Differential regulation of AMP-activated protein kinase in healthy and cancer cells explains why V-ATPase inhibition selectively kills cancer cells

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The cellular energy sensor AMP-activated protein kinase (AMPK) is a metabolic hub regulating various pathways involved in tumor metabolism. Here we report that vacuolar H+-ATPase (V-ATPase) inhibition differentially affects regulation of AMPK in tumor and nontumor cells and that this differential regulation contributes to the selectivity of V-ATPase inhibitors for tumor cells. In nonmalignant cells, the V-ATPase inhibitor archazolid increased phosphorylation and lysosomal localization of AMPK. We noted that AMPK localization has a prosurvival role, as AMPK silencing decreased cellular growth rates. In contrast, in cancer cells, we found that AMPK is constitutively active and that archazolid does not affect its phosphorylation and localization. Moreover, V-ATPase-independent AMPK induction in tumor cells protected them from archazolid-induced cytotoxicity, furtherunderlining the role of AMPK as a prosurvival mediator. These observations indicate that AMPK regulation is uncoupled from V-ATPase activity in cancer cells and that this makes them more susceptible to cell death induction by V-ATPase inhibitors. In both tumor and healthy cells, V-ATPase inhibition induced a distinct metabolic regulatory cascade downstream of AMPK, affecting ATP and NADPH levels, glucose uptake, and reactive oxygen species production. We could attribute the prosurvival effects to AMPK's ability to maintain redox homeostasis by inhibiting reactive oxygen species production and maintaining NADPH levels. In summary, the results of our work indicate that V-ATPase inhibition has differential effects on AMPK-mediated metabolic regulation in cancer and healthy cells and explain the tumor-specific cytotoxicity of V-ATPase inhibition.

In the 1920s Otto Warburg discovered that tumor cells have an altered metabolism by using glycolysis as the main energy source even in the presence of oxygen, called aerobic glycolysis or the Warburg effect. Since then, many modifications in oncogenes and tumor suppressors, like HIF1α, Akt, Ras, and p53, have been directly connected to regulation of the expression and activity of important components of tumor metabolism, presenting tumor metabolism as one hallmark of cancer (1). Despite ongoing research to identify the different aspects of cancer metabolism, the metabolic alterations that are critical for tumor progression remain largely unknown.

Recently, the highly conserved energy sensor AMP-activated protein kinase (AMPK)2 came into focus as a metabolic hub regulating many different pathways involved in tumor metabolism. It belongs to a family of serine/threonine kinases and consists of a catalytic α subunit and regulatory β and γ subunits (2). AMPK is activated by a variety of metabolic or oncogenic stress conditions, like nutrient starvation or hypoxia, and directs the cell toward metabolic changes that produce ATP and restore energy homeostasis (3). By sensing the AMP:ATP ratio, it can increase catabolic processes that generate ATP, like fatty acid oxidation and glycolysis, and inhibit anabolic processes that consume ATP, such as protein and lipid synthesis. Based on the finding that LKB1, the upstream kinase activating AMPK, is frequently inactivated in tumor cells, AMPK has historically been proposed as a tumor suppressor. This assumption was confirmed by showing that genetic loss of AMPK accelerates tumor growth in an experimental model of lymphangiomia and by the fact that pharmacological activation of AMPK leads to growth inhibition of several tumor cell lines (4, 5). However, accumulating evidence shows that AMPK has a dual role in cancer and can have a pro-tumorigenic role, as shown for different tumors, especially under stress conditions like glucose deprivation or oxidative stress (6, 7).

Interestingly, it was recently discovered that the vacuolar H+-ATPase (V-ATPase) is needed for activation of AMPK during glucose starvation by forming a complex with the scaffolding protein AXIN1 and LKB1 (8). V-ATPase is a highly conserved multisubunit proton pump located at endolysosomal membranes of most eukaryotic cells. It is responsible for maintaining pH homeostasis and essential for intracellular trafficking and receptor recycling (9). It has been introduced as a promising antitumor target, as inhibition of V-ATPase by several

1 The abbreviations used are: AMPK, AMP-activated protein kinase; V-ATPase, vacuolar H+-ATPase; ACC, acetyl-CoA carboxylase; ROS, reactive oxygen species; arch, archazolid; AICAR, 5-aminooimidazole-4-carboxamide ribonucleotide.

