ASB13 inhibits breast cancer metastasis through promoting SNAI2 degradation and relieving its transcriptional repression of YAP

Huijuan Fan,1 Xuxiang Wang,1 Wenyang Li,2 Minhong Shen,2 Yong Wei,2 Hanqiu Zheng,1,4 and Yibin Kang2,3,4

1Department of Basic Medical Sciences, School of Medicine, Tsinghua University, Beijing 100084, China; 2Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA; 3Cancer Metabolism and Growth Program, Rutgers Cancer Institute of New Jersey, New Brunswick, New Jersey 08903, USA

Transcription factor SNAI2 plays key roles during development and has also been known to promote metastasis by inducing invasive phenotype and tumor-initiating activity of cancer cells. However, the post-translational regulation of SNAI2 is less well studied. We performed a dual-luciferase-based, genome-wide E3 ligase siRNA library screen and identified ASB13 as an E3 ubiquitin ligase that targets SNAI2 for ubiquitination and degradation. ASB13 knockout in breast cancer cells promoted cell migration and decreased F-actin polymerization, while overexpression of ASB13 suppressed lung metastasis. Furthermore, ASB13 knockout decreased YAP expression, and such regulation is dependent on an increased protein level of SNAI2, which in turn represses YAP transcription. YAP suppresses tumor progression in breast cancer, as YAP knockout increases tumorsphere formation, anchorage-independent colony formation, cell migration in vitro, and lung metastasis in vivo. Clinical data analysis reveals that ASB13 expression is positively correlated with improved overall survival in breast cancer patients. These findings establish ASB13 as a suppressor of breast cancer metastasis by promoting degradation of SNAI2 and relieving its transcriptional repression of YAP.

[Keywords: breast cancer; ASB13; SNAI2; ubiquitin proteasome system; Hippo–YAP pathway; migration; metastasis]

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The majority of cancer-related deaths result from distant metastasis (Valastyan and Weinberg 2011; Wan et al. 2013). As the first step in metastasis, local and lymph node invasion is a strong poor prognosis marker for patient survival (Rakha et al. 2012). SNAI2 (SLUG) is a C2H2 zinc finger transcriptional repressor belonging to the three-member family of SNAIL protein [SNAIL, SNAI2, and Smuc] (Nieto 2002). Although best known for its role in orchestrating epithelial–mesenchymal transition program (EMT), during which tumor cells lose their epithelial properties and resemble fibroblast-like cell phenotypes with increased cell migration ability, more recent studies reveal that SNAI2 has a much broader effect on cancer progression, including many functions independent of its role in regulating EMT. SNAI2 has been linked to the induction of tumor initiation cell, cell cycle regulation, as well as invasion and metastasis (Mittal et al. 2011; Guo et al. 2012; Nieto and Cano 2012; Uygur et al. 2015). A better understanding of the regulatory mechanisms for SNAI2 will provide critical information on how to block cancer progression and metastasis.

The SNAI2 protein, like SNAIL, is rapidly turned over by the ubiquitin proteasome system (UPS) (Zheng and Kang 2014). Previous results suggested β-TrCP1/FBXW1, CHIP (C terminus of Hsc70-interacting protein), MDM2, and FBXL14 as E3 ligases for SNAI2 degradation. However, none of these E3s have been tested for their roles in regulating endogenous SNAI2, especially in the context of cancer cell migration and metastasis. It is still not clear which E3 ligases are the major mediators regulating endogenous SNAI2 ubiquitination and degradation in breast cancer (Vernon and LaBonne 2006; Wang et al. 2009; Wu 2019). The available data and our results suggest that ASB13 is a novel and potent E3 ligase that targets SNAI2 for ubiquitination and degradation, thereby relieving its transcriptional repression of YAP in breast cancer cells.
et al. 2012; Kao et al. 2014). Identification of such E3 ligases and relevant signaling pathways will provide a comprehensive understanding of the regulatory mechanisms for SNAI2 in cancer. To this end, we performed a dual-luciferase-based E3 siRNA screening similarly as in our previous study (Zheng et al. 2014) and tested functions of the identified E3 ligase ASB13 in breast cancer tumorigenesis, migration, and metastasis.

ASB13 belongs to the ankyrin repeat and suppressor of cytokine signaling (SOCS) box (Asb) E3 ligase protein family (Liu et al. 2019). It has been implicated to be involved in tumor progression, although its functional role in cancer has not been directly tested (Blenk et al. 2007; Chi et al. 2018). Through stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative proteomic profiling, several potential interacting proteins for ASB13 have been identified, including POLR3A, TCEB2, SSBP1, CCT8, etc. (Andresen et al. 2014). However, none of these proteins have been biochemically validated as direct protein substrate for ASB13.

