Supporting Information

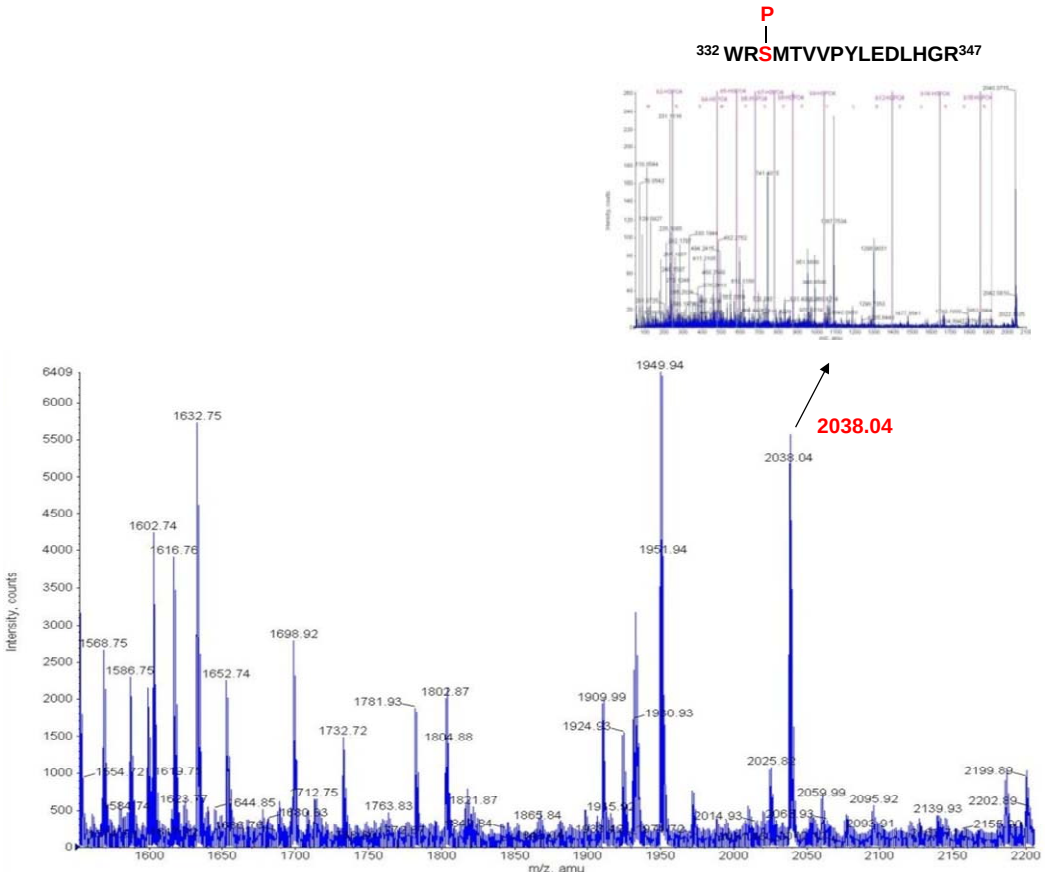
Material and Methods:

Mass spectrometry analysis. Protein bands were excised from coomassie brilliant blue-stained gels. After reduction with DTT and alkylation with iodoacetamide, the gel pieces were digested with trypsin and the peptide fragments desalted with C18 Ziptip (Millipore, Billerica, MA, USA). The tryptic mixture was mixed at 1:1 ratio with CHCA matrix and spotted onto a MALDI target. Mass spectra (MS) were acquired using a 4800 MALDI-TOF/TOF instrument (Applied Biosystems, Foster City, CA). External calibration was performed using Calmix 1 and 2 (Applied Biosystems) at a mass tolerance of 100 ppm. For MS analysis, laser intensity of 2500 was used and 8 sub-spectra with 50 shots each were acquired for every sample spot. Peak lists were created from the raw data by 4000 series Explorer™ software (v3.5, Applied Biosystems) using the following peak filter settings: mass range 900–4000 Da (MS), and minimal S/N 5. Keratin and trypsin autodigestion peaks were excluded. For MS/MS analysis, laser intensity of 3100 was used. The precursor tolerance was 0.2 Da and the MS/MS precursor resolution was set at 350. Twenty-five sub-spectra with 2500 shots were acquired for each sample spot with a metastable suppressor on. The MS/MS data were analyzed using GPS explorer software (v3.6, Applied Biosystems) and the MS/MS peptide identifications were achieved by database comparisons using an in-house MASCOT v2.1 (Matrix Science) searching engine. Trypsin was selected as enzyme taking into consideration of one missed cleavage site and variable protein modifications were allowed. For protein and peptide identification, the significant probability scores with a *p* value of less than 0.05 were accepted. All identified peptides were confirmed by manual *de novo* sequencing using the QSTAR XL hybrid quadrupole-TOF mass spectrometer (Applied Biosystems). The peptides were electrosprayed into the mass spectrometer with a distally applied spray voltage of 1.8 kV. MS/MS analysis was performed with one full-range mass scan (*m/z* 400–3000) followed by information-dependent data acquisition scan for the selected ion. MS/MS control, data acquisition, and spectral processing were carried out using Analyst and BioAnalyst™ software (Applied Biosystems). Three independent experiments were conducted using different batches of protein samples.

