Supplementary Materials and Methods for

ANCCA/ATAD2 overexpression identifies breast cancer patients with poor prognosis and controls B-Myb and EZH2 for proliferation and survival of triple-negative cells

Kalashnikova et al

Cell culture. Triple negative human breast cancer cells (MDA-MB 231, MDA-MB 436, MDA-MB 468, MDA-MB 453 and HCC1937) were obtained from ATCC in 2002 and 2008, and cultured in DMEM supplemented with 10% FBS (Omega) for MDA-MB 231, or RPMI supplemented with 10% FBS for the other cell lines. Frozen stock was made and stored in liquid nitrogen until the initiation of this study. After thawing frozen stock, the cells were kept at low passage (usually within 10 passages and within 4 months) throughout the study. The cell lines were tested recently (in 2009-2010) for authenticity by monitoring the cell morphology using microscope, growth rate using cell counting, response to factors or hormones for growth, expression of genes/proteins including the hormone receptors and members of the HER2/ErbB2 family using Western and RT-PCR, and were confirmed to be in line with the ATCC descriptions and with the literature.

Normal human mammary epithelial cells (HMECs) were obtained from Lonza/Clonetics and cultured in MEGM with BulletKit supplied from the manufacture. HMECs were harvested for Western analysis after being passed three times since the initial thaw and plating. The HMEC cells were used within 4 months after their receipt.

ChIP. ChIP assays were performed essentially as described previously (1) with the following modifications. Briefly, about 3 x 10^6 cells were fixed with 1% formaldehyde for 10 min at room temperature and lysed in the lysis buffer containing 0.2% SDS. The lysate was then sonicated as previously described (1) or for 10 min on Bioruptor (Diagenode Inc.), diluted and centrifuged. The cleared chromatin solutions were incubated overnight at 4°C with anti-ANCCA or pre-immune serum

(described below) at approximately 2 μg/ml (4 μl per ml) of diluted chromatin solution. Precipitated DNA were reverse cross-linked overnight at 65°C, purified and analyzed as previously described (2). PCR primers are available upon request.

Antibodies for Western blotting and ChIP. Antibodies against Cdc6 (D-1), Cdc2 (17), Cyclin D1 (H-295), Cyclin E1 (E-4), Cyclin E2 (N-20), E2F1 (C-20 or KH-95), MCM7 (G-7), PCNA (sc-56), VEGF (sc-152) and SUZ12 (P-15) were from Santa Cruz Biotechnology. Antibodies against Cyclin A2 (611269) and Cyclin B1 (610219) were from Beckton Dickinson. Antibodies against EZH2 (AC22), AKT1 (9272), pAKT1 (40513), IRS2 (4502), SGK (3272) were from Cell Signaling Technology. Top2A (AK5) was from MBL. Beta-Actin (AC-74) was from Sigma. Anti-ANCCA antibody was raised in rabbit (Covance) and affinity-purified by using GST-ANCCA N-terminus (aa 2-264) expressed and purified from *E. coli*.

Analysis of ANCCA/ATAD2 expression association with other genes in microarray gene expression data sets. In order to investigate ANCCA/ATAD2 transcript expression patterns in clinical breast cancer tissues, we utilized the dataset obtained in a study which profiled 198 patient samples from the TRANSBIG Consortium with Affymetrix HG-U133A GeneChip arrays (3, 4). The raw data (CEL files) and the sample and data relationship information were downloaded from ArrayExpress (European Bioinformatics Institute, Cambridge, UK) (5). Data analysis was performed using GeneSpring GX (version 10) software (Agilent Technologies, Santa Clara, CA). Briefly, probe-set expression intensities were obtained using robust multi-array average (RMA) (6) for probe summarization of quantile-normalized, background-adjusted, and log-transformed perfect match (PM) probe intensity values. The data was then filtered to retain only those probesets having expression values that exceeded the 20% lower cut-off threshold in at least one of the samples. Subsequently, transcripts co-expressed with ANCCA/ATAD2 were identified using the Spearmann rank correlation test. Hierarchical clustering was

then performed to further organize the ATAD2 co-expression cluster based on similarities of expression between genes and conditions (i.e., ER status).

