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Knockdown of BAG3 induces epithelial–mesenchymal transition in thyroid cancer cells through ZEB1 activation

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The process by which epithelial features are lost in favor of a mesenchymal phenotype is referred to as epithelial–mesenchymal transition (EMT). Most carcinomas use this mechanism to evade into neighboring tissues. Reduction or a loss of E-cadherin expression is a well-established hallmark of EMT. As a potent suppressor of E-cadherin, transcription factor ZEB1 is one of the key inducers of EMT, whose expression promotes tumorigenesis and metastasis of carcinomas. Bcl-2-associated athanogene 3 (BAG3) affects multifaceted cellular functions, including proliferation, apoptosis, cell adhesion and invasion, viral infection, and autophagy. Recently, we have reported a novel role of BAG3 implicated in EMT, while the mechanisms are poorly elucidated. The current study demonstrated that knockdown of BAG3 induced EMT, and increased cell migratory and invasiveness in thyroid cancer cells via transcriptional activation of ZEB1. We also found that BAG3 knockdown led to nuclear accumulation of β-catenin, which was responsible for the transcriptional activation of ZEB1. These results indicate BAG3 as a regulator of ZEB1 expression in EMT and as a regulator of metastasis in thyroid cancer cells, providing potential targets to prevent and/or treat thyroid cancer cell invasion and metastasis.

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The incidence of thyroid cancer, the most common neoplasm of the endocrine system, has been rising steadily during past decades.[1] The prognosis of thyroid cancer is highly correlated with the progression of localized primary tumors to advanced stages, which ultimately metastasize to multiple organs.[1] Therefore, dissecting the molecular mechanisms that regulate thyroid cancer invasion may facilitate the advancement of clinical treatment.

Carcinoma tumors use unique mechanisms to evade from the primary tissue into surrounding tissues. Molecular reprogramming as part of the epithelial–mesenchymal transition (EMT) is considered to be a crucial step involved in this process in most carcinomas.[2] EMT may facilitate epithelial cells to escape from the rigid structural constraints provided by the tissue architecture, during which an orchestrated series of transcriptional and morphological program take place, resulting in the loss of epithelial differentiation and acquisition of mesenchymal-like cellular competence of tumor cells.[3–5] During EMT procedure, the actin cytoskeleton is reorganized and cells acquire increased cell–matrix contacts, leading to dissociation from surrounding cells and enhanced migratory and invasive capabilities.[6] Reduction or a loss of E-cadherin expression has a crucial role in tumor progression to invasive cancer and is also one of the well-established hallmarks of EMT.[7] In line with a role for EMT in cancer progression, E-cadherin is often lost in cancer tissues and its reduction is associated with a poor clinical prognosis of many cancers.[8] A set of transcriptional factors such as ZEB1, ZEB2, Snail, E12/E47, and Twist have been identified as direct repressors of E-cadherin transcription and are among the most potent inducers of EMT in a variety of physiological and pathological contexts.[9] Furthermore, many of these E-cadherin repressors are frequently overexpressed in many cancers.[7]

Bcl-2-associated athanogene 3 (BAG3) is a 74-kDa cytoplasmic protein mainly localized in the rough endoplasmic reticulum, which has been identified as a Bcl-2-associated protein in protein interaction techniques.[10–12] BAG3 expression is very low in normal cells, while its expression is increased in many tumors, including thyroid, leukemia, pancreatic, glioblastoma, melanoma, and ovarian tumors.[13–18] BAG3 has been implicated in the survival of human cancer cells, and its expression levels influence apoptosis of tumor cells induced by various anticancer drugs.[14–16,19–24] In addition, it has been reported that BAG3 affects adhesion, migration, and invasion of tumor cells, and thereby contributes to tumorigenesis and metastasis of malignant tumors *in vivo*.[22,25–27] Very recently, we have reported a novel function ascribed to BAG3 involved in EMT regulation in thyroid cancer cells.[28] However, the molecular mechanisms by which BAG3 regulated EMT are not fully understood. In this

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Abbreviations: BAG3, Bcl-2-associated athanogene 3; ChIP, chromosomal immunoprecipitation; EMT, epithelial–mesenchymal transition; IF, immunofluorescence; RT-PCR, reverse transcription-polymerase chain reaction; shRNA, short hairpin RNA; siRNA, small interfering RNA; WT, wild type
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study, we report, for the first time, a role of BAG3 in EMT regulation and metastasis in thyroid cancer cells through the activation of ZEB1. We reported that BAG3 knockdown induced EMT and increased migration and invasion in epithelial thyroid cancer cells. In addition, we demonstrated a mechanism whereby BAG3 regulated EMT through modulation of the EMT transcription factor ZEB1. Finally, we demonstrated that BAG3 promoted nuclear accumulation of β-catenin and increased its transcriptional activity, which was responsible for the upregulation of ZEB1 at the transcriptional level. Collectively, the current study identified BAG3 as a novel regulator of EMT and metastasis in thyroid cancer cells via modulation of ZEB1.

Results

BAG3 downregulation induces morphological changes in thyroid cancer FRO cells. To investigate the function of BAG3 in thyroid cancer, BAG3 was downregulated by short hairpin RNA (shRNA) against BAG3 (shBAG3). Western blot analysis demonstrated that two of the independent shRNA (shBAG3 nos. 2 and 4) successfully downregulated BAG3 expression (Figure 1a). No obvious influence on BAG1 expression was observed upon transient transfection with shBAG3 (Figure 1a). We then generated FRO subline cells stably expressing shBAG3 nos. 2 or 4 and got six distinct clones in which BAG3 was downregulated (Figure 1b). Stable knockdown of BAG3 resulted in a slight increase in BAG1 expression (Figure 1b). Cell count using Trypan blue exclusion demonstrated that stable knockdown of BAG3 had no obvious effect on proliferation of FRO cells (Figure 1c). Under phase contrast microscopy, we noticed that BAG3-knockdown cells exhibited a loose cell contact and elongated morphology, compared with FRO or scramble shRNA-transfected cells (data not shown). Staining cytoskeleton of cells with phalloidin confirmed that knockdown of BAG3 resulted in a change to cellular fibroblast-like morphology (Figure 1d). Quantitative morphometric analysis showed that non-transfected and mock-transfected FRO cells demonstrated similar ratio of major axis *versus* minor axis, while BAG3 knockdown significantly increased the ratio of major axis *versus* minor axis, confirming that cells with BAG3 knockdown exhibited more elongated morphology (Figure 1e).

BAG3 downregulation induces EMT in thyroid cancer cells. Consistent with a mesenchymal-like morphological

