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The PxxP motif of Ypk1: a potential missing link between TORC2 and endocytosis

by

Anton Shuster

Director:
Prof. Dr. Robbie Loewith

Supervisor:
Dr. Manoël Prouteau

Department of Molecular Biology
Faculty of Sciences

Abstract

After a brief illustration of **AGC kinase superfamily** general structure and functioning we presented in more detail specific features and functions of its members, **Ypk1/2** and its mammalian homologue **Akt**, important conserved regulators of multiple cell processes, including actin polarization and endocytosis. We took the historical standpoint to trace the milestones in research on major controllers of cell growth, **TOR** kinases and then more precisely on **TOR2**-specific membrane-associated complex **TORC2** implicated in endocytosis. This developed into description of coalescence of **Ypk1** and **TORC2** research fields through identification of **Ypk1** as **TORC2** substrate and effector for endocytosis-related tasks. Further on we introduced the **SH3** protein interaction domain and its binding motif **PxxP** highlighting the significance of their binding for **AKT** physiology in mammals and the endocytosis machinery proteins in yeast. We performed an alignment, which showed that **PxxP** motif is highly conserved from **Akt** to **Ypk1**. The hypothesis that **SH3/PxxP** interface could be mediating **Ypk1** interactions with its yet undescribed endocytosis-linked partners has been emitted. Our *in silico* prediction and external experimental data provided an array of strongly interrelated **SH3-containing endocytosis proteins**. Among them we identified **Rvs167** and **Sla1** and their partners as excellent candidates for SH3/PxxP-mediated interaction with **Ypk1**. The experimental protocol has been proposed for both candidate approach and independent partner identification trials. Finally, we speculated over various scenarios of **Ypk1** potential novel PxxP-related interactions and proposed corresponding models.

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0. Abbreviations

Abl : Abelson murine leukemia viral oncogene homolog 1
Abp1p : actin binding protein 1
AGC superfamily : named after protein A, G and C families(PKA,PKC,PKG)
AST : active site tether
ATP : adenosine triphosphate
Bck1 : bypass of C Kinase
BLAST : basic local alignment search tool
Btk : Bruton's tyrosine kinase
c-Src : cellular Src
cAMP : cyclic adenosine monophosphate
Cdc15 : cell division cycle 15
CLT : C-lobe tether
EGF : epidermal growth factor
Ena/VASP : Ena/Vasodilator-stimulated phosphoprotein
Ent1/2 : epsine N-terminal homology
Eps8 : Epidermal growth factor receptor kinase substrate 8
EVH1 : enabled VASP homology 1
FKBP12 : FK506 binding protein 12
Fpk1: flippase kinase 1
FRET : Fluorescence resonance energy transfer
GFP : Green fluorescent protein
GHS-R1a : Growth hormone secretagogue receptor 1a
GPCR : G protein-coupled receptor
Grb2 : growth factor receptor-bound protein 2
GRK : G protein-coupled receptor kinase
GST : gluthathione S-transferase
HM : hydrophobic motif
K.D. : kinase dead = with lost kinase function
KESTREL : kinase substrate tracking and elucidation
Lsb1p : Las seventeen binding protein
MAGUK : Membrane-associated guanylate kinase
MCT : membrane containing TORC2
mRNA : messenger RNA
mSos1 : mammalian son of sleeveless 1
mTORC: TOR complex in mammals
Myo3/5 : myosin 3/5
NIH 3T3 : a fibroblast cell line
NLT : N-lobe tether
NVP-BHS345 : 2-methyl-2-{4-[3-methyl-2-oxo-8-(pyrimidin-5-yl)-2,3-dihydro-1H-imidazo[4,5-c]quinolin-1-yl]phenyl}propanenitrile
Pan1 : Poly(A)-binding protein-dependent poly(A) ribonuclease
Pdk1 : Phosphoinositide dependent kinase 1
Pex13 : peroxin 13
PH : pleckstrin homology domain
PI3K : Phosphoinositide 3-kinase
PID : protein interaction domain
PIF : Pdk1 interacting fragment
PIKK : PI-Kinase related protein kinase
PIP3 : Phosphatidylinositol (3,4,5)-trisphosphate
PKA : protein kinase A
PKB : protein kinase B, also known as AKT
PKC : protein kinase C
PKG : protein kinase G
Pkh1 : Pkb-activating kinase homologue 1
PPII : poly-proline type II
Prk1 : p53 regulatory kinase 1
PWM : position weight matrix
PX : phox homology domain
RHO : Ras homologue
RIE : an epithelial cell line
Rvs167p : reduced viability upon starvation 167
Sf9 : a clonal isolate of Spodoptera frugiperda Sf21 cells
SGK : Serum/glucocorticoid-regulated kinase
SH3/2 : SRC homology 3/2
SKOV3 : ovarian carcinoma cell line
Sla1 : synthetic lethal with ABP1
Slm1 : synthetic letal with Mss4
SMART : simple modular architecture research tool
SPOT : specificity prediction of target
Syk : spleen tyrosine kinase
TCP-1 : T-complex protein 1
TOR : target of rapamycin
TORC:TOR complex in yeast
UBPY : ubiquitin specific peptidase 8
v-Src : viral Src
Vrp1 : verprolin 1
WASP : Wiscott-Aldrich syndrome protein
WT : wild type
YAP65 : Yes-associated protein 65
Ypk1 : yeast protein kinase

1. Ypk1/2 and TORC2: the love story

1.1. A brief introduction to the AGC/PKB kinase family

The essential role of kinases in cell life is well illustrated by their abundance and conservation in eukaryotic genomes. The first massive sequencing study in *S. cerevisiae* identified that around 2% of all genome coded for kinases, the roles of 60% being already known at that time (1). The AGC superfamily of related serine/threonine kinases represents 15-20% of the yeast kinome and shows high level of conservation in all eukaryotes, including mammals (2,3). The birth of the kinase research dates back to 1958, when Krebs and Fischer first demonstrated that the protein activity can be altered by covalent phosphorylation by a kinase, so called “converting enzyme”, in rabbit skeletal muscle (4). The cAMP has been discovered and identified as a secondary messenger in 1958 (5). This enabled identification of the first member of PKA kinase family, phosphorylase kinase kinase A, and discovery of its dependence on cAMP using the same rabbit muscle model in 1968 (6). Many further studies concentrated on the understanding of PKA catalytic mechanisms and its activation. It has since then become one of the most studied kinases, hence a structural and functional prototype for AGC group (7). PKA is also notably the first kinase to have been completely sequenced (8).

The AGC group is generally subdivided into 14 families, where the main difference lays in regulation strategies. Thus, these families are regrouped in 3 molecular mechanism classes (MMC) defined by general activation principles: activated by dimerization and GRKs, activated by GPCRs directly or activated by phosphorylation (2). The latter class features, among others, both PKA and PKB families, containing Ypk1 and AKT, and will be therefore further presented.

1.2. AGC/PKB Zoom in: structure, functioning and regulation

The AGC superfamily has been first discovered, named and described in a PK**A**, PK**G** and PK**C** catalytic kinase core sequence alignment study by Hanks and Hunter in 1995 (9). It pointed to important conservation of structure in the whole group (2,10). The main AGC core consists of a small N-lobe and a large C-lobe. The catalytic site and its ATP molecule, the phosphate donor, is positioned between two lobes by the DFG motif of the activation loop (11). This ensures phosphorylation of the substrate peptide upon its docking to the activation site. An important structural element is the αC helix of the N-lobe, which enables catalytic activity by switching the kinase from “opened” to “closed” conformation(12). The stabilization of this helix and the catalytic site in general is the main constraint for AGC kinase activation (13). The pocket created by the residues of the DFG loop, the αC helix of the N-lobe, the AST region of the C-tail (14) and the C-lobe promotes reception of ATP, non-covalent interactions with its phosphates and subsequent substrate phosphorylation (Fig. 1A)(13–16). This environment is attained through conformational changes imposed by multiple regulation strategies, more or less specific to each AGC kinase family (Fig. 1B)(2,10). The N-terminal and C-terminal tails are variable in AGC kinases from different families and determine this specificity through multiple functional domains (16).