References

1. Louie MC, Yang HQ, Ma AH, et al. Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. Proc Natl Acad Sci U S A 2003;100:2226-30.
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3. Desmedt C, Piette F, Loi S, et al. Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series. Clin Cancer Res 2007;13:3207-14.
4. Buyse M, Loi S, van't Veer L, et al. Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer. J Natl Cancer Inst 2006;98:1183-92.
5. Brazma A, Parkinson H, Sarkans U, et al. ArrayExpress--a public repository for microarray gene expression data at the EBI. Nucleic Acids Res 2003;31:68-71.
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Primers used for RT-PCR analysis:

TCCTATCTTTTACTTGCTCC	RT-NCAPG-r1
CAAGACTTCCCAAGATTATC	RT-NCAPG-f1
GAGTCAAAGGTTGGGTTTTC	RT-TOP2A-r1
GTGGGAAGTGTGTTTAACTAT	RT-TOP2A-f1
GTTGGGGTTATTTAAGGCTG	RT-MCM10-r1
TGAATACCACTGGCATGATG	RT-MCM10-f1
TTCTTCCTTACCTGAATGAC	RT-SMC2-r2
TTATGGTCCAATTATTGTGG	RT-SMC2-f2
TTTGTTGCTTGAGACTCATC	RT-DLGAP5-r2
AAATGGAAAACTTACCTGAG	RT-DLGAP5-f2
TTTTTGCCTTTCAGATCCTG	RT-KIF15-r1
GAAAGAGTTTCCTTTGTAAGT	RT-KIF15-f1
ACTCTGCACCATCTGGTTGG	RT-KIF23-r1
CATGCCATCACAGTATCTGTT	RT-KIF23-f1
GCAAACATTACTCAAGGTAT	RT-SMC4-r2
CTTTCTCTGAAGGAATCATG	RT-SMC4-f2
AAGGGTATTTCTTGTAAAGTC	RT-DLGAP5-f1
AAAAGCATTGGCACTTCTGG	RT-DLGAP5-r1
AATGAGCATGAGGATGGTGAT	RT-KIF4A-f1

TCCGTTCAACAGTGCCCAAG	RT-KIF4A-r1
GCAATAGCATCTGGTGGATC	RT-BUB1-r2
CAAGGGATGGAAAATTCAGTC	RT-BUB1-f2
AAAGTGTGAAGTACAAGTCC	RT-TAF2-r1
ACTAAAGTGGACAGAAGTTAT	RT-TAF2-f1
ATTATACCAGCCAAGGGATC	RT-KIF11-r1
TCCTGTACGAAAAGAAGTTAG	RT-KIF11-f1
GAGTCCTGAAGAAAAGACCG	RT-DSCC1-f1
AAGAAGTGTGGCAGCAGAGTG	RT-DSCC1-f1
AGGACATGGTAATGGATTCC	RT-RAD51AP1-f1
TAGCAGAAGTAGCAGCAGCC	RT-RAD51AP1-f1
AAGGAGGTAATGTGTGTTTC	RT-GPSM2-r1
ACAATCTTGGGAATGTGTATC	RT-GPSM2-f1
AGCCATTAGTCACTTTCCAAG	RT-CEP55-f1
CACAGCAGCCTAAAAGATAC	RT-CEP55-r1
CTTTACAAAGCCCACTTTAA	RT-ECT2-f1
AGAGTGCTGATTTAGAAGAA	RT-ECT2-f1
CCCCCAGAAGTAGCAGAGTTTGTG	RT-cycA2-f1
GCTTTGTCCCGTGACTGTGTAGAG	RT-cycA2-r1
GAGAAAATCTGGCACCACACC	RT-actin-f1
ATACCCCTCGTAGATGGGCAC	RT-actin-r1
GAAATCCCATCACCATCTTCCAG	RT2-GAPDH
ATGAGTCCTTCCACGATACCAAAG	RT1-GAPDH
ACTGACTGCTGCTGCCTTGTGC	cycE2-RT-s
TCGGTGGTGTCATAATGCCTCC	cycE2-RT-as
CACCGAGTACTCCTGTGGCTTG	RT-ANCCA-f1
TCTAGCTCGAGTCATTCGCAGAACAC	RT-ANCCA-r1
GCACTTGGCTTCAAAGCTG	RT-CDC2/A
CCAAGTATTTCTTCAGATCCATGG	RT-CDC2/C

Figure Legends for Supplementary Figures

Supplementary Figure 1. Validation of anti-ANCCA antibody for IHC staining. A, IHC procedures were performed on a section of human breast cancer FFPE specimen with either anti-ANCCA antibody absorbed with a GST-fusion protein that was not used as an antigen for anti-ANCCA antibody

generation (a), or with the antibody absorbed with GST-ANCCA (amino acids 2-264) that was used as antigen for the anti-ANCCA antibody production (b). B, Western blotting analysis using the anti-ANCCA antibody shows one major band of molecular weight approximately 170kDa in the whole lysates of indicated breast cancer cell lines. Note the lower bands in the middle lane are likely derived from ANCCA protein as they are suppressed upon ANCCA-RNAi treatment of cells (data not shown).

