Identification of LKB1 kinase substrates involved in suppression of lung adenocarcinoma cell growth

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Master's Thesis

International Master's Degree Programme in Translational Medicine
Faculty of Medicine
University of Helsinki
2021

Abstract

STK11/LKB1 is a tumor suppressor gene and mutated in 18% of lung adenocarcinomas. Tumor suppressor liver kinase B1 (LKB1) is known to activate adenosine monophosphate-activated protein kinase (AMPK) and 12 AMPK-related kinases (ARKs) by phosphorylating a conserved threonine residue in their T-loop region. A number of studies focused on investigating the influence of LKB1-AMPK signaling on cancer cell proliferation. However, there is no systematic study for identifying the critical LKB1 kinase substrates in suppressing lung cancer cell growth. In this project, the LKB1-deficient lung adenocarcinoma cell line A549 cells were sequentially overexpressed with constitutively active mutants of AMPKα1, AMPKα2, MARK1, MARK2, MARK3, MARK4, NUAK1, NUAK2, SIK1, SIK2, SIK3. The overexpression status was confirmed at both genetic and protein levels by qPCR and Western blotting, correspondingly. *In vitro* growth assays demonstrated up to 33% reduced growth rate of A549 cells overexpressing AMPKα1, AMPKα2 and NUAK1. Furthermore, siRNA knockdown of the selected substrates in LKB1-overexpressing A549 cells significantly rescued the cell growth defect. These findings suggest, that AMPKα1, AMPKα2 and NUAK1 kinases are critical for LKB1-mediated cell growth defect in lung adenocarcinoma.

Keywords: LKB1; AMPK; NUAK1; lung adenocarcinoma; ARKs; growth defect; tumor suppressor.

List of abbreviations

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
C-term.	Carboxyl-terminus
cAMP	Cyclic adenosine monophosphate
dNTP	Deoxynucleoside triphosphate
DC	Detergent compatible
DMEM	Dulbecco`s Modified Eagle Media
emGF P	Emerald green fluorescent protein
FBS	Fetal Bovine Serum
g	Gram
G	Gauge
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hour
HRP	Horseradish peroxidase
Ig	Immunoglobin
kDa	Kilodalton
L	Liter
LSB	Laemmli sample buffer
M	Molar
min	Minute
mL	Milliliter
mM	Millimolar
N	Number
NADPH	Nicotinamide adenine dinucleotide phosphate
OAZ1	Ornithine Decarboxylase Antizyme 1
°C	Degree Celsius
P	*p*-value
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PKA	Protein kinase A

RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolution per minute
RT buffer	Reverse transcription buffer
RT-qPCR	Real-time quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
SEM	Standard error of the mean
Ser	Serine
siNT	Non-targeting small interfering ribonucleic acid
siRNA	Small interfering ribonucleic acid
TBS	Tris-buffered saline
Thr	Threonine
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
U	Enzyme unit
v/v	Volume per volume
V	Volt
WT	Wild type
w/v	Weight per volume
α-	Anti-
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar

Table of Contents

1 Introduction

Tumor suppressor LKB1 along with its 13 downstream substrates of AMPK subfamily comprise a signaling pathway that maintains a broad spectrum of functions within a cell – from the cell cycle control to the regulation of the cell metabolism[1,2].

Antitumor properties of LKB1 were first identified in Peutz-Jeghers syndrome, a hereditary cancer susceptibility disease, caused by germline truncating mutations of LKB1/STK11 gene[3,4]. Later, LKB1 mutations were also identified in sporadic tumors with different tissue localization[5–7], including lung adenocarcinomas, the most common form of lung cancer[8], where LKB1 loss-of-function mutation is detected in 18% of cases[9]. However, it remains unclear, which of the LKB1 downstream kinases are crucial for LKB1 mutant lung tumorigenesis.

To elucidate this mechanism, I conducted a systematic screening of LKB1 kinase substrates for their tumor suppressive functions and selected LKB1-deficient human lung adenocarcinoma cell line A549 for this purpose. While previous reports demonstrated, that reintroduction of the wild type LKB1 in A549 cells rescued the cell growth defect[10], in this study, *in vitro* growth assays were performed for 11 LKB1 kinase substrates that are expressed in A549 cells, including AMPKα1, AMPKα2, NUAK1, NUAK2, SIK1, SIK2, SIK3, MARK1, MARK2, MARK3, MARK4.

Altogether, this study identifies LKB1 downstream kinases with growth suppressive potential in lung cancer cell lines and provides evidence to the LKB1 tumor suppressive properties in lung cancer.

2 Literature review

2.1 Lung cancer: epidemiology and classification

Lung cancer has the highest incidence rate and mortality among cancers killing 1.8 million people annually[11,12]. In 90% of lung cancer cases the primary cause is tobacco consumption. Despite this fact, no reduction in tobacco smoking has been observed. Moreover, a number of new lung cancers will arise in former smokers within the next 20 years because of the time needed for lung tumors to develop[13].

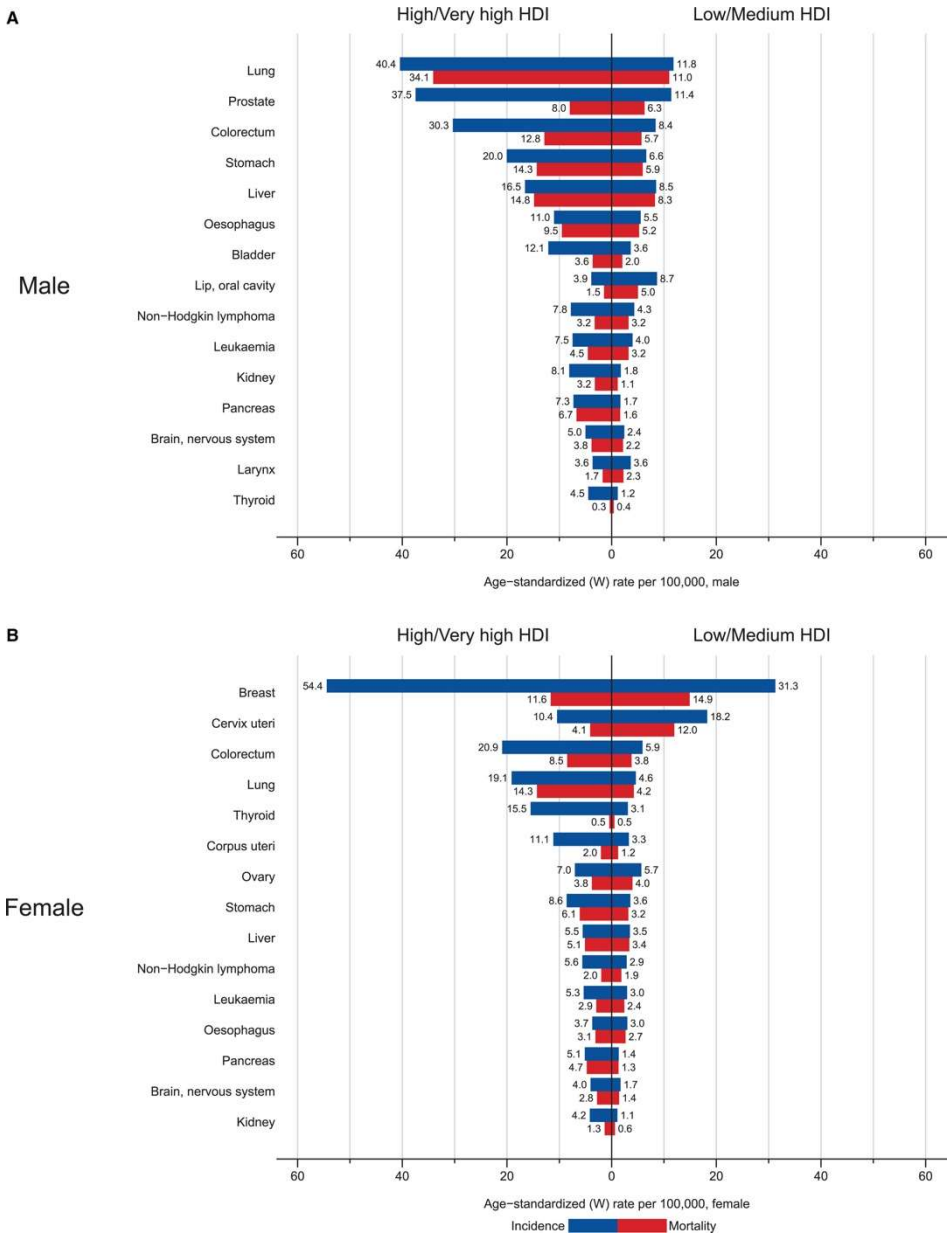

Fig.1 Bar Charts of Incidence and Mortality Age-Standardized Rates in High/Very-High Human Development Index (HDI) Regions Versus Low/Medium HDI Regions Among (A) Men and (B) Women in 2018[12]

Based on histopathological observations, primary lung tumors can be classified in non-small cell lung carcinomas (NSCLCs) and small cell lung carcinomas (SCLCs). NSCLC, which is responsible for 85% of lung cancers[14,15], can further be divided into squamous cell carcinomas (SCCs, ~28%), large-cell carcinoma (LCC, ~24%) and adenocarcinomas (ACs, ~48%)[8]. There is strong evidence that differences in morphology stand in line with genetic variations of these tumors[16–19].

A number of specific genes is known to be involved in lung cancer onset, both oncogenes altered by activating mutations: KRAS, NRAS, EGFR, BRAF, ERBB2, PIK3CA, MYC; and tumor suppressor genes affected by inactivating mutations: LKB1, PTEN, MYC, TP53, P16, RB, BRG1[20–22].

One of these genes, LKB1/STK11, has been reported to harbor inactivating biallelic somatic mutations in NSCLC[23–25], being the third most frequently modified gene in this type of lung cancer after TP53 and P16[22]. The protein coding gene is located at chromosome 19p, where loss of heterozygosity is observed in up to 80% of NSCLC cell lines[26]. Interestingly, LKB1 mutations not only predominate in adenocarcinomas from smokers but also tend to accompany KRAS mutations, resulting in more aggressive forms of cancer with shorter tumor latency, more common metastasis and increased tumorigenesis compared to cases when P16 or TP53 are missing[25,27,28].

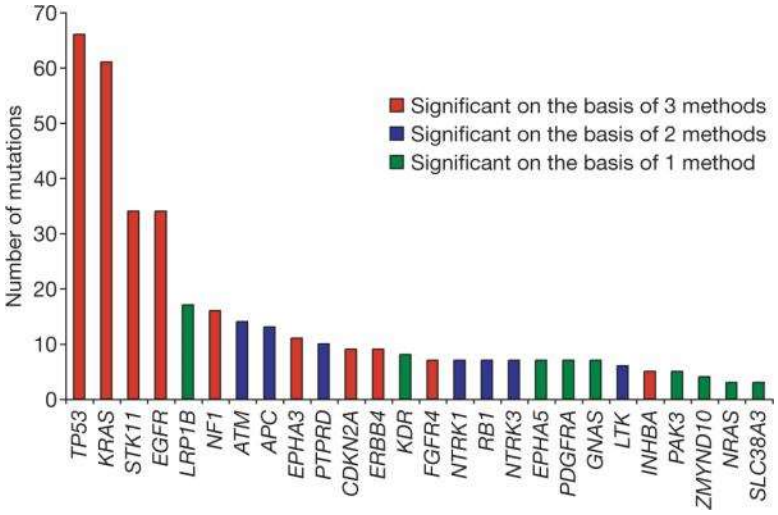

Fig.2 Significantly mutated genes in lung adenocarcinoma[21]

2.2 LKB1: functions and signaling pathways

Liver kinase B1 (LKB1) is a serine-threonine kinase that activates a group of kinases that belong to the AMPK subfamily. This group consists of AMPK and 11 AMPK-related kinases: NUAK1, NUAK2, SIK1, SIK2, SIK3, MARK1, MARK2, MARK3, MARK4, BRSK1, BRSK2. More than 50 fold activation of these substrates is achieved by LKB1 phosphorylating a conserved threonine residue in their T-loop region[1]. Later study revealed sucrose non-fermenting related kinase SNRK as another LKB1 substrate[29].

An interaction of LKB1 with STE-20-related adaptor (STRAD)[30] and mouse protein 25 (MO25)[31] is necessary to activate the kinase and relocate it from the nucleus to cell's cytoplasm[30–33], which is an important condition for expressing its tumor suppressor properties[34].

LKB1 inactivating mutations were first identified in context of Peutz-Jeghers syndrome, a hereditary condition characterized by high susceptibility to cancer[3]. LKB1 has versatile functions in the cell, such as controlling cell growth, regulating cell polarity and autophagy, managing cell metabolism.

LKB1 has been reported to cause cell cycle arrest at the G1 phase by interacting with TP53 and phosphorylating its Ser15 and Ser392 residues as well as activating its target gene - cyclin-dependent kinase inhibitor p21/WAF1[35]. It was also proposed that interaction with TP53 enables LKB1 to promote apoptosis in epithelial cells[36]. Later, however, evidence of the contrary effect was obtained. Cells lacking LKB1 appear to become especially susceptible to apoptosis when the AMP:ATP ratio is raised as they cannot stop their anabolic processes due to impaired LKB1-AMPK signaling[37,38]. This indicates that LKB1 might prevent apoptosis caused by nutrient starvation through its downstream target AMPK.

Regulation of the autophagy by LKB1 was also suggested to be mediated by AMPK as it can suppress mTOR pathway, which inhibits the autophagy when activated. Phosphorylation of cyclin-dependent kinase inhibitor p27 by LKB1-AMPK signaling is needed to initiate autophagy and improve the endurance of the cells[39]. LKB1 was shown to be entangled in the autophagy process also by other mechanisms: either by ataxia

telangiectasia (ATM) following the increase in reactive oxygen species (ROS) level [40], or by Poly(ADP-ribose) polymerase-1 (PARP-1) after exposure to hydrogen peroxide[41]. Later study revealed that autophagy activating kinase ULK1/2 was directly phosphorylated by AMPK, and knockdown of these kinases led to impaired autophagy and build-up of abnormal mitochondria in mouse embryonic fibroblasts. Defective AMPK-ULK1 interaction showed similar phenotype in LKB1-deficient NSCLC cells. These findings represent the protective role LKB1 exerts by means of autophagy in cells that undergo metabolic stress.

LKB1 induces the differentiation of the epithelial cells by promoting the association of the polarity complex[30] and counteracts the transforming growth factor-β (TGF-β) signaling which is responsible for the initiation of the epithelial-mesenchymal transition (EMT) in epithelial cells[42]. This is achieved through blocking the Smad4 transcription which is an important part of TGF-β signaling pathway. Smad4 is recruited to the complex with LKB1 and scaffolding protein LKB1-interacting protein 1 (LIP1), that results into blocking TGF-β effect, maintaining cell polarity and preventing the increased migration and invasiveness of the cells[43,44]. LKB1 was also reported to induce the establishment of the brush border in epithelial cells through the interaction with MST4 kinase and further phosphorylation of cytoskeletal peripheral protein Ezrin[45].

The regulation of the cell metabolism by LKB1 is mostly mediated by AMPK and described below.

2.3 The role of LKB1 in cancer

The contribution of LKB1 to the induction of tumors in various epithelial cell types was previously reported in different studies. LKB1 deletion *in vivo* could promote the formation of gastrointestinal polyps[46], breast tumors[47], as well as the development of hepatocellular carcinoma[48], pancreatic serous cystadenoma[49] and invasive endometrial cancer[50]. However, this effect might be tissue specific, as LKB1 inactivation in mouse lungs was not sufficient to cause the induction of a malignancy[27].

Rather intriguing is the observation that in lung adenocarcinoma with KRAS mutation, LKB1 could induce the trans-differentiation towards squamous cell carcinoma[27]. However, more research is needed to define whether this happened due to the stemness of cancer cells provided by LKB1, or because of its effect on basal cell differentiation.

There is evidence that LKB1 has a prominent role in suppressing cancer metastasis *in vivo*[27], and interaction with tumor stroma was marked a significant part of this process. An upregulation of lysyl oxidase (LOX), caused by mTOR-HIF-1α signaling, induces extracellular matrix (ECM) remodeling with the redundant deposition of collagen, that together with the activation of v-src sarcoma viral oncogene homolog (SRC) and focal adhesion kinase (FAK) supports the spreading of cancer cells.[51] Additionally, dysregulated cyclooxygenase-2 (COX-2), that is normally suppressed by LKB1 through the phosphorylation of polyomavirus enhancer activator-3 (PEA3), grants beneficial environment for tumor development by mediating prostaglandin E_2 synthesis[52].

