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Cancer Research

Metastasis-Associated Protein 1 and Its Short Form Variant Stimulates *Wnt1* Transcription through Promoting Its Derepression from *Six3* Corepressor

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Abstract

Although Wnt1 downstream signaling components have been well studied and activated in human cancer, the pathways that regulate *Wnt1* itself have not been explored in depth. Here, we provide gain-of-function, loss-of function, and molecular evidence supporting functional interactions between metastasis-associated protein 1 short-form (MTA1s), metastasis-associated protein 1 (MTA1), and Wnt1 signaling components during mammary gland development and tumorigenesis. Using multiple model systems involving overexpression or knockdown of MTA1s or MTA1, we discovered that MTA1s and MTA1 hyperactivate the Wnt1 pathway due to increased expression of *Wnt1* transcription. MTA1s and MTA1 physically interact with *Six3* chromatin, a protein product of which is a direct histone deacetylase inhibitor–dependent repressor of *Wnt1* transcription. Deletion of the MTA1s and MTA1 allele in murine embryonic fibroblasts resulted in the upregulation of Six3 and downregulation of Wnt signaling. In addition, mammary glands from the MTA1s/MTA1$^{-/-}$ mice exhibited increased recruitment of Six3 corepressor complex to the *Wnt1* promoter and inhibition of Wnt1 pathway in mammary glands. These findings identify MTA1s and MTA1 as important upstream modifiers of the *Wnt1* transcription, and consequently its functions, by directly inhibiting the transcription of *Six3*, allowing derepression of *Wnt1* transcription. *Cancer Res; 70(16); 6649–58. ©2010 AACR.*

Introduction

The Wingless (*Wnt*) genes encode a family of secreted glycoproteins with roles in normal and pathologic processes, including cancer. For example, Wnt1 represents one of the earliest pathways linked with the development of hyperplasia and cancer (1). Once upregulated, secreted Wnt1 acts as an autocrine and/or paracrine factor and initiates a cascade of cytoplasmic signaling events leading to the phosphorylation of glycogen synthase kinase 3β (GSK-3β) and inhibition of its ability to phosphorylate β-catenin. Stabilized β-catenin translocates to the nucleus, leading to the stimulation of Wnt-target genes (2, 3).

Although Wnt1 is widely upregulated in human cancer including breast cancer, much of the work on Wnt signaling research mainly focused on the action of Wnt1 from the plasma membrane to the nucleus. However, the regulation of *Wnt1* transcription continues to be poorly understood, particularly in the context of mammary gland. One of the best-characterized direct coregulators of *Wnt1* transcription is the Six3 homeodomain protein in the retinal or neuronal cells (4). Six3 interacts with the Groucho family of corepressors that associate with histone deacetylase inhibitors (HDAC; ref. 5). Interestingly, *Six3* transcription is tightly regulated by metastatic tumor antigen 1 (MTA1) containing the Mi-2/nucleosome remodeling and deacetylase (NuRD) complex (6) in retinal cells. The NuRD complexes are abundant deacetylase complexes in mammalian cells and have been implicated in chromatin remodeling in normal as well as in cancerous cells (7). The NuRD complex couples histone deacetylation and ATP-dependent chromatin remodeling in the same complex and is involved in chromatin compaction and transcriptional repression. The MTA1 was initially cloned from highly metastatic mammary adenocarcinomas (8), and its expression correlated with the aggressiveness of several human cancers (7). MTA1 acts a potent repressor of estrogen receptor-α (9) and of BRCA1 (10). In contrast of

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MTA1, its naturally occurring variant MTA1s primarily localizes in the cytoplasm (11) and participates in the stimulation of canonical Wnt1 signaling in breast cancer cells (12). MTA3, another member of the MTA family, was reported to physically interact with the *Wnt4* chromatin in a histone deacetylase–dependent manner resulting in the suppression of the Wnt4-dependent morphogenesis (13).

Most of our current understanding of the Wnt1 functions in mammary epithelial cells and in other systems is derived from the membrane-initiated signaling pathways feeding into target gene expression. However, in spite of widely reported increased Wnt1 expression in cancer, the nature of coregulators that regulate the transcription of *Wnt*1 gene itself in mammary epithelial cells remains unknown. Herein, we provide gain-of-function, loss-of function, and molecular evidence supporting the regulatory roles for MTA1 and its variant isoform MTA1s in the transcriptional stimulation of the *Wnt1* gene through the Six3 pathway in mammary epithelial and cancer cells.

Materials and Methods

Cell line authorization statement

All the cell lines used in this study are from Dr. Rakesh Kumar's laboratory and have been tested, authenticated, and previously used in the peer-reviewed articles from the laboratory (6, 9–13).

Cell culture

HC11, MCF-7, SKBR3 and MDA-MB-435 cells, and murine embryonic fibroblasts (MEF) from mice wild-type (WT), heterozygous, or homozygous for MTA1s cells were cultured in as described in the Supplementary Methods section.

Small interfering RNA transfection and luciferase reporter assays

The ΔSix3-luc was constructed by deleting the regions containing the three clustered Six3 recognition sequences (5). Small interfering RNA (siRNA) transfections and luciferase reporter assays were carried out as described elsewhere (11).

Immunofluorescence and confocal microscopy studies

Cellular localization of proteins was determined by using indirect immunofluorescence as previously described (11). Confocal scanning analysis was done as described in the Supplementary Methods section.

Immunohistochemistry and mammary gland whole mounts and histology

Detailed experimental procedures were described in the Supplementary Methods section.

Chromatin immunoprecipitation assay and ChIP-qPCR assay

Chromatin immunoprecipitation assay (ChIP) analysis and ChIP-relative quantitative PCR (qPCR) was carried out as described by Kumar and colleagues (11), and detailed procedure is described in Supplementary Methods. Primers used for ChIP analysis for Six3 promoter walk, and for ChIP analysis on Wnt1 promoter and enhancer regions are presented in Supplementary Table S2. Primers and Taqman probes used for ChIP-qPCR assay are presented in Supplementary Table S3.

Statistical analysis and reproducibility

Statistical analysis of the data were performed using the GraphPad Prism software (GraphPad Software, Inc.). Statistical analysis of reporter assays was performed using Student's *t* test, and the results were presented as mean ± SEM.

