Supporting Materials and Methods

Cell Culture. Human carcinoma cell lines were obtained from the American Type Culture Collection (ATCC; www.atcc.org) and cultured according to standard mammalian tissue culture protocols and sterile technique. SKOV-3 (ATCC no. HTB-77) and ES-2 (ATCC no. CRL-1978) cells were cultured in McCoy's 5A medium, the MDA-MB-231 (ATCC no. HTB-26) and A2058 (ATCC no. CRL-11147) cells were cultured in Dulbecco's Modified Eagle Medium. DU-145 (ATCC no. HTB-81) cells were cultured in RPMI medium 1640. All media was supplemented with 10% Australian-sourced FBS/ 100 units/ml penicillin/100 μg/ml streptomycin/2 mM L-glutamine. The RPMI media was also supplemented with 1 mM sodium pyruvate/10 mM Hepes buffer. All tissue culture media and supplements were obtained from Invitrogen.

Automated Wound-Healing Assay. *Hardware*. An automated 384-well plate-based assay device was built to specifications by the Engineering Department at the Genomics Institute of the Novartis Research Foundation. Briefly, the device consists of a machined aluminum block into which 384 holes are drilled wide enough to accommodate 12.5-μl pipette tips (Matrix Technologies, catalog no. 5302, Hudson, NH). The sterilized pipette tips are inserted into the block and suspended on a vertical tracking arm, and the tip block is raised against a top plate to stabilize the tip position and prevent any movement upon scratching. Clear bottom tissue culture plates (384-well; Greiner Bio-One, Frickenhausen, Germany; see below) are placed on a level platform below the aluminum block. Once the equipment is initiated, the aluminum block is automatically lowered to a point at which the pipette tips touch the bottom of each of the 384 wells (after plate-specific calibration). With the pipette tip block engaged, the platform is shifted ≈3 mm (well diameter = 3.70 mm) by hydraulic pressure, resulting in uniform "scratches" in each of the 384 well plates. After scratching, the tip block is raised up from the plate, and the plate holder returns to the start position where the plates are manually switched by the user.

small interfering RNAs (siRNA) Libraries, 384-Well Scratch Assay, and siRNA Transfection. siRNAs were purchased from Dharmacon (Lafayette, CO) or Qiagen

(Valencia, CA), prepared and dispensed into 384-well plates as described in ref. 1. Kinase-focused siRNAs were deposited at 8 ng/μl (1); other siRNAs were deposited at14 ng/μl. Resynthesized siRNAs for additional experiments were also purchased from Dharmacon or Qiagen and used at a final concentration of 2 pmoles per well. Motile cells were plated at high density (4,000–5,000 cells per well) in 384-well clear bottom tissue culture plates (Greiner Bio-One) in cell-specific media supplemented with 10% FBS. Cell density was determined to result in >95% confluence at the time of scratching, accounting for the toxicity of the transfection reagent.

For the 384-well siRNA transfections, cells were added directly to an siRNA/transfection reagent mixture, resulting in a “reverse transfection” as described in ref. 1. For these studies, 0.1 μl Lipofectamine 2000 in 10 μl of Opti-Mem reduced serum media (Invitrogen) was added to the preplated siRNA in each well, and allowed to incubate for 1 h at room temperature before the addition of the cells. For the small molecule experiments, cells were seeded into empty 384-well plates by using 10% fewer cells than for the transfection experiments. Compounds were added onto the cell layers 12 h before scratching, with a final concentration of 0.5% DMSO for all conditions. Media was changed in all experiments at 24 h after transfection/cell seeding by aspirating the old media from each well and adding 50 μl of fresh, prewarmed media per well. Assay plates were fitted with metal low-evaporation covers and incubated at 37°C, 5% CO_2 in humidified tissue culture incubators. All liquid dispensing steps were performed by using a Multidrop 384 dispenser (Titertek, Huntsville, AL), and all liquid aspiration steps were done by using an EMBLA plate washer (Molecular Devices).

After a 48-hour incubation, confluent cell layers were scratched using the automated device described above. Plates were returned to the incubator, and the cells were allowed to traverse the wound, resulting in closure within 12–14 h. The time to closure is influenced by the cell type and assay cell density and is typically less than one complete cell cycle, thus avoiding proliferation based reseeding of the wound area. After wound closure, all wells were fixed at the same time point. For fixation, formaldehyde (Sigma-Aldrich) was added directly to the cell media, for a final concentration of 3.7%. After

fixation, the cells were washed two times with PBS, permeabilized with Triton X-100 (Sigma-Aldrich), and stained with the nuclear stain DAPI (Molecular Probes). The wells were again washed two times with PBS after DAPI staining, with a final dispensing volume of 50 μl PBS per well. All PBS was supplemented with 0.9 mM calcium and 0.5 mM magnesium, and all washing steps were carried out by using the EMBLA plate washer (Molecular Devices).

