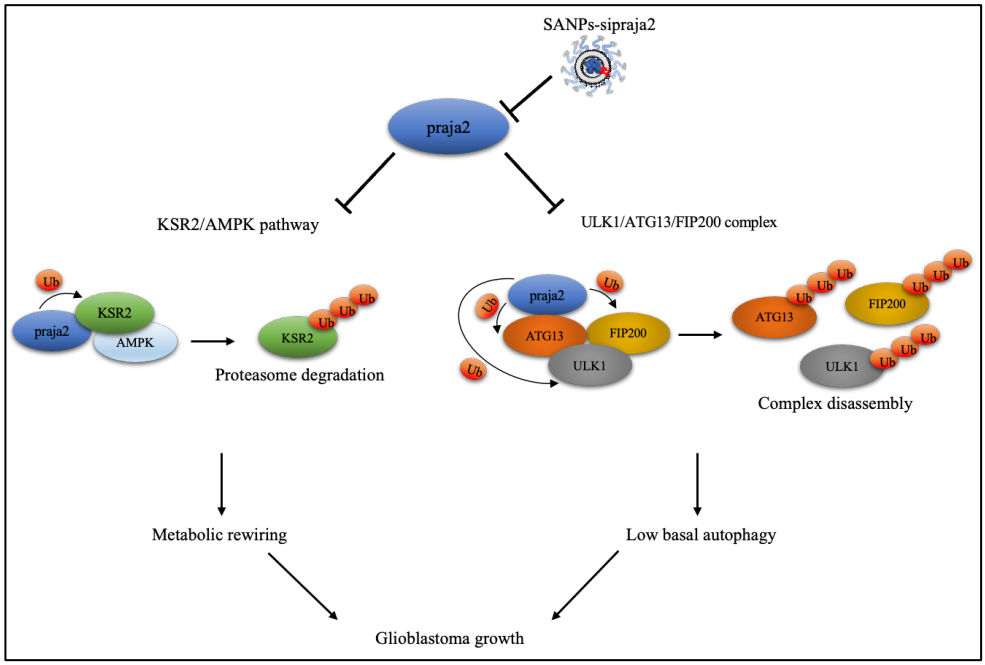
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXVI CYCLE

Rosa Iannucci

"CONTROL OF METABOLIC REWIRING AND AUTOPHAGIC PATHWAY IN GLIOBLASTOMA BY THE E3-UBIQUITIN LIGASE PRAJA2"



Year 2024

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"CONTROL OF METABOLIC REWIRING AND AUTOPHAGIC PATHWAY IN GLIOBLASTOMA BY THE E3-UBIQUITIN LIGASE PRAJA2"

Tutor
Prof. Antonio Feliciello

Candidate
Rosa Iannucci

Year 2024

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List of abbreviations

AICAR	5-aminoimidazole-4-trifluoromethoxy phenylhydrazone
AID	autoinhibitory domain
AMPK	AMP-activated protein kinase
AKAPs	A-kinase anchoring protein
α-KG	α-ketoglutarate
ATG13	autophagy-related protein 13
α-tub	α-tubulin
BAFA1	bafilomycin A1
BBB	blood-brain barrier
BLI	bioluminescent intensity
CAMKKII	calcium/calmodulin-dependent kinase kinase II
CBM	carbohydrate binding domain
CBS	cystathionine-β-synthase
CHX	cycloheximide
CMA	chaperone-mediated autophagy
CNS	central nervous system
Co-Ip	co-immunoprecipitation
CP	proteolytic core
CPT1	carnitine-palmitoyltransferase I
CRD	cysteine-rich domain
CTD	C-terminal domain
DEPTOR	DEP domain containing mTOR-interacting protein
DUBs	Deubiquitinating enzymes
4-EBP1	4E-binding protein 1
ECAR	extracellular acidification rate
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
FACS	fluorescent-activated cell sorter
FAO	fatty-acid oxidation
FCCP	carbonylcyanide p-trifluoromethoxyphenylhydrazone
FIP200	focal adhesion kinase family interacting protein 200
GAP	GTPase activating protein
GBM	Glioblastoma multiforme
GPCRs	G-protein-coupled receptors
GSEA	gene set enrichment analysis
HBSS	Hanks' balanced salt solution
2-HG	2-hydroxyglutarate
HMGCR	β-hydroxylβ-methylglutaryl-COA reductase

Hsc70	heat shock-cognate chaperone 70
Hsp90	heat shock protein 90
IDH1	isocitrate dehydrogenase I
IPA	ingenuity pathway analysis
KBD	kinase binding domain
KSR	kinase suppressor of Ras
LAMP2A	lysosome-associated membrane protein type 2A
LKB1	liver-kinase B1
MFSHA1	malignant fibrous histiocytoma amplified sequence 1
MGMT	O^6-methylguanine-DNA methyltransferase
MOB1	Mps one binder 1
mTORC1	mammalian target of rapamycin complex 1
OCR	oxygen consumption rate
OFD1	orofacial digital type I
O^6-MeG	O^6-methylguanine
OXPHOS	oxidative phosphorylation
PAS	phagofore assembly sites
PE	phosphatidylethanolamine
PFK1/2	phosphofructokinase 1/2
PGC1α	proliferator-activated receptor-γ-co-activator 1α
PI	polydispersity index
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PP	protein phosphatases
PPI	protein-protein interaction
PRAS40	proline-rich AKT substrate 40
PRD	proline-rich domain
PROTOR	protein binding Rictor
PTEN	phosphatases and TENsin
RBR	ring between ring
RNAseq	RNA sequencing
RPs	regulatory particles
RT	radiotherapy
RTKs	tyrosine kinase receptors
RTN	reticulon
SANPs	self-assembling nanoparticles
S6K1	S6 kinase 1
STRAD	kinase-dead STE20-related kinase
TMZ	temozolomide

TSC2	tuberous sclerosis complex 2
UBL	ubiquitin-like proteins
ULK1	Unc-51-like kinase 1
UPS	ubiquitin-proteasome system
WHO	World Health Organization

Abstract

Glioblastoma multiforme (GBM) is the most malignant form of primary brain tumor, whose high recurrence rate and resistance to standard treatments demand an urgent need for novel therapeutic strategies. The ubiquitin-proteasome system (UPS) is an important control mechanism of cell growth, metabolism, and alterations of this circuitry are linked to the onset and progression of various human cancers, including GBM. Thus, the ubiquitin-proteasome system represents a potential target for GBM treatment. In this study, we found that levels of praja2, a RING E3 ligase, are markedly enhanced in primary GBM lesions expressing the wild-type isocitrate dehydrogenase 1 gene (IDH1) and it is involved in the regulation of cell growth and metabolism. By using biochemical assays, we identified the 5'AMP-activated protein kinase (AMPKα1 and AMPKγ1) and Kinase Suppressor of Ras 2 (KSR2) as putative partners of praja2. In particular, praja2 binds to, ubiquitylates, and degrades KSR2 attenuating the activity of the downstream effector AMPK. As a consequence, cells undergo a metabolic switch from oxidative respiration to glycolytic pathway, supporting GBM proliferation. Since AMPK exerts a role in autophagy regulation, we have established a functional link between the two degradative systems. Indeed, we demonstrated that praja2 negatively regulates basal autophagy machinery by binding to and ubiquitinating components of the ULK1/FIP200/ATG13 complex. Finally, we used transferrin-targeted self-assembling nanoparticles (SANPs) to deliver inhibitory RNA molecules targeting praja2 into the brain, observing a reduction in tumor growth and an increase in the survival rate of treated mice. Altogether, our findings identify praja2 as an essential regulator of cancer cell metabolism, and as a potential therapeutic target for GBM treatment.

