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Small C-terminal Domain Phosphatase Enhances Snail Activity through Dephosphorylation*

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Down-regulation of E-cadherin plays an important role in epithelial-mesenchymal transition (EMT), which is critical in normal development and disease states such as tissue fibrosis and metastasis. Snail, a key transcription repressor of E-cadherin, is a labile protein with a short half-life and is regulated through phosphorylation, ubiquitination, and degradation. Previously, we showed that GSK-3β phosphorylated two stretches of serine residues within the nuclear export signal and the destruction box of Snail, provoking its cytoplasmic export for ubiquitin-mediated proteasome degradation. However, the mechanism of Snail dephosphorylation and the identity of the Snail-specific phosphatase remain elusive. Using a functional genomic screening, we found that the small C-terminal domain phosphatase (SCP) is a specific phosphatase for Snail. SCP interacted and co-localized with Snail in the nucleus. We also found that SCP expression induced Snail dephosphorylation and stabilization *in vitro* and *in vivo*. However, a catalytically inactive mutant of SCP had no effect on Snail. Furthermore, we found that Snail stabilization induced by SCP enhanced snail activity in the suppression of E-cadherin and increased cell migration. Thus, our findings indicate that SCP functions as a Snail phosphatase to control its phosphorylation and stabilization, and our study provides novel insights for the regulation of Snail during EMT and cancer metastasis.

Metastasis is the leading cause of human cancer deaths. The pathogenesis and mechanism underlying this process remain poorly defined. Epithelial-mesenchymal transition (EMT),[3] a vital event, which is required for large-scale cell movements during morphogenesis in embryonic development, is attracting increasing attention as an important mechanism for the initial step of metastasis, because the genes implicated in EMT during embryogenesis have been shown to control metastasis (1, 2). In this EMT process, epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility (1–3). A hallmark of EMT is the loss of E-cadherin expression (3, 4). E-cadherin is a cell-cell adhesion molecule that participates in homotypic, calcium-dependent interactions to form epithelial adherent junctions (5, 6). Loss of E-cadherin expression is consistently observed at sites of EMT during development and cancer, and E-cadherin expression levels are often inversely correlated with tumor grade and stage (5, 6).

Several transcription factors have been implicated in the transcriptional repression of E-cadherin, including the Snail/Slug family, Twist, δEF1/ZEB1, SIP1, and the basic helix-loop-helix factor E12/E47 (7, 8). Snail, a zinc finger transcriptional repressor, was identified in *Drosophila* as a suppressor of *shotgun* (an E-cadherin homologue) transcription in the control of embryogenesis. The absence of Snail is lethal, because it results in severe defects at the gastrula stage during development (9). Snail expression represses E-cadherin expression and induces EMT in Madin-Darby Canine Kidney and breast cancer cells (10–12), indicating that Snail plays a fundamental role in EMT and breast cancer metastasis by suppressing E-cadherin expression. In fact, Snail overexpression was recently found in both epithelial and endothelial cells of invasive breast cancer but was undetectable in normal breast tissue (13, 14). Our findings and those of others show that Snail expression is correlated with the tumor grade and nodal metastasis of invasive ductal carcinoma and predicts a poor outcome in patients with breast cancer (12, 13, 15, 16). In addition to being a crucial regulator of EMT and cell migration, Snail overexpression induces breast cancer recurrence; this spontaneous breast cancer recurrence is accompanied by EMT (17, 18). Furthermore, Snail overexpression induces apoptosis resistance in breast cancer cells (19, 20). The Snail-mediated survival may thus enhance the ability of tumor cells to invade and metastasize.

Snail is a critical regulator of multiple signaling pathways that lead to EMT and cell migration (8, 21, 22). Its expression is tightly regulated during development; however, this regulation is often disrupted in metastasis. For example, loss of estrogen receptor expression or metastasis-associated gene 3 (MTA3) function leads to aberrant up-regulation of Snail, resulting in EMT and breast cancer metastasis (23). In addition, the epidermal growth factor (EGF) receptor pathway can activate signal transducer and activator of transcription 3 (STAT3), which enhances Snail function by upregulating the zinc-transporter LIV1 (24), expression of which is induced by estrogen and

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[3] The abbreviations used are: EMT, epithelial-mesenchymal transition; HA, hemagglutinin; GST, glutathione *S*-transferase; WT, wild type; SCP, small C-terminal domain phosphatase; DAPI, 4′,6-diamidino-2-phenylindole; EGF, epidermal growth factor; IGF, insulin-like growth factor; CTD, C-terminal domain.

shown to be associated with metastasis in breast cancer (25). Furthermore, expression of stromal matrix metalloproteinase (MMP3), through the generation of Rac1b, causes an increase in cellular reactive oxygen species, which stimulates Snail expression (26). Previously we demonstrated that Snail activity is controlled by its stability and cellular location (12, 27). Glycogen synthase kinase-3β (GSK-3β) binds to and phosphorylates Snail at two consensus motifs to dually regulate its function; phosphorylation at the first motif regulated its ubiquitination mediated by β-Trcp, whereas phosphorylation at the second motif controlled its subcellular localization. A non-phosphorylated variant of Snail, 6SA, is more stable and resides in the nucleus exclusively to induce EMT. These results demonstrate that EMT induction and metastasis in breast cancer require both the protein stabilization and nuclear localization of Snail (12, 22). However, phosphorylation is a dynamic and reversible modification. The protein phosphatase that counteracts the phosphorylation and degradation of Snail remains elusive.

In the human genome, there are 36 protein-tyrosine phosphatases (PTPs), 16 dual-specific protein phosphatases (DUSP), and 39 protein Ser/Thr phosphatases (PPs), which remove phosphate molecules from serine and threonine residues in target protein. PPs can be further divided into PPM, PPP, and FCP/SCP families. The small C-terminal domain (CTD) phosphatases (SCPs) are localized to the nucleus and negatively regulate RNA polymerase II (RNAPII) by dephosphorylating its CTD on Ser-2 and Ser-5 (28). SCPs are widely expressed in human tissue and have a role in neuronal gene silencing and attenuating androgen receptor transcriptional activity (29, 30). Recent studies have also demonstrated that SCPs act as specific linker phosphatases of Smad1–3 to enhance BMP and TGF-β signaling (31–33). Whether SCP has other substrates remains unknown. In this study, we used functional genomic screening to identify SCPs as the phosphatase of Snail. SCPs interacted with and dephosphorylated Snail at the GSK-3β phosphorylation motif and regulated its stability and location. In addition, SCP expression correlated with the level of Snail in cancer cell lines and enhanced cell migration and E-cadherin promoter suppression. Our study uncovered the important aspect of Snail regulation in controlling cell migration and tumor metastasis.

