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Coupling of dephosphorylation and nuclear export of Smads in TGF- β signaling

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Abstract

In eukaryotes, regulation of signaling mediators/effectors in the nucleus is one of the principal mechanisms that govern duration and strength of signaling. Smads are a family of structurally related intracellular proteins that serve as signaling effectors for TGF- β and TGF- β -related proteins. Accumulating evidence demonstrate that Smads possess intrinsic nucleocytoplasmic shuttling capacity, which enables them to transmit TGF- β signals from cell membrane to nucleus. We recently identified two important steps in the termination of nuclear Smad signaling. The first step is initiated by a serine/threonine phosphatase PPM1A that dephosphorylates Smad2/3 in the nucleus, thereby shutting down signaling capacity of phosphorylated Smad2/3. The second step involves nuclear export of dephosphorylated Smad2/3 with the aid of nuclear protein RanBP3 to terminate Smad signaling. This chapter introduces methods for examining nuclear export of Smad2/3 in TGF- β signaling.

Keywords

Smad; PPM1A; RanBP3; nuclear phosphatase; nuclear export; signal transduction

1. Introduction

Smad proteins are critical intracellular signaling mediators for the transforming growth factor beta (TGF- β) superfamily. Eight Smads have been identified in mammals, including five receptor-activated Smads (R-Smads), one common mediator Smad (Co-Smad, i.e. Smad4) and two inhibitory Smads (I-Smads). Within R-Smads, Smad2 and Smad3 transduce signals from TGF- β and activin, while Smad1, Smad5 and Smad8 transduce signals from members of the Bone Morphogenetic Proteins/Growth Differentiation Factors (BMP/GDF) family. At the resting state, Smad2/3 are generally diffused within cells. Upon ligand-induced activation of TGF- β receptors, Smad2/3 become phosphorylated by the TGF- β type I receptor (T β RI), dissociate from the receptor, oligomerize with Co-Smad4 and translocate to the nucleus, where the Smad complex regulates transcription of TGF- β target genes^{1, 2}. At the end of active signaling, phospho-Smad2/3 are dephosphorylated³ and exported from nucleus to cytoplasm.

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During the activation-inactivation cycles of TGF- β signaling, TGF- β -induced Smad nuclear accumulation has been the subject of particular scrutiny. The subcellular distribution of Smads depends on their association with nuclear import or export factors and retention proteins^{4–8}. Although it has been reported that TGF- β -induced phosphorylation favors the nuclear import of phosphorylated R-Smads by enhancing its association with importin- β ^{9, 10} and/or disassociation with cytoplasmic retention factors such as SARA¹¹, in-depth analysis suggests a more pivotal role of export process in Smad nuclear accumulation. It appears that TGF- β does not affect the nuclear import rate of Smad2⁸, instead it decreases Smad2 nuclear export and therefore retains more Smad2 in the nucleus^{8, 12}.

Our recent studies investigate the functional roles of Smad2/3 export in TGF- β signaling. Examination of Smad2/3 export with the methods introduced in this chapter has revealed that Ran-binding protein 3 (RanBP3) negatively regulates TGF- β signaling by facilitating Smad2/3 nuclear export. In the nucleus, RanBP3 recognizes dephosphorylated Smad2/3, which results from the activity of nuclear Smad phosphatases and exports Smad2/3 in a Ran-dependent manner. As a result, increased expression of RanBP3 inhibits TGF- β -induced Smad2/3 nuclear accumulation. Conversely, depletion of RanBP3 expression or interference of RanBP3-Smad2/3 interaction retains more Smad2/3 in the nucleus. The methods for analyzing Smad2/3 nuclear export are presented here to illustrate the identification and characterization of RanBP3 as the Smad2/3 nuclear export mediator. We hope these methods would provide researchers with necessary concepts and tools for studying subcellular distribution, in particular nuclear export of proteins.