Results and Discussion

MTA1 and MTA1s are upstream positive modifier of the Wnt1 transcription

Although MTA1s predominantly localizes in the cytoplasm (11), an albeit but easily detectable amount of T7-MTA1s was also found in the nucleus of HC11 murine mammary epithelial cells expressing MTA1s cells (Fig. 1A). This observation combined with the fact that MTA1s overexpression in HC11 cells was accompanied by increased secretion of Wnt1 (12) suggested the possibility of increased expression of *Wnt1* by MTA1s. Indeed, we found that MTA1s overexpression in the HC11 cells was accompanied by increased expression of *Wnt1* mRNA and protein (Fig. 1B). As MTA1s is a differentially spliced, naturally occurring variant of MTA1 (11), we next wished to evaluate whether Wnt1 pathway and targets are also regulated by MTA1, a predominantly nuclear protein. We found that stable overexpression of T7-MTA1 in the HC11 murine mammary epithelial cells (HC11/MTA1) also resulted in a significant increased level of Wnt1 (Fig. 1C and D) as well as β-catenin–associated increased phosphorylated GSK-3β signaling (Fig. 1D) and Top-TK luc activity as readouts of β-catenin transactivation (Fig. 1E). Conversely, silencing of MTA1 by MTA1-specific siRNA in the MCF-7 cells resulted in downregulation of the Wnt signaling components (Fig. 1F), suggesting that MTA1-mediated increased *Wnt1* expression is also accompanied by increased stimulation of the cytoplasmic Wnt signaling. Together, these findings suggested that both MTA1s and MTA1 may be upstream modifiers of the *Wnt1* transcription in mammary epithelial and breast cancer cells.

MTA1 and MTA1s target Six3 expression in the mammary epithelial and breast cancer cells

Because Wnt1 expression itself is upregulated in breast cancer (3), we next explored the possibility whether MTA1 and MTA1s stimulate the Wnt1 expression by facilitating the stimulation of *Wnt1* transcription through repressing the expression of its negative regulator. We turned our attention to a direct known repressor of *Wnt1* transcription, namely Six3, in the neuronal and retinal systems (4, 6).

We found that upregulation of MTA1 or MTA1s in the HC11 cells led to a substantial reduction in the level of Six3 mRNA and protein (Fig. 2A), suggesting corepressive

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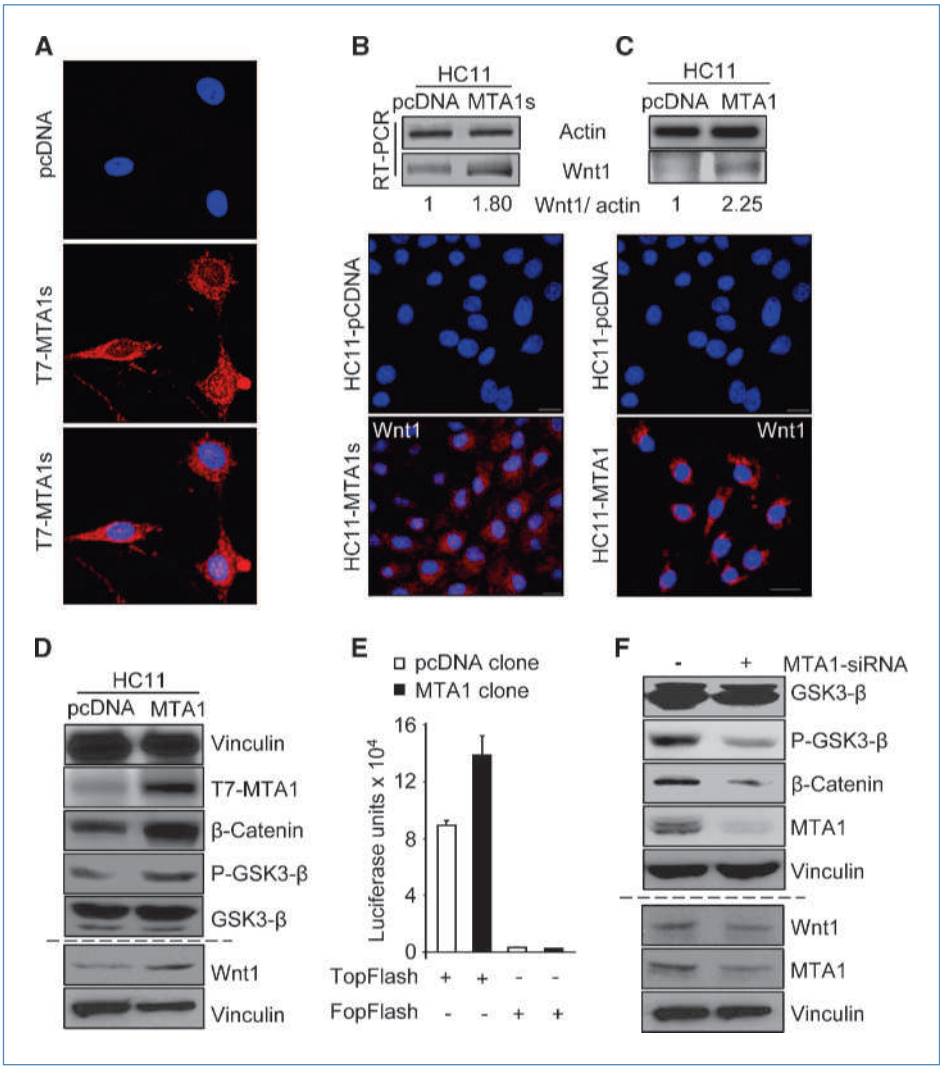

Figure 1. MTA1s overexpression upregulates Wnt1 signaling pathway in mammary epithelial cells. A, expression of T7-MTA1s by confocal microscopy; red, T7-MTA1s. Scale bar, 10 μm. B, RT-PCR and confocal analysis of Wnt1 in HC11/pcDNA and HC11/MTA1s cells. C, RT-PCR and confocal analysis of Wnt1 in HC11/pcDNA and HC11/MTA1 cells. D, Western blot analysis of β-catenin, Phospho-GSK3-β, GSK3-β, Wnt1, and T7 expression in HC11/pcDNA and HC11/MTA1 clones. E, transcription status of Top-flash, Fop-flash in HC11/pcDNA, and HC11/MTA1 clones. F, Western blot analysis of β-catenin, Wnt1, GSK-3β, Phospho-GSK3-β, and MTA1 expression in MCF-7 cell line following transfection with MTA1 or control siRNA for 48 h.

activity of MTA1 and MTA1s for Six3. Indeed, we found that stable upregulation of MTA1 or MTA1s in mammalian cells led to a substantial reduction in the *Six3* promoter activity (Fig. 2B). We next explored the roles of MTA1 or MTA1s complexes in the repression of *Six3*. Treatment of HC11/MTA1 and HC11/MTA1s cells with a general HDAC inhibitor TSA resulted in an increased Six3 promoter activity (Fig. 2C), suggesting that MTA1 and MTA1s repress Six3 expression in an HDAC-dependent manner in mammary epithelial cells. Because previous studies (11) have not delineated the basis of albeit levels of MTA1s in the nucleus (Fig. 1A), we decided to understand the biochemical basis of nuclear localization of MTA1s and found a role of endoplasmic reticulum membrane retention signal motif RTKP (K428) in this process (Supplementary Fig. S1).

