Molecular Basis of Cell and Developmental Biology:
Wnt-dependent Regulation of the E-cadherin Repressor Snail

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Down-regulation of E-cadherin marks the initiation of the epithelial-mesenchymal transition, a process exploited by invasive cancer cells. The zinc finger transcription factor, Snail, functions as a potenti repressor of E-cadherin expression that can, acting alone or in concert with the Wnt/β-catenin/T cell factor axis, induce an epithelial-mesenchymal transition. Although mechanisms that coordinate signaling events initiated by Snail and Wnt remain undefined, we demonstrate that Snail displays β-catenin-like canonical motifs that support its GSK3β-dependent phosphorylation, β-TrCP-directed ubiquitination, and proteasomal degradation. Accordingly, Wnt signaling inhibits Snail phosphorylation and consequently increases Snail protein levels and activity while driving an in vivo epithelial-mesenchymal transition that is suppressed following Snail knockdown. These findings define a potential mechanism whereby Wnt signaling stabilizes Snail and β-catenin proteins in tandem fashion so as to cooperatively engage transcriptional programs that control an epithelial-mesenchymal transition.

E-cadherin, the prototypic member of the cadherin single-pass transmembrane glycoprotein family, regulates cell adhesion in epithelial cells in a Ca2+-dependent manner via homotypic interactions with E-cadherin molecules on opposing cell surfaces (1). The adhesive function of E-cadherin is dependent on its binding to the cytoplasmic α- and β-catenin proteins, which serve to tether the cadherin to the actin cytoskeleton (1). During embryonic development, down-regulation of E-cadherin function marks the onset of a complex program wherein epithelial cells adopt a fibroblast-like phenotype and display tissue-invasive activity, a process termed the epithelial-mesenchymal transition (EMT) (1, 2). Likewise, E-cadherin repression is thought to play a major role in the abnormal manifestation of EMT in epithelial-derived cancer types (3–5). In both development and cancer, the zinc finger transcription factor, Snail, has been implicated in E-cadherin repression via its binding to elements in the E-cadherin promoter (6). Indeed, during development, Snail plays a required role in driving the EMT programs that mark gastrulation as well as neural crest development (1, 3–6). In a similar, but misdirected fashion, neoplastic cells co-opt Snail function to adopt a mesenchymal cell-like invasive phenotype that characterizes their aberrant behavior (1, 3–7).

Although Snail plays a critical role in both physiologic and pathologic EMT (5–8), E-cadherin repression frequently occurs in tandem with activation of the Wnt signaling cascade (9–14). Of note, independent of its well defined role in E-cadherin-dependent adhesion, β-catenin also participates in Wnt signaling (1, 2). In the absence of a Wnt signal, cytosolic β-catenin is normally phosphorylated by glycogen-synthase kinase 3β (GSK3β) at one or more serine or threonine residues in its N-terminal domain (1, 2). The N-terminally phosphorylated β-catenin is then recognized and ubiquitinated by a multiprotein complex containing the F-box protein β-TrCP, with resultant degradation of the poly-ubiquitinated β-catenin in the proteasome (1, 2). Alternatively, a subset of the 19 known Wnt genes found in mammals (e.g. Wnt-1) can activate a pathway that inhibits the ability of GSK3β to phosphorylate target substrates via a process dependent on the protein Dvl (disheveled) with resultant increases in β-catenin levels (1, 2). The stabilization of β-catenin consequently leads to both enhanced nuclear accumulation and enhanced binding to members of the T cell factor (TCF) family of transcription factors (1, 2). In turn, β-catenin-TCF complexes regulate the expression of a panoply of gene products that direct cell fate, polarity, and proliferation (1, 2).