2. Materials

Cell Culture reagents

Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), Phosphate buffered saline, Calcium and Magnesium free (PBS), Fetal bovine serum (FBS), penicillin /streptomycin sulfate, Non-essential amino acids (NEAA), and 0.25% Trypsin/EDTA are purchased from Hyclone.

Chemicals

TGF- β 1 is purchased from R&D systems, T β RI inhibitor SB431542 from CalBiochem, and protease inhibitor cocktail from Roche. ATP, creatine phosphate and creatine phosphokinase are purchased from Sigma.

Antibodies

The following antibodies are used in our experiments (vendors in the bracket): Anti-PPM1A antibody (Abcam), anti-Flag affinity gel (Sigma), anti-GADPH antibody (Research Diagnostics), anti-Lamin A/C (E-20) (Santa Cruz Biotechnology), anti-Smad2 antibody (Zymed), anti-Smad2/3 antibodies (Santa Cruz Biotechnology), anti-phospho-Smad2 and phospho-Smad3 antibodies (Cell Signaling Technology).

3. Methods

The nucleocytoplasmic shuttling of Smad2/3 is governed by several mechanisms involving nuclear import/export mediators and/or retention factors that tend to retain Smad2/3 in the cytoplasm or nucleus^{4–8}. Often, retention factors interact with Smad2/3 and function through interfering with the recognition of Smad2/3 by import/export mediators^{11, 12}. Thus the nuclear Smad2/3 level at a specific time is determined by the net effect of its import and export. Three assays introduced here, including cell fractionation assay, *in vitro* export assay and quantitative Smad2 export assay are all aimed at examination of Smad2/3 nuclear

export. The first two assays determine the nuclear Smad2/3 level under manipulated experimental conditions so that Smad2/3 import could be neglected (cell fractionation assay) or is disrupted (*in vitro* export assay). The quantitative Smad2 export assay utilizes a reporter system that produces chloramphenicol acetyltransferase (CAT) activity proportional to the nuclear export of Smad2. In the end of this chapter, we will also introduce the *in vitro* phosphatase assay, which we used to determine whether phosphorylated Smad2/3 are direct substrates of phosphatase PPM1A. This chapter assumes the familiarity of the readers with several common techniques including production of recombinant protein, cell culture, SDS-PAGE, Western blot analysis and RNA interference.

3.1 Mammalian cells culture

HaCaT cells are propagated in MEM supplemented with 10% FBS, 100 μ M NEAA, 100 μ g/ml streptomycin sulfate and 100U/ml penicillin (10% FBS MEM). To maintain HaCaT cells in the resting state, cells are cultured in MEM with 0.2% FBS for 24 hours before the experiments (0.2% FBS MEM).

Human embryonic kidney (HEK293T) cells are propagated in DMEM with 10% FBS, 100 μ g/ml streptomycin sulfate and 100U/ml penicillin (10% FBS DMEM).

3.2 Cell fractionation Assay

Subcellular fractionation is an extremely useful method to produce extracts enriched for proteins from the specific cellular compartments. The separation of nuclear and cytoplasmic fractions via this method allows the determination of Smad2/3 levels in each fraction by Western blot analysis. As the dysfunction of Smad2/3 nuclear export would alter its cellular distribution, here we compare the re-distribution of nuclear accumulated Smad2/3 in RanBP3-depleted HaCaT cells and its parental HaCaT cells.

Fractionation buffer: 10 mM HEPES [pH7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5% NP-40.

Lysis buffer: 25 mM Tris [pH7.5], 300 mM NaCl, 1% Triton X-100.

Western blot Sample buffer: 2X SDS Sample buffer (Bio-Rad);

Experimental Procedures:

1. HaCaT cells, and HaCaT cells stably expressing short hairpin RNA (shRNA) against human RanBP3 at 75–85% confluency are cultured in 0.2% FBS MEM for 24 hours to maintain cells in the resting state. HaCaT cells stably expressing shRNA against human RanBP3 are established beforehand by using standard method (see Note 1).
2. Treat cells (1×10^5) with TGF- β (2ng/ μ l) in 0.2% FBS MEM for 1 hour to induce nuclear accumulation of Smad2/3 (see Note 2).

