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FAK signaling pathways are modulated by HSPB8 and BAG3 in breast cancer

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Abstract

Background Breast cancer (BC) is a widespread and heterogeneous disease in which autophagy plays an essential role in tumor development and progression. It has been suggested that autophagy activation may prevent tumor development in the initial stages of the disease, while in more advanced stages, autophagy might activate survival mechanisms for cancer cells. Moreover, autophagy may be involved in developing therapies resistance and in forming metastases. Some Heat Shock Proteins (HSPs) play an important role in autophagy. The small HSPB8 draws attention because it is generally highly expressed in Estrogen Receptor positive (ER+) BC and its over-expression increases autophagic flux, proliferation, migration and survival of BC cells under stress conditions. HSPB8 mediates the autophagic degradation of client proteins via the chaperone-assisted selective autophagy (CASA) complex in which it binds the BAG cochaperone 3 (BAG3), the HSP70 and the E3-ubiquitin ligase STUB1. Similarly to HSPB8, BAG3 is also highly expressed in BC cells, and its unregulated expression is linked to a poor prognosis. In our previous studies, we showed that HSPB8 and BAG3 silencing reduces proliferation and migratory capacities of hormone-sensitive MCF-7 BC cells.

Methods Here, we analyzed the signal transduction mechanisms involved in HSPB8- and BAG3-mediated regulation of metastatic potential. We evaluated the function of protein tyrosine kinase 2 (PTK2 also known as FAK), known to activate several downstream signals controlling PI3K, AKT and MAP kinases.

Results We demonstrated that HSPB8 and BAG3 downregulation correlated with a significant reduction in the phosphorylated and active form of FAK which, in a cascade mechanism, induces a decrease in the proliferative, migratory, and adhesive capacity of MCF-7 and T47D ER⁺ human BC cell lines. Furthermore, co-immunoprecipitation studies demonstrated a co-localization between BAG3 and FAK, also confirmed by Immunofluorescence staining in BC specimen sections.

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Conclusion Based on our results, we suggest that BAG3 associates with FAK and, together with HSPB8, modulates the signal transduction mechanisms controlling the survival of the hormone-sensitive human ER⁺, PR positive (PR⁺), HER2 negative (HER2⁻) BC cell lines MCF-7 and T47D.

Keywords HSPB8, BAG3, FAK, Breast cancer

Introduction

Breast cancer (BC) is the most common malignancy worldwide and the leading cause of cancer-related deaths in women [1]. The complexity of the molecular mechanisms regulating tumor initiation and progression determines BC heterogeneity [2]. Based on several aspects such as histological type, tumor grade, lymph node status, and the presence of predictive markers including Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2), BC is classified into five subtypes: luminal A, luminal B, HER2, basal, and Claudin-low. Each subtype has a different prognosis and response to treatment [3]. The mechanisms underlying the development of BC are complex, differing in their various forms and involving genetic and epigenetic alterations that modify the activity of signaling pathways [4]. Numerous studies have demonstrated that endocrine factors play a critical role in the etiology and progression of human BC, and the effects are mainly mediated by their interaction with the α and β forms of the ER, a ligand-activated transcription factor. However, the estrogenic response is complex, both in terms of responsive genes, regulatory mechanisms, and molecules involved, as well as consequences for tumor growth [5].

Autophagy is a complex and multifaceted process in which a cell destroys old or defective cellular components, recycling them to meet its metabolic needs [6, 7]. During autophagy, cellular proteins and organelles undergo a catabolic process in which they are engulfed by autophagosomes, digested in lysosomes, and recycled to support cellular metabolism [7, 8]. In cancer cells, autophagy may have a suppressive role in the early stages of tumor growth, whereas in later stages, autophagy may increase tumor cell survival [9, 10]. Since autophagy plays a role in tumor suppression, its regulation may represent an important strategy for cancer treatment, and several autophagy modulators have been tested in preclinical and clinical trials [7, 11]. Several types of autophagy pathways have been described; among them, chaperone-assisted selective autophagy (CASA) is a peculiar form that exerts protective mechanisms against different human diseases [12]. The major player in CASA is the small Heat Shock Protein B8 (HSPB8) which acts as a facilitator of autophagy [10, 13, 14]. A HSPB8 dimer binds to the BAG cochaperone 3 (BAG3), recognizing aberrant proteins and interacting with the preformed heterodimer of Heat Shock Proteins 70 (HSP70) and STIP1 homology and U-box containing protein 1 (STUB1), forming the

so-called CASA complex. Once the client is bound, the CASA complex guides it to autophagosomes for clearance [10]. Clients include misfolded proteins, damaged proteins of the cytoskeleton, and actin during mitosis [12, 15]. Therefore, HSPB8-BAG3 can mediate quality control mechanisms during different cell events, including mitotic processes activated in proliferating cells [10, 15].

Notably, HSPB8 is induced by estrogens and is a rate-limiting component of the CASA complex; its expression in BC is associated with increased cell proliferation and migration, thus enhancing tumor aggressiveness [5, 9, 16]. Indeed, HSPB8 is highly expressed in BC, both in ER⁺ and in hormone-resistant cell lines. On these bases, it has been suggested that HSPB8 may mediate the acquisition of resistance to hormone therapy [5, 9], like for instance, the development of tamoxifen-resistance in BC [17]. Of note, BAG3 is expressed at high levels in several cancer cell lines and solid tumors, correlating with poor survival. Like HSPB8, BAG3 regulates cell proliferation and motility in several cancer cell lines, influencing the migratory phenotype through interaction with the SH3 domain-containing proteins involved in focal adhesion formation [18]. BAG3 has also been reported to regulate epithelial-mesenchymal transition (EMT) and metastasis formation in a variety of tumor models [18].

Protein tyrosine kinase 2 (PTK2 also known as FAK) is a non-receptor tyrosine kinase acting as an adaptor protein that mediates and integrates signals from growth factors, integrins, and G protein-coupled receptors [19]. FAK is a protein complex central to mesenchymal cell migration. Integrin receptor cluster on the extracellular matrix (ECM) induces the formation of focal adhesion on the cytoplasmic side of the plasma membrane [20]. FAK activates downstream effectors, such as the phosphoinositide 3 kinase (PI3K)/ protein-kinase B (AKT)/ mammalian target of rapamycin (mTOR) complex and MAP kinases to regulate intracellular functions. Furthermore, several pieces of evidence showed that increased FAK expression characterizes many human tumors [21–27], in which it exerts a key role in tumorigenesis [19]. FAK is expressed at low levels in normal or benign breast epithelium, while it is moderately or highly expressed in most malignant breast tissues, correlating with the metastatic process and with a lower survival. Generally, high FAK expression is found in ER-negative (ER⁻), PR-negative (PR⁻) BCs, or in triple-negative BCs (TNBC). The high FAK levels in early metastatic tissues suggest its involvement in BC metastasis, and its expression

correlates with lower survival. Accordingly, FAK inhibition leads to loss of tumor cell adhesion and apoptosis [19].

We already demonstrated that HSPB8 silencing inhibits proliferation and migration of the ER⁺ MCF-7 cell line [9]. Here, we investigated the role of HSPB8 and BAG3 in promoting cancer cell adhesion in ER⁺/HER2⁻ luminal BC. Using MCF-7 and T47D as an in vitro model of ER⁺/HER2⁻ luminal BC, we showed that HSPB8 and BAG3 contribute to the regulation of proliferation, migration, and adhesion processes through FAK-mediated signaling pathways. Supporting the existence of this crosstalk, we demonstrated a co-localization between BAG3 and FAK in BC cell models and their co-expression in patient-derived BC histological analyses. Our observations were further corroborated by BC dataset analyses, revealing that BAG3 and FAK expression are significantly associated with poorer survival. Our results support the clinical relevance of the HSPB8–BAG3–FAK axis in Luminal disease and provide a rationale for therapeutic targeting of this pathway in appropriately selected patients.

Materials and methods

Public dataset analysis

RNA-seq expression data for HSPB8 and BAG3 of BC cell lines from Cancer Cell Lines was queried from DepMap [28].

Gene expression data (Illumina HT-12 v3 microarray) from primary BCs was queried from the METABRIC cohort [29]. Out of the 1980 patients, we retained 1974 breast adenocarcinoma samples, excluding 6 cases diagnosed as phyllodes tumors or angiosarcoma. The molecular subtyping of the patients was inferred from the transcriptome with the SCMOD2-robust algorithm implemented in the “genefu” [30] package (version 2.4).

Difference in expression of HSPB8 and BAG3 across different molecular subtypes was assessed by ANOVA followed by Tukey post-hoc tests implemented in the “rstatix” package (version 0.7.2). Survival analysis was performed on ER⁺/HER2⁻ patients ($n=1376$) stratified over the median expression levels of FAK, BAG3 and HSPB8 and respective disease-specific survival was computed through Kaplan-Meier estimation. Cox proportional hazards models were employed to compute hazard ratios (HR) and confidence intervals (CI); significance was tested using the log-rank test. Kaplan-Meier estimation and Cox model are both implemented in the “survival” R package (version 3.8).

Cell culture

MCF-7 and T47D ER⁺, PR⁺, HER2⁻ cell lines were originally obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were routinely grown in RPMI 1640 medium (ECB9006L, Euroclone, Pero,

Italy), supplemented with 5% foetal bovine serum (FBS; 10270106, Gibco, Life Technologies Waltham, MA, USA), L-glutamine 1mM (ECB3004D, Euroclone) and antibiotics penicillin G 100 U/ml (31749.04, SERVA, Electrophoresis GmbH, Heidelberg, Germany), streptomycin 100 U/ml (S9137-25 G, Sigma-Aldrich-Merck Life Science, Waltham, MA, USA) in a humidified atmosphere of 5% CO₂, 95% air at 37°C.

Plasmid and siRNA

The pCMV-PTK2(human)-3xFLAG-Neo plasmid encoding for human PTK2 (FAK) has been obtained by PTK2 gene synthesis (Neo Biotech, Nanterre, France); an empty vector, pcDNA3.1 (EV) was used as control.

To silence endogenous HSPB8 and BAG3 expression, we used a custom siRNA duplex for HSPB8 (AGA GCA GUU UCA ACA ACG AUU, Dharmacon, Thermo Scientific Life Sciences Research, Waltham, MA, USA); a custom siRNA duplex for BAG3 (GCA UGC CAG AAA CCA CUC AUU, Dharmacon). A non-targeting siRNA (Scramble - Scr) was used as negative control (UAG CGA CUA AAC ACA UCA A, D-001210-01-05, Dharmacon, Waltham, MA, USA).

All plasmids and siRNAs were transfected using Jet-Prime Transfection Reagent and Jet-Prime Buffer (Kit Polyplus, Polyplus-transfection® SA New York, NY, USA), according to the manufacturer's instructions.

Cell growth studies

Cell growth/viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, culture medium was replaced with fresh medium containing MTT (1.5 mg/ml) and the cells were incubated at 37°C for 1 h; then, the medium was removed and 2-propanol (500 µl) was added to solubilize formazan. The absorbance was read at 550 nm with an Enspire 2300 Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA).

To study the effect of HSPB8 and BAG3 silencing on MCF-7 and T47D, cells were seeded in 24-well plates at 40,000 cells/well and transfected with siRNA-HSPB8 and siRNA-BAG3 for 48–72 h; cell viability was analyzed as described above.

