Small C-terminal Domain Phosphatases Dephosphorylate the Regulatory Linker Regions of Smad2 and Smad3 to Enhance Transforming Growth Factor-β Signaling

Katharine H. Wrighton, Danielle Willis, Jianyin Long, Fang Liu, Xia Lin, and Xin-Hua Feng

Transforming growth factor-β (TGF-β) controls a diverse set of cellular processes, and its canonical signaling is mediated via TGF-β-induced phosphorylation of receptor-activated Smads (2 and 3) at the C-terminal SXXS motif. We recently discovered that PPM1A can dephosphorylate Smad2/3 at the C-terminal SXXS motif, implicating a critical role for phosphatases in regulating TGF-β signaling. Smad2/3 activity is also regulated by phosphorylation in the linker region (N terminus) by a variety of intracellular kinases, making it a critical platform for cross-talk between TGF-β and other signaling pathways. Using a functional genomic approach, we identified the small C-terminal domain phosphatase 1 (SCP1) as a specific phosphatase for Smad2/3 dephosphorylation in the linker and N terminus. A catalytically inactive SCP1 mutant (dnSCP1) had no effect on Smad2/3 phosphorylation in vivo or in vitro. Of the other FCP/SCP family members SCP2 and SCP3, but not FCP1, could also dephosphorylate Smad2/3 in the linker/N terminus. Depletion of SCP1/2/3 enhanced Smad2/3 linker phosphorylation. SCP1 increased TGF-β-induced transcriptional activity in agreement with the idea that phosphorylation in the Smad2 linker must be removed for a full transcriptional response. SCP1 overexpression also counteracts the inhibitory effect of epidermal growth factor on TGF-β-induced p15 expression. Taken together, this work identifies the first example of a Smad2/3 linker phosphatase(s) and reveals an important new substrate for SCPs.

4 The abbreviations used are: TGF-β, transforming growth factor-β; SCP, small C-terminal domain phosphatase; EGF, epidermal growth factor; CTD, C-terminal domain; HA, hemagglutinin; WCL, whole cell lysate; GST, glutathione S-transferase; SBE, Smad-binding element; siRNA, small interfering RNA; RT, reverse transcription; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MOPS, 4-morpholinepropanesulfonic acid; GFP, green fluorescent protein; CDK, cyclin-dependent kinase; RNAPII, RNA polymerase II; BMP, bone morphogenetic protein.

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result in extracellular signal-regulated kinase (ERK)-mediated phosphorylation of Smad3 at Ser-204, Ser-208, and Thr-179 and of Smad2 at Ser-245/250/255 and Thr-220. Mutation of these sites increases the ability of Smad3 to activate target genes, suggesting that MAPK phosphorylation of Smad3 is inhibitory (11, 12). However, in contrast, ERK-dependent phosphorylation of Smad2 at Thr-8 enhances its transcriptional activity (13). Phosphorylation of Smad3 by p38 MAPK and ROCK (Ser-204, Ser-208, and Ser-213) and c-Jun N-terminal kinase (JNK) (Ser-208 and Ser-213; analogous Ser-250 and Ser-255 in Smad2) may enhance Smad2/3 transcriptional activity, suggesting that Smads and the p38/ROCK/JNK signaling pathways might cooperate in generating a more robust TGF-β response (14–16). A significant increase in Ser-208/Ser-213 phosphorylation of Smad3 is associated with late stage colorectal tumors, suggesting that the linker-phosphorylated Smad3 may mediate the tumor-promoting role of TGF-β in late tumorigenesis (17).

Additional kinases, e.g. MEKK-1, CaMKII, protein kinase C, and CKe, target R-Smads and regulate Smad-dependent transcriptional responses (18–21), and TGF-β may also induce Smad phosphorylation in the linker region as well as at the SXS motif (15, 16). Thus, the Smad linker region is emerging as an important and critical regulatory platform in TGF-β signaling.

Phosphorylation state and the subsequent activity of many proteins are controlled by dynamic interplay between kinases and phosphatases. Protein-serine/threonine phosphatases (PS/TPs) cleave phosphate molecules from serine and threonine residues in target proteins and can largely be grouped into the PPM, PPP, or FCP/SCP families (22).

TFII-F-associating C-terminal domain (CTD) phosphatase (FCP1) and small CTD phosphatases (SCPs, SCP1, SCP2, and SCP3) are characterized by a conserved DXDX(T/V) motif that is essential for their activity and that they share with a superfamily of phosphotransferases and phosphohydrolases (23–26). FCP/SCPs are well known for their ability to negatively regulate RNA polymerase II (RNAPII) by dephosphorylating its CTD at Ser-2 and Ser-5 (25–27). SCPs have also been shown to silence neuronal genes in non-neuronal tissues (28) and to attenuate androgen-dependent transcription (29). Recently, SCPs were reported to dephosphorylate Smad1 in the BMP pathway (30). It is likely that other as yet unidentified proteins involved in gene regulation are also targeted by SCPs.

We recently discovered that Smad2/3 can be dephosphorylated by the PPM family phosphatase PPM1A at the C-terminal SXS motif, to terminate Smad signaling, implicating a critical role for Smad2/3 dephosphorylation in the regulation of TGF-β signaling (31). Using a similar functional genomic approach and taking advantage of phospho-Smad2/3-specific antibodies, we undertook a screen for PS/TPs whose expression reduced Smad2/3 linker/N-terminal phosphorylation. Here we present data identifying SCPs as specific Smad2/3 linker/N-terminal phosphatases, and we show that SCP1 functions to enhance TGF-β signaling and counteract the inhibitory effect of EGF on TGF-β-induced p15 expression. In addition to providing the first example of a Smad2/3 linker phosphatase(s), we reveal an important new SCP substrate and identify the first scenario in which an SCP positively regulates gene transcription.

### EXPERIMENTAL PROCEDURES

**Construction of Human PS/TP cDNA Expression Library**—Full-length cDNAs were synthesized from HaCaT cell total RNA, by high fidelity PCR, as described previously (31).

**Expression Plasmids**—pCDNA3-FLAG-SCP1, pCDNA3-FLAG-dnSCP1, and pCDNA3-FLAG-SCP2 were a generous gift from Dr. Soo-Kyung Lee (Baylor College of Medicine). HA-Smad2/3 and FLAG-Smad2/3 plasmids were described previously (32, 33). pX2F2 and pX3F3H are derived from the cytomegalovirus-driven vector pRK5 (Genentech).

