Supporting Information

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SI Materials and Methods

shRNA, Lentiviruses, and Transduction. Oligonucleotides encoding shRNA targeting HIF-1α (nucleotides 2,123–2,141, GenBank NM_181054) and HIF-2α (nucleotides 1,992–2,012, GenBank NM_001430) were inserted into pRRL-luciferase-shRNA-GFP vector. pLKO.1-puro vectors encoding shLOX, shLOXL2, and shLOXL4 were purchased from Sigma–Aldrich: shLOX-90 (clone ID NM_002317.3-1506s1c1), shLOX-91 (clone ID NM_002317.3-810s1c1), shLOXL2-96 (clone ID NM_002318.1-962s1c1), shLOXL2-97 (clone ID NM_002318.1-2237s1c1), shLOXL4-05 (clone ID NM_032211.5-1387s1c1), and shLOXL4-06 (clone ID NM_032211.5-811s1c1). pLKO.1-puro vector carrying NTC sequence (Sigma–Aldrich) and pRRL-GFP EV were used as negative controls. shRNA-encoding lentiviruses were packaged in 293T cells by cotransfection with plasmid pCMV-dR8.91 and plasmid encoding vesicular stomatitis virus G protein using Lipofectamine 2000 (Invitrogen). Culture supernatants containing viral particles were collected 48 h after transfection and filtered through 0.45-μM filters (Millipore). MDA-231 and MDA-435 cells were transduced with viral supernatant in the presence of 8 μg/mL Polybrene (Sigma–Aldrich). After 24 h, cells were replenished with fresh medium (for cells transduced with virus generated from pRRL plasmids) or fresh medium containing 0.6 μg/mL puromycin (for cells transduced with virus generated from pLKO.1-puro plasmids). Cells were maintained in puromycin medium for selection of cells stably transfected with pLKO.1-puro constructs.

ChIP and Luciferase Reporter Assays. For ChIP assay, MDA-231 cells were cross-linked with formaldehyde and lysed with SDS lysis buffer. DNA was sheared by sonication. Sonicated lysates were precleared with salmon sperm DNA/protein A agarose slurry (Millipore). Immunoprecipitating antibodies against HIF-1α (Santa Cruz), HIF-2α (Novus Biologicals), HIF-1β (Novus Biologicals), or IgG (Santa Cruz and Novus Biologicals) were incubated with precleared lysates. Salmon sperm DNA/protein A agarose slurry was added to the antibody/protein/DNA complex. The agarose beads were washed sequentially with low- and high-salt wash buffers [0.1% SDS/1% Triton X-100/2 mM EDTA/20 mM Tris (pH 8.0) with 0.15 M or 0.5 M NaCl, respectively], LiCl wash buffer [0.25 M LiCl/1% NP-40/1% deoxycholate/10 mM Tris (pH 8.0)/1 mM EDTA], and TE buffer [10 mM Tris (pH 8.0)/1 mM EDTA]. DNA was eluted in 1% SDS/0.1 M $NaHCO_3$ and crosslinks were reversed by addition of NaCl to 0.2 M. For luciferase reporter assays, MDA-231 cells were cotransfected with pGL2 firefly luciferase reporter vector with HRE-WT or HRE-Mut and pRL Renilla luciferase reporter vector. Luciferase activities were determined using the Dual-Luciferase Reporter System (Promega).

Real-Time PCR and Western Blots. RNA was extracted by TRIzol (Invitrogen), and cDNA was synthesized by means of a cDNA synthesis kit (BD Biosciences). qPCR was performed with SYBR Green qPCR Master Mix (Fermentas) and appropriate primers (nucleotide sequences are provided in Table S1). The following primary antibodies were used for Western blot analysis: HIF-1α (BD Transduction Laboratory); HRP-conjugated β-actin (Santa Cruz); LOX (Santa Cruz); and HIF-2α, LOXL2, and LOXL4 (Novus Biologicals). HRP-conjugated secondary antibodies were purchased from Santa Cruz.

In Vitro Collagen Remodeling Assay. CM was concentrated by means of Amicon Ultra 15 centrifugal devices (Millipore). Type I collagen gel (2 mg/mL) was mixed with concentrated CM and incubated at 37 °C for 30 min. Additional concentrated CM was added on top of the gelled collagen for further incubation at 37 °C for 16 h. The gel was analyzed under reflection confocal microscopy.

