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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Basal-like **T**riple **N**egative **B**reast **C**ancers (B-TNBC) are highly lethal; yet effective treatments are lacking. The purpose of this grant is to investigate if Limb-Bud-and-Heart (LBH), a basal mammary stem cell transcriptional regulator and WNT target aberrantly overexpressed in 50% of B-TNBC, is a new molecular determinant driving the development of this aggressive breast cancer (BrCa) subtype. We are testing the hypothesis that LBH drives basal-like TNBC, which are highly metastatic and endocrine-resistant, by: (Aim 1) reprogramming luminal cells-of-origin into basal stem-like cancer cells; (Aim 2) increasing the abundance of tumor/metastasis-initiating cancer stem cells (CSC); and (Aim 3) promoting hormone receptor negativity.

During year 1, we generated multiple LBH-dependent BrCa cell models, including patient-derived TNBC cells with inducible LBH knockdown and luminal MCF7 cells with inducible ectopic LBH expression. Focusing on Aim 2, we found LBH upregulates CSC abundance, sphere formation, and tumorigenicity in both B-TNBC and luminal BrCa, demonstrating LBH is a potent CSC driver. Ongoing RNA-Seq transcriptomic analysis (Aim 1) indicates LBH activates basal stem cell programs but represses luminal lineage/epithelial differentiation genes. Importantly, LBH downregulation decreases tumor initiation and metastasis of B-TNBC cells *in vivo*. This identifies LBH as B-TNBC oncogene and putative molecular target for anti-CSC therapy.

15. SUBJECT TERMS

Triple negative breast cancer; biomarker; cancer stem cells; metastasis; cell lineage determination; tumor heterogeneity; tumor progression; endocrine resistance; Limb-Bud-and-Heart; transcriptional regulator; WNT signaling; breast cancer cell lines; patient-derived breast cancer cell models; RNA-Sequencing

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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Basal-like **T**riple **N**egative **B**reast **C**ancers (B-TNBC) are highly lethal; yet effective treatments are lacking. The purpose of this grant is to investigate how Limb-Bud-and-Heart (LBH), a basal mammary stem cell transcriptional regulator in the oncogenic WNT stem cell signal transduction pathway, may drive the development of clinically aggressive B-TNBC. The WNT pathway is hyperactivated in B-TNBC and represents a prime therapeutic target; however, no WNT-targeted therapies have yet been approved for clinical use due to the notorious cytotoxicity of inhibiting WNT signaling components. Our published and preliminary work suggests, LBH is essential for mediating the cancer stem cell (CSC)-promoting effects of WNT, whereas it appears not required for normal WNT stem cell function in development or adult tissue homeostasis. Thus, inactivation of LBH may provide a unique therapeutic opportunity for developing B-TNBC and/or CSC-specific drugs. We are testing in subtype-specific breast cancer cell lines and patient-derived breast cancer cell models the hypothesis that LBH drives basal-like TNBC, which are highly metastatic and endocrine-resistant, by: (Aim 1) reprogramming luminal cells-of-origin into basal stem-like cancer cells; (Aim 2) increasing the abundance of tumor/metastasis-initiating CSCs; and (Aim 3) promoting hormone receptor negativity. Importantly, we are testing if LBH inhibition induces differentiation of basal-like breast CSCs into better prognosis ER+ luminal tumor cells that may render B-TNBC patients susceptible to endocrine therapy. As LBH is aberrantly overexpressed in 50% of TNBC, in association with basal lineage markers and WNT pathway hyperactivation, these studies, if successful, will identify LBH as a new biomarker and molecular target for basal-subtype TNBC. Moreover, our study may benefit the 30% of patients with Luminal or HER2+ breast cancers, which acquire treatment-resistance due to de novo appearance of treatment-resistant basal subclones during tumor progression.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Triple negative breast cancer; biomarker; cancer stem cells; metastasis; cell lineage determination; tumor dedifferentiation; tumor heterogeneity; tumor progression; endocrine resistance; Limb-Bud-and-Heart; transcriptional regulator; WNT signaling; breast cancer cell lines; patient-derived breast cancer cell models; RNA-Seq global gene expression analysis; differentiation therapy

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

STATEMENT OF WORK – May 20, 2019 **START DATE - July 01, 2019**

Specific Aim 1: To determine if LBH promotes the development of basal subtype breast cancer from luminal cells	**Time line**	**% Completion**
Major Task 1: Generate LBH overexpressing (OE) luminal BrCa and LBH knockdown (KD) basal TNBC cell models	**Months 1-12**	**70%**
Subtask 1: Generate lentiviruses containing inducible LBH expression constructs or inducible LBH shRNA knockdown constructs under the control of inducible Tet-ON-promoters	Months 1-3	100%
Subtask 2: Generate human luminal and basal breast cancer cell lines/primary cell cultures (2 each) transduced with LBH OE or LBH KD lentiviruses respectively = total of eight LBH-dependent BrCa cell models	Months 3-12	70%
Milestone(s) Achieved: Primary breast cancer and cell line models for Tetracycline (Tet)-inducible LBH overexpression (OE) and LBH knockdown (KD) for pre-clinical studies testing of the LBH lineage- and stem cell-reprogramming activity in breast cancer oncogenesis	**Months 1-12**	**70%**
Major Task 2: Characterize the LBH-induced luminal-to-basal cell lineage phenotypic changes	**Months 11-24**	**10%**
Subtask 1: In vitro studies to characterize the LBH-induced luminal-basal cell lineage transitions by FACS, qPCR, Western Blot and Co-immunofluorescence quantification of lineage-specific marker expression; and by functional 3D Matrigel organoid cultures	**Months 11-18**	**10%**
Subtask 2: Characterize in vivo LBH effects on luminal-basal cell differentiation in Xenograft tumors developed in task 5.1	**Months 19-24**	**10%**
Milestone(s) Achieved: Identification of LBH as novel driver of basal subtype BrCa development from luminal cells; Test efficacy of LBH downmodulation in reprogramming B-TNBC into less malignant luminal tumors	**Months 11-24**	**10%**
Major Task 3: Determine LBH effects on luminal-basal gene signatures	**Months 10-22**	**25%**
Subtasks 1-3: RNA-Sequencing analysis of two LBH overexpressing (OE) and two LBH depleted KD breast cancer cell models plus data analysis	**Months 10-22**	**50%**
Subtask 4-5: Validation of LBH regulated luminal-basal lineage genes/microRNAs and functional characterization of two candidate LBH-regulated genes/pathways.	**Months 13-16**	**10%**
Milestone(s) Achieved: Identification of LBH molecular pathways in luminal-basal progression with focus on targetable targets	**Months 10-22**	**25%**
Milestone(s) AIM1: Publication of 1-2 peer reviewed papers.		**0%**

Specific AIM 2: To determine if LBH promotes a cancer stem cell (CSC) phenotype and the aggressiveness of basal TNBC (B-TNBC)		
Major Task 4: In vitro CSC assays	**Months 10-20**	**100%**
Milestone(s) Achieved: Will have shown if LBH is required for promoting a CSC phenotype		
Major Task 5: In vivo tumor initiation/metastasis assays	**Months 10-26**	**30%**
Subtask 1: Orthotopic bilateral implantation of limiting cell dilutions of four luciferase-tagged cell lines/primary cultures produced in *task 1.2* in NSG mice; IVIS monitoring of tumor formation and metastasis	Months 10-20	30%
Subtask 2: Detailed immunological and molecular characterization of Xenograft tumors to determine LBH effects on histopathology, and CSC/EMT marker expression	Months 13-22	10%
Milestone(s) Achieved: Production of preclinical in vivo BrCa mouse models for LBH overexpression and LBH knockdown; Identification of LBH as a novel breast CSC and metastasis driver; Assessment of efficacy of LBH down modulation on blocking tumor initiation and metastasis	**Months 10-26**	**50%**
Milestone(s) AIM2: Publication of 1-2 peer reviewed paper		1 paper has been submitted
Specific AIM 3: To determine if LBH promotes hormone receptor negativity; and if down modulation of LBH upregulates ER and sensitizes B-TNBC to ER-targeted endocrine therapy		
Major Task 6: Endocrine studies	**Months 13-36**	**0%**
Subtask 1: ***In vitro* endocrine studies** using the LBH OE luminal and LBH KD B-TNBC cell models produced in task 1.2	Months 13-20	0%
Subtask 2: ***In vivo* endocrine studies**: Orthotopic bilateral injections of two LBH OE luminal and two LBH KD B-TNBC cell models produced in task 1.2 into the 4th pair of mammary fat pads of female NSG mice; monitoring of tumor formation by calipering and IVIS	Months 21-30	0%
Milestone(s): Will have determined if LBH repression of ER leads to endocrine resistance; and if LBH downmodulation induces ER expression and sensitizes B-TNBC to ER-targeted endocrine therapy		
Major Task 7: Characterize the molecular mechanisms underlying LBH-mediated ER repression	**Months 30-36**	**0%**
Milestone(s) Achieved: Identification of potentially targetable LBH targets in ER repression	Months 30-36	0%
Milestone(s) AIM3: Publication of 1-2 peer reviewed papers	Months 30-36	0%

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) Major Activities: During the 1st year of this grant, we have generated and characterized multiple LBH-dependent BrCa cell models as part of AIM1 (Task 1). These include two luminal, ER+ BrCa cell lines (MCF7, T47D) with inducible ectopic LBH expression (OE) and two basal TNBC lines (HCC1395, MDA-MB-231) with inducible LBH knockdown (KD). We also generated a new patient derived TNBC cell model with both inducible LBH KD and OE. Focusing on AIM 2, we show LBH upregulates the abundance of cancer stem cells (CSC), sphere formation, and tumorigenicity in both B-TNBC and luminal BrCa, demonstrating LBH is a potent CSC driver (See *Appendix 1*). Ongoing RNA-Seq transcriptomic analysis (AIM 1; Task 3) indicates LBH activates basal stem cell programs but represses luminal epithelial differentiation genes. Importantly, we found LBH depletion decreases tumor initiation and metastasis of B-TNBC cells *in vivo* (see below; and See *Appendix 1*). This identifies LBH as a novel B-TNBC oncogene and putative molecular target for anti-CSC therapy. Studies in AIM3 will begin in the year 2 of this grant (see SOW).

2-4) Specific objectives/results-outcomes/other achievements

Aim 1: To determine if LBH promotes the development of basal subtype breast cancer from luminal cells

Task 1: Generate LBH overexpressing (OE) luminal BrCa and LBH knockdown (KD) basal TNBC cell models

Subtask 1: Generate lentiviruses containing inducible LBH expression constructs or inducible LBH shRNA knockdown constructs under the control of inducible Tet-ON-promoters:

To generate a Doxycycline (DOX)-inducible LBH expression vector for overexpression studies, the coding region of human LBH flanked by a N-terminal FLAG epitope tag was subcloned unidirectionally into the EcoRI-BamHI sites of lentiviral vector, pLVX-TetOne-Puro (**Fig. 1A**). Lentiviral particles for pLVX-TetOne-Puro-LBH and a pLVX-TetOne-Puro vector control were generated by our *Vector Shared Resource* under BL2 conditions. For LBH knockdown studies, transduction-ready lentiviruses expressing three different LBH-targeted DOX-inducible small hairpin RNAs (shRNAs) (Tet-shLBH-A, Tet-shLBH-B, Tet-shLBH-C; **Fig. 2A**), or non-target (NT) shRNA were purchased (Horizon Discovery).

Subtask 2: Generate human luminal and basal breast cancer cell lines/primary cell cultures (2 each) transduced with LBH OE or LBH KD lentiviruses respectively = total of eight LBH-dependent BrCa cell models

To test (i) if LBH reprograms luminal into basal stem-like BrCa cells (Aims 1&2), and (ii) if LBH induces endocrine resistance (Aim 3). We first engineered luminal, ER+ MCF7 BrCa cells with inducible LBH overexpression, MCF7-luc+Tet-LBH. Briefly, MCF7 cells (ATCC) were stably transduced sequentially with lentivirus expressing luciferase (luc), and then with lentiviruses expressing either pLVX-TetOne-Puro-LBH (hereafter: Tet-LBH) or pLVX-TetOne-Puro vector only control. After growth in selective medium, individual colonies of MCF7+Tet-LBH cells were isolated and induction of LBH expression by DOX was tested by Western Blot (WB) and quantitative Realtime-PCR (qPCR) analysis (**Fig. 1B**). Treatment of cells with 1 μg of DOX/ml resulted in strong induction of Tet-LBH expression, whereas uninduced (UI) MCF7 do not express LBH (**Fig. 1B**), due to epigenetic silencing of LBH in this BrCa line (unpublished). WB analysis further showed upregulation of basal stem cell markers (SLUG, ZEB2) in DOX-induced MCF7-luc+Tet-LBH, confirming that LBH is active in these cells (**Fig. 1C**).

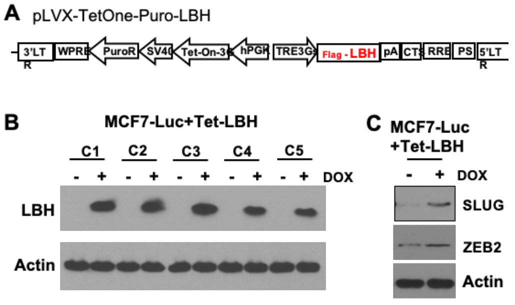

Figure 1: Ectopic, inducible LBH expression in luminal, ER+ MCF7 BrCa cells. A. Schematic of Tetracycline (Tet)/Doxycycline(DOX)-inducible LBH lentiviral expression vector. B. Western Blot analysis of LBH expression in luciferase (Luc)-positive MCF7 stably transduced with pLVX-TetOne-Puro-LBH. 5 individual MCF7-Luc+Tet-LBH colonies (C1-5) were isolated and treated for 5 days with 1μg/ml DOX (+) or vehicle (-).

We have generated one additional DOX-inducible Tet-LBH expressing luminal, ER+ BrCa lines (T47D), which will be used in concert with MCF7-luc+Tet-LBH to characterize LBH function in lineage reprogramming (AIM 1) and in promoting endocrine resistance (AIM 3) both *in vitro* and *in vivo*. Patient-derived, luminal ER+ BrCa cell models are currently in development in the laboratory of my Co-I, Dr. Tan Ince, and will be transduced with Tet-LBH, once they become available. Additionally, we plan to stably transduce Tet-LBH into: (i) luminal, ER+ MDA-MB-361 cells, because we found this BrCa line shows stronger Estrogen (E2)-dependent growth *in vivo* than T47D; and (ii) MCF12A, a normal-derived luminal, ER+ human breast epithelial cell line. Thus, we will have generated a total of four new luminal ER+ breast epithelial cell models with DOX-inducible ectopic LBH expression.

To test if inducible LBH depletion in basal TNBC cells leads to luminal differentiation (Aim 1), and (ii) sensitizes B-TNBC cells to ER-targeted drugs by restoring luminal ER expression. During year 1, we generated two inducible LBH knockdown (KD) B-TNBC cell line models (HCC1395 & MDA-MB-231-luc). These cell lines were stably transduced with three different DOX-inducible LBH-targeted shRNAs (Tet-shLBH-A, Tet-shLBH-B, & Tet-shLBH-C; Fig. 2A), or non-target (NT) shRNA control. Two of the LBH-targeted shRNAs, Tet-shLBH-B (hereafter: KD1) and Tet-shLBH-C (hereafter: KD2), yielded the best LBH KD efficiencies (50% and 80% respectively) in HCC1395 and MDA-MB-231-luc at DOX concentrations of 1-2μg/ml (Fig. 2B, C; and data not shown). Hence, these two LBH shRNAs and B-TNBC lines will be used for functional studies (Aims 1-3). Since HCC1395 (ATCC) is a poorly characterized BRCA1-deficient TNBC line, it was not known if it forms tumors *in vivo*. To test this, we transplanted 2 x 10^6 parental HCC1395 cells into NSG mice and monitored tumor formation over time by calipering. We found HCC1395 efficiently form K5/6+ basal-like tumors that spontaneously metastasize to the lungs > 2 months after cell engraftment (Fig. 2D). Hence, HCC1395 is a suitable primary tumor-derived B-TNBC model system. We also tested, if MDA-MB-231, which is a claudin-low, mesenchymal TNBC line, has basal characteristics. IHC analysis of MDA-MB-231 Xenograft tumors shows K5+ basal subclones in these tumors (Fig. 2D), confirming MDA-MB-231 cells are suitable for these studies.

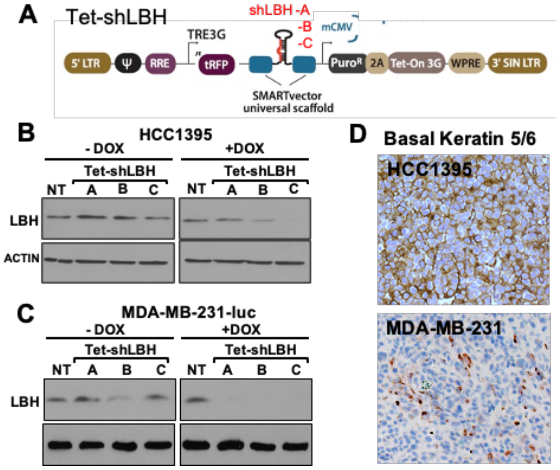

Figure 2: Inducible LBH knockdown (KD) in basal TNBC lines. A. Schematic: LBH-targeted Tetracycline(Tet/DOX)-inducible small hairpin RNA (shRNA) lentiviral expression vector. tRFP = Tomato Red Fluorescence Protein for cell labeling studies. B, C Western bolt analysis of LBH in B. HCC1395 and C. luciferase (luc)-expressing MDA-MBA-231 TNBC lines stably transduced with three independent LBH-targeted shRNAs (Tet-shLBH-A/-B/-C) or a non-target shRNA (NT) expressing lentiviruses 5 days -/+ 1 μg DOX. D. Immunohistochemical (IHC) analysis for basal stem-like markers, Cytokeratin K5/6. Top panel: HCC1395, a primary B-TNBC-derived tumor line, efficiently forms K5/6+ basal-like tumors, and lung metastases (not shown) after orthotopic cell injections into NSG female mice. Bottom panel: basal K5+ subclones in MDA-MB-231 Xenograft tumors.

To validate findings in primary TNBC models, we had proposed to use two LBH+ basal TNBC subtype patient derived Xenograft (PDX) models from the Baylor PDX biospecimen collections. However, one of these PDX (MC1) is contaminated with endolytic viruses and can no longer be used. Instead, we obtained resected, discarded tumor tissues from the *Biospecimen Shared Resources* of the Sylvester Comprehensive Cancer Center (SCCC) at the University of Miami. After primary tumor cell dissociation, we successfully established both, long-term cell cultures and a PDX model from a primary TNBC tumor originating from a 44-year-old African-American female patient who received neoadjuvant chemotherapy. This new patient derived TNBC cell model (TNBC1) and PDX represent invaluable new tools for breast cancer research, especially since PDX from chemo-treated tumors are rare.

As proof-of-principle, we transduced primary TNBC1 cells with LBH-targeted shRNA lentiviruses (Tet-shLBH-B = KD1, Tet-shLBH-C = KD2), or with pLVX-TetOne-Puro-LBH (Tet-LBH) expressing lentiviruses and assessed DOX-inducible LBH KD or LBH OE, respectively after 10 days by qPCR (Figure 3). Consistent with our results in BrCa cell lines, Tet-shLBH-C gave the best LBH KD efficiency, followed by Tet-shLBH-B, whereas Tet-LBH transduction of TNBC1 cells resulted in a 10,000- fold induction of LBH upon treatment of cells with 1μg/ml DOX. In this patient derived TNBC model LBH potently induced CSC activity (see *Appendix 1*, page 63, Figure 4N).

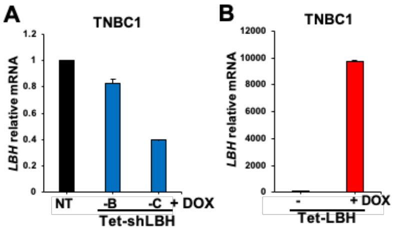

Figure 3: DOX (1 mg/ml) induction of LBH KD and OE in a new patient derived TNBC cell model. TNBC1 cells spin-transduced with Tet-shLBH or Tet-LBH expressing lentiviruses.

