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XXX CICLO

Regulation of PTP1B stability and signaling by the PKA scaffold protein praja2

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ABSTRACT

Protein tyrosine phosphatase 1B (PTP1B) is a non-transmembrane protein implicated as negative regulator in the insulin and leptin pathways. PTP1B is also implicated in the development of breast cancer due to its dephosphorylating role on the proto-oncogene Src. During these years, PTP1B has emerged as a promising potential therapeutic target for the treatment of the type 2 diabetes. PTP1B control mechanisms are still controversial and not well understood. Here, I've contributed to identify a novel mechanism of regulation of PTP1B mediated by the ubiquitin-proteasome pathway. We identify the E3 ligase praja2 as novel interactor and regulator of PTP1B. Thus, praja2 ubiquitinates PTP1B and sustains the insulin pathway, interfering with praja2 expression or activity negatively impacts on the insulin pathway. This different control mechanism of the insulin pathway through PTP1B ubiquitination adds a new dowel to the comprehension of the molecular basis of metabolic disorders.

1. Introduction

cAMP and the Ubiquitin-Proteasome System (UPS)

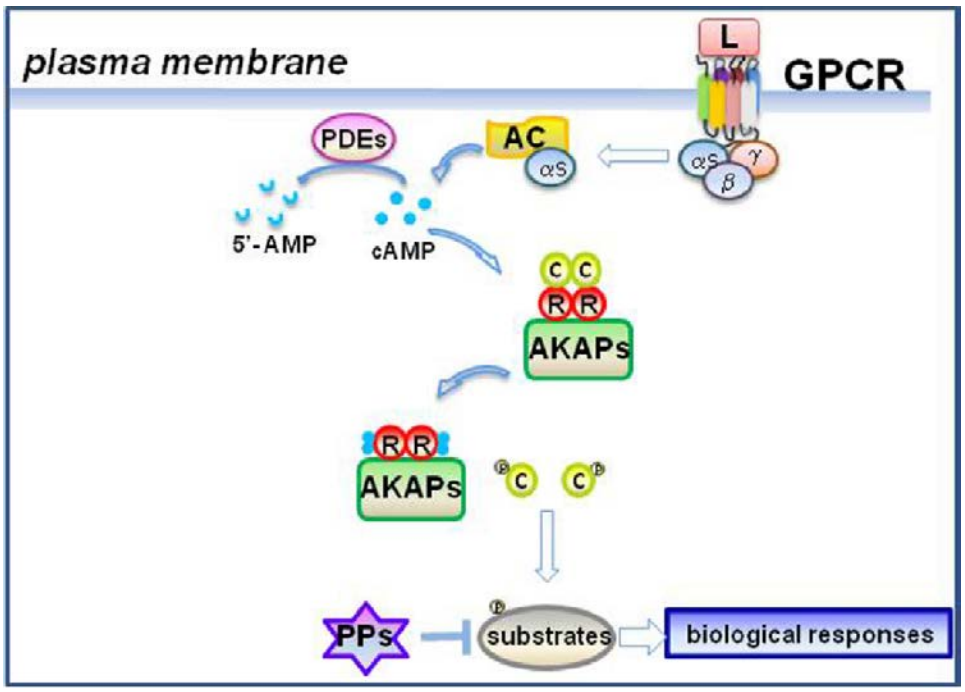
1.1 cAMP Signaling

Second messengers has a pivotal role in the transduction signal in response to hormones and growth factors. Cyclic adenosine 3′-5′-monophosphate (cAMP) represents an ancient second messenger implicated in crucial biological function. cAMP levels, duration and amplitude of propagating signal are tightly regulated by different classes of enzymes: the adenylyl cyclases (ACs), the cyclic nucleotide phosphodiesterases (PDEs), protein kinases, phosphatases (PPs) and scaffold proteins. The main effector of cAMP is protein kinase A (PKA), whose role is fundamental in the propagation of the signal downstream to target substrates/effectors (1).

cAMP is tightly linked to a large family of membrane proteins, G protein–coupled receptors (GPCRs) which transduce the signal from the extracellular microenvironment to inside the cells (Rosenbaum et al., 2009). GPCRs are a heterotrimeric complex composed by three sub unites, GαS, β and γ. Once the receptor is activated the α subunits is released from β/γ sub unites, so it can active the AC driving the formation of the cAMP (2). The activation of different isoforms of ACs are mediated by kinases, like PKA, PKC, Calmomodulin Kinase or Ca^{2+} (3, 4).

One of the most important target of the cAMP is the Protein Kinase A (PKA). This protein is a tetramer composed by two regulatory subunits (R) and two catalytic subunits (C), the binding of the cAMP to the R subunits causes a conformational change to the holoenzyme that leads to dissociation of the C subunits, which are able to phosphorylates different substrates, controlling different aspects of cell physiology (5). Scaffold proteins, called A-kinase anchoring proteins (AKAPs), drive the

localization of PKA in the different compartments of the cells, close to its substrates(6). Each AKAP contains two important domains; one is implicated into the association to regulatory subunits of PKA, and the other is a targeting domain that directs the kinase to specific subcellular compartments **(Fig.1)**. Biochemical and structural studies identified a conserved PKA-binding domain of AKAPs that forms an amphipathic helical wheel composed of 14–18- residues (7). The helical wheel binds with high affinity the N-terminal docking/dimerization (D/D) domain of the PKA-R dimer (8, 9). The assembled components of the cAMP such as, receptors, conjugated to the activation of AC, effectors, like AKAPs-PKA and attenuating enzymes like PDEs and PPs, lead to the formation of the transduceosome, thanks to which distinct signaling pathways converge and are locally attenuated or amplified, optimizing the biological response to extracellular stimuli (10-13).



Rinaldi et al. 2015

Figure 1. Activation of cAMP signaling. Ligand-induced activation of GPCR dissociates Gαs subunit which in turn activates the adenylyl cyclase (AC) (14). AC converts ATP into cAMP. cAMP binds the regulatory subunits of PKA, which is anchored to AKAPs, and releases the catalytics subunits (C). The C subunits phosphorylate different PKA substrates (1). Protein phosphatases (PPs) contributes to attenuate and regulates the signal (4).

1.2 Ubiquitin-Proteasome System

The ubiquitin–proteasome system (UPS) has emerged as an important control mechanism of cell metabolism, growth and survival. The UPS is the principal pathway to eliminate unneeded or damaged proteins (15). The ubiquitylation is mediated by the attachment of ubiquitin to the "γ-amine of lysine residues of target proteins. This process requires a series of ATP-dependent enzymatic steps catalyzed by E1 (ubiquitinactivating), E2 (ubiquitinconjugating), and E3 (ubiquitinligating) enzymes (16). E3-ubiquitin ligases fall into two main classes characterized by a HECT domain or a RING domain (17, 18). HECT domain E3 ligases act catalytically to form a thioester intermediate during ubiquitin transfer to substrate, whereas RING E3 ligases attend as a scaffold that brings together the substrate and the E2 ligase. The result of this sequential cascade of events is the covalent attachment of ubiquitin molecules to lysine residues on the target protein. These modifications can involve either a single ubiquitin (mono-ubiquitylation) or a chain of ubiquitin (poly-ubiquitylation (19) **(Fig.2)**. Polyubiquitylation of substrates in many cases drives through proteasome degradation (15), but polyubiquitylated proteins can also follow a non-degradative pathway (20). This mechanism may control the intracellular trafficking of the target protein or its activity (21). The UPS can be reverted thanks to classes of proteins named deubiquitinates enzymes (DUBs), by removing the ubiquitin moieties to restore the localization/activity of the modified protein (20).

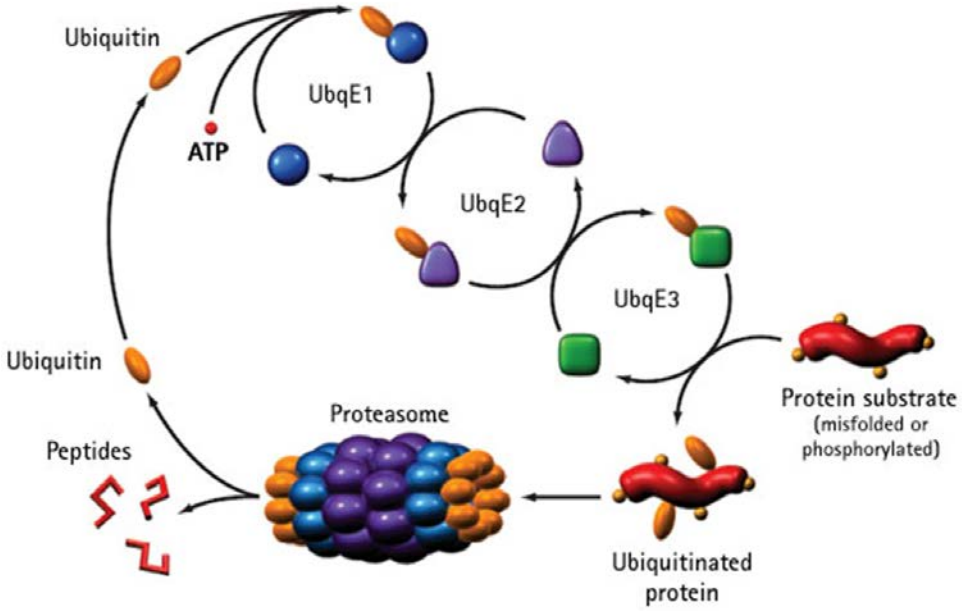


Figure 2. Schematic representation of ubiquitin system. Several enzymes compose the ubiquitin proteasome system (UPS). E1, the ubiquitin activating enzyme, E2, the ubiquitin conjugating enzyme and the E3 ligase, which are specific for precise substrates and are responsible for associating the ubiquitin molecules to the substrates. After the action of these three enzymes, the ubiquitinates proteins are degraded via proteasome. The ubiquitinated proteins can be de-ubiquitinated by a specific DUBs enzyme.

Interestingly, the cAMP-PKA signaling can regulate and be regulated by UPS at different steps, therefore controlling different aspects of cell fate. For example, the β-adrenergic receptor2 (β-2AR) can be desensitized thanks to UPS, indeed the adaptor protein ARRDC3 (arrestin domain containing 3) recruits the E3 ligase NEDD4 (neural precursor development down regulated protein 4) close to β-2AR. Concomitant inhibition of the deubiquitinase USP20 (Ubiquitin-specific-processing protease 20) by PKA favors ubiquitination and degradation of the receptor by NEDD4 (22, 23)

1.3 RING ligase praja2

Praja1 and praja2 are two members of praja E3 ligase family, they share 52.3% of sequence homology. The E3 ubiquitin ligase praja2 is a protein of 708 amino acids with an estimate weight of 78 kDa. However, there is a discrepancy between the praja2 estimate weight and the molecular mass show on SDS-polyacrylamide, infact praja2 is estimated to be 140 kDa (24). Praja2 is encoded by a gene localized on the long arm of the human chromosome 5 (5q21) (25). Praja1 is encoded by a gene mapped on human chromosome X encoding for a protein of 643 amino acids, with a molecular weight of 71 kDa (26). Booth proteins contain a well conserved domain at COOH-terminal end (aa 629-678), this domain is the RING-H2-finger motif. The RING-H2-finger motif is similar to the RING-finger motif, but with the substitution of the Cys4 replaced by His (27). Praja2 is characterized by a particular motif at the COOH terminal domain (aa583-600), encoding an α-amphipathic helix. This domain is characteristic of AKAPs family proteins, fundamental for PKA anchoring