Although Wnt signaling could conceivably stabilize the pool of cytosolic β-catenin that is released from E-cadherin-bound sites as a consequence of Snail-mediated E-cadherin repression (1, 2, 9), direct interplay between the Wnt and Snail systems has remained a subject of conjecture. Herein, we demonstrate that the Snail transcript encodes a series of β-catenin-like canonical motifs that support its GSK3β-dependent phosphorylation, β-TrCP-directed ubiquitination, and proteasomal degradation via a Wnt-1-regulatable process. Because increasing evidence indicates that Wnt signals can impact on multiple cell functions in neoplastic tissues (12–16), these findings support a model wherein activation of the Wnt-GSK3β signaling cascade regulates carcinoma cell phenotype by controlling β-catenin-TCF- and Snail-dependent transcriptional programs in tandem fashion.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**A human Snail cDNA with a C-terminal FLAG (Flg) epitope tag was subcloned into three expression vectors: pCR3.1, to
generate pCR3.1-Snail-Flg; a bicistronic pCMS-EFGP, to generate pCMS-EFGP-Snail-Flg; and the retroviral vector pBabePuro (5). A human Snail cDNA with both a His$_6$ and Flag C-terminal epitope tag was cloned into the pET21 vector to generate pET21-Snail-His for expression of recombinant Snail protein in Escherichia coli. Snail mutant proteins with Flag epitope tails, including the S100A, S104A, S107A, S100A/S104A, S104A/S107A, S100A/S107A, and S96A mutants, were constructed by PCR-based methods using a wild-type Snail cDNA as a template, followed by subcloning into the pCR3.1 vector. His$_6$/Flag-tagged Snail mutants (i.e., S104A/S107A and ∆S1–109) were also generated by PCR-based methods for expression in bacteria using the pET21 construct. The E-cadherin reporter gene constructs Ecad(−108)–Luc and Ecad(−108)/EboxA.MUT/EboxB.MUT/EboxC.MUT-Luc were described previously (5). A β-TrCP-cDNA was obtained by reverse transcription-PCR-based methods, using the total mRNA of 293 cells and the β-TrCP-cDNA subcloned into pCR3.1. The dominant negative mouse β-TrCP vector (mβ-TrCP) was kindly provided by Dr. J. Z. Chen (University of Texas-Southwestern, Dallas) (44). A pCR3.1 expression vector encoding HA-tagged ubiquitin was generated by reverse transcription-PCR-based methods using HeLa total RNA. HA-tagged Wnt-1 retroviral expression vector (pLNCF-mWnt-1-HA) was a gift from O. MacDougall (University of Michigan). To generate the pSUPER-Shairna, annealed oligonucleotides 5′-GACACCTCCTAATGCTTCATTGGTCGTTATATATTGCACTTTGTTAGGGCTGGGCTCTTTTGGAAAATGCGCTTTCAATGCAGACAACATCTTTGAGGCTGAGGCTCTTACTGGAA-3′ and 5′-AGCCTTTTTCACTTTATGCAATTTTTACCTGGTTTTTTTTTTTACACACACTTTTGTGGTTGAGGCGTGCTGTCTGTTTATTTGCACTTTGTTAGGGCTGGGCTCTTTTGGAAAATGCGCTTTCAATGCAGACAACATCTTTGAGGCTGAGGCTCTTACTGGAA-3′ were inserted into pSUPER-retro vector (OligoEngine). The 19-nt human Snail target sequence (indicated by underline) was designed and verified to be specific for Snail by Blast search against the human genome and reverse transcription-PCR, respectively. A control shRNA was also used to generate snail shRNA expression constructs (45) and added to cells at a final concentration of 30 μg/ml.

**Antibodies, Immunoprecipitation, and Immunoblot Analysis**—Snail-Fig proteins were detected with anti-FLAG-M2 antibody (Sigma), Snail-His/Flg proteins were detected with either anti-His$_6$ or anti-Flag antibodies, anti-β-catenin (BD Bioscience), anti-E-cadherin (Zymed Laboratories Inc.), or anti-E-cadherin (BD Bioscience) and anti-FLAG-M2 antibodies. MCF-7-Wnt-1 cells were transfected with 1.0 μg of reporter gene only (5). Reporter gene activities were measured with a luciferase assay system (Promega) 48 h after transfection and normalized by measuring β-galactosidase activities of co-transfected pSV-gal (0.25 μg/mg Clontech) with a β-galactosidase enzyme assay system (Promega). Reporter gene activities were presented as relative light units to that obtained from mock transfected cells. The results are expressed as the averages of the ratios of the reporter activities.