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Figure 1. V-ATPase inhibition differentially regulates AMPK activation. A and B, the tumor cell lines MCF7, MDA-MB-231 (MDA), and T24 (A) and the nontumor cell lines HEK293, MCF10A, and HMLE (B) were treated with 10 nM archazolid for 24 h, and phosphorylation of AMPK at Thr-172 and total AMPK protein were analyzed by Western blotting. C, HEK293, MCF-7 and MDA-MB-231 were incubated in glucose-free medium for 24 h, and phosphorylation of AMPK was analyzed. The whole-protein level (LC) served as a loading control. D, phosphorylation of AMPK was analyzed in patient-derived breast cancer tissue (T) and normal breast tissue (N) from the same patient. One representative picture of three independent experiments is shown.

In this study, we used archazolid as a highly potent tool to specifically block V-ATPase and found a differential effect on AMPK activation in tumor and nontumor cells that results in different metabolic regulation and sensitivity to apoptosis induction. In nontumor cells, AMPK is mostly inactive. Treatment with archazolid, however, led to profound activation of AMPK with a protective effect against oxidative stress induced by the drug. Tumor cells, on the contrary, showed constitutive activation of AMPK irrespective of archazolid treatment, but V-ATPase–independent activation of AMPK also protected them of AMPK-mediated protection and rendering them more sensitive to cytotoxicity induced by V-ATPase inhibitors. Hence, distinct AMPK regulation in cancer and nonmalignant cells accounts for the tumor cell specificity of V-ATPase inhibitors.

Results

V-ATPase inhibition activates AMPK in nontumor cells

To test whether inhibition of V-ATPase leads to AMPK activation, we treated different tumor (MDA-MB-231, MCF7, T24, and HUH7) and nontumor (HEK293, MCF10A, and HMLE) cells with archazolid and analyzed phosphorylation of AMPK on Thr-172. We found that all tumor cells had constitutively activated AMPK and that archazolid had no effect on the activation level (Fig. 1A and Fig. S1A). Astonishingly, however, V-ATPase inhibition led to profound activation of AMPK in all nontumor cells, which typically showed low basal AMPK activation (Fig. 1B and Fig. S1B). To test whether this effect is specific for V-ATPase inhibition or a general stress-induced effect, we starved the cells of glucose and subsequently analyzed AMPK activation. As shown in Fig. 1C and Fig. S1C, there was activation of AMPK in all tested cell lines and no difference between tumor and nontumor cells. These results suggest that the differential effect seen on AMPK phosphorylation is not induced by a general stress response but specific for V-ATPase inhibition. To further confirm archazolid-induced AMPK activation, we analyzed phosphorylation of acetyl-CoA carboxylase (ACC). As a downstream target of AMPK, ACC is a good marker for AMPK activity. Here we could show the same effect as with AMPK: activation in nontumor HEK293 cells and no change in MDA-MB-231 cells (Fig. S2). To make sure that V-ATPase was inhibited in all cell lines to the same extent, we used a pH-sensitive Lysotracker and could see a similar effect with archazolid treatment (Fig. S3). To further analyze AMPK status in tumor and nontumor tissue, we used patient-derived breast tumor samples and normal breast tissue from the same patients, respectively, and analyzed the activity of AMPK via Western blotting. Interestingly, we found that tumor cells had an increased level of total AMPK and also a slightly higher level of phosphorylated AMPK (Fig. 1D and Fig. S1D), indicating that AMPK activation is beneficial for cancer cells.

