A small interfering RNA screen for modulators of tumor cell motility identifies MAP4K4 as a promigratory kinase


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Communicated by Webster K. Cavenee, University of California at San Diego, La Jolla, CA, January 3, 2006 (received for review December 5, 2005)

Cell migration is a complex biological process, involved in development, inflammation, homeostasis, and pathological processes such as the invasion and metastatic spread of cancer. Here, we describe a genomic screen designed to identify inhibitors of cell migration. A library of 10,996 small interfering RNAs (targeting 5,234 human genes) was screened for their ability to block the migration of a highly motile ovarian carcinoma cell line, SKOV-3, by using a 384-well wound-healing assay coupled with automated microscopy and wound quantification. Two or more small interfering RNAs against four genes, CDK7, DYRK1B, MAP4K4 (NIK/HGK) (MAP4K4), mitogen-activated protein 4 kinase 4), and SCCA-1 (SerpinB3), potently blocked the migration of SKOV-3 cells, consistent with reduced transcript levels. Further studies of the promigratory role of MAP4K4 showed that the knockdown of this transcript inhibited the migration of multiple carcinoma cell lines, indicating a broad role in cell motility and potently suppressed the invasion of SKOV-3 cells in vitro. The effect of MAP4K4 on cellular migration was found to be mediated through c-Jun N-terminal kinase, independent of AP1 activation and downstream transcription. Accordingly, small molecule inhibition of c-Jun N-terminal kinase suppressed SKOV-3 cell migration, underscoring the potential therapeutic utility of mitogen-activated protein kinase pathway inhibition in cancer progression.

Results

Development of an Automated Cell Migration Assay. Measurement of cell migration by the wound-healing assay (4) was automated by using a custom-built, 384-well scratch device, coupled to automated image capture and quantification of wound closure (see Fig. 7 and Supporting Materials and Methods, which are published as supporting information on the PNAS web site). The assay system utilizes a precision-drilled aluminum block into which 384 pipette tips are inserted and fastened. The block is lowered on a static arm into the wells of a 384-well plate containing confluent cell monolayers and shunted ∼3 mm by hydraulic pressure. After the wounds are allowed to close, the cells are fixed and stained with the nuclear stain, DAPI, to enable rapid image capture by automated microscopy.

To validate the assay, the temporal migration of a highly motile ovarian carcinoma-derived cell line, SKOV-3, was monitored in the presence of siRNAs, small molecules and appropriate controls. The efficacy of siRNA-mediated migratory inhibition was assessed by using siRNAs homologous to Rac1, one of three Rho GTPases (RhoA, Rac1, and Cdc42) that integrates promigratory signals with dynamic reorganization of the actin cytoskeleton (ref. 2; Fig. L4). In addition, we tested known small molecule inhibitors of c-Src (SKI-606, ref. 6; and a 2-phenylaminomidazo-[4,5-h]-isoquinolin-9-one, termed compound 43, ref. 7), the activated form of which plays a central role in the motility and invasion of cancer cells, including ovarian (refs. 7 and 8; Fig, L4). Using a pixel density-based quantitative measure of cell migration (see Supporting Materials and Meth-

Conflict of interest statement: No conflicts declared.

Freedly available online through the PNAS open access option.

Abbreviations: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA.

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ods), the addition of the Rac1 siRNA pool or the Src inhibitors retarded SKOV-3 cell migration by 60–70%, respectively, at 12 h after scratching (relative to control wells at the same time points; Fig. 1B). Although migratory inhibition is evident at 8 and 16 h, 12 h represented the largest difference between controls and test siRNAs at a time point where control cells closed the wound. In parallel, we measured cell viability in identically treated 384-well plates by using an ATP-based luminescent assay to monitor potential toxic effects of siRNA transfection and small molecule inhibition on SKOV-3 cells. All screen controls shown in Fig. 1 did not have an appreciable effect on cell viability (luminescent signal was ≥90% of untreated well signal).

The reproducibility of the assay was tested by using a diverse subset of 384 preplated siRNAs targeting 192 genes (two siRNAs per gene plated in duplicate). For these experiments, SKOV-3 cells were reverse transfected on each of three replicate plates, grown to confluency, wounded, and incubated for a further 12 h. After image capture, wells from each of the three replicate plates were scored by the quantitative algorithm (see Supporting Materials and Methods) and the score from each individual well in each of the three replicate runs was compared to the mean well score by using the Pearson correlation coefficient. In each case, r² was >0.87, demonstrating a high degree of well-to-well consistency (Fig. 8, which is published as supporting information on the PNAS web site).

