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## **Supplemental Information**

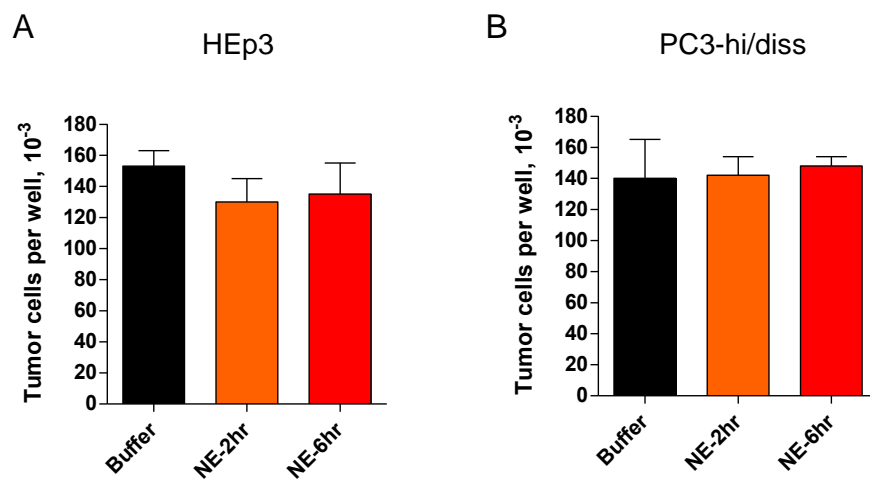
**Neutrophil Elastase Facilitates**

**Tumor Cell Intravasation**

**and Early Metastatic Events**

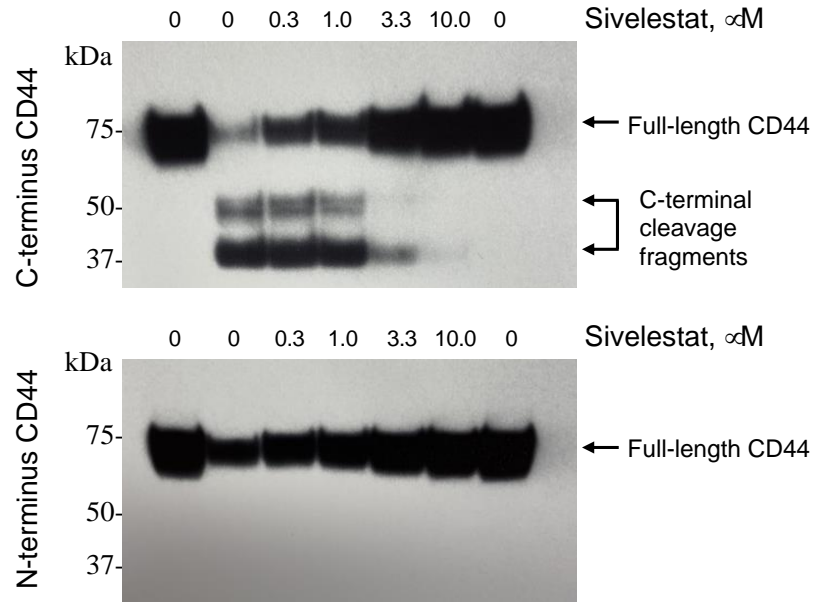
**Elena Deryugina, Alexia Carré, Veronica Ardi, Tomoki Muramatsu, Jonas Schmidt, Christine Pham, and James P. Quigley**

## Supplemental Figures



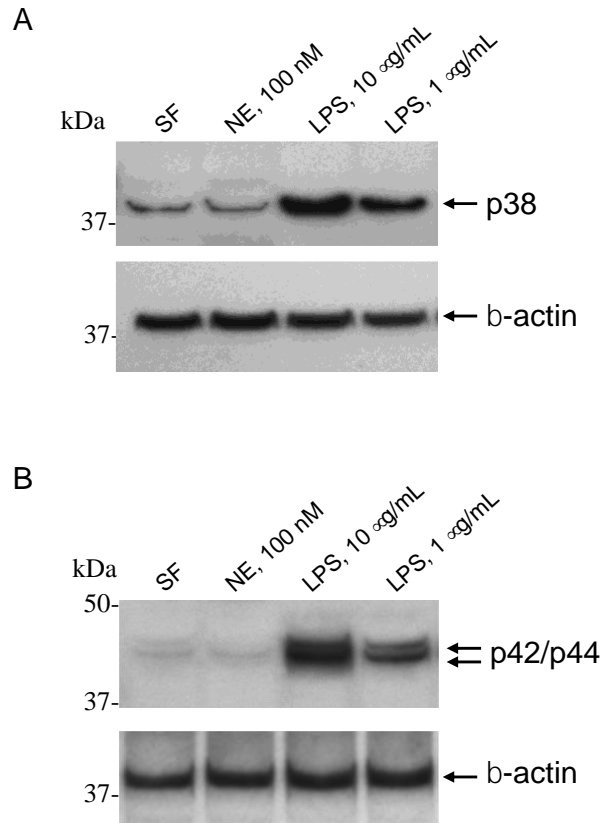
**Figure S1. NE does not affect tumor cell proliferation. Related to Figure 5.**