Status of Six3 in cells depleted of MTA1 and MTA1s

To establish a causative relationship between the levels of MTA1, MTA1s, and Six3 expression, we studied Six3 expression in MEFs from WT, heterozygous, and homozygous mice for MTA1 and MTA1s (6). Results indicate that loss of MTA1 and MTA1s expression in MEF resulted in a distinct upregulation of Six3 protein levels (Fig. 3A) and decreased TOP-Flash luciferase activity (Fig. 3B). In addition, selective silencing of MTA1s in MDA-MB-435 (Fig. 3C), and of MTA1 silencing in MCF-7 cells (Fig. 3D), also resulted in a distinct upregulation of Six3. To implicate a role for Six3 expression in the observed increased Wnt1 pathway by MTA1 or MTA1s, we selectively silenced endogenous Six3 by using Six3 specific siRNA in HC11 cells, which expresses high levels of Six3, and studied the ability of transfected MTA1s or MTA1 to upregulate β-catenin protein expression as well as TOPFLASH activity. Results showed that selective silencing of endogenous Six3 by Six3-specific siRNA potenitated the ability of both MTA1 and MTA1s to upregulate β-catenin protein expression as well as TOP-FLASH activity (Fig. 3E). Because Wnt1 pathway could be activated at multiple levels, these results suggested that MTA1s and MTA1

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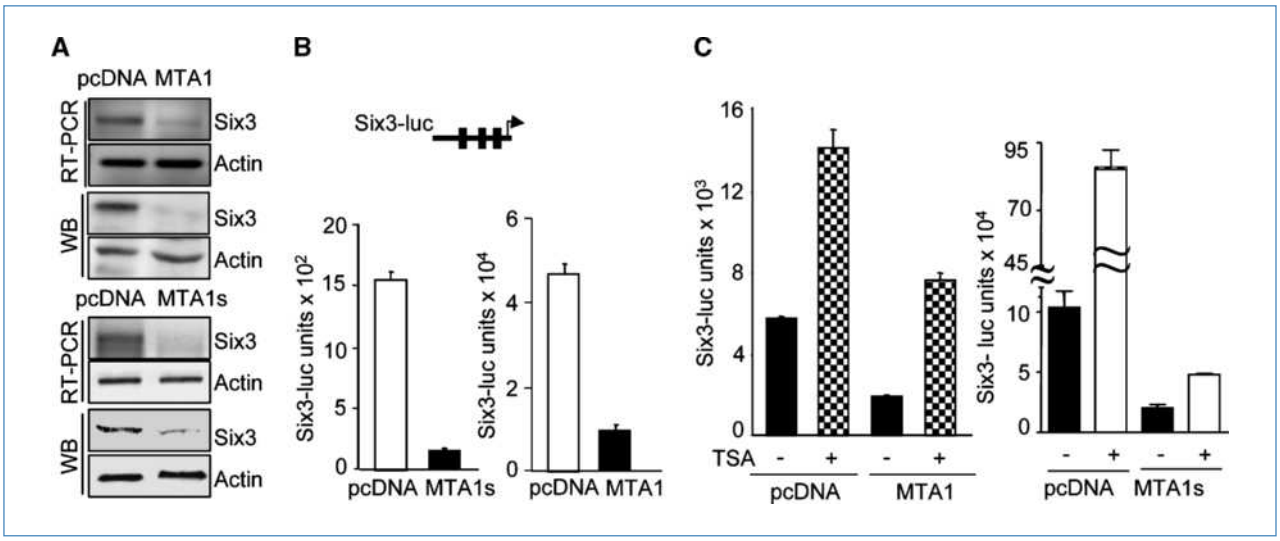

Figure 2. MTA1s and MTA1 targets Six3 expression in the mammary epithelial and breast cancer cells. A, RT-PCR and Western blot analysis for Six3 in HC11/pcDNA, HC11/MTA1, and HC11/MTA1s cells. B, MTA1s and MTA1 downregulates Six3 promoter-luc activity in HC11/MTA1s or HC11/MTA1 cells. C, Six3 promoter luc activity in HC11/pcDNA and HC11/MTA1 or HC11/MTA1s cells following treatment with HDAC inhibitor, TSA.

contribute to observed enhancement of the Wnt1 pathway by repressing the expression of a direct *Wnt1* transcriptional repressor Six3, in addition to the stabilization of β-catenin. Furthermore, these data also showed for the first time a significant biological role for Six3 outside of the retinal and neuronal systems, and identified MTA1 and MTA1s as regulators of Wnt1 expression in mammary epithelial cells.

MTA1s and MTA1 as upstream activators of the Wnt1-β-catenin pathway

To conclusively establish a temporal relationship between MTA1s and Wnt1 signaling components such as phospo-GSK-3β (P-GSK-3β), phospo-ERK (P-ERK), and β-catenin, we found that the loss of MTA1s and MTA1 expression in MEFs from MTA1/MTA1s homozygous mice resulted in a clearly reduced levels of phosphorylated GSK-3β, ERK, as well as β-catenin and *Wnt1*expression (Fig. 4A). Interestingly, reconstitution of Myc-MTA1s or T7-MTA1 but not control pcDNA in MTA1s/MTA1-null MEFs was accompanied by increased expression of phosphorylated GSK-3β, β-catenin, *Wnt1*, and downregulation of Six3 (Fig. 4B), implicating MTA1s and MTA1 as upstream determinants of the β-catenin. Next, we generated MTA1s/MTA1$^{-/-}$ MEFs stably expressing V5-tagged MTA1s and MTA1 protein. Stable expression of V5-tagged MTA1 and MTA1s resulted in the upregulation of β-catenin, phosphorylated GSK-3β, and *Wnt1*, and downregulation of Six3 (Fig. 4C). These findings identified MTA1s and MTA1 as upstream activators of the Wnt1-β-catenin pathway.

Mechanism of Six3 regulation by MTA1 and MTA1s

Next, we wished to gain a deeper insight about the mechanism by which MTA1 and MTA1s downregulates Six3 expression. The negative regulatory region of the mouse *Six3* has been mapped up to 5 kb upstream of the start site (4). To examine the physical interaction of MTA1 and MTA1s with the *Six3* chromatin, we carried out a promoter walk for the 5.5-kb region upstream of the *Six3* gene using ChIP analysis, with primers specifically designed against every 500-bp region of the *Six3* promoter. Initially, ChIP lysates from the HC11/T7-MTA1 and HC11/T7-MTA1s cells were immunoprecipitated with monoclonal anti-T7 antibody to pull down T7-MTA1s/MTA1 or with IgG as a control, and eluted DNA was tested by PCR analysis. This analysis identified one *Six3* promoter MTA1s-interacting region, encompassing roughly a 1.5-kb region (+1 to −1,434 bp, i.e., region 1, 2, and 3) and two *Six3* promoter MTA1-interacting regions, a 1.0-kb region (+1 to −954 bp, i.e., regions 1 and 2) and a 0.5-kb region (−3,834 to −4,314 bp, i.e., region 9; Fig. 5A and B). There was no other specific binding to other regions. Region 6 of the *Six3* chromatin, in which no association of was seen in ChIP assays, were used as negative controls for MTA1s and MTA1, respectively. In addition to this, ChIP-qPCR analysis also showed similar results when carried out under identical conditions (Supplementary Fig. S2A).

