Membrane targeting of inhibitory Smads through palmitoylation controls TGF-β/BMP signaling

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TGF-β/BMP (bone morphogenetic protein) signaling pathways play conserved roles in controlling embryonic development, tissue homeostasis, and stem cell regulation. Inhibitory Smads (I-Smads) have been shown to negatively regulate TGF-β/BMP signaling by primarily targeting the type I receptors for ubiquitination and turnover. However, little is known about how I-Smads access the membrane to execute their functions. Here we show that Dad, the *Drosophila* I-Smad, associates with the cellular membrane via palmitoylation, thereby targeting the BMP type I receptor for ubiquitination. By performing systematic biochemistry assays, we characterized the specific cysteine (Cys556) essential for Dad palmitoylation and membrane association. Moreover, we demonstrate that dHIP14, a *Drosophila* palmitoyl acyl-transferase, catalyzes Dad palmitoylation, thereby inhibiting efficient BMP signaling. Thus, our findings uncover a modification of the inhibitory Smads that controls TGF-β/BMP signaling activity.

palmitoylation | *Drosophila* germ-line stem cell | inhibitory Smads | Dad

Signaling of TGF-β/BMP (bone morphogenetic protein) is triggered by ligand–receptor binding that induces the complex assembly of activated heterotetrameric receptors, including type I and type II receptors with intrinsic kinase activity. The phosphorylated type I receptor subsequently transmits the signals to the receptor-regulated Smads (R-Smads) and the common-mediator Smad (Co-Smad) to regulate a variety of target-gene expressions (1, 2). Genetic studies have suggested that TGF-β/BMP signaling pathways play evolutionarily conserved roles in controlling embryonic development, tissue homeostasis, and stem cell regulation (3). Misregulation of TGF-β family pathways leads to developmental defects and has been linked to many human diseases, including cancers (3). It has been proposed that TGF-β/BMP signaling activity is finely balanced to trigger distinct target-gene expression via multiple mechanisms, such as regulations of their receptors and Smads (4). The inhibitory Smads (I-Smads) play a negative role in antagonizing the TGF-β/BMP signaling activity in a feedback-regulatory manner (5, 6). Previous studies have suggested several mechanisms by which the I-Smads (Smad6/7 in mammals) antagonize TGF-β/BMP signaling at the Smad level. For example, Smad7 inhibits R-Smad–Smad4 complex formation by competitively interacting with R-Smads (7). Moreover, Smad7 has been suggested to directly bind DNA, and thus impeding the binding of the R-Smad–Smad4 complex with their target DNA (8). In addition to their roles in affecting R-Smad proteins, I-Smads have been shown to act in concert with Smurfs and other ubiquitin E3 ligases to antagonize TGF-β/BMP signaling by mediating degradation of the type I receptors via the ubiquitin proteasome system (6, 9).

Protein *S*-palmitoylation is a type of posttranslational modification by addition of a 16-carbon fatty acid palmitate to substrate proteins via a covalent thioester bond, and this modification is dynamically regulated by palmitoyl acyl-transferases (PATs) and thioesterases (10, 11). Palmitoylation enhances the affinity of substrate proteins tethering to membranes, thus affecting their functions at membranes (12). Plasma membrane localization of receptors is important for ligand–receptor binding and subsequent downstream signaling transduction. Additionally, internalization of receptors from the cell surface into intracellular membrane compartments (e.g., endosomes) also contributes to the signal transduction (13). Receptor internalization occurs via two major routes: namely, clathrin-mediated and clathrin-independent endocytic pathways. Of note, the clathrin-independent endocytic pathway is lipid raft-dependent, which is regulated by various cellular components including caveolin-1 (Cav1), cholesterol, dynamin, and regulators of the actin cytoskeleton (14, 15). It has been reported that the caveolae/raft-dependent vesicles are associated with TGF-β receptor and function in its degradation via binding the Smad7-containing complex (16, 17). However, the issue of how the I-Smads access the membrane to regulate the turnover of TGF-β receptors remains elusive.

Here we employed *Drosophila* as a model to investigate how Dad, the *Drosophila* homolog of I-Smad, is regulated. We provide evidence that Dad is modified through palmitoylation to target the BMP type I receptor at membranes for ubiquitination in germ-line stem cell (GSCs). Moreover, we show that dHIP14, a PAT, in *Drosophila* promotes Dad palmitoylation, thereby

Significance

Inhibitory Smads (I-Smads) play important roles to negatively regulate TGF-β/BMP (bone morphogenetic protein) signaling, thus controlling numerous cellular and developmental processes. Recent studies have suggested that Smad7, a member of I-Smads, is overexpressed in numerous cancer types and its abundance is positively correlated to the malignancy. However, the molecular mechanism underlying action of I-Smads in cells remains poorly understood. Here we show that the *Drosophila* I-Smad, Dad, accesses the membrane via palmitoylation to target the BMP type I receptor for ubiquitination. Importantly, we show that the palmitoyltransferase dHIP14 catalyzes Dad palmitoylation and antagonizes BMP/Dpp signaling. Our findings uncover a mechanism by which I-Smad controls TGF-β/BMP signaling.

