p38 Stabilizes Snail by Suppressing DYRK2-Mediated Phosphorylation That Is Required for GSK3β-βTrCP-Induced Snail Degradation

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Abstract

Snail is a key regulator of epithelial–mesenchymal transition (EMT), which is a major step in tumor metastasis. Although the induction of Snail transcription precedes EMT, posttranslational regulation, especially phosphorylation of Snail, is critical for determining Snail protein levels or stability, subcellular localization, and the ability to induce EMT. To date, several kinases are known that enhance the stability of Snail by preventing its ubiquitination; however, the molecular mechanism(s) underlying this are still unclear. Here, we identified p38 MAPK as a crucial posttranslational regulator that enhances the stability of Snail. p38 directly phosphorylated Snail at Ser107, and this effectively suppressed DYRK2-mediated Ser104 phosphorylation, which is critical for GSK3β-dependent Snail phosphorylation and βTrCP-mediated Snail ubiquitination and degradation. Importantly, functional studies and analysis of clinical samples established a crucial role for the p38–Snail axis in regulating ovarian cancer EMT and metastasis. These results indicate the potential therapeutic value of targeting the p38–Snail axis in ovarian cancer.

Significance: These findings identify p38 MAPK as a novel regulator of Snail protein stability and potential therapeutic target in ovarian cancer.

Introduction

Ovarian cancer is the leading cause of death from gynecological malignancy (1). Despite introduction of targeted therapies, survival has not significantly improved in the last decade (2). The 5-year survival for ovarian cancer is remarkably low, as this disease frequently recurs and quickly metastasizes throughout the peritoneal cavity. Therefore, a better understanding of the molecular events that contribute to tumor invasion and metastasis is crucial for developing novel treatment strategies for ovarian cancer. Most cancer-related deaths are attributable to local invasion and distant metastasis of tumor cells (3). During metastasis, epithelial cells initially lose apical–basal polarity and cell–cell contact while shifting to a mesenchymal phenotype (4). This loss of epithelial features is often accompanied by increased cell motility and expression of mesenchymal genes, a process that is collectively referred to as the epithelial–mesenchymal transition (EMT) and considered a key step during the progression of tumors toward metastasis (5, 6). Thus, EMT regulators, which likely play important roles in cancer progression, have been extensively studied. One such regulator is the zinc finger protein Snail, which induces EMT by directly repressing E-cadherin transcription during development or tumor progression (7, 8).
Snail expression is controlled at the transcriptional level by many growth factors and cytokines including HGF, TNFα, or TGFβ (9–11); however, Snail mRNA is constitutively present in many cell types, even in the absence of activation of these signaling pathway (12). Snail is an extremely unstable protein, and its subcellular level or protein stability is mainly regulated by the many different kinases. Snail phosphorylation by GSK3β, for example, negatively regulates Snail function by inducing nuclear export and ubiquitination-dependent cytosolic degradation (12–14). In contrast, other kinases positively regulate Snail function by inducing nuclear import, nuclear retention, and enhancing its stability (15–19).

To date, three kinases (ATM, Erk2, and DNA-PKcs) have been known to enhance the stability of Snail preventing its ubiquitination (17–19); however, the molecular mechanism whereby these kinases prevent Snail ubiquitination is unknown. Here, we show that p38 MAPK suppresses DYRK2-mediated prime phosphorylation required for GSK3β–βTrCP–mediated Snail ubiquitination and degradation, and the resulting stabilized Snail protein can promote EMT and tumor metastasis.

Materials and Methods

Cell culture
All cell lines used in this study, except 4T1 cells (ATCC), were obtained from the Korean Cell Line Bank, where they were characterized by DNA-fingerprinting and isozyme detection, and cultured according to ATCC instructions. All cell lines were used within 3 to 20 passages of thawing the original stocks and were tested every 3 months for mycoplasma contamination. The cell lines were maintained for no more than 3 passages between experiments. Human HEK293T, HeLa, A549, human colon cancer cell lines (HCT116, SW480, SW620, DLD-1, KM-12), and human breast cancer cell lines (MCF-7, MDA-MB-231, T47D) were cultured in DMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin and streptomycin.

Immunoprecipitation
Cells were lysed in lysis buffer: 20 mmol/L Tris pH 7.4, 2 mmol/L EDTA, 150 mmol/L sodium chloride, 1 mmol/L sodium deoxycholate, 1% Triton X-100, 10% glycerol, 2 pills protease inhibitor cocktail (Roche), mixed by vortexing and incubated 30 minutes on ice. Lysates were precleared using protein A/G beads (Santa Cruz Biotechnology), incubated with primary antibodies for 2 hours at 4°C, and then incubated with beads for overnight at 4°C with gentle mixing. Beads were then washed five times with lysis buffer and eluted with 30 µL of 2 × SDS sample buffer. Western blot analysis was then performed.

Western blot analysis
Protein samples were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were incubated with indicated primary antibodies for overnight at 4°C. After washing with TBS-T (TBS containing 0.1% Tween-20), membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (1:5,000) for 1 hour at room temperature. Blots were developed with enhanced chemiluminescence (ECL, GE Healthcare) reaction according to manufacturer’s instructions. Primary antibodies used for immunoprecipitation (IP) were as follows: Flag (Cell Signaling Technology, No. 14793; 5 µg/mL), HA (Abm G036; 5 µg/mL), Snail (Cell Signaling Technology, No. 3879; 5 µg/mL), and p38 (Cell Signaling Technology, No. 9212; 5 µg/mL).

