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Supplemental Data

Role of Nucleosomal Occupancy

in the Epigenetic Silencing of the *MLH1* CpG Island

Joy C. Lin, Shinwu Jeong, Gangning Liang, Daiya Takai, Merhnaz Fatemi, Yvonne C. Tsai, Gerda Egger, Einav Nili Gal-Yam, and Peter A. Jones

Supplemental Experimental Procedures

Cell culture

Human melanoma cells (A375), a breast cancer cell line (T47D) and an ovarian cancer cell line (Caov3) were cultured as recommended by ATCC.

Plasmid construction

Genomic DNA was used for PCR to prepare various lengths of insert covering the intergenic region. In the *MLH1* direction, the amplified fragments were from nucleotide -324 to -324 to +10, -286 to +10, -261 to +10, -236 to +10 and -211 to +10 (relative to *MLH1* 1a): forward (Fwd) primers containing an XhoI site (underlined): 5'-AACTCGAGCACAAGCCCGGTTCCGGCAT-3' (-324 to +10), 5'-AACTCGAGATATTTCGTATTCCCCGAG-3' (-286 to +10), 5'-AACTCGAGAAAAACGAACCAATAGGAAGAGCG-3' (-261 to +10), 5'-AACTCGAGACAGCGATCTCTAACGC-3' (-236 to +10) and 5'-AACTCGAGATATCCTTCTAGGTAGCGGGCA-3' (-211 to +10); the reverse (Rev) primer containing a HindIII site (underlined), 5'-ATTAAGCTTGAAACGTCTAGATGC TCAACGG-3'. In the *EPM2AIP1* direction, the amplified fragments were from nucleotide -324 to +10, -324 to -183, -324 to -208, -324 to -232 and -324 to -258 (relative to *MLH1* 1a): Fwd primers containing an XhoI site (underlined), 5'-AACTCGAGAAACGTCTAGATGCTCAACGG-3' (-324 to +10), 5'-AACTCGAGTACTGCCCGCTACCTAGAAGGAT-3' (-324 to -183), 5'-AACTCGAGCGCTTGCGCGTTAGAGATCGCT-3' (-324 to -208), 5'-AACTCGAGCCGCTCTTCCTATTGGTTCGTTTTTA-3' (-324 to -232) and 5'-AACTCGAGAGGAGCTCGGGGAATACGAAAT-3' (-324 to -258); the Rev primer containing a HindIII site (underlined), 5'-ATTAAGCTTCACAAGCCCGGTTCCG GCAT-3'. PCR products were double digested with XhoI and HindIII, gel purified and ligated in the XhoI and HindIII sites of pGL3-Basic (Promega) using T4 DNA ligase. Plasmids were purified with a miniprep kit (Promega) and sequenced by an automated DNA sequencer at Laragen (Los Angeles).

Cell transfection and dual luciferase reporter assay

HeLa cells were seeded (1.2 x 10^4 cells per well, 96-well dish) 24 h before transfection. Transient transfections were performed by Lipofectamine Plus (Invitrogen). *MLH1* and *EPM2AIP1* promoter/firefly luciferase fusion vector (1 μg per well) and pRL-SV40 vector (10

ng per well, Promega) were cotransfected into HeLa cells. Cells were incubated until 24 h post-transfection and Luciferase assay was performed (Dual-Luciferase Reporter Assay System, Promega). The firefly luciferase activity of each test plasmid was normalized with the Renilla luciferase activity, which was used as an internal control to correct for the variation of transfection efficiency.

Methylation-sensitive single nucleotide primer extension (Ms-SNuPE)
Ms-SNuPE assays were done as described in the main text and all primer sequences are available upon request.

RT-PCR
Quantitative RT-PCR was performed usingn an Opticon light cycler with SYBR green I (Sigma). All values were normalized to –ACTIN expression ratios and a set of known amounts of standards was used for quantitation. Primer sequences are available upon request.

Mononucleosomal DNA preparation and analysis
Detailed protocols were published previously (Gal-Yam et al., 2006). Quantitative PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) and SYBR green I with DNA Engine Opticon Sysem (MJ Research, Cambridge, MA). The primers sequences and PCR conditions are described previously (Ozsolak et al., 2007).

ChIP assays
ChIP analyses were performed as described previously (Nguyen et al., 2001). Antibodies used were: 10 μl of either anti-Histone H3 (Abcam) or anti-acetylated Histone H3 (Upstate) and 1 μl of rabbit IgG (Upstate) as nonspecific antibody control.

