# Supporting Information

## Castellano et al. 10.1073/pnas.0906947106

### SI Text

### SI Methods

**Cell Culture.** MCF-7 and MELN cells were maintained in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, and 2% glutamine. MCF-7-Tet-Off (MCF-7-TO) cells (BD Biosciences, Clontech) were maintained in DMEM supplemented with 10% FCS, 100 $\mu$g/mL G418, and 1 $\mu$g/mL of doxytetracycline (Tet). MCF-7-TO-PLZF-ER$\alpha$ cells were maintained as described previously (1).

**Cell Treatments and RNA Isolation.** For E2 treatment, $1.5 \times 10^6$ of MCF-7 cells were deprived of hormones by growing them in DMEM lacking phenol red, supplemented with 10% dextran-coated charcoal-treated FCS (DSS) for 72 h, and stimulated with E2 (10 nM) for 0, 3, 6, or 12 h, after which total RNA was isolated using TRIzol (Invitrogen). For the MCF-7-TO and the MCF-7-TO-PLZF-ER$\alpha$ lines, $1.5 \times 10^6$ cells were seeded in 10-cm plates. After 24 h, the cells were washed with PBS and DMEM lacking phenol red, supplemented with 10% DSS and G418. Cells were cultured for a further 72 h, at which time E2 (10 nM) and Tet (1 $\mu$g/mL) were added as indicated. These cells were maintained in E2 for 24 h before TRIzol RNA extraction.

For separation of small ($< 200$ nt) and large ($> 200$ nt) RNA fraction the miRNeasy mini kit was used for obtaining the larger fraction, followed by the RNeasy MinElute Cleanup kit to isolate the smaller fraction, according to the manufacturer's instructions (Qiagen). Separation of RNA between the large and small fractions was confirmed by gel electrophoresis. After extraction the quality and levels of RNA were determined using an Agilent Bioanalyzer (Agilent Technologies) and a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific).

**Transfection and Luciferase Reporter Assays.** MELN cells ($5 \times 10^5$) were plated in 24-well plates in medium containing DMEM/10% DSS for 24 h, transfected with either the pre-miR miRNA precursor or an anti-miR miRNA inhibitor (Applied Biosystems) for 48 h using HiPerFect Transfection Reagent (Qiagen) and treated with E2 (10 nM) or vehicle (ethanol) for 24 h before luciferase measurement. For the 3′-UTR reporter assay, 200 ng pMIR-REPORT *firefly* luciferase vector, including the various fragments of *ER*$\alpha$ 3′-UTR, 100 ng of *Renilla* luciferase vector (pRL-TK) and the pre-miRNAs or negative control oligonucleotide, were transfected using Lipofectamine 2000 (Invitrogen). Forty hours after transfection, cells were harvested and luciferase activity measured using the Dual-Glo luciferase assay system (Promega).

**MiRNA Northern Blotting.** Northern blot analysis was performed as described previously (2) with minor modification. After electrophoresis the RNA was transferred from the gel to Hybond-N+ (GE Healthcare) membrane using a wet transfer apparatus. DNA oligonucleotide probe complementary to miR-18a was 5′-end-labeled with [$\gamma$-$^{32}$P]ATP, and hybridization was performed using Miracle hyb buffer according to the manufacturer's instructions (Thermo Scientific).

**Western Blotting.** Whole cell lysates were prepared in Nonidet P-40 lysis buffer [50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10% (vol/vol) glycerol, 1% Nonidet P-40, 5 mM DTT (DTT), 1 mM EDTA, 1 mM EGTA, 50 $\mu$M leupeptin, and 30 $\mu$g/mL aprotinin]. Lysates were subjected to SDS/PAGE and blotted on a Hybond C super nitrocellulose membrane (GE Healthcare). The intensity of bands was quantified using Image J software (National Institutes of Health). We used mouse monoclonal AIB1, ER$\alpha$, and $\beta$-actin antibodies purchased from Abcam.

### SI Discussion

Recently, a study undertaken by Bhat-Nakshatri et al. (3) showed that, after stimulation of MCF7 cells for 4 hours with E2, three miRNAs were up-regulated: let-7f, miR-98, and miR-21. Because these miRNAs are previously known to regulate the estrogen-responsive c-MYC, Bhat-Nakshatri el al. also suggested that the estrogenic response is regulated by miRNAs: therein E2F2, another E2-modulated gene, was found to be a new target of the let-7/miR-98 miRNA family. Herein, we also find that let-7g, miR-98, and miR-21 were significantly up-regulated by E2 between 3 and 6 h after E2 stimulation, but at a lower level (Table S1), probably reflective of different variants of MCF-7 cells expressing different levels of coactivators implicated in ER-mediated gene expression (4). In support of their data, we found that miR-27a and miR-27b were among the miRNAs down-regulated by E2, and in addition, we include all miR-181 family members (Table S1).


