Supplementary information on materials and methods (MS-word format)

Plasmids

The pCBASce (I-*Sce*I expression plasmid) plasmid was obtained from Dr. Maria Jasin, the Memorial Sloan-Kettering Cancer Center, USA. The expression plasmids to express Flag-tagged wild-type and D176N kinase-dead mutant LKB1 described previously (Setogawa et al 2006) were obtained from Dr. Tetsu Akiyama, Institute for Molecular and Cellular Biosciences, The University of Tokyo. The pEGFP-LKB1 and pEGFP-LKB1-N, -C1, and C2 plasmids were constructed by subcloning corresponding cDNA fragments amplified by PCR from the Flag-LKB1 plasmids into the pEGFP-C1 vector (BD Bioscience Clontech, Mountain View, CA) to express LKB1 polypeptides as fusions to the C-terminus of the EGFP polypeptides. Plasmid DNAs were propagated in and purified from DH10B E. *coli* cells (Invitrogen, Carlsbad, CA).

Cell lines

H1299 human lung cancer cell lines were obtained from Dr. John D. Minna, UT Southwestern Medical Center, USA. These cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. A H1299 clone, H1299dA3-1#1, with stably retaining a plasmid DNA containing two recognition sites for *I-Sce*I endonuclease (Jasin 1996) in a chromosome DNA was recently described (Ogiwara et al 2011).

Antibodies

Antibodies against LKB1 raised in mouse (mLKB1: sc-32245) or raised in rabbit (rLKB1: sc-28788), KU70 (sc9033) and KU80 (sc-9034) were purchased from Santa Cruz biotechnology (San Diego, CA). Other antibodies used were those against γH2AX (#05-636) from Upstate, BRM (ab15597), 53BP1 (ab36823) and AMPK2 (ab3760) from Abcam (Cambridge, MA), phospho-S36-H2B from ECM Bioscience (HP4331) and β-actin (A 5441) from Sigma (St Louis, MO). An antibody against mouse IgG conjugated with Alexa488 (A11029) and that against rabbit IgG conjugated with Alexa568 (A11011) (Molecular Probes/Invitrogen) were also used.

siRNAs

siRNAs for LKB1 were purchased from Invitrogen (siLKB1#1, 12938-048) and Santa Cruz (siLKB#2, sc-35816). siRNA for BRM (sc-29831) were also purchased from Santa Cruz. siRNAs for KU70, KU80, RAD52 and AMPK2were purchased from Dharmacon (ON-TARGETplus SMARTpool, Yokohama, Japan). We also used another set of siRNA (QIAGEN SI02758595 and SI02758602) for AMPK2 in Supplementary Fig. S3. Non-targeting siRNAs were also purchased from the same suppliers; Invitrogen (VHS50414), Santa Cruz (sc-35816) and Dharmacon (ON-TARGETplus SMARTpool).

Western blot analysis

Cells were harvested, washed with PBS and lysed in a lysis buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1 % SDS and 0.1 % Na deoxycholate) containing 1 mM PMSF, proteinase inhibitor cocktail and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). After 30 min of incubation on ice, the samples were centrifuged for 10 min at 14,000 rpm at 4°C. Supernatants were

mixed with SDS sample buffer, boiled and subjected to SDS–PAGE electrophoresis followed by blotting to a PVDF membrane. The membranes were blocked overnight with TBS containing 0.1% Tween 20 and 0.5% BSA, and probed with primary antibodies. After washing with TBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies and treated with an enhanced chemoluminescence reagent (GE Healthcare, Tokyo, Japan).

NHEJ assay

0.8 μg of pCBASce plasmid (i.e., *I-Sce*I expression plasmid) DNA was introduced by transfection with Lipofectamine 2000 reagent into 1.5 x 10^5 H1299dA3-1#1 cells pre-transfected with siRNA for 48 hr using Lipofectamin RNAiMAX. For FACS analysis, cells were harvested by trypsinization, washed with PBS, and applied to the FACS caliber apparatus (Beckton Dickinson, Franklin Lakes, NJ). EGFP positive cells were counted by using the Cellquest software. For quantitative PCR analysis, genomic DNA was purified from cells by an Illustra Tissue and Cells GenomicPrep Mini Spin kit (GE Healthcare), and 20 ng of DNA were subjected to quantitative PCR to quantify the freeform of uncut and joined DNAs using an ABI 7900HT fast real-time PCR system analyzer (Applied Biosystems, Foster City, CA). PCR was performed for 45 cycles consisting of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec and extension at 72 °C for 30 sec by using a QuantiTect Probe PCR kit (Qiagen, Valencia, CA). *GAPDH* DNA was co-amplified and quantified as an internal control.