**Cell Transfection, Immunoprecipitation, and Western Blotting**—Cell transfection, immunoprecipitation, and Western blotting were performed essentially as described previously (34, 35). In brief, for phosphatase screening HEK293T cells were co-transfected with tagged Smad2/3 and the relevant phosphatase using Lipofectin (Invitrogen). After 48 h cells were lysed in FLAG lysis buffer (25 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1% Triton X-100, with the addition of protease (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) and phosphatase inhibitors (NaF, Na3VO4)), and subjected to anti-FLAG or anti-HA immunoprecipitation. Products were analyzed by Western blotting using phospho-Smad2/3-specific antibodies. Phospho-Smad3 sera (anti-pS204, anti-pS208, and anti-pS213) and sera recognizing both phospho2 and phosphorylated (anti-PT8 and anti-pT179/T220) were described previously (10). The anti-pS2pS245/250/255 antibody was purchased from Cell Signaling Technology®. Smad SXS motif phosphorylation was assessed using anti-P-Smad2 (Cell Signaling) or anti-P-Smad3 sera, which was a kind gift from Ed Leof (Mayo Clinic). Total Smad2/3 levels were assessed by using anti-HA (Mouse 1.1; Babco), anti-FLAG (M2; Sigma), or anti-Smad2/3 antibody (E20; Santa Cruz Biotechnology) as per the manufacturer’s instructions. Equal protein loading was assessed using an anti-β-actin antibody (Sigma).

Immunoprecipitation of endogenous proteins was performed as described (35) using anti-SCP1 sera (a generous gift from Dr. Soo-Kyung Lee, Baylor College of Medicine). Precipitated proteins were subjected to Western blotting using an anti-Smad2 monoclonal antibody (Cell Signaling).

For growth factor treatment, cells were treated with 2 ng/ml TGF-β and/or 50 ng/ml EGF (in Dulbecco’s modified Eagle’s medium with 0.2% fetal bovine serum), for the indicated times, prior to harvest for immunoprecipitation, Western blotting, and/or in vitro phosphatase assay. EGF activity was confirmed by Western blot analysis of lysates with anti-ERK (M12320; Transduction Laboratories) and anti-P-ERK (9106; Cell Signaling) antibodies. TGF-β activity was confirmed by assessing Smad2/3 SXS phosphorylation as described above.

Nucleofection was used to examine the effect of SCP1 expression on endogenous TGF-β target gene expression. HaCaT cells were nucleofected with GFP or FLAG-SCP1 DNA using the Amaxa Biosystems Nucleofector™ with solution V and program U-020 (Amaza Inc.). After 24 h, cells were treated with TGF-β and/or EGF for 20 h prior to harvest. Samples were subject to Western blotting using anti-p15 (C-20; Santa Cruz Biotechnology) and antibodies as described above.
In Vitro Protein Binding—Recombinant GST-SCP1 and GST-dnSCP1 were generated by purification of bacterially expressed GST fusion proteins. HEK293T cells were lysed in Myc-lysis buffer (20 mM Tris (pH 8.0), 138 mM NaCl, 1% Nonidet P-40) and lysates pre-cleared with 5 μg of glutathione-Sepharose-bound GST. Lysates were subsequently rotated with 2 μg of glutathione-Sepharose-bound GST, GST-SCP1, or GST-dnSCP1 for 1 h at 4 °C. After washing (10 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40), samples were subject to Western blot analysis using anti-Smad2/3 and anti-GST (Babco) antibodies.

In Vitro Phosphatase Assays—FLAG-Smad2/3 and FLAG-SCPs (or negative control FLAG-SnoN) were immunoprecipitated from separately transfected HEK293T cells. FLAG-SCPs (or SnoN) were eluted from protein A-Sepharose using FLAG peptide (Sigma) and incubated with protein A-Sepharose-bound FLAG-Smad2/3 in phosphatase buffer (50 mM Tris (pH 7.5), 60 mM MgCl₂, 2.5 mM dithiothreitol) for 1.5 h at 37 °C. Alternatively, protein A-Sepharose-bound FLAG-Smad2/3 was incubated with recombinant GST-SCP1 or GST-dnSCP1, in phosphatase buffer, for 1.5 h at 37 °C. Assay products were analyzed for Smad2/3 phosphorylation by Western blotting.

In Vitro Kinase Assay/SCP1 Phosphatase Assay—Recombinant GST-Smad3 and GST-SCP1/dnSCP1 were generated by purification of bacterially expressed GST fusion proteins. Glutathione-Sepharose-bound GST-Smad3 (1 μg per reaction) was incubated in kinase buffer (20 mM MOPS (pH 7.4), 10 mM MgCl₂, 1 mM EGTA, 0.5 μM ATP) in the presence or absence of recombinant activated ERK2 (Upstate; 15 ng per 1 μg of GST-Smad3) for 30 min at 30 °C. Glutathione-Sepharose-bound GST-Smad3 was subsequently washed twice in phosphate-buffered saline, and once in phosphatase buffer, prior to incubation with recombinant GST, SCP1, or SCP1dn for 1.5 h at 37 °C. Assay products were analyzed by Western blotting using phosphorylated Smad2/3-specific antibodies.

RNA Interference and RT-PCR—siRNA duplexes were commercially synthesized (Sigma-Proligo). Sense strands (5' to 3') for the indicated targets are as follows: SCP1 (siSCP1, GCCG-GUGGGGUCGAGACCUTT; published previously (30)), SCP2 (siSCP2, GGAUCUGUGUGGUCAUUGAUU), and SCP3 (siSCP3, CUGCCAGCUUGGCCAAGUAUU). HaCaT cells were nucleofected with 3 μg each of siSCP1, siSCP2, and siSCP3 siRNA oligonucleotides or 9 μg of control siRNA, using the Amaxa Biosystems Nucleofector™ as described above for DNA. Cells were harvested for Western blot analysis or for RNA extraction (Trizol®, Invitrogen) and RT-PCR using MultiScribe™ reverse transcriptase (Applied Biosystems) as per the manufacturer’s instructions. The SCP primers (5’ to 3’) are as follows: SCP1 (forward, ATCCCTAAGCAGACCCCAGT; reverse, GTGGAAGACGCAGACTTCTC), SCP2 (forward, CGCTGCGTATAAGGAGGAAG; reverse, GTCACAG...
**SCPs Dephosphorylate Smad2/3 in the Linker**