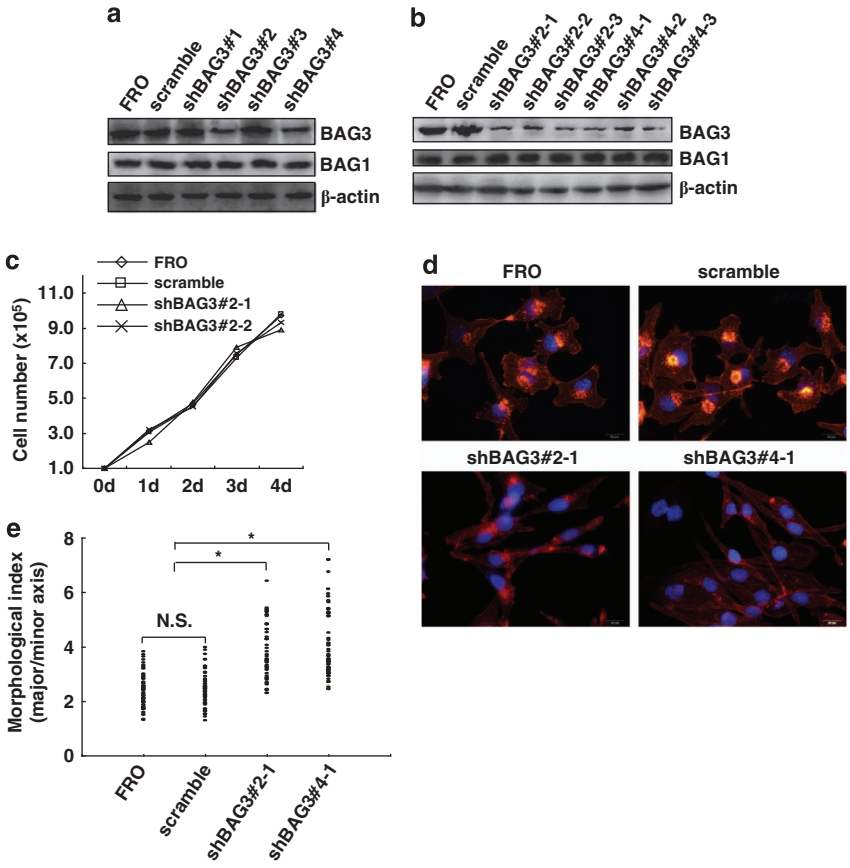


Figure 1 Knockdown of BAG3 induces EMT in thyroid cancer cells. (**a**) FRO cells were transfected with scramble shRNA or shRNA specific against BAG3 (shBAG3), and western blot analysis was performed using the indicated antibodies. (**b**) FRO cells were transfected with scramble shRNA, shBAG3 no. 2, or shBAG3 no. 4, stable clones were selected using G418, and BAG3 expression was investigated using western blot analysis. (**c**) Cell number was counted every 24 h, and plotted on the graph. (**d**) Cells were stained with phalloidin (red) and the nucleus with 4′,6-diamidino-2-phenylindole (DAPI) (blue). (**e**) Quantitative analysis of the degree of elongated cell morphology or morphological index as in (**d**). Representative data shown are from a single experiment, for which *n* was at least 50 for each cell type. Similar data were obtained from three independent cell preparations

change, compared with parental or scramble shRNA-transfected FRO cells, shBAG3 subline FRO cells demonstrated lower mRNA expression levels of epithelial markers E-cadherin and occludin (Figure 2a), while higher mRNA expression levels of mesenchymal markers vimentin, fibronectin and N-cadherin (Figure 2b). Consistent with mRNA expression, western blot analysis demonstrated that knockdown of BAG3 decreased E-cadherin and increased vimentin protein levels, respectively (Figure 2c). We further investigated the distribution of E-cadherin using cellular immunofluorescence (IF). Cellular IF demonstrated that E-cadherin was predominantly located at the membrane of parental and scramble shRNA-transfected FRO cells, while complete lack of periphery distribution was observed in BAG3-knockdown cells (Figure 2d). Similar distribution of another epithelial marker β-catenin was observed, as it was predominantly located at the membrane of parental and scramble shRNA-transfected FRO cells, while its periphery distribution was significantly attenuated and nuclear distribution was markedly increased in BAG3-knockdown FRO cells (Figure 2e). Western blot analysis confirmed that β-catenin was barely detected in the nuclear fraction of FRO or scramble shRNA-transfected FRO cells, while it was obviously distributed to the nuclear fraction in BAG3-downregulated cells (Figure 2f). Lack of LDH in nuclear fraction excluded contamination with cytoplasmic proteins (Figure 2f). Knockdown of BAG3 by a combination of shRNA nos. 2 and 4 also increased and decreased vimentin and E-cadherin expression, respectively, in other thyroid cancer cell lines, including KTC1 and KTC3 cells (Figure 2g). Real-time reverse transcription-polymerase chain reaction (RT-PCR) confirmed that BAG3 downregulation altered vimentin and E-cadherin mRNA expression in KTC1 and KTC3 cells (Figure 2h).

BAG3 downregulation promotes motion, migration, and invasion of thyroid cancer cells. To determine whether BAG3-knockdown-induced EMT cells had increased invasiveness as conferred by EMT, we performed scratch wound-healing assay and Boyden chamber migration/invasion assay. An area devoid of cells was created at time 0 h by scraping a monolayer of the indicated cells and followed by incubation of the cells in the absence of serum for 24 h (Figure 3a). Parental FRO cells and scramble shRNA-transfected FRO cells demonstrated no obvious difference in the rate of wound-healing ability, while BAG3-knockdown FRO cells showed a significantly higher rate of wound-healing capacity (Figure 3a). Boyden chamber migration assay demonstrated that BAG3-knockdown cells showed faster migration through the uncoated membrane in the Boyden chamber compared with their parental or scramble shRNA-transfected counterparts (Figure 3b). In addition, cells migrated through the Matrigel-coated membrane (invasion assay) significantly increased in BAG3-knockdown cells (Figure 3c). We also investigated whether BAG3 knockdown influenced invasiveness of KTC1 and KTC3 cells. MTT assay demonstrated that knockdown of BAG3 exhibited no obvious effect on cell viability in KTC1 and KTC3 (Figure 3d). Similar to that in FRO cells, Boyden chamber invasion assays demonstrated that knockdown of BAG3 increased invasive capacities in both KTC1 and KTC3 cells (Figure 3e).

Knockdown of BAG3 upregulates ZEB1 via transactivation of β-catenin. As reduction or loss of E-cadherin is a well-established hallmark of EMT, to examine the mechanisms of BAG3-knockdown-induced EMT, we next focused on how knockdown of BAG3 decreased E-cadherin expression. cDNA array demonstrated that mRNA expression level of ZEB1 was significantly increased in BAG3-knockdown cells (data not shown). Real-time RT-PCR confirmed the increase in ZEB1 mRNA expression in BAG3-knockdown FRO cells (Figure 4a). No obvious alteration was observed in other E-cadherin suppressors, including ZEB2, Twist1, Twist2, Snail1, Snail2, and E12/E47 (data not shown). Western blot analysis confirmed that ZEB1 was significantly increased in shBAG3-transfected cells (Figure 4b). ZEB2 expression exhibited no obvious difference among parent, scramble-transfected, and shBAG3-transfected FRO cells (Figure 4b). Cellular IF demonstrated that ZEB1 was predominantly located in the cytoplasm of parental FRO or scramble shRNA-transfected FRO cells, while obvious nuclear distribution of ZEB1 was observed in shBAG3-transfected cells (Figure 4c). Cellular IF demonstrated that BAG3 knockdown exhibited no effect on distribution of ZEB2 (Figure 4c). Redistribution of ZEB1 to the nuclei in shBAG3-transfected cells was also confirmed using nuclear fractionation followed by western blot analysis (Figure 4d).

To investigate whether knockdown of BAG3 increased ZEB1 expression at the transcriptional or post-transcriptional levels, nascent RNA was labeled and isolated from cells using Click-iT Nascent RNA Capture Kit (Invitrogen, Carlsbad, CA, USA). Real-time RT-PCR demonstrated that nascent ZEB1 mRNA was significantly increased in shBAG3-transfected FRO cells (Figure 4e), indicating that BAG3 knockdown affected ZEB1 expression at the transcriptional activation. It has been reported that β-catenin/TCF4 activates its transcription via binding directly to the ZEB1 promoter at -578 and -161 sites.[29] The fact of obvious nuclear accumulation of β-catenin in shBAG3-transfected FRO cells (Figures 2d and e) prompted us to investigate the involvement of β-catenin in the upregulation of ZEB1 by BAG3 knockdown. The TOP-Flash reporter system (Promega, Madison, WI, USA) demonstrated that β-catenin-TCF/LEF transcriptional activity was significantly increased in shBAG3-transfected FRO cells (Figure 4f). Chromosomal immunoprecipitation (ChIP) analysis demonstrated that interaction of β-catenin with the promoter sequence of *ZEB1* gene was significantly increased in BAG3-knockdown cells (Figure 4g). β-Catenin antibody demonstrated no obvious effects on recruitment of the promoter of *GAPDH* gene, which lacks TCF binding sites, excluding the nonspecific precipitation by the β-catenin antibody (Figure 4g).

