Supplementary data for:

Identification of WEE1 as a potential molecular target in cancer cells by RNAi screening of the human tyrosine kinome

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Supplementary Materials and Methods

RNAi screening. The primary RNAi screen of the human tyrosine kinome (89 genes) was performed using a pool of four siRNAs corresponding to each tyrosine kinase (TK) (part of Qiagen kinase set v2.0, Qiagen Inc, Germantown, MD) and the MB231 cell line. To maximize the potential effect of TK loss of function on our end-point assay of cell viability, we conducted pilot experiments that established that the growth of the MB231 cell line in media containing 1% FBS accentuated the effects of loss of selected kinases, compared to cells grown in 5% FBS (data not shown). Thus all RNAi experiments in the MB231 cell line including the primary RNAi screen were conducted using reduced (1%) serum conditions. Each pool of synthetic siRNAs (four siRNAs per TK gene target) was arrayed in 96 well plates using the interior 60 wells only, and transfections were performed using Oligofectamine (12252011, Invitrogen, Carlsbad, CA). Briefly, siRNA and Oligofectamine (7 µl/ml) in 50 µl of serum free RPMI 1640 were incubated for 30 min at room temperature. MB231 cells were added to the siRNA/lipid complex in 50 µl RPMI 1640 supplemented with 2% FBS to give a final concentration of 50 nM siRNA in RPMI plus 1% FBS. Transfections were performed in antibiotic-free media. This final mixture was incubated at room temperature for 30 min to ensure uniform plating before incubation at 37ºC in a humidified atmosphere containing 5% $C0_2$. After three days, cell viability was assayed by MTS assay using CellTiter 96 Aqueous One Solution reagent (G3581, Promega, Madison, WI) as previously described [1]. Each gene target was screened in triplicate on each plate and each experiment was performed three times. Each plate contained a non-targeting siRNA as a negative control (siNEG) and an siRNA

corresponding to ribonucleotide reductase subunit 2 (siRRM2), a non-kinase reference gene (siPOS), known to inhibit the viability of MB231 cells by approximately 50% [2]. We chose to use a non-kinase reference gene for (RRM2) as comparison for TK selection rather than a statistical deviation from the screen mean or median because we anticipated that the silencing of most TKs would alter cell viability to some degree resulting in too great a variation for whole screen normalization. The mean MTS values per gene target per plate were normalized first using the mean value for the siNEG for each plate. The values for each gene target across all three screens were averaged, and the delta positive values with standard error were calculated as follows: [] – 100. This provided a value for each target relative to the growth inhibition by siRRM2. The data were plotted as percentage change relative to siRRM2 (Supplementary Fig. 1).

For the secondary screen, siRNA pools that induced a more inhibitory phenotype than siRRM2 were selected and were screened again in MB231 cells and two additional cell lines, BT20 and HCC1937, as described above except for supplementation of growth media with 2.5% FBS. As siRRM2 did not inhibit the growth of HCC1937, the data for the secondary screen was normalized only to the values for siNEG and plotted as the percent change in growth relative to siNEG (Supplementary Fig. 2).

Gene specific RNAi analysis. The following ON-TARGETplus SMARTpool siRNAs and the transfection reagent, Dharmafect 1 were purchased from Dharmacon, (Chicago, IL): ABL2 (L-003101-00), JAK2 (L-003146-00), MET (L-003156-00), YES1 (L-003184-00), RYK (L-003174-00), WEE1 (L-005050-00), and PDGFRB (L-003163-00) along with a non-targeting siRNA control pool (D-001810-10). Cells were transfected

in solution with siRNA (pool of four siRNAs or individual siRNAs) at a final concentration of 50 nM using Oligofectamine (MB231: 7 µl/ml, BT20 and HCC1937: 8 µl/ml) or Dharmafect 1 (MCF10A: 2 µl/ml). Transfected cells were plated in either 96-well plates (3000 cells/well) or 6-well plates ($2x10^5$ cells/well); MB231 cells were grown in the presence of 1% serum, BT20 and HCC1937 cells in the presence of 2.5% serum and MCF10A in 5% serum. Three days post-transfection, MTS reagent was added to the cells in the 96-well plates and viability was measured by MTS assay (in replicates of six wells for each target). Results shown represent mean ± SE for three experiments. Cells plated in the 6-well plates were harvested for protein analysis at the same time point.