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inhibiting the efficient BMP signaling. Thus, our findings uncover a mechanism by which I-Smad proteins access membrane via palmitoylation to control TGF-β/BMP signaling.

Results

Dad Associates with Cellular Membranes in a Palmitoylation-Dependent Manner. Dad, the sole I-Smad homolog in *Drosophila*, plays a role in negatively controlling TGF-β family BMP/Dpp signaling (18). To better understand the mechanism of how Dad is regulated, we searched for Dad-associating factors by performing immunoprecipitation (IP) experiments followed by MS analysis. In this assay, we used overexpressed Myc-Dad as bait, and identified a number of potential Dad-associated proteins (Table S1). Among these candidates, we found that one SNARE binding protein, Rop, was present in the Dad complex. To confirm this observation, we carried out further co-IP experiments in transfected S2 cells, and found that Dad and Rop could be reciprocally immunoprecipitated in transfected cells (Fig. 1 *A* and *B*). Since SNARE binding proteins are engaged in vesicle transport processes (19, 20), we reasoned that Dad could be associated with cellular membranes through an uncharacterized mechanism. We therefore homogenized S2 cells that expressed Myc-Dad, and then performed fractionation assays using differential centrifugation to separate the membranes including the plasma and internal membranes from the cytosolic soluble compartments (Fig. S1*A*). As shown in Fig. 1*C*, similar to Rop, a significant portion of Dad was present in the membrane fractions. Because no apparent transmembrane domain exists in the Dad protein, it was of interest to determine how Dad is present in the membrane fraction. It has been suggested previously that Munc18c, the mammalian homolog of Rop, could be palmitoylated (21). To test whether Dad is palmitoylated, we treated the S2 cells expressing Dad with or without 2-bromo-palmitate (2-Brp), an effective inhibitor of palmitoylation (22). As shown in Fig. 1 *D* and *E*, treatment of 2-Brp significantly reduced levels of Dad in the membrane fraction, compared with the control, indicating that Dad associates with the membrane in a palmitoylation-dependent manner. To gain more supportive evidence, we next performed the thiopropyl captivation of *S*-palmitoylated protein assays, as described previously (23), to test whether Dad is palmitoylated in cells. As shown in Fig. 1*F*, the Dad proteins were reliably observed to be associated with thiopropyl beads when purified Dad proteins were treated with hydroxylamine, but not the control Tris·HCl (detailed methods shown in *SI Materials and Methods*), suggesting Dad could be present in a palmitoylated form. In support of these findings, we observed that the association of Dad with thiopropyl beads was significantly reduced even under hydroxylamine treatment, when the transfected S2 cells were treated with the palmitoylation inhibitor 2-Brp (Fig. 1*F*). Of note, in our control experiments, we found that Rop was also palmitoylated in S2 cells (Fig. 1*G*). Taken together, our findings support a notion that the Dad is palmitoylated to associate with membrane.

