

BAG3 is a negative regulator of ciliogenesis in glioblastoma and triple-negative breast cancer cells

Benedikt Linder¹  | Caterina Klein^{1,2}  | Marina E. Hoffmann³ | Florian Bonn³ | Ivan Dikic^{3,4}  | Donat Kögel^{1,5,6} 

¹Department of Neurosurgery, Experimental Neurosurgery, University Hospital, Goethe University, Frankfurt am Main, Germany

²Faculty of Biosciences, Goethe University, Frankfurt am Main, Germany

³Institute of Biochemistry II, Goethe University, Frankfurt am Main, Germany

⁴Buchmann Institute for Molecular Life Sciences, Goethe University, Frankfurt am Main, Germany

⁵German Cancer Consortium (DKTK), Partner Site Frankfurt, Frankfurt am Main, Germany

⁶German Cancer Research Center DKFZ, Heidelberg, Germany

Correspondence

Donat Kögel, Department of Neurosurgery, Neuroscience Center, Experimental Neurosurgery, University Hospital, Goethe University, 60528 Frankfurt am Main, Germany.
Email koegel@em.uni-frankfurt.de

Funding information

Deutsche Forschungsgemeinschaft, Grant/Award Number: KO 1898/11-1

Abstract

By regulating several hallmarks of cancer, BAG3 exerts oncogenic functions in a wide variety of malignant diseases including glioblastoma (GBM) and triple-negative breast cancer (TNBC). Here we performed global proteomic/phosphoproteomic analyses of CRISPR/Cas9-mediated isogenic BAG3 knockouts of the two GBM lines U343 and U251 in comparison to parental controls. Depletion of BAG3 evoked major effects on proteins involved in ciliogenesis/ciliary function and the activity of the related kinases aurora-kinase A and CDK1. Cilia formation was significantly enhanced in BAG3 KO cells, a finding that could be confirmed in BAG3-deficient versus -proficient BT-549 TNBC cells, thus identifying a completely novel function of BAG3 as a negative regulator of ciliogenesis. Furthermore, we demonstrate that enhanced ciliogenesis and reduced expression of SNAI1 and ZEB1, two key transcription factors regulating epithelial to mesenchymal transition (EMT) are correlated to decreased cell migration, both in the GBM and TNBC BAG3 knockout cells. Our data obtained in two different tumor entities identify suppression of EMT and ciliogenesis as putative synergizing mechanisms of BAG3-driven tumor aggressiveness in therapy-resistant cancers.

KEYWORDS

BAG3, cell migration, epithelial to mesenchymal transition, glioblastoma, primary cilium, triple-negative breast cancer

1 | INTRODUCTION

The multifunctional HSP70 co-chaperone BAG3 has a central physiological role in regulating cellular proteostasis. BAG3 competes with the BAG family member BAG1 for

binding to the ATPase domain of the heat shock protein HSP70, thus preventing HSP70/BAG1-dependent delivery of client proteins to the proteasomal pathway.¹ By functionally interacting with HSP70 and LC3, BAG3 can also selectively target polyubiquitinated client proteins to autophagy,

Benedikt Linder and Caterina Klein contributed equally to this study.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2021 The Authors. *Journal of Cellular Biochemistry* Published by Wiley Periodicals LLC

the second major cellular protein degradation pathway. In particular, BAG3 has been shown to target misfolded proteins to aggresomes, a perinuclear compartment with high autophagic activity, via microtubules.² BAG3-mediated regulation of client protein levels is involved in many physiological processes, including apoptosis, development, and cytoskeletal dynamics/organization. In addition, BAG3 is also correlated to several pathological conditions including age-related neurodegenerative diseases, cardiomyopathies, and malignant diseases, where BAG3 exerts oncogenic functions by regulating key cancer hallmarks, including cell survival, cell adhesion, metastasis, and angiogenesis.^{3,4}

Glioblastoma (GBM; grade IV glioma, WHO) is the most malignant primary brain tumor that mainly occurs in adults. Despite continuous efforts to improve clinical treatment success, GBM patients still have a dismal prognosis with a median survival barely exceeding one year.⁵ One cardinal problem for the treatment of these tumors is their diffuse and highly infiltrative growth into the brain parenchyma, making a complete surgical resection practically impossible.⁶ Based on these growth characteristics and the high resistance of GBM to therapy, the tumors will quickly relapse following surgery. Another highly aggressive tumor is triple-negative breast cancer (TNBC), a subtype of epithelial breast cancer that doesn't express estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).⁷ Similar to GBM, TNBC has a highly pro-migratory and pro-invasive phenotype and standard treatment is restricted to surgery and various types of chemotherapy, routinely leading to the development of therapy resistance and recurrent disease. Previous work from our and other groups has demonstrated that BAG3 is a potential therapeutic target in both tumor entities. In particular, we could show that overexpression of BAG3 contributes to cell-matrix adhesion and apoptosis resistance of GBM cells. Furthermore, genetic depletion of BAG3 led to reduced *in vivo* tumor growth in orthotopic mouse glioma models and limited clonogenic survival after short-term treatment with the anticancer agent AT-101.⁸ In analogy, BAG3 is frequently overexpressed in breast cancer and high BAG3 expression levels are correlated with a poor prognosis,⁹ as well as with chemotherapy resistance of TNBC.⁷ More precisely, we were able to confirm that BAG3 overexpression stabilizes the antiapoptotic Bcl-2 family members Mcl-1, Bcl-2, and Bcl-xL and could demonstrate that BAG3 contributes to apoptosis resistance of TNBC cells adapted to growth in the presence of different chemotherapeutic agents in our own previous work.^{7,10}

The primary cilium is an immobile, solitary membrane-protrusion that can occur in most somatic cell types. It has been shown that many signaling pathways that are important for development and whose misregulation have been

associated with numerous cancer types are localized at the primary cilium. These pathways include the Hedgehog, Wnt, Notch, Hippo, and PDGF signaling pathways. It has been further shown that mTOR and multiple G protein-coupled receptors like dopamine, serotonin, and melanin receptor signal through the cilium.¹¹ Defects in the cilia in particular ciliogenesis and cilium disassembly are associated with numerous human diseases collectively referred to as ciliopathies.¹¹ However, it has been shown in several studies that a defective ciliogenesis is associated with cancer aggressiveness.¹² In particular it has been shown that most cancers lack cilia, because ciliogenesis is linked to the cell cycle and is normally associated with quiescence.¹³

To unravel new mechanisms underlying BAG3-driven malignization of therapy-resistant cancer, we established CRISPR/Cas9-mediated isogenic BAG3 knockouts of two parental GBM lines (U343, U251) and performed an unbiased global proteomic/phosphoproteomic analysis in comparison to parental controls. Here we demonstrate that depletion of BAG3 has major effects on proteins involved in ciliogenesis/ciliary function and identify BAG3 as a negative regulator of ciliogenesis. These findings could be confirmed in BT-549 TNBC cells carrying a CRISPR/Cas9-mediated BAG3 knockout. Furthermore, we demonstrate that enhanced ciliogenesis is correlated to decreased protein levels of epithelial to mesenchymal transition (EMT) markers and reduced cell migration/invasion, both in the GBM and TNBC BAG3 knockout cells.

2 | MATERIALS AND METHODS

2.1 | Cells and cell culture

U251-MG (hereafter called U251) and U343 GBM cell lines¹⁴ were cultured in DMEM Glutamax including 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Gibco). The triple-negative (parental) human breast cancer cell line BT-549^{15,16} was cultured in IMDM including 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Gibco). The cells were cultured in a humidified incubator kept at 37°C and 5% CO₂.

2.2 | Antibodies

The following primary antibodies and dilution were used for Western blot (WB) or immunofluorescence staining (IF): rabbit-anti-BAG3 (Biozol/Abnova; PAB0330; 1:5000; WB); mouse-anti-GAPDH (Calbiochem; CB1001; 1:20,000; WB); rabbit-anti-IFT88 (Proteintech; 13967-1-AP; 1:100; IF);

mouse-anti-Tubulin (Sigma-Aldrich; T6199 – cloneDM1A; 1:10,000; WB).