Wound healing migration assay

To study cell migration abilities in conditions of HSPB8 and BAG3 downregulation, a scratch wound healing assay was performed. The T47D cells were seeded into 6-well plates at a density of 200,000 cells per well. 24 h post-transfection, a cross-shape scrape was made on the monolayer with a p-200 pipette tip; then, the medium was replaced. The wounded areas were marked for observation and photographed at the indicated time (0 and 48 h) after scratch (magnification 100x). The micrographs

of the scratch wound healing assay are representative of three independent experiments ($n = 3$). The migrated cells were quantified by measuring wound closure areas after injury by ImageJ software (version 1,51k, National Institutes of Health); all cells were counted in this area. Each bar represents mean $\pm$ SD ($n = 3$). The results were shown relative to the Non-Targeting siRNA (Scramble) cells. $*p < 0.05$ vs. Scramble.

Adhesion assay

MCF-7 and T47D cells were seeded into 6-well plates at a density of 300,000 cells per well and transfected for 48–72 h with siRNA-HSPB8 and siRNA-BAG3. After transfection, each plate, containing both silenced and non-silenced cells, was trypsinized, counted, resuspended in RPMI 1640 medium and seeded into the 48-well flat-bottomed plastic plate pre-coated with 20 μ g/mL laminin (L2020, Sigma-Aldrich, Saint Louis, MI, USA) or 20 μ g/mL fibronectin (ECM001, Sigma-Aldrich, Saint Louis, MI, USA) at 200,000 cells per well. Cells were let adhering for 4 h (MCF-7) or 5 h (T47D) at 37°C, based on our preliminary experiments we performed to set up the assay. At the end of the incubation, cells were fixed in methanol, and stained with Diffquik (Biomap, Italy); later, absorption was measured at 595 nm with an Enspire 2300 Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA).

mRNA expression analysis

For quantitative Polymerase Chain Reaction (qPCR) analyses, MCF-7 and T47D cells were seeded in 6-well plates at 300,000 cells/well and transfected as described above. 48 h after transfection, cells were collected and total RNA extracted using TRI Reagent® (T9424, Sigma-Aldrich, Saint Louis, MI, USA) and 1-bromo-3-chloropropane (B9673, Sigma-Aldrich, Saint Louis, MI, USA) following the manufacturer’s instructions. RNA quantification was performed using a NanoDrop 2000 (Thermo Fisher Scientific). RNA samples (1 μ g) were treated with DNase I (AMPD1, Sigma-Aldrich-Merck), and reverse transcribed to cDNA with the High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific

Inc., Waltham, MA, USA). Subsequently, qPCR was performed using the iTaq SYBR Green Supermix (1725124, Bio-Rad Laboratories, Hercules, CA, USA) in a total volume of 10 μ L with 500 nmol primers. A CFX96 Real-Time System (Bio-Rad Laboratories) was used according to the following cycling conditions: 94°C for 10 min and 40 cycles at: 94°C for 15 s (40 cycles), 60°C for 1 min. Data were transformed using the Eq. 2^{-DDCt} to give N-fold changes in gene expression; all statistical analyses were performed with DCT values. Primers for qPCR were synthesized by Eurofins Genomics (Ebersberg, Germany) with the sequences showed in Table 1.

Each sample was analyzed in triplicate ($n = 3$); *HSPB8*, *BAG3*, *e-Cadherin*, and *Vimentin* values were normalized with those of *Ribosomal Protein Lateral Stalk Subunit P0* (*RPLP0*).

Data Availability

The RNA expression and associated clinicopathological data for the METABRIC cohort used in this study are available on cBioPortal. [<http://www.cbioportal.org/>].

The RNA expression of HSPB8 and BAG3 in cell lines and associated metadata are available on DepMap. [<https://depmap.org/>].

Western blotting analyses

For Western Blot (WB) analyses, MCF-7 and T47D cells were seeded in 6-well plates at 300,000 cells/well and transfected for 48–72 h with the specific siRNAs. After transfection, cells were harvested in RIPA buffer (150mM NaCl, 0.5% Na-deoxycholate, 100 μ M Na-orthovanadate, 50mM NaF, 50mM M Tris-HCl pH 7.7, 10mM EDTA pH 8, 0.08% SDS, 0.8% Triton X-100) added with cOmplete Protease Inhibitor Cocktail (4693116001, Sigma-Aldrich-Merck) and centrifuged to remove the RIPA-insoluble pellet. Protein concentration was determined through bicinchoninic acid assay with QPRO BCA Kit Standard (PRTD1, Cyanagen Srl, Bologna, Italy), following the manufacturer’s instructions. Equal amounts of total proteins (20 μ g) were resolved on a 7, 10 and 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose 0.45- μ m membranes using a Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were washed with 10mM Tris-HCl, 150mM NaCl, 0.1% Tween 20 (TBS-T) for 30 min, blocked in TBS-T and 5% (w/v) dry skimmed milk, and then incubated with the primary antibody at 4°C o/n. Then, the membranes were washed and incubated for 1 h at room temperature (RT) with the secondary antibody conjugated with peroxidase. Primary and secondary antibodies are listed in Table 2. Immuno-reactive bands were visualized using enhanced chemiluminescence detection kit reagents (Westar Cyanagen, Cyanagen Srl). Images were acquired using a ChemiDoc™

Table 1 Primers used in qPCR

Primers	Forward (5'-3')	Reverse (5'-3')
HSPB8	AGAGGAGTTGATGGTGAAGACC	CTGCAG-GAAGCTGGATTTTC
BAG3	GGGTGGAGGCCAAACACTAA	AGACAGTGCACACACAGC
e-Cadherin	TGAAGGTGACAGAGCCTCTGGAT	TGGGTGAATTC-GGGCTTGTT
Vimentin	AGGCCAAAGCAGGAGTCCACTGA	ATCTGGCGTTC-CAGGGACTCAT
RPLP0	GTGGGAGCAGACAATGTGGG	TGCGCATCATGGT-GTTCTTG

Table 2 List of antibodies used in this study

Antibody	Host species	Dilution	Source
HSPB8	Mouse monoclonal	1:1,000	R&D System, MAB-4987
BAG3	Rabbit polyclonal	1:10,000	Abcam, ab47124
AKT	Rabbit polyclonal	1:1,000	Cell Signalling, #9272
pAKT	Rabbit polyclonal	1:1,000	Cell Signalling, #4060
FAK	Mouse monoclonal	1:200	Santa Cruz Biotechnology, sc-271,126
pFAK	Mouse monoclonal	1:200	Santa Cruz Biotechnology, sc-81,493
e-Cadherin	Mouse monoclonal	1:200	Santa Cruz Biotechnology, sc-8426
Vimentin	Rabbit polyclonal	1:200	Santa Cruz Biotechnology, sc-5565
PI3Kp110	Rabbit polyclonal	1:1,000	Cell Signalling, #4255
PI3Kp85	Rabbit polyclonal	1:1,000	Cell Signalling, #4292
mTOR	Rabbit monoclonal	1:1,000	Cell Signalling, #2983
pmTOR	Rabbit monoclonal	1:1,000	Cell Signalling, #5536
ERK	Rabbit polyclonal	1:1,000	Santa Cruz Biotechnology, sc-94
pERK	Mouse monoclonal	1:100	Santa Cruz Biotechnology, sc-7383
GAPDH	Mouse monoclonal	1:5,000	Immunological Sciences, MAB-10,578
II antibody			
anti-rabbit IgG-HRP	Goat	1:10,000	Jackson ImmunoResearch Laboratories, Inc., 111-035-003
anti-mouse IgG-HRP	Goat	1:10,000	Jackson ImmunoResearch Laboratories, Inc., 115-035-003
VeriBlot det. reagent		1:500	Abcam, ab131366

XRS+ System (Bio-Rad Laboratories), and optical density was analysed with Image Lab™ Software version 6.0.1 (Bio-Rad Laboratories).

Co-immunoprecipitation (Co-IP)

MCF-7 and T47D cells were seeded in 6-well plates at 300,000 cells/well. The next day, cells were harvested in RIPA buffer added with cOMplete Protease Inhibitor Cocktail (4693116001, Sigma-Aldrich-Merck), incubated on ice for 20 min, and centrifuged at 16,000 *g* for 15 min at 4°C for clarification. For the immunoprecipitation, 50 µL/sample DynaBeads Protein G Magnetic Beads (10004D, Invitrogen) were used, following the manufacturer's instructions. Anti-BAG3, anti-FAK, anti-HSPB8, anti-AKT and anti-PI3Kp110α and p85 antibodies were used for Co-IP. After washing in PBS-Tween, immunoprecipitated proteins were eluted from the beads with Laemmli Sample Buffer (1610737, Bio-Rad Laboratories) added with 5% β-mercaptoethanol by incubating samples for 10 min at 70°C. Immunoprecipitation, input, and output samples were then loaded onto 7.5–10% gels and analyzed through WB (see dedicated section). 2 control

conditions have been tested: *i*) no IgG and *ii*) no whole cell lysate (WCL). No IgG is a sample in which the Co-IP was performed without the antibody against the protein of interest (MCF-7 cell lysate + Beads) to exclude the non-specific binding with the Beads or with the tubes; instead the no WCL sample indicates a Co-IP performed without cell lysate (Beads + Antibody), to recognize and exclude the signal related to the light and heavy chains of the denatured antibodies during the detection. We used an antibody (VeriBlot for IP secondary antibody HRP, cat. N. ab131366, Abcam) that specifically recognizes non-denatured IgG. This antibody allowed us to detect the target proteins by recognizing only the antibody used for immunodetection and avoiding the signal related to the binding to small and heavy chains of the antibodies used to perform the immunoprecipitation.

Immunofluorescence (IF) studies

Formalin-fixed, paraffin-embedded (FFPE) tissue Sect. (3 µm thick) from four patients were deparaffinized in 100% xylene, rinsed in ethanol, and rehydrated in distilled water. Antigen retrieval was performed by heating the sections at 95°C for 50 min in Tris-EDTA buffer (T9285, Sigma). Sections were then washed for 5 min in washing buffer (pH 7.4; 24.7 mM Trizma base, 0.149 M NaCl, 100 mM sucrose, 0.01% NaN₃, 0.02% Tween-20) and permeabilized with 0.5% Triton X-100 in PBS for 30 min at RT. After blocking with 5% BSA in PBS for 1 h at RT, slides were incubated overnight at 4°C with primary antibodies diluted in antibody solution (2% BSA, 0.05% NaN₃, 100 mM trehalose, TBS): FAK (1:200) and BAG3 (1:1000). The following day, sections were incubated with the appropriate secondary antibodies and DAPI for nuclear counterstaining for 1 h at RT and then mounted using SlowFade Gold solution. Fluorescence images were acquired with a Leica SP8 confocal microscope equipped with a 63×/1.4 NA oil-immersion objective (pixel size, 360×360 nm²). Images were processed using Fiji software. The images shown represent single confocal planes with optimized brightness and contrast and are representative of four independent patients (*n* = 4).

Statistical analysis

Unpaired t-test was used when two groups were present. Statistical analysis was performed by one-way ANOVA followed by Bonferroni or Tukey multiple comparison tests. A *p*-value of 0.05 or less (*) was considered statistically significant. All analyses were performed using GraphPad PRISM (version 8.0.2).

