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The Small C-terminal Domain Phosphatase 1 Inhibits Cancer Cell Migration and Invasion by Dephosphorylating Ser(P)^{68}-Twist1 to Accelerate Twist1 Protein Degradation*

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Twist1 is a basic helix-loop-helix transcription factor that strongly promotes epithelial-to-mesenchymal transition, migration, invasion, and metastasis of cancer cells. The MAPK-phosphorylated Twist1 on its serine 68 (Ser(P)^{68}-Twist1) has a significantly enhanced stability and function to drive cancer cell invasion and metastasis. However, the phosphatase that dephosphorylates Ser(P)^{68}-Twist1 and destabilizes Twist1 has not been identified and characterized. In this study, we screened a serine/threonine phosphatase cDNA expression library in HEK293T cells with ectopically coexpressed Twist1. We found that the small C-terminal domain phosphatase 1 (SCP1) specifically dephosphorylates Ser(P)^{68}-Twist1 in both cell-free reactions and living cells. SCP1 uses its amino acid residues 43–63 to interact with the N terminus of Twist1. Increased SCP1 expression in cells decreased Ser(P)^{68}-Twist1 and total Twist1 proteins, whereas knockdown of SCP1 increased Ser(P)^{68}-Twist1 and total Twist1 proteins. Furthermore, the levels of SCP1 are negatively correlated with Twist1 protein levels in several cancer cell lines. SCP1-dephosphorylated Twist1 undergoes fast degradation via the ubiquitin-proteasome pathway. Importantly, an increase in SCP1 expression in breast cancer cells with either endogenous or ectopically expressed Twist1 largely inhibits the Twist1-induced epithelial-to-mesenchymal transition phenotype and the migration and invasion capabilities of these cells. These results indicate that SCP1 is the phosphatase that counterregulates the MAPK-mediated phosphorylation of Ser^{68}-Twist1. Thus, an increase in SCP1 expression and activity may be a useful strategy for eliminating the detrimental roles of Twist1 in cancer cells.

Nowadays more than 90% of cancer deaths are attributed to metastasis acquired through multiple steps, including cancer cell local migration and invasion, intravasation into and extravasation out of the circulating systems, and colonization in a distant organ site (1–3). It is generally believed that cancer cells with a complete or partial epithelial-to-mesenchymal transition (EMT)[3] phenotype possess largely enhanced capabilities to go through some of the metastatic steps, such as migration and invasion into the circulating systems, survival in the circulating systems, and dissemination into a metastatic organ site (4, 5). EMT is mainly induced by stromal cell-derived factors in the tumor environment through further induction and/or activation of a group of transcription factors in the tumor cells. These transcription factors, such as Twist1, Snail, Slug, Zeb1, and Zeb2, can either activate or repress their target genes to promote EMT, migration, invasion, and metastasis of various cancer cells (3, 4).

Twist1 expression has been detected in certain breast, prostate, gastric, ovarian, and cervical cancers as well as esophageal squamous cell cancers. Twist1 expression in these cancers has been associated with low rates of overall survival and progression-free patient survival (5–7). Twist1 is a member of the basic helix-loop-helix transcription factor family. Twist1 can prevent cells from p53-induced apoptosis; repress E-cadherin expression; up-regulate N-cadherin expression; promote breast cancer cell migration, invasion, and metastasis; increase cancer cell resistance to chemotherapy; and even induce tumor cell differentiation into endothelial cells (8–13). Twist1 can also promote EMT, cancer stem cell features, cell migration, and cell invasion through multiple pathways such as up-regulating the expression levels of Snail, Bmi1, AKT2, and PDGFRα; synergizing with Artemin, a glial cell-derived neurotrophic factor; and enhancing the TGF-β/PI3K/Akt signaling cascade (10, 11, 14–18). In addition, we have reported that Twist1 can directly silence the expression of E-cadherin and ERα by recruiting NuRD complex containing Rb-associated protein 46 (RbAp46), Mi2, histone deacetylase 2 (HDAC2), and metastasis-associated protein 2 (MTA2) (12, 19).

The small C-terminal domain phosphatases (SCPs) contain a conserved D*X*D*X*(T/V) motif and are members of the haloacid

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[3] The abbreviations used are: EMT, epithelial-to-mesenchymal transition; SCP, small C-terminal domain phosphatase; dn, dominant negative; DOX, doxycycline; IP, immunoprecipitation; ERα, estrogen receptor α.

dehalogenase superfamily (20). SCPs were initially found to be able to preferentially dephosphorylate the Ser(P)[5] on the C-terminal domain of RNA polymerase II to regulate RNA polymerase II recycling (21). Recently, it has been shown that SCP1 is required for RE-1 silencing transcription factor-repressed neuronal gene expression in non-neuronal cells (22). SCP1 can also dephosphorylate Smad1, Smad2, and Smad3 to potentiate TGF-β signaling (23, 24). In addition, SCP1 has been shown to dephosphorylate Snail1 at a GSK-3β-targeted phosphorylation site to regulate Snail1 protein stability (25).

The concentration and function of Twist1 are regulated by multiple posttranslational modifications. Tip60 acetylates Twist1 at Lys[63] and Lys[67], which allows Twist1 to interact with BRD4 for transcriptional activation of *Wnt5a* (26). Furthermore, the counterregulation between PKA-mediated phosphorylation and protein phosphatase 2A-mediated dephosphorylation of Twist1 on Thr[125] and Ser[127] plays crucial roles in Saethre-Chotzen syndrome. PKB-mediated phosphorylation of Ser[42] in Twist1 enables cells to evade the DNA damage-induced p53 response. In a cell line derived from squamous cell carcinoma of the head and neck, casein kinase 2 (CK2) induced by IL-6 can phosphorylate Twist1 at Ser[18] and Ser[20] to stabilize Twist1 and promote cell motility. The inhibitor of κB kinase β can also phosphorylate Twist1 at multiple sites to arrest Twist1 in the cytoplasm for ubiquitination and degradation (27–30).

Our laboratory has previously reported that the Ser[68] residue in Twist1 is highly phosphorylated by MAPKs in both breast cancer cell lines and human breast tumors, and this phosphorylation strongly stabilizes Twist1 protein to enhance breast cancer cell migration, invasion, and metastasis (31). In this study, we screened a serine/threonine phosphatase cDNA library to identify phosphatases that specifically dephosphorylate Ser(P)[68]-Twist1. We found that SCP1 specifically interacts with Twist1 and dephosphorylates Ser(P)[68]-Twist1. The SCP1-mediated dephosphorylation of Ser(P)[68]-Twist1 accelerates Twist1 ubiquitination and degradation, resulting in significant decreases in both the EMT phenotype and the migration and invasion capabilities of cancer cells. These findings suggest that activation of SCP1 and inactivation of MAPKs may be a useful approach for eliminating the detrimental role of Twist1 in cancer metastasis.

Experimental Procedures

Plasmids—Human SCP1 and its dominant negative mutant (dnSCP1) cDNAs in the pGEX-4T-1 plasmid were described previously (24). The N-terminal FLAG-tagged or HA-tagged SCP1 and dnSCP1 were subcloned into the pcDNA3.1-Hygro or pLenti6/TR plasmid. The N-terminal HA-tagged Twist1 and C-terminal FLAG-tagged Twist1 were subcloned into the pSG5 and pcDNA3.1 plasmids, respectively. SCP1 cDNA fragments were generated by PCR and subcloned into the pGEX-4T-1 plasmid. Full-length Twist1 and its fragments were tagged by a C-terminal FLAG sequence and subcloned into the pGEX-4T-1 plasmid. SCP1 deletion mutants were generated by PCR-based mutagenesis methods and subcloned into the pGEX-4T-1 plasmid. All expression vectors were confirmed by DNA sequencing.

Antibodies—This study used antibodies against SCP1 (NBP1-55978, Novus Biologicals), Twist1 (ab50887, Abcam), GAPDH (ab9484, Abcam), HA (C29F4, Cell Signaling Technology), vimentin (5741, Cell Signaling Technology), GST (sc-33613, Santa Cruz Biotechnology), E-cadherin (610181, BD Biosciences), tubulin (T-8203, Sigma-Aldrich), FLAG (F-1804, Sigma-Aldrich), and β-actin (A5441, Sigma). The antibody against Ser(P)[68]-Twist1 was described previously (31).

