ULK1 inhibition overcomes compromised antigen presentation and restores antitumor immunity in LKB1-mutant lung cancer

Jihui Deng1, Aatish Thennavan2, Igor Dolgalev3, Ting Chen1, Jie Li3,4, Antonio Marzio3,4, John T. Poirier5, David H. Peng1, Mirna Bulatovic1, Subhadip Mukhopadhyay6, Heather Silver1, Eleni Papadopoulos1, Val Pyon1, Cassandra Thakurdin1, Han Han1, Fei Li1, Shuai Li1, Hailin Ding1, Hai Hu1, Yuanwang Pan1, Vajira Weerasekara7,8, Baishan Jiang9,10, Eric S. Wang9,10, Ian Ahearn11, Mark Philips11, Thales Papagiannakopoulos3,12, Aristotelis Tsirigos3, Eli Rothenberg4, Justin Gainor5, Gordon J. Freeman13, Charles M. Rudin14, Nathanael S. Gray15, Peter S. Hammerman13, Michele Pagano3,4,6, John V. Heymach17, Charles M. Perou2, Nabeel Bardeesy3,7,8 and Kwok-Kin Wong1

Inactivating mutations in LKB1/STK11 are present in roughly 20% of nonsmall cell lung cancers (NSCLC) and portend poor response to anti-PD-1 immunotherapy. Unexpectedly, we found that LKB1 deficiency correlated with elevated tumor mutational burden (TMB) in NSCLCs from nonsmokers and genetically engineered mouse models, despite the frequent association between high-TMB and anti-PD-1 treatment efficacy. However, LKB1 deficiency also suppressed antigen processing and presentation, which are associated with compromised immunoproteasome activity and increased autophagic flux. Immunoproteasome activity and antigen presentation were restored by inhibiting autophagy through targeting the ATG1/ULK1 pathway. Accordingly, ULK1 inhibition synergized with PD-1 antibody blockade, provoking effector T-cell expansion and tumor regression in Lkb1-mutant tumor models. This study reveals an interplay between the immunoproteasome and autophagic catabolism in antigen processing and immune recognition, and proposes the therapeutic potential of dual ULK1 and PD-1 inhibition in LKB1-mutant NSCLC as a strategy to enhance antigen presentation and to promote antitumor immunity.

LKB1-deficient NSCLC presents a clinical challenge, with notably worse overall outcomes compared to other NSCLC subsets and notable resistance to current immunotherapies as demonstrated in both patients and genetically engineered mouse models (GEMMs)1,2. LKB1 is frequently comutated with KRAS in NSCLC, and these tumors (designated KL) display an objective response rate to immune checkpoint inhibition of <10%, whereas KRAS/TP53-mutant NSCLC (designated KP) show a >30% objective response rate1,4. However, both KL and KP NSCLCs often arise in patients with a history of heavy smoking, which drives increased levels of nonsynonymous mutations, a feature that in NSCLC in patients with a history of heavy smoking, which drives increased

LKB1 mutation increases tumor mutational burden

Previous studies of human lung cancers not stratified by smoking status revealed comparably high tumour mutational burden (TMB) in KL and KP tumors. We used spontaneous Kras-driven NSCLCs arising in GEMMs to more readily resolve the impact of Lkb1 loss on TMB. We found that Lkb1 comutation was associated with fivefold increase in TMB compared with Trp53 comutation in both cell lines and lung nodules generated from GEMMs (Fig. 1a and Extended Data Fig. 1a). This effect was attributable to Lkb1 status not Trp53 status, since mouse cell lines with comutated Lkb1,

1Division of Hematology & Medical Oncology, Laura and Isaac Perlmutter Cancer Center, New York University Langone Medical Center, New York, NY, USA. 2Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA. 3Laura and Isaac Perlmutter Cancer Center, NYU Langone Medical Center, New York University, New York, NY, USA. 4Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine, New York, NY, USA. 5Molecular Pharmacology Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA. 6Department of Radiation Oncology, Laura and Isaac Perlmutter Cancer Center, New York University School of Medicine, New York, NY, USA. 7Center for Cancer Research, Massachusetts General Hospital, Boston, MA, USA. 8Department of Medicine, Harvard Medical School, Boston, MA, USA. 9Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA. 10Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA, USA. 11Department of Pathology, New York University School of Medicine, New York, NY, USA. 12Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA. 13Memorial Sloan Kettering Cancer Center, New York, NY, USA. 14Department of Chemical and Systems Biology, Chem-H and Stanford Cancer Institute, Stanford School of Medicine, Stanford University, Stanford, CA, USA. 15Howard Hughes Medical Institute, New York University School of Medicine, New York, NY, USA. 16Departments of Thoracic/Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA. 17Departments of Thoracic/Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA. E-mail: Kwok-Kin.Wong@nyulangone.org
**Fig. 1 | Increased TMB in LKB1-mutant NSCLC tumors.** a, Comparison of TMB of nonsynonymous mutation using cell lines derived from \textit{Kras}\textsuperscript{G12D}\textit{Lkb1}\textsuperscript{fl/fl} (KL) or \textit{Kras}\textsuperscript{G12D}\textit{Trp53}\textsuperscript{R172P} (KP) NSCLC GEMMs lung nodules. Left panel, whole-exome sequencing (WES) of total nonsynonymous mutations per megabase (Mb) (KP, \(n = 6\) cell lines; KL, \(n = 5\) cell lines) (mean \(\pm\) s.d., unpaired two-sided Student’s t-test). Right panel, RNA-seq for the total number coding region mutants of each cell line (KP, \(n = 5\) cell lines; KL, \(n = 5\) cell lines) (mean \(\pm\) s.d., two-sided Student’s t-test). b, TMB score from patients with NSCLC with or without \textit{Lkb1} mutation (HS.LKB1wt \(n = 682\); HS.LKB1mut \(n = 263\); NS.LKB1wt \(n = 510\); NS.LKB1mut \(n = 10\) patients each group: NS, never smoked; HS, heavy smoker) (Mann–Whitney test of \(\log_{10}\)-transformed TMB, two-tailed). Boxes show the 25th to 75th percentiles with the line showing the median, and whiskers show the 10–90th percentiles. The Shapiro–Wilk test was used for normality test of transformed TMB score, two-tailed). c, Top suppressed biological process pathways from a patient with the TCGA KRAS/LKB1 mutant compared with patients with the KRAS/TP53 mutant. x-axis, –\(\log_{10}\)(q) value (Bonferroni) between the two groups (KP, \(n = 22\); KL, \(n = 19\) patients each group). d, Number of coding indel variants from either KP or KL cell lines of either short (1–2 basepairs (bp)) or long (\(\geq 3\) bp) indels (KP, \(n = 6\); KL, \(n = 5\) cell lines each group) (mean \(\pm\) s.d., unpaired two-sided multiple t-test, FDR < 0.05). e, GSEA of HR repair of KL and KP cells (KP, \(n = 5\); KL, \(n = 5\) cell lines each group).

\textit{Kras} and \textit{Trp53} (KL) had comparable TMB to KL lines (Extended Data Figs. 1b,c). Similar to \textit{LKB1}-mutant (LKB1mut) tumors from patients with cancer and KL GEMMs, KL cell lines generated from GEMMs showed a mixture of adenocarcinoma and squamous carcinomas phenotypes (Extended Data Fig. 1d).

Based on these findings in GEMMs, we analyzed a cohort of patients with NSCLC who had a known \textit{Lkb1} mutational status, smoking history and TMB measurement\textsuperscript{31}. Notably, \textit{LKB1} mutations in patients who had never smoked (NS.LKB1mut) were associated with a twofold increased TMB compared to a group who had never smoked with wildtype \textit{LKB1} (NS.LKB1wt). TMB was comparable in \textit{LKB1}-mutant (LKB1mut) and \textit{LKB1}-wildtype (LKB1wt) NSCLCs in patients who were heavy smokers, who have a higher TMB in general\textsuperscript{44} (Fig. 1b). As expected, both LKB1wt and LKB1mut heavy smoking groups had increased frequency of \textit{Kras} mutations compared to the groups who had never smoked. Furthermore, the \textit{TP53} mutation percentage was higher in the LKB1wt group than the LKB1mut group for both patients who had never smoked and heavy smokers, consistent with previous studies (Extended Data Fig. 1e).

Despite suppressive immune response pathways in KL tumors (Fig. 1c and Extended Data Fig. 1f), KL tumors also had an increase in total number of coding insertion–deletions (indels) and nonsynonymous single-nucleotide variations (Fig. 1d and Extended Data Fig. 2a), although the percentage of indels among total nonsynonymous mutations was slightly decreased in KL (Extended Data Fig. 2b). Indels have been implicated in driving immunogenic responses due to the generation of immunogenic neoantigens\textsuperscript{20,26}. \textit{LKB1}-mutant NSCLCs show increases in COSMIC mutational signatures 20 and 26, which are associated with frequent small insertions and deletions\textsuperscript{17}. Conversely, they show a decrease in signature 3, which suggests failure of DNA double-strand break (DSB) repair by homologous recombination (HR)\textsuperscript{18}, and signatures 7 and 22, which correlate with defective nucleotide excision repair\textsuperscript{19} (Extended Data Fig. 2c,d). Consistent with increased nonsynonymous mutations, gene set enrichment analysis (GSEA) demonstrated the association between \textit{LKB1} mutation and both the replication-dependent and -independent HR repair pathways (Fig. 1c and Extended Data Fig. 2e), but not other DNA repair-related pathways or others (Extended Data Fig. 2f). Collectively, these analyses suggest a link between \textit{LKB1} inactivation and defective nucleotide excision repair and HR.

