

# Dual regulation of Snail by GSK-3 $\beta$ -mediated phosphorylation in control of epithelial–mesenchymal transition

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The phenotypic changes of increased motility and invasiveness of cancer cells are reminiscent of the epithelial–mesenchymal transition (EMT) that occurs during embryonic development. Snail, a zinc-finger transcription factor, triggers this process by repressing E-cadherin expression; however, the mechanisms that regulate Snail remain elusive. Here we find that Snail is highly unstable, with a short half-life about 25 min. We show that GSK-3 $\beta$  binds to and phosphorylates Snail at two consensus motifs to dually regulate the function of this protein. Phosphorylation of the first motif regulates its  $\beta$ -Trcp-mediated ubiquitination, whereas phosphorylation of the second motif controls its subcellular localization. A variant of Snail (Snail-6SA), which abolishes these phosphorylations, is much more stable and resides exclusively in the nucleus to induce EMT. Furthermore, inhibition of GSK-3 $\beta$  results in the upregulation of Snail and downregulation of E-cadherin *in vivo*. Thus, Snail and GSK-3 $\beta$  together function as a molecular switch for many signalling pathways that lead to EMT.

Approximately 90% of cancer deaths result from the local invasion and distant metastasis of tumour cells; therefore, an understanding of metastasis is urgently needed. One important insight came from the discovery that the increased motility and invasiveness of cancer cells is reminiscent of the EMT that occurs during embryonic development<sup>1,2</sup>. In EMT, epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility. This process is associated with the functional loss of E-cadherin<sup>1,2</sup>. In cancer, downregulation of E-cadherin is the key step towards the invasive phase of carcinoma, and dominant transcriptional repression is largely responsible for the loss of E-cadherin expression<sup>3–7</sup>.

Several transcription factors have been implicated in this repression, including zinc-finger proteins of the Snail/Slug family<sup>8–10</sup>,  $\delta$ EF1/ZEB1, SIP1 (ref. 11) and the basic helix–loop–helix E12/E47 factor<sup>12</sup>. Snail was first identified in *Drosophila*, where it downregulates the transcription of shotgun (an E-cadherin orthologue) to control gastrulation<sup>2</sup>. Snail homologues have subsequently been found in many other species, including humans<sup>2</sup>. Recently, Snail was shown to repress the expression of E-cadherin and induce EMT in MDCK cells<sup>8–10</sup>, indicating that Snail has a fundamental role in EMT through its suppression of E-cadherin. Although TGF- $\beta$  and the oestrogen receptor were shown to regulate Snail transcription<sup>13,14</sup>, the mechanisms that modulate the concentration, location and function of Snail have remained elusive.

Here, we report that Snail is regulated by GSK-3 $\beta$ <sup>15–18</sup>. We further show that two phosphorylation motifs within Snail, one for protein degradation and the other for subcellular localization, dually regulate the function of this protein.

## RESULTS

### Snail is a labile protein

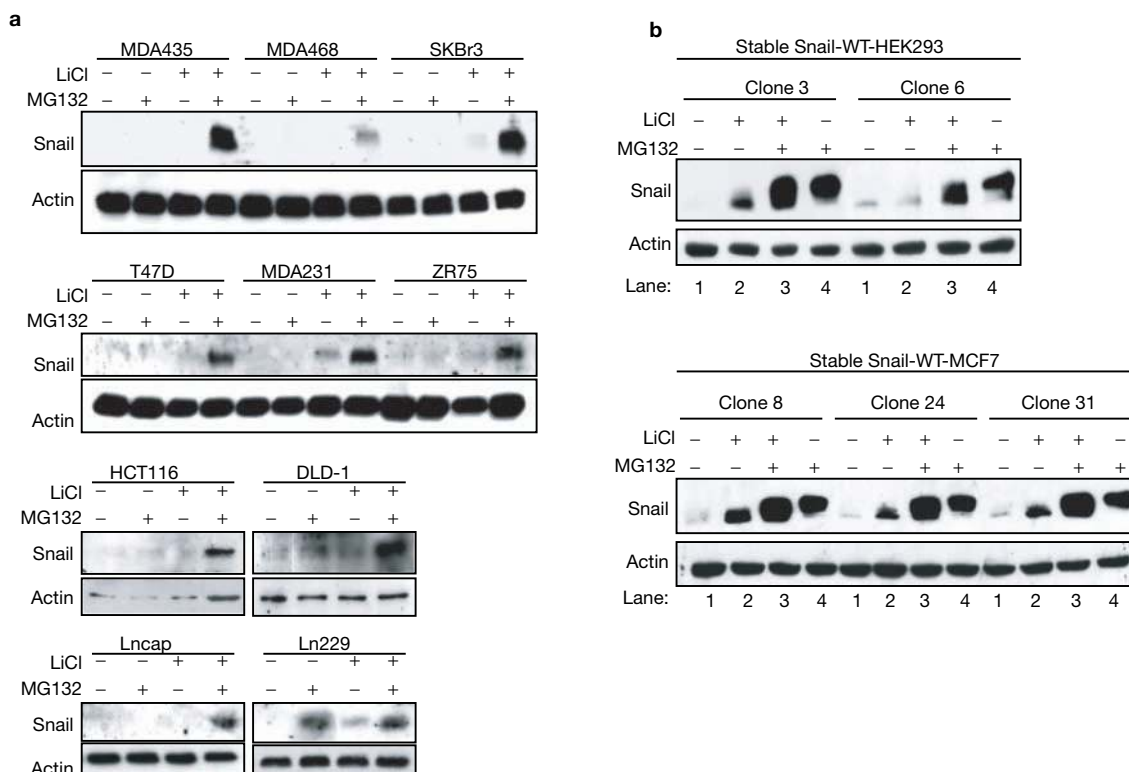
Snail is a transcriptional repressor of E-cadherin. To study the causal relationship between the two, we examined the expression of Snail in 26 cancer cell lines and initially detected it in none (data not shown). We reasoned that Snail might be tightly regulated by the ubiquitin–proteasome pathway<sup>19</sup>. Indeed, there was a slight detection of Snail in cells pre-treated with a proteasome inhibitor, MG132, or a potent GSK-3 $\beta$  inhibitor, lithium<sup>20</sup> (Fig. 1a). Surprisingly, the concentration of Snail was markedly elevated in cells that were pre-treated with both lithium and MG132 (Fig. 1a). To extend these observations, we generated stable Snail transfectants in HEK293 cells (Snail–HEK293 cells) and MCF7 cells (Snail–MCF7 cells). Similarly to before, Snail was marginally detected in the Snail–HEK293 and Snail–MCF7 stable clones. However, the amount of Snail was greatly increased in cells pre-treated with lithium or MG132, and even further increased in cells pre-treated with both lithium and MG132 (Fig. 1b). Moreover, Snail in cells pre-treated with MG132 was slow in migration on an SDS–PAGE gel (Fig. 1b). The slow migration of Snail was greatly inhibited after treatment of the cells with both lithium and MG132, compared with that treated with MG132 alone (Fig. 1b). We speculated that the slowly migrating band represented phosphorylated Snail. The fact that Snail was regulated by lithium and MG132 indicated that GSK-3 $\beta$  phosphorylation and the proteasome pathways were involved in the regulation of Snail.

### Snail is a GSK-3 $\beta$ substrate

We noticed that Snail contains two GSK-3 $\beta$  phosphorylation motifs

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**Figure 1** Suppression of the GSK-3 $\beta$  and proteasome pathways stabilizes Snail. (a) Cells were treated with or without 10  $\mu$ M MG132 or 40 mM LiCl, or both, for 5 h and analysed by western blotting with antibodies

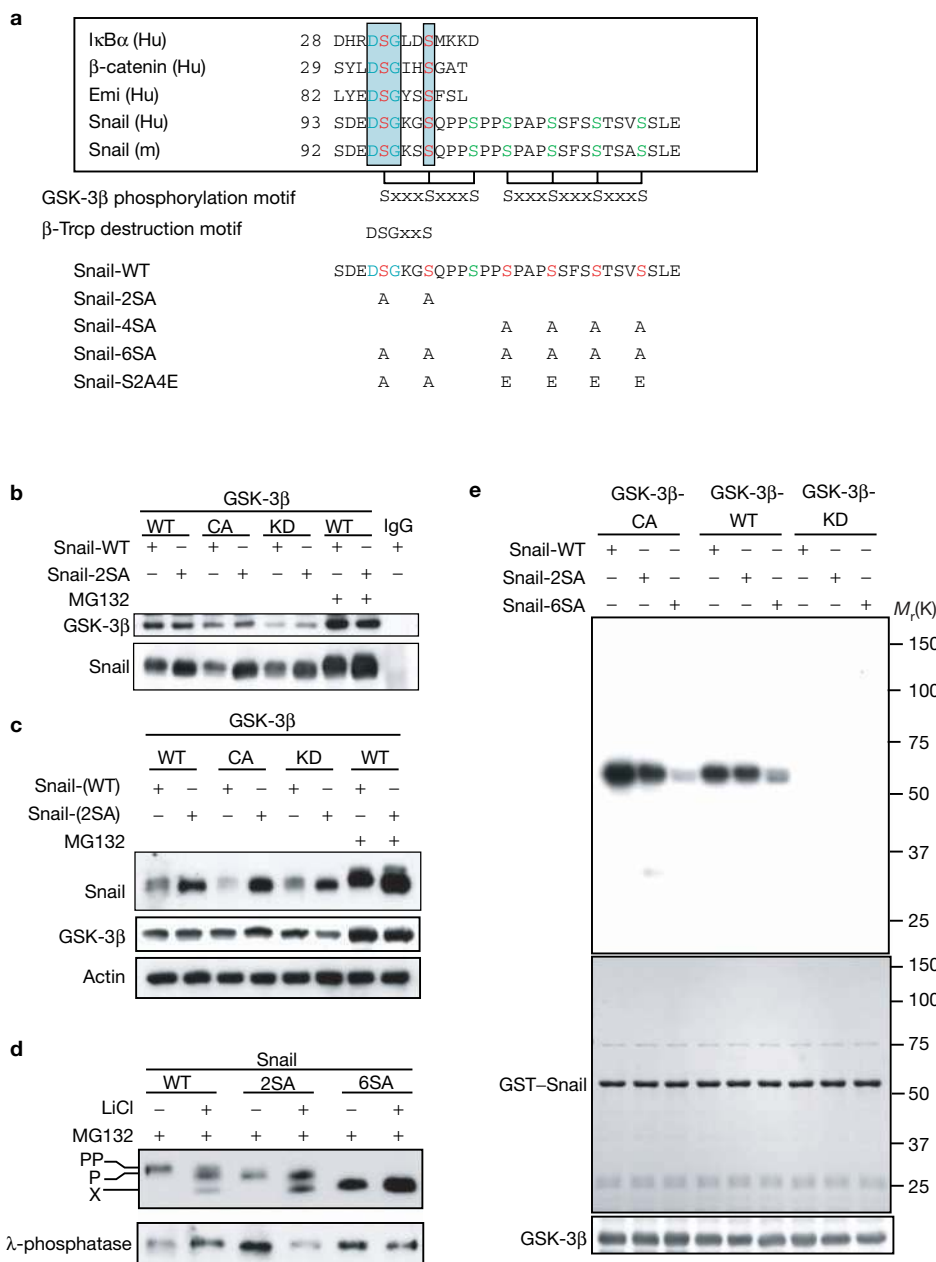
against Snail. (b) Cells from two clones of Snail-WT-HEK293 and three clones of Snail-WT-MCF7 cells were treated, and Snail was examined as described in a.

