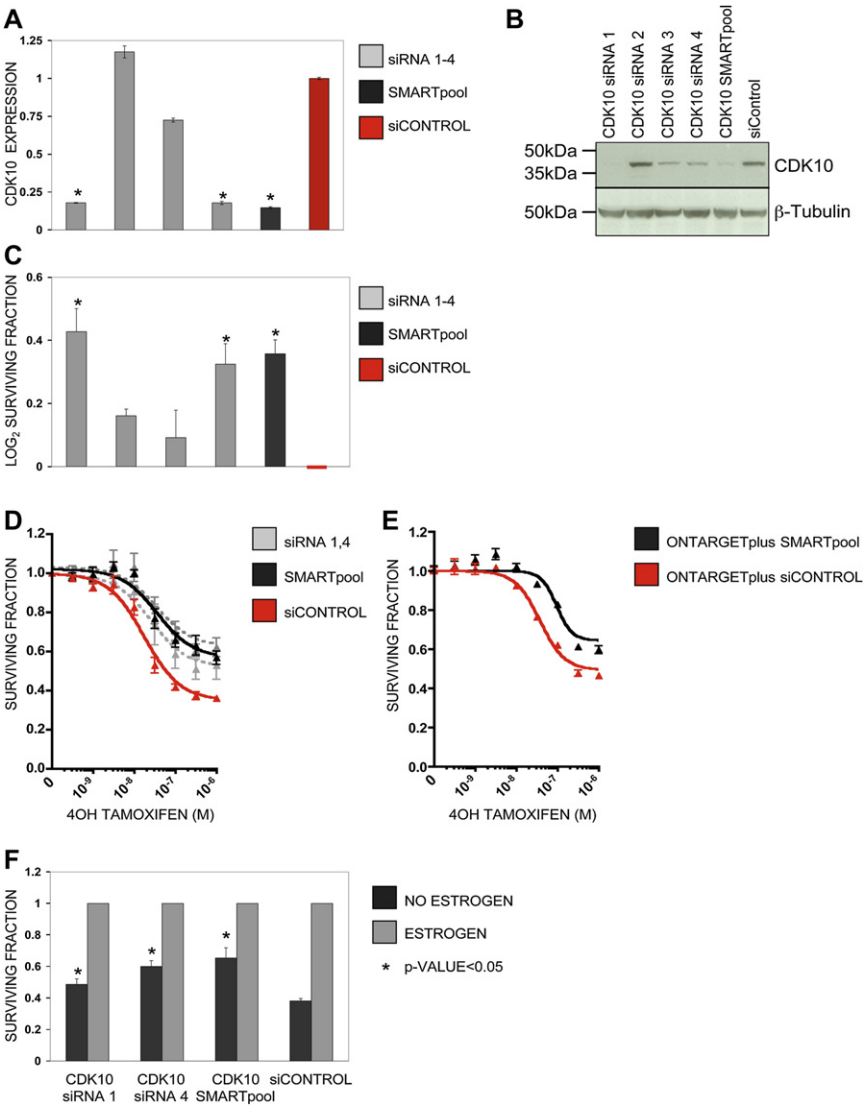

Figure 2. CDK10 Silencing Decreases Sensitivity to Tamoxifen and Estrogen Deprivation

(A) CDK10 mRNA levels quantified by qPCR following transfection of siRNA into MCF7 cells. CDK10 expression is shown relative to expression in siControl-transfected cells. Individual siRNAs 1 and 4 and the CDK10 SMARTpool caused the most significant reduction in CDK10 mRNA expression. *$p < 0.01$ compared to siControl-transfected cells (Student's t test). Error bars represent the SEM.

(B) Western blot analysis of lysates prepared from MCF7 cells transfected with pReceiver CDK10 HA and siRNA. Antibodies recognizing CDK10 were used with β-tubulin as a loading control. Individual siRNAs 1, 3, and 4 and the CDK10 SMARTpool significantly reduced CDK10 protein expression compared to siControl-transfected cells.

(C) Surviving fractions following 4OH tamoxifen treatment with the four individual siRNAs and the SMARTpool targeting CDK10, and siControl. Individual siRNAs 1 and 4 and the CDK10 SMARTpool most significantly reduced tamoxifen sensitivity. *$p < 0.01$ compared to siControl-transfected cells (Student's t test). Error bars represent the SEM.

(D) Cell viability assay in MCF7 cells transfected with CDK10 SMARTpool, individual CDK10 siRNAs 1 and 4, or siControl and treated with 4OH tamoxifen. CDK10-silenced cells have significantly decreased sensitivity to 4OH tamoxifen. Error bars represent the SEM.

(E) Cell viability assay in MCF7 cells transfected with CDK10 ONTARGETplus SMARTpool, or ONTARGETplus siControl and treated with 4OH tamoxifen. CDK10-silenced cells have significantly decreased sensitivity to 4OH tamoxifen. Error bars represent the SEM.

(F) Cell viability assay in MCF7 cells transfected with CDK10 SMARTpool, individual CDK10 siRNAs 1 and 4, or siControl and deprived of estradiol. CDK10 silenced cells have significantly decreased sensitivity to estrogen deprivation. *$p < 0.05$ compared to siControl (Student's t test). Error bars represent the SEM.

estrogen deprivation (Figure 2F), indicating that suppression of CDK10 causes resistance to inhibition of ERα signaling generally, rather than tamoxifen specifically.

A Reduction in CDK10 Expression Circumvents G_1 Cell Cycle Arrest in Cells Deprived of Estrogen Signaling

The antiproliferative effect of antiestrogens results in the induction of cell cycle arrest at the G_1 checkpoint, as characterized by an increase in cells in the G_1 phase of the cycle and a consequential decrease in the S phase proportion (Wilcken et al., 1997). We explored the possibility that a reduction in CDK10 expression circumvents this tamoxifen-induced cell cycle arrest. siControl-transfected cells treated with tamoxifen exhibited the expected increase in G_1 and decrease in S phase populations (Figures 3A and 3B and Table S2). In contrast, tamoxifen-treated, CDK10-silenced cells showed significantly reduced G_1 increase, and a significantly smaller decrease in the number of S phase cells (Figures 3A and 3B and Table S2), indicative of a decreased tamoxifen-induced G_1 arrest.

CYCLIN D1 expression actively drives transit through the G_1 checkpoint. CYCLIN D1 binds to and activates CDK4/6, which phosphorylate and inactivate the Retinoblastoma 1 protein (RB1), allowing progression through the restriction point within G_1 (Sherr, 1996). Overexpression of CYCLIN D1 has also been shown to cause entry into S phase of cells previously arrested at the G_1 checkpoint, reversing the growth inhibitory effects of antiestrogens (Wilcken et al., 1997). Both mRNA and protein levels of CYCLIN D1 were significantly elevated in CDK10-silenced cells (Figures 3C and 3D), and in addition, the levels of phosphorylated RB1 were also significantly elevated (Figure 3E). These results were consistent with the hypothesis that G_1 cell cycle arrest in tamoxifen-treated cells is circumvented by a reduction in CDK10 expression, resulting in decreased sensitivity to tamoxifen. This effect appears to be mediated by an increase in CYCLIN D1 expression and subsequent phosphorylation of RB1.