The following secondary antibodies and dilutions were used: IRDye 800CW goat-antirabbit 1:10,000 (926-32211) and IRDye 680RD goat-antimouse 1:10,000 (926-68070; both LI-COR Biosciences) for WB and F(ab')₂-goat-antirabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 1:500 (A-11070; Thermo Fisher Scientific) for IF.

2.3 | Generation of CRISPR/Cas9-knockouts

Generation of CRISPR/Cas9-mediated knockout was achieved as described previously¹⁷ according to published protocols¹⁸ by cloning the BAG3-specific guide RNA sequences into pSpCas9(BB)-2A-Puro (PX459) using the following sequences: sgBAG3_1f: 3'-AAACCACTGTTTATCTGGCTGAGTC-5'; sgBAG3_1r: 3'-CACCGACTCAGCCAGATAAACAGTG-5'; sgBAG3_2f: 3'-AAACCAGAGGTCCCAGTCACCTCTC-5'; sgBAG3_2r: 3'-CACCGAGAGGTGACTGGACCTCTG-5'; sgBAG3_9f: 3'-AAACCAGTTCGGAATCGCTGCATC-5'; sgBAG3_9r: 3'-CACCGATGCAGCGATTCCGAACTG-5'. The resulting plasmids PX459_sgBAG3_1, PX459_sgBAG3_2, and PX459_sgBAG3_9 were transfected pairwise (9/1 and 9/2) into cells using Lipofectamine 2000 (DNA:reagent-ratio 1:1 for U251 and U343 and 1:2 for BT-549) according to the manufacturers protocols. Two days hereafter the cells were put under puromycin (Puromycin dihydrochloride, Santa CruzBiotechnology, Inc.)-selection (1 µg/ml for U251 and BT-549 and 10 µg/ml for U343) for 48 h and finally seeded as single cells into 96-well plates for expansion and knockouts were confirmed via Western blot analysis. pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid #62988; <http://n2t.net/addgene:62988>; RRID:Addgene_62988).

2.4 | Phosphoproteomic and proteomic sample preparation and data analyses

A detailed description is available as Supporting Information. Briefly, the cells were grown until they reached confluence and were thereafter lysed and the proteins were extracted. The obtained proteins were digested using Trypsin (Promega; V5113) and LysC (Wako Chemicals). After purification using SepPak C18 columns (Waters; WAT054955) the peptides were TMT-labeled (Thermo Fisher Scientific; 90061; TH266884) and fractionated with the High pH Reversed-phase Fractionation Kit (Thermo Fisher Scientific). After HPLC the samples were directly sprayed into a QExactive

HF mass spectrometer and the RAW data was processed with Proteome Discoverer 2.2 software (Thermo Fisher Scientific). TMTpro reporter abundances were extracted and used for plotting and statistical analysis. Further analysis was done on Perseus (v.1.6.5.0). The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE^{19,20} partner repository with the data set identifier PXD024802.

2.5 | Cell-based assays

2.5.1 | Migration assay

One Ibidi Culture Insert was used per well and 20,000 BT-549 cells and 30,000 U251 and U343 cells were seeded in each Ibidi pocket in a 24-well culture plate. After 24 h of incubation, the inserts were removed and the 500 µm gap was photographed immediately with a Tecan SPARK plate reader (Tecan) and at the indicated time points. The gap width was measured using the line tool of FIJI.²¹

2.5.2 | Invasion assay (modified Boyden Chamber assay)

Modified Boyden chamber assays have been performed as described.⁷ Briefly, 20,000 cells were seeded into the insert and after 48 h the cells were methanol-fixed, stained using crystal violet and seven vision fields (×20) were counted.

2.6 | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Cell lysis, SDS-PAGE, and Western blot analysis were performed as described.²² Membranes were blocked either in 5% bovine serum albumin (BSA)/TBS-Tween-20 (TBS-T) or in 5% milk/TBS-T for 1 h at room temperature and primary antibodies were incubated overnight at 4°C. Secondary antibodies were diluted in 5% BSA/TBS-T for 1 h and detection was achieved using a LI-COR Odyssey reader (LI-COR Biosciences).

2.7 | Immunofluorescence staining and fluorescence microscopy

Cells were seeded (U343: 150,000 cells, U251: 120,000 cells, BT-549: 150,000 cells) on chamber slides and were fixed with 4% paraformaldehyde after 48 h of cultivation.

The staining was done as described.²² Slides were then stored at 4°C until analysis using a Nikon Eclipse TE2000-S inverted fluorescence microscope operated by the NIS Elements AR version 4.2 (both Nikon Instruments Europe B.V.) at ×60 magnification.

2.8 | Taqman-based quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Taqman-based qRT-PCR was performed as described previously,²² with the modification that the RNA isolation was done using the ExtractMe Total RNA Kit (Blirt S.A.). The following FAM-MGB probes were used: CDH1: Hs01023895_m1; CDH2: Hs00169953_m1; SNAI1: Hs00195591_m1; TBP: Hs00427620_m1; TWIST1: Hs01675818_s1; ZEB1: Hs01566408_m1.

2.9 | Statistics

The GBM and TNBC data are presented as mean ± SEM and all statistical analyses were done using GraphPad Prism 7 (GraphPad Software). The applied test is stated in the respective figure legend. $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Alterations of global proteomes and phospho-proteomes in BAG3-depleted GBM cells point towards increased ciliogenesis

We have recently shown that BAG3 plays a pivotal role in tumor cell aggressiveness in GBM as well as in TNBC^{7,8} which is in line with findings from other studies.¹ In fact, analyses of the publicly available databases TCGA (The Cancer Genome Atlas)²³ and CGGA (Chinese Glioma Genome Atlas)²⁴ accessed via the GlioVis-portal (<http://gliovis.bioinfo.cnio.es/>)²⁵ revealed that BAG3 mRNA expression is higher in tumor tissue compared to healthy tissue according to the TCGA data set (Figure S1A). Similar findings have also been described by Festa et al.²⁶ However, based on the TCGA-data set, no survival difference can be noted based on BAG3 mRNA expression levels (Figure S1B). The CGGA data set additionally shows that BAG3 expression is enhanced in recurrent tumors compared to the original tumors, supporting the possible role of BAG3 in therapy resistance (Figure S1C). Based on the CGGA data set, there is a trend towards a

negative association of BAG3 with patient survival (Figure S1D). Similarly, an interrogation of the data from the human protein atlas (<https://www.proteinatlas.org/ENSG00000151929-BAG3/pathology/glioma>)²⁷ revealed that BAG3 protein expression is negatively associated with survival across all glioma (data not shown).

To analyze the molecular events underlying BAG3-driven malignization in GBM in more detail, we generated isogenic BAG3-knockout cell lines (BAG3-KO) in the cell lines U251 and U343, and successfully confirmed KOs via Western blot analysis (Figure 1A). In both lines a complete loss of BAG3 protein expression is apparent. Next, we employed these cell lines for a combined phospho-proteomic and proteomic analysis in comparison to parental control cells. This proteomic approach (Figure 1B,C) revealed that a total of 7352 proteins could be quantified of which 263 and 78 were significantly decreased, while 250 and 141 were significantly increased in U251 BAG3 KO cells (Figure 1B) and U343 BAG3 KO cells (Figure 1C), respectively. Phospho-proteomic analysis (Figure 1D,E) led to the detection of 24,641 phospho-sites of which 3,115 and 3,550 were significantly decreased, while 3,627 and 3,526 were significantly increased in U251 BAG3 KO (Figure 1D) and U343 BAG3 KO (Figure 1E) versus controls, respectively. For each condition, the top five proteins and phospho-sites are depicted in the volcano plots. The obtained data were used to subsequently perform pathway analyses. A 1D analysis using the Perseus software^{28,29} was used to analyze which processes are increased or decreased in the BAG3 KO cells compared to the wildtype GBM cells. This 1D analysis revealed that in U251 BAG3 KO cells, many processes related to mitochondria were altered (Figure 1F). The 1D-analyses of the data derived from U343 BAG3 KO GBM cells (Figure 1G) revealed that many proteins in cell cycle-related processes, but also in cell adhesion are decreased, which is in agreement with our previous reports.^{7,8} Processes related to the mitochondria and the citrate cycle were also found to be altered in U343 BAG3 KO cells versus BAG3-proficient controls. Interestingly, we found a significant enrichment in processes related to the primary cilium, such as “cell projection,” “cilium assembly,” and “cilium membrane,” pointing towards a novel and as-of-yet undescribed function for BAG3 in regulating ciliogenesis (Figure 1G).

