Epigenetic Activation of TWIST1 by MTDH Promotes Cancer Stem-like Cell Traits in Breast Cancer

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Abstract

Cancer stem–like cells (CSC) are a cell subpopulation that can reinitiate tumors, resist chemotherapy, and give rise to metastases. Metadherin (MTDH) contributes widely to tumor growth, drug resistance, relapse, and metastasis, but its molecular mechanisms of action are not well understood. Here, we report that MTDH drives CSC expansion by promoting the expression of TWIST1, a transcription factor critical for cancer cell stemness and metastasis. MTDH activates TWIST1 expression indirectly by facilitating histone H3 acetylation on the TWIST1 promoter, a process mediated by the histone acetyltransferase CBP. Mechanistic investigations showed that MTDH interacts with CBP and prevents its ubiquitin-mediated degradation, licensing its transcriptional activation of TWIST1. In clinical specimens of breast cancer, MTDH expression correlates positively with TWIST1 expression and CSC abundance. Overall, our work revealed that MTDH promotes CSC accumulation and breast tumorigenicity by regulating TWIST1, deepening the understanding of MTDH function in cancer. Cancer Res; 75(17): 3672–80. ©2015 AACR.

Introduction

Tumor cells are highly heterogeneous and cancer stem–like cells (CSC) arise as the subpopulation that can perpetuate the growth of malignant cells indefinitely (1). Previous studies demonstrated that breast cancer contains CSCs that are responsible for tumor initiation, chemoresistance, and metastasis (2, 3). It is of biologic and clinical importance to elucidate the mechanisms of CSC maintenance and expansion in breast cancer to identify effective strategies for cancer treatment.

MTDH (also called AEG1 or LYRIC/3D3) is a single-pass transmembrane protein with the encoding gene located at chromosome 8q22, a genomic region that is frequently amplified in cancers (4, 5). The link of MTDH with cancer was first uncovered by a phage cDNA screening for cancer cell proteins that bound to epigenetic factors such as HMGA2, KLF17, and STAT3 (24, 25). Epigenetic modifications, including DNA and histone methylation, also play roles in the activation of TWIST1 expression (28, 29). However, the regulation of the histone acetylation status of TWIST1 has not been reported. In addition, although recently MTDH has been predominantly localized in the cytoplasm and nucleus rather than cell surface, thus not likely to directly mediate the interaction of cancer cell and lung endothelium (7), its function in cancer metastasis, as well as chemoresistance, was revealed again by an independent approach combining bioinformatic screening and experimental validation (5). Since then, rapidly accumulated data have documented its multifaceted roles to help tumors grow, evade apoptosis, resist chemo- or radiotherapy, and metastasize to distant organs (5, 6, 8–11). Gene amplification of MTDH is often observed in breast cancer, prostate cancer, colorectal carcinoma, and hepatocellular carcinoma, especially in metastatic tumors (5, 12–14). The expression of MTDH is also aberrantly elevated in a broad range of cancers (5, 15, 16), and correlates with clinical stage, tumor size, lymph node spread, distant metastasis, and poor survival (17). However, so far the functional mechanism of MTDH in cancer is not clear. Although studies have reported the regulation of a number of genes and pathways by MTDH (9, 18–20), little is known about how MTDH delivers oncogenic signals to the downstream molecules and performs the pluripotent functions in cancer.

TWIST1 is a basic helix–loop–helix transcription factor that potentially drives the EMT process in development and cancer (21). Activated TWIST1 upregulates N-cadherin (CDH2), downregulates E-cadherin (CDH1), and leads to morphologic change, migration, and metastasis of cancer cells. In addition, the acquisition of mesenchymal traits resulted from TWIST1 activation confers CSC characteristics, including tumorsphere formation, and in vivo tumor initiation, on cancer cells (22, 23). Transcriptionally, TWIST1 can be regulated by miRNAs and transcription factors such as HMGAl2, KLF17, and STAT3 (24–27). Epigenetic modifications, including DNA and histone methylation, also play roles in the activation of TWIST1 expression (28, 29). However, the regulation of the histone acetylation status of TWIST1 has not been reported. In addition, although recently MTDH has been...
indicated to promote cancer cell EMT (30–33), a direct link of MTDH to prominent EMT regulators, such as TWIST1, is still unknown. In this study, we demonstrate a MTDH–TWIST1 signal axis that drives CSC expansion and tumorigenesis of cancer cells. Mechanistically, the interaction between MTDH and the histone acetyltransferase CBP prevents CBP from degradation, thus facilitating the epigenetic activation of TWIST1 and CSC expansion in breast cancer as well as prostate cancer.