¹Notes

To evaluate the function of RanBP3 in Smad2/3 export and subsequent TGF- β signaling, stable HaCaT cell lines with depleted expression of RanBP3 are established. Several shRNAs against RanBP3, whose expression is controlled by RNA polymerase III promoter H1, are tested for their knockdown efficiency on RanBP3 expression. Three highly effective shRNAs are then selected to stably transfect HaCaT cells. Pools or multiple clones of each shRNA-transfected stable cells can be selected for confirmation of knockdown of RanBP3 expression and for subsequent analyses.

²To allow the examination of Smad2/3 nuclear export, Steps 1 & 2 induce the Smad2/3 nuclear accumulation in the cultured cells in a “synchronized” manner by stimulating the cells in the resting state with TGF- β .

3. Wash cells with PBS three times to remove TGF- β , treat with SB431542 (5 μ M) in 0.2% FBS MEM to inhibit TGF- β type I receptor kinase activity for up to 4 hours (see Note 3).
4. At each chosen time point (e.g. 0, 2, 4 hours after SB431542 treatment), wash cells with PBS once, detach cells with 0.25% Trypsin/EDTA. After cells are detached, inactivate Trypsin activity by the addition of 1ml regular culture medium (i.e. 10% FBS MEM).
5. Re-suspend cells in 1 ml of 10% FBS MEM and split cell suspension into Eppendorf tubes with 200 μ l in one tube and 800 μ l in the other. Collect cell pellets by centrifugation at 500 $\times$ g, 4°C, for 5 minutes. Pellets from 200 μ l cells are lysed directly with 100 μ l 1X SDS sample buffer (50 μ l H₂O and 50 μ l 2X SDS sample buffer) as whole cell lysates (see Note 4).
6. For subcellular fractionation, pellets from 800 μ l cells was washed with cold PBS once, cell pellets were gently re-suspended in 100 μ l fractionation buffer and kept on ice for 20 minutes.
7. Collect cells by centrifugation at 500 $\times$ g, 4°C, for 5 minutes. Transfer the supernatant (cytoplasmic fraction) in a new tube.
8. Wash the pellets (nuclei) with 500 μ l fraction buffer twice to eliminate cytoplasmic material contamination and then lyse the samples with 100 μ l lysis buffer.
9. Add 100 μ l 2 $\times$ SDS Sample buffer into the subcellular fractions for sample preparation.
10. To evaluate the separation quality of subcellular fractions, examine GADPH (cytoplasmic protein marker) and Lamin A/C (nuclear protein marker) protein levels in each sample by Western blot analysis (see Note 5). These protein markers also serve as loading control for each fraction.
11. After successful separation of the cytoplasmic and nuclear fractions is confirmed, determine the Smad2/3 level in nuclear and cytoplasmic fraction as well as in whole cell lysates by Western blot analysis. An example of the result produced is shown in Figure 1.

3.3 *in vitro* export assay

In vitro export assay utilizes digitonin to permeabilize cell plasma membranes for macromolecules, yet leave the nuclei structurally and functionally intact. Washing the permeabilized cells removes majority of the cytoplasmic proteins, which prevents the cytoplasmic Smad2/3 being imported into the nucleus again. Thus, the level of Smad2 in samples represents nuclear Smad2 that has not been exported. We use this assay to compare the effect of RanBP3 and its Ran-binding mutant RanBP3-wv (see Note 6) on Smad2 export.

³SB431542 almost instantaneously inhibits TGF- β receptor I kinase activity. It effectively blocks TGF- β -induced Smad2/3 phosphorylation in the cytoplasm and nuclear accumulation thereafter⁶. The re-distribution of nuclear accumulated Smad2/3 during this period is mainly determined by Smad2/3 nuclear export.