To quantify both HR and nonhomologous end joining repair (NHEJ) ratios in KL lines, we used a green fluorescent protein- (GFP)-based reporter system\textsuperscript{20,21}. KL cells showed greatly suppressed HR and NHEJ compared with KP lines (Fig. 2a). KL triple-mutant lung cancer cells also showed reduced HR but not NHEJ (Extended Data Fig. 3a). HR plays a dominant role during the late S/G2 and G1/early S cell cycle phases to repair DSBs and is typically nonmutagenic as compared with NHEJ\textsuperscript{22,23}. HR
Fig. 2 | LKB1 is critical for maintaining HR during DNA DSB repair.  

a, Flow analysis of HR (left) and NHEJ (right) rate in both KP and KL cells through quantifying repaired HR or NHEJ reporter GFP+ cells among total I-SceI expressing BFP+ cells. Left, representative flow panels; right, quantification of HR (KP n = 3; KL n = 3 cell lines each group) or NHEJ (KP n = 3; KL n = 3 cell lines each group) percentage. Data are representative one of three independent experiments (mean ± s.d., two-tailed t-test, unpaired). b, Percentage of HR and NHEJ in KP and KL isogenic cells with wildtype or kinase-dead (KD) LKB1 overexpression. n = 3 independent experiments for each group (mean ± s.d., two-tailed t-test, unpaired). c, HR ratio changes in human NSCLC LKB1-mutant cell lines H23, A427 and H460 with empty vector (pBABE), LKB1 or KLB1-KD overexpression. n = 3 independent experiments for each group (mean ± s.d., two-tailed t-test, unpaired). d, Chromatin binding assay of either KP or KL cells with LKB1 overexpression after NCS induced DNA damage. Upper panel, chromatin fraction of bound proteins; lower panel, total soluble fraction of the proteins examined. Data represent one of two independent experiments. Blots are cropped and uncropped images can be found in the source data. e, Immunofluorescent staining of pH2AX and Rad51 foci at DNA DSB sites after NCS-induced damage in KP and KL cells with or without LKB1 overexpression. UT, untreated. NCS, NCS treated. Scale bars, 5 μm. Data are representative of three independent experiments. f, Quantification of Rad51 and pH2AX positive and negative cells as determined in e representative photos, and quantified as percentage among total cells. UT and NCS groups, KP n = 6, KL n = 6, KL + LKB1 n = 6 independent experiments (mean ± s.d., two-tailed t-test, unpaired).
was rescued by LKB1wt overexpression in KL lines, but not by kinase-dead LKB1 (LKB1-KD) (Fig. 2b, left). By contrast, neither LKB1wt or LKB1-KD reconstitution restored NHEJ in LKB1-deficient cells (Fig. 2b, right). These data indicate that LKB1 is important for maintaining homology directed repair but not NHEJ for DSBs. We further confirmed this phenomenon using human NSCLC cell lines. In particular, LKB1 overexpression increased HR, but not NHEJ levels, in the human LKB1-deficient lung cancer lines H23, A427 and H460 (Fig. 2c and Extended Data Fig. 3b). Thus, the increase in the HR signature in LKB1-deficient tumors correlates with a critical requirement for LKB1 kinase activity for HR-mediated repair of DSBs, whereas the increase in NHEJ signatures is not directly related to LKB1 function.

On a DSB, PARP1 activity and a H2AX phosphorylation (pH2AX) signal can be detected at the DSB. This is followed by the recruitment of BRCA1 to promote DNA-end resection to generate single-strand DNA (ssDNA) for strand invasion and HR repair22. In turn, Rad51 recombinase is recruited at DSB sites to catalyze strand annealing and HR-mediated repair of DSBs, whereas the increase in NHEJ signatures is not directly related to LKB1 function.
confirmed compromised in Rad51 recruitment to pH2AX foci in LKB1-deficient cells and rescue on LKB1 re-expression, despite comparable levels of pH2AX positivity (Fig. 2e,f and Extended Data Fig. 3c). Moreover, immunoprecipitation assays demonstrated the presence of LKB1 in complex with BRCA1, but not to RAD51, AMPK or RPA, in response to NCS-induced damage (Extended Data Fig. 3d), suggesting that the functions of LKB1 in HR may involve complexing with BRCA1 with compromised RAD51 recruitment.

**LKB1 loss suppresses antigen presentation**

The relationship between DNA repair deficiency and immunotherapy response in the clinic is complex and incompletely understood. For example, while tumors with mismatch repair deficiency are more susceptible to immune checkpoint blockade⁷,⁸, BRCA1 mutated tumors have suppressed antigen presentation and require additional immune stimulating agents to engender sensitivity to immunotherapy⁹. Pathway analysis from both The Cancer Genome Atlas (TCGA) and mouse cell-line datasets by gene set enrichments showed that LKB1 mutations were associated with transcriptional signatures for suppressed immune response in cancer cells, including pathways involved in host defense response, immune response, regulation of immune system process, leukocyte activation and innate immune responses are suppressed in KL tumors compared to KP tumors (Fig. 1c, Extended Data Fig. 1f and Supplementary Table 1).

We reasoned that impaired neoantigen presentation by MHCI at the cell surface could function as a mechanism to promote immune evasion in the context of the HR defects and high TMB that are present in LKB1mut NSCLC. We found that patients with KL NSCLC have a decreased expression of the machinery for neoantigen processing and presentation, with reduced mRNA levels of B2M, HLA-A, HLA-B, TAP1 and immunoproteasome subunit PSMB9 compared to KP tumors (Fig. 3a and Extended Data Fig. 4a). By contrast, no differences were observed in the expression of the catalytic subunits of conventional proteasomal PSMB5 and PSMB6 (Fig. 3a). Similarly, KL mouse cancer cell lines and GEMM lung tumor nodules exhibited decreased expression of the immunoproteasome subunits PSMB8 and PSMB9 at the mRNA levels (Fig. 3b) and protein levels (Extended Data Fig. 4b) compared to murine KP tumors. TAP1 and Tapasin protein levels were reduced, where PSMB5 and PSMB6 were at comparable or increased levels in KL tumors (Extended Data Fig. 4b).

IFNγ stimulation induces the activity of the immunoproteasome subunits LMP7/PSMB8 (Ac-ANW) and LMP2/PSMB9 (Ac-PAL) along with upregulating the mRNA expression of classical class I MHC genes and processing factors, including HLA-A, HLA-B, HLA-C, TAP1 and TAPASIN26–29. Notably, IFNγ-induced PSMB8 and PSMB9 activity was greatly attenuated in KL cells compared to KP cells, whereas there was no compromise in these transcriptional changes (Fig. 3c and Extended Data Fig. 4b). The impairment in PSMB8 and PSMB9 activity was specifically due to LKB1 loss, since KLP cells showed comparable levels to KL cells (Extended Data Fig. 4c,d) and since wildtype LKB1 reconstitution increased immunoproteasome activity in KL cells (Extended Data Fig. 4e). Finally, while we observed decreased cell surface MHCI expression in KL tumors in vivo, MHCI levels were comparably induced on
IFNγ stimulation in vitro (Extended Data Fig. 5a–c). Thus, uptake of the MHCI complex from the endoplasmic reticulum to the cell surface remains functional in LKB1-mutant tumors, whereas there is compromised generation of immunogenic peptides through the immunoproteasome.

**Autophagy inhibition enhances immunoproteasome activity**

Suppressed antigen processing for MHCI presentation could promote immune evasion in LKB1-deficient tumors. Hence, therapeutic strategies that increase antigen presentation might restore antitumor immunity and compensate for LKB1 loss. Autophagy and proteasomal degradation are the two main pathways for quality control of cellular protein homeostasis, and reduced proteasome activity can induce autophagy as a compensatory process. Notably, KRA8-mutant cancers, including those with LKB1 mutations, have been shown to depend on autophagy-lysosomal catabolism for tumor growth via both cell-autonomous and non-autonomous mechanisms. GSEA comparison of lung cancers from the GEMMs showed enrichment of autophagy pathways in the KL tumors compared to KP tumors (Extended Data Fig. 2e,f). Although there was also a trend toward enrichment in human KL lung tumors, it did not reach statistical significance, possibly due to interference of other comutations in patient tumors (Extended Data Fig. 2e,f).

Unfolded protein pathways (UPR), which have been linked to autophagy and MHCI expression, did not show consistent differences in human and murine KL tumors (Extended Data Fig. 2e,f), indicating that UPR may not play a major role in antigen presentation defects resulting from of LKB1 inactivation.

Consistent with increased activity of the autophagy-lysosomal catabolic system in KL tumors and its requirement for in vitro proliferation, KL cells showed elevated sensitivity to autophagy inhibitors chloroquine and MRT68921 (Fig. 3d), which inhibit lysosomal acidification and ULK1/ULK2 kinase activity, respectively, compared to KP tumors. Using transmission electron microscopy (TEM) to quantify the number of autophagic vacuoles in KL cells, we found that reconstitution of LKB1, but not LKB1-KD, suppressed the number of autophagic vacuoles (Fig. 3e), consistent with increased autophagic catabolism in the absence of LKB1. Accordingly, inhibition of autolysosomal acidification with bafilomycin A1 promoted accumulation of p62 and LC3II levels in both KL-EV and KL-LKB1-KD cells, but not in KL cells expressing LKB1. Therefore, we found that MRT68921 treatment increased immunoproteasome activity in KL tumors treated with another autophagy inhibitor chloroquine (CQ) (Fig. 3c). By contrast, KP tumors did not respond to either MRT68921 or PD-1 treatment alone or in combination (Extended Data Fig. 7f). Neutralizing antibodies against CD8+ T cells blocked the effects of MRT68921 plus PD-1 combinatorial treatment on antitumor immunity in LKB1-mutant tumors (Fig. 6b), confirming that the efficacy of the combination involves CD8+ cytotoxic T cell activation rather than tumor intrinsic effects. Combination treatment resulted in an increased CD44+ CD62L− population within tumor infiltrating CD8+ T cells (Fig. 6c, upper panels). The IL-7Rα subunit CD127 was reduced, while CD69, CCR7, and B2M levels did not change after treatment (Fig. 6c, lower panels, and Extended Data Fig. 8d). CD127 is reported to be critical for CD8+ T cell homeostasis, and reduced expression of CD127 was associated with CD8+ T cells among total CD45+ T cells (Fig. 6c, upper panels), suggesting that CD127 reduction could be a consequence of CD8+ T cell depletion.

The block in autophagy caused by MRT68921 has been reported to involve targeting of ULK1 specifically, and not ULK2 (ref. 49). Using a tandem fluorescent reporter of autophagic flux (GFP-RFP-LC3) (Extended Data Fig. 6a,b), we confirmed that either short-hairpin RNA-mediated knockdown of Ulk1 or MRT68921 treatment reduced autophagic flux in KL cells (Extended Data Fig. 6b–d). MRT68921 can also target TBK1 (ref. 48), which has been implicated in LKB1-mediated immune suppression through the TBK1/STING pathway. However, we found that MRT68921 treatment increased TBK1 activity, as reflected by pTBK1 levels, in LKB1 isogenic lines of human NSCLC, probably due to the immune stimulating effects of ULK1 inhibition (Extended Data Fig. 6e). Furthermore, inhibition of autophagy regulators downstream of ULK1—including Atg7, Atg13 and Atg4—caused similar effects of increases in antigen processing via enhanced immunoproteasome activity (Fig. 4c–e and Extended Data Fig. 6f–i).