Hippo pathway is a major regulator for organ size by controlling cell proliferation, differentiation, and migration in developing adult tissues (Meng et al. 2016). This pathway is controlled by intrinsic cell machineries, such as cell polarity and actin cytoskeleton, as well as a wide range of paracrine signals, including cell–cell contact, cellular energy status, mechanical cues, and hormonal signals that act through G-protein-coupled receptors (Zhao et al. 2007; Dupont et al. 2011; Yu et al. 2012). As an important downstream mediator, yes-associated protein (YAP) was originally found to promote cell proliferation and transformation. Overexpression of YAP has been linked to tumor progression and worse patient survival in many cancer types (Xu et al. 2009; Wang et al. 2010; Song et al. 2012; Su et al. 2012; Liu et al. 2013). However, in breast cancer, YAP has been suggested as a tumor suppressor gene, as it is located in a chromosome region with frequent loss of heterozygosity (LOH) (Carter et al. 1994; Hampton et al. 1994; Tomlinson et al. 1995). Decreased expression of YAP is correlated with tumor progression and worse survival in breast cancer (Gudmundsson et al. 1995; Winqvist et al. 1995; TuFAIL et al. 2012). However, the exact role of YAP in breast cancer progression and metastasis and whether YAP can cross-talk with critical metastasis determinants like SNAI2 is still not clear. Understanding such cross-talk may reveal possible windows for therapeutic targeting of SNAI2 and Hippo pathway in cancer.

**Results**

A dual-luciferase system for a genome-wide screen to identify E3 ligases targeting SNAI2 protein

We first confirmed that the SNAI2 protein, like its family member SNAIL, is degraded through UPS (Zheng et al. 2014). The SUM159 breast cancer cell line (Forozan et al. 1999) culture was treated with cycloheximide (CHX) to block protein synthesis and the endogenous SNAI2 protein degradation was chased afterward [CHX chase assay]. Indeed, the SNAI2 protein was degraded rapidly and kept at a low baseline expression level within 2.5 h of CHX treatment in SUM159 cells [Fig. 1A; Supplemental Fig. S1A,B]. To confirm that SNAI2 degradation is mediated by the 26S proteasome, we blocked UPS with the proteasome inhibitor MG132, and the endogenous SNAI2 protein level was significantly increased [Fig. 1A; Supplemental Fig. S1A,B]. Similarly, SNAI2 was also degraded rapidly in a proteasome-dependent manner in LM2 cell line, a highly lung-metastatic subline of the MDA-MB-231 breast cancer cell line [Fig. 1B; Supplemental Fig. S1C–D; Minn et al. 2005]. To identify E3 ligase(s) responsible for SNAI2 ubiquitination and degradation, we generated a dual-luciferase-based siRNA library screening system that we developed in our previous study (Zheng et al. 2014). We fused the coding sequence of SNAI2 in-frame with the Firefly Luciferase coding sequence to produce lentiviruses containing this fusion gene (SNAI2-Luc). Breast cancer cell line SUM159 was then transduced with SNAI2-Luc lentiviruses and retroviruses constitutively expressing renilla luciferase to generate a dual-luciferase reporter stable cell line. In this cell line, firefly luciferase activity reliably represents the expression level of the SNAI2-Luc protein, while renilla luciferase activity is used as internal control. This reporter cell line, denoted as “SUM-SNAI2-Luc/rLuc” to facilitate descriptions below, allowed us to monitor SNAI2 stable level and its degradation dynamics by measuring luciferase activities [Fig. 1C]. When transfected and expressed in HEK293T cells, SNAI2-Luc mostly localized in the nucleus, similar to the nuclear localization of endogenous SNAI2 (Fig. 1D). In both HEK293T cells and SUM159, the SNAI2-Luc protein was degraded through UPS as demonstrated by CHX chase assay and proteasome inhibition assay [Fig. 1E,F; Supplemental Fig. S1E,F].

siRNA library screening identifies potential E3 ligases for targeting SNAI2 degradation

We followed the procedures outlined in Figure 2A to identify potential E3 ligase candidates for SNAI2 protein. Individual human E3 ligase was knocked down [KD] using pooled siRNAs [three siRNAs per gene] in SUM-SNAI2-Luc/R-Luc cells and those E3 ligase genes whose KD resulted in more than twofold increase in firefly luciferase/renilla-luciferase ratio were identified as candidate SNAI2 targeting E3s [Fig. 2B]. Among those E3 ligases, there were several ASB [ankyrin repeat and suppressor of cytokine signaling box] family members and TRIM [tripartite motif] family members [pink dots on the graph]. We next attempted to clone these E3 ligases and validate their interactions with SNAI2. Among the ones we were able to clone, ASB13 and TRIM3 were validated to interact with SNAI2 based on coimmunoprecipitation (co-IP) assay [Fig. 2C; Supplemental Fig. S2A]. Immunofluorescence staining showed that ectopically expressed HA-ASB13 was mostly localized in the nucleus of HEK293T cells, and colocalized with SNAI2. In contrast, TRIM3 was not colocalized with SNAI2, as it mostly resided in
ASB13 suppresses metastasis by SNAI2 degradation