1. Introduction

1.1 Glioblastoma

Glioblastoma multiforme (GBM) represents the most aggressive type of primary brain tumor and it is a form of glioma, a group of cancers that originate from the glial cells of the central nervous system (CNS)(Perrin 2019). Histologically, two major classes of infiltrative gliomas have been identified that resemble normal glial populations, such as astrocytes (astocytomas) and oligodendrocytes (oligodendrogliomas). Based on this classification, GBM is an astrocytoma (Pisapia 2017).

GBM is a disease that accounts for 17% of all brain cancers and 54% of glial tumors, with a median survival of 14,6 months. The annual incidence is 4-5 cases per 100.000 individuals with a peak at age 45-75 years and a prevalence in males (Wirsching 2016).

According to histopathology, cytoarchitecture, and immunological marker profile of the cells, the World Health Organization (WHO) divided glioma lesions into four subgroups (I, II, III, and IV) for astrocytomas and two subgroups (II and III) for oligodendrogliomas and oligoastrocytomas (Louis 2016). Grade I and II tumors are characterized by genetic alterations and some cellular anomalies or atypia; grade III tumors are characterized by anaplastic signs and elevated mitotic activity; grade IV tumors, including GBM, are characterized by nuclear atypia, high mitotic activity, vascular endothelial proliferation, and necrosis (Vigneswaran 2015). Recently, the WHO classification system has been integrated with the prognostic molecular markers isocitrate dehydrogenase 1 gene (IDH1) and co-deletion of chromosomes 1p–19q (**Fig.1)**. IDH1 is an enzyme involved in the citric acid cycle, whose activity is the conversion of the isocitrate to a-ketoglutarate (a-KG) (Jin 2012). Mutations in the IDH1 gene can lead to a loss of normal activity of the enzyme and abnormal production of an oncometabolite 2-hydroxyglutarate (2-HG) (Zhao 2009). It is an inhibitor of a-KG-dependent dioxygenases and lysine histone demethylases. Thus, 2-HG affects several metabolic and epigenetic pathways involved in tumor growth and development (Chou 2021). Therefore, tumors exhibiting both 1p–19q co-deletion and IDH1 gene mutation are categorized as oligodendroglial forms; on the other hand, tumors lacking 1p–19q co-deletion and exhibiting IDH1 gene mutation can be classified as astrocytic forms (Van Den Bent 2017; Trautwein 2022). Based on these molecular data, GBM is mainly divided into two groups: GBM IDH wild-type and GBM IDH-mutant. The majority of glioblastomas (90% of cases) with IDH1 wild-type corresponds to primary or de novo GBM and occur in patients over 55 years of age. Mutation of Phosphatases and TENsin homologous

(PTEN) and amplification of epidermal growth factor receptor (EGFR) are also found in primary GBM. Glioblastomas taking IDH1 mutations (10% of cases) correspond to secondary GBM that arise from low-grade diffuse astrocytoma or anaplastic astrocytoma and occur in younger patients. Mutations in TP53 and 19q loss are also found in secondary GBM (Ohgaki 2013).

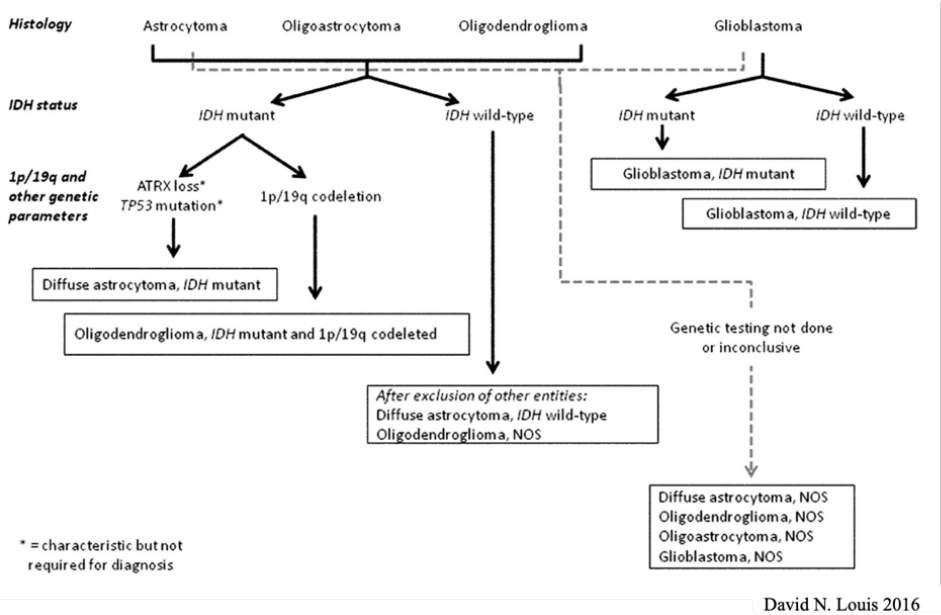

Figure 1. Classification of gliomas. Gliomas are classified into oligoastrocytoma, oligodendroglioma, and astrocytoma, which includes glioblastoma, based on histological features. Further classification involves dividing them into IDH wild-type and IDH mutant groups, based on genetic features.

Cellular heterogeneity, necrosis, high neo-angiogenesis, elevated mitotic activity, and invasiveness are histopathological features contributing to the poor prognosis of patients affected by GBM (Garcia 2021). Additionally, the capacity of tumor cells to modify their metabolism to survive in new microenvironments has recently received attention. It is commonly known that the primary energy source in normal cells is the glycolytic pathway, followed by mitochondrial oxidative phosphorylation (OXPHOS) under aerobic conditions. When the amount of oxygen is limited, cells mainly obtain energy from glycolysis. However, metabolic processes are completely different in tumor cells (Lapa 2020). In 1920s, the German biochemist Otto Warburg and his colleagues discovered that cancer tissues sustained a high rate of glycolysis, even in oxygen-abundant environments (Park 2020). This rise in glycolytic activity was coupled with enhanced lactate secretion and was termed aerobic glycolysis, also known as the ‘Warburg effect’ (Hanahan 2011). This phenomenon is well studied in glioblastoma since tumor cells have a strongly

increased glycolytic pathway and it is correlated to tumor proliferation, aggressive invasion, and resistance to chemo- and radio-therapies (Strickland 2017).

Despite recent advances in diagnostics techniques and cancer biology, the standard care of GBM is surgical resection followed by radiotherapy alone or in combination with radiotherapy (RT) and temozolomide (TMZ) (Davis 2016) **(Fig.2)**. However, the patient's age affects the course of treatment. Patients under 70 years of age receive traditional therapy consisting of surgery, radiotherapy, and chemotherapy. Elderly patients who are unable to receive both RT and TMZ are given TMZ alone (Alifieris 2015).TMZ is an alkylating agent with high capability to penetrate the blood-brain barrier (BBB). The mechanism of action of TMZ is the introduction of mismatch repair in DNA by methylation at the O^6 position of guanine (O6-MeG) which triggers cell apoptosis because of alterations in DNA replication (Fisher 2021). Nevertheless, some patients develop resistance to the treatment mediated by the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT), which removes methyl groups from O6-MeG. Radiotherapy and TMZ chemotherapy show their beneficial effects only in tumors with methylated MGMT (Davis 2016). Numerous novel strategies, such as immunotherapies, personalized medicine, and surgical refinement techniques, are being investigated due to the high heterogeneity of GBM, which affects the efficacy of therapy (Scholz 2020).

