Cancer Cell
Article

Stabilization of Snail by NF-κB Is Required for Inflammation-Induced Cell Migration and Invasion

Yadi Wu,[1,4] Jiong Deng,[5] Piotr G. Rychahou,[2] Suimin Qiu,[3,4] B. Mark Evers,[2,4] and Binhua P. Zhou[1,4,*]
[1]Department of Pharmacology and Toxicology
[2]Department of Surgery
[3]Department of Pathology
[4]Sealy Center for Cancer Cell Biology
The University of Texas Medical Branch, Galveston, TX 77555, USA
[5]Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA
*Correspondence: bpzhou@utmb.edu
DOI 10.1016/j.ccr.2009.03.016

SUMMARY

The increased motility and invasiveness of tumor cells are reminiscent of epithelial-mesenchymal transition (EMT), which occurs during embryonic development, wound healing, and metastasis. In this study, we found that Snail is stabilized by the inflammatory cytokine TNFα through the activation of the NF-κB pathway. We demonstrated that NF-κB is required for the induction of COP9 signalosome 2 (CSN2), which, in turn, blocks the ubiquitination and degradation of Snail. Furthermore, we showed that the expression of Snail correlated with the activation of NF-κB in cancer cell lines and metastatic tumor samples. Knockdown of Snail expression inhibited cell migration and invasion induced by inflammatory cytokines and suppressed inflammation-mediated breast cancer metastasis. Our study provides a plausible mechanism for inflammation-induced metastasis.

INTRODUCTION

It has become increasingly clear that chronic inflammation is tightly correlated with tumorigenesis, as cancer has been viewed as "a wound that never heals" (Coussens and Werb, 2002; Jackson and Evers, 2006; Karin and Greten, 2005). Mounting evidence from various clinical and experimental studies has demonstrated that the inflammatory tumor microenvironment plays a pivotal role not only in tumor development but also in metastasis. The inflammatory tumor microenvironment evolves as tumors grow; this includes the infiltration of immune cells and the activation of the inflammatory response. Inflammatory cells, particularly tumor-associated macrophages (TAMs), are usually found at the invasive front (the tumor-host interface) of more advanced tumors (Condeelis and Pollard, 2006). They facilitate angiogenesis, extracellular matrix breakdown, and tissue remodeling, and thus promote tumor cell motility. These TAMs also secrete such proinflammatory cytokines as tumor necrosis factor-α (TNFα), to activate the major inflammatory response NF-κB pathway, which facilitates both tumor development and metastatic progression (Karin, 2006; Karin and Greten, 2005).

The increased motility and invasiveness of tumor cells are reminiscent of the events at EMT, which is characteristic of embryonic development, tissue remodeling, and wound healing (Nieto, 2002; Peinado et al., 2007; Thiery and Sleeman, 2006). In the EMT process, epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility (Thiery and Sleeman, 2006). A hallmark of EMT is the loss of E-cadherin expression. Loss of E-cadherin expression is often correlated with the tumor grade and stage (Cowin et al., 2005). Under physiological conditions, EMT takes place at the edge of injury during wound healing (Neilson, 2006).

SIGNIFICANCE

A major challenge in cancer research is to identify the extrinsic signals and intrinsic factors that initiate metastasis. In this study, we found that the informatory cytokine TNFα dramatically enhanced the protein stabilization of Snail through NF-κB mediated CSN2 induction. We showed that the expression of Snail correlated with the activation of NF-κB and that knockdown of Snail expression significantly inhibited cell migration and invasion induced by inflammation. Our finding demonstrates that TNFα-mediated Snail stabilization plays critical role in cell migration, invasion, and metastasis. Our study not only reveals a critical mechanism underlying inflammation-induced metastasis but also has important implications in the development of treatment strategies for metastatic cancers.

Similarly, this process occurs at the invasive front of many metastatic cancers (Christofori, 2006; Franci et al., 2006). These observations suggest that the migratory and invasive ability of tumor cells at the invasive front are initiated and propelled by an inflammatory microenvironment through the induction of EMT. Several transcription factors have been implicated in the control of EMT, including Snail/Slug, Twist, Goosecoid, δEF1/ZEB1, SIP1, and E12/E47 (Hartwell et al., 2006; Nieto, 2002; Yang et al., 2004). Snail was identified in *Drosophila* as a suppressor of the transcription of *shotgun* (an E-cadherin homolog) in controlling the formation of the mesoderm and neural crest (Nieto, 2002; Thiery and Sleeman, 2006). The absence of Snail is lethal because of severe defects at the gastrula stage during development (Carver et al., 2001). Expression of Snail represses expression of E-cadherin and induces EMT in MDCK and breast cancer cells, indicating that Snail plays a fundamental role in EMT and breast cancer metastasis (Batlle et al., 2000; Cano et al., 2000; Zhou et al., 2004). In addition, overexpression of Snail correlates with tumor grade, nodal metastasis, and tumor recurrence and predicts a poor outcome in patients with various cancers (Blanco et al., 2002; Cheng et al., 2001; Moody et al., 2005; Zhou et al., 2004). Furthermore, expression of stromal matrix metalloproteinase (MMP3) stimulates expression of Snail through the increased cellular reactive oxygen species (Radisky et al., 2005). This interesting discovery highlights the importance of the microenvironment in regulating Snail and in the initiation of EMT at metastasis. We recently showed that Snail is highly unstable, with a short half-life, and is regulated by protein stability and cellular location though GSK-3β-mediated phosphorylation (Zhou et al., 2004). However, it is unclear what extrinsic signals regulated the activity of Snail and the induction of EMT at the tumor invasive front. Here, we examine the regulation of Snail and its role in cancer cell migration, invasion, and metastasis mediated by inflammation.

RESULTS

TNFα Is the Major Inflammatory Cytokine to Induce Snail Stabilization

Because EMT occurs at the edges of the wound during healing and at the invasive front of metastatic cancers (Christofori, 2006; Franci et al., 2006; Neilson, 2006), both processes are influenced by stimuli that emanate from the inflammatory microenvironment, and because Snail is a major transcription factor for EMT induction (Nieto, 2002; Thiery and Sleeman, 2006; Zhou and Hung, 2005), we reasoned that tumor cell migration and invasion at the tumor-host boundary are induced by inflammation through Snail-mediated EMT induction. To test this hypothesis, we first examined the invasive ability of 18 cancer cell lines from breast, prostate, and colon cancers in response to macrophage-conditioned medium. We found that the invasive ability for the majority of these cancer cell lines (15 of 18 cell lines) was dramatically increased, compared with cells cultured in regular medium (Figure 1A; see Figure S1 available online; data not shown). Similar findings were also obtained when these cancer cells were directly cocultured with macrophages (data not shown). Interestingly, stable expression of Snail in two nonmetastatic breast cancer cell lines (MCF7 and T47D, both of which contain little endogenous Snail) greatly increased the invasive ability of these cells (Figure 1A), indicating that Snail is a critical molecule for mediating inflammatory cytokine-induced invasion. Furthermore, when MCF7 and HEK293 GFP-Snail stable transfectants were cultured with macrophage-conditioned medium, cell detachment and acquisition of an EMT-like morphology were noted (Figure 1B). Surprisingly, the intensity of the GFP-Snail in these cells was dramatically elevated (Figure 1B). We previously demonstrated that the half-life of Snail is about 25 min and is mainly regulated through β-Trcp-mediated proteasome degradation (Zhou et al., 2004). The enhanced intensity of GFP-Snail, after culture with macrophage-conditioned medium, suggested that unknown cytokines from the conditioned medium induced protein stabilization of Snail. Of the many inflammatory cytokines that macrophages secrete, we tested the major cytokines—TNFα, IL-1β, IL-2, IL-6, and IFNγ—for their ability to stabilize Snail. When Snail/HEK293 cells were treated with different cytokines for 6 hr, we found that TNFα and proteasome inhibitor MG-132 induced the stabilization of Snail to a similar level (Figure 1C, lane 2 versus lane 7). Similar results were also found in Snail/MCF7, Snail/T47D, and Snail/ZR75 cells (Figure S2A). The cytokine-induced Snail stabilization appears to correlate with NF-κB activation (Figures S2B–S2D). IL-1β also induced Snail stabilization in Snail/MCF7 cells, but this effect depended on cell types and IL-1β concentration (Figure S2A and S2B). We also combined the ineffective cytokines with TNFα and found no synergistic effect between TNFα and the other cytokines (Figure 1C), indicating that the effect on Snail stabilization by macrophage-conditioned medium is mainly mediated by TNFα. To further confirm this observation, we treated four different Snail-expressing stable cell lines with TNFα and found that TNFα induced the stabilization of Snail to a similar level as did the proteasome inhibitor MG-132 (Figure 1D). Moreover, we treated several cancer cell lines with TNFα and found that TNFα also induced the stabilization of endogenous Snail in these cells (Figure 1E). Interestingly, both endogenous and exogenous Snail induced by TNFα migrated faster on an SDS-PAGE gel than did Snail induced by MG-132 (Figures 1D and 1E), suggesting that the mechanism of Snail stabilization appears to be different with these two agents. To determine whether the different migrating bands represent different phosphorylated states of Snail, we immunoprecipitated Snail and treated it with protein phosphatase (Figure 1F). Similar to the findings of our previous report, Snail stabilized by MG-132 was in a phosphorylated form. However, Snail stabilized by TNFα remained in a nonphosphorylated form. We next measured the time course for Snail stabilization and found that the level of Snail was increased after 1 hr of TNFα stimulation and reached a maximum at about 4 hr (Figure 1G). However, the mRNA level of Snail did not demonstrate a significant elevation within 6 hr of TNFα treatment in MDA-MB231 and Snail/HEK293 cells (Figure 1H; data not shown). Thus, the initial and rapid induction of Snail suggested that the elevation of Snail by TNFα was mainly due to protein stabilization. Furthermore, the TNFα-stabilized Snail is functionally important for cell migration and invasion, because the stabilization of Snail in Snail/MCF7 cells by TNFα enhanced migration and invasion of these cells (Figure 1A; Figures S1 and S3). Collectively, these results clearly indicate that the inflammatory cytokine TNFα induces the stabilization of Snail in a nonphosphorylated, functional form and thus enhances cell migration and invasion.