**A Large-Scale siRNA Screen for Inhibitors of SKOV-3 Cell Motility.** We used a preplated library of 10,996 siRNAs, targeting 5,234 genes (described in Supporting Materials and Methods) to identify inhibitors of cellular motility in SKOV-3 cells (Fig. 2). Cells were reverse transfected as described above and incubated for 48 h before wounding. The screen was performed in duplicate (∼22,000 wells) and quantitatively scored. Measurement of cell viability was performed in a set of duplicate siRNA library plates, and the luminescence of each well was compared to the normalized mean well intensity of each 384-well plate. To eliminate siRNAs that induced cell growth arrest or cell death and would not migrate as a consequence, we adopted a viability cutoff score of 0.9 (10% deviation from the plate mean), below which siRNAs were disregarded.

The top 5% of wells in which SKOV-3 cells migrated the least (n ≥ 532), were chosen for further analysis (Table 1, which is published as supporting information on the PNAS web site), based on a statistical review of the screen (see Supporting Materials and Methods). Because of the significant potential for off-target effects when considering the phenotypic effects of single siRNAs, we focused only on those transcripts targeted by at least two independent siRNA sequences (n ≥ 22), with the assumption that a similar phenotypic effect observed with two siRNAs would be less likely to occur by chance (Table 2, which is published as supporting information on the PNAS web site). To test this assumption, we resynthesized the siRNAs from the library sequences and monitored transcript knockdown by semi-quantitative RT-PCR in parallel with migratory inhibition. Of the 46 siRNAs targeting 22 genes (20 genes targeted by 2 siRNAs and 2 genes targeted by 3 siRNAs), 36 (78%; 17 unique genes) yielded migratory phenotypes similar to those observed in the primary screen. In contrast to the high degree of concordance at a phenotypic level, independent siRNAs against only 4 of the 22 genes; MAP4K4, DYRK1B, CDK7, and SerpinB3, exhibited similar reductions in transcript levels, consistent with phenotypic inhibition (Fig. 3).
relative transcriptional knockdown was quantified by using IMAGEJ (National Institutes of Health). RT-PCR analysis is shown for each transcript, and the data were compared to control siRNA and quantified by the automated algorithm (black bars, migration score; white bars, relative cellular viability). The extent of transcript knockdown is shown as a ratio of migratory inhibition relative to control siRNA-transfected cells, was consistent with the degree of transcript knockdown determined by RT-PCR (Fig. 3B), which ranged from 64% to 94%. The effect of the two most potent siRNAs (si.1 and si.2) on other highly motile carcinoma cells, and their associated transcriptional inhibition, are depicted in Fig. 4. Migratory inhibition is evident in all four cell lines relative to control siRNA (si.C), with variable potency. We assume that the variability reflects the following: (i) variable transfection efficiencies, (ii) differences in the relative expression of the gene, (iii) differences in signaling pathway dynamics in the different cell types, and (iv) the effects of variable cellular densities on the ability of the automated scoring algorithm to comparably score different cell types relative to SKOV-3.

Because of the strong relationship between increased cancer cell motility, tissue invasion, and metastasis (3), we next asked whether transient MAP4K4 knockdown could affect cell invasion. SKOV-3 cells were transfected by using si.1, si.2, or a control scrambled siRNA, and invasion was monitored by using a matrigel-coated (Boyden) chamber assay. Invasion was inhibited by 76% and 52% with si.2 and si.1, respectively, relative to control transfected cells (Fig. 5).