Involvement of V-ATPase inhibition in AMPK activation

It is known that V-ATPase interacts with the scaffolding protein AXIN1 in nontumor cells starved of glucose at the lysosome and that this interaction is required to activate AMPK (8). Therefore, we tested whether archazolid leads to interaction of V-ATPase and AXIN1 in tumor cells. We found that treatment with archazolid leads to increased interaction of AXIN1 and V-ATPase in the nontumor cell line HEK293 but not in the breast cancer cell line MDA-MB-231 (Fig. 2A). A similar effect could be shown for colocalization of AXIN1 and lysosomes (Fig. S4). Furthermore, using lysosomal fractionation, we could show that AMPK is increasingly located at the lysosome in archazolid-treated HEK293 cells but not in MDA-MB-231 cells (Fig. 2B). For AMPK activation, it is essential that LKB1 is also increasingly located at the lysosome. Finally, we found that archazolid leads to profound activation of AMPK in all nontumor cells, which typically showed low basal AMPK activation (Fig. 1B and Fig. S1B). To test whether this effect is specific for V-ATPase inhibition or a general stress-induced effect, we starved the cells of glucose and subsequently analyzed AMPK activation. As shown in Fig. 1C and Fig. S1C, there was activation of AMPK in all tested cell lines and no difference between tumor and nontumor cells. These results suggest that the differential effect seen on AMPK phosphorylation is not induced by a general stress response but specific for V-ATPase inhibition. To further confirm archazolid-induced AMPK activation, we analyzed phosphorylation of acetyl-CoA carboxylase (ACC). As a downstream target of AMPK, ACC is a good marker for AMPK activity. Here we could show the same effect as with AMPK: activation in nontumor HEK293 cells and no change in MDA-MB-231 cells (Fig. S2). To make sure that V-ATPase was inhibited in all cell lines to the same extent, we used a pH-sensitive Lysotracker and could see a similar effect with archazolid treatment (Fig. S3). To further analyze AMPK status in tumor and nontumor tissue, we used patient-derived breast tumor samples and normal breast tissue from the same patients, respectively, and analyzed the activity of AMPK via Western blotting. Interestingly, we found that tumor cells had an increased level of total AMPK and also a slightly higher level of phosphorylated AMPK (Fig. 1D and Fig. S1D), indicating that AMPK activation is beneficial for cancer cells.

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to transfer of LKB1 to the lysosome in HEK293 cells, in contrast to tumor cells, where no difference between treated and nontreated cells could be observed, as shown by confocal microscopy (Fig. 2C). These results further suggest that there is a difference in AMPK activation by V-ATPase inhibition in tumor and nontumor cells.

V-ATPase inhibition alters AMPK-related metabolic parameters

AMPK is activated because of an increased ratio of AMP:ATP. Therefore, we analyzed the effect of V-ATPase inhibition on ATP content. We found that tumor cells were only slightly affected, but the nontumor cell lines HEK293 and MCF10A showed a significant reduction in ATP levels after archazolid treatment, as shown by a luminescence assay (Fig. 3A). Furthermore, classical AMPK activation is induced by an increased level of AMP. Therefore, we analyzed changes in AMP by HPLC (Fig. S5). Interestingly, we could not find a clear increase in either nontumor HEK293 cells or in MDA-MB-231 cells, which might indicate a different activation mode by V-ATPase inhibition. However, we found increased glucose uptake and slight but nonsignificant up-regulation of glucose receptor 1 (GLUT1) in HEK293 cells, which can be a consequence of AMPK activation. In contrast, the tumor cell lines showed only a slight effect (MDA-MB-231) or even a decrease (MCF7) in glucose uptake (Fig. 3, B and C), indicating distinct metabolic regulation of tumor and nontumor cells by V-ATPase inhibition.