Supplementary Results

An RNAi screen of the human tyrosine kinome identifies WEE1 as an essential kinase for the viability of breast cancer cells. We performed a synthetic siRNA based RNAi screen of the human tyrosine kinome in cell lines representative of triple-negative/basal-like breast cancer. For the primary screen we used the MB231 cell line (BaB subtype), a pool of four synthetic siRNAs per tyrosine kinase (TK) gene, and an MTS assay (for assessment of metabolic activity) as an estimate of cell viability following gene specific loss-of-function. The MB231 cell line has been characterized as triple-negative by measurement of hormone receptors and *ERBB2* amplification and as a basal-like (BaB) breast cancer cell line by cDNA microarray analysis [3, 4]. Previously, we confirmed the receptor status of the MB231 and other cell lines used in this study [1]. For target selection we used a non-TK reference gene, the ribonucleotide reductase subunit 2, *RRM2*, whose loss has previously been shown to reduce cell viability [2]. Silencing of *RMM2* in MB231 resulted in a significant inhibition of cell viability compared to a nontargeting pool of siRNA (siNEG) (49.7±3.5%; $p=5.4x10^{-7}$). The silencing of 16 TKs reduced cell viability as much or more than inhibition of RRM2 (Supplementary Fig. 1). For the secondary screens we transfected the pools of siRNAs corresponding to these 16 tyrosine kinases into the MB231 cells and two additional triple-negative/basal-like (BaA subtype) breast cancer cell lines, BT20 and HCC1937. Published work has suggested that the EGFR and ABL-kinases may be molecular targets in triple-negative breast cancer [5-9]. We therefore continued evaluation of EGFR and ABL TKs, *EGFR, ERBB3*, *ABL1*, and *ABL2*, in the secondary screens even though the silencing of each of these gene targets mediated a less inhibitory effect on MB231 cell

growth than that seen for the *RRM2* reference (Supplementary Fig. 1). The secondary screen data was normalized to the non-targeting siRNA (siNEG; Supplementary Fig. 2) and the target selection focused on the generation of a consistent loss of cell viability phenotype in more than one of the breast cancer cell lines. RNAi mediated silencing of seven TKs (red bars in Supplementary Fig. 2) inhibited the growth of all three breast cancer cell lines with at least a 30% decrease in viability seen in two of three cell lines. These seven TK genes, *ABL2*, *JAK2*, *MET*, *PDGFRB*, *RYK*, *WEE1,* and *YES,* were selected for further characterization.

To substantiate the effect of loss of function of the seven TKs on cell viability we obtained an independent set of synthetic siRNAs targeting each of the selected TK genes (a pool of four siRNAs per TK gene). The follow up experiments in MB231 cells were performed to allow for the correlation of the decrease in target protein levels and the effect of loss of function of cell viability. A substantial decrease in protein levels for each of the seven TKs was observed in cells transfected with the independent pool of siRNAs targeting each gene when compared with siNEG-transfected cells, with the cells transfected with the siRNA pools corresponding to *ABL2* and *WEE1* showing the greatest decrease (Supplementary Fig. 3). However, in contrast to the screening results, only the silencing of *WEE1* with this independent pool of siRNAs resulted in a significant decrease in cell viability ($52.05 \pm 4.11\%$). Thus *WEE1* was chosen for more extensive evaluation.

Supplementary References

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Supplementary Table 1

Antibodies used in the study are listed here.

