Cell Reports
Article

PLK1 Signaling in Breast Cancer Cells Cooperates with Estrogen Receptor-Dependent Gene Transcription

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http://dx.doi.org/10.1016/j.celrep.2013.05.024

SUMMARY

Polo-like kinase 1 (PLK1) is a key regulator of cell division and is overexpressed in many types of human cancers. Compared to its well-characterized role in mitosis, little is known about PLK1 functions in interphase. Here, we report that PLK1 mediates estrogen receptor (ER)-regulated gene transcription in human breast cancer cells. PLK1 interacts with ER and is recruited to ER *cis*-elements on chromatin. PLK1-coactivated genes included classical ER target genes such as *Ps2*, *Wisp2*, and *Serpina3* and were enriched in developmental and tumor-suppressive functions. Performing large-scale phosphoproteomics of estradiol-treated MCF7 cells in the presence or absence of the specific PLK1 inhibitor BI2536, we identified several PLK1 end targets involved in transcription, including the histone H3K4 trimethylase MLL2, the function of which on ER target genes was impaired by PLK1 inhibition. Our results propose a mechanism for the tumor-suppressive role of PLK1 in mammals as an interphase transcriptional regulator.

INTRODUCTION

The steroid hormone estradiol (E2) acting via the estrogen receptor-α (ER) has essential functions in growth and development of both normal and tumorigenic breast tissue. ER is a nuclear receptor that mediates its functions through the transcriptional activation of target genes and by crosstalk with kinase cascades, which activate both receptors (Kato et al., 1995) and transcriptional cofactors (Wu et al., 2004) needed for target gene activation. Several kinase pathways have been identified as participants in estrogen-mediated gene regulation, including ERK/MSK1 (Migliaccio et al., 1996), PI3K/AKT (Castoria et al., 2001), PKC (Castoria et al., 2004), and IKKα (Park et al., 2005).

Target gene activation by nuclear receptors is a multistep process that includes the recruitment of coregulatory proteins to enhancer and promoter sites on the DNA. Coregulatory proteins function in chromatin remodeling and histone modification and as bridging factors to the RNA polymerase II initiation complex to activate or repress gene transcription (Kato et al., 2011). The two main classes of histone-modifying enzymes are histone acetyltransferases and methyltransferases together with their enzymatic counterparts. For instance, ER-activated genes are commonly associated with elevated levels of trimethylation on H3K4 (H3K4me3) (Mo et al., 2006), increased acetylation of several lysine residues on H3 and H4 (Métivier et al., 2003), and decreased levels of the repressive histone mark H3K9me3 (Shi et al., 2011). Kinases can also directly act as steroid receptor cofactors to locally modify other transcriptional regulators as well as chromatin components (Vicent et al., 2006; Devaiah et al., 2012).

The serine/threonine kinase Polo-like kinase 1 (PLK1) is a key regulator of mitotic cell division and is overexpressed in many types of human cancers, including breast cancer (McInnes and Wyatt, 2011). Conversely to its accepted role as proto-oncogene, evidence exists for an additional tumor-suppressive role of PLK1. PLK1 knockout mice are early embryonic lethal, while heterozygous knockout mice developed tumors at a 3-fold-higher frequency than wild-type mice, indicating a role of PLK1 in early development and tumor suppression (Lu et al., 2008). Genetic screens of cancer cell lines further identified several mutations in the PLK1 gene that impaired its function (Simizu and Osada, 2000). The molecular mechanisms involved in these tumor-suppressive roles of PLK1 are unknown.

We report here that PLK1 regulates estrogen-mediated gene expression by interaction with ER and corecruitment to ER target sites on chromatin. We also identified ER-independent transcriptional effects. Both ER-dependent and ER-independent PLK1 functions have tumor-suppressive properties, as indicated by correlation analysis to a published breast cancer cohort. Global phosphoproteomic analysis of PLK1 targets in interphase breast cancer cells identified several coregulatory proteins as well as transcription factors that potentially mediate ER-dependent and ER-independent transcriptional functions, providing a valuable resource for future studies on breast cancer physiology.

RESULTS

PLK1 Coregulates Estrogen-Mediated Gene Expression

As expression levels of PLK1 and ER were previously shown to be correlated in breast cancer (Wolf et al., 2000), we wondered whether a functional relationship exists between both proteins.

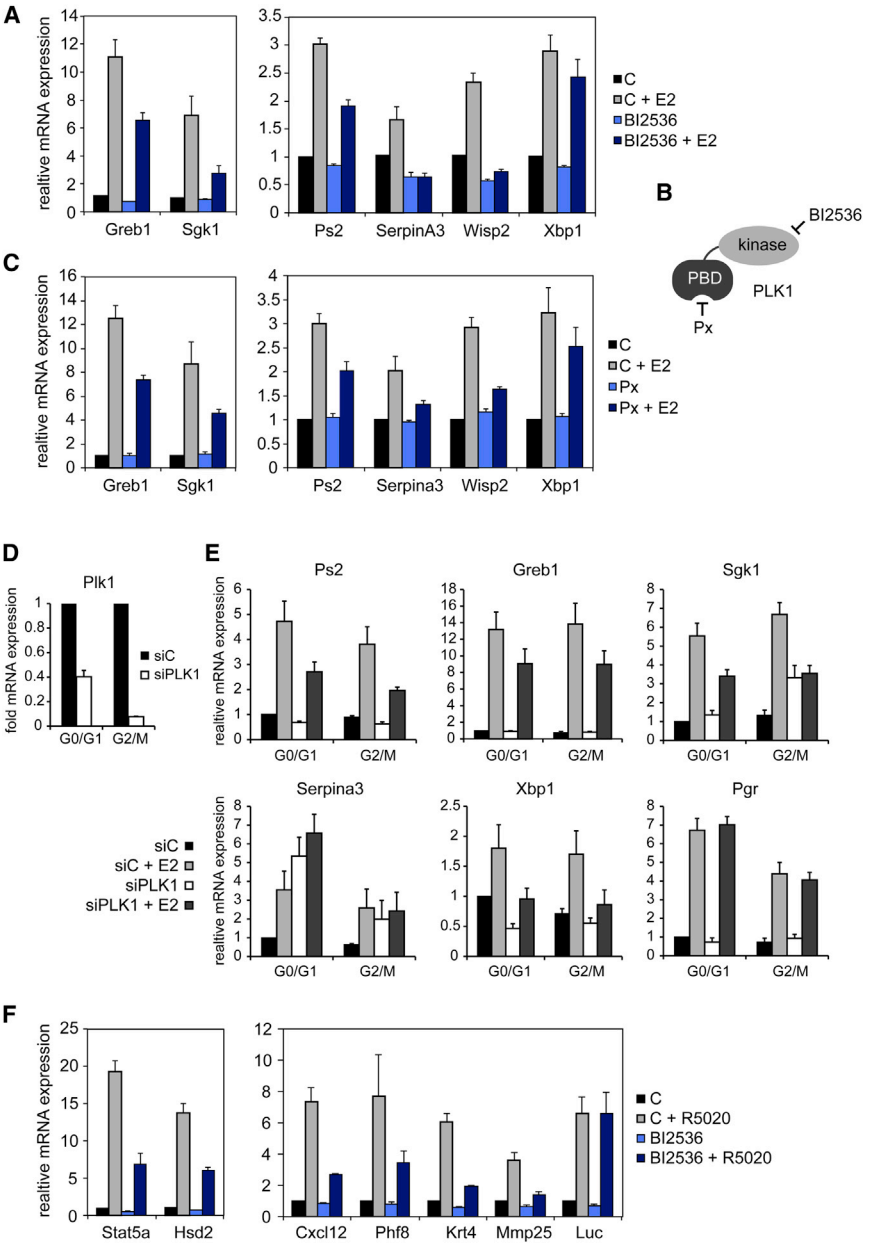

Figure 1. PLK1 Coactivates ER Target Gene Expression

(A) Hormone-deprived MCF7 cells were pretreated with 100 nM BI2536 or vehicle (DMSO) for 1 hr, followed by induction with E2 (10 nM) or EtOH for 6 hr. Gene-specific mRNA expression levels were measured by quantitative RT-PCR (qRT-PCR) and normalized to *Gapdh* expression and are represented as relative values to the vehicle control.