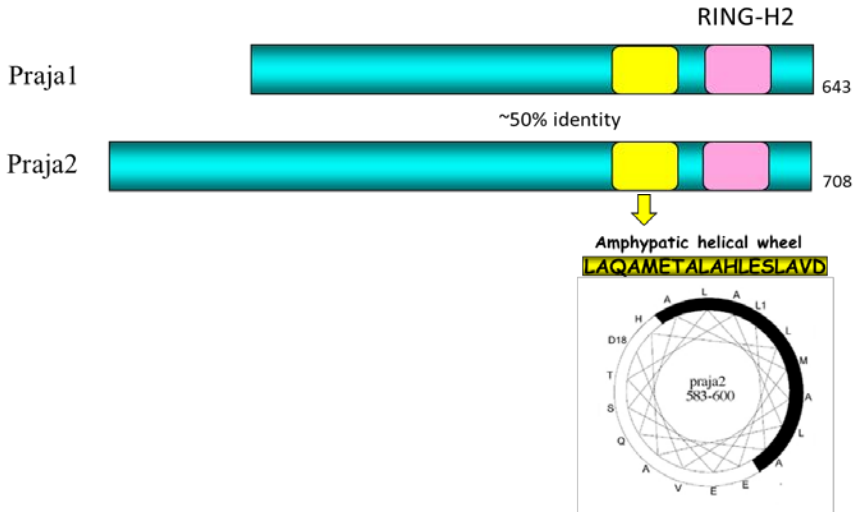
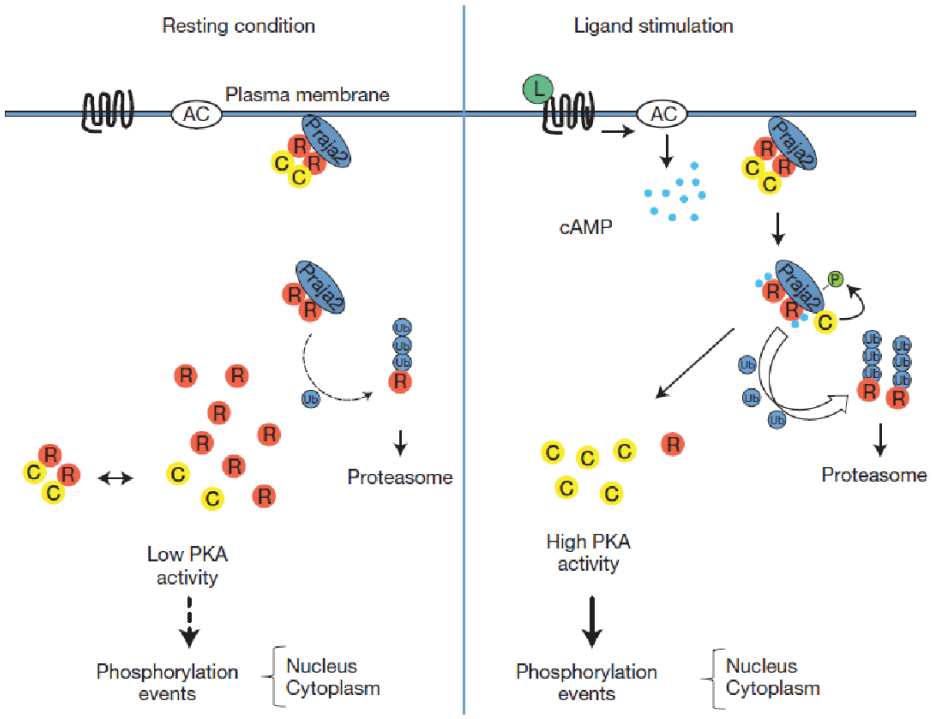


Figure 3. Schematic representation of praja proteins. Praja1 is a protein of 643 amino acids, while praja2 of 708 amino acids. They share 52.3% of sequence homology and, both, are characterized by a RING-H2 domain (in rose) and an α-amphipathic helix (in yellow) at COOH-terminal.

It is demonstrated that, in mice, praja1 is important for the neuronal plasticity, which is significant for learning and memory (26). Praja1 is expressed in the basolateral complex of the amygdala during formation of the fear memory (28). Downregulation of praja2 levels are correlated, in rat, with axonal degeneration and neuronal cell death (25). It is demonstrated the crucial function of praja2 involved in the cAMP-dependent signal transduction pathway. Praja2 controls the stability of mammalian regulatory subunits (R) of Protein Kinase A (PKA), regulating the strength and duration of the PKA signal output in response to cAMP, governing efficient nuclear cAMP signal and PKA-mediated long-term memory (29) **(Fig.4)**.



Lignitto et al. 2011

Figure 4. Schematic representation of the role of praja2 in PKA signaling. Activation of the PKA by the ligand drives the dissociation of the catalytic sub-unites (C) of the PKA holoenzyme from the regulatory (R) sub-unites which lead to the phosphorylation of Praja2 by the PKAc (C) sub-unites. Phosphorylates Praja2 is able to ubiquitinates and degrades R subunits through the proteasome pathway, with consequently accumulation of free active PKAc (C) sustaining the duration of cAMP signalling.

Praja2 is also involved in cAMP-induced neurite outgrowth, promoting proteasome degradation of NOGO-A, a crucial inhibitor of neurite outgrowth in mammalian brain (30) **(Fig.5)**.

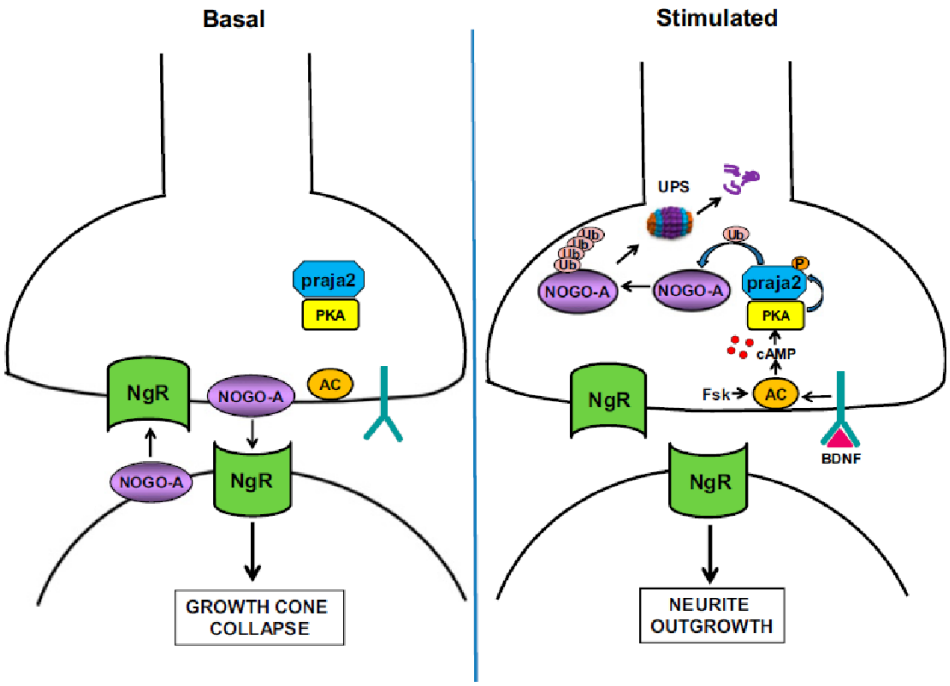


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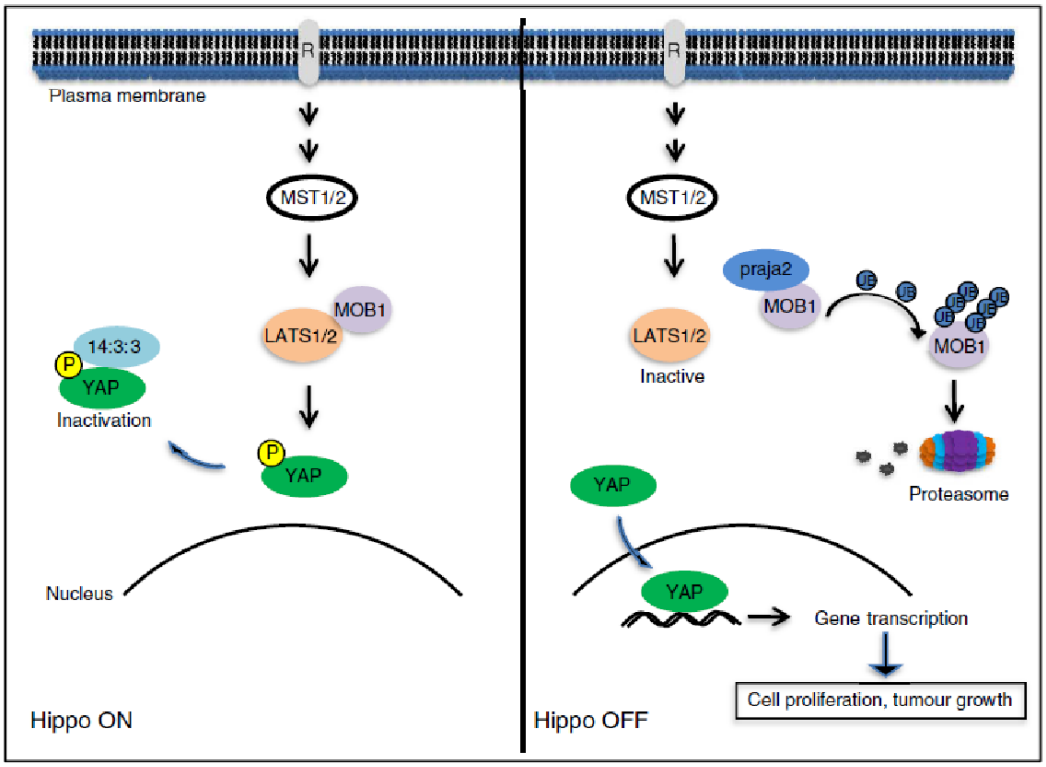
Figure 5. Schematic model of Praja2-NOGO-A pathway. Under basal condition, NOGO-A regulates and inhibits neurite outgrowth. External stimuli, like BDNF or Forskolin (Fsk) activate the adenylate cyclase (AC), increasing the cAMP levels, dissociation from PKA holoenzyme of the catalytic sub-unites (C), triggering the phosphorylation of Praja2 which is able to ubiquitinate and degrade via UPS NOGO-A, promoting neurite outgrowth.

Moreover, praja2 has a role in tumor growth, it controls the organ size and sustains glioblastoma growth *in vivo*, through the ubiquitination and degradation of MOB1, a core component of NDR/LATS kinase and a positive regulator of tumor-suppressor Hippo cascade (31) **(Fig.6)**.



