Supplemental Figures and Tables

Figure S1. Tamoxifen sensitivity in breast cancer cell lines. Indicated cell lines were treated with different concentrations of tamoxifen for 72 hours and then were assayed for cell viability.

Figure S2. Knockdown of Aurora-A sensitizes cells to tamoxifen in MCF7-TamR cells. The cells were transfected with two siRNAs of Aurora-A and control siRNA. Following 72 hours of incubation, cells were immunoblotted with indicated antibodies (A). The control siRNA and Aurora-A-knockdown MCF7-TamR cells were treated with and without tamoxifen for 5 days and then assessed apoptosis (B), and focus formation (C) as described in the “Materials and Methods”. The asterisks denote significance (*$P < 0.05$ and **$P < 0.01$).

Figure S3. Aurora-A has no effects on tamoxifen sensitivity in ERα-negative cells. MDA-MB-468 cells were transfected with 2 Aurora-A siRNAs and control siRNA (A) whereas MDA-MB-231 cells were introduced with HA-Aurora-A and vector control (D). After 72 hours of incubation, cells were treated with different concentration of tamoxifen and then were assayed for cell survival (B, E) and focus formation (C, F).

Figure S4. Aurora-A inhibitor MLN8237 synergizes with tamoxifen. Tamoxifen resistant BT474 and MCF7-TamR cells were seeded in 96-well plate and treated with different concentrations of MLN8237 and tamoxifen for 72 hours. The cell viability was
evaluated by MTT assay (A and B). The effects of drug combinations were evaluated with Calcusyn software (Biosoft). CI analysis to determine synergy (defined as CI values < 1) was carried out using Calcusyn software as described in Methods (C and D). The curves were generated by Calcusyn software to fit the experimental points. The effect ranges from 0 (no inhibition) to 1 (complete inhibition). Each data point is the average of 4 wells each from 3 independent experiments. Error bars indicate standard error.

**Figure S5. Orthotopic breast tumours from MCF7 cells are sensitive to tamoxifen.** MCF7 cells (5 x 10^6) were injected into mammary fat pad of nude mice. When tumour reached 100mm^3, mice were treated with tamoxifen and vehicle as described in “Materials and Methods”. The tumour growth (A) and tumour weight (B) were examined.

**Figure S6. Aurora-A increases E2-stimulated ERE reporter activity but Aurora-A-induced reporter activation could not be inhibited by tamoxifen. (A and B) MCF7 cells were transfected and treated with indicated plasmids and agents and then were subjected to luciferase assay.**

**Figure S7. Aurora-A phosphorylates ERα.** (A) GST-fused truncation mutations of ERα and location of three putative Aurora-A phosphorylation residues of ERα. (B and C) *In vitro* Aurora-A kinase was carried out by incubation of recombinant Aurora-A with indicated GST-ERα fusion proteins (top panel). Panel 2 shows GST-ERα proteins and panel 3 is recombinant Aurora-A used for *in vitro* kinase assay. (D) Cold *in vitro* Aurora-A kinase. Recombinant ERα was incubated with and without Aurora-A and then
immunoblotted with phospho-ERα-Ser167 and –Ser305 antibodies (panel 1 and 2). Panels 3 and 4 are Western blot showing ERα and Aurora-A used for *in vitro* kinase.

**Figure S8. Aurora-A inhibitor MLN8237 inhibits p-ERα-Ser167 and -Ser-305.** Tamoxifen resistant BT474 (A) MCF7-TamR (B) cells were treated with MLN8237 and then immunoblotted with indicated antibodies. (C) Western blot analysis of orthotopic tumours, which were treated with and without MLN8237, with indicated antibodies.

**Figure S9. Aurora-A does not induce CCND1 expression in ERα-negative cells.** MDA-MB-231 cells were transfected with Aurora-A or pHM6 vector alone. After 72 hours of incubation, cells were subjected to semi-quantitative RT-PCR (top 1 and 2 panels) and Western blot (panels 3-5) analyses.

**Figure S10. Aurora-A is positively correlated with CCND1 levels in ERα-positive breast tumours.** The expression of Aurora-A and CCND1 was analyzed in 2 independent datasets. CCND1 levels were significantly correlated with Aurora-A expression in ERα-positive (left panels) but not ERα-negative tumours (right panels).

