SUPPLEMENTARY METHODS

Antibodies and other reagents

Primary antibodies used for immunoblotting include: CARMA3 (catalog no: 111222, GeneTex), Bcl10 (catalog no: 5611, Santa Cruz), MALT1 (catalog no: 2494, Cell Signaling Technology), GAPDH (catalog no: 5174, Cell Signaling Technology), β-actin (catalog no:4970L, Cell Signaling Technology), Snail (catalog nos: 3895 and 3879, Cell Signaling Technology), ZEB1 (catalog nos: 3396 and 70512, Cell Signaling Technology), Vimentin (catalog no: 5741, Cell Signaling Technology), Ki-67 (catalog no: 9027, Cell Signaling Technology), E-cadherin (catalog no: 610181, BD Biosciences), N-cadherin (catalog no: 610920, BD Biosciences), PAR1 (catalog no: MABF244, EMD Millipore), ERα (catalog no: 8002, Santa Cruz), IKKα (catalog no: 7218, Santa Cruz), IKKβ (catalog no: 8014, Santa Cruz), CYLD (catalog no: 74435, Santa Cruz). Secondary anti-mouse (catalog no: W4021) and anti-rabbit (catalog no: W4011) antibodies were obtained from Promega. Goat anti-Rabbit IgG (H+L) Secondary Antibody Alexa Fluor 488 (catalog no: A11034) and Goat anti-Mouse IgG (H+L) Secondary Antibody Alexa Fluor 568 (catalog no: A11031) were purchased from Thermo Fisher Scientific.

IKK2 inhibitor VI (IKK-VI) was purchased from Cayman Chemical (catalog no: 17276). Mepazine hydrochloride was purchased from EMD Millipore (catalog no: 500500). Thioridazine hydrochloride was purchased from Sigma-Aldrich (catalog no: T9025). Puromycin Dihydrochloride was purchased from Thermo Fisher Scientific (catalog no: A1113803). Doxycycline hyclate was purchased from Sigma-Aldrich (catalog no: D9891). Nuclear Fixed Cell Stain (DAPI) was purchased from Thermo Fisher Scientific (catalog no: R37606). S-mepazine was provided by author, Dr. Daniel Krappman. MLT-748 was provided by author, Dr. Frédéric Bornancin, and Novartis Pharmaceuticals.

Quantitative RT-PCR

Total RNA was isolated from cell cultures using RNeasy Plus Mini Kit (Cat No: 74136, Qiagen) and reverse-transcribed into cDNA using the ABI High Capacity RT Kit (Cat No: 74136, Applied Biosystems). Real-time PCR was performed using TaqMan gene expression assay primer sets (AGTR1, Hs01096941_m1; ESR1, Hs00174860_m1) and TaqMan 2X master mix, in a Via7 real-time PCR system (Applied Biosystems). Relative expression of gene targets was determined using the ΔΔCT method as described (1).

Cell migration and invasion assays

Serum Starved MCF7, MCF7-PAR1, ZR75-1, ZR75-1-AT1R, BT549, and MDA-MB-231 cells were used in migration and invasion assays, as indicated. In some cases, cells were transiently transfected with control or MALT1 siRNA prior to initiating the assays. When using MALT1 inhibitors, cells were pre-treated with or without mepazine (5 μM) or MLT-748 (20 μM) for 24-48 hrs prior to the start of the assays.

2D migration assays were performed following the IncuCyte ZOOM 96-well Scratch Wound Cell Migration assay protocol, according to the manufacturer's instructions (Sartorius). siRNA transfected-cells or MALT1 inhibitor pre-treated cells were seeded at 40,000 cells/well in a 96-well ImageLock plate (Cat No: 4379, Sartorius) and incubated for 24 hours to reach confluence. A uniform scratch wound was generated in each well using the IncuCyte WoundMaker, a 96-pin mechanical device designed to create homogeneous, 700-800 micron-wide wounds in ImageLock 96-well plates. Wells were then rinsed with fresh medium to remove floating cells. Remaining adherent cells were maintained in serum free or low serum (eg, 0.25% FBS) conditions, and the wound healing process was monitored continuously using the IncuCyte

ZOOM Live-cell Imaging System (Sartorius). Images were acquired every 2-3 hours over a 24-48 hour time period using a 10X objective and analyzed using the IncuCyte Cell Migration Software module. Relative wound density (RWD) was used to quantify wound closure.

