UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXIV CYCLE

The role of FKBP51 in the activation of Akt oncogenic pathway

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2018-2022

List of Abbreviations

FKBP = FK506 binding protein
PPIase = peptidyl-prolyl cis-trans isomerase activity
CaN = calcineurin
mTOR = mammalian target of rapamycin
FKBP51 = FK506 binding protein 51
FK = FKBP-like domain
TPR = tetratricopeptide repeat
Hsp = heat shock protein
NF-κB = Nuclear factor kappa-light-chain-enhancer of activated B cells
FKBP51s = FKBP51 short
PD-L1 = Programmed cell death ligand 1
Akt/PKB = Protein Kinase B
HM = hydrophobic motif
T308 = threonine 308
S473 = serine 473
mTORC2 = mammalian or mechanistic target of rapamycin complex 2
PHLPP = PH domain leucine-rich repeat protein phosphatase
GSK-3 = Glycogen synthase kinase 3
mTORC1 = mammalian or mechanistic target of rapamycin complex 1
p70S6K = ribosomal protein S6 kinase beta-1
17-AAG = 17-N-allylamino-17-demethoxygeldanamycin
TRAF6 = tumor necrosis receptor-associated factor 6
siRNA = small interfering RNA
pAkt = phosphorylated Akt
EV = empty vector
IP = immunoprecipitation
IB = immunoblot

Abstract

FKBP51 is an immunophilin with a relevant role in sustaining cancer cell growth and aggressiveness of different human tumors and, particularly, melanoma. The protein contains two N-terminal FKBP-like domains FK1 and FK2, with FK1 exerting an isomerase activity, and a C-terminal region with a tetratricopeptide repeat (TPR) domain for protein/protein interaction. Based on its scaffold and isomerase activities, it participates in several signaling pathways, including NF-κB and Akt signaling. The role of FKBP51 in the Akt pathway is controversial. In 2009 Pei et al. proposed a role for FKBP51 as a scaffold promoting interaction between Akt and PHLPP phosphatase, thus negatively regulating Akt activation, in a pancreatic cancer context. In 2010, Romano et al. showed an association between FKBP51 upregulation and high pAkt levels in different tumor types. In 2013, Fabian et al. reported an increase in pAkt S473 upon FKBP51 overexpression. Deregulation of the Akt pathway is one of the major mechanisms that sustain cancer survival and progression. Especially in an incurable tumor as melanoma, in depth knowledge of the mechanisms underlying Akt activation is needed. In this thesis work, the interactions of FKBP51 with Akt and PHLPP are investigated along with the mechanism of Akt activation by the immunophilin. The study benefited from the recent identification of the FKBP51 spliced isoform (lacking the TPR domain), that reconciled the diverging results, generating the hypothesis that a unique gene regulated phosphorylation and de-phosphorylation of Akt.

1. Background

1.1. FK506 binding proteins

FK506 binding proteins (FKBPs) are highly conserved proteins that belong, along with cyclophilins (Cyp), to the immunophilins family. The designation "immunophilins" for this protein family is due to the immunosuppressive character of the complex formed by the immunosuppressant drugs FK506 or Rapamycin with FKBPs or by the cyclosporine A with cyclophilins. This drug/protein binding inhibits their enzymatic activity which consists in a peptidyl-prolyl cis-trans isomerase activity (PPIase) deputed to catalyze the isomerization of peptidyl-prolyl imide bonds, from cis to trans, and vice versa, in protein substrates (Dornan 2003; Fischer 2003). The complex formation is favored by the greater stability that FKBPs acquire when they are bound to their ligands, thus remaining more resistant to the proteolytic cleavage and creating an appropriate binding surface for binding to calcineurin (CaN) (in the case of FK506 ligand) and to the mammalian target of rapamycin (mTOR) (in the case of rapamycin ligand) (Yamamoto 1995; Dornan 2003; Fischer 2003). These enzymes are ubiquitous and abundant in subcellular compartments of virtually all organisms (Fisher 2003). In humans, there are at least seventeen FKBPs (Fig.1), that possess one or more PPIase domains with other functional polypeptide segments including: tetratricopeptide repeat (TPR) motif involved in protein interactions; EF-hand calcium-binding domain containing helix-loop-helix topology, in which Ca^{2+} ions are coordinated within the loop; nucleic acid binding regions; transmembrane domain; nuclear localization and endoplasmic reticulum signal sequence (Fisher 2003). The variety of FKBPs domains provide these proteins with a wide range of cellular functions as the co-chaperone activity which acts in protein folding; improvement of kinase performance (Romano 2015a); receptor signaling; protein trafficking and transcription (Fisher 2003).

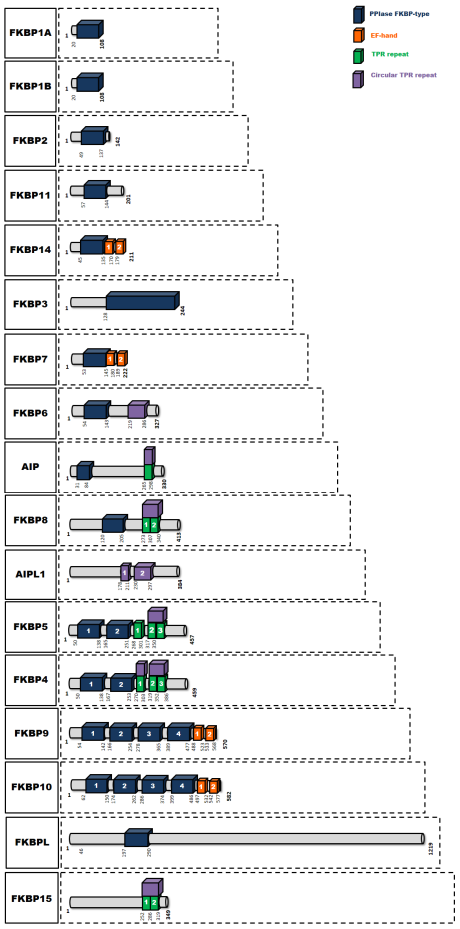

Figure 1. *Representation of all seventeen human immunophilins with all their domains: in blu PPIase FKBP-like domain; in orange EF-domain; in green TPR repeats, in purple circular TPR repeats (modified from D'Arrigo 2016).*