S.No	Antibody	Host	Catalog/Clone	Company
1	JAK2	Rabbit	3230, clone D2E12	Cell Signaling, Danvers, MA
2	CDC2	Rabbit	9112	Cell Signaling, Danvers, MA
3	pY15 CDC2	Rabbit	4539, clone 10A11	Cell Signaling, Danvers, MA
4	histone 2AX	Rabbit	07-627	Millipore, Temecula, CA
5	pS139 H2AX	Biotin conjugate	16-193, clone JBW301	Millipore, Temecula, CA
6	YES	Rabbit	sc8403, F7	Santa Cruz Biotechnology, Santa Cruz, CA
7	MET	Rabbit	sc-161, c-28	Santa Cruz Biotechnology, Santa Cruz, CA
8	ABL2	Rabbit	sc-20708	Santa Cruz Biotechnology, Santa Cruz, CA
9	RYK	Rabbit	ab5513	Abcam Inc, Cambridge, MA
10	WEE1	Mouse	sc-5285, clone B11	Santa Cruz Biotechnology, Santa Cruz, CA
11	YES	Mouse	sc8403, F7	Santa Cruz Biotechnology, Santa Cruz, CA
12	Tubulin	Mouse	T9026	Sigma, St Louis, MO
13	PDGFRB	Mouse	3175	Cell Signaling, Danvers, MA
14	Rabbit IgG-HRP		NA934V	GE Healthcare, UK
15	Mouse IgG-HRP		NA931V	GE Healthcare, UK
16	Streptavidin-HRP		RPN1231V	GE Healthcare, UK

Supplementary Figure legends

Supplementary Figure 1. Summary of the primary RNAi screen of the human tyrosine kinome. MB231 cells were transfected with a siRNA pool (50 nM) corresponding to each of the stated TKs (X axis), a non-targeting negative control siRNA (siNEG, shown in red) and a positive control siRNA targeting RRM2 (also shown in red). Cell viability was measured by MTS assay 3 days post-transfection. Results are plotted as a percent change relative to the RRM2 reference gene target and were calculated as [(MTS value) / (RRM2 MTS value) x 100] – 100. Candidate TKs chosen for secondary screen are indicated in blue.

Supplementary Figure 2. Secondary RNAi screens of tyrosine kinases performed in other breast cancer cell lines. Parallel RNAi screens using a pool of siRNA (50 nM) corresponding to each of the 20 selected TKs were conducted in MB231, BT20 and HCC1937. Cell viability was measured by MTS assay three days post-transfection. Results are plotted as a percent change ($\pm$ SE) relative to cells transfected with a control siRNA (siNEG). Values greater than zero indicate increased viability (proliferation) and values less than zero indicate reduced viability compared to siNEG-transfected cells. Seven of the TKs (shown in red) whose silencing inhibited cell viability by more than 30% in two of the three cell lines were selected for further study.

Supplementary Figure 3. Identification of WEE1 as a molecular target in breast cancer cells. MB231 cells were transfected with gene specific pools of siRNAs (50 nM) corresponding to seven selected TKs or a siNEG pool (50 nM) control using an

independent set of siRNAs from Dharmacon. Cell viability was measured 72 h post-transfection by MTS assay and is plotted as a percent change from the siNEG pool control. Silencing of *WEE1* inhibited cell viability significantly ($p<0.05$). Protein levels for each TK were assessed 72 h post-transfection with either the gene specific pool of siRNAs or the siNEG pool control. Immunoblots were performed using antibodies against the stated TKs and the loading control tubulin.

Supplementary Figure 4. **Expression and activity of WEE1 in breast cancer cells.** Breast cancer cells and the non-transformed basal cell line MCF10A were assessed for the expression of WEE1 and phosphorylation levels of Tyr15-CDC2, a measure of WEE1 activity and total CDC2 levels using immunoblotting. ERK2 is included as a loading control.

Supplementary Figure 5. Inhibition of WEE1 in breast cancer cells causes cell cycle arrest. MB231 (**a**), HCC1937 (**b**) and MCF10A cells (**c**) were treated with or without WEE1 inhibitor II for 24 h, incubated with BrdU, fixed and stained with anti-BrdU and analyzed by flow cytometry as described in the Materials and Methods. Histograms obtained after analysis from FLOWJO software are shown. Percentages of cells in various phases are indicated. Breast cancer cells, but not the non-transformed cells, were arrested in S-phase of the cell cycle.

