SUPPLEMENTAL FIGURES 1-15

Rab7-mediated neuropilin-1 degradation by LKB1 inhibits angiogenesis in vivo

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Conflict of interest: The authors declare that no conflict of interest exists.

Patient	Age	Gender	Smoking Status
1	59	Male	10/d x 45 yrs
2	50	Female	no
3	59	Male	no
4	51	Male	no
5	49	Female	no
6	48	Male	20/d x 30 yrs
7	57	Male	20/d x 30 yrs
8	72	Male	20/d x 10 yrs

Supplementary table 1. Patient information
Information of lung adenocarcinoma specimens showing gender, age and smoking status of patients.

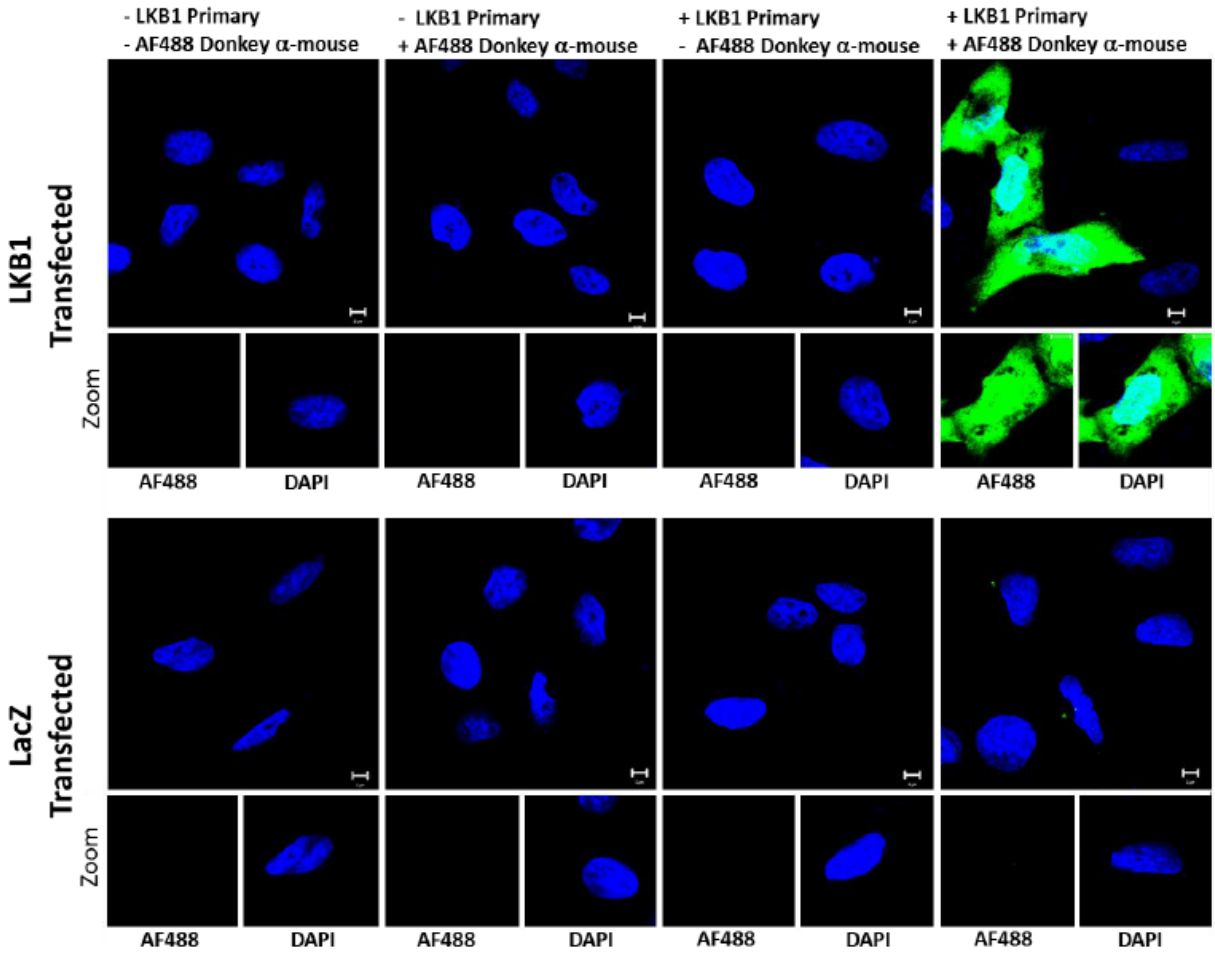
Table S1

Cell Line	Gender	Age	Stage	Tissue type
Healthy Cell Line				
MRC9	Female	*Fetal	Normal	Lung
Lung Cancer Cell Line				
A549	Male	58	NA	Carcinoma, lung
H1703	Male	54	I	Adenocarcinoma; Non-small cell lung
H1792	Male	50	IV	Adenocarcinoma
H1299	Male	43	NA	Carcinoma; Non-small cell lung
H1650	Male	27	IIIB	Adenocarcinoma; Bronchoalveolar carcinoma
H1975	Female	NA	NA	Adenocarcinoma; Non-small cell lung
H1734	Female	56	NA	Adenocarcinoma; Non-small cell lung

Source: ATCC, Manassas, VA 20108
*** NA= Information not available**
***Fetal: 15 weeks gestation**

Supplementary table 2. Information on commercially obtained lung ('normal' and cancer) cell lines