1. Buluwela L, et al. (2005) Inhibiting estrogen responses in breast cancer cells using a fusion protein encoding estrogen receptor-alpha and the transcriptional repressor PLZF. *Gene Ther* 12:452–460.
2. Yu Z, et al. (2008) A cyclin D1/microRNA 17/20 regulatory feedback loop in control of breast cancer cell proliferation. *J Cell Biol* 182:509–517.
3. Bhat-Nakshatri P, et al. (2009) Estradiol-regulated microRNAs control estradiol response in breast cancer cells. *Nucleic Acids Res* 37:4850–4861.
4. Kishimoto H, et al. (2005) The p160 family coactivators regulate breast cancer cell proliferation and invasion through autocrine/paracrine activity of SDF-1alpha/CXCL12. *Carcinogenesis* 26:1706–1715.

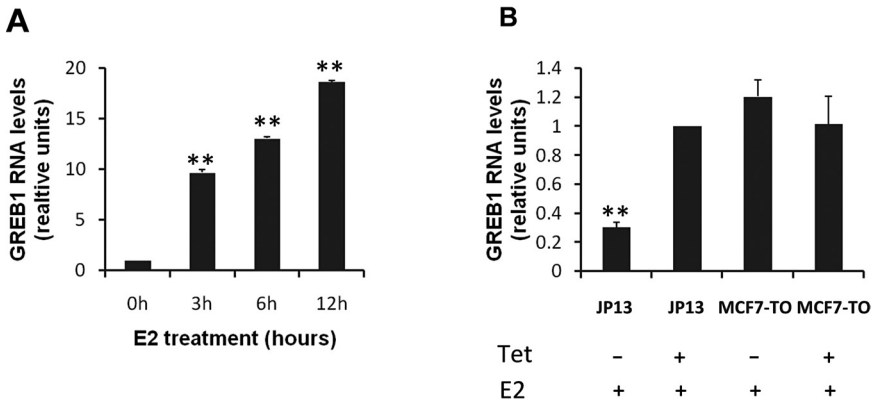


**Fig. S1.** *GREB1* is modulated by E2 treatment and overexpression of PLZF-ER$\alpha$. (*A*) MCF-7 cells were maintained in DMEM (minus phenol red) supplemented with 10% (vol/vol) charcoal-dextran FBS for 3 days and then were either left untreated or treated with 10 nM E2 for the indicated time periods. After total RNA extraction, expression of *GREB1* was analyzed by qRT-PCR using SYBR green and normalized to *GAPDH*. The mean of 3 experiments each performed in triplicate are presented, error bars represent SEM, the double asterisk represents $P < 0.005$ in comparison to time 0 h. *P* values were obtained using a 1-tailed Student's *t*-test. (*B*) JP13 and MCF-7-TO cells were cultured in the presence or absence of Tet for 72 h, followed by the addition of 10 nM E2 for 24 h. After total RNA extraction, expression of *GREB1* was analyzed by qRT-PCR using SYBR green and normalized to *GAPDH*.

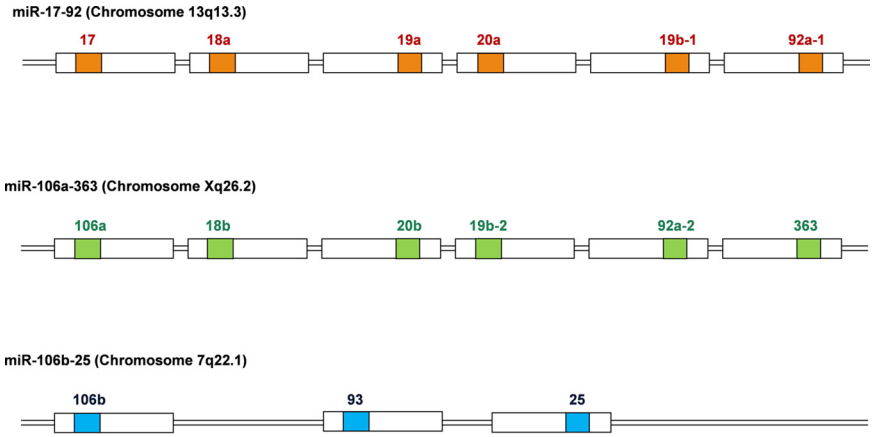


**Fig. S2.** Primary miRNA transcript structures of the 3 paralogous families. Human mir-17–92 (*Top*), mir-106a-363 (*Middle*), and mir-106b-25 (*Bottom*) clusters.