Supplementary Figure 6. Effects of WEE1 inhibition in non-transformed NIH/3T3 cells. NIH/3T3 cells were treated with or without 10 µM WEE1 inhibitor and the number

of viable (**a**) and the percentage of dead cells (**b**) were assessed after 48 h. The dotted line in (**a)** represents the number of cells plated at the start of the experiment. Data represents mean cell counts $\pm$ SE from three experiments. (**c**) Cell cycle of NIH/3T3 cells was assessed by flow cytometry 24 h after WEE1 inhibition. Percentages of cells in various phases are indicated. (**d**) NIH/3T3 cells treated with (+) or without (-) WEE1 inhibitor for 24 h were analyzed for γH2AX levels by immunoblotting and WEE1 inhibition was assessed by phosphorylation of CDC2 (Tyr15).

Supplemental Figure 1

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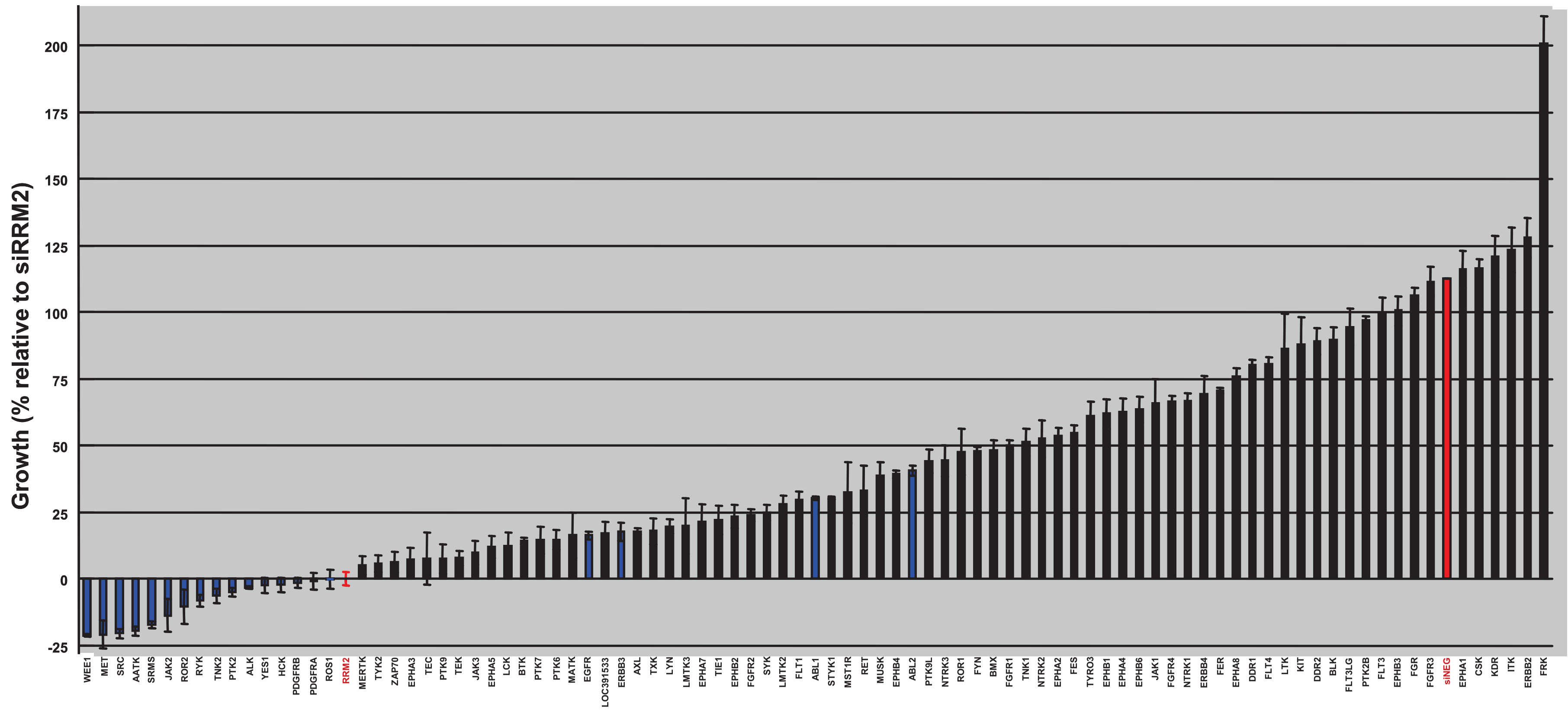

