RHAMM inhibits cell migration via the AKT/GSK3β/Snail axis in luminal A subtype breast cancer

Juan Wang[1], Dan Li[1], Wenzhi Shen[2], Wei Sun[3], Ruifang Gao[1], Pengling Jiang[4,5] , Liang Wang[6], Yanhua Liu[1], Yanan Chen[1], Wei Zhou[1], Rongrong Wang[7], Rong Xiang[1*], Dwayne Stupack[8*], Na Luo[1*]

1. Department of Anatomy and Histology, School of Medicine, Nankai University, Tianjin 300071, China
2. Department of Pathology and Institute of Precision Medicine, Jining Medical University, Jining 272067, China
3. Department of Molecular Genetics, School of Medicine, Nankai University, Tianjin 300071, China
4. Third Department of Breast Cancer, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin 300060, China
5. Qingdao Municipal Hospital, Qingdao 266011, China
6. Tianjin Institute of Urology, Tianjin Medical University General Hospital, Tianjin, 300052, China
7. Department of Obstetrics and Gynecology, Tianjin Medical University General Hospital, Tianjin, 300052, China
8. Department of Reproductive Medicine, School of Medicine and Moores Cancer Center, University of California, San Diego, La Jolla, California 39216, USA

Juan Wang, 393698719@qq.com
Dan Li, 920046101@qq.com
Wenzhi Shen, shenwenzhi2011@126.com
Wei Sun, sunweibio@nankai.edu.cn
Ruifang Gao, 945350619@qq.com
Pengling Jiang, jpl10177777@163.com
Liang Wang, aliangws@163.com
Yanhua Liu, liuyh23@nankai.edu.cn

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ar.24321

Yanan Chen, chenyanan851013@163.com
Wei Zhou, zealot_c@126.com
Rongrong Wang, jr_wang1213@126.com
Rong Xiang, rxiang@nankai.edu.cn
Dwayne Stupack, dstupack@ucsd.edu
Na Luo, luon11@nankai.edu.cn

Running title: RAHMM inhibits cell migration in luminal A subtype breast cancer

Correspondence to: Rong Xiang, School of Medicine, Nankai University, 94 Weijin Road, Tianjin, China. Email: rxiang@nankai.edu.cn; Dwayne Stupack, Department of Reproductive Medicine, School of Medicine and Moores Cancer Center, University of California, San Diego, La Jolla, California 39216, USA. E-mail: dstupack@ucsd.edu; Na Luo, School of Medicine, Nankai University, 94 Weijin Road, Tianjin, China. Email: luon11@nankai.edu.cn.

Grant sponsor: National Natural Science Foundation of China; Grant number: 81872325

ABSTRACT

Breast cancer is one of the most common types of cancer in women. Although the mortality rate of breast cancer has fallen over the past 10 years, effective treatments that reduce the occurrence of breast cancer metastasis remain lacking. In this study, we explored the role of RHAMM and the associated signaling pathway in cell migration in luminal A breast cancer. We first examined RHAMM expression levels using human breast tissue microarray and patient breast tissues. We then studied the role of RHAMM in migration in luminal A breast cancer using loss-of-function and gain-of-function strategies in *in vitro* models and confirmed these findings in an *in vivo* model. Finally, we investigated signaling molecules that play a role in cell migration using Western blot. Our results demonstrated the following: 1) RHAMM shows high expression levels in malignant breast tissue, 2) RHAMM shows low expression levels in luminal A breast cancer compared to other subtypes of breast cancer, 3) RHAMM inhibits cell migration in luminal A breast cancer and, 4) RHAMM inhibits cell migration via the AKT/GSK3β/Snail axis in luminal A breast cancer. This study demonstrates a novel role of RHAMM in cell migration in luminal A breast cancer and suggests that therapeutic strategies involving RHAMM should be considered for various subtypes of breast cancer.

Key Words: RHAMM, AKT/GSK3β/Snail, luminal A subtype

INTRODUCTION

Receptor for hyaluronan (HA) mediated motility [RHAMM; also known as hyaluronan-mediated motility receptor (HMMR)] is a hyaluronan receptor that lacks a classical membrane spanning domain and plays a role in cell motility. RHAMM is also a soluble protein and is highly expressed on the cell surface (where it is designated as CD168), in the cytoplasm, and associated with the cytoskeleton (Hardwick et al., 1992). RHAMM forms an intracellular complex with BRCA1 and BRCA2 and may therefore contribute to a higher risk of breast cancer (Joukov et al., 2006; Pujana et al., 2007). RHAMM also forms an extracellular complex with HA to affect the activity of extracellular-regulated kinase (ERK) and may, therefore, influence cell transformation and cell migration (Tolg et al., 2010). Previous studies have indicated that RHAMM facilitates cell migration in many breast cancer cell lines and that RHAMM is over-expressed at the invasive front of breast cancer and in tumor cell subsets (Assmann et al., 2001; Wang et al., 2009).

Breast cancer is one of the most common types of cancer in women. Although the mortality rate of breast cancer has fallen over the past 10 years, effective treatments that prevent metastasis of breast cancer remain lacking. In order to assist physicians to determine regimens and to develop new therapies, breast cancers have been categorized into 5 major molecular subtypes which

include: 1) luminal A, 2) luminal B, 3) HER2-enriched, 4) triple negative/basal-like and, 5) and normal breast-like (Sorlie et al., 2001). Luminal A breast cancer exhibits a tendency towards estrogen receptor-positivity/HER2 receptor-negativity, the best prognosis, high survival rates, and low recurrence rates (Inic et al., 2014).

Glycogen synthase kinase-3beta (GSK3β) is a serine-threonine kinase belonging to the glycogen synthase kinase family. GSK3β plays a role in energy metabolism, inflammation, ER-stress, mitochondrial dysfunction, and the apoptotic pathway (Hardt and Sadoshima, 2002; Beurel et al., 2015). Ser9 phosphorylation of GSK3β induces a pseudo-substrate conformation in the substrate docking motifs of GSK3β and thereby silences GSK3β. This results in GSK3β inactivation by proteasomal degradation which has been associated with many pathological conditions, including cancer. However, the role of GSK3β in cancer is not only complex but also controversial in that GSK3β may function either as a tumor promoter or tumor suppressor (Wang et al., 2004; Ougolkov et al., 2005; Shakoori et al., 2005; Shakoori et al., 2007). Importantly, regulatory interactions exist between GSK3β and the Ras/PI3K/PTEN/AKT/mTOR signaling pathway and the Ras/Raf/MEK/ERK signaling pathway (Hardt and Sadoshima, 2002; Yan et al., 2012). Both of these signaling pathways play pivotal roles in cell proliferation, cell survival, and often become dys-regulated in cancer.

