INTRODUCTION
Breast cancer affects more than one in ten women worldwide [1]. Currently, neoadjuvant chemotherapy is extensively used to treat breast cancer patients as it reduces tumor burden, thus downstaging the disease [2]. However, most non-targeted anticancer agents do not only trigger cytotoxicity in dividing cells, but also engage specific cellular response programs, including senescence, on both cancer cells and the tumor microenvironment [3].

Cellular senescence refers to the stable cell proliferation arrest caused by either telomere shortening, oncogene activation or genotoxic stress, all of which converge towards the activation of a sustained DNA damage response (DDR) [4]. Because of its engagement in preneoplastic lesions, senescence was originally hypothesized to serve as a barrier to malignant transformation, by preventing the proliferation of cells harboring an altered genetic content [5]. Additionally, senescent cells accumulate over time in mammals and contribute to the health defects associated with aging [6, 7]. The beneficial impact of senescence as a tumor suppressor mechanism early in life along with its detrimental impact on aging phenotypes led to the theory of antagonistic pleiotropy of senescence [8]. One of the hallmarks of senescence that could rationalize these otherwise contradictory features is the senescence-associated secretory phenotype (SASP) [9].

The SASP consists of a discrete set of pro-inflammatory cytokines, chemokines and growth factors secreted by senescent cells, in a cellular and senescence inducer-specific manner. As such, the SASP may contribute to the “inflamm-aging”, a sterile inflammation that develops as individuals age. In addition to its potential impact on aging phenotypes, exposure to the SASP has been reported to promote aggressive traits in tumor models, including increased cellular proliferation [10], enhanced angiogenesis [11], and activation of the epithelial-to-mesenchymal transition (EMT) [12]. However, the molecular mechanisms engaged by senescent cells to drive tumorigenesis remain poorly understood.

Lipocalin-2 (LCN2), also referred to as neutrophil gelatinase-associated lipocalin (NGAL), is a 25 kDa secreted glycoprotein involved in iron metabolism and inflammation [13]. LCN2 protein expression levels are found increased in various cancer types including breast [14, 15], colon [16], and pancreatic [17], the basis for which remain unexplored. Accordingly, high LCN2 expression has been detected in carcinoma tissues, sera, and urine of breast cancer patients [18].

Here, we demonstrate that exposure to the SASP or senescence-inducing neoadjuvant chemotherapy results in the potent upregulation of LCN2 expression in breast cancer cells in vitro and in human cancer samples, respectively, which correlates with increased cellular plasticity and poor prognosis.
The IL-1-dependent SASP promotes cellular plasticity in breast cancer cells.

We have previously demonstrated that inactivation of the IL-1 pathway can be used to uncouple SASP production from senescence-associated cell cycle exit [19]. We leveraged this property of IL-1α-inactivated cells to determine the impact of SASP exposure on cancer cells’ properties. MCF7 breast cancer cells were exposed to conditioned media (CM) from wild-type (WT) TERT-immortalized IMR90 (IMR90T) fibroblasts, senescent WT IMR90Ts (through ectopic expression of oncogenic Ras<sup>G12V</sup>) and senescent IL-1α<sup>−/−</sup> IMR90Ts. B Representative images of scratched MCF7 cells cultured in conditioned media (CM) from growing (Grow CM) or Ras-senescent (Ras CM) IMR90T cells with or without IL-1α for 3 days. Scale bar = 200 μm. C Quantification of relative gap width of MCF7 cultures treated with the indicated CM. D, E Representative images and quantification of transwell migration assays of MCF7 cells treated with the indicated CM. F Representative images of MCF7 cells treated with CM from 2 IMR90T clones, either growing or Ras-induced senescent with or without IL-1α. Inset: magnified images. Scale bar = 200 μm. G Representative images and quantification of E-cadherin immunofluorescence staining in MCF7 cells treated with the indicated CM. Scale bar = 50 μm. H Quantification of CD44<sup>+</sup> MCF7 cells after being treated with the indicated CM as analyzed by flow cytometry. n = 3, *p < 0.05, **p < 0.01.