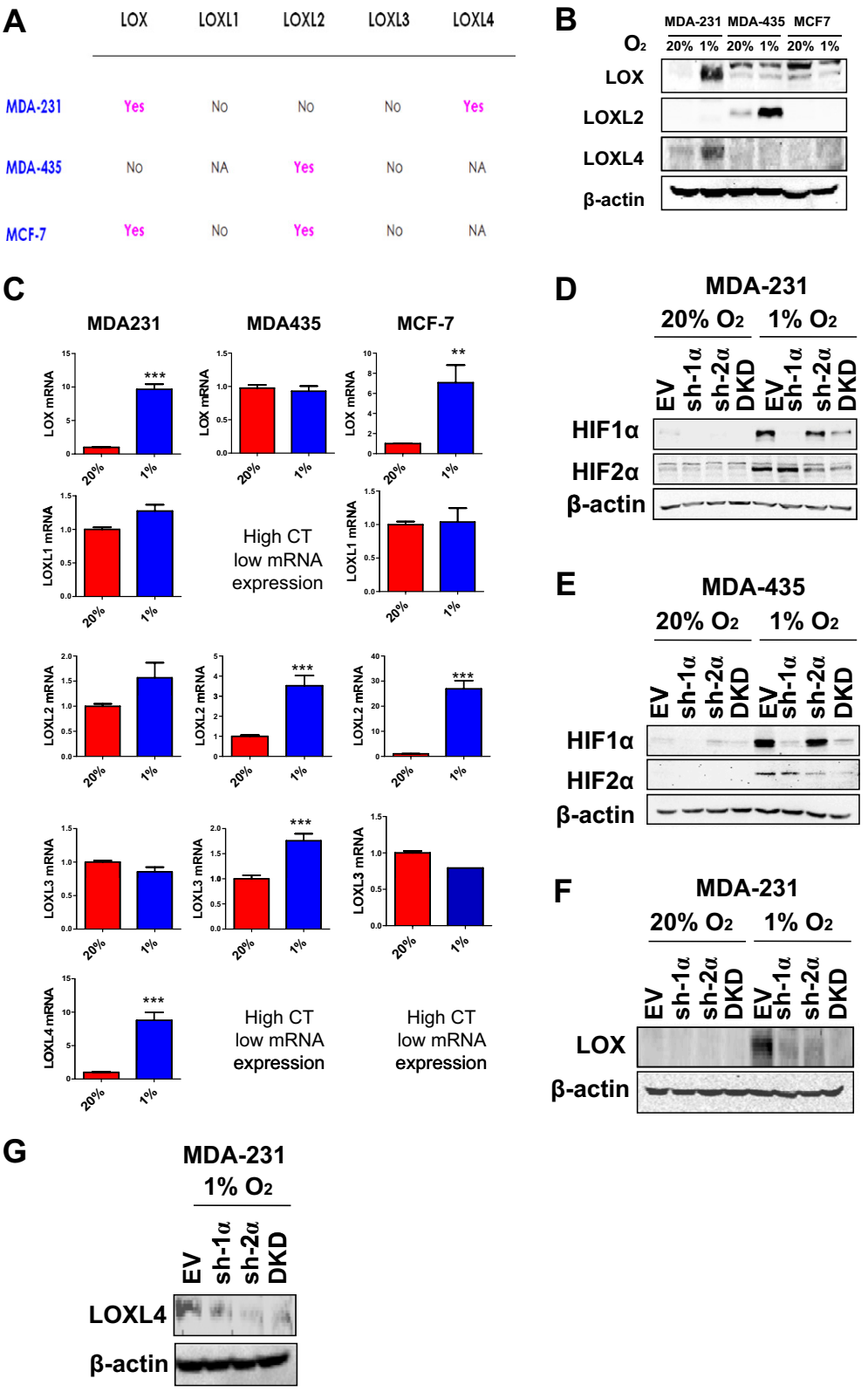

Fig. S1. LOX, LOXL, HIF-1α, and HIF-2α expression in breast cancer cell lines. (*A*) Summary of LOX/LOXL mRNA expression in MDA-231, MDA-435, and MCF-7 cells cultured in 20% or 1% O_2. "Yes" indicates that mRNA expression was increased at least twofold by hypoxia. mRNA expression that was not increased at least twofold by hypoxia is labeled as "No." mRNA expression could not be accurately determined for cycle number beyond 40, and this is indicated as "NA." (*B*) MDA-231, MDA-435, and MCF-7 cells were cultured in 20% or 1% O_2 for 48 h. LOX (55 kDa), LOXL2 (72–95 kDa), LOXL4 (72–95 kDa), and β-actin protein (45 kDa) levels were determined by Western blot analysis. (*C*) Histograms show LOX, LOXL1, LOXL2, LOXL3, and LOXL4 mRNA expression in MDA-231, MDA-435, and MCF-7 cells. All values were normalized to 20% O_2. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. 