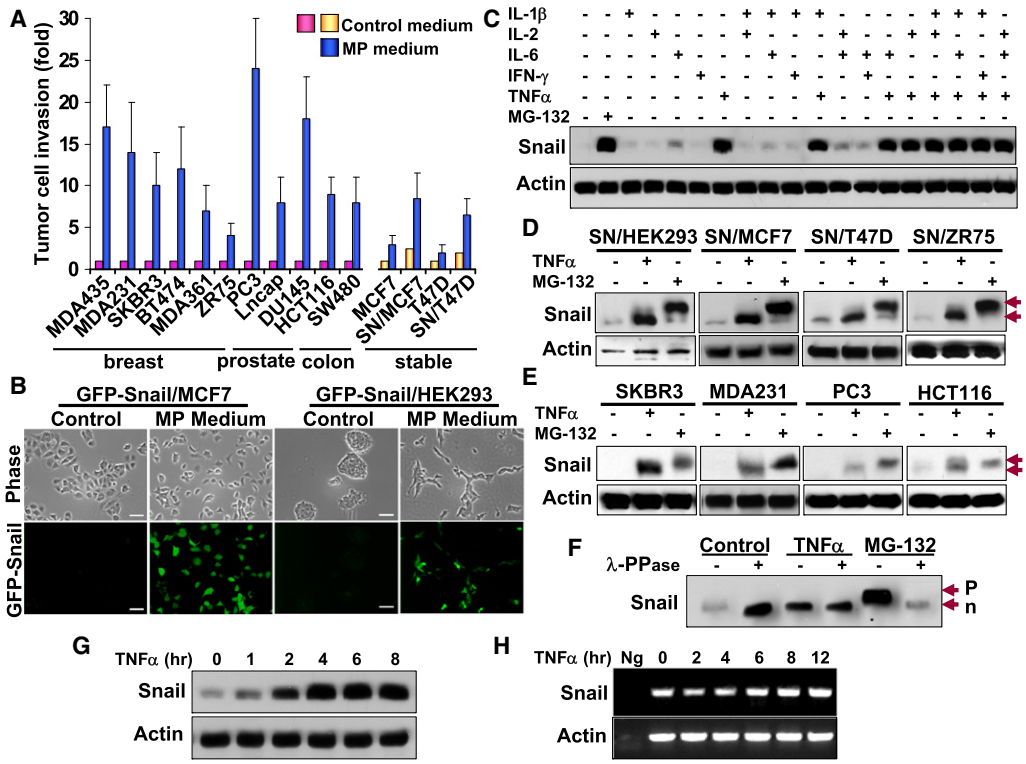

Figure 1. The Inflammatory Cytokine TNFα Induced Cell Migration and Invasion via Protein Stabilization of Snail

(A) The invasive ability of various cancer cell lines was examined by an invasion assay with regular culture medium (control medium) or macrophage-conditioned medium (MP medium), as described in Experimental Procedures. Error bars show standard deviation of three independent experiments in duplicate.

(B) MCF7 and HEK293 GFP-Snail stable cell lines were cultured with control or MP medium overnight; the morphology and the intensity of GFP were examined under a fluorescent microscope. Scale bar, 100 μm.

(C) Snail/HEK293 cells were treated with different cytokines (10 ng/ml for each) for 6 hr, and Snail was analyzed by western blotting.

(D) HEK293 and breast cancer MCF7, T47D, and ZR75 Snail stable cells were treated with either TNFα or MG-132 (10 μM) for 6 hr, and Snail was separated on 14% SDS-PAGE and examined by western blotting.

(E) Cells were treated with TNFα or MG-132, for 6 hr and endogenous Snail was analyzed by western blotting as described in (D).

(F) Snails were immunoprecipitated from Snail/HEK293 cells, and the immunocomplex was then incubated with or without λ-phosphatase for 30 min and analyzed by western blotting. p and n indicate phosphorylated and unphosphorylated Snail, respectively.

(G) Snail from Snail/HEK293 cells treated with TNFα for different time intervals was analyzed by western blotting.

(H) mRNA from MDA-MB231, treated with TNFα for different time intervals, was analyzed by RT-PCR. A reaction without template served as a negative control (Ng).

TNFα-Mediated Snail Stabilization Is Dependent on NF-κB Activation

Previously, we demonstrated that GSK-3β is a major kinase that phosphorylates Snail and induces the protein degradation of Snail (Yook et al., 2005; Zhou et al., 2004). To examine whether the stabilization of Snail by TNFα is mediated by regulation of GSK-3β activity, we treated Snail/HEK293 cells with the PI3K inhibitor LY294002 to activate GSK-3β prior to TNFα treatment (loss of GSK-3β phosphorylation at Serine 9 enhances GSK-3β activity). We found that TNFα induced stabilization of Snail regardless of the phosphorylated status of GSK-3β (Figure 2A), suggesting that TNFα-mediated Snail stabilization is independent of GSK-3β phosphorylation. To further investigate our findings, we knocked down the expression of GSK-3β in Snail/HEK293 cells using specific GSK-3β siRNA (Figure 2B). Down-regulation of GSK-3β partially elevated the level of Snail (Figure 2B, lane 2 versus lane 1). However, this down-regulation did not affect TNFα-mediated Snail stabilization (Figure 2B, lane 3 versus lane 4). Similarly, when Snail was expressed in *GSK-3β*$^{-/-}$ MEFs, it also became partially stabilized (Figure 2C, lane 5 versus lane 2), which is consistent with our previous finding that GSK-3β is the major kinase for mediating Snail degradation. Interestingly, TNFα-mediated Snail stabilization was further elevated in the absence of GSK-3β (Figure 2C, lane 6 versus lanes 5 and 3), indicating that TNFα and GSK-3β are two key factors for stabilization of Snail.

To determine which signaling pathway was involved in TNFα-mediated Snail stabilization, we next treated the cells with inhibitors of the MAPK/ERK, mTOR, p38, and JNK kinases, since TNFα can induce the activation of these pathways. Although the stabilization of Snail by TNFα was not affected by these inhibitors, we found that an NF-κB inhibitor (Sanguinarine) completely blocked TNFα-stabilized Snail (Figure 3A). Similar results were also noted when these cells were treated with another commonly used NF-κB inhibitor, BAY 11-7082 (Figure S4), suggesting that the activation of the NF-κB pathway is important for TNFα-mediated

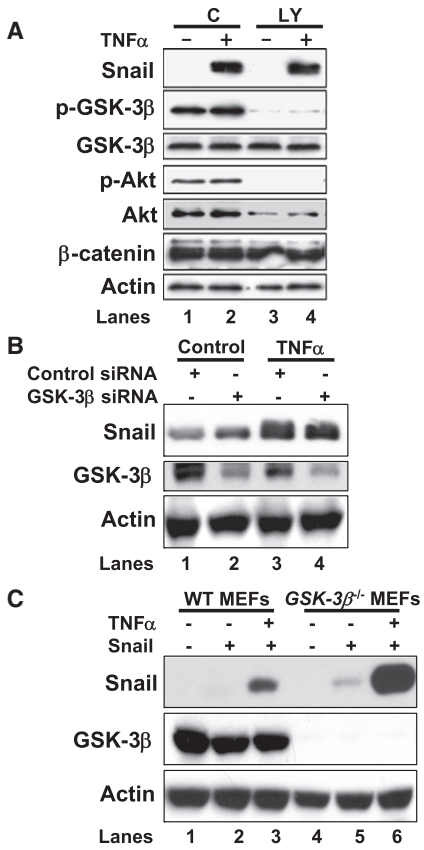

Figure 2. TNFα-Mediated Snail Stabilization Was Parallel with GSK-3β

(A) Snail/HEK293 cells were pretreated with or without LY294002 (20 μM) for 1 hr, followed by stimulation with or without TNFα for 6 hr. The expression of Snail and the activation of Akt and GSK-3β were examined by western blotting.

(B) Control and GSK-3β siRNA were expressed in Snail/HEK293 cells for 42 hr, followed by treatment with or without TNFα for additional 6 hr. The expression of Snail, GSK-3β, and actin was examined by western blotting.

(C) Snail was expressed in WT or *GSK-3β*$^{-/-}$ MEF cells for 42 hr followed by treatment with or without TNFα for additional 6 hr. The expression of Snail, GSK-3β, and actin was examined by western blotting.

Snail stabilization. To further investigate this finding, we expressed wild-type (WT) or kinase-dead (KD) IKKα or IKKβ, p65, or IκB mutant in Snail/HEK293 cells, followed by treatment with TNFα. Kinase-dead IKKβ partially suppressed the stabilization of Snail mediated by TNFα (Figure 3B, lane 10). Expression of the super-suppressor of IκB (Karin, 2006) completely inhibited the TNFα-stabilized Snail (Figure 3B, lane 14), whereas expression of p65 alone in these cells induced Snail stabilization (Figure 3B, lane 11). We also coexpressed p65 with GFP-Snail in HEK293 cells and found that the expression of p65 dramatically enhanced Snail protein levels as the expression of p65 was colocalized with GFP-Snail in the nucleus (Figure 3C). Collectively, these results indicate that the activation of the NF-κB pathway was critical for the stabilization of Snail. To confirm a causal relationship between p65 and Snail, expression of p65 was knocked down by siRNA in Snail/HEK293 cells. Knockdown of p65 expression suppressed the stabilization of Snail mediated by TNFα (Figure 3D). Similarly, knockdown of p65 expression in PC3 and HCT116 cells also blocked TNFα-induced endogenous Snail stabilization (Figure 3D). To rule out off-target effects caused by the siRNA, relevant data were also confirmed with a second siRNA that depletes p65 with a similar efficiency (Figure S5). In addition, when Snail was expressed in *p65*$^{-/-}$ MEF cells, we did not find the stabilization of Snail by TNFα (Figure 3E). However, when Snail was coexpressed with p65 in these cells, the stabilization of Snail by TNFα was restored (Figure 3E), confirming that p65 is required for the stabilization of Snail. Furthermore, when Snail was coexpressed with the E-cadherin promoter-driven luciferase construct, a mild suppression of E-cadherin luciferase was noted. Treatment with TNFα or coexpression with p65 greatly enhanced Snail-mediated E-cadherin promoter suppression, indicating that the enhanced suppression of the E-cadherin promoter was due to the stabilization of Snail mediated by TNFα or the coexpression of p65 (Figure 3F).