| PS/TPs tested for Smad2/3 linker-specific dephosphorylation | PS/TPs (phosphatase) | Smad2/3 linker dephosphorylation* |
|-------------------------------------------------------------|-----------------------|----------------------------------|
| SCP1, NM_182642                                            | Yes                   |                                   |
| SCP2, NM_005730                                            | Yes                   |                                   |
| SCP3, NM_001008392                                        | Yes                   |                                   |
| SCP4, NM_016396                                            | No                    |                                   |
| FCP1a, NM_004715                                           | No                    |                                   |
| PPP1CA, NM_002708                                         | No                    |                                   |
| PPP1CB, NM_002709                                         | No                    |                                   |
| PPP1CC, NM_002710                                         | No                    |                                   |
| PPP2CA, NM_002715                                         | No                    |                                   |
| PPP2CB, NM_004156                                         | No                    |                                   |
| PPP3CA, NM_000944                                         | No                    |                                   |
| PPP3CB, NM_021132                                         | No                    |                                   |
| PPP3CC, NM_005605                                         | No                    |                                   |
| PPP4C, NM_003720                                          | No                    |                                   |
| PPP5C, NM_006247                                          | No                    |                                   |
| PPP6C, NM_002721                                          | No                    |                                   |
| PPP7C, NM_006240                                          | No                    |                                   |
| PPP7C2, NM_152225                                         | No                    |                                   |
| PPM1A2, NM_177951                                         | No                    |                                   |
| PPM1B, NM_002706                                          | No                    |                                   |
| PPM1D, NM_003620                                          | No                    |                                   |
| PPM1E, NM_014906                                          | No                    |                                   |
| PPM1F, NM_014634                                          | No                    |                                   |
| PPM1G, NM_177983                                          | No                    |                                   |
| PPM1H, NM_350880                                          | No                    |                                   |
| PPM1I, NM_139245                                          | No                    |                                   |
| PDP1/PMP4C2, NM_018444                                     | No                    |                                   |
| PDP2, NM_020415                                           | No                    |                                   |
| TA-P2C, NM_139283                                         | No                    |                                   |
| ILKAP1, NM_037668                                         | No                    |                                   |
| ILKAP2, NM_176799                                         | No                    |                                   |
| PSPH, NM_004577                                           | No                    |                                   |
| PP2Czeta, NM_001617                                        | No                    |                                   |
| PDXP, NM_020415                                           | No                    |                                   |
| PHLP, NM_194449                                           | No                    |                                   |
| DUSP1/MKPI, NM_004417                                      | No                    |                                   |
| DUSP6/MKPA, NM_001396                                      | No                    |                                   |
| DUSP9/MKPA, NM_001395                                      | No                    |                                   |
| DUSP10/MKPI, NM_007208                                     | No                    |                                   |

*Yes indicates detectable dephosphorylation; no indicates undetectable dephosphorylation.*

GGTCCGCGACTATT, and SCP3 (forward, CTGCTGTTCCGTGATTACA; reverse, TTCCACATGAAAAAACACA).

**Transcription Reporter Assays**—Plasmid SBE-luc (containing the luciferase gene under the control of Smad-binding elements (SBE)), p15-luc, and p21-luc were used to assess TGF-β signaling. As Smad2/3 linker phosphorylation occurs on serine/threonine residues, we screened 40 PS/TPs, including 5 SCPs, 13 PPs, 18 PPMs, and 4 DUSPs (Table 1), all of which were assessed for Smad SXS motif phosphatase activity in our previous screen (31). HEK293T cells were co-transfected with FLAG-tagged Smad2/3 and the specified phosphatase for 48 h, and cells were lysed for anti-FLAG immunoprecipitation. Samples were subject to Western blotting with antibodies specific for the linker/N-terminal Smad phosphorylation sites. As shown in Fig. 1C (lane 7), Smad2/3 were phosphorylated in the linker/N terminal in HEK293T cells cultured in regular growth medium alone. SCP1 was clearly able to dephosphorylate Smad2 at Ser-245/250/255 and Thr-8, and Smad3 at Ser-204/208/213 and Thr-8 (Fig. 1C, lane 6). Interestingly, SCP1 was unable to dephosphorylate Smad2 or Smad3 at Thr-220 or Thr-179 respectively. GFP as a negative control (lane 7) and other phosphatases such as PPP1CC (lane 2) and PPP2CB (lane 4) were unable to dephosphorylate Smad2/3 at the linker/N terminal (Fig. 1C; Table 1). All of the phosphatases screened were untagged to ensure no interference with their phosphatase activity, although a tagged set was also made to examine and normalize their expression level in cells (31).

**SCP1 Specifically Targets the Linker Regions and Not the SXS Motif of Smad2/3**—We next examined the specificity of SCP1 for the Smad2/3 linker by testing its ability to dephosphorylate Smad2/3 at the C-terminal SXS motif. HEK293T cells were co-transfected with FLAG-Smad2/3 and FLAG-SCP1 or FLAG-PPM1A phosphatase. Transfected cells were cultured in the presence or absence of TGF-β for 1 h prior to harvest, to stimulate SXS phosphorylation, and lysed for immunoprecipitation and Western blotting as described above. PPM1A failed to dephosphorylate Smad2/3 in the linker/N terminus, in the presence or absence of TGF-β, as determined by immunoblotting with phospho-specific Smad2/3 antibodies (Fig. 2A, lanes 4 and 5). The FLAG-PPM1A protein was active as judged by its ability to dephosphorylate Smad2/3 at the SXS motif (Fig. 2A, lanes 4 and 5). Conversely, SCP1 was unable to dephosphorylate the Smad2/3 SXS motif but was efficient at dephosphorylating the other Smad2/3 Ser(P)/Thr(P) residues, with the exception of Thr(P)-220 (Smad2) and Thr(P)-179 (Smad3) (Fig. 2A, lanes 6 and 7). The ability of SCP1 to dephosphorylate Smad2/3 at linker/N-terminal Ser/Thr residues was independent of SXS motif phosphorylation (Fig. 2A, lanes 6 and 7). Equal levels of FLAG-Smad2/3 immunoprecipitated product, and expression levels of FLAG-PPM1A and FLAG-SCP1, were confirmed by immunoblotting with an anti-FLAG antibody.

**Results**

**SCP1 Dephosphorylates Smad2/3 in the Linkers**—Our laboratory recently discovered that PPM1A can dephosphorylate Smad2/3 at the C-terminal SXS motif (31). Smad2/3 activity is also regulated by phosphorylation in the linker/N terminus at Thr-220 and Ser-245/250/255 in Smad2, the comparable sites Thr-179 and Ser-204/Ser-208/Ser-213 in Smad3, and at Thr-8 in both Smad2/3 (Fig. 1A). Several kinases responsible for Smad linker phosphorylation have been identified (Fig. 1B), whereas the identity of a phosphatase responsible for dephosphorylating these sites remains elusive. In this study, we sought to determine which phosphatase(s) might dephosphorylate Smad2/3 in the linker region and what effect this would have on TGF-β signaling. As Smad2/3 linker phosphorylation occurs on serine/threonine residues, we screened 40 PS/TPs, including 5 SCPs, 13 PPs, 18 PPMs, and 4 DUSPs (Table 1), all of which were...
immunoblotting of whole cell lysates. We next determined whether the ability of SCP1 to dephosphorylate Smad2/3 is dose-dependent. Almost no linker Smad2/3 phosphorylation was detected on co-transfection with 0.5 μg of SCP1, as represented by immunoblots for pS245/250/255 (Smad2) and pS213 (Smad3) in Fig. 2C (lane 3). However, phosphorylation levels increased with decreasing concentrations of SCP1 (Fig. 2C, lanes 4–6), providing more evidence that SCP1 is a Smad2/3-specific phosphatase. Finally, to confirm that the SCP1-induced decrease in pSmad2/3 is because of phosphatase activity and not because of pSmad2/3 degradation, we examined the effect of SCP1 on Smad2 linker/N-terminal phosphorylation in the presence or absence of the proteasome inhibitor MG132. MG132 treatment did not block the SCP1-induced decrease in linker/N-terminal phosphorylated Smad2 (Fig. 2D, lane 4), suggesting that SCP1 reduces pSmad2/3 levels independently of degradation via the 26 S proteasome.