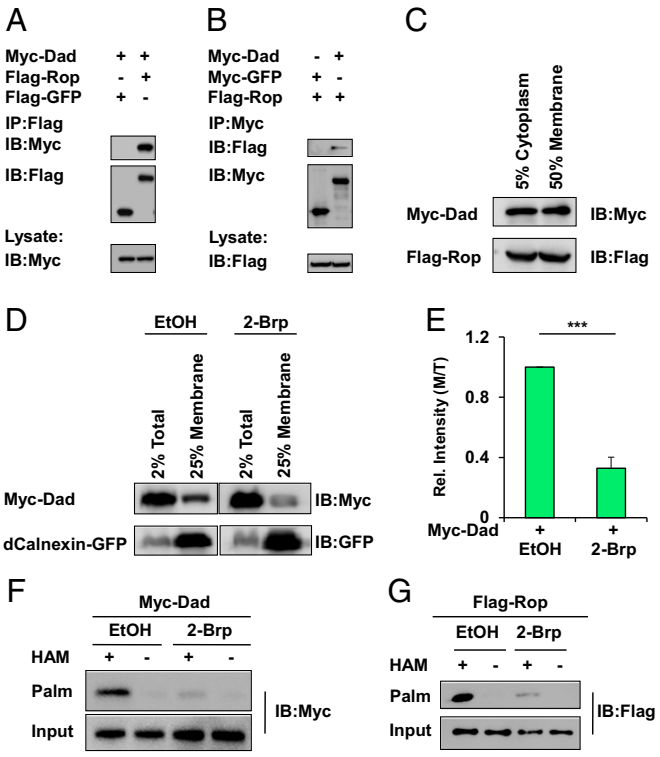

Fig. 1. Dad associates with membrane proteins in a palmitoylation-dependent manner. (*A* and *B*) S2 cells were transfected with the indicated combinations of plasmids. At 48-h posttransfection, cell lysates were immunoprecipitated with anti-Flag (*A*) or anti-Myc (*B*) beads. Western blots were performed to analyze the presence of Myc or Flag-tagged proteins. (*C*) S2 cells were transfected with the indicated plasmids. At 48-h posttransfection, cell lysates were fractionated as described in Fig. S1. Western blots were performed to analyze the presence of Myc or Flag-tagged proteins. (*D* and *E*) S2 cells were transfected with plamids expressing Myc-Dad and dCalnexin-GFP. dCalnexin-GFP was used as an internal reference of membrane fraction. At 42-h posttransfection, the cells were treated with ethyl alcohol (EtOH, as a control) or 2-Brp (25 μM) for 6 h, and then lysed for fractionation. Western blots were performed to analyze the presence of Myc or GFP-tagged proteins (*D*). Densitometric analyses to quantify levels of Dad in membrane in *D* are shown in *E*, Error bars represent SD ($n = 3$). (*F* and *G*) S2 cells were transfected with the Myc-Dad or Flag-Rop expression construct. At 42-h posttransfection, the cells were further treated with ethyl alcohol (as a control) or 2-Brp (25 μM) for 6 h; cell lysates were immunoprecipitated with anti-Myc or anti-Flag beads, and then subjected to the *S*-palmitoylation assay to measure palmitoylation levels of Dad (*F*) or Rop (*G*). HAM and Palm are abbreviations for hydroxylamine and palmitoylation, respectively. All of the biochemical experiments were performed at least three times. In *E*, the Student's *t* test was used to analyze statistical significance. ***$P < 0.001$ vs. the control groups. IB, immunoblotting; IP, immunoprecipitated; M, membrane; Rel., relative; T, total.

Dad Forms a Complex with Tkv to Mediate Its Ubiquitination. Given a significant portion of Dad present in the membrane fraction, we reasoned that membrane-associated Dad regulates Dpp/BMP signaling by primarily targeting the BMP type I receptor, Tkv in *Drosophila*. Consistent with previous findings (24), we observed that Dad formed a complex with Tkv or Sax (Fig. 2*A* and Fig. S2*A*). It has been previously shown that ubiquitin-mediated turnover of Tkv by Smurf, a ubiquitin E3 ligase, is an important mechanism for homeostasis of BMP/Dpp signaling (25). Therefore, we performed ubiquitination assays, according to the method described previously (26), to test whether Dad is engaged in Tkv ubiquitination in S2 cells. As shown in Fig. 2 *B*–*D*, overexpression of Dad significantly increased Tkv ubiquitination, and vice versa. Consistently, our pulse-chase analysis revealed that overexpression of Dad reduced the half-life of Tkv (Fig. 2 *E* and *F*). Of note, overexpression of Dad did not significantly affect Sax ubiquitination (Fig. S2*B*). Thus, our findings suggest that Dad primarily contributes to the regulation of Tkv ubiquitination and degradation.

We next tested whether the Dad-mediated Tkv ubiquitination pathway had an in vivo function by using a *Drosophila* GSC system. In *Drosophila* ovaries, two to three GSCs are in direct contact with niche cap cells at the tip of the germarium (Fig. 2 *G* and *H*). Previous studies have demonstrated that niche-dependent BMP/TGF-β (Dpp) signaling plays a critical role to repress *bam* transcription in GSCs, allowing for a proper asymmetric division of GSCs (27, 28). Overexpression of constitutive form of Tkv, Tkv(ca), by the *nanos* promoter led to tumorous

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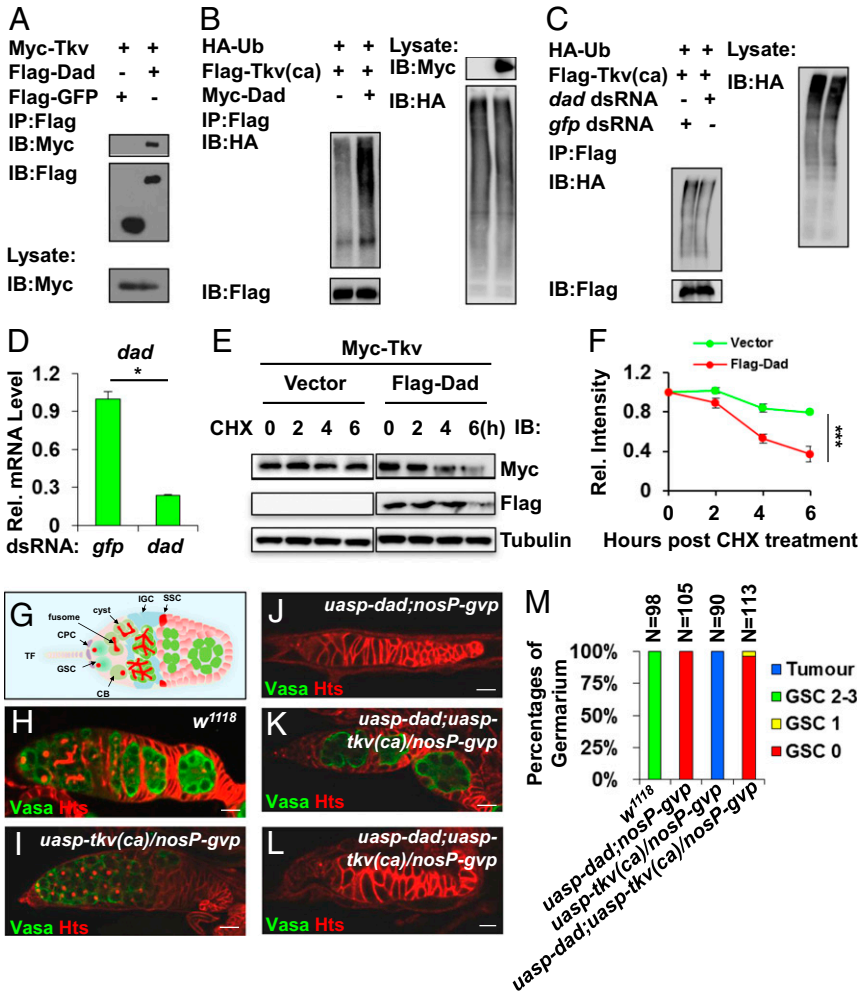