separated by two proline residues (Fig. 2a). The first GSK-3 $\beta$  phosphorylation motif overlapped with a well characterized  $\beta$ -Trcp destruction box that is also found in I $\kappa$ B,  $\beta$ -catenin, and Emi (Fig. 2a)<sup>21–24</sup>. To determine whether GSK-3 $\beta$  interacts with Snail, Snail was immunoprecipitated. The bound GSK-3 $\beta$  was clearly detected and their association was markedly stabilized in the presence of MG132 (Fig. 2b). To determine whether GSK-3 $\beta$  influences the stability of Snail, GSK-3 $\beta$  and Snail were co-expressed in 293T cells. We found that constitutively active GSK-3 $\beta$  (GSK-3 $\beta$ -CA) induced more Snail degradation than the wild-type and kinase-dead GSK-3 $\beta$  (GSK-3 $\beta$ -WT and GSK-3 $\beta$ -KD, respectively; Fig. 2c). Thus, GSK-3 $\beta$  physically interacts with Snail and induces Snail proteolysis. Interestingly, the Snail variant in which two serine residues that correspond to the first GSK-3 $\beta$  and  $\beta$ -Trcp destruction motif are replaced by alanine (2SA; Fig. 2a), was much more stable, indicating that the first GSK-3 $\beta$  phosphorylation motif regulates the stability of Snail. To examine whether GSK-3 $\beta$  can affect the phosphorylation of Snail *in vivo*, wild-type Snail (Snail-WT) and variant Snail proteins (Snail-2SA or Snail-6SA) were immunoprecipitated and subjected to immunoblotting. Snail-WT proved to be the slowest to migrate, whereas Snail-6SA was the fastest (Fig. 2d). In addition, three bands were observed when Snail-WT was immunoprecipitated from cells treated with lithium, corresponding to Snail-WT, Snail-2SA and Snail-6SA, respectively. Similarly, two bands were observed when Snail-2SA was immunoprecipitated, corresponding to Snail-2SA and Snail-6SA, respectively. However, when Snail-6SA was immunoprecipitated, only one band was observed. The slowly migrating bands observed after immunoprecipitation of Snail-WT and Snail-2SA were caused by GSK-3 $\beta$  phosphorylation, as these bands completely disappeared when the immunoprecipitated Snails were treated with protein phosphatase

(Fig. 2d). An immuno-complex kinase assay was also performed to further confirm that Snail was indeed phosphorylated at the two consensus motifs by GSK-3 $\beta$ -WT or GSK-3 $\beta$ -CA, but not by GSK-3 $\beta$ -KD (Fig. 2e). These results show that GSK-3 $\beta$  physically interacts with Snail and specifically phosphorylates it at two consensus motifs.

### The stability of Snail is dependent on its interaction with $\beta$ -Trcp

Because Snail was highly unstable (Fig. 1 and 2c), we examined the stability of Snail-WT, Snail-2SA and Snail-6SA. Initially, Snail-WT was barely detectable, but the concentration of the protein gradually increased and reached a maximum 5 h after MG132 treatment (Fig. 3a, b). However, variant Snails (Snail-2SA and Snail-6SA) were much more stable than the Snail-WT, as their protein concentration did not change significantly after MG132 treatment. We also measured the half-lives of wild-type and mutant Snails. Snail-WT had a half-life of about 25 min, but the variant Snails (Snail-2SA and Snail-6SA) had a half-life of 180 min (Fig. 3c, d). The same half-lives for both Snail-2SA and Snail-6SA indicated that the first GSK-3 $\beta$  phosphorylation motif was important in regulating the stability of Snail. The first GSK-3 $\beta$  phosphorylation motif overlaps with the  $\beta$ -Trcp destruction box, DSGXXS, which is also present in  $\beta$ -catenin, I $\kappa$ B and Emi (Fig. 2a)<sup>21–24</sup>. To determine whether  $\beta$ -Trcp regulates the stability of Snail,  $\beta$ -Trcp or  $\beta$ -Trcp $\Delta$ F (a  $\beta$ -Trcp variant that lacks the F-box domain) was expressed in Snail-MCF7 and Snail-HEK293 cells.  $\beta$ -Trcp induced the degradation of Snail, but not  $\beta$ -Trcp $\Delta$ F (Fig. 3e). To further examine whether the DSGXXS motif within Snail was required for the  $\beta$ -Trcp-mediated degradation,  $\beta$ -Trcp and Snail proteins (wild-type and variants) were co-expressed in 293T cells. Wild-type Snail was much more unstable when excess  $\beta$ -Trcp was present (Fig. 3f) and  $\beta$ -Trcp only induced the proteolysis of Snail-WT

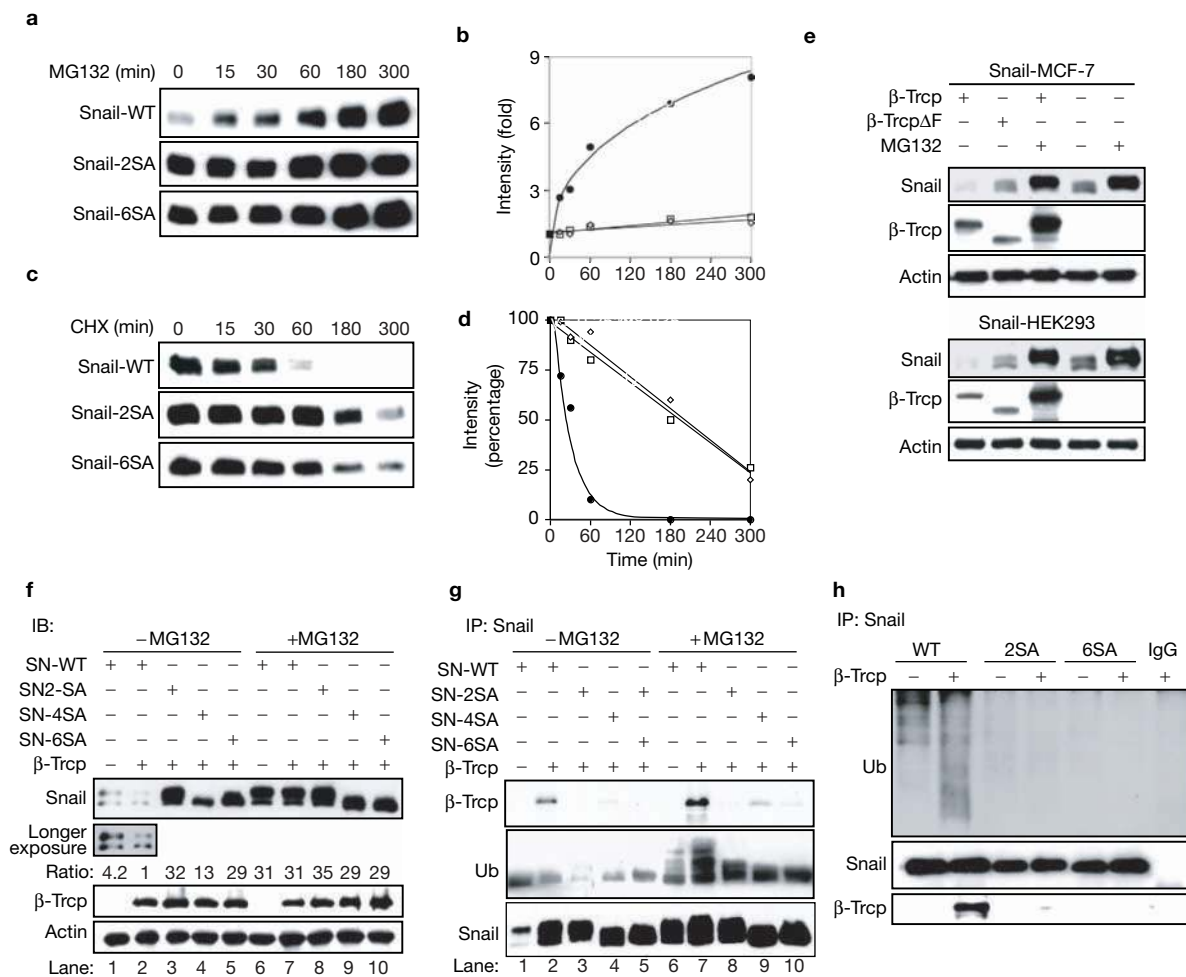


**Figure 2** GSK-3 $\beta$  interacts with Snail and phosphorylates it at two consensus motifs. **(a)** Structure of Snail showing the positions of two GSK-3 $\beta$  phosphorylation motifs and the  $\beta$ -Trcp destruction box. The corresponding constructs that were used in this study are also shown. **(b)** Flag-tagged Snail-WT or Snail-2SA coexpressed with Myc-tagged GSK-3 $\beta$  in 293T cells. Snail was immunoprecipitated with anti-Flag antibody and analysed by western blotting. GSK-3 $\beta$  was detected with an anti-Myc antibody. **(c)** One-fifth of cell lysate for each sample in **b** was subjected to western blot analysis to examine the expression of Snail and GSK-3 $\beta$ . Actin was used as a control for equal

loading. **(d)** Snail was immunoprecipitated from Snail-WT-HEK293, Snail-2SA-HEK293 or Snail-6SA-HEK293 cells treated with or without LiCl for 5 h. The immunocomplex was then incubated with or without  $\lambda$ -phosphatase for 30 min, resolved on a 14% SDS-PAGE gel and analysed by western blotting. **(e)** Myc-tagged GSK-3 $\beta$  was expressed and immunoprecipitated from 293T cells. The kinase reaction was performed as described in the Methods section. The two lower panels show the SDS-PAGE result (gel stained with Coomassie brilliant blue) for GST-Snail proteins and western blot for GSK-3 $\beta$  used in the phosphorylation reaction, respectively.