Snail is a zinc-finger transcription factor that induces epithelial-mesenchymal transition (EMT) which accompanies increased cell migration and metastasis in cancer cells (Wang et al., 2013). Snail may undergo phosphorylation by GSK3β which leads to Snail nuclear exportation and cytoplasmic translocation. In addition, Snail stabilization due to increased phosphorylation by GSK3β occurs during EMT in various solid cancer types (Zhou et al., 2004).

In this study we demonstrated the following: 1) RHAMM shows high expression levels in malignant breast tissue, 2) RHAMM shows low expression levels in luminal A subtype breast cancer compared to other subtypes of breast cancer, 3) RHAMM inhibits cell migration in luminal A subtype breast cancer and, 4) RHAMM inhibits cell migration via the AKT/GSK3β/Snail axis in luminal A subtype breast cancer. This study demonstrates a novel role of RHAMM on cell migration in luminal A subtype breast cancer and suggests that therapeutic strategies involving RHAMM should consider the various subtypes of breast cancer.

MATERIALS AND METHODS

Cell lines and treatment. Human breast cancer MDA-MB-231 cells were obtained from ATCC and cultured in Leibovitz's L-15 medium (Hyclone, Logan, UT) with 10% fetal bovine serum (FBS) (Biological Industries, Israel), 100 unit/ml penicillin and streptomycin (Hyclone, Logan, Utah), 100 unit/ml Non-Essential Amino Acid (NEAA) (Hyclone, Logan, Utah) at 37°C in a humidified atmosphere with 5% CO_2. Human breast cancer T47D, MCF7 and SUM159 cells were gifts from the laboratory of Dr. Peiqing Sun (Wake Forest University) and cultured in high glucose Dulbecco's Modified Eagle's Medium (Hyclone, Logan, Utah) with 10% FBS, 100 unit/ml penicillin and streptomycin, 100 unit/ml NEAA at 37°C in a humidified atmosphere with 5% CO_2. When necessary, 20μg/ml cycloheximide (Sigma-Aldrich, St. Louis, MO), 3μM IM-12 (Selleckchem, Houston, TX), 10 μM of LY294002 (CST, Danvers, MA) or 10 μM of PD98059 (Sigma-Aldrich, St. Louis, MO) was added to the medium in order to inhibit protein synthesis, GSK3β, AKT or ERK activities, respectively.

Tumor specimens and tissue microarray. Matched pairs of human breast cancer samples were obtained from Tianjin Medical University Cancer Institute & Hospital. This study was approved by the institutional ethics committees of Tianjin Medical University Cancer Institute & Hospital. Human breast cancer tissue microarray BR8011 (Alenabio Company, Shanxi, China) includes 80 samples (54 human breast cancer samples and 26 cancer-adjacent tissue samples) and BR1504A

(Alenabio Company, Shanxi, China) include 75 samples (70 human breast cancer samples and 5 normal/ cancer-adjacent tissue samples).

Immunohistochemistry. The human breast tissue microarray was incubated with anti-RHAMM antibody (Sigma, St. Louis, MO) at 4°C, overnight. The tissue sections were then incubated with a secondary antibody followed by peroxidase-anti-peroxidase (PAP) complex. The tissue sections were then incubated in a diaminobenzidene (DAB)/hydrogen peroxide substrate solution to obtain a brown reaction product. The tissue sections were counterstained with hematoxylin. RHAMM expression levels in human breast tissue microarray were scored according to the percentage of RHAMM-positive cells and the staining intensity of each breast tissue sample. Specifically, 0%–25%, 26%–50%, 51%–75%, and 76%–100% were scored as 0, 1, 2, and 3, respectively. Tissues with brown, light brown, intermediate brown, and dark brown staining intensities were scored as 1, 2, 3, and 4, respectively. The two scores were multiplied, and a score of 0–2 was considered negative, a score of 3–4 was considered weak, a score of 6–8 was considered intermediate, and a score of 9–12 was considered strong. Two independent observers performed the immunohistochemical evaluation. Photomicrographs were taken with an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan).

Western blot. Twenty microgram of protein isolated from T47D, MCF7, MDA-MB-231 and SUM159 cells was loaded to 10% sodium dodecyl sulphate-polyacrylamide gel for electrophoresis. The protein was then transferred to a polyvinylidene fluoride membrane. After blocking with 5% nonfat milk, the membrane was incubated with a primary antibody to either of the following: RHAMM (EMD Millipore, Burlington, MA), E-Cadherin (CST, Danvers, MA), Vimentin (CST, Danvers, MA), Snail (CST, Danvers, MA), Zeb1 (CST, Danvers, MA), Twist (CST, Danvers, MA), P-GSK3β (CST, Danvers, MA), GSK3β (CST, Danvers, MA), P-AKT (Santa Cruz, Dallas, TX), AKT (Santa Cruz, Dallas, TX), P-ERK (Santa Cruz, Dallas, TX), ERK (Santa Cruz, Dallas, TX), and β-actin (Santa Cruz, Dallas, TX) overnight at 48C. The membrane was exposed to chemiluminescence after incubation with corresponding secondary antibodies to detect specific signals.

TCGA data analysis. The TCGA breast invasive carcinoma RNA seq dataset (Level 3 data) was downloaded from the UCSC Cancer Genomics Browser (https://genome-cancer.ucsc.edu/). The mRNA expression data (log2FPKM value) and clinical information were matched by sample ID for each sample. Statistical analysis of RHAMM expression was determined by Tukey HSD test using R software and images were drawn by R_ggplot2.

