

# **The lysine methyltransferase SMYD2 methylates the kinase domain of type II receptor BMPR2 and stimulates bone morphogenetic protein signaling**

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# Equal contribution

**Running title:** SMYD2 regulates BMP but not TGF $\beta$  signaling transduction

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## ABSTRACT

**Lysine methylation of chromosomal and nuclear proteins is a well-known mechanism of epigenetic regulation, but relatively little is known about the role of this protein modification in signal transduction. Using an RNAi-based functional screening of the SMYD family of lysine methyltransferases (KMTs), we identified SMYD2 as a KMT essential for robust bone morphogenic protein (BMP)- but not TGF $\beta$ -induced target gene expression in HaCaT keratinocyte cells. A role for SMYD2 in BMP-induced gene expression was confirmed by shRNA knockdown and CRISPR/Cas9-mediated knockout of SMYD2. We further demonstrate that SMYD2 knockdown or knockout impairs BMP-induced phosphorylation of the signal-transducing protein SMAD1/5 and SMAD1/5 nuclear localization and interaction with SMAD4. The SMYD2 KMT activity was required to facilitate BMP-mediated signal transduction, as treatment with the SMYD2 inhibitor AZ505 suppressed BMP2-induced SMAD1/5 phosphorylation. Furthermore, we present evidence that SMYD2 likely modulates the BMP response through its function in the cytosol. We show that, although SMYD2 interacted with multiple components in the BMP pathway, it specifically methylated the kinase domain of BMP type II receptor BMPR2. Taken together, our findings suggest that SMYD2 may promote BMP signaling by directly methylating BMPR2, which, in turn, stimulates BMPR2 kinase activity and activation of the BMP pathway.**

Lysine methylation has been extensively studied in the context of histone proteins as a mechanism of epigenetic

regulation (1-3). With the presence of large number of lysine methyltransferases (KMTs) in mammalian genomes (4,5), it is not surprising that an increasingly larger number of non-histone chromosomal, nuclear and cytoplasmic proteins have been found to be lysine methylated (6-9). Lysine methylation can be dynamically removed by the action of demethylases (10,11), making it a feasible mechanism for signal transduction. However, up to now lysine methylation has been shown to regulate signaling proteins only in a limited cases. For instances, methylation of MAP3K2 by SMYD3 has been shown to increase MAP kinase signaling and promotes the formation of Ras-driven carcinomas (12), whereas methylation of VEGFR1 by SMYD3 activates its kinase activity (13), indicating that lysine methylation can play important roles in regulation of signal transduction.

TGF- $\beta$ /BMP superfamily of cytokines play pleiotropic roles in embryonic development, differentiation, organ morphogenesis and tissue homeostasis (14,15). These cytokines bind the cell surface type I and type II receptors that are also serine-threonine kinases (16,17). Upon ligand binding, the type II receptor activates the type I receptor by phosphorylating the GS motif of type I receptor (18). The activated type I receptor in turn phosphorylates regulatory SMADs (R-SMADs) at the C-terminal SSXS motif. This phosphorylation disrupts inhibitory intramolecular interaction between MH2 and MH1 domains of R-SMADs and stimulates R-SMADs to form a heteromeric complex with the Co-SMAD protein SMAD4, which can translocate to the nucleus and regulate target gene expression (19,20). The specificity of TGF $\beta$  and BMP pathways are largely mediated by distinct R-SMADs. TGF $\beta$  pathway typically

activates SMAD2/3, while BMP pathway typically activates SMAD1/5/8 (14,21). Given its functional significance, the TGF $\beta$ /BMP pathway is tightly regulated at different levels through various mechanisms to ensure that signaling is controlled in a spatial and temporal manner (21,22). However, except that the methyltransferase SET9 has recently been reported to potentiate TGF $\beta$  signaling by methylating SMAD7 (23), it is not known prior to our study if other component(s) of the TGF $\beta$ /BMP signaling pathway is also regulated by lysine methylation.

To investigate if the TGF $\beta$ /BMP pathway is regulated by lysine methylation, we focused our attention on the SMYD family KMTs. Characterized by a split SET catalytic domain inserted with a zinc finger MYND motif, the SMYD family KMTs include five members, SMYD1-5 (24). Unlike many other KMTs that are localized mainly in the nucleus, the SMYD KMTs are abundantly cytoplasm and thus could have a regulatory role in early stages of signal transduction (12,24,25). In support of this idea, SMYD3 has been shown to methylate MAP3K2, linking MAP3K2 lysine methylation to Ras-driven cancer (12). Using an RNAi-based functional screening, here we identified SMYD2 as a positive regulator for BMP2- but not TGF $\beta$ -induced target gene expression. We provided evidence that SMYD2 promotes BMP2-induced SMAD1/5 phosphorylation, SMAD1/5 nuclear localization and interaction with SMAD4. We show that SMYD2 specifically methylates the kinase domain of BMP type II receptor BMPR2. Taken together, our study suggests a working model that SMYD2 may positively regulate BMP signaling by directly methylating BMPR2.

## RESULTS

*An RNAi-based screening reveals a role for SMYD2 in BMP- but not TGF $\beta$ -induced target gene expression* - To investigate the potential function of SMYD family KMTs in TGF $\beta$  and/or BMP signaling transduction, we performed RNA interference to knockdown individual SMYD in HaCaT cells, a spontaneously immortalized, human keratinocyte line widely used for studies of TGF $\beta$ /BMP response, with specific siRNAs. To minimize the off-target effect of siRNA screening, two different siRNA were employed for each SMYD gene and the result was scored positive only when it was observed for both siRNAs. Two days after siRNA treatment, HaCaT cells were cultured with serum free medium overnight and then treated with TGF $\beta$ 1 or BMP2 for 4h before harvested for analysis of TGF $\beta$  or BMP-induced target gene expression by quantitative reverse transcription PCR (qRT-PCR). Through multiple experiments, we did not observe a reproducible effect of SMYD knockdown on TGF $\beta$ 1-induced expression of three representative TGF $\beta$  target genes p21, PAI1, and SMAD7 (26) (Fig. 1A). However, siRNA knockdown of SMYD2 consistently impaired basal and BMP2-induced transcriptional activation of three BMP target genes ID1, ID2 and SMAD6 (27), while knockdown of other SMYD proteins had no clear effect (Fig. 1B). These results suggest a potential role for SMYD2 in promoting BMP2-induced target gene expression.

*Validating the role of SMYD2 in BMP signaling pathway* - To validate the results of our RNAi-based screen, we constructed two shRNAs against SMYD2 that targeted at different sequences of SMYD2 from the two siRNAs used in our screen in lentiviral vector. Stable infection of HaCaT cells with

lentiviral shRNAs resulted in substantial knockdown of SMYD2 as revealed by Western blotting analysis (Fig. 2A). Importantly, we observed a significantly diminished induction of BMP target genes ID1, ID2, ID3 in both shSMYD2 stably transfected HaCaT cells in comparison to the control HaCaT cells (Fig. 2B). In contrast, the expression of TGF $\beta$  downstream genes p21, PAI1 and SMAD7 were not significantly affected by SYMD2 knockdown (Fig. 2C). Thus, consistent with our siRNA screening results, SMYD2 knockdown by distinct shRNAs also selectively impairs the BMP signaling pathway without affecting the TGF $\beta$  pathway. To further substantiate this observation, we made use of CRISPR/CAS9 technology to knockout SMYD2 in HaCaT cells. We verified deletion of various fragments in SMYD2 coding region by DNA sequencing (data not shown) and confirmed the loss of SMYD2 proteins by Western blotting analysis in isolated clones #2 and #16 (Fig. 2D). Subsequent qRT-PCR analysis demonstrated that SMYD2 knockout indeed impaired BMP2-induced downstream gene activation but had no effect on TGF $\beta$ -induced target gene activation (Fig. 2E and 2F). Together these data reveal a unique role of SYMD2 in the BMP but not TGF $\beta$ -induced signaling pathway.

*SMYD2 knockdown or knockout impairs BMP2-induced SMAD1/5 phosphorylation*  
- To investigate the molecular mechanism(s) by which SMYD2 regulates the BMP signaling pathway, we first compared the status of BMP-induced SMAD1/5 phosphorylation by using a commercial antibody that recognizes both phosphorylated SMAD1 and SMAD5 in control HaCaT cells and HaCaT cells stably

expressed shSMYD2-1, which down-regulated SMYD2 more effectively than shSMYD2-2 (Fig. 2A). As expected, BMP2 treatment resulted in increased levels of SMAD1/5 phosphorylation in a time course from 20 min to 60 min and maintained a high level of phosphorylation upon to 150 min (Fig. 3A). Knockdown of SMYD2 substantially impaired BMP-induced SMAD1/5 phosphorylation (Fig. 3A). However, no significant difference in the levels of SMAD5 and the BMP type2 receptor BMPR2 was observed between the control and SMYD2 knockdown cells. Consistent with these observations, additional experiments in Fig. 3B show a reduced BMP2-induced SMAD1/5 phosphorylation in SMYD2 knockdown cells, yet there was no difference in the levels of SMAD1, SMAD5 and SMAD4 between the control and SMYD2 knockdown cells. We further tested the role of SMYD2 in BMP-induced SMAD1/5 phosphorylation in SMYD2 knockout HaCaT cell lines #2 and #16. As shown in Fig. 3C, SMYD2 knockout had no effect on the levels of SMAD1, SMAD5, type 2 receptor BMPR2 and type 1 receptor ALK3 proteins. However, SMYD2 knockout markedly reduced the levels of BMP2-induced SMAD1/5 phosphorylation. Together these data clearly reveal a role for SMYD2 in promoting BMP2-induced SMAD1/5 phosphorylation.

