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Henry Huang April 15th, 2015

Ectopic Overexpression of MEK5 Suppresses LKB1 Activity:

A New Mechanism of Regulating LKB1

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Abstract

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The inactivation of LKB1 kinase has been shown to play a key role in lung cancer cell metastasis. The LKB1 gene is frequently inactivated at the DNA level in non-small cell lung cancer by somatic mutation. LKB1 can also be inactivated at the protein level by a B-RAF V600E mutation through MEK1/2 and ERK1/2 signaling in melanoma cells. Here we introduce a third possible mechanism of LKB1 inactivation at the protein level, through the MEK5 pathway. The overexpression of MEK5 was found to attenuate LKB1-mediated AMPK phosphorylation in H1299 cells under glucose-free conditions. MEK5 was found to bind to LKB1 in a GST pull-down assay, and both kinase-dead and phosphomimetic MEK5 mutants were found to have increased binding affinity for LKB1. BIX02188, a MEK5 kinase inhibitor, was found to disrupt the interaction between LKB1 and MEK5. Surprisingly, neither of the MEK5 mutants was capable of suppressing LKB1 kinase activity, suggesting that the increase in binding affinity of MEK5 to LKB1 does not play a role in suppressing LKB1 kinase activity. Depletion of MEK5 isoform C promoted the half-life of LKB1, suggesting that MEK5 may play a role in promoting the degradation of LKB1. LKB1 is known to be a tumor suppressor in breast cancer but the mechanism of LKB1 inactivation in that tumor type is currently unknown. Our data suggest that the recently identified MEK5 amplification in breast xenografts may be responsible for LKB1 inactivation in breast tumors.

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Tables and Figures

Introduction

Lung cancer is the leading cancer death in both genders in the U.S. and is estimated to account for about 27% of cancer deaths, with an estimated 158,260 deaths in 2014 [1]. Lung cancer can be divided into two subgroups: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC contributes to approximately 85% of lung cancer. While deaths due to lung cancer have been decreasing over the years, patients with NSCLC continue to have a low (18%) five-year survival rate due to cell metastasis [1, 2].

In approximately 20-30% of NSCLC patients, the tumor suppressor liver kinase B1 (LKB1) is found to be inactivated at the DNA level [3]. Located on chromosome 19, LKB1 is a gene that was initially found to be compromised in the inherited cancer disease Peutz-Jeghers syndrome [4, 5]. The inactivation of LKB1 can be the result of either direct inactivation of the LKB1 gene, resulting in no LKB1 or nonfunctional LKB1 protein, or epigenetic hypermethylation of the gene, resulting in suppression of gene expression [6, 7]. The loss of LKB1 function has been shown to play a key role in formation of lung adenocarcinoma [8]. Mice expressing an oncogenic KRas GTPase protein were shown to develop tumors with low latency and low aggressiveness. However, when LKB1 function was removed from the KRas mutant mice, there was accelerated tumor formation and increased metastasis, suggesting that LKB1 inactivation plays a key role in formation and metastasis of lung cancer [9].

LKB1 is a serine threonine kinase that plays a role in many cellular functions, including cell polarity and cell detachment [4, 10]. In its active form, LKB1 is found as a trimer with two other proteins, STRAD and MO25. STRAD serves as a nuclear exportation protein and MO25 serves as a scaffolding protein that stabilizes the complex [11, 12]. LKB1, MO25 and STRAD bind to each

other to form the LKB1/STRAD complex and activate LKB1's kinase activity [13]. LKB1 can target many downstream target proteins, including several kinases, one of which is AMP-dependent protein kinase (AMPK) [14]. When cells are under energy stress, LKB1 directly acts on AMPK through phosphorylation of the threonine 172 loop. Phosphorylation of AMPK increases its activity [15, 16]. Under energy stress conditions, AMPK plays a role in the inhibition of cellular growth by serving as a checkpoint and as a regulator for metabolites, including glucose and lipids (Figure 1) [15, 17]. AMPK is also responsible for regulating tight junction assembly during polarization and has been shown to play a key role in the inactivation of oncogenic cell proliferating pathways such as mTOR [18, 19]. Thus, abnormal activity of LKB1 and AMPK can lead to improper cell polarization in NSCLC and cause cell metastasis [10].

While LKB1 can be inactivated at the DNA level, recent experiments show another mechanism where LKB1 kinase activity is suppressed at the protein level through the B-RAF-MEK1/2-ERK1/2 protein signaling cascade (Figure 2). Constitutively active mutant B-RAF V600E was shown to cause downstream phosphorylation in its signaling cascade. Downstream of B-RAF is a class of proteins called mitogen-activated protein kinase kinases (MEK) [20]. MEK pathways are responsible for extracellular signaling in pathways such as migration and cell proliferation. MEK can phosphorylate its pathway-specific downstream protein, extracellular signal regulated kinase (ERK) [21]. In this mechanism, B-RAF activates MEK1 and MEK2, which can phosphorylate their pathway-specific downstream proteins, ERK1 and ERK2. Once phosphorylated, ERK1 and ERK2 can phosphorylate their various downstream targets such as transcription factors and proteins, activating signaling cascades [21]. In the case of B-RAF V600E, the active catalytic site targets the MEK1/2-ERK1/2 pathway and increases the activity

of ERK1/2 and ribosomal s6 kinase (RSK). The phosphorylation of LKB1 by ERK and RSK attenuates the LKB1/AMPK interaction. Therefore, in melanoma cells with the B-RAF V600E mutation, abnormal MEK1/2 and ERK1/2 activity leads to low pAMPK levels, which has been shown to cause changes in both cell proliferation and anchorage, leading to an increase of tumorigenesis [20].

MEK5 also belongs to the MEK pathway and targets its downstream protein ERK5 through the binding of upstream MEKK2 and MEKK3 to MEK5's PB1domain. MEK5 is comprised of 3 major domains: phox and BEMP1 (PB1) domain, an ATP binding domain, and a kinase domain. The PB1 domain plays a role in allowing MEK5 to bind with its upstream kinases MEKK2 and MEKK3 [22]. The MEK5 kinase domain amino acid residues S^{311} and T^{315} become phosphorylated to activate MEK5's downstream target protein ERK5, allowing ERK5 to trigger other downstream signaling pathways [23].

MEK5 has many transcript variants and isoforms. Unigene reports indicate that the MEK5 gene generates three different transcript variants in *Homo sapiens*. This is due to alternative splicing of RNA and differences in exon length of the MEK5 gene [24]. They are also described as MEK5 isoform A, B, and C. Isoform A is the longest at about 50 kiloDaltons (kD) , isoform B is 49 kD, and isoform C is 46 kD. MEK5 isoform A is expressed mainly in the brain and liver and is a strong activator of ERK5 [24]. Of the 3 isoforms, A is the longest and contains all exons. Isoform B has all exons isoform A contains except exon 17, accounting for the difference of 1 kD. Isoform C contains all of the exons found in isoform A , but utilizes a much shorter exon 1, accounting for its lower molecular weight. Studies have shown that the MEK5 pathway induces

cell proliferation, EMT signaling, and cell survival, suggesting that MEK5 may potentially be an proto-oncogene [23].

It has been hypothesized that MEK1 and MEK2 play key roles as upstream regulators of LKB1 in other cancer cell types. LKB1 activity was found to be suppressed in SW480 colorectal cancer cells, and the addition of U0126, a drug that inhibits the kinase activity of MEK1 and MEK2, restored phosphorylated AMPK levels [25]. However, experiments with CI-1040, a specific MEK1 and MEK2 inhibitor, showed no restoration of pAMPK. This data suggests that MEK1 and MEK2 did not play a role in the inhibition of LKB1 in SW480 cells. U0126 may also have the ability to decrease MEK5 and ERK5 activity, suggesting that U0126 may facilitate pAMPK restoration by inhibiting MEK5. In addition, data from Hai-an Fu's laboratory showed that MEK5 was pulled down with glutathione S-transferase (GST) tagged LKB1. We therefore aimed to determine if MEK5 plays a role in the inhibition of the LKB1-AMPK pathway.