AIM1; Task 3; Subtasks 1-3: **RNA-Sequencing analysis** of two LBH overexpressing (OE) and two LBH depleted KD breast cancer cell models plus data analysis.

During YR1, we performed whole transcriptomic RNA-Seq analysis to elucidate LBH controlled molecular mechanisms that may underly its potential stem cell- and basal lineage-promoting roles in breast cancer. For these studies, we used (i) two LBH+ B-TNBC cell lines (HCC1395, MDA-MB-231) that we transiently transfected with small inhibitory RNAs (siRNAs) to target LBH or with scrambled non-target siRNA control; (ii) one LBH-negative TNBC-line (BT549) engineered to stably express ectopic LBH; and (iii) one luminal, ER+ BrCa cell line (MCF7) with stable ectopic LBH overexpression and respective pCDNA3 vector only controls for (ii) and (iii). We elucidated the CSC-promoting function of LBH in these four LBH-dependent BrCa cell models as part of AIM2 (see below; and *Appendix 1*). Hence these LBH-knockdown and LBH overexpression BrCa cell lines are well characterized and provide excellent model systems to unravel LBH molecular mechanisms.

Although LBH-dependent gene expression changes were variable among these BrCa lines dependent on their intrinsic molecular subtype, bioinformatics analysis of differentially expressed genes (p<0.05) followed by gene set enrichment analysis (GSEA), identified 14 significantly LBH up-regulated and 65 LBH down-regulated gene signatures (p<0.05; FDR<0.1-0.25) that were consistent among all four LBH-dependent BrCa cell lines. Among the 14 LBH upregulated gene sets, five were stem cell gene signatures, whereby the 'mammary stem cell' (MaSC) gene signature by Pece et al. was most strongly upregulated by LBH (Fig. 4A,B). In contrast, LBH repressed epithelial E-cadherin adhesion complex genes and E-cadherin signaling (Fig 4A,C), suggesting LBH activates stem cell programs, but represses epithelial differentiation genes.

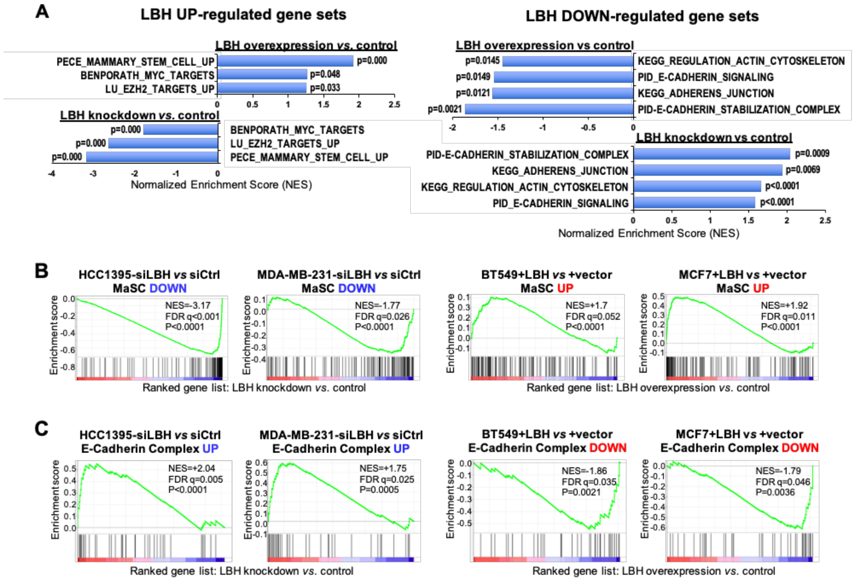

Figure 4: RNA-Seq analysis of LBH transcriptomes in human BrCa cell lines. A. GSEA analysis of LBH up-regulated and LBH down-regulated gene signatures in the MSigDB data base commonly and significantly enriched (p<0.05; FDR<0.1 – 0.25) among LBH-regulated genes in two LBH knockdown B-TNBC tumor cell lines (HCC1395, MDA-MB-231) and two LBH overexpressing BrCa cell models (BT549, MCF7). B, C Enrichment analyses of the top LBH regulated gene sets: B. the mammary stem cell (MaSC) gene signature from Pece et al., 2019) was up-regulated, and C. E-Cadherin complex genes were down-regulated by LBH. NES, Normalized Enrichment Score; FDR, False Discovery Rate.

Aim 2: To determine if LBH promotes a cancer stem cell (CSC) phenotype and the aggressiveness of basal triple-negative breast cancers (B-TNBC)

Cancer stem cells (CSC) initiate tumors, resist treatment, and seed lethal metastases; yet CSC-specific treatments are lacking. Triple-negative breast cancers (TNBC) are highly lethal and CSC enriched. During the past year, we focused on functional studies addressing the potential role of LBH in the regulation of breast CSCs as part of AIM 2. We found LBH upregulates CSC abundance, CSC markers, sphere formation, and tumorigenicity in both B-TNBC and luminal BrCa, demonstrating LBH is a potent CSC driver. Importantly, we show, LBH depletion abolishes tumor initiation and metastasis of MDA-MB-231/4175 B-TNBC cells *in vivo*. This identifies LBH as B-TNBC oncogene and putative molecular target for anti-CSC therapy. These studies have led to a manuscript (*Appendix 1*), which has been submitted for publication. I am listing below the completed tasks for this AIM2, with a reference to key research data shown in the manuscript. Please see the appended manuscript for a detailed description of experiments.

Task 4: *In vitro* CSC assays & Task 5: In vivo tumor initiation/metastasis assays.

Subtask 1: Characterize LBH effects on CSC phenotypes by FACS, in *vitro* tumor sphere formation, cell motility/invasion, cell proliferation, apoptosis assays, and stem cell marker expression.

Please see manuscript (*Appendix 1*), Results (pages/P 28-34) & Figures 2-5 (P 58-67) & Figures S2-S5 (P 71-74).

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Aim 1: In the next year, we will focus on studies addressing the role of LBH in luminal-to-basal lineage reprogramming. This epithelial cell plasticity process has recently gained attention in the breast cancer research field, as it is thought to underly both the development of basal-like TNBC from luminal breast epithelial cells, as well as the acquisition of endocrine resistance of luminal-type breast cancers. We will begin functional studies using our inducible LBH knockdown and overexpression breast cancer cell models (SOW: Major Task 2) as well as continue with our mechanistic studies (SOW: Major Task 3) to identify the mechanisms involved. In addition to the proposed RNA-Seq analysis (Task 3, Subtasks 1-3), which we have completed, it will be necessary to perform global ChIP-Seq analysis of LBH chromatin binding and ATAC-Seq analyses of chromatin structures to refine LBH transcriptional programs and epigenetic changes that may underly LBH-mediated reprogramming of cell lineage/stem cell states. These analyses will also facilitate the selection of LBH targets to carry out Major Task 3, Subtasks 4-5: functional characterization of two candidate LBH regulated genes/pathways in LBH-mediated luminal-to-basal progression.

Aim 2: While the current manuscript (see Appendix) on research performed during the first year of this grant provides functional evidence that LBH promotes a cancer stem cell phenotype and therefore aggressiveness (invasion, metastasis, chemoresistance) of TNBC, the mechanisms underlying the CSC promoting effects of LBH need to be further investigated. In the coming year, we will perform a detailed analysis of LBH-dependent changes in CSC/EMT markers both in vitro (e.g. FACS analysis) and *in vivo* (IHC, IF anlaysis of Xenograft tumor sections) as part of Major Tasks 4 & 5. As the tumor microenvironment (i.e. immune cells) plays an important role in metastasis, we will perform additional Xenograft experiments in immunocompetent murine hosts, as the reviewers of this grant proposal suggested.

Aim 3: We will initiate *in vitro* and *in vivo* endocrine studies (Major Task 6) using Doxycline-inducible LBH-expressing luminal MCF7 and LBH-knockdown basal HCC1395 cells that we generated during the past funding period. This will include Fulvestrant and Tamoxifen treatment of cells in culture and Fulvestrant treatment of Xenograft mice engrafted with these cells.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

1) A major accomplishment is the submission of a manuscript (see Appendix) describing the novel finding that LBH, a target gene of the oncogenic WNT signaling pathway implicated in normal breast stem cell regulation, is a potent cancer stem cell-driving oncogene in breast cancer that is critically required for the high metastatic potential and chemoresistance of lethal triple negative breast cancers. This manuscript further shows that LBH depletion reduces tumor initiation and metastasis of TNBC tumor cells in preclinical breast cancer mouse models, thus, identifying LBH as a potential molecular target to treat TNBC and other, treatment-refractory metastatic breast disease.

2) We submitted high-throughput RNA-Sequencing data of LBH regulated genes to the NCBI GEO database. This data provides new understanding of the mechanisms underlying breast tumor heterogeneity and tumor progression.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

1) The RNA-Seq data of LBH transcriptomes in human breast cancer cell lines that we submitted to the NCBI GEO database is the first in any cancer type and relevant to other cancer types as well.

2) The manuscript under submission (see Appendix) is the first work describing novel roles of LBH as a cancer stem cell and metastasis-promoting gene. Since LBH is also frequently overexpressed in other cancer types correlating with poor outcome (liver, gastric, pancreatic, glioblastoma, leukemia), this finding is of broad impact for understanding LBH function in cancer, at the same time offering a new rationale for inhibiting LBH to combat cancer metastasis and chemoresistance.

3) Our RNA-Seq LBH transcriptomic analysis is also expected to make an impact on autoimmune disease research (i.e. lupus erythematosus, rheumatoid arthritis) as LBH loss-of-function mutations confer autoimmunity.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

1) Delay of research proposed in Aim 1, because of a delay in hiring a 2nd research personnel (postdoc). It took several months to identify a suitable candidate. Unfortunately, the recruitment of this postdoc, Dr. In-Chi Young, got further delayed due to a temporary hiring freeze because of the COVID-19 pandemic. This got resolved in July 2020, and Dr. Young will be working with 100% effort on this grant starting in the 2nd year of this grant. Dr. Young has a strong background in molecular cancer biology, stem cell analysis through a previous postdoc in a prostate cancer lab at George Washington University, Washington DC. Hence these studies are expected to progress rapidly.

2) Delay of grant-related research (Aim1, Task 1: Generation of cell lines/primary breast cancer models) due to the COVID-19 pandemic. All non-critical research activity at the University of Miami was suspended from March 23 – May 18, 2020 due to a COVID-19-related lockdown in Miami-Dade, Florida.

3) Our research operation is currently in Phase I and expected to move to Phase II in Fall. Potential further delays are expected until full operation can resume.

4) To maintain productivity during the lockdown, we have concentrated on bioinformatics analysis of RNA-Seq data and public tumor gene expression data as well as written a manuscript on grant-funded research that we have submitted for publication (see *Appendix 1*).

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Delay in hiring a 2nd research personnel (postdoc): it took several months to identify a suitable candidate (Dr. In-Chi Young), followed by a hiring freeze due to the COVID-19 pandemic which postponed the recruitment of the research personnel to July 2020.

Two months lockdown due to the COVID-19 pandemics during which all non-critical research was suspended.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Koteswararao Garikapati, Kilan Ashad-Bishop, Pingping Chen, Megan E. Rieger, Linsey E. Lindley, Bin Wang, Diana J. Azzam, Mahsa Khanlari, Mehrad Nadji, Chaitanya Jain, Deukwoo Kwon, Yuguang Ban, Zhen Gao, Steven X. Chen, Andrew H. Sims, Susan Kesmodel, Joyce M. Slingerland, and Karoline J. Briegel. LBH is a key factor for cancer stemness promoting metastasis and chemoresistance in Triple-Negative Breast Cancer. (2020); submitted.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a* *periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Noting to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Noting to Report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

1) NCBI GEO accession number GSE151206 (Password: ixytqekqpxedtet)
https://nam01.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.ncbi.nlm.nih.gov%2Fgeo%2Fquery%2Facc.cgi%3Facc%3DGSE151206&data=02%7C01%7Czhen.gao%40med.miami.edu%7Ca4532b458d4e4996287208d805b2ce00%7C2a144b72f23942d48c0e6f0f17c48e33%7C0%7C0%7C637265612806877956&sdata=i1U6QfOjVVmNKpySmXdEifgbARsxO5tX36hvr6N4paE%3D&reserved=0

Description: RNA-Sequencing data of LBH transcriptomes in human breast cancer cell lines (HCC1395, MDA-MB-231, BT549, MCF7).

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

1) RNA-Sequencing data of LBH transcriptomes in human breast cancer cell lines (HCC1395, MDA-MB-231, BT549, MCF7) that we generated during the current funding period are available at NCBI GEO under accession number GSE151206 (Password: ixytqekqpxedtet)

https://nam01.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.ncbi.nlm.nih.gov%2Fgeo%2Fquery%2Facc.cgi%3Facc%3DGSE151206&data=02%7C01%7Czhen.gao%40med.miami.edu%7Ca4532b458d4e4996287208d805b2ce00%7C2a144b72f23942d48c0e6f0f17c48e33%7C0%7C0%7C637265612806877956&sdata=i1U6QfOjVVmNKpySmXdEifgbARsxO5tX36hvr6N4paE%3D&reserved=0

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student Researcher Identifier (e.g. ORCID ID): 1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

(1) Name: Dr. Karoline Briegel
Project Role: PI
ORCHID ID: 0000-0003-2661-509X
Nearest person month worked: 4 months
Contribution to Project: Dr. Briegel has supervised all aspects of this breast cancer research, including experimental design, training/supervision of lab personnel, data analysis, and written a manuscript on the funded research work.

(2) Name: Dr. Koteshwararao Garikapati
Project Role: Postdoc
Nearest person month worked: 12 months
Contribution to Project: Dr. Garikapati has generated a new primary TNBC cell model and PDX, inducible LBH-knockdown and overexpression breast cancer cell lines, RNA for RNA-Sequencing analysis, and performed functional cancer stem cell/metastasis analyses.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

APPENDIX 1 – Manuscript Submitted (2020)

Koteswararao Garikapati, Kilan Ashad-Bishop, Pingping Chen, Megan E. Rieger, Linsey E. Lindley, Bin Wang, Diana J. Azzam, Mahsa Khanlari, Mehrad Nadji, Chaitanya Jain, Deukwoo Kwon, Yuguang Ban, Zhen Gao, Steven X. Chen, Andrew H. Sims, Susan Kesmodel, Joyce M. Slingerland, and Karoline J. Briegel. LBH is a key factor for cancer stemness promoting metastasis and chemoresistance in Triple-Negative Breast Cancer. (2020); Submitted.

LBH is a key factor for cancer stemness promoting metastasis and chemoresistance in Triple-Negative Breast Cancer

Koteswararao Garikapati[1,2,8], Kilan Ashad-Bishop[1,2,3,8], Pingping Chen[1,2,8], Megan E. Rieger[2,8,10], Linsey E. Lindley[2,8,11], Bin Wang[2,8], Diana J. Azzam[2,8,12], Mahsa Khanlari[4,8], Mehrad Nadji[4,8], Chaitanya Jain[5,8], Deukwoo Kwon[6,8], Yuguang Ban[6,8], Zhen Gao[6,8], Steven X. Chen[6,8], Andrew H. Sims[9], Susan Kesmodel[1,8], Joyce Slingerland[2,5,7,8], and Karoline J. Briegel[1,2,8,13,*]

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[2]Braman Family Breast Cancer Institute, Sylvester Comprehensive Cancer Center
[3]Sheila and David Fuente Graduate Program in Cancer Biology
[4]Department of Pathology
[5]Department of Biochemistry and Molecular Biology
[6]Biostatistics and Bioinformatics Shared Resources, Sylvester Comprehensive Cancer Center, and Department of Public Health Sciences
[7]Department of Medicine
[8]University of Miami Miller School of Medicine, Miami, Florida, USA
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[12]Present address: Department of Environmental Health Sciences, Florida International University, Miami, Florida, USA
[13]Lead Author

[*]**Correspondence:** kbriegel@med.miami.edu

Running Title: LBH promotes cancer stem cells, metastasis, and chemo-resistance

Key Words: LBH; WNT; breast cancer; cancer stem cells; tumor progression; metastasis; chemoresistance; RNA-Seq; patient-derived tumors; mouse models

SUMMARY

Cancer stem cells (CSC) initiate tumors, resist treatment, and seed lethal metastases; yet CSC-specific treatments are lacking. Triple-negative breast cancers (TNBC) are highly lethal and CSC enriched. Here, we show that Limb-Bud-and-Heart (LBH), a WNT target required for normal mammary stem cell self-renewal, is a novel TNBC oncogene, critical for stem-like activity, cell motility, invasion, tumor initiation, metastasis and chemoresistance. LBH is expressed in tumor-initiating $CD44^{+}CD24^{-/low}$ breast CSC (TI-SC), but not in $CD44^{-}CD24^{high}$ non-CSC. LBH overexpression upregulates CSC abundance, self-renewal, stem cell gene signatures, and tumorigenicity in both TNBC and non-TNBC breast cancer cells, while *LBH* depletion decreases TI-SC and metastasis *in vivo*. *In vivo* stem cell analysis of LBH-deficient MMTV-Wnt1 mammary tumors expressing a SHIP-GFP+ stem cell reporter, further demonstrates LBH is essential for WNT-driven CSC expansion. Our findings identify LBH as an essential CSC driver downstream of WNT, and a new molecular target for anti-cancer stem cell therapy.

INTRODUCTION

Triple-negative breast cancers (TNBC) have the worst prognosis of all breast cancer subtypes and contribute disproportionally to cancer death, owing in part to their propensity to metastasize and resist treatment. TNBC lack key targetable markers, Estrogen Receptor (ER), Progesterone Receptor (PR), and/or Human Epidermal Growth Factor Receptor 2 (HER2) (Foulkes et al., 2010). Consequently, the mainstay of therapy remains chemotherapy, to which >50% of TNBC patients exhibit either *de novo* resistance or rapidly acquire it during an aggressive disease course, often culminating in death within 5 years of diagnosis (Dent et al., 2007; Foulkes et al., 2010). Thus, novel TNBC-specific biomarkers and molecular targets are needed.

TNBC are highly undifferentiated (high-grade) cancers, and >80% exhibit immunopositivity for basal cytokeratin 5/6 (K5/6) (Foulkes et al., 2010; Lehmann et al., 2011), a marker usually found in basal/myoepithelial cells of the normal breast. Intriguingly, basal-like TNBC show frequent hyperactivation of stem cell signaling pathways, including the WNT (Geyer et al., 2011; Khramtsov et al., 2010) and TGFβ pathways (Shipitsin et al., 2007), and greater enrichment for malignant cancer stem/progenitor cells than observed in other breast cancer types (Honeth et al., 2008; Ricardo et al., 2011). Cancer stem cells (CSC) are a subset of stem-like tumor cells that express specific markers (CD44^{+}CD24$^{-/low}$; ALDH1^{+}) and gene signatures reminiscent of normal mammary stem/progenitor cells (Al-Hajj et al., 2003; Ginestier et al., 2007). CSC show increased self-renewal *in vitro* and tumor initiating and metastatic potential *in vivo* (Al-Hajj et al., 2003; Ginestier et al., 2007), as well as resistance to chemo/radiation therapy (Fillmore and Kuperwasser, 2008; Li et al., 2008; Phillips et al., 2006); thereby driving tumor formation, treatment resistance, cancer recurrence, and metastasis, the main cause of cancer death. While it is thought that CSC are responsible for the innate aggressiveness of TNBC (O'Conor et al., 2018), the mechanisms sustaining high CSC abundance in TNBC remain poorly understood. Furthermore, although the outgrowth of treatment-resistant CSC represents a grave clinical problem, no CSC-specific therapies have yet been approved in the clinic.

We previously identified Limb-Bud-and Heart (LBH), a highly conserved, tissue-specific transcription co-factor in vertebrates (Briegel et al., 2005; Briegel and Joyner, 2001), that forms its own protein class (Al-Ali et al., 2010). We showed *LBH* is a direct WNT/ß-catenin target gene, induced by canonical WNT signaling in epithelial tissues (Rieger et al., 2010), including in TNBC cells (Maubant et al., 2015; Rieger et al., 2010). LBH is also a TGFß/SMAD3 target gene, however, induction of LBH by TGFß is context specific (Tufegdzic Vidakovic et al., 2015). In normal mammopoiesis, LBH is expressed in WNT-responsive LGR5+ mammary stem cells (MaSC) located in the basal epithelium of the postnatal mammary gland (Lindley et al., 2015). *Lbh* knockout (KO) studies in mice have demonstrated that LBH is critically required for MaSC self-renewal and differentiation (Lindley et al., 2015). Notably, LBH is upregulated in mammary tumors of MMTV-WNT1 transgenic (Tg) mice (Ashad-Bishop et al., 2019; Rieger et al., 2010), and *Lbh* KO in this WNT-driven breast cancer mouse model attenuated mammary hyperplasia and tumor onset (Ashad-Bishop et al., 2019).