*SMYD2 knockdown and knockout impair BMP2-induced SMAD1/5 nuclear translocation, complex formation with SMAD4 and inhibition of cell proliferation*  
- Phosphorylation of SMAD1/5 promotes translocation of SMAD1/5 from cytoplasm to nucleus and interaction with Co-SMAD SMAD4 (14). Having observed that knockdown or knockout of SMYD2 impaired BMP2-induced SMAD1/5 phosphorylation, we next tested its effect

on SMAD1/5 nuclear entry and interaction with SMAD4. As shown in Fig. 4A, we observed that knockdown of SMYD2 indeed reduced BMP2-induced SMAD1 accumulation in nucleus. Similarly, we found that BMP2-induced SMAD1 nuclear accumulation was substantially reduced in SMYD2 KO #16 cells. Furthermore, by co-immunoprecipitation assay we observed that, while BMP2 treatment stimulated the association of SMAD4 with SMAD1 in the control wild-type cells, much less SMAD4 was found to co-immunoprecipitate with SMAD1 in BMP2-treated SMYD2 KO #16 cells (Fig. 4C). Thus, consistent with an impaired BMP2-induced SMAD1/5 phosphorylation in SMYD2 knockdown or knockout cells, BMP2-induced SMAD1 nuclear entry and the interaction with SMAD4 are also impaired upon SMYD2 knockdown or knockout.

We next tested if SMYD2 knockout affected BMP-induced biological effect such as inhibition of cell proliferation in HaCaT cells. Compared to parent HaCaT cells, SYMD2 KO reduced cell proliferation (Fig. 4D), suggesting that SMYD2 may regulate cell proliferation through unknown mechanism. Nevertheless, while addition of BMP2 inhibited the proliferation of the wild-type HaCaT cells, addition of BMP2 had no significant inhibition on the proliferation of SMYD2 KO cells (Fig. 4D). Thus, SMYD2 not only promotes BMP2-induced gene expression but also BMP2-induced inhibition of cell proliferation.

*SMYD2 regulates BMP signaling in cytoplasm and requires its KMT activity* - Our findings that SMYD2 knockdown or knockout impairs BMP2-induced SMAD1/5 phosphorylation suggest that SMYD2 is likely to regulate BMP signaling at a step(s) in cytoplasm before SMAD1/5

nuclear translocation. To pinpoint its role in BMP signaling pathway, we first examined the subcellular localization of SMYD2 and the effect of BMP2 treatment on its subcellular localization in HaCaT cells. As expected, we observed that BMP2 treatment led to time-dependent reduction of cytosol SMAD1 and accumulation of nuclear SMAD1 (Fig. 5A). Also as expected, BMP2 treatment led to increased levels of phosphorylated SMAD1/5 both in cytosol and nucleus. However, SMYD2 remained in cytoplasm through BMP2 treatment (Fig. 5A). The appropriate fractionation of cytoplasm and nucleus fraction was confirmed by Western blotting analysis of cytosol marker GAPDH and nuclear marker Lamin A/C (Fig. 5A). Furthermore, by immunofluorescent staining we observed that, although BMP treatment induced rapid SMAD1 nuclear accumulation (Fig. 4A and 4B), SMYD2 remained in cytoplasm throughout the treatment (Fig. 5B). Together these data suggest that SMYD2 regulates BMP signaling in the cytoplasm and is unlikely to exert its regulatory role through a nuclear function.

To examine if the KMT activity is required for SMYD2 to regulate the BMP signaling pathway, we resorted to a recently identified potent and selective SMYD2 inhibitor AZ505 (28). AZ505 was shown to inhibit SMYD2 KMT activity with an IC<sub>50</sub> of 0.12  $\mu$ M, whereas the IC<sub>50</sub> for other KMTs is more than 83.3  $\mu$ M (28). We found that treatment of HaCaT cells with 1.2  $\mu$ M and 12  $\mu$ M of AZ505 was sufficient to inhibit BMP2-induced SMAD1/5 phosphorylation without affecting the protein levels of SMYD2 and SMAD5 (Fig. 5C). Considering that knockdown of SMYD2 but not other SMYD proteins impaired the BMP signaling pathway (Fig.



1), these results provide evidence that inhibition of SMYD2 KMT activity is sufficient to impair BMP signaling transduction. To substantiate this further, we tested if ectopic overexpression of an enzymatic inactive form of SMYD2, Y240A mutant (M), would act as a dominant negative to inhibit BMP2-induced SMAD1/5 phosphorylation. As shown in Fig. 5D, we observed that while ectopic expression of the wild-type SMYD2 further enhanced BMP2-induced SMAD1/5 phosphorylation, ectopic expression of SMYD2 mutant actually inhibited the BMP2-induced SMAD1/5 phosphorylation (Fig. 5D). Taking the consideration that SMYD2 mutant was expressed only 2-3 fold more than the level of endogenous SMYD2 proteins (Fig. 5D), the observed inhibition of BMP2-induced SMAD1/5 phosphorylation provides compelling evidence for a dominant negative effect of the SMYD2 KMT-deficient mutant.

*SMYD2 interacts with multiple components in the BMP pathway* - As SMYD2 requires its catalytic activity to facilitate BMP signaling transduction, we predicted that SMYD2 may regulate BMP signaling through methylating one or more components in the BMP signaling pathway. In order to identify potential SMYD2 substrates, we first aimed to examine the interaction of SMYD2 with various components of the BMP/TGF $\beta$  signaling pathways including SMADs, SMURFs and BMPR2. To this end, 293T cells were cotransfected with SMYD2 and Flag-tagged SMADs, SMURF1/2 or BMPR2, and co-immunoprecipitations were performed with anti-Flag antibody (Fig. 6A). Interestingly, we found that SMYD2 co-immunoprecipitated with multiple proteins in the BMP and TGF $\beta$  pathways, including SMAD1-4, SMAD7, SMURF2

and BMPR2. The observed interaction was likely authentic since SMYD2 was not precipitated by anti-Flag antibody when expressed alone (Fig. 6A, lane 1). As SMYD2 specifically affected BMP but not TGF $\beta$  pathway, we further analyzed if BMP2 treatment regulated the interaction of SMYD2 with SMAD1, SMAD4 and inhibitory SMAD7 by co-immunoprecipitation assay. We confirmed that SMYD2 co-immunoprecipitated with Flag-tagged SMAD1, SMAD4 and SMAD7 (Fig. 6B). Furthermore, we observed that BMP treatment enhanced the interaction between SMYD2 and SMAD1, but not the interaction between SMYD2 and SMAD4 and SMAD7 (Fig. 6B). Such a BMP-induced SMYD2-SMAD1 interaction was further confirmed by reciprocal co-immunoprecipitation using HA-SMAD1 and Flag-SMYD2 (Fig. 6C). Finally, the interaction between SMYD2 and BMPR2 was also confirmed by reciprocal co-immunoprecipitation using HA-BMPR2 and Flag-SMYD2, although in this case the interaction was not enhanced by BMP treatment (Fig. 6D).

We further characterized if the endogenous SMYD2 and BMPR2 interacted with each other by co-immunoprecipitation assay. The representative results in Fig. 6E show reciprocal co-immunoprecipitation of SMYD2 and BMPR2. Furthermore, this interaction appears to be constitutive and not affected by BMP2 treatment (Fig. 6F).

*SMYD2 methylates BMPR2 at its kinase domain* - Next, we investigated which component(s) of BMP/TGF $\beta$  pathway could be methylated by SMYD2. 293T cells were co-transfected with SMYD2 and Flag-tagged SMADs, SMURFs or BMPR2 for 48 h. Subsequently Flag-SMADs, SMURFs or BMPR2 were

immunoprecipitated and examined for methylation by Western blotting analysis using a pan specific mono/dimethylated lysine antibody (Fig. 7A). Despite of the broad interaction as observed above, none of the proteins except for BMPR2 were found to be methylated by SMYD2 under our experimental conditions (Fig. 7A). We confirmed that SMYD2 methylated BMPR2 and this methylation was dependent on the enzymatic activity of SMYD2, as it was not observed when the enzymatic inactive SMYD2 Y240A mutant was co-expressed with BMPR2 (Fig. 7B). In addition, under the same conditions SMYD2 did not appear to methylate type I receptors ALK1, ALK3, ALK5, ALK6 and ALK7 (data not shown). These results indicate that SMYD2 specifically methylates BMPR2. To lend further support that SMYD2 could methylate BMPR2, we expressed and purified recombinant 6xHis-SMYD2 from bacteria and performed *in vitro* methylation assay with Flag-BMPR2 immunoprecipitated from transfected 293T cells as substrate. The *in vitro* methylation reaction showed that purified SMYD2 methylated BMPR2 (Fig. 7C).

We also mapped which domain of BMPR2 could be methylated by SMYD2. As SMYD2 is cytoplasmic and thus most likely methylates the intracellular portion of BMPR2, we divided the intracellular part of BMPR2 into three fragments, namely kinase domain (KD, residues 179-504), C-terminal I (CT1, 504-800) and C-terminal II (CT2, 791-C). We also generated additional BMPR2 constructs without or with the kinase domain as illustrated in Fig. 7D. When these constructs were co-expressed with SMYD2 or SMYD2 Y240A mutant in 293T cells, strong methylation was detected for the constructs containing BMPR2 kinase domain, whereas a weak

methylation signal was also detected for CT domain (Fig. 7D). Together these results demonstrate that SMYD2 preferentially methylates BMPR2 within its kinase domain.

## DISCUSSION

In recent years, although there have been more and more reports on regulatory functions of lysine methylation on non-histone proteins (6-8), very few have linked lysine methylation to membrane receptors and their downstream signaling. In this study, we chose to explore if primarily cytoplasmically localized SMYD family KMTs regulate TGF $\beta$ /BMP signal transduction. Via a functional siRNA screening, we identified SMYD2 as a regulator specific for the BMP but not TGF $\beta$  pathway. By various loss of functional assays, we demonstrate that SMYD2 has a role in BMP-induced SMAD1/5 phosphorylation. Consequently, loss of SMYD2 also impairs SMAD1 nuclear localization and SMAD1-SMAD4 interaction. Furthermore, we show that SMYD2 methylates the BMP type 2 receptor BMPR2 at its kinase domain. Together these results indicate that SMYD2 likely promotes BMP signaling through methylating BMPR2, which in turn facilitates SMAD1/5 phosphorylation, nuclear entry and interaction with SMAD4 and consequently BMP target gene expression.