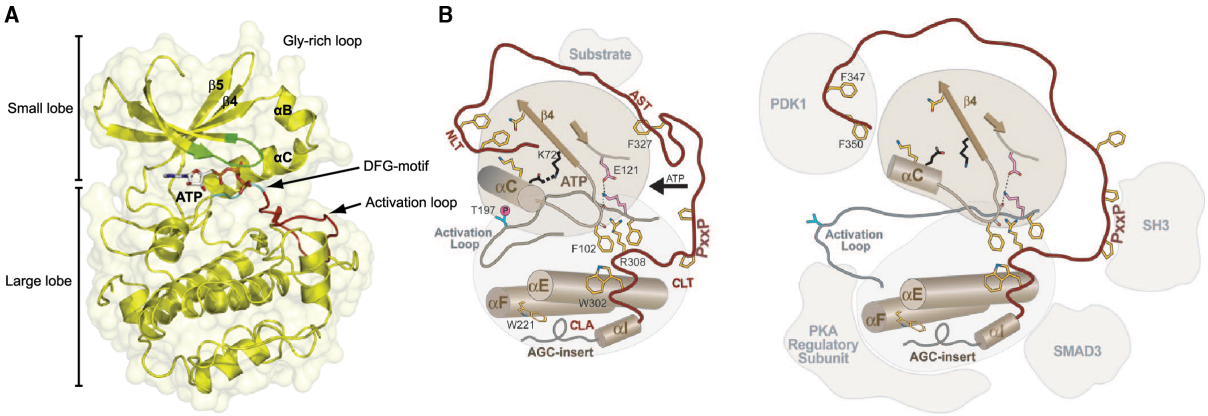

Figure 1. The structure of a prototypical AGC kinase. (A) The basic structural elements of AGC illustrated by PKA catalytic core crystal structure: Gly-rich loop (green), DFG-motif (cyan) and activation loop (red). (B) The active closed state (Left) and one of alternative disordered states (Right). Images taken from (2) (A) and (16) (B).

Phosphorylation is a key process for regulation of AGC activation state. Most AGC kinases feature 3 distinct phosphorylation sites that deliver concerted action to provide the active conformation: the main one at the activation loop (12) and both turn-motif and hydrophobic motif (HM) sites at the C-terminal tail (16,17).

The activation loop (T-loop) phosphorylation site stabilizes the loop's geometry and fixes the α-C helix, restricting movement to multiple stable conformations of the catalytic core observed in crystal structures (2). The C-terminal tail is divided into three segments **(a,b,c)**, each featuring conserved elements (14): **(a)** The N-lobe tether (NLT) is on the tail's C-terminal end. It contains the HM that interacts with important structural element of the N-lobe, the PIF-pocket (18); **(b)** The C-lobe tether (CLT) at the tail's N-terminal end interacts with the C-lobe and the lobe interlinker; **(c)** The active-site tether (AST) is placed between NLT and CLT. It assists ATP and substrate recruitment to the catalytic core and features a Zipper/turn-motif that stabilizes phosphorylated HM binding to its PIF-pocket (Fig. 2)(2,13,19). The HM is a conserved FXXFS/TY sequence, where the Ser/Thr phosphorylation site follows the hydrophobic pattern (20). When phosphorylated, HM interacts simultaneously with either the hydrophobic PIF-pocket and a separate phosphate-binding site of its own N-lobe (as in PKA) or with the pocket of an upstream activator kinase (such as Pdk1 activator for Akt). In this way it exerts the function of a "docking site" for the T-loop phosphorylation (15,21). The actions of HM and turn-motif phosphates are cooperative, as turn-motif provides a zipper-like association between the C-terminal tail and the N-lobe. This is achieved through turn-motif contact with phospho-Ser/Thr binding site from above the glycine-rich loop of the kinase domain. The resulting structural constraint favours HM-mediated interaction (17).

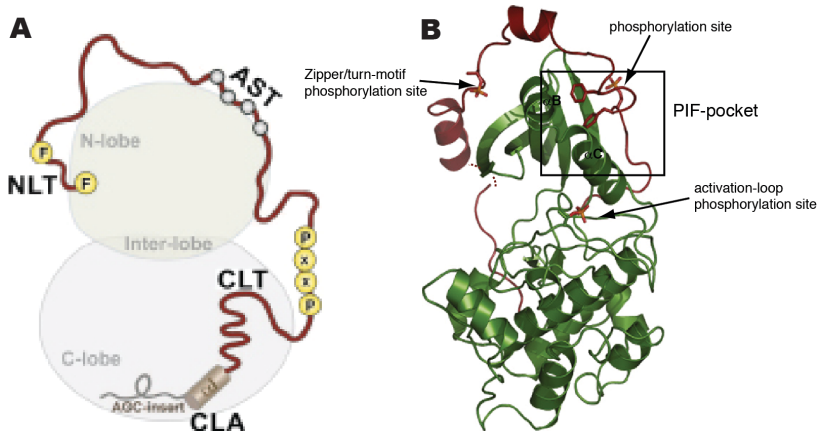

Figure 2. (A) Cartoon representation of an AGC kinase C-terminal tail showing its key regions and interaction sites on both lobes. (B) PKCβ II crystal structure illustrates the main phosphorylation sites of an AGC kinase and its PIF-pocket. The catalytic core is shown in green and the C-terminal tail in red. Images taken from (16) (A) and (2) (B).

1.3. Ypk1/2 and AKT: the AGC/PKB prototype members

The PKB/AKT kinases fulfil key regulator functions downstream of various signalling pathways (22). They activate numerous effectors implicated in vital processes, such as cell survival, growth, proliferation, angiogenesis, metabolism and migration (23,24). Hundred non-redundant substrates have been reported, where only approximately 25% contain the minimal Akt recognition pattern (R-X-R-X-X-S/T) (23). AKT are equally implicated in certain high impact human diseases, such as type-2 diabetes and cancer, which makes them prominent targets for various medical approaches (25–28).

X-ray crystallography gave evidence for pleckstrin homology (PH) domain presence in PKB, though kinase domain and PH had to be crystallized separately to obtain the image (22). An important insight has been given, when AKT/PKB activation was observed in presence of lipid vesicles containing PIP3 secondary messenger during *in vitro* assay with both mutant truncated PIF-pocket version of Pdk1 and with its wild-type version conjugated with PIFtide PIF-pocket inhibitor (29). The canonical Akt activation strategy describes PIF-pocket interaction of Akt HM motif with Pdk1 PIF-pocket, so a new PIF-pocket independent activation pathway was logically expected. The inositol phospholipid-mediated Pdk1-PKB interaction mechanism discovery resolved this enigma (30). It has been long known that PH is a lipid-binding domain specialized in signal transduction from membrane-bound lipids to cytosol proteins (19). The PH domains of Pdk1 and PKB enable their translocation to the plasma membrane, where they interact with PI3K inositol phospholipid products (Fig. 3, Fig. 6)(30–32). Both PH domains interact specifically with the phosphates in position D3 and D4 of the inositol ring, which explains their membrane colocalization related to preference for PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (22,30,33). The membrane-cytosol dynamics was followed in a FRET real-time *in vivo* assay. The inactive cytosolic PKB bared PH-in conformation, where its T-loop was obstructed and, thus, prevented Pdk1 from phosphorylating it. The membrane-localised PKB in PH-out confirmation was active, as its T-loop was liberated for phosphorylation (34). This means that membrane-associated PKB activation pathway consists of two steps: PKB membrane localization and conformational change that makes it a Pdk1 substrate (30). The structural feature that explains this particular complexity of PKB functioning is its auto-inhibition strategy. It is achieved through disordered kinase domain (helices αB and αC of the N lobe, activation loop), ordered steric blocks and extra disulphide bonds, when in free state. Upon Pdk1-dependant activation either through HM motif phosphorylation or PH-mediated translocation, the domain is restructured and becomes ordered and functional (22,35). Recent studies underline the redundant character of the 2 activation strategies of PKB/Akt and clarify the role of membrane nanorafts in their regulation (25,36). This structural metamorphosis is considered to be an extra measure of precaution, avoiding a palette of significant physiological consequences of undesired PKB activation.

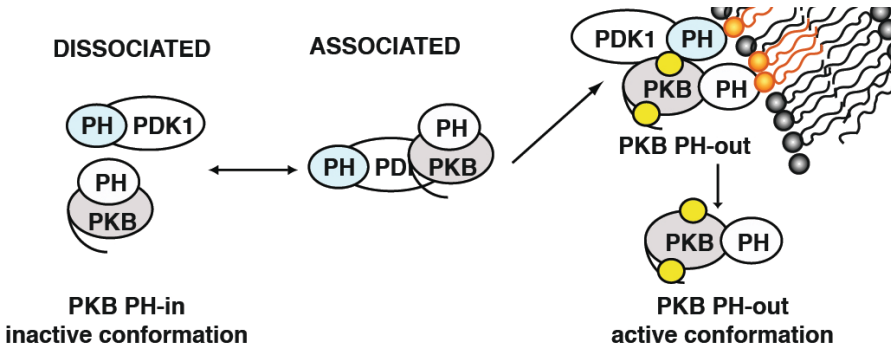

Figure 3. Schematic model of the interaction of PKB with PDK1. Image taken from (22) and modified.

