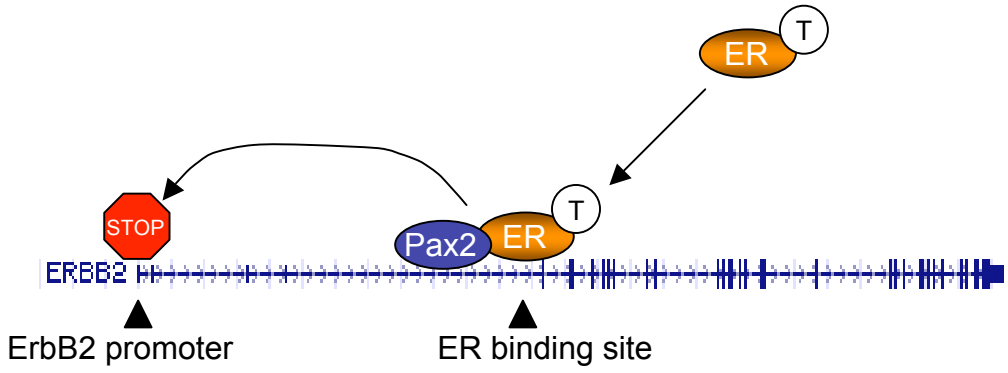
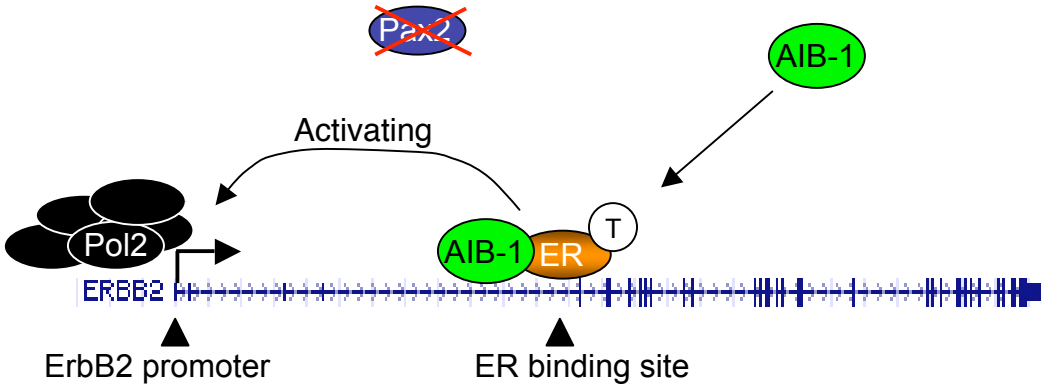
Supplementary data 1

Model of tamoxifen-ER transcriptional regulation of *ERBB2*. In ER positive breast epithelial cells, treatment with tamoxifen results in recruitment of ER and the putative repressor Pax2 to an ER binding site within the intron of the *ERBB2* genomic region. The presence of Pax2 results in active repression of ErbB2 transcription. Tamoxifen resistance can be potentially achieved by a number of mechanisms, including a decrease in Pax2 levels (which results in the opportunity for increased binding of the co-activator AIB-1), an increase in AIB-1 levels (which can out compete Pax2 for binding) or by amplification of the ErbB2 genomic locus, all of which increase ErbB2 levels. In resistant cells a chromatin loop can form between the ER binding site and the *ERBB2* A promoter, confirming that in wild type cells, *ERBB2* repression results from blocking this chromatin loop.

