The genetic and metabolic heterogeneity of RAS-driven cancers has confounded therapeutic strategies in the clinic. To address this, rapid and genetically tractable animal models are needed that recapitulate the heterogeneity of RAS-driven cancers in vivo. Here, we generate a *Drosophila melanogaster* model of Ras/Lkb1 mutant carcinoma. We show that low-level expression of oncogenic Ras (RasLow) promotes the survival of Lkb1 mutant tissue, but results in autonomous cell cycle arrest and non-autonomous overgrowth of wild-type tissue. In contrast, high-level expression of oncogenic Ras (RasHigh) transforms Lkb1 mutant tissue resulting in lethal malignant tumors. Using simultaneous multiview light-sheet microcopy, we have characterized invasion phenotypes of Ras/Lkb1 tumors in living larvae. Our molecular analysis reveals sustained activation of the AMPK pathway in malignant Ras/Lkb1 tumors, and demonstrate the genetic and pharmacologic dependence of these tumors on CaMK-activated Ampk. We further show that LKB1 mutant human lung adenocarcinoma patients with high levels of oncogenic KRAS exhibit worse overall survival and increased AMPK activation. Our results suggest that high levels of oncogenic KRAS is a driving event in the malignant transformation of LKB1 mutant tissue, and uncovers a vulnerability that may be used to target this aggressive genetic subset of RAS-driven tumors.
KRAS is the most commonly mutated oncogene in human cancer, and is frequently mutated in cancer types associated with high mortality such as non-small cell lung cancer. Efforts to directly target the KRAS protein have been challenging, although renewed efforts are currently in clinical trials. Large-scale sequencing of lung adenocarcinoma has uncovered heterogeneity in mutant KRAS tumors due to concomitantly mutated tumor suppressor genes such as TP53 and LKB1, genetic subtypes that are largely mutually exclusive and which harbor distinct biologies and therapeutic susceptibilities. An added layer of complexity arises due to the extensive metabolic rewiring observed in Ras-driven tumors, which can arise due to Ras-mutant dosage and alterations in signaling pathways downstream of mutated tumor suppressor genes. Increasingly, metabolic rewiring is known to be dependent on tissue-level dynamics within the tumor and the tumor microenvironment. Therefore, there is a need to develop rapid and powerful models of Ras-driven cancers that mimic the complex landscape of these tumors in vivo.

Liver Kinase B1 (LKB1) is a master serine/threonine kinase that phosphorylates 13 downstream kinases of the AMP-activated protein kinase family (AMPK) family to control cell growth and cell polarity. LKB1 activity is lost in a wide spectrum of human cancers and the gene that encodes LKB1 (STK11) is the third most frequently mutated tumor suppressor in human lung adenocarcinoma. Loss of LKB1 frequently occurs in KRAS-driven lung adenocarcinoma, and has been shown to promote metastasis, shorten overall survival, and confer resistance to targeted therapies and checkpoint inhibitors. Altogether, these differences in survival and treatment outcomes highlight the importance of in vivo models that recapitulate the complexity and heterogeneity of these tumors when developing and implementing cancer treatments.

Drosophila melanogaster is a powerful model system for studying cancer biology due to the high conservation of human oncogene and tumor suppressor pathways. Elegant genetic mosaic techniques in Drosophila allow tissue-specific overexpression of oncogenes and knockdown of tumor suppressors within distinct subpopulations of cells, which bestows the ability to build complex tumor landscapes in vivo. Seminal work using these methods has identified cooperating mutations that promote the metastasis of benign Ras-mutant tumors in vivo, and has identified such cooperating models as amenable to pharmacologic approaches. However, despite evidence from mouse models that loss of Lkb1 is sufficient to promote tumor progression and metastasis in Ras-mutant lung tumors, there has been no report of malignant synergy between Ras and Lkb1 using the rapid and genetically tractable Drosophila model.

Here, using a Drosophila model of Ras/Lkb1-driven malignant progression, we found that the relative levels of oncogenic Ras determine clonal growth dynamics in Lkb1 mutant tissue. Low levels of oncogenic Ras promote non-autonomous growth of surrounding wild-type tissue, while high levels promote malignant progression and organotypical lethality. To further characterize the metastatic capability of Ras/Lkb1 malignant cells we used simultaneous multiview light-sheet microscopy to image live tumor-bearing larvae for up to 48 h, and show that Ras/Lkb1 cells actively degrade basement membrane, and ultimately invade distant tissues. To further define the mechanism driving the progressive synergy between high oncogenic Ras and loss of Lkb1 we investigated signaling networks in mosaic tissue. We show that malignant Ras/Lkb1 tumors activate AMPK and are dependent on the activation of the Drosophila ortholog of CAMKK2. We validated the translational potential of our work by showing high-level KRAS with concurrent mutation in LKB1 represents a unique subset of patients with worse overall survival and increased AMPK activation. Our work uncovers oncogenic KRAS copy number gains or amplification as a synergistic mechanism that drives the aggressive nature of LKB1 mutant tumors. In addition, our work proves Drosophila as a powerful model for the rational design of targeted therapies for genetic subsets of RAS-driven cancers, and suggests that the LKB1 subset of KRAS-driven cancers may benefit from targeting of the CAMKK/AMPK circuit.

Results

Clonal loss of Lkb1 in vivo results in autonomous cell death.