**Figure 1.** A dual-luciferase system for the identification of SNAI2 targeting E3 ligase. [(A)] SUM159 cells were treated with 10 µM CHX for indicated time or MG132 for 6 h. The endogenous SNAI2 protein was detected by immunoblotting. β-ACTIN was used as internal loading control. (+) Treated with MG132, (−) no treatment. [(B)] LM2 cells were treated with 10 µM CHX for indicated time or MG132 for 6 h. The endogenous SNAI2 protein was detected by immunoblotting. β-ACTIN was used as internal loading control. [(C)] Illustration of the dual-luciferase reporter screening system for SNAI2 targeting E3 ligase. (CMV) Cytomegalovirus promoter, (LTR) long terminal repeat viral promoter. [(D)] Immunofluorescence staining of SNAI2-Luc in SUM159-SNAI2-Luc cell line with SNAI2 antibody. Scale bar, 50 µm. (E) In HEK293T cells stably expressing SNAI2-Luc, the turnover rate for SNAI2-Luc was determined by CHX pulse-chase assay and luciferase assay. Data are presented as mean ± standard error. (F) In SUM159 cells stably expressing SNAI2-Luc, the turnover rate for SNAI2-Luc was determined by CHX pulse-chase assays.

ASB13 targets SNAI2 for ubiquitination and degradation

To investigate whether ASB13 functions as a bona fide E3 ubiquitin ligase for SNAI2, Domain deletion mutants of ASB13 were generated and their interactions with SNAI2 were tested with co-IP experiment. Deletion of either ANK3-4 or ANK5-6 almost completely diminished ASB13’s interaction with SNAI2 [Supplemental Fig. S3A]. SNAI2 protein has been reported as a marker for poor prognosis in cancer (Hajra et al. 2002; Uchikado et al. 2005, 2011), with its high expression correlated with worse patient outcome, and SNAI2 is also expressed at a higher level in estrogen receptor-negative (ER−) patients than ER+ patients in breast cancer (Chakrabarti et al. 2012). Thus, we hypothesized that E3 ligase(s) targeting SNAI2 could be a good prognosis marker in breast cancer and might be expressed at a lower level in ER− patients, based on its negative regulation of SNAI2. Indeed, analyses of clinical breast cancer data sets confirmed that ER− breast cancer patients have lower ASB13 level in ER− breast cancer, and higher ASB13 expression correlates with longer overall patient survival [Fig. 3B,C].

Many signaling pathways like TGF-β, Wnt, and EGF pathways can regulate EMT and cancer metastasis (Ciruna and Rossant 2001; Kim et al. 2002; Lu et al. 2003; Gotzmann et al. 2004; Thuault et al. 2006; Yook et al. 2006; Leong et al. 2007). We found that ASB13 level was reduced after treatment by these EMT inducers in MCF10A breast epithelial cells and EpRas breast cancer cells [Fig. 3D,E], suggesting that ASB13 might work downstream from these EMT-inducing signals to stabilize endogenous SNAI2 protein. To directly test the effect of ASB13 on endogenous SNAI2 degradation, we generated ASB13-overexpressing stable cell lines in SUM159 and LM2 cells. In both cell lines, we observed a dramatic decrease of the endogenous SNAI2 protein level and the acceleration of its degradation when ASB13 was

the paranucleus region [Fig. 2D; Supplemental Fig. S2B]. Interestingly, in SUM159 cells stably expressing HA-ASB13, immunofluorescent staining demonstrated that ASB13 was localized in both nucleus and cytoplasm [Supplemental Fig. S2C]. Consistently, nuclear and cytoplasmic fractionation experiment demonstrated that ASB13 was partially localized in the nucleus and its expression reduced the SNAI2 protein stable level [Supplemental Fig. S2D]. The interaction between ectopically expressed ASB13 and endogenous SNAI2 was also observed in SUM159 cells as demonstrated by co-IP experiment [Supplemental Fig. S2E]. To functionally test the ability of ASB13 and TRIM3 to promote SNAI2 degradation, we cotransfected SNAI2-Luc with either ASB13 or TRIM3, and examined the degradation dynamics for SNAI2-Luc. ASB13 accelerated SNAI2-Luc protein degradation, whereas TRIM3 only had modest effect on SNAI2-Luc turnover [Fig. 2E,F]. Taken together, we considered ASB13 as the primary E3 ligase candidate for SNAI2.
overexpressed (Fig. 3F–I). To further test the effect of reducing endogenous ASB13 expression on SNAI2 degradation, we used siRNA to knock down ASB13 expression in SUM159 cells. SNAI2 protein was significantly stabilized after ASB13 KD in a pulse-chase assay (Supplemental Fig. S3B,C). Likewise, SNAI2 protein was significantly stabilized after ASB13 knockout (KO) by CRISPR-gRNA technology (Supplemental Fig. S3D,E).