**In Vivo Ubiquitination**—HA-tagged ubiquitin was co-transfected with each Flag-tagged Snail construct. After 48 h, the lysates were immunoprecipitated and immunoblotted with α-Flag or anti-HA antibodies.

**Wnt Treatment**—The cell culture and extracellular protein expression from Wnt-1 expressing RAC-311 and control MV7 cells (both cell lines kindly provided by L. R. Howe) were prepared from 0.4% NaCl buffer extract described previously (45) and added to cells at a final concentration of 30 μg/ml.

**RESULTS**

Following retroviral transduction of MCF-7 cells with a vector encoding a FLAG epitope-tagged form of human Snail (Snail-Fig), the cells displayed a morphology consistent with that seen in vitro (Fig. 1A). The expression of Snail induced cell invasion into the CAM interstitium (Fig. 1A). The expression of Snail strongly suppressed (Fig. 1A and B). To determine whether Snail can trigger tissue invasive activity in a manner consistent with the acquisition of a mesenchymal cell-like phenotype, fluorescence-labeled MCF-7 cells were cultured atop the chick chorioallantoic membrane. Whereas mock transduced MCF-7 cells were confined to the upper surface of the CAM, the Snail-expressing MCF-7 cells perforated the underlying basement membrane and invaded into the CAM interstitium (Fig. 1A). Interestingly, when the Snail protein was recovered from transfected MCF-7 (or 293) cells, it migrated as a closely spaced doublet, suggesting post-translational modifications (Fig. 1C). Although treatment with O- or N-deglycanase failed to alter the migration pattern of Snail (data not shown), treatment of protein extracts with λ protein phosphatase resulted in the loss of the more slowly migrating Snail isoform (Fig. 1C), consistent with notion that Snail is phosphorylated in MCF-7 as well as 293 cells (Fig. 1C).
human Snail amino acid sequence contains in excess of 20 serine and/or threonine residues, but the search for Snail domains that might be phosphorylated was considerably narrowed given that preliminary studies indicated that a mutant form of Snail lacking amino acids 92–120 migrated as a single band (data not shown). Of note, inspection of this region of the Snail protein revealed a conserved serine/threonine rich N-terminal phosphorylation motif found in β-catenin (Fig. 1D) (1, 2). Further, this region of the Snail protein also contained the DSGX5X5 destruction motif recognized by β-TrCP in β-catenin as well as the IκB protein (Fig. 1D) (1, 2).

Consistent with the possibility that Snail, like β-catenin, is recognized and bound by GSK3β (1, 2), immunoprecipitates of Snail-Flg recovered from transfected 293 cells contained endogenous GSK3β (Fig. 2A). In light of the existence of multiple potential phosphorylation sites within the amino acid 92–120 region of Snail, a series of single, or double, Ser → Ala substitutions were generated in the Snail-Flg expression vector. The constructs encoding the Snail mutants were then expressed in 293 cells, and the electrophoretic mobility of the Snail-Flg proteins was examined prior to and following Snail protein phosphatase treatment of the cell extracts. Although single Ser → Ala substitutions at Ser100 (S100A), Ser104 (S104A), or Ser107 (S107A) or double substitutions at Ser100/Ser104 (S100A,S104A) or Ser100/Ser107 (S100A,S107A) did not affect the λ protein phosphatase-induced gel shift, dual Ser → Ala mutations at Ser104 and Ser107 (S104A,S107A) ablated the gel shift associated with λ protein phosphatase treatment (Fig. 2B). A key role for the Ser104 and Ser107 residues was further corroborated by data showing that recombinant GSK3β directly phosphorylated wild-type Snail via a LiCl-sensitive process in vitro (17) but not the S104A,S107A double mutant form of Snail (Fig. 2C).

Indeed, whereas pulse-chase analysis demonstrated wild-type Snail was rapidly degraded in the course of a 4-h chase period, expression levels of the S104A,S107A mutant remained stable (Fig. 2D).