20% O_2; Student *t* test (mean ± SEM; $n = 3$). (*D*) Protein expression of HIF-1α and HIF-2α in MDA-231-EV, -sh-1α and -sh-2α, and -DKD subclones. (*E*) Protein expression of HIF-1α and HIF-2α in MDA-435-EV, -sh-1α, -sh-2α, and -DKD subclones. (*F*) LOX protein expression was analyzed in MDA-231 subclones. (*G*) LOXL4 protein expression was analyzed in MDA-231 subclones.

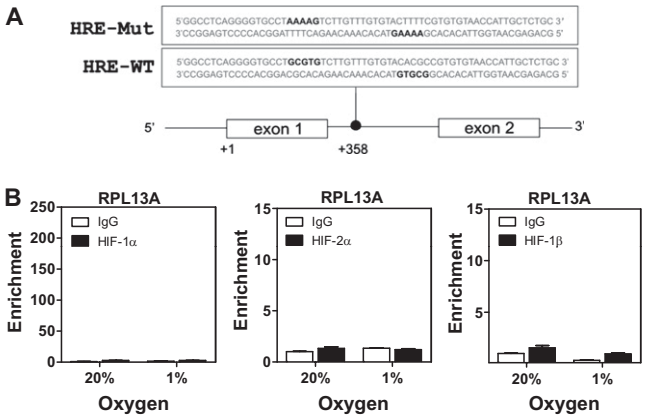

Fig. S2. (*A*) Candidate HRE was identified in intron 1 of the human *LOXL4* gene (HRE-WT). +1 designates the transcription start site. 5′-GCGTG-3′ WT sequences were mutated to 5′-AAAAG-3′ (HRE-Mut) to destroy HIF-1 binding sites (bold). (*B*) No significant hypoxia-induced binding at a non-HIF target gene, *RPL13A*. ChIP assay was performed using IgG, HIF-1α, HIF-2α, and HIF-1β antibodies. *RPL13A* primers were used for qPCR, and values were normalized to 20% O_2-IgG.