⁴The absolute nuclear or cytoplasmic Smad2/3 level is also determined by the total cellular Smad2/3 level. It is thus necessary to examine whether the altered experimental condition (in this case, the depletion of RanBP3 in HaCaT cells) affects the total Smad2/3 levels.

⁵The assay relies on successful separation of the nuclear and cytoplasmic fractions, which could be confirmed by examination of proteins with known subcellular localization through Western blot analysis. The existence of GADPH in the nuclear fractions or Lamin A/C in the cytoplasmic fractions all indicates the potential problems with the cell fractionation. It is critical to evaluate the quality of cell fractionation before any subsequent analyses.

⁶RanBP3-wv mutant (W352A/V353A) binds RanGTP 200-fold less than wild-type RanBP3 in RanGAP protection assay¹⁶ and is utilized here as RanBP3 Ran-binding mutant in comparison with wild-type RanBP3.

Transporting buffer: 20 mM HEPES [pH7.3], 110 mM KAc, 5 mM NaAc, 2 mM DTT, 1 mM GTP, 1.0 mM EGTA.

ATP regeneration system (Sigma): 1 mM ATP, 5 mM Creatine phosphate and 20 U/ml Creatine phosphokinase.

Experimental Procedures:

1. HaCaT cells stably expressing GFP-Smad2 (see Note 7) at 75–85% confluency are cultured in 0.2% FBS MEM for 24 hours to maintain cells in the resting state.
2. Treat cells (1×10^4 per well in a 12-well plate) with TGF- β (2 ng/ μ l) in 0.2% FBS MEM for 1 hour to induce nuclear accumulation of GFP-Smad2 (see Note 2).
3. Wash cells with cold PBS once, incubate cells with 250 μ l cold Transporting buffer containing digitonin (30 ng/ μ l) on ice for 5 minutes (see Note 8; Figure 2).
4. Wash cells with cold Transporting buffer three times, remove excess buffer after last wash.
5. Immediately incubate the permeabilized cells with 200 μ l Transporting buffer (pre-warmed at 30°C) supplemented with ATP regeneration system and 500 ng of recombinant protein RanBP3, RanBP3-wv or BSA as control (see Note 9). Perform the assay at 30°C for up to 60 minutes.
6. At each chosen time points (0, 30 and 60 minutes after incubation), quickly wash cells with the cold Transporting buffer three times, remove excess buffers after last wash.
7. Lyse cells in 100 μ l 1X SDS sample buffer (50 μ l H₂O and 50 μ l 2X SDS-sample buffer) for sample preparation.
8. Determine GFP-Smad2, GAPDH (cytoplasmic protein marker) and Lamin A/C (nuclear protein marker) by Western blot analysis. These protein markers also serve as loading control for each sample. An example of the result is shown in Figure 3.

3.4 Quantitative Smad2 export assay

This assay utilizes a CAT reporter system based on the observation that intron-containing mRNAs are exported out of nucleus only after splicing is completed¹³. As shown in Figure 4, the system consists of two components: (1) CAT reporter plasmid pDM128-8xMS2¹³. The reporter expresses an mRNA hybrid between CAT-encoding RNA and MS2 translation operator RNA, inserted between a pair of splicing donor/acceptor sites. Export of this hybrid mRNA depends on the specific recruitment of RNA-binding export factor to the unspliced CAT RNA. In the absence of export factor, CAT-encoding RNA is spliced out and no CAT is produced. (2) The second component is the MS2-Smad2 fusion protein, which contains a

⁷This assay could also be used to examine the endogenous Smad2 export in parental HaCaT cells. GFP-Smad2 allows direct visualization of nuclear GFP-Smad2 export process under fluorescence microscope, which is extremely convenient for optimizing the experimental conditions.