Materials and Methods

Plasmids and reagents

For MTDH knockdown, the sense and antisense shRNA oligonucleotides were annealed and cloned into the BglII and HindIII site of pSUPER.retro.puro (OligoEngine) as previously described (5). The same method was used for TWIST1 and CBP knockdown. For MTDH overexpression, the human MTDH gene was cloned into the pcDNA3.1 vector with BamHI and EcoRI digestion. For TWIST1 promoter reporter assays, a 2.3-Kb DNA fragment upstream of TWIST1 transcription start site was cloned into pGL3-basic (Promega) with EcoRI and HindIII digestion. All of these constructs were confirmed by sequencing. The sequences of primers and shRNA constructs were available in Supplementary Table S1. The mouse anti-human MTDH (Abnova H00092140-B01P), the mouse anti-human MTDH (Cell Signaling Technology 9596s), the rabbit anti-human CBP (Santa Cruz Biotechnology sc-369), the rabbit anti-human CD24 (Santa Cruz Biotechnology sc-11406), the rat anti-human CD4 (Santa Cruz Biotechnology sc-18849), the mouse anti-human TWIST1 (Santa Cruz Biotechnology sc-814417) antibodies, the rabbit anti-human p300 (Santa Cruz Biotechnology sc-584), and DAPI (Roche 10236276001) were used in this study for Western blot analysis or immunofluorescence analyses. The rabbit anti-acetyl-histone H3 antibody (Upstate 06-599) was used for chromatin immunoprecipitation (ChIP) assays. C646 (Seleckchem S1752, 25 μmol/L for 24 hours), cycloheximide (Beyotime s1560, 25 μg/mL for 4–24 hours), and MG-132 (Beyotime s1748, 100 μmol/L for 12 hours) were used to inhibit CBP, protein translation, and proteasome activity, respectively. The reagents used to inhibit DNA methylation and histone deacetylation were 5-Aza-2-deoxycytidine (2.5 or 5 μmol/L, 48–72 hours) and Trichostain A (300 or 600 nmol/L, 12–24 hours).

Tumorsphere culture

Cells were cultured as tumorspheres in DMEM containing 20 ng/mL rhEGF (R&D Systems), 10 ng/mL rhbFGF (R&D Systems), 4 μg/mL heparin sulfate (Sigma), B27 supplement, and 1% penicillin G-streptomycin. Five thousand cells were seeded in each well of a 48-well plate. After 2 weeks of culture, spheres with diameters larger than 50 μm were counted. When tumorspheres were passaged, sphere pellets were centrifuged and digested with trypsin-EDTA, and 5,000 cells were reseeded in a new plate.

FACS analyses

FACS analyses were performed using a MoFlo Astrios Flow Cytometer (Beckman). Nonviable cells were excluded from further analyses. The ALDEFLUOR Kit (Stem Cell Technologies) was used to analyze ALDH-positive subpopulation. The specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) was used to assess the background signal. For side population analyses, one million cells were incubated with 5 μg/mL Hoechst 33342 (Sigma) for 90 minutes at 37°C. Control samples were treated with 50 μmol/L verapamil (Sigma) to block the efflux of the dye.

Chemoresistance clonogenic assay

For chemoresistance analysis, 2 × 10^4 cancer cells were seeded in each well of a 48-well plate. Two days later when the culture grew to approximately 80% confluence, the cells were treated with 25 nmol/L paclitaxel for 24 hours, and reseeded to a 10-cm dish at various dilutions. Two weeks later, the colonies were counted with crystal violet staining.

 Luciferase reporter assay

293T cells were cotransfected with the control or MTDH overexpression/knockdown plasmids, the indicated firefly luciferase reporter plasmid and a Renilla luciferase plasmid with a ratio of 2:1:1. Lysates were collected at 48 hours after transfection, followed by analyses of firefly and Renilla luciferase activities with a Dual-Luciferase Reporter System (Promega).

RNA stability assay

Cancer cells of 80% confluence were treated with 5 μg/mL actinomycin D (Dalian Meilun Biology), and mRNA was collected at the indicated time and analyzed with real-time PCR.