**ULK1 inhibition restores antitumor immunity in KL**

Following our in vitro observations that KL cells are sensitive to ULK1 inhibition, and that targeting ULK1 restores antigen presentation with increased immunoproteasome activity, we sought to examine whether these effects translate to differential immune responses in vivo in LKB1-mutant and LKB1-wildtype cancer models. We confirmed that MRT68921 treatment blocked autophagic flux in KL lung tumors (Fig. 5a,b and Extended Data Fig. 7a,b). This led to increases in immunoproteasome activity (Fig. 5c–e and Extended Data Fig. 7c). MRT68921 treatment did not result in major toxicity (Extended Data Fig. 7d), and increased infiltration of both CD4+ and CD8+ T cells among total CD45+ immune cells in the tumor-bearing lungs (Fig. 5f,g and Extended Data Fig. 8a–c). MRT68921 treatment alone did not result in an antitumor effect (Fig. 6a and Extended Data Fig. 7e). Anti–PD-1 antibody mono-therapy had modest and inconsistent tumor responses. Conversely, combination MRT68921 treatment enhanced the efficacy of anti–PD-1 therapy, with evident tumor regression in eight out of 15 tumors (Fig. 6a and Extended Data Fig. 7e). We observed similar increased immunoproteasome activity in KL tumors treated with another autophagy inhibitor chloroquine (CQ) (Fig. 5c). By contrast, KP tumors did not respond to either MRT68921 or PD-1 treatment alone or in combination (Extended Data Fig. 7f). Neutralizing antibodies against CD8+ T cells blocked the effects of MRT68921 plus PD-1 combinatorial treatment on antitumor immunity in LKB1-mutant tumors (Fig. 6b), confirming that the efficacy of the combination involves CD8+ cytotoxic T cell activation rather than tumor intrinsic effects. Combination treatment resulted in an increased CD44+ CD62L− population within tumor infiltrating CD8+ T cells (Fig. 6c, upper panels). The IL-7Rα subunit CD127 was reduced, while CD69, CCR7, and B2M levels did not change after treatment (Fig. 6c, lower panels, and Extended Data Fig. 8d). CD127 is reported to be critical for CD8+ T cell homeostasis, and reduced expression of CD127 was associated with CD8+ T cells among total CD45+ T cells (Fig. 6c, upper panels), suggesting that CD127 reduction could be a consequence of CD8+ T cell depletion.

**Fig. 5 | Targeting ULK1 increases immunoproteasome activity and T cell activity in vivo.** a. Representative immunofluorescence image lung tumors of KL cells transduced with autophagy flux reporter GFP-LC3–RFP (GLR) after MRT68921 + PD-1 treatment. Data are representative of three independent experiments. Scale bar, 50 μm. b. Quantification of autophagic flux inhibition shown as GFP/RFP ratio for each immunofluorescence image after MRT68921 treatment. n = 7 fields from independent tumors each group. Data represent one of three independent experiments (data are presented as mean ± s.d., one-way ANOVA). c. Western blot shows antigen presentation related proteins, including PSMB9 and PSMB8, TAP1, TAPasin and B2M, increased in LKB1-mutant tumors with another autophagy inhibitor chloroquine (CQ) (Fig. 3c). By contrast, KP tumors did not respond to either MRT68921 or PD-1 treatment alone or in combination (Extended Data Fig. 7f). Neutralizing antibodies against CD8+ T cells blocked the effects of MRT68921 plus PD-1 combinatorial treatment on antitumor immunity in LKB1-mutant tumors (Fig. 6b), confirming that the efficacy of the combination involves CD8+ cytotoxic T cell activation rather than tumor intrinsic effects. Combination treatment resulted in an increased CD44+ CD62L− population within tumor infiltrating CD8+ T cells (Fig. 6c, upper panels). The IL-7Rα subunit CD127 was reduced, while CD69, CCR7, and B2M levels did not change after treatment (Fig. 6c, lower panels, and Extended Data Fig. 8d). CD127 is reported to be critical for CD8+ T cell homeostasis, and reduced expression of CD127 was associated with CD8+ T cells among total CD45+ T cells (Fig. 6c, upper panels), suggesting that CD127 reduction could be a consequence of CD8+ T cell depletion.
and T cell receptor engagement downregulates CD127 expression\(^1\). We also observed enhanced cytotoxicity, with increased levels of both CD107a and granzyme B after combination treatment group (Fig. 6d) as well as an increased ratio of CD8/Treg cells (Extended Data Fig. 8c). These data highlight increased CD8\(^+\) cytotoxicity on engagement of T cell receptors with tumor-derived neoantigens, with suppressed cell death through CD127 inhibition.

In summary, we discovered that LKB1 loss of function leads to DNA DSB repair deficiency with suppressed HR repair, which results in increased TMB in patients with cancer. However, despite
Fig. 6 | ULK1 inhibition restores antitumor immunity in LKB1-mutant lung cancers. a, Waterfall plot showing KL GEMM tumor volume changes 1 week after the drug treatment as indicated. Shown are the tumor volume percentage changes compared with week 0 tumor volume before the treatment. Each column represents one mouse (one-way ANOVA, Dunnett’s multiple comparisons test, two-sided). b, Tumor volume change of KL allograft tumor 1 week after CD8+ neutralizing antibody with or without MRT68921+PD-1 combinational treatment. n = 8 independent mice each group (mean ± s.d., one-way ANOVA, Dunnett’s multiple comparisons test, two-sided). c, FACS analysis of CD44+ CD62L+ (upper panel) and CD127+ (lower panel) cells among total tumor infiltrating CD8+ T cells in KL tumor after the treatment of drugs indicated. Contour plots representative of three independent experiments. Veh n = 8, MRT + PD-1 n = 9 independent tumors (mean ± s.d., two-tailed t-test, unpaired). d, Granzyme B (GranB) and CD107a levels within CD8+ T cells in KL tumor after the treatment of drugs indicated. Contour plots representative of three independent experiments. Veh n = 11, MRT + PD-1 n = 11 independent tumors (mean ± s.d., two-tailed t-test, unpaired).

high TMB and an increased number of neoantigens, patients with LKB1 mutations respond poorly to anti-PD-1 treatment. Our preclinical evidence indicates that LKB1 loss leads to restricted antigen presentation to MHC complexes due to increased autophagic flux and suppressed proteasomal degradation of antigenic peptides. Further studies will be needed to determine whether the DNA repair defects and reduced antigen presentation are functionally related or represent separate functions of LKB1. In this regard, LKB1 is the upstream kinase activating the 14 members of the AMPK-SIK-MARK family, which connect LKB1 to pleiotropic functions, including regulation of cell metabolism and cell polarity42. These functions generally favor restoration of cellular homeostasis in response to changes in nutrient cues and oppose growth. Overall, the integration of these activities, as well as DNA repair and immune regulation reported in the present study, is in keeping with the evolutionarily conserved roles of LKB1 in mediating nutrient stress responses. Inhibiting autophagy by targeting ULK1 or downstream key regulators restores suppression of antigen presentation through enhancing immunoproteasome activity, leading to increased T cell infiltration and enhanced response to anti-PD-1 treatment through the expansion of CD44+ CD62L+ effector CD8+ T cells in LKB1-mutant tumors. Potential dual effects of ULK inhibitors on both cancer cells and immune populations remain to be explored. Our findings provide the preclinical rationale for combining autophagy inhibition to enhance tumor neoantigen presentation and anti-PD-1 therapy in high-TMB tumors with inactivating mutations in LKB1.

Methods
RNA-sequencing and whole-exome sequencing (WES). For KP and KL cell lines and GEMMs lung nodules, RNA was extracted using RNaseasy Plus Mini Kit (Qiagen). RNA-sequencing (RNA-seq) libraries were prepared using the Illumina TruSeq Stranded mRNA Library Prep (for 96 samples, catalog no. 20020595), starting from 500 ng of total RNA, with ten cycles of PCR amplification. Total genomic DNA were extracted using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer’s manual. Mouse exome libraries were prepared using Roche SeqCap EZ mouse exome kit (54 Mb, catalog no. L2RD02), starting with 250 ng of genomic DNA, following the manufacturer’s protocol ( Nimblegen SeqCap EZ Library SR, v5.1). All libraries (exomes and RNA-seq) were uniquely barcoded with IDT 8 bp indices, pooled and sequenced on a NovaSeq 6000, on an S4 300 flow cell, as paired-end 150 reads or an Illumina HiSeq2500, producing an S4 300 flow cell, as paired-end 150 reads or an Illumina HiSeq2500, producing 2 × 50 bp paired-end reads with multiplexing.

Whole-exome sequencing and RNA-seq small nucleotide variants analysis. Sequencing results were demultiplexed and converted to FASTQ format using Illumina bcl2fastq software (v2.17). The reads were adapter and quality trimmed with Trimmomatic (v0.33)43.
For whole-exome samples, the sequencing reads were aligned to the mouse reference genome (build mm10/GRCm38) using the Burrows–Wheeler Aligner (v.0.7.17) with the BWA-MEM algorithm47. Low confidence mappings (mapping quality <10) and duplicate reads were removed using Sambamba48. Further local indel realignment and base-quality score recalibration was performed using the Genome Analysis Toolkit (GATK v.3.8)49.

For RNA-seq samples, the sequencing reads were aligned to the mouse genome (build mm10/GRCm38) using the splice-aware STAR aligner (v.2.5.3)50 discarding multi-mapped reads. MAPQ set to 60 for uniquely mapping reads. Duplicate reads were removed using Sambamba (v.0.6.7). GATK was used to split reads into exon segments and hard-clip any sequences overhanging into the intronic regions, followed by local indel realignment and base-quality score recalibration.

Single-nucleotide and small indel somatic variants were called with Mutect (v.2.1)51 and Strelka (v.2.9.2)52. Variants with at least five supporting reads and variant allele frequencies >5% were retained, with annotations for variants with genomic context such as functional consequence on genes. The MutationPatterns (v.1.4.3)53 R package was used to quantify the contribution of COSMIC mutational signatures.

**Patients’ mutational burden analysis.** STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.

**COSMIC mutational signatures.** Patients’ mutational burden analysis. STK11 somatic mutation status, TMB and smoking history were obtained for 1,497 patients with lung cancer from the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 December 2018 as previously reported13. The log10-transformed TMB values were calculated for the Memorial Sloan Kettering Cancer Center clinical sequencing cohort on 10 somatic mutation status, TMB.
Cell lysate immunoproteasome activity assay. Cells from individual cell lines were plated overnight, and stimulated with IFNγ at the indicated concentrations for 24 h. The immunoproteasomal activities of these cells were performed using the Immunoproteasomal Activity Fluorometric Assay Kit (UBPBio catalog no. J4170) according to the manual. Briefly, the lysates were generated by washing cells with cold PBS and lysing the resulting cell pellets, and lysates were obtained using i-cold cell lysis buffer according to the manufacturer's manual. Total cell lysate proteins (5 μg) were diluted in 1× assay buffer. Activity assay was carried out over 1 h following twofold sample dilution with 100 μM Ac-ANW-AMC, Ac-KQL-AMC or Ac-PAI-AMC substrate at 37 °C. Activity measurements were performed on a FlexStation 3 multi-mode microplate reader at wavelengths of excitation/emission 360 nm/415 nm. Data were analyzed with GraphPad Prism software. Each treatment condition was done in triplicate. Michaelis–Menten calculations were performed using a nonlinear fit in Prism v.8.2.0 to determine Vₘₐₓ.

