UNIVERSITY OF NAPLES FEDERICO II

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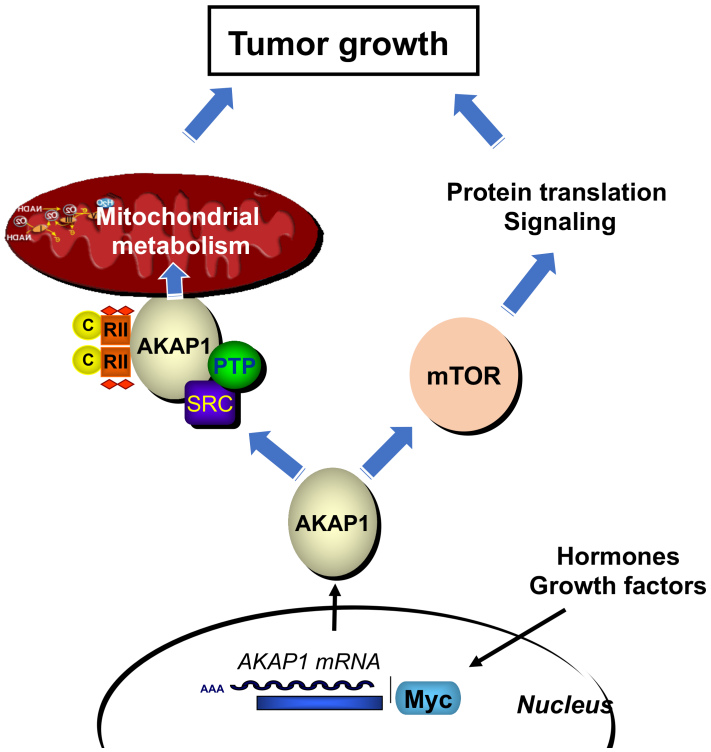
XXXIII CYCLE

Domenica Borzacchiello

"METABOLIC CONTROL OF TUMOR GROWTH BY MITOCHONDRIAL A-KINASE ANCHOR PROTEIN 1 (AKAP1)"



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“METABOLIC CONTROL OF TUMOR GROWTH BY MITOCHONDRIAL A-KINASE ANCHOR PROTEIN 1 (AKAP1)”

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Year 2021

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List of Abbreviations

AC	Adenylate Cyclase
AKAP	A-kinase Anchor Protein
AKT	Serine/threonine protein kinase
AMP	Adenosine Mono-Phosphate
AMPK	Adenosine Mono-Phosphate Kinase
ATM	Ataxia-Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3-related Protein
cAMP	Cyclic Adenosine Monophosphate
CHK	Checkpoint Kinase
ChChd3	Inner Mitochondrial Membrane Scaffold Protein
DDR	DNA Damage Response
DMEM	Dulbecco's Modified Eagle Medium
Drp1	Dynamin-Related Protein 1
ER	Endoplasmic Reticulum
FAO	Oxidation Fatty Acids
FCCP	Carbonylcyanide-4- (Trifluoromethoxy)-Phenylhydrazone
FACS	Fluorescence Activated Cell Sorting
GBM	Glioblastoma
GDH	Glutamate Dehydrogenase
GFP	Green Fluorescent Protein
GLS	Glutaminase
GPCR	G Protein–Coupled Receptor
GUS	β-glucuronidase
HER2	Human Epidermal Growth Factor Receptor 2
2-HG	2-Hydroxyglutarate
HIF1α	Hypoxia Inducible Factor 1α
IAP	Inhibitors of Apoptosis Proteins
IDH	Isocitrate Dehydrogenases
α -KG	alfa-ketoglutarate
OXPHO	Oxidative Phosphorylation
2-ME	2-Methoxyestradiol
MT	Mitochondrial Targeting Domain
MTDHD2	Methylen-Tetrahydrofolate Dehydrogenase 2
NRF1	Nuclear Respiratory Factor 1
NRF2	Nuclear Transcription Factor Erythroid 2-related Factor
OCR	Oxygen Consumption Rate
+4-OHT	4-Hydroxitamoxifen
OMM	Outer Mitochondrial Membrane
PDE	Phosphodiesterase
PGC-1α	Peroxisome Proliferator-Activated Receptor-Gamma Coactivator
PHD	Prolyl Hydroxylase
PI3K	Phosphatidylinositol-3 Kinase
PKA	Protein *Kinase A*
PP	Phosphatase Protein
PP2B	Protein Phosphatase 2B or Calcineurin
PTPD1	Tyrosine Phosphatase Protein
ROS	Reactive Oxygene Species
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

c-SRC	Proto-oncogene tyrosine-protein *kinase*
TCA	Tricarboxylic Acid Cycle
TGFβ	Tumor Growth Factor β
UPS	Ubiquitin–*Proteasome* System
UTR	3'-Untranslated Region
VEGF	Vascular Endothelial Growth Factor

ABSTRACT

Mitochondria are powerhouses present in all eukaryotic cells that play a fundamental role in energy production, survival and metabolism. In cancer cells, mitochondria provide the building blocks for the biogenesis of cellular organelles, cytoskeleton and membranes, and supply *all* the metabolic needs for cancer growth and spreading *in vivo*. AKAP1 is a scaffold protein that integrates and focus cAMP and src signaling on mitochondria, regulating protein synthesis, organelle biogenesis, oxidative metabolism and cell survival. During my thesis, I analyzed the mechanisms controlling the expression of AKAP1 in cancer cells and the role of this anchor protein in the control of metabolic pathways and cancer growth. I found that transcription and accumulation of AKAP1 are induced by the Myc proto-oncogene and by steroid hormones. I detected high levels of AKAP1 in a wide variety of high-grade human cancer tissues and cells, including prostate cancer, breast cancer and glioblastoma (GBM). I demonstrated that AKAP1 is required for mTOR pathway, oxidative metabolism and cancer growth, both in vitro and in vivo. Interestingly, I discovered a link between DNA damage pathways and AKAP1. In particular, I found that, in course of DNA damage, AKAP1 is phosphorylated by ATM/ATR kinase at its PKA binding domain. Phosphorylation of AKAP1 by ATM inhibits PKA targeting to mitochondria and downregulates oxidative metabolism. These data disclose a previously unrecognized role of AKAP1 in mTOR pathway and cancer growth AKAP1 and identify AKAP1 as a novel biologically relevant target of the DNA damage pathways.

1. INTRODUCTION

1.1 G protein-coupled receptor and cAMP signal transduction pathway. G protein–coupled receptors (GPCRs) are large family of membrane proteins that transduce signals generated at cell membrane to intracellular compartments[1 2]. Stimulation of the GPCR activates the adenylate cyclase, which in turn synthesizes cAMP at discrete points along the plasma membrane. cAMP is the prototypic second messenger generated at cell membrane by GPCR-activated adenylate cyclase. In eukaryotes, protein kinase A (PKA) represents the major cAMP-responsive enzyme and the principal effector of cAMP stimulation[3].

PKA is a tetrameric holoenzyme composed of two regulatory subunits (R) and two catalytic subunits (C). The binding of cAMP to R subunits dissociates the holoenzyme and releases the C subunit. The free C subunit phosphorylates a wide variety of intracellular substrates controlling essential aspects of cell biology, including oxidative phosphorylation, metabolism, differentiation, synaptic activity, growth and development[4]. Distinct R/C subunits have been identified and functionally characterized. This contributes to generate different PKA holoenzymes with distinct sensitivities to cAMP stimulation. Furthermore, the PKA holoenzymes are differentially distributed in cells and tissue. Accordingly, the differential rate, magnitude and persistence of PKA activation by cAMP signals contribute to enhance the specificity and sensitivity of cells to distinct GPCR ligands, inducing differential biological responses to the same second messenger, i.e. cAMP[5].

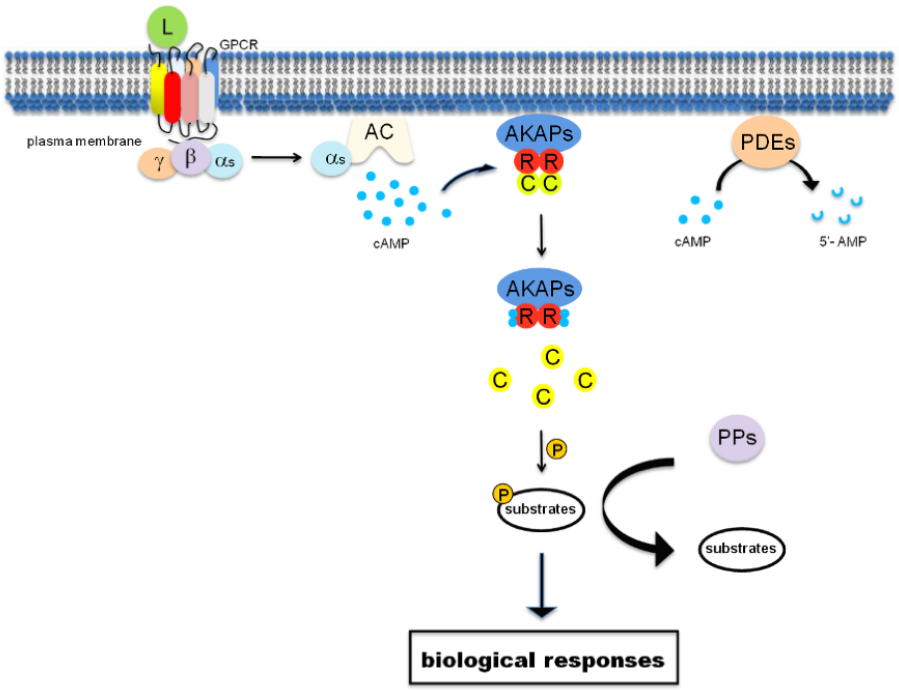


Figure 1. Model of the cAMP pathway. Activation of G protein-coupled receptor (GPCR) by a given ligand (L) induces dissociation of the heterotrimeric G protein and release of Gas subunit. The binding of Gas to the

adenylate cyclase (AC) stimulates the synthesis of cAMP. The binding of cAMP to the regulatory subunit dissociates the PKA holoenzyme and releases the active C subunit (PKAc). Phosphorylation of intracellular substrates by free PKAc controls a variety of biological responses. The activation of phosphodiesterase (PDE) and phosphatase attenuates the cAMP-induced signaling cascade.

1.2 Compartmentalized PKA pathway. PKA holoenzymes are targeted to discrete intracellular compartments through a direct interaction with a family of specific anchor proteins (A-kinase anchor proteins, AKAPs). AKAPs employ a PKA-binding motif that interacts with the R subunit of PKA holoenzyme and a targeting module that localizes the AKAP/PKA complex to intracellular membranes, organelles and cytoskeleton. The spatial distribution of PKA holoenzymes at discrete intracellular sites by AKAPs tightly controls the propagation of cAMP signals from sites of signal generation to PKA substrates/effectors[6]. AKAPs also assemble multienzyme complexes which include not only PKA holoenzymes, but also components of the cAMP generating systems (receptors and adenylate cyclase), effectors (PKA and Epac) and attenuating enzymes, such as cAMP-directed phosphodiesterases (PDEs) and protein phosphatases (PPs). Furthermore, adaptor molecules, mRNAs and effector enzymes distinct from PKA could also take part of the AKAPs complex. The signaling modules assembled by AKAPs work as 'transduceosomes' acting as highly specialized intracellular hubs where different signaling pathways converge and focus, generating biological responses to hormones, growth factors, neurotransmitters and cytokines. The signaling nodes assembled by AKAPs evolved from the ancient linear unicellular systems, where the activation of transduction enzymes and adapter molecules takes place in a single round within the same intracellular compartment. Interfering with the complexes assembled by AKAPs *in vivo* has major impact on essential physiological processes including differentiation, growth, metabolism and respiration[7].

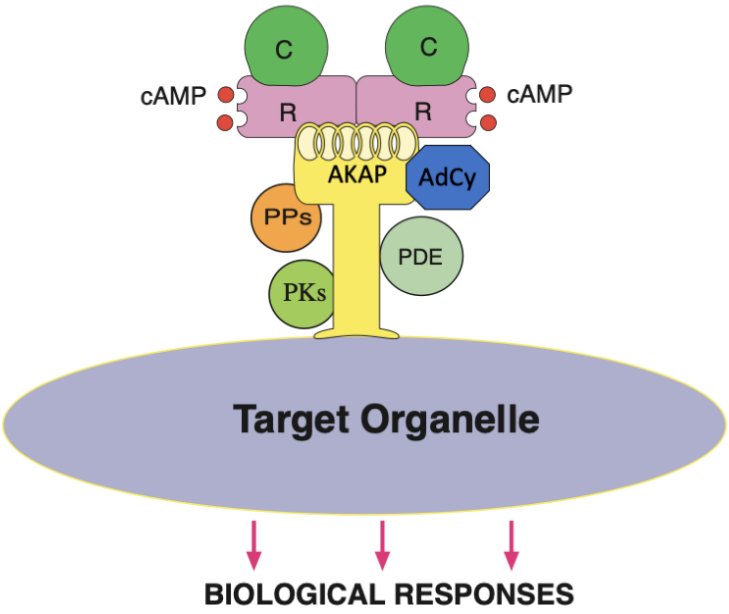


Figure 2. AKAPs complexes in cells. A schematic view of the transduceosome assembled by a prototypic AKAP at target organelle. The complex nucleated by AKAP is composed of cAMP-dependent protein kinase (R and C subunits), protein phosphatases (PPs), cAMP-adenylate cyclase (AdCy), cAMP-phosphodiesterase (PDE) and non-PKA protein kinases (PKs). (*Adapted from JMB 2001, 308, 99-114*)

1.3 cAMP signaling at mitochondria. Mitochondrial functions as respiration, survival, metabolism, organelle biogenesis and dynamics are tightly regulated by PKA. Several AKAPs have structurally and functionally characterized as resident of mitochondria[8]. AKAP1 is the prototypic mitochondrial AKAP that orchestrates many cAMP events at these organelles. AKAP1 binds and targets PKA to the outer mitochondrial membrane (OMM). AKAP1 mRNA undergoes alternative splicing that generates different variants of AKAP1 which include AKAP121, AKAP100 and AKAP84. All splice variants possess a common NH_2-terminal core domain that incorporates the PKA binding motif, but diverge at their C-terminal region. The mitochondrial targeting domain (MT) located at the extreme NH2-terminus of the AKAP1 splicing products mediates the interaction of the proteins to the OMM. A hydrophobic 33-residue modifier segment located upstream of the MT domain has been described as a product of alternative splicing that can target the protein to the endoplasmic reticulum (ER). Thus, using distinct targeting sequences, AKAP1 proteins can dynamically shift the focus of cAMP signaling between the ER and mitochondria, functionally coupling Ca^{2+} homeostasis, cell respiration and metabolism during stress conditions or changes in metabolic requirements.[9, 10]. However, the coding sequence of the ER domain has not been identified in the human genome, questioning the existence of ER-targeted AKAP1 in the human proteome [11].