Liginitto et al 2013

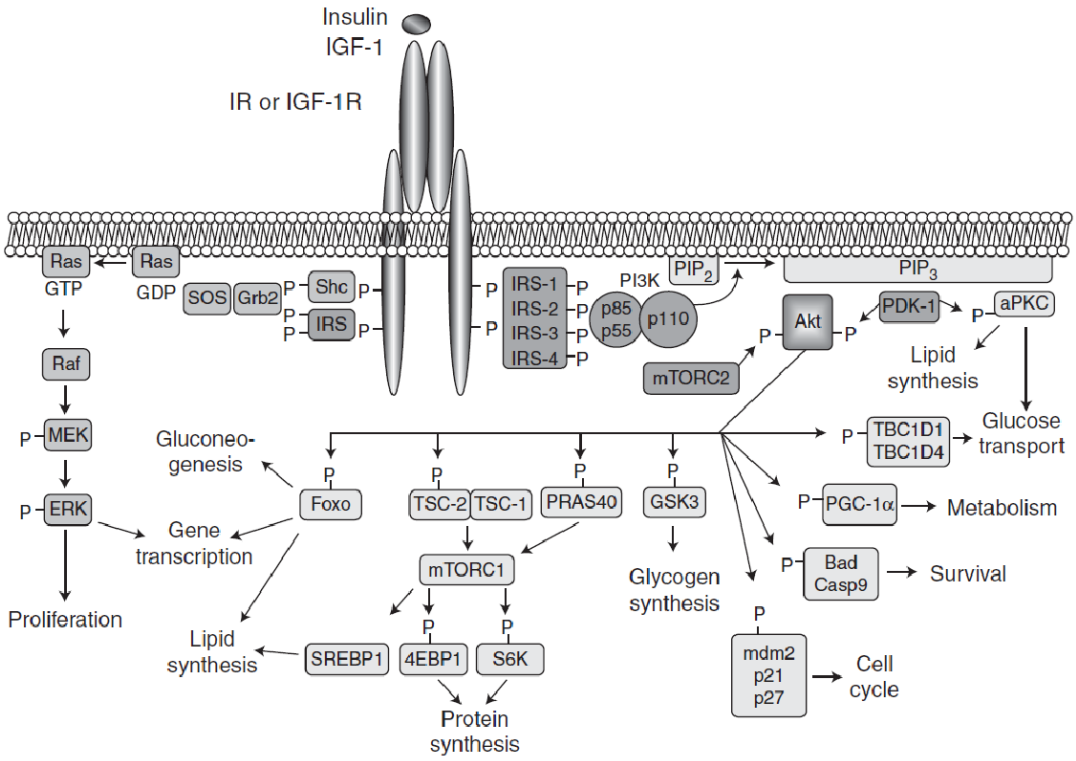
Figure 6. praja2 schematic representation in the regulation of the Hippo pathway. MOB1 is ubiquitinated and degraded via proteasome by praja2. Degradation of MOB1 leads to the attenuation of LATS1/2 kinases activity, preventing phosphorylation and inactivation of YAP, which are able to enter in the nucleus, driving gene transcription, promoting cells proliferation and tumor growth.

A study demonstrates a markedly praja2 over-expression in differentiated thyroids cancer, high levels correlated with the malignant phenotype of the tumor (32). In addition, Praja2 is involved in the attenuation of the MAP kinases cascade, regulating the ubiquitination and degradation of a scaffold protein called KSR1, governing the maintenance of undifferentiated pluripotent state in mouse embryonic stem cells (33). Finally, a recent study revealed a role of Praja2 in the regulation of the insulin secretion in response to glucose stimulation. Praja2 ubiquitinates and degrades p35, activating calcium signaling and promoting insulin secretion (34).

1.4 Insulin Pathway

Insulin biological effects are mediated by the binding of three highly homologous tyrosine kinase receptors, insulin, IGF-1 receptors (IR and IGF-1R) and the orphan insulin receptor-related receptor (IRR), which has been suggested to play a role in testis determination (35) and acts as an extracellular alkali sensor (36). Although insulin and IGF-1 preferentially bind to their own receptors, both ligands can also bind to the alternate receptor with reduced affinity (37). The IR, IGF-1R and IRR are tetrameric complex that consist in two α subunits, which are extracellular, and two transmembrane β subunits that are linked by disulfide bonds. The interactions between the hormones and the IR, IGF-1R lead to the activation of the pathway through a conformational change inducing the trans-phosphorylation of the β subunits further activating the kinase and allowing the recruitment of receptors substrates. A well known and characterized Insulin Receptors Substrates (IRSs) are a large family of proteins which act as scaffold to organize and mediate signaling complex (38) (39) (40-43) (44).

PI3-kinase (PI3K) is the critical protein involved in the signaling pathway of the insulin metabolic action. The class Ia PI3-kinases are heterodimers composed by a regulatory and catalytic subunit, each of which occurs in several isoforms (45). PI3K, thanks to two SH2 domains in the regulatory subunits, bind to tyrosine-phosphorylated IRS proteins (44, 46) and this results in activation of the catalytic subunit, which rapidly phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) in phosphatidylinositol (3,4,5)-triphosphate (PIP3) a crucial lipid second messenger. PI3K activity is reflected on a subset of AGC protein kinase family members, which includes isoforms of Protein Kinase B (Akt), 3-phosphoinositide-dependent Protein Kinase 1(PDK-1), p70 ribosomal S6 Kinase (S6K), Serum and Gluco-Corticoid Induced Protein Kinase (SGK) and several isoforms of the Protein Kinase C (PKC) **(Fig.7)**



J. Boucher et al. 2014

Figure 7. **Insulin- and IGF-1-signaling pathways**. Activation of the IR or IGF-1 receptors by their ligands. Conformational change and autophosphorilations of the receptors leading to the recruitments of receptor substrates, such as IRSs and Shc proteins. Shc is necessary for the activation of the MAP kinase cascade, whereas IRSs proteins leading to the activation of Pi3K-PDK-AKT or PKC. AKT activation regulates most of insulin's metabolic effects, such as glucose transport, cell cycle, lipid synthesis, gluconeogenesis, and glycogen synthesis.

PDK-1 is recruited under the plasma membrane binding PIP3 with a PH domain triggering its activation. PDK-1 phosphorylate and activate Akt in Thr-308, however, for a complete activation, Akt needs to be phosphorylate in Ser-473 by mammalian target of rapamycin complex 2 (mTORC2) (47-49). The activation of Akt leads to the regulation of several pathways involved in metabolism, protein synthesis and cell growth. Infact the phosphorylation of tuberous sclerosis complex protein 2 (TSC2), mediated by Akt, causes the degradation of tumor suppressor complex, TSC1 and TSC2, which activates the mTORC1 complex. The inhibition of 4E-binding protein 1 (4E-BP1), by phosphorylation from mTORC1 complex, activates ribosomal protein S6 kinase S6K1 and S6K2 and SREBP1, which regulates some genes associated to metabolism, protein synthesis and cell growth (50).

Phosphoprotein Phosphatases play a pivotal role in the tight regulation of all the insulin action at different levels of the pathway. Cytoplasmic protein tyrosine phosphatases such as, PTP1B and transmembrane phosphatases, such as LAR, have been shown to dephosphorylate the tyrosine residues on activated IR and IGF-1R, as well as IRS proteins, thereby reducing their activity (51). Serine/threonine phosphatase proteins are also involved, PP2A is the main serine/threonine phosphatase in cells that regulates the activity of several kinases, involved in insulin action, including Akt, PKC, S6K, ERK, cyclindependent kinases, and IKK (52).

1.5 Metabolic regulation by Ubiquitin-Proteasome System

Ubiquitin-Proteasome System has emerged as crucial regulation of several pathways involved in cell metabolism, growth and aging. Latest experimental evidence has described the pivotal role of the Ubiquitin-Proteasome System (UPS) in the regulation of the insulin pathway. Consequently, UPS alterations are involved in metabolic diseases.

Skeletal muscle is responsible for about 70–90% of insulin-stimulated glucose disposal **(**76**,** 77**)**, on account of this, insulin resistance in skeletal muscle is the earliest step in the pathogenesis of metabolic syndrome and type 2 diabetes **(**78-80**)** That muscle-specific mitsugumin 53 (MG53) is an E3 ubiquitin-ligase tripartite motif-containing (TRIM) family protein and is specifically expressed in skeletal muscle and heart (81, 82). MG53 is involved in the insulin cascade; because, directly, regulates the insulin receptor and IRS1 protein stability through ubiquitin-dependent degradation (80). Animal models for insulin resistance and metabolic disorders show abundant MG53 overexpression after high-fat diet (HFD) (83-86). The upregulation of MG53 was also confirmed in obese humans (80), underling the link between MG53 and metabolic diseases. The overexpression of MG53 is tightly linked to the insulin resistance in skeletal muscle, instead ablation of MG53 in $MG53^{-/-}$ mice fed with HFD, does not display any decrease of the insulin receptor and IRS1 levels in skeletal muscle.

The UPS is also involved in the regulation of the cellular growth played by the Cullin 7 (CUL7) E3 ubiquitin ligase complex containing the Fbw8-substrate-targeting subunit, Skp1, and the ROC1 RING finger protein (87). A recent study shows the insulin receptor substrate 1 (IRS-1), as a proteolytic target of the CUL7 E3 ligase (87). CUL7 is a protein composed of 1700 amino acids which assemble a complex with Skp1, the Fbw8 F-box protein and ROC1 (88-90), called SCF-like E3. The degradation of IRS-1 by CUL7 E3 ligase is a negative-feedback regulation by mTOR/S6K phosphorylations. IRS-1 is phosphorylated by S6K at Ser307 thanks to

which it can be ubiquitinated and degradated by the CUL7 E3 ubiquitin ligase complex (87). Genetic knock out of CUL7 leads cells to senescence, probably through sustained MAPK activation and/or increased Akt signaling (87, 91, 92).

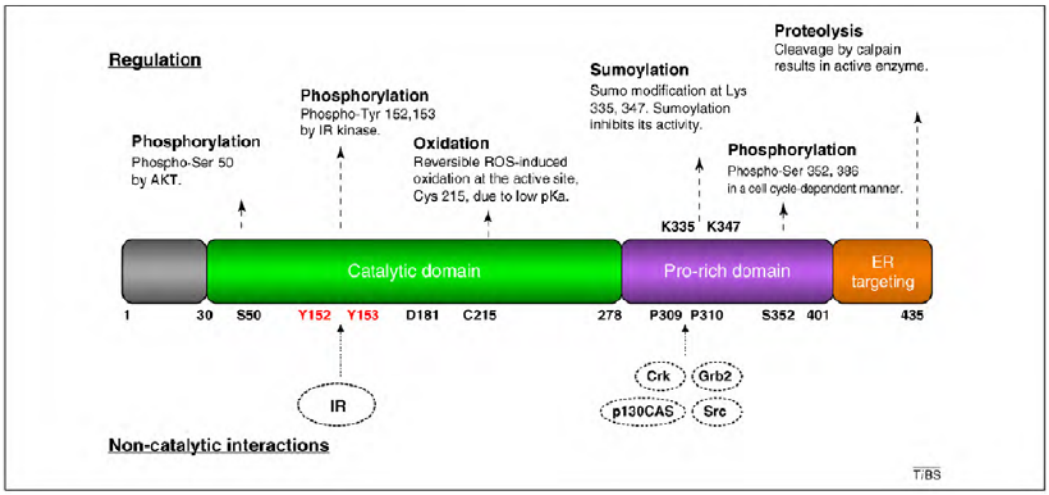
A recent study has described the regulation of insulin pathway, as a fundamental turning point for the balance between proteostasis and aging. The ubiquitin ligase CHIP plays a pivotal role in the cellular balance between protein folding and degradation (93-96). CHIP is fundamental for the ubiquitylation of damaged proteins relegated by chaperone partners to induce disposal through endocytic-lysosomal pathways (97, 98), proteasomal degradation (94-96), and autophagy (93). *Riga et al* study display the role of CHIP in the regulation of the ubiquitin-dependent degradation of the insulin receptor. Modulation of insulin signaling underlines a relationship between proteostasis and longevity (99).

1.6 Protein Tyrosine Phosphatase 1 B

Protein tyrosine phosphatase 1B (PTP1B) is a non-transmembrane protein tyrosine phosphatase implicated in the regulation of several pathways. PTP1B is monomeric protein of 435 amino acid with an estimated weight of 50 kDa. It is characterized by three region; Catalytic phosphatase domain at the N-terminus (residues aa1–300), a regulatory region (resides aa 80-100) and a region (residues aa 400–435) that tethers the enzyme to the cytoplasmic face of the endoplasmic reticulum (ER) (57, 58). PTP1B is tightly regulated by four known mechanism, oxidation, phosphorylation, sumoylation and proteolysis **(Fig.8)**. PTP1B activity is regulated *in vivo* by reversible oxidation involving Cys 215 at its active site, which temporarily abrogates its enzymatic activity (59). It is well known that the formation of transient ROS is generated by the activation of RTKs and integrin (59-61), PTP1B is regulated by both serine and tyrosine phosphorylation in multiple site, by several kinases such as protein kinase C (PKC) at S378 and at S352 and S386 by an unknown kinase (62, 63), at S50 by AKT (64), CLK1 and CLK2 (65). The effects of phosphorylations are either modest or controversial and not well known. PTP1B is also regulated by sumoylation in the last two lysine residues of the C-terminus by SUMO E3 PIAS1 (ligase protein inhibitor of activated STAT-1) (66). This post-translational modification is associated with a reduction in enzymatic activity. Proteolysis plays another important part in the activation and in the inactivation of PTP1B. The cleavage of the C-terminus domain by calpain-1 is required for its activation, interestingly the oxidized PTP1B form is the cleavage in small inactivate fragments (67-69).

