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14. ABSTRACT

Preliminary analysis in our laboratory reveals concurrent mutation of the LKB1 and KEAP1 tumor suppressors correlate with poor overall survival in lung adenocarcinoma (LA). Phenotypically, there is evidence to suggest that inactivation of KEAP1 may support adaptation to increased oxidative stress that results from LKB1 inactivation. We have found that inactivation of KEAP1 in the background of LKB1 inactivation results in increased growth and resistance to treatment. Further, we have evidence that cross-talk from the PERK kinase, may also further support adaptation to oxidative stress in combination with KEAP1 inactivation in LKB1-deficient LA. These findings suggest that KEAP1/LKB1 inactivation may represent a critical step in LA tumorigenesis and may have a role in therapeutic resistance.

15. SUBJECT TERMS

lung adenocarcinoma, lung cancer, LKB1, STK11, KEAP1, therapeutic resistance.

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1.Introduction

Lung cancers contribute to more deaths globally than any other malignancy[1]. Lung cancers are a histologically diverse tumor type, classified into small and non-small cell subtypes, with non-small cell lung cancers accounting for over 70% of all lung cancers[2]. Non-small cell lung cancers are further sub-classified into squamous cell, adenocarcinoma, large cell and neuroendocrine. Lung adenocarcinoma (LA) has become predominate (~70% of cases)[2], overtaking squamous cell carcinoma of the lung (SCC). The increase in LA has been attributed to several different factors, including changes in smoking behavior and environmental exposure. Genetic analyses indicate that LA and SCCs also differ considerably in regards to oncogenic mutations[2]. One of the most frequent mutations in LA are inactivating alterations to the *STK11/LKB1* gene[3-5]. Encoding a serine-threonine kinase, *LKB1* is a known tumor suppressor and *LKB1* inactivation is associated with poor overall survival in several different tumor types[3]. Deletion of the *Lkb1* gene simultaneously with expression of oncogenic KRAS ($KRAS^{G12}$) in murine lung potentiates aggressive LA, characterized by rapid growth, short overall survival (8 weeks vs 24 weeks in KRAS only mice) and local and distant metastasis[6]. *LKB1* regulates several fundamental processes, including growth and metabolism[3]. While it is apparent that *LKB1* inactivation contributes greatly to tumorigenesis, loss of *LKB1* function also results in a variety of distinct metabolic changes, consistent with its' regulatory function in cellular metabolism[3]. Although these changes in cellular metabolism are thought to enable more aggressive growth, *LKB1* loss has been shown by both our group and others to result in increased oxidative stress (i.e. reactive oxygen species [ROS]) and LKB1-deficient LA cells are more sensitive to pharmacological agents that aggravate oxidative stress levels[7,8]. Parallel studies indicate that aggravation of oxidative stress due to limited nutrients and chronic hypoxia also induces cell death[9]. Further, *LKB1* inactivation cooperates with oncogenic *KRAS* mutation[6], an alteration known to induce oxidative stress[10]. These conflicting effects (rapid growth vs increased cytotoxicity due to oxidative stress) resulting from *LKB1* inactivation highlight a potential requirement for additional genetic mutations in LKB1-deficient LA to overcome the negative effects of oxidative stress.
The *NFE2L2* gene encodes for the Nrf2 transcription factor[11,12]. Nrf2 regulates a gene expression program involved in detoxification of ROS and xenobiotic compounds, enabling adaptation and resistance to oxidative stress. Unsurprisingly, Nrf2 also functions as a potent oncogene, enabling resistance to oxidative stress and thus promotion of carcinogenesis. Activity of Nrf2 is regulated through association with KEAP1. Under homeostatic conditions, Nrf2 is tightly bound to KEAP1, which sequesters Nrf2 for degradation via the proteosomal degradatory pathway. Increases in ROS levels enables disassociation of Nrf2 from KEAP1, allowing Nrf2 to translocate to the nucleus and activate gene transcription. Consistent with the pro-tumorigenic function of Nrf2, *KEAP1* functions as a tumor suppressor and inactivating mutations to *KEAP1* are found in several tumor types, including LA[13]. While it is apparent that much of the benefit of dysregulated NRF2-KEAP1 signaling is related to detoxification of ROS, there is also evidence that aberrant Nrf2 signaling also promotes adaptation of tumor associated metabolism[11].

One of the conflicting paradigms regarding the contribution of *LKB1* to LA tumorigenesis has been how LA-deficient LKB1 maintain growth and adapt to increased sensitivity to oxidative stress. Based upon the known functions of Nrf2 and KEAP1, we have hypothesized that inactivation of *KEAP1* in concert with *LKB1* inactivation may work cooperatively to promote and support tumorigenesis in LA. Furthermore, we now have evidence that over-expression of the protein kinase, PERK may function as an alternate pathway driving Nrf2 activity towards ameliorating oxidative stress. We have designed experiments to test these hypotheses in order to understand how LKB1-deficient LA maintains growth and resist therapy.

2. Keywords
STK11, LKB1, KEAP1, Nrf2, PERK, oxidative stress, Lung adenocarcinoma, therapeutic resistance

3. Accomplishments
What were the major goals of the project?
Based upon our preliminary data, the primary goal for this project is to determine how LA lacking LKB1 resist and adapt to oxidative stress. Our data suggests that LKB1-deficient LA rely on two independent mechanisms that allow LKB1-deficient LA to blunt the negative effects of oxidative stress.
What was accomplished under these goals?