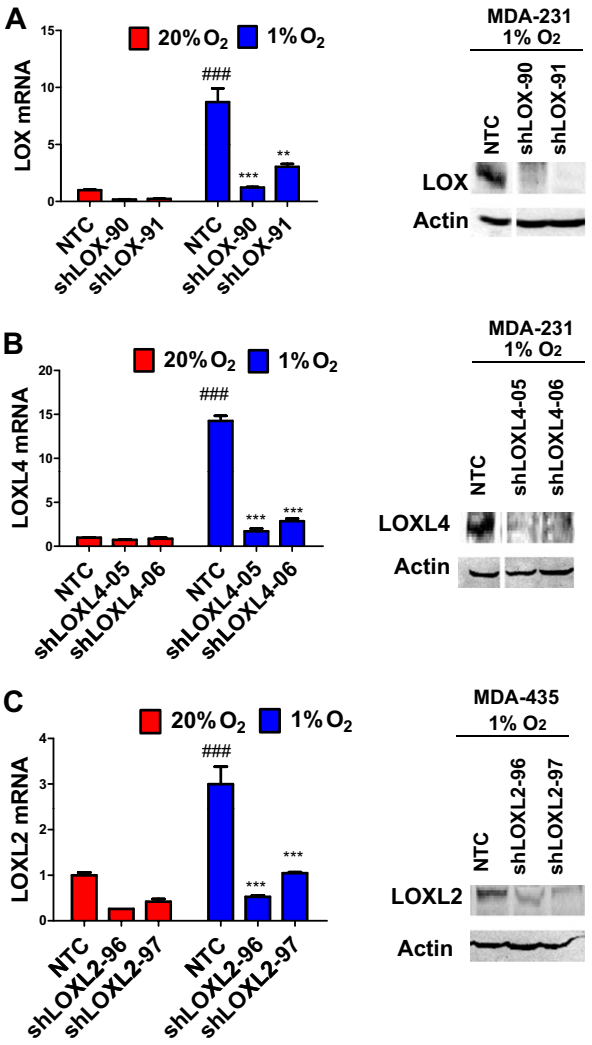

Fig. S3. Knockdown of LOX family members in breast cancer cells. mRNA expression was analyzed by RT-qPCR, and protein expression was detected by Western blot. (*A*) LOX mRNA (*Left*) and protein (*Right*) levels were reduced in shLOX-90 and shLOX-91 stable clones compared with NTC in MDA-231 cells. Western blot images were cropped from the same gel. (*B*) LOXL4 mRNA (*Left*) and protein (*Right*) levels were reduced in shLOXL4-05 and shLOXL4-06 stable clones compared with NTC in MDA-231 cells. Western blot images were cropped from the same gel. (*C*) LOXL2 mRNA (*Left*) and protein (*Right*) were reduced in shLOXL2-96 and shLOXL2-97 stable clones compared with NTC in MDA-435. $^{###}P < 0.001$ vs. 20% O_2 NTC; $^{**}P < 0.01$, $^{***}P < 0.001$ vs. 1% O_2 NTC; one-way ANOVA with Bonferroni correction (mean ± SEM; $n = 3$).

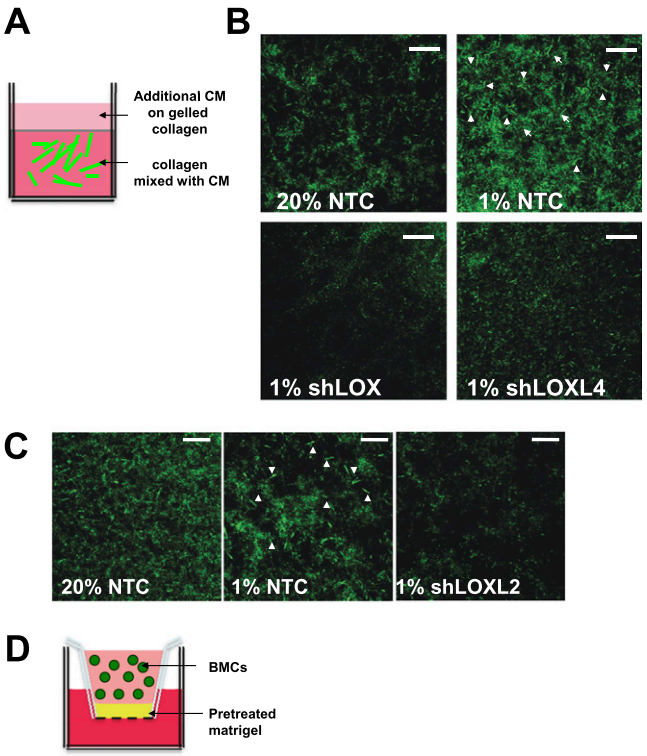

Fig. S4. LOX family members regulate collagen cross-linking. *(A)* Type I collagen was mixed with 10-fold concentrated CM. After 30 min of incubation at 37 °C, additional 10-fold concentrated CM was added on top of the gel for incubation at 37 °C for 24 h. *(B)* Collagen gel was incubated with CM generated by MDA-231-NTC, -shLOX, and -shLOXL4 cells that were cultured in 20% or 1% O_2. Representative photomicrographs of collagen gel under reflection confocal microscopy are shown. (Scale bar = 10 μm.) (*C*) Collagen gel was incubated with CM generated by MDA-435-NTC and -shLOXL2 cells that were cultured in 20% or 1% O_2. Representative photomicrographs of collagen gel under reflection confocal microscopy are shown. Arrowheads point to representative collagen fibers. (Scale bar = 10 μm.) (*D*) Transwell chambers were coated with matrigel. CM collected from breast cancer cells was incubated with matrigel for 16 h at 37 °C. BMCs in serum-free medium were seeded in the top chamber. Medium containing 10% (vol/vol) FBS was placed in the bottom chamber. BMCs that invaded through the pretreated matrigel to the bottom chamber were counted.

