BCL-2 hypermethylation is a potential biomarker of sensitivity to anti-mitotic chemotherapy in endocrine-resistant breast cancer.

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Abstract

Overexpression of the anti-apoptotic factor, BCL-2, is a frequent feature of malignant disease and is commonly associated with poor prognosis and resistance to conventional chemotherapy. In breast cancer, however, high BCL-2 expression is associated with favourable prognosis, estrogen receptor (ER) positivity and low tumour grade; whilst low expression is included in several molecular signatures associated with resistance to endocrine therapy. In the present study, we correlate BCL-2 expression and DNA methylation profiles in human breast cancer and in multiple cell models of acquired endocrine-resistance to determine whether BCL-2 hypermethylation could provide a useful biomarker of response to cytotoxic therapy. In human disease, diminished expression of BCL-2 was associated with hypermethylation of the second exon, in a region that overlapped a CpG island and an ER-binding site. Hypermethylation of this region, which occurred in 10% of primary tumours, provided a stronger predictor of patient survival ($p=0.019$) when compared to gene expression ($n=522$). In multiple cell-models of acquired endocrine-resistance, BCL-2 expression was significantly reduced in parallel with increased DNA methylation of the exon 2 region. The reduction of BCL-2 expression in endocrine-resistant cells lowered their apoptotic threshold to anti-mitotic agents: nocodazole, paclitaxel and the PLK1 inhibitor, BI2536. This phenomenon could be reversed with ectopic expression of BCL-2, and rescued with the BCL-2 inhibitor, ABT-737. Collectively, these data imply that BCL-2 hypermethylation provides a robust biomarker of response to current and next generation cytotoxic agents in endocrine-resistant breast cancer, which may prove beneficial in directing therapeutic strategy for patients with non-resectable, metastatic disease.

Introduction

At least 70% of breast cancers are classified as estrogen-receptor (ER) positive and consequently, the majority of patients with this disease receive some form of endocrine therapy as part of their adjuvant care (1). Whilst endocrine therapy reduces the risk of disease recurrence, 28% of luminal A and 43% of luminal B breast cancer patients will acquire drug resistance and develop distant metastases within 15 years of first receiving treatment (2). Metastases will most commonly form in the bone (19% of luminal A, 30% of luminal B), but also in the brain, lung and liver, ruling out surgical intervention for most patients (2). Current second-line therapeutic strategies remain limited to the sequential delivery of alternative endocrine therapies and in some instances, targeted therapies to growth-factor receptors (3-5). Responses, however, are often short-lived. The median duration of survival from time of relapse is 2.2 and 1.6 years for luminal A and luminal B breast cancer patients respectively (2); highlighting the need for more effective therapeutic approaches to metastatic disease, with companion biomarkers of response that can be profiled in the absence of a tumour biopsy.

In an effort to improve upon traditional chemotherapeutic agents that inhibit mitotic progression, such as taxanes and vinca alkaloids, small molecule inhibitors that target proteins specific to the mitotic spindle have been developed (6). These include inhibitors of the polo-like kinases (PLK), most commonly Plk1, which block mitotic spindle assembly in dividing cells, thereby causing arrest in the G_2/M phase of the cell cycle. Following mitotic arrest many cancer cells undergo cell death caused by the activation of the intrinsic apoptotic pathway (7). The activation of this pathway can be blocked by BCL-2, an anti-apoptotic factor that is overexpressed in many forms of cancer, including 90% of colorectal cancer, 70% of breast cancer, 30–60% of prostate cancer and 80% of B-cell lymphomas (8). In the majority of cancer-subtypes, the overexpression of BCL-2 is generally associated with poor survival and reduced sensitivity to cytotoxic chemotherapies (9, 10). In light of this, BCL-2 inhibitors that counteract its pro-survival function and serve to sensitise cancer cells to cytotoxicity have been developed, and are currently undergoing clinical analysis (11).

In breast cancer, however, high BCL2 expression is predictive of favourable prognosis and low tumour grade (12, 13). Whilst this may appear incongruent, in breast tissue, BCL-2 is regulated by estrogen and is associated with high ER expression, although it's expression is

not simply a surrogate for ER expression (13-15). Low BCL-2 expression is included in several molecular signatures associated with acquired resistance to endocrine therapy and poor prognosis in primary breast cancer (16-18). Since diminished BCL-2 expression in cancer confers increased sensitivity to cytotoxic chemotherapy, it is possible that breast cancer patients with endocrine-resistant disease could achieve significant therapeutic benefit from cytotoxic agents when used as a second-line treatment.

Although the mechanism for BCL-2 suppression in endocrine-resistant breast cancer has not been established, we and others have shown that following the acquisition of resistance, estrogen-regulated genes suppressed by tamoxifen are subject to permanent epigenetic silencing (19-22), so that well-characterised ER targets such PgR and pS2 become silenced by promoter hypermethylation in models of acquired tamoxifen resistance (19). Interestingly, a significant proportion of patients who receive adjuvant tamoxifen show reduced BCL-2 expression after 6 months treatment (23). Since patients typically receive tamoxifen for 5 years, there is considerable potential for further down-regulation and possible epigenetic modification in clinical breast cancer; however, there have been no reports linking BCL-2 methylation and expression in endocrine-resistant breast cancer to date.

With the emergence of epigenetic biomarkers for early detection of disease, tumour classification and response to treatment being explored in the clinic (24), it is possible that methylated BCL-2 could potentially provide a non-invasive biomarker for chemo-sensitivity in metastatic, non-resectable endocrine-resistant breast cancer. In the present study, we address whether promoter hypermethylation is associated with BCL-2 expression in breast cancer and whether this commonly occurs in anti-hormone resistant breast cancer cells. Additionally, since low BCL-2 expression is associated with increased apoptotic response in other cancer sub-types, we investigate whether the suppression of BCL-2 could be exploited to confer increased sensitivity to anti-mitotic agents, including the Plk1 inhibitor, BI2536.

Material and Methods

TCGA Breast cancer data analysis: Expression data in the form of RPKM-normalised RNA-Seq data for BCL-2 were obtained from TCGA breast cohort (25) via the cBio data portal (n=774 tumours) (http://www.cbioportal.org/public-portal/index.do (26). Methylation data in the form of probe-level beta-values from Infinium HumanMethylation450 BeadChips were obtained from the Data-Matrix section of the TCGA data portal (n=522 tumours, n=96 normal tissue) (https://tcga-data.nci.nih.gov/tcga/), and the subset of probes with valid data spanning BCL-2 was identified (n=42). Clinical data including follow-up were obtained from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) and analysed using R (v2.15.1). The association between methylation and RNASeq (n=485) was assessed using the maximal information coefficient (MIC) (27). We chose MIC rather than Spearman or Pearson's correlation because MIC is capable of identifying mutual exclusivity relationships (27), which matched our expectation that expression would only be seen when methylation is low, and vice versa. Survival analyses were performed using the survival package in R (28), using Cox-models built against penalised-spline predictors of methylation levels (n=522) or RNASeq data (n=774), with disease-specific survival as the clinical endpoint. Due to fitting penalised regression splines, we report the likelihood ratio test p-value, where $p<0.05$ defined our level of statistical significance. Correlation between BCL-2, PLK1 and ER expression levels was assessed using the Spearman's rank correlation test.

Cell culture and retroviral infection: MCF7 cells originally obtained from AstraZeneca and their tamoxifen-resistant (TAMR), fulvestrant-resistant (FASR) and estrogen-deprived (MCF7X) derivatives were cultured as previously described (29-31). All cell-lines were authenticated by STR profiling (CellBank, Australia) and cultured for less than 6 months after authentication. TAMR stably expressing the EcoR protein were generated by transfection with the retroviral vector pQCXIN (Clontech) (32) and subsequently infected with pQCXIP-BCL-2α retrovirus (33). Expression was confirmed by western blot.

Proliferation and apoptosis assays: Relative cell number was assessed using a crystal violet-based colorimetric assay. Data are presented as average absorbance per sample, corrected for background. Cell cycle analysis was achieved by flow cytometric analysis of propidium iodide-stained, ethanol-fixed cells. Apoptotic cell populations were determined by staining methanol-fixed cells with the M30 CytoDEATH antibody (Enzo Life Sciences). Inhibitors used

included BI2536 (Selleck Chemicals), paclitaxel (taxol) (LC Laboratories), nocodazole (Sigma), doxorubicin (Sigma) and ABT-737 (Selleck Chemicals).

Gene expression analysis: Quantitative reverse transcriptase PCR (RT-qPCR) used the High Capacity R.T Kit and inventoried TaqMan probes BCL-2 (Hs00608023_m1, which spans exon 2 and 3 i.e. specific for BCL-2α), Plk1 (Hs00153444_m1) and GAPDH (Hs02758991_g1) (Applied Biosystems).

Western blot analysis: Briefly, cell lysate samples (40 μg) were denatured, subjected to electrophoretic separation on SDS polyacrylamide gel and trans-blotted onto a PDVF membrane. Blots were blocked then incubated with the following primary antibodies: BCL-2 (α-isoform specific i.e. 26 kDa) (124, DakoCytomation), BCL-xL (#2764 Cell Signalling), ERα (sc-543 Santa Cruz), Plk1 (#4535 Cell Signalling), pPlk1 (Thr210) (#5472 Cell Signalling), PARP (#9542 Cell Signalling) and β-Actin (AC-15; Sigma). Secondary antibodies were applied (horseradish peroxidase–conjugated sheep α-mouse and donkey α-rabbit (Amersham Biosciences) and specific proteins were visualized by chemiluminescence.

Clonal bisulphite sequencing: The methylation status of a total of 32 CpG sites spanning the BCL-2 promoter from the 3' end of the (reverse strand) coding DNA sequence (CDS) of exon 2 and ending just after the upstream ER binding site (chr18: 60,985,184-60,985,640) (GRCh37/hg19) was interrogated. The bisulphite reaction was carried out on up to 1 μg of extracted genomic DNA for 12 hr at 55°C, under conditions previously described (19, 34). PCR amplifications were performed using the following bisulphite conversion primers: BCL-2 FW: 5'-gagaaatattgaaggggtattatttattattatattttat-3'; BCL-2 RV: 5'- acccrataccacctataatccaccta-3'. After cloning and sequencing the methylation state of the individual clones was analysed using BiQ Analyzer software.

Statistics: The statistical significance of pair-wise comparisons was derived using paired/unpaired t-tests where applicable. Where multiple independent factors were present, data were analysed using one-way ANOVA with post-hoc tests ($p<0.05$ was deemed statistically significant).