(B) Scheme depicting the different inhibitory modes of BI2536 and Poloxin on PLK1.

(C) Gene expression analysis as in (A) but with cells pretreated for 1 hr with 25 μM Poloxin.

(D) MCF7 cells were transfected with siRNA against PLK1 or control siRNA, sorted for different cell-cycle phases, and PLK1 mRNA expression levels analyzed by qRT-PCR.

(E) siRNA-transfected MCF7 cells were induced for 6 hr with E2 or EtOH control and subsequently sorted for different cell-cycle phases. Gene-specific mRNA expression levels were measured by qRT-PCR and normalized to *Gapdh* expression and are represented as relative values to the G1/G0 vehicle control.

(F) Hormone-deprived and serum-starved T47D-MVTL cells were pretreated with 100 nM BI2536 or DMSO for 1 hr, followed by induction with R5020 or EtOH for 6 hr, and gene expression levels analyzed by qRT-PCR. Error bars represent SEM of three independent experiments.

See also Figure S1.

To study whether PLK1 affects ER-dependent gene transcription, we treated MCF7 cells with the PLK1 inhibitor BI2536 or vehicle, followed by induction with E2 for 6 hr. Subsequent analysis of messenger RNA (mRNA) levels by quantitative RT-PCR (qRT-PCR) revealed a clear E2-mediated induction of classical ER target genes, including *Greb1*, *Sgk1*, *Ps2*, *Serpina3*, *Wisp2*, and *Xbp1*, in the control cells (Figure 1A). Notably, in the presence of BI2536, the induction of *Greb1*, *Sgk1*, *Ps2*, *Serpina3*, and *Wisp2* was strongly reduced, while *Xbp1* was only slightly affected (Figure 1A).

The inhibitor BI2536 is an ATP analog that inhibits PLK1 by binding to its catalytic domain and preventing binding of ATP (Steegmaier et al., 2007). To exclude the possibility of a nonspecific effect of BI2536 on other kinases, we repeated the experiments using the PLK1 inhibitor Poloxin, which blocks the Polo box domain needed for the interaction of PLK1 with its substrates (Figure 1B) (Reindl et al., 2008). Treatment of MCF7 cells with Poloxin inhibited E2-mediated induction of the same set of genes and to a similar extent, as observed for BI2536, but only slightly affected the induction of *Xbp1*, indicating that PLK1 functions as a target-gene-specific ER coactivator (Figure 1C).

Although both inhibitors are highly specific to Polo-like kinases, they are also able to inhibit the PLK1 isoforms PLK2 (SNK) and PLK3 (FNK) (Steegmaier et al., 2007; Reindl et al., 2008). To verify that our observed effects were due to inhibition of PLK1 and not other PLK isoforms, we reduced PLK1 levels with PLK1-specific small interfering RNA (siRNA) (Figure 1D). Under these conditions, about 45% of the cells arrested in G2/M phase due to the lack of mitotic signaling of PLK1 (Figures S1A and S1B). In order to evaluate the effect of PLK1 knockdown on gene expression without the possibility of cell-cycle-dependent expression changes, we sorted cells according to their cell-cycle phase after induction with E2 for 6 hr (Figure S1B). In accordance with its mitotic expression profile, PLK1 expression was 25-fold higher in G2/M compared to G1 (Figure S1C). In the presence of

PLK1 siRNA, PLK1 expression levels were effectively reduced in both cell-cycle phases (Figures 1D and S1A). Analyzing the expression of ER target genes that were sensitive to pharmacological PLK1 inhibition, we observed a strong reduction of E2-mediated gene induction in both G1 and G2/M populations of MCF7 cells (Figure 1E). While induction of *Ps2*, *Greb1*, *SerpinA3*, and *Sgk1* were affected in both cell-cycle phases, the E2-mediated upregulation of *Xbp1* expression was only affected in G2/M phase and the expression of *Pgr* was not affected by PLK1 knockdown (Figure 1E). In addition, siRNA-mediated knockdown of PLK2 or PLK3 in nonsorted MCF7 cells did not affect estrogen-mediated gene induction (Figures S1D and S1E). Taken together, these results further support the target-gene specificity of PLK1 action on E2-regulated gene transcription and support a direct effect of PLK1 signaling on the coactivation of ER target genes independently of the cell-cycle phase.

As a further proof of the ability of PLK1 to coactivate steroid receptor target genes, we also analyzed the effect of PLK1 inhibition on the induction of progesterone receptor (PR) target genes in T47D-MVTL breast cancer cells that carry a single copy of the MMTV promoter driving the luciferase gene (Truss et al., 1995). Treating T47D-MVTL cells for 6 hr with the synthetic progesterone analog R5020 in the presence or absence of BI2536, we observed that the induction of several PR target genes, including *Stat5a*, *Hsd2*, *Cxcl12*, *Phf8*, *Krt4*, and *Mmp25*, was strongly reduced (Figure 1F). Conversely, the induction of the MMTV promoter was not influenced by BI2536, indicating a target-gene-specific role of PLK1 rather than a general inhibition of the receptor also in the case of PR. As a control, the PR protein expression level was not affected by BI2536 (Figure S1F).

PLK1 Is Recruited to Estrogen Receptor Target Sites on Chromatin

To explore whether PLK1 acts at the level of chromatin, we performed chromatin immunoprecipitation (ChIP) experiments with MCF7 cells before and after 5, 30, and 60 min of E2 treatment. PLK1 was specifically recruited to estrogen responsive elements (ERE) in promoter or enhancer regions of *Ps2*, *Greb1*, *Wisp2*, and *Serpina3* as early as 5 min after hormone addition with similar kinetics as ER (Figure 2A). While PLK1 binding was maximal at 30 min of E2 treatment, it decreased toward 60 min for all binding sites. Meanwhile, we did not observe binding of PLK1 to a promoter proximal region in the gene body, indicating that the binding was specific to ER target sites (Figure 2A).

The similarity in the kinetics of PLK1 and ER recruitment to chromatin prompted us to analyze whether ER and PLK1 physically interact. We immunoprecipitated endogenous ER and immunoblotted for PLK1. Compared to the immunoglobulin G (IgG) control, PLK1 coimmunoprecipitated specifically with ER in a hormone-independent manner (Figure 2B). To assess whether PLK1 is corecruited to chromatin via ER, we performed a re-ChIP experiment (Figure 2C). In the presence of E2 treatment, the ER-PLK1 complex was 18.4-fold enriched at the *Ps2* promoter and 3.7-fold enriched at the *Greb1* ERE compared to both the nontreated cells and the ER-IgG control (Figure 2C). Likewise to the regular ChIP, the signal was specific for EREs, while we did not observe binding to the gene body.

As an additional proof of a combined function of ER and PLK1, we performed localization studies for both proteins using specific antibodies against the active phosphorylated forms of ER (pS118) and of PLK1 (pT210) (Kato et al., 1995; Jang et al., 2002). Notably, in a large subset of ER-positive cells, we also observed a clear nuclear signal for active PLK1 (Figure 2D). We conclude from these results that the interplay of ER and PLK1 takes place in the nucleus and that nuclear PLK1 is catalytically active.