Cell Culture, Transfection, and Stable Cell Lines—MDA-MB-436, MDA-MB-435, 4T1, MCF7, HeLa, Ishikawa, HEK293T, and HEK293 cells with doxycycline (DOX)-inducible Twist1-FLAG, S68A-Twist1-FLAG and S68E-Twist1-FLAG were cultured in DMEM supplemented with glucose (4.5 g/liter), L-glutamine, penicillin, streptomycin, and 10% FBS as described previously (12). Cells were transfected using Lipofectamine 2000 reagent (Life Technologies) or PEI reagent by following the instructions of the manufacturer. To generate stable MCF7 cell lines with Twist1 expression, MCF7 cells (10^6) were transfected with 1 μg of MfeI-linearized pcDNA3.1-Twist1-FLAG plasmid or pcDNA3.1 empty (control) plasmid. The transfected cells were selected in medium containing 500 μg/ml of G418 for 2 weeks. The surviving clones were individually isolated, expanded, and analyzed by Western blotting.

GST Pulldown Assay, Phosphatase Assay, and Immunoprecipitation—GST pulldown assays were performed as described previously (19). GST fusion proteins were individually expressed in BL21 *Escherichia coli* (Novagen). Bacteria were lysed in Sarkosyl buffer (0.5% Sarkosyl, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM EDTA) with sonication at 4 °C. Clear lysates containing 10 μg of protein were incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) at 4 °C for 1.5 h. The beads were washed three times and incubated with purified full-length Twist1 or Twist1 fragments at 4 °C for another 1.5 h. The beads were washed five times using buffer containing 20 mM Tris-HCl (pH 8.0), 5% glycerol, 1% Triton X-100, 0.05% SDS, 150 mM NaCl, 1 mM EDTA, and 0.1% β-mercaptoethanol. Proteins associated with the beads were heat-eluted in SDS-PAGE reducing sample buffer and subjected to Western blotting. The *in vitro* phosphatase assay was performed as described previously (24). For the *in vivo* phosphatase assay, cells were transfected with SCP1 or mutant SCP1 expression plasmids using Lipofectamine 2000 reagent (Life Technologies). Forty-eight hours later, cells were collected and subjected to Western blotting using appropriate antibodies. Immunoprecipitation assays were performed as described previously (12).

Ubiquitination Assay—HEK293 cell lines (10^5 cells) with DOX-inducible Twist1-FLAG expression were transfected with 1 μg of HA-tagged ubiquitin (HA-ubiquitin) plasmid and HA-SCP1 or HA-dnSCP1 plasmid. After 36 h, cells were treated with vehicle (H_2O) or 1 μg/ml DOX for 6 h and then with vehicle (DMSO) or 10 μg/ml MG132 for another 6 h. Cell lysates were prepared and mixed with anti-FLAG M2-agarose beads (Sigma) overnight at 4 °C. Beads were washed five times using ice-cold PBS. Bound proteins were heat-eluted in SDS-PAGE reducing sample buffer and analyzed by Western blotting.

shRNA-based Knockdown of SCP1—MDA-MB-436 and Ishikawa cells were infected with lentiviral particles of the GIPZ lentiviral shRNAmir-GFP system as described previously (19). These lentiviral vectors mediate the expression of non-targeting control shRNA or shRNAs (clone RHS4430-101068525 and clone RHS4430-101071006, Sigma) that target human SCP1 mRNA. To measure the knockdown efficiency, total RNA was isolated from the infected and growth-selected cell pools using TRIzol reagent (Thermo Fisher). Two micrograms of RNA were reverse-transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Universal Probe Library Center software (Roche Applied Science) was used to design primers and match the corresponding fluorescence-labeled probes for TaqMan real-time PCR. The relative mRNA concentration was presented after normalizing to the β-actin mRNA concentration. The protein levels of SCP1, Twist1, and Ser(P)^{68}-Twist1 in these cells were also assayed by Western blotting.

Cycloheximide Chase Assay—Cells expressing control shRNA or shRNA targeting SCP1 mRNA were treated with 250 μg/ml of cycloheximide for different periods of time. Cell lysates were prepared and subjected to Western blotting analyses of Twist1 and tubulin. Twenty micrograms of total protein were loaded in each lane. The relative level of Twist1 protein was obtained by normalizing to the level of tubulin protein in each sample.

Real-time Cell Growth, Migration, and Invasion Assays—Cells were transfected as described above. Forty-eight hours later, transfected cells were subjected to the growth, migration, and invasion assays using the RT-CES system (ACEA Biosciences) as described previously (12). For the growth assay, 5000 cells in medium containing 10% FBS were plated in each well. For migration and invasion assays, cells were washed five times with serum-free medium. Then, 50,000 cells in 100 μl of serum-free medium were plated in each upper chamber. Each of the lower chambers, which were separated by a membrane with 8-μm pores from the upper chamber, was filled with medium containing 10% FBS as a chemoattractant. For the invasion assay, the membrane of each chamber was precoated with 20 μl of 1:5 diluted growth factor-reduced Matrigel (BD Biosciences). The cell indices, which positively correlate with cell densities, were automatically recorded every 30 min during the entire monitoring process by the software of the RT-CES system. Each assay was performed in triplicate.

Results

Identification of SCP1 as the Phosphatase for Ser(P)^{68}-Twist1—We have previously reported that Twist1 is phosphorylated on Ser^{68} by MAPKs, which greatly stabilizes Twist1 protein to enhance cancer cell invasion (5, 31). To identify the phosphatase(s) that can counterbalance the detrimental role of MAPKs in Twist1 function by dephosphorylating Ser(P)^{68}-Twist1, we screened a phosphatase library consisting of 33 protein serine/threonine phosphatases and six dual-specific protein phosphatases (32). We co-transfected HEK293T cells with Twist1-FLAG (Twist-F) expression plasmid and one of the above 39 phosphatases and then examined the levels of each phosphatase, Ser(P)^{68}-Twist1-F, and total Twist1-F by Western blotting. We found that SCP1 expression significantly decreased the level of Ser(P)^{68}-Twist1-F as well as the level of total Twist1-F protein, whereas other phosphatases showed no obvious effect on the level of Ser(P)^{68}-Twist1-F (Fig. 1*A* and data not shown). These results suggest that SCP1 is a candidate phosphatase that dephosphorylates Ser(P)^{68}-Twist1.

To confirm that SCP1 expression caused dephosphorylation of Ser(P)^{68}-Twist1, we expressed Twist1-F together with different amounts of FLAG-tagged SCP1 (F-SCP1) in HEK293T cells. Western blotting analysis revealed that both Ser(P)^{68}-Twist1-F and total Twist1-F were decreased in a F-SCP1 dose-dependent manner when total protein inputs of all samples were normalized to GAPDH (Fig. 1*B*). Because Twist1 protein is less stable than Ser(P)^{68}-Twist1 (31), the decrease in total Twist1-F in cells with high F-SCP1 expression is expected. To further evaluate the ratio of Ser(P)^{68}-Twist1 to total Twist1 proteins, we co-expressed Twist1-F with different amounts of F-SCP1 in HEK293T cells and assayed the levels of Ser(P)^{68}-Twist1-F when comparable total Twist1-F protein was loaded to each lane. Again, the levels of Ser(P)^{68}-Twist1-F were markedly reduced when F-SCP1 expression levels were increased (Fig. 1*C*). To further evaluate the effect of SCP1 on endogenous Ser(P)^{68}-Twist1, we expressed F-SCP1 in MDA-MB-435 and 4T1 cancer cell lines with abundant endogenous Twist1 expression (12, 19, 31). Similarly, when similar amounts of total endogenous Twist1 protein in all samples were assayed by Western blotting, the levels of endogenous Ser(P)^{68}-Twist1 were drastically decreased in both of these cell lines with F-SCP1 overexpression (Fig. 1*D*). These results clearly demonstrate that the levels of either ectopically expressed or endogenous Ser(P)^{68}-Twist1 negatively correlate with the levels of SCP1 expression in multiple cell lines.