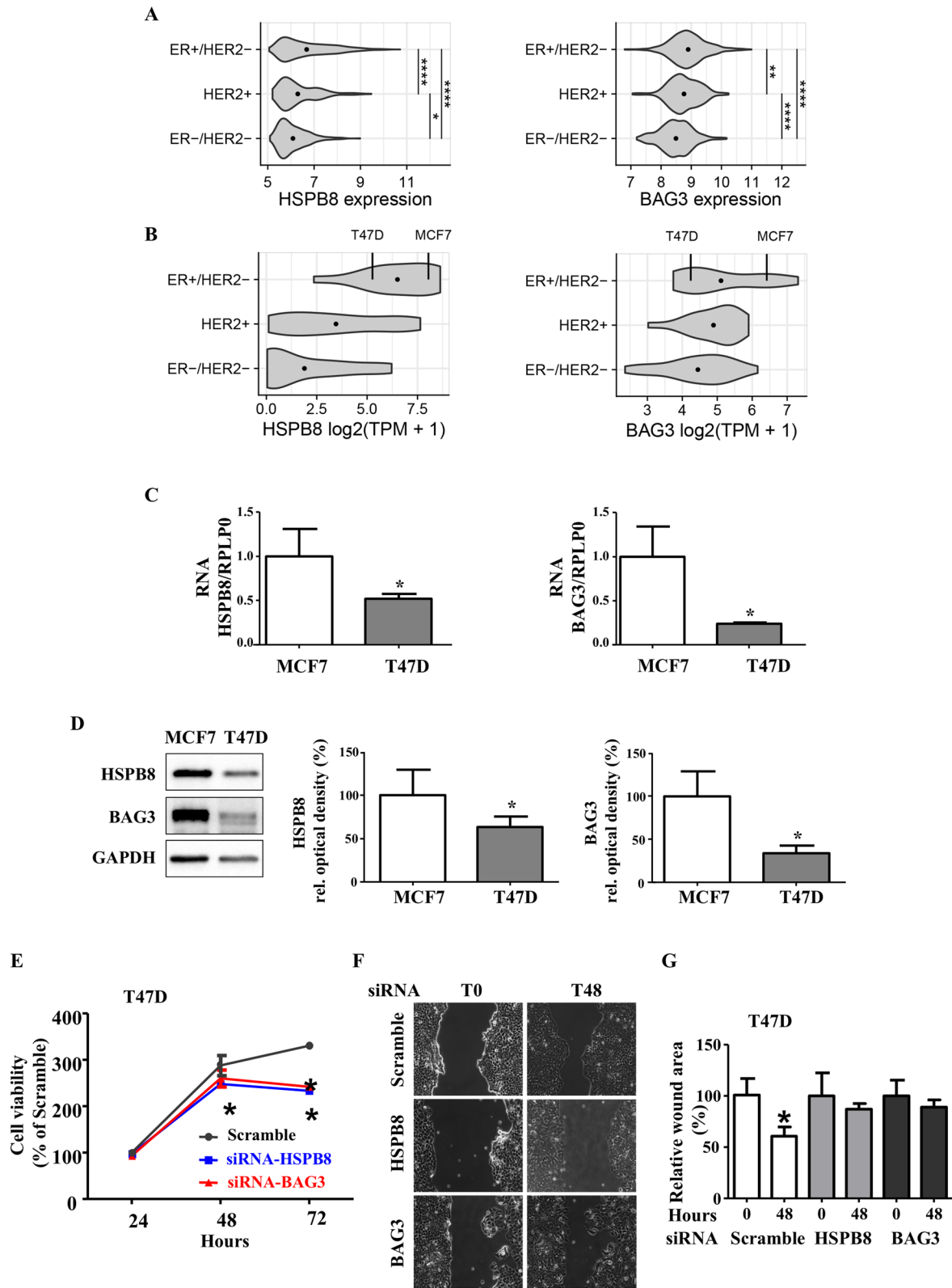


Fig. 1 (See legend on next page.)

(See figure on previous page.)

Fig. 1 Effect of HSPB8 and BAG3 silencing on T47D cell proliferation and migration. **A** HSPB8 (Left) and BAG3 (Right) expression in cell lines from Cancer Cell Line Encyclopedia/DepMap ($n = 68$) and **(B)** patients from the METABRIC cohort ($n = 1974$). HSPB8 and BAG3 mRNA and protein levels were measured by RT-qPCR analysis **(C)** and WB analysis **(D)** in MCF-7 and T47D BC cell lines. **E** T47D cells were transfected with siRNA-HSPB8, siRNA-BAG3 or NT-siRNA and the analyses were performed 24–48–72 h after transfection. Cellular proliferation was evaluated by MTT assay. **F** T47D cells were transfected with siRNA-HSPB8, siRNA-BAG3 or NT-siRNA and the analyses were performed 0–48 h after scratch. Cellular migration was evaluated by wound healing scratch test assay. Unpaired t test was used when two groups were present. Statistical analysis was performed by one-way ANOVA followed by Bonferroni or Tukey multiple comparison tests in the presence of 3 or more groups. $*p < 0.05$ vs. Scramble. Values represent the means of three independent experiments ($n = 3$). Non-Targeting siRNA (Scramble)

Results

Effects of HSPB8 and BAG3 silencing on MCF-7 and T47D cell proliferation and adhesion

Given the high variability of HSPB8 and BAG3 expressions in ER⁺/HER2⁻ luminal models, we selected T47D and MCF-7 cell lines as representative of two cell systems showing low- or high-expression levels of these two CASA members (Fig. 1, panel A–B). To assess differences in HSPB8 and BAG3 regulation in ER⁺, PR⁺ HER2⁻ BC cells, we initially compared their expression levels in MCF-7 and T47D tumour cells. The analysis performed by qPCR and WB, respectively (Fig. 1, panel C and D), showed that both mRNA and protein levels of HSPB8 and BAG3 are lower in T47D than in MCF-7 cells. Since we already found that HSPB8 silencing significantly reduced proliferation and migration of MCF-7 cells [9], we tested whether a similar effect was recapitulated in T47D cells. To this aim, T47D cells transfected with a siRNA-HSPB8 were analyzed with MTT assay (Fig. 1, panel E) finding that HSPB8 downregulation for at least 48 h was correlated with a significant reduction in cell viability. Of note, the silencing of BAG3 recapitulated these findings, although after 72 h of downregulation (Fig. 1, panel E). To study T47D cell migration abilities after HSPB8 and BAG3 downregulation, a scratch wound healing assay was performed. As visible in Fig. 1, panel F–G, HSPB8 and BAG3 silencing significantly reduced the migratory ability of T47D cells as compared to non-targeting siRNA (Scramble) cells. Next, we analyzed the effects of HSPB8 and BAG3 silencing on cell adhesion on laminin or fibronectin as alternative matrix substrates. Laminins are the major ECM protein components of the basal lamina. In general, laminins are heterotrimeric glycoproteins with binding regions for collagen, integrins, cellular domains, and proteoglycans. Laminin promotes adhesion, differentiation, migration, and growth on many cells in vitro [31, 32]. Fibronectin is an adhesion glycoprotein of the ECM involved in widespread interactions and functions, such as the attachment and migration of many cell types, cytoskeletal assembly, tyrosine phosphorylation, and metastasis [33, 34]. Our previous results indicated that the adhesive capacity of both MCF-7 and T47D cell lines significantly increases in the presence of laminin and fibronectin as coating proteins, reason why we chose these conditions to perform our cell adhesion studies (Supplementary Fig. 1, panel A–B). Therefore, after 48–72 h of

HSPB8 or BAG3 downregulation, MCF-7 and T47D cells were seeded on laminin- or fibronectin-coated plates and the adherent vs. floating cell ratios were evaluated at 4 h (MCF-7) or at 5 h (T47D) after plating. We observed that cells transfected with siRNA-HSPB8 or siRNA-BAG3 exhibited a decrease in the percentage of adherent cells on both laminin- and fibronectin-coated plates at all examined times points, compared to control and scramble cells. Therefore, our results demonstrate that HSPB8 or BAG3 depletion reduces the MCF-7 (Fig. 2, panel A and B) and T47D (Fig. 2, panel C and D) cell adhesion.

Effect of HSPB8 and BAG3 silencing on e-Cadherin, vimentin and FAK expression

The outcome of the adhesion assay prompted us to investigate which proteins and pathways HSPB8 and BAG3 interfere with, modulating the overall adhesive capacity in MCF-7 and T47D cells.

We initially measured the expression levels of e-Cadherin and Vimentin, two proteins highly expressed in the early stages of tumor formation. As reported in WB (Fig. 3, panel A, lines 1 and 2), the intensity of the immunoreactive bands of e-Cadherin and Vimentin remained similar in all conditions, including those in which HSPB8 and BAG3 were downregulated in MCF-7 cells (see also quantification in Fig. 3, panel B). As expected from the protein levels analysis, no changes in the mRNA levels of both e-Cadherin and Vimentin were observed in all conditions tested (Fig. 3, panel C). As for MCF-7 cells, also T47D cells exhibited no variations in e-Cadherin and Vimentin protein (Fig. 4, panel A, lines 1 and 2, and quantification in panel B) nor mRNA (Fig. 4, panels C) levels in all conditions analyzed. Thus, we then focused on FAK, a protein complex essential for cell adhesion and migration and investigated whether HSPB8 and BAG3 exert a modulatory effect on this protein complex. To this aim, BC cells transfected with the HSPB8 or BAG3 siRNAs were analyzed for FAK protein levels and phosphorylation to the active form pFAK. As shown in Fig. 3, panel A, control non-transfected and Scramble-transfected MCF-7 cells exhibited comparable levels of pFAK and FAK; instead, MCF-7 cells in which HSPB8 or BAG3 were downregulated displayed reduced pFAK levels at all time points considered (see also quantifications in panel B). No changes were observed for total FAK protein levels in all conditions examined (Fig. 3, panel B).

