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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. We are further exploring these findings using in vivo models.

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?

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

In our last report, we present data suggestive of a potential role for the protein kinase, PERK in ameliorating 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[14]. We and others have shown increased activity of the UPR in LKB1-deficient LA[8,15]. Direct evidence also indicates that PERK also functions in detoxification of reactive oxygen species[16-18]. 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[16-18]. 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 1A) and *in vivo* (Figure 2), and was independent of *Tp53* deletion. *Lkb1*-deficient murine NSCLC also displayed increased phosphorylation of eif2α, a target of Perk (Figure 1A). IRE1, a parallel regulator of theUPR, was found to be comparable across both genetic backgrounds (Figure 1A). Re-expression of LKB1 in a human *LKB1*-deficient NSCLC cell line reduced PERK protein expression levels (Figure 1B). We depleted PERK using RNAi in short-term cultures of *mtKras*/*Lkb1*+ and *mtKras*/*Lkb1*- murine NSCLC cells (Figure 6) 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 3A) and did not alter growth under hypoxia (0.1% O_2) (Figure 3A, B). However, depletion of PERK had a pronounced effect on the growth of *mtKras*/*Lkb1*- NSCLC cells, both at normoxia and hypoxia (Figure 3B). 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 3B). Inhibitors to PERK had a greater effect on the growth of *mtKras*/*Lkb1*- NSCLC cells compared to *mtKras*/*Lkb1*+ NSCLC cells (Figure 2C). PERK has been shown to function in scavenging of ROS[16,19]. Knockdown of PERK using shRNA significantly ($p<0.001$) increased basal ROS levels in *mtKras*/*Lkb1*-, relative to *mtKras*/*Lkb1*+ NSCLC cells (Figure 4A). 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[20-23], compared to the shRNA control and *mtKras*/*Lkb1*+ cell lines (Figure 4B). 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 2). Analysis of

ROS levels showed that treatment with bortezomib increased ROS levels in *mtKras*/*Lkb1*- but not *mtKras*/*Lkb1*+ NSCLC cells (Figure 4A). 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 4A). 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 preliminary 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 3D). The small molecule, bortezomib is a specific inhibitor of the 20S proteasome, blocking protein degradation and leading to aggravation of ER stress[24], part of a class of drugs referred to as ERSA(ER stress aggravator) . Our data (Figure 4A) shows that bortezomib increases ROS levels in the absence of PERK expression and has increased cytotoxicity in mtKras/LKB1-deficient LA (Figures 4A, 5A). Bortezomib is currently FDA-approved for the treatment of multiple myeloma[24], 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 5A-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 5D). Consistent with the role of these compounds in activating PERK, both bortezomib and NMS-873 stimulate PERK activation (Figure 6). 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 7). Furthermore, *mtKras*/*Lkb1*- murine NSCLC displayed cytotoxicity at sub-nanomolar concentrations of bortezomib in combination with GSK2606414 (Figure 7).

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.

What do you plan to do during the next reporting period to accomplish the goals?

We are working on completing our manuscripts for submission

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 (see Changes/Problems). Despite these hurdles, we are continuing to complete these studies (see Changes/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 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. The more recent findings in our laboratory suggesting that PERK provides a potential resistance mechanism for mtKras/LKB1- LA, represents a potential therapeutic vulnerability. 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 last report we experiments that outlines an approach of CRISPR-CAS9 deletion of KEAP1 *in vitro* followed by heterotopic implantation into immune-competent mice of the same background. 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. Thus we have turned to RNAi to inactivate KEAP1 in our short term mtKras/LKB1- LA cultures and subsequently use these cells in our proposed in *vivo* studies.

6. Products
Other Products: Our work over the past period 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 1B), 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: 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

a) Figure Legends:

Figure 1: **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 2: 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 3:. 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 4: 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 5:. 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 6: 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 7: 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.