Henry Huang performed all experiments except for experiments presented in Figure 3A and Figure 10, which were performed by XiuJu Liu.

Hypothesis

We postulate that MEK5 inhibits LKB1 kinase activity through its binding to LKB1.

Research Aims

Aim 1- To determine whether overexpression of MEK5 inhibits LKB1 activity.

Previous data from our collaborator Hai-an Fu demonstrated direct LKB1 and MEK5 interaction via a pull-down assay. Here, we will determine whether LKB1-dependent AMPK

phosphorylation under glucose-free (GF) conditions can be suppressed by the over-expression of MEK5. In addition, we will confirm their finding by determining if MEK5 can be pulled-down by GST-LKB1 and if LKB1 can be pulled-down by GST-MEK5.

Aim 2- To determine whether kinase-dead MEK5 and phosphomimetic MEK5 mutants will alter MEK5/LKB1 binding or LKB1 kinase activity.

To determine if the kinase domain of MEK5 is involved in negatively regulating LKB1 kinase activity, we will generate kinase dead and phosphomimetic mutants of GST-MEK5. This will be done by changing catalytic amino acids S^{311} and T^{315} on MEK5 into A^{311} and A^{315} for the kinase dead mutant and D^{311} and D^{315} for the phosphomimetic mutant. We will evaluate whether these mutants alter LKB1 binding affinity or are capable of suppressing LKB1 kinase activity.

Aim 3 -To determine how MEK5 regulates LKB1 protein level.

To test if MEK5 regulates the half-life of LKB1, H1299 cell lines will be generated that express shRNA against MEK5. Cells with depleted MEK5 will be compared to control cells for the expression of endogenous levels of MEK5. Both cells will then be subject to a time-course assay using cycloheximide to inhibit protein translation for 0, 15, and 45 hours. LKB1 levels will be evaluated to determine if depletion of MEK5 affects the half-life of the LKB1 protein.

Methods and Materials

Materials – Primary antibodies against pAMPK, LKB1, AMPK, actin, mouse and rabbit monoclonal antibodies, and 10x lysis buffer were purchased from Cell Signaling Technology Inc.

MEK5 antibody was purchased from BD Laboratories. Plasmid pDONR223-MAP2K5 (Addgene plasmid 23805) was purchased from Addgene [26]. Plasmid EGFP-LKB1 was a gift from Junying Yuan[27]. Venus-MEK5 (vMEK5) was a gift from Hai-an Fu. Gateway Vector pDEST27, Lipofectamine 2000, Opti-mem, RPMI 1640 medium, RPMI 1640 glucose-free medium, Omnimax 2T1 phage resistant cells, and LR Clonase II enzyme mix were purchased from Life Technologies. QuikChange II XL Site-Directed Mutagenesis Kit was purchased from Agilent Technologies. Primers were purchased from Integrated DNA Technologies. BIX02188 was purchased from Selleckchem [28] . Polyvinyl diflourde (PVDF) membrane and 30% Acrylamide/Bis solution 29:1 were purchased from Bio-Rad. Cycloheximide was purchased from Sigma-Aldrich. Gluthathione Sephorase 4B, RNAi Consortium Lentiviral shRNa TRC Human Map2K5 shRNA, clone ID TRCN0000001470, and TRC Lentiviral pLK0.1 empty vector control were purchased from GE Healthcare.

Cell Lines and Cell Culture - Cervical cancer (HeLa) and lung cancer (H1299) cell lines were purchased from American Type Culture Collection (ATCC) and were grown and maintained according to ATCC specifications.

Plasmid Recombination - Wild-type pDONR 223 MEK5, mutant pDONR MEK5AA, and MEK5 DD were recombined into pDEST27 through LR Clonase according to Invitrogen's specification. Recombined plasmids were then transformed into Omnimax 2T1 phage resistant cells according to Invitrogen specifications, selected for on Luria Broth agar plates with 100ug/mL ampicillin, inoculated in Luria Broth with 100ug/mL ampicillin, and placed into a shaker at 37˚C for 18 hours. Plasmids were then purified using Qiagen Midiprep and used for transfection.

Transfection - HeLa and H1299 cells were seeded on 6 well plates 12-18 hours prior to transfection with a cell density of $3x10^5$ cells per well. Transfection was performed with 1.5µg of DNA and 4µL of Lipofectamine 2000 per well in Opti-mem reduced serum media. Cells were incubated for six hours with the transfection mixture and replaced with RPMI 1640 medium. Cells were harvested after 24 hours post transfection for pull-down assays. Cells undergoing glucose-free medium treatment had RPMI 1640 medium replaced with RPMI 1640 glucose-free medium 24 hr post transfection and incubated for two hours in GF media.

GST Pull-down – Cells were transfected with respective plasmids and harvested using 300µL lysis buffer with 25µL PMSF per 2.5 mL of lysis buffer and 1 Protease Cocktail Inhibitor tablet per 10 mL of lysis buffer. Lysate was incubated in 4˚C on a rotator for 30 minutes efficient lysis and then measured for concentration. An amount 300µg was allocated for each pull-down and lysis buffer was added to make a total of 300µL. Beads were allocated at 100µL per sample, centrifuged at 500 RCF for 5 minutes, and washed with 500µL PBS. This was repeated twice. Beads were then mixed with the prepared cell lysate and incubated at 4˚C for 2.5 hours on a rotator. The sample was centrifuged at 500 RCF for 5 minutes and washed with cold 500µL PBS for a total of three times. After the third time, 50µL elution buffer was added and incubated at 4˚C on a rotator. SDS was added to the sample, heated to 95˚C for 10 minutes, and run on an 8% SDS page gel.

Site-directed Mutagenesis – Primers purchased from IDT were designed to mutate pDONR 223 MEK5 plasmid at the S^{311} and T^{315} position into A^{311} and A^{315}, and D^{311} and D^{315}. The primer sequences used for the amplification of mutation site for generation of MEK5 AA and DD are displayed in Table 1. Primers were first purified using a 12% native Polyacrylamide gel

electrophoresis. The gel was run at 80 volts for 2 hours. The gel was stained with SYBR Green for 10 minutes and observed under ultraviolet light. Primers of the right length were cut out and incubated in 500µL Tris-EDTA at 37˚C for two hours, and room temperature overnight. Primers and PCR reactants were prepared to concentration according to Agilent specifications.

The PCR reaction was performed with the following condition: initial denaturation at 95˚C for 2 minutes, then 18 cycles of 95˚C for 20 seconds, 60˚C for 10 seconds, 68˚C for 3 minutes, and final annealing at 68˚C for 5 minutes. The PCR product was a plasmid of 5629 base pairs and was run on a 1% agarose gel to ensure amplification. Restriction enzyme DPN I was used to digest methylated parental plasmid. The mutant non-methylated plasmid was then transformed into XL-Gold Ultracompetent Cells included in the site-directed mutagenesis kit. Bacteria colonies were then isolated and cultured. The plasmids were purified through mini-preparation. MEK5 mutant and wild-type (WT) plasmids were sequenced by Genewiz using the MEK5 sequencing primer. Wild-type pDONR 223 MEK5, mutant pDONR MEK5AA, and MEK5 DD were recombined into pDEST27 using LR Clonase. Recombined plasmids were then transformed into Omnimax 2T1 phage resistant cells, selected for, and cultured. Plasmids were purified using Qiagen Midiprep.

Generating H1299-1470 cell lines- Cell line H1299 was transfected with plasmids according the TRC lentiviral transfection protocol. Cells were selected for using puromycin and maintained using puromycin at a concentration of 2ug/mL in RMPI 1640.

Time-course analysis- Cells were grown on 6 well plates and allowed to adhere to the plate and grow to 90% confluency in 37˚C incubator overnight. Cells were then treated with 10µg

cycloheximide /mL and harvested at 0, 15 and 45 hours post treatment. Lysate was sonicated briefly on ice and centrifuged at 4°C for 10 minutes at 13,000 RCF. Concentration was then measured and samples were equalized to 40µg of total protein. Appropriate amounts of 6xSDS loading dye were added to each sample, heated to 95°C for 10 minutes, and run on a western blot.