RESULTS

The IL-1-dependent SASP promotes cellular plasticity in breast cancer cells

To begin to decipher the molecular mechanisms underlying the impact of exposure to the SASP on breast cancer cells’ properties, we profiled the transcriptome of MCF7 cells exposed to CM from growing or senescent IMR90Ts. Using a log<sub>2</sub> fold change cutoff and an adjusted p value of <0.05, 1981 genes were found differentially expressed between MCF7 cells exposed to growing CM and MCF7 exposed to senescent CM. Pathways upregulated in senescent CM samples include inflammatory response and extra-cellular matrix organization (Fig. 2A). Gene sets that were significantly enriched in senescent CM compared to growing CM samples included Epithelial-to-Mesenchymal Transition and Protein Secretion (Fig. 2B). These results are consistent with the previous demonstration that exposure to the SASP activates an inflammatory response.

Exposure to the SASP induces expression of Lipocalin-2 in breast cancer cells

Finally, the proportion of MCF7 cells expressing the EMT-associated surface marker CD44 [21] increased upon exposure to SASP (Fig. 1F). Accordingly, MCF7 cells exposed to senescent CM exhibited loss of expression of the epithelial marker E-cadherin (Fig. 1G). Collectively, these results indicate that exposure to the IL-1-dependent SASP induces cellular plasticity in breast cancer cells, as evidenced by increased migratory properties and the engagement of an at least a partial EMT program.
Exposure to the SASP induces expression of LCN2 in breast cancer cells. A Gene ontology analysis of genes that are upregulated in Ras CM compared to Grow CM samples with a log2 fold change > 2. B GSEA plots of pathways upregulated in Ras CM compared to Grow CM samples. C Volcano plot depicting differentially expressed genes in Ras CM samples compared to Grow CM samples. Blue dots: Transcripts with log2 fold change cutoff of 1 and adjusted p value < 0.05. Green dots: Transcripts with the same cutoffs with base mean > 100. Red dots: Transcripts with the same cutoffs with base mean > 500. D qRT-PCR analysis and E Western blot for LCN2 expression in MCF7 cells cultured with the indicated CM. F qRT-PCR analysis for LCN2 expression in MCF7 cells cultured with Grow CM or conditioned media from etoposide-induced senescent IMR90T cells (Etop CM) G Representative images and quantification of transwell migration assay of MCF7 cells treated with the indicated CM. Scale bar = 200 μm. H Western blot for LCN2 expression in MCF7, SKBR3, Hs578t, BT474, and MDA-MB-231 cells cultured with the indicated CM; G (Grow CM) and R (Ras CM). I Quantification of relative gap width of MDA-MB-231 cultures treated with the indicated CM. n = 3, **p < 0.01, ****p < 0.0001.

response and the initiation of an EMT program [22, 23]. The most upregulated transcript in MCF7 cells exposed to senescent CM encodes the protein Lipocalin-2 (LCN2, or NGAL) (Fig. 2C). We confirmed the upregulation of LCN2 mRNA and protein levels via qRT-PCR (Fig. 2D) and Western Blot (Fig. 2E). LCN2 was not upregulated in MCF7 cells treated with CM from senescent IL-1α−/− cells (Fig. 2D, E). Exposure to SASP from cells rendered senescent by etoposide (Etop) treatment resulted in a significant increase in LCN2.
mRNA levels, indicating that LCN2 upregulation was independent of the stimulus used to induce senescence (Fig. 2F). Of note, the LCN2 upregulation induced by exposure to genotoxic stress-induced SASP also correlated with increased migration (Fig. 2G). We extrapolated these observations to independent breast cancer cells with various ER, PR, and HER2 status, and detected a consistent upregulation of LCN2 upon exposure to the SASP (Fig. 2H). In these conditions, these cells also exhibited an elongated and mesenchymal-like morphology (Fig. S1). In addition, we observed an increase in the rate of migration of MDA-MB-231 and T47D cells exposed to the SASP for 2 days (Fig. 2I and Fig. S2). Taken all together, these results indicate that the SASP secreted from cells induced to senesce by various stimuli promotes migration and LCN2 upregulation in several breast cancer cell lines.