To determine whether β-catenin corresponds to BAG3-knockdown-induced ZEB1 in FRO cells, we sought to selectively suppress the expression of β-catenin using small interfering RNA (siRNA). Two independent siRNA against β-catenin (siβ-catenin nos. 1 and 2) significantly suppressed β-catenin expression, and also significantly decreased ZEB1 expression (Figure 4h). siβ-Catenin no. 3 also suppressed

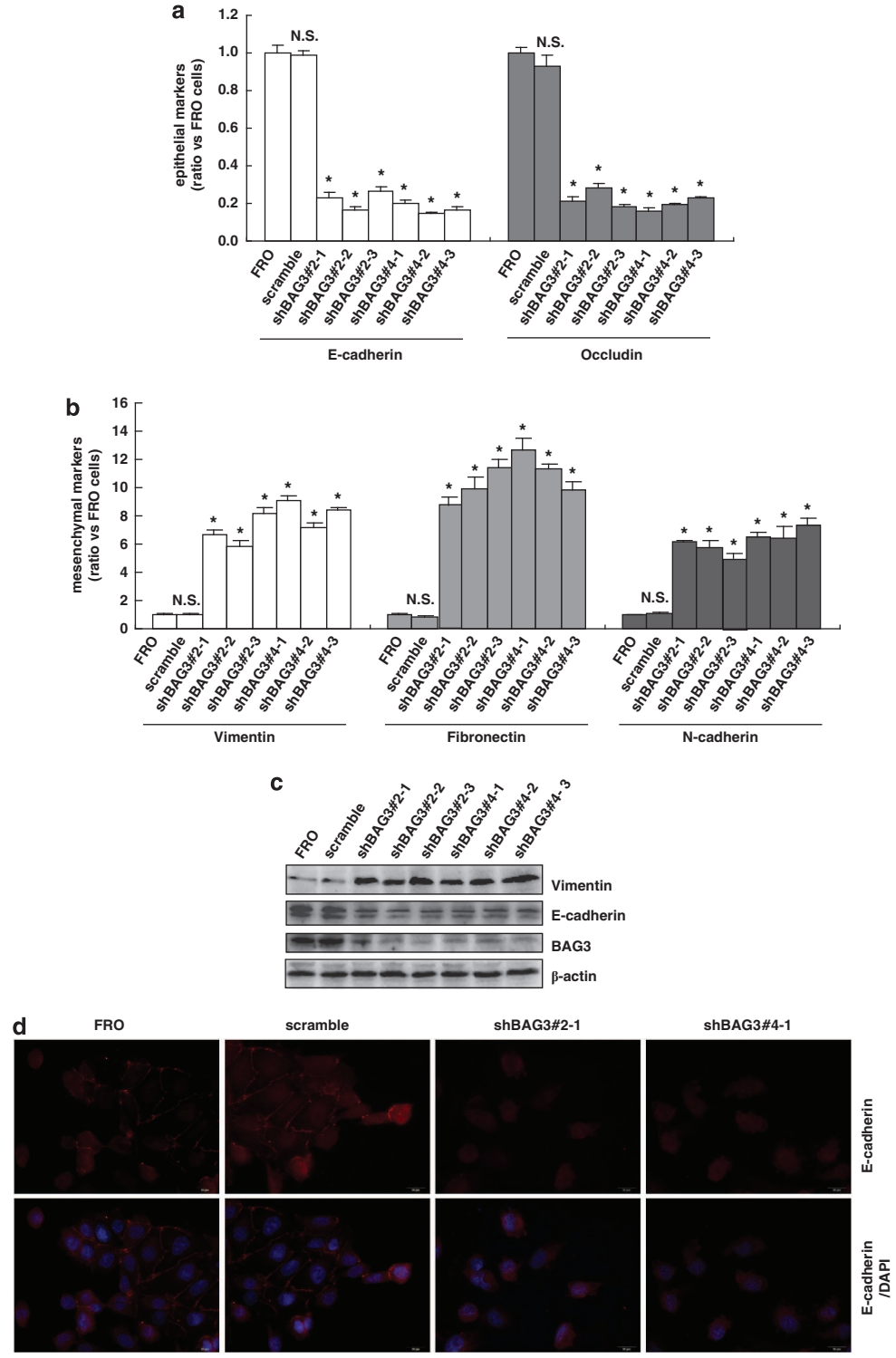


Figure 2 Knockdown of BAG3 induces EMT of thyroid cancer cells. (**a** and **b**) Total RNA was isolated from the indicated cells, epithelial (**a**) and mesenchymal (**b**) markers were analyzed using real-time RT-PCR. (**c**) Total protein was isolated form the indicated cells, and EMT markers were measured using western blot analysis. (**d**) IF of E-cadherin in the indicated cells. (**e**) IF of β-catenin in the indicated cells. (**f**) Nuclear proteins was isolated from the indicated cells, and western blot analysis was performed. (**g**) KTC1 and KTC3 cells were transfected with scramble shRNA, or combination of shBAG3 no. 2 and shBAG3 no. 4 (simply shBAG3 afterward). EMT markers were investigated using western blot analysis. (**h**) KTC1 and KTC3 cells were transfected with scramble shRNA or shBAG3, and real-time RT-PCR was performed to measure EMT markers. $^*P < 0.01$

β-catenin and ZEB1 expression, although to a much lesser extent (Figure 4h). Similarly, co-transfection of BAG3 shRNA and β-catenin siRNA significantly blocked the upregulation of ZEB1 mediated by BAG3 knockdown in KTC1 cells (Figure 4i).

ZEB1 is critical for E-cadherin reduction, migration, and invasion in BAG3-knockdown cells. To investigate the potential implication of ZEB1 in E-cadherin regulation mediated by BAG3 knockdown, we used siRNA-specific against ZEB1 (siZEB1) to suppress ZEB1 expression. Two independent siRNAs (siZEB1 nos. 1 and 3) decreased ZEB1 expression significantly in shBAG3 no. 1 subline FRO cells (Figure 5a). Importantly, knockdown of ZEB1 significantly increased E-cadherin expression (Figure 5a). Correlated with a sharp increase in E-cadherin expression, scratch wound-healing (Figure 5b) and Boyden chamber invasion assays (Figure 5c) demonstrated that knockdown of ZEB1 inhibited cellular motility and invasion, which were increased by BAG3 knockdown in FRO cells. Similar to that in FRO cells, E-cadherin expression was also increased in KTC1 cells co-transfected with shBAG3 and siZEB1, when compared with those transfected with shBAG3 alone (Figure 5d). In addition, Boyden chamber invasion assays demonstrated that downregulation of ZEB1 also decreased invasive capacity of KTC1 cells transfected with shBAG3 (Figure 5e).