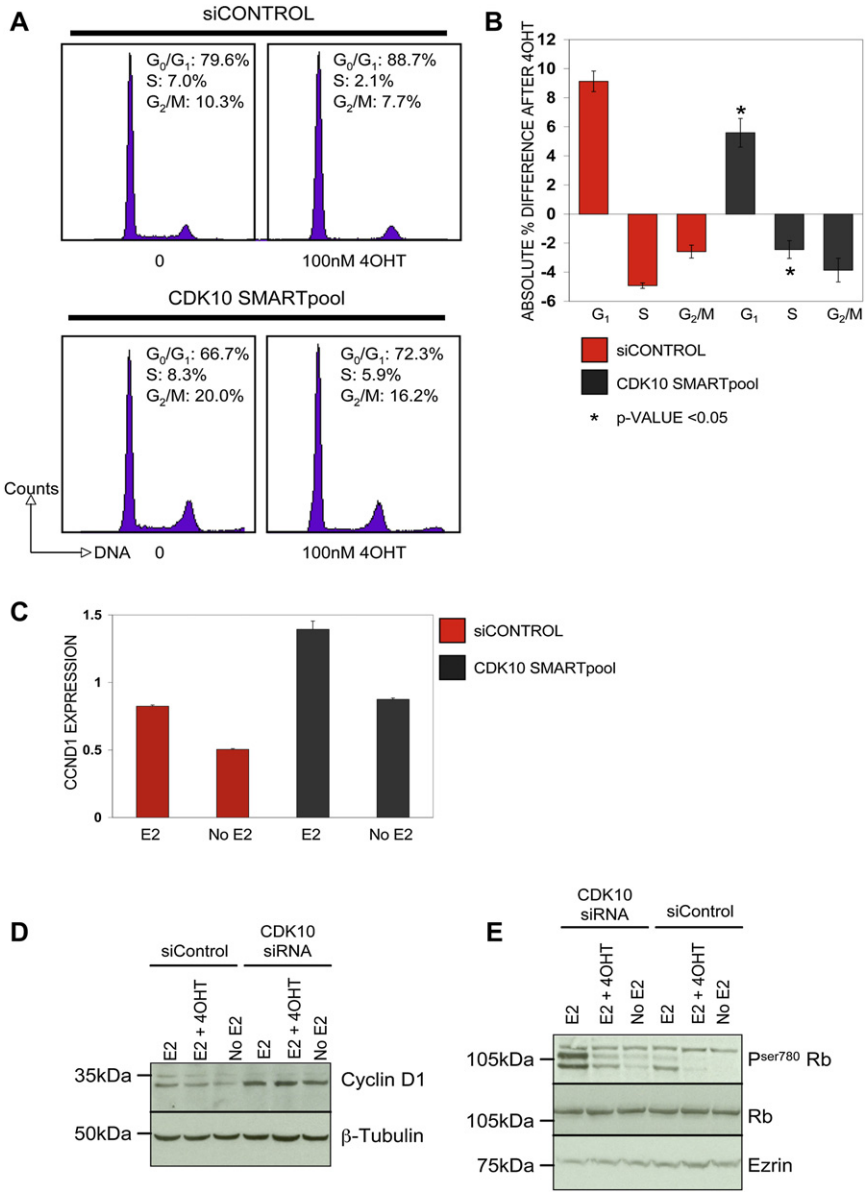

Figure 3. CDK10 Silencing Reduces Tamoxifen-Induced G_1 Arrest

(A) Resistance to tamoxifen in CDK10-silenced cells is characterized by decreased tamoxifen-induced G_1 arrest. Cells were transfected with either CDK10 SMARTpool or siControl. Cell cycle profiles were assessed by propidium iodide (PI) staining and fluorescence-activated cell scanning (FACS) from cell aliquots taken both before and after treatment with 4OH tamoxifen.

(B) Absolute differences were calculated by subtracting the percentage of cells in each phase of the cell cycle before 4OH tamoxifen treatment from the percentage of cells in each phase after 4OH tamoxifen treatment. Significantly smaller differences in G_1 and S phases after 4OH tamoxifen treatment were observed in CDK10-silenced cells, indicating decreased tamoxifen-induced G_1 arrest. *$p < 0.05$ compared to siControl (Student's t test). Error bars represent the SEM.

(C) CDK10 SMARTpool-transfected cells have significantly higher *CCND1* expression than siControl-transfected cells. qPCR on cDNA prepared from MCF7 cells transfected with siRNA for 48 hr followed by treatment with 1 nM estradiol, or left untreated for 24 hr. For CDK10 SMARTpool-transfected cells, $p < 0.05$ compared to matched siControl-transfected cells (Student's t test). Error bars represent the SEM.

(D) CDK10 SMARTpool-transfected cells have significantly higher expression of CYCLIN D1 protein than siControl-transfected cells. MCF7 cells transfected 48 hr earlier with siRNA were treated with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated. Lysates were made 24 hr following treatment. Antibodies recognizing CYCLIN D1 were used with β-tubulin as a loading control.

(E) CDK10 SMARTpool-transfected cells have significantly higher levels of phosphorylated RB1 than siControl-transfected cells. MCF7 cells transfected 48 hr earlier with siRNA were treated with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated. Lysates were made 24 hr following treatment. Antibodies recognizing phospho RB1 and total RB1 were used with Ezrin as a loading control.

Tamoxifen Resistance Is Not Caused by Ligand-Independent ERα Activation in CDK10-Silenced Cells

ERα stimulation causes cellular proliferation by increasing *CYCLIN D1* (*CCND1*) transcription (Altucci et al., 1996). A possible mechanism of tamoxifen resistance is ligand-independent ERα-induced transcription, resulting in continued ERα signaling in the presence of tamoxifen (Shou et al., 2004). We explored the possibility that the increase in *CCND1* observed in CDK10-silenced cells was due to ligand-independent ERα activation as indicated by ERα phosphorylation. There were no significant changes in either the expression or activation of ERα (Figure 4A). If ligand-independent activation of ERα was occurring, increases in the expression of other ERα regulated genes would also be expected. We showed that levels of the ERα target gene, the progesterone receptor (PR), were unaffected following CDK10 silencing (Figure 4B). We also assessed the expression of TFF1 (pS2), the product of one of the best-characterized ERα-regulated genes. Transcription of *TFF1* is tightly controlled by the binding of ERα to an ERE within the *TFF1* promoter (Jakowlew et al., 1984). There was no increase in TFF1 expression after CDK10 silencing, rather a reduction in TFF1 expression was observed (Figure 4C). These results suggested that ligand-independent activation of ERα signaling was unlikely to be the cause of tamoxifen resistance in CDK10-silenced cells. To further confirm that ERα was unlikely to be involved in resistance to tamoxifen in CDK10-silenced cells, we examined the effect of CDK10 silencing on sensitivity to ICI 182780, an agent that induces ERα degradation (Jones, 2002). CDK10 silencing significantly decreased sensitivity to ICI 182780 (siControl SF_{60} = 4 nM, CDK10 SMARTpool SF_{60} = 32 nM; an 8-fold reduction in sensitivity) (Figure 4D), further supporting the hypothesis that CDK10 silencing does not cause tamoxifen resistance by promoting ligand-independent ERα signaling.