Three AKT isoforms have been found in mammals: AKT1(PKBα), AKT2(PKBβ) and AKT3(PKBγ). They all present approximately 80% identity with one another and feature N-ter PH domains, kinase domains and C-ter regulatory tail (30). This high identity status explains redundancy in many cell processes and resemblance in regulation between the isoforms. Nevertheless, their distribution patterns in tissues differ and there is evidence of contribution of particular isoform to a particular health or disease pathway. It means that the isoforms possess certain differentiated roles, which is documented in multiple studies (26). Mammals equally feature a highly similar SGK1,2,3 family (37) of PDK-1- ,PIP3- dependant kinases. They are known to share many substrates with AKT, acting in a synergistic or redundant manner, depending on precise case (38,39). The major difference between SGK and AKT is the N-ter phox homology (PX) domain of SGK3, which confers it ability to localize to the endosome and other vesicle-like structures (40). Cell survival, proliferation and growth are fields, where SGK contribution is considered determining (38).

YPK1/2, the yeast functional counterparts of AKT, are the principal subjects of our study. The choice of the yeast *Saccharomyces cerevisiae* as a model organism is explained by high degree of evolutionary conservation of the growth control systems and related development of this model in the field. Since YPK gene first discovery in 1989 (41) and the protein's characterization (42), two closely related genes YPK1 and YPK2, coding for two 90% identical proteins, were reported (43). *ΔYpk2* mutant did not show altered phenotype, while *ΔYpk1* resulted in slow growth; *ΔYpk1ΔYpk2* double mutant was lethal due to severe defects in vegetative growth, illustrating functional overlapping and essential role of both genes in cell proliferation(43). Further study established close relation of YPK1/2 to mammalian homologues AKT and SGK. SGK was successfully phosphorylated by PKH1, PDK1 yeast homologue (a physiological activator of YPK). Even more, AKT1 and SGK1 were also partially able to rescue the *ΔYpk1ΔYpk2* phenotype (44). Many papers invested into accurate placing of YPK in the pathway context, which finally occurred highly homologous to the one of AKT (45,46). Since then, the expansion of interest for YPK led to characterisation of its role in multiple cellular activities including endocytosis (47), actin polarization (48), fatty acid uptake (49), autophagy (50), DNA stability (51), sphingolipid homeostasis (52) and sphingolipid-mediated pathway signalling (53).

1.4. Target of rapamycin complexes: major regulators of cell growth

Rapamycin, an anti-fungal agent from *Streptomyces hygroscopicus*, a bacterium found in a soil sample from Rapa-Nui in 1965, guided researchers to discovery of growth-related signalling pathways more than a decade after its first isolation in 1975 (54,55). Target of rapamycin genes, TOR1 and TOR2, were identified through isolation of rare dominant rapamycin-resistant yeast mutants (56). Their products have been later characterized as 282 kDa 68% identical atypical Ser/Thr-specific kinases, founders of PI-kinase related protein kinase family (PIKK) (57). Further performed double disruption of TOR1 and TOR2 showed a growth arrest phenotype similar to rapamycin treatment, establishing TOR as the main target of rapamycin (58). Afterwards, functional divergences between TOR1 and TOR2 have been elucidated. The double mutants arrested growth in G0 within one generation (59), while TOR1 mutants showed little-to-no effect and TOR2 mutants arrested growth in G2/M phase

showing randomized cytoskeleton and small-budded phenotype (55,58,60). Two distinct TOR pathways have been henceforth treated separately: TOR-shared rapamycin-sensitive pathway, where TOR1 and TOR2 fulfil redundant functions and TOR2-specific rapamycin-insensitive pathway, where TOR1 does not participate (60,61).

From there on TOR-shared signalling network was associated with mRNA and protein synthesis and degradation, ribosome biogenesis, nutrient transport and autophagy (62), while TOR2-specific branch was linked to actin cytoskeleton polarization, endocytosis and sphingolipid synthesis (47,63,64). In addition to this, co-immunoprecipitation experiments have shown that TOR1 and TOR2 permanently resided in 2 large molecular complexes of app. 2 MDa, TORC1 and TORC2. TORC1 equally accepts Tor1 and Tor2 but TORC2 is restricted to Tor2, giving evidence for previously observed bivalence (65). This was a major step, as numerous partners, common or specific to each complex, were identified and led further query. It is also remarkable that both TOR complexes are structurally and functionally conserved from algae and yeast to slime molds, worms, flies and mammals (66,67). Although mammals only possess one TOR gene, so only one TOR protein forms both mTORC1 and mTORC2 (68). TORC partners tagging with GFP demonstrated that TORC1 is permanently localized on the limiting membrane of the vacuole and that its “geography” does not obviously influence its functioning (69,70). TORC2 cellular localization experiments, featuring subcellular fractionation, indirect immunofluorescence, electron microscopy and GFP-tagging, were less reconciled and indicated TORC2 primary location at the plasma membrane and certain other sites, such as MCT (membrane containing TORC2). The finding appeared concordant with TORC2 already known functions (55). Most recent advances tend to approach TOR signalling in a systematic manner, giving insight into more complex roles and interrelationship between two complexes. For example, evidence has been collected that TORC1 could become not only a regulator downstream of environmental signals, but also an upstream cell metabolism manager (71,72); TORC1 relation to actin organisation and membrane-trafficking has also been established (73).

1.4.1. Downstream functions: Ypk1/2-mediated endocytosis

Nevertheless, cell growth related to Ypk1/2-mediated TORC2 regulated endocytosis canonical pathway remains our central preoccupation, as our ultimate ambition is pointing to possible structural interactions within this system.

Since realisation of existence of TOR2-specific functions mentioned above, its major role in actin organisation has rapidly been shown. Overexpression of TCP-1, cytosolic chaperonin responsible for actin structure biogenesis, led to recovery of growth and polarized actin distribution in a *tor2* mutant (63). Further paper by the same group specified the mechanism by identifying ROM2, a RHO1 and RHO2 GDP/GTP exchange factor, as a TORC2 substrate; while ROM2 activity is reduced in *tor2* mutants, its overexpression suppresses *tor2* mutation (74). This allowed tracing TORC2/Rho1 actin polarization pathway through Rho1 effector Pkc1, a yeast protein kinase C homologue (60). Parallel studies in mammals led to description of mTORC2 complex homologous to TORC2 in yeast. Its function has been equally placed upstream of Rho GTPases in actin skeleton regulation (64).

In the meanwhile, Pkh-Ypk cascade requirement for endocytosis has been elucidated. Genomic DNA plasmid library screening identified a plasmid carrying YPK1 gene that restored growth in *udi5-1* α-factor pheromone receptor internalization defective mutant (47). This receptor is physiologically downregulated by hyperphosphorylation of its Ste2 cytosolic tail, ubiquitination and internalization resulting in receptor degradation in endosome (75).

Subsequent Ypk1 “kinase-dead” (K.D.) assays indicated that its kinase activity is necessary for internalization but acts downstream of Ste2 phosphorylation; Pkh1-mediated Ypk1 activation has been proved necessary for this process (47). Another important screen by the same group associated a *udi11-1* ubiquitin-dependant internalization phenotype to a Tor2^{G2128R} single site mutant (76). Curiously, *tor2*^{G2128R} cells showed a certain degree of actin depolarization, in this way accurately placing Tor2 in already existing actin polarization-endocytosis tandem (77,78). At this point, the coalescence of Tor2 and Ypk signalling research fields appears indispensable. Ypk1 is presumably placed downstream of Tor2 but upstream of Rho. The confirmation did not take long to occur: a key study by Kamada et al. documents direct phosphorylation of Ypk2 by Tor2 in TORC2 (79). The yeast strain YYK241 *tor2*-associated lethality was suppressed by 5’-truncated Ypk2 starting at position 224 (Ypk2-224) isolated from yeast genomic library plasmid. An alignment with the homologues indicated a conserved region (^{237}TFDVT^{243}R), which inspired F238 and D239 Ypk2 substitutes. They showed influence on *tor2* phenotype similar to Ypk2-224, forging a later confirmed hypothesis of Ypk2 N-terminal auto-inhibitory domain. Tor2 *in vitro* phosphorylation of Ypk2 was successfully performed. Another AGC kinase, S6K1, has been already identified as a Tor2 substrate (80,81). This preliminary experience from its study gave a hint for identification of S641 turn-motif and T659 hydrophobic motif residues as direct substrates for Tor2 TORC2-specific phosphorylation of Ypk2 (79). A missing link in Ypk upstream regulator quiz has been replaced (Fig. 4). Almost simultaneously, the same has been shown for Akt/mTOR mammalian system. The rictor-mTOR complex phosphorylated directly Ser^{473} HM phosphorylation site residue in Akt *in vitro*, which enhanced the kinetics of T-loop phosphorylation by Pdk1 (82). Rictor has been characterized as obligatory partner for this process. A negative feedback loop through reorganisation of mTOR from mTOR-rictor (TORC2 analogue) to mTOR-raptor (TORC1 analogue) upon Akt activation has equally been assumed (83).