The first immunophilin that has been reported to be part of these complexes was FKBP12, the progenitor of FKBPs, whose binding with FK506 creates a complex that interacts with CaN, inhibiting calcium-dependent early events of T-cell activation (Fig.2) (Baughman 1995; Hogan 2003). In addition, FKBP12 binds to rapamycin (Fig.2) forming a complex that inhibits mTOR, the serine-threonine kinase that is activated after growth factors and nutrients stimuli to sustain cell cycle progression (Schmelzle 2000). Besides FKBP12, all human FKBPs (FKBP12;12.6;13;25;51;52) can form rapamycin-induced ternary complexes with the FKBP-rapamycin binding domain (FRB) of mTOR (Hausch 2013), while only FKBP12, FKBP12.6 and FKBP51 can mediate FK506 effects on CaN activity in human cells. Both mTOR and CaN are deregulated in many cancer types and responsible for tumor growth, metastasis and therapy resistance.

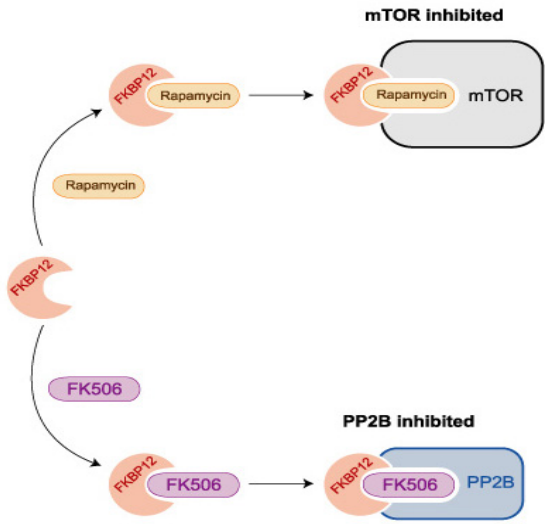

***Figure 2**. Mechanism of action of FK506 and rapamycin. Drugs binds to their natural ligand FKBPs (in this case, FKBP12 has been chosen in example) to form the immunosuppressant ternary complex with their specific substrates. Rapamycin/FKBP binds to mTOR; instead FK506/FKBP binds to CaN (also called PP2B). The function of this macro-complex is to inhibit the enzymatic activity of mTOR and CaN leading to an immunosuppression status.*

1.2. FKBP51 and its isoforms

FKBP51 belongs to the FKBPs family and it is a large immunophilin encoded by the *FKBP5* gene, localized on the short arm of chromosome 6 (6p21.31) (D'Arrigo 2016). The protein contains two N-terminal FKBP-like domains, named FK1 and FK2, separated by a short linker sequence (Fig.3); only FK1 exerts the PPIase and ligand binding activity, while FK2 is inactive but it contains an ATP/GTP-binding sequence and seems to retain an interaction ability and a structural role (D'Arrigo 2016). FKBP51 may function either as a scaffold or, because of its isomerase activity, by modulating conformation and function of partner proteins (Hausch 2013). The C-terminal region has a TPR domain (Fig.3), characterized by tandem repeats of 34 aminoacids with a defined helix-alpha-helix motif, which is involved in interaction with other proteins, including the chaperone heat shock protein 90 (Hsp90) (Cheung 2000). This structural feature suggests that FKBP51 may share some functions with the Hsps. FKBP51 was firstly cloned in lymphocytes and was found to be associated with immunosuppression mediated by CaN inhibition (Baughman 1995). Besides the immunoregulatory effects, FKBP51 exerts multiple physiological roles, such as i) it takes part and regulates the signaling of the steroid hormone

receptor, together with the heat shock proteins Hsp90/Hsp70 and the co-chaperone FKBP52 (Gallo 2007); ii) it protects against oxidative stress by exerting antiapoptotic mechanisms (Gallo 2011); iii) it exerts a neuroprotection function regulating the clearance of microtubule-associated protein tau and stabilizing microtubules (Jinwal 2010); iv) it is expressed in mitotically active mesenchymal cells in very early phases of differentiation (Hynes 2014). FKBP51 is broadly expressed in all tissues of mammalians, with the highest expression in active metabolically tissues, as adipocytes, skeletal muscle and lymphocytes (Pereira 2014) and in those regions of the brain that control stress response and anxiety-related behaviors. As such, the main effort of FKBP51 research *in vivo* is focused on its role in metabolic and stress-related disorders. Preclinical studies have shown that FKBP51 null mice had a reduced Peroxisome proliferator-activated receptor gamma (PPARγ) activity (Stechschulte 2016) , improved glucose tolerance and reduced weight gain (Hartmann 2012; Sanchez 2012), demonstrating the involvement of FKBP51 in adipocytes differentiation (Sidibeh 2018) and insulin resistance (Toneatto 2013). Overexpression of FKBP51 in animal studies causes increased anxiety and impaired extinction learning (Zannas 2016). Moreover, several studies have shown the association of abundant FKBP51 expression with schizophrenia (Sinclair 2013), bipolar disorders (Chen 2013) and post-traumatic demoralization syndrome (PTDS) (Young 2015).