Supplemental Figure 2

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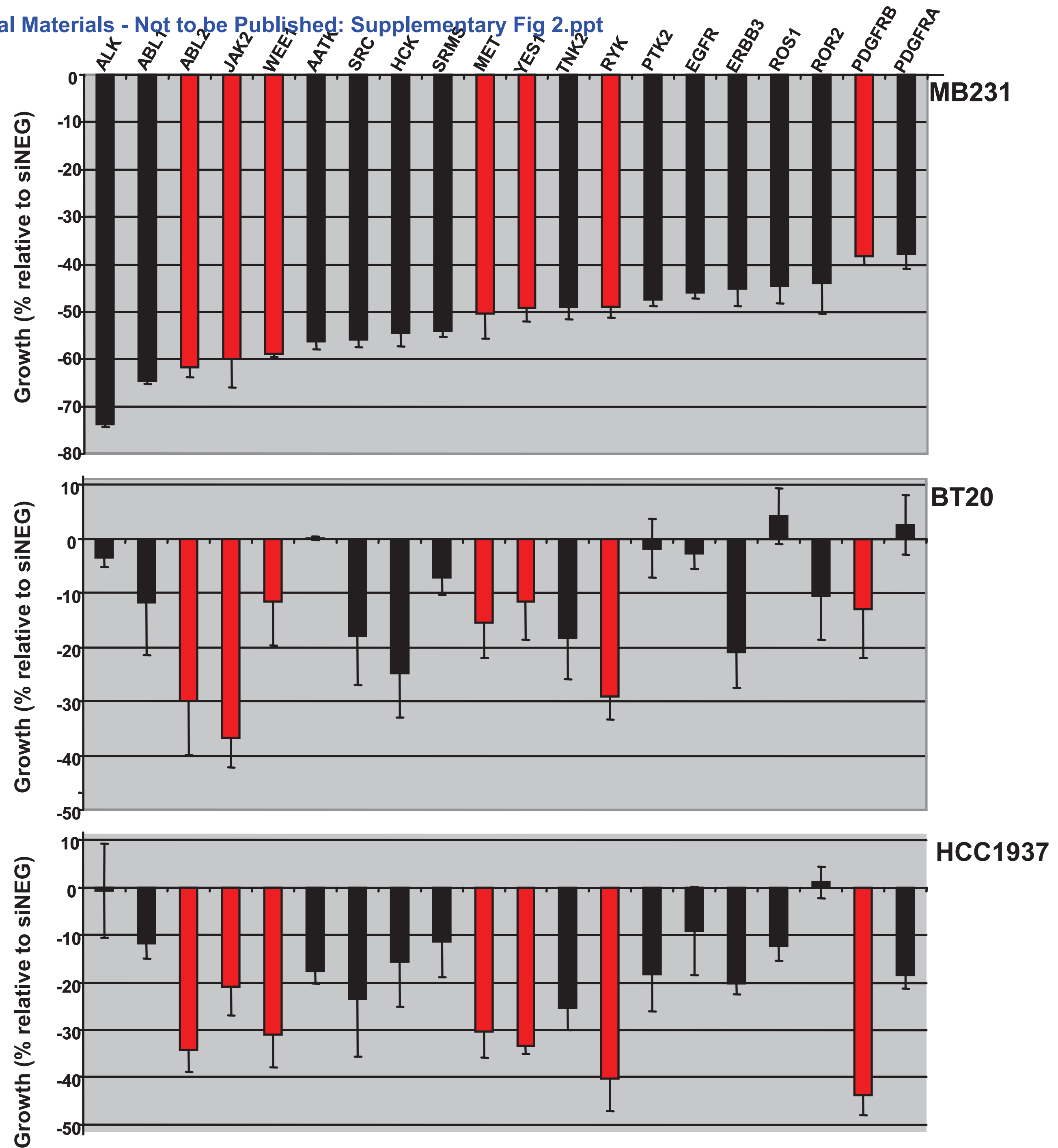