Tamoxifen-ER repression of ErbB2

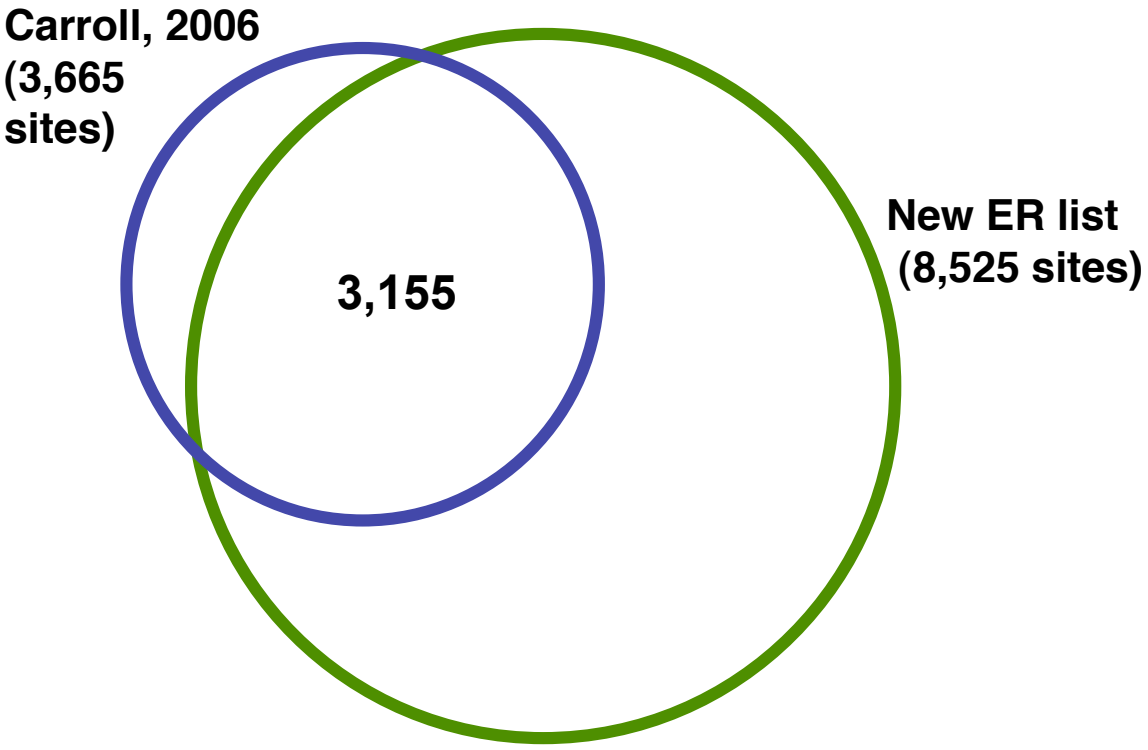


Tamoxifen resistance



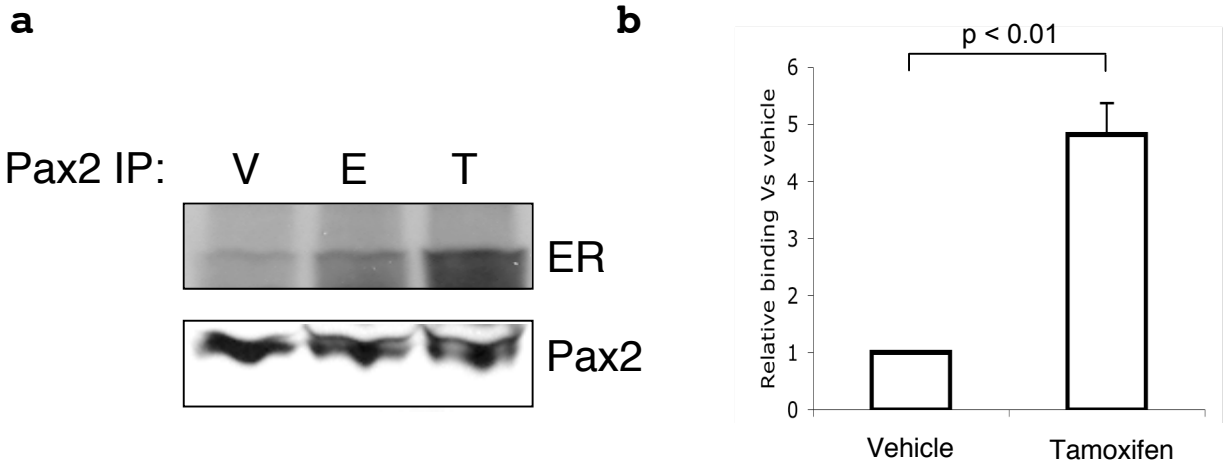
Supplementary data 2

ER ChIP-on-chip was performed as previously described (Carroll *et. al*, Nature Genetics, 2006, 38: 1289-97) and hybridised to Affymetrix human tiling microarrays 2.0. Data was analysed using MAT (Johnson, *et. al*, PNAS, 2006, 103:12457-62) with a False discovery rate (FDR) of 5%. This generated 8,525 ER binding sites within the non-repetitive human genome. A comparison between this list and the published list of 3,665 ER binding sites (Carroll *et. al*, Nature Genetics, 2006, 38: 1289-97) revealed an overlap of 3,155 sites (representing 86% of the original sites). The increase in total ER binding sites is a result of improved technical aspects and different statistical thresholds. The bed file with the new list of ER binding sites (EST_ER_FDR5_Hg18.bed) was converted to Hg_18 and can be loaded into UCSC genome browser (http://genome.ucsc.edu/) using the March 2006 version of the human genome.



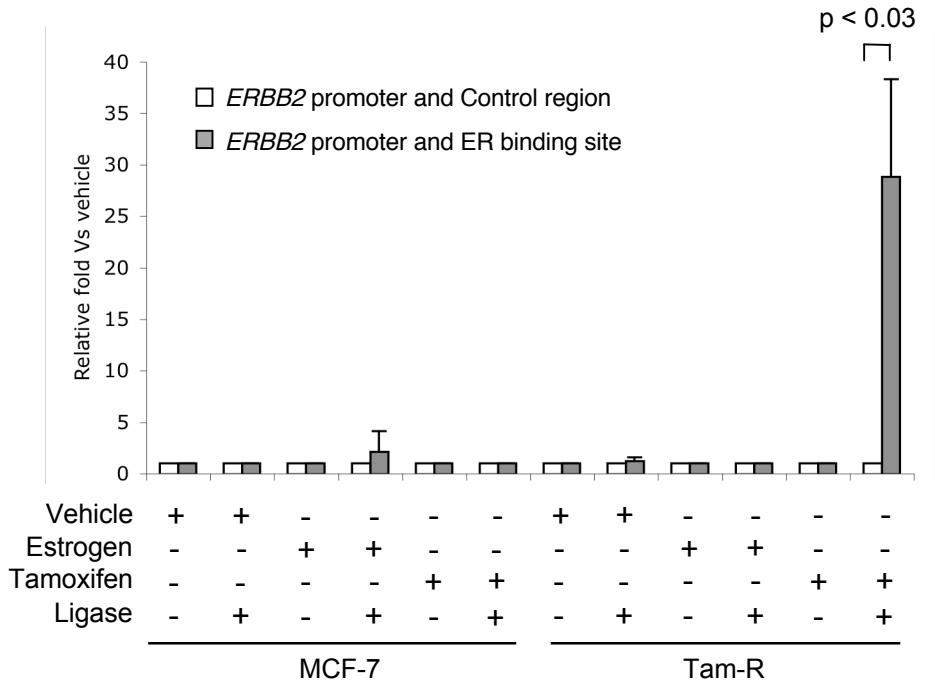
Supplementary data 3

ER and Pax2 form direct protein-protein interactions. **a.** Total protein lysate was collected from MCF-7 cells treated with vehicle (V), estrogen (E) or tamoxifen (T) for 1 hr. Pax2 IP was performed, followed by Western blotting for ER or Pax2 (as a loading control). **b.** Pax2 ChIP was performed, followed by 'release' of the chromatin and ER Re-ChIP. Real time PCR of the ER binding site within ERBB2 was performed.