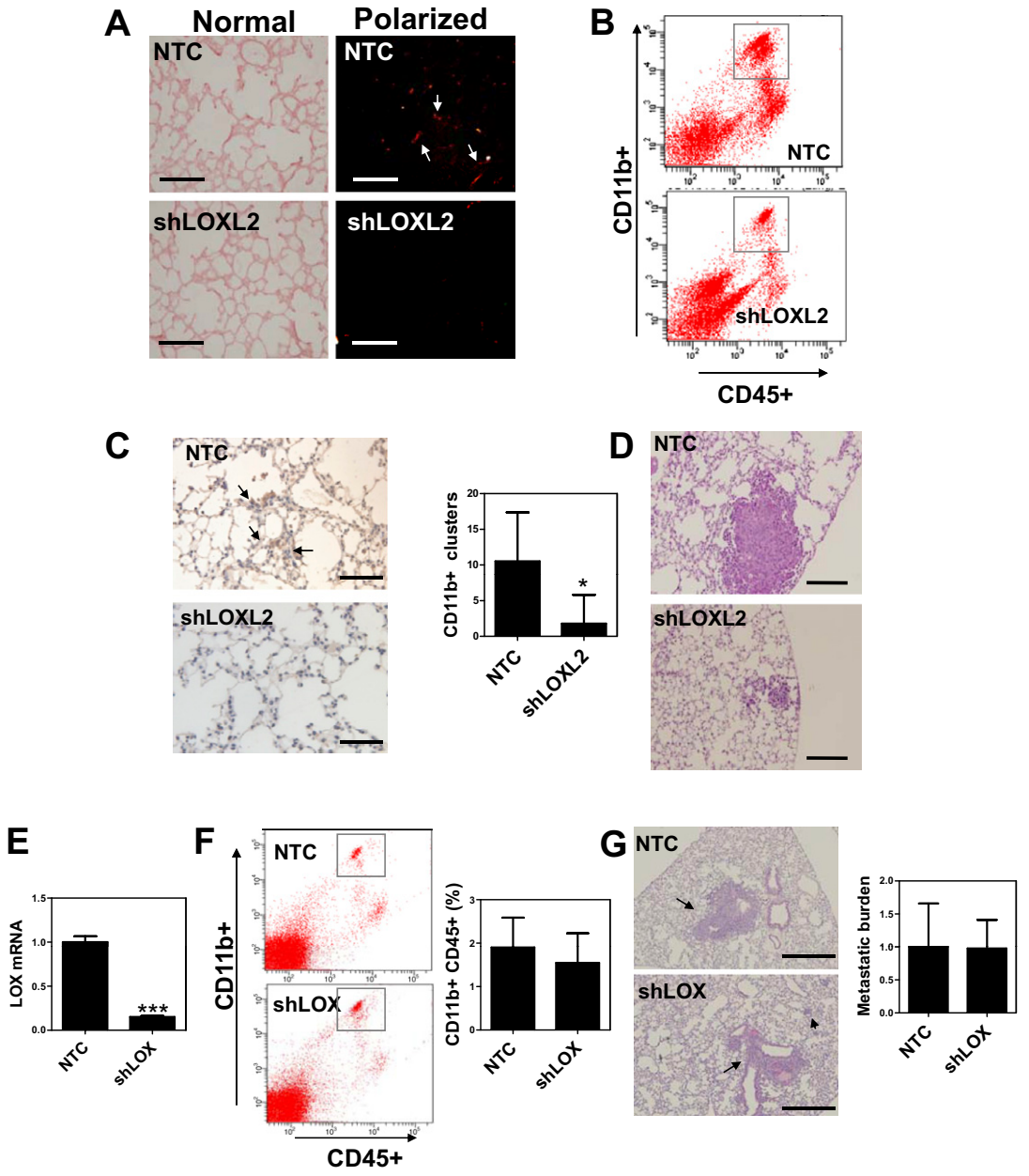

Fig. S5. Knockdown of LOXL2 but not LOX in MDA-435 cells suppresses metastatic niche formation. SCID mice were injected with MDA-435-NTC and -shLOXL2 subclones orthotopically and euthanized 42 d later. Representative photomicrographs show lungs that were analyzed by Picrosirius Red staining under polarized light, with arrows indicating crosslinked collagen fibrils (scale bar = 100 μm) (*A*), flow cytometry of $CD11b^{+}CD45^{+}$ BMDCs (*B*), immunohistochemical staining for CD11b (*Left*; scale bar = 100 μm), with arrows indicating stained cells that are quantified in bar graph (*Right*; $^{*}P < 0.05$, Student *t* test) (*C*), and H&E staining (*D*). (*E*) LOX mRNA expression in MDA-435-NTC and MDA-435-shLOX stable clones was determined by RT-qPCR (mean ± SEM; $n = 3$). (*F*) No difference in the percentage of BMDCs was found in the lungs of mice bearing MDA-435-NTC and -shLOX tumors as demonstrated by flow cytometry analysis. (*G*) No difference in lung metastasis in mice bearing MDA435-NTC and -shLOX subclones. (*Left*) Representative photomicrographs of H&E-stained lung sections. (Scale bar = 500 μm.) Arrows indicate metastatic foci. (*Right*) Metastatic burden of the lungs as determined by qPCR using human *HK2* primers. $^{***}P < 0.001$ vs. NTC, Student *t* test (mean ± SD; $n = 4$–5).