Consistently, the stable level of SNAI2 protein was found to be increased after ASB13 KO in both SUM159 and LM2 cells by three independent gRNA-mediated KOs (Fig. 4A,B; Supplemental Fig. S4A,B), while SNAI2 mRNA level was not changed after ASB13 KO [Fig. 4C]. Interestingly, when we KO other previously reported E3s for SNAI2 such as BTRC1 and FBXO11 (Vernon and LaBonne 2006; Wang et al. 2009; Wu et al. 2012; Kao et al. 2014) in SUM159 or LM2 cells, the endogenous SNAI2 level was not changed significantly [Supplemental Fig. S4C,D]. Taken together, our results suggest that ASB13 is a bona fide E3 ubiquitin ligase targeting endogenous SNAI2 protein for ubiquitination and degradation.
ASB13 inhibits breast cancer metastasis through promoting SNAI2 degradation

SNAI2 has been reported to induce cell migration in cancer cells (Kurrey et al. 2005; Martin et al. 2005; Shih et al. 2005; Peinado et al. 2007). As ASB13 KO increased the SNAI2 protein level, we speculated that ASB13 KO may increase cell migration ability. Indeed, in both SUM159 and LM2 cells, there were significantly more migrated cells in ASB13 KO compared with control cells in the transwell migration assay (Fig. 4D,E). To further investigate the functional impact of ASB13 on SNAI2-induced breast cancer metastasis, we intravenously injected 6-wk-old female athymic nude mice with vector control or ASB13-overexpressed LM2 cells [labeled with luciferase for in vivo tracking]. Overexpression of ASB13 significantly reduced lung metastasis progression as compared with that of control group by bioluminescence imaging (BLI) (Fig. 4F). There were also significantly less lung metastasis nodules in ASB13 overexpression group (Fig. 4G, H). In summary, these results suggest that ASB13 inhibits migration and metastasis by promoting the ubiquitination and degradation of the SNAI2 protein.

SNAI2 promotes cell migration through negatively regulating YAP expression

ASB13 KO or SNAI2-overexpressing cells displayed decreased cell spreading (Fig. 5A), promoting us to examine the F-actin polymerization status. Indeed, we noticed a significant decrease of F-actin fiber staining in ASB13 KO cells; this was also phenocopied in cells overexpressing SNAI2-Luc (Fig. 5A). Similarly, immunoblotting confirmed ASB13 KO or SNAI2-Luc expression significantly reduced F-actin polymerization compared with that in control cells, while there was slight increase of in G-actin level (Fig. 5B,C). These results thus suggest ASB13 KO induces F-actin depolymerization through up-regulating SNAI2 protein expression.
Previous studies have shown that F-actin cytoskeleton reorganization and cell detachment can be mechanical upstream signals of the Hippo–YAP pathway, which is a critical regulator for organ size, tumorigenesis, and metastasis (Gaspar and Tapon 2014; Yu et al. 2015). It is expected that decrease of cytoskeleton and F-actin would lead to decreased YAP protein level through post-translational modification and protein degradation (Aragona et al. 2013). To this end, we tested YAP protein level after ASB13 KO or SNAI2 overexpression in SUM159 cells. Indeed, YAP level was significantly reduced in ASB13 KO and SNAI2-Luc overexpressing cells (Fig. 5D; Supplementary Fig. S5A). Likewise, overexpression of SNAI2 in SUM159 cell line led to down-regulated YAP protein level.
Interestingly, the decrease of YAP protein level was also reflected at its mRNA level when we knocked out ASB13 or overexpressed SNAI2-Luc in SUM159 and LM2 cells (Fig. 5F; Supplemental Fig. S5C,D). Our results thus suggest that YAP expression is regulated at least in part at transcriptional level, likely mediated by transcriptional repression by SNAI2 protein. To further assess this hypothesis, we used a reporter system consisting of a 2-kb YAP promoter region containing five E-box sequences (Supplemental Fig. S5E). In the absence of SNAI2, pGL3-YAP displayed relative high luciferase activity. However, when SNAI2 was present, the reporter luciferase activity was strongly repressed (Fig. 5G). Furthermore, chromatin immunoprecipitation assay also confirms that SNAI2 directly binds to the YAP promoter through its first E-box region (Supplemental Fig. S5F). The reduction of YAP expression after ASB13 KO could also be rescued by exogenous expression of gRNA-resistant ASB13, suggesting that YAP was indeed a downstream gene of the ASB13-SNAI2 regulatory circuit (Fig. 5H). Since ASB13 gene expression was decreased [Fig. 3D,E] and SNAI2 was well known to be upregulated by EMT inducing pathways, we tested whether YAP was also regulated during EMT. Indeed, in EpRas cells undergoing EMT after TGF-β treatment, there is a significant decrease in YAP protein expression level (Supplemental Fig. S5G,H).