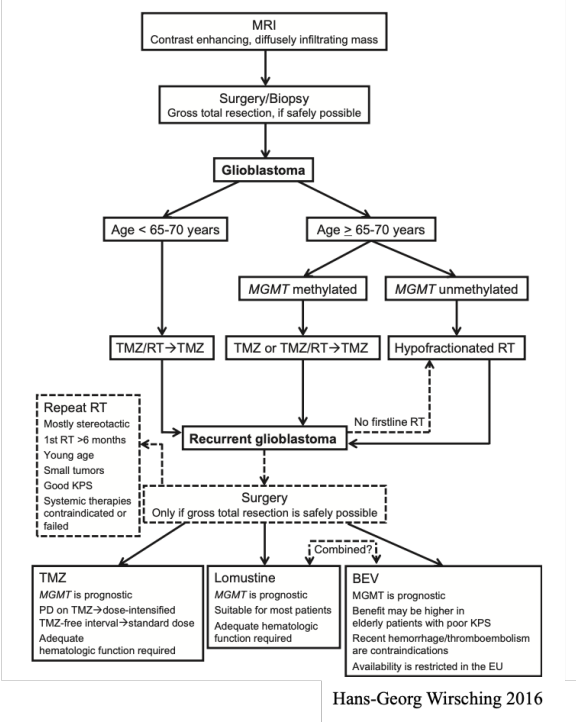

Figure 2. Therapeutic strategies for glioblastoma. Glioblastoma is diagnosed using MRI (magnetic resonance imaging). Standard treatment involves surgical resection followed by radiotherapy alone or in combination with chemotherapy. TMZ: temozolomide; MGMT: O6-methylguanyl DNA methyl- transferase; RT: radiotherapy; BEV: bevacizumab.

1.2 The ubiquitin-proteasome system

Protein homeostasis (proteostasis) is a complex network that coordinates protein synthesis, conservation, and degradation through the action of molecular chaperones, proteolytic systems, and their regulators (Hipp 2019). These mechanisms work in concert to prevent the buildup of potentially hazardous protein aggregates. Many pathologies, including neurodegenerative diseases, are caused by alterations in the proteostasis process (Labbadia 2015).

The ubiquitin-proteasome system (UPS) is a cellular pathway responsible for the degradation of proteins that are damaged, misfolded, or no longer needed by the cell (Jinyoung Park 2020). It plays a crucial role in some biological functions such as growth, metabolism, and proliferation. Dysregulation of the UPS has been implicated in numerous diseases, including glioblastoma (Popovic 2014; Maksoud 2021).

The ubiquitylation process requires the action of three enzymes that successively bind a molecule of ubiquitin to lysine residues exposed at the surface of targeted substrates (Callis 2014). Ubiquitin is a heat-stable and conserved protein of 76 aminoacids expressed in all eukaryotes. It is firstly activated by binding the ubiquitin-activating enzyme (E1) in the presence of ATP, through a thioester bond between the cysteine residue present in the active site of the enzyme and the carboxyl terminus of the Ub. The ubiquitin is then transferred from E1 to the ubiquitin-conjugating enzyme (E2). Finally, a ubiquitin ligase (E3) couples the Ub molecule to a target protein through a peptide bond (Lilienbaum 2013). The E3 ligases are divided into three groups: HECT domain E3 ligases, RING domain E3 ligases and ring between ring (RBR) ligases. The HECT E3 ligase forms a cysteine-dependent intermediate with the ubiquitin attached to the E2 enzyme and then transfers it to the substrate. The RING E3 ligase directly transfers the ubiquitin from the E2 enzyme to a substrate (Mani 2005). The RBR ligase has two RING domains: RING1 and RING2. The first interacts with the E2-ubiquitin complex and the second offers a cysteine residue to form an intermediate with the ubiquitin before it is transferred to the substrate (Wenzel 2011).

Ubiquitin is added to a target protein through a peptide bond between its C-terminal glycine residue and the ε-amino group of a lysine residue of a substrate. Furthermore, ubiquitin has seven lysine residues in positions 6, 11, 27, 29, 33, 48, and 63 (Akutsu 2016).

Three types of ubiquitylation can be distinguished based on the length of the ubiquitin chain: monoubiquitylation, multi-monoubiquitylation, and polyubiquitylation. The first is characterized by the addition of a single molecule of ubiquitin to the lysine of the substrate; the second is characterized by the addition of multiple single ubiquitin molecules onto different lysine

residues of the substrate; the third is characterized by the addition of a ubiquitin polymer to one or more lysine residues of the substrate (Livneh 2017). Moreover, the substrate's destiny is dictated by the type of ubiquitin-ubiquitin binding. While the Lys48-based chains represent the canonical proteasomal degradation signal, the Lys63-based chains are related to non-proteolytic functions (Kwon 2017).

The process of ubiquitylation is reversible. Ubiquitin molecules can be eliminated from the substrate by enzymes known as deubiquitinating enzymes (DUBs). There are about 90 DUBs encoded by the human genome, which fall into two main groups: metalloproteases and cysteine proteases (Amerik 2004).

Proteins targeted to the proteolysis are degraded via 26S proteasome (**Fig.3)**. The proteasome catalyzes the digestion of polypeptides into small peptides of 2-10 residues (Collins 2017). It consists of two large complexes: 20S proteolytic core (CP), which digests the substrate through proteases, and 19S regulatory particles (RPs), which recognize, unfold, and translocate the polypeptide into the CP. There are two subunits Rpn10 and Rpn13 that have ubiquitin-binding domains and therefore act as receptors for ubiquitinated proteins. Once the substrate is bound, three main DUBs such as Rpn11, Usp14, and Uch2 release the ubiquitin, which can be reused (Dikic 2017).

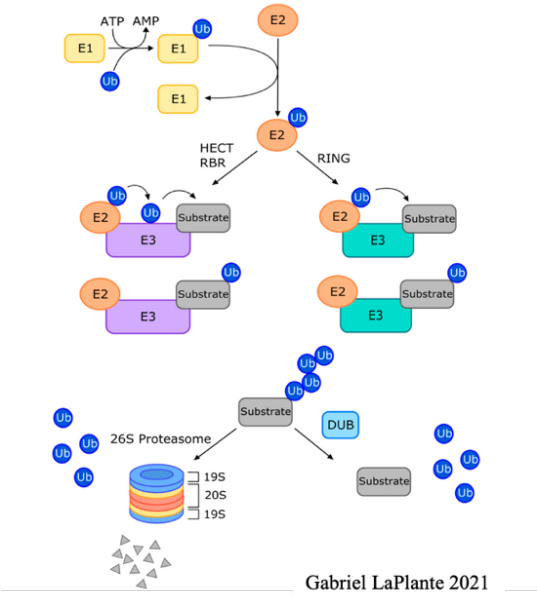

Figure 3. Ubiquitin-proteasome system. Ubiquitin conjugation to substrates involves a three-step process: binding of ubiquitin to a ubiquitin-activating enzyme (E1), transferring from E1 to a ubiquitin-conjugating enzyme (E2), and attachment of ubiquitin to substrates by an E3 ubiquitin ligase. HECT-E3 ligases receive the ubiquitin from E2 and transfer it to substrates. RING-E3 ligases directly transfer the ubiquitin to a substrate. Ubiquitylated proteins are targeted for degradation in the proteasome. Deubiquitinating enzymes (DUBs) play a role in removing ubiquitin from substrates.

1.3 E3 ubiquitin ligase praja2

Praja, a RING H2 finger E3 ligase, is one of the ubiquitin E3 ligases highly expressed in brain tissues including the cerebellum, cerebral cortex, and frontal lobe (Shin 2017). RING-H2 is a motif similar to the RING finger motif, with a change of Cys4 to His (Freemont 1993). Praja family is composed of two members, praja1 e praja2, which occur with high expression in glioblastomas and share 52,3% of sequence homology (Shin 2017). Praja1 gene located on the Xq12 chromosome encodes a protein with a molecular weight of 71 kDa. Furthermore, praja1 has an important role in neuronal plasticity which is the basis for learning and memory (Yu 2002).

Instead, praja2 gene is located on the long arm of chromosome 5 and it encodes a protein with a molecular weight of 78 kDa. Praja2 is characterized by a RING-H2 domain at the COOH-terminal end (aa 634-675) and an amphipathic helix (aa 583–600), that is characteristic of A-kinase anchoring protein (AKAPs) family proteins (Lignitto 2011). Indeed, praja2 regulates the strength and the duration of the Protein Kinase A (PKA) signal controlling the stability of the regulatory subunits RI and RII. Particularly, praja2 ubiquitinates and degrades them during cAMP stimulation, thus amplifying downstream signaling (Lignitto 2011). Moreover, praja2 has a role in cAMP-mediated neurite outgrowth. It ubiquitinates and degrades NOGO-A, a member of the reticulon (RTN) family of integral membrane protein and a potent inhibitor of axonal growth (Sepe 2014).

