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Myotubularin-related Protein 4 (MTMR4) Attenuates BMP/Dpp Signaling by Dephosphorylation of Smad Proteins*[S]

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Background: The intensity and duration of phosphorylation levels of R-Smads are required for precise control of BMP signaling.
Results: MTMR4 associated with and dephosphorylated the activated R-Smads in cytoplasm.
Conclusion: MTMR4 attenuates BMP signaling via its DUSP activity.
Significance: This study describes a novel role of MTMR4 as a negative modulator essentially involved in homeostatic BMP signaling.

Bone morphogenetic proteins (BMPs) signaling essentially regulates a wide range of biological responses. Although multiple regulators at different layers of the receptor-effectors axis have been identified, the mechanisms of homeostatic BMP signaling remain vague. Herein we demonstrated that myotubularin-related protein 4 (MTMR4), a FYVE domain-containing dual-specificity protein phosphatase (DUSP), preferentially associated with and dephosphorylated the activated R-Smads in cytoplasm, which is a critical checkpoint in BMP signal transduction. Therefore, transcriptional activation by BMPs was tightly controlled by the expression level and the intrinsic phosphatase activity of MTMR4. More profoundly, ectopic expression of MTMR4 or its *Drosophila* homolog CG3632 genetically interacted with BMP/Dpp signaling axis in regulation of the vein development of *Drosophila* wings. By doing so, MTMR4 could interact with and dephosphorylate Mothers against Decapentaplegic (Mad), the sole R-Smad in *Drosophila* BMP pathway, and hence affected the target genes expression of Mad. In conclusion, this study has suggested that MTMR4 is a necessary negative modulator for the homeostasis of BMP/Dpp signaling.

Bone morphogenetic proteins (BMPs)[4] belong to the TGFβ super family, and have a diverse array of functions in the development of mutilcellular organisms (1). Recent evidence has also shown that BMP signaling regulates the self-renewal and proliferation of stem cells (2, 3), and prolongs the lifespan of stem cells (4). The hierarchy and function of BMP signaling pathway are highly conserved in the metazoan organisms (5–7). After binding to BMPs, the serine-threonine kinase transmembrane receptors activate a signal cascade through intracellular regulatory Smad proteins (R-Smads). Phosphorylated R-Smads form a ternary complex with the common partner Smad (Co-Smad) and translocate to the nucleus to activate transcription of a spectrum of effector genes (8). There are two members of BMPs in *Drosophila.* Decapentaplegic (Dpp) is the homolog of BMP2/4 (9) and glass bottom boat (Gbb) is the homolog of BMP5/6/7 (10). These two *Drosophila* BMP members play a major role in cell proliferation and/or cell survival (11), and are required for the vein formation (12) during *Drosophila* wings development (13). Thickveins (tkv) serves as the type I BMP receptor that mediates Dpp signaling during wing morphogenesis and other stages of fly development (11, 14, 15), and Mathers against Decapentaplegic (Mad) turns out as the sole R-Smad in BMP signaling of *Drosophila* (16, 17).

The intensity and duration of phosphorylation levels of BMP receptors and R-Smads are required for precise control of BMP signaling (18, 19). A few nuclear phosphatases for R-Smads have thus far been identified, such as pyruvate dehydrogenase phosphatase (PDP), PPM1A (protein phosphatase magnesium-dependent 1A), and small C-terminal domain phosphatase (SCP2/Os4) (19–21). However, the existence and roles of cytoplasmic phosphatases have remained elusive; it has been recently revealed by us that a new endosomal phosphatase, myotubularin-related protein 4 (MTMR4), can specifically temper TGFβ signaling (22). MTMR4 belongs to myotubularin family and contains tyrosine/dual-specificity phospha-

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[4] The abbreviations used are: BMP, bone morphogenetic proteins; MTMR4, myotubularin-related protein 4; DUSP, dual-specificity protein phosphatase; Mad, Mothers against Decapentaplegic; Dpp, Decapentaplegic; Gbb, glass bottom boat; PCV, posterior crossvein; ACV, anterior crossvein.

tase (DUSP) activity (23) and functions in early endosomes (22, 24). MTMR4 specifically interacts with and dephosphorylates the activated R-Smads to keep TGFβ signaling in homeostasis (22). It therefore would be highly interesting to determine whether MTMR4 is also critically involved in BMP signaling.

In this study, we managed to demonstrate that MTMR4 is also an essential negative regulator of BMP signaling pathway. MTMR4 directly bound to and dephosphorylated the activated Smad1 via the DUSP enzymatic domain. Thus, overexpression of MTMR4 inhibited BMP-induced gene expression by accelerating Smad1 dephosphorylation, whereas knockdown of MTMR4 by siRNA enhanced BMP signaling with sustained Smad1 phosphorylation. We further demonstrated *in vivo* that MTMR4 and its *Drosophila* homologous gene CG3632 critically modulated the vein formation of *Drosophila* wings, by specifically targeting Mad activation. Therefore, MTMR4 may possess a conserved attenuator activity on R-Smads activation that is required for homeostatic BMP/Dpp signaling.

EXPERIMENTAL PROCEDURES

Plasmids—Expression of plasmids for YFP-tagged MTMR4 and its truncations/point mutation (MTMR4C407S) has been previously described (22). pCMV-Flag-Smad1, pCMV-HA-Smad5, GCCG-luc, and BRE-luc were described elsewhere (19, 25, 26). HA-MAD (27) was kindly provided by Dr Xin-hua Feng. The various truncations of Smad1 (pCMV-Flag-Smad1N/L/C) were obtained by PCR and cloned inbetween BglII (5′) and KpnI (3′) sites of the pCMV-HA-Flag vector derived from pCMV-HA (Clontech, Japan). Each derivative clone was verified by DNA sequencing analysis. pAGW-MTMR4 was constructed by the Gateway cloning technique (Invitrogen).

Cell Culture, Transfection, and Reporter Assays—HEK293T, HeLa, and HepG2 cells were grown in DMEM (Invitrogen), and S2 cells were cultured in Schneider's insect medium (Sigma). All media were supplemented with 10% heat-inactivated FBS (Invitrogen), 1% penicillin, and streptomycin (Invitrogen). The lipofectamine method (Invitrogen) was used to transfect HeLa and *Drosophila* S2 cells and calcium-phosphate method for 293T cells. Transient co-transfection and Luciferase reporter assays were performed as previously described (28). Data represent the average of triplicate experiments (mean ± S.D.). Recombinant BMP2 and Dpp ligand proteins were purchased from R&D (Minneapolis, MN).

Immunoprecipitation and Western Blotting—Immunoprecipitation was carried out exactly as previously described (22). Antibodies against pSmad1/5/8 (phosphor-SXS) and Smad1 were from Cell Signaling Technology (Beverly, MA). GFP antibody was from Zymed Laboratories Inc. (San Diego, CA) and MTMR4 antibody from Abgent (San Diego, CA). Flag and β-actin antibodies were from Sigma.