Significantly, LBH is frequently overexpressed in human cancers, correlating with poor outcome (Chen et al., 2018; Jiang et al., 2019; Rieger et al., 2010; Yu et al., 2019). *In silico* analyses of published cancer gene expression data indicate that in breast cancer, *LBH* is most prevalent in basal-like TNBC, in association with WNT pathway activation (Lamb et al., 2013; Rieger et al., 2010). However, no study to date has evaluated the functional consequences of aberrant LBH overexpression in human breast cancer. Moreover, while LBH appears critical to MaSC maintenance in the normal breast (Lindley et al., 2015), and for mammary tumorigenesis induced by WNT (Ashad-Bishop et al., 2019), its potential role as a downstream effector of WNT-controlled stem cell regulation has not yet been characterized.

Since *Lbh*-null mice develop normally and are fertile (Lindley and Briegel, 2013), indicating that LBH is not essential for normal physiologic function, targeting LBH for therapy may have few adverse effects in cancer patients. For these reasons, we undertook a detailed analysis of LBH protein expression in primary breast cancers, and sought to determine the involvement of LBH protein function in human breast carcinogenesis, and in WNT-mediated CSC regulation *in vivo*.

RESULTS

LBH protein is highly expressed in TNBC

To date there has been no analysis of LBH protein expression in clinical breast cancer (BrCa) samples; thus we performed tissue microarray immunohistochemical (IHC) analysis comprising 250 newly diagnosed invasive primary BrCa (Table 1). LBH expression in tumor cells strongly correlated with hormone receptor (ER, PR) negativity (p=0.0002 for ER-, and p=0.0030 for PR-; respectively), and was most frequent in ER-/PR-/HER2- triple-negative BrCa, whereby 55% of highly LBH-positive tumors were K5/6-positive basal-like TNBC (p<0.0001; Table 1). High LBH immunostaining (defined as immunostaining intensity of >+2 and >50% nuclei positive for LBH) was especially prominent in medullary (3/3) and aggressive metaplastic (4/4) TNBC tumors. While basal epithelial cells of normal breast tissues express moderate, exclusively nuclear LBH (Lindley et al., 2015), in basal-like TNBC, LBH was expressed at higher levels in both tumor nuclei and in cytoplasm (representative images, Figure 1A). In contrast, LBH was expressed at much lower frequency in tumor cells of Luminal and HER2+ breast cancers (20% and 25%, respectively; p<0.0001) (Table 1; Figure 1A). Moreover, abundant LBH expression was detected in tumor-infiltrating lymphocytes and tumor-associated stroma in all BrCa subtypes (Figure 1A). Notably, LBH strongly associated with high tumor grade (III) (p=0.0092), but not with TNM stage or lymph node status in this breast cancer cohort. Overall high LBH protein expression was detected in 16% of breast tumors (40/250; p<0.001; Table 1), a significant number reminiscent of the frequency of TNBC in the total breast cancer patient population (Foulkes et al., 2010).

Bioinformatic interrogation of two independent data sets, the TCGA (Cancer Genome Atlas, 2012) and the METABRIC (Curtis et al., 2012) breast cohorts, that together include over 2,778 primary BrCa, further showed that *LBH* mRNA levels were significantly higher in TNBC relative to non-TNBC cancers (Figure 1B). When grouped into intrinsic molecular subtypes (Sorlie et al., 2003), *LBH* was highest in basal, normal-like, and claudin-low BrCa (Figure S1A), which are enriched in TNBC (Herschkowitz et al., 2007; Sorlie et al., 2003), consistent with previous data (Rieger et al., 2010; Tufegdzic Vidakovic et al., 2015). PAM50 classification of the METABRIC data into prognostic Integrative Clusters (IntClust = IC) demonstrated greater *LBH* expression in TNBC clusters, IC4ER- and IC10 (basal-like) (Figure S1B), with the most cancer-related deaths occurring in the first 5 years (Rueda et al., 2019). As *LBH* maps to chromosome 2p23 (Briegel et al., 2005), a region amplified in leukemia and childhood cancers (Rieger et al., 2010), we also investigated *LBH* gene copy number status. We found *LBH* copy numbers modestly, but significantly increased in TNBC compared to non-TNBC in both datasets (Figure 1C). Thus, LBH is upregulated in TNBC at the protein, RNA, and gene levels.

TNBC is a heterogeneous disease, comprising at least four different subtypes distinctive in histopathology, molecular signatures, clinical outcome, and response to treatment (Burstein et al., 2015; Geyer et al., 2017; Lehmann et al., 2011; Lehmann et al., 2016). To ascertain whether LBH correlates with specific TNBC subgroups, we performed meta-analysis of *LBH* expression across the TNBC molecular subtypes identified by Lehmann et al. (Lehmann et al., 2016). This study clusters TNBC into luminal androgen receptor (AR) positive (LAR), two basal-like (BL1, BL2), and mesenchymal (M) TNBC, similar to (Burstein et al., 2015). *LBH* gene expression levels did not differ significantly between these TNBC subgroups (Figure S1C), although it was slightly higher in poorer prognosis basal-like (BL1, BL2) and mesenchymal (M) TNBC subgroups compared to the more indolent LAR subgroup (Santagata et al., 2014).

Analysis of LBH protein expression in a panel of 21 BrCa cell lines corroborated our findings in primary tumors (Figures 1D and S1D). LBH was detected exclusively in TNBC lines (7/14 TNBC *vs.* 0/7 non-TNBC lines), and at higher levels than in normal-derived, basal mammary epithelial lines (0/2) (Figures 1D,E and S1D). However, there was no apparent correlation between LBH protein and specific TNBC subgroups: among the TNBC lines positive for LBH, there was a similar distribution of basal-like (BL1, BL2) and mesenchymal, and claudin-low subtypes were not overrepresented (Figures 1D and S1D), in discrepancy with *in silico* data, showing highest *LBH* mRNA levels in this rare, highly metastatic TNBC subtype (Figures S1A and (Tufegdzic Vidakovic et al., 2015). There was also no apparent relationship between LBH and *BRCA1* mutant status, a characteristic of basal-like TNBC (Foulkes et al., 2010), although 4/7 LBH-positive TNBC lines were basal marker K5+ (Figures S1D).

However, nearly all LBH-positive TNBC lines (6/7) expressed high levels of the CSC marker, CD44 (Figures 1D and S1D). To further evaluate stem cell features of LBH-positive TNBC, we performed gene set enrichment analysis (GSEA) of LBH^{high} tumors in the TCGA BrCa dataset (Figure 1F,G). Remarkably, 4 of the top 10 significantly enriched signatures were stem cell signaling pathways (WNT, Hedgehog, TGFß, Notch). WNT/ß-catenin signaling genes were most significantly enriched (NES = 2.09, $p<0.0001$, $q=0.034$; Figure 1G), consistent with *LBH* being a WNT target gene (Rieger et al., 2010). Collectively, these data demonstrate that LBH is a putative biomarker for TNBC, and its expression might herald the presence of stem-like breast cancer cells.

LBH is required for clonogenicity and tumor growth of TNBC cells

To elucidate the role of LBH in TNBC, we knocked down LBH in two TNBC cell line models with high endogenous LBH expression, HCC1395 and MDA-MB-231 (Figure 1B), derived from two different aggressive TNBC subgroups. HCC1395 originates from a primary *BRCA1*-mutated basal-like TNBC, whereas MDA-MB-231 is a claudin-low, mesenchymal TNBC model derived from pleural metastases. We first assessed the immediate effects of LBH on tumorigenicity *in vitro* by transient transfection of cells with LBH-targeted siRNAs (siLBH) compared to a non-targeted siRNA (siCtrl) control. qPCR and Western blot (WB) analyses confirmed efficient LBH knockdown (KD) in both TNBC lines (Figure 2A). LBH depletion in HCC1395 and MDA-MB-231 significantly diminished cell proliferation (Figure 2B), clonogenicity in 2D culture (Figure 2C), and anchorage-independent growth in soft agar (Figure 2D), a key feature of malignant transformation (Mori et al., 2009).

To test the effects of LBH on tumorigenicity of TNBC cells *in vivo*, a luciferase-positive subclone of MDA-MB-231, 4175 (Minn et al., 2005), was stably transduced with either non-target shRNA control (shCtrl), or with one of four independent LBH-targeted shRNAs (Figure 2E). Two different LBH shRNA transduced 4175 populations (shLBH-1, shLBH-2) with the greatest LBH KD efficiencies (85-90%; Figure 2E), or shCtrl transduced cells were implanted into the mammary fat pads of immunocompromised NOD/SCID-*Il2Rgamma-/-* (NSG) mice (10^5 cells/mouse; 12 mice/group). Whole animal *in vivo* Bioluminescence Imaging (BLI) analysis revealed that LBH KD significantly attenuated *in vivo* tumor growth (Figure 2F,G).

High LBH levels promote breast cancer growth *in vivo*

Conversely, we examined whether LBH overexpression would increase breast tumor development. LBH was transfected stably into BT549, a TNBC line with low to undetectable endogenous LBH (Figures 1B and 3A). Furthermore, to test if oncogenic effects of LBH are TNBC-specific, LBH was also introduced into MCF7, an ER+ luminal (= non-TNBC) BrCa cell model, lacking LBH expression (Figures 1B and 3A). Ectopic expression of LBH in BT549 and MCF7 yielded LBH expression levels comparable to LBH-positive TNBC lines, as confirmed by qPCR and WB analysis (Figures 3A and 4I); thus, these lines represent valid models for LBH overexpression (OE) in breast cancer.

Surprisingly, while LBH OE did not increase cell proliferation in adherent culture (Figures 3B), it significantly and consistently increased anchorage-independent growth of both BT549 and MCF7 (Figures 3C). Importantly, orthotopic injection of luciferase-tagged MCF7+LBH cells into NSG hosts ($2x10^6$, n=12 mice/group) resulted in increased tumor formation (Figure 3D), tumor volumes (Figure 3E,F), tumor weight and size (Figure 3G) compared to empty vector expressing MCF7+vector cells, demonstrating that LBH is sufficient to increase the tumorigenic potential of BrCa cells. When fewer cells were injected orthotopically, the differences in tumor volumes between MCF7+LBH and MCF7+vector control became even more notable: MCF7+LBH tumors formed from 5×10^5 cells injected had a 2.1 fold greater volume, from 5×10^4 cells – ca. 3 fold, and from 5×10^3 cells – ca. 6 fold greater volume than controls ($p<0.05$; Figure 3H). Thus, LBH not only is required for TNBC tumorigenesis, but also promotes *in vivo* tumor growth of non-TNBC cells, reinforcing the notion that LBH is a potent breast cancer promoter.

LBH is enriched in breast CSC and sufficient to augment CSC abundance and self-renewal

Since LBH controls MaSC in the normal breast (Lindley et al., 2015), we next considered the possibility that LBH might promote breast tumorigenesis by modulating CSC, or tumor-initiating stem cells (TI-SC). To this end, we first examined whether LBH was expressed in breast CSC. Analysis of published gene signatures of $CD44^{+}CD24^{-/low}$ CSC, which have increased TI-SC activity (Al-Hajj et al., 2003), from 14 primary human BrCa (Creighton et al., 2009) showed that *LBH* is elevated in this CSC-enriched population compared to non-CSC populations from the same tumors (Figure 4A). Moreover, qPCR analysis of FACS-purified stem cell-enriched and non-stem-like populations from BrCa cell lines (Figure S2A) revealed much higher *LBH* levels in the CD44$^+$CD24$^{-/low}$ TI-SC than in the non-TI-SC CD44$^-$CD24^{high} population (Figure 4B).

To further validate the relationship between LBH and cancer stemness, we compared expression of *LBH* and key stem cell transcription factors (SC-TFs) in BT549 and MCF7 BrCa cell lines grown in standard 2D tissue culture *vs.* in spheres formed in serum-free, low-attachment mammosphere cultures, which enriches for CSC (Dontu et al., 2003). Remarkably, both *SOX2, NANOG, OCT4, KLF4* SC-TF and *LBH* expression were dramatically increased in CSC-enriched tumor spheres compared to 2D cultures from both BT549, and MCF7 lines (Figure 4C). These data show that LBH is highly expressed in TI-SC.

In keeping with *LBH* being upregulated in CD44$^+$CD24$^{-/low}$ CSC, FACS analysis showed that TNBC lines with high endogenous LBH expression, HCC1395 and MDA-MB-231, had very high % CD44$^+$CD24$^{-/low}$ CSC (86.3±4.42 and 96.9±0.24, respectively; Table S1). In contrast, the BT549 TNBC line and MCF7, that were both devoid of LBH, had low CD44$^+$CD24$^{-/low}$ CSC populations (11.8±1.31 and 0.47±0.31, respectively; Table S1). Remarkably, LBH depletion in HCC1395, decreased the CD44$^+$CD24$^{-/low}$ CSC population by ca. 20% (from 83% to 65%, p=0.03 Figure 4D), suggesting LBH is required to maintain high TI-SC frequency in TNBC. Conversely, LBH OE in BT549, caused a significant increase in the % CD44$^+$CD24$^{-/low}$ TI-SC population (from 13.4% to 20.1%; p=0.01, Figure 4E). Notably, ectopic LBH in MCF7 non-TNBC cells also increased CD44$^+$CD24$^{-/low}$ TI-SC by over five-fold (from 0.2% to 1.13%; p=0.019; Figure 4F), consistent with the observed increased tumorigenicity of these cells *in vivo* (Figure 3E-H).

Interestingly, LBH KD in MDA-MB-231 and its sister cell line, 4175, did not shift the CD44$^+$CD24$^{-/low}$ CSC to the CD44$^+$CD24^{high} non-CSC population (Figure S2B), as observed for HCC1395 (Figure 4D). Rather, in these claudin-low TNBC lines, stable LBH KD with two independent shRNAs changed the distribution of CSC within the CD44$^+$CD24$^{-/low}$ subpopulation. LBH depletion caused a five-fold decrease in $CD44^{+}CD24^{low}$ CSC, which are on top of the CSC hierarchy and have the highest self-renewal and metastasis-initiating potential (Azzam et al., 2013; Fillmore and Kuperwasser, 2008) (from 10.0 to 2.3%; $p<0.01$; Figure S2B), whereas $CD44^{+}CD24^{-}$ CSC, with lower TI-SC activity and lacking metastasis-initiating potential (Azzam et al., 2013; Fillmore and Kuperwasser, 2008), were increased. In concordance with these surface marker changes, tumor sphere formation was markedly reduced in MDA-MB-231 upon LBH depletion compared to control MDA-MB-231 shCtrl cells (Figure S3A). Thus, LBH shifts surface CD44/CD24 markers towards a more stem-like phenotype and increases the abundance of TI-SC.

To address whether LBH affected CSC function, we performed serial mammosphere assays, a quantitative *in vitro* measurement of stem cell self-renewal (Dontu et al., 2003). KD of LBH in four independent TNBC lines (HCC1395, MDA-MB-231, MDA-MB-157, HCC1187) significantly reduced primary (Figures 4G and S3A-C), and to a greater extent, secondary sphere formation (2.7 fold in HCC1395/$p=0.00176$; 2.9 fold in MDA-MB-231/$p=0.00154$; Figure 4G). Importantly, LBH OE in both BT549, and MCF7 cells increased primary, and more notably secondary tumor sphere formation (~3.5 fold for BT549+LBH/$p<0.001$; ~2 fold for MCF7+LBH/$p=0.00024$) *vs.* the respective controls (Figure 4H), suggesting LBH increases the self-renewal potential of BrCa cells.

Consistent with the idea that LBH increases stemness, LBH KD in HCC1395 and MDA-MB-231 decreased, while LBH OE in BT549 and MCF7 increased SC-TF, SOX2, OCT4, MYC, expression (Figure 4I). RNA-Seq gene expression analysis and GSEA further revealed a significant enrichment of stem cell-associated gene signatures ($P<0.05$; FDR $q<0.25$) by LBH in all four BrCa cell models (Figures 4J and S3D). Notably, the MaSC gene signature from (Pece et al., 2019) was most strongly and significantly upregulated in LBH OE lines (MCF7+LBH/NES=+1.9; $q=0.011$ and BT549+LBH/NES=+1.7; $q=0.052$), whereas it was downregulated in LBH KD lines (HCC1395-siLBH/NES=-3.2; $q<0.001$ and MDA-MB-231-siLBH/NES=-1.77; $q=0.026$) vs. respective controls (Figure 4, J,K). Thus, LBH activates stem cell gene expression programs.

To definitively test the requirement for LBH in CSC self-renewal and TI-SC abundance, 4175-

shCtrl and 4175-shLBH cells were implanted at limiting dilutions (10^4, 10^3, 10^2), in either left or right inguinal mammary fat pads of NSG female mice. LBH depletion profoundly reduced the TI-SC frequency in 4175 cells ($p = 0.0152$; Fig. 4L), demonstrating LBH is essential for tumor initiation. Moreover, SC-TFs were downregulated in LBH KD 4175 lines at both the RNA and protein levels (Figures 4M and S3E).

Finally, to determine whether primary, patient-derived CSC also required LBH, we isolated cells from discarded tumor tissues resected from a TNBC patient, and grew these cells under sphere-forming conditions to enrich for CSC. These tumor cells (TNBC1) were then transduced with lentiviruses expressing inducible versions of two independent *LBH* shRNAs (KD1, KD2) or an inducible *LBH* transgene (+LBH) and plated in mammosphere cultures in the presence of Doxycycline (DOX) (Figure 4N). Similar to the results obtained in BrCa cell lines, LBH KD significantly reduced, whereas LBH OE increased sphere forming ability of patient-derived TNBC1 cells by over 2 fold ($p<0.001$; Figures 4N and S3F). Collectively, these data demonstrate that LBH is a novel breast CSC-specific factor necessary and sufficient to promote a CSC phenotype.

LBH promotes tumor cell motility, invasion, metastasis, and chemoresistance

CSC have increased invasive, metastatic properties (Azzam et al., 2013; Sheridan et al., 2006). Hence, we next explored how LBH affects BrCa cell motility and invasion. LBH KD in four independent TNBC lines (HCC1395, MDA-MB-231, MDA-MB157, HCC1187) markedly reduced tumor cell migration (Figure S4A,B), and decreased invasion by up to 10 fold (Figures 5A and S4C). In contrast, ectopic LBH expression in both TNBC and non-TNBC models, BT549 and MCF7 respectively, increased cell motility by >2-4 fold (Figure S4B), and BT549 invasiveness by over 5 fold (Figure 5B, left). Additionally, whereas control cells formed small spheres in three-dimensional (3D) Matrigel cultures, LBH expressing BT549+LBH formed spheroids with extensive, highly invasive protrusions (Figure 5B, right).

To examine the effects of LBH on metastasis *in vivo*, metastatic 4175 cells expressing shCtrl or two LBH shRNAs (shLBH-1, shLBH-2) were injected orthotopically into NSG hosts and spontaneous metastasis was monitored over 7 weeks by BLI. LBH KD drastically reduced the ability of 4175 TNBC cells to form distant metastases ($p<0.01$; n=6 per group; Figure 5C,D). Whereas 4175-shCtrl generated visible metastases in lungs, spleen, and liver, 4175-shLBH KD cells only formed micro metastases in the lungs or no metastases, as determined by *ex vivo* BLI imaging of organs (Figure 5D; left). Lung weights were also significantly decreased in 4175-shLBH-1 and 4175-shLBH-2 Xenografts, reflective of reduced lung metastatic burden (Figure 5D; right). Moreover, when injected directly into the blood stream via tail vein LBH KD drastically reduced the ability of 4175 TNBC cells (shLBH-2) to colonize the lung parenchyma (Figure 5E,F). Notably, the number of metastasis-initiating CD44^{+}CD24^{low} CSC (MI-CSC) was significantly reduced by >2.5 to 4-fold in 4175 KD compared to 4175-shCtrl cells ($p<0.01$; Figure 5G), indicating that reduced metastasis upon LBH KD was CSC-dependent. Thus, LBH is required for both metastasis from primary tumors and for extravasation, and establishment of tumor metastases in this highly aggressive TNBC model.