Vector construction and establishment of stable cell lines. For gene overexpression, DNA sequences encoding human RHAMM were PCR-amplified from MCF10A cDNA and cloned into the pLV-EF1α-MCS-IRES-Bsd plasmid (Biosettia, San Diego, CA, USA). For gene silencing, short hairpin RNAs (shRNAs) targeting human RHAMM were cloned into the pLV-H1-EF1α-puro plasmid (Biosettia, San Diego, CA, USA). The lentiviruses carrying the overexpression vectors, gene silencing vectors or empty vectors were produced according to the manufacturer's instruction. Lentivirus-containing medium was applied to cells in the presence of 8 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO) for 48 h, prior to selection with 10 μg/mL blasticidin (Sigma-Aldrich, St. Louis, MO) or 1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO) for a week to establish stable cell lines. The primers and shRNAs are listed in Table 1, and the RHAMM cDNA (NM_012485 & NM_001142556) sequence is in Supplementary Material.

Wound healing and transwell invasion assays. *Wound healing assay,* the wound was generated using a pipette tip to scratch across the confluent cell monolayer. The healing process was allowed to occur under the condition of high glucose DMEM with 2% FBS and monitored microscopically after 8hr (MDA-MB-231), 24hr (SUM159 and T47D) or 48 hr (MCF7). The migration ability was defined as an average closed area of the wound relative to the initial wound area. *Transwell assay,* one hundred thousand cells were seeded to the upper chamber of 24-well transwell chambers (8μm

pore size) filling with high glucose DMEM and 2% FBS. The lower chamber was filled with high glucose DMEM and 10% FBS. After 8hr (MDA-MB-231), 10hr (SUM159), 12hr (T47D) or 48 hr (MCF7), the chambers were fixed in 4% paraformaldehyde and stained with crystal violet. Bright-field photomicrographs were taken using an Olympus BX51 microscope and the number of migrated cells was counted.

In vivo mouse models. Ten female NOD/SCID mice of 6–8 weeks old (Beijing Vital River Laboratory Animal Technology, Beijing, China) were housed under sterile conditions, kept on a 12 hr/12 hr light/dark cycle, and fed with sterile chow and water. The mice were randomly separated into two groups (n=5/group). Two million RHAMM-competent (sc) and RHAMM-deficient (shRHAMM) T47D cells were injected subcutaneously into the second pair of the fat pad and allowed to grow for 8 weeks. On day 40, the mice were euthanized following Nankai University IACUC-approved procedures. Tumor volume was measured twice a week and calculated using the standard formula: length × width^2/2. The tumors and lungs were harvested and fixed in 4% paraformaldehyde. Hematoxylin and eosin (H&E) staining were performed on the harvested tissues. Metastasis was evaluated by counting the metastatic foci in three randomly selected fields. Animal use complied with Nankai University Animal Welfare Guidelines.

Statistics. All data were analyzed using GraphPad Prism5 software (GraphPad Software, San Diego, CA). Results are presented as the mean ± SD with the exception of human samples and animal model data, which are presented as mean ± SEM. Statistical significance was determined by the Student's *t*-test or one-way ANOVA.

RESULTS

RHAMM shows high expression levels in malignant breast cancer tissue and low expression levels in luminal A subtype breast cancer compared to other subtypes of breast cancer

We first examined RHAMM levels in a human breast tissue microarray comprised of 124 malignant breast cancer tissues and 31 normal/cancer adjacent tissues using immunocytochemistry. Our results showed that RHAMM immunocytochemical staining increases in malignant breast cancer tissue versus normal/cancer adjacent tissue (Figs 1A, 1B). We next examined RHAMM expression in 6 pairs of breast cancer tissue and matched normal/cancer adjacent tissue obtained from breast cancer patients using Western blot. Our results indicated that RHAMM shows high

expression levels in breast cancer tissue versus normal/cancer adjacent tissue (Fig 1C). Furthermore, we analyzed RHAMM expression in different breast cancer subtypes using the TCGA dataset. Our results indicated that RHAMM shows low expression levels in the luminal A subtype versus the basal, her2, and luminal B subtypes (Fig 1D).

RHAMM inhibits cell migration in luminal A subtype breast cancer

To investigate the role of RHAMM in cell migration, we knocked down RHAMM expression in luminal A human breast cancer T47D and MCF-7 cell lines and measured cell migration using wound healing and transwell cell migration assays. Interestingly, our results demonstrated that RHAMM knockdown increases cell migration in luminal A subtype breast cancer which contradicts the established role of RHAMM in regards to cell migration i.e., RHAMM facilitates cell migration (Fig 2A-F). In addition, we found that RHAMM knockdown decreases E-cadherin (epithelial marker) expression in luminal A human breast cancer T47D and MCF-7 cell lines using Western blot (Fig 2M).

In order to corroborate the above-mentioned unique finding, we knocked down RHAMM expression in basal-like human breast cancer MDA-MB-231 and SUM159 cell lines and measured

cell migration using wound healing and transwell cell migration assays. Our results demonstrated that RHAMM knockdown decreases cell migration in basal-like subtype breast cancer which comports with the established role of RHAMM in regards to cell migration i.e., RHAMM facilitates cell migration (Fig 2G-L). In addition, we found that RHAMM knockdown decreases vimentin (mesenchymal marker) expression in basal-like human breast cancer MDA-MB-231 and SUM159 cell lines using Western blot (Fig 2N).

To confirm the above-mentioned results, we ectopically expressed shRNA-resistant RHAMM 1) in the luminal A human breast cancer T47D cell line and measured cell migration using wound healing and transwell cell migration assays. Our results showed that RHAMM ectopic expression decreases cell migration in the luminal A subtype human breast cancer T47D cell line (Fig 3A&C). In addition, we found that RHAMM ectopic expression increases E-cadherin (epithelial marker) expression in the luminal A human breast cancer T47D cell line using Western blot (Fig 3E).

In order to confirm the above-mentioned results, we ectopically expressed shRNA-resistant RHAMM in the basal-like human breast cancer MDA-MB-231 cell line and measured cell migration using wound healing and transwell cell migration assays. Our results showed that RHAMM ectopic expression increases cell migration in the basal-like subtype human breast

cancer MDA-MB-231 cell line (Fig 3B&D). In addition, we found that RHAMM ectopic expression increases vimentin (mesenchymal marker) expression in basal-like human breast cancer MDA-MB-231 cell line using Western blot (Fig 3F).