Recent work has highlighted effects of the dosage of oncogenic Ras on the progression of Ras-dependent cancers. Previous work in Drosophila has identified myriad pathways that collaborate with Ras to promote tumor progression and metastasis, but how the dosage of Ras affects tumor progression in these multiple hit models is unknown. To address this question, we identified oncogenic Ras transgenes with differing expression levels. One expresses oncogenic Ras at levels similar to endogenous Ras (RasLow). The other expresses Ras at levels several-fold higher (RasHigh) (Fig. 1b and Supplementary Fig. 4). To mimic the genetic landscape of human KRAS-driven cancers we chose to co-mutate the tumor suppressor LKB1 in RasLow and RasHigh tissue. Most tumor-specific LKB1 mutations are homozygous deletions or loss-of-heterozygosity with somatic mutation. Among the latter, nonsense or frameshift mutations leading to protein truncation are the most common. To identify the Drosophila Lkb1 loss-of-function allele with the strongest reduction in Lkb1 protein levels we first generated an antibody to Drosophila Lkb1. We then assayed for Lkb1 protein in transheterozygous larvae using three previously published Lkb1 loss-of-function alleles (X525, 4B1-11, and 4A4-2) over a large deletion that removes the Lkb1 gene. The Lkb1X525 and Lkb1IB1-11 loss-of-function alleles reduced Lkb1 protein expression by 60% compared to control. However, the Lkb1AA4-2 allele reduced protein expression by 80% (Fig. 1a and Supplementary Fig. 4), which agrees with prior published genetic data suggesting Lkb1IB1-11 as having residual protein activity. The Lkb1AA4-2 allele was chosen for further study and will be referred to as Lkb1−/−.

We used the GFP-labeled eye expression system to express RasLow in discreet patches or ‘clones’ of developing eye epithelial tissue. Expression of RasLow resulted in ablation of eye tissue and benign outgrowths of eye cuticle similar to what has been reported in prior reports using a UAS-RasV12 transgene (Fig. 1c). We then used the GFP-labeled eye expression system to inactivate the Lkb1 tumor suppressor (Lkb1−/−) in clones of cells in the developing eye. Inactivation of Lkb1 in clones resulted in adult flies with small, rough eyes (Fig. 1c), suggesting high levels of apoptosis. To test this, we assayed for cleaved death caspase 1 (DCP1) in mutant clones using immunofluorescence in wandering 3rd instar eye-imaginal discs. As expected, loss of Lkb1 (marked by GFP+ tissue) resulted in a large increase in autonomous cleaved DCP1 expression as compared to discs carrying control FRT82B clones (Fig. 1d, e and Supplementary Data 1). These data suggest that homozygous loss of Lkb1 within an otherwise wild-type epithelium can result in a high level of apoptosis in vivo.

Low-level Ras and loss of Lkb1 synergize to promote non-autonomous benign overgrowth. Data from genetically engineered mouse models suggests loss of Lkb1 is sufficient to promote the progression and metastasis of nascent Kras-mutant lung adenocarcinoma. Due to the redundancy of the vertebrate genome and paucity of rapid genetic mosaic analyses
in mice, we sought to use the GFP-labeled *Drosophila* eye expression system to build a Ras/Lkb1 model of cooperative tumorigenesis. We simultaneously expressed Ras Low and depleted Lkb1 (RasLow/Lkb1−/−) in clones of developing eye epithelial tissue, and found that autonomous DCP1 levels returned to those observed in control eye-imaginal disks (Fig. 1e; Δ mean = +41.7 [95% CI, 26.0–57.4]). These data suggest that low levels of oncogenic Ras promote the survival of Lkb1−/− mutant tissue in vivo. In addition, eye-imaginal disc complexes carrying RasLow/Lkb1−/− clones were larger than mosaic control discs but contained only a small amount of mutant GFP+ tissue compared to the expression of Ras Low alone. In agreement with these results, analysis of adult RasLow/Lkb1−/− mosaic eyes revealed a large, overgrown eye phenotype composed of mostly GFP− wild-type cells (Fig. 1c, f). To confirm the overgrown eye phenotype was due to synergy between Ras and Lkb1 and not to simply preventing cell death in Lkb1 mutant cells we expressed the baculoviral caspase inhibitor p35 in Lkb1 mutant clones. Expressing p35 in Lkb1 mutant clones resulted in a majority of flies with eyes that are phenotypically similar to expression of p35 alone (normal size eye), with 20% of flies exhibiting a more severe smaller malformed eye (Supplementary Fig. 1).