While EMT is a genetic program associated with metastasis and acquisition of stemness (Mani et al., 2008; Morel et al., 2008), and primary LBH^{high} TNBC were enriched in EMT genes (Figure 1D), we did not detect EMT-like changes in cell morphology or consistent changes in the expression of EMT markers, EMT-TFs, and EMT gene signatures following LBH up- or downmodulation (Figure S5A-C; data not shown), except for epithelial marker, E-cadherin. E-cadherin was significantly upregulated upon LBH KD in HCC1395 and MDA-MB-231 and downregulated following LBH OE in MCF7 and BT549 cells (Figure S5B,C). Moreover, unbiased RNA-Seq and GSEA analysis identified E-cadherin complex stability and signaling among the top 7 enriched canonical pathways inhibited by LBH (Figure S5D,E). This suggests LBH increases cell motility, invasion, and metastasis by downregulating epithelial cell adhesion.

Importantly, multivariate meta-analyses of *LBH* gene expression in primary breast tumors (Figure 5H-K) support our *in vivo* findings in the 4175 TNBC model that LBH is required to sustain metastasis. High *LBH* expression in primary BrCa from patients associated significantly with metastatic disease recurrence ($p<0.05$; Figure 5H), and patient death ($p=0.027$; Figure 5I) 3-5 years after diagnosis. Moreover, BrCa patients with LBH^{high} tumors (expressing LBH above the median) had reduced relapse-free [RFS] (p=0.038) and overall survival [OS] (p=0.013) compared to those with LBH^{low} tumors (Figure 5J,K).

Notably, in a cohort of 3,951 primary BrCa patients, of which 602 were treated with neoadjuvant or adjuvant chemotherapy, high intra-tumoral *LBH* expression associated significantly with reduced relapse-free survival after chemotherapy (Figure 5L). Since high *LBH* levels correlated with chemoresistance in patients, we further examined the effects of LBH in treatment-resistant 4175 TNBC cells. Notably, LBH KD in two independent stable lines significantly increased sensitivity of 4175 to paclitaxel (3-4 fold; $p<0.01$; Fig. 5M,N), a chemotherapy drug widely used in the treatment of TNBC. Collectively, these data uncover a novel role for LBH in malignant tumor progression that by promoting expansion of CSC, mediating chemoresistance and metastatic disease recurrence, drives the aggressive TNBC clinical course.

LBH is required for WNT-driven breast CSC expansion *in vivo*

WNT/ß–catenin is a key MaSC self-renewal and CSC-promoting signaling pathway (Jang et al., 2015; Schwitalla et al., 2013; Zeng and Nusse, 2010), implicated in TNBC, metastasis, and chemoresistance (Dey et al., 2013; Geyer et al., 2011; Khramtsov et al., 2010; Xu et al., 2015). However, while our present (Figure 1F,G) and prior work (Ashad-Bishop et al., 2019; Rieger et al., 2010) indicate a strong link between WNT and aberrant LBH OE in TNBC, to date the role of LBH in WNT-induced breast CSC expansion has not been defined.

To address this, we tested the effects of *Lbh* KO in a mouse model of WNT-driven breast cancer, MMTV-Wnt1^{Tg} mice (Tsukamoto et al., 1988). These mice exhibit abnormal amplification of self-renewing MaSC in pre-neoplastic mammary glands (Shackleton et al., 2006) and enrichment of CSC in tumors (Cho et al., 2008) due to the stem cell-promoting effects of WNT, which drive early tumor onset and malignancy in this mouse model (Liu et al., 2004). In contrast, LBH KO in mice reduces MaSC frequency and activity (Lindley and Briegel, 2013). MMTV-Wnt1^{Tg} mice were crossed with LBH KO mice (ROSA26[R26]-Cre;Lbh$^{-/-}$) (Lindley and Briegel, 2013), and a SHIP-GFP stem cell reporter line (Figure

6A), expressing GFP specifically in activated MaSC (Bai and Rohrschneider, 2010), to allow *in vivo* stem cell analysis. Loss of LBH profoundly delayed tumor formation, with a median tumor-free survival of 50 weeks in MMTV-Wnt1+;SHIP+;Lbh$^{-/-}$ KO compared to 10.5 weeks in MMTV-Wnt1^{+};SHIP+;Lbh$^{+/+}$ wild type (WT) mice ($p=0.0026$; Figure 6B). Notably, sphere formation of FACS-purified MaSC (CD29^{high}CD24^{+}) from pre-neoplastic mammary glands (Figure 6C), and the frequency of SHIP-GFP+ tumor stem cells in mammary tumors from LBH-deficient MMTV-Wnt1 mice were significantly and importantly reduced (Figure 6D,E). Thus, LBH not only promotes CSC in human BrCa models, but is also an essential effector of WNT-driven CSC expansion.

DISCUSSION

A serious clinical problem underlying cancer recurrence, metastasis, and mortality is the innate resistance of tumor-/metastasis-initiating CSC to current treatments. Lethal TNBC breast cancers are enriched in CSC. While previous work has implicated hyperactivation of the WNT/ß-catenin stem cell signaling pathway as causative for CSC expansion, metastasis, and treatment resistance in TNBC, little is known about the effectors downstream of WNT signaling that mediate its CSC-promoting effects. Our study identifies LBH as a novel CSC-intrinsic WNT effector that potently promotes the expansion of breast CSC, and is required for metastasis, and chemoresistance in TNBC (Figure 6F). Mechanistically, we found LBH activates stem cell gene expression programs, but inhibits epithelial adhesion genes, e.g. E-cadherin.

While *LBH* gene expression has been shown to be elevated in TNBC-enriched molecular subtypes of BrCa (Rieger et al., 2010; Tufegdzic Vidakovic et al., 2015), we uniquely report the ability of LBH to stratify TNBC into a subset of stem-like cancers, independent of other molecular and histopathological subclassifications. LBH^{high} TNBC tumors were enriched in stem cell pathways, most significantly WNT, consistent with *LBH* being a WNT/ß-catenin target gene (Rieger et al., 2010). Thus, LBH may have unique utility as a novel biomarker to detect CSC-enriched breast cancers in the clinic.

Consistent with our finding that LBH is upregulated in stem-like TNBC, we found LBH highly expressed in $CD44^{+}CD24^{-/low}$ breast CSC. This TI-SC population is enriched in TNBC (Honeth et al., 2008), and in residual cancers of luminal and HER2+ subtypes after chemotherapy (Creighton et al., 2009). Notably, KD of LBH in multiple TNBC cell lines, and in primary patient-derived TNBC tumor cells reduced $CD44^{+}CD24^{-/low}$ CSC numbers and/or sphere formation. In contrast, ectopic expression of LBH in BT549 and primary TNBC cells increased these CSC parameters, demonstrating LBH promotes CSC abundance and self-renewal. Importantly, depletion of LBH in highly malignant 4175 TNBC cells inhibited chemoresistance *in vitro*, and TI-SC frequency, tumor growth, and metastasis *in vivo*, indicating LBH has potential as therapeutic target for anti-cancer stem cell therapy.

Remarkably, the CSC-promoting effect of LBH was not limited to TNBC; instead LBH may have a universal effect in promoting stemness during BrCa progression. This notion is supported by our findings that ectopic expression of LBH in the non-TNBC, luminal ER+ MCF7 breast cancer model also increased the $CD44^{+}CD24^{-/low}$ breast CSC population, as well as self-renewal *in vitro* and tumor formation *in vivo*. Moreover, high intra-tumoral *LBH* expression, correlated with increased metastasis, chemoresistance, and death in the BrCa patient population as a whole.

We also investigated the potential causative role of LBH in WNT-mediated CSC expansion. Using an *in vivo* mouse model for WNT-driven TNBC (Herschkowitz et al., 2007), MMTV-$Wnt1^{Tg}$, and stem cell reporters, we show that LBH KO in MMTV-$Wnt1^{Tg}$ mice significantly reduced MaSC activity at early stages and frequency of SHIP-GFP+ tumor stem cells at later stages, resulting in a profound delay in tumor onset. Thus, LBH not only promotes CSC in human BrCa models, but is also an essential effector mediating WNT-induced CSC amplification.

In embryonic development, *Lbh* is expressed in stem cell niches and organizer regions controlling tissue development, i.e. pluripotent Xenopus blastomeres (Paris and Philippe, 1990), chick dorsal mesoderm (Alev et al., 2010), murine gastrula and the Apical Ectodermal Ridge (AER) (Briegel and Joyner, 2001), where *Lbh* expression is controlled by WNT signaling (Rieger et al., 2010). Moreover, *Lbh* gain-of-function genetic studies in mouse and chick have shown LBH promotes an undifferentiated, proliferating fetal progenitor state, while blocking cell differentiation (Briegel et al., 2005; Conen et al., 2009). Yet, *Lbh* KO in mice does not impair fetal development, vitality, or essential adult organ function (Lindley and Briegel, 2013). This indicates that, unlike other WNT target genes, LBH is not essential for embryonic stem cell development or adult tissue homeostasis. However, LBH, which is expressed in mouse and human MaSC after birth (Lim et al., 2010; Lindley et al., 2015), is critical for rapid MaSC expansion that drives the extensive mammary tissue growth during puberty and pregnancy (Lindley et al., 2015). Thus, LBH appears to be a unique stem cell regulator needed only in situations, requiring increased adult stem cell activity.

Increased stem cell function and tumor dedifferentiation are also hallmarks of cancer (Hanahan and Weinberg, 2011). It is therefore notable, that we found LBH is a CSC-specific gene OE in CSC-enriched BrCa, and crucial for breast CSC expansion as well as WNT-induced tumor stem cell amplification. Despite the paramount importance of WNT as CSC-promoting pathway (Schwitalla et al., 2013), there are currently no WNT-specific treatments approved in the clinic, as targeting WNT signaling components is often cytotoxic due to the pleiotropic essential roles of WNT in tissue development and homeostasis (Clevers and Nusse, 2012). Our work provides a novel rationale for targeting LBH to inhibit the CSC-promoting effects of WNT, with likely little side effects in patients.

LBH is also a direct TGFß/SMAD3 target gene, however induction of *LBH* by TGFß appears limited to rare claudin-low TNBC (Tufegdzic Vidakovic et al., 2015). Interestingly, in claudin-low TNBC lines, TGFß promotes CSC (Bruna et al., 2012), and LBH KD studies in claudin-low MDA-MB-231 suggest that LBH is required for the CSC-promoting effect of TGFß in this TNBC subtype (Tufegdzic Vidakovic et al., 2015). In contrast, in luminal BrCa cells (i.e. MCF7), that lack LBH, TGFß acts as tumor suppressor and inhibits TI-SC activity (Bruna et al., 2012). Thus, LBH may be required downstream of multiple CSC-inducing signaling pathways.

Importantly, our work uncovered a critical requirement for LBH in metastasis, the main cause of cancer deaths. LBH potently induced BrCa cell motility, invasion, whereas LBH KD abolished metastasis from primary tumors, and after tail vein injection, which was accompanied by a reduction in metastasis-initiating CSC. Our notion that LBH may be a metastasis-promoting gene is also supported by a recent study in pancreatic cancer mouse models highlighting *LBH* as a gene epigenetically activated during metastatic tumor progression (Roe et al., 2017).

In further support of our work, functional studies in gastric and glioblastoma cell lines have shown LBH promotes invasion *in vitro*, and tumor growth *in vivo* (Jiang et al., 2019; Yu et al., 2019). Notably, in glioblastoma cells LBH was induced by hypoxia (Jiang et al., 2019), a feature of stem cell niches promoting CSC. In discrepancy with our results, studies in nasopharyngeal, lung and prostate cancer cell lines suggest LBH is tumor suppressive, arrests cell proliferation in G1/S-phase, and inhibits tumor invasion (Deng et al., 2018; Liu et al., 2015; Liu et al., 2018). Unlike our investigation, however, none of these studies examined LBH effects in different tumor subtypes. Thus, LBH may have dual roles in cancer depending on the tissue- and/or cell-of-origin.

Collectively, our study calls attention to LBH as a novel CSC-intrinsic WNT effector predominantly expressed in TNBC that is key to promoting CSC and metastatic traits in BrCa. Importantly, our findings suggest that targeting LBH may provide a novel means of eliminating treatment-resistant CSC and lethal metastases, with likely little side effects in patients. Future studies are needed to decipher the precise mechanisms underlying the CSC- and metastasis-promoting function of LBH and to test the potential therapeutic benefit of LBH inhibition in diverse cancer types driven by WNT.

Author Contributions

Conceptualization, K.J.B.; Methodology, K.J.B., K.G., K.A-B., M.E.R., and C.J.; Investigation, K.G., K.A-B., P.C., M.E.R., L.E.L, B.W., D.A., and K.J.B.; Validation, K.G., K.A-B., and K.J.B.; Formal Analysis, C.J., D.K., Y.B., Z.G. and K.J.B. ; Data Curation, K.G., K.A-B., A.H.S, D.K., and Y.B.; Resources, M.N., S.K., and K.J.B.; Writing – Original Draft, K.J.B. and M.E.R.; Writing – Review & Editing, K.J.B, K.G., and J.M.S.; Visualization, K.J.B., K.G., M.R., L.E.L., B.W.; Supervision, K.J.B, M.N., S.X.C., and J.M.S.; Project Administration, K.J.B.; Funding Acquisition, K.J.B.

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Declaration of Interests

The authors declare no competing interests.

STAR * METHODS

MATERIALS AND METHODS

Cell lines

The breast cancer cell lines MCF7, T47D, ZR751, SKBR3, BT474, BT549, BT20, MDA-MB-361, MDA-MB-231, MDA-MB157, MDA-MB-436, MDA-MB-468, HCC1395, HCC1937, HCC1187, HCC1500, DU4474, and the normal-derived MCF10A cells were newly purchased from ATCC. The breast cancer cell lines, SUM52, SUM149, SUM 1315 were from Asterand. Primary HMEC cells were from Lonza. CAL51 was kindly provided by Dr. Chris Lord (UK), and MDA-MB-231 derivative, 4175 (Minn et al., 2005), expressing luciferase by Dr. Joan Massague (Sloan Kettering Institute). All cell lines were maintained as recommended by the suppliers. Specifically, BT549, MDA-MB-231, 4175, MDA-MB-157 were grown in DMEM+10% FBS, Non-essential Amino Acids and Pen-Strep; HCC1395, HCC1187 in RPMI+10% FBS, Pen-Strep; and MCF7 in IMEM+10%FBS, Insulin and Pen-Strep in a 5% CO_2 incubator at 37°C. Mycoplasma tests were routinely performed using MycoAlert Mycoplasma Detection Kit (Lonza). Stable LBH-overexpressing BT549 cell lines were generated by nucleofection of $2x10^6$ BT549 cells with 2 µg linearized pCDNA3 or pCDNA3+Lbh (Briegel and Joyner, 2001) and 1 µg pEGFP (Lonza) in Solution V (Lonza), using program A-023 on an Amaxa nucleofector. 48 hours post nucleofection, growth media containing 350 µg/ml G418 was applied to the cells to select for stably integrated transfectants. MCF7 cells were stably transfected with pCDNA3 or pCDNA3+Lbh (Briegel and Joyner, 2001) using Lipofectamine (Invitrogen), and after selection in 300 µg/ml G418 cells were tagged with luciferase by transduction with lentiviruses expressing pFU-Luc2-eGFP (Addgene).

Small interfering RNA (siRNA) and short hairpin RNA (shRNA)-mediate gene knockdown

For RNAi studies, triplicate samples of cells were transiently transfected with 2 nM of synthetic siRNA specific for *LBH* or a scrambled control sequence (Dharmacon SmartPool) using Dharmafect #1 transfection reagent (Dharmacon) according to manufacturer's protocol. Cells were incubated with siRNA containing media for 72 hours prior to splitting for other studies. For stable LBH knockdown, MDA-MB-231 (ATCC) and MDA-MB-231 variant, 4175 (J. Massague), were transduced with ready-made Mission shRNA lentiviral particles (Sigma) expressing four different *LBH*-specific shRNAs (TRCN0000107525-shLBH#1, TRCN0000107529-shLBH#2, TRCN0000107538-shLBH#3 and TRCN0000107533-shLBH#4) or a non-targeting control shRNA (Sigma, SHC002V) at MOI=5 and in the presence of 8 µg/ml polyprene, as in (Wang et al., 2012). Individual MDA-MB-231 and 4175 polyclonal cultures stably expressing LBH shRNA or control shRNA were obtained by selection in 2-5 µg/ml puromycine for > 10 days.

Immunohistochemistry (IHC)

Breast cancer tissue microarrays (Biomax BR961, BRC962, BR1503e) and paraffin sections from 24 clinical breast cancer samples representing five different tumor subtype (luminal hormone receptor/HR+ - low grade; luminal $\mathrm{HR}^{\mathrm{neg}}$ - high grade; TNBC-metaplastic; TNBC-medullary; and TNBC-atypical medullary) were either incubated overnight at 4°C with custom-made, affinity-purified anti-LBH antibody, as described (Lindley et al., 2015), or stained with commercial anti-LBH antibody (Sigma) using an automated Leica IHC staining platform. LBH immunostaining was scored by two pathologists blinded to the identity of the specimens. Scores were given as the percentage of carcinoma cell nuclei staining positive, with an absolute intensity on a scale of 0-3 (0, none; +1, low; +2, moderate; +3, strong homogenous immunostaining). Tumors with LBH immunostaining scores of 0 and +1 in tumor cell nuclei were considered $\mathrm{LBH}^{\mathrm{low}}$, whereas tumors with immune staining scores of +2 and +3 and >50% of carcinoma cell nuclei staining positive for LBH were considered $\mathrm{LBH}^{\mathrm{high}}$.

Bioinformatics Analysis of Public Data

Gene expression data for *LBH* in primary human breast cancers were obtained from the following published datasets: TCGA (Cancer Genome Atlas, 2012), METABRIC (Curtis et al., 2012), Lehmann (Lehmann et al., 2016), NKI-295 (van de Vijver et al., 2002), Hatzis (Hatzis et al., 2011) (GSE25066), and Creighton (Creighton et al., 2009) (GSE7513). DNA copy number information was extracted from the normalized SNP6.0 data downloaded from the METABRIC data set (Margolin et al., 2013). These cohorts were divided into TNBC and non-TNBC tumors according to their IHC-defined biomarker status.

TCGA gene expression values are log2 transformed RSEM values and downloaded from UCSC Xena (https://xenabrowser.net). TCGA breast cancer subtypes were identified based on the gene expression using TNBCtype (Chen et al., 2012). LBH^{high} and LBH^{low} samples were defined relative to the median LBH expression level of TCGA samples of the selected TNBC subtypes (BL1 and BL2). Gene set enrichments in LBH^{high} vs. LBH^{low} TNBC tumors in the TCGA data set (Cancer Genome Atlas, 2012) was analyzed using Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005). LBH^{high} and LBH^{low} patients were defined relative to the median LBH value of all patients in the NKI-295 data set. Kaplan-Meier analysis and log-rank test of survival difference between LBH^{high} and LBH^{low} patients were performed using the *survival* package from R (ver. 3.5.0). For meta-analysis of correlation with chemo resistance in breast cancer, we used the KM plotter (http://kmplot.com/analysis) (Gyorffy et al., 2010). Patient samples were selected based on whether they received neoadjuvant or adjuvant chemotherapy and grouped for high or low mRNA expression of LBH. All percentiles between the lower and upper quartiles were computed, and the best performing threshold was used as cutoff.

Quantitative Real-time PCR (qPCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen), and reverse transcribed with M-MLV Reverse Transcriptase (Promega). qPCR was performed using SsoFast Evagreen PCR master Mix (BioRad) and a Bio-Rad CFX96 Thermal Cycler. mRNA expression was normalized to the expression of *GAPDH* using standard comparative C_t method.