Real-Time PCR amplification of immunoprecipitated DNA
Quantitative PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) and SYBR green I with DNA Engine Opticon Sysem (MJ Research, Cambridge, MA). The primers sequences and PCR conditions are described previously (Ozsolak et al., 2007). For each PCR, a set of known amounts of DNA were included as quantitation standard and immunoprecipitated samples with nonspecific antibody were also included. The fraction of immunoprecipitated DNA was calculated as percentage of input DNA.

Supplemental References

Fatemi, M., Pao, M. M., Jeong, S., Gal-Yam, E. N., Egger, G., Weisenberger, D. J., and Jones, P. A. (2005). Footprinting of mammalian promoters: use of a CpG DNA methyltransferase revealing nucleosome positions at a single molecule level. Nucleic Acids Res *33*, e176.

Gal-Yam, E. N., Jeong, S., Tanay, A., Egger, G., Lee, A. S., and Jones, P. A. (2006). Constitutive nucleosome depletion and ordered factor assembly at the GRP78 promoter revealed by single molecule footprinting. PLoS Genet *2*, e160.

Nguyen, C. T., Gonzales, F. A., and Jones, P. A. (2001). Altered chromatin structure associated with methylation-induced gene silencing in cancer cells: correlation of accessibility, methylation, MeCP2 binding and acetylation. Nucleic Acids Res *29*, 4598-4606.

Ozsolak, F., Song, J. S., Liu, X. S., and Fisher, D. E. (2007). High-throughput mapping of the chromatin structure of human promoters. Nat Biotechnol *25*, 244-248.

Figure S1. Promoter deletion analysis. Top is the map of the region analyzed. Promoter DNA fragments inserted upstream of the firefly luciferase gene in the promoterless pGL3- basic vector (pGL3-B) are shown on the left as gray (*MLH1*) and hatched (*EPM2AIP1*) areas. The orientation of transcription in each construct is depicted as an arrow. On the right are luciferase activities corresponding constructs. The mean activity of construct #1 was set at 100% and the relative activity of each construct was calculated. Data shown on the right are the means +/- SD (n=12).