and Snail-4SA, both of which possess an intact  $\beta$ -Trcp motif (Fig. 3f). Moreover, when Snail was immunoprecipitated, bound  $\beta$ -Trcp was only found in Snail-WT and Snail-4SA transfectants, although at a reduced level (Fig. 3g). The inability of  $\beta$ -Trcp to interact with Snail-2SA and Snail-6SA was consistent with previous findings that the phosphorylation of the DSGXXS motif is required for the binding of  $\beta$ -Trcp with its substrates. When the same membrane was re-blotted with an

anti-ubiquitin antibody, more ubiquitination was seen with wild-type Snail than the variant Snails. Similar results were obtained when  $\beta$ -Trcp was expressed in Snail-HEK293 cells (Fig. 3h). Taken together, these results indicate that Snail is highly unstable, with a half-life of about 25 min. Mutation of the first GSK-3 $\beta$  phosphorylation motif markedly stabilized Snail by inhibiting the binding of  $\beta$ -Trcp, thus suppressing the ubiquitination of Snail.



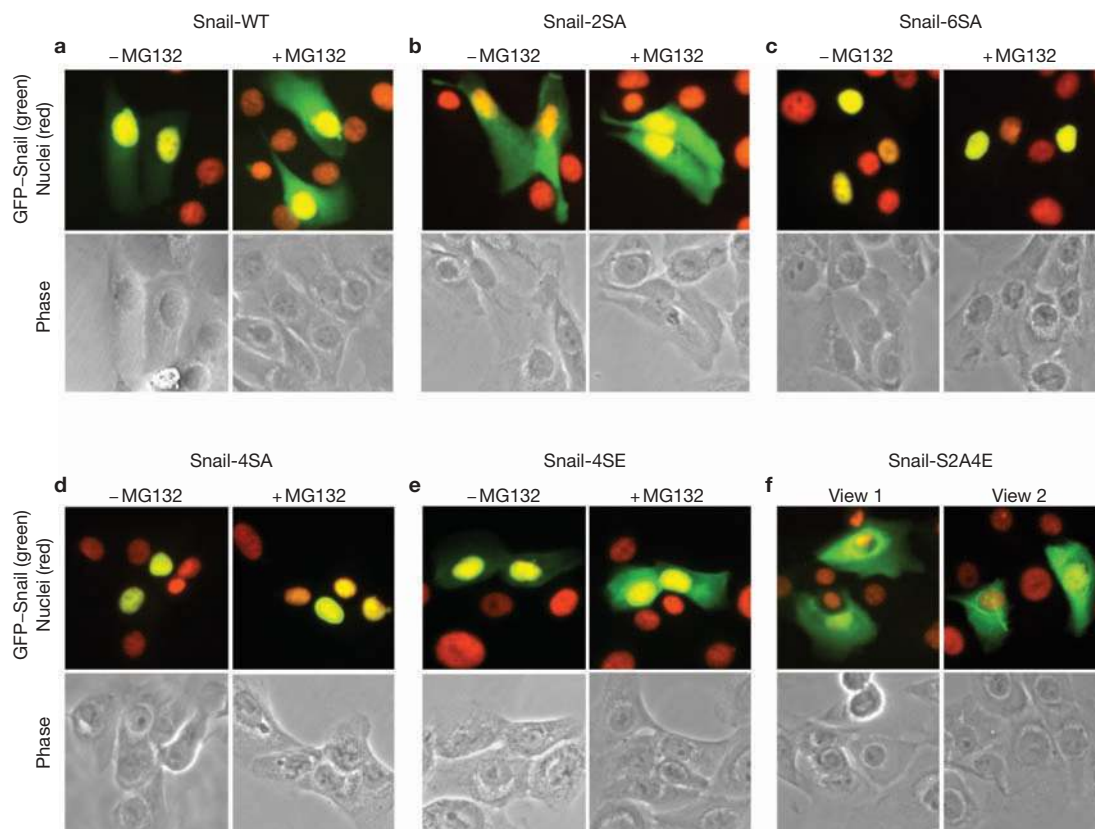
**Figure 3** Wild-type Snail is unstable and is ubiquitinated by  $\beta$ -Trcp. **(a)** WT or variant Snail proteins from Snail-WT-HEK293, Snail-2SA-HEK293 or Snail-6SA-HEK293 cells that were treated with MG132 for different time intervals were analysed by western blotting. Equal amounts of protein were subjected to western blot analysis, as determined by comparing amounts of actin (data not shown). **(b)** Densitometry of WT and variant Snail forms, after MG132 treatment, were plotted (filled circle, WT; open square, 2SA; open diamond, 6SA). **(c)** WT or variant Snail proteins from Snail-WT-HEK293, Snail-2SA-HEK293 or Snail-6SA-HEK293 cells treated with 20  $\mu$ M cycloheximide for different time intervals were analysed by western blotting. Equal amounts of protein were subjected to western blot analysis, as determined by comparing amounts of actin (data not shown). **(d)** Densitometry results for WT and variant Snails after cycloheximide treatment were plotted (filled circle, WT; open square, 2SA; open diamond,

6SA), and the half-lives of Snails were determined. **(e)** Myc-tagged  $\beta$ -Trcp or Myc-tagged  $\beta$ -Trcp $\Delta$ F was expressed in Snail-MCF7 and Snail-HEK293 cells that were treated with or without MG132 for 5 h. Expression of Snail was analysed by western blotting. **(f)**  $\beta$ -Trcp induces Snail degradation. Flag-tagged Snail proteins (SN) were coexpressed with Myc-tagged  $\beta$ -Trcp in 293T cells, and cells treated with or without MG132 for 5 h. The protein concentrations of Snail proteins were analysed by western blotting, and the densitometry results are listed below. **(g)** Snail proteins and  $\beta$ -Trcp were coexpressed in 293T cells as described in **f**. Snail proteins were immunoprecipitated and the bound  $\beta$ -Trcp and ubiquitination of Snail proteins examined by western blotting. **(h)**  $\beta$ -Trcp was expressed in Snail-WT-HEK293, Snail-2SA-HEK293 or Snail-6SA-HEK293 cells. After Snail proteins were immunoprecipitated, the bound  $\beta$ -Trcp and ubiquitination of Snail was examined as described in **g**.

### Phosphorylation of Snail regulates its subcellular localization

We next tested whether phosphorylation of Snail regulates its subcellular localization by using Snail proteins (Snail-WT, Snail-2SA and Snail-6SA) tagged with green fluorescent protein (GFP). GFP-Snail-WT mainly localized to the nucleus, with a weak signal in the cytoplasm. Treatment with MG132 greatly increased the amount of Snail in the cytoplasm (Fig. 4a; also see Supplementary Information, Fig. S1). In contrast, GFP-Snail-2SA, which was resistant to degradation, was found in both the cytoplasm and the nucleus, and treatment with MG132 did not affect its subcellular localization (Fig. 4b), indicating that the mutation of the first GSK-3 $\beta$  phosphorylation motif in Snail stabilized it in the cytoplasm.

Surprisingly, GFP-Snail-6SA, which was also resistant to degradation, was found only in the nucleus, and treatment with MG132 did not induce its cytoplasmic localization (Fig. 4c), indicating that the second GSK-3 $\beta$  phosphorylation motif controls the subcellular localization of Snail. To further examine this finding, we looked at the subcellular localization of two additional variants of Snail, GFP-Snail-4SA (in which the second GSK-3 $\beta$  phosphorylation motif was replaced with alanine) and GFP-Snail-4SE (in which a serine in the second GSK-3 $\beta$  phosphorylation motif was replaced with glutamic acid to mimic the phosphorylation of Snail by GSK-3 $\beta$ ). As expected, Snail-4SA predominantly localized to the nucleus, and treatment with MG132 did not enhance its signal in the