To investigate the mechanism that results in an increase in organ size in RasLow/Lkb1−/− flies, we analyzed BrdU incorporation in mosaic RasLow/Lkb1−/− eye-imaginal disc tissue. Eye disc tissue carrying RasLow/Lkb1−/− clones exhibits BrdU incorporation in GFP− wild-type cells surrounding mutant clones (Fig. 2a). In addition, we analyzed mosaic RasLow/Lkb1−/− eye-imaginal disc tissue by fluorescence-activated cell sorting (FACS). This analysis revealed an increase in the percentage of GFP+ mutant cells in G1 when compared to GFP+ cells from control FRT82B discs (Fig. 2b, c and Supplementary Data 2). Altogether, these data suggest that although RasLow/Lkb1−/− mutant cells survive, they undergo G1 arrest while promoting the increased hyperplastic proliferation of surrounding wild-type tissue.
High-level oncogenic Kras promotes the neoplastic transformation of \( \text{Lkb1} \) mutant tissue. Previous studies have implicated the dose of mutant Kras in tumor progression, cell motility, and metabolic reprogramming\(^{18,19,29} \), therefore we used the GFP-labeled eye expression system to clonally express Ras\(^{\text{High}} \) and mutate \( \text{Lkb1} \) in developing eye epithelia (Ras\(^{\text{High}} \)/Lkb\(^{-}\)). When combined with \( \text{Lkb1} \) loss-of-function, expression of Ras\(^{\text{High}} \) resulted in severely overgrown and disorganized 3rd instar larval eye-imaginal disc tumors composed of mostly GFP\(^+\) mutant tissue (Fig. 3a). FACS analysis of mutant tissue revealed a shift in cell-cycle phasing that favored G2/M, suggesting that mutant cells were precociously completing G1 (Fig. 2b, c). The majority of larvae carrying Ras\(^{\text{High}} \)/Lkb\(^{-}\) mosaic discs did not pupate but continued to grow into ‘giant larvae’ while expression of Ras\(^{\text{High}} \) alone resulted in late pupal lethality (Fig. 3b). The giant larval phenotype is shared by loss-of-function mutations in the \( \text{Drosophila} \) neoplastic tumor suppressor genes\(^{30} \) and suggests that Ras\(^{\text{High}} \)/Lkb\(^{-}\) tumors are malignant. To test this, we performed an allograft assay by implanting control, Ras\(^{\text{Low}} \)/Lkb\(^{-}\), and Ras\(^{\text{High}} \)/Lkb\(^{-}\) GFP\(^+\) tumor tissue in the abdomens of wild-type hosts. Transplanted control and Ras\(^{\text{Low}} \)/Lkb\(^{-}\) tissue failed to grow in host abdomens (Fig. 3e). Surprisingly, the lifespan of hosts with transplanted Ras\(^{\text{Low}} \)/Lkb\(^{-}\) tissue was shortened which suggests that residual GFP\(^-\) ‘wild-type’ tissue from the transplant could be partially transformed. In contrast, only transplanted Ras\(^{\text{High}} \)/Lkb\(^{-}\) tissue was able to grow into visible secondary tumors that significantly shortened host survival (Fig. 3e, f and Supplementary Data 3) thus confirming the malignancy of Ras\(^{\text{High}} \)/Lkb\(^{-}\) tumor tissue.

High-level Ras promotes the invasion and metastasis of Lkb1 mutant tissue. Mutations in cell polarity proteins cooperate with oncogenic Ras to drive tumor cell invasion and metastasis\(^{20} \).

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**Fig. 2** The level of oncogenic Ras determines distinct autonomous vs. non-autonomous cell-cycle phenotypes in Lkb1 mutant tissue. a Confocal images of mosaic eye-imaginal discs carrying GFP\(^+\)-clones of the indicated genotypes (control = FRT82B), and stained for BrdU incorporation (magenta). Images are representative of \( n = 10 \) independent eye-imaginal discs per genotype. Scale bar, 100 \( \mu \)m. b Fluorescence-activated cell sorting (FACS) analysis of mosaic eye-imaginal discs with GFP-labeled clones of the indicated genotypes. Black arrows point to shifts in relative cell-cycle phasing. Analysis is representative of \( n = 3 \) independent experiments of 20 mutant imaginal discs/genotype and 40 imaginal discs/genotype for control. c Histogram showing percentage of GFP-labeled control or mutant cells in each phase of the cell cycle.
Previous studies have shown that Lkb1 regulates cell polarity and epithelial integrity across species, therefore, we hypothesized that malignant Ras\textsuperscript{High}/Lkb1\textsuperscript{−/−} tumors would have invasive properties. To test this, we first examined whether Ras/Lkb1 mutant cells compromised basement membrane structure by examining the expression of GFP-tagged Collagen IV (Viking (Vkg)-GFP) using conventional fixation and confocal microscopy. Compared to control and Ras\textsuperscript{Low}/Lkb1\textsuperscript{−/−} tissue which shows contiguous Vkg-GFP expression in epithelia, Ras\textsuperscript{High}/Lkb1\textsuperscript{−/−} tissue exhibits breaks in Vkg-GFP expression (Fig. 3c). Expressing Ras\textsuperscript{High} on its own is lethal (albeit at the pharate adult stage), so we investigated Vkg-GFP in this genotype and once again found no breaks in the structure of Vkg-GFP. We next assayed matrix metalloproteinase (MMP) expression, as MMPs degrade basement membrane. Compared to control, Ras\textsuperscript{Low}/Lkb1\textsuperscript{−/−}, and Ras\textsuperscript{High} clones, Ras\textsuperscript{High}/Lkb1\textsuperscript{−/−} mutant tissues express high levels of autonomous MMPs (Fig. 3d). Last, we measured the extent to which Ras\textsuperscript{High}/Lkb1\textsuperscript{−/−} cells invade local tissues by dissecting cephalic complexes and assaying extent of migration over the ventral nerve cord (VNC). Compared to Ras\textsuperscript{High} control tissue which exhibits benign overgrowths confined to the eye-antennal discs, Ras\textsuperscript{High}/Lkb1\textsuperscript{−/−} cells completely invade and obscurbs contiguous organs. Scale bar, 1 mm. Images are representative of n = 10 cephalic complexes/genotype.