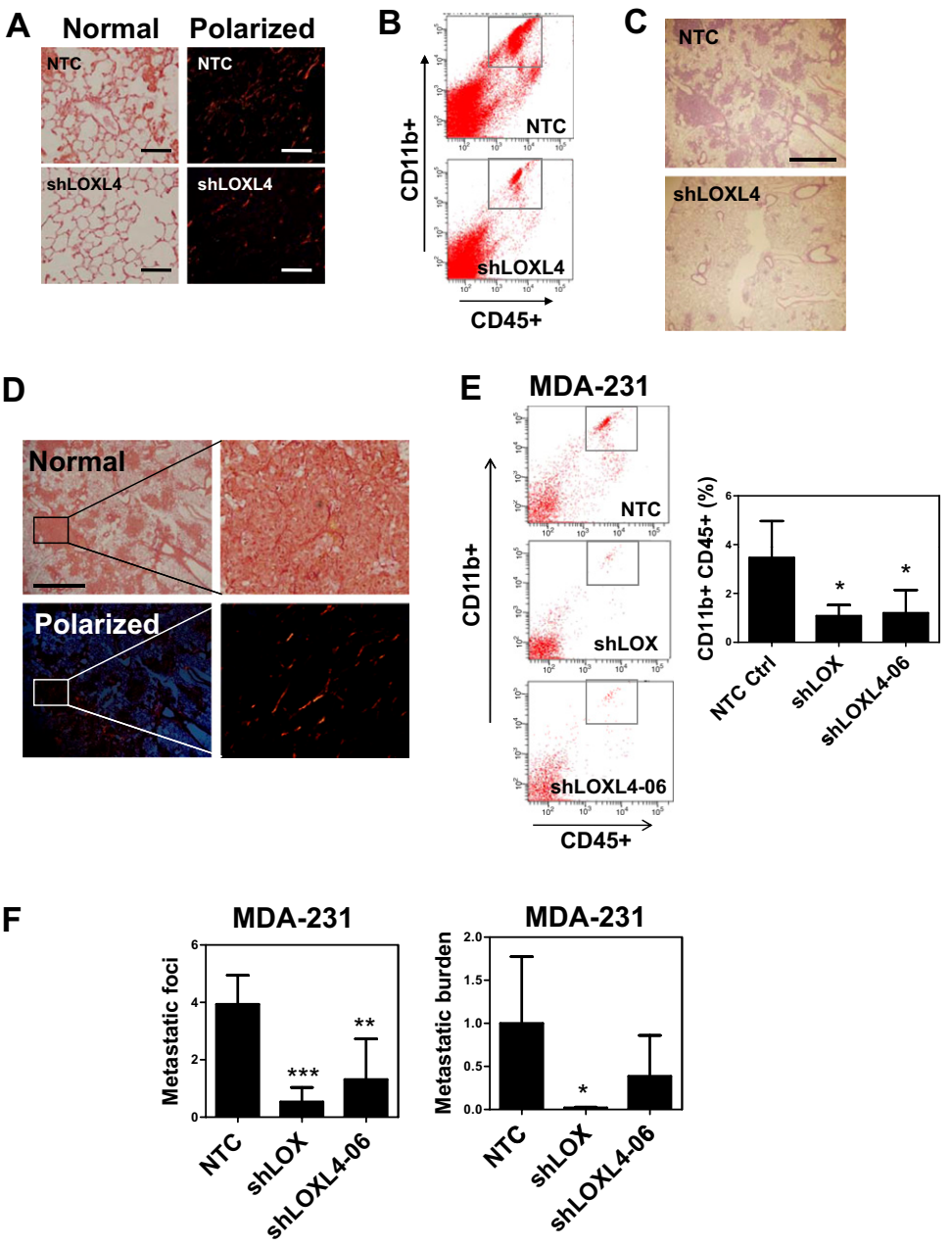

Fig. S6. LOXL4 expression is required for metastatic niche formation and subsequent metastasis in MDA-231 tumor-bearing mice. Nonobese diabetic-SCID mice were injected with MDA-231-NTC or -shLOXL4 subclone orthotopically. (*A*) Representative photomicrographs of lung sections that were stained with Picrosirius Red. (Scale bar = 100 mm.) (*B*) Flow cytometry analysis of $CD11b^{+}CD45^{+}$ BMDCs. (*C*) H&E staining. (Scale bar = 1 mm.) (*D*) Picrosirius Red staining was performed to analyze cross-linked collagen in the lung metastases. (Scale bar = 1 mm.) (*E*) Mice bearing shLOX or shLOXL4-06 tumors exhibited a reduction of BMDCs in the lungs compared with mice bearing NTC tumors. (*F*) Mice bearing shLOX or shLOXL4-06 tumors had decreased lung metastasis relative to the NTC group, as demonstrated by the number of metastatic foci on H&E-stained sections, and total lung metastatic burden, as determined by qPCR. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NTC; one-way ANOVA with Bonferroni correction (mean ± SD; $n = 4–5$).

