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# Arginine methylation initiates BMP-induced Smad signaling

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## Summary

Kinase activation and substrate phosphorylation commonly form the backbone of signaling cascades. Bone morphogenetic proteins (BMPs), a subclass of TGF-β family ligands, induce activation of their signaling effectors, the Smads, through C-terminal phosphorylation by transmembrane receptor kinases. However, the slow kinetics of Smad activation in response to BMP suggests a preceding step in the initiation of BMP signaling. We now show that arginine methylation, which is known to regulate gene expression, yet also modifies some signaling mediators, initiates BMP-induced Smad signaling. BMP-induced receptor complex formation promotes interaction of the methyltransferase PRMT1 with the inhibitory Smad6, resulting in Smad6 methylation and relocalization at the receptor, leading to activation of effector Smads through phosphorylation. PRMT1 is required for BMP-induced biological responses across species, as evidenced by the role of its ortholog Dart1 in BMP signaling during *Drosophila* wing development. Activation of signaling by arginine methylation may also apply to other signaling pathways.

## Introduction

Many signaling pathways are initiated, executed and controlled by kinases, supporting the view that substrate phosphorylation provides a universal language in activating and controlling signal transduction. Indeed, many transmembrane receptors are either kinases or act through associated cytoplasmic kinases (Feng and Derynck, 2005; Lemmon and Schlessinger, 2010; Platanias, 2005).



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TGF-β family proteins are secreted proteins that regulate cell function in all metazoans (Feng and Derynck, 2005). Among these, the BMPs direct differentiation and morphogenesis, playing important roles in development (Katagiri et al., 2008). For example, in *Drosophila*, BMP-like factors such as Dpp and Gbb regulate the establishment of body plan and wing development (Affolter and Basler, 2007; O'Connor et al., 2006), while, in vertebrates, BMPs direct mesenchymal differentiation along distinct lineages (Miyazono et al., 2010). TGF-β family proteins act through tetrameric cell surface complexes of two types of transmembrane kinases, the RII and RI receptors. Upon ligand binding, the RII receptors activate the RI receptors through phosphorylation, and RI receptors in turn activate Smads, i.e. Smad1 and Smad5, through C-terminal phosphorylation. The activated Smads then dissociate from RI receptors, form trimeric complexes with Smad4, and translocate into the nucleus to regulate target gene transcription. The inhibitory Smad6, antagonizes Smad1/5 activation by competing with Smad1/5 binding to the activated RI receptor (Feng and Derynck, 2005; Massague et al., 2005; Miyazono, 2008). Activation of Smad1/5 in response to BMPs occurs with much slower kinetics (Gromova et al., 2007) than the rapid substrate phosphorylation by ligand-activated receptor tyrosine kinases (Lemmon and Schlessinger, 2010; Smith et al., 2010), or receptor-associated tyrosine kinases (Shuai et al., 1993), which raises the question whether Smad phosphorylation is preceded by another step of signaling initiation.

Protein methylation on lysine (Lys) or arginine (Arg) has primarily been studied as a mechanism of epigenetic regulation focusing on histone methylation (Margueron and Reinberg, 2010), yet also non-histone proteins were found to be methylated (Bedford and Richard, 2005; Huang et al., 2010). The discovery of demethylases showed protein methylation to be reversible, allowing for epigenetic plasticity (Shi et al., 2004; Shi and Whetstine, 2007), and making methylation a feasible mechanism in signal transduction. For example, in NFκB signaling, the stability and transcription function of RelA is defined by lysine methylation (Huang et al., 2010; Levy et al., 2011; Lu et al., 2010). Additionally, arginine methylation of the transcription factor FOXO1 precludes its phosphorylation by Akt, and regulates its nuclear retention (Yamagata et al., 2008). Smad6 was also found to be methylated on arginine, which was attributed to PRMT1, based on *in vitro* methylation assays (Inamitsu et al., 2006). However, there has been no evidence that protein methylation initiates or activates signaling pathways from cell surface receptors, as has been well documented for phosphorylation.

We now show that Arg methylation of Smad6 by PRMT1 initiates BMP signaling through Smads. In response to BMP binding, the BMP receptor complex presents PRMT1 to Smad6, resulting in Smad6 methylation and dissociation from the RI receptor, allowing derepression of BMP-induced Smad activation by phosphorylation. Signaling initiation through Arg methylation may explain the slow kinetics of Smad activation, and defines PRMT1 as a positive regulator of BMP-induced Smad activation. We propose that the role of PRMT1 is conserved across species, as the *Drosophila* PRMT1 ortholog Dart1 methylates the Smad6 ortholog Dad and regulates BMP signaling in wing development.

## Results

### PRMT1 promotes BMP signal activation

Exploring possible roles of methylation in Smad activation, we found that the arginine methyltransferase PRMT1 is required in BMP signal activation. PRMT1 is expressed in multiple isoforms, and v1 is the common isoform in most cells and tissues (Goulet et al., 2007), including HaCaT, A549 and HepG2 cells, which are used in this study. We silenced *PRMT1* expression using siRNAs that target all PRMT1 isoforms, and assayed for BMP4-activated signaling and transcription responses. Silencing *PRMT1* expression by 95%

(Figure S1A–B) dramatically decreased BMP-induced C-terminal phosphorylation of Smad1 and Smad5, detected by phospho-Smad1/5 antibody (Figure 1A), and nuclear translocation of Smad1 (Figure 1B). Furthermore, silencing *PRMT1* repressed the transcription of the BMP target gene inhibitor of differentiation 1 (Id1) (Miyazono and Miyazawa, 2002) (Figure 1C). Likewise, in A549 lung adenocarcinoma cells, silencing *PRMT1* also impaired BMP-induced Smad1/5 activation (Figure S1C). A549 cells show a higher level of Smad6 expression than HaCaT cells, consistent with the frequently observed upregulation of Smad6 expression in lung cancer cells that correlates with poor prognosis (Jeon et al., 2008). These observations suggest that PRMT1 is required for BMP-induced activation of Smad1/5.

To address whether the methyltransferase activity of PRMT1 plays a role in facilitating BMP-induced Smad1/5 activation, we used a novel chemical inhibitor, DB867 (Ismail et al,. 2003) (Figure S1D), which inhibits PRMT1 activity with an $IC_{50}$ of 9.5 μM. At 50 μM, DB867 decreased BMP-induced C-terminal phosphorylation of Smad1/5 (Figure 1D, top) to a similar extent as the decrease in asymmetric dimethylation of histone H4 Arginine 3 (H4R3) (Figure 1D, third panel down), which is catalyzed by PRMT1. This observation suggests that BMP-induced activation of Smad1/5 requires the enzymatic activity of PRMT1.

## PRMT1 associates with and methylates Smad6

Since PRMT1 is required for efficient BMP-induced Smad1/5 activation, we evaluated whether it targets Smad1 or Smad5. PRMT1 did not co-immunoprecipitate with Smad1, Smad5 or Smad8, nor did it associate with Smad2 or Smad3, effectors of TGF-β and activin signaling, or Smad4, which associates with the TGF-β- and BMP-activated Smads (Feng and Derynck, 2005; Massague et al., 2005). Instead, PRMT1 associated with Smad6 (Figure 1E, S1E), which inhibits BMP signaling and prevents BMP-induced Smad1 or Smad5 activation (Miyazono, 2008), confirming the reported interaction of PRMT1 with Smad6 in transfected cells (Inamitsu et al., 2006). PRMT1 also weakly associated with Smad7 (Figure 1D), which inhibits both BMP and TGF-β signaling (Miyazono, 2008). PRMT4, a common PRMT also known as CARM1 (Bedford and Richard, 2005), did not interact with Smad6 (Figure 1F).

We examined whether PRMT1 can methylate Smad6 *in vitro* and *in vivo*. *In vitro*, recombinant PRMT1, but not PRMT4, methylated GST-fused Smad6 (Figure 1G, left panels), but not GST itself (data not shown), whereas PRMT4 and PRMT1 methylated their known substrates, histones H3 and H4, respectively (Figure 1G, right panels). These findings confirm the methylation of Smad6 by PRMT1 *in vitro* (Inamitsu et al., 2006). Smad6 was also methylated in 293T cells, as assessed by $^{3}$H-methyl incorporation, and this methylation was blocked by the general methyltransferase inhibitor adenosine dialdehyde (Adox) (Figure 1H). Increased PRMT1 expression enhanced Smad6 methylation (Figure 1I), and, conversely, silencing *PRMT1* expression by RNAi markedly decreased Smad6 methylation (Figure 1J). We conclude that Smad6 is methylated *in vivo*, as an endogenous PRMT1 substrate.

## Smad6 is methylated on arginines 74 and 81

Next, we identified which residues of Smad6 are methylated by PRMT1 *in vivo*. The substrate recognition and specificity of PRMT1 are incompletely understood, but most arginines methylated by PRMT1 reside in “RG” sequences with a preference in the surrounding amino acids (Bicker et al., 2011; Wooderchak et al., 2008). By mutagenesis, we replaced each of the predicted arginine residues with alanine (Figure 2A). Only the R74A mutation strongly decreased Smad6 methylation (Figure 2B), suggesting that Arg74 is a major methylation site. Since methylation of other sites could be masked by Arg74

methylation, we generated the 7RA Smad6 mutant, in which all seven arginines were replaced by alanines. This mutant was not detectably methylated, and reintroducing Arg74, in the A74R-7RA mutant, rescued Smad6 methylation, unlike the other arginines (Figure 2C). Using mass spectrometry we identified methylation at two arginines of Smad6 in multiple assays. One of these was Arg74, the site identified through mutation analysis and identified as the major site in Smad6 methylated by PRMT1 *in vitro* (Inamitsu et al., 2006), whereas Arg81 was also methylated (Figure 2D). Both residues were mono- (data not shown) and dimethylated (Figure 2D).