Early studies showed PTP1B as a negative regulator of insulin and leptin signaling pathways. Recent studies have revealed that PTP1B has a positive role in tumorigenesis. Infact, PTP1B acts as a positive regulator of Src in breast cancer, dephosphorylating the inhibitory Y529 site, thereby activating this kinase (53) (54). In the insulin pathway, PTP1B dephosphorylates the insulin receptor (IR) as well as the IRS proteins (55),

furthermore in the leptin signaling the regulation is through the dephosphorylation of the tyrosine kinase JAK2 (Janus kinase 2) (56).



Yip SC. 2010

Figure 8. Structural representation of PTP1B domain. PTP1B is a protein of 435 amino acids composed by different domain. At N-terminal is present the catalytic domain (green) at C-terminal the ER targeting domain (orange), bordering two proline-rich domains (PRD: 278-401AA; purple), which are crucial for protein–protein interactions.

2. Aim of the Study

Protein tyrosine phosphatase 1B (PTP1B) is implicated as a negative regulator of the Insulin and Leptin pathways, controlling the correct activation and propagation of the signal transduction inside the cell.

Ubiquitination is a post-translational mechanism that controls the activity and the stability of wide number of proteins, regulating key physiological processes, such as survival, differentiation and cell proliferation (15, 16, 70).

In the last years, the research line of our laboratory was focused on the regulation of different biological events elicited by the E3 ubiquitin ligase praja2. During my PhD program, I focused my attention on identifying and characterizing a novel interactor of praja2, namely PTP1B. The impact of such a regulation on metabolic pathway was the key issue. Below are the principal aims:

- Analyze the role of praja2 in the insulin pathway and identify its relevant target (PTP1B).
- Characterize praja2/PTP1B complex in cells.
- Test if praja2 ubiquitinates PTP1B and how this impact on the downstream pathway.

3. Materials and Methods

3.1 Cell lines. Human embryonic kidney cell line (HEK293) were cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 2mM L-glutamine, 100 IU/ml penicillin, in an atmosphere of 5% CO_2 at 37°C.

3.2 Plasmids, siRNAs and transfection. Vectors encoding for flag-praja2 (wild type) were purchased from Genecopeia. Flag-praja2rm and Flag-praja2 mutants were generated by site-directed mutagenesis (GenScript), whereas praja2-deletion mutants were generated by PCR with specific oligonucleotide primers and subcloned into the same vector of wild-type praja2 cDNA. Myc-Ub, were provided by Dr Carlomagno F. Vectors encoding for PTP1B-HA and PTP1B-GST were purchased from addgene (nonprofit plasmid repository).

siRNAs 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 or four or two independent siRNAs. Transfection efficiency was monitored by including a GFP vector in the transfection mixture. Below are there the siRNA sequences (Thermo Scientific; LU-006916-00-10) targeting human praja2:

Sequence 1: 5′-GAAGCACCCUAAACCUUGA-3′;

Sequence 2: 5′-AGACUGCUCUGGCCCAUUU-3′;

Sequence 3: 5′-GCAGGAGGGUAUCAGACAA-3′; and

Sequence 4: 5′-GUUAGAUUCUGUACCAUUA-3′.

3.3 Antibodies and chemicals. Monoclonal antibody directed against PTP1B were purchased from Millipore Merck and used at working concentration of 1:1000. Rabbit Praja2 were purchased from Bethyl Laboratories, mouse GST, Actin and ERK1 from Santa Cruz Biotechnology. Polyclonal antibodies directed against GSK3β, p- GSK3β, S6K and p-S6K were purchased from Cell Signaling.

Monoclonal antibodies directed against flag and myc epitope used at working diluition 1:3000 were purchased from Sigma. PTP1B XXII Inhibitor were purchased from Millipore Merck. Forskolin and Insulin were purchased from Sigma.

3.4 Immunoprecipitation and pull down assay. Cells were washed twice with phosphate-buffered saline and lysed in a buffer (50mM TRIS–hydrogen chloride, pH 7.4, 150mM sodium chloride, 5mM magnesium chloride, 5mM dithiothreitol, 1mM ethylene diamine tetraacetic acid, 1% Triton X-100,containing aprotinin (5 ⌈g/ml), leupeptin (10 ⌈g/ml), pepstatin (2 ⌈g/ml), Na3VO4 and 1mM phenylmethylsulfonyl fluoride and protease inhibitors. The lysates were cleared by centrifugation at 15,000 g for 15 min. Cell lysates (2 mg) were immunoprecipitated in rotation at 4 °C overnight with the indicated antibodies. Pellets were washed four times in lysis buffer and eluted in Laemly buffer. An aliquot of whole cell lysates (WCE) (100 ⌈g) or immunoprecipitates were resolved on sodium dodecyl sulfate polyacrylamide gel and transferred on nitrocellulose membrane using iBLOT2 technology from Thermo Fisher Scientific. Filters were blocked for 1 hour at room temperature in Tween-20 Phosphate buffer saline (TPBS) (PBS- Sigma, 0, 1% Tween 20, pH 7.4) containing 5% nonfat dry milk. Blots were then incubated O/N with primary antibody. Blots were washed three times with TPBS buffer and then incubated for 1 hour with secondary antibody (peroxidase-coupled anti rabbit (GE-Healthcare) in TPBS. Reactive signals were revealed by enhanced ECL Western Blotting analysis system (Roche).

GST-fusions were expressed and purified from BL21 (DE3) pLysS cells. GST hybrid proteins immobilized on glutathione beads were incubated for 3 hours with cell lysates from HEK293 cells transiently expressing praja2-rm-FLAG and mutants constructs in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 5mM DDT, 1 mM EDTA, 1% triton X-100) in rotation at 4 °C for 4 hours. Pellets were washed four times in lysis buffer supplemented with NaCl (1 M final concentration) and eluted in Laemmli buffer. Eluted samples were size-fractionated on SDS-PAGE and immunoblotted.

3.5 Immunofluorescence and confocal analysis. For immunofluorescence studies, HEK293 cells were plated on poly-L-lysine (10µg/ml) coated glass coverslips. Cells were fixed with Paraformaldehyde for 20 minutes. After three washes, cells were immunostained with polyclonal antibody directed against praja2 (Betyl) used at working dilution of 1:50, or with monoclonal antibody directed against PTP1B (Millipore Merck) and used at working dilutions of 1:100. High-resolution images were acquired with a Zeiss LSM 880 confocal microscope equipped with Airyscan super-resolution imaging module, using a 63×/1.40 NA Plan-Apochromat Oil DIC M27 objective lens (Zeiss MicroImaging, Jena, Germany)

3.6 Statistics. Data were analyzed using analysis of variance (ANOVA) for each region and *post hoc* repeated-measure comparisons (Least Significant Difference (LSD) test). Rejection level was set at $P < 0.05$.

4. Results

4.1. Identification of PTP1B as a novel praja2 interacting partner.

In our previous studies, we demonstrated the important role of praja2 in the regulation of several intracellular pathways (29-31, 33). Given the importance of this E3 ligase in the cell homeostasis and physiology, I decided to investigate the role of praja2 in cell metabolism. Specifically, I analyzed whether praja2 regulates insulin signaling. As readout, I monitored the phosphorylation of GSK3β at serines 9/21, a well known insulin regulated kinase. I transfected HEK293 cells with a vector encoding for a dominant negative mutant of praja2, called praja2-rm. The mutant protein is characterized by point mutations in the RING-H2 domain (C634/671A). Praja2-rm binds its substrates but it is not able to transfer the ubiquitin molecules on them, acting as a dominant inactive mutant towards the endogenous protein. Cells were treated with insulin (10 μM) for 15, 30, 45 minutes, then were harvested and lysates subjected to western blot assay **Fig.9**. The experiments in **Fig.9** show that cells transfected with praja2-rm vector displayed a decreased insulin-stimulated phosphorylation of GSK3β compared to control cells. This finding suggested that praja2 is, indeed, involved in the regulation of the insulin signaling.

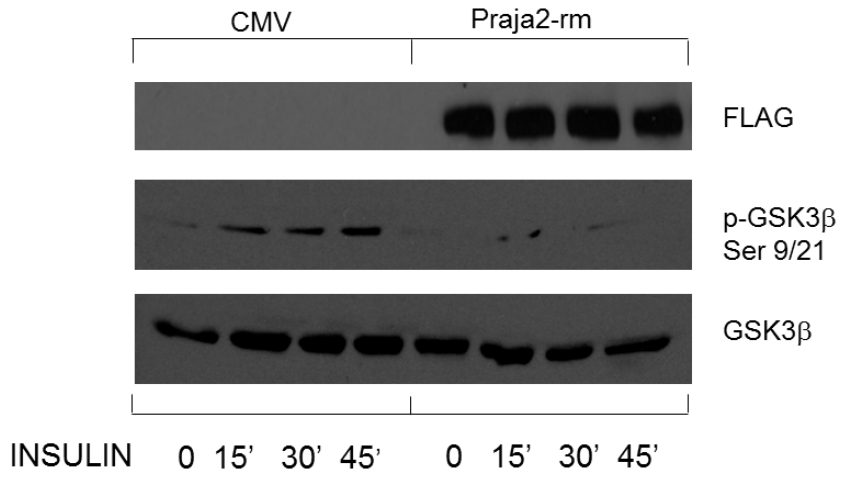


Figure 9. praja2-rm mutant inhibits GSK3β phospshorylations. HEK293 were transiently transfected with an empty vector (CMV) or with a vector encoding for praja2 RING mutant (C634, 671A; praja2-rm). Cells were serum deprived overnight, and then treated with insulin (10 μM) at indicated times. Lysates were separated on SDS-page and immunoblotted with anti-GSK3β and anti- pGSK3β.

Impairment of insulin signaling by praja2 inactive mutant suggested that a negative regulator of the insulin signaling could be a target of praja2. To identify this praja2 target, I looked for complexes between praja2 and different well known negative regulators of the insulin pathway, like PP2A, PTEN or PTP1B (data not shown). Using specific antibodies, I performed co-immunoprecipitation experiments using lysates from HEK293 cells. Among the potential partners analyzed, I found PTP1B as a novel praja2 interactor. **(Fig 10A)**. To support the co-immunoprecipitation data, I performed pull-down experiments using purified recombinant GST-PTP1B fusion protein on HEK293 lysates expressing praja2-rm. The GST-precipitates were immunoblotted with anti-Flag and anti-GST antibodies. This experiment confirmed that praja2 binds PTP1B **(Fig 10B)**. I used GST-beads as negative control.