Non-phosphorylated, but not phosphorylated, BAG3 at Ser187 site can rescue the effect of BAG3 knockdown in FRO cells. Previously, we have reported that BAG3 is phosphorylated at Ser187 site, and the phosphorylated form of BAG3 promotes EMT and invasiveness of thyroid cancer cells.[28] To investigate whether BAG3 could rescue the effect mediated by BAG3 knockdown, we transfected BAG3-knockdown FRO cells with wild-type-BAG3 (WT-BAG3), non-phosphorylated mimetic BAG3 mutant (S187A-BAG3) or phosphorylated mimetic BAG3 mutant (S187D-BAG3) expression vector (Figure 6a). WT-BAG3 and S187A-BAG3 decreased ZEB1 and increased E-cadherin expression, while S187D-BAG3 demonstrated no obvious effect (Figure 6a). Boyden

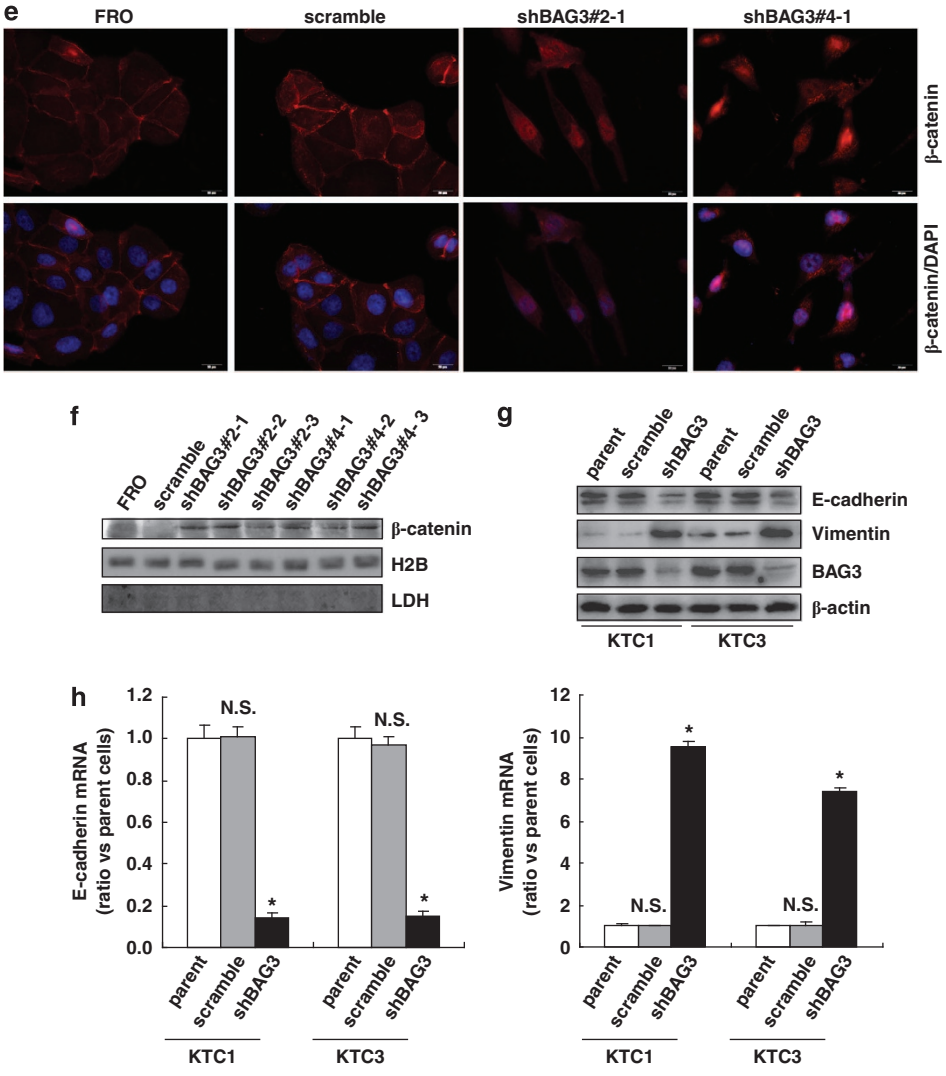


Figure 2 (Continued)

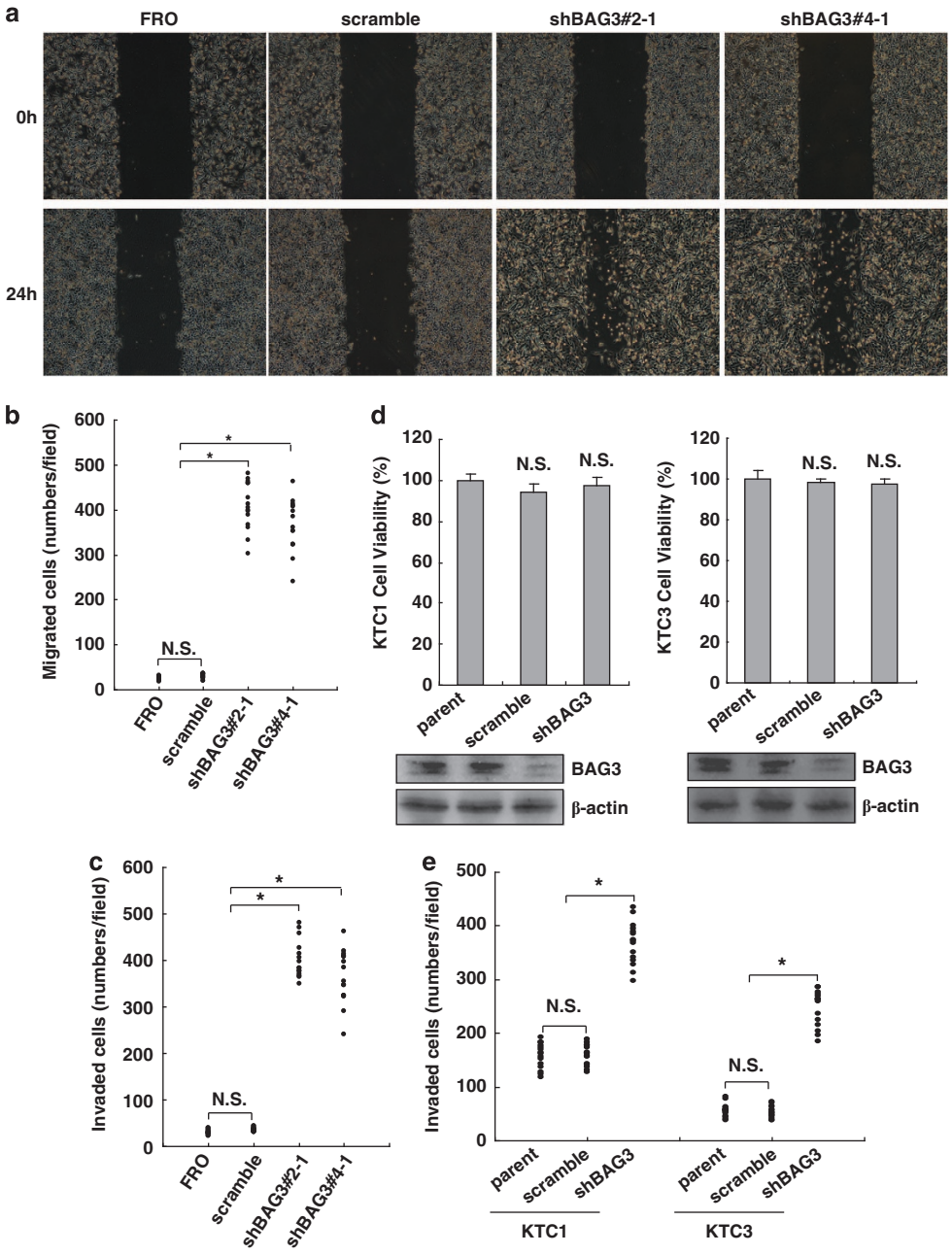


Figure 3 Knockdown of BAG3 increases motion, migration, and invasion of thyroid cancer cells. (**a**) The motion of the indicated cells was evaluated using wound-healing assays. (**b**) The migration of the indicated cells was evaluated by a Transwell assay, cells that have passed through Matrigel for 24 h were counted in five representative microscopic fields, and three independent experiments were performed. Cell numbers for each count were plotted in the graph. (**c**) The invasiveness of the indicated cells was evaluated by a Matrigel-coated Transwell assay, cells that have passed through Matrigel for 24 h were counted in five representative microscopic fields, and three independent experiments were performed. Cell numbers for each count were plotted in the graph. (**d**) KTC1 and KTC3 cells were transfected with scramble or shBAG3, andcell viability was measured using MTT assay. The expression of BAG3 was measured using western blot analysis and representative images were provided at the *bottom* of the graph. (**e**) KTC1 and KTC3 cells were transfected with scramble or shBAG3, and cell invasion was measured using a Matrigel-coated Transwell assay. $^*P<0.01$

chamber invasion assays demonstrated that WT-BAG3 and S187A-BAG3 significantly decreased invasiveness of BAG3-knockdown FRO cells, while S187D-BAG3 exhibited no obvious effect (Figure 6b). In addition, the suppressive effect of S187A-BAG3 was higher than those of WT-BAG3 (Figure 6b).