Plasmid construction and transfection
The full-length human Snail gene was subcloned into a pDONR207 vector (Entry vector) using the Gateway Cloning System (Invitrogen) following the manufacturer’s instructions. The entry clones were converted into several destination vectors, pDEST-GFP-C, pDEST-FLAG-C, and pDEST-HA-C. Site-directed mutagenesis was performed with a QuickChange Mutagenesis Kit, according to the manufacturer’s instructions. For transient transfection, HEK293T cells were seeded in 6-well or 100-mm-diameter dish for 24 h and transfected with the indicated plasmid by using Fugene 6 transfection reagent (Roche) following manufacturer’s instruction. After 48 hours, the cells were harvested and used for Western blot analysis. Two different siRNA oligo duplexes for targeting human p38 or murine p38, respectively, were purchased from Bioneer. Transient transfection of siRNA oligo duplex was accomplished using siLentFect Reagent (Bio-Rad) following manufacturer’s instruction. For stable transfection, SKO3 cells were transfected with Flag-tar (Con), Flag-WT-Snail, or Flag-S107A-Snail expressing plasmid by using the Fugene 6 transfection reagent (Roche). After 48 hours incubation, 500 µg/mL of G418 was added to the cultures to select for G418-resistant clones. Three to four weeks later, independent colonies were picked using cloning cylinder (Sigma), subcultured, and tested for Snail expression by Western blot analysis.

Kinase library construction and yeast two-hybrid screening
The full-length human kinase genes (approximately 300 kinase clones; Supplementary Table S1) were subcloned into a pDONBR207 vector (Entry vector) using the Gateway Cloning System (Invitrogen) following the manufacturer’s instructions. The entry clones were converted into a destination vector, pDEST–GADT7, which expresses fusion protein with the GAL4 activation domain. The full-length Snail protein was produced as a fusion protein with the GAL4 DNA-binding domain in plasmid pGBK7 and used as a bait for yeast two-hybrid screening. Yeast two-hybrid screening was performed with the MATCHMAKER two-hybrid system (Clontech) following the manufacturer’s instructions. The pGBK7-Snail plasmid was transformed into the MATa strain Y187 and human kinase library-containing pDEST–GADT7 plasmids were transformed into MATa strain AH1109. When two transformants were mated to each other, diploid cells were formed that contained reporter gene. If the fusion proteins interact each other, mated cells were grown in the selection medium. A total of 1.5 × 10⁷ transformants were screened. Positive colonies were selected based on their capacity to express the markers, such as ADE2, HIS3, and LacZ. The pDEST–GADT7 plasmids were isolated from the positive colonies, and transformed into Escherichia coli to amplify, and then cDNA inserts were sequenced.
p38 Regulates Snail Protein Stability

HEK293T cells were transfected with HA-Ub, GFP-Snail (WT or mutant Snail proteins (S82A-, S104A-, and S107A-Snail)), which were expressed in pDEST-MBP vector in E. coli. The structure of DYRK2 (PDB ID: 3K2L) was used for docking as a template for docking. The reaction was carried out for 30 minutes at 30 °C. The lower chambers were filled with 500 μL of prewarmed Cell Detachment Solution, and the upper chambers were removed and washed with PBS for 3 times to rinse off the detached cells. Cells were then cultured with appropriate fresh media for 24 hours. The wound closure was observed and photographed at 0 and 16 hours, using a phase-contrast microscope with digital camera.

Docking calculations

Docking simulations were done using AUTODOCK 4.2 (20). The structure of DYRK2 (PDB ID: 3K2L) was used for docking as a target with a peptide (residues 100-SQPPSSPPS-108 of Snail) carrying either unphosphorylated or phosphorylated Ser107. The peptide was generated by using Coot (21). The AutoDockTools program (20) was used to generate the docking input files using the implemented empirical free energy function and the Lamarckian genetic algorithm. In all docking, a grid box size of 60 × 60 × 60 pixels in x, y, and z directions was built and the maps were centered in the catalytic site of the protein. All of the other docking parameters were used with the default values according to the program manual. A hundred independent docking runs were performed. The best docked conformations with the lowest binding and highest scores were found. Figures were prepared using PyMOL (22).

Migration assay

Cells were seeded into Culture-Insert (Ibidi) at 5.0 × 10^5 cells insert. After the cells were confluent, the Culture-Insert was removed and washed with PBS for 3 times to rinse off the detached cells. Cells were then cultured with appropriate fresh media for further 24 hours. The wound closure was observed and photographed at 0 and 16 hours, using a phase-contrast microscope with digital camera.

Invasion assay

Invasion assays were assessed using QCM 24-Well Cell Invasion Assay (Fluorometric) Kit (Millipore) following the manufacturer’s instruction. Cells were serum-starved for 24 hours and 2.5 × 10^5 cells in 250 μL of serum-free medium were seeded into upper chambers in the absence or presence of 50 ng/mL of EGF. The lower chambers were filled with 500 μL of appropriate media containing 20% EBS. Twenty hours after incubation, noninvaded cells/medium remaining on the upper chambers were removed by pipetting. The upper chambers were transferred into a clean well containing 225 μL of prewarmed Cell Detachment Solution, and incubated for 30 minutes at 37°C. The upper chambers were removed from the well. Seventy-five microliters of lysis buffer/dye solution (CyQuant GR Dye 1:75 with 4 μL/lysis buffer) was added into each well and incubated for 15 minutes at room temperature. Two hundred microliters of the mixture were transferred into a 96-well plate and assessed with a fluorescence plate reader using a 480/520 nm filter set.