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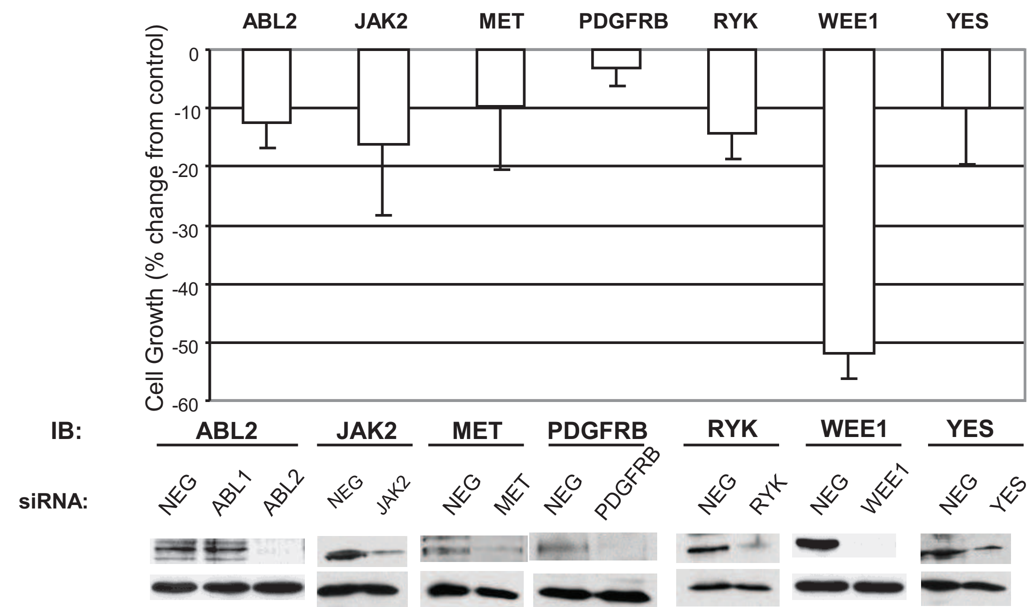

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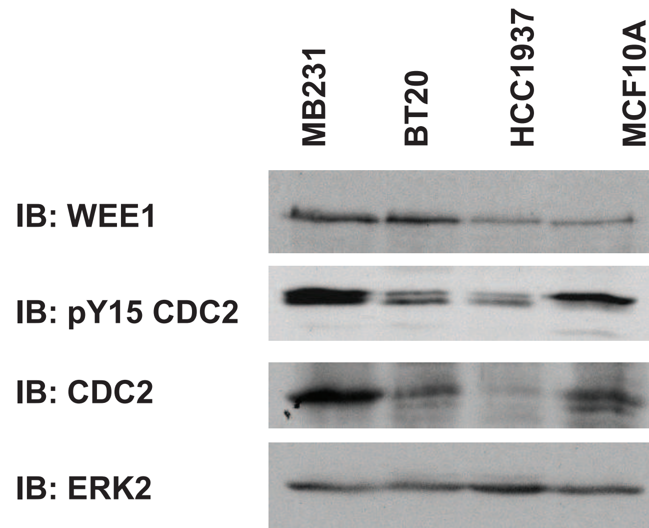