Real-time PCR—Total RNA was extracted with TRIzol reagent (Invitrogen) from HeLa or S2 cells treated with or without BMP2/DPP for the times indicated. Quantitative RT-PCR (ABI7500, Invitrogen) was carried out as previously described (22) using GAPDH or rp49 as the internal controls, respectively. Primer sequences for p21, forward: 5′-GGCAGACCAG CATGACAGATT-3′; reverse: 5′-GCGGATTA GGGCTTCC-TCTT-3′; Id1, forward: 5′-AACCGCAAGGTGAGCAAGG-TGG-3′; reverse: 5′-ACGCATGCCGCCTCGGC-3′; GAPDH, forward: 5′-CTGGGCTACACTGAGCACCAG-3′; reverse: 5′-CCAGCGTCAAAGGTGGAG-3′; Brinker, forward: 5′-CGG-CAATCAACGAACAAAGG-3′; reverse: 5′-TGAAAGCTGC-TGGTGATCGA-3′; Omb, forward: 5′-AAGTGCGTAAAGT-GTGGAGT-3′; reverse: 5′-ATATTCTTTGGACCTCCCAC-3′; Rp49, forward: 5′-AGATCGTGAAGAAGCGCACCAAG-3′; reverse: 5′-CACCAGGAACTTCTTGAATCCGG-3′. Data represent the average of three independent experiments (mean ± S.D.).

RNA Interference—The 21-nucleotide siRNA duplexes targeting MTMR4 and scrambled siRNA were synthesized and purified as previously described (22). The efficiency was measured by Western blot using MTMR4 antibody.

Fluorescence Microscopy—S2 cells in 35-mm plates were transfected with various RFP/GFP fusion plasmids indicated (1 μg each). After 36 h, the transfectants were seeded at 80% confluence on glass bottom tissue culture dishes and serum-starved overnight. Cells were then exposed to Dpp (40 ng/ml) for 2 h before fixed in 4% formaldehyde for 15 min at room temperature. Images were taken using a Zeiss fluorescent microscope (Observer Z1, Germany) and analyzed by Imag J software. DAPI (1:1000 dilution) was used to counter stain the nuclei.

Drosophila Stocks and Experimental Genotypes—All stocks were cultured at room temperature in standard cornmeal/molasses/agar media. The *Drosophila* stocks used in this study were as follows: *UAS-gbb* and *UAS-dpp* (described in Flybase), *UAS-tkvRNAi* (II), *UAS-tkvRNAi* (III), and *vg-gal4* (kind gifts from Matthew Gibson, Stowers Institute for Medical Research). The Gateway cloning system (Invitrogen) was used to generate *UAS-CG3632*, *UAS-MTMR4*, and *UAS-MTMR4C407S* constructs according to manufacturer's manual, after *cg3632*, *mtmr4*, and *mtmr4-C407S* gene fragments were PCR amplified. Different plasmid constructs were injected into *w1118* embryos to generate transgenic lines. To determine whether a moderate reduction of BMP signaling and overexpression of MTMR4 has any effect on *Drosophila* wing patterning, the *vg-gal4* virgins were crossed with *UAS-tkvRNAi* (II), *UAS-tkv RNAi* (III), *UAS-CG3632*, *UAS-MTMR4*, *UAS-MTMR4C407S*, and *w1118* males. To investigate how *gbb* overexpression affects wing pattern formation, the *vg-gal4* virgins were crossed with *UAS-gbb* males. To test whether MTMR4, MTMR4-C407S could interfere with BMP signaling, the *vg-gal4* virgins were crossed with *UAS-CG3632/Cyo*;*UAS-tkvRNAi(III)/TM6*, *UAS-MTMR4/Cyo*;*UAS-tkvRNAi(III)/TM6*, *UAS-MTMR4C407S/Cyo*;*UAS-tkvRNAi(III)/TM6*, *UAS-gbb/FM7*; *UAS-CG3632/Cyo*, *UAS-gbb/FM7*;*UAS-MTMR4/Cyo*, *UAS-gbb/FM7*;*UAS-MTMR4C407S/Cyo* males. All wings demonstrated here are from adult females and were mounted in Hoyer's medium.

RESULTS

MTMR4 Attenuates BMP Signaling—Because BMP and TGFβ both belong to TGFβ superfamily, that MTMR4 attenuates R-Smads activation in TGFβ pathway (22) prompted us to