AMPK activation protects nontumor cells from archazolid-induced cytotoxicity

As AMPK activation is quite controversially discussed as either a tumor suppressor or oncogene, we analyzed whether archazolid-induced AMPK phosphorylation is prosurvival or proapoptotic. Therefore, we silenced AMPK in HEK293 cells (Fig. 4B) and treated the cells with archazolid. We found that HEK293 cells with silenced AMPK had a decreased growth rate after archazolid treatment compared with control cells (Fig. 4A), which suggests a protective role for AMPK activity. If AMPK activation protects cells from archazolid induced cytotoxicity, then further inducing AMPK in tumor cells should decrease archazolid-induced apoptosis. Therefore, we treated MDA-MB-231 cells with the AMP analog AICAR in combination with archazolid, which increased phosphorylation of AMPK (Fig. 4C and Fig. S6A). Interestingly, we found that apoptosis induction was significantly reduced by combining archazolid with AICAR compared with archazolid-treated cells alone (Fig. 4D). This was accompanied by decreased cleavage of PARP-1, confirming decreased apoptosis induction and a protective role for AMPK (Fig. 4E and Fig. S6B). To confirm that this effect is really AMPK-dependent, we repeated the experiment with silenced AMPK and found that AICAR does not protect MDA-MB-231 cells from archazolid-induced apoptosis, confirming the protective role of AMPK (Fig. 4F and Fig. S6C).

The prosurvival role of AMPK results from different stress responses and maintaining redox homeostasis

As one known prosurvival role of AMPK is the control of redox homeostasis by preventing ROS accumulation and maintaining NADPH levels, we analyzed whether archazolid treatment leads to disturbance of redox homeostasis. As shown in Fig. 5A, V-ATPase inhibition led to induction of ROS in tumor cells but a decrease in ROS in nontumor cells. Furthermore, archazolid led to an increase in the NAPD/NDPH ratio in HEK293 cells, whereas MDA-MB-231 cells showed a decreased ratio (Fig. 5B), suggesting a disturbed redox balance in tumor cells. Activation of AMPK keeps the redox balance by decreasing fatty acid synthesis (7). Therefore, we analyzed the transcription of fatty acid synthase (FAS) and Stearyl-CoA-Desaturase 1 (SCD1), two major players in the fatty acid synthesis.
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Figure 3. V-ATPase inhibition differentially regulates metabolic parameters. The indicated tumor and nontumor cell lines were treated with 10 nM archazolid for 24 h or left untreated. A, ATP levels were measured by luminescence. B, glucose uptake was determined using the fluorescent glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose and subsequent FACS analysis. C, glucose receptor expression was analyzed by Western blotting. Error bars are the S.E. of three independent experiments. *, p < 0.05, Student’s t test. ns, not significant.

Figure 4. Nontumor cells are protected from arch-induced apoptosis via AMPK activation. HEK293 cells were transfected with siRNA against AMPK (siAMPK) or nontargeting siRNA (siNT). 48 h after transfection, cells were treated with 10 nM arch or left untreated. A, after 48 h of incubation, cytotoxicity was measured via cellTiter Blue assay. B, a representative Western blot showing down-regulation of AMPK. C, MDA-MB-231 cells were treated for 24 h with 10 nM arch, 0.5 mM AICAR, or a combination, and phosphorylation of AMPK was determined by Western blotting. D, MDA-MB-231 cells were treated for 72 h with 10 nM arch, 0.5 mM AICAR, or a combination, and apoptosis induction was analyzed by propidium iodide staining and flow cytometry. E, PARP-1 cleavage was determined by Western blotting after 48 h of treatment. F, MDA-MB-231 cells were transfected with siNT or siAMPK. 48 h after transfection, cells were treated as indicated, and PARP-1 cleavage was determined by Western blotting. One representative image of three independent experiments is shown. Quantification represents three different experiments. Error bars are the S.E. of three independent experiments. *, p < 0.05 (Student’s t test).
pathway, in tumor and nontumor cells. Interestingly, we found that archazolid treatment led to strong up-regulation of both enzymes in tumor cells but not in nontumor cells (Fig. 5C). To connect these effects to the prosurvival effect of AMPK, we treated MDA-MB-231 cells with the ROS scavenger tiron and analyzed apoptosis induction (Fig. 5D). We found that combination of archazolid with tiron decreased apoptosis induction. Further supporting this hypothesis is that the combination of AICAR and archazolid decreases ROS production in MDA-MB-231 cells (Fig. 5E). Finally, silencing AMPK in HEK293 cells led to up-regulation of ROS after archazolid treatment, confirming that AMPK activation is responsible for protection from ROS-induced apoptosis via V-ATPase inhibition (Fig. 5F).