More recently, it has been demonstrated the role of praja2 in ciliogenesis. Praja2 is recruited to the centrosome by TBC1D31 protein, which forms a multimeric complex including PKA and Orofacial digital type I (OFD1) proteins. OFD1 is an important component of the centrosome/basal body involved in primary ciliogenesis. Under cAMP stimulation, PKA phosphorylates OFD1, which is ubiquitinated and degraded by praja2 (Senatore 2021). Furthermore, praja2 regulates the cilium formation and trafficking of cargo and receptors through non-proteolytic ubiquitination of the core components of the BBSome complex. In particular, praja2 ubiquitylates BBS1 and BBS2 in response to cAMP stimulation, modulating the BBSome complex assembly and/or its targeting to the ciliary compartment. Downregulation of praja2 or expression *in vivo* of a ubiquitylation-defective BBS1 mutant impairs cilium biogenesis, ciliary trafficking and signaling, mimicking some of the Bardet-Biedl syndrome phenotypic alterations, such as defects in embryonic development and degeneration of retinal cells (Chiuso *et al.*, 2023).

Praja2 also regulates macrophage polarization. It is well known that pro-inflammatory mediators like TNF-α, IL-6, iNOS, and IL-1β are secreted more frequently by M1 macrophages, whereas M2 macrophages have anti-

inflammatory properties. MFSHA1 (Malignant Fibrous Histiocytoma Amplified Sequence 1), a putative oncogene, has been shown to interact with and ubiquitinated by praja2, activating the TLR2/JNK/p38/NF-kB pathway and promoting macrophage M1 polarization (Zhong 2017).

Finally, praja2 exerts an important role in glioblastoma growth, through the regulation of Mob1 (Mps one binder 1). Mob1 is a core component of NDR/LATS kinase and a positive regulator of the tumor-suppressor Hippo pathway. The ubiquitination and degradation of Mob1 by praja2 attenuate the Hippo cascade, sustaining glioblastoma growth in vivo (Lignitto 2013). In addition, the binding between praja2 and Mob1 is mediated by the focal adhesion molecule Kindlin-2, which inhibits the phosphorylation of LATS1 and YAP, and promotes YAP translocation into the nucleus, thus activating downstream Hippo target gene transcription. Inhibition of the Hippo signaling by Kindlin-2 enhances the development and progression of renal fibrosis (Song 2019)

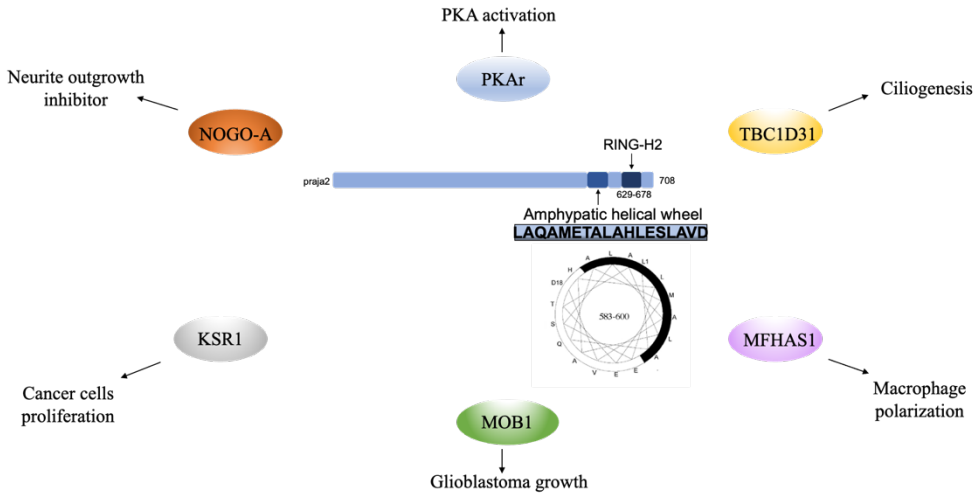

Figure 4. Schematic representation of praja2 structure. Praja2 is composed of 708 aminoacids. It is characterized by a RINH-H2 motif and a putative amphipathic helical wheel at COOH-terminal end. Praja2 ubiquitinates several proteins involved in different biological processes.

1.4 Kinase Suppressor of Ras2 (KSR2)

The kinase suppressor of Ras (KSR) is a scaffold protein that regulates the RAS/RAF/MEK/ERK signaling, which is involved in cellular proliferation, differentiation, and metabolism (Frodyma 2017). This protein family is constituted by two main members called KSR1 and KSR2, highly conserved in invertebrates and mammals (Roy 2002). They are characterized by five domains, namely CA1-CA5 (Therrien 1995) (**Fig.5)**. The CA1 domain is located at the N-terminus end, and it comprises 40 aminoacids that contribute to B-Raf binding to KSR1 and KSR1 membrane association (Koveal 2012). The CA2 domain is a proline-rich region whose function is unknown. Between the CA2 and CA3 domains of KSR2 is a region that interacts with AMP-activated protein kinase (AMPK). Actually, changes in this area have a detrimental effect on the interaction (Guo 2017). The CA3 domain is a cysteine-rich region that contributes to the membrane localization of KSR proteins by recruiting phospholipids (Karthik 2014). The CA4 domain is a serine/threonine region containing an essential *FXFP* (Phe-Xaa-Phe-Pro) motif for interaction with ERK (Fantz 2001). The CA5 domain is homologous to the kinase domain of Raf protein, but in KSR proteins it has substitutions of lysines with arginines which are required for catalytic activity (Sundaram 1995).

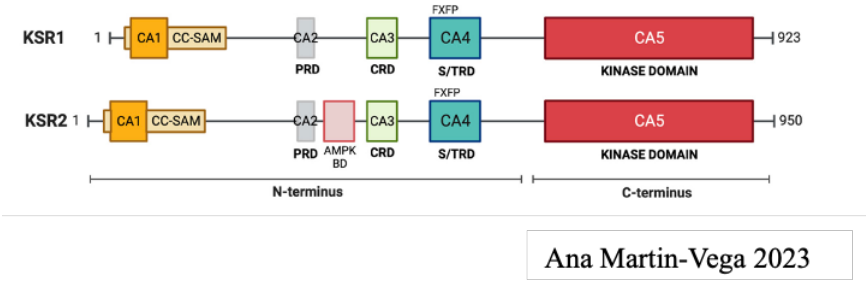

Figure 5. Schematic of KSR2 protein structures. KSR1 and KSR2 are composed of five domains called CA1-CA5. CA1 is involved in the formation of a KSR1-MEK-RAF complex. CA2 is a proline-rich domain (PRD) with unknown function. CA3 is a cysteine-rich domain (CRD) responsible for membrane localization of KSR proteins. CA4 is a serine/threonine domain needful for ERK interaction. CA5 is a putative kinase domain.