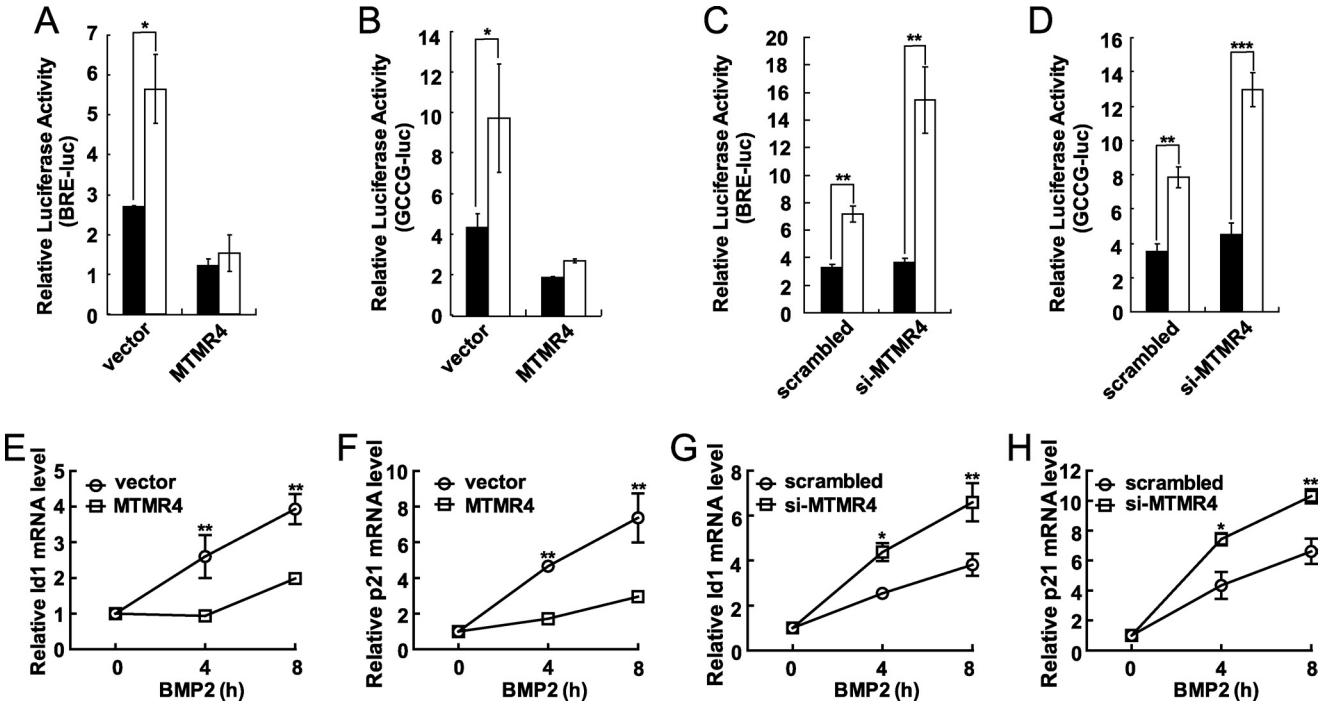

FIGURE 1. **MTMR4 inhibits BMP2-induced reporter activities and target genes activation.** *A* and *B*, ectopic expression of MTMR4 inhibited BMP2 signaling. HeLa cells were co-transfected with pEGFP vector or pEYFP-MTMR4 and BRE-Luc or GCCG-Luc reporter. pEGFP parental vector was used as a control. BMP2 (100 ng/ml) was added for 12 h before luciferase activities were measured. *C* and *D*, knockdown of MTMR4 in HeLa cells enhanced BMP2 signaling. HeLa cells were transfected with RNAi for MTMR4 or scrambled control for 72 h before cells were treated with BMP2 (100 ng/ml) for additional 12 h. All reporter assays were normalized to co-expressed *Renilla* activities. ■, w/o BMP2 stimulation; □, with BMP2 stimulation. *E–H*, MTMR4 affected BMP2 target genes expression. Hela cells were transfected with pEYFP-MTMR4 (*E* and *F*) for 48 h or MTMR4 RNAi (44) for 72 h before treated with BMP2 (100 ng/ml) for 0, 4, 8 h. Quantitative RT-PCR were performed for induction of Id1 (*E*, *G*) and p21 (*F*, *H*) gene expression. All data represent the average of triplicate independent experiments (mean ± S.E.). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, Student's *t* test.

test whether the conserved DUSP activity of MTMR4 would also play a role in BMP signaling. To test this hypothesis, first we transiently transfected two BMP-responsive luciferase reporters in HeLa cells, namely BRE-lux and GCCG-lux which contained multiple BMP-specific R-Smads binding elements GCCG (25, 29). The R-Smad responsive reporter activities increased by ~2.5-fold post-BMP2 treatment for 16 h, which was effectively suppressed by co-expressed MTMR4 (Fig. 1, *A* and *B*; supplemental Fig. S1*A* for expression levels of MTMR4 in transfectants). This result thus suggested that MTMR4 plays an inhibitory role in BMP signaling. Moreover, when we knocked down the endogenous MTMR4 gene expression by siRNA (supplemental Fig. S1*B* for the knockdown efficiency), as expected, cells became more responsive to BMP2, as demonstrated by much higher BRE/GCCG reporter activities than those treated with scrambled RNAi (Fig. 1, *C* and *D*). Therefore, MTMR4 would be required to attenuate BMP signaling. To substantiate this notion, we went on to further test whether MTMR4 affects the expression of BMP target genes. For this purpose, we examine the transcriptional levels of Id-1, a regulator of cell proliferation and differentiation (26, 30), and p21, a cell cycle inhibitor (31), both of which are well-known target genes of BMP/R-Smads signaling. Quantitative PCR analysis showed that the expression levels of Id1 and p21 was effectively suppressed by overexpressed MTMR4 (Fig. 1, *E* and *F*) and enhanced by knockdown of endogenous MTMR4 (Fig. 1, *G* and *H*) after BMP2 stimulation. Taken together, these results suggested that MTMR4 could attenuate BMP signaling, possibly via directly interrogating R-Smad activation.

MTMR4 Physically Interacts with Smad1/5—To validate the aforementioned hypothesis, we set out to examine whether MTMR4 interacts with those R-Smads of BMP pathway. Co-immunoprecipitation experiments showed that YFP-MTMR4 transiently expressed in 293T cells readily associated with Flag-Smad1 (Fig. 2*A*) or HA-Smad5 (Fig. 2*B*), two of R-Smads in BMP signaling pathway. Such intermolecular interactions also occurred under physiological conditions since the endogenous MTMR4 and Smad1 associated with each other, and interestingly enough, only after BMP2 stimulation (Fig. 2*C*). Moreover, the amount of MTMR4 associated with Smad1 reduced proportionally to the phosphorylation levels of Smad1, suggesting that MTMR4 might preferentially interact with the activated Smad1 (Fig. 2*C*).

To map the regions of contact between MTMR4 and Smad1, we further performed co-immunoprecipitation experiments using an array of truncational mutants of MTMR4 and Smad1 that transiently co-expressed in 293T cells (supplemental Fig. S2, *A* and *B* for the scheme of different mutants). The results showed that MTMR4 utilized its N-terminal region (MTMR4ΔFYVE, amino acids 1–591), which contains the conserved DUSP phosphatase domain, to contact Smad1 (Fig. 2*D*). Reciprocally, the MH2 domain, but not MH1 domain or linker region, of Smad1 was involved in contact with MTMR4 (Fig. 2*E*). These results suggested that the phosphorylated MH2 domain of Smad1 might serve as a physiological substrate of MTMR4.

MTMR4 Accelerates Smad1 Dephosphorylation—The aforementioned physical interaction between MTMR4 and the acti-