PLK1-Dependent Genes Are Enriched in Developmental Functions

To investigate the physiological role of PLK1 coactivation of ER target genes in breast cancer cells, we analyzed the effect of PLK1 on the E2-mediated transcriptional response at the global level. Using MCF7 cells treated for 6 hr with E2, we identified 638 upregulated genes and 498 downregulated genes. Pretreatment of cells with the PLK1 inhibitor BI2536 significantly inhibited the induction of 33% of E2-responsive genes (Figure 3A). Using gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, we observed that those genes were significantly enriched in developmental functions, while PLK1-independent E2-responsive genes were enriched for genes involved in cell-cycle and cancer pathways (Figure 3B; Table S1).

As in cancer biology developmental genes often act as tumor suppressors, we wondered whether PLK1-dependent E2-induced genes are involved in tumor suppression. To answer this question, we integrated the gene set with a previously published cohort of breast cancer samples from 268 tamoxifen-treated breast cancer patients (Loi et al., 2007) (Figure 3C). Out of 213 PLK1-dependent E2-induced genes, twice as many genes correlated with positive clinical outcome than with a negative outcome (Figure 3C). On the other hand, PLK1-independent E2-induced genes preferentially correlated with a negative clinical outcome (Figure 3C), supporting the results of the GO analysis. Moreover, breast cancer patients with high average expression of the PLK1-dependent E2-induced gene set had a clear clinical benefit as compared to patients with low expression ($p = 0.017$, hazard ratio [HR] = 0.47) (Figure 3D). Conversely, E2-upregulated genes that were not sensitive to BI2536 did not have any clinical impact (Figure 3D). To verify this observation, we also correlated expression of the PLK1-ER-dependent genes to a previously compiled data set of 1,881 breast cancer patients with mixed background (Ringnér et al., 2011) and obtained similar results (Figure S2A).

We next asked whether PLK1 has additional transcriptional functions in MCF7 cells aside from its coactivating function with ER. To this end, we searched our global gene expression data set for genes that were differentially regulated between the control and the BI2536-treated cells in the absence of estradiol. We identified 370 genes that were upregulated by BI2536 treatment and 874 that were downregulated. Performing GO enrichment analysis on the BI2536-downregulated gene set, we identified a significant ($p = 4.7E-5$) enrichment of proapoptotic genes, including *Tgfb3*, *Trail*, *Gpr109b*, and *Mcf2l*, which we

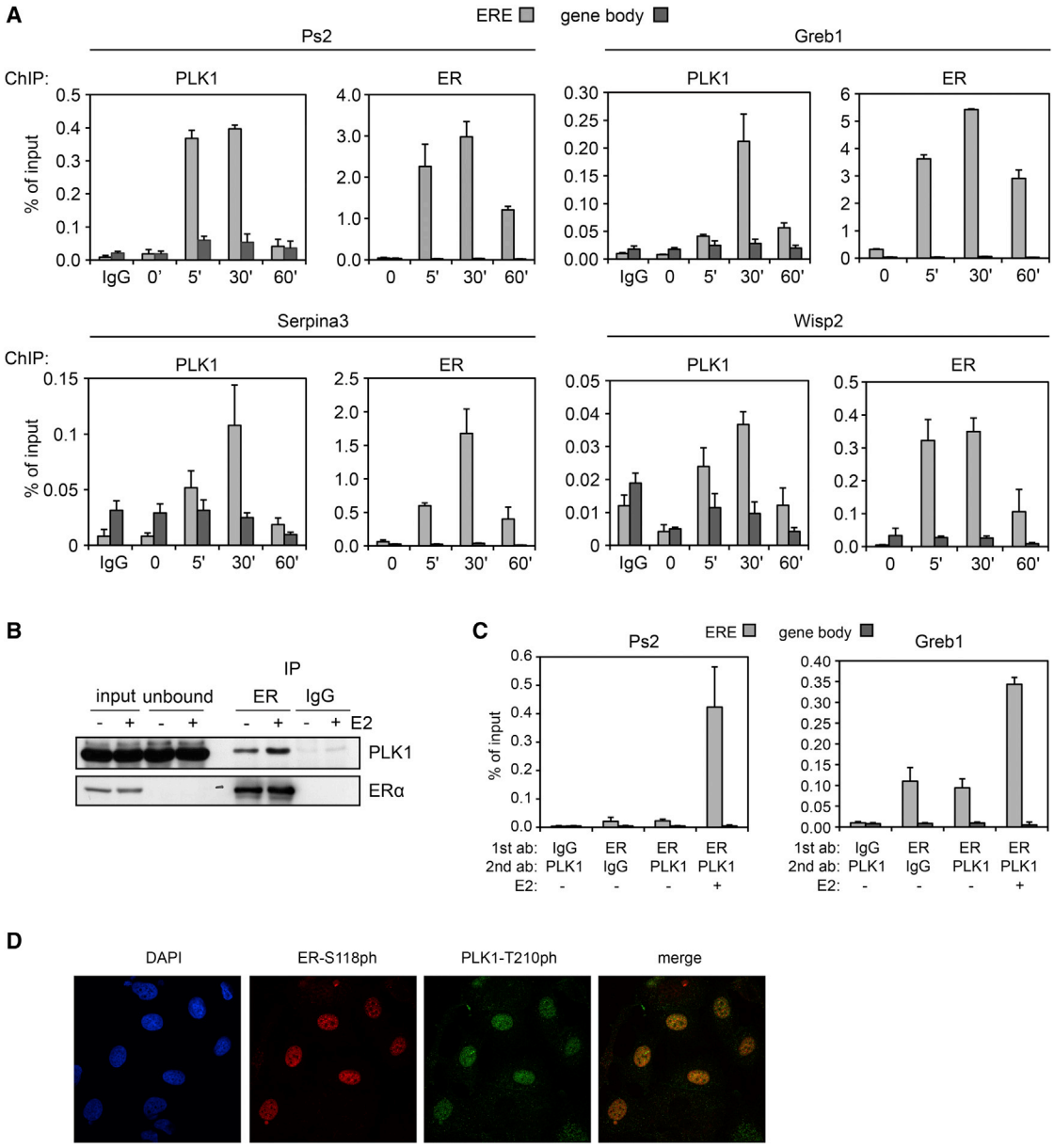

Figure 2. PLK1 Is Recruited to ER Target Sites on Chromatin and Interacts with ER

(A) Hormone-deprived MCF7 cells were induced with E2 for the indicated amounts of time, and ChIP assay was performed with specific antibodies against PLK1 and ER or total rabbit IgG. Specific binding was assessed by qPCR amplification of ER binding sites (ERE) or control regions in the promoter proximal gene body. Binding is represented as the percentage of input. Error bars represent the SEM of three independent experiments.

(B) Endogenous ER and PLK1 interact. Hormone-deprived MCF7 cells were induced with E2 or EtOH for 1 hr, immunoprecipitation was performed with ER or rabbit IgG, and protein levels were analyzed by western blot.

(C) PLK1 and ER are corecruited at EREs. re-ChIP experiments were performed with the indicated antibodies. Specific cobinding was assessed by qPCR amplification of ERE or control regions in the promoter proximal gene body. Target regions were amplified by quantitative real-time PCR. Binding is represented as the percentage of input. Error bars represent the SEM of three independent experiments.

(D) Active ER and PLK1 colocalize in the nucleus. Localization of PLK1-pT210 (green) and ER-pS118 (red) in MCF7 cells after 30 min E2 treatment were determined by immunofluorescence microscopy. DAPI staining is shown in blue.

also validated by qRT-PCR (Figures 3E and 3F). As these genes were also sensitive to Poloxin, we concluded that the transcriptional effect by PLK1 was specific (Figure S2B). Furthermore, the average expression of this gene set positively correlated with clinical outcome in both the tamoxifen-treated patient cohort as well as the group of 1,881 breast cancer patients, which is proof of the tumor-suppressive characteristics of this gene set (Figures 3G and S2C).