These findings could be further underscored by an additional analysis using STRING (v11; <https://string-db.org/>)³⁰; Figure S2) of the significantly changed proteins after BAG3-KO. Here, we found especially for the U251 data set that many signaling pathways that are known to be associated with the primary cilium and are known to require a cilium, such as Hedgehog, WNT, and Notch

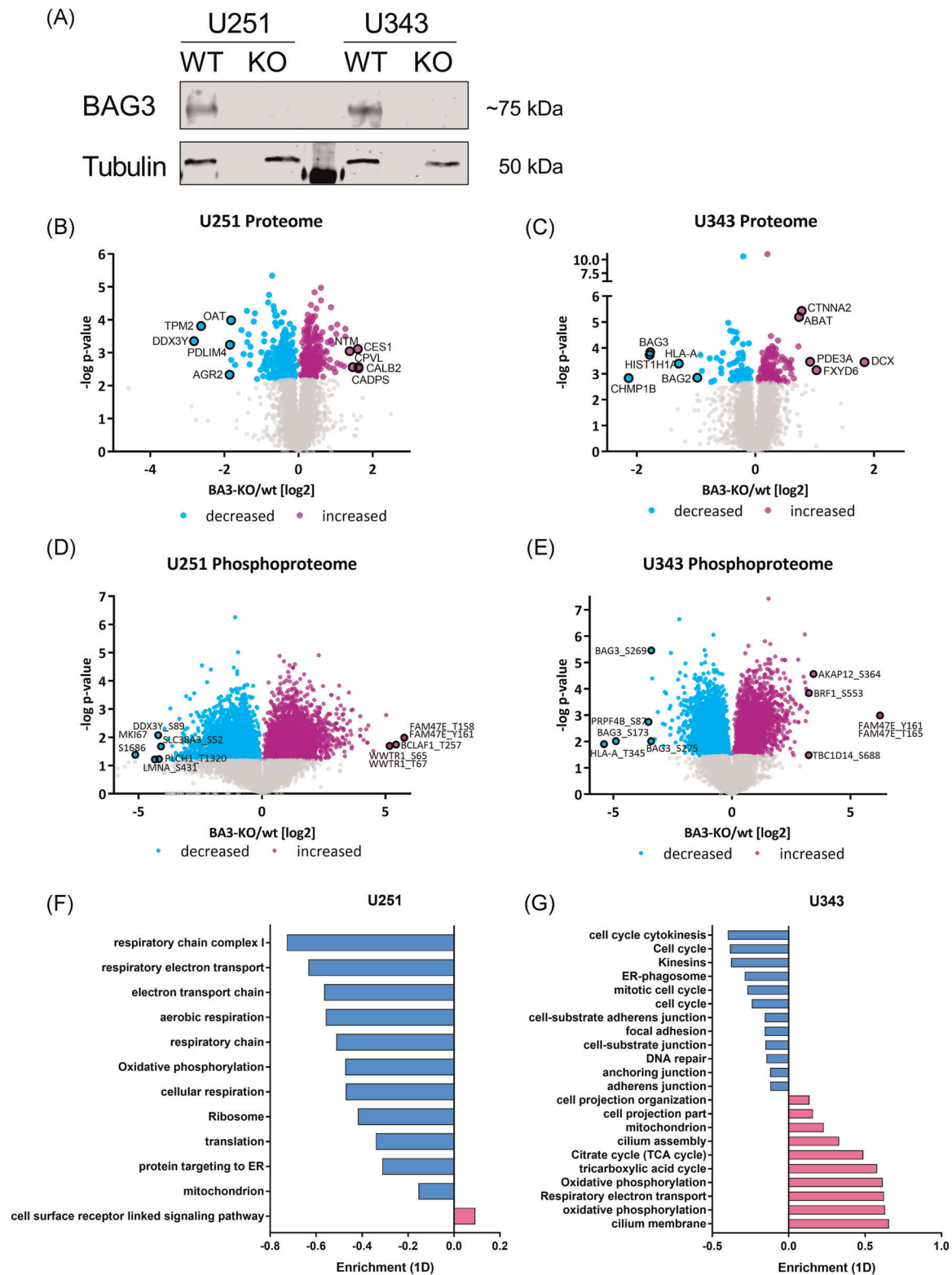


FIGURE 1 Generation of BAG3-knockouts (BAG3-KO) and phospho-proteomic analyses. (A) Western blot analysis of U251 and U343 parental controls (WT) and isogenic BAG3-KO showing complete absence of BAG3 protein; tubulin served as housekeeper. (B–E) Volcano plots of (B, C) proteomic and (D, E) phosphoproteomic data of (B, D) U251 and (C, E) U343 upon depletion of BAG3. The depleted and increased proteins are shown in blue and purple, respectively; the Top5 proteins/phosphosites are depicted in each volcano plot. (F, G) 1D annotation analyses using the Perseus software²⁸ of the proteomic data of (A) U251 and (B) U343 glioblastoma (GBM) cells after BAG3-depletion. The data are presented as bar graphs of the 1D score. The complete analyses are available as Supporting Information files

pathways^{13,31} are among the enriched pathways. These findings support the notion that loss of BAG3 also affects ciliogenesis and/or ciliary function in U251 cells, indicating that the findings obtained in U343 cells can be generalized.

To analyze the phospho-proteomic datasets, we first performed a 1D analysis, which revealed that in U251 (Figure 2A) multiple signaling pathways that are mediated through the primary cilium seemed to be activated in the BAG3-KO cells, as also indicated in the U343 proteomic data. The involvement of changes in ciliogenesis and/or ciliary function is underscored by an enrichment of the process “ciliary rootlet” in the KO enriched phospho-sites. Similar to our findings in the proteome, the phospho-proteomes of U343 GBM (Figure 2B) cells contain many decreased phospho-sites in processes related to DNA repair and cell cycle. Of the increased phospho-sites are again multiple processes related to cilia-dependent signaling pathways as well as the process “cell projection morphogenesis.” Lastly, we employed our phospho-proteomic data set to predict kinase activity using the freely available tool KSEA (Kinase-Substrate Enrichment Analyses; <https://casecpb.shinyapps.io/ksea/>³²). We inferred kinase activity by analyzing our data set using the annotated database PhosphoSitePlus and using the kinase-substrate prediction tool NetworKIN with a score cutoff of 2. This approach revealed that CDK1 and aurora-kinase A (AURKA), which both belong to the cilium disassembly complex,^{13,33,34} are among the most downregulated kinases after BAG3-KO in U251 (Figure 2C) and U343 (Figure 2D). Collectively, our multi-proteomic approach accumulated several lines of evidence suggesting that BAG3 is an as-of-yet unknown molecular component in ciliogenesis.

3.2 | BAG3-KO increases the amount of ciliated cells and reduces migration and invasion of GBM cells

All of the performed proteomic analyses suggested that BAG3 is involved in ciliogenesis and/or ciliary function. Although it was initially described that GBM cells harbor either no or only few cilia,³⁵ recent reports suggested that some tumor cells can develop cilia.³⁶ To address changes in ciliogenesis upon BAG3-KO, we performed immunofluorescence stainings of BAG3-KO and WT cells using the ciliary marker IFT88 (Figure 3).