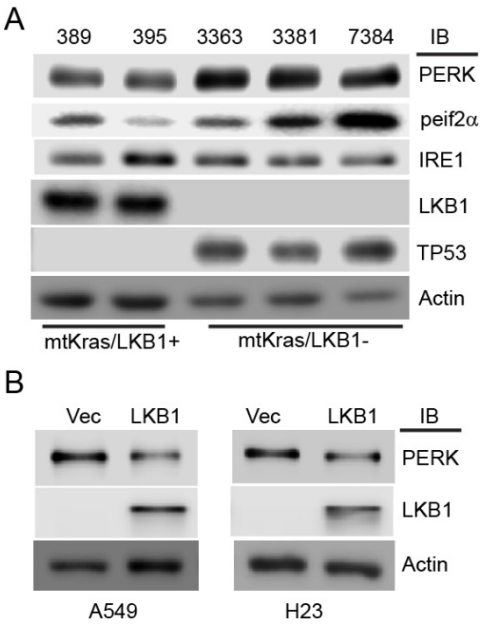


Figure 2

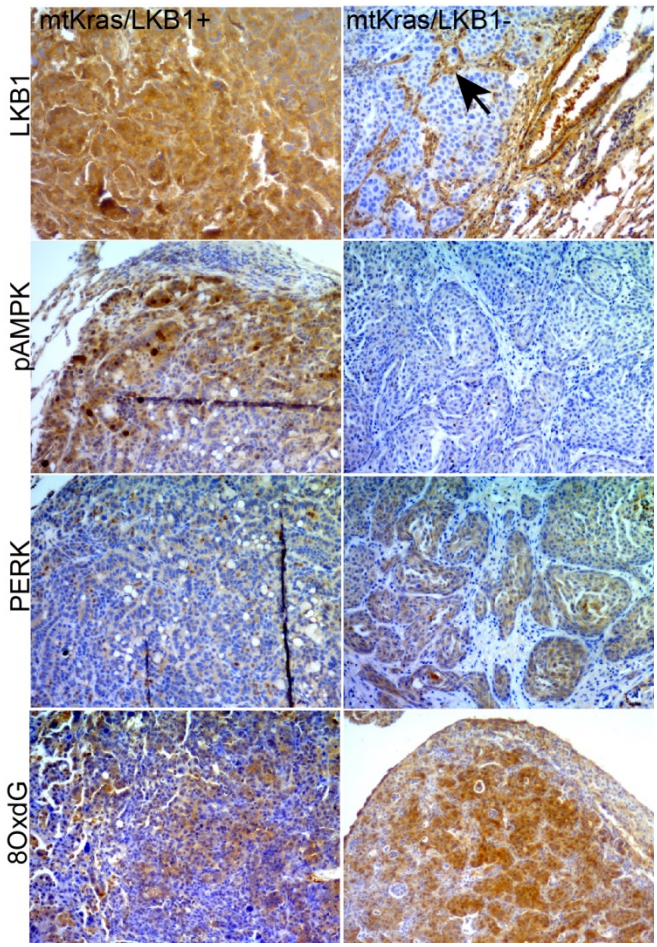


Figure 3

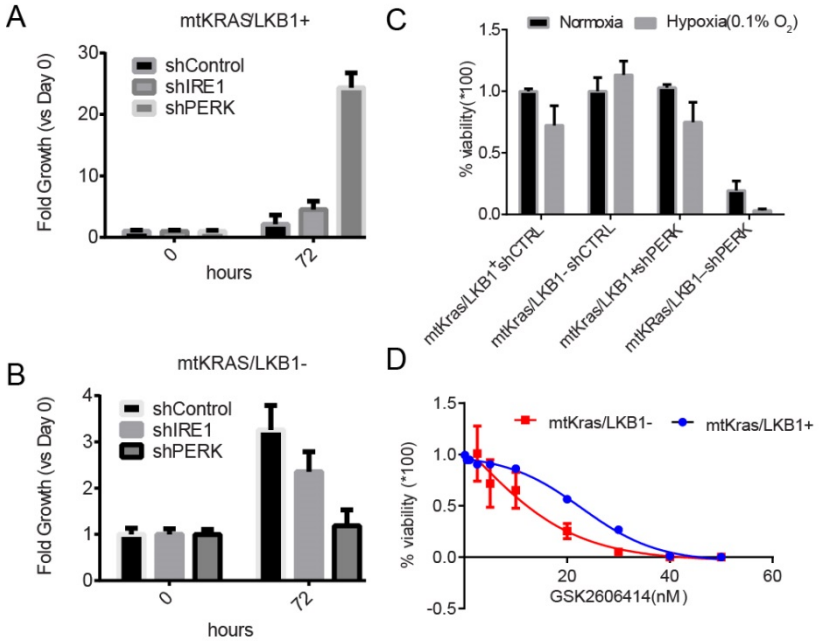


Figure 4

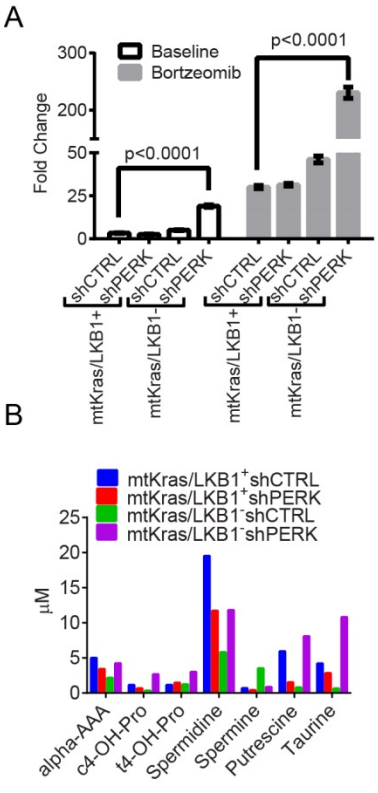


Figure 5

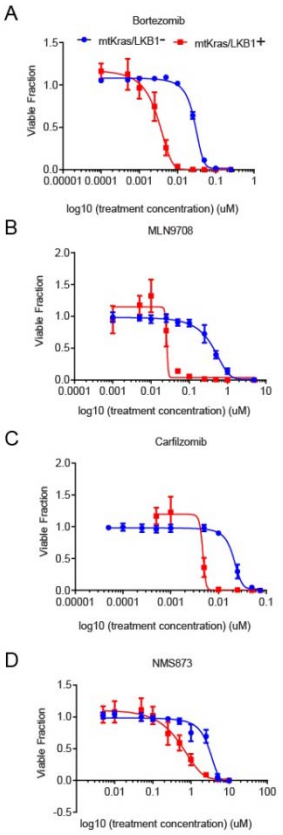


Figure 6