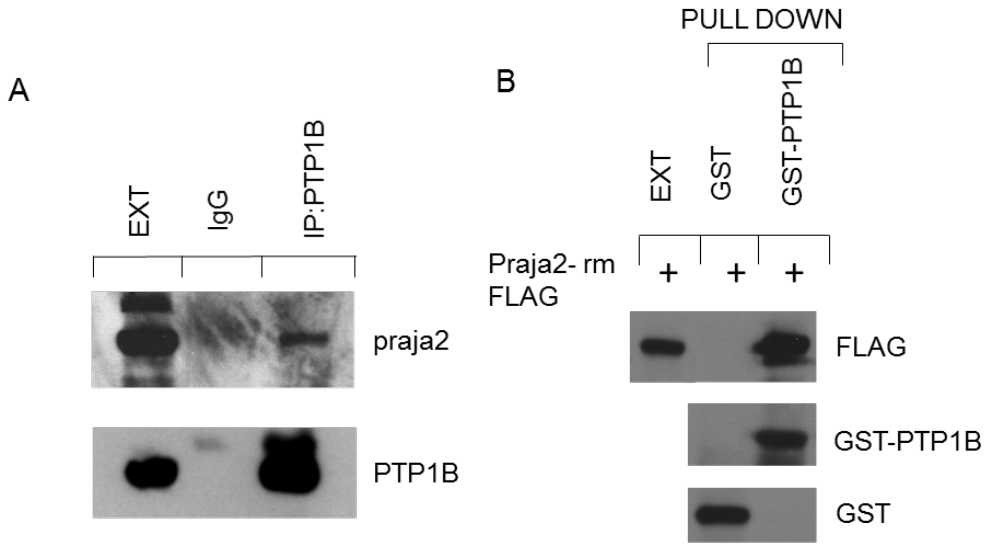


Figure 10. praja2 binds PTP1B. (A) HEK293 lysates were immunoprecipitated (IP) with anti-PTP1B or with control IgG. The precipitates and an aliquot of lysates (EXT) were immunoblotted with anti-praja2 and anti-PTP1B antibodies. **(B)** HEK293 cells were transiently transfected with praja2 RING mutant (praja2-rm) and lysed. Total lysates were subjected to pull down assay with beads linked to GST-PTP1B recombinant protein or GST as control. Precipitates and aliquot of the lysates were immunoblotted with anti-Flag and anti-GST antibodies.

Next, to demonstrate that PTP1B and praja2 are located within the same subcellular compartment, I performed an immunofluorescence experiment using anti-PTP1B and anti-praja2 antibodies in formalin-fixed HEK293 cells. The results indicate that praja2 and PTP1B partly colocalized within the endoplasmic reticulum and perinuclear region, as shown by the Pearson's coefficient analysis **(Fig.11)**

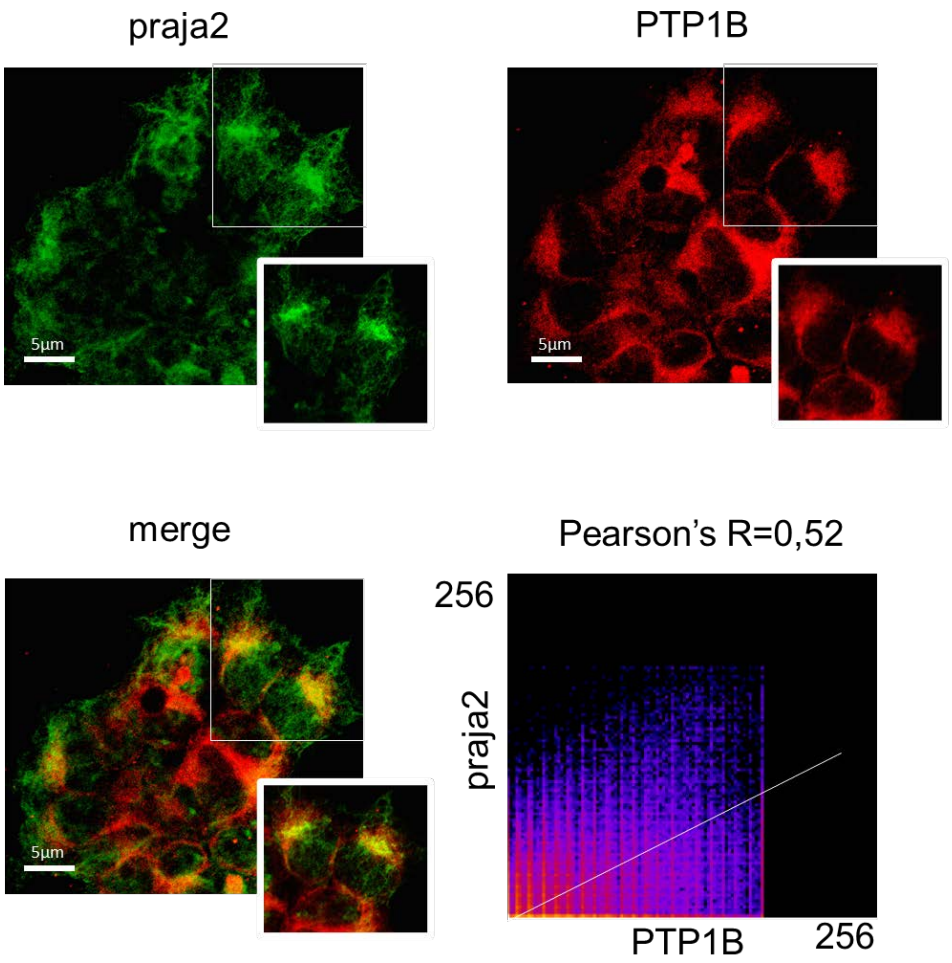


Figure 11. praja2 and PTP1B partly co-localize in cells. (A) HEK293 cells were subjected to double immunostaining with anti-PTP1B and anti-praja2 antibodies. Images were analyzed and collected by confocal microscopy. Magnification of selected areas is shown (insets). A Pearson's coefficient praja2-PTP1B was calculated: 0.52. Scale bar: 5µm.

4.2 Identification of the PTP1B-binding domain on praja2

The next step was to identify the PTP1B binding site on praja2. Therefore, we generated three praja2 deletion mutants at the C-terminus region, using as template flag-praja2 (praja2$_{1\text{-}630}$ Flag, praja2$_{1\text{-}530}$ Flag and praja2$_{1\text{-}401}$ Flag) and tested the ability of these mutants to bind PTP1B. I transfected HEK293 cells with praja2-rm or praja2 deletion mutants described above and performed co-immunoprecipitation assays. Lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-Flag and anti-PTP1B antibodies. As **Fig.12** shows, praja2$_{1\text{-}401}$ mutant was not able to bind PTP1B, while praja2$_{1\text{-}530}$ was, suggesting that the residues between 401-529 of praja2 are required for PTP1B binding.

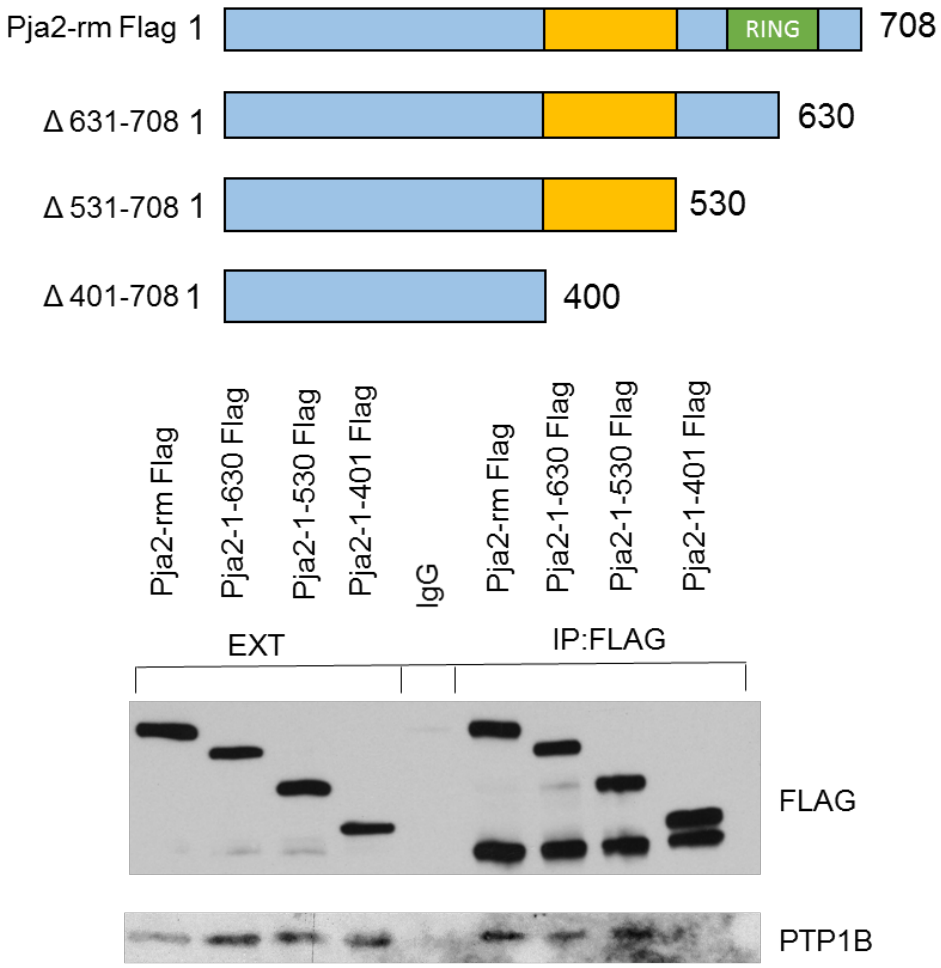


Figure 12. Identification of PTP1B binding domain on praja2. Schematic representation of human praja2 and its deletion mutants. RING domain and PTP1B binding domain are shown in green and in yellow (upper panel), respectively. HEK293 cells were transfected with praja2 RING mutant (praja2-rm), or with each of the three deletion mutants (praja2 1-630 Flag, praja2 1-530 Flag, and praja2 1-401 Flag). Lysates from transfected cells were immunoprecipitated with anti-flag antibody. The precipitates and aliquot of the lysates (EXT) were immunoblotted with anti-Flag and anti-PTP1B antibodies.

To further prove that the region spanning residues 401-529 of praja2 is, indeed, required to bind PTP1B, I generated a praja2 deletion mutant lacking residues 401-530 (praja2-Δptp1b Flag), and tested its ability to interact with PTP1B by pull down and co-immunoprecipitation assays. HEK293 were transiently transfected with praja2-rm or praja2-Δptp1b vector and harvested. Total lysates were subjected to pull down assay using GST-PTP1B or GST as control. Precipitated complexes were washed three times using 1M NaCl lysis buffer and immunoblotted with anti-Flag and anti-GST antibodies. The results shown in **Fig.13A** indicate that praja2-Δptp1b mutant does not bind PTP1B. Next, I performed co-immunoprecipiation assays using lysates prepared from HEK293 cells transiently transfected with praja2-rm, praja2-Δptp1b or PTP1B-HA vectors. The epitope-tagged praja2 variants were immunoprecipitated with anti-Flag antibody. Immunoprecipates were immunoblotted with anti-Flag and anti-HA antibodies. The results assay confirmed that residues 401-530 of praja2 are required for PTP1B binding **(Fig.13B**).

