Supplemental Methods

Western blot analysis and antibodies

Cells were treated in 6.0 cm diameter plates (80–90% confluence), washed three times with PBS, and lysed for 10 min on ice in RIPA buffer (Cat#: 89900, Thermo Scientific, Waltham, MA) containing a protease inhibitor mixture (Roche, Mannheim, Germany). Protein concentration was measured using the BCA assay (Thermo Scientific, Waltham, MA). The protein fractions were resuspended in loading buffer and denatured at 100°C for 10 min. Total proteins (20 µg/lane) were separated on 10% SDS polyacrylamide gels and transferred to PVDF membranes. The membranes were then blocked in 5% fat-free milk in TBST buffer (0.1% Tween-20) for 2 h at room temperature. For detection of HIC1, 0.2 µg/ml of rabbit anti-human HIC1 polyclonal antibody (1:5000, Cat#: H8539, Sigma, St Louis, MO, USA) was used in conjunction with 0.4 µg/ml of anti-species conjugated horseradish peroxidase (Upstate, Lake Placid, NY), and bands were detected by chemiluminescence (Amersham Pharmacia Inc, Piscataway, NJ).

Other commercial antibodies used were as follows: GAPDH (1:5000, Cat#: KC-5G4, Kangchen, Shanghai, China), anti-human p-AKT(Ser 473) (Cat#: 9271), AKT (Cat#: 9272), p-P70S6K (Cat#: 9204), P70S6K (Cat#: 9202), p-ERK1/2 (Cat#: 9101), ERK1/2 (Cat#: 9102), p-NF-κB p65 (Cat#: 3033), NF-κB p65 (Cat#: 4764), E-cadherin (Cat#: 9135), vimentin (Cat#: 3390), and MMP9 (Cat#: 3852). Antibodies used for detection of cell signaling pathway proteins were purchased form Cell
Tissue microarray and Immunohistochemistry

High-density tissue microarrays were constructed by US Biomax Inc using clinical samples obtained from a cohort of 192 patients including 32 benign and 160 breast cancer patients (Cat#: BR1921, Rockville, MD, USA). Paraffin-embedded tissue sections (5 μm) were deparaffinized by xylene and rehydrated in a graded alcohol series (100%, 95%, 80% and 70%, 5 min each). After antigen retrieval in sodium citrate buffer (10 mM, pH=6.0), endogenous peroxidase was blocked by 0.3% H2O2 in methanol for 10 min. Tissue sections were blocked with normal goat serum (NGS, 5%) for 30 min at room temperature and then incubated with rabbit anti-human HIC1 antibody (1:200, Cat#: H8539, Sigma) at 4°C overnight. After washing (1×PBS, three times, 5 min each), tissue sections were incubated with biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, USA) at room temperature for 30 min. Then, tissue sections were incubated with the avidin-biotin-complex-PO (Vector Laboratories, California, USA) and developed in DAB Coloring Agent (Sigma, St Louis, MO, USA). Negative controls were generated by omission of the primary antibody.

The staining intensity was scored on a scale of 5 as follows: negative (-), negligible (±), weak (+), moderate (++) and strong (+++). Negative (-) and negligible (±) were defined as negative staining for HIC1, and the others were defined as the positive group. Images were obtained using a microscope (Zeiss) with a 40× objective lens.
Cell migration and invasion assays

A wound healing assay was used to examine cell migration. The migration status was determined by measuring the movement of cells into a scraped area created by a 200 μl pipette tip. After wound scratching, cells were cultured in media supplemented with 0.1% FBS to eliminate the effect of cell proliferation. The process of wound closure was photographed at 24, 48 and 56 h under a 5× objective lens.

Cell invasion was examined using a reconstituted extracellular matrix membrane (BD Biosciences, San Jose, CA). Cells were suspended at a density of 4×10^4 cells/0.5 ml in serum-free medium and placed in the top chambers, and complete medium containing 10% FBS and 1% antibiotics (Invitrogen Corp, Carlsbad, CA) was added to the bottom chambers. The chambers were then incubated for 18-24 h at 37°C with 5% CO₂. After incubation, the noninvasive cells were gently removed from the top wells with a cotton-tipped swab and the chambers were fixed with methanol for 30 min. The chambers were then stained with crystal violet for another 30 min. Cell counting was facilitated by photographing the membrane through a microscope (Zeiss) under a 10× objective lens.

