Supplementary Methods for Janowski et al.

Methods

Double-Stranded RNAs

21-nucleotide RNAs were synthesized by Integrated DNA Technologies or Dharmacon. Oligonucleotides contained two 2'-deoxythymidine on the 3' end and were deprotected and desalted. Sequences are listed in Table I and identified relative to the transcription start site as described (AR: GenBank M58188, MVP: GenBank AJ238509-AJ238519, Htt: GenBank NM002111). Ago1 was silenced by RNA duplex 5'-TTAACCGGUCCUCGAACUAAC-3'/ TTGUUAGUUCGAGGACCGGUU. Ago2 was silenced by an siRNA pool (Dharmacon).

Cell Culture

MCF-7 cells (America type cell culture collection, ATCC) were maintained in RPMI-1640 media (ATCC) supplemented with 10% fetal bovine serum, 0.5% nonessential amino acids, 0.4 units mL^{-1} bovine insulin, 100 units mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin (pen/strep). Cells were cultured at 37°C and 5% CO_2.

Lipid-mediated transfection

Cells were plated at 80,000 cells per well in 6-well plates (Costar) two days before transfection without antibiotics. Transfection with agRNA was performed using Oligofectamine (Invitrogen) following manufacturer's instructions. Per well, 25nM duplex (0.9 μl lipid) in Optimem (Invitrogen) to a final volume of 250 μl. Media was added to the duplex/lipid mixture for a

final volume of 1.25 ml then added to cells. Media was changed 24 h later, and cells were harvested five days after transfection unless otherwise indicated.

Western blotting

Cell pellets were lysed and protein concentrations were quantified using BCA assay (Pierce). Westerns were performed on protein lysates (30 μg/well). Primary antibodies (Ab) included: AR-Ab (Cell Signalling), MVP-Ab (BD Transduction Laboratories) and, Htt (Chemicon). β-actin-Ab (Sigma) was used as an internal control and for quantitation. Protein was visualized using anti-mouse or anti-rabbit secondary antibody (Jackson Immunolabs) and Supersignal developing solution (Pierce).

RNA Analysis

Total RNA from treated MCF-7 cells was extracted using trizol (TRIzol, Invitrogen). 4 μg were reverse transcribed by random primers using High Capacity cDNA Archive kit (Applied Biosystems). Primers were supplied by Applied Biosystems. Detection employed TaqMan Gene Expression Assays (Applied Biosystems) with 100 ng of cDNA. Data was normalized relative to measured levels of GAPDH RNA.

Treatment with Trichostatin A

T47D cells were plated in 6-well plates at 120,000 cells per well. After adhering, cells are treated with complete RPMI media supplemented with TSA at 500 nM and 250 nM for 18 hours. TSA containing media was then removed and cells are transfected with either a noncomplementary duplex RNA or PR-26 duplex RNAs

for 24 hours. After transfection, cells are incubated with complete RPMI media for five days at which time cells are harvested for Western analysis.

Quantitative-RT-PCR

Total RNA from T47D breast cancer cells was extracted by the one-step method of Chomczynski and Sacchi (TRIzol, Invitrogen). RNA was treated with deoxyribonuclease to remove any contaminating DNA, and four micrograms were reversed transcribed using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen). Primer sets directed against human COX-2, ERα, PR, along with h368B against ribosomal RNA were generated utilizing Primer Express™ software (PE Applied Biosystems) based on published sequences (Table 1).

The relative abundance of each transcript was determined by real-time quantitative PCR using a modification of previously published methods (4). For the quantitative analysis of mRNA expression, the ABI Prism 7700 Detection System (Applied Biosystems) was employed using the DNA binding dye SYBER Green (PE Applied Biosystems) for the detection of PCR products. The cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The cycle threshold was set at a level where the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. We calculated the relative fold changes using the comparative cycle times (Ct) method with cyclophilin as the reference guide. Over a wide range of known

cDNA concentrations, all primer sets were demonstrated to have good linear correlation (slope = -3.4) and equal priming efficiency for the different dilutions compared to their Ct values (data not shown). Given that all primer sets had equal priming efficiency, the ΔCt values (primer – internal control) for each primer set were calibrated to the experimental samples with the lowest transcript abundance (highest Ct value) and the relative abundance of each primer set compared to calibrator was determined by the formula, $2^{\Delta\Delta Ct}$, whereby $\Delta\Delta$Ct is the calibrated Ct value.