One of the interesting observation in our study is that, while SMYD2 interacts with multiple components of the BMP and TGF $\beta$  signaling pathways (Fig. 6), it only methylates BMPR2 (Fig. 7). So far SMYD2 has been identified as a KMT catalyzing specific lysine monomethylation in diverse proteins (28,29), including the famous

tumor suppressor proteins p53 (30), RB (31,32) and PTEN (33). A recent comprehensive, large-scale proteomic study of lysine mono-methylation has identified several hundreds of potential SMYD2 methylation sites, and a subset of 35 sites were confirmed by SMYD2 knockdown (34). Although several studies have provided insights into the diversity of SMYD2 substrates, these studies also underscore a striking substrate selectivity for SMYD2, manifested as the binding of substrate peptides to the deep pocket of SMYD2 and extensive interaction of substrate peptides with SMYD2 (28,29). We suggest that the multiple interactions with BMP pathway components may help the recruitment of SMYD2 into the signaling transduction process and facilitate its methylation on BMPR2. The physiological role for the interaction between SMYD2 and multiple TGF $\beta$ /BMP pathway components, if any, remains to be investigated in future study.

It is well-established that the BMP signaling cascades begin with binding of ligands and activation of type 2 receptor kinase activity (14). Given our compelling evidence that SMYD2 methylates BMPR2 and remains in the cytoplasm upon BMP induction and that loss of SMYD2 impairs BMP2-induced SMAD1/5 phosphorylation, it is tempting to propose a working model in Fig. 7E as how SMYD2 selectively regulates the BMP pathway. In this model, SMYD2 methylates BMPR2 at its kinase domain to promote its phosphorylation on a type 1 receptor. The activated type 1 receptor then phosphorylates SMAD1/5 and promotes SMAD1/5 nuclear entry and interaction with SMAD4, and consequently promotes BMP-induced target gene expression. SMYD2-catalyzed BMPR2 methylation is likely functionally important for the BMP signaling pathway, since

inhibition of SMYD2 using a SMYD2-specific inhibitor AZ505 or ectopic expression of a KMT-deficient SMYD2 mutant both impaired BMP2-induced SMAD1/5 phosphorylation (Fig. 5C and 5D). At this stage, it is not known if SMYD2 constitutively methylates BMPR2 or methylates BMPR2 in a BMP-induced manner. So far we have not been able to convincingly detect endogenous methylated BMPR2 in the presence or absence of BMP treatment. Although SMYD2 interacts with BMPR2 constitutively (Fig. 6E and 6F), we could not rule out the possibility that SMYD2 methylates BMPR2 only when it is activated by ligands. In this regard, it is noteworthy that arginine methylation by PRMT1 has been shown to regulate BMP signaling (21). PRMT1 was shown to bind BMPR2, and BMP treatment activates complex formation between type I and II receptors and brings PRMT1 and inhibitory SMAD6 into proximity. PRMT1 then methylates SMAD6 on Arg 74 and Arg 81 and releases SMAD6 from the receptors, allowing SMAD1/5 to bind type I receptor and be activated (21).

The identification of SMYD2 as a positive regulator of the BMP pathway and BMPR2 as a substrate for SMYD2 methylation provides new insight into the signaling pathway of BMP. However, multiple questions remain to be addressed. For example, it is necessary in future to identify the methylation site(s) in BMPR2 kinase domain and investigate how methylation regulates BMPR2 kinase activity. In addition, the finding that BMPR2 can be methylated by SMYD2 also raises the question if BMPR2 is regulated by dynamic methylation/demethylation, and if does, the demethylase(s) involved.



A previous study has reported lysine methylation of membrane receptor VEGFR1 by SMYD3 (13). Methylation at K831 of VEGFR1 by SMYD3 enhances VEGFR1 tyrosine autophosphorylation and kinase activity (13). Here we show BMPR2 methylation by SMYD2 and a novel role for SMYD2 in promoting BMP signaling pathway. Thus, consistent with their primary cytoplasmic localization, the SMYD family KMTs may play unique roles in methylation of membrane receptors and regulation of signaling transduction.

## EXPERIMENTAL PROCEDURES

**Cell lines and Reagents** - Human HaCaT and 293T cell lines are from ATCC. Culture medium for HaCaT cells were RPMI 1640 (Gibco) and 10% fetal bovine serum, and culture medium for 293T cells were high glucose DMEM (Gibco) plus 10% fetal bovine serum. Reagents include Total RNA Extraction (Biorezyme), reverse transcription, quantitative real time PCR (qRT-PCR) and DNA purification kits (TransGen), DNA transfection kit and protease and phosphatase inhibitor cocktails (Biotool), Lipofectamine 2000 (Invitrogen), BMP2 (GenScript), TGF $\beta$ 1 (BD Biosciences), restriction enzymes and T4 DNA ligase and PNK (NEB), G-10 agarose (BioWest), Flag M2 and HA affinity purification beads (Abmart), and Ni<sup>+</sup> affinity purification column (Qiagen). The sequence information for siRNAs and shRNAs are as follow: siSMYD1-1, 5'-GGA GGA UGG UGG ACG GCU AUA-3'; siSMYD1-2, 5'-AGA ACG AAU UCA UGU ACU ACA-3'; siSMYD2-1, 5'-GGA AAG AAG GAU UGU CCA AAU-3'; siSMYD2-2, 5'-GGC AGA AGU CAG AGC UGU ACA-3'; siSMYD3-1, 5'-ACU GUU CGA UUG UGU UCA AUG-3'; siSMYD3-2, 5'-GGA GUC AAA UAU

UAA CAA ACU-3'; siSMYD4-1, 5'-CCA AGA UUA UGU UAC GUA AAG-3'; siSMYD4-2, 5'-GGC GAU GAC CAC CAU ACA ACA-3'; siSMYD5-1, 5'-GCA ACU GGA GAG UUU CUU AAC-3'; siSMYD5-2, 5'-GGA GGA AAU UGU CCA UAA ACU-3'; shSMYD2-1, 5'-CGG CAA AGA TCA TCC ATA TAT-3'; shSMYD2-2, 5'-GCT GTG AAG GAG TTT GAA TCA-3'. Guide RNA sequence for CRISPR/CAS9 SMYD2 knockout: 5'-GTT AGT CTT ACA GTC TCC GA-3'. Primers for RT-PCRs: GAPDH-qPCR-F, 5'-AGC CTC AAG ATC ATC AGC AAT G-3'; GAPDH-qPCR-R, 5'-ATG GAC TGT GGT CAT GAG TCC TT-3'; ID1-qPCR-F, 5'-CTG CTC TAC GAC ATG AAC GG-3'; ID1-qPCR-R, 5'-GAA GGT CCC TGA TGT AGT CGA T-3'; ID2-qPCR-F, 5'-AGC ACT GTG TGG CTG AAT AAG-3'; ID2-qPCR-R, 5'-AGT AAG AGA ACA CCC TGG GA-3'; ID3-qPCR-F, 5'-ATC CTA CAG CGC GTC ATC G-3'; ID3-qPCR-R, 5'-CTT CCG GCA GGA GAG GTT C-3'; SMAD6-qPCR-F, 5'-CCT ACT CTC GGC TGT CTC CT-3'; SMAD6-qPCR-R, 5'-GAA TTC ACC CGG AGC AGT GA-3'; PAI-1-qPCR-F, 5'-ACC GCA ACG TGG TTT TCT CA-3'; PAI-1-qPCR-R, 5'-TTG AAT CCC ATA GCT GCT TGA AT-3'; p21-qPCR-F, 5'-TGA GCC GCG ACT GTG ATG-3'; p21-qPCR-R, 5'-GTC TCG GTG ACA AAG TCG AAG TT-3'; SMAD7-qPCR-F, 5'-TGC TCC CAT CCT GTG TGT TAA G-3'; SMAD7-qPCR-R, 5'-TCA GCC TAG GAT GGT ACC TTG G-3'.

**Plasmids and antibodies** - ppY-CAGIP-SMYD2, ppY-CAGIP-SMYD2-Y240A mutant, Flag-SMAD1/2/3/4/6/7, Flag-SMURF1/2, Flag-BMPR2, HA-BMPR2, Myc-BMPR2 truncation constructs, HA-SMAD1 and Flag-SMYD2 were either

constructed by standard recombinant DNA techniques or provided by Dr. Xin-Hua Feng. The antibodies include mouse HA, Flag, Myc, SMYD2 and GAPDH (Abmart) and rabbit SMYD2 (ABclonal), SMAD1 and BMPR2 (Cell Signaling), SMAD4, SMAD5 and SMAD1/5p (Abcam), ALK3 (Protein Tech), and Pan lysine mono/dimethylation (Pan-Kme1/2) (PTM Biolabs) antibodies.

*The siRNA transfection and TGF $\beta$ 1/BMP2 treatment* – The siRNA transfection was performed using lipofectamine 2000 essentially according to manufacturer's instruction. The final concentration for each siRNA is 100 nM. For siRNA-based screen, HaCaT cells were transfected with the siRNAs against SMYD1 to SMYD5 as indicated. Two days later, cells were starved overnight with non-serum medium and then treated with 5 ng/ml TGF $\beta$ 1 or 25 ng/ml of BMP2 for 2 or 4 hours as indicated.

*Total RNA isolation and qRT-PCR* - Total RNA isolation and qRT-PCR were all performed according to manufacturers' procedures.

*Co-immunoprecipitation assay* - For co-immunoprecipitation assay, cells were lysed in buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM KCl, 1% NP40, 8% glycerol, 1 mM EDTA, 1 mM DTT and protease and phosphatase inhibitor cocktails. The cell lysates were centrifuged at 12000 rpm for 20 min at 4°C. The supernatants were diluted with up to three volumes of 50 mM Tris·HCl (pH 7.5), 150 mM KCl, 8% glycerol, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail, mixed with antibody-loaded Protein A beads, and rotated at 4°C for 3 h or overnight. The beads were then retrieved by centrifugation at 2000 rpm for 1 min at 4°C, washed for three or four times with buffer containing

50 mM Tris·HCl (pH 7.5), 150 mM KCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, and protease inhibitor cocktail by rotation at 4°C for 5 min. The beads were boiled in 20 ml SDS-loading buffer for 5 min and samples were subjected to standard SDS-PAGE and Western blotting analysis.