Fig. 3 Oncogenic Ras\textsuperscript{High} promotes the malignant transformation of Lkb1 mutant tissue. a Fluorescent images of 3rd instar larval eye-imaginal discs (exception labeled ‘13d AEL’) carrying GFP+ clones of the indicated genotypes (control = FRT82B). Images are representative of n = 10 independent eye-imaginal discs per genotype. Scale bar, 20 μm. AEL = after egg-lay. The stage ‘13 days AEL’ is indicative of a larva that failed to pupate at day ~5d AEL, and is a classic neoplastic phenotype. b Representative brightfield image of the lethal stage of a fly carrying Ras\textsuperscript{High} clones (left) and Ras\textsuperscript{High}/Lkb1\textsuperscript{−/−} clones (right). Note that both age-matched third instar and giant larvae are shown for the Ras\textsuperscript{High}/Lkb1\textsuperscript{−/−} genotype. c Confocal images of eye-imaginal discs carrying RFP+ clones (magenta) of the indicated genotypes and expressing type IV collagen-GFP (Vkg-GFP). White arrow indicates breaks in Vkg-GFP. a = apical, b = basal. Images are representative of n = 5 independent eye-imaginal discs per genotype. Scale bar, 100 μm. d Confocal images of third instar eye discs carrying GFP+ clones of the indicated genotypes, and stained for matrix metalloproteinase 1 (MMP1, magenta in overlay). Images are representative of n = 10 independent eye-imaginal discs per genotype. Scale bar, 100 μm. e Fluorescent images of w\textsuperscript{1118} adult virgin female hosts carrying transplanted allografts of 3rd instar eye-imaginal discs with GFP+ clones of the indicated genotypes. Scale bar, 100 μm. f Quantification of survival post-transplant in allograft assay. Survival was measured from 7 days post-transplant to time of death. CTRL (FRT82B, n = 7), KL\textsuperscript{Low} (Ras\textsuperscript{Low}/Lkb1\textsuperscript{−/−}, n = 8), and KL\textsuperscript{High} (Ras\textsuperscript{High}/Lkb1\textsuperscript{−/−}, n = 13) (CTRL-KL\textsuperscript{Low}, ***p = 0.00030, CTRL-KL\textsuperscript{High}, ***p = 0.00001, KL\textsuperscript{Low}- KL\textsuperscript{High}, *p = 0.0129, Log-rank test). g Representative fluorescent images of dissected cephalic complexes and ventral nerve cord (VNC) from larvae carrying GFP+ clones (white) of the indicated genotypes. BH = brain hemispheres; E/A = eye/antennal discs, MH = mouth hooks. The Ras\textsuperscript{High}/Lkb1\textsuperscript{−/−} tissue completely invades and obscures contiguous organs. Scale bar, 1 mm. Images are representative of n = 10 cephalic complexes/genotype.
intravital imaging and used simultaneous multiview (SiMView) light-sheet microscopy33 to visualize tumor cell and collagen IV dynamics for up to 48 h. SiMView allowed imaging of rapid cellular processes over time on an organismal scale, with minimal photobleaching. We collected image stacks in the z range every 60-s on two individual ‘giant’ tumor-bearing larvae with RFP-tagged RasHigh/Lkb1−/− mutant cells and Vkg-GFP expressed in the basement membrane of all epithelial tissues. Breakdown of Vkg-GFP was visible over time in each individual larva, especially in overlying tracheal branches dorsal to the tumor surface (Fig. 4a and Supplementary Movie 1). We defined two independent regions of interest in each larva that encompassed a tumor-adjacent tracheal branch and calculated Vkg-GFP pixel intensity every 2 h over a 14 h imaging window. Using the wing disc of each animal as an internal control, we observed a statistically significant difference in the change in levels of Vkg-GFP over the imaging window in the tracheal branches (Fig. 4b). Volumetric rendering and surface reconstruction of the tracheal branches revealed tumor cells in contact with trachea at several hundred µm away from the primary tumors (Fig. 4c–e) and on rare occasions were found on the ‘interior’ surface of Vkg-GFP. These data suggest RasHigh/Lkb1−/− mutant cells actively invade tracheal vascular cells to potentially spread to distant organs.