This approach revealed significantly more ciliated cells after BAG3-KO, indicating that BAG3 indeed acts as a negative regulator of ciliogenesis. In a recent preprint, it was shown that blockade of cilium disassembly in

glioma stem-like cells (GSCs) by depletion of key components of the cilia-disassembly complex caused these cells to differentiate and prevented their invasion into iPSC-derived brain organoids and in xenotransplantation experiments using mice,³⁷ indicating a central role for cilia regulating migration/invasion in GBM. The involvement of the primary cilium in these processes appears to be highly dependent on cellular context as both activating and inhibitory effects have been observed.³¹

Therefore, we next determined the migratory and invasive capacity of BAG3-depleted cells (Figure 4). A wound-healing assay revealed that BAG3-KO in U251 GBM cells (Figure 4A) significantly reduces the gap closure speed at all analyzed time points. Similarly, U343 WT cells (Figure 4B) can close the gap faster than BAG3-KO cells. This reached statistical significance after 40 h with a further increased difference after 48 h. The invasive capacity of U251 and U343 cells with and without BAG3-KO was analyzed using modified Boyden Chamber assays. Here, we observed that after 48 h significantly fewer U251 and U343 BAG3 KO cells could invade into the lower chamber (Figure 4C,D). These findings suggest that BAG3-depletion reduces the migratory and invasive capacity of GBM cells and prompted us to determine the expression of SNAI, TWIST1, and ZEB1, three known key regulators of EMT,^{6,31,38} as well as E and N-cadherin (expressed by *CDH1* and *CDH2*) via qPCR. BAG3-depletion induced a pronounced decrease in SNAI1 and ZEB1-expression in both U251 (Figure 4E) and U343 (Figure 4F) cells, whereas TWIST1 is strongly reduced in U251 BAG3-KO cells. *CDH1*-expression was found to be robustly induced in U343, and to a lesser extent in U251 BAG3-KO cells. Collectively, our data suggest that downregulation of key transcription factors known to promote EMT contributes to the prevention of migration and invasion following BAG3 depletion.

3.3 | Negative regulation of ciliogenesis by BAG3 in TNBC cells is associated with diminished cell migration

The mRNA expression and the protein level of BAG3 in breast cancer patient samples are significantly upregulated in tumor versus peritumoral areas. Liu et. al showed by data set analysis that a high BAG3 expression correlates with a lower overall survival.^{1,9} Moreover we could previously show that BAG3 plays a central role in therapy resistance and the aggressiveness of the two TNBC cell lines BT-549 and MDA-MB-468 and their derivatives adapted to growth in the presence of different chemotherapeutics.⁷ To further investigate the BAG3-related tumor properties that underlie TNBC

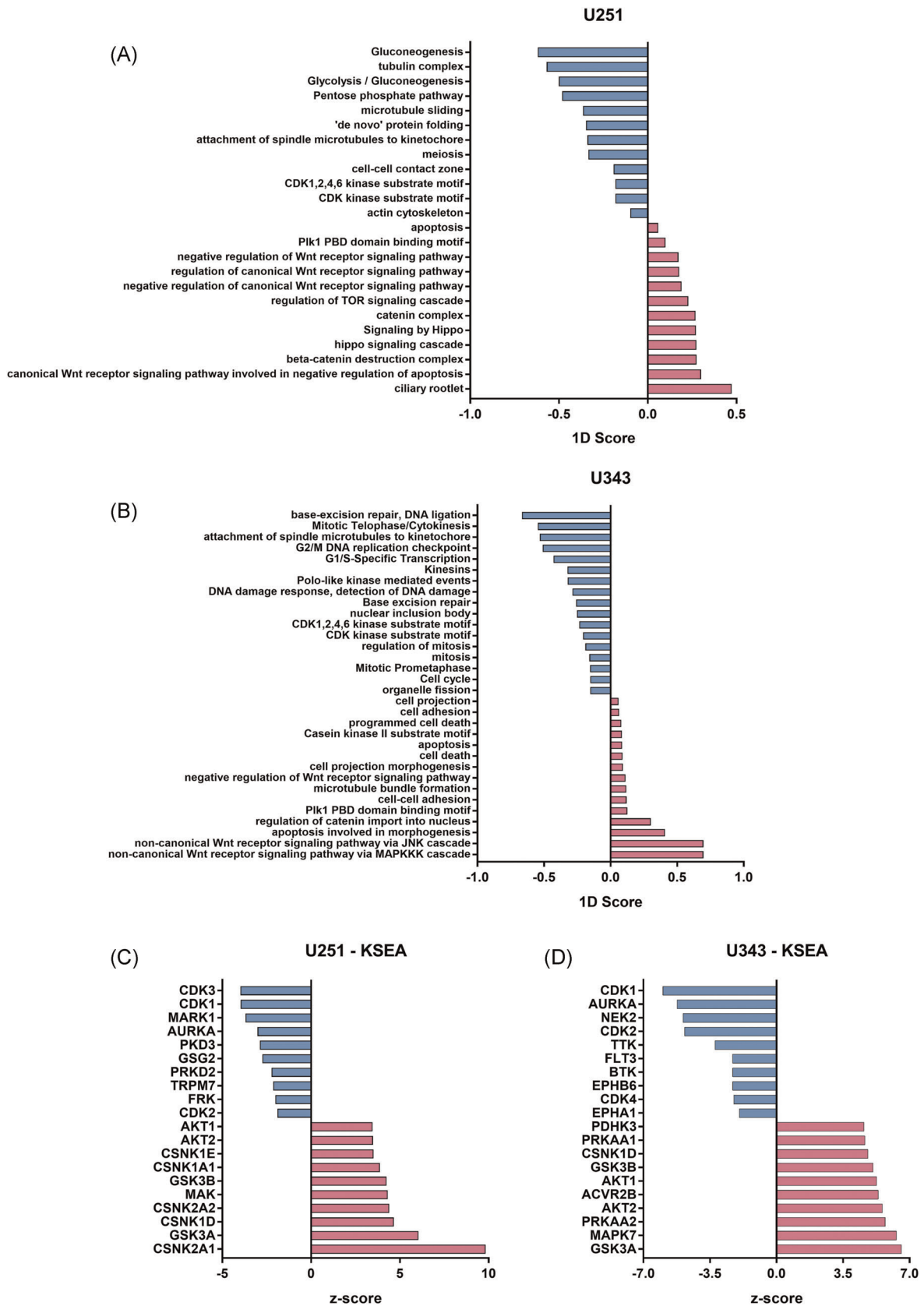


FIGURE 2 (See caption on next page)