Mouse AKAP121 and its human (AKAP149) and Drosophila ortholog (MDI) possess a KH domain located at their C-terminus which mediates interaction with nuclear-encoded mRNAs[12-14]. AKAP1 binding to mRNA and ribosomes at the OMM promotes efficient translation and co-import of mitochondrial proteins into the organelles, with important implications for global protein synthesis and mitochondrial physiology[15-17]. Importantly, AKAP1 complexes include not only PKA, but also the protein tyrosine kinase src, phosphatases (PP1, PTPD1), cAMP-phosphodiesterases, messenger RNA and adenylate cyclases. The 'transduceosome' assembled by AKAP1 on mitochondria controls respiration, metabolism, mitochondrial homeostasis and cell survival.

Different mitochondrial PKA substrates have been identified, including components of the oxidative phosphorylation machinery (OXPHO). Phosphorylation of OXPHO proteins by PKA, positively impacts on global respiration, thermogenesis and ATP synthesis[18]. AKAP1 plays also a major role in cell survival, promoting PKA phosphorylation and inhibition of proapoptotic proteins, as BAD. BAD is a BH3 proapoptotic Bcl-2 family member that interacts with- and inactivates anti-apoptotic Bcl-2 homologues. Under stress conditions, inhibition of Bcl-2 activity by BAD induces mitochondrial cristae disruption, mitochondrial swelling and release of cytochrome c, which in turn activates the proapoptotic pathway. Phosphorylation of BAD by ser/thr protein kinases, including PKA, inhibits BAD preventing cell death. An AKAP1 complex containing PKA and BAD has been isolated from cells and tissues. In presence of survival signals, PKA within the AKAP1 complex phosphorylates the co-assembled BAD at Ser155, preventing its association to the anti-apoptotic Bcl-2 protein and inhibiting cell death[19].

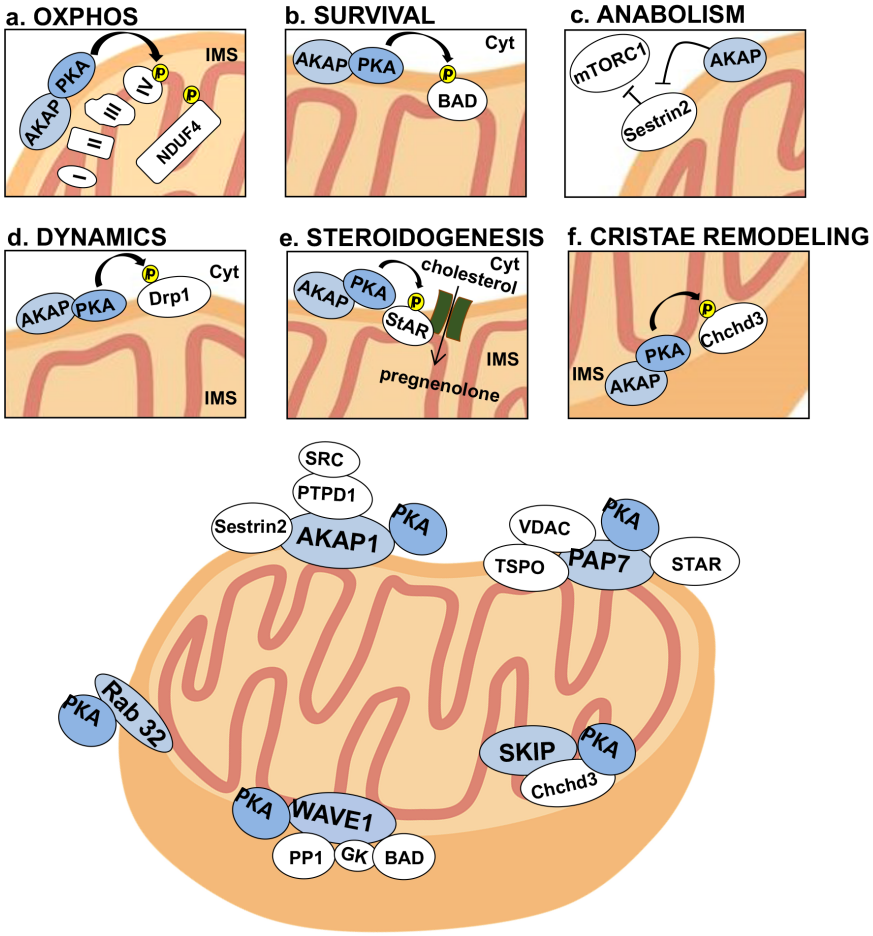


Figure 3. Role of AKAP1-PKA complex in mitochondrial activity. Distinct families of AKAPs have been identified at the mitochondrial compartments. AKAPs assemble multifunctional molecular platforms at the OMM and within the mitochondrial compartment that include not only PKA, but also other non-PKA partners. Phosphorylation of mitochondrial substrates by PKA controls essential aspects of organelle physiology. **a**. PKA phosphorylation of components of the respiratory chain positively regulates oxidative ATP synthesis.**b**. BAD binds to- and inactivates anti-apoptotic Bcl-2 homologs, promoting apoptosis. Phosphorylation and inactivation of BAD at the OMM by AKAP-anchored PKA prevents BAD/Bcl-2 interaction and inhibits apoptosis. **c**. Sestrin2 is a stress-induced anti-oxidant gene product that acts as inhibitor of mTORC1. By targeting sestrin2 to mitochondria, AKAP1 relieves the inhibitory constrain on mTORC1 and promotes anabolism and tumor cell proliferation. **d**. Phosphorylation and inactivation of the pro-fission protein Drp1 promotes mitochondrial fusion. **e**. Transport of cholesterol from cytosol (cyt) to intermembrane space (IMS) is stimulated by PKA-dependent phosphorylation of mitochondrial StAR protein. Consequent conversion of cholesterol to pregnenolone by the mitochondrial sidechain cleavage enzyme system (P450scc) is required for steroid biosynthesis. **f**. Phosphorylation of ChChd3 by SKIP-associated PKA within the mitochondrial compartment is essential for maintaining mitochondrial cristae integrity/remodeling (*BBA Rev.Cancer 2018 1869(2):293-302*).

1.4 Control of AKAP1 'transduceosome' by the ubiquitin proteasome system (UPS). The complex assembled at mitochondria by AKAP1 is controlled by the UPS. Following hypoxic insult, the levels of RING E3 ubiquitin ligase Siah2 rapidly rise within the cells, leading to ubiquitination and proteolysis of the prolyl hydroxylase PHD2. This enzyme, in the presence of physiological concentrations of oxygen, negatively controls the stability of the hypoxia sensor HIF-1α (Hypoxia Inducible Factor-1α)[20]. Under hypoxic conditions, PHD2 is degraded by Siah2-UPS pathway, promoting accumulation of HIF-1α and the transcriptional activation HIF1α-dependent genes, such as vascular endothelial growth factor (VEGF), tumor growth factor β (TGFβ) and erythropoietin. Upregulation of these factors induces vascularization, erythropoiesis and the metabolic rewiring of the ischemic tissue, preventing irreversible tissue damage. During hypoxia, mitochondria are also subjected to significant metabolic changes. As consequence of low oxygen availability, mitochondrial activities and oxidative stress are reduced. Mechanistically, hypoxia induces ubiquitination of AKAP1 by Siah2 and its consequent proteolysis through the proteasome. Downregulation of AKAP1 by the hypoxia-UPS pathway subserves as a major regulatory mechanism to adapt cells in course of hypoxic/ischemic conditions[21].

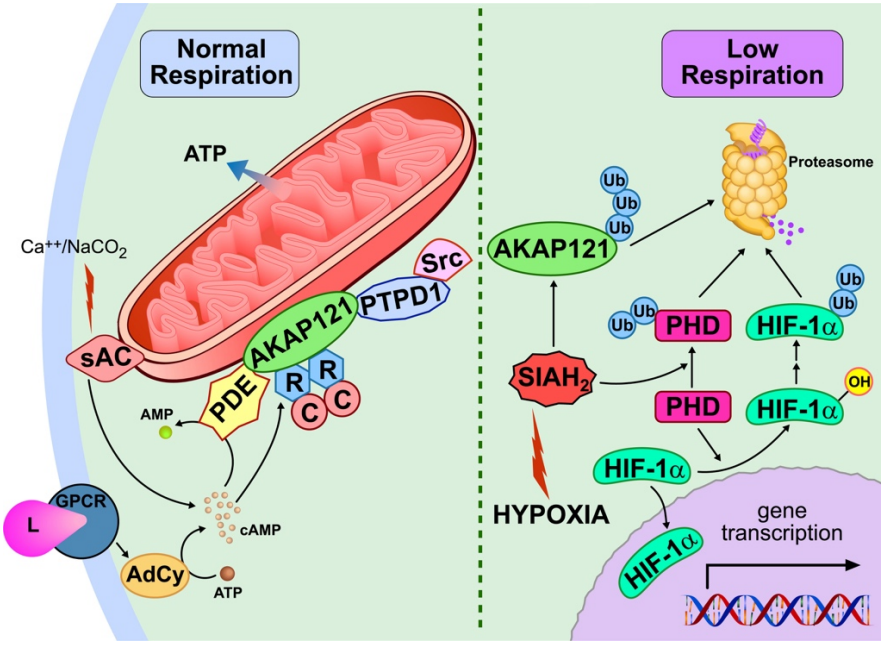


Figure 4. Control of AKAP1 by the hypoxia-UPS pathway. AKAP121 assembles a multivalent signaling complex on mitochondria that includes PKA, PDE and PTPD1–Src molecules. Local activation of these signaling enzymes is required for oxidative metabolism and ATP synthesis. Under normoxic conditions, HIF-1α hydroxylation by prolyl hydroxylase (PHD) promotes HIF-1α binding to von Hippel-Lindau complex and rapid degradation by the ubiquitin-proteasome pathway. Hypoxia induces expression and accumulation of

Siah2. Siah2 induces ubiquitination and proteasomal degradation of PHD and AKAP121. As a result, HIF-1α accumulates and activates transcription of hypoxia-induced genes, whereas degradation of AKAP121 attenuates mitochondrial respiration (*TiCB2008, 18,604-613*).

1.5 Role of AKAP1 in mitochondrial dynamics. AKAP1 plays an important role in mitochondrial dynamics. In response to changes of metabolic needs, mitochondria undergo to fusion or fission, changing their shape, number and intracellular distribution. This is a highly dynamic and rapid process that controls mitochondrial activities and cell viability[22]. The correct balance between fusion and fission is maintained by the coordinated action of key regulators that actively participate in each step of the mitochondrial dynamics. Work from several groups identified the mitochondrial AKAP1-PKA complex as a central hub in the control of mitochondrial dynamics. Thus, phosphorylation and inactivation of the dynamin-related protein 1 (Drp1), a pro-fission mitochondrial protein, by AKAP1-bound PKA induces mitochondrial elongation and increases cellular resistance to apoptotic signals[23]. The PKA action on mitochondrial fission can be counteracted by the calcium-activated ser/thr phosphatase, calcineurin (also known as PP2B). Thus, under stress conditions, calcium released from intracellular stores induces dephosphorylation and activation of Drp1 by PP2B, which promotes mitochondrial fission and ultimately cell death. Following hypoxic injury, proteolysis of AKAP1 by the Siah2-UPS pathway attenuates the mitochondrial PKA signaling and induces Drp1-dependent mitochondrial fission and cell death[24]. The regulatory mechanism controlled by AKAP1/PKA at mitochondrial compartment allows the cells to efficiently adapt under physiological conditions in response to specific metabolic needs but also following environmental stresses that would otherwise irreversibly damage the cells.

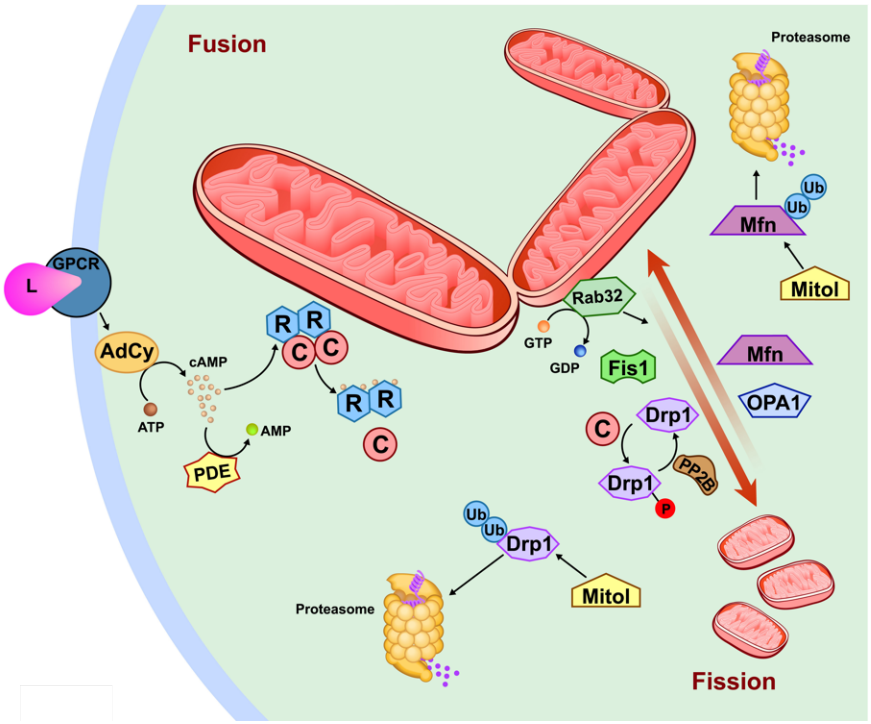


Figure 5. Control of mitochondrial dynamics by AKAP1 and UPS. Mitochondrial fusion is regulated by two distinct GTPase family proteins: mitofusins (Mfn1 and Mfn2) and OPA1. Mitochondria fission is controlled by Drp1 and hFis1, an integral protein of the outer membrane of mitochondria. Mitol, a mitochondrial E3 ubiquitin ligase, promotes ubiquitin-dependent degradation of Drp1 and Mfn2 through the proteasome pathway. Ligand (L)-mediated stimulation of GPCR activates adenylate cyclase (AdCy) and increases intracellular levels of cAMP. cAMP binds to the regulatory subunit (R) of PKA, dissociating the holoenzyme and activating PKA catalytic subunit (C). The PKA C subunit phosphorylates and inactivates Drp1, thereby promoting mitochondria fission. cAMP phosphodiesterase (PDE) converts cAMP to adenosine mono-phosphate (AMP). Drp1 is reversibly dephosphorylated and activated by the calcium-dependent Ser/Thr phosphatase calcineurin (PP2B) (*TiCB2008, 18,604-613*).

