Development of a RSK Inhibitor as a Novel Therapy for Triple Negative Breast Cancer

by

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Supplementary Materials and Methods

Velocity Determination

For motility assays the cells that did not proliferate or go out of the field of view were selected. In each frame the cell nucleus position was identified and a dot was placed in this spot. The dots were connected over frames and the tracks created represent the distance. This distance was divided by the time over which the tracks occurred.

Immunostaining

Human breast cancer tissue removed during breast reduction or breast cancer surgery was collected as waste tissue with institutional review board approval. A list of the age and race for each of the patient samples used in this study is provided (Supplementary Table S1).

For detection of pS6, mouse tibiae were fixed in 4% buffered formalin for 3 d and decalcified in 20% EDTA pH 7.4 at r.t. for 3-4 d. Decalcified samples were then placed in 70% ethanol, paraffin-embedded, and 5-μm sections were cut. MCF-7 cells plated on poly-lysine coated coverslips were fixed with 4% paraformaldehyde, washed with PBS, permeabilized with 0.5% Triton X, and blocked in 10% bovine serum albumin (BSA) in PBS.

For detection of pRSK, human breast tissue was fixed in buffered 10% formalin for 2 d and placed in 70% ethanol, paraffin-embedded, and 5-μm sections were cut. The sections were deparaffinized and blocked in 10% BSA in PBS.

Sections and fixed cells were incubated with primary antibody in 3% BSA/PBS overnight at 4°C, washed with 3% BSA/PBS, and incubated with secondary antibody for 1 h at r.t. The sections and cells were washed and DNA stained with Hoechst for 10 min. The coverslips were mounted using Fluoro-Gel (EMS).

Antibodies

Primary antibodies used for immunofluorescence: chicken anti-keratin-14 (Abcam plc); rat anti-keratin 8 (University of Iowa); anti-phospho-S6 (Cell Signaling Technology, Inc.); rabbit anti-p-Thr359/Ser363-RSK

(EMD Milipore). Secondary antibodies for immunofluorescence: AlexaFluor goat anti-rat 546 IgG (H+L), goat anti-chicken IgG 488 (H+L), goat anti-rabbit 647 IgG (H+L) (Thermo Fisher Scientific, Inc.).

Antibodies used for immunoblotting: rabbit anti-pSer167-ERα, rabbit anti-pSer51-eEF2, rabbit anti-eEF2, mouse anti-cyclin D1, rabbit anti-pSer235/236-S6, rabbit anti-S6, rabbit anti-pSer473-AKT, rabbit anti-AKT, rabbit anti-pThr359/Ser363-RSK, rabbit anti-pSer380-RSK, rabbit anti-caspase3, rabbit anti-cleaved caspase-3 (Asp175), rabbit anti-PARP (Cell Signaling Technology, Inc.); rabbit anti- ERα (Thermo Fisher Scientific Inc.); rabbit anti-Ran (provided by I.G. Macara, Vanderbilt University); mouse anti-RSK2, and rabbit anti-RSK1 (Santa Cruz Biotechnology, Inc.). Secondary antibodies used were HRP- conjugated donkey anti-rabbit and goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc.).

Supplementary Figure Legends

Figure S1 Schematic of the human kinome was reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com). Kinases screened with (**1b**) are listed.

