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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[14].

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. We have designed experiments to test this hypothesis and have evidence that inactivation of KEAP1 promotes increased growth in LKB1-deficient LA and provides resistance to therapy.

2. Keywords

STK11, LKB1, KEAP1, Nrf2, oxidative stress, Lung adenocarcinoma, therapeutic resistance

3. Accomplishments

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* (Table 1)[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).

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. While our *in vitro* work still needs to be confirmed *in vivo* (see Changes/Problems), our collective data support this hypothesis. Using both chemo-nieve short term cultures of murine LA cells and established human LA cells, we have found that expression of KEAP1 has a pronounced effect upon the growth of LKB1-deficient LA cells. It is notable that the effects of KEAP1 in LKB1-deficient LA appear at manifest at higher cell numbers, suggesting that the effects of KEAP1 upon the growth of LKB1-deficient LA may be related to metabolic alterations. As cells proliferate and become denser in culture, nutrients expectedly become more limited. Deletion of KEAP1 is known to potentiate changes in cellular metabolism, specifically driving processes towards anabolic pathways that support proliferation[14] that are independent of ROS detoxification. Thus, it is quite possible that deletion of KEAP1 and the subsequent shift towards anabolic metabolism may offset metabolic defects associated with LKB1 inactivation unrelated to oxidative stress. We are currently designing experiments to test this possibility and are working with a longtime collaborator (Patrick Pirrotte) at the Translational Genomics Research Institute (Phoenix, AZ) to undertake MALDI based analysis of metabolites in our sgKEAP1 murine LA cell lines.
Current treatment of LA relies upon DNA damaging agents, specifically cisplatin and its analogues (carboplatin). We have found that loss of LKB1 imparts sensitivity to DNA damaging treatment and more importantly found that a specific inhibitor to wee1 kinase, a protein involved in DNA repair, synergized with cisplatin to markedly improve the survival of mtKras/LKB1-deficient NSCLC *in vivo*[19]. We found that mtKras/LKB1-deficient NSCLC tumors displayed defects in DNA repair[19], however the exact mechanism/s behind this phenotype remained elusive. Our finding that KEAP1 expression resulted in reduced survival and increased DNA damage from cisplatin, suggests that having an intact Nrf2-KEAP1 regulatory pathway may play a critical role in mediating the effects of cisplatin and other DNA damaging treatments. Based upon our findings, we are currently testing whether KEAP1 and Nrf2 are involved. This has substantial clinical impact, as the frequent incidence of concurrent *KEAP1/LKB1* inactivation in LA suggests that inhibition of Nrf2 may enable improved responses to DNA damaging treatments. Indeed, efforts are underway to develop Nrf2 inhibitors and we are investigating potential collaborations to test these compounds in our models.

We and others have shown increased activity of the Unfolded Protein Response (UPR) in LKB1-deficient LA[8,15]. The UPR functions as an adaptive stress for the Endoplasmic Reticulum (ER) and is activated by perturbations to protein synthesis within the ER[21]. Due to the highly oxidative environment of the ER, the UPR and specifically the UPR regulator, PERK also functions in detoxification of ROS[22-24]. 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[22-24]. We have previously found that LKB1-defcient LA display increased PERK signaling[8]. Recently, analysis of short term cultures of mtKRAS/LKB1-deficent LA cells has revealed that expression of PERK is highly upregulated (Figure 6). 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/KEAP1-deficient LA. This has become an additional area of focus for our laboratory and we are in process of applying for RO1 funding from NIH.