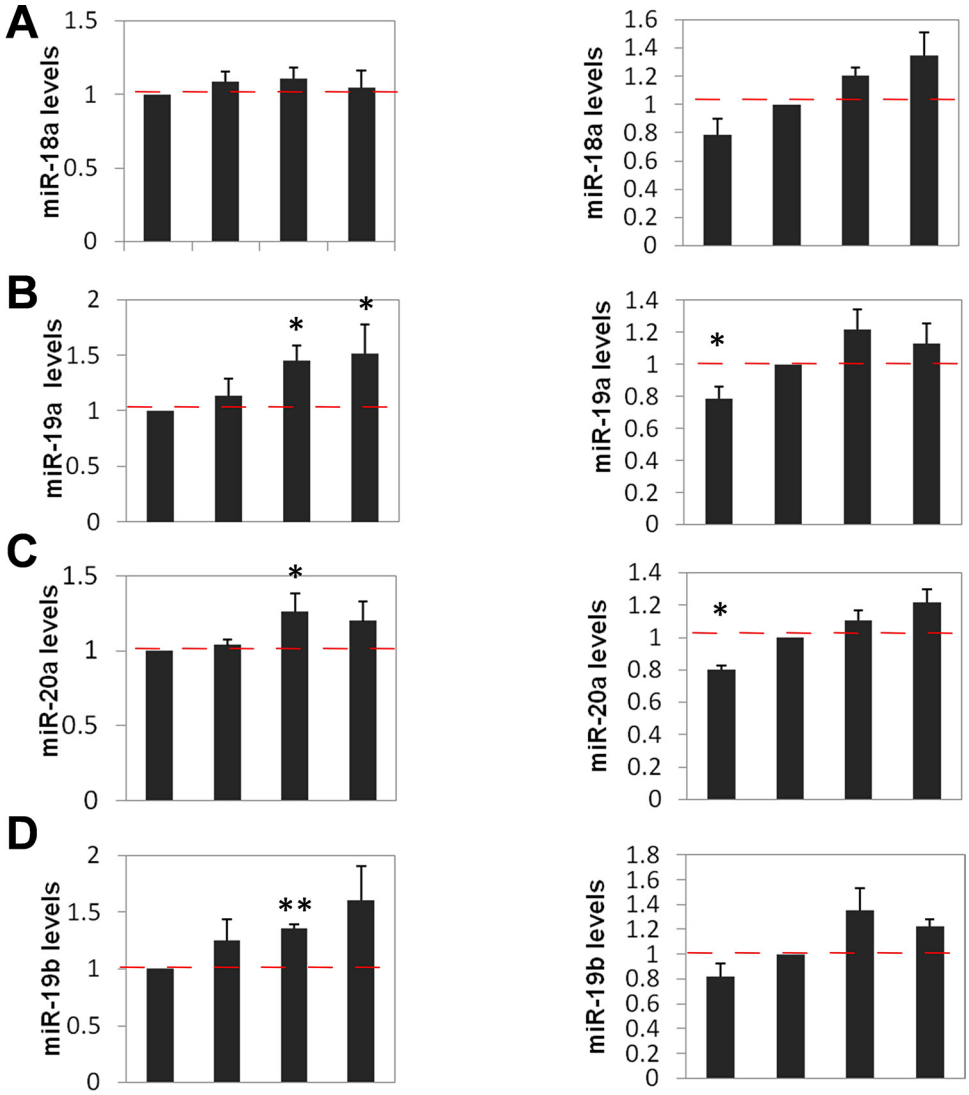


**Fig. S3.** Continued.

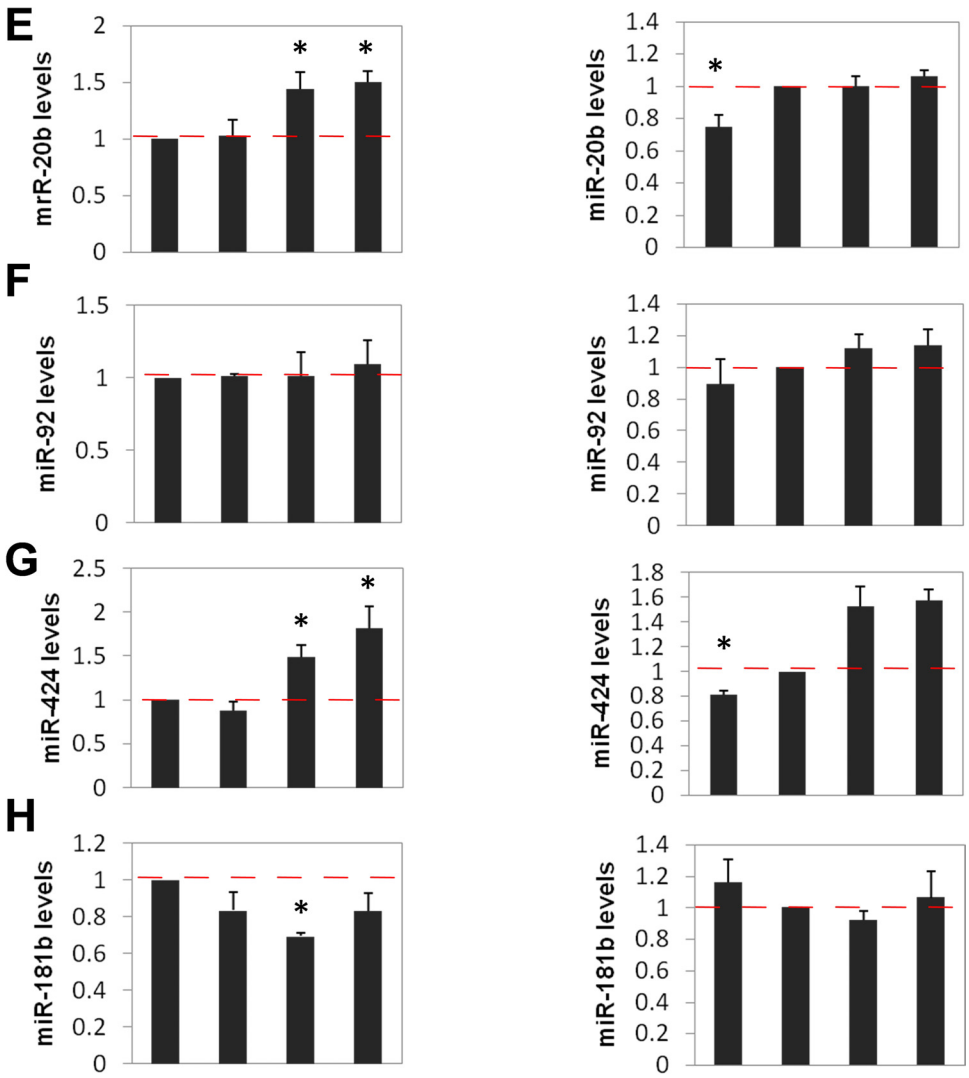


**Fig. S3.** Continued.

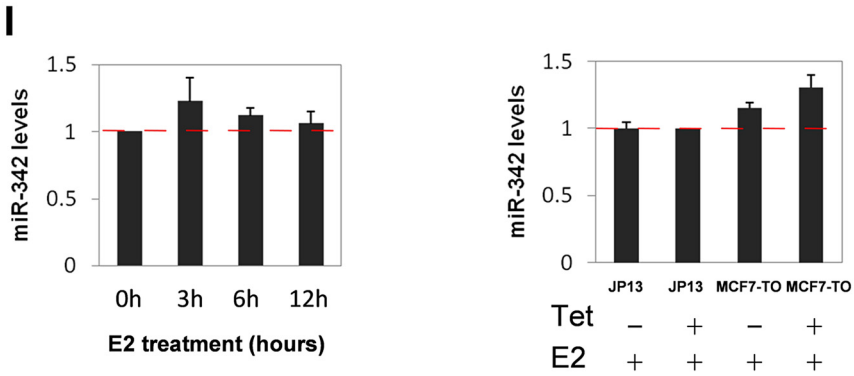


**Fig. S3.** Microarray validation by qRT-PCR. MiRNAs up- and/or downregulated were validated by Taqman qRT-PCR after 0, 3, 6, and 12 h of E2 treatment and after PLZF-ER$\alpha$ overexpression: (*A*) miR-18a, (*B*) miR-19a, (*C*) miR-20a, (*D*) miR-19b, (*E*) miR-20b, (*F*) miR-92, (*G*) miR-424, (*H*) miR-181b, and (*I*) miR-342 (used as a negative control). The mean of 3 experiments each performed in triplicate are presented, error bars represent SEM. The asterisk represents $P < 0.05$ in comparison to time 0 h. The double asterisk represents $P < 0.005$. *P* value was obtained using a 1-tailed Student's *t*-test.