The inability of GSK3β to phosphorylate the S104A,S107A mutant Snail protein in vivo could conceivably reflect nonspecific alterations in the conformation of the mutant Snail (18). However, consistent with the notion that Snail is phosphorylated in vivo by GSK3β, phosphorylation of wild-type Snail in 293 cells was blocked by treatment of the cells with the GSK3β inhibitor, LiCl (Fig. 2E) (17, 18). Coincident with the LiCl-dependent inhibition of Snail phosphorylation, steady state levels of Snail protein were observed in the presence of LiCl (Fig. 3A).
endogenous GSK3β levels in 293 cells engineered to ectopically express Snail, the steady state levels of Snail protein were likewise increased (Fig. 2F). To further determine whether GSK3β regulates the endogenous level of Snail in a fashion similar to that observed for the exogenously expressed protein, MCF-7 cells were cultured in the presence of either LiCl or the GSK3-specific inhibitor, CHIR99021 (19). As expected, GSK3 inhibition by either reagent induced significant increases in the steady state nuclear levels of endogenous Snail in tandem with similar increases in β-catenin (Fig. 2G).

**In vivo**, a subset of Wnt family members trigger largely undefined pathways that lead to the inhibition of GSK3β activity (1, 20). Because Wnt expression occurs frequently in a temporal fashion coincident with developmental EMT programs (1, 20–22), we sought to determine whether GSK3β-dependent phosphorylation of Snail could be inhibited by Wnt-1. Indeed, the phosphorylation of ectopically expressed wild-type Snail was suppressed markedly by treatment of the cells with Wnt-1-conditioned medium, and in concert, Snail half-life was increased (Fig. 3, A and B). Although Wnt-1 also increased the steady state expression levels of wild-type Snail, no effect was observed on the levels of the S104A,S107A mutant (Fig. 3C).

Consistent with the premise that GSK3β regulates Snail and β-catenin levels in a cooperative fashion, Wnt-1 signaling mediated tandem increases in the nuclear levels of endogenous Snail as well as β-catenin (Fig. 3D).

Following the GSK3β-dependent phosphorylation of β-catenin, a 32P-GSKXpS motif in the post-translationally modified protein is recognized by the E3 ubiquitin ligases β-TrCP1 or β-TrCP2, which mediate the ubiquitination of phosphorylated β-catenin and target the protein for subsequent degradation in

![Figure 2: GSK3β-dependent Snail phosphorylation](https://www.jbc.org/content/early/2014/07/01/jbc.M114.620787/F2.large.jpg)

**Fig. 2. GSK3β-dependent Snail phosphorylation.** A, GSK3 (both α and β) were detected in control and Snail-Flg-transfected 293 cell lysates by immunoblot analysis (*top panel*). GSK3β/Snail complexes were isolated in immunoprecipitates recovered from lysates of control or Snail-Flg-transfected 293 cells following pull-down with α-Flg-agarose beads, SDS-PAGE, and immunoblot with anti-GSK3 antibody (*middle panel*). Snail protein levels in the control or Snail-Flg-transfected cells were assessed by α-Flg blot (*bottom panel*). B, α-Flg-tagged Snail mutants harboring Ser → Ala substitutions were expressed in 293 cells, lysates from the transfectants incubated with or without λ protein phosphatase, and changes in Snail mobility analyzed by α-Flg immunoblot. C, His-tagged recombinant wild-type Snail, S104A,S107A mutant Snail, or a Δ31–109 Snail deletion mutant (2 μg of protein each) were incubated with recombinant GSK3β and (γ-32P)ATP for 2 h at 25 °C. Phosphorylated and total Snail protein were resolved by SDS-PAGE and visualized by autoradiography or α-Flg blot (*top panel*). Snail phosphorylation by GSK3β was inhibited by LiCl (20 mM) (*bottom panel*). D, wild-type or S104A,S107A mutant Snail-Flg was expressed in 293 cells, immunoprecipitated (IP) with α-Flg, and immunoblotted (IB) with either anti-phosphoserine (anti-pSer) or α-Flg antibodies (*top panel*). Following a 20-min pulse with [35S]Met/Cys, 293 cells transiently transfected with an GFP-Snail-Flg expression vector and without Wnt-1-conditioned medium for 18 h (top panel). Snail half-lives in LiCl-treated cells were measured by pulse-chase analysis (*bottom panel*) as described above. E, 293 cells were transiently transfected with an GFP-Snail-Flg expression vector and either GSK3β or scrambled siRNA duplexes. GSK3αβ and Snail levels were monitored by anti-GSK3 and α-Flg immunoblot. G, MCF-7 cells (1 × 10⁶) were incubated in the absence or presence of LiCl (40 mM) or CHIR99021 (2 μM) for 8 h. Following SDS-PAGE of nuclear extracts, endogenous Snail and β-catenin and actin were detected by immunoblot. Nuclear β-actin was used as a loading control.