1.6 Mitochondrial metabolism and signaling in cancer cells. Extracellular stimuli and intracellular signaling inputs can integrate and focus on mitochondria. The dynamic and synergistic interaction between the signaling events underlying glycolysis, oxidative phosphorylation, tricarboxylic acid cycle (TCA), beta oxidation of fatty acids (FAO) and biosynthetic pathways occurring within the mitochondrial compartment are essential for cancer cell growth. Within the tumor, cancer cells undergo a profound metabolic reprogramming that primarily involves mitochondria and metabolic pathways. Metabolic requiring supports the high energetic requirements of cancer cells. Interfering with this intricate metabolic-signaling network profoundly impacts on tumor development and cancer cells spreading[8].

One of the well-known metabolic change that takes place within most of the malignant tumors is the aerobic glycolysis, also known as 'Warburg effect'. This is a major metabolic shift that allows tumor cells to use glucose trough the fermentative pathway, regardless of oxygen availability. As consequence, large amounts of lactate are produced by tumor cells. Although initially supposed, the Warburg effect does not imply genetic defects of the components of the respiratory chain. In some tumors, the activity of the oxidative phosphorylation machinery is even increased[25]. Several mechanisms have been identified as responsible of the metabolic rewiring of cancer cells. The transcription factor HIF-1α, which accumulates within the tumor cells as consequence of oxidative stress or hypoxia, upregulates the glycolytic pathway[26]. Activation of several oncogenes, as K-Ras, c-Myc, phosphatidylinositol-3(PI3) kinase, or genetic inactivation of tumor suppressor genes, including p53 and PTEN (phosphatase and tensin homolog), increase glycolysis and promote mitochondrial fission and mitophagy[27]. Moreover, PI3K/AKT signaling pathway positively regulates mTOR-dependent anabolic pathway and mitochondrial biogenesis [28, 29]. mTOR also increases the levels of the glutaminase (GLS) and glutamate dehydrogenase (GDH), two enzymes involved in the conversion of glutamine to glutamate, and glutamate to α-ketoglutarate (α-KG), respectively. α-KG fuels the TCA cycle and can be also converted to isocitrate by the isocitrate dehydrogenases (IDHs). Isocitrate is used for biosynthetic and redox reactions[30, 31]. PI3K/AKT/mTOR pathway upregulates the expression of methylen-tetrahydrofolate dehydrogenase 2 (MTDHD2), the rate-limiting enzyme of the folate pathway, thereby stimulating the nucleotide synthesis. [32].

Nutrient limitation that often occurs within cancer cells leads to the activation of nutrient-sensing AMP-regulated kinase (AMPK). Activation of AMPK induces mitochondrial biogenesis and energy production. AMPK activation also controls the autophagic clearance of damaged mitochondria (mitophagy) that might otherwise negatively affect cancer cell growth[33, 34]. Interestingly, under conditions of increased energy demands, AMPK directly phosphorylates AKAP1, thus stimulating both the respiration and mitochondrial metabolic activities[35]. This represents a fundamental mechanism that allows normal tissues to adapt under increased energy demands, and it may also sustain tumor growth and widespread dissemination of cancer cells in the presence of limited nutrients availability.

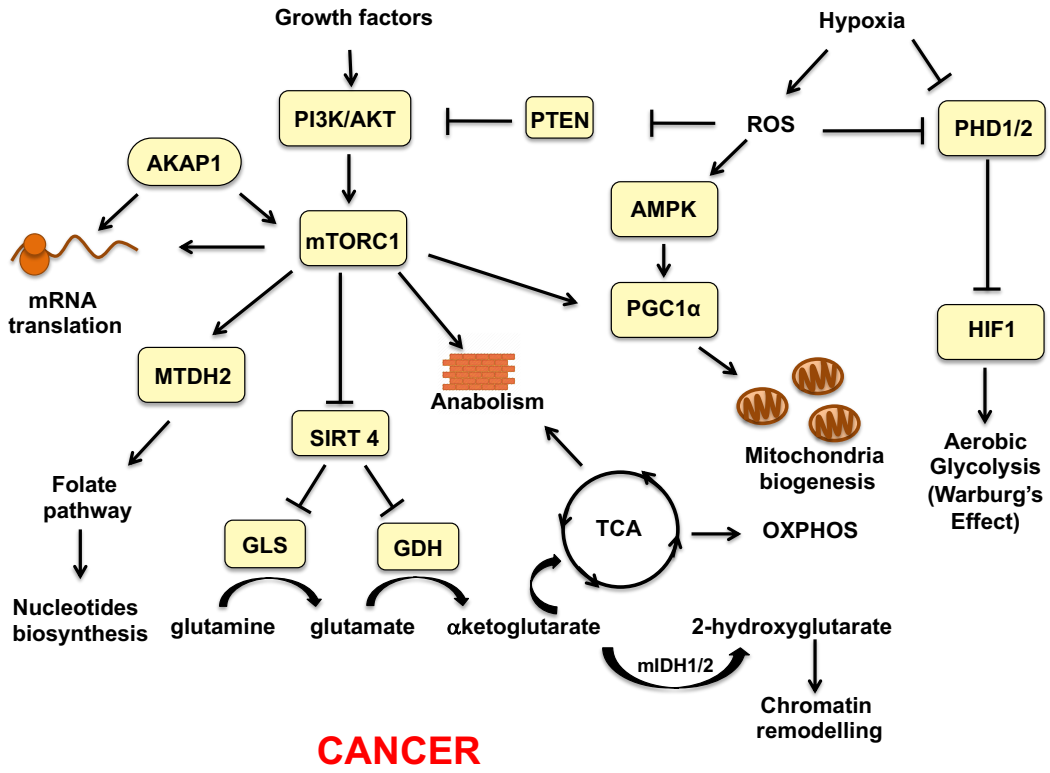


Figure 6. Mitochondria metabolism in cancer cells. Aberrant activation of the PI3K/AKT pathway increases the mTOR-dependent anabolic route, inducing mitochondrial biogenesis and transcriptional repression of SIRT4. By removing the inhibitory constrain of SIRT4 on glutaminase (GLS) and glutamate dehydrogenase (GDH), mTOR supports α-ketoglutarate production and the TCA cycle. In some cancer types, aberrant conversion of α-ketoglutarate to 2-hydroxyglutarate (2-HG) by mutated IDH1/2 induced chromating remodeling and transcription of cancer-associated genes. PI3K/AKT/mTOR pathway also activates the methylene-tetrahydrofolate dehydrogenase 2 (MTDHD2)-dependent folate pathway, thereby promoting nucleotide biosynthesis. mTOR upregulates PGC1a-dependent mitochondrial biogenesis and anabolism. Mitochondrial AKAP1 promotes translation and positively contributes to mTOR-dependent signaling cascade. Activation of AMP-regulated kinase (AMPK) by ROS stimulates a PGC-1α-dependent antioxidant circuitry that further supports mitochondrial homeostasis and metabolism. Elevation of ROS levels also inhibits prolyl hydroxylases (PHDs) and leads to accumulation of HIF1. HIF1-depedent upregulation of glycolytic enzymes contributes to the Warburg's effect (*BBA Rev.Cancer 2018 1869(2):293-302*).

1.7 Role of ATM/ATR kinases in mitochondrial metabolism. ATM (Ataxia-telangiectasia mutated kinase) and ATR (ATM- and RAD3- related kinase) are protein kinases belonging to the superfamily of phosphatidylinositol 3-kinase-related kinases (PIKKs). These are serine/threonine kinases that are activated following the DNA damage induced by chemicals or ionizing radiations. Once activated, ATM/ATR phosphorylate a wide array of cellular substrates, including p53, BRCA1, NBS1, histone

H2AX, CHK and other tumors suppressors, leading to cell cycle arrest which can culminate with DNA repair or apoptotic cell death[36]. Mutation of ATM/ATR pathway, identified in the human Ataxia-telangiectasia disorder, thus, affects the ability of cells to cop the oxidative stress and DNA damage, leading to premature senescence and cell death[37]. The mechanism of ATM/ATR kinase activation and downstream signaling pathways have been largely described. Additionally, recent evidence indicate that ATM activation can also be triggered by free oxygen radicals (ROS). ROS-mediated oxidation of ATM monomers leads to dimerization and consequent activation of ATM kinase in a DNA damage-independent manner. Active ATM reduces ROS levels, induces elimination of damaged mitochondria (mitophagy), regulates protein homeostasis and supports ROS-induced autophagy pathway[38]. ATM also work as a positive regulator of mitochondrial activity. Thus, active ATM phosphorylates and activates the nuclear respiratory factor 1 (NRF1), a nuclear transcription factors that control the expression of genes involved in mitochondrial biogenesis, respiratory chain activity and metabolic activity[38]. ATM also works as a major controller of the anti-oxidant pathway. ATM-mediated phosphorylation of the nuclear transcription factor erythroid 2-related factor (NRF2) activates the transcription of antioxidant genes that counteract the effects of the oxidative stress. Therefore, given the wide array of cellular activities controlled by the ATM/ATR pathways, deregulation of the ATM pathway, as it occurs in A-T patients, deeply impacts on different cellular mechanisms, as DNA repair, mitochondrial biogenesis and metabolism, stress responses and anti-oxidant defenses. Although ATM plays a pleiotropic and a fundamental role in the metabolic pathways, however the role of DNA damage-induced kinases in GPCR-induced signaling events at mitochondrial compartment and the impact on cAMP-regulated metabolic rewiring in cancer cells are still unknown.

1.8 Targeting mitochondria in cancer therapy. The metabolic and anabolic pathways in cancer cells are supported by the energy production within the mitochondrial compartment. Mitochondria also produce the majority of reactive oxygene species (ROS), which underlie to the high mutagenic rate and genome instability of cancer cells that contributes to cancer growth and widespread metastasis. However, ROS accumulation may also lead to apoptotic tumor cell death. Accordingly, a strategy to kill cancer cells is based on the use of oxidants. Thus, co-treatment with 2-methoxyestradiol (2-ME) and the microtubule-disrupting agent CYT997 or with metformin enhances ROS production and induces tumor cell death [39, 40]. Since some cancers carry overexpression of anti-apoptotic proteins (Bcl-2 or IAP family members) or mutations of pro-apoptotic factors (BAX and BAK) the pro-oxidant treatment may have no effects on tumor development. For these reasons, it is important to identify novel therapeutic strategies to target the apoptotic pathway in cancer cells.

Clinical trials based on Bcl-2 inhibitors (Venetoclax) and molecules that interfere with the anti-apoptotic activity of IAP (Inhibitors of Apoptosis Proteins) are currently at work [41, 42].

The identification of genetic mutations of metabolic enzymes, as IDH1 and IDH2, pathogenically linked to tumor growth and development, allowed the generation of new set of tumor inhibitors that selectively target mutant IDH1/IDH2. Inhibiting glycolytic or respiratory enzymes, as well as interfering with the activity of components of the OMM permeability system, opened novel therapeutic windows for cancer treatment[43-45].

Given the importance of AKAP scaffolds in the control of signaling pathways in normal as well as in cancer cells, modulating AKAP signaling is expected to deeply impact on cancer cell biology and tumor growth. Therapeutic approaches with AKAP inhibitors have been experimentally tested in translational medicine and the results strongly encouraged in considering AKAP modules as potential targets for cancer therapy. Accordingly, synthetic peptides spanning the amphipathic helix of AKAP, when perfused in cells and in tissues, displace PKA from intracellular pools and inhibit cAMP-dependent events on target proteins. As consequence, disrupting AKAP-regulated targeting of PKA signals affects ion channel activities, neuronal transmission, synaptic plasticity, learning and memory, immune responses, metabolism and cell survival[8, 46-48]. The major problem with this approach relies mostly on the off-target effects of the displacing peptides that limit their use in clinical trials. A more direct and safe procedure is the use of inhibitors for selected classes of AKAPs, such as PPI disruptors that interfere with the regulation of the AKAP module by disease-activated pathways. In the case of AKAP1, delocalization of AKAP1 complex from mitochondria using synthetic peptide spanning the mitochondrial targeting domain of AKAP1 had been successfully used to interfere with respiration and metabolism, and to induce oxidative stress and apoptosis in cardiomyocytes, both in cell cultures and in vivo[49]. This approach might be even more efficient in inhibiting oncogenic pathways and metabolic activities, ultimately leading to cancer cell death. Downregulation of AKAP levels represents an alternative strategy to interfere with AKAP-regulated oncogenic pathways. As example, the use of small RNA molecules (siRNAs and miRNAs) targeting mRNA for selected classes of AKAPs, including AKAP1, can be employed to inhibit the growth and progression of AKAP-addicted cancers. Selective downregulation of AKAP1 decreases mitochondrial respiration and ATP synthesis[50]. In neurons and in cardiomyocytes, prolonged downregulation of AKAP1 induces oxidative stress and mitochondrial fragmentation, that eventually leads to mitophagy and cell death[51, 52]. Based on these findings, we envisaged the possibility to selectively inhibits mitochondrial metabolism in cancer cells, thereby affecting the tumor aggressiveness. Accordingly, we provided proof-of principle of the validity of this strategy, by demonstrating that downregulation of AKAP1 by RNAi significantly inhibited cancer growth in an

orthotopic mouse model of human glioblastoma[53]. This finding indicates that mitochondrial AKAP1 constitutes the Achille's heel of chemo-resistant cancer cells. Pharmacological interference with signaling events regulated by AKAP1 at mitochondria may likely interrupt the trophic signals of oncogenic pathways that are directed by the organelles, preventing the metabolic rewiring occurring in cancer cells and tumor growth and dissemination.