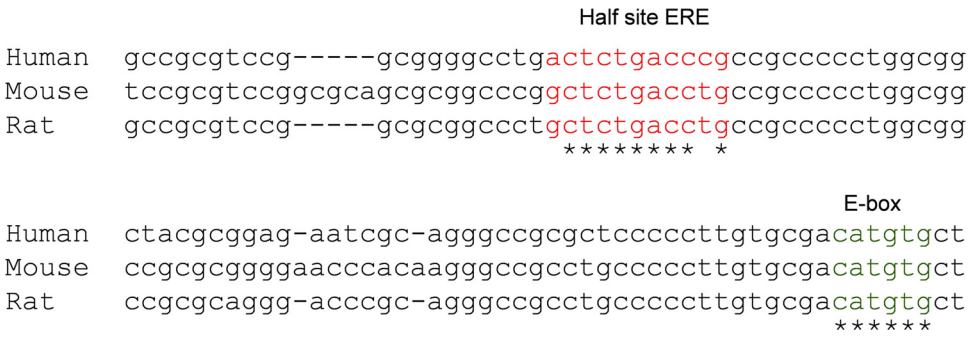


**Fig. S4.** Putative ERE and c-MYC consesus elements in mir-17–92 promoter are located in close proximity and conserved among species. Alignment between human, mouse, and rat of the genomic sequence of mir-17–92 promoter containing both a putative half-site ERE and the E-box (c-MYC binding site). The putative half-site ERE is indicated in red, the E-box in green.

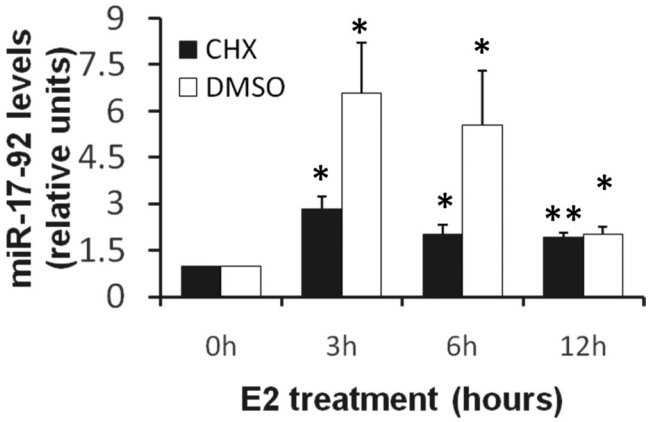


**Fig. S5.** New protein synthesis is not required exclusively for E2-induced mir-17–92 expression. MCF-7 cells were maintained in DMEM (minus phenol red) supplemented with 10% (vol/vol) charcoal-dextran FBS for 3 days and then pretreated with either CHX or DMSO (vehicle) as indicated, 1 h before 10 nM E2 treatment. After total RNA extraction, the expression of mir-17–92 was analyzed by qRT-PCR using SYBR green and normalized to *GAPDH.* The mean of 3 experiments each performed in triplicate are presented, error bars represent SEM. The asterisk indicates $P < 0.05$ in comparison to time 0 h, the double asterisk represents $P < 0.005$ in comparison to time 0 h. *P* values were obtained using a 2-tailed Student's *t*-test.