Discussion

BAG3 has a significant role in cell proliferation, apoptosis, migration, and invasion.[22,25,26] In a previous study, we found that BAG3 is a phosphorylation substrate of PKCδ, and phosphorylation of BAG3 at Ser187 sites induces EMT in

thyroid cancer.[28] EMT is a crucial step in the initiation of the metastatic spread of many tumor cells into distal organs.[7] In our present study, we found that knockdown of BAG3 significantly decreased expression of epithelial marker E-cadherin, but increased the expression of mesenchymal marker vimentin. Cytoskeleton IF demonstrated that BAG3-knockdown cells exhibited elongated morphology. In addition, knockdown of BAG3 significantly increased cell motility and invasion ability in thyroid cancer cells. These data indicated that knockdown of BAG3 may induce alterations in thyroid cancer cells resembling that of EMT procedure. In contrast to the current study, Iwasaki *et al.*[25] reported that BAG3 was localized to the leading edge of migrating cells, and BAG3 shRNA retrovirus-mediated the downregulation of BAG3, which in turn decreased the migration of COS7, ALVA31, Du145, and MCF7 cells using transwell chambers precoated with fibronectin. The different methodologies, as well as cell lines, used may contribute to the obviously paradoxical actions of BAG3 on invasiveness. BAG3 is a modular protein containing WW, PXXP, and BAG domains, which can interact with different partners. Therefore, BAG3 may influence invasion of cancer cells dependent on the tissue type and environment. For example, BAG3 was identified as a interacting partner of MMP-2, knockdown of BAG3 reduced MMP-2 expression, and invasiveness in MMP-2-positive ES2 ovarian cancer cells.[22] BAG3 was also identified to interact with PDZGEF2 via its WW domain, BAG3 overexpression increased cell invasion in control COS7 cells, but not in PDZGEF2-knockdown COS7 cells.[27] In addition, they reported that WW domain of BAG3 appeared to have a critical role, as a mutant BAG3 with deletion of WW domain-suppressed invasion of COS7 cells.[27] Furthermore, Kassis *et al.*[26] found that overexpression of WT-BAG3 decreased invasiveness, while overexpression of a mutant BAG3 with deletion of PXXP domain promoted adhesion and migration of MDA-MB-435 cells. Therefore, the apparent different interacting partners might influence the function of BAG3 in the regulation of invasiveness. Alternatively, phosphorylated status of BAG3 may reflect the paradoxical phenomena observed in different systems, as the non-phosphorylated and phosphorylated BAG3 at Ser187 site appear to function opposite in EMT and invasion of thyroid cancer cells, as the phosphorylated mimetic mutant S187D-BAG3 induces EMT and promotes migration and invasion; on the contrary, non-phosphorylated mimetic mutant S187A-BAG3 suppresses EMT and inhibits migration and invasion of thyroid cancer cells.[28] In the current study, we found that WT-BAG3 and S187A-BAG3 blocked induction of EMT and promotion of invasiveness mediated by BAG3 knockdown, while S187D-BAG3 could not rescue these effects of BAG3 knockdown. In some circumstances, BAG3 knockdown seemed to copy the phenotype of S187D-BAG3, such as induction of EMT, promotion of migration, and invasion in thyroid cancer cells, indicating that S187D-BAG3 might represent a mutant with loss of function. However, some difference also exists between BAG3-knockdown and S187D-BAG3 cells. For example, in the current study, we found that apparent nuclear accumulation of β-catenin was observed in BAG3-knockdown cells under basal culture condition. In S187D-BAG3 cells, β-catenin was accumulated in the cytoplasm, but rare nuclear distribution was observed under basal culture condition.[28] In addition, although E-cadherin mRNA levels were decreased in both S187D-BAG3- and shBAG3-transfected cells, its protein levels were only suppressed in shBAG3-transfected cells.[28] Thus, S187D-BAG3 may not a complete loss of function mutant, which might have some function under some circumstance.

As a cell adhesion molecule, the loss or reduction of E-cadherin expression is a key event in the initiation of EMT; transcription factors that suppress E-cadherin have been defined as potent drivers of EMT.[7] The major transcriptional repressors that are recognized to repress E-cadherin expression include those in the Snail and ZEB families, which are all able to activate EMT through direct binding to the E-cadherin promoter and repressing its transcription.[9] In the current study, ZEB1 expression was significantly increased in BAG3-knockdown cells at the transcriptional level. However, we could not detect obvious alteration in ZEB2, Snail1, Snail2, Twist1, Twist2, and E12/E47 expressions. This result suggests that BAG3 may specifically regulate ZEB1, but we cannot exclude the possibility that BAG3 regulates these other EMT transcription factors in a cell type- or cell-context-dependent manner. The importance of ZEB1 in BAG3-knockdown-induced EMT was further highlighted by the fact that knockdown of ZEB1 significantly increased E-cadherin expression, and reduced cell migration and invasion ability. In addition, the current study demonstrated that ZEB1 was not present in the nuclei of parental or scramble shRNA-transfected FRO cells, but its obvious nuclear accumulation was observed in BAG3-knockdown FRO cells. Further investigation is required to clarify the mechanisms by which BAG3 regulates nuclear translocation of ZEB1. The upstream signals implicated in the regulation of ZEB1 are not fully characterized. It is only recently reported that ZEB1 is induced by several pathways triggering EMT processes.[30,31] It has been reported that β-catenin/TCF4 directly binds to the ZEB1 promoter and activates ZEB1 transcription.[29] Apparent nuclear accumulation of β-catenin in BAG3-knockdown cells prompted us to investigate the possibility of its involvement. The current study demonstrated that BAG3 knockdown resulted in the activation of β-catenin/TCF4 transcription. Importantly, silence of β-catenin significantly suppressed the upregulation of ZEB1 mediated by BAG3 knockdown. These results indicate that the activation of β-catenin transcription is highly important for ZEB1 induction in BAG3-knockdown thyroid cancer cells. Currently, the mechanisms underlying activation of β-catenin by BAG3 knockdown remain poorly understood.

Previously, it has been shown that knockdown of BAG3 by adenoviral vectors reduces proliferation of thyroid cancer 8505C cells.[23] The current study demonstrated that stable knockdown of BAG3 had no obvious influence on proliferation of thyroid cancer FRO cells. Different methodologies and cell lines may contribute to the different observations by others and us. Alternatively, stable cell lines may acquire some adaptation during selection. For example, the current study found that transient knockdown had no obvious influence on the expression of antiapoptotic protein BAG1, while its expression was increased in stable BAG3-knockdown cells.