HEp3 (A) and PC3-hi/diss (B) tumor cells were treated with 100 nM NE for 2 or 6 hr, washed and plated at  $5 \times 10^4$  cells per well in triplicate. After 48-hr incubation, the cells were detached and counted. Two independent experiments were performed for each tumor cell type. Data are presented as mean  $\pm$  SEM.



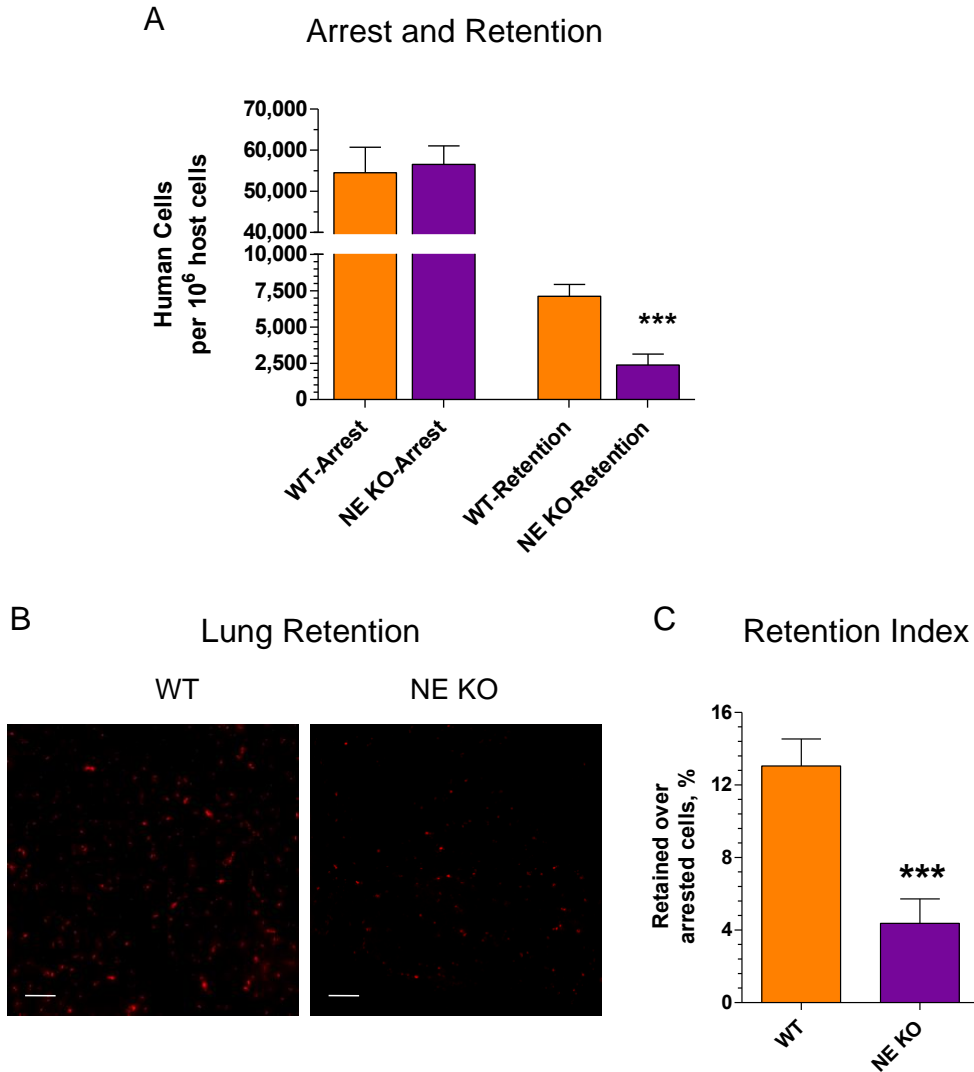
**Figure S2. Inhibition of NE-mediated cleavage of CD44 by Sivelestatat. Related to Figure 5.**