Since lysine is similarly charged as arginine, we also generated Smad6 mutants in which either or both Arg residues were replaced with Lys. However, the R81K mutation conferred nuclear localization of Smad6, making it difficult to interpret for the study of cytoplasmic functions of Smad6 (Figure S2). Replacing Arg74 with lysine strongly decreased methylation, phenocopying the R74A mutant, whereas the Arg81 substitution resulted in a milder decrease, and double mutation abolished Smad6 methylation (Figure 2E). Previous studies localized Smad6 primarily in the nucleus (Lin et al., 2003), yet it inhibits R-Smad activation at the receptor level (Miyazono, 2008; Miyazono et al., 2010), suggesting regulated export from the nucleus toward the receptors. Our results raise the possibility that the R81K mutation generated a nuclear localization sequence KRRR, and that Arg81 methylation controls subcellular localization, which is pursued separately from this report.

We finally assessed whether Arg74 methylation is mediated by PRMT1, using the A74R-7RA mutant as substrate. *PRMT1* downregulation strongly decreased A74R-7RA methylation (Figure 2F), indicating that PRMT1 methylates Smad6 on Arg74.

## BMP induces transient PRMT1 association and Smad6 methylation

We next examined whether BMP signaling regulates Smad6 methylation. Considering the difficulties in detecting endogenous Smad6, we first tested the effects of an activated BMP type I receptor on methylation of transfected Smad6. This receptor, caBMPRIB, has a Q203D mutation that activates its kinase (Imamura et al., 1997), mimicking BMPRI activation by BMPRII, albeit to a lower level. Activation of the BMP type I receptor resulted in increased methylation of transfected Smad6 (Figure 3A). Consistent with this observation, BMP4 increased endogenous Smad6 methylation in HaCaT cells, which was decreased following *PRMT1* downregulation (Figure 3B). Using HaCaT cells with increased BMPRI, BMPRII and Smad6 expression, allowing for higher levels of Smad6 methylation, we observed BMP-induced Smad6 methylation at its highest after 5 min of stimulation, and then decreased (Figure 3C–D), suggesting Arg demethylation.

Since BMP induced Smad6 methylation, we examined the kinetics of association between endogenous Smad6 and PRMT1 in HaCaT cells in response to BMP4 (Figure 3E). A low level interaction of PRMT1 and Smad6 was apparent in the absence of added BMP4, consistent with the association of PRMT1 and Smad6 when expressed at higher levels (Figure 1E, F). BMP4 induced a rapid association of Smad6 with PRMT1 within 5 min, followed by dissociation after 10 min (Figure 3E). This transient association peaked at a similar time as Smad6 methylation (Figure 3C), and preceded the activation of Smad1/5 by C-terminal phosphorylation at 20–30 min after adding BMP4 (Figure 3E, bottom panel). A similar kinetics of endogenous Smad6 interaction with endogenous PRMT1 was observed in A549 cells (Figure S3).

These results indicate that BMP-induced receptor activation induces a rapid and transient association of PRMT1 with Smad6, with consequent Arg methylation of Smad6, preceding Smad1/5 activation. The interaction of PRMT1 with Smad6, resulting in Smad6 methylation, implies a direct interaction, which may require accessory proteins. These data

further suggest that methylation of Smad6 by PRMT1 results in its dissociation from PRMT1. We therefore hypothesized that, in response to ligand, the activated BMP receptor complex presents PRMT1 to Smad6, resulting in Smad6 methylation and dissociation. We next examined this hypothesis stepwise, evaluating (1) the association of PRMT1 with the BMP receptors, (2) the interaction of Smad6 with the receptors, and (3) the effects of methylation on Smad6 localization and receptor association.

### PRMT1 associates with the BMP type II receptor

First we tested whether the BMP receptors present PRMT1 to Smad6. After reversible chemical crosslinking, PRMT1 coimmunoprecipitated with BMPRII, but not BMPRIB, and coexpression of BMPRIB and BMPRII resulted in association of PRMT1 with both receptors (Figure 4A, left panels). This suggested that either PRMT1 associates with RII, and that RII-RI stabilization presents PRMT1 to RI, or that PRMT1 associates with activated RI, upon activation by RII. To distinguish between these two possibilities, we used the caBMPRIB mutant that mimics activation of BMPRIB by BMPRII. PRMT1 did not interact with the activated BMPRIB alone, and only interacted with it in the presence of BMPRII (Figure 4A, right panels). We conclude that the RII receptor presents PRMT1 to the RI receptor.

Consistent with this result, endogenous PRMT1 interacted in HaCaT and A549 cells with endogenous BMPRII in the absence of ligand, and remained associated in response to BMP4 (Figure 4B, S4). In contrast, BMP4 induced a rapid association of PRMT1 with BMPRIB (Figure 4C). Concomitant with the increased association with BMPRI, the association of PRMT1 with BMPRII decreased slightly and gradually (Figure 4B, C). This may reflect a conformational change upon RII-RI complex formation and activation that results in decreased molecular contact of PRMT1 with BMPRII and affects the co-immunoprecipitation efficiency. These results indicate that PRMT1 is pre-associated with BMPRII in unstimulated cells, and presented upon BMP stimulation by the RII to the RI receptor as a result of RII-RI receptor complex formation (Figure 4D).

### Smad6 associates with the unstimulated BMP type I receptor and localizes to the cell surface

We next determined whether Smad6 also associated with the BMP receptor complex, as this would explain the rapid BMP4-induced interaction of PRMT1 with Smad6. The model in which Smad6 inhibits BMP-induced Smad activation invokes association of Smad6 with type I BMP receptors in response to ligand, thus providing a negative feedback loop (Miyazono, 2008; Miyazono et al., 2010). This model has not been verified at the level of endogenous Smad6 and receptors, due to the challenges to show Smad interactions with receptors, and in particular endogenous Smad6 associations. To evaluate whether Smad6 already associates with the BMPRI receptors in the absence of BMP4, we generated HaCaT cells that express tagged Smad6 at four-fold the endogenous level, corresponding to the range of Smad6 expression in many tumor cell lines (Jeon et al., 2008). Smad6 interacted with endogenous BMPRIB in the absence of BMP4, and remained associated with BMPRIB for 5–10 min after adding BMP4, but then dissociated from BMPRIB to more closely associate with BMPRII (Figure 5A). A similar yet somewhat faster kinetics of endogenous Smad6 dissociation from BMPRIB was observed in A549 cells (Figure S5A), presumably related to the higher endogenous Smad6 level in A549 cells, when compared to HaCaT cells. Strikingly, the kinetics of Smad6 dissociation from BMPRIB resembles that of BMP4-induced Smad6 methylation by PRMT1 (Figure 3C). Smad6 dissociation from BMPRIB was followed by increased Smad6 recruitment after prolonged exposure to BMP4 (data not shown), which is consistent with the model of BMP-induced negative feedback loop through association of Smad6 with BMPRIB (Miyazono, 2008; Miyazono et al., 2010).

The rapid association of Smad6 with PRMT1 in response to BMP4 (Figure 3E), and inhibition of BMP-induced Smad1/5 activation by Smad6 (Miyazono, 2008; Miyazono et al., 2010), require that Smad6 localizes close to the cell surface where BMP receptors are available for ligand binding. As shown by immunofluorescence, a low level of Smad6 was apparent at or close to the cell surface, with most of it in the nucleus, and BMP4 stimulation increased the Smad6 staining at the plasma membrane, especially in lamellipodia (Figure 5B). Following chemical stabilization of protein interactions and cell surface protein biotinylation, Smad6 was detected in biotinylated protein complexes (Figure 5C), confirming its association with cell surface proteins. Cell surface association of Smad6 was already apparent in the absence of BMP4, but BMP4 induced more stable or further recruitment of Smad6 (Figure 5C), consistent with the increased binding of Smad6 to BMPRII (Figure 5A). BMP4 also induced increased cell surface levels of BMPRII and BMPRIB (Figure 5C, top panels), with a corresponding decrease of intracellular receptors (Figure 5C, middle panels). As expected, PRMT1 also associated with biotinylated cell surface proteins (Figure 5C, top panels). Assessed by subcellular fractionation, an estimated 20% of total PRMT1, increasing in response to BMP4, localized to the membrane fraction (Figure S5B). Cell surface PRMT1 declined at 30 min after BMP treatment (Figure 5C), whereas PRMT1 in the membrane fraction increased at 30 min after BMP treatment (Figure S5B), suggesting that BMP may induce PRMT1 internalization and enrichment in intracellular vesicles. Collectively, these data demonstrate colocalization of PRMT1 and Smad6 with the cell surface BMP receptors. Since BMPRI-bound Smad6 already localizes at the cell surface without BMP stimulation, BMP4 may induce the interaction of RI-bound Smad6 with RII-bound PRMT1 through BMP-induced RI/II complex formation.

