The FBXW7 tumor suppressor inhibits breast cancer proliferation and promotes apoptosis by targeting MTDH for degradation

X. CHEN¹, X. Y. LI², M. LONG¹, X. WANG¹, Z. W. GAO¹, Y. CUI⁴, J. REN⁵, Z. ZHANG¹, C. LIU¹, K. DONG¹,*, H. ZHANG¹,*

¹Department of Medical Laboratory and Research Center; Tangdu Hospital; Fourth Military Medical University; Xi’an, China; ²Department of Ophthalmology; Xi’an No. 4 Hospital; Xi’an, China; ³Department of Ophthalmology, Eye Institute of Chinese PLA, Xijing Hospital, Fourth Military Medical University, Xi’an, China; ⁴Department of Blood Transfusion, Tangdu Hospital, The Fourth Military Medical University, Xi’an, China; ⁵Department of Medical, AstraZeneca China

*Correspondence: td_zhz@163.com, tddk01@126.com

Received February 28, 2017/ Accepted June 9, 2017

Metadherin (MTDH) is an oncoprotein and is expressed at high levels in a wide variety of human carcinomas, which represents an important genetic determinant and regulates multiple events in tumorigenesis. MTDH promotes breast cancer cell proliferation and tumorigenesis through the activation of numerous signaling pathways. Currently, the mechanism regulating MTDH expression is poorly understood. Here we identified that FBXW7, a component of E3 ubiquitin ligase, targets MTDH for ubiquitin-mediated degradation. Forced overexpression of FBXW7 could decrease the level of MTDH protein, and inhibition of endogenous FBXW7 expression remarkably increases the MTDH protein abundance. More importantly, overexpression of FBXW7 could lead to proliferation arrest and apoptosis in breast cancer cells through targeting MTDH degradation. These data suggest that FBXW7, a tumor suppressor, inhibits breast cancer cell proliferation and promotes apoptosis at least partially through targeting MTDH for proteolysis. This new regulatory mechanism of MTDH by FBXW7 represents a new pathway for malignant phenotype turnover in human breast cancer.

Key words: MTDH, FBXW7, Ubiquitin-proteasome degradation, cell proliferation, cell apoptosis, breast cancer

Metadherin (MTDH), also known as AEG-1, LYRIC or 3D3, is a transmembrane protein containing 582 amino acids [1]. MTDH is an oncoprotein and is expressed at high levels in a wide variety of human carcinomas, including glioma, melanoma and neuroblastoma, as well as carcinomas of the breast, prostate, liver and esophagus [2–4]. Studies have shown that MTDH plays a crucial role in regulation of the breast cancer development, progression and metastasis, and it is a prognostic factor in breast cancer [5]. Statistical analyses have shown a significant correlation of MTDH expression with the clinical stages and prognosis of the patients. In normal human breast tissues, the expression of MTDH is decreased or is completely absent, while it is widely overexpressed in nearly all of breast tumor tissues and tumor cell lines examined [1, 6–8]. In addition, inhibition of MTDH expression in breast cancer cells can interfere with the proliferation of these cells [9], indicating its potential as a specific new target in clinical-targeted therapeutics of breast cancer.

In these studies, we wanted to find the proteins capable of interacting with MTDH and FBXW7 was identified as a candidate of such proteins. FBXW7 (F-box and WD repeat domain-containing 7, also known as FBW7, cdc4) is one of the F-box proteins that function as substrate for the SCF³βγ⁷ (a complex of Skp1, CUL1 and F-box proteins) E3 ubiquitin ligase. The ubiquitin proteasome system regulates the turnover of a number of proteins and plays an essential role in maintaining normal cellular function [10]. Dysregulation of ubiquitin-mediated proteolysis results in the development of a variety of human cancers [11]. FBXW7, as an E3 ligase, plays an important role in mediating the ubiquitination and subsequent proteolytic turnover of protein substrates [12]. Our previous studies have shown that FBXW7 is a tumor suppressor and is inactivated in numerous human malignancies, including breast cancer, by gene mutation and expression downregulation [13, 14]. The FBXW7 gene encodes three protein isoforms with distinct subcellular localization, as the FBXW7α (nuclear) FBXW7β (cytoplasmic) and FBXW7γ (nucleolar) [15]. FBXW7 is also known to regulate the degradation of several oncoproteins, such as MYC [16, 17], cyclin E [15, 18], Notch [19], KLF-5 [20], and PGC1α [21, 22], many of its substrates have yet to be identified.