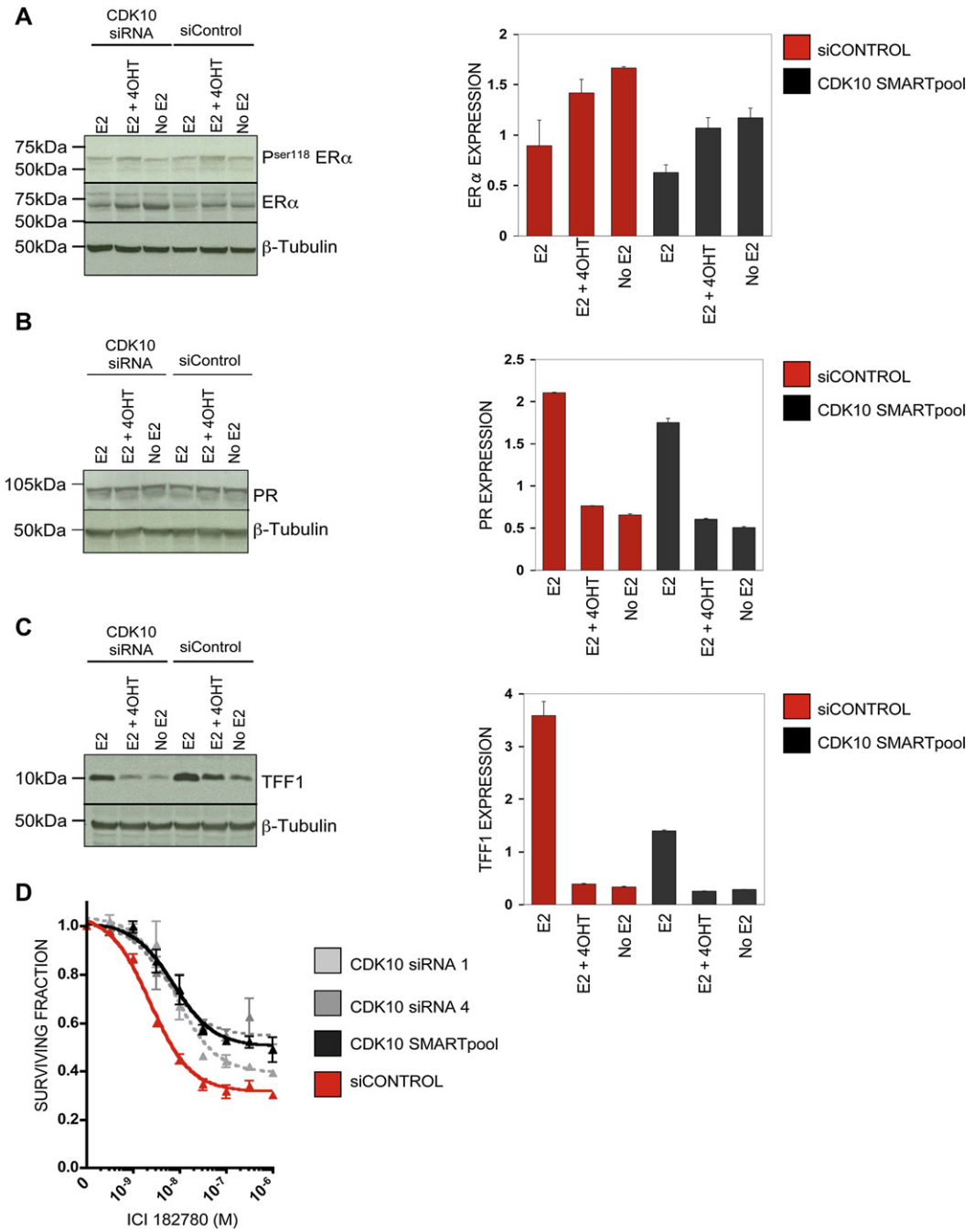

Figure 4. CDK10 Silencing Does Not Cause Ligand-Independent ERα Activation

(A) CDK10 SMARTpool-transfected cells do not have altered activation or expression of ERα compared with siControl-transfected cells. Left panel: MCF7 cells transfected 48 hr earlier with siRNA were treated with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated. Lysates were made 24 hr following treatment. Antibodies recognizing phosphoserine 118 and total ERα were used with β-tubulin as a loading control. Right panel: qPCR on cDNA prepared from MCF7 cells transfected with siRNA for 48 hr followed by treatment with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated for 24 hr. Error bars represent the SEM.

(B) CDK10 SMARTpool-transfected cells do not have increased expression of the ERα-regulated gene *PR* compared with siControl-transfected cells. Left panel: MCF7 cells transfected 48 hr earlier with siRNA were treated with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated. Lysates were made 24 hr following treatment. An antibody recognizing PR was used with β-tubulin as a loading control. Right panel: qPCR on cDNA prepared from MCF7 cells transfected with siRNA for 48 hr followed by treatment with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated for 24 hr. Error bars represent the SEM.

(C) CDK10 SMARTpool-transfected cells have significantly lower expression of ERα-regulated gene *TFF1* compared with siControl-transfected cells. Left panel: MCF7 cells transfected 48 hr earlier with siRNA were treated with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated. Lysates were

CDK10 Suppression Activates the p42/p44 MAPK Pathway

The reduction observed in expression of TFF1 (Figure 4C) supported the hypothesis that tamoxifen resistance occurs through activation of growth factor signaling pathways that circumvent tumor reliance on ERα signaling. Previous studies have shown that transfection of constitutively active MEK1 or c-RAF into MCF7 cells, which results in hyperactivation of p42/p44 MAPK, caused loss of ERα-mediated gene expression, characterized by TFF1 suppression and acquisition of antiestrogen resistance (El-Ashry et al., 1997; Oh et al., 2001). In addition, activation of p42/p44 MAPK has been shown to cause an increase in CYCLIN D1 expression (Lavoie et al., 1996). Therefore, we examined whether the p42/p44 MAPK pathway was activated in CDK10-silenced cells as measured by the phosphorylation of p42/p44 MAPK and MEK1,2. This indicated that phosphorylation of these MAPK pathway components was enhanced following CDK10 silencing (Figures 5A and 5B). To determine whether this activation was the cause of tamoxifen resistance in cells with reduced CDK10 expression, p42/p44 MAPK and MEK1,2 were silenced by siRNA in conjunction with suppression of CDK10 expression. Sensitivity to tamoxifen in this situation was restored (Figures 5C and 5D), suggesting that a reduction of CDK10 expression causes tamoxifen resistance by activation of the p42/p44 MAPK signaling pathway, circumventing the reliance upon ERα signaling.

CDK10 Silencing Increases c-RAF Expression

To determine the mechanism by which CDK10 silencing modifies p42/p44 MAPK signaling, the activation of the upstream pathway components c-RAF and Ras was examined. Phosphorylation of c-RAF was significantly increased in CDK10-silenced cells, and levels of total c-RAF protein were also increased (Figure 6A). *c-RAF* mRNA levels were also significantly increased in CDK10-silenced cells (Figure 6B). To determine whether this increase in expression was the cause of tamoxifen resistance in cells with reduced CDK10 expression, c-RAF expression was silenced by siRNA in conjunction with suppression of CDK10 expression. Sensitivity to tamoxifen in this situation was partially restored (Figure 6C and Figure S2C), suggesting that the effects of CDK10 silencing were c-RAF dependent. Ras was not activated by CDK10 silencing (Figure 6D). Therefore, activation of the MAPK pathway seemed likely to be mediated by c-RAF levels, caused by the increased transcription of *c-RAF*. To confirm that overexpression of c-RAF was sufficient to increase the levels of activated c-RAF and to activate downstream components of the MAPK pathway, wild-type c-RAF was overexpressed in MCF7 cells. Overexpression of wild-type c-RAF also resulted in increased levels of phosphorylated c-RAF (Figure 6E) and caused increased levels of phosphorylated p42/p44 MAPK (Figure 6F) suggesting that the increased MAPK activity that characterizes tamoxifen resistance in CDK10-silenced cells was driven by elevated c-RAF expression.