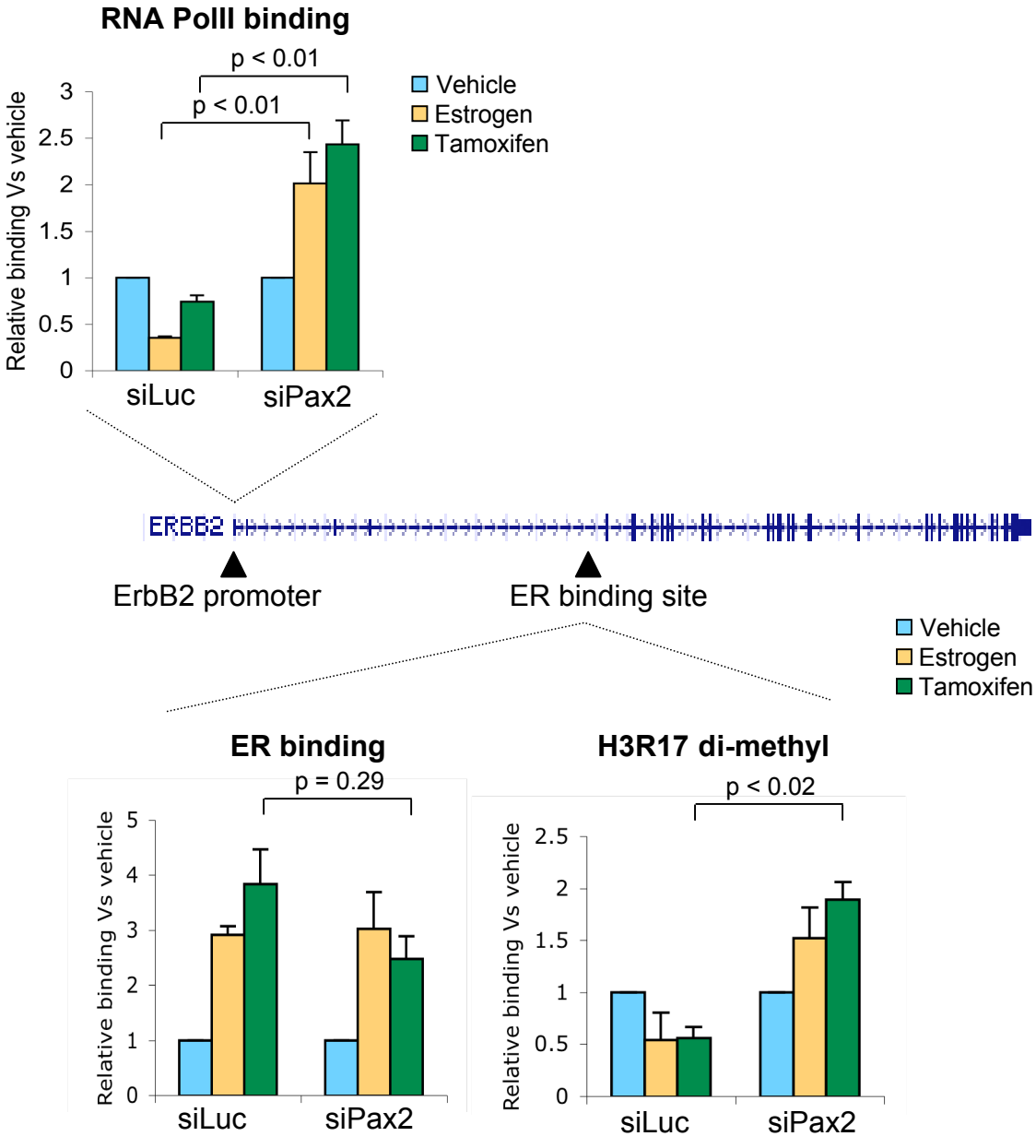
Supplementary data 4

Chromosome conformation capture (CCC) analysis confirming a chromatin loop between the ER binding site within the *ERBB2* intron and the *ERBB2* promoter region. Wild type and Tamoxifen-resistant (Tam-R) MCF-7 cells were treated with vehicle, estrogen or tamoxifen for one hour, after which CCC was performed. Taqman real time PCR was performed and the data are shown as the fold enrichment versus control. A control reverse primer against another PstI site closer to the *ERBB2* promoter is included. No chromatin loops form between the ER binding site and the *ERBB2* promoter in wild type cells suggesting that repressive chromatin loops do not occur. However, a chromatin loop was formed in the Tam-R cells, only in the presence of tamoxifen treatment, suggesting that in the resistant cells, an active transcriptional loop can form between the cis-regulatory element and the promoter.



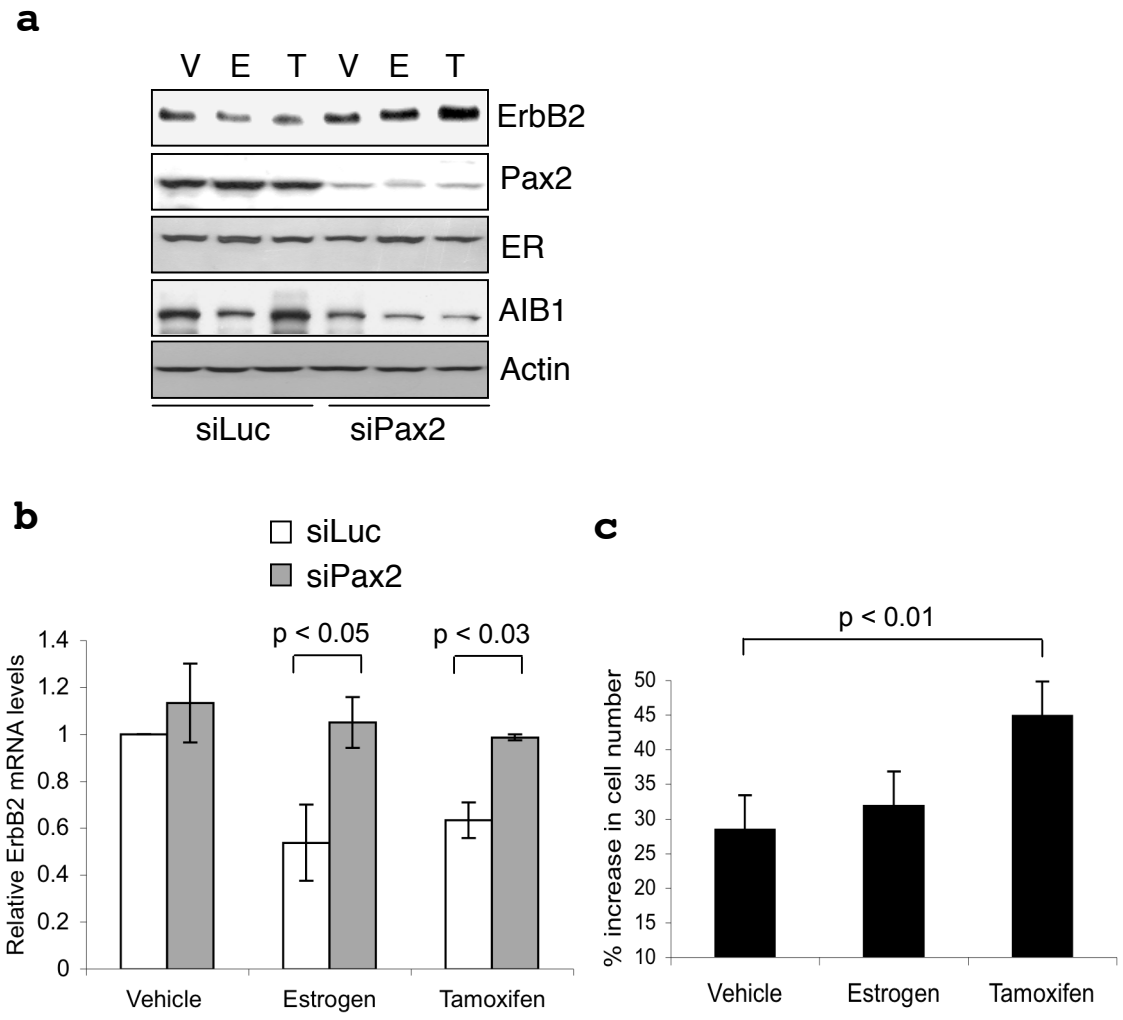
Supplementary data 5

Additional data for Figure 2. Specific silencing of Pax2 reverses the tamoxifen-mediated decrease in RNA PolII binding and alters the chromatin structure at the ER binding site within *ERBB2*. Control siRNA (siLuc) or siPax2 was performed as described and cells were treated with vehicle, estrogen or tamoxifen for 45 minutes. RNA PolII binding to the promoter of the *ERBB2* gene was determined by ChIP followed by real time PCR. ER and H3R17 dimethyl enrichment at the ER binding site was determined by ChIP, followed by real time PCR of the *ERBB2* intronic region. The data are the average of three independent experiments ± Std. Dev.