MAP4K4 Signals Through c-Jun N-Terminal Kinase, Independent of AP1 Activation and Downstream Transcription. To address the underlying signaling mechanism(s) through which MAP4K4 mediates its effects on cell migration, we asked whether MAP4K4 signaling converges on one or more of the key MAPK transduction pathways, i.e., c-Jun N-terminal kinase (JNK), p38, or Erk MAPK (10). Knockdown of MAP4K4 by two independent siRNAs had no appreciable effect on the phosphorylation of Erk1/2 (Fig. 6), consistent with a lack of migratory inhibition observed by using the MEK inhibitor, U0126 (11), or siRNAs specific to the MEK1 kinase (data not shown). Similarly, cells transfected with MAP4K4 siRNAs did not show a decrease in detectable levels of phosphorylated p38 MAPK, which were low in SKOV-3 cells. In contrast, phosphorylation of JNK was significantly decreased in MAP4K4 siRNA transfected cells, consistent with reports showing that MAP4K4 can phosphorylate JNK in vitro (12). That JNK plays a significant role in mediating MAP4K4 signaling was demonstrated by inhibition of SKOV-3 migration with the JNK-specific inhibitor, SP600125 (Fig. 7). Migration was potently inhibited at concentrations of 30 μM and above without any significant effect on the viability of siRNAs targeting the MAP4K4 gene (three from the primary screen and one additional siRNA, hereafter termed si.1–si.4; sequences are listed in Supporting Materials and Methods) on SKOV-3 migration. The migratory inhibition, which ranged from 50% to 66% relative to control siRNA-transfected cells, was consistent with the degree of transcript knockdown determined by RT-PCR (Fig. 3B), which ranged from 64% to 94%. The effect of the two most potent siRNAs (si.1 and si.2) on other highly motile carcinoma cells, and their associated transcriptional inhibition, are depicted in Fig. 4. Migratory inhibition is evident in all four cell lines relative to control siRNA (si.C), with variable potency. We assume that the variability reflects the following: (i) variable transfection efficiencies, (ii) differences in the relative expression of the gene, (iii) differences in signaling pathway dynamics in the different cell types, and (iv) the effects of variable cellular densities on the ability of the automated scoring algorithm to comparably score different cell types relative to SKOV-3.

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the cells. This effect on SKOV-3 migration is consistent with the effect of SP600125 on JNK-dependent cellular migration in NBT-II bladder tumor cells, MDA-MB-231 cells, fish keratinocytes (50 μM) (13), and fibroblasts (20 μM) (14). These results, together with the observation that siRNAs targeting Rac1 inhibit SKOV-3 migration (Fig. 1), tentatively suggest a constitutively active Rac1-MEK1-JNK pathway in SKOV-3 cells. However, additional work is required to substantiate whether the major effect is mediated via JNK and whether constitutively activated JNK can rescue the migration of cells with diminished MAP4K4.

We next asked whether the effect of JNK on SKOV-3 migration was mediated through activation of AP1 transcription. To first address this question, the expression profile of cells transiently transfected with two MAP4K4 siRNAs (si.1 and si.2) was assessed by oligonucleotide microarray hybridization on a 22,500-member array. We did not observe modulation of any known AP1 target genes; indeed, the only significantly altered probe sets when compared to control cells were those homologous to the MAP4K4 transcript, strongly indicating a lack of AP1-mediated transcription. Basal c-jun protein and phosphoprotein levels were essentially undetectable by Western blot in SKOV-3 cells (data not shown), further confirming the lack of AP1 activation.

MAP4K4 has also been reported to activate a number of other proteins, including kinases within the JAK/STAT pathway. Because JAK/STAT signaling is highly active in high grade ovarian carcinomas and has been shown to be important for SKOV-3 cell motility by RNA interference-mediated knockdown (15), we examined the phosphorylation of STAT-3 in the presence or absence of MAP4K4 siRNAs. No significant changes were observed (Fig. 7), further supporting a major role for JNK in mediating SKOV-3 cell motility.

Discussion

Epithelial tumor progression depends on the sequential acquisition of multiple cellular traits, including the dissolution of cell–cell contacts and increased motility, enabling the invasion of adjacent tissues and, ultimately, the colonization of distant body sites. To address the underlying molecular mechanisms that promote cancer cell motility, we developed an automated 384-well wound-healing assay, coupled with automated microscopy and quantification.

We queried a custom library of 10,996 synthetic siRNAs targeting 5,234 transcripts to identify genes involved in the migration of SKOV-3 cells, a highly motile cell line derived from the ascites of a patient with metastatic ovarian adenocarcinoma. Although we identified 532 siRNAs with highly significant effects on migration (i.e., the highest ranked 5%; see Supporting Materials and Methods), which included many genes known to play a role in cell motility, we chose to examine the 22 genes targeted by at least two siRNAs, because of the significant potential for off-target phenotypic effects elicited by a single siRNA. Evaluation of resynthesized siRNAs targeting these 22 genes showed a relatively high degree of phenotypic consistency (74%), but a much lower consistency in terms of correlated transcriptional knockdown and migratory suppression of both siRNAs. Four genes, CDK7, MAP4K4, DYRK1B, and SerpinB3, were validated by these criteria. Given the apparently high off-target rate, we anticipate that future screens should encompass additional siRNAs against each target or secondary screens designed to interrogate the subgroup of identified hits (532 in this case) with additional, independent siRNAs.