EXPERIMENTAL PROCEDURES

Antibodies and Plasmids—Human cDNA for SCP1, SCP2, and SCP3 was cloned into pcDNA3 with N-terminal hemagglutinin (HA) tag. Catalytically inactive mutants of SCP1 (D97E), SCP2 (D107E), and SCP3 (D112E) were generated by mutating a conserved aspartate residue to glutamate at the active site within the phosphatase domain using the QuikChange Mutagenesis kit (Stratagene, La Jolla, CA) (31–33). Human SCP1 and mutant SCP1 (dominant-negative [DN]-SCP1) were also subcloned into the GST expression vector pGEX-6P-3. All sequences were verified by DNA sequencing. Anti-SCP1 and anti-Snail antibodies were purchased from Abgent (San Diego, CA) and Cell Signaling (Danvers, MA), respectively. Anti-Flag and anti-HA antibodies were obtained from Sigma-Aldrich and Roche Applied Science (Indianapolis, IN), respectively.

Cell Cultures, Transfections, and Reporter Assays—The human embryonic kidney HEK293, cervical cancer Hela, breast cancer MCF7, and other cell lines were purchased from the American Type Culture Collection (Manassas, VA) and grown in DMEM/F12 plus 10% fetal bovine serum. Snail/HEK293 cells were cultured as described previously (12). SCP and Snail were transiently transfected into cells using FuGENE 6 (Roche Applied Sciences). For the luciferase assay, cells were plated in 6-well plates at a density of 2×10^5 per well. Cells were transfected with 0.3 μg of the pGL3-E-cadherin promoter-luciferase plasmid, 3 μg of the pCMV-Tag2B-Snail, and 3 μg of the pcDNA3-HA-SCP1 (WT or DN) in each well. To normalize transfection efficiency, cells were also co-transfected with 0.1 μg of the pRL-CMV (*Renilla* luciferase). Forty-eight hours after transfection, luciferase activity was measured using the Dual-Luciferase Assay kit (Promega) in a Monolight TM-20 luminometer for 10 s. Three independent experiments were performed, and the calculated means and S.D. are presented.

Western Blotting and Immunoprecipitation—For protein extraction, 5×10^5 cells per well were plated onto 6-well plates and transiently transfected with 0.5 μg of pCMV-Tag2B-Snail and 0.5 μg of pcDNA3-HA-SCP or vector. Forty-eight hours after transfection, cells were incubated with or without proteasome inhibitor MG132 (10 μmol/liter) for an additional 6 h before protein extraction and Western blot analysis. Primary antibodies against Flag (M2, 1:1000) and HA (3F10, 1:4000) were used for protein detection and immunoprecipitation. For immunoprecipitation, HEK293 cells transfected with the indicated expression plasmids were lysed in buffer (50 mmol/liter Tris at pH 7.5, 150 mmol/liter NaCl, 5 μg/ml aprotinin, pepstatin, 1% Nonidet P-40, 1 mmol/liter EDTA, and 0.25% deoxycholate). Cell lysates (1.0 mg; 2–3-fold more lysate for control and DN-SCP samples) were incubated with 1 μg of anti-HA or anti-Flag antibody conjugated to agarose beads (Roche Applied Science) overnight at 4 °C. Beads were then washed with lysis buffer, and the immunoprecipitated protein complexes were resolved by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Isolation of GST-SCP1 and Phosphatase Assays—Expression vector pGEX-6P-3 containing SCP1 (WT or DN) was transformed into *Escherichia coli* BL21 cells and cultured at 37 °C until A_{600} reached 0.6. GST-tagged SCP1 was induced by isopropyl-β-D-galactosidase (100 μM) at room temperature for 4 h. Cells were sonicated in ice-cold Tris-EDTA buffer containing 0.5 M NaCl, and GST-SCP1 was isolated by glutathione-Sepharose beads (Amersham Biosciences) according to the manufacturer's protocol. Proteins were eluted with 20 mM glutathione and dialyzed in Tris-EDTA buffer containing 0.27 M sucrose prior to being snap-frozen in liquid nitrogen and stored at −80 °C. Approximately 10 pmol each of GST-WT-SCP1 and DN-SCP1 were incubated in a 50-μl phosphatase assay mix (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 20 mM $MgCl_2$) containing immunoprecipitated Snail. The assays were performed at 30 °C for 1 h with constant rocking and were stopped by boiling the mixtures in SDS sample buffer for 5 min. The reaction mixture was analyzed by Western blotting with anti-Flag and anti-GST antibodies.

GST Pull-down Assay—GST proteins were expressed as described above. Cells were lysed in GST pull-down buffer (20 mM Tris, 150 mM NaCl, and 1% Nonidet P-40 with protease mixture) and rotated with glutathione-Sepharose-bound GST, GST-SCP1 (WT), or GST-SCP1 (DN). The binding complexes were eluted with SDS-PAGE sample buffer and analyzed by Western blotting.

Kinase Assay—The GSK-3β kinase assay was performed as described previously (12). Briefly, WT or kinase dead (DN) GSK-3β was expressed in HEK293 cells and immunoprecipitated from cell lysate. After incubation with 5 μg of GST-Snail (WT or S6A) in a kinase reaction buffer at 30 °C for 30 min, the GST-Snail was purified by glutathione beads and subjected to protein dephosphorylation assay by λ-phosphatase or GST-SCP1 (WT) as described above. Phosphorylation of Snail was analyzed on Western blotting by an antibody against pan-serine phosphorylation (Calbiochem, Gibbstown, NJ).

In Vitro Translation and Autoradiography—[^{35}S]Met-labeled β-Trcp was *in vitro* translated from pcDNA3-β-Trcp (T7) using a TNT T7 quick-coupled translation/transcription system (Promega). *In vitro* translated proteins were mixed with immunoprecipitated Snail bound to the Flag M2 beads for 2 h at 4 °C. The beads were washed four times with lysis buffer, and the bound proteins were eluted with sample buffer and subjected to 10% SDS-PAGE and autography.

Subcellular Fractionation—Cytoplasmic and nuclear fractions were prepared as described previously (34). In brief, cultured cells were lysed in buffer A (50 mM NaCl, 10 mM HEPES at pH 8.0, 500 mM sucrose, 1 mM EDTA, and 0.2% Triton X-100) on ice and dounced by a homogenizer. The nuclear pellet was washed and isolated. The nuclei were lysed in radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris at pH 7.5, 25 mM NaF, 2 mM Na_3VO_4, 5 mM phenylmethylsulfonyl fluoride, and 2 μg/ml of aprotinin). Poly(ADP-ribose) polymerase (PARP) and β-tubulin were used as markers of the nuclear and cytoplasmic fractions, respectively.

Immunofluorescence Imaging Analysis—For immunofluorescence microscopy, cells were grown on coverslips, fixed with 4% paraformaldehyde, and incubated with anti-HA monoclonal antibody overnight. Proteins were visualized by incubation with goat anti-mouse conjugated with Alexa Fluor 568 (Invitrogen, Carlsbad, CA). Finally, coverslips were incubated with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 20 min and visualized under a fluorescent microscope.