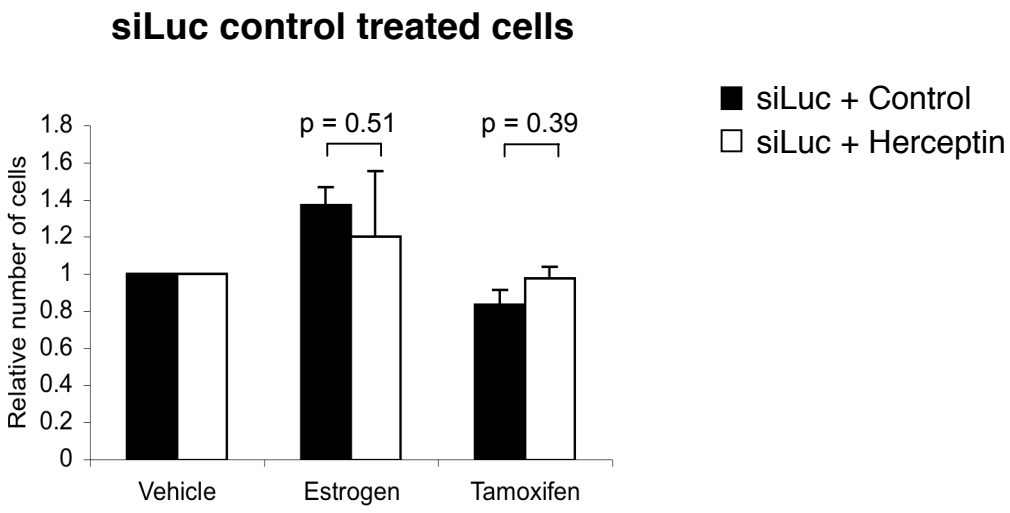
Supplementary data 6

Targeted silencing of Pax2 with an additional independent siRNA. Specific silencing of Pax2 reverses the tamoxifen-mediated repression of ErbB2 and growth arrest. **a.** An independent siRNA to Pax2 was transfected into hormone-depleted MCF-7 cells and stimulated with vehicle (V), estrogen (E) or tamoxifen (T). Total protein was collected and immunoblotted. Actin functioned as a loading control. **b.** Control siRNA (siLuc) (white bars) or siPax2 (gray bars) was transfected and total RNA was collected after 6 hrs treatment. ErbB2 mRNA was assessed by quantitative RT-PCR. The data are the average of three independent experiment ± Std. Dev. **c.** Control siRNA (siLuc) or siPax2 was performed as described and cells were treated for 24 hr, after which cells were collected and total cell number was determined. The data are shown as the relative increase in cell number after siPax2 compared to siLuc control and are the average of three independent experiment ± Std. Dev.



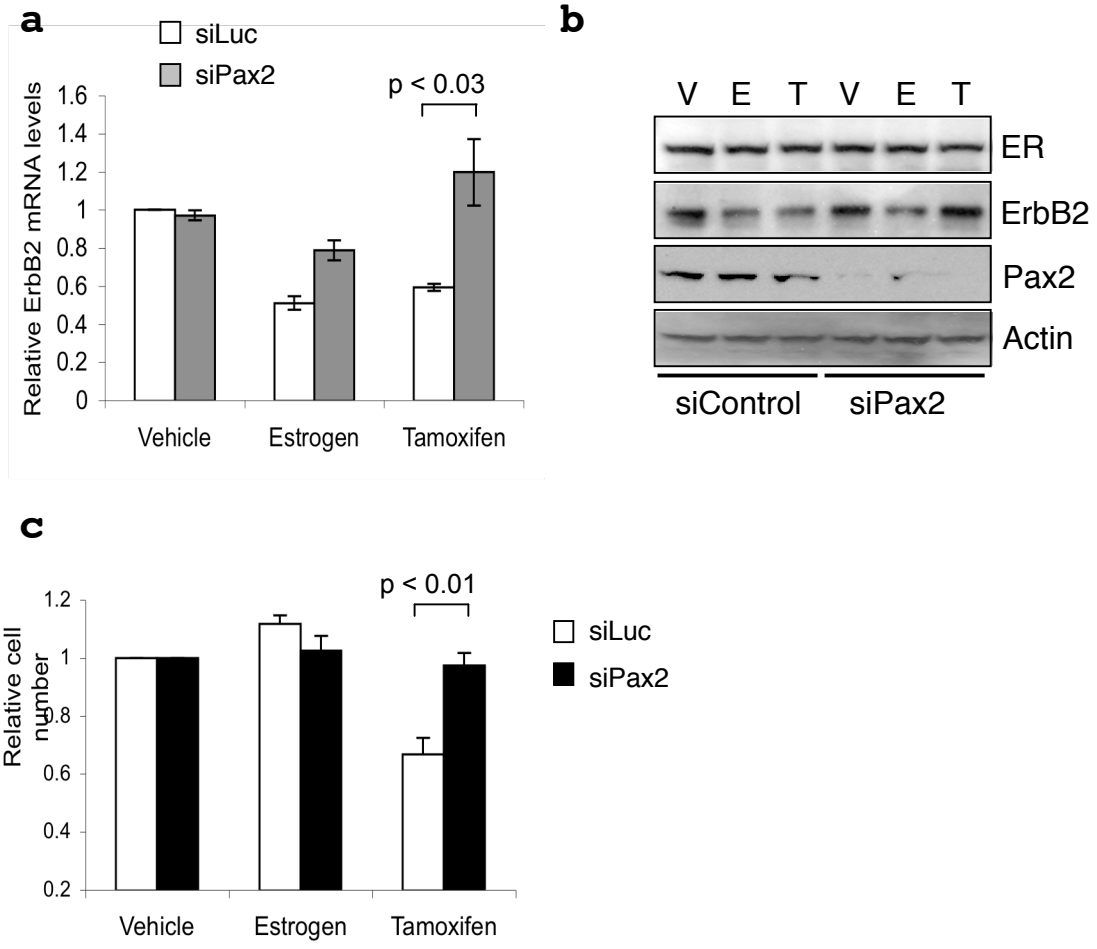
Supplementary data 7

Additional data for Figure 2c. Cell growth of MCF-7 cells after siLuc (control) in the presence or absence of Herceptin. Control siRNA (siLuc) was transfected as described and cells were treated with vehicle, estrogen or tamoxifen for 24 hr. The cells were also pretreated with Herceptin or vehicle control. Cells were collected and total viable cell number was determined. The data are normalised to vehicle treatment and are the average of three independent experiment ± Std. Dev.