YAP functions as a suppressor of breast cancer metastasis

YAP is located on chromosome 11q22-23, a site of frequent loss of heterozygosity in breast cancer. Rather than being considered as an oncogene in many other cancer types, YAP was suggested to be a tumor suppressor in breast cancer with limited reports (Yuan et al. 2008). We sought to assess the functional impact of decreased YAP expression in breast cancer progression. We constructed YAP KO cells by CRISPR–Cas9 and examined its impact on cell migration (Supplemental Fig. S6A). Similar as ASB13 KO cells, YAP KO cells also increased cell migration significantly (Fig. 6A,B). Tumorsphere formation assay and soft agar colony formation assay suggested that YAP KO led to significantly more sphere formation and colony formation (Fig. 6A,C,D). We further investigated the impact of YAP loss on tumor progression and especially spontaneous metastasis in vivo. YAP KO and the vector control SUM159 cells were injected orthotopically into NSG mice, and tumor growth and lung metastasis were
recorded. Interestingly, YAP KO cells formed smaller tumors than vector control cells, consistent with many previous reports of the tumor-promoting role of YAP (Lee et al. 2010; Wu et al. 2018). However, loss of YAP expression significantly increased spontaneous lung metastasis in vivo, indicating YAP might inhibit spontaneous lung metastasis (Fig. 6F,G). Finally, to test whether ASB13’s tumor-suppressive function is mediated through its targeting of SNAI2, we knocked out both ASB13 and SNAI2 simultaneously in SUM159 cells (Supplemental Fig. S6B,C) and found that the migration ability of the double KO cells was dramatically decreased compared with ASB13 KO alone, and reduced to nearly baseline level (Supplemental Fig. S6D). Furthermore, the ability of tumorsphere formation was also significantly reduced in the ASB13/SNAI2 double KO cells (Supplemental Fig. S6E,F). Furthermore, when we ectopically expressed YAP in ASB13 knockout cells, the rescue of YAP expression significantly reduced the cell migration (Supplemental Fig. S6G,H). Collectively, these findings demonstrate that YAP potentially functions as a metastasis suppressor and reveal a new connection of the ASB13–SNAI2–YAP axis in the regulation of breast cancer metastasis [Fig. 6H].

Discussion

SNAI2 has a broad effect on cancer progression, including cell migration, cell cycle regulation, and apoptosis as well as drug resistance [Wu et al. 2005; Haslehurst et al. 2012; Ye et al. 2015]. In this study, we focused on identifying endogenous E3 ligases for SNAI2 degradation. Conventionally, identifying such E3 ligases is a difficult process. We previously developed a dual-luciferase-based screening method to streamline the E3-substrate pair discovery process (Zheng et al. 2014). Through the unbiased siRNA library screening and by additional confirmation steps, we discovered ASB13 as a bona fide E3 ubiquitin ligase for SNAI2. Functionally, knockdown of ASB13 stabilized the endogenous SNAI2 protein and increased the migration ability for SUM159 and LM2 cells, while overexpression of ASB13 promoted SNAI2 degradation and inhibited lung metastasis. Consistent with these results, higher ASB13 expression level correlates with longer survival time in breast cancer patients. Notably, ASB13 KO cells have much higher stable SNAI2 protein level compared with that of β-TrCP1/FBXW1 or FBXO11 KO cells, suggesting that ASB13 may be a major E3 ligase for endogenous SNAI2 in breast cancer.
Best known for its role in inducing EMT program, the mechanisms and downstream genes by which SNAI2 regulating tumorigenesis, migration, and metastasis independent of EMT are much less appreciated (Guo et al. 2012; Ye et al. 2015). We observed that there was a moderate increase in mesenchymal phenotype after ASB13 KO in SUM159 cells (data not shown), while no significant changes were observed after ASB13 overexpression in SUM159 and LM2 breast cancer cells, possibly because these cells were already locked in a relatively mesenchymal-like state. Instead, we noticed two important changes after ASB13 KO (and SNAI2 up-regulation). Firstly, F-actin polymerization was significantly decreased; and secondly, the YAP protein level was decreased after ASB13 KO or SNAI2 overexpression. The inhibition of YAP expression likely happened at the transcriptional level, as the mRNA level of YAP was also significantly reduced after ASB13 KO or SNAI2 overexpression, and a YAP promoter region containing multiple E-boxes were conferred transcriptional repression of the luciferase reporter by SNAI2. Changes in F-actin polymerization might additionally affect YAP protein level at the post-translational level as previously reported (Aragona et al. 2013).