Cell lines were screened for protein and/or messenger expression levels of various targets, including, LKB1, NRP-1, VEGF and VEGFR2.

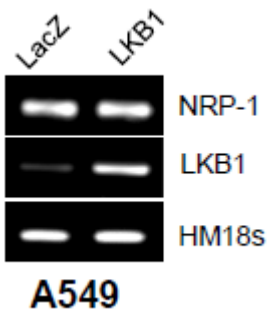
Table S2



Supplementary figure 1. Immunofluorescence detection of LKB1 expression upon transient transfection (24 hours) with LacZ or LKB1 (green) in A549 cells

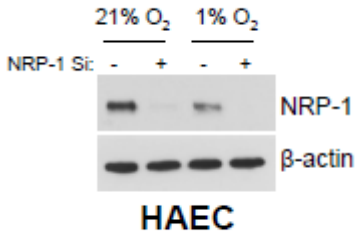
Appropriate controls with LKB1 antibody were used to exclude false-positive staining and demonstrate specificity of LKB1 expression in transfected cells. DAPI represents nuclear staining (Blue). Scale bar 5 μM.

Figure S1



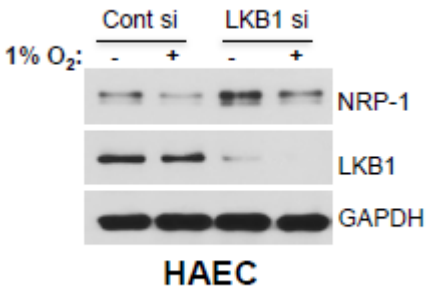
Supplementary figure 2. Messenger RNA levels of NRP-1 in A549 cells
Complementary DNA (cDNA) from LacZ or LKB1-transfected A549 cells were amplified by Real-time PCR using NRP-1, LKB1 or HM18s primers.

Figure S2



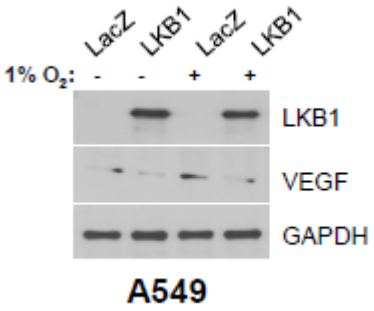
Supplementary figure 3. Immunoblot analyses following NRP-1 knockdown (siRNA) for 48 hours in human aortic endothelial cells (HAEC)
Cells were exposed to normoxia or hypoxia (3 hours) and lysates blotted for NRP-1 expression.

