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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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Running title: *Tumor-specific effect of V-ATPase inhibition*

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Abstract

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 non-tumor cells and that this differential regulation contributes to the selectivity of V-ATPase inhibitors for tumor cells. In non-malignant cells, the V-ATPase inhibitor archazolid increased phosphorylation and lysosomal localization of AMPK. We noted that AMPK localization has a pro-survival 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 the tumor cells protected them from archazolid-induced cytotoxicity, further underlining the role of AMPK as a pro-survival 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 (ROS) production. We could attribute the pro-survival effects to AMPK's ability to maintain redox homeostasis by inhibiting ROS 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.

Already in the 1920ies Otto Warburg discovered that tumor cells show an altered metabolism by using glycolysis as the main energy source even in the presence of oxygen, named aerobic glycolysis or the Warburg effect. Since then many modifications in oncogenes and tumor suppressors like HIF1alpha, Akt, Ras or p53 could be directly connected to regulate the expression and activity of important components of tumor metabolism, presenting tumor metabolism as one hallmark of cancer (1). But 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) came into focus as metabolic hub showing to regulate 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 towards 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 been historically proposed as a tumor suppressor. This assumption was confirmed by showing that genetic loss of AMPK accelerates tumor growth of an experimental model of lymphangioma 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). The 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 is essential for intracellular trafficking and receptor recycling (9). It has been introduced as a promising anti-tumor target as inhibition of the V-ATPase by several drugs like concanamycin or the myxobacterial compound archazolid leads to apoptosis induction, inhibition of migration and invasion in a variety of tumor cells *in vitro* and *in vivo* without affecting non-malignant cells (10,11). Furthermore, it was also revealed lately that the V-ATPase has additional functions besides just regulating pH and endocytosis. Apart from being involved in AMPK regulation it was shown to play a role in mTOR-mediated amino acid sensing and leads to an induction of glycolysis (12,13), revealing to play a role in metabolism.

The important role of V-ATPase for AMPK homeostasis and the controversial discussion of AMPK activity in tumor context prompted us to investigate the connection of V-ATPase inhibition and AMPK activation in tumor cell survival in detail.

In this study we used archazolid as a highly potent tool to specifically block the V-ATPase and found a differential effect on AMPK activation in tumor and non-tumor cells which results in different metabolic regulation and sensitivity to apoptosis induction. In non-tumor cells, AMPK is mostly inactive, treatment with archazolid however, lead to a profound activation of AMPK having a protective effect against oxidative stress induced by the drug. Tumor cells on the contrary, showed a constitutive activation of AMPK irrespective of archazolid treatment, yet V-ATPase independent activation of AMPK also protected from apoptosis induction. We propose that AMPK regulation in tumor cells is uncoupled from V-ATPase function depriving them from AMPK-mediated protection and renders them more sensitive to cytotoxicity induced by V-ATPase inhibitors. Hence, distinct AMPK regulation in cancer and non-malignant cells accounts for the tumor cell specificity of V-ATPase inhibitors.

Results

V-ATPase inhibition activates AMPK in non-tumor cells

To test whether inhibition of V-ATPase leads to AMPK activation we treated different tumor (MDA-MB-231, MCF7, T24, HUH7) and non-tumor cells (HEK293, MCF10A, HMLE) with archazolid and analyzed phosphorylation of AMPK on Thr172. We found that all tumor cells have constitutively activated AMPK and that archazolid had no effect on the activation level (Fig. 1A and S1A). Astonishingly however, V-ATPase inhibition lead to a profound activation of AMPK in all used non-tumor cells, which typically showed a low basal AMPK activation (Fig. 1B and 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 Figures 1C and S1C, there is an activation of AMPK in all tested cell lines and no difference between tumor and non-tumor 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 than with AMPK – an activation in non-tumor HEK293 cells and no change in MDA-MB-231 cells (Fig. S2). To make sure that V-ATPase is inhibited in all cell lines to the same extend we used a pH sensitive lysotracker and could show a similar effect by archazolid treatment (Fig. S3). To further analyze AMPK status in tumor and non-tumor tissue we used patient derived breast-tumor samples and normal breast tissue from the same patients, respectively, and analyzed activity of AMPK via Western Blot. Interestingly, we found that tumor cells showed an increased level of total AMPK and also a slightly higher level of phosphorylated AMPK (Fig. 1D and 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 non-tumor cells starved of glucose at the lysosome and that this interaction is required to activate AMPK (8). Therefore, we tested if archazolid leads to an interaction of the V-ATPase and AXIN1 in tumor cells. We found that treatment with archazolid leads to an increased interaction of AXIN1 and the V-ATPase in the non-tumor 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 also LKB1 is located at the lysosome. We finally found that archazolid leads to a transfer of LKB1 to the lysosome in HEK293 cells in contrast to tumor cells where no difference between treated and non-treated 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 non-tumor cells.

V-ATPase inhibition alters AMPK related metabolic parameters

AMPK is activated due to 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 non-tumor cell lines HEK293 and MCF10A showed a significant reduction of ATP level after archazolid treatment as shown by a luminescent 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 neither in non-tumor HEK293 cells, nor in MDA-MB-231 cells – which might indicate a different activation mode by V-ATPase inhibition. But we found an increased glucose uptake and a slight but not significant upregulation 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. 3B, C) showing a distinct metabolic regulation of tumor and non-tumor cells by V-ATPase inhibition.