SNAI2 possibly inhibits the transcription of YAP to execute its function in cell migration. Indeed, KO of YAP protein in SUM159 directly promoted cell migration. As a well-characterized core member of Hippo pathway, the role of YAP in cancer development is context-dependent. YAP was originally found to promote cell proliferation and transformation. Its expression was amplified in human hepatocellular carcinoma (HCC) and many other malignancies (Zender et al. 2006) and functional studies also confirmed its oncogenic role in tumorigenesis in many types of cancer (Lee et al. 2010; Wu et al. 2018). On the other hand, YAP has also been suggested to be a tumor suppressor in some cancer types. For example, YAP induces apoptosis in response to DNA damage in collaboration with p73 and promyelocytic leukemia (PML) [Strano and Blandino 2007; Lapi et al. 2008; Bertini et al. 2009]. YAP is located on chromosome 11q22-23, a locus where frequent loss of heterozygosity (LOH) occurs in breast cancer. Reduced expression of YAP was significantly associated with ER and/or PR negativity in invasive carcinoma, suggesting that YAP acts as a tumor suppressor in invasive breast carcinomas (Tufail et al. 2012). However, most of the functional studies of YAP only focus on primary tumor growth but not metastasis. In our study, we noticed that ASB13 KO led to increased SNAI2 expression and decreased YAP expression, which was accompanied by increased cell migration. Depletion of YAP in breast cancer cells also led to increased cell migration. Interestingly, our in vivo primary tumor growth and spontaneous metastasis experiments revealed that YAP KO led to decreased primary tumor growth but significantly more lung metastasis. This result thus suggests that YAP pathway might functions similarly as TGF-β pathway in cancer progression, display dichotomous functions in primary tumor growth, and metastasis of breast cancer. Our findings may help explain the seemingly conflicting observation of YAP being a good prognosis marker in breast cancer [Gudmundsson et al. 1995; Winqvist et al. 1995; Tufail et al. 2012] despite its well-established function in promoting growth [Meng et al. 2016]. However, the detailed molecular mechanisms mediating the metastasis-suppressing role of YAP in breast cancer need further investigation. Nevertheless, our study calls for caution and careful design in targeting YAP pathway for cancer therapy in breast cancer.

Materials and methods

Animal studies

All procedures involving mice and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Princeton University or Tsinghua University. For experimental lung metastasis assay, female athymic nude mice at 6–8 wk old were anaesthetized and tail vein-injected with 1×10^5 indicated tumor cells. Tumor cells were suspended in 100 µL of cold PBS before injection. Successful injections were confirmed by BLI imaging immediately after injection and lung metastasis progression was monitored by weekly BLI. By the experimental endpoint, mice were euthanized and lung tissues were dissected out and fixed for metastatic nodule counting. For primary tumor growth and spontaneous lung metastasis experiments using SUM159 cells in NSG mice, five female NSG mice aged 6–8 wk were used in each group. A total of 2×10^6 vector control cells or YAP KO cells was mixed 1:1 by volume with Matrigel (BD Biosciences) per injection. Each mouse was injected orthotopically in both flanks. Primary tumor growth was measured and tumor volume was calculated as volume = (length × width^2)/2. At the experimental endpoint, mice were sacrificed and lung samples were removed and fixed for counting of lung metastasis nodules.

Actin segmentation by ultracentrifugation

Actin segmentation assay was performed following the protocol described in previous studies [Qiao et al. 2017]. Cells were lysed directly in dishes using actin stabilization buffer [50 mM PIPES at pH 6.9, 50 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 2 mM ATP, 5% glycerol, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, 1:100 protease inhibitor mixture, 1:100 phosphatase inhibitor mixture] for 10 min at 37°C and collected into Eppendorf tubes by scraping, followed by centrifugation at 300g at room temperature to remove insoluble particles. An aliquot of the diluted cell lysates [10% of the volume] was kept separately as “total protein inputs,” to be analyzed by immunoblotting. The F-actin and G-actin pools of the diluted cell lysates were separated by ultracentrifugation at 100,000g for 1 h at 37°C. After centrifugation, F-actin precipitated at the bottom of the tube while G-actin remains in the supernatant. The supernatant containing the G-actin pool was removed to a fresh tube, while the pellet containing F-actin was resuspended in cold distilled water with 1 mM cytochalasin D (Sigma-Aldrich) and kept on ice for 45 min to dissolve F-actin. F-actin and G-actin fragments were used for immunoblotting by antibody against β-actin.