Proliferation assay

Cells were seeded in 6-well plates at 2 × 10^4 cells/well. After incubation for 1 to 4 days, cells were trypsinized and resuspended in 1 mL of appropriate medium. The viable cells were stained with trypan blue and counted with a hemocytometer.

Mice and animal housing

Female BALB/c nude mice at 6 to 8 weeks of age were purchased from Charles River Laboratories and housed in a pathogen-free...
barrier room in Animal Care Facility at Korea Research Institute of Bioscience and Biotechnology (KRIIBB). All experiments using animals were conducted under the Institutional Animal Care and Use Committee (IACUC)-approved protocols at KRIIBB in accordance with institutional guidelines.

Xenografts studies
For peritoneal dissemination analysis of SKOV3 human ovarian cancer models, 2 × 10^6 of SKOV3 (Con), WT-Snail-expressing SKOV3 (WT-Snail), or S107A-Snail-expressing SKOV3 (S107A-Snail) cells were injected intraperitoneally into BALB/c female nude mice (n = 6 for each group). Five weeks after the injection, the mice were euthanized, and primary tumor masses in the peritoneum for ovarian cancer were excised and weighed.

Ovarian cancer tissue specimens
De-identified and paraffin-embedded human ovarian cancer tissue specimens (39 cases) were collected from 2001 to 2012 at Gyeongsang National University Hospital, Jinju, Korea. These clinical ovarian cancer tissue specimens were examined and diagnosed by pathologists at Gyeongsang National University Hospital. Tumor collections with written informed consents and analyses were approved by the Institutional Review Board at Gyeongsang National University Hospital, Jinju, Korea.

Immunohistochemistry
Three-millimeter-diameter core tissues were obtained from individual formalin-fixed and paraffin-embedded tissue, and arranged in new recipient paraffin blocks. Two tissue cores from the most representative tumor areas were analyzed. IHC was performed on 4-μm-thick paraffin sections using a Benchmark ULTRA (Ventana Medical Systems Inc.) and Optiview DAB IHC Detection Kit (Ventana Medical Systems Inc.). Polyclonal antibodies specific to Snail2 (1:750) and phospho-p38 (Santa Cruz Biotechnology, sc-166182; 1:50), and a polyclonal antibody for DYRK2 (LifeSpan, LS-B7095-50; 1:200) were used for IHC. ULTRA Cell Conditioning 1 (Ventana Medical Systems Inc.) was used (56 minutes, 37°C) for antigen retrieval for Snail and phospho-p38. Antigen retrieval was not done for DYRK2. Incubation time for primary antibodies was 32 minutes.

Expression of each proteins was examined with blindness to each IHC results. Snail, phospho-p38, and DYRK2 were expressed in nucleus, nucleus and cytoplasm, and cytoplasm, respectively. Staining intensity was scored as 0 (negative), 1 (mild), 2 (moderate), or 3 (marked). Proportional score of stained tumor cells was classified into 0 (<5%), 1 (5%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (76%–100%). Expression score was calculated by multiplying the intensity score by the proportional score (0–12; Supplementary Table S2). An expression score higher than 4 was considered high expression, if not low expression.

Statistical analysis
Quantitative data in this study are presented as means ± SD and were analyzed by a 2-tailed unpaired Student t test to compare the difference between groups. P < 0.05 was considered statistically significant. For quantification of protein stability following treatment of CHX, Snail and α-tubulin proteins detected by immunoblotting were quantified using ImageJ software. For normalization, α-tubulin expression was used as a control. GraphPad Prism version 7 and SPSS version 21 software were used in this study. All experiments were repeated at least 3 independent times. Animal studies were performed with adequate n numbers to ensure statistical evaluation. No statistical method was used to predetermine sample size. Sample size was chosen on the basis of literature in the field.

Results

p38 interacts with Snail and phosphorylates residue Ser107
To find novel Snail-interacting kinases that could influence its function, we performed yeast two-hybrid screening with home-made human kinase cDNA library (composed of approximately 300 kinase clones; Supplementary Table S1) by using human Snail as the bait. Through this screening, we identified 13 independent kinases that might interact with Snail. Four of these, namely GSK3β (12), PAK (15), PKA (23), and PKD1 (24), have already been shown to influence Snail function by direct phosphorylation. In this study, we focused on p38 MAPK, which is known to induce EMT in many cancer cell types (25–27). We first confirmed the interaction between p38 and Snail by reciprocal immunoprecipitation in HEK293T cells cotransfected with vectors encoding HA-Snail and/or Flag-p38 (Fig. 1A). Next, we confirmed the interaction between endogenous p38 and Snail in SW620 and HCT116 colon cancer cells, both of which express high levels of Snail (Fig. 1B). Finally, to verify the direct binding of p38 with Snail in vitro, a commercially available, purified recombinant p38 protein was tested for its ability to bind to HA-Snail and MBP-Snail on proteins. The results clearly showed that p38 directly interacts with Snail without the need for any other proteins (Fig. 1C).