TEM. Cultured cells were treated with EBS5 for 1 h before being fixed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde and 2% paraformaldehyde for 2 h, and then post fixed with 1% osmium tetroxide for 1 h at 4 °C; later they were blocked in 0.25% aqueous uranyl acetate, processed in a standard manner and embedded in EMBed 812 (Electron Microscopy Sciences). Ultrathin sections (60 nm) were cut on copper grids and stained with uranyl acetate and lead citrate. Stained grids were examined under a Philips CM-12 electron microscope and photographed with a Gatan (4,000× x 2,700) digital camera.

Autophagy analysis. For investigation of autophagy flux, a tandem autophagy flux reporter Ad5::mCherry-EGFP-LC3 (purchased from Addgene as a gift from J. Debnath) was used to generate the indicated cells that were treated with MRT followed by imaging of the LC3 puncta using a Leica DM6, and image acquisition was done using LAS X software (v.2.0.14332.2). For quantification of autophagic flux, tandem GFP-RFP-LC3 reporter construct p6-1LC3 plasmid (Addgene catalog no. 21074) was transfected into indicated cells. Cells were treated with either vehicle control or MRT68921 (MCE, catalog no. HY-100006A) for 24 h, followed by EBS5 nutrient deprivation for 3 h, and collected for quantification of autophagy flux as determined by the fluorescent signal of the red fluorescent protein (RFP) to GFP ratio within live cells. Data were acquired by FACS using FACS Fortessa X-20 (BD) and analyzed using FlowJo software (BD v.10.6.1). For autophagy flux analysis using western blot, each group of cells was treated with Bafilomycin A1 (Cayman Chemical Company, catalog. no. 11038) and/or MRT68921 with EBS5 (ThermoFisher Scientific, catalog no. 24010043) for 1 h. Cells were collected and protein extractions were quantified for immunoblotting with the indicated antibodies. For autophagy flux analysis in vivo, tumors with GFP+/- EBSS-RFP reporter were collected after indicated drug treatment and quantified by ratio of RFP to GFP using immunofluorescence staining or FACS analysis.

Animal studies. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the New York University School of Medicine. The GEMM harboring a conditional activating mutation of endogenous Kras (KrasLSL-L56C) crossed with Lkb1 or P53 conditional knockout (Lkb1WW or Trp53WW) has been previously described. All the mice were crossed and confirmed with C57BL/6 genetic background by single-nucleotide polymorphism analysis, 99.99% for KP (KrasLSL-L56C), 94.46% for KL (KrasLSL-L56C), CRE recombinase was induced through intranasal inhalation of 1×106 plaque forming units of adeno-Cre (University of Iowa adenoviral core). The GEMM harboring a conditional activating mutation of endogenous Kras (KrasLSL-L56C) crossed with Lkb1 or P53 conditional knockout (Lkb1WW or Trp53WW) has been previously described. All the mice were crossed and confirmed with C57BL/6 genetic background by single-nucleotide polymorphism analysis, 99.99% for KP (KrasLSL-L56C), 94.46% for KL (KrasLSL-L56C). CRE recombinase was induced through intranasal inhalation of 1×106 plaque forming units of adeno-Cre (University of Iowa adenoviral core). The induced mice were evaluated by MRI imaging to quantify the lung tumor burden, and KP and KL lung tumor nodules were obtained and tumor cell lines were generated from these nodules ex vivo. Briefly, lungs were harvested and washed twice in 1× PBS, and then the tumors were cut into small pieces using scissors. The shredded tissues were cultured in an incubator at 37 °C (with 5% CO₂). Fresh medium was changed every other day. The cells were cultured for at least five passages to establish the stable cell lines. In this study, five KL cell lines were generated using the above method. The Kras mutated cell lines were obtained from Dr. J. Debnath as a gift. The KrasLSL-L56C induction was performed using the MRT68921 with EBSS (ThermoFisher Scientific, catalog no. 24010043) for 1 h. For investigation of autophagy flux, a tandem autophagy flux reporter Ad5::mCherry-EGFP-LC3 (purchased from Addgene as a gift from J. Debnath) was used to generate the indicated cells that were treated with MRT followed by imaging of the LC3 puncta using a Leica DM6, and image acquisition was done using LAS X software (v.2.0.14332.2). For quantification of autophagic flux, tandem GFP-RFP-LC3 reporter construct p6-1LC3 plasmid (Addgene catalog no. 21074) was transfected into indicated cells. Cells were treated with either vehicle control or MRT68921 (MCE, catalog no. HY-100006A) for 24 h, followed by EBS5 nutrient deprivation for 3 h, and collected for quantification of autophagy flux as determined by the fluorescent signal of the red fluorescent protein (RFP) to GFP ratio within live cells. Data were acquired by FACS using FACS Fortessa X-20 (BD) and analyzed using FlowJo software (BD v.10.6.1). For autophagy flux analysis using western blot, each group of cells was treated with Bafilomycin A1 (Cayman Chemical Company, catalog. no. 11038) and/or MRT68921 with EBS5 (ThermoFisher Scientific, catalog no. 24010043) for 1 h. Cells were collected and protein extractions were quantified for immunoblotting with the indicated antibodies. For autophagy flux analysis in vivo, tumors with GFP+/- EBSS-RFP reporter were collected after indicated drug treatment and quantified by ratio of RFP to GFP using immunofluorescence staining or FACS analysis.

Statistics and reproducibility. No statistical methods were used to predetermine sample size. The group size of mice and samples were chosen based on our previous publications that we used to generate statistically significant results. The experiments for the animal study were not randomized and the investigators were not blinded during the experiment and outcome assessment. Randomization is not relevant to other cell-based studies since they require different treatment conditions. For immunofluorescence microscopy, one sample (KL + LKB1) was excluded during confocal quantification due to cell contamination (Fig. 7). For comparison of normal distribution samples, a two-tailed t-test was used for statistical analysis using Prism software. For multiple comparisons, a one-way analysis of variance (ANOVA) or multiple t-test was used as specified for each experiment in the figure legend with P values calculated in Prism. For patient samples that are not in a normal distribution, the Shapiro–Wilk test was used for a normality test and a Mann–Whitney test was used for statistical analysis among different groups.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data generated and supporting the findings of this study are available within the paper. The RNA-seq data have been deposited in the Gene Expression Omnibus accession number GSE137244 and GSE137396. The whole-exome sequencing data have been deposited in the NCBI Sequence Read Archive accession number PRJNA643985. TCGA data used are publicly available at the Genomic Data Commons portal (https://portal.gdc.cancer.gov/). Source data are available for this paper. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

Received: 17 November 2020; Accepted: 9 April 2021; Published online: 17 May 2021

References

1. Stroblida, E. et al. STK11/LKB1 mutations and PD-1 inhibitor resistance in KRAS-mutant lung adenocarcinoma. Cancer Discov. 8, 822–835 (2018).
2. Kadara, H. et al. Whole-exome sequencing and immune profiling of early-stage lung adenocarcinoma with fully annotated clinical follow-up. Ann. Oncol. 28, 75–82 (2017).
3. Rizvi, H. et al. Molecular determinants of response to anti-programmed cell death (PD)-1 and anti-programmed-death-ligand 1 (PD-L1) blockade in patients with non-small-cell lung cancer profiled with targeted next-generation sequencing. J. Clin. Oncol. 36, 633–641 (2018).
4. Herter-Sprie, G. S. et al. Synergy of radiotherapy and PD-1 blockade in Kras-mutant lung cancer. JCI Insight 1, e87415 (2016).
5. Xu, C. et al. Loss of Lkb1 and Pten leads to lung squamous cell carcinoma with elevated PD-L1 expression. Cancer Cell 25, 590–604 (2014).

6. Koyama, S. et al. STK11/LKB1 deficiency promotes neutrophil recruitment and proinflammatory cytokine production to suppress T-cell activity in the lung tumor microenvironment. Cancer Res. 76, 999–1008 (2016).

7. Deng, J. et al. CDK4/6 inhibition augments antitumor immunity by enhancing T-cell activation. Cancer Discov. 8, 216–233 (2018).

8. Cancer Genome Atlas Research. N. Comprehensive molecular profiling of lung adenocarcinoma. Nature 511, 453–530 (2014).

9. Rizvi, N. A. et al. Cancer immunology. Mutualistic landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 348, 124–128 (2015).

10. McGranahan, N. et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. Science 351, 1463–1469 (2016).

11. Schumacher, T. N. & Schreiber, R. D. Neoantigens in cancer immunotherapy. Nat. Rev. Immunol. 16, 144–154 (2016).

12. McFadden, D. G. et al. Mutational landscape of EGFR-, MYC-, and KRAS-driven lung cancer. Cancer Discov. 5, 150–165 (2015).

13. Hisamatsu, H. et al. Newly identified pair of proteasomal subunits regulated by post-translational modification. Proc. Natl. Acad. Sci. USA 113, E6911–E6920 (2016).

14. Le, D. T. et al. PD-1 blockade in tumors with mismatch-repair deficiency. Cancer Discov. 8, 585–598 (2014).

15. Turajlic, S. et al. Insertion-and-deletion-derived tumour-specific neoantigens for immunotherapy. Nature 511, 581–587 (2014).

16. Mandal, R. et al. Genetic diversity of tumors with mismatch repair deficiency influences anti-PD-1 immunotherapy response. Science 364, 485–491 (2019).

17. Alexandrov, L. B. et al. Signatures of mutational processes in human cancer. Nature 500, 415–421 (2013).

18. Davies, H. et al. HRDetect is a predictor of BRCA1 and BRCA2 deficiency based on mutational signatures. Nat. Med. 23, 517–525 (2017).

19. Helleday, T., Eftagd, S. & Nik-Zainal, S. Mechanisms underlying mutational signatures in human cancers. Nat. Rev. Genet. 15, 585–598 (2014).

20. Pierie, A. J., Johnson, R. D., Thompson, L. H. & Jasin, M. XRC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev. 31, 2633–2638 (1999).

21. Bennardó, N., Cheng, A., Huang, N. & Stark, J. M. Alternative-NHEJ is a mechanistically distinct pathway of mammalian chromosome break repair. PLoS Genet. 4, e1000110 (2008).

22. Jasin, M. & Rothstein, R. Repair of strand breaks by homologous recombination. Cold Spring Harb. Perspect. Biol. 5, a012740 (2013).

23. Panier, S. & Dourouck, D. Push back to respond better: regulatory inhibition of the DNA strand-break response. Nat. Rev. Mol. Cell Biol. 14, 661–672 (2013).

24. Le, D. T. et al. PD-1 blockade in tumors with mismatch-repair deficiency. Nature 437, 2509–2520 (2015).

25. Noham, E. et al. Combined immune checkpoint blockade as a therapeutic strategy for BRCA1-mutated breast cancer. Sci. Transl. Med. https://doi.org/10.1126/scitranslmed.aal4922 (2017).