PC3-hi/diss cells were left untreated or treated with 50 nM NE alone or pre-mixed with Sivelestatat at the indicated increasing concentrations (0.3 to 10 mM). After 8-hr-incubation, the cell in each treatment culture (detached and still attached) were washed and lysed. Equal amounts of cell lysates (40 μg/lane) were probed under non-reducing conditions for the C-terminus and N-terminus CD44 with the corresponding terminus-specific antibodies. The blots indicate that CD44 is efficiently cleaved as evidenced by the diminishment of the extracellular N-terminal portion of 75 kDa full length CD44 and appearance of C-terminal cleavage fragments. At 3-10-μM range, Sivelestatat efficiently blocked NE-mediated cleavage of CD44.



**Figure S3. NE does not induce activation of p38 and Erk1/2 MAPKs. Related to Figure 5.**

(A) HEp3 cells were serum starved and then treated with 100 nM NE for 20 min or 1 or 10 mg/ml LPS for 6 hours as a positive control for activation of p38 (A) or Erk1/2 (B). Equal amount of protein in cell lysates were loaded per lane (40  $\mu$ g) of a gel run under reduced conditions. The phosphorylation of p38 and Erk1/2 was analyzed with the corresponding specific antibodies against phosphorylated p38 and p42/p44. Equal load was confirmed by probing blots for  $\beta$ -actin.

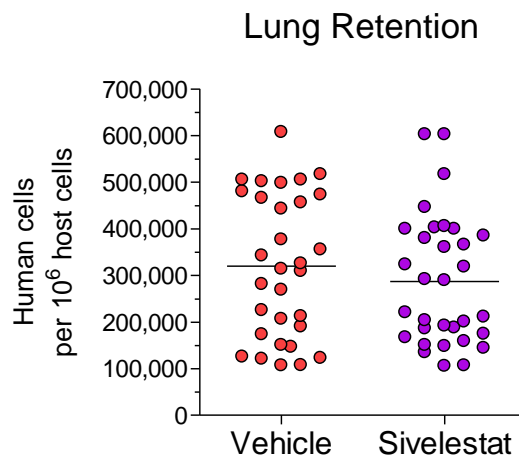


**Figure S4. NE is required for efficient lung retention of vascular-arrested PC3-hi/diss cells. Related to Figure 6.**

(A) PC3-hi/diss cells were stained with red-fluorescence dye (Abcam) and inoculated into the tail vein of C57BL/6 mice, WT or NE-KO ( $1 \times 10^6$  cells per mouse). The numbers of tumor cells arrested in the lung vasculature and cells retained in the lung tissue were measured within 20 min and 14 hr after cell inoculations, respectively, with human-specific *Alu*-qPCR. Two independent experiments were performed involving a total of 10 to 12 mice per variable. \*\*\*,  $P < 0.001$ , two-tailed Student's *t*-test. Data are presented as mean  $\pm$  SEM.

(B) Red-fluorescent PC3-hi/diss tumor cells were imaged in the lungs in an immunofluorescence microscope equipped with a digital video camera. Original magnification, 200x. Bar, 100  $\mu$ m.

(C) Retention indices were quantified for each mouse as the fraction (%) of PC3-hi/diss cells retained in the lung compared to the number of inoculated cells initially arrested in the lung vasculature. \*\*\*,  $P < 0.01$ , two-tailed Student's *t*-test. Data are presented as mean  $\pm$  SEM.



**Figure S5. Lack of inhibitory effects of Sivelestat on lung retention. Related to Figure 6.**

HEp3 cells were inoculated into the tail vein of immunodeficient SCID mice at  $1 \times 10^6$  cells per mouse. The mice were treated with a total of 1 to 3.5 mg Sivelestat per mouse, administered by i.p. or i.v. routes, 2-3 times within the 12 hr period before lung harvest. Control mice received equal volume of vehicle (4% DMSO in PBS). The number of HEp3 cells retained in the lung after 12 hr following cell inoculation was measured with human-specific *Alu*-qPCR. Four independent experiments involving a total of 30 and 31 mice per vehicle control and Sivelestat were performed. The individual mouse data from these experiments were pooled since there were no effects of Sivelestat regardless of the delivery route, number of inoculations, or dose.