Supplementary Table 1. Sequences of primers used for site-directed mutagenesis

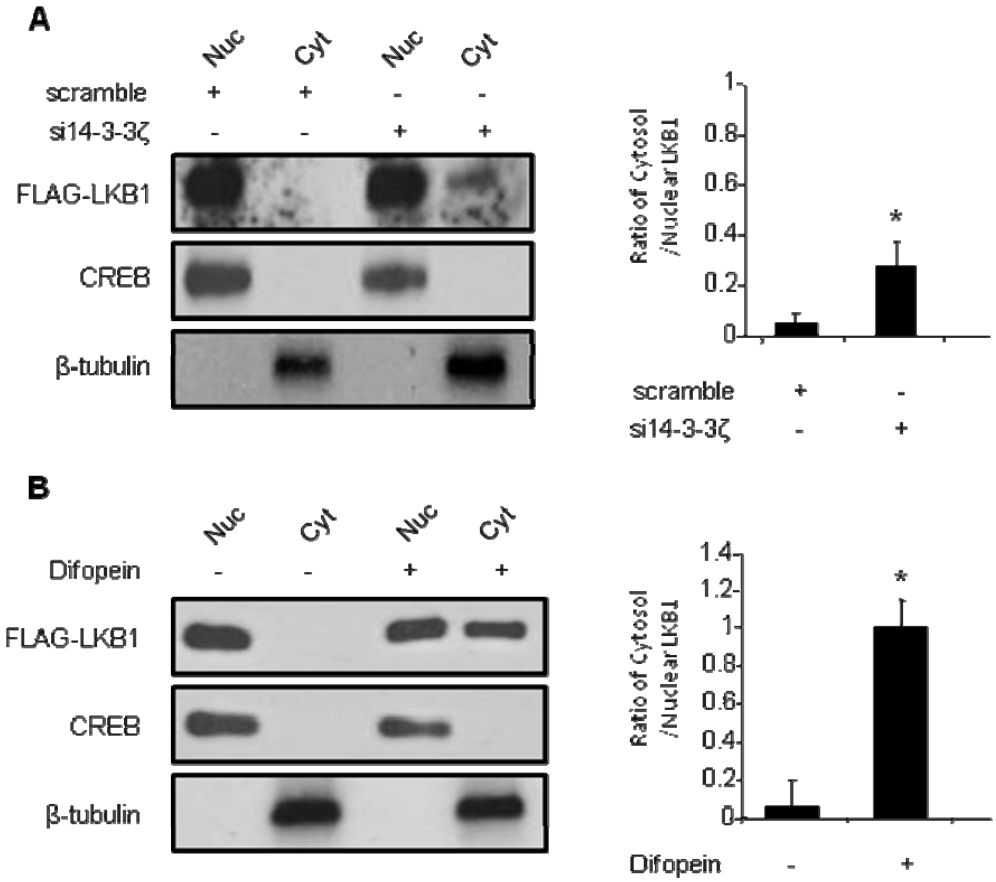
Mutants	Sequences
S334A	Forward: 5'AAGGACCGCTGGCGCGCTATGACTGTAGTGCCCTAC 3' Reverse: 5' GTAGGGCACTACAGTCATAGCGCGCCAGCGGTCCTT 3'
S334D	Forward: 5' AAGGACCGCTGGCGCGATATGACTGTAGTGCCCTAC 3' Reverse: 5' GTAGGGCACTACAGTCATATCGCGCCAGCGGTCCTT 3'