We found that FBXW7 could interact with MTDH in breast cancer cells MDA-MB-231. However, whether and how FBXW7 affects MTDH protein levels and further influ-
ences the MTDH functional roles within breast cancer cells has not been cleared so far. In this article, we found that FBXW7 negatively regulated MTDH protein level via the ubiquitination pathway. Depletion of endogenous FBXW7 stabilized MTDH protein, whereas overexpression of FBXW7 promoted degradation of MTDH and further impaired cell proliferation and apoptosis. In sum, our results showed that FBXW7, as an ubiquitin ligase of MTDH, was a critical negative regulator of MTDH protein abundance.

Materials and methods

**Antibodies and chemicals.** Antibodies against FBXW7 (ab171961), Flag tags (ab122902), Ubiquitin (ab7780), were purchased from Abcam (UK). The antibody against MTDH (13860-1-AP) was obtained from Proteintech (USA). The antibodies against His (sc-804), GST (sc-459) as well as all secondary antibodies were from Santa Cruz Biotechnology (CA, USA). Cycloheximide (CAS Number: 66-81-9) and MG132 (CAS Number: 133407-82-6) were purchased from Sigma (MO, USA). Cycloheximide was dissolved in water and the concentration for cell treatment was 25 μM at indicated time points. MG132 was dissolved in DMSO and dosage was 10 μM.

**Cell cultures and transfection.** Human breast cancer cell lines MDA-MB-23, SKBR3 and human embryonic cells HEK293 were purchased from ATCC (American Type Culture Collection) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2mM L-glutamine. All cells were incubated at 37°C with 5% CO2.

The cells were seeded in 6-well plates at 2.5×10^4 cells per well and grown overnight to 80% confluence prior to transfection. All transfections for plasmid and shRNA were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. To select neomycin-resistant cells, 500 μg G418 (Invitrogen, Carlsbad, CA, USA) was applied.

**Plasmid construction.** The pCDNA3.1-MTDH and pCDNA3.1-FBXW7α recombination vectors were constructed by inserting the full-length MTDH and FBXW7α cDNAs (generated by RT-PCR from total mRNA of MDA-MB-231 cells) into pCDNA3.1(+) vectors (Invitrogen), respectively [16]. The prokaryotic expression vectors for pGStag-FBXW7α were constructed by cloning the FBXW7α cDNAs into pGStag vectors (Amersham Biosciences). The MTDH, FBXW7α, FBXW7β and FBXW7γ cDNAs were inserted into pFlag-CMV vectors (Invitrogen) to construct the pFlag-CMV-MTDH, pFlag-CMV-FBXW7α, pFlag-CMV-FBXW7β and Flag-CMV-FBXW7γ recombination vectors, respectively. The primer sequences used in this part of experiments are shown in Table 1.

DNA template encoding FBXW7 shRNA was designed and synthesized (GenePharma Inc, Suzhou, China) as follows: the 21-nt target sequence (FBXW7, NM_033632, 1661-1681 bp) was chosen as a sense strand followed by a 9-nt spacer and a complementary antisense strand sequence as shown in Table 1 [23]. The shRNA was annealed and subcloned into pSilencer4.1-CMV neo (Ambion, Austin, TX, USA) BamHI and HindIII enzyme sites downstream of CMV promoter, and recombinant vector was named as pSilencer4.1-siFBXW7. The shRNA designed towards a non-specific (NS) sequence was also used as a negative control and named as pSilencer4.1-siNC. BLAST search against EST libraries was performed to confirm that no other human gene was targeted.