Microarray hybridization and data analysis

Total RNAs of MCF7 with or without MTDH overexpression were subjected to hybridization to Affymetrix U133 plus 2.0 arrays, performed by Shanghai Biotechnology Corporation. The microarray data were normalized according to the median intensity of each sample. The genes with expression ratios between the overexpression and control cells greater than 4 were defined as gene candidates regulated by MTDH. The microarray data were available in the Gene Expression Omnibus database (GSE67249).

Chromatin immunoprecipitation

For ChIP assays, cell lysates were cross-linked with 1% formaldehyde, and 125 mmol/L glycine was used to quench the formaldehyde. The nuclear extracts were sonicated and incubated with control IgG or anti-acetyl-histone H3 antibody for immunoprecipitation. The precipitated complexes were eluted and reverse cross-linked. The captured genomic DNA was purified with the silica membrane purification kit (TIANGEN) and was used for PCR analyses. One percent of total genomic DNA from nuclear extracts was used as input.

Animal studies

All animal experiments were performed according to the guidelines for the care and use of laboratory animals and were approved by the institutional biomedical research ethics committee of Shanghai Institutes for Biological Sciences (Shanghai, China). NOD/SCID or Balb/c nude mice at the age of 4 to 6 weeks were used in all studies. Orthotopic injection was performed to study primary tumor growth as previously described (11). Starting from one week before cancer cells injection, the mice were treated with subcutaneous injection of estradiol cypionate (5 mg/kg in 100 μL sesame oil, Dalian
Meliun Biology) every week for 4 weeks in MCF7 tumorigenesis assays.

Clinical analyses

Frozen and paraffin-embedded breast tumor specimens were obtained from Qilu Hospital of Shandong University (Jinan, China) with informed patient consent and approval from the Institutional Review Board. Frozen tissues were used for RNA extraction, followed by qPCR analyses of MTDH and TWIST1 expression levels. For Cox survival analysis, the patients were classified according to MTDH median expression level.

For immunofluorescence analysis, paraffin-embedded specimens were first deparaffinized. Then the antigen was unmasked with 10 mmol/L sodium citrate at a sub-boiling temperature for 10 minutes. After blocking, the specimens were incubated with a primary antibody of CD44, CD24, or MTDH overnight at 4°C. The samples were washed with PBS for three times and then a fluorochrome-conjugated secondary antibody was incubated with samples. After washing, the samples were mounted with coverslips and the immunofluorescence signal was collected with confocal microscopy. We distinguished the tumor epithelial and stromal cells with H/E staining. For statistic analyses, ten random view fields of each sample were assessed for fluorescence intensities with the ZEN blue edition software. Each sample was scored as low and high according to MTDH staining intensities and CD44+/CD24+ cells were counted in the tumor region.

Statistical analyses

Unless stated otherwise, data are presented as mean ± SD in the figures. A Student t test was performed to compare the in vitro data. A log-rank test was performed to compare tumor-free survival. P values less than 0.05 were considered statistically significant.

Results

MTDH knockdown inhibits CSC expansion and tumorigenicity of breast cancer cells

To study the functional role of MTDH in CSCs of breast cancer, MTDH was knocked down by two different shRNA in LM2 (Fig. 1A), a lung-metastatic subline derived from MDA-MB-231 cells (34), and the in vitro stemness of the cells were assessed. As showed in Fig. 1B, MTDH knockdown significantly inhibited tumorsphere formation of cancer cells. Through FACS analyses, we also found that the ALDH-positive CSC subpopulation was significantly reduced in the knockdown cells (Fig. 1C and D).

To examine the effects of MTDH knockdown on tumorigenicity, we performed serial transplantations of limiting dilutions of the cells into the mammary fat pads of NOD/SCID mice and assessed the tumor growth. Interestingly, when 10,000 cells were injected, no difference in tumor growth was observed between the control and MTDH knockdown groups. However, tumor inhibition by MTDH knockdown was revealed with 1,000 or 200 cells injected (Fig. 1E). We further analyzed the difference in tumor incidence and growth by injecting only 40 cells. MTDH knockdown significantly delayed the appearance of tumors (Fig. 1F). At the sixth week, 6 of 6 injected mice developed tumors in the control group, whereas only one tumor formed in the 12 mice injected with the MTDH knockdown cells (Fig. 1G). The tumor sizes were also significantly reduced in the knockdown groups when some tumors eventually appeared after 8 weeks (Fig. 1E and H). Hence, MTDH inhibition significantly impaired CSC expansion and tumorigenicity of breast cancer cells.