In order to confirm the above-mentioned *in vitro* results, we established a T47D *in vivo* orthotopic breast cancer model. We implanted RHAMM-deficient T47D cells or control cells into the 4th pair fat pad of NOD/SCID mice and measured tumor volume and metastasis. Our results indicated that tumor volume remains unchanged in the RHAMM-deficient T47D cells versus controls (Fig 4A&B). However, metastasis to the lung increases in the RHAMM-deficient T47D cells versus controls (Fig 4C).

Snail contributes to RHAMM deficiency-induced migration in luminal A subtype breast cancer

In order to investigate the mechanism of RHAMM deficiency-induced migration in luminal A subtype breast cancer, we examined EMT-promoting transcription factor (i.e., Snail, Twist, Zeb1) expression in luminal A human breast cancer T47D cells and basal-like human breast cancer

MDA-MB-231 cells using Western blot. Our results showed that RHAMM knockdown increases Snail and Zeb1 expression in luminal A human breast cancer T47D cells. However, RHAMM knockdown elicits no change in Snail, Twist, and Zeb1 expression in basal-like human breast cancer MDA-MB-231 cells (Fig 5A). We also treated luminal A human breast cancer T47D cells with cyclohexamide (a protein synthesis inhibitor) and measured Snail degradation using Western blot. Our results indicated that Snail degradation decreases in RHAMM-deficient luminal A human breast cancer T47D cells in the presence of cyclohexamide, thereby increasing the half-life of Snail in the RHAMM-deficient T47D cells versus controls (Fig 5B).

AKT-mediated GSK3β phosphorylation is the upstream regulator of Snail in luminal A subtype breast cancer

In order to examine the role of GSK3β-mediated phosphorylation in the regulation of Snail, we measured GSK3β phosphorylation in the luminal A human breast cancer T47D and MCF-7 cell lines and in the basal-like human breast cancer MDA-MB-231 and SUM159 cell lines using Western blot. Our results showed that RHAMM knockdown increases GSK3β phosphorylation which would thereby decrease Snail degradation and lead to Snail accumulation in luminal A human breast cancer T47D and MCF-7 cell lines (Fig 5C). To confirm this finding, we treated

luminal A human breast cancer T47D cells with IM-12 (a GSK3β inhibitor) and measured Snail expression using Western blot. We found that GSK3β inhibition decreases Snail expression (Fig 5D). However, our results also showed that RHAMM knockdown does not alter GSK3β phosphorylation which would thereby lead to no change in Snail degradation or Snail accumulation in the basal-like human breast cancer MDA-MB-231 and SUM159 cell lines (Fig 5C).

In order to explore the upstream regulator of GSK3β phosphorylation, we treated luminal A human breast cancer T47D cells with either LY294002 (a AKT inhibitor) or PD98059 (a ERK inhibitor) and measured GSK3β phosphorylation using Western blot. Our results indicated that AKT inhibition attenuates GSK3β phosphorylation in luminal A human breast cancer T47D cells (Fig 5E). However, ERK inhibition does not alter GSK3β phosphorylation in luminal A human breast cancer T47D cells (Fig 5F). These findings suggest that AKT is the upstream regulator of GSK3β phosphorylation in luminal A subtype breast cancer cells.

DISCUSSION

In 2000, Perou et al first proposed a classification of intrinsic subtypes of breast cancer based on variation in gene expression pattern (Perou et al., 2000). In 2001, these subtypes were further classified into various groups which include the following; 1) estrogen receptor-positive (luminal A and luminal B), 2) HER2-overexpressing, 3) basal-like, and 4) normal breast-like (Sorlie et al., 2001). Among these breast cancer subtypes, the luminal A subtype is relatively common. Furthermore, the luminal A subtype is characterized by high expression levels of the estrogen receptor and the progesterone receptor, a low expression level of Ki67 (a proliferation marker), a negative expression level of HER2, slow growth, and a good prognosis (Inic et al., 2014). In this study, we found that generally RHAMM shows high expression levels in malignant breast cancer which comports with previous reports. In contrast, however, we also found that RHAMM shows low expression levels specifically in luminal A breast cancer compared to other breast cancer subtypes. Along these lines, a large tumor size in luminal A subtype breast cancer correlates with an escalated metastasis to axillary lymph nodes (5). Consequently, our novel finding that RHAMM (a motility-related protein) shows low expression levels in luminal A subtype breast cancer appears reasonable.

The consensus concerning RHAMM states that RHAMM facilitates cell migration and invasion when studied in various breast cancer cell lines (Schwertfeger et al., 2015). In addition, RHAMM shows high expression levels at the invasive front of breast cancer and in tumor cell subsets. More importantly, high expression levels of RHAMM correlate with lower, metastasis-free survival rates in breast cancer patients(Wang et al., 1998). However, our novel finding showed that RHAMM inhibits (i.e., does not facilitate) cell migration in luminal A subtype breast cancer. This novel finding contradicts the consensus concerning RHAMM in regards to cell migration. Upon further investigation, we revealed that Snail (a zinc-finger transcription factor) plays a major role in this process. In this regard, RHAMM deficiency causes a decrease in Snail degradation which increases the half-life of Snail. The increased half-life of Snail results in a high accumulation of Snail associated with RHAMM deficiency which promotes not only an epithelial-mesenchymal transition but also cell migration in luminal A subtype breast cancer.

Studies concerning the connectivity between RHAMM and Snail are currently ongoing in many laboratories. In this regard, HA induces an interaction between RHAMM and TGFβ receptor I in human umbilical vein endothelial cells (HUVECs). HA also induces Snail as a downstream target of TGFβ signaling. RHAMM, TGFβ receptor I, and Snail all play a necessary role in the induction of plasminogen activator-inhibitor-1 (PAI-1) which is important for HA-induced angiogenesis

(Park et al., 2012). In addition, RHAMM expression levels are concordant with Snail expression levels in HT1376 breast cancer cell line and 253J lung metastasis cells (Morera et al., 2017).