For MCF-10A cells, BD Falcon cell culture inserts (8 μm, not matrigel-coated) were used for the cell migration assay (Cat#: 353097, BD) and the cell invasion assay was performed as described above. Cells at a density of 6×10^4 cells/0.5 ml in serum-free medium were placed in the top chamber, and complete medium was added to the bottom chamber. After incubation, the chambers were fixed, stained, and the
migrated or invasive cells were photographed and counted through the microscope under a 10× objective lens.

**ELISA**

LCN2 secretion levels in cell-conditioned medium and preoperative human serum were detected by using Quantikine Human LCN2 ELISA Kits (Cat#: DLCN20, R&D systems) according to the manufacturer’s instruction. Each experiment was performed in 4 wells and repeated three times.

**RNA extraction and real-time RT-PCR**

Total RNA was extracted using the Trizol reagent (Cat#: 15596-026, Invitrogen) and reverse transcribed using the transcriptase cDNA synthesis kit (Fermentas) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed into cDNA and an equal volume of cDNA was used as the PCR template using specific primers for LCN2 (Genbank No: NM_005564.3) and HIC1 (Genbank No: NM_006497.3). Real-time PCR analysis was performed by SYBR Premix Ex Taq™ (Cat#: RR420A, TaKaRa, Dalian, China) in an Applied Biosystems 7500 Fast Real-Time PCR System (ABI, USA) according to the manufacturer’s instructions. Primers were used at a concentration of 0.5 µM. According to the melting point analysis, only one PCR product was amplified under the following conditions: initial denaturation at 95°C for 30 sec, denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec, for a total of 40 cycles. Results were normalized to the internal controls. The primers used were as follows:

**HIC1:**  
*sense:* 5'-GTCGTGCGACAAGAGCTACAA-3',
antisense: 5'-CGTTGCTGTGCGAACTTGC-3'.

**LCN2:** sense: 5'-GTTCACGCTGGGCAACATTA-3',
antisense: 5'-GATTGGGACAGGGAAGACGA-3'.

**GAPDH:** sense: 5'-ACGGATTTGCTGATTTGGG-3',
antisense: 5'-CGCTCCTGGAAGATGGTGAT-3'.

**NGALR:** sense: 5'-GTTACCCCCCGACAGATTTG-3',
antisense: 5'-CCCCAGAGGAAATCGGAGGG-3'.

**MMP9:** sense: 5'-AGTCCACCCTTGTGTCTTCC-3',
antisense: 5'-TGCCACCCCGAGTGTAACCAT-3'.

### siRNA transfection

Three siRNA duplexes targeting LCN2 and control scramble siRNA duplexes were purchased from GenePharma (Shanghai, China). The effective sequences were as follows: si-238 (5'-CCUGGCAGGGAAUGCAAUUTT-3'), si-335 (5'-UCCGUCCUGUUUAGGAAAATT-3'), and si-480 (5'-CAGCAUGCUAUGGUGUUCUTT-3'). The nonspecific scramble siRNA duplexes (5'-UUCUCCGAACGUGUACGCUTT-3') were used as the normal control.

The siRNA duplexes were transduced into MDA-231 cells at a final concentration of 50 nmol/L using Lipofectamine 2000 (Invitrogen Corp, Carlsbad, CA). To detect the efficiency of these siRNAs, the conditioned medium of the transfected cells was collected to measure LCN2 secretion, and total RNA was extracted to analyze mRNA levels.

The siRNA sequences targeting NGALR were as follows: si-671
(5'-CCUCAAGGAUUGGGACUAUTT-3'), si-934
(5'-GAUUCCUCUUGGGCUUUCUTT-3') and si-1612
(5'-GCCUGUGGGAUUAUCUGAATT-3').

**Construction of lentiviral vectors**

For generation of a stable HIC1 knockdown cell line, GV248 lentiviral vectors expressing short hairpin RNAs targeting HIC1 were purchased from GeneChem Company (Shanghai, China). Lentiviruses were produced as described above and infected cells were selected by puromycin (1μg/ml). Three shRNAs (clone ID: HIC1-RNAi (9633-1, 9634-1, and 9635-1) were used in the experiment, namely shHIC1-1, 2, and 3.

