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Metastasis associated protein 1 and its short form variant stimulates *Wnt1* transcription via promoting its derepression from *Six3* corepressor

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Supplementary Figure legends

Supplementary Fig. S1. (**A**) Localization of MTA1s and mutant MTA1s (K428A) in HC11 cells . (**B**)Western blot analysis for the T7-MTA1s in the nuclear and cytoplasmic fractions isolated from MTA1s and MTA1s (K428A) stable clones of HC11 cells. (**C**) Construction of wild type and mutant MTA1s stable clones in HC11 cells. (**D**) Localization of MTA1s and mut MTA1s (K428A) in the ER membrane in HC11 cells. (**E**) ChIP analysis showing the recruitment of T7-MTA1s or Lamin B receptor followed by T7-MTA1s on to the Six3 promoter in MTA1s and MTA1s (K428A) stable clones of HC11 cells. (**F**) Western blot analysis for the β-catenin, P-GSK-3β, GSK-3β, Wnt1 and Six3 in MTA1s and MTA1s (K428A) stable clones of HC11 cells.

Biochemical basis for low levels of MTA1s in the nucleus

A careful analysis of the MTA1s protein sequence using PSORT program revealed a potential endoplasmic reticulum (ER) membrane retention signal motif RTKP (Supplementary Fig. S1A). We hypothesized that a portion of MTA1s sitting on the inner membrane of ER might be translocated to the nucleus. To test this possibility, we mutated the ER membrane retention signal motif RTKP to RTAP in MTA1s and generated stable pooled HC11 clone expressing the mutant MTA1s (Supplementary Fig. S1C). Immunoflorescence studies with these stable clones found a reduced accumulation of the mutant MTA1s (K428A) in the ER membrane when compared to the wild-type MTA1s (Supplementary Fig. S1D). Like-wise, there was a reduced recruitment of the mutant MTA1s (K428A) onto the *Six3* chromatin when compared to the wild-type MTA1s in stable clones (Supplementary Fig. S1E). Since there was no recruitment of

Lamin B receptor, an inner nuclear membrane protein, onto the Six 3 promoter in HC11 stable clones expressing MTA1s or mutant MTA1s (K428A), these results suggest that Lamin-B receptor-independent localization of MTA1s into the nucleus. As expected from these results, mutation in MTA1s (K428A) also affected the Wnt1 signaling molecules (Supplementary Fig. S1F), as well nuclear accumulation of MTA1s in HC11 stable clones (Supplementary Fig. S1B). These results suggest that the noticed MTA1s-ER interaction may play a role in the noticed albeit low levels of MTA1s in the nucleus.

Supplementary Fig. S2. (**A**) Quantitation of the real time qPCR-ChIP analysis showing differential recruitment of T7-MTA1s to regions 2 and 3 of *Six3* chromatin, and T7-MTA1 to region 2 and 9 of *Six3* chromatin in HC11-pcDNA, HC11-MTA1s and HC11-MTA1 cells. (**B**) Quantitation of the real time qPCR-ChIP analysis showing differential recruitment of HDAC2 to regions 2, 3, 6 and 9 of *Six3* chromatin in HC11-pcDNA, HC11-MTA1s and HC11-MTA1 cells. (**C**) Quantitation of the real time qPCR-ChIP analysis showing differential recruitment of Mi-2 to regions 2, 3, 6 and 9 of *Six3* chromatin in HC11-pcDNA, HC11-MTA1s and HC11-MTA1 cells.

Supplementary Fig. S3. (**A**) Quantitation of the real time qPCR-double ChIP assay, first with T7-Ab followed by the HDAC2-Ab for the regions 2, 3, 6 and 9 regions of the *Six3* chromatin in HC11-pcDNA, HC11-MTA1s and HC11-MTA1 cells. (**B**) Quantitation of the real time qPCR-ChIP analysis showing differential recruitment of acetyl-H3 to regions 2, 3, 6 and 9 of *Six3* chromatin in HC11-pcDNA, HC11-MTA1s and HC11-MTA1 cells.

Supplementary Fig. S4. Sequence analysis of the *Six3* promoter regions 1, 2 and 9. ATTA sequences in the promoter are boxed and colored red. Primer sequences used for ChIP analysis to amplify the promoter regions are highlighted purple.