Figure S3



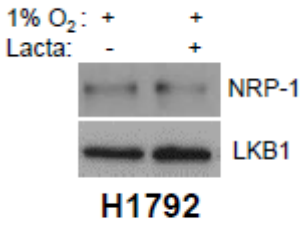
Supplementary figure 4. Effects of LKB1 and/or hypoxia on NRP-1 protein expression
LKB1 expression was silenced (siRNA) in HAEC prior to hypoxia treatment (3 hours). Immunoblot analysis was used to detect NRP-1 protein expression.

Figure S4



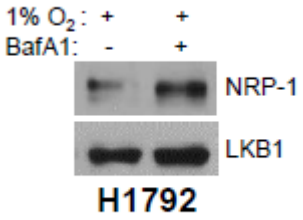
Supplementary figure 5. LKB1 inhibits VEGF expression
Under normal or hypoxia treatment (3 hours), VEGF protein expression was assessed by Western blot analyses in LacZ or LKB1-transfected A549 cells.

Figure S5



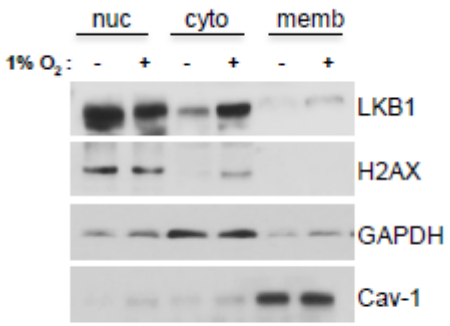
Supplementary figure 6. Proteosome inhibition fails to block LKB1-mediated abrogation of NRP-1
Attenuated NRP-1 protein expression was not rescued upon proteosome inhibition. Proteosome inhibition in H1792 cells with lactacystin (Lacta, 20 μM) in the presence of hypoxia (3 hours). Immunoblot analysis was used to detect NRP-1 expression.

Figure S6



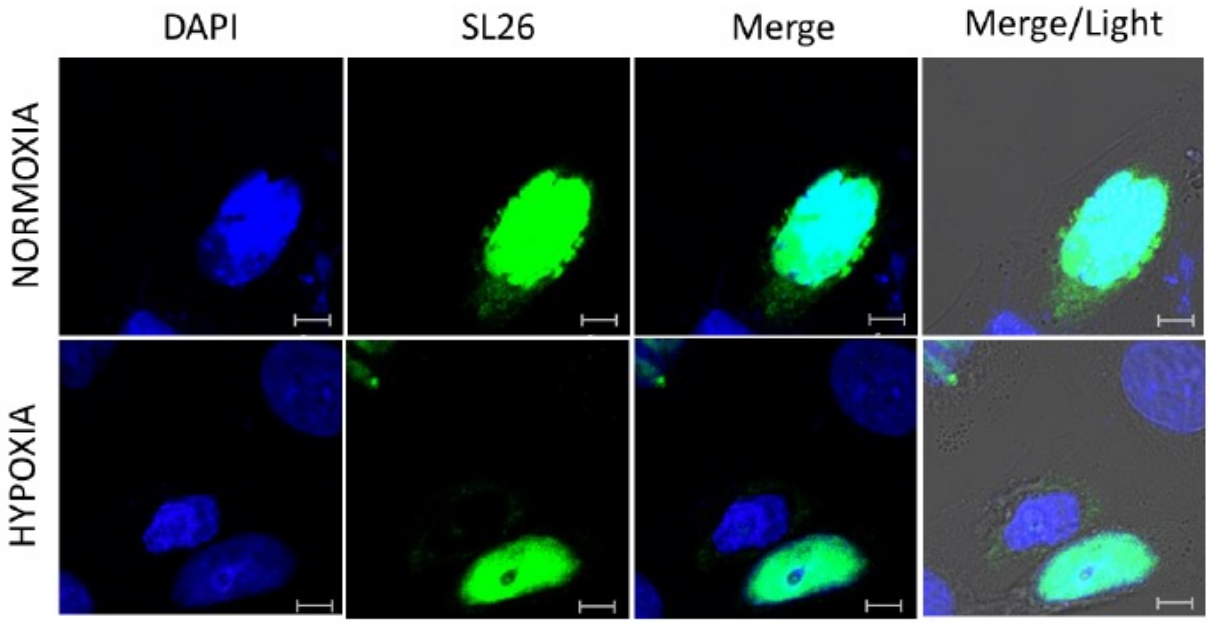
Supplementary figure 7. Lysosome inhibition correlated with rescued NRP-1 expression
In H1792 cells, inhibition of the lysosome degradation pathway with BafilomycinA1 (BafA1, 0.1 μM) in the presence of hypoxia (3 hours) and detection of NRP-1 expression by western blot.