Overall, our study demonstrates that BAG3 is a negative regulator of EMT and metastasis in thyroid cancer cells, specifically through transcriptional repression of E-cadherin via ZEB1. BAG3 is thus an attractive therapeutic target for reversing EMT and tumor metastasis, both key events in thyroid cancer progression that lead to mortality.

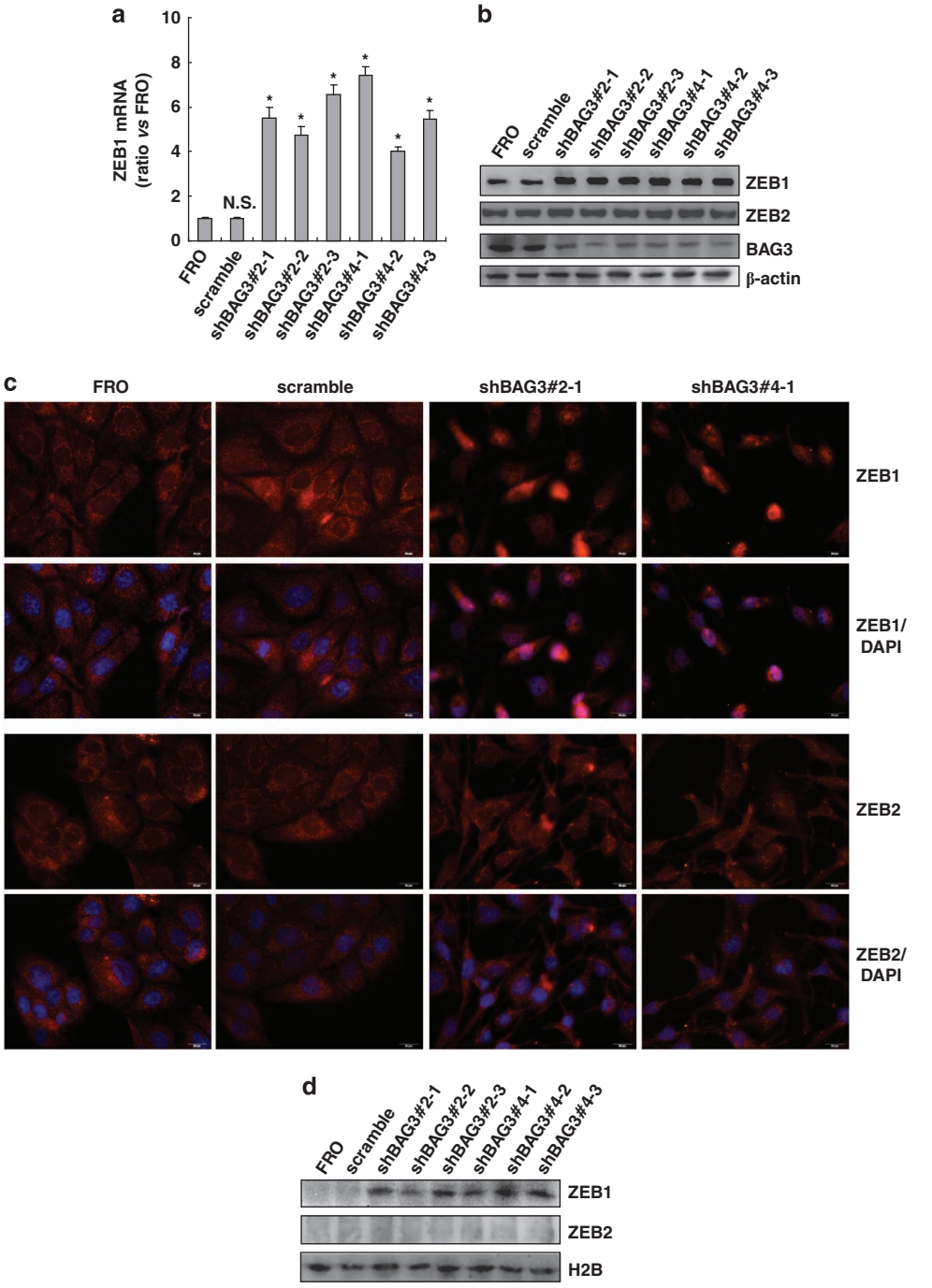


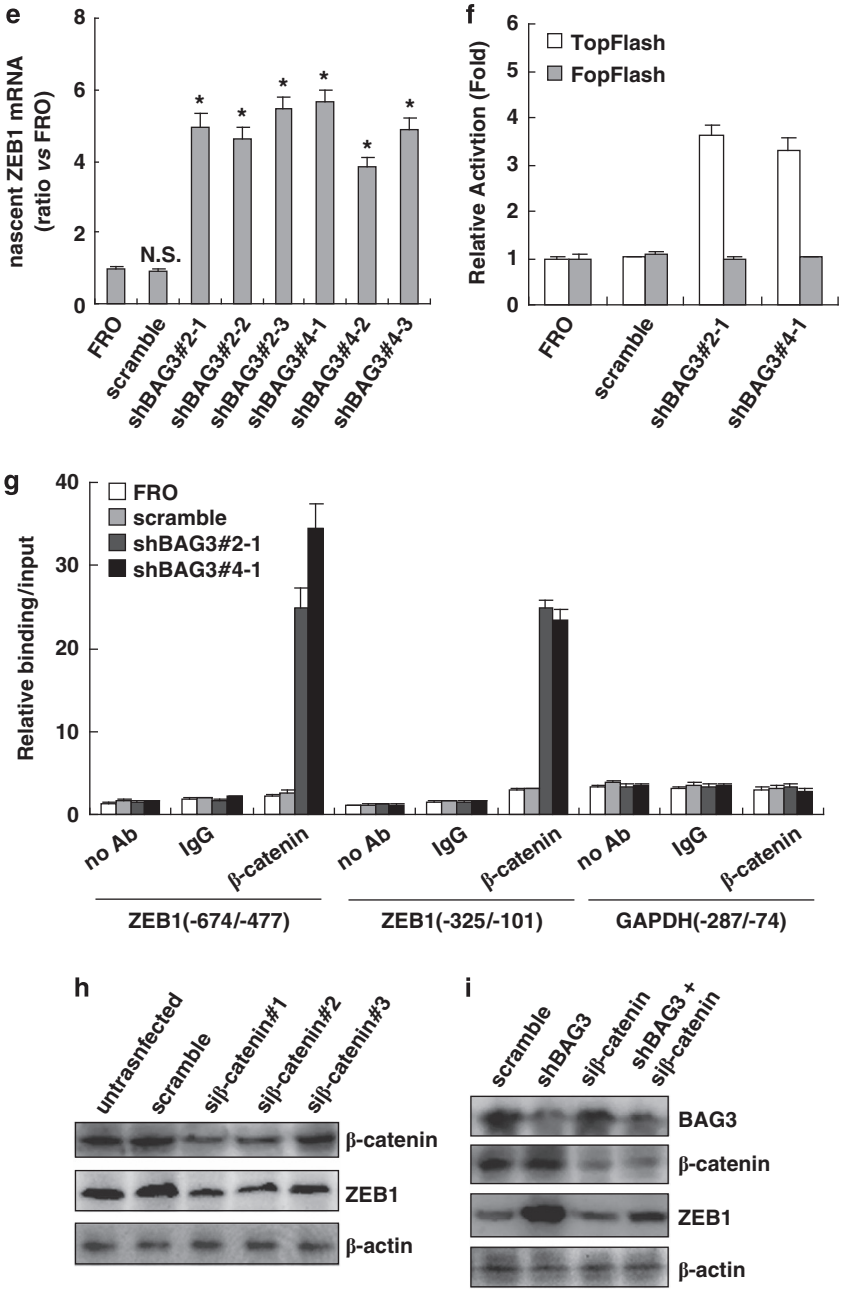
Figure 4 Knockdown of BAG3 upregulates ZEB1 via β-catenin activation. (**a**) Total RNA was isolated from the indicated cells, and ZEB1 mRNA expression was measured using real-time RT-PCR. (**b**) Total protein was isolated from the indicated cells, and ZEB1 and ZEB2 protein expression was analyzed using western blot analysis. (**c**) IF of ZEB1 and ZEB2 in the indicated cells. (**d**) Nuclear proteins were isolated from the indicated cells and western blot was performed using the indicated antibodies. (**e**) Nascent RNA was labeled and isolated from the indicated cells using Click-iT Nascent RNA Label and Capture Kit (Invitrogen); nascent ZEB1 mRNA was measured using real-time RT-PCR. (**f**) Cells were co-transfected with TOP-Flash or FOP-Flash reporter construct with *Renilla* luciferase reporter construct for 48 h; *Firefly* and *Renilla* luciferase activities were analyzed using Dual-Luciferase Reporter Assay System. (**g**) ChIP analysis was performed using a specific anti-β-catenin antibody and immunoprecipitated DNA was amplified by real-time PCR. (**h**) Stable BAG3-knockdown shBAG3 no. 2-1 FRO subline was transfected with scramble siRNA or siRNA-specific β-catenin (siβ-catenin), and western blot analysis was performed using the indicated antibodies. (**i**) KTC1 cells were transfected with shBAG3 and siβ-catenin alone or combination, and western blot analysis was performed using the indicated antibodies. $^*P<0.01$