RESULTS

SCPs Stabilize Snail Expression—Because Snail is phosphorylated on serine residues, we examined the PPs family members that can target Snail for dephosphorylation and stabilization. We tested the stabilization of Snail by 11 phosphatases in HEK293 cells. As we showed previously, Snail is a highly unstable protein, treatment with the proteasome inhibitor MG132 substantially enhanced Snail level (*lane 1 versus lane 13*, Fig. 1*A*). Interestingly, SCP1–3 expression also dramatically stabilized Snail to a level similar to that by MG132 (*lanes 9–11*, Fig. 1*A*). In contrast, expression of another member of the CTD phosphatase, FCP1, did not induce Snail stabilization (*lane 12*,

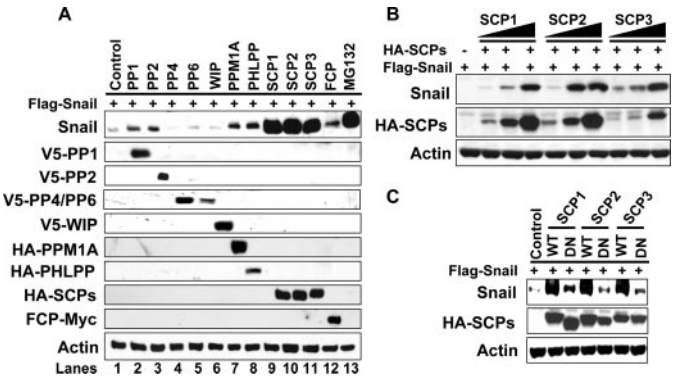

FIGURE 1. **SCP stabilizes Snail.** *A*, Flag-tagged Snail and specified phosphatases were co-expressed in HEK293 cells. Snail and individual phosphatases were examined by anti-Flag or respective anti-tag antibodies on Western blotting. *B*, Snail was co-expressed with increasing amounts of SCPs in HEK293 cells. Lysates were subjected to Western blotting with the indicated antibodies. Actin served as a loading control. *C*, Snail was co-expressed with SCPs (WT or DN) in HEK293 cells. Expression of Snail and SCPs were examined by Western blotting.

Fig. 1*A*). The PPM1A (PP2C) and PHLPP had a mild effect on the level of Snail, whereas other phosphatases such as PP4, PP6, and WIP had no effect (Fig. 1*A*). We also found that Snail stabilization by SCP1–3 occurred in a dosage-dependent manner; Snail levels increased as SCP1–3 expression increased (Fig. 1*B*). To examine whether SCP phosphatase activity is required for Snail stabilization, we generated the catalytically inactive SCP mutants DN-SCP1 (D97E), DN-SCP2 (D107E), and DN-SCP3 (D112E). These mutants lost their ability to stabilize Snail (Fig. 1*C*), indicating that Snail stabilization by SCPs is specific and depends on the catalytic activity of SCPs. Collectively, these results suggest that expression of SCPs can stabilize Snail *in vivo*.

SCPs Physically Interact with Snail—The interaction and cellular co-localization of a phosphatase with its substrates are critical in determining the substrate specificity of phosphatase. To determine whether Snail interacts with SCPs, we co-expressed Flag-Snail and HA-SCPs in HEK293 cells and performed a co-immunoprecipitation experiment. After immunoprecipitating SCPs, we detected the association of Snail and *vice versa* (Fig. 2*A*), indicating that these two molecules are physically associated. Interestingly, this association depended on the catalytic activity of SCPs. Wild-type SCPs associated more strongly with Snail than did DN-SCPs (Fig. 2*A*). To further confirm this finding, we generated recombinant GST-WT-SCP1 and GST-DN-SCP1 fusion proteins and tested their ability to bind to Snail. When GST-SCP1 was pulled-down, SCP1, but not GST, bound to Snail (Fig. 2*B*). Again, wild-type SCP1 interacted with Snail more strongly than did DN-SCP, suggesting that the catalytic activity of SCP is required for the interaction between SCP and Snail. The equal amount of GST protein used in the GST pull-down assay was confirmed with an anti-GST antibody, and the equal amounts of Snail in each cell lysate were verified by Western blotting (*bottom panels* of Fig. 2*B*). We also immunoprecipitated endogenous Snail from MDA-MB231 cells and detected the presence of endogenous SCP1; no endogenous SCP1 was detected using a nonspecific immunoglobulin (Fig. 2*C*). These results indicate that SCP interacts with Snail both *in vitro* and *in vivo*.

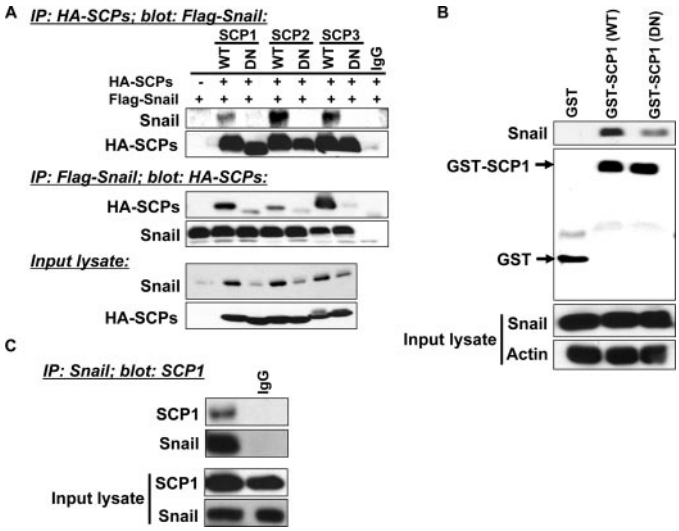

FIGURE 2. **SCP interacts with Snail.** *A*, Flag-tagged Snail was co-expressed with HA-tagged SCPs (WT or DN) in HEK293 cells. Snail and SCPs were immunoprecipitated with Flag or HA antibody, respectively, and analyzed by Western blotting. One-fortieth of cell lysate for each sample was subjected to Western blotting to examine the expression of Snail and SCP (*input lysate*). Actin served as a control for equal loading. *B*, Snail expressed in HEK293 cells was mixed with GST-SCP (WT or DN) or GST alone, pulled down by glutathione-agarose, and subjected to Western blotting. Equal amounts of Snail or GST protein were confirmed by anti-Flag or anti-GST antibodies. *C*, endogenous Snail was immunoprecipitated from breast cancer MDA-MB231 cells, and the associated endogenous SCP1 was detected by Western blotting.