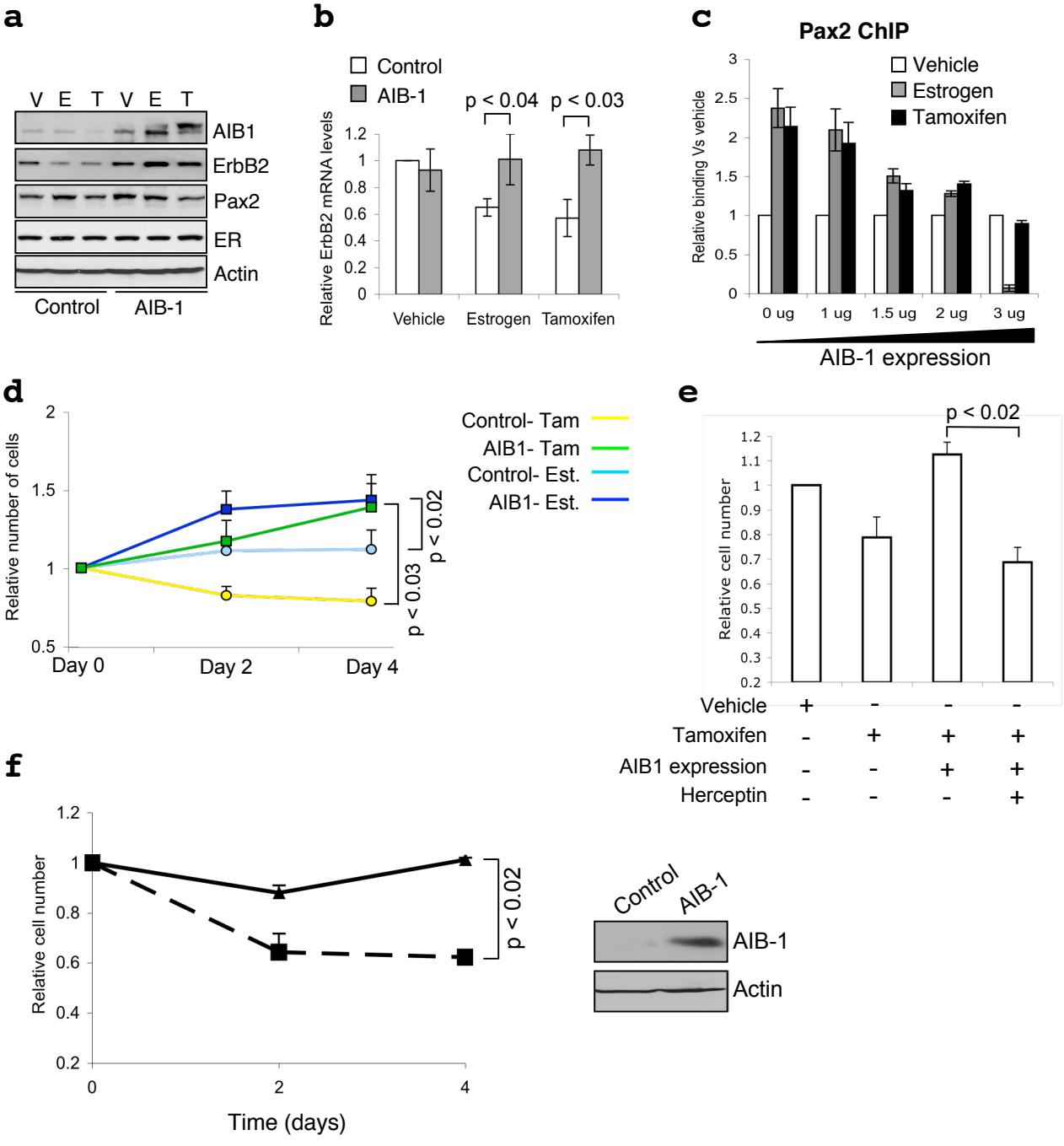
Supplementary data 8

Pax2 is required for tamoxifen repression of ErbB2 transcription and inhibition of proliferation in ZR75-1 breast cancer cells. **a.** siLuc (Control) or siRNA to Pax2 was transfected into hormone-depleted ZR75-1 cells and cells were stimulated with vehicle, estrogen or tamoxifen. Total RNA was collected and ErbB2 mRNA was measured by real-time RT-PCR. **b.** After siLuc or siPax2 transfection, hormone depleted ZR75-1 cells were stimulated with vehicle (V), estrogen (E) or tamoxifen (T) and total protein was collected for immunoblotting. **c.** Proliferating ZR75-1 cells were transfected with siPax2 (or control siRNA) and stimulated with vehicle, estrogen or tamoxifen for 24 hr and total cell number was determined.