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<th>Site</th>
<th>GC (%)</th>
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For generation of a stable NGALR knockdown cell line, four shRNAs (clone ID: SLC22A17-RNAi (23181, 23182, 23183 and 23184), namely shNGALR-1, 2, 3 and 4, were used in this study. shNGALR-4 was more efficient than the others.

<table>
<thead>
<tr>
<th>ID</th>
<th>Target sequence :</th>
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cDNA microarray analyses

Agilent SurePrint G3 Human Gene Expression microarray 8×60K was used in this study. This chip targets > 20,000 genes with >40000 probes and > 7,000 lincRNA derived from a broad survey of well known sources such as RefSeq, Ensembl, Unigene and others. The resulting view of the human genome covers 20K unique genes and transcripts that have been verified and optimized by alignment to the human genome assembly and by Agilent's Empirical Validation process. Total RNA (>300 ng) was extracted from four independent cultures of MDA-231\textsuperscript{GFP}, MDA-231\textsuperscript{HCl} cells, sh-control (HBL-100), and shHIC1-2 (HBL-100) cells. Microarray hybridization, data collection and analysis were performed at Oebiotech Biotechnology Corporation (Shanghai, China). Statistically significant differential expression was determined according to the fold change (×1.5) in transcript levels and Diffscore value between MDA-231\textsuperscript{GFP} and MDA-231\textsuperscript{HCl} cells, sh-control and shHIC1-2 cells. The Diffscore represents the significance of the difference in gene expression between two samples. It is defined as a method of measuring significant differential expression for a gene in two samples by Illumina BeadStudio, with the following levels of significance: Diffscore of > 13 or < -13 correspond to \( p \) values of 0.05. Hence the genes with Diffscore values (MDA-231\textsuperscript{HCl} \textit{versus} MDA-231\textsuperscript{GFP} cells, sh-control \textit{versus} shHIC1-2 cells) between -13 and +13 were eliminated. According to
functional annotation of the genes by Agilent, these gene chips showed HIC1-upregulated and downregulated target genes.

**Tail vein injections**

For tail vein injection, *balb/c* nude mice (Slaccas Laboratory Animal, Shanghai, China) were anesthetized, and their tails were cleaned with 75% ethanol. Luciferase tagged MDA-231\(^{\text{GFP}}\), MDA-231\(^{\text{HIC1}}\), MDA-231\(^{\text{LCN2}}\) and MDA-231\(^{\text{HIC1/LCN2}}\) cells were respectively injected into the tail vein using a 1 ml syringe (29G, BD Insulin Syringe). A total of 100 µl of cell suspension was injected (10\(^7\) cells/ml) into mice. Growth and metastasis of the tumor were examined using the Xenogen IVIS cryogenically cooled imaging system.

After 4 weeks, bioluminescence imaging was used to follow breast cancer-derived lung metastases as a primary outcome. The *balb/c* nude mice were injected intraperitoneally with luciferin (200 µl at 15 mg/ml in PBS) before imaging. This dose and route of administration have been shown to be optimal for rodent studies at 10–20 min after luciferin injection. Mice were anesthetized with 1.5% isoflurane/air, and the Xenogen IVIS cryogenically cooled imaging system was used as described previously (1). Mice were imaged weekly after tumor injection to monitor tumor development. Bioluminescence generated by the luciferin/luciferase reaction served as a locator for cancer growth and was used for quantification using the Living Image software on a red (high intensity/cell number) to blue (low intensity/cell number) visual scale. A digital grayscale animal image was acquired followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photon counts emerging from active luciferase within the animal. Signal intensity was quantified as the sum of all detected photons within the region of interest during a
1-minute luminescent integration time.

**Luciferase reporter assays**

Constructs of the LCN2 promoter region at -2955/+18, -1427/+18, -774/+18, -252/+18 and -62/+18 were generated from genomic DNA of MDA-MB-231 cells. All constructs and the LCN2 promoter containing mutations at -252/+18 to generate the ΔM1, ΔM2 and ΔM3 mutants were cloned into the pGL3 basic reporter gene vector and verified by sequencing to produce LCN2 promoter truncation constructs.