26. Viswanathan, H. et al. The influence of tumor microenvironment on the immune response to cancer. Nature 524, 53–59 (2015).

27. Celis, J. M. et al. Interleukin-7 receptor expression: intelligent evolutionary design. Cell. Biol. 129, 100–107 (2018).

28. Kozubski, L. et al. The mammalian target of rapamycin (mTOR)-dependent autophagy. J. Biol. Chem. 290, 28726 (2015).

29. Kimura, S., Noda, T. & Yoshimori, T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 3, 452–460 (2007).

30. Kitajima, S. et al. Pharmacological inhibition of ULK1 kinase blocks mammalian target of rapamycin (mTOR)-dependent autophagy. J. Biol. Chem. 290, 999–1005 (2014).

31. Shintani, T. & Kioshimsy, D. J. Autophagy in health and disease: a double-edged sword. Science 306, 990–995 (2004).

32. Petherick, K. J. et al. Pharmacological inhibition of ULK1 kinase blocks mammalian target of rapamycin (mTOR)-dependent autophagy. J. Biol. Chem. 290, 28726 (2015).

33. Kitajima, S., Noda, T. & Yoshimori, T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 3, 452–460 (2007).

34. Dorfman, N. et al. Genomic landscape of non-small cell lung cancer in smokers and never-smokers. Cell 150, 1121–1134 (2012).

35. Turajlic, S. et al. Insertion-and-deletion-derived tumour-specific neoantigens and the immunogenic phenotype: a pan-cancer analysis. Lancet Oncol. 18, 1009–1021 (2017).

36. Osorio, F., Lambrecht, B. N. & Janssens, S. Antigen presentation unfolded: identifying convergence points between the UPR and antigen presentation pathways. Curr. Opin. Immunol. 52, 100–107 (2018).

37. Shintani, T. & Kioshimsy, D. J. Autophagy in health and disease: a double-edged sword. Science 306, 990–995 (2004).

38. Petherick, K. J. et al. Pharmacological inhibition of ULK1 kinase blocks mammalian target of rapamycin (mTOR)-dependent autophagy. J. Biol. Chem. 290, 28726 (2015).

39. Kimura, S., Noda, T. & Yoshimori, T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 3, 452–460 (2007).

40. Kitajima, S. et al. Pharmacological inhibition of ULK1 kinase blocks mammalian target of rapamycin (mTOR)-dependent autophagy. J. Biol. Chem. 290, 28726 (2015).
patients with NSCLC. J.D., H.S. and C.T. performed HR and microhomology-mediated end joining analysis. A.M. performed chromatin binding assay, immunoprecipitation and DSB foci analysis. M.B. and D.H.P. performed western blot for the antigen presentation pathway. M.B. and D.H.P. generated the Ulk1 shRNA and Atg cell lines. F.L. and H.Hu. generated the KL cell lines. F.L., Y.P. and H.D. generated and characterized KLP cell lines. J.D., H.D., D.H.P. and H.Han performed the immunoproteasome activity assay. J.D., T.C., E.P., VP., C.T., S.L. and H.Hu. performed animal experiments, treatment studies and MRI imaging and analysis. J.D. and E.P. performed immune analysis of the animal models. T.C. performed the cell growth assay. J.L., J.D. and S.M. performed autophagy flux analysis. J.L. performed electron microscopy experiments and analysis. J.D., B.J. and N.S.G. participated in the Ulk1 inhibitor experiment. J.D. and N.B. drafted the paper. J.D., K.-K.W., N.B., V.W., E.S.W., P.S.H., N.S.G., T.P., A.T., M.Pagano, E.R., J.G., G.J.F., C.M.R., J.V.H., C.M.P., I.A. and M.Philips. conceptually designed and edited the paper. K.-K.W. conceived, designed and supervised all the experiments. All authors reviewed and discussed the final version of the paper.

**Competing interests**

The authors declare the following competing interests: K.-K.W. is a founder and equity holder of GI Therapeutics. K.-K.W. has sponsored Research Agreements with MedImmune, Takeda, TargImmune, Mirati, Merus, Alkermes and BMS. K.-K.W. has consulting and sponsored research agreements with AstraZeneca, Janssen, Pfizer, Novartis, Merck, Ono, Array. C.M.P is an equity stock holder and consultant, and Board of Director Member of BioClassifier LLC and GeneCentric Diagnostics. C.M.P. has consulted and served as a compensated consultant or received honoraria from Bristol-Myers Squibb, Genentech, Ariad/Takeda, Loxo/ Lilly, Blueprint Oncorux, Regeneron, EMD Serono, Gilead, AstraZeneca, Pfizer, Incyte, Novartis, Merck, Agios, Amgen and Array; research support from Novartis, Genentech/Roche and Ariad/Takeda; institutional research support from Bristol-Myers Squibb, Tesaro, Moderna, Blueprint, Jounce, Array Biopharma, Merck, Adaptimmune, Novartis and Alexal; and has an immediate family member who is an employee of Ironwood Pharmaceuticals. G.J.F. has patents/pending royalties on the PD-1/PD-1 pathway from Roche, Merck, Bristol-Myers-Squibb, Merck KGA, Boehringer-Ingelheim, AstraZeneca, Dako, Leica, Mayo Clinic and Novartis. G.J.F. has served on advisory boards for Roche, Bristol-Myers-Squibb, Xios, Origimed, Triursus, iTeos, NextPoint, IgM, Jubilant and GV20. G.J.F. has equity in NextPoint, Triursus, Xios, iTeos, IgM and GV20.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s43018-021-00208-6.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s43018-021-00208-6.

Correspondence and requests for materials should be addressed to K.-K.W.

**Peer review information** Nature Cancer thanks Andrew Thorburn and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at www.nature.com/reprints.

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2021
Mutational burden and signature analysis for LKB1 mutant tumors. 

Extended Data Fig. 1 | Mutational burden and signature analysis for LKB1 mutant tumors. 

**a.** Comparison of tumor mutational burden (TMB) of nonsynonymous mutation using freshly isolate NSCLC GEMMs lung nodules from Kras\(^{G12D}\)Lkb1\(^{fl/fl}\) (KL) or Kras\(^{G12D}\)Trp53\(^{fl/fl}\) (KP) from RNA sequencing. 

![RNA seq graph](image)

\(n=5\) lung nod each group. (unpaired t-test, two sided, FDR < 0.05) 

**b.** TMB analysis of cell lines derived from Kras\(^{G12D}\)Lkb1\(^{fl/fl}\)Trp53\(^{fl/fl}\) (KLP) NSCLC GEMM nodules using RNA sequencing (Upper table) or whole exome sequencing (WES) (lower table) for each cell line. 

| Coding Muts | KLP T1 | KLP T2 |
|-------------|--------|--------|
| RNA seq     | 313    | 305    |
| WES seq     | 28.3   | 28     |

**c.** Validation of KP, KL, KLP lines used. 

Upper panel, PCR result of indicated primers for each cell line. Lower panel, western blot result of LKB1, TRP53 and P16 protein levels of each cell line. Blots are cropped and that uncropped images can be found in Source Data. Data represents one of three independent experiments. 

**d.** Representative IHC images of KL allograft lung tumors showing adenocarcinoma (left panels) or squamous tumor (right panels). Scale bar, 100\(\mu m\). 

**e.** Co-mutational analysis of NSCLC patients analyzed in Fig. 1b, including KRAS, STK11 and TP53 as total patient number and percentage within each group. 

**f.** Top suppressed biological process pathways from Kras/Lkb1 comparing with Kras/Trp53 mutant mouse cell lines. x-axis, -log,q value (Bonferroni) between the two groups. 

\(n=5\) cell lines each group. 

---

Extended Data Fig. 1 | Mutational burden and signature analysis for LKB1 mutant tumors. 

**a.** Comparison of tumor mutational burden (TMB) of nonsynonymous mutation using freshly isolate NSCLC GEMMs lung nodules from Kras\(^{G12D}\)Lkb1\(^{fl/fl}\) (KL) or Kras\(^{G12D}\)Trp53\(^{fl/fl}\) (KP) from RNA sequencing. 

![RNA seq graph](image)

\(n=5\) lung nod each group. (unpaired t-test, two sided, FDR < 0.05) 

**b.** TMB analysis of cell lines derived from Kras\(^{G12D}\)Lkb1\(^{fl/fl}\)Trp53\(^{fl/fl}\) (KLP) NSCLC GEMM nodules using RNA sequencing (Upper table) or whole exome sequencing (WES) (lower table) for each cell line. 

| Coding Muts | KLP T1 | KLP T2 |
|-------------|--------|--------|
| RNA seq     | 313    | 305    |
| WES seq     | 28.3   | 28     |

**c.** Validation of KP, KL, KLP lines used. 

Upper panel, PCR result of indicated primers for each cell line. Lower panel, western blot result of LKB1, TRP53 and P16 protein levels of each cell line. Blots are cropped and that uncropped images can be found in Source Data. Data represents one of three independent experiments. 

**d.** Representative IHC images of KL allograft lung tumors showing adenocarcinoma (left panels) or squamous tumor (right panels). Scale bar, 100\(\mu m\). 

**e.** Co-mutational analysis of NSCLC patients analyzed in Fig. 1b, including KRAS, STK11 and TP53 as total patient number and percentage within each group. 

**f.** Top suppressed biological process pathways from Kras/Lkb1 comparing with Kras/Trp53 mutant mouse cell lines. x-axis, -log,q value (Bonferroni) between the two groups. 