Concurrent mutation of KEAP1 and LKB1 are common in lung adenocarcinoma and are associated with poor survival
Analysis of LA carrying oncogenic mutations to *KRAS* revealed frequent co-mutation of *LKB1* and *KEAP1* in patient tumors and LKB1-deficient LA display a gene signature associated with Nrf2 gene transcription[15]. Based upon these observations, we analyzed the Cancer Genome Atlas LA dataset[5] for mutations to *KEAP1*, *LKB1* or co-mutations of *KEAP1* and *LKB1* and 5-year survival. Consistent with published data, co-occurrence of mutations to *KEAP1* and *LKB1* is highly significant ($p<0.001$). In analysis of COSMIC (Catalogue of Somatic Mutations in Cancer) cell line DNA sequencing data, concurrent mutation of *KEAP1* and *LKB1* in 14% of established LA cell lines (Table 1). More importantly, analysis of 5-year survival in LA patients from the TCGA dataset revealed reduced survival in LA patients with concurrent mutations to *KEAP1* and *LKB1* compared to LA patients harboring mutations to *LKB1* or *KEAP1* (Figure 1). Collectively, these data show that concurrent mutation of *LKB1* and *KEAP1* is common in LA and the presence of these mutations is associated with poor overall survival.

Deletion of KEAP1 promotes growth of LA in vitro

To test cooperativity between *LKB1* and *KEAP*1 inactivation, we chose to utilize short-term cell cultures of murine LA cells collected from a well-characterized conditional transgenic model of LA. Developed by Jacks and collogues, the KRAS mouse harbors an oncogenic *Kras* (*Kras*^{G12D} or mtKras) gene flanked proximally by a floxed transcriptional stop sequence (lox-STOP-lox or LSL) knocked into the wild-type *Kras* gene locus[16]. Expression of oncogenic Kras is conditional, as the stop sequence in the LSL cassette prevents expression of oncogenic Kras. Transient expression of the Cre recombinase in the lung, via intranasal inhalation of a Cre adenovirus (adenoCre) results in DNA deletion of the LSL cassette and expression of mtKRAS. As mtKRAS expression is driven by its normal promoter, protein levels of mtKRAS are at normal physiological levels, unlike other approaches that utilize over-expression of mtKRAS via a viral promoter. mtKRAS mice display NSCLC pathogenesis comparable to human disease, with mtKRAS mice harboring early pre-neoplastic lesions (atypical adenomatous hyperplasia, epithelial hyperplasia, adenomas) 2 weeks post infection and high-grade NSCLC at later time points (~16 weeks post-infection). Adjusting the multiplicity of infection alters the number of lesions within the lung. Inclusion of floxed alleles of tumor suppressor genes (i.e. *Tp53, Lkb1*) via selective breeding allows for simultaneous deletion of tumor suppressors and activation of mtKras gene expression. Our laboratory currently maintains an active breeding colony of mtKras, mtKras/tp53$^{fl/fl}$ and mtKras/Lkb1$^{fl/fl}$ mice on a FVB/n background. mtKras/LKB1$^{fl/fl}$ mice (6-8 weeks old) were infected with adenoCre ($1x10^6$ pfu) via inhalation and

monitored. 8 weeks post-infection, mice were sacrificed and tumor nodules dissected from the lungs. Tumor nodules were minced and suspended in media and tumor cells allowed to attach under standard cell culture conditions. After one week of growth, tumor cells were checked for fibroblast contamination. If present, fibroblasts were removed by incubating samples with 0.5% trypsin/EDTA for 1-2 minutes and rinsed with warm media. Samples were further trypsinized and expanded for experiments. We have found that these procedures produces cultures comprised entirely of LKB1-deficient LA tumor cells. Consistent with Cre-mediated deletion of LKB1, these cells lack LKB1 (Figure 6) and still maintain expression of KEAP1 and low levels of Nrf2 at normal cell culture conditions (Figures 2A, 6). To test the effects of KEAP1 deletion in LKB1-deficient LA (LKB1-), we utilized CRISPR (Clustered regularly interspaced short palindromic repeats) to specifically delete *KEAP1* in the DNA of our short-term murine cultures of mtKras/LKB1- LA cells. sgRNA sequences to murine *Keap1* using an online design tool (http://crispr.mit.edu/) that were cloned into the pLentiV2 CRISPR vector. Following puromycin selection, immunoblotting for Keap1 and Nrf2 in short-term cultures from mtKras/LKB1- tumor cells showed reduced Keap1, with a corresponding increase of Nrf2 (Figure 2A) in cells infected with sgRNA targeting Keap1. Infection with a non-targeting sgRNA had no effect. For comparison, we performed CRISPR mediated deletion of *Keap1* in a murine LA cell line wildtype for *Lkb1*, but carrying an oncogenic mutation to *Kras* and found t increased Nrf2, consistent with reduced KEAP1 expression (Figure 2A). We compared growth of LKB1- (3363D) and LKB1+ (CMT64) to test the effects of *Keap1* deletion on growth. As shown in figure 2B, deletion of *Keap1* in the 3363D (LKB1-) LA cells resulted in increased cell growth compared to 3363D cells infected with the non-targeting sgRNA. Surprisingly, CRISPR mediated deletion of *Keap1,* resulted in reduced growth in the LKB1-expressing CMT64 LA cells (Figure 2B). To confirm these observations, we chose to test the effects of KEAP1 expression in human LA lines. Consistent with LA patient tumors[15], concurrent inactivation of *KEAP1* and *LKB1* are commonly present in established LA cell lines. We chose two lines, H2030 and A549, both shown to harbor inactivation mutations in *LKB1* and *KEAP1* [13,17] and stably re-expressed either KEAP1 or LKB1 using retroviral infection, followed by selection with puromycin. Notably, both H2030 and A549 also carrying oncogenic *KRAS*, but differ in regards to *TP53* status (H2030-*TP53*-; A549-*TP53*+)[18]. In addition, A549 harbor an activating mutation in *Nrf2*. Following puromycin selection, H2030 LA cells containing LKB1, KEAP1 or the empty vector (pBabe) were treated with the known LKB1 activators, phenformin and metformin. These agents indirectly activation LKB1 signaling by inhibiting mitochondrial complex I and LKB1 activity can be assayed by immunoblotting for phosphorylated AMPK (pAMPK), a downstream target of LKB1[3]. As expected, re-expression of LKB1 restored AMPK phosphorylation with phenformin and metformin treatment (figure 3A). Notably, neither drug induced pAMPK in H2030 cells re-expressing KEAP1 or pBabe. Re-expression of KEAP1 was found to reduce both Nrf2 and its downstream target HO-1 (Figure 3A), consistent with KEAP1's regulatory function. Interestingly, LKB1 re-expression increased Nrf2 expression in the H2030 cells (Figure 3A). We next compared the growth of H2030 and A549 LA cell lines re-expressing KEAP1, LKB1 or empty vector. As shown in Figure 3B, re-expression of KEAP1 reduced the growth of A549 and H2030 cells, compared to pBabe cells. LKB1 re-expression was found to also reduce the growth of H2030 LA cells, but had no effect upon A549 (Figure 3B).