To examine whether Ser(P)^{68}-Twist1 is a direct substrate of SCP1, we purified total Twist1-F protein from HEK293 cell lysate containing the phosphatase inhibitor and recombinant GST-SCP1 and GST-dnSCP1 fusion proteins from the bacterial lysates and performed dephosphorylation assays under cell-free conditions (Fig. 2*A*). The dnSCP1 contains an Asp-to-Asn phosphatase-dead mutation (24), and we used it as a negative control. After total Twist1-F was incubated with GST-dnSCP1 in the dephosphorylation reaction, a strong band of Ser(P)^{68}-Twist1-F was detected by using the Ser(P)^{68}-Twist1-specific antibody. However, after total Twist1-F was reacted with GST-SCP1 under the same conditions, the level of Ser(P)^{68}-Twist1-F was markedly reduced (Fig. 2*B*). Accordingly, the ratio of Ser(P)^{68}-Twist1-F to total Twist1-F, as measured by band intensities from Western blotting, was reduced 78% by GST-SCP1 *versus* GST-dnSCP1 (Fig. 2*C*). These results prove that SCP1 directly dephosphorylates Ser(P)^{68}-Twist1.

SCP1 Physically Interacts with Twist1—To investigate whether SCP1 dephosphorylates Ser(P)^{68}-Twist1 through direct interaction, we examined the interaction between SCP1 and Twist1. We first co-expressed F-SCP1 and HA-Twist1 in HEK293T cells and performed co-immunoprecipitation (co-IP) using HA antibody or non-immune IgG as a negative control, which was followed by Western blotting analysis with SCP1 and HA antibodies. We detected both F-SCP1 and HA-Twist1 in the cell lysate (input) and the co-IP sample by HA

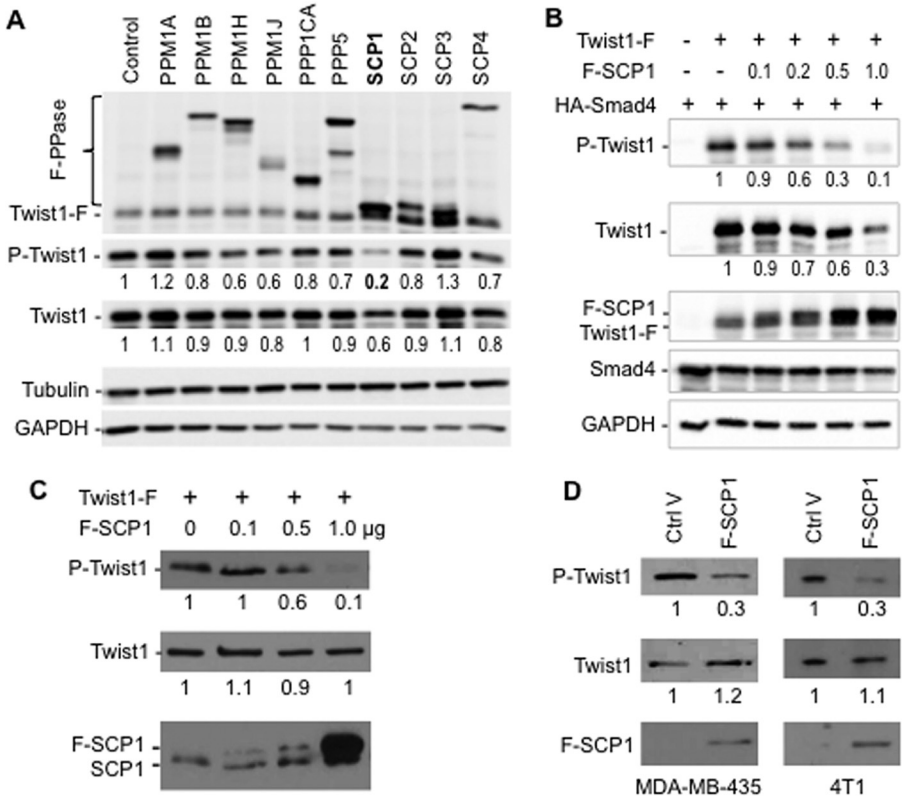

FIGURE 1. **Identification of SCP1 as a phosphatase that dephosphorylates Ser(P)^{68}-Twist1 in cells.** *A*, screening the phosphatase expression library. HEK293T cells were co-transfected with the empty vector, Twist1-F, and one of the FLAG-tagged phosphatases as indicated (only partial screening data are shown). Forty micrograms of protein were loaded in each lane. The expression levels of Twist1-F and FLAG-tagged phosphatases were analyzed by Western blotting using FLAG antibody. Ser(P)^{68}-Twist1 (*P-Twist1*) and total Twist1 (*Twist1*) were analyzed by Western blotting using Ser(P)^{68}-Twist1 and Twist1 antibodies, respectively. Relative band intensities measured by densitometry are indicated. Tubulin and GAPDH served as loading controls. *B*, SCP1 dephosphorylates Ser(P)^{68}-Twist1 in a dose-dependent manner. HEK293T cells were co-transfected with empty vector (−), Twist1-F, HA-Smad4, and increasing amounts of F-SCP1 as indicated. Smad4 served as a negative control. Ser(P)^{68}-Twist1 and total Twist1 were measured by their specific antibodies. Relative band intensities are indicated. F-SCP1 was analyzed by FLAG antibody, which partially overlapped with Twist1-F because of similar molecular weights. *C*, overexpression of SCP1 decreases the ratio of Ser(P)^{68}-Twist1 to total Twist1. HEK293T cells were transfected with Twist1-F and increasing amounts of F-SCP1 plasmids. Similar amounts of total Twist1 (*center panel*) were loaded from individual samples for analysis of Ser(P)^{68}-Twist1 (*top panel*). F-SCP1 and endogenous SCP1 were detected by SCP1 antibody (*bottom panel*). Relative band intensities are indicated. *D*, SCP1 dephosphorylates endogenous Ser(P)^{68}-Twist1. MDA-MB-435 and 4T1 cells were transfected with the empty vector or F-SCP1 plasmid. Transfected cells were analyzed by Western blotting using Ser(P)^{68}-Twist1, Twist1, and FLAG antibodies. Inputs were adjusted to similar levels of total Twist1 (*center panel*). Relative band intensities are indicated. *Ctrl*, control.

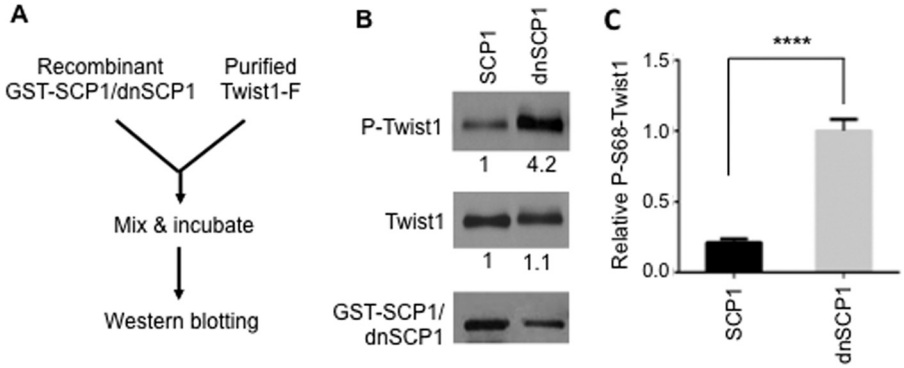

FIGURE 2. **SCP1 dephosphorylates Ser(P)^{68}-Twist1 under cell-free conditions.** *A*, Twist1-F protein purified from HEK293 cells was mixed with purified GST-SCP1 or GST-dnSCP1 and incubated in a phosphatase buffer. *B*, Ser(P)^{68}-Twist1, total Twist1, GST-SCP1, and GST-dnSCP1 were analyzed by Western blotting with their specific antibodies. Forty micrograms of protein were loaded in each lane. *C*, band intensities of Western blots were measured by densitometry and presented as relative ratios of Ser(P)^{68}-Twist1 to Twist1. The experiments were repeated three times. ****, $p < 0.0001$ by Student's *t* test.

antibody but not in the co-IP sample by IgG (Fig. 3*A*), suggesting that F-SCP1 is associated with HA-Twist1 in HEK293T cells. Furthermore, co-IP followed by Western blotting also revealed that endogenous Twist1 and SCP1 in 4T1 mammary tumor cells could be efficiently co-precipitated by each other (Fig. 3*B*), indicating that these two proteins form a steady protein complex. Moreover, we expressed GST, GST-SCP1, and GST-dnSCP1 in *Escherichia coli* and isolated these recombinant proteins by using glutathione beads. We also purified Twist1-F protein from HEK293 cells and incubated the purified Twist1-F with GST, GST-SCP1, or GST-dnSCP1 protein immortalized on the glutathione beads in an interaction buffer.