5. Changes/Problems

We proposed several parallel *in vivo* studies to support our *in vitro* experiments. These experiments were designed to take advantage of a publically available lentiviral Cas9/CRISPR construct that also includes a Cre recombinase cassette. This vector (pSECC) was designed to allow for cloning of user designed sgRNA and sgRNA, CAS9 and Cre expression *in vivo*[25]. The pSECC construct has been shown to successfully delete sgRNA targeted genes with simultaneous deletion of floxed DNA seuqences[25]. Our proposed experiment was designed to target Keap1 in our mtKras/LKB1$^{fl/fl}$ transgenic model and downstream analysis of tumorigenesis. This approach would have been particularly valuable, as it would have been able to determine whether co-mutation of KEAP1 and LKB1 were involved in initiation of LA and also assess the effects of LKB1/KEAP1 co-mutation in the context of fully competent immune system. While we have successfully deleted *Keap1 in vitro*, our attempts to apply the pSECC vector in our murine model has been problematic. Despite generating lentiviral particles at titers sufficient for *in vivo* infection (10^6-10^8), we have to date been unable to achieve both CAS9 deletion of Keap1 or Cre-mediated recombination of the LSL-mrKras and floxed LKB1 alleles in our mouse model. We have begun alternative experiments using short-term cultures of mtKras/LKB1- LA cell lines. These experiments (recently approved by the ACURO) will entail CAS9 deletion of KEAP1 *in vitro* followed by heterotopic implantation into immune-competent mice of the same background. These studies will enable validation of our *in vitro* studies and we have approval for a no-cost extension to complete this work.

6. Appendices

a) 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: Immunoblot analysis of short term cultures of LA cells of tumor nodules collected from mtKras/Tp53- (395, 389) and mtKras/Lkb1- (3363D, 3381A, 7384) mice. Total protein lysates from cells established after two weeks in culture were probed using the indicated antibodies. Actin was used as a loading control.

Figure 1.