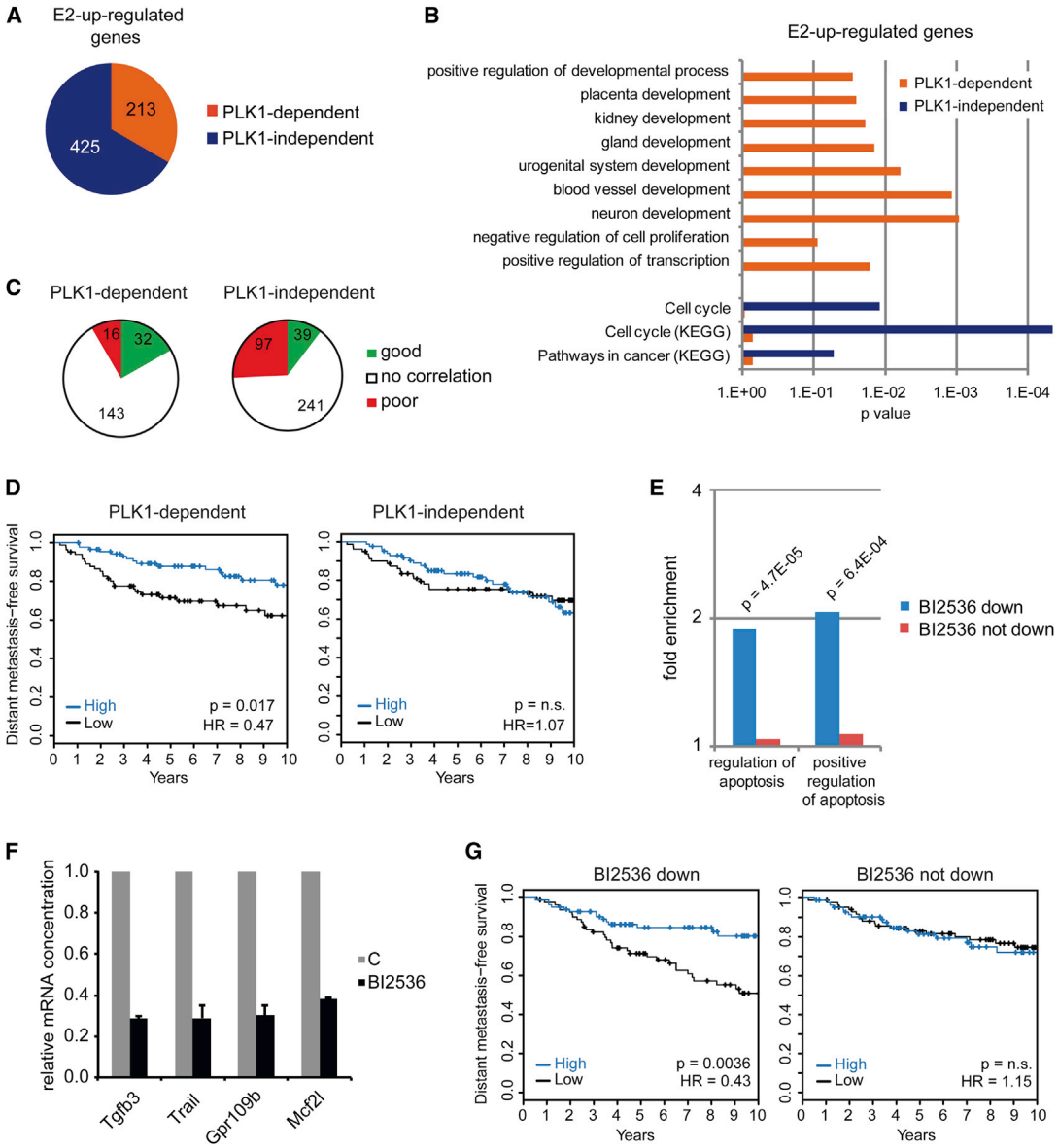

Figure 3. PLK1 Regulates Tumor-Suppressive Gene Transcription

(A) Hormone-deprived MCF7 cells were pretreated with 100 nM BI2536 or the vehicle (DMSO) for 1 hr, followed by induction with E2 (10 nM) or EtOH for 6 hr. Cells were harvested and mRNA levels analyzed by Agilent gene expression microarrays. The fraction of E2-regulated genes that were significantly inhibited by BI2536 is shown.

(B) GO-term enrichment analysis for E2-upregulated, BI2536-inhibited genes using Fisher's exact test. The entire list of significantly enriched GO terms can be found in Table S1.

(C) Correlation of E2-upregulated, BI2536-inhibited (left) or BI2536-noninhibited (right) genes with distant metastasis-free survival in a cohort of 268 tamoxifen-treated breast cancer patients (Loi et al., 2007). Correlations were scored as good (green), poor (red), or nonsignificant (white) using a cutoff of $p < 0.05$ for each gene, as determined by recursive portioning (RP) based on a log-ranked (Mantel-Cox) test.

(D) The average expression levels of the E2-upregulated, BI2536-inhibited, or BI2536-noninhibited gene sets were correlated with distant metastasis-free survival in the same breast cancer cohort used in (C). Patients were ranked according to the average expression values (top 33% versus bottom 33%), after which the distant metastasis-free survival was determined for both groups. The p value was determined by a log-ranked (Mantel-Haenszel) test, and the hazard ratio (HR) was determined with the Mantel-Cox method.

(E) GO-term enrichment analysis for BI2536-downregulated and BI2536-nondownregulated genes independent of E2. A complete list of significantly enriched terms can be found in Table S1.

(F) Hormone-deprived MCF7 cells were treated with BI2536 or vehicle (DMSO) for 7 hr, and gene expression levels were analyzed by qRT-PCR. Error bars represent SEM of three independent experiments.

(G) Kaplan-Meier plot as calculated in (D) for the BI2536-downregulated and BI2536-nondownregulated gene sets.

See also Figure S2.

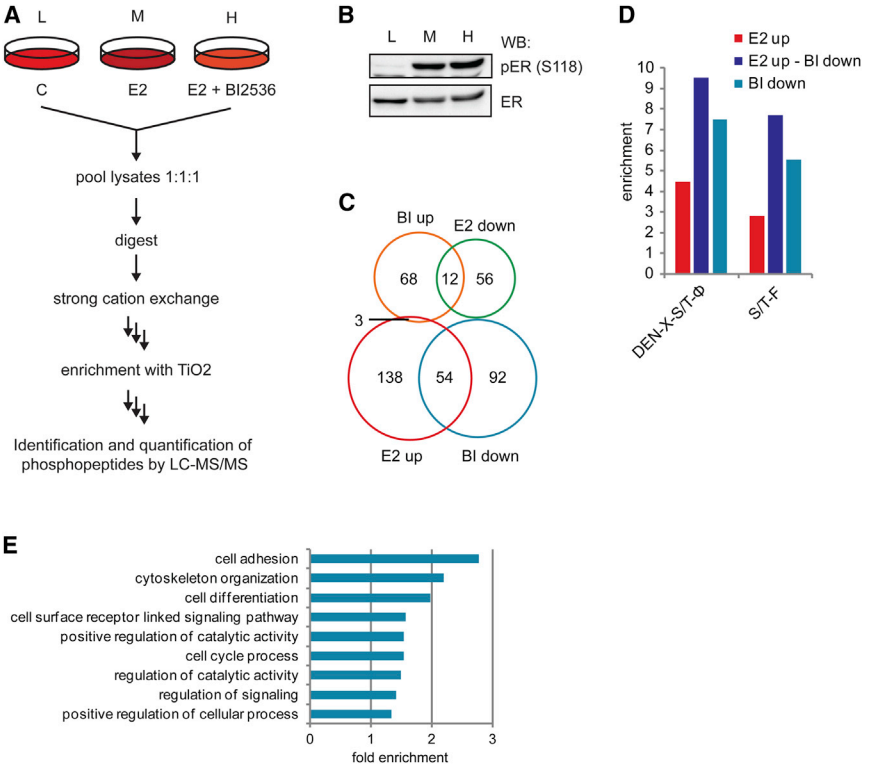

Figure 4. PLK1 Is a Key Component of E2-Mediated Signaling

(A) Schematic depiction of the experimental setup for the global phosphoproteome analysis. MCF7 cells were SILAC-labeled in three different populations: light (L), medium (M), and heavy (H). Cells were pretreated for 1 hr with BI2536 (H) or vehicle (L and M) following stimulation with E2 (M and H) or EtOH (L) for 30 min. Cells were lysed, combined, enzymatically digested, and phosphopeptides were enriched and analyzed by mass spectrometry.

