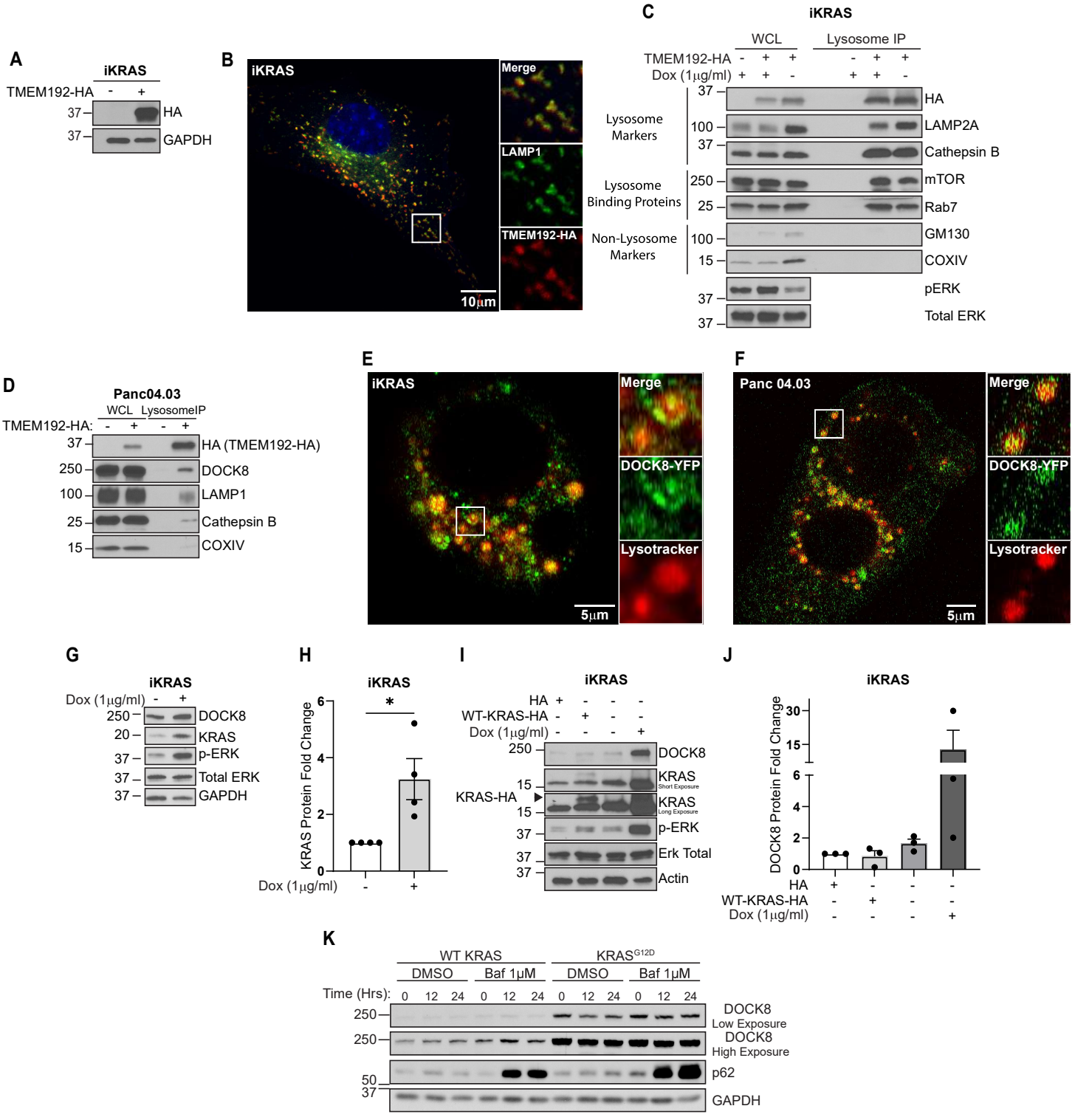
Cell Reports, Volume *42*

Supplemental information

Ectopic expression of DOCK8 regulates lysosome-mediated pancreatic tumor cell invasion

Omar L. Gutierrez-Ruiz, Katherine M. Johnson, Eugene W. Krueger, Roseanne E. Nooren, Nicole Cruz-Reyes, Carrie Jo Heppelmann, Tara L. Hogenson, Martin E. Fernandez-Zapico, Mark A. McNiven, and Gina L. Razidlo

Supplementary Figure S1



Supplementary Information

Figure S1. Generation of TMEM192-HA stable cells for lysosome proteomic analysis and validation of KRAS-mediated DOCK8 expression and localization to lysosomes. Related to Figure 1.

A, Immunoblotting for HA following lentiviral transduction of TMEM192-HA construct into iKRAS^{G12D} cells. GAPDH serves as loading control. **B,** Immunofluorescence staining of LAMP1 (green) and HA (TMEM192-HA, red) in TMEM192-HA iKRAS^{G12D} cells. Nuclei were labeled with Hoechst (blue). Scale bar, 10μm. **C**, Immunoblotting of whole cell lysates (WCL) and lysosomes isolated from control or TMEM192-HA iKRAS^{G12D} cells cultured with doxycycline (KRAS^{G12D}, 1μg/ml) or following 72-hour doxycycline withdrawal (WT). Purity and integrity of isolated lysosomes was validated by immunoblotting for lysosome markers (TMEM192-HA, LAMP2A, cathepsin B), lysosome binding proteins (mTOR, Rab7), and non-lysosome markers (GM130, Golgi; COXIV, Mitochondria). Induction of KRAS^{G12D} expression was validated by immunoblotting of phosphorylated ERK (pERK) and total ERK. **D**, Immunoblotting of DOCK8 in whole cell lysates (WCL) and lysosomes isolated from control or TMEM192-HA Panc04.03 cells. Purity and integrity of isolated lysosomes was validated by immunoblotting for lysosome markers (LAMP1, cathepsin B), and non-lysosome marker (COXIV, Mitochondria). Images of DOCK8-YFP (green) localizing to lysotracker labeled lysosomes (red) in **E,** iKRAS^{G12D} and **F,** Panc04.03 cells. Scale bar, 5μm (E) or 10μm (F). **G**, Immunoblotting and **H,** Quantitation of KRAS protein levels in iKRAS^{G12D} cells cultured with or without doxycycline as indicated above. Induction of KRAS^{G12D} expression was validated by immunoblotting of pERK and total ERK, and GAPDH served as loading control. **I**, Immunoblotting and **J,** Quantitation of iKRAS^{G12D} cells transfected with HA control or WT-KRAS-HA or treated with and without doxycycline as described above. **K**, DOCK8 is not degraded by lysosomes. Immunoblotting of DOCK8 in TMEM192-HA iKRAS^{G12D} expressing WT or KRAS^{G12D} following treatment with BafilomycinA1 (Baf, 1μM) for 0, 12, and 24 hours. p62 is a positive control for a cargo degraded by lysosomes, and GAPDH serves as a loading control. Graphs show the mean ± S.E.M of at least three independent biological replicates. P-values were calculated using a Students t-test. ns, $p > .05$; *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$.