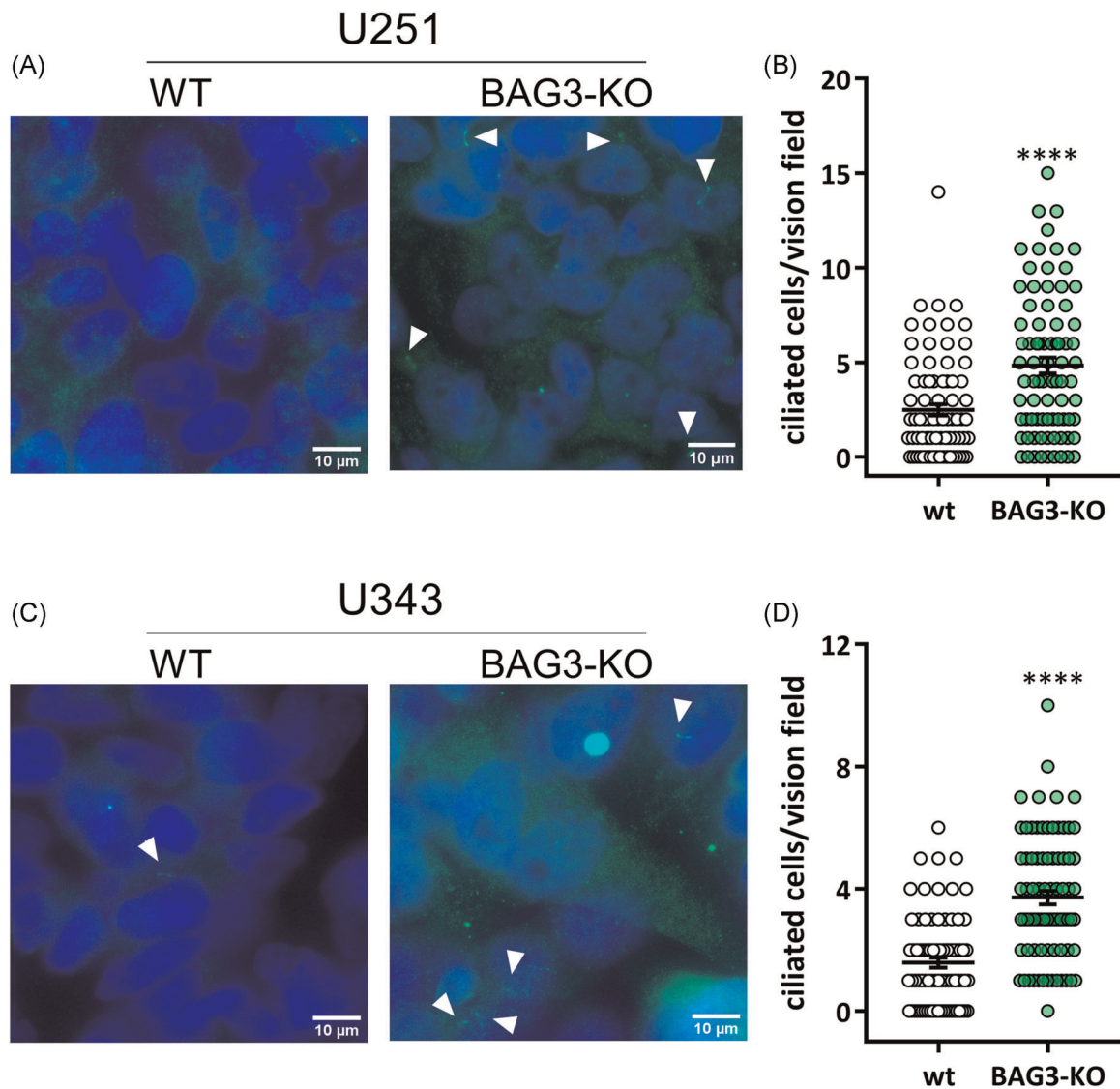


FIGURE 3 BAG3-knockout promotes ciliogenesis. (A) Representative Images of IFT88-staining of (upper row) U251 and (lower row) U343 glioblastoma (GBM) WT and BAG3-KO cells 48 h after seeding. Arrow heads: Cilia. (B) Point plots of the quantification of at least 20 vision fields of at least three independent experiments. **** $p < .0001$, two-tailed Mann–Whitney test (GraphPad Prism 7)

malignancy, we generated three CRISPR/Cas-mediated BAG3 knockout cell lines derived from BT-549 cells. The successful BAG3-KO was validated by Western blot analysis, where a complete loss of BAG3 protein expression was evident in all three KO cells (Figure 5A).

In the following, we aimed at cross-checking the main findings we obtained in the GBM cell lines in TNBC cells, in particular the novel role of BAG3 in the regulation of ciliogenesis. In a study using the Polyoma Middle T (PyMT)-induced breast cancer mouse model, it

FIGURE 2 Bioinformatic analyses of phosphoproteomic data point towards increased cilia-related processes after BAG3-depletion. (A, B) 1D-analyses²⁹ using the Perseus software²⁸ of the phosphoproteomic data set of (A) U251 and (B) U343 glioblastoma (GBM) cells after BAG3 depletion. (C, D) Kinase-substrate enrichment analyses (KSEA) of phosphoproteomic data sets after BAG3 depletion. Kinase-substrate predictions were inferred from phosphoproteomic data sets of (C) U251 and (D) U343 GBM cells after BAG3 depletion using the KSEA-App (<https://casecpb.shinyapps.io/ksea/>).³² Kinase-substrate searches included PhosphoSitePlus and NetworKIN with scores 2 or greater. Only the Top 10 depleted (blue) and enriched (pink) kinases are displayed. The data are presented as bar graphs of the 1D-score and KSEA z-score, respectively

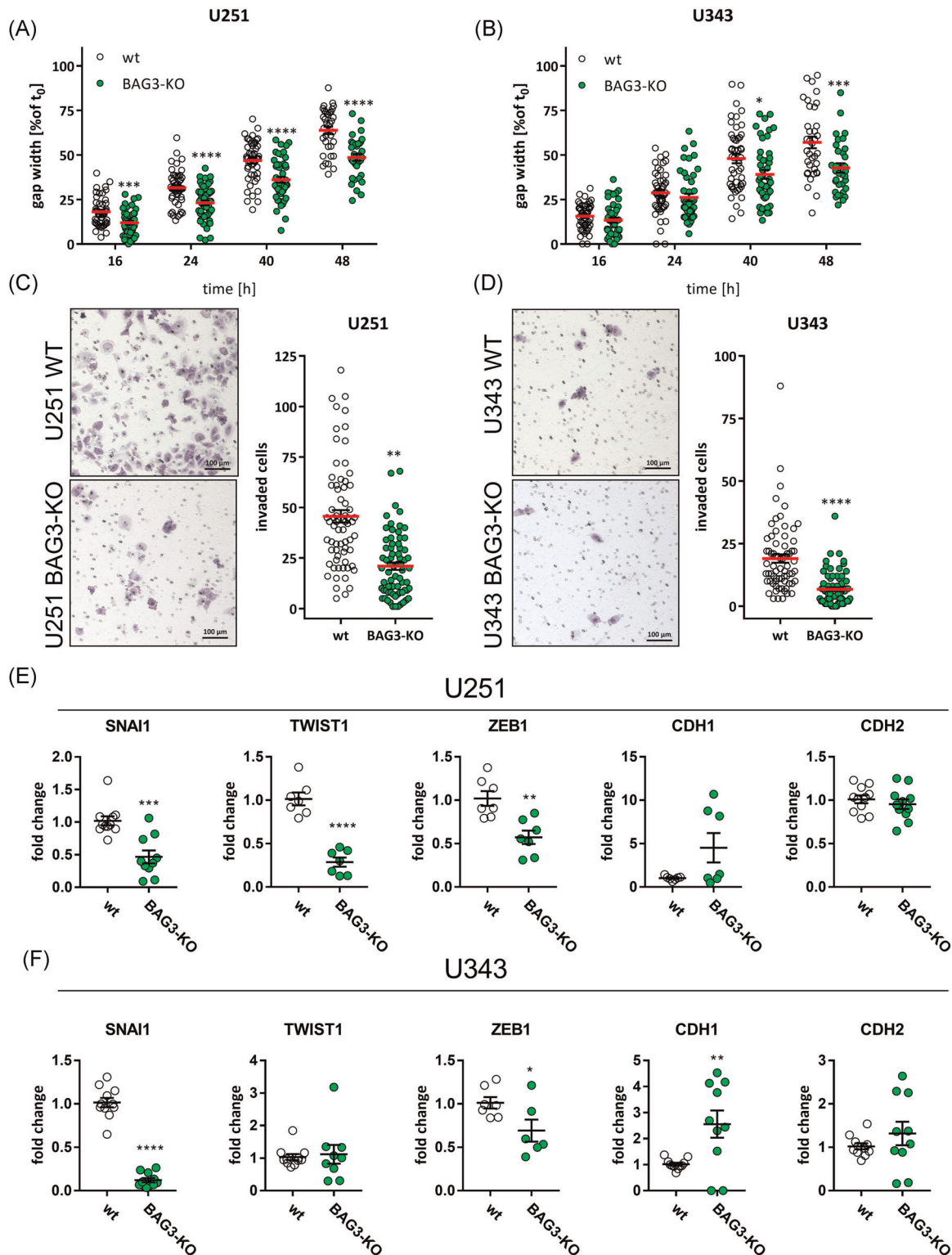


FIGURE 4 BAG3 depletion reduces migration and invasion in GBM cells via downregulation of SNAI1 and ZEB1. (A, B) Point-plots of wound-healing assays performed using Ibidi Culture Inserts of (A) U251 WT and BAG3-KO and (B) U343 WT and BAG3-KO cells. (C, D) Modified boyden chamber assays of (C) U251 and (D) U343 GBM cells 48 h after seeding and crystal violet-stained. The experiments were repeated at least three times, performed in triplicates and per replicate seven vision fields ($\times 20$ magnification) were counted. Taqman-based quantitative reverse-transcription polymerase chain reaction (qRT-PCR) of (E) U251 and (F) U343 GBM cells after BAG3-depletion of epithelial to mesenchymal transition (EMT)-associated marker genes. $*p < .05$; $**p < .01$; $***p < .001$; $****p < .0001$; (A, B, E and F) multiple t test corrected for multiple comparisons using the Holm-Sidak method; (C, D) unpaired t test with Welch's correction (GraphPad Prism 7)