AMPK activation protects non-tumor cells from archazolid induced cytotoxicity

As AMPK activation is quite controversially discussed as either tumor suppressor or oncogene we analyzed if archazolid induced AMPK phosphorylation is pro-survival or pro-apoptotic. Therefore, we silenced AMPK in HEK293 cells (Fig. 4B) and treated the cells with archazolid. We found that HEK293 cells with silenced AMPK showed a decreased growth rate after archazolid treatment compared to 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 induction. Therefore, we treated MDA-MB-231 cells with the AMP analogue AICAR in combination with archazolid which increased phosphorylation of AMPK (Fig. 4C and S6A). Interestingly we found that apoptosis induction was significantly reduced by combining archazolid with AICAR compared to archazolid treated cells alone (Fig 4D). This was accompanied by a decreased cleavage of PARP-1 confirming a decreased apoptosis induction and a protective role for AMPK (Fig. 4E and S6B). To confirm that this effect is really AMPK dependent we repeated the experiment with a silenced AMPKα1 and

found that AICAR does not protect MDA-MB-231 cells from archazolid induced apoptosis confirming the protective role of AMPK (Fig 4F and S6C).

Pro-survival role of AMPK results from different stress response and maintaining redox homeostasis

As one known pro-survival role of AMPK is the control of redox homeostasis by preventing ROS accumulation and maintaining NADPH levels, we analyzed if archazolid treatment leads to a disturbance of redox homeostasis. As shown in Fig. 5A, V-ATPase inhibition leads to an induction of ROS in tumor cells but rather to a decrease of ROS in non-tumor cells. Furthermore, archazolid lead to an increase of NADPH/NADP ratio in HEK293 cells while 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 FASN and SCD1 – two major players in the fatty acid synthesis pathway – in tumor and non-tumor cells. Interestingly we found that archazolid treatment lead to a strong upregulation of both enzymes in tumor cells but not in non-tumor cells (Fig. 5C). To connect these effects with the pro-survival 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 of AMPK in HEK293 cells leads to an upregulation of ROS after archazolid treatment confirming that AMPK activation is responsible for the protection of ROS induced apoptosis via V-ATPase inhibition (Fig. 5F).

Discussion

This work provides evidence that V-ATPase inhibition by archazolid leads to a differential metabolic regulation in tumor and non-tumor cells which results in an increased sensitivity of tumor cells towards 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 Figure 6, this protection is missing which results in increased apoptosis induction caused by a distinct effect on AMPK downstream cascade including ATP, glucose uptake, NADPH level and ROS production. As these effects could be abrogated by activating AMPK in tumor cells independently of V-ATPase, a novel role for AMPK in V-ATPase inhibition induced cytotoxicity was unraveled. This provides a novel interesting insight 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 non-malignant cells unaffected and thereby minimizing toxic side effects and make chemotherapy 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 (EGFR) (14), trastuzumab (HER2) (15) or imatinib which targets the bcr-abl tyrosinkase, a tumor-specific mutation (16). But despite the success experienced with these compounds selectivity of chemotherapeutics is still a major challenge.

The V-ATPase is a promising anti-tumor target and its inhibition was shown to have a pronounced effect on tumor cells, but leaves non-tumor cells almost unaffected as we and others could already show (11,17). We could even introduce more tumor and non-tumor cells lines to prove a clear difference in sensitivity towards V-ATPase inhibition in tumor versus non-tumor cells (Fig. S7). The anti-tumor effect of V-ATPase inhibition was mostly contributed to the disturbance of endocytotic recycling processes and several publications by our group and others could elucidate several underlying mechanisms (11,13,18,19). In recent years however, it became obvious that the V-ATPase also plays a pivotal role in nutrient sensing. Zoncu et al. showed that the V-ATPase is inevitable for mTOR-mediated amino acid sensing at the lysosome, by building a complex between mTORC1, the V-ATPase and Ragulator (12). Interestingly, Zhang et al. found that the 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 V-ATPase-Ragulator/LKB1-AMPK complex at the lysosome (8). Finally, McGuire et al. showed that glucose starvation leads to an increased V-ATPase assembly which is preceded by AMPK activation (20). These findings strengthen the hypothesis that V-ATPase is involved in metabolic regulation of the cell.

Zhang et al showed that an intact V-ATPase is needed to build the complex of LKB1/AXIN1/AMPK and V-ATPase and that inhibiting the V-ATPase with concanamycin mimicks glucose starvation and therefore activates AMPK in HEK293 cells (8). It was also shown that another V-ATPase inhibitor, bafilomycin, can activate AMPK in myoblasts (21). Consistent with their findings we could show that inhibiting the V-ATPase function pharmacologically with archazolid lead to an activation of AMPK in HEK293 and other non-tumor cells, to an increase in the binding of V-ATPase to AXIN1 and to an increased lysosomal localization of the AMPK-LKB1 complex. Interestingly, in tumor cells archazolid had no influence on AMPK phosphorylation and binding of V-ATPase to AXIN1 was rather decreased after archazolid treatment, though all tumor cells already showed a phosphorylated AMPK in controls. This finding is consistent with other groups showing high constitutive AMPK activity in tumor cells like prostate cancer metastasis or glioblastoma tumors (22,23).