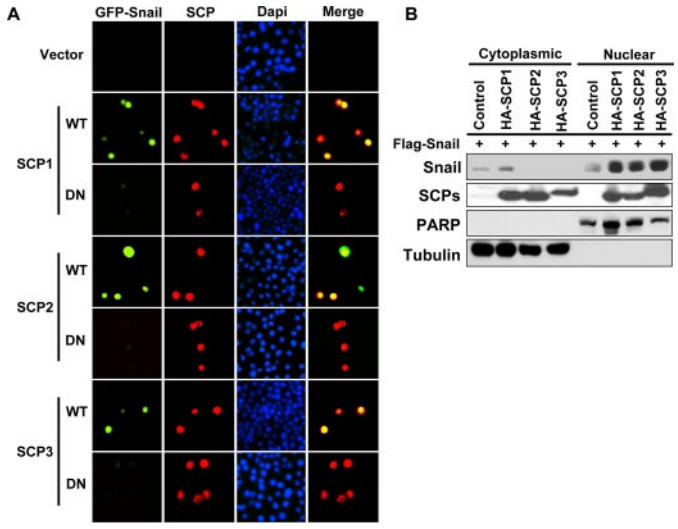

FIGURE 3. **SCP stabilizes Snail in the nucleus.** *A*, GFP-Snail was co-expressed with HA-tagged SCPs (WT or DN) in Hela cells. After fixation, the cellular location of Snail (*green*) and SCP (*red*) was examined by immunofluorescent staining using anti-HA antibody and visualized under a fluorescent microscope (nuclei were stained with DAPI, *blue*). *B*, cellular fractionation was carried out to determine the cellular localization of Snail after co-expressing of Snail with SCPs in HEK293 cells. Equal amounts of cytoplasmic fraction and nuclear fraction from each sample were analyzed by Western blotting. Tubulin and poly-(ADP-ribose) polymerase (PARP) are used as markers of the cytoplasmic and nuclear fractions, respectively.

SCPs Co-localize with Snail in the Nucleus—We next determined whether SCP affects the cellular localization of Snail. We co-expressed GFP-Snail with wild-type SCPs or DN-SCPs in Hela cells and evaluated its cellular localization by immunofluorescence analysis. As shown in Fig. 3*A*, both SCPs (WT or DN) were mainly localized in the nucleus. However, expression of WT-SCPs, but not DN-SCP, enhanced the intensity of GFP-

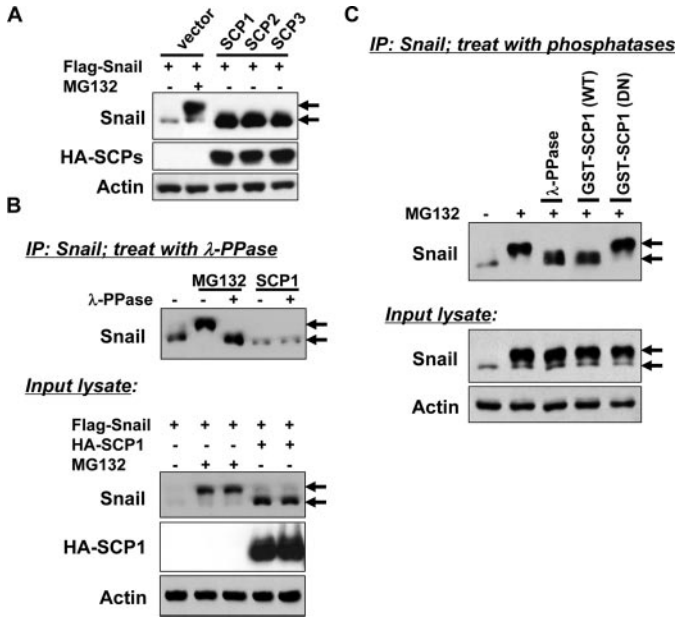

FIGURE 4. **SCP dephosphorylates Snail.** *A*, Flag-tagged Snail was co-expressed with vector or SCPs in HEK293 cells. After 42 h of post-transfection, cells were treated with or without MG132 for 6 h. Expression products of Snail and SCP were separated on a 14% SDS-PAGE and analyzed by Western blotting. *B*, Snail was immunoprecipitated from Snail/HEK293 cells treated with MG132 or expressed with SCP1 (*Input lysate, bottom panel*). The immunocomplex was then incubated with or without λ-phosphatase for 30 min, separated on a 14% SDS-PAGE, and analyzed by Western blotting. *C*, Snail was immunoprecipitated from Snail/HEK293 cells treated with MG132 (*Input lysate, bottom panel*), mixed with λ-phosphatase or GST-SCP1 (WT or DN) as described in *B*, and then separated on a 14% SDS-PAGE and subjected to Western blotting. Phosphorylated and nonphosphorylated forms of Snail are indicated by an *arrow*.

Snail in the nucleus. These results are consistent with the previous finding that Snail is unstable and localizes in the nucleus when it is non-phosphorylated, where it functions actively as a transcription repressor (12). Our results also indicate that catalytically active SCP is required for the stabilization of Snail in the nucleus. We further performed the experiments described above using biochemical cellular fractionation to determine the localization of Snail and SCPs. We found that Snail was mainly in the nuclear fraction when co-expressed with SCPs (Fig. 3*B*). As a control, we used β-tubulin and poly(ADP-ribose) polymerase as cytoplasmic and nuclear markers, respectively, to confirm that the observed cellular localizations of Snail and SCPs were not due to contamination. Together, the immunofluorescent analysis and cellular fractionation study indicate that SCPs co-localize with Snail and stabilize it in the nucleus.

SCPs Dephosphorylate Snail—We previously showed that Snail is highly unstable and is phosphorylated by GSK-3β and thus provokes its cytoplasmic translocation and degradation by E3 ligase β-Trcp. Treatment with proteasome inhibitor MG132 stabilizes Snail in a hyperphosphorylated form. Because SCP expression also induced Snail stabilization, we next examined the mechanism underlying this stabilization. First, we expressed Snail with SCPs or vector in HEK293 cells and treated the cells with or without proteasome inhibitor MG132. Although both MG132 and SCP expression induced Snail stabilization, the mechanisms appeared to differ, Snail stabilized by SCPs migrated faster on an SDS-PAGE gel than did Snail induced by MG132 (*lanes 3–5 versus lane 2*, Fig. 4A). To deter-

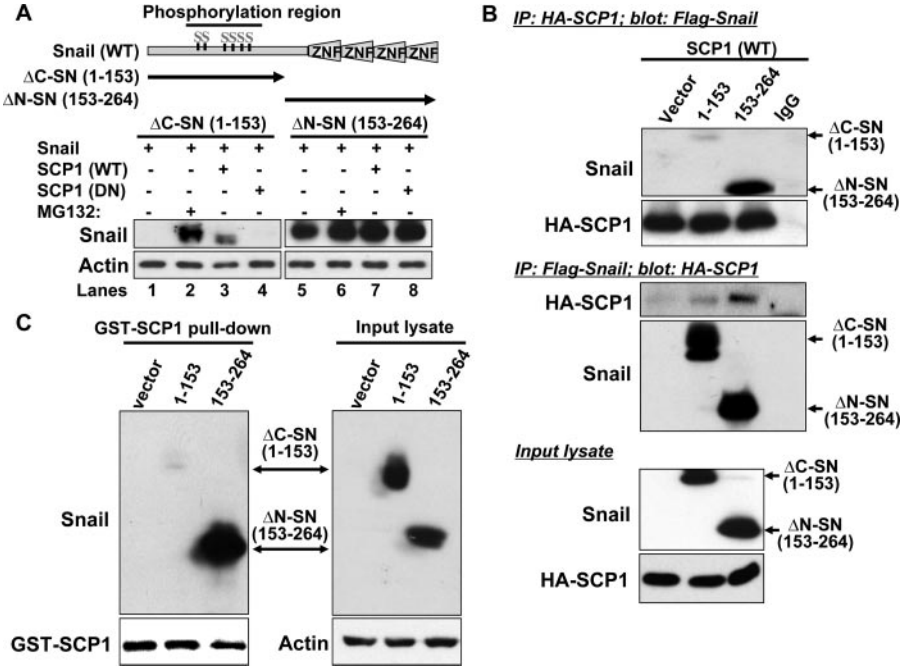

FIGURE 5. **SCP binds the C terminus of Snail.** *A*, *top*, schematic representations of WT and deletion mutants of Snail. *Bottom*, Snail deletion mutants were co-expressed with SCP1 (WT or DN) or treated with MG132. The expression level for each Snail deletion mutant was examined by Western blotting. *B*, Snail deletion mutants were co-expressed with SCP1 in HEK293 cells. SCP1 and Snail deletion mutants were immunoprecipitated with HA or Flag antibody, respectively, and analyzed by Western blotting. Input lysate used for the immunoprecipitation is shown on the *bottom panel*. *C*, Snail deletion mutants were expressed in HEK293 cells and mixed with GST-SCP1 (WT), pulled-down by glutathione-agarose, and subjected to Western blotting.