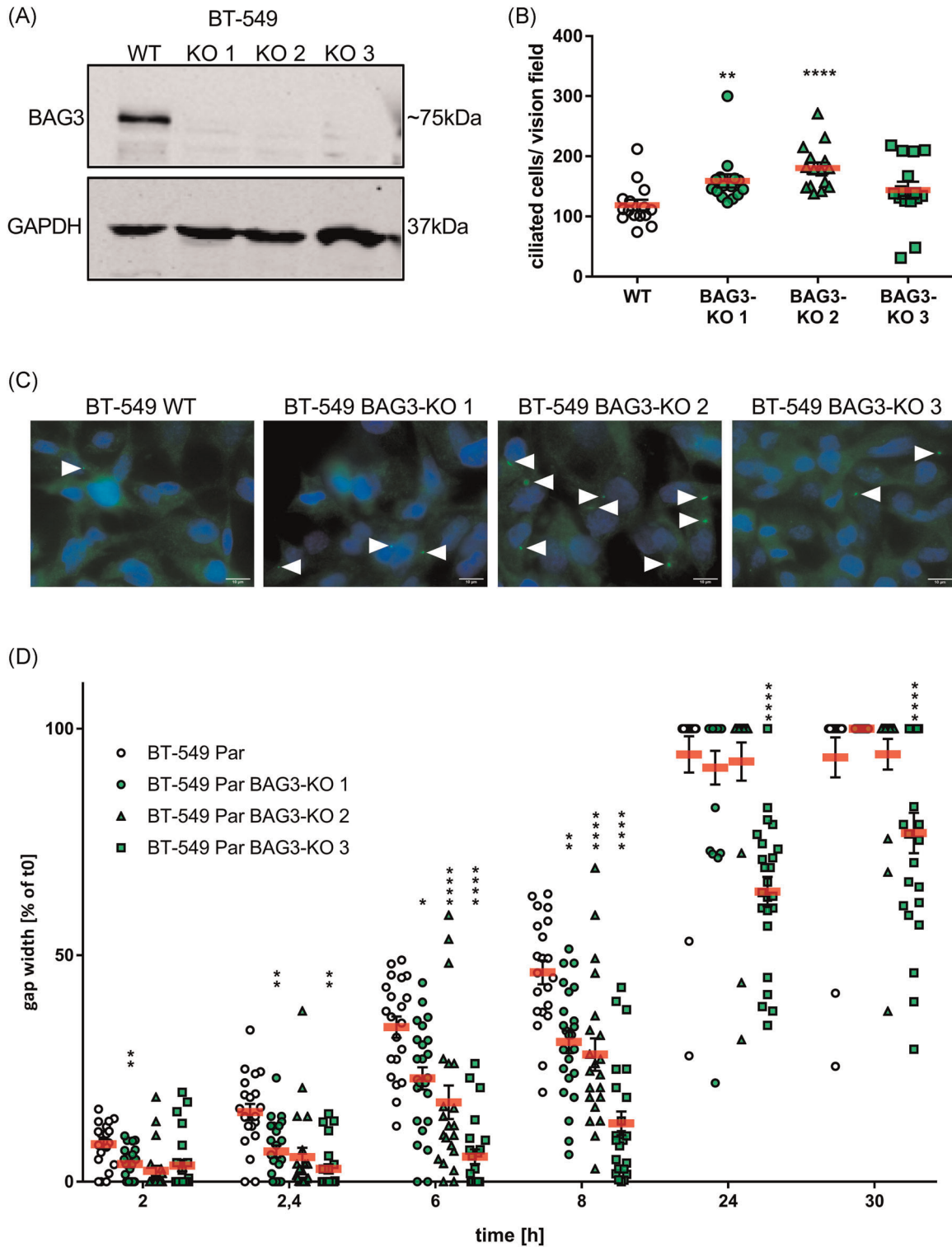


FIGURE 5 BAG3-knockout (BAG3-KO) increases ciliogenesis and diminishes cell migration of TNBC cells. (A) Western blot analysis of BT-549 parental controls (WT) and three CRISPR/Cas9-mediated isogenic BAG3-KO lines showing complete absence of BAG3 protein expression; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as housekeeper. (B) Point plot of the quantification of IFT88-positive cilia in BT-549 BAG3-KO and WT cells. The experiments were repeated at least five times and per replicate three vision fields ($\times 60$ magnification) were counted. (C) Representative pictures of IFT88 cilia staining in BT-549 BAG3-KO and WT cells 48 h after seeding. Arrow heads: Cilia. (D) Wound-healing assay performed using Ibidi culture inserts of BT-549 WT and three BAG3-KOs. * $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$; multiple t test corrected for multiple comparisons using the Holm-Sidak method

was shown that inhibition of ciliogenesis leads to earlier onset of tumor formation. An increased tumor growth rate, a higher tumor grade and an enhanced metastatic potential of these tumors could also be demonstrated.³⁹ The fact that a lack of ciliogenesis and overexpression of BAG3 both lead to enhanced breast cancer aggressiveness raised the question whether the functional connection between BAG3 and ciliogenesis observed in GBM is also existing in TNBC. It was originally described that TNBC cells have either no or few cilia compared to breast fibroblasts and epithelial cells, both in vitro and in human patient samples. The morphology of the cilia is also different in breast cancer cells, as they tend to have straight and short cilia.⁴⁰ To investigate the influence of BAG3 on ciliogenesis, we conducted an immunofluorescence staining of BAG3-KO and BAG3-proficient control cells utilizing the ciliary marker IFT88 (Figure 5B,C). Counting of the cilia revealed that cultures of all three BT-549 BAG3-KO cells contain more ciliated cells than WT cells, with statistically significant differences observed in two of the three KO cell lines. In analogy to GBM, this suggests that BAG3 also acts as a negative regulator of ciliogenesis in TNBC.

Given the proposed central role for cilia in regulating migration/invasion in GBM cells (see above), the influence of BAG3 on the migration capacity was subsequently investigated in the TNBC cells. KO of BAG3 significantly reduced the speed of gap closure, with all three BAG3-KO lines showing statistically significant differences between 6 and 8 h (Figure 5D). Of note, our previous findings had shown similar effects of BAG3 depletion on the expression of EMT regulators in BT-549 and MDA-MB-468 cells⁷ as observed in GBM in the present study, an event which may further contribute to diminished migration of BAG3 KO TNBC cells.