Differential activation was not due to a stress resistance or other resistance mechanisms in tumor cells as glucose deprivation lead to an increased activation of AMPK in all cell lines. Therefore this effect is specific for V-ATPase inhibition and suggests a differential regulation of V-ATPase inhibition induced stress responses in tumor and non-tumor cells. Interestingly, we did not find an increase of AMP, neither in tumor nor non-tumor cells. This effect might support the special role for V-ATPase induced AMPK activation.

Regarding this distinct effect we wanted to investigate if the activation of AMPK in non-tumor cells plays a role for archazolid-induced cytotoxicity.

Looking at the role of AMPK in tumor context makes clear that AMPK regulation is highly complex and depends on the cell type, the severity and the kind of the stress signal and its role as tumor suppressor or oncogene is discussed controversially (24).

In the tumor context AMPK has long been seen as an anti-tumor target which originated from the discovery of the AMPK upstream kinase LKB1, the loss of which results in the development of Peutz-Jergher syndrome that comes along with an increased cancer risk (25). Furthermore, the use of - often unspecific - AMPK-activators, were shown to inhibit tumor growth in a variety of studies (26-28). Yet, 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 (23,29,30) or myc overexpressing tumors (31). In that regard, most data showing a tumor-suppressive role for AMPK rely on pharmacological studies, whereas experiments using genetic tools mostly support a pro-survival role for AMPK. Several oncogenes like Src or Myc were shown to activate AMPK (31,32) and silencing of an AMPK subunit impairs the ability of cells to form tumors *in vivo* (33). In addition, loss of the tumor suppressor follicullin also activates AMPK (34). All 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 to wild type cells suggesting a pro-survival function of AMPK activation. Moreover, increasing AMPK activity in MDA-MB-231 breast cancer cells by 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 non-tumor 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 in periods of poor nutrition or other metabolic stresses. The best known pro-survival functions of AMPK activation are autophagy, macromolecule import, redox homeostasis and enhanced glycolysis (35-37). In regard to glucose metabolism, we found an increased glucose uptake and slightly higher GLUT1 expression

selectively in non-tumor cells and an unvarying or even decreased glucose uptake in tumor cells which correlates with the differential activation of AMPK upon V-ATPase inhibition. This is in coherence with the data of others, showing an AMPK dependent increased uptake of glucose and glucose receptor expression to promote breast cancer growth (38) and protection from oxidative stress (39). Concerning redox homeostasis, Jeon et al showed that AMPK activation protects from ROS production and maintains NADPH levels constant during glucose deprivation leading to cell survival (7) – therefore providing evidence that AMPK activation promotes cell survival during energy stress. Additionally AMPK was shown to be responsible for the anti-oxidant effects of resveratrol (40) and essential for the redox balance in leukemia cells where AMPK inhibition results in reduced leukemic 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 V-ATPase inhibitor. ROS production in non-tumor cells on the other hand was not affected. These differences came along with a decreased NADPH/NADP level and synthesis of two major enzymes of lipid synthesis selectively in tumor cells. Importantly the archazolid induced disturbance in redox homeostasis was overcome by AMPK activation in non-tumor cells, while tumor cells failed to activate AMPK upon V-ATPase inhibition. Similar effects were also reported for Myc-positive melanoma where AMPK activation could protect the cells from ROS induced apoptosis (44). We found that scavenging ROS by 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 non-tumor cells. This points to the importance of the redox homeostasis maintained by AMPK under metabolic stress which is important for alleviating cytotoxic effects of V-ATPase inhibition.

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

Experimental procedure

Cell Culture and Compounds

MDA-MD-231, HEK293, T24 and MCF-7 cells were obtained from DSMZ (Heidelberg, Germany) and MDA-MB-231 and HEK293 cells were cultured in DMEM medium supplemented with 10% FCS. MCF-7 cells were maintained in RPMI1640 medium supplemented with 10% FCS, Insulin and 1% non-essential amino acids. MCF10A cells were purchased from 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 cell were a kind gift of Dr. Christina Scheel (Helmholtz Center Munich) and cultured in MEGCM medium obtained from PromoCell (Heidelberg, Germany).

Starvation experiments were performed in glucose-free DMEM supplemented with 10% of 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 non-profit organization PATH biobank (Augsburg, Germany) (45). The collection of samples was approved by the local ethics committee in Bonn and all patients provided written informed consent. The sample collection was carried out according to the declaration of Helsinki.