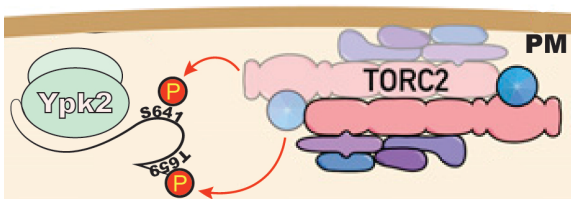

Figure 4. The plama membrane (PM) -associated TORC2 complex directly phosphorylates the turn-motif residue S641 and the HM residue T659 of Ypk2. Image taken from (55) and modified.

To date, most recent advances have brought more precision to TORC2/Ypk-related signalling cascades. The plasma membrane is the sole location for Ypk1 TORC2-mediated phosphorylation. The recruitment of Ypk1, curiously lacking PH domain, to plasma membrane is dependent on binding to inositol phospholipids. It has been proved to be realized through association of Ypk1 with Slm1. Slm1 is another important TORC2 downstream effector, which previously figured in calcineurin TORC2-related negative regulation (84); Slm1/2 have been also identified as major membrane stress sensors upstream of TORC2, adding membrane tension response and sphingolipid synthesis to the list of TORC2 goals (85). Ypk1/TORC2 implication in sphingolipid homeostasis has been further detailed through observation of direct Ypk1 phosphorylation of Orm1/2 upstream sphingolipid biosynthesis switches (52). The ROS pathway canonically controlled by Fpk1 and sphingolipids has also been placed downstream of TORC2-Ypk1 signalling in actin polarization pathway (48). Two very up-to-date discoveries revolutionized the field by allowing very specific TORC2 inhibition, either using novel small molecule inhibitor (86) or by genetic manipulation of Avo3 TORC2 component (87). The rapamycin target FKBP12 is sterically protected from rapamycin binding by Avo3 in TORC2. *AVO3*$^{\Delta CT}$ *TOR1-1* yeast strain, where TORC2 but not TORC1 is inhibited by rapamycin, has been created. Rapid actin depolymerization and G2/M cell-cycle arrest upon rapamycin treatment confirmed TORC2-specific inhibition by this new method (87). TORC2 inhibition by a new ATP-competitive small molecule TOR inhibitor NVP-BHS345 and subsequent engineering of BHS345-resistant TORC1 strain is one more novel strategy. It enabled differentiation of a fast Fpk-related actin polarization pathway and a slow Orm-mediated membrane

homeostasis pathways as two independent directions of TORC2 downstream regulation strategy (86). The long lasting polemics, whether the role of Ypk1 in endocytosis is limited to its function in actin organisation, has been resolved by the same paper. Fpk-independent phosphorylation of core endocytosis machinery proteins Pan1 and Ent1/2 by TORC2 has been depicted, proving separate function for TORC2 in endocytosis regulation.

However, the precise mechanisms of how Ypk1 interacts with its downstream effectors to maintain actin polarization and endocytosis pathways remain uninvestigated. Discovery of precise structural interactions data of Ypk1 and its partners could elicit insight into their more intimate relations.

2. SH3 domain-containing proteins: roles in Akt/Ypk regulation

2.1. Best friends forever: SH3 domains and PxxP motifs

Directing associations of actors, be it polypeptides, phospholipids, small molecules or nucleic acids, in a vast majority of cellular processes requires protein interaction domains (PIDs) (88). These can target proteins to specific subcellular locations, control the assembly of multiprotein complexes, help recognize posttranscriptional modifications and secondary messenger small molecules, regulate activity, conformation and substrate specificity of enzymes (88–90).

2.1.1. SH3/PxxP interaction and examples

The SRC homology 3 (SH3) is probably one of the best characterized members of the protein interaction domains family (91). Both SH3 and PxxP are abundantly distributed in genomes of most living beings from prokaryotes, such as *M. tuberculosis*, to yeast and metazoans, including, worms, flies, mice and human (92–94). Sequence similarity region observed in alignment between phospholipase C, Src family kinases and viral genes has long ago aroused curiosity, guiding the first steps to SH3 discovery (95,96). The concerned region seemed too small for enzymatic activity, which is why the main focus of both random peptide and expression library screenings has been placed on potential protein interactions (97,98). A 9 to 10 amino acid long proline-rich sequence, later called PxxP (where x indicates random residue), has been identified as SH3 conserved binding motif (99). At the same time, a series of studies concentrated on accurate functional and structural description of SH3/PxxP interaction in particular context of different cellular systems *in vivo* (100,101). One of the most preliminary demonstrations is the description of SH2- and SH3- containing Grb2 adaptor protein that transduces EGF reception signal through mSos1 leading to Ras activation (102,103). Other SH3-containing Rho kinase regulating proteins (104), as well as complex formation between SH3-bearing Abl kinase and its adaptor Crk (105) are among famous examples. At this point, cooperativity of protein interaction domains has been first shown through SH2-SH3 interaction, providing evidence for a mechanism conceiving new protein interactions upon phosphorylation; cassette-like organisation of regulatory modules started making sense (88,91,106).

2.1.2. Structural insights

Earliest structural resolution studies of SH3-containing tyrosine kinases and PI3K demonstrated an overall conserved character of PxxP/SH3 interface (107–109). The relatively flat hydrophobic ligand-binding surface of SH3 features 3 defined pockets or grooves. Two of these pockets feature conserved aromatic residue, which binds ligand's proline residues. The third pocket, formed by more variable RT and n-Src loops is especially significant for specificity and binds a basic residue distant to the PxxP core (Fig.

5A,B)(91,94,101). The ligand adopts a PPII (polyproline type II) left-handed helical structure with 3 residues per turn and resembles a triangular prism, where the imaginary triangle base sits on the surface of the SH3 plain (94,107,108). This unusual secondary structure of the ligand is explained by special steric and hydrogen-bonding properties of proline-rich motifs (94,110,111). Two main features contribute to PPII successful recognition: the side chains and the backbone carbonyls both point out from the main axis with regular intervals; the entropic cost of binding is reduced for PPII helix, which is constitutively restricted in its backbone geometry (101). All these characteristics result in two possible orientations of the ligand on SH3 later referred to as types or classes: class I K/RxxPxxP or class II PxxPxK/R, where K/R represents flanking residue (Fig. 5C). The preference of particular SH3 for a distinct orientation is dictated case-specifically (107,108,112,113).

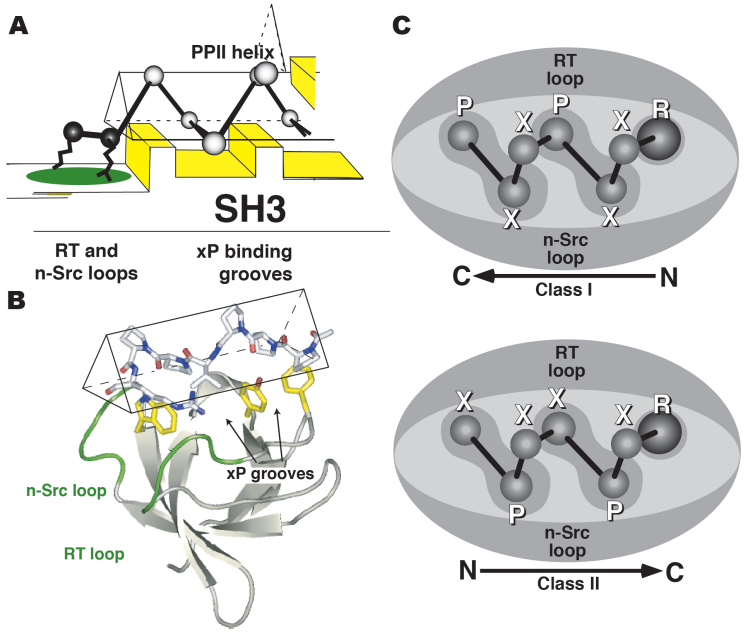

Figure 5. (A) Cartoon representation of PxxP motif and SH3 domain structures and interaction geometry and its (B) 3D version. (C) Illustration of two possible orientations of PxxP motif on SH3 domain surface. Images taken from (94) (A,B) and (91) (C).