(B) PLK1 inhibition does not affect phosphorylation levels of ER in S118. Extracts from the SILAC experiment depicted in (A) were analyzed by western blot.

(C) Overlap of E2- and BI2536-regulated phosphosites.

(D) Motif-enrichment analysis of E2-upregulated and BI2536-downregulated class I phosphosites. For D/E/N/-X-S/T-Φ: E2 upregulated: p = 1.3E-04, BI downregulated: p = 3.9E-08, E2 upregulated/BI downregulated: p = 3.0E-05; for S/T-F: E2 upregulated: p = 8.4-03, BI downregulated: p = 3.1E-06, E2 upregulated/BI downregulated: p = 9.8E-05.

(E) Fisher's test for significantly overrepresented GO terms ($p < 0.05$) among BI2536-downregulated phosphoproteins.

See also Figure S3.

Taken together, we conclude that transcriptional regulation by PLK1 of both ER-dependent and ER-independent genes in interphase MCF7 cells is tumor suppressive.

Global Phosphoproteome Analysis Reveals Involvement of PLK1 in Estrogen-Mediated Signaling

After identifying a key role of PLK1 in ER target gene activation, we decided to explore its involvement in E2-induced signaling in a global manner using a stable isotope labeling by amino acids in cell culture (SILAC)-based high-resolution mass spectrometry approach (Figure 4A) (Ong and Mann, 2007). Western blot analysis demonstrated that BI2536 did not influence ER activation, as measured by the level of phosphorylation at S118 (Figure 4B). Performing two independent experiments, we were able to identify and quantify 17,448 phosphorylation sites on 3,463 proteins, with 8,251 phosphosites common to both replicate experiments (Table S2). Applying a threshold of 1.5-fold regulation that had to be fulfilled in both replicates, we identified 195 sites that were upregulated by E2 and 68 that were downregulated (Figure 4C). Using a similar filter for the effect of BI2536 (ratio H/M), we reproducibly identified 146 sites that were reduced in response to BI2536 while 80 sites were enhanced, likely due to secondary effects of PLK1 inhibition on phosphatases (Figure 4C). Notably, we observed a strong overlap (54 sites) between E2-upregulated and BI2536-sensitive phosphosites, representing 28% and 40% of each population, respectively (p = 1.7E-51). This indicates that PLK1 is involved in a substantial subset of E2-mediated signaling events and further suggests that E2 has a strong effect on the PLK1-regulated phosphosites. This mutual regulation is further evidenced by an 18% overlap of E2-downregulated sites with sites that are enhanced by BI2536 (p = 2.1E-12) and the lack of a substantial overlap between phosphosites that are either upregulated by E2 and enhanced by BI2536 or downregulated by E2 and reduced by BI2536 (Figure 4C).

To address the specificity of BI2536 inhibition, we performed a consensus-motif enrichment analysis using Fisher's exact test and a sequence window of 15 amino acids after filtering all identified phosphopeptides for a localization probability of greater 75% (Olsen et al., 2006) (Figure S3E). We included the modified classical PLK1 motif D/E/N/-X-S/T-Φ as well as the alternative motif S/T-F (X represents any amino acid and Φ represents a hydrophobic amino acid), both of which were defined in a recent phosphoproteome study (Santamaria et al., 2011). Both motifs were highly enriched in the BI2536-sensitive set of phosphorylation sites, with p values of 1.9×10^{-8} and 3.1×10^{-7}, respectively (Figures 4D; Table S3). Interestingly, both motifs were also significantly enriched among the E2-upregulated sites (Figure 4D; Table S3). Most strikingly, however, the enrichment was highest in E2-upregulated phosphosites that were inhibited by BI2536 (Figure 4D; Table S3). These findings support an implication of PLK1 in E2-mediated signaling. Of note, both PLK1 motifs were also significantly enriched in a SILAC-based phosphoproteomic data set of T47D cells stimulated for 5 and 30 min with R5020 (Figures S3F and S3G), indicating a conserved role of PLK1 signaling in estrogen and progestin responses.

To gain deeper insight into the biological processes mediated by PLK1 in interphase breast cancer cells, we performed Fisher's exact test for GO terms for proteins containing one or more BI2536-sensitive phosphorylation sites. As expected, significantly enriched terms included those associated with PLK1

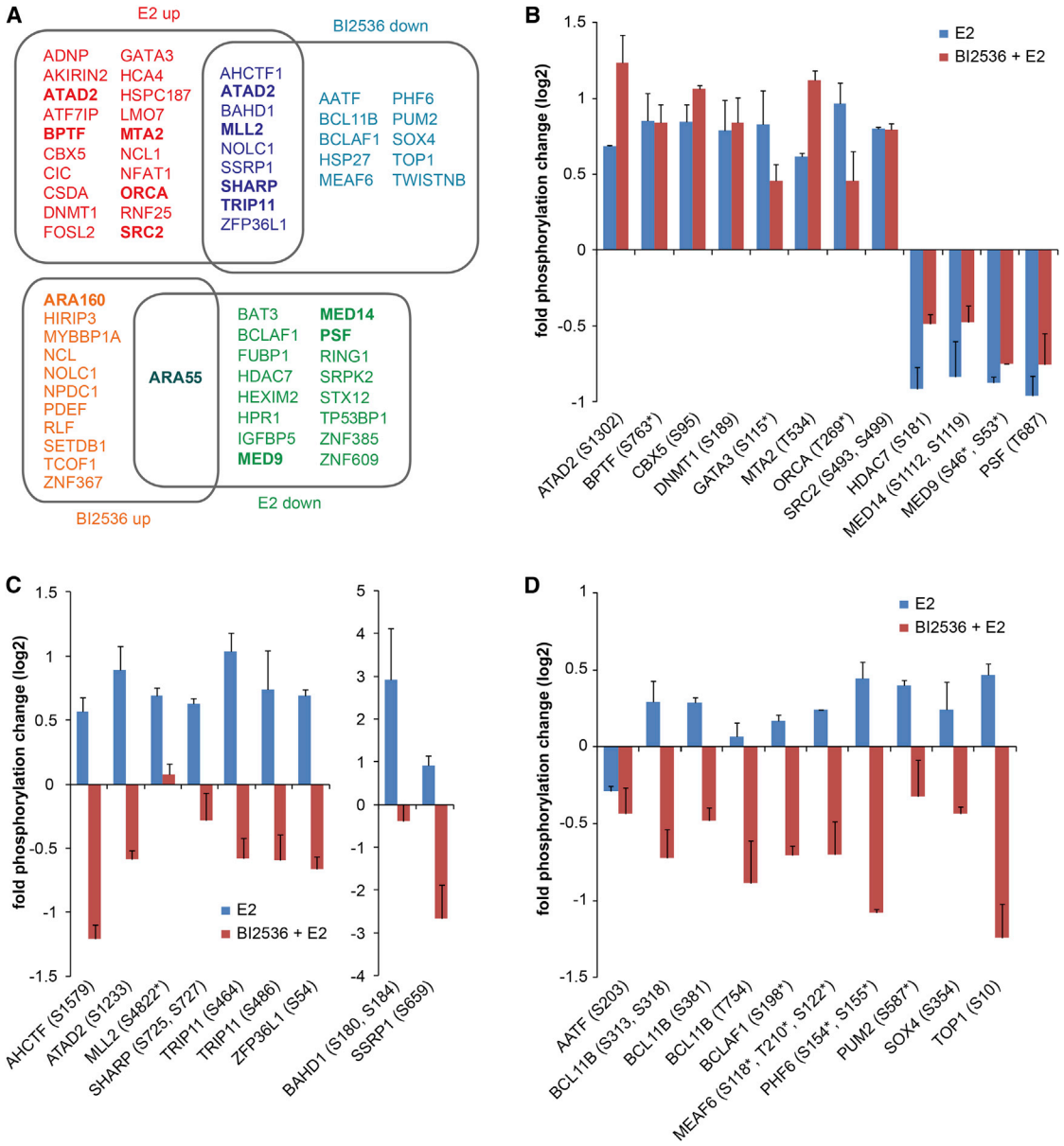

Figure 5. PLK1 and Estradiol Signaling Results in Phosphorylation of Transcriptional Regulators