Results

BCL-2 methylation in human breast cancer

To determine whether BCL-2 expression in human breast cancer correlated with DNA methylation, we examined Illumina 450k Methylation Array and RNASeq data from The Cancer Genome Atlas (TCGA) breast cohort (25). Forty two methylation probes spanned the BCL-2 gene (Supplementary Fig S1A), with 27 overlapping the large promoter-associated CpG island (CpG 133; Supplementary Fig S1B), 7 overlapping the second exon and a smaller CpG island (CpG 27; Supplementary Fig S1C), 6 spanning the intron and the remaining 2 overlapping exon 3 (Supplementary Fig S1A). The relationship between BCL-2 gene expression and methylation status is shown for each probe in Supplementary Fig S2 (n=485), which illustrates the marked variability in both the extent of methylation, and the association with gene expression. This variability occurred in a manner that was consistent with the genomic location of the individual probes. Probes overlapping the small CpG island within exon 2 (CpG_27, probes 11-13) featured a clear inverse relationship between methylation and expression, with a distinct subgroup of patients exhibiting hypermethylation and complete repression of expression (illustrated for probe 11 in Fig 1A). Interestingly, probes 11 and 12 are in close proximity to an ER-binding site within exon 2 (Supplementary Fig S1C). The remaining probes spanning exon 2 (i.e. probes 9, 10, 14 and 15) showed a similar relationship between hypermethylation and loss of expression (Supplementary Fig S2). In marked contrast, the promoter-associated CpG island (spanned by probes 16 to 42) exhibited low overall methylation and no correlation with expression, while the intron and 3rd exon (spanned by probes 1 to 8) displayed near-maximal hypermethylation but no correlation with expression (Supplementary Fig S2).

To determine whether this was a cancer-specific event, the methylation level of each probe spanning exon 2 was compared in cancer (n=522) and normal (n=96) samples. Using a conservative cut-off of the 95th percentile of methylation in the normal samples (dashed red line in Figure 1A and 1B), the region of DNA interrogated by probe 11 was hypermethylated in 55/522 (10.5%) of breast cancers (Figure 1B), with a similar proportion for probes 12 and 13 (7.5 and 13.4%). As with the relationship between hypermethylation and loss of expression, this pattern was only observed for probes overlapping exon 2, and

was strongest for those probes that overlapped the CpG island (i.e. probes 11-13) (Supplementary Fig S3).

To establish if BCL-2 promoter methylation was significantly associated with disease-specific survival (DSS), Cox regression models were fitted to the tumour-specific methylation level of each individual probe across the cohort (n=522). A p-spline transformation of the methylation data was applied to permit non-linear relationships between methylation and hazard to be detected, and to smooth out the variation in the methylation data, thus providing a more robust predictor. The region of hypermethylation most significantly associated with DSS was that spanning exon 2, with $p<0.05$ for 3 of the 7 probes (e.g. $p=0.02$ for probe 11, likelihood ratio test) and $0.05<p<0.1$ for a further 2 probes (Fig 1C – dashed red line $p=0.05$). Outside this region, just three of the remaining 35 probes (probes 16, 25 and 41) reached borderline-significance ($0.05<p<0.1$); however, each of these probes displayed low levels of maximal methylation (Fig 1C - purple line) and thus these results are unlikely to represent a true association between hypermethylation and DSS at these loci.

To examine the relationship between methylation and DSS more closely, the Cox regression results were visualised in a term plot (Fig 1D, Supplementary Fig S4). For probes spanning the CpG- island within exon 2, a non-linear trend of increasing hazard with increasing methylation was identified, i.e. the risk of hazard accelerated with increasing methylation. This relationship was statistically significant for probe 11 ($p=0.02$) (Fig 1D) and probe 13 ($p=0.019$), and borderline significant for probe 12 ($p=0.06$). Term-plots for the majority of probes outside of exon 2 approximated a horizontal line, indicating that there was no strong relationship between increasing methylation and DSS (Supplementary Fig S4). The relationship between BCL-2 expression by RNA-Seq and DSS, whilst approaching significance ($p=0.078$), was weaker and showed no trend towards increasing hazard with lower expression levels (Fig 1D), indicating that BCL-2 methylation was a more precise predictor of survival.

BCL-2 methylation in cell models of endocrine resistance

Collectively, our analyses of TCGA data led to the identification of a specific region of the BCL-2 gene whose methylation status correlated with reduced gene expression and reduced patient survival in breast cancer. Data permitting the analysis of the relationship between BCL-2 methylation and response to endocrine therapy were, however, not available for this cohort. Thus, to determine whether BCL-2 was silenced by DNA methylation in endocrine

resistance, we used well-characterised cell-line models of endocrine resistance. These models were generated by long-term culture of MCF7 hormone-responsive breast cancer cells in the presence of the clinically relevant anti-estrogens tamoxifen (TAMR) (29) or fulvestrant (FASR) (30), or in the absence of estrogen (MCF7X) to provide a model of acquired resistance to estrogen-deprivation strategies (31). In contrast to the parental MCF7 cells, BCL-2 expression was significantly suppressed in the MCF7X cell-model and barely detectable in TAMR and FASR cell-lines (Fig 2A and 2B). Critically, BCL-2 suppression was not associated with reduced expression of BCL-xL, a closely related anti-apoptotic factor, nor did it correlate with ER status (Fig 2B).

The methylation status of 32 CpG residues in a 456bp region of DNA which spanned from the 3' end of exon 2 to beyond the ER-binding site (overlapping 450K methylation probes 9 to 11 - chr18:-60,985,184-60,985,640, GRCh37/hg19) was interrogated using clonal bisulphite sequencing (Fig 2C). Relative to the MCF7 cells, there was an increase in DNA methylation detected in the resistant cell-lines, particularly at the first 4 CpG sites (Fig 2C). Of the first 4 CpG sites interrogated, only 5 of 72 (i.e. 18 clones per CpG site) were methylated in the MCF7 cells (7%), in contrast to the MCF7X (24/72 – 33%), TAMR (43/72 – 60%) and FASR cells (14/44 (11 clones per CpG site) – 32%). The greatest increase in methylation was observed in the TAMR cells, particularly at the BCL-2 ER-binding site, which was almost entirely methylated (Fig 2C).

Effects of anti-mitotic agents on endocrine-resistant cell-lines

Since treatment options are limited in endocrine-resistant breast cancer, we next investigated whether BCL-2 suppression in cell line models of endocrine resistance potentiated the effects of anti-mitotic agents. We focussed on the Plk1 inhibitor, BI2536 (Fig 3A), one of a new generation of anti-mitotic therapies specifically targeting mitotic proteins, thus potentially offering therapeutic advantages over currently used anti-mitotic therapies that target tubulin (6, 7). The therapeutic value of Plk1 inhibition in endocrine-resistant breast cancer is further highlighted by the strong association of PLK1 expression with early relapse in ER-positive breast cancer patients receiving endocrine therapy (Cox $p=1.13x10^{-8}$) (see Supplementary Methods and Supplementary Fig S5). Furthermore, whilst TCGA data showed a positive correlation between BCL-2 and ER expression ($\rho= 0.61$) and a negative correlation between PLK1 and ER expression ($\rho= -0.35$), BCL-2 and PLK1 expression were also inversely related ($\rho= -0.43$) (Spearman's rank correlation test). Of the 46 breast cancers

in which low BCL-2 expression was associated with DNA methylation (Fig 1A), 36 (i.e. 78%) had robust PLK1 expression (Supplementary Fig S5B).

The anti-proliferative effect of BI2536 (1-100 nM) was assessed in all cell lines after 48 h treatment (Fig 3B). Maximal inhibition was achieved following treatment with 10 nM Bl2536; however, the magnitude of the maximal response varied (Fig 3B). The parental, endocrine-sensitive MCF7 cells were the least responsive, displaying a 30% decrease in cell number (compared to non-treated control cells), whilst cell number decreased by approximately 45%, 55% and 60% for MCF7X, FASR and TAMR cells respectively (Fig 3B). This difference in sensitivity was not due to the overexpression of Plk1 in the resistant cells, or the failure of the drug to reduce the expression of active, phosphorylated Plk1 (Fig 3C). All cell lines arrested in the G_2/M phase of the cell-cycle in response to BI2536 (Fig 3D). Although there was little difference in the cell cycle phase distribution between the cell lines, there was a greater increase in the sub-G_1 population of endocrine-resistant cell-lines compared to the MCF7 cells following treatment (Fig 3D), suggesting that increased apoptosis might account for the greater decrease in cell number.

Increased apoptosis in Bl2536-treated endocrine-resistant cells was confirmed by flow cytometric analysis of cells stained using an antibody specific for caspase-cleaved cytokeratin 18 (M30) (Fig 4A) and by measurement of PARP cleavage (Fig 4B). Although the percentage of M30-positive MCF7 cells increased 6-fold following 48 h incubation with BI2536 (10 nM), the increase in M30 positivity was more marked in the MCF7X (8-fold), TAMR (13-fold) and FASR (11-fold) cells (Fig 4A). In MCF7 cells, PARP cleavage was not observed following Bl2536 treatment, consistent with the muted cytotoxic response of these cells. However, PARP cleavage was apparent in Bl2536-treated MCF7X cells, and readily detectable in TAMR and FASR cells (Fig 4B), paralleling the relative increase in M30 detection (Fig 4A). To determine whether the enhanced response of the endocrine-resistant cell lines to Bl2536 was characteristic of other anti-mitotic agents, we treated cells with paclitaxel (TAX) and nocodazole (NOC) (Fig 4C). Again, a significant increase in apoptosis was observed in all three endocrine-resistant cell lines compared to the parental MCF7 cells (Fig 4D). However, there was no significant change in the apoptotic response to cell-cycle non-specific chemotherapeutic agent, doxorubicin, in the endocrine-resistant cell-lines compared to the endocrine-sensitive MCF7 cells (Fig 4D).

Relationship between BCL-2 expression and sensitivity to anti-mitotic agents

To determine whether increased apoptosis following treatment with anti-mitotic agents was dependent on BCL-2 expression, TAMR cells expressing BCL-2 (TAMR-BCL-2) were generated (Fig 5A). TAMR-BCL-2 cells were significantly less responsive to all anti-mitotic agents compared to empty vector-transduced TAMR cells (TAMR-EV) (Fig 5B & 5C) and their apoptotic response comparable with that of the MCF7 cells, as determined by either M30 flow cytometry (Fig 5B) or PARP expression (Fig 5C). In contrast, TAMR-BCL-2 and MCF7 cells pre-treated with the BCL-2 inhibitor, ABT-737 (Fig 5D), for 24 h prior to exposure to either BI2536, TAX or NOC (for a further 24 h) exhibited dramatically increased apoptotic responses, comparable to parental TAMR (Fig 4D) or TAMR-EV cells (Fig 5B & 5C). These cells constitutively express BCL-xL (Fig 2B & Fig 5A), highlighting BCL-2 expression as a critical determinant of cytotoxic response in these cells (summarised in Supplementary Fig S6).

Discussion

Despite decades of research dedicated to deciphering the mechanisms by which endocrine-resistant cells evade the inhibitory effects of anti-hormone drugs, effective therapeutic strategies for the management of endocrine-resistant breast cancer have yet to be identified. In the present study, we provide evidence that the expression of BCL-2 is diminished in cell models of acquired endocrine-resistant breast cancer in association with increased DNA methylation of the second exon; a region that contains a CpG island and an ER-binding site. The reduced expression of BCL-2 conferred increased sensitivity to cytotoxic agents including the Plk1 inhibitor, BI2536 and paclitaxel; highlighting BCL-2 methylation as a potential biomarker of response to current and next generation cytotoxic chemotherapy in non-resectable metastatic endocrine-resistant breast cancer.