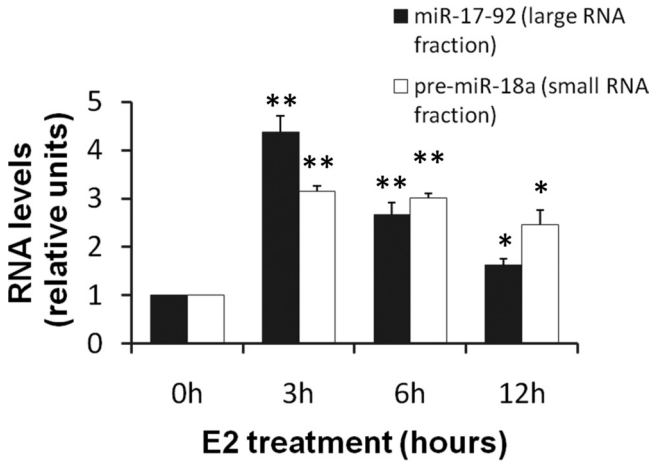


**Fig. S6.** Mir-17–92 is promptly processed by DROSHA complex that releases pre-miR-18a. After MCF-7 hormone starvation and treatment with 10 nM of E2 for 0, 3, 6, and 12 h, RNA fractions were separated and used for qRT-PCR. Mir-17–92 expression levels from large RNA fraction (>200 nt), normalized to *GAPDH* levels are presented with white columns, whereas pre-miR-18a expression levels from small RNA fraction (<200 nt), normalized to *U6* are presented with dark columns. Average of 3 experiments each performed in triplicate are presented, error bars represent SEM.

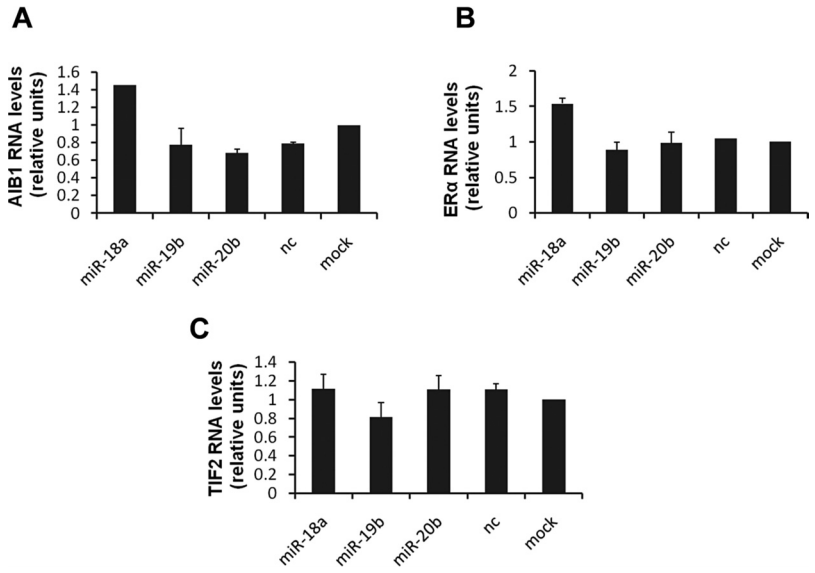


**Fig. S7.** *ERα* and *AIB1* mRNA levels do not change upon pre-miRNA overexpression. qRT-PCR analyses showing (*A*) *AIB1*, (*B*) *ERα*, and (*C*) *TIF2* (negative control) in MCF-7 cells untransfected or transiently transfected with pre-miR-18a, pre-miR-19b, pre-miR-20b, and pre-miR-n.c.