Fig. 2. Dad forms a complex with Tkv to mediate its ubiquitination. (*A*) S2 cells were transfected with plasmids as indicated. At 48-h posttransfection, cell lysates were prepared and immunoprecipitated with anti-Flag beads, followed by Western blot analyses. (*B*) S2 cells were transfected with plasmids as indicated. At 48-h posttransfection, cells were treated with MG132 (50 μM) for 6 h, then lysed and immunoprecipitated with anti-Flag beads, followed by Western blot assays to detect the ubiquitination status of Tkv(ca). (*C* and *D*) S2 cells were treated with dsRNAs targeting *gfp* (as a control) or *dad* for 24 h, followed by transfections with the indicated plasmids. At 48-h posttransfection, cells were treated with MG132 (50 μM) for 6 h, then lysed for IP assays to detect the ubiquitination status of Tkv(ca) (*C*) or cells were harvested for quantitative real-time PCR (qRT-PCR) assays to examine relative mRNA levels of *dad* (*D*), Error bars represent SD ($n = 3$). (*E* and *F*) S2 cells were transfected with plasmids as indicated. At 48-h posttransfection, cells were treated with CHX (50 ng mL^{-1}) for various times, followed by immunoblotting to examine levels of Tkv protein. Densitometric analyses to quantify Tkv expression in *E* are shown in *F*. Error bars represent SD ($n = 3$). (*G*) A schematic diagram of the germarium with different cell types and organelles indicated as follows: cystoblast cells (CB), cap cells (CPC), germ-line stem cells (GSC), inner germarium sheath cells (IGC), somatic stem cells (SSC), terminal filament (TF), and cyst (differentiated germ cells with extended or branched fusomes). Among these, TFs, CPCs, and IGCs produce Dpp ligands. (*H–L*) Ovaries collected from indicated genotypes were stained with anti-Vasa (green) and anti-Hts (red) antibodies. Anti-Hts was used to outline the germarium and the morphology of the fusome, and the staining of anti-Vasa was used to visualize all germ cells in the germarium and egg chambers. (Scale bars, 10 μm.) (*M*) Quantification of the germarium phenotypes of ovaries in *H–L*. All of the biochemical experiments were performed at least three times. In *D*, the Student's *t* test was used to analyze statistical significance. In *F*, the log-rank test was used to analyze statistical significance. $*P < 0.05$; $***P < 0.001$ vs. the control groups. IB, immunoblotting; IP, immunoprecipitated; Rel., relative.

germaria that are filled with GSC-like cells (Fig. 2 *I* and *M*), whereas overexpression of Dad in germ cells caused a germ-cell–loss phenotype (29, 30) (Fig. 2 *J* and *M*). To explore the genetic relationship between Dad and Tkv, we generated P {*uasp-dad*}; P {*uasp-tkv(ca)*}/P {*nosP-gal4:vp16*} flies to cooverexpress Tkv(ca) and Dad specifically in germ cells. As shown in Fig. 2 *K–M*, cooverexpression of Tkv(ca) and Dad again caused a germ-cell–loss phenotype. Collectively, our findings suggest that Dad primarily targets the type I receptor Tkv for its ubiqitination and degradation.

The Cys556 Site Is Essential for the Palmitoylation of Dad. Since the cysteine residues in the substrate proteins are the target sites modified by the palmitate donor, we investigated which cysteine sites are important for Dad palmitoylation. We performed a sequence alignment analysis by comparing the amino acid sequences of *Drosophila* Dad with Smad6/7 in vertebrates, and identified several conserved cysteine residues in Dad (Fig. S3). To assess whether these residues are required for Dad palmitoylation, we generated a series of mutant forms of Dad in which cysteine (C) sites were individually mutated to alanine (A), and then performed palmitoylation assays. As shown in Fig. 3*A*, while levels of palmitoylation signal from Dad mutants—such as Dad^{C180A}, Dad^{C306A}, Dad^{C401A}, and Dad^{C558A}—were comparable with that from the wild-type Dad, the Dad^{C556A} mutant exhibited much less levels of palmitoylation, suggesting that the 556 cysteine residue (Cys556) is important for Dad palmitoylation.