Increased *c-RAF* Transcription Is Mediated by ETS2

The ETS2 transcription factor has previously been shown to interact with CDK10. ETS2 transactivation is repressed by CDK10 binding (Kasten and Giordano, 2001), therefore it is possible that aberrant ETS2 activity could drive the increased expression of *c-RAF* in CDK10-silenced cells. To determine whether the increase in *c-RAF* transcription observed in CDK10-silenced cells was dependent on ETS2, ETS2 was silenced in combination with CDK10, and the levels of *c-RAF* mRNA were measured using quantitative PCR. *c-RAF* mRNA levels were decreased when ETS2 was silenced in combination with CDK10 (Figure 7A). c-RAF protein levels were also decreased when ETS2 was silenced in combination with CDK10, as were the levels of phosphorylated p42/p44 MAPK (Figure 7B). To determine whether the decrease in tamoxifen sensitivity observed in cells with reduced CDK10 expression was dependent on ETS2, ETS2 expression was silenced by siRNA in conjunction with suppression of CDK10 expression. Sensitivity to tamoxifen in this context was partially restored (Figure 7C and Figure S2D), suggesting that a reduction of CDK10 expression caused tamoxifen resistance by relieving the inhibition of ETS2 transactivation of *c-RAF*. We confirmed that CDK10 binds to ETS2 using coimmunoprecipitation (Co-IP) (Figure 7D and Figure S5) and identified a putative ETS2-binding site in the *c-RAF* promoter (Figure 7E). We demonstrated using chromatin IP (ChIP) that ETS2 and CDK10 bind to this site (Figure 7F) and showed that silencing of CDK10 results in increased binding of ETS2 to the ETS2 binding site in the *c-RAF* promoter (Figure 7G). These results suggest that CDK10 silencing relieves repression of ETS2 transactivation of the *c-RAF* promoter, resulting in increased recruitment of ETS2 to the *c-RAF* promoter and increased expression of *c-RAF.*

Low CDK10 Expression Is Associated with Clinical Resistance to Tamoxifen

To assess the clinical significance of our observations, *CDK10* expression levels were analyzed in 87 ERα-positive breast tumors from patients treated with adjuvant tamoxifen (Loi et al., 2007). Low *CDK10* expression was associated with a statistically significantly shorter time to distant relapse of disease ($p = 0.0205$, Figure 8A), and there was a trend to shorter overall survival ($p = 0.0793$, Figure 8B), suggesting that reduced *CDK10* expression is associated with clinical resistance to tamoxifen in patients, consistent with our in vitro studies. Low *CDK10* expression was not associated with well-established prognostic factors, including age, tumor size, grade, or node positivity (Table S3). To confirm these findings, *CDK10* expression was measured using quantitative PCR in a second independent set of ERα-positive breast tumors from patients treated with adjuvant tamoxifen. Consistent with the data presented above, low *CDK10* expression was strongly associated with a statistically significantly shorter time to disease progression ($p < 0.0001$, Figure 8C) and significantly shorter overall survival ($p < 0.0001$, Figure 8D), further suggesting that reduced *CDK10* expression

made 24 hr following treatment. An antibody recognizing TFF1 was used with β-tubulin as a loading control. Right panel: qPCR on cDNA prepared from MCF7 cells transfected with siRNA for 48 hr followed by treatment with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated for 24 hr. For CDK10 SMARTpool-transfected cells treated with E2, $p < 0.05$ compared to matched siControl-transfected cells (Student's t test). Error bars represent the SEM. (D) CDK10 silencing decreases sensitivity to ICI 182780. Cell viability assay in MCF7 cells transfected with CDK10 SMARTpool, individual CDK10 siRNAs 1 and 4, or siControl and treated with ICI 182780. CDK10-silenced cells have significantly decreased sensitivity to ICI 182780. Error bars represent the SEM.

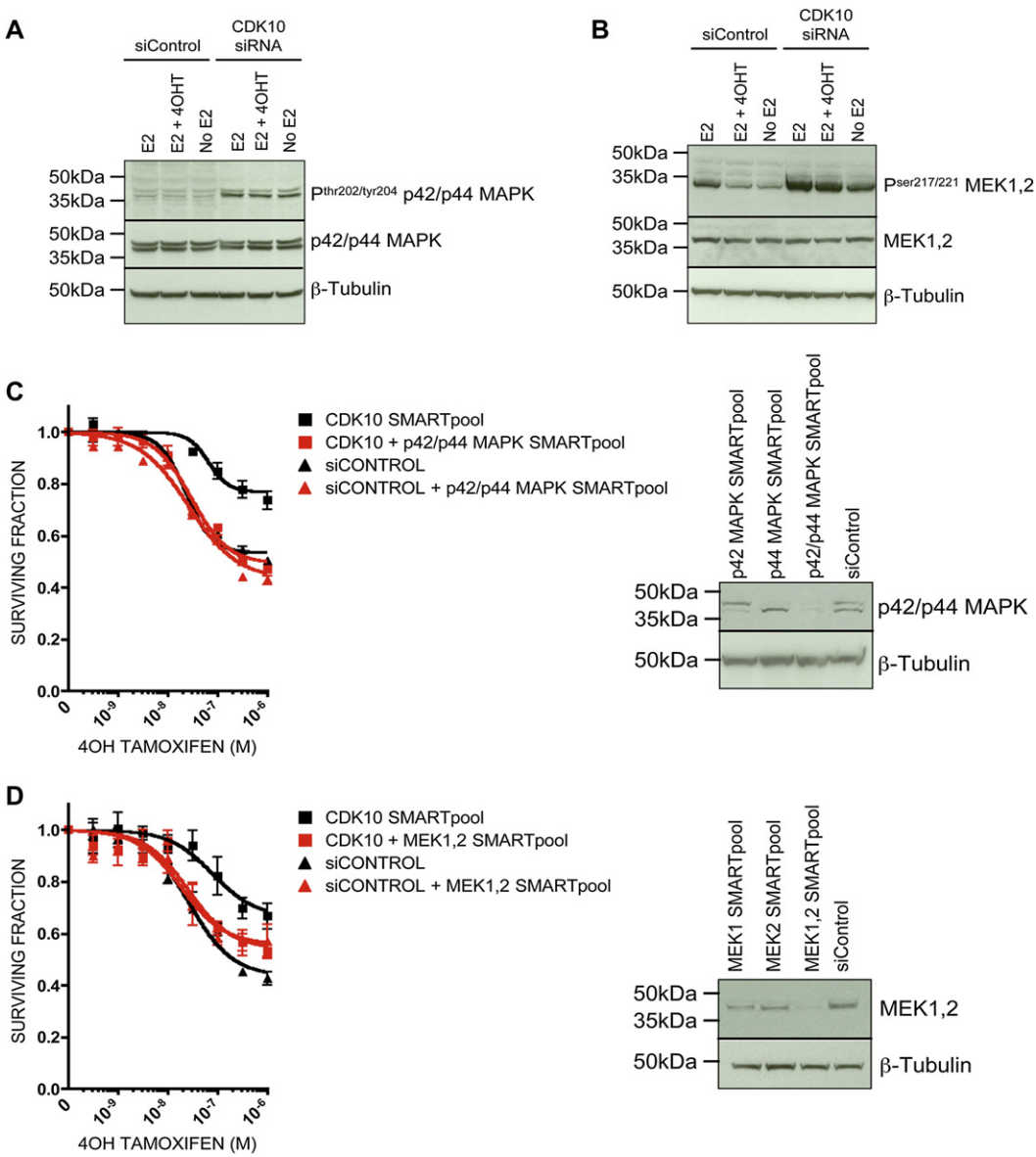

Figure 5. CDK10 Silencing Activates the p42/p44 MAPK Pathway, Causing Resistance to Tamoxifen

(A) Increased levels of phosphorylated p42/p44 MAPK in CDK10 silenced cells. MCF7 cells transfected 48 hr earlier with siRNA were treated with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated. Lysates were made 24 hr following treatment. Antibodies recognizing phospho and total p42/p44 MAPK were used with β-tubulin as a loading control.