The Transcriptional Activity of NF-κB Is Required for TNFα-Mediated Snail Stabilization

The p65 protein contains an N-terminal Rel-homolog domain (RHD) that is conserved among all five Rel family members and is required to associate with IκB and to form homo- or heterodimers among Rel family transcription factors (Chen and Greene, 2004; Gilmore, 2006). The C terminus of p65 contains a DNA-binding region and is required for its transcription activity (Figure 4A) (Chen and Greene, 2004; Gilmore, 2006). To analyze the molecular mechanism for Snail stabilization by p65, we coexpressed WT or different deletion mutants of p65 with Snail in HEK293 cells. Interestingly, when the C-terminal region of p65 was deleted, the ability of p65 to induce the protein stability of Snail was completely abolished (Figure 4A), indicating that the transcription activity of p65 was critical for the stabilization of Snail. Consistent with this notion, we found that the Snail stabilization by TNFα was completely blocked when cells were pretreated with a transcription inhibitor actinomycin D (Act D) (Figure 4B). Because we showed that TNFα-stabilized Snail is mediated at the posttranslational level (Figures 1G and 1H), the transcriptional dependence of p65 suggests that a mediator is involved in the stabilization of Snail and that this potential mediator is induced by TNFα or p65 at the early time point. To further define the underlying mechanism, we washed TNFα away from culture medium after 6 hr of cell incubation with TNFα and chased the degradation of Snail (Figures 4C and 4D). We found a slow down-regulation of Snail (with a half-life more than 4 hr) after removal of TNFα, suggesting that TNFα treatment has initiated a program for the stabilization of Snail. However, this slow down-regulation of Snail was suppressed by Act D even in the presence of TNFα, indicating that a mediator, which is transcriptionally induced by TNFα but is blocked by Act D, was required for the stabilization of Snail. Compatible with this idea, the accelerated down-regulation of Snail by Act D was completely blocked by a proteosome inhibitor, MG-132 (Figures 4C and 4D). In addition, we incubated cells with TNFα for 6 hr to induce a potential mediator and then treated the cells with protein translational inhibitor cycloheximide (Figure S6). We found that the rate of Snail degradation was slower than that in cells without TNFα pretreatment (Figure S6). Together, these results strongly suggest that a mediator was transcriptionally/translationally induced by TNFα or p65 to block the proteasome degradation of Snail.

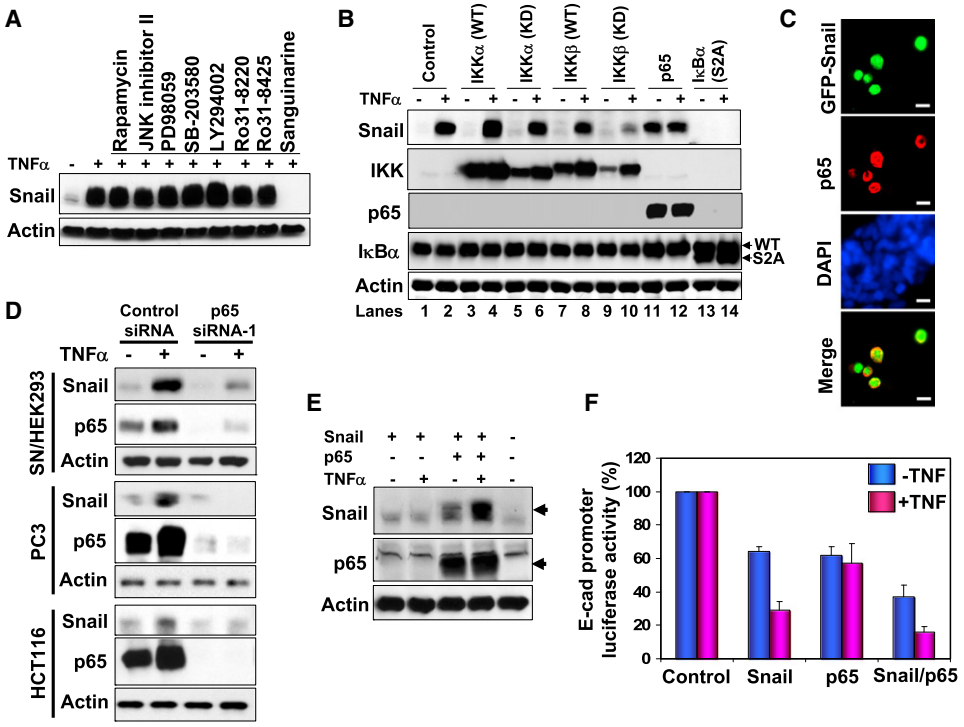

Figure 3. Activation of NF-κB Pathway Was Required for the Stabilization of Snail

(A) Snail/HEK293 cells were pretreated with various inhibitors for 1 hr followed by stimulation with TNFα for 6 hr. The level of Snail was examined by western blotting.

(B) FLAG-tagged IKK or Myc-tagged p65 or mutant IκB was coexpressed with Snail in HEK293 cells that were treated with or without TNFα for 6 hr. Expression of Snail, IKK, p65, and IκB was analyzed by western blotting.

(C) GFP-Snail was coexpressed with p65 in HEK293 cells. After fixation, the cellular location of Snail (green) and p65 (red) was examined by immunofluorescent staining (nuclei were stained with DAPI; blue). Scale bar, 20 μm.

(D) Control or p65 siRNA was expressed in Snail/HEK293, PC3, and HCT116 cells followed by TNFα or vehicle treatment for 6 hr. The levels of Snail and p65 were examined by western blotting.

(E) Snail was either expressed with or without p65 in $p65^{-/-}$ MEF cells treated with TNFα or vehicle for 6 hr. Snail and p65 were analyzed by western blotting.

(F) Snail and/or p65 was coexpressed with the E-cadherin promoter luciferase construct in HEK293 cells. After 40 hr, cells were treated with or without TNFα for 6 hr and luciferase activity was measured by using a Dual-Luciferase Reporter Assay (Promega) (mean ± SD in three separate experiments).

CSN2 Induces Snail Stabilization by Inhibiting Its Ubiquitination

Approximately 140 genes are regulated by p65, and some of them are early response genes that are induced within 1 to 4 hr (Zhou et al., 2003). Because the protein stabilization of Snail by TNFα begins at 1 hr and reaches a maximum at 4 hr (Figure 1G), the induction of genes that regulate Snail should occur within 1 hr of TNFα stimulation. There are about 50 genes that are induced within 1 hr of TNFα stimulation, such as IκBα (Zhou et al., 2003). We screened the majority of these genes for their ability to induce the stabilization of Snail (data not shown) and found that the expression of COP9 signalosome 2 (CSN2) was of particular interest. CSN2 is the second and most conserved subunit of the COP9 signalosome in all eukaryotes (Cope and Deshaies, 2003; Richardson and Zundel, 2005; Wolf et al., 2003). The eight CSN subunits share significant sequence homologies with the eight subunits of the 26S proteasome lid and are thought to cooperate with the ubiquitin/proteasome system in the regulation of protein stability by interacting with cullin-based ubiquitin ligases to control their activity. To test whether CSN2 is involved in the regulation of Snail mediated by TNFα or p65, we first examined the expression of CSN2 in cells by semiquantitative PCR after TNFα stimulation. Consistent with the findings of a previous report (Zhou et al., 2003), the induction of CSN2 expression occurred within 1 hr of TNFα treatment and reached a maximum at 4 hr in several cell lines, including HEK293, SKBR3, and MDA-MB231 cells (Figures 5A and S7). The induction of IκB by TNFα, as a positive control, also began within 1 hr of stimulation. Similar results were also obtained when we performed Q-PCR analysis (data not shown). Consistent with the change in mRNA, TNFα also induced endogenous CSN2 expression in a time-dependent manner in SKBR3 and MDA-MB231 cells (Figures 5A and S7). In addition, we noticed that the promoter region of CSN2 contains 4 conserved NF-κB binding sites. Expression of p65 induced the luciferase activity driven by the CSN2 promoter (Figure 5B). Deletion of the C terminus of p65 abolished its ability to induce CSN2 (Figure 5B). Moreover, when CSN2 was coexpressed with Snail in HEK293 cells, it dramatically stabilized Snail protein to a similar degree as did TNFα or MG-132 (Figure 5C). Surprisingly, the stabilized Snail mediated by CSN2 was in a nonphosphorylated form that is similar to TNFα but different from MG-132