## **Transparent Methods**

### ***Chemicals and reagents***

Purified NE was purchased from Athens Research & Technology, Inc. (Athens, Georgia). Natural inhibitor  $\alpha$ 1PI and protease and phosphatase inhibitor cocktails were from Sigma (St Louis, MO). Synthetic NE inhibitor Sivelestat was from Cayman Chemical. Interleukin 8 (IL-8) was purchased from Peprotech (Rocky Hill, NJ). Antibodies against p38 MAPK phosphorylated at Thr180/Tyr182 (#9216) and p44/42 (Erk1/2) phosphorylated at Thr202/Tyr 204 (#9106). were from Cell Signaling.

### ***Isolation of human neutrophils and preparation of nitrogen cavities***

Human neutrophils were isolated from whole blood obtained from healthy donors at the Scripps Research Institute. Two volumes of anti-coagulated blood (50mM EDTA) were layered over a double gradient formed by carefully layering one volume of Histopaque-1077 over one volume of Histopaque-1119 (both from Sigma-Aldrich). Blood cells were separated by centrifugation for 30 min at  $700 \times g$ . The upper plasma layer and mononuclear cells at the plasma/Histopaque-1077 interface were removed. The granulocytes were harvested from the Histopaque-1077/1119 interface and washed twice with cold Hanks Balanced Salt Solution (HBSS, Thermo Fisher Scientific) by centrifugation for 10 min at  $400 \times g$ . Washed granulocytes were re-suspended at the desired concentration in serum-free (SF) DMEM supplemented with gentamicin (10  $\mu$ g/mL). The purity of neutrophil fraction was verified with HEMO staining (Protocol) and was  $>95\%$ . To open granule contents, isolated neutrophils placed into the cryogenic tubes at  $1 \times 10^7$  cells/mL HBSS. Tubes were subjected to 3 freeze/thawing cycles in liquid nitrogen/warm water bath. After centrifugation at 4 min,  $4000 \times g$ ,  $4^\circ\text{C}$ , the cell debris was removed and the nitrogen cavities (NC) were kept at  $-20^\circ\text{C}$  until use.

### ***NE activity assay***

To determine the NE activity in NC preparations, 30  $\mu$ L of the sample were mixed with 350 nM of the elastase colorimetric substrate (Elastase substrate I, Calbiochem) in ammonium bicarbonate buffer (50 nM). The enzyme activity was assayed by a colorimetric detection at 405 nm (SynergyMX, BioTek) using a calibration curve generated by titration of purified NE. Kinetic assays were run for 1h, allowing for the determination of  $V_{\text{max}}$  values and calculation of NE activity using the Gen5 2.0 software.

### ***Tumor cells and culture conditions***

Human epidermoid HEp3 carcinoma cell line (Toolan, 1954) and an aggressive variant of human PC3 prostate carcinoma cell line possessing high disseminating potential, PC3-hi/diss (Conn et al., 2009), were used in this study. Murine carcinoma cells, MOC2, generated to study head and neck cancer in syngeneic mouse models ((Judd et al., 2012), were received from Dr. R. Upalluri of Washington School of Medicine (St. Louis, MO). Cells were cultured in Dulbecco's modified Earle's medium (DMEM), supplemented with glucose, sodium pyruvate, non-essential amino acids, gentamycin and 10% FBS (D10). For using in experiments, the cells were detached with enzyme-free buffer, washed in serum-free (SF) DMEM, and re-suspended in desired concentration.

### ***Cell proliferation and chemotactic cell migration***

HEp3 and PC3 cells were treated with purified NE at 100 nM for 2 or 6 hours, washed and suspended at  $1 \times 10^6$  cells/mL SF-DMEM. For cell proliferation assays, washed cells were plated at  $5 \times 10^5$  per well of 24-well cluster in D10. After 48 hours, cells were detached with trypsin-EDTA and counted. For cell migration assays, washed cells were plated at  $1 \times 10^5$  per insert of a 6.5- $\mu$ m Transwell with 8- $\mu$ m pore size membrane. Cells were allowed to migrate towards chemoattractant in an outer chamber, 5% FBS or 100 nM of hepatocyte growth factor (HGF). Transmigrated cells were harvested after overnight incubation and counted. For NE inhibition experiments, the cells were first pretreated with NE inhibitors,  $\alpha$ 1PI and Sivelestat, or signaling inhibitors, Dasatinib and Wortmannin, at the indicated concentrations for 20 min, and then the cultures were treated with NE.