### BMP4 induces Smad6 methylation at the cell surface

To specifically address whether cell surface-associated Smad6 is methylated in response to BMP4, we generated antibodies to mono-methylated R74 (R74me1) or asymmetric di-methylated R74 (R74me2) Smad6 (Figure S5C, D, E), and examined cell surface-associated Smad6, isolated after chemical stabilization of protein interactions and cell surface protein biotinylation, in HaCaT cells. No methylation was apparent in unstimulated cells, but BMP4 induced rapid mono- and di-methylation of cell surface Smad6 at Arg74 by 10 min, which decreased by 30 min (Figure 5D). Total Smad6 associated with cell surface also decreased by 30 min (Figure 5D), although Smad6 continued to enrich at the lamellipodia during this time (Figure 5B). Immunofluorescence confirmed these findings, revealing prominent di-methyl R74 Smad6 staining, and some mono-methylated R74 staining, in the lamellipodia of BMP-stimulated cells (Figure 5E). Both mono- and di-R74 Smad6 co-localized with Smad6 (Figure 5E). These observations support the model that BMP induces association of RII-bound PRMT1 with RI-bound Smad6 at the cell surface, resulting in PRMT1-mediated Smad6 methylation on Arg74 (Figure 5F).

### Methylation of Arg74 enables Smad6 dissociation from the RI receptor

Since the dissociation of Smad6 from RI (Figure 5A) occurred with similar kinetics as BMP-induced Smad6 methylation (Figure 3C, 5D) and dissociation from PRMT1 (Figure 3E), we examined whether Arg methylation decreases the affinity of Smad6 for BMPRIB using an *in vitro* binding assay (Figure 6A). Purified GST-Smad6, methylated *in vitro* by PRMT1, was compared with unmethylated GST-Smad6 for binding to immobilized, HA-tagged caBMPRIB with an activating Q203D mutation, purified from transfected cells (Figure 6A, diagram). Following Smad6 dimethylation by PRMT1 (Figure 6A, lowest panels), methylated Smad6 did not associate with caBMPRIB, even when Smad6 was used in excess (Figure 6A, second panel), and unmethylated Smad6 associated with caBMPRIB. At a lower PRMT1:Smad6 ratio, presumably resulting in incomplete methylation, most of the methylated Smad6 was found in the flow-through fraction with some binding to

caBMPRIB, whereas unmethylated Smad6 again associated nearly completely with caBMPRIB, with only little of it in the unbound flow-through fraction (Figure S6A). These results provide evidence that Arg methylation decreases the affinity of Smad6 for activated BMPRIB, and that Smad6 methylation by PRMT1 leads to its dissociation from the RI receptor.

Since endogenous Smad6 transitioned into a closer association with RII as it dissociated from RI (Figure 5A), we examined whether Arg methylation increased the association of Smad6 with BMPRII using a similar binding assay, except that HA-tagged BMPRII was purified from transfected cells stimulated with BMP4, resulting in interaction of endogenous RI with RII. Following Smad6 dimethylation by PRMT1, methylated Smad6 associated more efficiently with BMPRII than unmethylated Smad6 (Figure S6B). The association of unmethylated Smad6 resulted most likely from association with BMPRI that coimmunoprecipitated with BMPRII and remained associated under conditions to evaluate Smad6 interaction with BMPRII. These results suggest that Arg methylation enhances the affinity of Smad6 for BMPRII, enabling Smad6 transfer from the RI to RII receptor.

Supporting the notion that Arg methylation by PRMT1 decreases the affinity of Smad6 for the RI receptor, increasing PRMT1 expression decreased the interaction of Smad6 with caBMPRIB (Figure 6B). Moreover, binding of Smad6 R74A with the receptor was not affected by increased PRMT1 expression in both HaCaT or HepG2 cells, despite its less efficient association compared to wild type Smad6 (Figure 6C and S6C). Similar results were obtained using the R74K mutant of Smad6 (data not shown). Furthermore, Smad6 R74K repressed BMP-induced Smad1/5 phosphorylation more efficiently than wild-type Smad6 in HepG2 and HaCaT cells (Figure S6D, E). Together, these results support a scenario that, in response to BMP, RII-bound PRMT1 methylates RI-bound Smad6 at Arg74, and that this methylation decreases the affinity of Smad6 for RI, resulting in its dissociation, and derepression of the receptor complex required for activation of Smad1/5 (Figure 6D).

## Smad6 acts downstream from PRMT1

To confirm that Smad6 acts epistatically downstream of PRMT1, we performed combinatorial siRNA experiments in HaCaT cells. Specifically, we examined whether reducing Smad6 expression would rescue the impaired BMP-induced Smad1/5 activation, resulting from *PRMT1* silencing (Figure 1A). *PRMT1* expression was silenced efficiently (Figure S6F), but *Smad6* expression could not be reduced below 25% (Figure S6G, H). While silencing of *PRMT1* reduced the BMP4-induced Smad1/5 phosphorylation (Figure 6E, compare lanes 5–8 with lanes 1–4), a concomitant moderate decrease in *Smad6* expression relieved this inhibition (Figure 6E, compare lanes 9–12 with lanes 5–8). This result indicates that Smad6 acts downstream of PRMT1, and that PRMT1 relieves the repression of BMP receptor signaling through Smad6.

## PRMT1 and Smad6 cooperate in the control of biological responses

Considering the epistatic interaction of PRMT1 and Smad6 in BMP-induced Smad1/5 activation, we examined their cooperation in biological responses controlled by BMP. In mammalian cells, Id genes are direct targets of BMP-activated Smads and coordinate cell proliferation and differentiation (Miyazono and Miyazawa, 2002). We quantified the BMP4-induced expression of Id1, Id2 and Id3 mRNAs, which is moderate in HaCaT cells. Silencing *PRMT1* expression (Figure S6F) dramatically decreased the induction of *Id* gene expression, but a concomitant decrease in *Smad6* expression (Figure S6G) alleviated this inhibition (Figure 7A-C). This cooperation of *PRMT1* and *Smad6* illustrate the consequences of PRMT1-mediated Smad6 methylation in BMP-induced Smad responses.

Lastly, we addressed whether the relationship between *PRMT1* and *Smad6* in BMP signaling is conserved and functional in other metazoans. We chose to study wing development in *Drosophila melanogaster*, as it is regulated by the BMP homologs Decapentaplegic (Dpp) and Glass bottom boat (Gbb), homologs of BMPs 2 and 4, and BMPs 5, 6, 7 and 8, respectively (Affolter and Basler, 2007; Blair, 2007; O'Connor et al., 2006; Wartlick et al., 2011). Like in mammalian cells, these BMPs induce complexes of the type II receptors, Punt (Put), with the type I receptors, Thickveins (Tkv; homologous to BMPRI) or Saxophone (Sax), resulting in RI activation, and activation of the Smad1/5 ortholog Mothers against dpp (Mad), which then regulates transcription. The *Drosophila* counterpart of Smad6 is Daughters against dpp (Dad), which antagonizes Mad activation (Tsuneizumi et al., 1997). Just like the BMP pathway, the PRMT methyltransferase family is well conserved in *Drosophila*, with *Drosophila* arginine methyltransferase 1 (Dart1) as ortholog of PRMT1 (Boulanger et al., 2004). Illustrating the conservation of PRMT1-mediated Smad6 methylation in *Drosophila*, coexpression of Dart1 resulted in Dad methylation in 293T and *Drosophila* Kc cells (Figure 7D, E).

To examine whether *Dart1* regulates *Drosophila* BMP signaling through Dad, we tested its ability to rescue the BMP pathway repression that results from overexpressing *Dad*. Using the UAS-GAL4 system, we increased *Dad* expression in wing discs, which results in a dramatic wing size reduction and patterning defects (Tsuneizumi et al., 1997) (Figure 7F, top right), reflecting the role of *Drosophila* BMP in wing disc cell proliferation and morphogenesis. When expressing one or two copies of *Dart1* in addition to *Dad*, *Dart1* rescued the wing size and morphology of *Dad* transgenic flies in a dose-dependent manner (Figure 7F, G). This provides evidence that the PRMT1 ortholog antagonizes the effects of the Smad6 ortholog and de-represses Drosophila BMP signaling, similarly to our results in mammalian cells, suggesting evolutionary conservation of this functional relationship.

## Discussion

This study provides evidence for arginine methylation of an inhibitory Smad as initiating step in BMP-induced signaling. This process involves three steps: (1) BMP-induced interaction of RII-associated PRMT1 with RI-associated Smad6; (2) Smad6 methylation on Arg74 by PRMT1; (3) release of methylated Smad6 from the RI receptor to derepress downstream Smad1/5 signaling. The requirement for Smad6 methylation and dissociation may explain the slow kinetics of BMP-induced C-terminal phosphorylation of Smad1/5, starting at around 15 min, compared with the rapid phosphorylation of growth factor-induced Erk MAPK (Smith et al., 2010) or cytokine-induced STAT (Shuai et al., 1993), starting already at 5 min after ligand addition. Consistent with this model, lowering Smad6 expression conferred a more rapid BMP4-induced Smad1/5 phosphorylation (Figure 6E). The mechanism of BMP-induced Smad6 methylation as initiator of Smad signaling raises questions for further studies. Most intriguingly, ligand-induced Smad6 methylation is followed by a decrease in methylation, without change in Smad6 level. However, no arginine demethylase has been identified with certainty (Mantri et al., 2011; Webby et al., 2009), raising the question of the mechanism of Smad6 Arg demethylation. Also, the mechanism and role of the transition of methylated Smad6 from RI to RII remain to be further explored.