Supplementary Figure 2. ANCCA expression is significantly elevated in high grade tumors. A, representative images of anti-ANCCA IHC analysis of tumors in the TMA cores, which show different levels of ANCCA expression in different grades or normal breast tissue.

Supplementary Figure 3. Box-and-whisker plots of ANCCA mRNA expression in normal and breast carcinoma tissues. The analysis was performed using www.oncomine.org. Data sets of Richardson AL et al., 2006 are from a study in Cancer Cell. 2006 Feb;9(2):121-32; and data sets of Sorlie T et al., 2003 are from a study in Proc Natl Acad Sci U S A. 2003 Jul 8;100(14):8418-23.

Supplementary Figure 4. Representative IHC images from adjacent sections of different tumor tissues for comparison of distribution of anti-ANCCA or anti-Ki67 IHC positive cells in the tumor.

Supplementary Figure 5. Kaplan-Meier analysis of ANCCA protein expression and disease-free survival in ER-positive or ER-negative tumors.

Supplementary Figure 6. ANCCA expression promotes anchorage-independent growth. MDA-MB 453 cells were transfected with ANCCA siRNA or control siRNA, plated in soft agar for colony

formation. Representative images of colonies stained with MTT at 4x and 10x magnification, number of colonies formed from cells with different treatments obtained from three experiemts, and Western analysis of ANCCA two days after siRNA transfection are shown.

Supplementary Figure 7. Knockdown of ANCCA via a different siRNA demonstrated an inhibitory effect on cell proliferation and gene expression. MDA-MB 468 and MDA-MB 231 cells were transfected with si-control, or si-ANCCA #6 and/or #7, and harvested for cell numeration (A and B) and Western analysis (C) 2 days after the transfection. ANCCA si-RNA #7 targets an ANCCA mRNA sequence different from that of siANCCA #6, which was used for the experiments presented in the other figures.

Supplementary Figure 8. ANCCA is required for survival of triple negative breast caner cells. MDA-MB 231 cells were transfected with ANCCA siRNA or control siRNA, or mock treated, and processed for TUNEL assay. Representative images of TUNEL staining with cells with different treatments are shown.

Supplementary Figure 9. Unsupervised hierarchical clustering of the 25 probesets (representing 20 genes in addition to ANCCA) exhibiting expression patterns most highly correlated with that of ANCCA overexpression in a microarray data set of 198 patients described in the study by Desmedt C *et al.* (Clin Cancer Res 2007; 13:3207-14). Spearmann correlation coffecients (*i.e.*, with respect to ANCCA expression) are indicated adjacent to the gene symbols in the heat map. The magnitude of increased or decreased expression (relative to the mean) for each gene across the entire panel of samples is depicted by increasingly darker shades of red or blue, respectively; yellow is indicative of mean

expression. Tumor ER status is indicated below by blue bars for ER+ tumors (total of 134) or red bars for ER- tumors (total of 64).

Supplementary Figure 10. ANCCA controls the expression of the top 20 genes with aberrant expression associated with elevated levels of ANCCA in the tumors. A, MDA-MB436 cells were transfected with siRNA targeting ANCCA (black bars) or control siRNA (yellow bars) and harvested 48 hrs later for real-time RT-PCR analysis. Relative transcript levels were obtained by normalization of expression units for each gene with that of GAPDH. B, MDA-MB468 cells were transfected as above and harvested 72 hrs later for Western analysis for indicated protein expression. C. ChIP assay was performed with asynchronously growing MDA-MB 436 cells as described in Supplementary Materials & Methods. qPCR data were normalized and converted to percentage of input for anti-ANCCA enrichment of promoter sequences at the indicated genes, as compared to pre-immune control serum.

Supplementary Figure 11. ANCCA overexpression correlates strongly with EZH2 in breast cancers. Sections from tissue microarrays (BR1004 from U.S. Biomax) were processed for IHC analysis with specific antibodies as indicated. Examples of IHC staining of primary tumors were shown either in low magnification (top panels) or with the areas indicated by dashed frames displayed in high magnification (bottom panels).