(B) Increased levels of phosphorylated MEK1,2 in CDK10-silenced cells. MCF7 cells transfected 48 hr earlier with siRNA were treated with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated. Lysates were made 24 hr following treatment. Antibodies recognizing phospho and total MEK1,2 were used with β-tubulin as a loading control.

(C) Silencing of p42/p44 MAPK restores 4OH tamoxifen sensitivity to CDK10-silenced cells. Left panel: Cell viability assay in MCF7 cells transfected with CDK10 SMARTpool or siControl alone or in combination with p42/p44 MAPK SMARTpool siRNAs and treated with 4OH tamoxifen. Error bars represent the SEM. Right panel: Western blot analysis of lysates prepared from MCF7 cells transfected with siRNA. An antibody recognizing p42/p44 MAPK was used with β-tubulin as a loading control. The p42/p44 MAPK SMARTpools significantly reduced p42/p44 MAPK protein expression compared to siControl-transfected cells.

(D) Silencing of MEK1,2 restores 4OH tamoxifen sensitivity to CDK10-silenced cells. Left panel: Cell viability assay in MCF7 cells transfected with CDK10 SMARTpool or siControl alone or in combination with MEK1,2 SMARTpool siRNAs and treated with 4OH tamoxifen. Error bars represent the SEM. Right panel: Western blot analysis of lysates prepared from MCF7 cells transfected with siRNA. An antibody recognizing MEK1,2 was used with β-tubulin as a loading control. The MEK1,2 SMARTpools significantly reduced MEK1,2 protein expression compared to siControl-transfected cells.

is associated with clinical resistance to tamoxifen. As with the previous data set, low *CDK10* expression was not associated with well-established prognostic factors, including age, tumor size, grade, or node positivity (Table S4). In addition, low *CDK10* expression was not associated with the expression of the biomarkers HER2, MIB1, p53, ERα, or PR (Figures S6A–S6F).

Promoter methylation is a common mechanism of transcriptional repression. The methylation status of the CpG island in

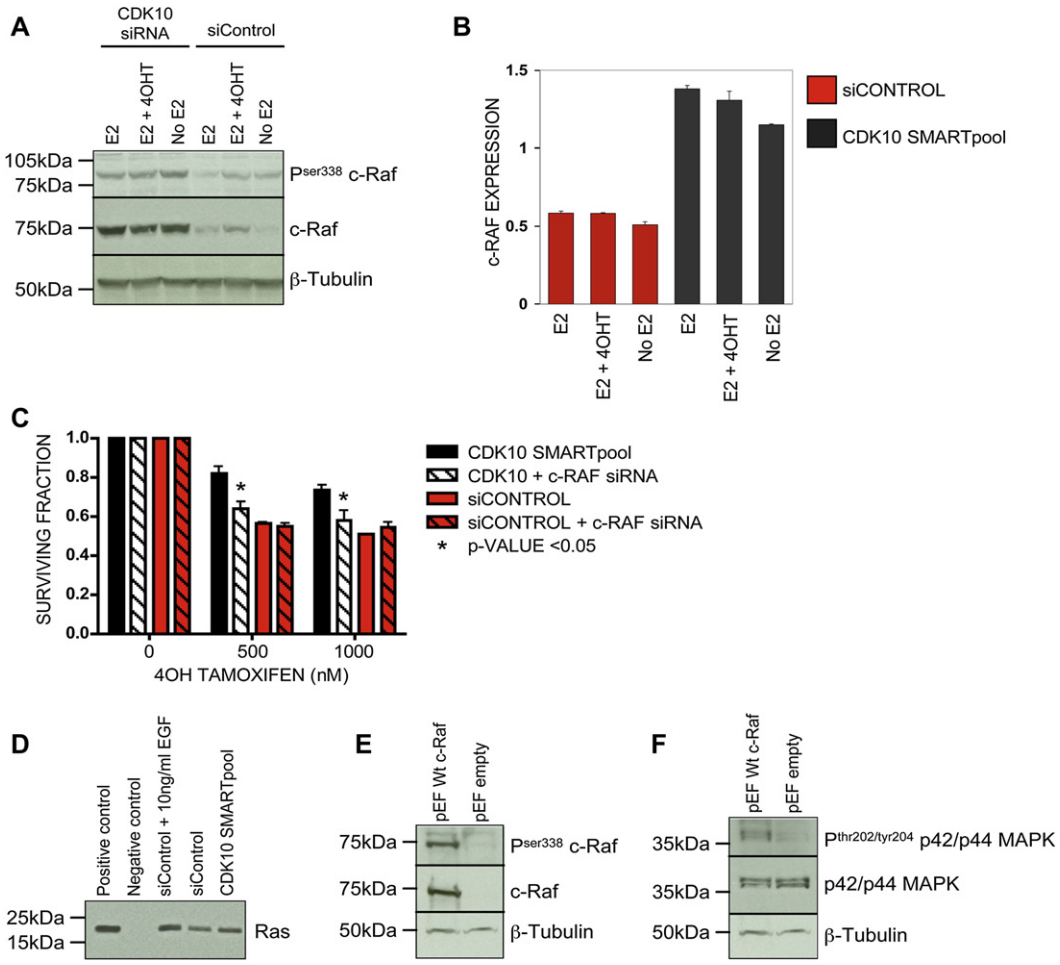

Figure 6. Activation of the p42/p44 MAPK Pathway in CDK10 siRNA-Transfected Cells Occurs from Overexpression of c-RAF

(A) Increased levels of phosphorylated and total c-RAF in CDK10-silenced cells. MCF7 cells transfected 48 hr earlier with siRNA were treated with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated. Lysates were made 24 hr following treatment. Antibodies recognizing phospho and total c-RAF were used with β-tubulin as a loading control.

(B) Significantly higher expression of *c-RAF* in CDK10-silenced cells. qPCR on cDNA prepared from MCF7 cells transfected with siRNA for 48 hr followed by treatment with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated for 24 hr. For CDK10 SMARTpool-transfected cells, $p < 0.05$ compared to matched siControl-transfected cells (Student's t test). Error bars represent the SEM.

(C) Silencing of c-RAF restores 4OH tamoxifen sensitivity to CDK10-silenced cells. Cell viability assay in MCF7 cells transfected with CDK10 SMARTpool or siControl alone or in combination with c-RAF siRNA and treated with 4OH tamoxifen. *$p < 0.05$ compared to matched CDK10 siRNA sample (Student's t test). Error bars represent the SEM.

(D) CDK10-silenced cells do not have significantly higher levels of activated Ras. Ras activation assay on lysates prepared from MCF7-transfected with siRNA for 72 hr.

(E) Overexpression of wild-type c-RAF increases the levels of phosphorylated c-RAF. MCF7 cells were transfected with pEF wild-type c-RAF or pEF empty vector control, and lysates were made 24 hr later. Antibodies recognizing phospho and total c-RAF were used with β-tubulin as a loading control.

(F) Overexpression of wild-type c-RAF increases the levels of phosphorylated p42/p44 MAPK. MCF7 cells were transfected with pEF wild-type c-RAF or pEF empty vector control, and lysates were made 24 hr later. Antibodies recognizing phospho and total p42/p44 MAPK were used with β-tubulin as a loading control.

the *CDK10* promoter was assessed in genomic DNA extracted from biopsies taken from the second set of breast tumors, using methylation-specific PCR (MSP). Seven of thirty-eight (18%) cases had methylation of the *CDK10* promoter, and *CDK10* promoter methylation was strongly associated with low *CDK10* expression (Figure 8E). Methylation of the *CDK10* promoter was also associated with a statistically significantly shorter time to disease progression ($p < 0.0001$, Figure S7A) and significantly shorter overall survival ($p < 0.0001$, Figure S7B). This suggests a mechanism by which tumors develop low CDK10 expression levels resulting in tamoxifen resistance.