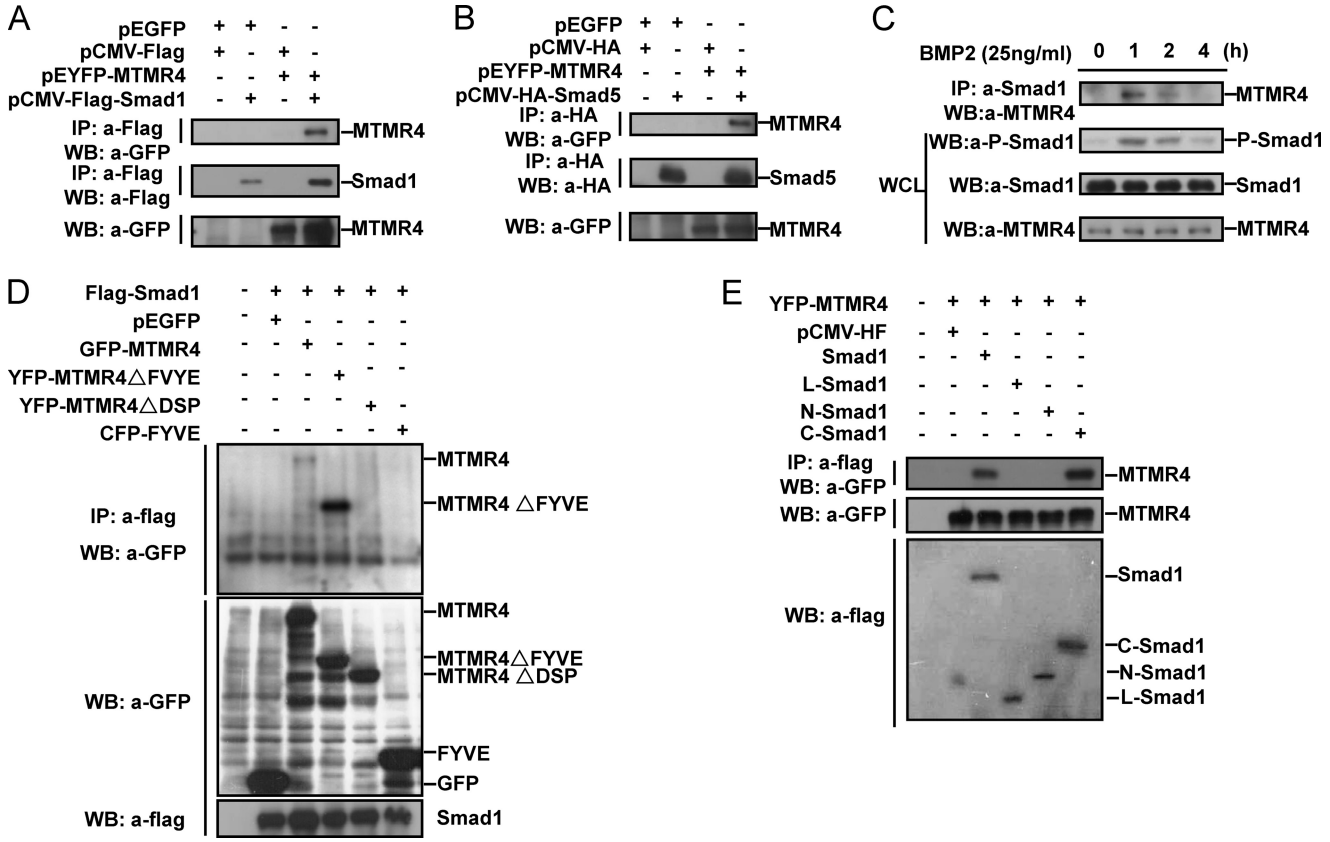

FIGURE 2. **MTMR4 interacted with R-Smad proteins of BMP signaling.** *A* and *B*, MTMR4 interacted with Smad1 and Smad5. HEK293T cells were co-transfected with pEYFP-MTMR4 and pCMV-Flag-Smad1 (*A*) or pCMV-HA-Smad5 (*B*) for 48h. Indicated parental vectors were transfected as negative control. YFP-MTMR4 was co-immunoprecipitated with Flag (*A*) or HA (*B*) antibody and visualized with GFP antibody. Protein expression levels were verified by Flag, HA, or GFP antibodies as indicated. *C*, endogenous MTMR4 and Smad1interacted depending upon BMP2 stimulation. HepG2 cells at 90% confluency were treated with BMP2 (25 ng/ml) for indicated time. Whole cell lysates (WCL, 300 μg total proteins) were prepared and immunoprecipitated with anti-Smad1 antibody. MTMR4 antibody was then used for immunoblotting of MTMR4. The WCL was immunoblotted with phosphor-Smad1, Smad1 or MTMR4 anitbody, respectively, for loading control. *D*, DUSP domain of MTMR4 was required for interaction with Smad1. HEK293T cells were co-transfected with pCMV-Flag-Smad1 and pEYFP-MTMR4 or its mutant derivatives as indicated for 48 h. Cell lysates were immunoprecipitated with M2 Flag antibody and visualized with GFP antibody (*top panel*). Expression levels of Flag-Smad1 (*middle*) and YFP-MTMR4 and various mutants (*bottom*) are also shown. *E*, MH2 domain of Smad1 was required for interaction with MTMR4. HEK293T cells were co-transfected with pEYFP-MTMR4 and pCMV-Flag-Smad1 or its mutant derivatives as indicated for 48 h. Cell lysates were immunoprecipitated with M2 Flag antibody and blotted with anti-GFP antibody (*top panel*). Expression levels of YFP-MTMR4 (middle) and Flag-Smad1 and various mutants (*two bottom panels*) are also shown.

vated Smad1 suggested that BMP signaling could be modulated by MTMR4 via targeting Smad1 phosphorylation. To test this hypothesis, HeLa cells were co-transfected with MTMR4 and Smad1, and the phosphorylation status of Smad1 was measured by a phosphor-SXS antibody specific to phosphorylated S463/S465 residues in the MH2 domain of Smad1, which are essential for Smad1 activation (6). In the absence of MTMR4, Smad1 was phosphorylated rapidly, and it reached a peak around 30 min post-BMP2 treatment. Then the phosphorylation levels of Smad1 gradually decayed up to 4 h. Overexpression of MTMR4 did not alter the onset of Smad1 phosphorylation, however, it accelerated Smad1 dephosphorylation just after the peak time (30 min) of BMP2 treatment (Fig. 3*A*). This enhanced dephosphorylation of Smad1 was not due to its proteasomal degradation, because the levels of Smad1 protein remained the same in the presence or absence of overexpressed MTMR4 (Fig. 3*A*). Moreover, siRNA knockdown of the endogenous *Mtmr4* gene in HeLa cells significantly attenuated Smad1 dephosphorylation when compared with the scrambled siRNA control (Fig. 3*B*). Consistent with the action of MTMR4 after phosphorylation of Smad1, overexpressed MTMR4 affected little BMP-target gene expression in the early time points and became more inhibitive in the later phase (supplemental Fig. S3). Therefore, these results suggested that MTMR4 control the velocity of Smad1 dephosphorylation, which could serve as a necessary mechanism to keep BMP activation signaling in homeostasis.

To further detect the function of MTMR4 in dephosphorylation of Smad1, we then transiently expressed the "phosphatase dead" mutant (MTMR4-C407S) or the mutant defective in early endosome localization (MTMR4ΔFYVE) of MTMR4 (supplemental Fig. S2, *A* and *C* for the scheme of different mutants). Measurement of phosphorylation levels of Smad1 upon BMP2 stimulation indicated that neither the onset nor the decay of Smad1 phosphorylation was affected by MTMR4-C407S or MTMR4ΔFYVE (Fig. 3*C*). Indeed, both BRE and GCCG reporter assays showed that overexpressed MTMR4-C407S or MTMR4ΔFYVE (Fig. 3, *D* and *E*) could no longer suppress BMP activation as compared with wild type MTMR4 (Fig. 1, *A* and *B*). These results therefore suggested that Smad1 dephosphorylation would require an endosome-localized phosphatase activity of MTMR4.