Smad6 binding to the RI receptor in response to BMP was proposed as negative feedback loop that attenuates Smad activation (Miyazono, 2008; Miyazono et al., 2010). We now provide evidence for another mechanism, i.e. that Smad6 already associates with the RI receptor in the absence of ligand, and that ligand induces its dissociation, enabling Smad activation in response to ligand. As the BMP RII and RI receptors have an inherent affinity for each other to form complexes in the absence of BMP (Ehrlich et al., 2011; Miyazono et

al., 2010), binding of Smad6 to the BMP RI receptors in the absence of ligand may act as a mechanism to keep the receptor complexes silent and prevent leaky signaling. Thus, Smad6 binding to the receptors in the absence of ligand may resemble the role proposed for FKBP12 in silencing TGF-β receptor complexes (Chen et al., 1997). Regulation of the Smad6 level at the cell surface may enable cells to adjust the threshold of BMP responsiveness, which may be critical in development, whereby BMP signals act as dose-dependent morphogens. Furthermore, transfer of Smad6 to RII upon dissociation from RI maintains Smad6 in the receptor complex, which may facilitate Smad6 function as a negative feedback regulator.

Mammalian cells express two inhibitory Smads, Smad6 and Smad7. Smad6 targets the BMP pathway, whereas Smad7 targets both TGF-β/activin signaling through Smad2/3, and BMP signaling through Smad1/5 (Miyazono et al., 2010). The N-terminal regions preceding the MH2 domains in Smad6, Smad7 and *Drosophila* Dad diverge highly (Miyazono, 2008; Miyazono et al., 2010) and, accordingly, Smad6 Arg74 is not conserved in Smad7 or *Drosophila* Dad. Furthermore, these regions lack conserved domains and are predicted to be inherently structurally disordered, i.e. their structures may be defined by interacting proteins and post-translational modifications. Thus, the structure of this segment of Smad6, and of Smad7 and Dad, may be defined by its interaction with the type I receptor (Hanyu et al., 2001). In rapidly evolving, disordered sequences, sites for phosphorylation by a defined kinase were shown to rapidly shift during evolution and lack sequence conservation (Holt et al., 2009). By analogy, the evolutionary divergence of this disordered sequence may explain the lack of sequence conservation of Arg74 in Smad6, when compared to Dad, especially given the minimal target recognition sequence of PRMT1 (Bicker et al., 2011; Wooderchak et al., 2008). Dissecting the role and mechanism of Smad7 methylation in TGF-β signaling will shed light on parallels with Smad6 methylation in BMP signaling.

PRMT1 shows diverse functions as it targets various transcription factors and signaling molecules (Bedford and Clarke, 2009; Bedford and Richard, 2005). PRMT1 shuttles between cytoplasm and nucleus (Herrmann et al., 2009), yet most functional studies have focused on the nuclear functions of PRMT1. We now present evidence that a considerable fraction of PRMT1 is associated with the membrane (Figure S5B, 5C), suggesting membrane-associated functions, consistent with a role of PRMT1 in initiating Smad activation. Considering the diverse targets of PRMT1, it was somewhat surprising that an epistatic relationship between PRMT1, Smad6 or Dad, and BMP-induced Smad signaling could be uncovered in *Id* gene expression or *Drosophila* wing development. The complex roles of PRMT1 may obscure its functional epistasis in other contexts of BMP-induced processes in development.

The function of Smad6 and PRMT1 in the context of the BMP cell surface receptors is likely independent from the role of Smad6 in the nucleus, where Smad6 is an as yet poorly defined transcription repressor (Ichijo et al., 2005; Lin et al., 2003), and PRMT1 is also found (Herrmann and Fackelmayer, 2009). Studies on the role of Smad6 methylation in the nucleus may reveal key aspects of the control of nuclear export of Smad6, possibly involving Arg81 methylation, and BMP-regulated transcription and epigenetic responses.

In summary, we demonstrated that Smad6 methylation by PRMT1 initiates BMP signaling. To our knowledge, this is the first example of methylation as signaling initiator of a cell surface receptor, raising the possibility that ligand-induced methylation may play a role in the activation or execution of other signaling pathways.

## Experimental Procedures

Please refer to the Extended Experimental Procedures for more extensive information.

### *In vitro* methylation assays

Immunopurified Flag-tagged PRMT1 or PRMT4 were incubated with GST-Smad6 or histone in reaction buffer in the presence of 2 μCi $^{3}$H-labeled S-adenosyl-methionine (Perkin-Elmer) at 30°C for 90 min. The reaction mixture was quenched with 5 X SDS buffer and separated by SDS-PAGE. The gel was stained, scanned, fluorographed, dried and exposed to Kodak film at −80°C.

### *In vivo* methylation assays

Transfected 293T, HaCaT or Kc cells were pretreated with inhibitors as specified, switched to medium without methionine, containing the same inhibitors and cycloheximide for 30 min, and then incubated with 10 μCi/ml of L-[methyl-$^{3}$H]methionine (Perkin-Elmer) for an additional 1–6 h. The cells were lysed in lysis buffer. The Smad6 complexes were immunopurified with anti-Flag M2, anti-Myc 9E10, or anti-Smad6 antibody and separated by SDS-PAGE. The protein gel was then processed as for *in vitro* methylation assays.

### GST adsorption assays

Immunopurified GST-tagged Smad6 was *in vitro* methylated by GST-PRMT1 in the presence or absence of S-adenosyl-methionine. Methylated or unmethylated Smad6 was incubated with immobilized HA-tagged caBMPRIB or BMPRII in lysis buffer in the presence of MG132 and chloroquine. After pull-down, precipitates and flow through were separated and subjected to SDS-PAGE followed by immunoblotting.

### Cell-surface BMP receptor complex crosslinking, biotinylation and precipitation

Tranfected 293T or HaCaT cells were labeled with sulpho-NHS-LC biotin in the presence of crosslinking reagent DSP at 4°C for 10–30 min, washed with 100 mM glycine and lysed in lysis buffer. Biotinylated cell-surface proteins were precipitated with neutravidin-agarose resin and subjected to immunoblotting.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Highlights

- PRMT1 is critical for BMP-induced Smad activation.
- BMP signaling induces Smad6 methylation by PRMT1 at the receptor level.
- Methylation on Arg74 enables Smad6 dissociation from the RI receptor.
- PRMT1 and Smad6 cooperate in the control of biological responses.

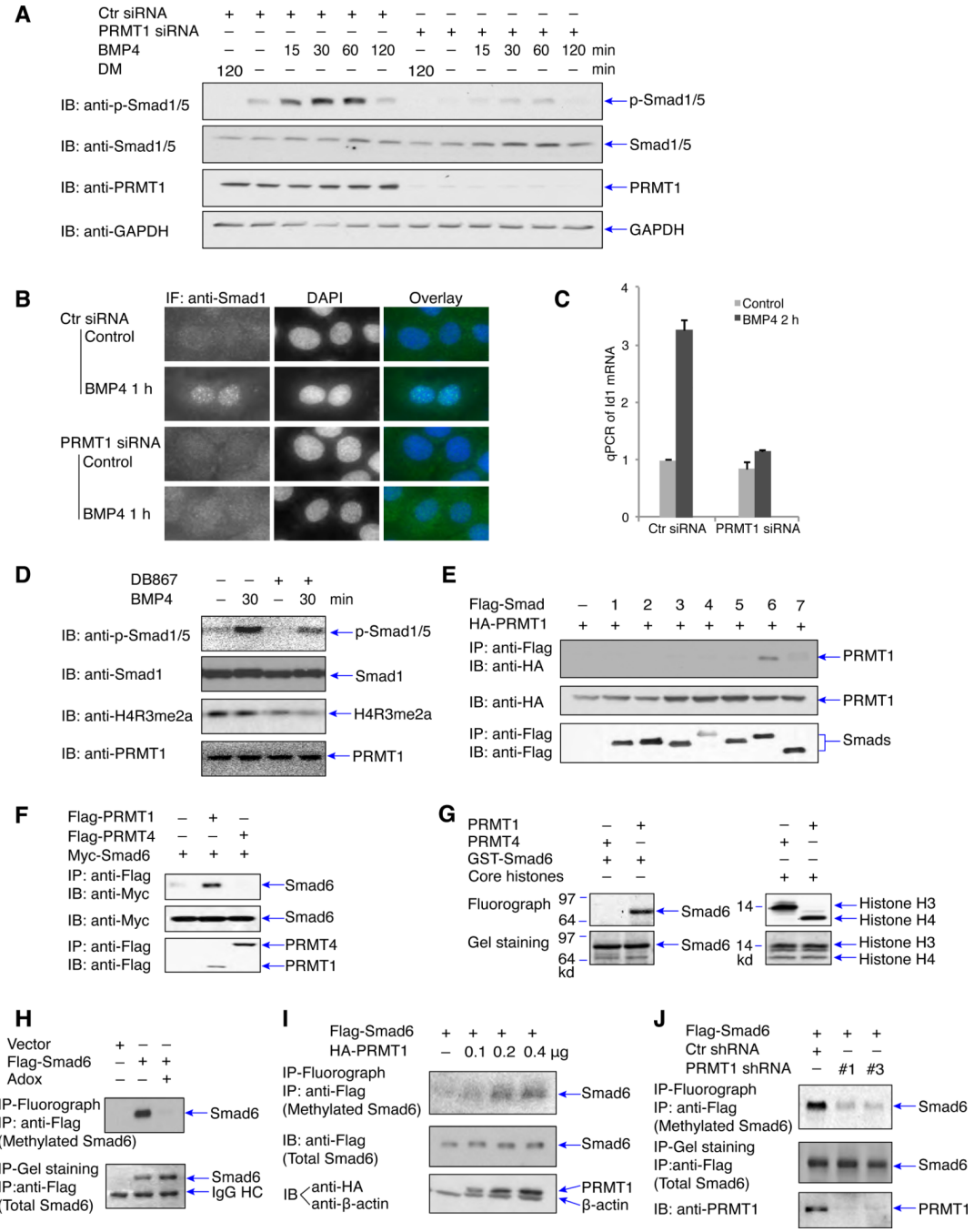


**Figure 1.**

PRMT1, a methyltransferase critical for BMP-induced Smad activation, associates with and methylates inhibitory Smad6.