RasHigh/Lkb1−/− malignant tumors depend on CaMK/Ampk signaling in vivo. Targeting effector signaling in KRAS-driven non-small cell lung cancer has resulted in limited efficacy in the clinic. In addition, previous studies have highlighted the additional complex transcriptional and signaling network changes in KRAS-driven tumors co-mutated for the tumor suppressor LKB15. Therefore, rapid and genetically tractable models of Kras/Lkb1 tumors may shed light on the complex rewiring of signaling pathways and highlight novel targeting approaches. To probe effector pathways in our tumor model we used Western analysis on a panel of Drosophila epithelia harboring mutant clones for RasLow, RasLow/Lkb1−/−, RasHigh, and RasHigh/Lkb1−/−. Similar
to human KRAS/LKB1 tumors, increases in the activation of the RAS effector circuit Erk/Mek were observed along with S6K and 4EBP1 suggesting increased mTOR pathway activity (Fig. 5a and Supplementary Fig. 5). Compared to all other genotypes AKT is not active in RasHigh/Lkb1−/− cells most likely owing to sustained pS6K signaling resulting in a negative feedback loop by ribosomal protein S6. Previous studies have attributed increased TOR pathway activity in LKB1 mutant tissue to loss of mTOR pathway inhibition by AMPK34. Therefore, we tested for loss of AMPK activity in our panel of Lkb1−/− mutant Drosophila tissue. We observed basal activation of Ampk in control tissue, followed by minimal activation in RasLow/Lkb1−/− mutants, most likely resulting from the overgrowth of surrounding wild-type epithelial tissue (Fig. 5b and Supplementary Fig. 5). However, in RasHigh/Lkb1−/− tissue we observed sustained pAmpk levels by Western blot, which was confirmed by immunofluorescence in mosaic imaginal discs (Fig. 5b, c). Recently, the presumed role of Ampk as a tumor suppressor has been challenged by evidence that...
Ampk can promote metabolic adaptation to effect tumor growth and survival. To test whether RasHigh/Lkb1−/− tumors are dependent on the genetic dose of ampk we expressed an RNAi transgene to ampk (knockdown efficiency of 50%; Supplementary Figs. 2a and 6) in developing GFP+ RasHigh/Lkb1−/− tissue. Inhibition of Ampk via RNAi in RasHigh/Lkb1−/− mutant clones resulted in a statistically significant percentage of flies surviving to adulthood (Δ mean = +8; 95% CI, 3.697–12.3) (Fig. 5e). Interestingly, surviving flies exhibited hyperplastic outgrowth similar to that of RasLow/Lkb1−/− adult flies (compare Figs. 5d to 1c). A recent study from the Guo group found that autophagy may sustain AMPK activity upon growth. In support of this, we detected increased lipidated s6K upon feeding developing RasHigh/Lkb1−/− larvae with the inhibitor torin1 (Fig. 5a and Supplementary Fig. 5). Altogether, these data support the conclusion that activation of Ampk is maintained in RasHigh/Lkb1−/− tumors and is required autonomously to promote malignant progression of Kras/Lkb1 tumors in vivo.

The Ca2+/calmodulin-dependent protein kinase kinase (CaMKK2) is a nucleotide-independent activator of AMPK37, therefore we assayed activation of the Drosophila ortholog CamkIIB (48% identical/63% similar to CaMKK2) in our panel of mutant tissue. We found that activation of CamkIIB was elevated in RasHigh/Lkb1−/− tumors (Fig. 5a and Supplementary Fig. 5), suggesting a conserved role for this kinase in activating Ampk in the presence of oncogenic Ras tumors lacking Lkb1. To test whether RasHigh/Lkb1−/− tumors are dependent on CamkIIB activity we performed pharmacological inhibition of the CaMK cascade feeding developing RasHigh/Lkb1−/− larvae with the inhibitor KN-9338, which in our model inhibited activation of the Drosophila CamkIIB by 47% (Supplementary Figs. 2b and 6). Treatment of RasHigh/Lkb1−/− larvae resulted in a rescue of whole-organismal lethality, with an increase in the number of flies surviving to the pupal and adult stage (6.5% adult survival for KN-93 vs. 0% adult survival for vehicle control) (Fig. 5f–h and Supplementary Data 4). Taken together, these data suggest that in the context of loss of Lkb1, high levels of oncogenic Ras result in activation of Ampk by the alternative sole Drosophila CAMKK2 ortholog. Moreover, our pharmacologic results suggest that targeting the upstream AMPK/CAMKK complex may offer therapeutic benefit to Kras/Lkb1 mutant lung adenocarcinoma patients.

High levels of oncogenic KRAS and loss of LKB1 result in decreased patient survival and AMPK signaling circuit activation in the TCGA lung adenocarcinoma cohort. To test the translational relevance of our findings in Drosophila we analyzed human lung adenocarcinoma genomic and clinical data using cBioPortal39,40 to study how differences in levels of oncogenic KRAS affect tumor progression in Lkb1 mutant patients. We used the TCGA Lung Adenocarcinoma PanCancer Atlas and TCGA Provisional Lung Adenocarcinoma datasets to select the proportion of patients with KRAS mutations in codon 12 (G12C, G12D, or G12V) for further study. We then used available RNA-sequencing data to stratify patients as either KRASlow or KRASHigh. We next investigated overall patient survival by comparing cohorts of KRASLow or KRASHigh alone, to those that contained mono, biallelic loss, and/or loss-of-function mutations in LKB1. We found no difference in overall survival in KRASlow/LKB1Mut vs. KRASlow patients (HR 2.181 95% CI, 0.9136–5.205), but strikingly KRASHigh/LKB1Mut patients exhibited significantly worse overall survival when compared with RASHigh patients (HR 2.72; 95% CI, 1.132–6.546) (Fig. 6a, b). We then tested whether KRAS copy number changes could account for the change in overall survival. Similar results were obtained when patients were stratified into either oncogenic KRASDiploid (HR 2.048; 95% CI, 0.8241–5.090) or KRASGain/Amp (HR 4.993; 95% CI, 2.057–2.12) (Fig. 6c, d). Interestingly, the ability of high level vs. low-level KRAS to drive survival differences did not extend to patients with TP53 mutations (Supplementary Fig. 3).