mine whether the different migrating bands represented different phosphorylated states of Snail, we immunoprecipitated Snail and treated it with λ-phosphatase (Fig. 4*B*). Snail stabilized by MG132 became migrating faster after λ-phosphatase treatment (*lane 3*, Fig. 4*B*), indicating that the slow migrating of Snail was mediated by protein phosphorylation. However, treatment with λ-phosphatase did not affect the band migrating of Snail stabilized by SCP (*lanes 4* and *5*, Fig. 4*B*), indicating that Snail stabilization by SCP remains in a non-phosphorylated form. To determine whether SCP can directly dephosphorylate Snail *in vitro*, we immunoprecipitated Snail from cells treated with MG132 and incubated with λ-phosphatase or bacterially purified GST-SCP1 (wild-type or DN-SCP) immobilized on glutathione beads (Fig. 4*C*). Wild-type GST-SCP1 and λ-phosphatase induced Snail dephosphorylation and enhanced Snail migration on SDS-PAGE (*lanes 3* and *4*, Fig. 4*C*). However, DN-SCP1 lost its ability to dephosphorylate Snail (*lane 5*, Fig. 4*C*), supporting the notion that the catalytic activity of SCP is required for the dephosphorylation process. Taken together, these data suggest that SCP1 can stabilize Snail by dephosphorylating it both *in vitro* and *in vivo*.

SCPs Associate with Snail at the C-terminal Region—To identify the region in Snail that associates with SCP, we generated two deletion mutants of Snail; the N-terminal region of Snail (ΔC-Snail; amino acids 1–153) that contains the serine-rich residues of Snail and the C-terminal region of Snail (ΔN-Snail; amino acids 153–264) that includes the conservative zinc-finger motif (*top panel*, Fig. 5*A*). When these two deletion mutants of Snail were co-expressed with WT- or DN-SCP1 in cells, we found that ΔN-Snail was more stable than ΔC-Snail (*lane 1 versus lane 5*, Fig. 5*A*). MG132 treatment or SCP1 expression did not change the level of ΔN-Snail (*lanes 6* and *7*, Fig. 5*A*), indicating that the C-terminal region of Snail is not important in controlling the stability and phosphorylation of Snail. However, MG132 treatment substantially stabilized ΔC-Snail (*lane 2 versus lane 1*, Fig. 5*A*). WT-SCP1 expression also induced ΔC-Snail stabilization, although to a much lesser extent (*lane 3 versus lane 2*, Fig. 5*A*). These results indicate that the N-terminal region of Snail is critical for the stabilization and phosphorylation of Snail and that SCP only affects the stability of the N-terminal portion of Snail (Fig. 5*A*). To determine which region of Snail interacts with SCP, we co-expressed Snail deletion mutants with HA-SCP1 in HEK293 cells. After immunoprecipitating SCP1, we detected the association between SCP1 and ΔN-Snail; however, a much weaker association between ΔC-Snail and SCP1 was noted (*top panel*, Fig. 5*B*). On a reverse co-immunoprecipitation assay, ΔN-Snail bound tightly with SCP1. Similarly, ΔC-Snail loosely affiliated with SCP1 (*middle panel*, Fig. 5*B*), indicating that the C-terminal region of Snail is mainly responsible for the association with SCP1. In line with this observation, when GST-SCP1 was pulled down from two deletion mutants of Snail, we found that GST-SCP1 interacted strongly with ΔN-Snail, but it lost its affinity for ΔC-Snail (Fig. 5*C*). These results demonstrate that the C-terminal region of Snail is critical for providing the docking site for SCP and suggest that the association between SCP and Snail facilitates the dephosphorylation of Snail at the N terminus. Loss of the C terminus of Snail decreases its interaction with SCP, leading to inhibition of Snail dephosphorylation and stabilization (*lane 3 versus lane 2*; Fig. 5*A*).

SCPs Dephosphorylate Snail at the GSK-3β Motif—We previously demonstrated that Snail has two GSK-3β phosphorylation motifs; the first motif regulates the stability of Snail, and the second one controls its cellular localization (*left panel*, Fig. 6*A*). Both GSK-3β phosphorylation motifs are located in the N-terminal region of Snail (*left panel*, Fig. 6*A*). We next examined whether Snail stabilization by SCP is mediated by the dephosphorylation of these two GSK-3β motifs, and thus controls the stabilization and cellular location of Snail. We co-expressed WT and mutant Snail with HA-SCP1 in HEK293 cells (*right panel*, Fig. 6*A*). As expect, SCP1 greatly enhanced the stabilization of WT Snail as that by proteosome inhibitor MG132 (*lanes 2* and *3 versus lane 1*, Fig. 6*A*). Interestingly, SCP1 also enhanced the stability of Snail-4SA (*lane 11 versus lane 9*, Fig. 6*A*) but not Snail-2SA and Snail-6SA, which were