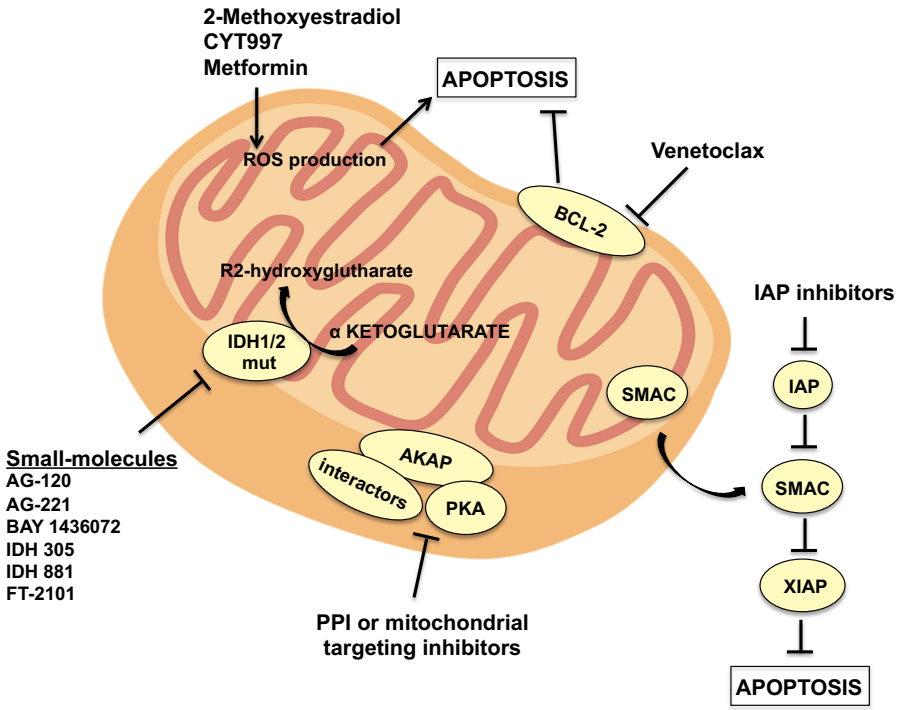


Figure 7. Targeting mitochondria and AKAP1 for cancer therapy. To selectively kill cancer cells, different experimental approaches have been designed to inhibit mitochondrial metabolism. Thus, forced overeproduction of mitochondrial ROS by treatment with 2-methoxyestradiol (2-ME), microtubule-disrupting agents (CYT997) or anti-diabetic drugs (metformin) efficiently enhances tumor cell killing. Similarly, inhibiting the anti-apoptotic machinery of cancer cells using bcl-2 antagonists (Venetoclax) or IAP inhibitors have been successfully used as cancer chemotherapeutics. Moreover, highly selective inhibitors of mutated IDH1/2 have been recently developed and are currently being used as chemotherapeutics in several clinical trials. Finally, targeting compartmentalized cAMP signaling at mitochondria using protein-protein interaction (PPI) inhibitors or small molecules interfering with mitochondrial localization of AKAP complexes have potential relevant implications as novel anti-cancer chemotherapeutics (*BBA Rev.Cancer 2018 1869(2):293-302*).

2. AIM OF THE STUDY

Mitochondria are essential organelles for all eukaryotic cells and control many aspects of cell biology, respiration, survival and metabolism. Proteomic analysis and functional screening identified a variety of signaling molecules, adapter proteins, receptors, channels, transcription factors and regulators as components of the mitochondrial compartment. The correct integration of distinct signaling events and metabolic pathways with the respiratory chain activity supports and sustains cell growth and survival. This aspect is of relevance for the development, progression and widespread dissemination of different types of cancers. The metabolic reprogramming that characterizes most of the human tumors provides all the energetic needs to fuel the cellular activities and to support the production of basic building blocks for cancer cells. The identification of genetic mutations involving mitochondrial metabolic enzymes that are essential for the growth of certain types of cancer further supports the concept that mitochondria actively and primarily participate in oncogenic pathways. Thus, mitochondria and its relevant players in signaling and metabolism, including AKAP1, represent valuable targets for novel therapeutic approaches for cancer treatment.

Accordingly, my thesis was aimed to:

1. Characterize the mechanism(s) regulating the expression of AKAP1 in cancer cells.

2. Define the role of AKAP1 in the metabolic rewiring of cancer cells.

3. Provide proof-of principle that inhibition of AKAP1 affects cancer growth and metabolism.

4. Demonstrate that AKAP1 pathway can be regulated by cellular stress in course of DNA damage

3. MATERIALS AND METHODS

Cells and tissues. The human glioblastoma cell line U87MG (grade-IV) and the human epithelial cancer cells, derived from breast and prostate tissues were purchased from the American Type Culture Collection (ATCC) and maintained in modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum and 2mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 1x non-essential amino-acid, 1mM sodium pyruvate, at 37°C, 5% CO2 and 95% of humidity. MCF10A were cultured in 1:1 mixture DMEM-F12 supplemented with 5% horse serum, 10 µg/ml insulin, 0,5 µg/ml hydrocortisone, 100 ng/ml cholera enterotoxin, and 20 ng/ml epidermal growth factor, and incubated at 37°C in humidified atmosphere with 5% CO2. MCF10a-MycER cells were starved by growth in minimal medium (1:1 mixture DMEM-F12 supplemented with 5% horse serum) for two days. Myc induction was obtained by treating the cell for the indicated time with complete medium with 600nM of 4-hydroxytamoxifen (OHT).

Antibodies and chemicals. Anti-human AKAP1 antibody was purchased from Bethyl Laboratories; anti-pT389-S6K antibody was purchased from Cell Signaling; anti-S6K and anti-RIIβ antibodies were purchased from Santa Cruz Biotechnology; anti-phospho(Ser345)CHK1 and anti-phospho-ATM/ATR substrates antibodies were purchased from Cell Signaling Technologies; anti-c-Myc (N262) was purchased from Santa Cruz Biotechnologies;

Transfection of plasmids and siRNAs. Vectors encoding for wild-type or mutant AKAP1 were previously described[53]. Transfection efficiency was monitored by including a GFP vector in the transfection mixture. ON-TARGET plus siRNA targeting coding regions of human AKAP1 was purchased from Dharmacon. The siRNA sequence (Thermo Scientific) targeting human AKAP1 is the following: GGGAGCAUGUCUUGGAAUU. Control and siRNA targeting AKAP1 were transiently transfected using Lipofectamine 2000 (Invitrogen) at a final concentration of 100 pmol/ml of culture medium. For siRNA experiments, similar data were obtained using a mixture of four or two independent siRNAs.

Immunoprecipitation and western blot analysis. Cells were washed twice with phosphate-buffered saline and lysed in Tris-buffered saline buffer-1% Triton-X 100 (NaCl, 150 mM; Tris-HCl, 50 mM, ph 7,5; EDTA, 1 mM; NaF, 1 mM; Na4P2O7, 1 mM; Na3VO4, 0.4 mM). Whole cell lysates (WCE) (100 µg) were resolved on sodium dodecyl sulfate polyacrylamide gel and transferred on nitrocellulose membrane (Biorad, Milan, Italy) for 3 h. Filters were blocked for 1 h at room temperature in Tween-20 Phosphate buffer saline (TPBS) (PBS- Sigma, 0,1% Tween 20, pH 7.4)

containing 5% non-fat dry milk. Blots were then incubated O/N with primary antibody. Blots were washed three times with TPBS buffer and then incubated for 1 h with secondary antibody (peroxidase-coupled anti-rabbit (GE-Healthcare) in TPBS. Reactive signals were revealed by enhanced ECL Western Blotting analysis system (Roche). Mitochondrial and cytosolic fractions were prepared as described[54].

qRT-PCR. RNA was extracted from MCF10-MycER cells using EuroGold Trifast (EuroClone). cDNA was generated using Quantitec Reverse Transcription Kit (Qiagen), according to manufacturer's protocol. Quantitative analysis was performed using SYBR Green 2X PCR Master Mix (Applied Biosystem). Each sample was run in triplicate and normalized to the expression of housekeeping beta-glucoronidase (GUS) gene as previously described[55]. The primers used in qPCR are: AKAP1, TTCTCTGCCGATGACATCCT and CATTGACCTGGTTGACC ACA; GUS GGAATTTTGCCGATTTCATGA and CCGAGTGAAG ATCCCCTTTTT.

Implantation of U87MG cells in mouse brain. Male CD1 nude mice (Charles River Laboratories; 20–22 g body weight) were housed in controlled conditions (temperature 22°C; humidity 40%) on a 12-hour light/dark cycle with food and water ad libitum and observed daily. The experiments were performed according to the guidelines for the care and use of animals promulgated by the National Institutes of Health (Bethesda, MD). 48 hours before implantation, U87MG cells were transfected with control siRNA or siRNAs targeting AKAP1. The cells ($4x10^5$ cells/animal) were then resuspended in DMEM without FCS and implanted in the caudate nucleus of the mice using the following stereotactic coordinates: 0.6 mm anterior to the bregma, 1.7 mm lateral to the meridian line, 3.5 mm in depth from the surface of the skull. Animals were anesthetized intraperitoneally with ketamine (100 mg/kg) and xylazine (10 mg/kg). Twenty-eight days after inoculation, mice were sacrificed by cervical dislocation after anesthesia, and their brains removed, fixed in formalin, dehydrated in ethanol at increasing concentrations, and embedded in paraffin. From each brain, 10-μm-thick serial sections, from the beginning of the striatum to the hippocampus (1section every 400 μm), were sliced. Subsequently, the sections were stained with Mayer's hematoxylin and eosin (both from Diapath) and subjected to analysis for the quantification of tumor volume. The volumetric analysis was performed using software that measures the tumor area in each section and calculates the total volume of the tumor according to the Cavalieri method by using the following formula: V= S(A)i x TS x n., where (A)i is the area of the tumor in level i, TS is the section thickness, and n is the number of sections disposed between the 2 levels[56].

Immunohistochemistry. Briefly, formalin-fixed and paraffin-embedded tissue sections (4 μm) were deparaffinized in xylene and rehydrated with graded ethanol. 60 samples of prostatic tumors, 10 cases of benign prostatic hyperplasia, and 10 cases of breast carcinomas were collected from the archive of the dept of Advanced Medical Science, section of the Anatomic Pathology, University Federico II Naples-Italy. Relatively to the prostatic tumors 30 cases were high grade PCa of which 20 cases with 4+4=8 Gleason score, and 10 cases with 5+4=9 Gleason score. 30 cases were low-grade PCa with 3+3=6 Gleason score. Moreover, 10 cases of benign prostatic hyperplasia were selected (**Fig.10B**). About the breast carcinomas: 5 cases luminal B $HER2^+$, 5 cases luminal B $HER2^-$ and 5 cases basal-like triple negative. Bioptic samples of GBM were surgically removed from Neuromed patients. All patients gave their informed consents and were shown to carry glioblastoma multiforme (according to WHO classsification). All tumors were positive for vimentin, GFAP (glial fibrillary acidic protein) and EGFR. Immunostaining for AKAP1 of all cases was evaluated. Antigen retrieval was carried out in citrate buffer (pH = 6.0, 12 min, microwave oven). Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide for 12 min. Non-specific binding sites were blocked with 5% normal horse serum in TBS-Tween (Wash buffer, Dako, Glostrup, Denmark) for 30 minutes. Sections were incubated with primary antibody (AKAP1) overnight at 4°C. All sections were visualized using the Liquid DAB Substrate Chromogen System for peroxidase (DakoCytomation) and were counterstained with hematoxylin, dehydrated and mounted. In particular, a score of 0–3 was given: 0, negative staining; 1, weak expression in 20% of the cytoplasm of tumor cells; 2, moderate or strong expression in 20–75% of tumor cells; 3, strong expression in > 75% of tumor cells. A score of 0 or 1 was considered as negative, and a score of 2 or 3 was considered positive (see **Figs. 8, 10A and 11A**).

Statistical Analysis. Statistical analysis of tumor volume in CD1 nude mice was performed using Student's *t*-test. All data were expressed as mean ± SEM. Statistical comparisons between experimental groups were performed using the *t*-test or one-way analysis of variance when required. *P value* < 0.05 was considered statistically significant.

Metabolic assays. The metabolic profile was monitored in control U87MG cells or in cells subjected to AKAP1 silencing. Real-time measurements of oxygen consumption rate (OCR) were made using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were plated in XF-96 plates (seahorse Bioscience) at the concentration of 2 x 10^4 cells/well and cultured for the last 12 hours in DMEM, 10%FBS. OCR was measured in XF media (non-buffered DMEM medium, containing 10 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate), under basal conditions and in response to 5 μM oligoMycin, 1.5 μM of carbonylcyanide-4- (trifluoromethoxy)-phenylhydrazone (FCCP) and

1 μM of AntiMycin and Rotenone (all from Sigma Aldrich). Indices of mitochondrial respiratory function were calculated from OCR profile: basal OCR (before addition of oligomycin), ATP-linked OCR (calculated as the difference between basal OCR rate and oligomycin-induced OCR rate) and maximal OCR (calculated as the difference of FCCP rate and antimycin+rotenone rate).

BrdU staining and FACS analysis Immunostaining analysis of incorporated bromo deoxyuridine (BrdU), an analog of the DNA precursor thymidine, and of 7-aminoactinomycin D (7-AAD), a DNA staining marker, allow the cell cycle phase distribution of the cells (G0/1, S, or G2/M phase) by florescence-activated cell sorting analysis (FACS). The human glioblastoma cell line U87MG was transiently transfected with control siRNA or with siRNA targeting the endogenous AKAP1. Thirty-six hours from transfection, cells were processed according to the BrdU Flow Kit protocol BD Pharmingen by FACS analysis. The U87MG cell culture was stained with BrdU (final concentration of 10 μM). 10 μL of the 1 mM solution BrdU was added to each mL of culture medium. The cell culture density should not exceed 2 x 10^6 cells/mL. Cells were maintained for 30-45 minutes (ie, when the cells are in the logarithmic phase of cell proliferation) at 37°C, 5% CO2 and 95% of humidity and then collected in 1 ml PBS1X, centrifuged and resuspended in BD cytofix/cytoperm buffer (100 μl sample) and incubated for 30 minutes on ice. Cells were washed with 1 mL of 1X BD Perm/Wash Buffer, collected by centrifugation for 5 minutes at 250*g*, resuspended in 100 μL of BD Cytoperm Permeabilization Buffer Plus (a staining enhancer and secondary permeabilization reagent) and incubated for 10 minutes on ice. Cells were washed in 1 mL of 1X BD Perm/Wash Buffer, re-fixed as in the previous step and treated with DNase (30 μL of DNase/106 cells) at 37°C for 1 hour. Cells were washed in 1 mL of 1X BD Perm/Wash Buffer and stained with FITC anti-BrdU antibody and 7-AAD solution. The cell cycle positions and DNA synthetic activities of U87MG cells was determined by analyzing the correlated expression of total DNA and incorporated BrdU levels by FACS. The measurement of cell-incorporated BrdU (with anti-BrdU FITC) and total DNA content (with 7-AAD) are displayed on a linear scale, as shown on the x and y-axis. The regions for the quantitative cell cycle analysis of cell populations are: R3 (G0/G1), R4 (S phase), R5 (G2 + M).

4. RESULTS

4.1 AKAP1 is overexpressed in human cancer tissues. Given the role of AKAP1 in mitochondrial metabolism and cell survival, I sought to investigate whether AKAP1 levels are, thus, regulated in human cancers. I analyzed AKAP1 accumulation in different human cancer cells and also in human samples from tissue biopsy by immunostaining analysis using specific anti-AKAP1 antibody. As shown in **Fig.8,** AKAP1 is expressed at high levels in human breast cancer tissues. In particular, I observed overexpression of AKAP1 protein in luminal B breast cancer tissue samples and no major differences was seen between luminal B HER2^{+} and luminal B HER2^{-} histologic subtypes. A similar overexpression pattern of AKAP1 was observed in triple negative basal-like cancer tissue samples.

