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Published in:
Cellular Signalling

DOI:
[10.1016/j.cellsig.2012.07.003](https://doi.org/10.1016/j.cellsig.2012.07.003)

Publication date:
2012

Document Version
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Bruce, D. L., Macartney, T., Yong, W., Shou, W., & Sapkota, G. P. (2012). Protein phosphatase 5 modulates SMAD3 function in the transforming growth factor-beta pathway. *Cellular Signalling*, 24(11), 1999-2006. [10.1016/j.cellsig.2012.07.003](https://doi.org/10.1016/j.cellsig.2012.07.003)

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Protein phosphatase 5 modulates SMAD3 function in the transforming growth factor β pathway.

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Running Title: PP5 regulates TGF β Signalling

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ABSTRACT

Protein phosphatases play a key role in balancing cellular responses to transforming growth factor- β (TGF β) signals. Several protein phosphatases have been attributed roles in the regulation of the TGF β pathway. Among these, PPM1A is the only phosphatase reported to dephosphorylate SMAD2/3 in the nucleus. However we observed PPM1A exclusively in the cytoplasmic fractions independently of TGF β treatment in all cells tested. These observations imply that a *bona fide* nuclear SMAD2/3 phosphatase remains elusive. In this study, we report a role for protein phosphatase 5 (PP5) in the TGF β pathway. We identified PP5 as an interactor of SMAD2/3. Interestingly, in mouse embryonic fibroblast cells derived from PP5-null mice, TGF β -induced transcriptional responses were significantly enhanced. This enhancement is due to the increased levels of SMAD3 protein observed in PP5-null MEFs compared to the wild type. No differences in the levels of SMAD3 transcripts were observed between the wild type and PP5-null MEFs. While PP5 is capable of dephosphorylating SMAD3-tail in overexpression assays, we demonstrate that its activity is essential in controlling SMAD3 protein levels in MEFs. We propose that PP5 regulates the TGF β pathway in MEFs by regulating the expression of SMAD3 protein levels.

1. Introduction

TGF β signalling plays a pivotal role through vertebrate development and tissue homeostasis. Concise control of cellular responses to TGF β signals is essential to ensure proper developmental progression and tissue differentiation. Upon binding their respective receptor kinase pairs at the cell-surface, TGF β ligands exert cellular responses by inducing a cascade of phosphorylation of key intra-cellular signal-mediators, including receptors and SMAD transcription factors (1). Dephosphorylation of these mediators by protein phosphatases is a key step in balancing the potency and duration of TGF β signalling (2). Protein phosphatases that indirectly modulate the expression, localisation or stability of key TGF β pathway mediators could also influence the outcome of TGF β signalling (2).

SMAD transcription factors mediate the TGF β signals inside the cell upon phosphorylation of the C-terminal tail SXS motif (hereafter referred to as tail) by the type I receptor kinases (1). The tail-phosphorylated SMADs then translocate to the nucleus, where they interact with SMAD4 and other co-factors to initiate or repress transcription of hundreds of target genes (1). The fate of SMADs following signal initiation, their nuclear translocation and transcriptional control of the target genes is generally well established. However, the precise details of the subsequent fate of the tail-phosphorylated SMADs are not completely clear. The polyubiquitin-mediated degradation and/or recycling by dephosphorylation of SMADs are two outcomes that have received the most scrutiny (2-11). As the tail-phosphorylated SMADs are observed in the nucleus, the phosphatases that catalyse their dephosphorylation are most likely nuclear. Further observations, including that receptor kinase inhibition results in pronounced cytoplasmic distribution of dephosphorylated SMAD2/3, support the existence of R-SMAD phosphatases in the nucleus (12-14). Consistent with this notion, PPM1A (also known as PP2C) was reported to be a SMAD2/3-tail phosphatase in the nucleus (11). In contrast, two cytoplasmic phosphatases, namely PP2A and MTMR4, have been proposed to act as SMAD phosphatases (15,16). PP2A has been reported to act as SMAD3-tail phosphatase only under hypoxia (15). MTMR4 has been reported to dephosphorylate SMAD2/3 in the cytoplasm and attenuate TGF β signalling (16). Despite these reports, evidence for the role of these phosphatases on the TGF β pathway has not been confirmed in knockout mouse models or cells.

In order to uncover novel regulators of the TGF β pathway, including protein phosphatases, a proteomic approach was employed to identify protein interactors of SMAD2/3. From these screens, protein phosphatase 5 (PP5) was identified as an interactor of SMAD2 and SMAD3. The proteomic screen did not identify PPM1A, PP2A and MTMR4 as interactors of SMAD2/3. PP5 is a serine/threonine phosphatase that belongs to the phospho-protein phosphatase (PPP) family of phosphatases (17). Many members of the PPP family are oligomeric holoenzymes that require a number of regulatory, catalytic and scaffold subunits to be active. PP5, however, has its catalytic, regulatory and substrate-binding domains within one polypeptide (17,18). PP5 is reported to mediate the dephosphorylation of a number of proteins including GR α , Chk1, 53BP1 and IKK β (19-22). However, its role in TGF β signalling has not been investigated. PP5 has been shown to localise in both the nucleus and cytoplasm (23,24). Mutation of its nuclear localisation motif located in the C-terminal region abolishes its ability to localise to the nucleus (25).

In this study, we evaluate the roles of SMAD-interacting phosphatases, in particular PP5, in the TGF β pathway. We also investigate how the localisation of reported nuclear SMAD phosphatase PPM1A, and a close family member PPM1B, is affected upon TGF β induction.

2. Materials and methods

2.1. Materials and general methods

Mammalian expression constructs for PPM1A, PP5, SMAD1, SMAD3, SMAD4 and SMAD7 were cloned into pCMV5 vectors expressing N-terminally FLAG- or HA-tagged proteins. Untagged PP5 and a catalytically inactive mutant of PP5 based on the structure of PP5 (26)(a kind gift from Tricia Cohen; the Asp residue at position 274 of PP5 was mutated to Ala; unpublished findings) were cloned into a pBABE-hygro retroviral vector (Cell Biolabs). pCMV-Gag-Pol and pCMV-VSVG constructs were purchased from Cell Biolabs, Inc. All DNA constructs used were verified by DNA sequencing, performed by DNA Sequencing & Services (MRC PPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. pGL2.11 3TP-Lux reporter construct was a kind gift from Joan Massagué. The pGL4.11 LUC2p-BRE (BMP-Response Element) and pGL4.11 LUC2p-SRE (SMAD-Response Element) reporter constructs were based on SMAD-binding sequences within ID1 (BMP-target gene) and PAI-1 (TGF β target gene) promoters respectively. Renilla luciferase reporter was used as transfection control. After stimulation, cells were lysed under passive conditions and the reporter activity assayed with Dual-Luciferase™ kit (Promega). Recombinant Activin A, TGF β 1 and BMP-2 were purchased from R&D Systems, MG-132 from Tocris, Bortezomib from LC Laboratories and Okadaic acid from Merck. The antibodies recognising P-SMAD1 (Ser463/465), P-SMAD2 (Ser465/467), SMAD2/3, SMAD3, Lamin A/C and GAPDH were purchased from Cell Signalling, Histone 1B from Abcam, P-SMAD3 (Ser423/425) was purchased from Rockland Inc., PPM1B from R&D Systems (IB) and Bethyl Laboratories (IF). Peptide antibodies against human PPM1A (aa 369-382), PP5 (aa 471-485), SMAD1 (aa 144-268), SMAD2 (aa 87-107) and SMAD3 (aa 158-178) were generated by injecting peptides into sheep and affinity purified; some have been described previously (27,28). HRP-coupled anti-FLAG (Sigma) and anti-HA antibodies (Roche) were also employed. Proteins were detected by HRP-coupled secondary antibodies (Pierce) or protein G-HRP antibodies (BioRad) and visualised with enhanced chemiluminescence (Pierce).