MTDH overexpression promotes CSC properties of breast and prostate cancer cells

Next, we analyzed the function of MTDH by overexpressing it in MCF7 cells (Fig. 2A). Compared with the cells expressing the
MTDH overexpression promotes CSC properties of breast cancer cells. A, MTDH overexpression in MCF7 cells. B, quantitation and representative images of sphere formation in MCF7 cells. Scale bars, 200 μm. C, relative sphere numbers of MCF7 cells in successive passage experiments. D, side population analyses of MCF7. Verapamil was used for negative control. E, side population quantitation of MCF7. F, tumor incidence of the mice injected with MCF7 cells at week 7 (n ≥ 6 in each group). G, tumor images of the mice injected with MCF7 cells at the sixth month. ***, P < 0.001.

Figure 2.

MTDH overexpression promotes CSC properties of breast cancer cells. A, MTDH overexpression in MCF7 cells. B, quantitation and representative images of sphere formation in MCF7 cells. Scale bars, 200 μm. C, relative sphere numbers of MCF7 cells in successive passage experiments. D, side population analyses of MCF7. Verapamil was used for negative control. E, side population quantitation of MCF7. F, tumor incidence of the mice injected with MCF7 cells at week 7 (n ≥ 6 in each group). G, tumor images of the mice injected with MCF7 cells at the sixth month. ***, P < 0.001.

empty vector, the overexpression cells formed more and larger tumorspheres (Fig. 2B). This enhancement was stable for at least three passages of the spheres (Fig. 2C). By Hoechst efflux analysis, we found that MTDH overexpression expanded the side population of the cells for more than two folds (Fig. 2D and E). The CD44^+CD24^- population was also upregulated by MTDH (Supplementary Fig. S1A). In addition, ectopic expression of MTDH correlated with increased expression of ABC transporter genes, including ABCB1 and ABCG2 (Supplementary Fig. S1B), and enhanced cell resistance to chemotherapeutic drugs such as paclitaxel and doxorubicin (Supplementary Fig. S1C), as well as cancer cell EMT (Supplementary Fig. S2). We also tested the in vivo tumorigenicity of DU145 cells by transplanting the cells subcutaneously into NOD/SCID mice. Similar to the observations of LM2 cells, there was no difference in tumor growth between the control and MTDH knockdown groups when 1 × 10^6 cells were injected (Supplementary Fig. S3C). However, when fewer cells, including 1 × 10^5, 1 × 10^4, 2 × 10^4, 4 × 10^4 cells, were injected, MTDH knockdown significantly reduced tumorigenicity and suppressed tumor growth compared with the control group (Supplementary Fig. S3C–S3E). Overall, these data demonstrated that MTDH was a pivotal factor for CSC regulation.

TWIST1 mediates the role of MTDH in CSCs

Given the fundamental role of MTDH in CSCs, we searched for the downstream genes mediating its function by performing a microarray analysis of MCF7 cells with or without MTDH overexpression (Fig. 3A). The microarray data showed that TWIST1 ranked at the top of the candidate genes regulated by MTDH, which was confirmed in the mRNA and protein levels by qPCR and Western blot analyses in MCF7 (Fig. 3B). We also found that MTDH knockdown led to the downregulation of TWIST1 in LM2 (Fig. 3B). Because TWIST1 is an important factor to promote EMT and CSC in breast cancer (22, 34–36), we examined whether TWIST1 mediated the function of MTDH in driving CSC expansion and tumorigenicity. We stably silenced TWIST1 in the MTDH-overexpressing MCF7 cells (Fig. 3C). TWIST1 knockdown significantly impaired side population abundance, tumorsphere formation, and in vivo tumorigenicity that were enhanced by
**MTDH overexpression.** Notably, the cells with both *MTDH* overexpression and TWIST1 inhibition displayed a tumor-initiating ability that was comparable with that of the control cells without *MTDH* overexpression (Fig. 3D and E).