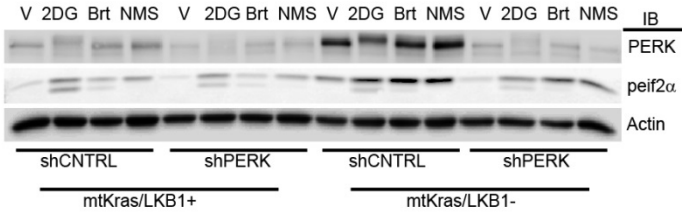
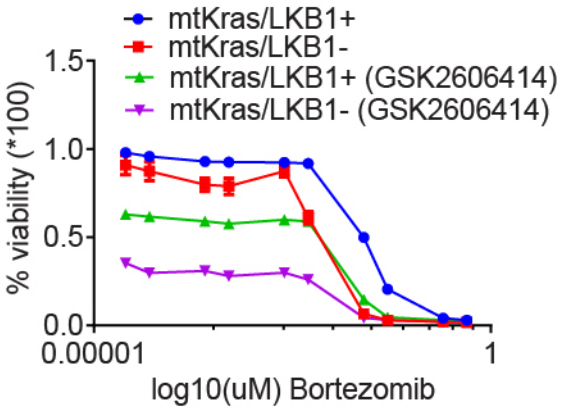


Figure 7


mtKras/LKB1+
mtKras/LKB1-
mtKras/LKB1+ (GSK2606414)
mtKras/LKB1- (GSK2606414)
% viability (*100)
1.5
1.0
0.5
0.0
0.00001
1
log10(uM) Bortezomib

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