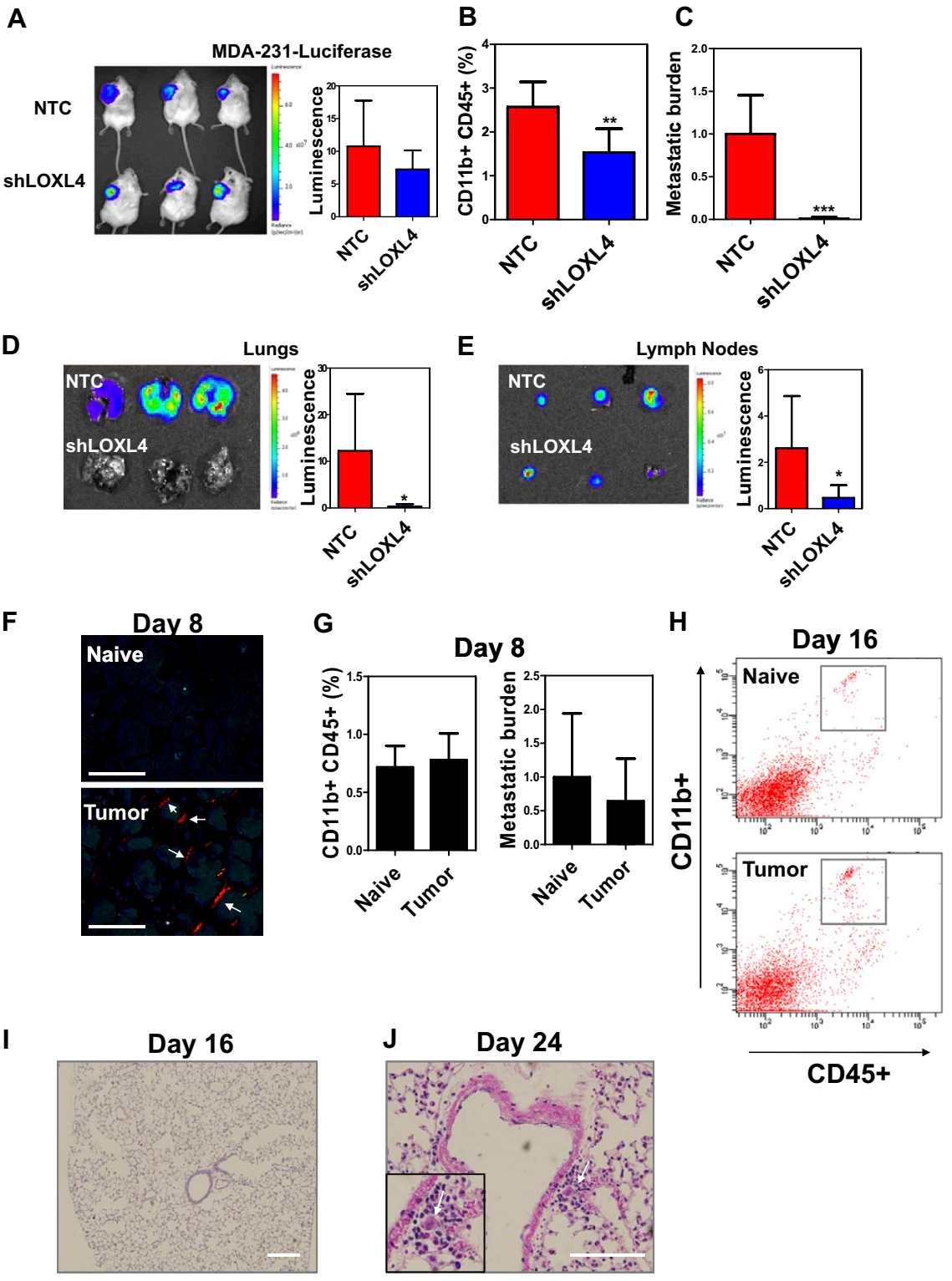

Fig. S7. LOX family members and metastatic niche formation. (*A–E*) SCID mice received MFP injection of luciferase-expressing MDA-231-NTC or -shLOXL4 cells. On day 45, mice were injected with D-luciferin and subjected to imaging (Xenogen). (*A*) Luminescence ($\times 10^8$ photons per second per square centimeter of steradian) generated by primary tumors was plotted. (*B*) Flow cytometry analysis of $CD11b^+CD45^+$ BMDCs in lungs. (*C*) Metastatic burden was determined by *HK2* qPCR. Luminescence ($\times 10^7$), generated in the lungs (*D*) and lymph nodes (*E*) harvested from mice, was plotted. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NTC; Student *t* test (mean ± SD; $n = 6$). (*F–J*) Kinetics of metastatic niche formation. MDA-435 cells were injected into the MFP of SCID mice, which were euthanized 8, 16, or 24 d later. (*F*) Representative photomicrographs of Picrosirius Red staining of the lungs from tumor free (Naive) and tumor-bearing (Tumor) mice on day 8, with arrows indicating crosslinked collagen fibrils. (Scale bar = 50 mm.) (*G*) Percentage of $CD11b^+CD45^+$ BMDCs (*Left*) and metastatic burden (*Right*) in the lungs of mice on day 