2.4 Description of LKB1 substrates and their role in disease

2.4.1 AMPK

One of the most studied downstream targets of LKB1, 5'AMP-activated protein kinase (AMPK), serves as an important cellular energy sensor and maintains ATP homeostasis in the cell. It consists of three subunits, and each of them is encoded by several genes: catalytic α-subunit (encoded by PRKAA1 and PRKAA2 genes), regulatory γ-subunit (encoded by PRKAG1, PRKAG2, PRKAG3) and scaffolding β-subunit (encoded by PRKAB1 and PRKAB2)[53]. Activity of the kinase depends on the [AMP]:[ATP] ratio in the cell in directly proportional manner. Upon energy deprivation, AMPK restrains anabolic reactions in the cell, such as protein and lipid synthesis, and promotes catabolic processes like oxidative phosphorylation, glycolysis and fatty acid oxidation to retain ATP level needed for the normal functioning of the cell[54–57].

There are two major upstream kinases that target AMPK – LKB1 and calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ)[58]. Both of them phosphorylate Thr172 residue in N-terminal domain of the α-subunit that results into more than several hundred-fold AMPK activation[59–61]. AMPK is additionally activated allosterically by AMP molecules that bind to its γ-subunit, but the increase in activity is much more modest in this case - only 2-5 fold[62]. What is more important, AMP prevents Thr172 from dephosphorylation by protein phosphatase 2Cα[60,63].

Considering the severe demand of cancer cells for nutrients and energy resources needed for extensive proliferation, AMPK must play a crucial role in the tumor development and maintenance.

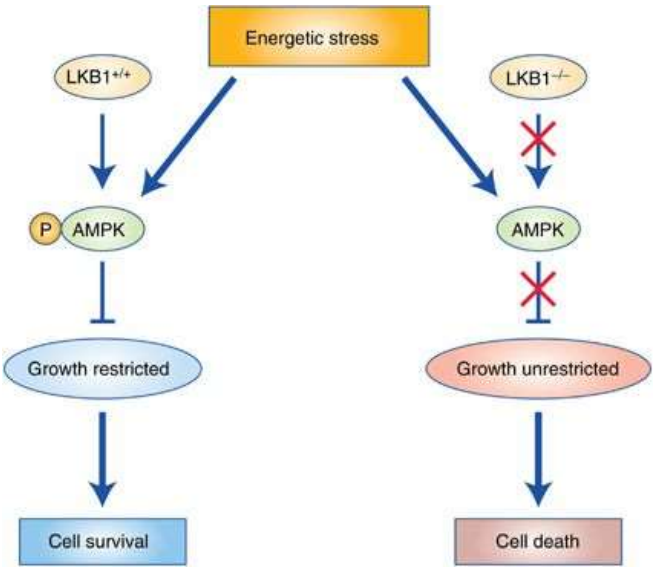

Fig.3 LKB1-deficient cells show select sensitivity to energetic stress[64]

Upon energy stress, AMPK is known to inhibit mechanistic target of rapamycin complex 1 (mTORC1), which is often upregulated in cancer, through the activation of its negative regulator tuberous sclerosis complex (TSC)[65]. mTORC1 promotes protein translation through its downstream targets - ribosomal S6 kinase (p70S6K1) and 4EBP1[66]. mTORC1 regulates several transcriptional factors, needed for the cell growth, that are inhibited by AMPK as well. One of them is sterol-regulatory element binding protein 1 (SREBP-1), responsible for the lipid synthesis[67], and the other is hypoxia-inducible factor 1a (HIF-1α), which promotes the activity of various glycolytic enzymes, glucose transporters and angiogenic factors[68–70].

The activation of AMPK by hypoxia and elevated levels of reactive oxygen species (ROS) independently of changes in AMP:ATP ratio is another noteworthy observation as these factors can facilitate angiogenesis and acquisition of new mutations resulting in the increased chance of metastasis[71–73]. AMPK was reported to secure the levels of antioxidant through the suppression of acetyl-CoA carboxylase 1/2 (ACC1/2). This way, the expenditure of NADPH is decreased due to the inhibition of fatty acid synthesis, while its production is boosted because of the activated fatty acid oxidation[74]. Another discovery in favor of protective role of AMPK against ROS concerns folliculin, a tumor suppressor, that was shown to inhibit AMPK, and whose depletion prevented cell death caused by hydrogen peroxide and other stimuli. AMPK-induced autophagy might be a part of this response[75,76].

Several studies also noted the potential involvement of AMPK in controlling cell polarity. Raise in the amount of polarized cancer colon cells under metabolic stress and higher number of tight junctions in kidney cells were observed upon the activation of AMPK[77–79].

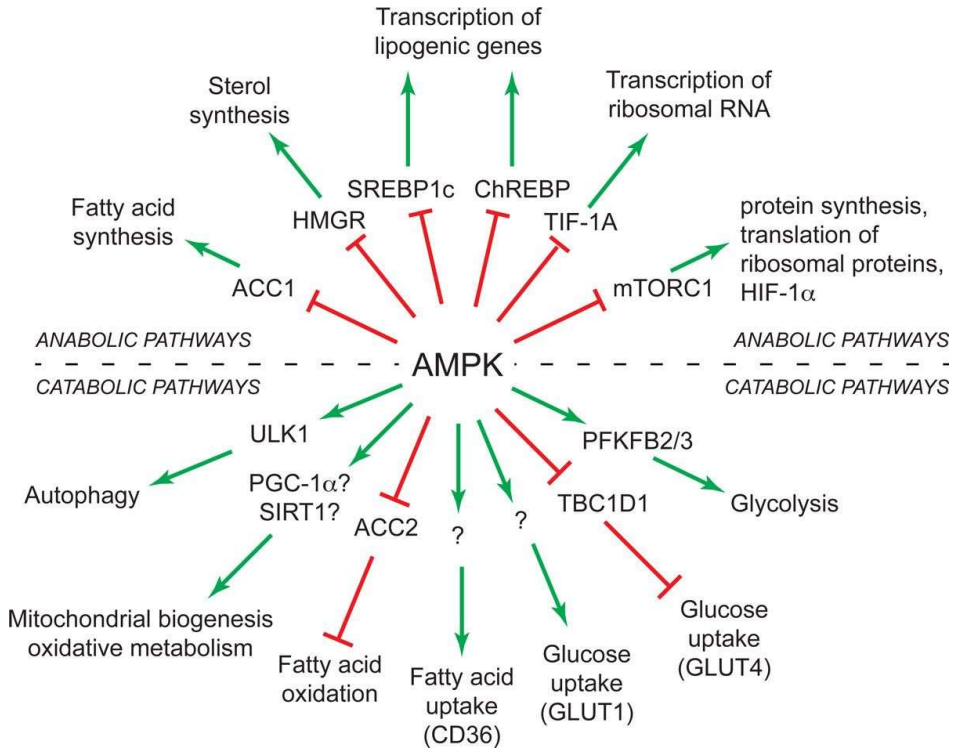

Fig.4 Summary of a selection of target proteins and metabolic pathways regulated by AMPK[80]

Recent study also showed that melanoma-associated antigen A3/6 (MAGE A3/6) upregulated mTORC1 activity and endorsed AMPKα1 degradation whereas its depletion had a contrary outcome[81]. These findings allow to assume that LKB1 might exert its tumor suppressor properties via the same role of AMPK, especially knowing that the activity of this kinase is severely diminished in mouse and human LKB1-deficient tumors[82,83]. However, there are several factors that make reevaluate this point of view more carefully.

One of them is the finding that AMPK activation can be provoked in LKB1-deficient cells (A549, HeLa) with chemical compounds of either direct action, such as A769662, or indirect one, like 2-Deoxy-D-glucose or Ca^{2+} ionophore[74,84–86]. It was also observed that α-subunit of AMPK can be directly phosphorylated at Thr172 not only by LKB1 but by CaMKKβ and TGFβ-activated kinase TAK1 as well[84,87]. Furthermore, the data shows PRKAA1 and PRKAB2 upregulation is a common event in different cancer types and correlates with the mutations of principal oncogenes (KRAS, AKT, BRAF, MYC)[88,89]. Lastly, the abundance of

genes encoding AMPK subunits makes it possible for 12 different variants of AMPK complexes to be assembled that potentially may have different functions and downstream effectors. For instance, a novel study discovered the prevalence of AMPKα1 over AMPKα2 subunits in the kinase located in Golgi apparatus, plasma membrane and lysosomes[90]. Also, the activity of AMPKα2 is downregulated in LKB1-depleted cardiac myocytes in case of ischaemia while AMPKα1 activity remains unchanged[91].

These revelations may hold back from making univocal conclusions about the definitive role of AMPK in mediating LKB1 tumor suppressor properties.

2.4.2 NUAK1

NUAK1 (ARK5) was the fifth kinase discovered in the ARK family. Early evidence suggested that this enzyme benefited cell survival in the condition of nutrient deprivation after phosphorylation at Ser600 by protein kinase B (Akt)[92]. More studies revealed that this effect was achieved due to the ability of NUAK1 to inhibit caspase 8 and negatively phosphorylate procaspase 6[93,94]. Also, clinical data showed robust correlation between NUAK1 expression and increased invasiveness and metastatic capacity of the tumors[95–97] as well as the link with poor prognosis in patients with pancreatic cancer[95], colorectal cancer[97,98], hepatocellular carcinoma[99,100], glioma[101] and multiple myeloma[102]. In addition, NUAK1 was reported to phosphorylate the myosin phosphatase target subunit 1 (MYPT1) and regulate cell adhesion. Cell adhesion was enhanced with the LKB1-NUAK1 signaling impaired.[103] These findings seem paradoxical, considering that NUAK1 is activated by the tumor suppressor kinase. However, there is evidence on the tumor suppressing properties of this kinase as well.

One of them is the induction of aneuploidy and premature senescence through the interaction with large tumor suppressor kinase 1 (LATS1). The phosphorylation of NUAK1 by LKB1 at Thr211 and the kinase properties of the enzyme are needed in this case[104]. Another discovered role of NUAK1 might explain the growth restricting property of LKB1 – apparently, G1/S cell cycle arrest caused by the master kinase is mediated by NUAK1 and its association with p53 and p21/WAF promoter[105].

2.4.3 NUAK2

Little is known about NUAK2 functions, but it can be regarded as part of the cellular stress response in different cell types[106] and seems to cause cell detachment under metabolic stress[107]. It might also contribute to the apoptosis resistance as decreased activity of NUAK2 makes cells less motile after death receptor CD95 stimulation. Consistently, NUAK2 was upregulated in several apoptosis-resistant cell lines by CD95 triggering[108]. NUAK2 depletion shortens the S phase, triggers senescence, inhibits migration and mTOR activity in melanoma cells[109]. Upregulation of NUAK2 predicts poor clinical outcome in melanoma patients[109], whereas in ovarian cancer patients it correlates with a better survival[110].

2.4.4 MARK subfamily

Microtubule affinity regulating kinases (MARK) are a family of serine-threonine kinases comprised of 4 related isoforms: MARK1, MARK2, MARK3, MARK4.
They are known to be crucial for the regulation of the cell polarity and microtubule organization[111]. MARK kinases phosphorylate microtubule associated proteins (MAPs), reducing the dynamic stability of the microtubules, and Dishevelled (Dvl) proteins, which are part of Wnt signaling pathway[112,113].

MARK1 and MARK4 have recently been reported to downregulate SNAIL1 expression through the phosphorylation of the scaffolding protein DIXDC1, possibly explaining the ability of LKB1 to abolish epithelial to mesenchymal transition and, consequently, the progression of metastasis[114]. LKB1-dependent activation of MARK2 was shown to inhibit tubulin polymerization via phosphorylation of microtubule-associated protein Tau and promote further proteasomal degradation of Tau. Respectively, the depletion of LKB1 causes the increased growth of the microtubules[115]. In addition, MARKs appear to also participate in the regulation of metabolism as the deletion of MARK2, MARK3 and MARK4 *in vivo* develops a phenotype characterized by increased metabolic rate, insulin hypersensitivity and resistance to adiposity, implying its potential link with the advancement of diabetes[116–118].

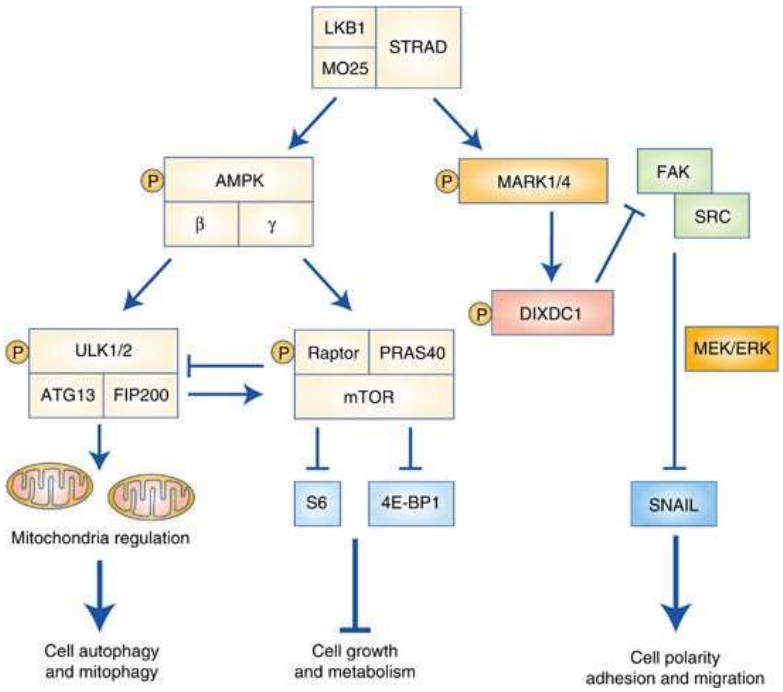

Fig.5 LKB1 regulation of AMPK and MARK signaling pathways[64]

2.4.5 SIK subfamily

This group of enzymes consists of 3 salt-inducible kinases - SIK1, SIK2, SIK3, - that are responsible for suppressing the activity of cAMP response element-binding protein (CREB) via phosphorylation of its transcriptional co-activators (CRTCs) and transcriptional regulators class II histone deacetylases (HDACs)[119]. Apart from regulating the gene expression, SIK subfamily of kinases was shown to be involved in glucose metabolism[120]. However, their cancer-related functions are still not very clear.

There is evidence that SIK1 is required for LKB1 ability to encourage p53-dependent anoikis and restrict metastasis progression in human mammary epithelial cells and lung adenocarcinoma cells[121]. SIK1 was also reported to suppress the migration of gastric adenocarcinoma cells[122]. Recent discovery shed light on the role of SIKs in pancreatic cancer: the inhibition of SIK signaling caused by mutated G protein αs (GNAS) within cAMP-PKA pathway promotes tumor development and extensive rewiring of lipid metabolism[123].

At the same time, closely related SIK2 kinase was reported to phosphorylate centrosome protein CEP250 (C-Nap1) and promote centrosome splitting and mitosis in ovarian cancer cells. Elevated expression of SIK2 is also linked to a poor prognosis for high-grade ovarian cancer patients[124]. SIK3 was also identified to maintain mitosis and ensure correct mitotic exit. The duration of the mitosis in cells with SIK3 knockdown is extended[125]. Moreover, SIK2 together with SIK3 kinase were shown to upregulate myocyte-specific enhancer factor 2C (MEF2C) transcription factor through the phosphorylation of its repressive cofactor histone deacetylase 4 (HDAC4). As a result, proliferation of acute myeloid leukemia cells was stimulated[126]. SIK2 was also reported to be crucial for the growth of prostate cancer cells[127].

In addition to this controversial evidence about the role of SIKs in human cancer, two latest studies claim that SIK1 and SIK3 might be in charge for the tumor suppressor functions of LKB1 in KRAS-driven lung adenocarcinoma as the genetic loss of these kinases leads to enhanced cell growth both *in vivo* and *in vitro*[119,128].

2.5 LKB1 interaction with the Hippo pathway

The LKB1 pathway was observed to cross over the Hippo pathway, which is known to regulate the organ volume and possess both pro- and antitumorigenic properties[129,130]. In the Hippo pathway, MST1/2 kinases phosphorylate LATS1/2 kinases, that, in turn, provide negative regulation for the transcription unit comprised of yes-associated protein 1 (YAP1) and taffazin (TAZ). Interestingly, other AMPK-related kinases apart from AMPK are suggested to participate in YAP1 regulation by LKB1[131].