Materials and Methods

Culture of multiple cancer cell lines. FRO82-1 (simply FRO) cell lines were initially obtained from Dr. James A Fagin (University of Cincinnati College of Medicine, Cincinnati, OH, USA) and provided to us by Dr. Shunichi Yamashita (Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan). KTC1 and KTC3 cell lines were generously provided by Dr. Junichi Kurebayashi (Kawasaki Medical School, Kurashiki, Japan).

Generation of stable BAG3-knockdown FRO cells. EGFP-tagged shRNA against BAG3 (shBAG3) was purchased from GeneChem Corporation (Shanghai, China). The following sequences were used to target BAG3: shBAG3 no. 1, 5′-GATACACGAGCAGAACGTTA-3′; shBAG3 no. 2, 5′-GACCAGGCTACA TTCCCATTCCT-3′; shBAG3 no. 3, 5′-GCAAAGAGGTGGATTCTAAACCTGTTT-3′; and shBAG3 no. 4 5′-GCAGGCTGTAGACAACTTTGAA-3′. FRO cells were transfected with Lipofectamine 2000 reagent (Invitrogen) as instructed by the supplier. Stable clones were selected with 1 μg/ml of G418 and maintained with 200 ng/ml of G418.

RNA isolation and real-time RT-PCR. Total RNA was isolated using TRIZOL Reagent (Invitrogen) and first-strand cDNA was synthesized from 2 μg of total RNA using SuperScript II Rnase H$^-$ Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time RT-PCR analysis was performed in triplicate on the ABI prism 7000 sequence detection system (Applied Biosystems, Eugene, OR, USA) using the SYBR Green PCR Master Mix (Applied Biosystems). Results were normalized against those of 18S rRNA and presented as ratio *versus* vehicle-treated control.



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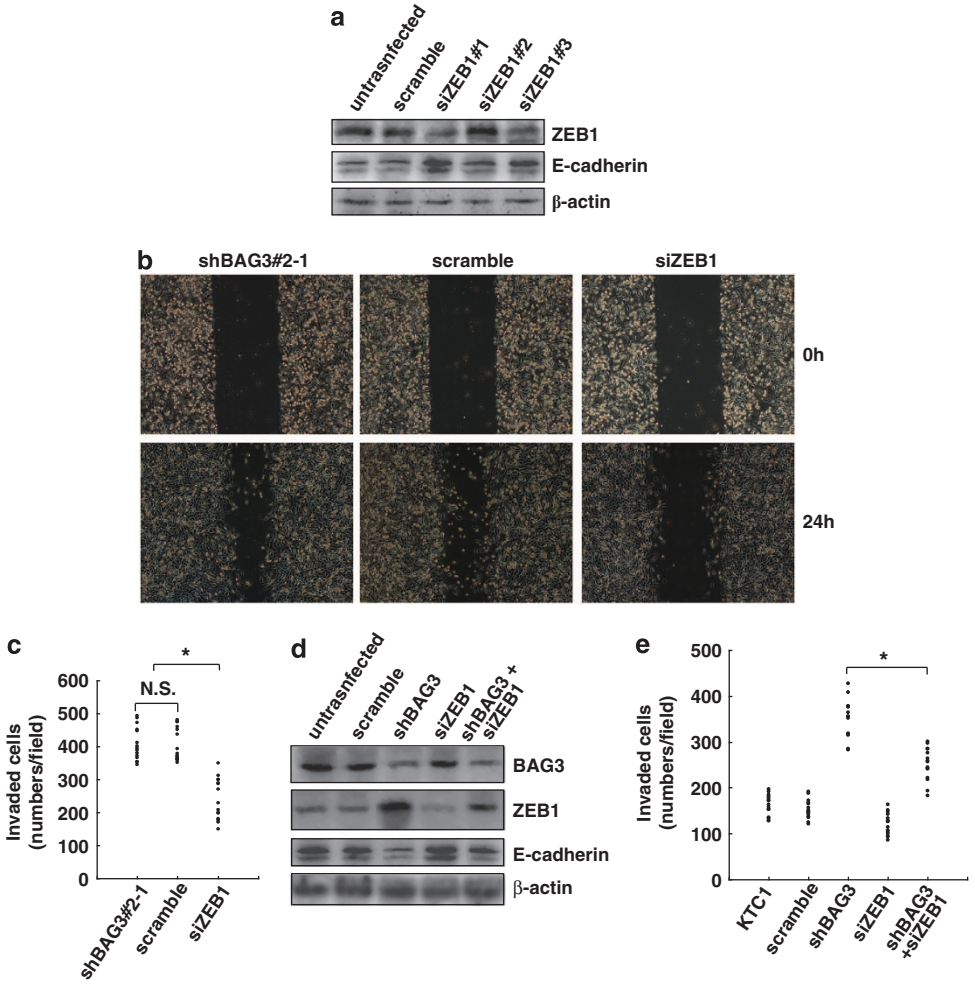

Figure 5 ZEB1 is implicated in the regulation of EMT, and knockdown of BAG3 promotes motion and invasion. (**a**) Stable BAG3-knockdown shBAG3 no. 2-1 FRO subline cells were transfected with scramble siRNA or siRNA-specific ZEB1 (siZEB1), and western blot analysis was performed using the indicated antibodies. (**b**) shBAG3 no. 2-1 FRO subline cells were transfected with scramble siRNA or siZEB1, and cell motion was measured using scratch wound-healing experiment. (**c**) shBAG3 no. 2-1 FRO subline cells were transfected with scramble siRNA or siZEB1, and cell invasiveness was evaluated by a Matrigel-coated Transwell assay. (**d**) KTC1 cells were transfected with shBAG3 and siZEB1 alone or in combination, andwestern blot analysis was performed using the indicated antibodies. (**e**) KTC1 cells were transfected with shBAG3 and siZEB1 alone or in combination, andcell invasiveness was evaluated by a Matrigel-coated Transwell assay. $^{*}P<0.01$

IF staining and fluorescence microscopy. Cells were fixed with 4% paraformaldehyde, permeabilized for 5 min with PBS containing 0.1% Triton X-100, and blocked with 1% BSA. Immunostaining was performed using the appropriate primary and secondary antibodies, and images were acquired using an Olympus fluorescence microscope (Osaka, Japan).