(A) siRNA-mediated decrease in *PRMT1* expression inhibited BMP4-induced Smad1/5 activation, assessed by immunoblotting (IB) for C-terminally phosphorylated Smad1/5. HaCaT cells were transfected with PRMT1 siRNA or control siRNA, and treated with the BMP signaling inhibitor dorsomorphin (DM) or BMP4 for the indicated times, or untreated. (B) Decreasing *PRMT1* expression using siRNA inhibited BMP4-induced nuclear translocation of Smad1, assessed by immunofluorescence (IF). HaCaT cells were transfected

with siRNA, as in (A), treated with BMP4 for 1 h, and immunostained with Smad1 antibody, or stained with DAPI.

(C) Decreasing *PRMT1* expression inhibited BMP4-induced Id1 mRNA expression, assessed by qRT-PCR. HaCaT cells were transfected with siRNA as in (A), and treated with BMP4 for 2 h. Data are represented as mean +/− SEM.

(D) A chemical inhibitor of PRMT1, DB867, inhibited BMP4-induced Smad1/5 activation and asymmetric dimethylation of Arg3 on histone 4 (H4R3). HaCaT cells were treated with 50 μM DB867 or solvent for 4 h, treated with BMP4 for 30 min, or left untreated, and assessed by immunoblotting (IB).

(E) PRMT1 associated with Smad6 and Smad7, but not Smads 1–5. Flag-tagged Smads and HA-tagged PRMT1 were expressed in 293T cells. PRMT1 association with the individual Smads was analyzed by immunoprecipitation (IP) of the Smads, followed by immunoblotting (IB).

(F) PRMT1, but not PRMT4, interacted with Smad6. Flag-tagged PRMT and Myc-tagged Smad6 were expressed in 293T cells. PRMT1 or 4 was immunoprecipitated, and immunoblotted for associated Smad6.

(G) PRMT1 methylated Smad6 *in vitro*. GST-Smad6 (left) or core histones (right) were incubated with PRMT1 or PRMT4 in the presence of $^{3}$H-SAM, separated by SDS-PAGE, and visualized by Gelcode blue staining and radiography.

(H) Smad6 was methylated *in vivo*. Flag-Smad6 was expressed in 293T cells in the presence of $^{3}$H-methionine, and absence or presence of the methyltransferase inhibitor Adox, immunoprecipitated, and subjected to SDS-PAGE, staining and autoradiography to visualize $^{3}$H incorporation. (I) Increasing PRMT1 expression, indicated by the increasing μg amounts of PRMT1 expression plasmid, enhanced Smad6 methylation *in vivo*, as determined by *in vivo* labeling using $^{3}$H-methionine, as in (H). (J) Downregulating *PRMT1* expression using lentiviral shRNA decreased the methylation of Flag-tagged Smad6 in 293T cells. Two different PRMT1 shRNAs (#1, #3) were compared with a control shRNA. $^{3}$H-methylation of Smad6 was visualized as in (H). See also Figure S1.

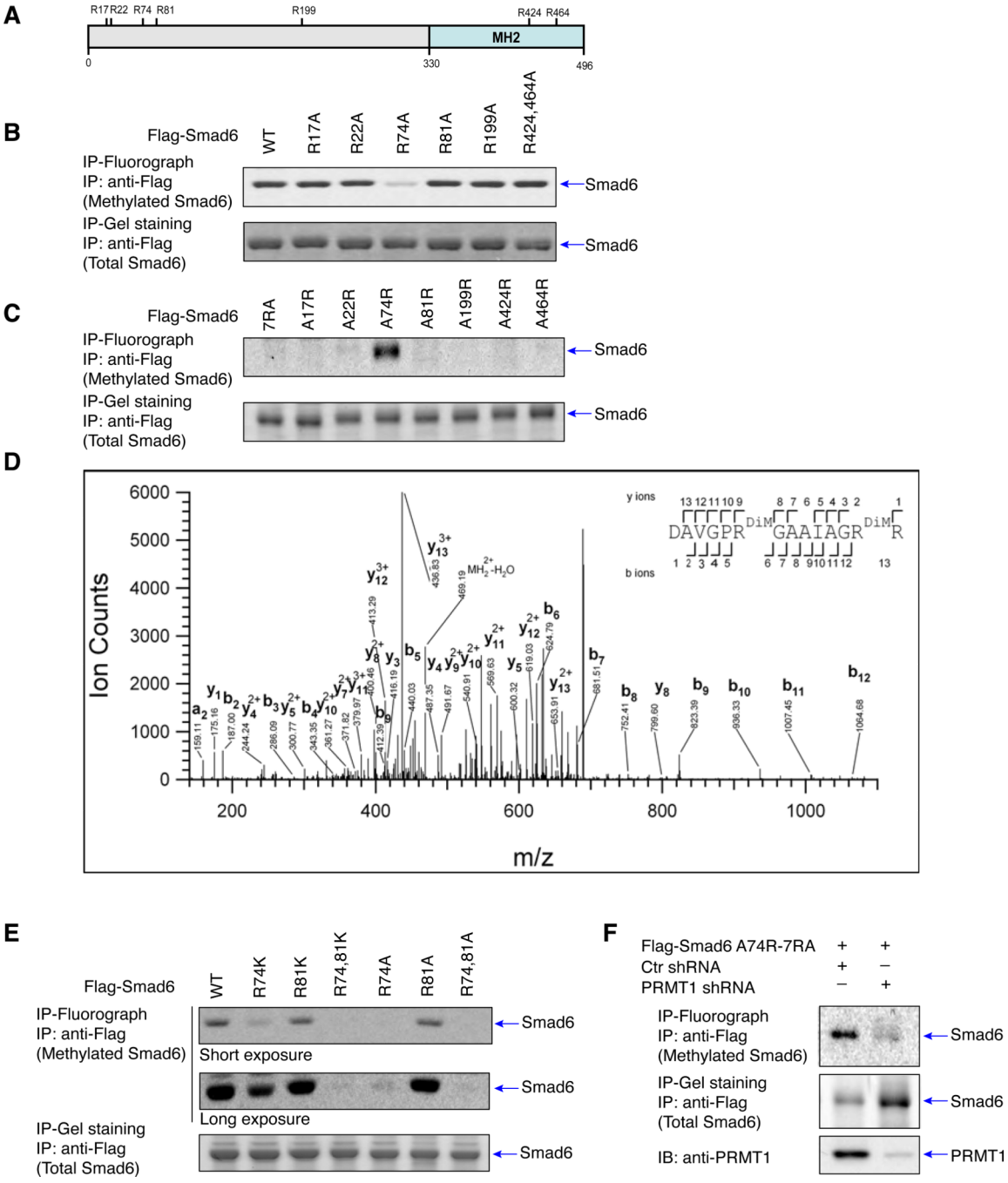


**Figure 2.**

Smad6 is methylated on arginines 74 and 81.

(A) Schematic diagram of Smad6 with seven arginines predicted as potential PRMT1 methylation sites.

(B) Alanine replacement of Arg74 (R74A), but not other arginines, decreased Smad6 methylation. 293T cells expressing wild-type or mutant Smad6 were labeled in the presence of $^{3}$H-methionine, immunoprecipitated with anti-Flag antibody, and visualized by SDS-PAGE, gel staining and $^{3}$H-autoradiography.

(C) Re-introducing Arg74 in the 7RA mutant, generating the A74R-7RA mutant, restored Smad6 methylation. *In vivo* $^{3}$H-methylation assay was done as in (B).

(D) CID tandem mass spectrometry identified R74 and R81 dimethylation in 293T cells expressing Flag-Smad6. CID tandem mass spectrum obtained from a precursor ion with m/z= $474.9506^{+3}$ corresponded to a tryptic peptide spanning residues Asp69 to Arg82 of mouse Smad6 (theoretical mass 474.9478; measured mass error, 5.89 ppm). The observed sequence ions are labeled in the figure and over the sequence. Masses of the C-terminal fragment ions from $y_2$ to $y_8$ show a shift of 28 D relative to the calculated masses of these product ions for the non-modified peptide. This shift increases to 56 D above $y_9$. N–terminal fragment ions from $b_6$ to $b_{12}$ are shifted 28 Da. This is congruent with Arg74 and Arg81 being dimethylated in this peptide.

(E) Arg74 and Arg81 were methylated in Smad6. Arg74 and Arg81 were substituted with Lys, individually or together, generating the R74K, R81K and R74,81K Smad6 mutants. These and the corresponding Ala mutants, R74A, R81A and R74,81A, were expressed and subjected to methylation assays as in (B). Mutation of both Arg74 and Arg81 was required to abolish Smad6 methylation.

(F) PRMT1 methylated Arg74 in Smad6 *in vivo*. The Smad6 mutant A74R-7RA was expressed in 293T cells stably expressing *PRMT1* shRNA, or control shRNA, and $^{3}$H-methylation was done as in (B). See also Figure S2.