Supplementary data 9

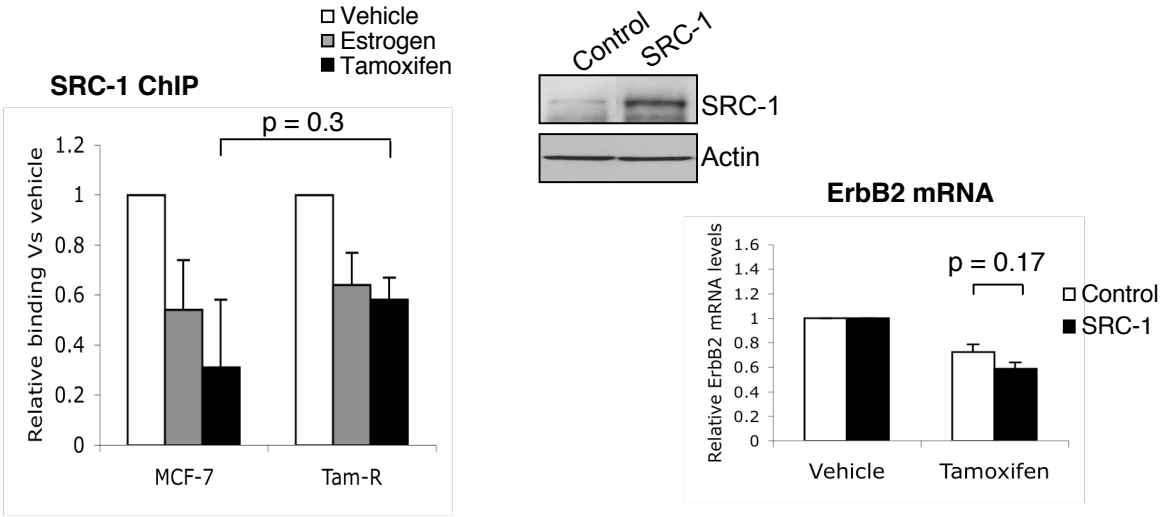
Expression of AIB-1 can out-compete Pax2 to induce tamoxifen resistance **a.** MCF-7 cells were transfected with control or AIB-1 expression constructs and total protein was Western blotted. **b**. Control (white bars) or AIB-1 (gray bars) expressing constructs were transfected and total RNA was collected after 6 hours treatment and ErbB2 mRNA was assessed by RT-PCR. **c.** Increasing AIB-1 expression results in decreasing Pax2 binding to the ER binding site within the *ERBB2* intron. MCF-7 cells were transfected with 0, 1, 1.5, 2 or 3ug of AIB-1 expressing plasmid and Pax2 ChIP was performed, followed by real time PCR of the ER binding site in *ERBB2*. **d**. Total viable cell numbers were determined following treatment in the presence of control or AIB-1 expressing vectors. **e**. Increased cell growth mediated by AIB-1 overexpression is ErbB2 dependent. Tamoxifen inhibition of cell growth is reversed by overexpression of AIB-1, although pretreatment with Herceptin blocks the AIB-1 mediated cell growth. **f**. AIB-1 expression was performed in T47D cells that do not contain an amplification of the AIB-1 genomic locus. Proliferating cells were treated with tamoxifen and total cell number was determined. Dashed line is overexpression of control vector and solid line in AIB-1 expressing construct.



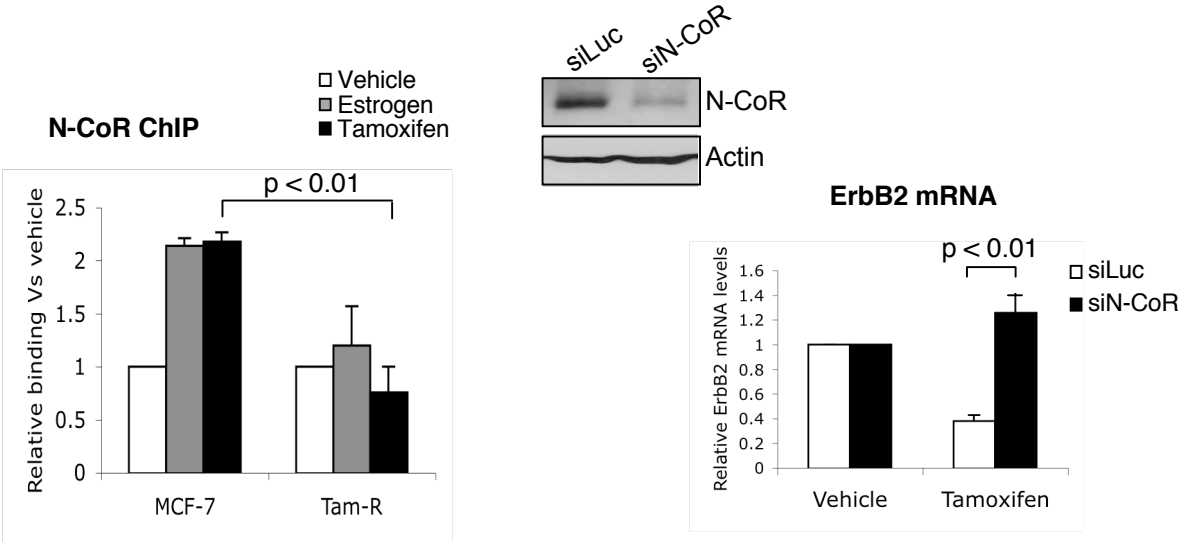
Supplementary data 10

Role of N-CoR and SRC-1 in ErbB2 transcriptional regulation. **a**. To assess if another p160 family member, SRC-1, was involved in regulation of ErbB2, we performed ChIP using an SRC-1 antibody in MCF-7 and Tam-R cells. Real time PCR data of the *ERBB2* intronic ER binding site was performed. SRC-1 is not recruited to the binding site in *ERBB2* in Tam-R cells. Overexpression of SRC-1 confirmed that unlike AIB-1, SRC-1 does not reverse the tamoxifen mediated repression of ErbB2 mRNA. **b**. ChIP of the co-repressor N-CoR was performed and confirmed that in wild type MCF-7 cells, N-CoR is recruited to the ER binding site within *ERBB2* with both estrogen and tamoxifen treatment. siRNA to N-CoR was transfected into MCF-7 cells and ErbB2 mRNA was assessed. The data confirm that N-CoR is required for tamoxifen mediated repression of ErbB2.

a



b



Supplementary data 11

Original immunoblots from Figure 3a and Figure 3c. The Western blots originally included lanes for estrogen treatment, but these specific bands were removed for the final figures.