Immunoblotting

Cells were lysed in a buffer containing EDTA 2 mM, NaCl 137 mM, Glycerol 10 %, $Na_4P_2O_7$ 2 mM, Tris-Base 20 mM, Triton X-100 1 % and Na-Glycerolphosphate 20 mM at pH 7,5. For immunoprecipitation cells were lysed and incubated with an antibody against

AXIN1 overnight. Subsequently the lysate was incubated with Protein Agarose A beads (Sigma Aldrich, Taufkirchen, Germany), washed with lysate buffer and beta-mercaptoethanol containing sample buffer was added. Equal amounts of protein were separated on a SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 5% milkpowder and then probed with primary antibodies.
For loading control 2,2,2-Trichloroethanol (TCE) was added to the polyacrylamide gel before polymerization. The tryptophans of proteins undergo an ultraviolet light-induced reaction with TCE which produces light in the visible range so that proteins can be detected (46). In some cases actin was used as loading control.
Following antibodies were used: pAMPK Thr172, AMPK, AMPK1alpha, pACC Ser79, ACC, PARP1 (Cell Signaling Technology, Danvers, MS), GLUT1 (Novus Biologicals), actin, rab7, AXIN1, V-ATPase D, (Santa Cruz Biotechnology, Dallas, TX), HRP-goat-anti-rabbit (Bio-Rad, Munich, Germany) and HRP-goat-anti-mouse (Santa Cruz Biotechnology).

Isolation of Lysosomes

Lysosomes were isolated as previously described (47). Briefly, 1,5 x 10^6 cells were seeded in 10 cm dishes and were treated as indicated. Subsequently medium was removed and 250 µl homogenization buffer was added to the cells. The cell suspension was transferred to a glas grinding vessel and homogenized using a potter homogenizer. Next, the homogenate was centrifuged 15 min at 14.000 g at 4°C. The supernatant was transferred to a 2 ml polycarbonate centrifugation tube and an equal volume of 16 mM $CaCl_2$ to precipitate the lysosomes was added. Then the homogenate was centrifuged at 25.000 g for 15 min. Next, supernatant is discarded and the pellet resuspended in one volume of washing buffer and again centrifuged. Now the pellet containing lysosomes is resuspended in 20 µl of Washing buffer and used for Western blot analysis.

Transfection

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

Immunocytochemistry

Cells were seeded on µ-slides 8-well ibidiTreat (IBIDI, Martinsried, Germany) and were treated as indicated. Cells were fixed and stained as described before (48) and analyzed with a Zeiss LSM 510 Meta confocal microscope (Jena, Germany). Following antibodies were used: LKB1 (Cell Signaling Technology, Dancers, MS), Lamp1 (Developmental Studies Hybridoma Bank Iowa, IA) AlexaFluor 488-goat-anti-rabbit, AlexaFluor 543-goat-anti-mouse (Invitrogen, Waltham, MA).

Apoptosis Assay

Subdiploid DNA content was determined according to Nicoletti et al (49). Briefly, cells were treated as indicated, harvested, permeabilized with Natriumcitrate containing Triton X-100, stained with 25 µg/ml propidiumiodide and analyzed by flow cytometry (Beckton Dickinson, Heidelberg, Germany). Subdiploid cells left of the G1-peak were considered as apoptotic.

Cytotoxicity assay

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

qRT-PCR analysis

Total mRNA was isolated from cell culture samples using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For cDNA synthesis, the High Capacity cDNA Rerverse Transcription Kit (Applied Biosystems, Foster City, CA) was used. qRT-PCR was performed with the AB 7300 RealTime PCR system, the TaqMan Gene Expression Master Mix (Applied Biosystems) and the 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-NBDG (2-[N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino]-2-Deoxy-D-Glucose) (Invitrogen, Waltham, MA) 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 of 2′,7′-dichlorofluorescein diacetate (Sigma Aldrich, Taufkirchen, Germany) 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 level was determined using the CellTiter-Glo assay (Promega) according to the manufacturer's instruction and measured in a luminometer (Berthold Technologies, Bad Wildbad, Germany) according to manufacturer's instructions.

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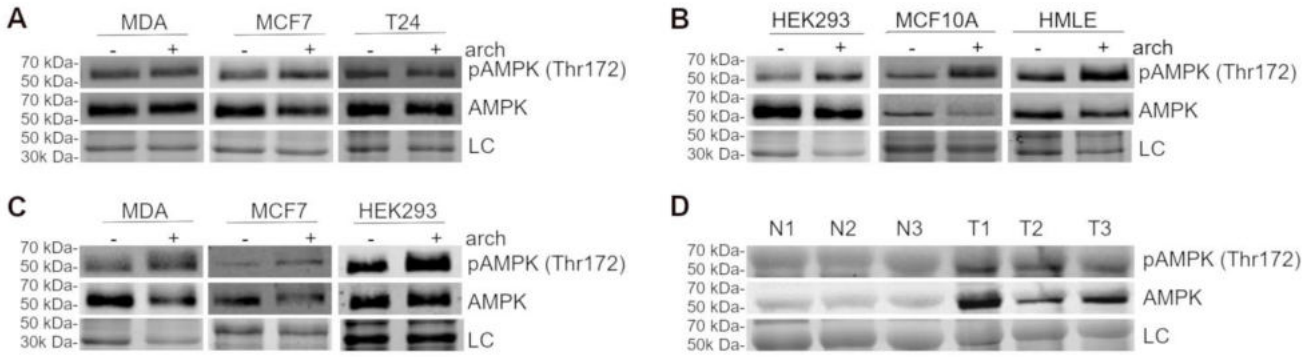

Figure 1: V-ATPase inhibition differentially regulates AMPK activation. The tumor cell lines MCF-7, MDA-MB-231 (MDA) and T24 **(A)** and the non-tumor cell lines HEK293, MCF10A and HMLE **(B)** were treated with 10 nM archazolid (arch) for 24 hours and phosphorylation of AMPK at Thr172 and total AMPK protein were analyzed by Western Blot. **(C)** HEK293, MCF-7 and MDA-MB-231 were incubated in glucose-free media for 24 hours and phosphorylation of AMPK was analyzed. Whole protein level (LC) served as loading control. **(D)** Phosporylation of AMPK was analyzed in patient-derived breast cancer tissue and normal breast tissue from the same patient. One representative picture out of three independent experiments is shown.