**Co-immunoprecipitation.** Co-immunoprecipitation assay was performed as previously described [24]. In brief, 48h after transfection, the cells were washed with PBS and lysed in modified RIPA buffer (20 mM Tris (pH 7.5), 150 mM NaCl,

<table>
<thead>
<tr>
<th>vector</th>
<th>primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1-MTDH</td>
<td>Forward: 5’-CCGAATTCATGGCTGCACGGAGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CGTCTAGATCACGTTTCTCGTCTG-3’</td>
</tr>
<tr>
<td>pcDNA3.1-FBXW7α</td>
<td>Forward: 5’-CCGAAGCTTATGAATCAGGAACTGCTCTCTCTG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CGTCTAGATCACTTCATGTCCACATCAAAGTCC-3’</td>
</tr>
<tr>
<td>pFlag-CMV-MTDH</td>
<td>Forward: 5’-CCGAATTCATGGCTGCACGGAGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CGTCTAGATCACGTTTCTCGTCTG-3’</td>
</tr>
<tr>
<td>pFlag-CMV-FBXW7α</td>
<td>Forward: 5’-CCGAAGCTTATGAATCAGGAACTGCTCTCTG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CGTCTAGATCACTTCATGTCCACATCAAAGTCC-3’</td>
</tr>
<tr>
<td>pFlag-CMV-FBXW7β</td>
<td>Forward: 5’-CCGAAGCTTATGAATCAGGAACTGCTCTCTG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CGTCTAGATCACTTCATGTCCACATCAAAGTCC-3’</td>
</tr>
<tr>
<td>pFlag-CMV-FBXW7γ</td>
<td>Forward: 5’-CCGAATTCATGGCTGCACGGAGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CGTCTAGATCACTTCATGTCCACATCAAAGTCC-3’</td>
</tr>
<tr>
<td>pGSTag-MTDH</td>
<td>Forward: 5’-CCGAATTCATGGCTGCACGGAGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CGTCTAGATCACGTTTCTCGTCTG-3’</td>
</tr>
<tr>
<td>pSilencer4.1-ShFBXW7</td>
<td>Sense: 5’-GATCCCCGGCTGTTCAATTATATGTTAAGAAGAATCATATTGAACAGCGGACA-3’</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-AGCTTGTCCGCTGTTCAATTATATGTTAAGAAGAATCATATTGAACAGCGGACA-3’</td>
</tr>
</tbody>
</table>
0.5% Triton X-100 and 1 mM protease inhibitor PMAF) in the cold. The lysates were incubated with antibodies (5 μg/sample) overnight at 4°C with gentle agitation. The next day, protein A/G Plus Agarose (Santa Cruz, CA, USA) was added to the mixture of lysates and antibodies (20 μl/tube), followed by a 1 h incubation in the cold. The samples were washed and boiled for 5 min at 95°C in 60 μl 2xSDS loading buffer. Then the beads were collected by brief centrifugation and the supernatant fraction was assessed by SDS-PAGE.

**Protein identification by mass spectrometry.** The analysis of proteins by liquid chromatography combined with random mass spectrometry (LC/MS/MS) was performed as previously described [24], and it was conducted by Beijing Proteome Research Center, China.