DISCUSSION

Resistance to endocrine therapies is one of the major limiting factors in the successful treatment of breast cancer, and strategies to enhance the utility of these agents would be of significant clinical benefit. Intracellular signaling pathways and their effects on sensitivity to endocrine therapies have been the subject of considerable study for some time. While much of this work has been highly informative, it has relied on the study of proteins whose function is consistent with the existing understanding of intracellular signaling. As a complement to these

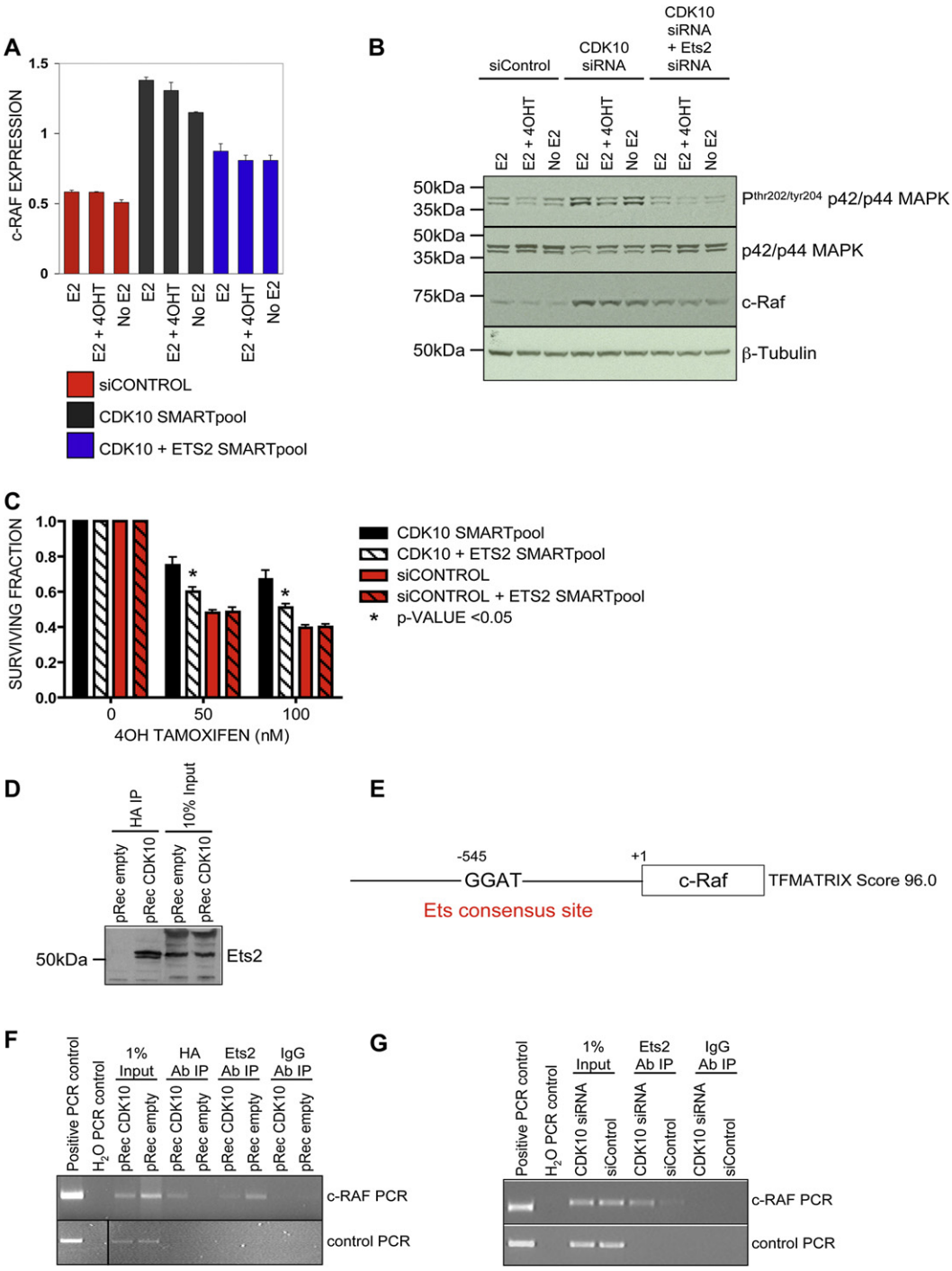

Figure 7. Increased *c-RAF* Expression in CDK10-Silenced Cells Is Regulated by ETS2 Transcription Factor

(A) Expression of *c-RAF* in CDK10-silenced cells can be partially restored to normal levels by silencing of ETS2. MCF7 cells were transfected with CDK10 SMARTpool alone or in combination with ETS2 SMARTpool siRNA or siControl and 48 hr later treated with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated. RNA was made 24 hr following treatment and used for qPCR. The levels of *c-RAF* mRNA were significantly decreased when ETS2 was silenced in combination with CDK10. For siControl- and CDK10 + ETS2 SMARTpool-transfected cells, $p < 0.05$ compared to matched CDK10 SMARTpool-transfected cells (Student's t test). Error bars represent the SEM.

(B) Levels of phosphorylated p42/p44 MAPK and c-RAF protein can be partially restored to normal levels by silencing of ETS2. MCF7 cells transfected 48 hr earlier with siRNA were treated with 1 nM estradiol or 1 nM estradiol plus 100 nM 4OH tamoxifen, or left untreated. Lysates were made 24 hr following treatment. Antibodies recognizing phospho and total p42/p44 MAPK and total c-RAF were used with β-tubulin as a loading control.

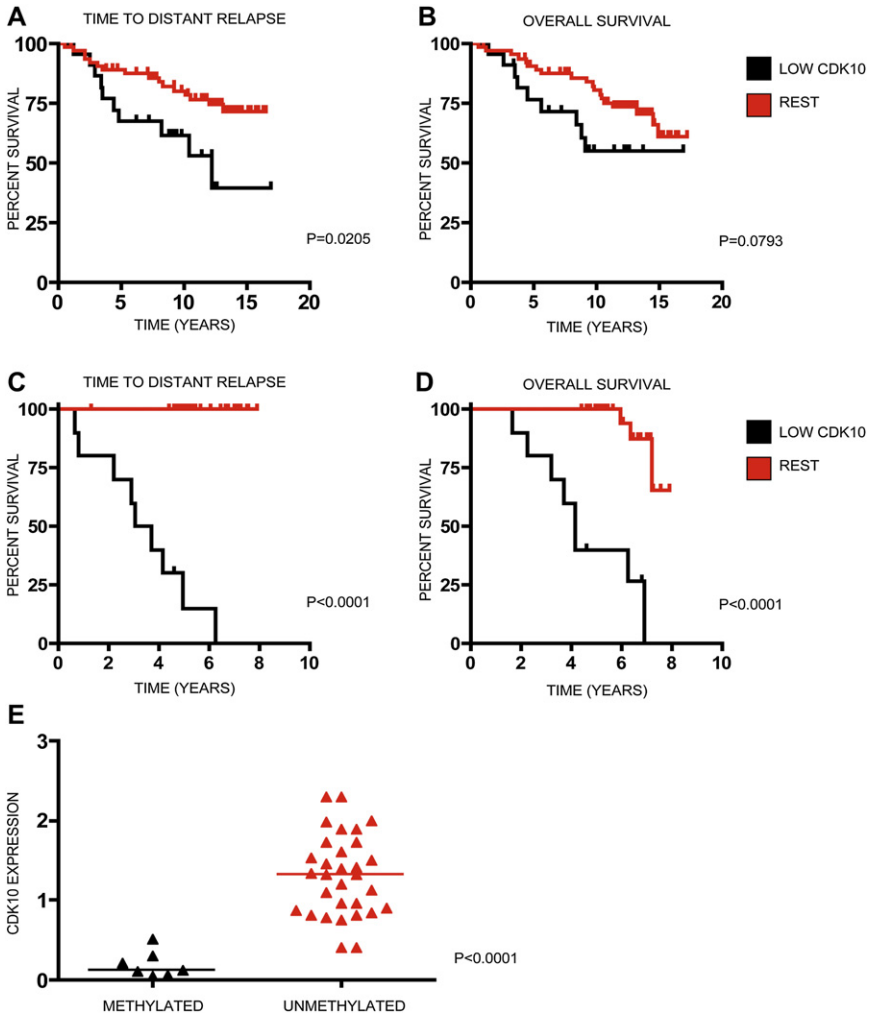

Figure 8. Low CDK10 Expression Is Associated with Significantly Poorer Survival in Patients Treated with Tamoxifen