8 were analyzed. (*H*) Percentage of $CD11b^+CD45^+$ BMDCs in the lungs of the mice on day 16 was analyzed by flow cytometry. (*I*) H&E-stained lung section from a tumor-bearing mouse on day 16. (Scale bar = 200 mm.) (*J*) H&E staining of the lung of a tumor-bearing mouse on day 24. (Scale bar = 100 mm.) Human cancer cells are indicated by white arrow in the photomicrograph and magnified inset.

Table S1. Oligonucleotide sequences of PCR primers

Target gene	Primer sequences
Human LOX	Forward: gttccaagctggctactc
	Reverse: gggttgtcgtcagagtac
Human LOXL1	Forward: cagaccccaactatgtgcaa
	Reverse: atgctgtggtaatgctggtg
Human LOXL2	Forward: ggaaagcgtacaagccagag
	Reverse: gcactggatctcgttgaggt
Human LOXL3	Forward: atgggtgctatccacctgag
	Reverse: gagtcggatcctggtctctg
Human LOXL4	Forward: accgaagacaaagccacaac
	Reverse: cacacgacactggcagagat
Human LOXL4 (ChIP)	Forward: tgcttccaactggacaagtg
	Reverse: ggcagagcaatggttacaca
Human HK2	Forward: ccagttcattcacatcatcag
	Reverse: cttacacgaggtcacatagc
Mouse 18S rRNA	Forward: cggcgacgacccattcgaac
	Reverse: gaatcgaaccctgattccccgt
Human 18S rRNA	Forward: gaggatgaggtggaacgtgt
	Reverse: agaagtgacgcagccctcta
Human RPL13A	Forward: gaggcgagggtgatagag
	Reverse: acacacaagggtccaattc