**Protein purification and GST-pulldown.** The prokaryotic recombination expression vectors of GST-FBXW7α and GST were expressed in BL21 (DE3) pLysS Escherichia coli, respectively. To obtain the expressed proteins, the cell lysates were prepared following manufacturer’s protocol (GE Healthcare Bio-Sciences AB) and were allowed to bind to Glutathione Sepharose 4B for 30 min at room temperature, washed 3 times with buffer-A (2.7 mM KCl, 140 mM NaCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3), and then eluted with 600 μl of buffer-B (50 mM Tris-HCl pH 8.0, 1 mM reduced glutathione, pH 8.0). The eluted proteins GST-FBXW7α and GST were analyzed by SDS-PAGE and stained with Coomassie Blue to check for the protein purity [27]. Then, 50 μg of GST or GST-MTDH were incubated with 50 μl (50% slurry) of Glutathione HiCap Matrix (Qiagen) in buffer-C (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM DTT, 10 mM MgCl2, pH 8.0 and protease inhibitor PMSF) at room temperature with rocking. 2 h later, the mixtures were collected in a similar interaction with MTDH in vitro, we used purified GST-MTDH in a pull-down assay and found that FBXW7 specifically interacted with MTDH (Figure 1D). Collectively, it could be concluded that FBXW7 had an interaction specifically interacted with MTDH (Figure 1D). Collected GST-MTDH in a pull-down assay and found that FBXW7 specifically interacted with MTDH (Figure 1D). Collectively, it could be concluded that FBXW7 had an interaction.
Figure 1. FBXW7 was a partner of MTDH protein. A) FBXW7 was identified as one of MTDH complexes from human breast cancer cell line MDA-MB-231. Affinity purified Flag-MTDH complexes were silver stained from MDA-MB-231 cells. Specific MTDH-interacting proteins were identified by mass spectrometry and were indicated. B) Proteins were immunoprecipitated from MDA-MB-231 cell lysates with antibodies against MTDH and checked for FBXW7. Western blotting results showed positive bands in both anti-MTDH and anti-FBXW7 antibodies panels. C) Proteins were immunoprecipitated from MDA-MB-231 cell lysates with antibodies against FBXW7 and checked for MTDH. Western blotting results showed positive bands in both anti-MTDH and anti-FBXW7 antibodies panels. D) GST and GST-MTDH (500 μg) were used for GST pull down assay with MDA-MB-231 cell lysates, transfected with pcDNA3.1-MTDH-Flag vector, and western blotting results showed positive bands in all three different primary antibodies of anti-MTDH, anti-FBXW7, and anti-GST.

Figure 2. FBXW7 negatively regulated MTDH protein stability. A) Left figure, MDA-MB-231 cells were transfected with different combinations of expression plasmids (Flag-CMV, Flag-CMV-FBXW7α, β and γ) and the MTDH protein levels were all decreased compared to control group, especially in the MDA-MB-231 cells transfected with Flag-CMV-FBXW7α. Right figure, densitometry of the immunoblots shown in B left figure (*p<0.05, **p<0.01 vs control, n=3). B) Left figure, MDA-MB-231 cells were transfected with 1.0, 2.0 and 3.0 μg Flag-CMV-FBXW7α respectively, western blotting showed an inverse expression of MTDH. Right figure, densitometry of the immunoblots shown in B left figure (*p<0.05, **p<0.01 vs control, n=3). C) Left figure, knock-down of FBXW7 with shRNA increases the MTDH protein levels in HEK293 cells compared to control cells. Right figure, densitometry of the immunoblots shown in C left figure (*p<0.05, **p<0.01 vs control, n=3).
demonstrated that FBXW7 overexpression led to decreasing abundance of MTDH protein level. To further study the role of FBXW7 in the regulation of MTDH stability under endogenous conditions, we knocked down the FBXW7 expression in HEK293 cells by shRNA. Western blotting analysis revealed that depletion of endogenous FBXW7 expression elevated MTDH abundance when compared with control cells (Figure 2C). These results suggest that FBXW7 is a negative regulator of MTDH.

**FBXW7 regulates MTDH protein stability through ubiquitination.** Since FBXW7, an E3 ubiquitin ligase, negatively regulates MTDH stability, we thought it was a matter of interest to check whether FBXW7 played an E3 ubiquitination. To confirm this possibility, we overexpressed FBXW7 together with or without Flag-FBXW7α in MDA-MB-231 cells. Whole cell lysates were treated with proteasome inhibitor MG132 48h after transfection, and then they were immunoprecipitated using anti-MTDH antibody. Western blotting results showed the level of poly-ubiquitination MTDH was markedly increased in the presence of FBXW7 compared to control groups (Figure 3A). To certify the role of FBXW7 in mediating MTDH ubiquitination under physiological conditions, we knocked down the FBXW7 gene expression in HEK293 cells by shRNA, and then conducted the immunoprecipitation assay with anti-MTDH antibody using the HEK293 cell lysates which were pre-treated with the proteasome inhibitor MG132. Western blotting analysis showed that deletion of endogenous FBXW7 significantly reduced the MTDH ubiquitination level compared to control groups (Figure 3B). Altogether, these results demonstrate that FBXW7 could ubiquitinate the MTDH.

