(clone 41), p38 MAPK (pT180/pY182) phospho-specific antibody (clone 36), p38α antibody (clone 27), MKP2 antibody (clone 48) and pan-JNK/SAPK1 antibody (clone 37), from BD Transduction Laboratories (San Jose, CA, USA); MKP-1 antibody (C-19), from Santa Cruz Biotechnology (Santa Cruz, CA, USA); α-tubulin antibody (clone B-5-1-2) and MAP kinase antibody, from Sigma; phospho-SEK1/MKK4 (Ser254/Thr261) antibody and phospho-MKK7 (Ser271/Thr275) antibody, from Cell Signaling Technology (Danvers, MA, USA); swine horseradish peroxidase (HRP)-linked anti-rabbit Ig, from DAKO (Glostrup, Denmark); and sheep HRP-linked anti-mouse Ig, from GE Healthcare UK Ltd (Amersham, UK). Plasmid pcMKP1 was generated from *Homo sapiens* dual-specificity phosphatase 1 cDNA, MGC clone (ID 4794895) purchased from Invitrogen (Carlsbad, CA, USA). The MGC clone had been cloned into pBluscriptR. This clone was digested with *Ava*I, treated with T4 DNA polymerase, ligated to the pcDNA 3.1 mammalian expression vector (Invitrogen) prepared by digestion with *Eco*RV and treated with calf intestinal phosphatase to produce pcMKP1. Plasmid DNA was prepared by standard techniques (Qiagen Plasmid Midi Kit). pBabePuro, a puromycin-resistant vector, was kindly provided by K. Shuai (UCLA, USA). pcDL-SRα296JNK2(VPF), a dominant-negative JNK expression vector, was kindly donated by E. Nishida (Kyoto University, Japan).

## Cell culture and transfection

Human non-small cell lung cancer cell line PC-9 was cultured to subconfluence in RPMI-1640 medium supplemented with 5% fetal calf serum and used for all of the experiments. PC-9 cells were plated 24 h before transfection and co-transfected with 8.5 μg of pcDL-SRα 296JNK2(VPF) or pcMKP-1 and 1.5 μg of pBabePuro by using the Lipofectamine reagent, and the transfected cells were selected by exposure to 2.5 mg of puromycin (Sigma) per mL of medium for 3 weeks. Empty vector and pBabePuro were used for co-transfection as a negative control. The expression of JNK protein and MKP-1 protein were verified by immunoblot analysis using anti-(pan-JNK/SAPK1 aa264–415) and anti-(MKP-1) (Santa Cruz Biotechnology), respectively.

### Determination of cell viability

The anti-proliferative effect of AG1478 on PC-9 cells was assessed by using a Cell Counting Kit-8 (DOJIN, Kumamoto, Japan) according to the manufacturer's instructions. The Cell Counting Kit-8 is a colorimetric method in which the intensity of the dye is proportional to the number of the viable cells. Briefly, 200 μL of a suspension of PC-9 cells was seeded into each well of a 96-well plate at a density of 2000 cells·well$^{-1}$. After 48 h, the culture medium was replaced with 100 μL of AG1478 solution at various concentrations. After incubation for 48 h at 37 °C, 10 μL of WST-8 solution was added to each well, and the cells were incubated for a further 40 min at 37 °C. $A_{450}$ was measured using a Bio-Rad microplate reader model 550. Each experiment was performed by using six replicate wells for each drug concentration and was carried out independently three times.

## Preparation of cellular lysates and immunoblotting

Preparation of cellular lysates and immunoblotting were performed as described previously [57]. Briefly, cells were lysed with buffer A (20 mM Tris/HCl, pH 7.4, containing 137 mM NaCl, 2 mM EGTA, 5 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, 100 μg·mL$^{-1}$ phenylmethanesulfonyl fluoride, 1 μg·mL$^{-1}$ pepstatin A, 1 μg·mL$^{-1}$ *p*-toluenesulfonyl-L-arginine methyl ester, 2 μg·mL$^{-1}$ leupeptin, 1 mM sodium orthovanadate, 50 mM sodium fluoride and 30 mM $Na_4P_2O_7$). Lysates were then incubated on ice for 30 min, and the insoluble material was cleared by centrifugation. Samples were normalized for protein content and separated by SDS/PAGE, after which they were transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) for immunoblotting with antibodies.

### Caspase 3 activity assay

Caspase activity was assayed as described previously [57]. Briefly, cells were lysed with buffer A, and the protein concentration in each sample was adjusted to 100 μg·50 μL$^{-1}$ of buffer A. Fifty microliters of 2× Reaction Buffer (0.2 M Hepes/NaOH, pH 7.4, containing 20% sucrose, 0.2% Chaps and 1 mM dithiothreitol) was added to each sample, which was then incubated with Z-DEVD-AFC substrate (50 μM final concentration) at 37 °C for 1 h. The samples were read in a fluorometer (VersaFluor; Bio-Rad) equipped with a 340–380 nm excitation filter (EX 360/40) and 505–515 nm emission filter (EM 510/10).

## JNK assay

PC-9 cells were cultured in RPMI-1640 supplemented with 5% fetal calf serum at a density of $6.0 \times 10^5$ per 100 mm dish for 2 days and then assayed for JNK activity. JNK assays were performed by using a SAPK/JNK Assay kit (Cell Signaling Technology) according to the manufacturer's specifications. In brief, after various times of treatment with AG1478, adherent cells and floating cells were harvested by centrifugation and washed once in NaCl/$P_i$. Subsequently, the cells were lysed with lysis buffer (consisting of 20 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 nM

deltamethrin, 180 nM nodularin, 100 $\mu g \cdot mL^{-1}$ phenylmethanesulfonyl fluoride, 25 $\mu g \cdot mL^{-1}$ aprotinin, 25 $\mu g \cdot mL^{-1}$ leupeptin and 25 $\mu g \cdot mL^{-1}$ pepstatin), and scraped into microcentrifuge tubes. Extracts were prepared by sonicating each sample on ice (BRANSON SONIFIER 250, Danbury, CT, USA), and insoluble material was removed by microcentrifugation. Soluble fractions were mixed with 2 μg glutathione *S*-transferase–c-Jun (1–89) agarose beads (Cell Signaling Technology) and rotated overnight at 4 °C. JNK–c-Jun complexes were collected and washed with lysis buffer followed by kinase buffer, consisting of 25 mM Tris/HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM Cleland's reagent, 0.1 mM $Na_3VO_4$ and 10 mM $MgCl_2$. The *in vitro* kinase reaction was initiated by the addition of kinase buffer containing 100 μM ATP, samples were incubated at 30 °C for 45 min, and reactions were terminated by the addition of SDS sample buffer and heating to 95 °C for 5 min. Phosphorylated c-Jun was detected by western blotting using a phospho-specific c-Jun antibody (Cell Signaling Technology).

### Hoechst- PI staining

For the study of nuclear morphologic changes induced by AG1478, PC-9 cells were seeded on coverslips, grown to sub-confluence, and treated with AG1478 for the desired times. After fixation with formalin solution, the cells were stained with 10 μM Hoechst33342 and 10 μM PI in 5% fetal calf serum/RPMI. Coverslips were mounted on slides by using Dakocytomation Fluorescent Mounting Medium (DAKO) and observed under a fluorescence microscope (Axioskop; Carl Zeiss, Jena, Germany).

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