RHAMM knockdown increases GSK3β phosphorylation which then inactivates GSK3β in the cancer stem cell population of head and neck cancers ($CD44^{high}/ESA^{high}$) (Shigeishi et al., 2013). In our study, we also found that RHAMM knockdown increases GSK3β phosphorylation which then decreases Snail degradation resulting in a high accumulation of Snail. GSK3β binds and phosphorylates Snail at 2 consensus motifs thereby dually regulating Snail function. The phosphorylation of motif 1 regulates Snail ubiquitination and causes Snail to undergo degradation. The phosphorylation of motif 2 causes Snail to localize in the cytoplasm (Zhou et al., 2004). GSK3β controls both the stability and transcription of Snail whereby GSK3β inhibition results in the up-regulation of Snail (Zhou et al., 2004; Yook et al., 2007; Zheng et al., 2013; Liu et al., 2014).

The regulation of GSK3β activity occurs via many signaling pathways. In our study, we chose two crucial signaling pathways, that is, protein kinase B (PKB/AKT) signaling pathway and the extracellular signal-regulated kinase (ERK) signaling pathway. In our study, we demonstrated that AKT (but not ERK) is the upstream regulator of GSK3β that controls Snail degradation/ transcription and mediates cell migration in luminal A subtype breast cancer. However, AKT and

ERK phosphorylate GSK3β at different sites to inactivate GSK3β. In this regard, AKT phosphorylates GSK3β at Ser9 whereas ERK phosphorylates GSK3β at Thr43. ERK phosphorylation of GSK3β at Thr43 primes GSK3β for its subsequent phosphorylation at Ser9 which results in GSK3β inactivation (Ding et al., 2005). This study provides evidence for a novel role of RHAMM in regards to cell migration in luminal A subtype breast cancer. Furthermore, this study implies that therapeutic cancer strategies involving RHAMM should consider the various subtypes of breast cancer.

REFERENCES

Assmann V, Gillett CE, Poulsom R, Ryder K, Hart IR, Hanby AM. 2001. The pattern of expression of the microtubule-binding protein RHAMM/IHABP in mammary carcinoma suggests a role in the invasive behaviour of tumour cells. The Journal of pathology 195:191-196.

Beurel E, Grieco SF, Jope RS. 2015. Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. Pharmacology & therapeutics 148:114-131.

Ding Q, Xia W, Liu J-C, Yang J-Y, Lee D-F, Xia J, Bartholomeusz G, Li Y, Pan Y, Li Z, Bargou RC, Qin J, Lai C-C, Tsai F-J, Tsai C-H, Hung M-C. 2005. Erk Associates with and Primes GSK-3β for Its Inactivation Resulting in Upregulation of β-Catenin. Molecular Cell 19:159-170.

Hardt SE, Sadoshima J. 2002. Glycogen synthase kinase-3beta: a novel regulator of cardiac hypertrophy and development. Circulation research 90:1055-1063.

Hardwick C, Hoare K, Owens R, Hohn HP, Hook M, Moore D, Cripps V, Austen L, Nance DM, Turley EA. 1992. Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. J Cell Biol 117:1343-1350.

Inic Z, Zegarac M, Inic M, Markovic I, Kozomara Z, Djurisic I, Inic I, Pupic G, Jancic S. 2014. Difference between Luminal A and Luminal B Subtypes According to Ki-67, Tumor Size, and Progesterone Receptor Negativity Providing Prognostic Information. Clin Med

Insights Oncol 8:107-111.

Joukov V, Groen AC, Prokhorova T, Gerson R, White E, Rodriguez A, Walter JC, Livingston DM. 2006. The BRCA1/BARD1 heterodimer modulates ran-dependent mitotic spindle assembly. Cell 127:539-552.

Liu Z-c, Chen X-h, Song H-x, Wang H-s, Zhang G, Wang H, Chen D-y, Fang R, Liu H, Cai S-h, Du J. 2014. Snail regulated by PKC/GSK-3β pathway is crucial for EGF-induced epithelial-mesenchymal transition (EMT) of cancer cells. Cell and Tissue Research 358:491-502.

Morera DS, Hennig MS, Talukder A, Lokeshwar SD, Wang J, Garcia-Roig M, Ortiz N, Yates TJ, Lopez LE, Kallifatidis G, Kramer MW, Jordan AR, Merseburger AS, Manoharan M, Soloway MS, Terris MK, Lokeshwar VB. 2017. Hyaluronic acid family in bladder cancer: potential prognostic biomarkers and therapeutic targets. British journal of cancer 117:1507-1517.

Ougolkov AV, Fernandez-Zapico ME, Savoy DN, Urrutia RA, Billadeau DD. 2005. Glycogen Synthase Kinase-3β Participates in Nuclear Factor κB–Mediated Gene Transcription and Cell Survival in Pancreatic Cancer Cells. Cancer Research 65:2076.

Park D, Kim Y, Kim H, Kim K, Lee YS, Choe J, Hahn JH, Lee H, Jeon J, Choi C, Kim YM, Jeoung D. 2012. Hyaluronic acid promotes angiogenesis by inducing RHAMM-TGFbeta receptor interaction via CD44-PKCdelta. Molecules and cells 33:563-574.

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D. 2000. Molecular portraits of human breast tumours. Nature 406:747-752.

Pujana MA, Han JD, Starita LM, Stevens KN, Tewari M, Ahn JS, Rennert G, Moreno V, Kirchhoff T, Gold B, Assmann V, Elshamy WM, Rual JF, Levine D, Rozek LS, Gelman RS, Gunsalus KC, Greenberg RA, Sobhian B, Bertin N, Venkatesan K, Ayivi-Guedehoussou N, Sole X, Hernandez P, Lazaro C, Nathanson KL, Weber BL, Cusick ME, Hill DE, Offit K, Livingston DM, Gruber SB, Parvin JD, Vidal M. 2007. Network modeling links breast cancer susceptibility and centrosome dysfunction. Nature genetics 39:1338-1349.

Schwertfeger KL, Cowman MK, Telmer PG, Turley EA, McCarthy JB. 2015. Hyaluronan, Inflammation, and Breast Cancer Progression. Frontiers in Immunology 6:236.