After staining, each well of the 384-well plate was photographed by a fluorescent microscope retooled by Q3DM, Inc., (San Diego) to automate image capture. The ×4 objective lens was used to visualize a majority of the space of each well within one field of view.

Sequences for MAP4K4 siRNAs are: si_1, 5'-CGCAAUGACAAGGUGUUCU-3'; si_2, 5'-GGGAAGGUCUAUCCUCUUA-3'; si_3, GATGACCAACTCTGGCTTG-3'; and si_4, 5'-TAAGTTACGTGTCTACTAT-3'

Quantitative Scoring Method for Cell Migration. The raw assay data comes in the form of one grayscale image per well. Bright regions represent cells and black regions represent background; pixel intensities vary (Fig 9*a*). The grayscale image is first converted into a binary black and white mask image, where cells are shown in white pixels and background in black pixels (Fig. 9*b*). Contaminations show up as unusually large blocks of continuous white regions and can be identified and excluded from our analysis. The initial scratch goes from left to right; however, sometimes a scratch does not start or end beyond the left and right image borders. To avoid taking unscratched regions into account, the left and right 25% of the original image are cropped out (Fig 9*b*).

The program then calculates the number of white pixels for every row in the image, as shown in Fig. 9*c*, and the resultant curve represents cell density as a function of vertical locations. The scratched zone contains much fewer white pixels compared to the rest of the image. For a hypothetical scratch window, the green line represents the average

number of white pixels outside the scratch window, the rectangular purple dashed area A_S is proportional to the number of cells being removed by the scratch. The yellow area, A_M, is proportional to the number of cells moving back into the scratched zone as the result of cell migration. The motility score is defined as:

$S = A_M/A_S$.

A score close to 1 is assigned to cells with high motility, and a score close to 0 to those with low motility. Because the score has been self normalized by cell density, it is comparable across wells and across plates.

The center of the scratch may vary from well to well; therefore, the algorithm does not assume a fixed scratch location. The above *S* score is iteratively calculated for every possible scratch center within a given range, as shown in Fig. 9*d*. Only the minimal possible *S* score is reported, and the corresponding location is the optimal guess of the scratch center.

As input parameters, the method only takes the width of the scratch window and a possible range of scratch center. It does not require any training data and is insensitive to variations in cell density. Analysis of randomly selected wells shows good correlation between the computer calculated *S* score and human visual inspection. The algorithm was implemented by using MATLAB 6.5 (Image Processing Toolbox).

Statistical Analysis for Hit Identification. The majority of the genes in our screen have at least two independently designed siRNAs; therefore, scores from multiple siRNAs targeting the same gene provide a basis for a natural self-validation. As one lowers the threshold for migration score S_0, more genes with $S < S_0$ can be hit picked. We assume that genes with both siRNA wells scoring better than S_0 are likely to be true hits, otherwise, they are likely to be false positives. We only report genes that are double-confirmed in this study.

Assuming N_{gene} is the total number of genes in the screen, and $2N_{gene}$ is the total number of siRNAs. For a given cutoff score S_0, N_{top} siRNAs are hit picked, among them $2N_{two}$ siRNAs provide double confirmation for N_{two} genes. If one randomly selects N_{top} siRNAs out of a pool of size $2N_{gene}$, the odds of having at least $2N_{two}$ siRNAs provide double-confirmation hits can be calculated as:

$$P = \sum_{i=N_{two}}^{N_{top}/2} \frac{\binom{N_{gene}}{i}\binom{N_{gene}-i}{N_{top}-2i}}{\binom{2N_{gene}}{N_{top}}}.$$

In this screen, we chose a hit rate of 5%, $S_0 = 0.362$, we obtained 23 double-confirmed genes. I.e., $N_{gene} = 4{,}766$, $N_{top} = 574$, $N_{two} = 23$, the P value is essentially zero (1.0×10^{-150}). The above P value is estimated based on the assumption that signals from any two siRNAs per gene are independent; however, correlation among siRNAs will require subjective modeling and computer simulations that are beyond the scope of this study. With the extremely low P value we obtained here, we believe the true P value is likely to be statistically significant as well.

Cell Viability. Cells were plated into an identical set of assay plates and processed identically to the sister wound closure plates, up until the point of plate scratching. Viability was measured by using Cell Titer Glo (Promega), a reagent with a luminescent readout that reflects cell viability via the measurement of ATP metabolism. At the approximate time of plate scratching, the media from the viability plates was removed by aspiration and 30 μl of Cell Titer Glo was added per well. After incubation, the luminescent intensities of the wells were measured by using an Acquest (Molecular Devices) multiwell plate reader. The mean intensity of each plate was calculated, and the percent of the plate mean was calculated for each well. siRNAs or molecules that resulted in a percent mean of <90% were considered to negatively effect viability and were eliminated from further followup.