KSR1 and KSR2 have phosphorylation and protein-protein interaction sites that mediate their peculiar subcellular localization (Volle 1999). For example, KSR1 interacts with an E3 ubiquitin ligase IMP which sequesters the scaffold in triton-resistant structures and therefore prevents ERK activation (Matheny 2004). Phosphorylation of KSR1 on Ser297 and Ser392 residues and of KSR2 on Ser310 and Ser469 residues by the C-TAK1 kinase retains KSR proteins in the cytosol through a binding with the 14-3-3(Xing 1997; Müller 2001; Gao 2022). Upon Ras stimulation, the IMP ligase is polyubiquitinated and degraded, while

KSR1 is dephosphorylated by the PP2A phosphatase (Ory 2003). Instead, KSR2 is dephosphorylated by calcineurin (Dougherty 2009).These events relocate the scaffolds in the membrane, favoring the RAS/RAF/MEK/ERK pathway (**Fig.6)**. There are different mechanisms of attenuation of the kinase cascade. It has been observed that locally activated ERK phosphorylates KSR1 and B-RAF causing a dissociation of the complex and an attenuation of the mitogenic signal (McKay 2009). Furthermore, B-RAF and KSR1 are also regulated through phosphorylation mediated by protein kinase A (PKA) (Smith 2010). Indeed, in response to growth factor or cAMP stimulation, the E3 ubiquitin ligase Praja2 ubiquitinates KSR1 attenuating the ERK1/2 cascade (Rinaldi 2016; Zhao 2021).This regulation is crucial to maintain the Embryonic Stem Cells (ESCs) in the pluripotent state and to drive their differentiation into epiblast-like cells (Rinaldi 2016).

Genetic studies demonstrate that KSR1 knock-out mice develop normally and are fertile but have an impaired immunological response (Fusello 2006). Conversely, KSR2 knock-out mice exhibit a different phenotype which is reduced fertility and spontaneous obesity, indicating an important role of KSR2 in energy homeostasis (Revelli 2011).

Patients affected by severe and early obesity carry multiple KSR2 mutations. Heterozygous frameshift, nonsense and missense mutations were the most common types. Additionally, few of these mutations affect the protein's kinase domain, which impairs the protein's ability to interact with MEK and B-RAF (Pearce 2013). Furthermore, some mutations fall in the region between the CA2 and CA3 domains, impairing the binding to AMPK which is a regulator of energy homeostasis (Hardie 2007). Knockout mice with deletions in the KSR2 or AMPKα2 subunit are hyperphagic and rapidly gain body weight around 20-24 weeks of life despite being fed the same way as wild-type mice(Henry 2014). They also show a reduction in energy expenditure, and this is due to an impairment of mitochondrial Fatty Acid Oxidation (FAO) (Guerra 1998). The inability to maintain a normal body temperature is a sensitive indicator of compromised fatty acid oxidation. Indeed, KSR2 knock-out mice, at 10 weeks of age, became intolerant to cold temperatures (Pearce 2013). Finally, deletions of KSR2 that modify its interaction with AMPK impair glucose oxidation, leading to a decrease in glucose consumption (Costanzo-Garvey 2009). This metabolic phenotype mimics the clinical characteristics of young, obese patients with KSR2 mutations, who are marked by severe insulin resistance, downregulation of basal metabolism, and a low heart rate (Pearce 2013). According to clinical reports, the administration of the antidiabetic drug metformin in certain children carrying KSR2 mutations leads to a significant weight reduction, stimulating AMPK activation (Rena 2017).

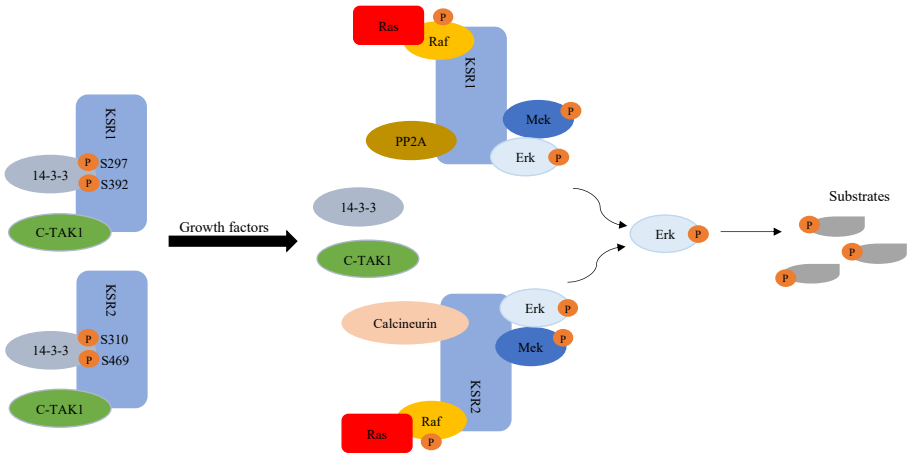

Figure 6. KSR1 and KSR2 regulation in the Ras/Raf/MAPK pathway. In resting conditions, KSR1 and KSR2 are phosphorylated by c-TAK1 kinase at S297 and S392, and S310 and S392, respectively. Under stimulated conditions, KSR1 is dephosphorylated by PP2A phosphatase, and KSR2 is dephosphorylated by calcineurin. KSR proteins are relocated in the membrane and promote the Ras/Raf/MEK/ERK pathway.

1.5 AMP-activated protein kinase (AMPK)

AMPK is the main metabolic sensor that regulates energy homeostasis and cellular responses to environmental stresses such as hypoxia and nutrient deprivation, autophagy, apoptosis, and cell proliferation (Yuan 2020). It belongs to a family of serine/threonine kinases highly conserved in eukaryotes and has two orthologues SNF1 and SnRK1, in yeast and plants, respectively. AMPK is expressed in different tissues, such as the brain, liver, and skeletal muscle, and is considered a potential target for treatment of pathologies associated with metabolic disorders such as diabetes, obesity, and fatty liver disease (Herzig 2018).

1.5.1 The structure of AMPK

AMPK is a heterotrimeric protein complex composed of three subunits: one catalytic α-subunit and two regulatory subunits, β and γ (Davies 1994)(**Fig.7)**. In humans, these subunits can be found in different isoforms. The catalytic α-subunit can exist as two isoforms, α1 and α2, encoded by the PRKAA1 and PRKAA2 genes (Stapleton 1996). Furthermore, the two isoforms have a different cellular distribution. The α1 isoform is widely expressed in heart, liver, lung, and brain, while the α2 isoform is mainly expressed in neuron and muscle cells (Quentin 2011). The α-subunit contains a kinase domain located at the N-terminus and five phosphorylation sites Thr172, Ser173, Thr258, and Ser485/491(Woods 2003). The residue Thr172 is phosphorylated by upstream kinases to promote the activity of the enzyme, while the other residues are auto-phosphorylated by PKA or AKT, thus inhibiting AMPK activity (Djouder 2010). Downstream of the kinase domain, there is an autoinhibitory domain (AID) that inhibits the kinase activity, followed by a β/γ interaction domain located at the C-terminus (Hardie 2012).

The regulatory β-subunit can exist as two isoforms, β1 and β2, encoded by the PRKAB1 and PRKAB2 genes (Hardie 2007). The β1 isoform is expressed mostly in the liver, while the β2 isoform is mainly expressed in skeletal muscle (Thornton 1998). Furthermore, it contains a carbohydrate-binding module or CBM which allows the association of AMPK to glycogen particles most likely to regulate target substrates such as glycogen synthase (Hudson 2003). Moreover, the β-subunit has a C-terminal domain (β-CTD) that mediates the binding with the α-CTD and the γ subunit. The regulatory γ-subunit can exist as three isoforms, γ1, γ2 and γ3, encoded by PRKAG1, PRKAG2 and PRKAG3 (Cheung 2000). The γ1 and γ2 isoforms are widely expressed in human tissues, while the γ3 isoform is only detected in skeletal muscle. It contains at the C-terminus an interaction region with the β-subunit, followed by four tandem

cystathionine-β- synthase (CBS) domains called Bateman domains. They allow the binding to adenine nucleotides enabling AMPK to respond to changes in ATP/AMP ratio (Xiao 2007).