A recent study has reported that Ampk has a pro-tumorigenic role in lung cancer genetically engineered mice with Kras and p53 mutations. Moreover, data from our Drosophila Lkb1 mutant tumor model indicate that halving the genetic dose of ampk is sufficient to partially reverse whole-organism lethality. To test whether AMPK signaling may be involved in human Kras/Lkb1 mutant lung adenocarcinoma we performed a correlation analysis between pAMPK and oncogenic codon 12 KRAS mRNA for LKB1 loss-of-function and LKB1 wild-type patients using TCGA data. We detected a positive correlation trend between pAMPK and oncogenic KRAS levels, but only in LKB1 mutant patients (Spearman’s correlation coefficient = 0.3, p = 0.068 for LKB1 loss-of-function vs. coefficient = −0.076, p = 0.683 for LKB1 wild-type patients) (Fig. 6e, f). To further test our hypothesis, we used canonical circuit activity analysis42 which recodes gene expression data into measurements of changes in the activity of signaling circuits, ultimately providing high-throughput estimations of cell function. We performed the analysis to estimate the activity of the AMPK pathway in KRASHigh/LKB1Mut lung adenocarcinoma patients compared to KRASHigh patients. The activity of three effector circuits is significantly (FDR < 0.05) upregulated in KRASHigh/LKB1Mut patients, one ending in the node that contains PPARGC1A (encodes PGC1alpha), the second one ending in the node with the MLYCD gene, and the third ending in the node containing EIF4EBP1 (Fig. 6g). These three genes control the cellular processes of circadian control of mitochondrial biogenesis, fatty acid metabolism, and translation regulation, and are known to be upregulated in various cancers43–45. These data confirm the translational relevance of our Drosophila model, and suggest that high oncogenic KRAS levels, perhaps through copy number gains and amplifications, activate specific sub-circuits of the AMPK signaling pathway to drive the malignant progression of LKB1 mutant tumors.

Discussion
It has been proposed that RAS-induced senescence functions as a tumor-suppressive mechanism46. More recent data have built upon these studies to show that high levels of Hras are required to activate tumor suppressor pathways in vivo, and that doubling the levels of oncogenic Kras is sufficient to cause metabolic rewiring leading to differences in therapeutic susceptibilities. Mutant Kras copy gains are positively selected for during tumor progression in a p53 mutant background; however, our results analyzing survival in patients indicate that unlike Kras/Lkb1 mutant patients, high levels of KRAS in TP53-mutant lung adenocarcinoma patients may not be a key factor in determining overall survival. In contrast, high-level KRAS and loss of LKB1 leads to significantly decreased overall survival in lung cancer. Interestingly, LKB1 has been shown to control genome integrity downstream of DNA damaging agents and cellular accumulation of ROS. Moreover, alterations in LKB1 occur more frequently in patients with no known mitogenic driver. Future work should uncover whether Kras copy number gains and amplifications are positively selected for due to the role of LKB1 as a gatekeeper of genome integrity.

Seminal work in Drosophila identified the loss of epithelial polarity genes as key cooperating events in Ras-driven tumors in vivo. In addition to its role in regulating cell growth, the Lkb1 protein is required to establish and maintain cell polarity...
across eukaryotes. However, alleles of Lkb1 were not reported to synergize with oncogenic Ras in these studies, the reason possibly due to insufficient oncogenic Ras levels. The fact that loss of Lkb1 behaves differently than other known polarity mutants suggests that an alternate function underlies the aggressive nature of RasHigh/Lkb1−/− tumors in vivo. Moreover, our findings in lung adenocarcinoma patients suggest that increased oncogenic KRAS is associated with increased activation of pAMPK in Ras/Lkb1 mutant patients. The fact that pharmacologic inhibition of the CaMK pathway to activate Ampk in RasHigh/Lkb1−/− mutant lung adenocarcinoma. In addition, work is needed to elucidate the mechanism used by high-level Ras signaling to engage the CaMK pathway. Last, our work is the first study to show that Ampk can have a pro-tumorigenic role in Lkb1 mutant cancer in vivo, and suggests that KRAS/LKB1 mutant lung adenocarcinoma patients may benefit from CAMKK inhibitors.

Methods

Drosophila stocks and maintenance. Flies were grown on a molasses-based food at 25°C.

The following Drosophila stocks were used: (i) w1118, FRT82B, (ii) Df(3R) Exel169,P[XP-U]Exel169/TM6B,Tb (#7648), (iii) UAS-RasV12, FRT82B (rasHigh—modified from stock #4847), (iv) FRT28B, UAS-P35, (v) UAS-AmpkTrip20 (RNAi) (#517795)—all provided by the Bloomington Drosophila Stock Center. UAS-RasV12, FRT28B (rasHigh)14 was provided by Tian Xu. w1118 and Viking-GRF51 were gifts from K. Moberg (Emory University). Lkb14A2 and Lkb1−/− were gifts from J. McDonald (Kansas State University). Lkb14B1-11 was a gift from W. Du (University of Chicago). Fluorescently labeled mitotic clones were induced in larval eye-imaginal discs using the following strain: y,w,Ey, Act>y+ >Gal4, UAS-GFP (or RFP); FRT82B, Tub-Gal80 (provided by Tian Xu).