Quantification of elongated cell morphology. Elongated cell morphology was measured as reported previously.[32] Briefly, cells were stained for F-actin with rhodamine-labeled phalloidin and nuclei with DAPI, and images of cells were acquired using a $\times 40$ objective. The lengths of the major and minor cell axes were measured using DP2-BSW software (Olympus, Osaka, Japan). The ratios of the major axis to the minor axis of cells were used to determine the degree of elongated cell morphology. For each experiment, at least 50 cells were measured.

Subcellular fractionation. Nuclei were isolated using a nuclear extract kit from Active Motif North America (Carlsbad, CA, USA) and the 60 μg of nuclear proteins were used in western blot analysis.

Western blot analysis. Cells were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA). Cell extract protein amounts were quantified using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Equivalent amounts of protein (25 μg) were separated using 12% SDS-PAGE and transferred to PVDF membrane (Millipore Corporation, Billerica, MA, USA).

Label and capture nascent RNA. Newly synthesized RNA was isolated using Click-iT Nascent RNA Capture Kit (Invitrogen) according to the manufacturer's instruction. Briefly, cells were incubated in 0.2 mM of 5-ethymyl uridine (EU, an alkyne-modified uridine analog, which is efficiently and naturally incorporated into the nascent RNA) for 4 h and total RNA labeled with EU was isolated using TRIzol reagent (Invitrogen). Then, EU-labeled RNA was biotinylated in a Click-iT reaction buffer (Invitrogen) with 0.5 mM of biotin azide, and subsequently captured on streptavidin magnetic beads.

Scratch wound-healing assay. Cells were seeded in a six-well plate and grown overnight to confluence. The monolayer cells were scratched with a sterile pipette tip to create a wound, and cells were washed twice with serum-free DMEM to remove floating cells and then replaced with medium without serum. The cells migrating from the leading edge were photographed at 0 and 24 h. Multiple views of each well were documented, and three independent experiments were performed.

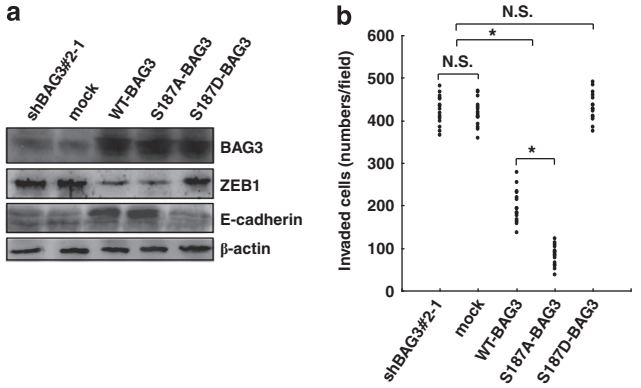

Figure 6 Non-phosphorylated, but not phosphorylated, BAG3 at Ser187 site rescues the effects of BAG3 knockdown. (**a**) Stable BAG3-knockdown FRO subline was transfected with the indicated expression vector, and western blot analysis was performed. (**b**) Stable BAG3-knockdown FRO subline was transfected with the indicated expression vector, and cell invasion was measured using a Matrigel-coated Transwell assay. $^*P<0.01$

Transwell migration and invasion assays. *In vitro* Transwell migration assays were performed in modified Boyden chambers with 8-μm pore filter inserts in 24-well plates (BD Biosciences, Eugene, OR, USA). Briefly, the lower chamber was filled with DMEM containing 10% FBS. Cells were collected after trypsinization, resuspended in 200 μl of DMEM medium, and transferred to the upper chamber (1×10^5 cells per well). After 24 h of incubation, the filter was gently removed from the chamber and the cells on the upper surface were removed using a cotton swab. Cells were fixed and nuclei were stained with DAPI. Cells were counted in five representative microscopic fields and photographed. Three independent experiments were performed.

The invasion assay was performed using BD BioCoat Matrigel invasion chambers (BD Biosciences; 8-μm pore size). The same procedures described above were used, except that the filters were precoated with 100 μl Matrigel at a 1 : 4 dilution in DMEM to form a genuine reconstituted basement membrane.

TOP-Flash assay. The TCF/LEF reporter plasmid, TOP-Flash, and its mutant control, FOP-Flash, were kindly provided by Prof. F Li (China Medical University, Shenyang, China). In brief, cells were co-transfected with 2 μg of reported construct pTOP-Flash, which contains consensus sequence of TCF/LEF enhancer upstream of the luciferase enzyme coding region, or pFOP-Flash (control). To serve as internal control for transfection, cells were co-transfected with non-TCF/LEF-dependent *Renilla* luciferase coding vector (PGL4.74[hRluc/TK]; Promega). Luciferase activities were measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Experiments were performed in triplicate and repeated at least three times.

ChIP assay. ChIP assays were performed using a kit from Upstate Biotechnology Inc. (Lake Placid, NY, USa) according to the protocol supplied. In brief, cells were fixed with 1% formaldehyde in PBS to crosslink chromatin. Cell lysates were prepared and sonicated on ice to break chromatin DNA to an average length of 400 bp. After a preclearing step, IP was carried out at 48 °C overnight with anti-β-catenin antibody or normal goat IgG (negative control antibody). Immune complexes were collected with salmon sperm DNA saturated protein A-agarose beads. After extensive washing, the immunoprecipitated complexes were eluted with 0.1 M $NaHCO_3$ and 1% SDS, and then protein–DNA crosslinks were reversed by incubating at 65 °C for 5 h. DNA was purified using proteinase K digestion, phenol: chloroform extraction, and ethanol precipitation. Real-time PCR was performed using primers specific for the ZEB1 promoter sequence. For the TCF binding site at position -578 of the human ZEB1 promoter, the -674 and -477 region was amplified with the primer pairs 5′-TGGAAGGGAAGGGAAGGGAGTC-3′ (forward) and 5′-AGGCAGGGCTACCA TCAGTC-3′ (reverse). For the TCF binding site at position -161 of the human ZEB1 promoter, the -325 and -101 region was amplified with the primer pairs 5′-TTTACCTTTCCAACTCCGACAGC-3′ (forward) and 5′-GGCTTTACGACATCA CCTTCCTTAC-3′ (reverse). The amount of ZEB1 promoter that was present in the IP and input fractions was calculated from the standard curve. The input represents 1% of the material used in the IP assay. The results were expressed as the IP/input ratios of the PCR products that were used for comparison.

siRNA. The siRNA sequences used here were as follows: siRNA against ZEB1 (siZEB1), 5′-AAUGAAGAUAACUUUAGUUGdTdT-3′ (no. 1); 5′-ACAGGACUCAA GACAUCUdTdT-3′ (no. 2); 5′-UUGAAAGUGAUCCAGCCAAAUdTdT-3′ (no. 3). siRNA against β-catenin (siβ-catenin), 5′-UUGUACGUACCAUGCAGAAUdTdT-3′ (no. 1); 5′-AGGCUCUUGUGCGUACUGUdTdT-3′ (no. 2); 5′-GUUGCUUGUUCGUGC ACAUdTdT-3′ (no. 3). The scramble nonsense siRNA (scramble; 5′-UUCUCCG AACGUGUCACGUTT-3′) that has no homology to any known genes was used as control. Transfection of siRNA oligonucleotide was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Statistics. The statistical significance of the difference was analyzed by ANOVA and *post hoc* Dunnett's test. Statistical significance was defined as $P<0.05$. All experiments were repeated three times, and data were expressed as the mean ± s.d. from a representative experiment.

Conflict of Interest

The authors declare no conflict of interest.

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