**SCP1 Physically Interacts with Smad2/3**—The above data suggest that SCP1 is a specific phosphatase for the Smad2/3 linker/N-terminal region. To determine whether Smad2/3 interacts with SCP1, we first generated recombinant GST-SCP1 and GST-dnSCP1 fusion proteins and assessed them for their ability to bind to endogenous Smad2/3 in HEK293T cell lysates. Western blot analysis revealed that GST-SCP1, but not GST alone, could specifically bind to Smad2/3 in HEK293T lysates (Fig. 3A). Use of an even amount of GST, as compared with GST-SCP1 and GST-dnSCP1, was confirmed by using an anti-GST antibody. Equal amounts of total protein and Smad2/3 in each cell lysate were confirmed by immunoblotting with anti-actin and anti-Smad2/3 antibodies, respectively (Fig. 3A). The catalytically inactive mutant dnSCP1 was still able to interact with Smad2/3 (Fig. 3A, lane 3), suggesting that SCP1 phosphatase activity is not essential for Smad2/3 binding. We next wished to determine whether Smad2/3 might interact with SCP1 in vivo. HEK293T cells were co-transfected with FLAG-SCP1 and Myc-Smad3, and co-immunoprecipitation experiments were performed. FLAG-SCP1 co-precipitated Myc-Smad3 (Fig. 3B, lane 3) as did the catalytically inactive mutant dnSCP1 (lane 5). We next immunoprecipitated endogenous SCP1 from HEK293T cell lysates, using an anti-SCP1 sera, to confirm that these proteins interact at the physiological level. Western blot analysis of products using anti-Smad2 antibody revealed that Smad2 interacts with SCP1 at the endogenous level (Fig. 3C, lane 2). No Smad2 was detected in the IgG control lane (Fig. 3C, lane 1). Taken together, the data in Figs. 2 and 3 suggest that SCP1 is a specific phosphatase for the Smad2/3 linker and N-terminal regions.

**SCP1 Dephosphorylates Smad2/3 Linkers in Vitro**—To rule out the possibility that SCP1 activates another phosphatase in the cell, which in turn dephosphorylates the Smad2/3 linker, we carried out several in vitro cell-free dephosphorylation assays. In the first assay, HEK293T cells were transfected separately with FLAG-SCP1/dnSCP1, FLAG-SnoN (which should not harbor phosphatase activity), or HA-Smad2/3. After 48 h, cells were lysed, and anti-FLAG and anti-HA immunoprecipitations were carried out for SCP1/dnSCP1 or SnoN and Smad2/3, respectively. The ability of SCP1/dnSCP1 and SnoN to dephosphorylate immunopurified Smad2/3 was analyzed. Equal levels of total HA-Smad2/3 were used in each assay reaction, as deter-

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**FIGURE 2. SCP1 requires its catalytic activity to specifically dephosphorylate the linker/N terminus, and not the SXS motif, of Smad2/3.** A, SCP1 specifically dephosphorylates Smad2/3 in the linker/N terminus. HEK293T cells were transfected with FLAG-Smad2/3 and the phosphatase FLAG-SCP1 or FLAG-PMM1A. Where indicated, 48 h after transfection cells were treated with TGF-β for 1 h prior to harvest, immunoprecipitation (IP), and Western blot analysis as in Fig. 1C. FLAG-phosphatase levels were determined by anti-FLAG immunoblotting of whole cell lysates (WCL). B, Smad2/3 dephosphorylation requires SCP1 catalytic activity. HEK293T cells were co-transfected with FLAG-Smad2/3 and FLAG-SCP1 or catalytically inactive FLAG-dnSCP1. Cells were harvested and subject to immunoprecipitation and Western blot analysis as in Fig. 1C. C, SCP1 dephosphorylates the Smad2/3 linker in a dose-dependent manner. HEK293T cells were transfected with FLAG-Smad2/3 and decreasing concentrations of FLAG-SCP1. Cells were harvested and subject to immunoprecipitation as in Fig. 1C. FLAG-SCP1 levels were determined by anti-FLAG immunoblotting of WCL. D, Smad2 dephosphorylation occurs in the presence of MG132. HEK293T cells were transfected with HA-Smad2 and FLAG-SCP1. Where indicated cells were treated with MG132 for 4 h prior to harvest and analysis as in Fig. 1C.
SCPs Dephosphorylate Smad2/3 in the Linker

A.

FIGURE 3. SCP1 Interacts with Smad2/3. A, HEK293T cell lysates were subject to incubation with glutathione-Sepharose-bound GST, GST-SCP1, or GST-dnSCP1. After washing, precipitation samples were analyzed by Western blotting using an anti-Smad2/3 antibody and anti-GST. Equal Smad2/3 levels and total protein across samples were confirmed by immunoblotting of WCL samples with anti-Smad2/3 and anti-actin antibodies, respectively. B, SCP1 and dnSCP1 interact with Smad3. HEK293T cells were co-transfected with Myc-Smad3 and FLAG-SCP1 or FLAG-dnSCP1. wt, wild type; dn, mutant. SCP-bound Smad3 was detected by anti-FLAG immunoprecipitation (IP) and anti-Myc Western blotting. C, endogenous SCP1 and Smad3 interact. HEK293T cell lysates were subject to anti-SCP1 immunoprecipitation. SCP1-bound Smad2 was detected by anti-Smad2 immunoblotting.

Dephosphorylation of Smad2/3 Linkers by SCP2 and SCP3—We next examined other members of the FCP/SCP family and found that SCP2 and SCP3 could also dephosphorylate Smad2/3 at all non-S sites apart from Thr-220/Thr-179 (Table 1; Fig. 5A). A putative additional member of the FCP/SCP family, CTD small phosphatase like 2 (CTDSPL2), has also been identified recently (36, 37). We also tested this protein, which we refer to as SCP4 in this study, in parallel with SCP2 and SCP3 and found that SCP4 expression did not result in dephosphorylation of the linker/N terminal (Fig. 5A, lane 7). FLAG-SCP phosphatase expression was confirmed by immunoblotting with anti-FLAG. The founding member of the FCP/SCP family, FCP1, was also unable to dephosphorylate Smad2/3 (Fig. 5B, lane 3) when assayed in parallel with FLAG-SCP1 as a positive control.

To further establish the function of SCPs as Smad2/3-specific phosphatases, we examined the effect of SCP overexpression and SCP depletion on endogenous Smad2/3 linker/N-terminal phosphorylation. First, FLAG-SCP1 and FLAG-SCP3 significantly reduced endogenous Smad2 phosphorylation at Ser-245/250/255 and Thr-8 (Fig. 5C, lanes 2 and 4). As expected and consistent with our data presented in Fig. 5, A and B, FLAG-dnSCP1 and FLAG-SCP4 expression had no effect on endogenous Smad2 linker phosphorylation, as is the case for the negative control FLAG-SnoN (Fig. 5C, lanes 1, 3, and 5). Total immunoprecipitated endogenous Smad2 was determined by immunoblotting with an anti-Smad2 antibody, and FLAG-SnoN and FLAG-SCP expression levels were determined by anti-FLAG immunoblotting. Second, as SCP1, SCP2, and SCP3 could all dephosphorylate the Smad2/3 linker, to perform loss-of-function analysis we nucleofected HaCaT cells with siRNA knockdown of SCP RNA resulted in the reduction of SCP2 and SCP3 RNA levels by ~70 and 100%, respectively, although the SCP1 RNA level was only marginally reduced (Fig. 5D, lower panel). Despite only partial depletion of SCP1/2/3, endogenous Smad2 (Ser-245/250/255) and Smad3 (Ser-208) linker phosphorylation was increased in siSCP1/2/3 nucleofected cells as compared with control cells (Fig. 5D, upper panel). Thus, SCP1, SCP2, and SCP3 appear to be genuine Smad2/3 phosphatases that are specific for the linker region and N terminus.