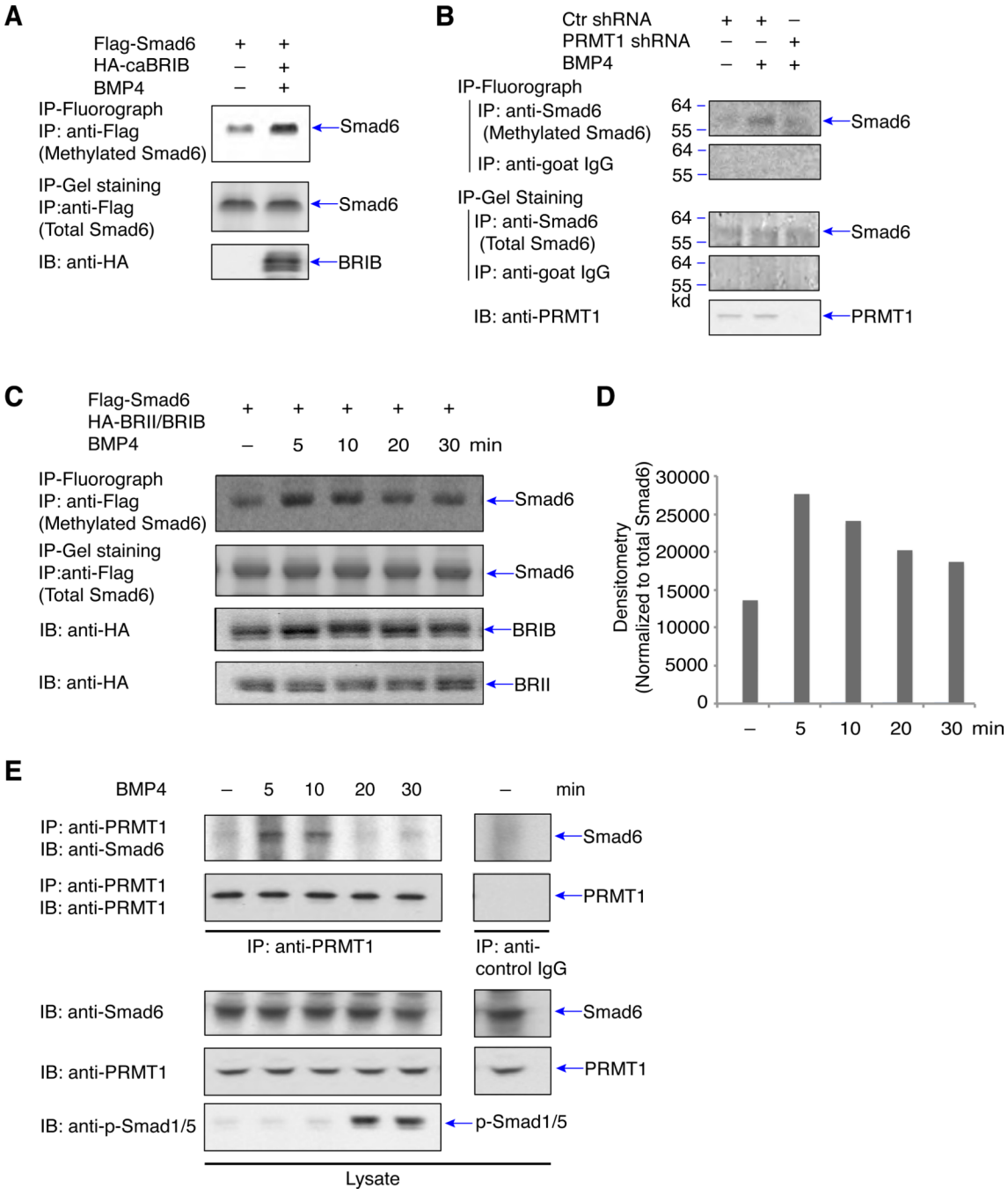


**Figure 3.**

BMP signaling induces PRMT1 association with Smad6, and Smad6 methylation.

(A) BMP receptor activation induced Smad6 methylation. 293T cells expressing Flag-tagged Smad6 in the absence or presence of activated BMPRIB and BMP4 were labeled using $^{3}$H-methionine, immunoprecipitated with anti-Flag, and visualized by SDS-PAGE, GelCode blue staining and $^{3}$H-autoradiography.

(B) BMP4 induced endogenous Smad6 methylation in HaCaT cells, and this methylation decreased when *PRMT1* expression was downregulated using lentiviral shRNA. Cells were incubated with $^{3}$H-methionine, treated with or without BMP4, followed by

immunoprecipitation with anti-Smad6, or goat IgG. $^{3}$H-methylated Smad6 was visualized as in (A).

(C) BMP4 induced enhanced Smad6 methylation. Smad6 was co-expressed with BMPRII and BMPRIB in 293T cells in the presence of $^{3}$H-methionine, preincubated with BMP inhibitor Noggin, treated with BMP4 for the indicated times, and subjected to *in vivo* methylation as in (A). Control panels show the levels of Smad6, BMPRIB and BMPRII.

(D) Quantification of the Smad6 methylation in (C) by densitometry, normalized to total Smad6.

(E) BMP4 induced rapid Smad6 association with PRMT1, followed by dissociation. HaCaT cells were treated with BMP4 for different times, PRMT1 was immunoprecipitated and the complexes were immunoblotted for associated Smad6 (top panel). Control panels show immunoprecipitated PRMT1, and total PRMT1 and Smad6 in the cell lysates. The bottom panel shows activation through C-terminal phosphorylation of Smad1/5, increasing by 20–30 min. A rabbit IgG was used as control for IP. No PRMT1 or associated Smad6 was detected in the control immunoprecipitation.

IP, immunoprecipitation; IB, immunoblotting. See also Figure S3.

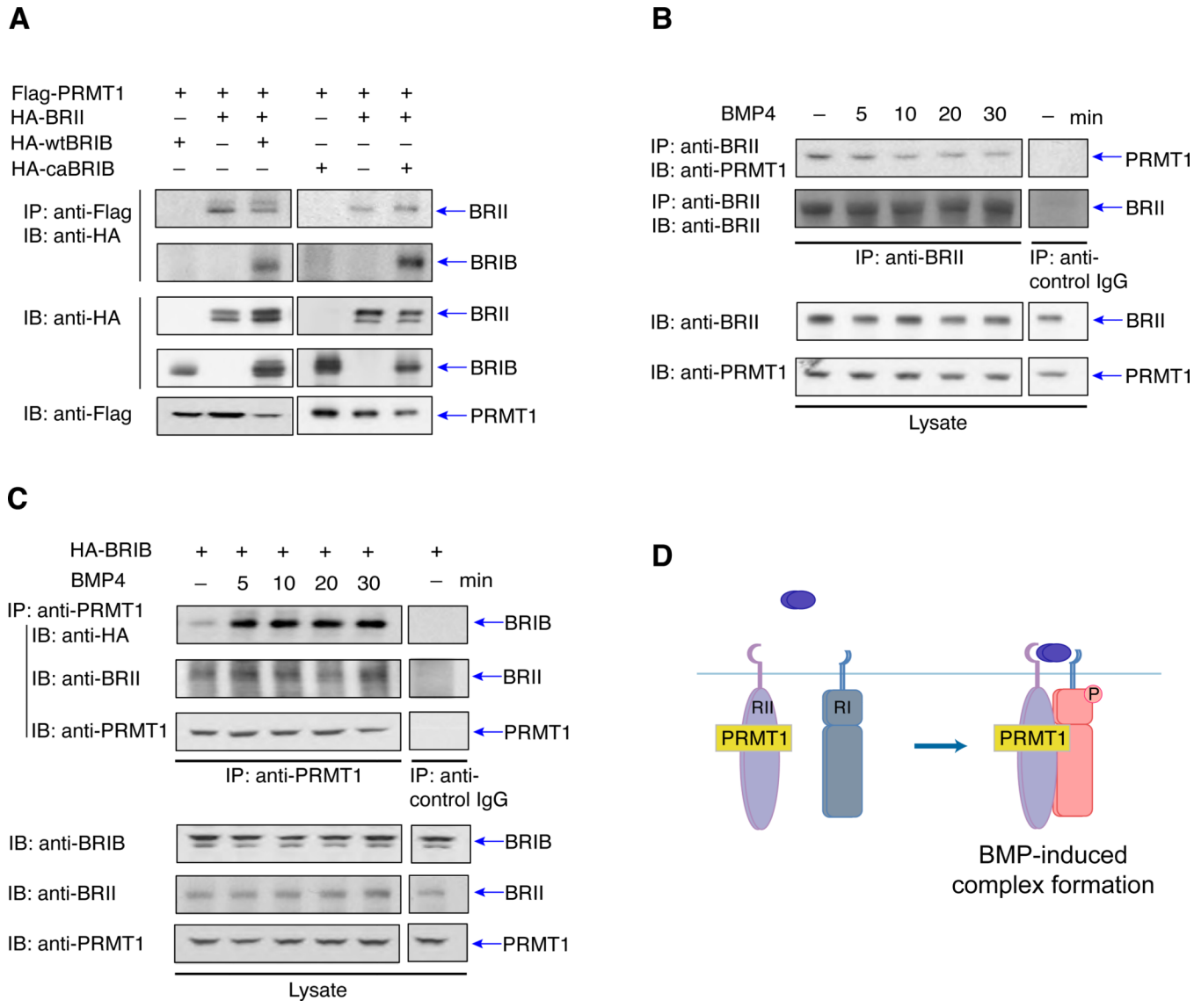


**Figure 4.**

PRMT1 is recruited by the BMP type II receptor.