2.2. Cell Culture, Transfections and Stimulations

HaCaT keratinocytes, HEK293 cells, HUVEC cells, HeLa cells, mouse embryonic fibroblasts (MEFs), Swiss 3T3 cells, HepG2 cells, Neuro2A cells, G361 cells, U87 cells, A549 cells and MDA231 cells were cultured at 37°C in an atmosphere with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1X Penicillin/Streptomycin solution and 1X L-glutamine. Sodium pyruvate (1X) and non-essential amino acids (0.1mM) were supplemented for Neuro2A, G361 and U87 cells. Mouse ES cells (29) and RAW macrophage cells (27) were maintained as described. The transfection of cDNA vectors was performed as described previously (3). Transfection of *siRNA* oligonucleotides against FoxO4, PPM1A and PPM1B was performed using Lipofectamine 2000 reagent as described previously (3). The

siRNA oligos used are as follows: PPM1A: GUAUCGCCAGAAGCAGUGATT; PPM1B: UAGACUGAAUCCACAUAGATT. The FoxO4 *siRNA* was described previously (7).

2.3. Generation of PP5 *-/-* MEFs stably expressing wild type PP5 or catalytically inactive PP5-PD mutant

Retroviral pBABE-hygro constructs (1 μ g each) encoding PP5 or PP5-PD were co-expressed with CMV-Gag/Pol (0.9 μ g) and CMV-VSVG (0.1 μ g) constructs in HEK-293T cells. Retroviruses were collected 48h-post transfection from the culture media by filtering it through 0.45 μ m filters onto sterile falcon tubes. PP5 *-/-* MEFs, plated at ~40% confluent, were infected by transferring the filtered retroviruses directly onto the cells and 8 μ g/ml Polybrene reagent was added to aid infection. 24h-post infection, cells were cultured in the presence of hygromycin B 100 μ g/ml for selection of infected cells. Western blots on cell extracts with a PP5 antibody was used to verify infection of the targets.

2.4. RNA isolation, cDNA preparation and analysis of transcripts by qRT-PCR

Total RNA was isolated from cells using the RNeasy kit (Qiagen). cDNA was prepared from 1 μ g of RNA using the i-Script Kit (Bio-Rad). qRT-PCR was performed in 96-well plate format using iQ5TM Real Time PCR detection system (BioRad), where each 20 μ l reaction included 1% cDNA preparation, 0.5 μ M primers and 10 μ l SYBR Green (Quanta). The mouse primer sequences (5'-3') used for qRT-PCR are as follows: GAPDH F: TATGATGACATCAAGAAGGTGG; R: CATTGTCATACCAGGAAATGAG; PP5 F: CTGGAGGAGAATCAACTGGA; R: CCATCTGGTCACAATAGTTGG; Gadd45 γ F: GACTTTGGCGGACTCGTAGA; R: ACTCTGGAAGAAGTCCGTGG; c-MYC F: CTCAGTGGTCTTTCCCTACC; R: CTTGCTCTTCTCAGAGTCG; SMAD3 F: CGTAATTCATGGTGGCTGTG; R: ACCAAGTGCATTACCATCCC; SnoN F: GAATGGAATTGCCATCATGG; R: GATTGATGAATTTGTCTCTGCC. Each qRT-PCR reaction was performed in triplicates and the data presented is a representative of three independent biological repeats.

2.5. Other Assays

Immunoblots were performed as described previously (27). For immunostaining, cells were fixed in 4% PFA and immunostained with the indicated antibodies as described previously (7). Nuclear and cytoplasmic fractionations were performed using a Nuclear and Cytoplasmic Extraction Kit (Pierce). For chemical cross-linking with Dithiobis (succinimidyl propionate) (DSP; Thermo), cells were lysed in ice-cold HEPES lysis buffer (40mM HEPES pH 7.4, 120mM NaCl, 1mM EDTA, 10mM sodium pyrophosphate, 50mM sodium fluoride, 1.5mM sodium orthovanadate, 1% Triton X-100, complete protease inhibitors (Roche)) containing 2.5mg/ml DSP, freshly added prior to lysis. Lysates were incubated at 4 $^{\circ}$ C for 30min before cross-linking was quenched with 1M Tris pH 7.5 at a final concentration of 0.2M. Extracts were further incubated for 30min at 4 $^{\circ}$ C before pre-clearing and immunoprecipitation (IP). For IPs, antibodies were pre-coupled to protein G-Sepharose beads. IPs were performed for 3h at 4 $^{\circ}$ C with constant agitation, beads were then washed twice with lysis buffer containing 0.5M NaCl and once with

TE buffer (50mM Tris pH 7.4 and 0.1mM EGTA) at 4°C before boiling for 5min in SDS sample buffer containing 50mM dithiothreitol (DTT).

2.6. Statistical analysis

Data are presented as the mean \pm SD. Statistical significance of differences between experimental groups was assessed using the two-way analysis of variance (ANOVA) test with Bonferroni posttests. Differences in means were considered significant if $p < 0.05$. Differences with $p < 0.05$ were annotated as “*”, $p < 0.005$ were annotated as “**” and $p < 0.001$ were annotated as “***”.

3. Results

3.1. PPM1A/B are cytoplasmic

In order to define the roles of phosphatases following TGF β -induced nuclear accumulation of tail-phosphorylated SMAD2/3, we initially sought to investigate and validate the roles of some known nuclear SMAD2/3 phosphatases in multiple cell systems. We separated human keratinocyte HaCaT cells, mouse ES cells, breast cancer MDA231 cells, HUVEC endothelial cells and HEK293 cells into nuclear and cytoplasmic fractions. Rather surprisingly, PPM1A was observed only in the cytoplasmic fractions in all of the cells (Figure 1A). We confirmed the PPM1A immunoreactive band by immunoblotting HaCaT extracts transfected with *PPM1A siRNA* or *FoxO4 siRNA* controls (Figure 1A; last two lanes). The transfections of *PPM1A siRNA* resulted in the reduction of the expression of PPM1A protein by ~90% (Figure 1A). As expected, GAPDH and Histone 1B fractionated with the cytoplasmic and nuclear fractions respectively (Figure 1A). We also investigated the localisation of PPM1B, which is closely related to PPM1A (17). Like PPM1A, PPM1B fractionated exclusively with the cytoplasmic fractions in all the cells tested (Figure 1A). The cytoplasmic localisation of PPM1A that we observed in various cell lines directly contradicted with published reports that have employed similar fractionation technique (11). Therefore we extended our investigation of PPM1A localisation to more cell types, including HeLa cells, mouse embryonic fibroblasts, Swiss 3T3 cells, liver hepatocellular carcinoma HepG2 cells, RAW macrophages, neuroblastoma Neuro2A cells, G361 melanoma cells, and U87MG glioblastoma cells. In all cases PPM1A was detected exclusively in cytoplasmic fractions (Figure 1B). In order to ensure that the cytoplasmic localisation of PPM1A/B was not due to inefficient fractionation techniques, we sought to verify their subcellular localisation by immunofluorescence. The PPM1A antibody used in our immunoblot assays yielded pan-cellular fluorescence in HaCaT cells transfected with or without *PPM1A siRNA* (data not shown). Therefore, we transfected HaCaT cells with N-terminal HA-tagged PPM1A, and demonstrate that the HA-antibody only stains the cytoplasm of the transfected cells while the adjacent untransfected cells are not stained (Figure 1C). Furthermore, HA-PPM1A was detected exclusively in the cytoplasmic fraction in these cells (Figure 1C, bottom panel). Using a PPM1B antibody for immunostaining, we demonstrate that in HaCaT cells endogenous PPM1B localises mainly to the cytoplasm (Figure 1D). The cytoplasmic staining was greatly reduced when cells were transfected with *PPM1B siRNA* (Figure 2B). Furthermore, PPM1B is detected only in the cytoplasmic fractions of HaCaT cells (Figure 1D; bottom panel). In HaCaT cells