Proof-of-concept analysis has demonstrated that circulating tumour DNA is an informative, inherently specific, and highly sensitive biomarker of metastatic breast cancer (35). Furthermore, recent publications have demonstrated the prognostic potential of methylated DNA, readily detected in serum samples from breast cancer patients (36, 37). The detection of methylated DNA as a biomarker of disease has several advantages over protein-based assays since defined regions of cancer-specific hypermethylated-DNA can be readily amplified from samples collected non-invasively using PCR-based technology (38). In addition, panels of DNA methylation probes specific to multiple genes can be readily assembled to further inform diagnosis, as demonstrated for lung cancer (39), ovarian cancer (40) and prostate cancer (41). Although the detection of methylated BCL-2 has not been assessed in breast cancer patient serum, recent publications describe the detection of BCL-2 methylation in blood samples derived from pancreatic cancer patients (42), and in urinary samples used to detect and monitor bladder cancer (43).

To our knowledge, the methylation of the DNA sequence encoding the second exon of BCL-2 has not been previously linked to gene expression in breast cancer. Interestingly, methylation of this region was observed in the development of colorectal carcinoma, however, it did not correlate with expression (44). This discrepancy between the relationship of BCL-2 methylation to gene expression in breast and colorectal cancer may be explained by the presence of an ER binding site within the small CpG island in exon 2. BCL-2 expression is largely dependent on estrogen regulation in breast cancer cells (15), and thus

hypermethylation of the ER-binding site is more likely to diminish BCL-2 expression in estrogen-responsive tissues.

BCL-2 expression was silenced in association with increased DNA methylation in approximately 10% of TCGA breast tumour samples. Although this equates to a substantial number of patients due to the high prevalence of the disease, this statistic is reflective of BCL-2 methylation incidence in non-stratified, untreated breast cancer patient samples. We hypothesise that the percentage of patients with silenced BCL-2 would be significantly greater in a cohort of patients with acquired tamoxifen resistance, as reflected in our *in vitro* models of endocrine resistance. Interestingly, BCL-2 methylation was a stronger molecular marker of disease specific death compared to gene expression. One possible explanation for this observation is that DNA hypermethylation is indicative of permanent epigenetic gene suppression, whereas in patients with no detectable BCL-2 methylation, but low BCL-2 expression, gene suppression may instead be a more transient result of altered regulation. Thus reduced BCL-2 expression likely combines two distinct phenotypes, one of which (BCL-2 promoter methylation) has a greater impact on prognosis, perhaps because it is a marker of more widespread epigenetic gene suppression.

All endocrine-resistant cell-lines exhibited increased methylation in the region of BCL-2 DNA interrogated by clonal bisulphite sequencing, and since even the smallest of changes in DNA methylation can have a significant impact on gene transcription, this likely contributes to diminished BCL-2 expression in these cell lines. However, the pattern of methylation varied. In contrast to the tamoxifen-resistant cells, the fulvestrant-resistant and estrogen-deprived cells did not exhibit DNA hypermethylation of the BCL-2 ER-binding site. This inconsistency may be attributed to the distinct mechanisms by which different endocrine therapies exert their anti-estrogen action. The presence of tamoxifen-bound ER at the gene promoter serves to accelerate the process of epigenetic silencing through the direct recruitment of co-repressors of transcription capable of recruiting DNA-methyl-transferases (DNMT) (45). In fulvestrant-resistant or estrogen-deprived cells, ER-binding sites remain unoccupied due to diminished levels of receptor or ligand, respectively, and thus epigenetic modifiers are not directly recruited to the site of transcription. It is possible, however, that the BCL-2 ER-binding site is suppressed by early epigenetic changes in the FASR and MCF7X cells, since repressive chromatin modifications are known to accumulate at ER-binding sites in the absence of ER-signalling, as demonstrated in proof of principle studies using ER siRNA (46).

Significantly, our data indicate that Plk1 inhibition may provide a novel therapeutic strategy in endocrine-resistant breast cancer, where low expression of BCL-2 serves to lower the apoptotic threshold of these cells (summarised in Supplementary Fig S6). The potential applicability of this therapeutic strategy to endocrine-resistant breast cancer is further highlighted by the strong association between Plk1 expression and tamoxifen failure in estrogen receptor-positive breast cancer patients. However, despite promising pre-clinical studies, Plk1 inhibitors have had limited success when used as single agents in patients. The most recent clinical trials in non-Hodgkin's lymphoma and pancreatic cancer have proven disappointing, with overall response rates of 9.8% and 2.3% respectively (47, 48). The failure of Plk1 inhibitors in the clinic is reflective of the lack of proven predictive biomarkers of response. Most recently p53 status was assessed as a predictor of response to Plk1 inhibition. No correlation between p53 expression/mutation and cytotoxic effect was observed, despite reports that the cytotoxicity induced by Plk1 inhibition is elevated in cancer cells with defective p53 (49). Our data suggest that BCL-2 status could potentially provide a robust predictive biomarker of response to Plk1 inhibition. Furthermore, Plk1 inhibitors could be used in combination with BCL-2 inhibitors, such as ABT-737 or the BCL-2 specific inhibitor, ABT-199, to maximise the cytotoxic effect on cancer cells that express BCL-2. Indeed, the anti-tumour effect of synergistic inhibition of BCL-2 and Plk1 expression has been previously demonstrated in xenograft models using antisense oligodeoxynucleotides (50).

Taken together, these data show that BCL-2 expression is associated with DNA methylation in human breast cancer and this represents a likely mechanism for diminished BCL-2 expression in a proportion of breast cancer patients with acquired endocrine-resistance. As such, BCL-2 hypermethylation may provide a robust biomarker of response to current and next generation cytotoxic agents, such as BI2536, which has significant therapeutic value in endocrine-resistant disease, given the strong association between Plk1 expression and early relapse in patients receiving endocrine therapy.

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Conflict of Interest

All authors declare they have no conflict of interest in relation to the submitted work.

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Figure Legends

Figure 1. ***BCL-2 expression is silenced by the DNA methylation of exon 2 in human breast cancer and correlates with poor survival.*** **A:** The relationship between BCL-2 methylation and expression is illustrated by data retrieved for probe 11. **B:** The methylation of BCL-2 in tumour (n=522) vs normal (n=96) samples (data from probe 11). The dashed red line represents the 95^{th} percentile of BCL-2 methylation in the normal samples. **C:** Cox proportional hazard p values for the 42 methylation probes that span BCL-2. The red dashed line represents p= 0.05. The purple line represents the maximal methylation recorded for each probe. **D:** Term-plot for BCL-2 methylation (probe 11) vs risk of hazard (left panel) and BCL-2 gene expression vs risk of hazard (right panel). The orange dashed lines represent 95% confidence intervals and the black bars represent individual samples.

Figure 2. ***BCL-2 expression is suppressed in cell-line models of endocrine-resistance and is associated with increased DNA methylation of exon 2 in endocrine-resistant cells.*** **A:** BCL-2 mRNA expression in MCF7 cells and the endocrine-resistant sub-lines, MCF7X, TAMR and FASR cells (****p<0.001, paired t-test). **B:** BCL-2, BCL-xL and ERα protein expression in MCF7 cells and endocrine-resistant sub-lines. **C:** Clonal bisulphite sequencing data for the interrogation of 32 CpG sites located in exon 2 of the BCL-2 gene (chr18:-60,985,184-60,985,640). Bisulphite maps determined by direct sequencing of individual clones (n=18 for MCF7, MCF7X and TAMR; n=11 for FASR) show the density of methylated CpG sites (black circles) and unmethylated CpG sites (white circles) at individual CpG residues. The red lines highlight 2 regions of differential methylation, i.e. the first 4 CpG residues of exon 2 and those that overlap the ERα binding site.

Figure 3. ***BI2536 causes G_2/M cell cycle arrest in endocrine sensitive and resistant cell-lines.*** **A:** The chemical structure of BI2536. **B:** The anti-proliferative effect of BI2536 (1, 3, 10, 30 and 100 nM) was assessed in MCF7, MCF7X, TAMR and FASR cells after 48 h using a crystal-violet based assay**.** Data points represent the mean ± SD (n=3). **C:** Plk1 mRNA expression (upper panel) and Plk1 and phosphorylated Plk1 (Thr 210) protein expression (lower panel) were measured by qRT-PCR and western blot respectively. Gene expression

data represent the mean ± SD (n=3) (***p<0.005, **p<0.01, *p<0.05, paired t-test). **D:** Flow cytometry was used to measure cell cycle phase distribution. Data are presented as percentage of cells in each phase relative to the total population (left panel - data represents mean ± SD n=3) and representative DNA histograms (right panel).

Figure 4. ***BI2536 and other anti-mitotic drugs induce apoptosis in endocrine-resistant, but not endocrine-sensitive cell lines.*** **A:** Flow cytometry was used to measure the percentage of cells undergoing apoptosis in response to BI2536 challenge (10 nM for 48 h). Data are presented as mean ± SD (****p<0.001, ***p<0.005, **p<0.01, *p<0.05, paired t-test) (n=3) (left panel) and representative DNA histograms (right panel). **B:** PARP cleavage was assessed in cells treated with BI2536 (10 nM for 48 h) by western blot. **C:** Chemical structures of paclitaxel (PAX), nocodazole (NOC) and doxorubicin (DOX). **E:** Flow cytometry was used to measure the percentage of cells undergoing apoptosis in response to BI2536 (10 nM), TAX (10 nM), NOC (100 ng/ml) and DOX (1 μM) 48 h challenge. Data are presented as mean ± SD (n=3) (****p<0.001, ***p<0.005, **p<0.01, *p<0.05, one-way ANOVA).

Figure 5. ***Anti-mitotic drug induced apoptosis is dependent on BCL-2 expression.*** **A:** TAMR cells were generated to ectopically express BCL-2 (TAMR-BCL-2) or empty-vector (TAMR-EV). The overexpression of BCL-2 had no impact on BCL-xL expression, as evidenced by western blot. **B:** Flow cytometry was used to measure the percentage of cells undergoing apoptosis in response to BI2536, TAX or NOC (24 h) following 24 h prior culture ± ABT-737 (1 μM). Data are presented as mean ± SD (n=3) (****p<0.001, one-way ANOVA). **C:** PARP cleavage was assessed in parallel by western blot. **D:** The chemical structure of ABT-737.

Supplementary Figure Legends

Supplementary Figure S1. UCSC Genome Browser images of Infinium Human Methylation 450 BeadChip (Illumina) probes that overlap the BCL-2 gene (A), the BCL-2 promoter region (B) and exon 2 (C).

Supplementary Figure S2. BCL-2 methylation vs mRNA expression for all 42 methylation probes that span the BCL-2 gene. Highlighted dot-plots are those for probes that overlap exon 2 i.e. probes 9 to 15. Those probes that overlap the exon 2 CpG island (i.e. probes 11 to 13) are enclosed by the dashed green lines.

Supplementary Figure S3. BCL-2 Methylation in tumour vs normal samples for all 42 methylation probes that span the BCL-2 gene. Highlighted box-plots are those for probes that overlap exon 2 i.e. probes 9 to 15. Those probes that overlap the exon 2 CpG island (i.e. probes 11 to 13) are enclosed by the dashed green lines.