**LCN2 upregulation is required for SASP-induced cell plasticity**

High expression of LCN2 results in increased migratory properties in cancer cells [24, 25]. However, the molecular basis for LCN2 upregulation remains unexplored. On account of the substantial LCN2 upregulation we detected in cells treated with senescent CM, we hypothesized that the SASP may enhance aggressive breast cancer phenotypes at least in part through upregulation of LCN2. We first successfully inactivated LCN2 in MCF7 cells by CRISPR/Cas9 induced gene deletion, as evidenced by Western Blot analysis (Fig. 3A, B). In agreement with the undetectable to low levels of LCN2 expressed in MCF7 cells grown in normal conditions, LCN2−/− MCF7 cells did not exhibit any proliferation defects (data not shown). Strikingly, scratch assays indicated that LCN2−/− MCF7 cells treated with the indicated CM for 2 days. Scale bar = 200 μm. **F** Volcano plot depicting differentially expressed genes in LCN2−/− MCF7 cells cultured with the indicated CM for growing (Grow CM) or Ras-senescent (Ras CM) IMR90T cells for 3 days. Scale bar = 200 μm. **G** Representative images and quantification of transwell migration assays of LCN2−/− and LCN2−/− MCF7 cells treated with the indicated CM for 2 days. Scale bar = 200 μm. **H** Gene ontology analysis of genes that are differentially expressed in LCN2−/− compared to LCN2−/− when treated with Ras CM. **I** GSEA plots of pathways affected by LCN2 knockout when treated with Ras CM. n = 3, *p < 0.05, **p < 0.01.

**SASP-induced LCN2 expression promotes breast cancer progression in vivo**

We next sought to assess the impact of LCN2 expression on tumor progression in vivo. We injected Luciferase-expressing MDA-MB-231 cells into mammary fat pads of nude mice and
followed tumor progression. Importantly, LCN2 status had no impact on MDA-MB-231 tumor growth (Fig. 4A, B). This observation is consistent with the undetectable to low LCN2 expression levels in these cells in the absence of a pro-senescence stimulus (Fig. 2H). Importantly, Western Blot analysis revealed that SASP-dependent upregulation of LCN2 levels was only transient, as removal of conditioned media from senescent fibroblasts resulted in a strong downregulation of LCN2 (Fig. 4C). Therefore, to ensure continuous SASP exposure and upregulation of LCN2 in tumors, we opted for a co-injection model with senescent human fibroblasts. MDA-MB-231 cells exhibited increased LCN2-dependent proliferation and tumor progression when co-injected with senescent fibroblasts (Fig. 4D–F). IHC revealed that LCN2+/+ tumors expressed reduced levels of E-cadherin and increased Ki67, compared to their LCN2−/− counterparts. Apoptosis could not account for the difference in tumor size between experimental groups since LCN2−/− tumors were negative for cleaved-caspase 3 (Fig. 4G). These results indicate that SASP-dependent LCN2 upregulation promotes tumor cell plasticity in vivo and results in the development of more aggressive tumors that are highly proliferative.

**SASP-induced LCN2 protects breast cancer cells from genotoxic stress**

Tumor cell plasticity and EMT are closely associated with therapy resistance in breast cancer [29–31]. Based on the phenotypes elicited by LCN2−/− breast cancer cells in vivo, we hypothesized that SASP-mediated LCN2 upregulation could confer breast cancer cells a chemo-protective phenotype. Indeed, cell viability and Annexin V assays indicated that LCN2+/+ MDA-MB-231 cells exposed to SASP were more resistant to doxorubicin than LCN2+/+ MDA-MB-231 cells exposed to normal medium. However, the increased resistance to doxorubicin upon exposure to the SASP was abrogated in LCN2−/− MDA-MB-231 cells (Fig. 5A, B). To validate these findings in vivo, we injected MDA-MB-231 cells into mammary fat pads of nude mice. While LCN2+/+ and LCN2−/− tumors grew at a comparable rate prior to doxorubicin treatment, doxorubicin injection resulted in a dramatic sensitization of LCN2−/− tumors (Fig. 5C, D). One week after doxorubicin treatment, LCN2+/+ tumors were significantly larger than their LCN2−/− counterparts, indicating that resistance to doxorubicin is enhanced by SASP-induced LCN2 upregulation. Together, these