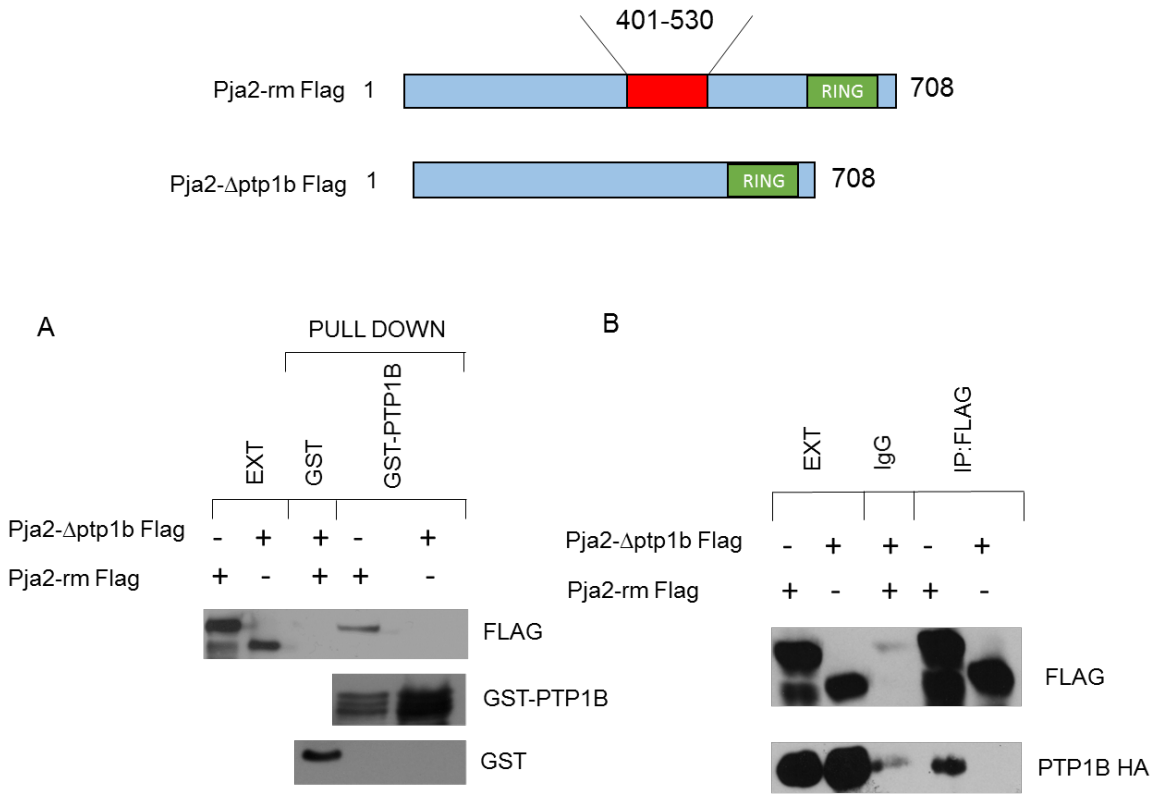


Figure 13. Praja2 deletion mutant Praja2-Δptp1b Flag (401-531) does not bind PTP1B. Schematic representation of human praja2 and its deletion mutants. RING domain and PTP1B binding domain are shown in green and in red (upper panel), respectively. **(A)** HEK293 cells were transfected with praja2 RING mutant (praja2-rm), or with pja2-Δptp1b Flag (401-531) vector. Lysates from transfected cells were subjected to pull down assays with GST-PTP1B recombinant protein or with GST beads. The precipitates and an aliquot of lysates (EXT) were immunoblotted with anti-Flag and anti-GST antibodies. **(B)** HEK293 cells were transiently co-transfected with praja2 RING mutant (praja2-rm), or with praja2-Δptp1b Flag (401-531) and PTP1B-HA vectors. Lysates were immunoprecipitates (IP) with anti-Flag antibody. The precipitates and an aliquot of the lysates were immunoblotted with anti-FLAG and anti-HA antibodies

4.3 PTP1B is ubiquitinated by praja2

The data indicate that PTP1B is a novel interactor of praja2. Since praja2 is an E3 ubiquitin ligase, I tested if PTP1B is also a substrate of praja2. For this purpose, I monitored the linkage of ubiquitin chain to PTP1B in cells expressing PTP1B-HA, myc-tagged ubiquitin and praja2 or praja2-rm. PTP1B was immunopurified from total cell lysates with anti-HA antibody. The precipitates were immunoblotted with anti-Myc, anti-HA and anti-Flag antibodies. The data show that overexpression of praja2, but not of its inactive mutant (praja2-rm), induced accumulation of polyubiquitinated forms of PTP1B **(Fig.14A)**. Given the role of PTP1B in the regulation of insulin signaling, I analyzed the effects of insulin stimulation on PTP1B polyubiquitination. As shown in **Fig.14B,** one hour stimulation with insulin was sufficient to induce PTP1B polyubiqutination. The effects of insulin were abrogated by expressing the praja2 inactive mutant (praja2-rm), supporting a role of endogenous praja2 in mediating PTP1B polyubiquitination.

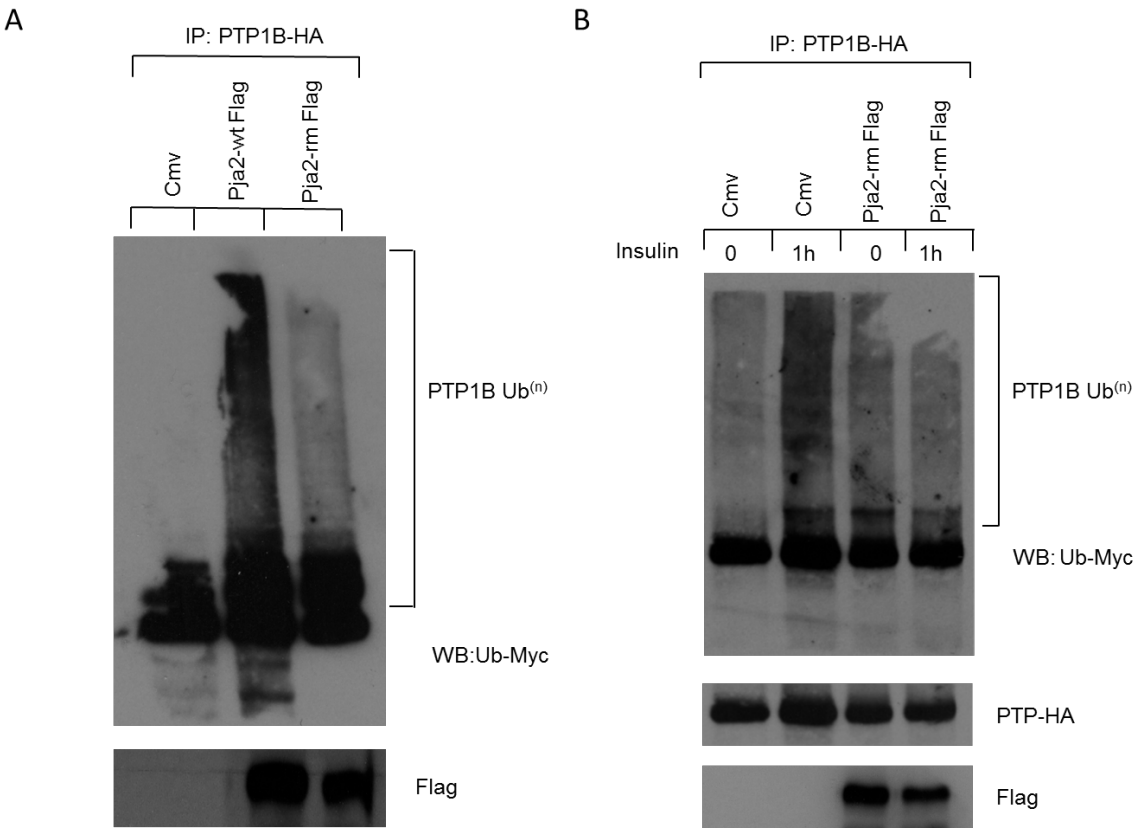


Figure 14. praja2 ubiquitinates PTP1B. (A) HEK293 cells were co-transfected with Myc-tagged ubiquitin and praja2 RING mutant (praja2-rm) praja2-wt Flag vectors. Lysates were immunoprecipitates (IP) with anti-HA antibody and immunoblotted with anti-Myc and anti-FLAG antibodies. **(B)** HEK293 cells were co-transfected with Myc-tagged ubiquitin, praja2 RING mutant (C634, 671A; praja2-rm) and an empty vector. After 24 hours of transfection cells were serum deprived for 12 hours, cells either were left untreated or stimulated with insulin (10 μM) at indicated time. Lysates were immunoprecipitates (IP) with anti-HA antibody and immunoblotted with Anti-Myc, anti-FLAG and anti-HA antibodies.

4.4 praja2 promotes PTP1B degradation by the proteasome.

praja2 is involved in the poly ubiquitination of PTP1B. The next step was to understand if polyubiquitination of PTP1B was linked to its proteasomal degradation. HEK293 cells were transfected with praja2-Flag or with praja2-rm-Flag and then treated with MG132 (10uM) (a proteasome inhibitor) for six hours. Cells were harvested and lysed. Lysates were immunoblotted with anti-Flag, anti-PTP1B and anti-ERK2 antibodies. The latter was used as loading control. The results show that expression of praja2, but not of its inactive mutant (praja-rm), caused a decrease of PTP1B levels that was prevented by the treatment with MG132, suggesting that the proteasome mediate praja2-dependent PTP1B degradation (**Fig 15**).

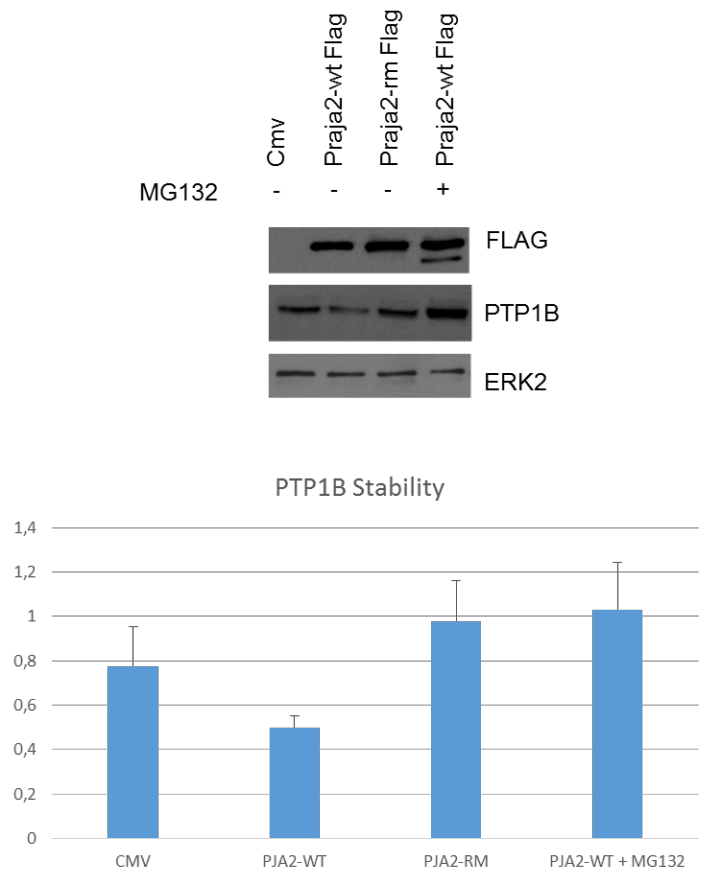


Figure 15. praja2 promotes PTP1B degradation through the proteasome degradation. HEK293 cells were transfected with an empty vector, praja2-wt Flag or with praja2 RING mutant (Praja2-rm) vector. Cells were left untreated or treated with MG132 (10 µM) for 6 hours before harvesting. Lysates were immunoblotted with anti-Flag, anti-PTP1B and anti-ERK2 antibodies. Lower panel shows cumulative data expressed as a mean value ± S.E.M of three independent experiments.

4.5 cAMP-praja2 axis regulates PTP1B Stability

Previous works demonstrated that praja2 acts in response to cAMP to ubiquitinate and degrade a variety of intracellular targets (29-31, 33). Accordingly, I analyzed the effects of cAMP stimulation on PTP1B stability. For this pupose, HEK293 cells were serum deprived overnight, treated with Forskolin (40µM), a potent agonist of the adenylyl cyclase, and harvested at different times from stimulation (0 30' 60' 90' 120'). Results shown in **Fig.16** indicate that stimulation of cAMP signaling decreased the levels of PTP1B.