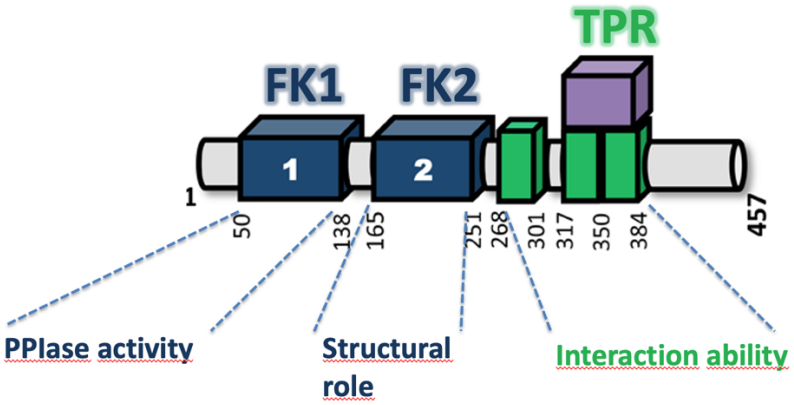

***Figure 3**. Schematic representation of the FKBP51 protein. FKBP51 contains two FKBP-like domains separated by a short linker sequence. The N-terminal FK1 is responsible for the PPIase- and ligand-binding activities. The second FK2 is inactive in those activities but seems to retain a structural role. The C-terminal contains TPR tandem repeats that confers the proteins the interaction ability (modified from D'Arrigo 2016).*

Besides in neuropathological disorders, FKBP51 has been found deregulated in a wide number of human cancers. In particular, for glioma (Jiang 2008), prostate cancer (Periyasamy 2010) and melanoma (Romano 2010a) a strict correlation between aggressiveness and protein abundance has been demonstrated. In gliomas, a study conducted by Jiang *et al.* (2008) on different specimens has shown that FKBP51 levels correlated with tumor grading and the enhanced FKBP51 expression is associated with apoptosis resistance and increased proliferation. In prostate cancer, FKBP51 is part of a super-chaperone complex that includes androgen receptor (AR) and androgen hormone (Periyasamy 2010). According to Ni *et al.* (2010), in this cancer, the immunophilin is upregulated and very important for determining the ligand-binding competence and transcriptional activity of the AR. In melanoma, FKBP51 sustains the activation of the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors and promotes resistance, cancer renewal and metastasis (Romano 2015a; Romano 2013). Also studies in precancerous conditions, such as myeloproliferative disorders, support the hypothesis that FKBP51 is essential for cancer cell proliferation. Giraudier (2002) firstly demonstrated that the overexpression of FKBP51 in idiopathic myelofibrosis, a chronic myeloproliferative disorder, regulates the growth factors independence of megakaryocyte progenitors and induces an apoptotic resistance to cytokine depletion, a condition that sustains cell survival. Immunohistochemistry studies of different human cancers also suggested that FKBP51 can be increased in lung, pancreas and ovary cancers (Staibano 2011; Romano 2010b).

In 2014, the research group to whom I belong identified a spliced isoform of FKBP51. Structurally, this isoform has multiple differences in the coding region and in 3' UTR, compared to the canonical isoform. The *FKBP5* gene generates, by alternative splicing, a truncated FKBP51 isoform lacking TPR domain because of a frameshift that generates an early STOP codon and consequentially, a different C- terminal sequence compared to the canonical one (Fig.4). For this reason, being the spliced isoform shorter than the canonical one, they decided to call it FKBP51 short (FKBP51s).

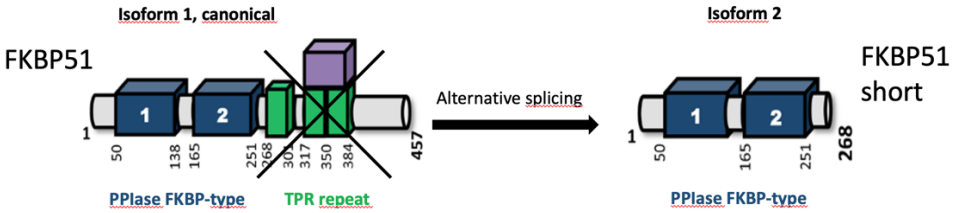

***Figure 4**. Representation of two FKBP51 isoforms. On the left side the canonical isoform, on the right side the short isoform. FKBP51s is generated by alternative splicing of the FKBP5 gene; it lacks the TPR domain and has a different coding region at C-terminal and in 3'-UTR.*

The functions of this spliced isoform are unknown and, since 2014, most efforts of our research team are focused on studying it. The research group demonstrated that the splicing isoform is opportunistically exploited by melanoma to suppress undesired immunity, through the interaction of the Programmed cell death ligand (PD-L1) with its receptor PD1 (Romano 2015a). Particularly, they found that FKBP51s expression in the tumor-infiltrating lymphocytes (TILs) of melanoma patients was influenced by the tumor expression of PD-L1 (Romano 2015a). In 2017, I also contributed to addressing the role of FKBP51s as foldase in PD-L1 post-translational modifications, occurring during protein maturation demonstrating that it supports the PD-L1 glycosylation and plasma membrane expression (D'Arrigo 2017). Moreover, FKBP51s enhances pro-tumoral properties of the glioblastoma cell improving its self-renewal and growth capacities both *in vitro* and *in vivo* (D'Arrigo 2019). Thus, for its role in PD-L1 maturation, FKBP51s contributes to an efficient inhibitory checkpoint signal and plays a relevant role in immune suppression and tumor resistance (D'Arrigo 2017; D'Arrigo 2019). Recent evidence by our group also showed a dynamism of PD-L1 expression that followed cyclin-D fluctuation in the glioblastoma cell and that this dynamic behavior is influenced by FKBP51s localization into the cell compartments (Tufano 2021).