**Fig. 4** SASP-induced LCN2 expression promotes breast cancer progression in vivo. A Representative images of tumor growth progression of LCN2+/+ and LCN2−/− MDA-MB-231 GFP-Luciferase injected into mammary fat pad of nude female mice. B Luminescence quantification for (A) using a PerkinElmer IVIS Spectrum system. n = 9 for LCN2+/+ tumors (Mean = 3.324 × 10^5) and n = 8 for LCN2−/− tumors (Mean = 3.5008 × 10^5), p = 0.9078. C Western blot analysis for LCN2 expression of MDA-MB-231 cells after removal or RasCM, G (Grow CM) and R (Ras CM). D Representative images of tumor growth progression of LCN2+/+ and LCN2−/− MDA-MB-231 GFP-Luciferase co-injected into mammary fat pad of nude female mice with 1 × 10^6 senescent fibroblasts. E Images of dissected LCN2+/+ and LCN2−/− tumor tissue. F Waterfall plot of the percentage change in tumor growth for LCN2+/+ and LCN2−/− MDA-MB-231 GFP-Luciferase co-injected with senescent fibroblasts. Tumor growth (%) was calculated dividing the bioluminescence intensity of each tumor on week 6 by the fluorescence intensity of the same tumor on week 2. n = 10. G Representative images of IHC for LCN2, E-cad, Ki67, and c-Caspase 3 of dissected LCN2+/+ and LCN2−/− tumors. Scale bar = 50 μm.
results suggest that the exposure to senescence-inducing stimuli promotes detrimental cellular plasticity in breast tumors through LCN2 expression.

**LCN2 expression is induced following chemotherapy and is a poor prognostic factor in breast cancer patients**

To assess the clinical relevance of the previous observations, we analyzed LCN2 levels in biopsy samples from individual breast cancer patients collected prior to or following neoadjuvant chemotherapy treatment. Upon chemotherapy treatment, we detected an increased positivity for the SASP marker IL-6 (Fig. 6A, B). Along IL-6 upregulation, samples collected after neoadjuvant chemotherapy treatment exhibited increased LCN2 positivity, while all biopsy samples collected before chemotherapy treatment displayed undetectable or low LCN2 expression (Fig. 6A, C). An opposite pattern of expression was observed for the epithelial marker, E-cadherin (Fig. 6A, D). We further analyzed the correlations between LCN2 levels and prognosis in breast cancer patients using publicly available expression databases. The analysis revealed that LCN2 was upregulated at the mRNA level in 125 of 2,507 patient samples (7%). Patients with increased levels of LCN2 have an inferior overall (data not shown) and relapse-free survival compared to those with unaltered levels of LCN2 (Fig. 6E). Additionally, 52.8% of patients with high LCN2 levels had received chemotherapy treatment prior to analysis, while only 18.5% of the patients with low levels of LCN2 had (Fig. 6F), further indicating a correlation between chemotherapy treatment and increased LCN2 levels. These data suggest that LCN2 could be a potential prognostic biomarker for breast cancer survival.

**DISCUSSION**

Cellular plasticity promotes tumor progression in breast cancer, through metastatic spread and resistance to conventional therapies. The molecular pathways that contribute to the plasticity of breast cancer cells, including EMT and dedifferentiation, are currently being uncovered. However, the stimuli leading to the acquisition of a cellular plastic phenotype within an established tumor remain largely unknown. We report here that exposure to the SASP induces breast cancer cell plasticity in an LCN2-dependent manner.

Our results are consistent with previous findings showing that breast cancer cells exposed to the SASP exhibit epithelial cell scattering and reduced cell-cell adhesions, features of EMT, and
increased expression of stemness markers [32, 33]. Strikingly, we demonstrate that LCN2 upregulation is critical for the engagement of an EMT-associated expression program in breast cancer cells upon SASP exposure. Furthermore, while we cannot conclude that all breast cancer cells are responsive to SASP-induced LCN2-upregulated migration, our results indicate that breast cancer cells of different molecular subtypes exhibit these properties. Such observation suggests that a large proportion of breast tumors could acquire more aggressive traits when surrounded by senescent cells. In the context of breast cancer, the source of the SASP can be several-fold: replicatively exhausted cells that accumulate in the organism with age secrete SASP factors that can reach tumor cells. Another source of the SASP may stem from pre-cancerous or cancerous cells themselves with constitutively active drivers of mitogenic signals, resulting in hyper-proliferation and fork collapse, known as oncogene-induced senescence (OIS). Finally, exposure to genotoxic therapies, including radiation therapy or chemotherapy, can drive both normal and tumor cells into senescence, in a process termed Therapy-Induced Senescence (TIS), leading to general and local SASP production. TIS has emerged as a novel functional target to improve cancer therapy [34]. However, accumulating evidence indicates that senescent cancer cells are capable of reentering the cell cycle and promoting tumor relapse and metastasis. Recent findings show that a senescence-like population of chemotherapy-resistant cells is capable of initiating cancer recurrence by increasing their stemness potential [35–37].