Next, I analyzed the contribution of praja2 in cAMP-induced decrease of PTP1B by performing a genetic knockdown of endogenous praja2 by an RNAi procedure. Thus, HEK293 cells were transiently transfected with siRNAs targeting endogenous praja2 or with control siRNAs. Following transfection, cells were serum deprived overnight and then treated with Forskolin (40µM) (0, 30', 60', 90', 120') as above. Lysates were immunoblotted with the indicated antibodies. **Fig. 17** shows that downregulation of praja2 by siRNAs efficiently prevented PTP1B degradation induced by the Forskolin treatment, promoting accumulation of the phosphatase at each time point of forskolin treatment, compared to controls. Altogether, these findings demonstrate that praja2 is an important regulator of PTP1B stability, constituting a nodal point where insulin and GPCR-cAMP pathways may converge and focus to control downstream metabolic pathways.

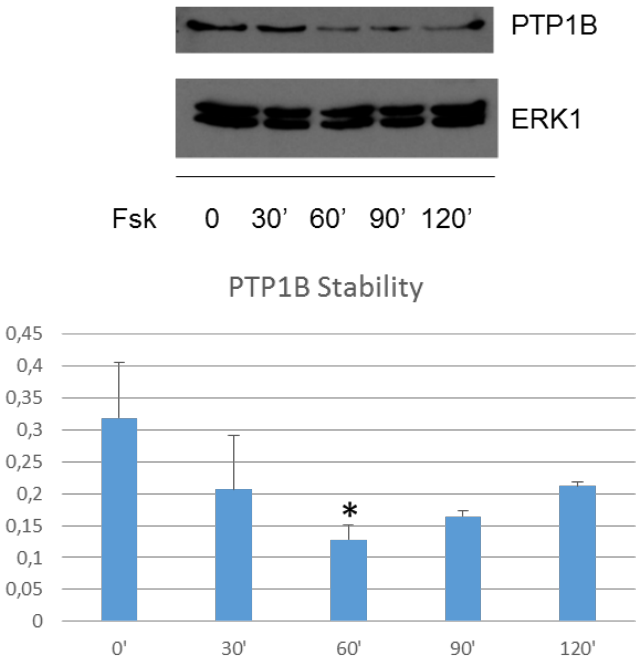


Figure 16. cAMP regulates PTP1B stability. Cells were serum deprived for 24 hours and then treated with Forskolin (40μM) at indicated time points. Lysates were immunoblotted with anti-PTP1B and anti-ERK1 antibodies. Lower panel shows cumulative data expressed as a mean value ± S.E.M of three independent experiments. *P<0.05 versus control.

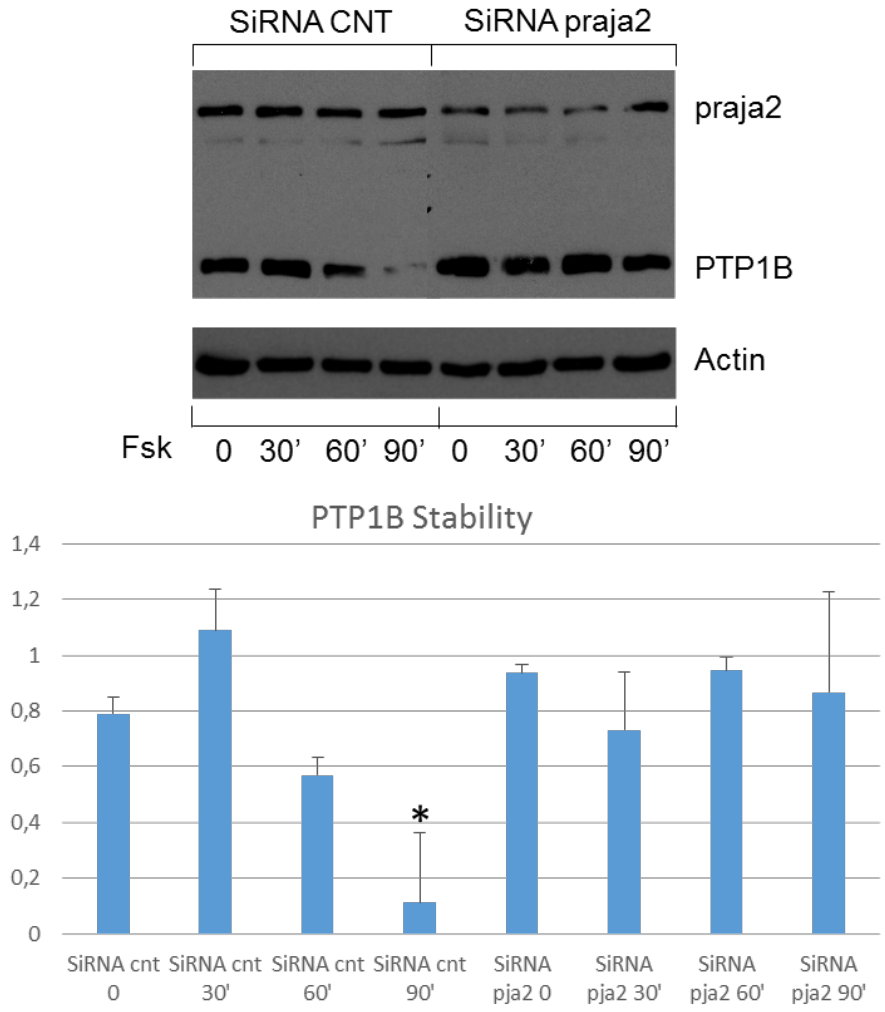


Figure 17. cAMP regulates PTP1B stability through praja2. HEK293 cells were transfected with control siRNA or with siRNA targeting praja2. Transfected cells were serum deprived overnight and then treated with forskolin (40µM) for the indicated time points. Lysate were immunoblotted with anti-PTP1B, anti-praja2 and anti-actin (loading control) antibodies. Lower panel shows cumulative data expressed as a mean value ± S.E.M of three independent experiments. *P<0.05 versus control.

4.6 Praja2 regulates insulin signaling.

The findings above indicate that praja2 regulates PTP1B stability. PTP1B is a negative regulator of the insulin signaling (71, 72). Accordingly, I tested the role of praja2 in the activation of the insulin pathway, focusing on p70S6Kinase. HEK293 cells were transfected with praja2-rm. 24 hours after transfection, cells were treated with insulin (10μM) at indicated times (0 15' 30' 45'), harvested and lysed. Lysates were subjected to immunoblot analysis with anti-p70S6K, anti-70S6K and anti-flag antibodies. The results in **Fig.18A** show that praja2 inactive mutant (praja2-rm) caused an impairment of insulin-induced p70S6K phosphorylation. I performed similar experiments by knocking down endogenous praja2 (**Fig.18B)**. The results supported the role of praja2 in regulating insulin-dependent phosphorylation of p70S6K.

Finally, to prove that praja2 acts through protein phosphatases in modulating insulin signaling, I repeated the experiments above by pretreating the cells with PTP1B XXII, a selective, reversible and non-competitive allosteric inhibitor of PTP1B. **Fig. 19** shows that the treatment with PTP1B XXII rescued the effect of praja2 genetic knock down on p70S6K phosphorylation**.**

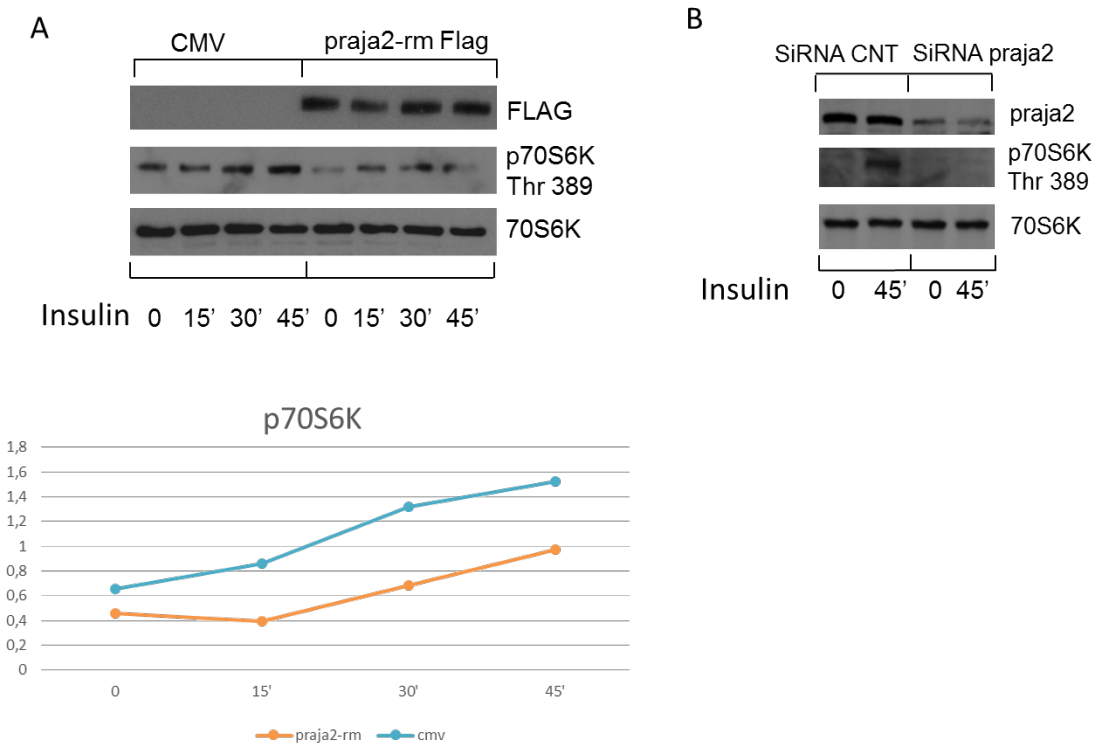


Figure 18. praja2 regulates phosphorylation of p70S6Kinase. (A) HEK293 cells were transfected with an empty vector or with praja2 RING mutant (praja2-rm) vector. Transfected cells were serum deprived overnight and then treated with insulin (10 µM) for the indicated time points. Lysates were immunoblotted with anti-Flag, anti-p70S6K and anti-70S6K antibodies. (**B**) HEK293 were transfected with control siRNA or praja2 SiRNA. Lysate were separated on SDS-page and immunoblotted with anti-praja2, anti-p70S6K and anti-70S6K antibodies.

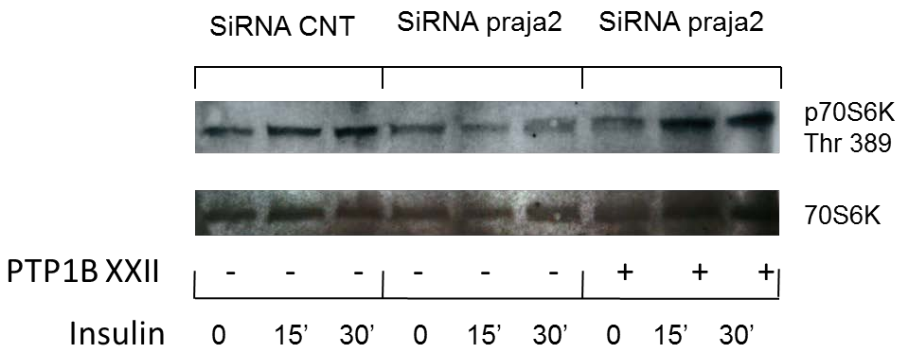


Figure 19. Praja2 is required for GSK3β phosphorylation. HEK293 cells were transfected with control siRNA or praja2 SiRNA. Transfected cells were then serum deprived overnight, pretreated (+) or not (-) with PTP1B XXII inhibitor (10μM) for 30' and then stimulated with insulin (10 μM) for 30 minutes. . Lysate were separated on SDS-page and immunoblotted with anti-p70S6K and anti-70S6K antibodies.