To evaluate the functional importance of the Cys556 in Dad, we employed a cell-based luciferase assay by using an AE-luciferase reporter (*dad-AE*-Luc), in which the promoter contained a *dad* activated element that responds to BMP/Dpp signaling (31). As shown in Fig. 3*B*, treatment of recombinant human BMP4 in S2 cells could significantly induce activation of the *dad-AE*-Luc reporter, whereas overexpression of the wild-type Dad inhibited the BMP4-induced luciferase activity. Using this reporter, we next expressed Dad mutants in S2 cells to investigate whether the Cys556 site is important for the function of Dad to antagonize the BMP signaling. As shown in Fig. 3*B*, like wild-type Dad, the expression of Dad mutants—including Dad^{C180A}, Dad^{C306A}, Dad^{C401A}, and Dad^{C558A}—significantly inhibited BMP4-induced luciferase activity. However, mutation of Cys556 in Dad significantly impaired the Dad function in antagonizing the BMP signaling. Collectively, our results suggest that Cys556 is the critical residue for Dad palmitoylation to antagonize BMP/Dpp signaling.

The Cys556 Residue Is Critical for Membrane-Function of Dad. We next tested whether the Cys556 palmitoylation functionally contributes to the membrane association of Dad, and performed the fractionation assays followed by Western blot assays. As shown in Fig. 3*C*, levels of DadC556A were present much lower

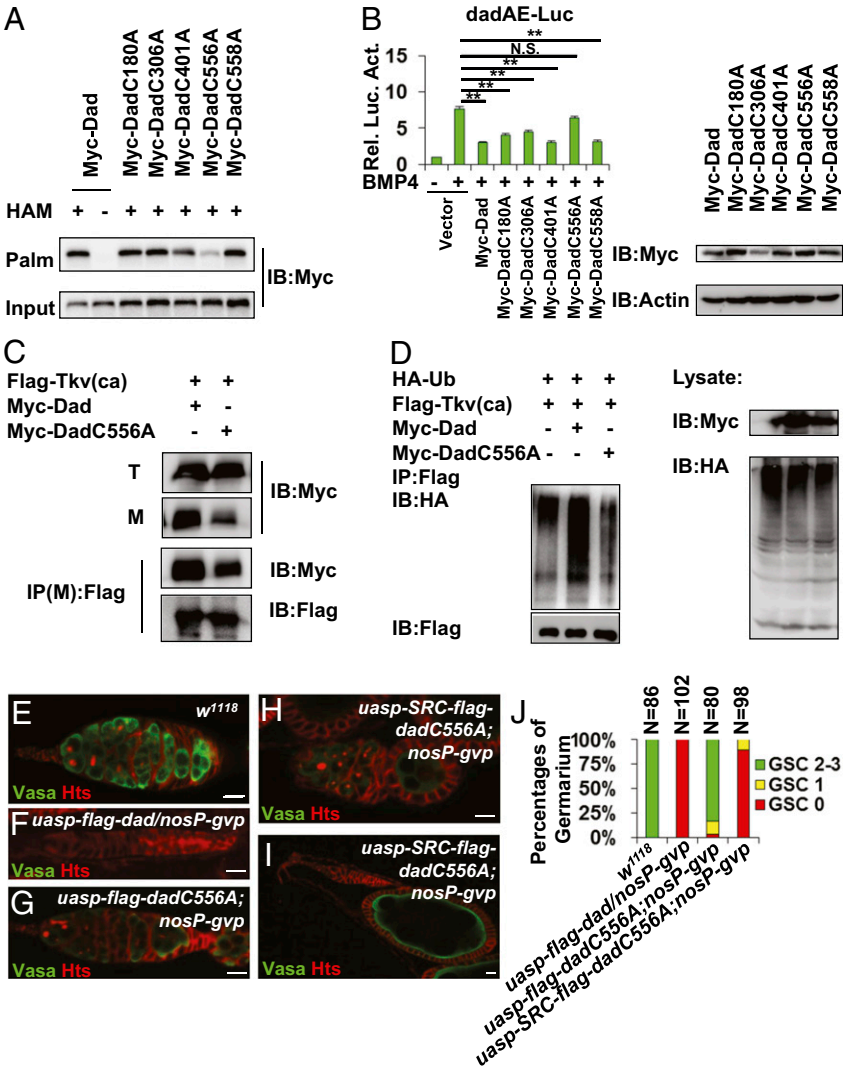

Fig. 3. The Cys556 site is important for Dad palmitoylation and antagonizes Dpp signaling. (*A*) S2 cells were transfected with expression plasmids encoding wild-type Myc-Dad or its mutants as indicated. Forty-eight hours after transfection, cell lysates were prepared for *S*-palmitoylation assay. Western blots were performed to detect the presence of Myc-tagged proteins. (*B*) S2 cells were cotransfected with Myc-Dad or its mutations, *dad-AE-luciferase* and *actinP-lacZ* (used as an internal control). At 36-h posttransfection, cells were treated with BMP4 (10 ng mL^{-1}) for 12 h, the cells were lysed for luciferase assays and immunoblotting assays. The Student's *t* test was used to analyze statistical significance. $^{**}P < 0.01$ vs. the control groups; N.S., not significant. (*C*) The S2 cells were cotransfected with Flag-Tkv(ca) and Myc-Dad or Myc-DadC556A mutant. At 48-h posttransfection, cell lysates were fractionated and the membrane fractions were immunoprecipitated with anti-Flag beads. Western blots were performed to analyze the presence of Flag- or Myc-tagged proteins. (*D*) S2 cells were transfected with the indicated plasmids. Forty-eight hours after transfection, cells were treated with MG132 (50 μM) for 6 h, then cell lysates were immunoprecipitated with anti-Flag beads, and subjected to immunoblotting analysis to detect the ubiquitination status of Tkv(ca). (*E–I*) Ovaries collected from indicated genotypes were stained with anti-Vasa (green) and anti-Hts (red) antibodies. (Scale bars, 10 μm.) (*J*) Quantification of the germarium phenotypes of ovaries in *E–I*. All of the biochemical experiments were performed at least three times. In *B*, the two-tailed Student's *t* test was used to analyze statistical significance. $^{**}P < 0.01$ vs. the control groups. IB, immunoblotting; IP, immunoprecipitated; M, membrane; Rel. Luc. Act., relative luciferase activity; T, total.