**FBXW7 promotes proteasome-mediated degradation of MTDH.** We have shown that FBXW7 negatively regulated the steady state level of MTDH protein. To confirm the involvement of the proteasome system in FBXW7 mediated degradation of MTDH protein, we treated MDA-MB-231 cells with MG132, which stabilized MTDH and eliminated the reduction of MTDH protein level even in the presence of FBXW7α (Figure 4A). To further study this function, we observed endogenous level of MTDH protein under the condition of overexpression of FBXW7α in MDA-MB-231 cells followed by treatment with CHX (cycloheximide, protein synthesis inhibitor). The results indicated that MTDH half-life was decreased in the condition of overexpression FBXW7α compared to control groups. The half-life of MTDH was ~12h in wild type cells, but it decreased to ~6h in the condition of overexpression FBXW7α cells (Figure 4B). Further, we also knocked down FBXW7 by shRNA in HEK293 cells and assayed the endogenous level of MTDH protein with CHX. The half-life of MTDH protein was increased in the condition of knock-down of FBXW7 (Figure 4C). All these results confirmed that FBXW7 negatively regulates MTDH stability by targeting it for ubiquitination and degradation through ubiquitin proteasome pathway.

**FBXW7 suppresses breast cancer cell proliferation and promotes cell apoptosis through promoting MTDH degradation.** We investigated the effects of overexpression of FBXW7 on cell proliferation in breast cancer cells. Cell proliferation was inhibited both in MDA-MB-231/FBXW7+ and SKBR3/FBXW7+ cells, and the inhibitory rate was 31.7±3.21% and 30.1±3.46% compared with mock groups, respectively on 72 h after transfection (p<0.05) (Figure 5A, 5B). Since FBXW7 also targets several other oncoproteins for degradation [29], it was important to illuminate that FBXW7 suppressed breast cancer cell proliferation through MTDH. When the MDA-MB-231/FBXW7+ and SKBR3/FBXW7+ cells were transfected with pCDNA3.1-MTD, the inhibitory rates were lower compared to MDA-MB-231/FBXW7+ and SKBR3/FBXW7+ cells respectively on 72h after transfection (Figure 5A, 5B). The cell proliferation of MDA-MB-231/FBXW7+ and SKBR3/FBXW7+ were reversed by forced expression of MTDH. These results show that FBXW7 suppresses breast cancer cell proliferation by targeting MTDH degradation.

Cell apoptosis was an important cause of viability suppression, so we also performed a cell apoptosis assay with a flow cytometer. The increased rate of apoptosis in MDA-MB-231/FBXW7+ and MDA-MB-231/FBXW7+ +MTDH cells was 15.38±1.63 times and 8.89±0.89 times compared to control cells. The increased rate of apoptosis in SKBR3/FBXW7+ and SKBR3/FBXW7+ +MTDH cells was 6.29±0.96 times and
3.20±0.54 times compared to control cells. FBXW7 overexpression induced cell apoptosis in MDA-MB-231 cells and SKBR3 cells. And the apoptosis of MDA-MB-231/FBXW7+ and SKBR3/FBXW7+ cells was reversed by forced expression of MTDH (Figure 5C, 5D). These results demonstrate that endogenous FBXW7 promotes cell apoptosis through targeting the endogenous MTDH for degradation. In sum, FBXW7 suppresses breast cancer cell progression through mediating MTDH degradation in some measure.