Figure S7



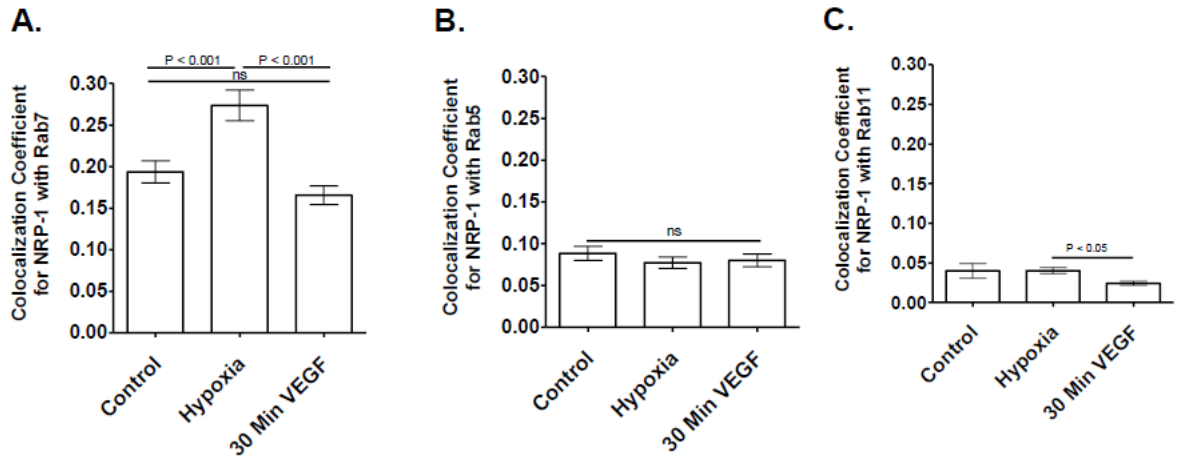
Supplementary figure 8. Hypoxia promotes nuclear LKB1 distribution to the cytosol
Lysates from membrane, cytosol and nuclear fractions of H1792 cells following normoxia or hypoxia (3 hours) were blotted for LKB1, and markers for different cell fractions.