(A) Kaplan-Meier survival curves of breast cancer patients from data set 1, n = 87, with tumors that have low CDK10 expression (defined as lowest quartile expression) were at significantly higher risk of distant relapse, hazard ratio 2.447, p = 0.0205 (log-rank test) when treated with adjuvant tamoxifen. CDK10 expression was analyzed using microarray analysis from a published study (Loi et al., 2007).

(B) Kaplan-Meier survival curves of breast cancer patients from data set 1, n = 87, with tumors that have low CDK10 expression had a trend of reduced overall survival, hazard ratio 1.992, p = 0.0793 (log-rank test) when treated with adjuvant tamoxifen. This is not statistically significant but shows a trend to significance. CDK10 expression was analyzed using microarray analysis from a published study (Loi et al., 2007).

(C) Kaplan-Meier survival curves of breast cancer patients from data set 2, n = 38, with tumors that have low CDK10 expression (defined as lowest quartile expression) were at significantly higher risk of distant relapse (hazard ratio not definable), p < 0.0001 (log-rank test) when treated with adjuvant tamoxifen. CDK10 expression was measured using quantitative PCR.

(D) Kaplan-Meier survival curves of breast cancer patients from data set 2, n = 38, with tumors that have low CDK10 expression have significantly reduced overall survival, hazard ratio 13.56, p < 0.0001 (log-rank test) when treated with adjuvant tamoxifen. CDK10 expression was measured using quantitative PCR.

(E) Methylation status of the *CDK10* promoter in data set 2 was significantly associated with expression of CDK10. Median CDK10 expression in methylated samples = 0.13, median CDK10 expression in unmethylated samples = 1.32, p < 0.0001 (Mann Whitney test).

candidate-based approaches, we identified determinants of response to tamoxifen using an unbiased loss-of-function screen. The identification of CDK10 demonstrated the utility of this approach, as this protein had not previously been implicated in resistance to endocrine agents. Furthermore, delineation of the mechanism by which CDK10 is likely to determine tamoxifen sensitivity, via ETS2 transactivation of the *c-RAF* gene, suggests a role for this protein in regulating activation of the MAPK pathway. Finally, the correlations between CDK10 expression, methylation, and clinical outcome suggest that such unbiased approaches can identify determinants that have clear clinical significance.

Integrating Complementary Functional Genomic and Clinical Approaches

One approach to the identification of determinants of endocrine therapy has been to use gene expression profiling, both on an individual gene and a genome-wide basis (Jansen et al., 2005). This approach, especially when carried out in a high-throughput

(C) Silencing of ETS2 restores 4OH tamoxifen sensitivity to CDK10-silenced cells. Cell viability assay in MCF7 cells transfected with CDK10 SMARTpool or siControl alone or in combination with ETS2 SMARTpool siRNA and treated with 4OH tamoxifen. * p < 0.05 compared to CDK10 siRNA (Student's t test). Error bars represent the SEM.

(D) CDK10 binds to ETS2. MCF7 cells were transfected with pReceiver CDK10 HA or the empty vector control pReceiver empty HA. Lysates were made 24 hr later, and CDK10 was immunoprecipitated with HA-conjugated beads. ETS2 binding was detected by western blotting of the immunoprecipitated lysate.

(E) Diagram illustrating an ETS2 consensus-binding sequence identified in the *c-RAF* promoter using TFMATRIX (Score 96.0).

(F) CDK10 and ETS2 bind to the *c-RAF* promoter at the ETS2-binding site. MCF7 cells were transfected with pReceiver CDK10 HA or empty vector control pReceiver HA for 24 hr and subsequently subjected to ChIP with the indicated antibodies. Shown are agarose gels with PCR products for the ETS2-binding site on the *c-RAF* promoter and PCR products for a control region.

(G) ETS2 binding to the *c-RAF* promoter at the ETS2-binding site is increased in CDK10-silenced cells. MCF7 cells were transfected with CDK10 SMARTpool siRNA or siControl for 48 hr and subsequently subjected to ChIP with the indicated antibodies. Shown are agarose gels with PCR products for the ETS2-binding site on the *c-RAF* promoter and PCR products for a control region.

fashion and with large cohorts, can be powerful in demonstrating correlations between transcript levels and clinical outcome. However, the interpretation of such analysis is limited by the inability to distinguish expression changes that are causative to the clinical phenotype from those that are merely consequential. The combination of functional analysis such as RNAi screening followed by gene expression analysis in clinical samples provides a powerful unbiased approach to the identification of the key genetic causes of phenotypes such as drug resistance.

CDK10 Regulates Expression of *c-RAF*

By integrating our data with previously published work, we suggest a model by which CDK10 modulates intracellular signaling and determines the response to tamoxifen and other endocrine therapies. We propose that CDK10 normally binds and represses the ETS2 transcription factor, in agreement with previously published work (Kasten and Giordano, 2001). We identified an ETS2-binding site in the *c-RAF* promoter and using ChIP demonstrated that both CDK10 and ETS2 bind to this site. In the absence of CDK10 activity, *c-RAF* transcription is significantly upregulated due to relief of ETS2 repression. This increase in c-RAF expression leads to activation of downstream components of the MAPK pathway, including MEK1,2 and p42/p44 MAPK, which increase the expression of CYCLIN D1 (Lavoie et al., 1996), resulting in tamoxifen resistance by circumventing the reliance upon estrogen signaling (Wilcken et al., 1997).

Clinical Significance of CDK10

Having identified CDK10 as a modifier of tamoxifen sensitivity, we examined CDK10 expression in tamoxifen-treated breast tumors to validate its clinical significance. Patients with low-CDK10-expressing tumors were resistant to tamoxifen, consistent with the functional effect of silencing CDK10 expression identified in our RNAi screen. Furthermore, we identified methylation of the *CDK10* promoter in a significant proportion of tumors (18%), suggesting a mechanism for suppression of CDK10 expression. The significant association of clinical outcome with methylation of the *CDK10* promoter provides further evidence that suppression of CDK10 is a key driver of resistance to tamoxifen. Given that the mechanism of tamoxifen resistance identified in tumors with low CDK10 suggests loss of reliance on estrogen signaling, low CDK10 expression may also be associated with clinical resistance to other endocrine therapies such as aromatase inhibitors. Our study identifies a subgroup of low-CDK10-expressing, ERα-positive breast cancer patients that respond poorly to endocrine therapies, and these patients may benefit from alternative therapeutic approaches, including the use of signal transduction inhibitors (Dancey and Sausville, 2003).