We chose to further examine the role of MAP4K4 in cancer cell motility primarily because the protein is involved developmentally in the motility of multiple cancer cell lines, signaling upstream of JNK, and that inhibition of MAP4K4 results in suppression of invasion in vitro. Activation of JNK by MAP4K4 has been shown previously; however, it is reported to be indirect, acting through TAK1, MKK4, and MKK7 (11). However, we have been unable to detect appreciable basal levels of phospho-MKK4 or phospho-MKK7 (data not shown), suggesting that MAP4K4 may activate JNK through other kinases, at least in this cell type. Downstream of JNK, we have shown that AP1-dependent transcription is not involved in SKOV-3 cell migration; however, the substrates through which MAP4K4-JNK mediates its effects are not yet defined. Multiple direct targets of JNK phosphorylation have been described (reviewed in ref. 9), including the microtubule-associated pro-
teins, MAP1B, DCX, and MAP2, and proteins involved in actin dynamics, such as Spir, and paxillin, which plays a key role in adhesion dynamics. Although all of these proteins are candidates for being targeted by JNK in SKOV-3 cells, a detailed examination of their possible role is beyond the scope of this report. The mouse homolog of MAP4K4, Nik, is reported to bind β1-integrin and associate with actin filaments. This observation might reflect a need to have MAP4K4 in close proximity to phosphorylation targets at points of substrate contact or that MAP4K4 also plays a structural role in cell movement.

Involvement of MAP4K4 in cell invasion has been suggested by one report in which rat intestinal cells overexpressing the protein were significantly more invasive in the presence of hepatocyte growth factor than parental rat intestinal epithelial cells (9). The same report also documented a striking complexity of MAP4K4 isoforms as well as an increased expression in tumor cells and primary tumors. Our own analysis has identified several splice variants of the gene in SKOV-3 cells, the dominant form of which is most similar to transcript variant 1 in the National Center for Biotechnology Information databases (data not shown). However, we have been unable to verify over-expression of MAP4K4 in primary tumor samples, either in our databases (http://symatlas.gnf.org) or the extensive tumor data sets available at Oncomine (www.oncomine.org). Notably, the expression of MAP4K4 in the data sets housed at Oncomine is measured both by cDNA and oligonucleotide microarrays, representing several different probes homologous to the gene. Thus, we suggest that MAP4K4 activity is triggered either by overactivity of an upstream kinase or by a constitutive autocontrol loop that involves receptors signaling into the Racl-MEKK1-MAP4K-JNK pathway.

In summary, we developed a fully automated approach to interrogate the molecular basis of cell motility. We exemplified this approach by screening a large siRNA library and identifying several genes with promigratory activity. One of these genes, MAP4K4, appears to play a central role in cell migration and invasion in vitro and most likely does so through the JNK pathway, independent of AP1-mediated transcription.

**Materials and Methods**

**Cell Culture.** Cells were obtained from the American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. Media was supplemented with 10% Australian-sourced FBS/100 units/ml penicillin/100 μg/ml streptomycin/2 mM L-glutamine. RPMI medium 1640 was additionally supplemented with 1 mM sodium pyruvate/10 mM Hepes buffer.

**Automated Wound-Healing Assay Device.** The automated 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). Sterilized pipette tips are inserted into the block and suspended on a vertical tracking arm. 384-well clear bottom tissue culture plates (Greiner Bio-One, Frickenhausen, Germany; see below) are placed on a level platform below the aluminum block. Upon initiation, the aluminum block is 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 (see also Supporting Materials and Methods).

**Wound-Healing Assays.** siRNAs were purchased from Dharmacon (Lafayette, CO) or Qiagen (Valencia, CA) and prepared and dispensed into 384-well plates as described in ref. 17. Motile cells were plated at high density (4,000–5,000 cells per well) in 384-well clear bottom tissue culture plates and cultured for up to 24 h. For 384-well siRNA transfections, cells were added directly to a siRNA/transfection reagent mixture, resulting in a ‘reverse transfection’ as described in ref. 17 and in detail in Supporting Materials and Methods. Compounds were added onto the cell layers 12 h before scratching at a final concentration of 0.5% DMSO for all conditions. Media was changed in all experiments at 24 h after transfection. Assay plates were fitted with metal low-evaporation covers and incubated at 37°C, 5% CO2 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). Confluent cell layers were scratched by using the automated device. Plates were reincubated to allow wound closure and then fixed with formaldehyde (Sigma-Aldrich) at a final concentration of 3.7%. Cells were washed two times with PBS, permeabilized with Triton X-100 (Sigma-Aldrich), and stained with the nuclear stain DAPI (Molecular Probes). Wells were further washed twice with PBS (supplemented with 0.9 mM calcium/0.5 mM magnesium) by using the EMBLA plate washer. Each well of the 384-well plate was photographed by a fluorescent microscope retooled by Q3DM, Inc. (San Diego) to automate image capture. A ×4 objective lens was used to visualize a majority of the space of each well within one field of view.