Supplementary Figure S2

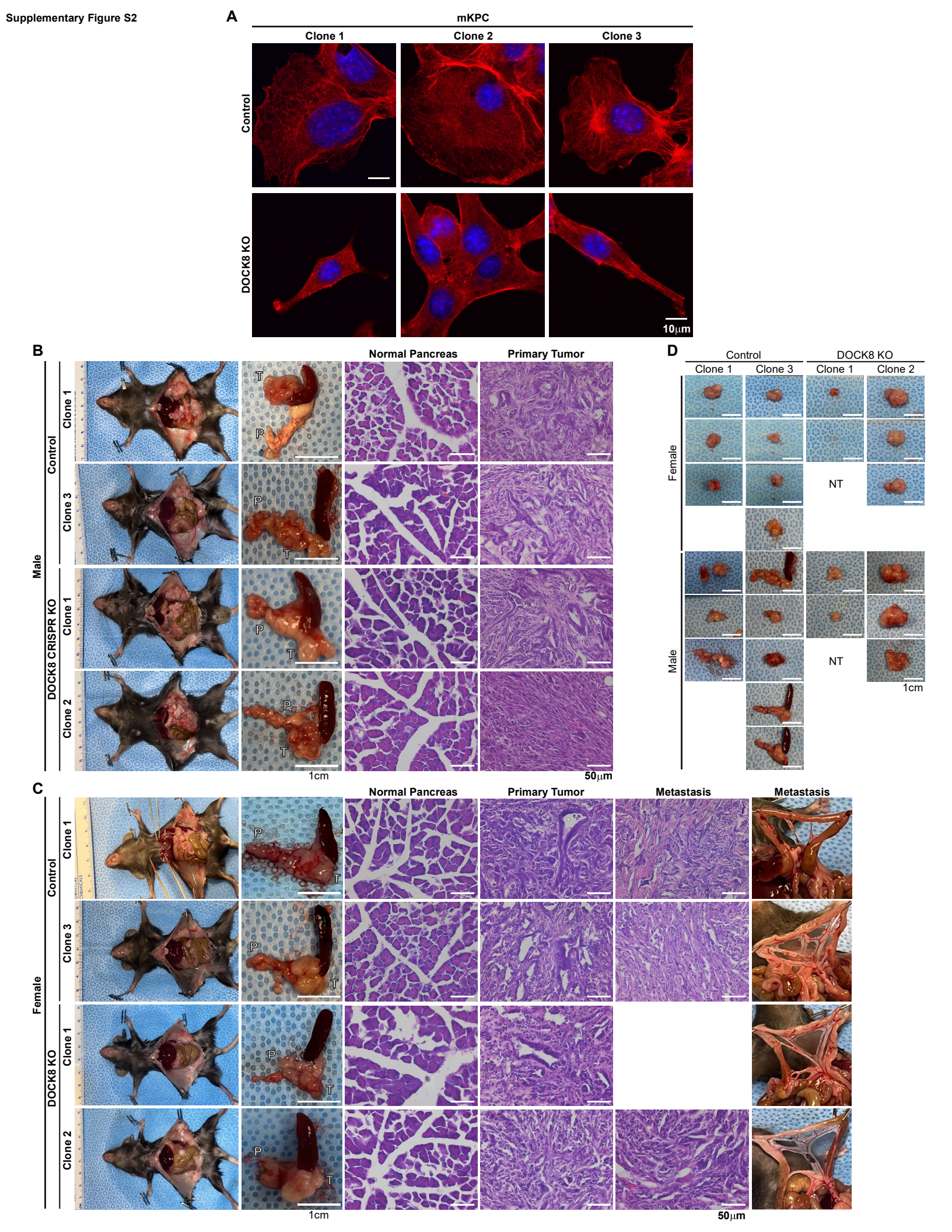


Figure S2. DOCK8 CRISPR knockout impairs tumor progression and metastatic invasion *in vivo*

Related to Figure 2

A, Immunofluorescence staining of Actin (red) in mKPC control or DOCK8 CRISPR knockout clones 1-3. Nuclei were labeled with Hoechst (blue). Scale bar, 10μm. **B**, **C**, Representative images of C57Bl/6J mice following pancreatic orthotopic injection of control (clone 1 or 3) or DOCK8 knockout (clone 1 or 2) in **B**, male and **C**, female. Image panel includes a general picture of each mouse, resected pancreas (P) with primary tumor (T) attached to the spleen (scale bar 1cm), H&E staining of normal pancreas, primary and metastatic tumors (scale bar 50μm). **D**, Control or DOCK8 knockout primary tumors from male and female mice. Scale bar, 1cm.