Given the interaction between p38 and Snail, we speculated that Snail might be a direct substrate of the p38 kinase. A search for the consensus p38 phosphorylation motif (SP/TP) revealed that Snail contains 3 potential p38-phosphorylation motifs (Ser 82, 104, and 107), and these serine residues are evolutionarily conserved in other species (Fig. 1D; Supplementary Fig. S1A). To determine which serine residue could be phosphorylated by p38, we performed in vitro kinase assays using commercially available recombinant active p38 kinase and purified MBP-fused wild-type (WT) or mutant Snail proteins. We found that WT-, S82A-, and S104A-Snail (but not the S107A-Snail mutant) were phosphorylated by active p38 in vitro (Fig. 1E; Supplementary Fig. S1B). We independently confirmed these results in experiments performed with a monoclonal antibody that recognizes phospho-serine or phosphothreonine, followed by a probe (pS/TP; Supplementary Fig. S1C). We also performed in vitro kinase assays using recombinant active p38 with a synthetic peptide (residues 96-SGKGSQPPSPSSPAPSSSFS-115 of Snail) in the absence of radioactive isotope. Analysis by mass spectrometry revealed clearly phosphorylation at Ser107 of Snail by p38 kinase (Supplementary Fig. S1D). Finally, to recapitulate p38-mediated Snail phosphorylation in vivo, we cotransfected WT-Snail or S107A-Snail with p38 and MKK6 (an upstream kinase required for p38 activation) and found that WT-Snail, but not S107A-Snail, was phosphorylated in HEK293T cells with activated p38 (Fig. 1F). The p38-binding ability of S107A-Snail was not different from that of WT-Snail (Supplementary Fig. S1E). Collectively, these results suggest that p38 directly interacts with Snail and phosphorylates residue Ser107.
p38 enhances Snail protein stability by suppressing ubiquitination-dependent Snail degradation

Because the phosphorylation status of Snail is important for regulating its stability (17–19), we next investigated whether p38 could affect Snail protein stability. In several cancer cell lines that we tested, the activation level of p38 (phosphorylation of p38 kinase at Thr180/Tyr182) correlated positively with the Snail expression level (Supplementary Fig. S2A). This positive relationship between p38 activity and Snail protein expression prompted us to study the ability of p38 to upregulate Snail stability. We noticed a significant increase in HA-Snail expression when cotransfected with Flag-p38 in HEK293T cells (Fig. 1A, right). Activation of p38 markedly enhanced endogenous Snail protein levels in HEK293T, SKOV3, and HeLa cells (Fig. 2A) without affecting their mRNA-expression levels (Supplementary Fig. S2B). This effect depends on p38 kinase activity, as cotransfection of a kinase-dead (KD) p38 form with MKK6 did not affect the Snail level (Fig. 2A). RNAi-mediated depletion of p38 or inhibition of p38 activity using a p38-specific inhibitor (SB203580) resulted in decreased endogenous Snail protein level in SW620, HCT116, and 4T1 cells, which have high expression of phospho-p38 and Snail proteins (Fig. 2B and C), without affecting the level of Snail mRNA (Supplementary Fig. S2C and S2D). The level of exogenously expressed Snail was also increased by cotransfection of the p38 expression vector and enhanced further still by transiently overexpressing both p38 and MKK6 in HEK293T cells (Fig. 2D). Importantly, inhibiting proteasome function with MG132 markedly enhanced exogenous Snail expression to the same level irrespective of p38 activation (Fig. 2D), suggesting that p38 activation could enhance Snail protein expression by inhibiting proteasome-dependent Snail degradation. Performing a CHX pulse-chase analysis demonstrated that p38 activation markedly extended the half-life of the WT-Snail protein (from 60 to 120 minutes, Fig. 2E).

To explore the effects of Snail phosphorylation at Ser107 more precisely, we transiently overexpressed WT- and S107A-Snail in HEK293T cells (Fig. 1A, right). Activation of p38 markedly enhanced endogenous Snail protein levels in HEK293T, SKOV3, and HeLa cells (Fig. 2A) without affecting their mRNA-expression levels (Supplementary Fig. S2B). This effect depends on p38 kinase activity, as cotransfection of a kinase-dead (KD) p38 form with MKK6 did not affect the Snail level (Fig. 2A). RNAi-mediated depletion of p38 or inhibition of p38 activity using a p38-specific inhibitor (SB203580) resulted in decreased endogenous Snail protein level in SW620, HCT116, and 4T1 cells, which have high expression of phospho-p38 and Snail proteins (Fig. 2B and C), without affecting the level of Snail mRNA (Supplementary Fig. S2C and S2D). The level of exogenously expressed Snail was also increased by cotransfection of the p38 expression vector and enhanced further still by transiently overexpressing both p38 and MKK6 in HEK293T cells (Fig. 2D). Importantly, inhibiting proteasome function with MG132 markedly enhanced exogenous Snail expression to the same level irrespective of p38 activation (Fig. 2D), suggesting that p38 activation could enhance Snail protein expression by inhibiting proteasome-dependent Snail degradation. Performing a CHX pulse-chase analysis demonstrated that p38 activation markedly extended the half-life of the WT-Snail protein (from 60 to 120 minutes, Fig. 2E).
Figure 2.
p38 enhances Snail protein stability by suppressing ubiquitination-dependent Snail degradation. A, The WT or KD form of p38 was cotransfected with MKK6 into HEK293T, SKOV3, or HeLa cells. Cell lysates were immunoblotted with the indicated antibodies. B, Immunoblot analysis in p38-depleted SW620, HCT116, or 4T1 cells. C, Immunoblot analysis in SB203580 (SB)-treated SW620, HCT116, or 4T1 cells. D, HA-Snail was cotransfected with plasmids expressing p38 or p38 and MKK6 into HEK293T cells (top left), and then treated with 10 μmol/L MG132 for 12 hours (bottom left). Cell lysates were immunoblotted with the indicated antibodies. The data are representative of three independent experiments and relative Snail levels were quantified using ImageJ software (right). For normalization, α-tubulin expression was used as a control. E, HA-WT-Snail or HA-S107A-Snail was cotransfected with plasmids expressing p38 and MKK6 into HEK293T cells in the presence of CHX (100 μg/mL) for the indicated times. Cell lysates were immunoblotted by antibodies as indicated (top). The data were quantified using ImageJ software (bottom). For normalization, α-tubulin expression was used as a control. F, HA-WT-Snail or HA-S107A-Snail was cotransfected with plasmids expressing the WT or KD form of p38 and MKK6 into HEK293T cells, and then the cells were treated with 10 μmol/L MG132 for 12 hours. Cell lysates were immunoblotted with the indicated antibodies (top). The data are representative of three independent experiments and relative Snail levels were quantified using ImageJ software (bottom). For normalization, α-tubulin expression was used as a control. G, GFP-WT-Snail or GFP-S107A-Snail was cotransfected with plasmids expressing HA-ubiquitin, p38, and MKK6 as indicated into HEK293T cells, and then the cells were treated with 10 μmol/L MG132 for 12 hours. Cell lysates were immunoprecipitated using an anti-GFP antibody and then analyzed by immunoblotting using an anti-HA tag antibody.
p38 regulates Snail protein stability