Generation of Drosophila Lkb1 antibody. ProteinTech was used to generate a custom Lkb1 polyclonal antibody specific to Drosophila using the following peptide sequence: VEDEMTVLLANKNFHYDV-Cys. Guinea Pigs were immunized and

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**Fig. 6 High levels of oncogenic KRAS drive decreased patient survival and is associated with AMPK activation in LKB1 mutant patients.**

a, b Analysis of patient survival using the TCGA Pan Lung Cancer study. Kaplan–Meier plots stratified by KRASLow (a) or KRASHigh (b) using oncogenic (codon 12) KRAS mRNA expression and further stratified based on LKB1 deletion and loss-of-function mutation status. c, d Analysis of patient survival using the TCGA Pan Lung Cancer study. Kaplan–Meier plots stratified by KRASLow or KRASHigh using oncogenic (codon 12) KRAS copy number data and further stratified based on LKB1 deletion and loss-of-function mutation status. e, f Analysis of phosphorylated AMPK (T172) expression as it correlates with KRAS mRNA expression and further stratified based on LKB1 deletion and loss-of-function mutation status. g, h Canonical circuit activity analysis was used to estimate the activity of AMPK signaling pathway (hsa04152) that result in functional cell activities. Red color represents significantly (p < 0.05) upregulated genes (or paths) in KRASHigh patients, and blue represents downregulated genes (or paths). The activity of three effector circuits is significantly (FDR < 0.05) upregulated in KRASHigh patients, one ending in the node that contains the protein PPARGC1A (p = 0.006; FDR = 0.037; Uniprot function Biological rhythms/ Mitochondrial biogenesis), the second one ending in the node with the MLYCD protein (p < 0.05), and the third ending in the node containing EIF4EBP1 (p = 0.001; FDR = 0.013; Uniprot function Translation regulation).
supplemented with booster immunizations before final antibody production after 102 days. Antibodies were affinity purified with Elisa confirmation of purification, and final antibody concentrations were estimated by SDS-PAGE.

**BrdU staining.** Third instar larval eye-imaginal discs were dissected in Grace's Insect Medium (ThermoFisher) then transferred into Grace’s Insect Medium containing 0.25 mg/ml BrdU (Invitrogen B23151) and incubated at 25 °C for 90 min. Discs were then washed in Grace’s Insect Medium for 5 min on ice followed by washing twice for 5 min each in 1× PBS on ice. Discs were then washed overnight (wrapped in foil) in 1% paraformaldehyde/0.05% Tween20. The following day discs were washed three times for 5 min each in 1× PBS and permeabilized for 20 min at RT in 0.3% PBSB. To remove detergent, discs were washed five times for 5 min each in 1× PBS and DNase treated for 30 min at 37°C. Discs were then washed three times for 10 min each in 0.1% PBSB and incubated overnight at 4°C in mouse anti-BrdU (1:250) secondary antibody (BA2700) and 0.88 μg/ml Z and rotational directions. The sample chamber is sealed using custom-made rubber gaskets and filled with Schneider’s Medium. The instrument is constructed as previously published with slight modification. All data were collected using a Nikon 16×/0.8 NA LWD Plan Fluorite water-dipping objective and Hamamatsu Orca Flash 4.0 v2 sCMOS cameras. Exposure time for all experiments was 15 ms per frame. We collected data using a single camera view in two illumination arms, exciting with each arm in sequence for each color and photometry. In our SIMView implementation for one-photon excitation, multiview image stacks are acquired by quickly moving the specimen over the desired z range and alternating light-sheet activation in the two illumination arms for each volume. This bidirectional illumination and detection capture recordings from two complementary views of each z plane in two illumination steps. Notably, no mechanical rotation of the specimen is required. The switching of laser shutters in the two illumination subsystems is performed within a few milliseconds. GFP and RFP fluorophores were excited using 488 and 561 nm Omicron Sole lasers, respectively.

**Analysis of SIMView data.** Following data acquisition, images were processed using Imaris 9.3.2. The raw data had been subtracted to account for dark counts of the sCMOS cameras. Images from each illumination arm corresponding to the same Z slice were merged and corrected for intensity variation. Details on these algorithms are previously published. Vkg-GFP pixel intensity over time was measured using maximum intensity projections of 3D volumes from eight consecutive time points between 0 and 1045 h. The pixel intensity over time within the region of interest was measured for each timepoint using FIJI/ImageJ. 3D volumetric time-lapse data were visualized using Bitplane Imaris 9 (Fig. 4c–e). Subsets of the entire 2000–3000 timepoint series (~3–5 TBs in size) were selected for 3D inspection and visualization from maximum intensity projection images. 3D regions of interest (3D-ROI) were created using Imaris’ intensity-based Surfaces function.

**Pharmacology.** Molas-based food was melted and 10 ml of food was aliquoted to vials. While warm, 10 μl of H2O or 10 μl of 5 mM KN-93 (Millipore Sigma, 422711) were added to vials, respectively. Food vials were cooled and allowed to solidify before use. Vials not immediately used were placed at 4°C. Adult y, w, eyFP1; Act > y > > Gαs, UAS-GFP, FRT282, Tub-Gal80 virgin female flies were crossed to FRT282 or UAS-RasV12M6;Lkb1Ac414.2 males, respectively. Flies were moved to embryo ‘egg-laying cups’ and allowed to egg-lay onto grape juice agar plates at 25°C. Flies were moved onto fresh agar plates every 24 h. After each 24 h period, embryos were collected using forceps and placed onto a fresh vial of food. Embryos were collected using forceps and placed onto a fresh vial of food. Once of age, 2nd-instar larvae were collected and placed onto drug-containing media at 25°C. Survival was quantified as the percentage of total embryos placed that survived to pupation and adulthood.