Figure S8



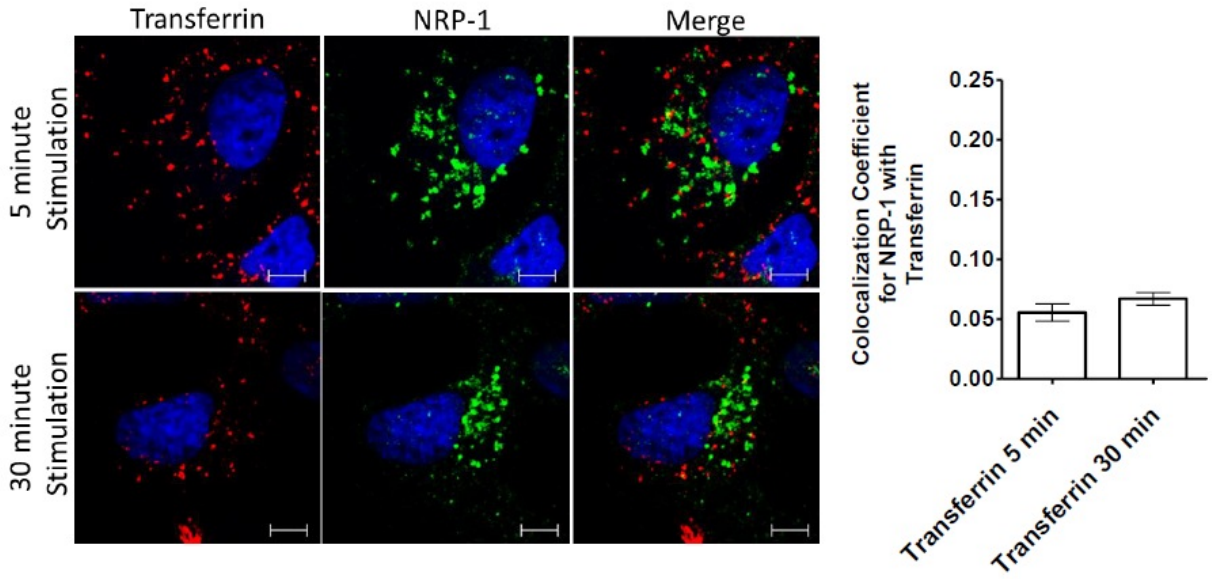
Supplementary figure 9. LKB1 SL-26 mutant is predominantly localized to the nucleus
Constitutively nuclear-localized LKB1 SL-26 mutant was transiently transfected into A549 cells, followed by hypoxia treatment for 3 hours. Immunofluorescence detection using LKB1 antibody (green). DAPI nuclear staining (Blue). Scale bar 5 μM.

Figure S9



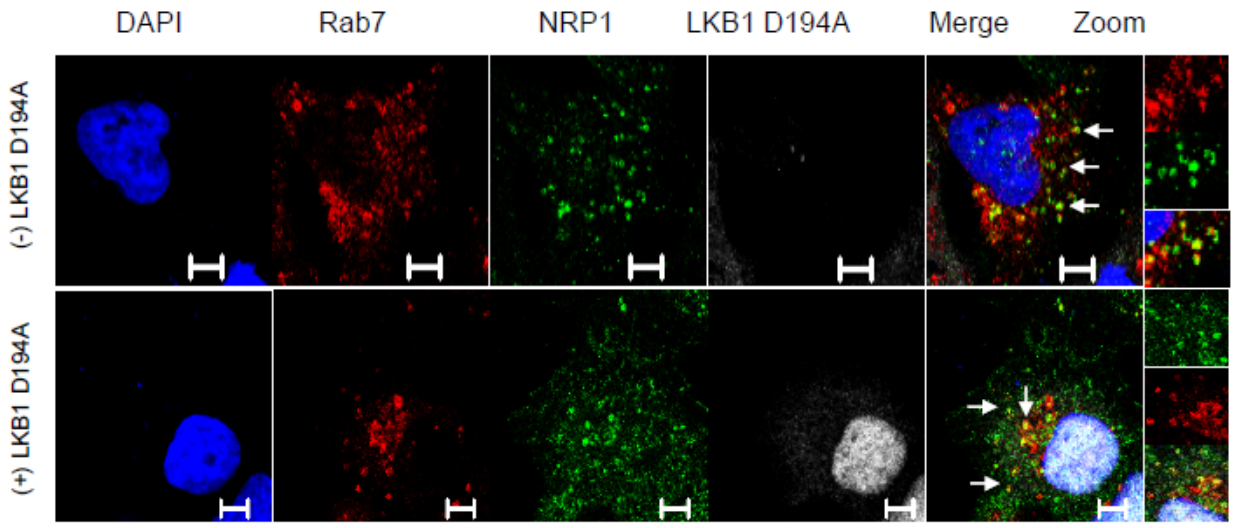
Supplementary figure 10. Hypoxia contributes to endosomal NRP-1 distribution
(**A**) Hypoxia promotes localization of internalized NRP-1 to Rab7-specific endosomes. Confocal microscopy was used to assess co-localization of NRP-1 (green) with Rab7 (red) in A549 cells following VEGF stimulation (25 ng/ml, 30 minutes), or hypoxia treatment (3 hours) in comparison with non-treated controls. (**B** and **C**) NRP-1 (green) localization with Rab5 (red) or Rab11 (red) vesicles was investigated under non-treated controls, VEGF or hypoxia conditions.