in the membrane fraction compared with the wild-type Dad, suggesting that the Cys556 site is important for the membrane-association of Dad. We then asked whether the Cys556 mutation affects the association of Dad with Tkv(ca) in the membrane fraction. We used the membrane fraction to perform Co-IP experiments, and found that mutation of Cys556 to alanine greatly reduced the Dad-Tkv(ca) association in the membrane fraction (Fig. 3*C*). Considering that Dad regulates Dpp primarily through ubiquitinating Tkv(ca), we then performed ubiquitination assays in S2 cells. As shown in Fig. 3*D*, overexpression of Dad^{C556A} resulted in a reduced ability to up-regulate the ubiquitination of Tkv(ca) compared with overexpression of the wild-type Dad. Taken together, these findings support a notion that Cys556 is important for Dad to regulate the type I receptor Tkv ubiquitination at membranes.

To determine the biological importance of the Cys556 site in Dad, we generated a series of transgenic fly strains, including P {*uasp-flag-dad*}, P {*uasp-flag-dad*^{C556A}}, in which the wild-type Flag-Dad or mutant Flag-Dad^{C556A} was controlled by the *uasp* promoter (32). We employed the germ-cell–specific driver, P{*nosP-gal4:vp16*}, to express Dad and its mutant form in germ cells. As shown in Fig. 3 *E–G* and *J*, overexpression of Flag-Dad in germ cells led to a germ cell-loss phenotype, whereas overexpression of the palmitoylation-deficient mutant, Flag-Dad^{C556A}, allowed 83.3% of germaria and had normal germ cell development ($n = 102$) (Fig. 3 *G* and *J*). To rule out the possibility that the differential phenotypes were attributed to the different expression levels of Flag-Dad or mutant Flag-Dad^{C556A}, we performed immunostaining experiments to measure relative levels of Flag-Dad and Flag-Dad^{C556A} proteins in primordial germ cells (PGCs) at the late third-instar larval stage, and found no significant difference in expression levels of two proteins. Thus, our findings suggest that mutation of the Cys556 site impaired the in vivo function of Dad. Given that palmitoylation could promote the affinity of soluble proteins with cellular membranes, we asked whether artificial membrane-targeting of palmitoylation-deficient Dad could restore the in vivo function of Dad. We generated another transgene, P {*uasp-SRC-flag-dad*^{C556A}}, in which the Flag-tagged Dad^{C556A} protein was fused with an SRC domain at its N terminus. The SRC was a signal peptide that could localize targeted proteins attached to both the plasma and cellular membrane locations. We expressed the SRC–Flag-Dad^{C556A} in germ cells, and found that expression of SRC–Flag-Dad^{C556A} caused a germ-cell–loss phenotype in adult females (Fig. 3 *H–J*).

To better understand how Flag-Dad and its membrane-tagged mutant affect germ-line development, we examined the behavior of female PGCs at the late third-instar larval stage, when Flag-tagged Dad or SRC–Flag-Dad^{C556A} was overexpressed. As shown in Fig. S4 *A–D*, most of PGCs in w^{1118} control female

gonads carried a single spherical fusome, and a few dividing PGC pairs contained elongated fusomes. In contrast, a considerable portion of PGCs had differentiated into germ-cell clusters, which were marked by branched fusomes, when Flag-Dad was overexpressed. Moreover, we found that SRC–Flag-Dad^{C556A} overexpression promoted most of PGCs differentiation, as indicated by the presence of many differentiated germ-cell clusters marked with branched fusomes in the tested gonads (Fig. S4*D*). In addition, Flag-Dad and SRC–Flag-Dad^{C556A} exhibited similar expression levels in PGCs (Fig. S4 *E–G″*). Thus, our findings suggest that the SRC–Flag-Dad^{C556A} had much stronger activities than the wild-type Dad in inhibiting Dpp signaling, likely because of its membrane-localization. In support of this notion, we found that overexpression of SRC–Flag-Dad^{C556A} led to more severe GSC-loss phenotype at the pupal stage, compared with wild-type Dad (Fig. S4 *H–L*). Taken together, our results further emphasize that membrane localization is critical for Dad to antagonize Dpp signaling.