Western Blot (WB) analysis

Cells were harvested in RIPA lysis buffer (Thermo Fisher Scientific, MA, USA) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, UK). Cell lysates were passed 5-8 times through a 26-gauge needle before centrifugation at high-speed. Cleared lysates were snap frozen until further use. 20-50 µg of total protein lysates were separated under reducing conditions (2.5% β-mercaptoethanol) by SDS-PAGE (SDS-Polyacrylamide Gel Electrophoresis) and transferred to nitrocellulose membrane (Amersham GE, UK) using Amersham ECL semi-dry blotter (GE). Membranes were incubated with primary antibodies to LBH (Sigma 1:1,000); CD44 (CST 1:1,000); K5 (Covance

1:2,000); K8 (Fitzgerald 1:2,000); SOX2 (CST 1:1,000); MYC (9E10; Roche; 1:2,000), OCT4 and NANOG (Novus 1:1,000); E-Cadherin (BD; 1:1,000); Vimentin (Sigma 1:1,000); or β-actin (Sigma, 1: 50,000) in TBST + 5% milk, followed by incubation with secondary HRP-coupled anti-rabbit or anti-mouse antibodies (Santa Cruz 1:10,000). Protein bands were detected using the West Femto Super Signal Kit (Thermo) on X-ray film, and quantified by densitometry and ImageJ analysis.

Proliferation Assay (MTS)

Cells were seeded in quadruplicates in 96 well plates at a density of 2×10^3 cells per well. Viable cell growth was determined using the CellTiter 96 AQ_{ueous} One Cell Proliferation Assay (Promega) with absorbance measurements taken daily at OD λ492nm. Data were normalized to values measured at day 0.

Clonogenicity assays

For two-dimensional (2D) colony formation assays, cells were plated in triplicate at low density (100 – 500 cells) in 6-well plates and grow for 2 - 3 weeks with regular media changes. Cells were fixed in methanol and stained with 0.35% crystal violet solution. Colonies were counted manually or using Gelcount (Oxford Optronix). For three-dimensional (3D) anchorage-independent soft agar growth assays, 0.6% Noble Agar (BD) was used as bottom layer in a 6-well plate and topped with a layer of 0.3% noble agar mixed with 5×10^3 (MDA-MB-231, HCC1395, MCF7) or 2×10^4 (BT549) cells in selective drug-containing growth medium. Triplicate samples of single cell suspensions were plated and grown for 21-28 days at 37°C in a 5% CO_2 incubator, with addition of 100 µl of fresh growth media every 2 days. Colonies were stained with 0.05% crystal violet for > 2 hours, imaged, and colonies larger than 50 cells or 8 megapixels at 100% magnification were counted.

Cell migration/invasion assays

Boyden chamber Transwell migration and Matrigel invasion assays were performed as in (Wang et al., 2012). For Matrigel colony invasion assays in 3D, single cell suspensions of 2.5×10^3 BT549 cells in 100 μl of complete growth media mixed with ice cold Matrigel (BD) (1:1) were plated in triplicate on 96-well plates. Plates were incubated at 5% CO_2, 37°C for 30 min to allow the Matrigel to solidify, where after 100 μl of complete media + 200 μg/ml G418 was added to each well. The culture media was changed every 2 days. Ten to 14 days after plating, pictures were taken under bright field at 20X magnification using a Leica DMIL inverted microscope.

Chemotherapy drug studies

MDA-MB-231-4175 (2.5×10^3 cells) were seeded on 96 well plates in triplicates in complete growth media. After 24 hours, media was replaced with media containing paclitaxel (Sigma) at different concentrations (1 μM; 0.33 μM; 0.11 μM; 0.037 μM; 0.01 μM; 0.004 μM and, 0.001 μM). Cells were grown for an additional 72 hours, after which cell viability was quantified using CellTiter 96 AQ_{ueous} One Cell Proliferation Assay (Promega). IC_{50} concentrations were calculated by standard dose response curve method using Graphpad Prism software.

Flow Cytometry, Cell Sorting

CD44-CD24 FACS analysis of human breast cancer cell lines was performed as in (Lindley and Briegel, 2010). Briefly, 1×10^6 cells from subconfluent cultures in triplicates were resuspended in 100 μl ice cold PBS+2%FBS. Cells were immunostained with 20 μl each of anti-CD24-PE (BD Biosciences) and anti-CD44-APC (BD Biosciences) antibodies for 30 min. on ice, washed with ice cold PBS+2%FBS, and resuspended in 500 μl final volume of PBS+2% FBS for analysis or sorting using LSR-II BD analyzer or FACS Arias, respectively. ALDH1+ stem cells were quantified using ALDEFLUOR kit (Stem Cell Inc.). Data were analyzed using FlowJo software.

Primary mammary epithelial cells (MEC) (2×10^6), isolated from 12-week-old females mice as previously described (Lindley et al., 2015), were blocked for 10 min in ice-cold PBS+2% HI-FBS containing anti-CD16/CD32 (BD Biosciences) and rat-γ-globulin (Jackson ImmunoResearch) antibodies. Cells were immunostained for 30 min with APC-conjugated CD45, CD31 and TER119 antibodies (BD Biosciences) specific to Lineage (Lin) markers in combination with anti-CD24-PE and anti-CD29-FITC antibodies (BD Biosciences). Labeled cells were washed with ice-cold PBS+2% HI-FBS, incubated for 30 min with Streptavidin-APC (Invitrogen) and with violet dead cell marker (Invitrogen) to exclude Lin+ and dead cells, filtered through a 40 µm filter (BD Falcon), and sorted using a FACS Aria-II (BD Biosciences). Sorted Mammary stem cell (MaSC)-enriched $CD29^{high}CD24^{+}$ cells were then plated in triplicates in low attachment 6-well plates at 10^4 cells per well for mammosphere assays (see above).

Mammosphere Assays

Single cells were plated in triplicates on low-attachment 6-well plates (Corning) at a density of 2–5 x 10^3 (human breast cancer lines) or 1x10^4 (primary mouse mammary epithelial cells) cells per well in serum-free DMEM/F12 medium supplemented with 20 ng/ml EGF, 20 ng/ml bFGF, B27 supplement [Invitrogen] diluted 1:50, and 1 mg/ml penicillin/streptomycin, as described (Lindley et al., 2015). Spheres were allowed to form for 5-14 days at 37°C in a 5% CO_2 incubator and then quantified. For serial passaging, primary tumor spheres after 7 days in culture were collected in culture media by centrifugation at 450 g for 5 min, washed with PBS, trypsinized, and mechanically dissociated into a single cell suspension using a 21-gauge syringe. After washing in media containing 2% heat inactivated FBS (HI-FBS), cells were resuspended in PBS for counting and re-plating in secondary mammosphere cultures (Lindley et al., 2015).

Infection and sphere assays of primary breast cancer cells

Fresh tissues from a primary triple-negative breast cancer (TNBC1) resected from a patient after neoadjuvant chemotherapy was obtained from the Biospecimen Shared Resource, Sylvester Comprehensive Cancer Center, University of Miami. Tumor tissues were minced into small pieces and dissociated into single cells using Milteny human tumor tissue dissociation kit (#130-095-929) and Milteny gentle MACS tissue dissociator at 37°C for 1 hour according to the manufacturer's protocol. Cells were treated with DNase I (StemCell Technologies #07900), washed with PBS, followed by RBC elimination using ACK Lysing solution (Thermo). Cells were washed again with PBS and passed through a 40-µm nylon strainer. Thereafter, cells were pelleted, resuspended in mammosphere medium and grown at low density (5,000 cells/ml) on ultralow-attachment plates (Corning) for 10-14 days to enrich for CSC. After four passages, TNBC1 spheres were dissociated into single cells by trypsinization for 5 min with 0.05% trypsin (Gibco, #25300-62). 1X trypsin inhibitor (1000X; 50mg/ml stock concentration; Sigma-Roche) was added to inactivate Trypsin, where after cells were washed two times with PBS and counted. 60,000 viable cells were resuspended in 5 ml mammosphere medium (see above) containing polybrene 1µg/ml (Sigma #107689). Cells were infected with 20 µl of concentrated lentiviruses expressing either LBH-specific Tet-shRNA vectors (KD1 and KD2) and non-target Tet-shRNA (Horizon Discovery), or pLVX TetOn-LBH expression vector (custom made) by spin transduction for 90 min at 1,000 g and 4°C, using a Thermo benchtop centrifuge equipped with a swing bucket rotor and 50ml tube adapters. After removing supernatants, cells were resuspended in fresh sphere medium and plated in triplicates -/+ 1 µg/ml Doxycycline (DOX) on 6-well ultralow attachment plates at 10,000 cells/well in 2 ml sphere medium. Sphere formation was assessed 9 days after Dox addition and growth at 37°C, 5% CO2 by counting and imaging (Fig. 4K). RNA was harvested from spheres to determine LBH knockdown and overexpression efficiencies by qPCR.

Mouse Studies

For Xenograft experiments, 5-week-old NOD-SCID IL2Rgamma^{null} female mice [= NSG; Stock No. 005557] were purchased from the Jackson Laboratories (Bar Harbor, ME). MDA-MB-231-4175 (1 x 10^5) or MCF7 (2 x10^6) cells suspended in 100 µl PBS containing 50% matrigel (BD) were injected into the inguinal mammary fat pad of NSG mice (n>6/group). For limiting dilution experiments 1x10^4/10^3/10^2 4175 or 5x10^5/10^4/10^3 MCF7 cells were injected. Additionally, MCF7 Xenografts received a 90-day slow-release estrogen implant (0.18 mg/pellet). Primary tumor growth was quantified weekly by caliper measurement and Bioluminescence (BLI) Imaging using IVIS Spectrum *In Vivo* Imaging System (Perkin Elmer). For orthotopic metastasis assays, primary tumors formed from orthotopic injection of 1 x 10^5 4175 cells (see above) were resected when they reached a size of 1 cm^3, where after mice were monitored weekly by BLI imaging to detect metastasis formation. For tail vein metastasis assays, 1 x

10^5 4175 tumor cells suspended in 100 µl PBS were injected intravenously (n=8 mice/group). At protocol-defined endpoints, primary tumors, lungs and other organs (spleen, liver) were dissected, weighed, and subjected to *ex vivo* IVIS analysis for the detection of metastases and histopathological analysis. For *in vivo* stem cell analysis in genetic mouse models, MMTV-Wnt1 transgenic (Tg) mice [B6SJL-Tg(Wnt1)1Hev/J; Stock No. 002870; Jackson Laboratories] were interbred with ROSA26(R26)Cre;Lbh$^{\Delta E2/\Delta E2}$ knockout (KO) mice (Lindley and Briegel, 2013) and SHIP-GFP transgenic mice [B6.Cg-Tg(Inpp5d-EGFP)DLrr/CprJ; Stock No. 024808; Jackson Laboratories] (Bai and Rohrschneider, 2010). All experiments and procedures involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Miami in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In situ GFP analysis

Mammary tumor tissues from MMTV-Wnt1Tg;R26-Cre;SHIP-GFP+;LBH$^{+/+}$ wild type (WT) and MMTV-Wnt1Tg;R26-Cre;SHIP-GFP+;LBH$^{-/-}$ KO female mice were fixed in 2% paraformaldehyde for 3 hours, immersed in 30% sucrose overnight at 4°C, embedded in O.C.T, snap frozen on dry ice, and sectioned (10 µm). Tissue cryo-sections were mounted in Slowfade Gold Mounting Media with DAPI counterstain (Thermo). Endogenous GFP expression was evaluated by Confocal Microscopy on a Leica SP5 Inverted Confocal Microscope and quantified using ImageJ software.

RNA-Seq Gene Expression Analysis

RNA from triplicate samples was extracted using Trizol (Invitrogen) and quantified by Agilent 2100 Bioanalyzer. RNA-Seq, including mRNA enrichment, library preparation, sequencing and data analysis, was performed by Novagen Inc. (California, USA). mRNA was enriched from 1 ug of total RNA starting material using Illumina TruSeq RNA Sample Prep Kit. RNA libraries were then prepared using NEBNext Ultra RNA-Seq library prep kit (New England Biolabs) followed by high-throughput sequencing on Illumina HiSeq 200. Between 38 and 60 million reads were obtained from each sample. Illumina Casava1.7 software was used for base calling. Raw sequence paired-ended data in FASTQ format were assessed for quality with FastQC (v11.5). Trimmomatic (ver.0.32) was used to remove adapters, Illumina-platform specific sequences, and low quality reads (Bolger, et al., 2014).

Reads were aligned to reference genome Homo sapiens GRCh37/hg19 (NCBI/UCSC/Ensembl) using STAR (v2.5) (Dobin et al., 2013). HTSeq v0.6.1 was used to count the read numbers mapped of each gene. Differential expression analysis for comparisons of two conditions (-/+LBH) per cell line (three biological replicates per group) with respect to their controls was performed using the DESeq2 R package (2_1.6.3) (Love et al., 2014). The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with FDR < 0.05 were assigned as differentially expressed.

GSEA analysis was conducted using the Wald statistic output from DESeq2 results and permutation by gene set for MSigDB v7.1 (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp) gene sets in the C2:CP, C2:CPG, C6, and Hallmark data sets (Subramanian et al., 2005). Only enriched gene sets commonly up- or down-regulated by LBH in all four cell lines (HCC1395, MDA-MB-231, BT549, MCF7) with p-values <0.05 and FDR q<0.25 were considered. The RNA-Seq data was deposited in the GEO database with accession number GSE151206 (see Key Resources Table for access information).

Statistical Analyses

Statistical analyses were performed using Microsoft Excel, GraphPad Prism Software, or statistical software package R (version 3.3.1). Each experiment was repeated three times or more and data are presented as mean ± standard deviation (SD) or standard error of the mean (SEM) unless otherwise noted. Data were analyzed by unpaired two-tailed Student's *t*-test, or analysis of variance (ANOVA) for more than two group comparison. Chi-square test was used to compare categorical clinical variables with LBH protein expression status (low vs. high) shown in Table 1. One sample proportion test was used to examine whether proportion of overall high LBH protein expression among IDC is 50%. Gene expression and copy number differences in clinical samples were evaluated by two-sided Mann-Whitney U-test. The log-rank test was used for Kaplan-Meier survival analysis. Results with $p<0.05$ were considered statistically significant.

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TABLES

Table 1.

Association of LBH expression with clinical parameters in breast cancer patients

			LBH		LBH		
Variable	**All**		**Low**		**High**		
	n	***%***	***n***	***%***	***n***	***%***	***p-value***$^{\#}$
Total number of IDCs	**250**	**100**	**210**	**84.0**	**40**	**16.0**	**<.0001**$^{\$}$
Estrogen Receptor							**0.0002**
Negative	145	58.0	111	52.9	34	85.0	
Positive	105	42.0	99	47.1	6	15.0	
Progesterone Receptor							**0.0030**
Negative	156	61.6	121	57.6	33	82.5	
Positive	94	38.4	89	42.4	7	17.5	
HER2*							0.6218
Negative	173	69.2	144	68.6	29	70.0	
Positive	77	30.8	66	31.4	11	27.5	
Triple-negative							**<.0001**
Yes	74	29.6	52	24.8	22	55.0	
No	176	70.4	158	75.2	18	45.0	
TNM stage							0.3128
Tis or T1 or T2	152	60.8	128	61.0	24	60.0	
T3 or T4	82	32.8	73	34.8	9	22.5	
Unknown	16	6.4	9	4.3	7	17.5	
Lymph Node status							0.4978
N0 or N1	194	77.6	168	80.0	26	65.0	
N2 or N3	40	16.0	33	15.7	7	17.5	
Unknown	16	6.4	9	4.3	7	17.5	
Tumor grade							**0.0092**
Low grade	31	12.4	31	14.8	.	.	
High grade	218	87.2	178	84.8	40	100.0	
Unknown	1	0.4	1	0.5	.	.	
Tumor Subtype							**0.0001**
Basal like	69	27.6	47	22.4	22	55.0	
*HER2 type^*	107	42.8	97	46.2	10	25.0	
Luminal A/B	74	29.6	66	31.4	8	20.0	

IDC = Invasive ductal carcinoma. Bold data indicate significant correlation.

[#] Chi-square test was used. [$] One proportion test was used.

* HER2 clinical status based on immunohistochemistry.

^ HER2 molecular subtype based on HER2 gene expression.

MAIN FIGURES

Figure 1

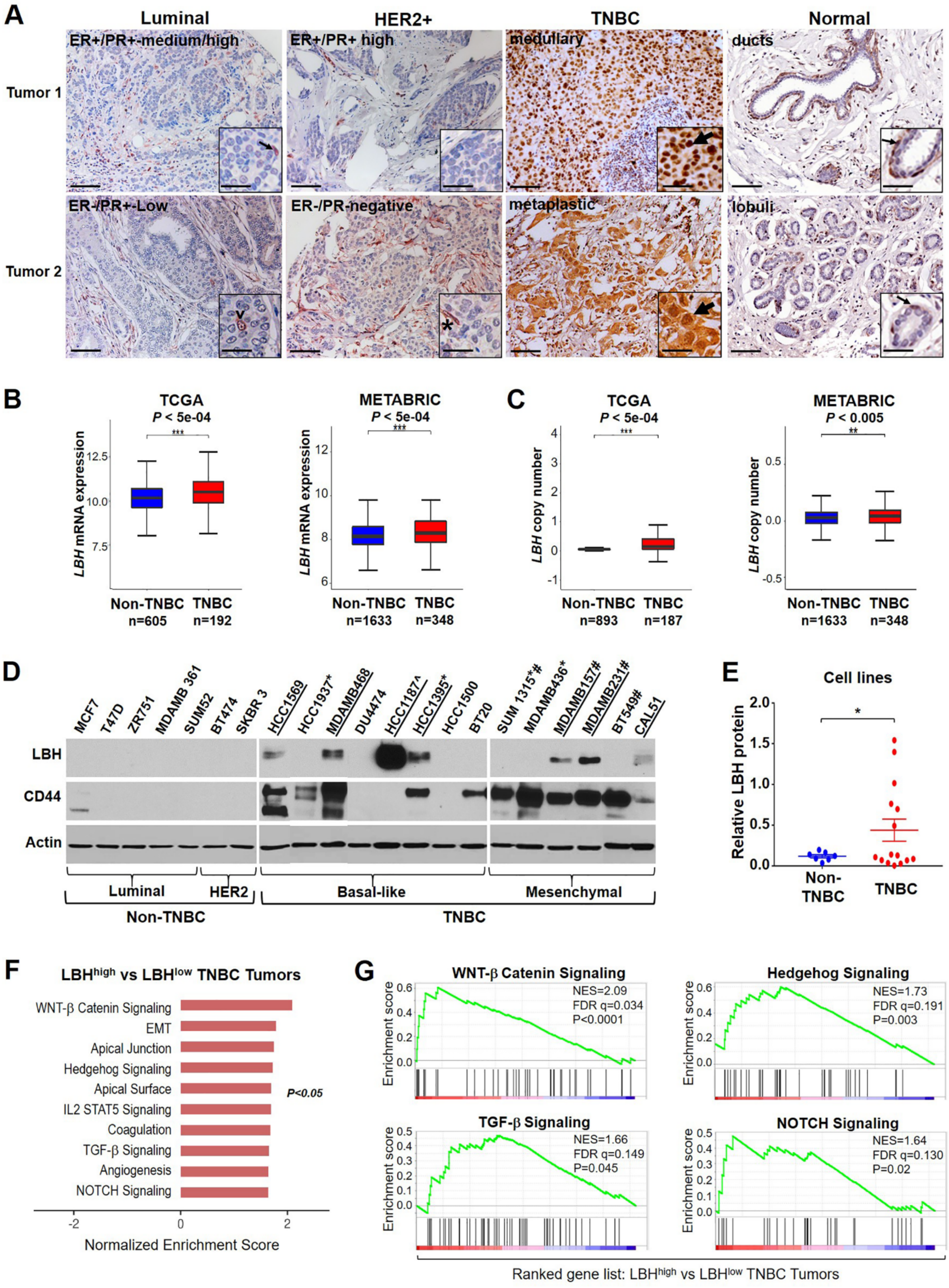


Figure 1: LBH is highly expressed in TNBC

(**A**) Representative IHC analysis of LBH in primary human BrCa, high-grade II-III) and normal breast tissues; Scale bars, 100 µm. Insets = close-ups; Scale bars 50 µm. Thin arrows = normal basal epithelial cells with nuclear LBH; Arrow head = tumor cell with nuclear LBH; Thick arrows = tumor cells with nuclear + cytoplasmic LBH; asterisk = LBH+ tumor stroma.