*Immunofluorescence staining*  
Immunofluorescent staining was performed essentially as described (36). In brief, HaCaT cells were washed with PBS for three times and fixed in 4% paraformaldehyde in PBS for 15-20 min. The cells were then washed with PBS three times and permeabilized with 1% Triton X-100 in PBS for 10 min, followed by washing with PBS three times and blocking with 5% BSA, 0.2% Triton X-100 in PBS for 1 h. Then, the cells were stained with the indicated primary and corresponding secondary antibodies, and nuclei were revealed by DAPI staining.

*Nucleocytoplasmic fractionation* - HaCaT cells were washed with cold PBS twice, lysed on ice for 15-20 min in lysis buffer (10 mM HEPES-NaOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM  $\beta$ -mercaptoethanol, and protease and phosphatase inhibitors). Then, NP40 was added to a final concentration of 0.5%. 2 min later, cells were centrifuged at 16000g for 10-15 min. The corresponding supernatant was cytoplasmic fraction. The pellet was washed with cold PBS twice, lysed in nuclear lysis buffer (10 mM Tris-HCl, pH 7.6, 420 mM NaCl, 0.5% NP-40, and 1 mM DTT, 1 mM PMSF, 2 mM MgCl<sub>2</sub>, and protease and phosphatase inhibitors) on ice for 20 min, and centrifuged at 16000g for 10-15 min to obtain the supernatant, which was nuclear fraction. Then, lower salt buffer (10 mM Tris-HCl, pH 7.6, 1 mM DTT, 1 mM PMSF, 2 mM MgCl<sub>2</sub>, and protease and phosphatase inhibitors) was

added to adjust the final concentration of NaCl to 150 mM.

*In vitro methylation assay* - *In vitro* methylation assay was performed essentially as described (37). In brief, 0.5 $\mu$ g bacterially purified His-SMYD2 and immunoprecipitated Flag-BMPR2 were incubated in 25 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM PMSF, 67  $\mu$ M S-adenosyl methionine at 37°C for 2 h. Flag-BMPR2 methylation was then detected by SDS-PAGE and Western blotting with Pan-Kme1/2 antibody.

*Stable SMYD2 knockdown and knockout cell lines* - To knockdown SMYD2 by shRNAs, SMYD2 shRNAs with the following targeting sequences 5'-CGG CAA AGA TCA TCC ATA TAT-3' (shSMYD2-1) and 5'-GCT GTG AAG GAG TTT GAA TCA-3' (shSMYD2-2) were cloned into lentiviral vector pLKO.1-puro. HaCaT cells were infected with lentivirus expressing shRNAs of SMYD2 and control vector. Two days after infection, the stable shRNA-infected cells were selected with addition of puromycin and

cultured for two more days and used for subsequent experiments.

To generate SMYD2 knockout cell lines by CRISPR/CAS9, a small-guide-RNA (5'-G TTA GTC TTA CAG TCT CCGA-3') targeting the exon3 of SMYD2 was cloned into pLKO.1-puro based sgRNA expression vector. Guide RNA of SMYD2 was selected through on line search at <http://crispr.mit.edu>. Stable cell lines expressing sgRNA was established by infecting HaCaT cells with sgRNA lentivirus and cell cultivation with puromycin. A second infection of adenovirus expressing Cas9 resulted in SMYD2 knockout. The individual SMYD2 knockout clones were isolated through limiting dilution and verified by DNA sequencing and Western blotting analysis.

*Cell proliferation assay* - One-thousand cells were seeded per well into a 96-well plate and cells were cultured for 24, 48, 72, 96, or 120 hours. The cells were labeled with Cell Counting Kit-8 (CCK8) (Bimake, B34302) for 2 hours and proliferation rates were determined at an absorbance of 450 nm.

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**Conflict of interest:** The authors declare that they have no conflict of interest with the contents of this article.

**Author contributions:** SG and ZW performed most of the experiments. XH and WW helped in siRNA screening and RT-PCR analysis. PC helped in *in vitro* methylation assay. JL, XF, JD and JW supervised the experiments and conceived the ideas. JD and JW wrote the manuscript with SG.

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### Figure Legends.

**Figure 1.** A siRNA-based screening reveals a specific role for SMYD2 in BMP2-induced target gene expression. (A) Knockdown of SMYD family KMTs by siRNAs did not affect TGF $\beta$ 1-induced target gene activation. HaCaT cells were transfected with the siRNAs against SMYD1 to SMYD5 as indicated. Two days later, cells were starved overnight with non-serum medium and then treated with 5 ng/ml TGF $\beta$ 1 for 4 h. The cells were harvested for preparation of total RNAs and subsequent qRT-PCR analysis of PAI1, p21 and SMAD7 transcripts. (B) Knockdown of SMYD2 but not other SMYDs specifically impairs BMP2-induced target gene expression. HaCaT cells were treated as above except that TGF $\beta$ 1 was replaced with 25 ng/ml of BMP2 and qRT-PCR analysis was performed for BMP target genes ID1, ID2 and SMAD6.

**Figure 2.** Validating the role of SMYD2 in regulating BMP but not TGF $\beta$  target gene expression. (A) Western blotting analysis showing substantially reduced levels of SMYD2 in HaCaT cells stably infected with two different lentiviral-based shRNAs (shSMYD2-1 and 2). (B) The shRNA infected HaCaT cells were serum starved overnight, and then stimulated with BMP2 for 0, 2 and 4h as indicated. The cells were harvested and qRT-PCR performed to detect BMP downstream genes ID1, ID2, and ID3. (C) The shRNA infected HaCaT cells were serum starved overnight, and then stimulated with TGF $\beta$ 1 for 0, 2 and 4h as indicated. The cells were harvested and qRT-PCR performed to detect TGF $\beta$  downstream genes p21, SMAD7, and PAI1. (D) Western blotting analysis showing the absence of SMYD2 proteins in SMYD2 knockout HaCaT cell lines #2 and #16. The HaCaT knockout cell lines were generated via CRISPR/CAS9 approach as described in Experimental Procedures. (E) The SMYD2 KO #2 and #16 cell lines were serum starved overnight, and then stimulated without or with BMP2 for 4h as indicated. The cells were harvested and qRT-PCR performed to detect BMP downstream genes ID1, ID2, and ID3. (F) The SMYD2 KO #2 and #16 cell lines were serum starved overnight, and then stimulated without or with TGF $\beta$ 1 for 4h as indicated. The cells were harvested and qRT-PCR performed to detect TGF $\beta$  downstream genes p21, SMAD7, and PAI1.

**Figure 3.** Impaired SMAD1/5 phosphorylation upon SMYD2 knockdown or knockout. (A) Knockdown of SMYD2 by siRNAs impaired SMAD1/5 phosphorylation induced by BMP2. HaCaT cells were transfected with SMYD2 siRNAs (siSMYD2-1 and 2). Two days later, the cells were serum starved overnight and stimulated with BMP2 for 0, 15, 30 and 45 minutes as indicated, and the levels of SMAD5, phosphorylated SMAD1/5 (SMAD1/5ph) and SMYD2 were analyzed by Western blotting. GAPDH was shown as the loading control. (B) Knockdown of SMYD2 by shRNAs impaired SMAD1/5 phosphorylation induced by BMP2. The stable shSMYD2-1 HaCaT cells were serum starved overnight and stimulated with BMP2 for 0, 2h and 4h and subsequently analyzed by Western blotting using antibodies as indicated. (C) The stable shSMYD2-1 HaCaT cells were serum starved overnight and stimulated with BMP2 for 0, 2h and 4h and subsequently analyzed by Western blotting using antibodies as indicated. (D) Knockout of SMYD2 by CRISPR/CAS9 impaired SMAD1/5 phosphorylation induced by BMP2. The control and SMYD2 KO #2 and #16 cell lines were serum starved overnight, and then stimulated without or with BMP2 for 0, 30 and 60 minutes and subsequently analyzed by Western blotting using antibodies as indicated.

**Figure 4.** Impaired SMAD1/5 nuclear entry and interaction with SMAD4 upon SMYD2 knockdown or knockout. (A) Immunofluorescence staining analysis showing impaired BMP2-induced nuclear translocation upon knockdown of SMYD2 by siRNA. HaCaT cells were treated with or without siSMYD2-1 for two days. The cells were then serum starved overnight, stimulated without or with BMP2 for 25 minutes and processed for immunofluorescent staining analysis using an anti-SMAD1 antibody. (B) Immunofluorescence staining analysis showing impaired BMP2-induced nuclear translocation upon knockout of SMYD2. The SMYD2 KO #16 cells were serum starved overnight, stimulated without or with BMP2 for 25 minutes and processed for immunofluorescent staining analysis. (C) Impairment of BMP2-induced interaction between SMAD1 and SMAD4 upon knockout of SMYD2. Control cells and SMYD2 KO 16# cells were serum starved overnight and stimulated with BMP2 for 40 minutes. The cells were harvested and co-immunoprecipitation assay was performed using anti-SMAD1 antibody followed by Western blotting analysis using anti-SMAD4 and anti-SMAD1 antibodies. (D) SMYD2 knockout diminished cell proliferation inhibition by BMP2. The wild-type and SMYD2 KO#16 cells were treated without or with 25 ng/ml BMP2 for up to 96 hours and cell proliferation was assayed by CCK8 assay at 0, 24, 48, 72 and 96 hours as indicated.

**Figure 5.** SMYD2 regulates BMP signaling transduction in cytoplasm in a KMT activity-dependent manner. (A) Nucleocytoplasmic fractionation showing that SMYD2 remained in cytoplasm regardless BMP treatment. HaCaT cells were serum starved overnight, treated with 25 ng/ml BMP2 for various times as indicated, and then subjected to nucleocytoplasmic fractionation and Western blotting with the antibodies against SMAD1, phosphorylated SMAD1/5 and SMYD2. GAPDH and Lamin A/C served as control for the cytoplasmic and nuclear fraction, respectively. (B) Immunofluorescent staining showing that SMYD2 remained in cytoplasm upon BMP treatment. HaCaT cells were transfected with Flag-SMYD2 for 24 h followed by serum starved overnight. The cells were then treated with 25 ng/ml BMP2 for various times as indicated and subjected to immunofluorescent staining using anti-Flag antibody. Nuclei were revealed by DAPI staining. (C) SMYD2 selective inhibitor AZ505 diminished BMP2-induced SMAD1/5 phosphorylation. HaCaT cells were serum starved for 4 h and then treated with an increasing concentrations of SMYD2 inhibitor AZ505 for 6 h. The cells were then treated with (+) or without (-) 25 ng/ml BMP2 for 1h before harvested for Western blotting analysis using antibodies as indicated. Note that BMP2-induced SMAD1/5 phosphorylation was inhibited by addition of 1.2  $\mu$ M and 12  $\mu$ M of AZ505. (D) An enzymatic activity-deficient SMYD2 acted as dominant negative inhibitor of the BMP signaling. HaCaT cells were transfected with ppY-CAGIP plasmids encoding the wild-type or SMYD2 Y240A mutant at conditions of high transfection efficiency. Two days after the cells were treated without or with 25 ng/ml BMP2 for 15 min before harvested for Western blotting analysis using antibodies as indicated.