dHIP14 Is a PAT and Palmitoylates Dad. We next sought to search for enzymes catalyzing Dad palmitoylation. A previous bioinformatics analysis identified 22 PATs in the *Drosophila* genome (33). As indicated in the FlyAtlas, genes encoding PATs, such as *CG1407*, *CG5196*, *CG5880*, *CG6017*, and *CG8314*, are highly expressed in adult ovaries. To identify the specific PAT for Dad palmitoylation, we coexpressed each of these PATs with Dad in S2 cells. As shown in a palmitoylation assay, overexpression of CG6017, which encodes a homolog of the human HIP14 (33), significantly increased Dad palmitoylation (Fig. 4 *A* and *B*). We then knocked down *Drosophila hip14* (*dhip14*) in S2 cells with Dad overexpression, and found that knockdown of *dhip14* evidently decreased levels of Dad palmitoylation compared with the control (Fig. 4*C*). In addition, immunoprecipitation assays revealed that dHIP14 forms a complex with Dad in S2 cells (Fig. 4*D*). Collectively, these findings together suggest that dHIP14 plays a role in regulating the palmitoylation of Dad.

We next tested whether dHIP14 contributes to the Dad-mediated regulation of BMP signaling using the cell-based *dad-AE*-Luc assay, and found that coexpression of dHIP14 with Dad enhanced the inhibition of Dad toward the BMP4-induced luciferase activity, suggesting that dHIP14 acts in concert with Dad to inhibit BMP/Dpp signaling in S2 cells (Fig. 4*E*). To determine the biological function of dHIP14, we knocked down *dhip14* in germ cells and found that, like *dad*, knockdown of *dhip14* increased the number of GSC-like cells in germaria (Fig. 4 *F*, *G*, and *J*). We then performed genetic-interaction experiments to test whether dHIP14 plays a role in affecting Dad function. As shown in Fig. 4 *H* and *J*, overexpression of *dad* driven by *nosP-gal4:vp16* led to complete loss of GSCs; however, as observed in the P {*uasp-dad*}; P {*dhip14RNAi*}/P {*nosP-gal4:vp16*} fly ovaries, GSCs and their differentiated lineage were restored in 23% of tested germaria (n = 266) (Fig. 4 *I* and *J*), suggesting that knockdown of *dhip14*, at least in part, rescued the phenotype induced by overexpression of *dad*. Collectively, our findings suggest that dHIP14 catalyzes Dad palmitoylation to regulate BMP/Dpp signaling.