Shakoori A, Mai W, Miyashita K, Yasumoto K, Takahashi Y, Ooi A, Kawakami K, Minamoto T. 2007. Inhibition of GSK-3β activity attenuates proliferation of human colon cancer cells in rodents. Cancer Science 98:1388-1393.

Shakoori A, Ougolkov A, Yu ZW, Zhang B, Modarressi MH, Billadeau DD, Mai M, Takahashi Y, Minamoto T. 2005. Deregulated GSK3β activity in colorectal cancer: Its association with

tumor cell survival and proliferation. Biochemical and biophysical research communications 334:1365-1373.

Shigeishi H, Biddle A, Gammon L, Emich H, Rodini CO, Gemenetzidis E, Fazil B, Sugiyama M, Kamata N, Mackenzie IC. 2013. Maintenance of stem cell self-renewal in head and neck cancers requires actions of GSK3beta influenced by CD44 and RHAMM. Stem cells 31:2073-2083.

Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lonning PE, Borresen-Dale AL. 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proceedings of the National Academy of Sciences of the United States of America 98:10869-10874.

Tolg C, Hamilton SR, Morningstar L, Zhang J, Zhang S, Esguerra KV, Telmer PG, Luyt LG, Harrison R, McCarthy JB, Turley EA. 2010. RHAMM promotes interphase microtubule instability and mitotic spindle integrity through MEK1/ERK1/2 activity. The Journal of biological chemistry 285:26461-26474.

Wang C, Thor AD, Moore DH, Zhao Y, Kerschmann R, Stern R, Watson PH, Turley EA. 1998. The overexpression of RHAMM, a hyaluronan-binding protein that regulates ras signaling, correlates with overexpression of mitogen-activated protein kinase and is a significant parameter in breast cancer progression. Clinical Cancer Research 4:567.

Wang L, Lin H-K, Hu Y-C, Xie S, Yang L, Chang C. 2004. Suppression of Androgen Receptor-mediated Transactivation and Cell Growth by the Glycogen Synthase Kinase 3β in Prostate Cells. Journal of Biological Chemistry 279:32444-32452.

Wang XY, Tan JX, Vasse M, Delpech B, Ren GS. 2009. Comparison of hyaluronidase expression, invasiveness and tubule formation promotion in ER (-) and ER (+) breast cancer cell lines in vitro. Chinese medical journal 122:1300-1304.

Wang Y, Shi J, Chai K, Ying X, Zhou BP. 2013. The Role of Snail in EMT and Tumorigenesis. Current cancer drug targets 13:963-972.

Yan D, Avtanski D, Saxena NK, Sharma D. 2012. Leptin-induced epithelial-mesenchymal transition in breast cancer cells requires beta-catenin activation via Akt/GSK3- and MTA1/Wnt1 protein-dependent pathways. The Journal of biological chemistry 287:8598-8612.

Yook J, Li X-Y, Ota I, Hu C, Kim HS, Hee Kim N, Young Cha S, Kyung Ryu J, Choi Y, Kim J, R Fearon E, J Weiss S. 2007. A Wnt–Axin2–GSK3 cascade regulates Snail1 activity in breast cancer cells.

Zheng H, Li W, Wang Y, Liu Z, Cai Y, Xie T, Shi M, Wang Z, Jiang B. 2013. Glycogen synthase kinase-3 beta regulates Snail and β-catenin expression during Fas-induced epithelial–

mesenchymal transition in gastrointestinal cancer. European Journal of Cancer 49:2734-2746.

Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC. 2004. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nature cell biology 6:931-940.

FIGURE LEGENDS

Figure1. RHAMM shows high expression levels in malignant breast cancer tissue and low expression levels in luminal A subtype breast cancer.

(A) The representative micrographs of RHAMM immunocytochemical staining in human breast tissue microarrays (including 124 human breast cancer tissue samples and 31 normal/cancer adjacent tissue samples). The lower panel micrograph shows increased RHAMM immunocytochemical staining in human breast cancer samples. The upper panel micrograph shows decreased RHAMM immunocytochemical staining in normal/cancer adjacent tissue samples. Upper panel= normal/cancer adjacent tissue (CAT); lower panel= breast cancer tissue. Scale bar= 50μm. **(B) Quantification of RHAMM immunocytochemical staining in human breast tissue microarrays (from 1A).** This bar graph shows that RHAMM immunocytochemical staining significantly increases in human breast cancer tissue samples versus normal/cancer adjacent tissue samples (6.1 ± 0.3 vs 2.23 ± 0.3, respectively;***$p<0.001$). Data are shown as mean ± SEM. Student's t-test was used for statistical analysis**. (C) Western blot analysis of RHAMM expression in 6 pairs of cancer tissue and matched CAT of breast cancer patients**. This Western blot qualitatively indicates that RHAMM shows high expression levels in breast cancer

samples versus CAT samples. B-actin serves as a loading control. **D) ggplot2 graph of TCGA breast invasive carcinoma RNA seq dataset.** This graph illustrates that RHAMM shows low expression levels in the luminal A subtype (blue) versus the basal (orange), her2 (green), and luminal B (purple) subtypes. Y-axis = RHAMM expression level; X= subtypes of breast cancer.

Figure 2. RHAMM inhibits cell migration in luminal A subtype breast cancer.

(A) Wound healing assay in luminal A human breast cancer T47D cells. The representative micrographs of 3 independent wound healing assays show that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) increases cell migration after 24 hours in luminal A breast cancer T47D cells versus the sc control group.