Supplementary Fig. S5. Stimulation of Wnt1 pathway in MTA1s- and MTA1-TG mice (**A**) RT-PCR analysis of the Wnt target gene *WISP-1* in mammary glands from separate 12-week-old WT and virgin MTA1s-TG mice. (**B**) RT-PCR analysis of the Wnt1 target gene *WISP-1* in mammary glands from 12-week-old WT and virgin MTA1-TG mice. (**C**) Immunohistochemical analysis of Wnt1, β-catenin, phospho-GSK-3β, phospho-ERK and Six3 expression in mammary glands of 12-week-old-virgin WT, MTA1s and MTA1-TG mice.

Supplementary Methods

Cell culture

HC11 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 10ng/ml epidermal growth factor, and 5μg/ml insulin. MCF-7, SKBR3 and MDA-MB-435 cells were cultured in DMEM supplemented with 10% fetal bovine serum. MEFs from mice WT, heterozygous, or homozygous for MTA1s were cultured in DMEM supplemented with 10% fetal bovine serum.

Generation of MTA1 null mice

Two mouse genomic Bac clones, 375/K3 and 552/L23, which contain mta1 locus were obtained by screening RPCI-22 129 mouse bac library with a mouse mta1 cDNA probe, and the clone 552/L23 was used in the subsequent work for constructing the targeting vector. Fragments of mta1 were subcloned into pBluescript (Stratagene), with 3 loxP sites

flanking the neo selection marker and exon 2. The targeting vector was transfected to PC3 ES cells by electroporation. Correct targeted clones were identified by double Southern blotting by using the left and right probes. Two clones, 1B7 and 3E6 were used to inject B6 blastocysts. Both injections yielded high rate chimera mice and successfully went germ line transmission. The line established from clone1B7 was designed M1, and the line from 3E6 designed as M2. Both the lines were kept at B6/129Sv mixed background. Genotyping of the mice were initially done by Southern blotting in the F1 progeny, and subsequently all done by using PCR.

Production of MTA1s/MTA1$^{-/-}$ MEFs stably expressing MTA1s

MTA1s/MTA1$^{-/-}$MEFs cells stably expressing V5-tagged MTA1s were generated by transfecting full-length pcDNA6-His-V5-MTA1s with the use of the FuGENE 6 transfection reagent (Roche Applied Science). Stable clones were selected by using blasticidin (10 μg /ml), and expression of MTA1s was verified by immunoblotting with an anti-V5 antibody.

Transfection and luciferase reporter assays

Six3-luc-reporter plasmid was kindly provided by Dr. G. Oliver, St.Jude Children's Research Hospital. The Six3-luc reporter plasmid was constructed by insertion of 1.36 kb mouse *Six3* genomic fragment which includes three clustered Six3 recognition sequences, a TATA box and the transcription start site of the Six3 promoter, and cloned into the NcoI site of pG5 luc vector. The ΔSix3-luc was constructed by deleting the regions containing the three clustered Six3 recognition sequences (5). Cells were plated in six-

well culture plates, and transfection was performed by using the FuGENE-6 transfection reagent.

Immunofluorescence and confocal microscopy studies

Cellular localization of proteins was determined by using indirect immunofluorescence as described previously (11). Confocal scanning analysis was performed with either a Zeiss LSM 510 (Carl Zeiss, Inc.) or Olympus FV300 (Olympus Corporation) confocal laser scanning microscope in accordance with established methods by using sequential laser excitation to minimize the possibility of fluorescent emission bleed-through. Images were exported to the Microsoft Photo Editor software program (Microsoft Corporation) for adjustment of the contrast prior to assembly into Figs with the use of the Microsoft PowerPoint software program.

Immunohistochemistry

For immunostaining, deparaffinized sections were subjected to antigen retrieval. This involved boiling the sections for 10 minutes and gradually cooling them for 30 minutes in 10 mM citric acid buffer (pH 6.0). Sections were then incubated with specific antibodies as indicated, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody.

siRNA transfection

siRNA against MTA1s was purchased from Ambion (catalog #16708A), and MTA1 siRNA, GSK3-β- siRNA, Six3 siRNA from Dharmacon as manufacturer's protocol.

Mammary gland whole mounts and histology

Briefly, the glands were fixed with acetic acid/ethanol (1:3) for 2 h and stained with 0.5% carmine/0.2% aluminum potassium sulfate for 16 h. After being rinsed briefly with distilled water, the glands were dehydrated with the use of graded ethanol, and lipids were removed with two changes of acetone. Finally, the glands were preserved in methyl salicylate. For histological analysis, mammary gland tissue was fixed in 10% neutral buffered formaldehyde and embedded in paraffin according to standard methods. Sections (4 μm each) were stained with hematoxylin and eosin.