The study showed that direct phosphorylation of LATS1 by NUAK1 and NUAK2 provoked its deterioration, potentially leading to the upregulation of the YAP1/TAZ transcription[104]. MARK1 and MARK4 were also reported to affect LATS1/2, but in a different fashion. MARKs promote the association of SCRIB factor with MST1/2 and, further, the phosphorylation of LATS1/2, thus restricting YAP1 activity[132]. Finally, AMPK was also observed to inhibit YAP1/TAZ transcription under metabolic stress[133–135].

More research on the extent of integration of these two intertwined pathways could provide exciting discoveries on their contribution to tumorigenesis.

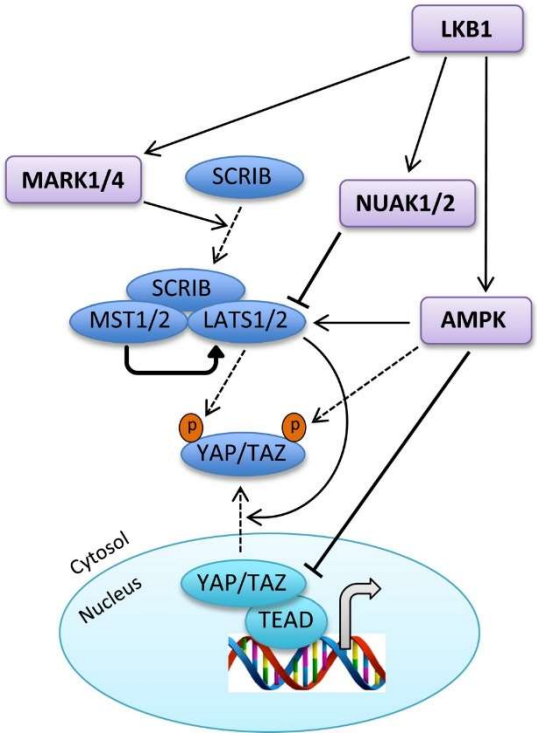

Fig.5. Regulation of Hippo signaling by AMPK and ARKs[136]

3 Hypothesis

While ectopic expression of LKB1 in LKB1-deficient A549 cells is demonstrated to have a kinase dependent suppression of its growth[10], it is plausible that this happens via one or several of its downstream kinase substrates. Therefore, gain-of-function experiments by overexpressing constitutively active mutants of these kinases in LKB1-deficient cells is a robust model for investigating the cell growth defect. After identifying the LKB1 substrates exhibiting the growth-restrictive properties, a loss-of-function rescue experiment utilizing RNAi-mediated silencing is expected to establish a LKB1-dependent growth suppression mechanism.

4 Aims and objectives

The aim of this study is to identify the LKB1 substrates that are crucial for the LKB1-caused cell growth defect in lung adenocarcinoma cells.

Therefore, the principal objective is to screen 11 LKB1 downstream kinases for their ability to affect the lung cancer cell growth in a series of gain-of-function experiments, concurrently verifying the genetic and protein expression. After detecting the potential candidates, a loss-of-function rescue experiment will be conducted to establish a LKB1-dependent growth suppression mechanism of lung adenocarcinoma cells.

5 Materials and Methods

5.1 Cell culture

The A549 and 293FT cells were obtained from American Type Culture Collection.

The A549 and 293FT cells were grown in DMEM high glucose (4.5 g/L) growth media (Lonza, USA) with the addition of 10% FBS (Gibco, USA), 1% L-glutamine (Lonza, USA) and antibiotics (penicillin and streptomycin) (Lonza, USA) in 10 cm CELLSTAR dishes (Greiner Bio-One, Germany) at 37°C, maintaining humidity and 5% CO_2 in the atmosphere.

5.2 Lentivirus production and transduction

Following human pLenti (pL) virus constructs were obtained from Mäkelä laboratory:

- pL6 emGFP empty vector
- pL6.3 emGFP empty vector
- pL6 LKB1-WT
- pL6 LKB1-K78M kinase dead mutant
- pL6.3 AMPKα1-WT
- pL6 AMPKα1-T174E constitutively active mutant
- pL6.3 AMPKα2-WT
- pL6 AMPKα2-T172E constitutively active mutant
- pL6.3 SIK1-T182E constitutively active mutant
- pL6.3 SIK2-T175E V5-tagged constitutively active mutant
- pL6.3 SIK3-T221E constitutively active mutant
- pL6 NUAK1-T211A kinase dead mutant
- pL6 NUAK1-T211E constitutively active mutant
- pL6 NUAK2-T208A kinase dead mutant
- pL6 NUAK2-T208E constitutively active mutant
- pL6.3 MARK1-WT
- pL6.3 MARK1-T215E constitutively active mutant
- pL6.3 MARK2-T175E V5-tagged constitutively active mutant

- pL6.3 MARK3-T211E V5-tagged constitutively active mutant
- pL6.3 MARK4-T213E V5-tagged constitutively active mutant

The 293FT cell line was used for lentivirus production. $7.5x10^6$ cells were plated in 10 cm CELLSTAR dishes (Greiner Bio-One, Germany) in DMEM high glucose (4.5 g/L) growth media (Lonza, USA) with the addition of 10% FBS (Gibco, USA) and 1% L-glutamine (Lonza, USA). The next day the expression construct (10.68 µg) with Delta 8.9 (7.98 µg) and VSVG (5.34 µg) packaging plasmids were diluted in 3 mL of Opti-MEM reduced serum media (Gibco, USA) and transfected into the cells using 60 µL of Lipofectamine 2000 (Invitrogen, USA). The supernatant was collected 48 h later and filtered through a 0.22 µm sterile filter (Jet Biofil, China).

For the transduction, the A549 cells were seeded in 6-well plates at the density of $3x10^5$ cells per well in DMEM high glucose (4.5 g/L) growth media (Lonza, USA) with the addition of 10% FBS (Gibco, USA), 1% L-glutamine (Lonza, USA) and antibiotics (penicillin and streptomycin) (Lonza, USA). After 24 h of incubation the media was removed; 1 mL of the same media, 1 mL of virus and 8 µg/mL of polybrene (Millipore, USA) were added into each well. After the 18 h incubation the media was changed. Next day the media was changed again with the addition of 7 µg/mL of blasticidine S hydrochloride (Sigma-Aldrich, USA). After 5 days of blasticidine selection the transduced cells overexpressing the according constructs were collected for protein and RNA samples as well as plated for the growth assay.

5.3 siRNA transfection

Following human siRNA constructs were obtained from Dharmacon:

- ON-TARGETplus Non-targeting Pool (Cat.nr. D-001810-10-20)
- ON-TARGETplus (SMARTpool) siRNA PRKAA1 (Cat.nr. L-005027-00-0005)
- ON-TARGETplus (SMARTpool) siRNA PRKAA2 (Cat.nr. L-005361-00-0005)
- ON-TARGETplus (SMARTpool) siRNA NUAK1 (Cat.nr. L-004931-01-0005)
- ON-TARGETplus (SMARTpool) siRNA NUAK2 (Cat.nr. L-005374-00-0005)

For the siRNA transfection, the A549 cells were seeded in 6-well CELLSTAR plates (Greiner Bio-One, Germany) at the density of $1.8x10^5$ cells per well in DMEM high glucose

(4.5 g/L) growth media (Lonza, USA) with the addition of 10% FBS (Gibco, USA) and 1% L-glutamine (Lonza, USA). The following day the 20 mM siRNA constructs were diluted in 200 µL of Opti-MEM reduced serum media (Gibco, USA) and transfected into the cells using 5 µL of Lipofectamine RNAiMAX (Invitrogen, USA). After 6 h incubation at 37°C with 5% CO_2 in the atmosphere, the transfection mixture was replaced with DMEM high glucose (4.5 g/L) growth media (Lonza, USA) with the addition of 10% FBS (Gibco, USA), 1% L-glutamine (Lonza, USA) and antibiotics (penicillin and streptomycin) (Lonza, USA) to neutralize any toxic effects of Lipofectamine. The next day the second round of siRNA transfection was conducted. After 18 h incubation, the cells were counted and plated for the growth assay, as well as plated in 6-well CELLSTAR plates (Greiner Bio-One, Germany) to be collected for RNA samples 72 h later.

5.4 Growth assay

5.4.1 Crystal violet

The cells were counted with Cellometer Auto T4 Plus Cell Counter (Nexcelcom Bioscience, USA) and seeded in 24-well CELLSTAR plates (Greiner Bio-One, Germany) at the density of $5x10^3$ cells per well in in DMEM high glucose (4.5 g/L) growth media (Lonza, USA) with the addition of 10% FBS (Gibco, USA), 1% L-glutamine (Lonza, USA), penicillin and streptomycin (Lonza, USA), and 7 µg/mL of blasticidine S hydrochloride (Sigma-Aldrich, USA). After 5 days of incubation, the cells were stained with 0.5% crystal violet solution (0.5% (w/v) crystal violet (Sigma-Aldrich, USA) and 20% (v/v) methanol (Fisher Scientific, USA)) and left to dry overnight. The plates were then scanned and quantified using ImageJ software, version 1.8.0 (National Institutes of Health, USA).

5.4.2 Incucyte

The cells were counted with Cellometer Auto T4 Plus Cell Counter (Nexcelcom Bioscience, USA) and seeded in 96-well COSTAR plate (Corning, USA) at the density of $2,5x10^3$ cells per well in DMEM high glucose (4.5 g/L) growth media (Lonza, USA) with the addition of 10% FBS (Gibco, USA), 1% L-glutamine (Lonza, USA), penicillin and streptomycine (Lonza, USA), and 7 µg/mL of blasticidine S hydrochloride (Sigma-Aldrich, USA), and after 4 h of incubation placed into the IncuCyte S3 Live-Cell Analysis System (Sartorius, Germany). The confluence of the wells was measured with 2 h interval for 72 h.

5.5 RNA isolation and cDNA synthesis

The RNA was extracted with NucleoSpin RNA Plus kit (Macherey-Nagel, Germany) and its concentration was measured with a NanoDrop-1000 spectrophotometer.

The cDNA synthesis was conducted in Eppendorf tubes. 0.2 µg of total RNA was mixed with HyClone HyPure Molecular Biology Grade water (GE Healthcare LifeSciences, USA) in the total volume of 7.5 µL, incubated at 65°C for 10 minutes and transferred on ice for 2 minutes. 2 µL of 10x RT buffer, 8.8 mM $MgCl_2$, 3.2 mM dNTP mix, 4 µM random hexamers and 0.2 µL of water were added to the tube. Lastly, 8 U of RNAse inhibitor and 25 U of MultiScribe Reverse Transcriptase were added to the solution, reaching the final volume of 20 µL. The reagents used for reverse transcriptase reaction were from TaqMan™ Reverse Transcription Reagents kit (Applied Biosystems, USA). After the short centrifugation, the tube was placed in Peltier Thermal Cycler PTC-2000 (MJ Research, USA) where it first underwent 10 min incubation at 25°C, then 30 min incubation at 48°C, and, finally, 5 min incubation at 95°C, after which it was cooled to 15°C and stored at -70°C.

5.6 Real-time polymerase chain reaction

The primers for the PCR reaction were obtained from Sigma-Aldrich. (Tab.1)

The polymerase chain reaction was performed in MicroAmp Fast 96-well reaction plate (0.1 mL) (Applied Biosystems, USA). A single well was loaded with 1 µL of cDNA, 7.6 µL of HyClone HyPure Molecular Biology Grade water (GE Healthcare LifeSciences, USA), 1 µL of 10 µM primer dilution (*Tab.1*), 10.4 µL of Kapa Sybr Fast Abi Prism master mix (Kapa Biosystems, South Afrcica). Each condition was plated in triplicates. The plate was then sealed with Q-Stick qPCR seal (4titude Ltd, UK), centrifuged in 5804R centrifuge (Eppendorf, Germany) at 2000 rpm for 2 min, and inserted into StepOnePlus real-time PCR system. The PCR run consisted of the holding stage (95°C for 3 min), followed by the cycling stage (40 cycles; each including 95°C for 3 sec and 60°C for 30 seconds), concluded by the melt curve stage (95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec).

Tab.1 qPCR primer information.

Target (species: human)	**Forward primer sequence 5' → 3'**	**Reverse primer sequence 5' → 3'**
LKB1	GCTCTTACGGCAAGGTGAAG	TTTTGTGCCGTAACCTCCTC
AMPKα1	GACAGCCGAGAAGCAGAAAC	CACATCAAGGCTCCGAATCT
AMPKα2	ACAGGCCATAAAGTGGCAGT	GTTGGAGTGCTGATCACCTG
NUAK1	ATGGAATATGCCAGCAAAGG	GTTGGAAAGCCCAAAGTCAG
NUAK2	ACCACCCTCACATCATTGCC	GGACAACTCTGTTCTGATGGC
MARK1	GGGAAATTTTGCCAAAGTCA	TCATTCTTCCATGGGCAACT
MARK2	CTCCCTTCCTCCAAGCTTCT	AGTCAAGGTGTCCCAAGGTG
MARK3	TGTTGAAAACAATCGGCAAG	TTGGATTCAACTGAGTTTTGTCA
MARK4	TCACTGGTCGGGAGGTTG	CCTTCATGATGCGGACTTCT
SIK1	GCTTCTGAACCATCCACACA	CCGTTGGAAGTCAAATAATCAAA
SIK2	GGGTGGGGTTCTACGACAT	GCATCCAGCTGAGACTTATCG
SIK3	AAGACCCAGCTGGATGAAGA	GCCTCCTTTTCTGCCATTCT
OAZ1	CAGCAGCAGTGAGAGTTCCAG	ATCTTCAGGGGTGGGTGAG

5.7 Protein isolation and processing

To collect protein samples, SDS lysis buffer was boiled at 98°C and added into wells, then the plate with the material was placed on ice. SDS boiling buffer composition: 1mM dithiothreitol (Applichem, Germany), 0.5mM phenylmethanesulonyl fluoride (Sigma-Aldrich, USA), 10mM glycerol 2-phosphate disodium salt hydrate (Sigma-Aldrich, USA), 2 µg/mL leupeptin trifluoroacetate salt (Santa Cruz Biotechnology, USA), 50mM sodium fluoride (Millipore, USA) diluted in SDS buffer (2.5% SDS, 0.25M Tris-HCl pH 6.8). After collecting protein samples into Eppendorf tubes, they were boiled at 98°C for 5 min and homogenized using 25G needle. To clear the sample, the tubes were centrifuged at 4°C and 12000 rpm for 30 min, and the supernatant was collected. Protein concentration was measured in 96-well CELLSTAR plates (Greiner Bio-One, Germany), using DC Protein Assay kit (Bio-Rad Laboratories, USA) with FLUOstar microplate reader (BMG Labtech, Germany).

Protein dilutions were prepared in final volumes of 30 µL, boiling SDS lysis buffer, 20 µg of protein and 7.5 µL of 4x Laemmli Sample Buffer (Bio-Rad Laboratories, USA) (with 2-mercaptoethanol (Sigma-Aldrich, USA) added freshly to LSB in 1:10 ratio) together at 98°C for 5 min.

5.8 SDS-PAGE and Western blotting

SDS-PAGE was performed in 4-20% Mini-PROTEAN® TGX™ Precast Gels, 10-well, 50 µL (Bio-Rad Laboratories, USA), at 75 V for 30 min, then switching to 130 V for 1 h, loading 20 µg of protein in each well.

The gel was transferred on 0.2 µm nitrocellulose membrane using Trans-Blot Turbo Transfer Pack (Bio-Rad Laboratories, USA) in Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA) at 25 V for 10 min.