(**B, C**) Box plots showing *LBH* gene expression (**B**) and copy numbers (**C**) in TNBC (red) and non-TNBC (blue) BrCa in the TCGA (Cancer Genome Atlas, 2012) (left) and METABRIC (Curtis et al., 2012) (right) data sets. Number (n) of patients per group as indicated. *P* values by two-sided Mann-Whitney U test. See also Figure S1A-C.

(**D**) WB analysis of LBH and CD44 in human BrCa cell lines. LBH expression (underlined), LBH gene amplification (triangle), BRCA1 mutant (asterisk) and claudin-low (number sign) status, as indicated. See also Figure S1D.

(**E**) Relative LBH protein in TNBC *vs.* non-TNBC lines (B), quantified by densitometry relative to β-actin loading control. Two-tailed Student t-test, n=3, *p<0.05.

(**F, G**) Gene set enrichment analysis (GSEA). (**D**) Top 10 gene signatures differentially expressed between LBH^{high} and LBH^{low} TNBC in the TCGA data set; p<0.05 and FDR<0.25.

(**G**) Enrichment plots of enriched stem cell signaling pathways in LBH^{high} vs. LBH^{low} TNBC. NES, Normalized Enrichment Score; FDR, False Discovery Rate.

Figure 2

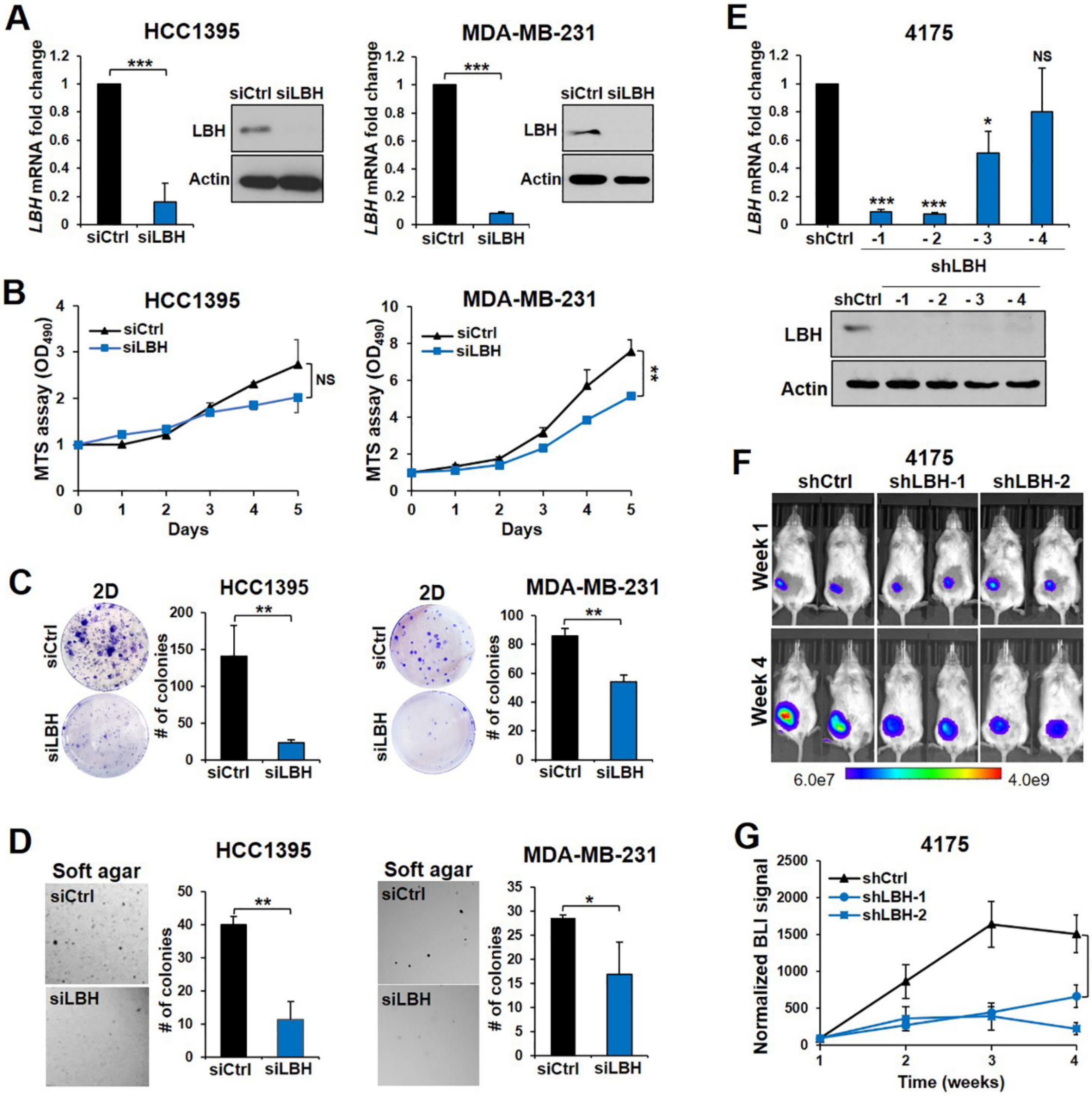

A
HCC1395
LBH mRNA fold change
1.2
1
0.8
0.6
0.4
0.2
0
***
siCtrl
siLBH
LBH
Actin
MDA-MB-231
B
HCC1395
MTS assay (OD490)
Days
NS
MDA-MB-231
**
C
2D
# of colonies
HCC1395
MDA-MB-231
D
Soft agar
*
E
4175
shCtrl
-1
-2
-3
-4
shLBH
NS
F
4175
shCtrl
shLBH-1
shLBH-2
Week 1
Week 4
6.0e7
4.0e9
G
4175
Normalized BLI signal
2500
2000
1500
1000
500
0
Time (weeks)
1
2
3
4
shCtrl
shLBH-1
shLBH-2

Figure 2. LBH is required for clonogenicity and tumor growth of TNBC cells

(**A-D**) TNBC cell lines, HCC1395 and MDA-MB-231, transiently transfected with a pool of four independent LBH-targeted siRNAs (siLBH) or scrambled siRNA control (siCtrl).

(**A**) qPCR (left) and WB analysis (right) of analysis of LBH expression 3-4 days after siRNA transfection.

(**B**) Proliferation (MTS) assays in adherent cell cultures (normalized to day 0).

(**C**) Two-dimensional (2D) colony formation.

(**D**) Anchorage-independent growth in soft agar. Representative images (left) and quantification of colony numbers (#) (right).

Experiments (A-D) were repeated > three times with n = 3 replicates per group.

(**E-G**) Luciferase-tagged MDA-MBA-231 variant, 4175, stably transduced with non-target control shRNA (shCtrl) or different *LBH*-targeted shRNAs (shLBH1-4)**.**

(**E**) qPCR (left) and WB analysis (right) show efficient LBH KD (>85%) in 4175 TNBC cells transduced with shLBH-1 and shLBH-2.

(**F**) Representative BLI images after orthotopic mammary fat pad injection of 4175 cells (10^5) expressing shCtrl, shLBH-1, or shLBH-2 into NSG female mice.

(**G**) Quantification of tumor growth by weekly BLI measurements (photons per second) normalized to BLI signals at day 1. n = 6 mice per group. Experiment E-G was repeated twice.

Data represent means ± SEM. NS, not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Significance determined by linear regression models (B, G) or Student’s *t*-test (A,C-E).

Figure 3

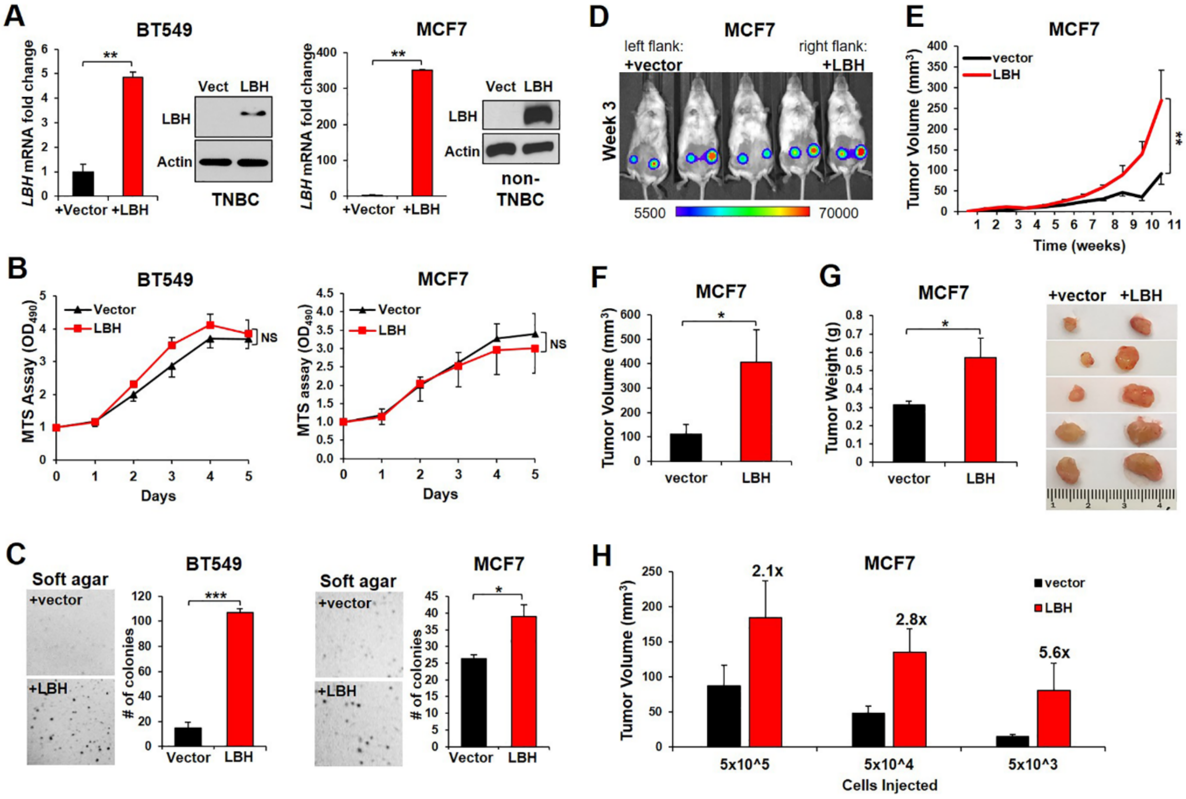

A
BT549
LBH mRNA fold change
**
+Vector
+LBH
Vect
LBH
LBH
Actin
TNBC
MCF7
LBH mRNA fold change
**
+Vector
+LBH
Vect
LBH
LBH
Actin
non-TNBC
D
MCF7
left flank:
+vector
right flank:
+LBH
Week 3
5500
70000
E
MCF7
vector
LBH
Tumor Volume (mm^3)
**
Time (weeks)
B
BT549
Vector
LBH
MTS Assay (OD$_{490}$)
NS
Days
MCF7
Vector
LBH
MTS assay (OD$_{490}$)
NS
Days
F
MCF7
Tumor Volume (mm^3)
*
vector
LBH
G
MCF7
Tumor Weight (g)
*
vector
LBH
+vector
+LBH
C
Soft agar
+vector
+LBH
BT549
# of colonies
***
Vector
LBH
Soft agar
+vector
+LBH
MCF7
# of colonies
*
Vector
LBH
H
MCF7
vector
LBH
Tumor Volume (mm^3)
2.1x
2.8x
5.6x
5x10^5
5x10^4
5x10^3
Cells Injected

Figure 3. LBH promotes tumorigenicity of TNBC and non-TNBC cells

(**A-D**) BT549 (TNBC) and MCF7 (non-TNBC) BrCa cell lines stably transfected with pCDNA3-LBH expression plasmid (+LBH) or empty vector control (+vector).

(**A**) qPCR (left) and WB analysis (right) show ectopic LBH expression in BT549+LBH and MCF7+LBH cells.

(**B**) Proliferation (MTS assays) in adherent cell cultures (normalized to day 0).

(**C**) Anchorage-independent growth in soft agar. Representative images (right) and quantification of colony numbers (#) (left). Experiments (A-D) were repeated > three times with n = 3 replicates per group.

(**D-G**) Luciferase-tagged MCF+LBH and MCF7+vector cells (2 x 10^6) were injected contralaterally into the mammary fat pads of NSG female mice, as indicated.

(**D**) Representative BLI images of *in vivo* tumor growth at 3 weeks.

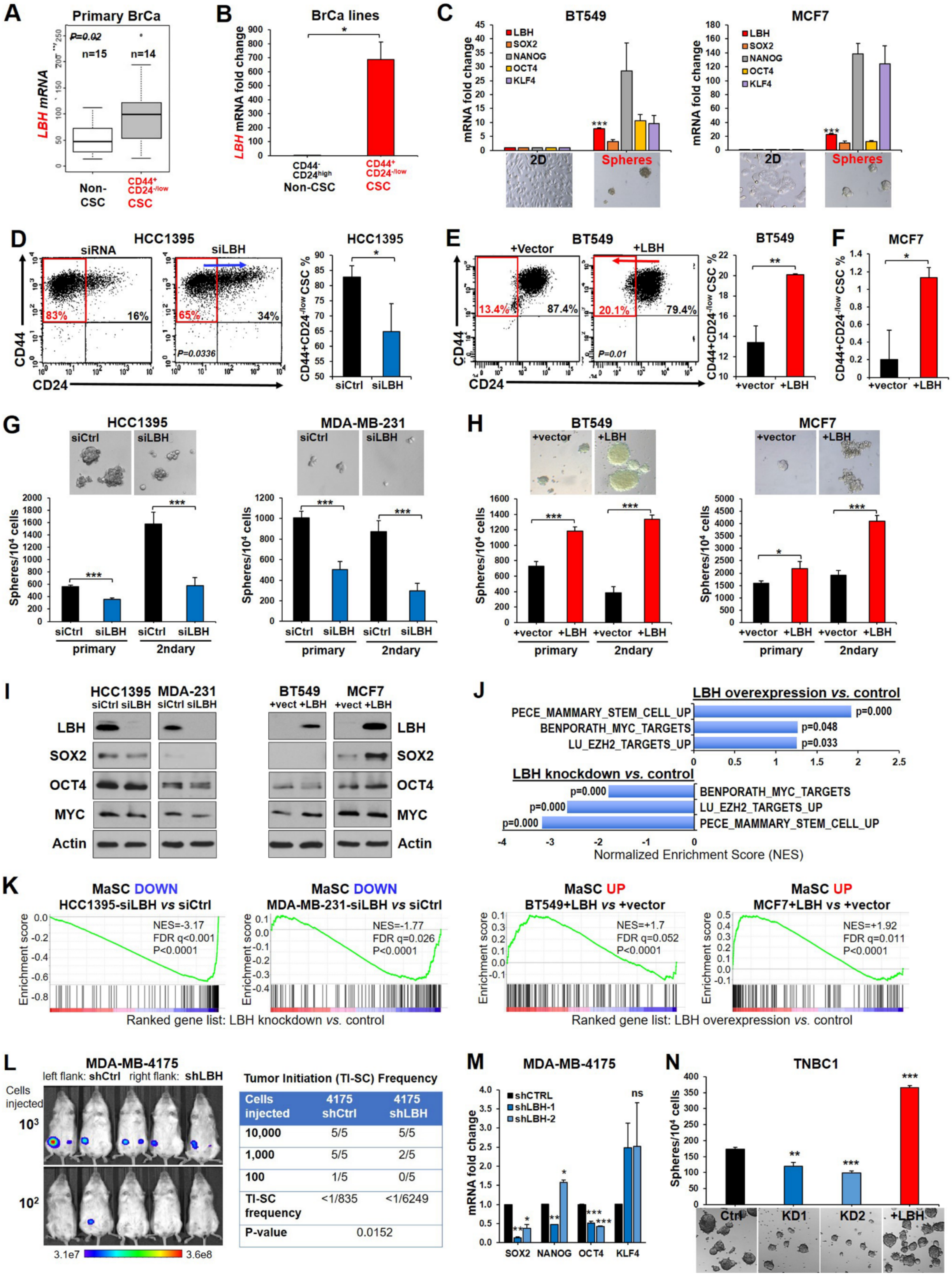
(**E**) Tumor volumes over time. n=6 mice per group.

(**F**) Tumor volumes and (**G**) Tumor weights at endpoint. n = 5 mice per group. Representative tumor images (right). Experiment D-G was repeated twice.

(**H**) Tumor volumes at 11 weeks post orthotopic injection of reducing concentrations of MCF+LBH or MCF7+vector cells into NSG female mice**.** n = 8 mice per group. $P < 0.05$.

Data represent means ± SEM. NS, not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Significance determined by linear regression models (B, E) or Student's *t*-test (A,C, F-H).

Figure 4



Cells injected	4175 shCtrl	4175 shLBH
10,000	5/5	5/5
1,000	5/5	2/5
100	1/5	0/5
TI-SC frequency	<1/835	<1/6249
P-value	0.0152	

Figure 4: LBH is expressed in breast CSC and promotes a self-renewing CSC phenotype.

(**A**) Gene profiling data for *LBH* in a TCGA data set (Creighton et al., 2009) comparing gene signatures of CD44^{+}CD24$^{-/low}$ CSC (grey bar) and non-CSC (white bar) populations from n > 14 primary human BrCa. *P* value by Mann-Whitney U test.

(**B**) qPCR analysis of *LBH* expression in FACS-sorted CD44^{+}CD24$^{-/low}$ CSC (red bar) and CD44^{-}CD24^{high} non-CSC (black bar) populations from BrCa lines. See also Figure S2A.

(**C**) qPCR analysis of *LBH* and pluripotency TFs (*SOX2, NANOG, OCT4, KLF4*) expression in CSC-enriched tumor spheres formed by parental BT549 TNBC and MCF7 non-TNBC cells in low-attachment suspension cultures *vs.* cells grown in 2D on adhesive plates.

(**D-F**) FACS quantification of CD44^{+}CD24$^{-/low}$ CSC populations in:

(**D**) HCC1395 cells 9 days after transient transfection with siLBH or scrambled siRNA (siCtrl);

(**E**) BT549, and (**F**) MCF7 stably expressing LBH (+LBH) or vector control (+vector). Representative FACS plots with gates for CD44^{+}CD24$^{-/low}$ CSC (red box) are shown on the right. Blue arrow (in D) indicates increased, red arrow (in E) reduced tumor cell differentiation.

(**G,H**) Quantitative serial sphere formation assays with representative tumor sphere images in:

(**G**) LBH knockdown HCC1395, MDA-MB-231 TNBC cells.

(**H**) LBH overexpressing BT549, MCF7 BrCa cells.

(**I**) WB analysis of LBH, SOX2, OCT4, and MYC expression.

(**J,K**) RNA-Seq gene expression and GSEA analyses:

(**J**) Bar diagrams depict stem cell gene signatures in the MSigDB C2:CPG data base commonly and significantly (P<0.05, FDR q<0.25) enriched among LBH-regulated genes in all four BrCa lines. Data for MCF7+LBH overexpression vs. MCF7+vector (top) and HCC1395-siLBH knockdown vs. HCC1395-siCtrl (bottom) are shown. Additional data for MDA-MB-231 and BT549 are shown in Figure S3D.

(**K**) Enrichment analysis of the MaSC gene signature from (Pece et al., 2019) (see J); NES, Normalized Enrichment Score. FDR, False Discovery Rate.

(**L**) Limiting cell dilution (10^5, 10^4, 10^3) orthotopic transplant assay of 4175 shCtrl (left flank) and 4175 shLBH-2 (right flank) cells. Representative BLI images at 5 weeks (right) and quantification (Table; left) of tumor initiating cell (TI-SC) frequencies (CI, 95% Confidence Interval; Chi Square Analysis/P = 0.0152) using http://bioinf.wehi.edu.au/software/elda/ software; n = 5 tumors per group.

(**M**) qPCR analysis of SC-TFs in 4175 shCtrl and LBH shRNA transduced lines.

(**N**) Tumor sphere formation of primary, patient-derived TNBC cells (TNBC1) transduced with two independent LBH shRNAs (KD1, KD2) and LBH cDNA (+LBH) expressing lentiviruses.

Data (B-H, M-N) represent means ± SEM of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$. $^{***}P < 0.001$. Student t-test; n >3 per group.