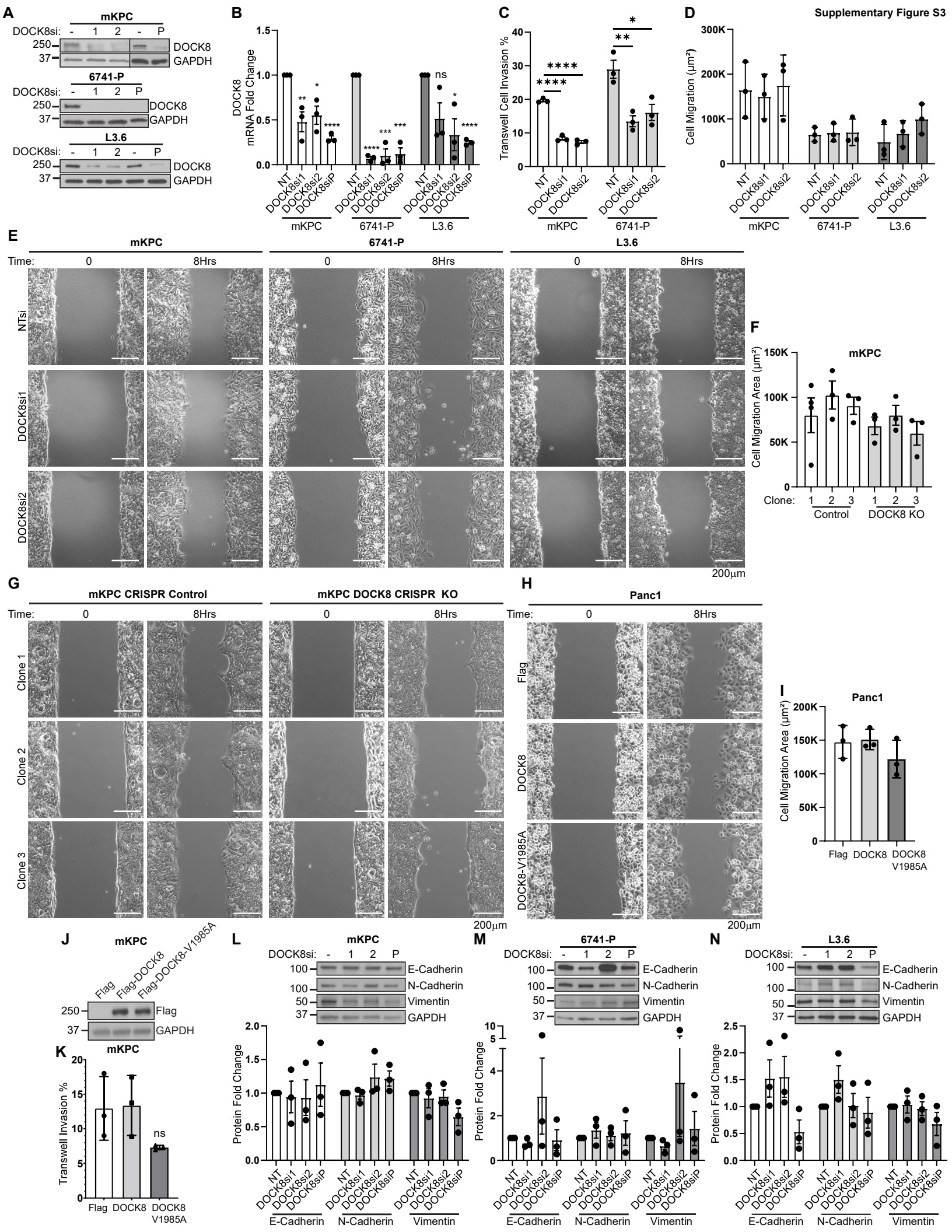

A
mKPC
DOCK8si: - 1 2 - P
250
37
DOCK8
GAPDH
6741-P
DOCK8si: - 1 2 P
L3.6
DOCK8si: - 1 2 - P
B
DOCK8 mRNA Fold Change
NT
DOCK8si1
DOCK8si2
DOCK8siP
mKPC
6741-P
L3.6
ns
C
Transwell Cell Invasion %
D
Cell Migration (µm²)
E
Time: 0 8Hrs
NTsi
DOCK8si1
DOCK8si2
200µm
F
Cell Migration Area (µm²)
Clone: 1 2 3 1 2 3
Control
DOCK8 KO
G
mKPC CRISPR Control
mKPC DOCK8 CRISPR KO
Clone 1
Clone 2
Clone 3
H
Panc1
Flag
DOCK8
DOCK8-V1985A
I
Flag
DOCK8
DOCK8 V1985A
J
Flag
Flag-DOCK8
Flag-DOCK8-V1985A
K
Transwell Invasion %
L
E-Cadherin
N-Cadherin
Vimentin
Protein Fold Change
M
N

Figure S3. DOCK8 is required for cell invasion but dispensable for cell migration. Related to Figure 2

A, Immunoblotting and **B**, Relative DOCK8 mRNA levels by qRT-PCR confirming DOCK8 knockdown by individual DOCK8si1 and DOCK8si2 and DOCK8siP in mKPC, 6741-P, and L3.6 cells. **C**, Quantitation of % transwell invasion following DOCK8 knockdown by the indicated siRNAs in mKPC, 6741-P, and L3.6 cells. A total of at least 500 cells were counted per condition on the top and bottom of the transwell filter followed by calculation of % of invasion. **D**, Quantitation and **E**, Images of wound healing cell migration at 0 and 8 hours in nontargeting (NT) control and DOCK8 knockdown (DOCK8si1 and 2) mKPC, 6741-P, and L3.6 cells. **F**, Quantitation and **G**, Images of wound healing cell migration at 0 and 8 hours in control and DOCK8 CRISPR knockout mKPC cells clones 1-3. **H**, Images and **I**, Quantitation of wound healing cell migration at 0 and 8 hours in Panc1 cells stably expressing Flag, Flag-DOCK8, or Flag-DOCK8-V1985A (GEF inactive). Cell migration area is graphed (μm^2). Scale bar, 200μm. **J**, Immunoblotting of DOCK8 in mKPC cells following lentiviral transduction of Flag, Flag-DOCK8, or Flag-DOCK8-V1985 (GEF inactive). **K**, Quantitation of transwell cell invasion assay of mKPC cells described in **J**. Cells were seeded on matrigel coated transwell filters and allowed to invade for 20 hours towards a high serum gradient. A total of 1500 cells were quantitated per condition across 3 experiments. **L-N**, Immunoblotting and quantitation of EMT markers E-Cadherin, N-Cadherin, and Vimentin in DOCK8 knockdown (DOCK8si1, 2, or DOCK8siP) mKPC, 6741-P, and L3.6 cells. GAPDH serves as loading control. Validation of DOCK8 knockdown in these cells is shown in figure S7H. Graphs show the mean ± S.E.M of at least three independent biological replicates. P-values were calculated using a Student's t-test. One-way ANOVA with the Tukey's post hoc test was used for comparison of the means of more than 2 datasets (**F**). ns, $p > .05$; *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$.