Figure S10



Supplementary figure 11. Exclusion of NRP-1 localization in the recycling pathway
Co-localization analysis in A549 cells using confocal microscopy between NRP-1 (green) and a recycling marker, rhodamin-labeled transferrin (red) at 5 or 30 minutes. DAPI represents nuclear staining (Blue). Scale bar 5 μM.

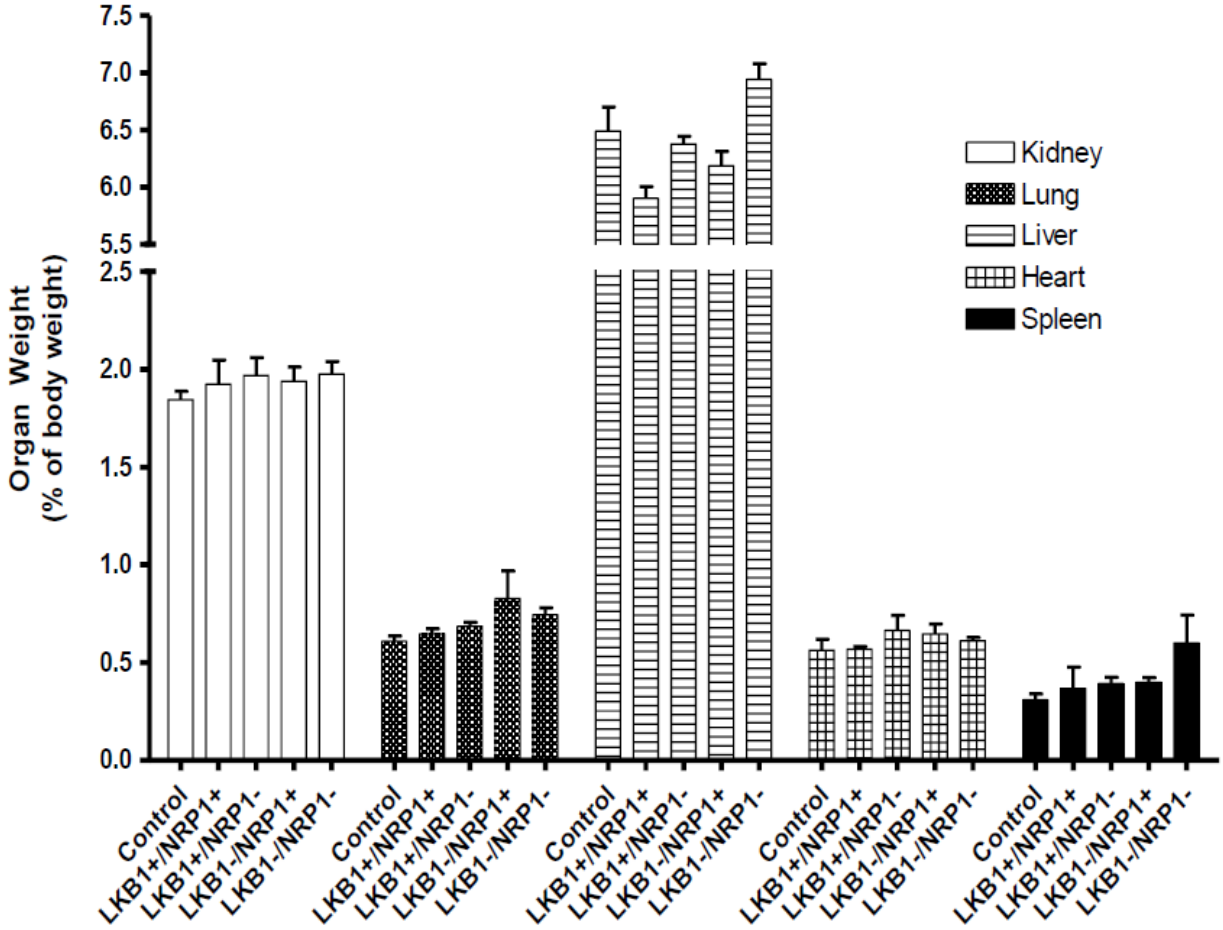