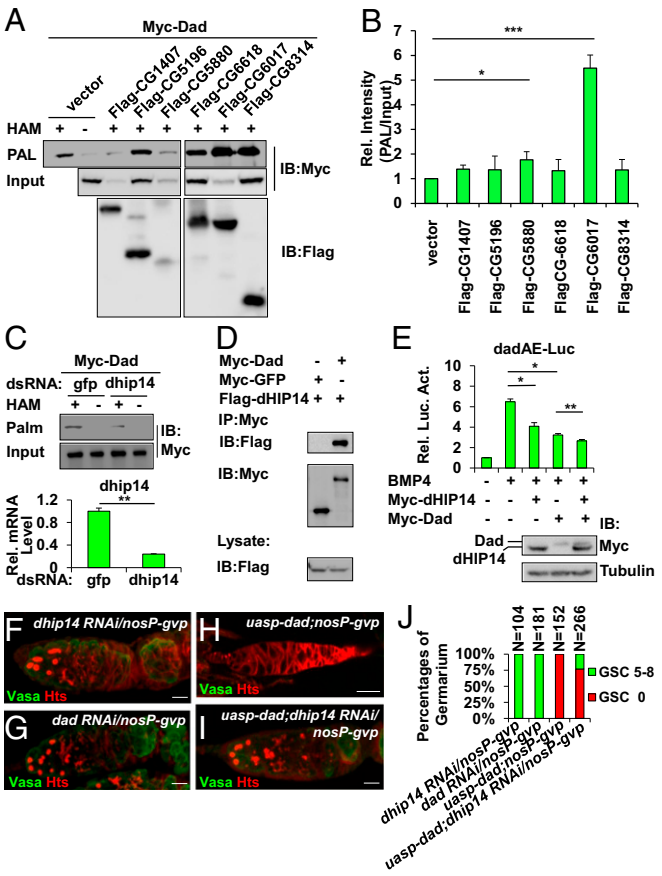

Fig. 4. PAT dHIP14 regulates Dad palmitoylation. (*A* and *B*) S2 cells were transfected with Myc-Dad together with empty vector or PAT expression constructs as indicated. Forty-eight hours after transfection, cell lysates were prepared for *S*-palmitoylation assay, followed by immunoblotting analysis (*A*). Densitometric analyses to quantify levels of palmitoylation of Dad in *A* are shown in *B*, Error bars represent SD (n = 3). (*C*) S2 cells were treated with dsRNAs targeting *gfp* (as a control) or *dhip14* for 24 h, followed by transfection with Myc-Dad. At 48-h posttransfection, cell lysates were prepared for *S*-palmitoylation assay to detect Dad palmitoylation levels, or qRT-PCR assays to examine relative levels of *dhip14* mRNA, Error bars represent SD (n = 3). (*D*) S2 cells were transfected with plasmids as indicated. At 48-h posttransfection, cell lysates were prepared and immunoprecipitated with anti-Myc beads, followed by Western blot analyses. (*E*) S2 cells were cotransfected with the indicated expression vectors together with *dad-AE-luciferase* and *actinP-lacZ* (used as an internal control). At 36-h posttransfection, cells were treated with BMP4 (10 ng mL^{-1}) for 12 h; the cells were lysed for luciferase assays (*Upper*) and immunoblotting assays (*Lower*). (*F–I*) Ovaries collected from indicated genotype females, were stained with anti-Vasa (green) and anti-Hts (red) antibodies. The flies were fed at 18 °C and dissected at 1-d-old. (Scale bars, 10 μm.) (*J*) Quantification of the germarium phenotypes of ovaries in *F–I*. All of the biochemical experiments were performed at least three times. In *B*, *C* and *E*, the Student's *t* test was used to analyze statistical significance. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. the control groups. IB, immunoblotting; IP, immunoprecipitated; Rel. Luc. Act., relative luciferase activity.

Discussion

TGF-β/BMP signaling pathways play evolutionarily conserved roles in regulating diverse developmental and homeostatic processes (1, 2, 34). Elucidating the mechanism of how TGF-β/BMP signaling is regulated is critical for developmental biology. In this study, we employed *Drosophila* as a model to study how Dad, a homolog of I-Smad proteins, in the TGF-β/BMP pathway, is regulated. We provided both biochemistry and genetic evidence showing that the Dad could be palmitoylated by a specific palmitoyl transferase. The palmitoylation modification allows Dad to target the type I receptors on the membrane compartment, thereby antagonizing TGF-β/BMP signaling. Our study reveals a mechanism by which I-Smad proteins execute their functions at membranes to regulate TGF-β/BMP signaling via a posttranslational modification.

Palmitoylation is a posttranslational covalent modification medicated by PATs, and this modification could enhance the binding affinities of target proteins with membranes, and consequently affect their functions at membranes (12). Previous studies have suggested that I-Smads act in concert with several ubiquitin E3 ligases to target the type I receptors for ubiquitination and degradation (6, 9). However, the molecular basis of

how I-Smads access membranes remains elusive. In this study, we have demonstrated that *Drosophila* I-Smad, Dad, targets the *Drosophila* BMP type I receptor, Tkv, for its ubiquitination. Our biochemistry analyses revealed that Rop, a protein engaged in vesicle transport processes, could form a complex with Dad. Although Rop does not apparently affect the function of Dad in regulating Dpp signaling, our results suggest that like Rop, Dad could associate with cellular membranes in a palmitoylation-dependent manner (Fig. S1 *B* and *C*). Importantly, we characterized that the Cys556 residue is important for Dad palmitoylation and membrane localization. Our genetic assays revealed the Cys556 is important for the in vivo function of Dad in antagonizing BMP/Dpp signaling, because overexpression of the wild-type form, but not the mutant form (Dad^{C556A}) of Dad in germ cells, led to a germ-cell–loss phenotype in *Drosophila*. Moreover, like wild-type Dad, expression of the membrane-targeted mutant form of Dad, SRC–Flag-Dad^{C556A}, in germ cells again caused a germ-cell–loss phenotype, emphasizing that membrane localization is critical for Dad to block BMP/Dpp signaling. Of note, in addition to their regulatory role in targeting the type I receptor, I-Smads have been proposed to antagonize TGF-β/BMP signaling by influencing R-Smad function via their MH2 domain (7). It would be interesting to test how membrane and nuclear functions of I-Smads coordinate to balance the TGF-β/BMP signaling activity in the future.

Unlike *N*-palmitoylation, *S*-palmitoylation modification is dynamically regulated by PATs and thioesterases, and *S*-palmitoyl acyl-transferases have a conserved "DHHC" motif (10, 11, 33). In this study, we identified dHIP14 as a specific PAT that catalyzes Dad palmitoylation in *Drosophila*, since overexpression of dHIP14 significantly increased levels of Dad palmitoylation in S2 cells and vice versa. Importantly, knockdown of *dhip14* in *Drosophila* germ cells significantly suppressed the germ-cell–loss phenotype induced by Dad overexpression. We noted that the "rescued germaria" phenotype looked more like that in knockdown of *dhip14*. The phenotype could be explained by two possible reasons. First, dHIP14 is one of *S*-palmitoyl acyl-transferases for Dad palmitoylation. Second, in addition to Dad, dHIP14 has other target proteins, whose function influences early germ cell differentiation. Nevertheless, given that the palmitoylation modification is conserved, it would be interesting to identify the specific PAT that targets I-Smads in mammals in the future.

Materials and Methods

Fly stocks used in this study were maintained under standard culture conditions. The w^{1118} strain was used as the host for all P element-mediated transformations. Strains P {*uasp-tkv(ca)*} has been described previously (29). Strains P {*uasp-flag-dad*}, P {*uasp-flag-dadC556A*}, and P {*uasp-SRC-flag-dadC556A*} were constructed for this study. The *dhip14* and *dad* knockdown transgene lines were obtained from the Tsinghua fly center. Additional materials and methods are available in *SI Materials and Methods*.

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