Discussion

This work provides evidence that V-ATPase inhibition by archazolid leads to differential metabolic regulation in tumor and nontumor cells, which results in increased sensitivity of tumor cells to the treatment. Our major findings are that V-ATPase inhibition leads to AMPK activation only in healthy cells, which protects them from archazolid-induced cytotoxicity. In tumor cells, as depicted in Fig. 6, this protection is missing, which results in increased apoptosis induction caused by a distinct effect on the downstream AMPK cascade, including ATP, glucose uptake, NADPH level, and ROS production. As these effects could be abrogated by activating AMPK in tumor cells independent of V-ATPase, a novel role of AMPK in V-ATPase inhibition–induced cytotoxicity was unraveled. This provides new and interesting insights into the regulation of stress responses in different cell types and a better understanding of the mechanism of action of V-ATPase inhibition regarding metabolism.

One major problem in cancer therapy is to find compounds that specifically target cancer cells and leave nonmalignant cells unaffected, minimizing toxic side effects and making chemo-
therapy more bearable for cancer patients. Intensive research has led to the introduction of several compounds that target, e.g., growth factor receptors that are overexpressed in tumors, like cetuximab (EGF-receptor) (14), trastuzumab (HER2) (15), or imatinib, which targets the bcr-abl tyrosine-kinase a tumor-specific mutation (16). Despite the success experienced with these compounds, selectivity of chemotherapeutic agents is still a major challenge.

V-ATPase is a promising antitumor target, and its inhibition has been shown to have a pronounced effect on tumor cells but leave nontumor cells almost unaffected, as we and others have shown (11, 17). We even introduced more tumor and nontumor cells lines to prove a clear difference in sensitivity toward V-ATPase inhibition in tumor versus nontumor cells (Fig. S7). The antitumor effect of V-ATPase inhibition was mostly attributed to disturbance of endocytotic recycling processes, and several publications by our group and others elucidated several underlying mechanisms (11, 13, 18, 19). In recent years, however, it became obvious that V-ATPase also plays a pivotal role in nutrient sensing. Zoncu et al. (12) showed that V-ATPase is required for mTOR-mediated amino acid sensing at the lysosome by building a complex between mTORC1, V-ATPase, and Ragulator. Interestingly, Zhang et al. (8) found that V-ATPase is not only required for mTOR regulation but also for AMPK activation under glucose deprivation. Their group impressively showed that the V-ATPase–Ragulator complex serves as a docking site for LKB1-mediated AMPK activation with the aid of the scaffolding protein AXIN1 by forming the V-ATPase–Ragulator/LKB1–AMPK complex at the lysosome (8). Finally, McGuire et al. (20) showed that glucose starvation leads to increased V-ATPase assembly preceded by AMPK activation. These findings strengthen the hypothesis that V-ATPase is involved in metabolic regulation of the cell.

Zhang et al. (8) showed that intact V-ATPase is needed to build the complex of LKB1/AXIN1/AMPK and V-ATPase and that inhibiting V-ATPase with concanamycin mimicks glucose starvation and therefore activates AMPK in HEK293 cells. It has also been shown that another V-ATPase inhibi-
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AMPK has long been seen as an anti-tumor target that originated from discovery of the AMPK upstream kinase LKB1, whose loss results in development of Peutz–Jeghers syndrome, which is accompanied by increased cancer risk (25). Furthermore, the use of often nonspecific AMPK activators has been shown to inhibit tumor growth in a variety of studies (26–28). However, in recent years it became obvious that AMPK activation can very well be tumorigenic and can even be essential for tumor progression, as shown for glioblastoma, prostate cancer (22, 23, 30), or myc-overexpressing tumors (31). In that regard, most data showing a tumor-suppressive role of AMPK rely on pharmacological studies, whereas experiments using genetic tools mostly support a pro-survival role of AMPK. Several oncogenes, like Sre and Myc, have been shown to activate AMPK (31, 32), and silencing an AMPK subunit impairs the ability of cells to form tumors in vivo (33). In addition, loss of the tumor suppressor folliculin also activates AMPK (34). All of these data suggest a context-specific role of AMPK activity.