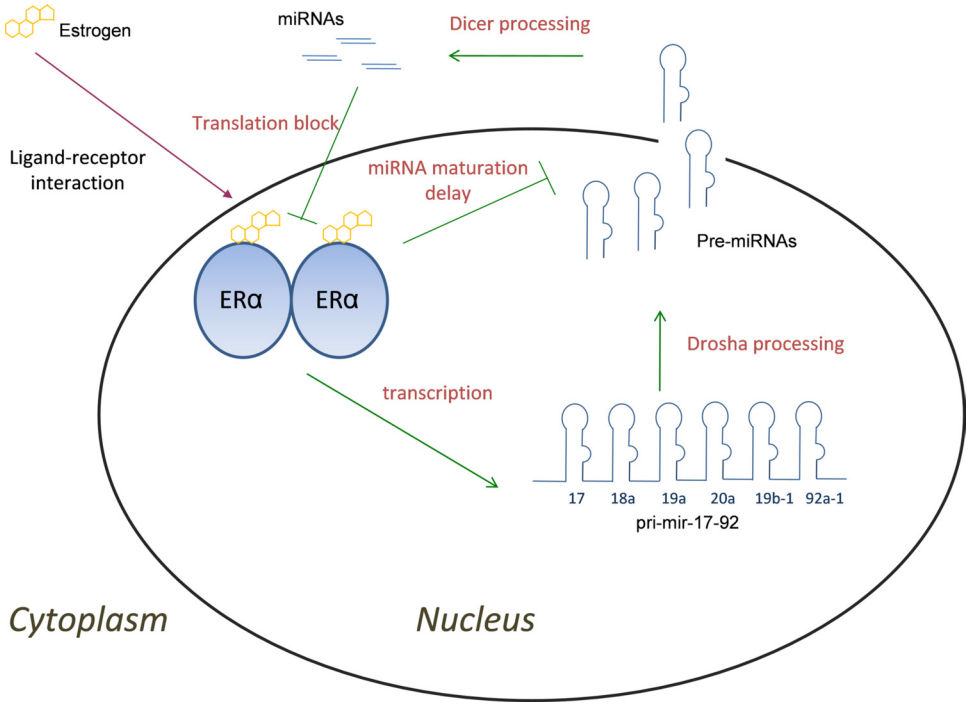


**Fig. S8.** Model of the negative feedback loop and miRNA maturation delay promoted by activated ER$\alpha$ in breast cells. After ligand binding, ER$\alpha$ induces the transcription of c-MYC that in turn directly activates pri-mir-17–92 transcription. The primary miRNA is promptly cleaved by DROSHA in the nucleus, but the processing from precursor to miRNA is delayed in an E2-dependent manner. The miRNAs derived from the precursors downregulate ER$\alpha$ and AIB1, therein fine tuning the ER$\alpha$ transcriptional response.

**Table S1. List of miRNAs significantly modulated by E2 treatment in MCF7 cells ($P < 0.05$)**

| miRNA | Fold change (0 h vs. 3 h) | *p* value | Fold change (0 h vs. 6 h) | *p* value | Fold change (0 h vs. 12 h) | *p* value | Fold change (jp13 vs. JP13 + Tet) | *p* value |
|---|---|---|---|---|---|---|---|---|
| hsa-miR-542–3p | | | | | 2.18 | 2.36E-01 | | |
| hsa-miR-424 | | | 1.48 | 2.00E-05 | 1.44 | 5.39E-10 | −1.16 | 4.77E-03 |
| hsa-miR-450 | | | 1.24 | 1.14E-03 | 1.3 | 1.13E-03 | | |
| hsa-miR-489 | 1.3 | 5.15E-03 | 1.29 | 1.85E-02 | 1.72 | 1.00E-05 | | |
| Let-7g | | | 1.18 | 1.67E-03 | | | | |
| hsa-miR-98 | 1.11 | 2.89E-03 | 1.15 | 4.21E-02 | | | | |
| hsa-miR-32 | | | 1.54 | 3.28E-03 | | | −1.16 | 4.28E-02 |
| hsa-miR-101 | | | 1.45 | 1.73E-03 | | | −1.15 | 1.32E-02 |
| *hsa-miR-19a | | | 1.36 | 4.04E-03 | | | −1.26 | 1.44E-03 |
| hsa-miR-92b | | | | | | | −1.32 | 4.81E-02 |
| *hsa-miR-20b | 1.2 | 9.13E-02 | 1.31 | 9.52E-08 | 1.24 | 7.40E-03 | −1.28 | 5.32E-06 |
| *hsa-miR-19b | 1.17 | 5.69E-03 | 1.29 | 5.02E-06 | 1.22 | 2.17E-23 | −1.28 | 2.62E-08 |
| *hsa-miR-20a | 1.13 | 1.55E-02 | 1.29 | 1.73E-07 | 1.24 | 9.26E-06 | −1.28 | 4.63E-13 |
| hsa-miR-7 | 1.23 | 4.01E-03 | 1.3 | 2.23E-06 | | | | |
| *hsa-miR-92 | | | | | 1.17 | 7.50E-04 | −1.27 | 2.62E-03 |
| *hsa-miR-106a | 1.16 | 3.95E-03 | 1.29 | 3.90E-04 | 1.26 | 1.95E-02 | −1.26 | 4.00E-05 |
| *hsa-miR-17–5p | 1.21 | 1.25E-02 | 1.25 | 4.35E-03 | 1.27 | 1.96E-02 | −1.25 | 6.96E-06 |
| *hsa-miR-18a | | | 1.27 | 3.36E-02 | | | −1.25 | 2.46E-07 |
| *hsa-miR-93 | 1.13 | 4.46E-03 | 1.12 | 1.00E-05 | | | −1.15 | 3.41E-06 |
| hsa-miR-301 | | | | | | | −1.13 | 1.00E-04 |
| *hsa-miR-25 | 1.1 | 2.36E-06 | 1.15 | 4.91E-03 | 1.12 | 1.14E-01 | −1.14 | 3.65E-07 |
| *hsa-miR-106b | | | 1.2 | 3.72E-02 | | | −1.13 | 4.21E-06 |
| has-miR-21 | | | 1.15 | 3.85E-02 | | | | |
| hsa-miR-181a | −1.11 | 2.60E-04 | −1.1 | 2.30E-03 | −1.17 | 9.50E-03 | | |
| hsa-miR-181b | −1.13 | 1.06E-07 | −1.13 | 4.58E-03 | −1.24 | 3.82E-03 | | |
| hsa-miR-181d | | | −1.15 | 9.51E-03 | −1.3 | 3.25E-03 | | |
| hsa-miR-181a* | −1.36 | 2.60E-03 | −1.72 | 1.98E-02 | −2.2 | 1.69E-02 | | |
| hsa-miR-27a | −1.27 | 1.54E-02 | | | | | | |
| hsa-miR-27b | −1.12 | 3.89E-02 | | | | | | |
| hsa-miR-22 | −1.2 | 1.00E-05 | −1.14 | 8.51E-03 | | | | |
| hsa-miR-487b | −1.32 | 1.26E-03 | −1.25 | 8.56E-03 | −1.17 | 2.17E-02 | | |
| hsa-miR-494 | −1.27 | 1.75E-03 | | | | | | |
| hsa-miR-198 | | | −1.34 | 3.77E-03 | | | | |
| hsa-miR-500 | | | −1.37 | 5.87E-03 | | | | |
| has-miR-584 | −1.29 | 1.30E-04 | −1.32 | 3.38E-02 | | | | |
| hsa-miR-663 | −1.37 | 5.00E-02 | −1.6 | 7.79E-06 | | | | |
| hsa-miR-671 | −1.29 | 7.23E-03 | −1.28 | 1.84E-02 | | | | |