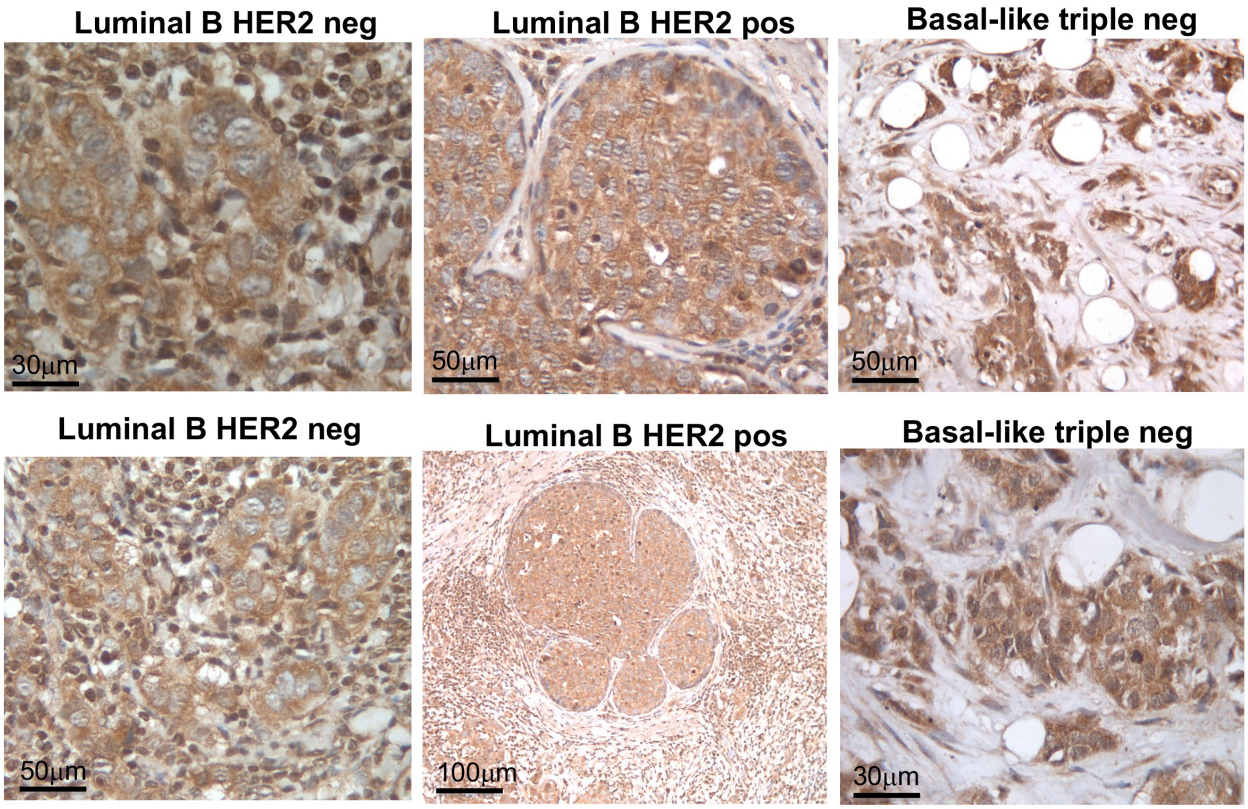


Figure 8. Expression analysis by Immunohistochemistry of AKAP1 in breast cancer tissues. Tissue sections from breast cancers (luminal B-HER2+ and luminal B-HER2^{-}, Basal-like triple negative) were formalin-fixed and immunostained with anti-AKAP1 antibodies. Size bars are indicated in the panels.

I also analyzed the expression of AKAP1 in breast cancer cells. As shown in **Fig. 9,** AKAP1 is expressed at significant levels in most of the breast cancer cells analyzed.

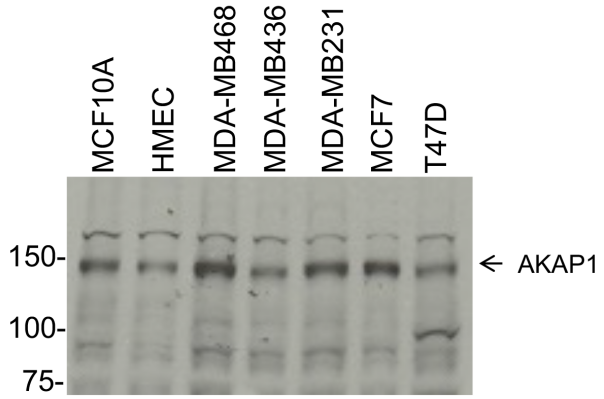
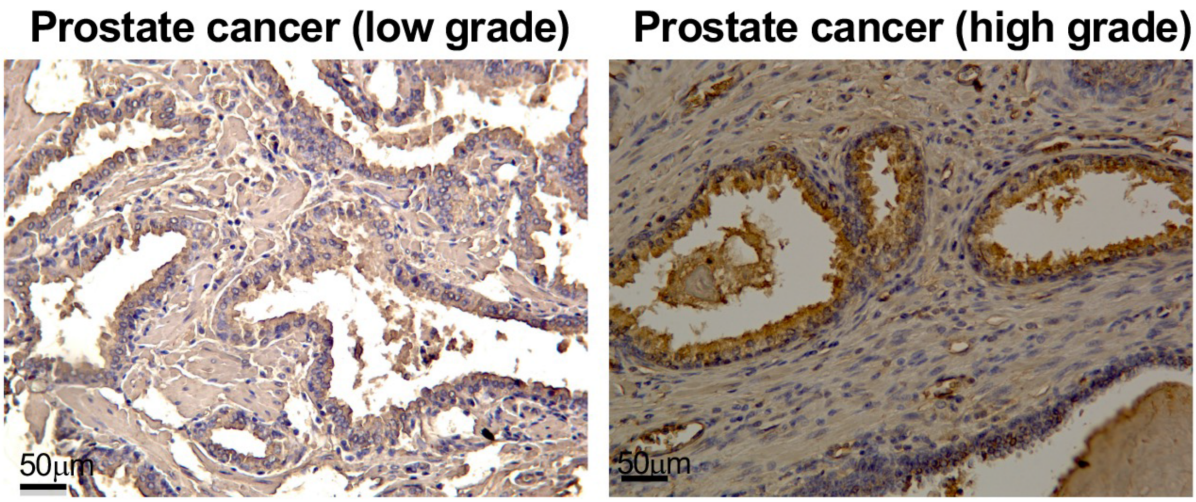


Figure 9. Immunoblot analysis of AKAP1 in breast cancer cells. Total lysates prepared from cultured breast cancer cells (HMEC, MDA-MB468, MDA-MB436, MDA-MB231, MCF7, T47D) and from a non-tumorigenic epithelial breast cell line (MCF10A) were size-fractionated on SDS-PAGE gel and immunoblotted with anti-AKAP1 antibodies.

Next, I reasoned that AKAP1 expression might also involve other epithelial malignant, hormone sensitive lesions, including prostate cancer. To this end, I performed an AKAP1 immunostaining analysis in tissue samples isolated from low-grade and high-grade human prostate cancer tissues. **Fig. 10A** shows that AKAP1 levels were less pronounced in low-grade tumors, whereas high levels of AKAP1 could be detected in high-grade prostate cancer tissues. Immunoblot analysis on total cellular lysates confirmed the expression of AKAP1 in several prostate cancer cells **(Fig. 10C)**.

A



B

Cases		GS	AKAP1 staining
HG-PCa	20	4+4=8	high
HG-PCa	10	5+4=9	high
LG-PCa	30	3+3=6	low
BPH	10	NA	low

C

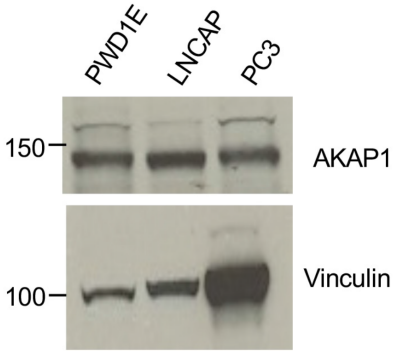


Figure 10. Expression analysis of AKAP1 in prostate cancer tissues and cell lines. A. Immunohistochemitry for AKAP1 in formalin-fixed high-grade (IHC score 3) and low-grade (IHC score 0/1) prostate cancer bioptic tissues. Size bars are indicated in the panels. **B**. Correlation of Gleason score and AKAP1 expression in PCa and BPH. PCa= prostatic carcinoma; HG= high-grade; LG= low-grade; BPH= benign prostatic hyperplasia; AKAP1 staining by immunohistochemistry; NA not applicable. **C.** Immunoblot analysis of AKAP1 in cultured prostate cancer cells (PWD1E, LNCAP, PC3).

I extended the expression analysis of AKAP1 also in non-epithelial cancer samples, such as glial tumours. I found a marked immunostaining of AKAP1 in high-grade glioma tissues (glioblastoma, GBM), whereas low levels of AKAP1 were evident to low grade lesions (glioma II, astrocytoma) (**Fig. 11A**). The overexpression of AKAP1 in GBM samples was confirmed by performing immunoblot assay on total lysates prepared from high-grade and low-grade glioma tissues (**Fig. 11B**). Moderate levels of AKAP1 protein could be detected in glioma III tissue samples.

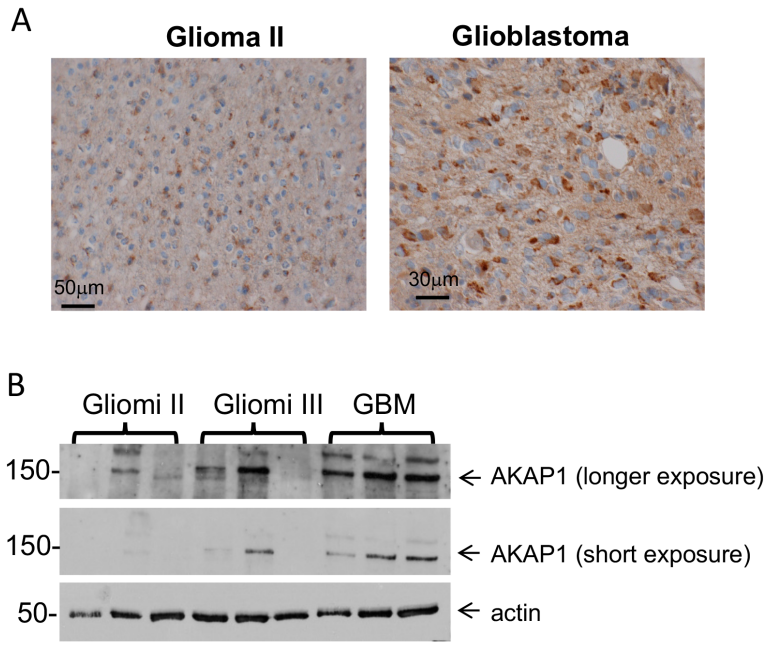


Figure 11. Expression analysis of AKAP1 in glioma tissues. A. Immunohistochemistry for AKAP1 on formalin-fixed tissue sections from astrocytoma (glioma II) and glioblastoma. Size bars are indicated in the panels. **B**. Total lysates prepared from low grade (glioma II) and high-grade gliomas (glioma III and glioblastoma) were immunoblotted with the anti-AKAP1 antibody. Actin levels were monitored as loading control.

4.2 Regulation of akap1 expression by Myc proto-oncogene and steroid hormones. Genomic analysis of the available ENCODE Data on AKAP1 gene allowed the identification of a putative Myc binding domain in the promoter region of AKAP1. Therefore, I analyzed whether Myc regulates AKAP1 transcription. To this aim, I used a non-transformed mammary epithelial cell line that stably express a chimeric gene composed of Myc fused to the estrogen receptor (ER) (MCF10A-MycER). In these cells, treatment with the ER inhibitor 4-hydroxytamoxifen (+4-OHT) activates Myc, which in turn stimulates Myc-dependent gene transcription. Cells were serum deprived overnight and then treated with serum containing +4-OHT for 12 and 24 hrs. **Fig. 10A** and **Fig. 10B** show that +4-OHT treatment induced a significant upregulation the mRNA and protein levels of AKAP1 which paralleled the accumulation of myc.

The data above indicate that AKAP1 is highly expressed in prostate cancer tissues. Since androgens play a pathogenic role in the development and progression of prostate cancer, I investigated if the androgen stimulation promotes AKAP1 accumulation. I used LNCAP cells that are androgen-sensitive and commonly used as prostate cancer cell model[57]. These cells were stimulated with the synthetic non-metabolizable androgen R1881 and harvested at the indicated time points from

stimulation. Immunoblot analysis on total cellular lysates showed a marked upregulation of AKAP1 expression by R1881 treatment (**Fig. 12C**). The effects of R1881 on AKAP1 expression were almost abrogated co-treating cells with Casodex, an anti-androgen medication that is primarily used for the treatment of cancer prostate patients (**Fig 12D**).

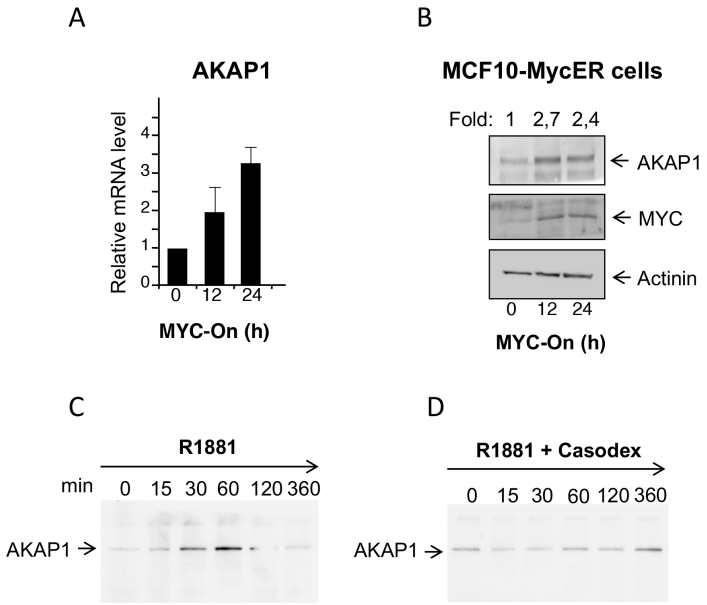


Figure 12. Transcriptional regulation of AKAP1 by Myc and steroids. A. MCF10-MycER cells were serum deprived overnight and then treated with the ER inhibitor 4-hydroxytamoxifen (+4-OHT) for the indicated time points. Accumulation of AKAP1 mRNA was monitored by quantitative RT-PCR. *p<0.01. **B**. Total lysates from cells treated as in A were immunoblotted for AKAP1, Myc and actinin (loading control). Fold changes over basal value (set as 1) are indicated. **C**. Prostate cancer cells (LNCAP) were left untreated or stimulated with R1881. Cells were harvested at the indicated time points from stimulation. Total lysates were immunoblotted with the anti-AKAP1 antibody. **D**. Same as in C, with the exception that cells were co-treated with the anti-androgen Casodex. Total lysates were immunoblotted with the anti-AKAP1 antibody.