We further overexpressed TWIST1 in LM2 with or without *MTDH* knockdown (Fig. 3C), and found that TWIST1 rescued the tumor-suppressing phenotypes of *MTDH* knockdown. More importantly, *MTDH* knockdown led to no obvious difference in tumor incidence or animal survival when TWIST1 was constitutionally expressed in the cancer cells (Fig. 3F and G). Therefore, TWIST1 acts downstream of *MTDH* to regulate CSC expansion and tumorigenicity of breast cancer cells.

**MTDH activates TWIST1 expression by upregulating histone acetylation.**

Next, we examined how TWIST1 was regulated by MTDH. Because the mRNA level of TWIST1 was extensively regulated by MTDH, we cloned the promoter of TWIST1 and performed luciferase reporter assays in 293T cells. The results showed that *MTDH* knockdown or overexpression failed to significantly affect the reporter activity (Fig. 4A), suggesting that MTDH cannot directly regulate the promoter activity of TWIST1. Next, we analyzed the mRNA stability of TWIST1 in breast cancer cell lines. Following actinomycin D treatment to inhibit de novo transcription, the mRNA level of TWIST1 declined within hours. However, *MTDH* knockdown or overexpression led to no significant changes in the speed of TWIST1 mRNA degradation (Fig. 4B). Therefore, *MTDH* was not likely to stabilize TWIST1 mRNA.

Then we analyzed whether epigenetic regulation was involved in TWIST1 activation by MTDH. We treated MCF7 cells with *MTDH* overexpression and control cells using the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) and the DNA methylation inhibitor 5-Aza-2'-deoxycytidine (Aza). The data showed that each of the chemicals was able to activate the expression of TWIST1 in the control cells, and the upregulation was more obvious when both chemicals were used together. However, in the TWIST1-expressing cells, TSA was less potent for TWIST1 induction than Aza (Fig. 4C). Further analyses demonstrated that when histone deacetylation was inhibited by TSA, the expression of TWIST1 was almost the same in control and *MTDH*-expressing cells (Fig. 4D). In contrast, Aza was not able to diminish the upregulation of TWIST1 by MTDH. The differential effects of these two inhibitors on TWIST1 expression was not related to the treatment duration or doses, as shown by repeating the experiments with different time courses and compound concentrations of the inhibitor treatment (Supplementary Fig. S4A and S4B). These data suggested that TWIST1 activation by MTDH might be due to regulation of histone acetylation. To verify this, we performed a ChIP assay to analyze the histone acetylation status around TWIST1 promoter and found that *MTDH* overexpression or knockdown led to no obvious changes in histone acetylation levels of the TWIST1 promoter (Fig. 4E). Moreover, the overall level of H3 acetylation was also increased after *MTDH* overexpression, as revealed by Western blotting (Fig. 4F). Notably, there was no obvious binding of acetylated histone on the TWIST1 reporter promoter ectopically introduced into the cells (Supplementary Fig. S4C), providing an explanation why the reporter construct was not responsive to *MTDH* overexpression or knockdown (Fig. 4A).