Supplementary Figure S4. BCL-2 Methylation vs disease specific survival for all 42 methylation probes that span the BCL-2 gene. Highlighted term-plots are those for probes that overlap exon 2 i.e. probes 9 to 15. Those probes that overlap the exon 2 CpG island (i.e. probes 11 to 13) are enclosed by the dashed green lines.

Supplementary Figure S5. A: The association between Plk1 expression and relapse-free survival in endocrine treated ER-positive breast cancer patients (n=287) using multiple quantiles (green – high expression, red – low expression). **B:** TCGA RNA-Seq data (n=774) was used to show a positive correlation between ER and BCL-2 expression (ρ= 0.61) (top left panel) and a negative correlation between ER and PLK1 (ρ= -0.35) (top right panel) and BCL-2 and PLK1 expression (ρ= -0.43) (bottom left panel) (Spearman's rank correlation test; red line = line of best fit). The association between BCL-2 methylation and PLK1 expression was assessed using TGCA samples with both RNASeq and methylation data present (n=485) (bottom right panel). The red dashed line represents the 95th percentile of BCL-2 methylation in the normal samples. The dashed blue line represents an arbitrary cut-off for PLK1 expression (i.e. >5).

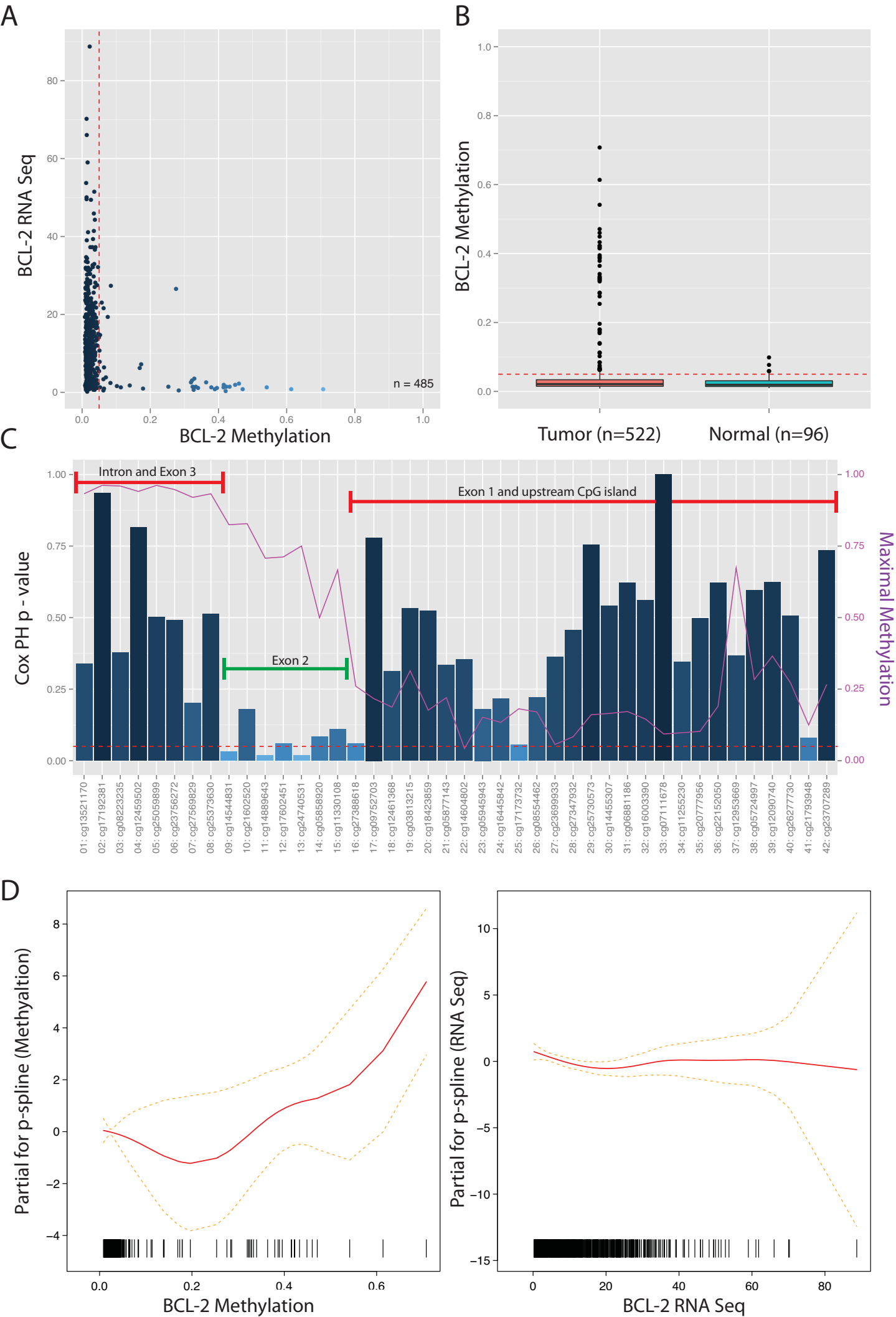
Supplementary Figure S6. A representation of the effect of BI2536 in endocrine-sensitive and endocrine-resistant breast cancer cells.

Supplementary Methods

Plk1 Expression/Survival Analysis in ER-Positive Patient Tumours: Affymetrix HG-U133A and HG-U133PLUS2 microarray data from a total of 863 breast cancers were normalized and combined into a single data set as previously described (1). Survival analyses were restricted to patients with unambiguous treatment and survival information who had not received any chemotherapy (n=287).

1. Caldon CE, Sergio CM, Kang J, Muthukaruppan A, Boersma MN, Stone A, et al. Cyclin E2 Overexpression Is Associated with Endocrine Resistance but not Insensitivity to CDK2 Inhibition in Human Breast Cancer Cells. Molecular Cancer Therapeutics. 2012;11(7):1488-99.

Figure 1


A
BCL-2 RNA Seq
BCL-2 Methylation
n = 485
B
BCL-2 Methylation
Tumor (n=522)
Normal (n=96)
C
Cox PH p - value
Maximal Methylation
Intron and Exon 3
Exon 1 and upstream CpG island
Exon 2
01: cg13521170
02: cg17192381
03: cg08223235
04: cg12459502
05: cg25059899
06: cg23756272
07: cg27569829
08: cg25373630
09: cg14544831
10: cg21602520
11: cg14889643
12: cg17602451
13: cg24740531
14: cg05858920
15: cg11330108
16: cg27388618
17: cg09752703
18: cg12461368
19: cg03813215
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21: cg05877143
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28: cg27347932
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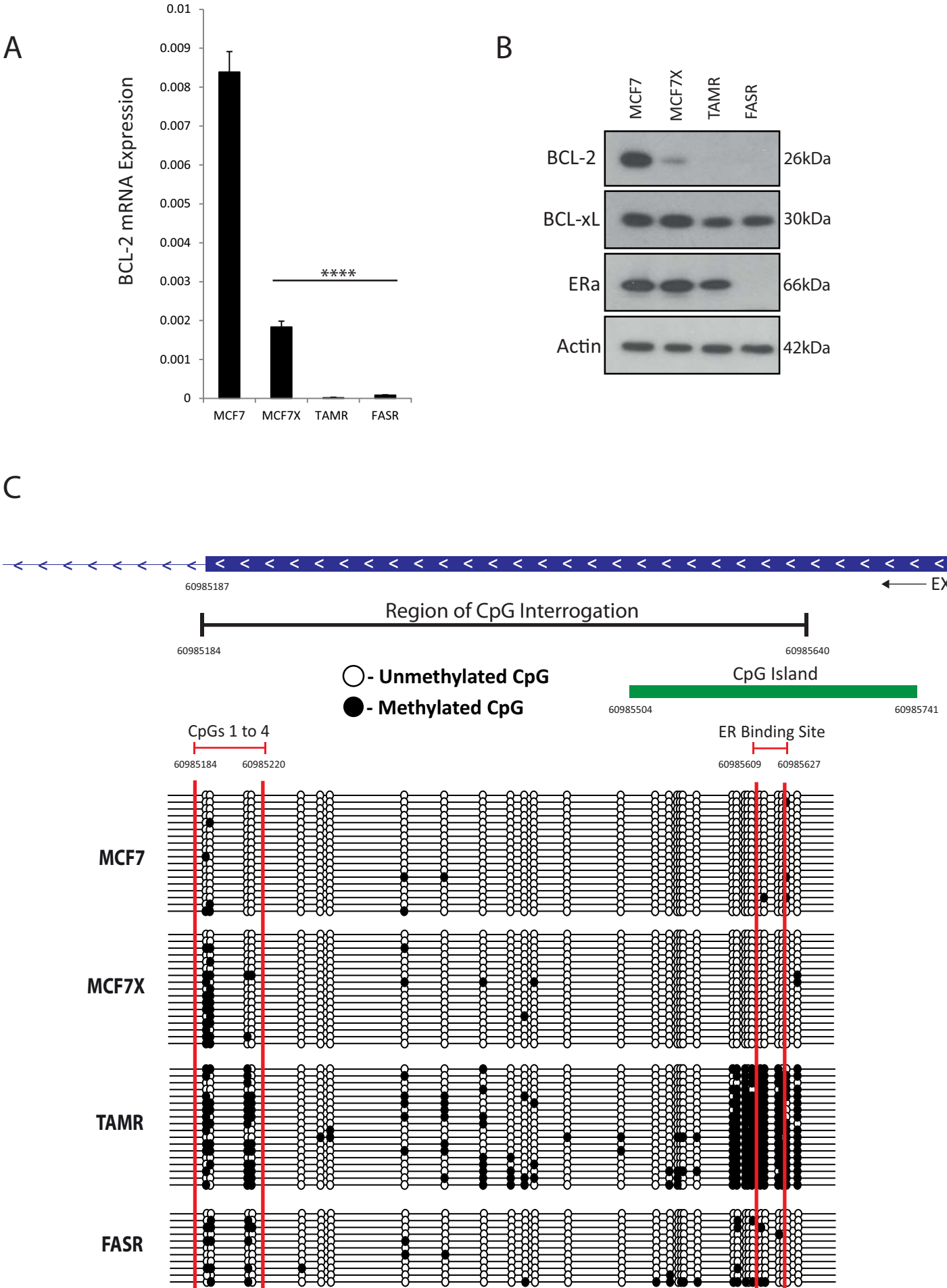
30: cg14455307
31: cg06881186
32: cg16003390
33: cg07111678
34: cg11255230
35: cg20777956
36: cg22152050
37: cg12953669
38: cg05724997
39: cg12090740
40: cg26277730
41: cg21793948
42: cg23707289
D
Partial for p-spline (Methyaltion)
BCL-2 Methylation
Partial for p-spline (RNA Seq)
BCL-2 RNA Seq