![Figure 3: Wnt-1 regulation of Snail phosphorylation](https://www.jbc.org/content/early/2014/07/01/jbc.M114.620787/F3.large.jpg)

**Fig. 3. Wnt-1 regulation of Snail phosphorylation.** A, 293 cells transfected with Snail-Flg were treated with control or Wnt-1-conditioned medium for 18 h. Phosphorylated Snail levels were determined following immunoprecipitation (IP, 1× and 0.5× volume of the respective cell lysates) with α-Flg and immunoblotted (IB) with anti-Ser(P) (anti-pSer). B, Snail half-life in control or Wnt-1-treated 293 cells was determined by pulse-chase analysis and SDS-PAGE/autoradiography of α-Flg immunoprecipitates. C, 293 cells were transfected with either GFP-Snail-Flg or S104A,S107A Snail-Flg and incubated with control or Wnt-1-conditioned cell extracts for 18 h. Snail, GFP, and β-actin were detected by α-Flg, α-GFP, and α-β-actin immunoblot. D, MCF-7 cells (1 × 10⁶) were incubated with control or Wnt-1-conditioned cell extracts for 18 h. MCF-7 nuclear extracts were immunoblotted for endogenous snail or β-catenin with nuclear β-actin serving as a loading control. WT, wild type.
the proteasome (1, 2, 23). To determine whether β-TrCP recognized the homologous DS Gerard motif found in Snail, a FLAG epitope-tagged wild-type Snail protein was ectopically expressed in 293 cells. Following immunoprecipitation of the extracts with an anti-FLAG antibody, endogenous β-TrCP1 protein was found to be specifically recovered in association with Snail (Fig. 4A). Furthermore, [35S]Met-labeled β-TrCP1 prepared by in vitro translation was shown to bind to the wild-type Snail protein recovered from lysates of transfected 293 cells (Fig. 4B). However, β-TrCP1 was unable to form a stable complex with either the phosphorylation-resistant S104A,S107A mutant Snail protein or the Snail mutant harboring a single Ser96 → Ala substitution within the putative β-TrCP1-binding domain (Fig. 4B). Consistent with these findings, the steady state levels of wild-type Snail were enhanced by co-expressing a dominant negative form β-TrCP1 in 293 cells (Fig. 4C) as well as MCF-7 cells (data not shown).