⁸Digitonin effectively solubilizes membrane proteins and permeabilizes the plasma membrane. It is thus very critical in this assay to avoid over-permeabilization that may disrupt the nuclear membrane and hence the functionality of nuclei. Proper cell membrane permeabilization could be evaluated by: 1) lack of GAPDH in the total lysates of permeabilized cells, indicating a clearance of all cytoplasmic proteins in permeabilized cells; 2) a comparable level of nuclear envelope marker Lamin A/C between digitonin treated and non-treated cells, indicating integrity of the nuclear envelope; 3) a comparable level of Smad2 before and immediately after the digitonin permeabilization (defined as time point 0 in the assay), as disruption of nuclear envelope would result in the loss of nuclear Smad2. For example, as shown in Figure 2, treatment with digitonin at 30 or 60 ng/ml (but not 10 ng/ml) for 5 minutes is able to permeabilize the HaCaT cell membrane, as both dosages resulted in the substantial loss of cytoplasmic GAPDH after washes.

⁹RanBP3 and RanBP3-wv are prepared from recombinant GST-RanBP3 or GST-RanBP3-wv proteins by removing GST with Precision proteinase (Amersham) according to the manufacture's instruction.

bacteriophage MS2 coat protein fused to Smad2¹². MS2 coat protein can bind to MS2 RNA. If the MS2 fusion partner undergoes nuclear export, the fusion protein binds to the unspliced CAT-MS2 RNA and transports the hybrid RNA into the cytoplasm, where the CAT RNA can be translated. Thus, production of CAT relies on the ability of Smad2, the fusion partner of MS2 coat protein, to undergo nuclear export. The activity of CAT quantitatively reflects the amount of Smad2 molecules exported. We use this assay to quantitatively determine the effect of RanBP3 and PPM1A on Smad2 nuclear export.

Plasmids and reagents:

MS2 coat protein-fused Smad2 plasmid (gift of Joan Massagué)¹².

pDM128/8xMS2 export reporter system (gift of Bryan Cullen)¹⁴.

pSVβgal (Promega).

ELISA-based Chloramphenicol acetyltransferase (CAT) assay kit (Roche).

ELISA-based β-galactosidase assay kit (BD Pharmingen).

Lysis buffer: 25 mM Tris [pH7.5], 300 mM NaCl, 1% Triton X-100.

Experimental Procedures:

1. HEK293T cells at 30% confluency are cultured in 12-well plates. Two or three wells (duplicate/triplicate) are required for each experimental data point.
2. Transfect HEK293T cells with plasmids encoding MS2-Smad2, pDM128/8xMS2, pSVβgal (Promega) (see Note 10) together with plasmids encoding RanBP3 or PPM1A by using LipofectAmine transfection reagent (Invitrogen).
3. Forty-five hours after transfection, wash cells with PBS once, scrape cells into 1ml PBS, harvest cells by centrifugation at 500×g, 4°C, for 5 minutes.
4. Lyse the cell pellets with 200μl lysis buffer on ice for 10 minutes.
5. Cell lysates are ready to be analyzed for CAT activity (150μl of cell lysates)/β-galactosidase activity (20μl of cell lysates) by using ELISA-based assay on a 96-well microreader according to manufacture's instructions.
6. Relative MS2-Smad2 export activity is calculated by normalizing the CAT activity with β-galactosidase activity in each sample. An example of the results produced is shown in Figure 5.