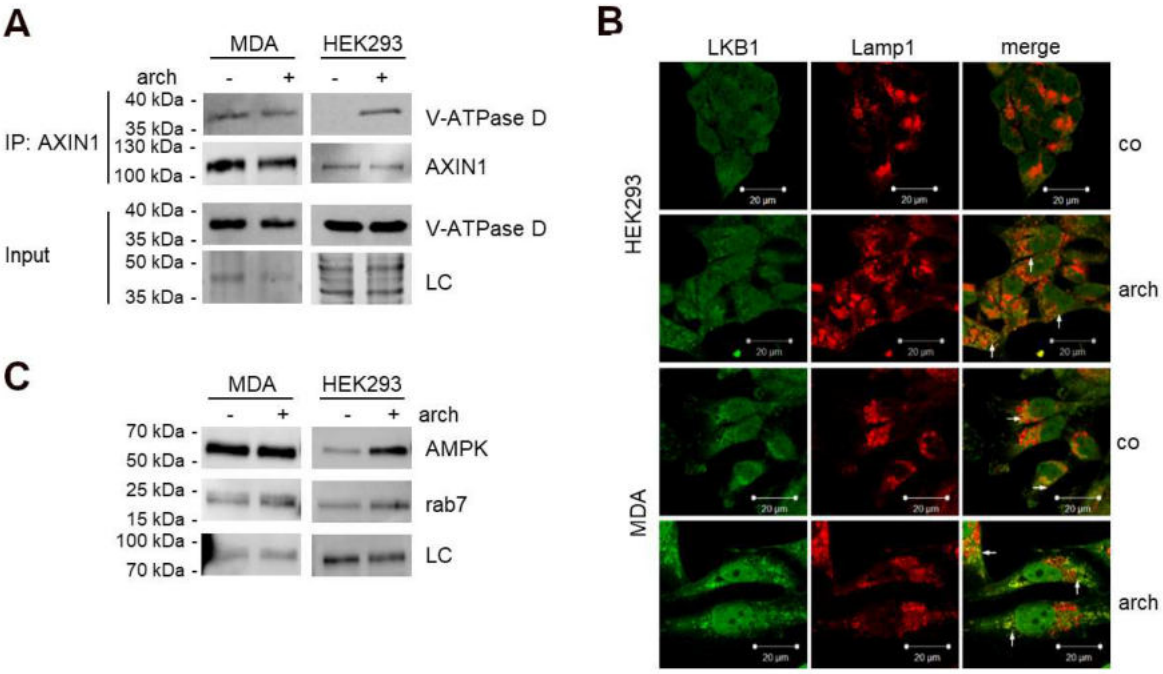

Figure 2: V-ATPase inhibition is involved in AMPK activation. MDA-MB-231 and HEK293 cells were treated with 10 nM archazolid for 24h or left untreated. **(A)** Binding of the V-ATPase subunit D to the chaperone AXIN1 was analyzed by immunoprecipitation and subsequent Western Blot. **(B)** Lysosomes were isolated and lysosomal localization of AMPK was analyzed by Western Blot. Rab7 and total protein (LC) served as loading control. **(C)** Cololocalization of Lamp1 and LKB1 was determined after treatment with 10 nM archazolid by confocal staining. One representative image out of three independent experiments is shown.