Supplementary Figure S4

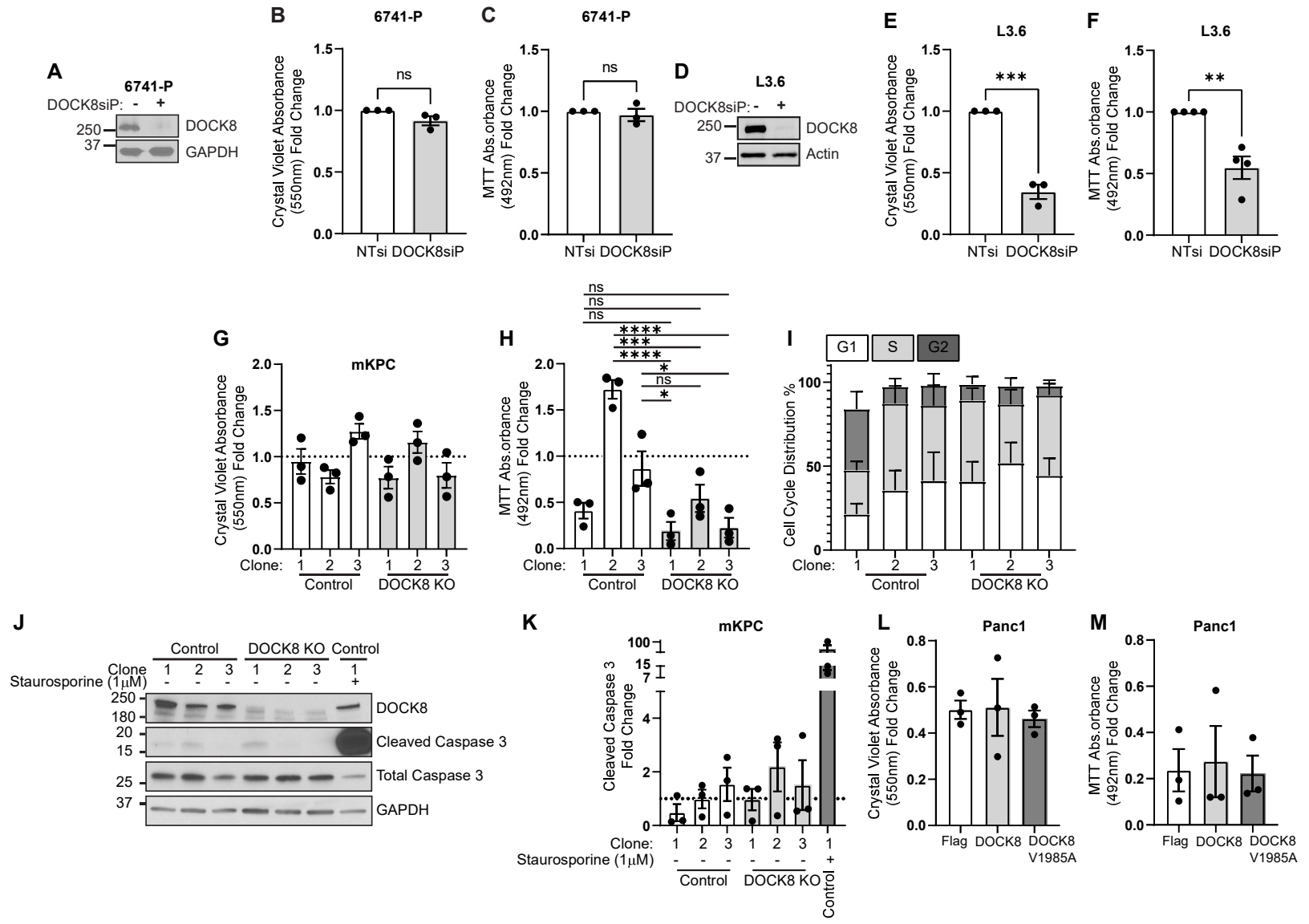


Figure S4. Minimal effects on cell viability upon DOCK8 depletion
Related to Figure 2

A, DOCK8 immunoblotting in 6741-P DOCK8 knockdown cells **B,** Quantification of crystal violet staining of 6741-P cells following DOCK8 knockdown. **C**, MTS cell viability assay of 6741-P cells following DOCK8 knockdown. **D**, DOCK8 immunoblotting in L3.6 DOCK8 knockdown cells **E,** Quantification of crystal violet staining of L3.6 cells following DOCK8 knockdown. **F**, MTS cell viability assay of L3.6 cells following DOCK8 knockdown. Quantification of **G**, Crystal violet staining and **H**, MTS cell viability assay of control and DOCK8 CRISPR knockout mKPC cells (clones 1-3). **I**, Propidium iodide FACS assessment of cell cycle distribution of control and DOCK8 CRISPR knockout mKPC cells. **J**, Immunoblotting for the apoptosis marker cleaved and total caspase 3 in control and DOCK8 CRISPR knockout mKPC cells (clones 1-3). Treatment of control clone 1 with staurosporine (1μM) for 16 hours served as an apoptosis positive control. GAPDH serves as loading control. **K**, Quantitation of Cleaved caspase-3 fold change normalized to GAPDH. Individual fold changes were normalized to the average of control clones 1-3 (dotted line). Quantification of **L**, Crystal violet staining and **M**, MTS cell viability assay of Flag, Flag-DOCK8, or Flag-DOCK8-V1985 Panc1 stable cells. Graphs show the mean ± S.E.M of three independent biological replicates. P-values were calculated using a Student's t-test. One-way ANOVA with the Tukey's post hoc test was used for comparison of the means of more than 2 datasets (**G, H K**). ns, $p > .05$; *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$.