Figure 3a

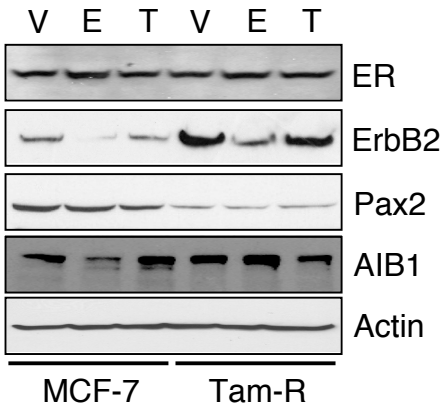
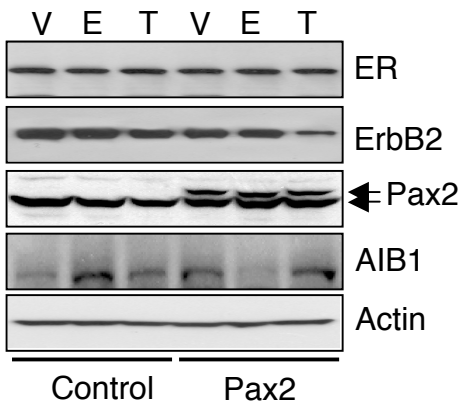
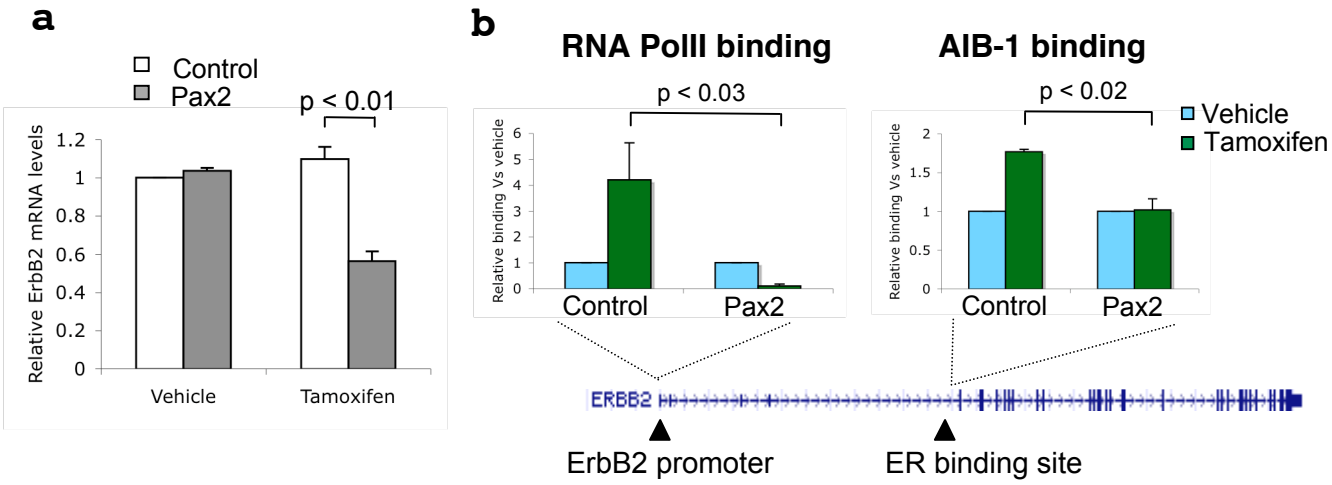


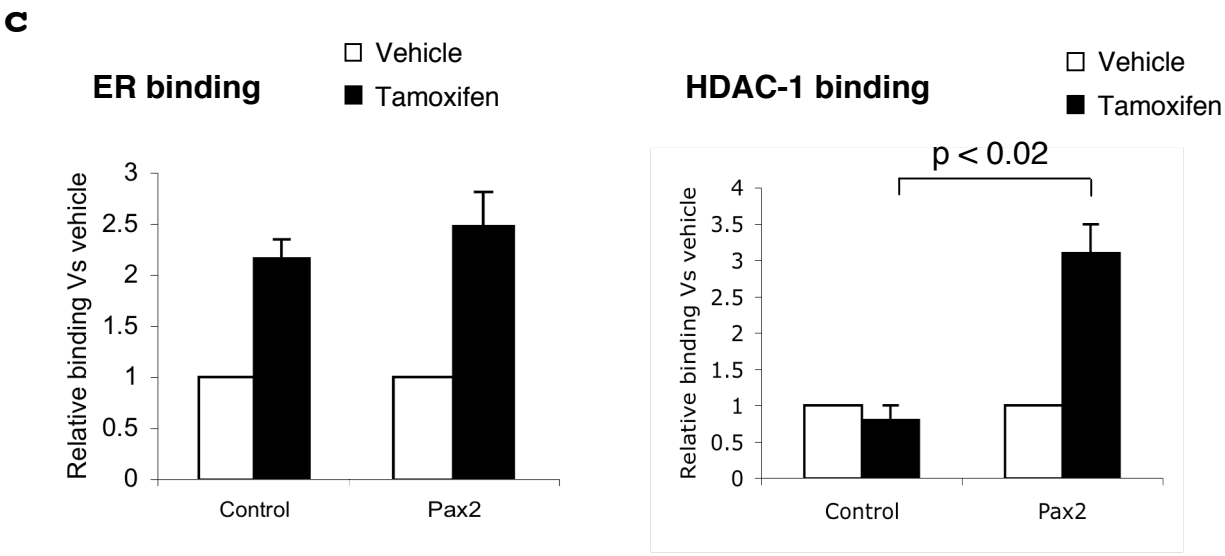
Figure 3c



Supplementary data 12

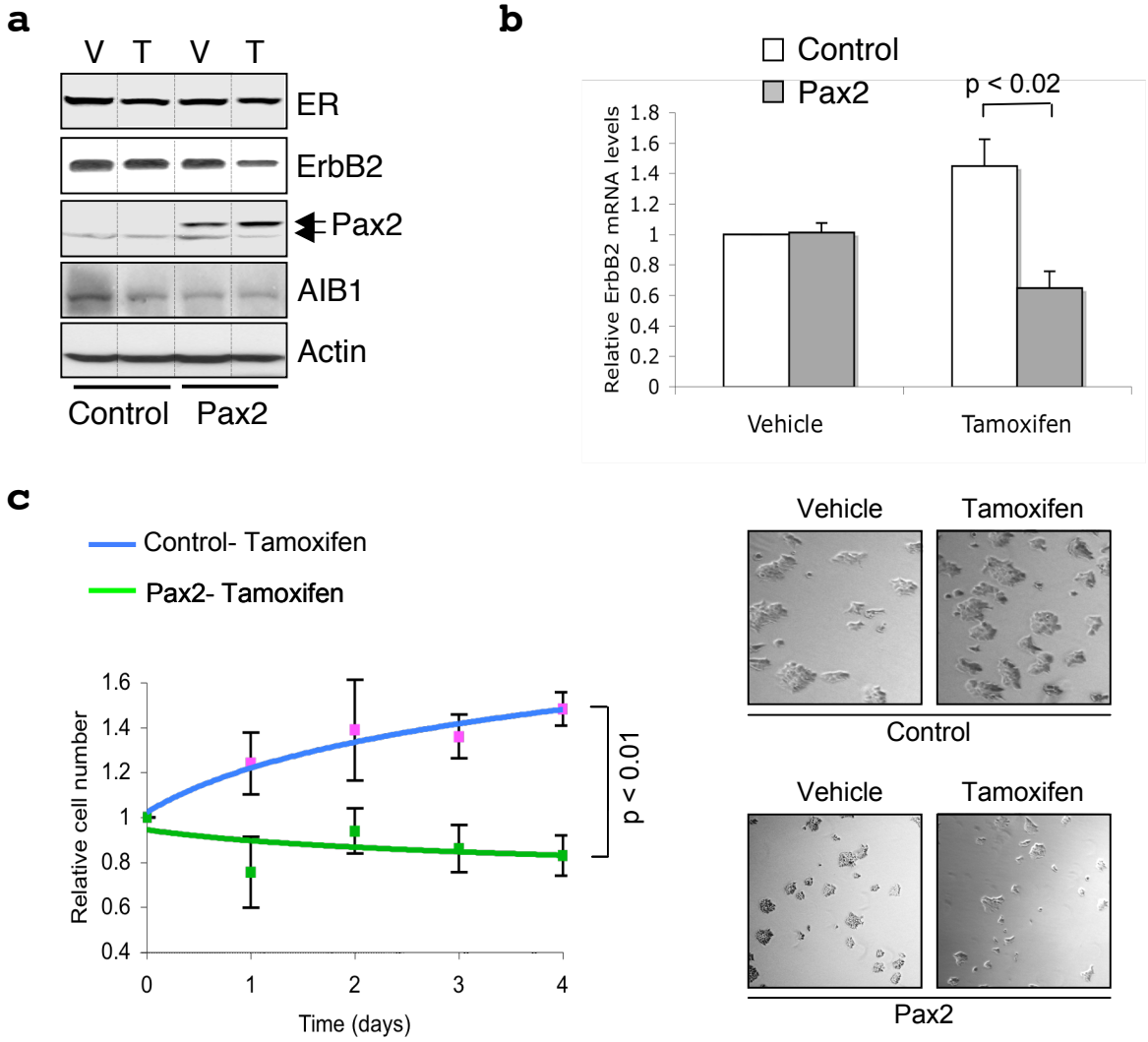
Additional data for Figure 3: Restoration of Pax2 levels in tamoxifen resistant cells reverses the tamoxifen resistant phenotype. **a.** A control expression plasmid (Control) or a Pax2 expressing plasmid was transfected into Tam-R cells, followed by vehicle or tamoxifen treatment for 8 hours. Total RNA was collected and ErbB2 mRNA levels were assessed by real time RT-PCR. **b.** Cells were transfected with control or Pax2 expressing constructs and chromatin was collected after vehicle or tamoxifen treatment. RNA PolII binding at the ErbB2 promoter and AIB-1 binding at the ER binding sites were determined by ChIP, followed by real-time PCR. The data are the average of three independent experiments ± Std. Dev. **c**. Tamoxifen resistant MCF-7 cells (Tam-R) were transfected with control plasmid or Pax2 expressing plasmid. Cells were stimulated with vehicle or tamoxifen for 45 minutes and ER and HDAC-1 ChIP was performed, followed by real time PCR of the ER binding site within the *ERBB2* gene. The data confirm that ER is recruited, even after Pax2 over-expression and that this interaction results in recruitment of HDAC-1, confirming that ER actively represses ErbB2 transcription and that this is dependent on Pax2.