transfected with *PPM1B siRNA*, the expression of PPM1B is depleted >80% over control cells (Figure 1D; bottom panel).

3.2. *TGFβ ligands do not alter the cytoplasmic localisation of PPM1A/B*

Faced with the observations from immunostaining and immunoblotting assays in HaCaT and other cells that PPM1A/B are cytoplasmic phosphatases, we sought to investigate whether treatment of cells with TGFβ (Figure 2A) or BMP (Figure 2B) altered their localisation. We performed time course stimulations in HaCaT cells with TGFβ or BMP for up to 20h prior to nuclear cytoplasmic fractionation. TGFβ or BMP treatment did not alter the cytoplasmic localisation of PPM1A, while inducing nuclear accumulation of phospho-tail SMAD2 or phospho-tail SMAD1 respectively (Figure 2A&B).

3.3. *PP5 interacts with SMAD2/3 but not SMAD1*

Our observations of cytoplasmic localisation of PPM1A suggested that a nuclear phosphatase for SMAD2/3 remained to be identified. Interestingly in a proteomic screen we undertook to identify novel interactors of SMAD2/3, we identified protein phosphatase 5 (PP5) in GFP-SMAD2 and GFP-SMAD3 immunoprecipitates (IPs) but not in GFP-alone IPs (data not shown). In order to verify the interactions between endogenous proteins, IPs of SMAD2 and SMAD3 or SMAD1 used as control were subjected to PP5 immunoblots. Endogenous PP5 was detected in SMAD2 and SMAD3 IPs but not in SMAD1 or control IgG IPs (Figure 3A&B). The levels of PP5 detected in the SMAD2/3-IPs did not change by TGFβ treatment, which resulted in phosphorylation of both SMAD2 and SMAD3 (Figure 3A). BMP treatment did not induce interaction between SMAD1 and PP5 (Figure 3B).

3.4. *PP5 overexpression leads to the dephosphorylation of SMAD3-tail*

PP5 belongs to the PPP family of phosphatases, which can be inhibited by okadaic acid (OA) (30). To study the effects of PP5 on TGFβ signalling, an *in vivo* dephosphorylation assay was established (Figure 4A). Firstly, HaCaT cells were treated with TGFβ for 45min in order to induce optimal phosphorylation of SMAD2/3-tail. At this point the ligands were removed by washing cells twice with DMEM. The subsequent rate of dephosphorylation of SMAD2/3 was tracked by immunoblotting cell extracts lysed at specific time points using P-SMAD3 antibody. Preincubating cells with OA in the above assay prolongs the phosphorylation of SMAD3 at 120min following ligand removal (Figure 4A). This indicates that an OA-sensitive phosphatase acts to inhibit the phosphorylation of SMAD2/3-tail, either directly or indirectly. In order to test whether PP5 could dephosphorylate TGFβ or Activin A-induced phospho-SMAD3, mammalian expression constructs encoding HA-PP5 and FLAG-SMADs 1, 3, 4 and 7 were co-expressed in HEK293T cells. Cells were treated with BMP or Activin A to induce tail-phosphorylation of FLAG-SMAD1 or FLAG-SMAD3 respectively. PP5 completely abolished Activin A-induced phosphorylation of Flag-SMAD3 but not BMP-induced phosphorylation of FLAG-SMAD1 or endogenous SMAD1 (Figure 4B).

3.5. *PP5 modulates TGFβ-induced responses in mouse embryonic fibroblasts*

In order to establish the role of PP5 on the TGF β pathway, we employed mouse embryonic fibroblasts (MEF) cells derived from both wild type (WT) and PP5 knockout (PP5-null) mice (31). We employed two methods: exploiting TGF β -responsive luciferase constructs and monitoring the changes in expression of TGF β -regulated genes by qRT-PCR. Significant increases in reporter activity were observed in the PP5-null cells after TGF β stimulation when compared to the WT cells (Figure 5A). This increase was detected with two different TGF β -responsive luciferase reporter constructs (Figure 5A). However, when a BMP-responsive luciferase construct (BRE) was employed as a negative control, the relative luciferase activity in BMP-stimulated PP5-null cells was similar to the WT cells (Figure 5B). Furthermore, PP5-null cells displayed an increased expression of three TGF β responsive genes (SnoN, c-MYC and Gadd45 γ) compared to the WT cells following stimulation with TGF β (Figure 5C). As expected, PP5 transcripts were absent from PP5-null MEFs. The levels of SMAD3 transcripts were similar in both PP5-null and WT MEFs (Figure 5D). These observations were consistent with the possibility of PP5 acting as a SMAD2/3 phosphatase.

3.6. PP5 modulates the expression of SMAD3 protein in mouse embryonic fibroblasts

Next we monitored the phosphorylation of SMAD3 upon TGF β stimulation (Figure 6A). When two concentrations of TGF β were used in a time course experiment, there was a modest increase in SMAD3 phosphorylation observed in extracts from PP5-null MEFs than WT. The difference was more prominent at latter time points, especially when cells were treated with 25pM TGF β (Figure 6A). Rather surprisingly, the protein levels of SMAD3 were observed to be consistently higher in PP5-null cells compared to WT MEFs while GAPDH levels were similar between all samples (Figure 6A). Next we tracked the levels of phospho-SMAD3 following removal of TGF β ligand from media as described above. The levels of phospho-SMAD3 were higher in PP5-null MEFs in comparison to the WT at both 0min and 120min time points following the removal of TGF β ligand (Figure 6B). However, the levels of SMAD3 protein were also consistently higher in PP5-null MEFs compared to the WT (Figure 6B). As the expression of SMAD3 transcripts in both WT and PP5-null MEFs were similar (Figure 5D), we investigated whether PP5 was inducing proteosomal degradation of SMAD3 in WT MEFs. Inhibition of the proteasome by inhibitors MG-132 and bortezomib did not rescue the levels of SMAD3 protein in the WT cells compared to the PP5-null cells (Figure 6C).