3.5 *in vitro* phosphatase assay

We found RanBP3 preferentially recognizes dephosphorylated Smad2/3, and PPM1A facilitates the interaction between RanBP3 and Smad2/3 in the nucleus¹⁵. These data indicate that dephosphorylated Smad2/3 serve as a proper cargo for RanBP3. Although both PPM1A and RanBP3 promote nuclear export of Smad2 (Figure 5), PPM1A dephosphorylates Smad2/3² and appears to act upstream of RanBP3 in the process of Smad2 export. To rule out the possibility that decreased P-Smad2/3 levels may be attributed to PPM1A phosphatase activity towards upstream activators of Smad2/3 (e.g. TGF-β receptor kinases) instead of Smad2/3 themselves, it is necessary to examine PPM1A phosphatase

¹⁰pSVβgal expresses β-galactosidase under the control of the SV40 early promoter. Measurement of β-galactosidase activity from the samples allows normalization of transfection efficiency.

activity in a cell-free system where only purified recombinant proteins are used. We use *in vitro* phosphatase assay to determine whether PPM1A directly dephosphorylates Smad2/3.

***In vitro* phosphatase reaction buffer:** 50mM Tris- HCl [pH7.5], 30mM MgCl₂, 5mM DTT and 1mg/ml of BSA.

Experimental Procedures:

1. To setup cell-free phosphatase reactions (see Note 11), add 100 ng of *E.coli*-expressed, purified recombinant His-tagged PPM1A protein and 100 ng of semi-synthetic recombinant phospho-Smad2 MH2 (P-S2MH2) peptide (see Note 12 & 13) in a 50 μ l phosphatase reaction buffer.
2. Perform the phosphatase reaction at 30°C for 30 min. Stop the reactions by addition of 50 μ l of 2X SDS loading buffer.
3. Examine the levels of total Smad2; P-Smad2 and PPM1A by Western blot analysis (see Note 2). Examples of the result are shown in Figure 6.

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¹¹PPM1A is a member of PPM family serine/threonine phosphatase. Its activity depends on metal ions Mg²⁺ or Mn²⁺ and could be inhibited by chelating agents (e.g. EDTA). Avoid using chelating agents in buffer and/or recombinant protein elutes in this assay.

¹²Please refer to a previous study (Wu, J. W. et al¹⁷) for the generation of phospho-Smad2 MH2 (241–462) *in vitro*. Of note, PPM1A can also dephosphorylate the C-terminal SSXS motif of Smad1/5/8, which is highly conserved in all R-Smads¹⁸.

¹³Alternatively, readers could use immunoprecipitated full-length Smad2/3 proteins as the substrate for this assay (see Fig 6B). To prepare the phosphorylated Smad2/3 proteins, transfect Flag-tagged Smad2/3 into HEK293T cells together with constitutively activated T β RI kinase. Phosphorylated Smad2/3 proteins could be immunoprecipitated with anti-Flag antibodies and eluted with Flag-peptide (Sigma) according to the manufacturer's instructions. Our lab uses rat T β RI-T202D¹⁹. The mutation of threonine to aspartic acid at the position of amino acid 202 in rat T β RI renders it constitutively active and able to signal in a ligand-independent manner, which is similar to the analogous mutation in the human T β RI-T204D²⁰.

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Figure 1. Depletion of RanBP3 blocks nuclear export of Smad2/3 in HaCaT cells

RanBP3-KD1 and parental HaCaT cells were treated with TGF- β for 1 h, cells were washed 3 times to remove TGF- β and treated with SB431542 up to 4 h. Cells were then harvested at indicated time, and the nuclear and cytoplasmic fractions were collected. Total Smad2/3 levels as well as phosphorylated Smad2 levels were examined by Western blot analysis. Detection of nucleus-localized Lamin A/C and cytoplasm-localized GAPDH demonstrates separation of the fractions and proper sample loading. Quantitation of image intensity was done using Image J software. The arbitrary unit for cytoplasmic Smad2/3 level in lane 4 was set to 1. N: Nuclear fraction, C: Cytoplasmic fraction. As shown here, at time point 0 (immediately after 60 min TGF- β treatment), a very low level of Smad2/3 in the cytoplasmic fractions of both wild-type and knockdown cell lines is detected, indicating most of the Smad2/3 resided in the nucleus. Although SB431542 treatment promotes the dephosphorylation of Smad2 in the nucleus and the cycling back of Smad2/3 in the cytoplasm in parental HaCaT cells, it exhibits a weaker effect on cytoplasmic accumulation of Smad2/3 in RanBP3-KD1 cells. The total Smad2/3 levels are comparable in RanBP3-KD1 and parental HaCaT cells.