(A) Proteins with E2- and/or BI2536-regulated phosphosites involved in transcriptional regulation. MLL2 S4822 was manually assigned to the E2-upregulated BI2536-inhibited gene set.

(B–D) Individual phosphosite regulations. Average normalized M/L ratios (blue) and H/L ratios (red) for E2-regulated BI2536-unaffected (B), E2-upregulated BI2536-downregulated (C), and BI2536-downregulated phosphosites (D). Phosphosites with localization probability below 75% are marked by an asterisk. Error bars represent the range of two independent experiments.

See also Figure S4.

functions, such as "cell cycle process" and "cytoskeleton organization" (Figure 4E; Table S4). Notably, BI2536-sensitive phosphoproteins were also significantly enriched for the term "cell differentiation," supporting the role of PLK1 in developmental processes. On the other hand, proteins containing E2-regulated phosphorylation sites were enriched in E2-signaling-associated GO terms, such as "positive regulation of transcription from RNA polymerase II promoter" and "ion transport," but also for "actin cytoskeleton organization" and "cell differentiation," similar to the BI2536-sensitive phosphosite population (Table S5).

PLK1 Targets Include Transcriptional Regulatory Proteins

We next searched our data set for transcriptional coregulators and transcription factors that contain one or more regulated phosphosites (Figure 5A). We first focused on proteins

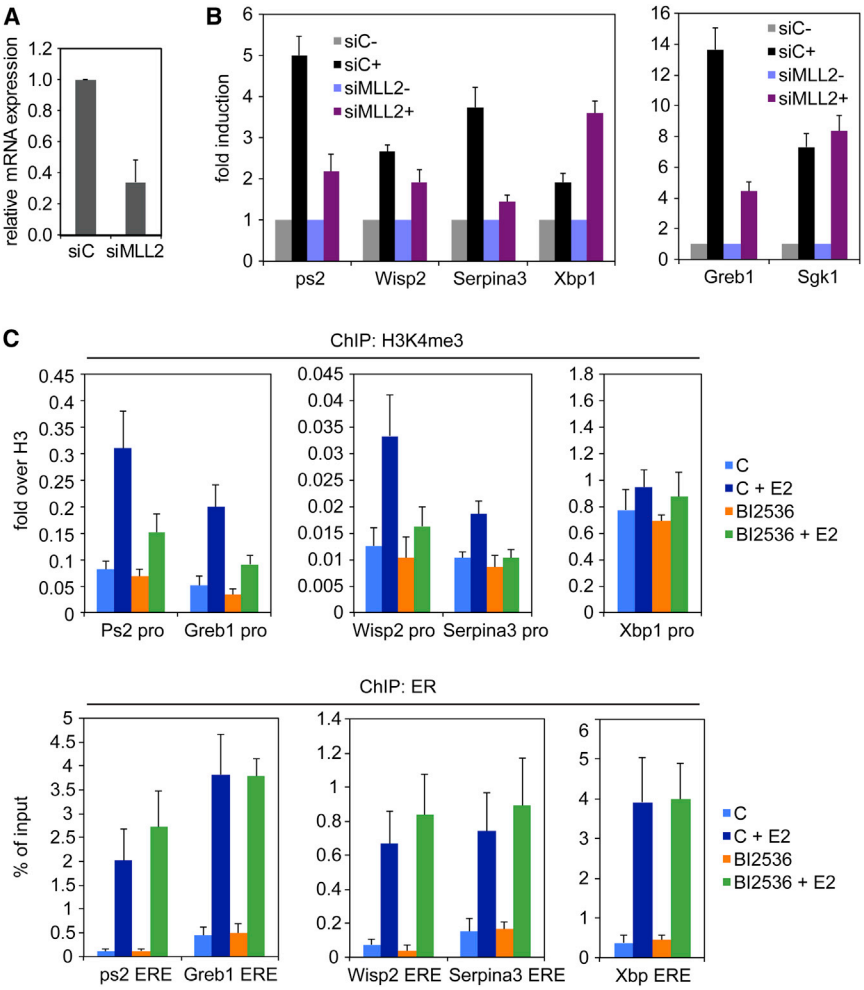

Figure 6. PLK1 Regulates the MLL2-Coactivating Function on ER Target Genes

(A) Hormone-deprived MCF7 cells were transfected with siRNA against MLL2 (siMLL2) or control siRNA (siC), and MLL2 mRNA levels were analyzed by qRT-PCR. RNA levels were normalized to *Gapdh* expression and represented as relative values to the control-siRNA transfected cells.

(B) Cells were transfected with siRNA as in (A) and treated for 6 hr with E2. Gene expression changes were analyzed by qRT-PCR.

(C) MCF7 cells were treated with BI2536 or vehicle (DMSO) for 1 hr followed by induction with E2 for 1 hr. Levels of H3K4me3 and ER were measured by ChIP in proximal promoter regions (top) and ER target sites (bottom), respectively.

Error bars represent SEM of three independent experiments.

containing E2 upregulated or downregulated phosphosites that were not affected by BI2536. We identified several known ER cofactors among this group of proteins, including SRC2, SRC3, BPTF, MTA, MED9, MED14, and PSF, indicating that our phosphoproteome analysis could represent known signaling events involved in steroid-receptor-mediated transcriptional regulation (Figures 5A and 5B).

Next, we focused on proteins with E2-upregulated BI2536-sensitive phosphosites to identify proteins that are potentially responsible for the coactivating function of PLK1 on ER target genes, identifying ten proteins with functions in gene regulation (Figures 5A and 5C). These included the ER coactivator ATAD2 (Zou et al., 2007), the thyroid receptor coactivator TRIP11 (Chang et al., 1997), the nuclear receptor corepressor SHARP (Shi et al., 2001), the p63 coactivator and transcriptional elongation factor SSRP1 (Zeng et al., 2002), and the ER-associated histone H3K4 trimethyltransferase MLL2 (Mo et al., 2006). The latter was reproducibly assigned to the BI2536-affected category by comparing the fold induction in response to E2 in the presence or absence of BI2536 (Figure S4A).

We also identified several proteins involved in transcriptional regulation among the proteins that contain BI2536-regulated phosphosites that were unaffected by E2 (Figures 5A and 5D). Interestingly, several of these have known functions in development or apoptosis regulation and could therefore potentially account for the ER-independent tumor-suppressive transcriptional effects of PLK1 described above (Figures 3E and 3G). These include the developmental transcription factor SOX4, the transcriptional repressor BCL11B, the apoptosis-antagonizing transcription factor AATF, and the death-promoting transcriptional repressor BCLAF1 (Figure 5D).