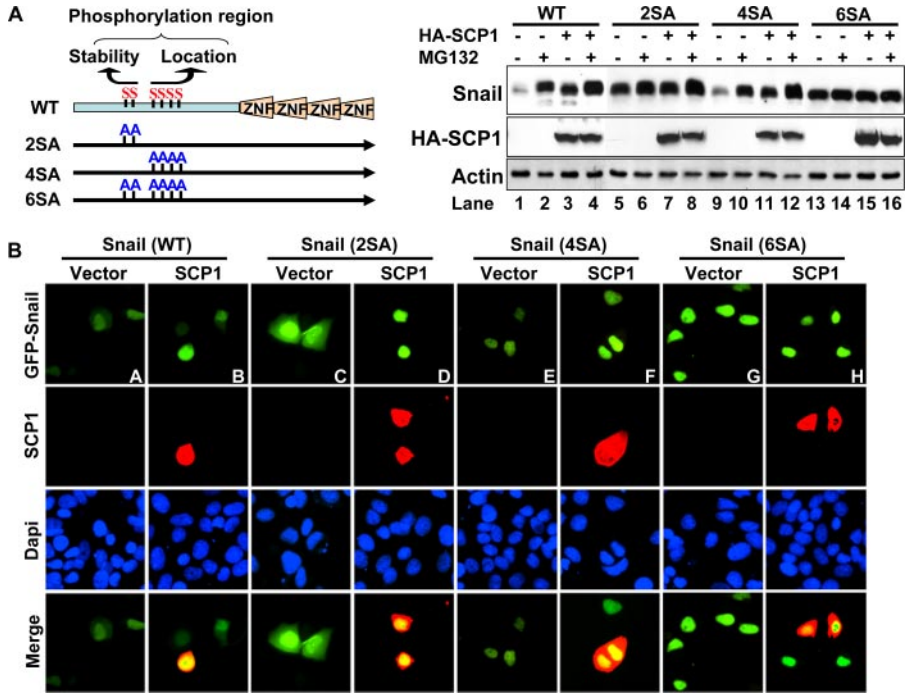

FIGURE 6. **SCP dephosphorylates two GSK-3β motifs on Snail.** *A*, *left*, schematic representation for sites of phosphorylation on Snail and mutant Snails used in this study. *Right*, WT or mutant Snails were co-expressed with SCP1 or vector in HEK293 cells followed by treatment with or without MG132 for 6 h. Expression levels of Snail and SCP1 were analyzed by Western blotting. *B*, GFP-tagged Snails (WT or mutants) were co-expressed with SCP1 or vector in breast cancer MCF7 cells. After fixation, the cellular location of Snail (*green*) and SCP (*red*) was examined by immunofluorescent staining using anti-HA antibody and visualized under a fluorescent microscope (nuclei were stained with DAPI, *blue*).

resistant to degradation as the degradation motif on these two mutants was changed from serine to alanine (12) (*left panel*, Fig. 6*A*). This result indicates that Snail stabilization by SCP is mediated by the dephosphorylation of the first GSK-3β motif. Furthermore, when SCP was co-expressed with WT Snail in MCF7 cells, it greatly stabilized Snail in the nucleus (*column B versus column A*, Fig. 6*B*). In agreement with previous data (12), when Snail-2SA was expressed alone, Snail was found in both the cytoplasm and the nucleus (*column C*, Fig. 6*B*); expressing of SCP induced the nuclear location of Snail-2SA (*column D versus column C*, Fig. 6*B*). Snail-4SA and Snail-6SA remained in the nucleus; expression of SCP1 did not change the nuclear location of these two mutants. As the second GSK-3β phosphorylation motif is responsible for the cellular location of Snail, the observation that SCP induced the nuclear location of Snail-2SA indicates that SCP1 also controls the dephosphorylation of the second GSK-3β motif on Snail. To provide the direct evidence that SCP indeed dephosphorylates Snail on the GSK-3β motifs, GST-Snail (WT and Snail 6SA) were phosphorylated by GSK-3β (WT and kinase dead mutant) *in vitro*. The phosphorylated GST-Snail was isolated by glutathione bead followed by incubation with either λ-phosphatase or GST-SCP1 (WT). Phosphorylation of Snail was further detected by anti-pan-serine phosphorylation antibody (Fig. 7*A*). Consistent with our previous finding, WT but not kinase dead (DN) GSK-3β-phosphorylated wild-type Snail. Mutation of serine residues to alanine on both GSK-3β motifs on Snail (Snail S6A) abolished the phosphorylation by GSK-3β. Strikingly, phosphorylation of Snail by GSK-3β was completely dephosphorylated by either λ-phosphatase or GST-SCP1 (WT). Together, these data indicate that SCP1 dephosphorylates both GSK-3β consensus motifs to keep Snail in a nonphosphorylated and nuclear-localized state, which is the exact opposite function of GSK-3β.

We and others showed previously that phosphorylation of the first GSK-3β motif induces the association of Snail with its E3 ligase β-Trcp and thus leads to degradation. Because SCP induced the dephosphorylation of Snail on both GSK-3β motifs and stabilized Snail, we reasoned that the association of Snail with β-Trcp and the ubiquitination of Snail will be suppressed when SCP is presented. When an equal amount of Snail was immunoprecipitated from the cell lysate, we found significantly high levels of Snail ubiquitination in cells treated with MG132 (*lane 2*, Fig. 7*B*). Expressing WT SCP1 completely suppressed the ubiquitination of Snail (*lane 3*, Fig. 7*B*) although it can also stabilize Snail to a similar extent as that of MG132 (*lane 3 versus lane 2*, *lysate panel* of Fig. 7*B*). Strikingly, the association of Snail with its E3 ligase, β-Trcp, was abolished in cells expressing SCP1 (*lane 3 versus lane 2*, Fig. 7*B*). We also measured the binding of phosphorylated (prevented by MG132 treatment) and non-phosphorylated (induced by expression of WT-SCP1) Snail with β-Trcp *in vitro* (Fig. 7*C*). Snail was immunoprecipitated from lysates treated with MG132 or expression with SCP1 and incubated with [^{35}S]methionine-labeled β-Trcp. After extensive washing, the bound β-Trcp was detected by radiography. We found that phosphorylated Snail (prevented by MG132, *lane 2*, Fig. 7*C*) bound tightly with β-Trcp, expression of WT SCP1 completely abolished the binding of Snail with β-Trcp (*lane 3*, Fig. 7*C*). These results clearly indicated that the dephosphorylation of Snail by SCP inhibits the interaction of Snail with β-Trcp and thus contributes to the stabilization of Snail.

SCPs Enhance the Snail Activity—Because Akt activation can stabilize Snail by suppressing GSK-3β activity, and SCP stabilizes Snail by dephosphorylating it and thus antagonizing GSK-3β activity, we expressed WT-SCP1 or DN-SCP1 in Snail/HEK293 cells and treated them with insulin-like growth factor 1(IGF-1) and epidermal growth factor (EGF) to activate the AKT and MAPK pathways. As expected, both growth factors and SCP1 stabilized Snail, although no synergic effect was observed (*lanes 2*, *4*, and *5*, Fig. 8A). When the AKT pathway was blocked with a phosphoinositide 3-kinase inhibitor (Wortmannin), which activates GSK-3β by releasing GSK-3β suppression (phosphorylation at serine 9), Snail became unstable (*lane 3 versus lane 2*, Fig. 8*A*). However, expression of WT-