Western Blot Analysis –Cells were lysed using 1x lysis buffer with 25µL PMSF per 2.5 mL per 10 mL of lysis buffer and 1 Protease Cocktail Inhibitor tablet per 10 mL of lysis buffer. Protein concentration was measured and 40µg of total protein was loaded into 10% polyacrylamide gels. After running for 30 minutes at 80 volts and 105 minutes at 120 volts, gels were transferred to a PVDF membrane, blocked with 10% milk for an hour, washed for 10 minutes 3 times with TBST, and probed for pAMPK, LKB1, MEK5 and total AMPK.

Results

Aim 1- To determine whether overexpression of MEK5 inhibits LKB1 activity

Glucose-free mediated AMPK phosphorylation is LKB1 dependent in our experimental systems.

H1299 cells originate from lung cancer cells that are wild-type for LKB1, and the glucose-free condition is sufficient to promote AMPK phosphorylation. In addition, we have previously shown in an experiment performed by X.J. Liu that the pAMPK stress induced response in H1299 cells is LKB1 dependent (Figure 3A). To show this, an H1299-LKB1shRNA cell line was created via lentiviral infection that introduced a plasmid containing small hairpin RNA (shRNA) against LKB1. H1299-pLK0.1 cell lines with an empty vector served as control cells. H1299-pLK0.1 and H1299-LKB1shRNA were both treated GF medium for 0, 2, 4, 6, and 12 hours.

Results show that H1299-LKB1shRNA had almost no LKB1 compared to H1299-pLK0.1. H1299-pLK0.1 with LKB1 had increased levels of pAMPK throughout GF treatment, while H1299-LKB1shRNA pAMPK levels were low throughout GF treatment. This shows that pAMPK cannot be induced in the absence of LKB1, demonstrating that LKB1 is required to mediate AMPK phosphorylation under glucose-free condition.

We also used HeLa cells as an alternative cell line model. HeLa cells originate from cervical cancer cells and have a homozygous deletion of the LKB1 gene [29]. Since HeLa cells do not have LKB1, glucose withdrawal will not cause AMPK phosphorylation [29] (Figure 4). X.J. Liu ectopically over-expressed LKB1 in HeLa cells to demonstrate that GF-induced AMPK phosphorylation is LKB1 dependent (See Figure 10, comparing lanes 2 and 4). In combination, these data provided by X.J. Liu indicate that glucose-free mediated AMPK phosphorylation is LKB1-dependent in our cell line models.

Overexpression of Wild-type MEK5 Inhibits LKB1-dependent AMPK phosphorylation.

To access how MEK5 affects LKB1 kinase activity, H1299 cells were transfected with GST-MEK5WT (Figure 3B). To ensure that lipofectamine reagents did not affect the LKB1 and pAMPK pathway, lane 1 and 2 cells were transfected without any DNA. Lane 2 and 4 cells were treated with GF medium for 2 hours and had an increase in pAMPK levels compared to cells with normal RMPI 1640 medium due to presence of endogenous LKB1. Lane 3 and 4 cells were transfected with GST-MEK5WT. Lane 3 showed no induction of pAMPK while Lane 4 showed induced pAMPK. Cells transfected with MEK5 and introduced to GF medium had significantly less pAMPK compared to control cells without transfection of MEK5. This suggests that the

overexpression of MEK5 may be inhibiting LKB1 kinase activity, preventing AMPK phosphorylation.

To investigate whether MEK5 and LKB1 interact, GST beads were used as our pull-down platform. In order to pull-down proteins, pDest27 was used as an expression vector because it encodes a GST tag at the N terminus of recombinant proteins. Venus-MEK5 has a yellow fluorescent protein attached to MEK5. GST-LKB1 and vMEK5 were co-transfected into the HeLa cells (Figure 5). Pull-down of GST-LKB1 was also able to bring down vMEK5, confirming that MEK5 interacts with LKB1. Addition of BIX02188, a selective MEK5 kinase inhibitor that acts on MEK5's catalytic site, to cells co-transfected with MEK5 and LKB1 in lane 4 showed much less binding than cells without the drug treatment in lane 3 [28, 30]. This suggests that BIX02188 disrupts LKB1 and MEK5 interaction, and that MEK5's kinase site may play a role in the binding interaction. Lanes 7 and 8 with cell lysate shows equal transfection of vMEK5 and lanes 1 to 4 showed approximately equal amounts of GST-LKB1 pulled down.

To further confirm our findings, we used GST-MEK5 as a bait and eGFP-LKB1 as the target. eGFP-LKB1 and GST-MEK5WT were co-transfected into HeLa cells (Figure 6). Pull-down of GST-MEK5WT was also able to pull-down eGFP-LKB1. Lanes treated with BIX02188 showed significantly less LKB1, suggesting again that BIX02188 disrupts the interaction. Lane 3 and 4 showed equal amounts of GST-MEK5 binding to the beads. In conjunction with our discovery that MEK5WT may decrease pAMPK, the fact that BIX02188 disrupts binding between MEK5 and LKB1, suggests that the MEK5 catalytic domain may be involved in the inhibition of LKB1 kinase activity.

Aim 2- To determine whether kinase-dead MEK5 and phosphomimetic MEK5 mutants will alter MEK5/LKB1 binding or LKB1 kinase activity.

To test if MEK5 kinase domain is involved in the binding between MEK5 and LKB1, kinase-dead and phosphomimetic MEK5 were generated using site-directed mutagenesis. The catalytic site of the MEK5 kinase domain was identified at S^{311} and T^{315} [31]. To inactivate kinase activity, amino acids S^{311} T^{315} were both mutated into non-polar $A^{311}A^{315}$, and to generate phosphomimetic MEK5, amino acids S^{311} T^{315} were both mutated into negatively charged $D^{311}D^{315}$ [31]. Sequencing results from Genewiz of the MEK5 plasmid show that MEK5AA had correct changes at the correct positions (Figure 7).

To investigate if changes to the catalytic site of MEK5 affect the binding affinity to LKB1, HeLa cells were co-transfected with EGFP-LKB1 and with either GST-MEK5WT or MEK5 mutants (Figure 8). In addition, we also tested BIX02188's effect on the binding between the different MEK5 constructs and LKB1. Similar to previous experiments, GST-MEK5WT was able to bind to eGFP-LKB1 while there was less eGFP-LKB1 bound in cells treated with BIX02188. Analysis of the pull-down between MEK5 mutants and LKB1 showed that kinase dead and phosphomimetic MEK5 pulled down similar amounts of eGFP-LKB1. The amount of LKB1 pulled down by both mutants was significantly greater than the amount of LKB1 pulled down by MEK5WT, demonstrating that the MEK5 mutants have a higher binding affinity that MEK5WT. Introduction of BIX02188 also disrupted binding between MEK5 mutants and eGFP-LKB1, suggesting that the kinase domain is involved in the binding.

To further test whether the increase in MEK5 binding leads to an alteration of LKB1 kinase activity, H1299 cells were transfected with MEK5 constructs and treated with GF medium for 2

hours (Figure 9). As expected, GF medium treatment caused increases in pAMPK while cells treated with normal medium did not show increased pAMPK levels. When comparing GF treated control cells to GF treated MEK5WT cells, cells with MEK5WT had significantly less AMPK phosphorylation consistent with our previous observation in Figure 3B, suggesting that MEK5WT inhibits LKB1-pAMPK activity. In contrast, cells with mutant AA or DD did not show changes in pAMPK levels under GF conditions compared to the control, indicating that these mutants are not capable of suppressing LKB1-mediated AMPK phosphorylation. Therefore, even though these mutants have high LKB1 binding affinity, our data suggests that the increase in the binding between MEK5 and LKB1 does not play a role in the suppression of LKB1.