\(n=5\) cell lines each group.
Extended Data Fig. 2 | Mutational signature analysis and gene set enrichment analysis (GSEA) from KL and KP tumors. **a.** Point nonsynonymous single-nucleotide variations (SNV) numbers from either KP or KL cells. KP n = 6, KL n = 5 cell lines each group. (mean ± sd, unpaired t test, two tailed). **b.** Percentage of Indels and SNVs in KP and KL cells. KP n = 6, KL n = 5 cell lines each group. (unpaired t test, two sided. P = 0.0032). **c.** Percentage of each COSMIC mutational signature detected in the cell lines examined. Each column represents one individual cell line. **d.** Fold change of the signatures shown in (a) and normalized to average levels of corresponding signature in KP. KL, KP n = 5 cell lines each group. (mean ± sd, multiple t test, FDR < 0.05). **e.** GSEA of DNA repair related pathways, including HR repair-replication independent DSB, non-homologous end joining, nucleotide excision repair and mismatch repair, and related autophagy pathway and unfolded protein response (UPR) pathway of KL and KP GEMM lung nodules. KL n = 5, KP n = 5 lung nodules each group. **f.** Gene set enrichment analysis (GSEA) of TCGA patients for autophagy pathway and UPR pathway. KL n = 19, KP n = 22 patients each group.
Extended Data Fig. 3 | LKB1 mutant tumor double strand break repair. a, HR and NHEJ levels in Kras/Lkb1/Trp53 mutant cell lines comparing with Kras/Trp53 cells. n = 3 cell cultures for each group. (mean±sd, two-sided t test, unpaired). b, NHEJ ratio changes in human NSCLC LKB1 mutant cell lines H23, A427 and H460 with empty vector (pBABE), LKB1 or KLB1-KD overexpression were determined by flow cytometry. n = 3 individual cell cultures for each group. (mean±sd, two-sided t test, unpaired). c, Quantification of Rad51 and pH2AX positive cells percentage in KLP cells. Result is combined from three independent experiments. KP UT, KP NCS and KLP UT n = 4, KLP NCS n = 3 individual cell cultures each group. Data shown one of two independent experiments. (mean ± sd, one-way ANOVA, multiple two-sided comparison, Tukey test). d, LKB1 forms complex with BRCA1 in response to DNA damage. Upper panel, immunoprecipitation assay showing LKB1 interacting proteins in response to neocarzinostatin (NCS) treatment induced DNA damage. Lower panel, western blot showing total level of input proteins from whole-cell extract (WCE). Data represents one of two independent experiments. Blots are cropped and uncropped images can be found in Source Data.
Extended Data Fig. 4 | Immunoproteasome activity changes in LKB1 mutant tumors upon IFNγ stimulation. **a**, Real-time PCR showing antigen presentation genes expressing in LKB1 mutant lines with LKB1 overexpression. Left, H460 human NSCLC cells. Right, mouse KL cells with or without LKB1 over-expression. *n* = 3 experiments each group. (mean ± sd, unpaired *t* test, two tailed). *P* < 0.05, **P** < 0.001. **b**, Western blot showing immunoproteasome subunits PSMB9 and PSMB8 levels, TAP1, Tapasin and B2M expression (Upper panel) and conventional proteasome subunits PSMB5 and PSMB6 expression (lower panel) from KP or KL lung nodules. Samples are derived from the same experiment and blots were processed in parallel. Blots are cropped and uncropped images can be found in Source Data. **c**, KL Ulk1 shRNA cell lines (upper panel) and KP Ulk1 shRNA cell lines (lower panel) were stimulated with IFNγ (10 ng/ml) for 24 hrs before the measurement of immunoproteasome activities showed as relative fluorescent units (RFU) per min (Vmax). *n* = 3 cell cultures for each group. (mean ± sd, multiple two-sided unpaired *t* test, two-stage step-up method of Benjamini, Krieger and Yekutieli). **d**, Immunoproteasome cleavage activity corresponding to indicated substrate for two KLP cells (KLP T1 and KLP T2) after IFNγ stimulation shown as Vmax fold change compared with KL vehicle control group. *n* = 3 cell cultures each group. (mean ± sd, multiple two-sided unpaired *t* test, two-stage step-up method of Benjamini, Krieger and Yekutieli). **e**, Immunoproteasome activity for KL (left) and H460 (right) with or without LKB1 over-expression. *n* = 3 cell cultures each group. (mean ± sd, two-sided unpaired multiple *t* test, two-stage step-up method of Benjamini, Krieger and Yekutieli).
Extended Data Fig. 5 | MHCI levels in KL and KP tumors. 

(a) MHCI levels from KP or KL allograft tumors as quantified as median fluorescent intensity (MFI) (left). Right, representative histograms depict MHCI level from mice with KP and KL tumors. n = 5 tumors each group. (mean ± sd, two-sided t test, unpaired) 

(b) MHCI levels from KP or KL tumor cell lines stimulated with IFNγ as shown MFI levels. Left panel, representative histograms. Right panel, quantification of MHC I expression levels in KP and KL after IFNγ (100 ng/ml) stimulation for 18 hrs. n = 3 cell cultures each group. Shown representative result of three independent experiments (mean ± sd, multiple t test, FDR < 0.05). 

(c) MHC I subunit H-2K and H-2D levels change in response to ULK1 inhibitor MRT68921 in mouse KL line in vitro 48hrs after the treatment. Data representative of two independent experiments.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Targeting autophagy pathway in LKB1 mutant NSCLC tumors. 

a, Representative immunofluorescent (IF) image of autophagy flux in KL and KL-LKB1 tumor cells in response to MRT using GFP-mCherry-LC3B reporter. Red, GFP-mCherry+ LC3 puncta; yellow, GFP + mCherry+ puncta. Scale bar, 15 μm. Data representative of 2 independent experiments.

b, Quantification of autophagic flux by GFP-RFP-LC3 reporter in KL tumors treated with MRT as left using flow cytometry analysis. Upper panel, gating strategy. Lower panel, quantification of autophagic flux shown as RFP:GFP ratios. n = 3 cell cultures each group. Data shown represents one of three independent experiments (mean ± sd, two-tailed t test for high group, unpaired).

c, Quantification of autophagic flux ratio (RFP:GFP) in KL cells with GFP-RFP-LC3 reporter transduced with Ulk1 shRNA. n = 3 cell cultures each group. Data shown one of two independent experiments. (mean ± sd, two-tailed t test for high group, unpaired). Samples were compared with NT shRNA cells for high group.

d, Western blot showing ULK1 protein levels in KL (left) and KP (right) stable cell lines with Ulk1 shRNA. Data represents one of two independent experiments.

e, Western blot of ULK inhibitor MRT68921 in human LKB1 isogenic lines for TBK1/STING pathway changes of H460 (left) and H23 (middle) and H1792 (right). Data represents one of two independent experiments.

f, g, h, Immunoproteasome activity for KL stable cell lines with Atg7 or Atg13 for ANW (f), KQL (g) or PAL (h) substrate. n = 3 independent experimental samples for each group. (data presented are shown as mean±sd, two-sided one-way ANOVA, Tukey test, statistics presented on top of each column is compared with vehicle group, and pairwise comparisons between groups after 100 ng/ml IFNγ treatment shown on top of columns). Data shown one of two independent experiments.

i, Western blot of KL stable cell lines generated with either Atg7 shRNA (left panel) or Atg13 shRNA (right panel). Shown one of two independent experiments. Blots in panels d, e and i are cropped and uncropped images can be found in Source Data.
Extended Data Fig. 7 | Targeting autophagy in LKB1 mutant NSCLC tumors in vivo. 
a, validation of KL cells transduced with autophagy flux reporter GFP–LC3-RFP (GLR). Generated KL GLR cells were starved in EBSS buffer for 24 hrs before FACS analysis for RFP/GFP signals. 
b, Representative FACS analysis of GFP/RFP ratio of KL tumors after indicated treatments. Data representative of 2 independent experiments. 
c, Western blot shows conventional proteasome subunits PSMB5 and PSMB6 expression levels from KL lung nodules after MRT + PD1 treatment. Each lane represents one individual mouse tumor nodule. 
d, Toxicity of MRT68921 shown as mouse body weight change percentage after the drug treatment. Each line represents one mouse. n = 9. 
e, Tumor volume changes after 2-week treatment of KL GEMM tumors with MRT + PD1. Veh n = 9, PD1 n = 14, MRT n = 8, MRT + PD1, n = 14 tumors each group. (mean ± sd, one-way ANOVA). 
f, Tumor volume change of KP allograft tumor 1 week after indicated treatment. Veh, MRT, MRT + PD1 n = 10; PD1 n = 8 tumors each group. (mean ± sd, one-way ANOVA).
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Immune infiltrates analysis in KL tumors after ULK1 inhibitor treatment. 

- **a.** Gating strategy used for immune analysis. 
- **b.** FACS analysis of CD4+ (left) and CD8+ (right) T lymphocytes among total tumor infiltrating CD45+ leukocytes in KL tumor after the treatment of ULK1 inhibitor MRT68921 (MRT). Veh n = 12, MRT n = 5 tumors each group. (mean ± sd, unpaired t test, two tailed). 
- **c.** CD8/T reg ratio of total tumor infiltrating leukocytes (TILs) after the indicated drugs treatment. veh n = 12, MRT n = 5, MRT + PD1 n = 12 tumors each group. (mean ± sd, unpaired t test, two tailed). 
- **d.** CD69, CCR7 and 2B4 levels within CD8+ T cells after MRT68921 (MRT) and anti-PD1 treatment. Left panel, veh n = 12, MRT + PD1 n = 10. Middle and right panel, veh n = 8, MRT + PD1 n = 10 tumors each group. (mean ± sd, unpaired t test, two tailed).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a  Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

RNA sequencing and whole exome sequencing results were collected from NovaSeq 600 or Illumina HiSeq2500, demultiplexed and converted to FASTQ format using illumina bc2fastq software. The reads were adapter and quality trimmed with Trimmomatic.

DNA repair analysis, autophagic flux and tumor immune infiltrate analysis were performed by flow cytometry using BD LSRfortessa X-20 flow cytometer (BD Biosciences) with FACS Diva 8.0.1

Immunofluorescence microscopy imaging was performed using a DeltaVision Elite inverted microscope system (Applied Precision) with SoftWorx imaging software version 5.0. Serial optical sections obtained 0.2-um apart along the z-axis were processed using the SoftWorx deconvolution algorithm and projected into one picture using SoftWorx software (Applied Precision).

Immunofluorescence microscopy analysis for autophagy flux of LC3 puncta were done using Leica DM6, and image acquisition was done using LAS X software (version 2.0.0.14332.2).

Immunoblotting of immunoreactive bands were visualized by enhanced chemiluminescence reagent (Thermo Fisher Scientific) and signal was acquired using ImageQuant LAS 400 (GE). Or secondary antibody conjugated with fluorescent dyes were used and the fluorescent signal on the membrane were imaged on Odyssey classic infrared imaging system (LI-COR) using Image Studio Lite (V 5.2).

Immunoproteasome activity measurements and cell growth assay were performed on FlexStation 3 multi-mode microplate reader using Softmax v5.4.6.005.

Mouse lung tumors development were examined by MRI scan by 7-T Bruker Biospec 70/30 MRI System and the images were acquired using ParaVision software (version 6).

Data analysis

Sequencing results were demultiplexed and converted to FASTQ format using illumina bc2fastq software (v2.17). The reads were adapter and quality trimmed with Trimmomatic (v0.33).

For whole exome samples, the sequencing reads were aligned to the mouse reference genome (build mm10/GRCm38) using the Burrows-Wheeler Aligner (version 0.7.17) with the BWA-MEM algorithm32. Low confidence mappings (mapping quality <10) and duplicate reads were removed using Sambamba33. Further local indel realignment and base-quality score recalibration was performed using the Genome Analysis Toolkit (GATK v3.8).