SCP1 Enhances TGF-β Transcriptional Responses—We next sought to determine the effect of SCP1-mediated dephosphorylation of the Smad2/3 linker on TGF-β transcriptional responses. We first used SBE-luc, a TGF-β-responsive synthetic reporter gene dependent on Smad activation. SCP1 was able to increase SBE-luc activity in HaCaT cells (Fig. 6A). Smad2/3 mediates the transcriptional regulation of p15 (38) and p21 (39, 40); thus we next compared the effects of SCP1 and dnSCP1 on these natural TGF-β-responsive promoters. TGF-β induced an ~35-fold increase in p15-luc activity in HaCaT cells, and expression of SCP1 further increased TGF-β-induced activity by ~2-fold, whereas dnSCP1 was unable to enhance TGF-β-induced activity, suggesting that the ability of SCP1 to increase the TGF-β response is because of its phosphatase activity (Fig. 6B). SCP1 was also able to induce ~1.5-fold more p21-luc activity than TGF-β alone (Fig. 6C). Taken together, these data suggest that SCP-induced dephosphorylation of
Smad2/3 can function to potentiate the Smad-mediated TGF-β response. This is in agreement with previous studies demonstrating that mutation of Smad2/3 linker phosphorylation sites increases the TGF-β response in Smad-specific transcriptional assays (10, 12).

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To determine whether the R-Smad-SCP1 interaction is TGF-β- or EGFR-regulated, HEK293T cells were co-transfected with FLAG-SCP1 and Myc-Smad3, treated with TGF-β or EGFR, and subjected to co-immunoprecipitation. We found that FLAG-SCP1 co-immunoprecipitated Myc-Smad3 regardless of growth factor treatment (Fig. 7A, lanes 3–5), as did the catalytically inactive mutant dnSCP1 (lanes 7–9). The EGF and TGF-β ligands were functionally active in this experiment, as judged by the presence of endogenous pERK (Fig. 7A, lanes 5 and 9) and pSmad2-SXS (Fig. 7A, lanes 4 and 8), respectively. We then immunoprecipitated Myc-Smad3 from the same cell lysates and assessed its linker dephosphorylation in the absence or presence of TGF-β and EGF. SCP1 was clearly able to dephosphorylate Smad3 at the representative linker/N-terminal sites Ser-208 and Thr-8, regardless of growth factor treatment (Fig. 7B, lanes 3–5). The dnSCP1 mutant was unable to dephosphorylate Smad3 at Ser-208 and Thr-8 under all conditions, as expected (Fig. 7B, lanes 7–9).

To confirm that TGF-β and/or EGF do not regulate the phosphatase activity of SCP1, we carried out an in vitro dephosphorylation assay using immunoprecipitated FLAG-SCP1/dnSCP1 and HA-Smad2/3 from separately transfected HEK293T cells. Cells transfected with FLAG-SCP1 were cultured in the absence or presence of TGF-β and/or EGF prior to anti-FLAG immunoprecipitations. An in vitro phosphatase assay was then performed as described for Fig. 4A. Equal levels of total HA-Smad2/3 were used in each assay reaction. SCP1 was able to dephosphorylate the Smad2/3 linker in vitro, and this was not affected by TGF-β or EGF treatment (Fig. 7C). Taken together the data in Fig. 7, A–C, suggest that TGF-β and EGF do not regulate R-Smad-SCP1 interactions and the ability of SCP to dephosphorylate Smad2/3 in the linker/N-terminal region. SCP1 expression level was also unchanged in the absence or presence of TGF-β and/or EGF treatment.

Finally, it has been reported that EGF can inhibit TGF-β-induced up-regulation of p15 mRNA and the TGF-β anti-proliferative effect (41). The authors found that this was not because of the abrogation of SX5 phosphorylation of R-Smads. As EGF is known to stimulate R-Smad linker phosphorylation, we sought to determine whether SCP1 could counteract the inhibitory effect of EGF on TGF-β-mediated p15 induction. In control cells (GFP-transfected) EGF treatment, both alone or in conjunction with TGF-β, resulted in a higher degree of Smad2 phosphorylation at Ser-245/250/255 and Thr-8, and Smad3 phosphorylation at Ser-208 and Thr-8, as predicted (Fig. 7D, compare lanes 1 and 2 with 3 and 4). In addition, EGF co-treatment greatly reduced TGF-β-induced p15 up-regulation in these cells (Fig. 7D, lane 4). Notably, expression of SCP1 induced higher levels of endogenous p15 than control cells when treated with EGF alone (compare lanes 3 and 7), and p15 levels were also partially restored in TGF-β/EGF co-treated...
cells overexpressing SCP1 (Fig. 7D, compare lanes 4 and 8). The increase in endogenous p15 in SCP1 overexpressing cells correlated with a decrease in Smad linker/N-terminal phosphorylation (Fig. 7D). Thus, these data suggest that the EGF-mediated inhibition of TGF-β-induced p15 up-regulation may be due, at least in part, to an EGF-mediated increase in Smad2/3 linker phosphorylation.

**DISCUSSION**

In addition to TGF-β-induced SXS motif phosphorylation, which is essential for Smad activation, Smad signaling is fine-tuned for diverse biological responses by phosphorylation in the linker region and N terminus. As Smad linker/N-terminal phosphorylation results from interaction with a variety of intracellular protein kinases, the Smad linker/N terminus also serves as a critical platform for modulating cross-talk between TGF-β and other signaling pathways. As a consequence, dephosphorylation of the Smad2/3 linker might also serve as an important mechanism for modulating signal cross-talk within cells. Here we present several lines of evidence to identify SCP1 as the first example of a Smad2/3 linker/N-terminal-specific phosphatase. First, of the 40 phosphatases screened, only SCP1, SCP2, and SCP3 were capable of dephosphorylating Smad2/3 in the linker region at sites Ser-245/250/255 (Smad2) or Ser-204/208/213 (Smad3) and at the N-terminal Thr-8. Second, recombinant SCP1, but not its catalytically inactive counterpart dnSCP1, was capable of dephosphorylating in vitro phosphorylated recombinant Smad3 in a cell-free assay. Third, SCP1 physically interacts with Smad2/3. Fourth, partial knockdown of endogenous SCP1/2/3 enhances Smad2 and Smad3 linker phosphorylation.