The membrane was then stained with Ponceau S (Sigma-Aldrich, USA) to assess the efficiency of the transfer and washed with water. Next, the membrane was incubated at the room temperature for 1 h in 5% milk (Valio, Finland) solution in TBS-T (1x TBS (Medicago AB, Sweden) with 0.1% Tween 20 (Sigma-Aldrich, USA)). After blocking, the membrane was incubated with primary antibody (*Tab.2*) at 4°C for 18 h. Then the membrane

was washed three times with TBS-T for 10 min each and incubated with corresponding HRP-conjugated secondary antibody (Goat anti-Rabbit IgG antibody (Millipore, AP132), Goat anti-Mouse IgG antibody (Millipore, AP308P), Goat anti-Chicken IgY (Abcam, ab6877)) diluted in 1:5000 ratio in 5% milk in TBS-T solution for 1 h. After that, the membrane was washed again with TBS-T three times for 10 min each. Finally, the membrane was incubated in Super Signal West Femto Maximum Sensitivity Substrate solution (Thermo Scientific, USA) in PBS (Medicago AB, Sweden) for 3 min, and developed on the Super RX-N Fuji Medical X-ray film (Fujifilm, Japan) using Optimax 2010 X-Ray Film Processor (Protec, Germany).

Tab. 2 Primary antibody information.

Target protein	Host species	Dilution	Manufacturer
LKB1	Mouse	1:1000, 5% w/v milk, 1xTBS, 0.1% Tween 20	Abcam (ab15095)
AMPKα1	Rabbit	1:1000, 5% w/v milk, 1xTBS, 0.1% Tween 20	Abcam (ab32047)
AMPKα2	Rabbit	1:1000, 5% w/v milk, 1xTBS, 0.1% Tween 20	Abcam (ab3760)
NUAK1	Rabbit	1:1000, 5% w/v BSA, 1xTBS, 0.1% Tween 20	Cell signaling (#4458)
NUAK2	Rabbit	1:250, 5% w/v milk, 1xTBS, 0.1% Tween 20	Abcam (ab107287)
MARK1	Rabbit	1:1000, 5% w/v milk, 1xTBS, 0.1% Tween 20	Proteintech (#21552-I-AP)
SIK1	Rabbit	1:1000, 5% w/v milk, 1xTBS, 0.1% Tween 20	Proteintech (#51045-I-AP)
SIK3	Chicken	1:1000, 5% w/v milk, 1xTBS, 0.1% Tween 20	Origene (TA319854)
V5 tag (C-term.)	Mouse	1:5000, 5% w/v milk, 1xTBS, 0.1% Tween 20	Invitrogen (#R960-25)
GAPDH	Rabbit	1:10000, 5% w/v BSA, 1xTBS, 0.1% Tween 20	Cell signaling (#2118)

5.9 Statistics

Statistical analyses were performed using GraphPad Prism, version 6.0 (GraphPad Software, San Diego, CA). Unpaired two-tailed Welch-corrected t test was applied to detect statistical difference between the two groups. Data are shown as mean ± standard error of the mean. Difference between means was considered not significant (ns) when $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$).

6 Results

6.1 LKB1 overexpression in A549 cells leads to the growth defect

To confirm tumor suppressive properties of LKB1, A549 cells were transduced with wild type LKB1 (LKB1-WT), kinase dead LKB1 (LKB1-K78M) and vector control (empty vector (EV)), and their growth potential was monitored (Fig.1).

The overexpression status of LKB1-WT and LKB1-K78M in the cells was supported both by quantitative RT-qPCR (on average, gene expression was elevated 39-fold and 29-fold, correspondingly) *(Fig.1a)*, as well as by Western blotting, where the level of LKB1 protein was also higher in the wild type sample than in the kinase dead mutant, and no LKB1 protein was detected in the empty vector control *(Fig.1b)*.

The further analysis of crystal violet staining showed a remarkable 51% reduction in confluence of wild type LKB1 compared to both empty vector *(P=0.0001)* and kinase dead control *(P=0.0001) (Fig.1c)*.

Though the observations from the crystal violet method indicated a significant growth defect in LKB1 overexpressing A549 cells, however, to mitigate any discrepancy in the initial seeding density and image quantification, we adapted continuous monitoring of the cell growth and well confluence changes using the IncuCyte system. This method enhanced the precision of the calculations by showing the actual starting cell density, and more data, such as growth rate of the cells, could be acquired. The growth rate was calculated by normalizing the difference in confluence between the endpoint and the starting point with the confluence in the beginning of the observation and dividing it by the duration of monitoring in hours.

The growth defect caused by the wild type form of LKB1 was confirmed by 16% reduction in the growth rate compared to the empty vector control *(P=0.0304)* and even higher 32% reduction compared to the kinase-dead mutant *(P=0.0021) (Fig.1d)*.

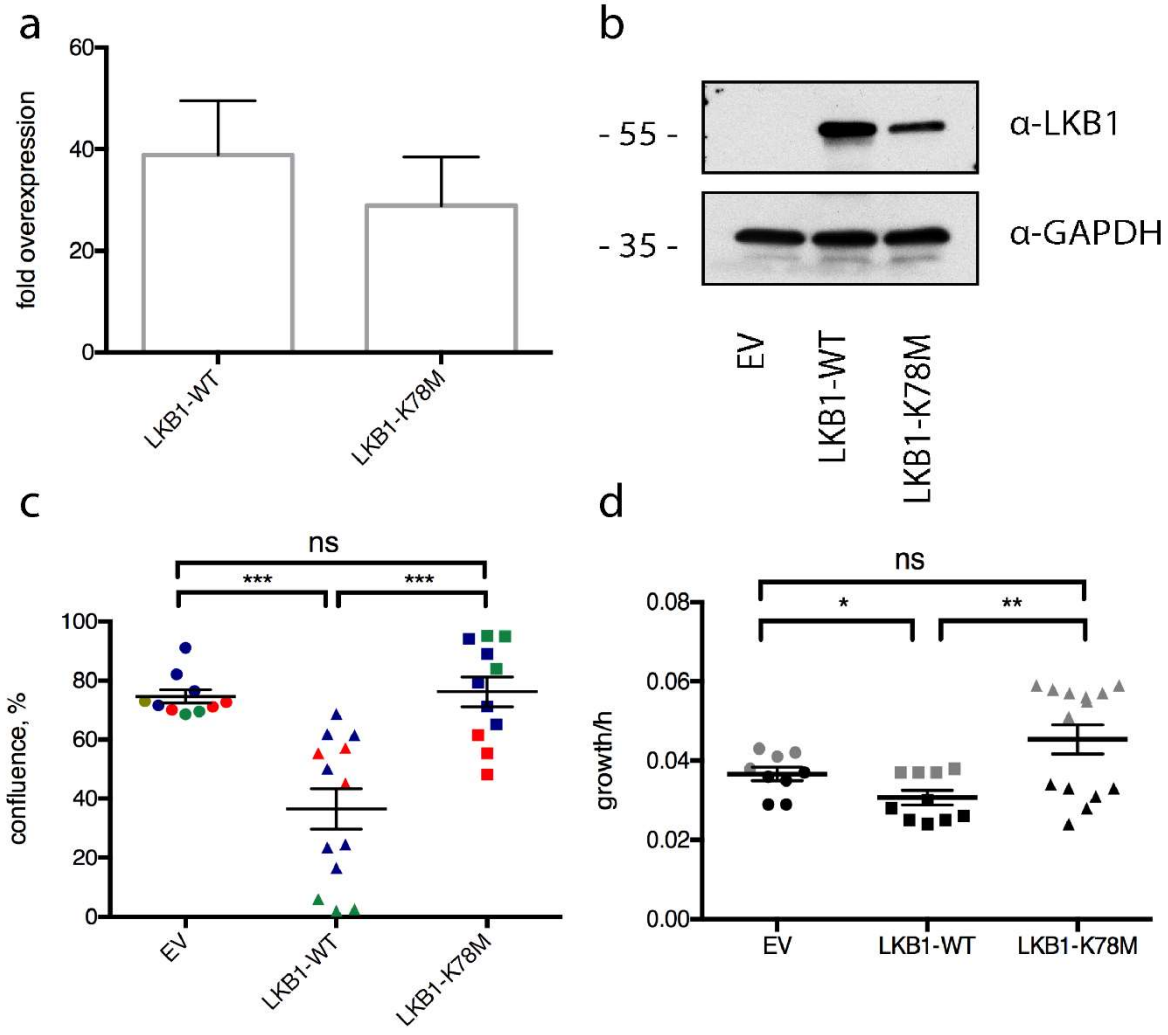

Fig.1
Ectopic expression of LKB1 in A549 cells results in diminished cell growth.
(**a**) Relative gene expression of LKB1 (normalized to empty vector control; ± SEM; N=4). (**b**) Western blotting of LKB1-WT and LKB1-K78M incubated with α-LKB1 primary antibody (Predicted molecular weight: 55 kDa; α-GAPDH is a loading control). (**c**) Crystal violet staining demonstrates the difference in confluence between wild type LKB1, kinase-dead mutant and empty vector control (*P=0.0001 (***)*, unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 10-13 wells from 3 experiments, colour-coded). (**d**) Growth rate of LKB1-WT is decreased in comparison to LKB1-K78M and EV control after 43-46h of incubation in IncuCyte (*P=0.0021 (**)* and *P=0.0304 (*)*, unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 10-14 wells from 2 experiments, colour-coded).

6.2 NUAK1 causes the impaired growth of A549 cells

To investigate the possible effect of NUAK kinases on the growth of A549 cells, a transduction of NUAK1 and NUAK2 constitutively active mutant constructs (NUAK1-TE, NUAK2-TE) was first performed in these cells and the growth assay was then conducted.

The crystal violet staining revealed an impressive 41% decrease in confluence for NUAK1 condition *(P<0.0001)* and similar 46% decrease for NUAK2 mutant *(P<0.0001)* compared to the empty vector condition *(Fig.2e)*.

Considering the significance of these results, as well as to investigate, whether the kinase property of the enzymes was involved in the observed cell growth defect, the experiment was repeated in the IncuCyte system, including also the kinase-dead mutants of NUAK1 (NUAK1-TA) and NUAK2 (NUAK2-TA). Strikingly, no reduction in cell growth rate of NUAK2 constitutively active mutant compared to either empty vector control or kinase-dead mutant was detected *(Fig.2f)*. However, the NUAK1-TE condition still demonstrated the inhibition of the cell proliferation: 33% reduction in growth rate in contrast to the empty vector control *(P=0.0214)* and 23% decline compared to the kinase-dead mutant *(P=0.0195)* *(Fig.2f)*.

Genetic overexpression of NUAK1 and NUAK2 was confirmed by RT-qPCR: 8-fold upregulation for NUAK1-TA, 34-fold increase for NUAK1-TE and 6-fold upregulation for both NUAK2 conditions was observed *(Fig.2a,b)*. This was also confirmed on the protein level with an obvious contrast between NUAK bands and the empty vector *(Fig.2c,d)*. Although NUAK1-TE on the gene level was expressed at least twice more than the kinase-dead mutant, almost no difference between these two conditions could be spotted on protein level, which might be due to the limited capacity of the cells to produce such amounts of the protein.

On the Western blots, the bands appear to have higher molecular weight than expected, with a pattern of TE-mutants having slightly bigger size than TA-mutants. This could be explained by potential post-translational modifications and incomplete denaturation of the proteins that could affect the protein separation during SDS-PAGE. Nevertheless, these results were reproducible across the experiments and the antibodies were tested with both positive and negative controls.

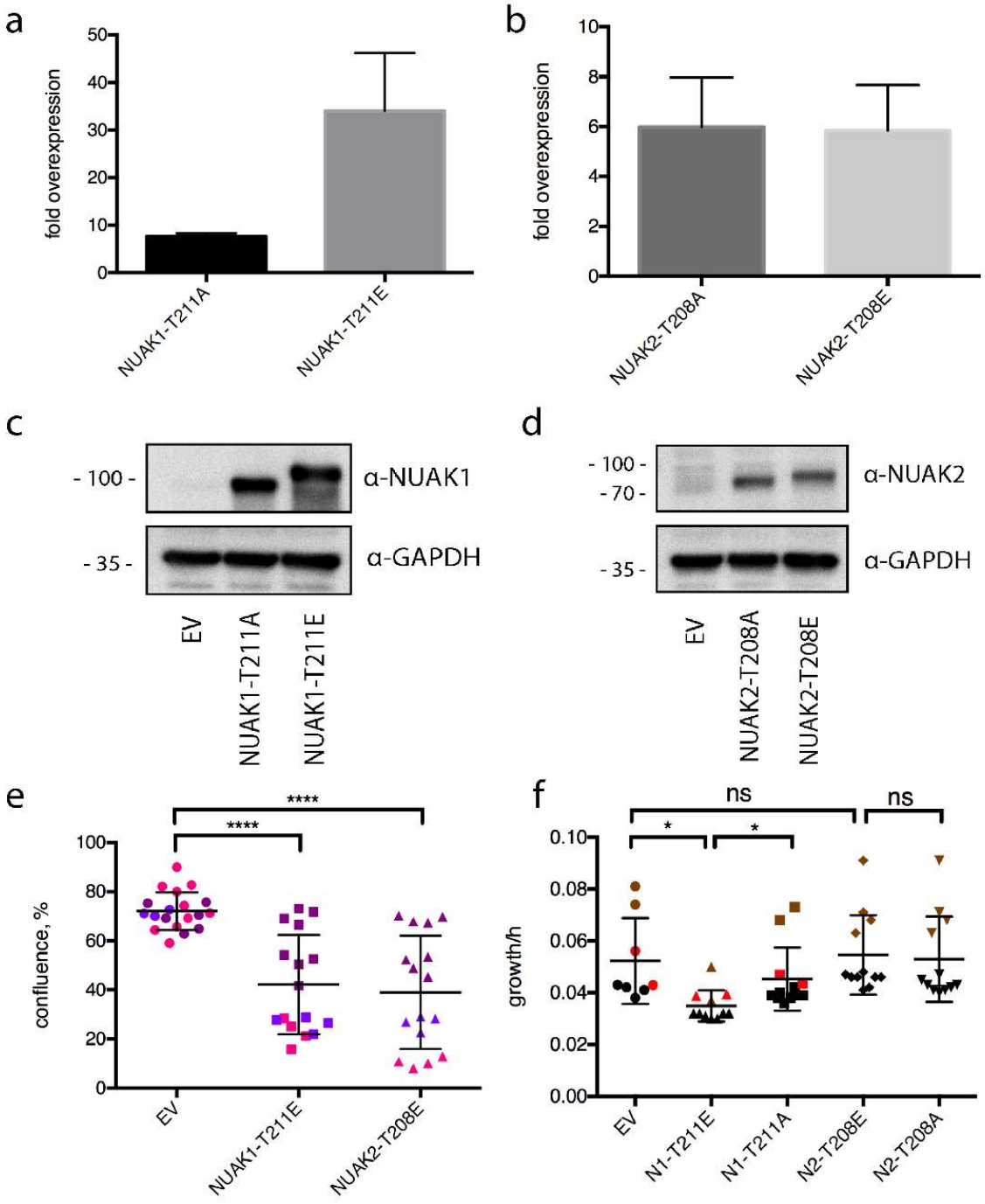

Fig.2
NUAK1 participates in A549 cell growth decrease.
(**a**) Relative gene expression of NUAK1 (normalized to EV control; ± SEM; N=2-4). (**b**) Relative gene expression of NUAK2 (normalized to EV control; ± SEM; N=2-4). (**c**) Western blotting of NUAK1 kinase-dead and constitutively active mutants incubated with α-NUAK1 antibody (Predicted molecular weight: 78 kDa; α-GAPDH is a loading control). (**d**) Western blotting of NUAK2 kinase-dead and constitutively active mutants incubated with α-NUAK2 antibody (Predicted molecular weight: 70 kDa; α-GAPDH is a loading control). (**e**) Crystal violet staining displays the difference in confluence between NUAK1-TE, NUAK2-TE and EV control (*P<0.0001 (****)*, unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 16-19 wells from 3 experiments, colour-coded). (**f**) Growth rate of NUAK1-TE is decreased in comparison to kinase-dead mutant and EV control after 46-70h incubation in IncuCyte (*P≤0.05 (*)*, unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 8-12 wells from 3 experiments, colour-coded).

6.3 AMPK is involved in the growth defect of A549 cells

The A549 cells were transduced with AMPKα1 and AMPKα2 wild type (AMPKα1-WT, AMPKα2-WT) and constitutively active mutant constructs (AMPKα1-TE, AMPKα2-TE), followed by the growth assay.

The gene expression was upregulated on average 5- to 6-fold for AMPKα1-TE and AMPKα1-WT, and 9- to 15-fold for AMPKα2-TE and AMPKα2-WT *(Fig.3a,b)*. The overexpression of AMPK was also reflected on the protein level – the bands of these conditions on the blots are thicker than ones that serve as the controls, however, they more likely demonstrate 2- to 3-fold difference *(Fig.3c,d)*. This discrepancy between the mRNA and protein levels could be explained by the inability of cells to produce as much protein as was genetically expressed. Another clarification would be the failure of the exceeding amount of AMPKα1/α2 subunits to associate with β- and γ-subunits, which led to their degradation in the proteasomes.