**Figure 6.** SMYD2 interacts with multiple components of the TGF $\beta$ /BMP signaling pathways. (A) Co-immunoprecipitation assay showing interaction between SMYD2 and various components of the TGF $\beta$ /BMP signaling pathways. 293T cells were co-transfected with SMYD2 and Flag-tagged SMADs, SMURFs and BMPR2. Two days after the cells were harvested for immunoprecipitations using anti-Flag antibody, followed by Western blotting analysis using anti-Flag and SMYD2 antibodies. (B) Co-immunoprecipitation



assay showing increased interaction between SMYD2 with SMAD1 upon BMP2 treatment. 293T cells were co-transfected with SMYD2 and Flag-SMAD1, Flag-SMAD4, or Flag-SMAD7 for 24 h. The cells were serum starved overnight and treated with (+) or without (-) 25 ng/ml BMP2 for 1 h, followed by co-immunoprecipitation assay using anti-Flag antibody. The co-immunoprecipitated SMYD2 were detected by Western blotting analysis. Note that BMP treatment only enhanced the SMYD2-SMAD1 interaction. (C) Reciprocal co-immunoprecipitation assay confirmed a BMP-induced interaction between SMYD2 and SMAD1. 293T cells were co-transfected with Flag-SMYD2 and HA-SMAD1 for 24 h, serum starved overnight, and treated with or without 25 ng/ml BMP2 for 1 h, followed by co-immunoprecipitation assay using antibodies as indicated. (D) BMP2 treatment did not affect the interaction between SMYD2 with BMPR2. 293T cells were co-transfected with Flag-SMYD2 and HA-BMPR2 for 24 h, serum starved overnight and treated with or without 25 ng/ml BMP2 for 1 h, followed by co-immunoprecipitation assay using antibodies as indicated. (E) Reciprocal co-immunoprecipitation assay showing the interaction between endogenous SMYD2 and BMPR2 in HaCaT cells. (F) The HaCaT cells were treated without or with 25 ng/ml BMP2 for 1 hour before harvested for co-immunoprecipitation assay as above.

**Figure 7.** SMYD2 methylates BMPR2 at its kinase domain. (A) Western blotting analysis using a pan mono/di-methylated lysine antibody revealed specific methylation of BMPR2 by SMYD2. 293T cells were co-transfected with SMYD2 and Flag-SMADs, SMURFs or BMPR2 for 48 h. Various Flag-tagged proteins were immunoprecipitated from the whole cell extracts with anti-Flag antibody, and methylation was detected by Western blotting analysis using a pan mono/di-methylated lysine antibody (Pan-Kme1/2). The methylated BMPR2 is indicated by bracket. Asterisks indicate two nonspecific bands, which were likely Hsp70, an abundant chaperone protein often heavily methylated endogenously, and IgG heavy chain. (B) Methylation of BMPR2 by SMYD2 required SMYD2 catalytic activity. 293T cells were co-transfected with Flag-BMPR2 and either wild-type SMYD2 (W) or SMYD2 Y240A mutant (M) for 48 h. The subsequent IP-Western analysis was as above. (C) SMYD2 methylated BMPR2 *in vitro*. Flag-BMPR2 was expressed in 293T cells via transient transfection and purified by anti-Flag antibody. The immune-affinity purified BMPR2 was subjected to *in vitro* methylation reactions using bacterially expressed and purified 6xHis-SMYD2 (SMYD2). The methylation of Flag-BMPR2 was detected by Western blotting analysis using Pan-Kme1/2. (D) SMYD2 methylates the BMPR2 kinase domain *in vivo*. The *Upper panel* shows various truncated BMPR2 constructs. Various Myc-tagged SMYD2 constructs were co-transfected with the wild-type (W) or SMYD2-Y240A mutant (M) for 48 h, followed by immunoprecipitation with anti-Myc antibody and Western blotting analysis with Pan-Kme1/2 and other indicated antibodies. (E) A working model illustrating how SMYD2 may potentiate BMP signaling transduction by methylating BMPR2. SMYD2 interacts with and methylates BMPR2 within its kinase domain. This methylation may promote the BMPR2 kinase activity and therefore phosphorylation of the type 1 receptor. The activated type 1 receptor then phosphorylates SMAD1/5 and promotes SMAD1/5 nuclear entry and interaction with SMAD4, and consequently promotes BMP-induced target gene expression.

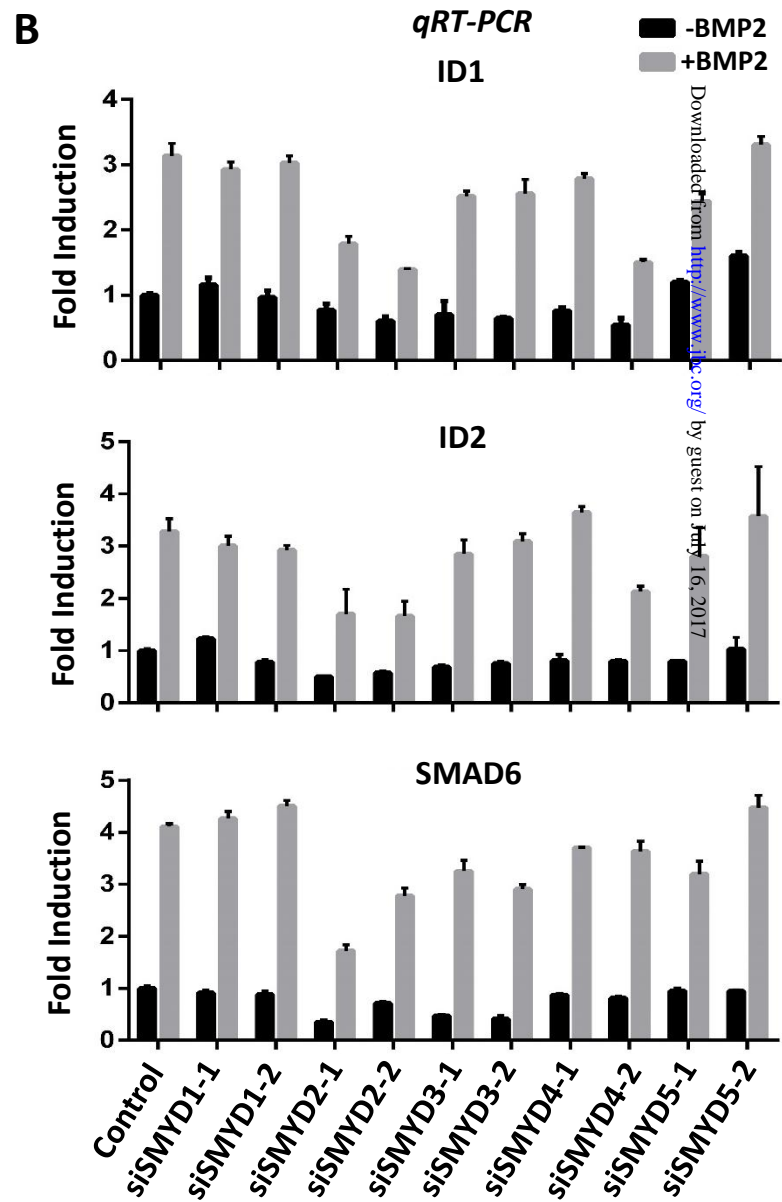
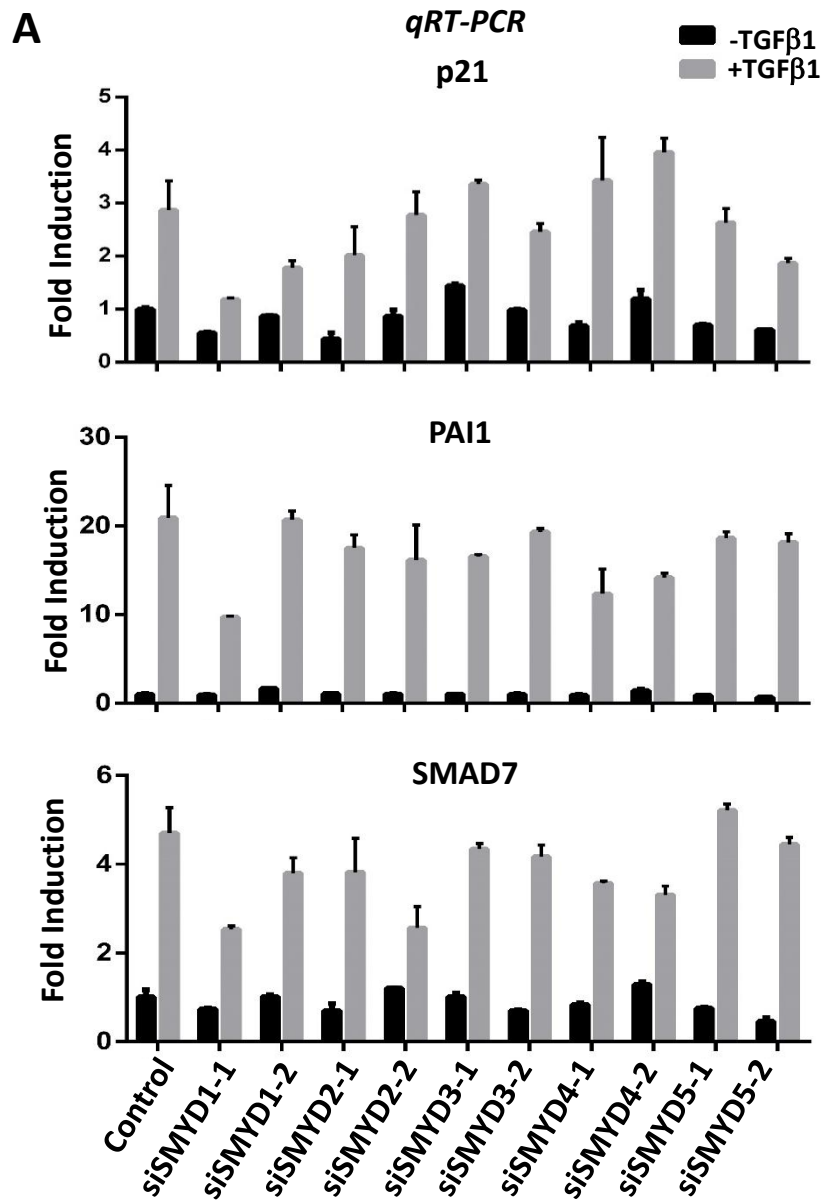