Figure 2. Permeabilization of the cells by digitonin
HaCaT cells stably expressing GFP-Smad2 were permeabilized with digitonin at 10, 30 or 60ng/ml for 5 min on ice. The *in vitro* export assay is performed by incubating permeabilized cells with 200μl Transporting buffer supplemented with ATP regeneration system and 500ng BSA at 30°C for up to 1 h. The levels of GFP-Smad2, Lamin A/C and GADPH were examined by Western blot analysis. Lamin A/C and GADPH indicate nuclear and cytoplasmic fractions, respectively. As shown, treatment with digitonin at 30 or 60 ng/ml (but not 10 ng/ml) for 5 min is able to permeabilize the HaCaT cell membrane, because both dosages result in the substantial loss of cytoplasmic GADPH after washes. The structural and functional integrity of the nuclear envelope is demonstrated by similar levels of GFP-Smad2 in digitonin-permeabilized and non-permeabilized cells at time point 0.

Figure 3. RanBP3 mediated Smad2 export depends on its Ran-binding ability

The ability of RanBP3 or RanBP3-wv protein on Smad2 export is examined by *in vitro* export assay. *In vitro* export of GFP-Smad2 is progressive over time as indicated by a decreased level of GFP-Smad2 (Figure 3, lanes 1–3) in the permeabilized cells. RanBP3 (Figure 3, lanes 4&5) but not RanBP3-wv (Figure 3, lanes 6&7) promotes nuclear export of Smad2.

Figure 4. Schematic diagram for MS2-based Nuclear Export Reporter Assay

In this cartoon, under normal conditions, the *SD-CAT-MS2-SA* RNA is only exported after splicing. In the presence of MS2-Smad2 fusion protein that can bind to the *MS2 RNA*, *SD-CAT-MS2-SA* RNA is exported and translated in the cytoplasm and CAT is then produced. SD, splicing donor site; SA, splicing acceptor site.

Figure 5. RanBP3 and PPM1A promote nuclear export of Smad2

HEK293T cells were transfected with plasmids for MS2-Smad2, CAT reporter pDM128/8xMS2 and β -galactosidase plasmid (for normalization) together with plasmids for RanBP3 (A) or PPM1A (B). 45 h after transfection, cell lysates were analyzed for CAT and β -galactosidase activity using ELISA-based assay. Relative export activity was calculated by normalizing the CAT activity with β -galactosidase activity in each sample. Values and error bars represent mean and standard deviation of three experiments. RanBP3 increases the Smad2 export by 5.2-folds, whereas RanBP3-wv fails to produce any effects. PPM1A increases the Smad2 export by 1.4-folds.

A **B**

Figure 6. PPM1A dephosphorylates the phospho-SXS motif in the Smad2 MH2 domain (P-S2MH2)

The phosphatase activity of PPM1A toward recombinant phosphorylated Smad2 MH2 domain (A) or immunoprecipitated phosphorylated Smad2 (B) is examined by *in vitro* phosphatase assay. The levels of PPM1A, Smad2-MH2, Smad2 as well as the phosphorylated Smad2-MH2 or Smad2 were examined by Western blot analysis. The results in Panel A indicate that equal amounts of semi-synthetic recombinant P-S2MH2 (lanes 1–5) and recombinant PPM1A (lanes 2–5) are loaded. Dephosphorylation of P-Smad2MH2 by PPM1A requires metal ion (lanes 3&4) and is abolished by 40 mM EDTA (lane 5). Similar results were also observed by using immunoprecipitated full-length Smad2 as shown in (B).