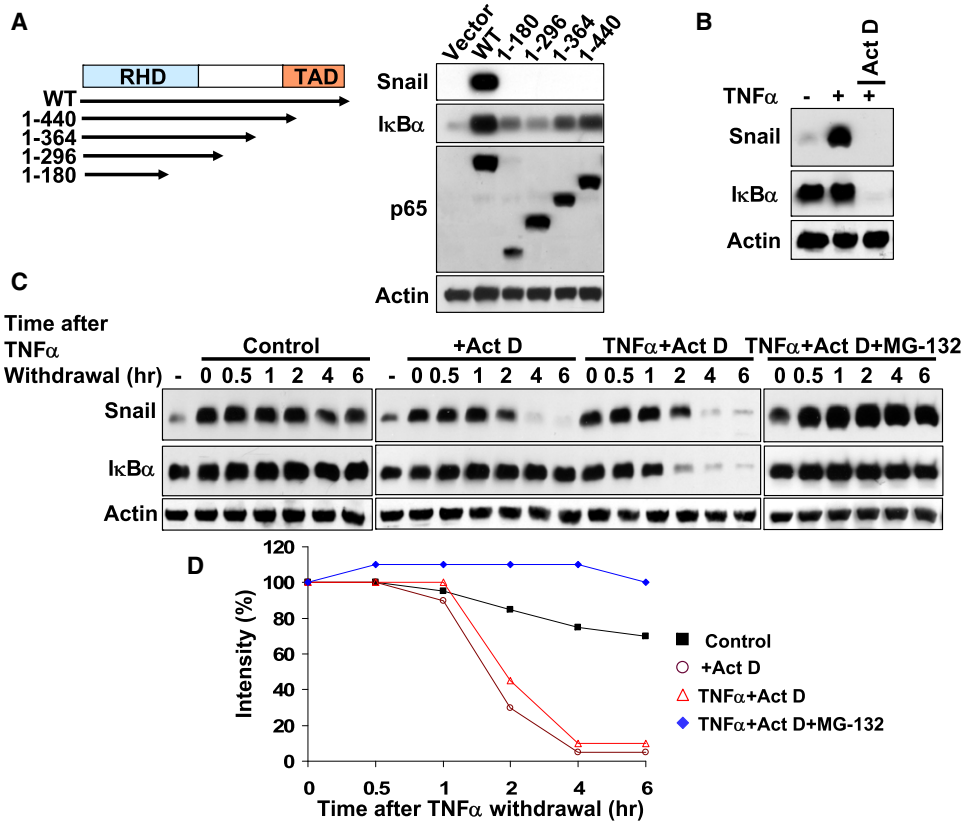

Figure 4. Transcriptional Dependence of NF-κB Was Involved in the Stabilization of Snail

(A) Wild-type (WT) or various deletion mutants of p65 (scheme diagram shown on left panel) were coexpressed with Snail in HEK293 cells and the expression of Snail, IκB, and p65 was analyzed by western blotting.

(B) Snail/HEK293 cells were pretreated with or without actinomycin D (Act D; 0.5 μg/ml) for 1 hr followed by TNFα stimulation for 6 hr. Snail or IκB was analyzed by western blotting.

(C) After stimulation with TNFα for 6 hr, Snail/HEK293 cells were washed twice with fresh medium to remove residual TNFα, followed by incubation without or with Act D in combination with TNFα or TNFα/MG-132 together over a time course. Snail or IκB was analyzed by western blotting.

(D) Densitometry results for the level of Snail described above were plotted.

(Figure 5C). Similarly, when CSN2 was coexpressed with GFP-Snail, it enhanced the intensity of GFP-Snail in the nucleus (Figure 5D). To further determine whether CSN2 is required for TNFα-mediated Snail stabilization, we treated cells with two known CSN inhibitors, curcumin or emodin (Berse et al., 2004) and found that the stabilization of Snail by TNFα was suppressed (Figure 5E). In addition, when CSN2 expression was knocked down by siRNA, the TNFα-stabilized Snail was greatly suppressed in Snail/HEK293 cells (Figure 5F). Knockdown of CSN2 expression also significantly inhibited the stabilization of endogenous Snail induced by TNFα in PC3 and SKBR3 cells (Figure 5F). This finding was not caused by the off-target effects of siRNA, because similar data were also obtained with a second siRNA that targets CSN2 with a similar efficiency (Figure S8). Furthermore, expression of exogenous CSN2 enhanced the steady-state of Snail, whereas knockdown of endogenous CSN2 expression facilitated the degradation of Snail (Figure S9). Together, these results indicate that CSN2 is the critical mediator and is required for TNFα-mediated Snail stabilization.

Cullin is an essential scaffolding component of ubiquitin E3 ligases, and it is modified covalently by neddylation, which blocks the association of cullin with a negative regulator, CAND1 (Goldenberg et al., 2004; Richardson and Zundel, 2005). The activity of CSN is to remove the neddylation from cullins and to facilitate the association of cullin with CAND1 and, therefore, inhibit the ubiquitin E3 ligase activity of SCF complex. CSN2 is the most conservative subunit of CSN, and it facilitates the functional assembly of CSN complex (Naumann et al., 1999; Schweitzer et al., 2007). The intrinsic de-neddylation function of CSN complex is executed by CSN5 that contains MPN metalloenzyme activity. Similar to CSN2, expression of CSN5 also induced the stabilization of Snail, indicating that the function of CSN complex is critical for Snail stabilization (Figure S10). Consistent with this finding, knockdown of CSN5 inhibited CSN2-mediated Snail stabilization (Figure S11). Interestingly, knockdown of CAND1, a negative regulator of cullins, also suppressed CSN2-mediated Snail stabilization (Figure S11). We previously demonstrated that the protein stability of Snail is regulated by the E3 ligase β-Trcp and the proteasome pathway; treatment with the proteasome inhibitor MG-132 stabilized Snail in a highly phosphorylated and ubiquitylated form (Zhou et al., 2004). Because Snail stabilized by TNFα or CSN2 occurs in

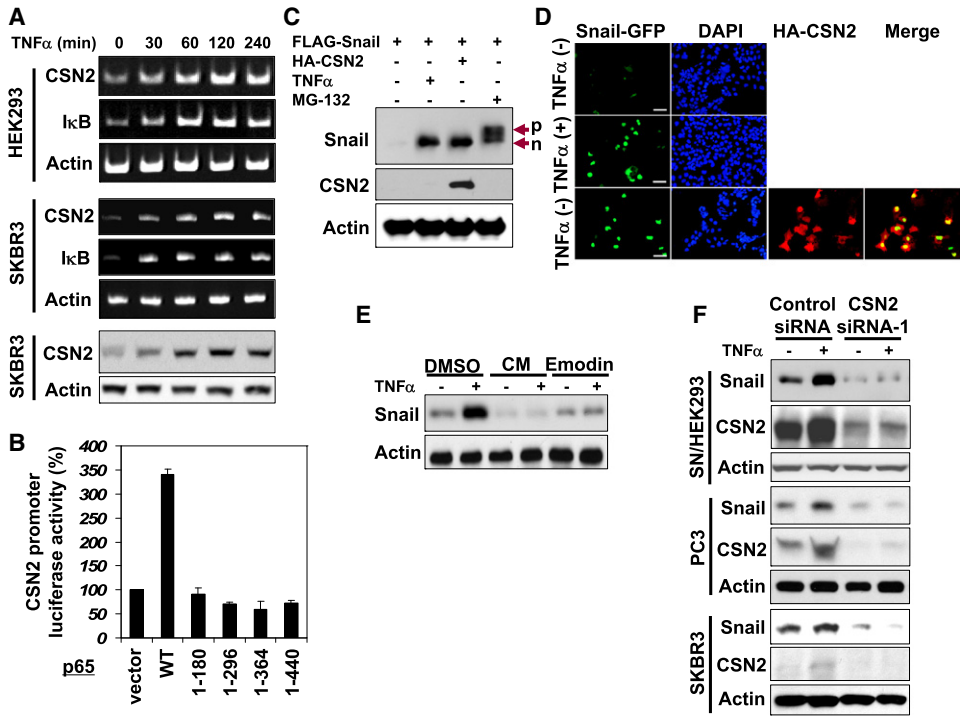

Figure 5. CSN2 Induced Snail Stabilization

(A) HEK293 and SKBR3 cells were treated with TNFα for different time intervals and mRNA from these cells was analyzed by semiquantitative RT-PCR. Expression of CSN2 was also analyzed in SKBR3 cells.

(B) The CSN2 promoter luciferase construct was coexpressed with WT or different deletion mutants of p65 in HEK293 cells. Luciferase activities were normalized and determined (mean ± SD in three separate experiments).

(C) Snail/HEK293 cells were either treated with TNFα or MG-132 for 6 hr or coexpressed with HA-CSN2. Expression of Snail and CSN2 was separated on 14% SDS-PAGE and analyzed by western blotting.

(D) GFP-Snail was coexpressed with CSN2 or empty vector in HEK293 cells followed by treatment with TNFα or vehicle for 6 hr. The cellular location of Snail (green) and CSN2 (red) was examined by immunofluorescent staining (nuclei were stained with DAPI; blue). Scale bar, 100 μm.

(E) Snail/HEK293 cells were pretreated with either DMSO, or Curcumin (CM; 50 nM) or Emodin (400 nM) for 1 hr followed by TNFα for 6 hr. Snail was examined by western blotting.

(F) CSN2 siRNA was expressed in Snail/HEK293, PC3, and SKBR3 cells followed by treatment with TNFα or vehicle. Snail and CSN2 were examined by western blotting.

a nonphosphorylated form (Figures 1F and 5C) and because CSN2 affects ubiquitin E3 ligase, we analyzed whether the protein stabilization is mediated by suppression of Snail ubiquitylation. When ubiquitin was immunoprecipitated from the cell lysate, we found significantly less Snail ubiquitylation in cells treated with TNFα than in cells treated with MG-132 (Figure 6A). Similarly, when Snail was immunoprecipitated, we also found that the ubiquitylation of Snail was dramatically suppressed in cells treated with TNFα or expressing CSN2, compared with cells treated with MG-132, although total stabilized Snail proteins were similar (Figure 6B). Moreover, we found that the association of Snail with its E3 ligase, β-Trcp, was abolished in cells treated with TNFα or expression with CSN2 (Figure 6B). Because TNFα- and CSN2-stabilized Snail are unphosphorylated, we also examined the association of Snail with GSK-3β since GSK-3β is the main kinase for mediating Snail phosphorylation and degradation. Surprisingly, we found that the association of Snail with GSK-3β was diminished in cells treated with TNFα or CSN2, compared with cells treated with MG-132 (Figure 6C). Consistent with these findings, when endogenous GSK-3β was immunoprecipitated, the associated endogenous Snail was significantly reduced in cells treated with TNFα, compared with that in cells treated with MG-132 (Figure 6D). Similarly, when endogenous Snail was pulled down, the bound endogenous GSK-3β was lost in cells treated with TNFα (Figure 6E). Together, these results clearly demonstrated that the stabilization of Snail by TNFα or p65 was mediated by the transcriptional induction of CSN2, which, in turn, inhibited the association of Snail with β-Trcp and GSK-3β and thus suppressed its ubiquitylation and phosphorylation.