**Discussion**

Here, we provide several lines of evidence to support that oncoprotein MTDH is targeted by E3 ubiquitin ligase FBXW7 for proteasome degradation. First, MTDH binds to FBXW7. Second, FBXW7 negatively regulates MTDH protein stability. Third, FBXW7 promotes MTDH protein stability through ubiquitination. Fourth, FBXW7 promotes proteasome-mediated degradation of MTDH. Addition-
ally, FBXW7 suppresses breast cancer cell proliferation and promotes cell apoptosis by targeting MTDH for degradation. These data indicate that FBXW7 is a negative regulator of MTDH stability and functions through down-regulation of the abundance of MTDH protein level in breast cancer cells.

MTDH, firstly cloned in 2002 by subtraction hybridization [1], has been shown to play an important role in the process of cancer development and progression, including proliferation, evasion of apoptosis, cell survival, migration, and chemoresistance [30]. Recent studies show that MTDH is widely overexpressed in the majority of human malignancies. The regulation of MTDH overexpression is very complicated, and the previous research about its overexpression has been focused mainly on the transcriptional regulation [31–33]. However, in this study, we have revealed the post-translational modifications of MTDH. The tumor suppressor FBXW7 is identified as a novel regulator of MTDH. Overexpression of any FBXW7 isoforms decreases the MTDH protein level. Importantly, knock-down of endogenous FBXW7 in HEK293 cells increases the endogenous MTDH protein level. In MDA-MB-231 cells, FBXW7α seems to be
the major functional isoform for MTDH on endogenous level. FBXW7a interacted with MTDH and promoted its ubiquitination in vitro and in vivo, while overexpression of FBXW7 reduced the half-life of endogenous MTDH. These results support the hypothesis that FBXW7 acts as a negative regulator of MTDH and plays a critical role in regulating the level of MTDH.

In addition, it was reported that MTDH played various roles in the regulation of cancer development and the inhibition of MTDH expression could lead to cell apoptosis and proliferation arrest. Our studies suggest that FBXW7 could inhibit breast cancer cells progression through degradation of MTDH. FBXW7 was reported to be a tumor suppressor controlling the level of several oncoproteins, such as MYC, Cyclin E, Notch, KLF-5. In this study, we showed that FBXW7 inhibits MTDH accumulation. Importantly, MTDH seemed to be a crucial substrate for FBXW7 to suppress breast cancer cell proliferation. Overexpression of FBXW7 could reverse the FBXW7-induced breast cancer cell proliferation inhibition and cell apoptosis. Thus, FBXW7 suppresses breast cancer cell progression in some measure by controlling MTDH accumulation.

We also showed that MTDH was subject to regulation via the ubiquitin proteasome pathway by interaction with the E3 ubiquitin ligase of FBXW7. The interaction between substrate protein and E3 ubiquitin ligase has been emphasized in research of the overexpression of oncoproteins, and it may play an important role in retaining cancer cell progression. FBXW7 is inactivated by mutation or decreased expression in various types of human malignances [34]. Previous studies also found that FBXW7 expression level was significantly reduced in breast cancer compared to the normal breast tissues [35]. Furthermore, we propose that FBXW7 could represent a new pathway for the regulation of MTDH accumulation.

In conclusion, our studies demonstrate that FBXW7 could inhibit MTDH accumulation by targeting it for degradation through ubiquitin proteasome pathway. The turnover of MTDH by FBXW7 led to proliferation arrest and apoptosis in breast cancer cells. Given the frequent inactivation of FBXW7 in breast cancer and other cancers, these findings may help us further understand the roles of FBXW7 and MTDH in cancer development. These findings confirm that the interaction between FBXW7 and MTDH will propose a promising strategy for clinical development in tumor treatment.

**Supplementary information** is available in the online version of the paper.

Acknowledgments: Thanks to everyone from the Department of Clinical Laboratory for their sincere help and excellent technical assistance. This study was supported by a grant from the National Natural Science Foundation of China (No. 81001195).

**References**