Because HDACs could interact with MTA1s or MTA1 and because MTA1s or MTA1 represses Six3 promoter activity in an HDAC-dependent manner (Fig. 2C), we next tested whether HDAC2 is also recruited to the MTA1s/MTA1-interacting *Six3* chromatin. ChIP analysis with an HDAC2-specific antibody showed that HDAC2 was present on the same region 1 to 3 (+1 to −1,434 bp, i.e., region 1, 2, and 3) of the *Six3* chromatin as MTA1s and also present on the same region 1 and 2 (+1 to −954 bp, i.e., regions 1 and 2) and region 9 (−3,834 to −4,314 bp) of the *Six3* chromatin as MTA1 (Fig. 5C), raising the possibility of

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the recruitment of the MTA1s/HDAC or MTA1/HDAC complex to the above-mentioned regions of the *Six3* chromatin. Similar results were obtained when we carried out ChIP analysis followed by a qPCR analysis under identical conditions (Supplementary Fig. S2B). Further ChIP analysis for Mi-2, the largest subunit of the NuRD complex, showed the recruitment of Mi-2 to region 1 to 3 (+1 to −1,434 bp, i.e., region 1, 2, and 3) of the *Six3* chromatin as MTA1s, and to region 1 and 2 (+1 to −954 bp, i.e., regions 1 and 2) and region 9 (−3,834 to −4,314 bp) of the *Six3* chromatin as MTA1 (Fig. 5D). ChIP analysis followed by a qPCR analysis carried out under the same conditions also yielded similar results (Supplementary Fig. S2C). These results suggest the possibility that MTA1s/MTA1 in association with Mi-2 and HDAC as a complex can be recruited to the *Six3* chromatin.

MTA1 or MTA1s corepressor complexes inhibit Six3-transcription

To understand the basis of MTA1 and MTA1s regulation of *Six3* transcription, we next performed a double ChIP assay in the HC11/MTA1s and HC11/MTA1 clones; the initial ChIP analysis was done with anti-T7 antibody to immunoprecipitate the T7-MTA1s– or T7-MTA1–bound DNA sequences, and the subsequent ChIP analysis was done using the anti-HDAC2 antibody. Results showed the simultaneous coassociation of MTA1s and HDAC2 with the region 1 to 3 (+1 to −1,434 bp, i.e., region 1, 2, and 3), but not to the regions 6 and 9, and coassociation of MTA1 with region 1 and 2 (+1 to −954 bp, i.e., regions 1 and 2) and region 9 (−3,834 to −4,314 bp) but not to the regions 3 and 6 (Fig. 6A). Double ChIP assay followed by a qPCR analysis under the same conditions also yielded similar results (Supplementary Fig. S3A).

The above-mentioned set of ChIP analyses suggested that the MTA1s/NuRD or MTA1/NuRD complexes associate with two distinct regions of *Six3* regulatory chromatin, bringing about histone deacetylation by the virtue of associated HDAC activity leading to chromatin compaction, and this would be reflected in the decreased acetylation status of the histones present in these two regions of the *Six3* chromatin. To explore the potential decreased acetylation status of the histones associated with the MTA1s- and MTA1-interacting *Six3* regions, we performed a ChIP assay with acetyl-Histone 3 (H3)–specific antibodies in HC11/pcDNA, HC11/MTA1s, and HC11/MTA1 clones. Results indicated a relative decrease in the association of the acetylation H3 with the 1.5-kb region (+1 to −1,434 bp,

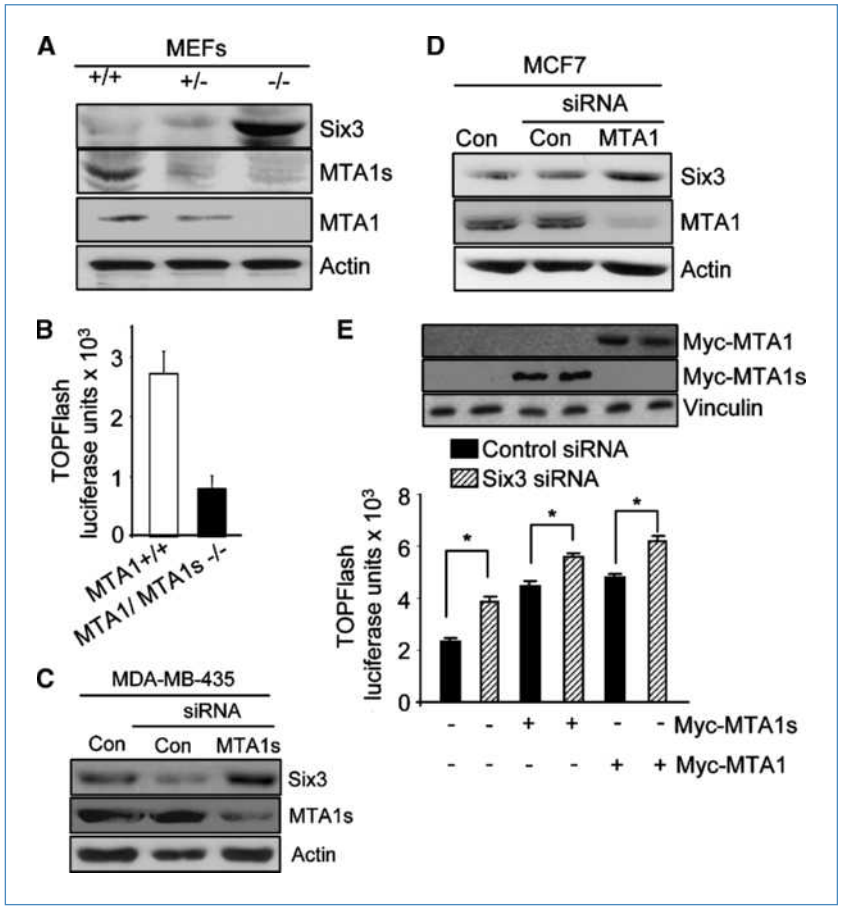

Figure 3. A, Western blot analysis of Six3, MTA1s, and MTA1 and in MEFs from WT (+/+), heterozygous (+/–), and homozygous (–/–) mice for MTA1s/MTA1. B, top-Flash luciferase activity in MEFs from WT (+/+) and homozygous (–/–) mice for MTA1s/MTA1. C, Western blot analysis of Six3 expression in MDA-MB-435 cell line following transfection with MTA1s or control siRNA for 48 h. D, Western blot analysis of Six3 in MCF-7 cell line following transfection with MTA1 or control siRNA for 48 h. E, Western blot analysis and Top-Flash luciferase activity in HC11 cells following transfection with myc-tagged MTA1s or MTA1 and Six3-siRNA.