Snail Is Required for Cell Migration, Invasion, and Metastasis Mediated by Inflammation

Having established that TNFα-stabilized Snail is mediated by NF-κB-induced CSN2, we next examined whether the stabilization of Snail was required for cancer cell migration, invasion, and metastasis mediated by inflammation. We first established stable shRNA Snail knockdown cells from two highly metastatic cancer cell lines, MDA-MB231 and MDA-MB435. We achieved about 90% knockdown of endogenous Snail expression (Figure 7A). Although we did not detect the gain of expression of E-cadherin (data not shown), the expression of N-cadherin

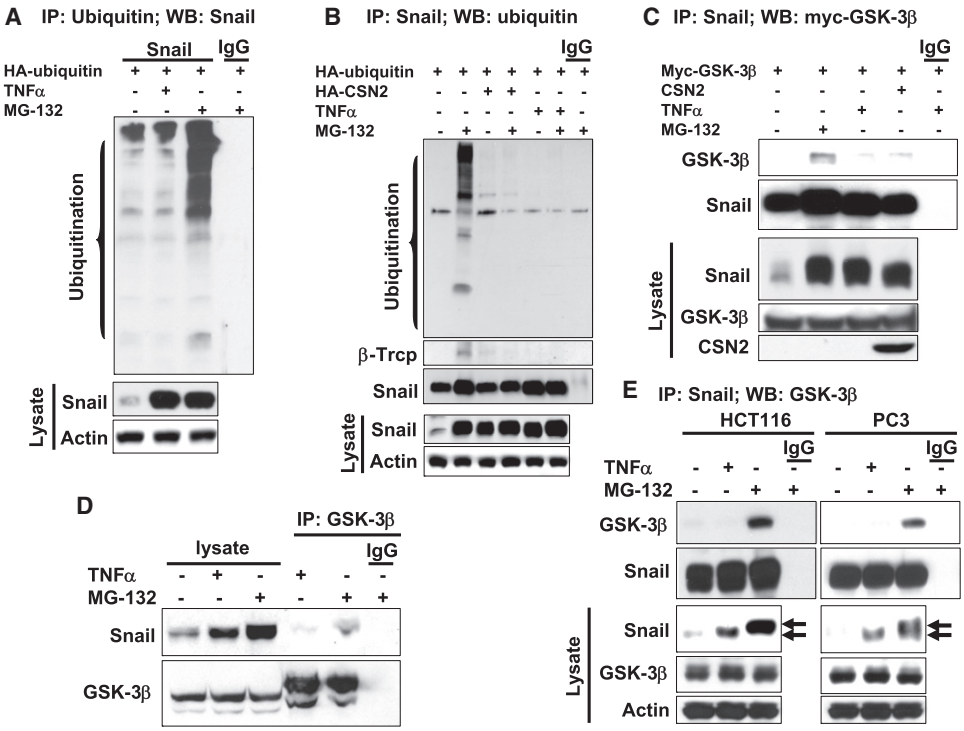

Figure 6. CSN2 Inhibited the Ubiquitination of Snail

(A) HA-ubiquitin was expressed in Snail/HEK293 cells followed by treatment with either TNFα or MG-132 for 6 hr. Ubiquitin was immunoprecipitated from equal amount of lysates (two lower panels) and the ubiquitinated Snail was examined by western blotting.

(B) HA-ubiquitin was coexpressed with HA-CSN2 or control vector in Snail/HEK293 cells followed by treatment with TNFα or MG-132 for 6 hr. After Snail was immunoprecipitated, the ubiquitination of Snail and the bound β-Trcp were examined by western blotting.

(C) Myc-GSK-3β was coexpressed with HA-CSN2 or control vector in Snail/HEK293 cells for 42 hr followed by treatment with MG-132 or TNFα for additional 6 h. Snail was immunoprecipitated and the association of GSK-3β was examined by western blotting.

(D) HCT116 cells were either treated with TNFα or MG-132. Endogenous GSK-3β was immunoprecipitated, and the bound endogenous Snail was detected by western blotting.

(E) Cells were treated with TNFα or MG-132. Endogenous Snail was immunoprecipitated and the associated endogenous GSK-3β was detected by western blotting.

was dramatically decreased, whereas the expression of two tight junction molecules, ZO-1 and claudin-1, was increased (Figure 7A). Consistent with these molecular changes, cell migration of both Snail knockdown cell lines was inhibited in a wound healing assay (Figures 7B and S12). Importantly, cell migration induced by TNFα was also significantly suppressed (Figures 7B and S12). To rule out off-target effects caused by the shRNA, relevant data were also confirmed with a second shRNA that depletes Snail with a similar efficiency in MDA-MB231 cells (Figure S13). On the invasion assays, although the invasiveness of MDA-MB231 and MDA-MB435 cells were dramatically increased when they were cocultured with macrophage-conditioned medium or macrophages (Figures 7C and S14), the addition of TNFα antibody, but not the control IgG, suppressed cell invasion induced by macrophages or macrophage-conditioned medium (Figures 7C and S14). These results further confirm our finding that TNFα is the major cytokine for inducing Snail stabilization and cell invasion (Figure 1). Most important, knockdown of Snail expression in these two cancer cell lines not only inhibited the invasiveness that is intrinsic to the metastatic cancers cells but also suppressed inflammation-enhanced invasion (Figures 7C and S14).

We also tested our findings in a xenograft metastasis model in which a cancer cell line, MDA-MB435, was used to generate pulmonary metastases, and the administration of lipopolysaccharide (LPS), an inducer of inflammation (Luo et al., 2004), greatly accelerated lung metastases in mice (Figures 8A, S15, and S16). Knockdown of Snail expression suppressed both the intrinsic and inflammation-enhanced metastasis in these mice. Similar results were also obtained when we used another metastatic breast cancer cell line, MDA-MB231, in the xenograft metastasis assay (Figure 8A). Together, our results demonstrated that Snail is required for cell migration, invasion, and metastasis mediated by inflammation.

To further extend our findings in vivo, we determined whether there is a correlation between the expression of Snail and the activity of p65 in cancer cell lines and resected human breast cancer specimens. Because p65 is active in the nucleus and Snail is localized in the nucleus when it is stabilized (Zhou et al., 2004), we measured the expression of Snail and p65 in nuclear extracts from 14 different cancer cell lines. We found that the nuclear level of p65 was highly correlated with the protein level of Snail (Figure 8B). Likewise, in 110 breast cancer specimens, the expression of p65 was highly correlated with the

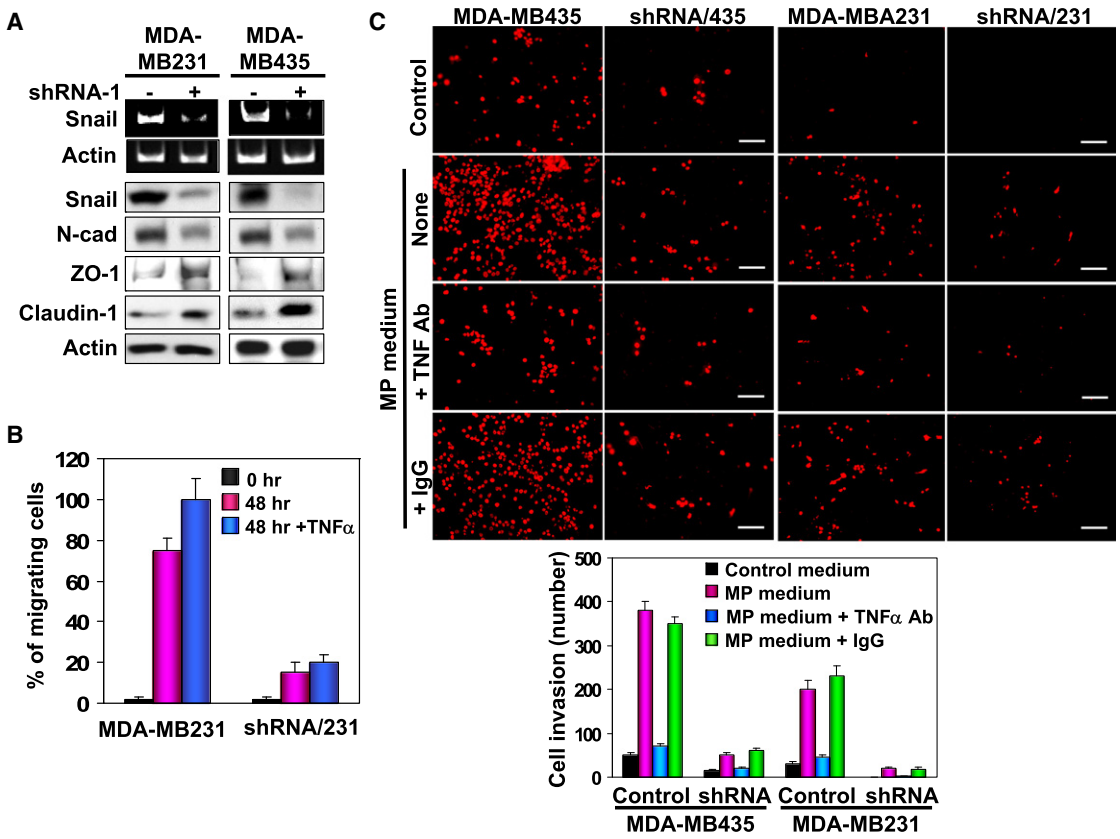

Figure 7. Knockdown of Snail Expression Suppressed Inflammation-Induced Cell Migration and Invasion In Vitro

(A) In the top two panels, mRNA from MDA-MB231 and MDA-MB435 cells and their corresponding stable transfectants with knockdown of Snail expression were analyzed by RT-PCR. In the bottom five panels, expression of Snail (treated with MG-132 for 6 hr), N-cadherin, ZO-1, Claudin-1, and actin was analyzed by western blotting.