Figure 2


A
BCL-2 mRNA Expression
0.01
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
****
MCF7
MCF7X
TAMR
FASR
B
MCF7
MCF7X
TAMR
FASR
BCL-2
26kDa
BCL-xL
30kDa
ERa
66kDa
Actin
42kDa
C
60985187
EXON2
Region of CpG Interrogation
60985184
60985640
- Unmethylated CpG
- Methylated CpG
CpG Island
60985504
60985741
CpGs 1 to 4
60985184
60985220
ER Binding Site
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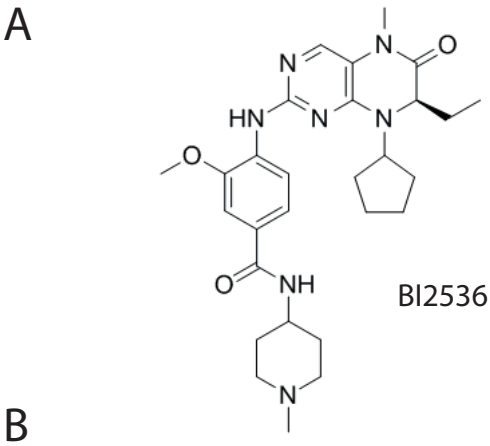
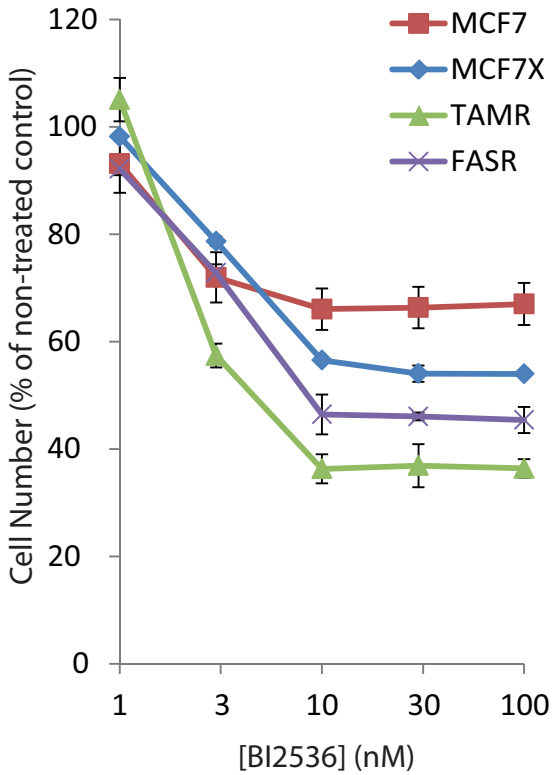
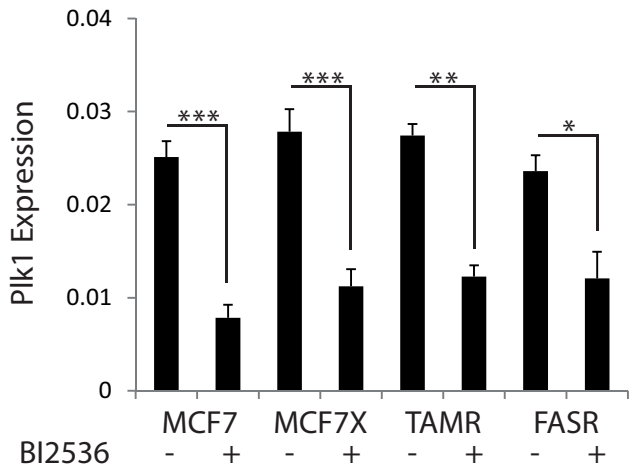
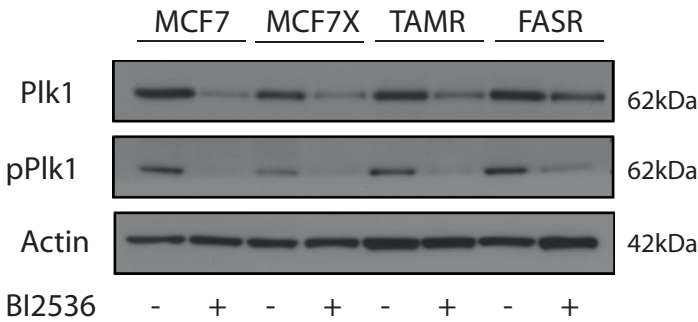
60985627
MCF7
MCF7X
TAMR
FASR

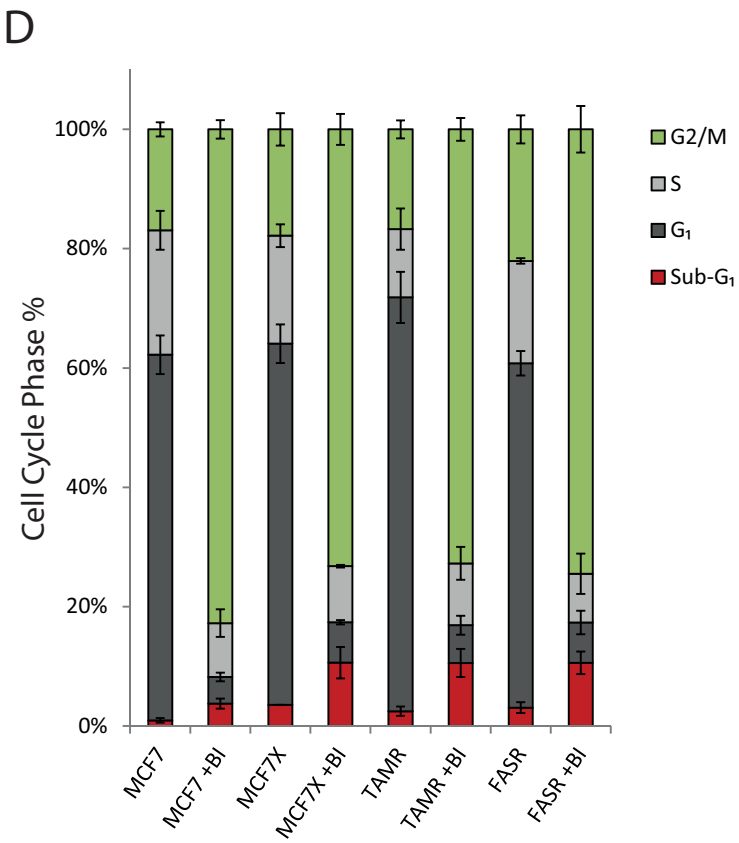
Figure 3


A
BI2536


B
MCF7
MCF7X
TAMR
FASR
Cell Number (% of non-treated control)
[BI2536] (nM)


C
Plk1 Expression
MCF7
MCF7X
TAMR
FASR
BI2536


MCF7
MCF7X
TAMR
FASR
Plk1
pPlk1
Actin
62kDa
62kDa
42kDa
BI2536


D
G2/M
S
G1
Sub-G1
Cell Cycle Phase %
MCF7
MCF7 +BI
MCF7X
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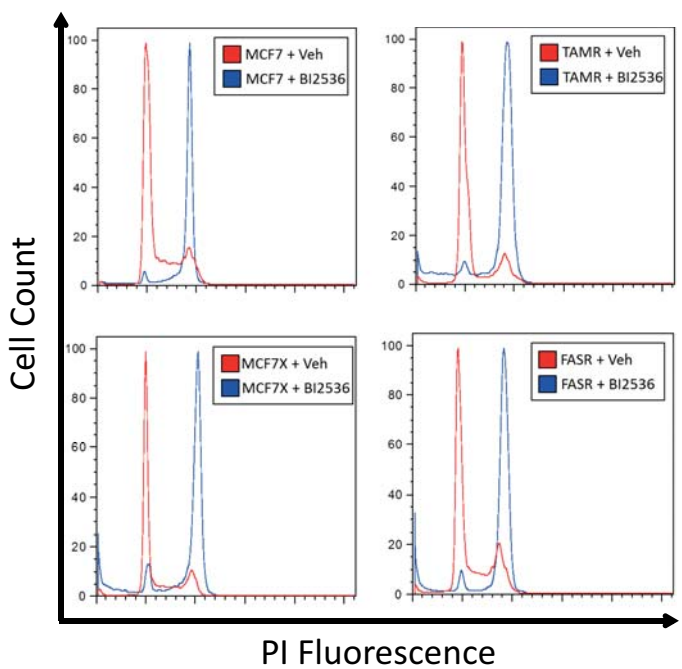
TAMR
TAMR +BI
FASR
FASR +BI


MCF7 + Veh
MCF7 + BI2536
TAMR + Veh
TAMR + BI2536
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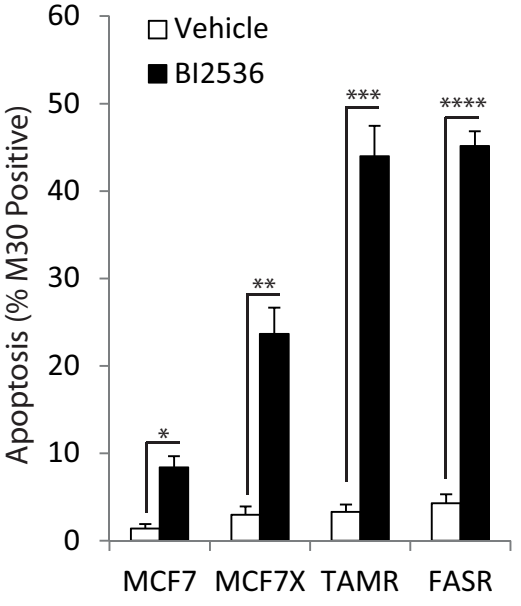
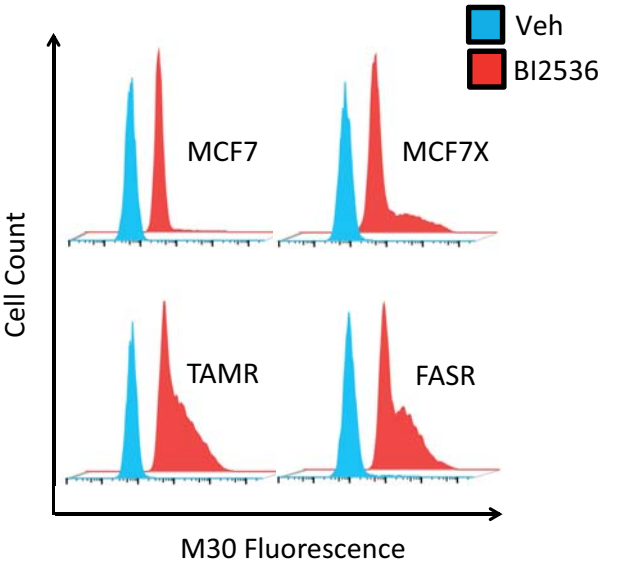
MCF7X + BI2536
FASR + Veh
FASR + BI2536
Cell Count
PI Fluorescence

Figure 4

A





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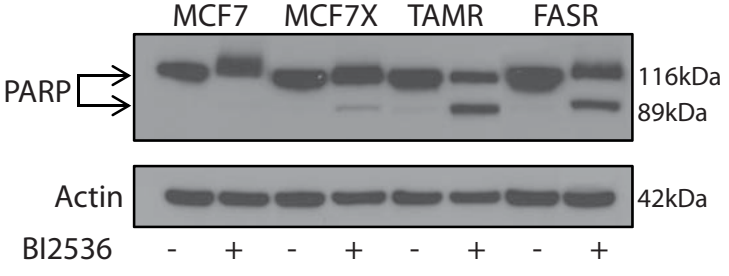