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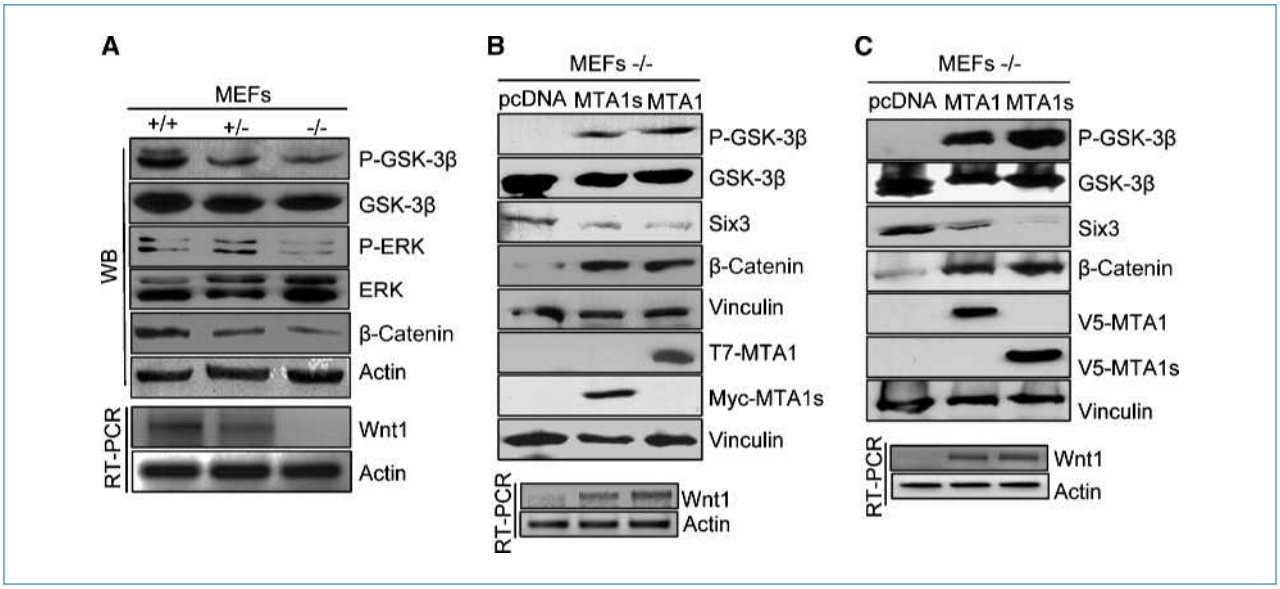

Figure 4. Status of Wnt signaling components. A, Western blot analysis of phospho-GSK-3β, GSK-3β, phospho-ERK, ERK, and β-catenin, and RT-PCR analysis of *Wnt1* in MEFs from WT (+/+), heterozygous (+/–), and homozygous (–/–) mice for MTA1s/MTA1. B, Western blot analysis of phospho-GSK-3β, GSK-3β, Six3, and β-catenin, and RT-PCR analysis of *Wnt1* in MTA1s/MTA1$^{-/-}$ MEFs transiently transfected with pcDNA, myc-MTA1s, and T7-MTA1. C, Western blot analysis of phospho-GSK-3β, GSK-3β, Six3, and β-catenin, and RT-PCR analysis of *Wnt1* in MTA1s/MTA1$^{-/-}$ MEFs stably expressing pcDNA, V5-MTA1, and V5-MTA1s.

i.e., region 1, 2, and 3) of the *Six3* chromatin in HC11/MTA1s cells (Fig. 6B). Similarly, the levels of acetylated H3 associated with the 1.0-kb (+1 to –954 bp, i.e., regions 1 and 2) and 0.5-kb (–3,834 to –4,314 bp) regions of the *Six3* chromatin in HC11/MTA1 cells were considerably decreased compared with the same regions in the HC11/pcDNA cells. ChIP followed by qPCR analysis carried out under identical conditions also yielded similar results (Supplementary Fig. S3B).

Overall, the above ChIP analysis provided clear evidence of distinct physical association of the MTA1s/HDAC or MTA1/HDAC complexes with two distinct regions of the *Six3* regulatory chromatin, resulting in greater levels of histone deacetylation and chromatin compaction, ultimately leading to transcriptional repression. Further analysis of the *Six3* promoter regions bound by MTA1s and MTA1 (promoter regions 1–3 and 9 of *Six3* chromatin) revealed the presence of core ATTA (DNA sequence bound by Six3) motifs in the regions 1, 2, and 9 but not in regions 3 and 6 of the *Six3* chromatin. Previous studies have reported the presence of three clustered ATTA core motifs in the Six3 promoter (5). In addition, Six3 has been shown to bind to its own promoter and regulate its own transcription (5). The presence of core ATTA motifs in regions 1, 2, and 9 of the Six3 promoter bound by MTA1s and MTA1 (Supplementary Fig. S4) suggested the possibility that Six3 binds to its own promoter and Six3 interaction with MTA1s/MTA1 family of corepressors along with the components of NuRD complex such as Mi-2 and HDAC2 and, thus, could facilitate autorepression of Six3 transcription. Furthermore, the presence of core ATTA motifs in the *Six3* promoter opens up the possibility that MTA1s or MTA1 may mediate *Six3* repression through the ATTA motif. It is interesting to note that the Six3 promoter luciferase construct used in the present study (Fig. 2B) were constructed by inserting the 1.36-kb mouse *Six3* genomic fragment including the three clustered Six3 recognition sequences (5).

To validate that the repression of *Six3* transcription by MTA1s or MTA1 is indeed mediated through the Six3 recognition sequences and/or other elements in the *Six3* promoter region, we made use of the Six3 reporter gene construct (Six3 pro-luc), which were generated by deleting the region containing the three Six3 recognition sequences. MTA1s or MTA1 showed increased Six3 promoter luciferase reporter activity compared with the WT Six3 promoter luciferase, but still reduced activity compared with the pcDNA controls, suggesting that MTA1s or MTA1 may not only use ATTA motifs to regulate Six3 (Fig. 6C).