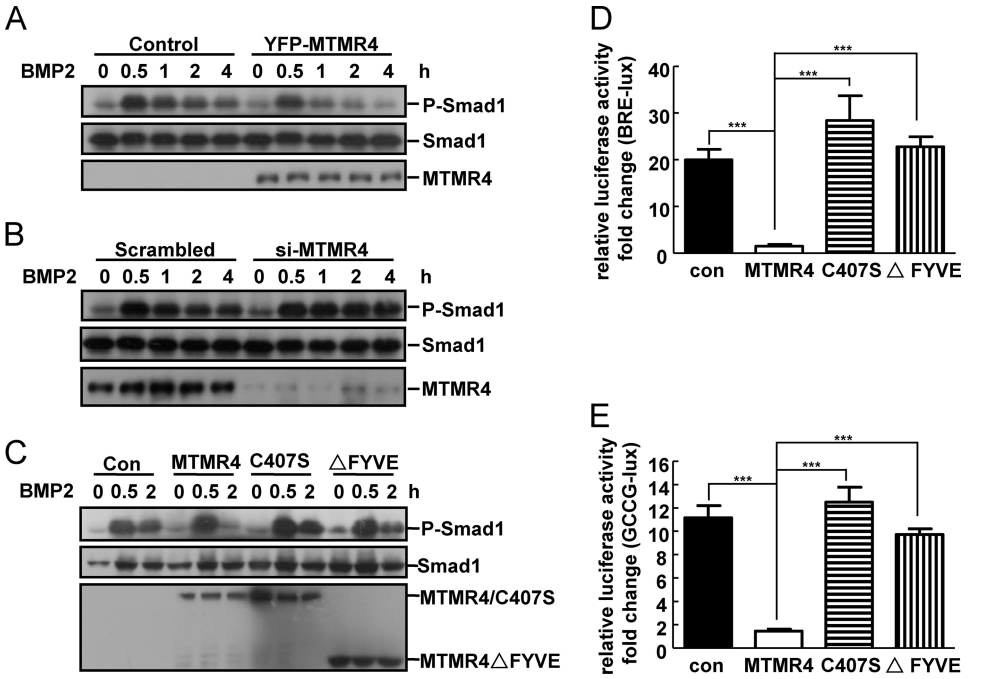

FIGURE 3. **MTMR4 accelerated the dephosophorylation of activated Smad1.** *A*, MTMR4 dephosphorylated the activated Smad1. HeLa cells were pretransfected with YFP-MTMR4 for 48 h before stimulated with BMP2 (100 ng/ml) for indicated time. WCL (30 μg total proteins) was separated by SDS-PAGE and endogenous Smad1 was immunoblotted with phosphor-Smad1 antibody (*top panel*), Smad1 antibody (*middle panel*), or anti-GFP antibody for expression of YFP-MTMR4 (*bottom panel*). *B*, down-regulation of MTMR4 resulted in sustained Smad1 phosphorylation. The experiment was performed as *A*, except that cells were transfected with MTMR4-specific siRNA or scrambled siRNA. *C*, endosomes localized DUSP activity of MTMR4 was responsible for Smad1 dephosphorylation. Hela cells were transiently expressed with pEYFP-MTMR4-C407S or pEYFP-MTMR4-ΔFYVE for 48 h. Immunoblotting was performed as in *A* to detect Smad1 phosphorylation. Protein loadings for endogenous Smad1 and exogenous YFP-MTMR4/C407S/ΔFYVE were performed as in *A*. *D-E*, endosomes localized DUSP activity of MTMR4 was required for Smad1 responsive gene activation. MTMR4-C407S and MTMR4ΔFYVE were transiently expressed in Hela cells as in *C*, and BRE-luc (*D*), and GCCG-luc (*E*) reporter activities were measured as in Fig. 1, *A* and *B*. All data represent the average of triplicate independent experiments (mean ± S.E.). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, Student's *t* test.

MTMR4 Impacts Vein Formation of Drosophila Wings—The regulatory specificity of BMP signaling can be conveniently scored by visual phenotypes of vein formation in *Drosophila* wings (13). Thus, to emphasize the biological significance of MTMR4 in the control of BMP signaling *in vivo*, we then assessed whether ectopically expressed MTMR4 might affect BMP signaling in the development of *Drosophila* wings. First we tissue-specifically knocked down BMP type I receptor *tkv* expression in wings by *UAS-tkvRNAi* driven by *vg-gal4* (32). Roughly 63% (n = 189) of *vg-gal4/+;UAS-tkvRNAi(III)/+* wings (Fig. 4*B*) showed branches at posterior crossvein (PCV) compared with normal wings of *vg-gal4/+* (Fig. 4*A*) *or UAS-tkvRNAi(III)/+* (supplemental Fig. S4*A*) controls (Fig. 4*J* for statistical analysis). The same branching phenotype was obtained in another independent strain, *UAS-tkvRNAi(II)/vg-gal4* (81% (n = 200), supplemental Fig. S4*B*, F for representative phenotype). These results would represent the first line of evidence that *tkv* was required for vein development in wings. We then generated three lines of transgenic flies, *UAS-MTMR4*, *UAS-MTMR4-C407S*, and *UAS-CG3632*, respectively. CG3632 is a *Drosophila* homologue of MTMR4 that shares all the conserved functional domains including the phosphatase domain (supplemental Fig. S2*C*) typified for the catalytic subgroup of MTM super family (23). Interestingly, wings containing overexpressed *mtmr4* (*UAS-MTMR4/vg-gal4*) or *cg3632* (*UAS-CG3632/vg-gal4*) also developed the similar branched PCV as to those in *tkv* knockdown lines, albeit at slightly lower rates (20.6% (n = 126) for *UAS-CG3632/vg-gal4* and 24.2% (n = 120) for *UAS-MTMR4/vg-gal4*, respectively) (Fig. 4, *C*, *D*, and *J*). In contrast, the wings developed normally in the *UAS-MTMR4-C407S/vg-gal4* line (Fig. 4*E*, J, n = 150). The phenotypic similarity in PCV development would suggest that MTMR4 might play a role in *tkv* signaling, and more importantly, by tempering *Dpp* activation signaling when MTMR4 was overexpressed.

To substantiate this notion, we went on to assess whether overexpression of *MTMR4* or *CG3632* could synergize wing patterning deficiency in the background of the reduced *tkv* expression. As expected, *vg-gal4*-driven simultaneous expression of *CG3632* and *tkvRNAi(III)* in wings (*UAS-CG3632/vg-gal4*; *UAS-tkvRNAi(III)/+*) caused 51.8% branched veins in PCV (n = 110, supplemental Fig. S5*A*). More profoundly, 14% showed intense deformity (classified as "enhanced" phenotype) with partial duplications and gaps (Fig. 4, *F*, *J*, and supplemental Fig. S5*B*). Similarly, *UAS-MTMR4/vg-gal4*; *UAS-tkvRNAi (III)/+* wings not only had the branched PCV (55.2%, n = 105, supplemental Fig. S5*C*) and enhanced phenotype (18.1%, n = 105, Fig. 4, *G* and *J* and supplemental Fig. S5*D*), but also displayed more severe deformation (4.8%, n = 105) with longer branches at PCV and L2 and partial duplications of L2 and L3 (Fig. 4, *H* and *J* and supplemental Fig. S5*E*) (classified as "severe" phenotype). To the contrary, only 25.8% (n = 129) *UAS-MTMR4-C407S/vg-gal4*; *UAS-tkvRNAi(III)/+* wings exhibited the branched PCV (Fig. 4, *I* and *J*), a much lower rate than that in *vg-gal4/+; UAS-tkvRNAi(III)/+* line (63%, Fig. 4, *B* and *J*). Provided that MTMR family proteins usually homo- or het-