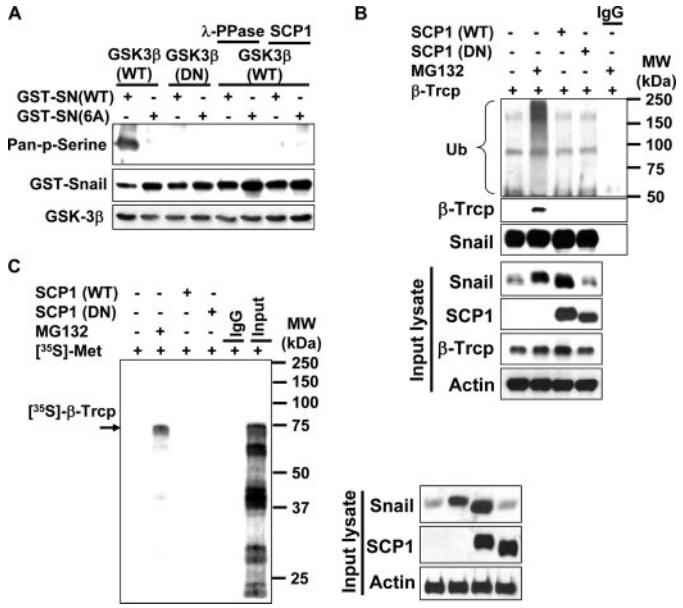

FIGURE 7. **SCP dephosphorylates and suppresses the ubiquitination of Snail.** *A*, after phosphorylated by GSK-3β, GST-Snail (WT or 6SA; 5 μg) were purified by glutathione beads and subjected to treatment with either λ-phosphatase or GST-SCP1 (WT) as described in the legend to Fig. 4*C*. Phosphorylation of Snail was detected by Western blotting. *B*, β-Trcp was co-expressed with vector or SCP1 (WT or DN) in Snail/HEK293 cells followed by treatment with or without MG132 for 6 h (*Input lysate panel*). Equal amounts of Snail were immunoprecipitated; the ubiquitination of Snail and the bound β-Trcp were analyzed by Western blotting. *C*, vector or SCP1 (WT or DN) was expressed in Snail/HEK293 cells followed by treatment with or without MG132 for 6 h. Snail was immunoprecipitated and mixed with *in vitro* translated [^{35}S]Met-labeled β-Trcp for 2 h at 4 °C. The beads were washed four times with lysis buffer, and the bound proteins were eluted with sample buffer and subjected to 10% SDS-PAGE and autoradiography.

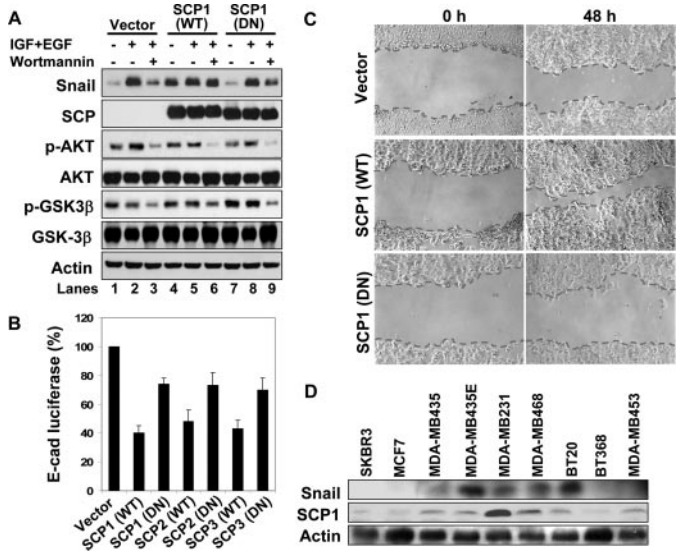

FIGURE 8. **SCP enhances the function of Snail.** *A*, Snail/HEK293 cells were transfected with vector or SCP1 (WT or DN). After 24 h, cells were starved overnight and then pretreated with or without Wortmannin (0.1 μM) for 1 h before stimulation with EGF (1 ng/ml) and IGF (10 ng/ml) for 6 h. The expression of Snail, SCP1, AKT (total and phosphorylated), and GSK-3β (total and phosphorylated) were examined by Western blotting. *B*, Snail and the indicated plasmids were co-transfected with the E-cadherin promoter luciferase reporter in HEK293 cells. The E-cadherin promoter luciferase activities were determined and normalized by *Renilla* luciferase activities (mean ± S.E. in three separate experiments). *C*, time-lapse photography of a scratch assay performed with Snail/MCF-7 cell transfected with vector or SCP1 (WT or DN). *D*, lysates from 9 different breast cancer cell lines were subjected to analysis for the expression of endogenous Snail and SCP1 by Western blotting.

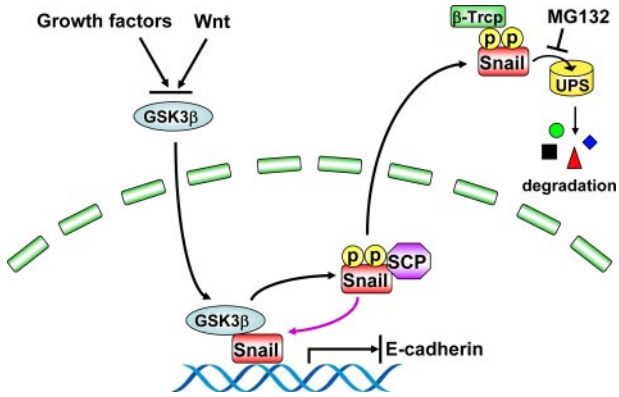

FIGURE 9. **SCP binds C terminus of Snail.** A model proposed to illustrate the dynamic regulation of Snail phosphorylation by GSK-3β and SCP.

SCP1 antagonized the activation of GSK-3β and maintained the stability of Snail (*lane 6 versus lane 3*, Fig. 8*A*), and this effect was lost when DN-SCP1 was used (*lane 9 versus lanes 3* and *6*; Fig. 8*A*). These results suggest that the stabilization of Snail can be regulated through two different routes; one is mediated by GSK-3β suppression (*lane 2*, Fig. 8*A*), and the other is through the dephosphorylation by SCP (*lane 4*, Fig. 8*A*). We also attempted to knockdown the expression of SCP1 in cells by specific siRNA approach and examined whether the down-regulation of SCP1 inhibited Snail stabilization mediated by the suppression of GSK-3β. However, the experiment was unsuccessful due to the highly conserved sequence among SCP1–3; siRNA target SCP1 also affected the other two members of SCP and caused massive apoptosis.

SCP expression significantly enhanced Snail ability to repress E-cadherin promoter, whereas DN-SCPs did not have this effect (Fig. 8*B*). Consistent with this observation, when WT-SCP1 or DN-SCP1 was expressed in Snail/MCF-7 cells and subjected to a wound-healing assay, SCP1 expression strongly enhanced cell migration to seal the wound (Fig. 8*C*). These results indicate that the dephosphorylation and stabilization of Snail by SCP plays a crucial role in cell migration and E-cadherin promoter suppression. To determine whether SCP1 expression is correlated with the level of Snail, we determined the expression level of Snail and SCP1 in 9 breast cancer cell lines (Fig. 8*D*). SCP1 expression was highly correlated with Snail levels, particularly in cells with high metastatic ability, such as MDA-MB231 cells. Collectively, our results indicate that SCP can dephosphorylate Snail and promotes tumor cell migration and metastasis through stabilizing Snail.