Figure 5

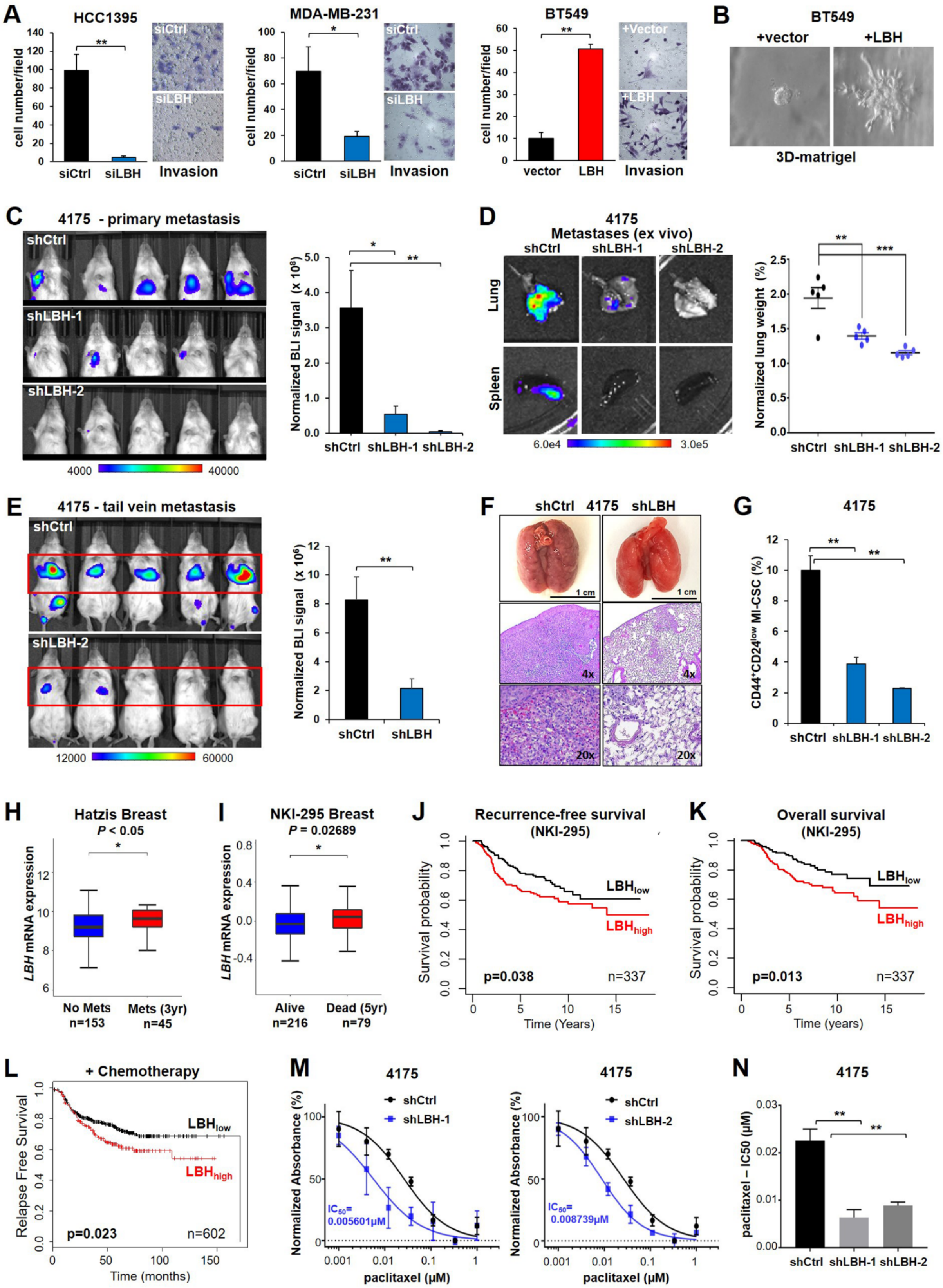

A
HCC1395
140
120
100
80
60
40
20
0
cell number/field
**
siCtrl
siLBH
Invasion
MDA-MB-231
*
BT549
**
vector
LBH
+Vector
+LBH
B
BT549
+vector
+LBH
3D-matrigel
C
4175 - primary metastasis
shCtrl
shLBH-1
shLBH-2
4000
40000
Normalized BLI signal (x 10^8)
D
4175
Metastases (ex vivo)
Lung
Spleen
6.0e4
3.0e5
Normalized lung weight (%)
***
E
4175 - tail vein metastasis
12000
60000
Normalized BLI signal (x 10^6)
shLBH
F
shCtrl 4175 shLBH
1 cm
4x
20x
G
4175
CD44+CD24low MI-CSC (%)
H
Hatzis Breast
P < 0.05
LBH mRNA expression
No Mets n=153
Mets (3yr) n=45
I
NKI-295 Breast
P = 0.02689
Alive n=216
Dead (5yr) n=79
J
Recurrence-free survival (NKI-295)
Survival probability
LBHlow
LBHhigh
p=0.038
n=337
Time (Years)
K
Overall survival (NKI-295)
p=0.013
n=337
Time (years)
L
+ Chemotherapy
Relapse Free Survival
LBHlow
LBHhigh
p=0.023
n=602
Time (months)
M
4175
shCtrl
shLBH-1
shLBH-2
Normalized Absorbance (%)
IC50= 0.005601µM
IC50= 0.008739µM
paclitaxel (µM)
N
4175
paclitaxel – IC50 (µM)

Figure 5. LBH promotes tumor invasion, metastasis, and chemoresistance

(**A**) Boyden chamber invasion assays in TNBC lines, HCC1395 and MDA-MB-231 transiently transfected with LBH siRNAs (siLBH) or scrambled siRNA (siCtrl), and BT549 stably expressing LBH (+LBH) or vector control (+vector). Representative images of crystal violet-stained invasive cells (right panels). n = 3 per group. See also Figure S4*.*

(**B**) Three-dimensional (3D) matrigel invasion assay. Note, only in the presence of LBH (+LBH), BT549 form spheres invading extracellular matrix.

(**C-D**) Orthotopic metastasis assay in 4175 TNBC cells stably expressing shLBH-1, shLBH-2 or non-target shCtrl.

(**C**) Representative BLI images (left) and quantification of *in vivo* metastatic burden (right) 7 weeks after orthotopic transplantation of cells (10^5). n = 6 mice per group.

(**D**) *Ex vivo* BLI images of lung and spleen metastases (left) and quantification of lung metastatic burden (right) by lung weight measurements (normalized to % of body weight). n=5 mice per group.

(**E, F**) Tail vein metastasis assay using 10^5 4175 control (shCtrl) and LBH KD (shLBH-2) cells.

(**E**) Representative BLI images (left) and quantification of lung metastatic burden (right) 5 weeks post engraftment. n=5 mice per group.

(**F**) Representative images (top) and H&E-stained tissue sections (middle, bottom) of lungs at endpoint. Scale bar, 1 cm. Magnifications as indicated.

(**G**) FACS quantification of metastasis-initiating $CD44^{+}CD24^{low}$ CSC (MI-CSC). n = 4 per group. See also Figure S2B.

(**H, I**) Box plots showing correlation of high *LBH* expression (red) with (**H**) metastatic event at 3 years (yr) and (**I**) patient death at 5 yr after diagnosis. Gene expression data were obtained from the Hatzis (Hatzis et al., 2011) and NKI-295 (van de Vijver et al., 2002) BrCa cohorts.

(**J, K**) Kaplan-Meier plots showing *greater-than-median* intra-tumor levels of *LBH* (LBH_{high}, red) associate with (**J**) reduced recurrence-free survival and (**K**) overall survival in the NKI-295 cohort.

(**L**) Top tier *LBH* expression (red) predicts reduced relapse-free patient survival after chemotherapy. Data were extracted from the Kaplan-Meier plotter dataset (http://kmplot.com/analysis).

(**M, N**) 4175 cells were treated with DMSO or increasing concentrations of paclitaxel (see Methods) for 3 days. (**M**) Standard dose response curves for 4175-shLBH1 and 4174-shLBH2 relative to control (shCtrl) and (**N**) Bar diagram comparing IC_{50} values of all three lines. IC_{50} concentrations for 4175 shLBH lines (blue) as indicated. IC_{50} for control cells was 0.02609 µM. n = 5 per group.

Data represent means ± SEM. *P<0.05; **P<0.01; ***P<0.001. P-vaules by Student's *t*-test (A,E), One-way ANOVA (C,D,G,N), Mann-Whitney U test (H,I), or log rank test (J-L).

Figure 6

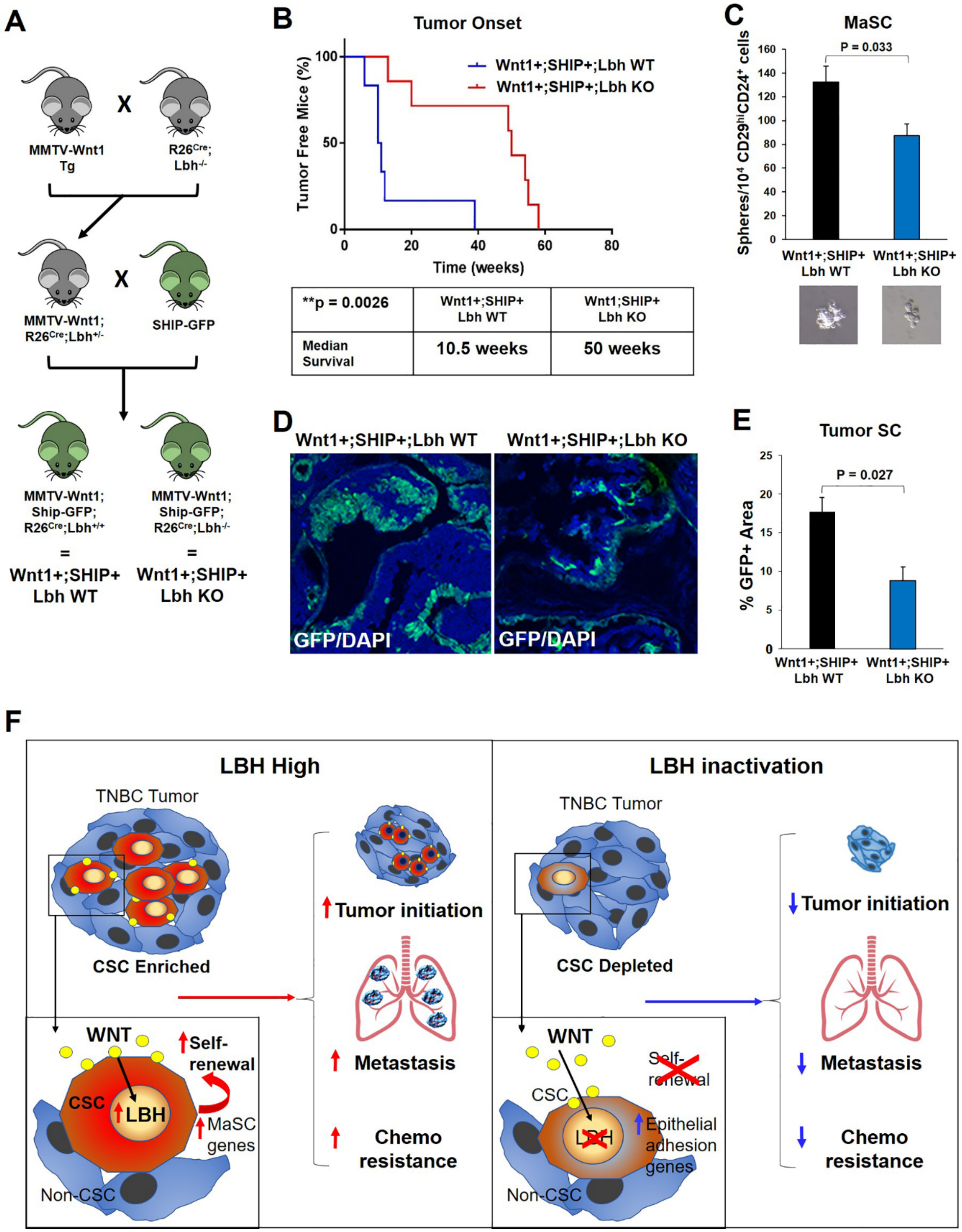

A
MMTV-Wnt1 Tg
X
R26^{Cre}; Lbh$^{-/-}$
MMTV-Wnt1; R26^{Cre};Lbh$^{+/-}$
X
SHIP-GFP
MMTV-Wnt1; Ship-GFP; R26^{Cre};Lbh$^{+/+}$
=
Wnt1+;SHIP+ Lbh WT
MMTV-Wnt1; Ship-GFP; R26^{Cre};Lbh$^{-/-}$
=
Wnt1+;SHIP+ Lbh KO
B
Tumor Onset
Wnt1+;SHIP+;Lbh WT
Wnt1+;SHIP+;Lbh KO
Tumor Free Mice (%)
100
50
0
0
20
40
60
80
Time (weeks)
**p = 0.0026
Wnt1+;SHIP+ Lbh WT
Wnt1;SHIP+ Lbh KO
Median Survival
10.5 weeks
50 weeks
C
MaSC
Spheres/10^4 CD29^{hi}CD24$^+$ cells
P = 0.033
160
140
120
100
80
60
40
20
0
Wnt1+;SHIP+ Lbh WT
Wnt1+;SHIP+ Lbh KO
D
Wnt1+;SHIP+;Lbh WT
Wnt1+;SHIP+;Lbh KO
GFP/DAPI
GFP/DAPI
E
Tumor SC
% GFP+ Area
P = 0.027
25
20
15
10
5
0
Wnt1+;SHIP+ Lbh WT
Wnt1+;SHIP+ Lbh KO
F
LBH High
TNBC Tumor
CSC Enriched
WNT
↑Self-renewal
CSC
↑LBH
↑MaSC genes
Non-CSC
↑Tumor initiation
↑ Metastasis
↑ Chemo resistance
LBH inactivation
TNBC Tumor
CSC Depleted
WNT
Self-renewal
CSC
LBH
↑Epithelial adhesion genes
Non-CSC
↓Tumor initiation
↓ Metastasis
↓ Chemo resistance

Figure 6. LBH is required for WNT-driven tumor stem cell expansion *in vivo*

(**A**) Experimental design: MMTV-Wnt1^{Tg} mice were crossed with ROSA26(R26)-Cre;Lbh-/- knockout (KO) mice and SHIP-GFP stem cell reporter mouse line.

(**B**) Kaplan-Meier plot of tumor-free survival in MMTV-Wnt1;R26Cre;SHIP+;Lbh$^{+/+}$ wild type (WT) (n=6) and MMTV-Wnt1; R26Cre;SHIP+;Lbh$^{-/-}$ KO (n=7) female mice. Median survival data (Table, bottom). P=0.0026; log rank test.

(**C**) Sphere formation of FACS-purified CD29^{high}CD24^{+} MaSC from 12-week-old mammary glands (n=3 mice/group and triplicate samples).

(**D**) Representative Immunofluorescence images and (**E**) quantification of SHIP-GFP+ tumor stem cells (SC) in tissue sections of mammary tumors from (B), using ImageJ software (>n=3 fields/section/group). Data represent means ± SEM. P values by Student's *t*-test (C,D).

(**F**) Schematic: Roles of WNT effector LBH in promoting breast CSC self-renewal, tumor initiation, metastasis and chemoresistance.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

Supplemental Figure S1

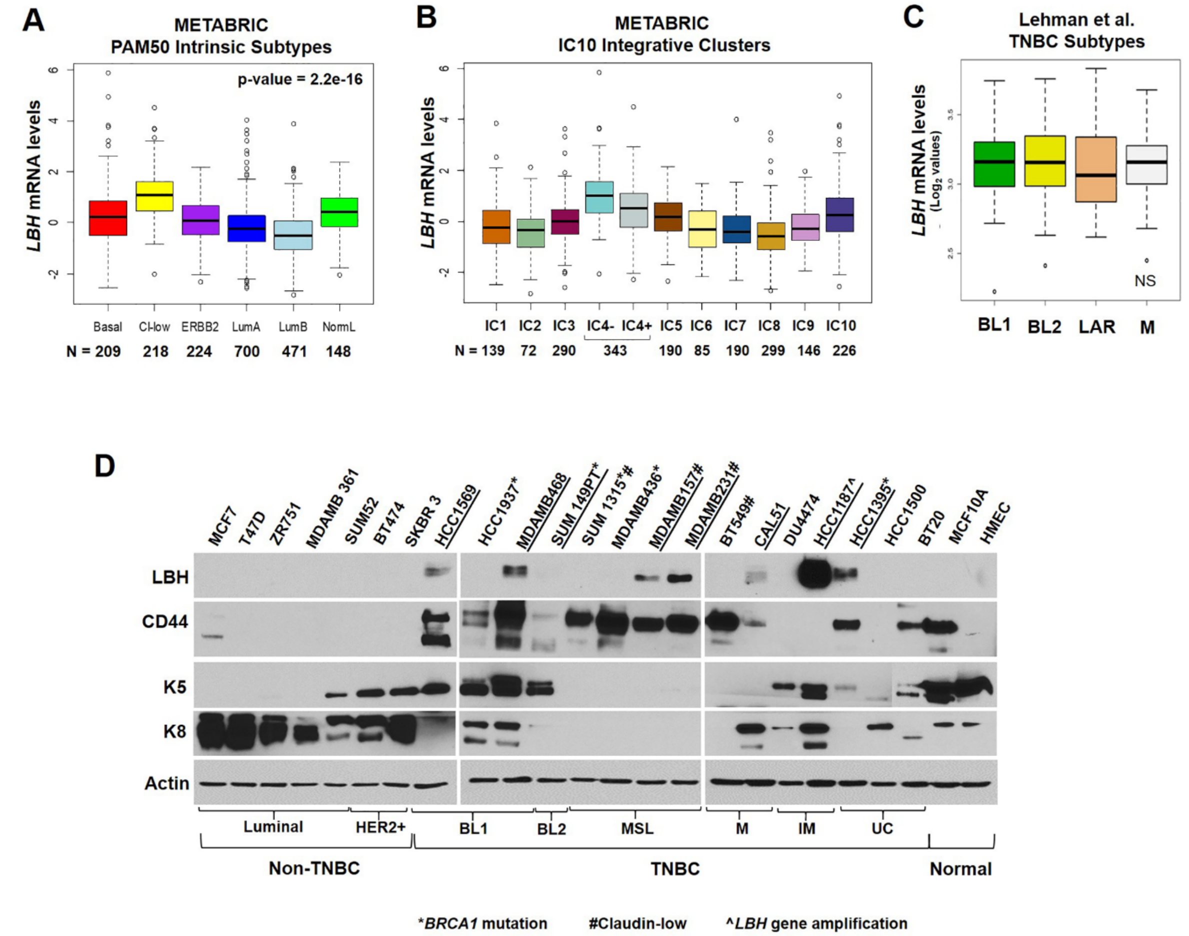


Figure S1 (related to Main Figure 1): LBH levels in primary human BrCa and BrCa cell lines
(**A,B**) Gene profiling expression data for *LBH* classified by: (**A**) PAM50 intrinsic molecular subtypes; (**B**) IC10 Integrative Subtypes in 1,980 tumors from the METABRIC dataset (Curtis et al., 2012).
(**C**) *LBH* expression in molecular subgroups of TNBC from the updated Lehmann data set (Lehmann et al., 2016), with highest *LBH* levels in basal-like (BL1, BL2) and mesenchymal (M) TNBC subtypes and lowest levels in the luminal androgen receptor positive (LAR) TNBC subgroup. P-values by Mann-Whitney U test as indicated. NS, Not significant.
(**D**) Extended WB data, with original loading sequence and including basal (K5) and luminal (K8) markers next to LBH and CD44. ß-actin = loading control. LBH-positive BrCa cell lines are underlined. BRCA1 mutant (Asterix), Claudin-low (number sign), and *LBH* gene amplification (triangle) are indicated. Molecular subclassification of TNBC lines according to Lehmann et al. 2011 (Lehmann et al., 2011): Basal-like 1 (BL1), Basal-like 2 (BL2), Mesenchymal-stem like (MSL), Mesenchymal (M), Immunomodulatory (IM), and Unclassified (UC). For the main Figure 1D, this data was grouped into basal-like (BL1, BL2, IM, UC) and mesenchymal (MSL, M) TNBC lines, according to revised TNBC subclassification data by Lehmann (Lehmann et al., 2016).