4.3 AKAP1 controls oxidative metabolism in cancer cells. AKAP1 is a mitochondrial scaffold protein that controls cAMP events on the organelles. In normal cells, AKAP1 plays a major role in cell survival and oxidative pathway[58]. Since AKAP1 was overexpressed in the majority of human cancer tissues and cells, I sought to investigate the role of AKAP1 in the oxidative pathway of cancer cells. As model system, we used U87-MG cells. This is a human glioblastoma cell line derived from a malignant glioma and when injected in the brain of nude mice reproduces a malignant glial tumour[56]. U87-MG cells were transfected with siRNA targeting human AKAP1 or with control siRNA. Immunoblot analysis confirmed the downregulation of AKAP1 by selective RNAi molecules

(**data not shown**). Controls and AKAP1-silenced cells were subjected to metabolic analysis using the Seahorse apparatus. As readout of mitochondrial oxidative capacity, I tested the Oxygen Consumption Rate (OCR) of siRNA-transfected cells under basal conditions or in the presence of oligomycin (ATP sintase inhibitor), carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (a mitochondrial protonophore uncoupler), as well as of rotenone and antimycin A (two mitochondrial transport chain inhibitors). The treatment is useful to define the contribution of AKAP1 silencing on the basal and ATP-linked OCR. As shown in **Fig. 13A** and **Fg.13B**, genetic silencing of AKAP1 dramatically impaired the mitochondrial respiration and the Spare Respiratory Capacity, compared to control cells. As expected, the ATP production (total and basal rate) was compromised in AKAP1-silenced cells (**Fig. 13C** and **Fig.13D**). These data indicate that AKAP1 is an important regulator of oxidative metabolism in U87-MG cells.

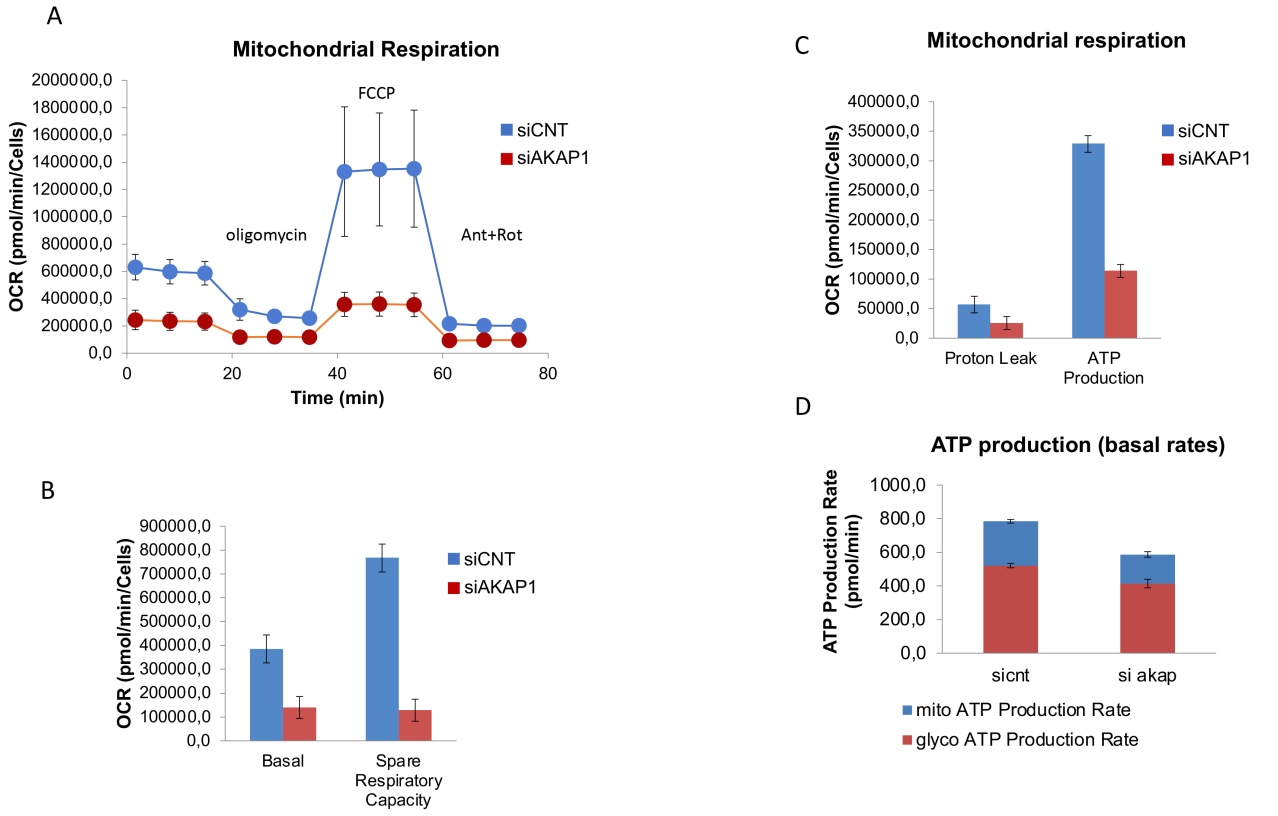


Figure 13. AKAP1 regulates mitochondrial respiration and ATP synthesis in glioblastoma cells. U87-MG cells were transiently transfected with siRNA targeting AKAP1 and then seeded on multi-well plates. **A.** Oxygen Consumption Rate (OCR) was dynamically measured in real time, both under basal condition (0) or in response to treatment with the following mitochondrial inhibitors: oligomycin, FCCP, Antimycin A and Rotenone. **B-C**. Indicated are the indices of mitochondrial respiratory function derived from the OCR profile of control (siCNT) and AKAP1-silenced cells (siAKAP1). **D**. Analysis of ATP (glycolytic and oxidative) production rate in control and AKAP1 silenced U87-MG cells.

4.4 AKAP1 is required for mTORC1 pathway and tumor growth. AKAP1 has been linked to the activation of protein translation at mitochondrial sites. Given the role of the mTOR protein kinase in the control of protein synthesis and cell growth, I tested if AKAP1 regulates mTOR activation. As suspected, I found that AKAP1 is required for proper activation of mTOR pathway. Thus, genetic silencing of AKAP1 dramatically impaired activation of mTORC1, as shown by reduced phosphorylation of the mTOR substrate p70S6K at threonine 389 in response to insulin stimulation (**Fig. 14A**)[53]**.** Activation of the mTOR has been mechanistically linked to tumor growth and inhibitors of mTOR pathway are currently used in clinical trials for cancer patients. Since AKAP1 is critical for mTOR activation and oxidative metabolism, I assessed the role of AKAP1 in cancer growth, both in cultured GBM cells and *in vivo*. U87-MG cells were transiently transfected with control siRNA or with siRNA targeting the endogenous AKAP1. Thirty-six hours from transfection, cells were harvested and subjected to Fluorescence-activated Cell Sorting (FACS) analysis. **Fig. 14B** show that AKAP1 silencing induced a discrete accumulation of cells in G1- and G2/M phase. Downregulation of AKAP1 had no major impact on cell viability of transfected cells, compared to controls (**data not shown**).

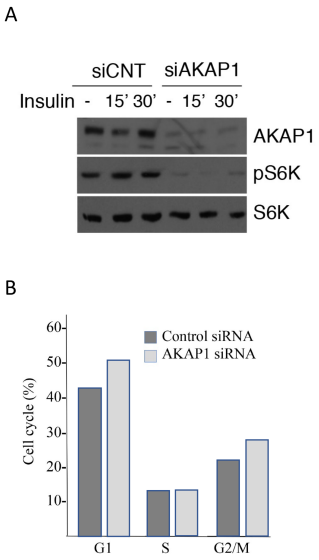


Figure 14. AKAP1 regulates mTORC1 activation and cell cycle progression. A. U87-MG cells were transiently transfected with control siRNA or siRNA targeting AKAP1. Twenty-four hours from transfection, cells were serum deprived and then stimulated with insulin for 15min and 30min. Cells were harvested and lysed. Lysates were immunoblotted with the indicated antibodies. **B**. U87-MG cells were transiently transfected with control (siCTRL) siRNA or with siRNA targeting AKAP1 (siAKAP1). Thirty-six hours from

transfection, cells were harvested and subjected to FACS analysis. A quantitative analysis of cell cycle phase distribution is shown.

Next, I examined the role of AKAP1 in cancer growth *in vivo*. To this aim, I used an orthotopic mouse model of human glioblastoma where U87-MG cells are implanted in the subventricular brain area of immune-compromised mice (nude mice) and tumor growth is analyzed four weeks later from injection. U87-MG cells transiently transfected with control siRNA or with siRNA targeting AKAP1, as above, were stereotaxically implanted into the left caudate nucleus of mouse brain. Four weeks later, the mice were sacrificed and total brain isolated and further analyzed. Histological analysis of post-mortem control brain revealed a homogenous tumor mass with sharp borders delimited from the adjacent normal brain tissue and composed of large pleomorphic cells with abundant eosinophilic cytoplasm. (**Fig. 15**). Interestingly, RNAi-mediated silencing of AKAP1 significantly reduced tumor size by about 2-fold compared to controls. These findings support a role of AKAP1 in cancer cell growth and tumour development *in vivo*.

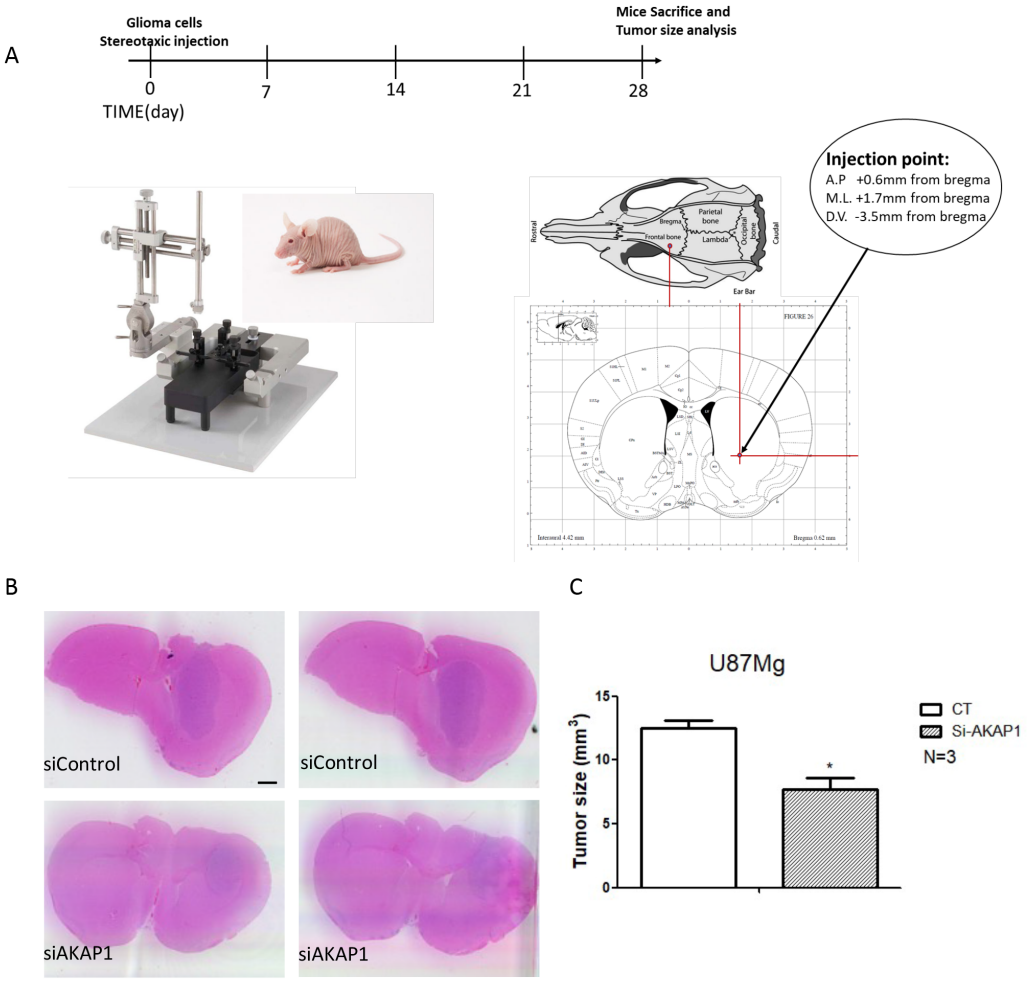


Figure 15. Genetic silencing of AKAP1 inhibits glioblastoma growth in vivo. A. Experimental design and stereotaxic injection apparatus with the indicated injection point in the brain of CD1 nude mice. **B**. U87-MG

cells were transiently transfected with control siRNA or siRNA targeting AKAP1. Twenty-four hours from transfection, cells were stereotaxically implanted into the brain (left caudate nucleus) of CD1 mice. Tissue coronal sections from tumor lesions were stained with hematoxylin/eosin; scale bar 500 μm. **C**. Volumetric analysis of tumor brain in nude mice. N, number of animals analyzed.

4.5 AKAP1 is a target of ATM/ATR kinases. An available phospho-proteomic data set analysis revealed that DNA damage induced by ultraviolet (UV) radiation promotes phosphorylation of AKAP1 by ATM/ATR kinases. Two highly conserved residues within the PKA-binding domain of human AKAP1 (ser315 and thr322) were identified as direct targets of ATM[59] (**Fig.16**).