In a fashion similar to that described for β-catenin (1, 2, 22), further studies demonstrated that wild-type Snail protein undergoes rapid ubiquitination in vitro (Fig. 4D). By contrast, ubiquitination of Snail proteins containing mutations at either key phosphorylation or recognition sites (i.e. Ser104,Ser107 or Ser96 within the DS Gerard motif) was strongly suppressed, as was the formation of high molecular weight Snail complexes in Wnt-1-treated cells (Fig. 4, D and E). Although the Ser96 → Ala mutant Snail protein was phosphorylated in 293 cells, the loss of this key phosphorylation site within the DSG Gerard destruction motif resulted in the failure of the protein to be recognized and ubiquitinated by β-TrCP, resulting in an increased steady state level and prolonged half-life of the mutant (Fig. 4, B, D, and F). Indeed, the half-life of the Ser96 → Ala mutant Snail protein was similar to that observed follow-
FIG. 5. Regulation of E-cadherin repressor and invasive activities by degradation-resistant Snail mutants. A, the E-cadherin repressor activity of wild-type, S104A, S107A, and S96A Snail were assessed with the reporter construct, Ecad(−108)-Luc, which contains the wild-type (WT) promoter sequence from nt −108 to +125 of the endogenous E-cadherin promoter or a control construct, Ecad(−108)/EboxA,MUT/EboxB,MUT/EboxC.MUT-Luc, which harbors mutations in all three E-boxes (3×Mut). Snail mRNA levels in each of the transfectants was monitored by reverse transcription-PCR. B, E-cadherin expression in MCF-7 cells transiently transfected with wild-type S104A, S107A or S96A Snail was assessed by indirect immunofluorescence with anti-E-cadherin mAb (green). Snail was stained with α-Fib rabbit polyclonal antibody (red). The images were obtained by confocal microscopy. C, MCF-7 cells were transiently transfected with control, wild-type, S104–107A, or S96A Snail, labeled with fluorescent beads (green) and cultured atop the chick CAM for 3 days. CAMs were fixed, cell nuclei were stained with DAPI (blue), and tissue sections were examined by fluorescence microscopy. The upper edge of the CAM surfaces is marked by the black arrows. The numbers of invaded cells were counted in 10 randomly selected fields for each experiment and expressed as the means ± S.D. (control), 0 ± 0; wild type, 4 ± 2; S104A, S107A, 30 ± 6; and S96A, 48 ± 4).

Snail has been proposed to play a key role during both development and cancer by virtue of its ability to repress E-cadherin expression and drive EMT (3–5). To assess the consequences of Snail stabilization on its E-cadherin repressor activity, we compared the relative abilities of wild-type Snail and the S104A, S107A or Ser96Ala mutants to regulate an E-cadherin promoter gene construct previously shown to be Snail-sensitive (3–5). Because Snail has been implicated in repression of E-cadherin transcription through the three E-box domains present in the proximal E-cadherin promoter region, a control version of the construct was employed wherein all three E-box domains were mutated (3×Mut) (5). Whereas limiting amounts (25 ng) of the expression vector encoding wild-type Snail exerted only modest inhibitory effects on the wild-type E-cadherin reporter gene construct in MCF-7 cells, equivalent amounts of the expression vectors encoding the Ser104/Ser107 or Ser96Ala mutants exerted stronger repression effects on E-cadherin promoter activity (Fig. 5A). An analysis of the levels of Snail transcripts expressed ectopically in the cells confirmed that the differences in repressor activity observed between the wild-type and mutant Snail constructs did not result from variations in the mass of DNA transfected or the levels of transcripts produced from the vectors (Fig. 5A). When compared with wild-type Snail, the Ser104/Ser107 and Ser96Ala mutants also displayed an enhanced activity to suppress endogenous E-cadherin expression in transfected MCF-7 cells (Fig. 5B). Although phosphorylation of Snail has been reported to regulate its nuclear localization and repressor activity by modulating the activity of a nuclear export sequence located between residues 132 and 143 (24), both the Ser104/Ser107 and Ser96Ala mutants were preferentially localized to the nuclear compartment in a fashion comparable with wild-type Snail following transfection into MCF-7 cells (Fig. 5B).

To address the possibility that stabilized Snail might play an exaggerated role in engaging an EMT program capable of promoting an invasive phenotype, MCF-7 cells were transiently transfected with wild-type Snail or either of the S104A, S107A or Ser96Ala mutants, and the in vivo invasive behavior of the MCF-7 transfectants was assessed by culturing the cells atop the chick CAM. Although control transfectants MCF-7 cells were unable to penetrate the CAM surface, both the S104A, S107A and the Ser96Ala mutants induced a more aggressive tissue invasive program than wild-type Snail (Fig. 5C).