(B) The motile behavior of MDA-MB231 and its corresponding Snail knockdown cells was analyzed by wound healing assay. The percentage of migrating cells was shown (mean ± SEM in three separate experiments) and the representative images were shown in Figure S12.

(C) The invasive ability of MDA-MB231 and MDA-MB435 cells and their corresponding stable transfectants with knockdown of Snail expression was analyzed by the invasion assay. The representative images were shown and the percentage of invasive cells was plotted in bottom graph (mean ± SEM in three separate experiments). Scale bar, 200 μm.

expression of Snail (Figure 8C and Table S1). Thus, these results in human breast cancer tissues confirmed our observations in cell culture and in an animal model, lending further support to our hypothesis that the stabilization of Snail by p65 is required for the cell migration and invasion induced by inflammatory cytokines.

DISCUSSION

In this study, we showed that TNFα-induced Snail stabilization plays a critical role in inflammation-induced EMT and cancer cell migration, invasion, and metastasis. Our study provides several insights into the regulation of EMT and metastasis. First, our study indicates that Snail-induced EMT is critical for inflammation-initiated invasion and metastasis. In the tumor-host boundary, the persistent recruitment of immune cells, such as macrophage and mast cells, is thought to establish an inflammatory tumor microenvironment. The remarkable interaction of macrophages with tumor cells has been visualized in vivo by multiphoton imaging and is found to enhance tumor cell dissemination and invasion at the invasive front (Condeelis and Pollard, 2006; Wyckoff et al., 2007). In addition, macrophage ablation in experimental tumor models greatly inhibited the occurrence of tumor metastasis (Lin et al., 2001). Although macrophages have been shown to enhance angiogenesis and MMP production and thus facilitate tumor metastasis (Lin et al., 2006; Luo et al., 2006), the underlying molecular mechanism for macrophage-induced dissemination and invasion of tumor cells at the invasive front remains unclear. In our study, we found that coculture of tumor cells with macrophages greatly enhanced the migration and invasion of tumor cells by inducing the EMT program through NF-κB-mediated Snail stabilization (Figure 1). Knockdown of Snail expression not only inhibits TNFα-induced cancer cell migration and invasion in vitro but also suppresses LPS-mediated metastasis in vivo (Figures 7 and 8). A recently study (Luo et al., 2004) elegantly demonstrated that injection of LPS induced an inflammatory condition and enhanced NF-κB-mediated tumor growth and metastasis of colon cancer in an experimental metastasis model. That study further demonstrated that blocking the NF-κB pathway converts inflammation-induced

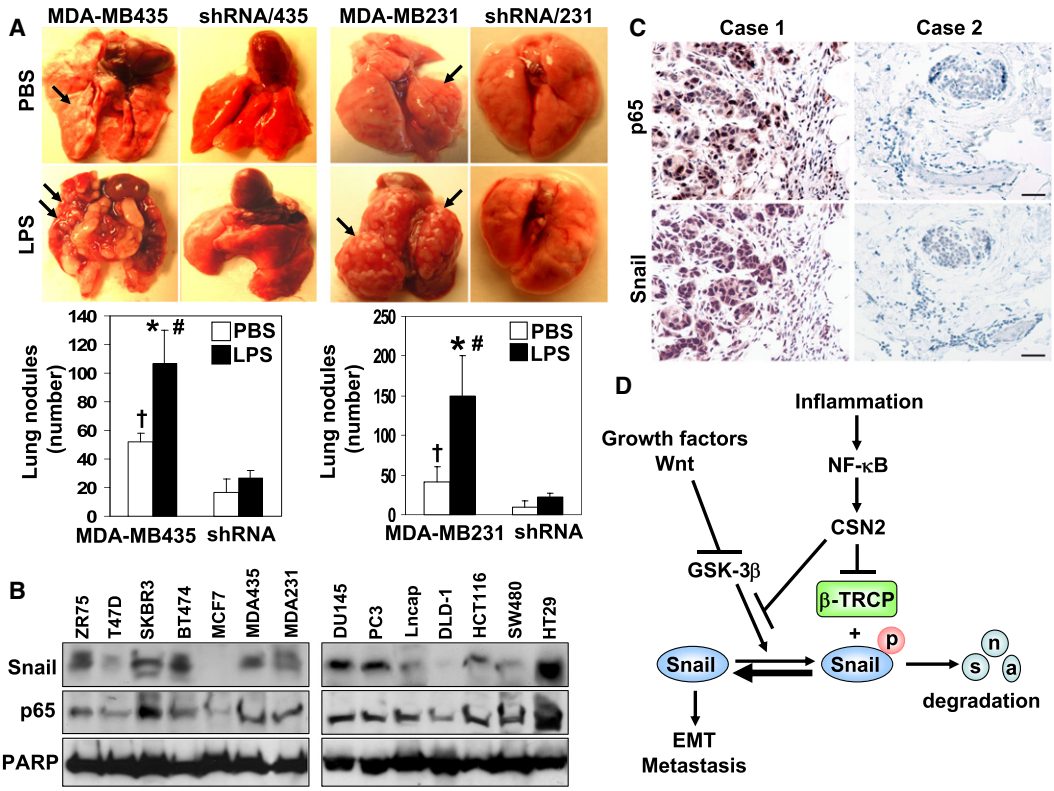

Figure 8. Knockdown of Snail Expression Suppressed Inflammation-Induced Metastasis In Vivo

(A) MDA-MB435 and MDA-MB231 cells and their corresponding stable transfectants with knockdown of Snail expression were injected into the tail vein of ICR-SCID mice. After 6–9 weeks, the number of lung metastases was plotted (mean ± SEM, n = 6) and the representative photography of lung was shown. For lung metastases, *p < 0.01 for parental cells compared with their stable transfectants with knockdown of Snail expression after treatment with LPS, #p < 0.05 when compared with PBS stimulation for parental cells, †p < 0.05 for parental cells compared with their stable transfectants with knockdown of Snail expression treated with PBS.

(B) Nuclear extracts were prepared from various cancer cell lines and the expression of Snail and p65 was analyzed by western blotting. Poly (ADP-ribose) polymerase (PARP) was used as a control for the equal loading of nuclear extracts.

(C) The 110 surgical specimens of breast cancer were immunostained using antibodies to Snail, p65, as well as the control serum (data not shown). Representative stains from the same tumor samples are shown. Scale bar, 100 μm.

(D) A proposed model to illustrate the stabilization of Snail by TNFα leading to EMT and metastasis.

tumor growth and metastasis to TRAIL-mediated tumor regression. This is an important finding because inflammation both promotes tumor growth and metastasis and kills cancer cells. Consistent with this observation, we found that the introduction of LPS in mice greatly enhanced lung metastasis of breast cancer cells (Figure 8A). Knockdown of Snail not only blocked metastasis that is intrinsic to the metastatic breast cancer cells but also greatly suppressed inflammation-accelerated metastasis (Figure 8A). Because Snail possesses an antiapoptotic function in addition to the induction of EMT (Kajita et al., 2004; Vega et al., 2004), future studies will explore whether knockdown of Snail induces apoptosis mediated by inflammation and thus contributes to the metastatic suppression. Similar to our findings, Olmeda et al. (2007) recently showed that silencing of Snail expression by shRNA inhibited tumor growth and invasiveness. It will be interesting to determine whether the silencing of Snail expression in their system also suppresses inflammation-mediated cell invasion and metastasis. Collectively, our study indicates that Snail stabilization and EMT induction, mediated by the inflammatory cytokine TNFα, are critical for metastasis and thus provides a plausible molecular mechanism for tumor cell dissemination and invasion at the invasive front.

Second, our study reveals a mechanism of Snail regulation by NF-κB in the process of inflammation-induced EMT and cancer metastasis. We and others previously showed that Snail is mainly regulated by GSK-3β-mediated phosphorylation and degradation (Yook et al., 2005; Zhou et al., 2004). In current study, we showed that TNFα-mediated Snail stabilization occurs through the induction of CSN2, which blocks the phosphorylation and ubiquitylation of Snail by disrupting its binding to GSK-3β and β-Trcp (Figures 5 and 6), resulting in Snail stabilization in a nonphosphorylated and nonubiquitylated state (Figure 8D). These findings are in agreement with the notion that Snail is a labile protein and is subjected to delicate regulation by multiple extracellular signaling pathways to control its ubiquitylation and degradation (Figure 8D). Interestingly, dorsal, a *Drosophila* homolog of p65, has been shown to be the key regulator of Snail and Twist during embryonic patterning and the innate immune response in fly (Furlong et al., 2001). In this study, we showed that activation of the NF-κB pathway is the

major signal for the stabilization of Snail and the induction of EMT (Figures 1 and 7). Activation of NF-κB correlated with the level of Snail in breast cancer cell lines and tumor samples (Figure 8). Thus, our results indicate that the signaling pathway between Snail and NF-κB is highly conserved from fly to mammal and highlight the notion that cancer cells utilize many developmental strategies for their proliferation and progression. Although Snail has recently been shown to be increased at the transcriptional level by the NF-κB pathway (Bachelder et al., 2005; Julien et al., 2007; Kim et al., 2007), our results indicate that the stabilization of Snail by TNFα also occurs at the posttranslational level. Therefore, it is likely that NF-κB regulates the activity of Snail through both transcriptional and posttranslational mechanisms at EMT (Huber et al., 2004). The posttranslational regulation provides a rapid induction of Snail that is urgently needed, whereas the transcriptional regulation offers a persistent and lasting activity of Snail during chronic inflammation.