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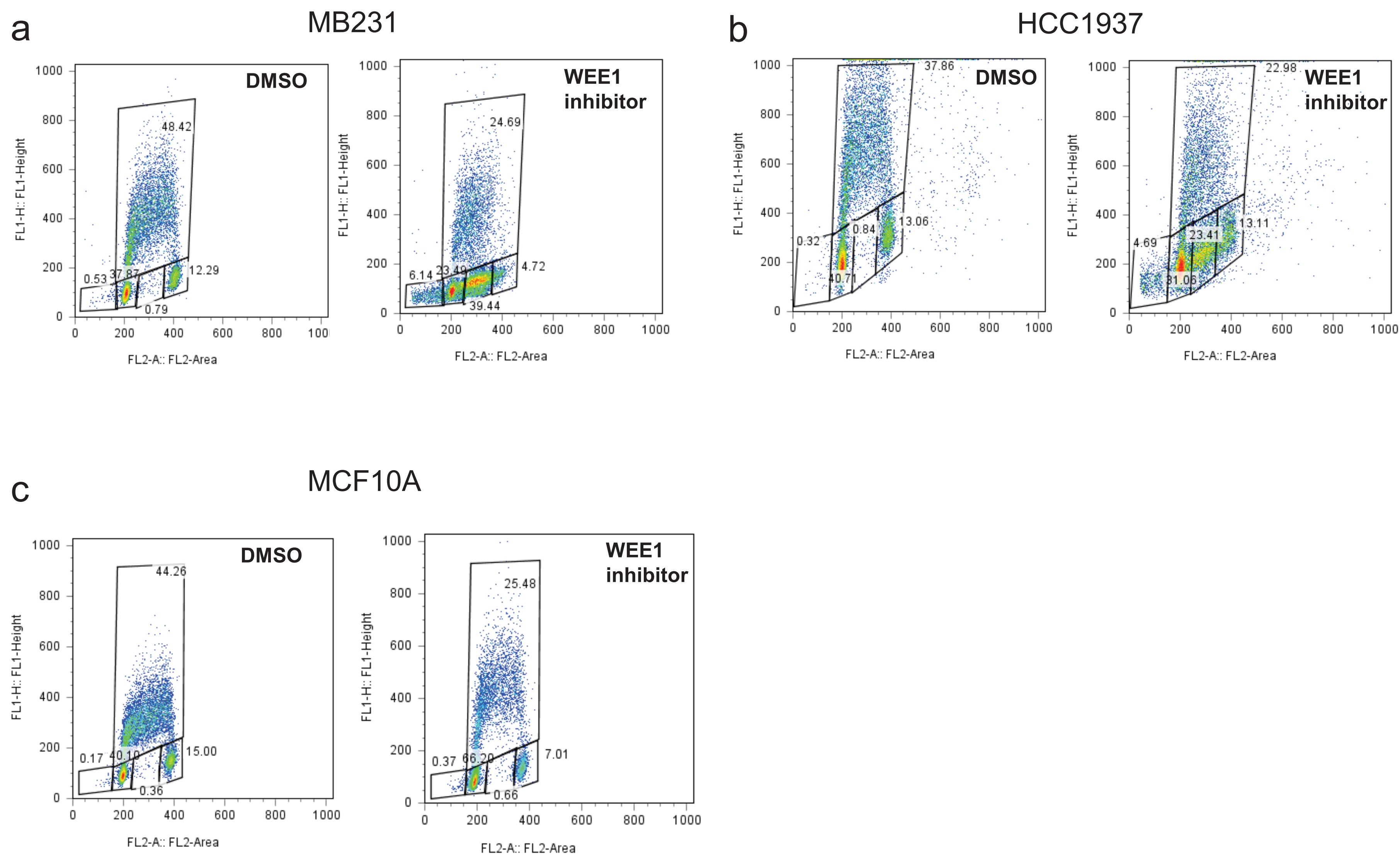

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