MDA-231 and MCF-7 cells were maintained in DMEM containing 10% FBS and transfected by Lipofectamine™ 2000 Reagent (Invitrogen Corp, Carlsbad, CA) in 12-well plates. The HIC1 plasmid: LCN2 promoter constructs: Renilla plasmid DNA (100ng: 200ng: 20ng) were co-transfected for 6–9 h and then incubated for 36–48 h in fresh complete medium. Cells were then rinsed in cold PBS and lysed with the luciferase assay buffer. Luciferase activities were measured by using a dual luciferase assay kit (Cat#: E1960, Promega, Wisconsin, USA) with a Berthold chemiluminometer (Berthold Detection Systems GmbH) (2). The results were expressed as ratio of firefly luciferase activity to Renilla luciferase activity. Data were expressed as the mean values and standard deviations from at least three independent transfections performed in triplicate.

**Chromatin Immunoprecipitation**

Chromatin Immunoprecipitation (ChIP) was performed according to published protocols with slight modifications (3-4). Briefly, formaldehyde was added directly to
the cultured BT474 and MCF-7 cells to a final concentration of 1% for 10 min at 37°C. The cross-linking was stopped by adding glycine to a final concentration of 0.125 M. After 5 min at 37°C, cells were lysed directly in the plates by resuspension in cell lysis buffer for 5 min. Then, the samples were pelleted, resuspended in nuclear lysis buffer, and sonicated to obtain chromatin fractions with an average size of 500 bp using a BioRuptor (Diagenode, Liege, Belgium). After preclearing with a 50% slurry of protein A-G beads (Santa Cruz, Delaware Ave, USA) preincubated with salmon sperm DNA and bovine serum albumin for 4 h at 4°C, the chromatins were incubated with anti-HIC1 antibody (Cat#: H8539, Sigma, St Louis, MO, USA), normal rabbit IgG or with no antibody overnight. The antibody bound chromatin was then pulled down for 60 min with protein A-G beads, washed extensively, and eluted two times with Elution buffer. After addition of 20 µl of 5 M NaCl, the cross-linking was reversed by overnight incubation at 65°C. The immunoprecipitated DNAs as well as whole cell extract DNAs (input) were preliminarily purified by treatment with RNase A and then proteinase K followed by further purification with an UltraClean TM 15 DNA Purification Kit (U11B10, MoBIO experiment diagnosis company, San Diego, USA). The purified DNA was used for PCR analyses using the relevant primers for LCN2 and GAPDH. The PCR specific primers for amplification of the LCN2 promoter region at -252/+18 containing HIC1-responsive elements (HiREs) were sense (5'-AACAGCACAAGGAAGGCACA-3') and antisense (5'-CAACTCCTGCGGAAACACTT-3'). Only one PCR product for LCN2 was amplified successfully under the following conditions: initial denaturation, 94°C for 3 min; denaturation, 94°C for 20 sec; annealing, 59°C for 30 sec, extension, 72°C for 30 sec, altogether 32 cycles; 72°C extension for 2 min, and 4°C forever. A control primer specific for the human GAPDH gene was used for monitoring the experiment.
primers used for GAPDH were sense (5'-TACTAGCGGTTTTACGGCG-3') and antisense (5'-TCGAACAGGAGGAGCAGAGCGA-3').

Exogenous LCN2 stimulation

One day before stimulation, 3×10⁵ cells were plated in 6-well culture plates to achieve 70–80% confluency at the time of stimulation. Cells were stimulated with 200 ng/ml Recombinant Human Lipocalin-2/NGAL (Cat#: 1757-LC-050, R&D, USA) for different times as follows: 0, 5, 15, 30, and 60 min. Then, cells were rinsed three times with ice cold-PBS and lysed in RIPA buffer (Cat#: 89900, Thermo Scientific, Waltham, MA). For cell invasion assay in MDA-231-HIC1 or GFP cells, 4×10⁴ cells in serum-free medium were placed in the top chamber, and complete medium supplemented with recombinant human LCN2 (200 ng/ml) was added to the bottom chambers. Then, chambers were fixed, stained and invasive cells were photographed and counted.

References