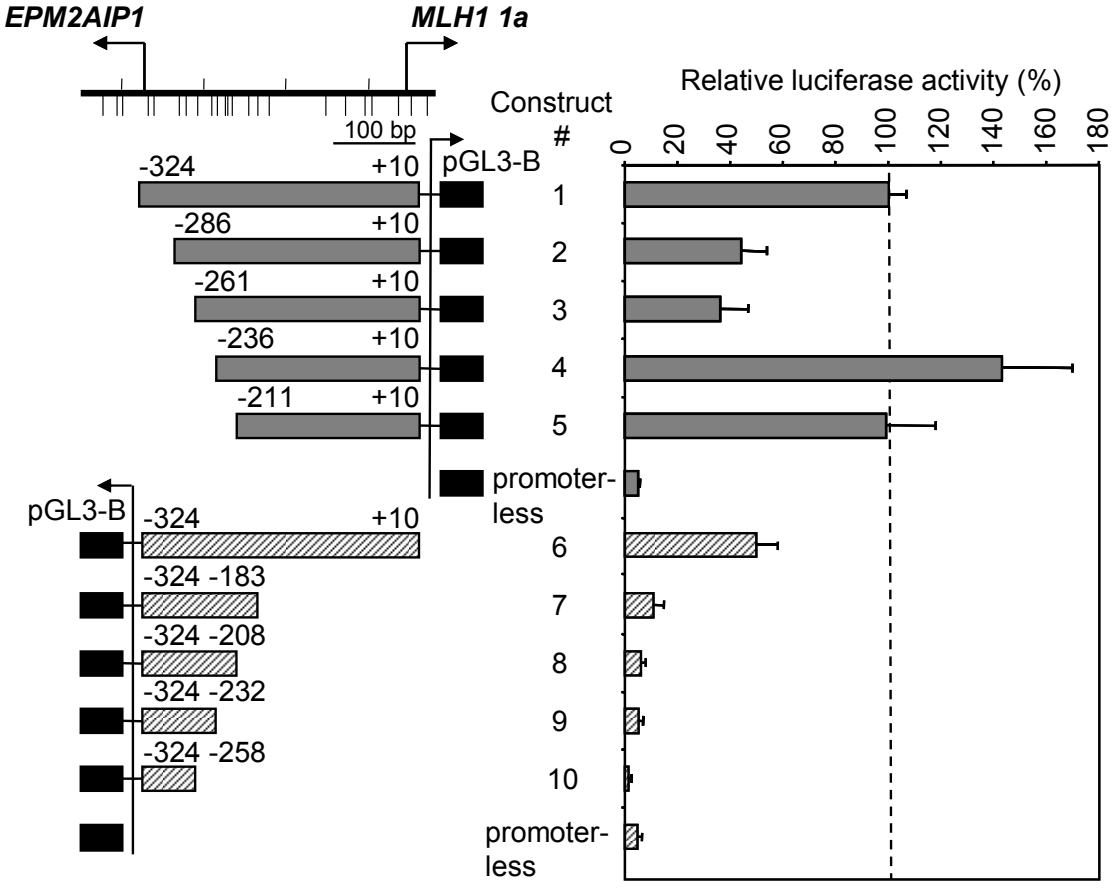

Figure S2. Correlation of methylation status of the bidirectional promoter and the expression patterns of *BRCA1* and *NBR2* in two cell lines. (A) The bidirectional promoter and the CpG island. Horizontal arrows show the transcriptional start sites (UCSC genome browser) and black tick marks indicate CpG dinucleotides. Horizontal bar underneath the tick marks represents the CpG island. The average methylation levels of 3 CpGs in the *BRCA1/NBR2* promoter region were analyzed by Ms-SNuPE (B) and the expression of both genes was determined by RT-PCR (C) in two human cancer cell lines. *β-ACTIN* expression served as a control for the input amount of cDNA. Results are shown as the mean (bar) +/- SD of two PCRs from two independent cDNA preparations.