**Survival analysis of patient data.** CbioPortal was used to obtain survival, copy number, mRNA expression, and RPPA expression data available through the PanCancer Genome Atlas (TCGA; PanCancer Atlas (2013) Cancer Genome Atlas (TCGA): PanCancer Atlas (2013) Cancer Genome Atlas (TCGA). Additional data on TCGA Lung Adenocarcinoma studies (Lung Adenocarcinoma studies (TCGA). Additional data on TCGA Lung Adenocarcinoma studies (Lung Adenocarcinoma studies (TCGA)), included: TCGA Pan-Lung Cancer study and TCGA Lung Adenocarcinoma studies (PanCancer Atlas and Provisional). Out of 1144 total samples, samples with
specific KRAS G12C, G12D, or G12V mutations were selected for further analysis (115 samples for mRNA analysis and 76 samples for copy number analysis). Stratification as KRAS<sup>wildtype</sup> or KRAS<sup>mutant</sup> was based on normalized (Log2) mRNA expression or relative copy number. Patients with KRAS normalized mRNA expression value <10.825 were designated as RAS<sup>low</sup>, while patients with a normalized mRNA expression value >10.825 were designated as RAS<sup>high</sup>. For copy number analysis, diploid patients were designated as KRAS<sup>Diploid</sup>, while patients with KRAS gains and amplifications were designated as KRAS<sup>Gain/Amp</sup>. Of these patients, mono or biallelic loss or predicted loss-of-function mutations in LKB1 or deletions or loss-of-function mutations in TP53 were also obtained. For pAMPK correlation analysis, specific studies used included: TCGA Pan-Lung Cancer study and TCGA Lung Adenocarcinoma study (PanCancer Atlas). Samples with specific KRAS G12C, G12D, or G12V mutations as well as RPPA expression data for pAMPK (T172) were selected for further analysis (n = 71).

**Canonical circuit activity analysis.** The HiPathia web-application (http://hipathia.babelomics.org/) was used to identify differentially expressed (activated or inhibited) pathways. RNA-sequencing raw RSEM count data based on human genome build hg19 was obtained for the TCGA lung adenocarcinoma patients from the Genomic Data Commons (GDG) legacy archive (https://portal.gdc.cancer.gov/legacy-archive). Patients without all data types were excluded. Patients were obtained and grouped based on KRAS status using CBioPortal as previously described and determined to be KRAS/LKB1<sup>WT</sup> if concomitant somatic mutations in LKB1 were absent, and as KRAS/LKB1<sup>Mut</sup> if secondary LKB1 mutations were present. Genes with average counts per million of >0.1 across all samples were kept. Normalization was done with a trimmed mean of M-values method and log2 transformed using edger package<sup>57</sup>. The normalized expression matrix was then used as input in HiPathia web-application to identify up- or downregulated pathways between the two groups (mutant vs. wild type) against all available pathways in HiPathia. Finally, differential gene fold change was estimated using the Limma R package.

**Statistics and reproducibility.** Statistical tests, sample size, and number of biological replicates are reported in the figure legends. In summary, GraphPad Prism 7 and 8 were used to generate P values using the two-tailed unpaired Student’s t-test to analyze statistical significance between two conditions in an experiment, ordinary one-way ANOVA with a Tukey’s multiple comparisons test for experiments with three or more comparisons, and Log-rank (Mantel-Cox) test for analysis of survival data. Significance was assigned to p values <0.05 unless otherwise indicated. For Fig. 6e–g, statistical analysis was conducted using RStudio. For Fig. 6e and f, data were divided into two groups, LKB1 loss of function (n = 40) and LKB1 wild type (n = 31). A single outlier sample in the LKB1 mutation category was excluded and calculated z-score for pAMPK and KRAS expression data was used. The correlation between the AMPK and KRAS was conducted and a Spearman’s correlation test. Due to the relatively small sample size, a p-value of ≤0.1 or 10% was considered significant.

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

**Data availability.** The molecular and clinical data used to support the conclusions of Fig. 6 are available from the GDC data portal (http://portal.gdc.cancer.gov/). Source data for Fig. 1e, Fig. 2b, c, Fig. 3f, and Fig. 5g, h are available in Supplementary Data 1–4. All other data that support the findings of this paper are available from the corresponding author upon request.

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Author contributions
M.G.R., B.R., C.S., and E.K. conceived and designed the project. B.R. and C.S. performed the Drosophila and molecular biology experiments. R.E.P. contributed to visualization and editing of the manuscript. E.K., N.A., J.M.H., T.L.C., and M.G.R. designed and performed the SimView imaging experiments. W.G., N.A., E.K., and M.G.R. analyzed the SimView data. B.D., M.R., and B.R. performed the bioinformatic, correlation studies, and statistical analysis using human patient data, and B.R. and M.G.R. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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