2.1.3. Selectivity and its prediction

There is very limited variability of SH3 domain and PxxP motif conserved sites and the affinity of SH3 to its natural ligands is moderate. In this light an impressive number of their copies in higher eukaryotes raised arguments about the ways of accurate selectivity (91). Combined approaches that enable interaction predictions between particular SH3/PxxP couples feature bioinformatics, bench assays and statistical analysis. Such predictions have been showing modest but increasing reliability, staying a valuable tool for interaction partners search (108,113,114). An historical example is SPOT (Specificity Prediction Of Target) assay, which uses information from structurally resolved SH3/peptide complexes to analyse alignments and organize phage panning peptide screenings. Their results are further arranged into matrices that describe the frequency of occurrence of specific residue pairs for each SH3/peptide contact site. The matrices are then used for estimation of probability of given SH3 and peptide interaction (114). Most modern studies follow the same strategy, though experimental double-checking, like yeast two hybrid assays, has become an obligatory step in pursuit of reliability (115). SH3 partner predictions are biased with assumption that every partner contains a proline-rich motif (113). This is almost always true, but a sufficient number of non-conventional SH3 binding motifs have been found. For example, Pix SH3-binding site (PPPVIAPRPETKS) in Pak (116), PxxDY consensus in Eps8 (117) and Hbp site (Px(V/I)(D/N)RxxKP) on UBPY (118). PxxP motifs also have alternative interaction domain partners, WW, EVH1 and GYF being the most documented (94). This

stays extremely rare and opting for classical PxxP partner identification for a novel SH3 still seems a valid strategy (91,113). Actual point of view considers that inherent specificity in most SH3-mediated interactions is insufficient for its observed selectivity. Stabilization by contact with neighbouring peptides (119), cellular co-localization, multiple protein interaction domains synergistic action, parallel processing of signalling pathways are common lines of thought towards elucidation of the matter (114,120).

2.2. SH3 domains and AKT: clues from mammalian example

The connection between tyrosine phosphorylation and AKT activation has been established in the end of the twentieth century in two fields simultaneously. The BCR-mediated pathway in mammalian B cells required Syk and was partially dependent on Btk tyrosine kinases for Akt activation (121,122). At the same time reduced Akt activity has been observed in c-Src deficient osteoclasts (123). Baculovirus-mediated transfection of Sf9, RIE and NIH 3T3 cell lines with v-Src raised Akt activity under various conditions, confirming a specific role for Src (124,125). Amino acid substitutions with phenylalanine indicated that Tyr^{315} and Tyr^{326} of Akt are necessary for Src phosphorylation. Subsequent kinase tests confirmed direct Src-mediated phosphorylation of these residues *in vitro* and *in vivo* in context of EGF-induced pathways (126). A parallel survey pointed to high populations of Tyr^{474}-phosphorylated Akt in SKOV3 ovarian carcinoma cells. They presented exaggerated Akt activity but Tyr^{474} substitution with phenylalanine resulted in its 55% decline (127).

More recent advances highlighted the role of β-arrestin in mediating ghrelin-stimulated Akt activation by c-Src. siRNA and co-precipitation experiments concluded that α-arrestin and β-arrestin make part of the GHS-R1a ghrelin receptor-based complex with c-Src and Akt but without mTOR or Rictor in a so-called late pathway. Arrestin presence has been proved to be necessary for successful Akt phosphorylation by c-Src (128). The mechanism seems to be widespread, as emerging papers keep detecting arrestin-scaffolded Akt activating complexes downstream of another receptors in different processes. Angiotensin AT1 receptors in protein synthesis (129) and PAR4-P2Y12 in platelet thrombi stabilization (130) make good examples. The function of arrestin in endocytic receptor internalization downstream of signal molecule receptors is well established. Its emerging role in regulating enzymatic activity through scaffolding receptor-based signalling complexes, “signalsomes”, appears noteworthy as part of our study (131–133). For example, in Akt pathway case, c-Src seems to be activated by conformational change upon β-arrestin scaffolding, while arrestin’s direct influence on Akt stays a mystery (132).

The *experimentum crucis* for our study has determined that Akt features PxxP motif that mediates docking with Src through its SH3, which is required for successful Akt phosphorylation. An alignment of multiple Akt’s from different organisms indicated conserved presence of PxxP in their C-ter tail regions (424-427). Alanine substitutions of Pro^{424} and Pro^{427} produced a mutant, where Thr^{308} and Ser^{473} lacked phosphorylation, so Akt was constitutively inactive. Further performed co-immunoprecipitation assays evidenced physical association between Src and Akt in response to EGF treatment and its absence in case of P424A/P427A mutant. However, the mutant could still be recruited to plasma membrane at the same pace as wild type. The conclusion that Akt is phosphorylated by membrane-bound Src upon its PH-mediated recruitment to membrane has been made. This tyrosine phosphorylation is followed by turn-motif and HM phosphorylations by classical activators, such as PDK1 and mTORC2 (Fig. 6)(134). Later field-specific studies equally emphasised

synergistic effects of mTOR and Src on Akt activation, as in case of lung cancer cell lines apoptosis induction experiments (135) or in leukemogenic potential studies of acute myeloid leukomia (AML) blasts (27). Latter survey indicates that Retinoic acid-inducible gene product (RIG-I), highly overexpressed in AML blasts, binds with its PxxP motif to Src SH3 domain. This happens upon RIG-I CARD association with Src SH1. This process is competitive with Akt PxxP-mediated docking to Src and seems to sequester Src, decreasing Akt activation. Altogether, we get a clear opinion that PxxP interactions play a significant role in Akt physiology, whereas their functional description remains incomplete.

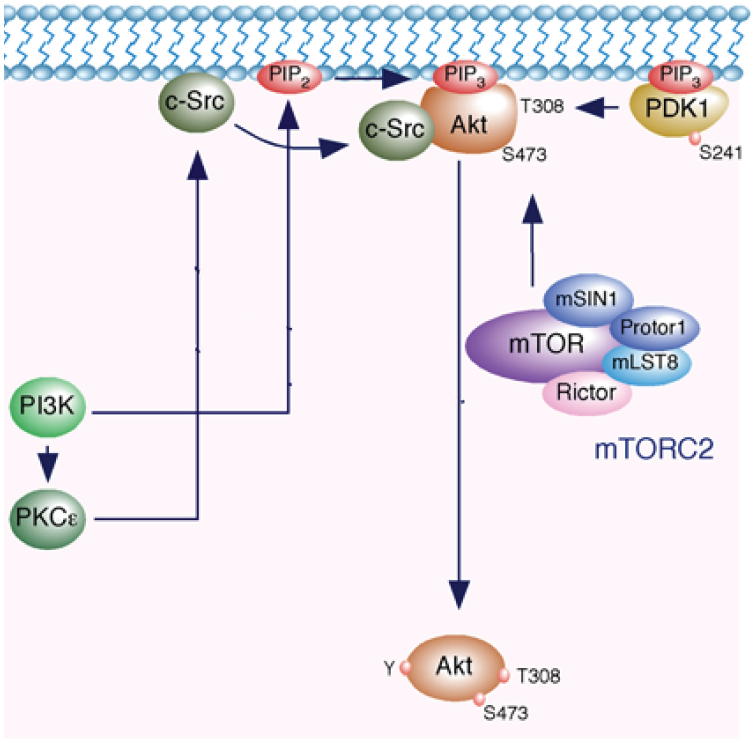

Figure 6. Illustration of the interface between 3 main AKT activation strategies known to date: related to PDK1, mTORC2 and c-Src. c-Src-mediated tyrosine phosphorylation requires AKT PxxP motif. Image taken from (128) and modified.

2.3. Ypk1 and SH3 domain-containing proteins in yeast

More than 1500 SH3 domains are registered in various domain and protein databases (113). To date, 27 unique SH3 domains (Fig. 7) from this pool have been identified by BLAST, PFAM and SMART bioinformatics analysis in *S.cerevisiae* genome and proteome (136). When organized by the ClustalW program, the SH3 domains from yeast repertoire are represented in most branches of the SH3 gene family phylogenetic tree settling yeast as auspicious model for SH3-related research (113).

2.3.1. SH3 domain-related functions: focus on endocytosis

Controlling assembly of membrane associated enzymes, such as MAGUKs, and actin nucleation through its core machinery, including WASP, Nck, WIP/verprolin, class 1 myosin and Pak, are amongst key goals of SH3 interactions in cellular physiology (91). Moderation of vesicular trafficking is also a well-recognized task of SH3. Its implication in interactions between most fundamental proteins of endocytosis scission module, such as dynamin, synaptojanin, amphiphysin I and II, has been elicited since long (137). Many more SH3-bearing endocytosis-related proteins have been identified through more recent protein interaction prediction trials in yeast (115).