Supplementary Figure S5

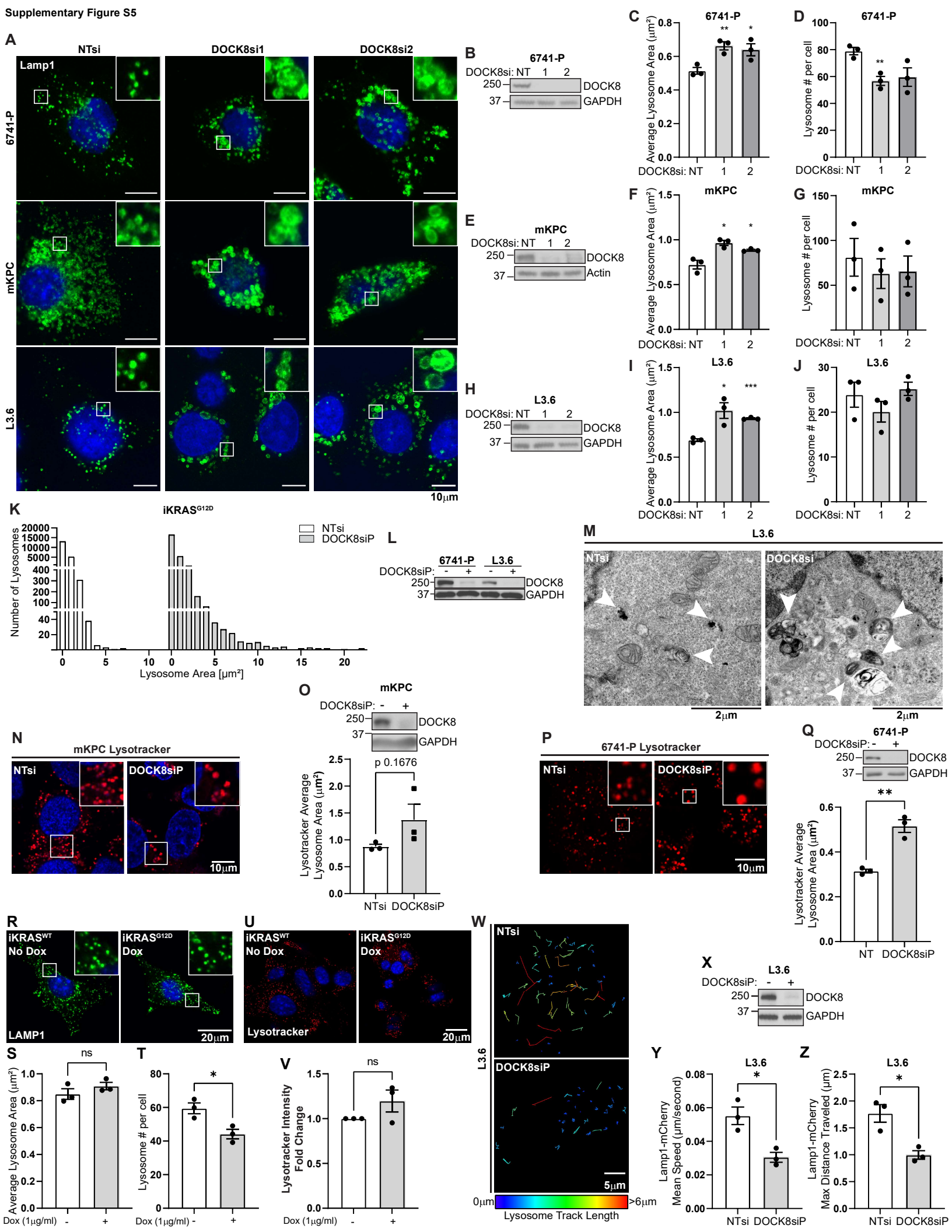


Figure S5. DOCK8 regulates lysosome size and motility in pancreatic cancer cells Related to Figure 3