Figure S1

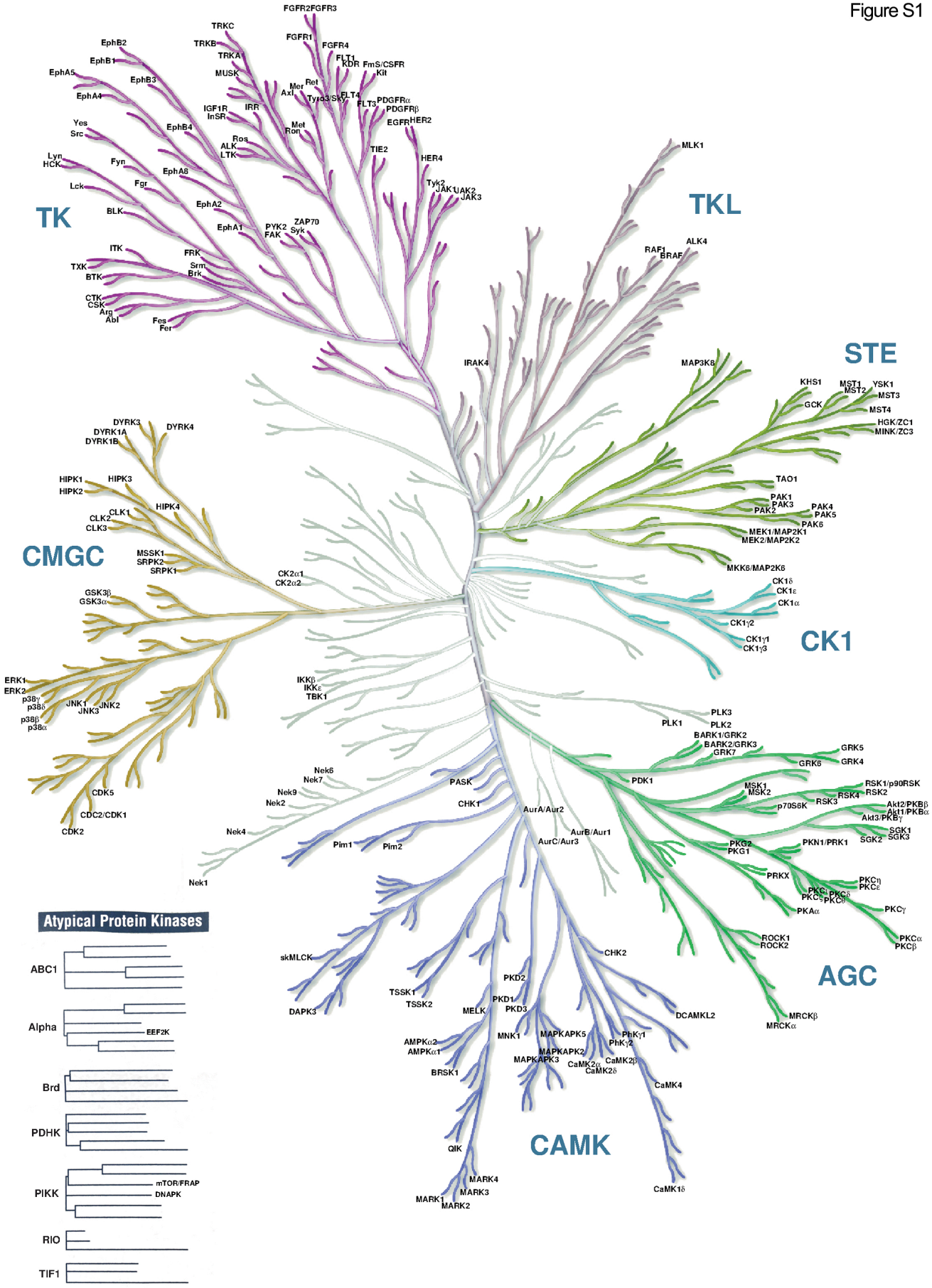

TK
TKL
STE
CMGC
CK1
AGC
CAMK
Atypical Protein Kinases
ABC1
Alpha
Brd
PDHK
PIKK
RIO
TIF1
EEF2K
mTOR/FRAP
DNAPK

Figure S2 RSK is required for TNBC proliferation. (A) Efficacy of (**1a**) and (**1b**) in inhibiting proliferation of various TNBC lines. Symbol, mean ± S.D. (n ≥ 2, triplicate). (B) Correlation of IC_{50} for inhibition of proliferation by (**1b**) versus activated RSK normalized to total RSK1 and RSK2 levels.