Next, we determined whether MTA1s or MTA1, and Six3 proteins physically interact in mammary cell lines. Because the MTA1s antibody used in our present study is not suitable for immunopreciptiation assays, we used Six3 antibody in the immunoprecipitation assay and found that the endogenous Six3 can physically interact with transiently transfected T7-tagged MTA1s and T7-MTA1 in the HC11 cells (Fig. 6D and E). In addition, endogenous Six3 was also found to physically interact with endogenous MTA1 and MTA1s in MD-MBA-435 cells (Fig. 6F). These findings provided a mechanistic basis of *Six3* gene repression upon deregulation of MTA1s or MTA1, suggesting that MTA1s or MTA1 complexes inhibit the

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expression of Six3, which in turn allows the upregulation of the Wnt1 transcription.

Activation of the Wnt pathway in MTA1s-TG mice

We recently generated transgenic mice with expression of MTA1s under the control of the mouse mammary tumor virus promoter long terminal repeat (MTA1s-TG mice; ref. 12). MTA1s-TG mammary glands were accompanied by precocious alveolar development (12) and stimulation of Wnt1-target gene WISP1 in the mammary glands of 12-week-old virgin mice (Supplementary Fig. S5A). In previous studies from this laboratory, we also observed increased expression of β-casein and β-catenin-Wnt1 signaling in virgin MTA1-TG mice (14). Similar to MTA1s-TG mice, mammary glands obtained from virgin MTA1-TG also exhibited upregulation of WISP1 (Supplementary Fig. S5B). Furthermore, mammary glands obtained from virgin MTA1s-TG and MTA1-TG mice exhibited significantly reduced expression of Six3, whereas the levels of Wnt1, β-catenin, P-GSK-3β, and P-ERK were upregulated (Supplementary Fig. S5C). These findings identified MTA1s and MTA1 as upstream repressor of Six3, leading to increased Wnt1 expression and resulting signaling.

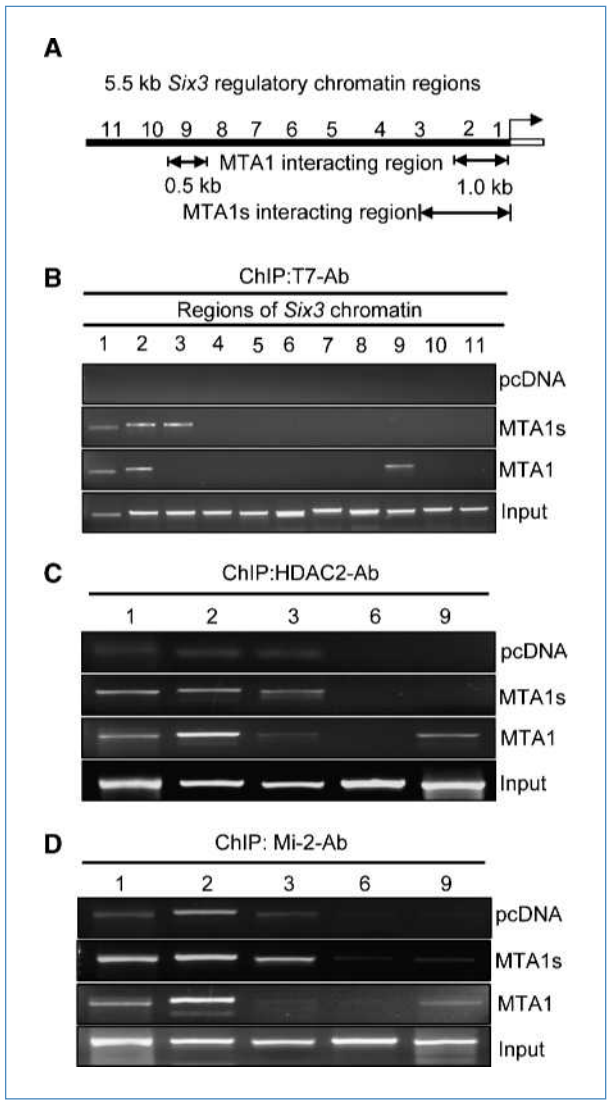

Figure 5. MTA1s/MTA1/NuRD complex targets *Six3* regulatory chromatin. A, schematic diagram of 5.5 kb regulatory chromatin of mouse *Six3* gene. B, ChIP showing differential recruitment of T7-MTA1s to regions 1 to 3 of *Six3* chromatin and T7-MTA1 to regions 1, 2, and 9 of *Six3* chromatin in HC11-pcDNA, HC11-MTA1s, and HC11-MTA1 cells. C and D, ChIP showing recruitment of HDAC2 and Mi-2 to regions *Six3* chromatin in HC11/pcDNA, HC11/MTA1s, and HC11/MTA1 cells.

Downregulation of the Wnt1 pathway in MTA1s/MTA1$^{-/-}$ mice

Because both MTA1s and MTA1-TG mice exhibited excessive hyperbranching, we examined the effect of MTA1s/MTA1 knockdown on mammary gland development using recently generated mice homozygous (−/−) for *MTA1/MTA1s* (6). We examined whole- mount preparations from littermates with matching estrous cycles at different developmental stages. We found that mammary glands from MTA1s/MTA1$^{-/-}$ mice exhibited hypobranching compared with control MTA1s/MTA1$^{+/+}$ mice (Fig. 7A). These findings implicated an important role for MTA1s/MTA1 in mammary gland development. Because both MTA1s-TG and MTA1-TG mice exhibited upregulation of the Wnt1 pathway, we examined the status of Wnt1 pathway components in MTA1s/MTA1$^{-/-}$ mice. Immunohistochemical analysis in virgin mammary glands from 12-week-old MTA1s/MTA1$^{-/-}$ mice showed upregulation of Six3 and downregulation of β-catenin (Fig. 7B), further validating a regulatory role for MTA1s and MTA1 in the Wnt1 pathway.