**Figure S11. Relationship of p-ERα-Ser167 or/and p-ERα-Ser305 with disease-free survival.** Kaplan-Meier curves revealed that phosphorylation of both Ser167 and Ser305 (A), especially co-occurrence with elevated Aurora-A (D) was significantly associated with short DFS. The p-ERα-Ser167 (B) but not p-ERα-Ser305 (C) alone was also associated with DFS.
Figure S12. Analysis for the Aurora-A expression levels with the recurrence in ERα-positive breast cancers. The analysis was conducted on a panel of 4 independent datasets summing-up more than ERα-positive 854 tumours (note: 3 of these datasets are also included in Kaplan-Meier Plotter database). Aurora-A expression values were used to separate tumour samples into “high” and “low” groups (see Method). Kaplan-Meier graphs represent the probability of cumulative recurrence-free survival in breast cancer datasets. The p-value of the log-rank test reflects a significant association between Aurora-A high and shorter survival.

Figure S13. The Receiver Operating Characteristic (ROC) curves for Aurora-A expression for prediction of recurrence from endocrine therapy. The curve describes the association between Aurora-A expression and the recurrence from endocrine therapy in ERα-positive breast cancers in 4 databases. Area under the curves (AUC) for Aurora-A range from 0.666 to 0.806.

Figure S14. Aurora-A is associated with disease-free survival in ERα-positive but not ERα-negative and basal-like breast cancers. Kaplan-Meier curves show the recurrence free-survival in breast cancer patients based on Aurora-A expression.

Figure S15. Co-localization of Aurora-A with p-ERα-Ser167 and -Ser-305. (A) MCF7-TamR cells were immunostained with anti-Aurora-A (green; b and f) and -p-ERα-Ser167 and -Ser-305 (red; c and g) antibodies, and counterstained with DAPI (blue; a and
e). The merged pictures (green, red and blue) were shown as $d$ and $h$. The magnified images of the indicated areas in panels i-iii are shown at the right side; Aurora-A: top panel (green), p-ER$\alpha$: middle panel (red), overlay: bottom panel. (B) MCF10A cells were transfected with GFP-ER$\alpha$ ($a$) and Aurora-A, and then were immunostained with antibody against $\gamma$-tubulin ($b$). Panel $c$ is counterstained with DAPI and panel $d$ shows the merged image.
Figure S1

Graph showing the effect of Tamoxifen (nM) on MCF-7, BT474, MCF-7 Tam R, and MDA-MB 231 cell lines.
Figure S2
Figure S3

A

MDA-MB-468

Control siRNA: + - -
siRNA-Aurora-A: - Si-1 Si-2

Aurora-A

ERα

Actin

B

Viability (%)

0 20 40 60 80 100 120

Tam. (μM): 0 0.1 0.5 1.0 2.0

Control Si-Aurora-A-1 Si-Aurora-A-2

C

Tam (μM) 0 0.5 1.0 2.0

Si-Control

Si-Aurora-A-1

Si-Aurora-A-2

D

MDA-MB-231

pHM6 vector: + -

HA-Aurora-A: - +

HA-Aurora-A

ERα

Actin

E

Viability (%)

0 20 40 60 80 100 120

Tam. (μM): 0 0.1 0.5 1.0 2.0

Control HA-Aurora-A

F

Tam (μM) 0 0.5 1.0 2.0

Control

HA-Aurora-A
Figure S4

A

B

C

D
Figure S5

MCF-7

A

B

Day

Volum (mm$^3$)

Weight (g)

p = 0.001

0 0.2 0.4 0.6 0.8 1.0

0 50 100 150 200 250 300

0 25 50 100 150 200

0 14 16 18 20 22 24 26

Ctl

Tam

Figures 14-26
Figure S6

Aurora-A: E2: Tamoxifen:

| Relative Luc activity | Aurora-A: | E2: | Tamoxifen: |
|-----------------------|-----------|-----|------------|
| - -                   | +         | -   | +          |
| ++                    | +         | -   | +          |
| ++                    | -         | -   | +          |
| ++                    | -         | +   | +          |
| +                     | -         | +   | +          |
| +                     | -         | +   | +          |
| +                     | -         | -   | +          |
| +                     | -         | +   | +          |

A

B

ERE-Luc: + + + + +
Aurora-A: - - + + +
E2: - - - + +
Tamoxifen: - - + - +

p=0.04
p=0.01
p=0.92
p=0.0003
Figure S7

**A**

- ERα 1-595
- ERα 1-150
- ERα 1-200
- ERα 1-318
- ERα 319-595

**B**

- GST only
- GST-ERα
- Aurora-A

**C**

|            | GST-ERα-1-200 | GST-ERα-168-218 | GST-ERα-208-318 |
|------------|---------------|-----------------|-----------------|
| 32p-ERα    |                |                 |                 |
| GST-ERα    |                |                 |                 |
| Aurora-A   |                |                 |                 |