Data from the crystal violet staining showed a remarkable drop in confluence for both AMPK isoforms: 48% in case of AMPKα1-TE compared to the wild type form *(P<0.0001)* and the empty vector condition *(P<0.0001)*, and 62% to 66% reduction in confluence of AMPKα2-TE condition compared to the wild type form *(P<0.0001)* and the empty vector *(P<0.0001)*, correspondingly *(Fig.3e)*.

To confirm these observations, the experiment was resumed in the IncuCyte system. AMPKα2 constitutively active mutant showed 26% lower growth rate than the empty vector *(P=0.1328)* and 18% less growth than the wild type form *(P=0.3345)*, however, these results were not statistically significant *(Fig.3f)*. At the same time, the growth rate of AMPKα1-TE condition was reduced by 27% in comparison to both wild type *(P=0.0208)* and empty vector controls *(P=0.0198)*, thus supporting the growth-restraining role of AMPKα1 *(Fig.3f)*.

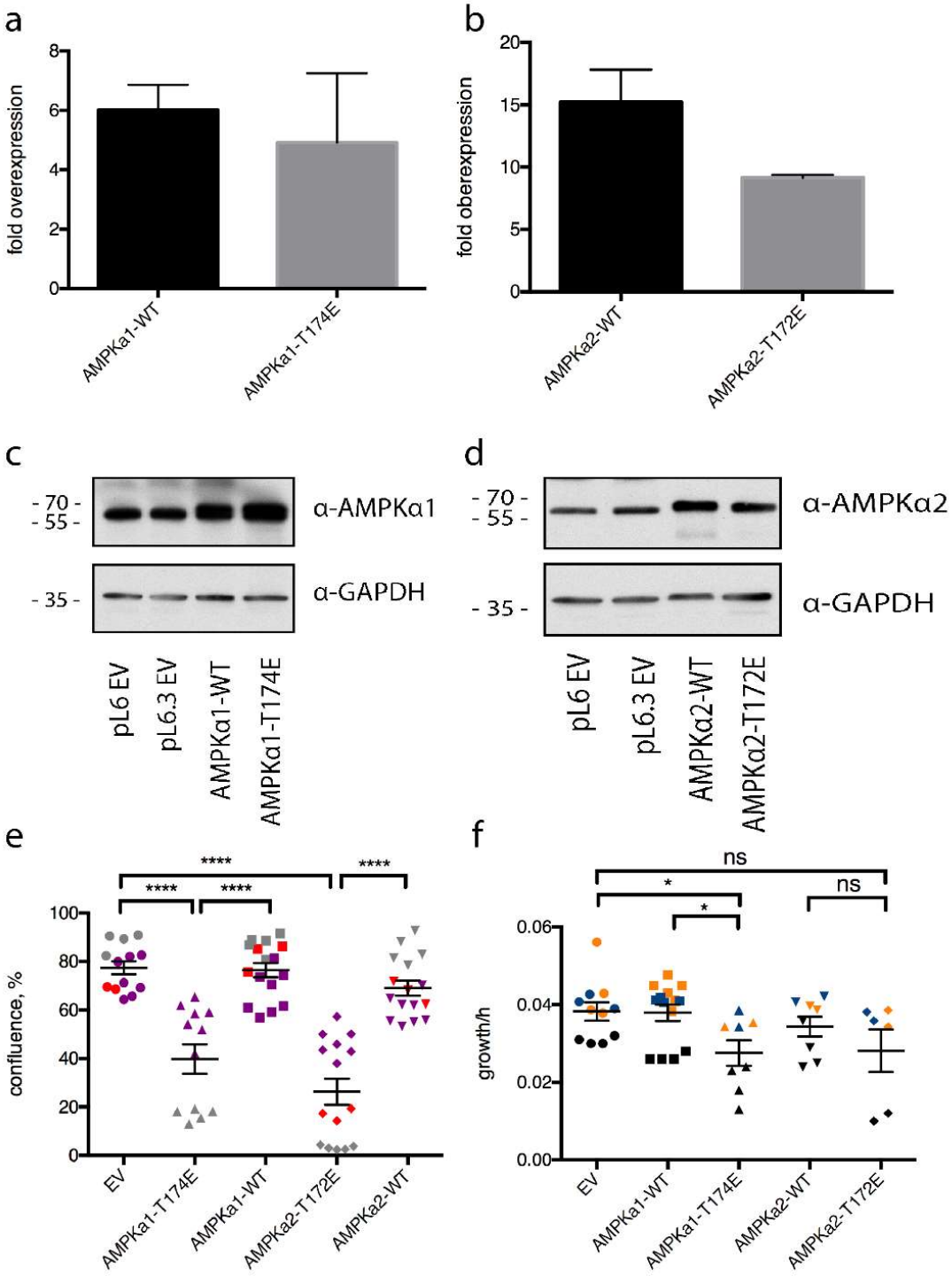

Fig.3

AMPK negatively affects the growth of A549 cells.

(**a**) Relative gene expression of AMPKα1 (normalized to EV control; ± SEM; N=2-3). (**b**) Relative gene expression of AMPKα2 (normalized to EV control; ± SEM; N=2). (**c**) Western blotting of wild type AMPKα1 and its constitutively active mutant incubated with α-AMPKα1 primary antibody (Predicted molecular weight: 63 kDa; α-GAPDH is a loading control). (**d**) Western blotting of wild type AMPKα2 and its constitutively active mutant incubated with α-AMPKα2 primary antibody (Predicted molecular weight: 63 kDa; α-GAPDH is a loading control). (**e**) Crystal violet staining reveals the difference in confluence of constitutively active AMPKα1 and AMPKα2 compared to EV and wild type controls

(*P<0.0001 (****)*, unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 12-16 wells from 3 experiments, colour-coded). (**f**) Growth rate of AMPKα1 and AMPKα2 constitutively active mutants is reduced in comparison to wild type forms and EV control after 48-70 hours of incubation in IncuCyte (*P≤0.05 (*)* and *P>0.05 (ns)*, unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 6-14 wells from 3 experiments, colour-coded).

6.4 MARK subfamily kinases do not affect the growth of A549 cells

To assess the role of MARK family regarding the growth of the A549 cells, a transduction with constitutively active mutant constructs of its four members (MARK1-TE, MARK2-TE, MARK3-TE, MARK4-TE) was first performed, and the growth assay was carried out.

Crystal violet staining method revealed that only one member of this kinase family had significant impact on the cell growth. MARK1 constitutively active mutant exhibited 27% confluence reduction compared to the empty vector control *(P<0.0001) (Fig.4e)*.

This kinase was then additionally tested in the IncuCyte system, and the wild type control (MARK1-WT) was also included to assess the role of the kinase activity of MARK1. Surprisingly, this time no evidence of growth arrest caused by MARK1 kinase was acquired. The growth rate of constitutively active MARK1 mutant was decreased insignificantly compared to the wild type form of the kinase and even exceeded the growth of the empty vector control *(Fig.4f)*.

RT-qPCR showed that, on average, the gene expression of MARK2 was elevated 7-fold, MARK3 – 14-fold, MARK4 – 27-fold *(Fig.4b)*. The average mRNA level of MARK1 seemed to be extremely upregulated: 887-fold in case of MARK1-WT and 510-fold for MARK1-TE condition *(Fig.4a)*, which was due to the low endogenous expression of MARK1 in A549 cells. The results from Western blots also demonstrated the overexpression of the substrates as their bands clearly exceeded the signal from the empty vector control *(Fig.4c,d)*. High genetic expression, however, could result in translation of abundant proteins to such an extent when the difference between MARKs could no longer be spotted on protein level. This, together with potential post-translational modifications, might also be the reason for the band size shift. In case of MARK2, MARK3 and MARK4, although the V5-tag in these constructs allowed to conveniently screen these substrates together, at the same time, it could contribute to a slightly increased molecular weight, but in a very humble manner (1-1.5 kDa). Still, the alignment pattern of the bands of these kinases was preserved and the results were reproducible across the experiments.

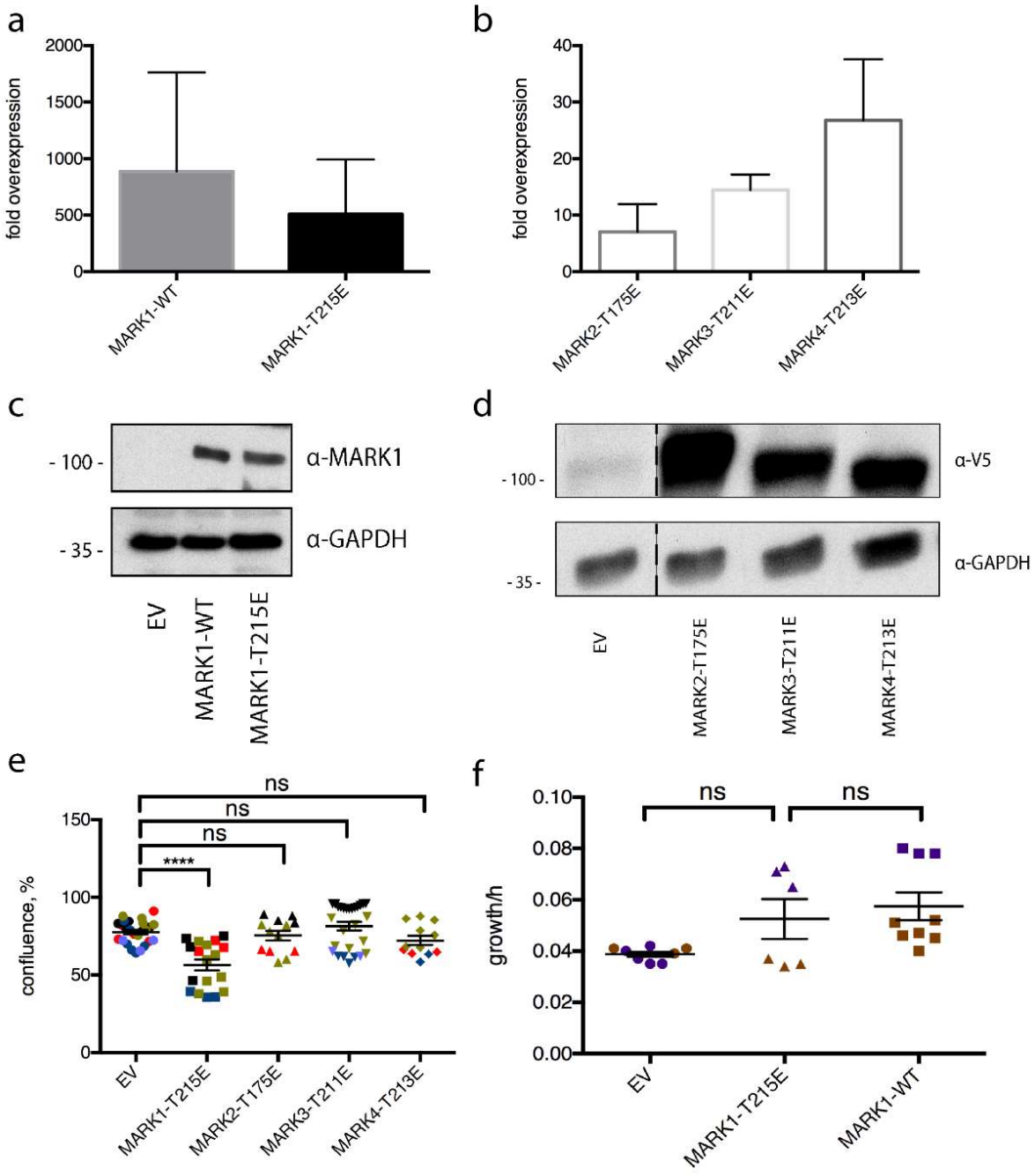

Fig.4
MARK subfamily is not involved in restricting A549 cell growth.
(**a**) Relative gene expression of MARK1 (normalized to EV control; ± SEM; N=2-4). (**b**) Relative gene expression of MARK2, MARK3 and MARK4 in corresponding constitutively active mutants (normalized to EV control; ± SEM; N=2-3). (**c**) Western blotting of wild type MARK1 and its constitutively active mutant incubated with α-MARK1 primary antibody (Predicted molecular weight: 85-89 kDa; α-GAPDH is a loading control). (**d**) Western blotting of MARK2-TE, MARK3-TE, MARK4-TE incubated with α-V5 C-term. primary antibody (Predicted molecular weight: MARK2 – 88 kDa, MARK3 – 85 kDa, MARK4 – 83 kDa; α-GAPDH is a loading control). (**e**) Crystal violet staining shows the difference in confluence between constitutively active mutant MARK1-TE and EV control (*P<0.0001 (****)*,

unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 18-25 wells from 4-5 experiments, colour-coded), but not for MARK2-TE, MARK3-TE, MARK4-TE. (**f**) Growth rate of MARK1-TE is not decreased in comparison to wild type MARK1 or EV control after 46-70h incubation in IncuCyte (*P>0.05 (ns)*, unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 6-9 wells from 2 experiments, colour-coded).

6.5 SIK kinases do not influence the growth of A549 cells

To evaluate the possible role of SIK subfamily kinases in diminishing A549 cell growth, the cells were transduced with SIK1, SIK2 and SIK3 constitutively active mutant contructs (SIK1-TE, SIK2-TE, SIK3-TE) and were subjected to the growth assay.

Genetic overexpression of the SIK kinases was confirmed by 8-fold upregulation of SIK1, 21-fold upregulation of SIK2 and 7-fold increase of SIK3 mRNA *(Fig.5a)*. This was reflected on the protein level in a similar way – with conspicuous difference between the empty vector and SIK2 constitutively active mutant, and less prominent distinction between the control and SIK1 and SIK3 mutants *(Fig.5b,c,d)*.

The analysis of crystal violet staining showed no reduction in confluence of SIK constitutively active mutants compared to the empty vector control *(Fig.5e)*.