(A) PRMT1 associated with the BMP receptor complex through its association with BMPRII. Flag-tagged PRMT1 was co-expressed with wild-type (wt) or activated (ca) HA-tagged BMPRIB, or BMPRII, or both. PRMT1 was immunoprecipitated following reversible protein crosslinking using the cell permeable crosslinker DSP, and the associated BMPRII or BMPRIB were visualized by immunoblotting. BMPRIB co-precipitated with PRMT1 only in the presence of BMPRII.

(B) PRMT1 associated constitutively with BMPRII. HaCaT cells were treated with BMP4 for the indicated times, immunoprecipitated with anti-BMPRII antibody following reversible crosslinking with the cell permeable crosslinker DSP and immunoblotted. A mouse anti-GFP antibody was used as control IgG for the IP. No BMPRII-interacting PRMT1 was detected in the control IP.

(C) BMP induced association of PRMT1 with BMPRIB. HaCaT cells with enhanced BMPRIB expression were treated with BMP4 for different times, immunoprecipitated with PRMT1 antibody following reversible crosslinking using DSP, and immunoblotted for HA-

tagged BMPRIB. A rabbit IgG was used as control for immunoprecipitation. No PRMT1-interacting protein was detected in the control IP.
(D) The constitutive interaction of PRMT1 with BMPRII enables BMP4 to induce presentation of PRMT1 to BMPRI.
IP, immunoprecipitation; IB, immunoblotting.
See also Figure S4.

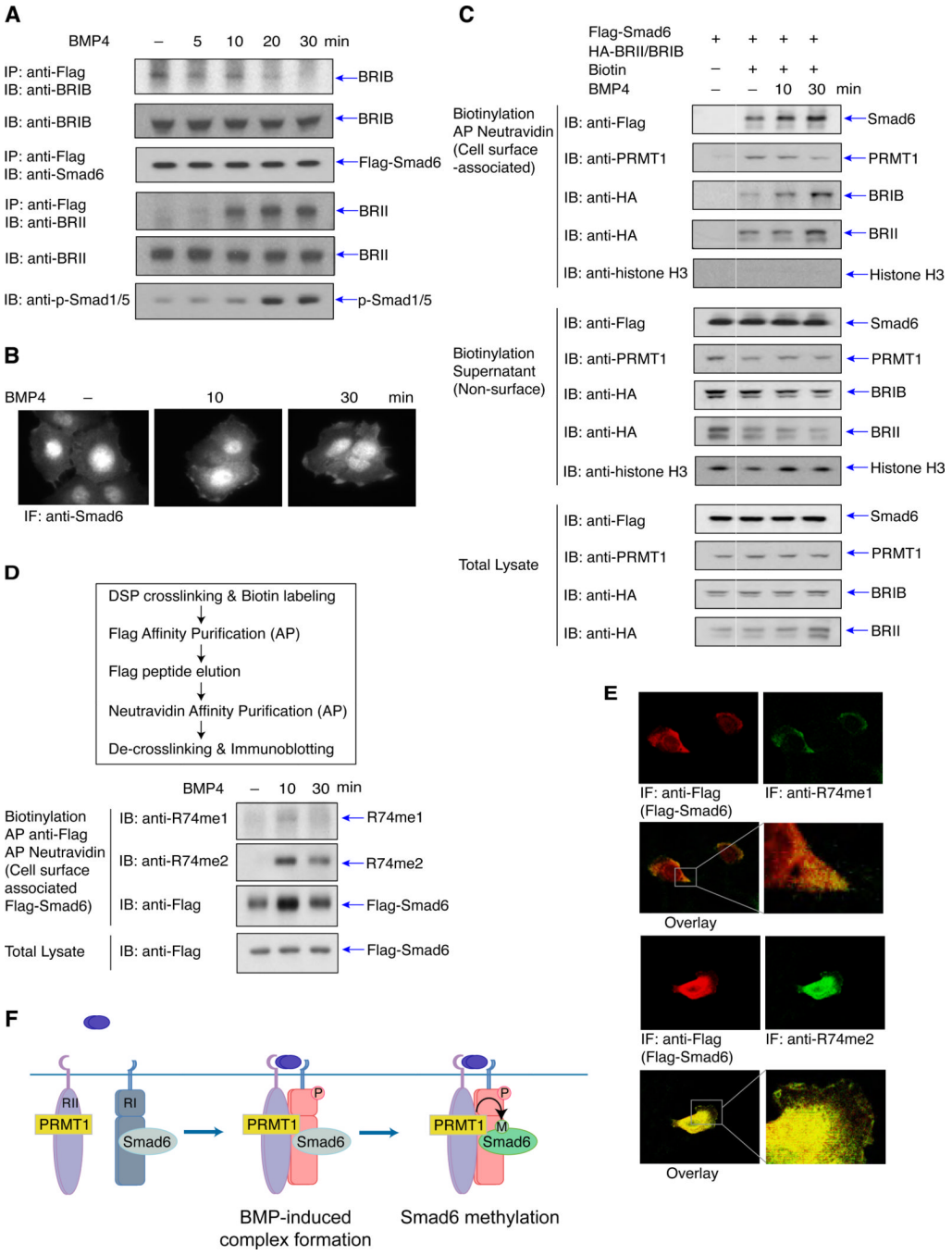


**Figure 5.**

Smad6 localization and BMP4-induced Smad6 methylation at the cell surface.

(A) BMP induced Smad6 dissociation from BMPRIB. HaCaT cells stably expressing Flag-tagged Smad6 were treated with BMP4 for the times shown. Smad6 was immunoprecipitated after reversible crossinking using the cell permeable crosslinker DSP, and the complexes were immunoblotted for associated BMPRIB (top panel) or BMPRII (4$^{th}$ panel down). The bottom panel shows activation through C-terminal phosphorylation of Smad1/5, increasing by 20–30 min, following Smad6 dissociation from BMPRIB at 10 min.

(B) Endogenous Smad6 localization to the cell surface increased in response to BMP4. HaCaT cells were treated with BMP4, methanol fixed and immunofluorescent (IF) stained for Smad6.

(C) Cell surface association of Smad6, visualized by cell surface protein biotinylation, was stabilized or increased in response to BMP4. 293T cells, transfected to express Smad6, BMPRII and BMPRIB, were treated with BMP4, or untreated, and subjected to cell surface protein biotinylation in the presence of the cell permeable, reversible crosslinker DSP, followed by affinity-precipitation (AP) with neutravidin beads to separate neutravidin-bound cell surface protein complexes and 'non-surface' protein complexes. These two fractions were analyzed by immunoblotting with the indicated antibodies. In control cells (first lanes), no EZ-link-sulfo-NHS-LC-biotin was added.

(D) Cell surface-associated Smad6 was rapidly methylated at R74 in response to BMP4. HaCaT cells expressing Flag-Smad6 were treated with BMP4 and subjected to biotinylation of cell surface proteins in the presence of the crosslinker DSP. Flag-Smad6 was isolated using adsorption to Flag antibody-conjugated beads and elution with competing 3XFLAG peptide. The cell surface-associated fraction of Smad6 was then isolated using neutravidin beads and analyzed by immunoblotting for Arg74 methylation using mono- (R74me1) or asymmetric dimethyl- (R74me2) Arg74-Smad6 antibodies.

(E) Methyl-R74-Smad6 was enriched at the cell membrane in response to BMP4. HaCaT cells expressing Flag-tagged Smad6 were treated with BMP4 for 10 min, methanol fixed and stained with antibodies to Flag, mono-methyl (R74me1) or asymmetric dimethyl- R74-Smad6 (R74me2). Note the co-staining at the cell membrane with both methyl R74 antibodies.

(F) BMP4 induces presentation of RII-bound PRMT1 to RI-associated Smad6, resulting in Smad6 methylation by PRMT1.

AP, affinity purification, IP, immunoprecipitation; IB, immunoblotting; IF, immunofluorescence.

See also Figure S5.