DOCK8 knockdown leads to enlarged lysosomes **A**, Immunofluorescence staining of Lamp1 (green) in nontargeting control and DOCK8 knockdown cells (DOCK8si1 and DOCK8si2) in 6741-P, mKPC, and L3.6 cells. Scale bar 10μm. DOCK8 immunoblotting in **B**, 6741-P, **E**, mKPC, and **H**, L3.6 DOCK8 knockdown cells. Quantitation of average lysosome area (μm^2) in **C**, 6741-P, **F**, mKPC, and **I**, L3.6 DOCK8 knockdown cells. Quantitation of average lysosomes per cells in **D**, 6741-P (NT control n=99 cells, DOCK8si1 n=101 cells, DOCK8si2 n=97 cells), **G**, mKPC (NT control n=149 cells, DOCK8si1 n=114 cells, DOCK8si2 n=155 cells), and **J**, L3.6 DOCK8 knockdown cells (NT control n=178 cells, DOCK8si1 n=100 cells, DOCK8si2 n=105 cells). Graphs show the mean ± S.E.M of at least three independent biological replicates. P-values were calculated using a Students t-test. ns, $p > .05$; *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$. **K**, Histogram showing frequency distribution of lysosome area (μm^2) in DOCK8 knockdown versus nontargeting (NT) control iKRAS^{G12D} cells, demonstrating an increase in the percentage of very large lysosomes following DOCK8 knockdown (iKRAS^{G12D} NT control n=168 cells, DOCK8 knockdown n=137 cells). **L**, Immunoblotting of 6741-P and L3.6 cells following DOCK8 knockdown. **M**, Transmission electron microscopy of L3.6 cells following DOCK8 knockdown. White arrows indicate lysosome compartments, which are enlarged in the DOCK8 knockdown cells. Scale bar 2μm. **N**, Images of lysotracker labeled lysosomes (red) in mKPC cells following DOCK8 knockdown. Scale bar 10μm. **O**, Immunoblotting of mKPC cells following DOCK8 knockdown. GAPDH serves as loading control, and area quantitation of average lysotracker-labeled lysosomes (μm^2). **P**, Images of lysotracker labeled lysosomes (red) in 6741-P cells following DOCK8 knockdown. Scale bar 10μm**. Q**, Immunoblotting of 6741-P cells following DOCK8 knockdown. GAPDH serves as loading control, and area quantitation of lysotracker-labeled lysosomes (μm^2). **R**, Immunofluorescence staining of LAMP1 in iKRAS^{G12D} cells cultured with doxycycline (KRAS^{G12D}) (1μg/ml) or following 72-hour doxycycline withdrawal (WT). Scale bar 20μm. Quantitation of **S**, Average lysosome area (μm^2), and **T**, Average lysosomes per cell (from **R**). **U**, Lysotracker labeling of iKRAS^{G12D} cells cultured with doxycycline (KRAS^{G12D}) (1μg/ml) or following 72-hour doxycycline withdrawal (WT). Scale bar 20μm. **V**, Quantitation of lysotracker mean fluorescence intensity fold change. Lysosome motility is reduced following DOCK8 knockdown. **W,** Track displacement of lysosomes over five minutes in LAMP1-mCherry labeled L3.6 cells. Tracks are pseudocolored to represent track length, 1 cell is shown. N=3 cells per condition in each of three independent experiments. Scale bar 5μm **X**, DOCK8 immunoblotting confirming DOCK8 knockdown in L3.6 cells. GAPDH serves as a loading control. **Y**, Quantitation of lysosome mean speed (of cells in **W**). **Z**, Quantitation of maximum lysosome distance traveled (of cells in **W**). Graphs show the mean ± S.E.M of three independent biological replicates. P-values were calculated using a Student's t-test. ns, $p > .05$; *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$.

Supplementary Figure S6

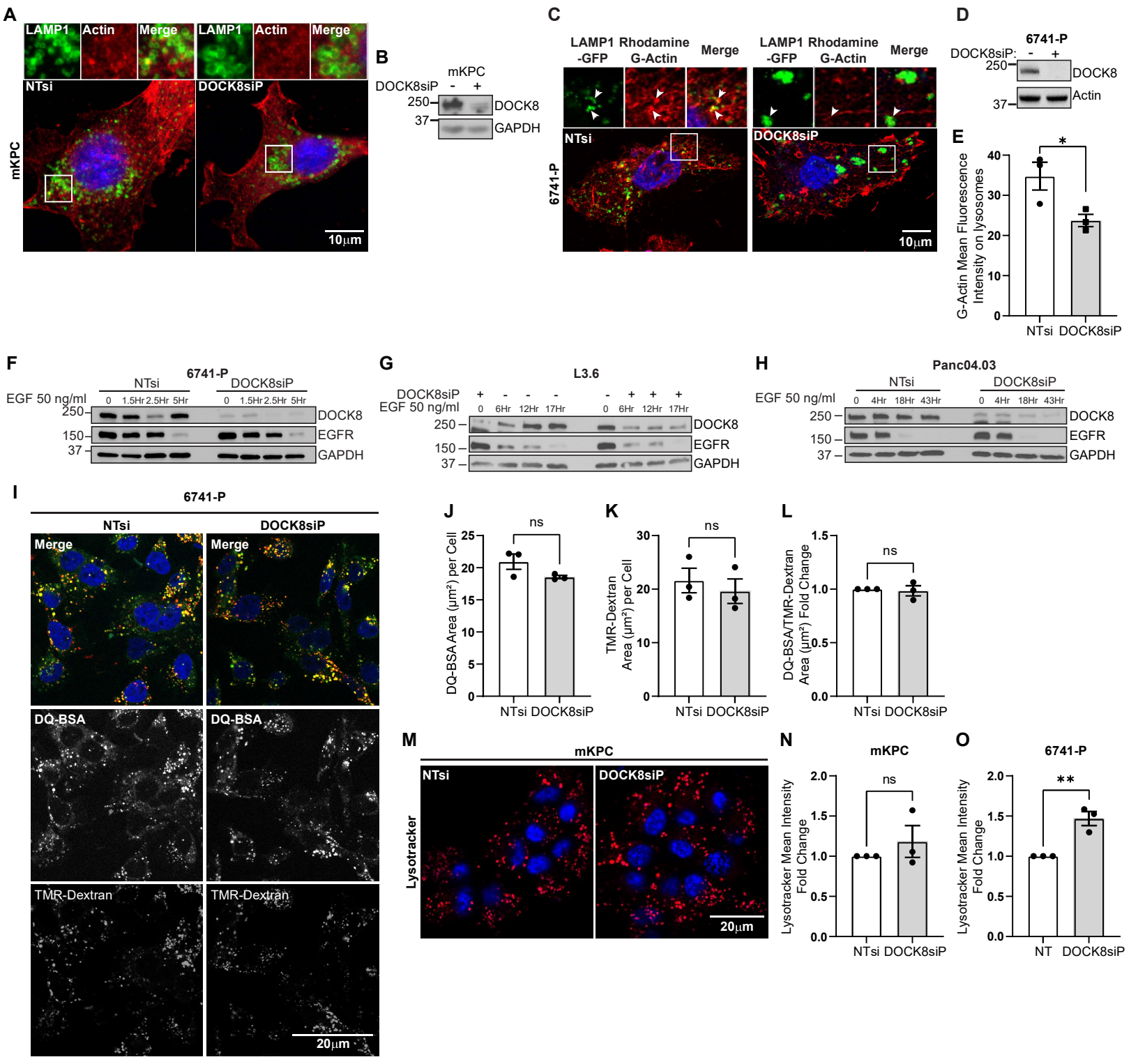


Figure S6. DOCK8 depletion reduces lysosomal actin without altering global lysosome function