ChIP assay

2 x 10^6 H1299dA3-1#1 cells cells plated in 100 mm dishes were transfected with or without 24 ug of the pCBASce plasmid using Lipofectamine 2000. At 0 or 18 hr after transfection, cells were treated with 1% formaldehyde for 10 min at room temperature to crosslink proteins to DNA, followed by addition of glycine (0.125 M) to stop crosslinking. Cells were collected with a cell scraper and washed in ice-cold PBS. ChIP assay was performed using a ChIP IT Express Enzymatic kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Briefly, re-suspended cells were subjected to DNA shearing for 10 min at 37°C, followed by addition of EDTA to stop the reaction. A portion of the samples was set aside for input control, while the remaining portion was subjected to immunoprecipitation performed at 4°C overnight. The crosslinks were reversed in a reverse cross-linking buffer for 15 min at 95°C, and the samples were de-proteinized for 1 hr at 37°C with Proteinase K. Aliquots of the samples were subjected to quantitative PCR using a described primer and probe set (Ogiwara et al 2011). and the ABI 7900HT fast real-time PCR system analyzer (Applied Biosystems). *GAPDH* DNA was co-amplified as an internal control of quantification. PCR was performed for 45 cycles consisting of denaturing at 95°C for 30 sec, annealing at 57°C for 30 sec and extension at 72 °C for 30 sec using a QuantiTect Probe PCR kit (Qiagen). Fractions of DNAs of test loci and the *GAPDH* locus crosslinked to proteins were calculated based on the quantity of DNAs in the input control samples at each time point. Relative enrichment of a protein at a test locus was calculated as a value of the fraction of DNAs of a test locus divided by that of the *GAPDH* locus, and plotted as ratios against values of samples without *I-Sce*I treatment.

Comet assay

The neutral comet assay was performed using a Trevigen's Comet Assay kit (4250-050-K, Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. H1299 cells were exposed to 20 Gy of γ-irradiation and subjected to comet analysis at the indicated time points. After staining with SYBR green, comet images were captured by fluorescence microscopy. Average Comet tail moment (percentage of DNA in tail x tail length) was scored for three fields using the CometScore software (TriTek, Sumerduck, VA). The values represent the mean± SD.

Clonogenic assay

H1299 cells pre-transfected with siRNA for 48 hr were subjected to (or not) irradiation. The cells were plated onto 60 mm dishes, and surviving colonies were counted after 10 days.

Laser micro-irradiation

Laser micro-irradiation was performed using the FV-500 confocal scanning laser microscopy system (Olympus, Tokyo, Japan) as reported previously(Lan et al 2005, Lan et al 2010, Nakajima et al 2006). Briefly, cells in glass-bottomed dishes were micro-irradiated with a 405 nm laser (Olympus). To examine endogenous LKB1, p53BP1 and γH2AX proteins, micro-irradiated cells were fixed and permeabilized with MeOH and acetone for 10 min at -20°C at 15 min after micro-irradiation. The cells were subjected to an immuno-fluorescence assay using primary antibodies and secondary antibodies. Samples were stained with 90% glycerol/PBS containing 1 mg/ml of paraphenylenediamine and 0.5 mg/ml of 4',6'-diamidino-2-phenylindole (DAPI), and

were observed using the FV-500 confocal scanning laser microscopy system. For the analysis of EGFP-LKB1 or EGFP-KU70 protein localization, H1299 cells pre-transfected with siRNAs for 48 hr were subjected to transfection with EGFP-LKB1, EGFP-KU70 or EGFP-BRM expression plasmid for 24 or 48 hr and treatment with photosensitizer, 8-methoxypsoralen (8-MOP) for 4hr.

I-SceI-induced multiple DSBs

Construction and methods of this assay was described previously (Lan et al 2010). Briefly, U2OS cells with more than 200 copies of the I-*Sce*I site and tetracycline-responsive elements at a genomic site were established by transfection of the plasmid pTRE/I-*Sce*I site and Zeocin selection in U2OS cells. pCMV-NLS-I-*Sce*I were introduced by electroporation into the cells, which have been transfected with pCherry-TBP and plasmid for expression of EGFP-tagged gene. Just after electropotation, Tamoxifen was added at a final concentration of 1mM for translocation of Cherry-TA-ER protein to the nucleus.

Immunoprecipitation

Cells were lysed in NETN420 buffer (20 mM Tris-HCl, pH 7.5, 420 mM NaCl, 0.5% NP-40, 1 mM EDTA) containing 1 mM PMSF, 10 mM sodium butyrate, a proteinase inhibitor cocktail and a phosphatase inhibitor cocktail (Roche). Insoluble fractions were removed by centrifugation at 14,000 rpm and the soluble fraction was diluted by three times with NETN dilution buffer (20 mM Tris-HCl, pH 7.5, 0.1% NP-40, 1 mM EDTA). Anti-LKB1 antibody or control IgG was added to the soluble fractions and the mixture was incubated at 4°C overnight. Magnetic bead-conjugated second antibodies (Invitrogen) were added to the mixture and incubated at 4°C for another 1 hr.

Antibody-protein-bead complexes were purified and subjected to western blot analysis for BRM.

References

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