It is surprising, given that Thr-220 (Smad2) and Thr-179 (Smad3) can be phosphorylated by the same kinases as other linker/N-terminal Thr/Ser sites, that Smad2/3 Thr(P)-220/Thr(P)-179 was not dephosphorylated by SCP1 (or SCP2/3) in vivo or in vitro. Phosphorylation at Thr-220 in Smad2 and Thr-179 in Smad3 could have a unique role, as compared with Ser-245/250/255 (Smad2) and Ser-204/208/213 (Smad3), which excludes it from dephosphorylation by SCPs. In fact, of the 40 phosphatases screened, none had phosphatase activity toward Smad2/3 Thr(P)-220/Thr(P)-179. Thus, it is likely that Smad2/3 can be dephosphorylated at this site by a phosphatase outside the

**FIGURE 5.** SCP1/SCP2/SCP3, but not SCP4 and FCP1, dephosphorylate Smad2/3 in the linker region and at the N terminus. **A,** SCP1/2/3 dephosphorylate Smad2/3. HEK293T cells were co-transfected with HA-Smad2/3 and the indicated FLAG-SCP. Cells were harvested and subjected to immunoprecipitation (IP) and Western blot analysis as in Fig. 1C. B, FCP1a does not dephosphorylate Smad2/3 in the linker. HEK293T cells were co-transfected with FLAG-Smad2/3 and His-FCP1a, or FLAG-SCP1 as a positive control. Cells were harvested and subjected to immunoprecipitation and Western blot analysis as in Fig. 1C. SCP1 and FCP1a expression was confirmed by immunoblotting of WCL with anti-FLAG and anti-His antibodies, respectively. C, SCPs can dephosphorylate endogenous Smad2. HEK293T cells were transfected with the indicated FLAG-tagged SCP or FLAG-SnoN as a negative control. After 48 h, cells were lysed and subjected to immunoprecipitation (IP) with anti-Smad2 antibody. Products were analyzed by Western blotting with anti-Smad2 and phospho-Smad2-specific antibodies. SCP expression was confirmed by immunoblotting of WCL samples with anti-FLAG. D, SCP reduction enhances linker phosphorylation of endogenous Smad2/3. HaCaT cells were nucleofected with control siRNA or combined siSCP1, siSCP2, and siSCP3 oligonucleotides. Cells were harvested 50 h after nucleofection for Western blot analysis using phospho-Smad2/3-specific antibodies, and antibodies against total Smad2/3 and actin (upper panels). Alternatively, cells were harvested for RNA extraction and RT-PCR analysis of SCPs and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (lower panels).
is interesting to note that we did not find SCP1 to be an efficient phosphatase at the SXS motif of Smad2/3 (31) and Smad1 (42), but we found it was specific for Smad2/3 linker dephosphorylation with the exception of Thr(P)-220/Thr(P)-179 (Fig. 2A). Additionally, we did not find the Smad2/3 C-terminal SXS phosphatase PPM1A to be a Smad2/3 linker phosphatase. We also did not find the SCP1-Smad3 interaction, or the Smad2/3-linker phosphatase activity of SCP1, to be regulated by TGF-β (which stimulates SXS motif phosphorylation) or EGF (which can stimulate linker phosphorylation) (Fig. 7, A–C).

In our study we found that SCP1, SCP2, and SCP3 were able to dephosphorylate Smad2/3 in the linker/N terminus, suggesting that all three SCPs can function in a similar manner. We found that unlike SCP1/2/3, the FCP/SCP founding family member FCP1 was unable to dephosphorylate the Smad2/3 linker/N terminus. The SCP1/2/3 proteins are homologous to the FCP1 catalytic domain, but they lack the BRCT binding domain of FCP1 (25). Although the difference in BRCT domain status does not prevent both SCPs and FCP from dephosphorylating the CTD of RNAPII, it is possible that in the context of Smad2/3 dephosphorylation the FCP1 BRCT domain acts in an inhibitory manner. SCP4/CTD-SPL2, a putative member of the FCP/SCP family, was also unable to dephosphorylate Smad2/3.

Our data suggest that SCP1-mediated dephosphorylation of Smad2/3 can function to potentiate the Smad-mediated TGF-β response. SCP1 expression increased transcription from a TGF-β-responsive synthetic reporter gene dependent on Smad activation and also from p15 and p21 gene-specific promoters. The dnSCP1 mutant was unable to enhance TGF-β-induced activity, suggesting the SCP1-mediated increase in TGF-β response is because of its phosphatase activity and, based on our data in this paper, likely a result of Smad2/3 dephosphorylation. This is in agreement with previous studies showing that mutation of CDK2/4 and ERK2 phosphorylation sites in the Smad2/3 linker increases the TGF-β response in Smad-specific transcriptional assays (10–12). However, as others find Smad2/3 linker phosphorylation enhances TGF-β-induced transcription (14–16), it is possible that the role of Smad2/3 linker phosphorylation is cell type/context-dependent, dependent on the phosphorylating kinase, and/or

scope of our screen. While this manuscript was under review, Knockaert et al. (30) reported that SCP1/2/3 dephosphorylate BMP-induced C-terminal SXS motif phosphorylation of Smad1. It

**FIGURE 6.** SCP1, but not dnSCP1, increases TGF-β-induced gene transcription. A–C, HaCaT cells were transfected with SBE-luc reporter (A), p15-luc (B), or p21-luc (C), and the indicated plasmid DNAs. TGF-β treatment and luciferase assay are described under “Experimental Procedures.”

**FIGURE 7.** SCP1 dephosphorylates the Smad linker independently of TGF-β and EGF. A, TGF-β and EGF do not affect the SCP1-Smad interaction. HEK293T cells were co-transfected with Myc-Smad3 and FLAG-SCP1 or FLAG-dnSCP1. Transfected cells were untreated or treated with TGF-β or EGF for 1.5 h prior to harvest. SCP-bound Smad3 was detected by anti-FLAG immunoprecipitation (IP) and anti-Myc Western blotting. Endogenous Smad2 and pSmad2-SXS and ERK and pERK, were assessed by immunoblotting of WCL. B, TGF-β and EGF do not affect SCP1-mediated dephosphorylation of the Smad2/3 linker in vivo. Using cell lysates from A, Myc-Smad3 was immunoprecipitated with anti-Myc antibody, Smad3 phosphorylation was detected by immunoblotting with phospho-Smad3 specific antibodies, and total Smad3 levels were analyzed using anti-Myc antibody. F-SCP1/dnSCP1 expression was confirmed by anti-FLAG immunoblotting with WCL. C, TGF-β and EGF do not influence SCP1-mediated dephosphorylation of the Smad2/3 linker in vitro. HEK293T cells were transfected with FLAG-SCP1 or FLAG-dnSCP1, and in a separate group with HA-Smad2/3. FLAG-SCP1-transfected cells were cultured in the absence or presence of TGF-β and/or EGF for 1.5 h prior to harvest, as indicated. FLAG-SCP1 proteins were immunoprecipitated and then eluted from protein A-Sepharose using FLAG-peptide. Separately immunopurified HA-Smad2/3 proteins were incubated with eluted SCP1 or dnSCP1. Products were analyzed by Western blotting using phospho-Smad2/3-specific antibodies. wt, wild type; dn, mutant. D, SCP1 counteracts the inhibitory effect of EGF on TGF-β-induced p15 expression. HaCaT cells were nucleofected with GFP or FLAG-SCP1 DNA. Cells were untreated or treated with TGF-β and/or EGF for 20 h prior to lysis as indicated. WCL were analyzed using antibodies against TGF-β–induced proteins (anti-p15) and total or phospho-Smad2/3 and ERK. FLAG-SCP1 expression was confirmed by immunoblotting with anti-FLAG.
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even dependent on the combination of linker Thr/Ser sites phosphorylated. In general, in this study SCP1-mediated Smad2/3 dephosphorylation *in vivo* cannot be linked to a specific kinase as the phosphorylation is stimulated under physiologically relevant conditions by growth medium, which could contain factors to activate all relevant kinases. However, SCP1 can be linked to *in vitro* dephosphorylation of ERK2 kinase-phosphorylated sites as recombinant ERK2 was used to phosphorylate Smad3 in this experiment. We also noticed that SCP1 could dephosphorylate EGF-induced endogenous Smad2/3 linker phosphorylation in HaCaT cells. This correlated with a higher level of TGF-β-induced p15 in the presence of EGF (Fig. 7D), suggesting that SCP1, and thus Smad2/3 linker dephosphorylation, results in a more robust TGF-β response.