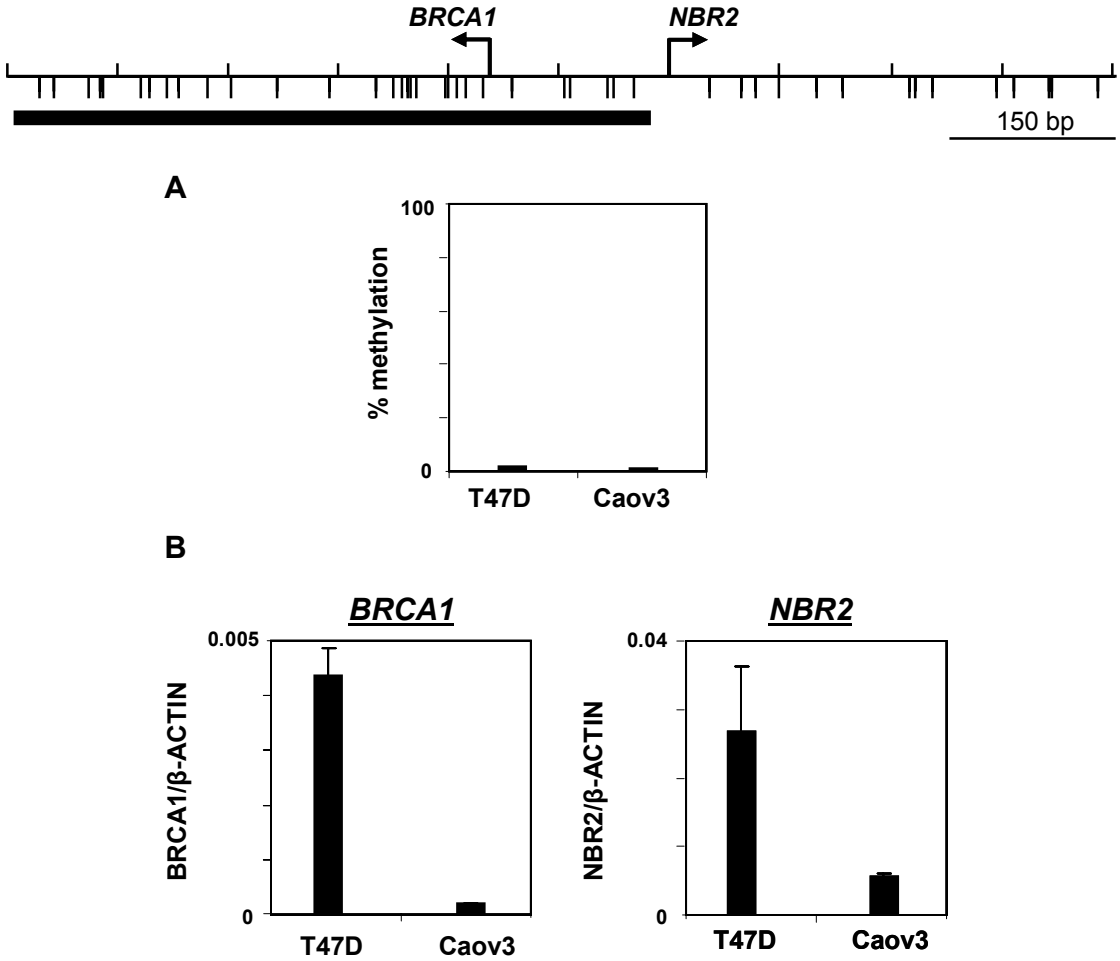

Figure S3. Nucleosomal occupancy at the *BRCA1/NBR2* promoter by mononucleosomal DNA analysis and ChIP. (A) Mononucleosomal DNA analysis. Nuclei from expressing T47D cells were digested partially with MNase and the reaction mixture was run on a sucrose gradient to isolate mononucleosomal DNA. Enrichment of mononucleosomal DNA was analyzed by real-time PCR using primers specific for five regions (R3 to R7) shown as black rectangles as depicted on top as described previously (Ozsolak et al., 2007). (B) Distinct chromatin structures at the *EPM2AIP1/MLH1* promoter in expressing T47D and Caov3 cells. ChIP analysis performed with antibodies against histone H3 and acetylated histone H3. Immunoprecipitated DNA was analyzed by real-time PCR as described in (A). The fraction of immunoprecipitated DNA was calculated as a percentage of input DNA. Results are shown as the mean (bar) +/- SD of two or three experiments from two independent chromatin preparations.

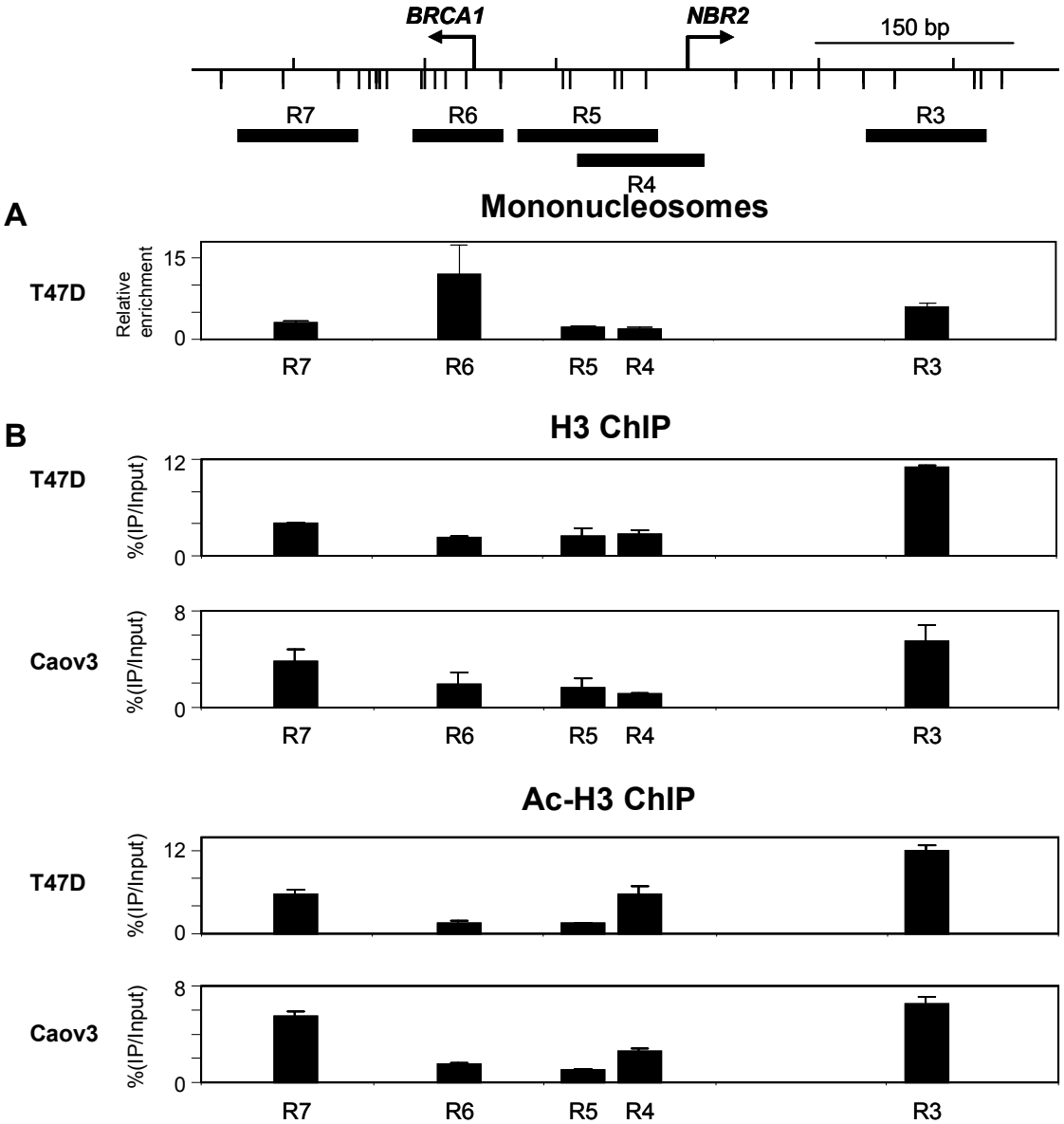

Figure S4. Accessibility of native chromatin to M.SssI at the *EPM2AIP1/MLH1* promoter region in expressing T24 cells. Nuclei were extracted from expressing unmethylated LD419