Intriguingly, we found that silencing AMPK1α in HEK293 cells treated with archazolid lead to a decreased growth rate compared with WT cells, suggesting a pro-survival function of AMPK activation. Moreover, increasing AMPK activity in MDA-MB-231 breast cancer cells with the AMPK activator AICAR protected the cells from archazolid-induced cytotoxicity, an effect that could be diminished by silencing AMPK1α. Hence, our findings support a pro-survival role of AMPK activation after V-ATPase inhibition. Importantly, treating nontumor cells with a combination of archazolid and AICAR had no effect on apoptosis induction (Fig. S8).

After showing that induction of AMPK by archazolid has a prosurvival effect, we wanted to elucidate the mechanism behind it. AMPK leads to a variety of metabolic changes facilitating survival during periods of poor nutrition or other metabolic stresses. The best known prosurvival functions of AMPK activation are autophagy, macromolecule import, redox homeostasis, and enhanced glycolysis (35–37). In regard to glucose metabolism, we found increased glucose uptake and slightly higher GLUT1 expression selectively in nontumor cells and unvarying or even decreased glucose uptake in tumor cells, which correlates with differential activation of AMPK upon V-ATPase inhibition. This is in agreement with the data of others showing that AMPK-dependent increased uptake of glucose and glucose receptor expression promotes breast cancer growth (38) and protection from oxidative stress (39). Concerning redox homeostasis, Jeon et al. (7) showed that AMPK activation protects from ROS production and maintains NADPH levels constant during glucose deprivation, leading to cell survival, evidence that AMPK activation promotes cell survival during energy stress. Additionally, AMPK has been shown to be responsible for the antioxidant effects of resveratrol (40) and essential for redox balance in leukemia cells, where AMPK inhibition results in reduced leukemia progression (41). Increased ROS levels have been reported to be a feature of V-ATPase inhibition in tumor cells (42, 43), which we could confirm in this study for MDA-MB-231 and MCF-7 cells using archazolid as a V-ATPase inhibitor. ROS production in nontumor cells, on the other hand, was not affected. These differences were accompanied by decreased NADPH/NADP levels and synthesis of two major enzymes of lipid synthesis selectively in tumor cells. Importantly, the archazolid-induced disturbance of redox homeostasis was overcome by AMPK activation in nontumor cells, whereas tumor cells failed to activate AMPK upon V-ATPase inhibition. Similar effects have also been reported for Myc-positive melanoma, where AMPK activation could protect the cells from ROS-induced apoptosis (44). We found that scavenging ROS with tiron decreased archazolid-induced apoptosis in tumor cells, and combination with AICAR decreased ROS production, whereas AMPK silencing resulted in increased ROS production after V-ATPase inhibition, even in nontumor cells. This points to the importance of redox homeostasis maintained by AMPK under metabolic stress, which is important for alleviating the cytotoxic effects of V-ATPase inhibition.

We propose that V-ATPase inhibition by archazolid leads to a stress response in nontumor cells, in which AMPK sustains cellular homeostasis, like energy supply and redox homeostasis. In tumor cells, however, V-ATPase does not lead to activation of AMPK-mediated metabolic changes, supposedly because of defective regulation of AMPK, which makes the cells more vulnerable to V-ATPase-induced metabolic stress (Fig. 6). These results demonstrate the significance of V-ATPase for tumor metabolism, give first insights into the mechanisms of tumor-specific effects of V-ATPase inhibition, and underline the importance of understanding the mechanistic differences of tumor and nontumor cells.
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Experimental procedures

Cell culture and compounds

MDA-MD-231, HEK293, T24, and MCF-7 cells were obtained from deutsche sammlung von mikroorganismen und zellkulturen (DSMZ) (Heidelberg, Germany), and MDA-MB-231 and HEK293 cells were cultured in DMEM supplemented with 10% FCS. MCF-7 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, insulin, and 1% nonessential amino acids. MCF10A cells were purchased from the ATCC and cultured in DMEM–F12 supplemented with 5% horse serum, 100 mg/ml epidermal growth factor, 10 mg/ml insulin, 1 mg/ml hydrocortisone, 1 mg/ml cholera toxin, and 1% penicillin/streptavidin. HMLE cells were a kind gift from Dr. Christina Scheel (Helmholtz Center Munich) and cultured in mammary epithelial cell growth media (MECGM) obtained from PromoCell (Heidelberg, Germany).