KEAP1 inactivation promotes resistance to chemotherapy

We have recently shown that *LKB1* loss results in increased sensitivity to DNA damaging chemotherapy in LA[19]. Based upon the frequent concurrent mutation of *KEAP1* and *LKB1* in LA and Nrf2-mediated resistance to therapy[20], we tested whether KEAP1 status had any role in these observations. As shown in Figure 4A, treatment with the DNA damaging agent, cisplatin, activated LKB1 signaling in A549 cells re-expressing LKB1 (A549-LKB1), but not A549 cells re-expressing

KEAP1 (A549-KEAP1) or pBabe (A549-pBabe). A549-LKB1 cells also showed reduced DNA damage compared to A549-pBabe cells, as marked by the DNA damage marker γH2AX, consistent with our previous work (Figure 4B). Surprisingly, A549-KEAP1 cells displayed considerably higher γH2AX and cleaved Parp (a marker of apoptosis) relative to both A549-pBabe and A549-LKB1, suggesting that KEAP1 expression imparted increased sensitivity to cisplatin treatment. To test confirm these findings, H2030 and A549 cells re-expressing LKB1, KEAP1 or pBabe were treated with increasing doses of cisplatin and cell viability was determined after 48 hours of treatment. Consistent with the presence of increased DNA damage, A549-KEAP1 and H2030-KEAP1 cells displayed significantly ($p<0.005$) reduced viability in response to cisplatin compared to H2030 and A549 cells expressing LKB1 or pBabe (Figure 5A,B).

LKB1-deficient LA relies on the protein kinase PERK to blunt oxidative stress.

PERK functions in direct activation of the Unfolded Protein Response (UPR), an adaptive stress for the Endoplasmic Reticulum (ER) and is activated by perturbations to protein synthesis within the ER[21]. We and others have shown increased activity of the UPR in LKB1-deficient LA[8,22]. Direct evidence also indicates that PERK also functions in detoxification of reactive oxygen species[23-25]. Deletion of PERK results in increased ROS and more importantly, PERK can directly phosphorylate Nrf2, which disassociates Nrf2 from KEAP1 and subsequently activating Nrf2 transcription[23-25]. We have previously found that LKB1-deficient LA display increased PERK signaling[8]. The increased expression of PERK, as well as our previous observations of increased PERK signaling may suggest that cross-talk between PERK and Nrf2 may be further supporting tumorigenesis of LKB1-deficient LA and work in concert with KEAP inactivation. We performed analysis of short term cultures of mtKRAS/LKB1-deficent LA cells to assess PERK protein expression. Deletion of *Lkb1* resulted in increased expression of *Perk* both *in vitro* (Figure 6A) and *in vivo* (Figure 7), and was independent of *Tp53* deletion. *Lkb1*-deficient murine NSCLC also displayed increased phosphorylation of eif2α, a target of Perk (Figure 6A). IRE1, a parallel regulator of the UPR, was found to be comparable across both genetic backgrounds (Figure 6A). Re-expression of LKB1 in a human *LKB1*-deficient NSCLC cell line reduced PERK protein expression levels (Figure 6B). We depleted PERK using RNAi in short-term cultures of *mtKras*/*Lkb1*+ and *mtKras*/*Lkb1*- murine NSCLC cells (Figure 11) and assessed the effects upon growth and survival. Depletion of PERK and IRE1 resulted in increased growth of *mtKras*/*Lkb1*+ NSCLC, at normal cell culture conditions (Figure 8A) and did not alter growth under hypoxia (0.1% O_2) (Figure 8A, B). However, depletion of PERK had a pronounced effect on the growth of *mtKras*/*Lkb1*- NSCLC cells, both at normoxia and hypoxia (Figure 8B). These effects were not observed with a non-targeting shRNA in both *mtKras*/*Lkb1*+ and *mtKras*/*Lkb1*- NSCLC cells. In *mtKras*/*Lkb1*-, reduction of IRE1 had no effects on cell growth (Figure 8B). Inhibitors to PERK had a greater effect on the growth of *mtKras*/*Lkb1*- NSCLC cells compared to *mtKras*/*Lkb1*+ NSCLC cells (Figure 7C). PERK has been shown to function in scavenging of ROS[23,26]. Knockdown of PERK using shRNA significantly ($p<0.001$) increased basal ROS levels in *mtKras*/*Lkb1*-, relative to *mtKras*/*Lkb1*+ NSCLC cells (Figure 9A). Consistent with these data, metabolomics analysis showed that PERK RNAi in *mtKras*/*Lkb1*- resulted in increases in metabolites associated with increased oxidative stress, specifically hydroxyprolines, putrescine and taurine[27-30], compared to the shRNA control and *mtKras*/*Lkb1*+ cell lines (Figure 9B). Furthermore, oxidative damage to DNA/RNA can be visualized by an antibody specific for the ROS-induced DNA/RNA adduct, 8-Oxo-7,8-dihydro-2'-deoxyguanosine (Ox8dG). Immunohistochemical staining of *in vivo mtKras*/*Lkb1*- and *mtKras*/*Lkb1*+ NSCLC tumor showed increased Ox8dG staining in *mtKras*/*Lkb1*- NSCLC tumors, compared to *mtKras*/*Lkb1*+ NSCLC tumors (Figure 7). Analysis of ROS levels showed that treatment with bortezomib increased ROS levels in *mtKras*/*Lkb1*- but not *mtKras*/*Lkb1*+ NSCLC cells (Figure 9A). In addition, RNAi depletion of PERK further increased ROS

levels (p<0.0001) upon bortezomib treatment in *mtKras*/*Lkb1*- NSCLC cells, relative to *mtKras*/*Lkb1*+ NSCLC cells depleted of PERK (Figure 9A). Collectively, our *in vitro* data demonstrated that *LKB1*-deficient NSCLC is reliant on PERK for both growth and survival.