Anchorage-independent growth

Cells [5×10^4] were added to 1.5 mL of growth medium with 0.3% agar and layered onto 1.5 mL of 0.5% agar base in six-well plates. Cells were fed with 500 µL of medium every 3 d for 3 wk, after
which colonies were photographed and counted. Assays were conducted in triplicate in three independent experiments.

**Cell culture**

HEK293T, EpRas, and LM2 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Corning) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gemini), penicillin/streptomycin (Corning). SUM159 cells were cultured in DMEM/F12 (1:1) medium added with 5% FBS, 5 µg/mL insulin, and 10 ng/mL EGF. MCF10A was cultured in DMEM/F12 [1:1] with 5% FBS, 20 ng/mL EGF, 0.5 µg/mL hydrocortisone and 0.1 µg/mL choler toxin. The medium for H29 (packaged for retrovirus) added 0.5 µg/mL doxycycline, 2 µg/mL puromycin, and 0.25 g/mL G418 based on the medium of HEK293T.

**Chromatin immunoprecipitation assay**

Four dishes of confluent SUM159 cells [10-cm dish] were fixed with 0.9% formaldehyde for 10 min and quenched in 0.125 M glycine for 5 min at 37°C. After centrifugation, cell pellets were re-suspended in lysis buffer [1% SDS, 50 mM Tris-HCl at pH 8.1, 10 mM EDTA] with freshly prepared 1 mM DTT, 1 mM PMSF, and protease inhibitor. Chromatin DNA was fragmented by sonication to an average length of 500–1000 bp. A small aliquot [20 µL] was taken to reverse crosslinks and be saved as input control. Lysates were then incubated with 5 µg of control IgG antibody (Santa Cruz Biotechnology sc-66902X) or SNAIL2 antibody (Santa Cruz Biotechnology sc-66902X) overnight at 4°C and then for 1 h with 30 µL of Dynabead protein G [Invitrogen]. Beads were washed with high-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris at pH 7.9, 500 mM NaCl] for seven times. Samples were eluted by IP elution buffer [1% SDS, 50 mM Tris at pH 8.1, 1 mM EDTA, 0.1 M NaHCO3, 0.25 M NaCl and Proteinase K] twice for 20 min at 65°C. Cross-linking was reversed overnight at 65°C. DNA fragments were purified using isopropyl alcohol precipitation. qPCRs were performed using 1 µL of DNA as template and the promoter fragment enrichment was calculated relative to input DNA. The primer sequences used were P1 (−2493 to −2257), ACAGAACATGATGCTCAACAG [forward] and CAGCCTGGGCAACAGGCAGA [reverse]; P2 (−2280 to −1950), TCAAGTGATTCATGCTCCAGC [forward] and AC TATGCTTTAAAATCTTCTCCTTCTCCT [reverse]; P3 (−1006 to −719), TGTACATCGTGCCTGGGATG [forward] and AGTACTC GAATTTGCAATAGT [reverse]; P4 (−786 to −579), AAGTTGA TGTATTTTAC [forward] and CCGCATTGATGGTTAAATA AA [reverse]; and P5 (−498 to −283), AGCCGAGGGAG AGTGCTGTT [forward] and CCGAACCACGAGACG [reverse].

**Clinical data set analysis**

Gene expression data from the Wang et al. [2005] data set (GSE2034) was used for ASB13 expression level analysis in subtypes of breast cancer patients. To analyze prognosis value of ASB13 expression with overall patient survival was presented as Kaplan-Meier plots and tested for significance using online KMplot database [http://www.kmplot.com] [Györrfy et al. 2010].

**Coimmunoprecipitation assay**

The SNAI2-FLAG (wild-type or mutant) plasmids were cotransfected with HA-tagged ASB13 or other E3 ligase candidate plasmids into HEK293T cells in six-well plates using PEI with the ratio 1:3. Two days after transfection, cells were treated with 10 µM MG132 for 4 h. Four-hundred microliters of cell lysates were then collected and immunoprecipitated with 20 µL of magnetic anti-FLAG-beads (Sigma) overnight at 4°C. After extensive washing [five times] with lysis buffer, the beads were spun down and resuspended with 50 µL of loading buffer. After boiling for 10 min, protein samples were run on SDS-PAGE along with 10% input sample and transferred to PVDF membrane to be detected with appropriate antibodies as described in each figure.

**CHX pulse-chase assay**

Cells were seeded on 12-well plates at a density of 2 × 10⁵ cells per well. After culturing overnight, the cells were transfected with plasmids as desired. Two days after transfection, cells were treated with 50 µg/mL CHX dissolved in DMSO, total protein lysates were collected at different time points and subjected to immunoblotting for SNAI2 and related protein loading controls.