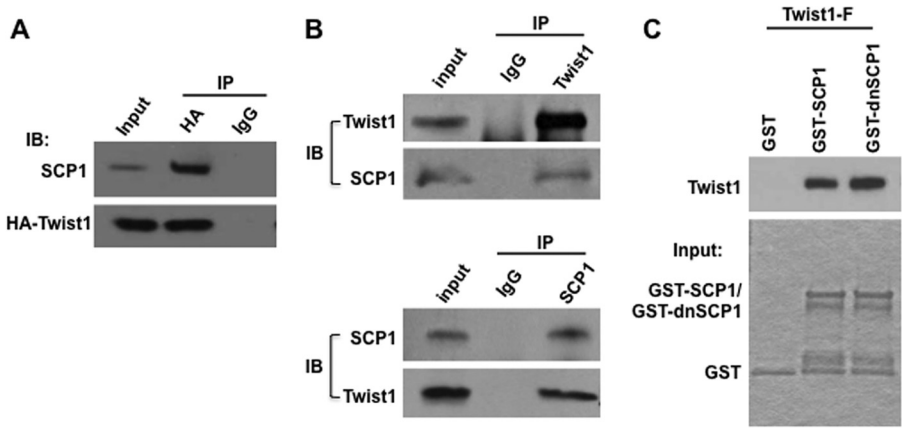

FIGURE 3. **SCP1 interacts with Twist1.** *A*, co-IP. HEK293T cells were co-transfected with 1 μg of FLAG-SCP1 plasmid and 1 μg of HA-Twist1 plasmid. Co-IP was performed with either HA antibody or IgG, followed by immunoblotting (*IB*) with SCP1 and Twist1 antibodies as indicated. *B*, co-IP assays for endogenous Twist1 and SCP1. Cell lysate containing endogenous Twist1 and SCP1 was prepared from 4T1 cells. Co-IP was performed with Twist1 (*top panel*) or SCP1 (*bottom panel*) antibody. IgG was used as a negative control. Western blotting analyses were performed using Twist1 and SCP1 antibodies as indicated. *C*, GST pulldown assays. Glutathione-Sepharose 4B bead-bound GST, GST-SCP1, and GST-dnSCP1 proteins were mixed and incubated with purified Twist1-F protein from HEK293 cells. The beads were washed and eluted by boiling in SDS-PAGE reducing buffer. Eluted proteins were analyzed by Western blotting using Twist1 antibody (*top*). The input proteins were visualized by Ponceau S staining (*bottom*).

After washing thoroughly, Western blotting analysis revealed that Twist1-F was co-precipitated with GST-SCP1 and GST-dnSCP1 but not with GST (Fig. 3*C*), indicating that Twist1 physically interacts with SCP1 regardless of its phosphatase activity.

SCP1 Uses its Amino Acid Residues 43–63 to Interact with the N Terminus of Twist1—After finding the direct interaction between SCP1 and Twist1, we further mapped their interaction regions by pulldown assays. We first expressed GST and five GST-SCP1 fusion proteins containing full-length SCP1, SCP1(1–115), SCP1(85–232), SCP1(180–261), SCP1(1–84), and SCP1(Δ85–232) in *E. coli* (Fig. 4*A*) and performed GST pulldown assays for their interactions with the full-length Twist1-F expressed in HEK293 cells. We found that GST-SCP1, GST-SCP1(Δ85–232), GST-SCP1(1–115), and GST-SCP1(1–84) strongly interacted with Twist1-F, whereas GST-SCP1(180–261) only showed very little interaction, and GST-SCP1(85–232) and GST showed no interaction (Fig. 4*B*, *left panels*). Because SCP1(1–84) contains the common region in these fragments, these results indicate that the N-terminal 84 amino acid region of SCP1 should contain a major interaction domain for Twist1. To further map this interaction domain, we expressed another four small recombinant GST-SCP1 fusion proteins containing SCP1(22–84), SCP1[1–84(Δ22–42)], SCP1[1–84(Δ43–63)], and SCP1(1–63) and examined their interactions with Twist1-F (Fig. 4*A*). GST pulldown assays revealed that GST-SCP1(22–84) and GST-SCP1(1–63) interacted with Twist1-F as strongly as GST-SCP1(1–84). GST-SCP1[1–84(Δ22–42)] still interacted with Twist1-F, although at a lesser degree. However, GST-SCP1[1–84(Δ43–63)] showed no interaction with Twist1-F (Fig. 4*B*, *right panels*). These results indicate that the 21 amino acid residues from amino acids 43–63 are responsible for SCP1 to interact with Twist1.

To define the region of Twist1 that interacts with SCP1, we expressed the full-length Twist1-F and three Twist1 fragments with FLAG tags in *E. coli* (Fig. 4*C*). When incubated with GST-SCP1(1–84), we found that full-length Twist1-F and Twist1(1–100)-F equally interacted with GST-SCP1(1–84). However, Twist1(80–170)-F and Twist1(140–202)-F did not interact with GST-SCP1(1–84). As expected, the full-length Twist1-F was not able to interact with GST-SCP1(85–232) and GST, the two negative controls (Fig. 4*D*). Taken together, these results demonstrate that the N-terminal 100 amino acid residues of Twist1 contain the domain for interacting with the region of amino acids 43–63 in SCP1.

SCP1 Accelerates Twist1 Degradation in Vivo—Ser(P)^{68}-Twist1 is known to be more stable than unphosphorylated Ser^{68}-Twist1 (31). The experiments shown in Fig. 1, *A* and *B*, demonstrated that ectopically expressed SCP1 reduced both Ser(P)^{68}-Twist1-F and total Twist1-F when normalized to the levels of cellular α-tubulin or GAPDH. To confirm that SCP1 indeed accelerates Twist1 degradation, we expressed HA-SCP1 and HA-dnSCP1 in a previously established HEK293 cell line with DOX-inducible expression of Twist1-F (12, 19, 33) and examined their effects on Twist1-F protein. Western blotting analysis revealed that expression of HA-SCP1 significantly reduced the levels of both Ser(P)^{68}-Twist1-F and total Twist1-F proteins, whereas expression of HA-dnSCP1 had no effect on these proteins compared with the empty vector transfection control (Fig. 5*A*). Furthermore, expression of HA-SCP1 in MDA-MB-436 cells decreased endogenous Ser(P)^{68}-Twist1 and total Twist1 (Fig. 5*B*), whereas stable knockdown of SCP1 in these cells by expressing either one of the two different shRNAs significantly increased endogenous Ser(P)^{68}-Twist1 and total Twist1 (Fig. 5*C*). Knockdown of SCP1 by either one of the two shRNAs also increased the levels of endogenous Ser(P)^{68}-Twist1 and total Twist1 in Ishikawa cells (Fig. 5*D*). These results suggest that SCP1-mediated dephosphorylation of Ser(P)^{68}-Twist1 plays an important role to control the cellular level of Twist1 protein.