For RNA-seq samples, the sequencing reads were aligned to the mouse genome (build mm10/GRCm38) using the splice-aware STAR aligner (v2.5.3) discarding multi-mapped reads and MAPQ set to 60 for uniquely mapping reads. Duplicate reads were removed using
Sambamba (version 0.6.7). GATK was used to split reads into exon segments and hard-clip any sequences overhanging into the intronic regions, followed by local indel realignment and base-quality score recalibration. Single-nucleotide and small indel somatic variants were called with MuTect (v2.1) and Strelka (v2.9.2). Variants with at least 5 supporting reads and VAF>5% were retained. ANNOVAR was used to annotate variants with genomic context such as functional consequence on genes. MutationalPatterns (v1.4.3) R package was used to quantify the contribution of COSMIC mutational signatures.

For the KL and KP cell-line and mouse nodule RNA-seq data, fastq files were aligned to the mouse mm10 reference genome using the STAR aligner algorithm. Resulting BAM files were sorted and indexed using Samtools (1.11) and quality control was performed using Picard (http://broadinstitute.github.io/picard/)(2.20). Transcript read counts were determined was performed using Salmon. Genes with no reads across any of the samples were removed. Salmon gene level counts upper quartile normalized. Genes were log2 transformed and filtered for 80% of expressed genes across all samples using Cluster 3.0 and zeros were preserved for signature analysis. Data was then median centered to establish the matrix in working form for statistical analyses.

To measure the association between biological states or pathways and the gene expression profiles of our KL and KP groups we used gene set enrichment analysis (GSEA) (version 3.0) through the GenePattern platform. For the enrichment results, we performed 1000 permutations using the curated set 5 gene ontology (GO) list (c5.all.v6.2.symbols.gmt), curated set 2 KEGG and REACTOME pathway lists (c2.cp.kegg.v6.2.symbols.gmt, c2.cp.reactome.v6.2.symbols.gmt) and hallmark list (h.all.v6.2.symbols.gmt). For GSEA analysis on the KL and KP human NSCLC RNAseq data we used the publicly available raw transcriptomic data matrix. The data matrix was upper quartile normalized, log transformed, filtered for 80% of expressed genes and median centered like the cell-line and mouse nodule data.

Besides GSEA, we also performed Differential gene expression (DE) analysis on our KP and KL cell-line, mouse nodule and LUNAD data matrices using DESeq2 R package. For the DE analysis we used raw matrices and performed the normalization within the DESeq2 workflow. For all 3 modes of comparison we compared the KL group against the KP group. Using these DESeq2 identified upregulated and downregulated genes in this supervised analysis. We interrogated and report their relevance in gene ontologies and pathways using the ToppGene Suite.

Immunofluorescence microscopy imaging acquired with SoftWorx imaging software version 5.0. Serial optical sections obtained 0.2 μm apart along the z-axis were processed using the SoftWorx deconvolution algorithm and projected into one picture using SoftWorx software (Applied Precision).

Immunoproteasome activity assay measurements were performed on FlexStation 3 multi-mode microplate reader using Softmax. Each treatment condition was conducted in triplicates. Michaelis-Menten calculations were performed using a non-linear fit in Prism v.8.2.0 to determine Vmax.

For cell growth assay, the data calculations were carried out as the percentage cell growth over the DMSO controls and IC50 was acquired using Calcsyn (version 2). The normalized data was then log transformed and plotted in GraphPad Prism v8.2.1.

Statistical analyses were performed using Prism v8.2.1. For all the t tests, F test was used to compare variance for the normal distribution as part of the t test analysis embedded in the software. For TMB analysis of NSCLC patients samples, Shapiro-Wilk normality test was performed to determine the normal distribution of each group (confidence level 95%). And log10-transformed TMB values were compared between STK11 mutant and wild-type tumors separately for either never smokers or current and former heavy smokers using an unpaired nonparametric Mann-Whitney test (two-tailed).

Flow cytometry: flowjo v10.6.1 and FACS Diva 8.0.1
Graphs: Graphpad Prism v8.2.1
Immunoblotting: Image Studio Lite v5.2 and ImageJ fuj. 1.51s

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data generated and supporting the findings of this study are available within the paper. The RNAseq data have been deposited in the Gene Expression Omnibus (GEO) accession number GSE137244 and GSE137396. The WESseq data have been deposited in NCBI Sequence Read Archive (SRA) accession number PRJNA64395. Additional information and materials will be made available upon request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [X] Life sciences - [ ] Behavioural & social sciences - [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/mr-reporting-summary-list.pdf
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
No statistical methods were used to predetermine sample size. The group size of mice and samples were chosen based on previous literature we used to generate statistically significant results. (Reference: Nature. 2007 448(7155):807-10)

**Data exclusions**
Immunofluorescence microscopy, one sample [X1-LKB1] was excluded during of foci quantification due to cell contamination (Fig. 2f). Since it has been reported previously that bacterial pathogens will affect cell DNA repair including RAD51 status, this contaminated sample was excluded from the analysis. (Reference: DNA damage repair and bacterial pathogens. PLoS Pathog. 2013;9(11):e1003711)

**Replication**
All experiments presented in this study were performed using at least 3 biological replicates. The precise number and repeats are provided in each figure legend. All attempts for replication were successful.

**Randomization**
For GEMM study, mice were randomized and sex- and age- matched to different experimental groups. For allograft study, mice with comparable tumor volumes at the time of enrollment were randomized into different treatment groups. Randomization is not relevant to other cell based study since they require different treatment conditions.

**Blinding**
Investigators for animal study were not blinded to group allocation during experiment set up, data collection and analysis. No blinding was performed per cage labeling requirement from NYU Langone Division of Comparative Medicine. The personnel processing the samples and collecting data were not aware of treatment conditions and performed as a blinded fashion.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☐   | Antibodies            |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology         |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

#### Antibodies

| Antibodies used |
|-----------------|
| Immunoblotting antibodies |
| LC3B (Cell signaling cat# 4108) lot 2 (1:10,000) |
| p62 (MBL cat# PM045) lot 019 (1:10,000) |
| LKB1 (Cell signaling cat# 3047) lot 2 (WB 1:1000) (1:1000) |
| beta-actin (Sigma Cat# A5441) lot 11S4835V (1:10000) |
| RAD51 (Genetex cat# GTX70230) lot 43459 (IF 1:200) |
| pH2AX (Cell Signaling cat# 97183) lot 13 (WB 1:1000) (IF 1:1000) |
| Histone H3 (Abcam cat# ab1791), lot GR252388-1 (WB 1:1000) |
| PARP1 (Cell Signaling cat# 95425), lot 14 (WB 1:1000) |
| BRCA1 (Bethyl cat# A301-377A, lot NA (WB 1:1000) |
| RP1 Millipore cat# NA191, lot NA (1:1000) |
| AMPKalpha Cell Signaling Technology #S832, lot 4 (WB 1:1000) |
| pCHK1 Cell Signaling Technology #12302, lot 11 (WB 1:1000) |
| SKP1 Michele Pagano’s lab, lot NA (WB 1:5000) |
| TAF1 (Cell Signaling cat# 123415) lot 1 (1:500) |
| Tapasin (Biolegend cat# 666702), lot 225360 (1:200) |
| BZM (R&D Systems MA98325) lot CIVK0217111 (1:500) |
| PSMB9/LMP2 (Abcam cat# ab3328) lot GR3244042-2 (1:1000) |
| PSMB8/LMP7 (cell signaling cat# 136355) lot 1 (1:1000) |
| PA28 (Cell signaling cat# 24095S) lot 1 (1:1000) |
| PSMB5 (Cell signaling cat# 129195S) lot 1 (1:1000) |
| PSMB6 (Cell signaling cat# 132675S) lot 1 (1:1000) |
| Ulk1 (Cell Signaling cat# 8054) lot 5 (1:1000) |
| p-TBK1 (Cell Signaling cat# 5483) lot 8 (1:1000) |
| TBK1 (Cell Signaling cat# 3504) lot 4 (1:1000) |
| STING (Cell Signaling cat# 13647) lot 4 (1:1000) |
| Atg7 (Cell Signaling cat# 8558) (1:500) |
Validation

All the antibodies except SKP1 used are commercially available and the validation of the antibodies are available on line from manufacture websites.

https://www.cellsignal.com/products/primary-antibodies/lc3a-b-antibody/4108
https://www.mlbbink.com/products/pm045/
https://www.cellsignal.com/products/primary-antibodies/ikkb1-d60c5-rabbit-mab/3047
https://www.sigmaldrich.com/catalog/product/sigma/a5441?ang=en&region=US
https://www.genetex.com/Products/Details/Rad51-antibody-1484_FT/10230
https://www.cellsignal.com/products/primary-antibodies/phospho-histone-h2a-x-ser139-2e3-rabbit-mab/9718
https://www.abcam.com/histone-h3-antibody-nuclear-marker-and-chip-grade-ab1791.html
https://www.cellsignal.com/products/primary-antibodies/parp-antibody/9542
https://www.bethyl.com/product/A301-377A/BRCA1-Antibody
https://www.emdmillipore.com/US/en/product/Anti-Replication-Protein-A-Antibody-clone-RPA34-20,MM_NF-MAB285
https://www.cellsignal.com/products/primary-antibodies/ampka-d63g4-rabbit-mab/5832
https://www.cellsignal.com/products/primary-antibodies/phospho-chk1-ser317-d12h3-3-rabbit-mab/13432
https://www.cellsignal.com/products/primary-antibodies/rap1-antibody/12341
https://www.cellsignal.com/products/primary-antibodies/phospho-chk1-1-ser139-2d14-rabbit-mab/13479
https://www.cellsignal.com/products/primary-antibodies/psmb8-lmp2-antibody-ab3326.html
https://www.cellsignal.com/products/primary-antibodies/psmb5-d11hb-rabbit-mab/12919
https://www.cellsignal.com/products/primary-antibodies/psmb6-eikb-rabbit-mab/13267
https://www.cellsignal.com/products/primary-antibodies/luk1-d8hs-rabbit-mab/8054
https://www.cellsignal.com/products/primary-antibodies/phospho-tbk1-nak-ser172-d52c2-3-rabbit-mab/5483
https://www.cellsignal.com/products/primary-antibodies/agg7-d12b1-rabbit-mab/8558
https://www.cellsignal.com/products/primary-antibodies/agg3-d4p1k-rabbit-mab/13273
https://www.cellsignal.com/products/primary-antibodies/p53-d2h9o-rabbit-mab-rodent-specific/32532
https://www.abcam.com/p16-arc-antibody-ep1551y-ab51243.html
https://www.lcor.com/bio/reagents/rd80r-d-goat-anti-rat-igg-secondary-antibody
https://rockland-inc.com/store/Whole-Igg-Affinity-Purified-Secondary-Antibodies-620-103-440-O4L_18013.aspx
https://www.lcor.com/bio/reagents/rd80rd-donkey-anti-mouse-igg-secondary-antibody
https://www.lcor.com/bio/reagents/rd800cw-donkey-anti-mouse-igg-secondary-antibody

https://www.bioreagent.com/en-us/products/brilliant-violet-605-anti-mouse-cd45-antibody-8721
https://www.bioreagent.com/en-us/products/brilliant-violet-785-anti-mouse-cd4-antibody-11548
https://www.bioreagent.com/en-us/products/brilliant-violet-711-anti-mouse-cd8a-antibody-7926
https://www.bioreagent.com/en-us/products/alexia-fluor-700-anti-mouse-cd3-antibody-3375
https://www.bioreagent.com/en-us/products/apc-cy7-anti-mouse-cd279-pd-1-antibody-9742
https://www.bioreagent.com/en-us/products/p-e-dazzle-594-anti-mouse-cd69-antibody-11763
https://www.bioreagent.com/en-us/products/p-e-dazzle-594-anti-mouse-cd197-ccr7-antibody-10378
https://www.bioreagent.com/en-us/products/fttc-anti-mouse-cd244-2-b2a6-anti-allotigen-antibody-5838
https://www.bioreagent.com/en-us/products/brilliant-violet-421-anti-mouse-human-cd44-antibody-7225
https://www.bioreagent.com/en-us/products/apc-anti-mouse-cd62l-antibody-381
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

KP and KL lung tumor nodules were obtained from genetically engineered mouse models (GEMM) harboring a conditional activating mutation of endogenous Kras (KrasL5L-G12D+) crossed with Lkb1 or p53 conditional knockout (Lkb1/+/l or Trp533/3). And tumor cell lines were generated from these nodules ex vivo.