PERK as a chemotherapeutic target in LKB1-deficent LA

Our data, as well as past work, suggests that aggravating ER stress with pharmacological compounds results in excessive ROS and cell death in mtKRAS/LKB1-deficient LA, indicative of a potential avenue for therapy. Concurrently, our preliminary data shows increase PERK signaling can reduce ROS and associated cytotoxic effects. This finding supports a hypothesis that inhibition of PERK would increase the cytotoxic effects of ER stress aggravation. Several small molecule inhibitors of PERK have been developed and are currently being explored for clinical use. We have found that one of these drugs (GSK2606414) displays preferential cytotoxicity in mtKras/LKB1-deficient LA cells (Figure 8D). The small molecule, bortezomib is a specific inhibitor of the 20S proteasome, blocking protein degradation and leading to aggravation of ER stress[31], part of a class of drugs referred to as ERSA(ER stress aggravator) . Our data (Figure 9A) shows that bortezomib increases ROS levels in the absence of PERK expression and has increased cytotoxicity in mtKras/LKB1-deficient LA (Figures 9A, 10A). Bortezomib is currently FDA-approved for the treatment of multiple myeloma[31], however concerns regarding limited uptake of bortzeomib into solid tumors has led to development of several second generation proteasome inhibitors that are being explored for clinical use. *Lkb1*-deficient LA are more sensitive to related second generation proteasome inhibitors MLN9708 and PR171 (Figure 10A-C). Likewise, the ERSA, NMS-873 that induces ER stress via inhibition of p97/VCP transport of misfolded proteins out of the ER, also shows increased *in vitro* efficacy in *LKB1*-deficient NSCLC (Figure 10D). Consistent with the role of these compounds in activating PERK, both bortezomib and NMS-873 stimulate PERK activation (Figure 11). Perhaps most critically, in a preliminary study of *mtKras*/*Lkb1*- and *mtKras*/*Lkb1*+ murine NSCLC cell lines co-treatment with 5μM GSK2606414 significantly (p<0.0001) enhanced the cytotoxic effects of bortezomib in *mtKras*/*Lkb1*-, but not *mtKras*/*Lkb1*+ murine NSCLC (Figure 12). Furthermore, *mtKras*/*Lkb1*- murine NSCLC displayed cytotoxicity at sub-nanomolar concentrations of bortezomib in combination with GSK2606414 (Figure 12).

What opportunities for training and professional development has the project provided?

Our data generated from this period has resulted in an award from the American Lung Association to develop therapeutic modalities targeting the PERK resistance mechanism in lung cancer (see Impact/future directions).

How were the results disseminated to communities of interest?

We are working on manuscripts that will disseminate our findings to the research community.

4. Impact/Future directions

The primary hypothesis of this work was that concurrent mutation to *KEAP1* and *LKB1* cooperate to promote tumorigenesis and resistance to therapy. Although we have *in vitro* data to support this hypothesis in our past report, our current efforts to perform confirming *in vivo* experiments have been problematic and we have yet to solve the technical issues with these proposed studies (see Problems).

In parallel, we have developed data around the role of PERK in mtKras/LKB1- LA. Notably, PERK may function as a parallel/alterative pathway that is used by mtKras/LKB1- LA to blunt oxidiative stress. This is of value, as therapeutic targeting of PERK is attainable using several clinically relevant small molecule inhibitors. As such we have developed a series of experiments that will allow us to 1)

explore the mechanism of action of PERK inhibition, both alone and in concert with ERSAs, 2) Test the pre-clinical *in vivo* efficacy of PERK inhibition/ERSA combinational therapy in mtKras/LKB1- LA. These studies will enable understanding towards the role of PERK in regulation of Nrf2 signaling and how activation of PERK-NRf2 signaling is supportive of mtKras/LKB1- LA tumorigenesis. Further, these studies will also allow us to understand how PERK-Nrf2 signaling functions in relation to KEAP1 and LKB1. We have successfully captured funding from the American Lung Association to pursue this work and are also working on a manuscript for submission in the coming year.

Collectively, these studies have substantial impact, both in our understanding of mtKras/LKB1- LA disease progression and approaches to therapy. As of this final report, patients with concurrent mtKRAS and LKB1 mutations lack options for therapy and have been found to harbor primary resistance to PD1/PDL1 immunotherapy. These factors highlight the critical need for developing alternate treatment strategies for these patients. Our data suggests that PERK provides a potential resistance mechanism for mtKras/LKB1- LA, and therapeutic targeting of the PERK protein may have therapeutic potential in treating mtKras/LKB1- LA. Publication and validation of this work and pre-clinical assessment of our treatment approach could result in a viable treatment for LA patients. Similarly, our work also addresses an outstanding issue regarding LKB1 inactivation. Specifically, how LKB1-deficient LA cells blunt oxidative stress resulting from dysregulation of cellular metabolism. In sum our work represents a novel area of work and holds potential to impact both clinical treatment and scientific understanding.