Next, we examined how SCP1 affects Twist1 stability by chasing Twist1 degradation after protein synthesis was blocked by cycloheximide treatment. We generated stable Ishikawa cell lines expressing a non-targeting shRNA or one of the two different shRNAs targeting SCP1 mRNA and treated these cells

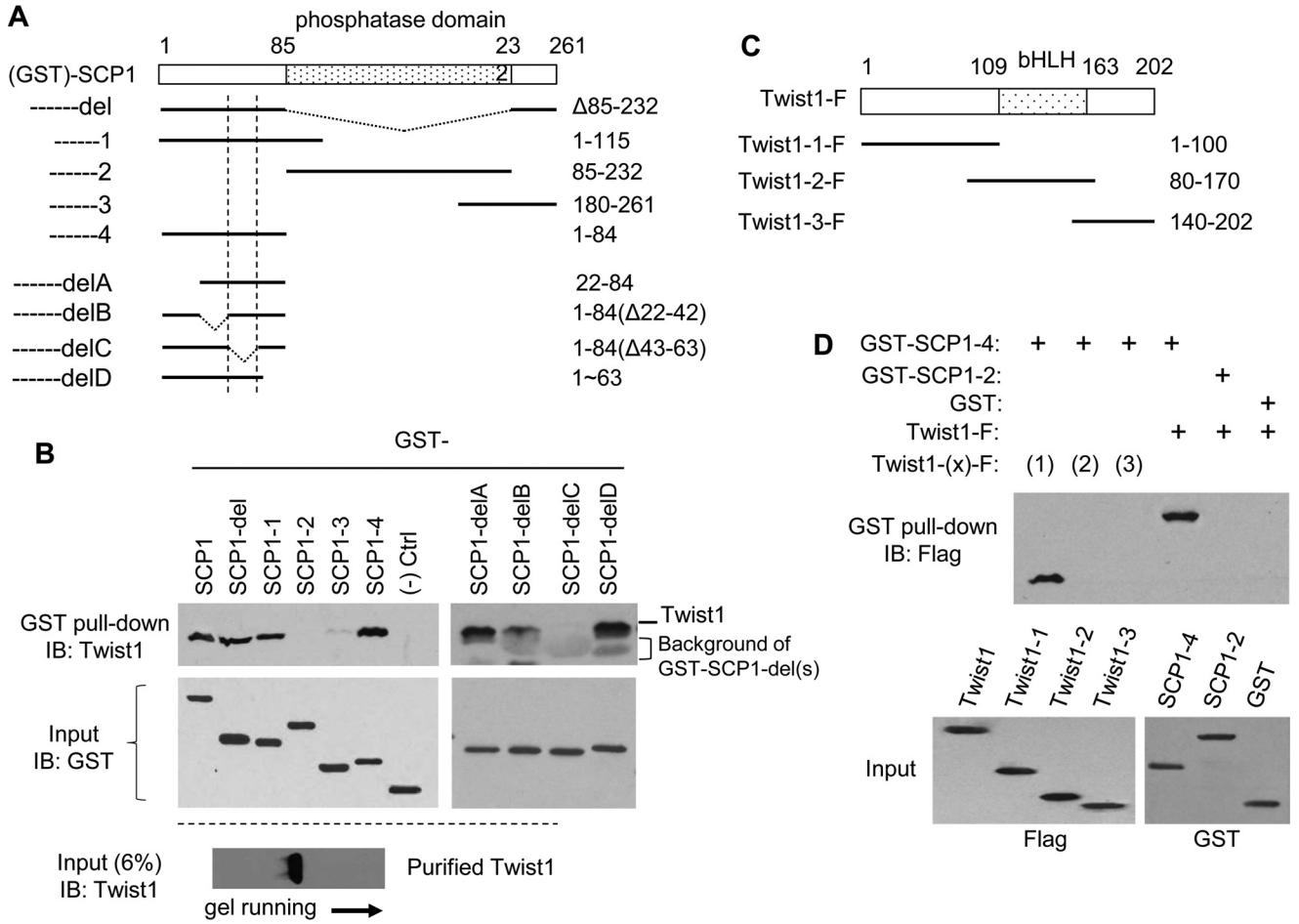

FIGURE 4. **The amino acid 43–63 region of SCP1 is responsible for interacting with the N terminus of Twist1.** *A*, GST-tagged full-length SCP1 and nine SCP1 fragments were purified from bacteria using glutathione-Sepharose 4B beads. *B*, Twist1-F protein was expressed in HEK293 cells and purified as described by Fu *et al.* (12). Equal amounts of the purified Twist1-F protein were incubated with beads carrying 10 μg of GST (a negative control), GST-tagged full-length SCP1, or one of the GST-tagged SCP1 fragments. Co-precipitated proteins were analyzed using Twist1 antibody. Input proteins were analyzed using GST or Twist1 antibody as indicated. *Ctrl*, control; *IB*, immunoblot. *C* and *D*, GST pulldown assays. Twist1-F and its N-terminal, medium, and C-terminal fragments with a C-terminal FLAG tag were produced in bacteria. The bacterial extracts were incubated with beads carrying GST, GST-SCP1–2, or GST-SCP1–4 (refer to *A* for SCP1 fragment nomenclature) as indicated. Co-precipitated proteins were assayed by Western blotting using FLAG antibody. Input proteins were assayed by Western blotting using FLAG or GST antibody as indicated. *bHLH,* basic helix-loop-helix.

with cycloheximide for different time periods. We found that Twist1 protein in cells with SCP1 knockdown degraded much slower than Twist1 in cells with non-targeting shRNA expression (Fig. 5*E*). To validate the negative correlation relationship between SCP1 and Twist1 proteins, we measured the relative levels of SCP1 and Twist1 proteins in MDA-MB-231, MDA-MB-435, BT549, and MDA-MB-436 human cancer cells. We found that the levels of SCP1 protein indeed inversely correlated with the levels of total Twist1 protein among these cell lines (Fig. 5*F*). In addition, considering the role of SCP1 in RNA polymerase II recycling, which might affect gene transcription, we measured Twist1 mRNA in cells with and without knockdown or overexpression of SCP1. We found that the expression level of Twist1 mRNA was not affected by SCP1 (data not shown). Taken together, these results demonstrate that SCP1 expression decreases the level of Twist1 by accelerating its protein degradation.

SCP1 Promotes Twist1 Ubiquitination—To examine whether SCP1-accelerated Twist1 degradation is dependent on the proteasome pathway, we transfected HEK293 cells with DOX-inducible Twist1 expression with HA-SCP1 expression plasmid or empty plasmid. Thirty-six hours later, when HA-SCP1 was steadily expressed, we treated these cells with DOX for 6 h to induce Twist1-F expression or without DOX as a blank control. After these cells were further treated with or without the MG132 proteasome inhibitor for another 6 h, we found that HA-SCP1 expression reduced both Ser(P)^{68}-Twist1-F and total Twist1-F in the absence of MG132. In the presence of MG132, total Twist1-F protein was markedly increased in both groups of cells with or without HA-SCP1 expression, whereas only Ser(P)^{68}-Twist-F but not total Twist1 was reduced in cells with HA-SCP1 expression *versus* cells without HA-SCP1 expression (Fig. 6*A*). These results indicate that SCP1-promoted Twist1 degradation is dependent on the normal function of the proteasome.

MAPK-mediated phosphorylation of Ser(P)^{68}-Twist1 stabilizes Twist1 by protecting Twist1 from ubiquitination (31). To address whether SCP1-mediated dephosphorylation could promote Twist1 ubiquitination, we co-expressed HA-ubiquitin with either SCP1 or dnSCP1 in MG132-treated HEK293 cells