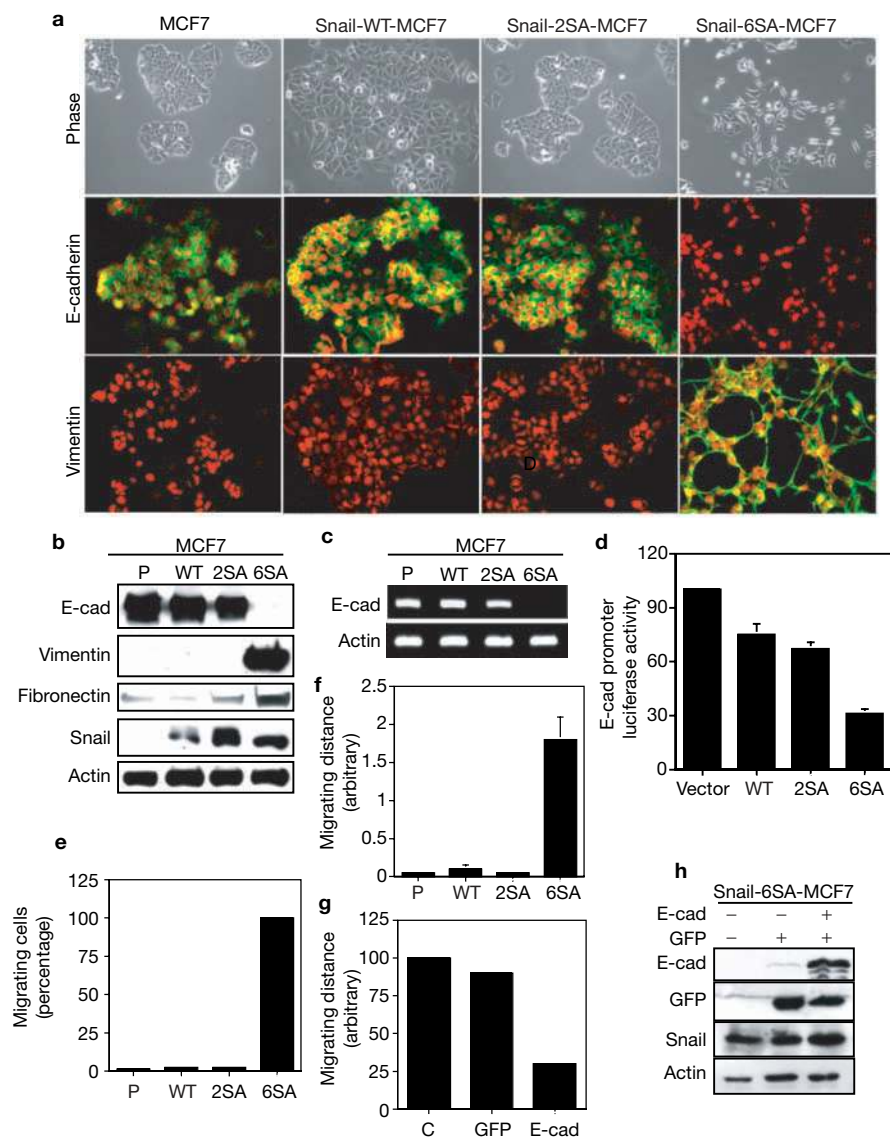
**Figure 4** Phosphorylation of Snail affects its subcellular localization. GFP-Snail proteins were expressed in breast mammary epithelial 184A1 cells. After fixation of the cells, the localization of Snail proteins (green) and nuclei

(red; DAPI) were examined under a fluorescent microscope. Panels for merge and phase images are also shown, whereas images for GFP-Snail proteins or nuclei alone are presented in the Supplementary Information (Fig. S1).

cytoplasm (Fig. 4d). However, although Snail-4SE was detected in the nucleus with a faint signal in the cytoplasm, treatment with MG132 greatly increased the amount of Snail-4SE in the cytoplasm (Fig. 4e). These results indicate several things: first, the second phosphorylation motif in Snail regulates its subcellular localization (for example, Snail-2SA compared with Snail-6SA); second, Snail-4SE moved out of the nucleus to the cytoplasm, where it was degraded; and third, abrogation of this motif in Snail-4SA caused Snail to reside in the nucleus. To confirm these notions, we generated another Snail variant, Snail-2A4E, in which the second motif was replaced with glutamic acid to mimic the phosphorylation by GSK-3 $\beta$  and the first motif was mutated to alanine to block  $\beta$ -Trcp-mediated ubiquitination. Snail-2A4E predominantly localized to the cytoplasm (Fig. 4f), indicating that the second GSK-3 $\beta$  phosphorylation motif was critical in determining the subcellular localization of Snail. The recent finding that the export of Snail is controlled by phosphorylation supports our results<sup>25</sup>. Taken together, our results show that GSK-3 $\beta$  might interact with Snail and phosphorylate it at two consensus motifs to dually regulate it in cells: phosphorylation of the second motif causes Snail to localize in the cytoplasm, whereas phosphorylation of the first motif causes Snail to undergo degradation. We also examined the subcellular localization of GSK-3 $\beta$  and found that GSK-3 $\beta$  existed in both the nucleus and the cytoplasm (see Supplementary Information, Fig. S2). All these results indicate that GSK-3 $\beta$  can interact with Snail and regulate its function through phosphorylation.

### Snail-6SA is a potent inducer of EMT

Because MCF7 breast cancer cells do not express endogenous Snail, we generated stable wild-type Snail transfectants in MCF7 cells (producing Snail-WT-MCF7 cells). Of 69 clones screened, more than 24 expressed Snail, but E-cadherin was not downregulated in any, nor were any morphological changes induced that resemble EMT (see Supplementary Information, Figs S3, S4). We also established stable transfectants of Snail-2SA that resisted proteolysis in MCF7 cells (Snail-2SA-MCF7 cells), but, again, no E-cadherin downregulation and morphological changes were found in any of these cells (16 positive clones; see Supplementary Information, Figs S4, S5a). However, when cells were screened to identify those with stable Snail-6SA expression in MCF7 cells (Snail-6SA-MCF7), one-half of the neomycin-resistant clones had morphological changes that resembled EMT (see Supplementary Information, Figs S4, S5), and these clones were later found to express Snail-6SA (Fig. 5a, b). Similar results were obtained in another breast cancer cell line, ZR75, which contains expression of wild-type E-cadherin (data not shown). Snail (Snail-WT, Snail-2SA or Snail-6SA) was also transiently expressed in MCF7 cells. Again, only Snail-6SA induced E-cadherin downregulation and morphological changes including disassociate from neighbouring cells (see Supplementary Information, Fig. S5). Indeed, the Snail-6SA-MCF7 cells had lost E-cadherin and expressed two fibroblast markers, vimentin and fibronectin (Fig. 5a, b). RT-PCR was also performed and showed that the mRNA of E-cadherin had disappeared in Snail-6SA-MCF7 cells (Fig. 5c), indicating that the loss of E-cadherin in these cells



**Figure 5** Snail-6SA that abolished GSK-3 $\beta$ -phosphorylation induced EMT. (a) The morphology of untransfected MCF7 cells, Snail-WT-MCF7, Snail-2SA-MCF7 or Snail-6SA-MCF7 cells was either visualized by phase-contrast microscopy (Zeiss), or the cells were stained with the epithelial marker E-cadherin (E-cad; 1:800) and the fibroblast marker vimentin (1:600). (b) The expression of Snail, E-cadherin, vimentin and fibronectin in MCF7 and mutant Snail-MCF7 cells was analysed by western blotting. (c) mRNA from MCF7 and mutant Snail-MCF7 cells was analysed by RT-PCR. (d) A 1:5 ratio of E-cadherin-promoter to luciferase was co-expressed with Snail proteins (Snail-WT, Snail-2SA or Snail-6SA) in MCF7 cells. Post-

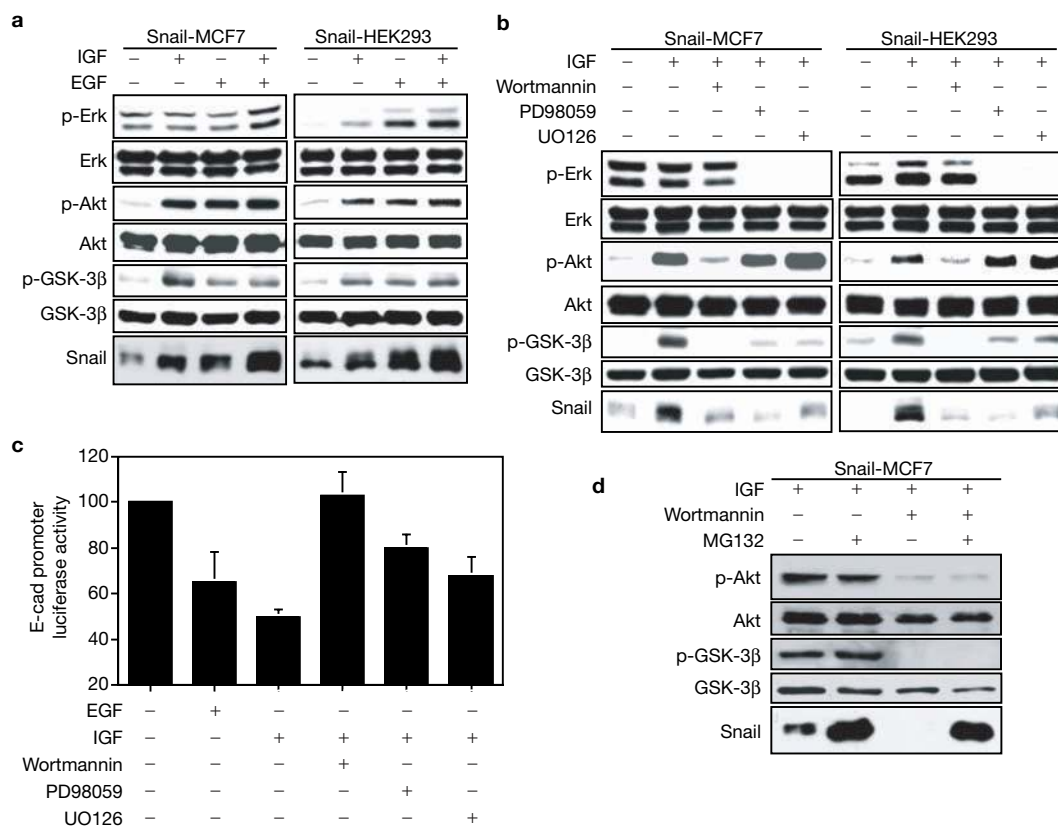
transfection (48 h), luciferase activities were determined and normalized by  $\beta$ -galactosidase activities (mean  $\pm$  s.e.m. in three separate experiments) (e, f) The motile behaviour of MCF7 and mutant Snail-MCF7 cells was analysed by time-lapse microscopy, and the population of migrating cells and the migrating distance (arbitrary units) are shown in e and f, respectively. (g, h) Downregulation of E-cadherin is required for the motile behaviour of Snail-6SA cells. E-cadherin or a vector control was co-expressed with GFP in Snail-6SA-MCF7 cells. After 24 h, the migration of GFP-positive cells was scored under a time-lapse fluorescent microscope (g) and the expression of GFP and E-cadherin in these cells was measured by western blotting (h).

resulted from suppression of its promoter by Snail-6SA. Furthermore, Snail proteins were transiently expressed along with a luciferase reporter driven by an E-cadherin promoter in MCF7 cells. Snail-6SA was more potent in suppressing the transcription of E-cadherin (Fig. 5d). Thus, Snail-6SA is the active form of a repressor for E-cadherin. Because E-cadherin is a cell-cell adhesion molecule and its downregulation enhances cell motility, we tracked the movement of Snail proteins in MCF7 cells by time-lapse microscopy. Snail-6SA-MCF7 cells were much more motile than the other three cells (Fig. 5e, f). When E-cadherin was expressed in

these cells, the motility of Snail-6SA-MCF7 cells were greatly suppressed (Fig. 5g, h), indicating that the downregulation of E-cadherin was required for the motile behaviour of these cells. Taken together, these results demonstrate that only Snail-6SA, which abolishes GSK-3 $\beta$ -mediated phosphorylation activities, could induce EMT in breast epithelial cells.