S107A-Snail (Fig. 2E). We also found that the degree of ubiquitination of S107A-Snail was significantly increased compared with that of WT-Snail (Supplementary Fig. S3C). Furthermore, p38 activation significantly increased the WT-Snail protein levels and decreased ubiquitination, but the expression and ubiquitination of S107A-Snail did not change significantly (Fig. 2F and G). Taken together, these results suggest that p38-mediated Ser107 phosphorylation leads to Snail stabilization by suppressing ubiquitination-dependent Snail degradation.

Phosphorylation of Snail at Ser107 by p38 inhibits DYRK2-mediated Snail phosphorylation at Ser104, which acts as a prime phosphorylation for GSK3β

Snail can undergo βTrCP-mediated ubiquitination and proteasomal degradation after GSK3β-dependent phosphorylation at Ser96/100 (12-14). Considering that p38-mediated phosphorylation of Snail at Ser107 decreased Snail ubiquitination (Fig. 2G) and that S107A-Snail showed increased ubiquitination compared with WT-Snail (Supplementary Fig. S3C), we next investigated whether p38 could impair the interaction between GSK3β and Snail, which is prerequisite for βTrCP-mediated Snail ubiquitination. We found that WT-Snail bound less to GSK3β than S107A-Snail (Fig. 3A) and that p38 activation markedly reduced GSK3β binding to WT-Snail, although S107A-Snail showed stronger binding regardless of p38 activation (Fig. 3B). In many cases, the association between GSK3β and its substrates requires the substrate to be phosphorylated at a priming site (28, 29), and in case of Snail, GSK3β can phosphorylate Snail at Ser96/100 only if Ser104 has already been phosphorylated by DYRK2 (30). We also found that the degree of binding of WT-Snail to GSK3β was strikingly increased by DYRK2, but decreased when p38 was activated (Fig. 3C). However, the interaction between Snail and DYRK2 was maintained even if the Snail protein was phosphorylated by p38 kinase (Fig. 3D). These results suggest that p38-mediated Ser107 phosphorylation of Snail likely inhibits the GSK3β–Snail interaction by preventing DYRK2-mediated prime phosphorylation of Snail at Ser104.

Because no site-specific phospho-antibody for Ser104 is available, to examine whether Ser107 phosphorylation of Snail by p38 could directly inhibit DYRK2-mediated Ser104 phosphorylation, we first performed an in vitro kinase assay with active p38 and WT-Snail in the presence of unlabeled cold ATP and then added a p38-specific inhibitor and DYRK2 with [γ-32P]–ATP (Fig. 3E). DYRK2 phosphorylated Snail at residue Ser104 (Fig. 3F), as also shown previously (30). The p38 inhibitor effectively suppressed its auto-phosphorylation, as well as Snail phosphorylation by p38 kinase (Fig. 3G). Although DYRK2-mediated phosphorylated Snail was not inhibited by the p38 inhibitor, pre-incubation with p38 completely reduced DYRK2-mediated Snail phosphorylation (Fig. 3H). These results, along with the results of Fig. 3D, suggest that the Snail protein phosphorylated by p38 kinase can interact with DYRK2 but not phosphorylated by DYRK2. Furthermore, the results of docking simulations using DYRK2 (PDB ID: 3K2L) with a peptide (residues 100-SPQPSPPSPP-108 of Snail) carrying either unphosphorylated or phosphorylated Ser107 predicted that the unphosphorylated peptide binds closely to the active site of DYRK2, but the phospho-Ser107-containing peptide cannot access the active site of DYRK2 due to steric hindrance by phosphorylated Ser107 residue (Fig. 3I). Finally, we found that DYRK2 markedly reduced WT-Snail expression, but this phenomenon was not observed in the presence of active p38 (Fig. 3J). In contrast, the expression of S107A-Snail was evidently reduced by DYRK2 but could not be recovered by active p38 expression (Fig. 3J). All of these results suggest that Ser107 phosphorylation of Snail by p38 directly inhibits DYRK2-mediated Ser104 phosphorylation, which is critical for GSK3β–βTrCP-induced Snail degradation, resulting in enhanced Snail expression.