Fig. 1

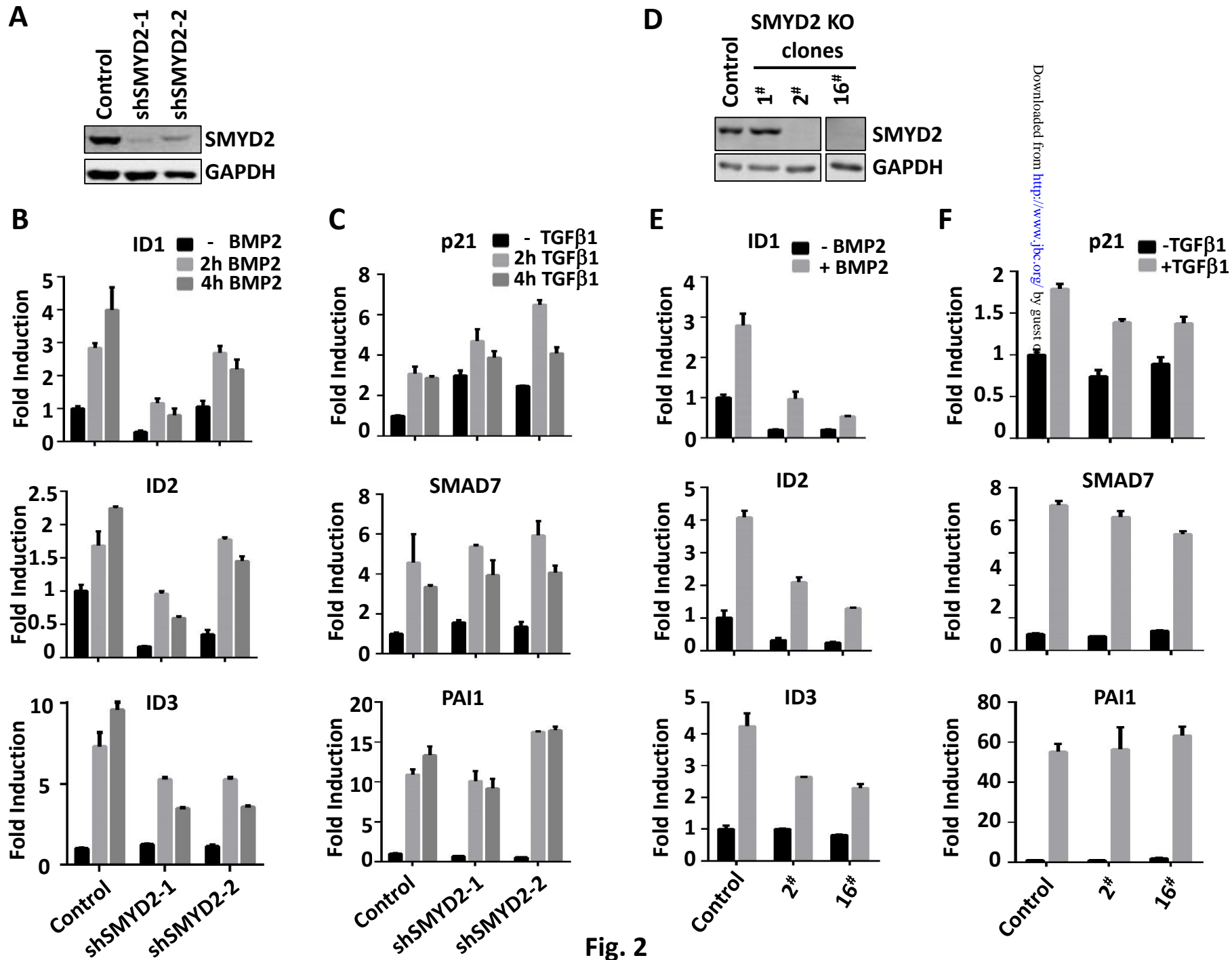
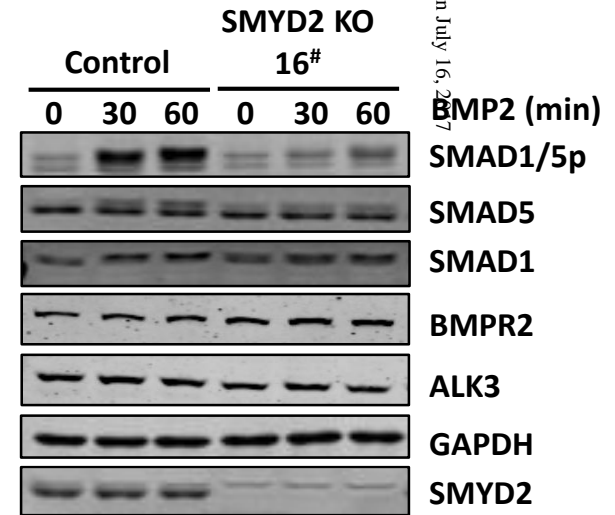
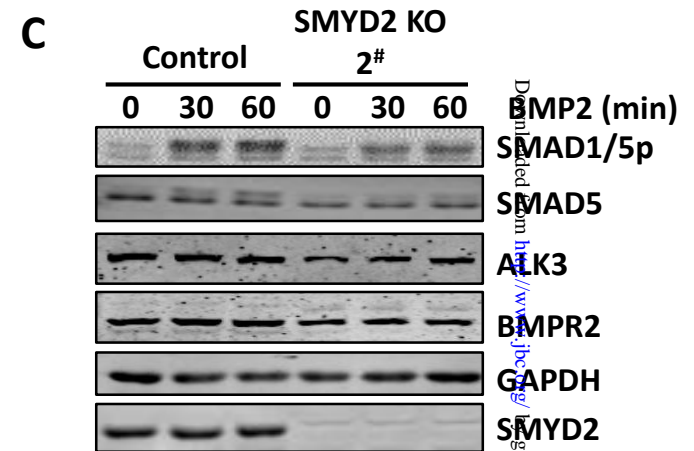
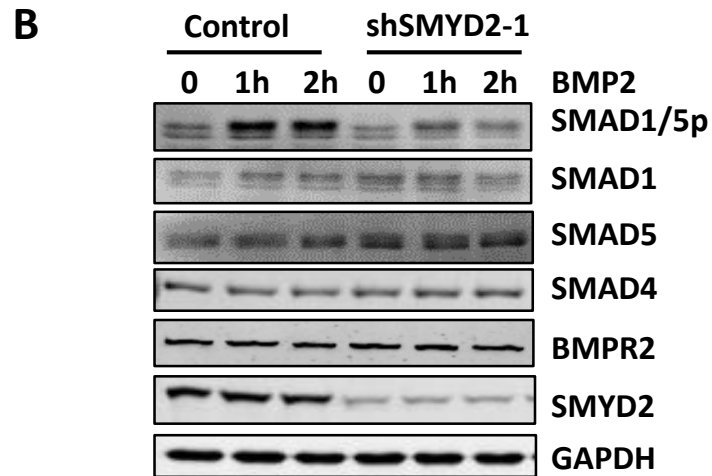
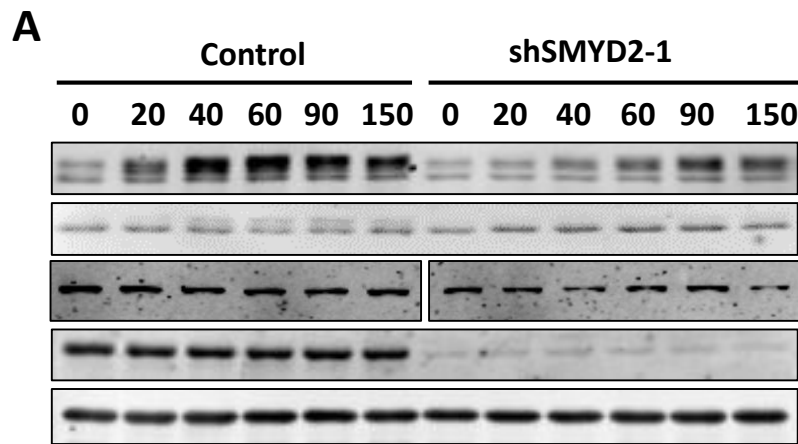