5. Changes/Problems

We proposed several parallel *in vivo* studies to support our *in vitro* experiments. In our efforts, we found that CRISPR-CAS9 deletion of KEAP1 *in vitro* was problematic. In developing these experiments, we have found following infection and puromycin selection protein levels of KEAP1 are reduced by CRISPR-CAS9. However, we have failed to observe complete loss of KEAP1 within our short term cultures of mtKRAS/LKB1- LA cells and this reduction is ultimately lost after two or more weeks. Notably, recent data has shown that the DNA cleavage by CAS9 within eukaryotic cells induces p53 activation. Subsequent activation of p53 DNA damage pathways results in apoptosis, severely limiting the efficacy of CRISPR-CAS9 efficiency. As our mtKras/LKB1- LA cells still maintain functional p53, we theorize that our observed results reflect this effect. We instead turned to RNAi approaches and were unable to find a suitable RNAi sequence that produced the necessary knockdown of KEAP1 that would enable our proposed experiments. As such, we have begun to investigate transgenic approaches that would enable our studies.

6. Products

Other Products: Our work has defined a new therapeutic avenue for LA tumor lacking LKB1. Specifically our data suggests that inhibitors to PERK and clinical ERSAs (bortezomib, MLN9708, PR171) may serve as potent therapeutics for LA tumors lacking LKB1. Furthermore, as we find that overexpression of PERK is also present in LA cells harboring concurrent mutations to KEAP and LKB1 (A549, H23 cell, Figure 6B), we also postulate that this approach may also serve to treat this patient population. Collectively, our proposed treatment approach of PERK inhibitors in combination with ERSA may serve as a new therapeutic modality for LA cancers.

7. Participants

Name: Vashti Carson
Project Role: Research Technician

Nearest Person Month Worked: 6
Contribution to Project: Performed in vitro experiments focused on PERK resistance mechanism. Performed in vivo and in vitro work focused on CRISPR-mediated deletion of KEAP in LKB1-deficient LA.
Funding Support: Intuitional support (SJHMC foundation) awarded to PI (Dr. Inge).

Name: Ye Lee
Project Role: Research Technician
Nearest Person Month Worked: 6
Contribution to Project: Performed in vitro experiments focused on PERK resistance mechanism. Performed in vivo and in vitro work focused on CRISPR-mediated deletion of KEAP in LKB1-deficient LA.
Funding Support: Intuitional support (SJHMC foundation) awarded to PI (Dr. Inge).

8. Appendices

Figure Legends:
Figure 1: Kaplan-Meier analysis of LA patient survival based on LKB1/STK11 and KEAP1 mutation from the TCGA dataset. Red-KEAP1 mutant patients, Blue-LKB1 mutant patients, Black-LKB1/KEAP1 co-mutant patients.

Figure 2: A) Immunoblot analysis of 3363D, 3381B (mtKras/LKB1-) and CMT64 (mtKras/LKB1+) murine cell lines following lentiviral infection with sgKEAP1 (sgKP1) or sgControl (sgCon) and selection in puromycin for one week. Total protein lysates were probed with antibodies specific to Nrf2, KEAP1 or Actin as a loading control. The human lung adenocarcinoma (*EGFR* mutant, *TP53* mutant) was used as a control for KEAP1 and Nrf2 expression. **B**) 50,000 cells (3363D, 3381B, CMT64) infected with the indicated sgRNAs were plated onto 6-well plates. Cells were trypsinized and counted on the indicated days using the Countess Cell Counter (Invitrogen).

Figure 3: A) Immunoblot analysis of H2030 (mtKRAS/LKB1-/TP53-) human LA cell lines after retroviral infection and puromycin selection. H2030 cells re-expressing KEAP1, LKB1 or empty vector (pBabe) were treated with vehicle (V), 2mM phenformin or 20mM metformin for 4 hours before lysis. Total protein lysates were probed with antibodies specific to Keap1, phosphorylated AMPK (pAMPK), AMPK, HO-1, Nrf2, LKB1 and Actin (as loading control). **B)** 50,000 A549 and H2030 LA cell lines stably expressing pBabe (blue), KEAP1 (red) or LKB1 (green) were plated and counted as described in Figure 1B.

Figure 4: A) A549-KEAP1,A549-LKB1 or A549-pBabe cells were treated with 4μM cisplatin for 6 hours before lysis. Total protein lysates were separated by SDS-PAGE and probed with antibodies specific to the indicated proteins. **B)** A549-KEAP1,A549-LKB1 or A549-pBabe cells were treated with 4μM cisplatin for 48 hours before lysis. Total protein lysates were probed for γH2AX (DNA damage) and cleaved Parp (clvd Parp, a marker of apoptosis). Actin was used as a loading control.

Figure 5: Cell viability of **(A)** A549-LKB1, A549-KEAP1, A549-pBabe and **(B)** H2030-LKB1, H2030-KEAP1 and H2030-pBabe treated with cisplatin at the indicated concentrations for 48

hours. Viability was determined using the CellTiterGlo™ kit (Invitrogen) according to manufacturer's instructions.

Figure 6: **A**) Immunoblot of mtKRas/Lkb1+ and mtKras/Lkb1- murine NSCLC cells for the indicated proteins. **B**) Immunoblot of LKB1-deficient human NSCLC cell lines (A549, H23) stably expressing empty vector (Vec) or vector encoding LKB1.

Figure 7: Immunohistochemical staining of mtKras/Lkb1+ (left) and mtKras/Lkb1- (right) for LKB1, phosphorylated AMPK (pAMPK), PERK and 8-Oxo-7,8-dihydro-2'-deoxyguanosine (Ox8dG). Arrow indicates normal tumor stroma expressing LKB1.

Figure 8:. Effects of PERK (shPERK), IRE1 (shIRE1) RNAi or non-targeting RNAi (shCTRL) on the growth of mtKras/Lkb1+ (**A**) and mtKras/Lkb1- (**B**) murine NSCLC cells. **C**) Viability of cells in normoxic or hypoxic conditions. **D**)Effect of the PERK inhibitor (GSK2606414) on viability of mtKRas/Lkb1+ and mtKras/Lkb1- murine NSCLC cells.