A specialized yeast SH3 domain interactome study has drawn our particular attention by dissecting a whole network of endocytosis proteins featuring SH3 domains through Bayesian modelling (136). 27 types of SH3 domains found in yeast genome have been used in GST-SH3 fusion form to isolate 1871 unique peptides from a collection of random and biased yeast peptide phage libraries (Fig. 7). The position weight matrixes (PWM) have been created from sets of ligands aligned for each domain, so that specificities could be calculated for each amino acid position of the ligand. Further on, the peptides from the real yeast proteome have been compared to PWM results and the endogenous SH3 partner protein list has been established. It is equally remarkable that phage-derived specificity profiles correlated with ligand affinities. Additional SPOT tests and yeast Two-Hybrid assays results have been combined into a Bayesian network along with phage-display derived information. This heterogeneous data has been integrated into a set of highly likely SH3-ligand interaction network.

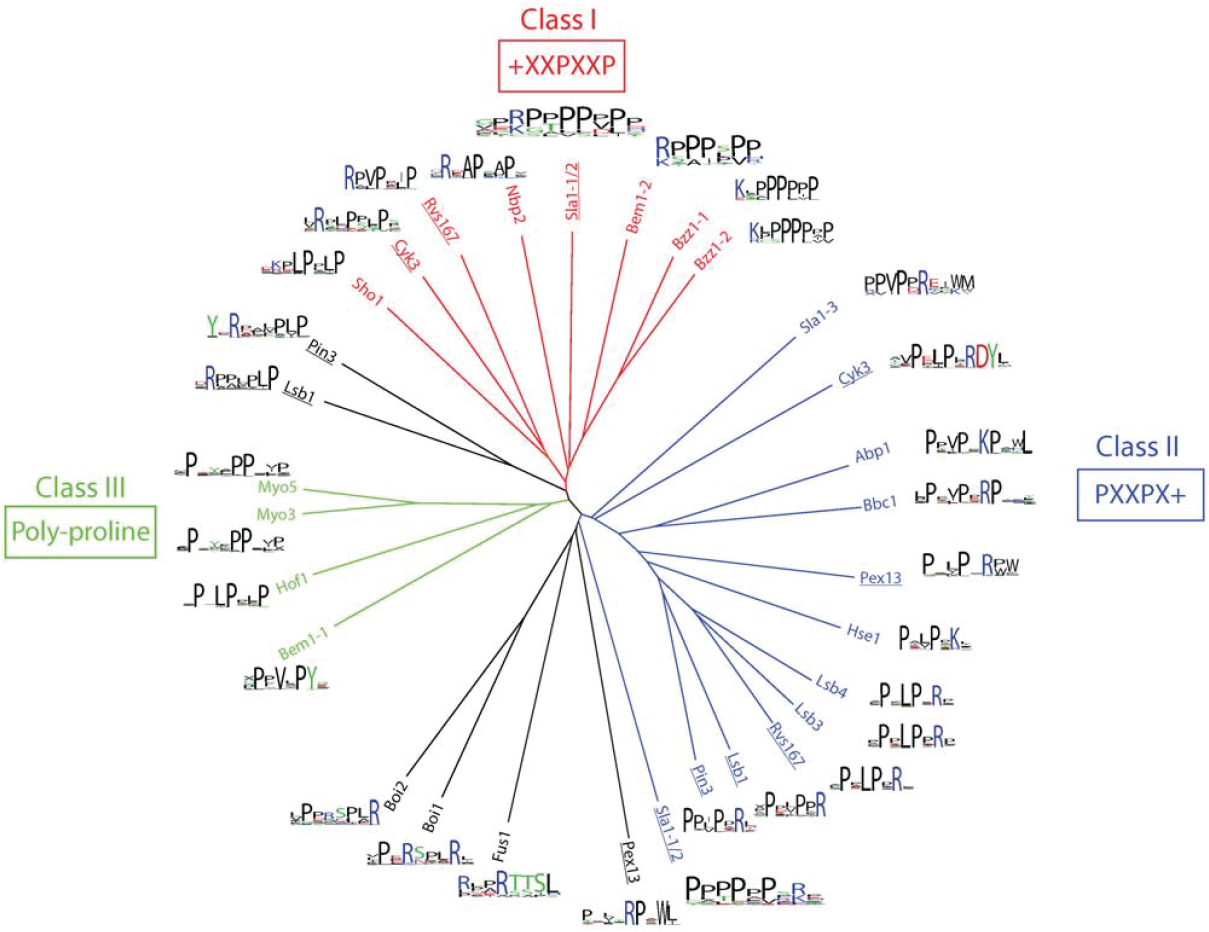

Figure 7. Endogenous specificity map for the yeast SH3 domain family. The specificity profile for each SH3 domain is represented next to the name. The SH3 domain specificity classes are colored as follows: I (red), II (blue), and III (green). Specificity profiles that could not be assigned to any class are shown in black. Underlined names indicate domains that exhibit two distinct specificity profiles. Image taken from (136).

This network contained 29 of the 60 endocytosis proteins known to date (138), confirming presumed importance of SH3 domain interactions for this process (136). All endocytosis proteins are traditionally divided into four dynamic modules based on their temporal (from early to late) and spatial preferences: the endocytic coat module, the WASP/Myo module, the scission module and the actin module (139–141). Aforementioned study suggested 53 connections between 19 endocytosis proteins from these four modules,

the majority being recruited 35 to 15 s prior to vesicle internalization, placing them downstream of clathrin (endocytic coat) module (136). The most significant among them are the following SH3-bearing WASP/Myo module proteins: Sla1, Abp1p, Bpc1p, Bzz1p, Las17p, Vrp1p, Myo3p and Myo5p. The highest Bayesian probability scores have been attributed to the proteins within a single module, which is rationally explained by resemblance in their spatio-temporal dynamics. This suggests that predicted specific SH3 interaction normally correlates with the time of arrival of particular protein to endocytosis patch.

2.3.2. Role of the conserved PxxP motif in Ypk1

We performed a ClustalX alignment of different copies of AKT from different species, Pkc-α, SGK, Pka-α and Ypk1 (Fig. 8). The alignment has shown that PxxP motif itself and the most significant flanking residues (for example lysine upstream of PxxP) are conserved in all of the studied proteins. This implies a highly probable physiological role of the motif in Ypk1. The PxxP motif of both Ypk1 and AKT can be attributed to specificity class I (+XXPXXP), as it only features a positive residue upstream of the core motif. However, the actual pattern is not entirely identical to the formula in brackets and no strict rules can be applied to PxxP interaction prediction. To our knowledge, unlike its mammalian counterpart, little to no literature contains the mention of the motif in context of Ypk1 in yeast to date. The nature of Akt and Ypk is highly identical, which is reinforced by earlier discovered functional parallelism. Most importantly, the significance of Akt regulation by PxxP/SH3-mediated interactions has been shown. Altogether this data suggests by way of analogy that the highly conserved PxxP motif could be implicated into Ypk1 functioning in yeast.

Figure 8. ClustalX multiple alignment of PxxP motifs. Parameters: Gap Opening:10, Gap Extension: 0.2, Delay divergent sequences : 30%, DNA transition weight : 0.5, PWM: Gonnet series.

As reviewed above, Ypk1 is a crucial element for achievement of TORC2-related endocytosis goals. However, the precise molecular mechanism of Ypk1 interactions, that promotes endocytosis, stays yet undescribed. Ypk1 also seems to be an only PxxP bearing protein in the array of TORC2 endocytosis-related downstream effectors. SH3-mediated interactions strongly contribute to the whole endocytosis machinery highly enriched in SH3-bearing proteins from early to late stages. The endocytosis proteins seem to make excellent candidates for SH3/PxxP interaction with Ypk1, in this way hopefully replacing the missing link between the TORC2-regulated pathways and one of their final goals, the endocytosis.

3. Research Plan/Discussion

3.1. PxxP called into question

We have documented that Ypk1 contains a PxxP motif highly identical to its mammalian homologue Akt. As a starting point, we considered essential a basic functional test of PxxP in Ypk1 in yeast. Ypk1 mutant yeast with AxxA motif instead of PxxP was unable to rescue ΔYpk1 slow growth phenotype (unpublished data from Loewith lab). This convinced us of general utility of the motif in Ypk1 in yeast and indicated necessity of further experiments.

3.2. Ypk1 partner prediction and its limitations

For higher reliability we decided to concentrate on the SH3 domain of c-Src in order to predict *in silico* interaction partners for yeast Ypk1 through the potentially conserved PxxP/SH3 interface. We performed an alignment of mouse c-Src SH3 with a complete database of S. *cerevisiae* proteins, which resulted in an array of SH3-containing candidates (Table 1). We noticed that this list is enriched by actin polarization- and endocytosis- related proteins.