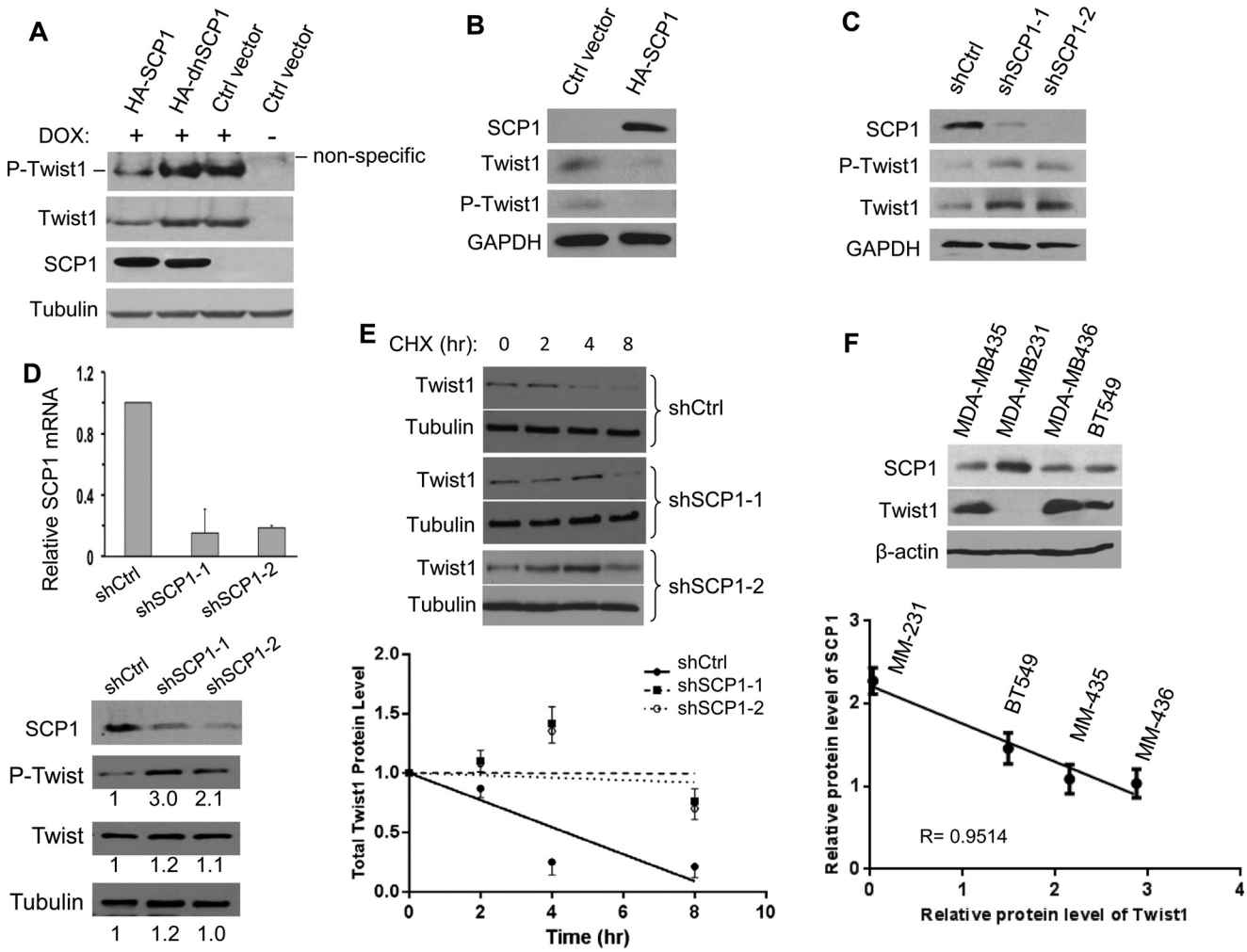

FIGURE 5. **SCP1-mediated dephosphorylation of Ser(P)^{68}-Twist1 accelerates Twist1 degradation.** *A*, HEK293 cells (10^5) with DOX-inducible Twist1 expression were transfected with 1 μg of HA-SCP1, HA-dnSCP1, or the empty vector. After 48 h, cells were treated with or without DOX for 6 h. Western blotting was performed to analyze the indicated proteins. *Ctrl*, control. *B*, MDA-MB-436 cells (10^5) were transfected with 1 μg of the empty or HA-SCP1 plasmid for 48 h before cells were harvested for Western blotting analyses for the proteins indicated. *C*, Western blotting analyses of SCP1, Ser(P)^{68}-Twist1, and Twist1 proteins in MDA-MB-436 cell lines expressing non-targeting shRNA (shCtrl) and SCP1 mRNA-degrading shRNAs (shSCP1-1 and shSCP1-2). *D*, real-time PCR analysis of SCP1 mRNA (*top panel*) and Western blotting analysis (*bottom panel*) of SCP1, Ser(P)^{68}-Twist1, and Twist1 in Ishikawa cells expressing shCtrl, shSCP1-1, or shSCP1-2 shRNAs. *E*, Ishikawa cells with shCtrl, shSCP1-1, or shSCP1-2 shRNA expression were treated with cycloheximide (*CHX*) for the time periods indicated. Cell lysates were analyzed by Western blotting using Twist1 and tubulin antibodies (*top panel*). Band intensities were measured by densitometry from three experiments. Relative band intensity was normalized to the band intensity at time zero for each group (*bottom panel*). *F*, Western blotting analysis of the endogenous SCP1 and Twist1 in the cell lines indicated and their negative correlation relationship. The average band intensities were obtained from three experiments and normalized to the band intensities of β-actin in each experiment. The R value was obtained from the linear regression analysis using GraphPad Prism software.

expressing DOX-inducible Twist1-F and performed a ubiquitination assay for Twist1-F. Western blotting analysis of immunoprecipitated Twist1-F revealed that Twist1-F is steadily polyubiquitylated in the cells without ectopic SCP1 or dnSCP1 expression. dnSCP1 expression reduced Twist1-F polyubiquitination because of its dominant-negative effect through interacting with Twist1-F, as shown in Fig. 3. Importantly, SCP1 expression robustly enhanced Twist1-F polyubiquitination compared with the empty vector control or dnSCP1 expression (Fig. 6*B*). These results demonstrate that SCP1 can significantly promote Twist1 ubiquitination. The ubiquitylated Twist1 is then rapidly degraded through the proteasome protein degradation pathway.

To rule out the possibility that SCP1 might dephosphorylate other sites rather than Ser(P)^{68}-Twist1 to promote Twist1 ubiquitination, we compared the levels of the ubiquitylated F-Twist1-F, S68A-Twist1-F (the serine 68-to-alanine mutant) and S68E-Twist1-F (the serine 68-to-glutamate mutant) induced by SCP1 expression in HEK293 cell lines with inducible expression of Twist1-F, S68A-Twist1-F, and S68E-Twist1-F. We found that ectopic expression of SCP1 robustly increased the ubiquitination level of Twist1-F but did not change the ubiquitination levels of the other two mutants (Fig. 6*C*). These results indicate that SCP1 promotes Twist1 ubiquitination through dephosphorylating Ser(P)^{68}-Twist1.

SCP1 Inhibits Cancer Cell Migration and Invasion by Suppressing Twist1 Function—Twist1 has been shown to promote the migration and invasion capabilities of various cancer cells (14, 34, 35). To examine whether SCP1-mediated dephosphorylation and destabilization of Twist1 inhibit cancer cell migration and invasion, we first expressed SCP1 in MDA-MB-436 human breast cancer cells to test whether SCP1 overexpression

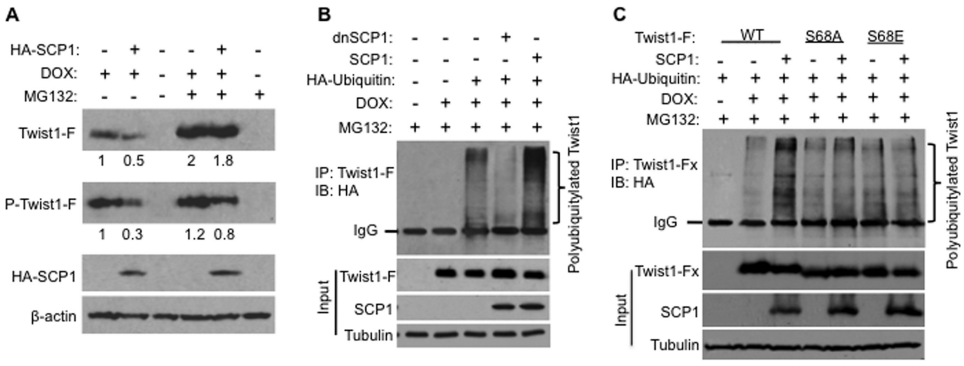

FIGURE 6. **SCP1 promotes Twist1 ubiquitination.** *A*, HEK293 cells with DOX-inducible Twist1-F expression were transfected with the empty vector (−) or HA-SCP1 plasmid. After 36 h, these cells were treated with DOX or vehicle (water) for 6 h and then with vehicle (DMSO) or MG132 for another 6 h. Cell lysates were analyzed by Western blotting using antibodies against Twist1, Ser(P)^{68}-Twist1, and HA (*HA-SCP1*) as indicated. The relative band intensities for Twist1-F and Ser(P)^{68}-Twist1-F are indicated. *B*, HEK293 cells with DOX-inducible Twist1-F expression were transfected with 1 μg of dnSCP1, SCP1, and/or HA-ubiquitin plasmids or the empty vector (−) as indicated. After 36 h, these cells were treated with vehicle (−) or DOX for 6 h and then with MG132 for another 6 h. Twist-F was precipitated using FLAG antibody and assayed by Western blotting using HA antibody to detect HA-ubiquitylated Twist1-F proteins. *IB*, immunoblot. *C*, three HEK293 cell lines with DOX-inducible Twist1-F (*WT*), S68A-Twist1-F, or S68E-Twist-F expression were transfected with 1 μg of the empty vectors or SCP1 and HA-ubiquitin plasmids as indicated. After 36 h, cells were treated with vehicle (−) or DOX for 6 h, followed by MG132 treatment for another 6 h. Immunoprecipitated Twist1-Fx proteins were assayed by immunoblotting using HA antibody to detect HA-ubiquitylated Twist1-F, S68A-Twist1-F, and S68E-Twist1-F (*Twist1-Fx*).