4 | DISCUSSION

Here, we describe a completely novel function of BAG3 as a negative regulator of ciliogenesis in GBM and TNBC, representing a possible mechanism driving BAG3-dependent malignization of therapy-resistant cancers. The roles of cilia in malignant diseases are hitherto poorly understood, but it is known that they are involved in multiple hallmarks of cancer, such as oncogenic signaling, cell cycle regulation, autophagy, cancer cell metabolism, EMT, apoptosis resistance, and angiogenesis.^{11,13,31,37,41} In line with our findings, primary cilia have been proposed to act as tumor suppressor organelles, suggesting that their presence is associated with a less malignant phenotype.⁴² Based on these observations, the function of primary cilia in the regulation of cancer

development is recently attracting increasing attention.³¹ In particular it has been shown that most cancers lack cilia, because ciliogenesis is linked to the cell cycle and is normally associated with quiescence¹³: Paradoxically, primary cilia appear to contribute to apoptosis resistance of cancer cells,^{31,43} at least in some reported cases. Therefore, it is currently unclear whether the inhibitory effects of BAG3 on ciliogenesis and apoptosis induction in TNBC and GBM cells as observed in the current study and our previous work^{7,8} are functionally correlated or whether these effects may rather reflect two distinct biological actions of the multifunctional hub protein BAG3 in cancer. In any case, our new data obtained in GBM and TNBC cells identify suppression of EMT and ciliogenesis as putative synergizing mechanisms of BAG3-driven tumor aggressiveness in both cancers. BAG3 depletion was associated with decreased expression of SNAIL and ZEB1, two key transcription factors known to regulate EMT in both GBM cell lines. This finding is consistent with the previously observed EMT-modulating role of BAG3 in other tumor entities^{44–46} and the established activating effect of EMT on the migratory, pro-invasive phenotype of tumors.^{6,31,38}

In line with the possible role of ciliogenesis suppression in tumor malignization, our proteomic and phosphoproteomic analyses detected a striking enrichment in processes and pathways related to the primary cilium in U251 and U343 BAG3 KO cells. Cilium disassembly is controlled by cell cycle-associated proteins such as AURKA and takes place in the G0-phase.¹³ A novel function of BAG3 for regulating the primary cilium is further corroborated by our analyses of the phosphoproteomic data using KSEA (https://bio.tools/KSEA_App),³² to determine kinases affected by BAG3-depletion. Here we observed that in both GBM cells the activity of AURKA and CDK1 was strongly reduced. AURKA is known to regulate the process of cilium disassembly^{13,33} and CDK1 has been shown to phosphorylate PLK1,³⁴ which can also promote cilium disassembly.^{3,21} Consistent with this notion, it was previously shown that cell cycle-related kinase (CCRK; now named CDK20) inhibited ciliogenesis and caused cell cycle progression in GBM cells, while CCRK-depleted cells exhibited cilia and growth arrest.⁴¹ Similarly, depletion of primary cilia via IFT88 knockdown in human primary astrocytes increased their proliferation indicating that ciliary defects might be an early event in gliomagenesis.⁴⁷ Using glioma stem-like, patient-derived cultures it was recently also demonstrated that enforced cilia formation via depletion of NEK2 blocked the cell cycle of these cultures and induced differentiation.³⁷ Based on our own data, BAG3 not only seems to regulate cilium formation, but also its disassembly via regulating

the levels of multiple client proteins. Nonetheless, future experiments are needed to experimentally validate these exciting novel findings and to determine if this is directly mediated via BAG3-dependent target protein stabilization/degradation or if additional regulatory proteins in-between are involved. Additionally, the possible physiological role of BAG3 in regulating ciliogenesis in non-transformed cells will be an interesting and worthwhile topic to be investigated in subsequent follow-up studies.

ACKNOWLEDGMENTS

We would like to thank Hildegard König and Gabrielle Köpf for their ongoing excellent technical assistance. This study is supported by a grant from the Deutsche Forschungsgemeinschaft (German Research Foundation, KO 1898/11-1) to Donat Kögel.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Conceptualization: Donat Kögel; data curation: Benedikt Linder, Caterina Klein, Marina E. Hoffmann, Florian Bonn; formal analysis: Benedikt Linder, Caterina Klein, Marina E. Hoffmann, Florian Bonn; investigation: Benedikt Linder and Caterina Klein; methodology: Benedikt Linder, Caterina Klein, Marina E. Hoffmann, Florian Bonn; resources: Donat Kögel and Ivan Dikic; funding acquisition: Donat Kögel; project administration: Donat Kögel; software: Benedikt Linder; Caterina Klein; Marina E. Hoffmann, Florian Bonn; supervision: Donat Kögel; validation: Benedikt Linder and Caterina Klein; visualization: Benedikt Linder and Caterina Klein; writing—original draft: Donat Kögel, Benedikt Linder, Caterina Klein, Marina E. Hoffmann, Florian Bonn; writing—review and editing: Donat Kögel, Benedikt Linder and Caterina Klein. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE^{19,20} partner repository with the data set identifier PXD024802. Any additional data can be made available upon reasonable request.

ORCID

Benedikt Linder  <https://orcid.org/0000-0003-0908-571X>

Caterina Klein  <https://orcid.org/0000-0002-7998-8837>

Ivan Dikic  <https://orcid.org/0000-0001-8156-9511>

Donat Kögel  <http://orcid.org/0000-0003-1209-0210>

REFERENCES

- Kogel D, Linder B, Brunschweiler A, Chines S, Behl C. At the crossroads of apoptosis and autophagy: multiple roles of the co-chaperone BAG3 in stress and therapy resistance of cancer. *Cells*. 2020;9(3), <https://doi.org/10.3390/cells9030574>
- Gamerding M, Kaya AM, Wolfrum U, Clement AM, Behl C. BAG3 mediates chaperone-based aggresome-targeting and selective autophagy of misfolded proteins. *EMBO Rep*. 2011; 12(2):149-156. <https://doi.org/10.1038/embor.2010.203>
- Rosati A, Graziano V, De Laurenzi V, Pascale M, Turco MC. BAG3: a multifaceted protein that regulates major cell pathways. *Cell Death Dis*. 2011;2:e141. <https://doi.org/10.1038/cddis.2011.24>
- Sturner E, Behl C. The role of the multifunctional BAG3 protein in cellular protein quality control and in disease. *Front Mol Neurosci*. 2017;10:177. <https://doi.org/10.3389/fnmol.2017.00177>
- Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization Classification of tumors of the central nervous system: a summary. *Acta Neuropathol*. 2016;131(6): 803-820. <https://doi.org/10.1007/s00401-016-1545-1>
- Liu CA, Chang CY, Hsueh KW, et al. Migration/invasion of malignant gliomas and implications for therapeutic treatment. *Int J Mol Sci*. 2018;19(4), <https://doi.org/10.3390/ijms19041115>
- Das CK, Linder B, Bonn F, et al. BAG3 overexpression and cytoprotective autophagy mediate apoptosis resistance in chemoresistant breast cancer cells. *Neoplasia*. 2018;20(3): 263-279. <https://doi.org/10.1016/j.neo.2018.01.001>
- Antonietti P, Linder B, Hehlhans S, et al. Interference with the HSF1/HSP70/BAG3 pathway primes glioma cells to matrix detachment and BH3 mimetic-induced apoptosis. *Mol Cancer Ther*. 2017;16(1):156-168. <https://doi.org/10.1158/1535-7163.MCT-16-0262>
- Liu BQ, Zhang S, Li S, et al. BAG3 promotes stem cell-like phenotype in breast cancer by upregulation of CXCR4 via interaction with its transcript. *Cell Death Dis*. 2017;8(7):e2933. <https://doi.org/10.1038/cddis.2017.324>
- Boiani M, Daniel C, Liu X, Hogarty MD, Marnett LJ. The stress protein BAG3 stabilizes Mcl-1 protein and promotes survival of cancer cells and resistance to antagonist ABT-737. *J Biol Chem*. 2013;288(10):6980-6990. <https://doi.org/10.1074/jbc.M112.414177>
- Wheway G, Nazlamova L, Hancock JT. Signaling through the primary cilium. *Front Cell Dev Biol*. 2018;6:8. <https://doi.org/10.3389/fcell.2018.00008>
- Mansini AP, Peixoto E, Jin S, Richard S, Gradilone SA. The chemosensory function of primary cilia regulates cholangiocyte Migration, invasion, and tumor growth. *Hepatology*. 2019; 69(4):1582-1598. <https://doi.org/10.1002/hep.30308>
- Liu H, Kiseleva AA, Golemis EA. Ciliary signalling in cancer. *Nat Rev Cancer*. 2018;18(8):511-524. <https://doi.org/10.1038/s41568-018-0023-6>
- Vaheri A, Ruoslahti E, Westermarck B, Ponten J. A common cell-type specific surface antigen in cultured human glial cells and fibroblasts: loss in malignant cells. *J Exp Med*. 1976; 143(1):64-72. <https://doi.org/10.1084/jem.143.1.64>
- Katayose Y, Kim M, Rakkar AN, Li Z, Cowan KH, Seth P. Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. *Cancer Res*. 1997;57(24): 5441-5445. <https://www.ncbi.nlm.nih.gov/pubmed/9407946>