Aim 3 -To determine how MEK5 regulates LKB1 protein level

An experiment carried out by X.J. Liu in HeLa cells where cells were co-transfected with eGFP-LKB1 and MEK5 constructs showed that overexpression of MEK5WT caused down-regulation of ectopic LKB1 protein expression (Figure 10). HeLa cells transfected with MEK5WT and LKB1 showed less LKB1 levels compared to control cells transfected with only LKB1 and cells co-transfected with LKB1 and MEK5AA. This suggests MEK5 may play a role altering the LKB1 protein level. Interestingly, there was no difference in LKB1 levels between cells exposed to normal medium and cells exposed to GF medium, suggesting that MEK5 acts on LKB1 under both normal and energy stress conditions. In addition, the fact that only MEK5WT was able to decrease LKB1 levels suggests that phosphorylation at the kinase domain of MEK5 may be needed to observe changes in LKB1 levels.

Since decreased LKB1 was observed in only cells overexpressed with GST-MEK5WT, we next tested what would happen to the LKB1 protein level if MEK5 were depleted. To do this, we used H1299-1470 cell lines that contained a constitutively expressed MEK5 shRNA. H1299-pLK0.1 cell lines again served as control cells. Upon immunoprobing, we discovered that the shRNA did not target all MEK5 isoforms. In H1299-pLK0.1 cells, MEK5 isoform C levels were present in higher levels than both isoform A and isoform B. In H1299-1470 cell lines, MEK5 isoform C level was significantly lower compared to H1299-pLK0.1 isoform C levels. Isoform A and isoform B were present at similar levels in both cell lines. In addition, we observed that after depletion of MEK5, there were no significant changes in LKB1 endogenous levels between the H1299-pLK0.1 cells and H1299-1470. Since LKB1 levels were consistent, this suggests that deletion of MEK5 isoform C does not affect steady state levels of LKB1.

To evaluate the half-life of LKB1, we performed a time course experiment using cycloheximide, a eukaryotic translation inhibitor. Cycloheximide stops any new production of LKB1 in the cell, and allows us to observe the change in endogenous LKB1 level in the presence and absence of MEK5 isoform C. H1299-pLK0.1 and H1299-1470 cells were subject to 10 μg cycloheximide per mL medium over the course of 45 hours and samples were harvested at 0, 15 and 45 hours (Figure 11). H1299-1470 shRNA targeted only MEK5 isoform C and abolished most traces of it. H1299-1470 cells showed stable levels of LKB1 throughout the 45 hour treatment. In contrast, H1299-pLK0.1 showed decreases in LKB1 levels at 45 hours. This difference may be explained by the depletion of MEK5 isoform C in H1299-1470 cells and the presence of MEK5 isoform C in H1299-pLK0.1.

Discussion

LKB1 is a tumor suppressor that is compromised in the hereditary disease Peutz-Jeghers syndrome [7]. Patients diagnosed with Peutz-Jeghers syndrome show greater risks of developing other cancers, suggesting that LKB1 plays a vital role as a tumor suppressor [32]. In NSCLC, the inactivation of LKB1 is known to cause an increase in tumor metastasis and aggressiveness as demonstrated by the loss of LKB1 in KRAS mutant mouse models [9]. Currently, there are two known mechanisms of LKB1 inactivation. LKB1 can be inactivated at the DNA level through somatic mutation or promoter hypermethylation, or at the protein level through the recently discovered B-RAF pathway where B-RAF activity inhibits the LKB1-AMPK pathway [6, 7, 20]. Here, we propose a new mechanism by which LKB1 can be inactivated at the protein level by MEK5 overexpression.

We found that overexpression of MEK5 suppressed GF induced pAMPK, suggesting that increased levels of MEK5WT may inhibit LKB1 kinase activity. One of our experiments also showed that overexpression of MEK5WT can also suppress LKB1 protein levels. In conjunction with our discovery that depletion of MEK5 leads to increased LKB1 half-life, the data from these results suggests that overexpression of MEK5WT in cells can promote LKB1 degradation.

Data from our collaborator, Hai-an Fu, suggests that MEK5 interacts with LKB1. To further confirm this discovery, we tested and demonstrated this through pull-down assay using either GST-LKB1 or GST-MEK5 as the pull-down vehicle in separate experiments. We observed that the addition of BIX02188, a specific MEK5 catalytic activity inhibitor, was able to disrupt binding between the two, suggesting that the kinase domain of MEK5 is involved in the interaction between MEK5 and LKB1.

To test if the kinase domain is involved, we generated kinase dead MEK5AA by site-directed mutagenesis where the catalytic hydroxyl groups of S^{311} and T^{315} were converted to nonpolar alanine. To generate the phosphomimetic and constitutively active MEK5, we changed the S^{311} and T^{315} both to aspartic acid. This change has been shown to activate MEK5 and increase phosphorylation of downstream target protein ERK5 [33]. We determined whether or not changes to MEK5 catalytic site would affect the binding to LKB1. Our findings were unexpected, as we discovered that both MEK5 mutants bound equally tightly to LKB1, and significantly more when compared to MEK5WT. This is unexpected because MEK5AA is a kinase dead mutant with no charge and has no catalytic capability, while MEK5DD is phosphomimetic with a negative charge and has increased catalytic capability [31]. This finding suggests that the MEK5 kinase domain is involved in the binding interaction between MEK5 and LKB1. The addition of BIX02188 also caused disruption in the interaction of all MEK5 constructs and LKB1. Since BIX02188 binds to MEK5's kinase domain, it further supports our finding above that the MEK5 kinase domain is involved in the binding interaction of MEK5 and LKB1.

We next assessed whether the increase in MEK5 mutant binding to LKB1 played a role in the suppression of LKB1's kinase activity. Since AMPK is a downstream target of LKB1, we measured pAMPK levels to monitor LKB1 activity [14]. Upon overexpression of MEK5 mutants, we discovered that cells with overexpression of MEK5 mutants did not have significant changes in pAMPK levels compared to a significant pAMPK level decrease in MEK5WT GF treated cells. Phosphorylated AMPK levels in MEK5 mutant expressing cells had similar pAMPK levels compared to control cells with no overexpression of MEK5. Since the MEK5 mutants bind equally tightly, and since the MEK5 mutants did not affect pAMPK levels, we concluded that the

increase in binding affinity between LKB1 and MEK5 was not sufficient for the suppression of LKB1 by MEK5.

Our findings that overexpression of MEK5WT can suppress the ectopic expression of LKB1 in HeLa cells (Figure 10) lead us to test how depletion of MEK5 may also affect LKB1 levels. We discovered that the depletion of MEK5 isoform C leads to increased LKB1 half-life while LKB1 half-life decreased due to the presence of MEK5 isoform C. This finding shows support that MEK5 may be suppressing LKB1 by targeting LKB1 for degradation.

There are several unresolved issues in our study that will require further evaluation. First, if MEK5 is targeting LKB1 for degradation, our next steps would be to add inhibitors of known degradation pathways to see if LKB1 may be rescued. Second, we noticed that there was no significant difference in endogenous LKB1 levels in cells with or without MEK5 isoform C, which suggests that the production of LKB1 may be down-regulated in MEK5-depleted cells. We will use quantitative real-time PCR to determine whether the transcription of LKB1 is altered in MEK5 depleted cells.

Our proposed mechanism suggests that the overexpression of MEK5 can have potential oncogenic effects through the inactivation of LKB1. For example, LKB1 was shown to have a low frequency of mutation in breast cancer, but the loss of LKB1 in mice mammary glands was shown to induce tumor formation in 46-65 weeks in mice [34, 35]. LKB1 was also shown to play a tumor suppressive role several breast cancer models [36]. Interestingly, MEK5 was found to be expressed at elevated levels in 50-70% of invasive breast cancer tissues compared to normal breast tissue [35]. MEK5 was also found to be amplified in 21% breast cancer xenografts in a case study [37]. Therefore, it may be possible that the amplification of MEK5 in breast tissue

may contribute to the inactivation of LKB1. This discovery may have potential therapeutic options as a better understanding of the mechanism between MEK5 and LKB1 can lead to drug discovery that can attenuate MEK5's inhibition on LKB1.