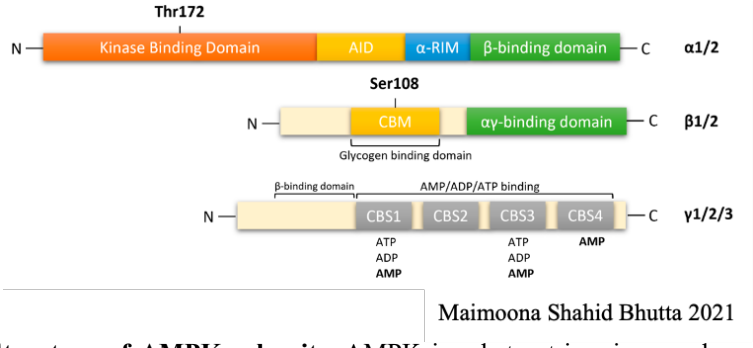

Figure 7. Structure of AMPK subunits. AMPK is a heterotrimeric complex composed of catalytic α-subunit and two regulatory subunits, β and γ. Catalytic α-subunit comprises a kinase binding domain (KBD) that contains Thr172, which is phosphorylated by an upstream kinase; an autoinhibitory domain (AID); a β-subunit binding domain at the C-terminal. Regulatory β-subunit comprises a carbohydrate-binding domain (CBM) and an αγ-binding domain. Regulatory γ-subunit comprises a β-subunit binding domain, followed by four cystathionine-β-synthase (CBS) domains.

1.5.2 AMPK activation

The AMPK complex is activated through the phosphorylation of Thr172 residue located in the activation loop of the catalytic α-subunit by upstream kinases. There are two major regulators for AMPK activation: liver kinase B1 (LKB1) and Calcium/calmodulin-dependent protein kinase kinase II (CAMKKII). LKB1 is a tumor suppressor that is mutated in the Peutz–Jeghers syndrome (Alessi 2006). It functions as a heterotrimeric complex associated with the kinase-dead STE20-related kinase (STRAD) and the STE20 family scaffolding protein MO25 (Hawley 2003). The LKB1 complex is not stimulated by AMP and is constitutively active in cell lines (Woods 2003). LKB1 phosphorylates and activates AMPK only after AMP binds to the γ subunit. LKB1/AMPK signaling protects cells from apoptosis induced by high AMP levels. Indeed, LKB1-deficient cells undergo rapid death because they are unable to reverse the AMP/ATP ratio (Shaw 2004). Recently, it has been demonstrated that AMPK activity is increased in cells undergoing senescence. For this reason, LKB1-deficient cells are also resistant to passage-induced senescence (Bardeesy 2002).

However, it is known that AMPK is also phosphorylated by CAMKKII (also called CAMKKβ) following intracellular calcium variations, and cellular stresses such as hypoxia, and aminoacid starvation (Hurley 2005). CAMKKII is a member of a calcium/calmodulin-dependent protein kinases (CAMKs) family, which are activated by increases in the concentration of intracellular

calcium and calmodulin (Hawley 2005). The increase in cytosolic calcium triggers processes that require the consumption of ATP and this leads to the activation of AMPK. This explains why the persistence of AMPK activity in LKB1-deficient cells (Woods 2005).

1.5.3 Downstream substrates of AMPK

AMPK is the principal metabolic sensor that inhibits the anabolic pathways to minimize the consumption of ATP and promotes the catabolic pathways to stimulate the production of ATP (Hardie 2006) (**Fig.8)**. The first identified downstream AMPK substrates are the acetyl- CoA carboxylases, ACC1 and ACC2. In particular, AMPK phosphorylates ACC1 at Ser79 residue and ACC2 at Ser212 residue and inhibits their function in the *de novo* lipids synthesis (Hardie 2002). Furthermore, ACC converts acetyl-CoA to malonyl-CoA promoting lipid synthesis. Malonyl-CoA is an inhibitor of the Carnitine Palmitoyltransferase I (CPT1) and prevents the entry of fatty acids into the mitochondria for β-oxidation (Ruderman 2003). Thus, by phosphorylating ACC, AMPK decreases fatty acid synthesis and increases fatty acid oxidation to generate more ATP. Moreover, it catalyzes inhibitory phosphorylation on the β-Hydroxyβ-methylglutaryl-CoA reductase (HMGCR), which is involved in the synthesis of cholesterol (Steinberg 2009).

AMPK has also an important role in glucose metabolism. It is well known that it increases glucose uptake by facilitating the translocation of the receptor GLUT4 in the plasma membrane (Fryer 2002). The membrane localization of the receptor is guaranteed by the inhibitory phosphorylation of TBC1D4, a Rab GTPase-activating protein (GAP) that suppresses the fusion of GLUT4 vesicles with the membrane (Chavez 2008). AMPK also increases the glycolytic pathway by phosphorylating and activating the phosphofructokinase 2 (PFK2), the enzyme responsible for the synthesis of fructose 2,6-bisphosphate (Marsin 2000). The latter activates the phosphofructokinase 1 (PFK1) of the glycolytic pathway and inhibits fructose- 1,6 bisphosphatase 1 of gluconeogenesis. Finally, AMPK phosphorylates and inhibits the glycogen synthases GYS1 and GYS2, indirectly fueling glycolysis (Bultot 2012).

AMPK is also involved in mitochondrial biogenesis. Indeed, mice lacking the AMPK β1/β2 subunits or the upstream kinase LKB1 have a reduced content of mitochondria in muscle (O'Neill 2011; Tanner 2013). Particularly, AMPK phosphorylates and activates the peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) at Thr177 and Ser538 residues, which controls mitochondrial biogenesis (Jäger 2007).

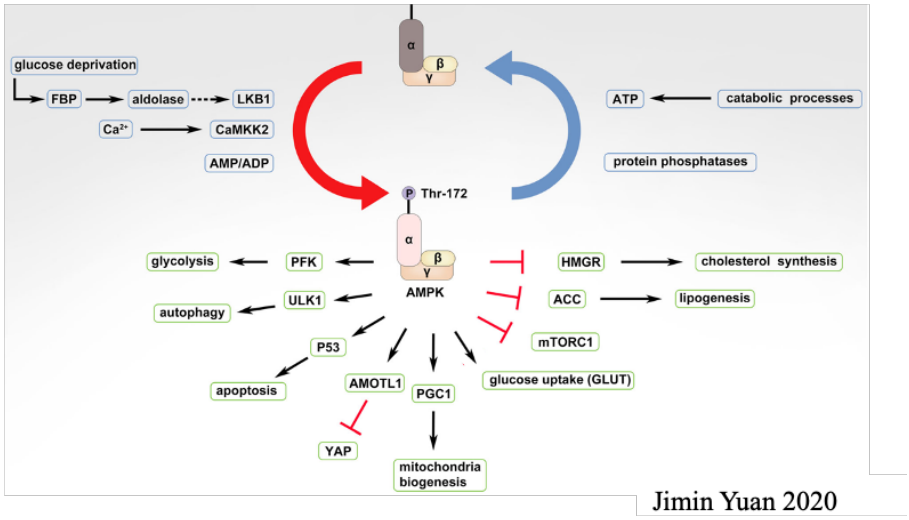

Figure 8. AMPK downstream effectors. Following the activation by LKB1 or CAMMKβ, AMPK activates different proteins involved in glycolysis, autophagy, apoptosis, mitochondria biogenesis, and glucose uptake, as indicated by the black arrows. Instead, AMPK inhibits proteins involved in cholesterol synthesis, lipogenesis, and mTORC1 signaling, as indicated by red bar-headed lines.