decreases cell migration and invasion. Furthermore, we overexpressed Twist1 in MDA-MB-436 cells with SCP1 overexpression to test whether overexpression of Twist1 could reverse the effects of SCP1 overexpression on cell migration and invasion. Again, SCP1 overexpression significantly reduced the endogenous Ser(P)^{68}-Twist1 and total Twist1 proteins. Co-expression of Twist1-F with SCP1 did not affect the protein level of SCP1. Co-expression of Twist-F rescued total Twist1-F and Ser(P)^{68}-Twist1-F to levels similar to the endogenous total Twist and Ser(P)^{68}-Twist1 (Fig. 7*A*, compare *lane 3* with *lane 1*). Overexpression of SCP1 alone or overexpression of both SCP1 and Twist1-F did not significantly alter the growth rate of MDA-MB-436 cells (Fig. 7*B*). However, SCP1 overexpression significantly decreased the migration and invasion capabilities of MDA-MB-436 cells and Twist1-F overexpression effectively reversed the SCP1 overexpression-reduced migration and invasion capabilities of these cells (Fig. 7, *C* and *D*). Similar effects were also observed in Ishikawa cells (data not shown). These results demonstrate that SCP1 inhibits cancer cell migration and invasion by dephosphorylating Ser(P)^{68}-Twist1 and reducing total Twist1 protein.

We next employed the MCF7 breast cancer cell model to test whether SCP1 overexpression could reverse the Twist1 overexpression-induced EMT phenotype and cell migration and invasion capabilities. We chose this ER-positive breast cancer cell model because it expresses little endogenous SCP1 and Twist1. MCF7 cells express epithelial cell proteins such as E-cadherin but not mesenchymal cell proteins, including vimentin and Twist1. As expected, Twist1 overexpression induced an EMT phenotype in two examined cell clones, as evidenced by the loss of E-cadherin expression and the acquisition of vimentin expression (Fig. 7*E*). Interestingly, SCP1 overexpression in both cell clones moderately restored E-cadherin expression and partially inhibited vimentin expression, which was accompanied by the SCP1-induced down-regulation of Twist1 in these cells (Fig. 7*F*). Although Twist1 overexpression slightly increased MCF7 cell growth *versus* the empty vector control cells, and ectopic SCP1 expression in MCF7 cells with Twist1 overexpression reversed this growth, the differences among the three cell type groups were not statistically significant (Fig. 7*G*). Strikingly, Twist1 overexpression robustly increased the migration and invasion capabilities of MCF7 cells, whereas SCP1 overexpression very effectively reversed the effects of Twist1 on the migration and invasion capabilities of MCF7 cells (Fig. 7*G*). These results indicate that SCP1 plays a critical function to inhibit Twist1-promoted EMT, migration, and invasion of MCF7 cancer cells.

Discussion

The death of most breast cancer patients is caused by metastasis, not by their primary tumor growth. Twist1 has been shown to be one of the master transcription factors driving EMT, migration, invasion, and metastasis of breast cancer cells (5, 10, 12). Thus, it is important to understand the structure, stability, and function of Twist1 in breast cancer cells. Similar to many important transcription factors, the stability and function of Twist1 are regulated by protein kinases though phosphorylation. We have previously shown that serine 68 in Twist1 is highly phosphorylated by MAPKs including JNK, ERKs, and p38 in cultured cells and that the levels of Ser(P)^{68}-Twist1 and total Twist1 are also positively correlated with JNK activities in human breast ductal carcinomas. We found that MAPK-mediated phosphorylation of Ser(P)^{68}-Twist1 strongly stabilizes Twist1 to enhance its function in promotion of TGF-β or active Ras-stimulated EMT, invasion, and chemoresistance of breast cancer cells (31). Based on these findings, we hypothesized that there is at least one phosphatase that can specifically dephosphorylate Ser(P)^{68}-Twist1 to destabilize Twist1 and thereby inhibit its detrimental roles in breast cancer. In this study, we carried out a large-scale functional screening assay to search for serine/threonine phosphatases that can specifically dephosphorylate Ser(P)^{68}-Twist1. We identified SCP1 as the phosphatase for Ser(P)^{68}-Twist1. We demonstrated that both bacterial and mammalian cell-produced SCP1 proteins are capable to dephosphorylate Ser(P)^{68}-Twist1 under both *in vitro* and *in vivo* conditions. Increased SCP1 expression also significantly acceler-