Protein name	Description	Score(bits)	E-value
Rvs167p	Implicated in actin polarization and endocytosis	46.2	9.3e-07
Sla1p	Involved in endocytosis progression and actin polymerization	40.6	4.7e-06
Pex13p	Peroxisome membrane importer component	39.9	7.7e-06
Ysc84p	Actin binding protein	41.3	2.2e-05
Sho1	Transmembrane osmosensor for filamentous growth and HOG pathways	38.1	2.6e-05
Lsb1p	Implicated in actin polarization and endocytosis	38.9	5.3e-05
Abp1p	Actin-binding protein of the cortical actin cytoskeleton	38.1	3.6e-04

Table 1. Best hits from NCBI BLASTP mouse c-Src SH3 domain query in S.*cerevisiae* protein database. Matrix used: BLOSUM62 (default settings).

The basis for any *in silico* prediction is the particular sequence of the motif. As mentioned above, the PxxP of Ypk1 (KGYIPPYKP) is classed in Group I (+XXPXXP), which normally determines the orientation of PxxP on the SH3 domain and limits the field of possible partners (108). Nevertheless, there is no strict rule and many exceptions exist in affinity of particular cases of PxxP motifs to their SH3 counterparts, as many motifs are similar, the selectivity is not robust, so predictions should be used with precaution (107,112,113). For example, Lsb1p and Pin3p yeast endocytosis proteins are capable to fix PxxP in both orientations, which means that they interact with both Class I and Class II PxxP motifs with their SH3 domains (136).

3.2.1. Potential non-SH3 partners review

The proline-rich motif interactions are not limited to SH3-containing partners. We evaluated the possible non-SH3 interactions in Ypk1 PxxP motif (KGYIPPYKP) case. WW domains classified in Group I have affinity for PPxY motifs, where the tyrosine residue have been documented to play a role of negative switch when phosphorylated (142). Among the illustrative members of this group are YAP65, a transcriptional co-activator capable of association with Src, dystrophine, a cytoskeleton associated complex, and Nedd4, a neural development protein (143). The relevance of WW-mediated interactions in yeast is yet to be determined. The Ypk1 PPYKP motif could become a recognition pattern for a Group I WW domain. The insight if the Ypk1 PPYKP tyrosine residue is phosphorylated *in vivo* under certain conditions could be instructive in respect of the mentioned mechanism of negative switch system. As there are no tyrosine kinases in yeast known to date, the isolated cases of dual-function kinase tyrosine phosphorylation seem to be the only possible way (144).

3.3. Experimental approach for partner identification

We propose an experimental protocol for partner identification. The simplest approach would be a GST pulldown of Ypk1 PxxP region that could isolate its physiological partners. The AxxA control must also be performed for this experiment. However, the contact between kinases and their substrates is often short-lived and unstable, complicating its detection by this method. Therefore we are inclined to use a new engineered peroxidase reporter APEX instead. APEX covalently tags its proximal partners with biotin upon H_2O_2 treatment in presence of biotin-phenol. This enables their subsequent enrichment on streptavidin beads and identification by mass spectrometry (145). Ypk1-APEX fusion protein would hopefully tag new physiological partners of Ypk1 and facilitate their isolation.

High throughput methods, such as protein chips or mass spectrometry-associated phosphoproteomics, are also among first line experiments. The array of multiple transgenic Ypk1 types should be constructed to differentiate PxxP related and non-related phosphorylations of different partners. For this purpose, the results from WT Ypk1, Ypk1-ΔPxxP and Ypk1 K.D. (or WT Ypk1 in presence of Ypk1-specific inhibitors, for example antibodies) can be compared: Ypk1 K.D. would indicate the false positive hits, whereas hits from WT but not Ypk1-ΔPxxP would be considered promising and further considered. Finer new generation tests, such as KESTREL, could also serve the same purpose (146). It has been documented that 39% of PxxP containing partners of SH3 containing endocytosis proteins in yeast interact with more than one partner (136). A high number of non-specific interactions in SH3/PxxP system means that a hit in a phosphoproteomics screening or pulldown cannot prove physiological significance of the discovered partner without following case specific in-depth consideration, which is why we propose a second line of experiments after sequencing and identification of potential partners.

The yeast two-hybrid assay would show if there is interaction at all before proceeding to more subtle tests. Following kinase assays *in vitro* featuring a purified Ypk1 or Ypk1-ΔPxxP and identified partners one by one would confirm, if the discovered partner contacts with Ypk1 through PxxP interaction and if it is directly phosphorylated by Ypk1. For more precise characteristics and spatio-temporal dynamics monitoring live imaging methods, such

as FRET, would be necessary, as *in vitro* phosphorylation tests are not always representative of what happens *in vivo*. In case of endocytosis related partners this step is particularly important, as different endocytosis modules have very exact temporal frame and this frame defines the molecular environment (136), so it would be essential to know at what step Ypk1 and, hence, TORC2 are implicated in the process to understand the control mechanisms.

3.3.1. Rvs167, Sla1 and related endocytosis proteins: excellent candidates for interaction with Ypk1

We opted to consider proteins predicted for Ypk1 PxxP interactions (Table 1) as basis for candidate approach procedures. Our further research revealed that a lot of biological data insists on their strong interrelations, which boosts the idea of their possible common regulation by a single control mechanism (Fig. 9).

The unpublished data from Loewith lab suggests that Rvs167 induced decrease in Ypk1 phosphorylation once overexpressed. Rvs167 is a membrane-associated amphyphisine homologue in yeast that polarizes actin upon initial bud emergence (147,148). It requires binding to Abp1, another protein whose SH3 is predicted to interact with Ypk1 PxxP (Table 1), through SH3 interaction to successfully polarize actin (149). Abp1 is known to cause lethal phenotype in *ΔAbp1/ΔSla1* double mutant (150).

The forementioned phosphoproteomics study of TORC2 lately performed by Loewith lab identified a list of proteins phosphorylated specifically by this complex, featuring key endocytosis proteins Ent1/2, Prk1, Pan1, and Sla1 among others (86). We noticed that Sla1 is the only protein from this list related to endocytosis and containing SH3 (136). Sla1 is a key actor and regulator of assembly of the late coat module and transition to the Myo/WASP module through actin polymerization (149,151). It contains 3 SH3 domains, presenting specificity for both Class I and II poly-proline motifs, and possesses an extensive range of interaction partners, mostly endocytosis-related (115,152). Sla1 interacts with ubiquitin PxxP-motif with its SH3-3 domain, the recognition pattern being reported Class I RXXPXXP (generally identical to Rvs167 SH3 domain) (153). This interaction is of moderate affinity and competitive with yet unidentified partners' PxxP motifs. The SH3-3 domain of Sla1 appears to be the most suitable for interaction with Ypk1 PxxP motif in terms of specificity. Another important Sla1 partner is a yeast N-WASP homologue, Las17. It interacts with Arp2/3 complex to nucleate and polymerize the actin tubules at the endocytosis patch at the end of the coat phase. SH3-bearing Lsb1, a potent Las17 inhibitor (154), and Ysc84, an actin-binding protein activated by Las17 (155), have been listed as candidates for Ypk1 PxxP binding (Table 1). There is a high affinity interaction between Sla1 SH3-1,2 domains and Las17 P10(VRLPAPPPPPRRG), P11(RRGPAPPPPPHRH) and P12(RRGPAPPPPPRAS) poly-proline unconventional motifs. Sla1 forms with Las17 a complex, called SLAC, through SH3/Proline-rich motifs association. SLAC localizes Las17 to endocytosis patches and inhibits it to prevent disordered actin polymerisation (152). Pan1, its binding partners Ent1/2 and Abp1 are all nucleation-promoting factors (NPFs) implicated in the actin polymerization stage of endocytosis; Prk1 is a regulator protein, which at the same stage disinhibits Las17, the strongest existing NPF, to allow polymerisation (149). The phosphorylation of all these intimately related proteins, as said earlier, has been proved TORC2-dependent (86). Sla1 exerts the function of a general switch in this system through Las17 control. Rvs167 is known to interact with Sla1 directly (156) and with its multiple partners, the SH3 domain of Rvs167

is appropriate for Ypk1 PxxP binding and Ypk1 phosphorylation state seems to be altered by Rvs167.

This made us distinguish Rvs167 and Sla1 as a possible bridge from TORC2 to its endocytosis final functionality through Ypk1 PxxP motif interaction. Could Sla1 cooperate with Slm1 to localize the famously PH-lacking Ypk1 to plasma membrane? Could phosphorylation by Ypk1 stimulate dissociation of Sla1 from SLAC complex and stimulate actin nucleation by Las17? Could Rvs167 and Sla1 be collaborating for signal transduction downstream of Ypk1 to endocytosis machinery? These questions drive our further curiosity.