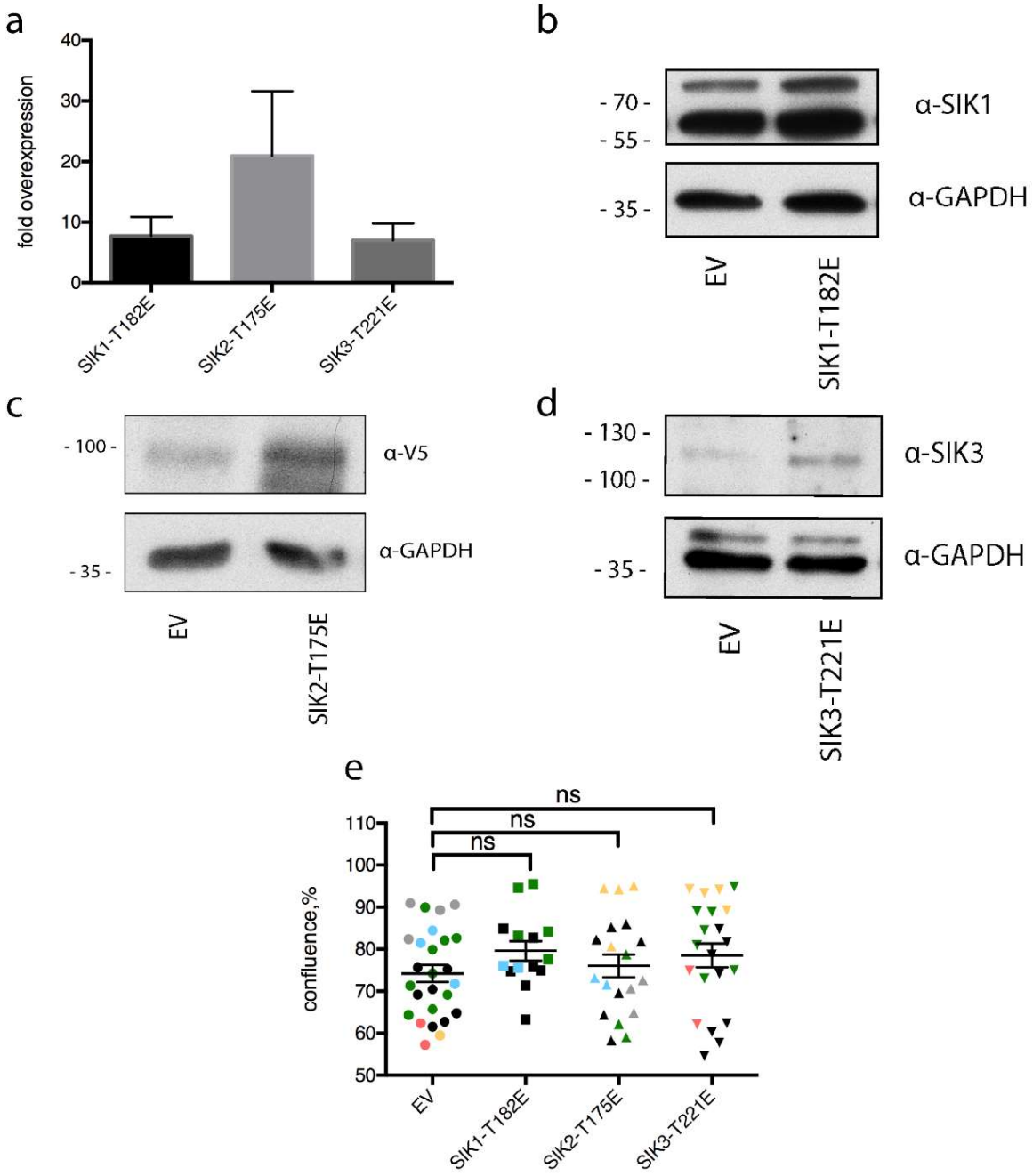

Fig.5
SIK subfamily does not alter the A549 cell growth.
(**a**) Relative gene expression of SIK1, SIK2 and SIK3 in corresponding constitutively active mutants (normalized to EV control; ± SEM; N=3). (**b**) Western blotting of SIK1 constitutively active mutant incubated with α-SIK1 primary antibody (Predicted molecular weight: 85 kDa; α-GAPDH is a loading control). (**c**) Western blotting of SIK2 constitutively active mutant incubated with α-V5 C-term. primary antibody (Predicted molecular weight: 103 kDa; α-GAPDH is a loading control). (**d**) Western blotting of SIK3 constitutively active mutant incubated with α-SIK3 primary antibody (Predicted molecular weight: 115 kDa; α-GAPDH is a loading control). (**e**) Crystal violet staining displays no reduction in confluence of constitutively active SIK1-TE, SIK2-TE, SIK3-TE compared to EV control (*P>0.05 (ns)*, unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 14-26 wells from 4-5 experiments, colour-coded).

6.6 AMPKα1, AMPKα2 and NUAK1 kinases cause cell growth defect in A549 cells in LKB1-dependent manner

To establish that the exhibited growth defect in AMPKs and NUAK1 overexpressing A549 cells is a LKB1-dependent mechanism, rescue experiments with siRNA-mediated loss-of-function of the kinase substrates in LKB1-overexpressing cells was conducted. siRNA-mediated knockdown of a kinase exerting growth-restricting properties in LKB1-reconstituted cells would lead to the rescue of the cell growth and indicate the LKB1-dependent manner of action of this substrate. Earlier, only AMPKα1, AMPKα2 and NUAK1 overexpressing cells showed growth suppressing potential, therefore these kinase substrates were considered for the rescue experiments.

First, A549 cells were transduced with constructs of empty vector, wild type LKB1 and kinase-dead LKB1, transfected with non-targeting siRNA (siNT) as knockdown control (EV+siNT, LKB1-WT+siNT, LKB1-K78M+siNT) and checked for the growth defect using IncuCyte. The growth rate of LKB1-WT was 65% lower than the kinase-dead mutant control *(P=0.0195)*, and 50% less than the empty vector control *(P=0.1155) (Fig.6c)*.

LKB1 gene expression in the studied cells was supported by RT-qPCR of the wild type and kinase-dead LKB1 knockdown controls, displaying 57-fold and 34-fold upregulation, correspondingly *(Fig.6a)*. RT-qPCR data also confirms the successful knockdown of AMPKs and NUAK1. On average, AMPKα1 was downregulated by 83-86%, AMPKα2 – by 96%. Double AMPK knockdown decreased the amount of AMPKα1 mRNA by 76-79% and the expression of AMPKα2 by 93-95% *(Fig.6b)*. NUAK1 was downregulated slightly less – by 62-66%, and in this case a trend for compensation of its activity by NUAK2 was noted *(Fig.6b)*. So, in LKB1-K78M+siNUAK1 condition the gene expression of NUAK2 was elevated by 50%, suggesting the tight balancing of these kinases in the cell.

siRNA knockdown of every tested condition showed the rescue of growth in A549 cells with reconstituted LKB1. AMPKα1 knockdown increased the growth rate by 57% *(P=0.0917)*, AMPKα2 knockdown - by 72% *(P=0.0254)*, double knockdown of both AMPK isoforms managed to raise this feature by 78% *(P=0.0282)*, and knockdown of NUAK1 caused 75% gain in growth rate *(P=0.0616)* compared to LKB1-WT with siNT knockdown *(Fig.6d)*.

Knockdown conditions in LKB1-WT cells demonstrated similar growth rate as kinase-dead mutant controls with identical knockdown of AMPKs and NUAK1 *(Fig.6d)*. Consistently, no significant difference in growth rate between knockdown control and studied conditions

was detected upon substrate inactivation in cells overexpressing LKB1 kinase-dead mutant as no growth defect could be observed there initially.

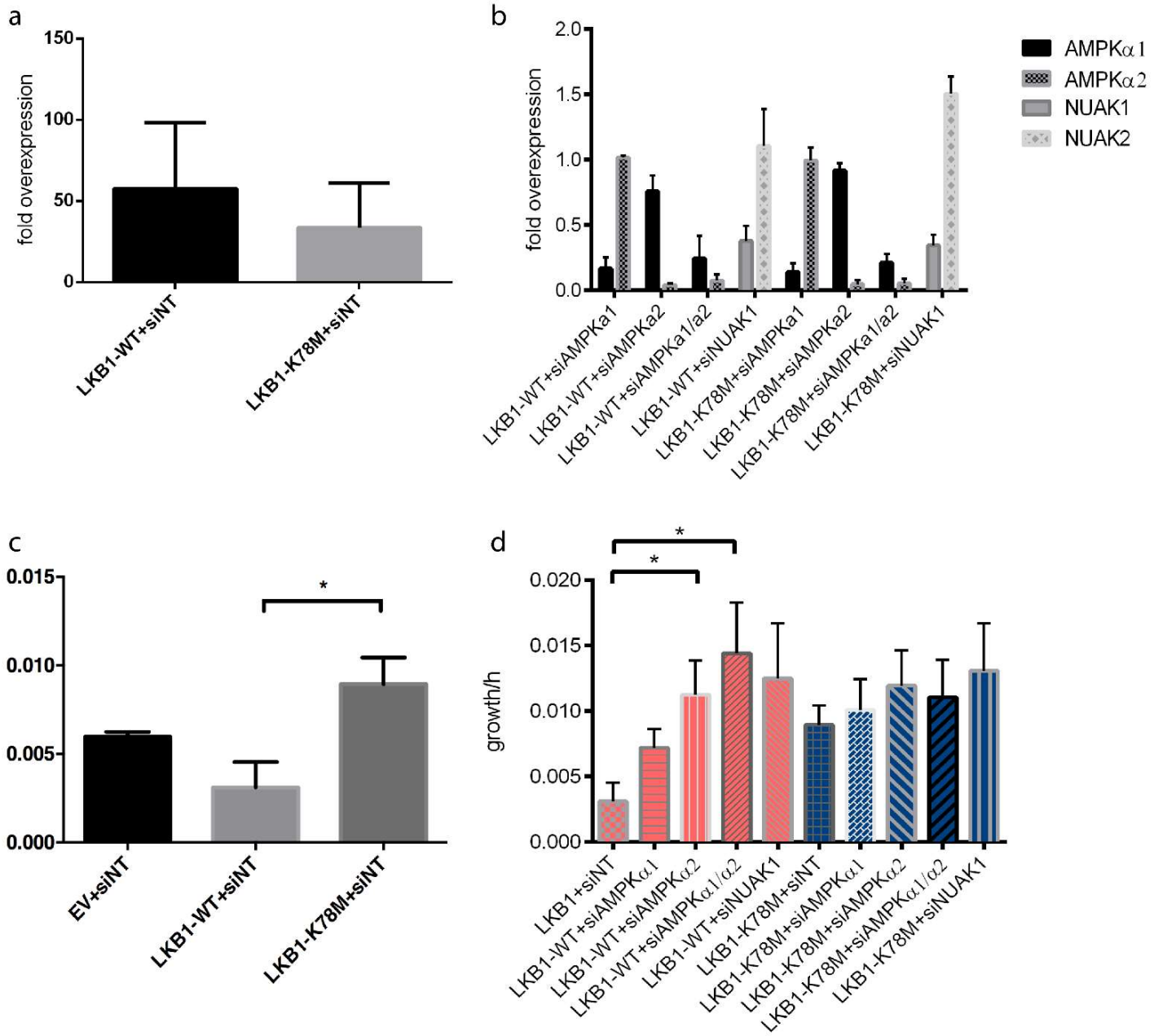

Fig.6

siRNA knockdown of AMPKs and NUAK1 rescues growth defect in A549 cells overexpressing wild type LKB1

(**a**) Relative gene expression of LKB1 (normalized to EV+siNT control; ± SEM; N=2).
(**b**) Relative gene expression of AMPKα1, AMPKα2 and NUAK1 (normalized to corresponding knockdown controls (LKB1-WT+siNT or LKB1-K78M+siNT); ± SEM; N=2).
(**c**) Growth rate of LKB1-WT+siNT condition is decreased in comparison to LKB1-K78M+siNT and EV+siNT controls after 48-72h of incubation in IncuCyte (*P=0.0195* (*) and *P=0.1155 (ns)*, unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 4-10 wells from 2 experiments). (**d**) Rescue of growth in A549 cells overexpressing wild type LKB1 after AMPK and NUAK1 knockdown and 48-72h of incubation in IncuCyte *(P≤0.05 (*)*, unpaired, two-tailed, Welch-corrected t test; ± SEM; N: 4-10 wells from 2 experiments).

7 Discussion

7.1 NUAK1 and AMPK are the key LKB1 effectors in lung cancer cell growth suppression

Tumor suppressor LKB1 is mutated in 18% of lung adenocarcinomas and its inactivation accelerates lung tumorigenesis in mouse model[9,27]. However, there is limited knowledge concerning the precise mechanism, by which LKB1 restricts the lung cancer cell growth. For years, researchers' attention was mostly drawn to the well-known metabolic regulator AMPK, and no systematic analysis of all LKB1 downstream targets has been performed to reveal their involvement in the lung cancer pathogenesis.

In this study, a total of 11 downstream LKB1 substrates were selected for examining their tumor suppressive functions. BRSK1, BRSK2 and SNRK kinases were excluded from the screening due to their low baseline expression in lung tissues[29,137]. The whole project was carried out in three steps: in the first phase of the screening, I used crystal violet staining method to spot the difference in A549 cell growth caused by constitutively active mutants of 11 LKB1 downstream kinases in the series of overexpression experiments. A remarkable and statistically significant cell growth reduction was observed for AMPKα1, AMPKα2, NUAK1, NUAK2 and MARK1 constitutively active mutants. Next, I repeated the experiments with these selected LKB1 substrates using the IncuCyte system and evaluated the cell growth. At this stage, all conditions were additionally compared either with the kinase-dead (for NUAK1 and NUAK2) or wild-type (for MARK1, AMPKα1, AMPKα2) controls to confirm that the kinase activity of the enzyme was causing the cell growth defect. The acquired data suggested that only NUAK1 and both AMPK isoforms are crucial for this process as only these conditions demonstrated the growth rate suppression, which also was quite similar - ranging from 18% to 33%. The final step was to link the well-known tumor suppressive properties of LKB1 with the collected data in growth rescue experiments. A549 cells with ectopically expressed LKB1 and knockdown of NUAK1, AMPKα1, AMPKα1 or double knockdown of both AMPK isoforms all showed increase in cell growth varying from 57% to 78%. These observations illustrate that the A549 cell growth defect caused by LKB1 is mediated by its downstream kinases NUAK1, AMPKα1 and AMPKα2.

The findings from this study in addition to the known functions of AMPK as a metabolic regulator might provide evidence for its tumor suppressive properties in cancer cells. The inhibition of mTORC1 pathway, which is achieved by AMPK through the phosphorylation of its substrates Raptor[65] and TSC2[138] upon energy stress, is believed to be one of the most significant growth-restricting aspects, as it halts the translation of several vital transcriptional factors, such as SREBP-1[67] and HIF-1α[70]. Downregulation of the latter one causes the inhibition of the glycolytic pathway[68,69], which, together with AMPK-provoked increase in mitochondrial growth[139] and enzymes[140], comprises the anti-Warburg effect. Consistently, the silencing of both AMPK α subunits in non-small cell lung carcinoma H1299 cell line demonstrated elevated basal extracellular acidification rate (ECAR) and lactate concentration, pointing at the involvement of AMPK in counteracting the Warburg effect, a distinctive feature of cancer cells[141,142]. Moreover, apart from the metabolic impact, AMPK was reported to induce the cell cycle arrest by activating the cell cycle regulating proteins p53 and p21[143,144].

A recent study[145] suggests that AMPK interrupts the cell cycle in LKB1-null cells upon phosphorylation by CAMKK2, its alternative upstream activator[84,146,147]. One of the study models in this project were A549 cells, and activation of AMPK by calcium ionophore A23187 did disrupt the G1/G2 transition, which stands in line with the results of my research. What remains unclear, is the authors' statement about AMPK being the only kinase responsible for the growth defect, even with the LKB1 expressed in the cell. In their paper, the authors provide evidence only for LKB1-independent AMPK activation and growth suppression. Also, their research is focused solely on AMPK, leaving AMPK-related kinases out of scope. This gave me additional motivation to apply more comprehensive approach in my study by including ARKs in the screening and investigating their effect on cell growth upon activation by LKB1.

My data on NUAK1 is consistent with the findings showing the ability of this kinase to directly phosphorylate p53 and to halt the cell cycle at the G1/S stage upon activation by the LKB1 in the same A549 cell line[105]. Surprisingly, the involvement of p53 suggests that NUAK1 and AMPK might share a common mechanism of interrupting the cell cycle progression.

My investigation shows no involvement of SIK or MARK kinases in cell growth control. However, two recent studies highlight SIK1 and SIK3 as the mediators of LKB1-caused cell growth defect in NSCLC[119,128]. In both projects, the mice models of KRAS-driven lung adenocarcinoma are used, which makes it rather challenging to compare the data from my *in vitro* experiments with potentially more reliable *in vivo* studies. Surprisingly, Hollstein et al. report, that, prior to starting *in vivo* studies, they failed to observe the LKB1-dependent cell growth defect in A549 cells cultured in standard adherent conditions, although the suppressing effect of ectopic LKB1 on A549 cell growth was first described by Jimenez et al. in 2003[10] and reproduced in my project. Also, some data from the paper by Murray et al. may raise questions. For instance, in addition to SIKs, NUAK and AMPK inactivation seems to also provoke an increased tumor burden in their mouse model, but this data is not considered. Unfortunately, no difference in cell growth caused by SIKs was detected in my *in vitro* study, and that could be one of the limitations of this project.

7.2 Limitations of the study

Although the *in vitro* overexpression experiments suit well for the screening purpose, they still have certain drawbacks. One such major shortcoming is the redundant expression of a gene of interest, which exceeds the level of its physiological expression. Consequent overexpression of an according protein may lead the cell into the state of protein burden, overloading the resources required for protein translation, folding and breakdown. This can also be caused by an overexpression of specific subunits of a protein complex (e.g. catalytic α-subunits in heterotrimeric AMPK complex) and following disturbance of the stoichiometric balance. Increased expenditure of cell's resources, accumulation of abnormal complexes and promiscuous protein interactions might impair the cell growth[148]. In this regard, a bias towards the false positive results could arise, considering the design of my project. At the same time, the discrepancies between gene and protein expression data observed in my experiments could be explained by the consequences of protein overexpression.