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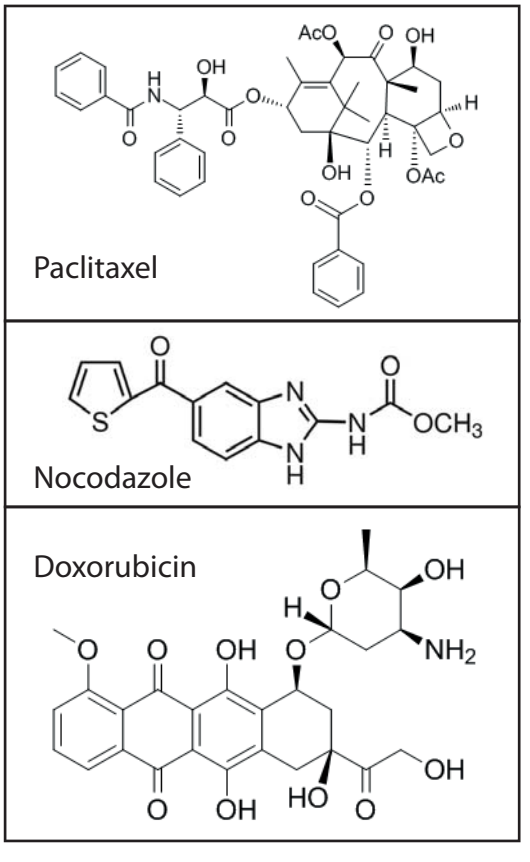


D

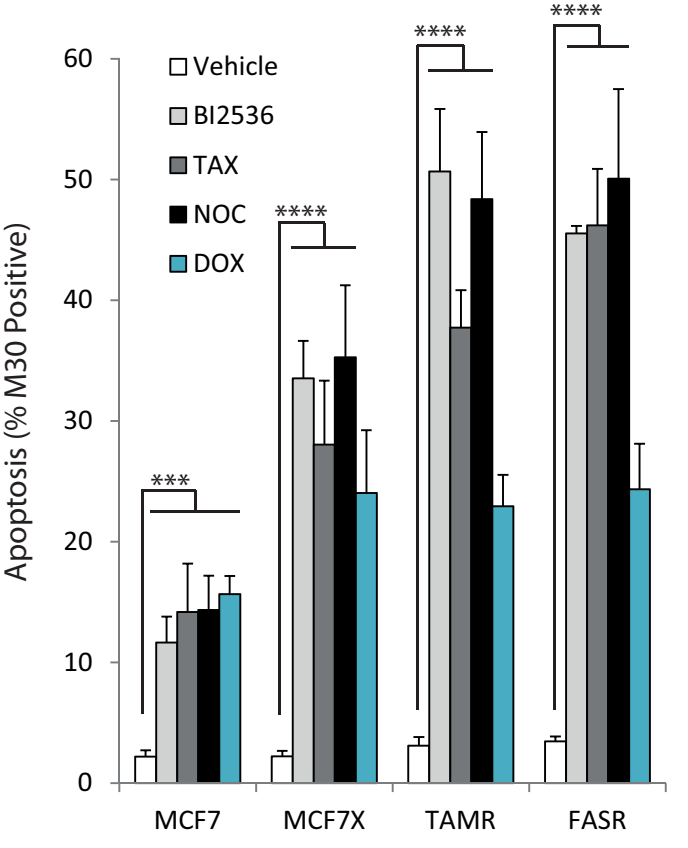
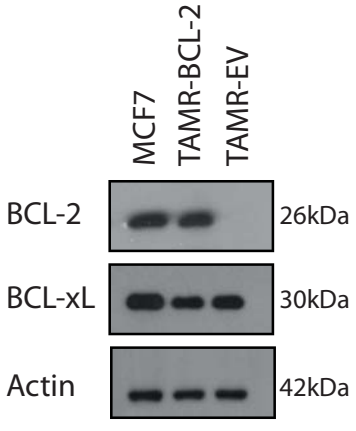


Figure 5

A



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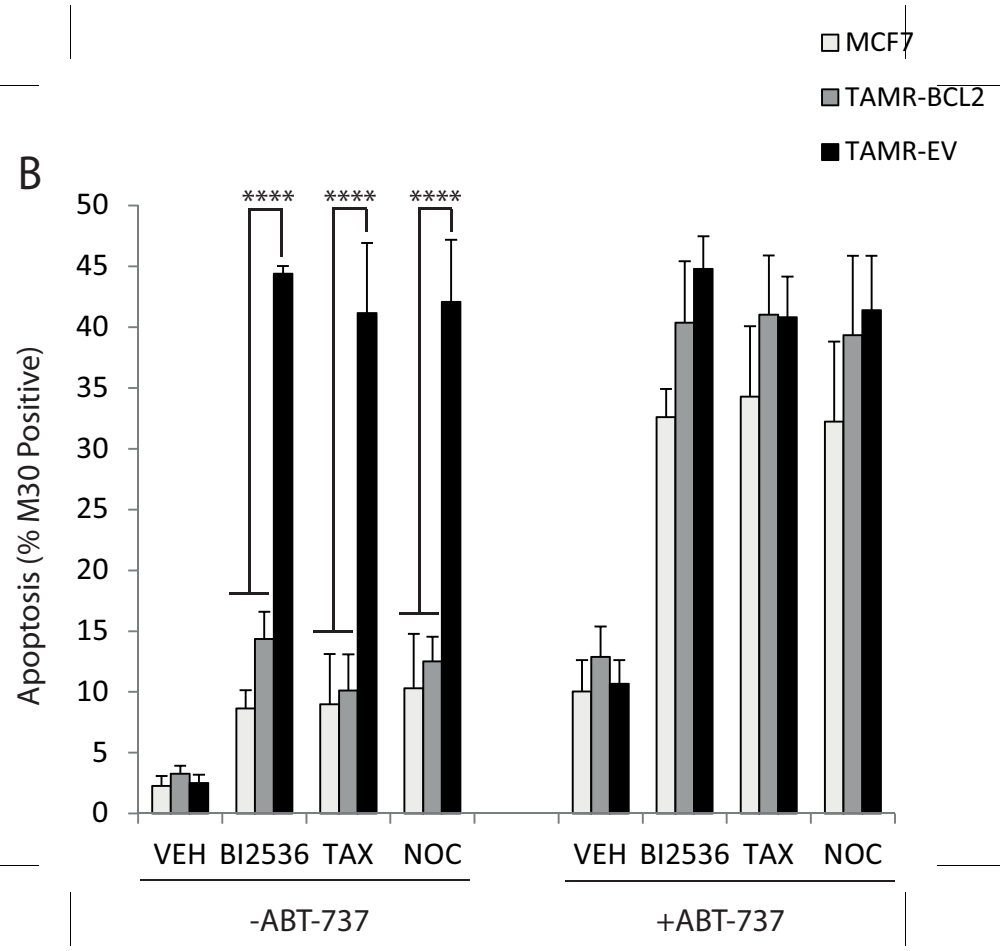

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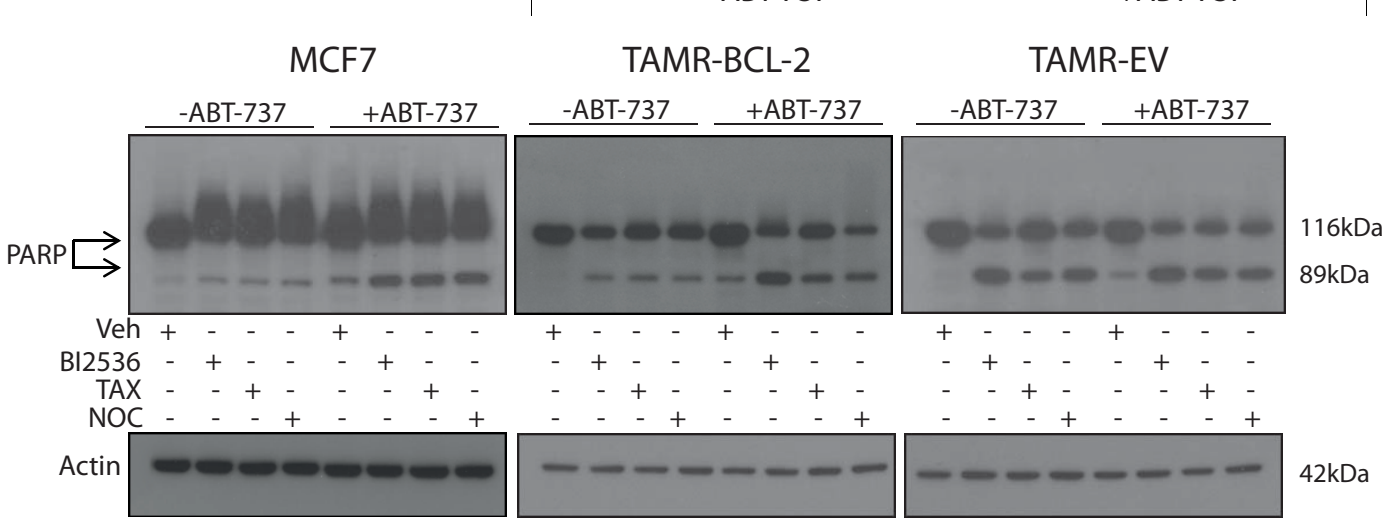