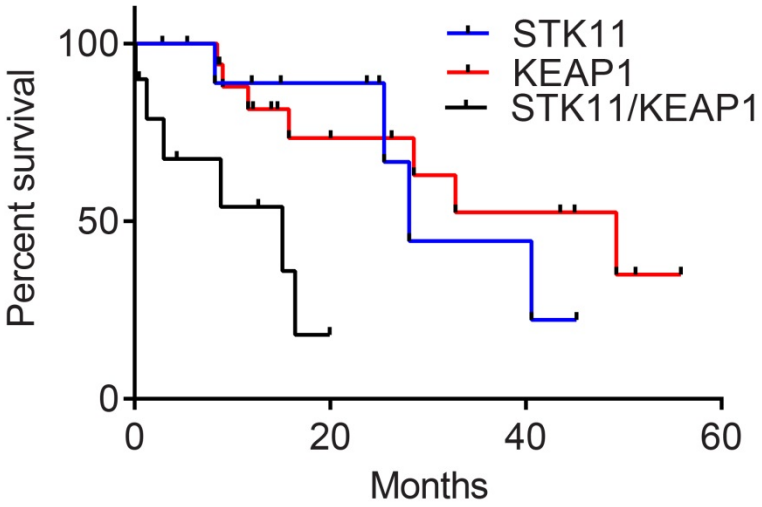


Figure 2

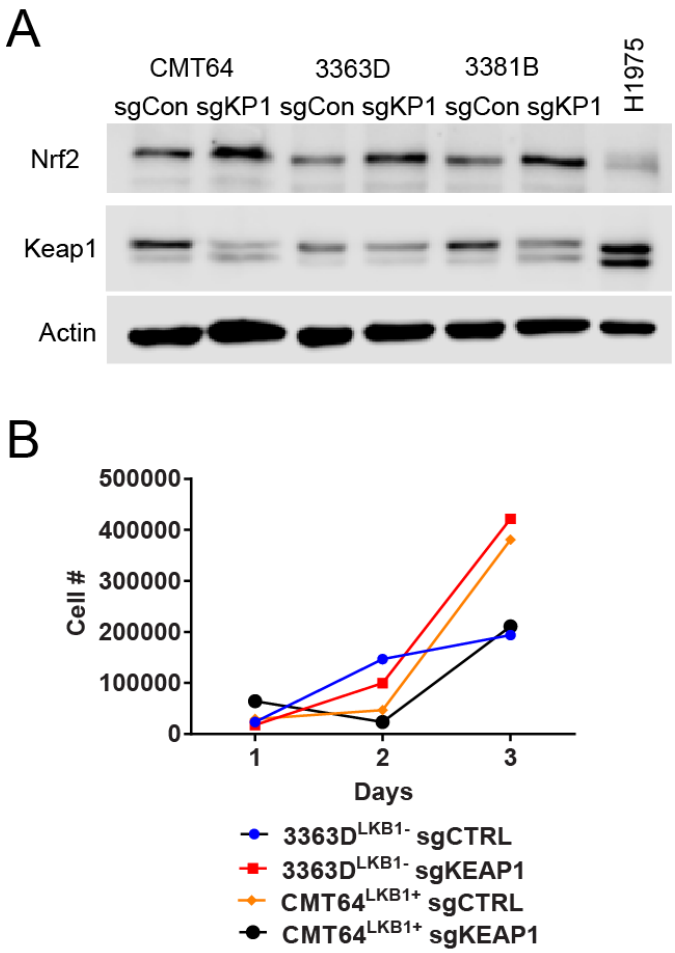


Figure 3

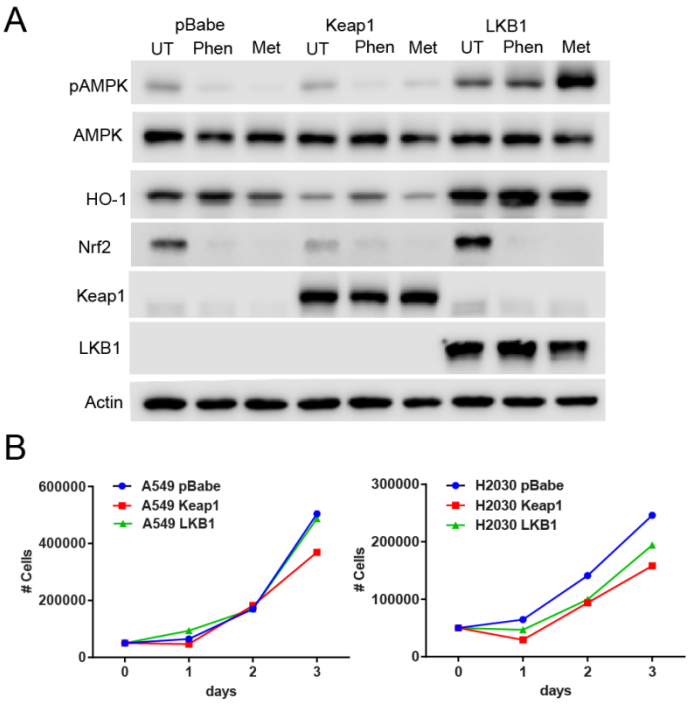


Figure 4

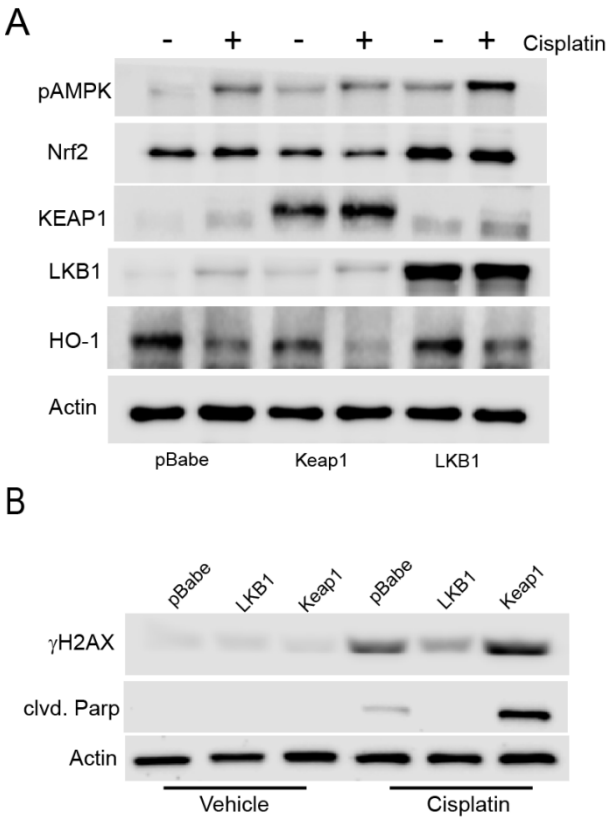


Figure 5

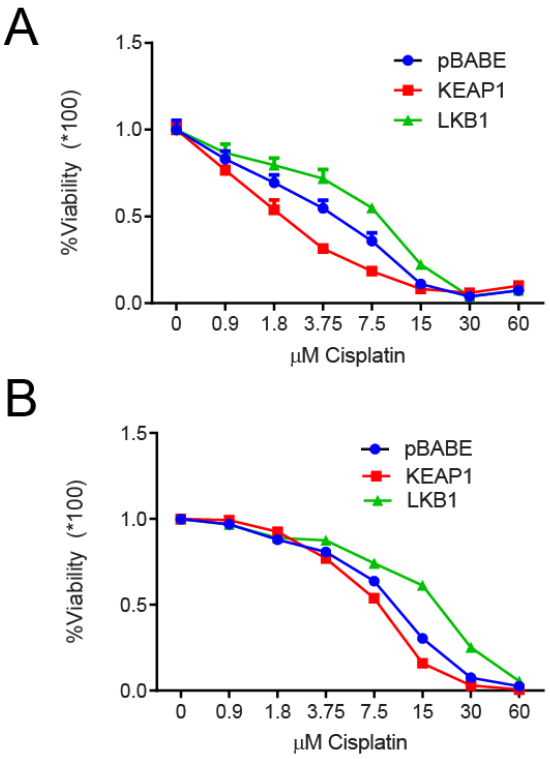
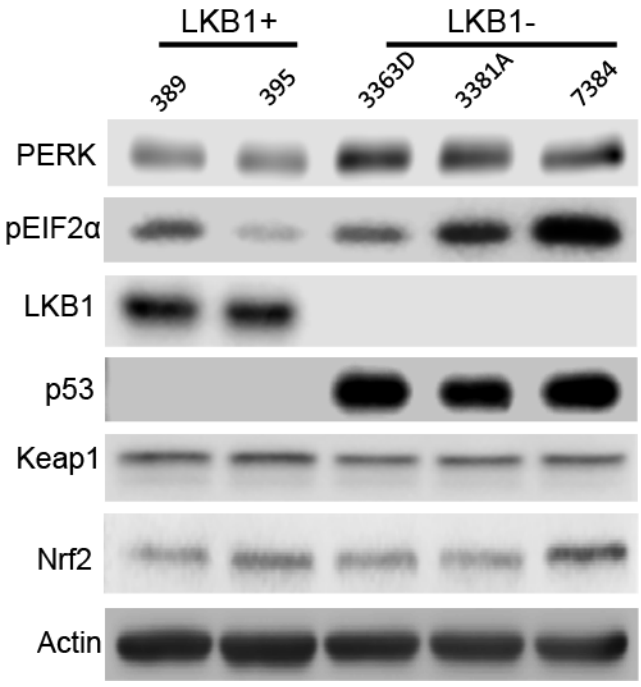


Figure 6



Sample Name	KEAP1 AA Mutation	STK11 AA Mutation
201T		p.L245F
ABC-1		
COR-L105		
EKVX		p.S216F
EMC-BAC-1		p.E165*, p.L164L
EMC-BAC-2		p.Q37*
H3255		
HCC-44	p.F211C	
HCC-78		
HCC-827		
HOP-62		
LC-2-ad		
LXF-289		
NCI-H1435	p.R413L	
NCI-H1563		p.Y272*,p.G242W
NCI-H1568		
NCI-H1573	p.A143P	p.S216F
NCI-H1651		
NCI-H1734		p.M51fs*14
NCI-H1755	p.E582*	p.P281fs*6
NCI-H1781		
NCI-H1793		
NCI-H1944	p.R272L	p.K78N, p.K62N
NCI-H1975		
NCI-H1993		p.E199*
NCI-H2023	p.W252C	
NCI-H2030	p.V568F	p.M392I, p.E357K, p.E317*, p.F157F
NCI-H2122	p.A170_R204del35	p.P281fs*6
NCI-H3122		
PC-14		
PC-3_[JPC-3]		
RERF-LC-KJ		
RERF-LC-MS	p.G119_M120>V	
SK-LU-1		
VMRC-LCD		

Table 1

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