Figure S11



Supplementary figure 12. Co-localization of NRP-1 with Rab7 is not affected by the loss of LKB1 activity

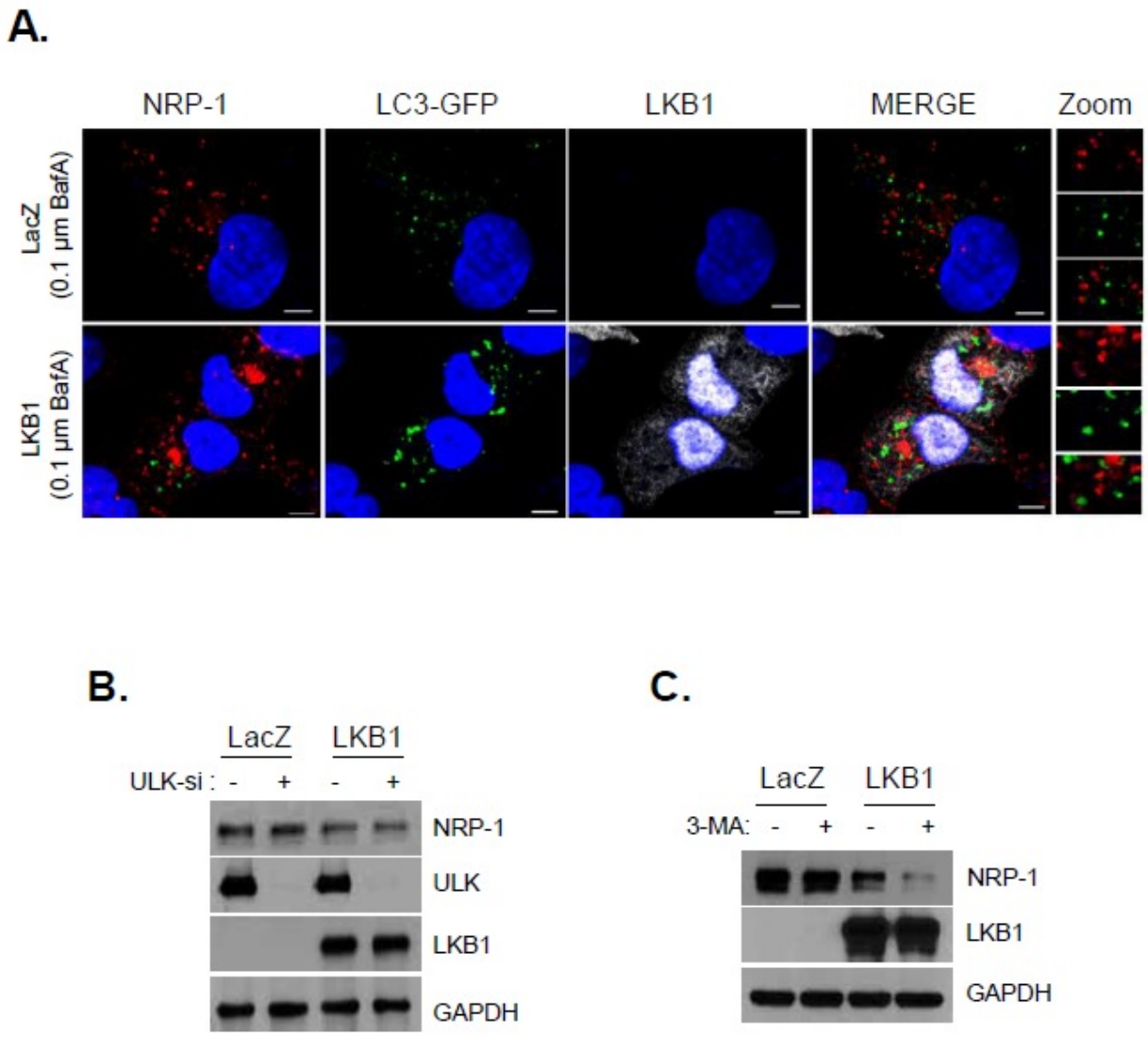
Co-localization of NRP-1 (green) with Rab7 (red) in A549 cells transiently transfected with kinase-inactive LKB1 D194A mutant (white). DAPI represents nuclear staining (Blue). Scale bar 5 µM.