DISCUSSION

In the current study, we showed that expression of human SCP can dephosphorylate and stabilize Snail. SCP specifically associated with Snail on the C-terminal region and dephosphorylated it at two GSK-3β motifs, thus inhibiting its association with E3 ligase β-Trcp and stabilizing it in a nonphosphorylated form in the nucleus. Stabilization of Snail by SCP enhances E-cadherin promoter suppression and promotes cell migration (Fig. 9). Our findings have several implications concerning the regulation of Snail, EMT, and metastasis. First, our study indicates that SCP has a substantial role in the regulation of EMT

and cancer metastasis. As protein phosphorylation and dephosphorylation are vital for the function of Snail, and we demonstrated previously that Snail is dually regulated by protein stability and cellular location through GSK-3β-mediated phosphorylation; thus, the identification of Snail phosphatase undoubtedly provides an opposite view for the dynamic regulation of EMT. Using a functional screening analysis, we identified that SCP dephosphorylates Snail and regulate its stability. SCP function is specific and unique because phosphatase-dead mutants of SCPs (DN-SCP) lost the ability to dephosphorylate Snail. In addition, FCP, another CTD phosphatase, was unable to dephosphorylate Snail. SCP and FCP are class C phosphatases that contain RNA polymerase II CTD phosphatase domain homology. However, FCP contains an additional BRCT domain, and it has been postulated that the BRCT domain plays an auto-inhibitory role in dephosphorylation (33). Interestingly, SCP1 interacted with the C terminus of Snail and dephosphorylated the serine residues on its N-terminal region. The observation that the C terminus is more stable than the N terminus of Snail is consistent with our previous finding that GSK-3β phosphorylation motifs play an essential role in regulating Snail phosphorylation and stability. The C terminus of Snail likely provides a docking site for SCP to facilitate Snail dephosphorylation at the N terminus. In addition, we found that SCP can antagonize the function of GSK-3β and stabilize Snail when GSK-3β is active (Fig. 8*a*). The mutual antagonization of GSK-3β and SCP indicates that these kinase/phosphatase switchers play a critical role in regulating Snail stability and activity. Many growth factor signaling pathways, such as HER2/neu and Wnt, can suppress the activity of GSK-3β and thus up-regulate the level of Snail (22). However, what signaling pathway controls the interaction of SCP with Snail remains unclear and will be important to determine in the future.

Second, our study also suggests that SCP cross-regulates Snail and the TGFβ signaling pathway. TGFβ cytokines transmit signals by activating and forming type I and II receptor complexes, leading to phosphorylation of receptor-regulated Smad (Smad2 and Smad3) at C-terminal serine residues (35, 36). Upon phosphorylation, Smad2 and 3 partner with cytoplasmic Smad4 and translocate to the nucleus, where Smad complexes control the transcription of target genes to regulate diverse cellular functions, such as EMT. Dephosphorylation of the C-terminal region of Smad2 and 3 recycles activated Smads out of the nucleus. However, many intracellular kinases, including MAPK, CDK2/4, p38MAPK, and JNK, phosphorylate the linker region (inhibitory) that connects the N-terminal DNA-binding domain with the C-terminal transcriptional domain (activating) of Smads for cross-talk between TGFβ and other signaling pathways (35, 36). Thus, phosphatases that dephosphorylate the linker region of Smad2 and 3 are required for a full transcriptional TGFβ response. Three research groups recently demonstrated that SCP physically interacts with and dephosphorylates the linker region of Smad2 and 3, increasing TGFβ-induced transcriptional activity (31–33). SCP1 overexpression also counteracts the inhibitory effect of EGF on TGFβ-induced p15 expression (33). Interestingly, Smad2 and 3 play essential roles in EMT induction and tumor progression (37). For example, Smad2 cooperated with H-ras to mediate EMT induction and metastasis formation (38). Overexpression of Smad2 and Smad3 resulted in increased EMT in a mammary epithelial model (39). In contrary, knock-out of Smad3 blocked TGFβ-induced EMT in primary tubular epithelial cells, and the reduction of Smad2 and Smad3 function was associated with the decreased metastatic potential of breast cancer cell lines in a xenograft model (40). These studies support the central role of Smads in TGFβ-induced EMT, which is associated with tumor progression and metastasis. In our study, we found that SCP also dephosphorylates Snail and stabilizes it in the nucleus to enhance E-cadherin promoter suppression and promote cell migration. However, whether SCP plays a synergistic role in dephosphorylating Smad2/3 and Snail, thus enhancing EMT induction, remains to be further characterized.

Third, our study suggests that Snail dephosphorylation is coupled to the transcriptional repression process. RNA polymerase II (RNAPII) consists of a folded region that is responsible for mRNA synthesis and a tail-like CTD. During the transcription elongation, the CTD undergoes hyperphosphorylation that binds mRNA processing factors for transcription-coupled mRNA maturation. SCP/FCP1 interacts with TFIIF in the nucleus and catalyzes the dephosphorylation of CTD serine residues to recycle RNAPII back to an inactive state. The x-ray structure of SCP1 reveals a core fold and an active center similar to those of other phosphohydrolases that share the D*X*D*X*(T/V) amino acid signature motif with SCP1 and FCP1. Although a conserved pocket in SCPs and FCP1, adjacent to the active site, has been proposed to bind the CTD of RNAPII and confer specificity, it is possible that other proteins involved in gene regulation are also targeted by SCP to enhance gene silencing. Indeed, elevated expression of SCP1 was found to repress transcription, whereas the phosphatase-inactive mutant, dominant negative SCP1 (dnSCP1), enhanced RNAPII activity. The mechanism that recruits SCP to the promoter and results in transcriptional repression by SCP has recently been established. Yeo *et al.* (30) found that SCP expression pattern was similar to that of the repressor element 1 (RE-1)-silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF), which is found in non-neuronal tissues but is excluded from neuronal cells. REST/NRSF is a DNA-binding protein that assembles a repressor complex on RE-1 elements present in more than 1000 neuronal genes. SCP interacts with REST/NRSF to form a physical complex at RE-1 elements of the promoter region of target genes; thus suppressing neuronal differentiation. The N terminus of REST/NRSF interacts with mSin3 and HDAC1 and 2, whereas its C terminus associates with Co-Rest (41). Interestingly, Snail has been shown to interact with mSin3 and HDAC1/2 for its transcription repression on the E-cadherin promoter (42). Using a protein purification coupled with mass spectrometry analysis, we recently found that Snail interacts with the Rest/Co-Rest complexes.[4] Consistent with our findings, the SNAG domain, which is highly conserved among Snail family members and GFI, has also been found to interact with Co-Rest (43). All these studies point to huge protein complexes that consist of REST/NRSF, SCP, Snail, mSin3, HDAC1/2, Co-

[4] B. P. Zhou, manuscript in preparation.

Rest, and RNAPII for transcription repression *in vivo*. The functional interactions among these molecules remain to be further characterized. We postulated that SCP dephosphorylates RNAPII to inhibit its activity, on the one hand. On the other hand, SCP dephosphorylates and stabilizes Snail, thus enhancing its interaction with Rest/mSin3/Co-Rest complexes to repress the transcription of Snail target genes.

The identification of SCP as a potential phosphatase of Snail provides considerable insight into the regulation of Snail during EMT induction and metastasis. Notably, the functional interactions between SCP and RNAPII, Smad, and Snail suggest that potentially valuable therapeutic strategies exist for targeting EMT induction and the tumor-promoting effect of TGFβ signaling.

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