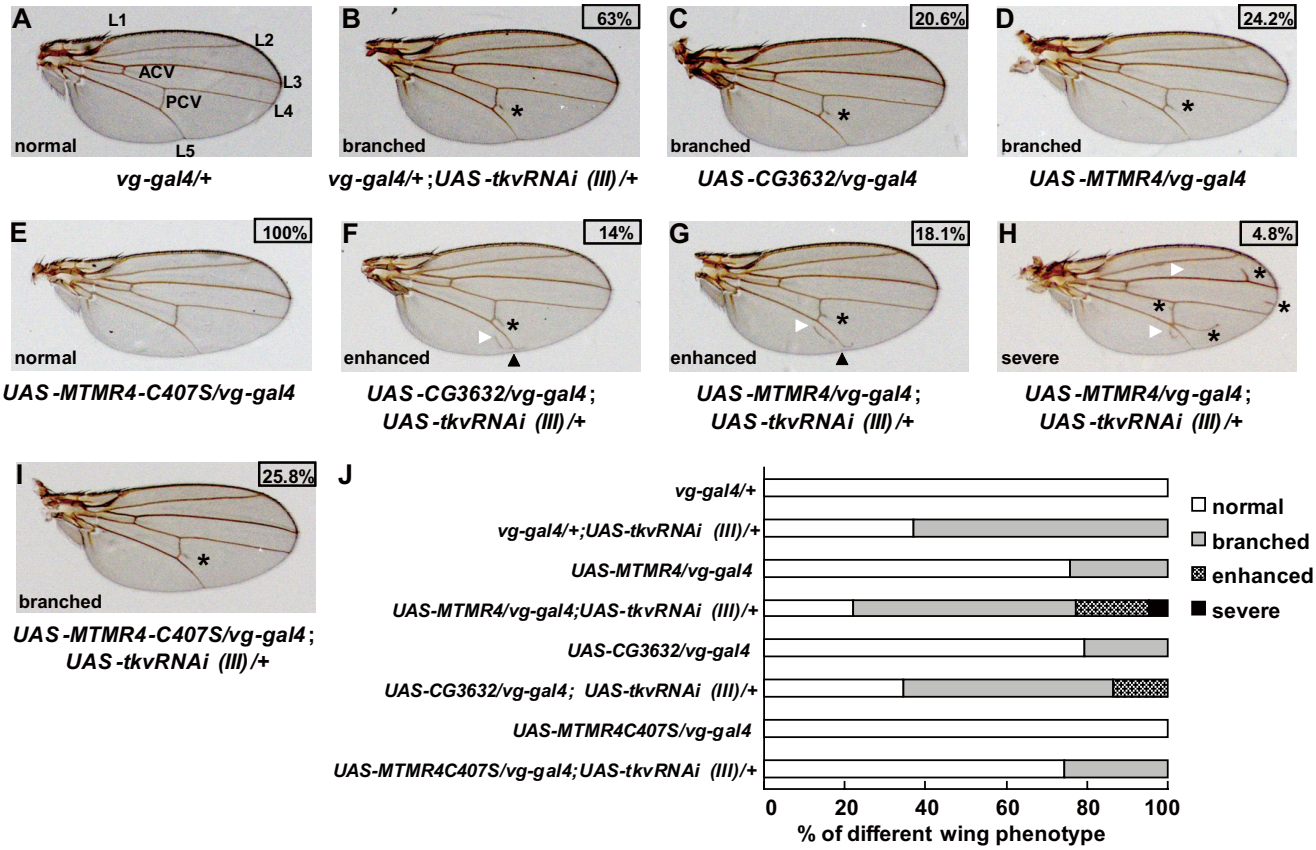

FIGURE 4. **MTMR4 and CG3632 potentiated Tkv knockdown in the vein formation.** *A*, representative genetic control wing (*vg-gal4/+*) with normal morphology. Longitudinal veins (L1, L2, L3, L4, and L5), anterior (*ACV*) and posterior crossveins (*PCV*) were indicated. *B–D*, MTMR4 and CG3632 transgenic flies had the similar vein abnormality to Tkv knockdown flies. Representative defective wings of *vg-gal4/+; UAS-tkvRNAi(III)/+* (n = 189), *UAS-CG3632/vg-gal4* (n = 126), and *UAS-MTMR4/vg-gal4* (n = 120) flies were shown, and the aberrant branches at PCV were marked by *asterisks*. Corresponding branching percentage derived from Fig. 3*J* are shown in *boxes*. *E*, loss of DUSP activity of MTMR4 failed to affect vein development. The representative wing of *UAS-MTMR4-C407S/vg-gal4* (n = 150) flies were shown, and the corresponding percentage of normal veins is shown in the *box*. *F–H*, MTMR4 and CG3632 enhanced vein abnormality of Tkv knockdown flies. Representative enhanced defective wings of *UAS-CG3632/vg-gal4; UAS-tkvRNAi(III)/+* (n = 110) and *UAS-MTMR4/vg-gal4; UAS-tkvRNAi(III)/+* (n = 105) flies were shown for branches at PCV (*asterisk*), partial duplicated vein of L5 (*white arrowhead*), and gaps at L5 vein junctions of the margin (*black arrowhead*). The representative wing of *UAS-MTMR4/vg-gal4; UAS-tkvRNAi(III)/+* (n = 105) flies with severe vein abnormality was shown in *H* for an increased number and places of branches (*asterisks*) and duplications (*white arrowheads*). Corresponding enhanced or severe percentage scores derived from Fig. 3*J* are shown in *boxes*. *I*, loss of DUSP activity of MTMR4 partially rescue Tkv knockdown in vein development. The representative defective wing of *UAS-MTMR4-C407S/vg-gal4; UAS-tkvRNAi(III)/+* flies (n = 129) exhibit fewer branched PCV (*asterisks*) and none enhanced or severe phenotypes. Corresponding branching percentage derived from Fig. 3*J* is shown in the *box*. *J*, statistics of various phenotypes of vein development of all flies in *A–I* is plotted in the bar graph.

erodimerize for function (33), a possible explanation could be that the null mutation of DUSP catalytic site (MTMR4-C407S) might inactivate the endogenous *CG3632*, which led to constitutive activation of R-Smads to partially rescue *tkv* deficiency. That overexpressed MTMR4 or CG3632 potentiated Tkv deficiency in wing development would suggest that these DUSPs could temper otherwise overactivated BMP signaling.

To address this issue, we first analyzed vein development with BMP signaling over-activated in wings by tissue-specific expression of Dpp or Gbb. To our disappointment, all of *vg-gal4/+*; *UAS-dpp/+* lines showed too severe phenotypes with round and blistered adult wings, probably due to the lack of adhesion of the dorsal and ventral wing compartments (supplemental Fig. S6*B*), to further analyze vein formation. Fortunately, *UAS-gbb/+; vg-gal4/+* strain was relatively milder with 29.7% (n = 155) wings showing moderate (ectopic vein branches near PCV and L5, Fig. 5, *B* and *J*) and 14.8% (n = 155) wings having severe defects (ectopic vein tissues nearby L5; Fig. 5, *C* and *J* and supplemental Fig. S6*D*). The ectopic vein tissue may be caused by abnormal cell proliferation induced by Gbb (34). We then co-expressed *UAS-gbb* with *UAS-CG3632 or UAS-MTMR4 in wings using vg-gal4* driver. The percentage of moderate defective wings was reduced from 29.7% (n = 155) in *UAS-gbb/+;vg-gal4/+* to 8.5% (n = 106) in *UAS-gbb/+*; *UAS-CG3632/vg-gal4*, and 4.5% (n = 110) in *UAS-gbb/+*; *UAS-MTMR4/vg-gal4*, respectively (Fig. 5, *B*, *D*, *F*, and *J*). Accordingly, the severe percentage was down from 14.8% (n = 155) to 8.5% (n = 106) and 5.5% (n = 110), respectively (Fig. 5, *C*, *E*, *G*, and *J*). In contrast, ectopically co-expression of MTMR4-C407S (*UAS-gbb/+*; *UAS-MTMR4- C407S/vg-gal4*) barely interfered with the Gbb overexpression phenotypes (27.3% (n = 139) with moderate defects (Fig. 5, *H* and *J*) and 14.4% severe defects (Fig. 5, *I* and *J*)). Together, these results suggested that MTMR4/CG3632 might be a functional component of BMP signaling and negatively controlled BMP/Tkv activation using its DUSP activity.