Our next goal in continuing to decipher this mechanism would be to identify the MEK5 domain that is involved in diminishing LKB1 levels. We cannot rule out the potential of other MEK5 domains such as MEK5's PB1 or ATP binding domain playing a role in the suppression of LKB1. In order to identify which domain may play a role, we will create truncations of MEK5 through site-directed mutagenesis in order to determine the domain needed for MEK5 to interact with LKB1. In addition, a truncation at MEK5's kinase domain will also be made to test if the loss of the MEK5 kinase domain will inhibit LKB1 kinase activity. To generate the truncations, stop codons will be encoded on primers that anneal to base pairs a few amino acids after each domain. The newly generated plasmid will have the stop codon encoded and can be transfected once the gene is moved into mammalian vector pDest27. We will check for correct truncations before analyzing the MEK5 molecular weight on western blots. Once the domain has been identified, we can begin targeting amino acids in those domains known to be important sites for MEK5 to interact with other proteins.

Other important experiments in the future will require further understanding of the different MEK5 isoforms and if knockout of MEK5 isoform A or isoform B produce similar effects as MEK5 isoform C. To do this, we will test other variants of MEK5 shRNA to try to knock down MEK5 isoform A and isoform B and perform cycloheximide time-course to discover if the half-life of LKB1 is affected. We will also need to determine if MEK5AA is kinase dead and if MEK5DD is constitutively active in our system. While it has been shown that conversion of catalytic

residues at S^{311} and T^{315} into D^{311} and D^{315} produce an active MEK5 that can phosphorylate downstream ERK5, we will analyze phosphorylated levels of MEK5 downstream target protein ERK5 in both HeLa and H1299 cells to ensure that our mutants have acquired their mutated functions [31].

Some limitations in our current study are that our current plasmid platform constitutively overexpresses MEK5 constructs. The overexpression of MEK5 does not produce a constant concentration, making it difficult to quantify the amount of MEK5 needed to inhibit LKB1 kinase activity. To do this, we will recombinate the MEK5 gene into inducible vectors where we can control the amount of MEK5 produced by altering concentrations of the inducing drug. We will also use real-time PCR to quantify the amount of MEK5 generated to determine if MEK5 requires a certain threshold to inhibit LKB1 kinase ability. Another limitation is that GST beads linked to prey protein interacting with fluorescently tagged bait protein may introduce artifacts that do not occur under normal cellular conditions. Our next step would be to pull-down endogenous LKB1 or MEK5 using an antibody against LKB1 or MEK5 to test for endogenous protein interaction between MEK5 and LKB1. This approach will decrease potential artifacts and allow us to analyze any proteins that are pulled down with our prey protein.

In summary, LKB1 is a key player in cellular response to energy stress. Loss of LKB1 function affects many of its downstream pathways, such as AMPK, which has been shown to play a role in regulating the oncogene mTOR [19]. Loss of LKB1 has also shown to result in accelerated tumorigenesis and metastasis in lung tissue [9]. Here, we propose a mechanism where MEK5 suppresses LKB1 function by decreasing its half-life and stability (Figure 12). Understanding this pathway is important and has potential therapeutic applications. The MEK5-LKB1 pathway may

be applicable to other types of cancer, such as breast cancer, where the MEK5 gene is amplified and LKB1 has been shown to be inactivated in invasive breast tissue [35]. Understanding the mechanism may open the road to development of drugs to inhibit MEK5 and restore LKB1 levels and tumor suppressor function.

Tables

Primer name	Primer (5’ => 3’)
MEK5AA-Forward	GCTGGTGAATGCTATAGCCAAGGCGTATGTTGGAAC
MEK5AA-Reverse	GTTCCAACATACGCCTTGGCTATAGCATTCACCAGC
MEK5DD-Forward	GCTGGTGAATGATATAGCCAAGGACTATGTTGGAACC
MEK5DD-Reverse	GGTTCCAACATAGTCCTTGGCTATATCATTCACCAGC
MEK5-F	GGAAGAATTGCAGTAGCAGTTG

Table 1. Primers used in this study.
The first four primers were used in site-directed mutagenesis to generate MEK5 mutant constructs. These primers were designed according to specifications provided by Agilent. MEK5-F was used as a sequence primer to validate the mutant constructs. Primers were purchased from Integrated DNA technology. Primers were purified using 12% PAGE gel and run at 120 volts for 2 hours. Gel was stained with SYBR Green and cut out primers were eluted in Tris-EDTA at 37˚C for 2 hours, then at room temperature overnight.

Figures

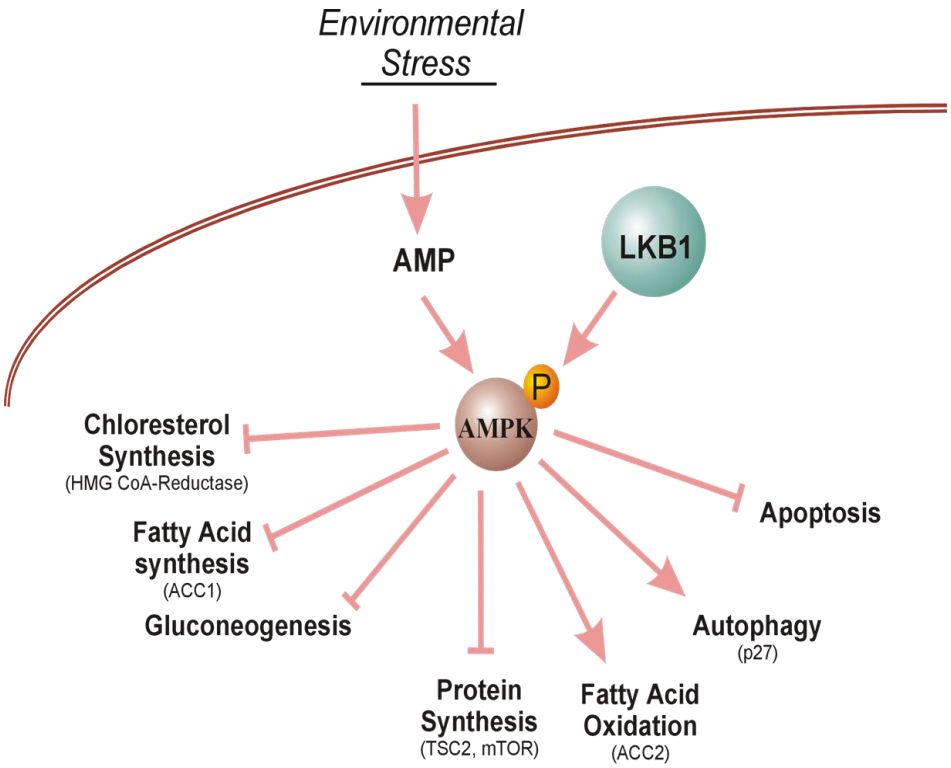

Figure 1. LKB1/AMPK metabolic checkpoint [17]
Energy stress increases cellular AMP concentration, leading to conformational change in AMPK, exposing phosphorylation site T172. LKB1 phosphorylates AMPK, which promotes catabolism and suppresses anabolism in order to conserve cellular energy consumption.

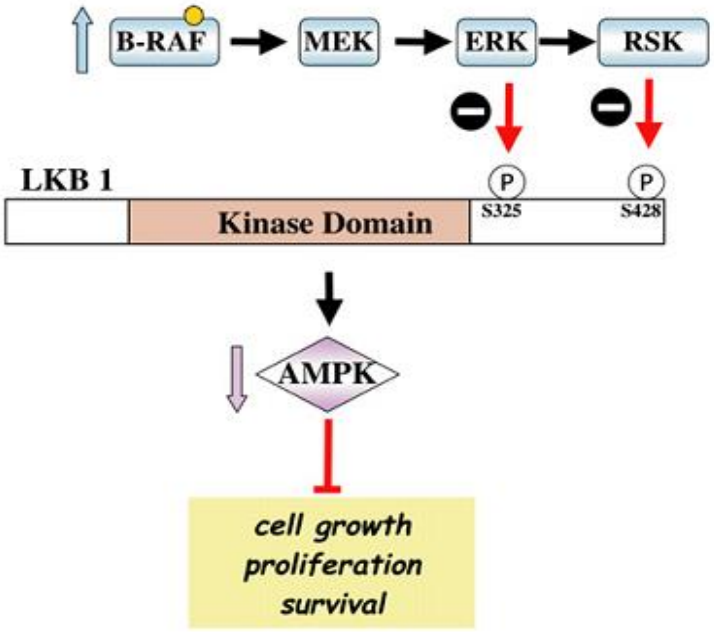

Figure 2. B-RAF negatively regulates LKB1.
B-RAF V600E mutants activate MEK/ERK/RSK signaling. The phosphorylation of LKB1 at S325 by ERK and at S428 by RSK attenuates LKB1-AMPK interaction, thus inhibiting LKB1-mediated AMPK phosphorylation [20].