3.7. PP5 activity is essential for modulation of the expression of SMAD3 protein

The changes in the SMAD3 protein levels observed in WT vs. PP5-null MEFs could be due to cell type differences. Therefore, in order to definitively establish the role for PP5 in the regulation of SMAD3 protein expression, we stably re-introduced WT (PP5 $^{-/-}$ WT) and catalytically inactive PP5-PD mutant (PP5 $^{-/-}$ PD) in PP5-null MEFs. The level of expression of PP5 protein in these MEFs was comparable to that seen in wild type MEFs (Figure 7A). Next we investigated the TGF β -induced SMAD3-tail phosphorylation in these cells. The levels of phospho-SMAD3 induced by 5pM or 25pM TGF β were lower in PP5 $^{-/-}$ WT cells compared to PP5 $^{-/-}$ empty or PP5 $^{-/-}$ PD cells (Figure 7B). Interestingly, the levels of SMAD3 protein were also lower in PP5 $^{-/-}$ WT cells compared to both PP5 $^{-/-}$ empty and PP5 $^{-/-}$ PD cells indicating that PP5 catalytic activity is responsible for regulating SMAD3 protein levels in these cells (Figure 7B).

4. Discussion

Identification and characterisation of a *bona fide* nuclear SMAD2/3-tail phosphatase capable of terminating TGF β signalling has long been one of the most sought-after challenges in the TGF β field (2). As such, when PPM1A was described as the nuclear SMAD1/2/3-tail phosphatase (11), this challenge appeared to have been finally resolved. However, our findings that PPM1A as well as the closely related PPM1B both localise to the cytoplasm independent of TGF β treatment raises doubts over the validity of PPM1A/B as nuclear SMAD2/3 phosphatases. In all of the 13 different cells that we fractionated, PPM1A was clearly only detected in the cytoplasmic fractions. These observations directly contradict the published findings using similar fractionation methodology (11). Our observations raise two possibilities on the role of PPM1A as SMAD2/3-tail phosphatases: i. PPM1A acts as SMAD2/3-phosphatases in the cytoplasm and affects cytoplasmic levels of phospho-SMAD2/3-tail thereby affecting the TGF β pathway. PPM1A could therefore have an analogous role to PP2A and MTMR4 that have been reported to act as cytoplasmic phosphatases against phospho-SMAD2/3-tail (15,16). ii. PPM1A does not target phospho-SMAD2/3-tail and any effects on the TGF β pathway signalling may be indirect. Both these possibilities could easily be confirmed in cells derived from PPM1A-knockout mice (32). As silencing of PPM1A alone by RNAi was clearly demonstrated to enhance TGF β pathway signalling (11), cells derived from PPM1A-knockout would be expected to display enhanced TGF β signalling. Indeed the authors of the original study describing PPM1A as a SMAD2/3-tail phosphatase have reported the isolation of mouse embryonic fibroblast cells (MEFs) from PPM1A-knockout mice. While the impact of PPM1A on RanBP3 phosphorylation was reported, its role on SMAD2/3 phosphorylation was not (32). It will be extremely interesting to find out how TGF β induced phosphorylation of SMAD2/3-tail is affected in these cells. Furthermore, PPM1A^{-/-} MEFs would also be ideal negative controls for investigating the subcellular localisation of PPM1A.

Upon identification of PP5 as an interactor of SMAD2/3 by mass spectrometry, we assessed its role in the TGF β pathway as a potential SMAD2/3-tail phosphatase. Any SMAD2/3 phosphatase would be expected to physically associate with its substrate. We verified the interactions between SMAD2/3 and PP5 at the endogenous level in the extracts. Furthermore, we were able to demonstrate that PP5, under overexpression conditions, was capable of dephosphorylating Activin A-induced phospho-SMAD3. This observation alone, however, does not provide any evidence for a role of PP5 in the TGF β pathway, as we would expect a similar observation with the overexpression of many phosphatases. In order to test whether PP5 had any effects on TGF β pathway, we employed MEFs derived from PP5-null mice. Interestingly, TGF β -induced transcriptional responses were enhanced in PP5-null MEFs compared to wild type. Consistent with this, the TGF β -induced phosphorylation of SMAD3 was also enhanced in PP5-null MEFs compared to the wild type. However, the levels of SMAD3 were also higher in PP5-null MEFs compared to the wild type. This suggested that PP5 could regulate the expression of SMAD3 protein in cells. Although the SMAD3 protein levels were affected in PP5-null cells compared to the wild type, the SMAD3 mRNA levels were unaltered suggesting PP5 might regulate SMAD3 levels post-transcriptionally. The proteasomal inhibitors MG-132 and Bortezomib did not significantly enhance the levels of SMAD3 in wild type MEFs implying that PP5 is unlikely to be promoting proteasome-mediated degradation of SMAD3. We wanted to ensure the differences

seen in the levels of SMAD3 in wild type and PP5-null MEFs was not due to differences in cell types. Therefore, we stably re-introduced wild type or a catalytically inactive mutant of PP5 in PP5-null MEFs. While re-introduction of wild type PP5 resulted in a decrease in levels of SMAD3, and consequently a decrease in levels of TGF β -induced phospho-SMAD3, the catalytically inactive mutant of PP5 did not. This indicated that the catalytic activity of PP5 is essential for the regulation of SMAD3 levels in MEFs. Precisely how PP5 regulates SMAD3 expression levels will be key to understanding its role in the TGF β pathway. A phosphatase controlling the TGF β pathway without directly dephosphorylating key TGF β pathway mediators has been reported recently. PP4 was demonstrated to interact with SMAD1/5 in a transcriptional complex to promote BMP signalling (33).

There are several issues to consider when contemplating the roles for PP5 in the TGF β pathway. Firstly, this study has analysed the roles of PP5 on the TGF β pathway primarily in MEFs. Experiments on other cells derived from PP5-null mice will help establish whether the roles of PP5 on TGF β pathway are ubiquitous or restricted to MEFs. Secondly, that the PP5 activity regulates SMAD3 levels in MEFs is quite clear, but whether PP5 also dephosphorylates SMAD2/3-tail is not. The fact that PP5 interacts with SMAD2/3 and is capable of dephosphorylating SMAD2/3-tail under overexpression conditions implies that PP5 could potentially act as SMAD2/3 phosphatase. However, the association of PP5 with SMAD2/3 was not dependent on TGF β -induction, suggesting that the interaction may have other cellular roles. It could be that other SMAD2/3 interacting partners could be dephosphorylated by PP5. Thirdly, PP5 has been implicated in the regulation of multiple pathways, including adipogenesis by controlling the GR α and PPAR γ phosphorylation (34), DNA-damage repair by controlling the ATM/ATR/Chk1 and p53 pathway components (21,31,34-37), MAPK-mediated growth and differentiation (38-44), and cell cycle arrest (45). Interestingly, GR α has also been implicated in the repression of the TGF β pathway by its association with SMAD3 (46). How these roles of PP5 relate to its potential impact on TGF β pathway is still unknown. Furthermore, the lack of severe and apparent phenotypes in PP5-null mice generated by two laboratories independently implies that during development PP5 may not play a critical role in regulating the extent and duration of the TGF β pathway (31,47).

5. Conclusions

- PPM1A/B are cytoplasmic phosphatases
- PP5 interacts with SMADs2 and 3
- TGF β pathway signalling is enhanced in PP5-null MEFs
- PP5 controls the levels of SMAD3 protein but not transcripts in MEFs
- PP5 catalytic activity is essential for its control of SMAD3 levels in MEFs

Acknowledgements:

We thank Claudio Alarcon and Joan Massague for reagents and technical support during the initial phases of the project. We thank Tricia Cohen for helpful discussions and invaluable reagents. We thank the Sequencing Service (School of Life Sciences, University of Dundee, Scotland) for DNA sequencing and the protein production teams [Division of Signal Transduction Therapy (DSTT), University of Dundee] coordinated by Hilary McLauchlan and

James Hastie for expression and purification of proteins and antibodies. We thank the Medical Research Council, and the pharmaceutical companies supporting the Division of Signal Transduction Therapy Unit (AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck-Serono and Pfizer) for financial support. DB is supported by MRC-Prize studentship.