**Quantitative Scoring Method for Cell Migration.** The derivation and use of the automated scoring algorithm to assess cell migration, and the statistical treatment of the screen data are described in detail in Fig. 9, which is published as supporting information on the PNAS web site.

**Cell Viability.** Cells were plated into an identical set of assay plates and processed identically to the sister wound closure plates, up to the point of plate scratching. Viability was measured by using Cell Titer Glo (Promega) according to the manufacturer’s protocol. The mean luminescent 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.** Invasion was measured by using an 8-μm pore, 24-well format matrigel-coated transwell chamber assay (Becton Dickinson Discovery Labware, Bedford, MA) according to the manufacturer’s recommended conditions (see also Supporting Materials and Methods). Membranes were photographed by using brightfield and 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.

**RT-PCR.** siRNA-treated cells were lysed in situ and RNA were isolated by using the Qiagen RNeasy kit. cDNA was prepared by using the Thermoscript RT-PCR system (Invitrogen). PCR was carried out for 26 cycles by using an annealing temperature of 60°C. The PCR buffer and Taq polymerase were from the PCR SuperMix High Fidelity reaction mixture (Invitrogen). 18S PCR primers were from the QuantumRNA Classic 18S kit (Ambion, Austin, TX). Gene-specific primers for MAP4K4 were 5'-GTGGTCAATGTGAATCCTACC-3' for the forward primer, and 5'-CCAAGTGAACAGTTCACGAG-3' for the reverse primer.

**Western Blotting.** Protein isolation, SDS/PAGE and Western blotting were all carried out by using standard techniques. Antibodies sources are described in Supporting Materials and Methods.
We thank Drs. Quinn Deveraux, Christopher Stroh, and Venkat Reddy for critically reading the manuscript and Drs. Pedro Aza-Blanc, Serge Batalov, and Sumit Chanda, Mr. Abel Gutierrez, and Mr. Paul deJesus for siRNA design, library construction, assay development, and advice.

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 at 14 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% CO2 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'-CGCAUGACAAAGGUGUUCU-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 9a). 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. 9b). 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 9b).

The program then calculates the number of white pixels for every row in the image, as shown in Fig. 9c, 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:

$$S = \frac{A_M}{A_S}.$$  

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. 9d. 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 $N_{\text{gene}}$ is the total number of genes in the screen, and $2N_{\text{gene}}$ is the total number of siRNAs. For a given cutoff score $S_0$, $N_{\text{top}}$ siRNAs are hit picked, among them $2N_{\text{two}}$ siRNAs provide double confirmation for $N_{\text{two}}$ genes. If one randomly selects $N_{\text{top}}$ siRNAs out of a pool of size $2N_{\text{gene}}$, the odds of having at least $2N_{\text{two}}$ siRNAs provide double-confirmation hits can be calculated as:

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

In this screen, we chose a hit rate of 5%, $S_0 = 0.362$, we obtained 23 double-confirmed genes. I.e., $N_{\text{gene}} = 4,766$, $N_{\text{top}} = 574$, $N_{\text{two}} = 23$, the $P$ value is essentially zero ($1.0 \times 10^{-150}$). 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 cm² 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 RNaseasy 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 spectrum 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 nitrocellulose 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.

Supplementary figure 1. Automated wound-healing assay device. (1) Sterilized pipette tips are inserted into an aluminum block and suspended on a vertical tracking arm. The tip block is raised against a top plate to stabilize the tip position and prevent any movement upon scratching. 384-well clear bottom tissue culture plates 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. (2) With the pipette tip block engaged, the platform is shifted approx. 3mm (well diameter = 3.70 mm) by hydraulic pressure, resulting in uniform "scratches" in each of the 384 wells. Following scratching, the tip block is raised up from the plate, and the plate holder returns to the start position to allow manual plate switching by the user.
Supplementary figure 2. Reproducibility of the 384-well scratch assay. The quantitative migration score of each well of a 384-well plate was first averaged across three replicate plates. Scores from each individual run were then compared to the average (represented by the different colors in "single score"), resulting in r^2 values > 0.87 for all comparison.