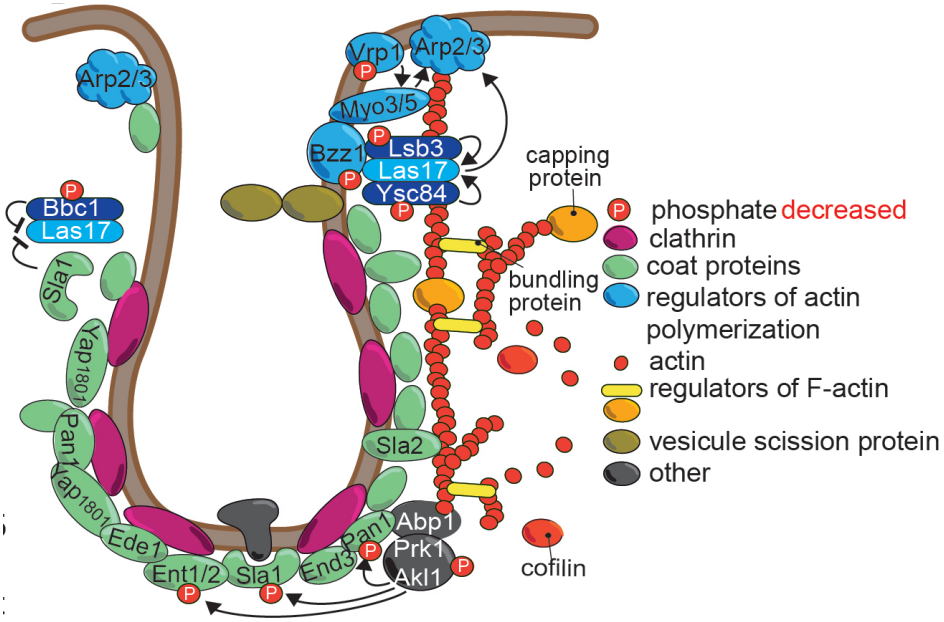

Figure 9. The main candidates for PxxP-mediated Ypk1 interaction form a group of intimately related SH3-containing endocytosis machinery proteins. Image taken from (86).

To test our hypothesis, we propose to overexpress the candidates with muted versions (or totally lacking) of SH3 domains (CANDIDATE $^{\Delta SH3}$) and observe if this brings altered phenotype. Simultaneous observation of TORC2 (Ypk1)-dependent phosphorylation levels of other mentioned proteins and/or modification of any existing relations between them could be instructive. Inverse control approach is also indispensable: if the yeast strain CANDIDATE;*Ypk1*$^{\Delta PxxP}$ shows the same phenotype as CANDIDATE $^{\Delta SH3}$, this will mean that the interaction between Ypk1 PxxP motif and candidate's SH3 domain is probably decisive for normal activity. Other approach would be to use Ypk1^{AS} analog-sensitive version. The candidates would be tagged with TAP tag. After elution with TAP-specific IgG beads they would be incubated with the antibody specific for phosphorylated sequence conserved in all AGC kinase substrates and detected by Western Blot. The Western Blot band from successful candidate would be expected to disappear upon Ypk1^{AS} inhibition by NM-PP-1 inhibitor. The already mentioned procedures, yeast two-hybrid, kinase assay and FRET, are obviously pertinent as confirmation and deepening experiments.

3.4. Modelling of potential novel Ypk1 pathways

The data gathered in our query inspired us to speculate over potential models of Ypk1 position in the signalling pathways according to our PxxP hypothesis. We therefore propose 3 scenarios to illustrate our main lines of approach:

a) PxxP-mediated interaction with a partner followed by its direct phosphorylation by Ypk1 (Fig. 10A)

The already activated Ypk1 uses its PxxP motif to bind its SH3-containing partner. Afterwards, the partner is directly phosphorylated by Ypk1, which modifies its activity promoting the cascade leading to endocytosis.

b) Ypk1 PxxP-mediated interaction with a partner allows Ypk1 to encounter its substrate and phosphorylate it (Fig. 10B)

The SH3 containing Ypk1 partner is not its substrate. Two options are possible: either the partner is initially in complex with Ypk1 substrate or the PxxP/SH3 interaction modifies the partner's structure (for example, a disordered SH3 domain becomes ordered), which leads to novel interactions and guides Ypk1 to its substrate. Then the substrate is phosphorylated by Ypk1 in this way promoting the endocytosis cascade. The partner's role in Ypk1 plasma membrane localization can also be imagined.

c) Ypk1 PxxP-mediated interaction modifies its conformation, catalysing its (in)activation through (de)phosphorylation by an upstream kinase (Fig. 10C)

The PxxP/SH3 interaction in Ypk1 exerts the same function as HM- and Turn Motif-phosphorylations: it allows its interaction with upstream partners through exposure of previously obstructed contact or phosphorylation sites. In this case PxxP-mediated interaction is not linked to downstream effectors.

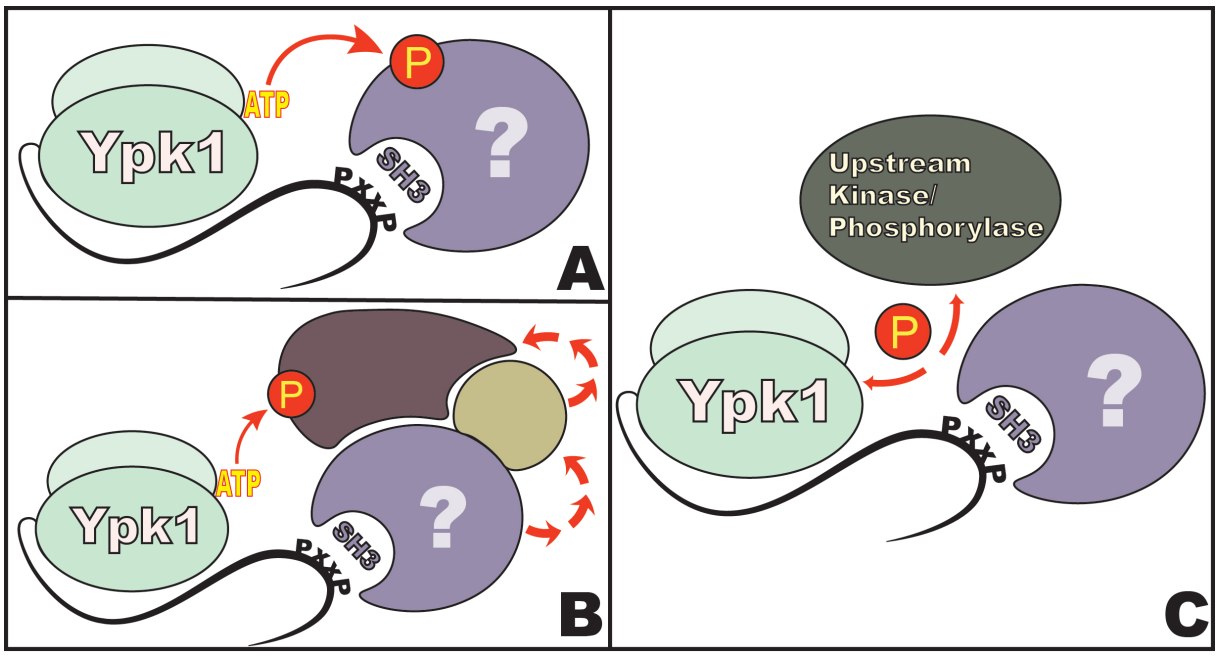

Figure 10. Three models for potential PxxP-mediated interactions of Ypk1 with its unknown partners. The illustrated SH3 domain might also be replaced by a less common PxxP-binding site, such as WW, EVH1 or other.

Obviously, the models can be combined, considering that PxxP motif's specificity often allows it to interact with multiple partners. Each interaction with each partner might follow a different plot.

4. Conclusion

Initially, we carried out our study in pursuit of discovery of Ypk1 connections to endocytosis machinery through its PxxP motifs but the subject opened up the whole new perspective for positioning of the motif in Ypk1 physiology. We think that not only Ypk1 interactions with its effector proteins but also its localization and its subsequent activation might be influenced by the PxxP-mediated interactions, which makes of PxxP a potential important basic structural element for Ypk1 functioning in general. In this way this paper inspires multiple directions for further research. The more precise knowledge on this subject is essential for finer understanding of cellular signalling cascades and their interrelations in the spirit of contemporary systematic approach but also for various valuable applications in the fields of biotechnology and medicine, such as anti-cancer therapy, cell growth regulation and many others.

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