In conclusion, both FKBP51 isoforms differentially act supporting cancer features such as cell survival, apoptosis resistance and cancer tolerance.

1.3. The Akt pathway

The Protein Kinase B (PKB/Akt) is a member of the serine-threonine kinase AGC superfamily and consists of three isoforms (Fig.5): Akt1, 2 and 3 that differ in their tissue expression. Akt1 is widely distributed across all the tissues and promotes cell growth and survival (Chen 2001); Akt2 is restricted to insulin-sensitive tissues where it regulates glucose homeostasis (Garofalo 2003); Akt3 is the least studied isoform and its expression is limited to the testis and

brain (Yang 2003). The Akt pathway is involved in a lot of cellular and physiological processes in mammals and plays an essential role in cell survival, growth, migration, proliferation, polarity, metabolism, and cell cycle progression. Deregulation or malfunction of Akt contributes to a wide variety of human diseases including cancers; glucose intolerance; schizophrenia; viral infections and autoimmune diseases (Manning 2007; Brazil 2004). Indeed, Akt is often constitutively active in tumors and assumes a relevant role in cancer growth and resistance (Fruman 2017; Manning 2007; Laplante 2012). Structurally, Akt has an amino-terminal lipid-binding pleckstrin homology domain (PH), the bilobal kinase core domain (KD) and a carboxyl-terminal regulatory domain with hydrophobic extension, also known as the hydrophobic motif (HM) (Fig.5).

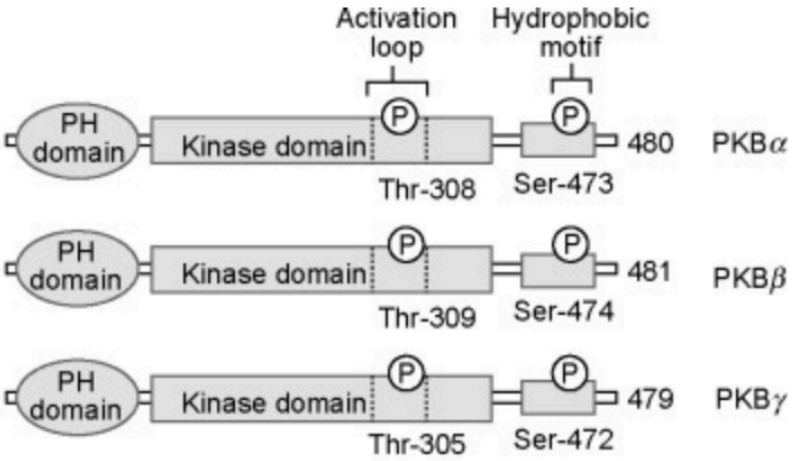

Figure 5. *Akt isoforms sequence: at the N-terminus PH domain serves to recruiting the kinase to the membrane; in the middle is shown the kinase domain that retain the enzymatic activity, with the phosphorylation site in threonine in activation loop; at the C-terminus the hydrophobic motif with the serine phosphorylation site. Both phosphorylation sites are slightly different in the three isoforms, but their relevance in the activation process of Akt is the same (Dummler 2004).*

The kinase is activated through the binding of its N-terminal PH domain to the phosphatidylinositol 3,4,5-triphosphate (PIP3), which affects the structure of Akt and recruits it to the plasma membrane, where phosphoinositide-dependent kinase 1 (PDK1) phosphorylates the activation loop in threonine 308 (T308) (Alessi 1997). Phosphorylation in T308 promotes the conformational change that enable Akt to bind to ATP allowing phosphate transfer (Yang 2002a). A further phosphorylation of the HM at serine 473 (S473) occurs by the mammalian or mechanistic target of rapamycin complex 2 (mTORC2) that, at

least *in vitro*, drives to the maximal Akt activation with an increase of its activity ten-to hundred-fold (Yang 2002b). There is a third site of phosphorylation in Akt sequence on threonine 450, but it occurs constitutively during translation and it is important for kinase stability (Oh 2010). Interestingly, a number of recent studies have demonstrated that, *in vivo*, S473 phosphorylation is not essential and T308 is sufficient to trigger Akt and led to downstream target activation (Balasuriya 2018). Indeed, ablation of mTORC2, or mutation of S473, did not affect that much the Akt activity (Biondi 2001) and it has been suggested that S473 role *in vivo* is to protect the kinase from dephosphorylation by cellular phosphatase (Chan 2015). Moreover, it has been shown that T308 phosphorylation is more strictly associated to Akt activity in tumor samples, rather than S473 (Lin 2005). It is, indeed, worth to mention that in acute myeloid leukemia, patients' sample analysis had revealed a strong association between cytogenetic high-risk and p-T308 (Gallay 2009). In Non-Small-Cell-Lung Cancer (NSCL) Tsurutani (2006) reported that only T308, but not S473 had a prognostic value. Moreover, a very recent work by Wei (2019) demonstrated that Tripartite Motif-Containing Protein 44 (TRIMM44) induce melanoma progression by enhancing phosphorylation in T308 of Akt. Upon phosphorylation, Akt is detached from the membrane and translocates to the target sites in the cytoplasm and nucleus. The Akt pathway (Fig.6) is tightly controlled by different phosphatases: protein phosphatase A (PP2A) dephosphorylates T308, whereas PH domain leucine-rich repeat protein phosphatase (PHLPP) dephosphorylates S473 (Resjö 2002), both events induce the inactivation of Akt, switching off kinase pro-survival and growth-promoting signaling (Fabian 2013).