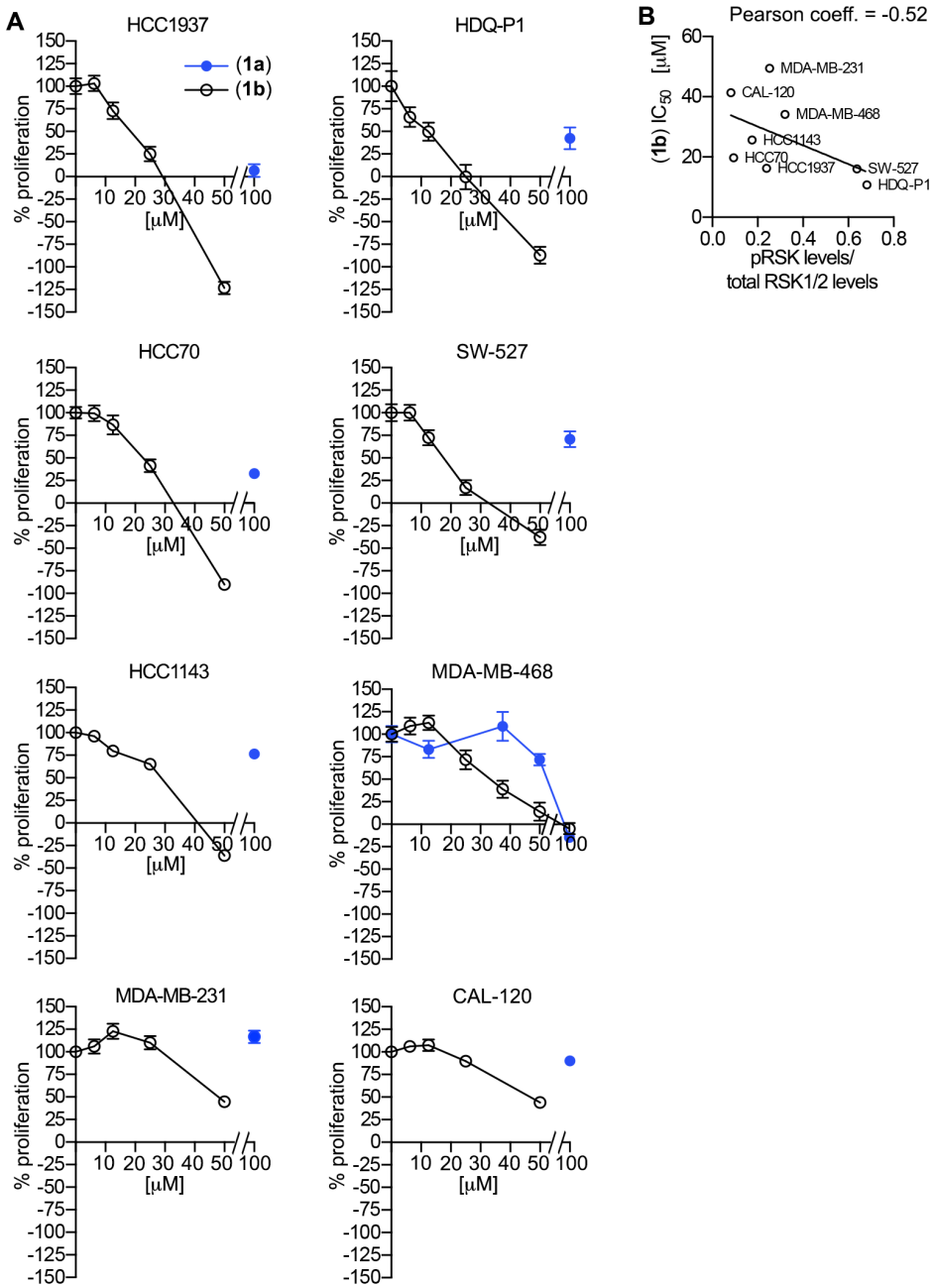