FCP/SCPs are well known for their ability to dephosphorylate the CTD of RNAPII at serines 2 and 5 (25–27), and until recently this appeared to be the only true target of FCP/SCPs identified. SCPs are transcriptional regulators that negatively regulate RNAPII (25–27), neuronal gene expression (28), androgen-dependent transcription (29), and BMP signaling (23, 24). We have identified that SCPs are transcriptional regulators that negatively regulate RNAPII (25–27), neuronal gene expression (28), androgen-dependent transcription (29), and BMP signaling (30). It has also been shown that SCP1 inhibits transcription from a variety of promoter-reporter gene constructs, while expression of the dnSCP1 mutant lacking phosphatase enhances transcription (25). Our finding that SCP1, but not the catalytically inactive dnSCP1, led to an enhanced TGF-β transcriptional response may be the first example of SCP positively regulating transcription. Thus, there appears to be specificity for SCP1 on TGF-β signaling, as we see the opposite effect to what is expected from the negative regulation of global gene transcription through dephosphorylating RNAPII.

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SCP2 was initially identified (and called OS4) in a chromosomal region frequently amplified in sarcomas and brain tumors (43) and was subsequently linked to TGF-β superfamily signaling in *Xenopus* (44). *Xenopus* SCP2/Os4 (XSCP2) was shown to doralize the ventral mesoderm, resulting in a phenotype similar to that initiated by activation of Activin (TGFB family) signaling or the inhibition of BMP signaling. Recently, published data suggest that secondary axis formation by XSCP2 is because of inhibition of BMP signaling via its ability to dephosphorylate the Smad1/5/8 motif (30). Our identification of SCP2 as a Smad2/3 linker phosphatase that can enhance Smad2/3 signaling is also consistent with the role of XSCP2 in promoting doralization of the ventral mesoderm. Thus, it seems possible that on some level SCPs could have a dual function in promoting secondary axis development in *Xenopus* embryos, simultaneously promoting Activin signaling (via dephosphorylation of the Smad2/3 linker) and inhibiting BMP signaling (via dephosphorylation of the Smad1/5/8 motif).

In conclusion, SCP1/2/3 are true phosphatases for the linker/N-terminal region of Smad2/3 and are likely to be involved in modulating cross-talk between signaling pathways that converge on R-Smads. The combination of linker (de)phosphorylation events is likely to contribute to the final gene responses to Smad signaling, and thus may ultimately be important in cancer and other diseases. To this end a significant increase in Ser-208/Ser213 phosphorylation of Smad3 is associated with late stage colorectal tumors (17), suggesting that understanding how (de)phosphorylation of the Smad2/3 linker occurs in normal cells could be clinically relevant. Our identification of Smad2/3 as a critical new substrate for SCPs opens the door to the possibility that these phosphatases may have many, as yet unidentified, key targets in cell signaling.

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REFERENCES

1. Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., and Wrana, L. J. (1997) *J. Biol. Chem.* 272, 27678–27685
2. Souchelnytskyi, S., Tamaki, K., Engstrom, U., Wernstedt, C., ten Dijke, P., and Heldin, C. H. (1997) *J. Biol. Chem.* 272, 28107–28115
3. Feng, X. H., and Derynck, R. (2005) *Annu. Rev. Cell Dev. Biol.* 21, 659–693
4. Massague, J., and Gomez, R. R. (2006) *FEBS Lett.* 580, 2811–2820
5. Massague, J., Seoane, J., and Wotton, D. (2005) *Genes Dev.* 19, 2783–2810
6. Izzl, L., and Attisano, L. (2004) *Oncogene* 23, 2071–2078
7. Feng, X. H., and Lin, X. (2006) in *Smad Signal Transduction* (Dijke, P. T., and Heldin, C.-H., eds) pp. 253–276, Springer-Verlag New York Inc., New York
8. Derynck, R., and Zhang, Y. E. (2003) *Nature* 425, 577–584
9. Moustakas, A., and Heldin, C. H. (2005) *J. Cell Sci.* 118, 3573–3584
10. Matsuura, I., Denissova, N. G., Wang, G., He, D., Long, J., and Liu, F. (2004) *Nature* 430, 226–231
11. Kretzschmar, M., Doody, J., Timokhina, L., and Massague, J. (1999) *Genes Dev.* 13, 804–816
12. Matsuura, I., Wang, G., He, D., and Liu, F. (2005) *Biochemistry* 44, 12546–12553
13. Funaba, M., Zimmerman, C. M., and Mathews, L. S. (2002) *J. Biol. Chem.* 277, 41361–41368
14. Engel, M. E., McDonnell, M. A., Law, B. K., and Moses, H. L. (1999) *J. Biol. Chem.* 274, 37413–37420
15. Mori, S., Matsuuzaki, K., Yoshida, K., Furukawa, F., Tahashi, Y., Yamagata, H., Sekimoto, G., Seki, T., Matsu, H., Nishizawa, M., Fujisawa, J., and Okazaki, K. (2004) *Oncogene* 23, 7416–7429
16. Kamaraju, A. K., and Roberts, A. B. (2005) *J. Biol. Chem.* 280, 1024–1036
17. Yamagata, H., Matsuuzaki, K., Mori, S., Yoshida, K., Tahashi, Y., Furukawa, F., Sekimoto, G., Watanabe, T., Uemura, Y., Sakaida, N., Yoshioha, K., Kamiyama, Y., Seki, T., and Okazaki, K. (2005) *Cancer Res.* 65, 157–165
18. Brown, J. D., DiChiara, M. R., Anderson, K. R., Gimbrone, M. A., Jr., and Topper, J. N. (1999) *J. Biol. Chem.* 274, 8797–8805
19. Wicks, S. J., Liu, S., Abdal-Wahab, N., Mason, R. M., and Chantry, A. (2000) *Mol. Cell. Biol.* 20, 8103–8111
20. Yakymovych, I., Ten Dijke, P., Heldin, C. H., and Souchelnytskyi, S. (2001) *FASEB J.* 15, 553–555
21. Waddell, D. S., Liberati, N. T., Guo, X., Frederick, J. P., and Wang, X. F. (2004) *J. Biol. Chem.* 279, 29236–29246
22. Gallego, M., and Virshup, D. M. (2005) *Curr. Opin. Cell. Biol.* 17, 197–202
23. Collet, J. F., Stroebant, V., Pirard, M., Delpierre, G., and Van Schaftingen, E. (1999) *J. Biol. Chem.* 274, 14107–14112
24. Chambers, R. S., and Dahms, M. E. (1994) *J. Biol. Chem.* 269, 26243–26248
25. Yeo, M., Lin, P. S., Dahms, M. E., and Gill, G. N. (2003) *J. Biol. Chem.* 278, 26078–26085
26. Kamenski, T., Heeimeier, S., Meinhart, A., and Cramer, P. (2004) *Mol. Cell* 15, 399–407
27. Meinhart, A., Kamenski, T., Hoepfner, S., Baumli, S., and Cramer, P. (2005) *Genes Dev.* 19, 1401–1415
28. Yeo, M., Lee, S. K., Lee, B., Ruiz, E. C., Pfaff, S. L., and Gill, G. N. (2005) *Science* 307, 596–600
29. Thompson, J., Lepikhova, T., Teixido-Travesa, N., Whitehead, M. A., Palvimo, J. J., and Janne, O. A. (2006) *EMBO J.* 25, 2757–2767
SCPs Dephosphorylate Smad2/3 in the Linker