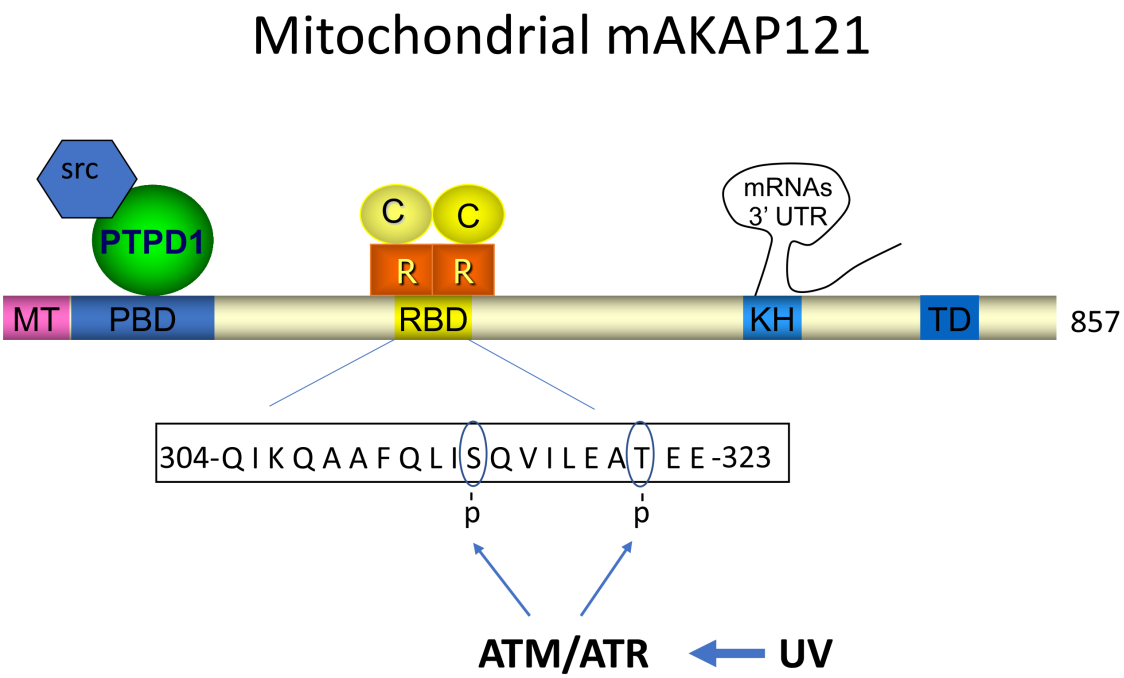


Figure 16. Mitochondrial AKAP1 protein. In the schematic view of mouse AKAP1, relevant domains are shown: mitochondrial targeting domain (MT), PKA binding domain (RBD), tyrosine phosphatase (PTPD1) binding domain (PBD), KH domain interacting with the 3'-untranslated region (UTR) of mRNA, tudor domain (TD) of unknown functions. Boxed is the RBD with the serine and threonine residues (circled) identified by phospho-proteomic screening in UV-irradiated cells[59].

Since DNA damage response plays a relevant role in mitochondrial metabolism, a glycolytic rewiring is usually observed in cells subjected to DNA damage[60]. Therefore, I hypothesized that ATM/ATR, by modulating AKAP1 scaffold activity, induces the glycolytic switch in DNA damaged-cells. Most of the ATM substrates, following phosphorylation, undergo to mobility shift on SDS-PAGE gel.

Accordingly, I analyzed the AKAP1 profile in cells treated with etoposide at different time points (1h-6h). As control of DNA damage kinase activation, I monitored the phosphorylation of Chk, a well-established ATM/ATR substrate. **Fig. 17** shows that etoposide treatment induced a mobility shift of AKAP1 starting at 1h from treatment, which long lasted for the next 3hrs. The mobility shift of AKAP1 observed in etoposide-treated cells was likely due to a phosphorylation event of the anchor protein by the ATM/ATR kinase pathway.

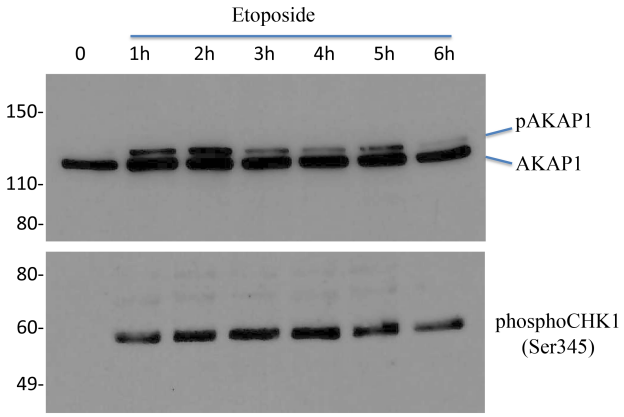


Figure 17. **Gel mobility shift AKAP1 following etoposide treatment.** Cells were left untreated or stimulated with etoposide and harvested at the indicated time points from treatment. Total lysates were size-fractionated and immunoblotted with anti-AKAP1 and anti-phosphoCHK1(ser345) antibodies. A representative set of experiments that gave similar results is shown.

To support this hypothesis and demonstrate that AKAP1 was, indeed, phosphorylated by ATM/ATR kinases, I monitored AKAP1 phosphorylation using anti-phospho-ATM substrate antibodies in cells exposed to etoposide, a topoisomerase II inhibitor that generates DNA double strand breaks and activates ATM/ATR kinases[61]. **Fig. 18** shows that short treatment with etoposide markedly induced AKAP1 phosphorylation at ATM/ATR sites.

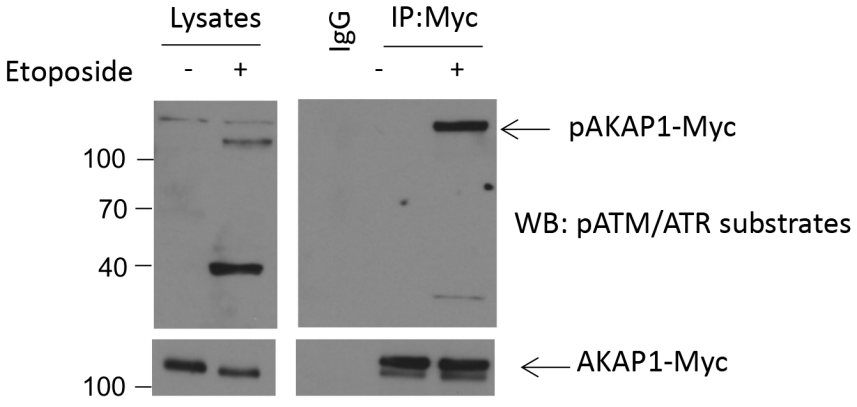


Figure 18. Phosphorylation of AKAP1 at ATM/ATR sites by etoposide. Cells were transiently transfected with AKAP1-Myc vector. Twenty-four hours later, cells were left untreated or treated with Etoposide for 60 min. Total lysates were immunoprecipitated with anti-AKAP1 antibody or with control IgG. The immunoprecipitates and an aliquot of lysates were immunoblotted with antibodies against phospho-ATM/ATR substrates and Myc epitope.

Placing a negatively charged phosphoryl group within the amphypatic helical wheel of the PKA binding domain of AKAP1, ATM/ATR activation should interfere with AKAP1/PKA interaction. I tested this hypothesis by monitoring the levels of PKA associated to mitochondria in cells treated with etoposide. **Fig. 19A** shows that treatment of cells with etoposide for 1h and 3h significantly reduced the levels of PKA-R subunits recovered in the mitochondrial fraction, which paralleled a concomitant increase of PKA-R subunits present in the cytosolic fraction. The distribution and accumulation of AKAP1 in mitochondrial and cytosolic fractions were largely unaffected by etoposide treatment. Next, I tested if ATM/ATR phosphorylation of AKAP1 affects its interaction with PKA-R. To this end, I generated a phospho-mimetic mutant of AKAP1 carrying substitution of Ser315 residue with aspartate (D) and tested its ability to bind PKA by co-immunoprecipitation assay. As shown in **Fig. 19C**, AKAP1-S315D mutant was nearly absent in the RIIβ immunoprecipitates, suggesting that AKAP1/PKA interaction is regulated by phosphorylation of the RII binding domain of AKAP1 by ATM/ATR kinases.

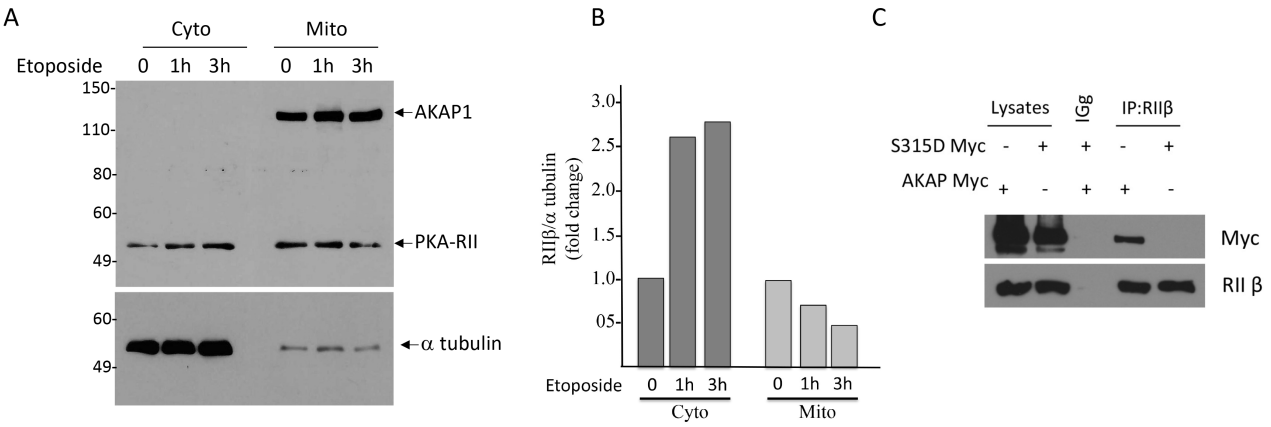


Figure 19. Etoposide treatment delocalizes PKA from mitochondria. **A.** Cells were left untreated or exposed to etoposide for the indicated time points, harvested and lysed. Mitochondrial and cytosolic fractions were prepared by differential centrifugation and immunoblotted with the indicated antibodies. **B**. Quantitative analysis of the experiments shown in A. A mean value of two independent experiments is shown. **C**. Total lysates from cells transiently transfected with Myc-tagged AKAP1, either wild type or S/D mutant, were

subjected to immunoprecipitation assays with anti-RIIβ antibody. Lysates and immunoprecipitates were immunoblotted with anti-Myc and anti-RIIβ antibodies.

4.6 DNA damage inhibits mitochondrial respiration in GBM cells. As shown above, AKAP1/PKA complex controls mitochondrial respiration and ATP synthesis in GBM cells. Since ATM/ATR phosphorylates AKAP1 and affects PKA targeting on mitochondria, I suspected that ATM/ATR activation by displacing PKA from mitochondria impacts on mitochondrial respiration. I tested this hypothesis by evaluating mitochondrial respiration in etoposide-treated cells. **Fig. 20A** and **Fig. 20B** show that treating U87-MG cells with etoposide for 6 hours reduced the mitochondrial respiration and ATP synthesis, compared to untreated, control cells. Pre-treating the cells with Kudos, a selective and potent inhibitor of ATM/ATR kinases, restored the ATP production rate in Etoposide-treated cells, suggesting a role of these kinases in mediating the metabolic changes induced by DNA damage (**Fig. 20C**).

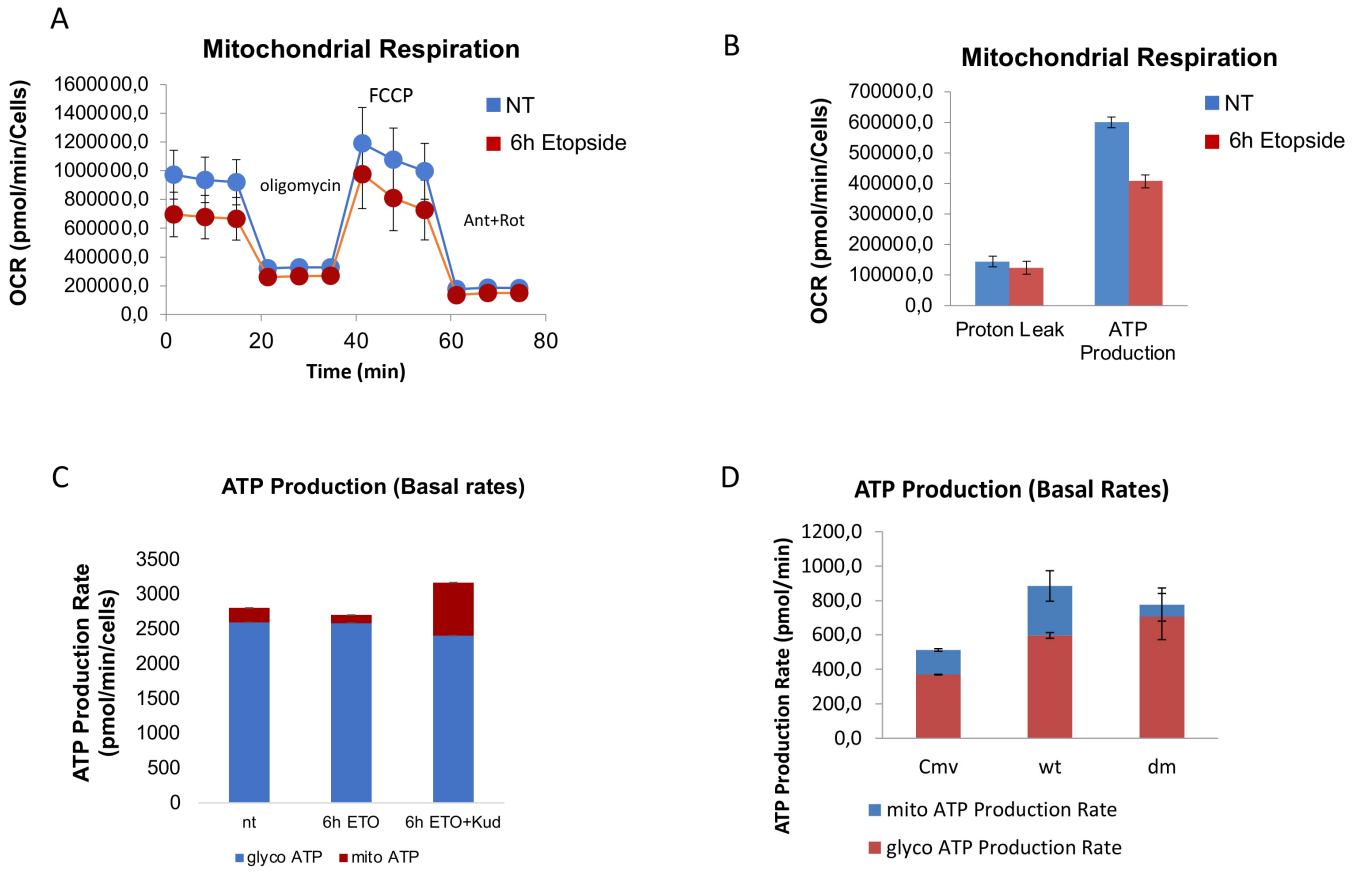


Figure 20. Etoposide treatment reduces mitochondrial respiration and ATP synthesis in GBM cells. A-C. U87-MG cells were left untreated or exposed to etoposide treatment for 6h. Oxygen Consumption Rate (OCR) was dynamically measured in real time, both under basal condition (0) or in response to treatment with

the following mitochondrial inhibitors: oligomycin, FCCP, Antimycin A and Rotenone. Indicated are the indices of mitochondrial respiratory function derived from the OCR profile and the basal rates of ATP production. Where indicated, cells were preatreated with the ATM/ATR inhibitor Kudos. **D**. Basal rates of ATP production (glycolytic and mitochondrial) in U87-MG cells transiently transfected with a control vector (CMV), a vector encoding for wild type AKAP1 or the PKA-binding mutant (dm).