Finally, given the ability of Wnt-1 to induce the up-regulation of endogenous levels of nuclear Snail protein, coupled with its ability to repress E-cadherin expression and support a tissue invasive EMT program, we sought to determine whether Wnt-1 drives EMT via a Snail-dependent process. Indeed, following Wnt-1-transduction, MCF-7 cells adopted a fibroblastic phenotype, down-regulated E-cadherin levels, increased nuclear concentrations of β-catenin and Snail proteins, and suppressed E-cadherin promoter activity (Fig. 6, A–C). Moreover, consistent with the proposition that a Wnt-driven EMT program has been induced, the Wnt-1-transduced MCF-7 cells expressed tissue invasive activity similar to that observed with Snail-transduced cells (compare Fig. 6C with Fig. 1A). If, however, Snail expression in Wnt-transduced MCF-7 cells was targeted with any one of four different shRNA constructs, Snail...
mRNA expression was inhibited completely (data not shown), the suppression of E-cadherin promoter activity was reversed (Fig. 6B), and tissue invasive activity was lost (Fig. 6C). By contrast, transfection with a control shRNA neither affected E-cadherin promoter activity or invasion (Fig. 6, B and C). Hence, Snail plays a key role in regulating Wnt-1-induced EMT in MCF-7 cells.

**DISCUSSION**

Snail plays a key role in EMT processes characterizing tumor progression, as well as developmental programs ranging from gastrulation to neural crest formation (6). The unexpected identification of a β-catenin-like consensus motif in the N-terminal domain of Snail that supports its GSK3β-dependent phosphorylation, β-TrCP-dependent ubiquitination, and proteasomal degradation highlights a new model wherein Wnt signaling participates in the co-regulation of Snail-driven as well as β-catenin/LEF-1-driven transcriptional programs. With regard to β-catenin, its dual phosphorylation by GSK3β and casein kinase-1 appears to take place in a multi-protein complex that includes adenomatous polyposis coli (APC) and Axin tumor suppressor proteins (1, 26, 27). Although a required role for casein kinase-1 can phosphorylate Snail in vitro.\(^2\) The ability of GSK3β to phosphorylate primed versus unprimed Snail in vivo, however, requires further study. These findings notwithstanding, it is conceivable that Snail, like β-catenin, might be stabilized by mutations in APC, Axin, or the Snail transcript itself, thus predisposing to the development of carcinogenic states (3–5, 29). However, because increasing evidence supports important roles for the activation of canonical Wnt signaling in normal as well as neoplastic states (12–15, 30, 31), inappropriate activation of Snail-dependent transcriptional programs are not necessarily constrained to pathological processes that require somatic mutations in the Snail gene itself or accessory molecules that regulate its phosphorylation and degradation.

During embryogenesis, E-cadherin down-regulation appears to be temporally linked to fibroblast growth factor signaling, Snail expression, and activation of the canonical Wnt signaling cascade (9–11, 20, 21, 32). Recent studies suggest that β-catenin, upon its association with the TCF transcription factor family member, LEF-1, can act in a cooperative fashion with Snail to suppress E-cadherin transcription via LEF-1/β-catenin

interactions with sequences upstream of the E-cadherin promoter (11). Our findings raise the possibility of a more complex and interdigitated scheme wherein exposure of normal or neoplastic cells to a combination of Snail-inducing growth factors and Wnts would stabilize the intracellular levels of the Snail and β-catenin proteins by as yet undefined mechanisms that serve to shield these regulatory molecules from GSK3β-dependent phosphorylation (1, 20, 33). In turn, Snail acting as a transcriptional repressor of E-cadherin potentially facilitates the intracellular transfer of E-cadherin-bound β-catenin to a Wnt-stabilized “signaling” pool (3–5, 9). Snail might further synergize with the Wnt/β-catenin axis by inducing LEF-1 expression either directly or by suppressing BMP expression (11, 29, 34). Additional studies are needed to determine the relative roles that stabilized Snail and β-catenin (and perhaps, γ-catenin) (35) play in coordinating Wnt-induced EMT programs, but Snail appears to play a dominant, if not required, role in driving the tissue invasive process in MCF-7 breast carcinoma cells. Given the parallels noted between developmental and neoplastic EMT programs (6), these findings are consistent with a required role for Snail during formation of the mesoderm germ layer (36).