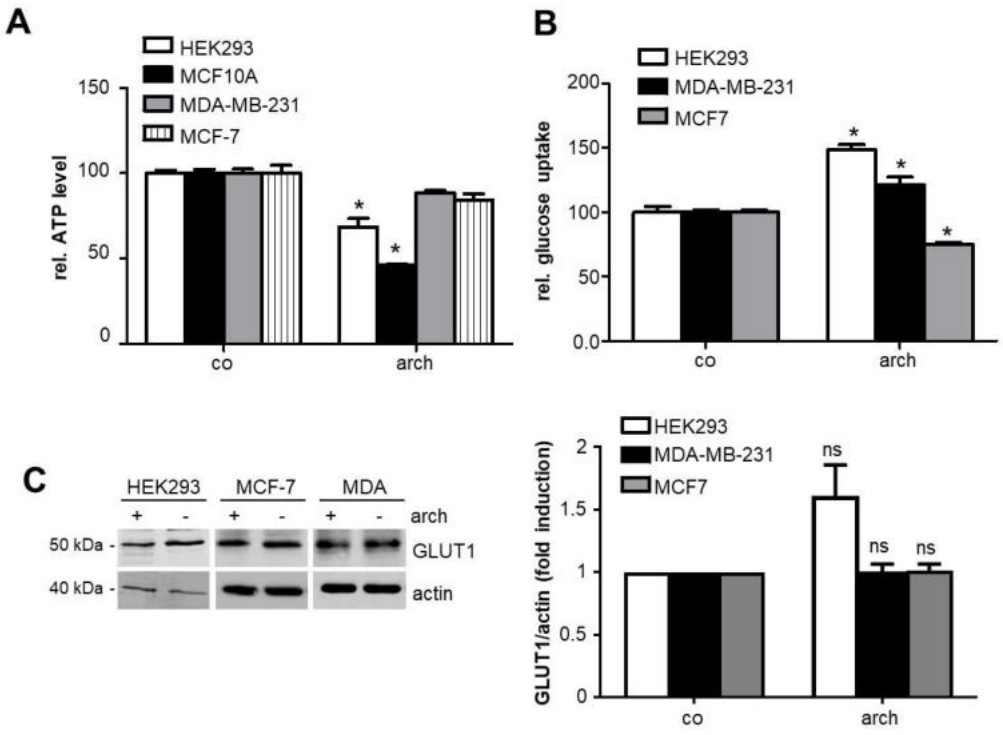

Figure 3: V-ATPase inhibition differentially regulates metabolic parameters. The indicated tumor and non-tumor cell lines were treated with 10 nM archazolid for 24 hours or left untreated. **(A)** ATP level was measured by luminescence. **(B)** Glucose uptake was determined using the fluorescent glucose-analogue 2-NBDG and subsequent FACS analyzes and **(C)** glucose receptor expression was analyzed by Western Blot. Bars are the SEM of three independent experiments. p*<0.05, student's t-test.