**D**

- r-Aurora-A: - +
- r-ERα: + +

- p-ERα-S167
- p-ERα-S305

**In vitro cold kinase**
Figure S8
Figure S9
Figure S10

**GSE29210**

**ERα-positive**

\[ P < 0.0001 \]

**ERα-negative**

\[ P = 0.4604 \]

**GSE4560**

**ERα-positive**

\[ P < 0.0001 \]

**ERα-negative**

\[ P = 0.0163 \]
Figure S11

A

Disease-free survival

0 50 100 150 200

0 20 40 60 80 100

Negative pERα-S167/S305
N=38

Positive pERα-S167/S305
N=34

p=0.017

Months

B

Disease-free survival

0 50 100 150 200

0 20 40 60 80 100

Negative pERα-S167
N=79

Positive pERα-S167
N=88

p=0.042

Months

C

Disease-free survival

0 50 100 150 200

0 20 40 60 80 100

Negative pERα-S305
N=95

Positive pERα-S305
N=71

p=0.204

Months

D

Disease-free survival

0 50 100 150 200

0 20 40 60 80 100 120 140

Low AurA/-pERα-S167/S305
N=35

High AurA/-pERα-S167/S305
N=32

p=0.006

Months

Figure S11
Figure S12
Figure S13
ERα-positive Breast Cancer/Endocrine Therapy

Recurrence-free survival

HR = 1.91 (1.57 - 2.34)
logrank P = 8.9e-11

ERα-negative Breast Cancer

Recurrence-free survival

HR = 0.69 (0.66 - 1.21)
logrank P = 0.47

Basal-like Breast Cancer

Recurrence-free survival

HR = 0.96 (0.72 - 1.27)
logrank P = 0.75

Figure S14
Table S1. Correlation of Aurora-A expression and ERα phosphorylation with clinicopathological characteristics in ERα-positive breast tumor patients

| Variables        | Cases (n) | Aurora-A Positive | p-S167-ERα Positive | p-S305-ERα Positive |
|------------------|-----------|-------------------|---------------------|---------------------|
|                  | n (%)     | P *               | n (%)               | P *                | n (%)               | P *               |
| **Age**          |           |                   |                     |                     |                     |                   |
| <50              | 61        | 29 (48)           | 34 (56)             | 23 (37)             |                     |                   |
| ≥50              | 106       | 44 (42)           | 54 (51)             | 48 (45)             |                     |                   |
| **Tumor size**   |           |                   |                     |                     |                     |                   |
| <2 cm            | 112       | 45 (40)           | 54 (48)             | 47 (42)             |                     |                   |
| ≥2 cm            | 55        | 28 (51)           | 34 (62)             | 24 (44)             |                     |                   |
| **Lymph node status** |         |                   |                     |                     |                     |                   |
| Negative         | 98        | 38 (39)           | 46 (47)             | 41 (42)             |                     |                   |
| Positive         | 69        | 35 (51)           | 42 (61)             | 30 (43)             |                     |                   |
| **Stage**        |           |                   |                     |                     |                     |                   |
| I/II             | 132       | 57 (43)           | 67 (51)             | 54 (41)             |                     |                   |
| III/IV           | 35        | 16 (46)           | 21 (60)             | 17 (49)             |                     |                   |
| **Grade**        |           |                   |                     |                     |                     |                   |
| I/II             | 119       | 49 (41)           | 53 (45)             | 46 (39)             |                     |                   |
| III/IV           | 58        | 22 (38)           | 35 (60)             | 25 (43)             |                     |                   |