Figure 9: A) mtKRas/Lkb1+ and mtKras/Lkb1- with shPERK or shControl were treated with 10nM Bortezomib for four hours and then stained with the ROS indicator, H2-DCF. Values were normalized to viable cells. **B**) Metabolomic analysis of mtKRas/Lkb1+ and mtKras/Lkb1- with shPERK or shControl murine NSCLC cells.

Figure 10:. Effect of ERSAs (**A**) Bortezomib,(**B**) MLN9708, (**C**) PR171 and (**D**) NMS873] on the viability of mtKRas/Lkb1+ (**blue**) and mtKras/Lkb1- (**red**) murine NSCLC cells.

Figure 11: Immunoblot of shCNTL and shPERK mtKRas/Lkb1+ and mtKras/Lkb1- murine NSCLC cells after 6 hours of treatment with 2DG (20mM), Bortezomib (Brt-10nM) or NMS-873 (NMS-700nM) for indicated proteins.

Figure 12: Combinational treatment of mtKras/Lkb1- and mtKras/Lkb1+ murine NSCLC cell lines with borteozmib and 5μM GSK2606414 for 72 hours. Treatments were normalized to vehicle (DMSO) control

Figure 1.

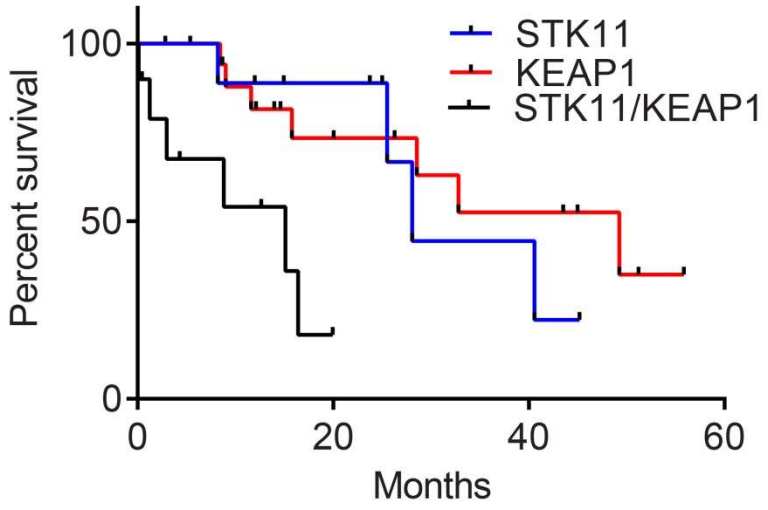
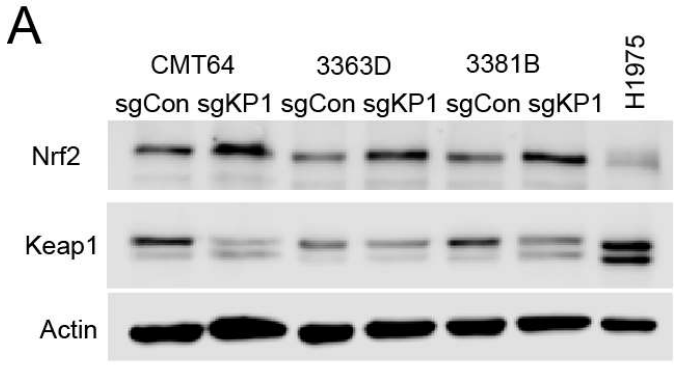