As was mentioned before, AMPK can be activated by either LKB1 or CAMKK2. In my experiments I did not use CAMKK inhibitors, so, possibly, the wild-type form of AMPK was left exposed to phosphorylation by this kinase and could continue affecting the cell proliferation. Since I used the wild-type AMPK as a control for the constitutively active mutant, blocking CAMKK2 could increase the detected difference in cell growth between these conditions and strengthen the evidence in favour of AMPK growth-suppressing properties.

This project assumes that several kinases might mediate the growth-restricting properties of LKB1 in lung cancer cells, however, the outline of this study does not provide the possibility to measure, whether this effect is individual or cumulative, as well as it does not answer the question about the exact molecular mechanism responsible for the cell growth defect.

Also, several experiments should have been repeated to improve the statistical significance of the results. This is especially relevant for the rescue experiments with siRNA knockdown.

8 Conclusions and prospects

The results of this project highlight that the kinase activity of NUAK1 and AMPKα isoforms is crucial for the LKB1-mediated suppression of the lung adenocarcinoma cell growth. The involvement of other AMPK-related kinases in the LKB1-mediated lung adenocarcinoma cell growth suppression appears to be insignificant.

Future studies should focus on investigating the detailed mechanism behind the LKB1-dependent observed cell growth defect in AMPKα, NUAK1 overexpressed lung adenocarcinoma cells. For instance, proliferation and apoptosis markers such as phospho-histone H3 and cleaved caspase 3 can be a good marker to reveal whether the restricted growth of cancer cells is due to a defect in the cell cycle or apoptosis. ROS assay could help better understand the metabolic profile of the studied conditions. Also, it would be extremely useful to adapt the results from this project and carry them over to the physiologically more relevant *in vivo* model for investigating functions of LKB1 downstream kinases in lung cancer.

In abundance of often contradictory evidence regarding the role of AMPK and AMPK-related kinases in cancer, the findings from this project will help to prioritize the aims and define future course of the research as well as action towards targeting LKB1 mutant lung cancer.

9 References

1. Lizcano, J. M. *et al.* LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J.* (2004). doi:10.1038/sj.emboj.7600110
2. Alessi, D. R., Sakamoto, K. & Bayascas, J. R. LKB1-dependent signaling pathways RID C-9450-2011. *Annu. Rev. Biochem.* **75**, 137–163 (2006).
3. Hemminki, A. *et al.* A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature* (1998). doi:10.1038/34432
4. Hemminki, A. The molecular basis and clinical aspects of Peutz-Jeghers syndrome. *Cellular and Molecular Life Sciences* (1999). doi:10.1007/s000180050329
5. Sato, N. *et al.* STK11/LKB1 Peutz-Jeghers gene inactivation in intraductal papillary-mucinous neoplasms of the pancreas. *Am. J. Pathol.* (2001). doi:10.1016/S0002-9440(10)63053-2
6. Wingo, S. N. *et al.* Somatic LKB1 mutations promote cervical cancer progression. *PLoS One* (2009). doi:10.1371/journal.pone.0005137
7. Liu, W. *et al.* LKB1/STK11 Inactivation Leads to Expansion of a Prometastatic Tumor Subpopulation in Melanoma. *Cancer Cell* (2012). doi:10.1016/j.ccr.2012.03.048
8. Tuveson, D. A. & Jacks, T. Modeling human lung cancer in mice: Similarities and shortcomings. *Oncogene* (1999). doi:10.1038/sj.onc.1203107
9. Jordan, E. J. *et al.* Prospective comprehensive molecular characterization of lung adenocarcinomas for efficient patient matching to approved and emerging therapies. *Cancer Discov.* (2017). doi:10.1158/2159-8290.CD-16-1337
10. Jimenez, A. I., Fernandez, P., Dominguez, O., Dopazo, A. & Sanchez-Cespedes, M. Growth and molecular profile of lung cancer cells expressing ectopic LKB1: Down-regulation of the phosphatidylinositol 3′-phosphate kinase/PTEN pathway. *Cancer Res.* (2003).
11. WHO. No Title. *Fact sheets* (2018).
12. Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA. Cancer J. Clin.* (2018). doi:10.3322/caac.21492
13. Maxwell Parkin, D., Bray, F., Ferlay, J. & Pisani, P. Estimating the world cancer burden: Globocan 2000. *International Journal of Cancer* (2001). doi:10.1002/ijc.1440
14. Jemal, A., Bray, F. & Ferlay, J. Global Cancer Statistics. *CA Cancer J Clin* (2011).
15. Soerjomataram, I. *et al.* Global burden of cancer in 2008: A systematic analysis of disability-adjusted life-years in 12 world regions. *Lancet* (2012). doi:10.1016/S0140-6736(12)60919-2
16. Travis, W. D., Brambilla, E., Müller-Hermelink, H. K. & Harris, C. World Health Organization classification of tumours; tumours of lung, pleura, thymus and heart. *World Heal. Organ. Classif. tumours; tumours lung, pleura, thymus Hear.* (2004).
17. Bhattacharjee, A. *et al.* Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc. Natl. Acad. Sci. U. S. A.* (2001). doi:10.1073/pnas.191502998
18. Garber, M. E. *et al.* Diversity of gene expression in adenocarcinoma of the lung. *Proc. Natl. Acad. Sci. U. S. A.* (2001). doi:10.1073/pnas.241500798
19. Angulo, B. *et al.* Expression signatures in lung cancer reveal a profile for EGFR-mutant tumours and identify selective PIK3CA overexpression by gene amplification. *J. Pathol.* (2008). doi:10.1002/path.2267
20. Sanchez-Cespedes, M. Dissecting the genetic alterations involved in lung carcinogenesis. *Lung Cancer* (2003). doi:10.1016/S0169-5002(03)00033-3
21. Ding, L. *et al.* Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* (2008). doi:10.1038/nature07423
22. Blanco, R. *et al.* A gene-alteration profile of human lung cancer cell lines. *Hum. Mutat.*

(2009). doi:10.1002/humu.21028
23. Sanchez-Cespedes, M. *et al.* Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. *Cancer Res.* (2002).
24. Carretero, J., Medina, P. P., Pio, R., Montuenga, L. M. & Sanchez-Cespedes, M. Novel and natural knockout lung cancer cell lines for the LKB1/STK11 tumor suppressor gene. *Oncogene* (2004). doi:10.1038/sj.onc.1207502
25. Sanchez-Cespedes, M. A role for LKB1 gene in human cancer beyond the Peutz-Jeghers syndrome. *Oncogene* (2007). doi:10.1038/sj.onc.1210594
26. Virmani, A. K. *et al.* Allelotyping demonstrates common and distinct patterns of chromosomal loss in human lung cancer types. *Genes Chromosom. Cancer* (1998). doi:10.1002/(SICI)1098-2264(199804)21:4<308::AID-GCC4>3.0.CO;2-2
27. Ji, H. *et al.* LKB1 modulates lung cancer differentiation and metastasis. *Nature* (2007). doi:10.1038/nature06030
28. Matsumoto, S. *et al.* Prevalence and specificity of LKB1 genetic alterations in lung cancers. *Oncogene* (2007). doi:10.1038/sj.onc.1210418
29. Jaleel, M. *et al.* Identification of the sucrose non-fermenting related kinase SNRK, as a novel LKB1 substrate. *FEBS Lett.* (2005). doi:10.1016/j.febslet.2005.01.042
30. Baas, A. F. *et al.* Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD. *EMBO J.* (2003). doi:10.1093/emboj/cdg292
31. Boudeau, J. *et al.* MO25α/β interact with STRADα/β enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. *EMBO J.* (2003). doi:10.1093/emboj/cdg490
32. Brajenovic, M., Joberty, G., Küster, B., Bouwmeester, T. & Drewes, G. Comprehensive Proteomic Analysis of Human Par Protein Complexes Reveals an Interconnected Protein Network. *J. Biol. Chem.* (2004). doi:10.1074/jbc.M312171200
33. Boudeau, J. *et al.* Analysis of the LKB1-STRAD-MO25 complex\r10.1242/jcs.01571. *J Cell Sci* (2004).
34. Tiainen, M., Ylikorkala, A. & Mäkelä, T. P. Growth suppression by Lkb1 is mediated by a G1 cell cycle arrest. *Proc. Natl. Acad. Sci. U. S. A.* (1999). doi:10.1073/pnas.96.16.9248
35. Zeng, P. Y. & Berger, S. L. LKB1 is recruited to the p21/WAF1 promoter by p53 to mediate transcriptional activation. *Cancer Res.* (2006). doi:10.1158/0008-5472.CAN-06-0999
36. Karuman, P. *et al.* The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death. *Mol. Cell* (2001). doi:10.1016/S1097-2765(01)00258-1
37. Shaw, R. J. *et al.* The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc. Natl. Acad. Sci. U. S. A.* (2004). doi:10.1073/pnas.0308061100
38. Inge, L. J., Coon, K. D., Smith, M. A. & Bremner, R. M. Expression of LKB1 tumor suppressor in non-small cell lung cancer determines sensitivity to 2-deoxyglucose. *J. Thorac. Cardiovasc. Surg.* (2009). doi:10.1016/j.jtcvs.2008.11.029
39. Liang, J. *et al.* The energy sensing LKB1-AMPK pathway regulates p27kip1 phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat. Cell Biol.* (2007). doi:10.1038/ncb1537
40. Alexander, A. *et al.* ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. *Proc. Natl. Acad. Sci. U. S. A.* (2010). doi:10.1073/pnas.0913860107
41. Huang, Q., Wu, Y. T., Tan, H. L., Ong, C. N. & Shen, H. M. A novel function of poly(ADP-ribose) polymerase-1 in modulation of autophagy and necrosis under oxidative stress. *Cell Death Differ.* (2009). doi:10.1038/cdd.2008.151
42. Moustakas, A. & Heldin, C. H. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. *Cancer Science* (2007). doi:10.1111/j.1349-7006.2007.00550.x
43. Morén, A., Raja, E., Heldin, C. H. & Moustakas, A. Negative regulation of TGFβ signaling

by the kinase LKB1 and the scaffolding protein LIP1. *J. Biol. Chem.* (2011). doi:10.1074/jbc.M110.190660
44. Wu, Y. & Zhou, B. P. New insights of epithelial-mesenchymal transition in cancer metastasis. *Acta Biochimica et Biophysica Sinica* (2008). doi:10.1111/j.1745-7270.2008.00443.x
45. ten Klooster, J. P. *et al.* Mst4 and Ezrin Induce Brush Borders Downstream of the Lkb1/Strad/Mo25 Polarization Complex. *Dev. Cell* (2009). doi:10.1016/j.devcel.2009.01.016
46. Miyoshi, H. *et al.* Gastrointestinal hamartomatous polyposis in Lkb1 heterozygous knockout mice. *Cancer Res.* (2002).
47. McCarthy, A. *et al.* Conditional deletion of the Lkb1 gene in the mouse mammary gland induces tumour formation. *J. Pathol.* (2009). doi:10.1002/path.2599
48. Nakau, M. *et al.* Hepatocellular carcinoma caused by loss of heterozygosity in Lkb1 gene knockout mice. *Cancer Res.* (2002).
49. Hezel, A. F. *et al.* Pancreatic Lkb1 Deletion Leads to Acinar Polarity Defects and Cystic Neoplasms. *Mol. Cell. Biol.* (2008). doi:10.1128/mcb.01621-07
50. Contreras, C. M. *et al.* Lkb1 inactivation is sufficient to drive endometrial cancers that are aggressive yet highly responsive to mTOR inhibitor monotherapy. *DMM Dis. Model. Mech.* (2010). doi:10.1242/dmm.004440
51. Gao, Y. *et al.* LKB1 inhibits lung cancer progression through lysyl oxidase and extracellular matrix remodeling. *Proc. Natl. Acad. Sci. U. S. A.* (2010). doi:10.1073/pnas.1004952107
52. Upadhyay, S. *et al.* LKB1/STK11 suppresses cyclooxygenase-2 induction and cellular invasion through PEA3 in lung cancer. *Cancer Res.* (2006). doi:10.1158/0008-5472.CAN-05-2902
53. Hardie, D. G. Molecular pathways: Is AMPK a friend or a foe in cancer? *Clin. Cancer Res.* (2015). doi:10.1158/1078-0432.CCR-14-3300
54. Hardie, D. G., Ross, F. A. & Hawley, S. A. AMPK: A nutrient and energy sensor that maintains energy homeostasis. *Nature Reviews Molecular Cell Biology* (2012). doi:10.1038/nrm3311
55. O'Neill, H. M., Holloway, G. P. & Steinberg, G. R. AMPK regulation of fatty acid metabolism and mitochondrial biogenesis: Implications for obesity. *Molecular and Cellular Endocrinology* (2013). doi:10.1016/j.mce.2012.06.019
56. Carling, D. The AMP-activated protein kinase cascade - A unifying system for energy control. *Trends in Biochemical Sciences* (2004). doi:10.1016/j.tibs.2003.11.005
57. Gowans, G. J., Hawley, S. A., Ross, F. A. & Hardie, D. G. AMP is a true physiological regulator of amp-activated protein kinase by both allosteric activation and enhancing net phosphorylation. *Cell Metab.* (2013). doi:10.1016/j.cmet.2013.08.019
58. Carling, D., Sanders, M. J. & Woods, A. The regulation of AMP-activated protein kinase by upstream kinases. *International Journal of Obesity* (2008). doi:10.1038/ijo.2008.124
59. Sanders, M. J., Grondin, P. O., Hegarty, B. D., Snowden, M. A. & Carling, D. Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *Biochem. J.* (2007). doi:10.1042/BJ20061520
60. Suter, M. *et al.* Dissecting the role of 5′-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. *J. Biol. Chem.* (2006). doi:10.1074/jbc.M606357200
61. Stein, S. C., Woods, A., Jones, N. A., Davison, M. D. & Cabling, D. The regulation of AMP-activated protein kinase by phosphorylation. *Biochem. J.* (2000). doi:10.1042/0264-6021:3450437
62. Cheung, P. C. F., Salt, I. P., Davies, S. P., Hardie, D. G. & Carling, D. Characterization of AMP-activated protein kinase γ-subunit isoforms and their role in AMP binding. *Biochem. J.* (2000). doi:10.1042/0264-6021:3460659

63. Davies, S. P., Helps, N. R., Cohen, P. T. W. & Hardie, D. G. 5′-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2Cα and native bovine protein phosphatase-2Ac. *FEBS Lett.* (1995). doi:10.1016/0014-5793(95)01368-7
64. Momcilovic, M. & Shackelford, D. B. Targeting LKB1 in cancer-exposing and exploiting vulnerabilities. *British Journal of Cancer* (2015). doi:10.1038/bjc.2015.261
65. Gwinn, D. M. *et al.* AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. *Mol. Cell* (2008). doi:10.1016/j.molcel.2008.03.003
66. Holz, M. K., Ballif, B. A., Gygi, S. P. & Blenis, J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* (2005). doi:10.1016/j.cell.2005.10.024
67. Porstmann, T. *et al.* SREBP Activity Is Regulated by mTORC1 and Contributes to Akt-Dependent Cell Growth. *Cell Metab.* (2008). doi:10.1016/j.cmet.2008.07.007
68. Denko, N. C. Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nature Reviews Cancer* (2008). doi:10.1038/nrc2468
69. Keith, B. & Simon, M. C. Hypoxia-Inducible Factors, Stem Cells, and Cancer. *Cell* (2007). doi:10.1016/j.cell.2007.04.019
70. Thomas, G. V. *et al.* Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. *Nat. Med.* (2006). doi:10.1038/nm1337
71. Sabharwal, S. S. & Schumacker, P. T. Mitochondrial ROS in cancer: Initiators, amplifiers or an Achilles' heel? *Nature Reviews Cancer* (2014). doi:10.1038/nrc3803
72. Emerling, B. M. *et al.* Hypoxic activation of AMPK is dependent on mitochondrial ROS but independent of an increase in AMP/ATP ratio. *Free Radic. Biol. Med.* (2009). doi:10.1016/j.freeradbiomed.2009.02.019
73. Nagata, D., Mogi, M. & Walsh, K. AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress. *J. Biol. Chem.* (2003). doi:10.1074/jbc.M300643200
74. Jeon, S. M., Chandel, N. S. & Hay, N. AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. *Nature* (2012). doi:10.1038/nature11066
75. Yan, M. *et al.* The tumor suppressor folliculin regulates AMPK-dependent metabolic transformation. *J. Clin. Invest.* (2014). doi:10.1172/JCI71749
76. Possik, E. *et al.* Folliculin Regulates Ampk-Dependent Autophagy and Metabolic Stress Survival. *PLoS Genet.* (2014). doi:10.1371/journal.pgen.1004273
77. Zhang, L., Li, J., Young, L. H. & Caplan, M. J. AMP-activated protein kinase regulates the assembly of epithelial tight junctions. *Proc. Natl. Acad. Sci. U. S. A.* (2006). doi:10.1073/pnas.0608531103
78. Zheng, B. & Cantley, L. C. Regulation of epithelial tight junction assembly and disassembly by AMP-activated protein kinase. *Proc. Natl. Acad. Sci. U. S. A.* (2007). doi:10.1073/pnas.0610157104
79. Lee, J. H. *et al.* Energy-dependent regulation of cell structure by AMP-activated protein kinase. *Nature* (2007). doi:10.1038/nature05828
80. Hardie, D. G. & Alessi, D. R. LKB1 and AMPK and the cancer-metabolism link - ten years after. *BMC Biology* (2013). doi:10.1186/1741-7007-11-36
81. Pineda, C. T. *et al.* Degradation of AMPK by a cancer-specific ubiquitin ligase. *Cell* (2015). doi:10.1016/j.cell.2015.01.034
82. Dupuy, F. *et al.* LKB1 is a central regulator of tumor initiation and pro-growth metabolism in ErbB2-mediated breast cancer. *Cancer Metab.* (2013). doi:10.1186/2049-3002-1-18
83. Shackelford, D. B. *et al.* mTOR and HIF-1α-mediated tumor metabolism in an LKB1 mouse model of Peutz-Jeghers syndrome. *Proc. Natl. Acad. Sci. U. S. A.* (2009). doi:10.1073/pnas.0900465106