Human NSCLC cell lines used in the study H23, H460 and A427 and HEK293T were purchased from ATCC.

For KL and KP Lkl shRNA stable cell line generation, lentiviral vector for Lkl shRNA (Sigma TRCN0000319764 and TRCN0000028768) or control pi.KO.1 vector (Sigma Cat # SHC001) was employed. For the lentivirus production, HEK-293T cells were co-transfected with the three-vector system using Lipofectamine 3000 (Cat #L3000008, Invitrogen) including pi.KO.1-shRNA vector and packaging vectors psPAX2 (Addgene #12260) and pMD2.G (Addgene #12259). Prior to infection, cell culture supernatant was passed through 0.45 μm syringe filter (Corning Cat #431220) and the filtered virus was added to KL cells in a 6-well plate in the presence of polybrene (10 μg/ml, Sigma Cat #TR-1003-G). The transduced cells were selected with Puromycin (Sigma Cat #P9620) 48 hrs post infection. And selected stable cell lines were validated by western blot and maintained in the cell culture media with 2 μg/ml Puromycin.

Generation of isogenic lines with empty vector (pBABE) (Addgene #1764), wild type LKB1 (pBABE-LKB1, Addgene #8592) and LKB1-KD (Addgene #8593) were performed similar as previously described. Briefly, HEK-293T cells were transfected with the pBABE-LKB1, LKB1-KD or pBABE vectors, along with pCL-Eco (Addgene Plasmid #12371) and pCMV-VSV-G (Addgene #8454) packaging vectors using Lipofectamine 3000. Supernatant containing virus were filtered before transduction of the cells. The positive clones were selected with puromycin and validated by western blot.

For Atg knockdown cell lines generation, shRNA vectors were obtained from Sigma MISSION TRC shRNA library with clone ID as below: shAtg7 #1 (mouse) TRCN0000305991, shAtg7 #2 (mouse) RCRN0000375444, shAtg13 #1 (mouse) TRCN0000277121, shAtg13 #2 (mouse) TRCN0000176029, shGFP TRCN000072186. GFP–LC3–RFP (Addgene, plasmid 117413), pINDUCER20-mStrawberry and pINDUCER20-mStrawberry-Atg4B4C4A were gifts from Alec Kimmelman. KL GFP shRNA, Atg7 shRNA, Atg13 shRNA and Atg4B4C4A stable cell lines were generated using lentiviral packaging system described above.

Authentication

All human NSCLC cell lines and derived isogenic lines were authenticated by STR at The Centre for Applied Genomics Genetic Analysis Facility (TCAG, Canada). HEK293T cells were authenticated by ATCC using STR profiling.

The mouse KP and KL cells were used were authenticated by PCR genotyping routinely run in the lab.

Mycoplasma contamination

All the cell lines used were tested for mycoplasma using Universal Mycoplasma Detection Kit (ATCC) with negative result.

Commonly misidentified lines

(See ICTAC register)

Cell lines used are not on the list of commonly misidentified lines.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

The genetically engineered mouse model (GEMM) harboring a conditional activating mutation of endogenous Kras (KrasL5L-G12D+) crossed with Lkb1 or p53 conditional knockout (Lkb1/+/l or Trp533/3) has been previously described. Briefly KrasL5L-G12D strain carries a Lox-Stop-Lox (LSL) sequence followed by the KrasG12D point mutation allele commonly associated with human cancer. Trp53 exons 2-10 are flanked by loxp sites in this conditional targeted mutation. For Lkb1/+/l mice, the transcript from the Lkb1 null allele eliminates exons 2-6, resulting in a translational frameshift. All the mice are crossed and confirmed with C57BL/6J genetic background by SNP analysis, 99.59% for KP (KrasG12DTrp533/3) and 94.46% for KL (KrasG12DLkb1/+/l). Age and sex matched mice (6-8 weeks, male and female mice) were induced with CRE recombinase using adeno-Cre virus for all mice. All mice were bred and maintained in the animal facility at NYU Langone School of Medical. For all clinical experiment of KL, tumors, female B6C3F-Tyr-2/J (B6-albino) mice (6-8 wks) were purchased from Jackson lab as host, and maintained at animal facility at NYU Langone. For additional information please contact corresponding author.

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve samples collected from the field.

Ethics oversight

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the New York University School of Medicine (NYU/SoM).
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g., CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Sample preparation is detailed in “Method: cell isolation for immune analysis” section

Instrument
BD LSFRFortessa X-20 and BD Biosciences LSRII

Software
FACSDiva (BD) was used for sample collection and Flowjo (BD) was used for data analysis

Cell population abundance
N/A. Total lung with tumor and immune cells were collected for FACS analysis and counted for total cell number. Each population analyzed were stained and analyzed. For each sample, total CD45 collected is higher than 10,000 cells to ensure enough target cells were obtained for analysis.

Gating strategy
For each sample, FSC-A and SSC-A were used to exclude cell debris and aggregates of the sample, followed by FSC-W/FSC-H positive and SSC-W/SSC-H positive to define single cells and exclude doublets, live/dead-aqua stain were used to exclude dead cells. Within all live/dead-aqua negative live cells, CD45+ -> CD3+ were used to define T cells, with CD4+ or CD8+ cells gated for further analysis. Gating strategy was demonstrated in Extended Data Fig. 8b.

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary information.

Magnetic resonance imaging

Experimental design

Design type
The genetically engineered mouse model (GEMM) harboring a conditional activating mutation of endogenous Kras (KrasLSL-G12D) was crossed with Lkb1 or p53 conditional knockout ([Lkb1f/f] or Trp53f/f)) were induced by CRE recombinase through intranasal inhalation of 1x107 p.f.u. adeno-Cre (University of Iowa adenoviral core). Kl cells were injected into female B6(Cg)-Tyr-c2J/J (B6-albino) mice via tail vein injection at 1x106/mice and the development of lung nodules. The developed lung nodules were evaluated by MRI imaging to quantify the lung tumor burden.

Design specifications
Mice are anesthetized (~5 min) initially in a chamber using a mixture of oxygen (1000 cc/min) and isoflurane (3%). Once an animal is fully anesthetized, a mixture of oxygen and isoflurane change to 500cc/min and 1.5%, optical lubricant is placed on each eye to prevent drying. The mouse is then placed in a prone position on a plastic bed fitted with a nose cone that continuously supplied oxygen (1000cc/min) and isoflurane (1.5%) during the scan. A respiration pad is taped over the mouse’s up part of abdomen to monitor the breathing rate for respiratory gating. The mouse is heated to maintain a core temperature of approximately 37°C by blowing thermally controlled warm air into the magnet’s bore. The anesthesia was adjusted to maintain a constant breathing rate so that there is little variability in MR characteristics, imaging time, and imaging quality during and between runs. Respiration pad is connected with interface modules to a computer that ran the PC-SAM small animal monitoring and gating software (SAIL Instruments). After centered the mouse’s chest with laser, the plastic bed is moved and placed in the RF coil so that the mouse’s thoracic cavity is positioned both in the center of the homogeneous RF field and at the isocenter of the magnet.

Behavioral performance measures
Animal is fully anesthetized, a mixture of oxygen and isoflurane and a respiration pad is taped to monitor the breathing rate for respiratory and controlled between 30-100.

Acquisition

Imaging type(s)
structural

Field strength
7.0 Tesla

Sequence & imaging parameters
Rapid Acquisition for positioning
A 3-plane localizer scout scan (~1 min) is acquired using an ungated Rapid Acquisition with Relaxation Enhancement (RARE) pulse sequence with the following parameters: repetition time (TR) = 15 ms, echo time (TE) = 1.7 ms, field of view (FOV) = 5 cm, matrix=256 x256, slice thickness = 1 mm, and flip angle=15.0 degrees. This scan verifies that the mouse’s thoracic cavity is at the isocenter of the magnet and RF coil. If not, the mouse’s
position was adjusted, and a new 3-plane scout scan (~1 min) is acquired.

High resolution scan
When the mouse is properly positioned, a set of slices (24 slices) short axial plane of the high-resolution scan is positioned using the 3-plane localizer scout images with the Gradient Echo, a Fast Low Angle Shot (FLASH) pulse sequence: TR=107.0 ms, TE effect = 1.7 ms, field of view (FOV) =3 cm, matrix=256 x256, slice thickness =1 mm, number of averages = 13, and flip angle=15.0 degrees, and with the number of slices sufficient to cover the entire lungs.

Whole lung area is imaged. The region of lung was determined by rapid acquisition, and verifies that the mouse’s thoracic cavity is at the isocenter of the magnet and RF coil.

Preprocessing
Preprocessing software
3D slicer 4.8.0
Normalization
Data acquired were not normalized
Normalization template
Data acquired were not normalized
Noise and artifact removal
Noise were removed by calculating below 20% of max threshold as background noise in 3D slicer.
Volume censoring
n/a

Statistical modeling & inference
Model type and settings
n/a
Effect(s) tested
n/a
Specify type of analysis:
□ Whole brain  ✗ ROI-based  □ Both
Anatomical location(s)
The location of the tumors quantified were manually annotated within lung region using paint effect within the threshold setting. Heart and liver area were excluded.

Statistic type for inference (See Klund et al. 2016)
n/a
Correction
n/a

Models & analysis
n/a | Involved in the study
✗ Functional and/or effective connectivity
✗ Graph analysis
✗ Multivariate modeling or predictive analysis