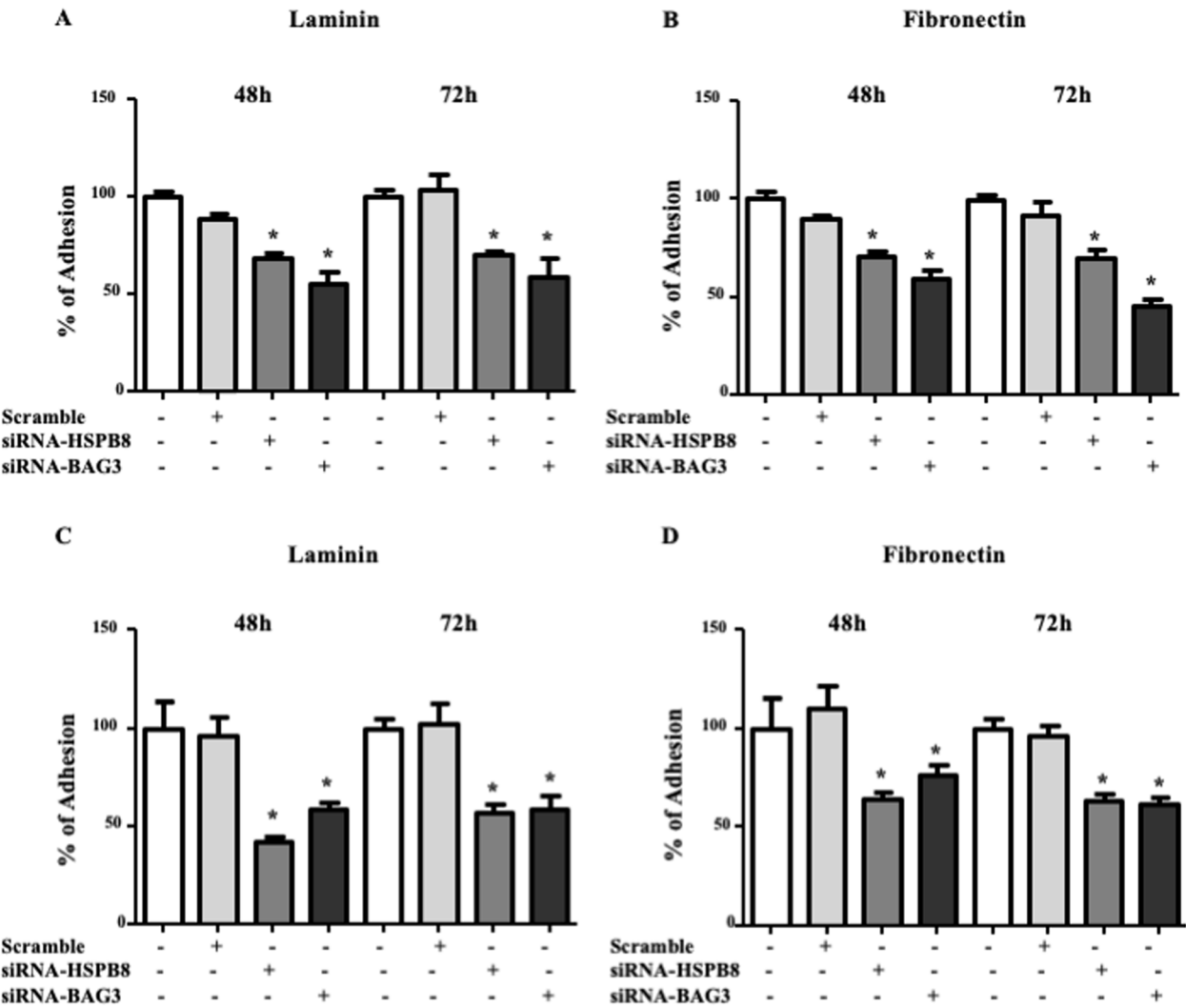


Fig. 2 Effect of HSPB8 and BAG3 silencing on MCF-7 and T47D cell adhesion. Effects of HSPB8 and BAG3 silencing on the adhesive capacity of MCF-7 (A-B) and T47D (C-D) cells in the presence of laminin and fibronectin. The adhesive capacity was evaluated on MCF-7 and T47D cells, transfected for 48-72 h with specific siRNAs, using the Cell Adhesion Test. The data collected represent the percentage change in the number of adherent cells compared to control and scramble cells. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparison tests. * $p < 0.05$ vs control, non-transfected cells were considered statistically significant. Values represent the means of three independent experiments ($n = 3$). Non-Targeting siRNA (Scramble), siRNA-HSPB8, siRNA-BAG3

A similar pattern was found in T47D cells. Indeed, as reported in Fig. 4 panel A, the total FAK protein levels were unaffected, while the phosphorylated form pFAK was significantly decreased in case of either HSPB8 or BAG3 downregulation. To strengthen the causal link between HSPB8, BAG3 and FAK activation, MCF-7 and T47D cells were transfected with pCMV-PTK2(human)-3xFLAG-Neo (FAK) plasmid, siRNA-HSPB8, siRNA-BAG3 or NT-siRNA and the analyses were performed 48 h after transfection. Our results indicate that FAK overexpression was effective, producing a significant increase in FAK levels in both BC cell lines. However, HSPB8 and BAG3 silencing effectively counteracted FAK overexpression in MCF7 and T47D cells, thus confirming

their efficacy not only on endogenous but also on induced levels of FAK (Supplementary Fig. 2). The observed reduction in pFAK protein levels upon HSPB8 or BAG3 silencing led to the hypothesis that these two proteins are essential for the activation of FAK in our ER⁺ BC cell lines. The efficiency of the siRNA was confirmed in all experiments measuring HSPB8 and BAG3 protein levels in WB analysis (Figs. 3 and 4, panel A, lines 5 and 6).

Effect of HSPB8 and BAG3 silencing on the PI3K, AKT and mTOR pathway
FAK activates downstream effectors, such as the PI3K/ AKT/mTOR pathway that are crucial in many physiological processes like cell cycle progression, differentiation,

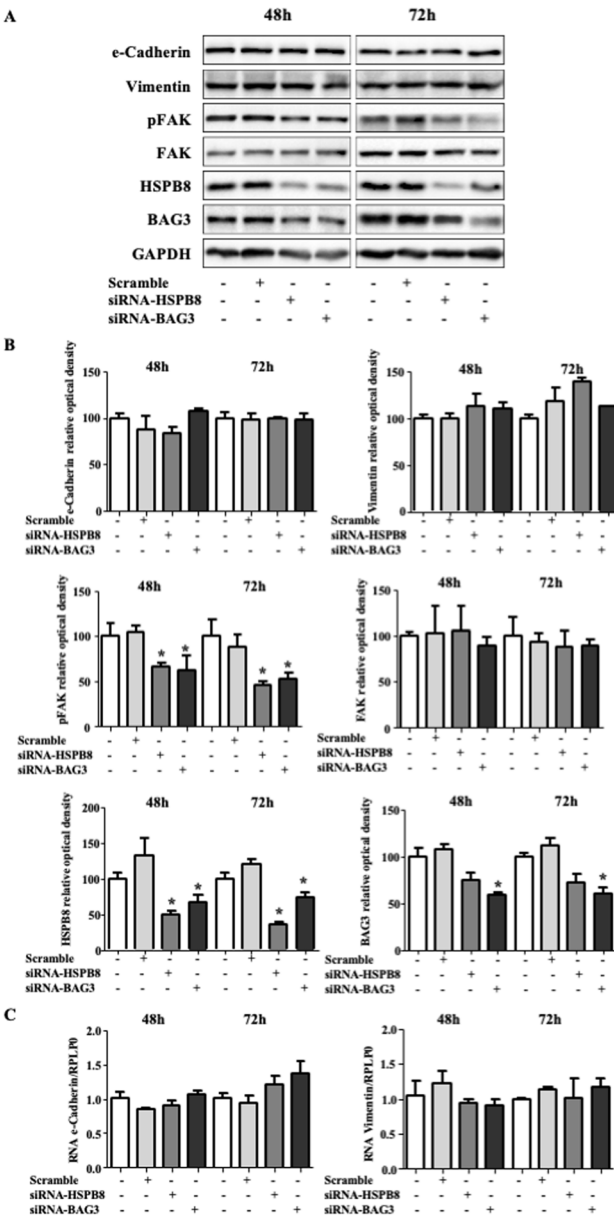


Fig. 3 Effect of HSPB8 and BAG3 silencing on e-Cadherin, Vimentin and FAK expression in MCF-7 cells. **A** MCF-7 cells were transfected with siRNA-HSPB8, siRNA-BAG3 or NT-siRNA and the analyses were performed 48-72 h after transfection. e-Cadherin, Vimentin, pFAK, FAK, HSPB8 and BAG3 protein levels were measured by WB analysis. **B** e-Cadherin, Vimentin pFAK, FAK, HSPB8 and BAG3 WB analysis quantification on MCF-7 cells. **C** e-Cadherin and Vimentin mRNA levels were measured by qPCR analysis 48-72 h after transfection. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparison tests. * $p < 0.05$ vs control, non-transfected cells. Values represent the mean of three independent experiments ($n = 3$). Non-Targeting siRNA (Scramble), siRNA-HSPB8, siRNA-BAG3

apoptosis, cell motility and metabolism [35]). This represents one of the main molecular pathways involved in cell survival, which is deregulated in many malignant tumors and, if altered, can contribute to both tumor pathogenesis and resistance to therapies. Thus, we investigated

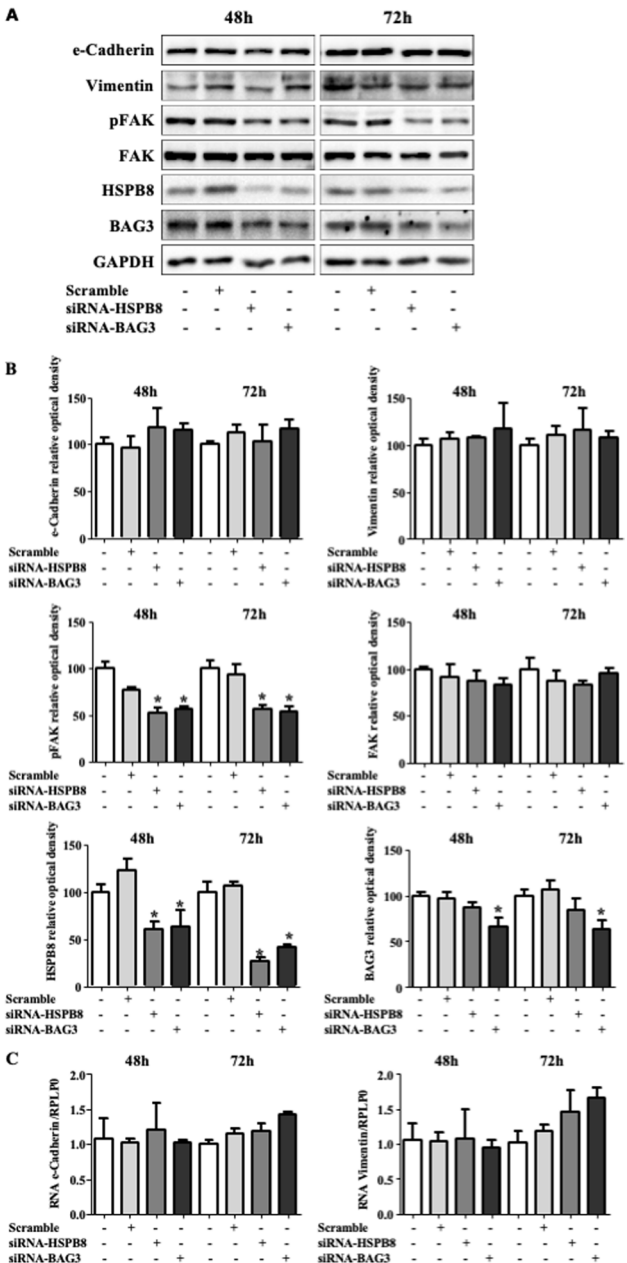


Fig. 4 Effect of HSPB8 and BAG3 silencing on e-Cadherin, Vimentin and FAK expression in T47D cells. **A** T47D cells were transfected with siRNA-HSPB8, siRNA-BAG3 or NT-siRNA and the analyses were performed 48-72 h after transfection. e-Cadherin, Vimentin, pFAK, FAK, HSPB8 and BAG3 protein levels were measured by WB analysis. **B** e-Cadherin, Vimentin, pFAK, FAK, HSPB8 and BAG3 WB analysis quantification on T47D cells. **C** e-Cadherin and Vimentin mRNA levels were measured by qPCR analysis 48-72 h after transfection. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparison tests. * $p < 0.05$ vs control, non-transfected cells. Values represent the mean of three independent experiments ($n = 3$). Non-Targeting siRNA (Scramble), siRNA-HSPB8, siRNA-BAG3

this signaling pathway starting from PI3Kp110 catalytic and PI3Kp85 regulatory subunits in MCF-7 and T47D cell lines in which HSPB8 and BAG3 levels were down-regulated as described above. By WB analyses, we found

no changes in the protein levels of PI3K p110 or p85 subunits at 48–72 h post-transfection in MCF-7 cells (Fig. 5, panel A, lanes 1 and 2) or in T47D cells (Fig. 6, panel A, lanes 1 and 2). Next, we examined the levels of total AKT and those of its activated phosphorylated form (pAKT), distinguishable based on their slightly different molecular weight in SDS-PAGE (due to the upshift associated with the phosphate residues). As shown in Fig. 5, panel A and B, WB results from MCF-7 cell line demonstrate that pAKT levels were similar in control and Scramble groups, while HSPB8 or BAG3 silencing caused pAKT level reduction at all times considered (Fig. 5, panel A, line 3). Remarkably, AKT remained equally present in all groups examined (Fig. 5, panel A, line 4). As seen in pFAK, also in this case a similar cell response was observed in T47D cells. In fact, pAKT levels were stable in all control cells, but largely decreased when HSPB8 or BAG3 were downregulated in T47D cells at all-time points considered (Fig. 6 panel A, line 3). Also, in the case of T47D cells, total AKT levels remained unchanged in all conditions tested (Fig. 6, panel A, line 4).