#### Inhibition of GSK-3 $\beta$ induces upregulation of Snail

Because the GSK-3 $\beta$  phosphorylation motifs in Snail are important in regulating Snail, we screened mutations in the *snail* gene, especially in



**Figure 6** Inhibition of GSK-3 $\beta$  correlates with upregulation of Snail.

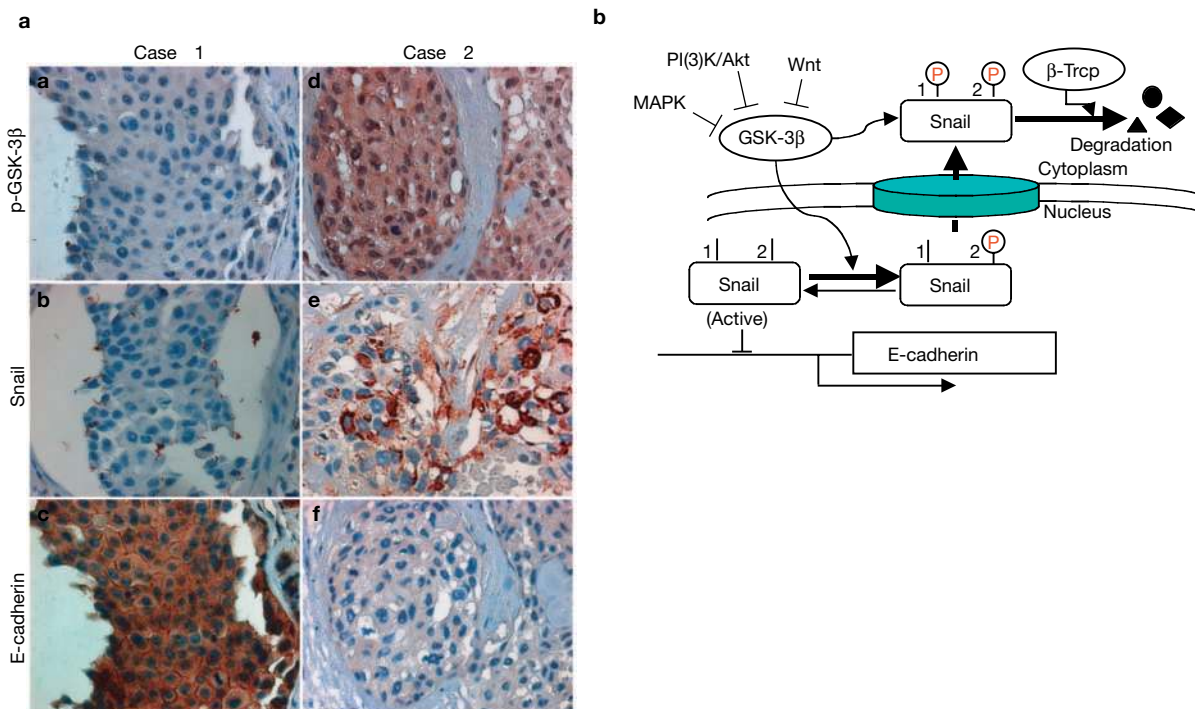
(a) Snail-MCF7 or Snail-HEK293 cells were either stimulated with EGF (1 ng ml<sup>-1</sup>), IGF-1 (10 ng ml<sup>-1</sup>) or in combination for 6 h. The concentration of Snail and the activation of Akt, GSK-3 $\beta$  and Erk were examined by western blotting. (b) Snail-MCF7 or Snail-HEK293 cells were pre-treated with either wortmannin (0.1  $\mu$ M), PD98059 (20  $\mu$ M) or UO126 (20  $\mu$ M) for 1 h before stimulation with IGF-1 as described in a. The expression of Snail and the activation of Akt, GSK-3 $\beta$  and Erk were examined by western blotting.

(c) The E-cadherin promoter fused to luciferase was expressed in Snail-MCF7 cells. After 36 h, the cells were either stimulated with ligands as described in a, or treated with inhibitor as described in b. The E-cadherin luciferase activities were determined and normalized by  $\beta$ -galactosidase activities (means  $\pm$  s.e.m. from three separate experiments). (d) Snail-MCF7 cells pre-treated with wortmannin for 1 h were stimulated with IGF-1 before treatment with or without MG132 for 5 h. The expression of Snail and the activation of Akt and GSK-3 $\beta$  were examined by western blotting.

exon 2, which contains the two phosphorylation motifs. We reasoned that mutations in this region might disrupt the phosphorylation of Snail and stabilize the protein, thus enhancing the invasive and metastatic potential of cancer cells. Of 26 cell lines screened, no mutations were found in exon 2 (see Supplementary Information, Fig. S6). The absence of mutations in Snail therefore suggested that the upstream signalling is much more important in controlling the function of Snail, and this prompted us to examine the relationship between the activation of GSK-3 $\beta$  and the stabilization of Snail. Because the activation of phosphatidylinositol-3-OH kinase (PI(3)K)/Akt and MAPK pathways has been implicated in the induction of invasion and EMT, we treated the cells (Snail-MCF7 and Snail-HEK293) with growth factors — insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF) or both — to activate the Akt and the MAPK pathways, and examined the status of Snail and GSK-3 $\beta$ . As expected, activation of Akt led to the suppression of GSK-3 $\beta$  (through phosphorylation of Ser 9) and the stabilization of Snail (Fig. 6a). When these pathways were blocked with a PI(3)K inhibitor (Wortmannin), MAPK inhibitor (PD98059) or MEK inhibitor (UO126), suppression of GSK-3 $\beta$  was released, which correlated with the downregulation of Snail (Fig. 6b). Similar results were obtained when the E-cadherin promoter was

transfected into Snail-MCF7 cells (Fig. 6c). The elevation of Snail concentration by growth factors was caused by protein stabilization, as the absence of Snail could be recovered when the proteasome pathway was blocked (Fig. 6d). Taken together, our results indicate that many upstream pathways can regulate the activity of GSK-3 $\beta$ , which in turn controls the phosphorylation of Snail, and thus regulate its protein stability and subcellular localization.

To examine whether this regulation also existed in tumour tissues, we compared the levels of inhibited GSK-3 $\beta$ , the amount of Snail and the expression of E-cadherin in 129 human breast tumours by immunostaining. Consistent with our observations in cell cultures, upregulation of Snail significantly correlated with the inhibition of GSK-3 $\beta$  and the downregulation of E-cadherin (Fig. 7a and Table 1). Although the concentration of Snail was not associated with the tumour grade, it significantly correlated with breast cancer metastases (Table 1). These results are consistent with a previous finding that the expression of Snail is found in biopsies that associate with malignant phenotypes from patients with ductal breast cancer, gastric cancer and hepatocellular carcinoma<sup>10,26–29</sup>. Thus, tumour-staining data confirmed our observations in cell cultures, and led further credence to our hypothesis that the inhibition of GSK-3 $\beta$  stabilizes Snail and downregulates E-cadherin.



**Figure 7** Inhibition of GSK-3 $\beta$  correlates with upregulation of Snail and downregulation of E-cadherin. **(a)** The 134 surgical specimens of breast cancer were immunostained for phosphorylated GSK-3 $\beta$  (**a** and **d**), Snail (**b** and **e**) and E-cadherin (**c** and **f**), as well as the control serum (data not shown).

Representative stainings from the same tumour samples are shown. **(b)** A model proposed to illustrate that the inhibition of GSK-3 $\beta$  by many extracellular pathways suppresses the phosphorylation of Snail and thus induces the nuclear localization and protein stabilization of Snail, which leads to EMT.

## DISCUSSION

In this study, we have shown that Snail is regulated by GSK-3 $\beta$ . We have found that two phosphorylation motifs within Snail dually regulate the function of this protein and our studies provide several insights into the regulation of EMT. First, E-cadherin downregulation in most carcinomas is a transient and dynamic event. This dynamic regulation of E-cadherin is seen during embryonic development, during which E-cadherin expression is lost when EMT occurs but returns when epithelial lineages from mesoderm layers are established. This mesenchymal–epithelial transition (MET), a reverse of EMT, occurs at various stages of morphogenesis and is also used by cancer cells that make up micrometastases to establish a secondary carcinoma<sup>1,30</sup>. This suggests that the initial invasion stage probably requires a rapid and effective repression of E-cadherin through the stabilization of Snail by inhibition of GSK-3 $\beta$ , whereas the re-expression and maintenance of E-cadherin in the secondary carcinoma requires the degradation of Snail through the activation of GSK-3 $\beta$ . The reversible regulation of EMT is consistent with the result that no mutation was found in 26 cancer cell lines (see Supplementary Information, Fig. S6). Thus, our study provides an explanation for the finely tuned events required to regulate EMT and MET in metastasis.