Modified Boyden chamber invasion assays were performed using transwell inserts (8.0 μm PET membrane; Corning, Cat No: 353097) coated with growth factor-reduced matrigel (0.75 mg/ml in RPMI SF Media; Cat No: 356231, Corning). Serum starved siRNA transfected or MALT1 inhibitor pre-treated cells were seeded ($1x10^5$ cells in RPMI/DMEM SF media) into the top chamber of transwell inserts, placed in a 24 well plate. Medium containing 1% FBS served as a chemoattractant in the bottom chamber of each transwell. For MALT1 inhibitor experiments, MALT1 inhibitors were also added to both upper and bottom chambers of the transwell during the duration of the assay. After 18 hours, residual, non-migrating cells on the top surface of transwell membranes were removed using cotton tips, and membranes were fixed and stained according to the Diff–Stain Kit staining protocol (Cat No: K7128, IMEB Inc.). 3-4 non-overlapping images covering the bottom surface area of each transwell were taken with a Zeiss Axiovert 200M inverted microscope connected to an AxioCamHR3 Color camera (4X objective, Zeiss). Cells in each image were counted using NIS Elements AR Analysis software (Nikon Instruments), with results from all images compiled for each transwell.

Immunohistochemistry and quantitative image analysis

Five-micron sections of xenograft tumors were deparaffinized in xylene and rehydrated with graded washes of ethanol and distilled water. Antigen retrieval was performed using citrate buffer (pH 6.0) for 30 minutes at 100°C. Tumor sections were then permeabilized and blocked with 5% normal goat serum and 1% BSA in PBS containing 0.05% Tween 20, for 30 mins. Slides were then incubated in a humidified chamber at 4°C overnight with primary antibodies against ZEB1

(Cell Signaling Technology, #70512; 1:100), followed by incubation with 3% hydrogen peroxide in distilled water for 10 min to block endogenous peroxidase activity. Sections were next incubated with biotin-conjugated anti-rabbit IgG (1:250) and horseradish peroxidase (HRP)-ABC Elite complex (Vector Labs, #PK-6101) according to the manufacturer's instructions. Slides were developed with DAB (3,3'-diaminobenzidine) using a DAB substrate kit, (Vector Labs, #SK-4100), according to the manufacturer's instructions. Finally, sections were counterstained with hematoxylin for 5s, followed by graded washes of ethanol and xylene, and then mounted using Richard-Allan Scientific Cytoseal™ 60 (Thermo Fisher, #8310-16). No significant staining was observed in negative controls, which were prepared using the same tissue sections and incubated in normal goat serum in lieu of the ZEB1 primary antibody. Lung and liver sections were similarly fixed and sectioned, followed by citrate buffer antigen retrieval and staining with a cocktail of primary antibodies against vimentin (D21H3; 1:200) and Ki-67 (D2H10; 1:400). Sections were developed as described above using the Vector Labs HRP and DAB kits.

Whole slide images of immunostained sections were generated at 20x magnification using an Aperio scanner in the tissue imaging facility of the Pitt Biospecimen Core. Resulting .svs files were viewed and analyzed in QuPath v2.0. Intensity threshold parameters for nuclear ZEB1 immunoreactivity (1+, 2+, 3+) were established under the positive cell detection settings of QuPath by a board-certified pathologist (PCL) and were applied uniformly across all immunostained images. H-scores for each sample were then calculated using QuPath after selecting all viable tissue from each whole slide image (including ~37,000 – 126,000 cells / section). Micrometastatic burden in liver and lung were quantified by first using the pixel classification setting in QuPath to define an appropriate threshold for immunoreactivity and then segmenting images into tumor

versus non-tumor areas. The percent tumor area was calculated using QuPath as the percentage of total tissue cross-sectional area occupied by IHC-positive cells.

Western blot analysis of xenograft samples

Excised flash-frozen MDA-MB-231 tumors (DMSO or mepazine treated) were homogenized in RIPA lysis buffer (Thermo Fisher, #89901) containing Halt Protease and Phosphatase cocktail (Thermo Fisher, #78440) using an M-tube and gentleMACS Octo-Dissociator (Miltenyi Biotech), according to the manufacturer's instructions. Homogenized lysates were cleared by centrifugation at 4000xg for 5 minutes and analyzed for protein content using a BCA Protein Assay Kit (Thermo Fisher, #23225). Western blotting was performed using anti-Snail (Cell signaling Technology, #3879; 1:1000) and anti-β-Actin (Cell signaling Technology, #4970; 1:1000). Bands were quantified using AlphaView Software (Protein Simple).

REFERENCES

1. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods **2001**;25:402-8