Therefore, we concluded that HSPB8 and BAG3 downregulation reduced FAK phosphorylation, which in turn reduces the levels of the active AKT protein form (pAKT), without interfering with the *de novo* synthesis or the clearance of the non-phosphorylated proteins. In fact, FAK overexpression in HSPB8 and BAG3 depleted cells, prevented pAKT reduction supporting that HSPB8 and BAG3 mediate AKT activation via FAK (Supplementary Fig. 2).

Following the same pathway, we next focused on mTOR, a highly conserved, atypical 289 kDa serine-threonine kinase of the PI3K-related kinase family involved in the regulation of cell growth and proliferation and cell cycle progression. Here, we analyzed the effect of HSPB8 and BAG3 downregulation on total mTOR protein levels and its phosphorylated form (p-mTOR) both in MCF-7 and T47D cells under the same conditions previously tested. The data in MCF-7 (Fig. 5, panel A, lane 5 and 6) and in T47D (Fig. 6, panel A, lane 5 and 6) cells indicate that neither mTOR phosphorylation, nor mTOR total protein levels are influenced by HSPB8 or BAG3 silencing. Thus, these results support the hypothesis that in both cell types AKT is activated in basal conditions and may exert its activity in an mTOR-independent manner. Therefore, the effects of HSPB8 and BAG3 depletion on adhesion properties of MCF-7 and T47D cells may be linked to a decrease in AKT phosphorylation.

Effect of HSPB8 and BAG3 silencing on ERK activation

As previously mentioned, FAK also activates downstream signaling molecules such as MAP kinases to regulate several intracellular functions. To clarify whether HSPB8 and BAG3 silencing was also able to modify ERK1/2

activation in BC cells, we analyzed the levels of total ERK1/2 protein and its phosphorylated form pERK1/2 using the same experimental paradigms described above. Despite we observed that ERK1/2 was already activated in control cells, we did not detect changes on ERK1/2 protein levels for both cell types and all time considered (Fig. 7, panels A-D). Instead, as for pFAK and pAKT, a decrease in ERK1/2 phosphorylation was observed after HSPB8 or BAG3 downregulation in both cell types and time analysed, although this decrease was non statistically significant in MCF-7 cells at 72 h post-transfection. Therefore, FAK overexpression in HSPB8 and BAG3 depleted cells, prevented pERK1/2 reduction supporting that HSPB8 and BAG3 mediate ERK1/2 activation via FAK (Supplementary Fig. 2).

Overall, our results show that in MCF-7 and T47D cells both ERK1/2 and FAK are activated under basal conditions. Moreover, the effects of HSPB8 and BAG3 silencing on proliferation and migration of MCF-7 and T47D cells may be linked to a decrease in FAK, AKT and ERK1/2 phosphorylation.

BC cell lines and patient-derived samples confirm a crosstalk between BAG3 and FAK

To finally assess whether HSPB8 and BAG3 affect the investigated pathways by direct interaction with the target proteins analyzed, we performed Co-IP assays. Figure 8, panel A shows that FAK specifically interacts with BAG3. In fact, by pulling down BAG3 with a specific antibody from total cell lysates of MCF-7 or T47D cells, we were able to detect an immunoreactive band for FAK. In Fig. 8, panel B shows that HSPB8 does not interact with AKT. In fact, we were unable to detect a band for HSPB8 by pulling down AKT with a specific antibody from total cell lysates of MCF-7 or T47D cells. This supports the notion that HSPB8 and possibly BAG3, that is normally bound to HSPB8, do not directly interfere with the AKT protein, despite HSPB8 silencing correlates with a reduction of pAKT levels. Similar results were obtained pulling down the PI3K subunits p110 and p85. As previously seen with AKT, also in this case we were unable to detect any immunoreactive HSPB8 band in the immunoprecipitated samples for each of these subunits, namely the p110 (p110 in Fig. 8, panel C; p85 in Fig. 8, panel D). Consequently, Co-IP results showed that BAG3 may contribute to the regulation of proliferation, migration and adhesion of ER⁺BC cell lines through the association with the FAK protein. This co-localization between FAK and BAG3 was also confirmed by IF staining of FAK and BAG3 in breast tumor sections (Fig. 8, panel E). To demonstrate the clinical relevance of the HSPB8–BAG3–FAK axis in real-world patients, we leveraged the METABRIC cohort. As shown in Fig. 8, panel F-G-H, we stratified ER⁺/HER2⁻ patients based on the expression levels of

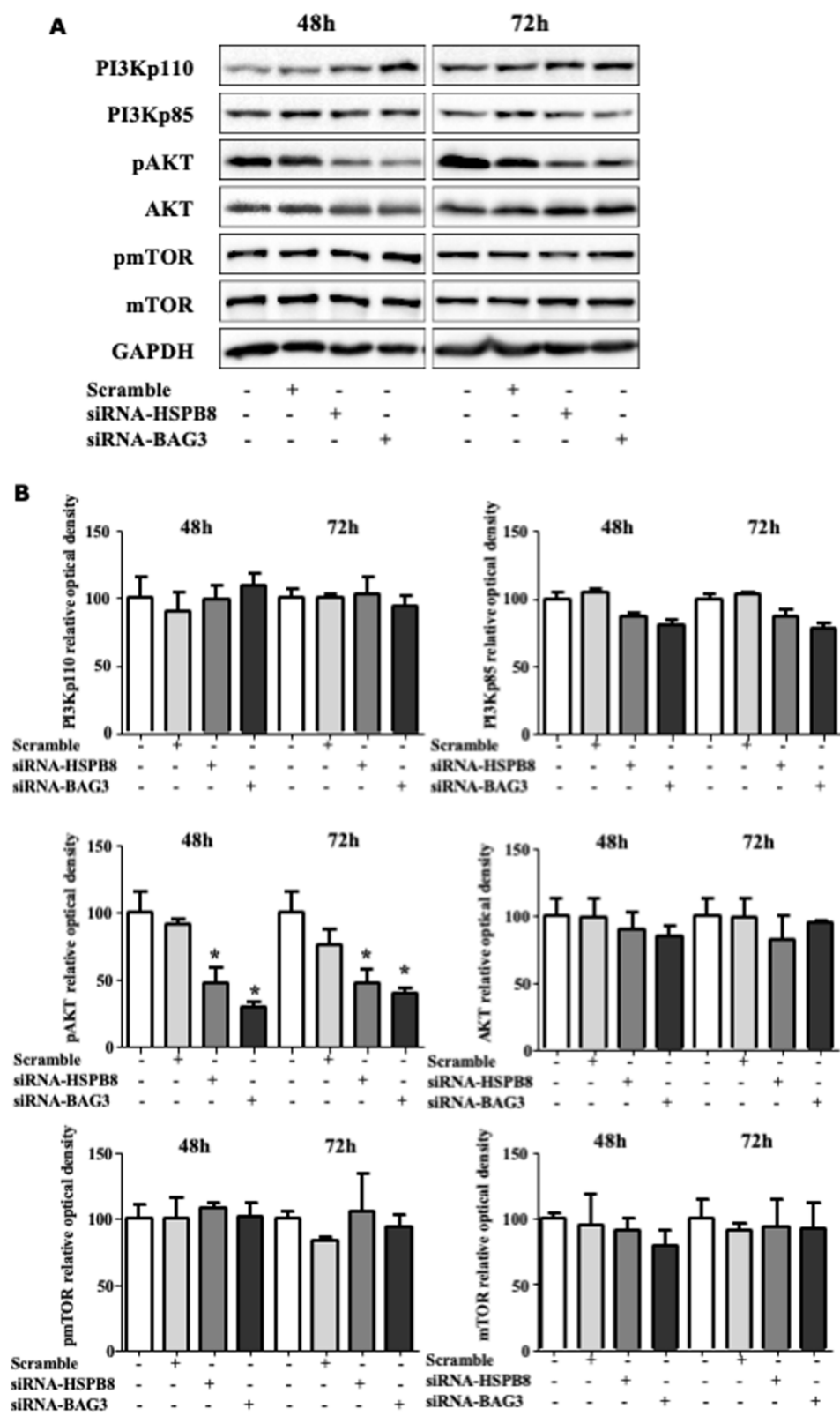


Fig. 5 Effect of HSPB8 and BAG3 silencing on PI3K, AKT and mTOR pathway in MCF-7 cells. **A** MCF-7 cells were transfected with siRNA-HSPB8, siRNA-BAG3 or NT-siRNA and the analyses were performed 48-72 h after transfection. PI3K, AKT and mTOR protein levels were measured by WB analysis. **B** PI3K, AKT and mTOR WB quantification analysis on MCF-7 cells. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparison tests. * $p < 0.05$ vs Control, non-transfected cells. Values represent the mean of three independent experiments ($n = 3$). Non-Targeting siRNA (Scramble), siRNA-HSPB8, siRNA-BAG3

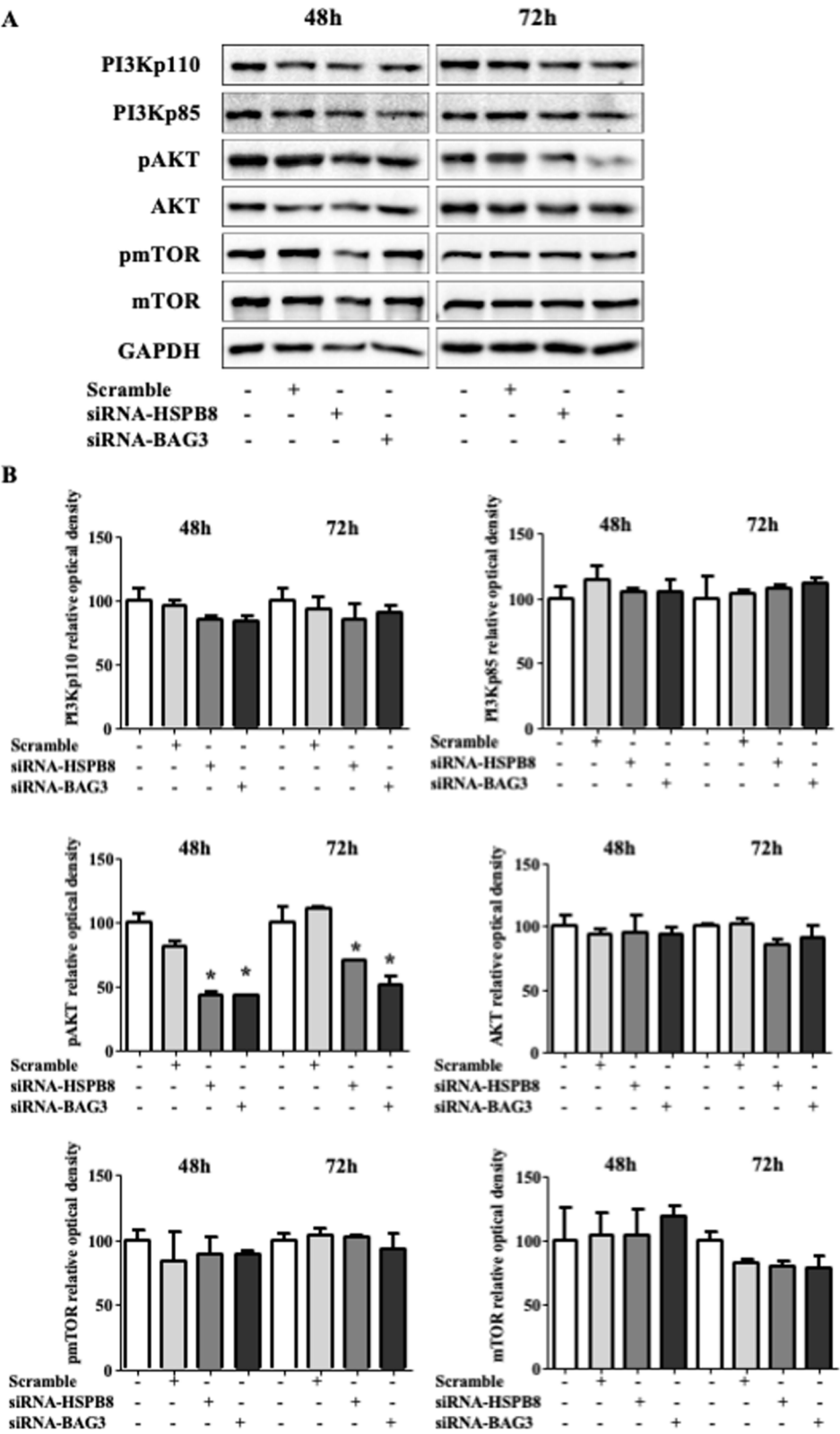


Fig. 6 Effect of HSPB8 and BAG3 silencing on PI3K, AKT and mTOR pathway in T47D cells. **A** T47D cells were transfected with siRNA-HSPB8, siRNA-BAG3 or NT-siRNA and the analyses were performed 48-72 h after transfection. PI3K, AKT and mTOR protein levels were measured by WB analysis. **B** PI3K, AKT and mTOR WB quantification analysis on T47D cells. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparison tests. * $p < 0.05$ vs Control, non-transfected cells. Values represent the mean of three independent experiments ($n=3$). Non-Targeting siRNA (Scramble), siRNA-HSPB8, siRNA-BAG3