(B) Wound healing assay in luminal A human breast cancer MCF7 cells. The representative micrographs of 3 independent wound healing assays show that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) increases cell migration after 48 hours in luminal A human breast cancer MCF7 cells versus the sc control group. **(C) Quantification of the wound healing assay in luminal A human breast cancer T47D cells.** This bar graph shows that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) increases cell migration after 24 hours in luminal A breast cancer T47D cells versus the sc control group (164.64$\pm$13.9%, 191.71$\pm$9.9%, versus

100±15.4%, respectively; ** p<0.01, *** p<0.001). **(D) Quantification of the wound healing assay in luminal A human breast cancer MCF7 cells**. This bar graph shows that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) increases cell migration after 48 hours in luminal A breast cancer MCF7 cells versus the sc control group (148.36±19.3%, 190.16±17.3%, versus 100±5.7%,, respectively; * p<0.05, *** p<0.001). **(E) Transwell assay in luminal A human breast cancer T47D cells.** This bar graph shows that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) increases cell migration in luminal A breast cancer T47D cells versus the sc control group (493.33±18.1, 762.67±33.3, versus 206.33±29.8, respectively; ***p<0.001). Representative micrographs of 3 independent transwell assays are shown beneath the bar graph. **(F) Transwell assay in luminal A human breast cancer MCF7 cells.** This bar graph shows that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) increases cell migration in luminal A breast cancer MCF7 cells versus the sc control group (56±5.3, 85.67±6.0, versus 39.67±8.0, respectively; *p<0.05, ***p<0.001). Representative micrographs of 3 independent transwell assays are shown beneath the bar graph. **(G) Wound healing assay in basal-like human breast cancer MDA-MB-231 cells.** The representative micrographs of 3 independent wound healing assays show that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) decreases cell migration after 8 hours in basal-like human breast cancer MDA-MB-231 cells versus the sc control group. **(H) Wound healing assay in basal-like human breast cancer SUM159 cells.** The representative micrographs

of 3 independent wound healing assays show that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) decreases cell migration after 24 hours in basal-like human breast cancer SUM159 cells versus the sc control group. **(I) Quantification of the wound healing assay in basal-like human breast cancer MDA-MB-231 cells.** This bar graph shows that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) decreases cell migration after 8 hours in basal-like human breast cancer MDA-MB-231 cells versus the sc control group (66.43$\pm$15.5%, 45.80$\pm$6.8%, versus 100$\pm$31.8%, respectively; not significant, * $p<0.05$). **(J) Quantification of the wound healing assay in basal-like human breast cancer SUM159 cells.** This bar graph shows that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) decreases cell migration after 24 hours in basal-like human breast cancer SUM159 cells versus the sc control group (47.62$\pm$6.1%, 44.24$\pm$7.8%, versus 100$\pm$15.6%, respectively; ** $p<0.01$). **(K) Transwell assay in basal-like human breast cancer MDA-MB-231 cells.** This bar graph shows that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) decreases cell migration in basal-like human breast cancer MDA-MB-231 cells versus sc control group (609.67$\pm$157.7, 413.33$\pm$92.7, versus 1288$\pm$81.2, respectively; **$p<0.01$, *** $p<0.001$). Representative micrographs of 3 independent transwell assays are shown beneath the bar graph. **(L) Transwell assay in basal-like human breast cancer SUM159 cells.** This bar graph shows that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) decreases cell migration in basal-like human breast cancer SUM159 cells versus sc control group (289.33$\pm$45.2,

137.78±17.5, versus 128±22.6, respectively; **p<0.01, ** p<0.001). Representative micrographs of 3 independent transwell assays are shown beneath the bar graph. **(M) Western blot analysis of E-cadherin expression in luminal A human breast cancer T47D and MCF7 cells.** This Western blot qualitatively indicates that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) decreases E-cadherin expression levels in both luminal A human breast cancer T47D and MCF7 cells. β-actin serves as a loading control. **(N) Western blot analysis of vimentin expression in basal-like human breast cancer MDA-MB-231 and SUM159 cells.** This Western blot qualitatively indicates that RHAMM knockdown (i.e., shRHAMM-1; shRHAMM-2) decreases vimentin expression levels in both basal-like human breast cancer MDA-MB-231 and SUM159 cells. β-actin serves as a loading control.

Figure 3. RHAMM ectopic expression reverses RHAMM deficiency-induced cell migration in luminal A subtype breast cancer.

(A) Wound healing assay and quantification in luminal A human breast cancer T47D cells. The representative photomicrographs of 3 independent wound healing assays show that RHAMM ectopic expression (i.e., shRHAMM2-RHAMM1; shRHAMM2-RHAMM2) decreases cell

migration after 24 hours versus the shRHAMM2-MCS group. The bar graph shows that RHAMM ectopic expression (i.e., shRHAMM2-RHAMM1; shRHAMM2-RHAMM2) decreases cell migration after 24 hours versus the shRHAMM2-MCS group (111.49±18.2%, 101.15±12.1%, versus 234.77±34.7%, respectively; **p<0.01). The wound healing assay and the quantification also reaffirm our previous finding that RHAMM knockdown (i.e., shRHAMM-2) increases cell migration after 24 hours in luminal A breast cancer T47D cells versus the sc group (181.18±45% versus 100±15.3%, respectively; * p<0.05). MCS= multiple cloning site **(B) Wound healing assay and quantification in basal-like human breast cancer MDA-MB-231 cells.** The representative photomicrographs of 3 independent wound healing assays show that RHAMM ectopic expression (i.e., shRHAMM2-RHAMM1; shRHAMM2-RHAMM2) increases cell migration after 8 hours versus the shRHAMM2-MCS group. The bar graph shows that RHAMM ectopic expression (i.e., shRHAMM2-RHAMM1; shRHAMM2-RHAMM2) increases cell migration after 8 hours versus the shRHAMM2-MCS group (80.09±15.1%, 104.5±30.4%, versus 51.68±5.1%, respectively; **p<0.01). The wound healing assay and the quantification also reaffirm our previous finding that RHAMM knockdown (i.e., shRHAMM-2) decreases cell migration after 8 hours in basal-like human breast cancer MDA-MB-231 cells versus the sc group (52.90±7.0% versus 100±20.3%, respectively; * p<0.05). **(C) Transwell assay in luminal A human breast cancer T47D cells.** This bar graph shows that RHAMM ectopic expression (i.e., shRHAMM2-RHAMM1;