84. Woods, A. *et al.* Ca2+/calmodulin-dependent protein kinase kinase-β acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab.* (2005). doi:10.1016/j.cmet.2005.06.005
85. Memmott, R. M. *et al.* Phosphatidylinositol ether lipid analogues induce AMP-activated protein kinase-dependent death in LKB1-mutant non-small cell lung cancer cells. *Cancer Res.* (2008). doi:10.1158/0008-5472.CAN-07-3091
86. Göransson, O. *et al.* Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. *J. Biol. Chem.* (2007). doi:10.1074/jbc.M706536200
87. Momcilovic, M., Hong, S. P. & Carlson, M. Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. *J. Biol. Chem.* (2006). doi:10.1074/jbc.M604399200
88. Gao, J. *et al.* Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci. Signal.* (2013). doi:10.1126/scisignal.2004088
89. Cerami, E. *et al.* The cBio Cancer Genomics Portal: An open platform for exploring multidimensional cancer genomics data. *Cancer Discov.* (2012). doi:10.1158/2159-8290.CD-12-0095
90. Miyamoto, T. *et al.* Compartmentalized AMPK Signaling Illuminated by Genetically Encoded Molecular Sensors and Actuators. *Cell Rep.* (2015). doi:10.1016/j.celrep.2015.03.057
91. Sakamoto, K. *et al.* Deficiency of LKB1 in heart prevents ischemia-mediated activation of AMPKα2 but not AMPKα1. *Am. J. Physiol. - Endocrinol. Metab.* (2006). doi:10.1152/ajpendo.00443.2005
92. Suzuki, A. *et al.* Identification of a novel protein kinase mediating Akt survival signaling to the ATM protein. *J. Biol. Chem.* (2003). doi:10.1074/jbc.M206025200
93. Suzuki, A. *et al.* ARK5 suppresses the cell death induced by nutrient starvation and death receptors via inhibition of caspase 8 activation, but not by chemotherapeutic agents or UV irradiation. *Oncogene* (2003). doi:10.1038/sj.onc.1206899
94. Suzuki, A. *et al.* Regulation of caspase-6 and FLIP by the AMPK family member ARK5. *Oncogene* (2004). doi:10.1038/sj.onc.1207963
95. Suzuki, A. *et al.* ARK5 Is a Tumor Invasion-Associated Factor Downstream of Akt Signaling. *Mol. Cell. Biol.* (2004). doi:10.1128/mcb.24.8.3526-3535.2004
96. Kusakai, G., Suzuki, A., Ogura, T., Kaminishi, M. & Esumi, H. Strong association of ARK5 with tumor invasion and metastasis. *J. Exp. Clin. Cancer Res.* (2004).
97. Kusakai, G. I. *et al.* ARK5 Expression in Colorectal Cancer and Its Implications for Tumor Progression. *Am. J. Pathol.* (2004). doi:10.1016/S0002-9440(10)63186-0
98. Roh, S. A. *et al.* Growth and invasion of sporadic colorectal adenocarcinomas in terms of genetic change. *J. Korean Med. Sci.* (2010). doi:10.3346/jkms.2010.25.3.353
99. Liu, L. *et al.* Deregulated MYC expression induces dependence upon AMPK-related kinase 5. *Nature* (2012). doi:10.1038/nature10927
100. Cui, J. *et al.* Overexpression of ARK5 is associated with poor prognosis in hepatocellular carcinoma. *Tumor Biol.* (2013). doi:10.1007/s13277-013-0735-x
101. Lu, S. *et al.* ARK5 promotes glioma cell invasion, and its elevated expression is correlated with poor clinical outcome. *Eur. J. Cancer* (2013). doi:10.1016/j.ejca.2012.09.018
102. Suzuki, A. *et al.* ARK5 is transcriptionally regulated by the Large-MAF family and mediates IGF-1-induced cell invasion in multiple myeloma: ARK5 as a new molecular determinant of malignant multiple myeloma. *Oncogene* (2005). doi:10.1038/sj.onc.1208844
103. Zagórska, A. *et al.* New roles for the LKB1-NUAK pathway in controlling myosin phosphatase complexes and cell adhesion. *Sci. Signal.* (2010). doi:10.1126/scisignal.2000616
104. Humbert, N. *et al.* Regulation of ploidy and senescence by the AMPK-related kinase NUAK1. *EMBO J.* (2010). doi:10.1038/emboj.2009.342

105. Hou, X. *et al.* A new role of NUAK1: Directly phosphorylating p53 and regulating cell proliferation. *Oncogene* (2011). doi:10.1038/onc.2011.19
106. Lefebvre, D. L. & Rosen, C. F. Regulation of SNARK activity in response to cellular stresses. *Biochim. Biophys. Acta - Gen. Subj.* (2005). doi:10.1016/j.bbagen.2005.03.015
107. Suzuki, A. *et al.* Induction of cell-cell detachment during glucose starvation through F-actin conversion by SNARK, the fourth member of the AMP-activated protein kinase catalytic subunit family. *Biochem. Biophys. Res. Commun.* (2003). doi:10.1016/j.bbrc.2003.09.184
108. Legembre, P., Schickel, R., Barnhart, B. C. & Peter, M. E. Identification of SNF1/AMP kinase-related kinase as an NF-κB- regulated anti-apoptotic kinase involved in CD95-induced motility and invasiveness. *J. Biol. Chem.* (2004). doi:10.1074/jbc.M404334200
109. Namiki, T. *et al.* AMP kinase-related kinase NUAK2 affects tumor growth, migration, and clinical outcome of human melanoma. *Proc. Natl. Acad. Sci. U. S. A.* (2011). doi:10.1073/pnas.1007694108
110. Emmanuel, C. *et al.* Comparison of expression profiles in ovarian epithelium in vivo and ovarian cancer identifies novel candidate genes involved in disease pathogenesis. *PLoS One* (2011). doi:10.1371/journal.pone.0017617
111. Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E. M. & Mandelkow, E. MARK, a novel family of protein kinases that phosphorylate microtubule- associated proteins and trigger microtubule disruption. *Cell* (1997). doi:10.1016/S0092-8674(00)80208-1
112. Biernat, J. *et al.* Protein kinase MARK/PAR-1 is required for neurite outgrowth and establishment of neuronal polarity. *Mol. Biol. Cell* (2002). doi:10.1091/mbc.02-03-0046
113. Sun, T. Q. *et al.* PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. *Nat. Cell Biol.* (2001). doi:10.1038/35083016
114. Goodwin, J. M. *et al.* An AMPK-independent signaling pathway downstream of the LKB1 tumor suppressor controls snail1 and metastatic potential. *Mol. Cell* (2014). doi:10.1016/j.molcel.2014.06.021
115. Kojima, Y. *et al.* Suppression of tubulin polymerization by the LKB1-microtubule-associated protein/microtubule affinity-regulating kinase signaling. *J. Biol. Chem.* (2007). doi:10.1074/jbc.M700590200
116. Sun, C. *et al.* Inactivation of MARK4, an AMP-activated protein kinase (AMPK)-related kinase, leads to insulin Hypersensitivity and resistance to diet-induced obesity. *J. Biol. Chem.* (2012). doi:10.1074/jbc.M112.388934
117. Hurov, J. B. *et al.* Loss of the Par-1b/MARK2 polarity kinase leads to increased metabolic rate, decreased adiposity, and insulin hypersensitivity in vivo. *Proc. Natl. Acad. Sci. U. S. A.* (2007). doi:10.1073/pnas.0701179104
118. Lennerz, J. K. *et al.* Loss of Par-1a/MARK3/C-TAK1 Kinase Leads to Reduced Adiposity, Resistance to Hepatic Steatosis, and Defective Gluconeogenesis. *Mol. Cell. Biol.* (2010). doi:10.1128/mcb.01472-09
119. Hollstein, P. E. *et al.* The AMPK-related kinases SIK1 and SIK3 mediate key tumor suppressive effects of LKB1 in NSCLC. *Cancer Discov.* CD-18-1261 (2019). doi:10.1158/2159-8290.cd-18-1261
120. Bardeesy, N. *et al.* The Kinase LKB1 Mediates Glucose Homeostasis in Liver and Therapeutic Effects of Metformin. *Science (80-.).* (2005).
121. Cheng, H. *et al.* SIK1 couples LKB1 to p53-dependent anoikis and suppresses metastasis. *Sci. Signal.* (2009). doi:10.1126/scisignal.2000369
122. Selvik, L. K. M. *et al.* Salt-inducible kinase 1 (sik1) is induced by gastrin and inhibits migration of gastric adenocarcinoma cells. *PLoS One* (2014). doi:10.1371/journal.pone.0112485
123. Patra, K. C. *et al.* Mutant GNAS drives pancreatic tumourigenesis by inducing PKA-mediated SIK suppression and reprogramming lipid metabolism. *Nat. Cell Biol.* (2018).

doi:10.1038/s41556-018-0122-3
124. Ahmed, A. A. *et al.* SIK2 Is a Centrosome Kinase Required for Bipolar Mitotic Spindle Formation that Provides a Potential Target for Therapy in Ovarian Cancer. *Cancer Cell* (2010). doi:10.1016/j.ccr.2010.06.018
125. Chen, H. *et al.* Salt-inducible kinase 3 is a novel mitotic regulator and a target for enhancing antimitotic therapeutic-mediated cell death. *Cell Death Dis.* (2014). doi:10.1038/cddis.2014.154
126. Tarumoto, Y. *et al.* LKB1, Salt-Inducible Kinases, and MEF2C Are Linked Dependencies in Acute Myeloid Leukemia. *Mol. Cell* (2018). doi:10.1016/j.molcel.2018.02.011
127. Bon, H. *et al.* Salt-inducible kinase 2 regulates mitotic progression and transcription in prostate cancer. *Mol. Cancer Res.* (2015). doi:10.1158/1541-7786.MCR-13-0182-T
128. Murray, C. W. *et al. An Lkb1-Sik axis suppresses lung tumor growth and controls differentiation*. *Cancer Discovery* **175336**, (2019).
129. Kodaka, M. & Hata, Y. The mammalian Hippo pathway: Regulation and function of YAP1 and TAZ. *Cellular and Molecular Life Sciences* (2015). doi:10.1007/s00018-014-1742-9
130. Moroishi, T., Hansen, C. G. & Guan, K. L. The emerging roles of YAP and TAZ in cancer. *Nat. Rev. Cancer* (2015). doi:10.1038/nrc3876
131. Nguyen, H. B., Babcock, J. T., Wells, C. D. & Quilliam, L. A. LKB1 tumor suppressor regulates AMP kinase/mTOR-independent cell growth and proliferation via the phosphorylation of Yap. *Oncogene* (2013). doi:10.1038/onc.2012.431
132. Mohseni, M. *et al.* A genetic screen identifies an LKB1-MARK signalling axis controlling the Hippo-YAP pathway. *Nat. Cell Biol.* (2014). doi:10.1038/ncb2884
133. Mo, J. S. *et al.* Cellular energy stress induces AMPK-mediated regulation of YAP and the Hippo pathway. *Nat. Cell Biol.* (2015). doi:10.1038/ncb3111
134. Wang, W. *et al.* AMPK modulates Hippo pathway activity to regulate energy homeostasis. *Nat. Cell Biol.* (2015). doi:10.1038/ncb3113
135. deRan, M. *et al.* Energy stress regulates Hippo-YAP signaling involving AMPK-mediated regulation of angiomotin-like 1 protein. *Cell Rep.* (2014). doi:10.1016/j.celrep.2014.09.036
136. Monteverde, T., Muthalagu, N., Port, J. & Murphy, D. J. Evidence of cancer-promoting roles for AMPK and related kinases. *FEBS Journal* (2015). doi:10.1111/febs.13534
137. Kishi, M., Pan, Y. A., Crump, J. G. & Sanes, J. R. Mammalian SAD kinases are required for neuronal polarization. *Science (80-.).* (2005). doi:10.1126/science.1107403
138. Inoki, K., Zhu, T. & Guan, K. L. TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival. *Cell* (2003). doi:10.1016/S0092-8674(03)00929-2
139. Cantó, C. *et al.* AMPK regulates energy expenditure by modulating NAD + metabolism and SIRT1 activity. *Nature* (2009). doi:10.1038/nature07813
140. Winder, W. W. *et al.* Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. *J. Appl. Physiol.* (2000). doi:10.1152/jappl.2000.88.6.2219
141. Faubert, B. *et al.* AMPK is a negative regulator of the warburg effect and suppresses tumor growth in vivo. *Cell Metab.* (2013). doi:10.1016/j.cmet.2012.12.001
142. Heiden, M. G. V., Cantley, L. C. & Thompson, C. B. Understanding the warburg effect: The metabolic requirements of cell proliferation. *Science* (2009). doi:10.1126/science.1160809
143. Imamura, K., Ogura, T., Kishimoto, A., Kaminishi, M. & Esumi, H. Cell cycle regulation via p53 phosphorylation by a 5′-AMP activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, in a human hepatocellular carcinoma cell line. *Biochem. Biophys. Res. Commun.* (2001). doi:10.1006/bbrc.2001.5627
144. Jones, R. G. *et al.* AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol. Cell* (2005). doi:10.1016/j.molcel.2005.03.027
145. Fogarty, S. *et al.* AMPK causes cell cycle arrest in LKB1-deficient cells via activation of CAMKK2. *Mol. Cancer Res.* (2016). doi:10.1158/1541-7786.MCR-15-0479

146. Hawley, S. A. *et al.* Calmodulin-dependent protein kinase kinase-β is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab.* (2005). doi:10.1016/j.cmet.2005.05.009
147. Hurley, R. L. *et al.* The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. *J. Biol. Chem.* (2005). doi:10.1074/jbc.M503824200
148. Moriya, H. Quantitative nature of overexpression experiments. *Molecular Biology of the Cell* (2015). doi:10.1091/mbc.E15-07-0512

10 Acknowledgements

This project was conducted in the laboratory of Tomi Mäkelä at the University of Helsinki.

First, I would like to express my sincere gratitude to Tomi Mäkelä for granting me the opportunity to work in his laboratory, learn from great scientists and witness how a high-class scientific research is done.

I am extremely grateful to my direct supervisor Sushil Tripathi for his infinite patience and guidance; for covering me at the lab meetings; for his hospitality and jokes; finally, for being as much a supervisor as being a friend.

I want to thank all members of Mäkeläb for creating nice friendly working environment; for being helpful, understanding and professional. You are great, and I admire all of you!

Special thanks to my study coordinator Tiina Immonen for great support at all times, for wise advice and caring attitude.

I am grateful to my friends, old and new, for unforgettable moments that happened during this journey and for the warm memories that I will keep for the rest of my life.

Finally, I express my deepest gratitude and love to my family for their faith in me. Thank you for making me work harder and not give up. Thank you for staying by my side along this way. All of this could be hardly possible without you.