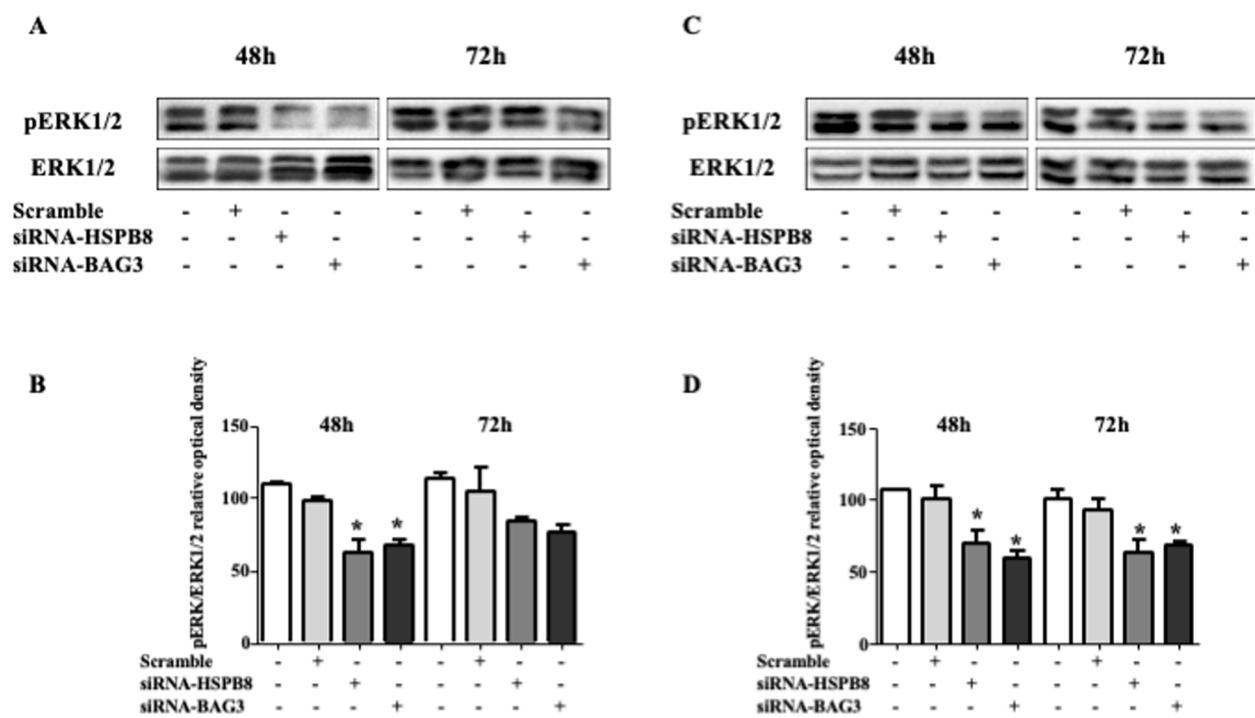


Fig. 7 Effect of HSPB8 and BAG3 silencing on ERK1/2 activation. **A–C** MCF-7 and T47D cells were transfected with siRNA-HSPB8, siRNA-BAG3 or NT-siRNA and the analyses were performed 48–72 h after transfection. pERK1/2 and ERK1/2 protein levels were measured by WB analysis. **B–D** pERK1/2 and ERK1/2 WB quantification analysis on MCF-7 and T47D cells respectively. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparison tests. * $p < 0.05$ vs Control, non-transfected cells. Values represent the mean of three independent experiments ($n = 3$). Non-Targeting siRNA (Scramble), siRNA-HSPB8, siRNA-BAG3

FAK, BAG3 and HSPB8, and evaluated the different disease-specific survival. Although HSPB8 showed no statistically significant correlation with survival, higher BAG3 and FAK expression were significantly associated with poorer survival.

Discussion

In this study, we analyzed how two major components of the CASA complex modulate the adhesion properties of two BC cell models. Our main aim was to better understand the molecular mechanisms that control one of the most common cancers among women and the leading cause of death from cancer in females [1]. Our study is based on our previous data [9] showing that HSPB8 silencing reduces proliferation and migration of MCF-7 cells. These cells express HSPB8 and BAG3 mRNA and protein at higher levels compared to the T47D cell line. Therefore, we evaluated if similar effects were present in T47D cells after HSPB8 and BAG3 silencing. MTT analyses demonstrated a decrease in cell viability in T47D cells following HSPB8 and BAG3 silencing, with a maximum effect at 72 h after transfection. In the same way, the scratch wound healing assay showed that HSPB8 and BAG3 downregulation significantly reduced the migratory ability of T47D cells, confirming the data previously obtained in the MCF-7 cell line. Cell adhesion studies

on laminin or fibronectin matrix substrates showed that HSPB8 or BAG3 silencing correlated with a decreased capability of MCF-7 and T47D cells to adhere to plates coated with both substrates. Our results corroborate and extend those reported by Shields and colleagues on different TNBC cell lines, who demonstrated that BAG3 silencing reduced the invasive capabilities of these cells [18]. Similarly, using human epithelial cancer cell lines, Iwasaki and colleagues showed that BAG3 overexpression increased cell motility while BAG3 silencing had the opposite effects [36].

E-Cadherin is a glycoprotein that mediates cell adhesion in the presence of calcium ions in epithelial tissues. Being localized at the level of adherent junctions, it plays a crucial role in tumor development and progression. Tumor cells are characterized by reduced intercellular adhesion, loss of normal morphology, and a consequent increase in cell mobility. In tumors, the loss of adhesion mediated by e-Cadherin causes the loss of epithelial morphology and the acquisition of metastatic potential [37].

Vimentin is a highly conserved protein expressed in functional mesenchymal cells, and it is known to maintain cellular integrity and provide resistance to stress [38]. Increased expression of Vimentin has been reported in various epithelial tumors. At the cellular level, Vimentin participates in cell migration, proliferation, adhesion, and

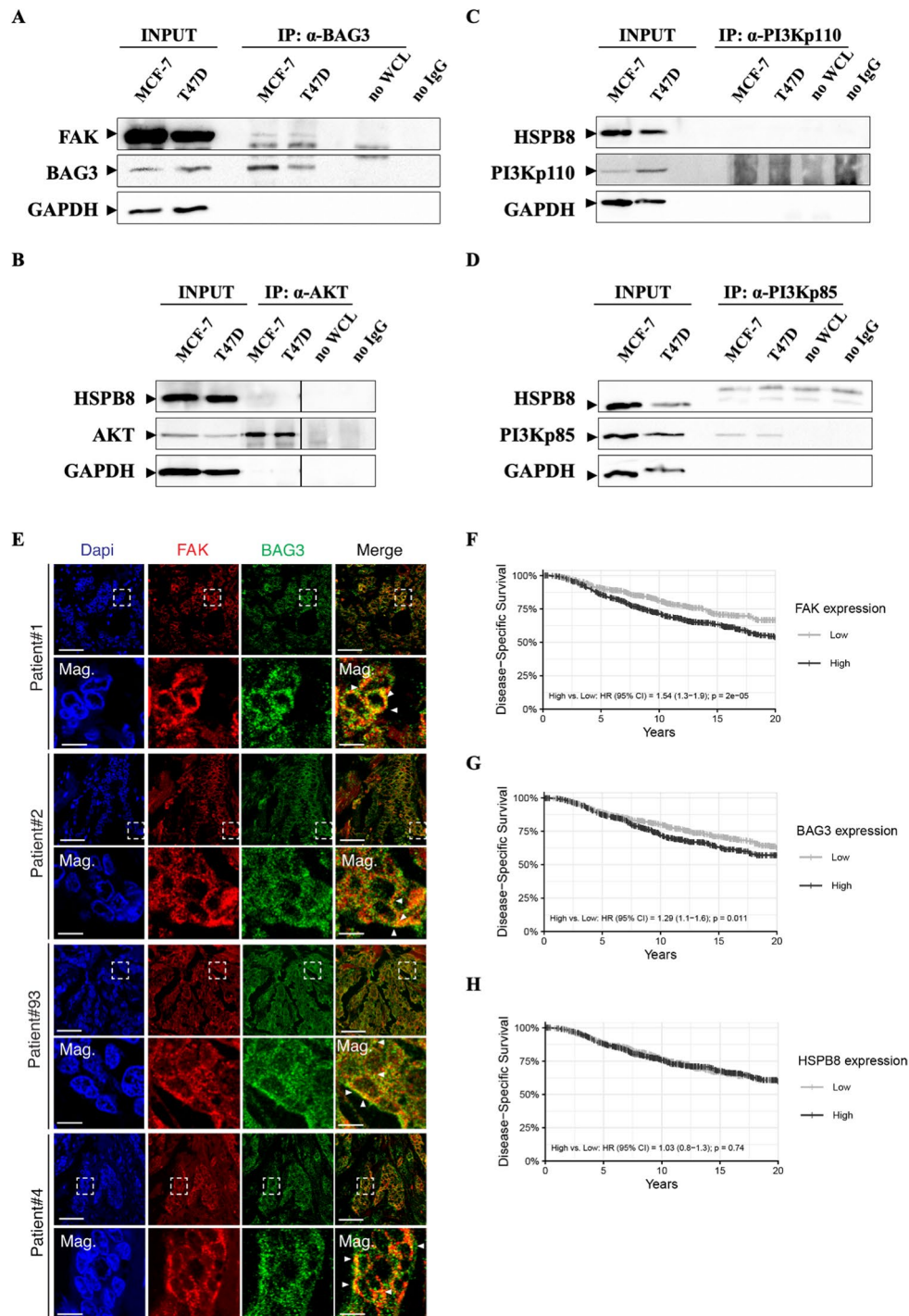


Fig. 8 BC cell lines and patient-derived samples confirm a crosstalk between BAG3 and FAK. WB analysis of protein lysates (INPUT) and Co-IP samples (IP) from MCF-7 and T47D cells, using an antibody (A) anti-BAG3; (B) anti-AKT; (C) anti-PI3Kp110; (D) anti-PI3Kp85. No IgG is a sample in which the Co-IP was performed without the antibody against the protein of interest (MCF-7 cell lysate + Beads) to exclude the non-specific binding with the Beads or with the tubes; instead, the no whole cell lysate (WCL) sample indicates a Co-IP performed without cell lysate (Beads + Antibody), to recognize and exclude the signal related to the light and heavy chains of the denatured antibodies during the detection. The Co-IP assays were repeated three times ($n=3$), each always showing the same results. **E** IF staining of FAK (red) and BAG3 (pseudocolored green) with DAPI nuclear staining (blue) in breast tumor sections. Scale bar, 50 μ m. Arrowheads indicate FAK/BAG3-colocalizing cells. Boxed regions are shown at higher magnification below (Mag., scale bar, 10 μ m). **F-H** Survival analysis was performed on ER⁺/HER2⁻ patients ($n = 1376$) stratified over the median expression levels of FAK (F), BAG3 (G) and HSPB8 (H) and respective disease-specific survival was computed through Kaplan-Meier estimation. Cox proportional hazards models were employed to compute hazard ratios (HR) and confidence intervals (CI); significance was tested using the log-rank test. Kaplan-Meier estimation and Cox model are both implemented in the “survival” R package (version 3.8)