It has been known that GSK3β could be inactivated by phosphorylation at Thr390 (human)/Ser389 (mouse) by p38 kinase (31). To verify whether p38 could directly phosphorylate and inactivate GSK3β, we checked the phosphorylation level of GSK3β and the expression level of the target protein for GSK3β when p38 is activated in HEK293T cells, but could not find any difference (Supplementary Fig. S4). These results suggest that p38 does not affect the activity of GSK3β under our experimental condition.

Ser107 phosphorylation is required for Snail-promoted EMT and metastasis in the SKOV3 ovarian cancer cell line

To understand the role of Ser107 phosphorylation in Snail function, we next examined whether this phosphorylation might contribute to Snail-promoted EMT. For this purpose, we used SKOV3 human ovarian epithelial cancer cells because exogenously expressed WT-Snail is more stable in this cell line and has a longer protein half-life, compared with S107A-Snail (Supplementary Fig. S3A and S3B). Interestingly, among 3 SKOV3 cell lines expressing Flag-tag (Con), WT-Snail, or S107A-Snail, the morphology of WT-Snail-expressing cells was distinct from that of the control and S107A-Snail–expressing cells. Confocal microscopy analysis of phalloidin-stained cells confirmed the presence of filopodia, lamellipodia, and microspikes in WT-Snail–expressing SKOV3 cells, whereas control cells and S107A-Snail–expressing cells exhibited less staining and no cellular protrusions (Fig. 4A). These results prompted us to measure the expression levels of EMT marker genes in these cells. WT-Snail protein was readily detected, whereas the S107A-Snail protein was barely detected because of its instability, even though both cell lines expressed comparable amounts of Snail protein in the presence of MG132 (Fig. 4B). We also found that depletion of p38 expression significantly reduced WT-Snail but did not alter S107A-Snail expression (Supplementary Fig. S5A), in contrast depletion of DYRK2 expression could only increase S107A-Snail expression (Supplementary Fig. S5B). These results suggest that the expression level of S107A-Snail protein is reduced by DYRK2-mediated phosphorylation of Snail and p38 can reverse this effect on WT-Snail. As expected, WT-Snail expression inhibited the expression of epithelial markers (including E-cadherin and Occludin), but promoted the expression of mesenchymal markers (including N-cadherin, fibronectin, and vimentin) in SKOV3 cells (Fig. 4B). However, S107A-Snail expression failed to regulate these markers and only inhibited E-cadherin expression (Fig. 4B). These results suggest that Ser107 phosphorylation is required for Snail-dependent EMT promotion. Next, we investigated whether Snail expression could alter the migratory properties of SKOV3 cells by conducting a wound-healing assay. After 16 hours, we found that when compared with control and S107A-Snail–expressing cells, an increased number of WT-Snail–expressing SKOV3 cells had migrated into the
Figure 3.
Phosphorylation of Snail at Ser107 by p38 inhibits DYRK2-mediated Snail phosphorylation at Ser104, which acts as a prime phosphorylation for GSK3β.

A, Flag-WT-Snail or Flag-S107A-Snail was cotransfected with plasmids expressing HA-GSK3β into HEK293T cells, and then the cells were treated with 10 μmol/L MG132 for 12 hours. Cell lysates were immunoprecipitated using an anti-HA antibody and then analyzed by immunoblotting using an anti-Flag antibody.

B, Flag-WT-Snail or Flag-S107A-Snail was cotransfected with plasmids expressing HA-GSK3β, p38, and MKK6 (as indicated) into HEK293T cells, and then the cells were treated with 10 μmol/L MG132 for 12 hours. Cell lysates were immunoprecipitated using an anti-HA antibody and then analyzed by immunoblotting using an anti-Flag antibody.

C, GFP-WT-Snail was cotransfected with plasmids expressing HA-GSK3β, Flag-DYRK2, p38, and MKK6 (as indicated) into HEK293T cells, and then the cells were treated with 10 μmol/L MG132 for 12 hours. Cell lysates were immunoprecipitated using an anti-HA antibody and then analyzed by immunoblotting with an anti-GFP antibody. (Continued on the following page.)
scratch wound (Fig. 4C). Similarly, in an in vitro invasion assay, we detected a meaningful increase in the number of invasive WT-Snail–expressing SKOV3 cells, relative to control and S107A-Snail–expressing cells (Fig. 4D). In addition, WT-Snail–expressing SKOV3 cells showed a slightly decreased growth rate under the same growth conditions (Fig. 4E), indicating that the increased migration and invasion observed with WT-Snail expression is independent of the growth rate. Furthermore, depletion of p38 expression significantly inhibited the migration ability of WT-Snail–expressing cells, but not that of S107A-Snail–expressing cells, in contrast depletion of DYRK2 expression could only increase the migration ability of S107A-Snail–expressing cells (Supplementary Fig. S5C). These results suggest that the migration ability of S107A-Snail–expressing SKOV3 cells is inhibited by DYRK2-mediated phosphorylation of Snail and p38 can reverse this effect on WT-Snail.