Previous studies have linked LCN2 and EMT or metastasis of breast cancer cells [15, 38], but the mechanisms involved remain unclear. Downregulation of the estrogen receptor ERα induces expression of the transcription factor Slug, driving EMT. Our transcriptome analyses indicate that in wild-type MCF7 cells, ERα is indeed downregulated and Slug is upregulated upon treatment with senescent CM compared to those treated with growing CM (data not shown). Studies have demonstrated that ERα signaling helps maintain the epithelial phenotype through inhibition of Snail activity [39]. However, based on our demonstration that SASP-induced LCN2 promotes migration in ERα-breast cancer cells, it is unlikely that LCN2 mediates its effects on breast cancer plasticity through the ERα pathway exclusively. Accordingly, our transcriptome analyses revealed that LCN2 levels show no correlation with ERα expression. Instead, LCN2 could drive EMT through its interaction with MMP9, reducing E-cadherin expression levels on the cell surface [40]. Further experiments will be necessary to elucidate the mechanism employed by LCN2 to promote EMT in breast cancer cells.

Lipocalin-2 (LCN2) was first characterized as an iron-binding protein, sequestering it from Gram-negative bacteria and inhibiting their proliferation. LCN2 iron-binding sequestration properties have also been implicated in the etiology of Leptomeningeal Metastasis (LM) [41]. Indeed, LCN2 upregulation allows metastatic cancer cells to thrive in an iron-limiting environment such as the cerebrospinal fluid. Accordingly, the administration of iron chelators slows cancer progression in a mouse model of LM [41]. Iron facilitates tumorigenesis by driving cell proliferation [42]. Therefore, LCN2 upregulation could result in an increased ability to sequester iron leading to an aggressive tumor growth in breast cancer cells [43].

The experiments presented and conclusions drawn here focus on the cancer cell autonomous impact of SASP-induced LCN2 upregulation. However, based on the pleiotropic impact of iron metabolism, it is also likely that LCN2 modulates the anti-tumor immune response in vivo. Indeed, LCN2 has the ability to upregulate human leukocyte antigen G (HLA-G), which can promote tumor immune escape in mouse models [44]. Furthermore, by conferring cancer cells with the ability to outcompete macrophages for iron, LCN2 may also contribute to the generation of a tumor microenvironment that promotes tumor growth, a possibility that remains to be investigated. For these reasons, therapeutic inhibition of LCN2 could provide a therapeutic relief for patients with breast tumors that are poised to undergo plasticity, for example following exposure to genotoxic therapies.

**MATERIALS AND METHODS**

**Cells**

IMR90 primary lung embryonic fibroblasts expressing hTERT (IMR90Ts) were obtained from S. Smith (NYU School of Medicine, New York, NY). Cells were cultured in MEM (Corning) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Cellgro). MCF7, SKBR3, Hs578T, and...
T47D cells were obtained from R. Possemato (NYU School of Medicine, New York, NY). MDA-MB-231 cells were obtained from E. Hernando (NYU School of Medicine, New York, NY). BT474 cells were obtained from B. Neel (NYU School of Medicine, New York, NY). Breast cancer cells (except T47D) were cultured in DMEM (Corning) supplemented with 10% FBS and 1% penicillin/streptomycin. T47D cells were cultured in RPMI 1640 media (Corning) supplemented with 10% FBS and 1% penicillin/streptomycin. HEK293T cells (ATCC) were used to generate retro- and lentiviruses and were cultured in DMEM (Corning), supplemented with 10% donor calf serum and 1% penicillin-streptomycin. IMR90T cells were maintained in 6% O2 and 5% CO2 at 37°C, while 293T and breast cancer cells were maintained in 5% CO2 at 37°C. Cells were regularly tested for mycoplasma.