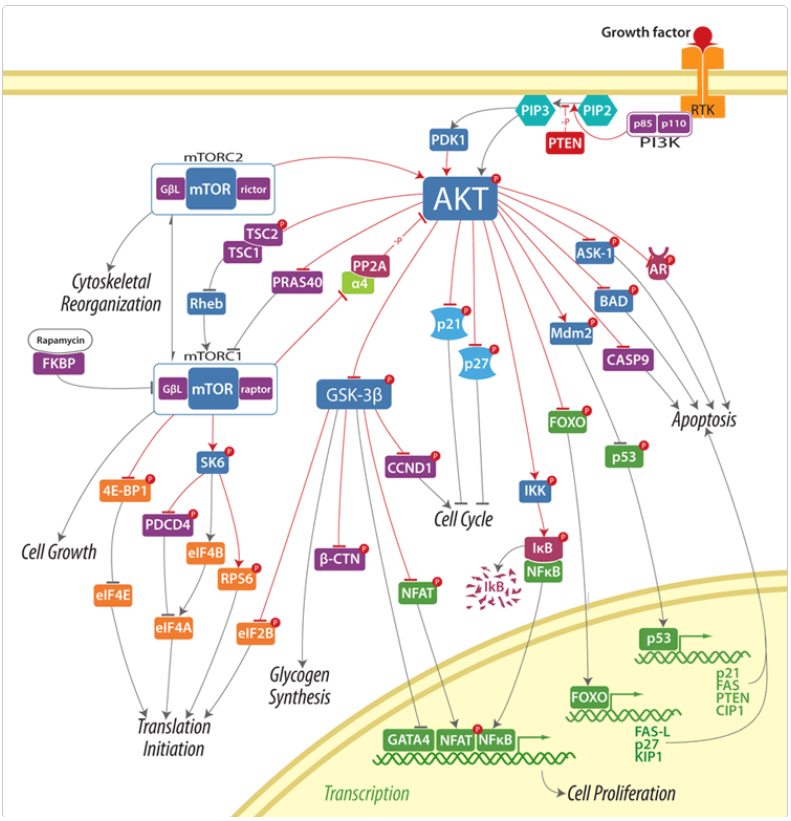

***Figure 6**. PI3k/Akt/mTOR pathway (from website http://www.naturalheightgrowth.com/2012/09/20/the-akt-signaling-pathway/).*

The oncogenic potential of this kinase is due mainly to its pro-survival effects. Akt phosphorylates the proapoptotic protein Bcl-2 associated agonist of cell death (Bad), preventing the binding to its target (Datta 1997). Akt also phosphorylates the Forkhead family of transcriptional factor (FOXO) inducing the inhibition of FOXO and the downregulation of its target genes as the proapoptotic Bcl-2-like protein 11 (Bim) (Brunet 1999). Another important target of Akt is the IκB kinase alpha (IKKα), whose phosphorylation leads to the activation of NF-κB (Nidai 1999). Akt also activates the mouse double minute 2 homolog (MDM2), whose phosphorylation results in translocation to the nucleus where MDM2 inhibits the tumor suppressor protein p53 (Zhou 2001). Among the plethora of Akt substrates, others that are worth to mention are those involved in cell cycle progression. For instance, the cyclin-dependent kinase inhibitor 1 ($p21^{Cip1}$), an inhibitor of cell cycle (Chang 2003); the X-linked inhibitor of apoptosis protein (XIAP), whose phosphorylation and stabilization, in turn, inhibits apoptosis (Dan 2016) and Glycogen synthase kinase 3 (GSK-3), whose role in cell cycle progression is crucial. GSK-3 plays a proapoptotic role by inhibiting the antiapoptotic protein-induced myeloid leukemia cell

differentiation protein (Mcl-1) (Maurer 2006), phosphorylating and inhibiting pro-survival proteins as β-catenin (Wu 2009) and cyclin D1 (Diehl 1998). Hence, the phosphorylation and inhibition of GSK-3 by Akt is a key event in promoting cell proliferation. Indeed, numerous studies showed that stabilization and increased levels of cyclins D1, D2 and D3 are a direct consequence of activation of Akt and inhibition of GSK-3 (Diehl 1998; Casanovas 2004; Kida 2007; Fatrai 2006). Furthermore, after GSK-3 degradation induced by Akt phosphorylation, β-catenin translocates in the nucleus where it interacts with different transcription factors as T cell factor/lymphoid enhancer factor-1 (TCF/LEF-1) to increase the expression of several genes, such as cyclin D1. A major downstream target of Akt is the mammalian or mechanistic target of rapamycin complex 1 (mTORC1), activated through the complex tuberous sclerosis protein 1 and 2 (TSC1-TSC2)/Ras homolog enriched in brain (Rheb), or by the Proline-rich Akt1 substrate 1 (PRAS40). In particular, Akt phosphorylates and inhibits the GTPase activating protein TSC2, which would otherwise block Rheb. Rheb in its active form is bound to GTP and can induce the activation of mTORC1, thus allowing mTOR to activate its pro-oncogenic pathway (Patel 2003). The kinase mTOR exists in at least two multiprotein complexes: mTORC1 and mTORC2, activated by different factors. MTORC1 is a sensor for cellular energy status and is activated by aminoacid and high ATP levels. The main function of mTORC1 is to coordinate nutrient and energy availability with extracellular growth signals to induce and control protein synthesis, proliferation, autophagy and cellular metabolism. The primary targets of mTORC1 are the ribosomal protein S6 kinase beta-1 (p70S6K), an AGC kinase, and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), a suppressor of protein translation (Sabatini 2006). Activation of mTORC1 promotes cell growth. Phosphorylation of S6K1 induces the activation of eukaryotic translational initiation factor 4B (eIF4B) which is a positive regulator of messenger RNA (mRNA) maturation (Holz 2005). Moreover, S6K1 supports the degradation of the proapoptotic proteins programmed cell death protein 4 (PDCD4) (Dorrello 2006). Besides its primary targets, mTORC1 promotes the expression of different metabolic enzymes (Peterson 2011) and suppresses the protein catabolism pathway and autophagy (Kim 2019). MTORC2 is instead activated by growth factors like insulin, high serum or nutrients levels and its best-characterized target is the S473 in Akt protein-sequence (Ikenoue 2008). Different studies have suggested that phosphorylation of the HM by mTORC2 acts as a docking site for PDK1 to allow the phosphorylation in the activation loop of the kinase (Mora 2004; Lien 2017). Besides mTORC1, also mTORC2 has a role in sustaining proliferation, it stabilizes the Insulin Receptor Substrate 1 (IRS1) by repressing its ubiquitin ligase F-box/WD repeat-containing protein 8 (Fbw8) (Kim 2012), activates the Oxidative stress-responsive 1 (OSR1) to respond to stress (Sengupta 2013) and, especially, activates Akt itself and its oncogenic pathway as mentioned above.