PLK1 Phosphorylation of MLL2 Affects Gene Regulation

The histone-lysine N-methyltransferase MLL2 is a key regulator of developmental genes essential for the early embryonic development of mice (Glaser et al., 2006). MLL2 interacts with ER and is essential for the transcriptional activation of ER target genes, including *Ps2* (Mo et al., 2006) and *Greb1* (Shi et al., 2011). Estrogen-mediated, PLK1-dependent phosphorylation of MLL2 occurs at S4822, which has been previously identified by mass spectrometry in various cell types (Dephoure et al., 2008; Mayya et al., 2009; Van Hoof et al., 2009). Interestingly, we also identified a strong induction of this site in a phosphoproteome analysis of T47D breast cancer cells in response to progestin (Figure S4B), indicating a conserved function in the action of steroid hormones in breast cancer cells.

Knocking down MLL2 by siRNA transfection (Figure 6A) inhibited E2-mediated induction of *Ps2*, *Wisp2*, *Serpina3*, and *Greb1*, while it did not inhibit the induction of *Sgk1* and *Xbp1* (Figure 6B).

The main described function of MLL2 is to catalyze trimethylation of histone H3 on K4 (H3K4me3), a histone marker linked to transcriptional activation (Glaser et al., 2006). Having identified phosphorylation of MLL2 by PLK1, we wondered whether PLK1 activity would be important for the estradiol-mediated increase in H3K4me3. To this end, we compared H3K4me3 levels

in the presence or absence of PLK1 inhibition using MCF7 cells that had been induced with E2 for 1 hr or left untreated (Figure 6C). E2 treatment mediated a 1.8- to 5-fold increase in H3K4me3 in proximal promoter regions of *Ps2*, *Greb1*, *WISP2*, and *Serpina3*. In the presence of PLK1 inhibition, this increase was strongly reduced for all four genes. Conversely, for the MLL2-independent ER target gene *Xbp1*, we did not observe an increase in H3K4me3 levels in response to hormone induction.

DISCUSSION

It is well established that the serine/threonine kinase PLK1 is a key regulator of mitotic cell division, and immunohistochemistry studies in breast cancer revealed a close correlation between ER and PLK1 expression levels (Wolf et al., 2000; Takai et al., 2005). We show here that PLK1 directly modulates estrogen-dependent gene transcription in breast cancer cells. PLK1 physically interacts with ER and is corecruited to the enhancer elements of ER-regulated genes. We also found that PLK1 regulates genes independently of ER. Surprisingly, both ER-dependent and ER-independent gene sets were enriched in tumor-suppressive functions, and high expression levels of each gene set correlated positively with a clinical benefit in breast cancer patients. Phosphoproteomics identified several targets for the transcriptional effects of PLK1, including the histone H3 K4 trimethyltransferase MLL2 phosphorylated on serine 4822. Although the large size of MLL2 did not allow us to assess its function in vivo due to technical limitations, we identified the same S4822 phosphorylation site to be strongly induced in T47D breast cancer cells in response to progestin, indicating a conserved role of MLL2 S4822 phosphorylation in the steroid hormone response. However, we cannot exclude that MLL2 is an indirect target of PLK1 kinase activity.

Regulation of MLL2 is not the only mechanism through which PLK1 potentially affects ER-dependent gene transcription. The E2-mediated transcriptional induction of *Sgk1*, for instance, was dependent on functional PLK1 but not on the presence of MLL2. Interestingly, in breast cancer cells, *Sgk1* expression was reported to depend on the AAA ATPase and bromodomain-containing protein ATAD2 (Kalashnikova et al., 2010), which we identified to be phosphorylated by E2 and to be sensitive to the PLK1 inhibitor BI2536. Thus, the induction of *Sgk1* could be due to regulation of ATAD2.

Global gene expression analysis identified 213 E2-induced genes that depend on PLK1 activity. Although for a set of classical target genes we proved that the effect of PLK1 inhibition was direct, we cannot exclude that BI2536-mediated changes in cell-cycle kinetics are responsible for this effect in some of the genes.

The observed enrichment of proapoptotic genes among genes upregulated by PLK1 was unexpected and controverts previous studies claiming a negative regulatory role of PLK1 on proapoptotic genes caused by inhibiting the transcriptional activity of the p53 family members p53 (Ando et al., 2004), Tap63 (Komatsu et al., 2009), and Tap73 (Koida et al., 2008; Soond et al., 2008). The discrepancy is likely due to the different experimental conditions. While in previous studies the transcriptional effects of PLK1 were assessed by ectopic coexpression of PLK1 and p53 family members in p53 null-cells or by siRNA-mediated knockdown of PLK1 in p53 wild-type cells, we targeted the endogenous levels of PLK1 by direct inhibition. If PLK1 were to negatively influence p53 family members in our system, then we should have observed effects in the expression of p53 family target genes. However, the expression levels of the classical p53 target genes *p21*, *14-3-3σ*, and *Bax* were not affected by BI2536 treatment (data not shown).

Among genes inhibited by BI2536, we identified the tumor necrosis factor-related apoptosis-inducing ligand (*Trail*), which in leukemic T cells was described to be a major target of the transcriptional repressor BCL11B (also known as CTIP2) (Grabarczyk et al., 2007). Notably, we identified BCL11B among the PLK1 phosphorylated proteins, with several BI2536-sensitive phosphorylation sites. BCL11B was described to repress target genes by recruiting the NuRD complex to promoter sites via direct interaction with the two NuRD complex members RbAp46 and RbAp48 (Cismasiu et al., 2005; Topark-Ngarm et al., 2006). Interestingly, the PLK1-dependent double phosphorylation site T313, S318 on BCL11B is located within the protein region identified to interact with RbAp46 and RbAp48 (Topark-Ngarm et al., 2006), while the PLK1-dependent phosphorylation site on S772 resides in a region known to be important for the interaction of BCL11B with the repressive factor HP1α (Rohr et al., 2003). PLK1-dependent phosphorylation in these regions might interrupt the interactions of BCL11B with the NuRD complex and HP1α, leading to derepression of BCL11B-repressed genes, including *Trail*.

Estrogen signaling via ER is key for the growth and development of breast cancer cells. Having identified a dependency of a quarter of E2-induced phosphorylation sites on PLK1, we propose that PLK1 fulfills a crucial role in the signaling effects of ER. Our phosphoproteomic study further provides a source of catalytic PLK1 targets in interphase-enriched cells, whereas previous studies specifically covered mitotic targets of PLK1 (Grosstessner-Hain et al., 2011; Kettenbach et al., 2011; Oppermann et al., 2011; Santamaria et al., 2011). Although we identified a significant enrichment for proteins involved in "cytoskeleton organization," these represented only 11% of all PLK1 targets. This observation is in close agreement with the study of Lowery and colleagues, who searched for Polo box domain (PBD)-interacting proteins using U2OS whole-cell extracts (Lowery et al., 2007). They found that only a small percentage were actually involved in classical PLK1-related mitotic functions, while the majority of PBD-interacting proteins were involved in other cellular processes, including transcriptional regulation. Using Poloxin to inhibit the PBD, we have now shown that the PBD has an essential function in the transcriptional regulatory activity of PLK1.

Identifying a tumor-suppressive role of PLK1 at the level of gene transcription raises the question of how to reconcile this with the tumor-progressive properties of PLK1. A possible explanation could be that overexpression of PLK1 drives cells faster through mitosis, while basal levels of PLK1 are needed to maintain a tumor-suppressive transcriptional program. Our findings therefore request a reconsideration of the role of PLK1 in breast cancer that takes into account the different levels of expression of the kinase in different tumor cell populations.