MTMR4 Is a Conserved BMP Attenuator—The genetic interaction between MTMR4 and Gbb/Tkv signaling pathway pro-

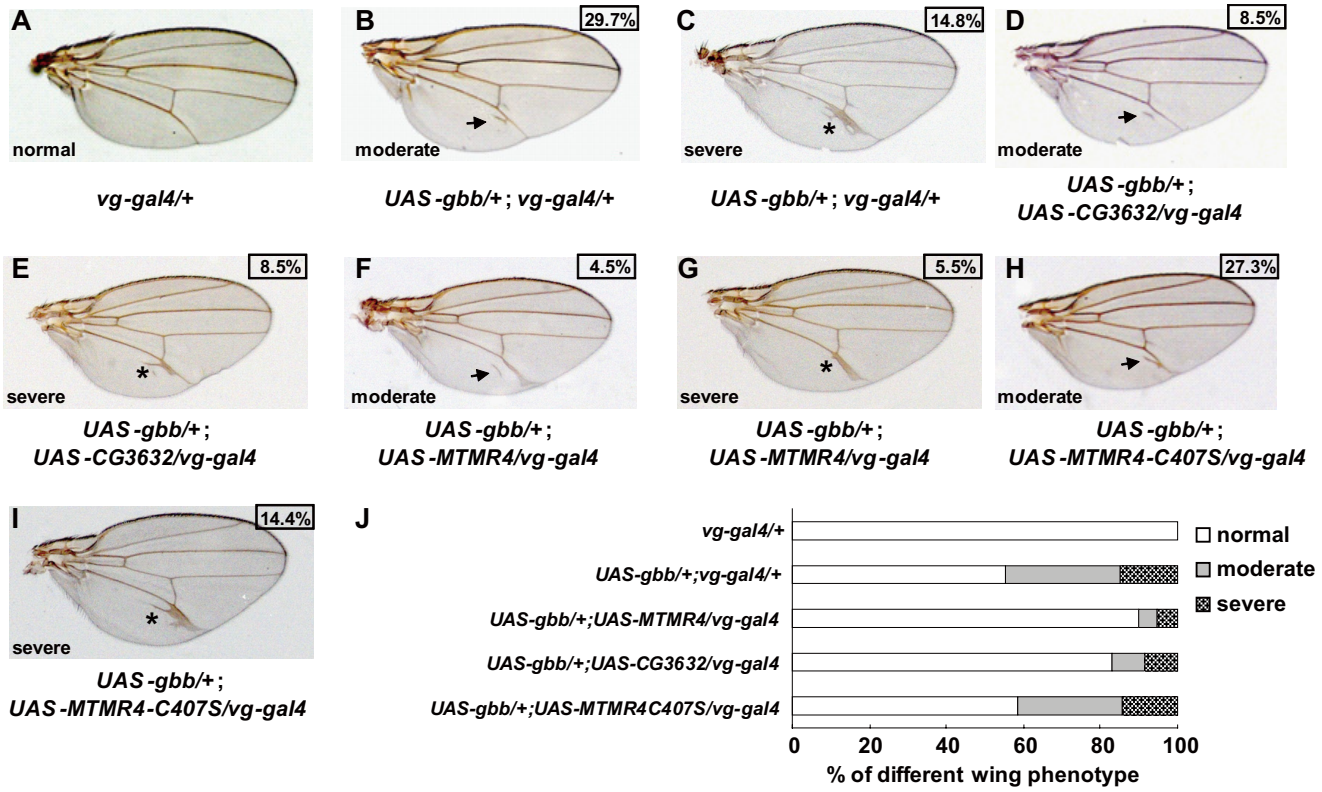

FIGURE 5. **MTMR4 and CG3632 ameliorate vein defectiveness induced by forced *Gbb* signaling.** *A*, representative genetic control wing (*vg-gal4/+*) with normal morphology. *B* and *C*, overexpression of Gbb caused deformity of vein development. Representative wings of *UAS-gbb/+; vg-gal4/+* flies ($n = 155$) showed moderate (*B*) and severe (*C*) vein defects. *D* and *E*, co-expression of CG3632 antagonized Gbb. Representative moderate (*D*) and severe (*E*) defective wings of *UAS-gbb/+; UAS-CG3632/vg-gal4* ($n = 106$) flies were shown. *F* and *G*, co-expression of MTMR4 antagonized Gbb. Representative moderate (*F*) and severe (*G*) defective wings of *UAS-gbb/+; UAS-MTMR4/vg-gal4* ($n = 110$) flies are shown. *H* and *I*, loss of DUSP activity of MTMR4 failed to antagonize Gbb. Representative wings of *UAS-gbb/+; UAS-MTMR4-C407S/vg-gal4* flies ($n = 139$) are shown for moderate (*H*) and severe (*I*) vein deformity. In the aforementioned figures, the *black arrow* indicates ectopic vein branches near PCV and L5, and the *asterisk* indicated ectopic vein tissue. Corresponding moderate or severe percentage scores derived from Fig. 4*J* are shown in *boxes*. *J*, statistics of various phenotypes of vein development of all flies in *A–I* is plotted in the bar graph.

moted us to test whether MTMR4 could interact with Mad, the only R-Smad in *Drosophila* BMP pathway. We transiently co-transfected HEK293T cells with YFP-MTMR4 and HA-Mad. Co-immunoprecipitation showed that there was indeed a intermolecular interaction between Mad and MTMR4 (Fig. 6*A*). We then analyzed whether the phosphorylation levels of the endogenous Mad was affected by MTMR4 in *Drosophila* S2 cells. Immunoblotting with a phosphor-Smad1 antibody showed that Mad phosphorylation peaked at 30 min after Dpp treatment of S2 cells, and gradually decreased till 2 h (Fig. 6*B*). Overexpression of MTMR4 carried by a *Drosophila* expression vector (pAGW-MTMR4) did not apparently affect the onset of Mad phosphorylation. However, it enhanced Mad dephosphorylation in response to Dpp stimulation (Fig. 6*B*). Co-transfection of the DUSP null mutant of MTMR4 (pAGW-C407S), as expected, failed to alter the activation kinetics of Mad (Fig. 6*B*). In agreement with the effect of MTMR4 on Mad dephosphorylation, fluorescent microscopy showed that nuclear translocation of RFP-Mad in response to Dpp stimulation (Fig. 6*C*, *top two panels*) was inhibited by co-expressed GFP-MTMR4 (*middle two panels*), but not by GFP-C407S (*bottom two panels*) in S2 cells. Finally, previous studies have established that gene expression of *Drosophila optomotor-blind* (*omb*) is induced by Dpp (35), while *brinker* (*brk*) is suppressed by Dpp (36). Overexpression of MTMR4, but not MTMR4-C407S, apparently inhibited *omb* (Fig. 6*D* and supplemental Fig. S7*B*) and promoted brk (Fig. 6*E* and supplemental Fig. S7*A*) gene expression, respectively, when S2 cells were treated with Dpp. Therefore, these results suggested that human MTMR4 possessed a conserved phosphatase activity on R-Smads including *Drosophila* Mad, and more importantly, was able to modulate BMP signaling homeostasis via targeting the activated Smad1 in mammalian cells or Mad in *Drosophila* cells.