The involvement of CSN2 in regulating the stability of Snail is intriguing. CSN2 is the second and most conserved subunit of the COP9 signalosome in all eukaryotes (Cope and Deshaies, 2003; Richardson and Zundel, 2005; Wolf et al., 2003). Recent evidence indicates that CSNs cooperate with the ubiquitin/proteasome system in the regulation of protein stability by interacting with cullin-based ubiquitin ligases to control their activity. In line with this concept, Chang and colleagues found a 512-gene expression pattern as a wound response signature and showed that this signature is a powerful predictor of metastasis and death in diverse types of primary human tumors (Chang et al., 2005; Chang et al., 2004). They demonstrated that amplification of c-Myc and another subunit of COP9 signalsome, CSN5, are the critical genetic regulators for these large-scale transcriptional wound response signatures in cancer (Adler et al., 2006). Strikingly, overexpression of c-Myc and CSN5 in nontransformed mammary epithelial MCF10A cells induce an invasive morphology and confer invasive ability on these cells to invade through a three-dimensional basement membrane matrix. In addition, expression of these two genes activates the wound response program with a similar wound response signature (Adler et al., 2006). Because c-*myc* is a transcriptional factor and CSN5 is a critical regulator of protein stability, we speculate that c-*myc* and CSN5 control the wound response program at transcriptional and posttranslational level, respectively. The critical role of CSNs in regulating protein stability is further supported by the observations that many important transcription factors and signal transduction regulators have short half-lives (such as p53, c-Jun, HIF1, and IκBα) and are regulated through ubiquitylation and degradation. Expression of CSN2 or CSN5 induces the protein stabilization of c-Jun, IκBα, and p27 (Huang et al., 2005; Yang et al., 2002). In our study, we found that expression of CSN2 induces stabilization of Snail and knockdown of CSN2 expression blocks TNFα-mediated Snail stabilization (Figures 5 and 6). Thus, the posttranslation regulation of Snail, mediated by CSN during the inflammatory response, provides a delicate control of Snail during EMT and metastasis. Interestingly, Schweitzer et al. (2007) recently found that CSN2 controlled NF-κB activity by de-ubiquitylating and stabilizing IκBα. They found that expression of CSN2 induced the functional assembly of CSN complex. In addition, CSN2 disrupted the association of IκBα with β-Trcp and enhanced the de-ubiquitylating of IκBα by de-ubiquitinylase USP15. On contrary, knockdown of CSN2 expression enhanced TNFα-mediated highly phosphorylated and highly ubiquitylated IκBα. Their results are consistent with our findings. However, whether CSN2 also stabilizes other β-Trcp substrates in a similar fashion requires systematic and thorough investigation.

Our also study suggests that EMT and MET (mesenchymal-epithelial transition) is a dynamic process that is controlled by an inflammatory microenvironment. By adopting a mesenchymal phenotype through EMT, individual carcinoma cells can infiltrate adjacent tissues, cross endothelial barriers, and enter the circulation through blood and lymphatic vessels. Once the cancer cells reach their secondary tissues or organs, they can revert back to an epithelial morphology through an MET process at the secondary tissues or organs that lack inflammatory stimuli. This model is consistent with the fact that, although some cancers are invasive and metastatic, their secondary metastasized lesions have well-differentiated epithelial characteristics. Our study also supports the view that EMT mainly occurs at the invasive front of metastatic carcinoma (Christofori, 2006; Franci et al., 2006). It is highly unlikely that EMT would occur in a whole tumor because there is lack of tumor-stroma interaction and an inflammatory microenvironment in the center of the tumor.

In summary, we showed that inflammation-triggered migration, invasion, and metastasis of tumor cells are mediated through NF-κB-induced Snail stabilization. Knockdown of Snail expression inhibits inflammation-induced cancer cell migration and invasion in vitro and metastasis in vivo. Our study not only reveals a critical mechanism underlying inflammation-induced metastasis but also has important implications in the development of treatment strategies for metastatic cancers.

EXPERIMENTAL PROCEDURES

Plasmids, siRNA, and Antibodies

GSK-3β siRNA expression plasmid pkD-GSK-3β-V1 was purchased from Upstate Biotechnology. Smart pool siRNA against human CSN2 (siRNA-1), p65 (siRNA-1), CSN5, and CAND1 were obtained from Dharmacon (Chicago, IL). A second validated siRNA against CSN2 (siRNA-2) or p65 (siRNA-2) was purchased from QIAGEN (Valencia, CA). Primers corresponding to the human Snail (from 507 to 525) were cloned into the pSUPER vector to generate pSUPER-shRNA-Snail-1 expression plasmid (shRNA-1) for RNAi silencing. A second shRNA against Snail (shRNA-2; TRCN0000063819) was purchased from MISSION shRNA at Sigma-Aldrich (St Louis, MO). Information for other plasmids and antibodies used in this study are described in Supplemental Experimental Procedures.

Macrophage Preparation and Culture

Buffy coats that contain mononuclear cells were collected from the blood of healthy individual donors at Gulf Coast Regional Blood Center (Houston, TX). Primary blood monocytes were isolated by density-gradient centrifugation through Ficol/Hypaque (Amersham Bioscience, Piscataway, NJ), suspended (8×10^6 cells/ml) in RPMI 1640 medium with 15% heat-inactivated human serum (Sigma, St. Louis, MO), and seeded in flasks (Borovikova et al., 2000). After incubation for 2 hr at 37°C, adherent cells were detached with 10 mM EDTA and resuspended (10^6 cells/ml) in medium supplemented with 40 ng/ml human MCSF (PeproTech Inc, Rocky Hill, NJ). Cells were allowed to differentiate for 7 days in the presence of MCSF. On day 7, fresh medium without MCSF was added to the cells, and the cells continued to culture for 24 hr. Human macrophages were finally exposed to LPS (100 ng/ml, Sigma) for 24 hr. The culture medium was collected, centrifuged, stored in aliquots at −80°C, and defined as macrophage-conditioned medium. For

coculture experiments, Transwell inserts (0.2 μm pores, Nunc) that contain 2 × 10^5/ml macrophages were placed inside an upper well of the Boyden chamber, which was coated with Matrigel and seeded with various cancer cell lines.

Experimental Lung Metastasis Model

Female ICR-SCID mice (6–8 weeks old) were purchased from Taconic (Germantown, NY) and maintained and treated under specific pathogen-free conditions. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and conform to the legal mandates and federal guidelines for the care and maintenance of laboratory animals. Mice (6 for each group) were injected intravenously with breast cancer cell lines MDA-MB-231 (1 × 10^6 cells/mouse) or MDA-MB-435 (2 × 10^6 cells/mouse) cells via the tail vein. Three weeks later, mice were injected intraperitoneally with 10 μg of LPS (serotype 055:B5; Sigma) in PBS or with PBS alone. After an additional 3 weeks (for MDA-MB435) or 9 weeks (for MDA-MB231), mice were killed by cervical dislocation, and lungs were removed and weighed. Visible lung metastatic nodules were examined macroscopically or detected in paraffin-embedded sections stained with hematoxylin and eosin. Data were analyzed by Student's t test; $p < 0.05$ was considered significant.

Statistical Analysis

The experiments were repeated at least two times. Results are expressed as mean ± SD or SEM, as indicated. An independent Student's t test was performed to analyze the luciferase assay. Two-tailed student's t test was used to compare the intergroup; $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL DATA

The Supplemental Data include one table, 16 figures, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00085-3.

ACKNOWLEDGMENTS

We thank Dr. Warner C. Greene for providing wild-type and deletion mutants of p65. We also thank Dr. James R. Woodgett for providing immortalized wild-type and *GSK-3*$\beta^{-/-}$ MEFs. This work was supported by the John Sealy Memorial Endowment Fund, a pilot award from the ACS (IRG-110376), grants from the Susan G. Komen Foundation (KG081310) and NCI (RO1-CA125454) (to B.P.Z.), and grants RO1 CA104748 and RO1 DK48498 from the NIH (to B.M.E.). Y.W. was supported by postdoctoral fellowship from NIH (T32CA117834).

Received: January 21, 2008
Revised: September 11, 2008
Accepted: March 12, 2009
Published: May 4, 2009

REFERENCES

Adler, A.S., Lin, M., Horlings, H., Nuyten, D.S., van de Vijver, M.J., and Chang, H.Y. (2006). Genetic regulators of large-scale transcriptional signatures in cancer. Nat. Genet. *38*, 421–430.

Bachelder, R.E., Yoon, S.O., Franci, C., de Herreros, A.G., and Mercurio, A.M. (2005). Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition. J. Cell Biol. *168*, 29–33.

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J., and Garcia De Herreros, A. (2000). The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat. Cell Biol. *2*, 84–89.

Berse, M., Bounpheng, M., Huang, X., Christy, B., Pollmann, C., and Dubiel, W. (2004). Ubiquitin-dependent degradation of Id1 and Id3 is mediated by the COP9 signalosome. J. Mol. Biol. *343*, 361–370.

Blanco, M.J., Moreno-Bueno, G., Sarrio, D., Locascio, A., Cano, A., Palacios, J., and Nieto, M.A. (2002). Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. Oncogene *21*, 3241–3246.

Borovikova, L.V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G.I., Watkins, L.R., Wang, H., Abumrad, N., Eaton, J.W., and Tracey, K.J. (2000). Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature *405*, 458–462.