Fig. 2



**Fig. 3**



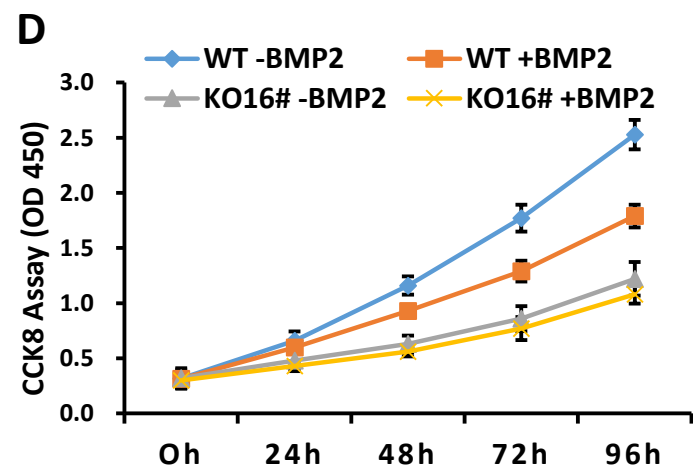
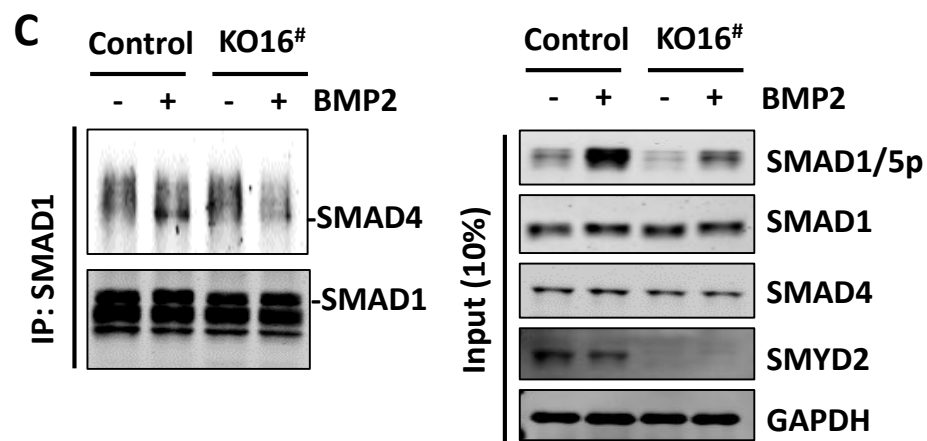
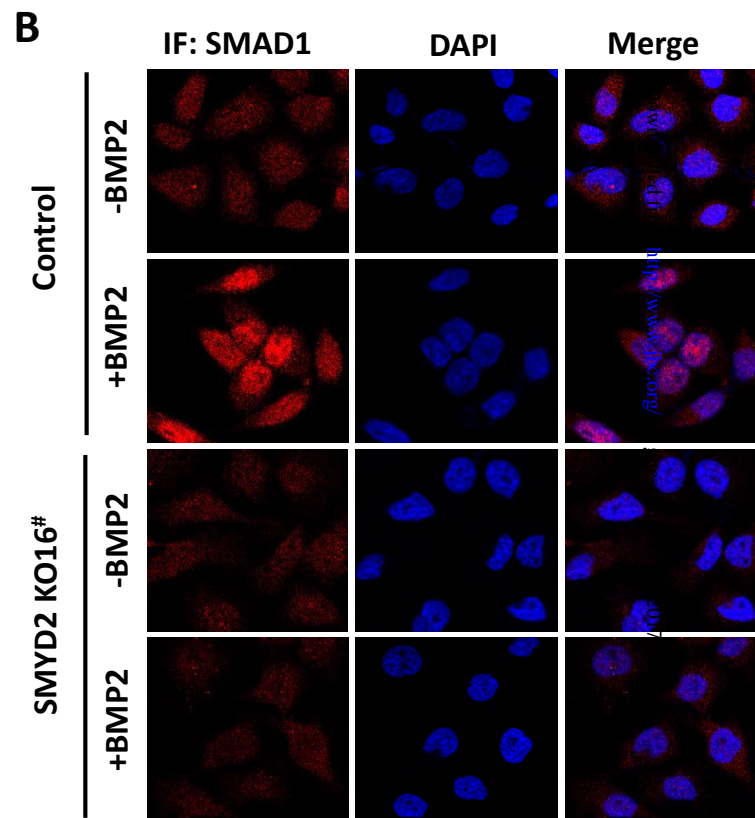
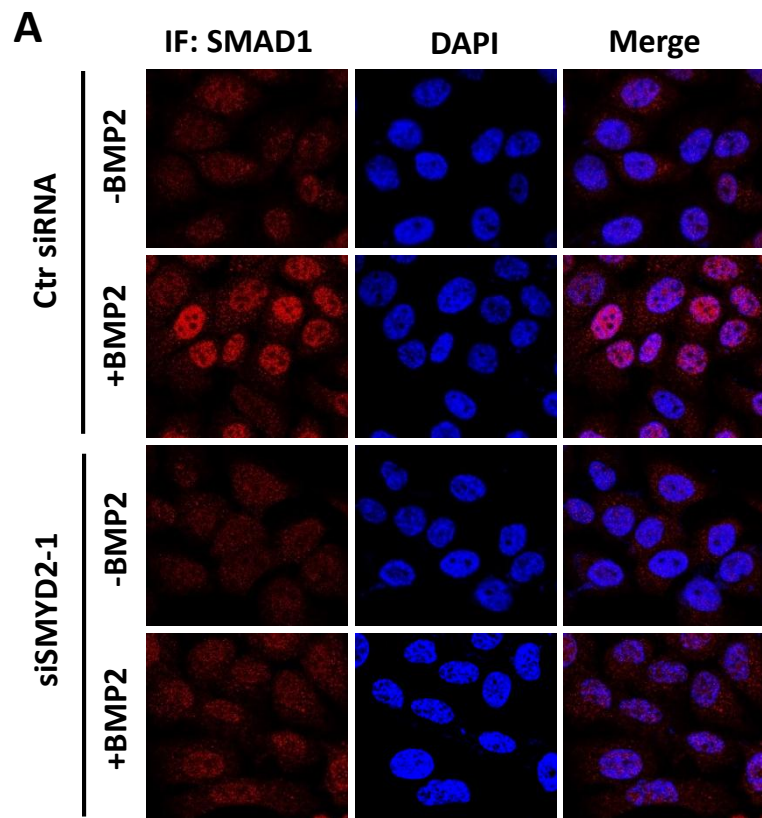


Fig. 4

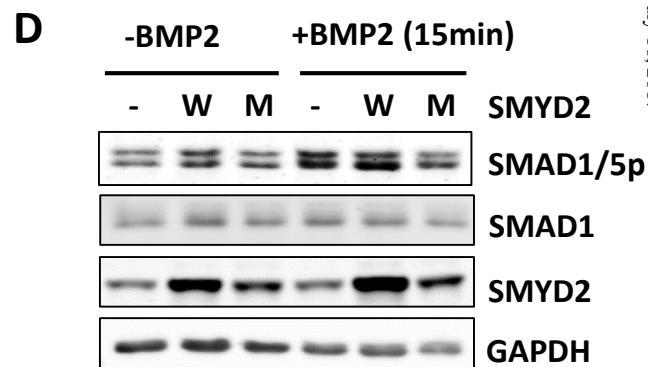
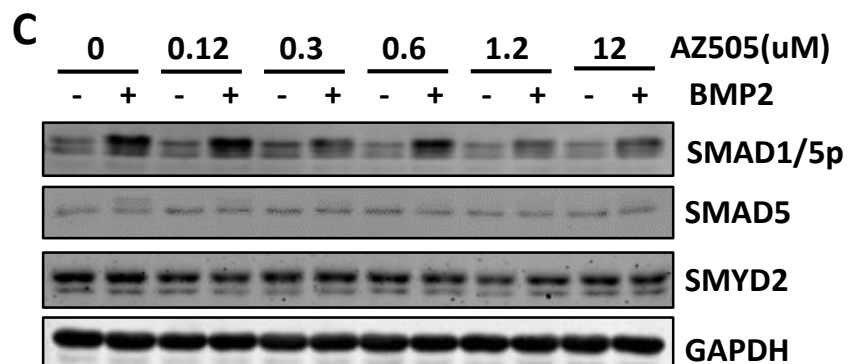
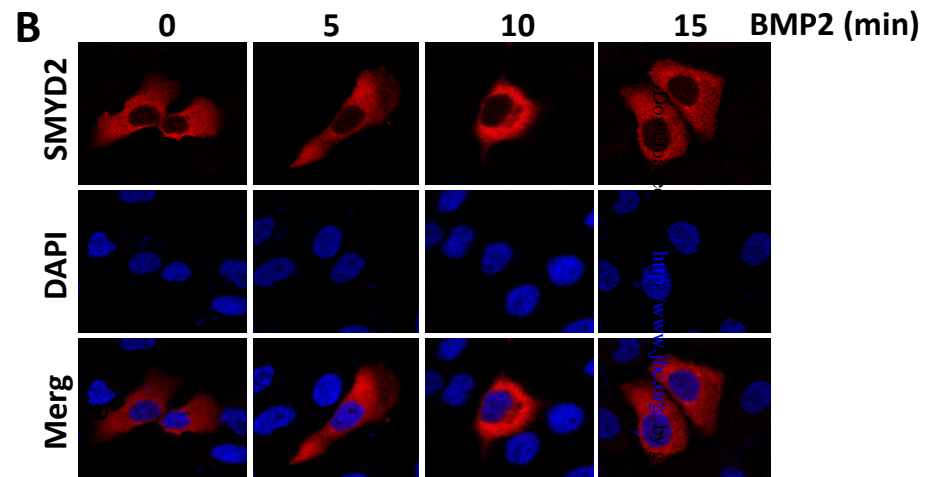
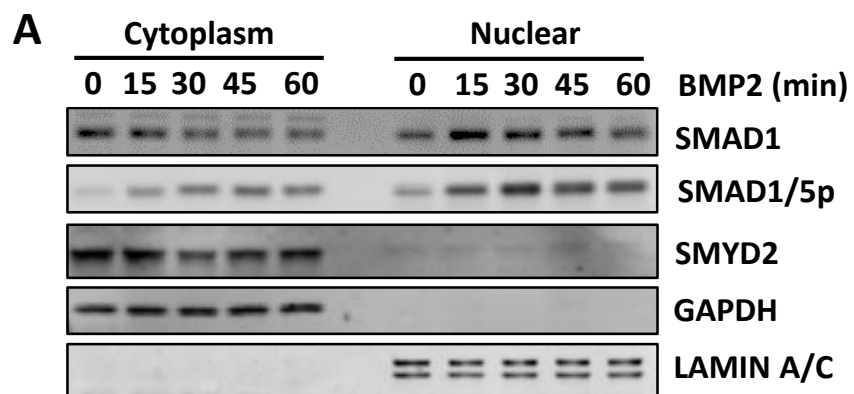
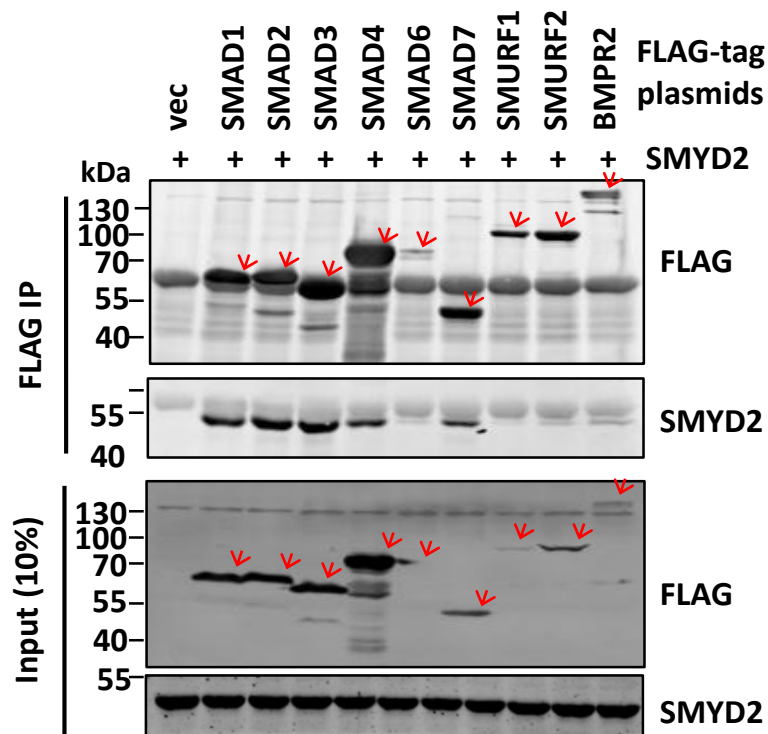
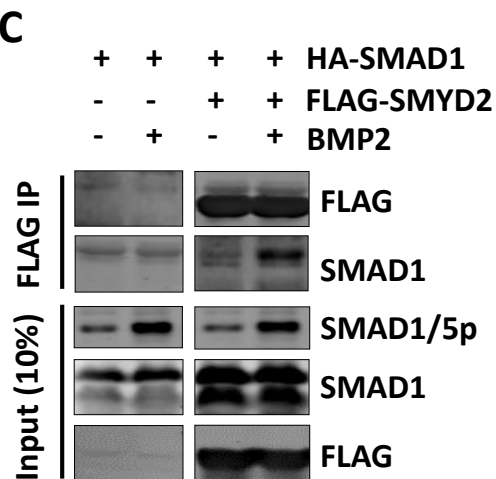