cells and then were treated with M.SssI for 15 min followed by bisulfite genomic sequencing. Two independent bisulfite-sequencing reactions were done to avoid introducing a bias in the analyses. Four PCR products of different sizes as indicated by the blue dotted lines, were included in the analysis. (A) Untreated nuclei (B) Nuclei treated with M.SssI. Horizontal lines with circles indicate individual molecules that were sequenced after PCR amplification and cloning of bisulfite-treated DNA. Solid circles, methylated CpG dinucleotides; open circles, unmethylated CpG dinucleotides. Pink bars indicate inaccessible areas or patches to M.SssI, suggesting presence of nucleosomes. Patches are defined as at least two consecutively unmethylated sites flanked on each side by least two consecutively methylated CpG sites (Fatemi et al., 2005). Blue rectangles show the putative protein binding regions.

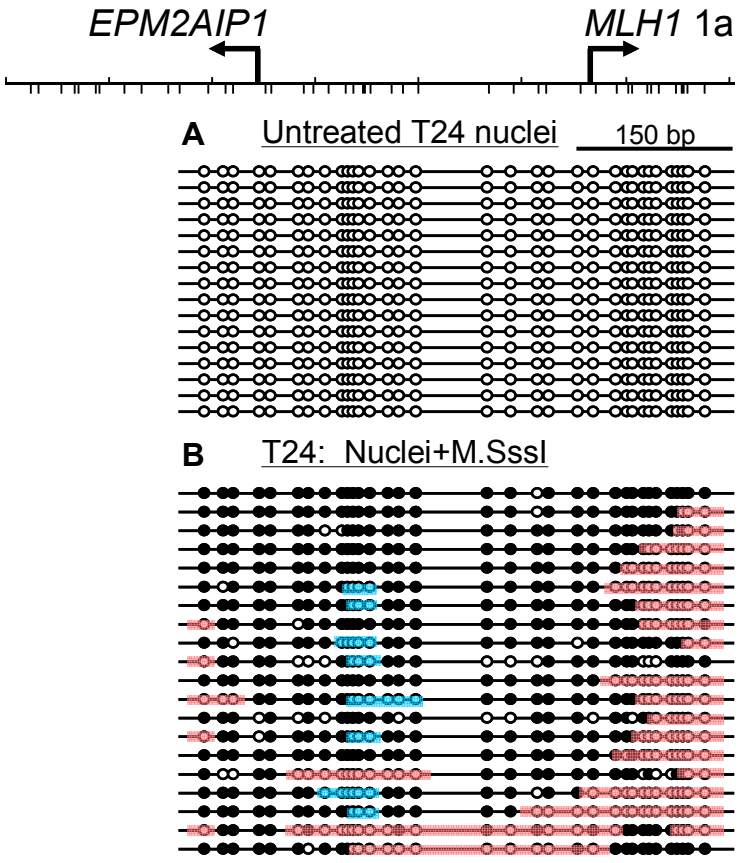

Figure S5. Methylase-based single promoter analysis (MSPA) of RKO cells following 5-aza-CdR treatment. (A) Nuclei from untreated RKO cells were extracted and subjected to bisulfite genomic sequencing. The promoter showed extensive endogenous methylation. (B)

RKO cells were treated with 5-aza-CdR for 24 h and harvested 72 h after drug treatment started. Naked DNA from drug-treated cells was extracted and then subjected to M.SssI treatment, followed by bisulfite genomic sequencing. The promoter was almost fully methylated after M.SssI treatment. Selective amplification with primers that only anneal to unmethylated molecules to filter out extensively methylated molecules which were not suitable for M.SssI treatment. To verify the specificity of the primers, selective amplifications were done on DNA samples from RKO cells 72h (C) and 44 days (D) after drug addition and only extensively unmethylated molecules were amplified. Horizontal lines with circles indicate individual molecules that were sequenced after PCR amplification and cloning of bisulfite-treated DNA. Solid circles, methylated CpG dinucleotides; open circles, unmethylated CpG dinucleotides.