Figure 2



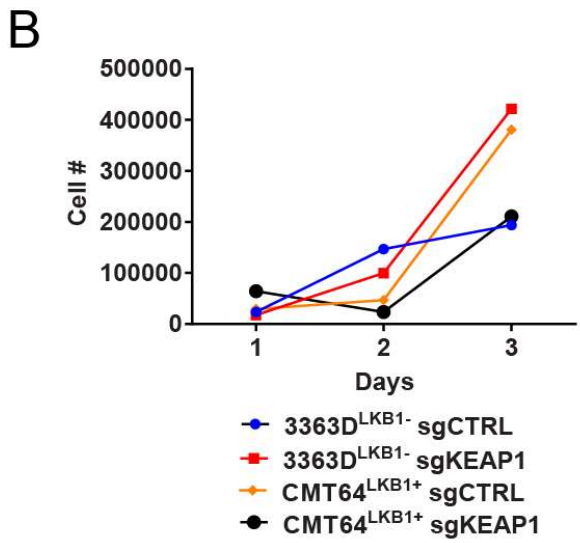


Figure 3

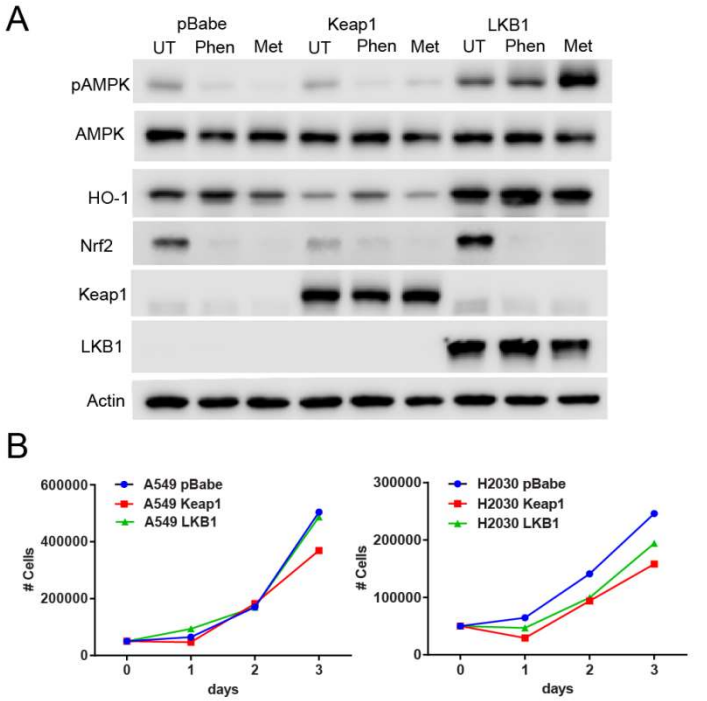


Figure 4

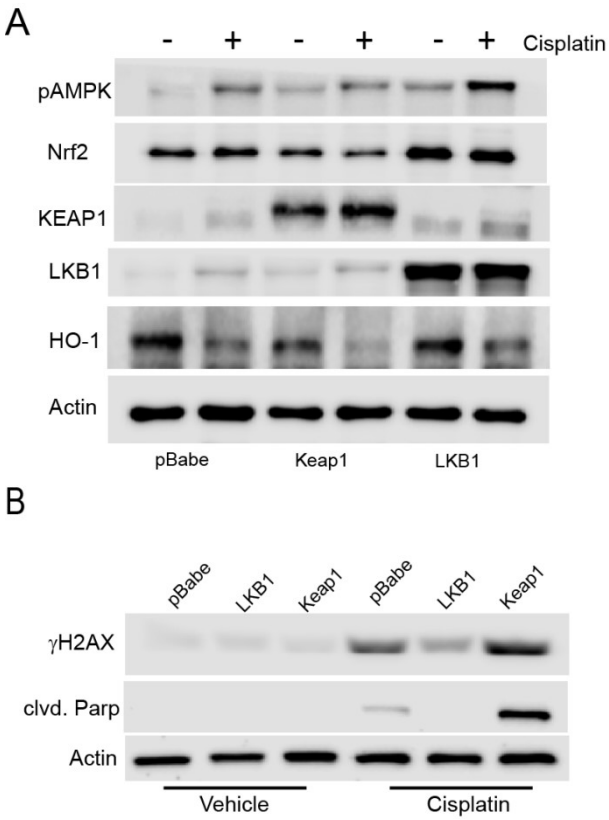


Figure 5

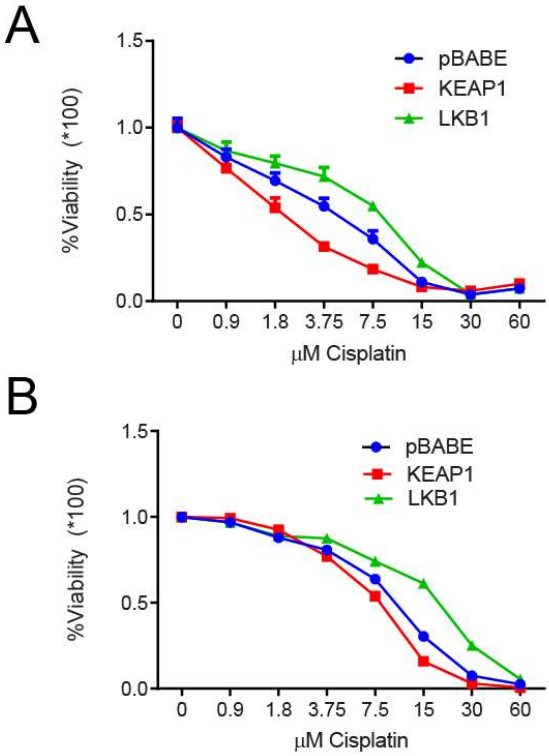

A
1.5
1.0
0.5
0.0
%Viability (*100)
pBABE
KEAP1
LKB1
0
0.9
1.8
3.75
7.5
15
30
60
μM Cisplatin
B
1.5
1.0
0.5
0.0
%Viability (*100)
pBABE
KEAP1
LKB1
0
0.9
1.8
3.75
7.5
15
30
60
μM Cisplatin