**MTDH interacts with CBP to prevent ubiquitin-mediated CBP degradation.**

Because *MTDH* regulated histone H3 acetylation on the promoter of TWIST1, we further searched for the executor of histone acetylation. Previous studies showed that MTDH was able to interact with the histone acetyltransferase CBP (9). In addition, CBP and its homolog p300 were connected to TWIST1 activation by MTDH. We treated MCF7 cells with *MTDH* overexpression and control cells using the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) and the DNA methylation inhibitor 5-Aza-2'-deoxycytidine (Aza). The data showed that each of the chemicals was able to activate the expression of TWIST1 in the control cells, and the upregulation was more obvious when both chemicals were used together. However, in the TWIST1-expressing cells, TSA was less potent for TWIST1 induction than Aza (Fig. 4C). Further analyses demonstrated that when histone deacetylation was inhibited by TSA, the expression of TWIST1 was almost the same in control and *MTDH*-expressing cells (Fig. 4D). In contrast, Aza was not able to diminish the upregulation of TWIST1 by MTDH. The differential effects of these two inhibitors on TWIST1 expression was not related to the treatment duration or doses, as shown by repeating the experiments with different time courses and compound concentrations of the inhibitor treatment (Supplementary Fig. S4A and S4B). These data suggested that TWIST1 activation by MTDH might be due to regulation of histone acetylation. To verify this, we performed a ChIP assay to analyze the histone acetylation status around TWIST1 promoter and found that *MTDH* overexpression or knockdown led to no obvious changes in histone acetylation levels of the TWIST1 promoter (Fig. 4E). Moreover, the overall level of H3 acetylation was also increased after *MTDH* overexpression, as revealed by Western blotting (Fig. 4F). Notably, there was no obvious binding of acetylated histone on the TWIST1 reporter promoter ectopically introduced into the cells (Supplementary Fig. S4C), providing an explanation why the reporter construct was not responsive to *MTDH* overexpression or knockdown (Fig. 4A).
involved in MTDH-regulated histone acetylation. First, we confirmed that MTDH interacted with the CBP protein with reciprocal coimmunoprecipitation assays in cancer cells (Fig. 5A). Then we found that CBP transcription was unaffected by MTDH in MCF7, but its protein level was obviously increased upon MTDH overexpression (Fig. 5B and C). CBP mRNA stability analysis showed that MTDH could not protect CBP transcript from degradation (Fig. 5D). However, when de novo protein synthesis was inhibited by cycloheximide (CHX), the degradation of CBP protein was pronouncedly slower in MTDH overexpression cells than the control cells (Fig. 5E and F). In further analyses, we blocked ubiquitin-mediated proteasome activity by treating cells with MG132 and analyzed the ubiquitination status of CBP. Accordantly, less CBP ubiquitination was observed when MTDH was overexpressed (Fig. 5G), arguing for the conclusion that MTDH protects the CBP protein from ubiquitin-mediated degradation.

To further verify that CBP was responsible for MTDH regulation of TWIST1 expression and CSC properties, we used a p300/CBP-specific inhibitor C646 to treat MCF7 and found that CBP inhibition blocked TWIST1 expression and sphere formation that were induced by MTDH overexpression (Fig. 5H and I). We also stably silenced CBP with a siRNA construct in MTDH overexpression cells (Supplementary Fig. S5A) and observed the same changes (Fig. 5I). In addition, side population analyses in these cells showed that although MTDH increased CSC fraction in MCF7, CBP silencing reversed the phenotype induced by MTDH (Fig. 5K). Notably, the CBP homolog p300 was not affected by MTDH overexpression or CBP shRNA. In addition, inhibition of p300 with a siRNA construct did not influence the expression of TWIST1 (Supplementary Fig. S5B and S5C). Collectively, these data demonstrated that CBP, but not p300, acted downstream of MTDH to regulate TWIST1 expression and CSC population in cancer cells.

MTDH correlates positively with CSC abundance and TWIST1 expression in breast cancer samples

Next, we studied the clinical significance of our findings. To investigate the correlation of MTDH with CSC abundance in human breast tumors, we assessed the expression of MTDH, CD44, and CD24 in 16 invasive ductal breast carcinomas by immunofluorescence staining (2). The analysis showed that higher expression of MTDH was significantly linked to increased presence of CD44+CD24−CSC population in the tumors (Fig. 6A and B). To unravel the clinical relevance of MTDH and TWIST1 expression, we collected a set of fresh-frozen breast tumors from Qilu Hospital and assessed the mRNA levels of the two genes. MTDH expression correlated positively with that of TWIST1 in these samples (Fig. 6C, Pearson r = 0.38). IHC analysis also showed that both MTDH and TWIST1 proteins were upregulated in breast tumor edges as compared with intratumor areas (Supplementary Fig. S6), suggesting the enrichment of the molecules in invasive front of tumors. Moreover, MTDH expression was also a prognostic factor of distant metastasis in this cohort (Fig. 6D). Thus, MTDH is clinically linked to TWIST1 expression, CSC abundance, and prognosis of breast cancer.