DISCUSSION

Homeostasis of BMP signaling is critical for numerous biological processes. Among various modulators, phosphorylation-dephosphorylation of R-Smads provides undoubtedly efficient fine tuning. The observations in this work suggest that MTMR4/CG3632 might serve as a critical endosomal phosphatase that controls activation status of R-Smads/Mad in the BMP signaling pathway. MTMR4/CG3632 probably binds to the phosphorylated SXS-motif of R-Smads, and such physical proximity allows DUSP domain to dephosphorylate the activated R-Smads/Mad and consequently, attenuate transcriptional activation of target genes of BMP/Dpp. Intriguingly, just because MTMR4 is early endosome localized, the physical constrain would prohibit MTMR4 from affecting the phosphorylation of R-Smads/Mad in cytosol, or the subsequent

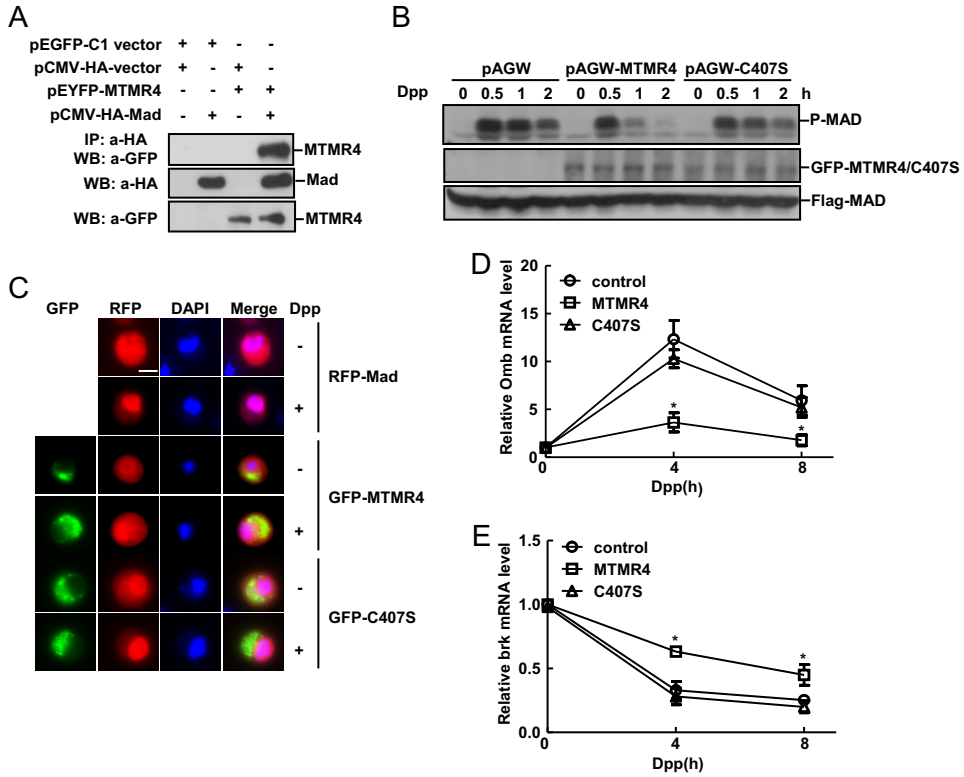

FIGURE 6. **MTMR4 interacts with *Drosophila* MAD and inhibits Dpp signaling.** *A*, MTMR4 interacted with Mad. HEK293T cells were transiently transfected with pCMV-HA-Mad and pEYFP-MTMR4, with each parental expression vectors as control. YFP-MTMR4 was co-immunoprecipitated with HA antibody and visualized by immunoblotting with GFP antibody (*top panel*). Protein expression levels were verified by anti-HA (*middle*) or -GFP (*bottom*) antibodies, respectively. *B,* MTMR4 dephosphorylated Mad in S2 cells. S2 cells were transiently co-transfected with pAC5.1-FLAG-Mad and pAGW parental vector, or pAGW-MTMR4, pAGW-MTMR4-C407S for 48 h. Dpp (40 ng/ml) was then added for indicated time. Phosphorylation of Mad was detected by immunoblotting with phosphor-Smad1 antibody (*top panels*). Expression levels of GFP-MTMR4, GFP-MTMR4-C407S, and Flag-Mad was verified with GFP (*middle panels*) and Flag (*bottom panels*) antibodies. *C*, DUSP activity of MTMR4 attenuated nuclear translocation of Mad. S2 cells were transiently co-expressed with RFP-Mad and GFP-MTMR4 or GFP-MTMR4- C407S for 48 h before Dpp (40 ng/ml) was added for an additional 2 h. Mad or MTMR4 was visualized under fluorescent microscopy using RFP and GFP channels, respectively. Scale bar: 5 μm. *D* and *E*, MTMR4 affected expression of Dpp target genes. S2 cells were transiently overexpressed with MTMR4 or MTMR4-C407S for 48 h. Dpp (40 ng/ml) was added for the indicated time before *Omb* (*D*) and *brk* (*E*) mRNA were measured by quantitative RT-PCR. All data represent the average of triplicate independent experiments (mean ± S.E.). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, Student's *t* test.

translocation and accumulation of phophorylated R-Smads/Mad in endosomes. The supportive evidence was shown by observation that Smad1 or Mad phosphorylation occurred normally (30 min after BMP2/Dpp stimulation) even in the presence of overexpressed MTMR4. Thus, overexpressing MTMR4 does not alter the onset of R-Smads phosphorylation. This indicates that MTMR4 inhibits BMP signaling might be not due to the redistribution of Endofin or related proteins (37) although some MTMs could result in PI3P diminution (33). Such an endosomal attenuator would be advantageous for cells to avoid R-Smads from being overactivated.

BMP activity gradients are critical for *Drosophila* wing patterning (38, 39). The finding that MTMR4/CG3632 modulated BMP signaling in vein formation strongly suggested that an endosomal DUSP activity would be critical in fine tuning of R-Smad activation status for proper BMP signaling. The precise spectrum of MTMR4/CG3632 target genes remains to be investigated. It is worthy of note that a great proportion of "severe" vein deformity appears in *UAS-MTMR4/vg-gal4;UAS-tkvRNAi(III)/+* wings. This is likely that the increased amount of dephosphorylated Mad could activate the canonical Wingless signaling, because Wingless and BMP signal pathways are by large partitioned by the phosphorylation status of Mad (40). Therefore, besides modulating the homeostasis of BMP/Dpp signaling, MTMR4 activity *per se* has to be tightly controlled to avoid unnecessary crosstalk with other signaling pathways. This speculation has yet to be experimentally confirmed.

The involvement of multiple protein phosphatases at multiple layers of R-Smads activation axis has presented a complex network of TGFβ/BMP signaling. It has become more complicated that some phosphatases are dual functional in both TGFβ and BMP signalings while some are unique to certain pathway. For example, PDP is a Mad-specific phosphatase in *Drosophila* BMP signaling (21), but with no effect on Smad2 dephosphorylation in TGFβ signaling pathway (21, 41). In contrast, PPM1A can dephosphorylate all R-Smads members in the nucleus (19, 41). MTMR4 functions on both Smad2/3 and Mad, suggesting a PPM1A-like dual player on both TGFβ and BMP pathways. Of course, MTMR4 differs from PPM1A for its subcellular localization, thus provides different checkpoint of BMP activation. Moreover, the new role of MTMR4 on vein development of *Drosophila* revealed by this study would provide insight into better understanding of hypoxic response that MTMR4

might be involved, such as the malignancy of papillary thyroid cancer (42) and hypertensive fibrogenesis (43).

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