**Generation of stable cell lines**

Stable overexpression of all genes was achieved using the pLEX lentiviral plasmids or pMSCV retroviral plasmids. Lentiviruses were packaged in HEK293T cells, while retroviruses were packaged by using H29 cell line. After 48 h, viruses were collected, filtered, and used to infect target cells in the presence of 8 µg/mL polybrene. The infected cells were selected with puromycin or hygromycin for 5–7 d.

**Immunofluorescence (IF) staining**

Cells were seeded onto sterile glass coverslip for experiments. Cells were washed with cold PBS, fixed with 4% formaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 5 min, blocked with 5% BSA in PBS for 1 h, and incubated with primary antibodies overnight at 4°C and then with secondary antibodies for 1 h at 4°C in the dark. The antibodies used in the IF staining were anti-HA [1:100, Roche 1186743001], anti-SNAI2 [1:100, Santa Cruz Biotechnology A1317]. Secondary antibodies used in the experiments were goat-anti-mouse, 488 and rabbit anti-rat, TRINC [Thermo].

**Luciferase reporter assay-based siRNA library screening**

SUM1315-SNAI2-Luc/R-Luc cells (1 × 10⁵) were seeded in 24-well plates. On the following day, 10 ng/mL total pooled siRNAs [three siRNAs/gene] were transfected using RNAiMAX reagent (Life Technology) into cells. Cell lysates were collected 2 d after transfection and assayed for luciferase activity using the Glomax 96 microplate luminometer [Promega]. The remaining cell lysates were used for immunoblotting to confirm SNAI2-Luc protein level changes indicated by luciferase assay.

**YAP promoter luciferase assay**

For the luciferase reporter assay, 2 × 10⁴ 293T cells were seeded in 12-well plates. pGL3-YAP or pGL3-Basic, pLEX-SNAI2 or pLEX-MCS, and pCMV-Renilla plasmids were cotransfected. Two days after transfection, cells were lysed and luciferase activity were assayed using a multiple microplate luminometer [EnVision]. All luciferase activities were normalized to pGL3-YAP promoter activity, Renilla-luciferase activity was used as internal control. Around 2 kb YAP promoter region (−2493 to −436 bp relative to the transcriptional start site) was cloned from SUM159.
ASB13 suppresses metastasis by SNAI2 degradation

Genomic DNA was purified from the indicated population, the targeted loci were amplified by PCR. The primers designed for PCR are ∼150 bp upstream of and 250 bp downstream from the targeted loci. KO efficiency was analyzed by the indel efficiency on the at https://tide.deskgen.com. Sequences used were as follows: hASB13[g1] (forward: 5′-CAAGACAAAAACGGTGCTTCT AA-3′; reverse: 5′-GCTTAGATCTCTCTGAGCTGAG-3′), hA S13[g2] (forward: 5′-ACGTCCTGAGGGCGCTTCG-3′; reverse: 5′-CGGAGTGACCCAAAGCATGT-3′), and hSNAI2 (forward: 5′-TAAAGAAAATCAGTGTTGTTGCTA-3′; reverse: 5′-TGAAACTTTTCAGCTTCAATGGCA-3′).

**Tissue culture**

Cells were maintained in DMEM/F12 (1:1) containing 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO2. For experiments, cells were grown to 80% confluence and treated with serum-free medium 15 h before harvesting.

**Reverse transcription and qPCR analysis**

Total RNAs were isolated using the RNeasy Mini Kit (Qiagen). cDNAs were synthesized using the iScript Select cDNA Synthesis Kit (Bio-Rad). Real-time quantitative PCR (qPCR) was performed using the SYBR Green PCR mix (DSBIO) with Bio-Rad CFX96 PCR machine. Primers were designed to span the gRNA target site using the AlignTop software (Takara). qPCR analysis was performed in triplicate and normalized to the expression of the reference gene, GAPDH.

**Immunoblot analysis**

Whole cell lysates were subjected to SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 5% non-fat milk and incubated with primary antibodies. Primary antibodies used were anti-β-ACTIN (1:5000; Abcam ab6276), anti-SNAI2 (1:1000; sc-166476), anti-HA (1:1000), anti-YAP1 (1:1000; Cell Signaling Biotechnology). Membranes were subsequently incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) for 1 h and then visualized using chemiluminescence detection reagents (DPI). Signals were quantified using ImageJ (NIH).

**Results**

Results were reported as mean ± SD (standard deviation) or mean ± SEM (standard error of the mean), as indicated in the figure legend. Statistical comparisons were performed using unpaired two-sided Student’s t-test with unequal variance assumption. Statistical comparison for LM2 lung metastasis assay was performed using the Student’s t-test with unequal variance assumption.
using Mann–Whitney U-test. All the experiments with representative images (including Western blot and immunofluorescence) were repeated at least twice and representative images are shown.

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