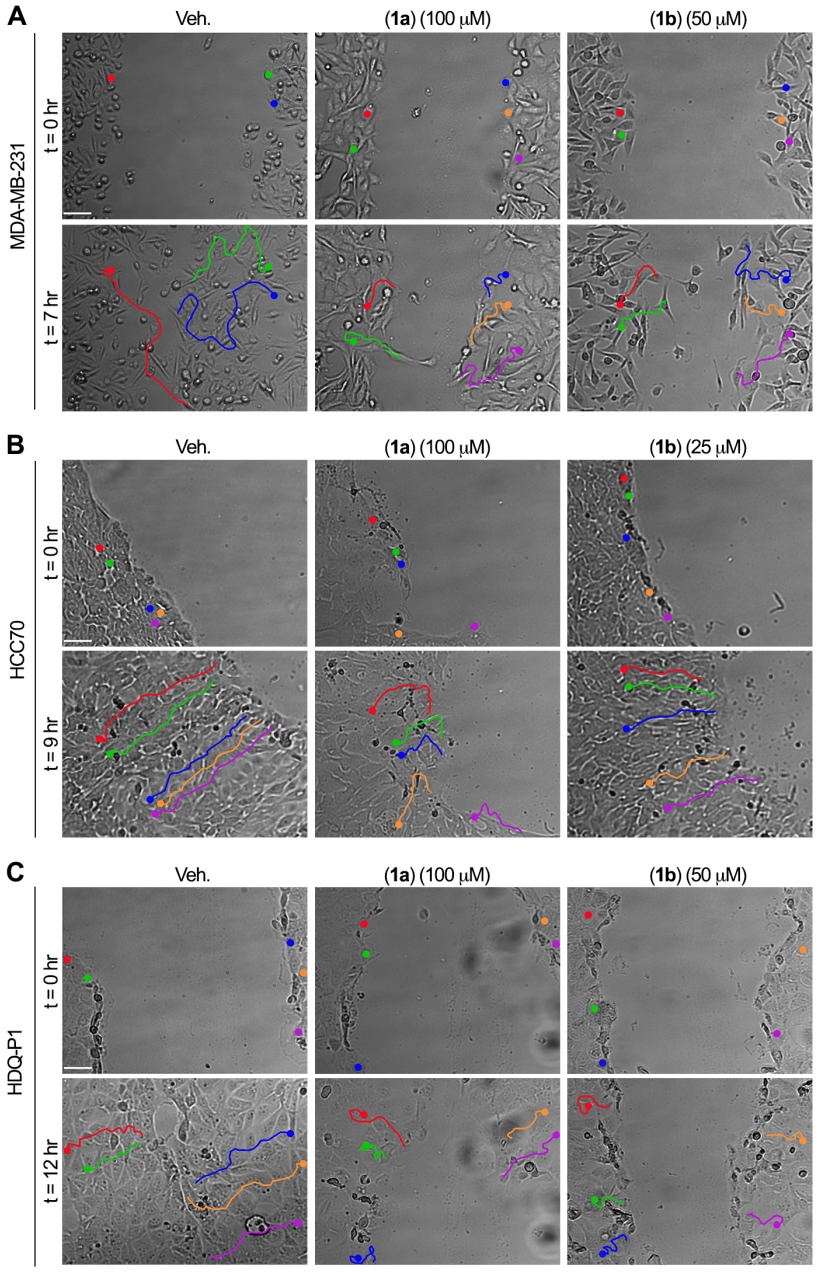