Related to Figure 4

A, Immunofluorescence staining of LAMP1 (green) and actin (red) in mKPC cells following DOCK8 knockdown. Scale bar 10μm. Quantitation in Fig. 4C. **B,** Immunoblotting validating DOCK8 knockdown in mKPC cells. **C**, Images of Rhodamine G-actin (red) nucleation at LAMP1-GFP (green) labeled lysosomes in 6741-P NT control and DOCK8 knockdown (DOCK8siP) cells. Scale bar 10μm. **D**, Immunoblotting validating DOCK8 knockdown in 6741-P cells. GAPDH serves as a loading control. **E**, Quantitation of Rhodamine G-actin fluorescence intensity overlapping with lysosomes (LAMP1-GFP). Nontargeting control n=47 cells, DOCK8siP n=59 cells. **F-H** Immunoblotting of EGFR degradation assay in **F,** 6741-P, **G,** L3.6, and **H**, Panc04.03 cells following DOCK8 knockdown (DOCK8siP) and treatment with 50ng/ml EGF for the indicated times. GAPDH serves as loading control. **I**, Images of DQ-BSA (green) and TMR-Dextran (red) in 6741-P cells following DOCK8 knockdown (DOCK8siP). Quantitation of **J**, DQ-BSA degradation (green, area per cell). **K**, Quantitation of macropinocytosis of TMR-Dextran (red, area per cell). Nontargeting control, n=252 total cells; DOCK8 KD, n=211. **L**, DQ-BSA fluorescence normalized to Macropinocytosis of TMR-Dextran. **M**, Images of lysotracker labeled lysosomes in mKPC cells following DOCK8 knockdown. Quantitation of lysotracker mean fluorescence intensity in **N**, mKPC (nontargeting control n= 414 total cells; DOCK8 KD, n= 496), and **O**, 6741-P cells (Images in S5P). Scale bars, 20μm (**I**, **M**). Graphs show the mean ± S.E.M of three independent biological replicates. P-values were calculated using a Student's t-test. ns, $p > .05$; *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$.