FIGURE LEGENDS

Fig. 1. PPM1A/B are cytoplasmic phosphatases. (A) HaCaT, mouse ES, MDA231, HUVEC and HEK293 cells were separated into cytosolic and nuclear fractions. Each fraction was immunoblotted with anti-PPM1A or PPM1B antibodies, as well as GAPDH (cytosol) and Histone H1B (nucleus) as markers of the fractionation. As a control, extracts from HaCaT cells transfected with *siRNAs* targeting *PPM1A* (*iPPM1A*) or *FoxO4* (*iFoxO4*) were immunoblotted as indicated. (B) As in A except HeLa, MEFs, Swiss 3T3, HePG2, RAW, Neuro2A, G361 and U87 cells were fractionated into nuclear and cytoplasmic fractions and immunoblotted with the indicated antibodies. The long exposures for PPM1A immunoblots are intentionally included to demonstrate very clear cytoplasmic localisation of PPM1A in all the cells. (C) HaCaT cells transfected with a construct encoding HA-tagged PPM1A were analysed by immunostaining with an antibody against the HA-tag (upper panel) or separated into nuclear and cytoplasmic fractions and immunoblotted with the indicated antibodies (lower panel). (D) HaCaT cells transfected with *siRNAs* targeting *PPM1B* (*iPPM1B*) or *FoxO4* (*iFoxO4*) were analysed by immunostaining with a PPM1B antibody (upper panel) or separated into nuclear and cytoplasmic fractions and immunoblotted along with the cell extracts (lower panel).

Fig. 2. The cytoplasmic localisation of PPM1A is unaffected by TGF β /BMP treatment. (A) HaCaT cells were stimulated with a time course of TGF β (50pM) and then fractionated into cytoplasmic and nuclear fractions. Expression of PPM1A was analysed by immunoblotting. Lamin A/C and GAPDH immunoblots were used as controls for efficient fractionation. The long exposures for PPM1A immunoblots are intentionally included to demonstrate very clear cytoplasmic localisation of PPM1A in all the cells. (B) As in A except that HaCaT cells were subjected to a time course of BMP (25ng/ml) treatment.

Fig. 3. PP5 selectively interacts with SMAD2/3 but not SMAD1. (A) HaCaT cells were treated with or without TGF β (50pM) for 1h prior to lysis in the presence of crosslinker DSP. 500 μ g protein lysates were subjected to immunoprecipitation with pre-immune IgG or antibodies against SMAD2 and SMAD3. Extracts (20 μ g) and IPs were subjected to SDS-PAGE and immunoblotted with the indicated antibodies. (B) As in A except that A549 cells were used and treated with or without Activin A (20ng/ml) and BMP (25ng/ml) as indicated for 1h before lysis. Extracts or the indicated IPs were subjected to SDS-PAGE and immunoblotted with the indicated antibodies.

Fig. 4. Role for okadaic acid (OA)-sensitive phosphatases in the TGF β pathway (A) Schematic diagram depicting the time points that cells were treated with ligands or inhibitors, washed and lysed. HaCaT cells were stimulated with TGF β (25pM) for 45min, in the presence or absence of OA (20nM), and then washed and incubated at 37 $^{\circ}$ C for a further 120min. Cells were lysed at each time point and extracts were subjected to immunoblotting with the indicated antibodies. (B) HEK293 cells were transfected with HA-tagged PP5, alone or with FLAG-

SMADs, for 48h. Cells were stimulated 1h before lysis with Activin A (20ng/ml). Extracts were immunoblotted with the indicated antibodies. (C) Same as C except cells were stimulated with BMP₂ (25ng/ml).

Fig. 5. TGF β -induced transcription is enhanced in PP5-null MEFs (A) PP5 wild type (+/+) and PP5-null (-/-) MEFs were transfected, in triplicate, with either 400ng/well of SRE-Lux or 3TP-Lux – both TGF β -responsive transcription reporter constructs. The transfections were controlled with 40ng/well Renilla. After 16h, cells were stimulated with or without TGF β (25pM) in the presence or absence of 10 μ M SB-431542 for 6h prior to lysis in passive conditions. Error bars represent the standard deviation of 3 independent experiments. (B) As in A except that BMP-responsive luciferase construct BRE-Lux was used in conjunction with BMP (25ng/ml) stimulation and the use of the BMP-type I receptor inhibitor (LDN-193189 at 100nM). (C) RNA was isolated from PP5+/+ or PP5-/- MEFs following a TGF β (25pM) time course. cDNA was synthesised and the expression of indicated TGF β target genes was assessed by qRT-PCR. The fold change in gene expression was measured relative to the untreated PP5+/+ MEFs. The experiment was repeated 3 times. (D) RNA was isolated from PP5+/+ or PP5-/- MEFs in the absence of TGF β stimulation and qRT-PCR was performed using SMAD3 specific primers (n=6).

Fig. 6. PP5 alters the levels of SMAD3 protein in MEFs. (A) Wild type (+/+) and PP5-null (-/-) MEFs were stimulated with TGF β (5pM or 25pM) for indicated times prior to lysis. Cell extracts were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. (B) As in A except cells were stimulated with 25pM TGF β for 45min, washed and either lysed or incubated in DMEM at 37°C for indicated times before lysis. (C) As in A except cells were pre-treated with the proteasome inhibitors Bortezomib (10 μ M) or MG-132 (10 μ g/ml) for 2h before stimulation with TGF β (25pM) for 1h.