Cano, A., Perez-Moreno, M.A., Rodrigo, I., Locascio, A., Blanco, M.J., del Barrio, M.G., Portillo, F., and Nieto, M.A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat. Cell Biol. *2*, 76–83.

Carver, E.A., Jiang, R., Lan, Y., Oram, K.F., and Gridley, T. (2001). The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. Mol. Cell. Biol. *21*, 8184–8188.

Chang, H.Y., Sneddon, J.B., Alizadeh, A.A., Sood, R., West, R.B., Montgomery, K., Chi, J.T., van de Rijn, M., Botstein, D., and Brown, P.O. (2004). Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. PLoS Biol. *2*, E7.

Chang, H.Y., Nuyten, D.S., Sneddon, J.B., Hastie, T., Tibshirani, R., Sorlie, T., Dai, H., He, Y.D., van't Veer, L.J., Bartelink, H., et al. (2005). Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival. Proc. Natl. Acad. Sci. USA *102*, 3738–3743.

Chen, L.F., and Greene, W.C. (2004). Shaping the nuclear action of NF-kappaB. Nat. Rev. Mol. Cell Biol. *5*, 392–401.

Cheng, C.W., Wu, P.E., Yu, J.C., Huang, C.S., Yue, C.T., Wu, C.W., and Shen, C.Y. (2001). Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene. Oncogene *20*, 3814–3823.

Christofori, G. (2006). New signals from the invasive front. Nature *441*, 444–450.

Condeelis, J., and Pollard, J.W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell *124*, 263–266.

Cope, G.A., and Deshaies, R.J. (2003). COP9 signalosome: a multifunctional regulator of SCF and other cullin-based ubiquitin ligases. Cell *114*, 663–671.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860–867.

Cowin, P., Rowlands, T.M., and Hatsell, S.J. (2005). Cadherins and catenins in breast cancer. Curr. Opin. Cell Biol. *17*, 499–508.

Franci, C., Takkunen, M., Dave, N., Alameda, F., Gomez, S., Rodriguez, R., Escriva, M., Montserrat-Sentis, B., Baro, T., Garrido, M., et al. (2006). Expression of Snail protein in tumor-stroma interface. Oncogene *25*, 5134–5144.

Furlong, E.E., Andersen, E.C., Null, B., White, K.P., and Scott, M.P. (2001). Patterns of gene expression during *Drosophila* mesoderm development. Science *293*, 1629–1633.

Gilmore, T.D. (2006). Introduction to NF-kappaB: players, pathways, perspectives. Oncogene *25*, 6680–6684.

Goldenberg, S.J., Cascio, T.C., Shumway, S.D., Garbutt, K.C., Liu, J., Xiong, Y., and Zheng, N. (2004). Structure of the Cand1-Cul1-Roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases. Cell *119*, 517–528.

Hartwell, K.A., Muir, B., Reinhardt, F., Carpenter, A.E., Sgroi, D.C., and Weinberg, R.A. (2006). The Spemann organizer gene, Goosecoid, promotes tumor metastasis. Proc. Natl. Acad. Sci. USA *103*, 18969–18974.

Huang, X., Hetfeld, B.K., Seifert, U., Kahne, T., Kloetzel, P.M., Naumann, M., Bech-Otschir, D., and Dubiel, W. (2005). Consequences of COP9 signalosome and 26S proteasome interaction. FEBS J. *272*, 3909–3917.

Huber, M.A., Beug, H., and Wirth, T. (2004). Epithelial-mesenchymal transition: NF-kappaB takes center stage. Cell Cycle *3*, 1477–1480.

Jackson, L., and Evers, B.M. (2006). Chronic inflammation and pathogenesis of GI and pancreatic cancers. Cancer Treat. Res. *130*, 39–65.

Julien, S., Puig, I., Caretti, E., Bonaventure, J., Nelles, L., van Roy, F., Dargemont, C., de Herreros, A.G., Bellacosa, A., and Larue, L. (2007). Activation of

NF-kappaB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. Oncogene *26*, 7445–7456.

Kajita, M., McClinic, K.N., and Wade, P.A. (2004). Aberrant expression of the transcription factors snail and slug alters the response to genotoxic stress. Mol. Cell. Biol. *24*, 7559–7566.

Karin, M. (2006). Nuclear factor-kappaB in cancer development and progression. Nature *441*, 431–436.

Karin, M., and Greten, F.R. (2005). NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat. Rev. Immunol. *5*, 749–759.

Kim, H.J., Litzenburger, B.C., Cui, X., Delgado, D.A., Grabiner, B.C., Lin, X., Lewis, M.T., Gottardis, M.M., Wong, T.W., Attar, R.M., et al. (2007). Constitutively active type I insulin-like growth factor receptor causes transformation and xenograft growth of immortalized mammary epithelial cells and is accompanied by an epithelial-to-mesenchymal transition mediated by NF-kappaB and snail. Mol. Cell Biol. *27*, 3165–3175.

Lin, E.Y., Nguyen, A.V., Russell, R.G., and Pollard, J.W. (2001). Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J. Exp. Med. *193*, 727–740.

Lin, E.Y., Li, J.F., Gnatovskiy, L., Deng, Y., Zhu, L., Grzesik, D.A., Qian, H., Xue, X.N., and Pollard, J.W. (2006). Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res. *66*, 11238–11246.

Luo, J.L., Maeda, S., Hsu, L.C., Yagita, H., and Karin, M. (2004). Inhibition of NF-kappaB in cancer cells converts inflammation-induced tumor growth mediated by TNFalpha to TRAIL-mediated tumor regression. Cancer Cell *6*, 297–305.

Luo, Y., Zhou, H., Krueger, J., Kaplan, C., Lee, S.H., Dolman, C., Markowitz, D., Wu, W., Liu, C., Reisfeld, R.A., and Xiang, R. (2006). Targeting tumor-associated macrophages as a novel strategy against breast cancer. J. Clin. Invest. *116*, 2132–2141.

Moody, S.E., Perez, D., Pan, T.C., Sarkisian, C.J., Portocarrero, C.P., Sterner, C.J., Notorfrancesco, K.L., Cardiff, R.D., and Chodosh, L.A. (2005). The transcriptional repressor Snail promotes mammary tumor recurrence. Cancer Cell *8*, 197–209.

Naumann, M., Bech-Otschir, D., Huang, X., Ferrell, K., and Dubiel, W. (1999). COP9 signalosome-directed c-Jun activation/stabilization is independent of JNK. J. Biol. Chem. *274*, 35297–35300.

Neilson, E.G. (2006). Mechanisms of disease: Fibroblasts–a new look at an old problem. Nat. Clin. Pract. Nephrol. *2*, 101–108.

Nieto, M.A. (2002). The snail superfamily of zinc-finger transcription factors. Nat. Rev. Mol. Cell Biol. *3*, 155–166.

Olmeda, D., Jorda, M., Peinado, H., Fabra, A., and Cano, A. (2007). Snail silencing effectively suppresses tumour growth and invasiveness. Oncogene *26*, 1862–1874.

Peinado, H., Olmeda, D., and Cano, A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat. Rev. Cancer *7*, 415–428.

Radisky, D.C., Levy, D.D., Littlepage, L.E., Liu, H., Nelson, C.M., Fata, J.E., Leake, D., Godden, E.L., Albertson, D.G., Nieto, M.A., et al. (2005). Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. Nature *436*, 123–127.

Richardson, K.S., and Zundel, W. (2005). The emerging role of the COP9 signalosome in cancer. Mol. Cancer Res. *3*, 645–653.

Schweitzer, K., Bozko, P.M., Dubiel, W., and Naumann, M. (2007). CSN controls NF-kappaB by deubiquitinylation of IkappaBalpha. EMBO J. *26*, 1532–1541.

Thiery, J.P., and Sleeman, J.P. (2006). Complex networks orchestrate epithelial-mesenchymal transitions. Nat. Rev. Mol. Cell Biol. *7*, 131–142.

Vega, S., Morales, A.V., Ocana, O.H., Valdes, F., Fabregat, I., and Nieto, M.A. (2004). Snail blocks the cell cycle and confers resistance to cell death. Genes Dev. *18*, 1131–1143.

Wolf, D.A., Zhou, C., and Wee, S. (2003). The COP9 signalosome: an assembly and maintenance platform for cullin ubiquitin ligases? Nat. Cell Biol. *5*, 1029–1033.

Wyckoff, J.B., Wang, Y., Lin, E.Y., Li, J.F., Goswami, S., Stanley, E.R., Segall, J.E., Pollard, J.W., and Condeelis, J. (2007). Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res. *67*, 2649–2656.

Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R.A. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell *117*, 927–939.

Yang, X., Menon, S., Lykke-Andersen, K., Tsuge, T., Di, X., Wang, X., Rodriguez-Suarez, R.J., Zhang, H., and Wei, N. (2002). The COP9 signalosome inhibits p27(kip1) degradation and impedes G1-S phase progression via deneddylation of SCF Cul1. Curr. Biol. *12*, 667–672.

Yook, J.I., Li, X.Y., Ota, I., Fearon, E.R., and Weiss, S.J. (2005). Wnt-dependent regulation of the E-cadherin repressor snail. J. Biol. Chem. *280*, 11740–11748.

Zhou, A., Scoggin, S., Gaynor, R.B., and Williams, N.S. (2003). Identification of NF-kappa B-regulated genes induced by TNFalpha utilizing expression profiling and RNA interference. Oncogene *22*, 2054–2064.

Zhou, B.P., and Hung, M.C. (2005). Wnt, hedgehog and snail: sister pathways that control by GSK-3beta and beta-Trcp in the regulation of metastasis. Cell Cycle *4*, 772–776.

Zhou, B.P., Deng, J., Xia, W., Xu, J., Li, Y.M., Gunduz, M., and Hung, M.C. (2004). Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat. Cell Biol. *6*, 931–940.