Figure S3 RSK regulates TNBC cell motility. (A) (**1b**) and (**1a**) inhibit proliferation of the indicated cell lines as measured by the scratch assay. The cells were plated as a confluent monolayer on fibronectin, pre-treated with vehicle or inhibitors for 2 h and a wound introduced into the monolayer. The movement of the cells was monitored over time. The velocity of individual cells was calculated from their distance traveled over the time and normalized to the vehicle control. Representative DIC images and cell traces (as shown by the colored lines) of (A) MDA-MB-231, (B) HCC70 and (C) HDQ-P1 cells. (D) Lysates were obtained from cells treated with vehicle or (**1b**) for a 10 h time period, which was the maximum length that the scratch assay was performed. Arrows indicate location of full length and cleaved products. The absence of cleaved products indicates that apoptosis was not occurring. MW markers are shown on the right. ns=nonspecific

Figure S3


A
Veh.
(1a) (100 μM)
(1b) (50 μM)
MDA-MB-231
t = 0 hr
t = 7 hr
B
Veh.
(1a) (100 μM)
(1b) (25 μM)
HCC70
t = 0 hr
t = 9 hr
C
Veh.
(1a) (100 μM)
(1b) (50 μM)
HDQ-P1
t = 0 hr
t = 12 hr

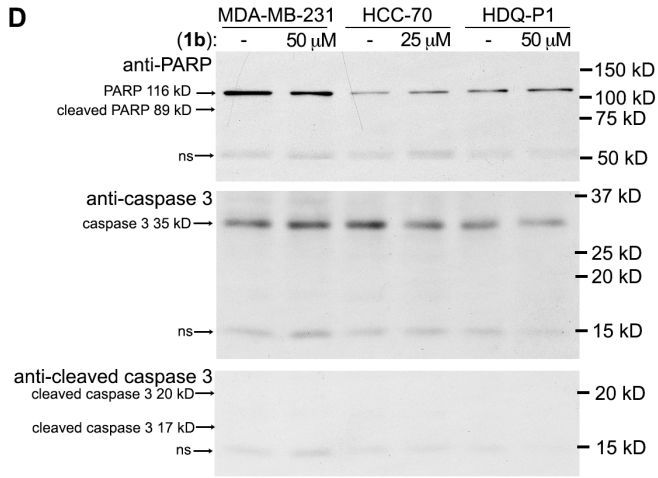

D
MDA-MB-231
HCC-70
HDQ-P1
(1b): - 50 μM - 25 μM - 50 μM
anti-PARP
PARP 116 kD
cleaved PARP 89 kD
ns
150 kD
100 kD
75 kD
50 kD
anti-caspase 3
caspase 3 35 kD
ns
37 kD
25 kD
20 kD
15 kD
anti-cleaved caspase 3
cleaved caspase 3 20 kD
cleaved caspase 3 17 kD
ns
20 kD
15 kD

Figure S4 RSK1 and RSK2 contribute to TNBC metastasis. (A) Analysis of lysates generated from MDA-MB-231 transduced with scramble (scrbl), RSK1 or RSK2-targeting shRNAs. (B) The luminescence signal correlates with the cell number of MDA-MB-231-Luc cells transduced with scramble (scrbl), RSK1- or RSK2-targeting shRNAs. (C) Bioluminescence images of NSG mice injected IC with MDA-MB-231-Luc cells transduced with scramble (scrbl), RSK1- or RSK2- targeting shRNAs. (D) The number of metastatic foci in livers in mice from (C) detected by histology correlates with the number of metastatic foci in livers detected by *ex vivo* bioluminescence imaging. (n= 8 mice/group, 4 sections/mouse) (E) Representative paraffin-embedded H&E sections of mouse livers from (C). Arrowheads indicate tumor foci. (F) Silencing RSK1 or RSK2 reduces the number of liver metastatic foci as detected by H&E. Bar, median $\pm$ quartile (n = 8 mice/group, ≥4 sections/mouse).