16. Littlewood-Evans AJ, Bilbe G, Bowler WB, et al. The osteoclast-associated protease cathepsin K is expressed in human breast carcinoma. *Cancer Res.* 1997;57(23):5386-5390. <https://www.ncbi.nlm.nih.gov/pubmed/9393764>
17. Meyer N, Zielke S, Michaelis JB, et al. AT 101 induces early mitochondrial dysfunction and HMOX1 (heme oxygenase 1) to trigger mitophagic cell death in glioma cells. *Autophagy.* 2018;14(10):1693-1709. <https://doi.org/10.1080/15548627.2018.1476812>
18. Ran FA, Hsu PD, Lin CY, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell.* 2013;154(6):1380-1389. <https://doi.org/10.1016/j.cell.2013.08.021>
19. Perez-Riverol Y, Csordas A, Bai J, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* 2019;47(D1):D442-D450. <https://doi.org/10.1093/nar/gky1106>
20. Vizcaino JA, Csordas A, del-Toro N, et al. 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* 2016;44(D1):D447-D456. <https://doi.org/10.1093/nar/gkv1145>
21. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nature Methods.* 2012;9(7):676-682. <https://doi.org/10.1038/nmeth.2019>
22. Linder B, Wehle A, Hehlhans S, et al. Arsenic trioxide and (-)-gossypol synergistically target glioma stem-like cells via inhibition of hedgehog and notch signaling. *Cancers.* 2019;11(3). <https://doi.org/10.3390/cancers11030350>
23. Cancer Genome Atlas Research, N. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* 2008;455(7216):1061-1068. <https://doi.org/10.1038/nature07385>
24. Zhao Z, Meng F, Wang W, Wang Z, Zhang C, Jiang T. Comprehensive RNA-seq transcriptomic profiling in the malignant progression of gliomas. *Sci Data.* 2017;4:170024. <https://doi.org/10.1038/sdata.2017.24>
25. Bowman RL, Wang Q, Carro A, Verhaak RG, Squatrito M. GliOVis data portal for visualization and analysis of brain tumor expression datasets. *Neuro-Oncology.* 2017;19(1):139-141. <https://doi.org/10.1093/neuonc/now247>
26. Festa M, Del Valle L, Khalili K, et al. BAG3 protein is overexpressed in human glioblastoma and is a potential target for therapy. *Am J Pathol.* 2011;178(6):2504-2512. <https://doi.org/10.1016/j.ajpath.2011.02.002>
27. Uhlen M, Zhang C, Lee S, et al. A pathology atlas of the human cancer transcriptome. *Science.* 2017;357(6352). <https://doi.org/10.1126/science.aan2507>
28. Tyanova S, Temu T, Sinitcyn P, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nature Methods.* 2016;13(9):731-740. <https://doi.org/10.1038/nmeth.3901>
29. Cox J, Mann M. 1D and 2D annotation enrichment: a statistical method integrating quantitative proteomics with complementary high-throughput data. *BMC Bioinformatics.* 2012;13(Suppl 16):S12. <https://doi.org/10.1186/1471-2105-13-S16-S12>
30. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 2019;47(D1):D607-D613. <https://doi.org/10.1093/nar/gky1131>
31. Fabbri L, Bost F, Mazure NM. Primary cilium in cancer hallmarks. *Int J Mol Sci.* 2019;20(6). <https://doi.org/10.3390/ijms20061336>
32. Wiredja DD, Koyuturk M, Chance MR. The KSEA App: a web-based tool for kinase activity inference from quantitative phosphoproteomics. *Bioinformatics.* 2017;33(21):3489-3491. <https://doi.org/10.1093/bioinformatics/btx415>
33. Boukhalfa A, Miceli C, Avalos Y, Morel E, Dupont N. Interplay between primary cilia, ubiquitin-proteasome system and autophagy. *Biochimie.* 2019;166:286-292. <https://doi.org/10.1016/j.biochi.2019.06.009>
34. Wang G, Chen Q, Zhang X, et al. PCM1 recruits Plk1 to the pericentriolar matrix to promote primary cilia disassembly before mitotic entry. *J Cell Sci.* 2013;126(Pt 6):1355-1365. <https://doi.org/10.1242/jcs.114918>
35. Moser JJ, Fritzlner MJ, Rattner JB. Primary ciliogenesis defects are associated with human astrocytoma/glioblastoma cells. *BMC Cancer.* 2009;9:448. <https://doi.org/10.1186/1471-2407-9-448>
36. Sarkisian MR, Siebzehrubl D, Hoang-Minh L, et al. Detection of primary cilia in human glioblastoma. *JNO.* 2014;117(1):15-24. <https://doi.org/10.1007/s11060-013-1340-y>
37. Goranci-Buzhala G, Mariappan A, Ricci-Vitiani L, et al. Cilium induction triggers differentiation of glioma stem cells. *bioRxiv.* 2020.2020.2012.2023.424140 <https://doi.org/10.1101/2020.12.23.424140>
38. Colella B, Faienza F, Di Bartolomeo S. EMT regulation by autophagy: a new perspective in glioblastoma biology. *Cancers.* 2019;11(3). <https://doi.org/10.3390/cancers11030312>
39. Hassounah NB, Nunez M, Fordyce C, et al. Inhibition of ciliogenesis promotes hedgehog signaling, tumorigenesis, and metastasis in breast cancer. *Mol Cancer Res.* 2017;15(10):1421-1430. <https://doi.org/10.1158/1541-7786.MCR-17-0034>
40. Yuan K, Frolova N, Xie Y, et al. Primary cilia are decreased in breast cancer: analysis of a collection of human breast cancer cell lines and tissues. *J Histochem Cytochem.* 2010;58(10):857-870. <https://doi.org/10.1369/jhc.2010.955856>
41. Yang Y, Roine N, Makela TP. CCRK depletion inhibits glioblastoma cell proliferation in a cilium-dependent manner. *EMBO Rep.* 2013;14(8):741-747. <https://doi.org/10.1038/embor.2013.80>
42. Peixoto E, Richard S, Pant K, Biswas A, Gradilone SA. The primary cilium: its role as a tumor suppressor organelle. *Biochem Pharmacol.* 2020;175:113906. <https://doi.org/10.1016/j.bcp.2020.113906>
43. Lee J, Park KC, Sul HJ, et al. Loss of primary cilia promotes mitochondria-dependent apoptosis in thyroid cancer. *Sci Rep.* 2021;11(1):4181. <https://doi.org/10.1038/s41598-021-83418-3>
44. Meng X, Kong DH, Li N, et al. Knockdown of BAG3 induces epithelial-mesenchymal transition in thyroid cancer cells through ZEB1 activation. *Cell Death Dis.* 2014;5:e1092. <https://doi.org/10.1038/cddis.2014.32>
45. Song F, Wang G, Ma Z, Ma Y, Wang Y. Silencing of BAG3 inhibits the epithelial-mesenchymal transition in human cervical cancer. *Oncotarget.* 2017;8(56):95392-95400. <https://doi.org/10.18632/oncotarget.20726>
46. Xiao H, Cheng S, Tong R, et al. BAG3 regulates epithelial-mesenchymal transition and angiogenesis in human hepatocellular carcinoma. *Lab Invest.* 2014;94(3):252-261. <https://doi.org/10.1038/labinvest.2013.151>

47. Loskutov YV, Griffin CL, Marinak KM, et al. LPA signaling is regulated through the primary cilium: a novel target in glioblastoma. *Oncogene*. 2018;37(11):1457-1471. <https://doi.org/10.1038/s41388-017-0049-3>

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Linder B, Klein C, Hoffmann ME, Bonn F, Dikic I, Kögel D.. BAG3 is a negative regulator of ciliogenesis in glioblastoma and triple-negative breast cancer cells. *J Cell Biochem*. 2021;1-14.
<https://doi.org/10.1002/jcb.30073>