Second, the identity of Snail in cells remains a mystery, as endogenous Snail is barely detectable and therefore the level of mRNA is widely used to represent its expression. The unstable nature of Snail may explain the difficulty in detecting this protein, and thus groups Snail with several other key short-lived transcription factors, such as c-Myc, p53 and HIF-1 that are critical in the control of cellular homeostasis. Moreover, Snail can also be regulated by its subcellular localization through GSK-3 $\beta$ -mediated phosphorylation. Thus, the dual regulation of Snail indicates that it can function as a molecular

**Table 1.** Relationships between Snail expression and p-GSK-3 $\beta$ , and E-cadherin in surgical specimens of breast cancer

	Snail expression			P-value
	Negative	Low	High	
<b>p-GSK-3<math>\beta</math></b>				
Negative	36	5	14	<0.001
Low	8	3	6	
High	13	9	35	
<b>E-cadherin</b>				
Negative	28	8	37	<0.001
Low	8	0	16	
High	22	9	1	
<b>Grade</b>				
1	12	4	8	0.1448
2	18	2	23	
3	28	11	23	
<b>Metastases</b>				
Negative	39	5	23	<0.0001
Positive	18	14	30	

The expression patterns of these three molecules in the 129 breast cancer samples were determined and summarized. The correlation between Snail and phosphorylated GSK-3 $\beta$  and E-cadherin was analysed using fisher's exact test ( $P < 0.001$ ). A P value of less than 0.05 was set as the criterion for statistical significance.



switch for EMT and reinforces the notion that cell migration and cancer metastasis are finely tuned events.

Third, the regulation of Snail by GSK-3 $\beta$  reveals that many oncogenic pathways could govern human cancer cell invasion and metastasis by modulating GSK-3 $\beta$  activity. GSK-3 $\beta$  is a multi-tasking kinase involved in the Akt, Wnt and Hedgehog pathways, all of which are engaged in the determination of cell fate and morphology<sup>15</sup>. GSK-3 $\beta$  is present in the nucleus and the cytoplasm (see Supplementary Information, Fig. S2)<sup>31</sup>. Moreover, it can phosphorylate several nuclear transcription factors, such as c-Myc, p53 and NF-AT, and induces the nuclear export of NF-AT<sup>15,18,32</sup>. Phosphorylation by GSK-3 $\beta$  is a sequential event that starts from the C-terminus of the molecule; typical examples are the phosphorylation of glycogen synthase and  $\beta$ -catenin<sup>18,33</sup>. Thus, we propose that GSK-3 $\beta$  binds and phosphorylates Snail (at motif 2) and thereby induces its nuclear export. Subsequent phosphorylation by GSK-3 $\beta$  (at motif 1) results in the association of Snail with  $\beta$ -Trcp and thus leads to the degradation of Snail (Fig. 7b). Furthermore, we have shown that many upstream signalling pathways regulate the function of Snail through modulating the activity of GSK-3 $\beta$  (Fig. 6). This observation was supported by our immunostaining analysis of 129 primary breast cancer samples, and is also consistent with previous findings. For example, activation of Akt in cancer cells leads to EMT and invasion *in vivo*<sup>34</sup>. Moreover, oncogenic Src and Ras can induce EMT through PI(3)K and MAPK pathways<sup>1,30</sup>. Therefore, we propose that many oncogenic signals, such as PI(3)K/Akt, MAPK and Wnt, inhibit GSK-3 $\beta$  and thus in turn result in the stabilization and nuclear localization of Snail to trigger cell migration and EMT (Fig. 7b). Because GSK-3 $\beta$  also regulates  $\beta$ -catenin, we speculate that the inhibition of GSK-3 $\beta$  by these pathways stabilizes both Snail and  $\beta$ -catenin, which induces cell migration, proliferation and oncogenesis in a coordinated fashion. This speculation is consistent with the fact that EMT also correlates with the presence of  $\beta$ -catenin in the nucleus, but the overexpression of  $\beta$ -catenin alone does not lead to EMT; it results in apoptosis<sup>35</sup>.

Although GSK-3 $\beta$  is expected to be inactive under many culture conditions, because Akt and MAPK are activated under serum stimulation, the failure to induce EMT under this culture condition suggests an additional layer of regulation inside the cell. Alternatively, there is a substantial level of GSK-3 $\beta$  activity inside the cells, which may be enough to phosphorylate Snail and induce its proteasomal degradation. Complete inhibition of GSK-3 $\beta$  may require the activation of multiple signal pathways simultaneously.

A fourth accomplishment of this study is that we induced complete EMT in non-motile MCF7 breast cancer cells by introducing an unphosphorylated form of Snail (Snail-6SA). The fact that only the Snail-6SA variant can induce EMT is consistent with the dual regulation of Snail that we propose, and therefore constitutes a useful model for the study of EMT. Future studies that make use of an inducible expression of Snail-6SA may shed new light on the dynamic event of cell migration.

Although there is a wealth of literature on the complex regulation of transcription factors, it essentially comes down to three areas: regulation of the location, regulation of the abundance and regulation of the activity of transcription factors<sup>19</sup>. In this study, we show that GSK-3 $\beta$  dually regulates the function of Snail in controlling EMT and thus put Snail at the crossroads of the regulation of many other transcription factors. Most importantly, the understanding of EMT in tumour invasion and metastasis that has been gained will improve our prospects for developing effective cancer treatments.  $\square$

## METHODS

**Antibodies.** The anti-E-cadherin and anti-Snail antibodies were obtained from Oncogene Research Products (Cambridge, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Antibodies against phosphorylated Akt, GSK-3 $\beta$  and Erk were purchased from Cell Signaling (Beverly, MA).

**Snail and GSK-3 $\beta$  plasmid constructs.** The coding sequences of *snail* and *GSK-3 $\beta$*  were obtained from SKBr3 and Hela cDNA. A *Bam*HI restriction site and an *Xho*I restriction site were generated near the start and termination codons of these genes, and the genes subcloned into CMV-Tag2B and CMV-Tag5B plasmids, respectively (Stratagene, La Jolla, CA). Site-directed mutagenesis was performed as described<sup>36</sup>. All sequences were verified by automated sequencing. The E-cadherin promoter fused to luciferase was provided by E. Fearon<sup>6</sup>, and the human E-cadherin expression plasmid was provided by B. Gumbiner. GST-tagged wild-type and mutant Snail proteins were purified as described<sup>36,37</sup>.

**Cell culture.** HEK293 and MCF7 cell lines containing Snail-WT, Snail-2SA or Snail-6SA were established as described<sup>38,39</sup> and were grown in DMEM/F12 supplemented with 10% fetal bovine serum and 400  $\mu$ g ml<sup>-1</sup> G418. Breast mammary epithelial 184A1 cells (Clonetics, Walkersville, MD) were grown according to the manufacturer's instructions.

***In vitro* kinase assays, immunostaining, immunoprecipitation and immunoblotting.** All these experiments were performed as described<sup>36–38</sup>. For immunoblotting, whole-cell lysates were used to detect the expression of Snail. Briefly, cells were lysed in lysis buffer that contained a cocktail of protease inhibitors. After being adjusted to equal protein concentration, samples were mixed with 4 $\times$  SDS-PAGE sample buffer and boiled for 5 min. For immunohistochemistry, each tumour sample was stained with specific antibodies as indicated and scored by an H-score method that combines the values of immunoreaction intensity and the percentage of tumour cell staining as described<sup>40,41</sup>.

**Mutational analysis of the *snail* gene.** The *snail* gene is composed of 3 exons. Because putative phosphorylation sites are found in exon 2 from codons 95–122, we only analysed exon 2 for somatic mutations, as described<sup>42</sup>. Sequences were examined by automatic sequencing.

**BIND identifiers.** One BIND identifier ([www.bind.ca](http://www.bind.ca)) is associated with this manuscript: 154061.

*Note: Supplementary Information is available on the Nature Cell Biology website.*

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## COMPETING FINANCIAL INTERESTS

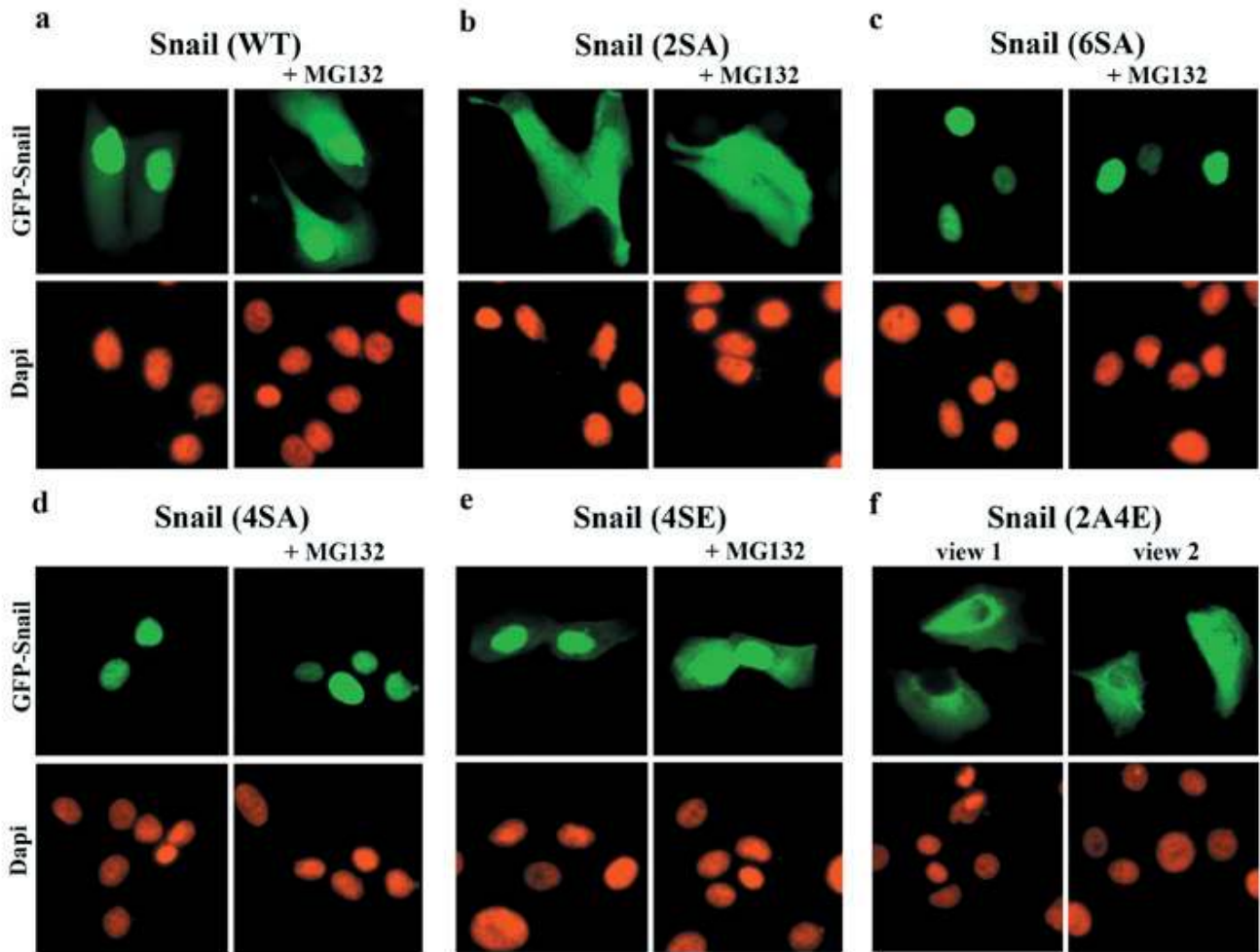
The authors declare that they have no competing financial interests.