MiRNAs encoded by the 3 paralogous clusters (miR-17–92, miR-106a-363, and miR-106b-25) are indicated by an * before the name. In our experiments, for each individual miRNA, at least 2 or 3 probes were spotted on the platform and data are shown for a representative miRNA but interestingly all the probes abundantly reach our statistical thresholds.

**Table S2. Primer sequences**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *GREB1* | CAAAGAATAACCTGTTGGCCCTGC | GACATGCCTGCGCTCTCATACTTA |
| *GAPDH* | TGAAGGTCGGAGTCAACGGATTT | GCCATGGAATTTGCCATGGGTGG |
| *miR-17–92* | AAAGGCAGGCTCGTCGTTG | CGGGATAAAGAGTTGTTTCTCCAA |
| *U6* | CTCGCTTCGGCAGCACA | AACGCTTCACGAATTTGCGT |
| *pri-miR-342* | GCCATTGCATCCTTCTCT | TCAATCACAGATAGCACCC |
| *pre-mIR-18a* | TAAGGTGCATCTAGTGCAGATAG | GAAGGAGCACTTAGGGCAGT |
| *HNRPA1* | ATACTGTGAATGGCCACAA | AACCACTTCGACCTCTTTG |
| *ERα* | GAATCTGCCAAGGAGACTCGC | ACTGGTTGGTGGCTGGACAC |
| *AIB1* | CGTCCTCCATATAACCGAGC | TCATAGGTTCCATTCTGCCG |
| *TIF2* | GCCCGATTTCTCTTGGATTTG | TGGAGGGGTCAGAGGTATTT |
| *SRC1* | ATGGTGAGCAGAGGCATGACA | AAACGGTGATGCTCATGTTG |
| *ER1* | CGCACTAGTAAATGGCTCTAAGAATAAGC | CGCACGCGTAGTGCTATTTTGTCTACTGT |
| *ER2* | CGCACGCGTGATGCCTATTGTTGGATACT | CGCACGCGTAATTGTTTACAGGTGCTCGA |
| *ERup* | CGCACGCGTAGTGCTATTTTGTCTACTGT | CGCACTAGTCCACACGGTTCAGATAAT |
| *ERfl* | CGCACGCGTAGTGCTATTTTGTCTACTGT | CGCACGCGTAATTGTTTACAGGTGCTCGA |
| *TFF1 promoter* | CACCCCGTGAGCCACTGT | CTGCAGAAGTGATTCATAGTGAGAGAT |
| *mir-17–92 promoter* | AAAGGCAGGCTCGTCGTTG | CGGGATAAAGAGTTGTTTCTCCAA |