5. Discussion

PTP1B emerged as a crucial protein in cell signaling and a potential target for metabolic diseases, such as type II diabetes, obesity and cancer. PTP1B could be regulated at different levels by mechanisms involving oxidation, phosphorylation and sumoylation (59, 64, 65, 67-69, 73). However, the role of the ubiquitin pathway in the control of PTP1B stability and signaling was still unknown. Here, I've identified a new regulatory mechanism that efficiently couples the ubiquitin/proteasome system to the insulin cascade that involves the E3 ubiquitin ligase praja2. I've found that praja2 interacts with- and regulates the stability of PTP1B, modulating its ubiquitination and stability, and exerts major effects on the insulin pathway.

The ubiquitin proteasome system (UPS) has emerged as an important control mechanism of cell growth, survival and metabolism. Degradation of a protein via UPS involves modification of the substrate protein by covalent attachment of multiple ubiquitin molecules (70, 74). The ubiquitin-tagged protein is eventually degraded by the proteasome. By modulating the levels of target proteins, the UPS controls essential aspects of cell biology. Emerging evidence indicates that changing in the levels, subcellular targeting and catalytic activity of the E3 ubiquitin ligases, enzymes involved in the ubiquitination of UPS targets, may exert major effects on metabolism, tumor cell growth and survival (75). The UPS is strongly involved in the correct homeostasis of the skeletal muscle. Thus, the E3 ubiquitin ligase Thatmuscle-specific mitsugumin 53 (MG53) regulates the insulin receptor and IRS1 protein stability through the ubiquitin pathway (80). MG53 overexpression is observed in several animal models and in obese humans. MG53 up-regulation is strongly related to the insulin resistance in skeletal muscle, which is responsible for 70–90% of insulin-stimulated glucose disposal (80).

Cell growth and senescence are crucial aspects of cellular homeostasis that are regulated and controlled by the UPS. Thus, Cullin 7 (CUL7) E3 ubiquitin ligase complex directly regulates the insulin pathway through the control of IRS-1 stability, constituting a relevant feedback control mechanism of mTOR/S6K pathway (87). Dysregulation of this signaling network leads to cell senescence, possibly through sustained MAPK activation and/or increased Akt signaling (87, 91, 92). Therefore, identification of the targets and effectors of UPS pathway and understanding the basic mechanism(s) governing protein stability represent important goals for the design of effective drugs for metabolic diseases and cancer.

Praja2 belongs to a growing family of widely expressed mammalian RING-H2 proteins with intrinsic E3 ubiquitin-ligase activity and plays an important role in different aspects of cell physiology (29, 34). Praja2 acts as an AKAP that binds and targets PKA holoenzyme to the cell membrane, perinuclear region and cellular organelles. Co-localization of praja2•PKA complexes with PKA substrate/effector molecules ensures efficient integration and propagation of the locally generated cAMP to distinct target sites. By regulating cAMP signaling, praja2 efficiently couples phosphorylation to ubiquitination of protein kinases, scaffolds and effectors, with important implication for differentiation and cell proliferation. Dysregulation of praja2-regulated signaling has been causally linked to GBM growth and progression (31).

Here, I report that PTP1B is a novel partner of praja2. PTP1B represents a crucial protein in the study of metabolic diseases such as type II diabetes, but it is emerging, also, as a controversial partner in cancer development. Several studies define PTP1B as negative regulator of cell growth and cells transformation. Thus, PTP1B has inhibitory effects on many RPTKs and it is involved in cell–cell adhesion promoting N-cadherin–β-catenin complex formation (100-102). In addition, PTP1B contributes to apoptotic cascade

enhancing IRE1-mediated ER stress signaling, as well as by dephosphorylating STAT3 during TRAIL-induced apoptosis (102-104).

PTP1B is also involved in the regulation of bone marrow cells. In this context, myeloid-specific deficiency of PTP1B is sufficient to promote the development of acute myeloid leukemia (AML) (105). Controversially, in many tumors PTP1B protein levels are increased, especially in breast and ovary cancer (102, 106, 107). Indeed, over-expression of PTP1B has an oncogenic role in ErbB2+ breast cancer, modulating Src Y529 phosphorylation and Src-related signaling pathways (108)**.** In the same way, PTP1B regulates positively cell proliferation and metastasis in non-small cell lung cancer, as much as in gastric cancer **(**109**,** 110**).**

In light of the role of PTP1B in important pathways involved in cell metabolism and proliferation, it became critical to study and understand how PTP1B is regulated. Different types of post-translational modifications are entailed in PTP1B modulation, such as: reversible oxidation (59), serine and tyrosine phosphorylations (62, 63)(64)(65), sumoylation by SUMO E3 PIAS1 (ligase protein inhibitor of activated STAT-1) (66) and proteolysis (67-69). In my thesis, I report a new mechanism of post-translational modifications of PTP1B, based on the ubiquitination and proteolysis of the phosphatase by the UPS.

Biochemical experiments demonstrated that praja2 interacts with PTP1B. Deletion mutagenesis and *in vitro* binding assays identified the domain spanning residues 400-530 of praja2 as responsible of PTP1B binding. This domain is just upstream the region of praja2 that mediates the interaction to other signaling proteins, as PKA and MOB1 (29, 31). However, if and how the binding of PT1B to praja2 affect interaction with other signaling relays needs further analysis.

Previous work demonstrated that direct phosphorylation of praja2 by PKA induces its E3 ligase activity. Thus, following cAMP stimulation, several praja2 partners undergo to extensive ubiquitination and subsequent

proteolysis through the proteasome, with important biological consequences on downstream signaling pathways involved in cell growth and differentiation. Similarly, I found that praja2 ubiquitylates and degrades PTP1B. The proteolysis of PTP1B can be induced by cAMP stimulation and it is mediated by praja2. This finding suggested that praja2 by modulating the ubiquitination and stability of PTP1B can elicit major effects on PTP1B-regulated signaling pathways. To prove this hypothesis, I analyzed the effects of praja2 downregulation on insulin pathway. In particular, I used two distinct approaches, such as genetic knockdown of praja2 or expression of dominant negative variants of praja2. In both experimental conditions, I found that, interfering with praja2 expression or activity, the phosphorylation of two relevant metabolic targets of the insulin pathway (GSK3b and p70S6K), is impaired. This strongly suggested that praja2, beyond its role in cell growth and differentiation, is also implicated in the key aspects of metabolic pathways regulated by insulin.

Another important observation that emerged from my studies relates to the cross-talk between GPCR-cAMP signaling and the insulin pathway. I found that cAMP stimulation promotes a rapid and transient degradation of PTP1B by the praja2-UPS pathway. Removing a negative regulator of the insulin pathway, such as PTP1B, GPCR signaling may contribute to sustain the insulin-dependent metabolic pathways. To formally prove that praja2 regulates metabolic pathways *in vivo*, we have generated an inducible praja2 knock out mouse line. The analysis of KO mouse line will reveal the role of praja2 in such metabolic pathways.

Collectively, the data reported in the thesis suggest a model **(Fig. 20)** whereby, as consequence of GPCR-cAMP, PTP1B undergoes to praja2-dependent ubiquitination and proteolysis through proteasome. By removing PTP1B, cAMP-praja2 axis positively regulates the transmission of insulin signals to downstream metabolic targets. This represents a novel point of intersection between both GPCR and insulin pathways, with

important implications in cell physiology. The molecular characterization of praja2-regulated signaling pathways, the generation of appropriate animal models of praja2 knockout and the identification of genetic mutations affecting praja2 function *in vivo*, will likely provide novel therapeutic targets for the treatment of human metabolic and proliferative disorders.

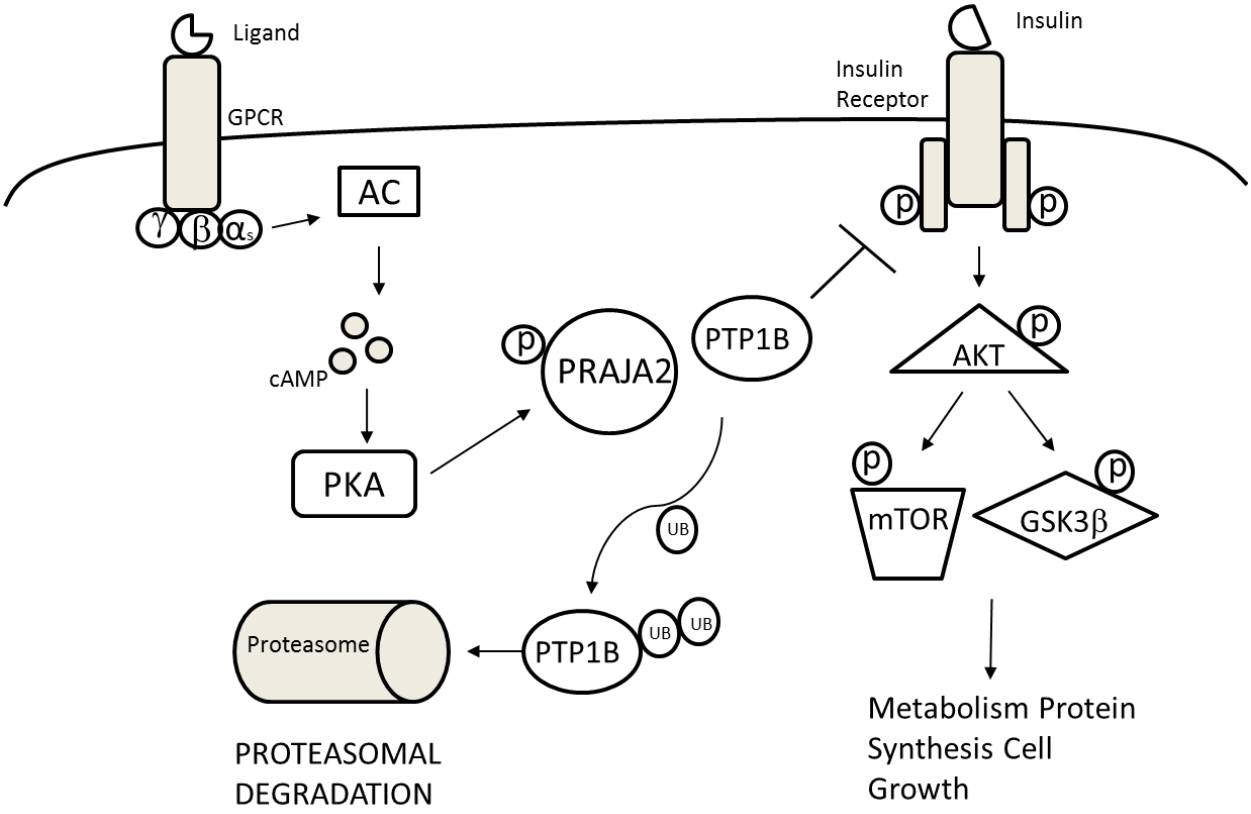


Figure 20. Working model. Schematic representation of PTP1B stability in the insulin cascade. Propagation of the cAMP waves and activation of the insulin receptor by its ligand leads to PTP1B ubiquitination by praja2 and its consequent degradation by the proteasome. Downregulation of PTP1B levels by praja2 would enhance the sensitivity of the insulin cascade to hormone stimulation.

6. Appendix

IR: Insulin Receptor

IGF-1: Insulin-like growth factor

GPCRs: G protein–coupled receptors

AD: Adenylate cyclase

cAMP: Cyclic-AMP

PDE: Phosphodiesterase

PKA: Protein Kinase A

GST: Glutathione S-transferase

FSK: Forskolin

mTOR: Target of rapamycin

GSK3β: Glycogen synthase kinase 3 beta

S6K: Ribosomal protein S6 kinase beta-1

7. Acknowledgments

I would like to thanks Professor Garbi for the immunofluorescence experiments.

8. References

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9. List of Publications

praja2 regulates KSR1 stability and mitogenic signaling.

Rinaldi L, Delle Donne R, Sepe M, Porpora M, Garbi C, **Chiuso F**, Gallo A, Parisi S, Russo L, Bachmann V, Huber RG, Stefan E, Russo T, Feliciello A.

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