Interestingly, our results complement and extend a very recent report by Zhou et al. (37) that similarly describes a role for GSK3β in regulating Snail phosphorylation and activity. In a variation of the theme presented here, these investigators proposed that Snail displays two distinct GSK3β consensus motifs. The first GSK3β recognition sequence, as in our study, centers on the putative β-TrCP binding site at Ser(P)96 and Ser(P)100. They then propose that a second motif, 107S

When a series of six Ser → Ala substitutions were introduced into the Snail protein at Ser96, Ser100, Ser107, Ser111, Ser115, and Ser119, and the mutant construct was overexpressed in target cells (37). Hence, our demonstration that Wnt signaling not only regulates endogenous Snail but also drives Snail-dependent EMT represents the first functional demonstration of the potential importance of this pathway in neoplastic cells.

By embedding within Snail a series of β-catenin-like recognition motifs for GSK3β and β-TrCP, a cooperative system appears to have evolved that allows for the tandem regulation of Snail- and β-catenin-TCF-regulated target genes as they converge on the EMT process. Of note, although the $^{96}S\times_2D\times_3S\times_2S\times_2S^{117}$ motif is conserved in human as well as other mammals (Fig. 1), substitutions are observed in more distinctly related vertebrate species (Table I) where it has already been noted that Snail family members display highly divergent N-terminal regions (6). Although differences in Snail function among species make direct comparisons between conserved regions difficult to interpret (e.g. Slug, rather than Snail, plays the dominant role in driving EMT in the chick) (6), the Ser96 → Glu substitution is consistent with more recent studies demonstrating the ability of β-TrCP to both recognize a charged glutamate residue in place of a Ser(P) moiety (39) as well as target substrates harboring a DSG$_2$ motif where n > 2 (23). We do note, however, that the bulk of the putative nuclear localization signal assigned by Zhou et al. to the $^{111}S\times_4X\times_5S\times_4X\times_5S^{119}$ motif (37) is not conserved in lower vertebrates (Table I). This caveat aside, it appears that a common but modified scheme for regulating Snail activity may extend beyond mammals to include less closely related vertebrate species where constraints specific to given organisms have resulted in the evolution of a set of similar, yet distinct, molecular solutions.

Until recently, attention has focused on the role of mutational defects in APC, β-catenin, and Axin in activating the Wnt/β-catenin-TCF pathway in human cancer. Intriguingly, although mutational defects in β-catenin, Axin, and APC are fairly common in certain cancer types, in other neoplastic states such as breast cancer, mutational defects in well defined components of the canonical Wnt pathway have only very rarely been identified (5, 40). These findings could be interpreted as evidence that Wnt signaling cascades play only a limited role in the development and progression of breast and other cancers where mutational defects in the canonical Wnt signaling pathway have rarely been seen. However, recent studies have suggested an important functional role for Wnt signals in various cancers and the possibility that nonmutational mechanisms may activate Wnt signaling via either enhanced local (autocrine or paracrine) expression of Wnts, epigenetic inactivation of the expression of Wnt antagonists (such as secreted frizzled-related proteins of Wnt inhibitory factor-1), or the up-regulation of potential GSK3β inhibitory factors (12, 14, 16, 40). Curiously, and inconsistent with the view that Wnt signals in cancer cells transmit their effects solely via stabili-
zation of β-catenin, a requirement for upstream Wnt ligand function/activity has even been implicated in cancers with constitutive deregulation of β-catenin resulting from APC or β-catenin mutations (13–15). Taken together, these data support a model wherein inappropriate activation of the Wnt-GSK3β signaling cascade regulates carcinoma cell phenotype not only via effects on β-catenin-TCP-dependent transcription but also through the ability of the Wnt-GSK3β signaling cascade to activate Snail-driven transcriptional programs. Indeed, given that Snail activity extends beyond the regulation of EMT-related processes to include cell death and growth (8, 41–43), our findings support a new operating platform by which canonical Wnt signals can regulate tumor cell phenotype.

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