AMPK regulates protein translation by controlling the activity of the mammalian target of rapamycin complex 1 (mTORC1), a master regulator of protein translation, cellular proliferation, and autophagy (Bar-Peled 2014). mTORC1 is composed of regulatory associated protein of mTOR (raptor), mTOR associated protein LST8 homolog (mLST8, also known as GβL) and DEP domain containing mTOR-interacting protein (Deptor) (Efeyan 2012). mTORC1 is regulated positively by the PI3K/AKT signaling. Following stimulation of the tyrosine kinase receptors (RTKs), phosphatidylinositol 3-kinase (PI3K) type I generates a second messenger PI (3,4,5)P3 that leads to the activation of the AKT kinase (Kim 2017). AKT phosphorylates and inhibits two suppressors of mTORC1 activity (**Fig.9)**. The first is the tuberous sclerosis complex protein 2 (TSC2), a GTPase-activating protein that converts the small GTPase Rheb from the active GTP-bound state to the inactive GDP-bound state (Inoki 2002; Inoki 2003). The GTPase Rheb binds mTORC1 and promotes its kinase activity. The second is proline-rich Akt substrate 40 kDa (PRAS40) which inhibits Rheb-mediated activation of mTORC1 by binding raptor (Sancak 2007). Once activated, mTORC1 stimulates protein synthesis by phosphorylating ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) (Ma 2009). AMPK is known to inhibit mTORC1 through the activation of the negative mTORC1

regulator TSC2 and the inhibition of the mTORC1 subunit Raptor. Indeed, AMPK phosphorylates TSC2 at T1227 and S1345 residues, which in turn blocks protein translation by inhibiting the phosphorylation of S6K and 4E-BP1 (Inoki 2003). Inactivation of the TSC1/TSC2 complex causes an autosomal dominant disorder called Tuberous Sclerosis and increases tumor incidence due to the hyperactivation of mTORC1(Kobayashi 2001). Furthermore, AMPK phosphorylates the scaffold protein Raptor at Ser722 and Ser792 residues, enabling 14-3-3 binding and inhibiting mTORC1 activation (Gwinn 2008). Thus, AMPK and mTORC1 signaling represent a fundamental tumor suppressor and a potential therapeutic target, respectively (Zheng 2021). Nevertheless, treatment of GBM patients with mTORC1 inhibitors has not been very successful due to the activation of compensatory mechanisms (Jhanwar-Uniyal 2019). Indeed, patients affected by PTEN null glioma develop chemotherapy resistance mediated by mTORC2 activation (Tanaka 2011). This is the second member of the mTOR family and is composed of Rapamycin-insensitive companion of mTOR (Rictor), stress-activated protein kinase-interacting protein 1 (Sin1) and protein-binding Rictor (Protor), Deptor and mLST8 (Gulati 2009). mTORC2 plays essential role in cell survival and cytoskeleton organization and is an upstream regulator of AKT (Sarbassov 2005). Recently, it has been demonstrated that AMPK phosphorylates and activates mTORC2, promoting cell survival (Chhipa 2018; Kazyken 2019).This suggests that AMPK may, in some circumstances, paradoxically encourage carcinogenesis and that glioblastoma treatment could be improved by simultaneously inactivating mTORC1 and mTORC2.

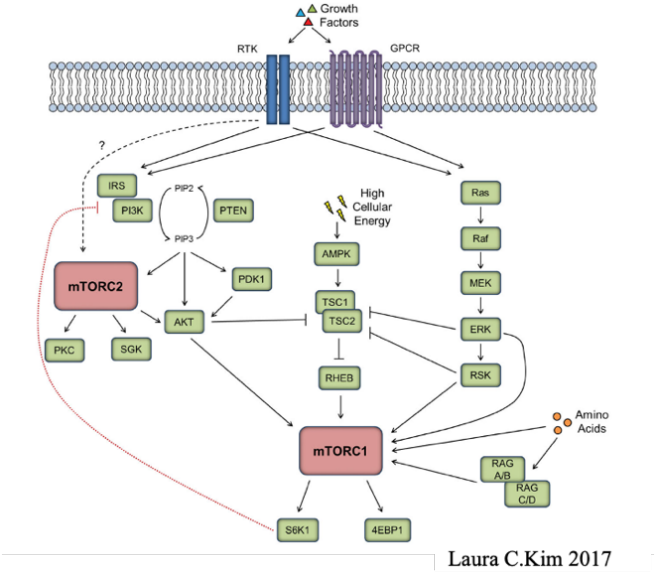

Figure 9. mTOR signaling pathway. Following stimulation of the tyrosine kinase receptors (RTKs), phosphatidylinositol 3-kinase (PI3K) phosphorylates PIP2, increasing the level of PIP3 in the membrane and facilitating the co-localization of AKT, PDK1, and mTORC2. This results in the activation of AKT, which in turn, activates mTORC1 by inhibiting TSC2, a GTPase-activating protein (GAP) for RHEB, an mTORC1 activator. AKT-mediated phosphorylation of PRAS40 promotes its dissociation from mTORC1, further enhancing mTORC1 activity. High cellular ATP levels inhibit AMPK, an activator of TSC2, leading to increased activities of RHEB and mTORC1.

1.6 AMPK, mTOR and autophagy

AMPK and mTORC1 exert an opposite role in the autophagy regulation. Autophagy is an evolutionarily conserved biological process that involves the degradation and recycling of cellular components, including proteins, organelles, and macromolecules (Parzych 2014). The turnover of old or damaged molecules occurs at a low level constitutively, but the autophagic pathway can be induced under stress conditions, such as nutrient or energy starvation, to provide nutrients useful for cell survival (Yorimitsu 2005). Alterations of the pathway have been linked with different human pathologies, including metabolic and neurodegenerative disease, as well as cancer (Wirawan 2012). In mammalian cells, there are three types of autophagy: microautophagy, chaperone-mediated autophagy, and macroautophagy (**Fig.10)**.

1.6.1 Microautophagy

The term microautophagy refers to a process in which cytoplasmic constituents are sequestered and engulfed by lysosomal vesicles. It has been extensively studied in baker's yeast, Saccharomyces cerevisiae, and the methylotrophic yeasts, Pichia pastoris and Hansenula polymorpha (Mijaljica 2011). Microautophagy can be divided into non-selective and selective forms. Non-selective microautophagy is involved in the degradation of randomly cytosolic components, whereas selective microautophagy is involved in the degradation of specific organelles such as mitochondria, nucleus, and peroxisomes (Farré 2009). Several studies demonstrate that microautophagy relies on morphological changes in lysosomes, vacuoles, or endosomes. Therefore, three types of microautophagy processes can be distinguished: type I, with protrusion of the lysosome or vacuole; type II, with invagination of the lysosome or vacuole; type III with endosomal invagination (Wang 2023). However, the mechanisms and biological functions are still poorly understood.

1.6.2 Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) is a highly specific process through which a wide range of substrates are degraded, including glycolytic enzymes, transcription factors and their inhibitors, and proteasome subunits (Arias 2011). CMA uses chaperone molecules to identify target substrates that contain in their sequence a consensus motif biochemically related to the pentapeptide KFERQ (Parzych 2014). This motif is recognized by the heat shock-cognate chaperone of 70kDa, HSC70, which unfolds and delivers the target protein to the lysosome surface (Agarraberes 2001). The substrate protein-chaperone complex binds to lysosome-associated membrane protein type 2A (LAMP2A), which acts as the

receptor for this pathway. This interaction leads to a multimerization of LAMP2A to form an active complex required for substrate translocation. Following translocation, the target proteins are degraded in the lumen of the lysosome and LAMP2A returns to its monomeric state to recognize and bind other substrates (Bandyopadhyay 2008).

1.6.3 Macroautophagy

Macroautophagy, also referred to as autophagy, differs from microautophagy and CMA because molecules, that are headed for degradation, are sequestered by double-membrane vesicles, termed autophagosomes. They are produced at various sites in the cytoplasm known as phagofore assembly sites (PAS) (Itakura 2010). During autophagosome formation, the membrane is supplied by different compartments such as plasma membrane, endoplasmic reticulum, Golgi apparatus, and the cargo substrate is surrounded (Weidberg 2011). Once it is formed, the autophagosome fuses with lysosomes forming autolysosomes. The autophagic cargo is thus degraded and the resulting macromolecules are recycled (Yorimitsu 2005).