Figure 6

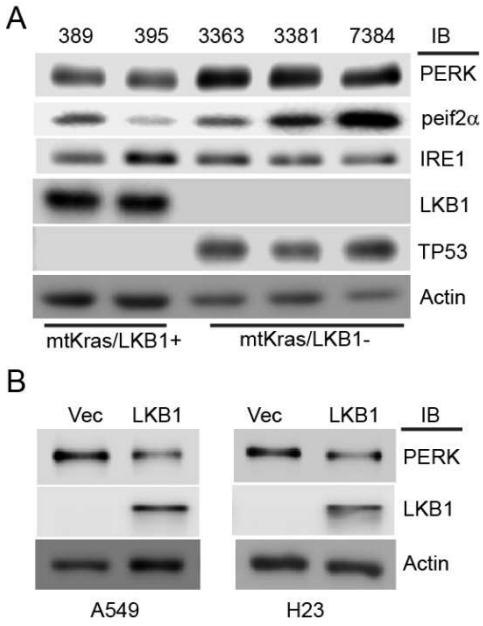


Figure 7

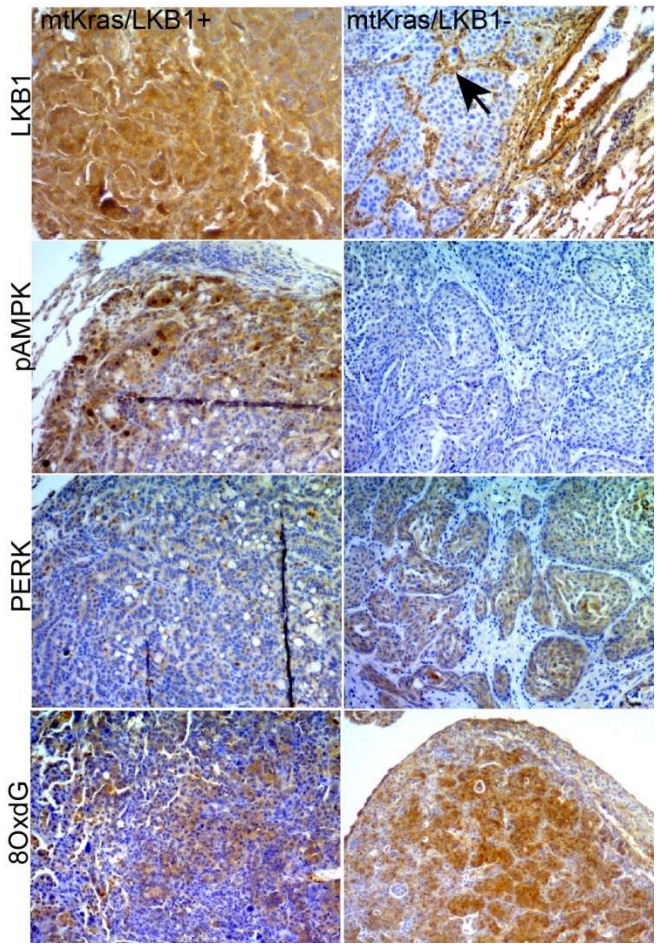


Figure 8

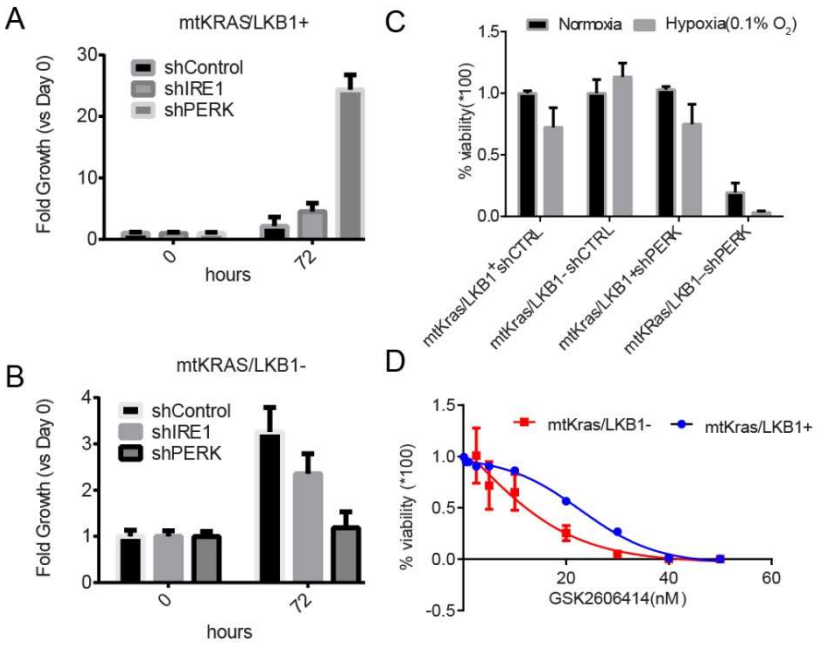


Figure 9

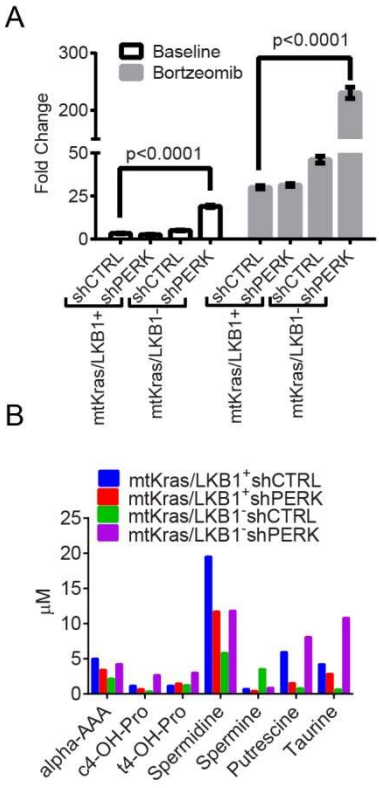


Figure 10

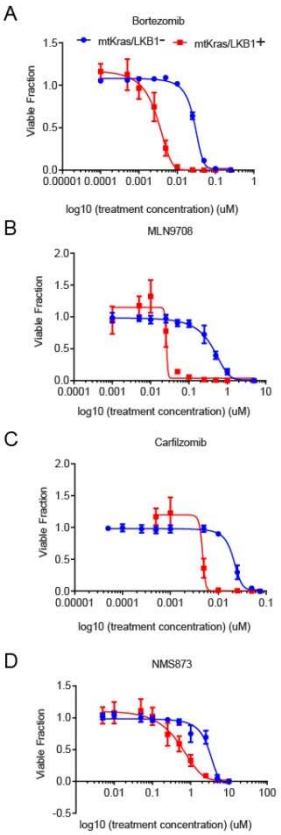


Figure 12

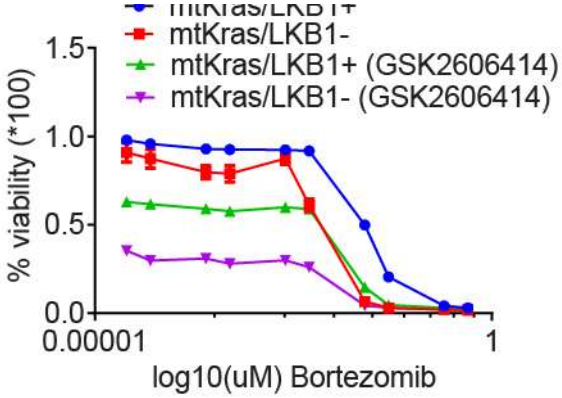
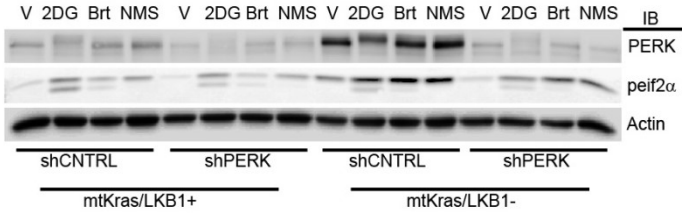


Figure 11



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