Cell Invasion. Invasive potential was measured by using an 8 μm pore, 24-well format matrigel coated transwell chamber assay (Becton Dickinson Discovery Labware, Bedford, MA). For the siRNA experiments, SKOV-3 cells were reverse transfected by using 200 pmol siRNA, 10 μl of Lipofectamine 2000 (Invitrogen), and 400,000 cells per 3.5 cm^2 well. Media was changed after 24 h, and the cells were harvested 48 h after reverse transfection. After harvesting, cells were washed and resuspended in media without FBS, supplemented with 0.2% protease free BSA (Jackson ImmunoResearch). Cell concentration was measured by using a Casy cell counter (Scharfe Systems, Reutlingen, Germany), and adjusted to 200,000 cells/ml. Sixty thousand cells (0.3 ml) were added to the top of each hydrated chamber, with 1 ml media plus 10% FBS used as chemoattractant in the well plate.

Chambers were incubated for 22 h in a standard tissue culture incubator. After incubation, nonmigrating cells were swabbed off the top of the membrane by using a cotton tipped applicator, and the remaining invading cells on the bottom of the membrane were fixed with methanol and stained by using the Diff-Quik staining kit (Dade Behring, Newark, DE). Membranes were photographed by using brightfield settings on a Nikon inverted microscope fitted with a digital camera. Pictures of five consecutive vertical fields of view were taken by using the ×10 objective lens. The number of cells in each field of view was counted manually. For all conditions, a minimum of four replicate chambers were assayed. The average total number of cells per chamber was calculated for each condition, and compared to scrambled siRNA control.

RT-PCR. siRNA-treated cells (as above for invasion experiment) were lysed *in situ*. Cell lysis/RNA isolation was performed by using Qiagen's RNeasy kit, according to the manufacturer's directions. RNA was quantitated and used to prepare cDNA by using Invitrogen's Thermoscript RT-PCR system. The resulting cDNA was analyzed by PCR with primers specific for the gene of interest and the 18S ribosomal subunit (to control for quantification). The 18S PCR primers were from Ambion's (Austin, TX) QuantumRNA Classic 18S kit. For mitogen-activated protein 4 kinase 4 (MAP4K4), the

gene specific primers were 5'-GTGGTCAATGTGAATCCTACC-3' for the forward primer, and 5'-CCAAGTGACCAGTTTCCACAG-3' for the reverse primer. PCR was carried out for 26 cycles by using an annealing temperature of 60°C. The PCR reaction buffer and *Taq* polymerase were from the PCR SuperMix High Fidelity reaction mixture (Invitrogen).

Western Blotting. siRNA-treated cells were reverse transfected as described for the invasion assays and harvested with a nonenzymatic EDTA based buffer 48 h after transfection. Whole cell protein lysates were prepared by adding RIPA lysis buffer plus broad sprectrum protease inhibitors (Complete mini tablets; Roche Applied Science, Indianapolis) to the cell pellets. After incubation on ice, the lysed cells were centrifuged at 14,000 x *g* for 15 min and the supernatant was collected and quantitated (BCA assay; Pierce). SDS/PAGE and Western blotting were performed according to standard technique by using Tris-glycine polyacrylamide gradient gels (Invitrogen) and nitrocelluose membrane (Schleicher and Schuell BioScience, Dassel, Germany). After antibody incubation, peroxidase activity was detected via chemiluminescence (ECL reagent; GE Healthcare, Piscataway, NJ) by using Kodak BioMax Light film. All Western blot blocking and incubation steps were carried out in 5% nonfat dry milk dissolved in Tris buffered saline plus 0.2% Tween-20 (Sigma-Aldrich).

Antibodies were obtained from the following sources: phospho-p38 mitogen-activated protein (MAPK) kinase (mouse monoclonal, clone 28B10), total p38 MAPK (rabbit polyclonal), phospho-pErk1/2 (mouse monoclonal, clone E10), total pErk2 (rabbit polyclonal), phospho-Stat3 (mouse monoclonal, clone 6E4), and total Stat3 (rabbit monoclonal, clone 79D7) antibodies were obtained from Cell Signaling Technology (Beverly, MA). The phospho-JNK (mouse monoclonal, clone 41) and total JNK (mouse monoclonal, clone G151-333) antibodies were obtained from BD Pharmingen. The total Rac1 (mouse monoclonal, clone 23A8) antibody was obtained from Upstate Biotechnology (Charlottesville, VA). The total β-1-Tubulin (mouse monoclonal, clone TUB 2.1) antibody was obtained from Sigma-Aldrich, and the total actin antibody (goat

polyclonal) was obtained from Santa Cruz Biotechnology. All secondary horseradish peroxidase-conjugated antibodies were obtained from Jackson ImmunoResearch.

1. Aza-Blanc, P., Cooper, C. L., Wagner, K., Batalov, S., Deveraux, Q. L. & Cooke, M. P. (2003) *Mol. Cell* **12,** 627–637.