Differential recruitment of Six3 to Wnt1 chromatin in WT and MTA1s/MTA1$^{-/-}$ mice

Results from MTA1s- and MTA1-overexpressing cell lines, and MTA1s/MTA1$^{-/-}$ mice implicated an important role for MTA1s and MTA1 in the Wnt1 pathway. To confirm whether *Wnt1* gene chromatin is a direct target of Six3 in the mammary epithelium and to determine whether MTA1s/MTA1 repression of Six3 expression compromises the recruitment of Six3 to the *Wnt1* promoter, we performed ChIP analysis in mammary glands from WT and MTA1s/MTA1$^{-/-}$ mice. In these assays, we used primers encompassing 720 bp of the *Wnt1* 5′-promoter region and also 280 bp of the *Wnt1* 3′-enhancer element (4; Fig. 7C). In MTA1s/MTA1$^{-/-}$ mammary glands, we observed an increased recruitment of Six3 to the promoter regions of Wnt1 compared with the corresponding control MTA1s/MTA1$^{+/+}$ mice (Fig. 7D). ChIP followed by qPCR analysis carried out under identical conditions also yielded similar results (Fig. 7D, bottom). In the *Wnt1* enhancer region, as in the promoter region, strong recruitment of Six3 in MTA1s/MTA1$^{-/-}$ mammary glands compared with its corresponding control MTA1s/MTA1$^{+/+}$ mice (Fig. 7E) was observed. ChIP assay followed by real-time quantitative PCR analysis carried out under identical conditions also yielded similar results (Fig. 7E, bottom). Western

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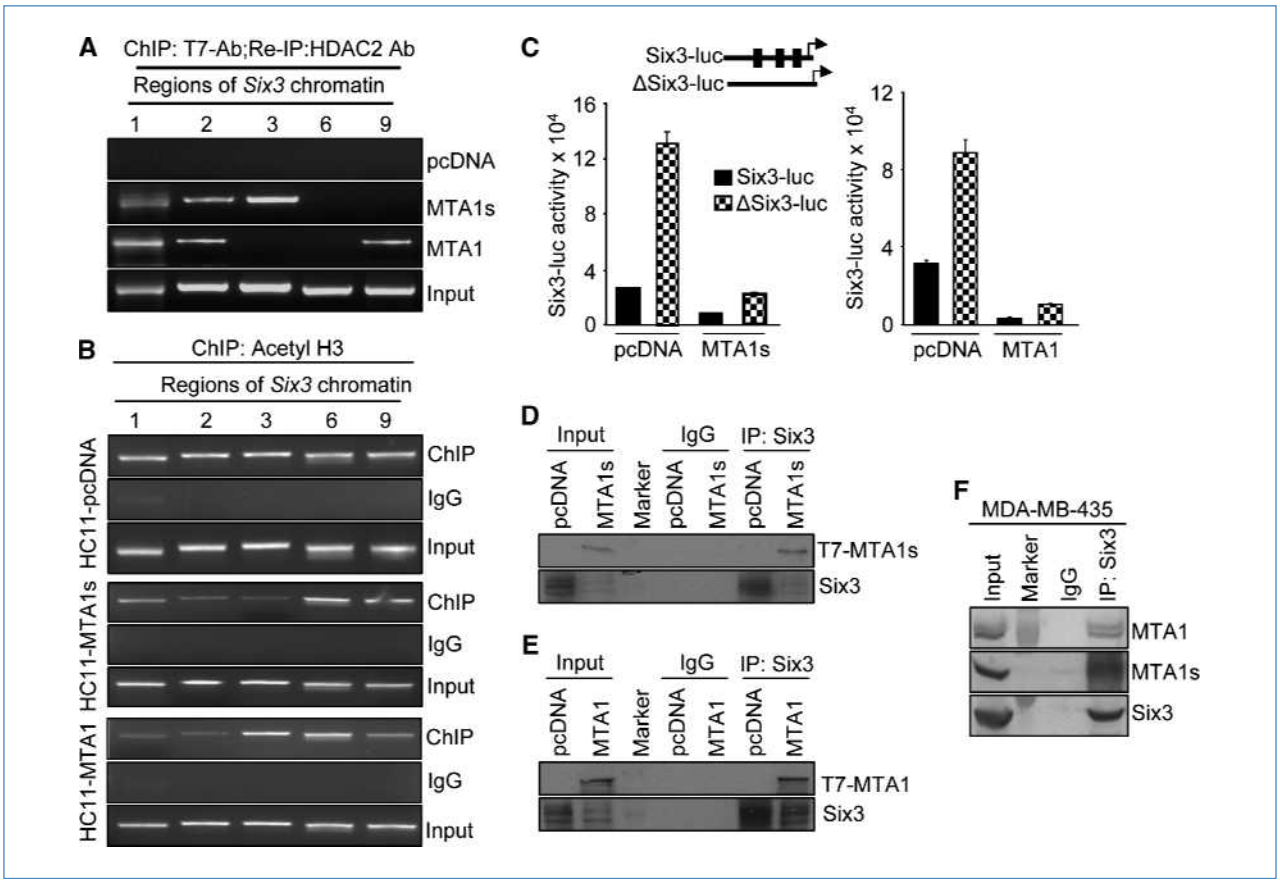

Figure 6. Transcriptional regulation of Six3 by MTA1 and MTA1s. A, double ChIP assay, first with T7-Ab followed by the HDAC2 Ab for the regions 1 to 3, 6, and 9 of the *Six3* chromatin in HC11/pcDNA, HC11/MTA1s, and HC11/MTA1 cells. B, status of the acetylated histone H3 in the MTA1s/MTA1-NuRD complex–bound *Six3* chromatin in the HC11/pcDNA, HC11/MTA1s, and HC11/MTA1 by ChIP analysis. C, Six3 promoter-luc and ΔSix3-promoter-luc activity in HC11/pcDNA and HC11/MTA1s cells. Boxes, schematic representation of the 1.36-kb *Six3* promoter-luciferase construct and the three Six3 recognition (ATTA) sequences. Schematic representation of the 1.36-kb Δ*Six3* promoter-luciferase construct without the three Six3 recognition (ATTA) sequences. D, endogenous Six3 interacts with transiently transfected T7-MTA1s in the HC11 cells. E, transiently transfected T7-MTA1 interacts with endogenous Six3 in the HC11 cells. F, endogenous interaction of MTA1 and MTA1s with Six3 in MDA-MB-435 cells.

analysis of Six3 expression and reverse transcription-PCR (RT-PCR) analysis of *Wnt1* in the same samples showed increased the expression of Six3 in MTA1s/MTA1$^{-/-}$ mice compared with its corresponding control mice (Fig. 7F). These findings suggest that Six3 may restrain the activation of *Wnt1* transcription in mammary gland and that MTA1s and MTA1 upregulate Wnt1 expression through downregulating the expression of Six3, a direct repressor of *Wnt1*.

Next, we attempted to investigate whether Six3 could be recruited to the *Wnt1* promoter in human breast cancer cell lines in an MTA1s- or MTA1-dependent manner. To examine this possibility, we carried out ChIP analysis of Six3 recruitment to the *Wnt1* promoter and enhancer regions in the MDA-MB-435 cell line following treatment with MTA1s-specific siRNA or control siRNA. Interestingly, our results showed an enhanced recruitment of Six3 to the *Wnt1* promoter and enhancer regions in MTA1s-siRNA–silenced MDA-MB-435 cells compared with control siRNA–treated cells (Fig. 7G), suggesting that Six3 may be recruited to *Wnt1* promoter in a MTA1s-dependent manner in breast cancer cell lines.