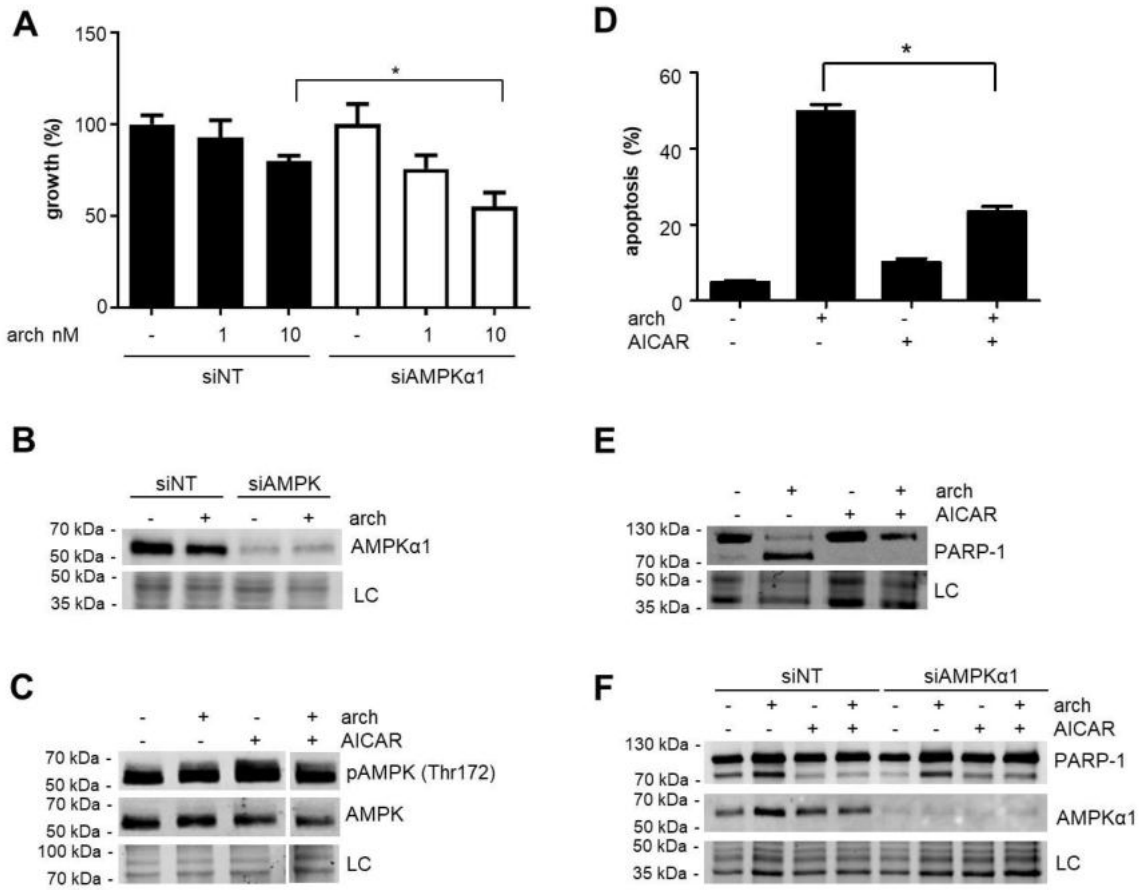

Figure 4: Non-tumor cells are protected from arch-induced apoptosis via AMPK activation. HEK293 cells were transfected with siRNA against AMPK (siAMPK) or non-targeting siRNA (siNT). 48 h after transfection cells were treated with 10 nM arch or left untreated. After 48 h of incubation **(A)** cytotoxicity was measured via cellTiter Blue assay, a representative Western Blot showing downregulation of AMPK is shown **(B)**. **(C)** MDA-MB-231 cells were treated for 24 h with 10 nM arch, 0.5 mM AICAR or in combination and phosphorylation of AMPK was determined by Western Blot. **(D)** MDA-MB-231 cells were treated for 72 h with 10 nM arch, 0.5 mM AICAR or in combination and apoptosis induction was analyzed by propidium iodide staining and flow cytometry or **(E)** PARP-1 cleavage was determined by Western Blot 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 Blot. One representative image out of three independent experiments is shown. Quantification represents three different experiments. Bars are the SEM of three independent experiments. p*<0.05 (student's t-test).

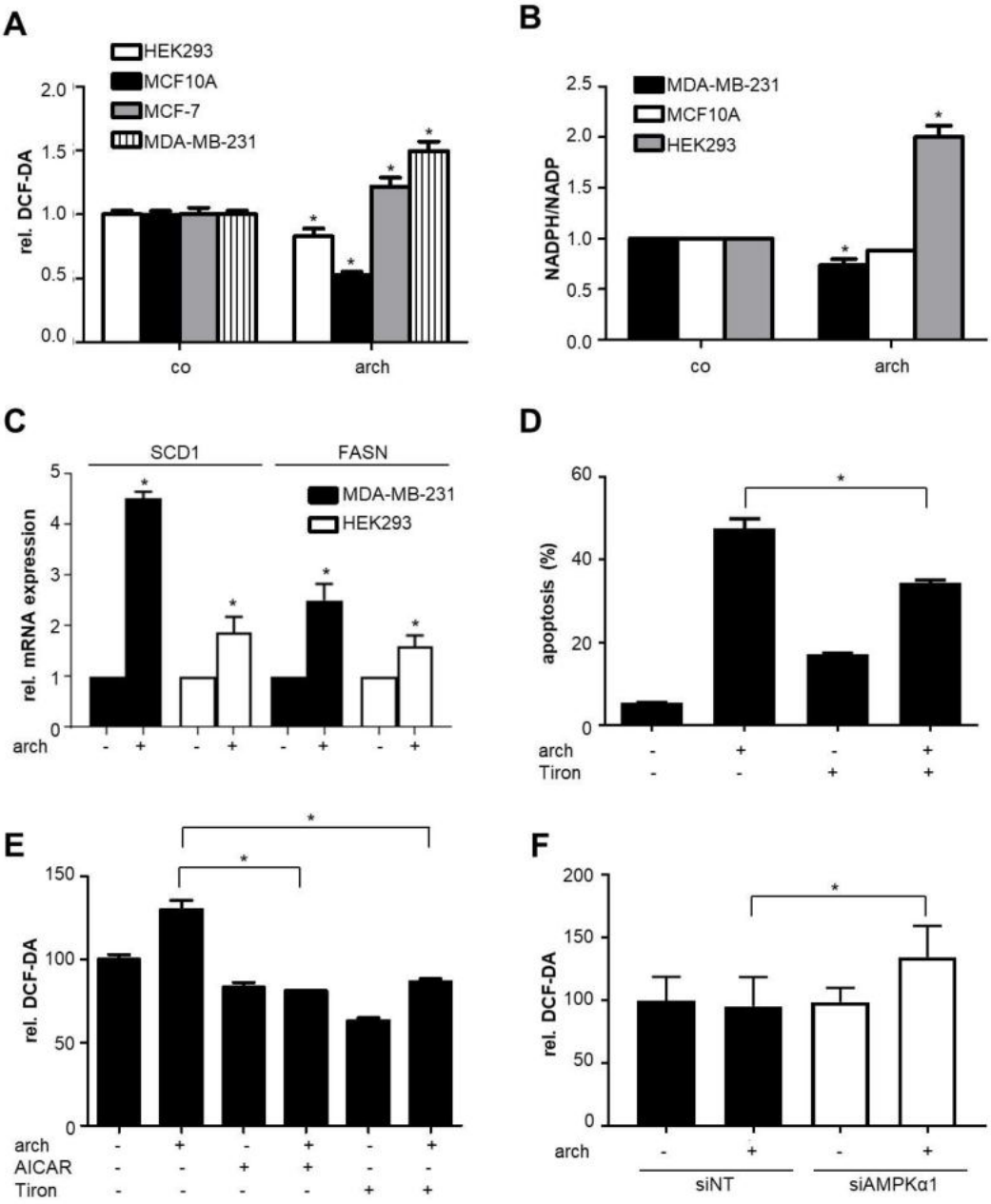

Figure 5: Redox homeostasis is involved in pro-survival role of AMPK. The indicated cell lines were treated with 10 nM arch for 24 h or were left untreated. **(A)** ROS induction was analyzed by staining the cells with DCF-DA and flow cytometry. **(B)** NADPH/NADP level was measured via a luminescence assay. **(C)** MDA-MB-231 and HEK293 cells were treated with 10 nM arch for 24 h and mRNA expression of FAS and SCD1 was analyzed by qPCR. **(D)** MDA-MB-231 cells were treated with 10 nM arch, 10 mM tiron or in combination and apoptosis induction was analyzed by propidium iodide staining and flow cytometry. **(E)** MDA-MB-231 cells were treated with 10 nM arch, 0.5 mM AICAR or in combination and ROS induction was analyzed by flow cytometry. **(F)** HEK293 cells were transfected with siNT and siAMPK. 48 h after transfection cells were treated as indicated and ROS induction was measured. Bars are the SEM of three independent experiments. p*<0.05, student's t-test.