Discussion

Accumulated data of functional studies and clinical analyses have firmly established MTDH as an important regulator of neurodegeneration, cancer metastasis, and chemoresistance (5, 11, 17, 39, 40). However, compared with the unanimous functional roles of MTDH in these processes, the underlying
mechanisms are far from clear. Indeed, the downstream events of MTDH remain fragmentary despite previous efforts to explain the potency of MTDH in cancer regulation. On one hand, although numerous studies reported that MTDH activated oncogenic pathways such as PI3K/Akt, NF-κB, and Wnt/β-catenin (9, 18–20), it is unclear how MTDH is involved in these signaling pathways. On the other hand, recent data demonstrated that the physical interaction of MTDH with the nuclease protein SND1 was critical for the action of MTDH with the nuclease protein SND1. Here, we reported a different and detailed pathway that can connect MTDH to the malignant behaviors of cancer cells. First, we provided evidence of the role of MTDH in CSC regulation by showing that MTDH elevated the abundance of ALDH-positive fraction and side population of cancer cell lines, and correlated with the CD44+/CD24− subpopulation in clinical samples. It also promoted sphere formation, drug resistance as well as in vivo tumorigenesis of cancer cells. Then we demonstrated that the regulation of CSC traits by MTDH was dependent on the downstream molecules CBP and TWIST1. MTDH interacted with CBP to prevent ubiquitin-mediated degradation of the histone acetyltransferase and enhanced histone H3 acetylation on TWIST1 promoter, thus leading to the transcriptional activation of TWIST1 and CSC promotion. Thus our study delineated a molecular pathway of MTDH in driving cancer progression (Fig. 6E) and will profoundly expand our understanding for this master regulator of cancer cell features.

Metastasis and chemoresistance are the two most malignant features of tumor cells that account for disease progression and eventually therapeutic failure of cancer. As we and many others have shown the dual roles of MTDH to promote metastasis and chemoresistance (11, 40), it would be interesting to test whether MTDH also regulates cancer initiation and recurrence. Previously Kang and colleagues demonstrated that MTDH promoted tumor initiation using oncogene-driven breast cancer mouse models (42). Here, we corroborated this conclusion with various CSC assays in cancer cell lines. In addition, our data uncovered the link of MTDH with TWIST1, the pivotal transcription factor in CSC expansion and cancer cell EMT (21, 22). Therefore, these results suggest that the pluripotency of MTDH in cancer can be explained by a fundamental role in CSC regulation, and support the rationale of MTDH-targeting strategies to prevent cancer initiation, recurrence, and metastasis.

The challenge in studying the functional mechanisms of MTDH is largely due to the lack of homology of MTDH to any known protein structures. However, among the misty data regarding the molecular nature of MTDH, the observations that MTDH plays its roles by protein–protein interactions seem to be increasingly convincing. In addition to CBP, a number of proteins, such as CEACAM1, BCCIPα, PLZF, p65, AGO2, and SND1, have been documented to interact with MTDH, and these interactions are indispensable for MTDH regulation of cancer cell features (7, 9, 19, 43–46). Notably, MTDH stabilizes some of these partner proteins, including BCCIPα and SND1. Here, we not only show that MTDH prevents the degradation of CBP through physical interaction, but also demonstrate the involvement of ubiquitin-mediated proteasome activity in this process. Therefore, disrupting the interaction of MTDH with its partner proteins may be useful to target MTDH in cancer therapeutics. However, further studies are required to illustrate how MTDH selects its interacting partners and interrupts protein degradation in order to rationalize such approaches.

Histone modifications are important epigenetic mechanisms to regulate a broad range of developmental and disease processes. In this study, we unravel a new role of MTDH in epigenetic regulation to interact and stabilize the CBP protein. As a core member of the p300/CREB histone acetyltransferase (HAT) family, CBP regulates gene expression as a transcriptional coactivator by acetylating histones and other transcription factors. CBP has been
widely known to be involved in normal stem cell regulation and cancer progression (47–49), but its role in CSC is seldom reported. Our data show that CBP directly regulates the expression of TWIST1 and promotes CSC traits, thus expanding the role of CBP in cancer. Our data, together with previous studies (37, 38), suggested that CBP acted upstream of TWIST1 and its homolog p300 was a downstream target of TWIST1, highlighting the connection of the MTDH–TWIST1 axis to histone modification. More importantly, as many histone modification inhibitors are being developed in therapeutics, our findings that link MTDH to epigenetic regulation may provide new opportunities to target CSC and MTDH signaling in metastatic cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: X. Kong, D. Hong, G. Hu
Development of methodology: D. Hong, Q. Yang

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Grant Support
This work was supported by National 973 programs (2011CB510105 and 2013CB910904) and grants from the National Natural Science Foundation of China (81430070, 81222032, 31371409) and the Ministry of Science and Technology of China (2012ZX09506-001-005).

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Received April 8, 2015; revised June 16, 2015; accepted June 29, 2015; published OnlineFirst July 3, 2015.

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