Supplemental Figure S2

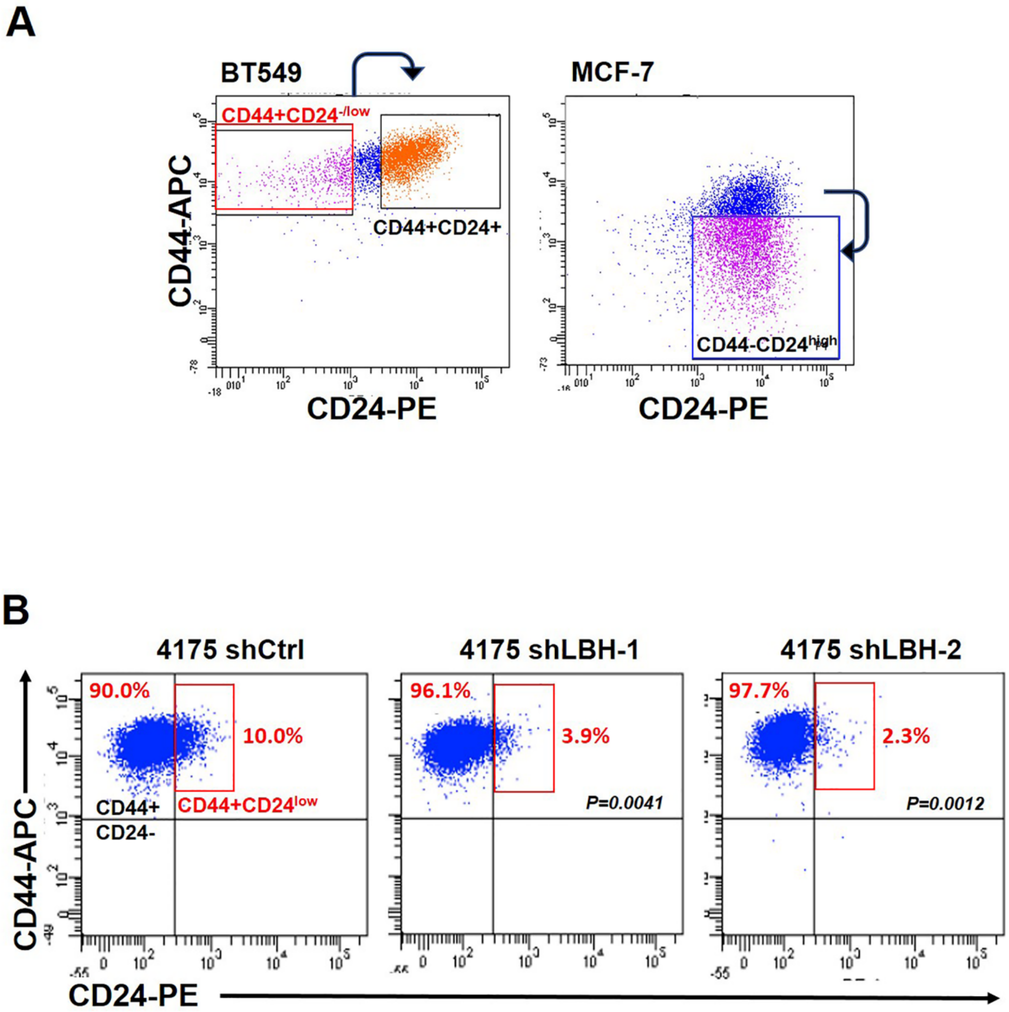


Figure S2 (related to Main Figure 4A-F): Extended FACS data

(**A**) CD44-CD24 FACS profiles of BT549 and MCF7 cells showing the distribution of breast CSC ($CD44^{+}CD24^{-/low}$; red rectangle) and non-CSC ($CD44^{-}CD24^{high}$ – blue rectangles) populations that were sorted for the gene expression analysis show in the main Figure 4B. Arrows indicate increasing degree of tumor cell differentiation.

(**B**) CD44-CD24 FACS profiles of MDA-MB-231 sister line, 4175, stably transduced with shCtrl and two independent LBH-targeted shRNAs (shLBH-1, shLBH-2). Percentages of different CSC-enriched populations ($CD44^{+}CD24^{-}$ and $CD44^{+}CD24^{low}$) as indicated. Quantification of metastasis-initiating $CD44^{+}CD24^{low}$ CSCs (red box) is shown in main Figure 5G.

Supplemental Figure S3

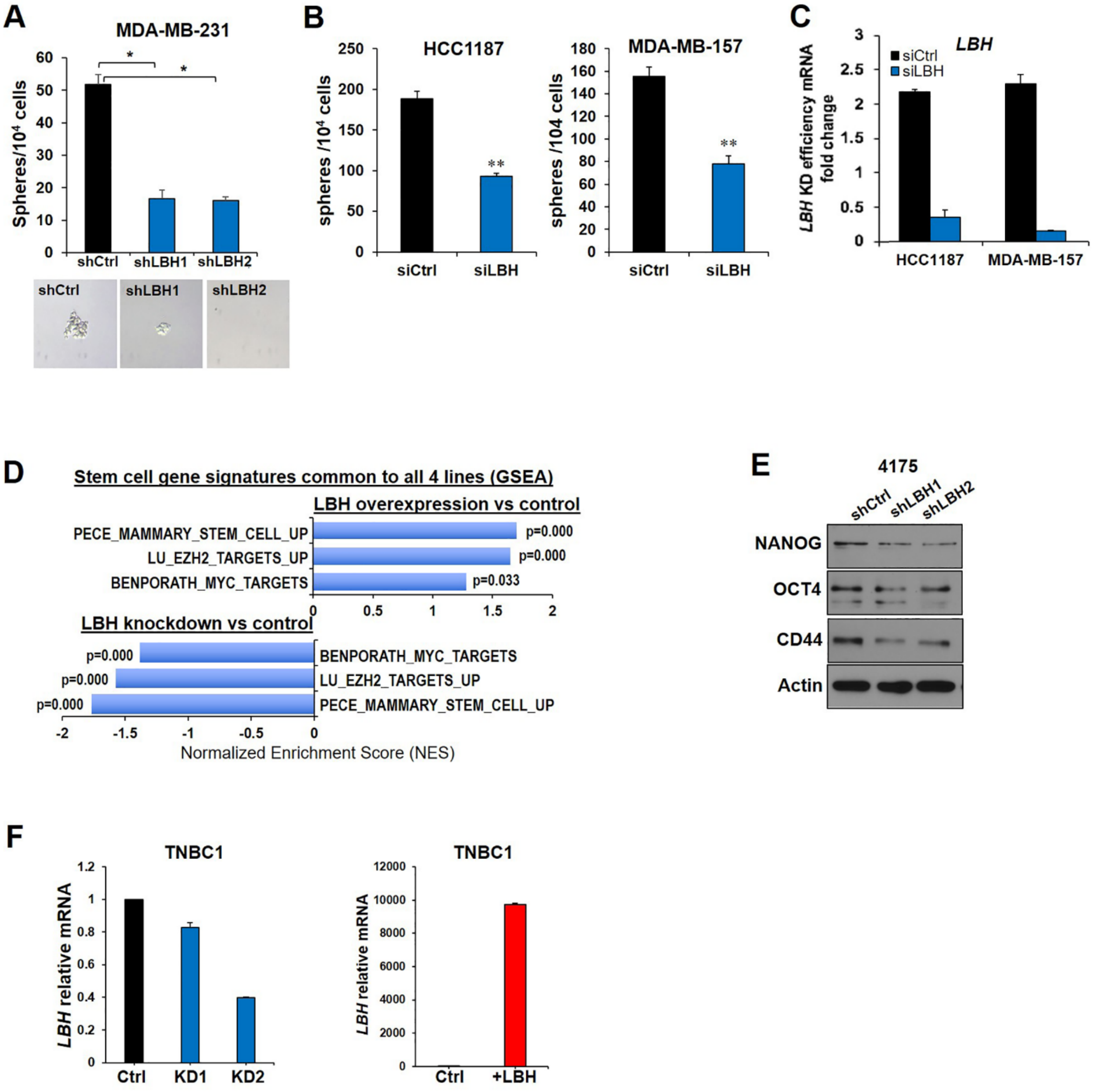


Figure S3 (related to Main Figure 4G-N): Sphere formation, LBH and stem cell marker analyses in additional BrCa cell models. (**A-B**) Tumor sphere formation of BrCa lines of different TNBC subtypes: (**A**) mesenchymal MDA-MB-231 stably expressing shCtrl or shLBH-1, shLBH-2; (**B**) basal HCC1187 and metaplastic MDA-MB-157 TNBC cells transiently transfected with non-target siRNA control (siCtrl) and LBH-targeted siRNAs (siLBH). (**C**) qPCR analysis of *LBH* normalized to *GAPDH*. Data represent means ± SEM (n=3, Student *t*-test; $P < 0.05$). (**D**) RNA-Seq gene expression and GSEA analyses: Bar diagrams depict stem cell gene signatures in the MSigDB C2:CPG data base commonly and significantly ($P<0.05$, FDR $q<0.25$) enriched among LBH-regulated genes in all four BrCa lines. Shown are data for BT549+LBH overexpression vs BT549+vector control (top) and MDA-MB-231-siLBH knockdown vs. MDA-MB-231-siCtrl control (bottom). See main Figure 4J. (**E**) WB analysis of stem cell markers in 4175 TNBC cells stably transduced with shCtrl and two independent LBH-targeted shRNAs (shLBH-1, shLBH-2). (**F**) qPCR analysis showing *LBH* knockdown and overexpression efficiencies in primary, patient-derived TNBC spheres 10 days after transduction with inducible shLBH (KD1, KD2) or LBH cDNA (+LBH) expression vectors grown under sphere forming conditions in the presence of 1 µg DOX. Data was normalized to *GAPDH* and represent means ± SEM (n=3, Student *t*-test; $P < 0.05$).

Supplemental Figure S4

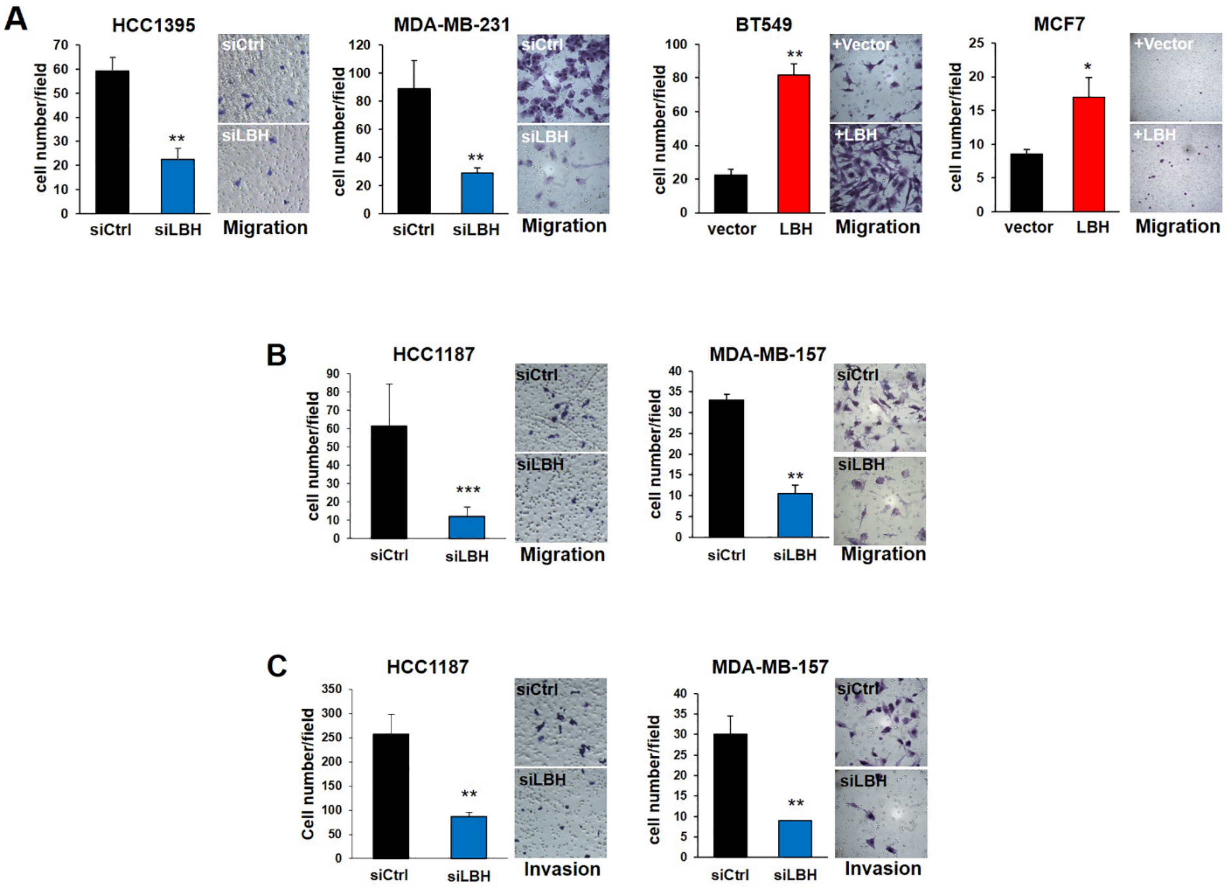


Figure S4 (related to Main Figure 5A): LBH induces BrCa cell motility and invasion
(**A**) *In vitro* Transwell migration assays 3 days after transient transfection of HCC1395 and MDA-MB-231 TNBC cells with LBH-specific siRNAs (siLBH) or scrambled siRNA (siCtrl); in comparison with BT549 TNBC and MCF7 non-TNBC cells stably expressing ectopic LBH (+LBH) or empty vector control (+vector).

(**B**) *In vitro* Transwell migration and (**C**) Boyden Chamber Matrigel Invasion assays 4 days after transient transfection of HCC1187 and MDA-MB-157 TNBC cells with LBH-specific siRNAs (siLBH) or scrambled siRNA (siCtrl).

Data represent means ± S.D. (n>3; Two-tailed unpaired *t*-test). *P*-values: **P < 0.05; **P < 0.01.* Representative images of crystal violet-stained migratory tumor cells are shown on the right.

Supplemental Figure S5

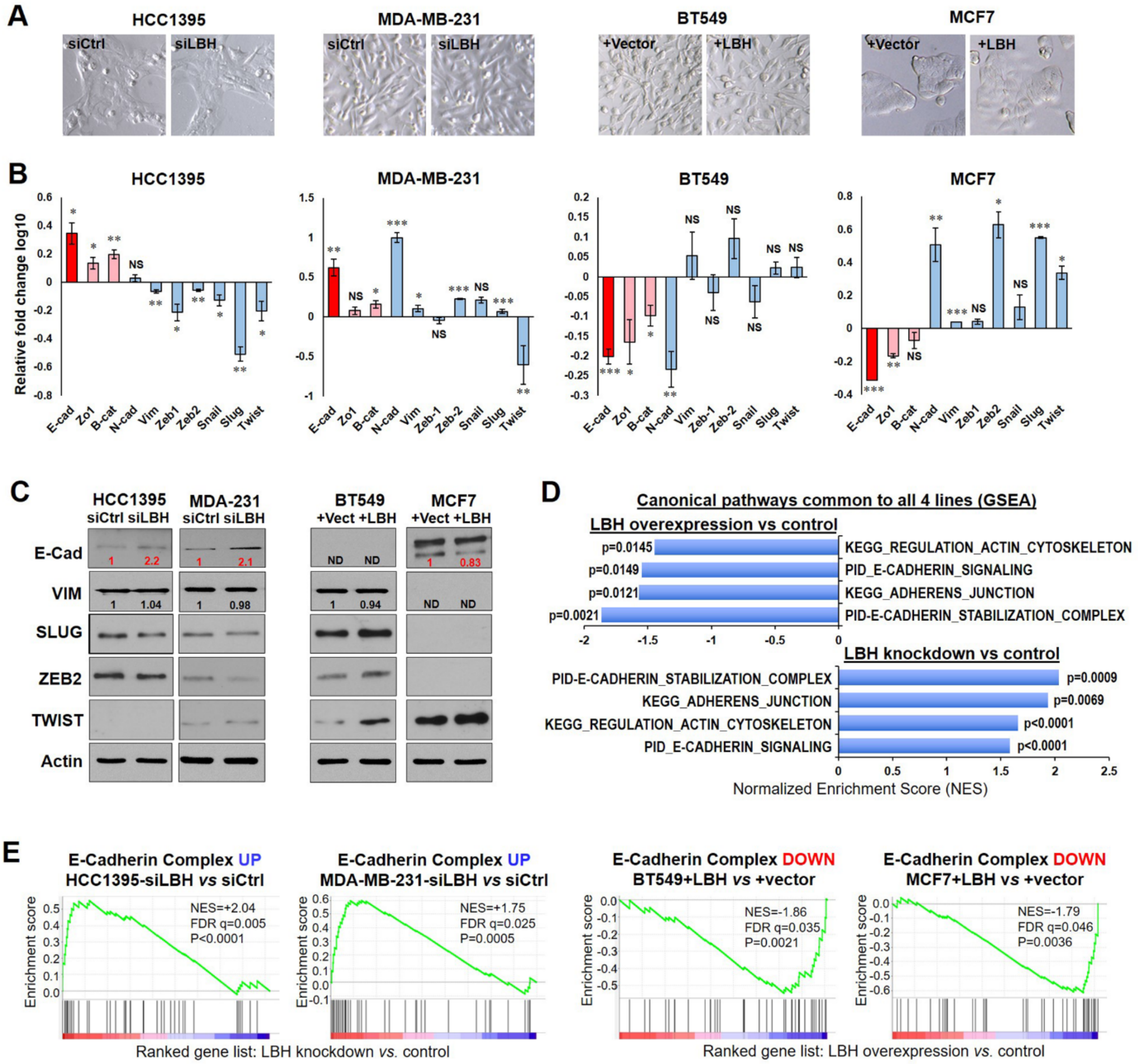


Figure S5 (related to Main Figure 5A-G): LBH inhibits epithelial adhesion genes

(**A**) Cell morphology did not visibly change following LBH knockdown in HCC1395, MDA-MB-231 TNBC and LBH overexpression in BT549 TNBC and MCF7-non-TNBC cells. (**B**) qPCR and (**C**) WB analyses of EMT markers and transcription factors. Fold changes in E-cadherin and Vimentin protein expression (normalized to ß-actin) were quantified by Densitometry. ND, not detectable. Data represent means ± SEM (>n=3, Student *t*-test). NS, not significant. *P < 0.05; **P < 0.01; ***P <0.001. (**D**) RNA-Seq and GSEA analyses: Gene signatures in the MSigDB C2:CP data base commonly and significantly (P<0.05, FDR q<0.25) enriched among LBH-regulated genes in all four BrCa lines. A total of 5 canonical pathways were consistently up- and 7 consistently down-regulated by LBH. Four of the top 7 LBH-downregulated pathways were E-cadherin and adherence junction gene signatures, of which 3 are shown in the bar diagram. The others 4 down-regulated gene sets were Regulation_of_Actin_cytoskeleton (also shown), MET signaling pathway and Hemostasis (not shown). Note, EMT gene signatures were not among consistently LBH up- or down-regulated genes (not shown). Shown are data for BT549+LBH overexpression vs BT549+vector control (top) and HC1395-siLBH knockdown vs. HCC1395-siCtrl control (bottom). (**K**) Enrichment plots for the top enriched PID_E-cadherin_Stabilization_Complex gene signature (see D); NES, Normalized Enrichment Score. FDR, False Discovery Rate.

SUPPLEMENTAL TABLES

Supplemental Table S1: Cancer stem cell populations (%) in various breast cancer cell lines

Cell line	$CD44^{+}CD24^{-/low}$	ALDH+	Tumor type	Tissue Source	Cell type classification	LBH expression
HCC-1395	86.3 ± 4.42	2.4 ± 0.15	DC	Primary	Basal/TN	+++
MDA-MB-231	96.8 ± 0.24	0.5 ± 0.32	AC	Pleural Effusion	Mesenchymal stem-like/TN	+++
BT549	11.8 ± 1.31	0.4 ± 0.15	IDAC	Primary	Mesenchymal/TN	(+)
MCF7	0.47 ± 0.31	2.12 ± 0.03	IDAC	Pleural Effusion	Luminal/ER+	-

TN triple-negative (ER-/PR-/HER2-); *AC* adenocarcinoma, *DC* ductal carcinoma, *IDAC* infiltrating ductal adenocarcinoma