invasion. The expression levels of Vimentin are closely related to tumor progression. It has been reported that the positive expression rate of Vimentin in patients with early BC was higher than in patients with benign BC [39]. To establish whether HSPB8 and BAG3 silencing have an antitumor effect also acting on these two proteins, we evaluated possible variations in e-Cadherin and Vimentin expression after HSPB8 and BAG3 downregulation, but the levels of both transcripts and of their coded proteins were not altered in both BC cells considered, excluding the role of the CASA in the EMT in our ER⁺ BC cell lines. It might be possible that HSPB8 and BAG3 downregulation reduces proliferation, adhesion and migration of MCF-7 and T47D cells using different mechanisms (pFAK-pAKT-pERK reduction), which probably precede the EMT normally occurring during the metastatic evolution of BC. For these reasons, both e-Cadherin and Vimentin are likely not modulated in these conditions by the CASA complex. The outcome of the adhesion assay prompted us to investigate the potential mechanisms by which HSPB8 and BAG3 exert their anti-cancer activity in MCF-7 and T47D BC cell lines. Hence, we analyzed FAK, a multifunctional tyrosine kinase protein, which is activated in most malignant breast tissue cancers and with an important role in promoting BC metastasis [40–43]. We found that HSPB8 or BAG3 downregulation correlates with a strong reduction in the phosphorylated active pFAK form, without affecting the total FAK protein levels. The decrease in pFAK levels suggests that these two CASA components are relevant for FAK activation in BC cell lines.

Since FAK activates the PI3K/AKT/mTOR signaling cascade, which is crucial in many physiological processes (e.g.: cell cycle progression, differentiation, apoptosis, cell motility and metabolism [44, 45]) and dysregulated in several malignant tumors, we investigated this pathway. We initially evaluated the involvement of the PI3Kp110 catalytic subunit, often mutated and amplified in many tumors [46], and its PI3Kp85 regulatory subunits, but our analyses excluded their contribution in mediating the effects of HSPB8 and BAG3 in BC cells.

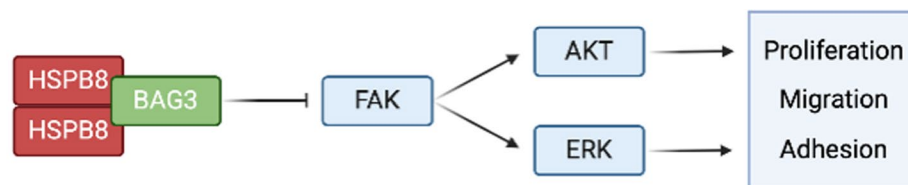
Therefore, we focused on AKT, a serine/threonine kinase essential in many processes that influence cell survival and differentiation, another candidate target of FAK. Notably, we found that, not only pFAK, but also pAKT is reduced in BC cells depleted of HSPB8 or BAG3, suggesting the existence of a link between the two phenomena. In addition, we performed FAK rescue experiments to see if some of the downstream effects (PI3K-AKT and MAPK pathways) returned to be active. Notably, we found that FAK overexpression with HSPB8 and BAG3 knockdown activated pERK and pAKT, confirming that the decrease in FAK phosphorylation, seen with HSPB8

and BAG3 silencing, is the cause of the decrease in ERK and AKT activation.

Our results are in part in line with previous data obtained on TNBC cell lines, which also found alterations in activation of both the AKT and FAK pathways induced by BAG3 silencing [18].

To consolidate the research strategy described above, we also tested a possible mTOR involvement in mediating the HSPB8/BAG3 effects on BC cells; in fact, this kinase plays a central role in the regulation of cell growth and proliferation and cell cycle progression. Importantly, we excluded that this serine-threonine kinase mediates the effects of HSPB8 or BAG3 downregulation in BC cells. In fact, the total mTOR levels and those of its phosphorylated form remained unchanged in all conditions tested in this study. Therefore, all together these data suggest that in both MCF-7 and T47D cells, AKT is normally hyperactivated, but HSPB8 and BAG3 silencing decreases AKT phosphorylation along with the capability of cells to adhere to substrate and to remain viable. These data differ from those collected in other cancer cell lines. For example, Matsushima-Nishiwaki and colleagues suggested that the migration of human hepatocellular carcinoma cells (HCC) stimulated by the transforming growth factor- α (TGF- α) or by the hepatocyte growth factor (HGF) significantly increase after HSPB8 silencing. This altered migratory behavior was accompanied by enhanced TGF- α - or HGF-induced AKT phosphorylation. Inhibitors of the PI3K/AKT pathway, which suppress migration induced by TGF- α , significantly reduced amplitude by HSPB8 silencing. Thus, HSPB8 may reduce the migratory capacity of HCC cells through the downregulation of the PI3K/AKT signaling pathway [47]. Although these observations seem conflicting, they underline the diverse roles exerted by HSPB8 and possibly BAG3 in different human tumours (see [10] for details).

As mentioned above, FAK is implicated in BC cell cycle progression through several mechanisms, including the activation of signal transduction pathways, such as the MAP kinases, starting from FAK phosphorylation mediated by integrin receptors [35]. In BC, the MAP kinases pathway is aberrantly activated and the ERK protein is overexpressed, even in the absence of genomic mutations [48]. Remarkably, the MAP kinase pathway is frequently activated especially in tumors in which growth factor receptors such as EGFR and HER2 are overexpressed. ERK hyper-phosphorylation is associated with a low response to tamoxifen in metastatic BC [35] and the increased phosphorylation of ERK has also been identified in the TNBC cancer cell line MDA-MB-231 [48]. Here, we found that ERK1/2 is already activated in both our control ER⁺ BC cell lines but, similarly to FAK and AKT, its phosphorylation status is significantly reduced



Schematic representation of HSPB8 and BAG3 hypothetical regulatory mechanism on cell proliferation, migration and adhesion of hormone-sensitive BC cells.

Fig. 9 Schematic representation of HSPB8 and BAG3 hypothetical regulatory mechanism on cell proliferation, migration and adhesion of hormonesensitive BC cells

when HSPB8 and BAG3 are silenced; again, no modulation of ERK1/2 total protein levels was observed in all samples analyzed. Our data agree with results obtained on ER⁺ and TNBC cell lines [18, 49], reinforcing the notion that in ER⁺ BC cells, ERK1/2, AKT and FAK are all already active in basal conditions, but much less in the absence of HSPB8 or BAG3. This also suggests that HSPB8 and BAG3 effects on proliferation, migration, and adhesion of BC cell lines may be linked to a decrease of ERK1/2, AKT and FAK phosphorylation.

In a study by Kassis and colleagues performed on the TNBC MDA-MB-435 cell line, a direct interaction between BAG3 and FAK was excluded, and the authors suggested that BAG3 modulated FAK signalling remotely from the cytoplasm [50]. Conversely, using Co-IP, we found a co-localization between BAG3 and FAK, also confirmed by IF staining of FAK and BAG3 in breast tumor sections, while the HSPB8 protein was not pulled down neither with PI3K (p85 and p110 subunits) nor with AKT. The discrepancy between these two studies might be attributed to different BC cell models, specifically an ER⁻ cell line in the Kassis study, while ER⁺ cell lines in our investigation. These results are also partially in contrast with those obtained in HCC-derived HuH-7 cells in which HSPB8 was found to directly interacts with PI3K, but not with AKT [47], thus reinforcing the notion of a cell specific response of the CASA components in different tumour cells. To expand the clinical implications of targeting the CASA pathway in ER⁺ BC, we utilize the METABRIC cohort, demonstrating that higher BAG3 and FAK expression were significantly associated with poorer survival, supporting the clinical relevance of the HSPB8–BAG3–FAK axis in Luminal disease and provide a rationale for therapeutic targeting of this pathway in appropriately selected patients. It remains to be determine if the co-localization of BAG3 and FAK observed by IF studies in specimens from patients and also by Co-IP studies from BC cell lysates are also related to a direct interaction between these two factors or are mediated by other proteins relevant in tumorigenesis.

In conclusion, our data demonstrate that HSPB8 and BAG3 proteins interact at FAK level with the signal

transduction mechanism we analyzed and suggest that BAG3 together with HSPB8 may be involved in FAK-mediated signal transduction mechanisms (Fig. 9). This also indicates that the two CASA components may participate in the main molecular pathways involved in the survival of the ER⁺ human BC cell lines MCF-7 and T47D.

Abbreviations

AKT	Protein-kinase B
BAG3	BAG cochaperone 3
BC	Breast Cancer
CASA	Chaperon-assisted selective autophagy
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ER	Estrogen Receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HCC	Human hepatocellular carcinoma cells
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HSPB8	Small Heat Shock Protein B8
HSP70	Heat Shock Protein 70
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PI3K	Phosphoinositide 3 kinase
PR	Progesterone Receptor
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0
STUB1	STIP1 homology and U-box containing protein 1
TGF- α	Transforming Growth Factor- α
TNBC	Triple-negative BC

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12964-026-02698-2>.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

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Authors' contributions

MP, BT, RC, VC and APo: study concept, design, and writing. MP, BT, MGF, FAT, APa, TD, PS, RC, VC and APo: revision of the paper. MP, BT, MGF, FAT, APa, TD, PS, RC and VC: acquisition of data and statistical analysis. MP, BT, VF, MGF, FAT, APa, EC, MCh, PP, LC, CM, MCo, RM, AM, MB, PK, PR, MG, DT, SP, RC, VC and APo:

analysis and interpretation of data. All authors have approved the final version of the manuscript.