To support the role of PKA delocalization from mitochondrial compartment in the metabolic rewiring of etoposide-treated cells, we evaluated the effects of the PKA binding mutant of AKAP1 in mitochondrial and glycolytic ATP synthesis. U87-MG cells were transiently transfected with control plasmid or with a plasmid encoding for the AKAP1 mutant carrying S/T to D mutations at the ATM sites (dm). Twenty-four hours from transfection, cells were metabolically profiled by the Seahorse apparatus. **Fig. 20D** shows that expression of wild type AKAP1 increased the global (glycolytic and oxidative) ATP synthesis. In contrast, the AKAP1dm mutant significantly reduced the mitochondrial ATP synthesis, compared to control cells (CMV-transfected) and also to cells expressing wild type AKAP1. A concomitant increase of the glycolytic pathway in AKAP1dm-expressing cells was observed (**Fig. 20D**).

Collectively, the data indicate that mitochondrial localization of PKA by AKAP1 in cancer cells constitutes a major regulatory mechanism for mitochondrial respiration and survival. Delocalization of PKA from mitochondria mediated by ATM phosphorylation of the PKA-binding domain of AKAP1 induces a biologically relevant metabolic rewiring in course of DNA damage, attenuating oxidation respiration and promoting glycolysis.

5. DISCUSSION

In my thesis, I report the identification of AKAP1 a novel transcriptional target of Myc oncogene and of steroid androgens in cancer cells. Expression analysis of AKAP1 revealed that the anchor protein is highly expressed in a variety of human cancers, including luminal and basal-like breast cancers, high-grade prostate cancer, and in highly malignant glioma tissues, such as glioma III and glioblastoma. I also found that AKAP1 is overexpressed in numerous cultured cancer cell types derived from breast, prostate and glioma cancer tissues. Forced downregulation of AKAP1 by RNAi-based silencing negatively impacted on cancer cell growth *in vitro* and significantly inhibited cancer growth *in vivo*. Metabolically, I found that genetic silencing of AKAP1 dramatically reduced the mitochondrial respiration, the spare respiratory capacity and the basal rate of ATP synthesis. Interestingly, I found that AKAP1-dependent mitochondrial metabolism is targeted by the DNA damage-induced ATM/ATR pathway in cancer cells. Thus, following DNA damage, ATM/ATR phosphorylation of AKAP1 at its PKA binding domain delocalized PKA from mitochondria and decreased mitochondrial respiration and oxidative ATP synthesis.

Compartmentalization of enzymes, adapter proteins, signaling regulators and effectors emerged as a fundamental mechanism underlying the biological responses to hormones, neurotransmitters and growth factors. AKAPs belong to a family of structurally different, but functionally homologous proteins that control the flux of cAMP signals generated at cell membrane by G-protein coupled receptor stimulation and travelling to specific intracellular targets[62]. The differential expression and regulation of AKAPs in distinct tissues, the peculiar intracellular distribution of AKAP-assembled complexes and the sensitivity of AKAP-bound PKA holoenzymes to cAMP molecules tightly control highly specialized biological cell functions. Changing the levels of a given AKAP by extracellular stimuli or by sudden changes of microenvironmental conditions further contributes to differentiate the biological outcomes of distinct tissues to hormones and neurotransmitters[8].

AKAP1 is the prototypic mitochondrial AKAP that controls essential functions related to respiration, survival, metabolism and organelle dynamics. Both PKA holoenzymes, type I and type II, have been identified as partners of AKAP1 at mitochondrial outer membrane. AKAP1 has been show to interact with nuclear-encoded mRNAs and ribosomes at the OMM, promoting efficient translation and co-import of mitochondrial proteins into the organelles, with important implications for global protein synthesis and mitochondrial physiology. Protein tyrosine kinases (Src), protein phosphatases (PP1 and PTPD1), phosphodiesterases, mRNA and adenylate cyclases have been identified in complex with AKAP1. The macromolecular complex assembled by AKAP1 at mitochondrial outer membrane finely couples cell signaling to metabolic pathways, playing a pivotal role in mitochondrial homeostasis and organelle physiology. During hypoxia or ischemic insult,

proteolysis of AKAP1 mediated by the ubiquitin-proteasome system attenuates mitochondrial respiration and preserve cell viability and tissue remodeling[63].

Here, I report that transcription of AKAP1 is regulated by Myc proto-oncogene. I identified a cis-acting element within the AKAP1 gene promoter that mediates the interaction with- and the transcriptional regulation by- Myc, both *in vitro* and *in vivo*. It is well known that Myc promotes mitochondrial biogenesis, linking increased metabolic needs of cancer cells to cell cycle progression. By enhancing mitochondrial mass, Myc ensures efficient energy (ATP) supply to anabolic processes required for the synthesis of building blocks in rapidly dividing cells[63]. Although several genes encoding for mitochondrial proteins have been identified as Myc targets, the role of this protooncogene in the transcriptional regulation of mitochondrial AKAPs was largely unknown. The findings indicate that AKAP1 transcription if upregulated by Myc adds a novel layer of complexity to the regulation of mitochondrial metabolism in the control of cancer cell growth.

Interestingly, I also found that steroid hormones, such as androgens, induce a time-dependent accumulation of AKAP1 in prostate cancer cells. It is well known that signaling pathways activated by androgen receptor play a critical role in the pathogenesis of prostate cancer. In prostate cancer cells, androgens regulate different aspects of mitochondrial dynamics and metabolism. Activation of androgen receptors increases glycolysis, tricarboxylic acid cycle, oxidative phosphorylation and lipid metabolism[64]. Given the important role of AKAP1 in some of these metabolic pathways, the findings in my thesis suggest that the effects of androgens on mitochondrial activities may be, at least in part, dependent on AKAP1. Upregulation of AKAP1 content by Myc or androgen stimulation may, thus, confer an energetic advantage to tumour cells, increasing the production of energy and promoting anabolic pathways. This regulatory mechanism may, thus, explain the upregulation of AKAP1 observed in a wide variety of metabolically active cancer cells and tumour tissues, supporting a fundamental role of this anchor protein in cancer growth *in vivo*. Accordingly, I found that genetic silencing of AKAP1 severely impaired oxidative metabolism, the spare respiratory capacity and ATP synthesis in cancer cells, and significantly inhibited cancer cell growth. *In vivo*, using orthotopic mouse models of human glioblastoma, I found that down-regulation of AKAP1 reduced cancer cell proliferation and tumor development in vivo.

Mechanistically, I contributed to identify sestrin2 as a novel partner of AKAP1[65]. Sestrin2 is a p53-induced scavenging protein that works as major regulator of antioxidant defenses in mitochondria. Sestrin2 also acts as regulator of anabolic pathways. Thus, under stress conditions, sestrin2 interacts with GATOR2, a component and positive regulator of mTORC1 complex. The interaction between sestrin2 and GATOR2 prevents the assembly of active mTORC1 complex and inhibits downstream activation of protein synthesis[66, 67]. I found that AKAP1 binds to- and targets

sestrin2 on mitochondria, thereby removing the inhibitory constraint of sestrin2 on mTORC1 and promoting anabolic pathway and cancer cell growth[53]. In cancer cells, AKAP1 is, indeed, required for mTORC1-dependent phosphorylation of its downstream targets. The recent identification of MDI, the Drosophila ortholog of AKAP1, as the principal regulator of protein translation during oogenesis and development of flies, supported an evolutionary conserved the role of AKAP1 in anabolic pathways[68]. Collectively, the data support a model whereby AKAP1 works at the crossroad between oxidative phosphorylation, anti-oxidant responses and mTOR-dependent anabolic pathways, dynamically and functionally linking energy production and scavenging system to the protein translational machinery, with important implications on cancer cell growth and tumor development.

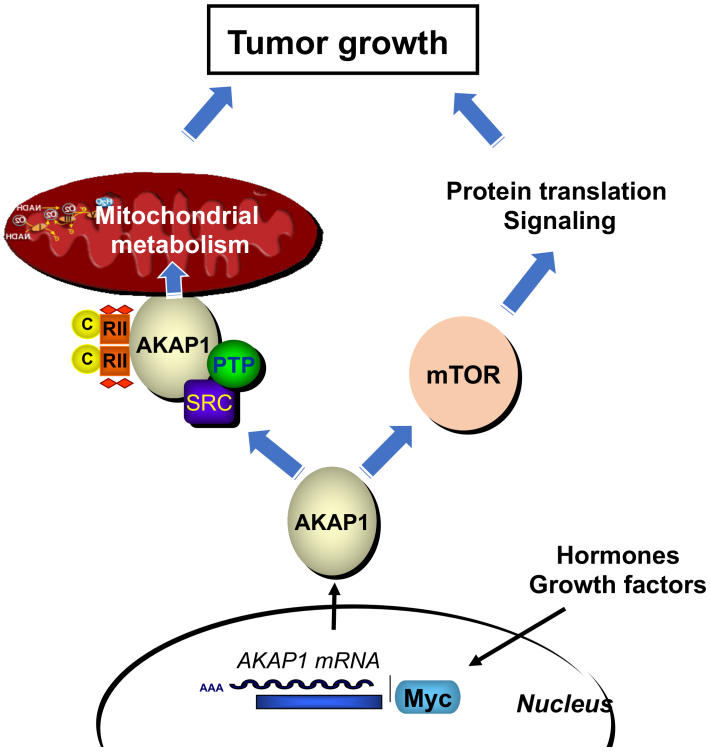
Interestingly, I found that mitochondrial pathways regulated by AKAP1 can be downregulated by DNA damage-induced ATM/ATR kinases. These are serine/threonine protein kinases that are activated by DNA double-strand breaks. Once activated, ATM/ATR phosphorylate intracellular proteins involved in DNA damage checkpoints, leading to cell cycle arrest, DNA repair and, in some circumstances, to apoptotic cell death[36]. A variety of ATM/ATR substrates have been identified and functionally characterized. However, the impact of ATM/ATR activation on the cAMP signal transduction pathway at mitochondrial compartment was largely unknown. Here, I report for the first time that AKAP1 is phosphorylated by ATM/ATR following DNA damage induced by etoposide treatment. Phosphorylation occurs at two conserved residues (ser315 and thr322) located within the PKA binding domain of AKAP1. Placing phosphoryl groups within the hydrophobic amphipathic helical wheel of AKAP1 affects the interaction to PKA and delocalizes the kinase from mitochondrial compartment. By attenuating PKA events at mitochondria, ATM/ATR activation negatively impacts on cAMP-regulated mitochondrial respiration and ATP synthesis. This regulatory system constitutes a safeguard mechanism that tightly controls mitochondrial respiration in course of DNA damage, limiting the production of excess free oxygen radicals by the respiratory chain reactions that would further damage cellular constituents and DNA. In cancer cells, this mechanism is of particular relevance since one of the hallmarks of cancers is the intrinsic genomic instability that allows the continuous accumulation of DNA damage in rapidly cycling cells[69]. Although DNA damage contributes to generate more aggressive cancer cells, genetic instability may also contribute to enhances the sensitivity of damaged cells to death stimuli. This evidence is in agreement with the some of the current therapeutic protocols adopted for cancer treatment that are mostly based on the use of DNA-damaging drugs and radiotherapy to induce cancer cell death. However, these treatments induce off-target effects and collateral damages to normal tissue that limit their use in some debilitated cancer patients[70]. For these reasons, novel strategies are needed to selectively target the DNA damage response (DDR) pathways in cancer cells. The identification of AKAP1 as a major regulator of

mitochondrial respiration and metabolism, and as a relevant target of ATM/ATR kinases in DNA-damaged cancer cells suggests that using selective inhibitors of the ATM/ATR-AKAP1 axis can enhance the sensitivity of cancer cells to chemotherapeutics.

Taken together, the findings reported in my thesis demonstrate that AKAP1 is a transcriptional target of Myc proto-oncogene and of androgens. Upregulation of AKAP1, thus, in normal cells controls mitochondrial activities and anabolic pathways under conditions of increased metabolic demands. The complex assembled by AKAP1 on mitochondria and its regulation by oncogenic pathways and hormones become particularly relevant in cancer cells, as it provides sufficient energy for the synthesis of the building blocks and cellular organelles in rapidly dividing cells. Furthermore, the regulation of AKAP1-dependent mitochondrial pathways by ATM/ATR kinases provides novel molecular clues to better understand the links between DNA damage checkpoints and mitochondrial metabolism, with important implications for cancer cell survival in the presence of DNA damaging therapeutics. Interfering with the signaling events regulated by ATM/ATR-AKAP1 axis at mitochondrial compartment and/or modulating its interaction with components of the metabolic pathways will pave the way for the development of novel molecular strategies for cancer therapy.

6. CONCLUSIONS

Mitochondria are intracellular sites at which oxidative, metabolic pathways and survival converge and focus. A role of mitochondria in different aspects of cell signaling and metabolism has been largely demonstrated. In this context mitochondria are the residence for a variety of signaling molecules, adapter proteins, ion channels, receptors, transcription factors and mRNAs, constituting a intracellular hubs able to integrate signaling, cell respiration and anabolic pathways that support normal cell growth and survival. These aspects are of particular relevance for cancer cells, where integration of different signaling pathways and metabolic needs are essential for cancer growth and progression. Mutations of metabolic enzymes working within the mitochondrial compartment have been mechanistically linked to the development and progression of aggressive human cancers. Therefore, understanding the molecular mechanisms controlling the metabolic rewiring of cancer cells and defining the key elements of the signaling pathways governing tumour cell biology, will pave the way for the design of novel therapeutic approaches for cancer treatment. The data reported in my thesis demonstrate that mitochondrial AKAP1 is the Achille's heel of aggressive cancer cells, as GBM cells. Targeted downregulation of AKAP1-controlled pathways at mitochondrial compartment provides a novel valuable target for the treatment of aggressive cancers.



Schematic model of myc-AKAP1 axis in the control of mitochondrial metabolism and tumor growth. Stimulation of receptors by hormones or growth factors induces myc-dependent transcription of AKAP1 gene. Accumulation of AKAP1 scaffold complex stimulates oxidative metabolism and mTOR pathway, supporting tumor growth.

7. ACKNOWLEDGMENTS

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8. LIST OF PUBLICATIONS

1. Sisalli MJ, Feliciello A, Della Notte S, Di Martino R, **Borzacchiello D**, Annunziato L, Scorziello A. (2020) Nuclear-encoded NCX3 and AKAP121: Two novel modulators of mitochondrial efflux in normoxic and hypoxic neurons. Cell Calcium, 87:102193.

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4. Rinaldi L, Delle Donne R, **Borzacchiello D**, Insabato L, Feliciello A. (2018) The role of compartmentalized signaling pathways in the control of mitochondrial activities in cancer cells. Biochim. Biophys Acta Review on Cancer. 1869(2):293-302.

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