shRHAMM2-RHAMM2) decreases cell migration versus the shRHAMM2-MCS group (214.3±16.9, 152.3±6.8, versus 263±17.6, respectively; *p<0.01, ***p<0.001). This bar graph also reaffirms our previous finding that RHAMM knockdown (i.e., shRHAMM-2) increases cell migration in luminal A breast cancer T47D cells versus the sc group (266±14.4 versus 126±15.1, respectively; *** p<0.001). Representative micrographs of 3 independent transwell assays are shown beneath the bar graph. **(D) Transwell assay in basal-like human breast cancer MDA-MB-231 cells.** This bar graph shows the RHAMM ectopic expression (i.e., shRHAMM2-RHAMM1; shRHAMM2-RHAMM2) increases cell migration versus the shRHAMM2-MCS group (738.67±40.1, 920±105.8, versus 506.67±40.4, respectively; **p<0.01, ***p<0.001). This bar graph also reaffirms our previous finding that RHAMM knockdown (i.e., shRHAMM-2) decreases cell migration in basal-like human breast cancer MDA-MB-231 cells versus sc control group (483.33±47.3 versus 824.33±75.5, respectively; ***p<0.001). Representative micrographs of 3 independent transwell assays are shown beneath the bar graph. **(E) Western blot analysis of cadherin expression in luminal A human breast cancer T47D cells.** This Western blot qualitatively indicates that RHAMM ectopic expression (i.e., shRHAMM2-RHAMM1; shRHAMM2-RHAMM2) increases E-cadherin expression levels versus the shRHAMM2-MCS group. This Western blot also reaffirms our previous finding that RHAMM knockdown (i.e., shRHAMM-2) decreases E-cadherin expression levels in luminal A human breast cancer T47D

cells versus the sc control group. β-actin serves as a loading control. **(F) Western blot analysis of vimentin expression in basal-like human breast cancer MDA-MB-231 cells.** This Western blot qualitatively indicates that RHAMM ectopic expression (i.e., shRHAMM2-RHAMM1; shRHAMM2-RHAMM2) increases vimentin expression levels versus the shRHAMM2-MCS group. This Western blot also reaffirms our previous finding that RHAMM knockdown (i.e., shRHAMM-2) decreases vimentin expression levels in basal-like human breast cancer MDA-MB-231 cells. β-actin serves as a loading control.

Figure 4. RHAMM knockdown promotes luminal A subtype breast cancer T47D cell metastasis to the lung.

(A) Photographs of xenograft T47D tumors formed in NOD/SCID mice harvested on Day 40. The photographs qualitatively indicate that T47D tumor size shows no apparent change in the RHAMM knockdown group (i.e., shRHAMM) versus the sc control group. **(B) Growth curve of xenograft T47D tumors formed in NOD/SCID mice.** This graph demonstrates that T47D tumor volume shows a similar growth pattern over a 40 day time period in the RHAMM knockdown group (i.e., shRHAMM) versus the sc control group. Tumor volume = length x width^2/2. **(C) Representative photomicrographs of the lung parenchyma and quantification.**

The micrographs demonstrate that T47D metastatic loci to the lung are enhanced in the RHAMM knockdown group (i.e., shRHAMM) versus the sc control group harvested on day 40. The bar graph demonstrates that T47D metastatic loci to the lung increase in the RHAMM knockdown group (i.e., shRHAMM) versus the sc control group harvested on day 40 (3.5 ± 0.96 versus 0.25 ± 0.25, respectively; $^*p<0.05$). Data are shown as mean ± SEM; Student's t-test was used for statistical analysis.

Figure 5. Snail contributes to RHAMM deficiency-induced migration and AKT-mediated GSK3β phosphorylation is the upstream regulator of Snail in luminal A subtype breast cancer.

(A) Western blot analysis of Snail, Zeb1, and Twist expression in luminal A human breast cancer T47D cells and basal-like human breast cancer MDA-MB-231 cells. This Western blot indicates that EMT transcription factors Snail and Zeb1 expression levels increase in the RHAMM knockdown (i.e., shRHAMM) group versus the sc control group in luminal A human breast cancer T47D cells. However, EMT transcription factors Snail, Zeb1, and Twist expression levels remain unchanged in the RHAMM knockdown (i.e., shRHAMM) group versus the sc control group in basal-like human breast cancer MDA-MB-231 cells. β-actin serves as a loading control. **(B)**

Western blot analysis and quantification of Snail degradation in cyclohexamide-treated luminal A human breast cancer T47D cells. This Western blot indicates that Snail degradation decreases in the RHAMM knockdown (i.e., shRHAMM) group versus the sc control group in cyclohexamide-treated luminal A human breast cancer T47D cells from 0 to 300 minutes. The graph also indicates that Snail degradation decreases in the RHAMM knockdown (i.e., shRHAMM) group versus the sc control group in cyclohexamide-treated luminal A human breast cancer T47D cells from 0 to 300 minutes based on a quantification of the Western blot intensity. β-actin serves as a loading control. CHX=cyclohexamide **(C) Western blot analysis of GSK3β phosphorylation (P- GSK3β) in luminal A human breast cancer T47D and MCF-7 cells and basal-like human breast cancer MDA-MB-231 and SUM159 cells.** This Western blot shows that GSK3β phosphorylation (P- GSK3β) increases in the RHAMM knockdown (i.e., shRHAMM) group versus the sc control group in luminal A human breast cancer T47D and MCF-7 cells. However, GSK3β phosphorylation (P- GSK3β) remains unchanged in the RHAMM knockdown (i.e., shRHAMM) group versus the sc control group in basal-like human breast cancer MDA-MB-231 and SUM159 cells. β-actin serves as a loading control. **(D) Western blot analysis of Snail degradation in IM-12 treated luminal A human breast cancer T47D cells.** This Western blot shows that Snail expression levels decrease in the IM-12 treated shRHAMM group versus the DMSO treated shRHAMM group in luminal A human breast cancer T47D cells. β-actin serves as

a loading control. **(E) Western blot analysis of GSK3β phosphorylation (P- GSK3β) in LY294002 treated luminal A human breast cancer T47D cells.** This Western blot shows that GSK3β phosphorylation (P- GSK3β) decreases in the LY294002 treated shRHAMM group versus the DMSO treated shRHAMM group in luminal A human breast cancer T47D cells. GSK3β, AKT, and β-actin serves as a loading control. **(F) Western blot analysis of GSK3β phosphorylation (P- GSK3β) in PD98059 treated luminal A human breast cancer T47D cells.** This Western blot shows that GSK3β phosphorylation (P- GSK3β) remains unchanged in the PD98059 treated shRHAMM group versus the DMSO treated shRHAMM group in luminal A human breast cancer T47D cells. GSK3β, ERK, and β-actin serves as a loading control.