RT-PCR analysis- For RT-PCR analysis, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and first-strand cDNA synthesis was carried out with SuperScript II reverse transcriptase (Invitrogen) using 2 μg of total RNA and poly (dT) primer. RT-PCR was performed with the gene-specific primers listed in supplementary table 1. The levels of mRNA of all the genes were normalized to that of β-actin mRNA.

Chromatin immunoprecipitation assay

HC11/pcDNA, HC11/MTA1s and HC11/MTA1 cells were cross-linked with formaldehyde (1% final concentration) and sonicated on ice to fragment the chromatin into an average length of 1-2 kb. Supernatants in the sonicated lysates were diluted 10-fold with chromatin-dilution buffer (0.01% SDS, 1.1% Triton X100, and protease inhibitor cocktail). Monoclonal T7 tag-specific antibody (Novagen), mouse IgG (Sigma), anti-HDAC2 and anti-acetyl Histone 3 (Santa Cruz Biotechnology, Santa Cruz, CA), were the antibodies used for ChIP. Immunoprecipitations were done at 4°C overnight.

Protein A Sepharose beads were added to the lysate to isolate the antibody-bound complexes. The beads were washed to remove nonspecific binding, and the antibody-bound chromatin was eluted. The eluate was "decrosslinked" by heating at 70°C for 6 h. RNase was added during this step to digest the RNA contaminants. Samples were then treated with proteinase K for 1 h at 40°C to digest the proteins pulled down by immunoprecipitation, and finally, the DNA was extracted using the phenol chloroform method. For the double ChIP experiment, an initial ChIP assay was done with HC11/pcDNA, HC11/MTA1s and HC11/MTA1 cell lysates with anti-T7 antibody to immunoprecipitate T7-bound chromatin, which was eluted from the protein A sepharose beads and subjected to a second ChIP assay with anti-HDAC2 antibody. With the DNA isolated at the end of the ChIP analysis, PCR was conducted.

For ChIP analysis in the mammary gland, mammary glands from 12 wk-old virgin WT, MTA1s-TG, MTA1s-TG, MTA1s/MTA1+/+ and MTA1s/MTA1-/- mice were collected. The lymph nodes were excised and snap-frozen in liquid nitrogen and ground into powder. The tissue was fixed in formaldehyde (1%) at room temperature. After several washes in ice-cold PBS, the tissue pellet was resuspended in NEBA buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) supplemented with complete protease inhibitor cocktail and 1 μg ml-1 pepstatin, dounce homogenized and incubated for 15 min on ice. NP40 (0.5%) was added and samples were vortexed for 30s before collecting the nuclei. Subsequently, the nuclear fraction was lysed in nuclear lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS, complete protease inhibitor cocktail, 1 mM vanadate and 1 μg ml-1 pepstatin). Chromatin was sheared by sonication to a size of approximately 1-2 kb, and samples were processed for PCR as described above. PCR

products were resolved on either 2% agarose gel and visualized with ethidium bromide. Images were quantified using Sigma gel-analysis software, version 1. Primers used for ChIP analysis for Six3 promoter walk, and for ChIP analysis on Wnt1 promoter and enhancer regions are presented in Supplementary Table 2. ChIP analysis with T7-MTA1s and Lamin B receptor followed by T7-MTA1s on Six3 promoter in HC11/pcDNA, HC11/MTA1s and HC11/MTA1s K428A was carriedout using the primers designed for the Region 2 of the Six3 promoter listed in supplementary table 2.

Chromatin immunoprecipitation-qPCR assay

For ChIP-qPCR assay, ChIP analysis was carried out as described above and quantitative PCR analysis were performed in real time using the ABI PRISM 7900 Sequence Detection System and Taqman assay with iQ Supermix (Biorad, Hercules, CA). Relative recruitment values were calculated by determining the apparent immunoprecipitation efficiency i.e., the ratios of the amount of immunoprecipitated DNA to that of the input sample, and normalized to the level observed at a control region. Primers and Taqman probes used for ChIP-qPCR assay are presented in Supplementary Table 3.

Site-directed mutagenesis.

Mutations in the MTA1s-pcDNA at K428 was carried out by using the site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) using the primers listed in supplementary table 4.

Statistical analysis and reproducibility

Statistical analysis of the data was performed using Graphpad Prism software (GraphPad Software Inc., San Diego, CA). Statistical analysis of reporter assays and Brdu

incorporation was performed using Student's *t*-test and the results were presented as mean ± standard error.