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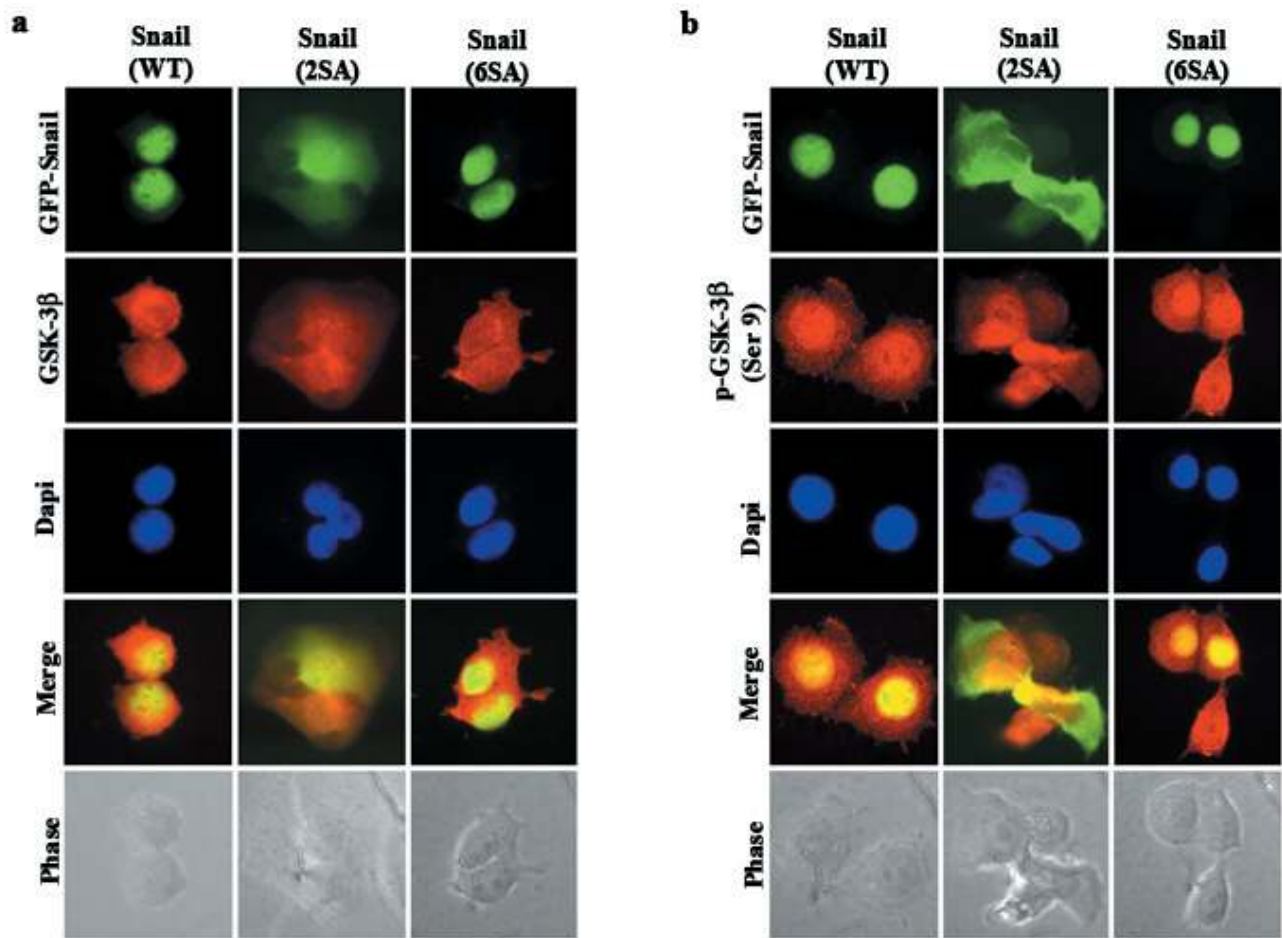
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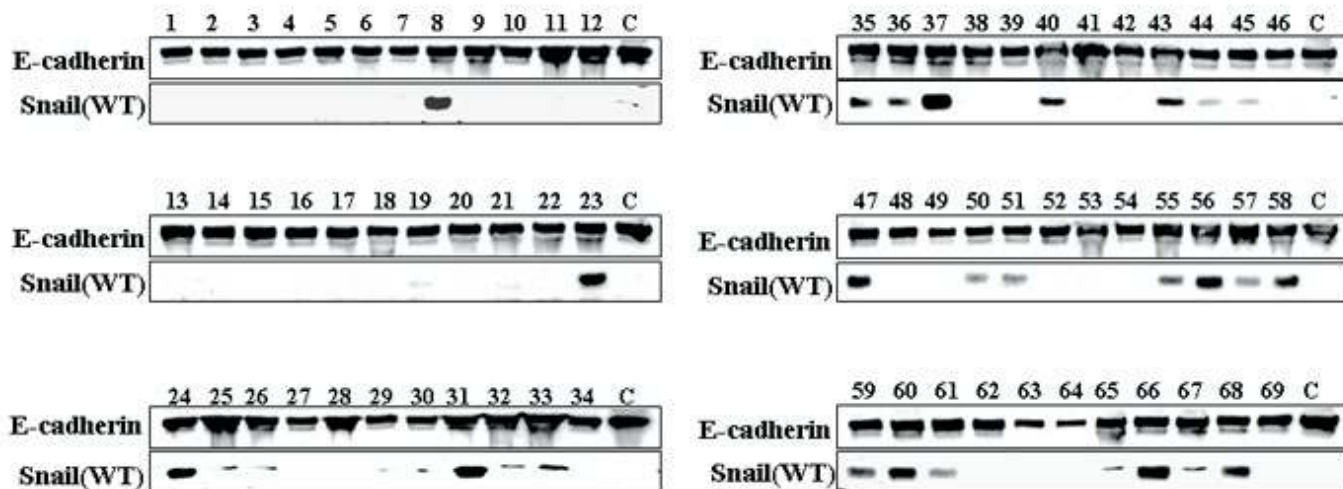
**Figure S1** Phosphorylation of Snail affected its cellular localization. GFP-Snails were expressed in 184A1 cells and the cellular locations of Snail

(green) and nuclei (dapi, red) were examined. The merge and phase images for this experiment were presented in Fig. 4.



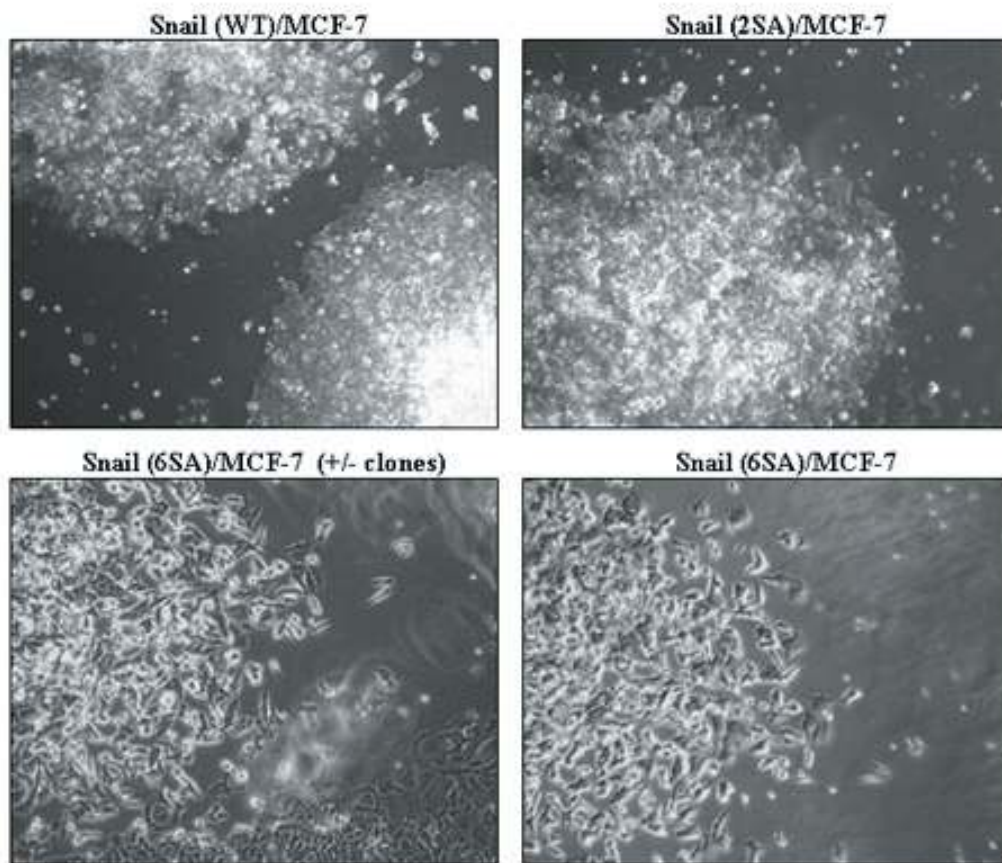
**Figure S2** GSK-3 $\beta$  and phosphorylation of GSK-3 $\beta$  (Ser 9) resided in the cytoplasm and nucleus. **(a and b)** GFP-Snails were expressed in breast cancer MCF-7 cells. After fixation of the cells, the cellular location of GSK-3 $\beta$  **(a)** or

phosphorylation of GSK-3 $\beta$  **(b)** was examined by immunofluorescent staining using specific antibodies. The cellular location of GSK-3 $\beta$  (red), Snails (green), and nuclei (dapi, blue) were examined under a fluorescent microscope (Zeiss).