2. Aims

My PhD project aimed to disclose the role of FKBP51 in Akt activation. In particular, I attempted to clarify the exact interaction mode of FKBP51 with Akt and PHLLP, the mechanism of Akt activation by the canonical immunophilin and the effect of the spliced FKBP5.

3. Materials and methods

3.1.Cellular culture and reagents

In this study we used A375 and A2058 human melanoma cell lines and D54MG, U251MG and GB138 human glioblastoma (GBM) cell lines, kindly provided by CEINGE cell bank (Cellular Technology Platform) at the Advanced Biotechnology Institute (Naples, Italy). The melanoma cell line A375 (Giard 1973) derived from a metastatic tumor, was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Corning, Glendale, Arizona, USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS)(Corning, Glendale, Arizona, USA), 200mM glutamine (Corning, Glendale, Arizona, USA), and 100U/ml penicillin-streptomycin (Biowest, Nuaillé, France). The melanoma cell line A2058 (Stetler-Stevenson 1989), derived from lymph node metastasis, and glioblastoma primary cell line GB138, established from acutely resected human GBM (Kroonen 2011) were cultured in DMEM (Corning, Glendale, Arizona, USA) supplemented with 10% heat-inactivated FBS (Corning, Glendale, Arizona, USA), 200mM glutamine (Corning, Glendale, Arizona, USA) and 100U/ml penicillin-streptomycin (Biowest, Nuaillé, France). D54MG (Jones 1981) and U251MG (Timerman 2014) originated from surgical resection of patients with malignant glioblastoma multiforme, were cultured in DMEM/Hams F-12 50/50 (Corning, Glendale, Arizona, USA) supplemented with 10% heat-inactivated FBS (Corning, Glendale, Arizona, USA), 200mM glutamine (Corning, Glendale, Arizona, USA) and 100U/ml penicillin-streptomycin (Biowest, Nuaillé, France). All the cell lines were kept at 37°C in 5% CO_2 humidified atmosphere and are mycoplasma free.

A375 cell line stably knocked down with an FKBP51 short hairpin RNA (Sh FKBP51) or with a control shRNA (Sh Ctrl) were obtained as previously described (Romano 2015b). For the establishment of the A375 and A2058 knockout cell lines, cells were transfected with a CRISPR/Cas9 KO plasmid along with an HDR plasmid for the puromycin resistance (Santa Cruz Biotechnology, Dallas, Texas, USA). Control cells were obtained transfecting HDR plasmid alone. After 24h from transfection, cells were selected with 200 ng/ml puromycin (Merck, Darmstadt, Germany). Transfected cells that survived to puromycin were furthered seeded with the limiting dilution technique to generate single FKBP51-KO clones (Tufano 2021).

For the experimental procedures, 17-AAG (Merck KGaA, Darmstadt, Germany) was diluted in DMSO to make a stock solution of 10 mg/ml, in accordance with the manufacturer's recommendations. Treatment with 0.5-1 μM of 17-AAG was performed 16h before cells were collected.

3.2.Western Blot and antibodies

To obtain whole lysates, cells were collected and cellular pellets were homogenized in v/v of modified RIPA buffer (50 mM Tris-HCl pH7.5; 125 mM

NaCl; 1% NP-40; 0.25% Na-deoxycholate; 1 mM Na-fluoride; 1 mM Na-orthovanadate; 1 mM phenylmethanesulfonylfluoride (PMSF); 1 mM dithiothreitol (DTT); protease inhibitor cocktail). After 30 minutes of incubation on ice, lysates were centrifuged ad 14.000 rpm for 15 minutes to remove cell debris and the supernatant was saved for immunoblot (IB) assay. Protein concentration was determined using the Bradford protein assay (Bio-rad, Hercules, California, USA) and the absorbance readings were taken at 595 nM. Cell lysates were equalized for total protein, and the final volume was levelled with water and Laemmli Buffer (LB) 4X [Tris-HCl 0.5 M pH 6.8; SDS 10%; Glycerol; Bromophenol blue (3′,3″,5′,5″-tetrabromophenolsulfonphthalein) in 10% of ethanol; beta-Mercaptoethanol 20%]. Samples were denatured for 5 minutes at 95°C, then loaded in 8/10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a methanol-activated polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Darmstadt, Germany). The membranes were incubated overnight at 4°C with a primary antibody. The primary antibodies against the M2-Flag (mouse monoclonal, Sigma-Aldrich, St. Louis, Missouri, USA) and γ-Tubulin (mouse monoclonal, Sigma-Aldrich, St. Louis, Missouri, USA) were diluted 1:5000. The primary antibodies against FKBP51 (rabbit polyclonal, Novus Biological, Abingdon, UK) and Vinculin (Santa Cruz Biotechnolgy, Dalla, Texas, USA) were diluted 1:3000. After washes membranes were incubated with secondary antibodies for 1h at room temperature. Anti-mouse and anti-rabbit secondary antibodies HRP-conjugated were purchased from ImmunoReagents (Raleigh, Nord Carolina, USA) and diluted 1:5000. IBs were revealed with Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Dallas, Texas, USA).