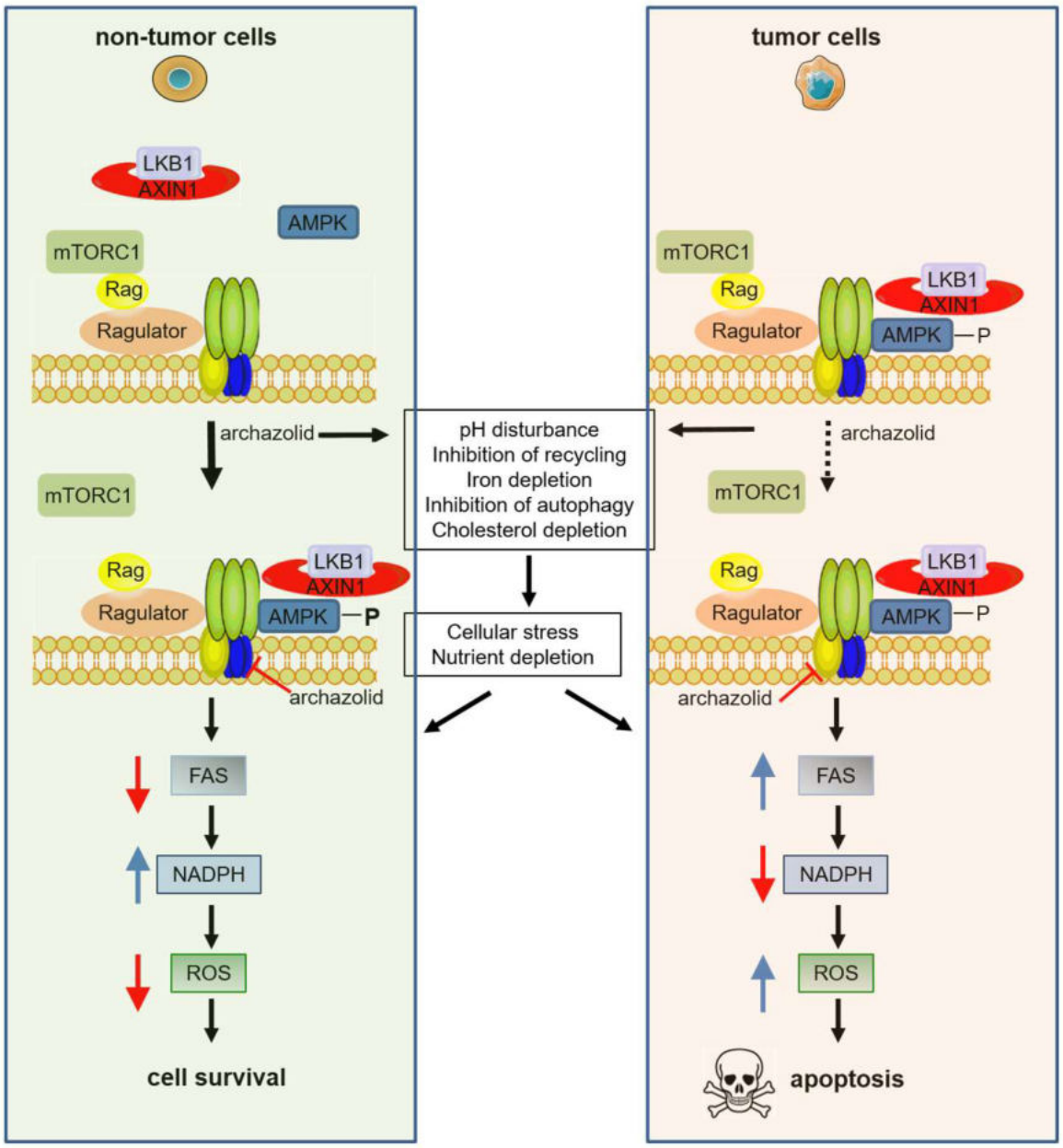

Figure 6: Schematic depiction of differential metabolic regulation in tumor and non-tumor cells by V-ATPase inhibition. In tumor non-tumor cells treatment with archazolid leads to cellular stress but only in non-tumor cells to an active induction of AMPK phosphorylation. This leads to a decreased fatty acid synthesis, increased NADPH/NADP ratio and thereby to a protection of ROS induced apoptosis. In tumor cells – although showing a phosphorylated AMPK in control – V-ATPase inhibition had no effect on pAMPK level and they displayed a decreased NADPH/NADP ratio, increased fatty acid synthesis and ROS level leading to cell death.

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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