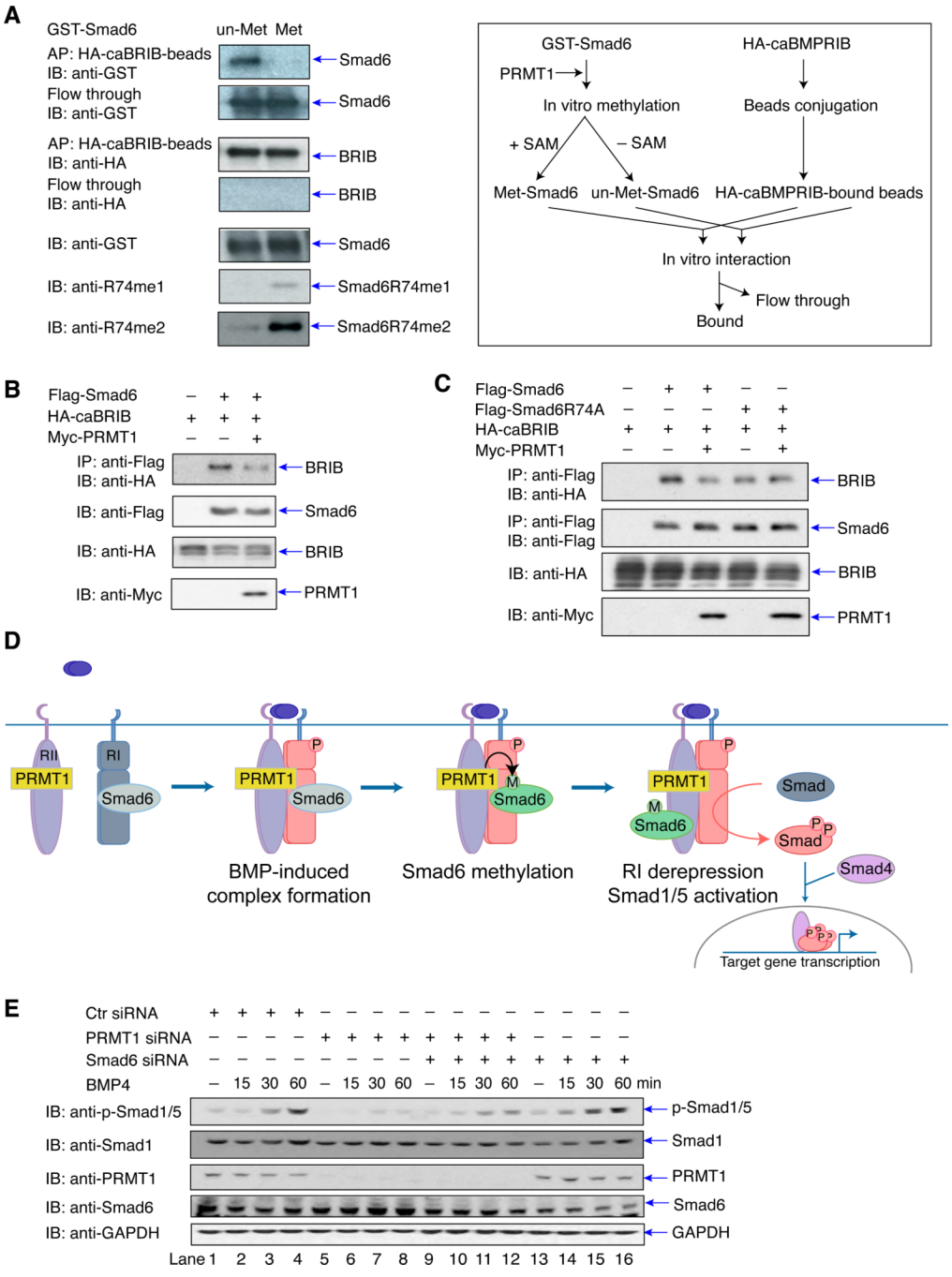


**Figure 6.**

BMP-induced methylation on Arg74 enables Smad6 dissociation from BMPRIB.

(A) Methylation by PRMT1 decreases the affinity of Smad6 for the activated (ca) type I receptor BMPRIB. GST-Smad6 was incubated with PRMT1 at a 1:20 ratio in the presence (Met) or absence (Un-Met) of S-adenosyl-methionine, followed by incubation with immunoprecipitated, Sepharose-bound HA-tagged activated BMPRIB. The association of methylated and unmethylated Smad6 with BMPRIB were analyzed by immunoblotting of the bound and flow-through fractions. The lower panels showed a low level of mono-, and a much higher level of dimethylation of Smad6, assessed by immunoblotting for R74me1 and R74me2.

(B) Increased PRMT1 expression decreased the association of Smad6 with activated (ca) BMPRIB. Smad6 was co-expressed with activated BMPRIB with or without PRMT1, and immunoprecipitated. BMPRIB association was revealed by immunoblotting.
(C) Increased PRMT1 expression decreased the association of wild-type Smad6 with activated (ca) BMPRIB, but did not affect the lower level association of the Smad6 R74A mutant with activated BMPRIB. Wild-type or R74A mutant Smad6 were co-expressed with activated BMPRIB with or without PRMT1. Cell lysates were subjected to immunoprecipitation and immunoblotting.
(D) The BMP-induced BMP receptor complex presents Smad6 to PRMT1, allowing PRMT1 to methylate Smad6, thus promoting dissociation of Smad6 from PRMT1 and the type I receptor, and allowing Smad1/5 activation by the type I receptor.
(E) Decreased Smad6 expression rescued the impaired Smad1/5 activation imposed by silencing PRMT1 expression, assessed by immunoblotting for C-terminally phosphorylated Smad1/5. HaCaT cells were transfected with control siRNA, PRMT1 siRNA and/or Smad6 siRNA, and treated with BMP4 for the indicated times.
AP, affinity purification, IP, immunoprecipitation; IB, immunoblotting.
See also Figure S6.

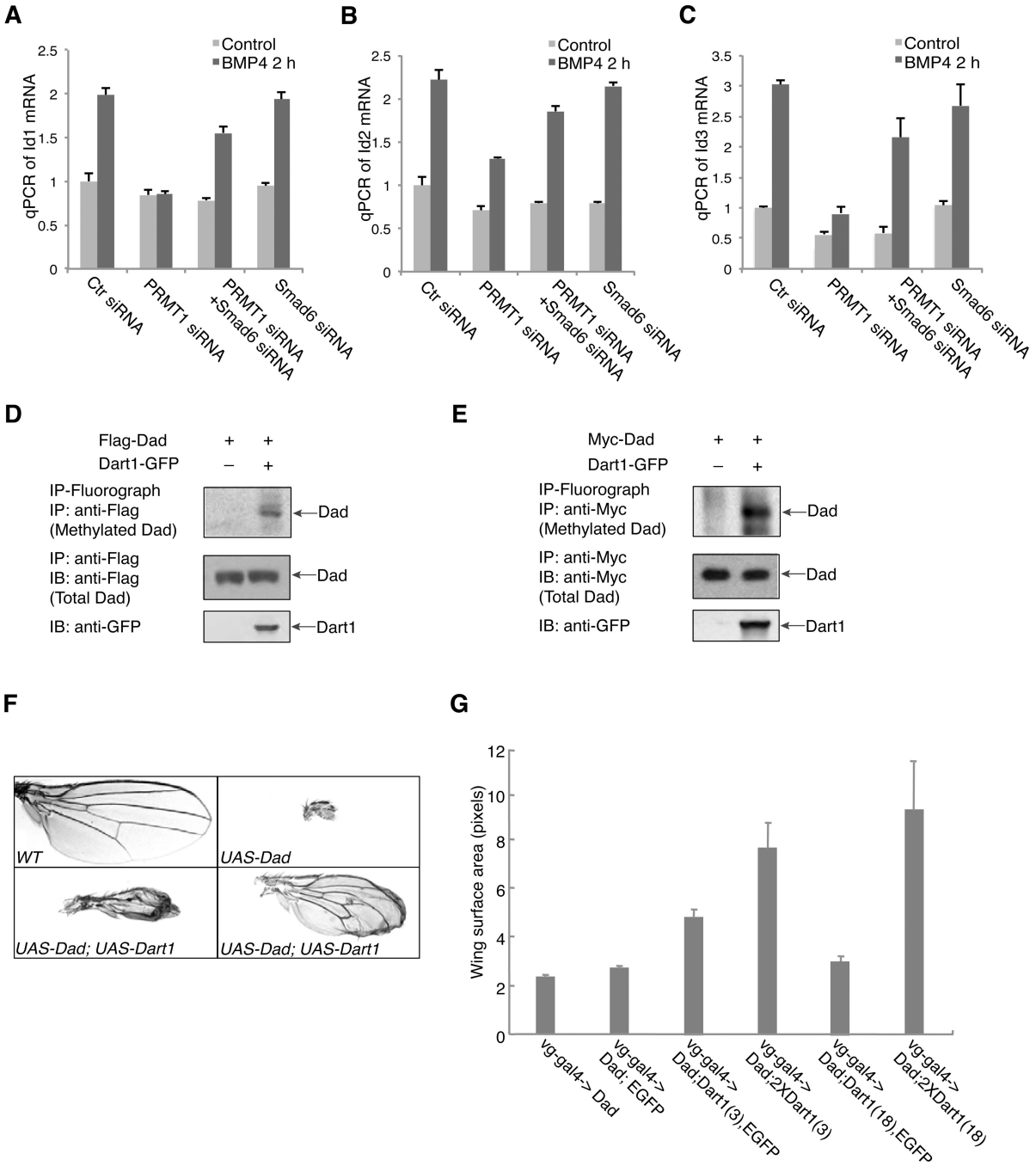


**Figure 7.**

PRMT1 and Smad6 cooperate in the control of BMP-induced biological responses.

(A-C) Decreasing Smad6 expression rescued the inhibition of BMP4-induced Id1 (A), Id2 (B) and Id3 (C) mRNA expression that resulted from silencing of PRMT1 expression. HaCaT cells were transfected with siRNA and treated with BMP4 for 2 h. mRNA levels were measured by qRT-PCR. Data are represented as mean +/− SEM.

(D-E) Dad was methylated by Dart1. Flag-Dad (D) or Myc-Dad (E) were expressed in 293T cells (D) or *Drosophila* Kc cells (E), respectively, in the absence or presence of Dart1-GFP,

and in the presence of $^{3}$H-methionine, immunoprecipitated, and subjected to SDS-PAGE, staining and autoradiography to visualize $^{3}$H incorporation.
(F-G) Increased *Dart1* expression rescued the wing phenotype of *Dad* overexpression. Two independent lines of UAS-Dart1-EGFP were crossed with Vg-Gal4 and UAS-Dad, In (D), the lower left and lower right panels showed an average rescue, while the right lower panel shows one of the best examples of rescued wings. UAS-Dart1-EGFP and Vg-Gal4;UAS-Dart1-EGFP have normal wings. (E) Quantification of wing size, scored in n=∼100 flies per group. Data are represented as mean +/− SEM.