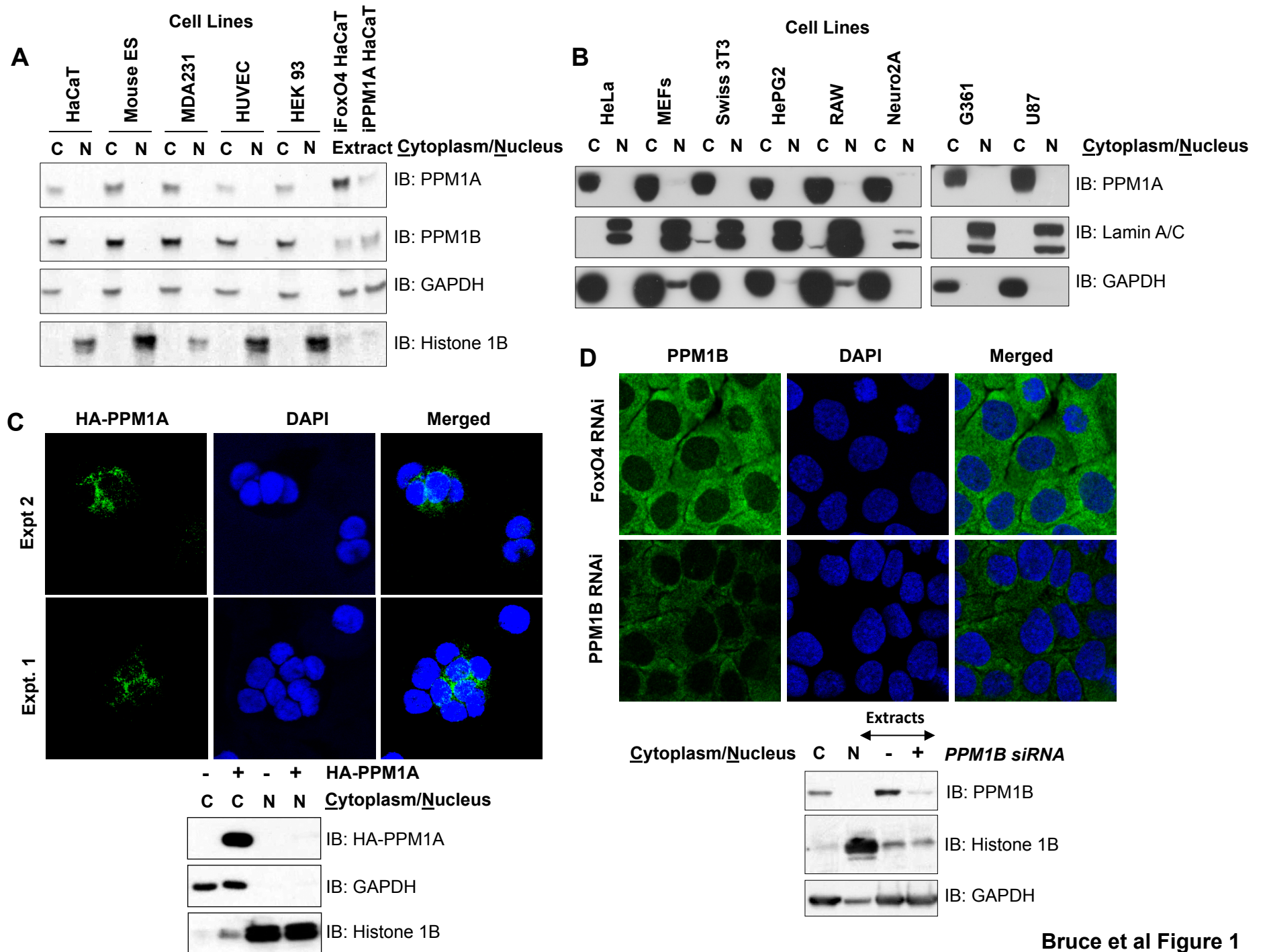
Fig. 7. PP5 catalytic activity controls the expression of SMAD3 protein in MEFs. (A) PP5 (-/-) MEF cells were infected with retroviruses encoding wild type (WT) PP5, catalytically inactive (PD) mutant of PP5 or empty vector controls. Cell extracts were subjected to SDS-PAGE and blotted with the PP5 antibody. (B) As in A except cells were stimulated with TGF β (5pM or 25pM) for 45min prior to lysis. The extracts were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