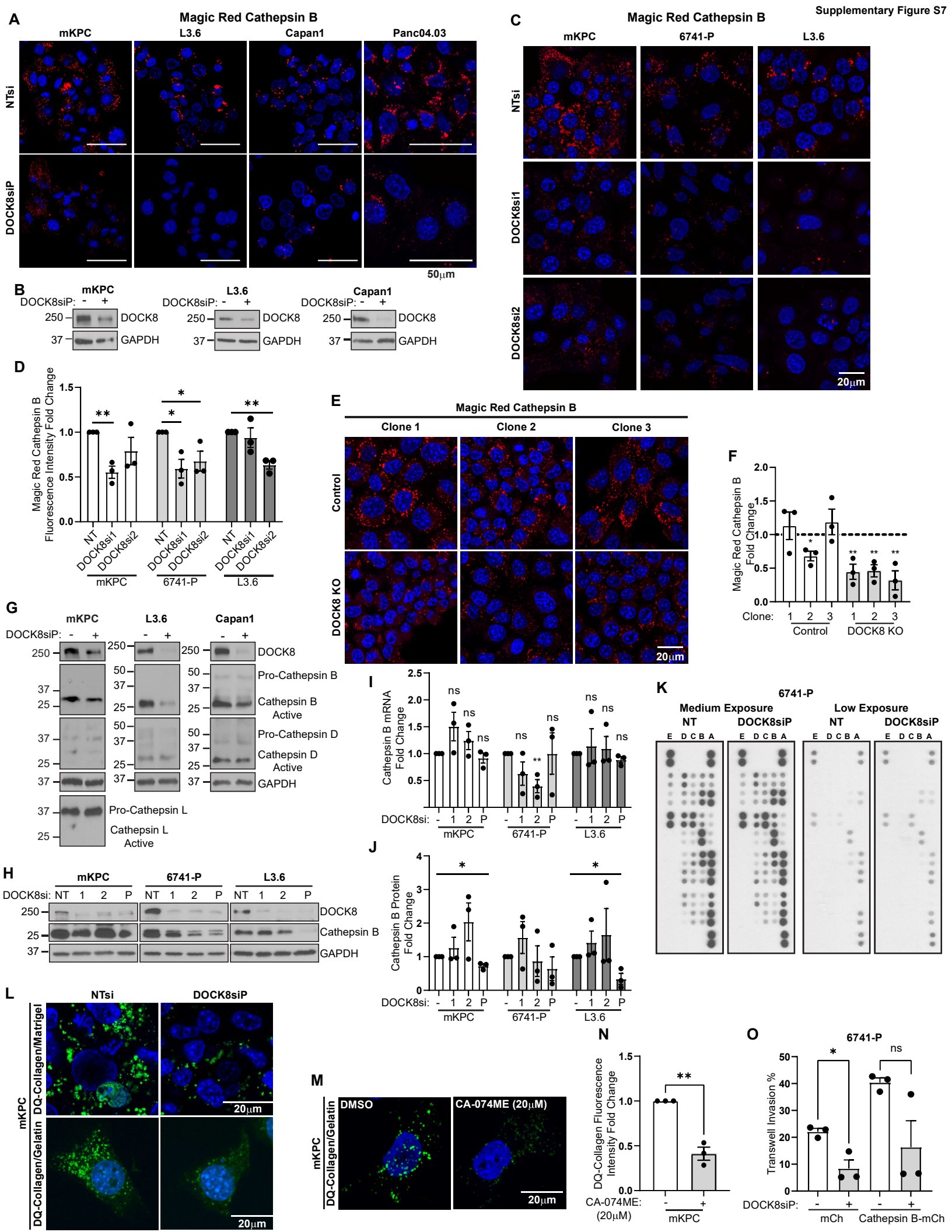

A
Magic Red Cathepsin B
mKPC
L3.6
Capan1
Panc04.03
NTsi
DOCK8siP
50μm
B
mKPC
L3.6
Capan1
DOCK8siP: - +
250
37
DOCK8
GAPDH
C
Magic Red Cathepsin B
mKPC
6741-P
L3.6
NTsi
DOCK8si1
DOCK8si2
20μm
D
Magic Red Cathepsin B Fluorescence Intensity Fold Change
NT
DOCK8si1
DOCK8si2
mKPC
6741-P
L3.6
E
Magic Red Cathepsin B
Clone 1
Clone 2
Clone 3
Control
DOCK8 KO
20μm
F
Magic Red Cathepsin B Fold Change
Clone: 1 2 3 1 2 3
Control
DOCK8 KO
G
mKPC
L3.6
Capan1
DOCK8siP: - +
DOCK8
Pro-Cathepsin B
Cathepsin B Active
Pro-Cathepsin D
Cathepsin D Active
GAPDH
Pro-Cathepsin L
Cathepsin L Active
H
mKPC
6741-P
L3.6
DOCK8si: NT 1 2 P
DOCK8
Cathepsin B
GAPDH
I
Cathepsin B mRNA Fold Change
DOCK8si: - 1 2 P
mKPC
6741-P
L3.6
J
Cathepsin B Protein Fold Change
DOCK8si: - 1 2 P
mKPC
6741-P
L3.6
K
6741-P
Medium Exposure
Low Exposure
NT
DOCK8siP
E D C B A
L
NTsi
DOCK8siP
mKPC
DQ-Collagen/Matrigel
DQ-Collagen/Gelatin
20μm
M
mKPC
DQ-Collagen/Gelatin
DMSO
CA-074ME (20μM)
20μm
N
DQ-Collagen Fluorescence Intensity Fold Change
CA-074ME: - +
(20μM)
mKPC
O
6741-P
Transwell Invasion %
DOCK8siP: - + - +
mCh
Cathepsin B-mCh

Figure S7. DOCK8 promotes pancreatic cancer cell invasion via cathepsin B
Related to Figure 4, 5

A, Images of Magic Red Cathepsin B fluorescence (red) indicating decreased active cathepsin B in mKPC, L3.6, Capan1, and Panc04.03 (immunoblot on figure 3A) cells following DOCK8 knockdown with a DOCK8siP. Scale bars, 50μm. **B**, Immunoblotting of DOCK8 in cells in **A** confirming DOCK8 knockdown. GAPDH serves as loading control. **C**, Images of Magic Red Cathepsin B fluorescence (red) indicating decreased active cathepsin B in mKPC, 6741-P, and L3.6 following DOCK8 knockdown with individual DOCK8si1, DOCK8si2, or nontargeting control. Scale bars, 20μm. **D,** Quantitation of Magic Red Cathepsin B fluorescence intensity in **C** (mKPC NT control n=411 cells, DOCK8si1 n=471 cells, DOCK8si2 n=441 cells; 6741-P NT control n=220 cells, DOCK8si1 n=247 cells, DOCK8si2 n=276 cells; L3.6 NT control n=263 cells, DOCK8si1 n=249 cells, DOCK8si2 n=272 cells). **E**, Images of Magic Red Cathepsin B fluorescence (red) in control and DOCK8 CRISPR knockout mKPC cells. Scale bars, 20μm. **F**, Quantitation of Magic Red Cathepsin B fluorescence intensity in **E** (Control clone 1 n= 404 cells; Control clone 2 n= 509 cells; Control clone 3 n= 292 cells; DOCK8 KO clone 1 n=630 cells; DOCK8 KO clone 2 n= 397 cells; DOCK8 KO clone 3 n= 683 cells). **G**, Immunoblotting of cathepsins in mKPC, (DOCK8, cathepsin B, D, L, and GAPDH), L3.6, and Capan1 cells (DOCK8, cathepsin B, D, and GAPDH) following DOCK8 knockdown. Quantified in figure **4G**. **H**, Immunoblotting of cathepsin B in mKPC, 6741-P, and L3.6 cells following DOCK8 knockdown with (DOCK8si1, DOCK8si2, and DOCK8siP). **I,** Relative cathepsin B mRNA levels by qRT-PCR in DOCK8 knockdown cells from **H**. **J**, Quantitation of cathepsin B protein fold change normalized to GAPDH and nontargeting control in cells from **H. K**, Protease arrays for secreted proteases in conditioned media from DOCK8 knockdown (DOCK8siP) 6741-P cells. Quantitation in figure **5A. L**, Images of cleaved DQ-Collagen (green) and nuclei (Hoechst, blue). mKPC Cells were seeded on coverslips coated with DQ-Collagen/Matrigel (top images) or DQ-Collagen/Gelatin mix (bottom images) following DOCK8 knockdown and were allowed to degrade ECM for two days. DOCK8 knockdown reduced ECM degradation. Quantified in figure **5D, E**. Scale bars, 20μm. **M**, Cathepsin B is required for ECM degradation. Images of cleaved DQ-collagen (green) by mKPC cells seeded on a DQ-collagen substrate with gelatin for two days following overnight treatment with the cathepsin inhibitor CA-074ME (20μM) or DMSO as control. Scale bars, 20μm. **N**, Quantitation of cleaved DQ-Collagen in the indicated cells. Cells were allowed to degrade ECM in the presence of CA-074ME for two days (DMSO, n= 208 total cells; CA-074ME, n=314). **O**, Transwell cell invasion of 6741-P cells following mCherry or cathepsin B-mCherry expression and overnight treatment with CA-074ME (20μM) or DMSO control. Cells were seeded on Matrigel coated transwell filters in the presence of CA-074ME and allowed to invade for 20 hours towards a high serum gradient. A total of 1500 cells were counted per condition across three experiments. Graphs show the mean ± S.E.M of three independent biological replicates. P-values were calculated using a Student's t-test. ns, $p > .05$; *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$.