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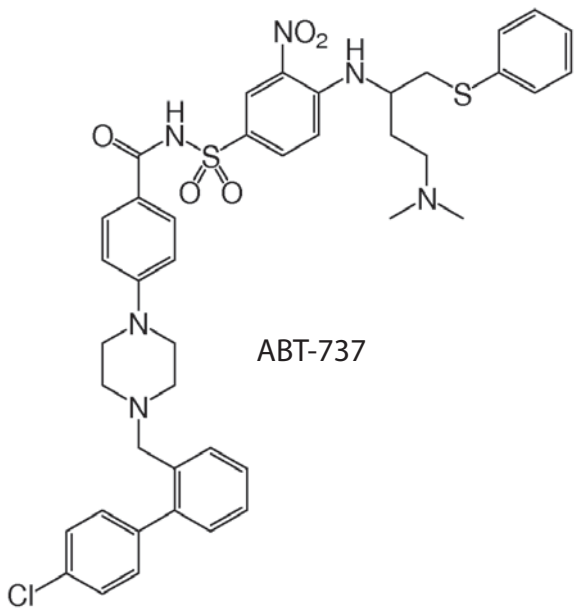
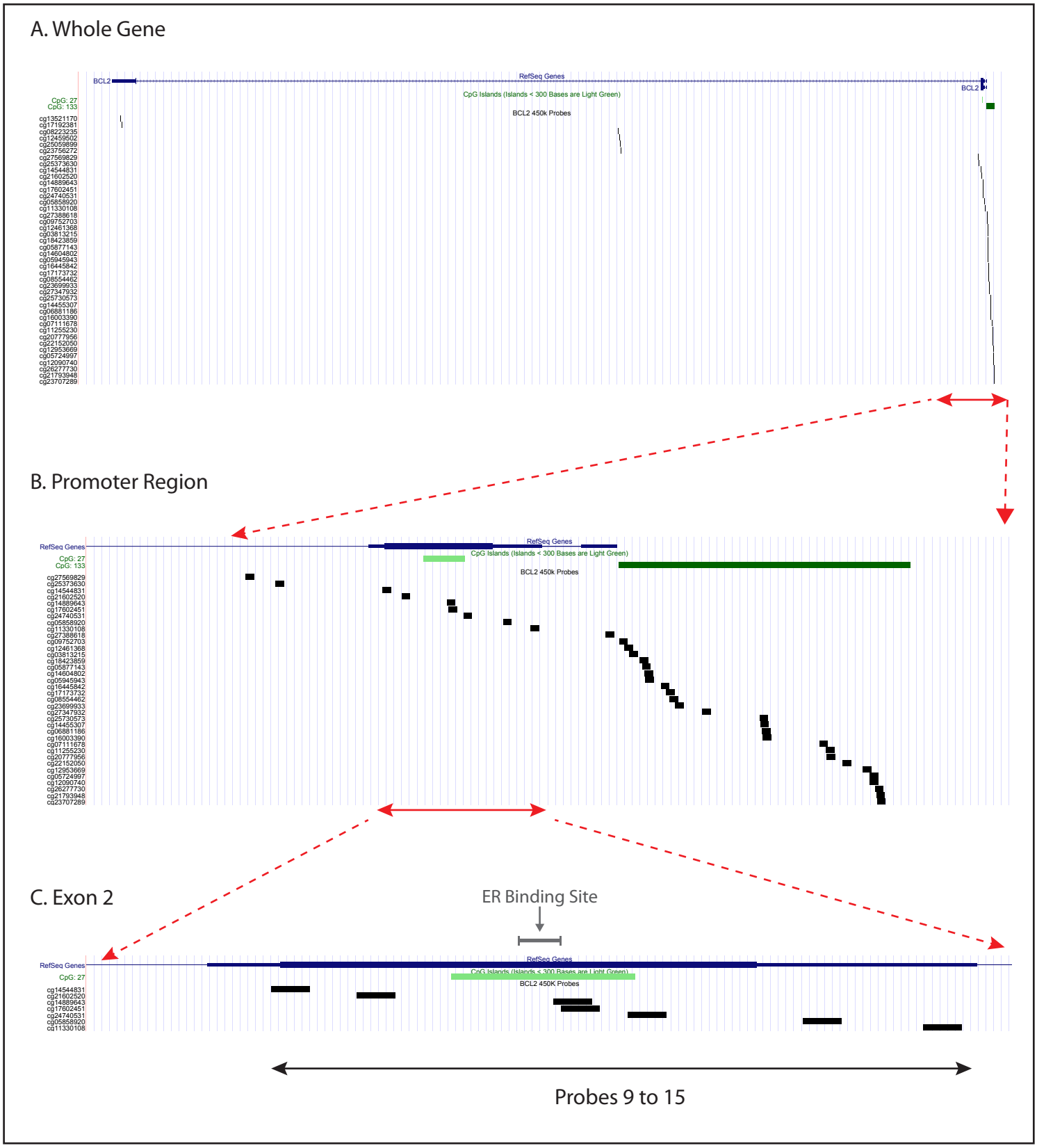
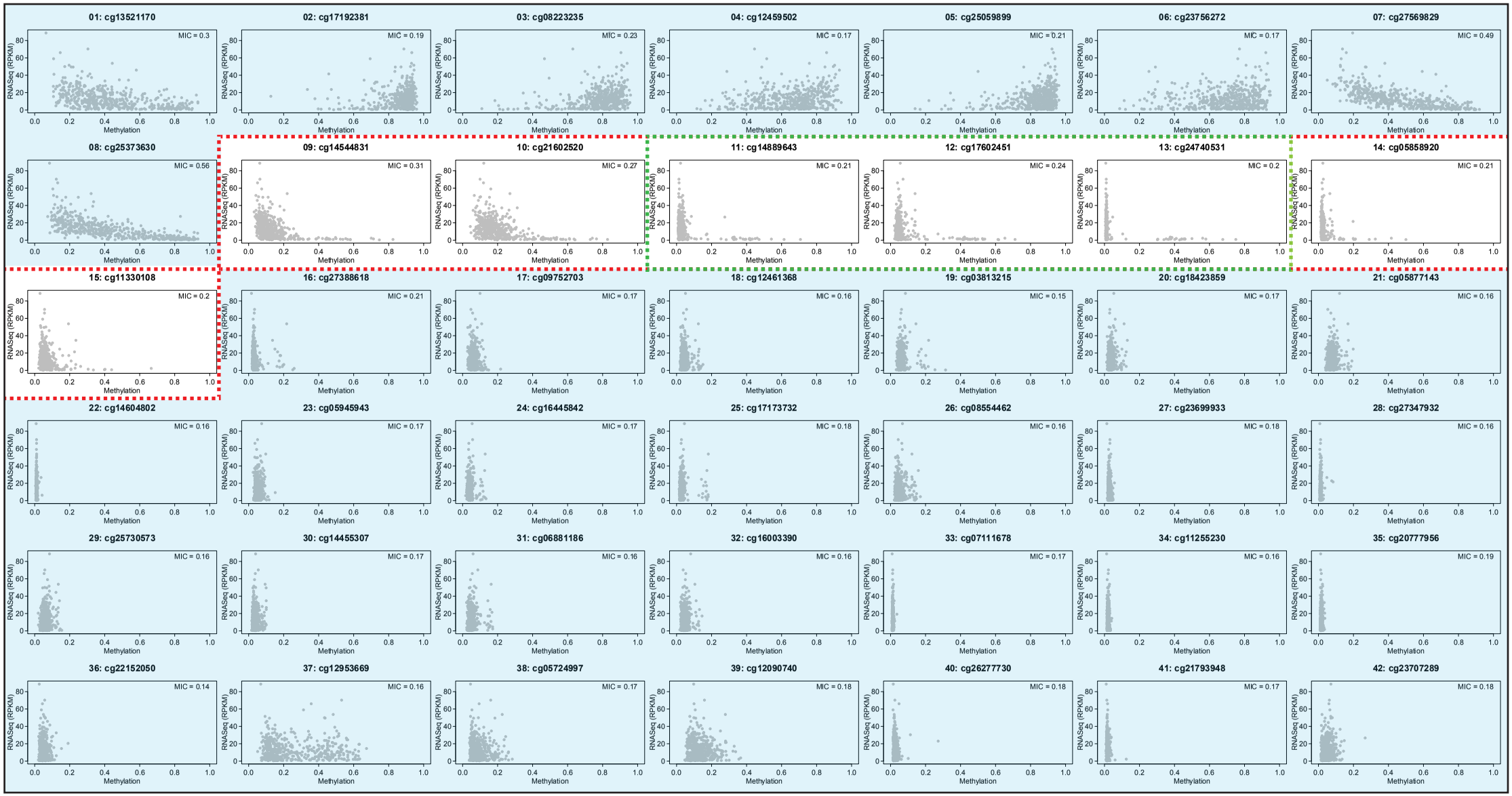


ABT-737

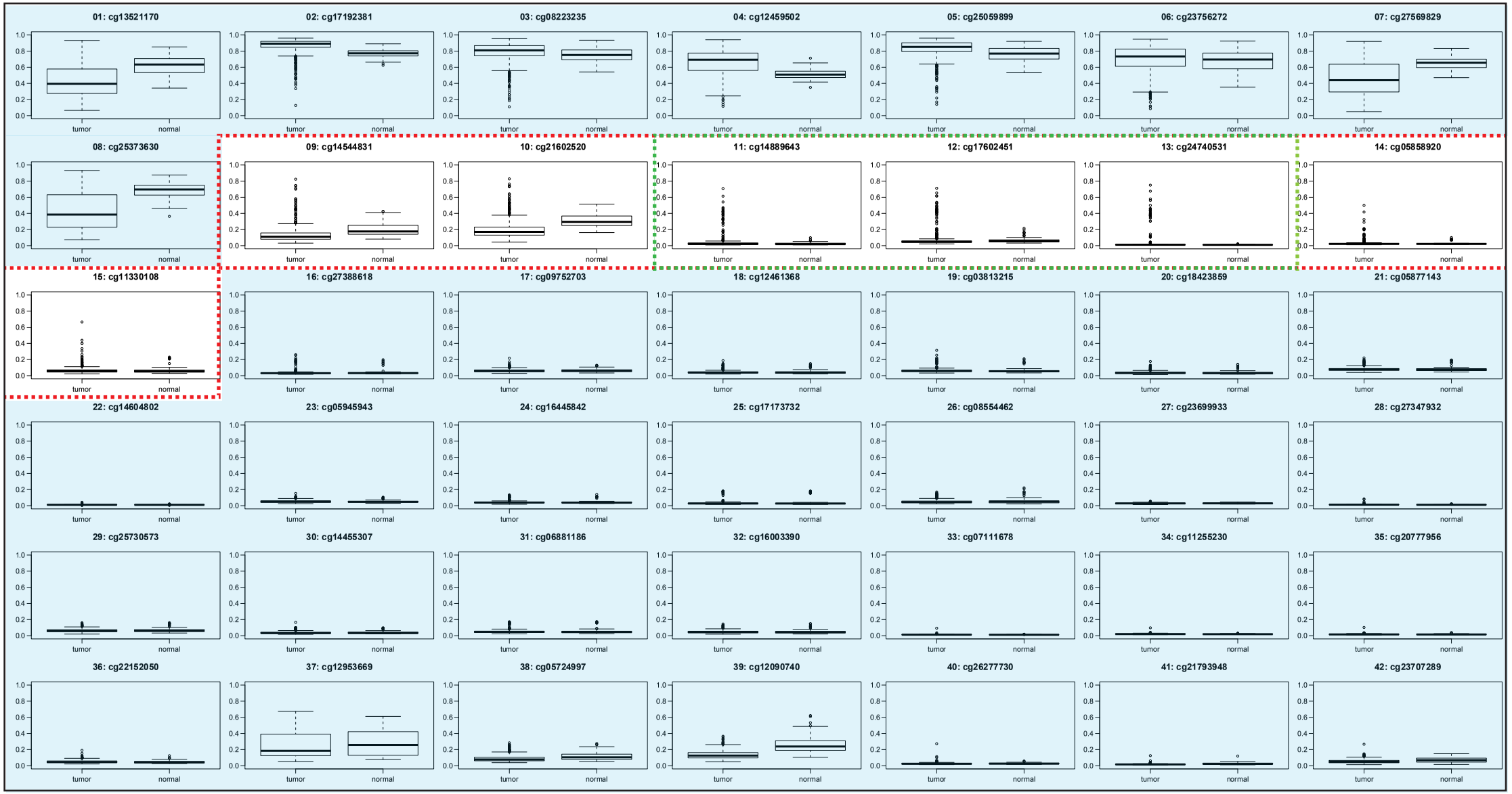
Supplementary Fig S1 - 450K Array Methylation Probes for BCL-2