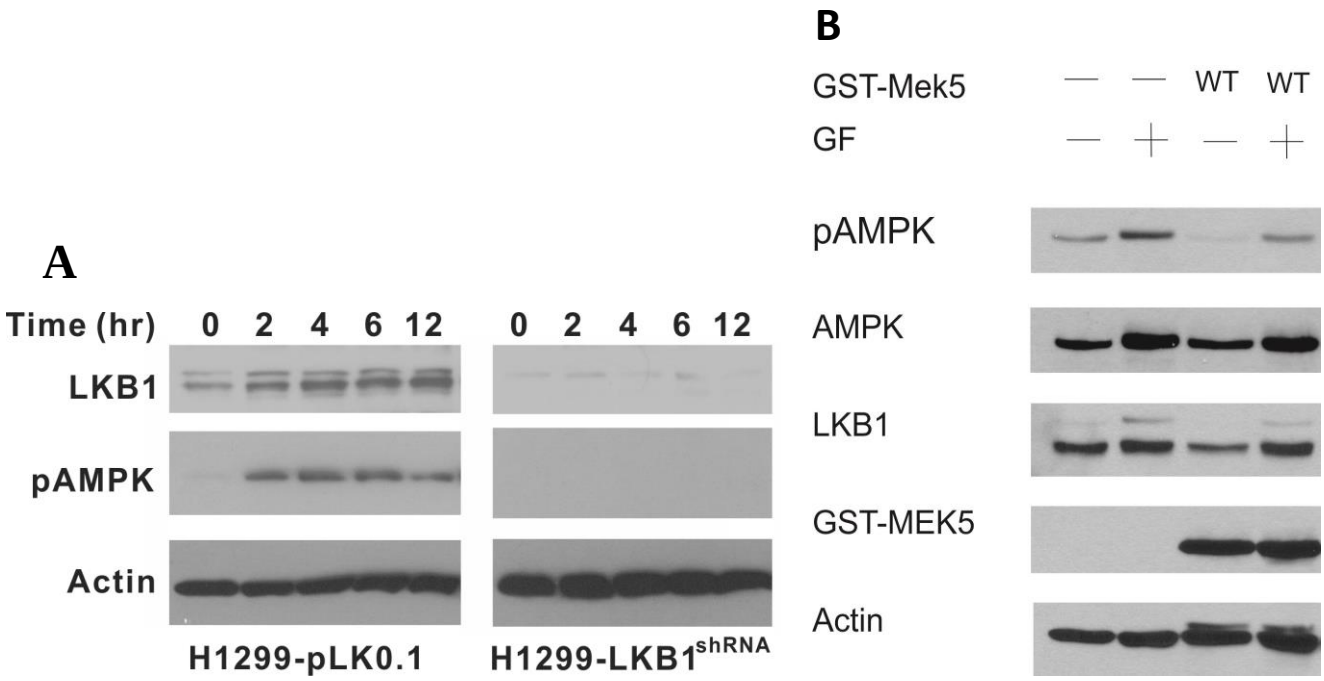

Figure 3. MEK5 over-expression suppresses LKB1-dependent AMPK phosphorylation under glucose-free condition in H1299 cells.

To show LKB1 mediates pAMPK in H1299, H1299-LKB1shRNA cells were generated by infection of lentivirus containing-LKB1-shRNA, followed by puromycin selection. H1299-pLK0.1 served as control cells. Cells were treated with GF medium for 0, 2, 4, 6, and 12 hours to induce pAMPK (A). H1299-LKB1shRNA was unable to induce pAMPK while H1299-pLK0.1 had increased pAMPK levels. This shows that LKB1 directly regulates pAMPK. This experiment was performed by XiuJu Liu. H1299 cells were transfected with GST-MEK5 for 6 hours and transfection medium was replaced with RPMI 1640 (B). 48 hours post transfection, cells were treated with GF medium containing 5% FBS for 2 hours in indicated lanes. Cells were lysed with 100µL lysis buffer, run on western, and probed with indicated antibodies.

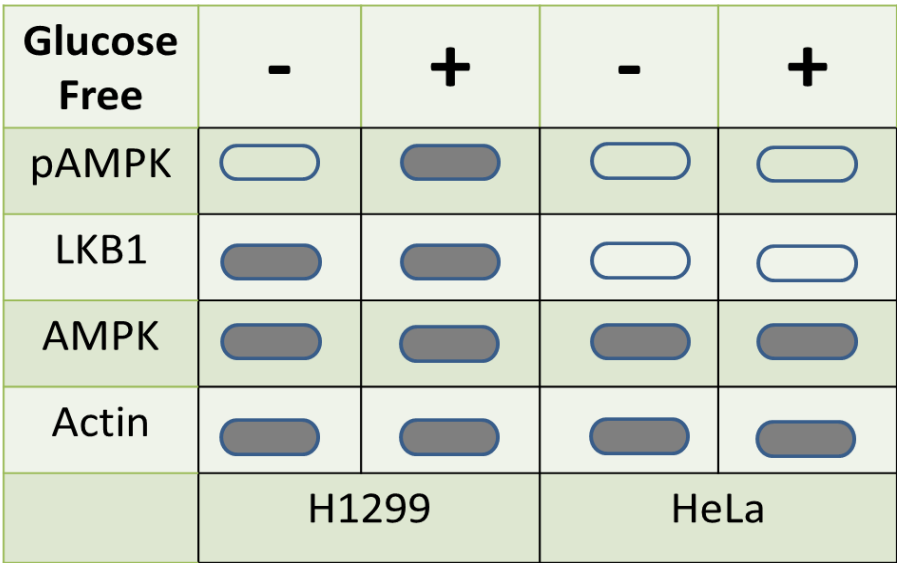

Figure 4. pAMPK is inducible in H1299 cells, but not in HeLa cells. Lung cancer H1299 cells contain endogenous LKB1. Under GF conditions, AMPK can be phosphorylated by LKB1. In cervical cancer HeLa cells, LKB1 is bi-allelic deleted. Under GF conditions AMPK cannot be phosphorylated due to the absence of LKB1. Both cells have endogenous AMPK despite HeLa cells not being able to induce pAMPK. Actin is used as a loading control in our experiments.

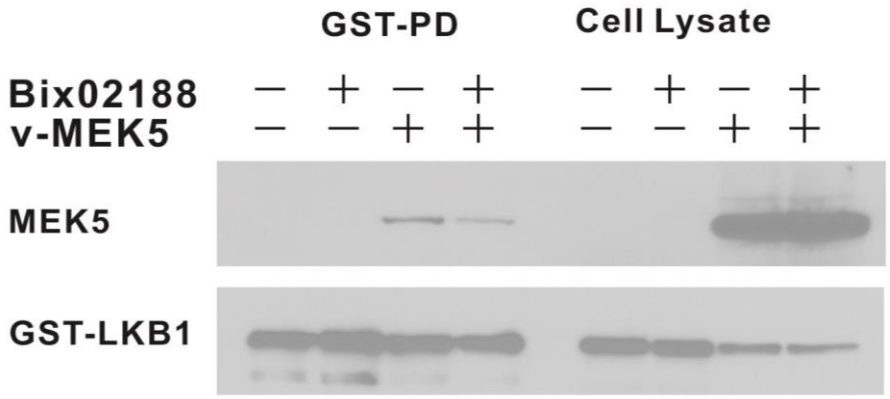

Figure 5. GST-LKB1 binds to vMEK5 and BIX02188 disrupts their interaction

HeLa cells were seeded with $3x10^5$ cells per well in 6 well plates. At 75% confluency, HeLa cells were co-transfected with indicated plasmids for 6 hours. After 24 hours of transfection, cells were treated with BIX02188 at 1µM for two hours, then harvested using 100µL prepared lysis buffer. Lysates were incubated with prepared GST-agarose beads at 4˚C on a rotator, then centrifuged and washed with PBS three times. The samples were eluted, run on a western blot, and probed using indicated antibodies.