### ***Western blotting***

Sub-confluent layers of tumor cells plated the day before experiment were washed in SF-DMEM and treated with 100 nM purified NE for 20 min. For NE inhibition experiments, the cells were first pretreated with NE inhibitors,  $\alpha$ 1PI and Sivelestat or signaling inhibitors Dasatinib and Wortmannin at the indicated concentrations for 20 min, and then the cultures were treated with NE. Cells were lysed on ice with mRIPA buffer supplemented with the protease and phosphatase inhibitors (Sigma). Cell lysates were clarified by centrifugation and equal protein contents (20-40  $\mu$ g/lane) were separated by SDS-PAGE on 4-20% gels. The separated proteins were transferred to a PVDF membrane, blocked with 1% BSA, and incubated overnight with the mouse monoclonal antibody against Akt, phospho Akt, phospho p38, phospho Erk1/2,  $\beta$ -actin (all from Cell Signaling), C-terminal CD44 (Rb antibody from Boster), or N-terminal CD44 mouse mAb 29-7 generated in our laboratory. The membranes were washed and incubated with the secondary antibodies conjugated with HRP (Cell Signaling). The bound secondary antibodies were visualized with West Pico substrate (Thermo Fisher Scientific).

### ***Animal studies***

All experiments involving animals were conducted in accordance with the Animal Protocol approved by TSRI Animal Care and Use Committee (IACUC).

### ***CAM angiogenesis model***

The effects of purified NE on tumor cell-induced angiogenesis were determined using a collagen onplant angiogenesis assay as described (Deryugina and Quigley, 2008a). Briefly, GFP-tagged HEp3 cells were incorporated at  $1 \times 10^6$  cells per mL into 2.2 mg/mL neutralized native type I collagen (BD Biosciences) and 30  $\mu$ L-droplets of the mixture were polymerized over grid meshes (3x4 mm) generating three-dimensional (3D) rafts (“onplants”) by incubating at 37°C for 20 min. Solidified onplants were grafted onto the chorioallantoic membrane (CAM) of 10 day-old chick embryos incubated *ex ovo* (6 onplants/embryo). The onplants were treated daily with 10  $\mu$ L of 100 nM of NE or vehicle (PBS/1% DMSO). Within 68 h to 72 h, newly developed blood vessels that had grown into 3D collagen rafts were scored over the lower mesh using a stereoscope. The angiogenic index was calculated for each onplant as the ratio of number of grids having newly formed blood vessels over the total number of scored grids. To visualize the angiogenic blood vessels within collagen rafts, the onplant-bearing embryos were injected via



the allantoic vein with 100 ug of Rhodamine-conjugated *Lens culinaris* agglutinin (LCA; Vector laboratories) in 0.1 mL PBS. Portions of CAM with onplants were visualized for fluorescence in an Olympus microscope equipped with a digital video camera. To measure tumor cell intravasation, portions of CAM, 1-5 cm distal to collagen onplants, were harvested on day 5 after onplant grafting and processed for quantitative human-specific *Alu*-qPCR as described (Minder et al., 2015).