We also investigated whether Ser107 phosphorylation in Snail is critical for the metastasis of ovarian cancer. Ovarian cancer primarily metastasizes to the peritoneum and rarely migrates to distant sites. The resulting peritoneal implants are characterized by the adhesion and invasion of tumor cells into the peritoneum, leading to peritoneal dissemination (32). Therefore, to analyze the in vivo roles of Ser107 phosphorylation of Snail in the peritoneal dissemination of ovarian cancer cells, female BALB/c nude mice were injected intraperitoneally with control SKOV3 (Con), WT-Snail–expressing SKOV3 (WT-Snail), or S107A-Snail–expressing SKOV3 (S107A-Snail) cells, after which, the tumors were allowed to grow for 5 weeks. After 5 weeks, the peritoneal surface tumor burden was significantly higher (P < 0.05) in mice that had received SKOV3 WT-Snail cells (1.22 ± 0.17 g) versus control cells (0.53 ± 0.12 g), but not in mice administered SKOV3 S107A-Snail cells (0.31 ± 0.08 g; Fig. 4F and G). These results indicate that Ser107 phosphorylation of Snail is critical for enhancing the metastatic potential of ovarian cancer cells.

EGF can activate MAPK pathways (such as p38 MAPK) and regulate gene expression to promote EMT and cancer metastasis (33–35). To examine whether EGF can enhance Snail stability and whether p38-mediated Ser107 phosphorylation is important for EGFr-induced increased Snail stability, we treated SKOV3 cell lines stably expressing either WT-Snail or S107A-Snail with EGF. Under the serum-starved condition, we observed similar, low-level basal expression levels of WT-Snail and S107A-Snail in each cell line; however, EGF treatment significantly increased WT-Snail protein levels and inhibiting p38 activity suppressed this effect, whereas S107A-Snail protein levels were not changed regardless of EGF treatment (Fig. 5A). These results suggest that activation of EGF signaling pathways stabilizes Snail protein expression by inducing p38-mediated Snail phosphorylation at Ser107. We next investigated the role of Snail Ser107 phosphorylation in EGF-induced cell migration and invasion. The migration and invasion abilities of EGF-treated S107A-Snail-expressing cells were not significantly different when compared with control cells, whereas WT-Snail–expressing cells showed robustly increased migration and invasion abilities (Fig. 5B and C). Together, these results suggest that Ser107 phosphorylation play an important role in mediating EGF-induced and Snail-promoted cancer cell migration and invasion.

Activation of p38 is the main cause of increased Snail expression in patients with human ovarian cancer.

Snail expression has been shown to be highly correlated with both the tumor stage and metastatic potential in human ovarian cancer (36–38). To verify whether p38 activation plays an important role in increasing the Snail expression in patients with ovarian cancer, we performed tissue microarray analysis of 39 ovarian cancer tissue specimens and observed a strong positive correlation between p38 activity (i.e., the phospho-p38 level) and the Snail protein expression. We found that 86% of the patient samples with high Snail expression also showed high p-p38 levels and that all patient samples with low Snail expression showed low p-p38 levels (Fig. 6A). However, we did not find any inverse correlation between DYRK2 and Snail expression. DYRK2 expression was high in 9 of 11 patients (82%) with low Snail expression and in 26 of 28 patients (93%) with high Snail expression (Fig. 6A). Interestingly, we observed that even though DYRK2 was highly expressed, all patient samples with high p-p38 levels showed high Snail expression and 69% of patient samples with low p-p38 levels showed low Snail expression (Fig. 6B). We also found that in patients with high DYRK2 expression, the level of Snail expression was higher in patients with high p-p38 levels than in patients with low p-p38 levels (Fig. 6C; Supplementary Table S2). All of these results indicate that activated p38 can enhance Snail protein levels in patients with ovarian cancer by suppressing DYRK2-mediated negative regulation of Snail, which is consistent with the results we have shown earlier.

In conclusion, our study reveals a functionally important posttranslational control mechanism for the EMT master regulator Snail. Our results suggest a model in which DYRK2 and GSK3β
are strongly activated in normal or early-stage tumor cells, where they effectively degrade Snail through βTrCP-mediated ubiquitination, thus suppressing EMT. In contrast, p38 MAPK is highly activated in malignant tumor cells and activated p38 can suppress DYRK2-mediated prime phosphorylation required for GSK3β-βTrCP-mediated Snail ubiquitination and degradation, where

Figure 4.
Ser107 phosphorylation is required for Snail-promoted EMT and metastasis in the SKOV3 ovarian cancer cell line. A, Morphologic changes of SKOV3 cells lines expressing Flag-tag (Con), WT-Snail, or S107A-Snail were visualized by confocal microscopy after staining with TRITC-conjugated phalloidin. B, Expression levels of EMT marker proteins or mRNAs in SKOV3 cell lines expressing Flag-tag (Con), WT-Snail, or S107A-Snail were analyzed by immunoblotting or RT-PCR analysis. C, Flag-tag- (Con), WT-Snail-, or S107A-Snail-expressing SKOV3 cells were analyzed in wound-healing assays by visualizing wound closure via phase-contrast microscopy (top). Wound areas were measured using WimScratch software (Wimasis). The data shown represent the percentage of the wound area and are expressed as the means ± SD of three individual experiments (bottom). *, P < 0.05 as determined by t test. ns, not significant. D, Flag-tag- (Con), WT-Snail-, or S107A-Snail-expressing SKOV3 cells were seeded onto Matrigel matrix-coated top chambers, and the fold-changes of invading cells were measured after 24 hours. The data shown are expressed as the means ± SD of three individual experiments, each performed in triplicate. **, P < 0.01 as determined by t test. E, The indicated cells were seeded in a 6-well plate at a concentration of 2 x 10⁴ cells per well. After incubation for 1 to 4 days, the viable cells were counted with a hemocytometer after trypan blue staining. F, In vivo roles of Snail phosphorylation at residue Ser107. Flag-tag- (Con), WT-Snail-, or S107A-Snail-expressing SKOV3 cells (2 x 10⁶) were suspended in 200 µL PBS and intraperitoneally injected into BALB/c nude mice (6 mice/group). After 5 weeks, tumor burdens and ascites formation were estimated. Arrows, disseminated tumors. G, At autopsy, tumors were excised and weighed. The data are shown as the means ± SD of 6 mice/group. Statistical significances were determined by t test.
the resulting high levels of Snail expression can promote EMT and tumor metastasis (Fig. 6D).