Figure S12



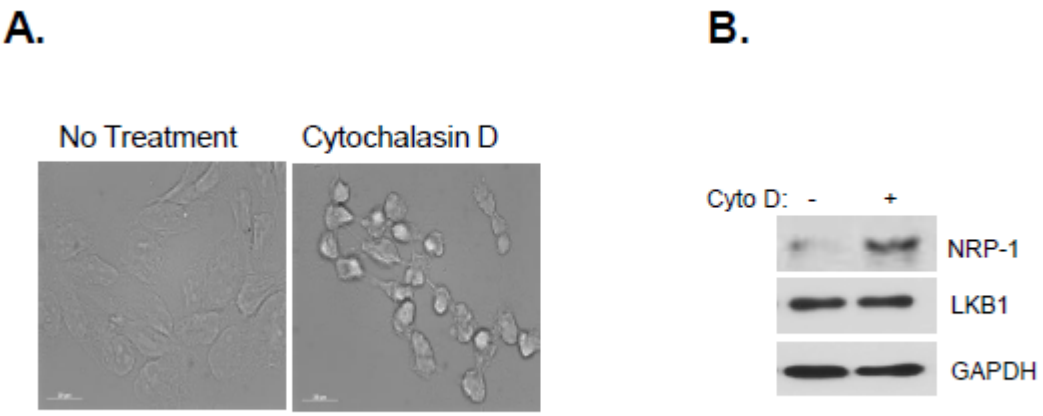
Supplementary figure 13. Selected mice organ weights
Mice organ weights were obtained from nude mice following subcutaneous implantation of LKB1 and/or NRP-1-expressing H1792 cells.

Figure S13



Supplementary figure 14. Autophagy is not involved in LKB1-mediated attenuation of NRP1
(**A**) Following lysosome inhibition with BafilomycinA (BafA), co-localization of NRP-1 (red) with autophagy marker LC3 (LC3-GFP, green) was assessed by confocal microscopy in A549 cells transiently transfected with LacZ or LKB1 (white). DAPI represents nuclear staining (Blue). Scale bar 5 μM. (**B**) Knockdown (siRNA) of autophagy regulator, ULK was undertaken in A549 cells transiently transfected with LacZ or LKB1, and NRP-1 protein expression was detected by western blot analysis. (**C**) Chemical inhibition of autophagy using 3-methyladenine (3-MA; 5 mM for 2 hours) in A549 cells transiently transfected with LacZ or LKB1.

Figure S14



Supplementary figure 15. Disruption of actin cytoskeleton rescues NRP-1 protein expression
(**A** and **B**) Actin cytoskeleton of A549 cells were disrupted using cytochalasin D (cytoD, 1 µg/ml for 30 minutes) and cell morphology visualized by light microscope (40x), and western blot detection of NRP-1 expression.

Figure S15