30. Knockaert, M., Sapkota, G., Alarcon, C., Massague, J., and Brivanlou, A. H. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 11940–11945

31. Lin, X., Duan, X., Liang, Y. Y., Su, Y., Wrighton, K. H., Long, J., Hu, M., Davis, C. M., Wang, J., Brunickardi, F. C., Shi, Y., Chen, Y. G., Meng, A., and Feng, X. H. (2006) J. Biol. Chem. 278, 18714–18719

32. Liang, M., Melchior, F., Feng, X. H., and Lin, X. (2004) J. Biol. Chem. 279, 22857–22865

33. Feng, X. H., Lin, X., Sun, B., Liang, M., Shi, Y., Brunickardi, F. C., and Feng, X. H. (2003) Mol. Cell. Biol. 23, 9081–9093

34. Ota, T., Suzuki, Y., Nishikawa, T., Otsuki, T., Sugiyama, T., Irie, R., Waki-matsu, A., Hayashi, K., Sato, H., Nagai, K., Kimura, K., Makita, H., Sekine, M., Obayashi, M., Nishi, Y., Shibahara, T., Tanaka, T., Ishii, S., Yamamoto, J., Saito, K., Kawai, Y., Isono, Y., Nakamura, Y., Nagahara, K., Murakami, K., Yasuda, T., Iwayanagi, T., Wagatsuma, M., Shiratori, A., Sudo, H., Hosoi, T., Kaku, Y., Kodono, H., Sugawara, M., Takahashi, M., Kanda, K., Yokoi, T., Furuya, T., Hikawa, E., Omura, Y., Abe, K., Kamihara, K., Katsuta, N., Sato, K., Tanikawa, M., Yamazaki, M., Ninomiya, K., Ishibashi, T., Yamashita, H., Murakawa, K., Fujimori, K., Tanai, H., Kimata, M., Watanabe, M., Hiraoka, S., Chiba, Y., Ishida, S., Ono, Y., Takiguchi, S., Watanabe, S., Yosida, M., Hotota, T., Kusano, J., Kanehori, K., Takahashi-Fujii, A., Hara, H., Tanase, T. O., Momura, Y., Togiyia, S., Komai, F., Hara, R., Takeuchi, K., Arita, M., Imose, N., Musashino, K., Yuuki, H., Oshima, A., Sasaki, N., Aotsuka, S., Yoshikawa, H., Matsunawa, H., Ichihara, T., Shiohata, N., Sano, S., Moriya, S., Moriyma, H., Satoh, N., Takami, S., Terasima, Y., Suzuki, O., Nakagawa, S., Senoh, A., Mizoguchi, H., Goto, Y., Shimizu, F., Wakebe, H., Hishigaki, H., Watanabe, T., Sugiyama, A., Takemoto, M., Kawakami, B., Yamazaki, M., Watanabe, K., Kumagai, A., Itakura, S., Fukuzumi, Y., Fujimori, Y., Komiyama, M., Togashii, A., Fujii-wara, T., Ono, T., Yamada, K., Fujii, Y., Ozaki, K., Hirao, M., Ohmori, Y., Kawabata, A., Hikiji, T., Kobatake, N., Inagaki, H., Ikema, Y., Okamoto, S., Okitani, R., Kawakami, T., Noguchi, S., Itoh, T., Shigeta, K., Senba, T., Matsumura, K., Nakajima, Y., Mizuno, T., Morinaga, M., Sasaki, M., Togashi, T., Oyama, M., Hata, H., Watanabe, M., Komatsu, T., Mizushima-Sugano, J., Satoh, T., Shirai, Y., Takahashi, Y., Nakagawa, K., Okamura, K., Nagase, T., Nomura, N., Kikuchi, H., Masuho, Y., Yamashita, R., Nakai, K., Yada, T., Nakamura, Y., Ohara, O., Iso, T., and Sugano, S. (2004) Nat. Genet. 36, 40–45

35. Kimura, K., Wakamatsu, A., Suzuki, Y., Ota, T., Nishikawa, T., Yamashita, R., Yamamoto, J. I., Sekine, M., Tsuritani, K., Wakaguri, H., Ishii, S., Sugiyama, T., Saito, K., Isono, Y., Irie, R., Kushida, N., Yoneyama, T., Onsuka, R., Saito, K., Yokoi, T., Kondo, H., Wagatsuma, M., Murakawa, K., Ishida, S., Ishibashi, T., Takahashi-Fujii, A., Tanase, T., Nagai, K., Kikuchi, H., Nakai, K., Isogai, T., and Sugano, S. (2006) Genome Res. 16, 55–65

36. Feng, X. H., Lin, X., and Derynck, R. (2000) EMBO J. 19, 5178–5193

37. Pardali, K., Kurisaki, A., Monen, R., ten Dijke, P., Kardassis, D., and Moustakas, A. (2000) J. Biol. Chem. 275, 29244–29256

38. Seoane, J., Le, H. V., Shen, L., Anderson, S. A., and Massagué, J. (2004) Cell 117, 211–223

39. Dunfield, L. D., and Nachtigal, M. W. (2003) Oncogene 22, 4745–4751

40. Duan, X., Liang, Y. Y., Feng, X. H., and Lin, X. (2006) J. Biol. Chem. 281, 36526–36532

41. Su, Y. A., Lee, M. M., Hutter, C. M., and Meltzer, P. S. (1997) Oncogene 15, 1289–1294