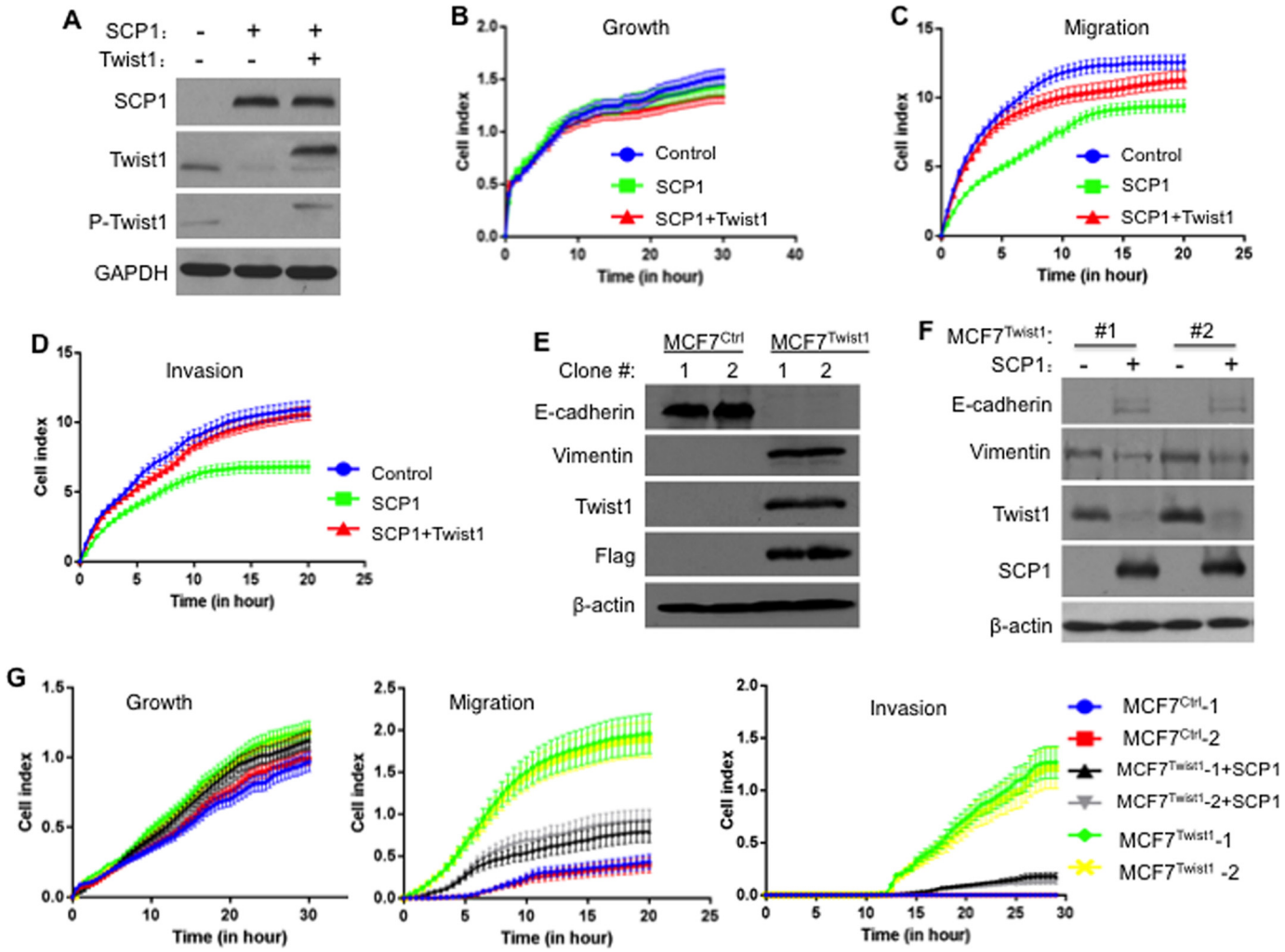

FIGURE 7. **SCP1 inhibits cell migration and invasion through an interfering Twist1-mediated effect.** *A*, MDA-MB-436 cells (10^5) were transfected with 1 μg of the empty vectors or SCP1 and/or Twist1 plasmids. Immunoblotting analyses of SCP1, Twist1, and P-S68-Twist1 were performed 48 h later. *B–D*, MDA-MB-436 cells (10^5) were transfected with 1 μg of the empty vector or SCP1 and/or Twist1 plasmids. The growth, migration, and invasion were measured in real time as described under "Experimental Procedures." The cell index is a relative measurement of cell density. *E*, Western blotting analysis of the indicated proteins expressed in stable MCF7 cell lines with a control vector (*MCF7^{Ctrl}*) or Twist1 overexpression (*MCF7^{Twist1}*). *F*, two lines of MCF7^{Twist1} cells were transfected with 1 μg of the empty or SCP1 expression plasmids and subjected to Western blotting analysis using the antibodies indicated. *G*, increased SCP1 expression inhibits the migration and invasion capabilities of MCF7^{Twist1} cells. Two lines of MCF7^{Ctrl} and two lines of MCF7^{Twist1} cells were transfected with 1 μg of the empty or SCP1 expression plasmids as indicated. Real-time cell growth, migration, and invasion assays were carried out as described under "Experimental Procedures." The transfection efficiency of these cells was more than 60%, as determined by co-transfecting a GFP expression plasmid.

ates Twist1 ubiquitination and degradation and suppresses Twist1-mediated breast cancer cell migration and invasion. These results clearly indicate that MAPKs and SCP1 reciprocally regulate Twist1 stability and function in cancer cells by a counterbalanced molecular mechanism between phosphorylation and dephosphorylation. In cancer cells that often possess highly activated MAPKs because of the stimuli of their upstream signaling pathways, such as hepatocyte growth factor, FGF, and EGF signaling pathways and active Ras mutations, the phosphorylation of Ser^{68}-Twist1 may become dominant to facilitate EMT, migration, invasion, and metastasis induced by these pathways. Therefore, finding a way to increase SCP1 expression and function may counteract the effects of MAPK-stimulated Twist1 activity on cancer cell migration, invasion, and metastasis.

We also demonstrated that SCP1 physically interacts with the N terminus of Twist1 by using its amino acid residues 43–63. This result indicates that SCP1 phosphorylates Ser^{68}-Twist1 by directly binding to Twist1. Based on this finding, we reasoned that mutation or deletion of this 21-amino acid motif of SCP1 could diminish its interaction with Twist1 and also inhibit Ser^{68}-Twist1 phosphorylation. This appears to be the case, as shown by our cell-free experiments using purified SCP1 and Twist1 proteins. However, when we replaced this 21-amino acid region with an HA antibody tag, the mutant SCP1 could still interact with Twist1, as assayed by co-IP in cells (data not shown). These results suggest that SCP1 can also interact with Twist1 *in vivo* through other protein(s). At this time it is unclear whether this indirect interaction is required and/or sufficient for SCP1-mediated dephosphorylation of Ser(P)^{68}-Twist1 in living cells. This indirect interaction between SCP1 and Twist1 may be related to the components of their protein complexes. SCP1 is involved in multiple protein complexes containing REST (RE-1 silencing transcription factor), Snail, mSin3,

HDAC1, HDAC2, CoREST (a corepressor for REST), and/or RNA polymerase II (25). Twist1 also interacts with multiple components of the NuRD complex, including MTA2, RbAp46, Mi2, and HDAC2 and with Snail1 or Snail2 (12, 36). The common components of these complexes, such as Snail1 and HDAC2, may allow SCP1 to interact with Twist1 indirectly.

SCP1 has been shown to dephosphorylate and stabilize Snail1 expressed ectopically in MCF7 cells to promote Snail-induced EMT and cell migration (25). However, in our study, SCP1 is demonstrated to dephosphorylate and destabilize Twist1 expressed in MCF7 cells to inhibit Twist1-induced EMT, cell migration, and invasion. Therefore, the roles for SCP1 in breast cancer cell EMT, migration, and invasion may depend on the relative expression levels and functional contributions of Twist1 *versus* Snail1. In agreement with this notion, we found that ectopically expressed SCP1 in cancer cells such as MDA-MB-436 and Ishikawa cells with high endogenous Twist1 expression reduces Ser(P)^{68}-Twist1 and total Twist1 and also suppresses the migration and invasion of these cancer cells (data not shown). Restored expression of Twist1 and Ser(P)^{68}-Twist1 in SCP1-overexpressing MDA-MB-436 cells partially rescues the migration and invasion of these cells. MCF7 cells are ER-positive luminal epithelial breast cancer cells with very little endogenous Twist1 and Snail1 expression. When Snail1 is ectopically expressed in MCF7 cells, SCP1 can dephosphorylate and stabilize Snail1 to further enhance Snail1 function to drive EMT, cell migration, and invasion. In contrast, when Twist1 is ectopically expressed in MCF7 cells, SCP1 can dephosphorylate and destabilize Twist1 to inhibit Twist1-promoted EMT, cell migration, and invasion. Therefore, depending on the cell context, with more Snail1 or more Twist1 SCP1 might play a promoting or inhibitory role in the regulation of EMT, migration, and invasion of cancer cells. This suggests that case-specific therapeutic strategies should be considered if SCP1 is to be used as a molecular target for breast cancer treatment.

There are 518 protein kinases in the human genome, 428 of which are Ser/Thr kinases. However, there are only 147 protein phosphatase catalytic subunits and ~200 protein phosphatases. Only ~40 of the 147 protein phosphatase catalytic subunits target Ser/Thr phosphorylation sites (20, 37). Although no consensus phosphopeptide sequences could be identified for the Ser/Thr phosphatase superfamily, it has been reported that the P*X*(S/T)PP sequence is an important motif in the substrates for the enzymatic catalysis of SCP1, and the two *X*aa-Pro peptide bonds flanking the Ser/Thr residue in this motif play an essential role for SCP1 to recognize the substrate site (38, 39). Importantly, the Ser^{68} in Twist1 is located in a P*X*(S/T)PP motif for SCP1 to recognize, which is consistent with our results showing that SCP1 can dephosphorylate Ser(P)^{68}-Twist1. In many cases, a phosphatase catalytic domain can be also guided to its substrate through complex protein interactions (37). Through these multiple mechanisms, protein phosphatases, especially Ser/Thr phosphatases, are involved in multiple signaling pathways that regulate various cellular events in both normal and cancer cells. Certain Ser/Thr phosphatases, such as protein phosphatase 2A (PP2A) and PP2C, are multitask phosphatases (40, 41). This and previous studies also suggest that SCP1 is a multitask Ser/Thr phosphatase with many more, yet-to-be-defined targets and biological functions.

Author Contributions—J. X., T. Sun, J. F., and X. H. F. designed the experiments. T. Sun, J. F., T. Shen, X. L., and L. L. performed the experiments and analyzed the data. T. Sun and J. X. wrote the manuscript, and the other authors read and approved the manuscript.

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Note Added in Proof—In the original version of this paper, published as a Paper in Press on March 14, 2016, Fig. 4*B* was assembled with the wrong immunoblot for "Input-Twist1." In Fig. 5*A*, a tubulin immunoblot panel was misidentified as β-actin. These errors have been corrected and do not affect the interpretations of the results or conclusions of the article.

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