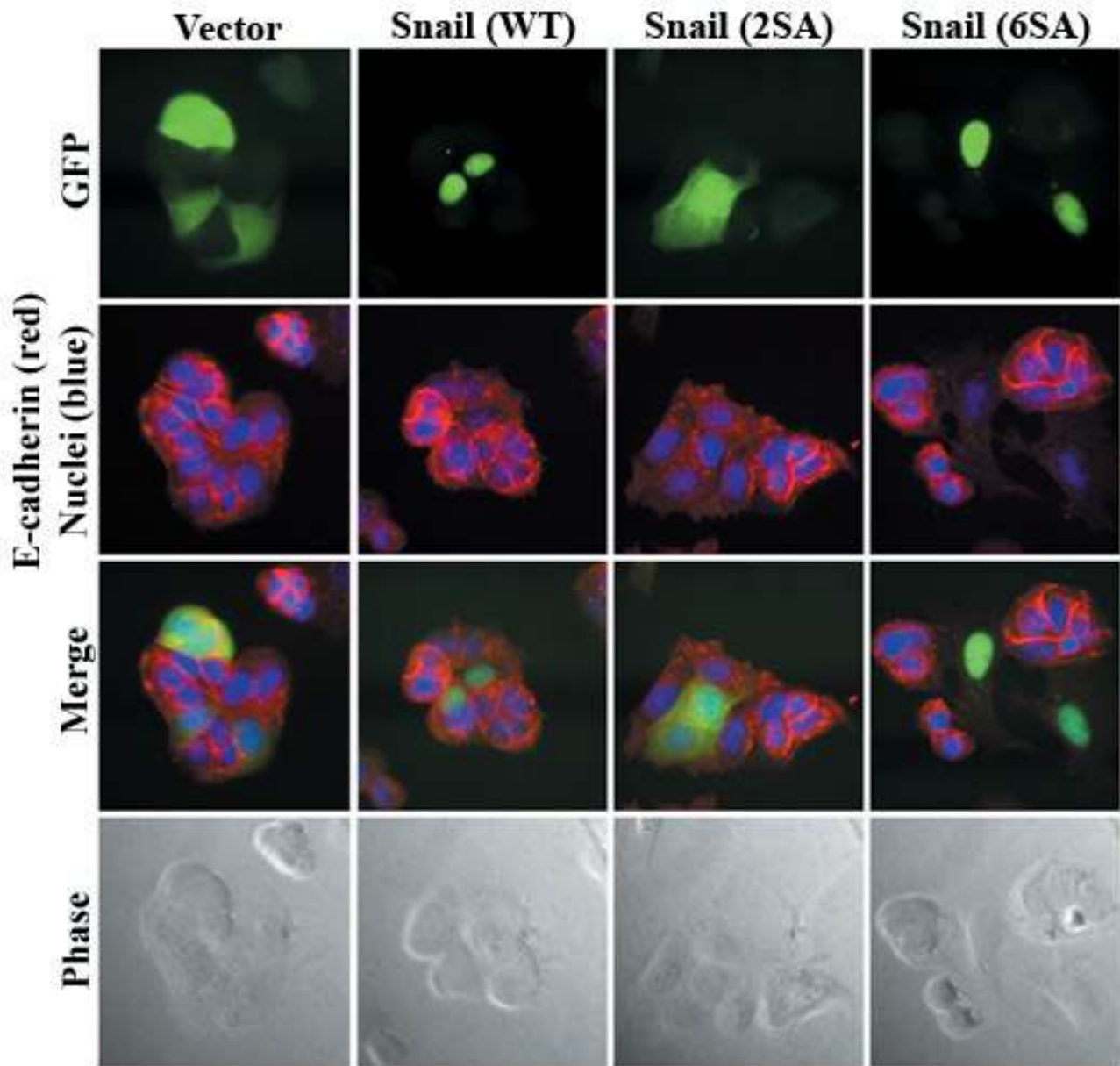
**Figure S3** WT-Snail did not induce the downregulation of E-cadherin. WT Snail was transfected into MCF-7 cells, and 69 neomycin resistant clones

were isolated. Expression of Snail and E-cadherin in these cells was examined by western blotting.



**Figure S4** Only Snail (6SA) induced EMT and suppressed the expression of E-cadherin. Snail (WT, 2SA, or 6SA) was transfected into MCF-7 cells, and selection was done with 800  $\mu$ g/ml neomycin for 4 weeks. The morphology of neomycin-resistant clones with the three types of Snail was examined

by microscopy. In the bottom left panel, two neomycin-resistant clones are shown. One shows morphological changes typical for EMT and expressed Snail (6SA); the other showed neither.



**Figure S5** Only Snail (6SA) suppressed E-cadherin expression and induced disassociation from neighboring cells. Snails with GFP-tagged were transiently expressed in MCF-7 cells. After culture for 60 h, the cells were fixed and the expression of E-cadherin was examined by immunofluorescent

staining using specific antibody. The cellular location of Snail (green), E-cadherin (red), and nuclei (dapi, blue) was examined under a fluorescent microscope (Zeiss).

**a** Cell lines examined for SNAIL (in exon 2)

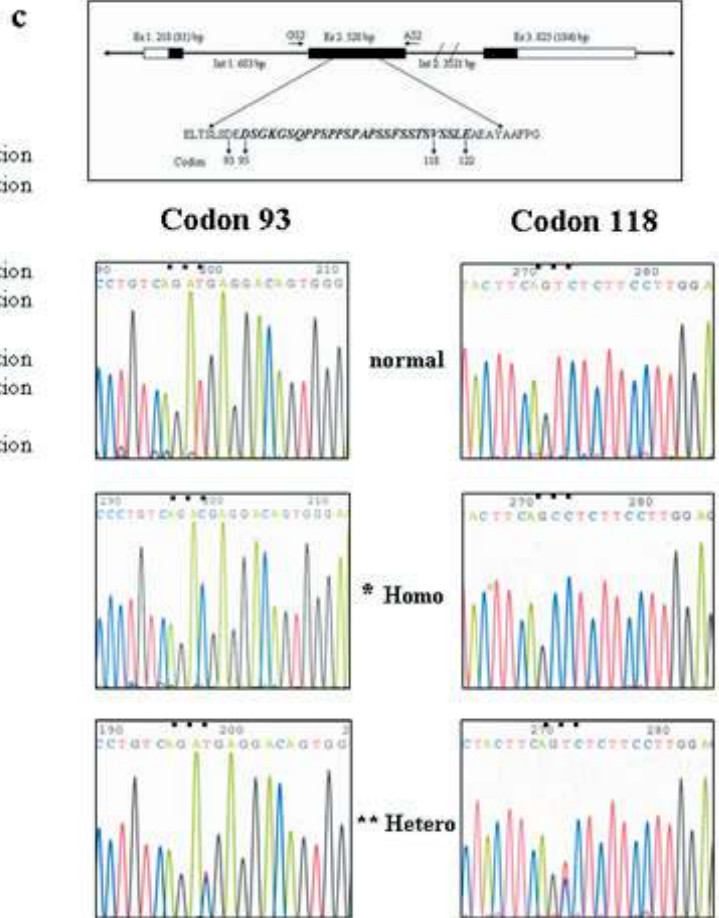
1. MDA453	No alteration	14. PC3	**
2. 293T	*	15. LnCap	No alteration
3. MDA231	No alteration	16. SW480	No alteration
4. T47D	No alteration	17. HEPG2	**
5. MCF7	No alteration	18. LiverChang	*
6. ZR75	No alteration	19. HUH7	No alteration
7. MDA468	*	20. BT474	No alteration
8. HCT116	No alteration	21. SKOV3	**
9. DLD1	No alteration	22. PA1	No alteration
10. RKO	**	23. MDA361	No alteration
11. MDA435	No alteration	24. SK-HEP1	**
12. SKBR3	No alteration	25. BT36	No alteration
13. DU145	No alteration	26. HEP3B	**

**b**

*	Codon 93	gat---gac	Homo
		D→D	
	Codon 118	gtc---gcc	Homo
		V→A	
	Codon 177	acg---aca	Homo
		T→T	
**	Codon 93	gat---gac	Hetero
		D→D	
	Codon 118	gtc---gcc	Hetero
		V→A	
	Codon 177	acg---aca	Hetero
		T→T	

**Figure S6** Mutation analyses of the putative phosphorylation sites in the *snail* gene. **a**, No somatic mutation was detected in most of the cells, but some cell lines showed a single-nucleotide polymorphism, either in the heterozygous state (RKO, PC3, HepG2, SKOV3, SK-HEP1, HEP3B) or the homozygous state (293T, MDA468, Liver Chang) at codons 93, 118, and



177. **b**, Genomic structure and putative phosphorylation sites of *snail* gene and the location of primers used for amplification of PCR. **c**, Representative sequence chromatograms showing the normal (MDA453), heterozygous (RKO), and homozygous (Liver Chang) appearances at codons 93 and 118.