In brief, results presented here provide new insight into the mechanism by which MTA1s and MTA1 regulate WNT signaling and for the first time identify MTA1s and MTA1 as novel upstream regulators of the Wnt1 transcription. These results suggest that MTA1s and MTA1 promote WNT signaling at multiple levels by inhibiting two known regulators of Wnt pathway namely Six3, as well as GSK3-β (Fig. 7H). As components of the Wnt1 pathway are established contributors to mammary gland tumorigenesis, and considering the fact that MTA1s and MTA1 are upregulated in breast tumors, the newly established dysfunction of the MTA1s- and MTA1-Wnt1 pathways in mammary epithelium may contribute to the pathogenesis of breast tumors. Aberrant Wnt1 pathway is implicated in a various diseases in addition to the breast cancer, and regulation of the Wnt

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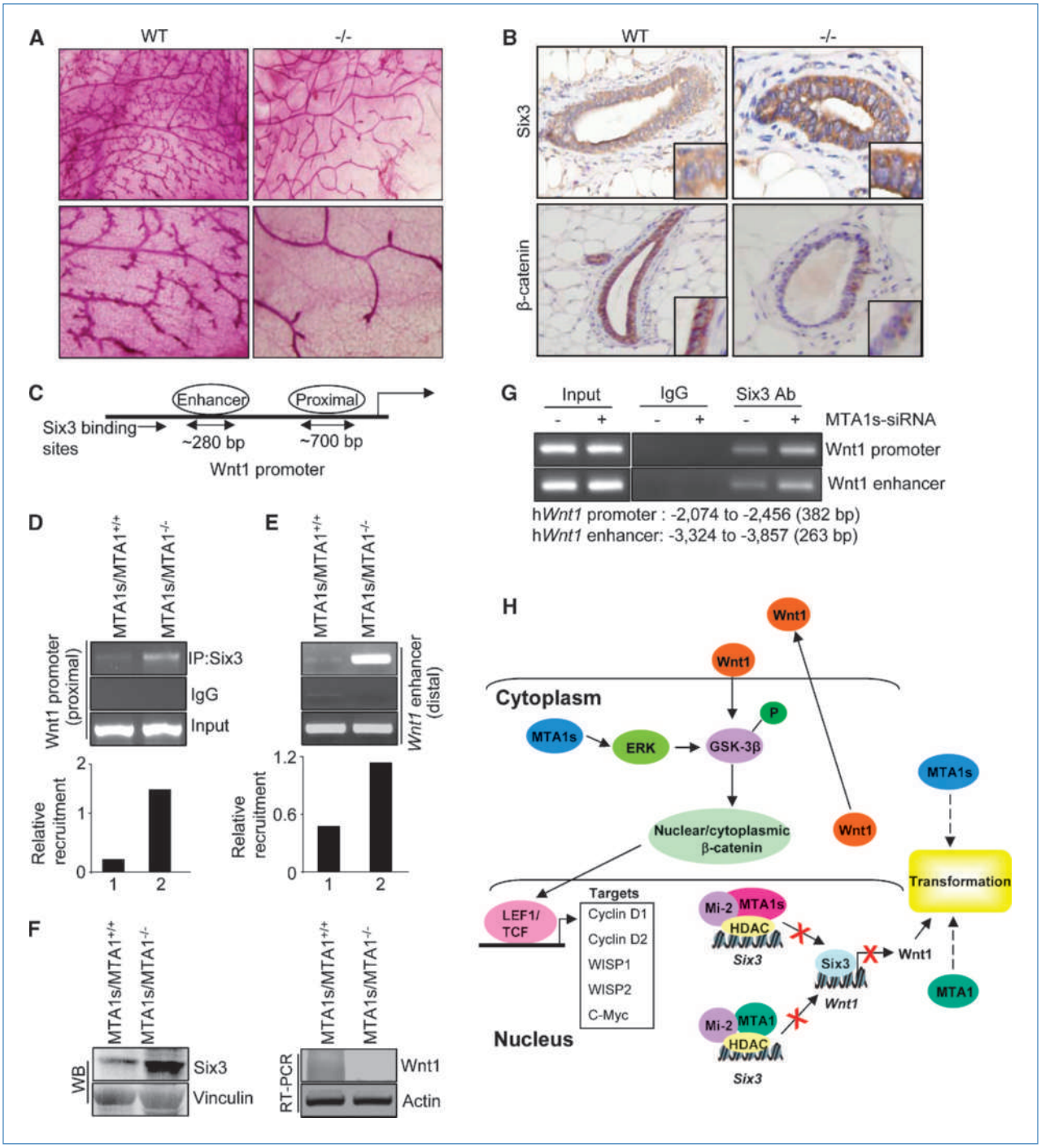

Figure 7. Mammary gland development and status of Wnt pathway in MTA1s/MTA1$^{-/-}$ mice. A, Carmine Red–stained whole mounts of inguinal mammary glands obtained from MTA1s/MTA1$^{+/+}$ mice and MTA1s/MTA1$^{-/-}$ mice at 12 wk of age. The images in the bottom panel are the images of the top panel at increased magnification. B, immunohistochemical analysis of Six3 and β-catenin expression in mammary glands of 12-wk-old virgin MTA1s/MTA1$^{+/+}$ and MTA1s/MTA1$^{-/-}$ mice. C, schematic representation of the Six3 binding sites in *Wnt1* promoter. D, ChIP analysis showing differential recruitment of Six3 to the *Wnt1* proximal promoter region in MTA1s/MTA1$^{-/-}$ mice and its corresponding MTA1s/MTA1$^{+/+}$mice. Bars below, the relative quantitation of Six3 recruitment to the *Wnt1* promoter using ChIP- q PCR assay. E, ChIP analysis showing differential recruitment of Six3 to the *Wnt1* enhancer region in MTA1s/MTA1$^{-/-}$ mice and its corresponding MTA1s/MTA1$^{+/+}$ mice. Bars below, the quantitation of Six3 recruitment to the *Wnt1* enhancer using ChIP-qPCR assay. F, Western blot analysis of Six3 expression and RT-PCR analysis of *Wnt1* expression in MTA1s/MTA1$^{-/-}$ mice and its corresponding MTA1s/MTA1$^{+/+}$ mice. G, ChIP analysis showing enhanced recruitment of Six3 to both *Wnt1* promoter (top) and *Wnt1* enhancer region in MTA1s-specific siRNA-transfected MDA-MB-435 cells compared with control siRNA–transfected cells for 48 h. H, schematic representation of Wnt1 expression and signaling by MTA1s or MTA1. Dotted lines going to transformation, other possible mechanisms not investigated here.

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pathway is emerging as an attractive target for molecular intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Metastasis-Associated Protein 1 and Its Short Form Variant Stimulates *Wnt1* Transcription through Promoting Its Derepression from *Six3* Corepressor

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