Starvation experiments were performed in glucose-free DMEM supplemented with 10% dialyzed FCS. Archazolid was provided by Prof. Dirk Menche (University of Bonn). AICAR was purchased from Tocris Bioscience (Bristol, UK).

Patient-derived breast tumor samples and healthy breast tissue samples were a kind gift from the nonprofit organization PATH Biobank (Augsburg, Germany) (45). Collection of samples was approved by the local ethics committee in Bonn, and all patients provided written informed consent. Sample collection was carried out according to the Declaration of Helsinki.

Immunoblotting

Cells were lysed in a buffer containing 2 mM EDTA, 137 mM NaCl, 10% glycerol, 2 mM Na2HPO4, 20 mM Tris base, 1% Triton X-100, and 20 mM Na-glycerophosphate (pH 7.5). For immunoprecipitation, cells were lysed and incubated overnight with an antibody against AXIN1. Subsequently, the lysate was incubated with Protein A–agarose beads (Sigma-Aldrich, Taufkirchen, Germany) and washed with lysate buffer. Then the homogenate was centrifuged. Next, the supernatant was discarded, and the pellet was resuspended in 1 volume of washing buffer and used for Western blot analysis.

Transfection

Cells were transfected with GenaxxonFect (Genaxxon Bioscience, Münster, Germany) according to the manufacturer’s instructions. Briefly, AMPKα1 was silenced using ON-TARGET-Plus SMARTpool siRNA from Dharmacon (Schwete, Germany) and nontargeting siRNA as a control and treated as indicated 48 h after transfection.

Immunocytochemistry

Cells were seeded on microslides (8-well ibidiTreat, IBIDI, Martinsried, Germany) and treated as indicated. Cells were fixed and stained as described before (48) and analyzed with an LSM 510 Meta confocal microscope (Zeiss, Jena, Germany). The following antibodies were used: LKB1 (Cell Signaling Technology), Lamp1 (Developmental Studies Hybridoma Bank), and Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 543 goat anti-mouse (Invitrogen).

Apoptosis assay

Subdiploid DNA content was determined according to Nicoletti et al. (49). Briefly, cells were treated as indicated, harvested, permeabilized with sodium citrate containing Triton X-100, stained with 25 μg/ml propidium iodide, and analyzed by flow cytometry (BD Biosciences). Subdiploid cells to the left of the G1 peak were considered apoptotic.

Cytotoxicity assay

Cells were seeded in a 96-well plate and treated as indicated, and growth inhibition was analyzed with the CellTiter Blue assay (Promega, Madison, WI) according to the manufacturer’s instructions.

Quantitative real-time PCR analysis

Total mRNA was isolated from cell culture samples using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. For complementary DNA synthesis, a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) was used. qRT-PCR was performed with the AB 7300 real-time PCR system, TaqMan Gene Expression Master Mix (Applied Biosystems), and SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. All designed primers were purchased from Metabion (Martinsried, Germany).
**Glucose uptake**

Cells were treated as indicated. Subsequently, cells were incubated with 100 μM 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (Invitrogen) in Hanks’ buffer for 30 min at 37 °C. Then cells were harvested, and changes in glucose uptake were measured by flow cytometry.

**ROS measurement**

Cells were treated as indicated, harvested, and incubated with 10 μM 2’,7’-dichlorofluorescein diacetate (Sigma-Aldrich) for 30 min at 37 °C. After a washing step with PBS, reactive oxygen species production was analyzed by flow cytometry.

**ATP**

Cells were seeded in a 96-well plate and treated as indicated. ATP levels were determined using the CellTiter-Glo assay (Promega) according to the manufacturer’s instructions and measured in a luminometer (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer’s instructions.

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