The following primers were used for Q-PCR analysis.

Gene	***Primer***	***Ref. #***
COX-2:	FWD 5'-TTC CAG ATC CAG AGC TCA TTA AA -3'	AY462100
	REV 5'-CCG GAG CGG GAA GAA CT -3'	
PR:	FWD 5'-TCA GTG GGC AGA TGC TGT ATT T-3'	NM000926
	REV 5'-GCC ACA TGG TAA GGC ATA ATG A-3'	
ERα:	FWD 5'-AGA GAA GTA TTC AAG GAC ATA ACG ACT ATA T-3'	M12674
	REV 5'-TCT TCC TCC TGT TTT TAT CAA TGG-3'	
36B4:	FWD 5'-TGC ATC AGT ACC CCA TTC TAT CA-3'	NM001002
	REV 5'-AAG GTG TAA TCC GTC TCC ACA GA-3'	

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using a modification of previously published methods. Briefly, T47D cells ($3 – 1 \times 10^7$ cells/treatment) were washed once with PBS and incubated with 1% formaldehyde (in control medium) for 10 min at room temperature to cross-link proteins and DNA. Crosslinking was terminated by the addition of glycine (0.125 M, final concentration). The cells were washed twice with cold PBS and placed in 500 μl of 'lysis buffer' (50 mM Tris pH 8.1, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail (Roche), and 5 mM EDTA). The lysates were sonicated on ice to produce sheared, soluble chromatin. The soluble chromatin was precleared with Protein A/G Plus agarose beads (60 μl) at 4°C for 1 h. The samples were microfuged at 14,000 rpm to pellet the beads, and the supernatant containing the sheared chromatin was placed in new tubes. The precleared chromatin was aliquoted into 300 μl amounts and incubated with antibodies for dimethyl-Histone H3 (Upstate) or Histone H3 (Upstate) at 4°C overnight. Two aliquots were reserved as 'controls' – one incubated without antibody and the other with non-immune IgG. Protein A/G Plus agarose beads (60 μl) were added to each tube, the mixtures incubated for 2 h at 4°C and the immune complexes collected by centrifugation. The beads containing the immunoprecipitated complexes were washed sequentially for 5-10 min in wash buffer I (20 mM Tris-HCl, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 150 mM NaCl), wash buffer II (same as I, except containing 500 mM NaCl), wash buffer III (10 mM Tris-HCl, pH 8.1, 1 mM EDTA, 1% NP-40, 1% deoxycholate, 0.25 M LiCl), and in 2 × TE buffer. The beads were eluted with 250

μl elution buffer (1% SDS, 0.1mM $NaHCO_3$ + 20 μg salmon sperm DNA (Sigma) at room temperature. This was repeated once and eluates were combined.

Crosslinking of the immunoprecipitated chromatin complexes and 'input controls' (10% of the total soluble chromatin) was reversed by heating the samples at 65°C for 4 h. Proteinase K (15 μg, Invitrogen) was added to each sample in buffer (50 mM Tris-HCl, pH 8.5, 1% SDS, 10 mM EDTA) and incubated for 1 h at 45°C. The DNA was purified by phenol-chloroform extraction and precipitated in EtOH for overnight at -20° C. Samples and 'input' controls were diluted in 10-100μl TE buffer just prior to PCR. Real-time PCR was employed using forward (5'-CCTAGAGGAGGAGGCGTTGTT-3') and reverse (5'-CATTGAGAA-TGCCACCCACAC-3') primers that amplify a ~100 bp region surrounding the area in the *hPR* promoter (NM_000926) where PR11 AgRNAs bind. Using serial dilutions of human chromosomal DNA, these primers were demonstrated to have equal efficiency in priming their target sequences.