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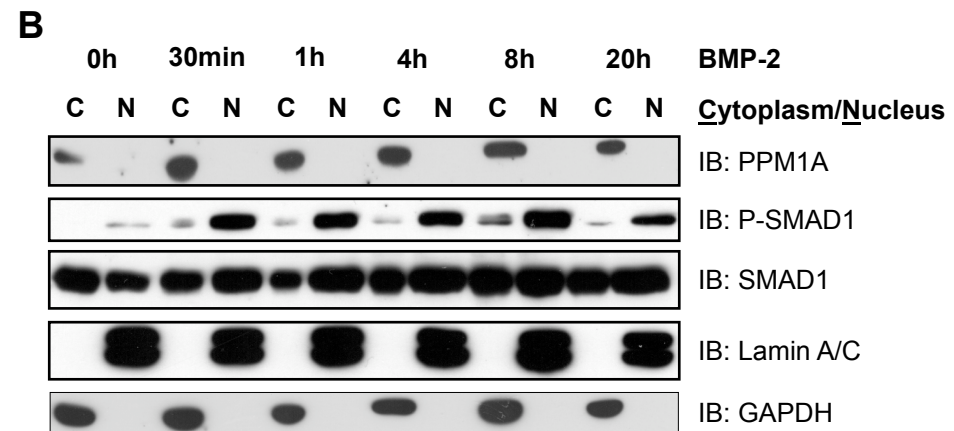
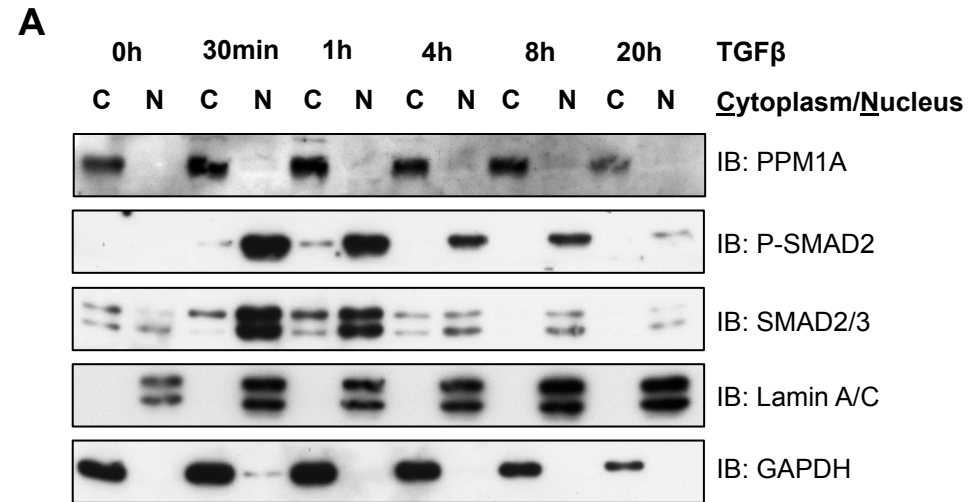
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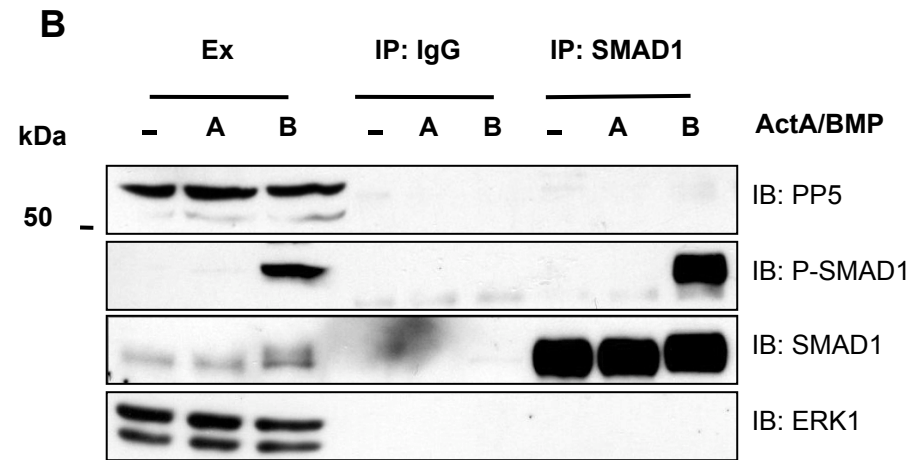
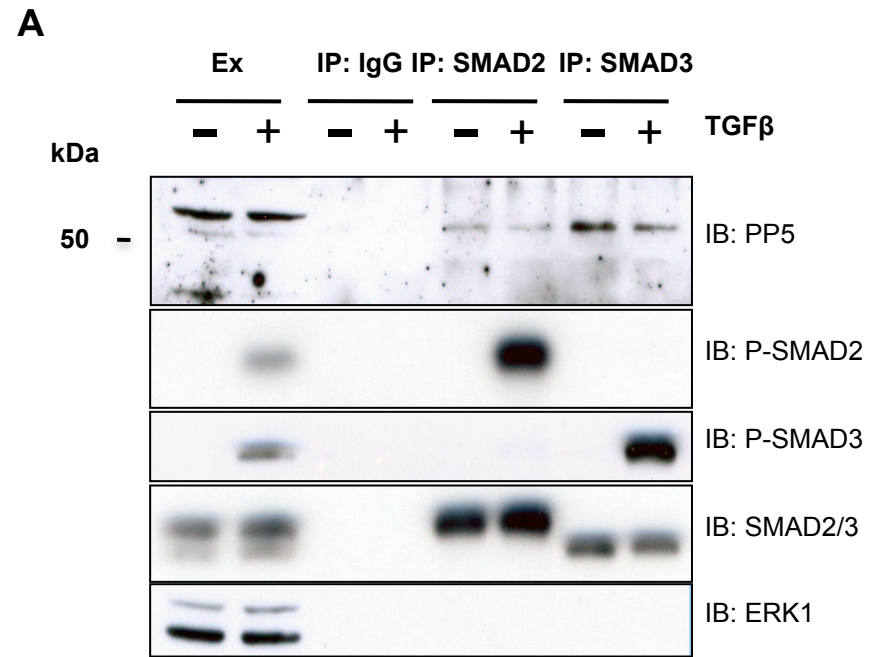
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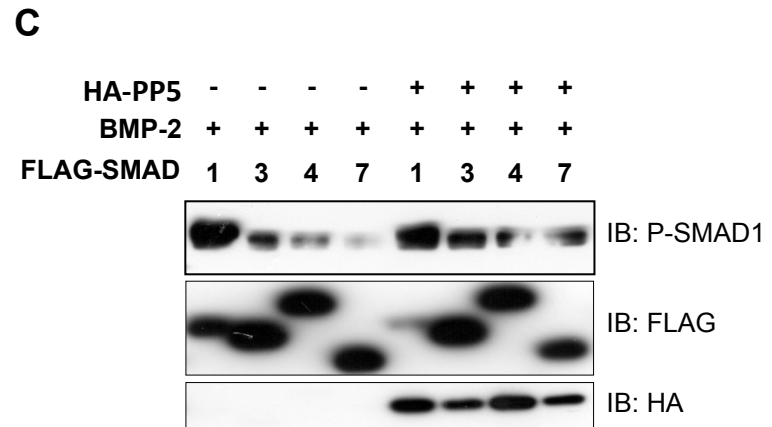
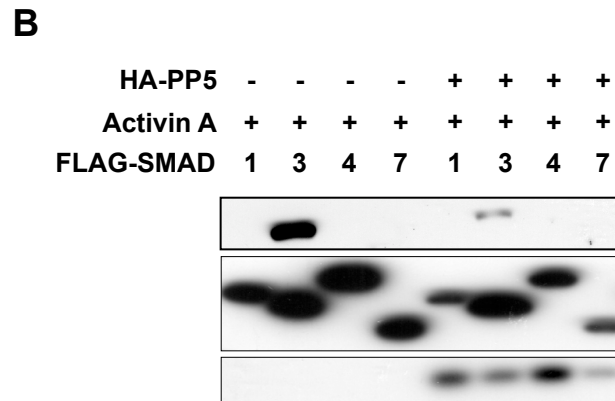
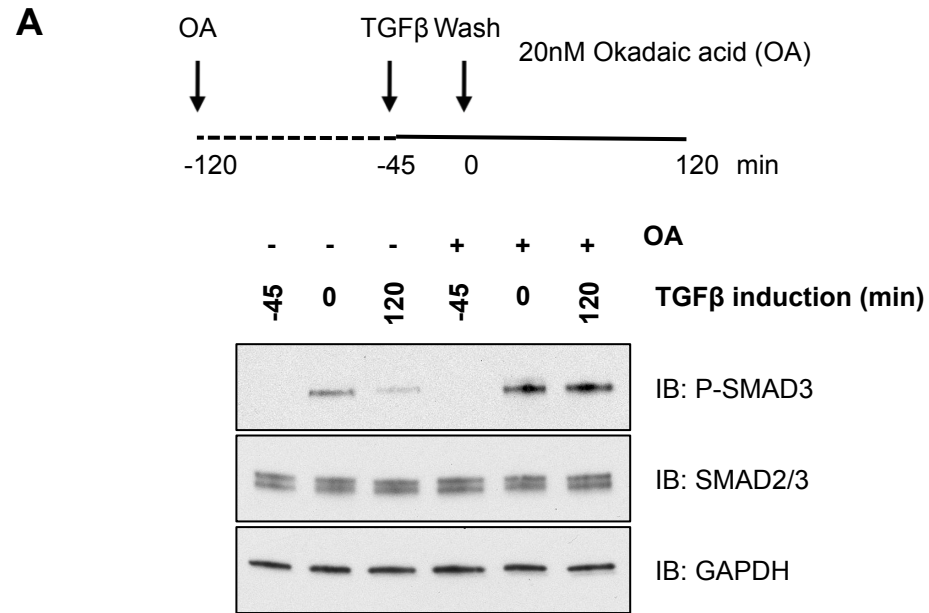
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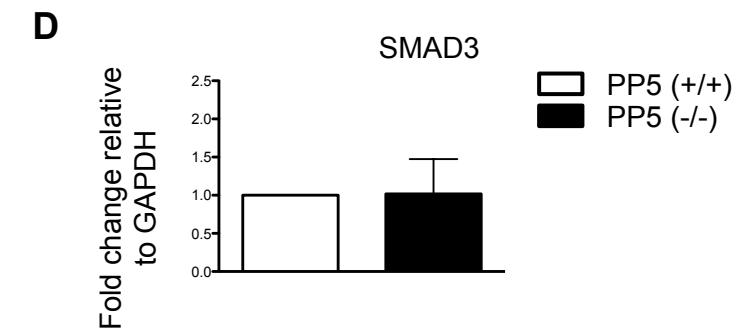
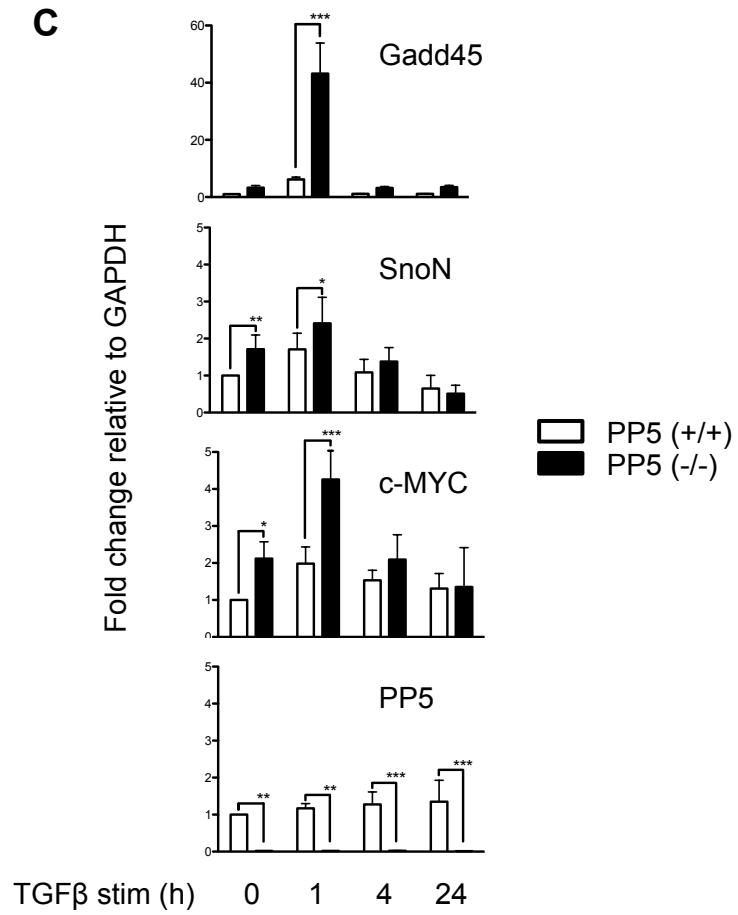
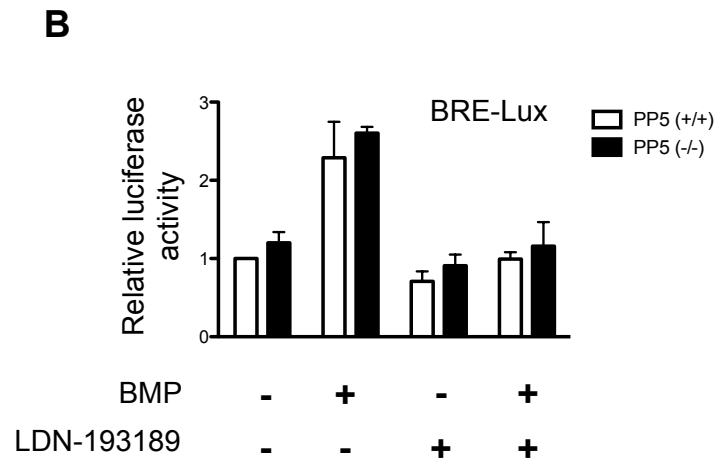
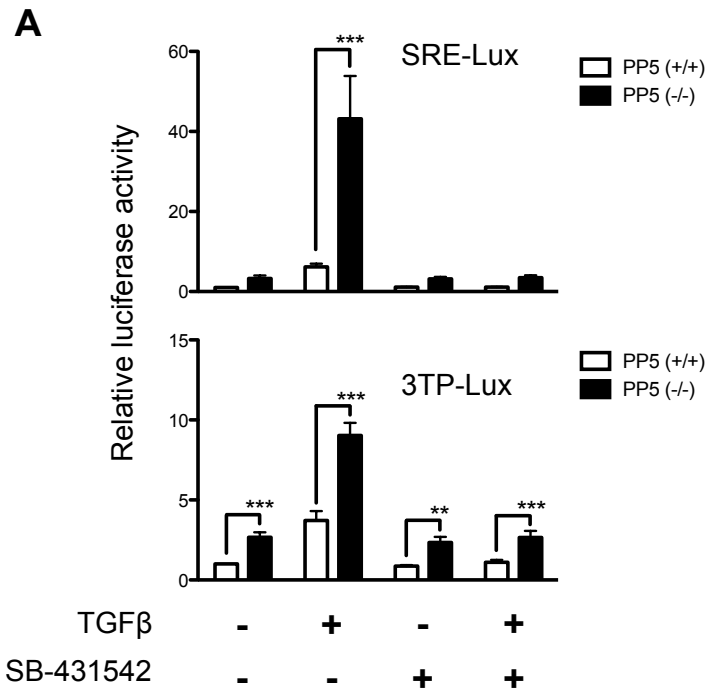