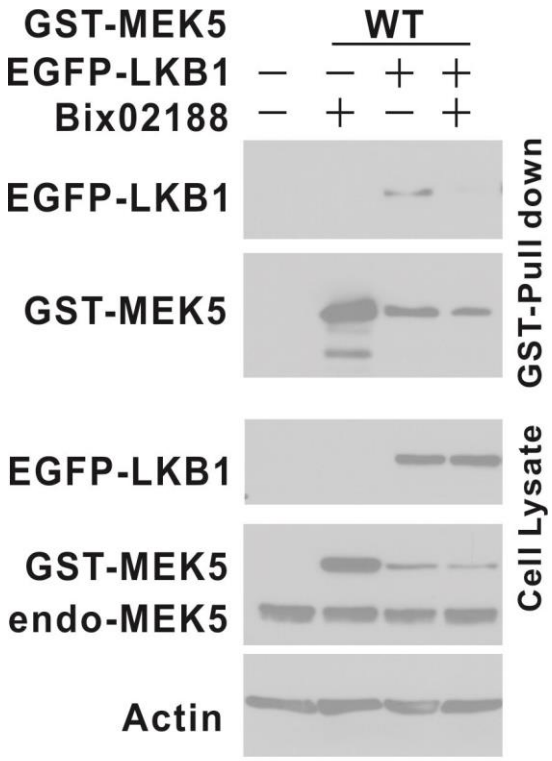

Figure 6. GST-MEK5 binds to eGFP-LKB1 and BIX02188 disrupts their interaction

HeLa cells were seeded with $3x10^5$ cells per well in 6 well plates. At 75% confluency, HeLa cells were co-transfected with indicated plasmids for 6 hours. 24 hours after transfection, cells were treated with BIX02188 at 1uM for two hours, then harvested using 100µL prepared lysis buffer. Lysates were incubated with prepared GST-agarose beads at 4˚C on a rotator, then centrifuged and washed with PBS three times. The samples were eluted, run on a western blot, and probed using indicated antibodies.

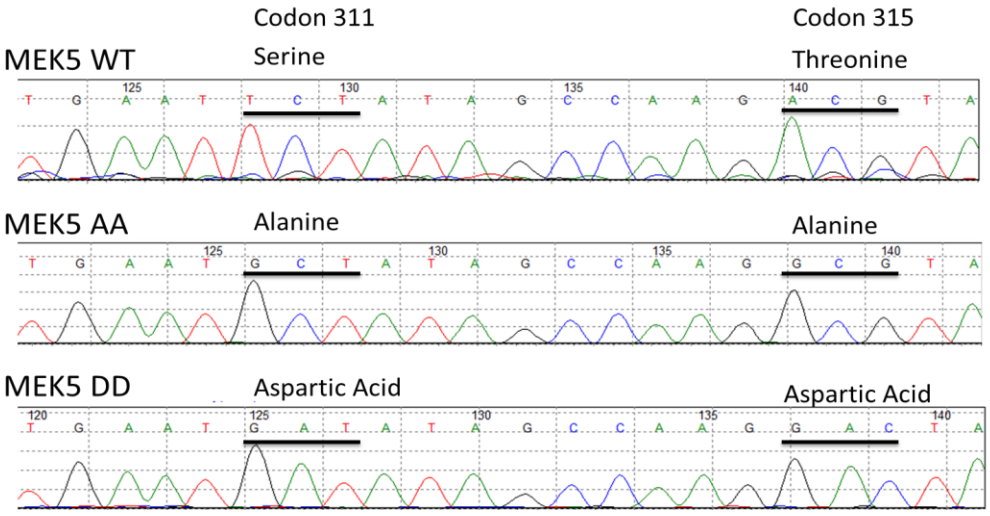

Figure 7. Genewiz sequencing confirms correct mutation in MEK5 mutants.

MEK5 mutants were sent to Genewiz to sequence with sequencing primer MEK5-F. MEK5AA had S311 and T315 changed to A311 and A315 using primers MEK5AA-F/R. MEK5DD had S311 and T315 were changed to D311 and D315 using MEK5DD-F/R. Analysis was done by aligning MEK5 mutants with MEK5WT using Mutation Explorer software.

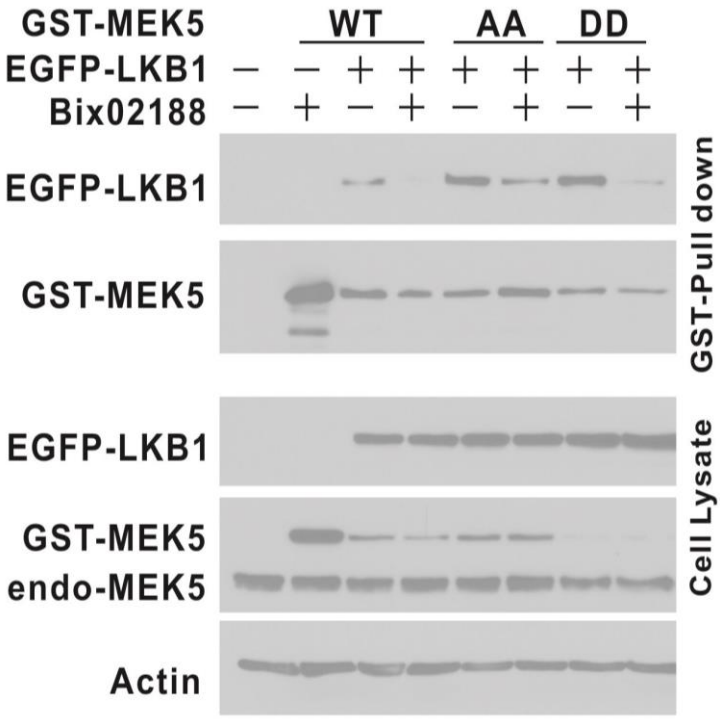

Figure 8. MEK5 mutants bind more tightly to LKB1 than MEK5WT does to LKB1.

HeLa cells were seeded with $3x10^5$ cells per well in 6 well plates. At 75% confluency, HeLa cells were co-transfected with indicated plasmids for 6 hours. 24 hours after transfection, cells were treated with BIX02188 at 1µM for two hours, then harvested using 100µL prepared lysis buffer. Lysates were incubated with prepared GST-agarose beads at 4˚C on a rotator, then centrifuged and washed with PBS three times. The samples were eluted, run on a western blot, and probed using indicated antibodies.

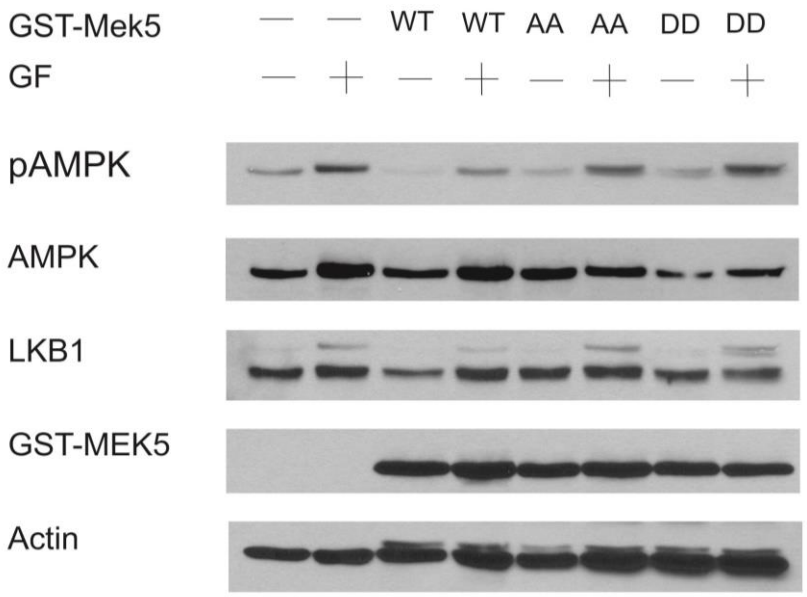

Figure 9. MEK5 mutants do not suppress LKB1 kinase activity. H1299 cells were seeded with $3x10^5$ cells per well in 6 well plates. At 75% confluency, cells were transfected with GST-MEK5 for 6 hours, then transfection medium was replaced with RPMI 1640. 48 hours post transfection, cells were treated with GF medium containing 5% FBS for 2 hours in indicated lanes. Cells were lysed with 100µL lysis buffer, run on western, and probed with indicated antibodies.