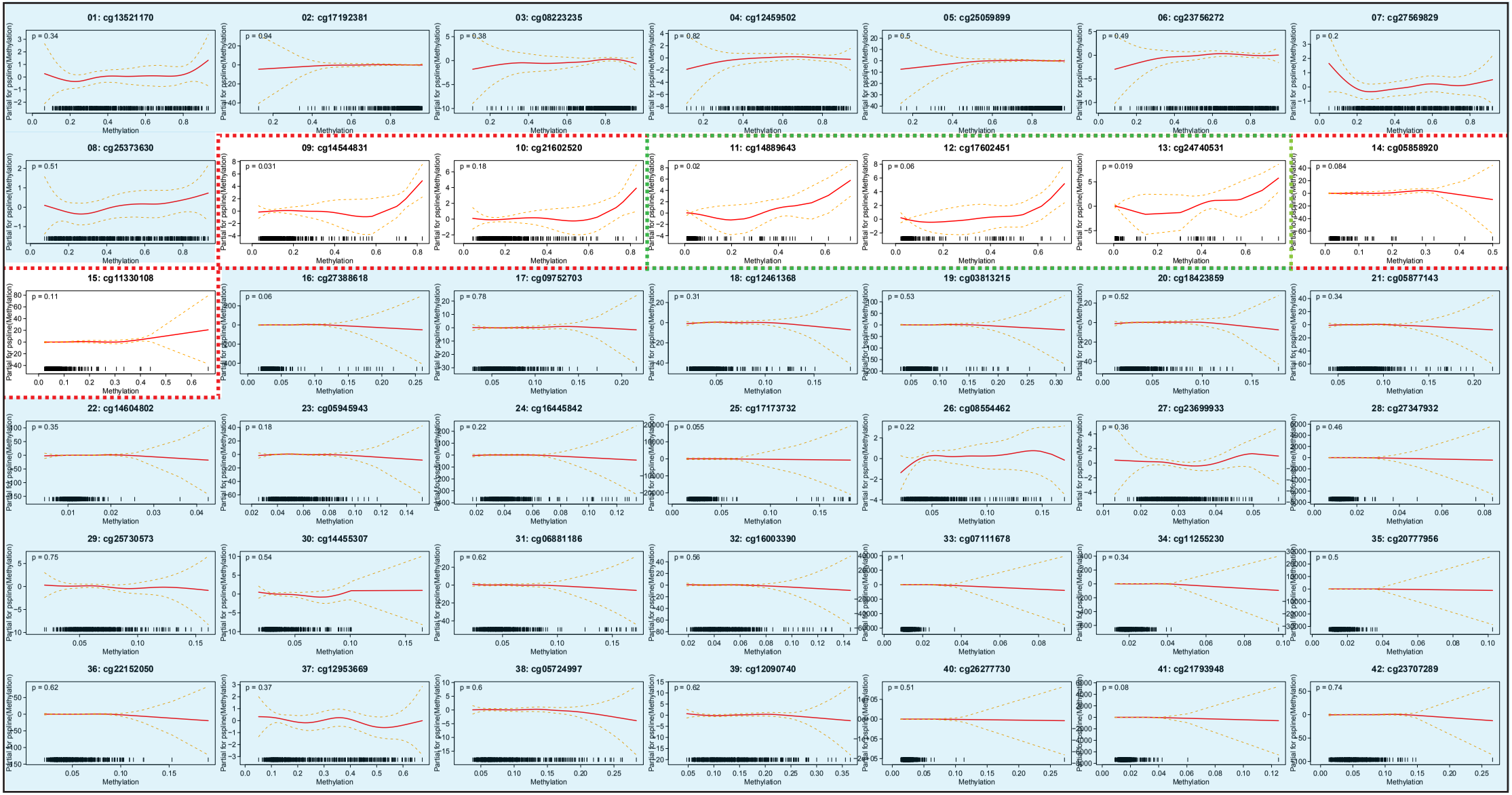
Supplementary Fig S2 - BCL-2 Methylation vs mRNA Expression



Supplementary Fig S3 - BCL-2 Methylation in Tumour vs Normal

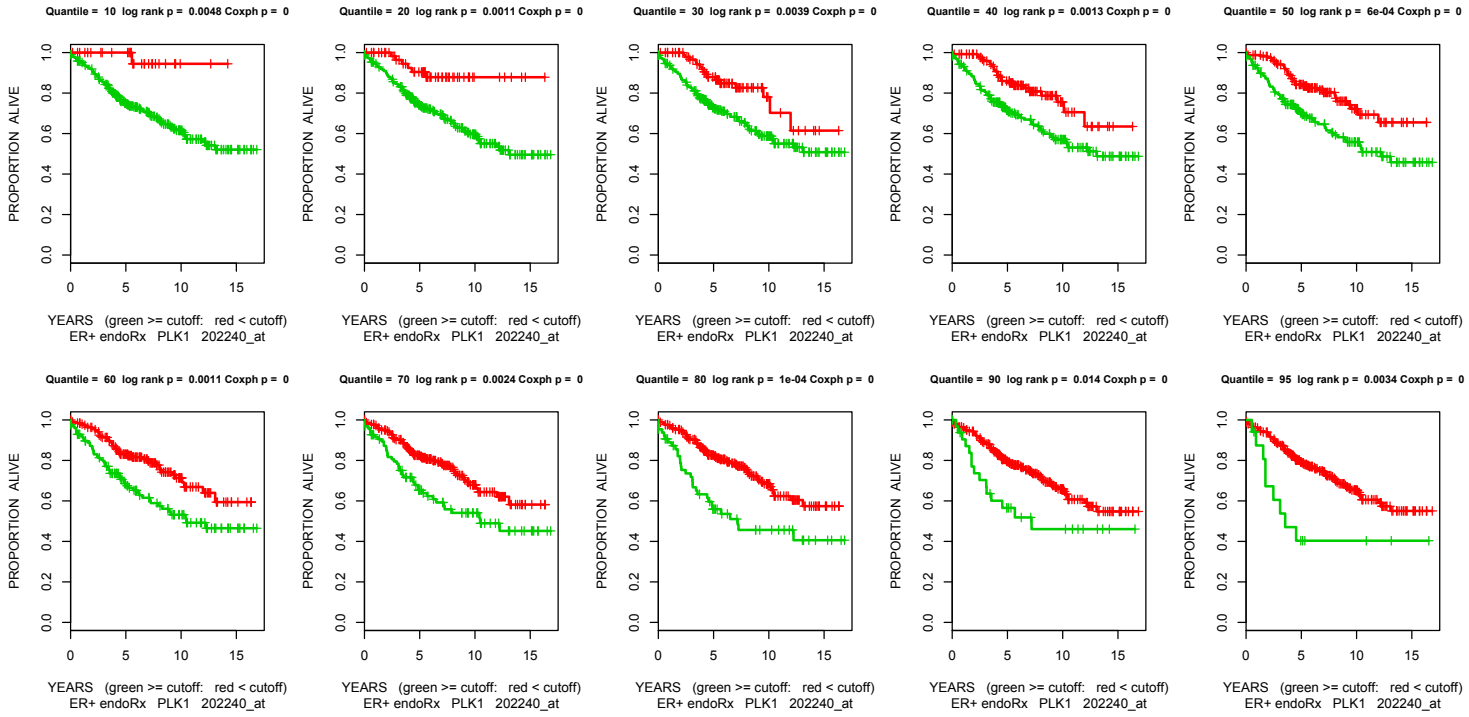


Supplementary Fig S4 - BCL-2 Methylation vs Risk.

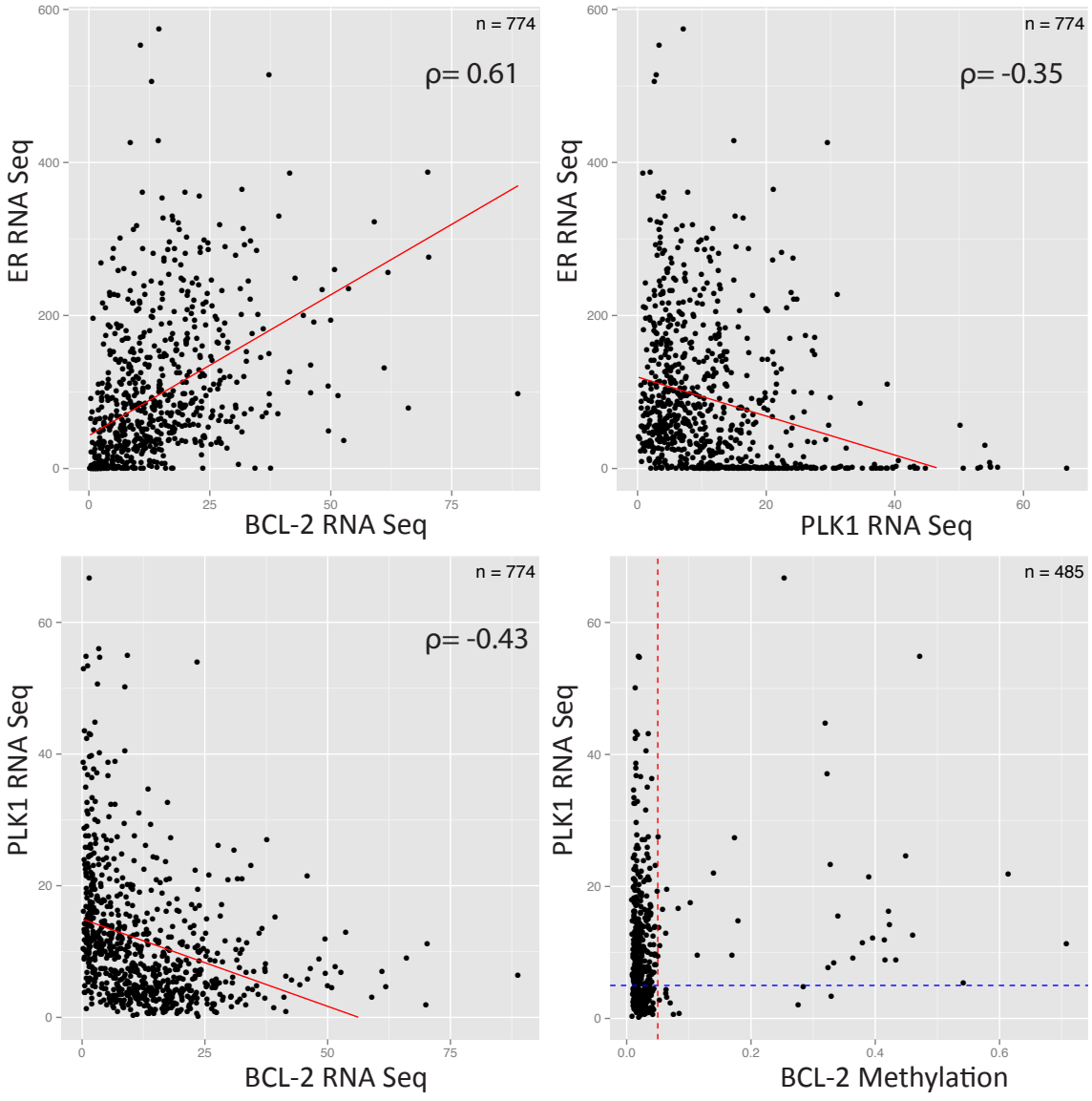


Supplementary Fig S5

S5A - PLK1 Expression is associated with relapse-free survival in ER-positive patients (n=287) (green = high, red = low).



S5B - PLK1 expression is inversely correlated with BCL-2 expression.



Supplementary Fig S6 - The effect of BI2536 on endocrine sensitive and resitant breast cancer cells.