### ***CAM tumor model to study spontaneous metastasis***

To analyze the effects of purified NE, NC and intact neutrophils on intratumoral angiogenesis and tumor cell intravasation, CAM microtumor assays was employed as described (Deryugina, 2016). Briefly, GFP-tagged HEP3 cells were suspended at  $1 \times 10^7$  cells per mL within 2.2 mg/mL neutralized native type I collagen (BD Biosciences). Six 15  $\mu$ L-droplets of cell-containing collagen mixtures were placed separately on the top of a CAM on 10 day-old chick embryos developing *ex ovo* (Deryugina and Quigley, 2008b). Developing tumors were treated daily by topical application of tested ingredients in PBS supplemented with 1% DMSO (vehicle). Purified NE was used at 100 nM, the NC was used at a concentration equivalent to 100 nM of NE activity, and  $\alpha$ 1PI was used at a final concentration of 300 nM. To assay the effect of intact neutrophils, freshly isolated neutrophils were injected on day 3 of microtumor development into the allantoic vein of chick embryos ( $2 \times 10^6$  neutrophils per embryo). After 5 days, 100  $\mu$ g of Rhodamine-LCA in PBS was inoculated within 0.1 mL PBS in an allantoic vein to highlight chick embryo vasculature. Within 10-30 minutes, the portions of the CAM distal to tumors were excised and processed for *Alu*-qPCR to determine the number of intravasated human cells, after which the CAM microtumors were imaged using an Olympus IX51 microscope equipped with a QuantiFire XI digital microscope camera. Images were acquired at the original magnification of 200X (20X objective and 10X eyepiece). In acquired images, intratumoral vasculature was analyzed using ImageJ for lumen size distribution. The lumen diameters were determined for 3-5 areas within a tumor, 2-6 tumors per embryo, 3-5 embryos per group, allowing for statistically significant number of measurements.

### ***Pulmonary tumor cell arrest and lung retention in mice***

Lung retention experiments were conducted as described (Oncogene, 2012). WT and NE-KO mice (6-to-8-week-old) were injected through a lateral tail vein with  $5 \times 10^5$  HEP3 cells or  $1 \times 10^6$  PC3 cells per mouse. Both sexes of mice were used throughout the study, although one the mice of one sex of similar age were used in an individual experiment. Tumor cell arrest in pulmonary vasculature and lung tissue retention of tumor cells was analyzed correspondingly after 30 minutes or 12 hours following cell inoculations. Where indicated, the mice following cell injections were treated with Sivelestat at 1-1.5 mg/kg administered up to three times in 0.2 mL PBS/4%DMSO i.p. or i.v. during 12-hr-long duration of lung retention assays. Control. Mice were treated with vehicle (PBS/4% DMSO). Lung tissue was harvested and processed for human-specific *Alu*-qPCR to determine actual numbers of human cells using a standard curve as described (Conn et al., 2009; Deryugina and Kiosses, 2017; Minder et al., 2015).

### ***Mouse orthotopic model for spontaneous metastasis of head and neck cancer***

Mouse model to study mechanisms of spontaneous metastasis of head and neck cancer was performed as described (Deryugina et al., 2018). Herein, we employed an aggressive murine head and neck carcinoma cell line, MOC2, a syngeneic setting using genetically compatible C57BL/6 mice, WT and NE KO. Both sexes of mice were used throughout the study, although

one the mice of one sex of similar age were used in an individual experiment. MOC2 cells were inoculated into buccal lining of the upper lip at  $1 \times 10^6$  per site (one site per mouse). After 12-14 days, mice were sacrificed when primary tumors reached end point-size of  $\sim 0.5$  cm in diameter. Tumors were excised and weighed. Lungs (the major organ for hematogenous dissemination of head and neck carcinomas in this model) were harvested and fixed for histological examination. Lung sections were stained with hematoxylin-eosin and digitally scanned. Areas in the lung tissue occupied by tumor cells were outlined and quantified using ImageJ software.

### ***Quantitative human-specific Alu-qPCR***

Dissemination of human tumor cells in CAM or mouse assays was measured by the quantification of human-specific *Alu* repeats in total DNA extracted from host tissue as described (Conn et al., 2009; Deryugina and Kiosses, 2017; Minder et al., 2015). The DNA was purified using the Puregene DNA purification kit from Qiagen. The *Alu*-qPCR was performed on 10 ng of genomic DNA in a Bio-Rad MyIQ LightCycler (Bio-Rad) using SYBR green (Life Technologies) as a binding dye. The Cq values were converted into number of human cells using a standard curve generated by spiking increasing numbers of human tumor cells (from 10 to  $10^4$  cells) into a fixed number ( $10^6$  cells) of chick embryo fibroblasts or murine B16 melanoma cells.

### ***Statistical analysis***

Data were analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). In bar graphs the data are presented as mean  $\pm$  SEM from either a representative experiment or several normalized pooled experiments. To normalize the data, the ratios of numerical values for each measurement over the mean of the control group of an individual experiment were calculated. The outliers were eliminated based on the Grubb's test incorporated in GraphPad Software. The differences (*P* values) between data sets were analyzed with the unpaired two-tailed Student's *t*-test. *P* values  $< 0.05$  were considered statistically significant.

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