3.3.Immunoprecipitation and Co-immunoprecipitation

For IP and Co-IP assays, 300 µg of whole lysates were essayed with 1 µg/mL of primary antibody and the samples were incubated in rotation overnight at 4°C; 10% of whole lysate was used as input fraction. The primary antibodies used for IP assay were: anti-FKBP51 (rabbit polyclonal, Santa Cruz Biotechnology, Dallas, Texas, USA); anti-FKBP51s (rabbit polyclonal home-made, raised against protein C-terminal); anti-Akt1/2/3 (mouse monoclonal, Santa Cruz Biotechnology, Dallas, Texas, USA) and anti-M2-Flag (mouse monoclonal, Sigma-Aldrich, St. Louis, Missouri, USA). The day after, 25 µL of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Dallas, Texas, USA) were added to the antigen-antibody (Ag-Ab) complex and the mix was incubated in rotation for 2h at 4°C. Then, samples were washed three times with modified RIPA buffer without protease inhibitors and Ag-Ab complex was eluted with 10 µL of LB 4X and loaded in 8/10% SDS-PAGE along with the input fraction, as described above.

4. Results

4.1. FKBP51 and FKBP51s differentially act on Akt phosphorylation

We overexpressed FKBP51 and FKBP51s in A375 melanoma cells and measured pAkt levels by IB. As shown in Fig.7a, pAkt was increased following FKBP51 overexpression, in comparison with EV-transfected cells. In FKBP51s overexpressing cells, pAkt levels remained comparable to those of the control. Interestingly, when we doubled concentration of Flag-FKBP51s, pAkt levels decreased (Fig.7b). We, then, generated two FKBP51 KO cell lines, through the CRISPR/Cas9 technology, using A375 and A2058 melanoma cell lines. FKBP51 KO cells and their respective control cells were assayed by IB for pAkt and its downstream targets, p70S6K and cyclin D1. IB showed impaired activation of the Akt pathway in KO cells (Fig.7c). FKBP51 rescue of A375 KO restored Akt activation (Fig.8a). Overexpression of FKBP51s in KO clone did not reactivate Akt phosphorylation (Fig.8b). By employing an A375 cell line stably downmodulated for FKBP51 by a short hairpin RNA (ShFKBP51) (Romano 2015b) and correspondent control cells transfected with a non-silencing Sh (ShCtrl) we show that pAkt levels are reduced in ShFKBP51 in comparison to ShCtrl (Fig.8c). In the same cell system, ectopic FKBP51s reduced Akt phosphorylation (Fig.8c).