Figure S4

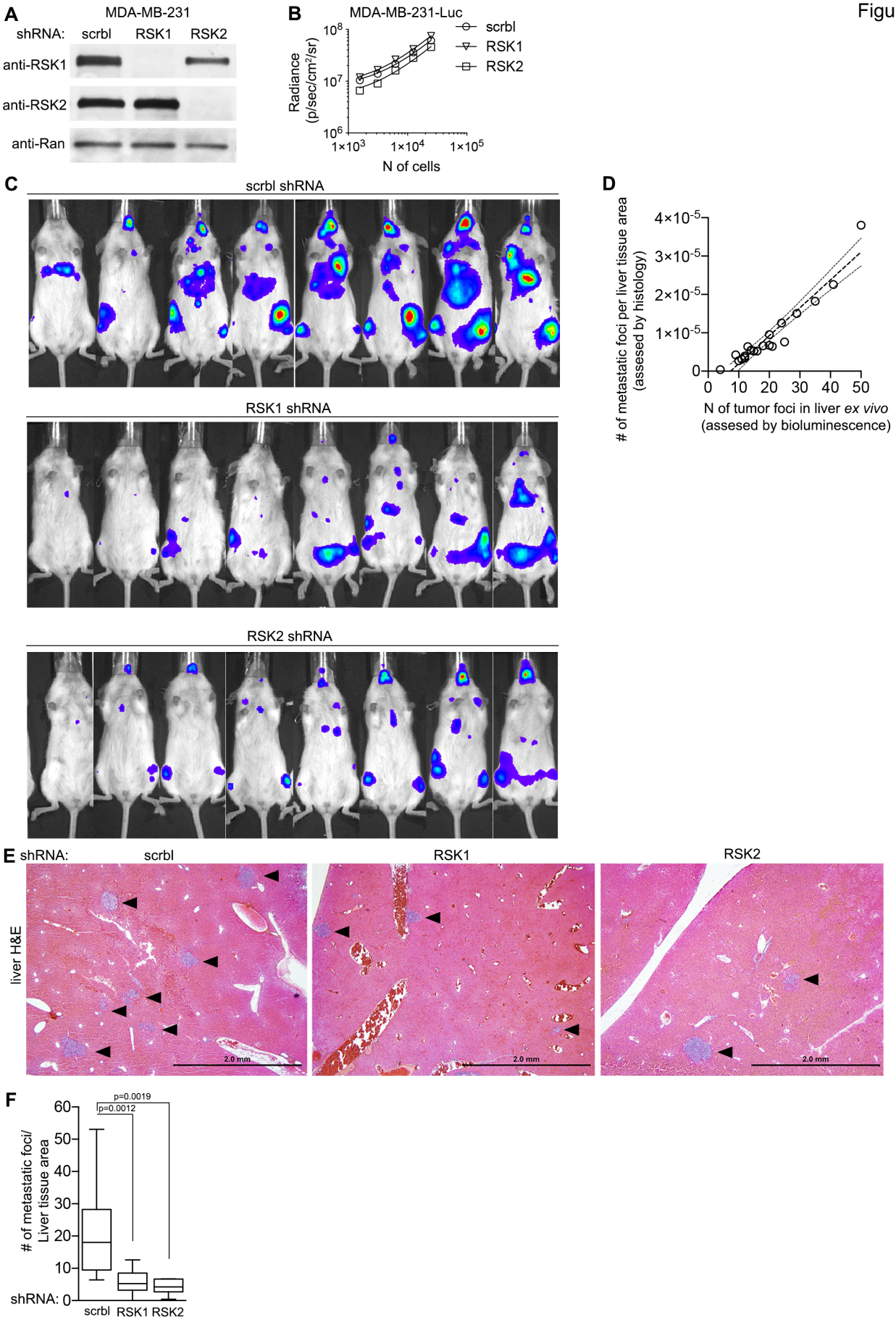
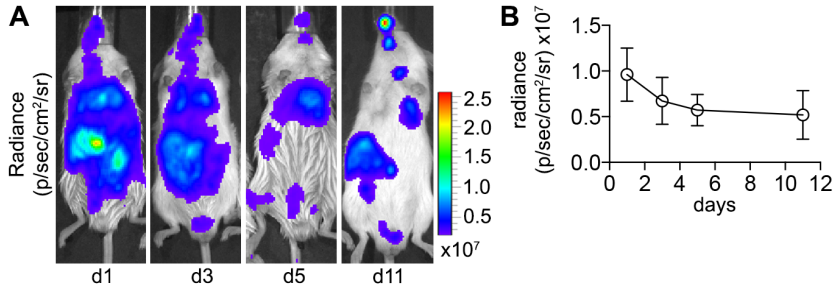

A
MDA-MB-231
shRNA: scrbl RSK1 RSK2
anti-RSK1
anti-RSK2
anti-Ran
B
MDA-MB-231-Luc
Radiance (p/sec/cm²/sr)
N of cells
scrbl
RSK1
RSK2
C
scrbl shRNA
RSK1 shRNA
RSK2 shRNA
D
# of metastatic foci per liver tissue area (assesed by histology)
N of tumor foci in liver ex vivo (assesed by bioluminescence)
E
shRNA: scrbl RSK1 RSK2
liver H&E
2.0 mm
F
# of metastatic foci/ Liver tissue area
p=0.0019
p=0.0012
shRNA: scrbl RSK1 RSK2

Figure S5 The HDQ-P1 line preferentially targets to the viscera. (A) Representative bioluminescence images at the indicated times of male NSG mice injected IC with HDQ-P1-Luc cells. (B) Total bioluminescence signal from HDQ-P1-Luc metastases stabilizes within the viscera in mice from (A). Symbol, mean ± S.D. (n= 4 mice)



Supplementary Tables

Table S1. Patient Statistics

Median age 53

Patient ID	Race	Age
T1	Caucasian	64
T2	African-American	52
T3	Caucasian	28
T4	African-American	60
T5	Other	43
T6	African-American	56
T7	African-American	53
T8	Caucasian	55
N1	Caucasian	62
N2	Caucasian	39
N3	Caucasian	35

Supplementary Movie Legends

Movie S1. MDA-MB-231 cells: top vehicle, bottom (**1b**) (50 μM) over a 7h time frame.

Movie S2. HCC70 cells: top vehicle, bottom (**1b**) (25 μM) over a 9h time frame.

Movie S3. HDQ-P1 cells: top vehicle, bottom (**1b**) (50 μM) over a 10h time frame.