**Discussion**

Similar to other signaling proteins, the activities of transcription factors are commonly regulated by phosphorylation in response to various cellular signals. In the case of Snail, phosphorylation can control the protein stability and function in various ways. Because Snail is known to be negatively regulated by ubiquitination-dependent proteasomal degradation in the cytoplasm, one way to positively increase Snail stability might be to promote its delivery into the nucleus. PAK1 phosphorylates Snail at Ser246, which is located in the zinc finger domain that is critical for its nuclear localization (39), which was found to enhance its protein-expression level and function (15). Although the protein machinery used for nuclear Snail import is not precisely known, Snail phosphorylation by PAK1 probably increases the interaction of Snail with the protein that is important for its nuclear trafficking. A second way of increasing Snail stability might be to allow it to stay in the nucleus longer. Lats2 phosphorylates Snail at Thr203 in the nucleus and prevents its nuclear export, thereby leading to its stabilization (16). Although the precise molecular mechanism explaining how Snail phosphorylation at Thr203 causes nuclear retention is unclear, Lats2-mediated Thr203 phosphorylation might alter Snail binding with the nuclear export machinery or with some resident nuclear proteins. A third way might be to prevent Snail ubiquitination. Phosphorylation of Snail at Ser100 by ATM and DNA-PKcs decreases Snail ubiquitination or reduces Snail interaction with GSK3β, which is critical for Snail degradation, thus enhances its protein stability (17, 19).
Figure 6.
The Snail protein expression level correlates positively with the phospho-p38 protein level in patients with ovarian cancer. A, Left, representative IHC images of phospho-p38 and Snail in ovarian tumors. Scale bars, 50 μm. Right, correlation of the Snail expression level with the phospho-p38 or DYRK2 levels, as determined using an ovarian tumor tissue microarray. Statistical significances were determined by Pearson $\chi^2$ test and Fisher exact test. B, Top, representative IHC images of DYRK2, phospho-p38, and Snail in ovarian tumors with high DYRK2 expression. Scale bars, 50 μm. Bottom, correlation of the Snail expression level with phospho-p38 levels in patients with ovarian cancer who had high DYRK2 expression. Statistical significances were determined by Pearson $\chi^2$ test and Fisher exact test. C, Scatter dot plots comparing Snail and phospho-p38 levels in patients with ovarian cancer who had high DYRK2 expression. Statistical significances were determined by $t$ test. D, Proposed model to illustrate how stabilization of Snail by p38 may lead to EMT.

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Erk2-mediated Ser82/Ser104 phosphorylation of Snail also protected it from ubiquitination and subsequent proteasomal degradation (18). However, none of these reports explained exactly how phosphorylation of Snail inhibits ubiquitination.

In this study, we showed that the precise molecular mechanism by which phosphorylation can inhibit Snail ubiquitination and thereby enhance its stability: p38 MAPK enhances Snail stability by suppressing the DYRK2-mediated prime phosphorylation for GSK3β, which is critical for βTrCP-mediated Snail ubiquitination and subsequent degradation. Recent studies have suggested that GSK3β-dependent phosphorylation of Snail is also crucial for SPSB3- and FBW7-mediated Snail ubiquitination and degradation (40, 41). These results suggest that p38-mediated Snail phosphorylation may also inhibit ubiquitination of Snail by these E3 ubiquitin ligases.

Besides phosphorylation, other posttranslational modifications also can control Snail stability. For example, jasplakinolide-like 2 (LOXL2) modifies Snail at Lys98/Lys137 and leads to increased protein stability by preventing Snail ubiquitination and its interaction with GSK3β (42). In addition, O-GlcNAc modification of Snail at Ser112 increases Snail protein stability by blocking Snail ubiquitination and its interaction with GSK3β (43). Recently, it was shown that A20-mediated monoubiquitination of Snail at Lys206/Lys234/Lys235 can increase Snail protein stability by reducing the binding affinity of Snail for GSK3β (44). Interestingly, most posttranslational modifications that can block Snail ubiquitination (phosphorylation at Ser100 by DNA-PKcs and ATM, phosphorylation at Ser104 by Erk2, jasplakinolide at Lys98, and O-GlcNAc modification at Ser112) occur near the phosphorylation sites for DYRK2 and GSK3β. These findings, combined with the results of this study, suggest that additional studies should be performed to determine whether these various modifications could inhibit DYRK2-mediated prime phosphorylation for GSK3β.

In this study, we showed the precise molecular mechanism by which p38 promotes the EMT and metastasis of tumor cells by regulating the stability of the Snail. Our study not only reveals a critical mechanism underlying p38-induced cancer metastasis, but also has important implications in the development of treatment strategies for metastatic cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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p38 Stabilizes Snail by Suppressing DYRK2-Mediated Phosphorylation That Is Required for GSK3 β-βTrCP–Induced Snail Degradation


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