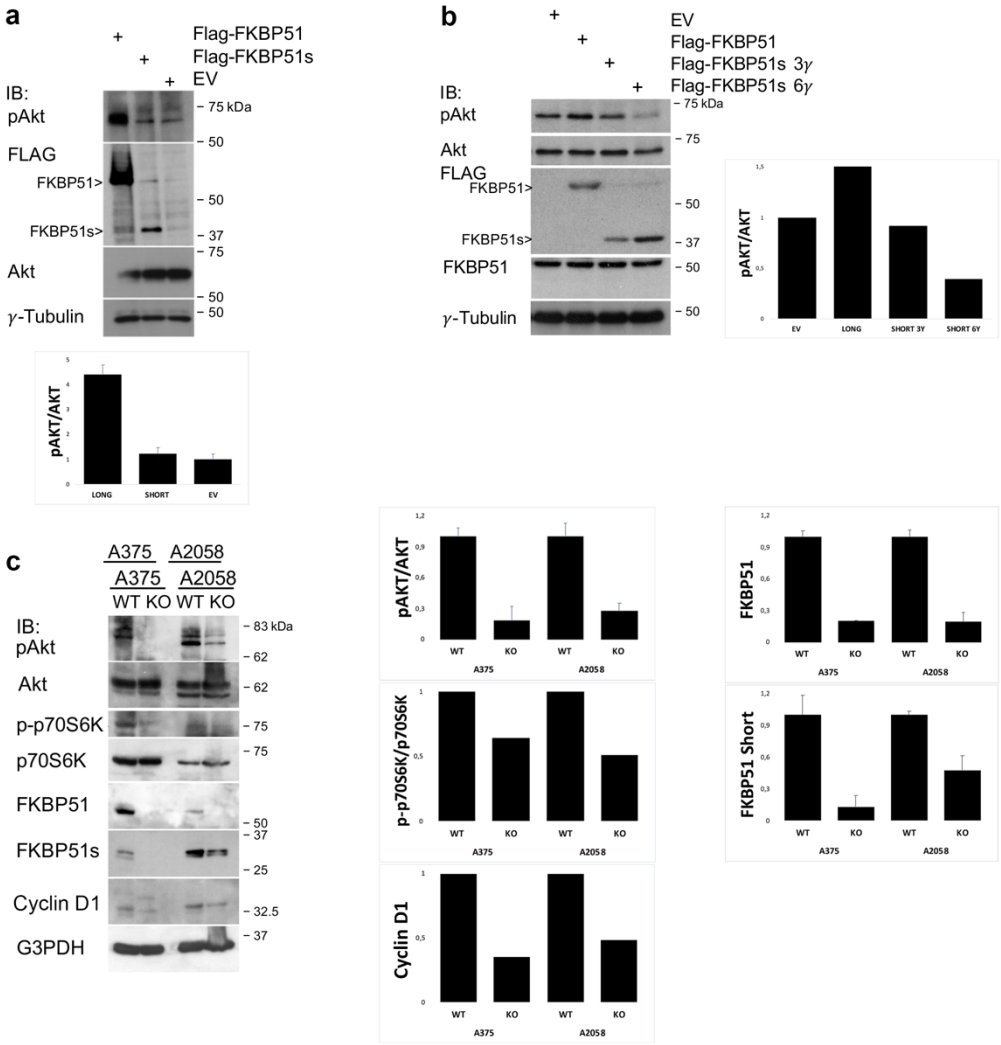

Figure 7. FKBP51 and FKBP51s effect on Akt phosphorylation. *(a) IB of A375 cell transfected with Flag-FKBP51, Flag-FKBP51s or correspondent EV as control. The canonical FKBP51, but not the spliced FKBP51, increased pAkt levels. γ-Tubulin was used as loading control. Data are representative of three independent experiments. (b) IB of A375 cell transfected with Flag-FKBP51 or increasing amounts (3γ and 6γ) of Flag-FKBP51s. PAkt levels decreased in FKBP51s overexpressing cells in a dose-dependent manner. γ-Tubulin was used as loading control. (c) IB assay of A375 and A2058 melanoma cell lines, stably knocked out for FKBP51 with the CRISPR/Cas9 technology. Compared to control (WT) cells, KO cells showed impaired levels of pAkt and its downstream targets. G3PDH was used as loading control. Data are representative of three independent experiments.*

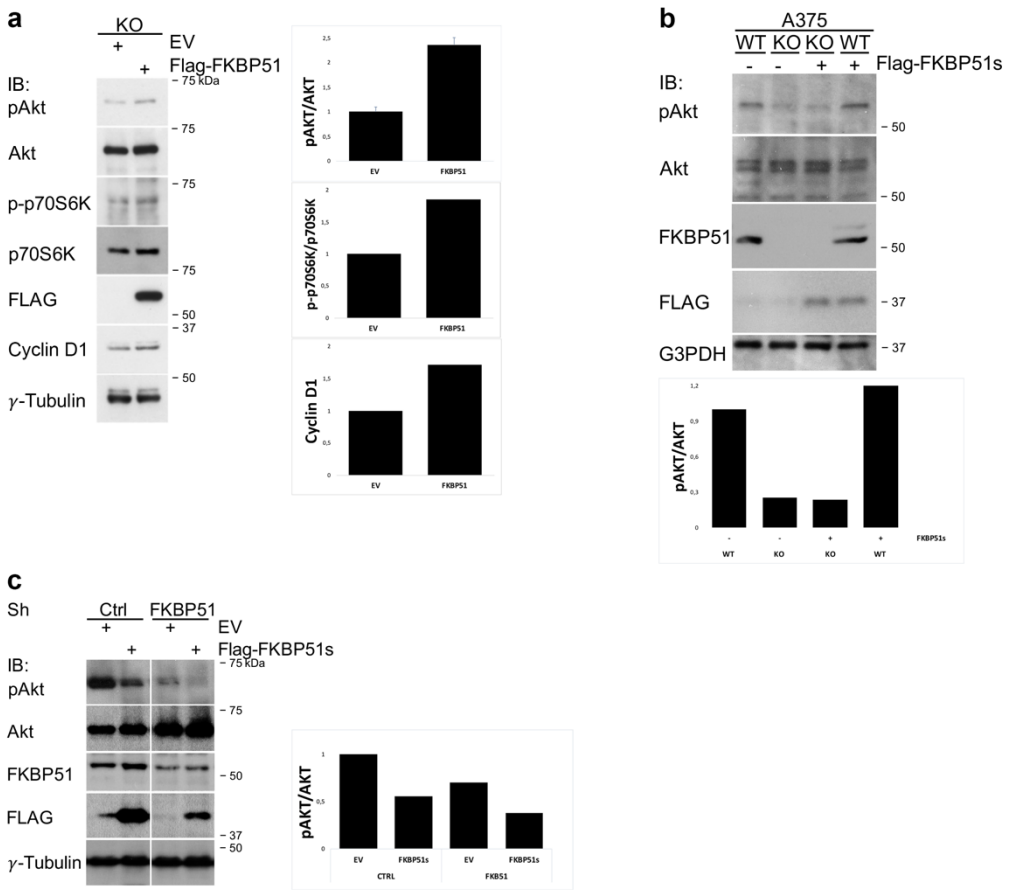

Figure 8. FKBP51 and FKBP51s effect on Akt phosphorylation. *(a) IB of pAkt, p-P70S6 and cyclin D1 levels in A375 KO cells, rescued or not with Flag-FKBP51. Activation of Akt pathway was restored upon FKBP51 rescue. γ-Tubulin was used as loading control. Data are representative of three independent experiments (b) IB of pAkt levels in WT and KO cells overexpressing Flag-FKBP51s. FKBP51s did not restore pAkt levels in A375 KO cells. G3PDH was used as loading control. (c) IB of A375 cell line stably knocked down with a FKBP51 shRNA (Sh FKBP51), or with a control shRNA (Sh Ctrl). Cells were transfected with Flag-FKBP51s or EV as control. PAkt levels were impaired in Sh FKBP51 cells, compared to Sh Ctrl cells; FKBP51s overexpression further decreased Akt activation levels. γ-Tubulin was used as loading control.*

5. List of publications

1. D'Arrigo P*, Russo M*, Rea A, **Tufano M**, Guadagno E, Del Basso De Caro ML, Pacelli R, Hausch F, Staibano S, Ilardi G, Parisi S, Romano MF and Romano S. A regulatory role for the co- chaperone FKBP51s in PD-L1 expression in glioma. *Equal contribution. Oncotarget. 2017 Jul 17;8(40):68291-68304. doi: 10.18632/oncotarget.19309. eCollection 2017 Sep 15 PMID:28978117.
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