Bruce et al Figure 1

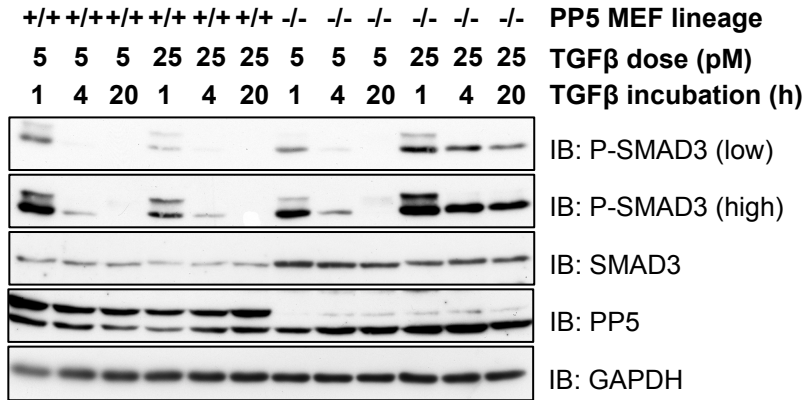




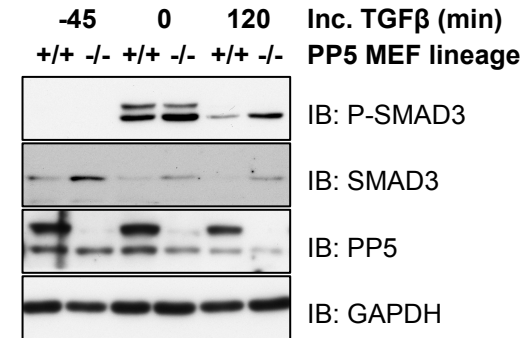




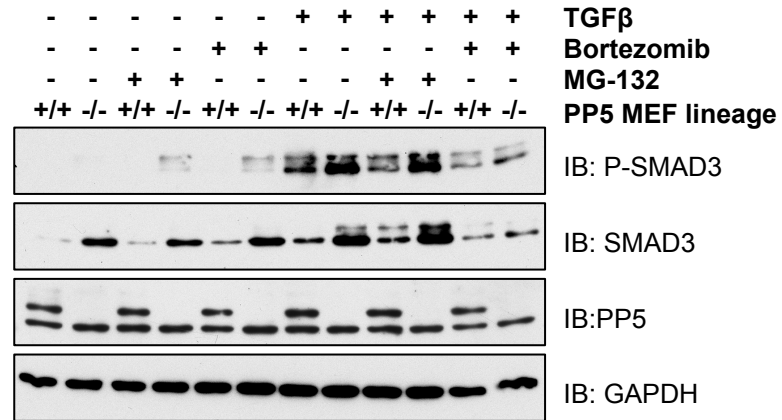
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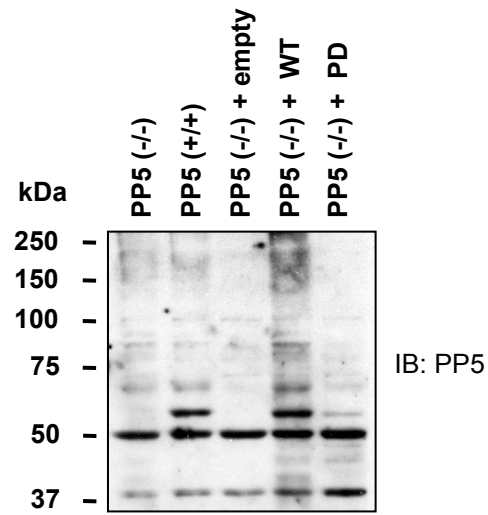
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