Supplementary Figure 1



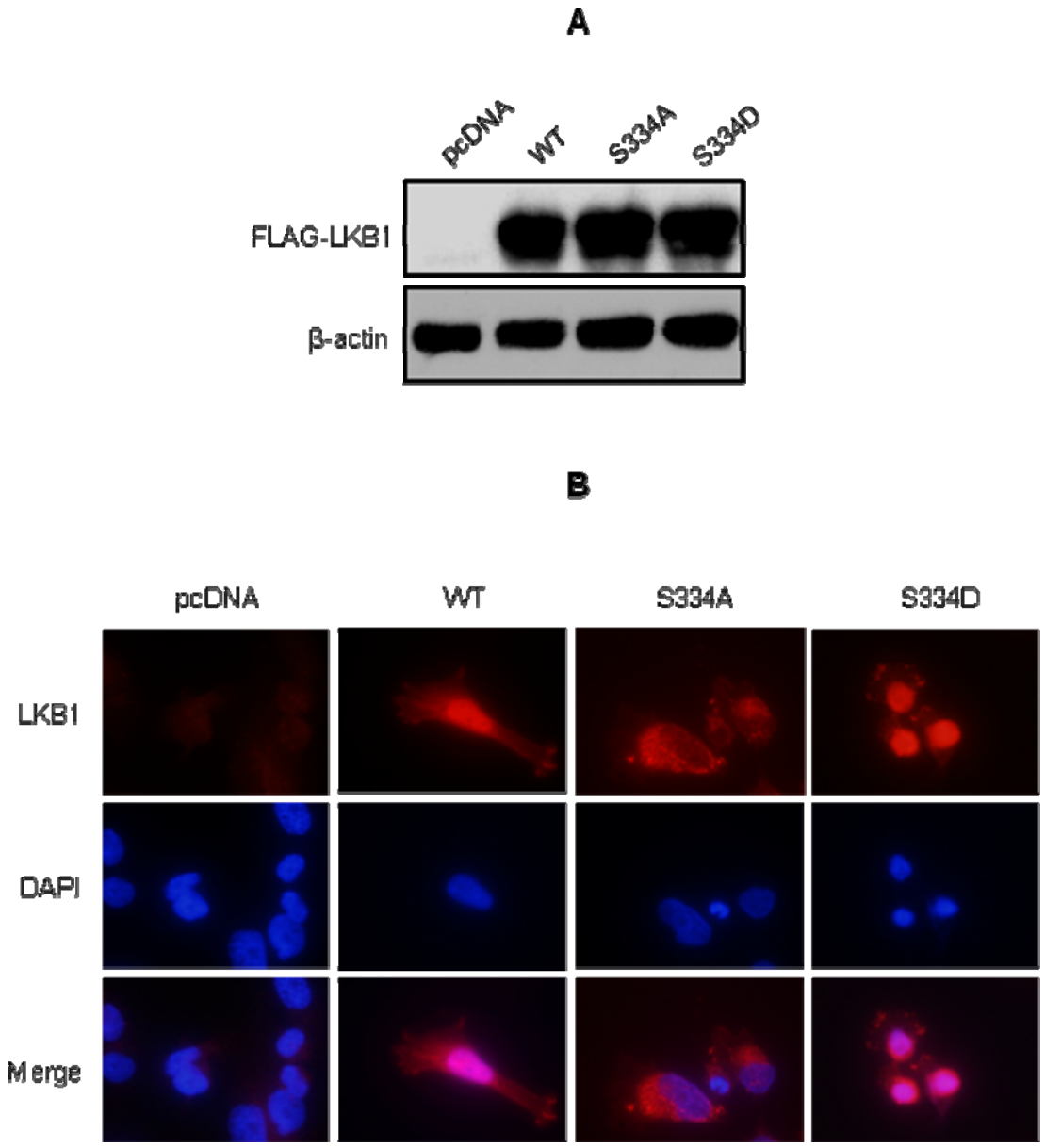
Supplementary Figure 1. Mass spectrometry and *de novo* sequencing analysis of tryptic peptides of LKB1 protein collected from the *in vitro* phosphorylation assay. Peptide mass finger printing was performed using a MALDI-TOF/TOF mass spectrometer and the differentially expressed peptides analyzed by tandem mass spectrometry using a QSTAR XL hybrid quadrupole-TOF mass spectrometer as described in Methods. The *de novo* sequencing results revealed a mono-phosphorylated peptide (MW: 2038.04 Da) containing the phosphorylated Ser334 residue.

Supplementary Figure 2



Supplementary Figure 2. 14-3-3 proteins are required for Akt-mediated nuclear accumulation of S334D mutant. (A). The effects of 14-3-3ζ knocking down on sub-cellular distribution of S334D were evaluated in the nuclear (Nuc) and cytosolic (Cyt) fractions of HEK293 cells stably overexpressing Flag-tagged LKB1 S334D mutant. Western blotting was performed using an anti-Flag antibody. (B). HEK293 cells stably overexpressing Flag-tagged LKB1 S334D mutant were transfected with or without difopein. The S334D contents in nuclear and cytosolic fractions were detected using an anti-Flag antibody. The relative ratios of the protein bands in Western blotting results were calculated and presented as the means ± SEM from at least three independent experiments. *, $P < 0.05$ vs corresponding controls.

Supplementary Figure 3



Supplementary Figure 3. Phosphorylation of LKB1 at Ser334 modulates its sub-cellular localization in MDA-MB-231 breast cancer cells. Stably-transfected MDA-MB-231 cells were established as described in Methods. (A). Western blotting was performed for measuring the protein expression levels of Flag-tagged wild type LKB1 and the two mutants (S334A and S334D) in the stably-transfected cells. (B). Intracellular localizations of the three types of LKB1 variants were analyzed by immunofluorescence staining using the anti-Flag antibody (red). The images were merged with those stained with DAPI (blue). Magnification, 400 ×.