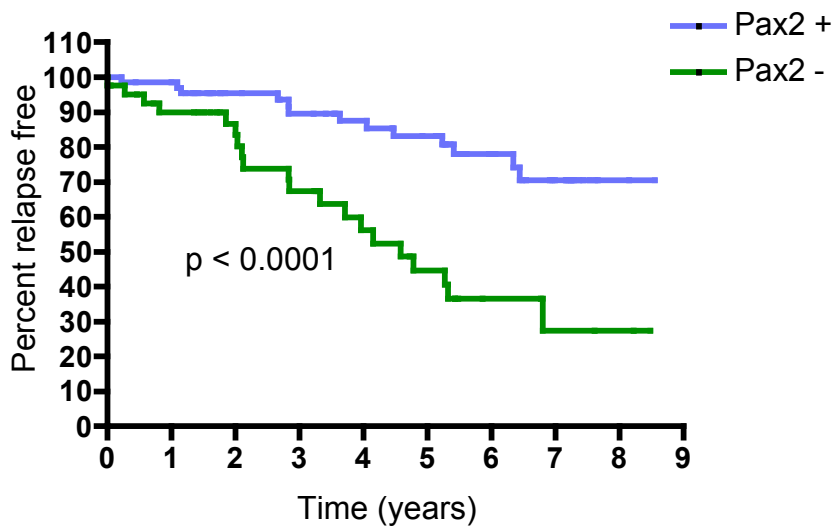
Supplementary data 13

Expression of Pax2 reverses the growth of *ERBB2* amplified BT-474 cells. **a.** BT-474 cells were transfected with Pax2 or control vector, cells were treated with vehicle (V) or tamoxifen (T) and total protein was collected and immunoblotted. Pax2 runs as a slower migrating band due to the presence of a tag. **b.** Control or Pax2 expressing vector was transfected into BT-474 cells and total RNA was collected after 8 hours of vehicle or tamoxifen treatment and ErbB2 mRNA levels were determined by RT-PCR. **c.** Following control or Pax2 expression in BT-474 cells, total viable cell numbers were determined after vehicle or tamoxifen treatment and normalised to vehicle treated conditions. All the data in these figures are the average of three independent experiments ± Std. Dev.



Supplementary data 14

Pax2 predicts clinical outcome in breast cancer patients. Kaplan-Meier curve representing percent relapse free survival in Pax2 positive and Pax2 negative tumours (n = 109).



Supplementary data 15

Primer sequences

ChIP primers

ErbB2 A promoter FWD	CACATCCCCCTCCTTGACTA
ErbB2 A promoter REV	TCCCTAGGCTGCCACTCTTA
ER binding site in ErbB2 FWD	GTTCCTCCCTCCTGTTCCTC
ER binding site in ErbB2 Rev	CCACAAACTGGTGGTCTCCT
GPR30 FWD	AGCTGCTGAGGGTCTGATGT
GPR30 REV	ATGAAGTGGGATTGGGTCAG
HDAC2 FWD	GCCCATGTCACTAAGCAGGT
HDAC2 REV	GTGGCTTGTGCAATGCTTTA
AIB1 FWD	GCAGTTTTGGTTCCTTGAGC
AIB1 REV	GCAGAATGGGAGCAAAGAAG
XBP-1 FWD	ATACTTGGCAGCCTGTGACC
XBP-1 REV	GGTCCACAAAGCAGGAAAAA
TFF-1 FWD	CACCCCGTGAGCCACTGT
TFF-1 REV	CTGCAGAAGTGATTCATAGTGAGAGAT
SDF1 FWD	AGGCATCACAATGCAAATCA
SDF1 REV	AGGCTGGTGAGATGCTGAGT

RT-PCR primers

ErbB2 mRNA FWD	AAAGGCCCAAGACTCTCTCC
ErbB2 mRNA REV	CAAGTACTCGGGGTTCTCCA

Supplementary data 16

Representative images of Pax2 immunohistochemistry. Two examples of tumours scored negative (a and b) and two examples called positive (c and d) are shown. Immunohistochemistry for Pax2 was performed on an automated BondMax Immunostainer (Leica, Germany) using Pax2 antibody (Abcam: ab38738) at a concentration of 1:100. Triplicate independent scoring was performed, scoring only nuclear positive or nuclear negative staining.