Senescence induction and condition media (CM) harvest
For Ras-induced senescence, IMR90T-Ras(27)−ERT2 were treated with 200 nM tamoxifen for 10 days. Fresh media and tamoxifen were added every 2–3 days. Cells were treated with an equal volume of ethanol as control. On day 8 of Ras induction, media was replaced with serum-free media and harvested after 48 h. For etoposide-induced senescence, cells were treated with 50 μM etoposide (Sigma) or an equal volume of DMSO as a control for 48 h. Etoposide-containing medium was then replaced by normal culture media for 5 days. 7 days after etoposide treatment, media was replaced with serum-free media and harvested after 48 h. Conditioned media was aliquoted and stored in liquid nitrogen before storing at −80°C.

Scratch assays
Cells were grown in six-well plates until confluent. A P200 tip was used to create vertical scratches. Media was then changed to media containing 1% FBS supplemented by CM from growing or senescent cells. The data are presented as relative gap width compared to the gap width of each sample at day 0. Values were subjected to two-way ANOVA followed by Tukey’s multiple comparisons test. Data are presented as means ± SEM (n = 3).

Transwell migration assays
MCF7 cells were exposed to 1–3 mL CM from growing or senescent cells. Fresh CM was added every day for a total of 2 days. A total of 50,000 cells in 100 μL serum-free DMEM were seeded on top of transwells containing 8 μm pores for use in 24-well plates. One milliliter of DMEM supplemented with 20% FBS was added to the bottom of the wells and cells were allowed to migrate for 48 h. Values are expressed as fold change in the number of cells compared to the number of cells cultured in the corresponding growing CM. Values were subjected to two-way ANOVA followed by Tukey’s multiple comparisons test. Data are presented as means ± SEM (n = 3).

Immunofluorescence
MCF7 cells were treated with CM from growing or senescent cells. CM was added every day for 2 days. Cells were then fixed and incubated with mouse anti-E-cadherin (Millipore, MAB1199) at 1:200 dilution in blocking solution at 37°C for 1 h. Cells were then washed and incubated in Cy3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch) for 1 h at RT. Cells were mounted with mounting medium containing Dapi (Vectashield). Slides were examined on a Zeiss Axiosmager A2 microscope. A total of 100 cells per cover slip were counted. Amount E-cadherin staining was quantified as number of red pixels per Dapi-positive cells using Image J to calculate pixels. Values were subjected to a paired t test. Data are presented as means ± SEM (n = 3).

Transcriptomics analysis
MCF7 cells were cultured in CM from growing or Ras-induced senescent cells for 2 days. MCF7 LCN2+/+ and LCN2−/− cells were cultured in CM from Ras-induced senescent cells for 2 days. RNA quality assessment, library preparation and sequencing were performed by the NYU School of Medicine Genome Technology Center or by Genewiz. Strand-specific libraries were prepared using the TrueSeq RNA library Prep kit, and libraries were sequenced on an Illumina HiSeq2500 using 50-bp paired-end reads. Sequences were mapped to the hg10 genome, and analysis was done as previously described (Proudhon, 2016).

Real-time PCR
The following sets of primers were used: hTubulin forward 5′-cttgctctgctgcatcag-3′, reverse 5′-tggctacagcttacgatca-3′, mLCN2 forward 5′-gaatgtgctactgttcgatca-3′, reverse 5′-gtacgcttgcggaggtta-3′, mGAPDH forward 5′-caggaatcccgccgagatc-3′, reverse 5′-accggtttgctccaccc-3′. Values were subjected to two-way ANOVA followed by Tukey’s multiple comparisons test. Data is presented as means ± SEM (n = 3).

CRISPR/Cas9 editing
sgRNAs were cloned into lentiCRISPR v2 (Addgene) and 48 h after infection, cells were selected with puromycin (1 μg/mL) for at least 4 days before plating single cell clones. The following sgRNA’s were used for hLCN2: forward 5′-ccacggaatcttcatgatttacg-3′, reverse 5′-aaagggcttaccagttacct-3′ and forward 5′-caccggtgtgccagctatct-3′, reverse 5′-aag gcggagaggtgtgac-3′.