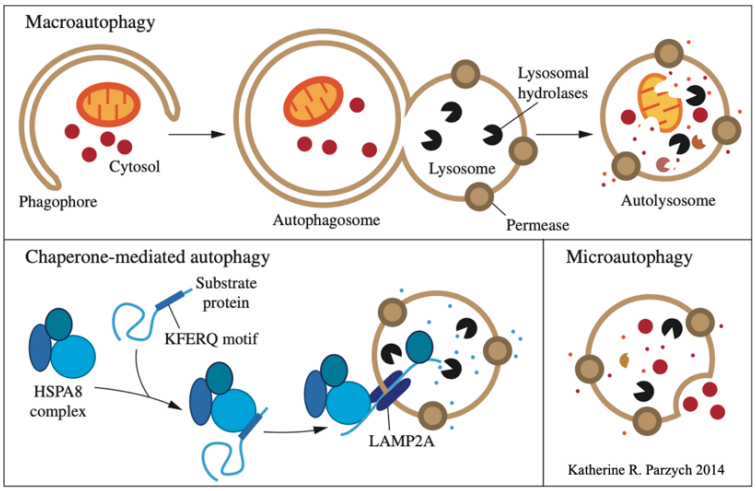

Figure 10. Different types of autophagy in mammalian cells. Macroautophagy is dependent on de novo generation of cytosolic double-membrane vesicles, known as autophagosomes, which serve to encapsulate and transport cargo to the lysosome. Chaperone-mediated autophagy uses chaperones to unfold and transport cargo proteins to the lysosome surface. Microautophagy involves the direct engulfment of cargo through the invagination of the lysosomal membrane.

The autophagic machinery involves several phases: induction, nucleation, elongation, autophagosome completion, and fusion, which involve different protein complexes (**Fig.11**). The first autophagic complex is Unc-51-Like Kinase 1 complex composed of the serine/threonine kinase ULK1, autophagy-related protein 13 (ATG13), focal adhesion kinase family interacting protein of

200 kDa (FIP200), and ATG101 (Zachari 2017). The interaction of ULK1 with ATG13 and FIP200 is essential to enhance ULK1 kinase activity. Indeed, the absence of either ATG13 or FIP200 reduces its activity and prevents its correct localization (Ganley 2009). Under nutrient conditions, mTORC1 associates with the complex and phosphorylates ULK1/2 and ATG13, thus inactivating both proteins (Hosokawa 2009). On the contrary, when cells are treated with rapamycin, a mTORC1 inhibitor, or starved for nutrients, mTORC1 dissociates from the complex and ULK1 phosphorylates its binding partners ATG13 and FIP200 to induce autophagy (Jung 2009). Instead, AMPK positively regulates autophagy through different mechanisms. As previously described, it phosphorylates and activates TSC2, a negative regulator of mTORC1 complex, and inhibits RAPTOR, a protein present within the complex, leading to impaired mTORC1 activity. AMPK also directly phosphorylates ULK1 on four residues Ser467, Ser555, Thr574, and Ser637, promoting autophagy (Egan 2011).Subsequently, the ATG14-containing class III phosphatidylinositol 3-kinase (PtdIns3K) complex is recruited to the site of autophagosome formation. This complex is composed of ATG14, PIK3C3/VPS34, PIK3R4/p150, and Beclin1 and is required for macroautophagy (Burman 2010). ATG14 can be replaced by UVRAG generating the UVRAG-associated PtdIns3K complex, which is involved in the later stages of autophagosome formation and in the endocytic pathway (Liang 2006). PtdIns3K is negatively regulated by the anti-apoptotic protein BCL2, which interacts with Beclin1 disrupting complex formation, while it is positively regulated by the AMBRA1 protein, which promotes autophagy activation by binding Beclin-1 (Pattingre 2005; Fimia 2007). The UVRAG-associated PtdIns3K complex is positively regulated by the SH3GLB1/Bif-1 protein, which interacts with Beclin-1 through UVRAG, activating PI3KC3 and inducing autophagosome formation (Takahashi 2007). On the contrary, this complex can be negatively regulated by the Beclin1-binding protein, Rubicon, which binds to UVRAG and Beclin-1, suppressing the autophagosome maturation (Matsunaga 2009). The PtdIns3K complex generates Phosphatidylinositol-3-phosphate (PtdIns3P), which recruits different proteins to the phagophore, such as WIPI1/2 and DFCP1, whose function is still little known (Jeffries 2004; Polson 2010). The phagophores expansion phase involves two conjugation systems consisting of ubiquitin-like proteins (UBL) (Weidberg 2011). The first is represented by Atg12–Atg5-Atg16 complex. The UBL protein ATG12 is covalently conjugated to ATG5 by the E1 activating enzyme ATG7 and the E2 conjugating enzyme ATG10 (Ohsumi 2001). Subsequently, the ATG12-ATG5 complex is bound by the UBL protein ATG16 via ATG5. ATG16 induces the dimerization of two complexes ATG12-ATG5-ATG16, which are associated with the phagophore, promoting membrane

expansion (Mizushima 2003). The second conjugation system is represented by the ATG8/LC3 system. The ATG8-like proteins are divided into LC3 and GABARAP subfamilies. The LC3 family includes three variants LC3A, LC3B and LC3C, while the GABARAP family includes GABARAP, GABARAPL1, GATE-16 (also known as GABARAPL2), and GABARAP-L3 (Hemelaar 2003). Although both proteins are localized on the autophagosome, they act at two different stages of autophagosome formation. LC3 proteins act during the elongation phase of the phagophore, while GABARAP proteins act during the maturation phase of the autophagosome(Weidberg 2010). The conjugation system begins with the exposure of a glycine residue at the C-terminus of LC3 by the action of a cysteine protease ATG4. Then, The E1-like enzyme Atg7 activates the processed LC3, named LC3-I, and transfers it to the E2-like enzyme Atg3. The exposed glycine of LC3 is conjugated to the lipid phosphatidylethanolamine (PE) by the dimeric complex ATG12-ATG5-ATG16, which functions as an E3 ligase. This process generates a lipidated LC3, called LC3-II (Parzych 2014). As soon as the autophagosome is formed, it fuses with the endosome or lysosome, generating the autolysosome. The fusion occurs through membrane proteins called SNAREs, such as VAMP7/9 and Syntaxin 17 (Fader 2009; Itakura 2012). Inside the lysosomes, the cargo is degraded by lysosomal proteases, and the degradation products, such as amino acids and fatty acids are recycled for metabolic activities (Panda 2015).

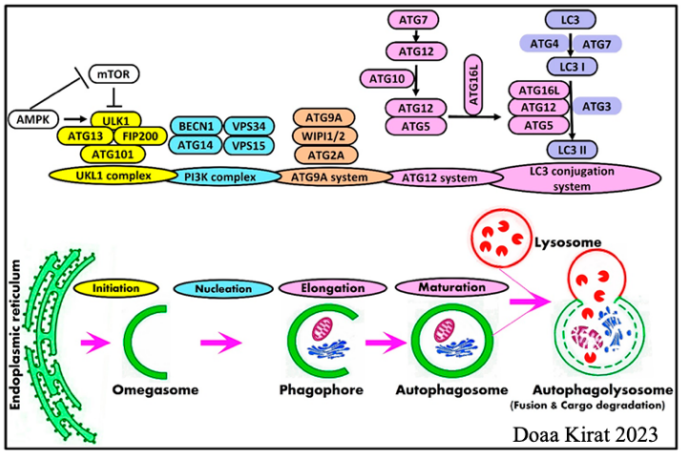

Figure 11. Schematic representation of macroautophagy pathway. Macroautophagy is initiated in response to cellular stress, nutrient deprivation, or other signals. The induction of autophagosome formation is regulated by ULK1 complex, which includes ULK1, FIP200, ATG13, and ATG101 proteins. Following initiation, the membrane begins to expand through the action of PI3K complex, including BECN1, VPS34, ATG14, and VPS15. The elongation and completion of phagophore formation occur through two conjugation systems, ATG12-ATG5-ATG16 complex and ATG8/LC3 complex. The mature autophagosome fuses with a lysosome, forming an autolysosome, in which the cargo is degraded.