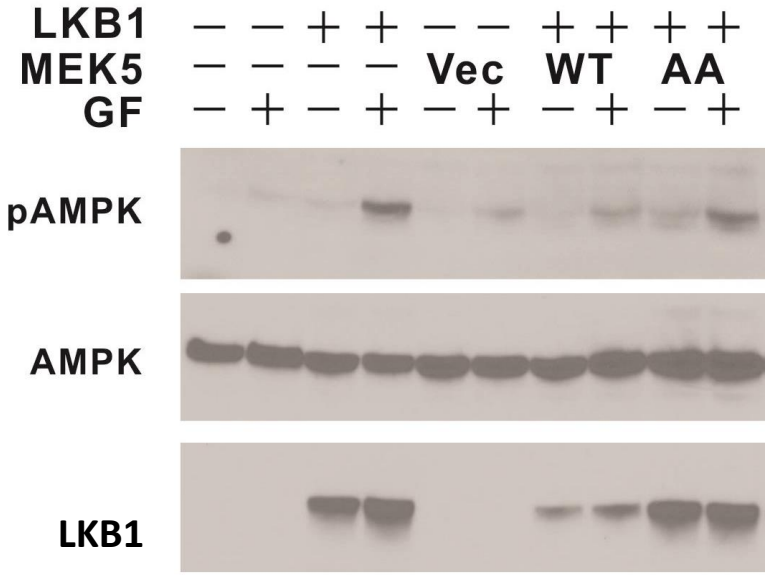

Figure 10. MEK5WT decrease LKB1 levels.
HeLa cells were seeded with $3x10^5$ cells per well in 6 well plates. At 75% confluency, HeLa cells were co-transfected with indicated plasmids for 6 hours. 24 hours after transfection, cells were treated with BIX02188 at 1µM for two hours, then harvested using 100µL prepared lysis buffer. Lysates were sonicated briefly on ice, run on a western blot, and probed using indicated antibodies. This experiment was performed by XiuJu Liu.

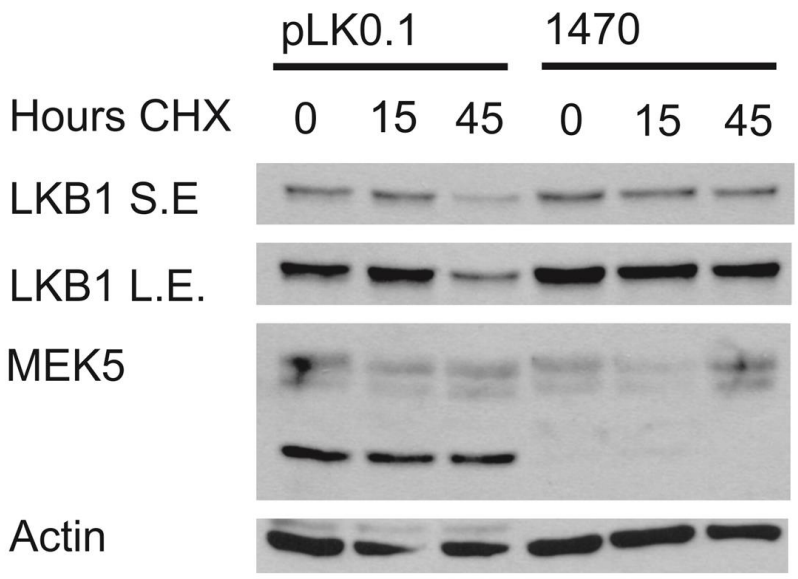

Figure 11. **MEK5 isoform C decreases LKB1 half-life**
H1299 cells were infected with a lentivirus containing MEK5-shRNA (1470) and selected with puromycin. Cells treated with pLK0.1 lentivirus were used as vector control. H1299-pLk0.1 and H1299-1470 cells were seeded at $3x10^5$ cells per well in 6 well plates. Cells were incubated in RPMI 1640 with 2µg/mL puromycin for selection. At 75% confluency, cells were treated with cycloheximide at 10µg/mL in RPMI 1640. Cells were harvested after 0, 15 and 45 hours of cycloheximide treatment. Lysates were sonicated briefly on ice, run on a western blot, and probed using indicated antibodies.

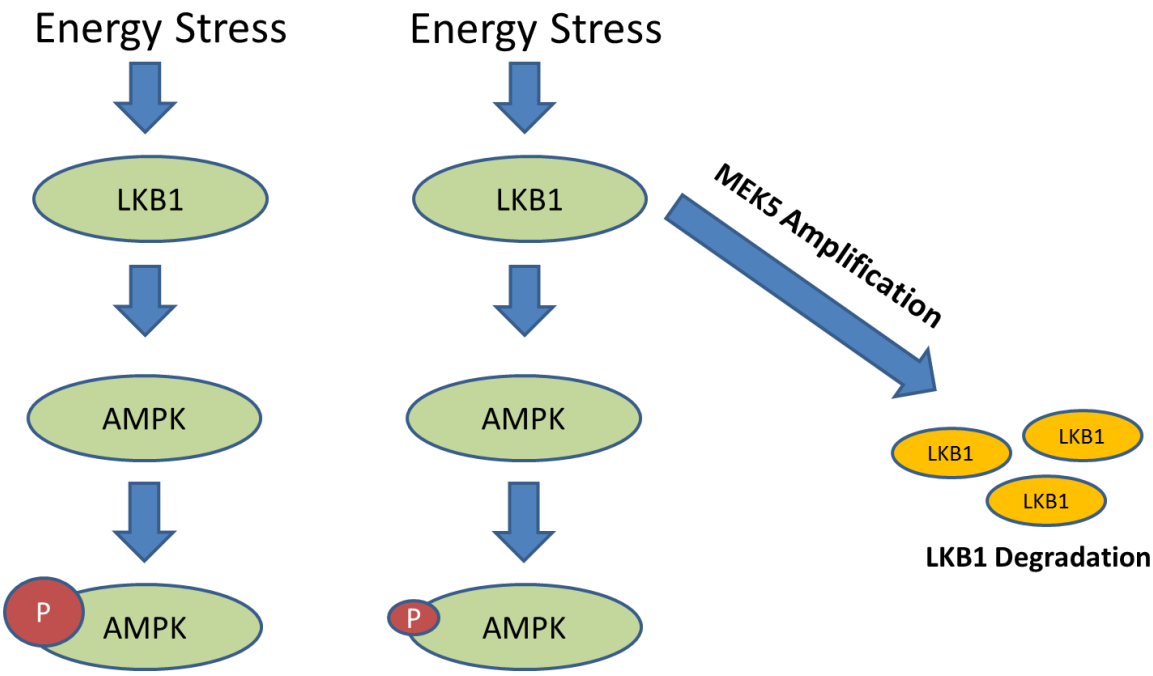

Figure 12. Overexpression of MEK5 inhibits LKB1 activity by decreasing LKB1 levels. Under normal endogenous protein levels, energy stress exposes AMPK phosphorylation site and allows LKB1 to activate pAMPK (Left). Overexpression of MEK5 inhibits LKB1 activity, causing decreases in pAMPK levels (Right). Our mechanism proposes that the overexpression of MEK5 potentially leads to LKB1 degradation, causing decreased LKB1 activity.

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