It is notable that previous studies have demonstrated an association between both p42/p44 MAPK activation and increased CYCLIN D1 expression and tamoxifen resistance (Gee et al., 2001; Kenny et al., 1999). Our data identify a possible mechanism explaining these associations. In addition, elevated ETS2 expression and phosphorylation have been associated with reduced disease-free survival in tamoxifen-treated patients (Myers et al., 2005; Svensson et al., 2005), supporting the hypothesis that increased ETS2 activity can cause resistance to tamoxifen.

Summary

This study reports a functional RNAi screen to systematically identify the causes of tamoxifen resistance. We identified CDK10 as a modifier of tamoxifen sensitivity and established its mechanism of action, regulation of the p42/p44 MAPK pathway. Importantly, we demonstrate the clinical significance of these findings. Our work illustrates the power of combining systematic functional approaches with gene expression profiling of tumor samples.

EXPERIMENTAL PROCEDURES

Detailed Experimental Procedures are available in the Supplemental Data.

Cell Lines, Compounds, Antibodies, Plasmids, and siRNA

MCF7 cells were obtained from ATCC (USA) and maintained in phenol-red-free RPMI 1640 (Invitrogen), supplemented with 10% dextran charcoal-treated FCS (10% (v/v)), 1 nM estradiol, glutamine, and antibiotics. 4OH tamoxifen and estradiol were obtained from Sigma. ICI 182780 was obtained from Tocris Bioscience UK. The pReceiver CDK10 HA plasmid (EX-Q0187-M08) was obtained from Genecopoeia (USA). The pEF wild-type c-RAF plasmid was a kind gift from Professor Richard Marais (ICR, UK). MCF7 cells were transfected with SMARTpool siRNAs using Dharmafect 3 transfection reagent according to the manufacturer's instructions (Dharmacon). The protein kinase siRNA library (siARRAY, targeting 779 known and putative human protein kinase genes) was obtained in ten 96-well plates from Dharmacon (USA). Each well in this library contained a SMARTpool of four distinct siRNA species targeting different sequences of the target transcript. Details of antibodies used are provided in the Supplemental Experimental Procedures.

HTS Method

MCF7 cells (2500 per well) were plated in 96-well plates and transfected 24 hr later with siRNA (final concentration 100 nM), using Dharmafect 3 (Dharmacon, USA). Twenty-four hours after transfection, cells were trypsinized and divided into four identical replica plates. At 48 hr following transfection, two replica plates were treated with 50 nM 4OH tamoxifen in media and two replica plates with 0.05% ethanol vehicle in media. Media containing 4OH tamoxifen or vehicle were replenished after 48 hr and 96 hr, and cell viability was assessed after 7 days 4OH tamoxifen exposure using CellTiter Glo Luminescent Cell Viability Assay (Promega, USA) as per the manufacturer's instructions. The luminescence reading for each well on a plate was expressed relative to the median luminescence value of all wells on the plate. The screen was completed in duplicate. For each transfection the effects on cell growth and tamoxifen sensitivity were calculated. Details of the calculations are described in the Supplemental Experimental Procedures.

Validation of HTS

Four distinct siRNA species targeting each gene were used to revalidate hits from the screen. A significance threshold of $p < 0.05$ (Student's t test) was used for each individual siRNA. Validation of RNAi gene silencing was measured by quantitative PCR as described in the Supplemental Experimental Procedures.

Cell Viability Assays to Measure Drug Sensitivity

MCF7 cells were transfected with siRNA using Dharmafect 3, divided 24 hr later into 96-well plates, and exposed to various doses of drug from 48 hr post-transfection. Cell viability was assessed by CellTiter Glo Luminescent Cell Viability Assay (Promega, USA) at 9 days posttransfection, and the surviving fraction for each dose of drug was calculated by dividing the luminescence value from drug-treated wells by the luminescence value in vehicle-treated wells.

Quantitative PCR

MCF7 cells were transfected with siRNA using Dharmafect 3, and after 24 hr the media were refreshed with no estradiol, 1 nM estradiol, or 1 nM estradiol with 100 nM 4OH tamoxifen. RNA was extracted 48 hr later and cDNA

synthesized using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) with oligo dT. Real-time qPCR was performed using Assay-on-Demand primer/probe sets from Applied Biosystems (Foster City, CA), with endogenous control GAPDH. Gene expression was calculated relative to expression of GAPDH endogenous control and adjusted relative to expression in siControl-transfected cells.

Fluorescence-Activated Cell Scanning Analysis

MCF7 cells were transfected with siRNA using Dharmafect 3, and after 24 hr the media were refreshed with no estradiol, 1 nM estradiol, or 1 nM estradiol with 100 nM 4OH tamoxifen. After 48 hr the cells were fixed in 70% ice-cold ethanol and stained with 4% propidium iodide (PI) and 10% RNase A in PBS. The sample readout was performed on the FACSCalibur (Becton Dickinson, USA), and the data were analyzed using CellQuest Pro (Becton Dickinson, USA).

Caspase 3/7 Activation Assay

MCF7 cells were transfected with siRNA using Dharmafect 3. Twenty-four hours after transfection, media were refreshed with various concentrations of 4OH tamoxifen. Forty-eight hours after transfection, caspase 3/7 activation was assessed using Caspase-Glo 3/7 Assay (Promega), as per the manufacturer's instructions.

Ras Activation Assay

MCF7 cells were transfected with siRNA using Dharmafect 3, and after 24 hr the media were refreshed with RPMI 1640 without phenol red. After 48 hr the cells were lysed. The activation of Ras was assessed by the use of a Ras Activation Assay Kit (Cell Biolabs, CA). In brief, activated GTP-Ras was isolated from cell lysates by the use of agarose beads conjugated to the Ras-binding domain of c-RAF. The amount of GTP-Ras was quantified by the western blotting of purified samples with a mouse monoclonal antibody recognizing all three isoforms of Ras.

Co-IP

MCF7 cells were transfected with pReceiver CDK10 HA or the empty vector control pReceiver empty HA, using Fugene 6 as per the manufacturer's instructions. After 24 hr the cells were lysed in lysis buffer and total cell lysates were used for Co-IP.

Identification of ETS2-Binding Site

The ETS2-binding site in the *c-RAF* promoter was identified using the TFMATRIX transcription factor-binding site database (Wingender et al., 1996).

ChIP Assay

MCF7 cells were transfected with pReceiver CDK10 HA or the empty vector control pReceiver empty HA using Fugene 6, or CDK10 SMARTpool siRNA or siControl using Dharmafect 3. After 24 hr, or 48 hr, respectively, the cells were lysed and the ChIP assay was performed using the ChIP Assay Kit according to the manufacturer's instructions (17-295, Upstate, UK). Antibodies used for immunoprecipitation were HA (sc-805, Santa Cruz, USA), ETS2 (ab37214, abcam, USA), and normal rabbit IgG (sc-2027, Santa Cruz, USA). PCR primers were designed to flank the putative ETS2-binding site of the *c-RAF* promoter at position −545. Control PCR primers flanked an amplicon of similar size in *EGFR* exon 21. Primer sequences are available upon request.

Patients and Clinical Tissue Samples for MSP and qPCR

Primary breast cancer samples were obtained as paraffin-embedded tissue sections. Tissues were obtained with fully informed consent and Ospedale Santa Croce e Carle Local Ethics Committee approval.

Bisulfite Modification and MSP

Genomic DNA was subjected to modification with sodium bisulphite as described previously (Smith et al., 2007). Methylation-specific PCR was performed with multiple primer pairs encompassing different regions of the CpG island located at the 5′ end of the *CDK10* gene. Primer sequences are available upon request.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, seven supplemental figures, and four supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/2/91/DC1/.

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