Fig. 5

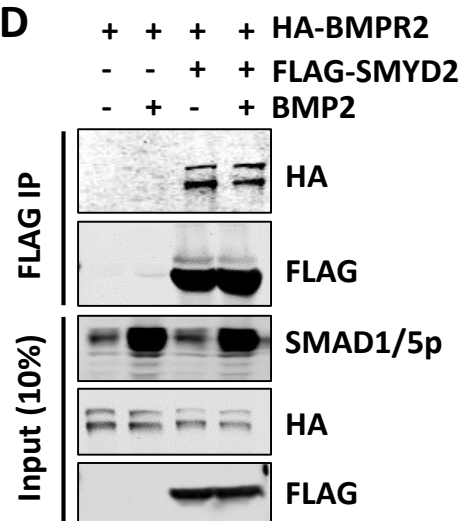
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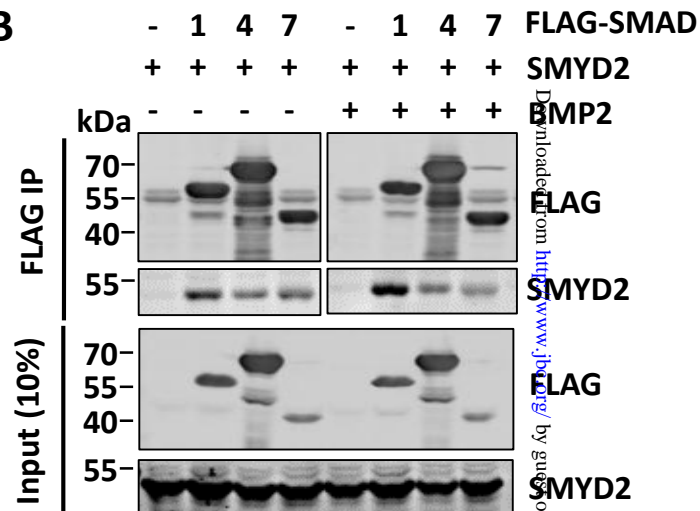
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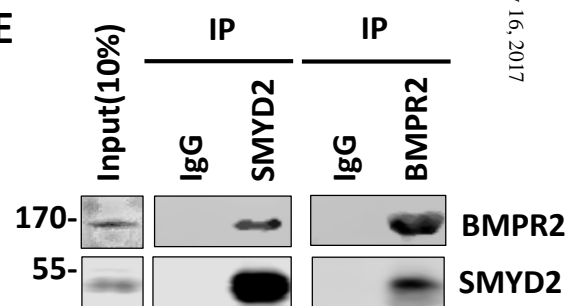
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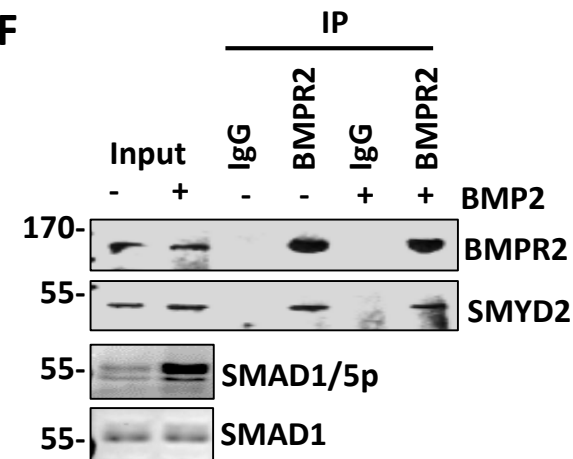
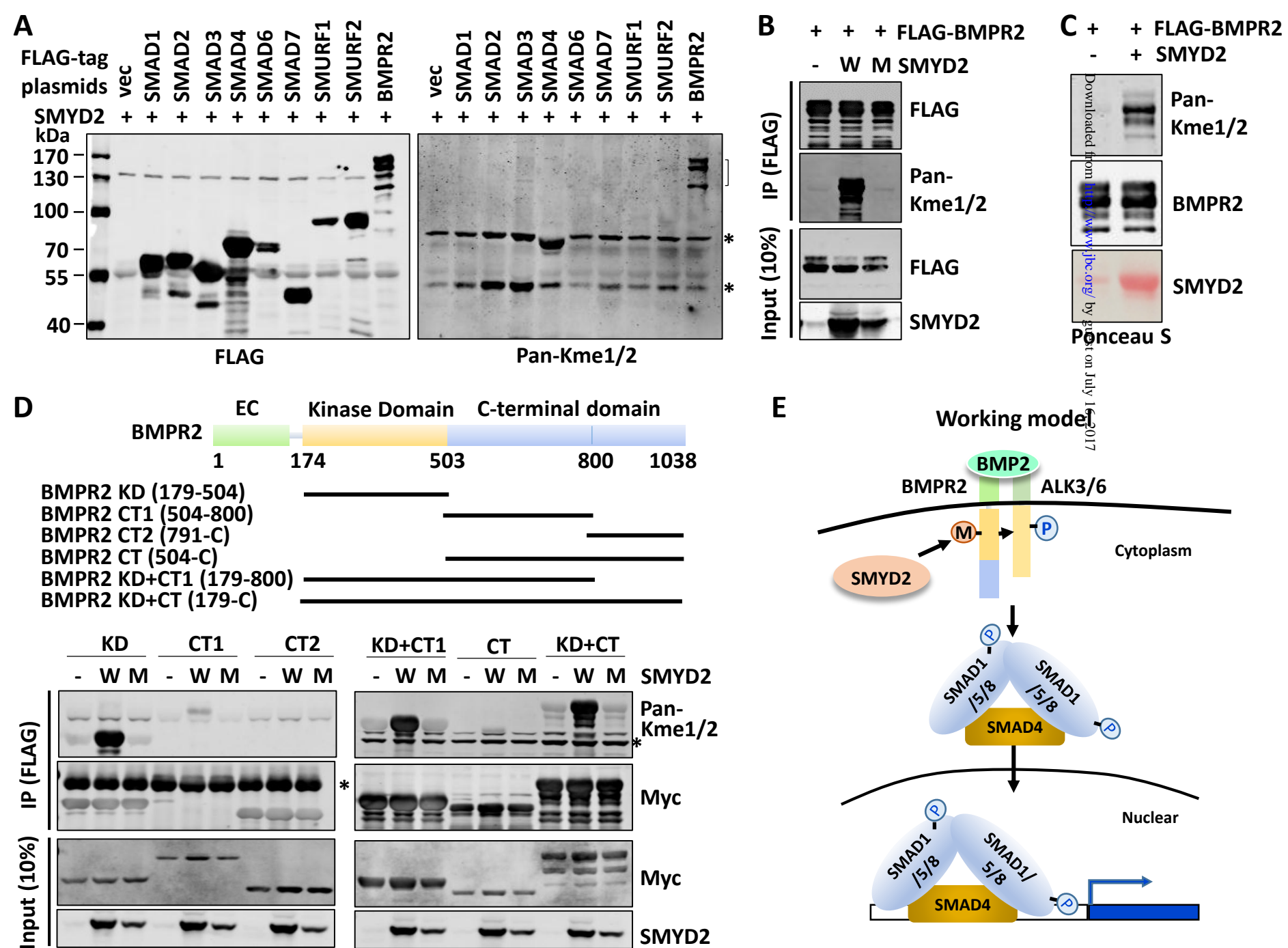


Fig. 6



**Fig. 7**



**The lysine methyltransferase SMYD2 methylates the kinase domain of type II receptor BMPR2 and stimulates bone morphogenetic protein signaling**

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