EXPERIMENTAL PROCEDURES

Cell Culture and Hormone Treatments

MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and standard antibiotics. For hormonal starvation, MCF7 cells were cultured for 3 days in phenol-red-free DMEM supplemented with 5% charcoal/dextran-treated FCS. T47D-MVTL cells were cultured and hormone deprived as described previously (Vicent et al., 2006). Cells were induced with E2 (10 nM), R5020 (10 nM), or vehicle (ethanol) for the indicated times. For knockdown experiments, siRNAs were transfected using Lipofectamine 2000 at day 1 of the starvation process. Control and PLK1 knockdown cells were harvested after hormone induction, stained with DAPI, and sorted by flow cytometry into G0/G1 and G2/M cell populations based on their DNA content.

RNA Extraction and RT-PCR

RNA was extracted with the RNeasy extraction kit (QIAGEN). Complementary DNA (cDNA) was generated from 100 ng of total RNA with the First Strand cDNA Superscript II Synthesis kit (Invitrogen) and analyzed by quantitative PCR (qPCR). Gene-specific expression levels were regularly normalized to *Gapdh* expression. Primer sequences are listed in Table S6.

Coimmunoprecipitation

Cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 0.5% Triton-X), protease inhibitor cocktail, and phosphatase inhibitors for 30 min at 4°C. Extracts were cleared by centrifugation and incubated overnight with anti-ER or normal rabbit IgG bound to protein A Dynabeads. Immunoprecipitated material was washed four times with Tris-buffered saline and analyzed by western blot.

ChIP and Re-ChIP Assays

ChIP assays were performed as described elsewhere (Strutt and Paro, 1999), with minor modifications. Cells were crosslinked in medium containing 1% formaldehyde for 10 min at 37°C. Crosslinking was quenched with 125 mM glycine for 5 min at room temperature. Cells were washed twice with PBS and collected by scraping in PBS supplemented with protease and phosphatase inhibitors on ice. Cells were lysed in hypotonic buffer and centrifuged and the pellet containing crude nuclei was lysed with SDS-lysis buffer. Chromatin was sheared by seven 30 s cycles in a Bioruptor (Diagenode). Around 20 μg of chromatin (DNA content) was diluted at least 10-fold with ChIP buffer and incubated with 5 μg of antibody overnight. Immunocomplexes were recovered with 40 μl of protein A agarose bead slurry for 2 hr with rotation and washed three times with ChIP buffer and once with ChIP buffer containing 500 mM NaCl. Eluted DNA complexes were incubated at 65°C overnight, and purified DNA was analyzed by qPCR. Primer sequences are listed in Table S6.

For the re-ChIP assays, after the washes of the first immunoprecipitation using ER or rabbit IgG antibodies, the immunocomplexes were eluted with 10 mM DTT at 37°C for 30 min, diluted 50-fold with ChIP buffer, and immunoprecipitated with PLK1 or rabbit IgG antibodies.

Agilent Gene Expression Profile and Gene Ontology

Labeled complementary RNA was hybridized to the Agilent Human whole genome 4X44k v2 microarray according to the manufacturer's protocol. Intensity data were extracted using Feature Extraction software (Agilent) and processed using the Agi4x44PreProcess-package of the Bioconductor project in the R statistical environment. Differential expression analysis was carried out on noncontrol probes with an empirical Bayes approach on linear models (limma) (Smyth, 2004). Results were corrected for multiple testing according to the false discovery rate (FDR) method (Benjamin-Hochberg). A cutoff of 1.5 and an FDR of 0.01 were chosen to analyze the regulation of gene expression. To address the effect of BI2536 on ER-mediated gene induction, the contrasts of regulation by E2 in the presence and absence of BI2536 were required to be 1.3-fold different with a p value <0.05. GO data were generated using the DAVID Bioinformatics Resources 6.7 (Huang et al., 2009).

Gene Expression Data from Human Breast Tumors

The microarray data set from 268 tamoxifen-treated patients was obtained from a previous study (Loi et al., 2007). All data were normalized previously by robust multiarray analysis. The preprocessed 1,881-sample breast tumor data set was previously described (Ringnér et al., 2011). For the integrated expression analysis, patients were ranked for the average expression of the studied gene set and top 33% versus bottom 33% analyzed for distant metastasis-free survival according to Zwart et al. (2011). The p values for Kaplan-Meier curves were calculated with a log-rank test (Mantel-Haenszel), and the corresponding HR was determined by the Mantel-Cox method.

SILAC Phosphoproteomics

SILAC-labeled MCF7 cells were cultured for 3 days in SILAC media without phenol red supplemented with 5% charcoal-treated dialyzed FCS. The unlabeled and the two metabolically labeled cell populations were tested individually for their ability to respond to E2 by measuring the levels of ER S118 phosphorylation without the detection of any difference (Figure S3B). Cells were treated with BI2536 (100 nM) or vehicle (DMSO) for 1 hr followed by induction with E2 (10 nM) or vehicle (ethanol) for 30 min. Cells were harvested on ice in PBS and lysed in modified RIPA buffer containing protease and phosphatase inhibitors. Extracts were separated by centrifugation. Proteins in the supernatant were acetone precipitated overnight and redissolved in urea buffer supplemented with phosphatase inhibitors. The insoluble pellet was resuspended in urea buffer with phosphatase inhibitors and benzonase and extracted under rotation at room temperature. Extracts of soluble and pellet extracts were each mixed in a 1:1:1 (w:w:w) ratio, reduced, alkylated, and subsequently digested with endoproteinase Lys-C and trypsin. Desalted peptides were separated by strong cation exchange chromatography and enriched for phosphorylated peptides using TiO_2 beads. Peptides were desalted on C18 StageTips, dried to almost completeness, and reconstituted in 4% FA for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

The peptides were analyzed on an Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) linked to an online nanoflow high-performance liquid chromatography (HPLC) system via a nanoelectrospray ion source and fragmented via higher-energy C-trap dissociation (HCD) (Nagaraj et al., 2010).

Bioinformatic Analysis of Phosphoproteome Data

Raw data were processed with MaxQuant software (volume 1.2.2.5) and processed as per the standard workflow previously described (Cox et al., 2011; Geiger et al., 2012). Bioinformatic analysis was performed using Perseus software (volume 1.2.0.17). For identification of regulated phosphosites, a threshold of 1.5-fold regulation was used, which had to be fulfilled in both replicate experiments. Regulation by E2 was measured using normalized M/L ratios, and regulation by BI2536 was measured using normalized H/M ratios. If a peptide contained several phosphorylation sites, regulatory information was included for all possible phosphorylation states. For motif-enrichment analysis, phosphosites were filtered for a localization probability >0.75 to qualify them as class I sites (Cox et al., 2011) (Figure S3E), and Fisher's exact test was performed for regulated phosphosites respective to all measured phosphosites. For GO-term enrichment analysis, Fisher's exact test was applied for all regulated phosphosites compared to all measured phosphosites at the protein level.

ACCESSION NUMBERS

Microarray data have been deposited in the Gene Expression Omnibus database under the accession number GSE46856. The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD000275.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.05.024.

LICENSING INFORMATION

ACKNOWLEDGMENTS

We would like to thank Thorsten Berg for kindly providing the Poloxin compound; Johan Staaf for the preprocessed 1,881-sample breast tumor data set; Giancarlo Castellano, Sarah Bonnin, and Andrew Pohl for bioinformatic support; Alessandra Ciociola and Roni Wright for experimental help; and Juan Valcarcel for critically reading the manuscript. Mass spectrometry, microarray, and cell sorting analyses were performed at the UPF/CRG proteomic, microarray, and flow cytometry core facilities, respectively. G.V. has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement number 299429 and from the European Molecular Biology Organization (EMBO long-term fellowship ALTF 1106-2011, cofunded with the European Commission EMBOCOFUND2010, GA-2010-267146). This study was supported by grants from the Spanish government (BMC 2003-02902 and 2010-15313; CSD2006-00049), the European Union (IP HEROIC), and the Catalan government (AGAUR).

Received: December 30, 2012
Revised: April 4, 2013
Accepted: May 13, 2013
Published: June 13, 2013

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