Protein extraction and western blotting
Cells were lysed in 1× RIPA buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA), 0.5 μM DTT, 25 mM NaF, 1 mM sodium vanadate, 1 mM PMSF, and cComplete protease cocktail inhibitor (Sigma). Samples were resolved by SDS-PAGE and analyzed by standard western blotting techniques. The following primary antibodies were used: mouse anti-tubulin (Sigma T9026) at 1:2000 dilution, goat anti-LCN2 (R&D AF1747) at 0.2 μg/mL, mouse anti-vinulin (Sigma V9131) at 1:1000 dilution and rabbit anti-EGFR (Cell Signaling 4267) at 1:1000 dilution. Each experiment was performed at least three times.

Bioluminescence
For in vivo luminescence of Luciferase, mice were injected i.p. with 100 μL of o-Luciferin (ThermoFisher) per kg of body weight. Fifteen minutes later, the mice were anesthetized with isoflurane and luminescence was measured with a PerkinElmer IVIS Spectrum system. Analysis of the tumor size was performed in a single-blind manner.

MDA-MB-231 co-injection with senescent fibroblasts
2.5 × 10^4 LCN2−/− or LCN2+/+ MDA-MB-231 GFP-Luciferase cells were pretreated with RasCM for 48 h before being injected into the inguinal mammary fat pad of 4–6 weeks old female nude mice with 10^5 senescent IMR90T cells in 50% Matrigel (Corning) (n = 10).

Immunohistochemical staining
Immunohistochemical staining was performed at the NYU Langone Experimental Pathology Research Laboratory as previously described (Rielland, 2014). The following antibodies were used: goat anti-LCN2 (R&D AF1747), E-cadherin (Cell Signanl, 3195T), IL-6 (Santa Cruz Biotechnolog, sc-1265). Analysis of human samples was performed in a double-blind manner.

Cell viability assay
400,000 cells were plated in triplicate in 12-well plates and allowed to adhere overnight. Cells were then treated with increasing concentrations of doxorubicin and CM for 24 h. Wells were washed with PBS and cells were fixed with 2% glutaraldehyde in PBS for 15 min. Cells were then stained with crystal violet (0.1% in 10% ethanol) for 30 minutes. After washing and drying, cells were stained in 10% acetic acid for 15 min. Optical density (OD) was measured at 595 nm absorbance. Values were subjected to two-way ANOVA followed by Tukey’s multiple comparisons test. Data is presented as means ± SEM (n = 3).

Annexin V staining
Treated and untreated cells were collected, without washing to collect all floating cells, centrifuged at 1300 rpm for 3 min. After discarding supernatant, 5 μL of Annexin V (BioLegend) was added to cells resuspended in 200 μL of binding buffer (BioLegend). Cells were then incubated for 30 min at room temperature in the dark before centrifuging them again. Cells were then resuspended in 200 μL of binding buffer after discarding the supernatant and analyzed via flow cytometry using a FACSCalibur Flow Cytometer (BD). Values were subjected to two-way ANOVA followed by Tukey’s multiple comparisons test. Data is presented as means ± SEM (n = 3).

MDA-MB-231 tumors and doxorubicin treatment
1 × 10^3 LCN2−/− or LCN2+/+ MDA-MB-231 GFP-Luciferase cells in 50% Matrigel (Corning) were injected into the inguinal mammary fat pad of
4–6 weeks old female nude mice. Once tumors were established, mice were treated with a single dose of 10 mg/kg of doxorubicin (Sigma) (n = 13).

Animal studies

The number of animals needed to achieve statistical power was calculated based on a two-sided Wilcoxon nonparametric test with a significance level of 5%. No animal was excluded from the study. Mice were randomly allocated to experimental and control groups.

Study approval

Animal work and human subject work were performed according to approved protocols from the NYU School of Medicine's IACUC and IRB. Written informed consent was obtained from each human participant before study procedure.

DATA AVAILABILITY

Raw transcriptomic data were deposited at GEO and are available under accession numbers GSE198661 and GSE198685. Additional data that support the findings of this study are available in figshare with the identifier: https://doi.org/10.6084/m9.figshare.2001763.

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AUTHOR CONTRIBUTIONS
The authors confirm contribution to the paper as follows: LL initiated the study under supervision of GD and contributed to Figs. 1 and 2 of the manuscript. JMV designed, performed, and analyzed data from the experiments presented in this manuscript. TMN contributed to sample preparation. UD provided patient samples and analyzed the data. GD designed the study and supervised the research. JMV and GD wrote the manuscript. All authors reviewed the results and approved final version of the manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
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