* p-value
| Variables                  | Recurrence-free survival |                |                |
|---------------------------|--------------------------|----------------|----------------|
|                           | Univariate analysis      | Multivariate analysis |
|                           | HR (95% CI) | p-value | HR (95% CI) | p-value |
| **Age**                   |              |        |              |        |
| <50 vs. ≥50               | 0.881 (0.583-1.493) | 0.861 | 1.101 (0.789-1.672) | 0.756 |
| **Tumor size**            |              |        |              |        |
| ≥2 cm vs. <2 cm           | 1.762 (1.091-2.531) | 0.010 | 1.489 (0.846-3.271) | 0.044 |
| **Lymph node status**     |              |        |              |        |
| Positive vs. Negative     | 2.472 (1.456-4.134) | 0.001 | 2.285 (1.270-3.531) | 0.012 |
| **Stage**                 |              |        |              |        |
| III/IV vs. I/II           | 2.310 (1.211-4.219) | 0.003 | 2.116 (1.412-4.010) | 0.001 |
| **Grade**                 |              |        |              |        |
| III/IV vs. I/II           | 0.912 (0.678-1.231) | 0.892 | 0.981 (0.579-1.872) | 0.612 |
| **Aurora-A**              |              |        |              |        |
| Positive vs. negative     | 2.313 (1.112-4.147) | 0.0012 | 1.99 (1.012-4.002) | 0.006 |
| **p-S167-ERα**            |              |        |              |        |
| Positive vs. negative     | 1.892 (0.981-3.257) | 0.035 | 1.451 (0.891-2.190) | 0.042 |
| **p-S305-ERα**            |              |        |              |        |
| Positive vs. negative     | 0.972 (0.687-2.192) | 0.421 | 1.108 (0.891-1.987) | 0.362 |
### Supplemental Table S3

| Cohort                                      | Platforms     | Samples          | Data source         | References                      |
|---------------------------------------------|---------------|------------------|---------------------|---------------------------------|
| Nuvera Biosciences                          | HG-U133A      | 298*             | GEO GSE17705        | Symmans WF, et, al 2010         |
| Veridex                                     | HG-U133A      | 208* (286†)      | GEO GSE2034         | Wang Y, et, al 2005             |
| Genome Institute of Singapore               | HG-U133A      | 214* (249†)      | GEO GSE4922         | Ivshina AV, et, al 2006         |
| University of Oxford                        | HumanRef-8 v1.0 | 134* (216†)     | GEO GSE22219        | Buffa FM, et, al 2011           |

†Total sample number.  *ERα-positive tumours.

Symmans WF, Hatzis C, Sotiriou C, Andre F et al. Genomic index of sensitivity to endocrine therapy for breast cancer. *J Clin Oncol* 2010;28:4111-9.

Wang Y, Klijn JG, Zhang Y, Sieuwerts AM et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* 2005; 365:671-9.

Ivshina AV, George J, Senko O, Mow B et al. Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer. *Cancer Res* 2006;66:10292-301.

Buffa FM, Camps C, Winchester L, Snell CE et al. microRNA-associated progression pathways and potential therapeutic targets identified by integrated mRNA and microRNA expression profiling in breast cancer. *Cancer Res* 2011;71:5635-45.
Table S4. ROC analysis of using Aurora-A expression for prediction of endocrine therapy outcome

| Data source   | AUC* | p value |
|---------------|------|---------|
| GEO GSE17705  | 0.666| 0.001   |
| GEO GSE2034   | 0.760| 0.0001  |
| GEO GSE4922   | 0.785| 0.0001  |
| GEO GSE22219  | 0.806| 0.0001  |

*AUC, area under the curve
Table S5. Association of Aurora-A expression levels with 15-years recurrence-free survival of breast cancer patients from the KM Plotter Database†

|                      | **Aurora-A Probe 1** |                      | **Aurora-A Probe 2** |                      |
|----------------------|----------------------|----------------------|----------------------|----------------------|
|                      | Number†‡             | HR‡                  | P value              | Number               | HR                  | P value              |
| **ERα-positive**     |                      |                      |                      |                      |                     |                     |
| Endocrine therapy    | 687/686              | 1.91 (1.57-2.34)     | **8.9e-11**          | 687/686              | 1.94 (1.58-2.36)    | **4.3e-11**          |
| Grade 1              | 94/94                | 1.55 (0.79-3.05)     | 0.2                  | 94/94                | 2.27 (1.11-4.62)    | **0.02**             |
| Grade 2              | 178/178              | 1.48 (1.02-2.14)     | 0.036                | 178/178              | 1.43 (0.99-2.07)    | 0.054                |
| Grade 3              | 98/98                | 1.29 (0.81-2.05)     | 0.27                 | 98/98                | 1.2 (0.76-1.90)     | 0.43                 |
| **ERα-negative**     | 222/222              | 0.89 (0.66-1.21)     | 0.47                 | 222/222              | 0.85 (0.63-1.16)    | 0.32                 |
| **Basal§**           | 234/234              | 0.96 (0.72-1.27)     | 0.75                 | 234/234              | 1.10 (0.83-1.46)    | 0.51                 |

†(http://www.kmplot.com) ‡Number of Aurora-A low/high patients. #HR, hazard ratio with 95% confidence interval. §Basal-like breast cancer.