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Data availability

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. Katsura C, Ogunmwoyi I, Kankam HK, Saha S. Breast cancer: presentation, investigation and management. *Br J Hosp Med (Lond)*. 2022;83:1–7.
2. Kawiak A. Molecular research and treatment of breast cancer. *Int J Mol Sci*. 2022;23 (17):9617. <https://doi.org/10.3390/ijms23179617>.
3. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. *Breast Cancer Res*. 2011;13:215.
4. Rosen LS, Ashurst HL, Chap L. Targeting signal transduction pathways in metastatic breast cancer: a comprehensive review. *Oncologist*. 2010;15:216–35.
5. Sun X, Fontaine JM, Bartl I, Behnam B, Welsh MJ, Benndorf R. Induction of Hsp22 (HspB8) by Estrogen and the metalloestrogen cadmium in Estrogen receptor-positive breast cancer cells. *Cell Stress Chaperones*. 2007;12:307–19.
6. Galluzzi L, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cecconi F, Choi AM, Chu CT, Codogno P, Colombo ML, et al. Molecular definitions of autophagy and related processes. *EMBO J*. 2017;36:1811–36.
7. Cocco S, Leone A, Piezzo M, Caputo R, Di Lauro V, Di Rella F, Fusco G, Capozzi M, Gioia GD, Budillon A, De Laurentis M. Targeting autophagy in breast cancer. *Int J Mol Sci*. 2020;21(21):7836. <https://doi.org/10.3390/ijms21217836>.
8. Levine B, Kroemer G. Biological functions of autophagy genes: A disease perspective. *Cell*. 2019;176:11–42.
9. Piccolella M, Crippa V, Cristofani R, Rusmini P, Galbiati M, Cicardi ME, Meroni M, Ferri N, Morelli FF, Carra S, et al. The small heat shock protein B8 (HSPB8) modulates proliferation and migration of breast cancer cells. *Oncotarget*. 2017;8:10400–15.
10. Cristofani R, Piccolella M, Crippa V, Tedesco B, Montagnani Marelli M, Poletti A, Moretti RM. The role of HSPB8, a component of the Chaperone-Assisted selective autophagy Machinery, in cancer. *Cells*. 2021;10(2):335. <https://doi.org/10.3390/cells10020335>.
11. Kocaturk NM, Akkoc Y, Kig C, Bayraktar O, Gozuacik D, Kutlu O. Autophagy as a molecular target for cancer treatment. *Eur J Pharm Sci*. 2019;134:116–37.
12. Tedesco B, Vendredy L, Timmerman V, Poletti A. The chaperone-assisted selective autophagy complex dynamics and dysfunctions. *Autophagy*. 2023;19:1619–41.
13. Arndt V, Dick N, Tawo R, Dreiseidler M, Wenzel D, Hesse M, Furst DO, Saftig P, Saint R, Fleischmann BK, et al. Chaperone-assisted selective autophagy is essential for muscle maintenance. *Curr Biol*. 2010;20:143–8.
14. Crippa V, Sau D, Rusmini P, Boncoraglio A, Onesto E, Bolzoni E, Galbiati M, Fontana E, Marino M, Carra S, et al. The small heat shock protein B8 (HspB8) promotes autophagic removal of misfolded proteins involved in amyotrophic lateral sclerosis (ALS). *Hum Mol Genet*. 2010;19:3440–56.
15. Varlet AA, Fuchs M, Luthold C, Lambert H, Landry J, Lavioie JN. Fine-tuning of actin dynamics by the HSPB8-BAG3 chaperone complex facilitates cytokinesis and contributes to its impact on cell division. *Cell Stress Chaperones*. 2017;22:553–67.
16. Piccolella M, Cristofani R, Tedesco B, Chierichetti M, Ferrari V, Casarotto E, Cozzi M, Crippa V, Rusmini P, Galbiati M, et al. Retinoic acid downregulates HSPB8 gene expression in human breast cancer cells MCF-7. *Front Oncol*. 2021;11:652085.
17. Gonzalez-Malerva L, Park J, Zou L, Hu Y, Moradpour Z, Pearlberg J, Sawyer J, Stevens H, Harlow E, LaBaer J. High-throughput ectopic expression screen for Tamoxifen resistance identifies an atypical kinase that blocks autophagy. *Proc Natl Acad Sci U S A*. 2011;108:2058–63.
18. Shields S, Conroy E, O'Grady T, McGoldrick A, Connor K, Ward MP, Usekaita Z, Dempsey E, Reilly R, Fan Y, et al. BAG3 promotes tumour cell proliferation by regulating EGFR signal transduction pathways in triple negative breast cancer. *Oncotarget*. 2018;9:15673–90.
19. Zhang Z, Li J, Jiao S, Han G, Zhu J, Liu T. Functional and clinical characteristics of focal adhesion kinases in cancer progression. *Front Cell Dev Biol*. 2022;10:1040311.
20. Tapia Martinez P, Lopez Navajas P, Lietha D. FAK structure and regulation by membrane interactions and force in focal adhesions. *Biomolecules*. 2020;10(2):179. <https://doi.org/10.3390/biom10020179>.
21. Zhou B, Wang GZ, Wen ZS, Zhou YC, Huang YC, Chen Y, Zhou GB. Somatic mutations and splicing variants of focal adhesion kinase in Non-Small cell lung cancer. *J Natl Cancer Inst*. 2018;110(2). <https://doi.org/10.1093/jnci/djx157>.
22. Zhang Y, Sun X. Role of focal adhesion kinase in head and neck squamous cell carcinoma and its therapeutic prospect. *Onco Targets Ther*. 2020;13:10207–20.
23. Ignjatovic VB, Jankovic Miljus JR, Roncevic JV, Tatic SB, Isic Dencic TM, Doric ID, Selemetjev SA. Focal adhesion kinase splicing and protein activation in papillary thyroid carcinoma progression. *Histochem Cell Biol*. 2022;157:183–94.
24. Roy-Luzarraga M, Abdel-Fatah T, Reynolds LE, Clear A, Taylor JG, Gribben JG, Chan S, Jones L, Hodivala-Dilke K. Association of low tumor endothelial cell pY397-Focal adhesion kinase expression with survival in patients with Neoadjuvant-Treated locally advanced breast cancer. *JAMA Netw Open*. 2020;3:e2019304.
25. Li M, Hong LI, Liao M, Guo G. Expression and clinical significance of focal adhesion kinase and adrenomedullin in epithelial ovarian cancer. *Oncol Lett*. 2015;10:1003–7.
26. Goto Y, Ando T, Izumi H, Feng X, Arang N, Gilardi M, Wang Z, Ando K, Gutkind JS. Muscarinic receptors promote castration-resistant growth of prostate cancer through a FAK-YAP signaling axis. *Oncogene*. 2020;39:4014–27.

27. Murata T, Naomoto Y, Yamatsuji T, Okawa T, Shirakawa Y, Gunduz M, Nobuhisa T, Takaoka M, Sirmali M, Nakajima M, et al. Localization of FAK is related with colorectal carcinogenesis. *Int J Oncol*. 2008;32:791–6.
28. Ghandi M, Huang FW, Jane-Valbuena J, Kryukov GV, Lo CC, McDonald ER 3rd, Barretina J, Gelfand ET, Bielski CM, Li H, et al. Next-generation characterization of the cancer cell line encyclopedia. *Nature*. 2019;569:503–8.
29. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012;486:346–52.
30. Gendoo DM, Ratanasirigulchai N, Schroder MS, Pare L, Parker JS, Prat A, Haibe-Kains B. Genefu: an R/Bioconductor package for computation of gene expression-based signatures in breast cancer. *Bioinformatics*. 2016;32:1097–9.
31. Timpl R, Rohde H, Robey PG, Rennard SJ, Foidart JM, Martin GR. Laminin—a glycoprotein from basement membranes. *J Biol Chem*. 1979;254:9933–7.
32. Durbeej M. Laminins. *Cell Tissue Res*. 2010;339:259–68.
33. Williams CM, Engler AJ, Slone RD, Galante LL, Schwarzbauer JE. Fibronectin expression modulates mammary epithelial cell proliferation during acinar differentiation. *Cancer Res*. 2008;68:3185–92.
34. Pankov R, Yamada KM. Fibronectin at a glance. *J Cell Sci*. 2002;115:3861–3.
35. Rigracciolo DC, Cirillo F, Talia M, Muglia L, Gutkind JS, Maggiolini M, Lappano R. Focal adhesion kinase fine tunes multifaced signals toward breast cancer progression. *Cancers (Basel)*. 2021;13(4):645. <https://doi.org/10.3390/cancers13040645>.
36. Iwasaki M, Homma S, Hishiya A, Dolezal SJ, Reed JC, Takayama S. BAG3 regulates motility and adhesion of epithelial cancer cells. *Cancer Res*. 2007;67:10252–9.
37. Riethmacher D, Brinkmann V, Birchmeier C. A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *Proc Natl Acad Sci U S A*. 1995;92:855–9.
38. Lepucki A, Orlinska K, Mielczarek-Palacz A, Kabut J, Olczyk P, Komosinska-Vaskev K. The role of extracellular matrix proteins in breast cancer. *J Clin Med*. 2022;11(5):1250. <https://doi.org/10.3390/jcm11051250>.
39. Chen Z, Fang Z, Ma J. Regulatory mechanisms and clinical significance of vimentin in breast cancer. *Biomed Pharmacother*. 2021;133:111068.
40. Cance WG, Harris JE, Iacocca MV, Roche E, Yang X, Chang J, Simkins S, Xu L. Immunohistochemical analyses of focal adhesion kinase expression in benign and malignant human breast and colon tissues: correlation with preinvasive and invasive phenotypes. *Clin Cancer Res*. 2000;6:2417–23.
41. Watermann DO, Gabriel B, Jager M, Orlowska-Volk M, Hasenburger A, zur Hausen A, Gitsch G, Stickeler E. Specific induction of pp125 focal adhesion kinase in human breast cancer. *Br J Cancer*. 2005;93:694–8.
42. Almstedt K, Sicking I, Battista MJ, Huangfu S, Heimes AS, Weyer-Elberich V, Hasenburger A, Schmidt M. Prognostic significance of focal adhesion kinase in Node-Negative breast cancer. *Breast Care (Basel)*. 2017;12:329–33.
43. Lightfoot HM Jr, Lark A, Livasy CA, Moore DT, Cowan D, Dressler L, Craven RJ, Cance WG. Upregulation of focal adhesion kinase (FAK) expression in ductal carcinoma in situ (DCIS) is an early event in breast tumorigenesis. *Breast Cancer Res Treat*. 2004;88:109–16.
44. Devaud C, Tilkin-Mariame AF, Vignolle-Vidoni A, Souleres P, Denadai-Souza A, Rolland C, Duthoit C, Blanpied C, Chabot S, Bouille P, et al. FAK alternative splice mRNA variants expression pattern in colorectal cancer. *Int J Cancer*. 2019;145:494–502.
45. Fan Z, Duan J, Wang L, Xiao S, Li L, Yan X, Yao W, Wu L, Zhang S, Zhang Y, et al. PTK2 promotes cancer stem cell traits in hepatocellular carcinoma by activating Wnt/beta-catenin signaling. *Cancer Lett*. 2019;450:132–43.
46. Mishra R, Patel H, Alanazi S, Kilroy MK, Garrett JT. PI3K inhibitors in cancer: clinical implications and adverse effects. *Int J Mol Sci*. 2021;22(7):3464. <https://doi.org/10.3390/ijms22073464>.
47. Matsushima-Nishiwaki R, Toyoda H, Takamatsu R, Yasuda E, Okuda S, Maeda A, Kaneoka Y, Yoshimi N, Kumada T, Kozawa O. Heat shock protein 22 (HSPB8) reduces the migration of hepatocellular carcinoma cells through the suppression of the phosphoinositide 3-kinase (PI3K)/AKT pathway. *Biochim Biophys Acta Mol Basis Dis*. 2017;1863:1629–39.
48. Rocca A, Braga L, Volpe MC, Maiocchi S, Generali D. The predictive and prognostic role of RAS-RAF-MEK-ERK pathway alterations in breast cancer: revision of the literature and comparison with the analysis of cancer genomic datasets. *Cancers (Basel)*. 2022;14(21):5306. <https://doi.org/10.3390/cancers14215306>.
49. Pasillas MP, Shields S, Reilly R, Strnadel J, Behl C, Park R, Yates JR 3rd, Klemke R, Gonias SL, Copping JA. Proteomic analysis reveals a role for Bcl2-associated athanogene 3 and major vault protein in resistance to apoptosis in senescent cells by regulating ERK1/2 activation. *Mol Cell Proteom*. 2015;14:1–14.
50. Kassir JN, Guancial EA, Doong H, Virador V, Kohn EC. CAIR-1/BAG-3 modulates cell adhesion and migration by downregulating activity of focal adhesion proteins. *Exp Cell Res*. 2006;312:2962–71.

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